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"Diferenciación miogénica de células madre pluripotentes inducidas de humano: una herramienta para la terapia celular y el modelamiento *in vitro* de la Distrofia Miotónica tipo 1"

"*Myogenic differentiation of human induced pluripotent stem cells: a tool for cell therapy and disease modelling of Myotonic Dystrophy type 1*

TESIS

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CONTENT

RESUMEN

Distrofia Miotónica tipo 1 es una enfermedad multisistémica que afecta principalmente al sistema nervioso central, corazón y músculo esquelético. Es causado por una expansión de los repetidos CTG en la región no traducida 3' del gen *DMPK*. Debido a la falta de terapias y tratamientos para DM1, evaluamos la efectividad del trasplante celular como enfoque terapéutico en el contexto del músculo esquelético. Cruzamos ratones HSA^{LR} (modelo de DM1) con ratones NSG para generar ratones inmunodeficientes-DM1 (NSG-HSA^{LR}). Corroboramos la inmunodeficiencia de estos ratones por la ausencia de células T, B y NK en sangre periférica, y validamos la presencia de agregados nucleares de RNA, el secuestro de MBNL1 y la afección en los eventos de *splicing* alternativo. Se trasplantaron progenitores miogénicos no afectados en músculos tibialis anterior de ratones NSG-HSA^{LR} y se observó adecuada adaptación al tejido. Inesperadamente, observamos que los núcleos provenientes de las células transplantadas mostraron la presencia de agregados nucleares de RNA, sugiriendo que la fusión de células trasplantadas con fibras musculares preexistentes promueve la transmisión intra-fibra de los agregados nucleares. Estos resultados no fueron observados en ensayos de co-diferenciación *in vitro*. En resumen, este nuevo modelo inmunodeficiente con DM1 permite estudios de trasplante de células humanas, lo cual representa una herramienta útil para estudiar el enfoque de trasplante celular en DM1.

La patología muscular de DM1 ha sido estudiada principalmente a través de mioblastos obtenidos de pacientes, sin embargo, el procedimiento de biopsia es invasivo y la disponibilidad de las muestras es limitada. Lo anterior genera la necesidad de desarrollar alternativas de modelos miogénicos para el estudio de la enfermedad. Establecimos dos líneas de células madre pluripotenciales inducidas a partir de fibroblastos de pacientes. Mediante el uso del sistema de expresión condicional de PAX7, diferenciamos las células iPS a progenitores miogénicos y, subsecuentemente a miotubos. Observamos que los progenitores miogénicos, así como los miotubos, muestran agregados intranucleares de RNA con el consecuente secuestro de MBNL1 y la desregulación de eventos de *splicing* alternativo relacionados a DM1. Utilizamos este modelo celular para probar la eficiencia del uso de oligonucleótidos antisentido (OAS) y encontramos una disminución significativa de los agregados de RNA y la recuperación en el *splicing* alternativo de *BIN1*. Los resultados demostraron que los miotubos derivados de células iPS recapitulan las características moleculares de DM1 y son útiles para modelaje *in vitro* de la enfermedad y para probar potenciales fármacos.

ABSTRACT

Myotonic Dystrophy 1 (DM1) is a multi-system disorder primarily affecting the central nervous system, heart and skeletal muscle. It is caused by an expansion of the CTG trinucleotide repeats within the 3' untranslated region of the *DMPK* gene. Due to the lack of therapies for DM1, we evaluated the effectiveness of cell transplantation as a therapeutic approach for the skeletal muscle pathology. We crossed HSA^{LR} mice (mouse model for DM1) with NSG mice to generate an immunodeficient-DM1 mouse model (NSG-HSA^{LR}), which allows us to evaluate the engraftment potential of myogenic progenitors. Immunodeficiency of NSG-HSA^{LR} mice was corroborated by a complete lack of NK, T and B cell populations in peripheral blood. Also, cryo-sections of NSG-HSA^{LR} tibialis anterior (TA) muscle showed the classic DM1 features, such as intranuclear RNA *foci*, MBNL1 sequestration by the foci and the consequent mis-splicing events. Transplantation of non-affected human iPS cell-derived myogenic progenitors in TA muscles of NSG-HSA^{LR} mice showed successful engraftment of the transplanted cells. Surprisingly, we observed that nuclei expressing human lamin A/C also showed positive staining for RNA *foci*, suggesting that upon fusion with preexistent fibers, non-diseased human nuclei acquired the RNA *foci* from NSG-HSA^{LR} endogenous nuclei. Interestingly, this was only observed *in vivo*, since *in vitro* co-differentiation studies did not show the RNA *foci* transmission. In summary, this novel immunodeficient mouse model for DM1 is suitable for the transplantation of human cells, thus representing a useful tool to study the feasibility of cell-based therapy for myotonic dystrophy 1.

Although patient-derived myoblasts have been used to study the muscle pathology of the disease, the invasiveness of the sample collection procedure, as well as the low accessibility to muscle biopsies, motivate the development of alternative myogenic models. We established two iPS cell lines from DM1 patient-derived fibroblast and, using the PAX7 conditional expression system, differentiated them into myogenic progenitors, and subsequently, terminally differentiated myotubes. Both, DM1 myogenic progenitors and myotubes, were found to express intranuclear RNA foci, exhibiting sequestration of MBNL1, along with DM1-related mis-splicing events. Treatment of iPS cell-derived DM1 myotubes with antisense oligonucleotides (ASO) efficiently abolished the RNA foci and rescued *BIN1* mis-splicing. These results demonstrate that myotubes derived from DM1 iPS cells recapitulate the main molecular features of DM1 and are sensitive to ASO treatment, confirming that these cells can be used for *in vitro* disease modeling and drug testing.

INTRODUCTION

1. Myotonic Dystrophy 1

Muscular dystrophies (MD) are an heterogenous group of hereditary diseases that cause progressive muscle weakness and wasting. Myotonic dystrophy type 1 (DM1) is the most prevalent type of muscular dystrophy in adults worldwide (affecting 1 in 8000 individuals)[1]. DM1 is an autosomal dominant disease first described in 1909 by Hans Steiner as a progressive muscle disorder causing myotonia (muscle hyperexcitability that causes a delay in the relaxation time upon muscle contraction)[2]. Nowadays, DM1 is described as a multisystemic disease that affects skeletal muscle, central nervous system and heart, primarily.

1.1. Clinical presentation of DM1

DM1 can cause clinical manifestations in a variety of organs and tissues, which complicates the proper diagnosis of the disease. Moreover, the severity of the symptoms varies among patients according to the age of onset. Therefore, DM1 can be classified as: congenital, childhood, adult/classical or late-onset[1]. Classical and congenital DM1 are of main interest as classical DM1 is the most frequent, while congenital DM1 has a higher mortality rate and more severe symptoms.

- Classic DM1

Besides myotonia, classic DM1 is characterized by causing weakness and wasting in distal muscles. It may also cause cardiac conducts abnormalities, arrhythmias, central nervous system damage and respiratory failure. Other symptoms include cataracts, mental retardation, gastrointestinal abnormalities, insulin resistance and testicular atrophy. The pattern of symptoms and their severity is variable among patients[1-3].

- Congenital DM1

The phenotype observed in congenital DM1 is usually different to the one observed in classic DM1. Symptoms before birth include decreased fetal movement and polyhydramnios. Newborns also show bilateral facial weakness that leads to sucking difficulties. Although symptoms are severe in most cases, congenital DM1 newborns do not show myotonia. Mortality rate is usually high in the first hours after born, mainly due to respiratory failure. After the neonatal stage, prognosis improves, although motor difficulty is still present. During the second decade of life,

patients show severe symptoms of classic DM1, including myotonia, and mortality increases mainly due to cardiomyopathies and cardiac arrhythmias[2-4]

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1.2. DM1 Genetics

Along the genome, there are short repeated sequences of DNA (1-6 nucleotides). These are referred as microsatellite DNA or short tandem repeats (STRs). In humans, most of the STRs are located within non-coding regions, mainly in chromosome 19. During cell division, the STRs length is generally stable. However, some of these sequences might expand in their number of repeats, causing polymorphisms among the population[5-7]. Interestingly, expansions of trinucleotide repeats are frequently associated with a pathology[8]. In 1992, it was described that DM1 is caused by a CTG triplet repeat expansion within the 3' untranslated region (UTR) of the dystrophia myotonica protein kinase (*DMPK)* gene, located on chromosome 19q13.3[9]. Non-affected individuals have between 5 and 37 CTG repeats, whereas DM1 patients can bear from 50 to several hundreds or thousands repeats. Expansions of 38-50 CTG repeats do not cause significant clinical manifestations. However, these repeats tend to be very unstable, leading to pathological expansions in the progeny. Classic DM1 symptoms are observed in patients bearing from 50 to 1,000-2,000 repeats, whereas congenital DM1 is usually related to expansions larger than 2,000 repeats. In general, the severity of the symptoms is directly proportional to the length of the CTG repeat expansion and inversely proportional to the age of onset.

