University of São Paulo

Igor Neves Barbosa

Gene editing with Crispr-Cas9 in dystrophic patients bearing an out of phase duplication

Edição de genes com Crispr-Cas9 em pacientes distróficos portadores de duplicação fora de fase

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EXEMPLAR CORRIGIDO

Igor Neves Barbosa

Gene editing with CRISPR-Cas9 in dystrophic patients bearing an out of phase duplication

Edição de genes com Crispr-Cas9 em pacientes distróficos portadores de duplicação fora de fase

Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Biologia Genética, na Área de Ciências Biológicas.

Orientadora: Mayana Zatz

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Mayana Zatz
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Introduction

1. Duchenne Muscular Dystrophy

1.1. The Disease

Duchenne Muscular Dystrophy (DMD) is a recessive X-linked genetic disease caused by the deficiency of the protein Dystrophin, in most cases due to frame-shifting or truncating mutations in the *DMD* gene sequence. It affects 1 in every 3500-5000 male births around the world. Male patients affected by the disease, suffer from progressive muscle loss, with symptoms arising at years 2 or 3 and wheelchair requirement at around age 11 (Figure 1). Most patients need assisted ventilation in the second or third decade. Life expectancy depends on the treatment and medical care, in some countries, with optimal care, patients can survive for more than 40 years of age (DUAN; GOEMANS; TAKEDA; MERCURI *et al.*, 2021; KIENY; CHOLLET; DELALANDE; LE FORT *et al.*, 2013). Death is usually due to the aggravation of cardio-respiratory problems. Females may be affected under exceptional circumstances such as: cases where chromosomal translocations involve the *DMD locus* leading to a preferential inactivation of the non-translocated X chromosome, Turner syndrome or bi-allelic *DMD* mutations (DUAN; GOEMANS; TAKEDA; MERCURI *et al.*, 2021; NOZOE; AKAMINE; MAZZOTTI; POLESEL *et al.*, 2016).

For diagnosis confirmation, DNA from patients is extracted for molecular analysis. Since the *DMD* is one of the largest genes in the human genome, with 2.2 megabases in length and 79 exons, most of the mutations may be detected either by MLPA (Multiplex

Ligation-dependent Probe Amplification), which is the gold standard, or by a custom designed CGH-array (Comparative Genomic Hybridization-array) for DMD, but in some cases other molecular analysis need to be carried out.

Duchenne's Muscular Dystrophy
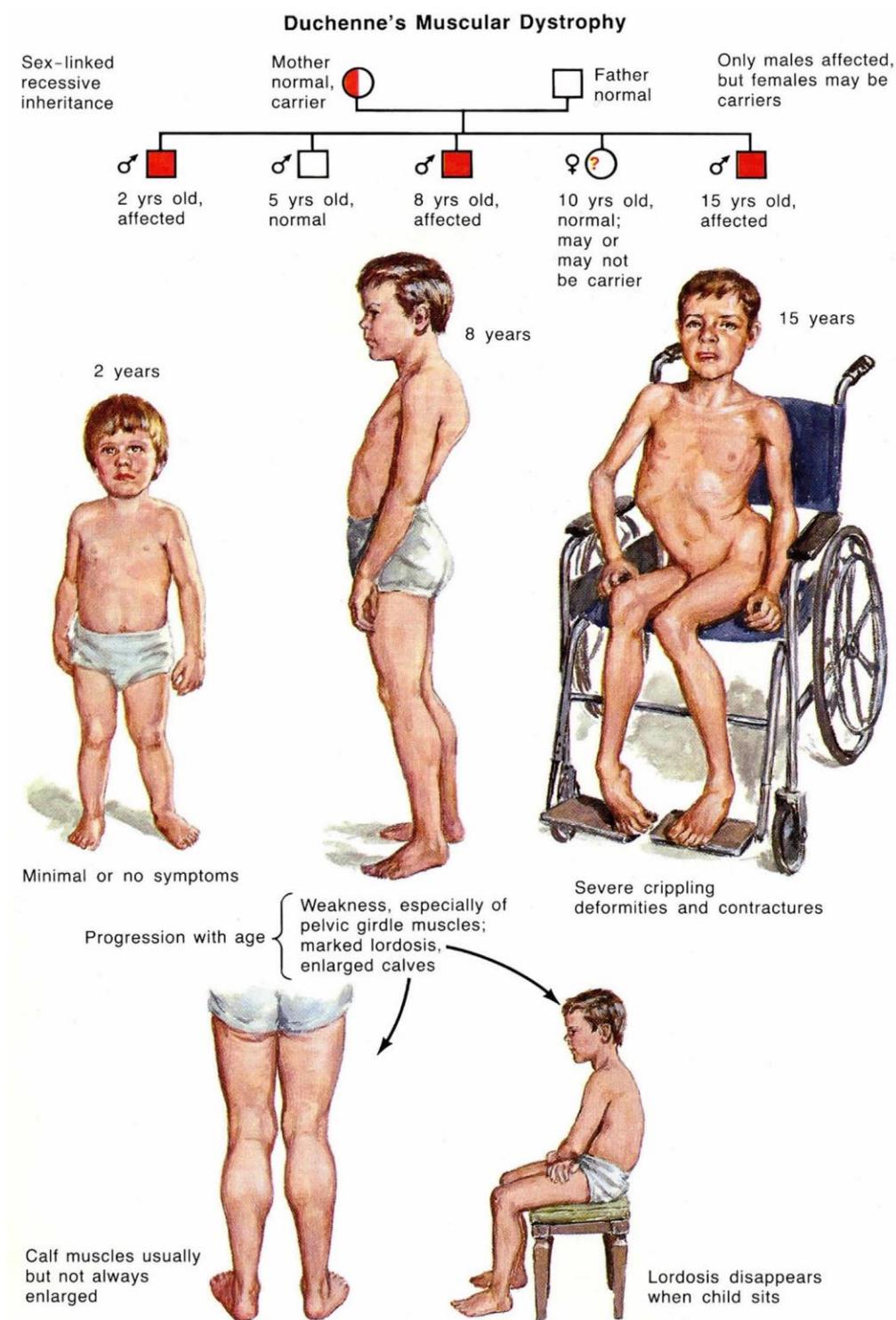
Figure 1: Schematic representation of the X-linked pattern of inheritance of DMD, as well as the physical progression of the disease. (HEALTHJADE)

Among the disease-causing mutations in DMD there are mostly large deletions (44,2%), nonsense mutations (27,4%), large duplications (13,5%), frame-shifting insertions, deletions or both (10,7%), and other changes like splicing changes, missense mutations and other small changes (4,2%) (FLANIGAN; DUNN; VON NIEDERHAUSERN; SOLTANZADEH *et al.*, 2009). Most of these mutations occur at exons 45-55 hotspot (47%) and exons 3-9 hotspot (7%). This mutational spectrum may vary slightly depending on the cohort analyzed and the classification of mutations (DUAN; GOEMANS; TAKEDA; MERCURI *et al.*, 2021). In some cases, mutations in the *DMD* gene can also lead to a milder type of muscular dystrophy, called Becker Muscular Dystrophy (BMD), which has a more varied phenotype and is usually characterized by later onset and loss of ambulation and longer life expectancy.

A third of DMD patients also have some kind of cognitive deficit, beyond IQ scores, there may be an association with attention deficit hyperactivity disorder (ADHD) and autism (DUAN; GOEMANS; TAKEDA; MERCURI *et al.*, 2021; LANGE; GILLHAM; ALKHARJI; EATON *et al.*, 2022). Distal mutations in the *DMD* gene are associated with lower IQ scores, while proximal mutations correlate with cardiac impairment. Furthermore, some mutations can cause an exclusively cardiac disease with no effect on skeletal muscles, called X-linked Dilated Cardiomyopathy, which can also affect DMD patients as the disease progresses (MAGRI; GOVONI; D'ANGELO; DEL BO *et al.*, 2011). *DMD* has eight promoters and several isoforms: the full-length isoforms of dystrophin are present in muscles, brain and Purkinje cells, but some smaller isoforms are present only in

specific tissues and cells, including the brain and astrocytes, or during the embryonic development, which supports the importance of dystrophin for brain function (LANGE; GILLHAM; ALKHARJI; EATON *et al.*, 2022).

The difference between DMD and BMD is often defined by the patient's clinical status throughout childhood to adolescence. DMD patients have a more rapid progression of symptoms and muscle wasting than a BMD. Although it is not always possible to predict whether a mutation will result in a DMD or BMD phenotype, most of the time, the underlying molecular mechanism can be explained by whether the mutation in *DMD* is completely truncating or not, as most of the truncating mutations are diagnosed in DMD, while most non-truncating mutations are found in BMD (in the latter case a partially functional dystrophin is produced) (FLANIGAN; DUNN; VON NIEDERHAUSERN; SOLTANZADEH *et al.*, 2009).

Muscle immunostaining shows, in most DMD/BMD patients, a few "revertant fibers" that stain strongly for dystrophin. These fibers are thought to harbor some sort of molecular mechanism that restores the dystrophin reading frame, such as the skipping of exons adjacent to the mutation (THANH; NGUYEN; HELLIWELL; MORRIS, 1995). In some patients, it is also possible that dystrophin is produced as a subfunctional protein and there is an association between the amount of this dystrophin and the rate of progression of the disease (DUAN; GOEMANS; TAKEDA; MERCURI *et al.*, 2021).

Utrophin is a paralogue of dystrophin that is upregulated in DMD/BMD patients, but previous work from our group showed that there is no correlation between the amount of utrophin and the

clinical course in DMD (VAINZOF; FEITOSA; CANOVAS; AYUB-GUERRIERI *et al.*, 2016).

Muscle damage in young DMD patients is sensed by the immune system, which acts at early stages by activating pro-inflammatory M1 macrophages promoting muscle cell lysis by production of Nitric oxide (MACMICKING; XIE; NATHAN, 1997; TIDBALL; WELC; WEHLING-HENRICKS, 2011). Since inflammation is a characteristic of the disease, the most commonly used treatment for DMD is the immunosuppressor corticosteroid prednisone, which slows the progression of the disease. However, whether this effect is due solely to the drug's anti-inflammatory properties or its set of other effects, such as affecting myogenesis and the metabolism, reducing proteolysis and myonuclear apoptosis or increasing the expression of the dystrophin paralog - utrophin, is still debatable. Still, it's worth mentioning that in an experiment aiming macrophages depletion in *mdx* mice resulted in an 80% reduction in dye influx into muscle cells at acute onset of pathology, indicating that the immune system and particularly macrophages are responsible for a large portion of the membrane damage in muscle (TIDBALL; WELC; WEHLING-HENRICKS, 2011). At later stages, chronic inflammation takes place and M2 macrophages promote regeneration and fibrosis. Other contributors to inflammation in DMD are CD4^{+} helper T and CD8^{+} cytotoxic T cells, which produce inflammatory cytokines and trigger muscle cell death, respectively. Other cells, like neutrophils, mast cells and eosinophils also take place in the immune-mediated pathology of DMD (DUAN; GOEMANS; TAKEDA; MERCURI *et al.*, 2021).

At early stages, the balance between degeneration and regeneration keeps muscle functioning, but as time goes by, muscle cells are

replaced by fibrosis and fatty cells due to the upregulation of TGFβ and the incapacity to regenerate the excessive damage in the chronically inflammatory DMD muscle. In short, the pathology in DMD is characterized by fragilized muscle cells that suffer excessive contraction-induced damage, which is then exacerbated by the immune system and cannot be regenerated properly along the years.

1.2. Muscle Regeneration

The role of dystrophin in muscles is to attach the cytoskeleton to sarcolemmal proteins, such as dystrobrevin, syntrophins and nitric oxide synthase (cytoplasmic), β-dystroglycan, sarcoglycans and sarcospan (transmembrane) and α-dystroglycan (extracellular). Laminin binds the α-dystroglycan and provides adhesion to the endomysium and basal lamina on the extracellular matrix, forming the dystrophin-associated protein complex (DAPC) (Figure 2). It provides anchorage during muscular contraction/relaxation protecting muscle cells from excessive contraction-induced damage. In the absence of a functional dystrophin, the DAPC loses its interaction between the cytoskeleton and the extracellular matrix leading ultimately to a fragilized sarcolemma (muscle cell plasma membrane) (GAO; MCNALLY, 2015).

Damage and repair are a normal process in skeletal muscle physiology. The main stimuli for muscle growth are micro ruptures on the sarcolemma of muscle cells caused by exercising, followed by regeneration by satellite cells (SCs), the muscle stem cells, known for their role in muscle growth, regeneration and maintenance (DUMONT; WANG; RUDNICKI, 2015).
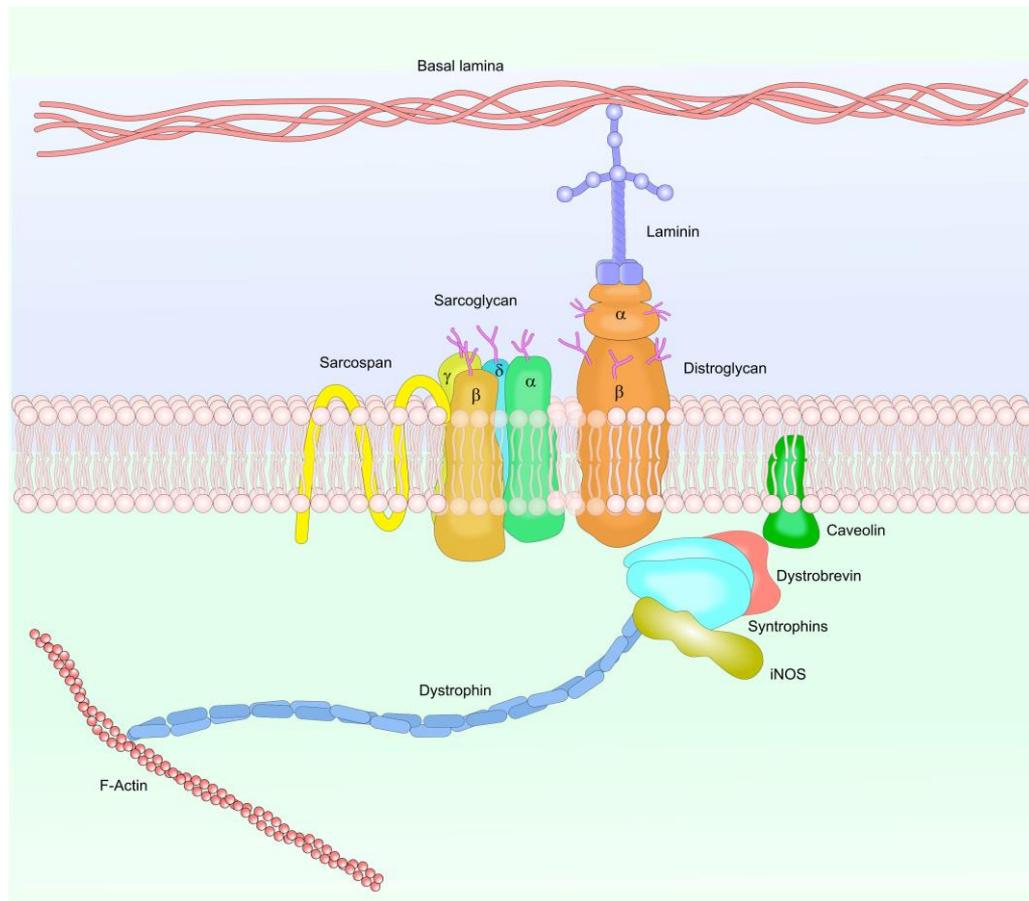
Figure 2: Schematic representation of the dystrophin-associated protein complex (DAPC). Dystrophin attaches the muscle cell's cytoskeleton (F-Actin) to sarcolemmal proteins around the plasma membrane. Laminin binds to the α-dystroglycan subunit and provides adhesion to the extracellular matrix (endomysium and basal lamina). Licensed by AdobeStock.

However, a recent study showed another mechanism of repair for minor injuries in muscle's sarcolemma (such as those caused by physical exercise in normal muscle). This mechanism works through the migration of myonuclei (muscle cell nucleus) to the site of injury (Figure 3) where they will deliver messenger RNAs (mRNA) of repair factors locally, accelerating the regeneration of the injured sarcolemma in a mechanism that is independent of SCs (ROMAN; PINHEIRO; PIMENTEL; SEGALÉS *et al.*, 2021).
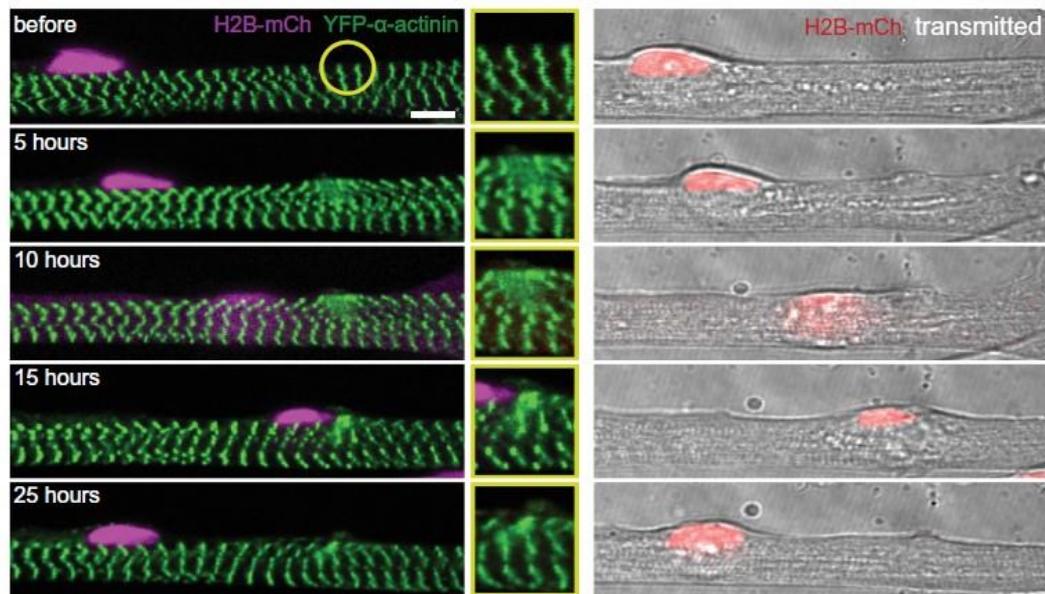
Figure 3: Time lapse of nuclear movement after laser-induced damage (yellow circle) in a myotube expressing YFP–a-actinin (z-line) and mCherry-H2B (nucleus). Adapted from (ROMAN; PINHEIRO; PIMENTEL; SEGALÉS *et al.*, 2021)

