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**microRNAs as molecular tools modulating
muscle differentiation and regeneration**

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ABBREVIATION

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AGO2: Argonaute 2	FSC: Founder Stem Cells
ATP: Adenosine Triphosphate	GLI3: GLI Family Zinc Finger 3
BMD: Becker Muscular Dystrophy	GM: Growth Media
Ccn: Cyclin	Hdac: Histone Deacetylase
Cdc: Cell division cycle	HEY1: Hesr1
Cdkn2a: Cycle regulator p16INK4a	HEYL: Hesr3
CHD4: Chromodomain Helicase DNA Binding Protein 4	HGFA: Hepatocyte Growth Factor Activator
CTX: cardiotoxin	hMuSC: Human Muscle Stem Cell
Cx43: connexin43	IGF: Insulin-like Growth Factor
DGC: Dystrophin associated Glycoprotein Complex	Igfbp5: Insulin Growth Factor-Binding Protein-5
DLL: Delta-Like	IL: Interleukin
DMD: Duchenne Muscular Dystrophy	JAG: Jagged
DMT: Dermomyotome	KDR: Kinase Domain Receptor
ECM: Extracellular Matrix	LRC: Label Retaining Cell
EDL: Extensor <i>Digitorum Longus</i>	Mcm7: Minichromosome maintenance Complex Component 7
EMT: epithelial-mesenchymal transition	miR: microRNA
eMyHC: embryonic Myosin Heavy Chain	miR-106b: miR-106b-5p
FBS: Fetal Bovine Serum	MMPs: Matrix Metalloproteinases
FISH: Fluorescence <i>In Situ</i> Hybridization	MRF: Myogenic Regulator Factor

mRNA: Messenger Ribonucleoprotein Acid

mRNP: Messenger Ribonucleoprotein

MT: Myotome

MyHC: Myosin Heavy Chain

MYOG: Myogenin

NF- κ B: Nuclear Factor-kappa β

NICD: Notch Intracellular Domain

PARD: Partitioning Defective Complex

PDGF: Platelet-Derived Growth Factor

Pdgfr β : Platelet-Derived Growth Factor
Receptor beta

PFA: Paraformaldehyde

PKC: Atypical Protein Kinase C

pri-miRNA: primary-miRNA

P/S: Penicillin/Streptomycin

PSF: P/S-Fungizone

RBPJ: Recombining Binding Protein
Suppressor of Hairless

RISC: RNA- Induced Silencing Complex

ROS: Reactive Oxygen Species

RT: Retrotranscription

S1p: Sphingosine-1-phosphate

SCs: Satellite Cells

Sfr: Splicing Factor

SIRT1: Sirtuin1

Srf: Serum Response Factor

TA: *Tibialis Anterior*

ULK1: Unc-51 Like Autophagy Activating
Kin

ABSTRACT

Satellite cells (SCs), the skeletal muscle stem cells, display behavioural heterogeneity, and several defects related to their regenerative potential are linked to muscle-wasting diseases, such as Duchenne Muscular Dystrophy (DMD) or ageing. SC behaviour is associated to endogenous expression of the myogenic transcription factor *Myf5* and their ability to activate and enter into the cell cycle. MicroRNAs (miRs) play an essential role regulating the regenerative potential of SCs in homeostasis and disease, as well as modulating developmental myogenesis. In this doctoral Thesis, we explore the role of miR-106b-5p (miR-106b) during muscle regeneration, muscular wasting diseases and developmental myogenesis. We demonstrate that the miR-106b acts reinforcing *Myf5* inhibition and preventing cell proliferation in a subset of highly quiescent SCs. miR-106b expression decrease during SC activation and its down-regulation is required for effective muscle repair. Interestingly, we also show that miR-106b expression levels are increased in mdx dystrophic mice. Intramuscular administration of antimiR-106b in two different injured dystrophic mouse models enhances muscle regeneration inducing transcriptional changes related to skeletal muscle differentiation and reducing inflammatory response. Additionally, antimiR-106b treatment enhances the engraftment of human muscle stem cells (hMuSCs). Importantly, miR-106b is also elevated in dystrophic hMuSCs and antimiR-106b treatment ameliorates intrinsic proliferative defects and enhances their myogenic potential. Besides, we demonstrate that miR-106b is also up-regulated in muscles from elderly mice, and miR-106b inhibition promotes proliferation and myogenic potential in aged SCs. Overall, our analyses reveal that miR-106b is an important regulator of SC behaviour, and miR-inhibition could be proposed as a new therapeutic approach to promote muscle regeneration and enhance the regenerative capabilities of SCs in the context of several muscular disorders such as DMD and sarcopenia. On the other hand, SC behavioural heterogeneity in adult skeletal muscles could be linked to their developmental origin. In this context, we observed that miR-106b is expressed in a subset of undifferentiated myogenic progenitor cell population during embryonic and fetal myogenesis indicating that miR-106b might be playing a role defining quiescence *versus* differentiation in myogenic precursors during development.

CHAPTER I: INTRODUCTION

1 - Skeletal muscle

1.1 - Structure and function

The skeletal muscle represents between 30-40% of our body mass, being the main tissue in the human body. It covers crucial functions like respiratory mechanics, metabolic functions, voluntary movements and help maintain posture and balance (Mukund and Subramaniam, 2020).

The basic unit of the skeletal muscle is the myofiber, a multinucleated syncytium resulted from the fusion of myoblasts (mononucleated muscle cells). The myofiber is surrounded by its plasma membrane, named sarcolemma, and its cytoplasm, denominated sarcoplasm. Each myofiber is formed by a set of myofibrils, bundles of proteins filament that will allow the muscle contraction. Several myofibers join to form fascicles and various fascicles bundle together to form the muscle (figure 1) (Mukund and Subramaniam, 2020). Skeletal muscle is an active tissue highly irrigated by blood vessels and surrounded by three layers of the extracellular matrix (ECM) that provide support: the endomysium which surrounds each myofiber, the perimysium that surrounds fascicles and the epimysium that surrounds the muscle and join it to the bone through the tendon (figure 1) (Biga et al., 2019; Gillies and Lieber, 2011).

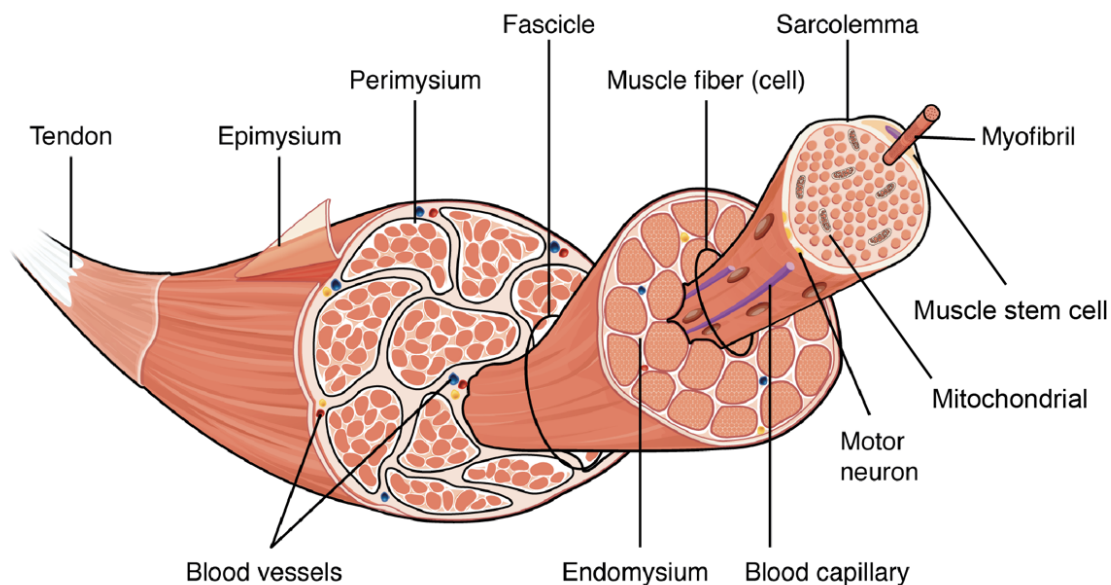


Figure 1. Skeletal muscle structure. The three connective tissue layers: each myofiber is covered by the endomysium; groups of myofibers (fascicle) are covered by the perimysium; and bundles of fascicles are covered by the epimysium. Modified from Biga *et al.*, 2019.

In the skeletal muscle, the smallest functional unit for contraction is the sarcomere, a complex structure inside the myofibrils consisting of intercalated thin filaments (formed by α -ACTIN, TROPONIN and TROPOMYOSIN proteins) and thick filaments (formed by MYOSIN and corresponding proteins) parallel to fiber axis. The alternation of thin and thick filaments leads the skeletal muscle a striated appearance that divide the myofibril in light and dark bands (figure 2A) (Biga et al., 2019; Fraile-Láiz et al., 2012). In the sarcomere, the MYOSIN filaments superposed to the ACTIN filaments constitute the dark A-band, with a central part denominated H-zone where thin filaments do not overlap. Besides, where the ACTIN filaments are not superimposed by the MYOSIN filaments constitute the light I-band, situated in the sarcomeres' edges and shared by adjacent sarcomeres. Accordingly, myofibrils are formed by sarcomeres connected in series that form an alternation of I-bands and A-bands (figure 2B) (Mukund and Subramaniam, 2020).

Myofiber sarcoplasm includes different cellular organelles that will contribute to muscle contraction, such as multiple mitochondria that produce energy (adenosine triphosphate, ATP) or the specialized smooth endoplasmic reticulum known as sarcoplasmic reticulum that is located around the myofibril (figure 2B). Sarcoplasmic reticulum is responsible for the storage, release, and retrieval of calcium (Ca^{2+}) ions, essential during muscle contractions. The mechanical contractions begin after excitation-contraction coupling at the neuromuscular junction, synaptic connection between the motor nerve and the myofiber. In this regard, muscle fiber receives the neural signal and subsequently Ca^{2+} ions are released from the sarcoplasmic reticulum to bind the TROPONIN-C, part of the thin filaments (figure 2B). Ca^{2+} ions binding to the TROPONIN-C implies a conformational change in the thin filaments (ACTIN subunits) and the binding sites for myosin are uncovered. Afterwards, ATP molecules bind to the MYOSIN filaments (thick filaments) and the MYOSIN head can finally bind to the ACTIN subunits (thin filament). When ATP is hydrolysed, the heads of MYOSIN filaments move forwards into the ACTIN filament, which results in ACTIN filaments slide through MYOSIN filaments. During ACTIN and MYOSIN filament sliding, I-bands get shorter and in the end, each sarcomere reduces its length, which collectively results in muscle contraction (Huxley and Kress, 1985; Kuo and Ehrlich, 2015).

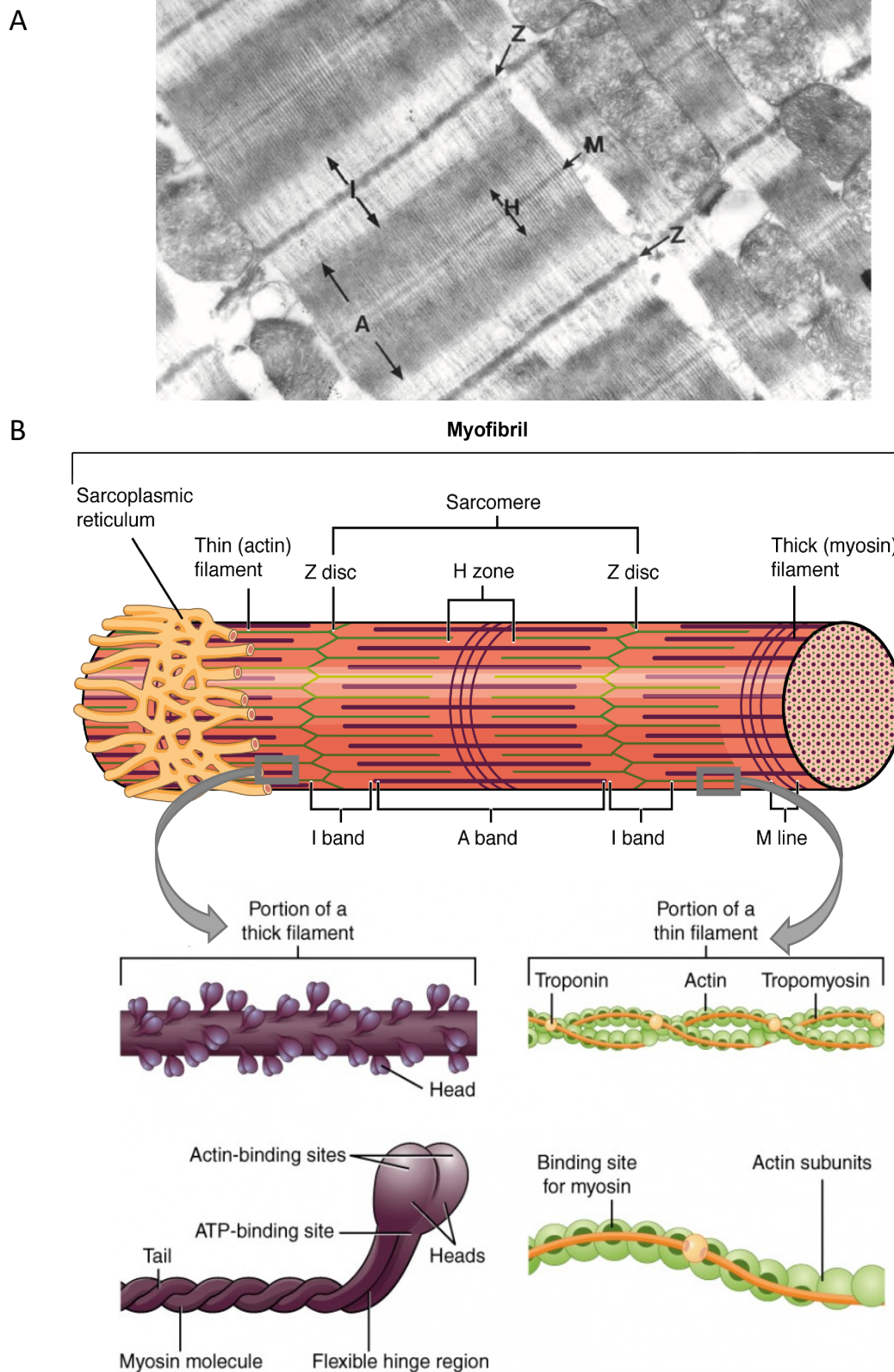


Figure 2. Striated skeletal muscle sarcomere. (A) Electron micrograph of striated skeletal muscle showing light bands (I-band with Z-disc in the middle) and dark bands (A-bands with H-zone in the middle and M-line in the centre). Taken from Fraile-Láiz et al., 2012. (B) Scheme of the sarcomere. The sarcoplasmic reticulum surrounds the myofibril. Thin filaments are formed by ACTIN proteins and thick filaments are formed by MYOSIN proteins. Thin and thick filaments intertwine to form the sarcomere. The striated appearance of skeletal muscle is due to the light and dark regions of the sarcomere that subdivide it into I-bands and A-bands, which in turn are constituted by the Z disc, M line and H zone. Modified from Biga et al., 2020.

2 - Muscle stem cells, satellite cells (SCs), and adult myogenesis

Skeletal muscle is one of the tissues in the human body that is capable of self-renewal and fully repair after a damage or an injury. Skeletal muscle regeneration is a complex and orchestrated process that include an inflammatory response and the intervention of several cellular types (Forcina et al., 2020). However, a successful skeletal muscle regeneration relies on SCs, the muscle stem cells, which in resting conditions, are located between basal lamina and sarcolemma (figure 3A-B) (Mauro, 1961). There are other types of resident cells in the skeletal muscle (interstitial cells, endothelial cells, pericytes, etc.) that can differentiate into muscle cell progenitors contributing to repair the tissue (Mitchell et al., 2010; Tedesco et al., 2010); nonetheless, in the absence of SCs, the muscle regeneration is compromised (Sambasivan et al., 2011).

In adult skeletal muscle, SCs remain in a quiescent status characterized by the expression of the paired box transcription factor *Pax7*, essential for its specification (figure 3C) (Seale et al., 2000). However, the role of PAX7 in SC biology has been largely controversial (Oustanina et al., 2004). In this regard, Lepper *et al.* have shown that *Pax7* conditional inactivation in adult SCs did not compromise SC function or its regenerative capability. They have observed that *Pax7* expression was only required during perinatal period to set quiescence status in juvenile SCs in mice (from birth to 21 days of age) (Lepper et al., 2009). Nevertheless, subsequent studies, using similar mouse models, called into question Lepper's observations, demonstrating that *Pax7* expression in SCs is crucial for regulate myogenic potential and SC function in the neonatal and adult skeletal muscle (Günther et al., 2013; von Maltzahn et al., 2013). Accordingly, full *Pax7* ablation in adult mice suppressed muscle regeneration after injury, identifying *Pax7* as an essential transcription factor for SC maintenance and function (Lepper et al., 2011; Sambasivan et al., 2011). These observations let to the conclusion that PAX7^{pos} SCs are an essential requirement to ensure muscle regeneration throughout life.

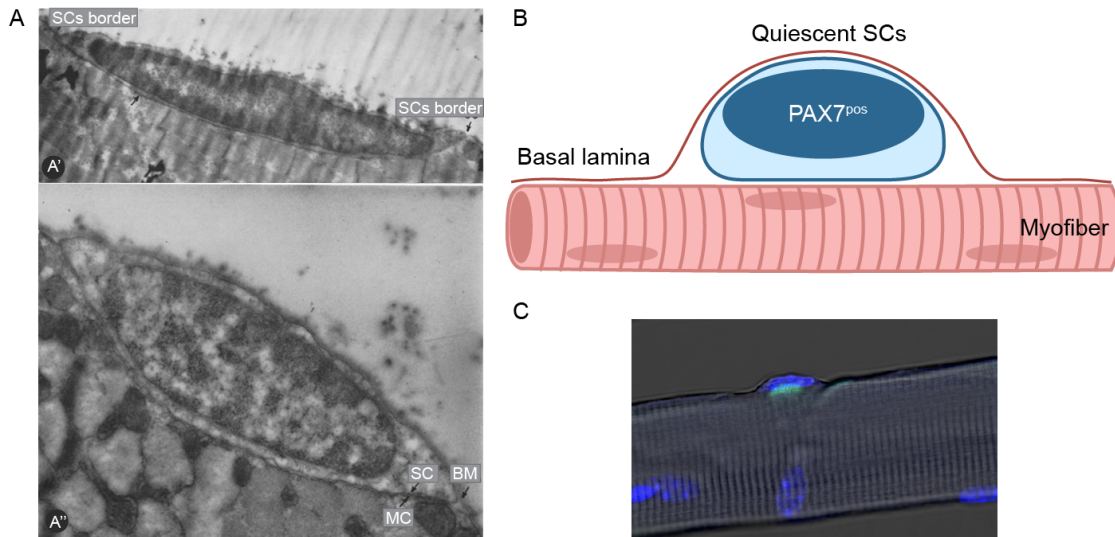


Figure 3. Quiescence satellite cell (SC). (A) Electron micrograph of the SC. (A') View of one SC in the periphery of the skeletal muscle fiber of the Tibialis anterior of the frog. (A'') Transversal section of a SC and skeletal muscle fiber from the rat sartorius. MC: muscle cell; BM: basal membrane. Modified from Mauro, 1961. (B) Scheme of quiescence SC between the basal lamina and the sarcolemma of the myofiber. In resting conditions, quiescent SCs are characterized by the expression of transcription factor PAX7. (C) Immunostaining of fresh isolated myofiber from C57BL/6 mouse showing a quiescent SC: PAX7 (green) and nuclei with DAPI (blue).

2.1 - SCs in quiescence

In resting conditions SCs remain in a reversible quiescence status termed as G_0 . These quiescent SCs display a low cytoplasmic-nuclear ratio and low metabolic activity (Cheung and Rando, 2013); however, its quiescence can be defined as an active status of inactivity that needs a complex surveillance mechanisms that involves around 500 genes (Fukada et al., 2007).

In this regard, Notch pathway is a well-known master regulator of SC quiescence (Gioftisidi et al., 2022). *Notch* gene family is formed by 4 transmembrane receptors (NOTCH1, 2, 3 and 4) that mediate cell-cell communication by binding to different ligands, such as Delta-Like (DLL)-1, expressed in myofibers, DLL-4, JAGGED (JAG)-1 and JAG-2. After Notch activation, the ligand-triggered intramembrane proteolysis cleaves the Intracellular Domain of NOTCH (NICD), that is translocated into the nucleus to act as a coactivator of gene transcription (Gioftisidi et al., 2022). Activation of *Notch* pathway promote SC quiescence status as, once NICD is released, it interacts with the Recombining Binding Protein Suppressor of Hairless (also referred as RBPJ) that directly upregulates the expression of transcription factor *Pax7*, essential in SC identity and self-renewal (Wen et al., 2012). Bjornson et al. have observed that after SC-specific deletion of *Rbpj*, required for Notch signalling, SC pool was depleted in skeletal muscles. Deficient *Rbpj* SCs were activated automatically and underwent myogenic differentiation without previous proliferation;

moreover, deficient *Rbpj* SCs couldn't re-enter into quiescence (Bjornson et al., 2012). This study supports the critical role of Notch signalling in SC self-renewal. Besides, Fukada *et al.* have shown that Notch pathway activation can promote the transcription of *Hesr* genes family, *Hesr1* (*Hey1*) and *Hesr3* (*Heyl*), that regulates the self-renewal of SCs during muscle homeostasis. HEY1 and HEYL maintain SCs in an undifferentiated quiescent status that preserve SC pool through life (Fukada et al., 2011). In accordance with these observations, when Notch signalling is permanent inhibited, SCs go through activation and myogenic differentiation, resulting in a fatal stem cell pool reduction that leads to muscle mass loss, similar to what is observed in sarcopenia (muscle mass loss due to ageing) (Bjornson et al., 2012; Fukada et al., 2011; Mourikis et al., 2012). Conversely, NICD overexpression in SCs downregulates *Myod1* expression, a master regulator of myogenic commitment, thus leading to a reduction of myogenic differentiation potential together with a decrease in SC proliferation (Wen et al., 2012). Altogether, these findings identify Notch pathway as a fundamental regulator of quiescence and self-renewal that maintain quiescence SC pool throughout life (figure 4).

There are other regulator factors that either maintain SC quiescence or promote cell-cycle exit to G_0 status after SC activation. For instance, the inhibitor of receptor tyrosine kinase signalling SPROUTY1 (SPRY1) maintains SC quiescence by inhibiting the ERK signalling pathway. *Spry1* is downregulated in activated SCs to allow proliferation of myogenic cells, and *Spry1* expression is reinduced again in some PAX7^{pos} cell, promoting cell-cycle exit and return activated SCs into quiescence status (figure 4) (Shea et al., 2010). Likewise, Sreenivasan *et al.* have shown that the chromatin remodel CHD4 (Chromodomain Helicase DNA Binding Protein 4), a member of the nucleosome remodelling and deacetylase repressive complex, plays a key role in maintaining transcriptional SC identity as stem cell during its activation, which ultimately avoid SC pool depletion. When CHD4 is conditional depleted, muscle repair fail after several rounds of damage since SC pool is exhausted (Sreenivasan et al., 2021). These studies emphasize the importance of maintaining stem cell identity in SC population for a correct muscle homeostasis.

After a stimulus, SCs can directly activate and re-enter in the cell cycle G_1 to proliferate and increase the number of myogenic progenitor, or remain in a pre-activated status prior to cell cycle re-entry named G_{Alert} (figure 4) (Rodgers et al., 2014; van Velthoven and Rando, 2019). Rodgers *et al.* have described that this G_{Alert} status allows a rapid SC response after an injury or muscle stress (Rodgers et al., 2014). They have also shown that after muscle injury in one mouse hindlimb, the SCs from the contralateral hindlimb reach the G_{Alert} status, which prepares them for an upcoming muscle repair if necessary, enhancing muscle regenerative potential (Rodgers

et al., 2014). Pre-activated G_{Alert} status is essential for a quicker response during muscle damage, as the first cell cycle takes much longer when SCs are in a quiescent G_0 state (Dumont et al., 2015a). Thereafter, Rodgers *et al.* shown that SC entrance in G_{Alert} state is controlled by mTORC1 signalling via activation of Hepatocyte Growth Factor Activator (HGFA), which is found in the ECM of many tissues upon injury. Active HGFA induces the receptor tyrosine kinase cMet activation, which finally triggers mTORC1 pathway and promotes SCs to enter in G_{Alert} state (Rodgers et al., 2017, 2014). Recently, Brun *et al.* have observed that GLI Family Zinc Finger 3 (GLI3), which participate in cilia SC function, is responsible to avoid that pre-activated SCs in G_{Alert} status return to quiescence G_0 status. GLI3 regulation is mediated by mTORC1 signalling, since GLI3 depletion in SCs induces mTORC1 pathway upregulation, thus prompting SCs to re-enter the cell-cycle through G_{Alert} state transition (Brun et al., 2022). These studies show that a fine balance between SC quiescence and pre-activation status is crucial for a quick response during muscle injury and that a precise regulation of SC quiescence status is necessary to maintain self-renewal capacity and avoid SC depletion.

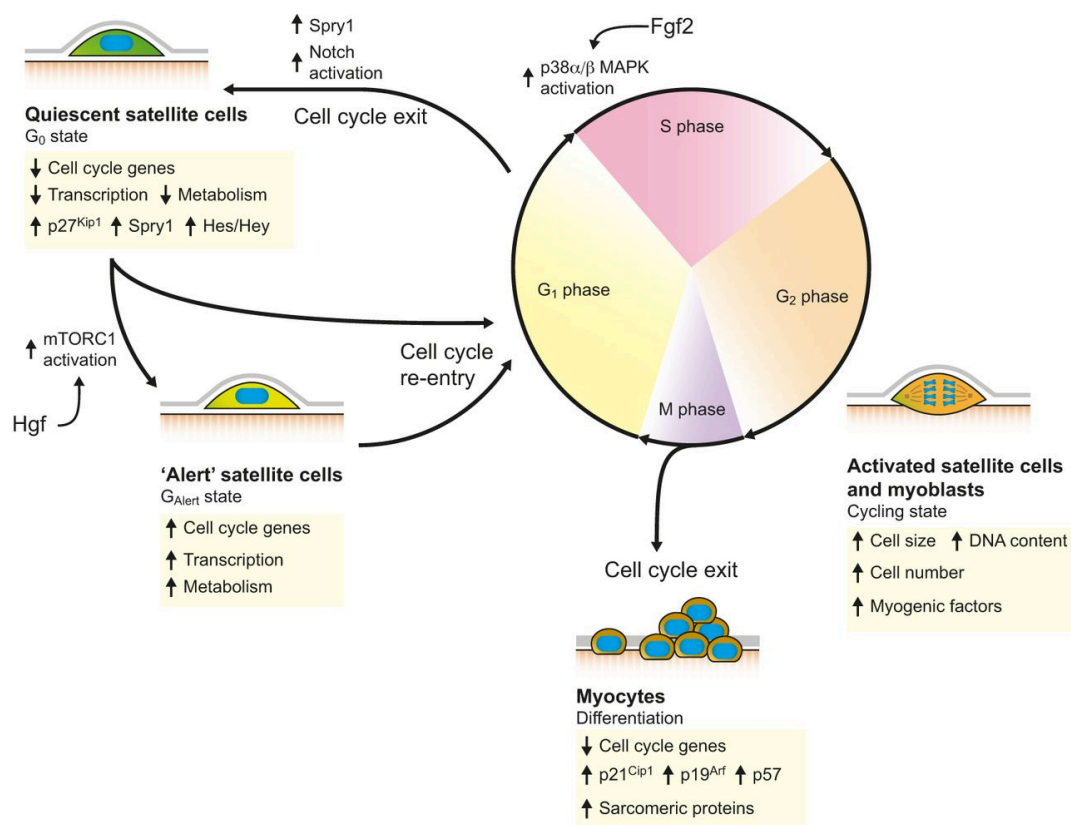


Figure 4. Cell cycle regulation in satellite cells. In resting conditions, SCs remain in a reversible quiescent status (G_0). After a stimulus SCs can re-enter the cell cycle directly (G_1) or via a pre-activated status term G_{Alert} . Notch signalling is one of the main responsible for maintain its quiescence and, conversely the activation of mTORC1 pathway activate SCs. Activated SCs can exit the cell cycle to return to a quiescence status by Notch activation or Spry1 upregulation. Additionally, activated SCs can also proliferate in order to increase the amount of myogenic committed progeny or exit the cell cycle to differentiate into myocytes. Taking from Dumont *et al.*, 2015a.

2.2 - SCs during activation and differentiation: regulatory pathways

SC activation and consecutive proliferation can occur in a symmetric or asymmetric way depending on the mitotic spindle orientation respect to the myofiber axis. During symmetric divisions the mitotic spindle is orientated parallel to the myofiber and SC will give rise to two identical daughter cells ($Pax7^{pos}$) (figure 5). This planar cell polarity symmetric expansion is regulated through Wnt7a/Fzd7 pathway, which allows an increase of SC number, hence maintaining the regeneration potential of skeletal muscle for next rounds of injuries and muscle homeostasis (Le Grand et al., 2009). Conversely, during SC asymmetric division the mitotic spindle is orientated perpendicular to the myofiber, and it gives rise to one stem cell ($PAX7^{pos}$) that remains in contact with the basal lamina, and one committed daughter cell that remains adjacent to the sarcolemma and starts to express the Myogenic Regulator Factors (MRFs) *Myf5* and/or *Myod1* ($MYF5^{pos}/MYOD1^{pos}$) (figure 5) (Cossu and Tajbakhsh, 2007). In asymmetric SC division, committed daughter cell controls the entrance into the myogenic program by PAX7 methylation through the arginine methyltransferase CARM1 that, in the end, activates the transcriptional expression of *Myf5* (Kawabe et al., 2012). Additionally, commitment myoblasts ($MYF5^{pos}/MYOD1^{pos}$) can also undergo on a symmetric division for myogenic progenitor expansion (figure 5) (Chang et al., 2016; Feige et al., 2018; Motohashi and Asakura, 2014).

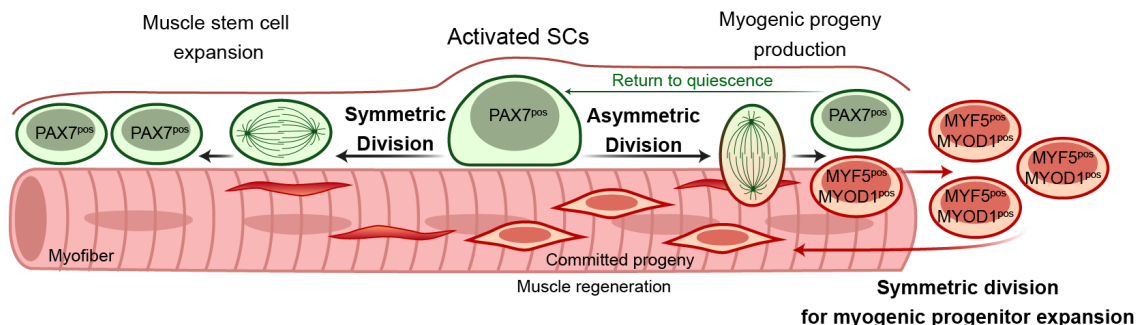


Figure 5. Scheme of satellite cell (SC) symmetric and asymmetric division. Symmetric divisions help to expand the stem cell pool ($PAX7^{pos}$) in the skeletal muscle. Asymmetric divisions give rise to both, a stem SC ($PAX7^{pos}$) and a myogenic cell daughter ($MYF5^{pos}/MYOD1^{pos}$) that allow myofiber repair. Committed myoblast ($MYF5^{pos}/MYOD1^{pos}$) can undergo symmetric expansion to enhance the number of myogenic progenitors available. SCs: satellite cells.

Asymmetric cell division requires prior cell polarization of different regulatory factors. In this regard, it has been shown that the Partitioning Defective complex (PARD3 and PARD6) and an atypical Protein Kinase C (PKC) are distributed asymmetrically in the committed daughter cells (Knoblich, 2008; Troy et al., 2012). PARD3 and PKC asymmetric distribution activates the p38 α / β -MAPK pathway in the committed SCs, which induces *Myod1* expression, allowing cell cycle entry and myogenic progenitor proliferation (Troy et al., 2012). Conversely, the absence

of PARD3 and PKC in the other daughter cell avoids *Myod1* expression, promoting SC re-quiescence (Troy et al., 2012). Notch pathway, essential for self-renewal, also regulates the cell fate during asymmetric divisions. In the daughter stem cell (MYF5^{neg}) the presence of NOTCH-3 receptor maintains it in a quiescent stemness status, meanwhile the Notch ligand DELTA1 and the Notch antagonist NUMB, which inhibit the Notch pathway, are asymmetrically located in the committed daughter cell (MYF5^{neg}) promoting myogenic differentiation (George et al., 2013; Kuang et al., 2007). A correct balance between symmetric and asymmetric division is essential as it will allow SC pool maintenance and it will ensure skeletal muscle repair and homeostasis.

During myogenic differentiation, SCs recapitulate embryonic myogenesis program (Hernández-Torres et al., 2017). Upon activation, SCs proliferate and undergo to myogenic commitment program, the transcription factor *Pax7* becomes downregulated whereas there is an upregulation of the MRFs, members of the basic-helix-loop-helix family. The MRFs regulate the myogenic commitment of SCs into myoblasts, differentiation towards myocytes, and finally fusion between them or with pre-existing myofibers to restore the damaged muscle (figure 6) (Dumont et al., 2015a; Relaix and Zammit, 2012). The MRFs include a family of muscle specific proteins: MYF5, MYOD1, MYOGENIN (MYOG) and MYF6 (also known as MRF4) (Hernández-Hernández et al., 2017). The first MRF that becomes upregulated is *Myf5*, which determines the myogenic commitment of activated SCs as pre-myoblast. MYF5 protein levels gradually increase promoting the activation of the following MRFs (Asfour et al., 2018). Additionally, committed PAX7^{pos} SCs start to co-express *Myod1*, which specifies their myogenic differentiation into myoblasts (Rudnicki et al., 1993, 1992). MYOD1 is a crucial factor for myoblast differentiation progression that target the cell-cycle inhibitors *p21* and *p57*, ceasing myoblast expansion as well as directly activating *Myog* expression (Halevy et al., 1995). MYOG has an antiproliferative activity through inactivation of the cell proliferation machinery, and it also allows myoblast final differentiation into myocyte throughout directly downregulation of *Pax7* and *Myod1* expression, and upregulation of several muscle specific genes (Asfour et al., 2018; Singh and Dilworth, 2013; Venuti et al., 1995).

After differentiation program begins, myocytes change its shape, function and they start to fuse to form multinucleated myotubes that will mature into myofibers. In this sense, MYOG increases the expression of genes involved in muscle contraction, such as *α-actin*, *Troponin*, *Myosin light* and *Heavy Chain (MyHC)* (Singh and Dilworth, 2013). Subsequently, transcription factors MYOD1 and MYOG activate the fusion of myocytes throughout the skeletal muscle-specific transmembrane MYOMARKER protein (Petrany et al., 2020). New generated myofibers are

characterized by the expression of the *embryonic MyHC (eMyHC)* and the presence of new added nuclei in the centre of the fiber, which will migrate to the margins in the mature myofibers (Schmalbruch, 1976; Sharma et al., 2018).

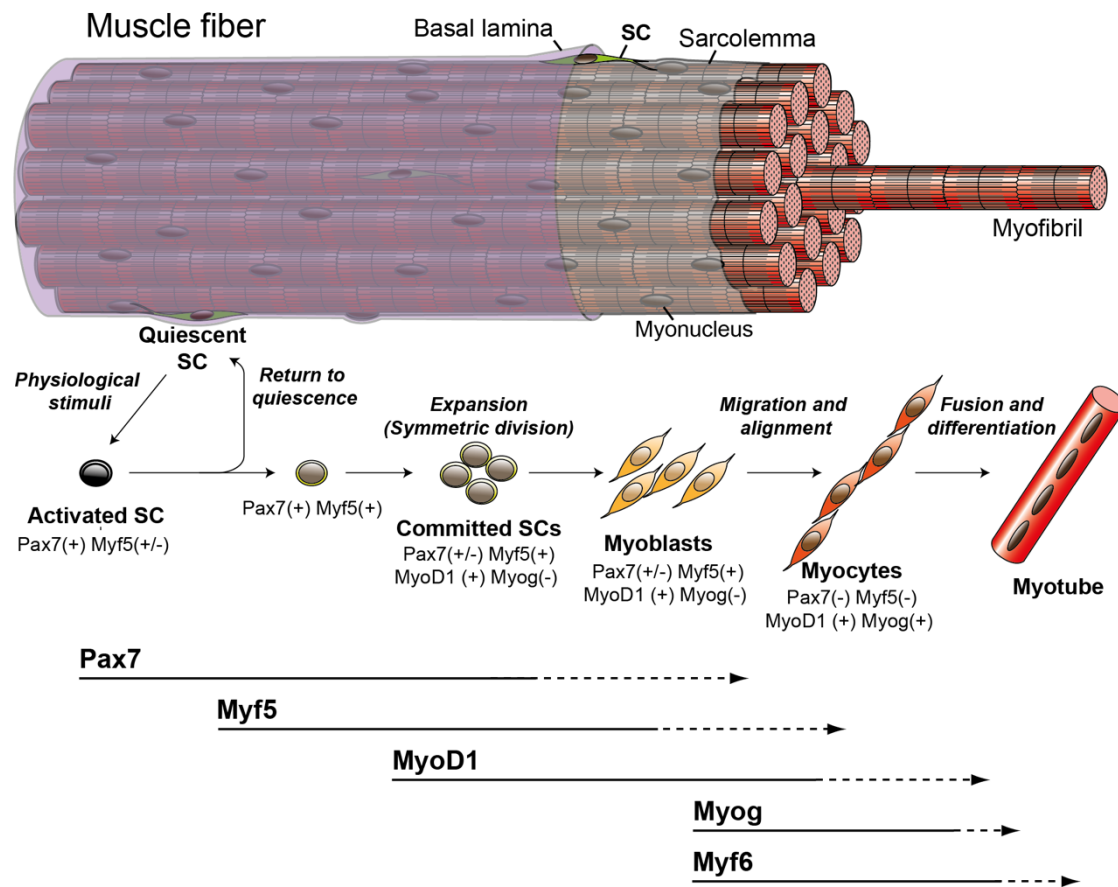


Figure 6. Adult myogenesis and the myogenic regulator factors (MRFs) implied in the process. Skeletal muscle regeneration includes quiescent SC activation, proliferation and commitment to myogenesis, differentiation and fusion to form myotubes that will finally mature to myofibers. SCs, satellite cells. Modified from Hernández-Torres *et al.*, 2017.

The factors and regulatory mechanisms that participate in SC activation, proliferation and differentiation just begin to become clarify. In the course of SC activation, all the mechanism that control quiescence status need to be switch to start the myogenic program. This process implies a major metabolic change of protein degradation and biosynthesis denominated autophagy. In this sense, Tang and Rando have shown that autophagy is crucial for a correct SC activation and it is regulated by the key nutrient sensor SIRTUIN1 (SIRT1) through the AMPK/mTOR pathway, well-known trigger of SC activation (Tang and Rando, 2014). Most often, activated SCs have to migrate to the damage area to contribute to muscle repair. Thus, activated SCs emerge from their niche between sarcolemma and basal lamina by the emission of Matrix Metalloproteinases (MMPs) that digest the ECM (Dumont et al., 2015a). Consecutively,

activated SCs migrate to the injured areas due to soluble factor such as HGF or adhesion factors located in the sarcolemma: $\alpha 7$ -INTEGRIN, CD34 (stem cell marker) or LAMININ, one of the most potent inducers of SC migration (Alfaro et al., 2011; Siegel et al., 2009). In addition, after activation, SCs undertake several rounds of proliferation to enhance the number of myogenic cells required for muscle repair. For instance, it has been shown that this process involves the upregulation of several cell cycle gene, like *Cyclins (Ccn) -A, B, D, E, F and G* (Fukada et al., 2007).

SC proliferation and progression through myogenic program involves stimulus from different tissues, ECM, damage myofibers and immune system (like macrophages) among others (Dumont et al., 2015a). In this regard, several proinflammatory cytokines (Interleukin (IL)-6, IL-1 β and TNF- α) secreted by myoblast, myofiber or immune system cells, stimulate SC activation and its differentiation (Tidball and Villalta, 2010). Other key activator of SCs in early stages is the HGF, released from ECM and diverse organs after a muscle injury. Additionally, members of the FGF family, secreted by myofibers, fibroblast or endothelial cells, participate in muscle regeneration (Sousa-Victor et al., 2022). FGFs, significant up-regulated during muscle regeneration, acts by activating pro-myogenic p38 α/β MAPKs pathway that allow SCs to enter into cell cycle and initiate myogenic commitment (Jones et al., 2005). For instance, FGF-2, founded in the basal lamina, participates in myoblast proliferation, and FGF-6 is involved in the expression of muscle specific genes (Yin et al., 2013). Insulin-like Growth Factor (IGF)-1 also induces the re-entry of SCs in the cell-cycle throughout the stimulation of Akt/mTOR pathway and downregulation of FOXO activity (García-Prat et al., 2020; Machida et al., 2003). Moreover, intrinsic SC signalling such as Sphingosine-1-Phosphate (S1P), in the inner leaflet of the SC plasma membrane, participates in SC activation and entry into the cell cycle (Nagata et al., 2006).

Understanding of the pathways involved in SC activation, proliferation and differentiation will shed light on the mechanisms involved in muscle homeostasis and it will open new approach of treatments to enhance muscle regeneration potential in muscle diseases.

3 - Behavioural heterogeneity in the SC population

The mechanism of SC activation and myogenic differentiation is well-known; however, several studies have pointed out the existence of different SC behaviour during skeletal muscle regeneration (Cornelison, 2018; Tierney and Sacco, 2016). In this sense, diverse SC subpopulations can be defined by their response to activation and differentiation during adult myogenesis.

Different proliferation capabilities have been described during SC activation. Accordingly, SCs are divided in populations of fast-dividing cells, representing the majority of them, and slow-dividing cells, least-represented and designated as the long-term muscle stem cells. An initial *in vivo* study of cell cycle timing carried out by Schultz *et al.* have revealed that 80% of SCs were fast-dividing cells that contribute to muscle repair, meanwhile only 20% of SCs were slow-dividing cells (figure 7). The slow-dividing cells were designed as “reserve cells”, since they tended to undergo asymmetric division, maintaining the stem cell pool (Schultz, 1996). In consonance with this study, Ono *et al.* have observed that fresh isolated SCs from mouse muscles contain a minor subpopulation of undifferentiated cells, characterized by high levels of ID1 protein, an inhibitor of myogenic differentiation. This minor undifferentiated SC population divides at lower ratio and presents a long-term self-renewal potential (Ono *et al.*, 2012). Additionally, when this undifferentiated and slow-dividing SC subpopulation is transplanted, it is able to contribute to myofiber regeneration *in vivo* (Ono *et al.*, 2012). These studies highlight that during muscle regeneration the slow-dividing SC population has a preferential disposition for engraftment, pointing out its relevance to preserve the muscle stem cell pool while contribute to myofiber regeneration in future waves of muscle damage. Similarly, García-Prat *et al.* have recently found that there is a minor SC subpopulation with CD34^{high} expression, regulated by FOXO signalling, that shows a ‘genuine’ state with stemness properties and no proliferative traits (figure 7). They have revealed that CD34^{high} SC subpopulation is firstly detected in juvenile mice (around 21 days post-partum) when quiescent SCs are established for the future adult muscle homeostasis. CD34^{high} SC population is only lost in extreme aged muscles, causing several regeneration muscle issues in geriatric mice, showing that SC heterogeneity is required for muscle homeostasis maintenance through life (García-Prat *et al.*, 2020).

As previously explained, asymmetric division generates two different cell daughters that will differ in their future fate: committed to myogenesis or remain stemness to expand and preserve the SC pool in skeletal muscle. In this sense, during asymmetric SC division the daughter cell nearer to the basal lamina, that will stay as stem cell (PAX7^{POS}), undergoes to a non-random DNA segregation to keep the original DNA template (Shinin *et al.*, 2006). This observation is in line with the hypothesis of the ‘immortal DNA strand’ which suggests that stem cells preserve the original DNA strand through non-random DNA segregation to significantly decrease the DNA replication errors in future rounds of regeneration (Cairns, 1975). Therefore, non-random DNA segregation in more stemness SC population will avoid DNA-replication errors during life. Using a transgenic Tg:*Pax7*-nGFP mouse model that marks all SCs, Rocheteau *et al.* have established

two population with different division capabilities and DNA segregation regarding *Pax7* expression levels: PAX7^{high} and PAX7^{low} SC subpopulations (figure 7) (Rocheteau et al., 2012). PAX7^{high} subpopulation shows higher non-random DNA segregation retaining the ‘original’ DNA strand, moreover, it presents a lower metabolic status like dormant SCs (Rocheteau et al., 2012). Furthermore, as slow-dividing SC subpopulation, when PAX7^{high} subpopulation is transplanted muscle regeneration takes place properly as it gives rise to both subpopulations: PAX7^{high} SCs (more stemness to replenish the niche) and PAX7^{low} SCs (more committed to myogenic differentiation) (Rocheteau et al., 2012). Recently, Evano *et al.* have described that the non-random DNA segregation in SCs can take place *in vivo* and, *ex vivo* analyses show that SCs can switch from asymmetric to symmetric division, suggesting a non-permanent cell fate (Evano et al., 2020). This observation is in accordance with their *in vivo* analysis demonstrating that SCs preferentially undergo through symmetric divisions at 3 days post injury and that they can switch to asymmetric divisions until 5 days after injury (Evano et al., 2020).

