# Lineage Plasticity and Regenerative Potential of Adult Muscle Stem Cells: Investigation of Satellite Cell Direct-Reprogramming and Pericyte Self-Renewal

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To my grandmothers

# DECLARATION OF AUTHORSHIP

I, Mattia Francesco Maria Gerli, confirm that the work presented in this thesis is my own. Where information has been derived from other sources or work has been performed in collaboration with other researchers, I confirm that this has been clearly indicated in the thesis.

# ABSTRACT

Satellite cells are responsible for most of adult skeletal muscle regeneration. Upon activation they differentiate into transient amplifying myoblasts that undergo cell fusion to form multinucleated fibres. Despite their remarkable differentiation ability and the positive outcomes obtained with transplantation in dystrophic mice and recently in patients with oculo-pharyngeal muscular dystrophy (OPMD), clinical trials in patients with Duchenne muscular dystrophy (DMD) showed limited efficacy, mainly ascribed to myoblasts low survival and poor migration ability. Muscle pericyte-derived mesoangioblasts (perivascular cells associated to the capillaries) also contribute to muscle regeneration and colonise the satellite cell niche. These cells can be injected systemically and migrate through the vascular endothelium, circumventing the necessity of multiple intra-muscular injections. Mesoangioblasts have been also tested in a recently completed phase I / II clinical trial to assess their safety profile in five DMD patients (EudraCT no. 2011-000176-33). We hypothesise that exploiting the key properties of myoblasts and mesoangioblasts may have the potential to produce clinically relevant cells, superior to those currently available. This work shows that exposure to molecules involved in pericyte specification such as the Notch ligand DLL4 and the growth factor PDGF-BB can induce direct reprogramming of primary satellite cells to pericyte-like cells. Reprogrammed cells acquire perivascular marker expression without losing the satellite cell marker Pax7. These highly myogenic cells can be expanded in culture and showed increased engraftment. In vitro and in vivo experiments also showed improved migration

ability, similar to what has been observed with mesoangioblasts. Additionally, this thesis includes a set of experiments aiming to assess the self-renewal potential of mesoangioblast-derived cells via serial transplantation assays. Overall, the results obtained improve our understanding of smooth / skeletal fate choice and self-renewal, providing evidence of the possibility of exploiting a direct reprogramming approach to allow systemic delivery of myoblasts for cell therapies of muscular dystrophies.

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# LIST OF ABBREVIATIONS

ADP: Adenosine diphosphate

ADSCs: Adipose-derived stem cells

ANOVA: Analysis of variance

aON: Antisense oligonucleotides

AP: Alkaline phosphatase

APC: Allophycocyanine

ASPA: Animal scientific procedures act

ATP: Adenosine triphosphate

BBB: Blood-brain barrier

bFGF: Basic fibroblasts growth factor

BMD: Becker muscular dystrophy

BMP: Bone morphogenetic protein

BSA: Bovine serum albumin

cDNA: complementary deoxyribonucleic acid

CMD: Congenital muscular dystrophy

CNF: Centrally nucleated fibres

CRISPR: Clustered regularly interspaced short palindromic repeats

CSA: Cross-sectional area

CT: Control

DAPC: Dystrophin associated protein complex

DLL1: Delta-like ligand 1

DLL3: Delta-like ligand 3

DLL4: Delta-like ligand 4

DMD: Duchenne muscular dystrophy

DMEM: Dulbecco's modified Eagle's media

DNA: Deoxyribonucleic acid

DYS-HAC: Human artificial chromosome containing the dystrophin locus

EDL: Extensor digitorium longus

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EGFP: Enhanced green fluorescent protein

EPC: Endothelial progenitor cells

ESCs: Embryonic stem cells

FACS: Fluorescent activated cell sorting

FAP: Fibro adipogenic progenitors

FBS: Foetal bovine serum

FSHD: Facioscapulohumeral muscular dystrophy

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GFP: Green fluorescent protein

HAC: Human artificial chromosome

HDAVs: Helper-dependent adenoviral vectors

HE: Haematoxylin and eosin staining

HS: Horse serum

HUVECs: Human umbilical vein endothelial cells

iPSCs: Induced pluripotent stem cells

ITS: Insulin transferrin selenium solution

JAG1: Jagged 1

JAG3: Jagged 3

LGMD: Limb girdle muscular dystrophy

MABs: Mesoangioblasts

MDSC: Muscle derived stem cells

miRNA: micro RNA

MRFs: Myogenic regulatory factors

mRNA: messenger RNA

MSC: Mesenchymal stem cells

MyHC: Myosin heavy chain

NICD: Notch intracellular domain

nLacZ: Nuclear LacZ

NMJ: Neuromuscular junction

OPMD: Oculopharyngeal muscular dystrophy

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PDGF: Platelet derived growth factor

PDGFrB: Platelet derived grow factor receptor beta

PE: Phycoerythrine

PECAM: Platelet endothelial cell-adhesion molecule

PFA: Paraformaldehyde

PIC: Pw1-positive interstitial cells

qRT-PCR: Quantitative real time polymerase chain reaction

rAAVs: recombinant adeno-associated vectors

RNA: Ribonucleic acid

Scid: Severe combined immune deficiency

SEM: Standard error mean

Sgca: $\alpha$-sarcoglycan

SMA: Smooth muscle actin

TALEN: transcription-activator like effector nucleases

tdTomato: Tomato red fluorescent protein

TGF: Transforming growth factor

TnAP: Tissue non-specific alkaline phosphatase

TNF: Tumour necrosis factor

VEGF: Vascular endothelial growth factor

VSMCs: Vascular smooth muscle cells

YFP: Yellow fluorescent protein

ZFN: Zinc-finger endonucleases

# CHAPTER 1:

# 1. INTRODUCTION

## 1.1. The skeletal muscle tissue

### 1.1.1. General architecture

Striated skeletal muscle is the most abundant tissue of the adult human body accounting for ≈38% of the total body mass (Relaix and Zammit, 2012). This system is composed by approximately 640 different muscles (depending upon anatomical classification) and is responsible for: 1) movement and posture; 2) supporting soft tissues; 3) guarding body openings; 4) maintaining body temperature; 5) storing nutrients (Janssen et al., 2000). The functional units of a skeletal muscle are cylindrical structures named myofibres. A myofibre contains up to hundreds of nuclei sharing a continuous cytoplasm. These syncytial cells are formed by the fusion of mononuclear progenitor cells termed myoblasts (Mintz and Baker, 1967). While the length of a human myofibre can easily sit in the range of centimetres, its diameter is normally around 10-100μm (Ropper A., 2014). Each myofibre contains bundles of myofibrils, which are composed of a repeated series of thousands of sarcomeres. Within each sarcomere are filaments of actin and myosin that interact to produce the force (Huxley, 1974). During postnatal growth the overall number of myofibres remains constant, but each myofibre grows in size by additional myoblast fusion (Yin et al., 2013). While the specific force of

a muscle fibre depends on its diameter, the strength of the whole muscle structure depends mainly on the combination of diameter and number of fibres composing it (Krivickas et al., 2011). The diameter of the fibres, the content of myoglobin, the presence of different myosin isoforms and the number of mitochondria determine the speed of contraction (Scott et al., 2001).

Muscle fibres are classified in two groups according to their speed of contraction: type 1, slow-twitch fibres characterised by an oxidative metabolism and type 2, fast-twitch fibres predominantly glycolytic. Type 2 fibres can be sub-grouped in 2a, 2b and 2x on the basis of the myosin isoform composing their sarcomeres that in turn influence the speed of contraction (2b being the fastest). Virtually all muscles are composed of a mixture of type 1 and 2 myofibres though in variable proportion (Scott et al., 2001).

***Figure 1. Structure of the skeletal muscle.*** *The image depicts the structure of a skeletal muscle, and its anchoring to the bone through a stiff connective tissue structure named tendon. The arrows point to the hierarchy of connective tissue structures, that contributes to the stability of the tissue embedding the muscle fibres in bundles named fascicles. (Illustration obtained from the "Biotechnology learning hub" public repository).*

The myofibres run in parallel along the muscle's axis while a layer of connective tissue named epimysium holds the whole structure together. Within the epimysium, myofibres are grouped in bundles (named fascicles) that are held together by an additional connective tissue layer named perimysium. This infiltrates between the myofibres, creating a thin scaffold

named the endomysium, which is also part of the basal lamina of the muscle fibres (Figure 1) (Light and Champion, 1984). This layered structure of connective tissue contributes to the stiffness and stability of the muscle tissue and creates the environment defined as the muscle stem cell niche (Yin et al., 2013). The muscular blood supply relies on the presence of an arterial tree that branches through the epimysium, generating a large number of branches through the perimysial layer. A tight capillary network originating from these arterioles surround the single muscle fibres running along the endomysium. These capillaries need to adapt their length depending on the contracted or relaxed status of the muscle, and for that purpose they are characterised by a sinusoidal spring-like shape (Korthuis, 2011). Myoglobin, a protein present in the muscle, is smaller but similar to haemoglobin (its circulating counterpart) and guarantees oxygen supply to the tissue. Myoglobin and mitochondria are abundant in slow-twitching myofibres, allowing the continuous production of energy through an oxidative metabolism. For this reason muscles rich in slow fibers do not undergo fatigue. In contrast, fast-twitching muscles allow rapid movements but their metabolism is glycolytic and thus they fatigue after repetitive contractions (Kendrew et al., 1958; Nelson, 2000).

### 1.1.2. Muscle contraction

The mechanics behind skeletal muscle contraction have been outlined by a theoretical approach developed independently by Rolf Niedergerke, Jean Hanson, Hugh and Andrew Huxley in the early 1950's and is commonly known as the *sliding filament theory* (Huxley and Niedergerke, 1954; Huxley

and Hanson, 1954). During the active phase of this process the sarcomeres shorten, generating what has been consequently defined as the contraction force. The contraction of a muscle relies on the simultaneous contraction of the single myofibres composing it.

***Figure 2. Schematic representation of the sarcomeric structure.*** *The thick myosin filaments (green) are the active component of the contractile apparatus. The thin filaments are composed of actin (red), troponin (orange) and tropomyosin (yellow). Titins (light blue) anchor the thick filaments to the alpha-actinin composing the Z-disk (purple). Thanks to the presence of a central spring-like domain (blue), titins are responsible for the passive elasticity and stiffness of the muscle tissue. T tubules are invaginations of the sarcolemma and are depicted at the edges of this scheme (light blue). The T tubule network mediates the transduction of the action potential through an acetylcholine-receptor-mediated depolarization. This depolarization causes the release of calcium ions from the sarcoplasmic reticulum cisternae located between the T tubules and the Z disk, initiating the muscle contraction. Adapted from Kobirumaki-Shimozawa et al. (2012).*

An action potential originated in the central nervous system reaches the muscles through a motor neuron, which is responsible for transmitting the stimulus down its axon to the muscle. In resting conditions, vesicles containing acetylcholine are localised where the motor neuron comes in close proximity with the myofibre forming an area with a convolute pretzel-shape structure named neuromuscular junction (NMJ) (Balice-Gordon and Lichtman, 1990; Sine, 2012). The action potential causes an influx of calcium ions towards specific voltage-gated channels present at the NMJ. This calcium influx causes the fusion of the acetylcholine-containing vesicles with the plasma membrane, leading to the release of its contents in the extracellular

space. For this reason, muscle contraction can be described as a calcium-mediated process (Szent-Gyorgyi, 1975). Here the acetylcholine associates with the nicotinic acetylcholine receptor, a pentameric trans-membrane ion channel present on the myofibre membranes in areas concomitant with the neuromuscular junction (Miyazawa et al., 2003). This activation causes an immediate influx of sodium through a $Na^+$ / $K^+$ channel, triggering an action potential which is then propagated through the myofibre membrane towards a network of T-tubules (light blue in figure 2). T-tubules are normally located in between two sarcoplasmic reticulum structures named terminal cisternae, to form a cluster known as the triad. This depolarisation activates voltage-dependent L-type calcium channels in the T tubule causing calcium release (via calcium releasing channels named ryanodine receptors) at each of its contact points with the sarcoplasmic reticulum. At this stage the sarcoplasmic reticulum releases calcium ions into the cytoplasm, which leads to the movement of troponin C and causes its conformational change and the dissociation of troponin from tropomyosin, a molecule composing the thin filaments (Figure 2). This conformational change exposes the myosin-binding domain of the actin filaments. Myosin is the engine of muscle contraction and the main component of the thick filaments (Figure 2). At this stage myosin, which has an ATPase domain, uses the energy obtained by hydrolysing it to ADP+P to bind to the actin filaments and pull them in a ‘power stoke’, generating the contraction. These movements cause a reduction in the distance between the Z-disks, shortening the sarcomere and consequently the whole muscle structure. This phenomenon has been for this reason

defined as ‘contraction’. This process, known as the sliding filament theory, is repeated as long as calcium is freely bound within the thin filaments and ATP is available. Relaxation occurs by a sequence of events including: 1) degradation of the acetylcholine operated by acetylcholinesterases, 2) termination of the action potentials; 3) sarcoplasmic reticulum stopping calcium release and starting to actively pumping it back within its compartments. A lack of calcium induces a conformational switch in the troponin / tropomyosin, which masks the myosin binding domain on the actin filaments, preventing the activity of myosin heads. At this stage, the elastic tension built on tendons leads to passive elongation of the muscle fibre that returns to its resting state.

### 1.1.3. Muscle repair / regeneration

Skeletal muscle fibres (also known as myofibres) are syncytial cells containing several hundreds of post-mitotic nuclei. Healthy muscles have large myofibres with peripheral myonuclei. Although skeletal muscle is a post-mitotic tissue, it preserves the ability to regenerate following injuries, intense exercise or as a consequence of degenerating pathologies such as muscular dystrophies (Carlson, 1973; Tedesco et al., 2010). The process through which muscle regeneration occurs has been a controversial topic for a long time; specifically, whether regeneration occurs due to fusion of mono-nucleated precursors, or by generation of mono-nucleated cells derived from fragmentation of the damaged myofibres. Various papers published across 1960 and 1961, provided compelling evidence that, at least in higher

vertebrates, myofibres originate and regenerate from fusion of mononuclear cells (Capers, 1960; Konigsberg, 1960; Pietsch, 1961; Stockdale and Holtzer, 1961).

Alexander Mauro reported the presence of mononuclear cells located beneath the ensheathing endomysial basal lamina that surrounds the myofibres. These mono-nucleated cells have their niche at the periphery of the myofibres on top of the sarcolemma and have been named satellite cells due to their anatomical position (Mauro, 1961) Satellite cells are considered the main players in post-natal skeletal muscle growth, tissue homeostasis and regeneration. Upon damage, these resident stem cells exit their quiescent status, undergo asymmetric cell division and generate transient amplifying progenitors named myoblasts, which expand upon symmetric cell division (Relaix and Zammit, 2012; Sambasivan and Tajbakhsh, 2015; Tedesco et al., 2010; Yin et al., 2013). Mintz and Baker formally demonstrated that after an initial proliferative phase, myoblasts contribute to muscle regeneration by differentiating and undergoing cell fusion to repair the damaged myofibres or to replace them with newly formed ones (Mintz and Baker, 1967). Satellite cell activation has been shown to support muscle regeneration even after repeated muscle-wide / toxin-induced injuries, requiring the generation of a large number of myoblasts on each occurrence (Luz et al., 2002). A study from the 1960's also demonstrated that this regeneration mechanism is so strong that is able to restore muscle function even if an entire muscle is explanted, minced and relocated back *in situ* (Studitsky, 1964).

The nuclei of mature healthy fibres are located at the periphery of the fibre, underneath the plasma membrane. Regenerating muscles, are characterised by fibre size heterogeneity, increased number of centrally nucleated myofibres and for the presence of an increased number of small regenerating fibres (identifiable by a reduced cross sectional area) (Maughan et al., 1983; Wroblewski et al., 1982). Skeletal muscle repair involves also the infiltration of inflammatory cells and fibroblasts contributing to the removal of the necrotic damaged fibres and to the tissue remodelling process (respectively). In patients with severe muscular dystrophies, the regeneration process fails as a consequence of the exhaustion/dysfunction of the stem cell pool. The infiltrate cells start depositing non-contractile extracellular matrix, collagen and fat in the interstitium in a process named fibrosis, interfering with the muscle function, strength and regeneration ability (Mann et al., 2011).

***Figure 3. Histological hallmarks of muscle degeneration, regeneration and fibrosis.*** *The panel shows microphotography images of canine skeletal muscle sections. The top images (**a**) represent sections stained with haematoxylin and eosin. The canine DMD model depicted on the right shows the presence of centrally nucleated myofibres indicating active muscle regeneration. Notably, the diameter of the fibres is less homogeneous than the one of the control section on the left. The bottom part of the panel (**b**) shows a Masson trichrome staining performed on control (left) and dystrophic (right) samples. This staining highlights in red the myofibres while connective tissue infiltrate is stained in blue showing clear signs of fibrosis in the*

*dystrophic sample on the right (Scale bar 100 µm). Adapted from Smith et al. (2011).*

In addition to satellite cells and myoblasts, several other progenitors such as interstitial cells and pericytes have been shown to undergo myogenic differentiation during development, regeneration or upon transplantation (reviewed in (Benedetti et al., 2013; Peault et al., 2007; Tedesco et al., 2010). A specific section of this thesis details these cell types, focussing on their role and possible applications for cell therapy.

## 1.2. Muscle satellite cells and myoblasts

Satellite cells were first observed via transmission electron microscopy by Alexander Mauro in 1961 and are located in a peripheral position to the muscle fibres. These cells have a high nucleus to cytoplasm ratio and their anatomical position is intimately juxtaposed to the muscle fibres, with their niche underneath the myofibre basal lamina (Mauro, 1961). As demonstrated with experiments performed in the early 1980's, using developing chick and quails, satellite cells originate from the somites, spheres of paraxial mesoderm that generate skeletal muscle, axial skeleton and dermis (Armand et al., 1983; Gros et al., 2005; Sambasivan and Tajbakhsh, 2007). A pioneering study by Michael Rudniki and colleagues linked satellite cells to the paired box transcription factor Pax7, showing that inactivation of this gene led to a severe depletion of this cell population (Seale et al., 2000). Different lineage tracing experiments ascribed the origin of satellite cells to the appearance of Pax7 or Myf5 positive cells at different stages of embryonic development; however the exact progenitors that give rise to satellite cells still remain to be identified (Biressi et al., 2013; Lepper and Fan, 2010; Tajbakhsh, 2009; Zammit et al., 2004). Quiescent satellite cells represent around 6% of the total number of nuclei of a healthy adult muscle and can be identified by the expression of Pax7 (Zammit et al., 2006b) (Figure 4). Pax7 is indeed present virtually in all the quiescent satellite cells of the adult muscle (Gnocchi et al., 2009) and has been shown to have an essential role for their lineage specification and survival (Kuang et al., 2006).

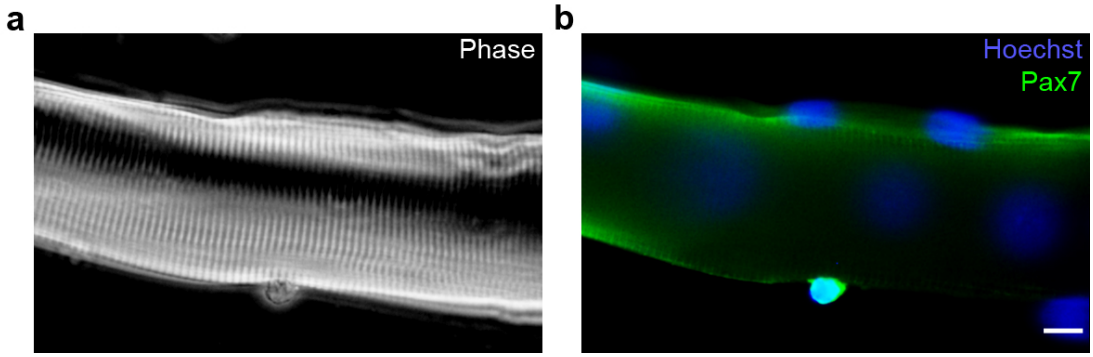



***Figure 4. Immunofluorescence for Pax7 on a single myofibre isolated from a murine skeletal muscle.*** *The image depicts a freshly isolated single myofibre obtained from a wild type murine muscle. In the phase contrast image on the left (a), it is possible to appreciate the striped sarcomeric structure and the presence of a single cell at the periphery. The image on the right (**b**) shows an immunofluorescence staining of the same fibre for the satellite cell marker Pax7 (depicted in green). The peripheral cell stains positive for Pax7 and is therefore identified as a satellite cell. The myofibre was counterstained with Hoechst (blue) to visualise the nuclei (Scale bar 15μm).*

Radioactive thymidine tracing experiments demonstrated that in the adult, satellite cells are mitotically quiescent but can undergo activation and rapidly enter the cell cycle following an injury (Snow, 1977). Satellite cell activation is a complex process that involves the combination of different signals coming from the damaged myofibres, but also from vasculature and innervation (Yin et al., 2013). Satellite cells undergo both symmetric and asymmetric cell division as shown in label retention experiments on purified cells (Rocheteau et al., 2012; Shinin et al., 2006). The whole regeneration process occurs in the mouse in approximately one week after toxin-induced damage during

which asymmetric and symmetric cell divisions are finely regulated to maintain the stem cell pool (Whalen et al., 1990; Zammit et al., 2002)

The differentiation process is mainly controlled in vertebrates by four myogenic regulatory factors (MRFs): MyoD, Myf5, Mrf4 and Myogenin (Buckingham, 2001; Hammond et al., 2007; Pownall et al., 2002). Myf5 and MyoD mainly control the progression of activated satellite cells towards the myogenic differentiation (Tajbakhsh et al., 1996). Subsequently these activated cells start expressing Myogenin and start fusing to form regenerating fibres that finally also express MRF4 (Montarras et al., 1991).

The status of a satellite cell can be defined according to its marker expression pattern. As shown in Figure 5, Pax7 positive / MyoD negative satellite cells can be defined as quiescent. Activated satellite cells, which undergo asymmetric and symmetric cell division, should be positive for both the markers (Pax7 positive / MyoD positive). However, MyoD positivity as defining criteria to identify activated satellite cells is still debated, suggesting a more relevant role for Myf5 (Kuang et al., 2008; Rudnicki et al., 2008). Myoblasts, canonically undergoing only symmetric expansion, progressively turn off Pax7 and activate Myogenin lately towards their transition to the “myocyte” status. These committed myocytes then differentiate fusing to form new myofibres and turn off MyoD (please refer to Figure 5 for a schematic overview on the process of satellite cell activation).

***Figure 5. Satellite cell activation.*** *The scheme depicts the process of satellite cell activation, which in vivo is followed by asymmetric cell division. Pax7, MyoD, and Myf5 are expressed in differentiating cells while only Pax7 is expressed in the cells returning to quiescence in order to replenish the pool of progenitors. Adapted from Tedesco et al. (2010).*

The regenerative potential of satellite cell-derived myoblasts was confirmed in the early nineties, with experiments showing that transplanted myoblasts can sustain host muscle regeneration upon serial injury (Morgan et al., 1993; Yao and Kurachi, 1993). The first formal evidence of quiescent satellite cell self-renewal was obtained with single fibre transplantations into regeneration-insufficient irradiated mice. This experiment demonstrated that as few as seven satellite cells can give rise to thousands of myonuclei and hundreds of satellite cells able to support subsequent rounds of muscle regeneration (Collins et al., 2005). The self-renewal potential of satellite cells was then confirmed with a lineage tracing experiment in which Pax7 / nGFP positive, freshly isolated satellite cells sustained up to 7 rounds of serial transplantation, contributing to muscle regeneration and to the maintenance of a pool of Pax7 / nGFP positive satellite cells (Rocheteau et al., 2012).

## 1.3. Pericytes and mesoangioblasts

Although firstly observed by Eberth in 1871 (Eberth, 1871), paternity of the pericyte identification is generally assigned to Rouget. The French scientist described these as a population of contractile cells which surrounds the blood vessel walls (Rouget, 1874, 1879). Rouget cells have since been re-named pericytes, referring to their anatomical localisation in close immediacy to the endothelial layer (Zimmermann, 1923). In the first half of the twentieth century, numerous publications described pericytes, questioning also their ability to contract. This partly reflects pericyte heterogeneity and the confusion about their cell identity (Armulik et al., 2011). Currently cells defined as pericytes are localised in the vascular basal membrane as seen via electron microscopy (Miller and Sims, 1986). These cells possess a prominent nucleus, a small content of cytoplasm and several large cytoplasmic protrusions. Nowadays, it is clear that different cell types are located in the perivascular compartment and the correct identification of the various sub-populations is still challenging (Krueger and Bechmann, 2010). The clear identification of these cells becomes even more difficult in conditions of active angiogenesis such as during embryogenesis and tissue regeneration. It is also widely accepted that pericytes are more frequent in the proximity of micro-vessels (capillaries, venules and terminal arterioles), although this has been recently challenged by the observations of sub-endothelial pericyte-like cells in large vessels (Diaz-Flores et al., 2009). Vascular smooth muscle cells (VSMCs), fibroblasts, and macrophages recurrently occupy the periendothelial position together with pericytes (Armulik et al., 2011). The

view that pericytes and vascular smooth muscle cells belong to the same lineage is commonly accepted. However, it is important to consider that no single molecular marker has been recognised as unequivocally identifying pericytes and distinguishing them from vascular smooth muscle cells (VSMCs) and other perivascular mesenchymal cells. The multiple markers generally utilised, are neither cell-specific nor constant in their expression (Armulik et al., 2011). Perivascular cells have been classified as pericytes (peri, around; cyte, cell) or alternatively as vascular mural cells or vascular smooth muscle cells (VSMC) depending on their morphology and location (Hirschi and D'Amore, 1996; Zimmermann, 1923). These cells are located around blood capillaries, arterioles and venules. In these vessels, endothelial cells and pericytes share the basal membrane and are connected by tight, gap, and adherens junctions. A single pericyte can be indeed connected with several endothelial cells by cell protrusions that wrap around and along the blood vessel (Gerhardt and Betsholtz, 2003; Kovacic and Boehm, 2009). Pericytes detected around large arteries not only occupy the periendothelial position, but can be found in the media and adventitia associated with the vasa vasorum (Andreeva et al., 1998). Distinguishing between pericytes and VSMC is a complicate; moreover it is not possible to exclude that one cell type may represent subtypes of the other or that these share the same progenitors. It has also been suggested that pericytes that reside in close proximity with the endothelium of large vessels could be the progenitors of VSMC (Armulik et al., 2005; Armulik et al., 2011). Pericytes number and distribution is highly variable according to size and vessel type. These cells

are frequently found on small venules and arterioles and more rarely on capillaries; they cover irregularly the veins with large membrane processes while they continuously cover the arteries where they form an embedding cell layer. Blood pressure has been indicated as one of the factors regulating their distribution, as observed in the retina (Wallow et al., 1993). The heterogeneity of pericytes is shown in the differences observed in correlation to the organ in which they are located. In the central nervous system, they interact via tight junction with the endothelial cells, composing the blood brain barrier (BBB) (Ballabh et al., 2004). Kidney pericytes are also known as mesangial cells (Betsholtz et al., 2004), while in the liver they are named Ito cells (Ito and Nemoto, 1952). In the murine and human skeletal muscle, 90% of the capillaries have been found to be associated with Neuro-glial 2 proteoglycan (NG2) positive pericytes (Kostallari et al., 2015).

***Figure 6. Schematic representation of pericyte location and functions.*** *The scheme depicts the most commonly recognised functions in which pericytes (in green in the scheme) have a crucial role (Winkler et al., 2011a).*

Pericytes have several functions in all tissues. In the central nervous system for example, pericytes are one of the main components of the blood-brain barrier (BBB) and together with vascular smooth muscle cells, have been indicated as potentially responsible for blood flow regulation through vasoconstriction / dilation mechanisms (Rucker et al., 2000) (Figure 6). They do this by directly interacting with the extracellular matrix and closely with endothelial cells via gap junctions (Li et al., 2011). Another process in which

pericytes have a crucial role is angiogenesis. This process requires both the recruitment of endothelial cells from pre-existing vessels (Lamalice et al., 2007) and of pericytes from the surrounding tissue (Armulik et al., 2005) via different signalling pathways such as delta-mediated Notch signalling, PDGF and TGF-β that will be further detailed in the next paragraphs of this thesis.

Because of all these different characteristics and locations, it is particularly difficult to concur on a universal definition of pericytes. Some cytoskeletal proteins such as Desmin, a muscle specific class III intermediate filament protein, and α-smooth muscle actin (SMA) have been used to identify pericytes. Desmin is, for example, normally also expressed by differentiated skeletal, cardiac and smooth muscle cells; whereas SMA is also known to be present on smooth muscle cells and myofibroblasts (Nehls et al., 1992; Ronnov-Jessen and Petersen, 1996). NG2 is a chondroitin sulphate proteoglycan strongly expressed by pericytes, in particular during angiogenesis and can bind basic-FGF, PDGF-AA, plasminogen and angiostatin (Abboud, 1995). Therefore NG2 has been commonly utilised to identify pericytes. NG2 knockout mice are viable, but show compromised revascularisation upon damage (Ozerdem and Stallcup, 2004; Rajantie et al., 2004). However, this proteoglycan is not specific for pericytes and is commonly expressed by other cell types such as immature neural stem cells capable of differentiation into neurons or glia. PDGFr-β (Platelet-Derived Growth Factor Receptor beta) is also a commonly utilised pericyte marker. This receptor is localised in the pericyte membrane and has a crucial role in the recruitment of these cells by the endothelium during angiogenesis

(Betsholtz, 2004). PDGFr-β deficient mouse embryos lack micro-vascular pericytes and develop frequent micro-aneurysms (Lindahl et al., 1997). Analysis of the gene expression profile of these PDGFr-β deficient mice highlighted the lack of RGS-5 (a regulatory G protein); for this reason RGS-5 has been recently proposed as a novel pericyte marker (Berger et al., 2005). CD13 is a surface marker that was utilised to identify pericytes in the early nineties (Kunz et al., 1994), is now less common because the expression of this protein is also observed in vascular smooth muscle cells, inflamed endothelium, myeloid, epithelial and gut cells (Armulik et al., 2011).

Alkaline phosphatases (APs) are a group of glycoproteins able to hydrolyse a large range of monophosphate esters and have their optima at an alkaline PH. The first evidence linking AP expression to the capillaries, was published in 1965 (Mizutani and Barrnett, 1965). In humans, four AP isoforms have been recognised while there are only three in the mouse (Stefkova et al., 2015). Although its physiological function is still unknown, AP is histochemically traceable in the mouse embryo as early as the 2-4 cell stage (Mulnard and Puissant, 1987). The tissue nonspecific isoform (TnAP) is conserved across the species and its sequence (composed of 12 exons) codes for an enzyme expressed by a subset of pericytes of the skeletal muscle (Grim and Carlson, 1990; Safadi et al., 1991) bone and heart (Schultz-Hector et al., 1993). AP-positive cells have been easily isolated from skeletal muscle and cultured *in vitro*, showing mesenchymal morphology and pericyte marker expression (Levy et al., 2001).