In DMD/BMD patients, the contraction-induced damage to muscle cells is increased even during normal physical activities and will require a more robust regeneration process that will activate SCs. However, the disrupture of the DAPC also negatively affects the regenerative potential of activated SCs, partially compromising SCs myogenic commitment, leading to the progressive muscle impairment observed in dystrophinopathies, with fibrosis and centrally located myonuclei (Figure 4) (CHANG; SINCENNES; CHEVALIER; BRUN *et al.*, 2018; DUAN; GOEMANS; TAKEDA; MERCURI *et al.*, 2021).
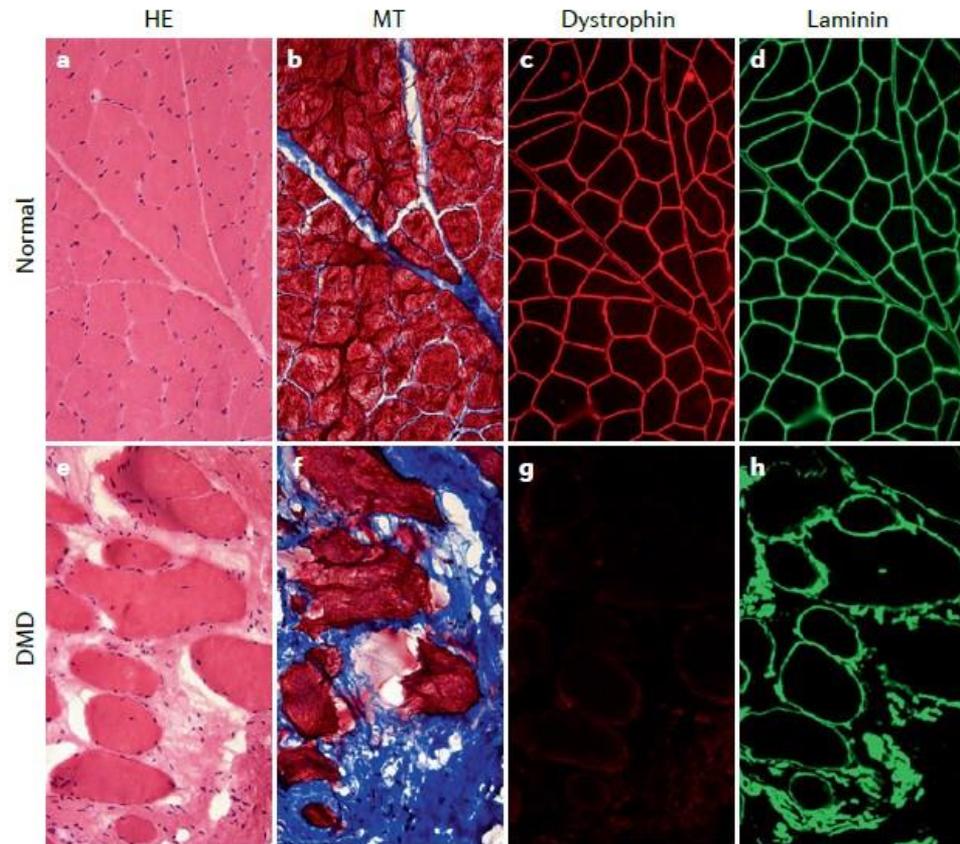
Figure 4: Healthy muscle and DMD muscle histology. Cross-sectional staining of healthy muscle (a–d) and DMD muscle (e–h) from muscle biopsies. Haematoxylin and eosin (HE) staining shows centrally nucleated myofibers, inflammatory cell infiltration, variable myofiber size, and endomysium and perimysium connective tissue deposition in DMD muscle. Masson trichrome (MT) staining shows increased fibrosis (blue staining) in a patient with DMD when compared with healthy muscle. Immunofluorescence labelling of dystrophin and laminin shows a lack of dystrophin in a patient with DMD compared with healthy muscle and variation in myofiber size in DMD muscle. Adapted from (DUAN; GOEMANS; TAKEDA; MERCURI *et al.*, 2021)

The skeletal muscle tissue is composed in most part by long multinucleated muscle fibers and a small population of mononucleated stem cells, the aforementioned satellite cells (SCs), which represent roughly 2-10% of total nucleus observed (Figure 5) (DUMONT; WANG; RUDNICKI, 2015). Myonuclei are mostly found on the periphery of normal muscle fibers, but in DMD/BMD myonuclei also appear centrally located, as shown in Figure 4, which is a result of hypernucleation. In DMD mouse model (*Mdx*

mice), muscle fibers contain twice as many myonuclei as wild type mice and the excess number of nucleus accounts for the centrally placed myonuclei. Whether hypernucleation contributes to the myopathic process, protects from further damage or is a result of the disease remains undetermined (DUDDY; DUGUEZ; JOHNSTON; COHEN *et al.*, 2015). Since myonuclei cannot replicate inside muscle fibers, one of the reasons for hypernucleation might be the overactivation of SCs, as they ultimately fuse to muscle fibers providing their plasma membrane and cytoplasmatic contents, including the nucleus (WANG; RUDNICKI, 2012).
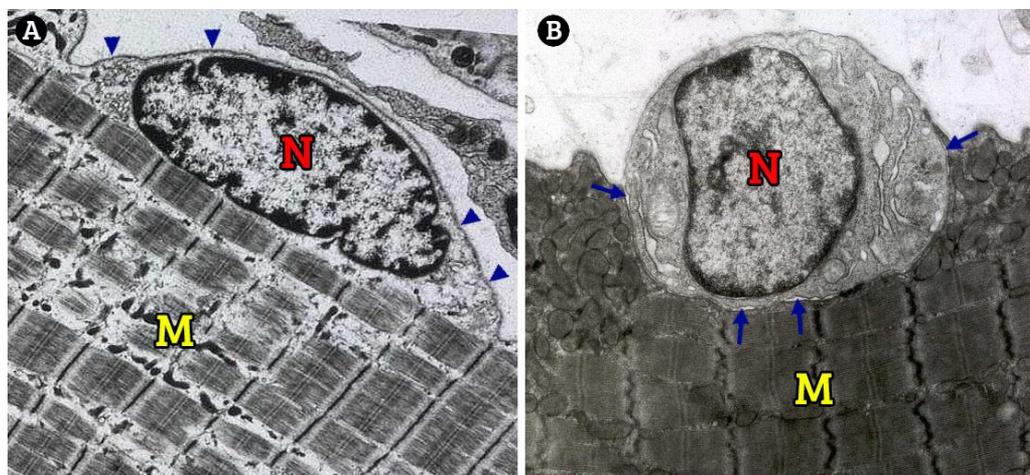
Figure 5: (**A**) Myonuclei at the periphery of a muscle fiber (M), note that the sarcoplasm involves the nucleus (N) (arrowheads). (**B**) Satellite cell has its own separate cytoplasm (arrows) and is localized adjacently to the muscle fiber. Adapted from (UNICAMP)

In adult skeletal muscle, satellite cells can be characterized by the transcription factor paired box 7 (PAX7), which specifies SCs myogenic identity and is a transcription factor required to maintain their undifferentiated status. PAX3 is also a known marker for satellite cells, but mainly during embryonic development and in some specific adult muscle tissues, like the diaphragm. On the other hand, PAX7 is required for the development and

maintenance of satellite cells in adult skeletal muscles and is expressed in all satellite cells and proliferating myoblasts until it gets down regulated to differentiate into myocytes (WANG; RUDNICKI, 2012). About 10% of the satellite cell population never leaves the quiescent state in normal muscle, being marked as PAX7$^+$MYF5$^-$, while the remaining are committed to the myogenic lineage expressing the myogenic factor 5 (MYF5), thus marked as PAX7$^+$MYF5$^+$, until activation. After activation, SCs become myoblasts by expressing the myoblast determination protein (MYOD), marked as PAX7$^+$MYF5$^+$MYOD$^+$. Subsequently these cells downregulate PAX7 and upregulate Myogenin (MYOG), MRF4 and other factors to differentiate into Myocytes, which will ultimately fuse to form new muscle fibers or help regenerate existing ones (Figure 6) (WANG; RUDNICKI, 2012).
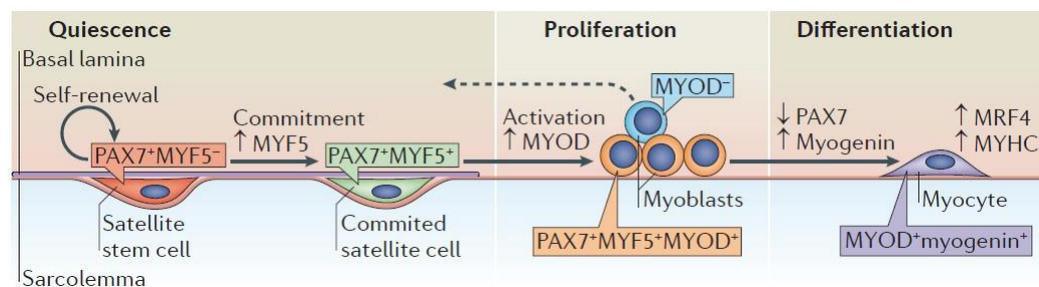
Figure 6: Quiescent satellite cells are capable of self-renewing by cell division, or can commit to the myogenic lineage, after injury or growth stimuli they can become activated and proliferate by cell division, some of the resulting cells can downregulate MYOD and return to quiescence or committed state. Others will downregulate PAX7 and upregulate Myogenin, MRF4 and MYHC and differentiate into myocytes. Adapted from (WANG; RUDNICKI, 2012)

One of the most important characteristics of SCs is their capability of self-renewing and expanding their cell pool in skeletal muscle tissue, but not in cardiac muscle where the regenerative capability is much more limited and still poorly understood (RASMUSSEN; RAVEENDRAN; ZHANG; GARRY, 2011). Satellite cells can go

under cell division in a symmetric way and expand their numbers, or divide in an asymmetric fashion and self-renewal the stem cell pool and produce committed SCs (Figure 7). In cases where constant activation stimuli are sent to SCs, such as DMD, it's possible that SCs increase the asymmetric formation of committed myogenic progenitors and the muscle stem cell pool is reduced with time until being eventually depleted (WANG; RUDNICKI, 2012).
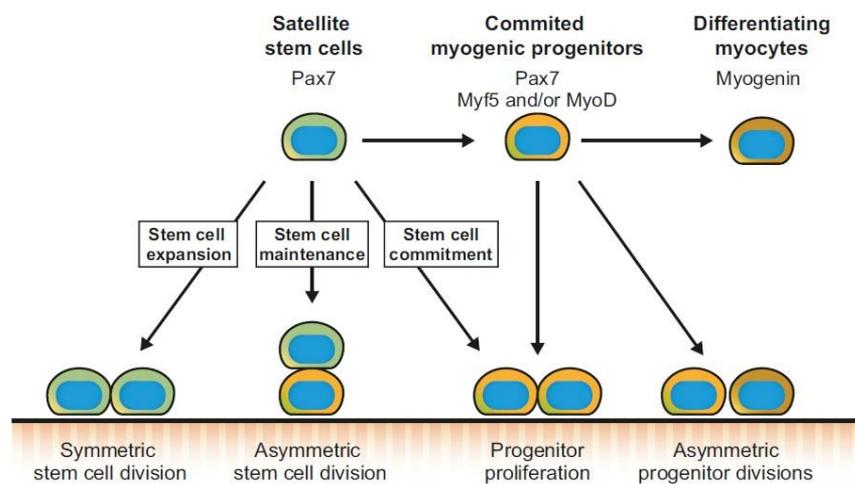
Figure 7: Overview of satellite cell self-renewal and myogenic commitment in asymmetric and symmetric cell divisions. Quiescent satellite cells can undergo cell division in a symmetric fashion to expand the satellite stem cell population, or in an asymmetric way to both maintain the stem cell population and generate myogenic progenitors. Alternatively, they can commit to the myogenic lineage and proliferate. Myogenic progenitors can proliferate, divide asymmetrically or directly differentiate into myocytes. Adapted from (DUMONT; WANG; RUDNICKI, 2015)

Mice studies show that these markers and their interactions with each other are very important for satellite cell function and muscle fiber development (WANG; RUDNICKI, 2012). Mice without PAX3 present with developmental defects, such as lack of limb muscles and myotome with reduced MYOD expression. Mice without both PAX3 and MYF5 have no muscle, since they cannot compensate the lack of MYF5 by upregulating MYOD, which

reinforces the role of PAX3 in activating MYOD during embryonic development. PAX3 or PAX7 are essential for muscle formation as mice without both of these genes are devoided of muscle fibers. Mice lacking only MYOD have normal muscle, and so do those lacking only MYF5, but when mice lack both of these proteins, they are completely devoided of muscle fibers, which shows the importance of satellite cell myogenic determination by MYOD or MYF5 in order for muscle fibers to be formed. MYOG is crucial for myoblast terminal differentiation, as shown in mice knockout for MYOG in which almost no muscle fiber is formed and undifferentiated myoblasts are accumulated. When mice lack both MYOD and MRF4, they present with a very similar phenotype to the MYOG-knockout mice, which shows that in absence of MYOD, MYF5 requires MRF4 for terminal differentiation (WANG; RUDNICKI, 2012). This demonstrates that these markers have an overlapping regulatory network that is influenced by many factors, including the Notch signaling pathway, which is essential to control proliferation and maintenance of satellite cells, as well as differentiation of myogenic progenitors (LIU; SATO; CERLETTI; WAGERS, 2010; MOURIKIS; SAMBASIVAN; CASTEL; ROCHETEAU *et al.*, 2012).

1.3. Notch Signaling Pathway

The Notch signaling pathway occurs through cell surface ligand-receptor interaction, where cells bearing a ligand will activate an adjacent cell with a Notch receptor in its surface, which then affects a cascade of intracellular events that will ultimately regulate the transcription of downstream genes. This type of

signaling controls the fate and differentiation of stem cells, playing a role in the development of most organs, as well as the maintenance of a stem cell pool in several adult tissues, including the muscle. Notch signaling is highly conserved and occurs in probably all multicellular organisms (GAZAVE; LAPÉBIE; RICHARDS; BRUNET *et al.*, 2009; SIEBEL; LENDAHL, 2017) Notch receptors are transmembrane proteins, that once synthesized are directed to the endoplasmic reticulum and Golgi apparatus, where they undergo their first proteolytic processing by a furin-like convertase (S1 cleavage). The Notch receptor has an extracellular domain (ECD) composed by large arrays of epidermal growth factor-like repeats and a negative regulatory region (NRR), which is important for the second receptor cleavage by the ADAM metalloproteases (S2 cleavage) upon ligand interaction. This processing step is essential for Notch activation, as it will release another portion of the receptor: the Notch intracellular domain (ICD), which is then cleaved again by the γ-secretase complex (S3 cleavage) before it is transported from the cytoplasm to the nucleus, where it interacts with the repressor CSL (also known as RBPJ), and forms a trimeric complex together with MAML (Mastermind-like). MAML converts CSL from repressor to activator of genes downstream of Notch (Figure 8) (SIEBEL; LENDAHL, 2017).

When the ligand interacts with the Notch receptor it is believed that it exerts a pulling force that alters the conformation of the NRR domain, making the cleavage by ADAM (A Disintegrin And Metaloproteinase) possible (GORDON; ZIMMERMAN; HE; MILES *et al.*, 2015). The NRR forms a hingelike structure, until ligand pulling alters its structure and exposes the S2 cleavage site.

Furthermore, there is evidence that the amount of exposition for cleavage might differ between Notch receptors and alter the ADAM metalloprotease cleavage site. The most easily cleaved of the Notch receptors is probably Notch3, as a study reported constitutive Notch3 activation that is ligand-independent in basal breast cancer (CHOY; HAGENBEEK; SOLON; FRENCH *et al.*, 2017; SIEBEL; LENDAHL, 2017).

After S2 cleavage the Notch ECD is trans-endocytosed together with the ligand into the ligand-expressing cell (PARKS; KLUEG; STOUT; MUSKAVITCH, 2000). Meanwhile, the Notch ICD suffers S3 cleavage and in certain cases goes directly from the cell membrane to the cell nucleus (Figure 8). However, there is emerging evidence that Notch ICD undergoes a more complex routing which involves the endosome as an intermediate station and that intracellular routing all the way to the nucleus is a regulated process in Notch signaling. For example, liberation of the Notch ICD by S3 cleavage leads to its nuclear localization, which is controlled by importin-α proteins. In Drosophila, mutations in Rab5 and Syntaxin7 lead to accumulation of Notch receptors at the cell surface and consequently reduce Notch signaling, suggesting these genes are involved in Notch receptor routing (SIEBEL; LENDAHL, 2017).

There is also an undergoing debate about where exactly the S3 cleavage occurs: at the cell surface level or in the acidic environment of endosomes (VACCARI; LU; KANWAR; FORTINI *et al.*, 2008). Depending on where inside the cell this cleavage occurs (at the cell membrane or after internalization), the position of the S3 cleavage site may alter, resulting in different Notch ICDs with variable stability (SIEBEL; LENDAHL, 2017).
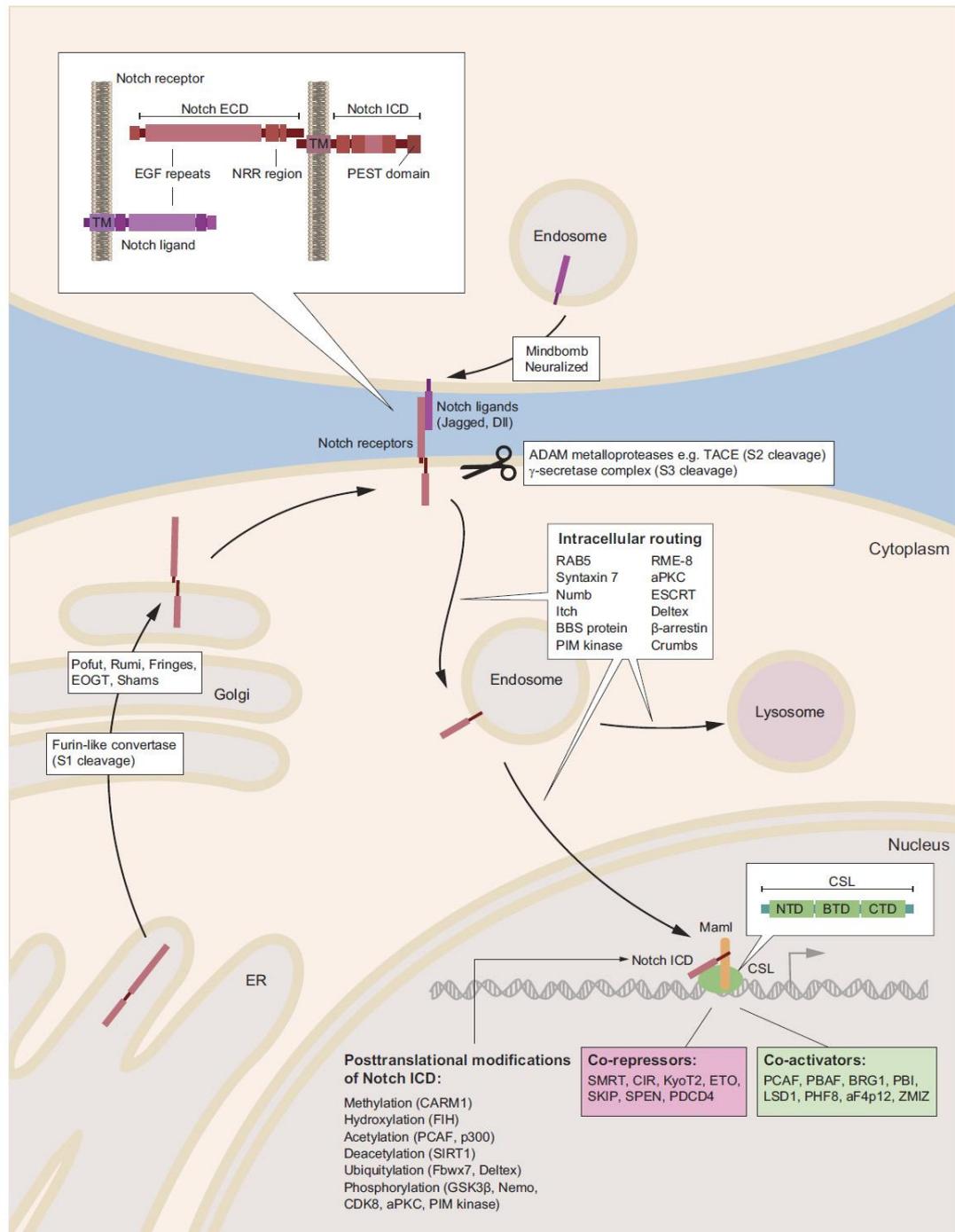
Figure 8: Overview of the Notch signaling pathway. The Notch receptor is synthesized in the endoplasmic reticulum and its first processing step is performed by a furin-like convertase (S1 cleavage) in the Golgi apparatus, then it is transported to the cell surface, where the Notch receptor interacts with a Notch-ligand presented by an adjacent cell. After ligand-interaction the second processing of the Notch receptor is carried out by ADAM metalloproteases (S2 cleavage), then the third cleavage is performed by the γ-secretase complex (S3 cleavage) and this step can occur in the endosomes or at the cell. The S2-cleaved receptor is then internalized into endosomes, where the Notch ICD will be released to be translocated to the cell nucleus. Alternatively, S2-cleaved receptor can be degraded if relocated to the lysosome.