To further explore the subpopulation dynamics in SCs, Chakkalakal *et al.* (2014) used a TetO-H2B-GFP reporter that allow to monitor cell proliferation. Low rates of cell division maintain the H2B-GFP label inside cells, defining them as label retaining cells (LRCs), and contrary, high rates of cell divisions dilute the H2B-GFP labelling, describing these cells as non-LRCs. They have observed that the majority of SCs (approx. 70%) are non-LRCs with tendency to undergo myogenic commitment and differentiation. Interestingly, only 30% of SCs are LRCs, being less differentiated and, as mentioned above, when transplanted they act as true stem cells generating self-renewal (PAX7^{pos}) and myogenic committed daughter cells (figure 7) (Chakkalakal et al., 2014). LRCs stemness stage is maintained through CIP/KIP family members p21cip1 (Cdkn1a) and p27kip1 (Cdkn1b). At birth, both subpopulations (LRCs and non-LRCs) can be distinguished, but only during juvenile period they are recognized as divergent SC population with different behaviours that persist in adult muscle (Chakkalakal et al., 2014). All together, these studies demonstrate that there are two different population of SCs that partly or fully overlaps in their characteristics. In simplified terms, there is a more stemness SC population that can be defined as the “true muscle stem cells” in a long-term period, characterized by: lower ratio divisions, non-random DNA segregation that keep the original DNA strand and, when transplanted, they act like the true stem cell population by contributing to stem cell pool through symmetric expansion and to myofiber repair by giving rise to myogenic committed cells.

The expression of MRFs can also define the fate of SCs towards a more stemness or myogenic commitment status. Using a *Myf5*^{nlacZ/+} mouse and the CD34 quiescent marker, Beauchamp *et*

al. have identified a minor subpopulation of CD34^{neg}/MYF5^{neg} SCs that maintain the myogenic committed-lineage, whereas CD34^{pos}/MYF5^{pos} cells are more prone to myogenic differentiation (figure 7) (Beauchamp et al., 2000). Afterwards, using two different *Pax7* mouse models: transgenic Tg:*Pax7*-nGFP mice and knock-in *Pax7*^{nGFP/+} mice, Gayraud-Morel *et al.* have analysed the role of MYF5 in SC behaviour. They observed that *Myf5* is expressed heterogeneously in PAX7^{pos} SC population and, when one *Myf5* allele is compromised (Tg:*Pax7*-nGFP:*Myf5*^{loxP/+}), SCs are transcriptionally committed to myogenic differentiation (*Myod1*, *Myog* and contractile protein gene expression) (Gayraud-Morel et al., 2012). Surprisingly, Tg:*Pax7*-nGFP:*Myf5*^{loxP/+} cells are able to contribute to *in vivo* muscle repair similar to wild type and they are more efficiently replenishing the SC pool than wild type, explained by higher expression levels of TIE2, a SC self-renewal factor (Gayraud-Morel et al., 2012). These studies demonstrate the importance of *Myf5* expression in SC behaviour heterogeneity during the first phase of activation.

Some studies shown that committed progenitors can also display behaviour heterogeneity. *In vitro* analysis in C2C12 myoblast cell line and isolated myofibers from mice have shown that when cultured, only a minor subpopulation of activated PAX7^{pos}/MYOD1^{pos} SCs can downregulate *Myod1* and remain in cell culture as PAX7^{pos}/MYOD1^{neg} reserve cells (figure 7) (Yoshida et al., 1998; Zammit et al., 2004). This PAX7^{pos}/MYOD1^{neg} reserve cell population is able to generate both: new reserve cells and committed to differentiation progeny (Yoshida et al., 1998; Zammit et al., 2004). These results are consistent with the observations of mouse myoblast lacking *Myod1* (*Myod1*^{-/-}), characterized by a stem cell phenotype with better engraftment after transplantation and a higher resistance to apoptosis than wild type (Asakura et al., 2007; Hirai et al., 2010). Hence, these observations highlight the ability of committed myoblast for revert to a quiescent status and contribute to stem cell pool maintenance.

It is interesting to note that in the adult muscle, there is a subpopulation of quiescent SCs that express *Pax3*, a paralog of *Pax7*, crucial during embryonic myogenesis (Relaix et al., 2006). Recently, this minor PAX3^{pos}/PAX7^{pos} SC subpopulation has been related to respond under environmental pollutants and radiation stress (figure 7) (Der Vartanian et al., 2019; Scaramozza et al., 2019). Der Vartanian *et al.* have demonstrated that *Pax3* expression protects SCs when they are exposed to toxic pollutants (Dioxin/TCDD exposure). PAX3 mediates this response by arresting activated SCs in G_{Alert} state through mTORC1 up-regulation. This pre-activated status avoids aberrant SC proliferation in a toxic environment (Der Vartanian et al., 2019). Similarly, using a *Mx1*-Cre transgenic reporter mouse that track resident stem cells, Scaramozza *et al.* have

demonstrated that there is a minor subpopulation of Mx1-Pax3-Pax7-expressing SCs with a quiescent behaviour. $MX1^{pos}/PAX3^{pos}/PAX7^{pos}$ SC population displays reduced levels of reactive oxygen species (ROS) and it mainly contributes to muscle repair only after radiation stress (Scaramozza et al., 2019). This study describes a minor subpopulation of radiotolerant reserve SCs in adult muscles that express PAX3 and PAX7 markers (Scaramozza et al., 2019). Both studies reveal the existence of a PAX3-expressing SC subpopulation that acts like the reserve stem cell under environmental insults, which will guarantee a stem cell reservoir in fatal circumstances.

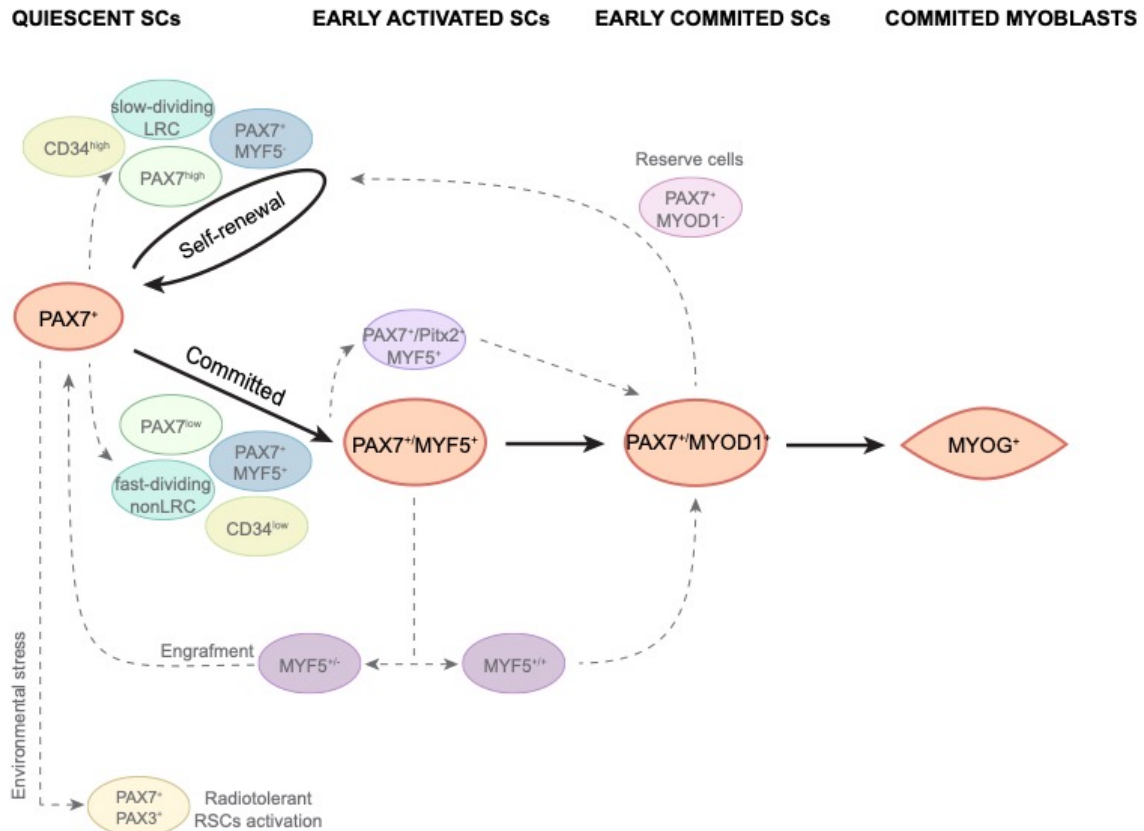


Figure 7. Functional heterogeneity behaviour in adult satellite cell (SC) population. SC activation and myogenic differentiation during adult muscle regeneration (black arrows). Representation of the different SC subpopulations described in the course of quiescence, activation and commitment during adult muscle regeneration (discontinued grey arrows). LRC, label-retaining cells; non-LRC, non-label-retaining cells; RSCs, reserve stem cells. Modified from Rodríguez-Outeiriño et al., 2021.

SC heterogeneity has been also related to their localization since SCs near the blood vessels maintain its stemness status through endothelial cell recruitment, via VEGFA secretion (Verma et al., 2018). Finally, Tierney et al. probed by multicolour lineage tracing that clonal SC dynamic, but not functional heterogeneity, is maintained through life allowing aged muscle to response to different insults due to its clonal heterogeneity. However, after repeated rounds of injury this clonal complexity is reduced, which can imply an issue in pathologies with high turnover ratios

(Tierney et al., 2018). García-Prat *et al.* have also noticed that loss of SCs heterogeneity, specifically the depletion of stemness CD34^{high} SC population, in extremely old mice drastically reduces the muscle regeneration potential during ageing (García-Prat et al., 2020).

Together, these studies show the essential role of SC heterogeneity in maintaining adult skeletal muscle homeostasis and its relevance to resist under different environmental insults. Understanding how these different subpopulations are generated and maintained through life could help us to comprehend the SC role in muscle regeneration and diseases.

4 - Embryonic origin of SC functional heterogeneity

4.1 – Origin of SCs: Embryonic myogenesis

In vertebrates, the skeletal muscle and the myogenic precursor cells originate from the paraxial mesoderm (Biressi et al., 2007a). In mice, two waves of myogenesis occurs during embryonic development, first or embryonic myogenesis takes place between embryonic day (E)8.5 (days post coitus) and E14.5, and second or fetal myogenesis takes place between E14.5 and E17.5 (Biressi et al., 2007a).

The skeletal muscle in the limbs and body trunk originates from the myogenic precursor cells that arise from somites, transient segmented structures of paraxial mesoderm placed on either side of the neural tube (figure 8) (Buckingham and Vincent, 2009; Hernández-Torres et al., 2017). In the mouse embryo the somites emerge around E8.0 and they present a spherical shape that before long polarize into two parts: the ventral part that will form the sclerotome and the dorsal epithelial region that will constitute the dermomyotome (DMT) (Buckingham and Vincent, 2009; Hernández-Torres et al., 2017). Whilst the sclerotome give rise to the progenitors to form the ribs and axial skeleton, the DMT form the progenitors of the skeletal muscle, dorsal dermis, vascular cells and brown fat cells (Buckingham and Vincent, 2009; Rodríguez-Outeiriño et al., 2021). Myogenic precursor cells from the epaxial and hypaxial lips of the DMT start to delaminate in an epithelial-mesenchymal transition (EMT) and accumulate underneath, where they differentiate to constitute the myotome (MT), the first muscular structure formed in the body (figure 8). The MT will function as a scaffold for consecutive waves of myogenesis. Subsequently, around E10.5 in mouse embryos, myogenic precursor cells from the central part of the DMT delaminate to reach the MT, where they differentiate into embryonic myoblast, intercalating within the primary MT and finally fusing to originate the embryonic fibers

(Bentzinger et al., 2012; Biressi et al., 2007a). The epaxial MT will give rise to deep back muscles, meanwhile the hypaxial MT will form the diaphragm, the rest of body wall muscles and the intercostal muscles (figure 8) (Bentzinger et al., 2012; Biressi et al., 2007a; Hernández-Torres et al., 2017). Simultaneously, at E9.5 in mouse embryos in limb regions, myogenic precursor cells from the hypaxial lip of the DMT undergo an EMT migrating to the limb buds (figure 8), where they originate the ventral and dorsal premyotome mass that will form the skeletal muscle and SCs in the adult limbs (Buckingham and Relaix, 2015; Cusella-De Angelis et al., 1994; Rodríguez-Outeiriño et al., 2021).

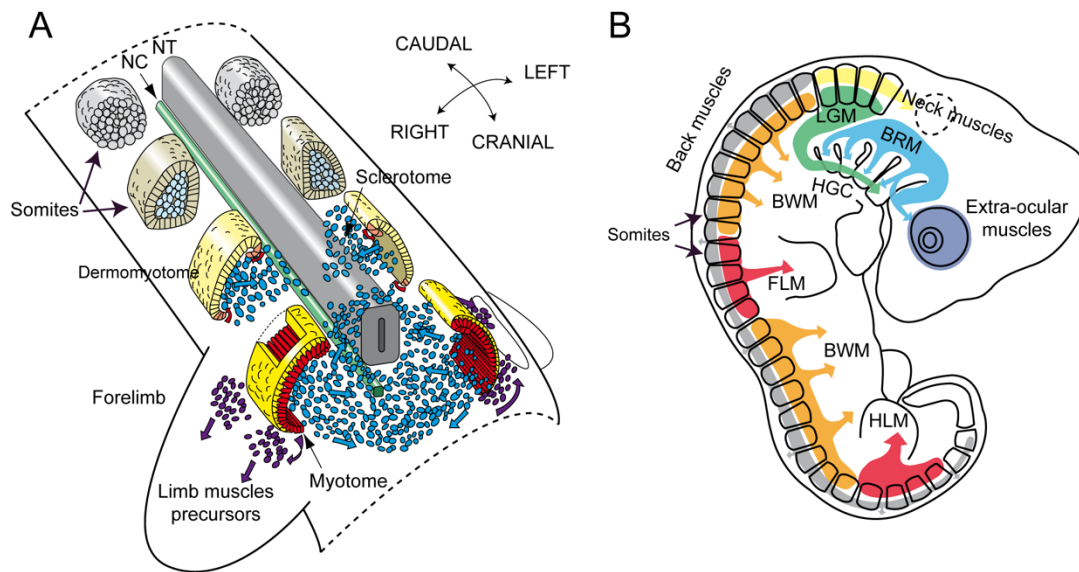


Figure 8. (A) Scheme of somite development. Somites develop following an anterior to posterior developmental gradient. Myogenic precursor cells originate from the lips of the dermomyotome to form the myotome. Later, a second wave of myogenic precursors from the central part of the dermomyotome migrate underneath to contribute to muscle development. In the limb regions, myogenic precursor arises from the hypaxial lips of the dermomyotome. **(B) Paraxial mesoderm origin of the skeletal muscles from the somites.** NT, neural tube; NC, notochord; BRM, branchial muscles; LGM, laryngoglossal muscles; HGC, hypoglossal cord; BWM, body wall muscles; FLM, forelimbs muscles; HLM, hind limbs muscles. Modified from Hernández-Torres et al., 2017.

In mouse embryos, fetal myogenesis begins around E14.5. At this stage, in the trunk and limb buds, the majority of myogenic precursor cells proliferate and differentiate into fetal myoblast that fuse with each other or with the earlier formed embryonic myofibers to form the secondary or fetal myofibers (Biressi et al., 2007a; Hernández-Torres et al., 2017). However, during this period a small population of non-proliferative myogenic precursors remain undifferentiated and at the end of fetal myogenesis they will settle between the basal lamina and sarcolemma to form the juvenile SCs found at birth. During postnatal period, from birth to 21 days in mice, juvenile SC population goes through a high proliferation phase to expand and add nuclei to growing muscle. Finally, most of this population enters into apoptosis while a few

proliferative juvenile SCs return into quiescence status and set as the main SC pool for the adult life (Sambasivan and Tajbakhsh, 2007).

At regulatory mechanism level, the transcription factors PAX3 and PAX7 govern the cell fate of myogenic precursor cells during embryonic and fetal myogenesis (Buckingham and Relaix, 2015; Seale et al., 2000). *Pax3* is firstly expressed in the pre-somitic mesoderm and later in the somites. As somite is polarized, *Pax3* expression become confined in the epithelial DMT (figure 9) (Buckingham and Relaix, 2015). During embryonic myogenesis, *Pax3* is expressed in the myogenic precursor cells, playing a key role in myogenic specification and precursors migration to more distant regions (Kassar-Duchossoy et al., 2005). The first round of myogenic precursors, that delaminate from the lips of the DMT to form the MT, decrease *Pax3* expression and they begin to express *Myf5/Mrf4* and *Myod1* factors in order to form the first differentiated myofibers of the MT (Buckingham and Relaix, 2015). Around E9.5, *Pax7* starts to co-express with *Pax3* in the central part of the DMT (figure 9) (Bentzinger et al., 2012; Buckingham and Rigby, 2014). These PAX3^{pos}/PAX7^{pos} myogenic precursors delaminate underneath the MT to give rise to the progenitors of the skeletal muscle in the trunk (Kassar-Duchossoy et al., 2005). Between E9.5 and E10.5, PAX3^{pos} cells from the hypaxial lips of the DMT start to undergo an EMT to reach the limb buds (figure 9). Afterwards, at E11.5 some of these migrating PAX3^{pos} cells in the limb bud start to co-express *Pax7* (Buckingham and Relaix, 2015). PAX3^{pos} cells in the limb buds can give rise to endothelial and myogenic cells, while PAX3^{pos}/PAX7^{pos} cells are restricted to myogenic population, thus the progenitors of the skeletal muscle in the limbs (Buckingham and Relaix, 2015; Kassar-Duchossoy et al., 2005). The transcription factor PITX2, which is involved in muscle patterning and muscle progenitor fate, is also present in some PAX3^{pos} myogenic precursors at the ventral lateral of the DMT as well as in a subpopulation of migrating PAX3^{pos} myogenic precursor cells into the limb buds (Hernández-Torres et al., 2017; Ramírez de Acuña et al., 2022). PITX2 is also the first regulator that specify the myogenic commitment in the progenitors of the head muscles (Hernández-Torres et al., 2017). During fetal period, *Pax3* expression becomes reduced and finally it is restricted to a minor SC subpopulation (PAX3^{pos}/PAX7^{pos}) in some adult muscles, like the diaphragm or the pectoralis (Relaix et al., 2006).

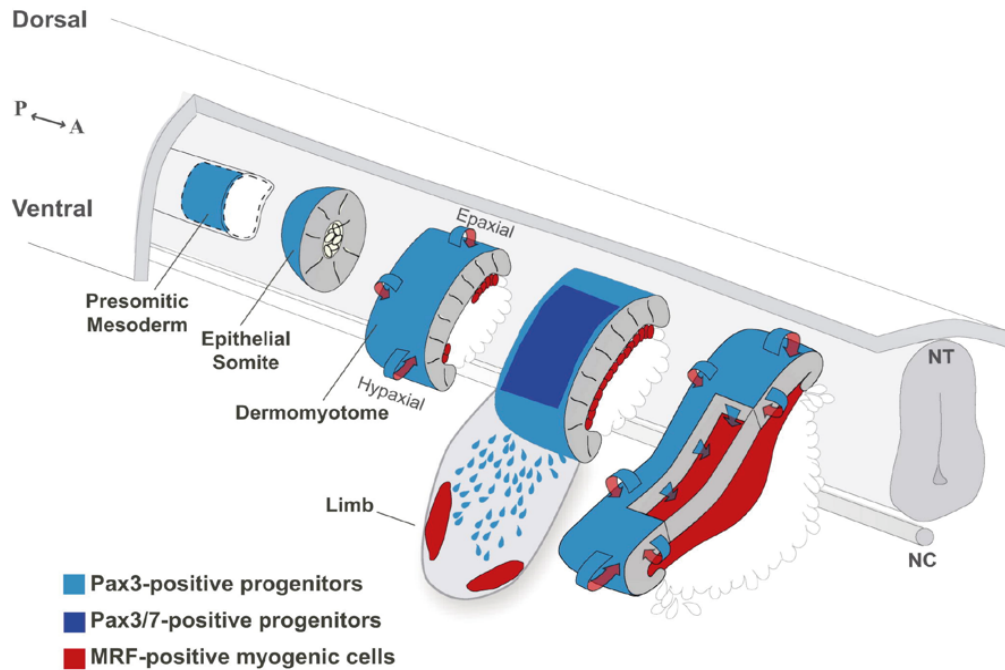


Figure 9. Schematic representation of somite development: $PAX3^{pos}$, $PAX7^{pos}$ and MRF^{pos} populations. Somites mature following antero (A) to posterior (P) gradient. PAX3 and PAX7 expression in muscle precursor cells. NT, neural tube; NC, notochord. Taking from Buckingham and Rigby, 2014.

Pax3 plays an essential role during myogenic specification as *Pax3*^{-/-} mutant mice show affected somitogenesis with MT malformation and muscle defects. Remarkably, in *Pax3* mouse mutants the limb muscles are absent (Buckingham and Relaix, 2015). Furthermore, it has been demonstrated that transcription factor PAX3 have an anti-apoptotic role in the hypaxial somite since *Pax3*-mutant mice present an increased ratio of apoptosis at E10.5 affecting the trunk muscle formation (Borycki et al., 1999). Meanwhile, *Pax7* mutant mice show no significant alteration during embryonic development; however, after birth, they do not live longer than 2-3 months, they present small muscles, the SC pool is extremely reduced and the SCs that are left tend to activation, differentiation and finally undergo apoptosis (Buckingham and Relaix, 2015; Mansouri et al., 1996; Relaix et al., 2005). In this sense, it was pointed out that PAX7 is essential for SC specification during embryonic myogenesis as well as SC maintenance during development and after birth (Messina and Cossu, 2009). In *Pax3/Pax7* double mutant mice the primary MT is not formed and there is a complete absent of muscle formation in the limbs (Relaix et al., 2006). These observations demonstrate the importance of PAX3 and PAX7 as regulator for initiation and formation of progenitors of muscle mass during embryonic development, and the relevance of PAX7 to ensure SC identity at birth and during adult skeletal muscle homeostasis.

4.2 - Does the embryonic origin of SCs underlie functional heterogeneity?

It is well accepted that in adult muscle SCs in the trunk arise from PAX3^{pos}/PAX7^{pos} cells in the central part of the DMT as well as SCs in the limbs are derived from the PAX3^{pos} migrating cells that later will co-express *Pax7*. However, how the heterogeneous SC subpopulations in adult muscle are formed remains unknown (Rodríguez-Outeiriño et al., 2021).

During embryonic myogenesis the different waves of myogenic precursor cells (embryonic and fetal myoblast as well as SC-progenitors) present heterogeneity features; for instance, they show different morphologies, divergent response to factors, different gene expression patterns (*Hox* or *Notch* genes) and they give rise to different slow and/or fast contracting fibers (Biressi et al., 2007a, 2007b; Cusella-De Angelis et al., 1994; Stockdale, 1992). Accordingly, when cultured *in vitro*, embryonic myoblasts present an elongated shape and they tend to differentiate into mono-/oligonucleated myotube; otherwise, fetal myoblasts present a more triangular shape, they proliferate in response to different growth factor such as TGF- β , BMP-4 or phorbol esters, and they differentiate into large multinucleated myotubes (Biressi et al., 2007a, 2007b; Cusella-De Angelis et al., 1994; Stockdale, 1992). In adult stages, SCs present a round shape, they differentiate into large multinucleated myotubes and they are sensitive to TGF- β and BMP-4 but not to phorbol esters. Additionally and contrary to fetal myoblast, SCs present high binding capacity to the Platelet-Derived Growth Factor (PDGF), which promotes their proliferation and represses its differentiation (Biressi et al., 2007a; Yablonka-Reuveni and Seifert, 1993). This different sensitivity to PDGF between fetal myoblast and adult SCs could allow the differentiation of fetal myoblast during fetal myogenesis meanwhile SCs remain undifferentiated in the postnatal and adult muscle (Biressi et al., 2007a). By RNAseq analysis Biressi *et al.* have shown that adult SC population is more related to fetal myoblast than to embryonic myoblast (Biressi et al., 2007b). These results led us wonder if SCs arise from a common precursor of embryonic and fetal myoblast or, otherwise, arise from the different populations defined during embryonic development. Regarding this concern, in chicken and mouse embryos, Picard *et al.* have identified two co-existing populations of myogenic cells during embryonic and fetal myogenesis. They have noted different proliferative capabilities: a minor subpopulation of resident PAX7^{pos} myogenic cells with slow-cycling divisions and a major PAX7^{pos}/MYF5^{pos} subpopulation that can divide faster (Picard and Marcelle, 2013). Overall, these observations demonstrate a complex heterogeneity in myogenic precursor cells during vertebrate skeletal muscle development. Furthermore, it still remains unknown whether this

heterogeneity is caused by intrinsic changes in the cells or if it could be related to extrinsic factors in the environment that determine their cell fate.

It has been shown that SCs derived from *Pax3/Pax7*-expressing cells in the DMT, but whether embryonic myoblast, fetal myoblast and SCs derive from common or different progenitors are still undetermined (Buckingham and Relaix, 2015; Hutcheson et al., 2009; Rodríguez-Outeiriño et al., 2021). Using a *Myf5*-Cre-stop-flox-YFP reporter mouse, Kuang *et al.* have shown that only 10% of adult SCs ($PAX7^{pos}$) have never expressed *Myf5* during development. The minor $PAX7^{pos}/MYF5^{neg}$ population has a more stemness status as it tends to divide asymmetrically, generating both committed cells ($MYF5^{pos}$) and stem cells ($MYF5^{neg}$) that allow muscle repair and maintain the SC pool. Furthermore, after transplantation, $PAX7^{pos}/MYF5^{neg}$ SC subpopulation, when compared to $PAX7^{pos}/MYF5^{pos}$ population, displays an increased engraftment repopulating the SC niche as they can contribute to myofiber repair as well as preserve the stem cell pool through self-renewal divisions (Kuang et al., 2007). However, later studies with a *Myod1*-Cre tracking mice show that almost all the adult SCs have expressed *Myod1* at some point during embryonic development (Kanisicak et al., 2009). These studies lead us wonder if SCs originate from a common progenitor in the DMT or arise from different populations already established at early stages that lead to a more stemness subpopulations which remains undifferentiated along the embryonic development (figure 10).

It is well-established that SCs originate from the same founders that form the skeletal muscle where they lie in (Tajbakhsh, 2009). In this sense, we can distinguish 3 different round of founder stem cells (FSC) during embryonic myogenesis (Tajbakhsh, 2009). The FSC1 population in the DMT that express *Pax3* and will migrate and differentiate to form the MT (Bentzinger et al., 2012; Buckingham and Relaix, 2015; Hernández-Torres et al., 2017; Sambasivan and Tajbakhsh, 2007; Tajbakhsh, 2009). The FSC2 population that corresponds to the $PAX3^{pos}/PAX7^{pos}$ progenitors in the central part of the DMT (Tajbakhsh, 2009). And the FSC3 population, that corresponds to the $PAX3^{pos}$ progenitors from the hypaxial lip of the DMT that migrate to form the progenitors of skeletal muscle in the limbs, diaphragm and tongue (Buckingham and Relaix, 2015; Cusella-De Angelis et al., 1994; Rodríguez-Outeiriño et al., 2021; Tajbakhsh, 2009). It has been suggested that only FSC2 and FSC3 population give rise to the skeletal muscle and the majority of SCs found after birth and during adult life (Tajbakhsh, 2009). Interestingly, after birth the juvenile SCs are also an heterogeneous population: $PAX7^{pos}/MYF5^{pos}/MYOD1^{pos}$ cells that will contribute to settle future SCs in adult tissue; $PAX7^{pos/neg}/MYF5^{pos}/MYOD1^{pos}$ myoblasts that can revert to form adult SCs or finally differentiate to form myofibers; and differentiated

PAX7^{pos}/MYOD1^{pos} myoblasts (figure 10) (Kassar-Duchossoy et al., 2005; Sambasivan and Tajbakhsh, 2007). At 21 days after birth, these juvenile subpopulations can be distinguished from the quiescent SC population found in adult muscles (Shinin et al., 2006). These observations call into question whether heterogeneous embryonic/fetal progenitors and/or juvenile SC populations give rise to the behavioural heterogeneous subpopulation of SCs described in the adult muscle (Rodríguez-Outeiriño et al., 2021).

It is important to remark that around 90% of quiescent PAX7^{pos} SCs in adult skeletal muscle have already expressed *Myf5*. In this regard, using a Tamoxifen-inducible *Myf5*^{CreER} mouse, Biressi et al. have demonstrated that a substantial amount of SCs arise from progenitors that expressed *Myf5* for the first time during fetal stage (E15.5) (Biressi et al., 2013). Therefore, it might be asked if MYF5^{neg} SCs arise from a different population of precursor cells during embryonic myogenesis. It is worth recall that some SCs in adult muscle express *Pax3*, and that this quiescent *Pax3*/*Pax7*-expressing subpopulation have been related to remain in a more stemness status as well as to respond as the reserve stem cell under environmental stress (Der Vartanian et al., 2019; Scaramozza et al., 2019). This observation leads us hypothesize if the origin of this PAX3^{pos}/PAX7^{pos} cell population share same progenitors than PAX7^{pos}/MYF5^{pos} SCs, which downregulated *Pax3* at the same point during development; or if PAX3^{pos}/PAX7^{pos} cell population originates from embryonic *Pax3*-expressing progenitors that remain MYF5^{neg} during muscle develop while PAX7^{pos}/MYF5^{pos} SCs originate from fetal progenitors (figure 10). Future works of regulatory mechanism and molecular signals will help us to better address how different progenitors during development are formed, and to elucidate the origin of SC heterogeneous populations in adult muscles from a common or divergent progenitors.

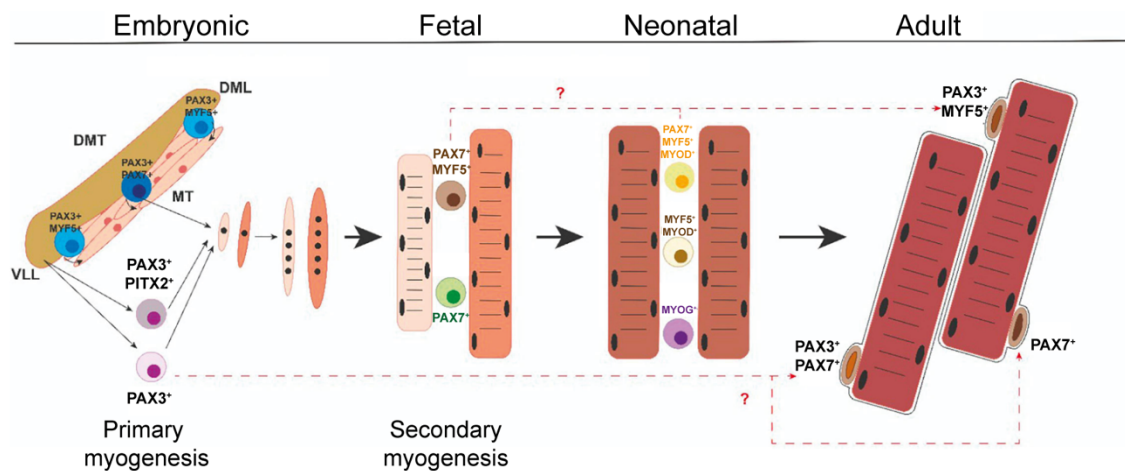


Figure 10. Myogenic progenitors' heterogeneity and different origins of adult satellite cells (SCs). Diverse populations of myogenic precursors are described during embryonic and fetal myogenesis that will give rise to different subsets of SCs in adult skeletal muscle. DMT, dermomyotome; MT, myotome; DML dorsomedial lip of the dermomyotome; VLL, ventrolateral lip of the dermomyotome. Taking from Rodríguez-Outeiriño et al., 2021.

5 – SC dysfunctions in muscular disorders

Several chronic and age-related pathologies course with impaired skeletal muscle regeneration. In this sense, muscular dystrophies and ageing sarcopenia are two pathologies characterized by flawed skeletal muscle regeneration that led to a loss of muscle mass, enhanced muscle atrophy, absence of neural re-innervation, increased intramuscular fibrosis and progressive muscle weakness. In both diseases, malfunctional skeletal muscle regeneration is exacerbated by dysfunctional SCs that cannot assist skeletal muscle loss (Sakuma et al., 2014). Currently, there is no cure or effective treatment for skeletal muscle wasting in sarcopenia or muscular dystrophies. However, the understanding of muscle regeneration defects linked to SC dysfunction could help us develop new and effective therapeutic approaches that potentiate intrinsic muscle regeneration.

5.1 - Muscular dystrophies: Duchenne Muscular Dystrophy (DMD)

Muscular dystrophies represent a group of diverse genetic diseases that are characterized by muscle degeneration, muscle mass loss and general muscle weakness. The most severe muscular dystrophy is DMD, that affects around 1 of 3500 males (Emery, 1991). First symptoms of muscle weakness in DMD appear at children ages 2 to 5. DMD patients show a rapid progression of the disease that confine them in wheelchairs around 12 years of age. During teen years, DMD patients start to suffer cardiorespiratory pathologies, which are the main cause of death in their early 20's (Broomfield et al., 2021).

DMD is caused by different mutations in the *Dystrophin* gene, located in the X chromosome, that encodes the DYSTROPHIN protein. *Dystrophin* gene is the largest known human gene with 79 exons, which make it susceptible to 91 different mutations (Koenig et al., 1988). DMD can be caused by disruption in the reading frame, deletion of one or several exons, duplications or punctual mutation that result in a non-functional or complete loss of DYSTROPHIN protein (Gao and McNally, 2015). There is a less severe dystrophic phenotype associated to *Dystrophin* mutations known as Becker Muscular Dystrophy (BMD). *Dystrophin* mutations in BMD patients lead to a truncated protein that remain partially functional, which lead to a later onset and lighter symptomatology compared to DMD patients (Gao and McNally, 2015).

DYSTROPHIN is a 427kDa cytoskeletal protein localized in the cytoplasmic face of the sarcolemma that interacts with ACTIN filament network connecting the contractile machinery to the ECM through the Dystrophin associated Glycoprotein Complex (DGC) (figure 11). DGC plays a pivotal structural role stabilizing the sarcolemma and protecting myofibers from contraction-induced damage (Gao and McNally, 2015). In DMD patients, lack of DYSTROPHIN causes alterations in the DGC that cannot accomplish its structural function, which entails that myofibers become vulnerable to contraction-induced membrane stress (Gao et al., 2015; Petrof et al., 1993). This scenario lead to continuous muscle damage with chronic inflammation, sequential cycles of necrosis and failed muscle repair attempts, in where dystrophic myofibers are finally replaced for fat and fibrotic tissue (Kharraz et al., 2014). As a consequence, DMD patients course with chronic inflammation and a general muscle mass loss entailing muscle weakness and fatal cardiorespiratory failures (Gao and McNally, 2015). Corticosteroids are administered to control the symptoms and slow the disease progression; however, they cause many undesirable side effects, driving the need of new successful treatments. In the last few years early diagnosed as well as new therapeutic approach have helped to extend the life expectancy of DMD patients to their first 30's, nonetheless there is still no cure or effective treatment available (Broomfield et al., 2021).

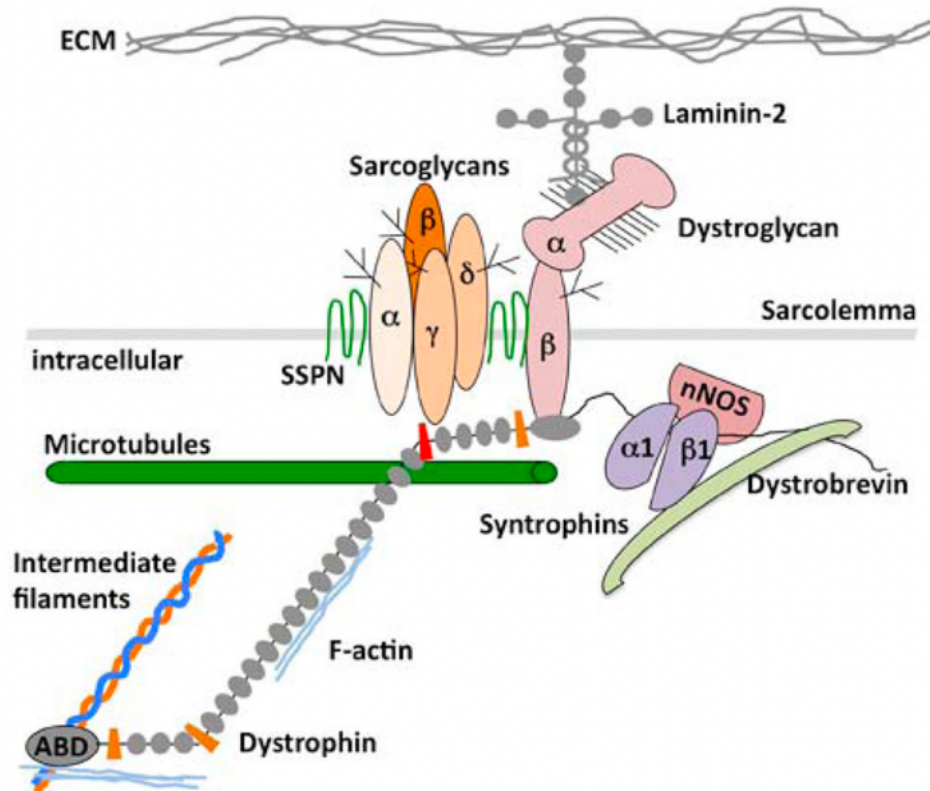


Figure 11. Dystrophin associated Glycoprotein Complex (DGC). DYSTROPHIN protein is located in the sarcolemma. DGC binds the intracellular cytoskeleton to the extracellular matrix (ECM). Taking from Gao and McNally, 2015.

It is interesting to remark that progressive muscle loss in DMD patients is frequently associated to the reduction of the muscle regeneration potential. In this sense, it was proposed that in DMD muscles repetitive rounds of muscle degradation and regeneration may cause a SC depletion (Sacco et al., 2010). However, the SC pool reduction in dystrophic muscles is widely controversial. Some studies shown that in different mouse models of DMD the number of SCs can vary on sex-depending manner, during ageing or due to the genetic background of the mouse strain (Boldrin et al., 2015; Jiang et al., 2014). Unexpectedly, in DMD patients the number of SCs was higher when compared to control patients (Bankolé et al., 2013; Kottlors and Kirschner, 2010). Additionally, SC number was also increased in the mdx mouse model, the most used mouse model for DMD (Jiang et al., 2014; McGreevy et al., 2015). Even though SCs are increased in mdx mice, the majority do not express *Myod1*, which indicate an incapacity of dystrophic SCs to properly conduct myogenic commitment (Boldrin et al., 2015). Moreover, Heslop *et al.* have shown that the radiotolerant SC subpopulation, the reservoir stem cell subpopulation essential for long-term muscle viability, is reduced in mdx mice (Heslop et al., 2000). This idea is in agreement with Jiang *et al.* study where they describe that young mdx mice display a downregulation in Notch signalling pathway, essential for self-renewal and maintenance of SC quiescence, that results in a significant reduction of non-committed PAX7^{pos}/MYF5^{neg} cell population (Jiang et al., 2014). Therefore, even though SC pool is enhanced in mdx mice and DMD patients, there is a reduction of the more quiescent SC subpopulation that led to a decrease of self-renewal divisions, and a lack of committed myogenic cells that reduce the contribution to regenerate dystrophic muscle.

The study carried out by Dumont *et al.* (2015b) described for the first time that DYSTROPHIN is also expressed in SCs, showing a polarized expression just before asymmetric division (figure 12A). They discovered that in asymmetric divisions, DYSTROPHIN binds to the Serine-Threonine Kinase MARK2, regulator protein of cell polarity, in the side of the SC nearer to the basal lamina. MARK2 polarization regulates asymmetric distribution of PARD3 in the opposite side of the cells (figure 12A). PARD3, an evolutionarily conserved regulatory protein complex of cell polarity during divisions, is located in the side of the SC nearer to the myofiber where it will activate the p38 α / β -MAPK pathway to start the myogenic commitment program in the daughter cell that will undergo myogenic differentiation (Dumont et al., 2015b). In dystrophic SCs the absence of DYSTROPHIN causes a downregulation of MARK2 protein expression that results in a no polarization of PARD3, thus dystrophic SCs lose the ability to conduct proper asymmetric divisions, causing aberrant divisions and a lack of committed muscle progenitors (figure 12B) (Chang et al., 2016; Dumont et al., 2015b; Keefe and Kardon, 2015). Moreover, the absence of

cell polarization in activated dystrophic SCs promotes anomalous symmetric divisions, which causes a stem cell hyperplasia of MYF5^{neg} cells that retain stemness properties but cannot generate myogenic progenitors to contribute for muscle repair (figure 12B) (Dumont et al., 2015b). More recently, Chen *et al.* have described that the Kinase Domain Receptor (KDR)-VEGFA pathway is also involved in asymmetric SC divisions, since KDR requires the presence of polarized DYSTROPHIN for a correct localization and function. As expected, in DYSTROPHIN-deficient SCs, impaired KDR signalling potentiate a reduction of asymmetric division and a loss of myogenic progenitors (Chen et al., 2022). Altogether, these results show that muscle loss in DMD patients is aggravated by dysfunctional SCs that cannot develop proper asymmetric divisions in order to repair the myofiber chronic damage.

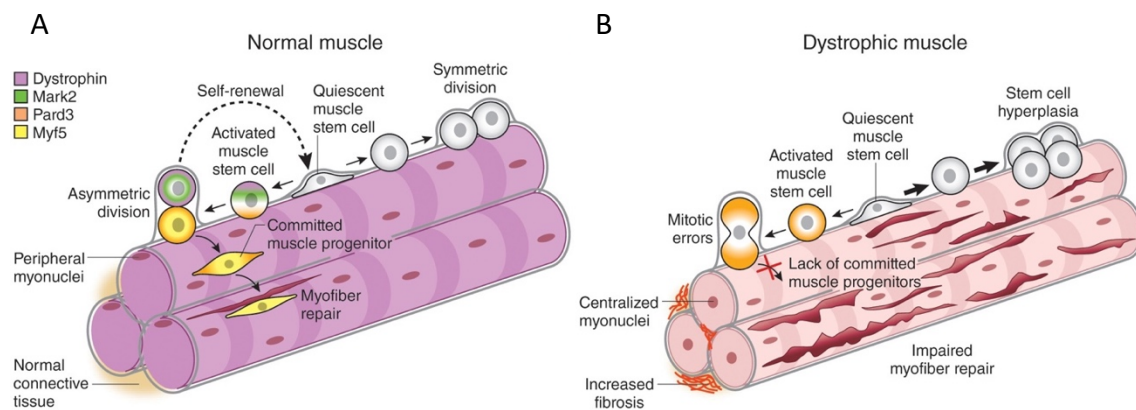


Figure 12. (A) Satellite cell (SC) polarization is essential for a correct asymmetric cell division. In SCs, DYSTROPHIN promotes the opposite polarization of MARK2 and PARD3 that allow a proper asymmetric cell division to generate committed muscle progenitors. **(B) The absence of polarization in dystrophic SCs lead to a lack of committed muscle progenitors.** Loss of DYSTROPHIN in dystrophic SCs lead to a loss of factor polarization required in asymmetric cell divisions. Dystrophic SCs cannot conduct asymmetric divisions which causes a decrease in myogenic progenitors that together with damaged myofiber exacerbates progressive muscle loss. Taking from Keefe and Kardon, 2015.

Therefore, new therapeutic approaches are focused in restoring intrinsic defects of dystrophic SCs to enhance the number of myogenic precursors available and, thereby, increase intrinsic muscle regeneration potential in the context of DMD.

5.2 - Sarcopenia

Sarcopenia is defined as the loss of skeletal muscle mass mostly due to ageing, but also as a result of other pathologies such as physical disabilities (Santilli et al., 2014). With an ageing population, sarcopenia is growing in prevalence, becoming a major concern with a high social

and economic burden. Sarcopenia courses with muscle denervation, enhanced frailty, muscle weakness and high risk of mortality (Chen et al., 2020; Iyer et al., 2021). Sarcopenia progression can be attenuated with exercise thanks to skeletal muscle ability for self-regeneration (Yoo et al., 2018). Nonetheless, intrinsic SC defects and altered niche in aged muscle limit muscle recovery, leading to a fibroadipogenic phenotype similar to the one observed in DMD patients (Chen et al., 2020).