1.3. DM1 molecular pathogenesis

Although various molecular events related to the CTG repeat expansion have been associated with the disease phenotype, the most relevant is a toxic RNA gain-of-function of the *DMPK* mutant transcripts[10-13]. *DMPK* mRNAs containing expanded CUG repeats fold into extended stem-loop structures that accumulate in the nucleus as RNA *foci*[14-16]. Within the nucleus, these RNA *foci* interact with RNA binding proteins, such as Staufen 1 (also known as STAU1), hnRNP H and members of muscleblind protein family (MBNL). MBNL1, MBNL2 and MBNL3 are alternative-splicing regulators that are sequestered by the intranuclear RNA *foci*, therefore their activity is altered[17,18]. Particularly, disruption in the alternative splicing of MBNL1 target genes is the main molecular feature associated with DM1 skeletal muscle pathology[19-21].

Accumulation of DM1 RNA *foci* has also been associated with an increased phosphorylation, and subsequent activation, of CELF1 (CUG-BP, Elav-like family member 1), another alternative

splicing regulator. Overexpression of CELF1 in a transgenic mouse model also resembles pathological features of DM1[20,22,23].

1.4. DM1 Diagnosis and treatment

Myotonia is the most evident sign suggesting DM1 in patients. However, the variability in the severity of the symptoms, organs affected and age of onset among patients may complicate the clinical diagnosis. Thus, molecular diagnosis (*e.g*. Southern blot, small pool PCR or triplet-repeat primed PCR) is needed to confirm the disease and to distinguish it from DM2 (myotonic dystrophy type 2).

To date, there is no cure for DM1, although recent reports have shown promising phenotypic rescue when using modified antisense oligonucleotides to eliminate the RNA *foci in vitro* and *in vivo* using animal models[24] . Alternative experimental approaches under investigation include decreasing *DMPK* transcripts (normal and mutant) or restoring MBNL1 proper function by inducing its overexpression[25,26]. More recently, gene therapy approaches have been developed to delete the CTG repeats directly at the *DMPK* 3'UTR or to add a polyadenylation signal upstream the repeats so that the transcripts do not contain the expanded CUG[27-30]. Nonetheless, there is still a need of developing novel therapeutic approaches that improve the quality of life of DM1 patients.

2. Cell-based therapy approach in muscular dystrophies

To date, there is no cure or efficient treatment that improves the muscle function in muscular dystrophies, including DM1. A promising approach involves transplanting healthy muscle cells that restore the damaged tissue. Skeletal muscle is made of aligned multinucleated cells called myofibers, which are the functional contractile units. Between the sarcolemma and the basal lamina of mature muscle cells, there is a population of undifferentiated quiescent cells called satellite cells. Satellite cells are activated under stress or injury and proliferate undergoing asymmetric division. A population of activated satellite cells differentiate into myoblasts that are able to fuse with existing myofibers or to generate new myofibers, whereas another population proliferate to maintain the satellite cell pool[31]. Due to the ability of myoblasts to fuse and form multinucleated myotubes *in vitro,* the first attempts of cell therapy for muscular dystrophies included intramuscular transplantations of non-affected mouse myoblasts (C2C12) into mdx mice (mouse model for Duchenne Muscular Dystrophy, DMD, where the protein dystrophin is not

properly expressed)[32]. Despite the success in recovering dystrophin expression in engrafted mdx muscles, clinical trials in DMD patients failed mainly due to low engraftment efficiency[33].

2.1. Embryonic stem cells and induced pluripotent stem cells for a cell-based therapy approach in MDs.

Embryonic stem cells (ES cells) are a population of cells derived from the inner cell mass of the blastocyst. These cells have unlimited self-renewal capabilities and pluripotency, i.e. they are able to differentiate into any cell type from the three germ layers, including skeletal muscle. Thus, there has been an increasing interest to differentiate ES cells to the myogenic lineage in order to generate a population of myogenic cells able to engraft upon *in vivo* transplantation. To achieve this, it was shown that inducible expression of Pax3 or Pax7 (iPax3 iPax7), two transcription factors key during myogenesis, under embryoid body (EB) culture conditions of mouse ES cells allowed the generation of a PDGFαR^{+}/Flk1^{-} population that gives raise to myogenic progenitors. These mES cells-derived myogenic progenitors were able to engraft upon intramuscular transplantation in mdx mice and recovered dystrophin expression along with an improvement in muscle force measurements[34-36].

Although ES-derived cell therapy approaches have shown promising results in mice models, there are ethic limitations on the use of ES cells for their potential applications in humans. To overcome these hurdles, reprogramming of somatic cells to induced pluripotent stem cells (iPS cells) has become of great interest. iPS cells can be derived from a variety of somatic cells through the exogenous expression of reprogramming factors, also called Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc), and resemble the self-renewal and pluripotent capabilities of ES cells[37,38]. Similar to ES cells, human iPS (hiPS) cells can also be differentiated into myogenic progenitors through an EB-iPax7 protocol that can either differentiate *in vitro* to myotubes or engraft upon intramuscular transplantation in immunodeficient mice, including immunodeficient mdx (NSG-mdx) mice[39].

Even though cell-based therapy approaches have been shown to be promising in the field of MDs, to date, there are no reports about the feasibility of this strategy in DM1.

3. DM1 *in vitro* disease modelling.

To better understand the molecular and cellular features of DM1, as well as to develop new therapeutic strategies, there is a necessity for reliable *in vitro* models of the disease. Particularly in the muscle context, myoblasts obtained from patient muscle biopsies have been widely used, as they reproduce the main molecular features and mechanisms of the disease, including the presence of intranuclear RNA foci that sequester MBNL and the subsequent alternative splicing alterations[40-42]. However, primary cells are known to undergo senescence upon passaging, which makes it a difficult model to expand. Although myoblast immortalization may overcome this limitation, cell cycle is altered and therefore it is abnormal in these cells[43,44]. Moreover, performing muscle biopsy is a painful invasive procedure and samples are usually not easy to access. In this regard, the myogenic differentiation of patient-derived human induced pluripotent stem (iPS) cells raises as a promising tool for drug screening or disease modelling. Reprogramming of DM1 patient-derived somatic cells to iPS cells has been previously described to study the central nervous system[45-48], but to date, studies aiming to model the DM1 skeletal muscle pathology are still lacking.

OBJECTIVES

- **Intramuscular transplantation of non-affected hiPS cell-derived myogenic progenitors in an immunodeficient-DM1 mouse model to evaluate the effectiveness of a cell therapy approach.**
    - Generate an immunodeficient mouse model resembling the molecular features of DM1 by crossing NSG mice with HSA^{LR} mice to obtain a homozygous colony of NSG-HSA^{LR} mice.
    - Validate immunodeficiency and DM1 features in NSG-HSA^{LR} mice.
    - Evaluate the engraftment efficiency upon intramuscular transplantation of hiPS cell-derived myogenic progenitors in NSG-HSA^{LR} mice.
    - Analyze the molecular features of DM1 in the transplanted immunodeficient/myotonic mice to evaluate the effectiveness of a cell therapy approach for DM1.

- **Differentiate DM1 patient-specific hiPS cells into myotubes in order to validate their potential as a tool for drug screening or *in vitro* disease modeling.**
    - Obtain and characterize DM1 patient-derived fibroblasts
    - Reprogram DM1 patient-derived fibroblasts to iPS cells and characterize their pluripotency.
    - Transduce DM1 hiPS cells with the PAX7 inducible system and perform EB-iPAX7 myogenic differentiation to obtain DM1 hiPS cell-derived myogenic progenitors and myotubes.
    - Characterize the main DM1 molecular features along the myogenic differentiation procedure, including fibroblasts, iPS cells, myogenic progenitors and myotubes.
    - Validate the use of antisense oligonucleotides to restore the molecular features of DM1 in hiPS cell-derive myotubes.