Notch ICD reaches the nucleus to form a trimeric complex with CSL and MAML, the latter is responsible to convert CSL from repressor to activator of transcription of genes downstream of Notch. Several post-translational modifications of Notch ICD have been observed: methylation, acetylation, ubiquitylation, phosphorylation, hydroxylation, which may affect Notch ICD longevity. Boxes show other aspects of intracellular routing. A box in the top left corner shows a more detailed representation of the domains in Notch receptors and ligands. A box in the bottom right corner shows the domain structure of CSL. Adapted from (SIEBEL; LENDAHL, 2017).

A curious fact about the Notch signaling pathway is that its ligands also have a complex intracellular routing: after being synthesized and directed to the cell membrane, ligands need to be endocytosed and recycled back to the cell surface. This recycling process occurs with the help of Mindbomb and Neuralized (two E3 ubiquitin ligases regulating Notch ligands) and is essential for ligands to mature and become signaling-competent. However, what molecular alterations the recycling process provides to activate ligand function is still not fully elucidated (SIEBEL; LENDAHL, 2017).

The function of Notch receptors (Notch1, Notch2, Notch3 and Notch4) and their ligands (Jagged1, Jagged2, Dll1, Dll3 and Dll4) are not all the same. For example, previous studies indicate that Notch2 is tumor-promoting and Notch1 is tumor-suppressive in bladder cancer. Notch3 on the other hand provides a number of distinct outputs depending on the situation when compared with other receptors. There is also the Notch4 receptor that, although has similar sequences to the other *Notch* genes, cannot be activated through ligand stimulation and may even inhibit Notch1 in *cis*. The same applies to ligands: a study expressing Dll4 from the Dll1 locus has shown that these ligands have different functions, as this

swap results in different outcomes in presomitic mesoderm(SIEBEL; LENDAHL, 2017).

The cross-talk between Notch signaling and other signaling mechanisms might be an explanation for the diversity of the signaling outputs observed in Notch. For example, the Wnt signaling pathway can interact with Notch in many ways, such as physical interaction between key regulators in the Wnt pathway and Notch ICD. There is also non canonical Notch signaling, which doesn't follow faithfully the canonical Notch signaling that was described previously. Apparently MAGP1, MAGP2 and YB1 can bind to Notch receptors, inducing cleavage and activation, but whether these alternative ligands act in the same way as canonical ligands, influence Notch signaling indirectly or if an alternative mechanism is involved is not yet elucidated (SIEBEL; LENDAHL, 2017).

In skeletal muscle, which is in part derived from the myotome compartment in the somite, myogenesis is controlled by Notch signaling at many steps. At the beginning of myogenesis, during embryonic development, the cell fate of $Pax3^+$ progenitor cells in the somite is controlled by Notch, as endothelial and smooth muscle cell fates are promoted by the activation of Notch1 ICD. When hypomorphic (partial loss-of-function) Dll1 allele or conditional CSL ablation are present, there is a loss of Notch signaling, which leads to the depletion of myogenic $Pax3^+$ and $Pax7^+$ progenitors (SIEBEL; LENDAHL, 2017).

In adult muscle, satellite cells and their commitment to muscle repair is controlled by Notch signaling. Muscle regeneration decreases with aging, and part of the reason for that might be the decrease in Notch signaling with age. In fact, when muscle is

injured, Notch signaling becomes elevated, which is evidence of its role in the repair process (SIEBEL; LENDAHL, 2017).

Previous studies have shown that by tweaking different Notch receptors and ligands, different outcomes for muscle development and maintenance are observed. In mice with constitutive Notch activation, Pax7 expression was upregulated and satellite cells self-renewal was increased, but muscle regeneration was impaired. That can be explained by the downstream gene regulation promoted by the constituently expressed Notch ICD, as CSL/Rbpj will promote Pax7 expression, as well as upregulate genes of the *Hes*/*Hey* family, which suppress the expression of MyoD and Myogenin, thus compromising muscle regeneration (Figure 9) (WEN; BI; LIU; ASAKURA *et al.*, 2012).
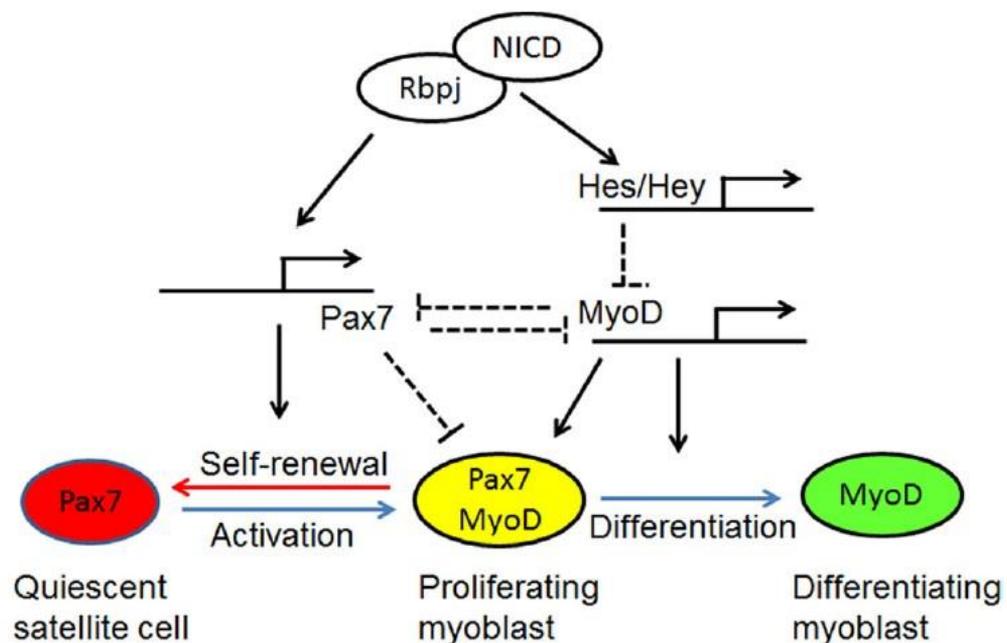
Figure 9: Schematic representation of the intracellular Notch signaling pathway and its implication in muscle satellite cell commitment. Quiescent muscle satellite cells express high levels of Pax7 and constitutive Notch signaling will upregulate this marker, promoting satellite cell self-renewal. Meanwhile constant Notch ICD will keep Hes/Hey proteins upregulated, which will inhibit MyoD gene transcription, blocking muscle differentiation. Adapted from (WEN; BI; LIU; ASAKURA *et al.*, 2012).

On the other hand, mutant mice with Notch signaling blockage in satellite cells showed clear signs of muscular dystrophy (Figure 10), with reduced survival, impaired muscle regeneration and reduction in satellite cell activation and proliferation, but with increased myoblast differentiation (LIN; SHEN; JIN; GU *et al.*, 2013)
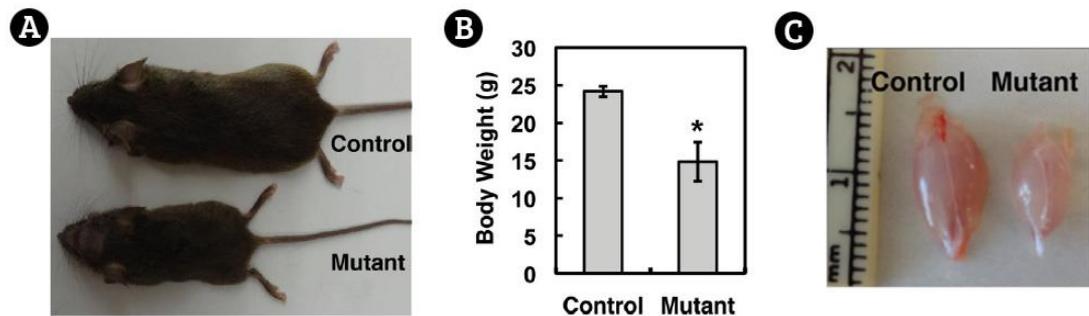
Figure 10: Notch blockage causes various phenotypes in Mutant mice when compared with Control mice: (A) Control and Mutant mice body sizes; (B) body weight of Control vs Mutant mice; (C) difference in size of gastrocnemius muscle between Control and Mutant mice. Adapted from (LIN; SHEN; JIN; GU *et al.*, 2013)

When mdx mice are knockout for Notch3, they develop muscle hypertrophy by muscle regeneration when there is repetitive muscle injury (Figure 11). In these mice, the number of quiescent and self-renewing satellite cells was increased and primary myoblasts collected from Notch3 deficient mice grew faster when compared to control cells. In the same study, an assay of overexpression of Notch3 showed the activation of *Nrarp* (NOTCH Regulated Ankyrin Repeat Protein), which acts as a negative feedback regulator in Notch signaling, suggesting that Notch3 may act as a repressor of Notch1 expression (KITAMOTO; HANAOKA, 2010).
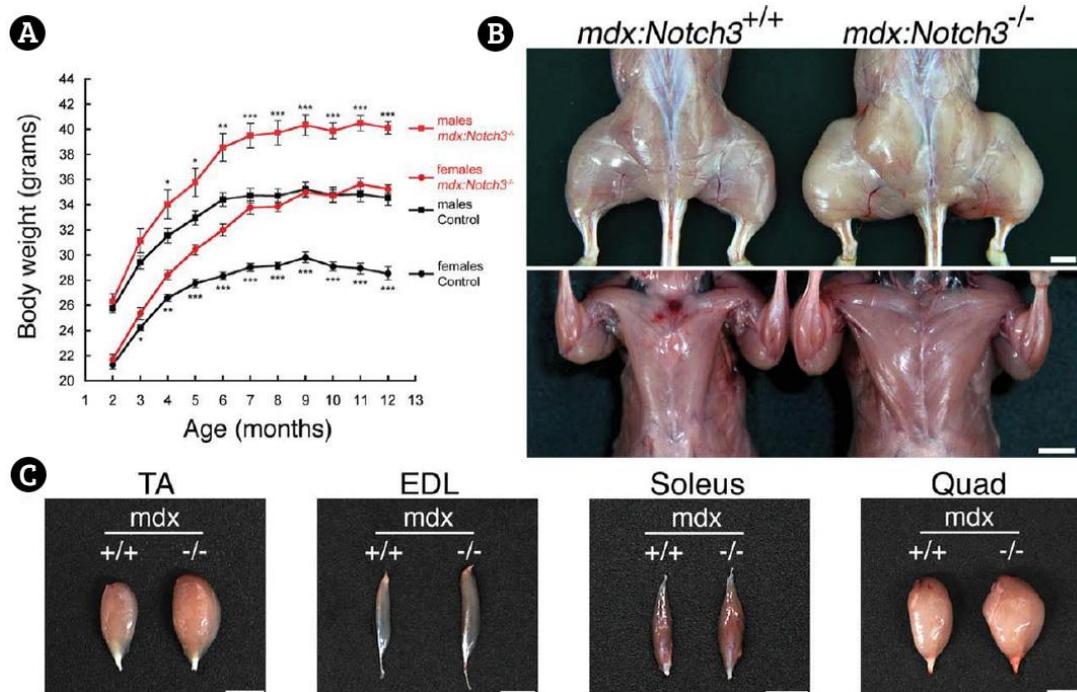
Figure 11: Physical comparison between *mdx* mice without Notch3 (*mdx:Notch3⁻ᐟ⁻*) and *mdx* mice with Notch3 (*mdx:Notch3⁺ᐟ⁺* and *mdx:Notch3⁺ᐟ⁻*): (A) body weight over 12 months; (B) qualitative comparison of body muscles, showing that *mdx* mice depleted of Notch3 have bigger muscles than control *mdx* mice; (C) Size comparison of Tibialis anterior (TA), extensor digitorum longus (EDL), soleus, and quadriceps femoris (Quad) muscles. Scale bar = 5mm. Adapted from (KITAMOTO; HANAOKA, 2010)

Our research group has previously reported a mutation on the promoter of *Jagged1* (*Jag1*) in exceptional Golden Retriever Muscular Dystrophy (GRMD) dogs with a milder course of disease. This mutation creates a *myogenin* binding site that leads to increased Jag1 expression and is responsible, at least in part, by the milder course in these "escaper" dogs (Figure 12) and suggested as a genetic modifier in GRMD (VIEIRA; ELVERS; ALEXANDER; MOREIRA *et al.*, 2015).
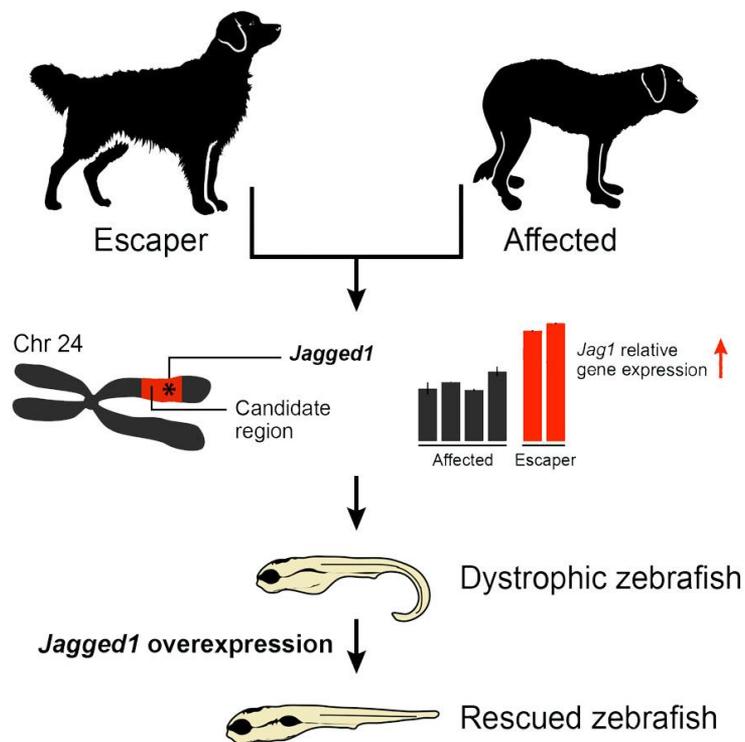
Figure 12: Escaper dogs with almost normal phenotype presented a genetical modifier at the promoter of Jagged1, that leads to its upregulation. Using transgene overexpression of Jagged1 in dystrophic zebrafish also lead to a rescued phenotype. Adapted from (VIEIRA; ELVERS; ALEXANDER; MOREIRA *et al.*, 2015).

These studies indicate that many factors are involved in the development of healthy and functional muscle and that modifications in those machineries can either cause disease, modify or prevent it. In the third chapter of this document, we discuss the development of an alteration in the Notch signaling pathway, by knockout of Notch3. The aim is to analyze its effect in muscle cell progenitors and differentiated muscle fibers *in vitro*, in order to further elucidate the biological pathways underlying muscle phenotype modification.

1.4. Case Study

In an exceptional case, reported by our research group in 2015, it was shown two half-brothers bearing the same exon 2 duplication in *DMD*, complete absence of dystrophin in muscle immunostaining and Western Blot (WB) and yet, they have a surprising discordant phenotype: the younger brother has a normal course of the disease, with loss of ambulation at age 9, while the older brother aged 13 at the time had only some mild weaknesses, such as difficulty for running and climbing stairs (Figure 13). Their muscle histology and utrophin expression were similar and could not explain the discordant phenotype (VAINZOF; FEITOSA; CANOVAS; AYUB-GUERRIERI *et al.*, 2016; ZATZ; PAVANELLO; LAZAR; YAMAMOTO *et al.*, 2014).
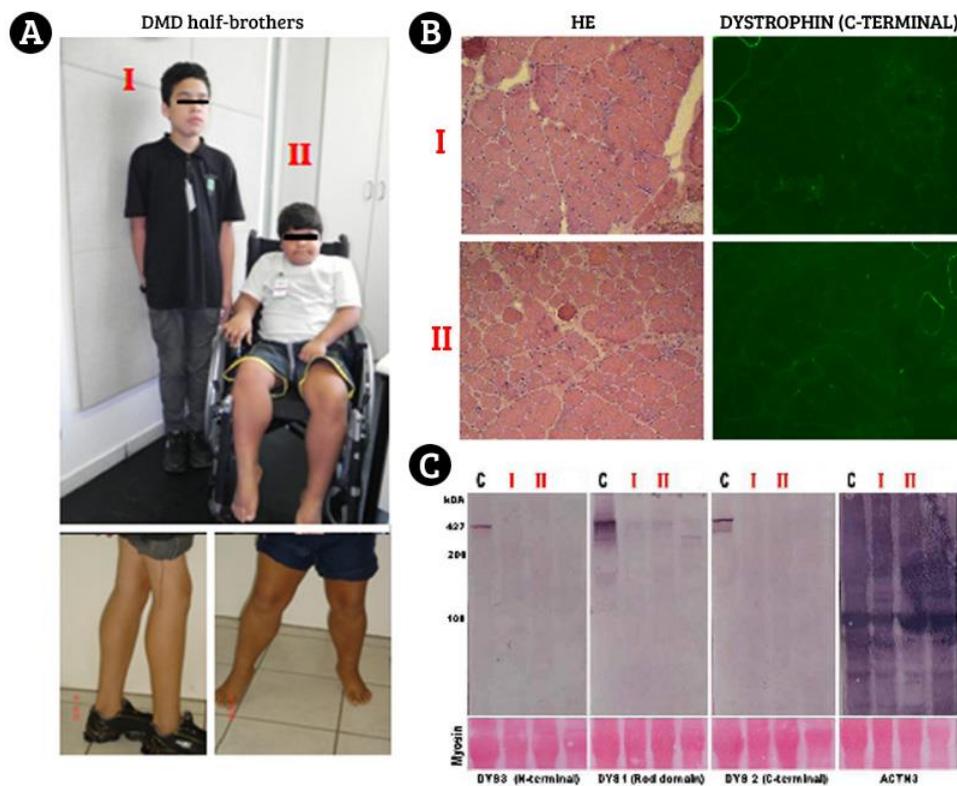
Figure 13: (A) DMD half-brothers at ages 15 (I) Mild and 11 (II) Severe, showing signs of calves' hypertrophy. (B) Muscle histology stained with hematoxylin and eosin (HE) and immunohistochemical analysis of dystrophin C-terminal antibody. (C) Western blot analysis

of dystrophin N-terminal, Rod domain and C-terminal antibodies and actinin 3 (ACTN3) antibody. Adapted from (ZATZ; PAVANELLO; LAZAR; YAMAMOTO *et al.*, 2014).

Today, these half-brothers are 19 and 23 respectively, and the older brother can still walk without assistance (unpublished observations).

Furthermore, a more recent study showed the phenotypic spectrum in DMD patients with exon 2 duplications (Dup2), where 61% were classified as DMD, 30% BMD and 9% were intermediary (IMD) (ZAMBON; WALDROP; ALLES; WEISS *et al.*, 2021). This is to show that the gene mutation or quantity of dystrophin present in the muscle are not always the determining factors for disease progression in DMD, as the patient's genetical background can also provide modifiers for the disease.