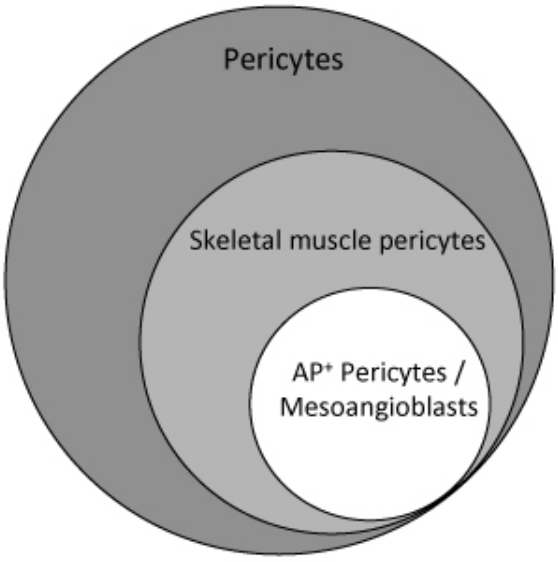



***Figure 7. Pericytes and mesoangioblasts.*** *The diagram depicts mesoangioblasts (in vitro counterpart of the AP+ pericytes), as a subset of muscle pericytes, subgroup of the general ensemble of vessel associated pericytes.*

A pioneering study published in 2002, in collaboration between the groups of Paolo Bianco and Giulio Cossu, reported that cells isolated from the embryonic murine dorsal aorta and ascribed to the perivascular lineage (by the expression of CD34, Flk-1, SMA and c-Kit), were able to generate *in vivo* different mesodermal tissues. For this reason, these cells have been named mesoangioblasts (Minasi et al., 2002). Similar cells have been subsequently isolated from murine (Sampaolesi et al., 2003), canine (Sampaolesi et al., 2006) and human skeletal muscle biopsies (Dellavalle et al., 2007) and resulted capable to contribute to skeletal muscle regeneration also upon intra-arterial delivery. Adult mesoangioblasts are defined as the *in vitro* counterpart of a subset of muscle pericytes expressing Alkaline Phosphatase (Figure 7). Taken together, these properties make mesoangioblasts an appealing

candidate for cell therapy purposes (Sampaolesi et al., 2005). Interestingly, recent lineage tracing experiments have shown that TnAP-positive pericytes and their progeny, contribute to postnatal muscle development and give rise to Pax7 positive satellite cells during growth and regeneration (Dellavalle et al., 2011).

## 1.4. The muscular dystrophies

Muscular dystrophies are a heterogeneous group of inheritable myopathies involving muscle tissue degeneration, with considerable variation in their clinical manifestation and severity (Emery, 2002). Overall, these pathologies are characterised by a progressive weakening and degeneration of the skeletal muscle tissue (Mercuri and Muntoni, 2013). The unifying feature of these pathologies is regarded as the altered muscle morphology. Histological studies have shown variations in fibre size, monocyte infiltration, necrosis and replacement by fat and connective tissue (Emery, 2002). These pathologies have been grouped in different categories in accordance with the predominant distribution of the main symptom, the muscle weakness (Figure 8). In some forms of muscular dystrophy a cardiac involvement is present with variable extent (Verhaert et al., 2011). Variable cognitive impairment has been also reported in some forms of muscular dystrophy such as in DMD (Bresolin et al., 1994; Wicksell et al., 2004), dystroglycanopathies (Waite et al., 2012), and in some forms of congenital muscular dystrophy (Cardamone et al., 2008). Different hypostesis have been formulated to explain the cognitive impairment observed in DMD. Clinical investigation of the role of dystrophin in the central nervous system suggested that its lack might have significant relevance in explaining this defect. Interestingly, a recent report highlighted that a three base-pair deletion affecting the brain isoform of dystrophin's binding site for the β-dystroglycan is sufficient to cause intellectual disabilitiy either in patients that shows no muscular symptoms (de

Brouwer et al., 2014). It has been postuated that lack of dystrophin correlates with alterations in the distribution and function of the $GABA_A$ inhibitory receptor impacting on the clinical efficacy of drugs, sleep disorders and motor control. These phenomena are frequently alterated in DMD patients (Anderson et al., 2002). Defects in the assembling and processing of the distroglycan complex in both neurons and glia are also associated with a spectrum of brain abnormalities. The role of dystrophin and the dystroglycan complex in the clustering of $GABA_A$ receptors may explain the association of *DMD* mutations with cognitive impairment in some patients (Waite et al., 2012).

***Figure 8. Distribution of predominant muscle weakness in different types of muscular dystrophy.*** *A, Duchenne-type and Becker; B, Emery-Dreifuss; C,limb-girdle; D, facioscapulohumeral; E, distal, F, oculopharyngeal. The affected areas are shaded in grey (Emery, 2002).*

Although some forms of muscular dystrophy affect nuclear membrane proteins, the majority of these pathologies involve, directly or indirectly, genes coding for components of the dystrophin associated protein complex (DAPC) (Figure 9), located on the myofibre membrane (Ervasti et al., 1990). This complex functions as a link between the intracellular F-actin cytoskeleton and the extracellular matrix (Davies and Nowak, 2006). Overall, this group of genetic defects causes alterations in the mechano-elastic support to the muscle contraction, leading to ruptures of the myofibre membrane.

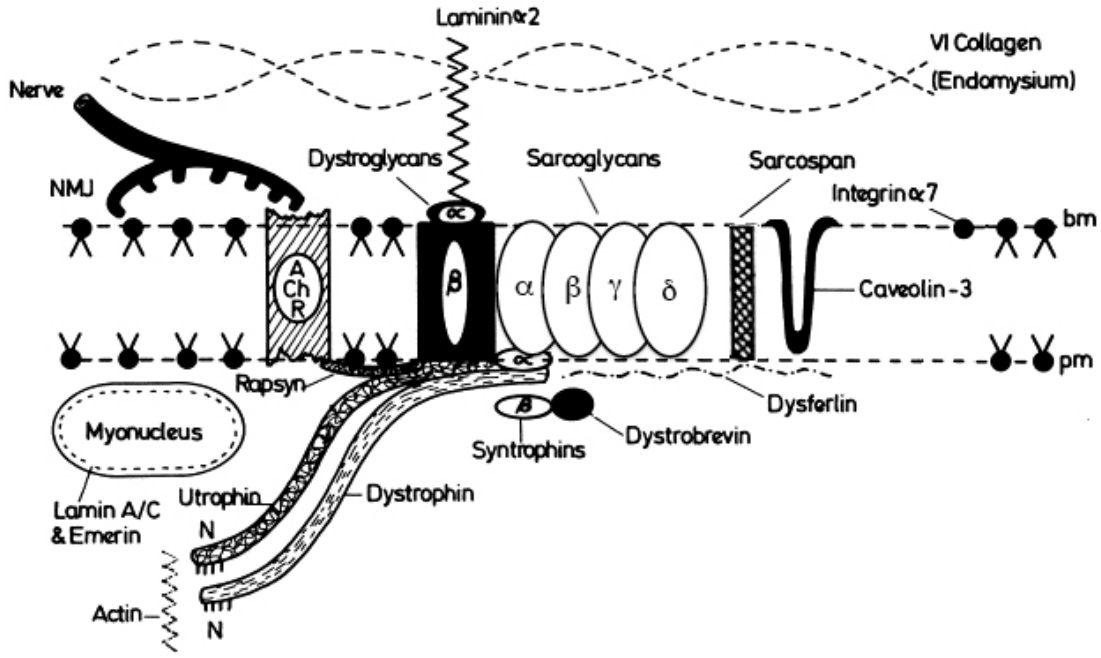



***Figure 9. Schematic representation of the Dystrophin associated protein complex (DAPC).*** *The scheme depicts the main components of the DAPC. Dystrophin is located inside the cell and its N-terminus is bound to the actin cytoskeleton. The C-terminus is associated to a large complex of membrane glycoproteins. This consists of sarcoglycans (α, β, γ, and δ subunits), dystroglycans (α- and β), sarcospan, and syntrophins (α- and β). Mutations in the dystrophin gene lead to BMD and DMD. Mutations in the sarcoglycan subunits cause various forms of limb girdle muscular dystrophy (LGMD). LGMD2B is caused instead, by mutation in the gene coding for Dysferlin. LGMD2I is a distinct form of LGMD caused by a mutation in the FKRP gene, encoding a Golgi apparatus protein. Mutations in the genes encoding the nuclear envelope protein emerin and lamin A/C cause Emery-Dreifuss muscular dystrophy and various forms of Laminopathy. Abbreviations: bm, basement membrane (basal lamina); pm, plasma membrane (plasmalemma); NMJ, neuromuscular junction; AChR , acetylcholine receptor. Adapted from Emery 2002.*

More than half of the identified forms of muscular dystrophy are grouped under the definition of limb-girdle muscular dystrophies (LGMD); other forms include Emery-Dreifuss, distal, facioscapulohumeral (FSHD) and oculopharingeal muscular dystrophy (OPMD), congenital muscular dystrophy (CMD). Auspiciously, the genetic defects causing the most common of these disorders have been already identified. This essential information allows accurate diagnosis making also feasible genetic counselling and prenatal screenings (Emery, 2002).

As mentioned above, some of these pathologies directly affect proteins composing the DAPC; this includes for example Duchenne muscular dystrophy (detailed in the next paragraph) and sarcoglycanopathies (forms of limb girdle muscular dystrophies involving the sarcoglycans) (Gordon and Hoffman, 2001). In some forms of LGMD and CMD the DAPC is indirectly affected. In dystroglycanopathies for example, mutations are mainly affecting genes like FKTN, FKRP, POMT1, POMT2 and POMGNT1, ISPD and LARGE causing a defect in the post-translational glycosylation of α-Dystroglycan. Dystroglycans are responsible of forming a link between the actin cytoskeleton and the extracellular matrix and alteration in its glycosylation status interfere with this interaction causing a broad spectrum of muscle and non muscle-related symptoms (Muntoni et al., 2011). Laminopathies are a heterogeneous group of diseases affecting the LMNA gene and its splicing products lamin A and C. These proteins, together with emerin are major components of the nuclear lamina and play a fundamental role in the organization of the nuclear architecture in all human cells. Laminopathies

cannot be defined as muscle disorders *sensu stricto*. Not all mutations in the LMNA gene results in a muscle disease phenotype. However, some forms of these diseases manifest predominantly with muscle weakness and degeneration, as in the case of LMNA-related congenital muscular dystrophy and Emery-Dreifuss muscular dystrophy. Notably in these pathologies, the DAPC is not involved at all (Bione et al., 1994; Brown et al., 2008; Mercuri et al., 2004; Scharner et al., 2010).

Dystrophic muscles are subjected to continuous degeneration and regeneration cycles. This dysfunction is initially compensated by the resident tissue progenitors that increase their number of symmetric and asymmetric divisions to regenerate the damaged tissue, leading to an initial phase of muscle pseudo-hypertrophy (Reimers et al., 1996). However, this compensatory mechanism is not sufficient to sustain long-term tissue regeneration, mainly because the newly formed muscle / myofibres carry the same genetic defects, resulting in the same level of fragility and subsequent degeneration. Additionally, this increased number of cell divisions causes the premature exhaustion of the resident muscle stem cell pool (Sacco et al., 2010). As mentioned above, the repeated cycles of degeneration-regeneration that the muscle tissue undergoes, with the constant presence of necrotic and damaged myofibres, result in a chronic inflammatory status, reflected by an increased resident monocyte presence, combined with fibrotic and adipose infiltration. Indeed, dystrophic patients in an advanced stage of the disease show extensive muscle fibrosis, where the myofibres are replaced with fat and connective non-contractile tissue, causing a permanent

loss of muscular function (Mann et al., 2011). This might be even worsened by transient aberrant vascular regulation observed in some DMD patients (Palmieri et al., 1988). Studies on transgenic mice ascribed this phenomenon to the lack of dystrophin in the vascular smooth muscle, causing the anomalous NO-dependent modulation of α-adrenergic vasoconstriction in the active muscles, leading to transient ischaemic events and contributing to patient's muscle degeneration (Ito et al., 2006; Thomas, 2013).

The lifespan and quality-of-life of the patients affected by muscular dystrophies has significantly improved in the last two decades, mainly thanks to advanced corticosteroid treatment regimes and improvements in the standards of care (Bushby et al., 2010a, b). It is important to mention that currently there is no definitive treatment that can affect the long-term progression of this group of diseases. Advancements in pharmacological approaches, gene manipulation and stem cell therapies are suggesting cautious optimism on the possibility of finding definitive cures for certain / specific muscular dystrophies in the not-too-distant-future (Emery, 2002).

### 1.4.1. Duchenne muscular dystrophy

The most common of these pathologies, with an incidence of up to 1:3,500 male children, is Duchenne muscular dystrophy (DMD; weakness distribution represented in Figure 8 A). The paternity on the identification of this pathology is still debated. Recent bibliographic research linked the discovery to an Italian physician named Gaetano Conte, who described two cases in a national scientific journal in 1836 (Nigro, 2010). The first international

publication describing this pathology appeared in 1851, authored by a British doctor named Edward Meryon (Emery, 2000; Meyron, 1851). This study highlighted the familial inheritance of the disease and that the vast majority of the patients affected were male. As a cause, Meryon, with an astonishing visionary intuition (given the then available methodology), excluded alteration in the spinal cord but linked the symptoms to rupture and destruction of the muscle membrane. Detailed histological analyses have since confirmed the fragility of the myofibres membranes and the presence of connective and adipose tissue infiltrate in the muscles of patient at the late stage of the disease (Meyron, 1851). This pathology owes its name to the French neurologist Duchenne de Boulogne, who detailed the clinical symptoms a few years later (Duchenne, 1868).

DMD has an early onset in childhood, with the child experiencing difficulties running and climbing stairs. In this disease the muscle weakness is mainly proximal and progressive with most of the affected individuals becoming wheelchair-bound by the age of 12-15. The premature death of individuals in late adolescence mainly occurs because of cardiac and respiratory complications. Better clinical care of these conditions has significantly prolonged life expectancy and delayed progressive immobilisation, but patients are still facing a long final period of almost complete inability to move.

It was only in 1987 that the DMD gene was identified as the defective molecule in DMD and its milder form, Becker muscular dystrophy (BMD)

(Hoffman et al., 1987). This gene is located in the Xp21 locus of the X chromosome. It is the largest gene known in nature (2.4Mb) and codes for a protein named dystrophin. Dystrophin is usually absent in DMD patients (as shown in the figure 10), whereas it can be present in a reduced amount or abnormal size in patients with BMD. The dystrophin protein is one of the main components of the dystrophin-associated protein complex (DAPC) and is required for the stability of the complex (Petrof et al., 1993). When absent, the structural link between the cytoskeleton and the plasmalemma becomes fragile. Upon contraction, this weakens causing ruptures and damages to the myofibres.

Clinical diagnosis is validated via immunohistochemistry and immunoblotting; further molecular analyses of the specific mutation revealed that most of the mutations causing DMD (60%) are intragenic deletions (Koenig et al., 1989). BMD is less frequent and less severe than DMD, although the gene and muscles affected coincide (Becker and Kiener, 1955). In BMD patients the production of dystrophin is not completely ablated, or the protein is present in a shorter isoform, thanks to spontaneous exon skipping events that exclude the exon containing the mutation at the transcriptional level. For this reason, progression and chronic muscular degeneration is generally slower in BMD patients, with a life expectancy that increases up to more than 50 years of age (Emery, 2002).

***Figure 10. Immunofluorescence staining for Dystrophin on a healthy donor and DMD patient muscle cryo-section.*** *The image shows an immunofluorescence staining performed using an anti-dystrophin monoclonal antibody. Healthy donor muscle biopsies show sharp dystrophin-positive rims at the periphery of the myofibres; this rim is generally absent in sections obtained from DMD patient biopsies. Adapted from Emery (2002)*

## 1.5. Therapeutic approaches to treat muscular dystrophies

Although no definitive cure for muscular dystrophies is available to date, a number of different experimental strategies aiming to treat dystrophic patients are currently under preclinical and clinical investigation. These strategies can be classified in three main different groups:

- Pharmacological approaches
- Exon skipping, gene editing and gene addition / replacement
- Stem / progenitor cell therapies

Each therapeutic approach aiming to treat dystrophic patients needs to face major challenges related to the nature of the disease itself. The need to target different muscles in the body, the cardiac and respiratory involvement (for DMD), the potential immune response (Maffioletti et al., 2014), the requirement of a long-term effect and the need to prevent or at least limit fibrotic tissue accumulation within the muscle all have to be taken into account (Mercuri and Muntoni, 2013; Tedesco and Cossu, 2012). The following paragraphs will give an overview on the mechanisms and results achieved with pharmacological and genetic correction strategies. A more comprehensive review on the cell therapy strategies adopted along the years will be provided. In the cell therapy section, additional information will be given on the physiological role of the stem cells and progenitors relevant for this work: satellite cells and pericytes.

### 1.5.1. Pharmacological approaches

Different pharmacological approaches have been attempted to directly address the primary defect in muscular dystrophies, or in alternative to provide a functional compensatory system. Gentamicin and Ataluren (known also as PTC124) have been shown to restore the dystrophin expression in both *in vitro* and *in vivo* studies by suppressing nonsense mutations that frequently occur in Duchenne patients (Barton-Davis et al., 1999; Welch et al., 2007). The mechanism underlying this strategy is the induction of a ribosomal read-through of the premature stop mutations, leading to the restored production of the full-length functional dystrophin. Despite a favourable pharmacodynamic response to the drug, recent clinical trials on Gentamicin have demonstrated modest beneficial effects accompanied by significant side effects (Malik et al., 2010). To reduce the toxicity of Gentamicin and increase its specificity, a hybrid liposomes-based drug-delivery system has been recently developed and might lead to a future clinical assessment (Yukihara et al., 2011). However encouraging results have been observed in a Phase II clinical trial with Ataluren, where it has been shown that this drug is beneficial at a relatively low dose, allowing its use in an on-going Phase III clinical trial where the long-term safety and efficacy will be assessed (Bushby et al., 2014). Most importantly, in May 2014 the European Medicines Agency granted conditional marketing authorisation for this drug, under the commercial name of Translarna™, making it the first drug approved for the treatment of DMD patients (Ryan, 2014).

An alternative pharmacological approach for DMD proposed in the early 1990's by the group of Kay Davis consists in a functional compensation

of the missing dystrophin protein up-regulating its autosomal paralogue: utrophin (Tinsley and Davies, 1993). Increased levels of utrophin, achieved upon the overexpression of a truncated version of the transgene, can restore the assembly of the dystrophin associated protein complex (DAPC) at the level of the sarcolemma and have been shown to alleviate the dystrophic pathology in pre-clinical studies on animal models of DMD (Tinsley et al., 1996). More recently, small molecule drugs have been shown to stimulate utrophin transcription. (Tinsley et al., 2011). Utrophin-based therapies have different advantages over the dystrophin-based ones. Being utrophin natively present in DMD patients, no immunological response is expected upon its up-regulation at variance with what previously observed upon the introduction of a functional dystrophin (Mendell et al., 2010a; Wells et al., 2002). Utrophin-based approaches might also be effective for all DMD patients, regardless of gene defect. Moreover, the use of small molecules open possibilities for systemic administration to the patients, since constitutive overexpression of utrophin in mdx mice resulted to be nontoxic (Fisher et al., 2001). These utrophin modulators have been recently tested in a Phase I clinical trial to investigate safety, tolerability and pharmacokinetics, with encouraging results (Tinsley et al., 2015).

Several other drugs have been proposed with the aim of treating dystrophic patients and are undergoing preclinical and clinical experimentation. Examples include anti-inflammatory molecules (Serra et al., 2012), nitric oxide (Brunelli et al., 2007), IGF1 (Barton et al., 2002), agents capable of neutralising / blocking Myostatin (Wagner et al., 2008) and more recently,

drugs to improve blood supply and muscle tissue oxygenation such as the PDE5 inhibitor Sildenafil, aiming to reduce the intrinsic muscle ischemia (Nelson et al., 2014).

### 1.5.2. Exon skipping, gene editing and gene addition / replacement

In order to induce *de novo* protein expression in patients affected by muscular dystrophies, exon skipping, gene correction and gene replacement therapies have been developed to: modulate the RNA processing; repair the underlying genetic defect or to replace the defective gene.

#### 1.5.2.1. Exon skipping

In exon-skipping synthetic antisense oligonucleotides (AONs) hybridise specific pre-mRNA targeted motif, causing the "skipping" of the mutation-containing exon. This process restores the normal mRNA reading frame leading to the production of a (slightly shorter) functional dystrophin protein, similar to what is found in BMD patients (Helderman-van den Enden et al., 2010). Based on successful results obtained in preclinical *in vivo* studies (Lu et al., 2005; Yokota et al., 2009), exon-skipping technology has progressed to two main clinical trials: a phase III study of the oligonucleotide PRO051 (Drisapersen) by Prosensa and GSK, and a phase IIb study of the oligonucleotide AVI-4658 (Eteplirsen) by Sarepta Therapeutics (Aartsma-Rus, 2010; Mendell et al., 2013). Encouraging results were obtained in the phase II clinical trial on Drisapersen, indicating a slight increase in dystrophin production and better performance in the six minutes walk test (6MWT)

(functional assay commonly utilised to evaluate the muscle function in dystrophic patients) (Voit et al., 2014). However the phase III clinical trial on this drug has been prematurely interrupted by GSK since it failed to meet the primary endpoint of a statistically significant improvement in the 6MWT, where a possible reason has been ascribed to the relatively rapid clearance of the compounds from the circulation. Moreover, controversial interpretation of the results coming from these studies (i.e. the non-correlation of amount of dystrophin positive fibres via immunohistochemistry and the level of protein detected via western blot), revealed the need to refine those analytical approaches (Lu et al., 2014). In order to avoid repeated administration of AONs and enable sustained levels of dystrophin protein for a long-term therapeutic efficacy, modified small nuclear RNAs (U1 and U7snRNA) have been designed to shuttle AONs via recombinant adeno-associated vectors (rAAV) (Benchaouir and Goyenvalle, 2012). In this way, AONs involved in the repairing of the mutated genes (and the consequent restoration of gene expression), are efficiently delivered *in vivo* through viral vectors by enhancing the efficiency in comparison with their original way of being delivered. The potential of this approach has been shown in preclinical studies in murine and canine DMD models, where a single systemically delivered dose of rAAV-snRNA–mediated exon skipping was able to restore near-normal levels of dystrophin improving function in the muscles examined, including the heart (Denti et al., 2008; Goyenvalle et al., 2012; Vulin et al., 2012).

Apart from DMD, the therapeutic potential of exon-skipping techniques has also been exploited for other muscular dystrophies, such as *in vitro* and *in vivo* studies conducted for myotonic dystrophy type 1 (Francois et al., 2011; Wheeler et al., 2012), limb-girdle muscular dystrophy 2B (Kergourlay et al., 2014; Wein et al., 2010) and congenital muscular dystrophy (Aoki et al., 2013; Taniguchi-Ikeda et al., 2011).

#### 1.5.2.2. Gene editing

Alternative gene correction strategies are based on the use of engineered nucleases for site-specific correction of mutated genes. Zinc-finger (ZFNs), transcription-activator like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPR) and mega nucleases have been recently exploited to genetically correct pluripotent stem cells and myogenic progenitors (Bertoni, 2014; Rousseau et al., 2011). Meganucleases have been successfully used to correct a specific mutation in the dystrophin gene, leading to the restoration of the reading frame and to the expression of a truncated form of the missing protein in myoblasts *in vitro* and *in vivo* (Chapdelaine et al., 2010). In another study, meganucleases have been used to target a specific deletion in the *DMD* gene of human patient myoblasts resulting in expression of a full-length dystrophin (Popplewell et al., 2013). Other endonucleases, more efficient than meganucleases, have been taken into consideration for gene therapy approaches for muscular dystrophies. Ousterout and colleagues used both ZFNs and TALENs to exclude the mutated exon 51 of the dystrophin gene in DMD myoblasts leading to the

production of the protein both *in vitro* and upon transplantation in immunodeficient animals (Ousterout et al., 2015b; Ousterout et al., 2013). TALENs are also capable of highly efficient manipulation in pluripotent stem cells, and are characterised by low toxicity (Hockemeyer et al., 2011). This genome editing system has been recently used to correct the mutation and revert the phenotype in neural stem cells derived from a myotonic dystrophy type 1 (DM1) patient induced pluripotent stem cells (iPSCs) (Xia et al., 2015). Li and colleagues utilised recently a TALEN / CRISPR-based gene editing approach to genetically correct DMD iPSCs restoring dystrophin expression upon myogenic differentiation (Li et al., 2015). However, this approach can correct only one specific mutation and would require a specific design for the vast majority of patients. To overcome this limitation, a multiplex CRISPR / Cas9-based system has been developed to target the mutational hotspot at exons 45-55 of *DMD* gene in patient myoblasts, offering a system that can correct up to 62% of the known DMD mutations. The efficiency of this method was proven by the restoration of dystrophin expression *in vitro* and *in vivo* upon transplantation of the genetically corrected cells in immunodeficient mice (Ousterout et al., 2015a). These results can be considered the first steps towards novel gene editing-based therapeutic approaches. However, extensive *in vivo* studies on functional correction of the dystrophic phenotype still need to be performed.

### 1.5.2.3. Gene replacement

Recent gene replacement approaches have successfully shown the possibility of providing an additional functional copy of the genes affected by the mutation causing the specific muscular dystrophy. These strategies aim to restore the production of the missing protein in order to recover the muscle function. Gene replacement methods do not face the mutation-specific limitations of the gene correction methods mentioned above, although safety and long-term efficiency of the gene delivery techniques are more challenging. Triggering an immune response against either the vector or the newly expressed protein is the main hurdle related to gene replacement methods. Moreover, the abundant volume of muscle tissue that needs to be treated in muscular dystrophies and its intrinsic feature of being composed of fibres surrounded by connective tissue affects the efficiency of delivering the gene of interest.

The types of vectors currently under investigation to treat skeletal muscles can be divided into viral and non-viral. Viral vectors have been largely exploited as gene transfer vehicle for *in vivo* and *ex vivo* gene therapy approaches for muscular dystrophies, due to their ability to transfect different cell types and stably integrate into the genome. For example, helper-dependent adenoviral vectors can accommodate the full-length dystrophin cDNA (14 kilobases) resulting in its efficient expression (Guse et al., 2012; Kawano et al., 2008), but the applicability of these vectors for a gene therapy purpose is limited by the need of multiple intramuscular administrations and

the high risk of immune response, highlighted in preclinical studies in non-human primates (Brunetti-Pierri et al., 2004).

Recombinant adeno associated virus (rAAV) vectors overcome some of these limitations and are suitable for systemic delivery (Gregorevic et al., 2004; Lai et al., 2005; Wang et al., 2005) also exhibiting lower immunogenicity (Zaiss et al., 2002). For these reasons rAAVs are currently considered an ideal delivery tool for gene therapy for muscular dystrophies. However, this promising therapeutic approach is challenged by the small packaging capacity of these vectors (~ 4.5 kilobases), restricting their use to the delivery of small therapeutic genes such as the sarcoglycans or shorter versions of the dystrophin (Fougerousse et al., 2007; Gregorevic et al., 2006; Koppanati et al., 2010; Krahn et al., 2010). These encouraging results validated the use of rAAV vectors in large dystrophic animal models as well as in patients (Koo et al., 2011; Mendell et al., 2010b; Rodino-Klapac et al., 2010; Wang et al., 2012). While rAAV resulted non immunogenic in mice, several studies have observed that rAAVs can elicit an immune response in a canine DMD model (Wang et al., 2007) and in humans (Mingozzi et al., 2009) leading to a re-evaluation of the role of these viral vectors for future trials. Micro / mini-dystrophin have been engineered to fit the small rAAV cargo size. However it has been reported in both dogs and DMD patients, that T lymphocytes against these transgenes appear likely hampering their long-term expression (Mendell et al., 2010a; Yuasa et al., 2007). This indicates that further studies will be needed to develop systemic treatments for patients who suffer from muscular disorders (Nayak and Herzog, 2010)

Differently from rAAV, lentiviral vectors have a relatively large size capability (~ 7.5 kilobases) and are characterised by low immunogenicity, as shown in *in vivo* and *ex vivo* pre-clinical studies (Modlich et al., 2009; Montini et al., 2006). Lentiviral vectors have been used to deliver micro / mini-dystrophin *in vivo* via intramuscular injection, showing successful genetic correction although with a low efficiency (Kimura et al., 2010; Kobinger et al., 2003). Consequently, these vectors have been exploited to develop *ex vivo* gene therapy strategies. In these approaches muscle progenitors, isolated from dystrophic animals have been genetically corrected as above. Upon transplantation in dystrophic recipients, the expression of missing protein was restored (Bachrach et al., 2004; Li et al., 2005; Pichavant et al., 2010; Quenneville et al., 2007). Although this approach might represent a valid platform for the development of an autologous stem cell-gene therapy, *in vivo* studies in DMD dogs have shown that micro-dystrophin does not replicate all of the essential functions of the full-length dystrophin (Sampaolesi et al., 2006). On the other hand, lentiviral vectors could be used for *ex vivo* gene therapy studies of other muscular dystrophies such as LGMD2D. Recently, we have transduced via a lentiviral vector the full cDNA of the α-sarcoglycan (disrupted in LGMD2D patients) in myogenic cells derived from patient-specific induced pluripotent stem cells (iPSCs). We subsequently transplanted these cells into ad hoc generated α-sarcoglycan-null immunodeficient mice (Sgca-null / scid / beige an animal model for LGDM2D disease optimised to improve the xenograft efficiency) where they restored

the α-sarcoglycan protein expression and the reconstitution of the DAPC ameliorating the phenotype of the animals (Tedesco et al., 2012).

Although lentiviral vectors are currently in use for gene therapy-based clinical trials (Aiuti et al., 2013; Biffi et al., 2013), their use is hampered by the risk of insertional mutagenesis (unfavourable integration in the host genome). To circumvent this problem, non-integrating lentiviral vectors have been recently generated based on the inactivation of the integrase (IDLV) (Matrai et al., 2011). This system is highly attractive for gene transfer in post-mitotic tissues such as muscles even though the levels of expression and transduction efficiency are generally low (Kaufmann et al., 2013; Kymalainen et al., 2014). However, a level of dystrophin production as low as 30% is reported to be sufficient to prevent muscular dystrophy in healthy mice and humans (Neri et al., 2007; Wells et al., 1995). For these reasons, the use of non-viral vectors for gene-transfer based therapies for muscular dystrophies can be considered as a valid alternative for gene replacement to overcome the limitations mentioned above.

Non-viral vectors evoke only limited immune response and allow transfer of genetic material of larger sizes, such as the dystrophin cDNA of 14 kilobases and even its whole locus of 2.4 megabases. These features allowed the use of these vectors in various gene therapy approaches, in particular for diseases caused by mutations in large genes such as DMD. Naked plasmids have been the first kind of these vectors considered attractive for gene delivery. Direct intramuscular injection of DNA constructs encoding for the full-length dystrophin, showed no adverse events in DMD

patients. However this approach was limited to a defined area and the expression of the protein was not sustained; in addition intra-muscular injection elicits a strong immune reaction against the transgene (Romero et al., 2004). Hydrodynamic limb vein injections have been suggested as an alternative to the intramuscular injections (Herweijer and Wolff, 2007). By applying these techniques, pre-clinical studies in dystrophic mice showed the restoration of the full-length dystrophin into the lower limbs and long-term protection of skeletal muscles (Zhang et al., 2010). Interestingly, studies on dose-response in rodents and nonhuman primates reached a transfection efficiency of 20-36%. These numbers are potentially sufficient to support the application of this method for possible therapeutic treatments of patients affected by DMD and other muscular dystrophies (Hegge et al., 2010; Wooddell et al., 2011).

Alternative non-integrating and non-viral vectors are the human artificial chromosomes (HACs) (Kazuki et al., 2011; Kazuki and Oshimura, 2011; Kouprina et al., 2013). HACs have a potentially unlimited cargo size and do not integrate in the host genome bypassing the risk of insertional mutagenesis (with possible activation of oncogenes). The use of HAC has been exploited for therapeutic approach for DMD where the entire 2.4 megabases human dystrophin gene, as well as its promoter and native regulatory elements, have been accommodated into the HAC (DYS-HAC) (Hoshiya et al., 2009). Our group provided the first evidence of HAC-mediated gene replacement therapy for DMD. Dystrophic murine mesoangioblasts have been genetically corrected with a DYS-HAC and

transplanted intramuscularly and intra-arterially into scid / mdx dystrophic immunodeficient mice. These genetically corrected cells supported the production of the human dystrophin in the engrafted skeletal muscle restoring a level of protein production up to 20% of control muscles, leading to a phenotypical and functional improvement of the dystrophic phenotype (Tedesco et al., 2011). Recent studies on DYS-HACs currently under development in our laboratory include the translation of this strategy to reversibly immortalised human cells (Benedetti et al., In preparation). In parallel, we derived myogenic progenitors from patient specific DMD iPSCs genetically corrected with a DYS-HAC that successfully differentiated into myotubes and produced human dystrophin *in vitro* (Tedesco et al., 2012). Further studies currently on going in the Tedesco laboratory will include transplantation studies based upon human (stem) cells containing the DYS-HAC. The main limitation of this approach is represented by the low efficiency achievable in transferring the chromosome to the target cells. This process, named microcell-mediated chromosome transfer (MMCT) still needs to be optimised and the current technology makes it costly and time-consuming. The pre-clinical studies performed so far showed stability and safety of the HAC. However, further studies will be required in order to see clinical application of this strategy (Tedesco, 2015).