MATERIALS AND METHODS

FACS analysis

White blood cells from peripheral blood of 4 weeks old HSA^{LR}, NSG or NSG-HSA^{LR} mice were analyzed using the following antibodies: CD3e (PE) and CD19 (PE-Cy7) or NK1.1 (APC) and CD49b/Dx5 (PE). Briefly, cells were washed with PBS and incubated with FcR blocker (CD16/CD32 antibody, Bioscience) for 5 minutes. Then, cells were incubated with proper antibodies diluted in FACS buffer (10% FBS, 1% Penicillin-Streptomycin in PBS) for 20 minutes on ice. FACS analysis was done using FACS Aria (BD Biosciences, San Diego, CA). Results were analyzed using FlowJo software.

Cell transplantation

Animal handling was performed according to protocols approved by the University of Minnesota Institutional Animal Care and Use Committee. Cells in culture (hiPS cell-derived myogenic progenitors or human skeletal myoblasts) were collected with cell dissociation buffer – enzyme free (Gibco) and $5x10^5$ cells in 10 µl of PBS were injected in TA muscles from NSG or NSG-HSA^{LR} mice that had been pre-injured with cardiotoxin 24 h prior to cell transplantation[39]. Satellite

cells from CAG::H2B-EGFP mice (The Jackson laboratory) bulk muscles were isolated and transplanted as previously described[49].

Muscle collection and processing

Tibialis anterior muscles were dissected and embedded in Tissue-Tek O.C.T. compound (Sakura). Cryomolds containing embedded tissues were snap frozen on isopentane pre-cooled with liquid nitrogen. Blocks were cryo-sectioned and 10 µm sections were collected on glass slides.

Patient samples

De-identified cryopreserved skin fibroblasts from two diagnosed DM1 patients (DM1-1 and DM1-2) were obtained through the Paul and Sheila Wellstone Muscular Dystrophy Center at the University of Minnesota, according to procedures approved by the Institutional Review Board of the University of Minnesota. Cells were expanded in high-glucose DMEM medium containing 10% FBS, 1% GlutaMax, 1% Penicillin-Streptomicin, 1% Sodium pyruvate and 1% Non-essential amino acids in under standard culture conditions.

Reprogramming

DM1 fibroblasts were reprogrammed into iPS cells using the CytoTune^{TM} -iPS 2.0 Sendai Reprogramming kit (Thermo Fisher Scientific) using feeder-free conditions according to manufacturer's instructions. Three to four weeks following the transduction of pluripotency factors, iPS cell colonies were picked, transferred to fresh dishes and expanded for 10 passages to eliminate the non-integrative Sendai virus from the cultures. DM1 iPS cells were passaged with ReLeSR^{TM} (STEMCELL Technologies) and cultured on Matrigel-coated dishes using mTeSR^{TM}1 medium (STEMCELL Technologies). For each patient-derived line (DM1-1 and DM1-2), three clones showing classic pluripotent stem cell colony morphology were used for pluripotency characterization, and one clone was used for subsequent studies.

Teratoma studies

Animal experiments were carried out according to protocols approved by the University of Minnesota Institutional Animal Care and Use Committee. iPS cells were injected at 1.5x10^6 in the quadriceps of 8 weeks old male NOD-scid IL2Rg^{null} (NSG – Jackson lab) mice. Before injection, cells were resuspended in 1:1 solution DMEM-F12 and matrigel (final volume including cells: 65µl).

Myogenic differentiation

DM1-1 and DM1-2 iPS cells were transduced with a doxycycline-inducible PAX7 system (iPAX7) and differentiated towards the myogenic lineage as previously described [50]. DM1 iPAX7-iPS or

unaffected iPAX7-iPS (hiPS) cells were dissociated with Accumax (Innovative Cell Technologies) and 1e6 cells were cultured in a 60 mm petri dish using mTeSR™1 medium supplemented with 10 µM Y-27632 (ROCK inhibitor) and incubated on a shaker (day 0). On day 2, medium was replaced with EB differentiation medium (15% FBS, 10% Horse Serum, 1% KnockOut Serum Replacement™ (KOSR), 1% GlutaMax, 1% Penicillin-Streptomicin, 50 µg/ml ascorbid acid and 4.5 mM monothioglycerol in IMDM) supplemented with 10 µM Y-27632 and 10 µM CHIR990217 (GSK3 inhibitor). Following incubation of EBs in suspension for three days, the medium was replaced with fresh EB differentiation medium containing 10 µM Y-2763 to withdraw GSK3i treatment. On day 7, EBs were collected and plated on gelatin-coated flasks to promote their adhesion and expansion as monolayer using EB differentiation medium supplemented with 10 ng/ml human basic fibroblast growth factor (bFGF). On day 10, medium was replaced with fresh EB differentiation medium supplemented with 10 ng/ml human bFGF + 1 µg/ml Doxycycline to promote PAX7-GFP expression. On day 14, GFP^+ cells ($PAX7^+$, myogenic progenitors) were sorted and expanded on gelatin coated flasks using the same medium. DM1 or control iPS cell-derived myogenic progenitors were terminally differentiated into myotubes by growing them to confluency and then switching to terminal differentiation medium (20% KOSR, 1% GlutaMax, 1% Penicillin-Streptomicin in KnockOut™ DMEM). Myotube characterization was performed after 5 days of terminal differentiation. Throughout the study, we used a previously established and validated human iPAX7-iPS cell line[39] as a non-disease control.

Southern blot

To determine the approximate length of the CTG triplet repeat in the DM1 fibroblasts samples, southern blot was performed. Briefly, genomic DNA was isolated from DM1-1, DM1-2 and control cells using the PureLink™ Genomic DNA Mini Kit (Invitrogen). Purified gDNA was digested with BamHI, which generates a fragment of about 1.4 kb from the 3' region of the *DMPK* gene considering a normal allele containing 20 CTG repeats. Digested gDNA was run in a 1 % agarose gel, denatured (1.5 M NaCl, 0.5 M NaOH), neutralized (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5), blotted to a positively charged nylon membrane by capillary transfer with 20 x SSC buffer, and fixed to the membrane by UV-crosslinking. To detect the BamHI-digested fragment spanning the CTG repeats, a probe was design using the following primers (Fwd: 5'-TCCCCAACCTCGATTCCCCTC-3'; Rev: 5'- GGCCACCAACCCAATGCAGC-3'). Labelling of the probe and detection were done following the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche).

RNA-FISH and immunofluorescence

To detect the intranuclear RNA foci, cells grown on coverslips or muscle sections collected on slides were fixed with 4% PFA for 10 min, permeabilized with 0.3% Triton X-100 for 15 min, and incubated with pre-hybridization buffer solution (2 x SSC and 30% formamide in DEPC water) for 10 min at room temperature (RT). Samples were then incubated with hybridization solution (2x SSC, 30 % formamide, 0.02% BSA, 2 mM ribonucleoside vanadyl complex, 66 µg/ml yeast tRNA and 0.1 ng/ul of a Cy3-labeled $(CAG)_7$ probe in DEPC water) for 2 h at 37 °C in a humid chamber. Samples were washed twice with pre-hybridization buffer solution for 7 min at 42 °C and two more times with 1x SSC in DEPC water for 5 min at RT. At this point, samples were either mounted with ProLong™ Gold antifade reagent with DAPI (Invitrogen) or processed for immunostaining. For the later, samples were blocked with 3% BSA in PBS for 30 min and incubated overnight with proper primary antibody at 4 °C. The following day, they were washed three times with PBS for 5 min and incubated with proper secondary antibody for 1 h at RT. Samples were washed again 3x with PBS for 5 min and mounted as described above. Analysis was done by confocal microscopy (Nikon NiE C2 upright confocal microscope).

The following antibodies were used for immunofluorescence: anti-OCT3/4 (C-10, Santa Cruz Biotechnology; 1:50), anti-SOX2 (Y-17, Santa Cruz Biotechnology; 1:50), anti-NANOG (H-2, Santa Cruz Biotechnology; 1:50), anti-PAX7 (PAX7, Developmental Studies Hybridoma Bank (DSHB); 1:50), anti-MBNL 1 (3A4, Santa Cruz Biotechnology; 1:75)), anti-MYHC (MF 20, DSHB; 1:100), anti-human DYS (2C6, Millipore), Alexa Fluor 488 goat anti-Mouse IgG (Invitrogen; 1:500), Alexa Fluor 555 goat anti-Mouse IgG (Invitrogen; 1:500).