In this study, we generated unedited and edited iPSCs cell lines from the aforementioned DMD half-brothers, classified as Severe and Mild, and the unaffected father of the mild patient who served as a normal control, named Father. The experiments with those cell lines will be discussed in chapters 2 and 3 (Figure 14).
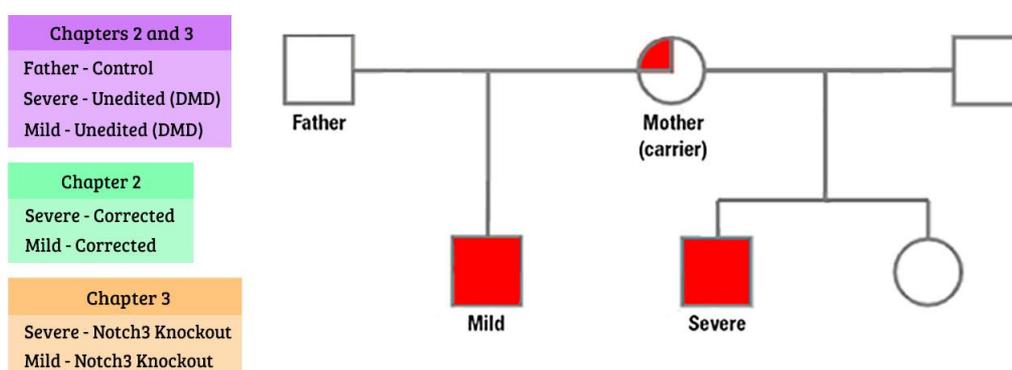
Figure 14: Heredogram of the discordant DMD half-brothers' family. Cell lines from Severe and Mild patients, as well as the Father were generated and discussed in chapters 2 and 3.

2. DMD Correction

Abstract: Several therapies have been developed and tested to treat Duchenne Muscular Dystrophy (DMD) in the past decades, but none of them had expressive effect in life expectancy or patients' quality of life, indicating that new approaches must be pursued. In this study, CRISPR technology was used aiming mutation correction in induced Pluripotent Stem Cells (iPSCs) derived from skin cells from two half-brothers bearing a duplication in exon 2 of *DMD* with a discordant clinical course. Unedited and edited cells were differentiated *in vitro* using a transgene-free method. We show that the duplication was removed and *DMD* reading frame was recovered, as observed through dystrophin immunostaining and Western Blotting in muscles differentiated *in vitro*. This supports the potential of using these cells for the development of new gene and cellular therapy.

Key words: DMD, CRISPR, Gene Editing, Exon 2 Duplication

Resumo: Diversas terapias foram desenvolvidas e testadas para tratar a Distrofia Muscular de Duchenne (DMD) nas últimas décadas, mas nenhuma delas foi capaz de aumentar expressivamente a expectativa de vida ou qualidade de vida dos pacientes, indicando que é neccessário buscar novas abordagens. Neste estudo, a tecnologia de CRISPR foi usada com a intenção de corrigir a mutação em células-tronco pluripotentes induzidas (iPSCs) derivadas da pele de dois meios-irmãos que possuem uma duplicação no exon 2 de *DMD*, mas com curso clínico discordante. Diferenciamos células não editadas e editadas *in vitro* usando uma metodologia livre de transgenes. Nós mostramos que a duplicação foi removida e o quadro de leitura em *DMD* foi recuperado, como observado na immunomarcação e *Western Blotting* da distrofina nos músculos diferenciados *in vitro*. Isso sustenta o potencial de utilizar estas células no desenvolvimento de uma nova terapia celular e gênica.

Palavras-chave: DMD, CRISPR, Edição Gênica, Duplicação do Exon 2

2.1. Introduction

Therapies seeking out the cure for DMD have been developed in the last decade, but have failed so far to significantly increase the patients' clinical course or life expectancy. One of those therapies is based on the synthetic oligomer Eteplirsen, which is designed to bind complementarily to the pre-mRNA transcripts of the *DMD* gene causing exon-skipping of exon 51, aiming to treat patients with mutations that are amenable to skipping of exon 51 (CHARLESTON; SCHNELL; DWORZAK; DONOGHUE *et al.*, 2018). Although this therapy successfully restored some expression of dystrophin in DMD patients, the clinical results over long-term follow-up of treatment show that the benefits are modest (MCDONALD; SHIEH; ABDEL-HAMID; CONNOLLY *et al.*, 2021; MENDELL; KHAN; SHA; ELIOPOULOS *et al.*, 2021) and do not justify its $750,000 to $1.5 million cost a year.

A clinical trial, first reported in 2004, has attempted an allotransplantation of myogenic progenitors derived from muscle biopsies of DMD patients' parents. In this experimental trial the cells were expanded *in vitro* and injected into patients' muscle, together with immunosuppression. There was some recovery of Dystrophin expression, but the long-term survival of the transplanted cells was compromised by acute immune rejection (SKUK; GOULET; ROY; CHAPDELAINE *et al.*, 2006; SKUK; ROY; GOULET; CHAPDELAINE *et al.*, 2004).

Recently another therapy has been developed based on gene transfer of a micro-dystrophin through an adeno-associated virus delivery. The micro-dystrophin is based on studies on BMD patients with very large "in-frame" deletions with a very mild BMD course (PASSOS-BUENO; VAINZOF; MARIE; ZATZ,

1994). The study of these patients made possible to establish which exons in *DMD* are the most essential for its function, and as *DMD* sequence is too large to fit inside the adenovirus capsid, researchers developed this shortened, but still functional, micro-dystrophin. Results shown so far demonstrate the expression of the micro-dystrophin in patients' muscle biopsies, but the clinical effects on patients seem modest (MENDELL; SAHENK; LEHMAN; NEASE *et al.*, 2020). Unfortunately, the gene therapy trial conducted by Pfizer using this approach has been interrupted by the FDA after an unexpected death of a patient occurred (KEOWN, 2021).

One of the reasons therapies based on dystrophin recovery have been failing repeatedly might be due to DMD patients' immune response to the protein Dystrophin. The immune system distinguishes "self" from "non-self" and most DMD patients have probably never produced full-length dystrophin in the first place. Evidence for Dystrophin immunity comes from *mdx* mice, that develop an anti-dystrophin antibody response to adenoviral-mediated dystrophin gene transfer (FERRER; WELLS; WELLS, 2000; GILCHRIST; ONTELL; KOCHANEK; CLEMENS, 2002). Furthermore, the immune system has been a hurdle for all CRISPR-based gene therapies, as it has been shown that humans and animal models can have inbuilt anti-Cas9 antibodies even before delivery of CRISPR-Cas9 therapies (HAKIM; KUMAR; PÉREZ-LÓPEZ; WASALA *et al.*, 2021).

These observations reinforce the need to continue seeking for alternatives to treat DMD. In this chapter, it is shown the results of an *in vitro* CRISPR strategy used to correct the exon 2 duplication in *DMD* of iPSCs generated from skin of two half-brothers with a

discordant clinical course, which are named Severe and Mild in this study (Figure 13). A previous study has shown that probably all exon 2 duplications have a minimal common duplication region that can be corrected using the same guide RNA (gRNA) (Figure 15) (LATTANZI; DUGUEZ; MOIANI; IZMIRYAN *et al.*, 2017).
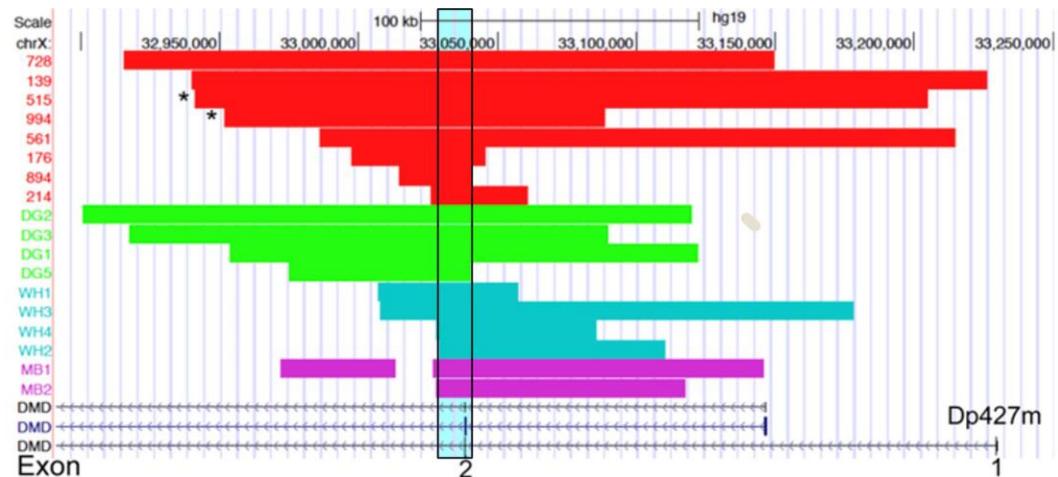
Figure 15: CGH-array profile of exon 2 duplication across patients (each line) of different studies (each color represents a different study). There is a minimal common duplication region of approximately 10 Kb (black box) that is found in all patients studied. Adapted from (LATTANZI; DUGUEZ; MOIANI; IZMIRYAN *et al.*, 2017).

The CRISPR machinery has completely changed the gene editing field since it has shown that the genome could be altered in a programmable and directed way, while also being relatively cheap and easy to use (JINEK; CHYLINSKI; FONFARA; HAUER *et al.*, 2012). The Cas9 protein works as a scissor causing a double-strand break in the DNA directed by a gRNA molecule that can be custom designed to be complementary to the sequence in the genomic DNA that should be cut and edited. Today, many modified versions of Cas9 and other variants found in different bacteria have been developed to expand the utilities of the CRISPR toolkit (ANZALONE; KOBLAN; LIU, 2020).

For the strategy discussed in this chapter the normal Cas9 was used to excise out one of the duplicated exon 2 in iPSCs from both Severe and Mild patients to recover the reading frame of *DMD* (Figure 16). The corrected cells were then differentiated into skeletal muscles *in vitro*, using a transgene-free method, to achieve dystrophin recovery.
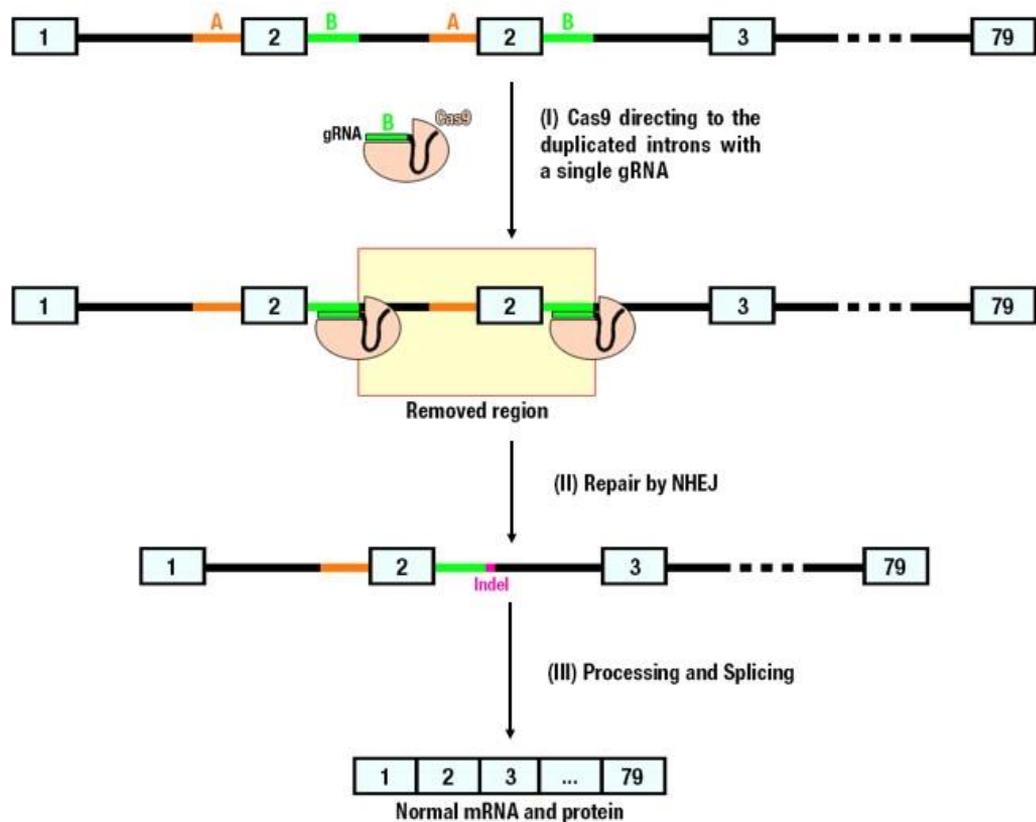
Figure 16: (I) Schematic representation of the *DMD* gene (79 exons) bearing an exon 2 duplication being corrected via CRISPR-Cas9 using a single gRNA targeting the adjacent intron B. (II) The duplicated exon is excised and the DNA is sewed back together by the mechanism of non-homologous end joining (NHEJ) introducing an intronic insertion or deletion (indel). (III) The normal reading frame of the gene is restored and normal mRNA and protein are produced.

2.2. Materials and Methods

2.2.1. Sample collection and patients

This research is being conducted at the Human Genome and Stem Cell Research Center (HUG-CEL) of the University of São Paulo, Brazil and was approved by the universities' ethics committee: CAAE 25342719.6.0000.5464.

Patients seen at HUG-CELL are followed by a multidisciplinary team composed of scientists, geneticists, physicians and other professionals. After clinical evaluation, the molecular diagnosis is first investigated through MLPA (multiplex ligation-dependent probe amplification) since about 60-70% of the patients carry deletions in the DMD gene. If no deletion is identified other types testing can be carried out such as CGH-array and WES (Whole exome sequencing). If they can contribute to new insights they are included into scientific studies.

After diagnosis confirmation, patients and their families receive genetic counseling and are invited to participate in the study. For this, a term of consent is signed either by the patient, if older than 18 years of age, or by their parents or guardians. When consent is granted, a blood sample and a 3 mm fragment of skin are collected by a trained health professional. These samples are then used to isolate peripheral blood cells (PBMCs) and fibroblasts respectively.

2.2.2. Cell culture reagents

Fibroblast medium: DMEM High, 10% FBS (Fetal Bovine Serum), 100 µg/ml Normocin.

PBMCs medium: StemSpan medium, 50 ng/ml SCF (Stem Cell Factor), 2U/ml EPO (erythropoietin), 1 μM Dexamethasone; 40 ng/ml of IGF1 (insulin growth fator-1), 10 ng/ml IL-3 (interleukin 3), 10 μg/ml Gentamicin.

Human Embryonic Stem Cell (hESC) medium (for reprogramming PBMCs): DMEM/F12 medium with Glutamax, 20% KSR (KnockOut™ Serum Replacement), 1% NEAA (MEM Non-Essential Amino Acids Solution), 100 μM β-mercaptoetanol, 100 μg/ml Normocin, 2 μM SB431542, 0,5 μM PD0325901, 2 μM Thiazovivin, 0,5 mM VPA (valproic acid), 0,25 mM NaB (sodium butyrate), 10 ng/ml FGF2 (fibroblast growth fator 2).

hESC medium (for reprogramming fibroblasts): DMEM/F12 medium with Glutamax, 20% KSR, 1% NEAA, 0,1% β-mercaptoetanol, 100 μg/ml Normocin, 10 ng/ml FGF2, 2 μM SB431542, 0,5 μM PD0325901, 500 μM VPA.

Medium for iPSCs culture: Essential 8™ (E8) Medium, 100 μg/ml Normocin.

DiCL and DiCLF medium: DMEM F12 medium with Glutamax, 1% ITS, 1% NEAA, 0,003 mM CHIR-99021 and 0,0005 mM LDN-193189. For DiCLF, also add 20 ng/ml of FGF2.

DKHIFL medium: DMEM F12 medium with Glutamax, 15% KSR, 1% NEAA, 0,2% PenStrep, 0,1 mM β-mercaptoetanol, 0,01 μg/ml HGF, 0,002 μg/ml IGF, 0,020 μg/ml FGF2 and 0,0005 mM LDN-193189.

DKI and DKHI medium: DMEM F12 medium with Glutamax, 15% KSR, 1% NEAA, 0,2% PenStrep, 0,1 mM β-

mercaptoetanol e 0,002 µg/ml IGF. For DKHI, also add 0,01 µg/ml HGF.

SKGM (for muscle progenitor cells expansion): SkGMTM-2 Skeletal Muscle Cell Growth Medium-2 BulletKitTM (Lonza) Terminal Differentiation medium: DMEM F12 medium with Glutamax, 2% KSR, 0,2% Pen/Strep, 1% ITS, 10 µM SB-431542.

2.2.3. Fibroblast isolation

The skin fragment collected from the patient is immediately transferred to DMEM/F12 medium with Normocin. Then it is washed with PBS and covered with a 25U/ml Dispase solution overnight at 4ºC. The skin is then cut and reduced into smaller fragments with the help of a scalpel. The fragments are scattered in a 60 mm plate without medium and after 5 minutes the fibroblast medium is added carefully to the plate. Fragments are incubated at 37ºC and 5% CO_2 for several days, and medium is changed every three days, until fibroblasts detach from the fragments. After reaching a 80% confluency the cells are treated with TryPLE for 5 minutes at 37ºC, resuspended in basal medium. After growing, cells are frozen in a solution of 10% DMSO and 90% FBS.

2.2.4. Cell reprogramming

The iPSCs produced from fibroblasts are electroporated using the "Human Dermal Fibroblast Nucleofector™" Kit (Lonza). Fibroblasts are cultured as described in 2.2.3, detached, centrifuged and mixed with a solution containing reprogramming plasmids: pCXLE-hOCT3/4-shp53-F,

pCXLE-hSK, pCXLE-hUL (Addgene), nucleofection solution and kit's supplements, The mix is then transferred to a cuvette where the cells are electroporated using the Nucleofector™ 2b Device.

After electroporation cells are immediately transferred to a plate covered by mouse embryonic fibroblasts (MEF) – Millipore A24903 – mitotically inactivated, then these cells are cultured using the hESC medium for fibroblasts, which is changed every two days.

Cells are observed daily until the formation of iPSC colonies, when they reach a certain size, they are mechanically detached using a pipette tip, collected and transferred to an individual well with iPSCs medium with ROCKi (5 μM). During the following days the medium is changed daily, but without ROCKi.

After reaching 80-90% confluency, the cells are detached either in colonies, if it's necessary to remove differentiated cells, or in single cell using an enzymatic reagent. For colony passaging, cells are washed with PBS twice and treated with EDTA and NaCl in PBS (Beers et al., 2012) for 10 minutes at room temperature (RT). The colonies that detach are collected and centrifuged briefly at 100g, 15-60 seconds. The supernatant is carefully removed and are plated in wells covered in Matrigel (10 μg/cm^2) in E8 medium with ROCKi. After the culture is free of differentiated cells, they are passaged in single cell by washing twice with PBS and treating with StemPro Accutase, incubated at 37ºC for 3-5 minutes, resuspended in E8 medium with ROCKi and replated at the desired density in a well, covered with Matrigel.

2.2.5. Gene editing – CRISPR

Cas9 and the gRNA (GATGATACTGGGACAAAG) were mixed in Buffer R to form a ribonucleoprotein complex (RNP complex) and delivered into iPSCs using the Neon Electroporation System. The molar proportion of Cas9 to gRNA used was 1:3 as described as being the most efficient (SEKI; RUTZ, 2018) (Table 1).

Reagent	Company	Qty (µl)	Conc.
HiFi Cas9	Produced by IDT	0.3 µl	18 pmol
Buffer R	Produced by Thermo Fisher	0.2 µl	-
gRNA	In vitro transcribed (IDT kit)	0.5 µl	54 pmol

Table 1: Reagents used for RNP mixture, companies that produced the reagents, quantity in µl used in the electroporation reaction and molar concentration.