Transposons are DNA-based mobile genetic elements able to integrate in the target genome, and guarantee a stable and prolonged expression of the gene of interest, showing low immunogenicity, an improved safety profile of integration and a reduced risk of oncogenic mutagenesis

compared to viral vectors (Di Matteo et al., 2012; Doherty et al., 2012; Huang et al., 2010; Yant et al., 2005). Transposons safety has been recently evaluated in a clinical trial based on genetic engineering of T cells (Kochenderfer et al., 2010; Singh et al., 2013). Sustained expression of the transgene has been shown in various proof-of-principle studies, showing transposons ability to target clinically relevant cells (Di Matteo et al., 2014; Mates et al., 2009). Within these, human satellite cells and pericyte / myogenic progenitors can be genetically manipulated with transposons (Ley et al., 2014). Interestingly, transposons carrying micro-utrophin have been successfully used to genetically correct dystrophic murine iPSC-derived myogenic cells (Filareto et al., 2013). Unlike viral vectors, transposons are not able to deliver genetic material directly to the nucleus. For this reason, invasive delivery methods (i.e. electroporation) are required, making the direct use of transposons *in vivo* more challenging than viral vectors. Interesting recent studies are focusing on the use of hybrid technologies combining the viral delivery ability to the advantages of transposons (de Silva et al., 2010; Staunstrup et al., 2009; Vink et al., 2009).

### 1.5.3. Stem / progenitor cell therapies

Satellite cells are considered the main player in skeletal muscle development, postnatal growth and regeneration of the damaged myofibres (Relaix and Zammit, 2012). Other myogenic progenitor cells located outside the basal lamina of the muscle fibres (e.g. pericytes, endothelial and interstitial cells)

have been shown to possess some myogenic potential *in vitro* and *in vivo* during growth and upon transplantation (Tedesco et al., 2010). Although the origin and the physiological contribution of these progenitors to muscle homeostasis is still unclear, lineage tracing experiments have demonstrated that some populations (e.g. pericytes) can contribute to the satellite cell niche during development and tissue regeneration This suggests a possible lineage relationship (or plasticity) between these cell types (Dellavalle et al., 2011).

Among the various strategies aiming to treat muscular dystrophies, several cell therapy approaches based on different stem / progenitor cell populations are undergoing preclinical and clinical investigation. This section will provide an insight on the cell types known to be involved in skeletal muscle regeneration, mainly focussing on satellite cells and pericytes (a central topic for this work), also giving an overview on the other cell types relevant for this process. A comprehensive list of the cell types proposed as cell therapy tools to treat muscular dystrophies, including details on the delivery route, preclinical and clinical studies performed is summarised in Table 1.

#### 1.5.3.1. Preclinical and clinical studies on myoblast transplantation

Because of their ability of regenerating skeletal muscle, myoblasts were the first cell type considered for transplantation, with promising results obtained since the late eighties transplanting wild type myoblasts in murine models of muscular dystrophy and achieving a level of dystrophin expression of 30-40%

of control non dystrophic muscles in the transplanted extensor digitorum longus (EDL) muscles (Partridge et al., 1989). In light of these preclinical achievements, several groups performed the clinical trials based on myoblast allogeneic transplantation in Duchenne Muscular Dystrophy (DMD) patients in the early nineties (Gussoni et al., 1992; Karpati et al., 1993; Tremblay et al., 1993b). These trials showed safety but very limited efficacy: death of injected cells and host immune-rejection were identified as the main causes of the poor outcome. This was confirmed following additional preclinical studies, showing improved engraftment upon immunosuppression (Kinoshita et al., 1994; Morgan and Partridge, 1992; Vilquin et al., 1994). To investigate this further and assess the efficacy of a syngeneic approach, a proof of principle transplantation was performed in twin monozygotic girls carrying Duchenne muscular dystrophy (Tremblay et al., 1993a). Mendell and colleagues then attempted to improve engraftment efficiency combining myoblast transplantation with a strong immunosuppressive regime. The achievements obtained with this strategy were far below the expectations, leading to a maximum of 10% donor-derived dystrophin-positive myofibres in one single patient out of 12 treated (Mendell et al., 1995). In addition to the issue of host immune-rejection, myoblast transplantation failures have been mainly ascribed to high cell-mortality rate upon transplantation and to the poor migration ability of these cells (Law et al., 1992; Partridge, 2002; Skuk et al., 2007b). More recently, intramuscular transplantation of myoblasts has been performed in patients affected by oculopharyngeal muscular dystrophy (OPMD) in a phase I / IIa clinical trial (Perie et al., 2014). In this particular

pathology, caused by heterozygous mutation in the gene encoding poly(A)-binding protein-2 (PABPN1), small muscles of the face and neck are specifically affected by the degeneration. Autologous myoblasts were isolated and cultured from less affected muscles (quadriceps or sternocleidomastoid) and transplanted in the pharyngeal muscles. The encouraging results achieved with local administration of myoblasts are paving the way to a possible novel treatment for this disease, though more stringent controls will be needed. However, this approach is so far inapplicable for DMD patients that require treatment of large muscular districts. Different groups have attempted to treat a larger area of the muscles, for example by performing clusters of intramuscular myoblast injections using grids of needles at a distance of 1mm each. This technique, named high-density injection (Skuk et al., 2007a), is far from solving the issue of myoblast migration. Moreover, the damage induced by the multiple injections (and the consequent appearance of fibrotic and inflammatory infiltrate) might even outweigh the advantage of having more and better-distributed cells in the transplanted muscle. Notably, this system does not allow the treatment of the diaphragm, one of the mostly affected muscles and responsible for respiratory failure in DMD patients.

An ideal delivery route to target all the muscle districts of the body would be to exploit the circulatory system. Although intravenous injection would be the simplest to approach, cells administered via this route will need to get through the filter organs (liver, kidney and lungs) causing a premature trapping before they reach their target organ (the skeletal muscles). For this reason, intra-arterial injection has been considered as a preferential route, offering the

possibility of firstly targeting the muscles, in a loco-regional fashion. However, myoblasts are considered unable to cross the blood vessel wall upon systemic delivery, therefore limiting their use for the treatment of patients affected with systemic myopathies (Sampaolesi et al., 2003). In light of the poor results achieved with myoblast-based clinical trials, various groups have looked to alternative cell types, for the development of novel strategies to treat muscular dystrophy patients.

### 1.5.3.2. Other myogenic cell types

To overcome the limitations that impede the clinical use of satellite cells and myoblasts for cell therapy purposes, other myogenic progenitors isolated from various tissues have been investigated exploiting the use of tissue-specific transgenic markers (Cossu, 1997). Diverse cell types have indeed been demonstrated to undergo myogenic differentiation (for a recent review, see Benedetti et al., 2013). Exposure to the myogenic regulator MyoD (via transfection, co-culture or transplantation in the muscle environment) has been indicated as the dominant factor able to direct these cells towards the myogenic lineage.

The only ectodermal cells that have been shown to have some myogenic potential when co-cultured with skeletal myoblasts or upon intramuscular transplantation have been neural stem cells (Galli et al., 2000). The presence of cells positive for the early myogenesis marker Myf5 within

the spinal cord and in the brain, suggested that a latent myogenic potency might be present in some of these cells and that is possible to restore it by exposing these cells to the muscle environment (Tajbakhsh et al., 1994).

In 1998 it was reported that bone marrow-derived cells have limited myogenic potential. The bone marrow-derived cells of donor transgenic animals carrying the nuclear LacZ reporter under the muscle specific promoter Myosin light chain (MLC3f), were transplanted into irradiated recipient animals. The muscles of these recipients were subsequently injured, and regenerated muscles showed expression of the bone marrow-derived beta galactosidase, indicating unequivocally bone marrow contribution to the tissue regeneration (Ferrari et al., 1998). This study highlighted for the first time the possibility (although with a very low efficiency) of recruiting circulating cells to the myogenic lineage. Further preclinical studies underlined that the efficiency of this phenomenon was too low to consider possible therapeutic applications (Ferrari et al., 2001; Gussoni et al., 1999). Interestingly this phenomenon has also been observed, again with a very low efficiency, with the presence of donor-derived nuclei in the muscles of patients that received bone marrow transplantation (Gussoni et al., 2002).

CD133 positive cells are a circulating population, ascribed to the haematopoietic stem cell compartment that has been reported to have myogenic potential (Torrente et al., 2004). These cells are able to contribute to muscle regeneration and to the satellite cell pool upon transplantation in a murine model of DMD (Benchaouir et al., 2007; Meng et al., 2014). A preliminary clinical trial performed in 2007 demonstrated the safety of

autologous CD133-positive cells transplantation (Torrente et al., 2007). Patient recruitment for a follow-up phase II clinical trial based on the transplantation of these cells is currently on going.

Muscle derived stem cells (MDSCs) have been isolated from skeletal muscle tissue using different techniques. These cells share some markers with other myogenic progenitors (such as satellite cells and mesoangioblasts) and have been transplanted in murine models of muscular dystrophy with variable outcome (Asakura et al., 2002; Gussoni et al., 1999; Meng et al., 2011; Qu-Petersen et al., 2002) and upon intraarterial delivery in dystrophic dogs (Rouger et al., 2011). Although these are interesting findings, MDSCs have not yet stepped into clinical experimentation.

PW1 positive interstitial cells (PICs), have been identified by Sassoon's group in the mouse as a subset of cells located in the muscle interstitium and capable of contributing to muscle growth, regeneration and to replenish the satellite cell pool. Lineage tracing experiments have shown that these cells do not share the origin with satellite cells (Mitchell et al., 2010). This study revealed the presence of PW1 positive cells with similar features, in different adult tissues (i.e. central nervous system, skin and bone). It has been then suggested the possibility of using PW1 as a marker for tissue-specific self-renewing progenitor cells (Besson et al., 2011). More recently, PW1 was found expressed also by mesoangioblasts, regardless of the species of origin and age of isolation (Bonfanti et al., 2015). This study showed also that PW1 expression is required for mesoangioblasts engraftment and extravasation.

### 1.5.3.3. Mesoangioblasts as therapeutic tool to treat muscular dystrophies

Mesoangioblasts are considered the *in vitro* counterpart of post-natal skeletal muscle Alkaline Phosphatase-positive pericytes. These cells have been proposed as an alternative to myoblasts for cell therapy protocols aiming to treat skeletal muscle diseases (Sampaolesi et al., 2005). At variance with myoblasts, mesoangioblasts have been shown to be capable of crossing the vessel wall upon intra-arterial delivery, actively contributing to muscle regeneration and ameliorating the dystrophic phenotype of murine and canine models of muscular dystrophy (Sampaolesi et al., 2006; Sampaolesi et al., 2003). These works indicated also that these cells are easily transduced with lentiviral vectors, making them suitable for gene and cell therapy approaches. Following these studies, Dellavalle and colleagues showed that mesoangioblasts could be also isolated from the human skeletal muscle, genetically corrected and engrafted in a murine model of DMD (Dellavalle et al., 2007). These cells, distinct from satellite cells, showed the presence of some pericyte markers (i.e. NG2 and AP) (Dellavalle et al., 2007; Tonlorenzi et al., 2007). Based upon these findings a series of studies investigated the possible applications of mesoangioblasts for other forms of muscular dystrophy (Diaz-Manera et al., 2010) and to correct aged dystrophic muscles (Gargioli et al., 2008) and cardiac defects (Galli et al., 2005). Notably, in 2011 we demonstrated the possibility of exploiting mesoangioblasts for a cell-mediated non-viral gene replacement approach. This study provided the first

evidence of on the *in vivo* use of a HAC containing the whole human dystrophin locus. Genetically corrected mesoangioblasts supported human dystrophin expression in recipient animals, leading to a long-term functional amelioration of the dystrophic phenotype in scid / mdx mice (Tedesco et al., 2011).

As mentioned above, immunological reactions play a key relevant in cell muscle cell therapy (Maffioletti et al., 2014). Indeed, similarly to what was observed with mesenchymal stem cells (English and Mahon, 2011), adult human mesoangioblasts have been also reported to have an immunomodulatory potential, inhibiting T cell proliferation (English et al., 2013). Allogeneic human mesoangioblasts have been shown to elicit an immune response only in presence of inflammatory cytokines (Noviello et al., 2014).

These encouraging findings have led to a first-in-man phase I / II clinical trial, in which allogeneic mesoangioblasts have been transplanted in DMD patients ("Cell Therapy Of Duchenne Muscular Dystrophy by intra-arterial delivery of HLA-identical allogeneic mesoangioblasts"; EudraCT no. 2011-000176-33). In light of this clinical study, a better understanding of self-renewal potential, mechanisms of fate choice and differentiation ability of these cells, will have a key importance aiming to improve the outcome of possible cell therapy protocols exploiting this cell type.

### 1.5.3.4. Myogenic progenitors from pluripotent stem cells

The derivation of myogenic progenitors from embryonic stem (ES) cells and induced pluripotent stem (iPS) cells could represent an alternative route for the cell therapy of muscular dystrophies. ES cells are pluripotent stem cells isolated from the inner cell mass of the blastocyst and have the ability to differentiate into any cell type (Evans and Kaufman, 1981; Thomson et al., 1998). However the generation of ES cell lines often involves the destruction of human blastocysts, although a recent study has shown the possibility to derive ES cells from single blastomere cells without destroying the embryo (Rodin et al., 2014). For this reason some ethical concerns raised from the use of ES cells (de Wert and Mummery, 2003). These concerns have been overcome with the generation of the iPS cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). iPS cells are adult somatic cells reprogrammed to pluripotency by the transient expression of four reprogramming factors octamer-binding transcription factor 4 (Oct4), kruppel-like factor 4 (Klf4), sex determining region Y-box 2 (Sox2) and c-Myc (2012 Nobel Prize for Physiology or Medicine). Differently from ES cells, iPS cells can be patient-specific and therefore their derivatives can be potentially more applicable for autologous transplantation thus avoiding immune rejection (de Almeida et al., 2013). Importantly, a pilot clinical study is currently on-going for the treatment of age-related macular degeneration to assess a first proof of safety in using iPS-derived cells (Nakano-Okuno et al., 2014).

Several protocols have been developed to derive transplantable myogenic cells from murine and human ES and iPS cells. Darabi, Perlingeiro and co-workers reported the derivation of myogenic progenitors from pluripotent stem cells, exploiting a combination of the overexpression of myogenic determinants (Pax3 and Pax7), differentiation into embryoid bodies and prospective isolation via FACS sorting (Darabi et al., 2012; Darabi et al., 2009; Darabi et al., 2008; Darabi et al., 2011a; Filareto et al., 2013). Cells derived with this methodology successfully engrafted in dystrophic mice and also showed a functional improvement of muscle function.

Alternative approaches exploited the upregulation of the myogenic regulatory factor MyoD, to trigger the terminal differentiation of the cells (Goudenege et al., 2012; Tanaka et al., 2013; Tedesco et al., 2012; Yasuno et al., 2014). Among these, our laboratory published in 2012 the first protocol to derive myogenic progenitors from human dystrophic iPS cells without the use of embryoid bodies or prospective isolation (Tedesco et al., 2012). These cells, defined as human iPS cell-derived mesoangioblast-like cells (HIDEMs) have been successfully transplanted intramuscularly and intra-arterially in an immunodeficient murine model of LGMD2D (as detailed in Gerli et al., 2014), engrafting the muscles and inducing the reassembly of the DAPC on the myofibres membranes. Interestingly, translating this protocol to an intraspecific setup we achieved functional amelioration of the dystrophic mice phenotype, with improvements in force and motor capacity. A recent follow up study extended the applicability of this protocol to human ES cells and iPS cells cultured in feeder-free conditions (Maffioletti et al., 2015). Considering

my significant contribution to these developments, more details on these strategies are reported in this thesis in chapter 4 and the recently published article has been included as an appendix. Interestingly, these cells have also been shown able to suppress T-cell proliferation (Li et al., 2013) as observed in mesoangioblasts, their tissue-derived counterpart (English et al., 2013). More in general, safety hurdles related to the possible use of iPS-derived cells in cell therapy approaches include immunogenicity, genetic instability and residual pluripotency (Tan et al., 2014), indicating that further studies will be needed to reduce immunogenicity and exclude eventual tumorigenic potential before extensive clinical translation.

| Cell type | Derivation | Delivery | Animal model (disease) | Clinical Trials |
|---|---|---|---|---|
| ***Satellite cells and myoblasts*** | Skeletal muscle | Local | mdx mice (DMD) (Partridge et al., 1989) | Phase II: completed (DMD) (Skuk et al., 2007a)*<br><br>Phase II: completed (OPMD) (Perie et al., 2014) |
| ***Pericytes and Mesoangioblasts*** | Vessel/ Skeletal muscle | Systemic / Local | scid/mdx mice (DMD) (Dellavalle et al., 2007; Tedesco et al., 2011); sgca-null mice (LMG2D) (Galvez et al., 2006; Gargioli et al., 2008; Sampaolesi et al., 2003); scid/BIAJ mice (LMG2B) (Diaz-Manera et al., 2010); GRMD dogs (DMD) (Sampaolesi et al., 2006) | Phase I/II: completed (DMD) (Cossu et al.,Submitted) |
| ***MDSCs*** | Skeletal muscle | Local | mdx mice (DMD) (Cao et al., 2003; Gussoni et al., 1999; Qu-Petersen et al., 2002); mdx nude mice (DMD) (Meng et al., 2011); GRMD dogs (DMD) (Rouger et al., 2011) | N/A |
| ***CD133 positive cells*** | Blood and skeletal muscle | Systemic / Local | scid/mdx mice (DMD) (Benchaouir et al., 2007; Torrente et al., 2004) | Phase I: completed (DMD) (Torrente et al., 2007);<br><br>Phase II: Recruiting (DMD) |
| ***PICs*** | Skeletal muscle | Local | Injured nude mice (Mitchell et al., 2010) | N/A |
| ***Mesenchymal stem cells (MSCs)*** | Bone marrow vessels[§] | Systemic / Local | Injured rats and mdx nude mice (DMD) (Dezawa et al., 2005); injured NOD/scid and scid/mdx mice (Crisan et al., 2008); injured $Rag2^{-/-}$ $Y_c^{-/-}$/C5 mice (Meng et al., 2010); injured nude mice (De Bari et al., 2003); mdx mice (DMD) (De Bari et al., 2003; Gang et al., 2009; Wernig et al., 2005) | N/A |
| ***Hematopoietic stem cells (HSCs)*** | Bone marrow and blood | Systemic /Local | mdx mice (DMD) (Gussoni et al., 1999); injured scid/beige mice (Ferrari et al., 1998); $mdx^{4cv}$ mice (Ferrari et al., 2001); injured mice (Corbel et al., 2003) | N/A |
| ***Amniotic fluid stem cells*** | Amniotic fluid | Systemic | HSA-Cre, $Smn^{F7/F7}$ mice (Piccoli et al., 2012)[⌘] | N/A |

| | | | | |
|---|---|---|---|---|
| ***ES cell-derived progenitors*** | Embryo | Systemic / Local | Injured scid/beige mice (Barberi et al., 2007); injured mdx mice (DMD) (Chang et al., 2009; Darabi et al., 2008; Darabi et al., 2011b); injured nude mice (Sakurai et al., 2008); injured $Rag2^{-/-}\gamma c^{-/-}$ mice (Darabi et al., 2008); injured NSG mice and NSG-$mdx^{4CV}$ (Darabi et al., 2012); $Rag2^{-/-}$/mdx and injured $Rag2^{-/-}$ /mice (Goudenege et al., 2012); | N/A |
| ***iPS cell-derived progenitors*** | Dermis, muscle and other tissues | Systemic / Local | sgca-null/scid/beige mice (LMG2D) ; sgca-null mice (LMG2D) (Quattrocelli et al., 2011; Tedesco et al., 2012); injured NSG mice and NSG-$mdx^{4CV}$ (Darabi et al., 2012); $Rag2^{-/-}$/mdx (DMD) (Goudenege et al., 2012); | N/A |

***Table 1. Summary of the reported preclinical and clinical studies on cell therapy approaches to treat muscular dystrophies.*** **Past clinical trials have been summarised and reviewed in (Tedesco et al., 2010) and (Tedesco and Cossu, 2012). §Although this is the standard tissue to derive MSCs, there is reported heterogeneity with regards to the tissue from which the cells have been isolated in the quoted articles. ⌘Model of spinal muscular atrophy carrying an homozygous deletion of Smn exon 7 presenting signs of muscular dystrophy. N/A: not available. This table has been updated and adapted from Benedetti et al. (2013).*

## 1.6. Cell fate plasticity

The ability of a committed cell to change fate (trans-differentiate) towards a different lineage is defined as fate plasticity (Bonfanti et al., 2012).


***Figure 11. Schematic representation of the meaning of fate plasticity.*** *The stem cell / progenitor A can trans-differentiate originating B. This phenomenon could be also bi-directional.*

A classic example of fate plasticity observed in patients is metaplasia. In this pathological condition one tissue type is partially or fully replaced by a different one in response to stress or abnormal stimuli (Mosby, 2009). In the intestinal metaplasia of the oesophagus (Barret's oesophagus) for example, the normal squamous epithelium of the oesophagus is replaced by intestinal columnar epithelium (Shaheen and Richter, 2009). However, whether Barret's oesophagus is indeed caused by a fate switch of differentiated cells or by selective proliferation of undifferentiated progenitors from different origin is still debated (Krishnadath and Wang, 2015).

The scientific community interest started focussing more on fate plasticity in the last two decades, mainly for its possible implications for regenerative medicine. Plasticity could indeed be re-defined as extrinsic factor-mediated reprogramming (Bonfanti et al., 2012). MyoD, the myogenic determination gene, provided the first and most remarkable example of

induced lineage plasticity or, as most recently defined, reprogramming. Upon transfection, this single transcription factor has been shown able to induce skeletal muscle differentiation in non-myogenic cells (Davis et al., 1987). A spontaneous example of this process has been indeed reported in 1995, in a paper reporting spontaneous smooth to skeletal muscle trans-differentiation in the murine oesophagus (Patapoutian et al., 1995), although this work was later questioned upon lineage tracing results (Rishniw et al., 2003). A milestone for the field was indeed a 1998 paper demonstrating that adult murine bone marrow contains cells that are capable of contributing to skeletal muscle regeneration (Ferrari et al., 1998). Following this study, many papers have been published showing that different cells of adult tissue can contribute to the regeneration of various tissues, mainly by adapting to the recipient environment (Bjornson et al., 1999; Krause et al., 2001; Lagasse et al., 2000; Orlic et al., 2001). However, some of these results were incorrect interpretations of the results, in which for example direct reprogramming was in fact mainly due to cell fusion, instead of an actual stimulation by extrinsic factors (Alvarez-Dolado et al., 2003; Balsam et al., 2004; Wang et al., 2003).

### 1.6.1. Pericyte multi-potency and plasticity

On top of covering the pericyte functions detailed above, recent studies suggest that pericytes have the ability to differentiate into various mesodermal lineages. Pericytes have been indicated as multi-potent resident tissue progenitors, able to differentiate both into smooth muscle and in the various

mesodermal cell types composing tissue in which are located (Bianco et al., 2008). In the kidney for example, pericytes play a crucial role during tissue regeneration as well as in pathologic fibrosis (Duffield and Humphreys, 2011; Kramann and Humphreys, 2014). Pericyte ability to differentiate into bone for example, opened the possibility of these cells having a role in ectopic calcification found in arteries and muscles (Johnson et al., 2006). Indeed, recent evidence linked pericytes to the muscular ossification observed in patients affected by fibrodysplasia ossificans progressiva (Hegyi et al., 2003). Additionally, in a publication where Annexin 5 was used to isolate pericyte cells from the brain, it was shown that these cells undergo osteogenic, chondrogenic and adipogenic differentiation *in vitro* (Brachvogel et al., 2005). This was further confirmed with differentiation assays *in vitro* and *in vivo*, performed in pericytes isolated from the retina (von Tell et al., 2006).

On the other hand, whether different cells/progenitors could acquire a pericyte fate is still under investigation. Glioblastoma stem cells for example have been observed to acquire pericyte functions to support vascular stabilization during tumour growth (Cheng et al., 2013). The acquisition of pericyte markers and properties has also been recently reported in cultured embryonic myoblasts upon stimulation of Notch and PDGF signalling (Cappellari et al., 2013). Further details on the observation of fate plasticity involving skeletal muscle pericytes are detailed in the next paragraph.

### 1.6.2. Muscle pericytes and satellite cells fate plasticity

An interesting aspect outlines the close relationship between satellite cells and pericytes and the possible fate plasticity across these two lineages. In a lineage tracing study, in which TnAP-positive pericytes were labelled with LacZ through a Tamoxifen-mediated Cre recombinase system, these cells were shown to contribute to the satellite niche, generating Pax7 positive cells during growth and regeneration (Dellavalle et al., 2011).

***Figure 12. Generation of satellite cells from AP positive pericytes during postnatal growth.*** *Immunohistochemistry on pectoralis muscles explanted from TnAP-CreERT2:R26R mice, one week (a) and one month (b) after Tamoxifen-mediated Cre-recombination. The blue staining has been obtained via LacZ enzymatic reaction and labels the TnAP pericyte-derived cells; in brown are highlighted the Pax7 positive satellite cells. The insets show high magnification images, in which is possible to observe pericytes (blue arrows), endogenous satellite cells (brown arrows) and pericyte-derived satellite cells (green arrows). Scale bar 100 μm. Adapted from Dellavalle et al. (2011).*

In line with these results, b-galactosidase-labelled mesoangioblasts, genetically corrected with a human artificial chromosome containing the whole dystrophin locus, have been shown to generate Pax7 positive satellite cells when transplanted in dystrophic animals. These cells correctly localised underneath the myofibre basal lamina, replenishing the satellite cell niche (Tedesco et al., 2011).

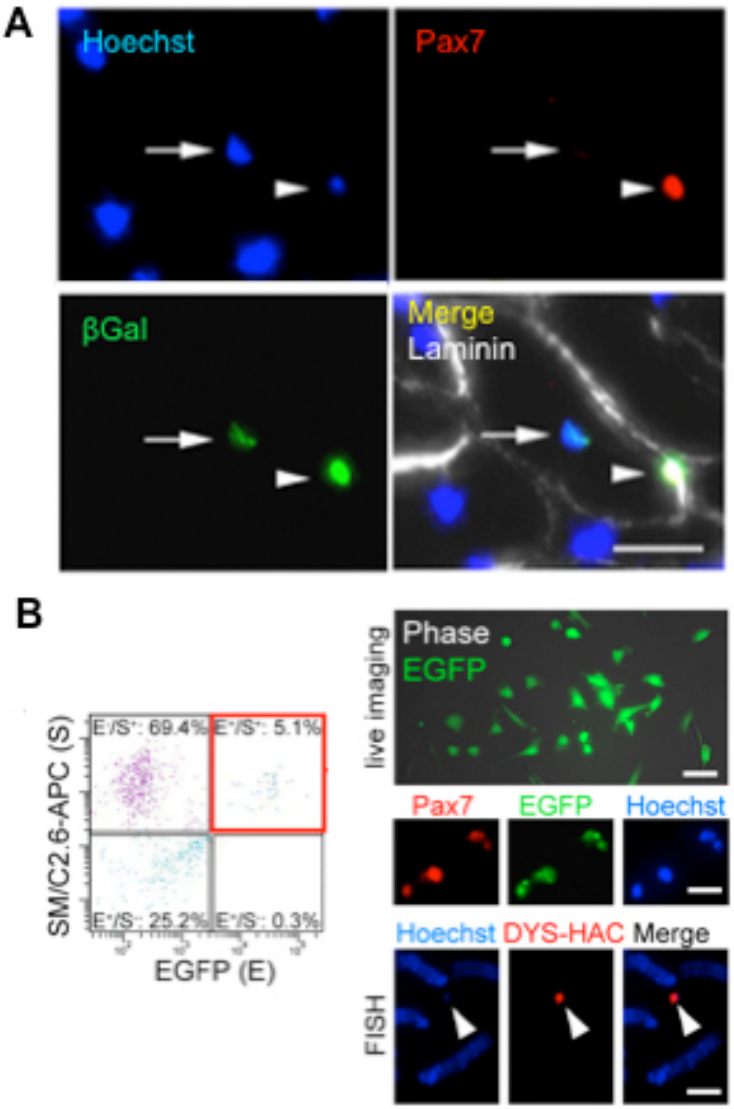



***Figure 13. Genetically corrected mesoangioblasts engraft the satellite cells niche upon transplantation. A.*** *Immunofluorescence staining showing donor derived βGal labelled cells in a transplanted scid / mdx muscle. The arrow shows a donor nucleus integrated in a skeletal muscle fibre. The arrowhead points to a donor-derived cell, located in the satellite cell position, underneath the muscle fibre basal lamina, expressing the satellite cell marker Pax7 (scale bar 100 μm).* ***B.*** *The left panel shows the FACS sorting plot obtained upon staining of a digested transplanted muscle with SM/C-2.6 to isolate the donor derived satellite cell fraction (gate outlined in red). Right panel shows a confirmation of the donor origin with the expression of EGFP from the sorted cells in live imaging (top). Central panel shows the*

*immunofluorescence that confirms the expression of Pax7 co-localised with the EGFP. The bottom part of the panel shows a FISH confirming the presence of the human artificial chromosome in the isolated cells (scale bars 50 µm, 50 µm, 4 µm). Adapted from (Tedesco et al., 2011).*

A recent publication from Cappellari and colleagues indicated for the first time that primary embryonic myoblasts could trans-differentiate to pericytes, indicating possible bi-directional lineage plasticity. Among the pathways that regulate this phenomenon in the embryo, this work highlighted that the Notch cascade, interplaying with PDGF signalling is of key importance. When primary embryonic myoblasts were exposed to Notch ligands and PDGF, known to regulate the inhibition of myogenesis and pericyte recruitment, these cells activated pericyte markers and started showing perivascular cell properties (Cappellari et al 2013).

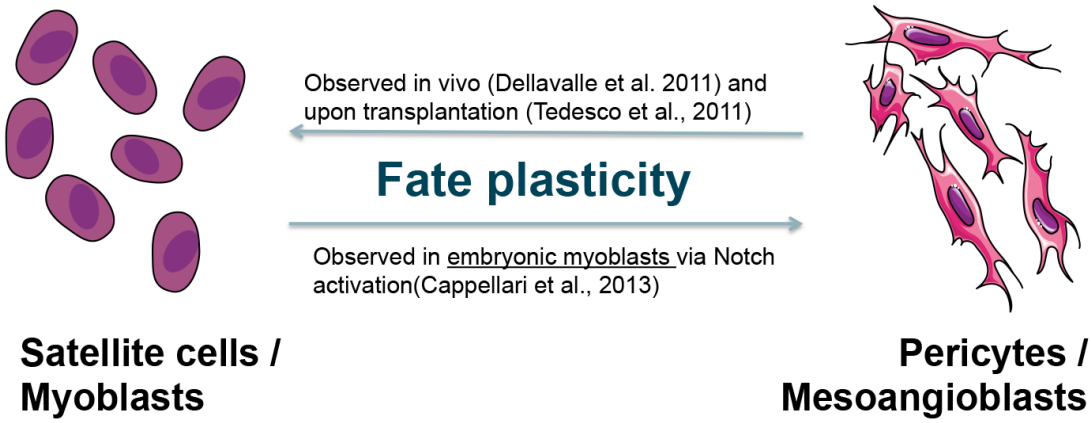



***Figure 14. Reported evidence of fate plasticity between satellite cells / myoblasts and pericytes / mesoangioblasts.***

It is therefore conceivable that the mechanisms of trans-determination (or fate plasticity) seen in the embryo might be preserved in the adult. Possible lineage plasticity between satellite cells and other myogenic progenitors, such as pericytes, still needs to be understood and the mechanisms driving this phenomenon to be elucidated.