RT-PCR

Samples were collected with TRIzol™ Reagent (Invitrogen) and RNA was purified using the Direct-zol™ RNA Miniprep Plus kit (Zymo Research) following manufacturer's instructions. Purified RNA was quantified with NanoDrop 2000 (Thermo Scientific) and retrotranscribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) following manufacturer's instructions. cDNA was used as template for PCR using GoTaq Flexi DNA polymerase (Promega). Previously reported primers were used for pluripotency characterization of DM1 iPS cells[51], splicing analysis of *BIN1* exon 11 in DM1 iPS cell-derived myotubes[19], and *Clcn1* exon 7A[52], *Nfix* exon 7[52], *Serca1* exon 22[53] and *Gapdh*[52] in mice TA muscles.

Antisense oligonucleotide treatment

DM1 iPS cell-derived myogenic progenitors were seeded onto gelatin-coated plates for terminal differentiation. On day 4 (after switching to terminal differentiation medium), myotubes were transfected with 2 µg/ml of 2-OMe-PT-(CAG)7 antisense oligonucleotides using RNAi Max transfection reagent as previously described [43]. Twenty-four hours after treatment, cells were processed for RNA-FISH or RT-PCR analysis. To test the effect of ASO treatment on myotube differentiation, DM1-1 and DM1-2 iPS-derived myogenic progenitors were transfected with 2-OMe-PT-(CAG)7 antisense oligonucleotides one day before switching to terminal differentiation medium, using the protocol described above. After five days in terminal differentiation conditions, cells were fixed and processed for MYHC immunofluorescence. Plates were imaged using the BioTek™ Cytation™ 3 Cell Imaging Multi-Mode Reader, and images were analyzed by quantifying the ratio of % MYHC area to % DAPI area using Fiji[54]

Statistical analysis

Statistical comparisons of values were done using the unpaired Student's-t test in Prism 7 software (GraphPad). *p* values < 0.05 were considered significant.

RESULTS

1. Intramuscular transplantation of non-affected hiPS cell-derived myogenic progenitors in an immunodeficient-DM1 mouse model to evaluate the effectiveness of a cell therapy approach.

1.1. Generation and characterization of an immunodeficient-myotonic (NSG-HSA^{LR}) mouse model suitable for human cells transplantation.

In order to test whether cell-based therapy is a feasible approach for DM1, we crossed an immunodeficient mouse model (*NSG)* with a widely used mouse model for DM1 (HSA^{LR})[12]. *NSG* mice carry the severe combined immune deficiency (*scid)* mutation in the DNA repair complex protein *Prkdc*, which leads to B and T cell deficiency, combined with the *IL2rg^{null}* mutation, which ablates functional NK cells. On the other hand, the HSA^{LR} mouse model contains a transgene

expressing 250 CTG repeats inserted in the final exon of the human skeletal actin *(HSA)* gene, between the termination codon and the polyadenylation signal[12]. Progeny was genotyped and crossed until a homozygous NSG-HSA^{LR} colony was obtained.

First, we characterized the immunodeficiency in NSG-HSA^{LR} mice by analyzing the presence of T, B and NK cells in peripheral blood. FACS analysis revealed the lack of NK 1.1^{+}CD49b^{+} cells (NK cells), as well as CD3e^{+} and CD19^{+} cells (T and B lymphocytes, respectively) in NSG and NSG-HSA^{LR} mice, contrary to immunocompetent HSA^{LR} mice (Fig. 1).

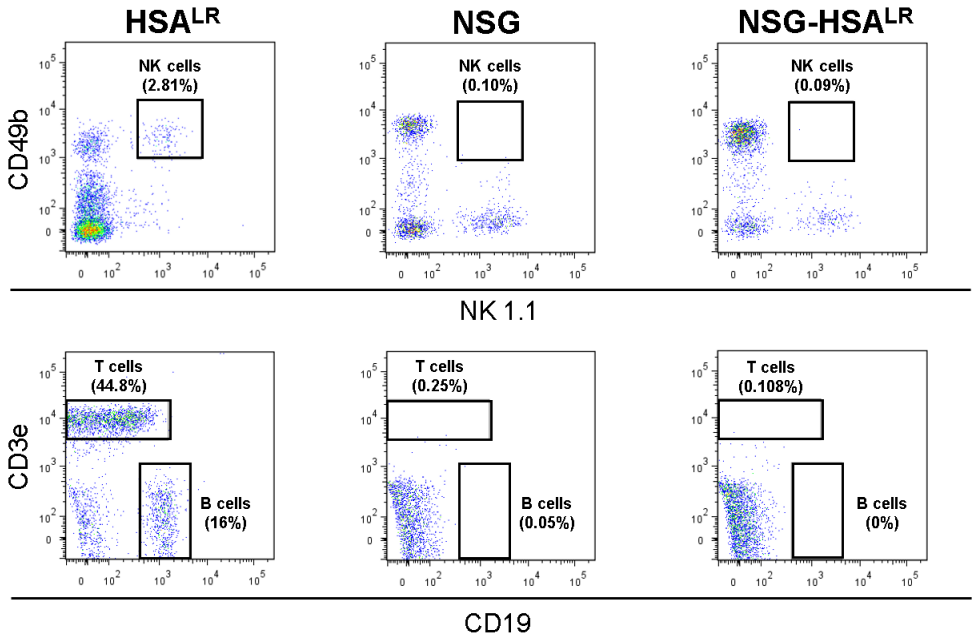

Figure 1. FACS analysis of NK, T and B cell fractions in HSA^{LR}, NSG and NSG-HSA^{LR} mice. Circulating white blood cells were purified from HSA^{LR}, NSG and NSG-HSA^{LR} mice and analyzed for DX5/NK1.1 or CD19/CD3e surface markers by FACS. Dx5/NK1.1 analysis shows the lack of NK cells in NSG and NSG-HSA^{LR} mice (upper panel), whereas CD19 and CD3e analysis demonstrates the absence of B and T cells, respectively, in NSG and NSG-HSA^{LR} mice. Immunocompetent HSA^{LR} mice were positive for the three cell populations analyzed.

Then, we set to determine whether NSG-HSA^{LR} mice preserved the molecular features of DM1 previously reported in HSA^{LR} mice. We dissected and analyzed tibialis anterior muscle cross-sections form NSG, HSA^{LR} or NSG-HSA^{LR} mice. RNA-FISH analysis using a Cy3-labeled $(CAG)_7$ probe that recognizes CUG expansions revealed the presence of intranuclear RNA *foci* in muscle sections of NSG-HSA^{LR} mice, similar to the ones typically observed in HSA^{LR}. NSG

sections were absent of RNA foci. Moreover, immunostaining of MBNL1 following RNA-FISH confirmed the sequestration of MBNL1 by RNA *foci* (Fig. 2).