Cells were detached using Accutase to release them as single cells and resuspended in 1 ml of E8 medium for counting in Countess™ II. For each cell line 1.6×10^5 cells were used, the right number of cells were collected and centrifuged, resuspended in PBS and centrifuged again. Then, supernatant was removed and cells were resuspended in 9 µl of Buffer R and mixed with the RNP complex. The mixture was pipetted using the 10 µl Neon Tip and electroporated using the following setup: 1200V, 20ms, 2 pulses. After electroporation, cells were put in recovery medium (E8 plus ROCK Inhibitor) and incubated at 37°C/5% CO_2. DNA for analysis of gene editing was collected after a few days of cell growth in culture.

2.2.6. Clonal isolation

After gene editing, edited cells had to be clonally selected in order to achieve a 100% edited cell culture. For that, cells were diluted in order to pipette roughly 1 cell per well in one 96-well plate for each lineage. For better survival of isolated cells, CloneR™ reagent from Stem Cell was added to the cell medium. After a week following medium change and cell growth, cells were observed under the microscope to find formed colonies. Those that are large enough are collected (around 1 mm in diameter) by physically detaching them with a pipette tip and pipetting them into a new well in a 24-well plate for growth. Then, DNA was collected and analyzed through PCR and gel electrophoresis to find gene corrected clones.

2.2.7. Myogenic differentiation of iPSCs

Plates covered in Matrigel are prepared to plate 2.9×10^5 iPSCs in E8 and ROCKi. The medium is changed the next day without adding ROCKi and when the cells reach a 60% confluency the primary differentiation starts in order to produce the myogenic progenitors. For 22 days, a series of medium are used and changed daily, according to the protocol published (CHAL; AL TANOURY; HESTIN; GOBERT *et al.*, 2016), using DiCL/DiCLF for iPSCs derived from fibroblasts, DKI/DKHI, DKHIFL. Then the cells are washed with PBS and incubated with Collagenase IV and TryPLE for 20 minutes at 37ºC. The cells are then homogenized, filtered, centrifuged and resuspended in SKGM with 10 µM of ROCKi. The myogenic progenitor cells are then plated in wells or

flasks covered in Matrigel and expanded in SKGM medium. At this stage, cells can be frozen, if necessary. When cells reach 80% confluency, they are ready for secondary differentiation or terminal differentiation, where myogenic progenitors are differentiated into muscle fibers over 10 days.

2.2.8. Immunofluorescence (IF)

For IF analysis, differentiated muscle fibers were fixated at day 6 of terminal differentiation and prepared for immunostaining. The antibodies used for Dystrophin and Titin were from Abcam and DSHB respectively. The nucleus was stained with DAPI.

2.2.9. Western Blotting (WB)

For WB analysis, samples were extracted in RIPA buffer, quantified using Qubit and loaded in Mini-PROTEAN TGX Precast Gels. The used antibody binds to the rod-domain of dystrophin and is produced by VectorLabs. GAPDH from ThermoFisher was used as an endogenous control. The ladder used was the Precision Plus Protein WesternC standards.

2.3. Results

To further analyze the duplication found in the half-brothers, a CGH-array was performed to measure its size and localization, verify if it would match the minimal common duplicated region and find its breakpoint through PCR. After confirmation, we used CRISPR to correct the duplication, screened edited clones using PCR and gel electrophoresis and selected one DMD corrected clone from each patient for further experiments and analysis (Figure 17).
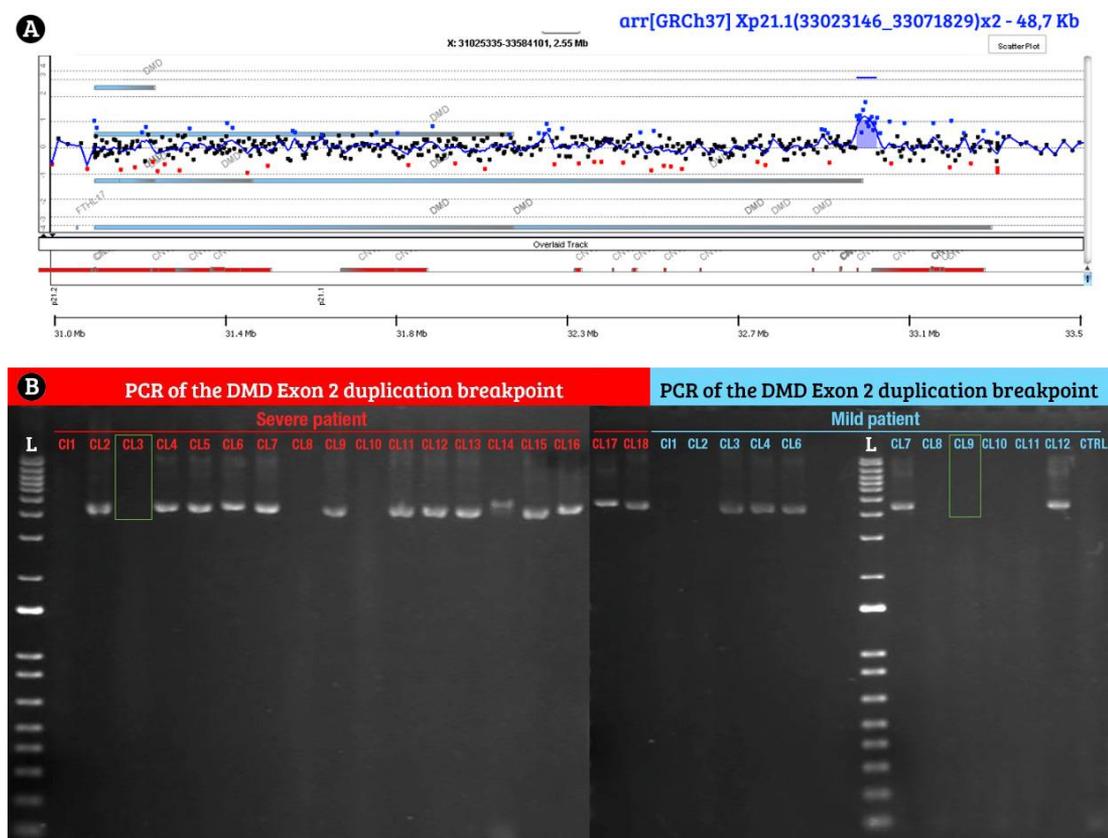
Figure 17: (A) CGH-array of *DMD* performed to assess the size of the duplication and its localization in the half-brother's genome (arr[GRCh37] Xp21.1(33023146_33071829)x2 - 48,7 Kb), confirming that it overlaps with the minimal common duplicated region found in other patients (B) PCR analysis at the site of the exon 2 duplication breakpoint of edited clones selected from both patients: Severe (red) and Mild (cyan). The DNA of clones that amplified a band at around 4.5 Kb did not have their duplication corrected, the clones without any band visible had their duplication corrected. The clones selected for further studies from both patients are highlighted (green box). CL, Clone; L, Ladder (1 Kb Plus DNA Ladder); CTRL, Control (individual without duplication).

Selected clones were analyzed by MLPA specifically for the *DMD* gene to assess the duplication status in corrected cells when compared to unedited cells, as well as to show that no other deletions or genetic anomalies were caused by CRISPR (Figure 18). A PCR analysis was performed for three of the top off-target regions in the genome and no off-target indels were found in patients' corrected clones (Sup. Fig. 1).
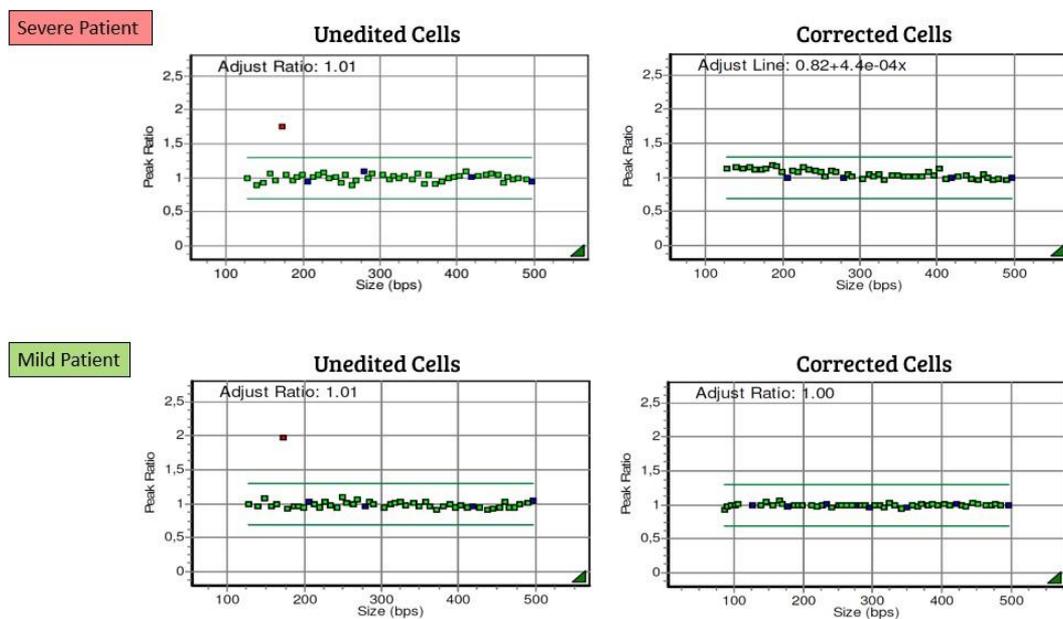
Figure 18: MLPA of the *DMD* gene in unedited and corrected cells from both Severe and Mild patients showing that the clones selected no longer present an exon 2 duplication, as observed in unedited cells.

Then, unedited and corrected cells were differentiated into skeletal muscles *in vitro*, using a transgene-free method. One differentiation failed to produce proper myogenic progenitors (Severe Unedited) and was replaced by the same lineage and clone, but from a previous primary differentiation (Severe Unedited MD25), which was also used for further analysis. Muscle fibers from unedited and corrected cells did not appear to have any observable qualitative differences during differentiation (Fig. 19).

A Severe Unedited MD25 (10X) | Severe DMD Correction (10X)

Day 0

Severe Unedited MD25 (10X) | Severe DMD Correction (10X)

Day 3

Severe Unedited MD25 (10X) | Severe DMD Correction (10X)

Day 6

Severe Unedited MD25 (10X) | Severe DMD Correction (10X)

Day 10

B | Mild Unedited (10X) | Mild DMD Correction (10X)

Day 0

Mild Unedited (10X) | Mild DMD Correction (10X)

Day 3

Mild Unedited (10X) | Mild DMD Correction (10X)

Day 6

Mild Unedited (10X) | Mild DMD Correction (10X)

Day 10

Figure 19: Terminal differentiation of iPSC-derived muscle progenitor cells from the (A) Severe and (B) Mild patient. Comparison of Unedited vs DMD Corrected muscle differentiation: At day 0 the cell culture is mostly composed of satellite cells, myoblasts and myocytes (myogenic progenitors), seen as single cells slightly elongated. After terminal differentiation medium is added to the cell culture, it is possible to observe the formation muscle fibers, seen as very elongated structures, which peak at day 3. As the differentiation continues some of the muscle fibers detach from the culture plate as seen at days 6 and 10. No qualitative difference in muscle differentiation was observed between Unedited and DMD Corrected cells in vitro. MD, Muscle Differentiation; Scale bar = 400 μm.

Muscle fibers were fixated and immunostained for dystrophin and titin, at day 6 of terminal differentiation. To confirm *DMD* correction by comparing protein expression in unedited (DMD), corrected cells and the unaffected father of the mild patient who was used as a normal control. (Sup. Fig. 2). The images show that dystrophin expression was in fact recovered (Figure 20). It was also observed that Pax7$^+$ cells are maintained throughout muscle differentiation (Sup. Fig. 3).
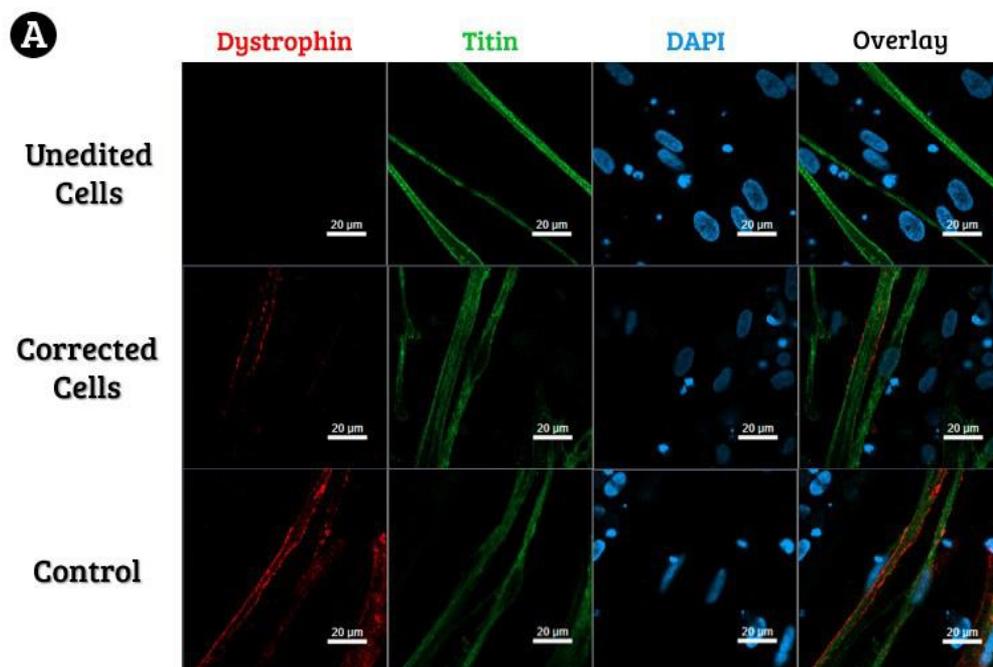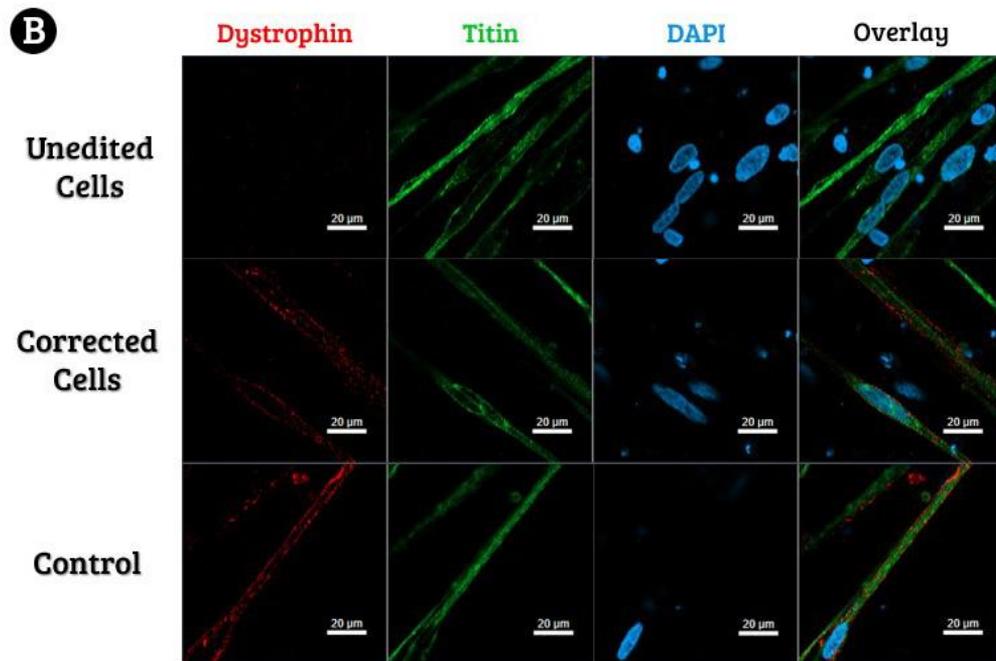
Figure 20: Immunofluorescence of dystrophin in iPSC-derived differentiated muscle fibers of (A) Severe patient and (B) Mild patient – comparing Unedited cells, Corrected Cells and Control (Father) without DMD. Corrected cells recovered dystrophin expression as shown by colocalization with titin, which is also part of the DAPC, when compared with the control. DAPI labels nuclear DNA. Scale bar = 20 μm.

Protein expression was also assessed by Western Blotting, using protein samples extracted from muscle fibers, at day 3 of terminal differentiation. Again, it confirmed the correction in *DMD* and the recovery of dystrophin by comparing the bands of dystrophin in Unedited (DMD), Corrected and Control cells (Figure 21)
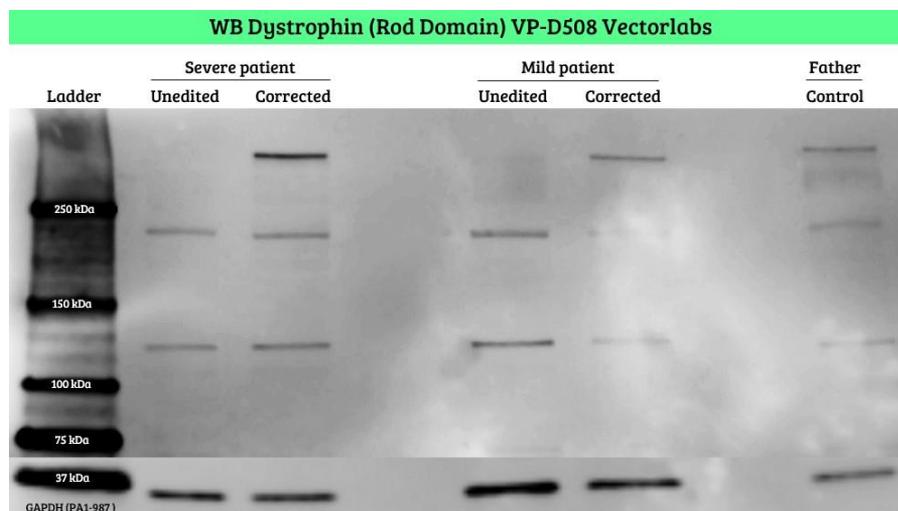
Figure 21: Western Blotting of dystrophin (427 kDa) in Unedited and Corrected muscle fiber samples from Severe and Mild patients, as well as the Father's sample as a control. Confirming that dystrophin expression was recovered in vitro. At the bottom (37 kDa) GAPDH was used as an endogenous control.

2.4. Discussion

In this study we show that it is possible to generate myogenic progenitors capable of differentiating into muscle fibers that produce dystrophin from own patients' gene corrected iPSCs. Furthermore, as muscle fibers are incapable of self-expansion and satellite cells are key in muscle regeneration and growth, it is possible that these *in vitro*-generated dystrophin-corrected myogenic progenitors could represent a promising alternative for treating DMD/BMD. Such transplantation could reduce chances of immune rejection, as the cells are derived from own patients, and deliver satellite cells that once fused to patient's muscle fibers would form myonuclei capable of expressing dystrophin, which could help strengthen the muscle's sarcolemma and deter degeneration, as well as regenerate the muscle. However, possible immune response to the newly introduced Dystrophin protein in patients' muscle must be observed and tackled. This approach could also be employed for other genetic diseases that can be corrected by gene editing techniques and that could benefit from some kind of cellular transplantation therapy.