## 1.7. Notch signalling

Notch signalling is an evolutionary conserved cell fate regulator that plays essential roles in the development and homeostasis of potentially all the tissues of the body (Artavanis-Tsakonas et al., 1999; Kimble and Simpson, 1997). In mammals four Notch heterodimers have been identified: Notch1, Notch2, Notch3 and Notch4. These receptors are large trans-membrane proteins. The extracellular domain of all the Notch receptors contains up to 36 EGF-like domains repeated in tandem. These domains mediate the dimerisation process and in particular the domains 11 and 12 are crucial to inhibit cis-activation, when a Notch ligand is expressed on the same cell membrane (Cordle et al., 2008). Five molecules have been recognised to act as Notch ligands: Delta-like ligand 1, 3, 4, Jagged 1 and Jagged 2. Because all these Notch ligands are trans-membrane proteins, cell-to-cell interaction is normally required to achieve an efficient signal transduction (Gerhardt and Betsholtz, 2003).

When the ligand-receptor interaction occurs, it causes a proteolytic cleavage operated by γ-secretase. As a result, a fragment of the Notch protein known as Notch intracellular domain (NICD) is released in the cytosol and subsequently translocate within the nucleus. Once in the nucleus, NICD interacts with DNA-binding proteins (such as the transcription factor RBP-J), which positively and negatively regulates the transcription of a wide range of downstream targets (Gridley, 2007).

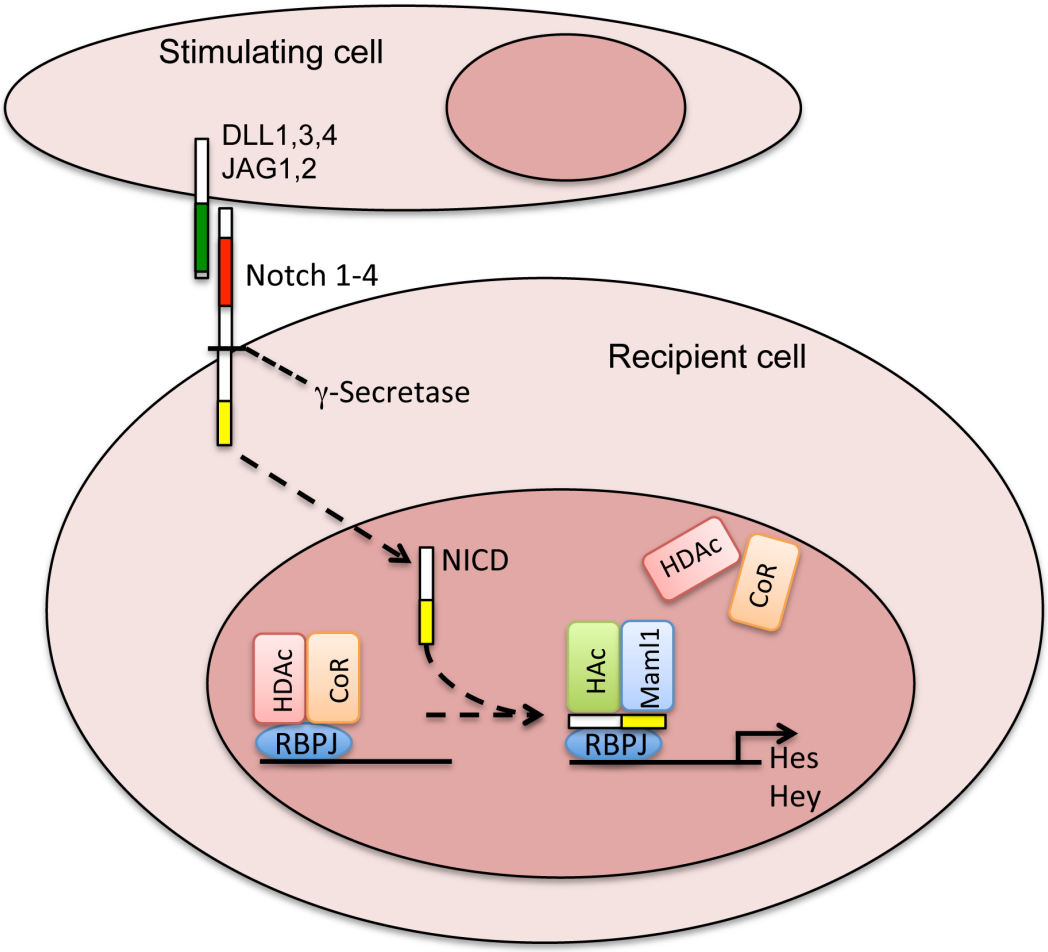



***Figure 15. Core components of the canonical Notch signalling pathway***. *The Notch ligands (DLL1, DLL3, DLL4, JAG1 and JAG2), present on the stimulating cell interact with the Notch family receptors (NOTCH1,2,3 and 4) of the recipient cell. The intracellular domain of the Notch receptor (NICD) undergoes a proteolytic cleavage operated by the γ-secretase and it is released in the cytosol. Then, the NICD translocate to the nucleus and forms a complex with the RBPJ protein. This is done displacing a histone deacetylase (HDAc)-co-repressor (CoR) complex from the RBPJ. Then, an activation complex, such as MAML1 and histone acetyltransferases (HAc), its recruited to the NICD-RBPJ complex, leading to the transcriptional activation of Notch target genes.*

It has been reported that Notch has a direct role as an initiator of the fate choice during asymmetric cell division. During this process both Notch ligands and receptors can be internalised into specific endosomes that are then differentially distributed in the daughter cells, directly acting as a fate determinant (Coumailleau et al., 2009). The Notch cascade is tightly modulated at various developmental stages by miRNA regulation, endosome trafficking and transcriptional target selection. Moreover, Notch cross talk with other signalling cascades such as BMP and Wnt maintains a delicate signalling homeostasis, which is still under investigation (Shin et al., 2009).

### 1.7.1. Roles of notch in the vasculature

Notch activity regulates embryonic vascular development, angiogenesis, adult vasculogenesis and arterial-venous specification (Hofmann and Iruela-Arispe, 2007; Regan and Majesky, 2009). Cell-to-cell interactions between pericytes and endothelial cells have been demonstrated to be sufficient to induce Notch3 up-regulation both in pericytes and vascular smooth muscle cells (Liu et al., 2009). Consistent with this, Notch3 has been reported to play a crucial role in pericyte and vascular smooth muscle cell survival (Liu et al., 2010; Walshe et al., 2011), in particular upon Delta-like ligand 4-mediated stimulation (Stewart et al., 2011). Indeed, mice with specific endothelial ablation of RBP-J showed impaired pericyte adhesion, reduced pericyte coverage of the blood vessels and a high frequency of perinatal haemorrhagic

events, confirming that Notch signalling has role in pericyte survival and cell-adhesion to the endothelial cells (Li et al., 2011; Winkler et al., 2011b).

### 1.7.2. Notch in skeletal muscle development and homeostasis

Notch signalling also plays a major role during skeletal muscle differentiation, from the early stages of the myogenic specification in the embryo through to involvement in postnatal muscle regeneration. During development, oscillatory waves of Notch signalling contribute in defining the antero-posterior patterning of somites (Lewis et al., 2009). Active Notch signalling has long been known to suppress myogenic differentiation. This has been observed in the murine myoblast cell line C2C12 (Kopan et al., 1994; Lindsell et al., 1995; Nofziger et al., 1999) and in primary satellite cells (Wen et al., 2012) where it acts with various mechanisms among which repression of the myogenic differentiation gene MyoD is one of the most studied (Buas et al., 2010; Hirsinger et al., 2001). In adult muscle, Notch regulates satellite cell activation during quiescence, niche colonisation and myogenic differentiation (Brohl et al., 2012; Conboy and Rando, 2002; Delfini et al., 2000; Mourikis et al., 2012; Mourikis and Tajbakhsh, 2014).

Notch signalling is crucial for normal developmental myogenesis. Indeed, animals with induced mutations in Notch Delta ligand 1 (DLL1) or in its downstream transcriptional mediator Rbpj present hypotrophic muscles and lack myogenic progenitors. Depletion of Notch signalling leads to a

premature exhaustion of the satellite cell pool, leading to an impairment of muscle regeneration (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). On the other hand repeated injuries in animals carrying mutations of Notch3 lead to muscle hypertrophy and to an increase in the number of muscle satellite cells indicating the presence of compensatory mechanisms (Kitamoto and Hanaoka, 2010).

Intramuscular injections of a Notch1 antibody activated Notch signalling, allowing the rescue of regenerative defects observed in aged muscles (Conboy et al., 2003) while sustaining Notch activation (via Delta Ligand 1; DLL1) in canine satellite cells during *in vitro* expansion improves their engraftment ability by promoting quiescence (Parker et al., 2012a). A recent report from Quattrocelli and colleagues demonstrated that the Notch cascade regulates also mesoangioblasts activity. DLL1-mediated notch depletion lead indeed to a reduction in mesoangioblasts engraftment ability while its activation has an opposite effect (Quattrocelli et al., 2014).

Notably, the above mentioned publication by Cappellari et al. showed that embryonic myoblasts undergo a fate switch towards the perivascular lineage upon DLL4 and PDGF-BB stimulation, implying that bidirectional plasticity between these two cell types is present in the embryo (Cappellari et al., 2013). The interplay between Notch and PDGF, and the mechanisms and roles of PDGF and its receptors, relevant for this project, are detailed in the next section.

## 1.8. PDGF signalling

Platelet derived growth factor (PDGF) was one of the first growth factors identified (Paul et al., 1971). This molecule mediates cell proliferation and plays a significant role during the angiogenesis, where it acts as a potent mitogen on fibroblast, smooth muscle and other cell types (Heldin, 1992). The PDGF family is divided in PDGF-A and PDGF-B according to the composition of their polypeptide chains. These chains are formed of a series of 100 amino acid residues with a 60% homology between the two types (Heldin and Westermark, 1999). Part of this chain is similar to the ones observed in the VEGF family (Joukov et al., 1997). PDGF-A and B associate in hetero- and homo-dimers (i.e. PDGF-AA, PDGF-BB, PDGF-AB) able to bind to three dimeric receptors: PDGFrα, PDGFrαβ and PDGFrβ. PDGF-AA has been reported active only on α-receptors, AB works on both α- and αβ-receptors while PDGF-BB is effective on all the three receptors (Hammacher et al., 1989; Kanakaraj et al., 1991; Seifert et al., 1989). These receptors are localised on the cell membrane and when activated show a tyrosine kinase activity (Matsui et al., 1989). Different factors have been reported to regulate isoform specific PDGF signals. IL1α, TNFα and basic-FGF for example specifically upregulate the receptors binding PDGF-AA (Bonner et al., 1996; Centrella et al., 1992), while IL1 and TGFβ induce a downregulation of the beta receptors (Bonner et al., 1995; Xie et al., 1994).

Ligand-receptor interaction activates PDGF signalling transduction. The ligand induces a conformational change that starts the intracellular signal transduction, mediated by proteins that dock to the tyrosine kinase domain upon via SH2/SH3 domains. This activation mediates the one of different signalling transduction pathways involving PI3-kinases, Phospholipase C, Src, GRB2, GTPases activating protein and Stat3 (Darnell, 1997; Erpel and Courtneidge, 1995; Heidaran et al., 1993; Hu et al., 1995; Kamat and Carpenter, 1997; Vanhaesebroeck et al., 1997). It is difficult to dissect the precise role of all these different effectors, however the main ascribed to these signalling pathways have been linked to cell division, actin reorganisation, chemotaxis and to the Akt/PKB anti apoptotic effect (Heldin and Westermark, 1999).

In addition to its mitogen activity, PDGF has a role in tissue remodelling, cell migration and differentiation (Hoch and Soriano, 2003). PDGF is considered a required element for the regulation of fibroblast proliferation, in particular during the wound healing process (Pierce et al., 1991). For this reason, PDGF overexpression has been linked to the development of different diseases, among which are atherosclerosis and fibrotic disorders (Abboud, 1995; Trojanowska, 2008).

PDGF-B and its receptor PDGFrβ are essential for pericyte recruitment in the CNS, kidneys and for the cardiovascular system (Leveen et al., 1994; Lindahl et al., 1997; Tallquist et al., 2003). During vessel growth, endothelial cells directly recruit mesodermal progenitors to the pericyte fate by secreting PDGF-BB (Hellstrom et al., 1999). In the embryo, Notch signalling works

together with PDGF-BB in driving fate choice between the smooth and skeletal muscle lineages, where it induces an up-regulation of PDGFrβ, leading to an increased cell response to PDGF-BB stimulation (Jin et al., 2008). Importantly, it has been recently shown that a combinatory treatment with DLL4 and PDGF-BB induces a lineage switch in embryonic myoblasts towards the pericyte lineage (Cappellari et al., 2013). These results set the basis for the hypothesis behind this thesis.

## 1.9. Other pathways involved in skeletal muscle regeneration and recruitment of mesodermal progenitors to the pericyte fate.

Several signalling pathways regulate recruitment of mesodermal progenitors to the pericyte fate. In particular, tissue culture studies have shown that the cytokine TGFβ1, in combination with PDGF, is involved in the recruitment of mesenchymal precursors to the smooth muscle lineage (Hellstrom et al., 1999; Hirschi et al., 1998). Moreover, TGFβ1 has been shown to induce vessel maturation, inhibiting endothelial cell proliferation and migration (Sato et al., 1990). Noggin, expressed from the newly formed myofibres in the embryo, acts in contrast with BMP 2 / 4 produced from the vessels (i.e. aorta), to play a fundamental role in mesodermal progenitors recruitment to the muscle fate (Ugarte et al., 2012). The progression from commitment to terminal differentiation of satellite cells relies on a temporal switch from Notch to Wnt signalling (Tsivitse, 2010). The crosstalk between these two pathways involves the activity of Gsk3β, that maintains Notch signalling by inhibiting the Wnt3a cascade (Brack et al., 2008). Notch has also been linked to TNF signalling. DMD patients show increased levels of TNF-α, which has also been proposed as a novel marker of the disease (Abdel-Salam et al., 2009). This signalling pathway has been shown to down-regulate Notch1 mRNA levels and inhibit the self-renewal capacity of cultured murine myoblasts (Acharyya et al., 2010).

## 1.10. Stem cell self-renewal

Self-renewal is defined as a series of cell division cycles that repeatedly generate at least one daughter cell equivalent to the mother with latent capacity for differentiation. This concept is considered to be a defining property of a stem cell (Smith, 2006).

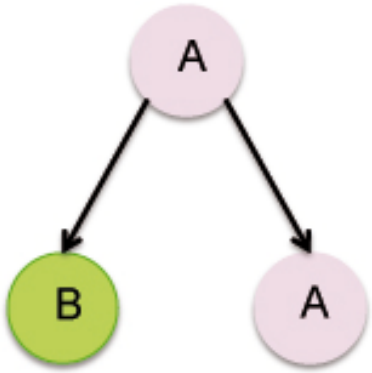



***Figure 16. Schematic representation of the meaning of self-renewal.*** *The "stem cell" A undergoes a cycle of asymmetric cell division that generates two daughter cells. Of these two cells, A is identical to the mother and will replenish the stem cell pool while B represents a more committed progenitor*.

In light of this definition, self-renewal is a phenomenon strictly linked to another fundamental concept of stem cell biology: asymmetric cell division (Figure 16). This property defines a cell able to generate progenies with distinct fate from a single mitosis (Jan and Jan, 1998). This oriented cell division may position daughter cells in different microenvironments (or anatomical compartments). Intrinsic determinants (i.e. transcription factors) may also be segregated into only one daughter cell. This property is observed in some, but not all stem cells and can occur in other types of progenitors

(Smith, 2006). Indications of self-renewal have been observed in different tissues including intestine, epidermis and testis (Simons and Clevers, 2011).

Serial transplantation of clonal stem cell populations is recognised as the most stringent assay to study stem / progenitor cell regenerative potential. This assay provides a thorough outcome for the investigation of the self-renewal capacity of a stem cell population. Serial transplantation assays have been utilised to prove the self-renewal potential of hematopoietic, skeletal and epidermal stem cells (Blanpain et al., 2004; Claudinot et al., 2005; Sacchetti et al., 2007) In the skeletal muscle field there is increasing evidence indicating that satellite cells retain self-renewal potential, together with other stem cell properties, such as asymmetric distribution of cell fate determinants and DNA strands (Kuang et al., 2007; Shinin et al., 2006). Recently, satellite cells have been formally proved capable of self-renewal with a serial transplantation assay (Rocheteau et al., 2012).

Various methods have been proposed aiming to isolate satellite cells via flow cytometry (Conboy et al., 2010; Motohashi et al., 2014; Pasut et al., 2012). The group of Sinhichi Takeda, reported in 2004 the possibility of isolating quiescent (i.e. non-committed and non-proliferating) satellite cells from digested adult skeletal muscle via fluorescence-activated cell sorting (FACS), using a monoclonal antibody against the surface antigen SM/C-2.6 (Fukada et al., 2004; Fukada et al., 2007; Ikemoto et al., 2007). Utilising this antibody, it has been possible to isolate donor-derived satellite cells from muscles transplanted with mesoangioblasts (Tedesco et al., 2011).

Although pericytes can give origin to Pax7-positive satellite cells during development (Dellavalle et al., 2011), whether myogenic progenitor different from satellite cells (i.e. mesoangioblasts) could generate myogenic progenitors capable of self-renewal still needs to be rigorously elucidated. Understanding the self-renewal potential of mesoangioblasts and their progeny is particularly relevant considering the role that self-renewal has in the long-lasting effectiveness of cell therapy protocols that exploit this cell type.

# CHAPTER 2:

# 2. OBJECTIVES OF THE RESEARCH

## Aim 1: Investigation of satellite cell fate plasticity

The main aim of my PhD project has been to investigate the fate plasticity of adult muscle satellite cells, focussing on the possibility to directly reprogram these cells towards the pericyte lineage.

Indications of fate plasticity between these two lineages have been reported in the literature. In particular, pericytes and mesoangioblasts have been shown to contribute to the satellite cell niche during growth, tissue regeneration and upon transplantation (Dellavalle et al., 2011; Tedesco et al., 2011). On the other hand, the only available evidence that this plasticity could be bidirectional is restricted to primary embryonic myoblasts stimulated in culture with a combination of cytokines. Upon treatment, these cells were able to activate pericyte genes and acquire rudimental pericyte features, providing the first evidence in this direction (Cappellari et al., 2013). Our hypothesis is that primary adult satellite cells and myoblasts may retain a level of plasticity similar to the one observed in the embryo.

The recruitment of adult cells to the perivascular fate and the idea that these cells might act as resident progenitors in various tissues has been hypothesised and indicated in some past work (Abou-Khalil et al., 2010; Bianco and Cossu, 1999; Cossu and Bianco, 2003; Minasi et al., 2002).

Notch and PDGF signalling have been shown to have a crucial role both in vascular homeostasis and in skeletal muscle biology, where cross-talk between these pathways regulates tissue regeneration, stem cells quiescence and activation. Delta-like ligand 4 is a Notch signalling stimulating molecule that is physiologically produced by the endothelial cells during vessel formation. In combination with a PDGF-B dimer (PDGF-BB), DLL4 is involved in the recruitment of mesodermal cells to the pericyte fate during vessel formation (Hellstrom et al., 1999; Stewart et al., 2011). Nevertheless, whether this process is active in adult skeletal muscle homeostasis and regeneration still needs to be elucidated.

Therefore it is conceivable that treating adult muscle satellite cells with DLL4 and PDGF-BB could induce in these cells a direct reprogramming to the pericyte-like fate. To our knowledge, at the time of this thesis, there are no examples of this in the literature.

If true, this work will provide indications of bi-directional fate plasticity between these two lineages, and insights into skeletal muscle homeostasis and regeneration. Importantly, the possibility of skeletal-to-smooth muscle lineage reprogramming could also be exploited to give to the cells beneficial properties for cell therapy purposes. For example, treated myoblasts might acquire the ability to cross the blood vessel wall upon systemic delivery (a particular property of pericyte-derived mesoangioblasts), while retaining the remarkable myogenic memory that characterises satellite cells and myoblasts.

The cellular response to the treatment has been characterised firstly by analysing phenotypic variations in terms of morphology, activation of pericyte markers, proliferation and the ability to undergo myogenic differentiation. The gene expression profile of the cells will then be assessed via quantitative real time PCR (qRT-PCR) analyses, to investigate variations in the genes targeted by the stimulation as in satellite cell and pericyte markers. Treated cells will then be tested for functional pericyte properties *in vitro*, investigating their ability to support endothelial network formation and stabilisation. Additional tests will include the assessment of the cell migration ability toward an endothelial cell layer *in vitro*. Tumour formation assays will ensure that the treatment does not alter the "non-transformed" state of the cells. Transplantation experiments will be performed with the aim of assessing the engraftment potential of these cells. Moreover, proof of principle experiments will be also performed, aiming to understand if reprogrammed cells gain the ability to migrate through the vessel wall *in vivo*.

If translated to human cells, this direct reprogramming mechanism may have the potential to produce a more clinically relevant stem cell product that shares both the beneficial properties of both satellite cells and pericytes. This might have a significant impact for the development of novel cell therapy strategies aiming to treat muscular dystrophies.

## Aim 2: Understanding the self-renewal potential of mesoangioblast-derived cells.

Previous observations showed that pericytes and mesoangioblast derivatives are capable of repopulating the satellite cell niche, generating Pax7 positive cells that correctly localise underneath the myofibres basal lamina during muscle regeneration and upon transplantation (Dellavalle et al., 2011; Tedesco et al., 2011). Tajbakhsh and co-workers have recently provided a formal demonstration of the long-term self-renewal capacity of satellite cells, combining lineage tracing and label retaining assays in an elegant serial transplantation approach (Rocheteau et al., 2012).

Whether pericyte-derived cells are able to self-renew as demonstrated for bona-fide muscle stem cells still need to be elucidated. A series of experiments performed in our laboratory have indicated that transplanted mesoangioblasts are able to sustain long-term engraftment (up to 8 months) and a subsequent round of regeneration upon acute myoinjury. This publication also showed the possibility of isolating donor-derived satellite-like cells from the transplanted muscles (Tedesco et al., 2011). Taken together, these data provided preliminarily indications on the self-renewal ability of pericytes / mesoangioblast-derived cells *in vivo*. However, this phenomenon needs to be formally proved via serial transplantation assays.

Based upon the above observations, our hypothesis is that upon transplantation, mesoangioblasts are able to generate cells that are capable of self-renewal.

To this aim, we performed a series of serial transplantation experiments to assess the self-renewal of these mesoangioblast-derived cells *in vivo*. Intramuscular injection was chosen over intra-arterial delivery for feasibility reason, given the complexity of the microsurgical technique required to transplant cells intra-arterially in mice (Gerli et al., 2014). Transplanted muscles will be subject to a mechanical and enzymatic digestion protocol implemented to isolate mononuclear cells from transplanted skeletal muscles. Donor derived cells, will be then subjected to FACS sorting purification exploiting also a monoclonal antibody (SM/C-2.6) reported to allow the separation of satellite from non-satellite cells (Fukada et al., 2004). Isolated cells will then be serially transplanted to investigate their potential *in vivo (Figure 17)*.

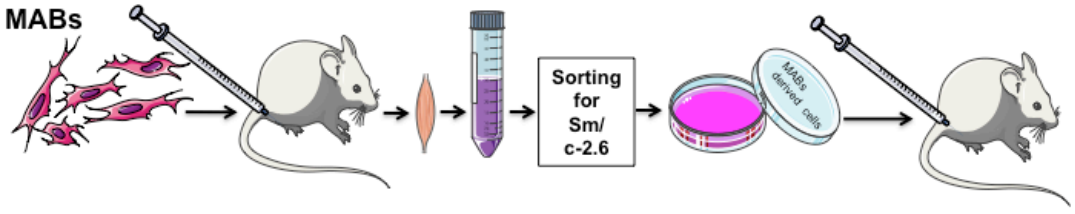



***Figure 17. Schematic representation of the serial transplantation strategy.*** *Mesoangioblasts (MABs) will be transplanted intramuscularly in dystrophic mice. One month after transplantation, muscles are harvested and digested. The single cell suspension obtained will be FACS-sorted for SM/C-*

*2.6 to identify and isolate mesoangioblast-derived satellite cells, which are subsequently cultured and serially transplanted intramuscularly into a new recipient (Illustrations obtained from Servier Medical Art).*

# CHAPTER 3:

# 3. MATERIALS AND METHODS

## 3.1. Cell isolation and culture

Primary satellite cells were isolated from adult muscles, mechanically fragmented and digested twice in an HBSS solution containing Collagenase D (0.1%; Roche, Switzerland; 11088882001), Dispase (0.24U / ml; Gibco, USA; 17105-041) and DNAse (0.1mg / ml; Roche, Switzerland; 11284932001) for 20 minutes at 37°C in a shaking waterbath. The digestion product was filtered through a 40 µm strainer (Corning, USA; 352340) and enzymatic activity was inhibited with 20% of Foetal Bovine Serum (FBS; Gibco, USA; 10270-106). The cell suspension was then centrifuged for 30' at 1100rpm in a centrifuge, pre-cooled to 4° C. The pellet was washed twice in Phosphate-Buffered Saline (PBS, Gibco, USA; 10010015), then re-suspended in culture medium and pre-plated on cell culture plastic and incubated for one hour at 37° C 5% $CO_2$ to remove eventual contaminant fibroblasts and enrich the floating fraction of satellite cells, typically low adherent on uncoated cell culture plastic. The supernatant containing the satellite cells was then collected and transferred to collagen-coated dishes (Sigma-Aldrich, USA; C8919) at a density of $1.2x10^3$ cells / $cm^3$. The cells were then cultured at low density in an ad-hoc defined satellite cell media (DMEM-F / 12, Gibco, USA, 11039-21; 10% Horse Serum, Euroclone, Italy, ECS0090L; 20% FBS and 5ng / ml basic-fibroblast growth factor, 5ng / ml b-FGF, Gibco, USA, AA10-155; 1%

penicillin / streptomycin / gentamicin). Mesoangioblasts / Pericytes utilised in this study were described, isolated and maintained in culture in DMEM (GIBCO, USA; 21063-029) containing 20% FBS as reported previously (Dellavalle et al., 2011; Tedesco et al., 2011; Tonlorenzi et al., 2007).

## 3.2. Fluorescence-activated cell sorting purification

Fluorescence activated cell-sorting purification of the Pax7nGFP (satellite cells), TnAP-YFP (freshly isolated pericytes) positive cells from bulk preparations were performed with a FACS Aria III (Becton Dickinson, USA) at the Institute of Child Health flow cytometry facility. When donor-derived satellite-like cells have been re-isolated from transplanted muscles (i.e. for the self-renewal project) these have been labelled using a biotinylated SM/C-2.6 monoclonal antibody (kindly provided by Prof. Shin'ichi Takeda, National Centre of Neurology and Psychiatry, Kodaira, Tokyo, Japan), as previously described (Fukada et al., 2004; Tedesco et al., 2011). After two washes in PBS (Gibco, USA; 10010015) containing 1% FBS and 0.5 mM EDTA (Gibco, USA; 15575-020), freshly digested cell suspensions were incubated with the SM/C-2.6 primary antibody, washed in PBS and incubated with the appropriate APC, PE or PeCy7-conjugated streptavidin. Cell sorting has been performed with a MoFlow (Beckman coulter, USA) or FACS Aria III (Becton Dickinson, USA) sorter.

## 3.3. DLL4 and PDGF-BB treatment

Murine recombinant delta-like ligand 4 (DLL4; R&D, USA: 1389-D4) was resuspended to a final concentration 10 µg / ml in sterile PBS containing 0.1%

wt / vol Bovine serum albumin (BSA; Sigma-Aldrich, USA; A9418-10G) as carrier protein. To induce reprogramming, standard cell culture plastic flasks were coated with the DLL4 solution and incubated at 37° C for 45 minutes prior use. The cells were then seeded on the coated flasks and the cultures were then supplemented with 50ng / ml of PDGF-BB (Sigma-Aldrich, USA; P4056) daily for the first week of treatment, then every other day together with medium changes. One week was considered the minimum duration of the treatment.

## 3.4. Myogenic differentiation assays

Myogenic differentiation assays were performed seeding the cells at a density of $1.2x10^4/cm^2$ as previously described (Tedesco et al., 2011). When confluecy was reached, the cultures were switched to a differentiation media (DMEM 2% Horse serum) replaced every other day for one week. For the γ-secretase inhibitor supplementation experiments, L-685,458 (Sigma-Aldrich, USA; L1790) has been added to the cultures in proliferation media one day before starting the differentiation protocol and then every other day together with the differentiation media changes as previously reported (Cappellari et al., 2013). The dishes were then fixed with 4% Paraformaldehyde (PFA; TAAB Laboratories Equipment, England; P001) for 5 minutes, washed in PBS and stored covered in PBS and refrigerated at 4°C until use for immunofluorescence analysis. If the cells already had a fluorescence reporter, the exposure to light was minimised by covering the samples with aluminium foil.

## 3.5. Immunofluorescence and enzymatic reaction staining

Cells and tissues were processed as previously reported (Tedesco et al., 2012). Firstly a 15 minutes permeabilisation in a solution of PBS containing 0.1% Triton (Sigma-Aldrich, USA; T8787) and 1% bovine serum albumin (BSA) was performed. Next, the non-specific binding sites were blocked by incubating the samples in PBS containing 0.1% Triton and 1% BSA, supplemented with 10% goat or donkey serum (Jackson ImmunoResearch Laboratories, USA, 005-000-121; Sigma-Aldrich, USA, D9663-10ML) for 30 minutes. The samples were then incubated overnight at 4° C with the following primary antibodies: mouse anti-myosin heavy chain (Developmental Studies Hybridoma Bank, USA; MF20), CD31 (Developmental Studies Hybridoma Bank, USA; PECAM), Pax7 (Developmental Studies Hybridoma Bank, USA; Pax7), mouse anti alpha sarcoglycan (Novocastra, UK; NCL-a-SARC); mouse anti-dystrophin Dys1 and Dys2 (Novocastra, UK; NCL-DYS1 and NCL-DYS2); mouse anti-MyoD1 (Dako, Denmark; M3512), rabbit anti-EGFP (Molecular Probes, USA; A-11122), chicken anti-EGFP (Millipore, Germany; AB16901), chicken anti-Laminin (Abcam, England; ab14055). To minimise the evaporation of the primary antibodies solution a humid environment was created placing a wet tissue in the box utilised for the staining. Following this, samples were washed twice in PBS. The samples were then incubated in the dark for 60 minutes at room temperature with the following secondary antibodies diluted 1:500 in PBS: Donkey and Goat IgG were used as secondary antibodies (Molecular Probes, USA; Alexa Fluor series), Hoechst 33342 (Sigma-Aldrich, USA; B2261) was used to highlight

the nuclei. Before use, secondary antibody solutions were centrifuged for 5 minutes at 12000 RPM to eliminate eventual antibody clusters, avoiding collecting the precipitate at the bottom of the tubes. After the incubation and two PBS washes, coverglasses were mounted using fluorescent mounting medium (DAKO, Denmark; S302380). The samples were stored at 4°C minimising exposure to light until use. The imaging of the stained samples was performed using Leica DM and DMI 6000 optical fluorescent microscopes, equipped with 405, 488, 546 and 647 filters. Alkaline phosphatase stainings were performed on fixed cells and tissue preparing the staining solution following the standard procedures available in the manufacturer kits (Roche, Switzerland, NBT / BCIP 11681451001). X-gal staining was performed following the standard manufacturer procedures (Invitrogen, USA, B-1690). The samples were then incubated with the staining solution for one hour at 37° C, washed in PBS and counterstained as required.