In parallel with the loss of self-renewal and committed SCs in DMD condition, aged SCs present similar intrinsic dysfunctions that reduce the muscle regeneration potential. In this regard, maintenance of SC quiescent is essential to preserve a stem cell pool that allows muscle repair and homeostasis during life (Chen et al., 2020). It has been described that mutations in Notch signalling, essential for a correct quiescence activity, lead to sarcopenic phenotype due to a depletion of SC pool (Gioftsidi et al., 2022). Chakkalakal *et al.* have shown that two-thirds of aged SCs manifest dysfunction mechanism linked to symmetric-asymmetric divisions. They have observed that *in vitro* aged SCs tend to myogenic commitment, presenting a lack of self-renewal divisions that in long-term will reduce the stem cell pool (Chakkalakal et al., 2012). Bernet *et al.* have evidenced that loss of self-renewal divisions occurs through alterations in Fibroblast Growth Factor Receptor-1, p38 α and p38 β /MAPK signalling (Bernet et al., 2014). Similarly, Cosgrove *et al.* have described that elevated activity of the p38 α and p38 β /MAPK pathway in aged SCs leads to an increase in their myogenic commitment (Cosgrove et al., 2014). As it was expected, inhibition of p38 α and p38 β /MAPK pathway in aged mice ameliorates self-renewal SC defects and enhances muscle strength after muscle damage (Bernet et al., 2014; Cosgrove et al., 2014). Additionally, Sousa-Victor *et al.* have described that fails in aged SCs to re-enter quiescence G₀ phase were caused by significantly upregulated levels of the cell *Cycle regulator p16INK4a (Cdkn2a)* that promote its activation, reducing the stem cell pool over time (Sousa-Victor et al., 2014). These observations show that loss of self-renewal divisions and quiescence status in aged SCs lead to a reduction in the stem cell pool in sarcopenic mice that, in the end, accelerate the loss of muscle regeneration potential.

During ageing, muscle inactivity results in long periods of SC quiescence that can be counterproductive (figure 13) (Chen et al., 2020). Quiescence is a status with reduced metabolic activity, however, basal activity still produce cellular waste and mitochondrial ROS that need to be appropriately processed (van Velthoven and Rando, 2019). Extremely long periods of quiescence cause an excessive accumulation of damage proteins, enhanced oxidative stress production and mitochondrial DNA damage that altogether cause cell proteotoxicity, that will

be the cause of SCs acquiring an irreversible senescent phenotype and, finally, apoptosis (figure 13) (Rubinsztein et al., 2011). Irreversible senescent SCs are not able to activate and generate progeny, which exacerbate stem cell number depletion and the lack of myogenic commitment progenitors that could repair aged muscle (Feige et al., 2018).

On the other hand, activation and consecutive cell division of aged SCs promote different metabolic pathways that reduce and dilute the cellular waste. In this context, despite the fact that autophagy was described as a cellular process induced by stress, it is crucial for SC maintenance during long periods of quiescence (figure 13) (Chen et al., 2020). Campanario *et al.* have shown that if autophagy is compromised in SCs from young mice, they displayed a similar senescent phenotype to aged SCs. In accordance, when autophagy is enhanced in aged SCs, its muscle regeneration potential is restored (Campanario et al., 2020). Additionally, SESTRINS, a family of stress-inducible metabolic regulators, have also been described by Segalés *et al.* as a crucial autophagy regulator that protect skeletal muscle from muscle mass loss. They have observed that SESTRIN1 protein expression is decreased during ageing as well as its overexpression in aged SCs prevents muscle atrophy (Segalés et al., 2020). Similarly, ablation of macroautophagic *Atg7* component, essential for autophagy process, turns SCs into a senescent status and the re-establishment of autophagy through *Atg7* overexpression rescues aged SCs and restores its regenerative potential (García-Prat et al., 2016). Altogether demonstrate that activation of aged SCs reduces its pre-senescent phenotype through increased autophagy and enhanced damage response, hence, it restores the ability to return into a reversible quiescence (figure 13) (Chen et al., 2020). In short, restoration of reversible quiescence in aged SCs maintain the stem cell pool over time and, consequently, improves the regeneration potential of sarcopenic muscle.

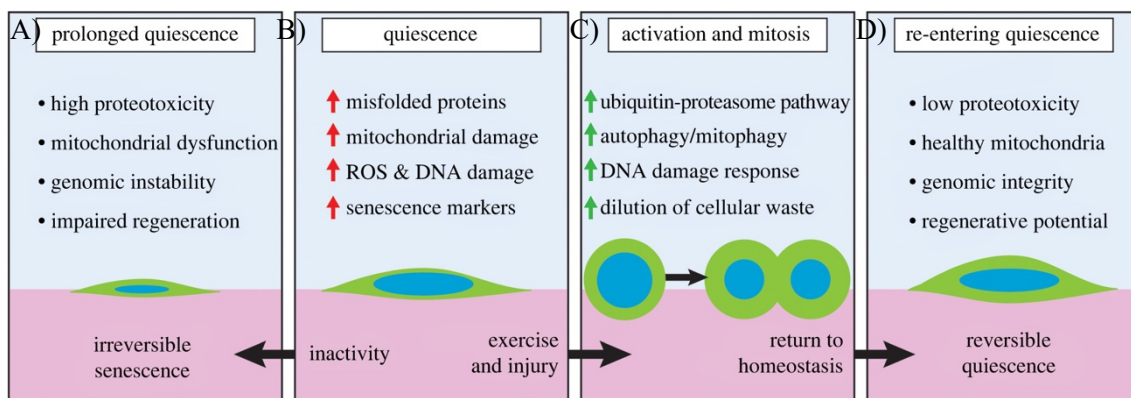


Figure 13. Healthy quiescent satellite cells (SCs) require occasional activation. (A) Long periods of quiescence lead SCs to an irreversible senescent status. (B) Quiescent SCs are susceptible to proteotoxicity due to accumulation of misfolded proteins, mitochondrial damage and reactive oxygen species (ROS) production. (C) After long periods of quiescence, SC activation through exercise or injury ameliorates the senescent phenotype by increasing autophagy, protein degradation and dilution of cellular waste. (D) Activated SCs can return to quiescence with lower levels of proteotoxicity than before its activation. Taken from Chen *et al.*, 2020.

Overall, a better understanding in mechanism involved in SC dysfunctions in muscle wasting pathologies, such as DMD and sarcopenia, will help us to uncover new therapeutic approaches that enhance intrinsic potential of SCs for muscle regeneration in these conditions.

6 - MicroRNAs (miRs) in the context of myogenesis and muscular diseases

Skeletal muscle development and regeneration are a well-orchestrated processes with a precise control of gene expression and post-transcriptional regulation. In the last decades, miRs, small non-coding RNAs, have emerged as crucial post-transcriptional regulators of gene expression in a sequence-specific manner in every biological process (Lee et al., 1993; Melo and Melo, 2014). In this context, miRs have been described to participate from embryonic myogenesis to adult muscle homeostasis and regeneration (Hernández-Torres et al., 2020).

MiRs canonical biogenesis start in the nucleus where they are transcribed by RNA polymerase II into primary-miRNA (pri-miRNA) transcripts (figure 14). Some miR loci can contain several miRs, named miR-clusters, that are transcribed together as a primary transcript (Hernández-Torres et al., 2020). Pri-miRs structure contain stem-loop recognized and cleavage by the RNase II endonuclease Drosha, resulting in miRs precursor, pre-miR (Lee et al., 2003). Afterwards, pre-miR is translocated to the cytoplasm by exportin 5 via Ran-GTP-depend mechanism and processed by RNase III Dicer into a mature double-stranded ~22-nucleotid miR (figure 14) (O'Brien et al., 2018). Half of the miRs are transcribed from introns of other non-coding RNAs or from protein coding genes following the canonical pathway for its biogenesis. However, some miRs are encode in the introns of host gene and they are transcribed at the same time as their host gene by the miRtron pathway (figure 14) (Hernández-Torres et al., 2020).

In order to carry out its post-transcriptional regulatory function, the mature miR (guide strand) is transferred to RNA-Induced Silencing Complex (RISC) containing Argonaute 2 (AGO2) and the RNA-binding protein TARBP2 (TAR (HIV) RNA-Binding Protein 2) (Hernández-Torres et al., 2020). The mature miR will guide the silencing complex to the target messenger ribonucleoprotein acid (mRNA) for its inhibition or degradation (figure 14). miR-mRNA interaction occurs in a complementary manner in a 6-8 nucleotides sequence named seed sequence, normally between the 3' UTR of the mRNA and 5' end of the miR (Melo and Melo, 2014; Sjögren et al., 2017). miRs can have several targets, as well as one mRNA can be post-transcriptional regulated

by several miRs. Even tough, sometimes the same miR and its target can have divergent effects depending on the tissue or context (Sjögren et al., 2017).

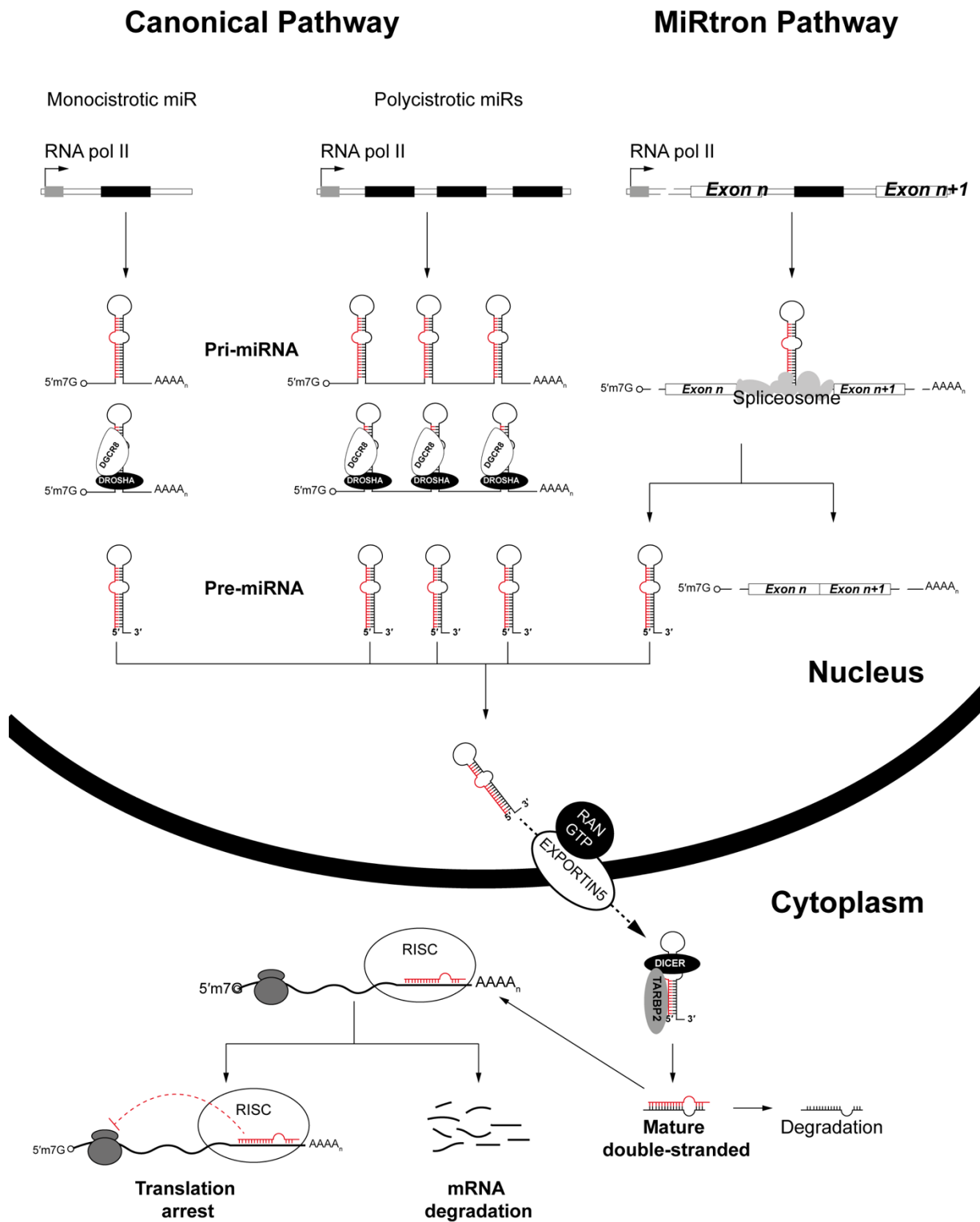


Figure 14. Schematic representation of miRs biogenesis. Taking from Hernández-Torres et al., 2020.

6.1 - miRs as regulators of skeletal muscle myogenesis

It has been proved that miRs are key regulators in SC maintenance and function since the deletion of *miRNA-processing endoribonuclease Dicer1* in SCs leads to an exhaustion of its pool and the lack of skeletal muscle regeneration (Cheung et al., 2012). There is a group of miR families named 'myomiRs' that are preferential or exclusive expressed in heart and skeletal muscle tissue: miR-1, miR-133a, miR-206, miR-143, miR-486, miR-499 and miR-208a/-208b (Hernández-Torres et al., 2020; Sempere et al., 2004). Among them, miR-208a is exclusive expressed in cardiac muscle and miR-206 in skeletal muscle. MyomiRs as well as other non-myomiRs have been described to play a critical role regulating SC behaviour during quiescence, activation and differentiation, from adult myogenesis to embryonic myogenesis (Hernández-Torres et al., 2020; Sjögren et al., 2017).

6.1.1 – miRs in adult myogenesis

SC quiescence in adult skeletal muscle is regulated by several miRs (Hernández-Torres et al., 2020). For instance, Cheung *et al.* have shown that miR-489 promotes SC quiescence by inhibiting the oncogene *Dek*, strongly upregulated during activation (Cheung et al., 2012). Similarly, miR-15 family (miR-195 and miR-497) inhibits myoblast proliferation by targeting *Igf-1* receptor and regulatory cell cycle genes (*Cell division cycle(Cdc)-25*, *Ccnd1*, *Ccnd2* and *Ccne1*), thus maintaining SC quiescence status (Sato et al., 2014). Recently, Baghdadi *et al.* have described that the Notch-miR-708-Tns3 axis actively represses SC migratory machinery, thereby, stabilizing the quiescent SCs in their niche (Baghdadi et al., 2018).

During adult myogenesis, miRs also have an active role in SC activation, proliferation and differentiation. Feng *et al.* have found that, in C2C12 myoblast cultured *in vitro*, the myomiRs, miR-1 and miR-133 target (mRNA) *Fgfr1* and *Pp2ac* promoting the myoblast (Feng et al., 2013). In the same line, *in vivo* analyses carried out by Zhang *et al.* revealed that after a muscle injury, FGF2 represses p38 signalling pathway leading to miR-1 and miR-133 downregulation and thus an increase of its targets: *Ccnd1* and *Sp1*, needed for SC activation and re-enter in the cell cycle (Zhang et al., 2012). On the other hand, miRs directly control the downregulation of transcription factor *Pax3* and *Pax7*, essential for SC commitment to differentiation. Crist *et al.* have showed that miR-27 directly tags (mRNA) *Pax3*. *In vitro* miR-27 inhibition in fresh isolated adult SCs preserves the levels of PAX3, resulting in a delay in myogenic differentiation and, *in vitro* anti-miR-27 administration after a muscle injury avoids PAX3 downregulation, delaying thus

skeletal muscle regeneration (Crist et al., 2009). miR-27 can also promote *in vitro* C2C12 myoblast proliferation by targeting *Myostatin*, a well-known negative regulator of myogenesis (Huang et al., 2012). Moreover, the levels of transcription factor *Pax7* can be directly regulated by miR-431, which controls the fine tune between the PAX7^{high} and PAX7^{low} SC populations, with heterogeneity behaviour towards myogenic differentiation or to stem like phenotype respectively (Wu et al., 2015). Additionally, it has been shown that *Pax7* is directly downregulated in mouse C2C12 myoblasts by miR-486 and miR-206 accelerating myoblast differentiation (Dey et al., 2011). Furthermore, in cultured myoblast miR-486 targeted several mRNAs promoting initial muscle differentiation: *Tensing homolog (Pten)*, *Platelet-derived growth factor receptor beta (Pdgfrβ)*, *Foxo1*, *Serine and arginine-rich splicing factor 1 (Sfrs1)* and *Sfrs3* (Hernández-Torres et al., 2020). Both transcription factors *Pax7* and *Pax3*, are directly tagged by the myomiRs: miR-1 and miR-206. Thus, Chen *et al.* have described that miR-1 and miR-206 are highly upregulated during SC activation and downregulated after muscle regeneration. miR-1 and miR-206 downregulate PAX7 protein expression, restricting SC proliferative potential and promoting its differentiation (Chen et al., 2010). Later, Goljanek-Whysall *et al.* have demonstrated in C2C12 myoblasts that miR-1 and miR-206b promote myogenic differentiation by targeting several regulatory factors, highlighting the essential role of multiple miR targets for muscle differentiation program as well as to prevent alternative cell fates (Goljanek-Whysall et al., 2012). Finally, different studies showed that miR-29 plays a key role in myoblast differentiation by inhibiting several mRNAs involved in the NF-κβ pathway, such as *Rybp*, *Yy1*, *Histone deacetylase (Hdac)-1/-4* and *Akt3* (Wei et al., 2013; Winbanks et al., 2011; Zhou et al., 2012).

It is important to stress that (mRNA) *Myf5*, the first MRF that control myogenic commitment of SCs, is also directly regulated by miRs, such as miR-31. Curiously, miR-31 sequesters (mRNA) *Myf5* in messenger ribonucleoprotein (mRNP) granules until SC activation (Crist et al., 2012). In this context, we have previously identified miR-106b as a member of a Pitx2-miR pathway regulating SC activation and myogenic commitment (Lozano-Velasco et al., 2015, 2011). We have demonstrated that one of the miRs regulated by PITX2c, miR-106b, displays a tissue expression pattern compatible with marked expression on SCs (Lozano-Velasco et al., 2015). In addition, our *in vitro* analysis in freshly isolated SCs have shown that miR-106b modulates cell proliferation by targeting *Ccnd1* and *Ccnd2* 3'UTRs (Lozano-Velasco et al., 2015). More importantly, in SCs miR-106b also targets the *Myf5*, that play a key role in the acquisition of a myogenic cell fate, and down-regulation of miR106b leads to an increase the MYF5^{pos} SC-derived population (Lozano-Velasco et al., 2015). These results indicated that miR-106b can modulate

the commitment to a myogenic cell fate, suggesting that it might play an important role in the regulation of SC activation and differentiation.

All together, these studies highlight the crucial regulator function of miRs for SC behaviour, proposing miRs as potential targets enhancing SC functionality in skeletal muscle regeneration (Hernández-Torres et al., 2020), aiming miR-106b as a novel candidate to enhance both SC proliferation and myogenic commitment.

6.1.2 – miRs in embryonic myogenesis

MyomiRs and other miRs are also essential during embryonic myogenesis for proper muscle development (Luo et al., 2021). For instance, Chen *et al.* have shown that in *in vivo* and *in vitro* analysis of *Xenopus laevis* embryos miR-133 promotes myoblast proliferation by repressing the *serum response factor (Srf)* (Chen et al., 2006). Crist *et al.* have discovered that miR-27 directly targets (mRNA) *Pax3* in the MT during embryonic myogenesis, promoting myoblast differentiation. Remarkably, the myomiRs miR-1 and miR-206b, upregulated during myogenic precursor cells activation by MRFs, participate in somite myogenesis by directly targeting (mRNA) *Pax3*, causing myogenic cell commitment and later differentiation (Goljanek-Whysall et al., 2011). Additionally, Anderson *et al.* have shown that, in perinatal muscles, miR-1 and miR-206 promote myoblast fusion through targeting *Connexin43 (Cx43)* (Anderson et al., 2006).

On this regard, our group has also identified that Pitx2 is essential in PAX3^{pos} uncommitted myogenic precursor cells during early embryonic muscle development mediating cell proliferation and migration (Ramírez de Acuña et al., 2022). However, *Pitx2* deletion in committed myogenic precursor cells (*Myf5^{Cre/Wt}/Pitx2^{-/-}* mutant mice) gives rise to normal skeletal muscle that contain SCs with higher PAX7 expression levels (PAX7^{high} SC subpopulation) that display functional regenerative defects (Ramírez de Acuña et al., 2022). In this scenario, SC population of *Myf5^{Cre/Wt}/Pitx2^{-/-}* homozygous mutant mice presents a reduced number of MYF5^{pos} SCs but an increased number of quiescent miR-106b^{pos} SCs. Additionally, two distinguishable PITX2^{pos} and miR-106b^{pos} cell populations are observed in the DMT during embryonic myogenesis (E10.5) (Ramírez de Acuña et al., 2022). All together indicates that miR-106b may have a role in myogenic precursor cell specification during embryonic development and SC heterogeneity origin in adult skeletal muscles.

6.2 - miRs in SCs in the context of muscular disorders

Several analyses of muscle samples from different mouse models and human patients of muscle wasting diseases have described that miR dysregulation promotes and/or exacerbates these pathological conditions (Aránega et al., 2021; Perry and Muntoni, 2016; Yedigaryan and Sampaolesi, 2021). In this circumstances, miR modulation has been proposed to restore different features of skeletal muscle regeneration that can reduce skeletal muscles loss.

6.2.1 - miRs in DMD

miRs dysregulations in DMD muscles worsen the diseases by enhancing muscle fibrosis, magnifying myofiber disruption and/or decreasing SC regenerative potential (Yedigaryan and Sampaolesi, 2021). In accordance, miR levels restoration can ameliorate several features of DMD condition, such as fibrosis, mitochondrial damage as well as to increase muscle regeneration potential.

It has been described that miR-21 and miR-29 regulate muscle fibrosis in mdx mice (Morgoulis et al., 2019; Zanotti et al., 2015). Zanotti *et al.* have shown that miR-21 is upregulated in muscle biopsies and fibroblasts isolated from DMD patients. After *in vivo* miR-21 silencing in mdx mice and *in vitro* inhibition in DMD fibroblasts, muscle fibrosis is reduced and fibrotic factors are downregulated respectively (Zanotti et al., 2015). Additionally, they have observed an opposite role for miR-29 which is significantly downregulated in DMD muscle and fibroblasts. Thus, *in vitro* miR-29 overexpression in DMD myoblasts reduces the profibrotic targets (Zanotti et al., 2015). Previously, Wang *et al.* have observed that *in vitro* and *in vivo* restoration of miR-29 (down-regulated in mdx mouse model) not only reduces fibrosis, but it also promotes muscle regeneration by inhibiting myoblast transdifferentiation into myofibroblast, route upregulated in DMD pathology (Wang et al., 2012).

Others miRs alterations have been described as related to exacerbate the loss of myofiber integrity and to reduce proper muscle function in DMD context. In a study carried out by Liu *et al.*, they showed that miR-499, which participates in Fnip1/AMPK signalling controlling mitochondrial function, is downregulated in muscle from DMD patients and mdx mice. *In vivo* miR-499 overexpression in mdx mice restores mitochondrial function in the dystrophic myofibers, which in the end reduces the myofiber turnover, improving the histological phenotype (Liu et al., 2016). Likewise, Alexander *et al.* have shown that miR-486 is down-

regulated in muscle from DMD patients and in $Dmd^{mdx-5Cv}$ mouse model. Interestingly, when miR-486 is overexpressed in $Dmd^{mdx-5Cv}$ mice, they observed a reduction in DOCK3/PTEN/AKT signalling leading to improving sarcolemma integrity, reducing thus the myofiber turnover (Alexander et al., 2014). Besides, Zhai *et al.* have observed that miR-127 promotes differentiation of C2C12 myoblasts, accelerates muscle regeneration in wild-type mice and promotes myogenic cell differentiation in transgenic mdx mice overexpressing miR-127 by reducing the levels of myofiber damage markers via *S1pr3* (Zhai et al., 2017). These studies demonstrate that miR modulation can reduce myofiber damage and its turnover ratio in dystrophic muscles.

Additionally, Wu *et al.* have observed that miR-431 controls the fine tune between the $PAX7^{high}$ and $PAX7^{low}$ subpopulation by targeting *Pax7*. miR-431 transgenic mouse model (overexpressing miR-431) presents a large number of committed $PAX7^{low}$ SC subpopulation, and they propose that its upregulation may enhance muscle myogenic commitment. Consistently, when miR-431 is overexpressed in mdx mice, the number of proliferating and committed myoblasts is increased (Wu et al., 2015). However, it may be asked whether miR-431 overexpression and subsequently reduction of $PAX7^{high}$ SC population, essential for muscle self-renewal capacities during life, could deplete the SC pool in a long-term period.

Interestingly, Cacchiarelli *et al.* have described that miR-31, which targets *Myf5*, could also directly inhibit (mRNA) *Dystrophin*. miR-31 levels are up-regulated in muscles from mdx mice and DMD patients. Moreover, *in vitro* miR-31 inhibition combined with exon splicing strategy (to produce functional DYSTROPHIN) in human DMD myoblasts, rescues higher DYSTROPHIN protein production than de-repression by miR-31 or exon skipping alone (Cacchiarelli et al., 2011). This study exemplifies that therapeutical approaches combining miR modulation and DYSTROPHIN rescue could be essential to improve DMD phenotype over the long term.

Within this context, previous data published by our group has shown that *Pitx2c* isoform is downregulated in SCs isolated from mdx mice (Vallejo et al., 2018). Interestingly, the activation of the *Pitx2*-miR-106b pathway in dystrophic SCs by overexpressing *Pitx2c* and subsequently transplanted into host mdx mice, significantly increased the number of proliferative myogenic committed cells after muscle injury, leading to an enhance of muscle regeneration (figure 15). We have also found that *Pitx2c* overexpression in mdx SCs increased DYSTROPHIN-positive (revertant) myofibers by *Pitx2c*-mediated miR-31 down-regulation (figure 15) (Vallejo et al., 2018).

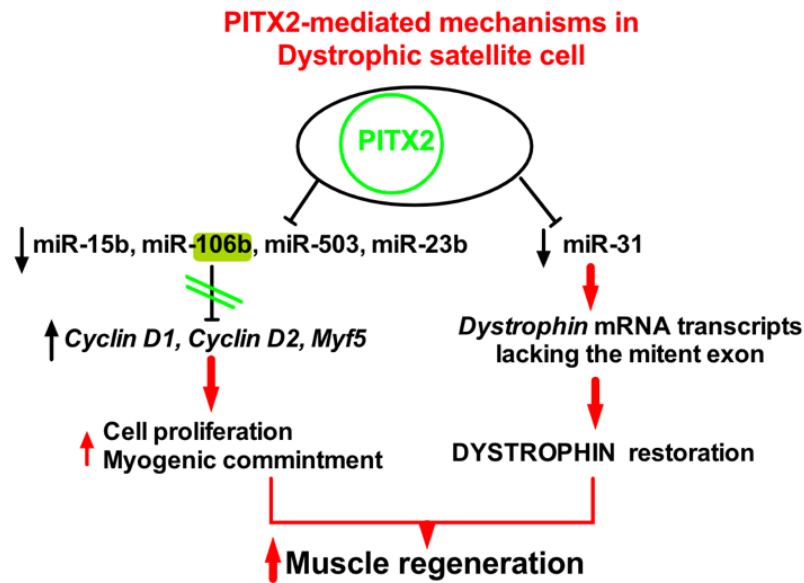


Figure 15. PITX2-miR pathway in dystrophic satellite cells (SCs) during muscle repair. *Pitx2* overexpression in dystrophic SCs enhance proliferation and myogenic commitment through miR-106b inhibition. Additionally, *Pitx2* overexpression represses miR-31, which lead to an increase in the amount of DYSTROPHIN-expressing myofibers. Altogether enhance muscle regeneration in mdx dystrophic mice. Taking from Vallejo *et al.*, 2018.

These results provide *in vitro* and *in vivo* evidence for a role of PITX2-miRNA pathway in skeletal muscle repairment and in degenerative muscle diseases. Since we previously identified miR-106b as a member of a Pitx2-miRNA pathway, which regulates the cell proliferation and myogenic commitment of SCs, we think that miR-106b may play a role in SC myogenic progression during muscle regeneration and could have an impact by modifying SC behaviour in the context of muscle dystrophies.

6.2.2 - miRs in sarcopenia

Similar to what observed in DMD, diverse studies have shown that several miRs are deregulated in muscles and SCs isolated from aged mice and in elderly patients compared to young controls (Drummond *et al.*, 2011; Yanai *et al.*, 2020). These miRs dysregulation worsen the sarcopenic phenotype, suggesting that miRs may be interesting therapeutic targets to ameliorate impaired skeletal muscle regeneration in ageing.

It is important to highlight that some miRs present similar dysregulations in both DMD and sarcopenic muscles. For instance, Hu *et al.* have found that miR-29, overexpressed in DMD context, is also upregulated in aged mouse muscles. *In vitro* miR-29 overexpression in primary myoblasts reduces myogenic proliferation and induces senescence throughout several targets

that contribute to muscle atrophy (*p85α*, *Igf-1* and *B-myb*). Additionally, miR-29 injection in young mice led to an aged muscle phenotype, suggesting that increased miR-29 levels in aged muscles potentiates sarcopenic phenotype (Hu et al., 2014). Furthermore, it is interesting to note that miR-431, which regulates *Pax7* expression levels in PAX7^{high} and PAX7^{low} SC subpopulation, is downregulated in aged myoblast (Lee et al., 2015). Overexpression of miR-431 improves myoblast proliferation in mdx, likewise, ectopic expression of miR-431 in aged myoblast enhances its differentiation potential by targeting (mRNA) *Smad4*, involved in TFG-β signalling during myogenic commitment (Lee et al., 2015).

Other miRs dysregulation can explain the pre-senesce phenotype observed in aged SCs. Soriano-Arroquia *et al.* have demonstrated that the myomiR miR-143 regulates *in vitro* muscle regeneration throughout tagging *Insulin growth factor-binding protein 5 (Igfbp5)*. miR-143 is down-regulated in aged SCs from mice, probably as a compensatory mechanism. Moreover, the miR-143 target (mRNA) *Igfbp5* is also associated with increased cell senescence, which in the end contribute to the senescent phenotype present in aged SCs (Soriano-Arroquia et al., 2016). Recently, those authors have described that miR-24 is also downregulated in old SCs. They proposed that miR-24 downregulation in aged SCs could be an adaptative response since its target *Prdx6*, upregulated during ageing, participates in the reduction of mitochondrial ROS production and DNA damage response (Soriano-Arroquia et al., 2021). Microarray analysis has shown that Let-7 miRNA family is significantly upregulated in muscle samples from elderly patients. Let-7 miRNA family inhibits SC proliferation in aged mice throughout targeting several cell cycle regulators (*Cdk6*, *Cdc25a* and *Cdc4*), which in the end suggests an impaired cell cycle regulation and reduction of self-renewal divisions in sarcopenic muscles (Drummond et al., 2011). Another study carried out by Onodera *et al.* have described that miR-155, up-regulated in aged mouse SCs, activates SC differentiation, reducing stemness cell pool during ageing (Onodera et al., 2018).

Similar to what has been described in DMD diseases, miR restoration has been proposed as novel therapeutic approach to ameliorate sarcopenic muscle phenotype. A study carried out by Goljanek-Whysall *et al.* have described that miR-181a, regulator of mitochondrial dynamics, is downregulated in muscle from aged mice and patients. After restoring miR-181a levels in old mice, mitochondrial quality and muscle function is improved by enhancing autophagy potential and reducing abnormal mitochondria generation (Goljanek-Whysall et al., 2020). Similarly, miR-195, up-regulated in aged myoblasts from mice, promotes senescent phenotype by targeting

the antisenesence (mRNA) *Sirt1*. After *in vitro* miR-195 inhibition in aged myoblast, the regenerative potential was restored (Kondo et al., 2016)..

Altogether those studies exemplify the potential of miR modulation in aged muscles and SCs as therapeutic approach to enhance its self-renewal loss and restore regeneration muscle potential in sarcopenia (Sannicandro et al., 2019). In this context, and as we have previously shown that miR-106b had a role in SC activation as well as myogenic commitment during muscle regeneration (Rodríguez-Outeiriño et al., 2022), we speculate that miR-106b modulation could improve aged SC self-renewal potential as well as myogenic commitment, consequently, increasing the myogenic potential in aged muscles.

CHAPTER II: HYPOTHESIS AND AIMS

Hypothesis

We have previously identified miR-106b as a member of a Pitx2-miRNA pathway that regulates cell proliferation and myogenic commitment of early activated SCs by targeting cell-cycle regulatory genes *Ccnd1* and *Ccnd2*, and also myogenic factor *Myf5*. Diverse muscle disorders, such as DMD and sarcopenia, are linked with the loss of the regenerative capabilities due to intrinsic SC defects to generate myogenic committed progeny, needed to successfully complete muscle regeneration. In addition, in the mouse embryos we have identified the presence of a miR-106b-expressing cell population of migrating myogenic progenitors in the limb buds. Therefore, in this doctoral Thesis we hypothesize that miR-106b may play a role in modulating SC behaviour in the context of muscle regeneration and disease. In this work we also initiate the analysis of miR-106b role during embryonic myogenesis.

Aims

1. To unravel the miR-106b function during skeletal muscle regeneration.
2. To evaluate if miR-106b regulation may play a role in the context of DMD.
3. To investigate miR-106b modulation of SCs in sarcopenia.
4. To analyze miR-106b expression profile during skeletal muscle development.

CHAPTER III: MATERIAL AND METHODS

All the employed reagents and materials are listed in section ‘13- supplementary information’ at the end of the chapter III: Material and methods.

1 – Animal samples, human samples and cell Lines

The animal procedures were approved by the University of Jaen (UJA) Ethics Committee, and were conducted according to national and European community guideline regulations for animal care and handling inside an animal facility at the UJA, University of Granada (UGR) and University of Galway, Ireland. C57BL/6J and C57BL/10ScSn-Dmd^{mdx}/J (henceforth mdx) mice were supplied by the Jackson Laboratory. CD1 mice were supplied by the Charles River Laboratories. DBA2J/mdx mice were kindly provided by Pura Muñoz-Cánoves’s Laboratory. NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (henceforth NSG) were provide by the UGR. The use of human sample collection was approved by the Human Research Ethics Committee of UJA and UGR. Biopsies were obtained from informed patients or their legal guardian’s consent. Sol8 and HEK293T cell lines were purchased from ATCC® and kept in culture in line with the manufacturer’s instructions. Detailed information is listed in table 1.

Experimental Model	Reference or Source	Identifier or Information
C57BL/6J mice	The Jackson Laboratory	nº 000664
C57BL/10ScSn-Dmd ^{mdx} /J mice	The Jackson Laboratory	nº 001801
CD1 mice (CD-1®)	Charles River Laboratories	nº 022
NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Wjl} /SzJ NSG TM , NOD scid gamma mice	Ceded the by University of Granada. From Jackson Laboratory.	nº 005557
DBA/2 mice	Kindly provided by Pura Muñoz’s laboratory. From Janvier Labs	DBA/2J
HEK293T cell line	ATCC®	CRL-3216 TM
Sol8 cell line	ATCC®	CRL-2174 TM
Wild type and DMD mouse primary satellite sells	from C57BL/6J and C57BL/10ScSn-Dmd ^{mdx} /J mice hindlimbs	N/A
Wild type skeletal muscle myofiber	from extensor <i>digitorum longus</i> (EDL) muscle of C57BL/6J mice	N/A

Human Plasma	Biobank of the Public Health System of Andalusia	N/A
Human biopsies from BMD patient and primary satellite cells	Hospital Univesitario San Cecilio, Granada, Spain	Quadriceps muscles. Male, 32 and 35 years old
Human biopsy from DMD patient and primary satellite cells	Hospital Univesitario Virgen del Rocío, Seville, Spain. Provided by Dr. Carmen Paradas	Quadriceps muscles. Male, 5 years old
Human biopsies from healthy patients and primary satellite cells	Hospital Univesitario San Cecilio and Biobank of the Public Health System of Andalusia. Granada, Spain	Sartorius and anterior rectus femoris muscles Male, 27 to 40 years old.
Human myoblasts from healthy patients and DMD male patients in Myoblot study	Queen Square Centre for Neuromuscular Disorders BioBank (CNMD Biobank, London, UK)	Males
BMD, DMD and healthy human primary satellite cells	from biopsies cited in this table	Males

Table 1. Cell Lines, Animals and Human samples. N/A - not applicable.

1.1 - Mouse and Human SCs isolation and culture

For mouse SCs isolation, hindlimb muscles from 4-5-month-old male (C57BL/6 and mdx) mice and 29-month-old male C56BL/6 mice were collected. Muscles were transferred and minced on a dish with DMEM:F12 (1:1). Minced muscles were dissociated 1 hour at 37 °C on an enzymatic solution containing DMEM:F12 (1:1) supplemented with 1 mg/mL collagenase-type D and 4% Trypsin solution. Afterwards, mechanical dissociation was performed through 14 G and 18 G needle up and down. For each step of mechanical dissociation, muscle mixture was decanted 5 min and supernatant was collected to a new tube with 5 mL of Fetal Bovine Serum (FBS). Supernatant-FBS suspension was filtered through 100 µm and 40 µm cell strainers. Suspension cells were centrifuged at 300 g for 5 min and then the pellet was resuspended with 1 mL of PBS. Red Blood Cells Lysis Solution was incubated for 2 minutes. Negative (MicroBeads Satellite Cell

Isolation Kit) and positive (Anti- Integrin α -7 MicroBeads Kit) selections were sequentially performed by incubation at 1:5 dilution with magnetic labelling for 15 min at 4°C. LS and MS Columns were pre-equilibrated and placed in appropriate magnets and cell suspension was applied onto the column. Purified mouse SCs (5000- 10000 cells) were seeded on 0.1% gelatin coated ibidi μ -Dish 50 mm and cultured in mouse growth medium (mouse-GM) containing DMEM GlutaMAX™ and DMEM:F12 50:50 supplemented with 20% FBS, 2% Ultrosor® G, 3% Penicillin/Streptomycin (P/S) and incubated at 37°C, 5% CO₂. For myoblast differentiation, when 70-80% confluence is reached, the medium was changed decreasing to 2% FBS.

For hMuSCs isolation, BMD biopsies and their controls were sorted by MACS as previously described for mice, in this case negative cell sorting was performed using CD45 and CD31 human MicroBeads and positive cell sorting was performed using CD56-human MicroBeads. DMD and control hMuSCs were isolated from fresh muscle biopsies as follows. Muscle fascicles were released and cut in 3 mm fragments in HBSS supplemented with 1% P/S-Fungizone (PSF). Fascicles were incubated overnight at 37 °C, 5% CO₂ in conditioning medium containing M199 supplemented with 37.5% FBS and 1% PSF. Next day, explants were seeded on plates coated with 25% v/v fresh human plasma until cell growth appeared. Subsequently, explants were cut in 1 mm length fragments and cultured on 33.3% v/v fresh human plasma-1.5% gelatine type-A coated plates until cell monolayer appeared around explants. Positive cell sorting was performed using CD56-human MicroBeads. CD56^{pos} hMuSCs were seeded on 0.1% gelatin type-A coated plate and cultured in human-GM containing 75% DMEM and 25% M199 supplemented with 10% FBS, 50 μ g/mL insulin, 0.25 μ g/mL human-FGF2, 0.02 μ g/mL human-EGF, 2 mM L-glutamine and 1% PSF. Cells were incubated at 37 °C, 5% CO₂ until 50-60% of confluence was reached. For myoblast differentiation 70%-80% confluence should be reached and human-GM was substituted with one supplemented with 2% FBS, 50 μ g/mL insulin, 2 mM L-glutamine and 1% PSF.

1.2 - Single myofiber isolation and culture

Isolated single myofibers were obtained from EDL of 4-month-old C57BL/6 mice as previously described by Zammit's lab with slight variations (Moyle and Zammit, 2014). After dissection, EDL muscles were incubated in 0.2% w/v collagenase type I /DMEM for 1 hours 30 min in a 37 °C water bath. Digestion was stopped when the muscle started to loosen up. EDLs were transferred to a pre-warmed dish with DMEM supplemented with 1% P/S. After 30 min of muscle rest,

mechanical dissociation was performed to release individual myofibers flushing with a bore glass pipette (dishes were returned to the incubator every 10 min for 10 min to keep them warm). Single myofibers were transferred through three new pre-warmed dishes for clean-up. Immediately, single myofibers were fixed for analysis at 0 hours or cultured for 48 hours in high serum medium (DMEM-high glucose, GlutaMAXTM supplemented with 10% Horse Serum, 0.5% Chicken Embryo and 1% P/S). Horse serum was used on plates and glass pipettes as coating substrate for floating condition and to avoid stuck fibers.

2 - *In vitro* miR modulation

2.1 - Lentiviral transduction

HEK293T cells (2×10^6) were plated in a 100-mm tissue culture dish and transfected the next day when they reached 75% confluent with plasmids harbouring mimic hsa-mir-106b (premiR), inhibitor hsa-miR-106b (antimiR) or control-scramble miR sequences (figure 16) by using Lenti-PacTM HIV Expression Packaging Kit. Infectious lentivirus were harvested at 48 hours post-transfection, filtered through 0.22 μ m and stored at -80 °C. Lentivirus were titrated by using the Lenti-PacTM HIV qRT-PCR Titration Kit.

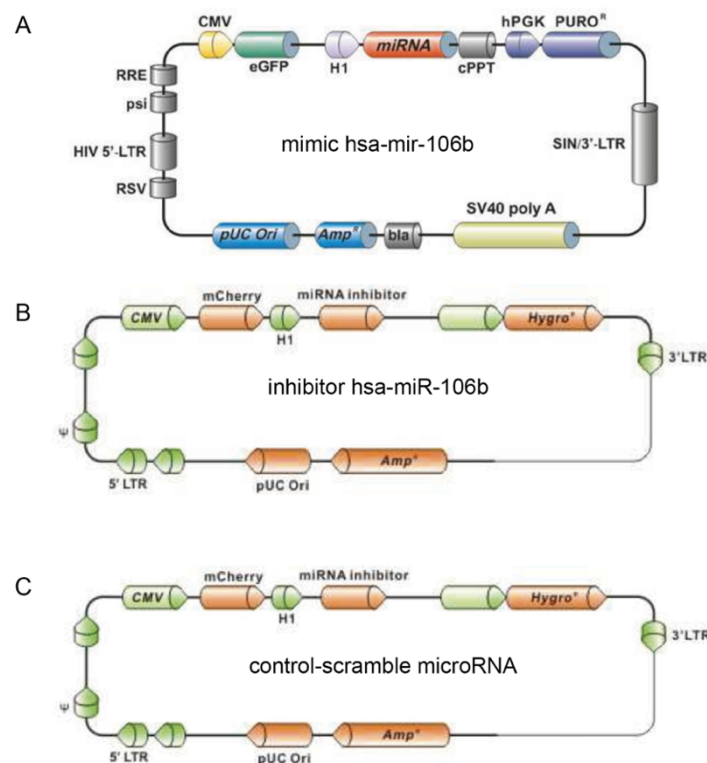


Figure 16. Plasmids maps for lentiviral particle production. (A) mimic has-miR-106b (premiR). **(B)** inhibitor has-miR-106b (antimiR). **(C)** control-scramble microRNA. From GenecopoeiaTM.

For *in vitro* miR gain-and-loss of function analysis, fresh isolated SCs from 3-month-old mice (C57BL/6 and mdx) and fresh isolated HMuSCs, from BMD DMD and control samples, were transduced at 200 MOI at day 0. Transduction was monitored by fluorescence microscopy. GM was refreshed at day 3. Subsequently, cells were fixed in PFA 4% at different days for immunostaining analysis in order to study proliferation (Ki67/MyoD1 staining) and differentiation (MYOG and/or MF20 staining).

For miR loss of function *in vitro* analysis in aging, fresh isolated SCs from two 29-month-old male C57BL/6 mice were transduced at day 0. GM was refreshed at day 4. Cells were fixed at day 6 for proliferation analysis (KI67/MYOD1 staining), differentiation media was changed at day 7 and cells were fixed at day 10 for differentiation analysis (MF20 staining).

2.2 - Lipofectamine transfections

Sol8 cell line were transfected with mirVana™ miRNAs sequences (premiR-31, premiR-106b, premiR-31 + premiR-106b and Scramble) at 80 nM by using Lipofectamine-2000 following manufacturer's instructions. After 24 hours, cells were collected for RNA extraction and retrotranscription (RT) -qPCR analysis.

3 - Cell cycle arrest on fresh isolated SCs

Fresh isolated SCs from 1-month-old male C57BL/6 mice were seed in two μ -Dish 50 mm for lentivirus transfection with scramble or antimiR-106b as described before. After 3 days of culture, SCs were attached, medium was refreshed and cycle arrest was started as previously described (Liu et al., 2012). Briefly, to inhibit DNA synthesis, SCs were treated with 2.5 mM thymidine for 16 hours and released for 8 hours, treated again with 2.5 mM thymidine for 16 hours and released for 4 hours. Afterwards, to arrest cells during mitosis, SCs were treated with 68 μ M monastrol for 8 hours, released and treated for 2 hours with 10 μ M MG132 to block the proteolytic activity of the 26S proteasome complex. Finally, cells were released for 1 hour to synchronize activated SCs at the late telophase stage, cells were then fixed in 4% paraformaldehyde (PFA) for 10 min and immunocytochemistry was performed.

4 - Human myoblast in-cell western assay (Myoblot)

For the in-cell western assay, human DMD patient's myoblasts (exon 48-50 deletion) were requested from the Queen Square Centre for Neuromuscular Disorders Biobank (CNMD Biobank, London, UK). Myoblasts were maintained in Skeletal Muscle Cell GM supplemented with 10% FBS, GlutaMAX™, 1x MEM NEAA, 1% P/S, and incubated at 37°C in 5% CO₂.