3. Notch3 Knockout

Abstract: The role of the notch signaling pathway, more specifically Notch3, in controlling the pool of satellite cells and other myogenic progenitors is not yet completely elucidated. Here, a knockout of Notch3 was performed in iPSCs from the two half-brothers with DMD. These cells were differentiated *in vitro* to generate myogenic progenitors and muscle fibers. We observed that Notch3 knockout myogenic progenitor cells show significantly higher gene expression of myogenic markers Pax7, MyoD and MyoG and also grew significantly more than non-edited cells. These observations suggest that Notch3 limits the population of both quiescent and activated satellite cells and might act as an inhibitor of growth by contact. Tweaking Notch3 expression can change the composition of myogenic progenitors during their formation, which could be beneficial when developing a cellular therapy based on these cells.

Keywords: Notch3 Knockout, Satellite Cells, Myogenic Progenitors

Resumo: O papel da via de sinalização do Notch, mais especificamente do *Notch3*, em controlar o *pool* de células satélite e outros progenitores miogênicos não foi totalmente elucidado. Neste estudo foi realizado o *knockout* de Notch3 em iPSCs de dois meio-irmãos com DMD e estas células foram diferenciadas *in vitro* para gerar progenitores miogênicos e fibras musculares. Nós observamos que progenitores miogênicos com *knockout* em *Notch3* possuem expressão gênica significativamente maior dos marcadores miogênicos *Pax7*, *MyoD* e *MyoG* e que elas crescem significativamente mais que células não editadas. Essas observações sugerem que o *Notch3* limita a população tanto de células satélites quiescentes quanto ativadas e pode agir como inibidir de crescimento por contato. Manipular a expressão de *Notch3* pode alterar a composição de progenitores miogênicos, o que pode ser vantajoso no desenvolvimento de terapias celulares baseadas nestas células.

Palavras-chave: *Notch3 Knockout*, Células Satélite, Progenitores Miogênicos

3.1. Introduction

Mouse studies have shown the importance of the Notch signaling pathway and its interacting genes in the development of muscle stem cells and muscle fibers. However, mutations in genes of the Notch signaling pathway are also known to cause genetic diseases in humans. For example, mutations in Notch1 have been linked with structural abnormalities of the aortic valve and are associated with several types of cardiac diseases (SIEBEL; LENDAHL, 2017). Mutations in Jagged1 and Notch2, on the other hand, are associated with another type of disease, known as Alagille Syndrome, defined clinically by cholestasis and possible cardiac, skeletal and ophthalmologic manifestations (MCDANIELL; WARTHEN; SANCHEZ-LARA; PAI *et al.*, 2006). Notch3 mutations are associated with CADASIL, which causes a progressive vascular pathology affecting mainly the brain (HOSSEINI-ALGHADERI; BARON, 2020). Jagged2 mutations were associated with a form of autosomal recessive muscular dystrophy in 13 unrelated families and seems to resemble a form of muscular dystrophy caused by mutations in POGLUT1 (COPPENS; BARNARD; PUUSEPP; PAJUSALU *et al.*, 2021; SERVIÁN-MORILLA; TAKEUCHI; LEE; CLARIMON *et al.*, 2016). In short, receptors, ligands and other agents involved in the Notch signaling pathway network have unique contributions to human health and disease.

In this study, Notch3 knockout cell lines from the two DMD half-brothers were developed to further understand the puzzle piece of the Notch signaling pathway.

3.2. Materials and Methods

3.2.1. Gene editing

To perform a knockout in Notch3 a gRNA targeting the exon 3 of Notch3 was used (CGCTCACCCACCCAGCCAGG).

3.2.2. Western Blotting

Two antibodies from Abcam, to assess Notch1 and Notch3, and two antibodies from Cell Signaling for Notch3 were used.

3.2.3. Real Time-PCR

384-well plates and SYBR Green were used to investigate gene expression. The genes studied were MyoD, MyoG, Notch3, Pax7 and as an endogenous control RPLP0 was used. QuantStudio 12K Flex system was used to run the plates.

3.2.4. xCELLigence

E-Plate 96 PET was used to run a proliferation assay on the xCELLigence RTCA System from Acea Biosciences.

3.2.5. Stastistical Analysis

Results are expressed as mean ± SEM (statistical error of the mean). Differences between groups were evaluated by one-way ANOVA, considering as statistically relevant those with $P < 0.05$, followed by tukey multiple comparison method with the value of 0.05 for alpha (confidence interval of 95%). Graphs and statistics were generated using GraphPad Prism 7.00 - Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3.3. Results

Notch3 knockout was performed on both DMD half-brothers iPSCs, and the genotype of the clones was assessed by Sanger sequencing. The results show that the Severe Notch3 knockout clone we selected had an insertion of one nucleotide in one allele and a minus four nucleotide deletion in the other, which results in a premature stop codon in exons 3 and 5 respectively. In the Mild Notch3 knockout clone, both alleles had a minus five nucleotide deletion that corresponds to a premature stop codon in exon 3. Three of the main off-target were analyzed through Sanger sequencing. In one of the clones, it was found an off-target in one region (RP11-238K6.2), where there is a long noncoding RNA (lncRNA) with no known function (supplementary image 4).

To further confirm whether the gene knockout resulted in the absence of the correspondent protein, WB analysis of Notch3 was performed in iPSCs, in *in vitro* differentiated Notch3 knockout muscle fibers and in myogenic progenitors. For this analysis, three different antibodies against Notch3 were used (Sup. Fig. 5, 6, 7 and 8). All three antibodies showed absence of the bands predicted for Notch3, but close bands above predicted bands were still visible suggesting they might correspond to one of the other, heavier, Notch receptors (Notch1 or Notch2).

Since Sanger sequencing, and absence of Notch3-predicted bands in WB matched, we assumed that the cell lines were knockout for Notch3 and proceeded with further analysis of gene expression and proliferation during terminal muscle differentiation *in vitro*. Unedited, DMD corrected and Notch3 knockout cells from both half-brothers were differentiated into muscle (Fig. 19 and 22), as well as the Father's cells as a control without DMD (Sup. Fig. 2).

Severe Notch3 KO (10X)	Mild Notch3 KO (10X)
Day 0	Day 0
Severe Notch3 KO (10X)	Mild Notch3 KO (10X)
Day 3	Day 3
Severe Notch3 KO (10X)	Mild Notch3 KO (10X)
Day 6	Day 6
Severe Notch3 KO (10X)	Mild Notch3 KO (10X)
Day 10	Day 10
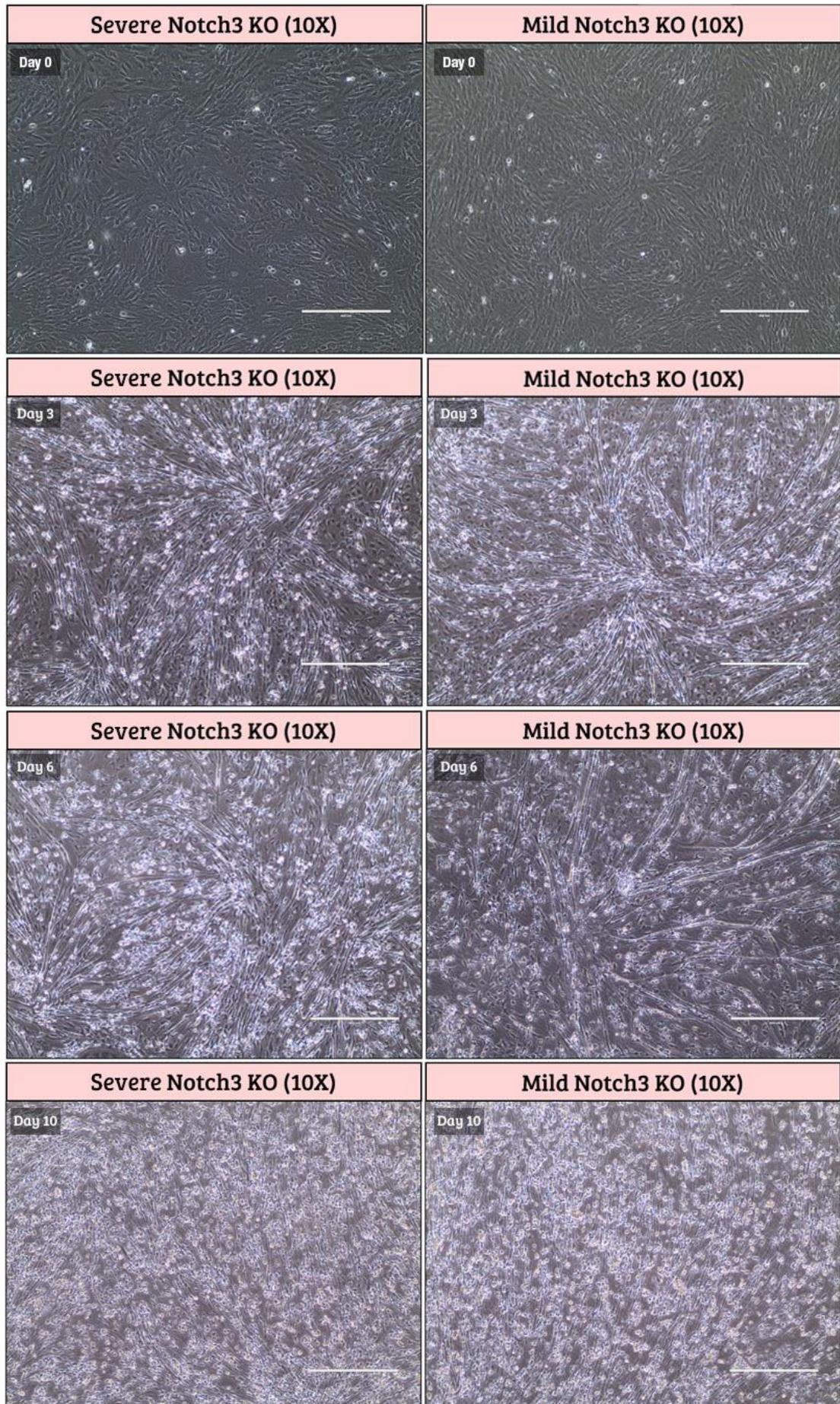
Figure 22: Terminal differentiation of Notch3 knockout muscle progenitor cells from (A) Severe and (B) Mild patients. At day 0 the cell culture is mostly composed of satellite cells, myoblasts and myocytes (myogenic progenitors), seen as single cells slightly elongated. After terminal differentiation medium is added to the cell culture, it possible to see the formation muscle fibers, seen as very elongated structures, which peak at day 3. As differentiation continues some of the muscle fibers detach from the culture plate as seen at days 6 and 10. No qualitative difference in muscle differentiation was observed between Severe and Mild patients, nor from Unedited and Notch3 knockout cells in vitro. MD, Muscle Differentiation; Scale bar = 400 μm.

RNA was collected from four timepoints along differentiation: at days 0, 3, 6 and 10. The expression of four genes were analyzed through RT-qPCR: Notch3, Pax7, MyoD and MyoG. Before differentiation, at day 0, no statistical difference was found in Notch3 expression, but as differentiation proceeded, it was observed that Notch3 knockout cell lines maintained low levels of Notch3 mRNA expression (Figure 23), which is probably the result of the mechanism of non-sense mediated decay due to the premature stop codons (HUG; LONGMAN; CÁCERES, 2016).
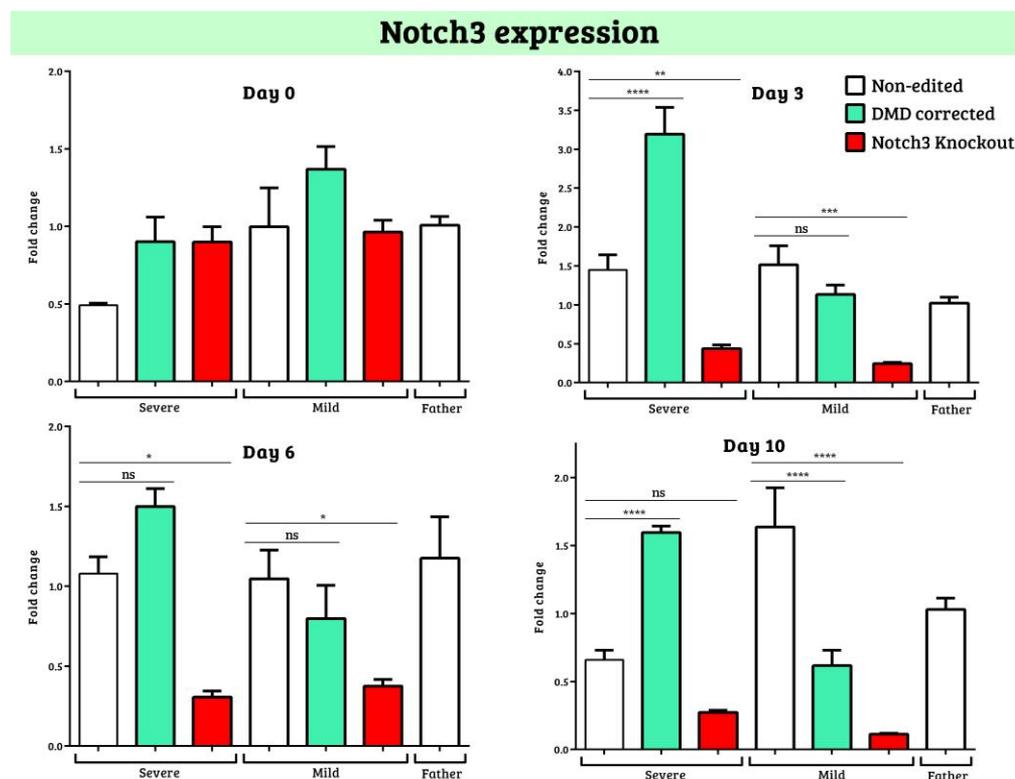
Figure 23: RT-qPCR of Notch3 at four time points in terminal muscle differentiation. Non-edited cells (white), DMD corrected cells (cyan) and Notch3 knockout cells (red) gene expression is compared and normalized by the Father's cell expression of its respective day; ns, not significant.

Pax7 expression was higher in Notch3 knockout cell lines at day 0 of differentiation, since this time point is when the pool of cells is composed mostly by myogenic progenitors. It suggests that a higher pool of quiescent satellite cells is present in Notch3 knockout cells when compared to unedited cells. Throughout terminal muscle differentiation, Pax7 expression is higher than in unedited cells, supporting that self-renewal of Pax7 positive cells is also more prominent in Notch3 knockout cells (Figure 24).
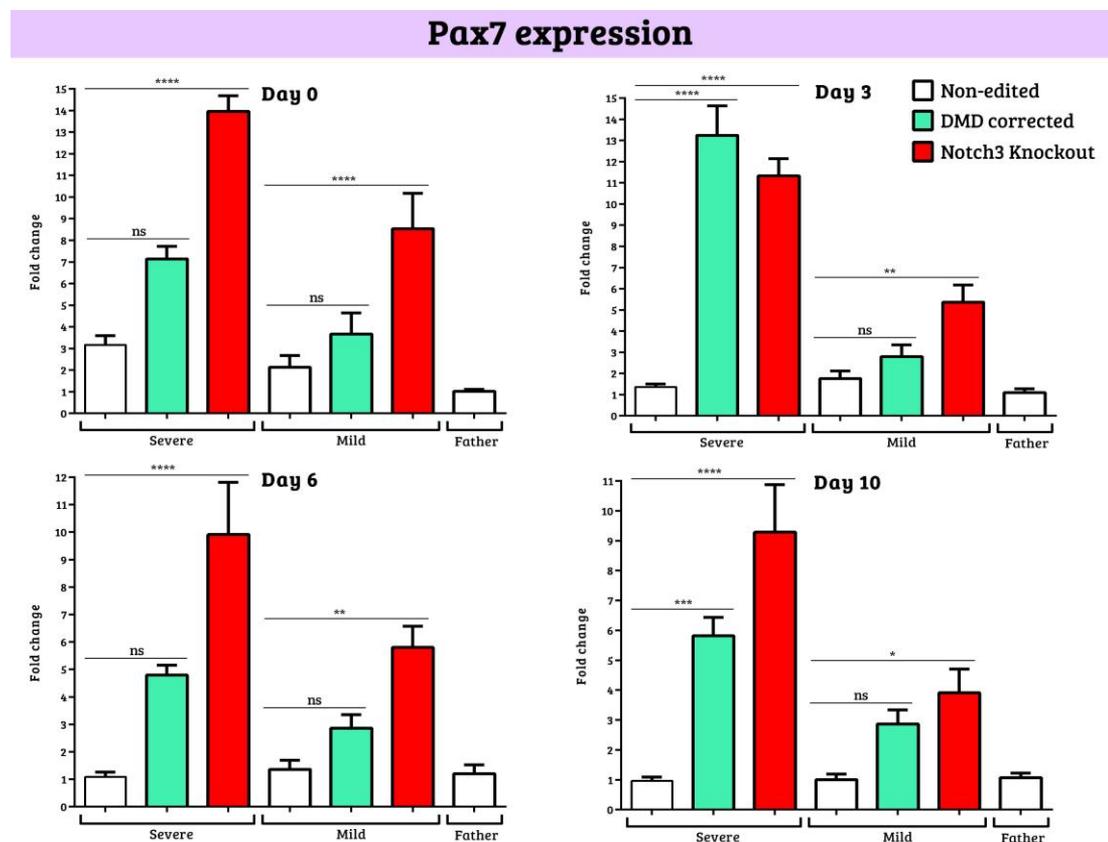
Figure 24: RT-qPCR of Pax7 at four time points in terminal muscle differentiation. Non-edited cells (white), DMD corrected cells (cyan) and Notch3 knockout cells (red) gene expression is compared and normalized by the Father's cell expression of the respective days; ns, not significant.

Myogenic progenitors, at day 0 of terminal muscle differentiation, showed higher expression of MyoD, suggesting that activated satellite cells (myoblasts) are increased in Notch3 knockout cells before differentiation into muscle, but not afterwards (Figure 25)
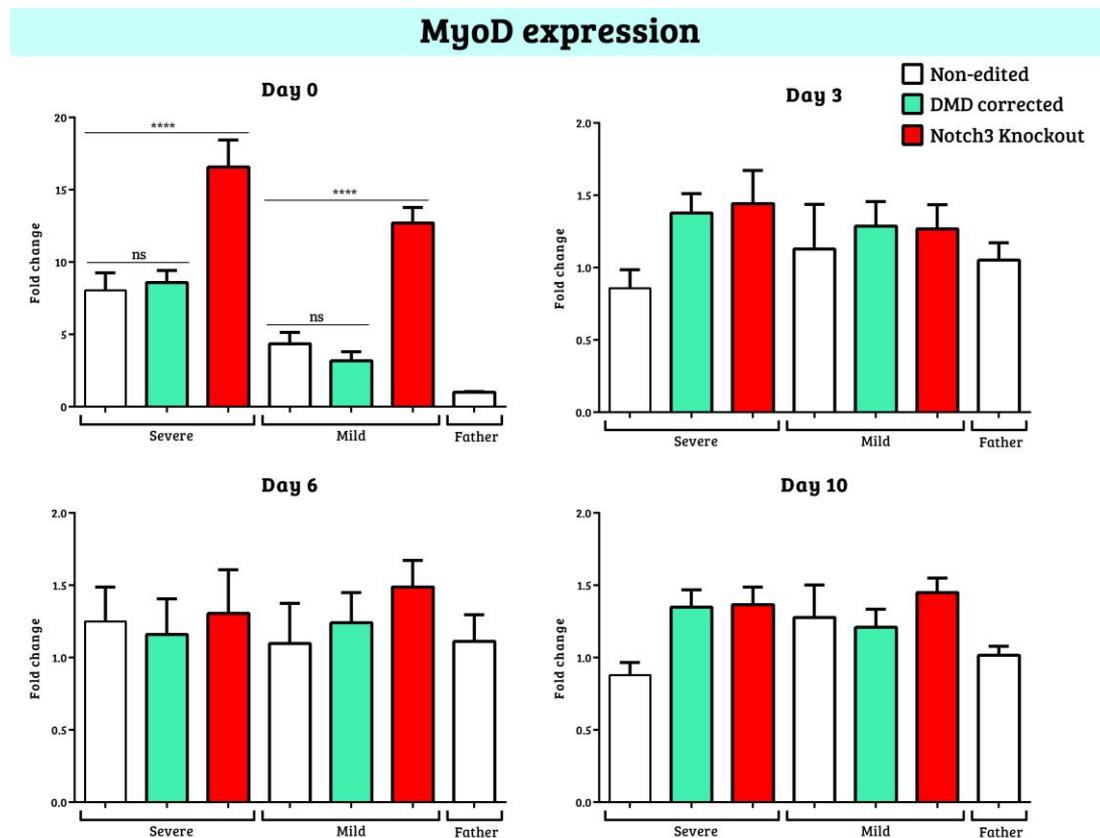
Figure 25: RT-qPCR of MyoD at four timepoints in terminal muscle differentiation. Non-edited cells (white), DMD corrected cells (cyan) and Notch3 knockout cells (red) gene expression is compared and normalized by the Father's cell expression of the respective day; ns, not significant.