## 3.6. Quantitative real time PCR and gene expression analyses

RNA extraction was performed with RNeasy kits (Qiagen, Germany; 74004), quantified with a Nanodrop 2000 (Thermo scientific) and reverse-transcription with Improm RT kit (Promega, USA; A3800) using a BioRad T100 thermocycler and following the standard manufacturers procedure. Samples were then processed for quantitative real time PCR using Real Time Master Mix (Promega, USA; A600A) on a BioRad CFX96 system. Annealing temperatures were adjusted to be optimal for each pair of primers (See Table

2 for details on the primers). qRT-PCRs were performed in triplicate on samples obtained from three independent experiments (with the exception of the one performed on purified Pax7nGFP cells for which a single cell preparation was available). GAPDH was used as normaliser gene for each run. Amplicons were then resolved by electrophoresis on a 2% agarose gel to confirm the fragment length. RNA extraction and amplification reactions have been performed in compliance with the MIQE guidelines (Bustin et al., 2013). Data analysis was performed with Microsoft Excel and GraphPad Prism 6 using a standard ΔΔCt / fold increase method (Schmittgen and Livak, 2008). Data were presented as mean ± standard deviation (SD). Significance was assessed on the ΔCt values using Student's t-test assuming two-tailed distribution and equal variances. Large standard deviations were observed trough the independent biological replicates. Therefore, to increase the power of the statistical analysis a Two-way ANOVA (using Fisher LSD uncorrected multiple) and a multiple regression analysis on the fold transformations have been implemented (Yuan et al., 2006).

| **Gene** | **Sequence (5'->3')** | **Annealing temperature (°C)** |
|---|---|---|
| **Notch1 FW** | TGGACGCCGCTGTGAGTCA | 55 |
| **Notch1 REV** | TGGGCCCGAGATGCATGTA | 55 |
| **Hesl FW** | ACACCGGACAAACCAAAGAC | 60 |
| **Hesl REV** | AATGCCGGGAGCTATCTTTC | 60 |
| **Hey1 FW** | CACCTGAAAATGCTGCACAC | 60 |

| | | |
|---|---|---|
| **Hey1 REV** | ATGCTCAGATAACGGGCAAC | 60 |
| **Pax7 FW** | ATGTTCAGCTGGGAAATCCGGG | 60 |
| **Pax7 REV** | TCCCGAACTTGATTCTGAGCACTCG | 60 |
| **MyoD FW** | GCCCGCGCTCCAACTGCTCTGAT | 60 |
| **MyoD REV** | CCTACGGTGGTGCGCCCTCTGC | 60 |
| **PDGFrβ FW** | GCTCACGGTCTGAGCCATTC | 60 |
| **PDGFrβ REV** | GCTCGGACATTAAGGCTTGCT | 60 |
| **TnAP FW** | GTGGATACACCCCCCGGGGC | 56 |
| **TnAP REV** | GGTCAAGGTTGGCCCCAATGCA | 56 |
| **SM22 FW** | CCAACAAGGGTCCATCCTACG | 60 |
| **SM22 REV** | ATCTGGGCGGCCTACATCA | 60 |
| **NG2 FW** | ACAAGCGTGGCAACTTTATC | 55 |
| **NG2 REV** | ATAGACCTCTTCTTCATATT | 55 |
| **GAPDH FW** | AGGTCGGTGTGAACGGATTTG | 60 |
| **GAPDH REV** | TGTAGACCATGTAGTTGAGGTCA | 60 |

***Table 2. List of the qRT-PCR primers utilised for this study.*** *Listed in the table the qRT-PCR primers utilised for this study with the optimum melting temperature calculated using PrimerBlast.*

## 3.7. Endothelial network formation assay

Endothelial network formation assays were carried out as previously described (Cappellari et al., 2013; Goodwin, 2007; Tedesco et al., 2012; You

et al., 2014). Primary human umbilical vein endothelial cells (HUVEC) were maintained in culture in EGM media (Lonza, Switzerland; CC-4133) on 1% gelatine-coated flasks (Sigma-Aldrich, USA; G9136). HUVECs were kept below 70% of confluence and used up to passage 7. For the network formation assays, cell culture dishes were coated with 25% of reduced-growth factors Matrigel resuspended in phenol red-free DMEM at 37°C for 30 minutes. HUVECs were then seeded on the Matrigel coated dishes at a density of $5x10^4$ cells / $cm^2$. After thirty minutes, the cells to be tested were added at a ratio of 1:10 to the HUVECs and the cultures were supplemented with 10 ng / ml of vascular endothelial growth factor (VEGF) (Sigma-Aldrich, USA; V7259) to stimulate the formation of the endothelial networks. Dishes were then monitored via fluorescent live imaging using a Leica DMI600b microscope equipped with a thermostatic chamber and an electronic CO2 regulator. The number of network branches / 20X field (0.31 $mm^2$) was quantified over time to assess the stability of the networks. The number of cells co-localised with the endothelial network branches was also quantified using image J.

## 3.8. *In vitro* migration assay

*In vitro* migration assay was performed using the H5V murine endothelial cell line (Garlanda et al., 1994). H5V were grown to full confluence on 1% gelatin-coated 8µm pore transwell membranes (BD Biosciences, USA; 353093). Confluence of the endothelial layers was assessed, by measuring the permeability of BSA with the Protein Assay Reagent Kit A-B-S (BioRad, USA) using an ELISA plate reader (iMark microplate reader; BioRad, USA). The

upper chamber of the transwell was loaded with the cells to be tested, re-suspended in a serum-free media. The lower chamber was loaded with a chemoattractant medium composed of 50% fresh growth media and 50% myoblasts conditioned media (previously exposed for 24 hours to differentiated C2C12 murine myoblasts to mimic the muscle environment). After six hours, the membranes were gently washed in PBS and fixed for 5 minutes in 4% PFA. The top of the membrane was scraped off with a cotton bud to remove the non-migrated cells. After an additional PBS wash, the membranes were mounted with fluorescent mounting media above glass microscope slides. The number of cells that migrated through the H5V endothelial layer was quantified by counting the number of fluorescent cells on the lower side of the membrane using an inverted microscope (Leica DMI6000B). A minimum of 30 random 20X field (0.31 $mm^2$) / condition was counted per each independent experiments.

## 3.9. Mice

All the animals used for this study were housed in specific pathogen free (SPF) conditions in clean ventilated racks at the UCL facility (London, United Kingdom). All procedures involving living animals have been approved by the UCL ethical committee and conformed to the Home Office regulations (ASPA 1986), PPL 70 / 7435 and PIL 70 / 24251. The use of animals has been rationalised following the Home Office guidelines and implementing the 3Rs (Reduction, Refinement, Replacement). Pax7-nGFP mice were bred at the Pasteur institute (Paris, France) complying the European legislation and under the approval of the institutional ethical committee.

## 3.10. Intramuscular cell transplantation

Three to five-month-old Sgca-null / scid / beige (Tedesco et al., 2012) and scid / mdx (Farini et al., 2007) immunodeficient dystrophic mice were used for the transplantation experiments listed in this thesis. After trypsinisation (Trypsin-EDTA solution, Sigma-Aldrich, USA; T3924), cells were washed twice in PBS to eliminate the residual FBS present in the media. The final cell preparation was then resuspended in $Ca^{++}$ / $Mg^{++}$ free PBS to a concentration of $5x10^5$ cells / 30ul. The skin of the animals was disinfected with a chlorexidine-based disinfectant. Injection was performed with a 30G needle syringe into tibialis anterior, gastrocnemius and quadriceps muscles. In detail For the tibialis anterior, 5 mm of the needle was inserted approximately 2 mm below the insertion of the proximal tendon (cranio-caudal direction) with a 15° inclination relative to the tibia and the cell suspension was slowly injected while retracting the needle emptying the syringe with 2 mm of the needle still inside the muscle. For the GC and QC, the same procedure was repeated as detailed for the tibialis anterior, with the main difference being the caudo-cranial insertion of the needle 2 mm above the myotendinous junction of the Achilles tendon for the GC and 2 mm above the distal tendon for the QC (15° inclination with respect to the femur). Further details on the intra-muscular cell transplantation procedure are reported in a protocol paper published during this PhD a copy of which is attached to this thesis (Gerli et al., 2014).

## 3.11. Intra-arterial cell transplantation

For intra-arterial transplantation, the mice were anesthetised using isofluorane (2 litres / min in $O_2$). Analgesia (Caprofen, Bayer) was administrated 40 minutes before the surgery to avoid to the animals further discomfort related to the surgical procedure. After trypsinisation (Trypsin-EDTA solution, Sigma-Aldrich, USA; T3924), cells were washed twice in PBS to eliminate the residual FBS present in the media and filtered through a 40um cell strainer to eliminate possible cell clusters from the cell suspension. The final cell preparation was then resuspended in $Ca^{++}$ / $Mg^{++}$ free PBS to a concentration of $5x10^5$ cells / 50ul. After having shaved and sanitised the skin of the inguinal region of the mice with a chlorexidine-based disinfectant, a 5 mm incision to access to the femoral artery was performed. The femoral bundle was exposed gently removing the connective tissue fascia overlaying it. The artery was separated from the femoral vein and nerve by gently introducing the tip of a forceps (or a 30 G needle) in between them and by progressively enlarging the hole. Transplantations were performed lifting the artery with one tip of a forceps and clamping it with the other tip. The artery was then punctured with a syringe equipped with a 30 G needle and injected with 50 ul of cell suspension, at a concentration of $2.5 \times 10^5$ cells / 50µl. The needle and the forceps were then gently removed from the artery to restore bloodstream in the limb. Pressure was applied with sterile gauze to avoid bleeding and/or cauterization was performed as required. The wounds were sutured and the animals monitored until recovery from anesthesia. Analgesia was administered for 3 additional days and the wound inspected daily for at

least one week. Further details on the intra-muscular cell transplantation procedure are reported in a protocol paper published during this PhD a copy of which is attached to this thesis (Gerli et al., 2014).

## 3.12. Tumour formation assay

Tumorigenic formation assays were performed injecting subcutaneously immunodeficient mice with 2 x $10^6$ cells washed in PBS as described above and resuspended in a volume of 100ul. After the injections, the animals were observed biweekly for a minimum of 2 months as a follow-up to verify the absence of tumour masses. At the end of the experiment, the animals were humanely culled and dissected to confirm the absence of tumour masses. An appropriate positive control for this experiment could be the subcutaneous injection of a tumorigenic cell line such as S180 murine sarcoma cells (ATCC, TIB-66). However, this control experiment has not been performed for this project, aiming to implement the 3Rs (reduction, refinement and replacement) taking in consideration that I have demonstrated to master this technique in a recently published article (Tedesco et al., 2012; Figure S1B).

## 3.13. Tissue explant and processing

At the suited time point, transplanted mice were humanely killed following the schedule 1 procedures approved by the UCL ethical committee and conformed to the Home Office regulations (ASPA 1986). Tissues were explanted applying aseptic techniques and removing the muscle fascia

(epimysium) with the forceps. Whenever the transplanted cells were labelled with a fluorescent protein, engraftment was assessed at first via direct fluorescent stereomicroscopy using an UV-equipped stereomicroscope (Leica Microsystems, Leica MZ10F). The tissue samples were then processed for cryopreservation as follow. 4% Tragachant gum was utilised as a sample holder for the inclusion. 1 g of gum was placed above a cork support. The tip of the muscle (possibly a tendon) was then embedded in the gum being careful that the myofibres were oriented vertically. The cork was then moved in a beaker containing 50 ml of isopentane pre-chilled in liquid nitrogen for one minute to achieve the snap-freezing of the specimen. The samples were then moved for two additional minute in liquid nitrogen to complete the freezing and then stored in a -80° C freezer until processing. Tissues were then cryosectioned with a cryostat (Leica biosystems, Leica CM1850) set to a temperature of -22°C and 7-10 µm thick sections were cut. Eight to ten replicate slides (25-40 sections / slide) were obtained from each tissue specimen. Slides were then placed into -80 C for long-term storage, and used for histological, immunohistochemistry and immunofluorescent stainings. For each sample, a series of sections have been collected into 1.5 ml tube and stored at -80° C to perform molecular biology assays.

# CHAPTER 4:

# 4. PROTOCOL DEVELOPMENT

This section aims outline my contribution to the development of protocols with relevance for the field and in light of the experiments detailed in this thesis. In particular the first section (4.1) details refinements introduced into the in vivo transplantation and surgical procedures aiming to reduce its impact on the animal's welfare. Section 4.2 details my contribution in the improvement and extension of a protocol to derive mesoangioblast-like cells from pluripotent stem cells. Although his protocol has marginal direct relevance in light of the work detailed in this thesis, this offers an alternative strategy to develop an efficacious cell therapy platform to treat muscular dystrophies. This paragraph has been included to underline that the way to achieve an effective treatment for these diseases is still long and my interest in these developments is not restricted to a single candidate technique.

## 4.1. Establishing and optimising the in vivo methodology of myogenic cell transplantation.

Together with Sara Martina Maffioletti, another PhD student from our laboratory and with the help and supervision of Francesco Saverio Tedesco, I have significantly contributed to the develop and improve of the available protocols and methodologies utilised to perform the in vivo experiments detailed in this thesis. The improvements have been introduced in the transplantation protocols, to allow fine tune of the transplantation and ameliorate the welfare of the animals utilised for the studies implementing the 3Rs (reduction, refinement and replacement).

The techniques utilised in this thesis included surgical procedures and cell transplantation in murine models of muscle regeneration and muscular dystrophy. These techniques require extensive training and supervision and for this reason, we decided to detail these methods in a protocol paper / video protocol highlighting the steps required for this approach (Gerli et al., 2014). This paper details the procedure of intramuscular and intra-arterial transplantation of myogenic progenitors, describing the steps required for the preparation of cultured cells, the transplantation procedure and possible ways to evaluate the functional amelioration of the animal's phenotype. The aim of this paper is not only to provide visual training material for researchers that aim to acquire these techniques, but will also help other scientist aiming to reproduce the experiments reported in this thesis and other works from our lab.

Amongst the various refinement of the protocols achieved along these years, recent updates include the use of gas anaesthesia (e.g. isofluorane) over the previously utilised injectable tribromethanol (Avertin) (Tedesco et al., 2011). Although Avertin is easily accessible and simple to administrate via intra-peritoneal injection, it's toxicity and side effects led some countries (including the United Kingdom) to ban its use for recovery procedures (Meyer and Fish, 2005). Isofluorane gas anaesthesia requires additional equipment and training but offers less side effects, lower toxicity and faster recovery, significantly improving the animal welfare (Ludders, 1992).

For intramuscular injection procedures, the volume in which the cells are re-suspended has been reduced from 50 μl to 30 μl aiming to reduce the volumetric stress to the muscles. On the other hand, a volume of 50 μl has been chosen for intra-arterial transplantation, since it has no reported influence on the total blood volume of the mice (1.5 - 2.5 ml). This more diluted cell suspension keeps the solution less thick, facilitating the flow through the small 30 G needle, reducing the stress for the cells and allowing a more precise volume dosing.

Administration of the non-steroidal anti-inflammatory analgesic Caprofen, previously performed via subcutaneous injection after the surgery has been anticipated on suggestion of the NACWO to 40 minutes before starting the procedure. This allows achieving the active analgesic effect concomitantly with the beginning of the procedure, further reducing the discomfort for the animals.

## 4.2. Derivation of mesoangioblast-like myogenic progenitors from pluripotent stem cells.

Cell therapy approaches aiming to treat pathologies that affect large body districts such as in the case of muscular dystrophies have to face the problem of the limited expansion potential of primary cells. The possibility of differentiating myogenic progenitors from pluripotent stem cells might help to overcome this limitation.

In parallel with the main line of research conducted during my PhD, starting from the year before registering to the graduate school, I have significantly contributed to the development of a method to differentiate mesoangioblast-like myogenic cells from human iPS cells (HIDEMs). With this newly established protocol we derived HIDEMs from healthy donor and patient specific LGMD2D dystrophic iPS cells. We then genetically corrected these patient-derived cells with a lentiviral vector containing the gene mutated in this pathology (the alpha-sarcoglycan) and successfully engrafted these cells in an ad-hoc generated dystrophic immunodeficient murine model of the disease. The results obtained were published in Science Translational Medicine during the first year of my PhD (Tedesco et al., 2012).

During the following years I provided equal contribution together with Sara Martina Maffioletti (PhD student in our group), in extending the applicability of this protocol to human embryonic stem cells and iPSCs cultured in feeder-independent conditions. The methodology required to be amended in several of the culture step, adjusting seeding density and culture conditions to accommodate the different cell mortality and adaptability to the

substrate observed in particular for the feeder-independent iPSCs. Once adapted, this protocol has been applied to generate new lines of patient specific and healthy donor HIDEMs, in light of a collaborative project carried out with Sumitava Dastidar, PhD student in Thierry VandenDriessche and Marinee K Chuah laboratory at the Free University of Brussels, Belgium.

With the aim of increasing the accessibility to the method to other groups and allow a better reproducibility, a detailed version of the protocol including these recent ameliorations, has been included in a recently published protocol paper in which I am listed as co-first author (Maffioletti et al., 2015). This protocol paper has been also selected as the cover story of the journal (Nature Protocols, July 2015, Volume 10 No 7).

As mentioned above, this methodology allows the production of a potentially unlimited amount of cells with a robust myogenic differentiation potential (triggered by synchronous activation of MyoD). This opens possibilities for further developments not only in the field of cell therapy, but also for the development of novel tissue engineering and drug development platforms.

# CHAPTER 5:

# 5. RESULTS: Direct reprogramming of adult muscle satellite cells to pericyte-like cells via Notch and PDGF signalling

The main aim of this chapter is to investigate the fate plasticity of adult muscle satellite cells, focussing on the possibility to directly reprogram these cells towards the pericyte lineage exploiting Notch and PDGF signalling. The experimental results reported in this section of the thesis aim to investigate this possibility, stimulating these two pathways with DLL4 and PDGF-BB, previously reported to exert a similar effect on embryonic myoblasts (Cappellari et al., 2013). Treated satellite cells have been evaluated for variation in morphology, gene expression pattern and ability to undergo myogenic differentiation in vitro. We have then assessed if treated cells acquire the pericyte abilities of stabilising endothelial networks and migrate through endothelial layers in vitro. To investigate if the cells acquire beneficial properties exploitable for cell therapy purposes, such as improved engraftment and the ability to cross the blood vessel walls, we report here the results of proof of principle transplantation experiments in dystrophic mice.

## 5.1. Primary satellite cells change their morphology when subjected to DLL4+PDGF-BB treatment

Primary satellite cell cultures were established from wild type mice and cultured on type I collagen-coated dishes in an ad-hoc defined satellite cell medium (please refer to the materials and methods section for further details on the primary cell isolation and culture). The cells were then seeded on DLL4-coated dishes and supplemented daily with PDGF-BB. After one week of treatment the cells, that initially showed a circular morphology typical of satellite cells, acquired a more flat elongated shape resembling the one of cultured control pericytes (Figure 18A). This phenomenon was shown to be consistent over three independent cell preparations. Cultured cells have been imaged and cell circularity was evaluated using the ImageJ measurement tool. Treated cells revealed that a remarkable decrease in the average cell circularity ratio indicating the consistent acquisition of a non-circular morphology over three independent cell preparations (Figure 18B).

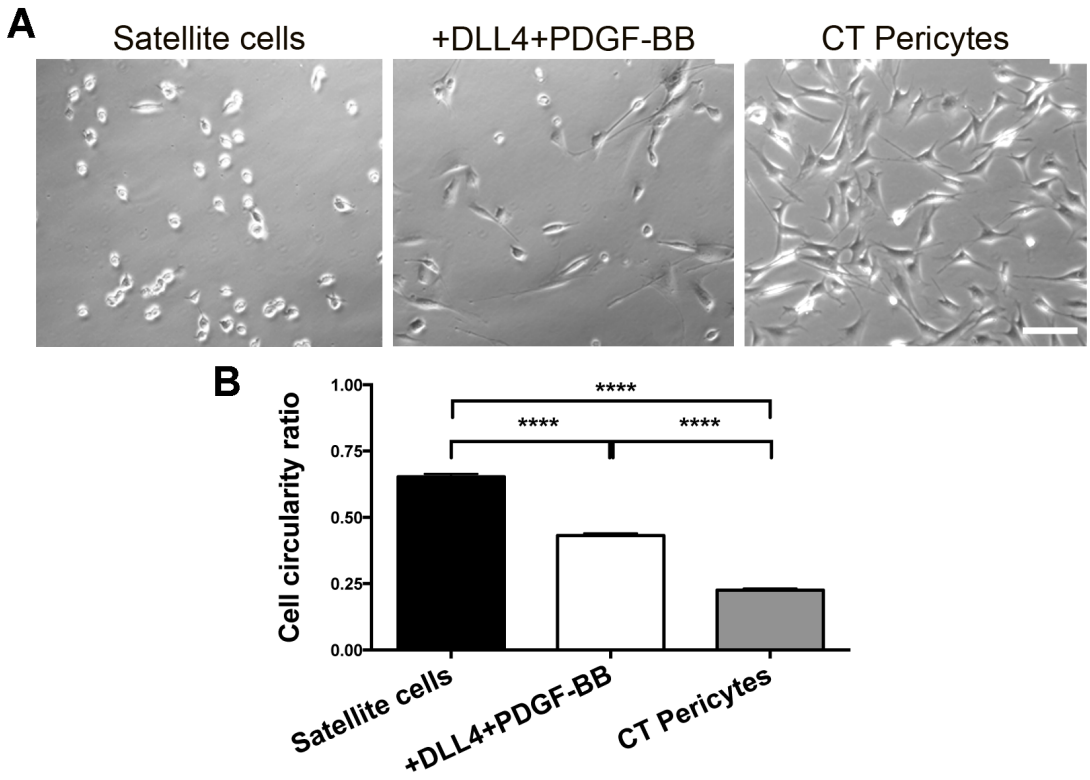



***Figure 18. Characterisation of the morphological properties of treated satellite cells.*** ***A*** *Phase contrast images showing the morphology of untreated satellite cells (Left), satellite cells treated with DLL4+PDGF-BB (Centre) and pericyte-derived mesoangioblasts (Right; scale bar 80μm).* ***B*** *To quantify the variation in morphology, the circularity ratio of the cells has been evaluated using ImageJ. A minimum of 500 cells / condition has been measured from three independent experiments. Data are represented as mean circularity ratio ± SEM; Satellite cells 0.66 ± 0.01; Treated 0.42 ± 0.01; CT Pericytes 0.23 ± 0.003. One-way ANOVA P-value 0.0001 (Pairwise comparisons have been performed using the Bonferroni's method and are reported on the graph as: **** P<0.0001).*

## 5.2. Treated cells show increased alkaline phosphatase activity

Alkaline phosphatase is a recognised marker of skeletal muscle pericyte-derived mesoangioblasts (Tonlorenzi et al., 2007). For this reason cells subjected to the DLL4 and PDGF-BB stimulation were assessed for the presence of alkaline phosphatase enzymatic activity using the NBT/BCIP kit (Roche; Please refer to the material and methods section for further details). This kit allows the formation of a black/purple precipitate in the cells expressing the enzyme (Figure 19A). The treated satellite cell cultures showed an increase in the percentage of AP-positive cells compared to the untreated population and even higher than the one observed in control pericytes (Figures 19B).

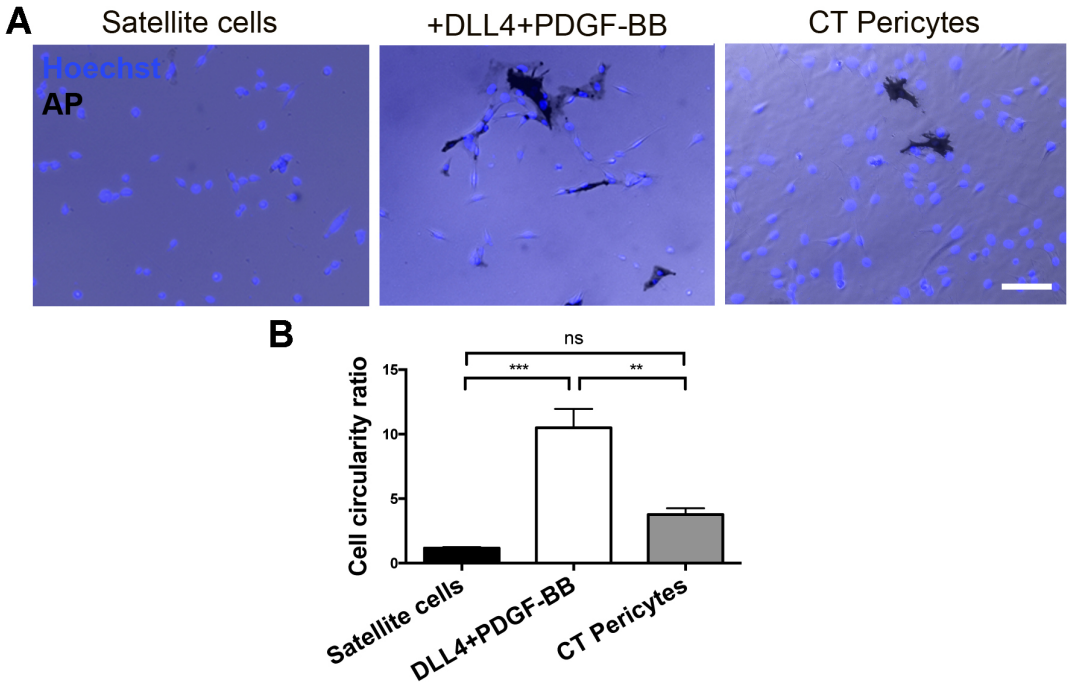



***Figure 19. Evaluation of the Alkaline Phosphatase enzymatic activity in treated and control cells. A*** *The panel depicts an overlay of phase contrast and Hoechst images of untreated satellite cells, treated satellite cells and control pericytes. Alkaline Phosphatase (AP) positive cells are identified by the formation of a black precipitate in their cytoplasm (Right; scale bar 90 μm).* ***B*** *The graph shows the percentage of AP positive cells. A minimum of nine 1.5 mm2 microscopic fields has been quantified from three independent experiments. The column bars represent the mean percentage of AP positive cells / field ± SEM: Satellite cells 1.16 ± 0.14; Treated 10.5 ± 2.53; CT Pericytes 3.76 ± 0.50; One-way ANOVA p-value: 0.0008. Pairwise comparisons have been performed using the Bonferroni's method and are reported on the graph as: ns not significant; ** P<0.01; *** P<0.001.*

## 5.3. DLL4+PDGF-BB treatment induces a reversible decrease in cell proliferation

Growth curve proliferation analyses revealed that DLL4+PDGF-BB treated satellite cells proliferate less in comparison with both satellite cell-derived myoblasts and control pericytes (Figure 20A). Notch signalling is well known to control satellite cell activation and quiescence (Brohl et al., 2012; Mourikis et al., 2012), therefore the reduction in cell proliferation observed with the DLL4 treatment is not surprising. Although this result appears to be in contrast with the short-term proliferation analyses performed on embryonic myoblasts (Cappellari et al., 2013), experiments on DLL1-mediated Notch activation (Parker et al., 2012a; Parker and Tapscott, 2013) and upon overexpression of the Notch intracellular domain (Wen et al., 2012) reported a similar proliferative slowdown in both canine and murine adult satellite cells. Importantly the reduction of proliferation observed did not impede the cell expansion required for transplantation purposes.

This reduction in the proliferative capacity has also been observed when the treatment was started on satellite cells that have been already cultured for two weeks in control conditions. This indicated that cultured satellite cells preserve responsiveness to the treatment at least up to 8 passages (Figure 20B, red inset). Interestingly, this phenomenon was reversible when the treatment was discontinued after two weeks, confirming its signal-dependence. Seeding treated cells in un-supplemented satellite cells culture condition promptly restored their proliferative potential (Figure 20B, red inset).

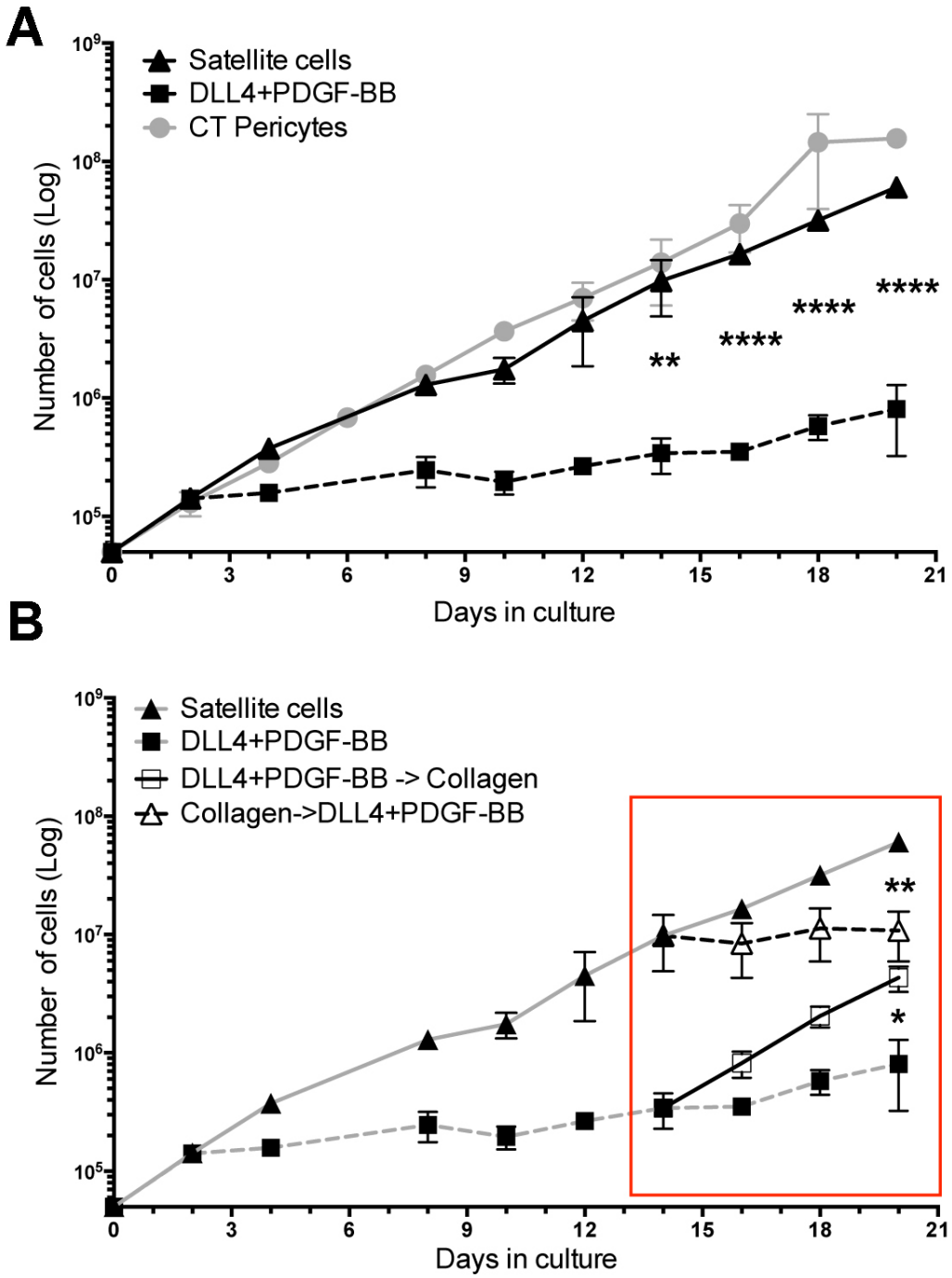



***Figure 20. Proliferation analysis on DLL4+PDGF-BB treated satellite cells. A*** *The graph shows the proliferation curve obtained culturing satellite cells, DLL4+PDGF-BB treated satellite cells and control pericytes. The cells have been counted every other day for three weeks. Each point represents the average counting of three independent experiments. Each point represents the average of the counting obtained from three independent*

*experiments; error bars represent the SEM. Statistical significance between the Satellite cell and DLL4+PDGF-BB group has been assessed with Two-way ANOVA (Bonferroni's multiple comparison) ** P<0.01; **** P<0.001* ***B*** *The graph shows the same proliferation curve in which an additional set of data has been added. Two weeks after having started the treatment or having cultured the cells un-supplemented, a fraction of each population was seeded in the opposite culture condition and its proliferation was assessed for an additional week to verify the reversibility of the treatment and the possibility of starting it on long-term cultured cells (red rectangle). Each point represents the average of the counting obtained from three independent experiments; error bars represent the SEM. Statistical significance between the two groups with the switched culture conditions has been assessed with a Two-way ANOVA (Bonferroni's multiple comparison) * P<0.05; ** P<0.01. Part of these proliferation analyses, have been performed under my supervision by James Lane and Ekin Ucuncu.*

## 5.4. Treated cells show Notch-dependent reduction of their myogenic differentiation potential

Notch activation has been widely reported to inhibit myogenesis in embryonic and adult myoblasts *in vitro* (Brohl et al., 2012; Conboy and Rando, 2002; Delfini et al., 2000; Mourikis and Tajbakhsh, 2014; Nofziger et al., 1999). To assess if this mechanism is also triggered upon the DLL4+PDGF-BB treatment, cells were seeded at high density ($1.2x10^4$ / $cm^2$) as previously reported (Tedesco et al., 2011), grown to confluence and kept for one week in myogenic differentiation medium (DMEM supplemented with 2% horse serum) as previously described. This myogenic differentiation assay revealed that satellite cells previously subjected to the DLL4+PDGF-BB treatment have a reduced myogenic differentiation ability compared to both untreated satellite cells and control pericytes (Figure 21A, left; Figure 21B, left plot)

To further investigate the possible Notch dependency of this phenomenon, replicate dishes of the differentiating cultures were supplemented with a γ-secretase inhibitor (L-685,458). This molecule blocks the Notch signalling cascade hampering the proteolytic activation of Notch receptor, operated by the γ-secretase, consequently preventing nuclear translocation of the Notch intracellular domain (Vilquin et al., 1994). Blocking the Notch cascade with the γ-secretase inhibitor treatment was sufficient to revert the functional impairment observed in the treated cells and restore the latent myogenic memory of this cells (Figure 21A, right; Figure 21B, right plot).