Human myoblasts were seeded at 7500 cells/well on a 96-well plate previously coated with Matrigel® in Skeletal Muscle Cell GM. After 24 h, when confluence was reached, medium was replaced with differentiation medium (DMEM-Lonza, 20% horse serum and 1% P/S). After 24 h, cells were transfected with mirVana™ miRNAs sequences (premiR-106b, antimiR-106b or Scramble) at 40 nM using Lipofectamine-2000. Briefly, the medium was replaced with fresh differentiation medium at 5 h post-transfection. After a 7-day incubation, period to allow myotube formation, cells were fixed by adding ice-cold methanol for 10 min at room temperature and washed with 1x PBS. For the in-cell western assay, cells were permeabilized with 1x PBS-Tween 0.1% Triton and then incubated with Odyssey® Blocking Buffer for 2 h. After blocking, MF20 primary antibody diluted 1:100 in Odyssey® Blocking Buffer was added for an overnight incubation at 4 °C. On the next day, plates were washed with PBS-Tween 0.1% and then treated with the secondary antibody mix IRDye 800CW goat anti-mouse and CellTag 700 Stain for 1 h at room temperature. Then, incubation plates were washed again with PBS-Tween 0.1% solution and were scanned by Odyssey® CLx Imager.

5 - *In vivo* gain and loss of function analysis

For gain and loss of function analysis mice were anesthetized by using isoflurane, 2-5% inhaled, to a surgical plane of anaesthesia. 24 C57BL/6, 15 mdx and 8 DBA2J/mdx mice were injected in each in each *tibialis anterior* (TA) muscles with 50 µL of cardiotoxin solution (CTX; 10 µM) together with 11.55 µg of mirVana™ miRNAs: hsa-miR-106b-5p mimic (premiR-106b), hsa-miR-106b-5p inhibitor (antimiR-106b) or negative control #1 inhibitor (Scramble). Samples were collected at different days during muscle regeneration.

Finally, an exhaustion treadmill trial was performed to evaluate the endurance of treated 4-month-old male mdx mice (C57BL/6 were used as control) as described previously (Benchouir et al., 2007). Briefly, the treadmill was run at 0° of inclination at 5 m/min for 5 min; subsequently,

the speed was increased 1 m/min every minute. The test ended when the mouse remained more than 20 seconds on the shocker plate.

6 – Human Muscle SCs Xenotransplants

Xenotransplants were performed on 3-month-old NSG mice by intramuscular injection of anti-miR-106b-transduced hMuSCs. Briefly, two days before transplantation, TA irradiation (12 Gy) was done to incapacitate endogenous SCs, and hMuSCs were transfected with scramble or anti-miR lentiviral particles *in vitro*. One day before transplantation, injury with CTX was done on each TA to induce regeneration. During transplantation day, 1000–3000 treated hMuSCs were collected on 50 μ L of DMEM and injected using a 25 G needle into regenerating TA. Mice were sacrificed 5 weeks after cell injection and TA were harvested for histological analysis.

7 - Sample collection

7.1 - TA muscle collection

For muscle collection, mice were sacrificed by cervical dislocation. TA muscles from mice were collected and frozen in liquid nitrogen-cooled isopentane for sectioning or in liquid nitrogen for total RNA isolation. For histological analysis tissue specimens were sliced to 8 μ m thick by using a cryostat microtome and mounted onto SuperFrost Ultra Plus™ Adhesion Slides.

7.2 - Embryo samples collection for RNA isolation

At the day a vaginal plug was observed during timed matings, noon was considered E 0.5. Female CD1 mice were sacrificed at E9.5, E10.5, E11.5, E12.5 and E14.5 stage and embryos were collected and rinse in ice-cold PBS 3 times. Afterwards, each embryo was transferred into an Eppendorf with 1 mL of RNAlater® overnight at 4 °C as described previously (Sun et al., 2011). Next day, embryos were stored at -20 °C until dissection.

Dissection was performed in P100 plate with RNAlater® under a magnifier as follow. The head was cut off and each embryo was eviscerated. Afterwards, embryos were placed in a fresh RNAlater®-plate and the different regions were collected as shown in table 2 and figure 17.

Region	E9.5	E10.5	E11.5	E12.5	E14.5
Forelimb buds	X	X	X	X	N/A
EASDS at forelimb level	X	X	X	X	X
EASDS at interlimb level	X	X	X	X	N/A
EASDS at hindlimb level	X	X	X	X	N/A
Hindlimb buds			X	X	X
Number of samples/embryo	4	4	5	5	2

Table 2. Embryonic regions of CD mouse embryos collected for RT-qPCR analysis. EASDS – Embryonic Area containing Somite-Derived Structures. X – region collected. N/A - not applicable.

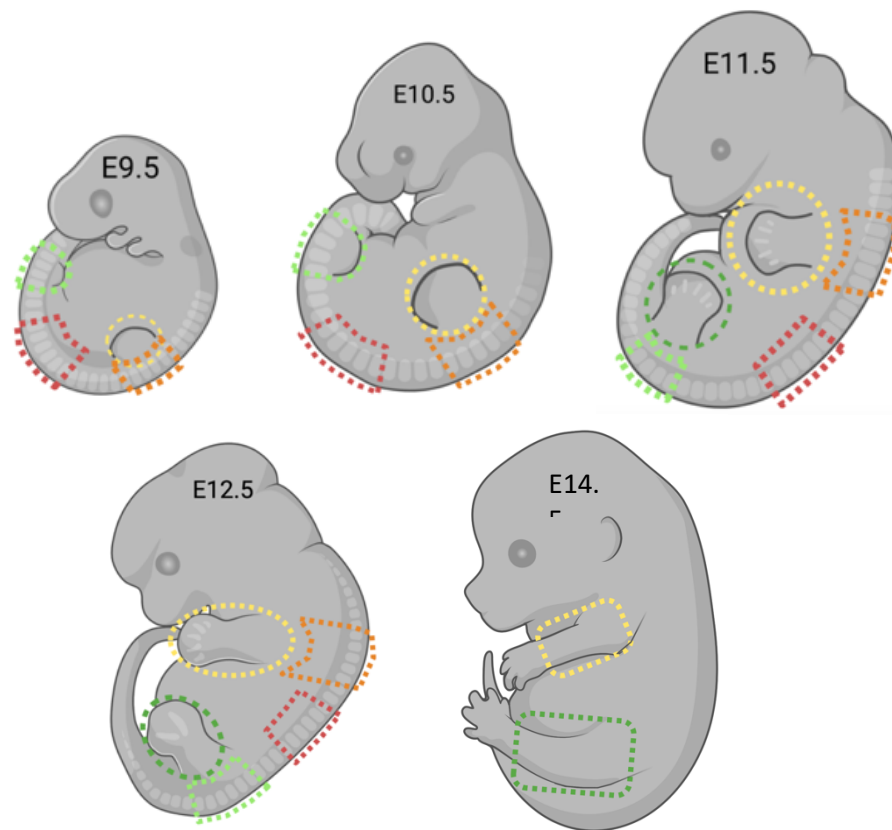


Figure 17. Embryos section recollected for RT-qPCR analysis of miR-106b expression. Yellow (forelimb buds), orange (area containing somite-derived structures at forelimb level), red (area containing somite-derived structures at interlimb level), light green (area containing somite-derived structures at hindlimb level), dark green (forelimb buds). At E9.5 and E10.5 stages area containing somite-derived structures at hindlimb level includes the hindlimb buds. Created by Biorender.com.

Each sample was placed in an individual Eppendorf with RNAlater®, briefly centrifuged to discard RNAlater and 100 µL of Lysis Buffer (from RNAqueous™-Micro Total RNA Isolation Kit) was added. Samples were vortex for 20 seconds, short-spin was done and they were stored at -80 °C at least on day prior to RNA isolation.

7.3 - Embryo samples collection for histological analysis

CD1 female mice were sacrificed at E9.5, E10.5, E12.5 and E14.5, embryos were collected and rinsed in ice-cold PBS 1x 3 times. Fixation was performed in 4% PFA overnight at 4 °C. Due to the size of the embryo, a hole in the head was performed at E10.5 stage as well as in older embryos to improve fixation step.

Afterwards, embryos were rinsed 3 times in PBS 1x for 10 min and plunged in increased dilutions of sucrose: PBS1x (10%, 20%, 30%, 40% and 40%-OCT). Finally, embryos were placed and embedded in OCT and frozen in liquid nitrogen-cooled isopentane for sectioning. Frozen embryos were sliced using a cryostat microtome to 10 µm thick and mounted onto SuperFrost Ultra Plus™ Adhesion Slides.

8 - Histological analysis

8.1 - Fluorescence *In Situ* Hybridization (FISH)

FISH for miR detection was performed in cryosections of TA muscles from C57BL/6 and mdx mice, mice embryos (CD1), human sartorius muscle, and isolated myofibers from mice (C57BL/6). All the samples were post-fixed in 4% PFA, followed by washes in DEPC-treated PBS. To block endogenous peroxidase activity, a 10-minute wash with 0.03% H₂O₂/PBS was performed. Subsequently, permeabilization was performed with 2% acetone/PBS for 5 min. After one wash with 30% formamide/2xSSC buffer (300 mM NaCl, 30 mM Na₃ Citrate-2H₂O, pH 5) for 10 min, pre-hybridization was carried out for 30 min at 61°C with the hybridization mix (50% formamide, 1.3x SSC, 0.5 mg/mL yeast tRNA, 0.2x CHAPS, 0.5M EDTA NaOH, pH 8, and 0.2% v/v Tween®-20). Afterwards, hybridization was performed with hsa-miR-106b-5p double DIG-labelled LNA probe at 25 nM diluted in hybridization mix at 61 °C for 2 h and 30 min. Samples were then washed in 2x SSC and 1x SSC, at 61 °C and 1x SSC at room temperature. Samples were rinsed with 1x PBS prior to Alexa Fluor™ Tyramide SuperBoost™ Kit protocol (B40943 or B40912, Invitrogen). Afterwards, samples were blocked for 1 h with Blocking Buffer (Invitrogen) and rinsed 3 times with 1x PBS. The corresponding anti-Digoxigenin antibody (rabbit or mouse) was incubated at 5 µg/mL diluted in Blocking Buffer overnight at 4 °C. On the next day, samples were rinsed 3 times with 1x PBS and then were incubated with the corresponding poly-HRP-conjugated secondary antibody (anti- rabbit or mouse) at room temperature for 60 min. Finally, samples were rinsed 3 times with 1x PBS before Tyramide Working Solution addition during 8

min at room temperature following the manufacturer's instructions. Stop Reagent was added on top for 30 seconds and samples were rinsed in 1x PBS prior to immunostaining.

8.2 - Haematoxylin-eosin staining

For Haematoxylin-eosin staining, TA cryosections samples were pre-fixed with 4% PFA for 10 min at room temperature. After several washes in water, samples were incubated for 10 min with Mayer's Haematoxylin Solution until nuclei were stained. Activated Eosin Y Solution 0.5% was incubated for 10 min. Then several washes with water were performed prior to dehydration and DPX was used as mounting media. Images were captured with a 2.0 MP digital microscope camera, which was attached via a c-mount to the side port of a Motic AE2000 microscope.

8.3 - Immunostaining

For immunocytochemistry purposes, cultured cells and isolated myofibers were fixed with 4% PFA for 10 min and permeabilized with PBS, 0.25% Triton X-100 and 50 mM NH_4Cl 10 min at room temperature. Samples were then blocked with Blocking Solution containing 0.2% gelatin/PBS (G1393) for 20 min at room temperature. Primary antibodies were added at 5-10 $\mu\text{g}/\text{mL}$ in Blocking Solution overnight at 4°C. AlexaFluor 488, 546 or 633-conjugated secondary antibodies diluted 1/300 in Blocking Solution were added for 30 min at room temperature.

For immunohistochemistry, the cryosections samples were fixed in 4% PFA for 10 min at room temperature. For PAX7, PS6 and MYOD1 immunostaining, epitopes were unmasked in citrate buffer (10 mM Sodium Citrate, 0.05% Tween-20, pH 6.0) in a pre-heat water bath for 30 min at 96°C. Afterward, all the samples were pre-incubated in TBSA-BSAT (10 mM Tris, 0.9% NaCl, 0.02% sodium azide, 2% BSA and 0.1% Triton-X100) for 30 min at room temperature. Primary antibodies were diluted to 5-10 $\mu\text{g}/\text{ml}$ in TBSA-BSAT and incubated overnight at 4°C in a humidified chamber. On the next day, AlexaFluor 488, 546 or 633-conjugated secondary antibodies diluted 1/200 in TBSA-BSAT were incubated for 2 h at room temperature.

Finally, for both immunostaining protocols, samples were incubated in a 1/2000 DAPI/PBS dilution for 15 min at room temperature. Finally, Hydromount was used for mounting. All images were acquired by a Leica TCS SPE-CTR6500 Confocal Microscope except for images taken for

miR-106b modulation in fresh isolated SCs from old mice (aging analysis) that were acquired in an Invitrogen EVOS M7000 microscope.

9 - RNA Isolation and cDNA synthesis

Total muscle RNA, except for aging analysis, was extracted from the treated TA muscles by the Directzol™ RNA MiniPrep Kit following the manufacturer's instructions. SCs' total RNA was extracted by the RNAqueous™-Micro Total RNA Isolation Kit following the manufacturer's instructions.

Aged TA muscles were collected from 25-months-old C57BL/6J male mice (6 months-old mice were used as control). Mechanical disruption was performed with sterile lab mortar and total RNA was extracted from TA muscles by the mirVana™ miRNA Isolation Kit. ezDNase treatment was performed following the manufacturer's instructions.

For embryo RNA isolation, frozen samples in Lysis Buffer (from RNAqueous™-Micro Total RNA Isolation Kit) were defrost and mechanical disruption was performed with sterile lab mortar and total RNA was extracted from samples by the RNAqueous™-Micro Kit following the manufacturer's instructions.

Either 500 ng of the total RNA from muscles or 50 ng of the total RNA from SCs were used as mRNA RT templates for Maxima First Strand cDNA Synthesis Kit from Thermo Fisher for RT-qPCR in line with the manufacturer's instructions.

For miR RT, 10 ng of total RNA from muscles, SCs or embryo samples were used as miR RT template for miRCURY LNA Universal RT Kit.

10 – qPCR analysis

The qPCRs for the mRNAs' measurements were performed by using SsoFast™ EvaGreen® Supermix. The qPCR program consisted in 95 °C for 30 seconds (initial denaturalization), followed by: 40 cycles of 95 °C for 5 seconds (denaturalization); 60 °C for 10 seconds (annealing); 75 °C for 7 seconds (extension). Finally, melt curves were determined by an initial step of 95 °C for 5 seconds, followed by 0.5 °C increments for 7 seconds from 65 °C to 95 °C. The relative

expression level for each gene was calculated as the ratio of the extrapolated levels of each gene expression, and Gapdh and Gusb genes were used as normalizers. The primers used for the mRNA qPCR are listed in table 3:

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
CD34 (NM_009911.3)	ACGGCTGTAGAGCGAGTGTT	AGGGTTCCTTGTGGAGTCA
Gapdh (NM_008084.2)	GGCATTGCTCTCAATGACAA	TGTGAGGGAGATGCTCAGTG
Gusb (NM_010368.1)	ACGCATCAGAAGCCGATTAT	ACTCTCAGCGGTGACTGGTT
Myf5 (NM_008656.5)	TGAGGGAACAGGTGGAGAAC	AGCTGGACACGGAGCTTTTA
Myod1 (NM_010866.2)	GGCTACGACACCGCCTACTA	TCCCTGTTCTGTGTCGCTTA
Myog (NM_031189.2)	CTACAGGCCTTGCTCAGCTC	ACGATGGACGTAAGGGAGTG

Table 3. Primers used for mRNA qPCR analysis.

The qPCRs for the miRs measurements (except for aging analysis) were performed by GoTaq® qPCR Master Mix at Bio-Rad's CFX Real-Time PCR system. The qPCR program consisted in 95 °C for 10 min (initial denaturalization), followed by: 40 cycles of 95 °C for 5 seconds (denaturalization); 60 °C for 1 min (annealing and extension). Finally, melt curves were determined by an initial step of 95 °C for 5 seconds, followed by 0.5 °C increments for 7 seconds from 65 °C to 95 °C. The relative level of each miR gene expression was calculated as the ratio of the extrapolated levels of each gene expression and U6 and 5S were used as the normalizers.

For miR expression analysis in aging, miRs measurements were performed by miRCURY LNA SYBR® Green PCR Kits at StepOne qPCR Systems (Applied Biosystems™). The qPCR program consisted in 95°C for 2 min (initial denaturalization), followed by: 40 cycles of 95°C for 10 seconds (denaturalization); 56°C for 1 min (annealing and extension). Finally, melt curves were determined by 0.5°C increments from 65°C to 95°C. The relative level of each miR gene expression was calculated as the ratio of the extrapolated levels of each gene expression and U6 and 5S were used as the normalizers. The primers used for the miR qPCR are listed in table 4:

Gene	Source, identifier
hsa-miR-17-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP02119304
hsa-miR-20a-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP00204292
hsa-miR-20b-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP00204755
hsa-miR-93-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP00204715
hsa-miR-106b-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP00205884
U6 snRNA (hsa, mmu) miRCURY LNA miRNA PCR Assay	Qiagen, YP00203907
5S rRNA (hsa) miRCURY LNA miRNA PCR Assay	Qiagen, YP00203906

Table 4. Primers used for miR qPCR analysis.

Each PCR reaction was performed in triplicate and repeated in at least three different biological samples to obtain a representative average. Relative measurements were calculated as described by Pfaffl M.W. (Pfaffl, 2001).

11 - RNA-Seq Analysis

For RNA-Seq analysis, TA muscle from treated mdx mice (CTX + scramble versus CTX + anti-miR-106b) were collected and total RNA was isolated using the Direct-zol™ RNA MiniPrep-Zymo Research Kit (three biological replicates). RNA samples were sent to the Centro de Análisis Genómico (CNAG, Barcelona Spain) for RNA sequencing. 200 ng of the total RNA were subsequently employed to prepare the RNA-Seq library with the TruSeq RNA sample prep kit (Illumina) according to the manufacturer's instructions. Paired-end (2x75) RNA sequencing was carried out by HiSeq 2000 (Illumina). For trimming and aligning raw data, fastq sequences reads were uploaded to the European version of the Galaxy platform (<https://usegalaxy.eu/>) (Afgan et al., 2018). Reads were trimmed with the Trim Galore software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and aligned to the built-in mouse reference genome mm10 with the RNA STAR Gapped-read mapper for the RNA-seq data (Galaxy Version 2.6.0b-2) (Dobin et al., 2013). For the gene expression analysis, bam files were downloaded from the Galaxy server and further analysed with the different RStudio packages downloaded from the Bioconductor website (<http://bioconductor.org>). Reads were assigned to genes by means of the "featureCounts" function of the "Rsubread" package, version 1.28.1 (Liao et al., 2019), and mouse annotation file release M19, GRCm38.p6 (ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_mouse/release_M19/). Only the mapped reads were used to calculate gene expressions. The library size of each experimental point ranged from 34816337 to 54599372 sequences.

The differential gene expression analysis was performed by the limma+voom linear modelling approach using the "edgeR" version 3.20.9 package, the "limma" version 3.34.9 package and an empirical Bayes method for differential expression (Law et al., 2014; Ritchie et al., 2015; Robinson et al., 2010). All the gene comparisons with a p-value <0.05 and a fold change >2 was considered differentially expressed under the experimental conditions.

A Functional Enrichment Analysis was performed by the Over Representation Analysis (ORA) Method using either the "enricher" function for the "H: hallmark gene set" of MSigDB or the

“enrichGO” function “GO Biological Process gene set”. Both functions appear in the ‘clusterProfiler’ package version 3.6.0 (Yu et al., 2012). The gene sets with a p-value <0.05 were considered overrepresented under the experimental conditions.

12 - Quantification and statistical analysis

Two-tailed unpaired Student’s t-test or Kolmogorov Smirnov test were performed to calculate p-values and to determine statistically significant differences. The number of independent experimental replications, mean $\pm$ SD and statistical tests (p-value) are reported in figure legends. Statistical analyses were performed with GraphPad Prism and R-studio. Images were processed for quantification by the ImageJ software.

13 - Supplementary information

All the employed reagents, materials and its references are listed in the table 5:

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Antibodies	Source	Identifier
Mouse Anti-Dystrophin human (2C6)	Developmental Studies Hybridoma Bank (DSHB)	MANDYS106; RRID: AB_2753249
Mouse Anti-eMyHC (F1.652)	Developmental Studies Hybridoma Bank (DSHB)	RRID: AB_528358
Rabbit Anti-Ki67 (SP6)	abcam	ab16667; RRID: AB_302459
Rabbit Anti-Laminin	Sigma-Aldrich	L9393; RRID: AB_477163
Mouse Anti-MF20	Developmental Studies Hybridoma Bank (DSHB)	RRID: AB_2147781
Rabbit Anti-Myf5	My BioSource	MBS9604552
Mouse Anti-MyoD1 (5.8A)	DAKO	M3512; RRID: AB_2148874
Mouse Anti-Myogenin (F5D)	Developmental Studies Hybridoma Bank (DSHB)	RRID: AB_2146602
Mouse Anti-Pax7	Developmental Studies Hybridoma Bank (DSHB)	RRID: AB_528428
Rabbit Anti-Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E)	Cell Signaling	4858; RRID: AB_916156
AlexaFlour® Goat Anti-mouse 488	Thermo Fisher Scientific	A-11001; RRID: AB_2534069
AlexaFlour® Goat Anti-mouse 546	Thermo Fisher Scientific	A-11030; RRID: AB_144695
AlexaFlour® Goat Anti-mouse 633	Thermo Fisher Scientific	A-21052; RRID: AB_2535719

AlexaFlour® Goat Anti-rabbit 488	Thermo Fisher Scientific	A-11008; RRID: AB_143165
AlexaFlour® Goat Anti-rabbit 546	Thermo Fisher Scientific	A-11035; RRID: AB_2534093
AlexaFlour® Goat Anti-rabbit 633	Thermo Fisher Scientific	A-21071; RRID: AB_2535732
IRDye® 800CW Goat anti-Mouse IgG	LI-COR® Biosciences	926-32210
DAPI	Thermo Fisher Scientific	D1306; RRID: AB_2629482
Hydromount	National Diagnosis	HS-106
Oligonucleotides and other sequence-based reagents		
mRNA RT-qPCR	This study	Materials and methods
microRNA RT-qPCR	This study	Materials and methods
Control microRNA vector	GeneCopoeia™	CmiR-AN0001-AM03
hsa-miR-106b-5p double DIG-labelled LNA oligos	Qiagen	339111 YD00618264-BCG
hsa-miR-106b inhibitor vector	GeneCopoeia™	HmiR-AN0029-AM03
hsa-miR-106b mimic vector	GeneCopoeia™	HmiR0111-MR11
mirVana™ miRNA Inhibitor, Negative Control #1	Thermo Fisher Scientific	4464077
mirVana® miRNA inhibitor, hsa-miR-106b-5p	Thermo Fisher Scientific	4464085
mirVana® miRNA mimic, hsa-miR-106b-5p	Thermo Fisher Scientific	4464067
mirVana® miRNA mimic, miR-31	Thermo Fisher Scientific	4464066
Chemicals, Enzymes and other reagents		
Albumin (BSA) Fraction V	PanReac AppliChem	A1391.0100
Anti-Integrin α -7 MicroBeads mouse	Miltenyi Biotec	130-104-261
CD31 MicroBead Kit, human	Miltenyi Biotec	130-091-935
CD45 MicroBeads, human	Miltenyi Biotec	130-045-801
CD56 MicroBeads, human	Miltenyi Biotec	130-050-401
Chicken Embryo Extract	Seralab	CE-650-J
Chicken Serum	Sigma-Aldrich	C5405
Collagenase D	Roche	11-088-866-001
Collagenase Type I	Gibco Life Technologies	17100017
DMEM, High Glucose	Gibco Life technologies	41965-039
DMEM, High Glucose, GlutaMAX™, Pyruvate	Gibco Life technologies	31966-021
DMEM	Lonza	12-741
DMEM:F12 (1:1)	Lonza	12-719F
EGF Human	Peptrotech	AF-100.15
Fetal Bovine Serum (FBS)	Sigma-Aldrich	F0804
FGF-2 Human	Prospec	cyt-218
Fungizone	Gibco Life Technologies	15290-018
Gelatin 2%	Sigma-Aldrich	G1393
Gelatin Type A	Sigma-Aldrich	G1890
HBSS	Gibco Life Technologies	24020-091
HBSS w/o Ca Mg	Gibco Life Technologies	14185-045
Horse Serum	Sigma-Aldrich	H1138

Insulin	Sigma-Aldrich	I5500
L-Glutamine	Sigma-Aldrich	G7513
Matrigel™	BD Biosciences	356234
MG-132	Sigma-Aldrich	M7449
Monastrol	Sigma-Aldrich	475879
M-199	Gibco Life Technologies	21180-021
MEM Non-essential Amino Acid Solution	Sigma-Aldrich	M7145
Opti-MEM™	Thermo Fisher Scientific	31985062
Penicillin/Streptomycin (P/S)	Gibco Life Technologies	15140-122
Red Blood Cell Lysis Solution (10×)	Miltenyi Biotec	130-094-183
Satellite Cell Isolation Kit mouse	Miltenyi Biotec	130-104-268
Skeletal Muscle Cell Growth Medium	PromoCell	C-23060
Thymidine	Sigma-Aldrich	89279
Trypsin	Sigma-Aldrich	T4424
Trypsin-EDTA	Sigma-Aldrich	T3924
Ultrosor® G	PALL Life Sciences	15950-017
Digoxigenin Recombinant Rabbit Monoclonal Antibody	Invitrogen™	9H27L19
Anti-Digoxigenin antibody mouse [21H8]	Abcam	ab420
Alexa Fluor™ 488 Tyramide SuperBoost™ Kit, goat anti-mouse IgG	Invitrogen™	B40912
Alexa Fluor™ 488 Tyramide SuperBoost™ Kit, goat anti-rabbit IgG	Invitrogen™	B40943
CTX, <i>Naja pallida</i>	Sigma-Aldrich	217503-1MG
CellTag™ 700 Stain	LI-COR® Biosciences	926-41090
CHAPS	Melford	75621-03-3
Eosin A 0,5%	Merck	1098441000
Formamide	PanReac AppliChem	141956.1211
Goat serum	Sigma-Aldrich	G9023
Haematoxylin Solution, Mayer's	Sigma-Aldrich	MHS32-1L
Isoflurin 1000mg/g	FATRO	585305-5
Isopentane	PanReac AppliChem	143501.1611
Maleic acid	Sigma-Aldrich	M0375
TRIsure™	Bioline	BIO-38032
Triton® X-100	PanReac AppliChem	A4975
Tween® 20 for synthesis	PanReac AppliChem	162312.1214
Odyssey® Blocking Buffer	LI-COR® Biosciences	927-40000
RNA from yeast	Roche	10109223001
RNAlater®	Invitrogen	AM7021
Sucrose	PanReac AppliChem	131621.1210
Software		
Galaxy platform	Open Source	https://usegalaxy.eu/
Galaxy Version 2.6.0b-2		
GraphPad Prism version 7.0a	GraphPad Software	https://www.graphpad.com
GRCm38.p6	Open Source	ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_mouse/release_M19/

ImageJ	Open Source	https://imagej.nih.gov/ij/download.html
R version 3.4.4	Open Source	https://cran.r-project.org/bin/macosx/
RStudio version 1.1463	Open Source	https://www.rstudio.com/
Trim Galore software	Open Source	http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
Other		
Direct-zol RNA Miniprep Kits	Zymo Research	R2050
Cryostat microtome	Leica	CM1510S
ezDNase treatment	Invitrogen	11766051
GoTaq [®] qPCR Master Mix for Dye-Based Detection	Promega	A6001
Lenti-Pac [™] HIV Expression Packaging Kit	GeneCopoeia [™]	LT001
Lenti-Pac [™] HIV qRT-PCR Titration Kit	GeneCopoeia [™]	217LT005
Lipofectamine 2000 Transfection Reagent	Invitrogen	11668019
Maxima First Strand cDNA Synthesis Kit for RT-qPCR	Thermo Fisher Scientific	K1642
miRCURY LNA [®] Universal RT microRNA PCR	Exiqon	203301
miRCURY LNA SYBR [®] Green PCR	Qiagen	339346
mirVana [™] miRNA Isolation Kit	Invitrogen	AM1560
RNAqueous [™] -Micro Total RNA Isolation Kit	Thermo Fisher Scientific	AM1931
SsoFast [™] EvaGreen [®] Supermix	Bio-Rad	1725201
Leica CTR 6500 confocal microscope	Leica Microsystems	CTR 6500
Panlab Treadmills, Harvard Apparatus	Panlab	LE8708 single mouse
LS Columns	Miltenyi Biotec	130-042-401
MS Columns	Miltenyi Biotec	130-042-201
μ-Dish 50 mm	ibidi	81136
NanoDrop OneC	Thermo Scientific	OneC
SuperFrost Ultra Plus [™] Adhesion Slides	Thermo Scientific	J3800AMNZ
HiSeq 2000	Illumina	
RNAseq Files	Gene Expression Omnibus GEO	GSE134111

Table 5. Reagents and materials.

CHAPTER IV: RESULTS

miR-106b is a novel target to promote muscle regeneration and restore satellite stem cell function in injured Duchenne dystrophic muscle

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Satellite cells (SCs), muscle stem cells, display functional heterogeneity, and dramatic changes linked to their regenerative capabilities are associated with muscle-wasting diseases. SC behavior is related to endogenous expression of the myogenic transcription factor MYF5 and the propensity to enter into the cell cycle. Here, we report a role for miR-106b reinforcing MYF5 inhibition and blocking cell proliferation in a subset of highly quiescent SC population. miR-106b down-regulation occurs during SC activation and is required for proper muscle repair. In addition, miR-106b is increased in dystrophic mice, and intramuscular injection of anti-miR in injured mdx mice enhances muscle regeneration promoting transcriptional changes involved in skeletal muscle differentiation. miR-106b inhibition promotes the engraftment of human muscle stem cells. Furthermore, miR-106b is also high in human dystrophic muscle stem cells and its inhibition improves intrinsic proliferative defects and increases their myogenic potential. This study demonstrates that miR-106b is an important modulator of SC quiescence, and that miR-106b may be a new target to develop therapeutic strategies to promote muscle regeneration improving the regenerative capabilities of injured dystrophic muscle.

INTRODUCTION

Muscle regeneration is affected in muscle-wasting diseases like Duchenne Muscular Dystrophy (DMD),¹ a severe genetic disorder that causes progressive muscle waste in affected individuals for which there is currently no cure. DYSTROPHIN, the protein product of the gene defective in DMD, plays a pivotal structural role in anchoring muscle fiber to the extracellular matrix in the muscle niche.² DYSTROPHIN is also expressed in muscle satellite cells (SCs). Previous works have shown that dystrophic SCs are impaired in several SC life cycle aspects, which impact their muscle regenerative functions.^{3,4}

Upon activation, SCs, which are characterized by Pax7 transcription factor expression, undergo symmetric and asymmetric divisions. Asymmetrically dividing cells generate both committed progeny (PAX7^{pos}/MYF5^{pos} cells) and offspring, whose stem cell identity remains (PAX7^{pos}/MYF5^{neg} cells), whereas symmetric expansion helps to replenish the stem cells pool.⁵ A fine balance between self-renewal and differentiation is necessary to preserve the satellite stem cell pool and to give rise to a suitable number of myogenic progenitors for proper muscle repair. Remarkably, it has been shown that dystrophic SCs display a few asymmetric divisions, which are needed to generate myoblasts and lead to a low generation rate of myogenic precursor cells.⁴ Moreover, dystrophic muscle fibers cultured for 72 h display an increase in the subset of PAX7^{pos} SCs that never express myogenic factor MYF5, with a reduction in the number of MYOG-expressing progenitors,⁶ which further suggests that dystrophic SCs can also display enhanced self-renewal. Hence, research to restore the SC function has attracted much interest in recent years, and the intention is to develop new strategies to treat muscle regeneration intrinsic defects in DMD.

In the context of SC biology, microRNAs (miRNAs) play a critical role in regulating muscle regeneration and SC behavior. Therefore, lack of miRNA-processing endoribonuclease DICER1 leads to the depletion of SCs and the quasi-absence of repair upon injury.⁷ miRNAs, like miR-195 and miR-497, are particularly involved in SC cell-cycle arrest during the juvenile to adult phase transition,⁸ and miR-489 targets oncogene Dek to prevent cell cycle re-entry.⁷

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miR-31 also binds to *Myf5* mRNA and is, thus, sequestered in messenger ribonucleoprotein (mRNP) granules, which represses its translation until SCs re-enter the cell cycle.⁹ Recently, a *Notch*-miR-708-*Tns3* axis has been described to modulate SC quiescence and transition to the activation state through the dynamic regulation of migratory machinery.¹⁰ These findings generally indicate the potential of miRNAs as powerful molecular tools to regulate the satellite function in the muscle regeneration and disease context.

As we previously identified miR-106b as a member of a *Pitx2*-miRNA pathway, which regulates the cell proliferation and myogenic commitment of SCs,^{11,12} we hypothesize that miR-106b may play a role in SC myogenic progression during muscle regeneration and could have an impact by modifying SC behavior in the muscle dystrophies context.

RESULTS

miR-106b reinforces quiescence in *Myf5*^{neg} SCs and its expression is down-regulated after muscle injury

We previously identified miR-106b as a member of a *Pitx2*-miRNA pathway that regulates the cell proliferation and myogenic commitment of early activated SCs by targeting cell-cycle regulatory genes *Ccnd1* and *Ccnd2*, and also myogenic factor *Myf5*.¹¹ This led us to wonder whether miR-106b plays a role in SC myogenic progression in the muscle injury and regeneration context. First, we further analyzed the expression pattern of miR-106b in mouse adult muscle. miR-106b expression was only detected in a proportion (~13%) of PAX7^{pos} SCs, we also observed that miR-106b^{pos} cells were MYF5^{neg} (Figure 1A). Next, we performed *in vitro* experiments of gain- and loss-of-function in freshly isolated SCs by using lentiviral particles bearing the miR-106b mimic (premiR), miR-106b inhibitory (antimiR) and Scramble-control molecules. After 3 days of premiR lentiviral transduction, only the non-transduced myogenic MYOD1^{pos} proliferating (KI67^{pos}) cells were observed (Figure 1B), as miR-106b overexpression led to inactive free-floating SCs after lentiviral transduction that remain floating after 5 days in culture while antimiR-transduced SCs display enhanced differentiation (Figure S1A). This cell phenotype might be a consequence of miR-106b effects that target cell-cycle regulatory genes, as we previously reported.¹¹ Consistently with this, antimiR-transduction clearly increased the pools of proliferating activated MYOD1^{pos}/KI67^{pos} cells in culture (Figure 1B). In addition, lentiviral antimiR-transduction in freshly isolated SCs led to *Myf5* up-regulation, which was accompanied by the down-regulation of quiescence marker *Cd34* mRNA,^{13,14} whereas premiR transduction induced low *Myf5* mRNA levels, but high *Cd34* mRNA levels (Figure S1B). This scenario suggests that miR-106b maintains SCs in a high quiescent state. If miR-106b can promote the quiescence of SCs, its down-regulation would be related to SC activation. According to this idea, we found that miR-106b was present in the PAX7^{pos} quiescent SCs of freshly isolated myofibers, but we did not detect any miR-106b expression in the activated SCs (MYOD1^{pos}) after 48 h of myofiber cultures (Figure 1C). We also checked the impact of miR-106b inhibition on asymmetric division in the isolated SCs following a previously described

cell-cycle synchronization strategy combined with Pax7 and MyoD1 labeling to examine the fate of newly divided daughter cells.¹⁵ We found that doublets of sister cells in the antimiR-106b transduced cells were predominantly PAX7^{pos}MYOD1^{pos}:PAX7^{pos}MYOD1^{neg} and PAX7^{pos}MYOD1^{pos}:PAX7^{neg}MYOD1^{pos}, which represent asymmetric self-renewal and asymmetric differentiation divisions and, as a consequence of an increase in cell division, the total number of MYOD1^{pos} was higher in the antimiR-transduced cells (Figure 1D). All these findings suggest that miR-106b expression confers a strong quiescent stemness status, while its down-regulation leads to SC activation and proliferation with predominant asymmetric cell division.

In addition, the relevance of miR-106b reinforcing quiescence in an SC population subset was highlighted by two facts; first, miR-106b was the main miRNA of its miR-family (miR17-family) to be expressed in freshly isolated quiescent SCs (Figures S2A and S2B); second, the high capability to modify *Myf5* mRNA stability versus other miRNAs, which also targeted this transcription factor, such as miR-31 (Figure S2C).⁹

Next, we analyzed miR-106b behavior during muscle regeneration in mice. Our results clearly showed that miR-106b was down-regulated within the time window that comprised days 3 and 7 after cardiotoxin (CTX) injection in the tibialis anterior (TA) muscle of the C57BL/6 mice (Figure 2A). Interestingly, a strong and statistically significant up-regulation of myogenic factors *Myf5*, *Myod1*, and *Myog* in regenerating muscle was observed on day 3 after CTX injection (Figure 2A). This finding supports the notion that miR-106b down-regulation is associated with myogenic differentiation during muscle regeneration.

miR-106b inhibition induces myogenesis differentiation and enhances muscle regeneration

In order to further characterize the role of miR-106b during muscle repair, we followed an *in vivo* miR-106b gain- and loss-of-function approach by injecting CTX together with premiR or antimiR-106b molecules in TA muscles (Figures 2B and S2D). Notably, miR-106b manipulation in TA did not bring about any significant compensatory changes in the levels of other miR-17 family members (Figures S2E and S2F). Three days after intramuscular injection, the number of proliferating MYOD1^{pos}/KI67^{pos} cells significantly lowered in the CTX- and premiR-injected muscles. On the contrary, the number of proliferating committed MYOD1^{pos}/KI67^{pos} cells significantly increased in the antimiR-treated muscles (Figure 2C). As a result, muscle regeneration was enhanced, as observed by the largest number of differentiating eMyHC^{pos} new myofibers on day 7 of antimiR and CTX injection (Figure 2D). The morphometric measurements revealed a narrower size variation for the regenerating eMyHC^{pos} myofibers, which became smaller in the TA treated with antimiR than in the control muscles (Scramble-treated) on day 7 of injury (Figure 2D). However, the fiber measurements taken on day 15 after muscle damage in the antimiR-treated muscles revealed an increase of the larger myofiber sizes (Figure 2E) and a higher fusion index (Figure 2F), which suggest more mature fiber formation. Nonetheless, premiR-injected muscles displayed an increase in the number of

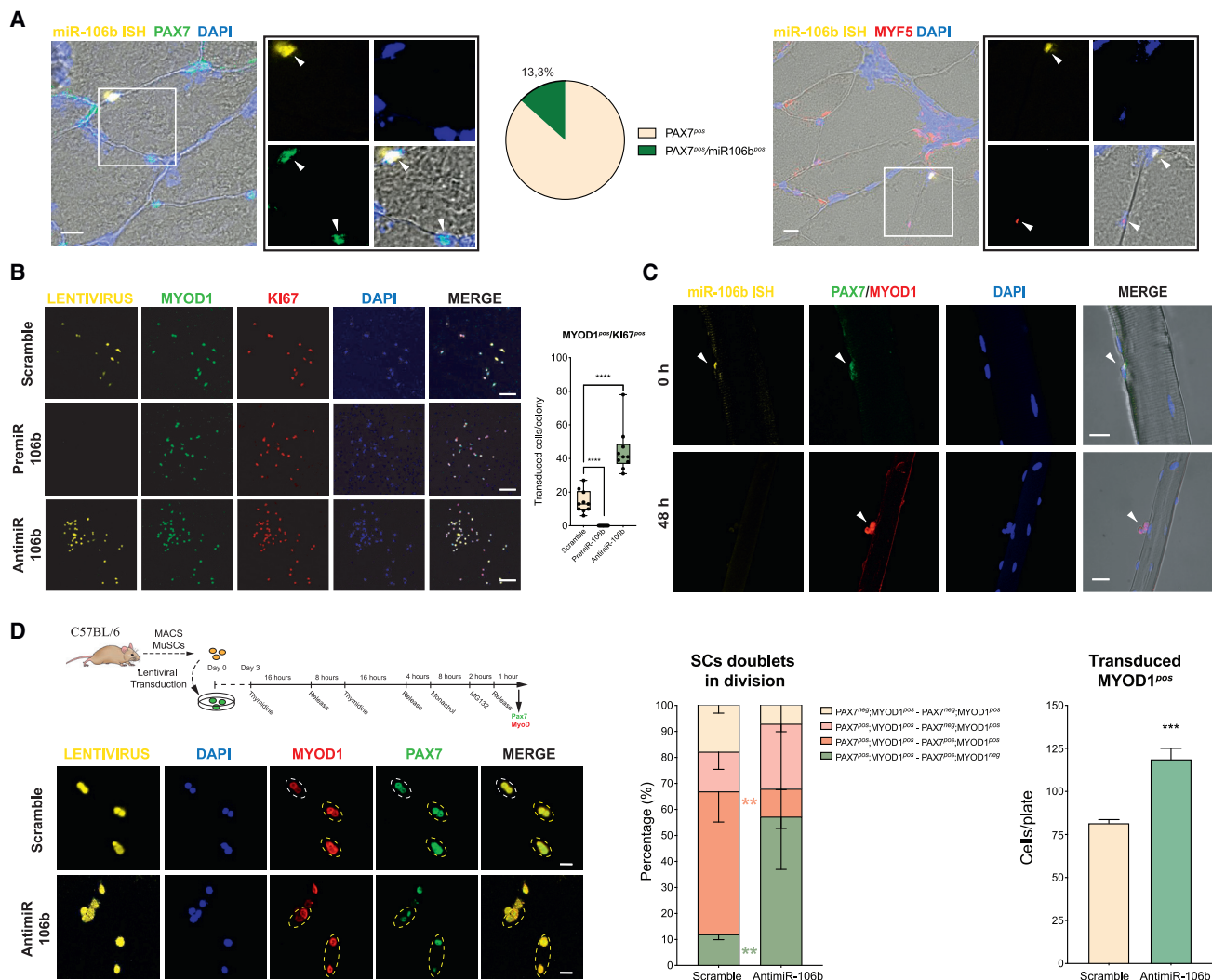


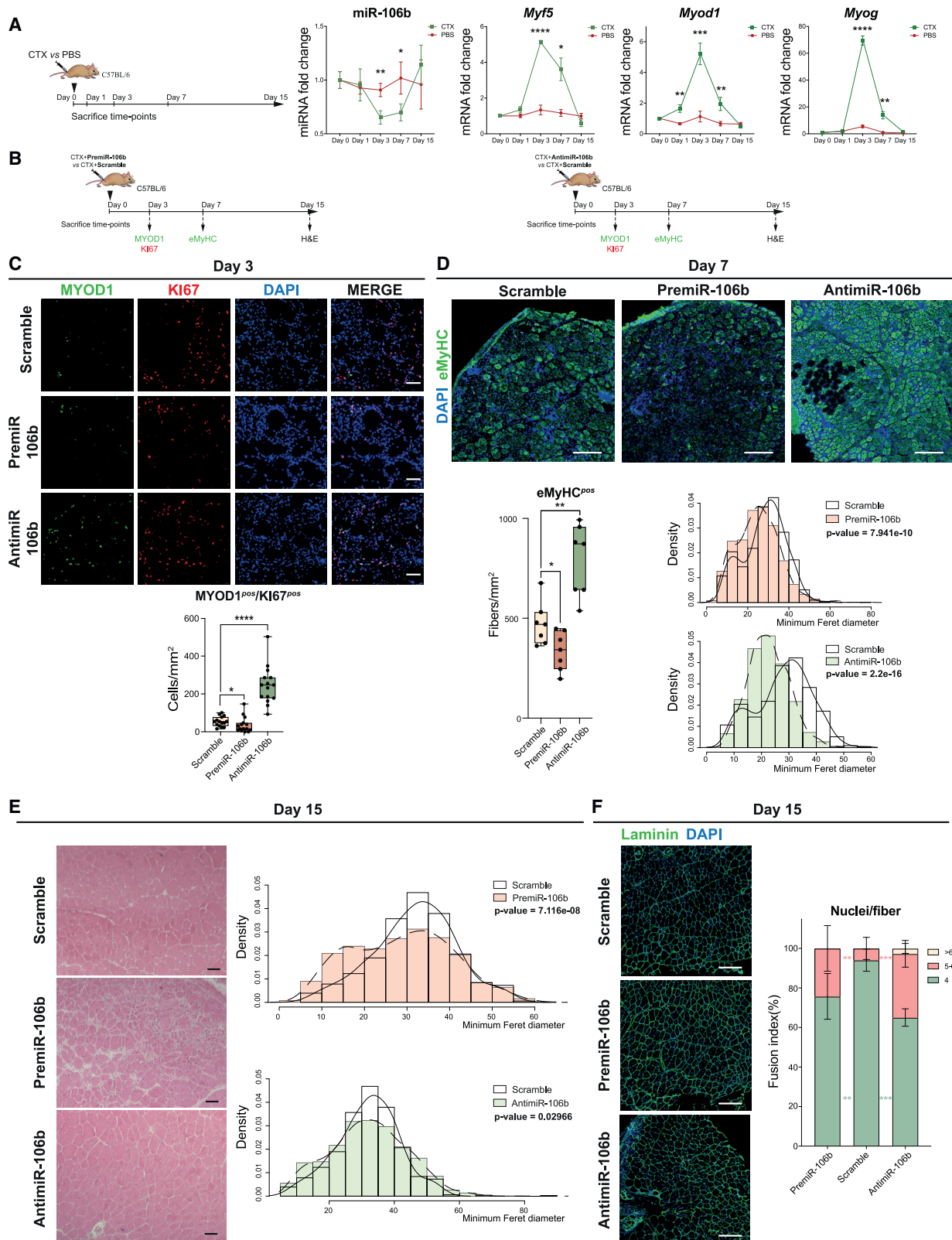
Figure 1. miR-106b is expressed in PAX7^{pos}/MYF5^{neg} SCs, represses proliferation and differentiation, and its inhibition leads to the cell activation state

(A) total of 18 C57BL/6 male mice, aged 4–6 months, were randomized into three groups: LNA *in situ* hybridization for miR-106b, miR-106b gain, and miR-106b loss-of-function experiments *in vitro*, cultured single myofibers at 0 and 48 h and synchronizing cultures in the late telophase stage (n = 3 per condition and time point). A fluorescence *in situ* hybridization for miR-106b and the immunofluorescence staining of PAX7 and MYF5 in the TA section from C57BL/6 mice. Quantification of the miR-106b^{pos}/PAX7^{pos} cells is shown (six sections per mouse; five fields per section). (B) The MYOD1 and Ki67 staining of the cultured SCs from C57BL/6 mice hindlimb muscles 72 h after premiR, anti-miR or control Scramble lentiviral transduction. Quantification of MYOD1^{pos}/KI67^{pos} cells per colony (n = 3 independent experiments; 20 cell colonies). (C) Fluorescence *in situ* hybridization for miR-106b and the immunofluorescence staining of PAX7 and MYOD1 in single EDL myofibers from the C57BL/6 mice cultured for 0 and 48 h (three mice; n = 15 fibers). (D) Strategy for synchronizing the cultured SCs in the late telophase stage after lentiviral transduction (yellow), and representative images of the lentiviral-transduced SCs synchronized in the telophase stage and stained for PAX7 (green) and MYOD1 (red). Percentages of the asymmetric self-renewal (PAX7^{pos}/MYOD1^{pos}; PAX7^{pos}/MYOD1^{neg}), symmetric proliferative (PAX7^{pos}/MYOD1^{pos}; PAX7^{pos}/MYOD1^{pos}), asymmetric differentiation (PAX7^{pos}/MYOD1^{pos}; PAX7^{neg}/MYOD1^{pos}), and symmetric differentiation (PAX7^{neg}/MYOD1^{pos}; PAX7^{neg}/MYOD1^{pos}) divisions are shown (n = 3 independent experiments, 15 doublets per condition). Quantification of the total number of transduced MYOD1^{pos} cells is shown. Scale bars, 20 μ m (A, C, and D), 100 μ m (B). Data are presented as mean $\pm$ SD. **p < 0.01; ***p < 0.001; p < 0.0001.

very small regenerating myofibers on 7 and 15 days of injury (Figures 2D and 2E). A lesser increase in fusion index was also observed in premiR-treated muscles with respect to control scramble-injected (Figure 2F), this fact might be due to premature myoblast fusion after accelerated exit of cell cycle induced by premiR-treatment. These results indicate that miR-106b inhibition facilitates muscle regeneration, while large doses of miR-106b lead to an

abnormal muscle repair pattern, which indicates that miR-106b decay is required for proper muscle repair.