A higher myogenin (MyoG) expression was found in myogenic progenitors at day 0, up to a hundred-fold more than in unedited cells (Figure 26), suggesting that Notch3 controls the commitment and maintenance of the muscle satellite cell pool. The absence of Notch3 results in increased numbers of myoblasts (MyoD$^+$) and myocytes (MyoG$^+$), without compromising quiescent satellite cells (Pax7$^+$) and even possibly increasing their numbers, as illustrated

by higher Pax7 expression before and after terminal muscle differentiation.
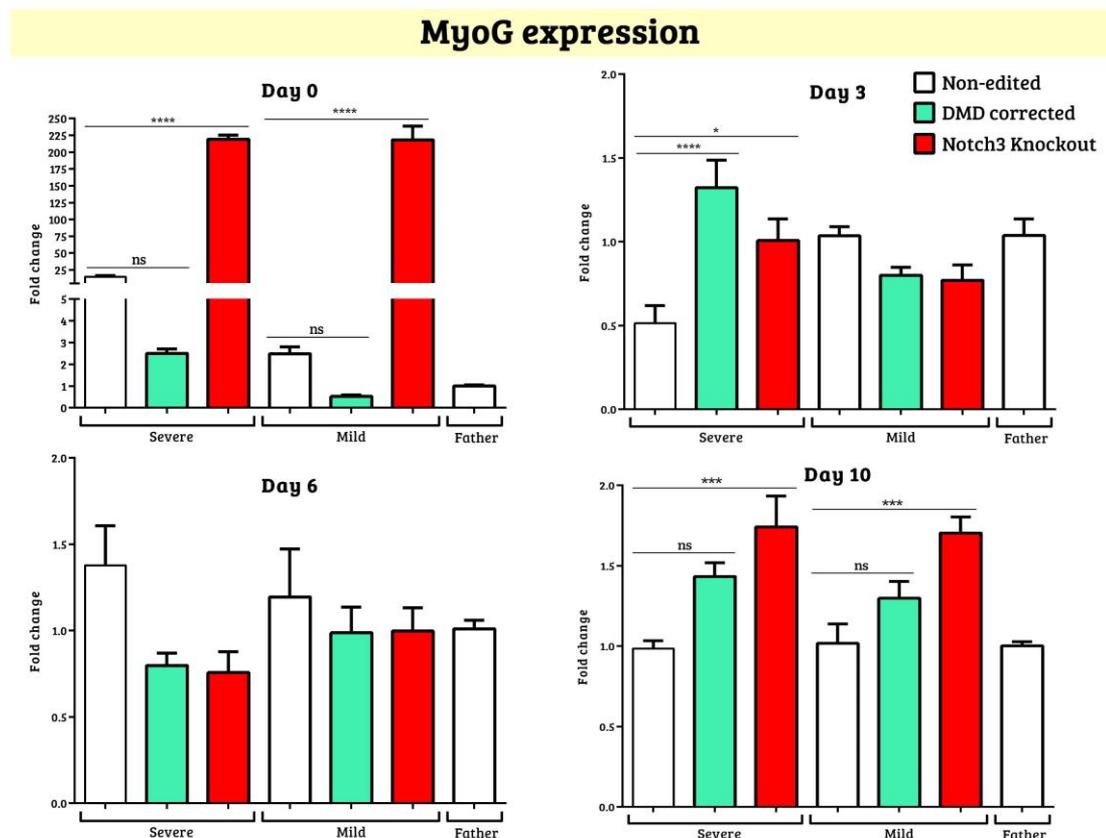
Figure 26: RT-qPCR of MyoG at four time points in terminal muscle differentiation. Non-edited cells (white), DMD corrected cells (cyan) and Notch3 knockout cells (red) gene expression is compared and normalized by the Father's cell expression of the respective days; ns, not significant.

To analyze how Notch3 affects proliferation of myogenic progenitor cells collected at day 0 of terminal differentiation, an assay using the xCELLigence RTCA System was run to monitor cell growth of these cells, without differentiating them, over the course of 10 days (Fig 27). It was found that Notch3 knockout myogenic progenitors were able to grow significantly more than unedited cells, suggesting that Notch3 limits the population of both quiescent and activated satellite cells and might act as an inhibitor of cellular growth by contact.
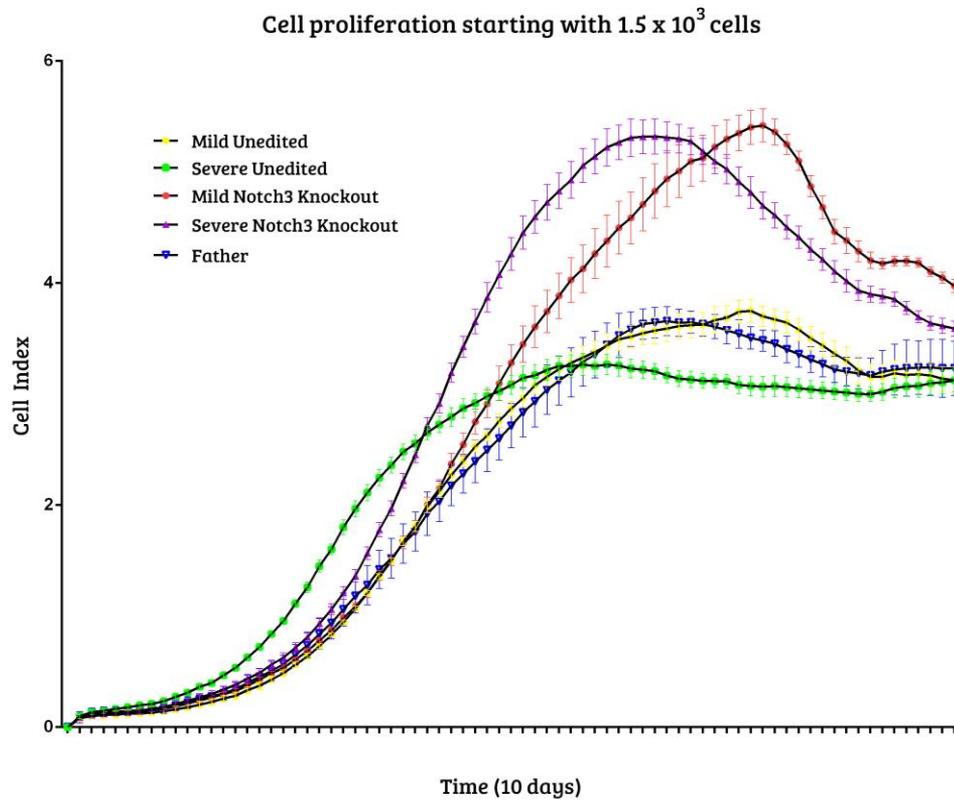
Figure 27: Cell proliferation of myogenic progenitors in unedited and edited cells, starting with 1.5×10^3 cells, over the course of 10 days. Cell index directly correlate with cell growth and proliferation.

3.4. Discussion

The experiments presented in this chapter reveal another puzzle piece of the Notch signaling pathway: Notch3 seems to have a unique control over the pool of myogenic progenitors by restricting their growth and commitment, and this might also be true *in vivo*, as evidenced by other studies using mice models. The absence of Notch3 resulted in a higher pool of activated satellite cells, but it was not in detriment of quiescent satellite cells, as Notch3 knockout cells were capable of increasing the overall pool of myogenic progenitors *in vitro*. Tweaking Notch3 expression might be a way to control the overall composition and relative activation of myogenic progenitors, which could be beneficial when developing a gene and cellular therapy based on these cells.

General Discussion and Conclusion

Here we show that it is possible to generate myogenic progenitors capable of differentiating into muscle fibers that produce dystrophin from own patients' gene corrected iPSCs. Our study suggests that Notch3 seems to have unique control over the pool of myogenic progenitors by restricting their growth and commitment, as the absence of Notch3 allowed for a higher pool of both quiescent and activated satellite cells, increasing the overall pool of myogenic progenitors *in vitro*. The combination of these two strategies could potentialize the use of gene corrected satellite cells for the treatment of dystrophinopathies, since controlling the expression of Notch3 might change the overall composition and relative activation of myogenic progenitors. Furthermore, it is possible that these in vitro-generated dystrophin-corrected myogenic progenitors could reduce immune rejection, as they are derived from own patients' cells. However, a possible immune response to the newly expressed Dystrophin protein in patients' muscle must be considered. Delivering satellite cells with corrected mutations could fuse to patient's muscle fibers, form myonuclei capable of expressing dystrophin, help strengthen the muscle's sarcolemma and decrease degeneration. They could also enhance muscle regeneration, considering that muscle fibers are unable of self-expansion and satellite cells are key in muscle regeneration and growth. This approach could also be customized to target other genetic diseases that can be corrected by gene editing techniques and that could benefit from some kind of gene and cellular transplantation therapy.

Resumo

Nas últimas décadas, diversas terapias foram desenvolvidas e testadas para tratar a Distrofia Muscular de Duchenne (DMD), no entanto nenhuma delas foi capaz de aumentar expressivamente a qualidade ou expectativa de vida dos pacientes, indicando a necessidade de novas abordagens terapêuticas. Além disso, o papel da via de sinalização do Notch, mais especificamente o *Notch3*, em controlar o *pool* de células satélite do músculo e outros progenitores miogênicos não foi totalmente elucidado. Aprofundar nossa compreensão sobre a via do Notch pode ser essencial para a criação de uma terapia capaz de transformar a vida dos pacientes. Neste estudo, CRISPR foi usado para corrigir geneticamente células-tronco pluripotentes induzidas (iPSCs), derivadas da pele de dois meios-irmãos com curso clínico discordante, portadores de uma duplicação no exon 2 de *DMD*. Nosso objetivo era investigar a viabilidade de se gerar células satélite corrigidas geneticamente e capazes de se diferenciar em músculo que produz distrofina. Paralelamente, para estudar os efeitos da via do Notch na formação dos progenitores miogênicos *in vitro*, foram diferenciadas células não editadas e *Notch3 knockout* dos mesmos pacientes através de uma metodologia livre de transgenes. Os resultados mostram que é possível gerar células satélite e outros progenitores miogênicos *in vitro* corrigidos geneticamente para o gene *DMD* e capazes de produzir distrofina, de forma que estas células podem ser potencialmente utilizadas no desenvolvimento de uma nova terapia celular e gênica, reduzindo-se as chances de uma rejeição imunológica por se tratarem de células do próprio paciente. Contudo, uma possível resposta imunológica à proteína Distrofina introduzida ao músculo dos pacientes precisa ser investigada. Também foi observado que progenitores miogênicos com *knockout* em *Notch3* possuem expressão gênica significativamente maior dos marcadores miogênicos *Pax7*, *MyoD* e *MyoG* e que estas células crescem notadamente mais que células não editadas, sugerindo que o *Notch3* limita a população de células satélites quiescentes e ativadas, podendo agir como um inibidir de crescimento por contato. Este estudo propõe que ao manipular a expressão de *Notch3* é possível alterar a composição dos progenitores miogênicos, o que pode ser vantajoso no desenvolvimento de uma terapia celular e gênica baseada nestas células.

Abstract

Over the last decades, several therapies have been developed and tested to treat Duchenne Muscular Dystrophy (DMD), yet none of them had expressive improvement in life expectancy or quality of life for patients, indicating the need of new treatment approaches. Furthermore, the role of the Notch signaling pathway, more specifically Notch3, in controlling the pool of muscle satellite cells and other myogenic progenitors is not completely elucidated. Enhancing our comprehension on the Notch pathway might be essential for the development of a therapy that could be life changing for patients. In this study, CRISPR was used to genetically correct induced Pluripotent Stem Cell (iPSCs), derived from the skin of two half-brothers with a discordant clinical course, carrying an exon 2 duplication in *DMD*. Our aims were to investigate the viability of generating genetically corrected satellite cells capable of differentiating into muscle that produces dystrophin. In parallel, to study the effects of the Notch signaling pathway in the formation of myogenic progenitors *in vitro*, non-edited and Notch3 knockout cells from the same patients were differentiated with a transgene-free method. The results show that it is possible to generate satellite cells and other myogenic progenitors *in vitro* that are genetically corrected for *DMD* and capable of producing dystrophin. Therefore, these cells can be potentially used in the development of a new gene and cellular therapy, reducing the chances of immune rejection as patients' own cells are used. However, a possible immune response to the Dystrophin protein introduced to patients' muscle must be considered. It was also observed that Notch3 knockout myogenic progenitors express significantly more the myogenic markers Pax7, MyoD and MyoG and that these cells grow notably more than non-edited cells, which suggests that Notch3 limits the population of quiescent and activated satellite cells, possibly acting as an inhibitor of growth by contact. This study proposes that by manipulation Notch3 expression it is possible to alter myogenic progenitors' composition, which may be advantageous in the development of a gene and cellular therapy based on these cells.