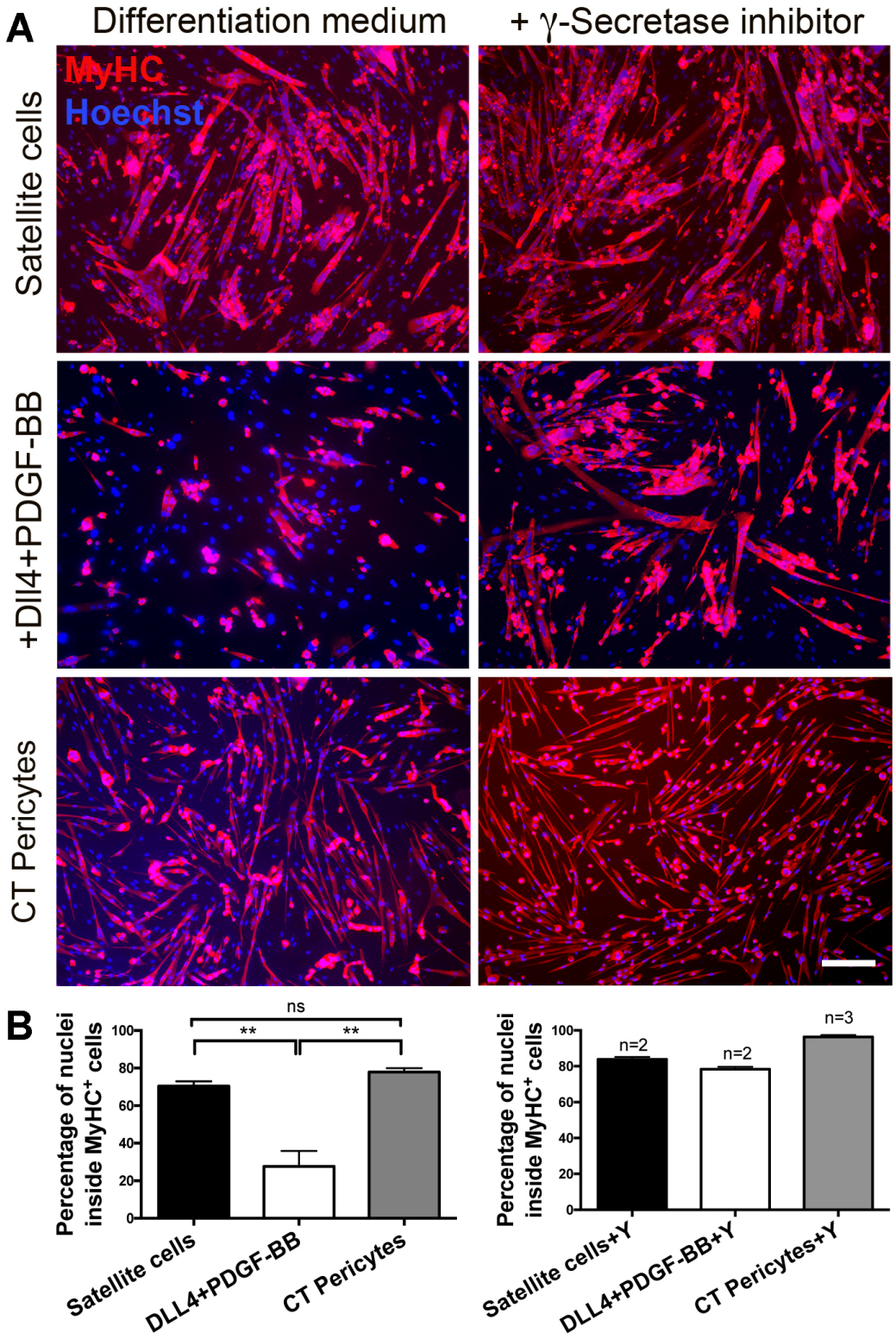


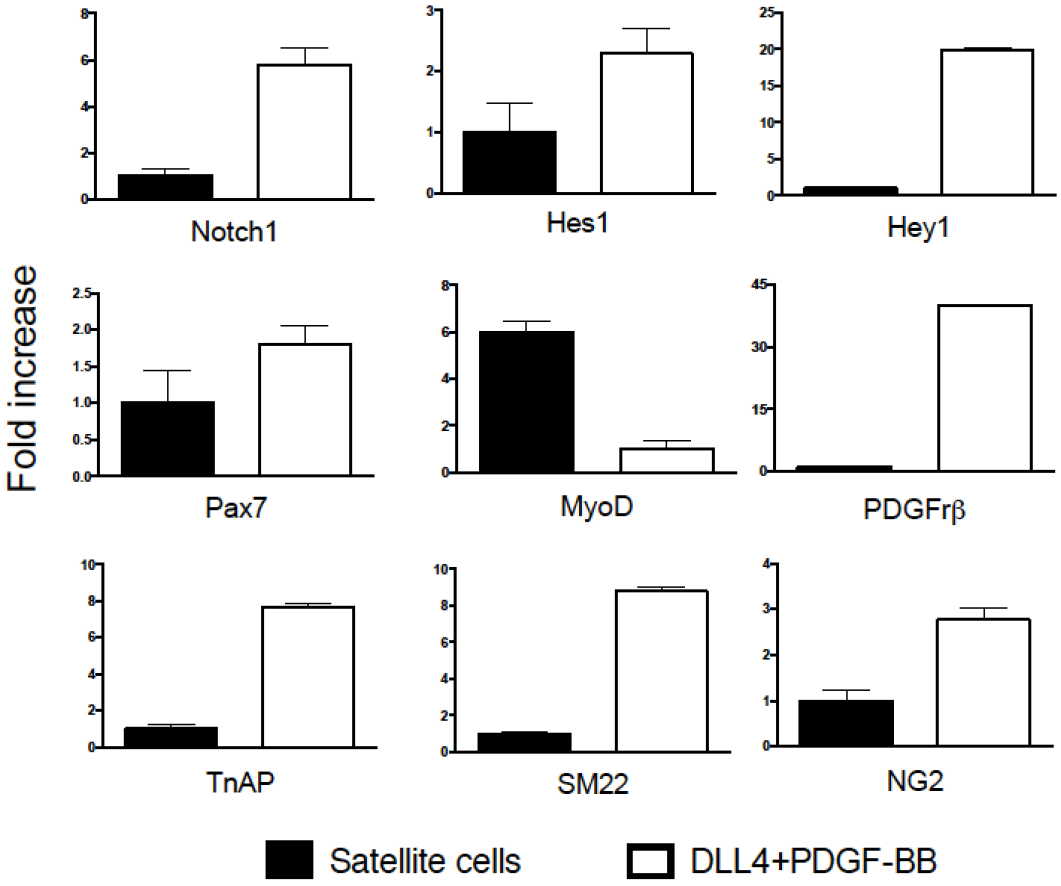

***Figure 21. Assessment of myogenic potential of DLL4+PDGF-BB treated cells, with and without γ-secretase-mediated Notch signalling***

***inhibition. A*** *Immunofluorescence staining for Myosin Heavy Chain (MyHC; marker of terminal myogenic differentiation) performed on untreated satellite cells, treated satellite cells and control pericytes subjected to a terminal myogenic differentiation assay with and without γ-secretase inhibitor supplementation (Scale bar 100μm).* ***B*** *The graph on the left shows quantification of the percentage of nuclei inside myosin heavy chain positive cells. The plot indicates that cells treated with DLL4+PDGF-BB shows reduced myogenic potential in comparison with untreated satellite cells and pericyte controls. The data are represented as mean percentage of nuclei inside Myosin Heavy Chain positive cells ± SEM: Satellite cells 70.39 ± 2.58; Treated 27.64 ± 8.23; CT Pericytes 77.88 ± 2.07; One-way ANOVA p-value 0.0009. Pairwise comparisons have been performed using the Bonferroni's method and are reported on the graph as: ns not significant; ** P<0.01. A minimum of 10 10x (1.5mm2) fields quantified in 3 independent experiments. The plot on the right shows the effect of γ-secretase inhibitor supplementation in reverting the myogenic inhibition trend as observed in two independent experiments. Data are represented as mean percentage of nuclei inside Myosin Heavy Chain positive cells ± SEM: Satellite cells 83.90 ± 1.3; Treated 78.35 ± 1.34; CT Pericytes 96.4 ± 0.94.*

## 5.5. Gene expression profile of treated satellite cells

To investigate a possible variation in the gene expression profile of the cells upon DLL4+PDGF-BB treatment and understand the reasons for the observed proliferation and myogenic potential reduction, a series of quantitative real time PCR (qRT-PCR) analyses was performed. After the treatment, RNA was extracted and reverse transcribed to cDNA. A first series of qRT-PCRs was performed aiming to verify the effect of DLL4 on the cells in activating the Notch signalling pathway. Analysis of the expression of its target gene Notch1 and of its downstream regulators Hes1 and Hey1 confirmed that these three genes were upregulated in treated cells (Figure 22 top row), validating the effectiveness of the treatment. The expression of the satellite cell marker Pax7 increased in treated cells (although without reaching statistical significance), giving a qualitative indication of an increased quiescence status. This hypothesis was confirmed by reduction of the MyoD transcript, indicating a less-activated status (i.e. more satellite cell-like and less myoblast-like). A remarkable increase in the expression of the PDGFrβ gene was observed upon treatment (Figure 22 central row). This may be because active Notch signalling is reported to be sufficient to induce an upregulation of this gene in vascular smooth muscle cells (VSMCs), leading also to an increased cell response to PDGF-BB (Jin et al., 2008). Further qRT-PCR analyses showed up-regulation of the mostly recognised pericyte markers TnAP, NG2, SM22 (Figure 22 lower row) and as mentioned above PDGFrβ.

**A**




**B**

| | Student's t-test | | 2-way ANOVA | | Multiple regression | |
|---|---|---|---|---|---|---|
| Notch1 | 0,0553923 | ns | 0,0473 | * | 1,43E-06 | *** |
| Hes1 | 0,0148241 | * | 0,3383 | ns | 0,02972 | * |
| Hey1 | 0,00909924 | * | 0,0013 | ** | 2,56E-13 | *** |
| Pax7 | 0,28906 | ns | 0,5001 | ns | 0,12335 | ns |
| MyoD | 0,00279303 | * | 0,0433 | * | 5,51E-06 | *** |
| PDGFRb | 0,0920756 | ns | 0,0001 | *** | 2,00E-16 | *** |
| TnAP | 0,0915078 | ns | 0,0222 | * | 1,29E-06 | *** |
| SM22 | 0,124432 | ns | 0,0154 | * | 2,22E-08 | *** |
| NG2 | 0,220125 | ns | 0,24 | ns | 0,00575 | ** |

***Figure 22. qRT-PCR analyses on treated and untreated satellite cells. A*** *The image shows the calculation of fold increase variation in gene expression assessed via qRT-PCR obtained trough the ddCT method. The*

*black bars depict the expression level of untreated satellite cells while the white bars depict satellite cells treated with DLL4 and PDGF-BB. Each bar represents mean ± SEM of the triplicate qRT-PCR analyses performed for three independent biological replicates (3 independent cell preparations analysed, each gene analysis run in triplicate).* ***B*** *The table present an overview on the statistical analysis performed before the fold transformation presented in A. Statistical significance on the dCT has been assessed implementing Student's t-test; Two-way ANOVA and with a multiple regression model.*

## 5.6. Gene expression profile of treated purified Pax7-nGFP satellite cells

To exclude the possibility that the effects observed were artefacts due to a possible contamination with other cell types that might be present in the primary cell preparations, these analyses were repeated using a purified satellite cell preparation. With this aim a collaboration with the group of Shahragim Tajbakhsh was established. This group developed in 2009 a murine model in which the Pax7 positive cells co-express the nuclear GFP (Sambasivan et al., 2009). Thanks to this collaboration, we obtained a muscle cell preparation from these Pax7-nGFP mice. Satellite cells were then purified from this preparation via fluorescent-activated cell sorting (FACS) exploiting their GFP fluorescence (Figure 23A). These cells were subsequently treated as described above and used for RNA extraction, reverse transcription and qRT-PCR analyses. Purified Pax7-GFP positive satellite cells showed a comparable response to the treatment with a gene expression profile consistent to what observed in non-purified cells (Figure 23B).

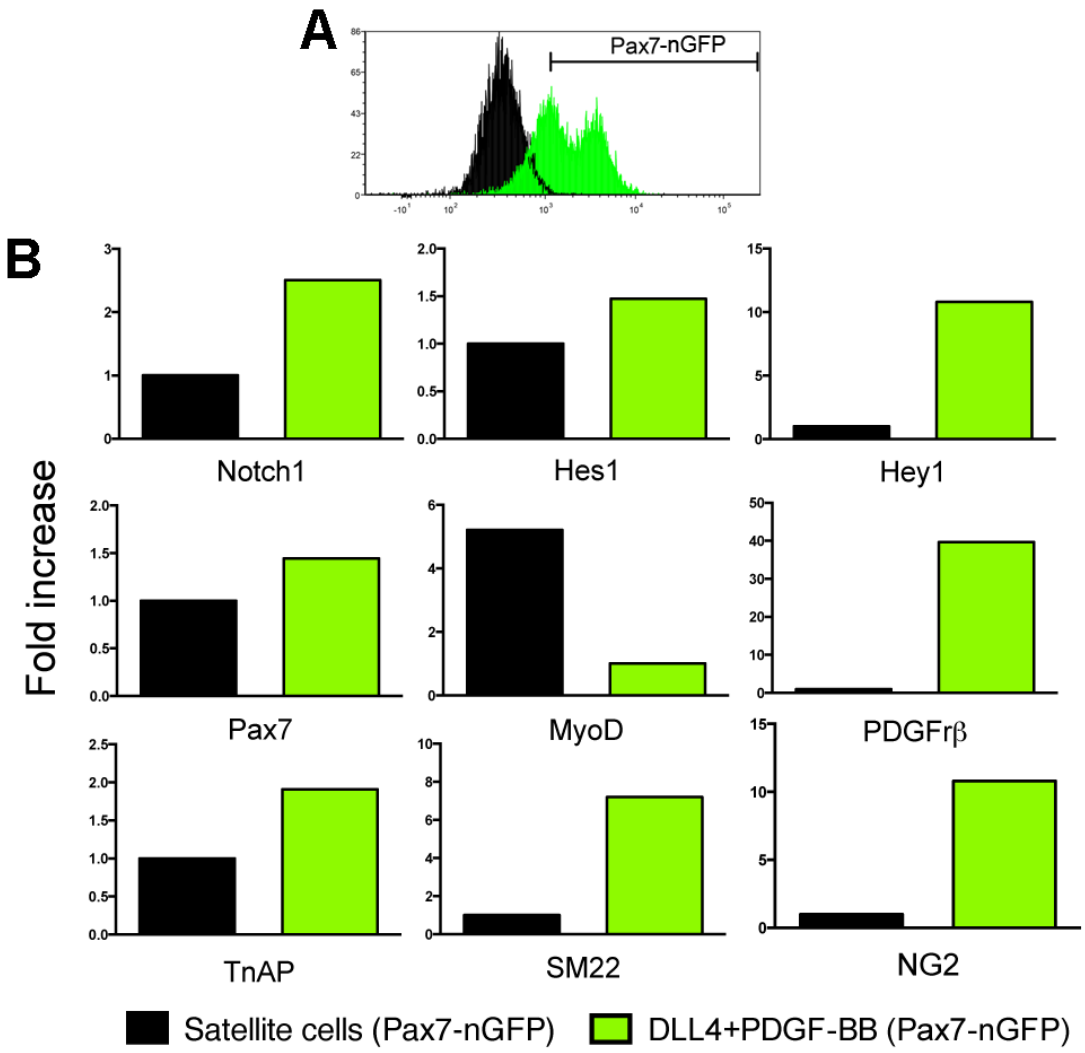



***Figure 23. qRT-PCR analyses on treated and untreated purified Pax7nGFP positive satellite cells. A*** *Histogram plot of the FACS sorting purification of the Pax7nGFP positive cells. In black the profile of a non-GFP control satellite cell preparation. The top bar indicates the gate utilised for the isolation.* ***B*** *The plots show the fold increase variation in gene expression assessed via qRT-PCR. The black bars depict the expression level of untreated Pax7nGFP positive satellite cells, while the green bars Pax7nGFP positive satellite cells treated with DLL4 and PDGF-BB. Each bar represents the average of a triplicate qRT-PCR run performed for each gene. A single cell preparation was available for this set of experiments (mice currently not available within UCL), therefore the lack of biological replicates impeded the assessment of statistical significance.*

## 5.7. Reprogrammed cells stabilise endothelial networks *in vitro*

Primary satellite cells were isolated from CAG-EGFP reporter mice. These animals ubiquitously express the green fluorescent protein under a strong beta-actin promoter (Okabe et al., 1997), thus allowing the isolation of fluorescent satellite cells, easily traceable in co-culture experiments. Fluorescent satellite cells were then treated as described above (DLL4 coating and PDGF-BB supplementation), and used for endothelial network formation assays (Figure 23A) This assay exploits the property of human umbilical vein endothelial cells (HUVECs) in forming vascular-like networks *in vitro* upon vascular-endothelial growth factor (VEGF) stimulation on Matrigel-coated dishes. In normal conditions, networks formed by the HUVECs are not stable and are disrupted in the first twenty-four hours. Among their various physiological functions, pericytes contribute to vessel stabilisation. This property, previously reported as preserved *in vitro,* helps the formation and stabilisation of HUVEC endothelial networks *in vitro* (Cappellari et al., 2013; Goodwin, 2007; Tedesco et al., 2012; You et al., 2014). We utilised this assay to investigate if treated and untreated satellite cells, are capable of behaving like *bona fide* pericytes stabilising endothelial networks *in vitro*.

In the first five hours after VEGF stimulation, HUVECs started forming the endothelial networks in all of the culture conditions: HUVEC; HUVEC+Satellite cells; HUVEC+Treated satellite cells; HUVEC+Control Pericytes. Twenty-four hours after the induction, HUVEC networks showed

partial disaggregation when cultured alone, they were destabilised when HUVECs when co-cultured with control satellite cells but found to be stable when HUVECs when co-cultured with satellite cells exposed to DLL4+PDGF-BB and control pericytes (Figure 24A and B). The number of network branches per each 10X microscopic field (1.5 $mm^2$) was quantified and no significant differences in its stability were detected at the various time points (Figure 24C; average number of branches / 1.5 $mm^2$ ± SEM: HUVEC only 4.75 ± 1.55; HUVEC+Satellite cells 0.00; HUVEC+Treated 24.25 ± 3.57; HUVEC+Treated 48h 19.17 ± 2.15; HUVEC+Treated 72h 23.00 ± 2.48; HUVEC+Treated 120h 20.6 ± 3.51; HUVEC+CT Pericytes 42.07 ± 2.26). In support to these results, treated satellite cells were found to be in close proximity to the HUVEC endothelial networks and were able to stabilise the structure for up to 120 hours after induction, consistent with what was observed with control pericytes (Figure 24D; number GFP positive cells co-localised with the network branches / 1.5 $mm^2$ ± SEM: HUVEC only 0; HUVEC+Satellite cells 0; HUVEC+Treated 96.99 ± 1.48; HUVEC+Treated 48h 97.51 ± 1.06; HUVEC+Treated 72h 100.00 ± 0.00; HUVEC+Treated 120h 99.13 ± 0.54; HUVEC+CT Pericytes 99.14 ± 0.39).

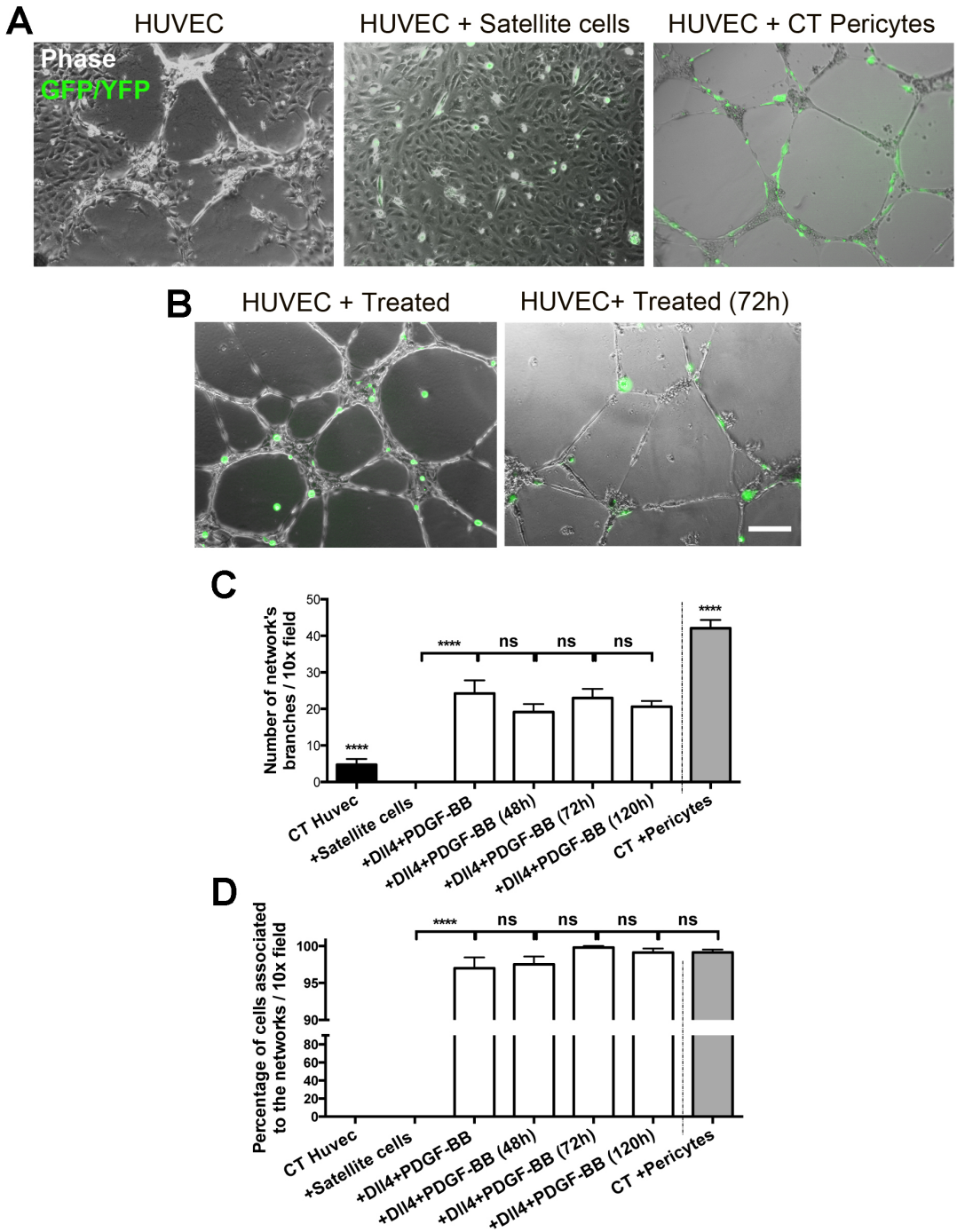



***Figure 24. Endothelial network formation assay. A*** *Images acquired twenty-four hours after the endothelial network induction. HUVECs have been utilised as a positive control for the formation of the network (top left). When mixed with untreated satellite cells, HUVEC formed an unstable endothelial network that dissociated in the first 24 hours while it was stabilised with primary pericytes, the positive control of the experiment (Scale bar 100 μm).*

***B*** *The images show that treated satellite cells contribute to the stability of the endothelial networks after 24 and 72 hours similarly with what observed with control pericytes in A.* ***C*** *The bars represent the quantification of the number of GFP positive endothelial networks branches ± SEM.* ***D*** *The bars represent the quantification of the average number of GFP positive cells that co-localised with an endothelial network branch in three independent experiments ± SEM. One-way ANOVA (Bonferroni's multiple comparisons test) **** p<0.0001; ns: not significant.*

## 5.8. Assessment of migration ability *in vitro*

The above experiments demonstrated that treated satellite cells acquire pericyte markers and network stabilisation properties. A transwell migration assay was performed with the aim of investigating if this switch towards the pericyte lineage was reflected also in increased migration ability. Treated and untreated GFP-positive satellite cells were seeded on a murine H5V endothelial layer previously grown on an 8 mm-pored transwell membrane coated with 1% gelatine. The upper chamber of the well was loaded with serum-deprived medium while the lower chamber of the transwell was loaded with myotube-conditioned medium to give the cells a chemoattractant stimulus to migrate through the endothelial layer (Figure 25A). The number of GFP-positive cells that migrated through the endothelial layer over 6 hours was calculated to be for the treated cells 2.4 folds more than the untreated control, indicating that treated cells have an improved migration ability, either superior to the one observed with control pericytes (Figure 25B; number of GFP-positive cells / 1.5mm$^2$ microscopic field ± SEM: H5V+Satellite cells 45.13 ± 1.83; H5V+Treated 113.7 ± 5.27; H5V+CT Pericytes 85.91 ± 1.89; H5V 0).

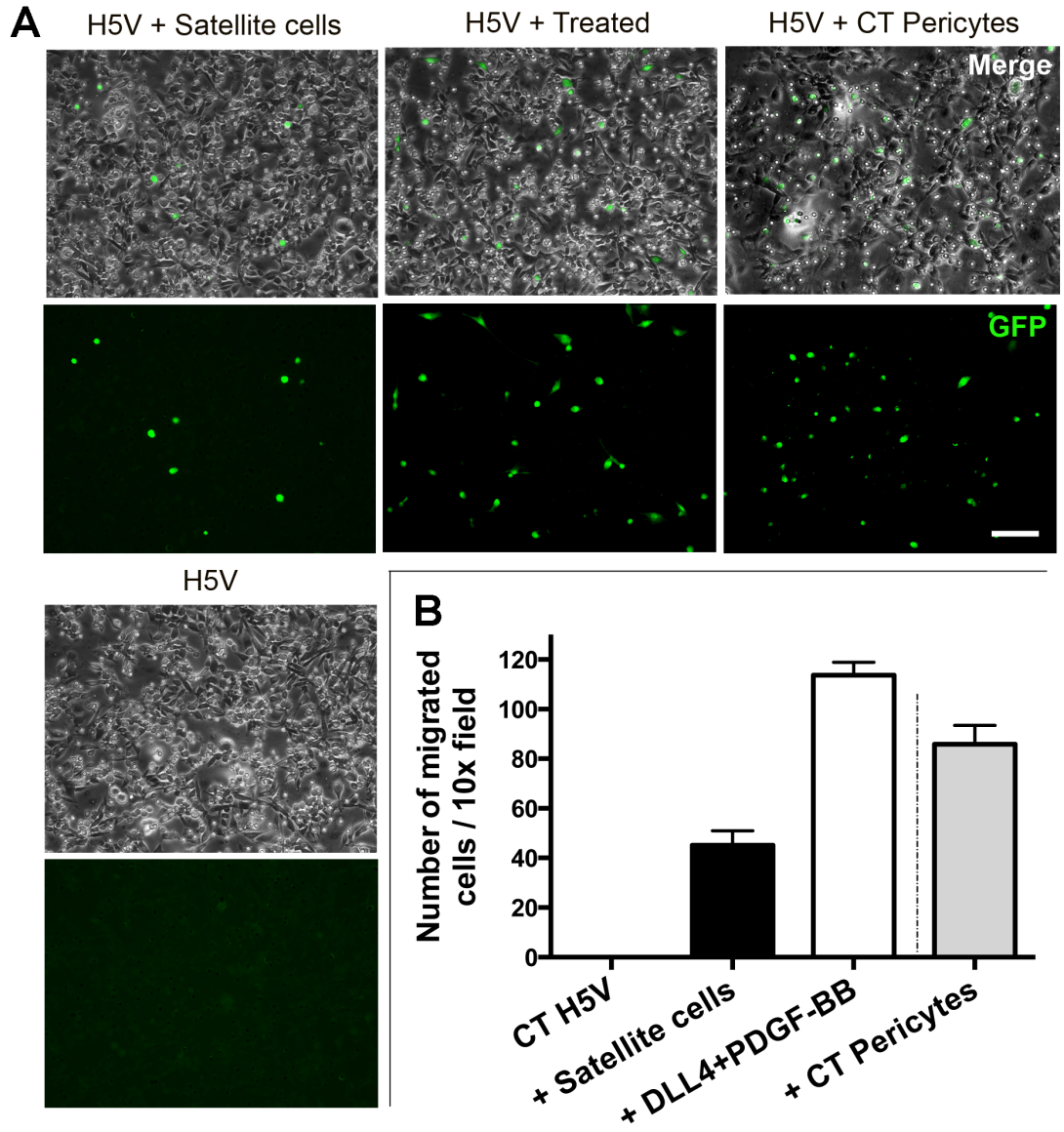



***Figure 25. Assessment of cell migration through a layer of endothelial cells.*** ***A*** *The panel shows images of the lower side of the transwell membranes utilised to assess the migration of treated and untreated GFP-positive satellite cells. The top part of the panel shows merged phase contrast and green fluorescence images of the lower side of the transwell membranes at a 6-hour time point. The non-fluorescent cells are murine endothelial H5V cells, which migrate freely through the pored membrane. The bottom images depicts the green fluorescent channel of the same fields, in which GFP-positive cells are those that migrated through the endothelial layer (Scale bar: 100 μm)* ***B*** *The bar graph depicts the quantification of the GFP-positive cells*

*that migrated through the membrane in 2 independent experiments performed in triplicate, in which a minimum of 15 1.5mm$^2$ microscopic field / condition were quantified. These data represent the outcome of 2 independent biological experiments; a third independent biological replicate will be performed within the project of Ekin Ucuncu, MRes student in the laboratory. The final results of this experiment will be then included in a manuscript describing this project currently under preparation.*

## 5.9. Treated cells show no evidence of tumourigenesis

One of the strengths of the above-described DLL4+PDFG-BB direct reprogramming approach resides in the lack of genetic manipulation. Compared to other gene-based reprogramming strategies, the methodology developed in this thesis aims to obtain a cell product with less safety hurdles. To confirm that the signalling manipulation did not induce cell transformation, tumour formation assay were performed injecting treated and untreated satellite cells subcutaneously in immune-deficient (scid/beige) mice (please refer to the material and methods section for further details). None of the animals that received the injections showed the presence of a tumour mass after 2 months of follow up (n = 5), providing preliminary indication of the safety of the strategy.