It was noteworthy that anti-miR-106b treatment did not affect the re-entry of SCs into quiescence after muscle repair, as revealed by a non-statistically significant trend of a larger total number of PAX7^{pos} sublaminal cells in the anti-miR-treated muscles 30 days after



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injury (Figures 3A and 3B), with no changes noted in the number of MYOD1^{pos} sublamellar cells (Figures 3A–3C), as previously described as committed SCs.¹⁶ Furthermore, the SCs in the TA treated with a single antimiR injection continued to robustly contribute to muscle repair after re-injury, as noted by an increase in both the fiber diameter and the number of centralized nuclei at day 7 after re-injury in the previously antimiR-injected muscles (Figure 3D). Similar to that found after first muscle injury, a non-significantly larger number of PAX7^{pos} sublamellar cells in the re-injured antimiR-treated muscles was observed (Figure 3E). Collectively, all these findings indicate the relevance of miR-106b inhibition as a potential valuable approach to induce muscle regeneration.

miR-106b is highly expressed in mouse dystrophic SCs and *in vivo* antimiR-106b treatment enhances the skeletal muscle regenerative potential in dystrophic mice

Next, we looked for the miR-106b expression in a context in which muscle regeneration is not successfully completed by using the dystrophic-deficient mdx mouse model of DMD.¹⁷ miR-106b was expressed in mdx mouse muscles (Figure 4A) in a similar proportion to that found in wild-type mice (data not shown). We also found that miR-106b had increased in the muscles and isolated SCs of the dystrophin-deficient mdx mice (Figures 4B and 4C) and, contrary to that observed for the wild-type SCs, high miR-106b expression levels were maintained on culture day 5 (Figure 4C), which is consistent with the maintenance of the quiescence and a previously reported reduced proliferating capacity for dystrophic SC populations.^{4,12,18} Furthermore, miR-106b loss-of-function clearly increased the number of MYOG^{pos} myoblasts (Figure 4D), which indicates that antimiR treatment enhances the onset of myogenic differentiation of dystrophic SCs.

In order to address whether miR-106b inhibition could modulate muscle regeneration in mdx mice, the TAs of the 4-month-old mdx mice were injected with CTX and antimiR-106b. The lower miR-106b levels induced by antimiR injection were concomitant with the up-regulation of Myf5, MyoD1, and Myog, which suggests that miR-106b inhibition could induce myogenic differentiation (Figure 5A). We further checked for cells in the G-Alert state, in which SCs were “primed” to rapidly respond under stress or injury conditions.¹⁹ We observed that the *in vivo* antimiR injection enhanced the number of SCs positive for G-Alert-marker PS6 (Figure S3), and also increased the number of proliferating MYOD1^{pos}/KI67^{pos}

3 days after antimiR injection (Figure 5B). These findings suggest that antimiR-106b treatment enhances the functional ability of dystrophic SCs to activate and participate in tissue repair.

The immunohistochemical and histological analyses revealed that the number of eMyHC^{pos} myofibers and the amount of regenerating larger myofiber sizes were enhanced in the dystrophic TA muscles on day 7 after antimiR injection (Figure 5C). In addition, an increase in the percentage of fibers with centralized nuclei and an enlargement in the larger myofibers sizes on day 15 in antimiR-treated compared with control scramble-injected muscles were observed (Figure 5D), as well as a highest fusion index (Figure 5E). These findings indicate that the regenerative potential was enhanced in the mdx-SCs after antimiR injection. Functional performance was assayed by treadmill tests in the mdx mice injected with CTX + antimiR-106b until exhaustion 30 days after antimir treatment. As illustrated in Figure 5F, both the running time and distance were respectively 50% and 95% longer in the antimiR-treated mice compared with the control, which indicates that the antimiR injection boosts physical performance.

To further elucidate the effects of antimiR-106b injection during muscle regeneration in a context in which miR-106b is deregulated, such as the dystrophic muscle, we examined the transcriptome profile changes mediated by antimiR treatment at day 3 and day 7 after CTX injection in mdx mice by the RNA-sequencing (RNA-seq) analysis (Figure 6). This analysis revealed that the number of differentially expressed genes between days 3 and 7 after CTX injection was higher (6,278) in antimiR-treated muscles than in the control Scramble-injected mice (3,244) (Figures 6A and 6B). AntimiR-106b and scramble-treated dystrophic muscles shared 2,985 differentially genes between day 3 and day 7 after CTX injection (Figures 6A and 6B). Note that 3,293 genes were only deregulated in antimiR-106b-treated muscles and 259 genes were only deregulated in scramble-treated muscles. Moreover, since the number of differentially expressed genes between antimiR-treated and scramble-injected muscles were clearly higher at day 3 (426) than at day 7 (9) (Figures 6A and 6B), we concluded that the changes in the transcriptome profile induced by antimir treatment occurred mainly on day 3. This is consistent with the transient down-regulation of miR-106b after antimir treatment is mostly observed at day 3.

Interestingly, the GO-biological process and cellular component ORA-analysis in the antimiR-treated dystrophic muscles after injury

Figure 2. miR-106b is down-regulated after muscle injury and its inhibition enhances muscle regeneration

(A) Scheme of CTX injection in the C57BL/6 male mice TA, and the transcript levels of miR-106b, *Myf5*, *MyoD1*, and *Myog* in TA muscle. CTX was injected in TA and PBS injected into contralateral muscle and used as a control. n = 15 mice randomized into five groups: days 0, 1, 3, 7, and 15 after injection (n = 3 per time point). (B) Scheme of the CTX + premiR-106b or antimiR-106b injection in the C57BL/6 mice TA. CTX + scramble was injected into contralateral muscle and used as a control. n = 18 mice randomized into three groups: days 3, 7, and 15 after intramuscular injections (n = 3 per group). (C) MYOD1 and KI67 immunostaining on the TA section from scramble and premiR or antimiR (3 days post-administration). Quantification of the percentage of the MYOD1^{pos}/KI67^{pos} cells is shown. (D) eMyHC staining on the TA section from scramble and premiR or antimiR (7 days post-administration) and quantification of the number per area and minimum Feret's diameter distribution (μm) of the eMyHC^{pos} myofibers. (E) H&E staining on the TA section from scramble and premiR or antimiR (15 days post-administration) and muscle fiber size distribution analyzed by Minimal Feret Diameter (μm) is shown. (F) Immunofluorescence for Laminin on the TA section from scramble and premiR or antimiR (15 days post-administration) and the fusion index is shown. Scale bars, 50 μm (C), 200 μm (D–F). Data are presented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

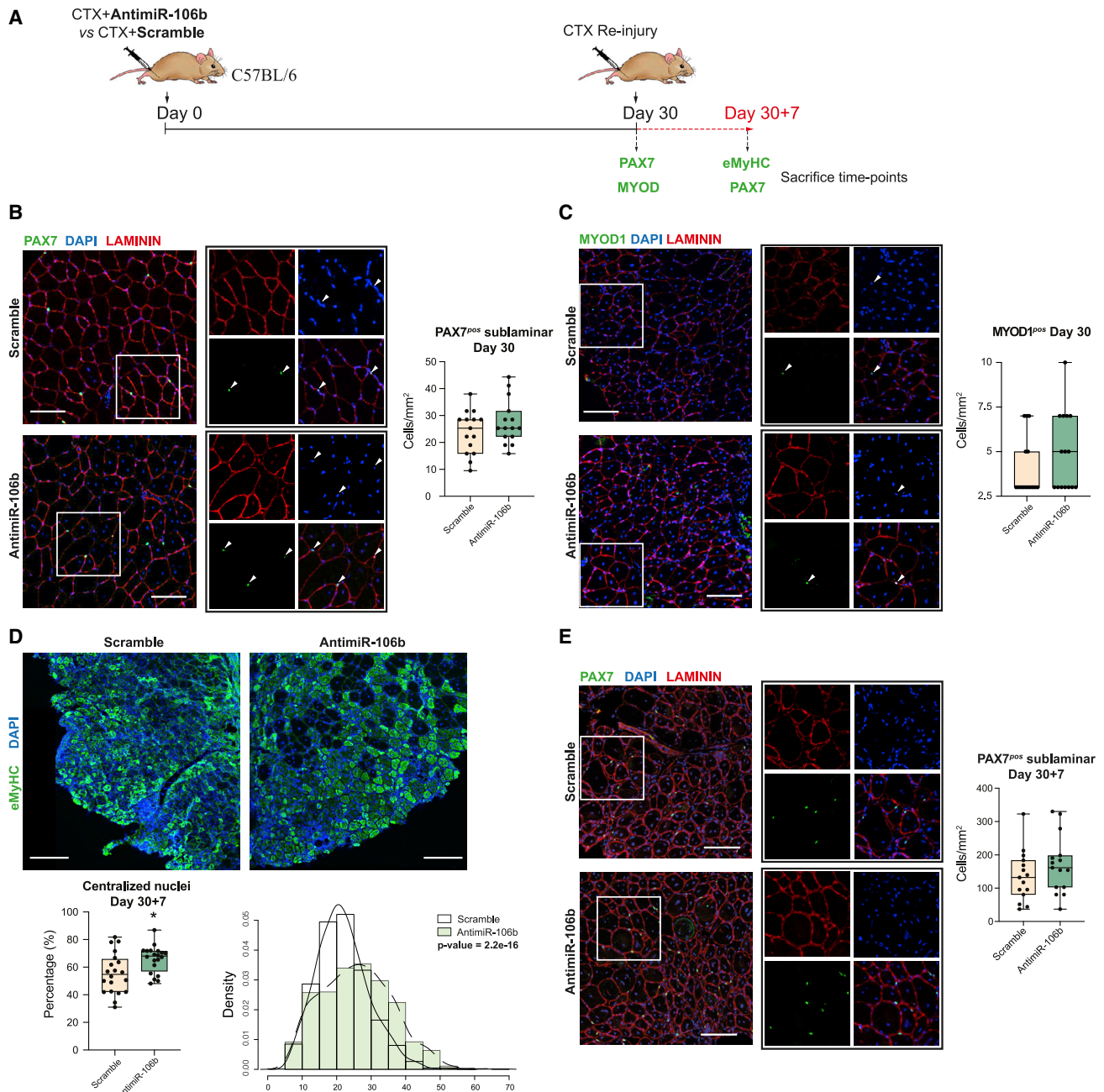


Figure 3. Single anti-miR-106b injection continues to contribute to muscle repair after re-injury

(A) Scheme of CTX+ anti-miR versus Scramble injection and re-injury in the C57BL/6 male mice TA. Six mice were randomized into two groups CTX + scramble versus CTX + anti-miR injected at 30 days post-injection and CTX + scramble versus CTX + anti-miR injected at 30 days post-injection + 7 days after re-injury ($n = 3$ per group). (B) Representative images of LAMININ and PAX7 staining on the TA sections from the scramble and anti-miR-injected TA muscles (30 days post-administration). Quantifications of the number of PAX7^{POS} is shown. Scale bars, 40 μm . (C) Representative images of LAMININ and MYOD1 staining on the TA sections from the scramble and anti-miR-injected TA muscles (30 days post-administration). Quantifications of the number of MYOD1^{POS} sublamellar cells are shown. Scale bars, 40 μm . (D) Immunofluorescence staining of eMyHC in the TA sections from scramble and anti-miR (30 days post-administration + 7 days after re-injury). The percentage of centralized nuclei and muscle fiber size distribution (analyzed by Minimal Feret Diameter [μm]) in eMyHC^{POS} myofibers are shown. (E) Representative image of the PAX7^{POS} sublamellar cells in the anti-miR-106b-treated re-injured muscles; quantification of the number of PAX7^{POS} sublamellar cells is shown. Scale bars, 200 μm . Data are presented as mean $\pm$ SD. * $p < 0.05$.

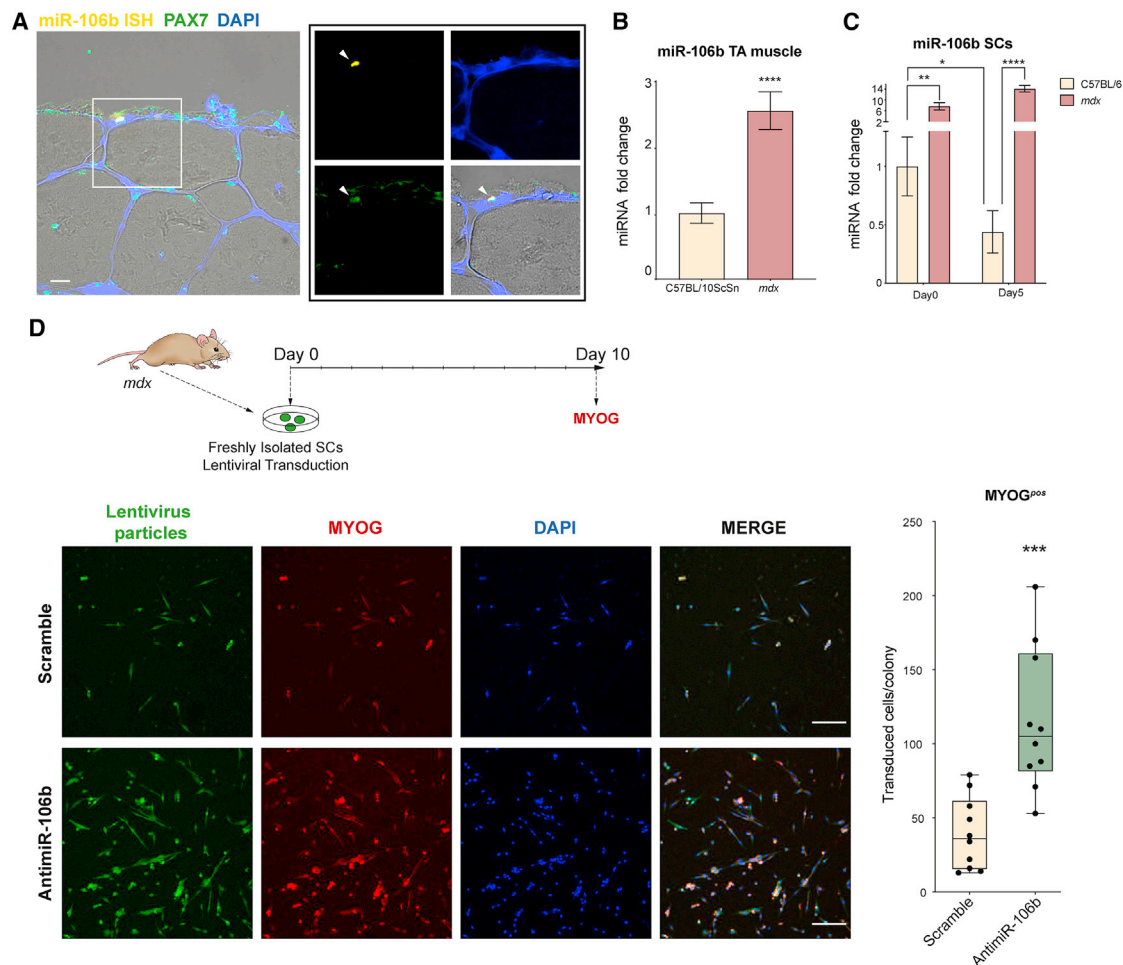


Figure 4. miR-106b is enriched in mdx-SCs and modulates their differentiation capabilities

A total of 15 male mdx and three C57BL/6 male mice, aged 4–6 months, were randomized into four groups: the LNA *in situ* hybridization analyses, RT-qPCR analyses in TA muscles, RT-qPCR analyses in cell cultures, miR-106b loss-of-function experiments *in vitro* ($n = 3$ per group). (A) Fluorescence *in situ* hybridization for miR-106b and the immunofluorescence staining of PAX7 on a TA section from the mdx mice. (B) Transcript levels of miR-106b in the TA from mdx mice hindlimb muscles. (C) Transcript levels of miR-106b in freshly isolated quiescent SCs from C57BL/6 and mdx mice on day 0 versus the 5-day cultured SCs (1,500–2,000 cells). (D) Scheme of anti-miR lentiviral transduction in freshly isolated mdx-SCs and MYOG staining; quantification of the number of lentiviral-transduced MYOG^{pos} nuclei per cell colony 10 days after treatment (10 colonies per mouse). Scale bars, 20 μ m (A), 100 μ m (D). Data are presented as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

revealed an enrichment in gene sets related to skeletal muscle differentiation, such as myofibril, striated muscle cell differentiation, actin filament organization, or actin filament-based (Figures 6C–6E). Nonetheless, in control Scramble-injected dystrophic muscle gene sets linked to inflammatory process, such as acute inflammatory response or regulation of production of molecular mediator of immune response, were enriched (Figures 6D and 6E). Those findings reinforce the notion that anti-miR treatment increases muscle regeneration in dystrophic mice after injury, but also indicate that the persistent inflammation associated to the dystrophic muscle might be attenuated,²⁰ a finding probably linked to enhanced muscle regeneration.

Finally, the impact of anti-miR-106b treatment on dystrophic muscles was further validated by the DBA/mdx mouse model, a DMD

model that better recapitulates several human characteristics of DMD myopathology (<https://www.jax.org/jax-mice-and-services>). Anti-miR-106b injection in the TA muscle of DBA2J/mdx mice also boosted the differentiation kinetics of SCs, as observed by an increased number of KI67^{pos}/MYOD1^{pos} cells on day 3 after induced muscle damage (Figures 7A and 7B). Because DBA/mdx mice exhibit a retarded and impaired muscle regeneration,^{21–23} eMyHC^{pos} myofibers were analyzed on day 15 post-administration; we found that both the number of eMyHC^{pos} myofibers and the regenerating larger myofibers sizes were increased (Figure 7C). Overall, these results reinforce the notion that anti-miR-106b injection enhances muscle regeneration in the dystrophic muscle by increasing the number of myogenic progenitors after SC activation.

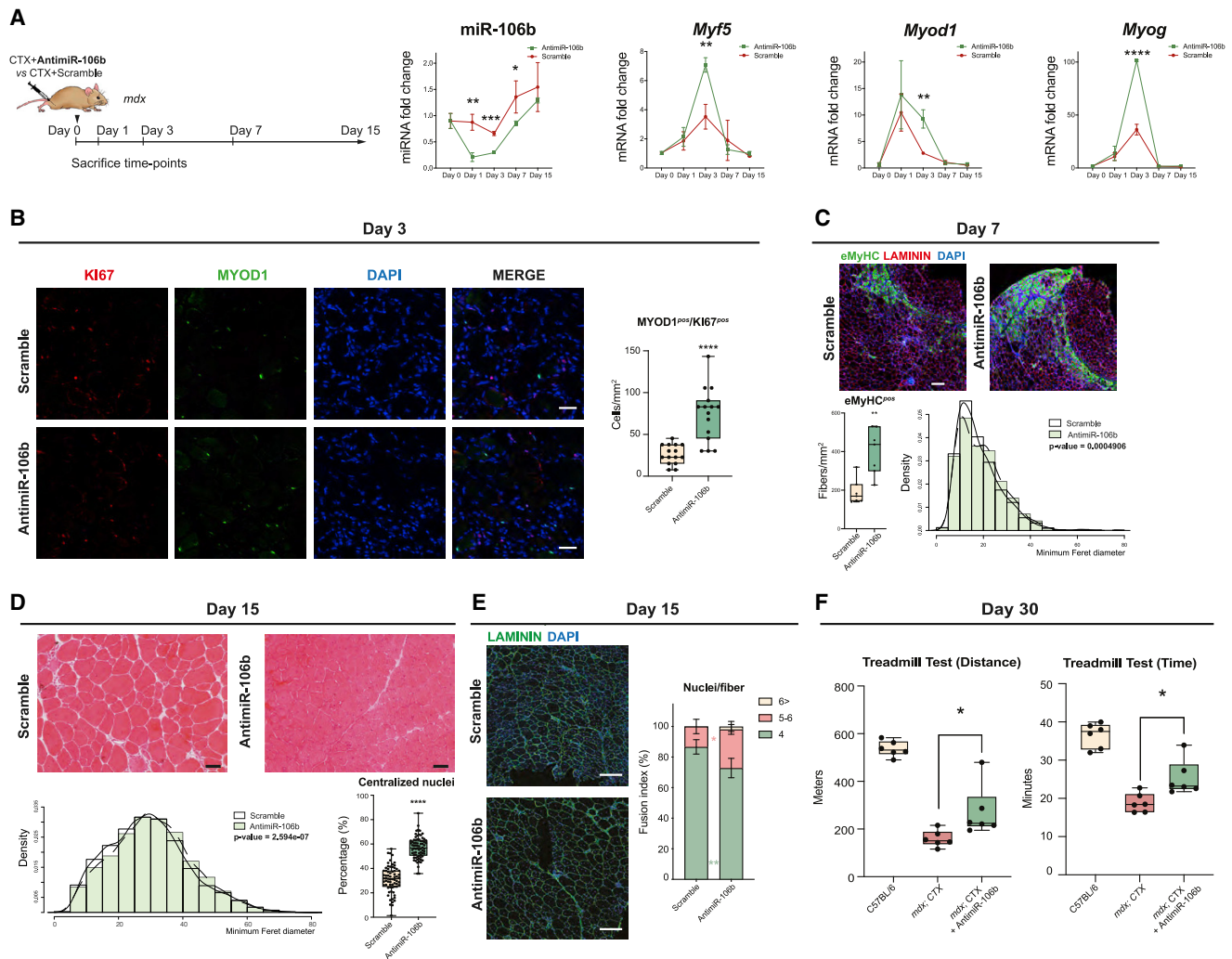


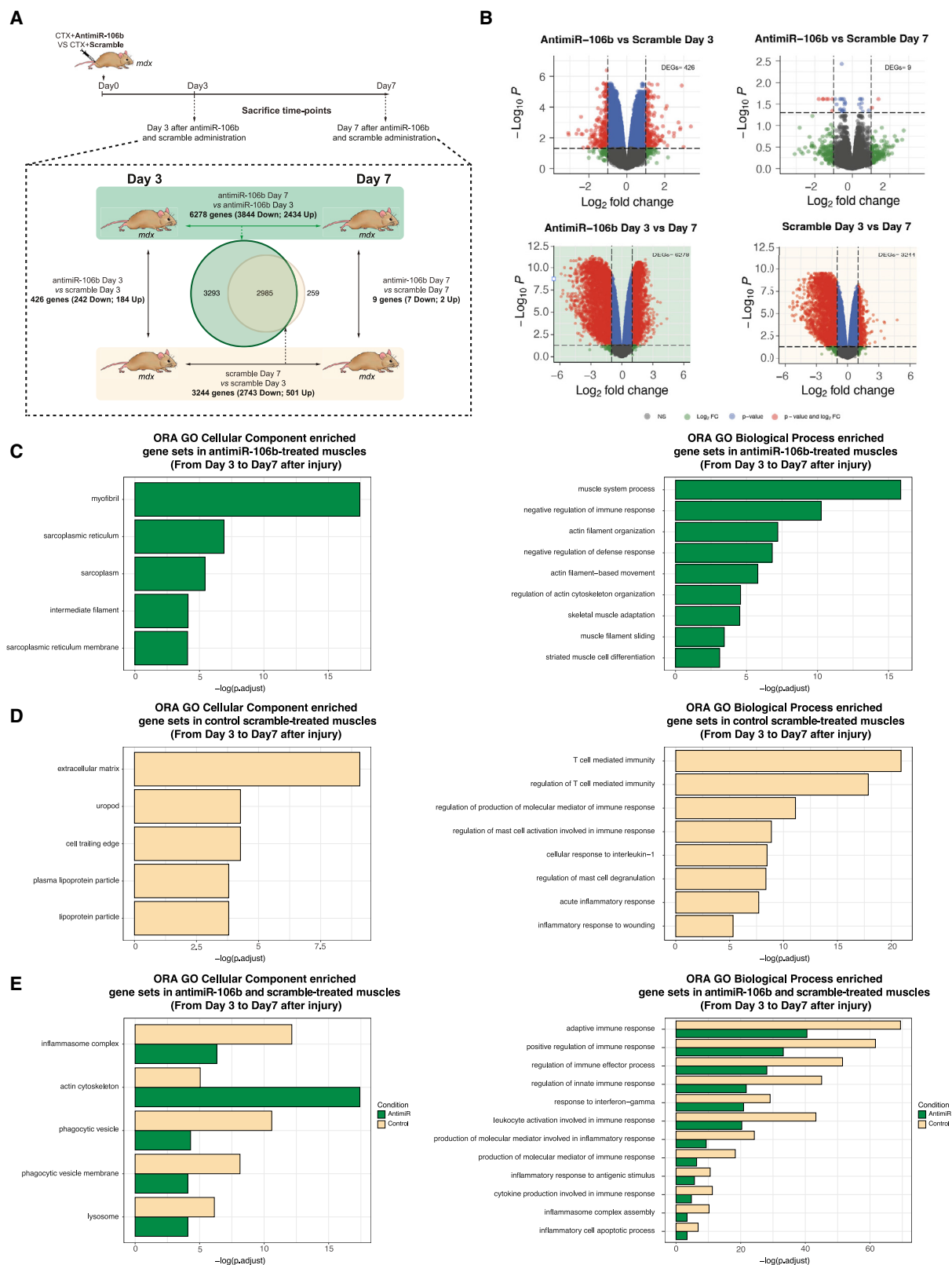
Figure 5. miR-106b inhibition enhances muscle regeneration in dystrophic mdx mice

n = 36 mdx mice, aged 4–5 months, were randomized into three groups: CTX-scramble/anti-miR-injected for the RT-PCR analyses (days 0, 1, 3, 7, and 15 after injection, n = 3 per time point and condition); CTX-scramble/anti-miR-injected for the immuno- and histochemical experiments (days 3, 7, and 15 after injection, n = 3 per time point and condition); CTX-Scramble and CTX-anti-miR injection for the Treadmill test (n = 6 per condition). A separate batch of 6 C57BL/6 male mice, aged 4–6 months, was used for the Treadmill test. (A) Scheme of the CTX + anti-miR injection in the mdx male mice TA and the transcript levels of miR-106b, *Myf5*, *MyoD1*, and *Myog* in the injured TA muscles. The control scramble + CTX were injected into the contralateral TA and used as a control. (B) MYOD1 and Ki67 immunostaining on the TA section from the scramble- and anti-miR-treated mdx mice (3 days post-administration). Quantification of the percentage of Ki67^{pos}/MYOD1^{pos} cells are shown. (C) Immunostaining for eMyHC^{pos} myofibers in the TA section of the anti-miR-treated mdx mice (7 days post-administration). The number of eMyHC^{pos} myofibers and muscle fiber size distribution (analyzed by Minimal Feret Diameter [μm]) are shown. (D) H&E staining on the mdx-TA section from scramble and anti-miR (15 days post-administration). Muscle fiber size distribution (analyzed by Minimal Feret Diameter [μm]) is shown. (E) Immunofluorescence of LAMININ on the mdx-TA section from scramble and anti-miR (15 days post-administration). Fusion index is shown. (F) The treadmill test in the CTX + scramble- and CTX + anti-miR-treated mdx and C57BL/6 mice (30 days post-administration). Scale bars, 50 μm (B), 200 μm (C and D). Data are presented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

miR-106b inhibition increases the *in vitro* myogenic differentiation of human muscle stem cells and enhances its contribution to muscle regeneration after intramuscular transplantation

We next tested if miR-106b could also act in human muscle stem cells (hMuSCs). *In situ* hybridization experiments in the sections of hMuSCs revealed that miR-106b was also present in a subset

of PAX7^{pos} hMuSCs (Figure 8A) at a similar ratio to that observed in mice (data not shown). The miR-106b effects on *in vitro* differentiation were evaluated by loss-of-function experiments by lentiviral transduction in freshly isolated hMuSCs from muscle biopsies. We observed that myotube formation was enhanced in the anti-miR-transduced cells given a significant increase in the fusion index, which suggests that anti-miR treatment also



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modifies the *in vitro* cell differentiation potential of hMuSCs (Figure 8B).

In order to check if miR-106b inhibition modulated the regenerative abilities of hMuSCs, we performed xenotransplantation of the hMuSCs transfected with anti-miR into the pre-injured TA of immunodeficient mice. Our results showed a larger number of human-DYSTROPHIN^{pos} myofibers for anti-miR-transduced cells after 5 weeks of xenotransplantation (Figure 8C). These results indicate that miR-106b inhibition promotes the engraftment and formation of human-derived myofibers.

miR-106b is high in human dystrophic muscles and modulates the myogenic potential of human dystrophic muscle stem cells

In order to investigate the role of miR-106b in the human muscular dystrophies context, we used the muscle biopsies obtained from patients with Becker Muscular Dystrophy (BMD) and DMD after obtaining written informed consent from all of them or from their legal representatives. Due to ethical issues linked with patients' age and/or the highly invasive nature of muscle biopsies, only a few patients were included in this analysis. The RT-qPCR analysis revealed higher miR-106b expression levels in the muscles of two BMD patients compared with the control muscle biopsies (Figure 9A). The hMuSCs isolated from the muscle biopsies of the BMD patients, and transduced with anti-miR, significantly increased the number of myoblasts after 14 days of *in vitro* culture (Figure 9B). However, consistently with the loss of proliferative capacity of the miR-106b-overexpressing cells, no premiR-transduced cell was observed at 14 culture days.

We also noticed that the hMuSCs freshly isolated from the muscle biopsy of one DMD patient displayed higher miR-106b expression levels (Figure 9C), and they generated a smaller amount of proliferating (MYOD1^{pos}/KI67^{pos}) cells after 2 days of *in vitro* culture compared with the control hMuSCs, which gave fewer MF20^{pos} myofibers after 6 culture days in differentiation medium (Figure S4A). Interestingly, anti-miR-lentiviral transduction in undifferentiated freshly isolated hMuSCs (Figure 9D) increased the number of MYOD1^{pos}/KI67^{pos} cells (Figure 9E), which led to both a larger amount and a longer length of the formed myotubes after 6 days in differentiation medium (Figure 9E). However, in immortalized human DMD-MuSC-derived myoblasts, treatment with pre-miR inhibited proliferation, further promoting differentiation, whereas the anti-miR treatment maintained cell proliferation, thus impeding further differentiation (Figure S4B). This indicates that miR-106b has no effect on differentiation of dystrophic hMuSC-derived myoblasts in which the myogenic program has already been initiated.

This scenario suggests that miR-106b inhibition enhanced the regenerative potential of undifferentiated dystrophic hMuSCs by increasing their capability to proliferate and to form myogenic precursors.

DISCUSSION

The understanding of muscle satellite stem cells' quiescence and activation is integral to regenerative medicine. Accumulating evidence reveals that SC behavior is widely heterogeneous in both its transition to a proliferative state and its decision to self-renew or differentiate.^{6,19,24} In line with this, several previous studies support a relation between MYF5 heterogeneous expression and SC functionality. Hence the SCs that never expressed MYF5 (MYF5^{neg}) were found to give rise to a MYF5^{neg} satellite stem cell and MYF5^{pos} committed SCs, whereas the transplanted MYF5^{pos} acted as myoblasts by failing to engraft, exhibited poor survival, and fused with one another to form new myofibers.⁶ Myf5 heterozygous SCs are more prone to a stem-like phenotype without it affecting their ability to generate new myofibers. However, Myf5-null SCs display a better self-renewal capacity than wild-type stem cells, but their ability to regenerate muscle is limited.²⁴ Despite the functional importance of these different SC subpopulations, the molecular mechanisms underlying the differences between them, and the existence of a practical marker, are still missing. Here we report the first example of an miRNA that defined a robust quiescent state in MYF5^{neg} cells. miR-106b was present in quiescent MYF5^{neg} SCs, but was down-regulated during SC activation. Moreover, miR-106b overexpression maintained SCs in a more stem-like dormant phenotype, but miR-106b inhibition facilitated asymmetric divisions by allowing them to increase the number of proliferative myogenic-committed cells. These results, together with the fact that miR-106b was down-regulated concomitantly with the first wave of myogenic differentiation during muscle regeneration, enable us to conclude that miR-106b inhibition is required for full and proper SC activation.

The normally quiescent state of SCs has also been shown to be regulated by miR-489, miR-195, and miR-497 by silencing cell-cycle genes *Dlk1*, *Ccnd*, and *Cdc25* in quiescent SCs.^{7,8} There are also reports that miR-431 mediates SC heterogeneity during muscle development and regeneration by targeting *Pax7*.²⁵ Other miRNAs, like miR-31, have been previously implicated in regulating the entry into the myogenic program during SC activation by targeting *Myf5* 3'UTR.⁹ In this study, we found that miR-106b regulated both SC proliferation and myogenic progression *in vitro* and *in vivo*. As miR-106b was present only in MYF5^{neg} SCs and we have previously demonstrated that this miRNA targets cell-cycle regulatory genes,¹¹ we concluded that this miRNA played a specific role by

Figure 6. RNA-seq analysis of anti-miR-106b and scramble administration effects during muscle regeneration in mdx mice after CTX injury

(A) Schematic representation of anti-miR-106b/scramble administration and RNA-seq analysis (n = 3 per time point and condition). (B) Volcano plots showing differentially expressed genes in Anti-miR-106b versus Scramble-treated dystrophic muscles at day 3 after CTX injection, Anti-miR-106b versus Scramble-treated dystrophic muscles at day 7 after CTX injection, and differentially expressed genes in anti-miR-106b-treated dystrophic muscles at day 3 versus day 7 after CTX injury and Scramble-106b-treated dystrophic muscle at day 3 versus day 7 after CTX injury. (C) GO-biological process and cellular component ORA-analysis in anti-miR-106b treated muscles between day 3 and day 7 after CTX injury. (D) GO-biological process and cellular component ORA-analysis in scramble-treated muscles between day 3 and day 7 after CTX injury. (E) GO-biological process and cellular component ORA-analysis shared by anti-miR-106b and scramble-treated muscles between day 3 and day 7 after CTX injury.

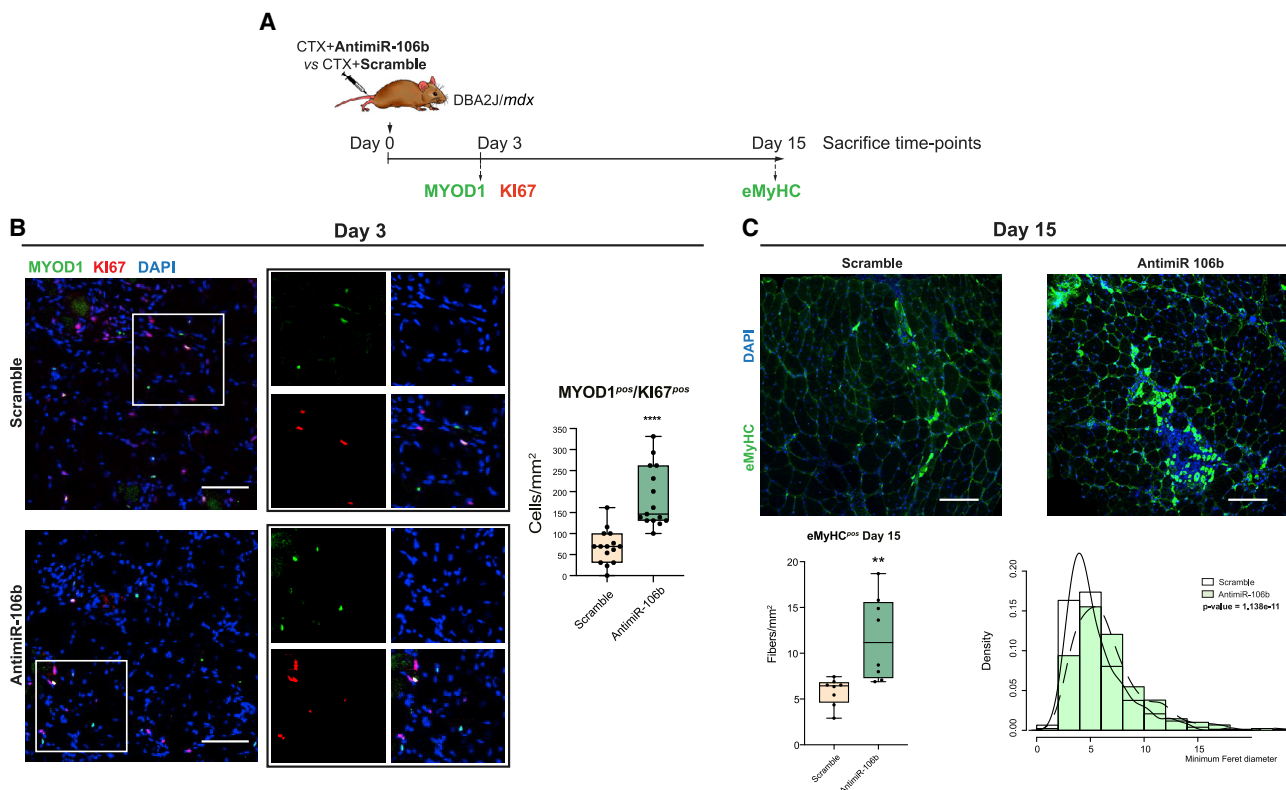


Figure 7. miR-106b inhibition *in vivo* increases muscle regeneration in the DBA/mdx mice

(A) Scheme of the CTX + anti-miR injection in the DBA2J/mdx male mice TA. CTX was injected together with anti-miR molecules in TA; scramble and CTX were injected into the contralateral muscle and used as a control. $n = 8$ DBA2J/mdx mice, aged 4–6 months, were randomized into two groups: CTX + scramble versus CTX + anti-miR injected at 3 days post-injection and scramble versus CTX + anti-miR injected at 15 days post-injury ($n = 4$ per time point in each group). (B) MYOD1 and KI67 immunostaining on the TA section from the scramble- and anti-miR-treated DBA2J/mdx mice (3 days post-administration). Quantification of the number of MYOD1^{pos}/KI67^{pos} cells is shown. (C) Immunostaining for eMyHC^{pos} in the scramble and anti-miR-treated DBA2J/mdx mice. Percentage of eMyHC^{pos} myofibers and muscle fiber size distribution analyzed by Minimal Feret Diameter (μm) are shown. Scale bars, 120 μm (B), 40 μm (C). Data are presented as mean $\pm$ SD. ** $p < 0.01$; **** $p < 0.0001$.

controlling quiescence versus cell activation in MYF5^{neg} SCs. As we observed the good capability of miR-106b compared with the other miRNAs (miR-31) targeting *Myf5*, we propose that miR-106b could act by reinforcing MYF5 inhibition in a subset of a highly quiescent SC population. However, as miRNAs may have multiple potential targets, the possibility that other miR-106b targets may contribute to the observed effects could not be excluded. In addition, because fluorescence *in situ* hybridization (FISH) amplification system used for miRNA detection in this study did not enable us to clearly discern miR-106b staining in cytoplasm and/or nucleus of SCs; we cannot rule out that the effects mediated by miR-106b in these cells can be induced both in nucleus and cytoplasm, as previously reported for other cell types.²⁶

Research conducted to understand the mechanisms controlling SC behavior in response to muscle injury has drawn considerable interest because emerging studies have pointed out that an altered SC function is associated with skeletal muscle atrophy in chronic illness, disuse, aging, and other muscle degenerative diseases.^{27–29} Therefore, the enhancement of intrinsic factors within SCs may

address impaired cell proliferative capacity and differentiation, and might improve skeletal muscles' regenerative response. We provide evidence that miR-106b inhibition by an anti-miR intramuscular injection enhanced the regenerative potential of mouse MuSCs by amplifying the proliferative myogenic-committed population in early muscle regeneration phases. Interestingly with a single anti-miR injection, muscle regeneration enhancement became even more pronounced after a second round of injury. In the adult muscle, muscle PAX7^{pos} SCs coexisted with a small subpopulation of committed MYOD1^{pos} SCs ready for immediate differentiation without preceding cell division.¹⁶ We found that the number of PAX7^{pos} sublamellar cells increased after 30 days of muscle injury in anti-miR-treated muscles, with no changes in MYOD1^{pos} sublamellar cells, plus a tendency to generate a large amount of PAX7^{pos} cells that was conserved after re-injury was also observed. Those results, together with the fact that miR-106b inhibition facilitates asymmetric division in cultured SCs, led us to think that anti-miR treatment could contribute mainly to extend stem SC populations, which display good engraftment capability, but also contribute to differentiated myofibers.⁶ We also show for the first time the

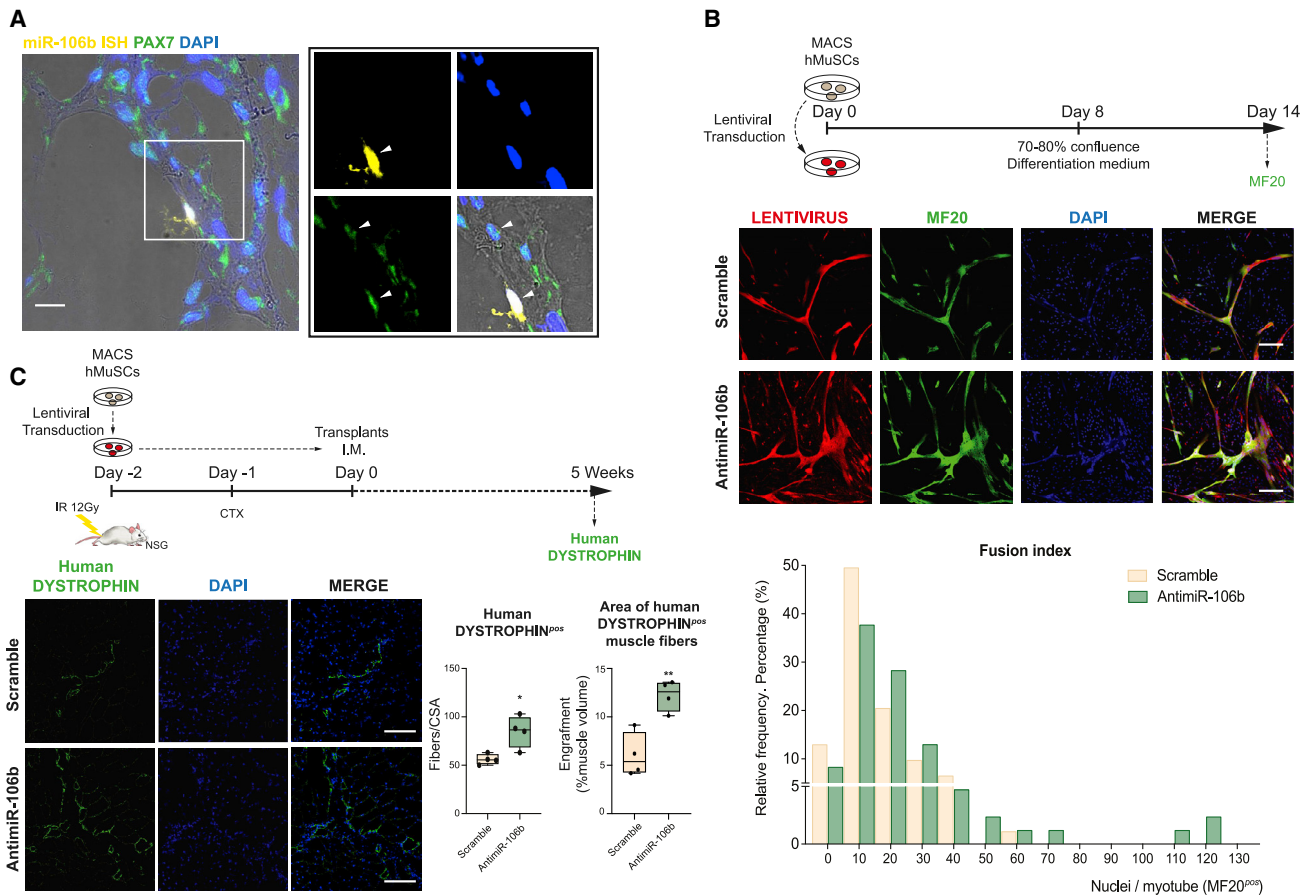


Figure 8. miR-106b is expressed in hMuSC and its inhibition enhances hMuSC myogenic differentiation potential and promotes their engraftment after cell transplantation *in vivo*

(A) Fluorescence *in situ* hybridization for the miR-106b and immunofluorescence staining of PAX7 on the human muscle section. (B) Scheme of the anti-miR-106b hMuSCs-treated cells (30,000–40,000 cells) and MF20 staining; the quantification of the fusion index is shown ($n = 3$ experimental replicates; 15 fields per plate). (C) Scheme of the anti-miR-106b hMuSC transplantation in NSG mice; $n = 4$ NSG male mice (Scramble-transfected cells were transplanted into the contralateral muscle and used as a control), and immunostaining for human DYSTROPHIN in the TA section from the transplanted NSG mice. The quantification of the human-DYSTROPHIN^{pos} myofibers and the extend of myoblast engraftment (area of human dystrophin positive muscle fibers) are shown. Scale bars, 20 μ m (A), 200 μ m (B), and 120 μ m (D). Data are presented as mean $\pm$ SD. * $p < 0.05$.

importance of miRNA inhibition for increasing the regenerative potential of hMuSCs, which indicates the relevance of miR-106b inhibition for the future development of novel therapeutic approach to improve muscle regeneration.