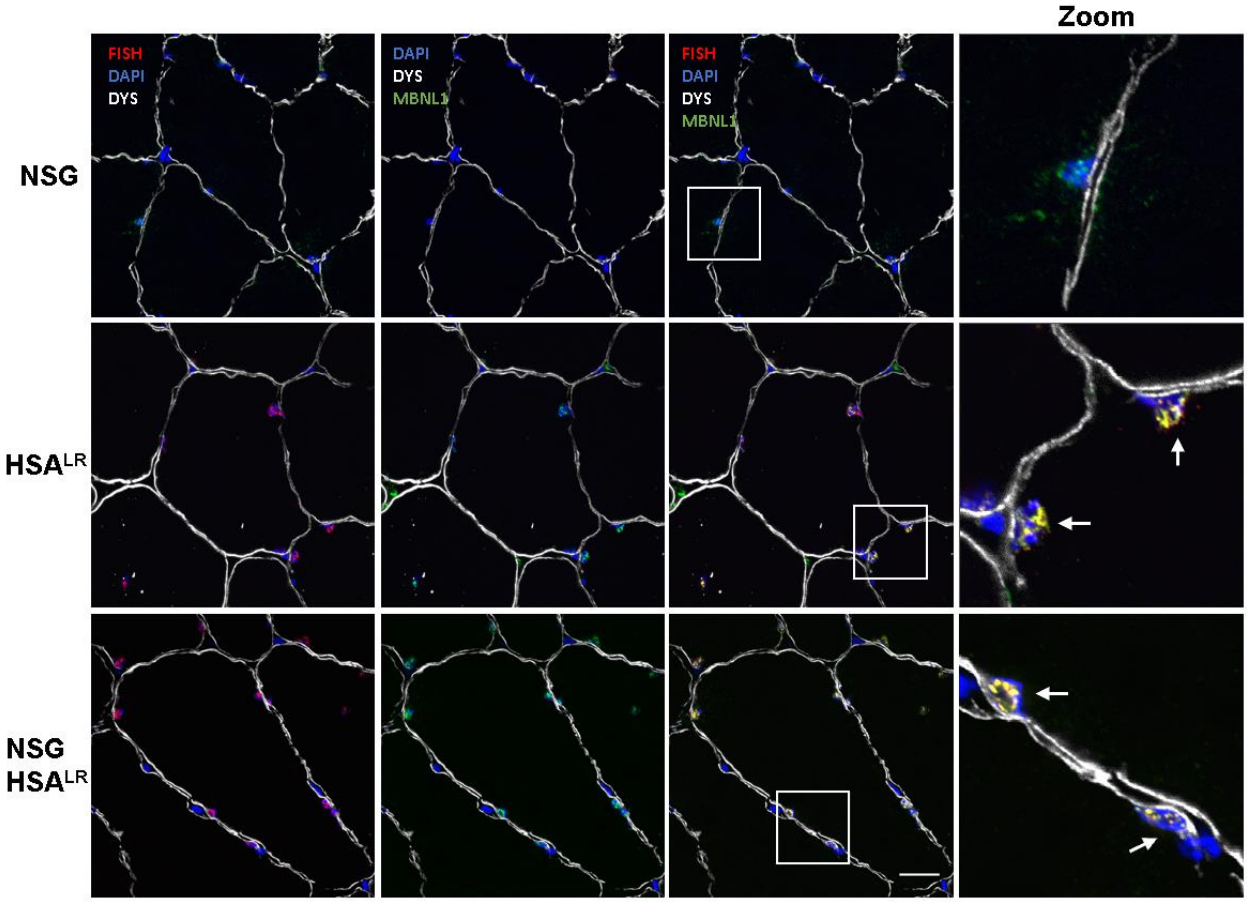

Figure 2. RNA-FISH and MBNL1 localization analysis in cross-sections of TA muscles from NSG, HSA^{LR} and NSG-HSA^{LR} mice. Representative images show RNA-FISH in combination with MBNL1 immunostaining. An anti-dystrophin antibody (white) was used to visualize the muscle fibers. RNA *foci* (in red) is observed in HSA^{LR} and NSG-HSA^{LR} and the pattern of *foci* co-localizes with MBNL1 distribution (in green), as indicated by arrows. Scale bar is 20 µm. Maximum projection is shown.

To evaluate whether sequestration of MBNL1 by RNA *foci* in NSG-HSA^{LR} led to alternative splicing alterations, we analyzed three genes in which alternative splicing has been shown to be affected in DM1 patients and HSA^{LR} mice: Inclusion of *Clcn1* exon 7A, inclusion of *Nfix* exon 7 and exclusion of *Serca1* exon 22. We observed that HSA^{LR} and NSG-HSA^{LR} showed a similar alternative splicing pattern when compared to NSG (Fig. 3, A-B).

Thus, we validated that NSG-HSA^{LR} mice show the immunodeficient phenotype of NSG while conserving the molecular features of DM1 found in HSA^{LR}.

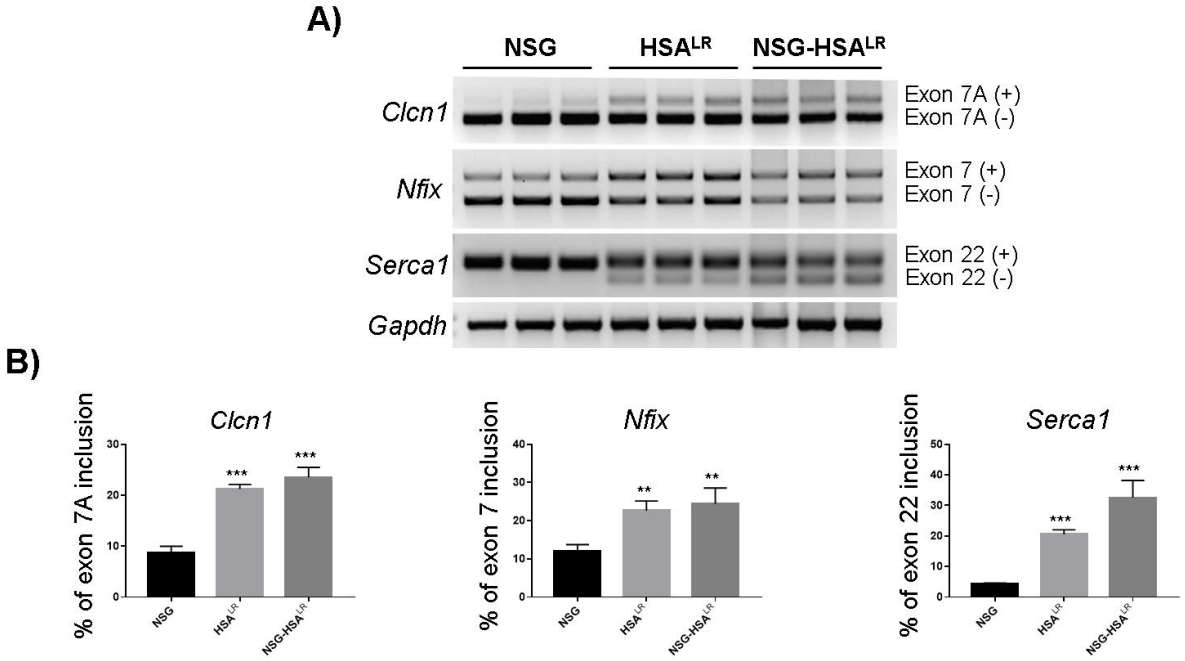

Figure 3. RNA splicing analysis of *ClCn1, Nfix* and *Serca1* in TA muscles from NSG, HSA^{LR} and NSG-HSA^{LR} mice. A) Total RNA was extracted from TA muscles collected from NSG, HSA^{LR} or NSG-HSA^{LR} mice. Then, RT-PCR was done to obtain cDNA, which was further used to detect the splicing variants from *ClCn1, Nfix* and *Serca1* transcripts. In HSA^{LR} and NSG-HSA^{LR} mice, we observe an increased inclusion of exon 7a in *ClCn1* and exon 7 in *Nfix*, and increased exclusion of exon 22 in *Serca1*. **B)** Bar graphs represent respective percentage of exon 7A inclusion (Clcn1), exon 7 inclusion (Nfix), or exon 22 exclusion (Serca1) from three independent replicates (from A). Data are shown as mean + SEM. ***p<0.001

1.2. Transplantation of non-affected human-derived muscle progenitors in NSG-HSA^{LR} mice

Based on the immunodeficiency observed in NSG-HSA^{LR} mice, we performed intramuscular transplantation of non-affected hiPS cell-derived myogenic progenitors[39] in TA muscles of NSG or NSG-HSA^{LR} pre-injured with cardiotoxin (CTX). Four weeks after transplantation, muscles were dissected and cryo-sectioned for analysis. To analyze the engraftment efficiency, we counted the fibers that were double positive for immunostaining using antibodies detecting human lamin A/C and human dystrophin. We observed successful engraftment of transplanted cells in both NSG and NSG-HSA^{LR} muscles (Fig. 4, A). NSG-HSA^{LR} transplanted muscles showed around 100 double positive fibers, very similar to the engraftment efficiency observed in NSG transplanted muscles (Fig. 4, B).