## 5.10. Increased engraftment in dystrophic animals upon intramuscular delivery

To assess whether the treatment with DLL4+PDGF-BB has a positive impact on myoblast engraftment potential a pilot intramuscular transplantation experiment was performed. Treated and untreated satellite cells were transduced with a nuclear LacZ lentiviral vector; this approach was then utilised to allow the detection of the donor-derived nuclei via X-Gal staining in an intraspecific mouse-into-mouse transplant. In this experiment 5 x $10^5$ cells were intramuscularly transplanted in two dystrophic immunodeficient α-sarcoglycan-null/Scid/Beige mice. Three weeks after transplantation, the animals were humanely culled and the transplanted muscles explanted. X-Gal and α-sarcoglycan staining were performed on cryosections of the transplanted muscles to evaluate cell engraftment and differentiation (Figure 26A). We observed an increase in the average number of X-Gal positive nuclei in the sections of the muscles transplanted with the DLL4+PDGF-BB treated cells, in comparison to the ones transplanted with untreated satellite cells (Figure 25B; 44 sections analysed, average number of Xgal positive cells / section ± SEM: Satellite cells 10.51 ± 1.74; DLL4+PDGF-BB 110.3 ± 8.08). Similarly, an increase in the number of donor derived α-sarcoglycan positive myofibres (completely absent in this murine model) was observed (Figure 25C; 24 sections analysed, average number of αSG positive fibres / section ± SEM: Satellite cells 3.58 ± 0.67; DLL4+PDGF-BB 36.96 ± 3.73). This data is purely qualitative considering the small sample size (n = 2) and

further biological replicates will be required to assess statistical significance and validate these preliminary encouraging results.

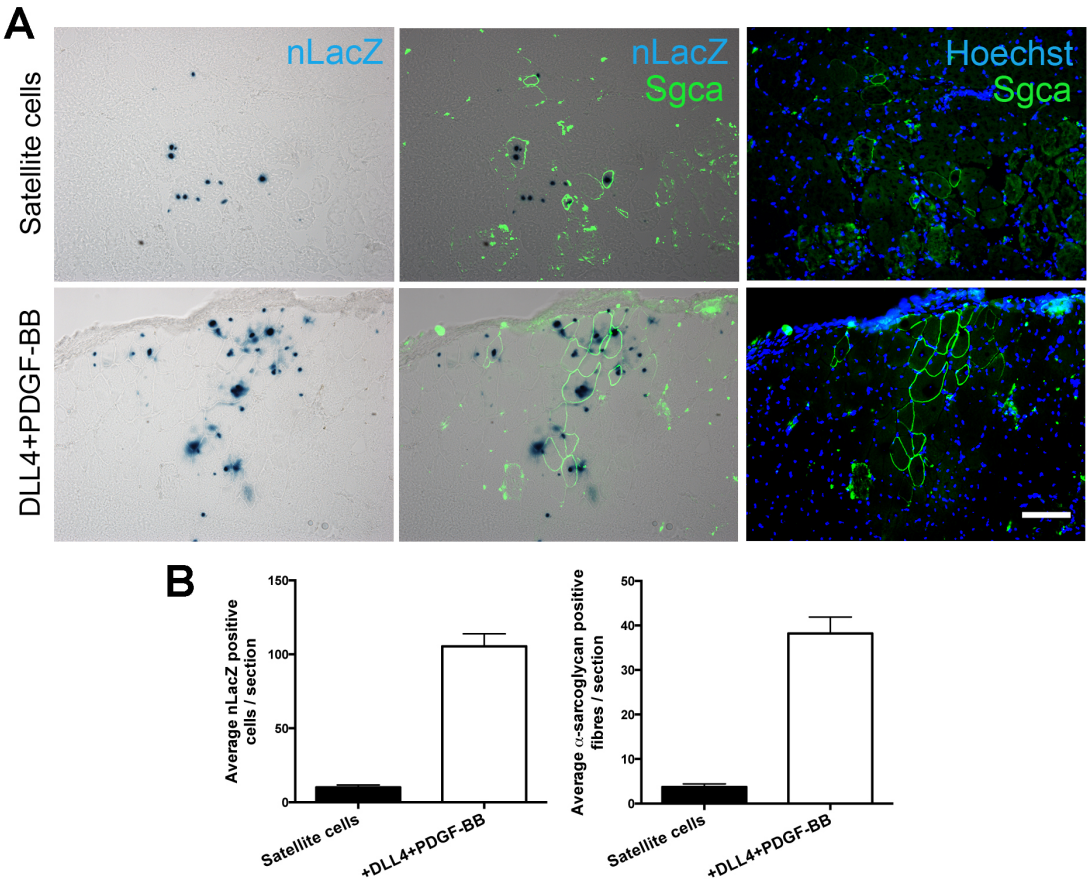



***Figure 26. Intramuscular transplantation of reprogrammed satellite cells.*** ***A*** *The panel shows on the left X-Gal staining to assess the presence of nLacZ positive donor derived cells. The central column depicts an overlay of the X-gal staining, with immunofluorescence staining to detect the presence of the α-sarcoglycan (Sgca), also depicted on the right as an overlay with the nuclear dye Hoechst (Scale bar: 180μm).* ***B*** *The graph on the left depicts the average number of nLacZ positive nuclei traced in each 7μm-thick muscle section analysed* ± SEM *(44 sections analysed). The graph on the right depicts the quantification of the average number of α-sarcoglycan positive fibres in each 7 μm-thick muscle section analysed* ± SEM *(24 sections analysed).*

## 5.11. Treated satellite cells acquire the ability to cross the vessel wall upon intra-arterial delivery

As an additional proof of principle experiment, we transplanted 2.5 x $10^5$ cells in the femoral arteries of two Sgca-null/Scid/Bg mice as previously detailed (Gerli et al., 2014). Two weeks after transplantation the presence of donor-derived nLacZ positive nuclei in the muscles downstream of the injection site was assessed via whole mount X-gal staining of the hind limbs. The presence of donor-derived nuclei was scarce in the animals transplanted with control satellite cells, with a total of 2 nuclei identified by stereoscopic microscopy (Figure 27A top row). Animals transplanted with treated satellite cells instead showed a more marked presence of donor cells at two weeks post-transplantation, with up to 53 nuclei identified (Figure 27A bottom row). Donor-derived myonuclei were identified upon X-Gal and eosin stainings on sections from muscles transplanted with treated satellite cells, indicating fusion of donor nuclei with the host myofibres (Figure 27B). Immunohistochemistry staining performed on the sections showed that the positive nuclei did not co-localise with PECAM-positive vessels (in brown; Figure 27C), indicating that treated cells has the ability to cross the blood vessel wall upon systemic delivery. Further experiments will be performed prior to submission of this project for publication, in order to quantitatively support these preliminary observations and assess statistical significance.

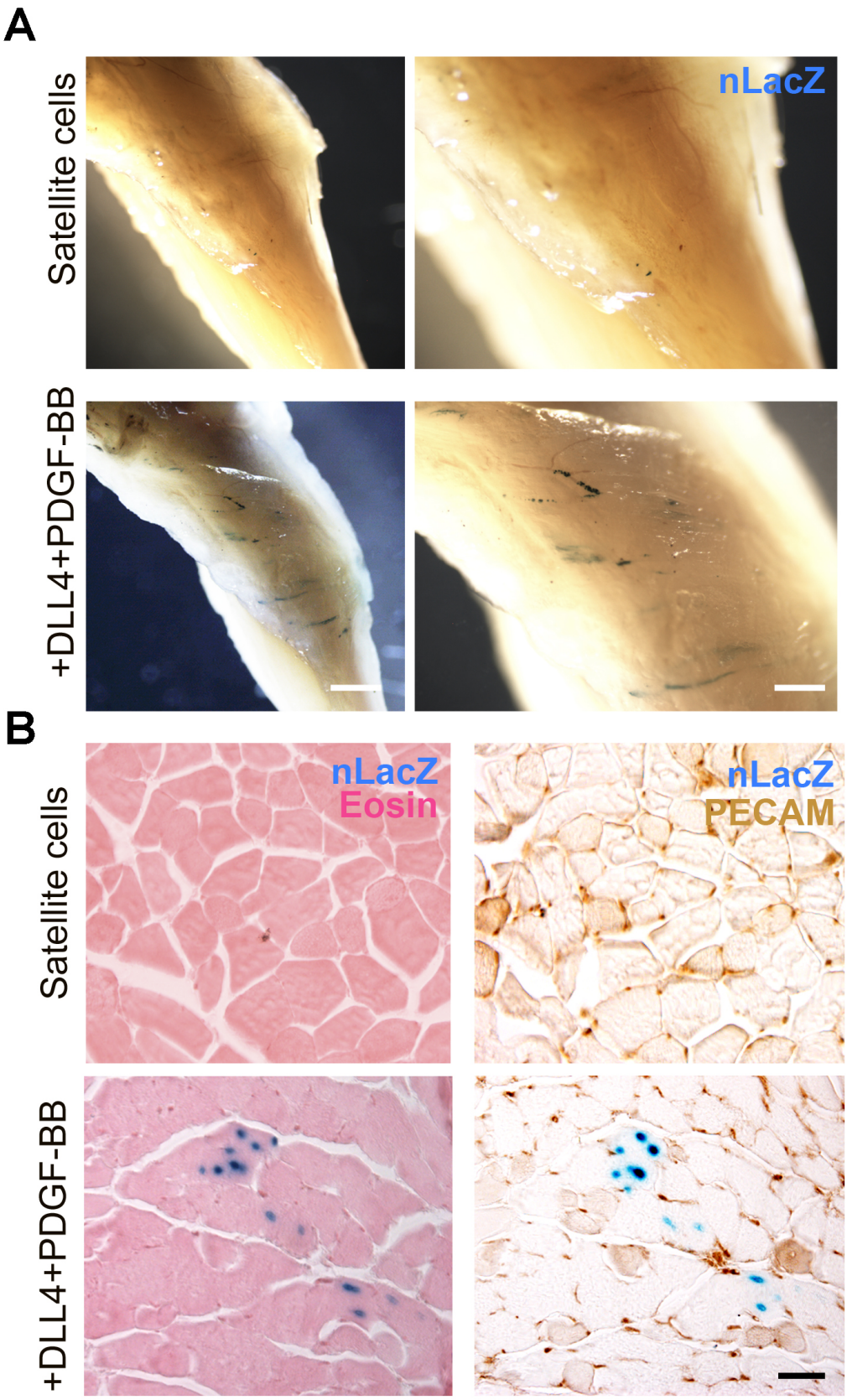

A
Satellite cells
nLacZ
+DLL4+PDGF-BB
B
Satellite cells
nLacZ
Eosin
nLacZ
PECAM
+DLL4+PDGF-BB

***Figure 27. Evidence of in vivo migration of DLL4+PDGF-BB treated cells engrafted upon intra-arterial delivery****. **A** Stereomicroscopic images of murine limbs that received untreated (top) and treated satellite cells (bottom). Donor-derived nuclei are labelled in blue upon whole mount X-gal staining giving preliminary indications of engraftment upon intra-arterial delivery (Scale bars: Left 1mm; Right 0.5mm). **B** The panel shows phase contrast images of sections of the muscles explanted from animals that received intra-arterial delivery of untreated (top) and treated satellite cells (bottom). The left images depict haematoxylin and eosin staining showing the presence of blue nuclei within the muscle fibres. On the right, immunohistochemistry staining for the endothelial marker PECAM (right) performed on the serial sections highlight the localisation of the donor-derived X-Gal-positive nuclei outside the PECAM positive vessel structures (Scale bar: 100 μm).*

## 5.12. Conclusions

The results reported in this chapter indicate that adult muscle satellite cells respond to a treatment with the Notch ligand DLL4 in combination with the growth factor PDGF-BB, acquiring a pericyte/mesoangioblast-like phenotype. The treatment induced a morphological switch from the classic round shape typical of satellite cells, to a more elongated morphology that resembles the one of fibroblasts / pericytes (Figure 18). An increase in the alkaline phosphatase enzymatic activity provided indication of a possible shift towards the pericyte lineage (Figure 19). This hypothesis was then verified via qRT-PCR analysis, showing an increased level of expression of all the pericyte markers analysed (TnAP, NG2, SM22, PDGFrβ; Figure 22 and 23).

The effectiveness of the Notch stimulation was also confirmed upon qRT-PCR analyses. The increased expression of Notch1 and its downstream targets Hes1 and Hey1, confirmed the presence in treated cells, of an active Notch signalling cascade (Figure 22 and 23). This activation led also to a Notch-dependent reduction in the myogenic potential of the cells. This phenomenon resulted to be fully reversible upon blocking of the Notch signalling cascade with a γ-secretase inhibitor (Figure 21).

Surprisingly, in contrast with our fate switch hypothesis, we observed an up-regulation of the satellite cell marker Pax7 indicating that upon treatment, the cells maintained a “satellite cell memory”. This Pax7 up-regulation, in combination with a down regulation of the activation marker MyoD, appears to recapitulate what is considered a “quiescence profile”

(Figure 22 and 23). In line with this possible “increased quiescence”, treated cells showed reduction in their proliferative ability (Figure 20A). This result is consistent with what previously reported in other studies involving Notch up-regulation in myogenic progenitors (Parker et al., 2012a; Quattrocelli et al., 2014; Wen et al., 2012). Interestingly we confirmed the signalling-dependence of this phenomenon easily reverting the reduced proliferation trend by discontinuing the treatment (Figure 20B).

Treated cells resulted capable in stabilising endothelial networks *in vitro* upon co-culture with primary endothelial cells (Figure 24). This assay models *in vitro* the ability of pericytes to contribute to vessel stabilisation and regulation (Andreeva et al., 1998). The localisation of the cells in proximity to the network branches indicated also the role of a physical interaction between the two cell types, resembling what happens during Notch/PDGF-mediated pericyte recruitment operated physiologically by the endothelial cells (Armulik et al., 2005; von Tell et al., 2006). Overall these experiments provided indication that upon treatment, satellite cells acquire functional pericyte properties *in vitro.*

Consistently with previous and recent observations on Notch-stimulated satellite cells and mesoangioblasts (Parker et al., 2012a; Quattrocelli et al., 2014), treated cells showed improved engraftment in our pilot transplantation experiments in dystrophic mice (Figure 26). Moreover, manipulating the Notch and PDGF-BB signalling cascades did not alter the safety of the cells upon tumor formation assays.

The ability of pericytes / mesoangioblasts to cross the vessel wall made this cell type appealing for the development of the cell therapy protocol recently exploited in a phase I/II clinical trial (EudraCT no. 2011-000176-33). Treated satellite cells showed *in vitro* ability to migrate through artificial endothelial layers superior to the untreated controls and surprisingly even to the one observed in control pericytes (Figure 25). Following this path we performed a proof-of-principle intra-arterial transplantation experiment in a murine model of muscular dystrophy. Three weeks after intra-arterial delivery of treated satellite cells, it was possible to identify donor-derived cells outside the PECAM-positive vessels and inside the host myofibres. This provided preliminarily indication on the possibility of delivering manipulated satellite cells/myoblasts through the arterial circulation (Figure 27).

In conclusion these results show for the first time that a DLL4+PDGF-BB treatment in adult muscle satellite cells induces a partial fate-switch towards the pericyte lineage. This triggers the acquisition of a hybrid pericyte / satellite cell phenotype that gives to the cells possible beneficial properties for cell therapy. Although further *in vivo* validation of this strategy will be required, the results obtained so far indicate that treated satellite cells acquire pericyte features and show an increased engraftment potential (Figures 26 and 27). Importantly, these reprogrammed cells acquired improved migration and extravasation properties superior to what observed with untreated satellite cells. Overall this project highlights that a satellite cell / pericyte conversion could be considered a new and exciting strategy to develop novel cell therapy strategies for muscle diseases.

# CHAPTER 6:

# 6. RESULTS: Investigation of self-renewal of pericyte / mesoangioblast derived myogenic progenitors

This chapter describes the result achieved in a set of analyses complementary to the study on fate plasticity outlined in Chapter 5. The experiments shown above provide evidence of inducible lineage plasticity, where satellite cells are reprogrammed to pericyte-like cells. After having defined the differentiation capacity of a putative stem/progenitor cell population, it is critical to investigate its self-renewal potential. While it is reported that satellite cells can self-renew *in vivo*, even upon serial transplantation (Rocheteau et al., 2012) it is not known if pericytes/mesoangioblasts could do the same. Pericytes and mesoangioblasts have been shown to contribute to the satellite cell pool upon transplantation (Dellavalle et al., 2011; Diaz-Manera et al., 2010; Tedesco et al., 2011). The set of experiments detailed in this chapter was mainly performed to investigate the self-renewal potential of mesoangioblast-derived satellite-like cells, using serial transplantation in dystrophic murine skeletal muscle.

## 6.1. Wild type mesoangioblasts engraft host dystrophic skeletal muscles and generate clonogenic SM/C-2.6 positive and negative cells

A first series of transplantation experiments has been conducted using previously characterised wild type adult mesoangioblasts (C57-MABs; Diaz-Manera et al., 2010) as a proof-of-principle of the feasibility of the strategy. These cells were previously transduced with a lentiviral vector encoding for the GFP (driven by a constitutive PGK promoter) in order to trace them in the host muscle (Figure 28A). Scid/mdx mice were used as recipient animals for these experiments. The Scid/mdx is an immune-deficient model of DMD that allows transplant of non-syngeneic cells (Farini et al., 2007). C57-GFP MABs were transplanted intramuscularly ($10^6$ cells / injection) into tibialis anterior, gastrocnemius and quadriceps muscles of scid/mdx mice (n=4). One month after transplantation, the muscles were explanted and analysed under a fluorescent stereomicroscope to confirm the presence of GFP-positive areas (Figure 28B). The samples were then digested mechanically and enzymatically with the aim of isolating mononuclear cells. The cell suspension obtained with the digestion was then FACS-purified to retrieve donor-derived GFP positive and SM/C-2.6 positive (13.9%; 1.4 x $10^5$ cells) and donor-derived GFP positive and SM/C-2.6 negative cells (0.13%; 1.3 x $10^3$ cells) (Figure 28C). SM/C-2.6 is a monoclonal antibody reported to allow the separation of satellite and non-satellite cells (Fukada et al., 2004). Previous experiments performed in our laboratory confirmed the identity of the cells isolated with this method through immunofluorescence staining for the satellite cell marker Pax7 (Tedesco et al., 2011; Figure 4J).

The clonogenic assay is a well-established method for testing survival and proliferative capability of cells. The ability to form single cell clones is a shared property of both satellite cells and mesoangioblasts (Dellavalle et al., 2007; Molnar et al., 1996; Zammit et al., 2006a). For this reason the two donor derived cell fractions were subjected to single cell cloning and showed respectively a 20.1% and 68% ability of generating single cell clones (29/144 and 98/144 of the single cells seeded generated a clonal colony).

Experimental replicates of the transplanted muscles have been also analysed via cryosectioning and immunofluorescence, to confirm the presence of GFP co-localised with donor derived dystrophin positive myofibres (Figure 28D). Two randomly picked clones were subsequently expanded in culture, assessed for myogenic differentiation *in vitro* and transplanted with the same setup utilised for the polyclonal population. After one month, the muscles were explanted and processed for imaging as described above (representative example in Figure 28E). The results with this experiment have indicated the feasibility of the serial transplantation approach and have provided preliminary indication that the regenerative potential of donor mesoangioblasts is retained also at the clonal level.

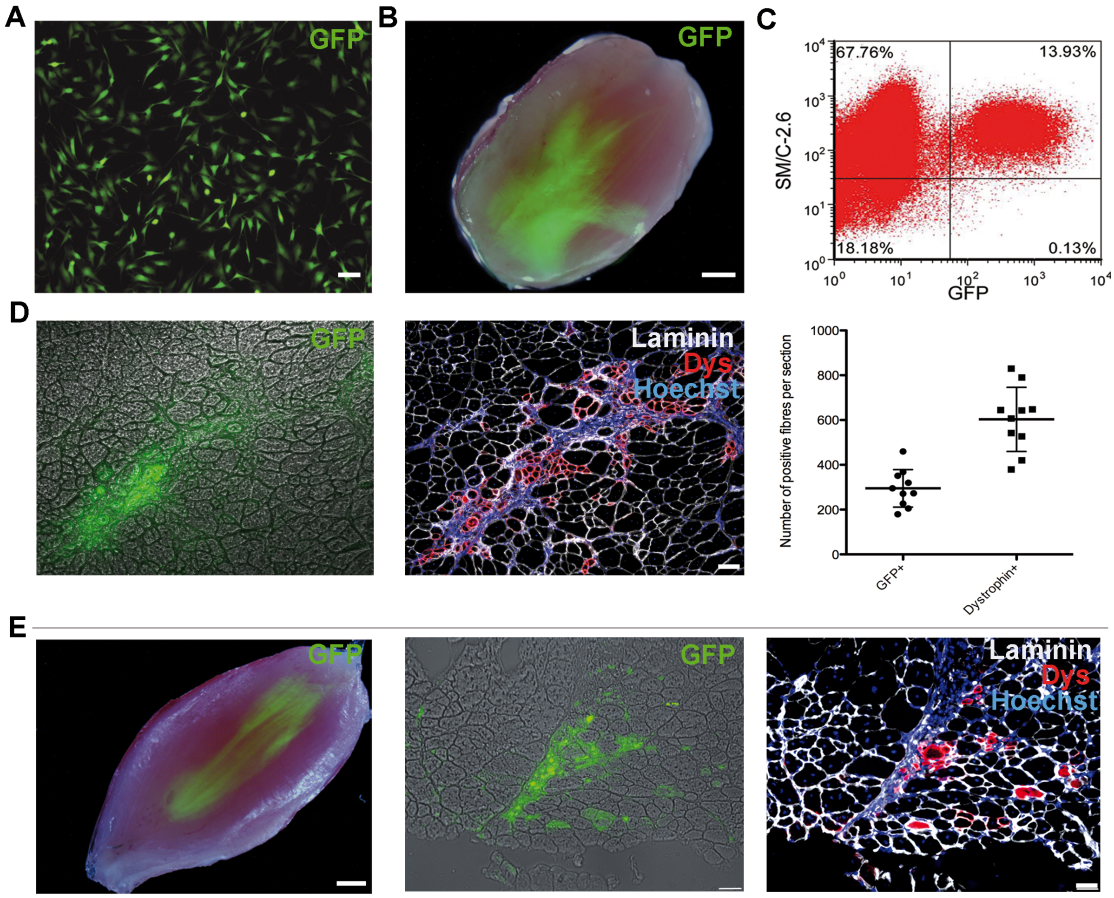



***Figure 28. Serial transplantation of wild type mesoangioblasts in dystrophic mice. A*** *Fluorescent microscopy image of the c57-GFP MABs utilised for this set of experiments (Scale bar: 50um).* ***B*** *Stereomicroscopic image showing the green area, engrafted by GFP-positive cells, in a tibialis anterior muscle explanted one month after transplantation (Scale bar: 0.5mm).* ***C*** *Dot plot of the FACS sorting performed on the digested muscles aiming to isolate the GFP and SM/C-2.6 fractions.* ***D*** *Section of a transplanted muscle showing the engrafted GFP positive area. This area co-localises with dystrophin-positive fibres traced via immunofluorescence (centre) and quantified on the plot on the right (Scale bar: 100μm)* ***E*** *The images show a muscle transplanted with a GFP positive SM/C2.6 positive clone. Sections of the muscles highlight co-localised positivity for GFP (central) and dystrophin (right) indicating the donor origin of the fibres (Scale bar: 0.5mm and 100μm).*

*This last part of the panel is part of the characterisation work currently on going, performed under my co-supervision by Chrystalla Constantinou.*

## 6.2. Dystrophic mesoangioblasts, genetically corrected with a human artificial chromosome, are serially transplantable

On the basis of the experiments with wild type mesoangioblasts, a second series of serial transplants utilising genetically corrected cells was performed. These cells, namely DYS-HAC mesoangioblasts, were previously generated from dystrophic mdx mesoangioblasts genetically corrected with a human artificial chromosome (HAC) containing the entire human dystrophin locus (Tedesco et al., 2011). EGFP was also included in the HAC as fluorescent marker to allow cell tracing in transplantation setups. These cells have been previously shown capable of long-term engraftment (8 months), generated Pax7 positive satellite cells and sustained muscle regeneration after acute injury of the transplanted muscles (Tedesco et al., 2011). Here we sought to formally assess the self-renewal capacity of these mesoangioblast-derived cells in a serial transplantation approach (For a schematic overview on the experiment see Figure 29A).

Transplantation of mesoangioblasts containing the DYS-HAC was performed in mdx/scid mice as described above. At variance with the experiments performed with wild type mesoangioblasts, in this series of transplants we administered 3 x $10^6$ cells / muscle, aiming to increase the number of isolated cells, to reduce the culture time required to obtain sufficient cells for the following transplantation. One month after the injection, the transplanted muscles were mechanically and enzymatically digested. The

derived cell suspension was FACS purified for EGFP and SM/C-2.6. Both SM/C-2.6 positive and negative fractions have been isolated, cultured and cryopreserved for future analyses. We then serially transplanted only the SM/C-2.6 positive donor derived fraction, assuming that this contained mostly satellite cell. The SM/C-2.6 negative population was harvested, cultured and cryopreserved for future analyses. The donor derived SM/C-2.6 positive cells were cultured and assayed for their myogenic potential *in vitro*. After one week in myogenic differentiation medium, cells generated large multinucleated myotubes positive for myosin heavy chain and human dystrophin (Figure 29B), indicating that a subpopulation of transplanted cells did not fuse with the host myofibres, maintaining an undifferentiated mononuclear state *in vivo* that allowed re-isolation. The transplanted muscles utilised to re-isolate the cells have been assessed for engraftment by stereomicroscopic fluorescence. This assay revealed the presence of EGFP positive areas in the transplanted muscles indicating the contribution of donor cells to the muscle regeneration (Figure 29C). As an additional confirmation of engraftment, a series of transplanted muscles was processed and cryo-sectioned in order to perform immunofluorescence staining to assess the expression of the human dystrophin (Figure 29D).

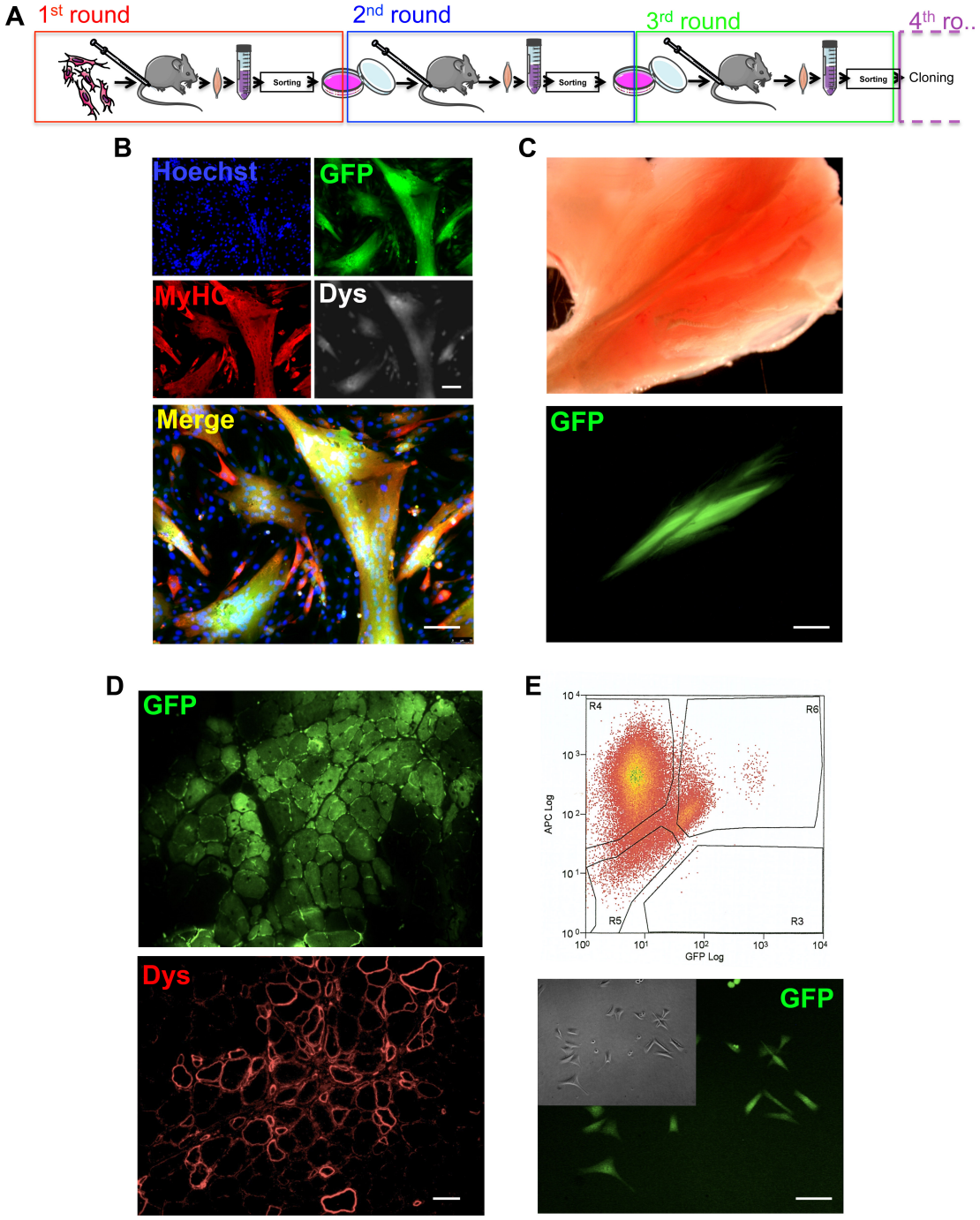



***Figure 29. Serial transplantation of genetically corrected DYS-HAC mesoangioblasts in scid / mdx mice. A*** *Experimental scheme of the serial transplantation of genetically-corrected DYS-HAC mesoangioblast-derived cells (Illustrations obtained from Servier Medical Art).* ***B*** *Immunofluorescence*

*staining of in vitro differentiated donor-derived Sm/c-2.6-positive cells isolated in light of this project. The cells show the expression of EGFP, myosin heavy chain (MyHC) and dystrophin (scale bars 120 μm).* ***C*** *Representative stereomicroscopic images showing bright field (top) and EGFP-positive area (bottom) of a gastrocnemius muscle explanted after the second round of transplant (scale bar 0.5 mm).* ***D*** *Cryostat section and immunofluorescent staining of the same muscle shows the expression of dystrophin co-localised with the EGFP engrafted area of a serial section (scale bar 100mm).* ***E*** *An example of a FACS-sorting dot plot obtained after digestion of replicates of the muscle shown in (C). The isolated double-positive cells are shown in the bottom part of the panel, both in phase contrast and by direct fluorescence (scale bar 120 μm).*

The SM/C-2.6 positive cells were also expanded in culture and serially transplanted intramuscularly in scid/mdx mice (n = 6). One month after the second round of transplantation, muscles were harvested. Some of the explanted muscles were included for histological analysis; the remainder were digested and isolated cells were FACS-sorted as described above. EGFP-positive areas visible upon analysis using the stereomicroscope suggested successful engraftment of donor cells in host muscles (Figure 30A). EGFP-positive and SM/C-2.6-positive cells were again cultured and used for a third round of serial transplantation in scid/mdx mice (n = 6), scaling down the cell number to 2 x $10^6$ cells / muscle. One month later, transplanted muscles were harvested, digested and FACS-sorted as described above (Figure 30B). Both the EGFP-positive / SM/C-2.6-positive and the EGFP-positive / SM/C-2.6-negative fractions were collected and amplified. We then performed a single cell cloning experiment with the aim of investigating if cells undergoing 3 rounds of serial transplantation retained their clonogenic ability. Both

populations generated single cell clones to a different extent (SM/C-2.6-positive: 23.61%, SM/C-2.6-negative: 65.6%). Interestingly, two clones (A9 and B5), obtained from the double positive fraction were expanded and transplanted into scid/mdx mice, scaling down the dose to $10^6$ cells / muscle (n = 4 / clone) (Figure 30C). Characterisation of the cryosectioned replicates from transplanted muscles and of the SM/C-2.6-negative fractions is on going as part of the project of Chrystalla Constantinou, an UCL MRes student that I am co-supervising, currently working in the laboratory. Overall these results indicate that genetically corrected mesoangioblast-derived satellite cells can support three rounds of serial transplantation, plus an additional one as single cell clones. Both SM/C-2.6 positive and negative (satellite-like and non-satellite-like) cells were generated at every step, providing indications on the ability of these cells to self-renew.