It is also apparent that myopathies like DMD significantly impair the regenerative process due to a low proliferative potential and poor progenitor formation from mdx-SCs.^{4,12} The importance of miRNA ameliorating muscular dystrophy in mdx mice has been previously highlighted in the search for new therapeutic strategies to target SC defects in DMD. miR-206 delays muscular dystrophy progression in mdx mice by promoting SC differentiation by targeting *Notch3*, *Pax7*, and *IGFbp5*.³⁰ Transgenic mdx mice that overexpress miR-431 lessen dystrophic phenotype severity.²⁵ Our analysis revealed miR-106b to be inherently up-regulated in dystrophic murine SCs.

As we previously documented that PITX2 represses miR-106b expression in SCs,¹¹ increased miR-106b levels could be due to the decreased *Pitx2* expression in mdx muscles, as our group also previously reported.¹² One remarkable finding in the present work was that miR-106b inhibition in mdx mice after muscle injury increased the number of myogenic progenitors, enhanced muscle regeneration, and promoted functional rescue. The transcriptomic analysis further emphasized the notion that miR-106b inhibition led to enrich the transcriptional program associated with skeletal muscle differentiation after injury in mdx mice. We also observed that biological process linked to impaired muscle regeneration in DMD, such as inflammatory response, are enriched in scramble-treated control but not in anti-miR-106b treated mdx mice after muscle injury. Therefore, we believe that the increased muscle regeneration after anti-miR-106b treatment in mdx mice might be also minimizing degenerative

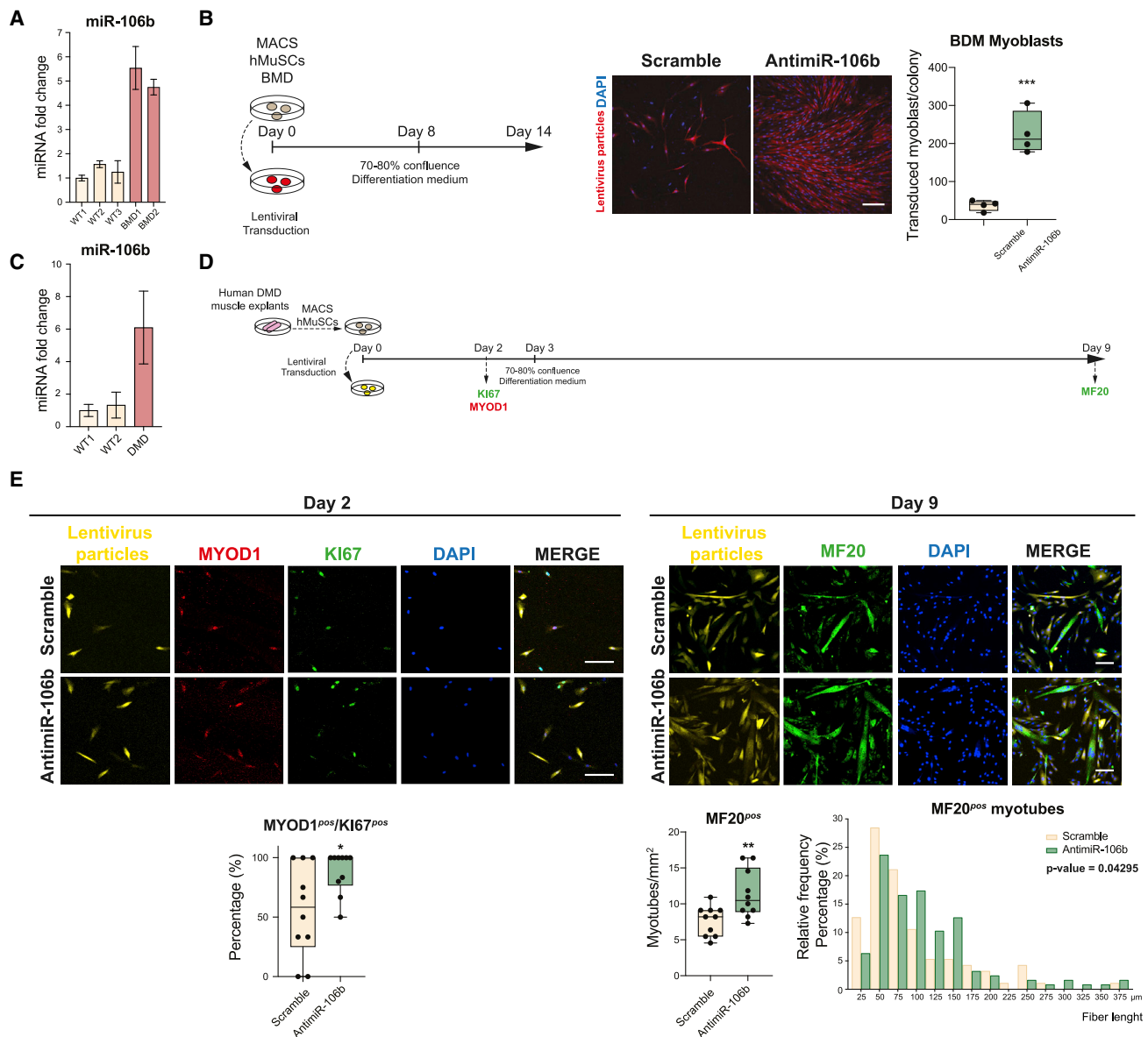


Figure 9. anti-miR-106b treatment increases the myogenic differentiation of BMD hMuSCs and increases the capabilities to proliferate and to form the myogenic precursors of DMD hMuSCs

(A) Transcript levels of miR-106b in muscle biopsies from control patients ($n = 3$ male, 31, 35, and 40 years old [CT1, CT2, and CT3, respectively]) and two BMD patients (male, 32 and 35 years old [BMD1 and BMD2, respectively]). Error bars in RT-qPCR analysis show the technical SD deviation of each patient. (B) Scheme of hMuSC isolation from BMD muscles biopsies, anti-miR treatment by lentiviral transduction, and the quantification of the number of transduced-myoblasts per cell colony (day 14 after lentiviral transduction). (C) Transcript levels of miR-106b in fresh isolated CD56^{pos} hMuSCs from control biopsies ($n = 2$ male, 35 and 40 years old [CT4 and CT5, respectively]) and from DMD biopsy (male, 5 years old). Error bars in RT-qPCR analysis show the technical SD deviation of each patient. (D) Scheme of hMuSCs isolation from the DMD muscle biopsies and anti-miR or Scramble treatment by lentiviral transduction. (E) Immunostaining for MYOD1 and KI67 on day 2 after lentiviral transduction. MF20 staining of the myotubes derived from the transduced dystrophic hMuSCs at 9 days post-transduction. The percentage of the KI67^{pos}/MYOD1^{pos} cells on day 2 and the number of MF20^{pos} myoblasts as well as fiber length distribution on day 9 are shown. 30,000–40,000 cells; $n = 3$ experimental replicates; 10 fields per plate. Scale bars, 50 μm . Data are presented as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

transcriptional pathway response previously identified in the muscles of dystrophic mice, such as the inflammatory response.³¹ More importantly, we demonstrated that miR-106b inhibition in hMuSCs

isolated from patients with BMD and DMD improved the inherent defects linked to their proliferation capability and proper differentiation.

In conclusion, we demonstrate that miR-106b is an important modulator of SC behavior, acting as a quiescence regulator in a subset of the SC population, and its inhibition is required for proper muscle regeneration. The main finding, based on miRNA modulation in mouse and human dystrophic SCs, identifies miR-106b as a new candidate to develop novel therapeutic strategies that increase the regeneration capacity of the damaged muscle in DMD. Although the newly generated muscle will still show lack of dystrophin, increased skeletal muscle regeneration after anti-miR treatment may compensate muscle fiber loss, ameliorating thus regenerative stimulus and minimizing the risk of SC depletion in DMD. Furthermore, the possibility of SC exhaustion after short-term anti-miR treatment is reduced since we observed that the amount of Pax7^{POS} cells in the muscle stem cell niche was enlarged in anti-miR-injected muscles after re-injury. Therefore, anti-miR-106b treatment could, at least temporarily, increase muscle mass, making it better able to respond to gene therapy.

MATERIAL AND METHODS

All the employed reagents and materials are listed in [Tables S1](#) and [S2](#).

Cell lines, animals, and human samples

Cell lines Sol8 and HEK293T were purchased from ATCC and kept in culture in line with the manufacturer's instructions. The animal procedures were approved by the University of Jaen Ethics Committee, and were conducted according to national and European community guideline regulations for animal care and handling inside an animal facility at the Universities of Jaen (UJA) and Granada (UGR). C57BL/6J and C57BL/10ScSn-Dmd^{mdx}/J (henceforth mdx) mice were supplied by the Jackson Laboratory. DBA2J/mdx mice were kindly provided by the laboratory of Pura Muñoz-Canoves. NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (henceforth NSG) mice were provided by the UGR. The use of human sample collection was approved by the Human Research Ethics Committee of UGR. Biopsies were obtained from informed patients or their legal guardian's consent. Detailed information is listed in [Table S1](#). The study was performed in accordance with the ethical standards laid down in the 1975 Declaration of Helsinki and approved by the Ethics Committee at the University of Jaen and Hospital Universitario Virgen de las Nieves from Granada. Informed consent was obtained from all participants.

Mouse and human SC isolation and culture

For mouse SC isolation, hindlimb muscles from 4- to 5-month-old male (C57BL/6 and mdx) mice were collected. Muscles were transferred and minced on a dish with DMEM:F12 (1:1). Minced muscles were dissociated 1 h at 37°C in an enzymatic solution containing DMEM:F12 (1:1) supplemented with 1 mg/mL collagenase type D and 4% Trypsin solution. Afterward, mechanical dissociation was performed through 14-G and 18-G needle up and down. For each step of mechanical dissociation, muscle mixture was decanted 5 min and supernatant was collected to a new tube with 5 mL of fetal bovine serum (FBS). Supernatant-FBS suspension was filtered through 100-µm and 40-µm cell strainers. Suspension cells were centrifuged at 300 × g for 5 min and then the pellet was resuspended with 1 mL of PBS. Red Blood Cells Lysis Solution (130-094-183; Miltenyi Biotec) was incubated for 2 min.

Incubation with magnetic labeling was performed for 15 min at 4°C of 1:5 dilution according MicroBeads Satellite Cell Isolation Kit (negative selection with Satellite Cell Isolation Kit-130-104-268, and positive selection with Anti-Integrin α -7 MicroBeads-130-104-261; Miltenyi Biotec). LS and MS Columns were pre-equilibrated and placed in appropriate magnets, and cell suspension was applied onto the column. Purified mouse SCs (5,000–10,000 cells) were seeded on 0.1% gelatin coated µ-Dish 50 mm (Ibidi, 81136) and cultured in mouse growth medium (mouse-GM) containing DMEM GlutaMAXTM (Life Technologies) and DMEM:F12 (Lonza) 50:50 supplemented with 20% FBS (Sigma), 2% Ultrosor G (Pall), 3% penicillin/streptomycin (P/S), and incubated at 37°C, 5% CO₂. For myoblast differentiation, when 70% to 80% confluence is reached, the medium was changed decreasing to 2% FBS.

For BMD, and control hMuSC experiments, isolation was done by MACS, as previously described for mice. In this case, negative cell sorting was performed using CD45 and CD31 human MicroBeads (130-045-801 and 130-091-935; Miltenyi) and positive cell sorting was performed using CD56-human MicroBeads (130-050-401; Miltenyi). DMD and their controls, hMuSCs were isolated from fresh muscle biopsies as follows. Muscle fascicles were released and cut into 3-mm fragments in HBSS supplemented with 1% PSF (P/S-Fungizone). Fascicles were incubated overnight at 37°C, 5% CO₂ in conditioning medium containing M199 (Gibco) supplemented with 37.5% FBS and 1% PSF. Next day, explants were seeded on plates coated with 25% v/v fresh human plasma until cell growth appeared. Subsequently, explants were cut into 1-mm-length fragments and cultured on 33.3% v/v fresh human plasma-1.5% gelatin type-A (Sigma) coated plates until the cell monolayer appeared around explants. Positive cell sorting was performed using CD56-human MicroBeads (130-050-401; Miltenyi). CD56^{POS} hMuSCs cells were seeded on 0.1% gelatin type-A coated plates and cultured in human growth medium (human-GM) containing 75% DMEM and 25% M199 supplemented with 10% FBS, 50 µg/mL insulin, 0.25 µg/mL human-FGF2, 0.02 µg/mL human-EGF, 2 mM L-glutamine, and 1% PSF. Cells were incubated at 37°C, 5% CO₂ until 50% to 60% of confluence was reached. For myoblast differentiation 70% to 80% confluence should be reached and human-GM was substituted with one supplemented with 2% FBS, 50 µg/mL insulin, 2 mM L-glutamine, and 1% PSF.

Single myofiber isolation and culture

Isolated single myofibers were obtained from extensor digitorum longus (EDL) of 4-month-old C57BL/6 mice as previously described in the protocol by Zammit's lab with slight variations.³² After dissection, EDL was incubated in 0.2% w/v collagenase type I (17100017, Gibco)/DMEM for 1 h 30 min in a 37°C water bath. Digestion was stopped when the muscle started to loosen up. EDLs were transferred to a pre-warmed dish with DMEM supplemented with 1% P/S. After 30 min of muscle rest, mechanical dissociation was performed to release individual myofibers, flushing with a bore glass pipette (the dishes were returned to the incubator every 10 min for 10 min to keep them warm). Single myofibers were transferred through three new pre-warmed dishes for clean-up. Immediately, single myofibers were fixed

for analysis at 0 h or cultured for 48 h in high serum medium (DMEM-high glucose, GlutaMAX supplemented with 10% horse serum, 0.5% chicken embryo, and 1% P/S). Horse serum was used on plates and glass pipettes as coating substrate for floating condition and to avoid stuck fibers.

Lentiviral transduction

Lentivirus particles expressing eGFP or mCherry Fluorescent Protein were produced using Lenti-Pac HIV Expression Packaging Kit (LT001, GeneCopoeia). Briefly, HEK293T cells (ATCC) were transfected with three plasmids encoding the vector components: mimic hsa-miR-106b (HmiR0111-MR11 from GeneCopoeia, onwards premiR-106b), inhibitor of hsa-miR-106b (HmiR-AN0029-AM03 from GeneCopoeia, onwards anti-miR-106b) or control microRNA (CmiR-AN0001-AM03 from GeneCopoeia, onwards Scramble), plasmid CMV-eGFP was used as a transfection control. Infectious lentivirus were harvested at 48 h post-transfection, filtered through 0.22 μ m and stored at -80°C . Lentivirus was titrated by using the Lenti-PACTM HIV qRT-PCR Titration Kit (217LT005, GeneCopoeia). To infect cells, lentivirus was added at 200 MOI and transduction was monitored by fluorescence microscopy.

Lipofectamine transfections

Sol8 cell line were transfected with mirVana miRNAs: premiR-31 (4464066; Thermo), premiR-106b (4464067; Thermo), premiR-31 + premiR-106b and Scramble (4464077; Thermo) at 80 nM using Lipofectamine-2000 (Invitrogen) following the manufacturer's instructions. After 24 h, cells were collected for RNA extraction and RT-qPCR analysis.

Cell-cycle arrest

Fresh SCs from 1-month-old male C57BL/6 mice were isolated as previously described in this article. SCs were seeded in two μ -Dish 50 mm for lentivirus transfection with scramble or anti-miR-106b diluted in mouse-GM. After 3 days of culture, SCs were attached, medium was refreshed, and cycle arrest was started as previously described.¹⁵ Briefly, to inhibit DNA synthesis, SCs were treated with 2.5 mM thymidine for 16 h and released for 8 h, treated again with 2.5 mM thymidine for 16 h and released for 4 h. Afterward, to arrest cells during mitosis, SCs were treated with 68 μ M monastrol for 8 h, released, and treated for 2 h with 10 μ M MG132 to block the proteolytic activity of the 26S proteasome complex. Finally, cells were released for 1 h to synchronize activated SCs at the late telophase stage, cells were then fixed in 4% paraformaldehyde (PFA) for 10 min and immunocytochemistry was performed.

Gain- and loss-of-function *in vivo* analysis

For gain- and loss-of-function analysis, mice were anesthetized by using isoflurane, 2% to 5% inhaled, to a surgical plane of anaesthesia. Twenty-four C57BL/6, 15 mdx, and eight DBA2J/mdx mice were injected in each TA muscle with 50 μ L of cardiotoxin solution (CTX; 10 μ M) together with 11.55 μ g of mirVana miRNAs (Thermo). This dose is equivalent to 0.57 mg per kg body weight.³³ Negative Control #1 Inhibitor (4464077) was used as Scramble, hsa-miR-

106b-5p mimic (4464067) was used as premiR-106b and hsa-miR-106b-5p inhibitor (4464085) was used as anti-miR-106b. Finally, an exhaustion treadmill trial was performed to evaluate the endurance of treated 4-month-old male mdx mice (and C57BL/6 mice were used as controls) fusing a motorized treadmill (Panlab Treadmills) supplied with a shocker plate as described previously.³⁴ The treadmill was run at 0° of inclination at 5 m/min for 5 min; subsequently, the speed was increased 1 m/min every minute. The test ended when the mouse remained more than 20 s on the shocker plate.

hMuSC xenotransplants

Xenotransplants were performed on 3-month-old NSG mice by intramuscular injection of anti-miR-106b-transduced hMuSCs. Briefly, 2 days before transplantation, TA irradiation (12 Gy) was done to incapacitate endogenous SCs, and hMuSCs were transfected with scramble or anti-miR lentiviral particles *in vitro*. One day before transplantation, injury with CTX was done on each TA to induce regeneration. During transplantation day, 1,000 to 3,000 treated hMuSCs were collected on 50 μ L of DMEM (Lonza) and injected using a 25-G needle into regenerating TA. Mice were killed 5 weeks after cell injection and TAs were harvested for histological analysis.

Muscle collection and processing

For tissue collection, mice were killed by cervical dislocation. TA from mice were collected and frozen in liquid nitrogen-cooled isopentane for sectioning or in liquid nitrogen for total RNA isolation. For histological analysis, tissue specimens were sliced using a cryostat microtome (CM1510S, Leica) to 8 μ m thick and mounted onto Thermo Scientific SuperFrost Ultra Plus Adhesion Slides.

Fluorescence *in situ* hybridization

FISH for miRNA detection was performed in cryosections of TA muscle from C57BL/6 and mdx mice, and also on human sartorius muscle and isolated myofibers from C57BL/6. All the samples were post-fixed in 4% PFA, followed by washes in DEPC-treated PBS. To block endogenous peroxidase activity, a 10-min wash was done with 0.03% H_2O_2 /PBS. Subsequently, permeabilization was performed with 2% acetone/PBS for 5 min. After one wash with 30% formamide/ $2\times$ saline sodium citrate (SSC) buffer (300 mM NaCl, 30 mM Na_3 Citrate- $2\text{H}_2\text{O}$, pH 5) for 10 min, pre-hybridization was done for 30 min at 61°C with the hybridization mix (50% formamide, $1.3\times$ SSC, 0.5 mg/mL yeast tRNA, $0.2\times$ CHAPS, 0.5M EDTA NaOH, pH 8, and 0.2% v/v Tween-20). Hybridization was performed with 25 nM of the hsa-miR-106b-5p double DIG-labeled LNA probe (339111 YD00618264-BCG; Qiagen) diluted in hybridization mix at 61°C for 2 h and 30 min. Samples were then washed in $2\times$ SSC and $1\times$ SSC, at 61°C and the last wash at room temperature (RT). Samples were rinsed with $1\times$ PBS prior to Alexa Fluor Tyramide SuperBoost Kit protocol (B40943 or B40912; Invitrogen). Briefly, samples were blocked for 1 h with Blocking Buffer (Invitrogen) and rinsed three times with $1\times$ PBS. The corresponding anti-Digoxigenin antibody (rabbit-9H27L19 or mouse-ab420) was incubated at 5 μ g/mL diluted in Blocking Buffer overnight at 4°C . The next day, samples were rinsed three times with $1\times$ PBS and then were

incubated with the corresponding poly-HRP-conjugated secondary antibody (rabbit-B40943 or mouse-B40912) at RT for 60 min. Samples were rinsed three times with $1\times$ PBS and the Tyramide Working Solution was added 8 min at RT following the manufacturer's instructions. Stop Reagent was added on top for 30 s and samples were rinsed in $1\times$ PBS prior to immunostaining.

Hematoxylin-eosin staining and immunostaining

For H&E staining, the cryosection samples were pre-fixed with 4% PFA for 10 min at RT. After several washes in water, samples were incubated for 10 min with Mayer's Hematoxylin Solution until nuclei were stained. Activated Eosin Y Solution 0.5% was incubated for 10 min. Then several washes with water were performed prior to dehydration and DPX was used as mounting media. Images were captured with a 2.0-MP digital microscope camera, which was attached via a c-mount to the side port of a Motic AE2000 microscope.

For immunocytochemistry purposes, cultured cells and isolated myofibers were fixed with 4% PFA for 10 min and were then permeabilized with PBS, 0.25% Triton X-100, and 50 mM NH_4Cl for 10 min at RT. Samples were blocked with Blocking Solution containing 0.2% gelatin/PBS (G1393) for 20 min at RT. Primary antibodies (listed in Table S2) were added at 5 to 10 $\mu\text{g}/\text{mL}$ in Blocking Solution overnight at 4°C . Alexa-conjugated secondary antibodies diluted 1/300 in Blocking Solution were added for 30 min at RT. For immunohistochemistry, the cryosection samples were fixed in 4% PFA for 10 min at RT. For the PAX7, PS6, and MYOD1 immunostaining, epitopes were unmasked in citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) in a pre-heated water bath for 30 min at 96°C . Afterward, all the samples were pre-incubated in TBSA-BSAT (10 mM Tris, 0.9% NaCl, 0.02% sodium azide, 2% BSA, and 0.1% Triton-X100) for 30 min at RT. Primary antibodies (listed in Table S2) were diluted to 5 to 10 $\mu\text{g}/\text{mL}$ in TBSA-BSAT and incubated overnight at 4°C in a humidified chamber. The next day, Alexa-Fluor-conjugated secondary antibodies diluted 1/200 in TBSA-BSAT were incubated for 2 h at RT. Finally, for both immunostaining protocols, samples were incubated in a 1/2,000 DAPI/PBS dilution for 15 min at RT. Hydromount was used for mounting and images were acquired by a Leica TCS SPE-CTR6500 Confocal Microscope.

Human myoblast in-cell western assay: myoblot

For the in-cell western assay, human DMD patient myoblasts (exon 48–50 deletion) were requested from the Queen Square Centre for Neuromuscular Disorders BioBank (CNMD Biobank, London, UK). Myoblasts were maintained in Skeletal Muscle Cell Growth Medium (PromoCell) supplemented with 10% FBS, GlutaMAX, $1\times$ MEM NEAA, 1% P/S, and incubated at 37°C in 5% CO_2 .

Human myoblasts were seeded at 7,500 cells/well on a 96-well plate previously coated with Matrigel in Skeletal Muscle Cell Growth Medium. After 24 h, when confluence was reached, medium was replaced with differentiation medium (DMEM-Lonza, 20% horse serum, and 1% P/S). After 24 h, cells were transfected with premiR-106b, antimiR-106b and Scramble at 40 nM using Lipofectamine

2000. Briefly, the medium was replaced with fresh differentiation medium at 5 h post-transfection. After a 7-day incubation period to allow myotube formation, cells were fixed by adding ice-cold methanol for 10 min at RT and were then washed with $1\times$ PBS. For the in-cell western assay, cells were permeabilized with $1\times$ PBS-Tween 0.1% Triton and then incubated with Odyssey Blocking Buffer (LI-COR Biosciences) for 2 h. After blocking, MF20 (DSHB) primary antibody diluted 1:100 in Odyssey Blocking Buffer was added for an overnight incubation at 4°C . The next day, plates were washed with PBS-Tween 0.1% and then treated with the secondary antibody mix IRDye 800CW goat anti-mouse and CellTag 700 Stain (LI-COR Biosciences) for 1 h at RT. Then, incubation plates were washed again with PBS-Tween 0.1% solution and were scanned by Odyssey CLx Imager (LI-COR Biosciences).

mRNA and miRNA extraction and RT-qPCR

Muscle total RNA was extracted from the treated TA muscles by the Direct-zol RNA MiniPrep-Zymo Research Kit (R2050; Zymo Research) following the manufacturer's instructions. Cells' total RNA was extracted from SCs by the RNAqueous-Micro Kit (AM1931; Thermo-Fisher Scientific) following the manufacturer's instructions. Either 500 ng of the total RNA from muscles or 50 ng of the total RNA from SCs were used as an mRNA retrotranscription template with the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (K1642; Thermo-Fisher Scientific) in line with the manufacturer's instructions. For miR retrotranscription, 10 ng of total mRNA from muscles or SCs were used as the miR retrotranscription template with the miRCURY LNA Universal RT Kit (203301; Qiagen).

The qPCRs for the mRNA measurements were performed by SsoFast EvaGreen Supermix (1725201; Bio-Rad). The qPCR program consisted of 95°C for 30 s (initial denaturalization), followed by 40 cycles of 95°C for 5 s (denaturalization); 60°C for 10 s (annealing); 75°C for 7 s (extension). Finally, melt curves were determined by an initial step of 95°C for 5 s, followed by 0.5°C increments for 7 s from 65°C to 95°C . The relative level of each gene expression was calculated as the ratio of the extrapolated levels of each gene expression, with the Gapdh and Gusb genes as normalizers (both transcripts present constant expression stability, indicating their suitability as reference genes, Figure S2G). Primers used for the mRNA qPCR are listed in Table S3.

The qPCRs for the miRs measurements were performed by GoTaq qPCR Master Mix (A6001, Promega). The qPCR program consisted of 95°C for 10 min (initial denaturalization), followed by 40 cycles of 95°C for 5 s (denaturalization); 60°C for 1 min (annealing and extension). Finally, melt curves were determined by an initial step of 95°C for 5 s, followed by 0.5°C increments for 7 s from 65°C to 95°C . The relative level of each miR gene expression was calculated as the ratio of the extrapolated levels of each gene expression and U6 as the normalizer. Primers were used for the microRNA qPCR are listed in Table S4.

All the qPCR reactions were performed in a Bio-Rad CFX384 Real-Time PCR system. Each PCR reaction was performed in triplicate and repeated in at least three different biological samples to obtain a representative average. Relative measurements were calculated as described by Pfaffl MW.³⁵

RNA-seq analysis

For RNA-seq analysis, TA muscles from treated mdx mice (CTX + scramble versus CTX + antimiR-106b) were collected and total RNA was isolated using the Direct-zol RNA MiniPrep-Zymo Research Kit (three biological replicates). RNA samples were sent to the Centro de Análisis Genómico (CNAG, Barcelona Spain) for RNA sequencing. Subsequently, 200 ng of the total RNA were used to prepare the RNA-seq library with the TruSeq RNA sample prep kit (Illumina) according to the manufacturer's instructions. Paired-end (2×75) RNA sequencing was carried out by HiSeq 2000 (Illumina). For trimming and aligning raw data, fastq sequence reads were uploaded to the European version of the Galaxy platform (<https://usegalaxy.eu/>).³⁶ Reads were trimmed with the Trim Galore software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and aligned to the built-in mouse reference genome mm10 with the RNA STAR Gapped-read mapper for the RNA-seq data (Galaxy Version 2.6.0b-2).³⁷ For the gene expression analysis, bam files were downloaded from the Galaxy server and further analyzed with the different RStudio packages downloaded from the Bioconductor website (<http://bioconductor.org>). Reads were assigned to genes by means of the “featureCounts” function of the “Rsubread” package, version 1.28.1,³⁸ and mouse annotation file release M19, GRCm38.p6 (ftp://ftp.ebi.ac.uk/pub/databases/gencode/Gencode_mouse/release_M19/). Only the mapped reads were used to calculate gene expressions. The library size of each experimental point ranged from 34816337 to 54599372 sequences.

The differential gene expression analysis was performed by the limma + voom linear modeling approach using the “edgeR” version 3.20.9 package, the “limma” version 3.34.9 package, and an empirical Bayes method for differential expression.^{39–41} All the gene comparisons with a p value <0.05 and a fold change >2 were considered differentially expressed under the experimental conditions.

A functional enrichment analysis was performed by the Over Representation Analysis (ORA) Method using either the “enricher” function for the “H: hallmark gene set” of MSigDB or the “enrichGO” function “GO Biological Process gene set.” Both functions appear in the “clusterProfiler” package version 3.6.0.^{42,43} The gene sets with a p value <0.05 were considered overrepresented under the experimental conditions.

Quantification and statistical analysis

Two-tailed unpaired Student's t test or Kolmogorov-Smirnov test were performed to calculate p values and to determine statistically significant differences. The number of independent experimental replications, mean ± SD, and statistical tests (p value) are reported in figure legends. Statistical analyses were performed with GraphPad Prism and RStudio. Images were processed for quantification by ImageJ software.

Data and software availability

The RNA-seq data reported in this paper are available under GEO accession number GSE134111.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2022.08.025>.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.E.A.; Methodology, L.R.O., F.H., A.R., P.S., and V.A. Writing – Original Draft, A.E.A., F.H., and L.R.O.; Writing – Review & Editing, D.F. and A.E.A.; Validation: L.R.O., F.H., A.R., and V.A.; Formal Analysis: L.R.O., F.H., A.R., F.R., and V.A.; Investigation: L.R.O., F.H., F.R.A., P.S., V.A., C.C., A.C., and A.E.A.; Resources: C.C., A.C., M.M., and L.S. Visualization: L.R.O., F.H., D.V., P.S., and V.A.; Supervision: D.F. and A.E.A.; Project Administration: A.E.A.; Funding Acquisition, D.F. and A.E.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental information

***miR-106b* is a novel target to promote muscle
regeneration and restore satellite stem cell
function in injured Duchenne dystrophic muscle**

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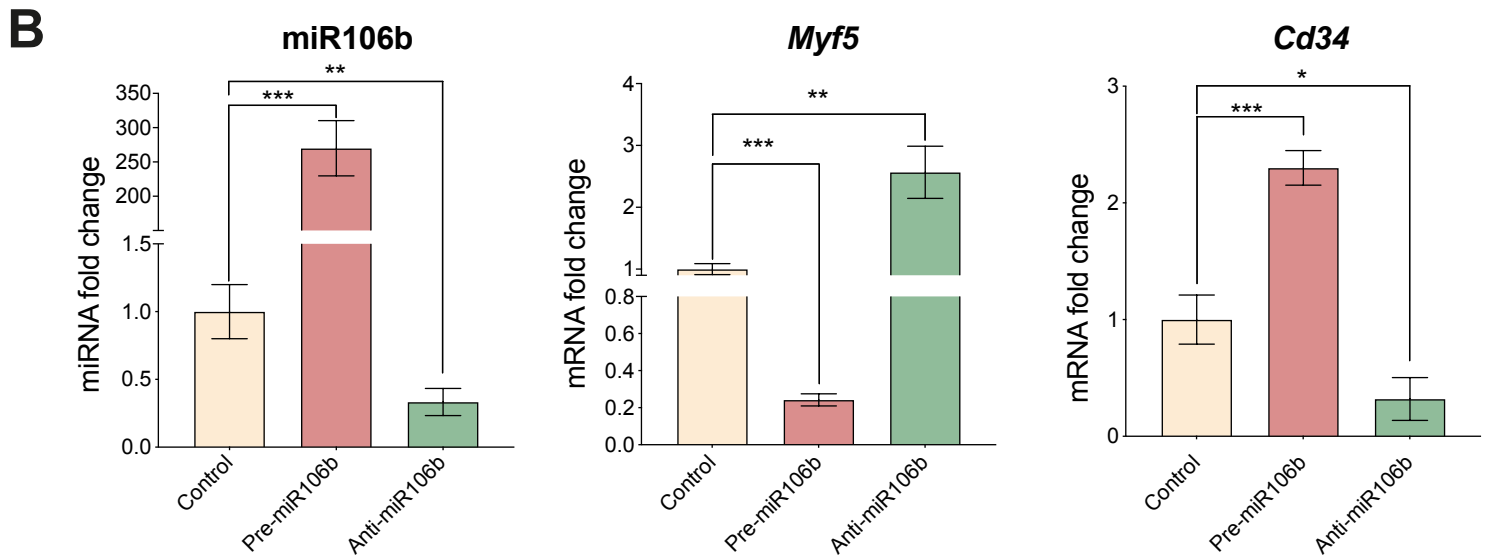
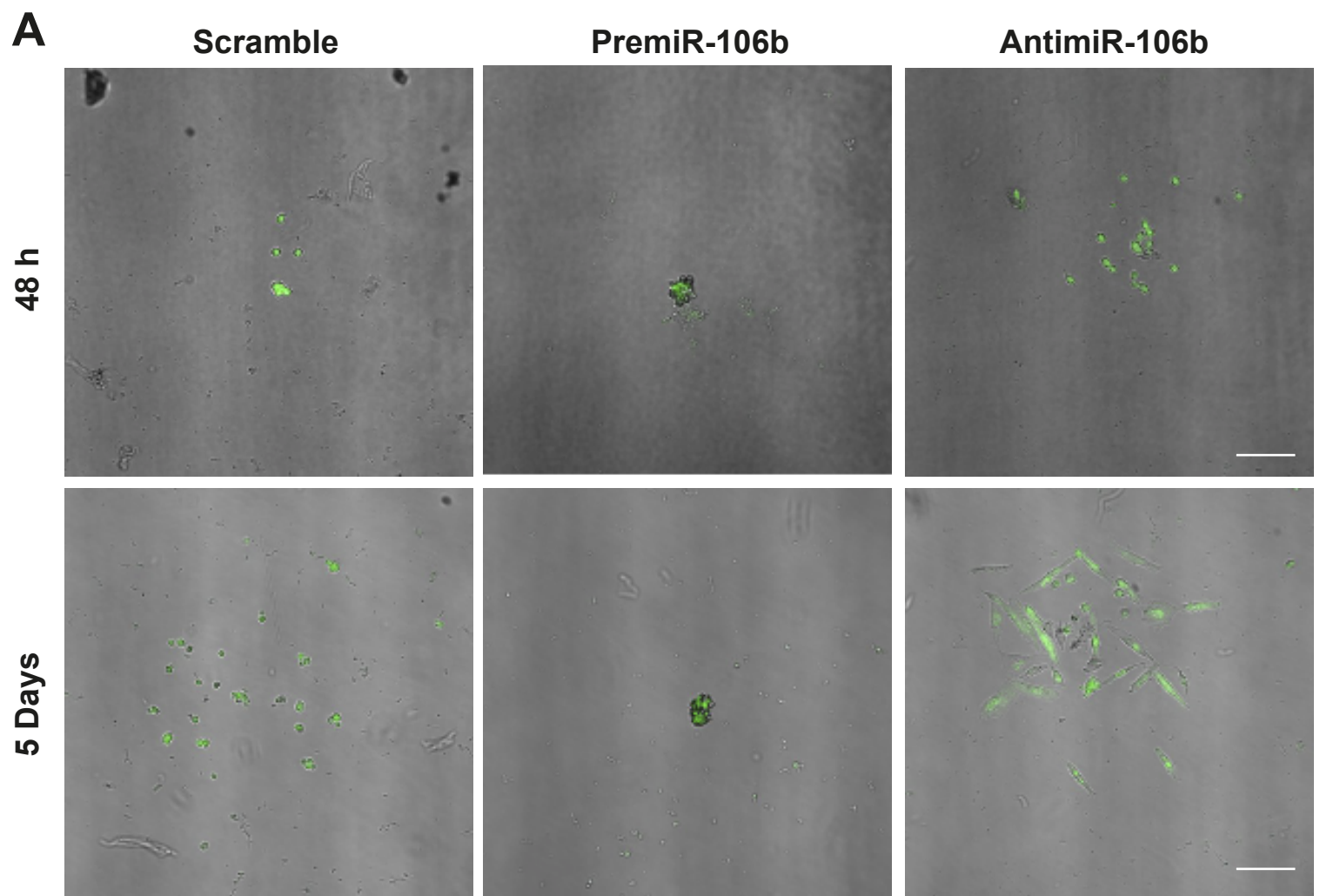


Figure S1. Phenotype of the premiR-106b transduced SCs and *Myf5* and *Cd34* expression after anti-miR lentiviral transduction **A.** Representative images of freshly isolated cultured SCs at 48 h and 5 days after premiR, anti-miR and control scramble lentiviral transduction. **B.** Transcript levels of miR-106b, *Myf5* and *Cd34* in freshly isolated SCs 2 days after premiR, anti-miR and control scramble lentiviral transduction, n=9 C57BL/6 male mice, aged 4-6 months, randomized into two groups: SCs cultures for the lentiviral transduction experiments and RT-PCR analyses (n=3 per condition). *p < 0.05; **p < 0.01; ***p < 0.001.

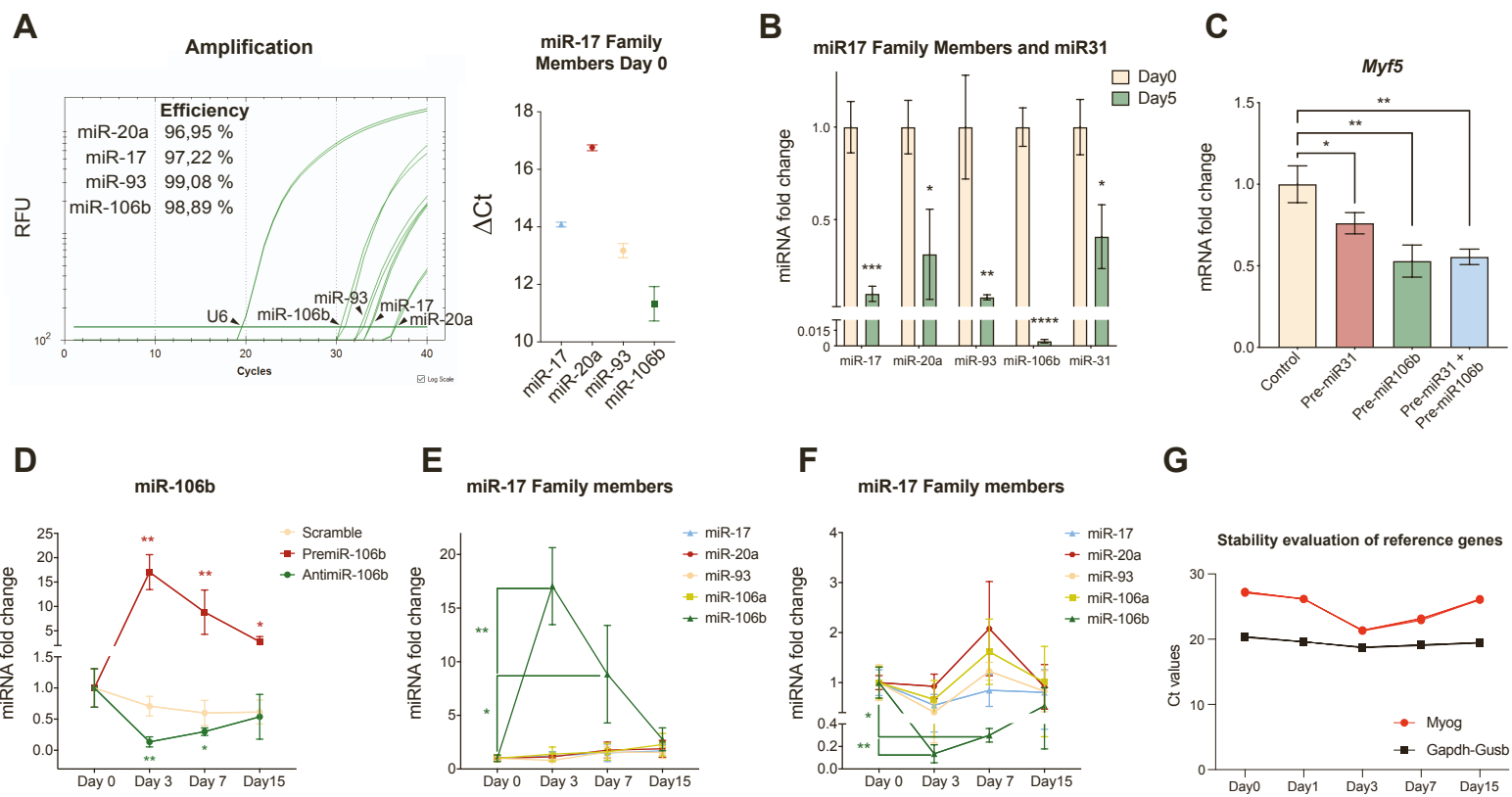


Figure S2. Myf5 transcript levels modulation by premiR-31 and premiR-106b transfection, expression levels of the miR-17 family members and miR31 in SCs, and after premiR-106b+CTX or antimiR106b+CTX intramuscular injections in mice, and stability evaluation of reference genes for RT-qPCR assays **A** Primer efficiency and ΔC_t of the miR-17 family members vs. the U6 normalizer in freshly isolated quiescent SCs (Day 0). Note that the lowest ΔC_t corresponds to miR-106b, which indicates that miR-106b is the main miR-17 member family expressed in SCs (D0). miR-20b and miR-106a were not detected (1500-2000 cells; n=3 independent experiments). **B** Transcript levels of the miR-17 family members and miR-31 in freshly isolated quiescent SCs (Day 0) (1500-2000 cells; n=3 independent experiments) vs. the 5-day cultured SCs (1000-15000 cells; n=3 independent experiments). miR-20b and miR-106a were not detected. Note that, in agreement with our results in myofiber cultures, miR106b expression levels drastically dropped after 5 days of cell culture. **C** Transcripts levels of Myf5 in the Sol8 myoblast culture after premiR-31, premiR transfection and premiR-31/premiR co-transfection (50000 cells; n=3 independent experiments). **D** The miR-106b transcript levels in the premiR or antimiR vs. the scramble-treated muscles after 3, 7 and 15 days of CTX injection. **E** Transcript levels of miR-17 family members in premiR-106b-treated muscles after 3, 7 and 15 days of CTX injection after 3, 7 and 15 days of CTX injection. **F** Transcript levels of the miR-17 family members in the antimiR-treated muscles after 3, 7 and 15 days of CTX injection. **G** Ct mean values of Gapdh-Gusb normalizers compared to Myog gene in RT-qPCR experiments (days 0, 3, 7 and 15 after CTX injection in TA muscles; n=3 mice per time point). Data are presented as mean $\pm$ SD. *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001 n=36 C57BL/6 male mice, aged 4-6 months, randomized into three groups: SCs cultures for the premiR-31, premiR-106b and premiR-106b +premiR-31 transfections; RT-PCR analyses in SCs (Day0 and 5); experiments and RT-PCR analyses (days 0, 3, 7 and 15 after the scramble and antimir injection in TA muscles) (n=3 per condition and time point).