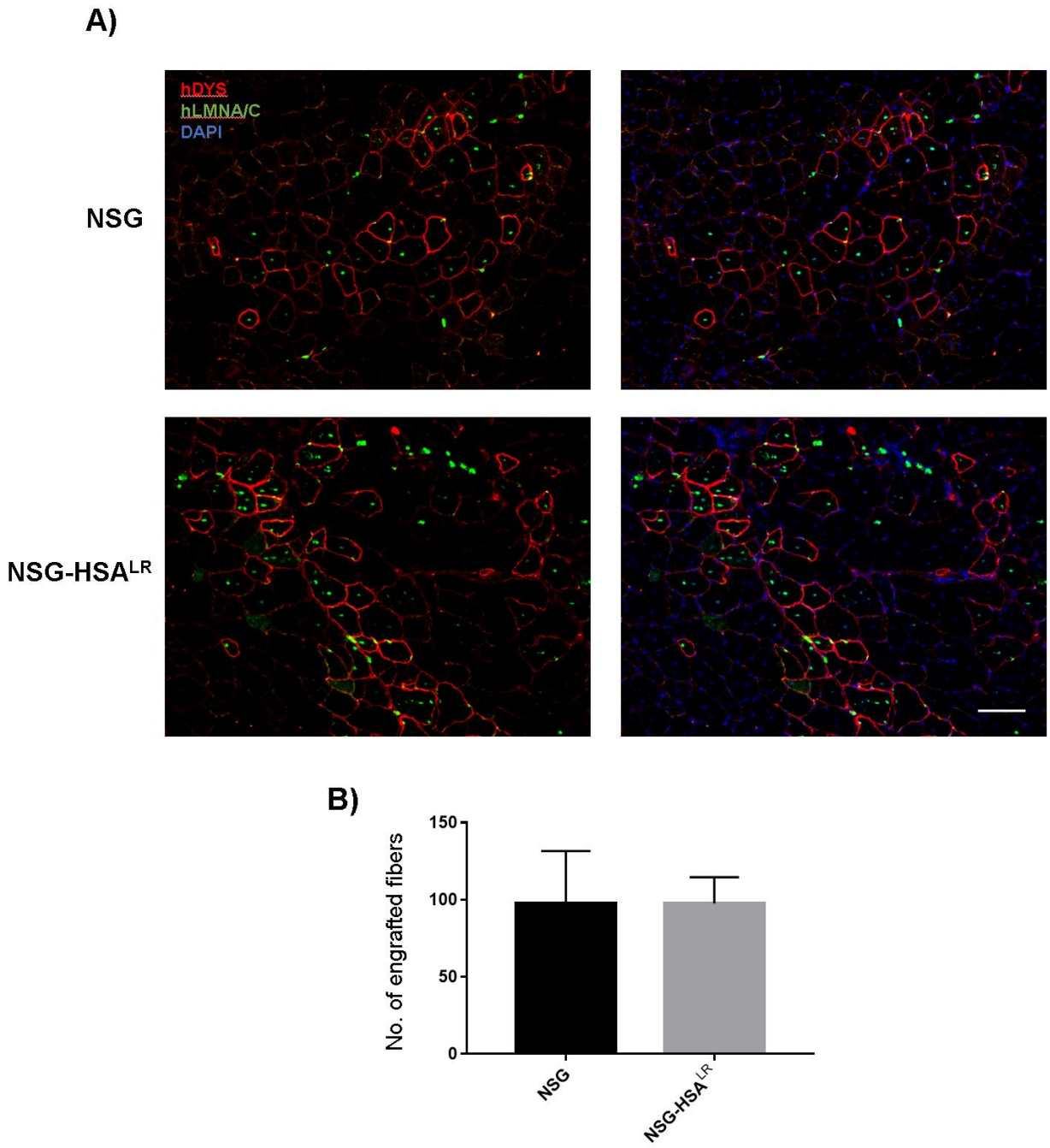

Figure 4. Engraftment of unaffected myogenic progenitors upon transplantation into TA muscles of NSG or NSG-HSA^{LR} mice. A) Non-affected human iPSC-derived iPax7 myogenic progenitors were transplanted into the TA muscles of NSG or NSG-HSA^{LR} mice that had been previously pre-injured with cardiotoxin. Four weeks later, transplanted muscles were collected for engraftment analysis. Antibodies specific for human Lamin A/C (green) and human Dystrophin (red) were used to detect the engraftment of human cells in the mouse tissue. Engraftment was detected in both NSG and NSG-HSA^{LR} muscles. Scale bar is 100 μm **B)** Quantification of engraftment (hDYS^{+}/hLMNA/C^{+}) in NSG or NSG-HSA^{LR} transplanted mice (N=4).

1.3. Analysis of DM1 RNA foci in NSG-HSA^{LR} muscles transplanted with non-affected myogenic progenitors

Upon confirming that NSG-HSA^{LR} mice are a suitable model for the transplantation of human cells in the context of a cell-based therapy approach, we analyzed the potential contribution of the engrafted cells to the DM1 phenotype. We first analyzed the RNA *foci* distribution in the transplanted muscles. Surprisingly, we observed that human lamin A/C (+) nuclei also contained intranuclear RNA *foci* evidenced by RNA FISH-Immunostaining analysis. This result was consistent in all the muscles analyzed. Moreover, cells from the same preparation were also transplanted in NSG mice, resulting in negative RNA *foci* detection (Fig. 5A). This finding led us to the hypothesis that this was a result of fusion of non-affected human nuclei with pre-existing mouse muscle fibers, in which human Lamin A/C proteins would be synthesized and directed to mouse nuclei within the fiber, therefore, giving a false positive for foci detection in human cells. To challenge this hypothesis, we performed DNA FISH using human Cot-1 as a probe to label specifically nuclei containing human DNA[55]. Our data showed that hLMNA/C^{+} nuclei are also positive for human DNA (hDNA), and again positive for RNA FISH (Figure 5B, yellow asterisk). Note the presence of mouse nuclei in the same fiber absent of hDNA or hLMNA/C labelling (Figure 5B, white asterisks). These results confirmed that non-affected human myogenic progenitors that fused to mouse muscle fibers, upon engraftment, contained intranuclear RNA foci similar to NSG-HSA^{LR} mice endogenous nuclei. To discard the possibility that this unexpected finding was exclusive to the transplanted cell type, we performed intramuscular transplantation of human skeletal myoblasts (HSkM) in NSG or NSG-HSA^{LR} mice. We observed hLMNA/C^{+} nuclei containing RNA foci in NSG-HSA^{LR} but not in NSG mice, corroborating our observations above (Fig. 6).

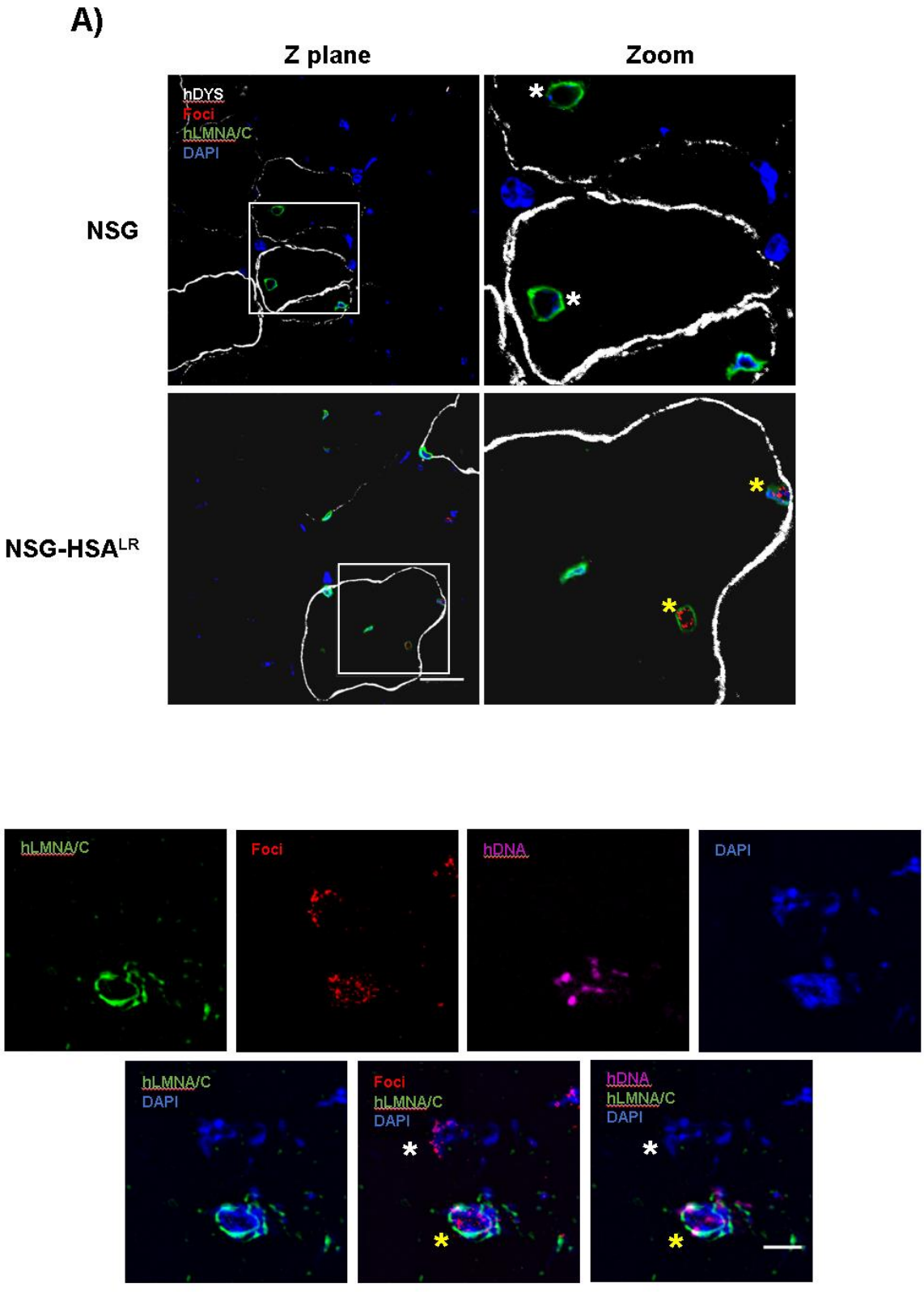

Figure 5. RNA-FISH analysis of engrafted muscles reveals that nuclei from transplanted cells are positive for mutant RNA foci upon their fusion with NSG-HSA^{LR} muscle fibers. (A) Cryo-sections from NSG or NSG-HSA^{LR}