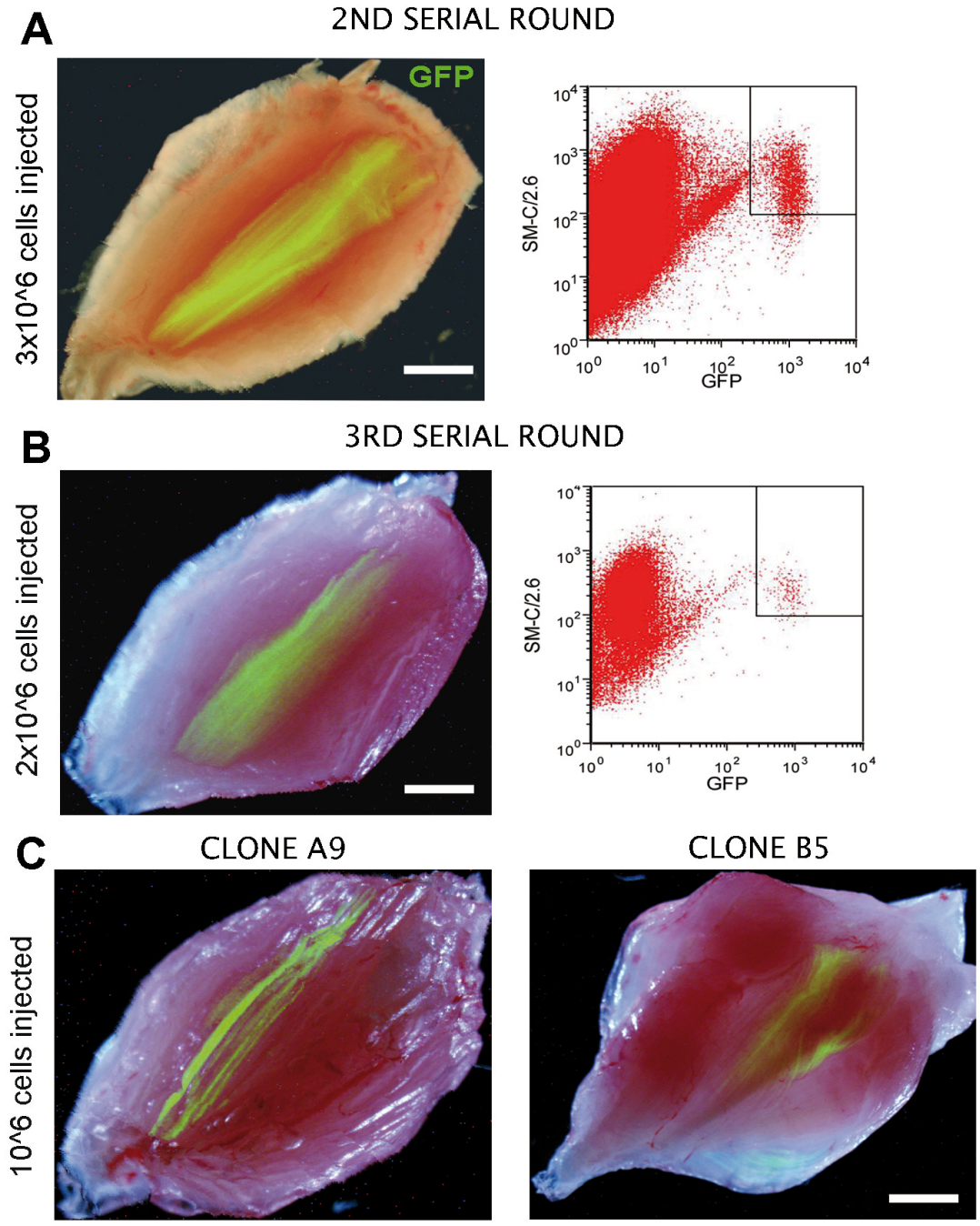



***Figure 30. Engraftment and isolation of DYS-HAC mesoangioblast-derived cells upon further rounds of serial transplantation.*** ***A*** *Stereomicroscopic image of a transplanted muscle at the 2nd round of transplantation, with the paired FACS sorting plot (Scale bar: 0.7 mm).* ***B*** *Stereomicroscopic image of a transplanted muscle at the 3nd round of transplantation, with the paired FACS sorting plot (Scale bar: 0.7 mm).* ***C***

*Stereomicroscopic image of two muscles that have been transplanted with cells expanded from single cell clones obtained with the sorting in B (Scale bar: 0.7 mm).*

## 6.3. Fresh isolation of primary pericytes for serial transplantation

To further test the hypothesis that pericytes are able to generate self-renewing cells *in vivo*, a series of experiments combining serial transplantation and lineage tracing experiments was recently established. Transplanting freshly isolated cells, we aim to eliminate the possible cell culture bias. Moreover, under physiological conditions, the number of resident myogenic progenitor is not as abundant as in a transplantation setup. For this reason, we decided to reduce the number of transplanted cells (down to $10^4$ cells / injection). Compared to the experiments based on cultured mesoangioblasts shown above, this fresh-isolation strategy aims to get closer to the natural process of muscle regeneration.

The isolation of primary pericytes from freshly explanted tissues was performed using the tissue non-specific alkaline phosphatase (TnAP)-CreERT2 mouse model (Dellavalle et al., 2011). In these animals the Cre recombinase is specifically expressed only in TnAP positive cells upon exposure to tamoxifen. To perform this series of experiments the TnAP-CreERT2 mice were crossed with a reporter mouse that carries a floxed fluorescent YFP cassette (Srinivas et al., 2001). Subcutaneous injection of the oestrogen receptor ligand Tamoxifen in juvenile mice activates the recombination and permanently label the TnAP positive cells with the YFP fluorescent marker as previously described (Dellavalle et al., 2011). The timeline of the administrations detailed in Figure 31A. Isolation of YFP-positive pericytes was performed using a FACS sorter; isolated cells were

viable and positivity for the YFP was confirmed by immunofluorescent staining (Figure 31B).

Transplantation of $10^4$ cells was performed in scid/mdx mice as outlined in the schematic representation in Figure 31C. One month after transplantation it was possible to isolate YFP-positive, SM/C-2.6-positive cells (representative sorting dot plot in Figure 31C. This confirmed that primary pericytes give origin to satellite-like cells *in vivo*, consistently with the data obtained in MAB cell lines.

A reported drawback of the YFP reporter is its faint brightness(Shaner et al., 2005). For this reason, together with the lower number of injected cells, made the identification of donor-derived cells within the transplanted muscles challenging (Figure 31D). However, the number of dystrophin-positive fibres in two transplanted tibialis anterior muscles was counted and found increased when compared to age-matched non-transplanted controls (Figure 31D, right; average number of dystrophin-positive fibres / section ± SEM: transplanted 55.43 ± 5.186; control 27.55 ± 2.38; 2 animals transplanted and analysed). Having age-matched controls (either littermates or an un-transplanted PBS injected contralateral muscle from the same animals) is relevant when working with scid/mdx mice. In this murine model, some muscle fibres undergo a spontaneous exon-skipping event that allows sporadic expression of a truncated form of dystrophin on the myofibres' sarcolemma. These myofibres, known as revertant fibres, create a "background noise" that might

be problematic in transplantation studies (Pigozzo et al., 2013), in particular when the number of transplanted cells is very limited as in this experiment.

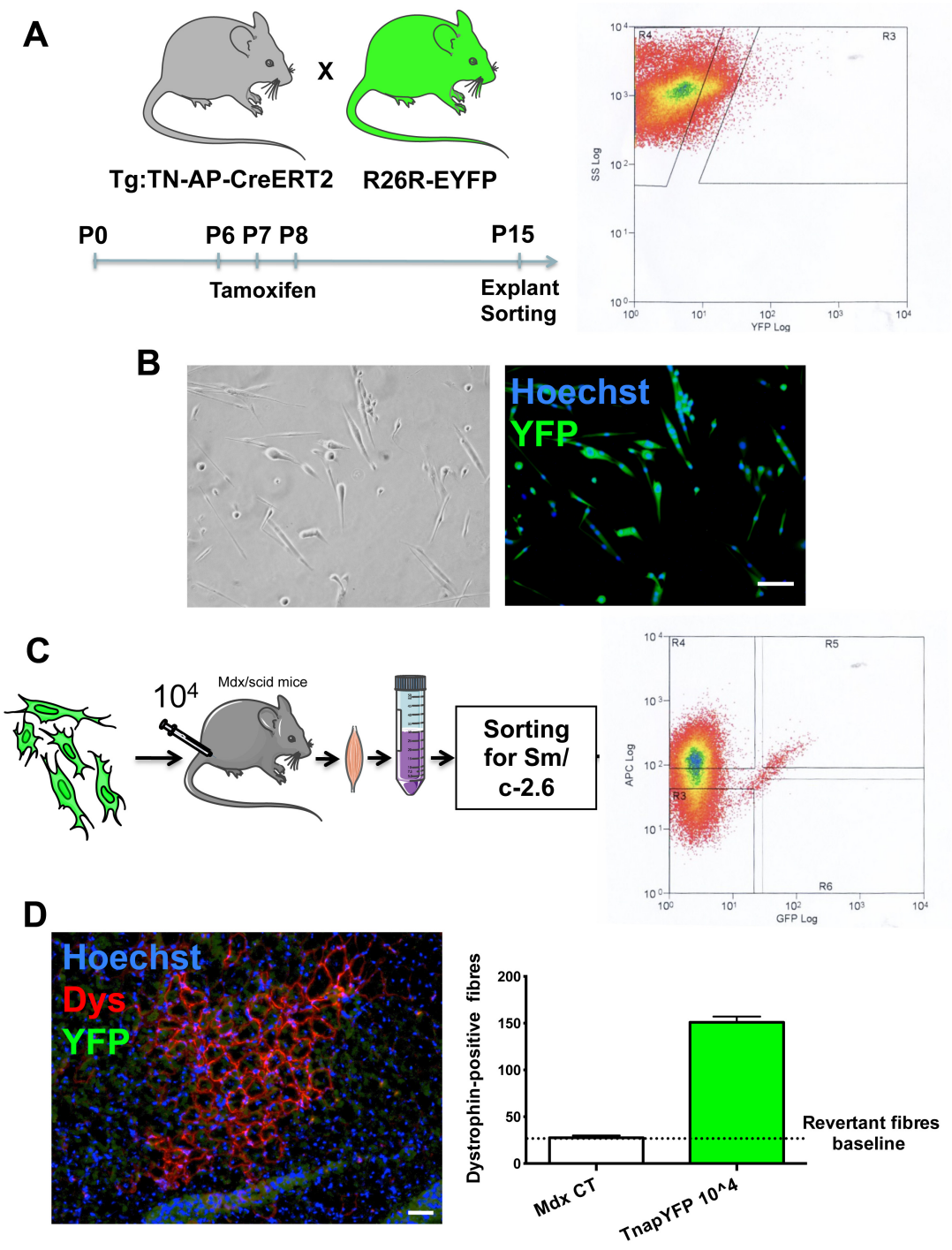



***Figure 31. Fresh isolation and transplantation of primary pericytes. A*** *Tg:TNAP-CreERT2 mice were crossed with R26R-YFP reporters (top),*

*Tamoxifen-induced Cre-Lox recombination was induced following the depicted timeline (bottom) and utilised to isolate primary pericyte. The right part of the panel depicts a representative sorting plot to isolate primary YFP-positive pericytes.* ***B*** *Phase contrast and immunofluorescence image of freshly-isolated YFP-positive pericytes obtained from the progeny of the crossing shown in A (scale bar 100 μm).* ***C*** *Schematic representation of the transplantation experiment performed using freshly-isolated pericytes. The right part of the panel shows the FACS-sorting dot plot of the cells obtained from the transplanted muscles. SM/C-2.6 positive satellite-like cells are labelled with an APC fluorochrome.* ***D*** *Immunofluorescence staining showing dystrophin and YFP expression on a section of muscle transplanted with TNAP-YFP pericytes (scale bar 100 μm). The graph on the right shows quantification of the number of dystrophin-positive fibres compared with the ones present in an age-matched untransplanted control, aiming to quantify the difference in comparison with the baseline number of revertant fibres normally present in the scid/mdx mice (Illustrations obtained from Servier Medical Art)..*

## 6.4. Conclusions

The serial transplantation experiments detailed in this chapter were designed to test the self-renewal potential of mesoangioblast-derived cells (presumably, donor-derived satellite-like cells). Wild type mesoangioblasts successfully engrafted the transplanted muscles giving rise to dystrophin positive fibres. These cells were able to generate *in vivo* SM/C-2.6 positive satellite-like cells (Figure 28A-D), which were able to generate single cell clones, capable to re-engraft dystrophic muscles also upon clonal expansion. This set of data provided indications on the self-renewal potential of mesoangioblasts *in vivo* (Figure E).

A more extensive series of serial transplantation experiments was performed using genetically corrected mesoangioblasts containing the DYS-HAC. These cells already showed a remarkable engraftment potential, the ability to regenerate the engrafted tissue upon serial injury and to contribute to the satellite cell niche (Tedesco et al., 2011). We report here the ability of these mesoangioblast-derived satellite-like cells to serially engraft the dystrophic muscles. These cells successfully supported three subsequent round of transplantation and re-isolation, generating at each round both donor-derived SM/C-2.6 positive and negative cells (Figure 29 and 30). Both fractions successfully generated single cell clones after three rounds of serial transplantation, indicating that the engraftment *in vivo* did not impair their clonogenic potential. Two randomly picked clones have also been expanded and transplanted. These cells engrafted the host muscles indicating their preserved myogenic potential (Figure 30C).

Overall these data indicate that mesoangioblast-derived satellite-like cells are capable of self-renewal upon serial transplantation. It is important to mention that due to the articulate process of chromosome transfer requiring long term culture and single cell cloning, the DYS-HAC mesoangioblasts have lost their spontaneous myogenic potential. For this reason, the starting cells have been previously transduced with a lentiviral vector containing MyoD as reported in Tedesco et al. (2011). Despite the constitutive expression of MyoD, we were able to isolate SM/C-2.6 positive and negative DYS-HAC mesoangioblasts-derived mononuclear cells at each round of transplantation. This indicates that a fraction of the donor-derived cells did not fuse with the host myofibres upon transplantation, acting as a progenitor-reservoir available for further rounds of regeneration despite the expression of a factor normally associated with commitment more than with self-renewal. This last observation is in line with what was published by Zammit and co-workers on the dynamics of the endogenous MyoD expression in primary mouse satellite cells (Zammit et al., 2004).

The preliminary results obtained with fresh isolation of TnAP-positive pericytes indicate that a serial transplantation approach could be used to investigate the self-renewal potential of these cells. However, indications obtained with the pilot experiment of transplantation / isolation of YFP cells suggested the use of a brighter fluorescent marker will be required to perform this series of experiments.

# CHAPTER 7:

# 7. Discussion

The data reported in this thesis aim to improve our understanding of myogenic progenitor biology, particularly focusing on muscle satellite cells and pericyte-derived mesoangioblasts, with the aim of developing efficacious and long-lasting cell therapy protocols to treat muscular dystrophies. A first phase of this project has been dedicated to establishing and optimising the available techniques to deliver cells via local intramuscular injection and through the vascular route of dystrophic mice. These improvements, detailed in chapter 4.1, allowed me to perform the in vivo experiments detailed in chapter 5 and 6.

The results reported in chapter 5 indicate that adult murine satellite cells can be conditionally reprogrammed to a pericyte-like fate, upon exposure to a combination of the Notch ligand DLL4 and the growth factor PDGF-BB. A similar approach, recently described by Cappellari and colleagues, demonstrated the acquisition of a pericyte-like phenotype by embryonic myoblasts (Cappellari et al., 2013). To our knowledge, this work represent the first report indicating that adult satellite cells retain a similar level of plasticity.

With this innovative approach we aimed to make the first steps to overtake the major hurdle faced in the development of cell therapy protocols

based on satellite cells / myoblasts. Although myoblasts are relatively easy to isolate and possess a remarkable myogenic potential, their limited migration ability compromised the results achieved in cell therapy protocols based on this cell type (Sampaolesi et al., 2003). Having cells with a strong migration potential is a crucial requirement to treat large muscular districts affected by pathologies like muscular dystrophies (Benedetti et al., 2013; Tedesco and Cossu, 2012). Pericyte-derived mesoangioblasts on the other hand, have been shown to contribute to muscle regeneration in different animal models of muscular dystrophy (Dellavalle et al., 2007; Diaz-Manera et al., 2010; Galvez et al., 2006; Gargioli et al., 2008; Giannotta et al., 2014; Sampaolesi et al., 2006; Sampaolesi et al., 2003) and are now undergoing clinical experimentation with a Phase I/II clinical trial (EudraCT no. 2011-000176-33).

Our results highlight that adult satellite cells treated with DLL4 and PDGF-BB show an increased pericyte marker expression (TnAP, NG2, SM22, PDGFr $\beta$ ), indicating that the level of plasticity so far observed only in embryonic myoblasts may be conserved in adulthood. Active Notch signalling was confirmed by the increased expression of Notch1 and its downstream targets Hes1 and Hey1, indicating the effectiveness of the treatment. The up-regulation of the PDGF receptors is a known phenomenon in cells with an active Notch signalling cascade (Jin et al., 2008). Our strategy might then activate a loop leading, through Notch stimulation, to an increased cell response to PDGF supplementation.

The gene expression analyses conducted by qRT-PCR (Figure 21 and 22) have been recently extended to a high-throughput gene expression profiling using RNA microarrays (Affymetrix GeneChip® *Mouse* Genome 430). This set of experiments, performed in collaboration with the team of Professor Enrico Tagliafico (Università di Modena e Reggio Emilia, Italy), aim to study the clustering of the treated cells in comparison with control untreated satellite cells and with control pericytes in a principal component analysis. Moreover, these on-going analyses will be extended to other muscle cell types, utilising dataset available in the literature (i.e. meta-analyses). Different sets of genes will then be analysed paying particular attention to molecules involved in cell migration, relevant signalling pathways, myogenic differentiation genes and other mesodermal progenitor markers. Novel potential pericyte marker genes, recently identified in a project currently on-going in the laboratory of Professor Giulio Cossu at the University of Manchester, will be also assessed for possible up-regulation (Moreno-Fortuny A. and Cossu G., unpublished results). The datasets generated in light of this collaboration will be also uploaded on the GEO repository to make it freely accessible to other researchers in the field. The results of these gene expression-profiling experiments will help to unravel the molecular mechanism of the fate switch observed in this project.

Several reports available in the literature have shown that transplanted freshly isolated cells (Montarras et al., 2005), myofibres (Collins et al., 2005) and even whole muscle biopsies (Zhang et al., 2014), have a superior engraftment ability when compared to cultured cells, mainly because

a more quiescent status (and/or the niche) is preserved. Our results suggest that, at variance with their embryonic counterpart (Cappellari et al., 2013), adult satellite cells may acquire a more “quiescent-like” gene expression profile upon DLL4 and PDGF-BB treatment. This is reflected in the down-regulation of the activation marker MyoD and in a low but consistent up-regulation of the quiescence marker Pax7 (no statistical significance observed for this gene). This gene expression profile resembles also the one observable in freshly isolated satellite cells. We believe this supported the improvement in the engraftment efficiency observed in our proof-of-principal transplantation experiments. This improved engraftment is also consistent with what observed in other studies on Notch-stimulated satellite cells and mesoangioblasts (Parker et al., 2012a; Quattrocelli et al., 2014). Consistently with our “increased quiescence” theory, treated cells showed also a reduction in their proliferation and myogenic potential in vitro. These observations complement what previously reported in other studies involving Notch up-regulation in myogenic progenitors (Parker et al., 2012a; Quattrocelli et al., 2014; Wen et al., 2012). Our results confirmed also that these phenomena are signalling-dependent. However, these variations did not affect the cell expansion required to perform in vivo experiments.

It has to be mentioned that for the reported series of qRT-PCR experiments GAPDH has been used as normalizer. Although its use is commonly accepted, a recent report from Hildyard and Wells indicated that this gene might not retain the same level of expression in differentiating myogenic cells and it might not be an ideal gene expression normaliser. For

this reason the use of Csnk2a2 and Ap3d1 will be taken into account for future experiments involving myogenic cells (Hildyard and Wells, 2014).

Similarly to *bona fide* pericytes, treated cells were capable to stabilise endothelial networks *in vitro.* This indicated that the treatment not only induced pericyte marker expression, but also that the cells acquired functional pericyte properties. As an additional validation experiment, future analyses might include *in vivo* Matrigel plug assays. In this technique, a mixture of HUVEC, treated / untreated cells and Matrigel is administered subcutaneously to immunodeficient mice to assess the formation of a vascular network (Akhtar et al., 2002).

The migration ability of pericyte-derived mesoangioblasts led to the development of a cell therapy protocol recently used in a phase I/II clinical trial (EudraCT no. 2011-000176-33). Our results indicated that the pericyte-like phenotype acquired by our treated satellite cells was reflected also in an improved migration through artificial endothelial layers *in vitro*. In light of these results we performed a proof-of-principle intra-arterial transplantation experiment. Notably, three weeks after transplantation we have identified donor-derived cells that migrated towards the blood vessel walls downstream of the injection site and contributed to muscle regeneration.

With the aim of improving the migration ability observed in treated satellite cells even further, a proposed follow up to this project could include testing a combination of molecules known to stimulate cell migration such as Diprotin A, Adiponectin or SDF1 (Fiaschi et al., 2010; Galvez et al., 2006;

Parker et al., 2012b). Moreover, a recent work published by Giannotta, Benedetti and colleagues showed that pharmacological inhibition of the GTPase Rap1 significantly increase engraftment and migration ability of intra-arterially transplanted mesoangioblasts impeding the tightening of endothelial cell junctions targeting the molecule JAM-A (Giannotta et al., 2014). It is not unconceivable that this mechanism, in combination with the strategies mentioned above, might also be exploited to improve the migration of cells obtained with the protocols detailed in this thesis.

It will be important, as a follow up of this study, to assess if the Notch / PDGF stimulation elicits a similar response in human cells. Human myoblasts, isolated on purpose or obtained from bio-banks are currently being subjected to the stimulation aiming to investigate their possible fate plasticity. A pilot experiment we recently performed treating human myoblast indicates that the treatment has no visible effect on morphology, induces an up-regulation of the alkaline phosphatase activity (Figure 32A) but appears not to alter the myogenic differentiation potential of the cells (Figure 32B). Although a more systematic analysis of additional cell population may be required, this preliminary experiment suggests that further studies are needed to adapt the treatment conditions to human cells.

If an adaptation of this protocol to human cells will be successful it may allow the generation of a novel hybrid human pericyte-like cell type with relevance for possible future clinical applications. The ideal cells will harbour, upon treatment, the key properties of both pericytes and satellite cells:

namely, the ability of crossing the vessel upon systemic delivery combined with high myogenic potential and ability to self-renew.

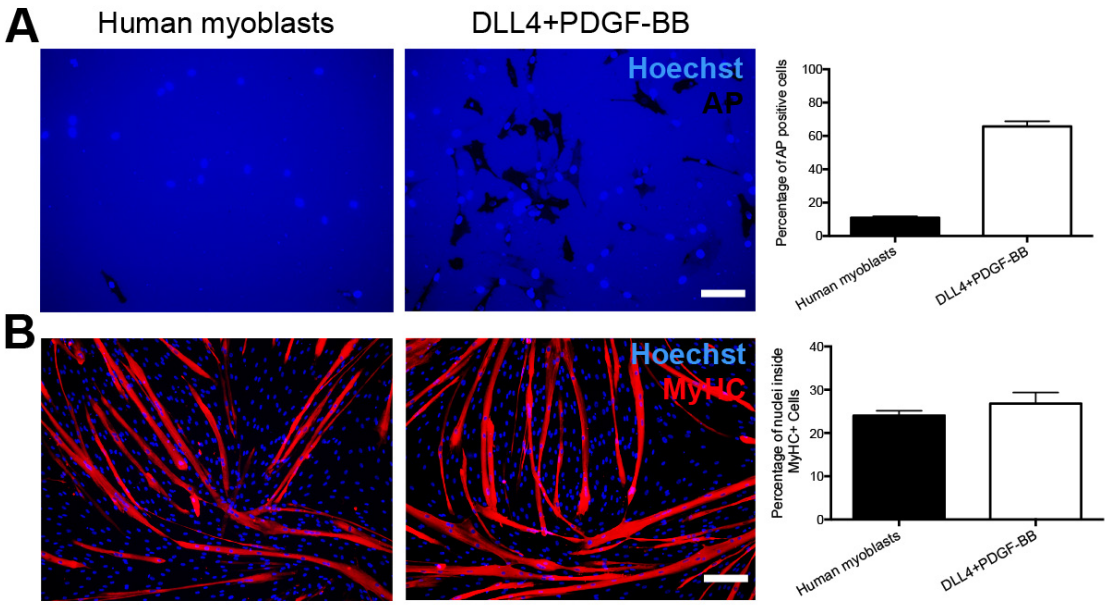



***Figure 32. Assessment of alkaline phosphatase activity and myogenic differentiation potential of treated human myoblasts.*** *The panel shows the results of a preliminary experiment conducted using one line of human myoblasts.* ***A*** *The image shows a staining for the alkaline phosphatase activity on untreated and treated human myoblasts (Scale bar: 100um). The percentage of AP positive cells is quantified on the right (Untreated 10.96% ± 0.84 SEM; Treated 65.72% ± 3.05 SEM).* ***B*** *Immunofluorescence staining for Myosin Heavy Chain performed on untreated and treated human myoblasts subjected to terminal myogenic differentiation assay (Scale bar: 140um). The graph depicts the percentage of cells within the (Untreated 24.03% ± 1.11 SEM; Treated 26.86% ± 2.51 SEM).*

The self-renewal potential of satellite cells has been formally demonstrated by Rocheteau and colleagues in a rigorous serial transplantation setup (Rocheteau et al., 2012). Previous reports from our group indicated that pericyte- and mesoangioblast-derived cells repopulate the satellite cell niche

and generate Pax7 positive cells during muscle regeneration and upon transplantation (Dellavalle et al., 2011; Tedesco et al., 2011). Mesoangioblasts were also capable of sustaining long-term engraftment and a subsequent round of regeneration upon acute myoinjury (Tedesco et al., 2011).

Understanding if pericytes are capable of generating self-renewing cells upon transplantation has a crucial importance, considering the impact this might have on novel cell therapy protocols arising for the recent mesoangioblast-based clinical trial. Moreover, investigating if pericyte-like cells possess this property, is also important in light of the results detailed in chapter 5 in which satellite cells are shifted towards that lineage. The experiments reported in chapter 6 were designed to investigate if mesoangioblasts are capable of generating self-renewing satellite-like cells by means of serial transplantation assays.

Wild type mesoangioblasts engrafted the host muscles and generated satellite-like cells. It was then possible to re-isolate these cells that re-engrafted dystrophic muscles and generate single cell clones. We extended these findings to genetically corrected mesoangioblasts, which allowed the generation of serially transplantable satellite-like cells up to three round of transplantation. We subjected the cell population isolated from the third round of transplants to single cell cloning and then re-transplanted two of these randomly picked clones, with the aim of confirming the potency of engraftment also at the clonal level. Genetically corrected mesoangioblasts

successfully supported three subsequent round of transplantation and re-isolation, generating at each round both donor-derived SM/C-2.6-positive (satellite) and negative (non-satellite) cells. With the exception of a single report from Sacco and colleagues (Sacco et al., 2008), transplantation of a single cell in the skeletal muscle is known to be extremely challenging (Rocheteau et al., 2012). Experiments using donor-derived single cell clones obtained from transplanted muscles could recapitulate the behaviour of a single cell, subjected to subsequent rounds of amplification during tissue regeneration, indirectly providing indications on the potential of these cells.

Replicates of the muscles serially transplanted with genetically corrected DYS-HAC mesoangioblast have been cryopreserved and are now being cryosectioned and analysed by immunofluorescence staining and molecular biology techniques (such as qRT-PCR) to evaluate the efficiency of the engraftment. This characterisation is part of a project developed under my co-supervision by Chrystalla Constantinou (UCL MRes student).

The main limitation of this set of experiments was the use of cells lines. Although these cells have been utilised and characterised from recent previous studies, the long-term culture might have introduced a bias in our evaluation of their potency. With the aim of overcome this limitation we decided to perform a series of serial transplantation experiments using freshly isolated cells, obtained crossing the TnAP-CreERT2 mouse with the R26R-YFP reporter. Isolation of YFP-positive pericytes was performed using the FACS sorter but although the cells were viable and positive for the YFP, the

weakness of the fluorescence of this reporter, impeded tracing the cells in the transplanted muscles.

To overcome the limitation in the ability to trace the cells, pericytes have been recently isolated in the laboratory from TnAP-CreERT2-R26R-tdTomato mice. These animals were obtained upon crossing the TNAP-CreERT2 mice with a reporter mouse that carries a tdTomato fluorescent cassette, whose fluorescence is brighter than the one of the YFP (Madisen et al., 2010; Shaner et al., 2005). With the aim of improving the detection of few donor cells in a dystrophic muscle, the freshly isolated TNAP-tdTomato pericytes will be transplanted in α-sarcoglycan-null/scid/beige mice (Tedesco et al., 2012), excluding the bias of the revertant fibres not present in this model.

Serial transplantation of freshly isolated pericytes will be performed for at least three rounds of transplantation / isolation aiming to validate what observed with the two mesoangioblast cell lines. A single-cell cloning assay will also be performed on the serially transplanted TnAP-tdTomato pericyte-derived cells to analyse if their clonogenic potential is in line with the observations reported in this thesis. Some of these experiments have been recently performed under my co-supervision as part of Chrystalla Constantinou MRes project. These results will be included as original data in her final dissertation.

The data obtained so far provided indications that mesoangioblast-derived satellite-like cells are capable of self-renewal in a serial

transplantation setup. Notably donor-derived SM/C-2.6-negative myogenic cells were present and have been isolated from each isolation experiment performed. The generation of pericytes/mesoangioblasts *in vivo* upon transplantation of satellite-like cells cannot therefore be excluded. While the possibility of performing serial transplantation experiments with these cells is currently under consideration, the characterisation of these negative fractions is in the pipeline. If these results will indicate the presence of satellite cell-derived mesoangioblasts in the negative fractions, the mechanism underlying this fate switch will be investigated aiming to dissect the biological relevance of this phenomenon.

Meaningful insights about the mechanisms guiding this fate plasticity *in vivo* will come also from the microarrays performed for the project described in chapter 5. These results might provide significant information that will help to unveil what rules the homeostatic balance between satellite cells and pericytes. Particular attention will be given in comparing the signals in use to induce the fate switch *in vitro* (Notch activation and PDGF-BB) with the ones involved in the self-renewal mechanism, aiming to investigate a possible correlation between the two phenomena.

# CHAPTER 8.

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# CHAPTER 9:

# 9. APPENDIX