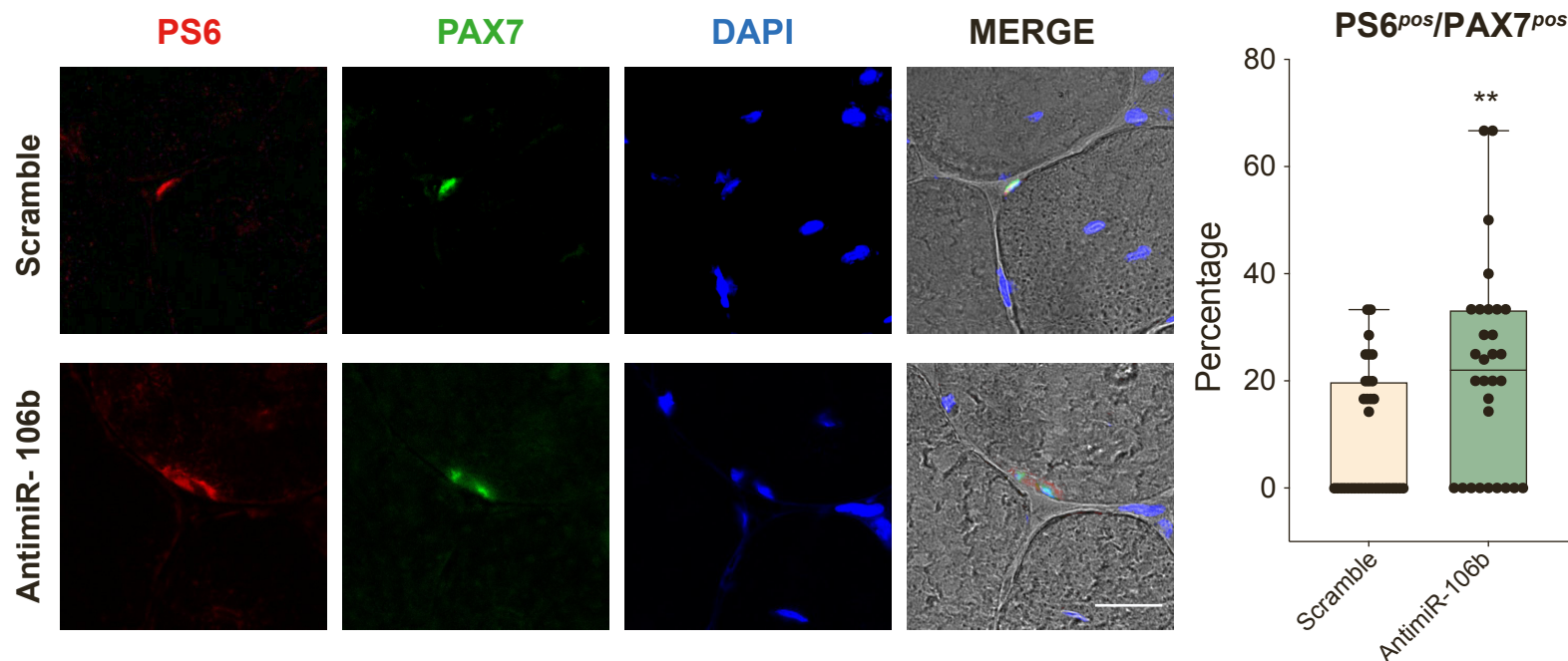
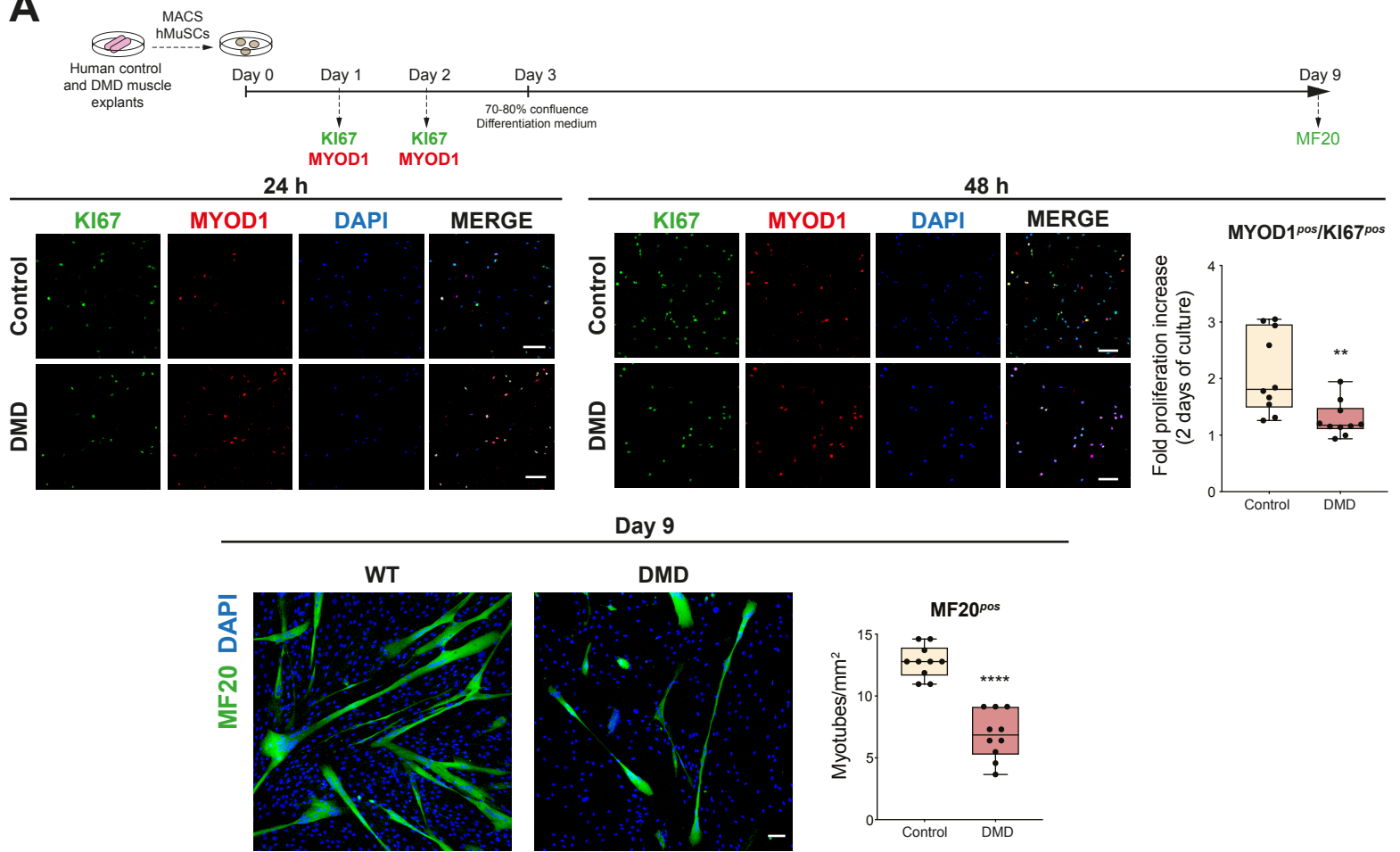


Figure S3. G-Alert marker PS6 and Treadmill test in the antimiR-treated mdx mice. n=12 mdx male mice, aged 4-6 months, randomized into two groups: the CTX-Scramble, and CTX-antimiR injection for immunostaining (n=3 per condition). PAX7, PS6 immunostaining on the TA section from the scramble- and antimiR-treated mdx mice (3 days post-administration). The percentage of the PS6^{pos}/PAX7^{pos} cells is shown. Scale bar 20 μ m (A). DAPI was used for nuclear staining. Data are presented as mean $\pm$ SD. **p < 0.01.

A**B**

Human dystrophic myoblasts
“in-cell” western analysis

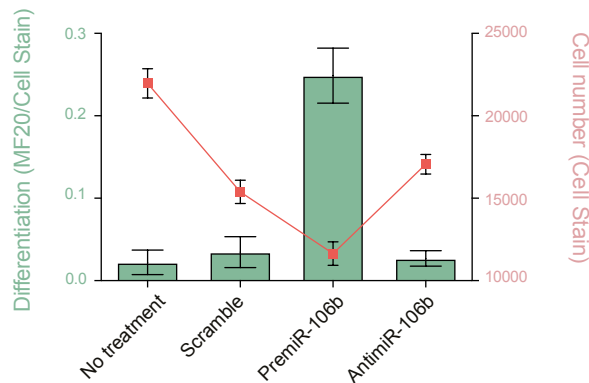


Figure S4. A Scheme of the hMuSCs isolation from the control and DMD muscles biopsies, and MYOD1 and KI67 staining. The proliferation increase ratio (evaluated by the KI67^{pos}/MYOD1^{pos} cells) between day 1 and day 2 is shown. MF20 staining in the control and dystrophic hMuSCs after 6 days cultured in differentiation medium. The quantification of the number of MF20^{pos} myotubes is shown. 30000-40000 cells; n=3 experimental replicates; 10 fields per plate. Scale bars 50 μ m. DAPI was used for nuclear staining. **B** The human dystrophic myoblast “in-cell” western analysis after premiR-106b and antiMiR-106b treatment. Green bars represent the anti-myosin heavy chain (MF20) antibody intensity signal as a marker of differentiation in the human DMD cultured myotubes (n=3 independent experiments). The red line represents the Cell Tag 700 signal and indicates the average number of cells per condition in the human DMD cultured myotubes (n=3). Data are presented as mean $\pm$ SD. **p<0.01; ***p<0.01; ****p<0.0001.

Table S1. Cell Lines, Animals and Human samples

Experimental Models	Reference or Source	Identifier or Information
C57BL/6J	The Jackson Laboratory	n° 000664
C57BL/10ScSn-Dmd ^{mdx} /J	The Jackson Laboratory	n° 001801
NOD.Cg-Prkdc ^{scid} Il2rg ^{tm1Wjl} /SzJ NSG TM , NOD scid gamma	Ceded the by University of Granada. From Jackson Laboratory.	n° 005557
DBA/2	Kindly provided by Pura Muñoz's laboratory. From Janvier Labs	DBA/2J
HEK293T	ATCC [®]	CRL-3216 TM
Sol8	ATCC [®]	CRL-2174 TM
DMD mouse primary Satellite Cells	This paper, from C57BL/6J mice hindlimbs	N/A
Wild type mouse primary Satellite Cells	This paper, from C57BL/10ScSn-Dmd ^{mdx} /J mice hindlimbs	N/A
Human Plasma	Biobank of the Public Health System of Andalusia	N/A
Human biopsies from BMD patient	Hospital Univesitario San Cecilio, Granada, Spain	Quadriceps muscles. Male, 32 and 35 years old
Human biopsy from DMD patient	Hospital Univesitario Virgen del Rocío, Seville, Spain. Provided by Dr. Carmen Paradas	Quadriceps muscles. Male, 5 years old
Human biopsy from healthy patients	Hospital Univesitario San Cecilio and Biobank of the Public Health System of Andalusia. Granada, Spain	Sartorius and anterior rectus femoris muscles Male, 27 to 40 years old.
Human myoblasts from healthy patients and DMD male patients	Queen Square Centre for Neuromuscular Disorders BioBank (CNMD Biobank, London, UK)	Male

N/A - not applicable

Table S2. Reagents and Material (Excel file see “Supplemental Videos and Spreadsheets”)

Table S3. Primers used for the mRNA qPCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
CD34 (NM_009911.3)	ACGGCTGTAGAGCGAGTGTT	AGGGTTCCTTGTTGGAGTCA
Gapdh (NM_008084.2)	GGCATTGCTCTCAATGACAA	TGTGAGGGAGATGCTCAGTG
Gusb (NM_010368.1)	ACGCATCAGAAGCCGATTAT	ACTCTCAGCGGTGACTGGTT
Myf5 (NM_008656.5)	TGAGGGAACAGGTGGAGAA C	AGCTGGACACGGAGCTTTTA
Myod1 (NM_010866.2)	GGCTACGACACCGCCTACTA	TCCCTGTTCTGTGTCGCTTA
Myog (NM_031189.2)	CTACAGGCCTTGCTCAGCTC	ACGATGGACGTAAGGGAGTG

Table S4. Primers used for the microRNA qPCR

Gene	Source, identifier
hsa-miR-17-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP02119304
hsa-miR-20a-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP00204292
hsa-miR-20b-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP00204755
hsa-miR-93-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP00204715
hsa-miR-106b-5p miRCURY LNA miRNA PCR Assay	Qiagen, YP00205884
U6 snRNA(hsa, mmu) miRCURY LNA miRNA PCR Assay	Qiagen, YP00203907

2 - miR-106b is upregulated in the muscles of elderly mice and its inhibition in aged SCs increases their myogenic differentiation potential

Our previous results showed that miR-106b reinforces quiescence in a particular subpopulation of MYF5^{neg} SCs in the adult muscle and that miR-106b inhibition is necessary for proper muscle regeneration (Rodríguez-Outeiriño et al., 2022). We also found that miR-106b is upregulated in the muscles and in SCs of the dystrophic mouse model mdx as well as in dystrophic hMuSCs (Rodríguez-Outeiriño et al., 2022). Importantly, we have demonstrated that miR-106b inhibition restores the intrinsic defects of dystrophic SCs related with regenerative capabilities, increasing their myogenic potential, what finally enhances muscle regeneration (Rodríguez-Outeiriño et al., 2022). Similarly to that observed in dystrophic SCs, progressive muscles mass loss in sarcopenia is linked to SC intrinsic dysfunctions: impaired balance between symmetric-asymmetric divisions that entails a lack of self-renewal and an irreversible quiescence status that lead in a loss of stemness and myogenic committed progeny (Chakkalakal et al., 2012; Feige et al., 2018; Filippelli and Chang, 2022). Based on that, we next explored miR-106b expression and function in sarcopenic muscles.

As it has been described that prolonged periods of SC quiescence are related with the development of sarcopenia (Chen et al., 2020; Rubinsztein et al., 2011) and we have previously demonstrated that miR-106b plays a role maintaining a strong quiescence status in SCs (Rodríguez-Outeiriño et al., 2022), we evaluated the expression levels of miR-106b in the TA muscles of elderly mice (>25-month-old) by RT-qPCR assay. We found that miR-106b expression displayed increased levels in the TA muscles of elderly mice when compared to adult mice (6-month-old) (figure 18A). This result is similar to those found in the muscles of dystrophic mice ((Rodríguez-Outeiriño et al., 2022), figure 4), suggesting that miR-106b dysregulation is also present in aged muscle. Additionally, since we have demonstrated that in skeletal muscle miR-106b is expressed only in a subpopulation of SCs ((Rodríguez-Outeiriño et al., 2022), figure 1A) and that it has been shown that elderly C57BL/j mice (24-month-old) experience a significative decrease (>50%) in the number of SCs in the TA muscles (Arpke et al., 2021), high levels of miR-106b in aged TA muscles (figure 18A) could correlate with high expression in SCs, reinforcing its quiescence, which in the end may worsen sarcopenic SC inactive phenotype.

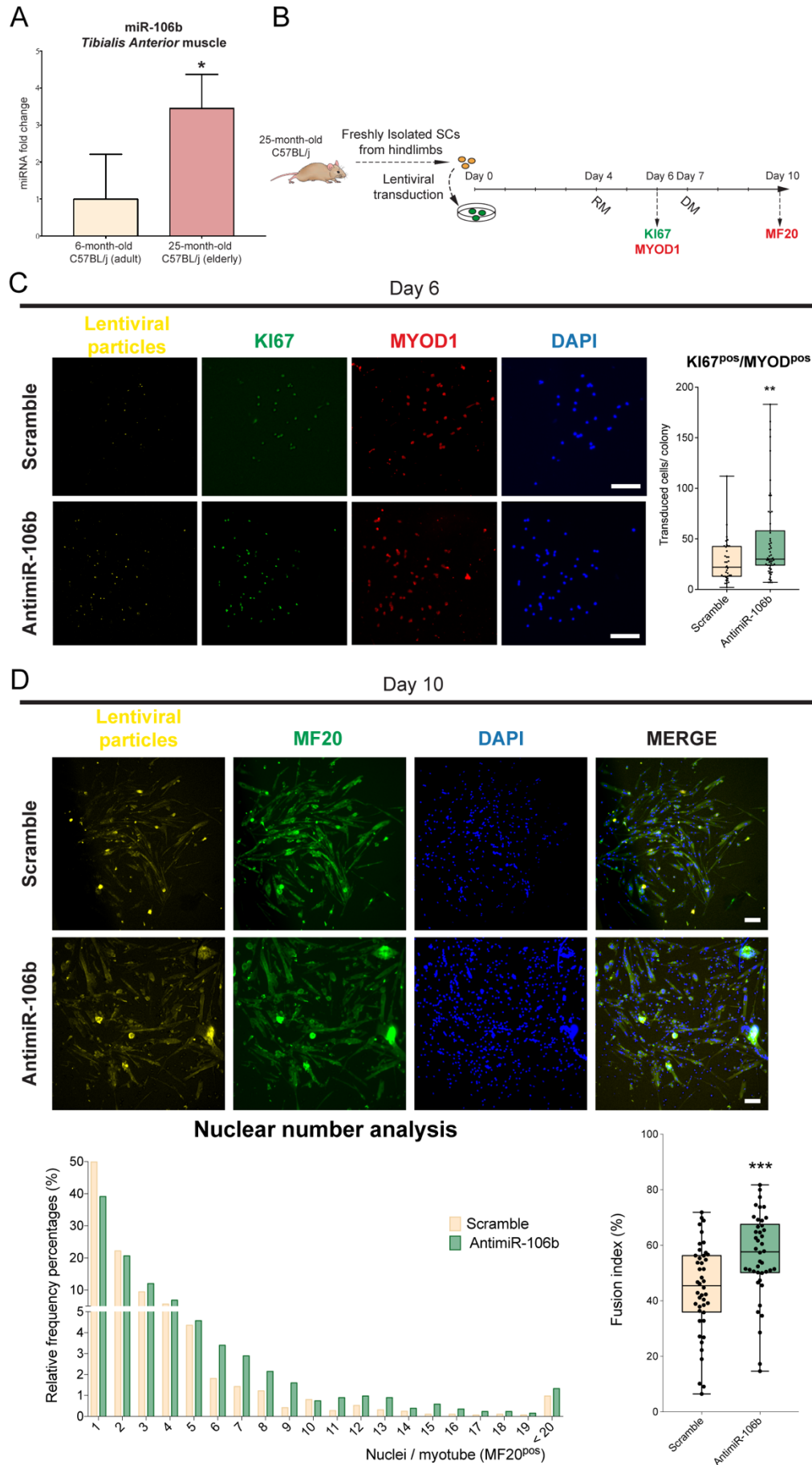


Figure 18. miR-106b is up-regulated in *tibialis anterior* (TA) muscles of 25-month-old C57BL/j and miR-106b *in vitro* inhibition in aged satellite cells (SCs) increases their proliferation and enhance myogenic differentiation potential. (A) Transcript levels of miR-106b in the TA muscles from C57BL/j mice (25-months-old as elderly mice and 6-months-old as control). (B) Scheme of SCs isolation from 25-month-old C57BL/j hindlimb muscles and anti-miR-106b or scramble treatment by using lentiviral particles. RM: Refresh Media; DM: Differentiation Media. (C) Immunostaining for MYOD1 and KI67 on day 6 after lentiviral transduction. Increased percentage of transduced KI67^{pos}/MYOD1^{pos} cells per colony after anti-miR-106b lentiviral treatment is shown. (D) MF20 staining of the myotubes derived from the transduced 25-month-old C57BL/j SCs at 10 days post-transduction. Myogenic differentiation potential: nuclear number analysis of transduced MF20^{pos} myotubes showed an increase after anti-miR-106b lentiviral transduction and it also showed an enhanced fusion index, counted as the number of nuclei inside transduced MF20^{pos} myotubes (≥ 2 nuclei) as a percentage of the total number of nuclei in the field. Scale bars 100 μ m. $n \geq 3$ mice. Unpaired t-test. Data are presented as mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

In order to evaluate if miR-106b inhibition might modulate aged-SC behaviour, we performed and *in vitro* loss-of-function analysis in freshly isolated SCs from hindlimb muscle of elderly mice (25-months-old) by using lentiviral particles bearing the miR-106b inhibitory (anti-miR-106b) and scramble (control miR) molecules (figure 18B). We first evaluated the anti-miR-106b impact in SC activation by quantifying the number of proliferative activated SCs by co-staining with KI67 proliferation marker and MYOD1. Similar to what we observed in dystrophic muscle stem cells, after 7 days of lentiviral transduction, anti-miR-106b treatment significantly enhanced the amount of proliferating activated KI67^{pos}/MYOD1^{pos} cell population when compared to control treatment (figure 18C). This observation indicates that miR-106b inhibition is able to enhance the amount of proliferating myogenic committed cells derived from aged SCs.

Subsequently, and to further study the effect of anti-miR-106b in myogenic differentiation potential in aged SCs, we evaluated the differentiation and fusion indexes in myotubes by using the MF20 antibody. After 5 days in differentiation media, anti-miR-106b treated aged-SCs displayed a significantly higher differentiation index, as measured by quantify the number of nuclei in the MF20^{pos} myotubes (figure 18D). Moreover, anti-miR treatment also enhanced their fusion index (figure 18D), evaluated as the percentage of SCs that differentiate and eventually fuse to form multinucleated myotubes. These findings indicate that, similarly to what we observed in dystrophic SCs, anti-miR-106b treatment enhances the myogenic potential of senescent aged SCs by increasing their capability to proliferate and to form myotubes.

3 - miR-106b is expressed during embryonic and fetal myogenesis in a subpopulation of myogenic precursor cells

A recent study performed by our laboratory has demonstrated that the lack of *Pitx2* in the committed myogenic progenitors (*Myf5^{Cre/Wt}/Pitx2^{-/-}* homozygous mutant mice) during development affects the number of SCs expressing MYF5 and/or miR-106b in the adult muscle (Ramírez de Acuña et al., 2022). Moreover, in accordance with the previously described role of PITX2 repressing miR-106b (Lozano-Velasco et al., 2015), we saw that, as expected, *Pitx2* deficiency in committed myogenic precursors leads to an increase in the number of miR-106b^{pos} cells in the skeletal muscle (Ramírez de Acuña et al., 2022). By contrast, the number of MYF5^{pos} SCs was clearly diminished in *Myf5^{Cre/Wt}/Pitx2^{-/-}* mutant mice (Ramírez de Acuña et al., 2022). These results suggest that the interplay between *Pitx2* and miR-106b expression in myogenic progenitors may be defining different subpopulations of SC precursors during skeletal myogenesis. In agreement with this idea, two clearly distinguishable PITX2^{pos} and miR-106b^{pos} independent cell populations were previously observed by us in the limb buds of the mouse embryo at E10.5 (Ramírez de Acuña et al., 2022). Taken together, these results suggest that *Pitx2* and miR-106b might be defining different myogenic progenitors that will give rise to different adult SC populations.

To further explore the role of miR-106b during embryonic myogenesis, we first analysed miR-106b expression profile at E9.5, E10.5, E11.5 and E12.5 stages (figure 19A). During this time window of embryonic muscle development, the somites have been divided in the DMT and the sclerotome. The DMT will give rise to the MT and subsequently, some multipotent myogenic progenitors arise from the hypaxial lip of the DMT and migrate to form the muscle mass in the limb buds (Hernández-Torres et al., 2017; Messina and Cossu, 2009). Thus, in order to characterize miR-106b expression in the different embryonic areas containing myogenic progenitors, somites, somitic-derived structures and limb buds' regions were dissected and used for RNA isolation and RT-qPCR analysis (figure 19A). This assay revealed that even though miR-106b was expressed in all developmental stages examined, there was a higher expression between E10.5 and E12.5 in all regions analysed (somitic-derived structures and limb buds) with a peak expression at E10.5 when compared to E9.5 (figure 19B).

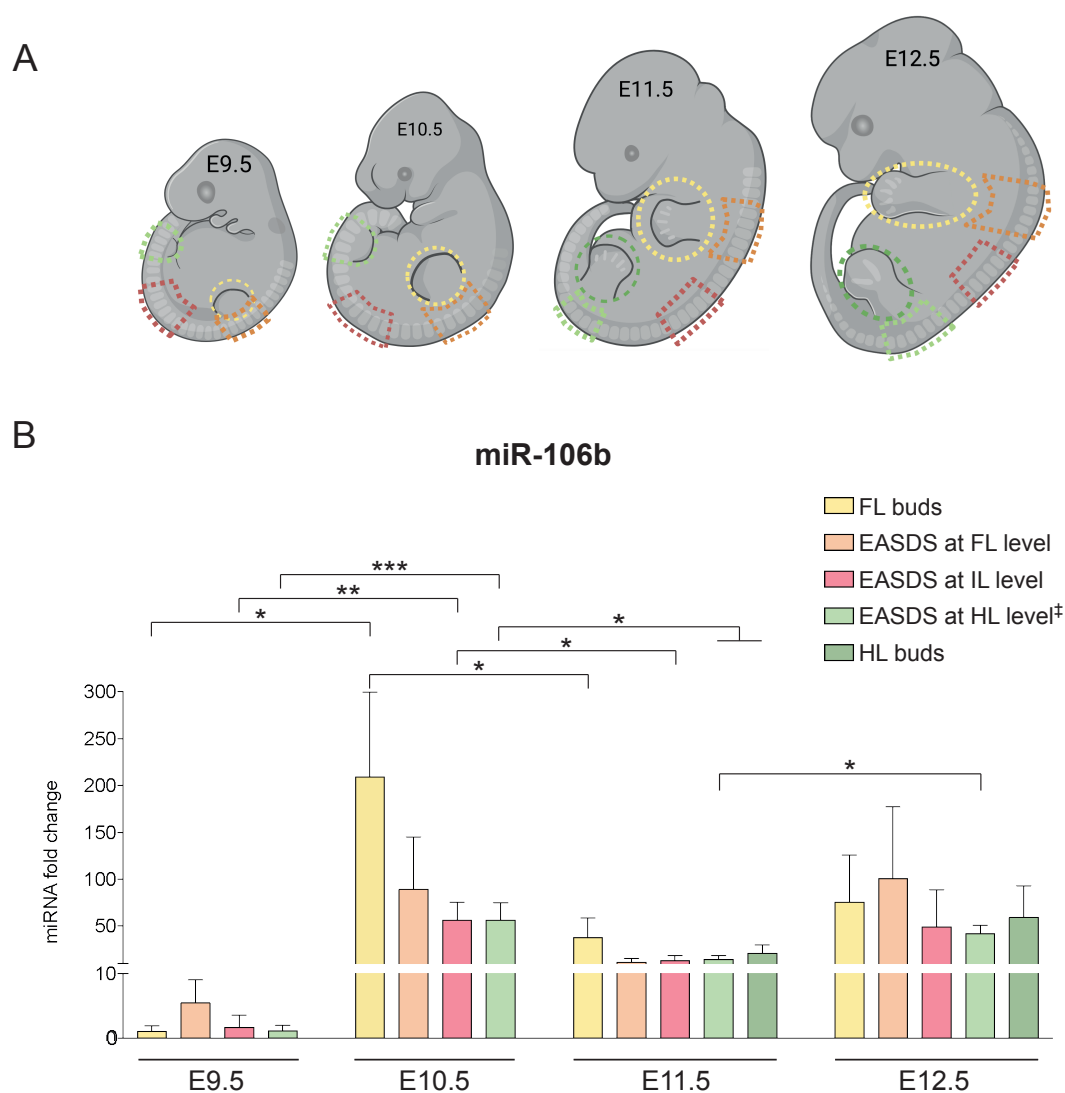


Figure 19. miR-106b expression is upregulated during embryonic myogenesis. (A) Scheme of limb buds and Embryonic Area containing Somitic-Derived Structures (EASDS) collected in CD1 mouse embryo at E9.5, E10.5, E11.5 and E12.5 stage for RT-qPCR analysis. Created by Biorender.com. (B) miR-106b transcript levels in the limb buds and EASDS of mouse embryo at E9.5, E10.5, E11.5 and E12.5 stage. [‡] At E9.5 and E10.5 stages EASDS at hindlimb level includes the hindlimb buds. $n = 3$ mice per experimental point. Data are presented as mean $\pm$ SD. FL: forelimb; IL: interlimb; HL: hindlimb. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

We found that miR-106b was expressed at low levels at E9.5 with no significant changes between the different embryonic regions analysed but with a weak non-significant tendency to increase in the somites present at forelimb buds' level (figure 19B). At E10.5 stage miR-106b expression levels, when compared to E9.5 stage, were significantly increased in the forelimb buds ($p < 0.05$), in the embryonic area containing somitic-derived structures at the interlimb buds' level ($p < 0.01$), in hindlimb buds and in the area containing somitic-derived structures at the hindlimb buds' level ($p < 0.001$) (figure 19B). Although non statistical significance, we also found miR-106b expression levels displayed a tendency to increase in the somites present at the

forelimb buds' level between E9.5 and E10.5 stage (figure 19B). We observed that miR-106b displayed a mild significant decrease between E10.5 and E11.5 ($p < 0.05$), although its expression levels at E11.5 remain elevated when compared to first stage of embryonic myogenesis analysed, at E9.5 (figure 19B). Finally, at E12.5, except a small significant increase ($p < 0.05$) at the somitic-derived structures located in hindlimb buds' level, miR-106b expression levels remained almost without significant fluctuations when compared to E11.5 (figure 19B). Based on our previous observation, these results could be indicating that miR-106b is present in a subpopulation of myogenic progenitors.

To further explore miR-106b expression in the first stage of fetal myogenesis at the limb buds' level, we analysed the expression profile at E14.5 stage in the forelimbs and hindlimbs of mouse embryos (figure 20A). From E14.5 to E16.5 proliferative PAX7^{pos} myogenic progenitors are present in the muscle masses to form new myofibers and contribute to muscle growth, however, a few non-proliferative PAX7^{pos} myogenic progenitors will remain undifferentiated to eventually set the SC pool found in the adult muscle (between the sarcolemma and basal lamina) (Sambasivan and Tajbakhsh, 2007). When compared to E12.5, miR-106b expression at E14.5 stage was significant reduced in the forelimb and hindlimb buds ($p < 0.01$) (figure 20B). Nonetheless, miR-106b expression was still detected at E14.5 in the limb buds (figure 20B). Assuming that miR-106b is expressed in quiescent myogenic progenitors, this result could be suggesting that miR-106b decreased expression levels at fetal stages may be due to a diminished number of quiescent and undifferentiated myogenic progenitors in the forming muscle at E14.5.

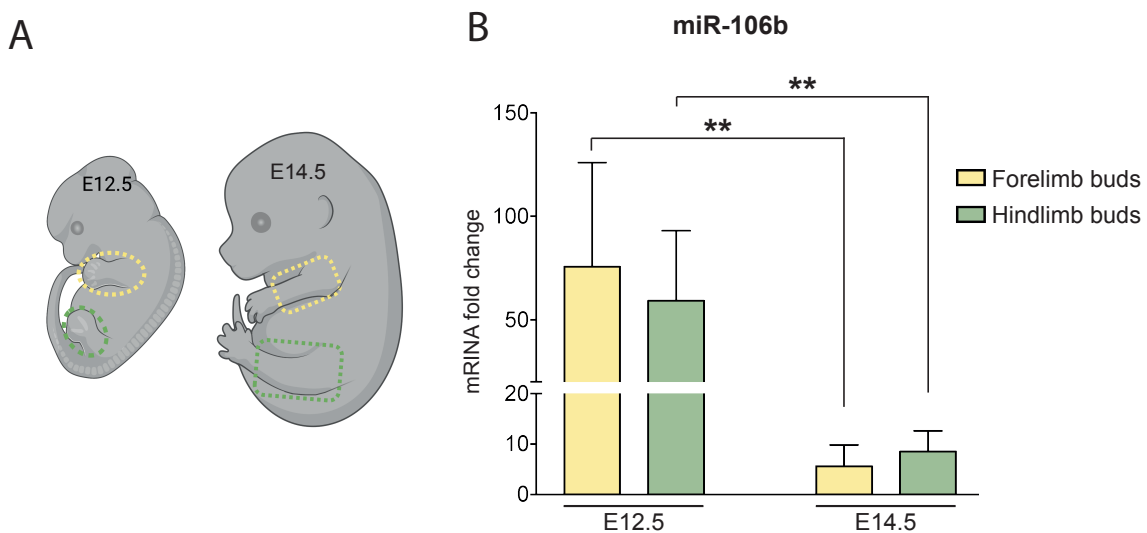


Figure 20. miR-106b expression is downregulated at the beginning of fetal myogenesis (E14.5). (A) Scheme of limb buds collected in CD1 mouse embryos at E12.5 and E14.5 stage for RT-qPCR analysis. Created by Biorender.com. (B) miR-106b transcript levels in the limb buds' regions of mouse embryos at E12.5 and E14.5. $n = 3$ mice per experimental point. Data are presented as mean $\pm$ SD. ** $p < 0.01$.

During embryonic development several miRs participate in the regulation of myogenic precursors, myoblast proliferation and differentiation (Luo et al., 2021). In this sense, two myomiRs, miR-1 and miR-133, have been previously described as essential regulators of MT formation, myoblast proliferation and differentiation during embryonic muscle development (Chen et al., 2006; Feng et al., 2013; Goljanek-Whysall et al., 2012; Luo et al., 2021; Mok et al., 2018; Sweetman et al., 2008). In order to evaluate the relevance of miR-106b with respect to these well-defined myomiRs (miR-1 and miR-133a) during embryonic myogenesis, we carried out a comparative analysis of the expression levels in the limb buds and in the areas containing somitic-derived structures at E10.5 and E12.5 in mouse embryos. As shown in figure 21, at E10.5 stage, miR-106 expression levels were significant higher in all areas analysed when compared to miR-1/miR-133a. Later on, at E12.5 stage, miR-106b expression was only significant higher in the limb buds (forelimbs and hindlimbs) when compared to both myomiRs (figure 21). These results may be indicating that miR-106b is playing a preponderant role in miR-mediated regulation in embryonic area containing somitic-derived structures and limb buds at early stages of embryonic myogenesis (E10.5), but also in the limb buds at later stages (E12.5).

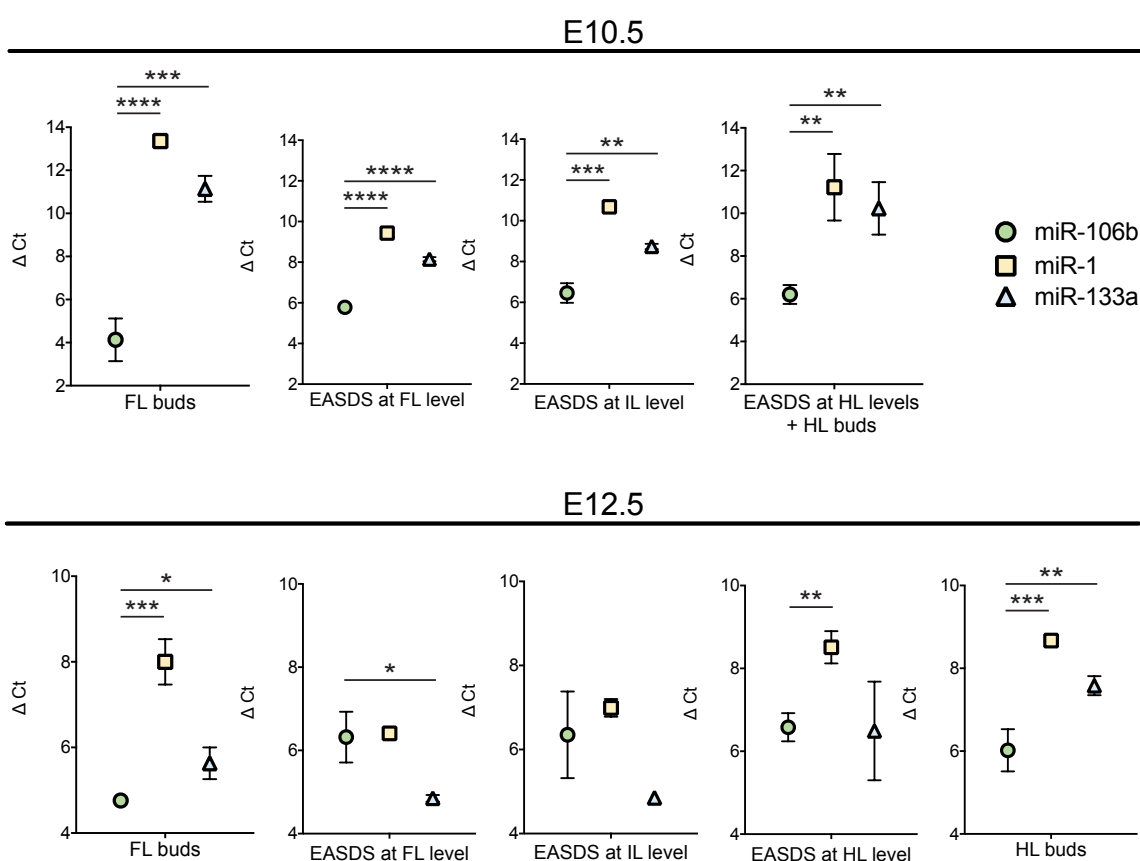


Figure 21. miR-106b presents higher expression levels than myomiRs miR-1 and miR-133a mostly during first stage of embryonic myogenesis. Δ Ct of the miR-106b vs. miR-1 and miR-133a in limb buds and Embryonic Area containing Somitic-Derived Structures (EASDS) of CD1 mouse embryos at E10.5 and E12.5 stages. Note that the lowest Δ Ct indicates that expression of the miR is higher. $n = 3$ mice per experimental point. Data are presented as mean $\pm$ SD. FL: forelimb; IL: interlimb; HL: hindlimb. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

We have observed high levels of miR-106b expression in the limb buds and embryonic area containing somitic-derived structures during embryonic myogenesis (E9.5 to E12.5) and in the limb buds at the beginning of fetal myogenesis (E14.5). Interestingly, we detected miR-106b-peak expression at E10.5 and E12.5. Moreover, previous work from our group have suggested that *Pitx2* and miR-106b expression in myogenic progenitors may be defining different subpopulations of SC precursors (Ramírez de Acuña et al., 2022). Accordingly, in the limb buds of the mouse embryo at E10.5 two different populations of PITX2^{pos} and miR-106b^{pos} cells have been previously identified (Ramírez de Acuña et al., 2022). In order to define the myogenic cell population that express miR-106b during embryonic and fetal myogenesis, we have performed miR-106b FISH analysis in mouse embryo cryosections ranging from E9.5 to E14.5 at the level of limb buds.

At E9.5, miR-106b-expressing cells were detected in somite regions, specifically in the DMT, and sparsely in the forelimb buds (figure 22A-A'). At this stage, cells delaminating from the DMT are forming the MT and some myogenic precursors are migrating from the hypaxial lips of the DMT to reach the limb bud (Besse et al., 2020; Buckingham and Relaix, 2015; Hernández-Torres et al., 2017; Rafipay et al., 2018). Therefore, at E9.5 miR-106b might be playing a role in regulating some myogenic precursor cells.

In this regard, transcription factor *Pax3* plays an essential role during somitogenesis and myogenesis development (Buckingham and Relaix, 2015; Seale et al., 2000). *Pax3* is expressed in the somites to later become confined in the epithelial DMT, where it regulates myogenic specification and migration of the myogenic precursor cells (Bentzinger et al., 2012). *Pax3* expression is also crucial in the precursor cells that arise from the hypaxial lip of the DMT and that will form the myogenic progenitors in the limb buds (Bentzinger et al., 2012). Thus, in order to better characterize miR-106b-expressing cell population, we have carried out miR-106b and PAX3 colocalization combining FISH and immunohistochemistry techniques. In mouse embryos at E9.5 we observed that almost all miR-106b^{pos} cells colocalize with PAX3^{pos} cells in the DMT, particularly in the hypaxial lip (figure 22B-B'). This result demonstrates that miR-106b is early expressed in undifferentiated PAX3^{pos} myogenic precursors cells. Moreover, these findings (figure 22) are consistent with our previous RT-qPCR analysis detecting miR-106b expression in somitic areas and limb buds of mouse embryos at E9.5 (figure 19B).

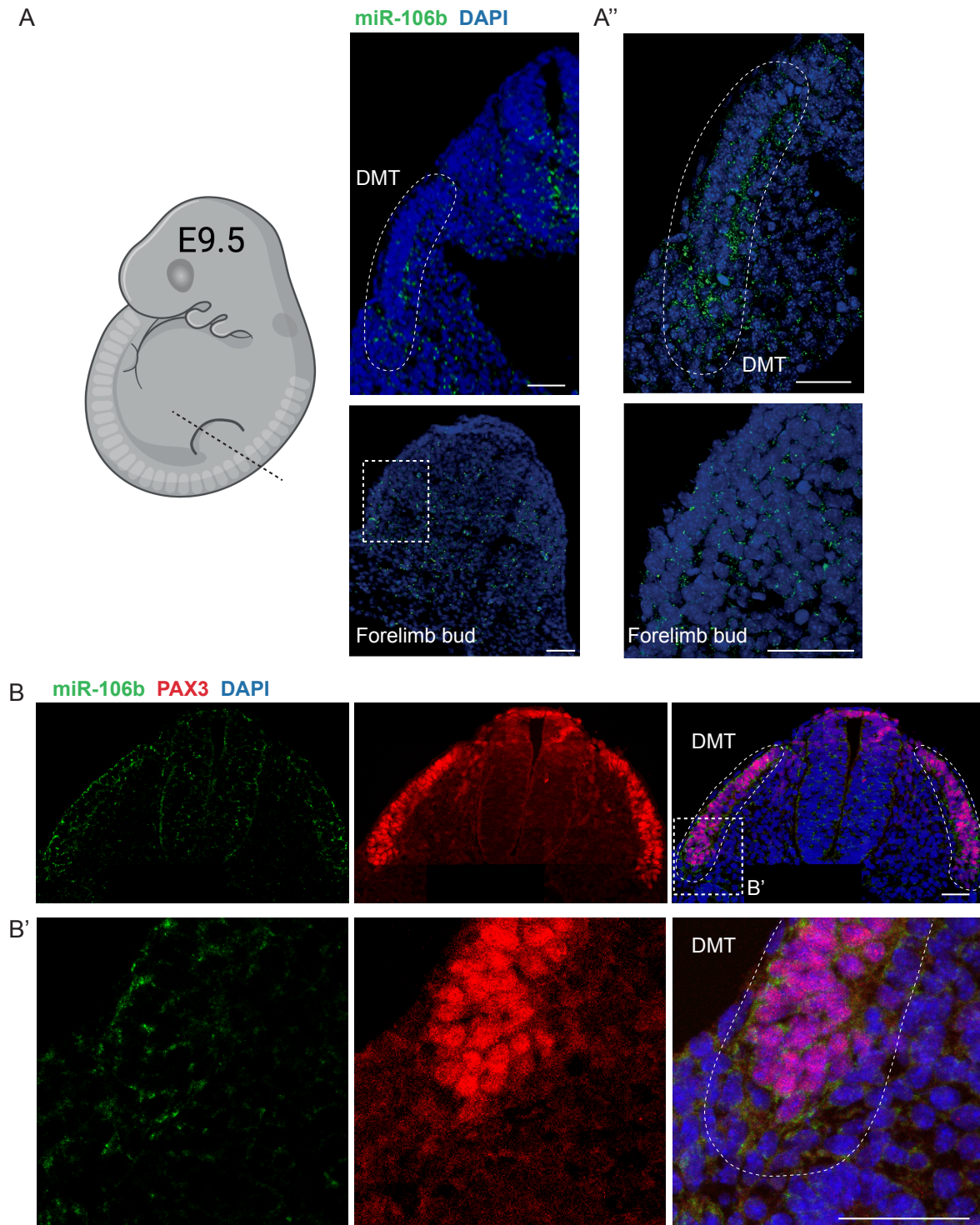


Figure 22. miR-106b expression in CD1 mouse embryos at E9.5 at the forelimb buds' level. (A) Scheme of section analysed in CD1 mouse embryos at E9.5. Created by Biorender.com. Representative images of miR-106b FISH and nuclear staining (DAPI) in 10 µm embryo sections at E9.5. **(A')** Magnifications of the dermomyotome (DMT) and forelimb bud. **(B)** Representative images of miR-106b FISH, PAX3 staining and nuclear staining (DAPI) in 10 µm embryos sections at E9.5. **(B')** Magnification of the hypaxial lip of the DMT. Scale bars: 50 µm.