muscles engrafted with non-affected myogenic progenitors were analyzed by RNA FISH (red). Antibodies specific for human Lamin A/C (green) and human Dystrophin (white) were used to detect human engraftment. Cells engrafted in NSG muscles were absent of RNA foci (white asterisks). Notably, intra-nuclear RNA *foci*, similar to host nuclei, were detected in human engrafted nuclei (hLamin A/C positive nuclei, yellow asterisks). Scale bar is 20 µm. Mid Z projection is shown. **(B)** Combination of DNA-FISH with RNA FISH and immunostaining shows that nuclei containing human DNA and positive for human Lamin A/C are positive for RNA foci (yellow asterisk), suggesting an intra- muscle fiber transmission of RNA *foci* from the host nuclei to the unaffected engrafted nuclei. Scale bar is 5 µm. Mid Z projection is shown.

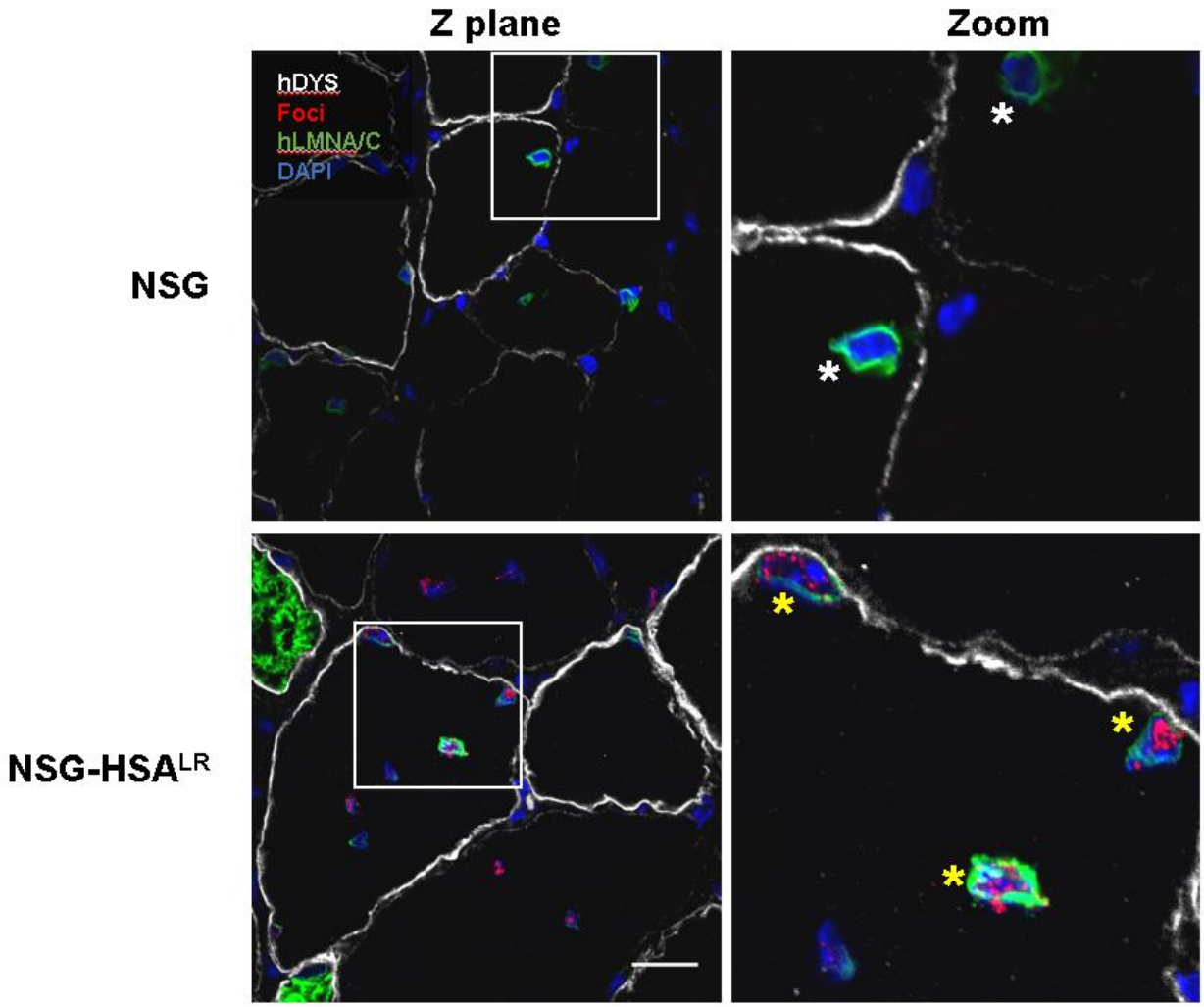

Figure 6. RNA-FISH analysis of NSG-HSA^{LR} muscles transplanted with non-affected human skeletal myoblasts (HSkM) corroborates RNA foci positivity in engrafted nuclei. Cryo-sections from NSG or NSG-HSA^{LR} muscles engrafted with non-affected human skeletal myoblasts were analyzed by RNA FISH (red). Antibodies specific for human Lamin A/C (green) and human Dystrophin (white) were used to detect human engraftment. HSkMs engrafted in NSG mice are absent in foci (upper panel) while cells engrafted in NSG-HSA^{LR} mice were positive for RNA foci (lower panel). Scale bar is 20 µm. Mid Z projection is shown.

Moreover, to rule out the possibility that the apparent inter-nuclear migration of RNA foci was related to a human-mouse interspecies engraftment, we isolated satellite cells from CAG::H2B-eGFP mice, which allowed us to identify donor-derived nuclei through nuclear GFP labelling, and transplanted them in NSG-HSA^{LR} mice . Accordingly, GFP^{+} nuclei were also positive for RNA foci (Figure 7A, lower, yellow asterisks). In agreement, no recovery in splicing alterations was observed in engrafted muscles compared to PBS-injected controls (Figure 7B and C).

In an effort to establish an *in vitro* model to study in detail a mechanism that would explain the results observed *in vivo*, we co-differentiated unaffected hiPS cell-derived myogenic progenitors expressing H2B-GFP with DM1-1 hiPS cell-derived myogenic progenitors. We observed hybrid myotubes containing GFP+ (unaffected) and GFP- (DM1) nuclei. However, we did not find any GFP+ nucleus showing RNA *foci* upon five days of terminal differentiation, suggesting that the transmission of RNA *foci* observed *in vivo* was not able to be reproduced *in vitro* (Fig. 8).

Overall, our results demonstrated that we successfully generated an immunodeficient-DM1 mouse model (NSG-HSA^{LR}) that is suitable for transplantation experiments of human or mouse cells. Unexpectedly, we observed that non-disease engrafted nuclei showed the presence of RNA *foci*, similar to the endogenous nuclei. Even though it is generally accepted that the RNA foci are retained inside the nucleus, in which the RNA was synthetized, our data suggests that in a chimeric fiber, RNA bearing the CTG expansion leave the nucleus and subsequently form aggregates in other nuclei within the fiber, even in nuclei that are not producing their own toxic RNA.