Supplementary Attachments and Appendices
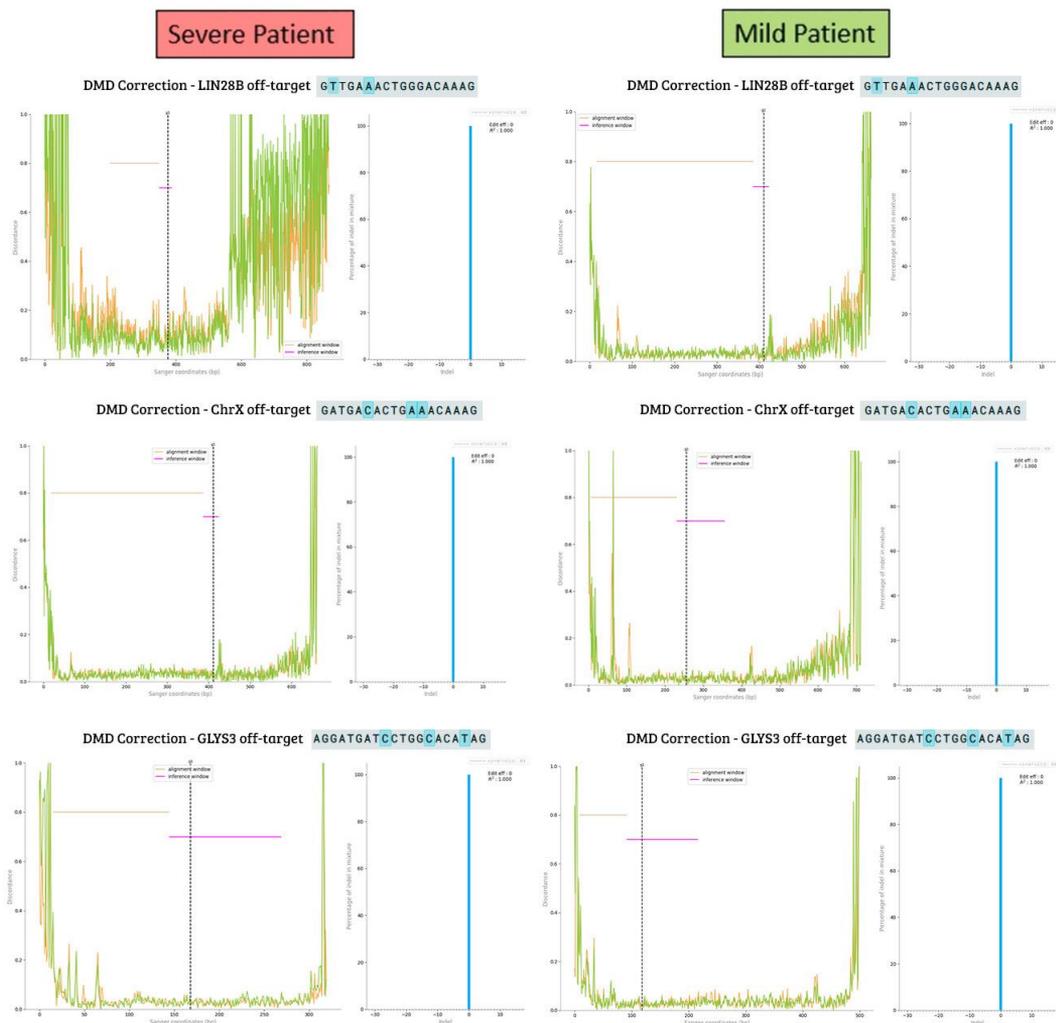
Supplementary Figure 1: Three off-targets were assessed in each patient's DMD corrected clones. The three main off-targets were in the regions LIN28B-AS1 (chr6), intronic ChrX and GLYS3 (chr9). The bases that differed from the gRNA in these regions are highlighted in the blue boxes above the graphs. On the left of each graph, the decomposition of the Sanger sequences compares the unedited and corrected clones by aligning the sequences (alignment window) and comparing the interference window around the cut site (dashed line), on the right you can see the indels found in the analysis, where 0 means no indel was found in that region.
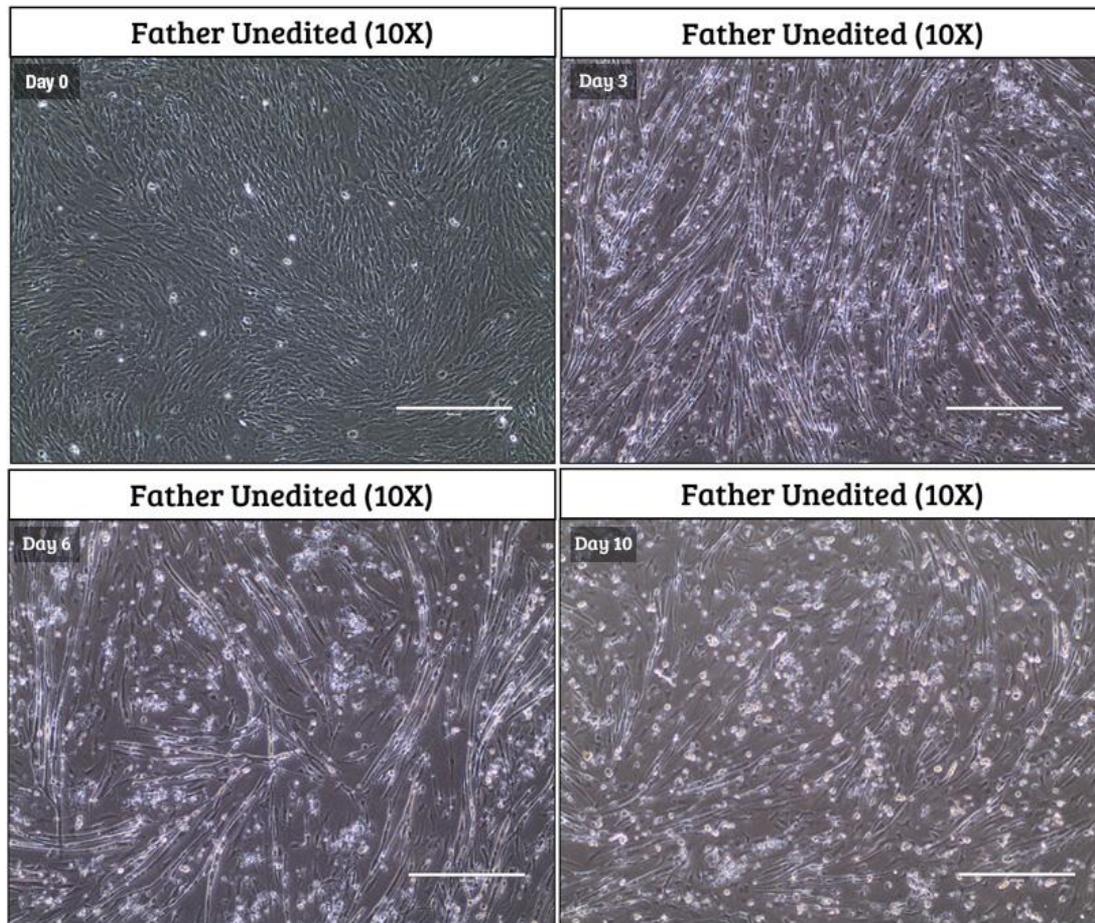
Supplementary Figure 2: Terminal differentiation of Father's muscle progenitor cells. At day 0 the cell culture is mostly composed myogenic progenitors, seen as single cells slightly elongated. Formation of muscle fibers peak at day 3, some of the muscle fibers detach from the culture plate as seen at days 6 and 10. MD, Muscle Differentiation; Scale bar = 400 μm.
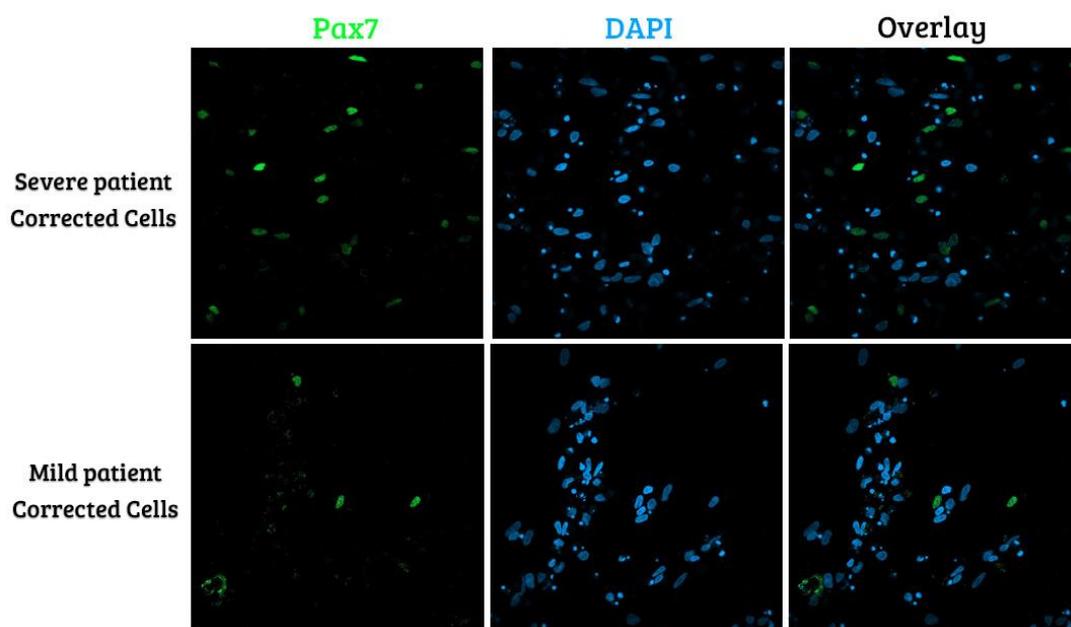
Supplementary Figure 3: Immunofluorescence of Pax7 in iPSC-derived differentiated muscle fibers and myogenic progenitors of Severe patient and Mild patients, showing the presence of Pax7$^+$ cells after 6 days of terminal muscle differentiation. DAPI labels nuclear DNA. 400x magnification.
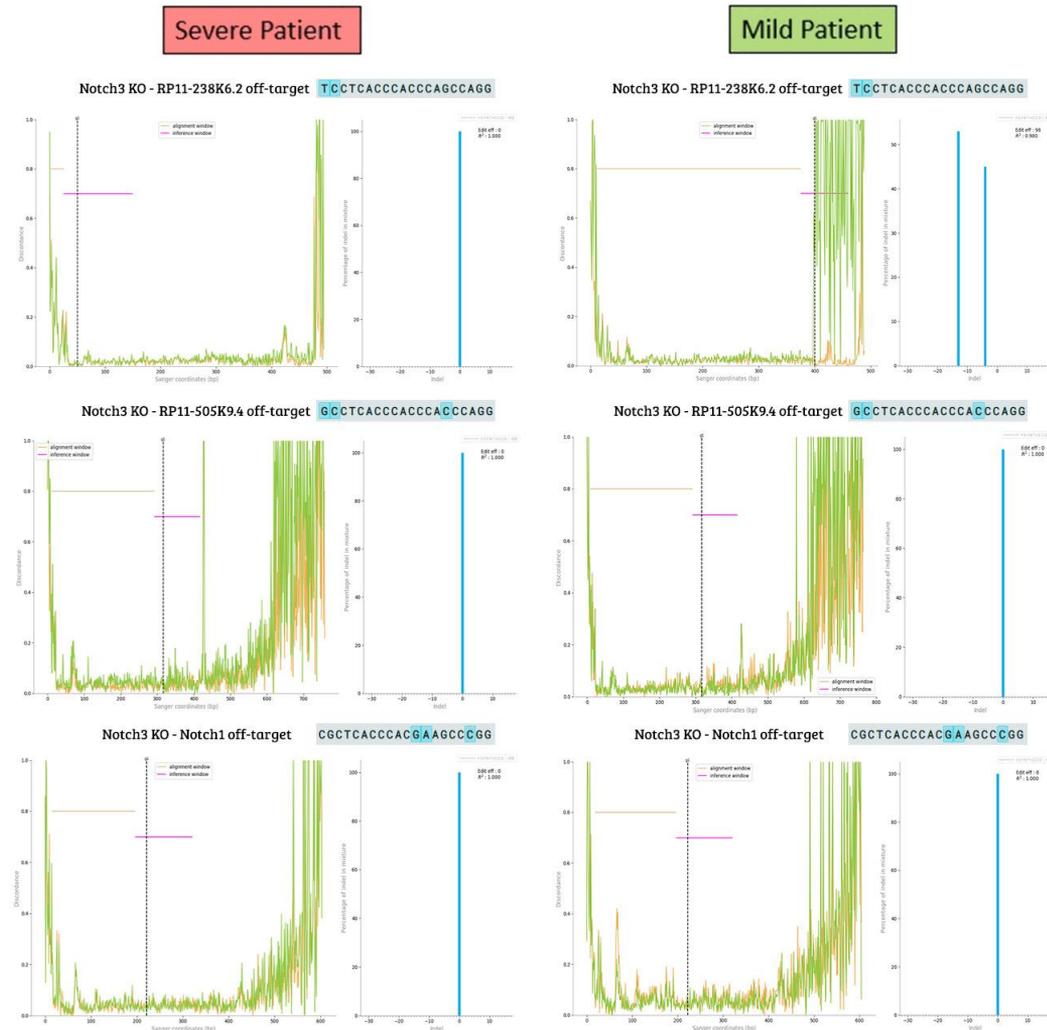
Supplementary Figure 4: Three off-targets were assessed in each patient's Notch3 knockout clones. The three main off-targets were in the regions RP11-238K6.2 (chr8), RP11-505K9.4 (chr16) and Notch1 (chr9). The bases that differed from the gRNA in these regions are highlighted in the blue boxes above the graphs. On the left of each graph, the decomposition of the Sanger sequences compares the unedited and corrected clones by aligning the sequences (alignment window) and comparing the interference window around the cut site (dashed line), on the right you can see the indels found in the analysis, where 0 means no indel was found in that region. In the RP11-238K6.2 region, the Mild patient's clone presented with off-target indels, in this region there is no protein transcript, but there is a lncRNA with no described function.
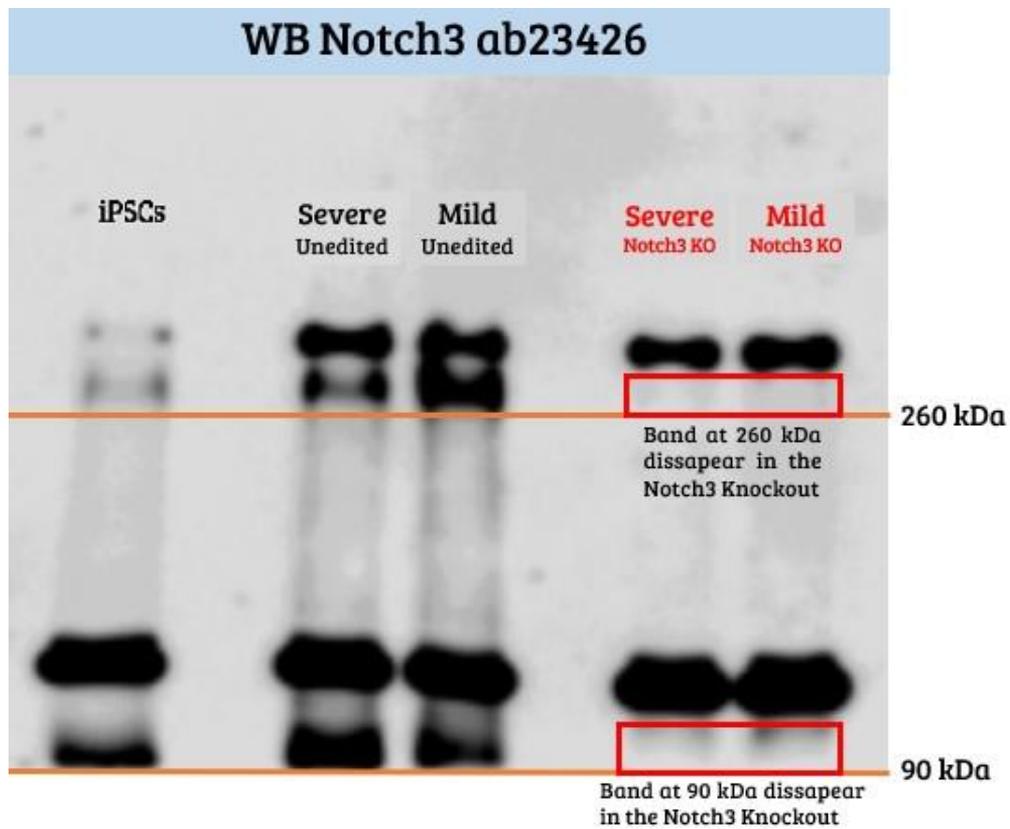
Supplementary Figure 5: Western Blotting of Notch3 using polyclonal antibody from Abcam in Unedited iPSCs, as well as in Unedited and Notch3 knockout cells, at day 6 of muscle differentiation, from Severe and Mild patients. It was observed that in Notch3 knockout cell lines, two bands were absent: one around 260 kDA and another at around 90 kDa, which might correspond to Notch3 extracellular domain (ECD) and intracellular domain (ICD) respectively (red boxes). The other unspecific bands might be from other Notch receptors.
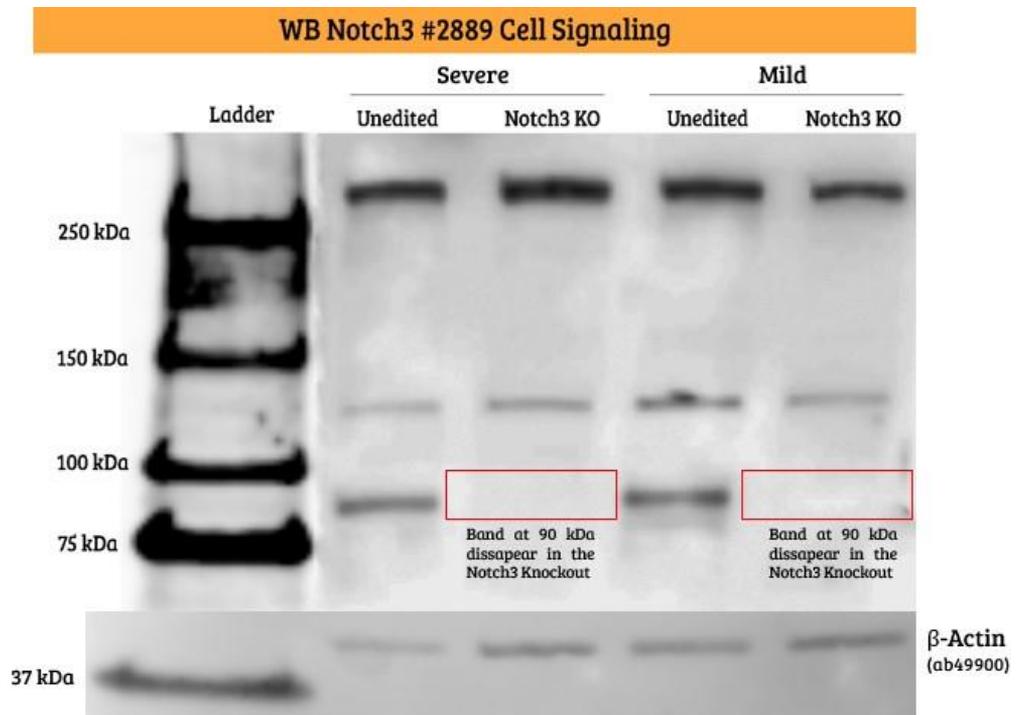
Supplementary Figure 6: Western Blotting of Notch3 using polyclonal antibody from Cell Signaling in Unedited and Notch3 knockout cells, at day 3 of muscle differentiation, from Severe and Mild patients. It was observed that in Notch3 knockout cell lines, one band is absent at around 90 kDa (red boxes), which might correspond to Notch3 ICD. The other unspecific bands might be from other Notch receptors.
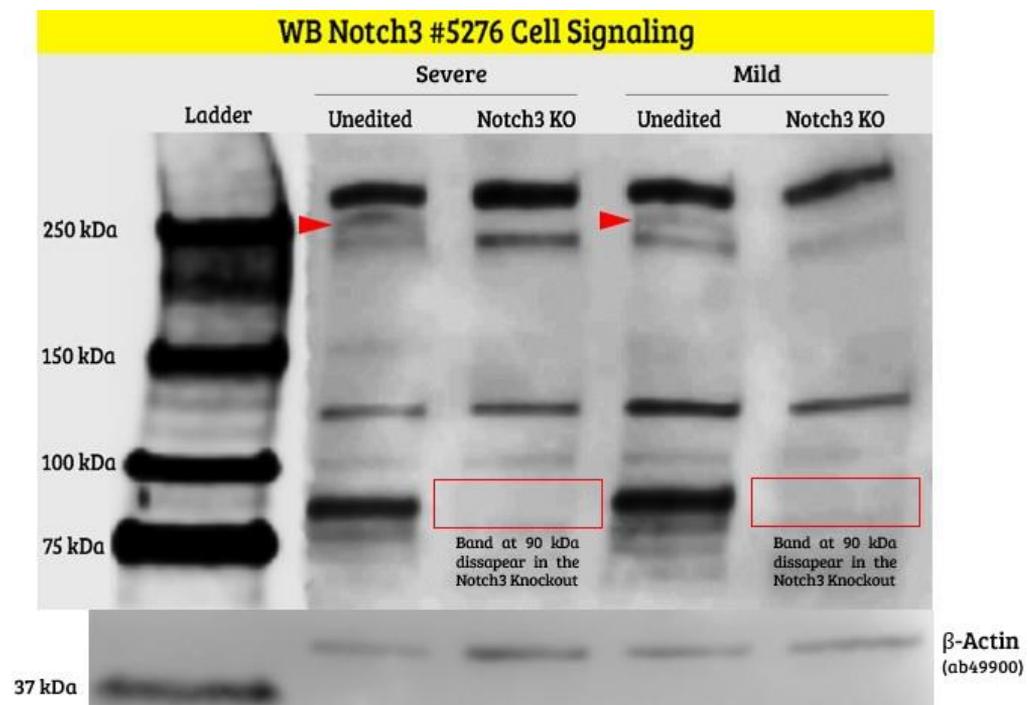
Supplementary Figure 7: Western Blotting of Notch3 using monoclonal antibody from Cell Signaling in Unedited and Notch3 knockout cells, at day 3 of muscle differentiation, from Severe and Mild

patients. It was observed that in Notch3 knockout cell lines, one band is absent at around 90 kDa (red boxes), which might correspond to Notch3 ICD, but it was also observed an intermediary band at around 260 kDa (arrowhead), that is absent in knockout cells and might correspond to the Notch3 ECD. The other unspecific bands might be from other Notch receptors.
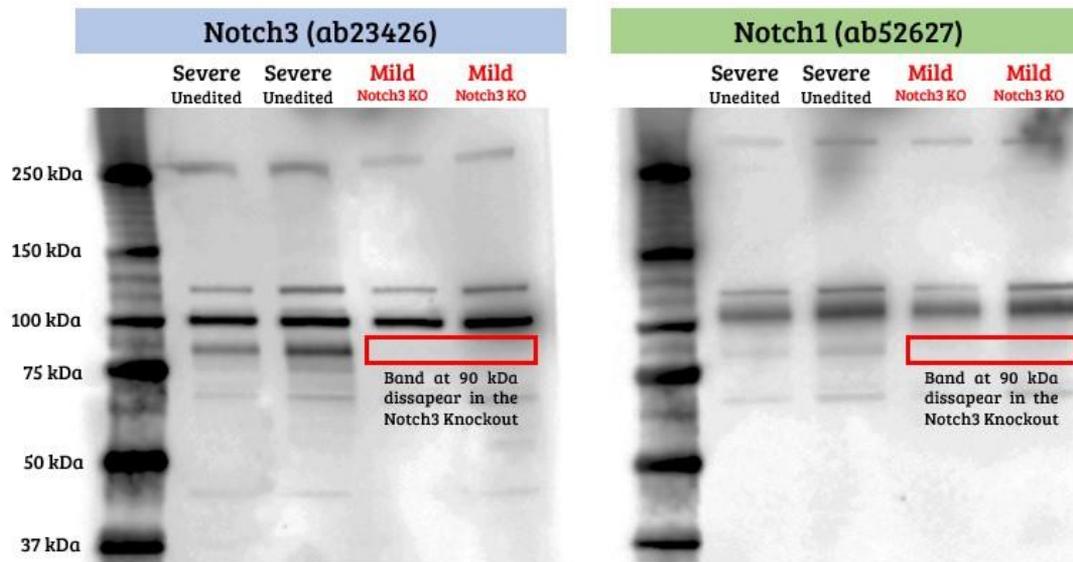
Supplementary Figure 8: Western Blotting of Notch3 using polyclonal antibody from Abcam and Notch1 using a monoclonal antibody from Abcam in Unedited and Notch3 knockout cells, at day 3 of muscle differentiation, from Severe and Mild patients. It was observed that in Notch3 knockout cell lines, one band was absent at around 90 kDa in both antibodies (red boxes), which might correspond to Notch3 ICD. There seems to be a pattern of unspecific staining of different Notch receptors among Notch antibodies.

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