In accordance with our RT-qPCR analysis, we observed miR-106b-expressing cells in the DMT area (figure 23A and 23A') as well as in the forelimb buds at E10.5 (figure 23A and 23A''). Because *Pax3* gene is a master regulator for myogenic lineage specification at early stages of myogenesis (Buckingham and Relaix, 2015), and with the aim to further identify this miR-106b^{pos} population in the context of myogenic lineages at E10.5, we colocalized it with the transcription factor PAX3. At E10.5 we found that some of the PAX3^{pos} cells in the DMT and some PAX3^{pos} migrating progenitors in the ventral and dorsal muscles masses at the forelimb buds were also miR-106b^{pos} (figure 23B-23B'''). These results showed that at E10.5 miR-106b expression is maintained only in some PAX3^{pos} cells in the DMT and in migrating myogenic progenitor cells in the forelimb bud area. Since we have previously defined that miR-106b inhibits proliferation and blocks myogenic progression in myogenic cells (Lozano-Velasco et al., 2015; Rodríguez-Outeiriño et al., 2022), we speculate that miR-106b could be playing a role in maintaining a subset of PAX3 precursor cell population as undifferentiated and quiescent cells during embryonic myogenesis.

It is interesting to note that our RT-qPCR analysis showed that miR-106b expression levels were higher at E10.5 respect to E9.5 (figure 19); however, we observe that the majority of PAX3^{pos} cell in the DMT are miR-106b^{pos} at E9.5 (figure 22) while at E10.5 only some PAX3^{pos} cell in the DMT co-express miR-106b (figure 23B). This apparent discrepancy could be artefactual due to, at E10.5, the total amount of myogenic cells is increased respect to E9.5 (Cinnamon et al., 1999) and it would be also occurring for the total miR-106b-expressing cells.

We further address miR-106b^{pos} cell distribution at the last stages of the embryonic myogenesis (E12.5) (figure 24A). We observed that miR-106b was expressed in cells that form different premuscle masses in the trunk (figure 24A-24A''), as well as in the dorsal and ventral premuscle masses in the limbs (figure 24A-24A''). Our results demonstrate that at E12.5 miR-106b expression remains located in cell population of premuscle masses.

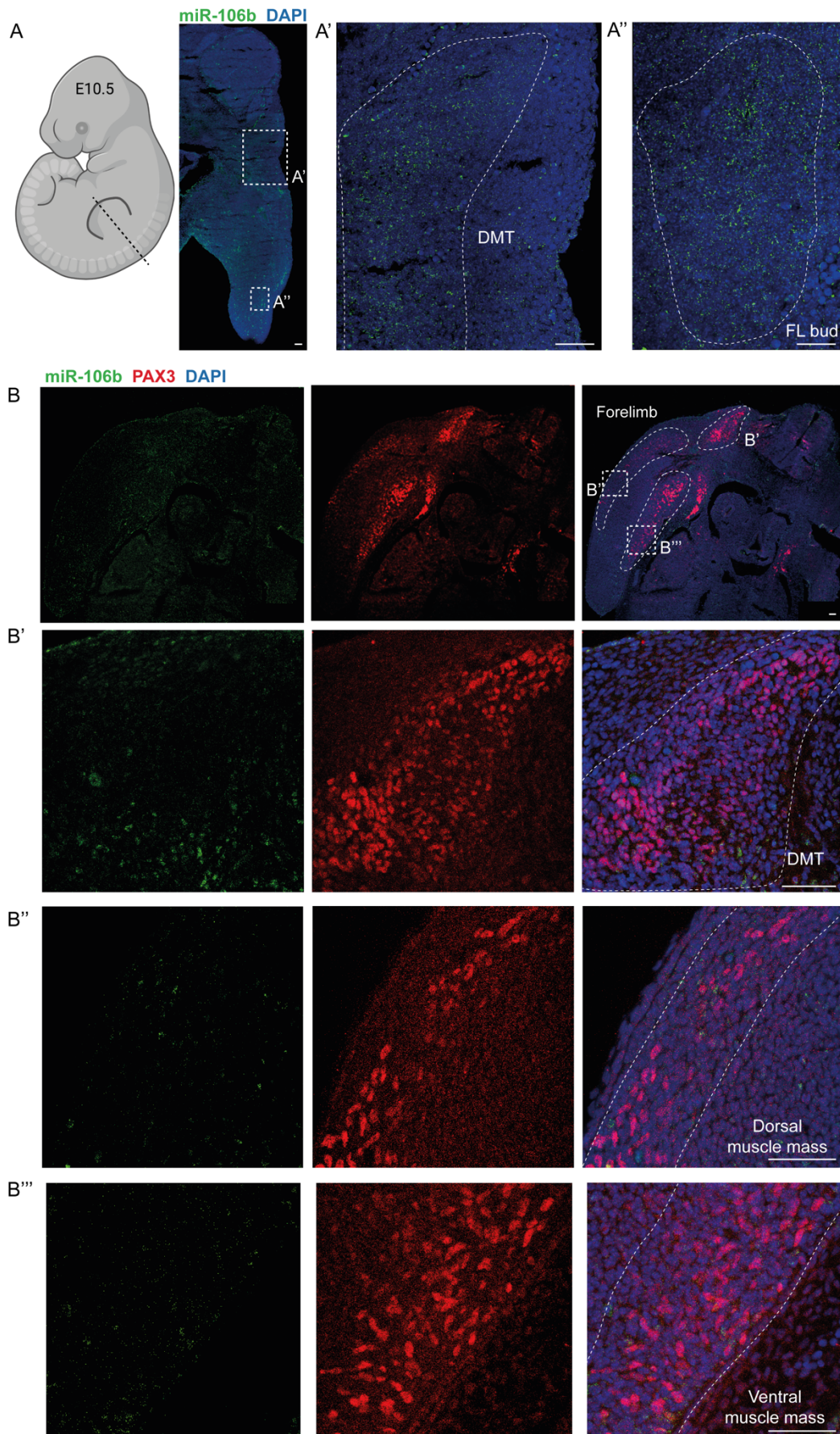


Figure 23. miR-106b expression in CD1 mouse embryos at E10.5 at the forelimb buds' level. (A) Scheme of section analysed in CD1 mouse embryos at E10.5. Created by Biorender.com. Representative images of miR-106b FISH and nuclear staining (DAPI) in 10 µm embryo sections at E10.5 and its magnifications for the dermomyotome (DMT) (A') and forelimb (FL) bud (A''). (B) Representative images of miR-106b FISH, PAX3 staining and nuclear staining (DAPI) in 10 µm embryo sections at E10.5 and its magnifications for the DMT (B') and migrating myogenic progenitors in the dorsal (B'') and ventral (B''') muscle masses at the forelimb bud. Scale bars: 50 µm.

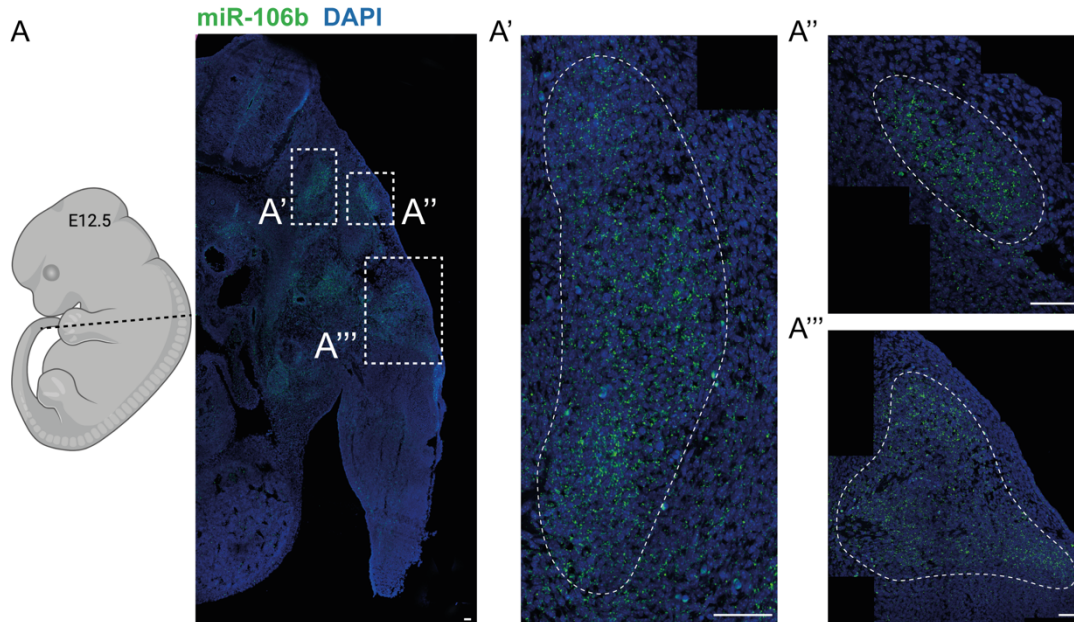


Figure 24. miR-106b expression in CD1 mouse embryos at E12.5 at the forelimb buds' level. (A) Scheme of section analysed in CD1 mouse embryos at E12.5. Created by Biorender.com. Representative images of miR-106b FISH and nuclear staining (DAPI) in 10 µm embryo sections at E12.5 and its magnifications for the premyotome masses in the trunk (A' - A'') and premyotome masses in the dorsal in the forelimb (A'''). Scale bars: 50 µm.

We have described that miR-106b expression levels significantly decrease at E14.5 in both limb buds as compared to E12.5 (figure 20). To determine the miR-106b^{pos} cells population during fetal myogenesis, we next analysed miR-106b-expressing cells in the hindlimbs of mouse embryos at E14.5. At this stage, undifferentiated muscle precursor cells downregulate *Pax3* and start to express *Pax7* (Relaix et al., 2004). Thus, to elucidate miR-106b expression in myogenic precursors at this stage, miR-106b and PAX7 co-staining was performed by FISH and immunohistochemistry. Interestingly, we noticed a clear miR-106b staining in a subset of PAX7-expressing cells in different muscle mass of the hindlimbs at E14.5 (figure 25A-A''). These results are congruent with our RT-qPCR analysis showing a significant decrease in miR-106b expression levels at E14.5 (figure 20), since, at this stage miR-106b expression seems to become restricted to a small population of Pax7^{pos} cells (figure 25A-A''). At E14.5 the majority of PAX7^{pos} muscle progenitor cells are in a proliferate status that contribute to generate new myogenic cells during secondary myogenesis. However, a few non-proliferating PAX7^{pos} will remain undifferentiated during the fetal period and probably they finally set between the basal lamina and sarcolemma

of the myofibers to give rise the juvenile SCs found at birth (Sambasivan and Tajbakhsh, 2007). These results, together with our previous findings, could be indicating that, at E14.5, miR-106b could be playing a function specifying a subpopulation of Pax7^{pos} myogenic precursors that would give rise to the miR-106b^{pos}/Pax7^{pos}/Myf5^{neg} SC population previously identified by us in the adult skeletal muscle ((Rodríguez-Outeiriño et al., 2022), figure 1 and S1).

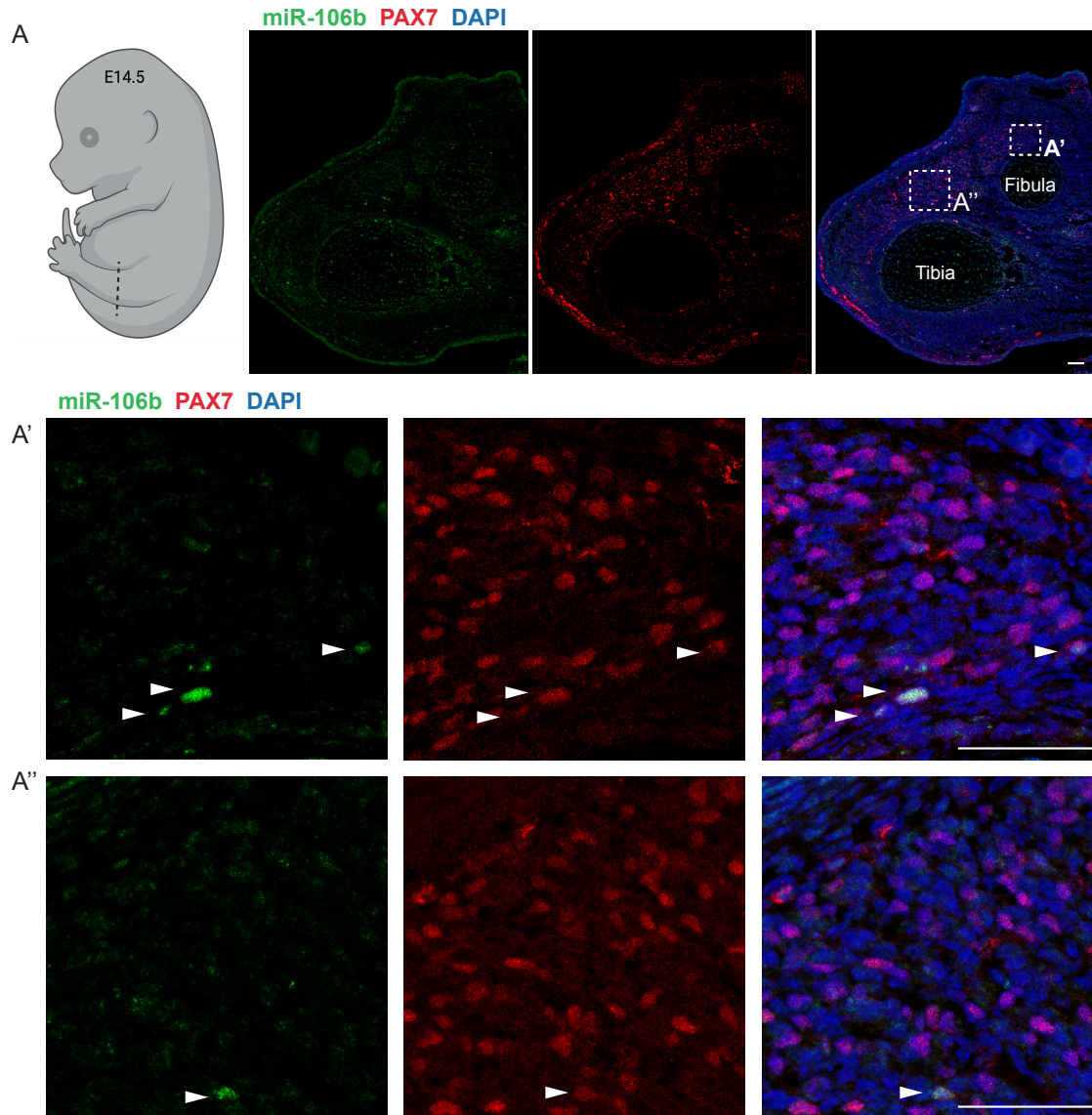


Figure 25. miR-106b expression in hindlimbs of CD1 mouse embryos at E14.5. (A) Scheme of section analysed in CD1 mouse embryos at E14.5. Created by Biorender.com. Representative images of miR-106b FISH, PAX7 staining and nuclear staining (DAPI) in 10 μm hindlimb sections at E14.5 and its magnifications for the extensor *digitorum longus* muscle (A') and *peroneus digiti quinti* muscle (A''). Arrows indicate miR-106b^{pos}/PAX7^{pos} cells. Scale bars: 50 μm.

CHAPTER V: DISCUSSION

1 - miR-106b is an important modulator of SC quiescence and a target to promote muscle regeneration potential and restore SC functions in DMD

SCs are essential for proper muscle regeneration; hence a better understanding of its quiescence maintenance and activation is crucial for a correct approach in regenerative medicine. Numerous research have highlighted that SC behaviour during muscle regeneration is broadly heterogeneous, with a minor subpopulation that tend to maintain the stem cell pool and major subpopulation that contribute to myogenic differentiation and muscle restoration (Cornelison, 2018; Rodríguez-Outeiriño et al., 2021; Tierney and Sacco, 2016). The preservation of this functional SC heterogeneity is required for a proper muscle homeostasis (Sutcu and Ricchetti, 2018). Some studies have correlated differential MYF5 expression with adult SC behaviour. In this sense, it has been described that when transplanted, a minor subpopulation of SCs (~10%) that have never expressed MYF5 ($PAX7^{pos}/MYF5^{neg}$) can give rise to $MYF5^{neg}$ SCs as well as committed $MYF5^{pos}$ SCs, while the majority subpopulation of SCs that have already expressed *Myf5* presents a committed fate, giving rise to differentiated myoblast with a lower engraft capacities (Kuang et al., 2007). Similarly, Beauchamp *et al.* have observed that $CD34^{neg}/Myf5^{neg}$ SC population contributes better to the committed-lineage population than the $CD34^{pos}/Myf5^{pos}$ SC population, which tends to myogenic commitment (Beauchamp et al., 2000). Nevertheless, the molecular mechanism that control these different SC subpopulations in adult skeletal muscles, and useful markers, are still unknown.

Adult SC heterogeneity can be regulated by miRs, such as miR-431, through *Pax7* targeting (Wu et al., 2015), or miR-31, which it has been described to modulate (mRNA) *Myf5* levels in SCs during activation (Crist et al., 2009). In line with this observation, our group has previously shown that Pitx2-miR-106b pathway regulates SC proliferation and myogenic commitment by targeting (mRNA) *ccnd1*, *ccnd2* and *Myf5* respectively (Lozano-Velasco et al., 2015, 2011). In this doctoral Thesis we took a closer look to the role of miR-106b role in SC quiescence-activation. We have observed that miR-106b is only expressed in a minor subpopulation of $Myf5^{neg}$ SCs and that miR-106b inhibition enhances SC asymmetric division, increasing the number of committed cell daughters. Additionally, we have shown that miR-106b downregulation is needed for SC activation, and during muscle regeneration its downregulation is concomitantly with the downregulation of quiescent marker *CD34* and the upregulation of the *MRFs*. Altogether, describe for the first time that miR-106b maintains the quiescent status in a small subpopulation of $Myf5^{neg}$ SCs and that miR-106b reduction is essential for a correct SC activation and

contribution to muscle repair (figure 26A) (Rodríguez-Outeiriño et al., 2022). We have also reported that in SCs miR-106b is the main miR expressed in its cluster family (miR-17). We explored the capacity of miR-106b targeting (mRNA) *Myf5* compared to other miRs, like miR-31, and we observed that miR-106b presents an enhanced ability for downregulate (mRNA) *Myf5* (Rodríguez-Outeiriño et al., 2022). Therefore, we purpose that miR-106b acts reinforcing quiescence status in a SC subpopulation by inhibiting *Ccnd1*, *Ccnd2* and *Myf5*, and that miR-106b inhibition can enhance SC myogenic commitment in mice and humans. Nevertheless, since miRs can have different potential mRNA targets, we cannot exclude that others miR-106b targets are contributing to the described effects. Additionally, since we used FISH amplification system for miR-106b detection, we cannot discern if the effects mediated by the miR is in the nucleus and/or the cytoplasm like some studies have reported for other cell types (Liu et al., 2018).

Talking into consideration that muscle wasting diseases are associated with disrupted SC behaviour that contributes and exacerbates the disease (Sakuma et al., 2014), new therapeutic approaches are focus on restore intrinsic SC dysfunctions to preserve and ameliorate muscle mass loss and function (Filippelli and Chang, 2022; McKenna and Fry, 2017; Sousa-Victor et al., 2022). In this concern, enhancing intrinsic SC potential would improve its proliferation and differentiation capabilities which will enhance skeletal muscle regeneration. In this doctoral Thesis we demonstrated that in wild type mice, *in vivo* antimiR-106b injection in TA muscles enhances the number of committed SCs (KI67^{pos}/MYOD1^{pos}), boosting muscle regeneration. In skeletal muscles there is a minor subpopulation of MYOD1-expressing SCs that present a more commitment to myogenesis (Yin et al., 2013). We noticed that after 30 days of antimiR-treatment in injured muscles there is no variation in the number of this sublaminal MYOD1^{pos} population as well as a small non-significative increase is observed in the number of sublaminal PAX7^{pos} SCs. After a second round of injury, muscle regeneration is also enhanced in the TAs treated with a single antimiR-106b injection, probably due to enhanced SCs in G_{Alert} state, since we demonstrate that miR-106b inhibition increases the number of cells in this pre-activated state ((Rodríguez-Outeiriño et al., 2022), figure S3). As miR-106b is expressed in quiescent MYF5^{neg} SCs and its inhibition increases SC asymmetric divisions ((Rodríguez-Outeiriño et al., 2022), figure 1), we presumed that antimiR-106b enhances the activation of the stemness SC population. In this sense, more stem-cell like SC subpopulations are consider the true stem cells since they are able to maintain stem cell pool as well as they contribute to muscle regeneration (Rodríguez-Outeiriño et al., 2021). Our results shown that a second injury with CTX after a single injection of antimiR-106b do not significantly alter the number of PAX7^{pos} SCs in the regenerated muscles ((Rodríguez-Outeiriño et al., 2022), figure 3E), meaning that SC pool is not compromised

when we activate it with miR-106b inhibition. Accordingly, we do not expect that SC pool will be reduced if anti-miR-106b treatment is delivered in the long-term, as the stemness SC population that is activated can re-enter in a sublamellar localization as quiescence population. We also described that miR-106 inhibition in hMuSCs can modulate its behaviour boosting its myogenic potential, which emphasizes the applicability of anti-miR to develop new treatments in muscle wasting diseases.

It is well studied that some muscle disorders such as DMD are correlated to intrinsic SC dysfunctions, reduced proliferative and myogenic commitment capabilities, that diminish muscle regeneration potential in dystrophic muscles (Chang et al., 2016; Dumont et al., 2015b). In this context, some miRs, such as miR-486, miR-431 or miR-127, has been proposed as interesting SC modulators in DMD disease for increasing their activation and ameliorating some features of dystrophic skeletal muscle regeneration (Alexander et al., 2014; Wu et al., 2015; Zhai et al., 2017). In connection, our results showed that miR-106b expression levels in dystrophic SCs (mice and humans) are highly increased. Since prior work from our group has demonstrated that miR-106b expression is downregulated by PITX2, and that *Pitx2* expression is decreased in mdx mouse model, elevated miR levels could be due to *Pitx2* dysregulation (Lozano-Velasco et al., 2015; Vallejo et al., 2018). We have explored the capacity of miR-106b inhibition to enhance SC myogenic commitment and muscle restoration capacities in DMD context by using mouse models displaying different stages of DMD-disease progression (mdx and DBA/mdx). Our results demonstrate that *in vivo* miR-106b inhibition in TA muscle of two dystrophic mouse models enhances the number of myogenic precursors and ameliorated muscle regeneration after an injury proposing anti-miR-106b treatment as a novel therapeutic target in dystrophic muscles (figure 26B).

As miRs can have different targets and in order to discard detrimental changes as part of miR therapeutic approach, we investigated by RNAseq analysis the pathways involved in miR-106b inhibition treatment in mdx mice. We found that the transcriptional program of muscle differentiation is enhanced, probably due to increased stemness SC activation. We also noted that the inflammatory response, which is normally upregulated in dystrophic patients due to the activation of the Nuclear Factor-kappa beta (NF- κ B) pathway is decreased in anti-miR treated muscles (Tulangekar and Sztal, 2021). This inflammatory reduction could be linked to anti-inflammatory response of miR-106b inhibition observed in other works. For instance, it has been described that miR-106b inhibition in macrophages or in microglia can block the Akt/NF- κ B signalling and reduces the pro-inflammatory cytokine levels in serum (Liu et al., 2021; Tao et al.,

2017; Yu et al., 2020). Since in DMD pathology there is a continuous activation of macrophages and unbalanced situation that aggravate the chronic inflammation (Tulangekar and Sztal, 2021), previously described anti-inflammatory response of anti-miR-106b might be ameliorating this chronic inflammation in dystrophic muscles.

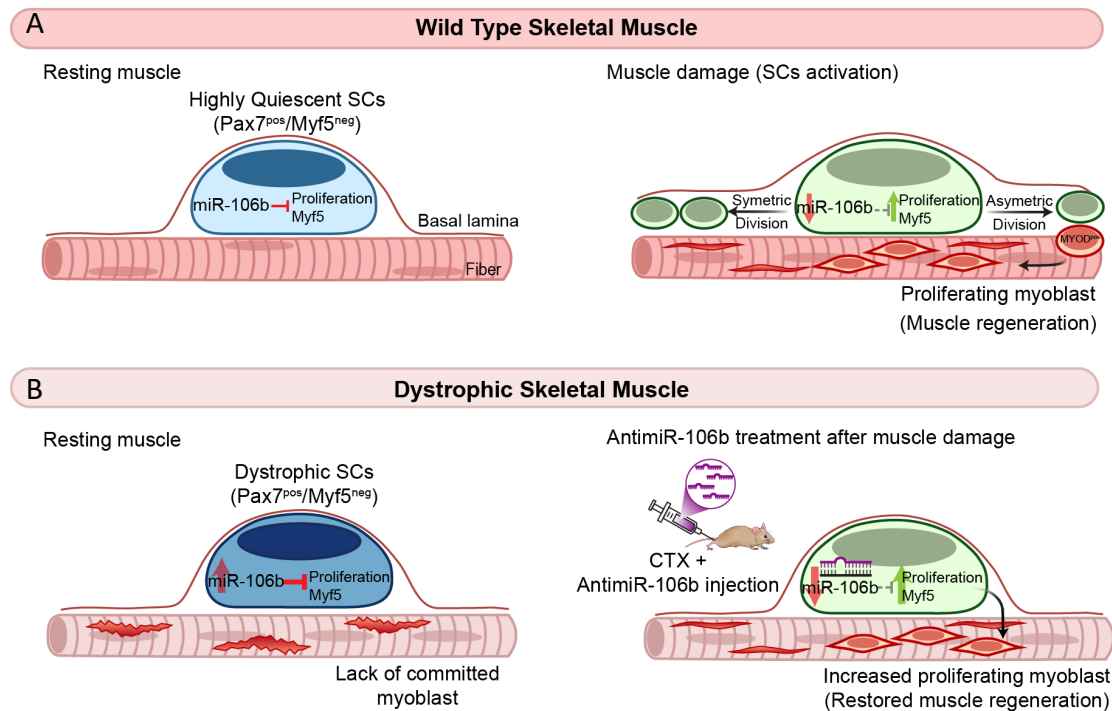


Figure 26. Representation of miR-106b role in SCs and anti-miR-106b treatment in dystrophic muscles. (A) miR-106b reinforces quiescence in SCs, and its down-regulation is required for proper muscle repair. (B) miR-106b is increased in dystrophic murine and human muscle stem cells. *In vivo* miR inhibition in injured dystrophic mice enhances SC regenerative potential. SCs: satellite cells; CTX: cardiotoxin. Taking from Rodríguez-Outeiriño, *et al.* 2022.

Altogether led us conclude that miR-106b inhibition in a dystrophy context potentiates muscle regeneration, improves the DMD phenotype and ameliorates muscle functionality. More importantly, we show that *in vitro* miR-106b inhibition benefits can be extrapolated to restore intrinsic defects of hMuSCs behaviour in DMD and BMD conditions.

In conclusion, our results demonstrated that miR-106b maintains SC quiescence status and its downregulation activates SCs for proper muscle repair. We evidenced that miR-106b is dysregulated in dystrophic SCs from mice and human samples. We identify anti-miR-106b treatment as a novel therapeutic strategy to restore SC dysfunction in DMD. Additionally, anti-miR-106b treatment can, at least temporarily, enhance muscle restoration in dystrophic condition. Even though dystrophic SCs will still lack of DYSTROHPIN, increased myofiber regeneration can reduce the replacement with fibrotic tissue, which will imply a huge progress

for DMD patients. Furthermore, it would be interesting to explore anti-miR-106b treatment as a combined therapy with gene correction strategies in DMD patients to generate new myofibers that do not lack on DYSTROPHIN protein, which will reduce sequential cycles of muscle degradation-regeneration and chronic inflammation (Blat and Blat, 2015; Gao et al., 2015).

2 - miR-106b modulates the myogenic potential of SCs in sarcopenia

SC dysfunction is a common characteristic of several muscle wasting diseases including sarcopenia. Aged SCs present a senescent phenotype characterized by G₀-irreversible quiescent state, failing to re-enter in the cell cycle, that leads to a decrease in the skeletal muscle regeneration potential in sarcopenia (Filippelli and Chang, 2022). SC senescent phenotype can be prevented if SCs maintain their capability to activate and differentiate (Chen et al., 2020; Filippelli and Chang, 2022; Sousa-Victor et al., 2022). As we previously shown that miR-106b has a role in SC activation during muscle regeneration, and that antimiR-treatment enhances SC proliferation and myogenic commitment without altering the SC pool, we explored if antimiR-106b modulation could enhance aged SC potential for muscle repair in sarcopenia.

In this doctoral Thesis we described that miR-106b is increased in TA muscles from aged mice (>25-months-old) (figure 18A). Since we previously observed that miR-106b expression in TA muscle is only present in a highly quiescent SC subpopulation (Rodríguez-Outeiriño et al., 2022), and the global SC pool is depleted in aged muscle (Arpke et al., 2021), the increased levels of miR-106b found in the aged TA muscles could be suggesting that the ratio of miR-106b^{pos} SC population is maintained during aging respect to the total SC pool. Future studies of miR-106b^{pos} cell quantification in aged muscles will help us to clarify miR-106b^{pos} cell population progression during ageing. In addition, as a quiescence irreversible status leads to a pre-senescent phenotype in sarcopenic SCs (Feige et al., 2018; Rubinsztein et al., 2011), and miR-106b acts reinforcing SC quiescence, the increased levels of the miR in aged muscles may be contributing to maintain this sarcopenic phenotype. Therefore, and similarly at what we observed in DMD condition, we wonder if miR-106b inhibition could enhance sarcopenic SC potential. Our results showed that antimiR-106 treatment in aged SCs enhances its proliferation and myogenic differentiation capabilities, highlighting its potential in these diseases context. Since sarcopenic muscle regeneration potential is compromised due to the absence of functional SCs, and that miR-106b inhibition ameliorates aged SC regenerative potential, further analyses such as *in vivo* miR inhibition in elderly mice will shed light on the potential of antimiR-106b treatment as a new approach that boosts pre-senescent aged SCs to maintain the muscle mass and to reduce muscle atrophy.

It is interesting to remark that in aged SCs, long periods of quiescence and disrupted autophagy result in proteotoxicity stress: increased levels of ROS and DNA damage that contribute to SC senescence (Chen et al., 2020; Muñoz-Cánoves et al., 2020; van Velthoven and Rando, 2019).

Some studies have demonstrated that miRs modulation can prevent proteotoxicity in aged SCs, which ultimately, improves muscle regeneration potential in sarcopenia (Goljanek-Whysall et al., 2020; Kondo et al., 2016). In this regard, it has been previously documented that *in vitro* miR-106b inhibition in myoblasts enhances autophagy through targeting *Unc-51 like autophagy activating kinase 1 (ULK1)* restoring mitochondrial damage repair response, and it also attenuates ROS production by targeting the mitochondrial transmembrane GTPase *Mitofusin 2* (Wu et al., 2012; Zhang et al., 2015, 2013). Therefore, in future studies it would be of interest to explore the possibility that miR-106b inhibition is also attenuating ROS production and mitochondrial damage in aged SCs. Thus, antimiR-106b treatment would be enhancing proliferation and myogenic commitment but also protecting SCs from irreversible senescence in aged muscles (figure 27).

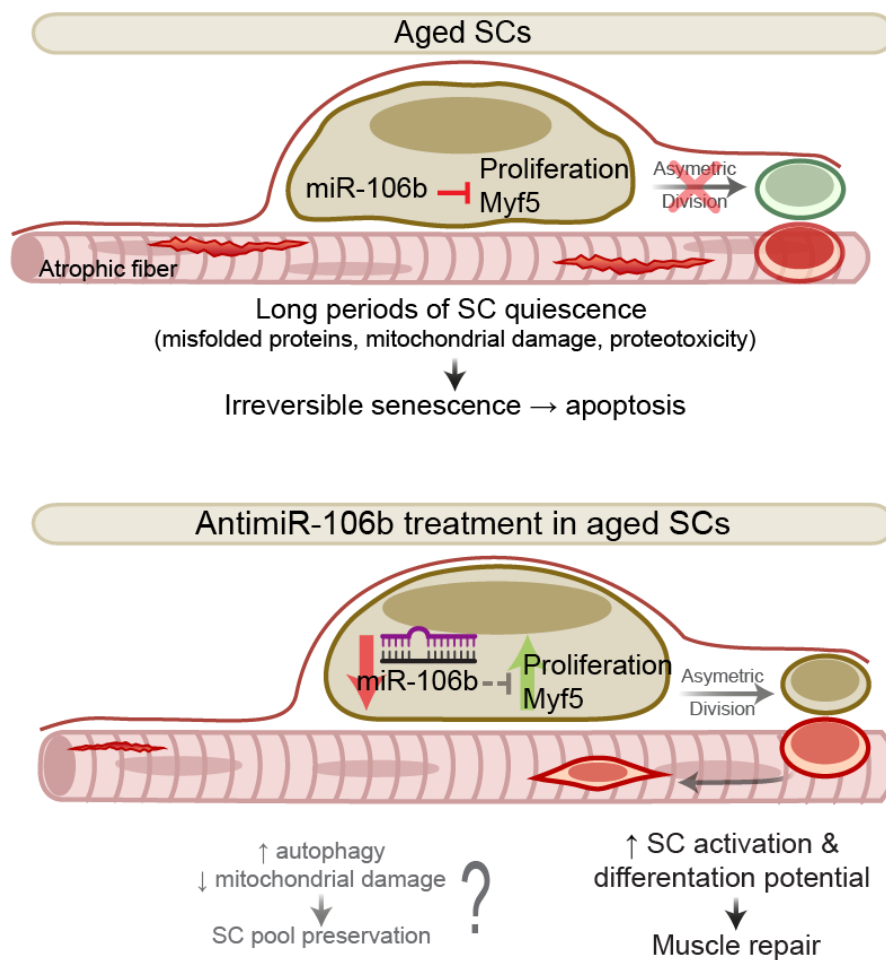


Figure 27. Model for antimiR-106b regulation in sarcopenia. Aged SCs present lack of asymmetric division. Additionally long periods of quiescence increase their proteotoxicity due to the accumulation of misfolded proteins and enhanced mitochondrial damage, leading to an irreversible quiescence and, finally, apoptosis. We observed that antimiR-106b treatment in aged SCs enhances their proliferation and differentiation potential. Additionally, other studies have shown that antimiR-106b treatment in myoblast increases autophagy, attenuates reactive oxygen species production and enhances mitochondrial damage response, thus, we wonder if antimiR-106b could have the same therapeutic potential in aged SCs, and therefore, preserve sarcopenic SC pool. SC: satellite cell.

In summary, our preliminary results in sarcopenia demonstrates that miR-106b is dysregulated in skeletal muscles from aged mice and that *in vitro* miR-106b inhibition in aged SCs enhances its proliferation and myogenic differentiation potential. Eventually, it would be interesting to explore antimiR-106b treatment in SCs as an enhancer of damage prevention (autophagy or DNA damage response) in sarcopenic context. Finally, we propose that miR-106b inhibition could be a novel therapeutic strategy in sarcopenic muscles, as we already showed in dystrophic context, and on the whole, it suggests that antimiR-106b treatment as a potential approach that can be extrapolated to other wasting muscular disorders.

3 - miR-106b is defining a subpopulation of myogenic progenitor cells during embryonic development

Different studies have highlighted that during muscle development there is a high heterogeneity among the muscle precursor populations at embryonic, fetal, neonatal and juvenile stages (Biressi et al., 2007a; Rodríguez-Outeiriño et al., 2021). However, it still remains unknown the molecular mechanisms underlying cell heterogeneity during development or how the origin of adult SC populations is related to development heterogeneity. Nor is it known how some muscle precursor cells remain in an undifferentiated status throughout embryonic, fetal and neonatal myogenesis, to finally settle as quiescent SCs in adult skeletal muscle. In this concern, previous results from our group showed that during embryonic myogenesis the loss of *Pitx2* in committed myogenic precursor cells alters the different adult SC population by increasing the number of miR-106b^{pos} SCs and reducing Myf5^{pos} SC population (Ramírez de Acuña et al., 2022).

Additionally, we have also described that PITX2 represses miR-106b expression (Lozano-Velasco et al., 2015) and we have identified two clearly different PITX2^{pos} and miR-106b^{pos} cell subpopulations during embryonic myogenesis (Ramírez de Acuña et al., 2022), indicating that miR-106b may be defining a particular subpopulation of SC precursors. To get insight into this issue, we explored miR-106b expression during embryonic and the beginning of fetal myogenesis in the mouse embryos. In this doctoral Thesis we revealed that miR-106b is expressed in some multipotent PAX3^{pos} myogenic progenitors during embryonic myogenesis. Additionally, we observed that at the beginning of fetal myogenesis, a few PAX7^{pos} muscle progenitors in the limbs highly express the miR-106b. These data suggest that miR-106b could have an important role during myogenesis regulating specific myogenic precursors population.

Our analysis shown that during embryonic myogenesis miR-106b is highly expressed between E10.5 to E12.5 stage in the embryonic area containing somitic-derived structures and limb buds. During these stages the DMT gives rise to the PAX3/PAX7-expressing myogenic precursor cells from the central part of the DMT and multipotent PAX3-expressing myogenic precursor cells in the hypaxial lip of the DMT that will migrate to the limb buds (Hernández-Torres et al., 2017). Several miRs control the process of myogenic precursor determination, activation and differentiation, such as myomiRs miR-1 and miR-133a, strong regulators of myoblast proliferation-differentiation during embryonic muscle development (Luo et al., 2021). Meanwhile miR-133 increases myoblast proliferation and inhibit its differentiation during embryonic development, miR-1 promotes its myogenesis differentiation (Chen et al., 2006; Feng et al., 2013; Goljanek-Whysall et al., 2012; Luo et al., 2021; Mok et al., 2018; Sweetman et al.,

2008). Additionally, Mok *et al.* have shown that in chicken embryos miR-133 is required for proper myogenic stabilization and MT growth (Mok *et al.*, 2018). Particularly, miR-133a isoform is necessary for the maintenance of muscle and myofiber identity in the adult stage (Liu *et al.*, 2011). When we compared miR-106b expression levels respect to miR-1 and miR-133a, we noted that at E10.5 miR-106b is expressed at higher rates in all regions analysed, but, at E12.5 stage miR-106b expression levels are only significantly enhanced in the limb buds. Our results suggest that miR-106b could be playing a preponderant role in early myogenesis and in the muscle formation at the limb levels.

On the other hand, our FISH analyses showed that, at E9.5, miR-106b is firstly expressed in the majority of PAX3^{pos} cells in the DMT, notably in the hypaxial lip. Pax3-expressing cells in the DMT are essential for myogenic specification and muscle progenitor migration to more distant regions (Buckingham and Relaix, 2015; Kassam-Duchossoy *et al.*, 2005). At E9.5 myogenic progenitors of the central part of the DMT start to delaminate to form the MT, and from the hypaxial lip of the DMT the precursor of muscle progenitors migrate to the limb buds (Rafipay *et al.*, 2018). Since miR-106b inhibits proliferation and myogenic commitment (Lozano-Velasco *et al.*, 2015), its expression in DMT at E9.5 could be regulating the maintenance and specification of myogenic precursors in an undifferentiated and non-proliferative status.

At E10.5 stage in the mouse embryo, all migrating myogenic progenitor cells have entered into the limb buds (Buckingham *et al.*, 2003). In our analysis we observed that at E10.5 there is an increase in miR-106b expression in the embryonic area containing somitic structures (figure 19), while only some PAX3-expressing cells in the DMT remain miR-106b^{pos} (figure 23). miR-106b increased expression in the PAX3-expressing cells in the DMT at E10.5 could be linked to the requirement of some myogenic precursor cells to remain undifferentiated and in a quiescence status during embryonic myogenesis process, but also later on during fetal and neonatal stages.

Similarly, at E10.5 stage we registered a significative upregulation of miR-106b expression levels in the limb buds and just a few PAX3^{pos} migrating cells that are co-expressing miR-106b. This data suggests that probably some PAX3^{pos}/miR-106b^{pos} cells from the hypaxial lip of the DMT (E9.5) have already migrated to the limb buds at E10.5 and they downregulated miR-106b expression. We have previously reported that Pitx2-miR-106b signalling could be defining specific subpopulations of muscle precursor in the limb buds at E10.5 (Lozano-Velasco *et al.*, 2015, 2011; Ramírez de Acuña *et al.*, 2022). Since miR-106b inhibits proliferation and differentiation, miR-106b downregulation in the limb buds at E10.5 would be necessary to allow myogenic cells proliferation and differentiation, while some PAX3^{pos}/miR-106b^{pos} myogenic cells

remain quiescent and undifferentiated (figure 23). These PAX3^{pos}/miR-106b^{pos} myogenic cells will persist undifferentiated to preserve myogenic precursor pool in later waves of myogenesis. This hypothesis would be in agreement with previous studies that shown that at E11 stage PAX3^{pos} cells in the limb buds also express PITX2, while not all PITX2^{pos} cells express PAX3 (L'Honoré et al., 2010, 2007), pointing out that some myogenic precursors cells enter the differentiation program through PITX2 factor while others remain undifferentiated (Bajard et al., 2006; L'Honoré et al., 2007). With further development (E12.5) myocytes fuse to form myofibers and the premuscle myofibers begin to emerge (Besse et al., 2020; Rafipay et al., 2018). We have observed a downregulation in miR-106b expression and diffuse miR-106b-expressing cells in the premuscle mass at E12.5 stage. At this period, miR-106b could be regulating myogenic progenitor quiescence for subsequently fetal myogenesis.

When fetal myogenesis begin (E14.5), individual muscles masses can be recognizable (Besse et al., 2020; Rafipay et al., 2018). At this stage, PAX7-expressing myogenic progenitors proliferate and differentiate to contribute to muscle development. Later on, some of these PAX7-expressing cells exit the cell-cycle and return to quiescence to adopt a SC fate (Esteves de Lima and Relaix, 2021). Interestingly, at E14.5 stage we detected that a few PAX7^{pos} cells in the hindlimbs express high levels of miR-106b, suggesting that this subpopulation could remain quiescent and undifferentiated, similar to what observed in adult SCs (Rodríguez-Outeiriño et al., 2021). Future analysis of this subpopulation will clarify if PAX7^{pos}/miR-106b^{pos} myogenic progenitor population remain undifferentiated and in a non-proliferative status until birth, when juvenile SC subpopulations appear.

Although it would be interesting to study the role of miR-106b during embryonic development by *in vivo* loss of function approach (for instance, generating a miR-106b knockout mice), it entails a problematic challenge due to miR localization. miR-106b is located on the 13th intron of the highly conserved *Minichromosome maintenance complex component 7 (Mcm7)* gene in chromosome 5 in mice, and chromosome 7 in humans (Sagar, 2022; Ventura et al., 2008). MCM7 is an indispensable protein involved in DNA replication initiation during cell activation (Sagar, 2022), therefore miR-106b inactivation would disrupt the function of *Mcm7* gene, with fatal consequences for the embryo development. However, despite of it, other future approaches such as miR-106b^{pos} myogenic precursors tracking studies or *ex vivo* loss of function essays in mouse embryos would clarify the role of this miR in myogenic precursor formation during embryonic, fetal, neonatal development and finally SC specification.

Overall, our preliminary results show that there is a subpopulation of myogenic precursor cells that express miR-106b during embryonic myogenesis. Later on, miR-106b expression becomes restricted to a few Pax7 myogenic progenitors at E14.5 in the muscles mass of the limb buds. Additionally, we demonstrated that miR-106b is only expressed in a small (13%) subpopulation of quiescent SCs in the adult skeletal muscle (Rodríguez-Outeiriño et al., 2022). In this scenario, several studies have shown that miR-106b and its cluster (miR-17) participate in the specification and maintenance of stem/progenitor cells in other cell types, such as: bronchioalveolar stem cells from mouse lung (Carraro et al., 2009; Qian et al., 2008), neuronal stem/progenitor cells (Brett et al., 2011; Mao et al., 2014; Xia et al., 2019), through miR-17/106–p38 axis (Naka-Kaneda et al., 2014), and nephron progenitors (Ho et al., 2011). These observations may be supporting the hypothesis of miR-106b as a novel regulator of stemness preservation in muscle progenitors during embryonic development.

CHAPTER VI: CONCLUSIONS

Conclusion 1: miR-106b is expressed in a subset of MYF5^{neg} SC population reinforcing its quiescence.

Conclusion 2: miR-106b down-regulation is required for effective skeletal muscle regeneration and *in vivo* miR-106b inhibition increases the regenerative potential of SCs.

Conclusion 3: miR-106b expression is increased in mouse dystrophic SCs and *in vivo* anti-miR-106b treatment enhances muscle regeneration in mouse models displaying different stages of DMD-disease progression.

Conclusion 4: miR-106b inhibition potentiates the *in vitro* myogenic differentiation of hMuSCs and enhances its contribution to muscle regeneration after intramuscular transplantation.

Conclusion 5: Dystrophic hMuSCs also display high levels of miR-106b expression and *in vitro* anti-miR-treatment enhances its myogenic potential.

Conclusion 6: SCs from elderly mice present high levels of miR-106b expression and *in vitro* miR-106b inhibition boosts their myogenic potential.

Conclusion 7: miR-106b expression profile during developmental myogenesis reveals that miR-106b is expressed at embryonic and fetal stages in somitic-derived areas and limb buds and comparative analyses with other myomiRs indicates a preponderant role of miR-106b at early stages of myogenesis.

Conclusion 8: A subset of PAX3^{pos} myogenic progenitor cell population expresses miR-106b during embryonic myogenesis, and a subpopulation of PAX7-expressing cells also maintain miR expression within the muscle masses at fetal stages. Further analyses are necessary to better delineate the role of miR-106b in different myogenic cell populations during development.

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