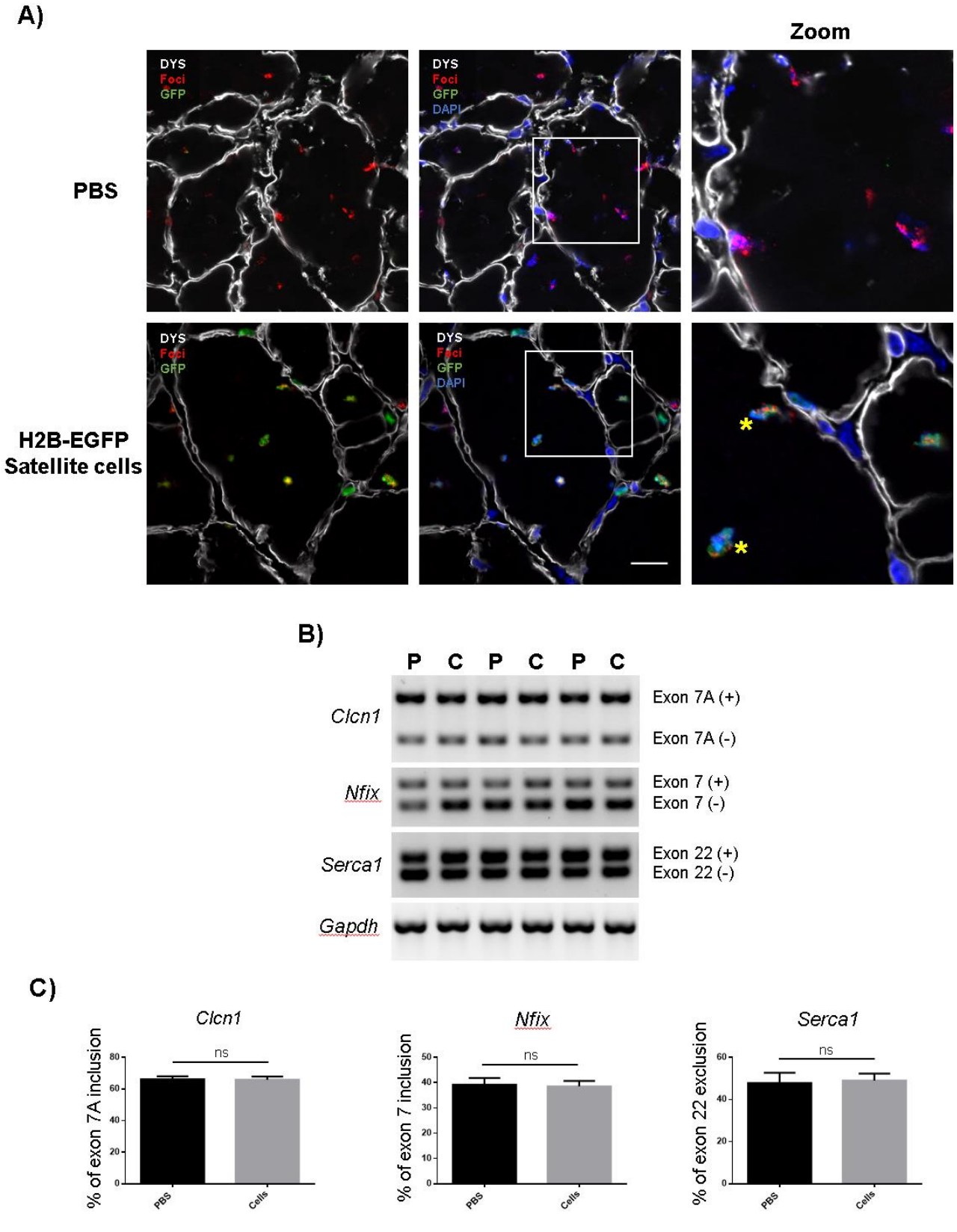

Figure 7. RNA-FISH analysis of NSG-HSA^{LR} muscles transplanted with H2B-eGFP satellite cells reveals that H2B-GFP+ nuclei are positive for mutant RNA foci and there is no recovery on the alternative splicing pattern.

(A) Cryo-sections from NSG-HSA^{LR} muscles injected with non-affected satellite cells expressing H2B-GFP or PBS were analyzed by RNA FISH (red). Antibodies specific for GFP (green) and pan-Dystrophin (white) were used to detect engraftment. RNA foci were observed in GFP+ nuclei (yellow asterisks). Scale bar is 20 μm. Mid Z projection is shown. **(B and C)** Alternative splicing pattern of *Clcn1*, *Nfix* and *Serca1* showed no significant difference between PBS or cell injected contralateral TA muscles of NSG-HSA^{LR} mice.

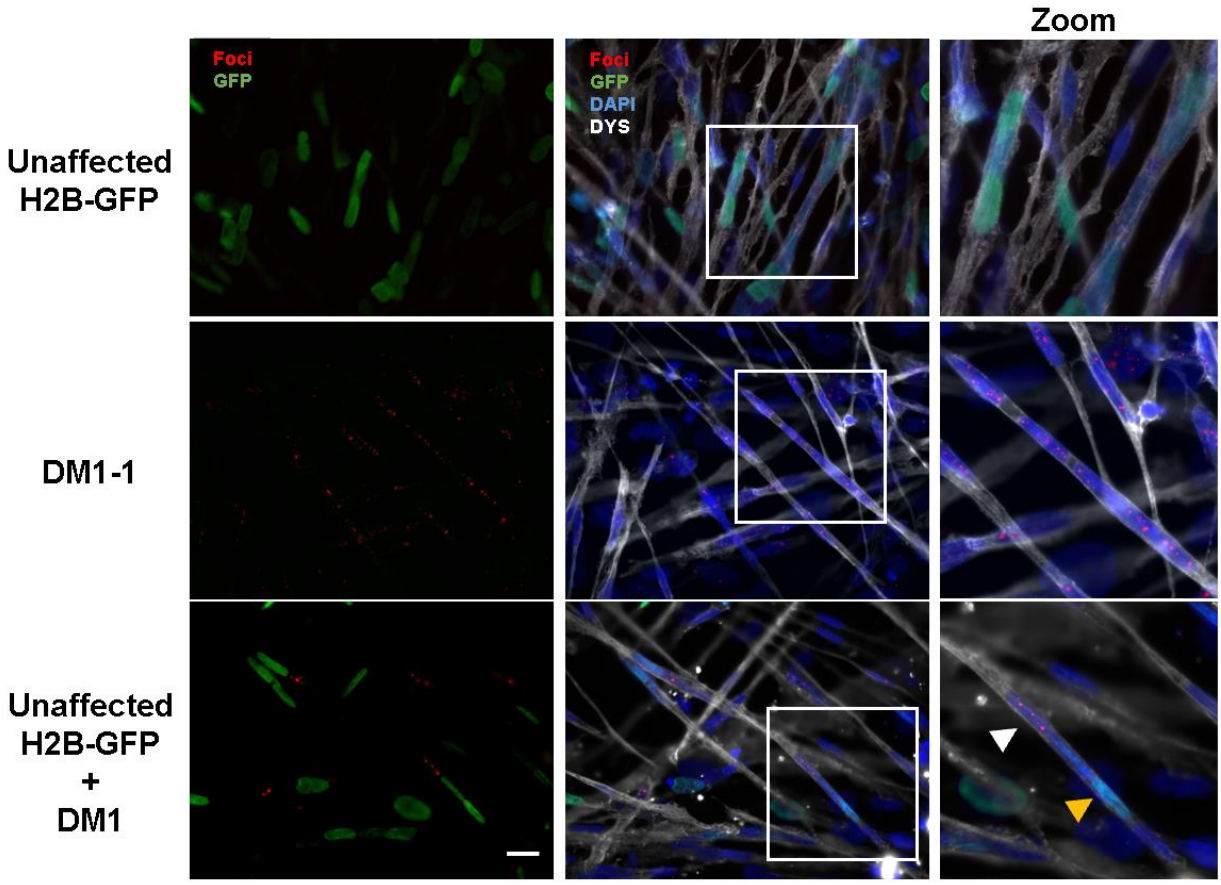

Figure 8. ***In vitro*** **co-differentiation of non-affected and DM1 myogenic cells.** Non-affected hiPS cell-derived myogenic progenitors were transduced with an H2B-GFP lentiviral vector. Then, non-affected myogenic progenitors were co-cultured with DM1 patient-derived myogenic progenitors and terminally differentiated to obtain hybrid fibers. In myotubes containing non-affected and DM1 nuclei, H2B-GFP+ nuclei were absent of RNA foci. Scale bar is 20 μm.

2. Myogenic differentiation of DM1 patient-specific hiPS cells

2.1 Characterization of DM1 patient-derived fibroblasts and iPS reprogramming

As a first step in assessing the potential of patient-specific iPS cell-derived myogenic derivatives for the *in vitro* modeling of DM1, we reprogrammed skin fibroblast samples obtained from two diagnosed DM1 patients into iPS cells. Sample DM1-1 was obtained from a 35-year-old male patient bearing an expansion of 716 CTG repeats, whereas DM1-2 was obtained from an 18 year-old-male patient with 473 CTG repeats, both in blood cells. The molecular features of DM1 were characterized in both fibroblasts samples. Southern blot analysis showed an expansion of about 2,000 and 1,500 CTG repeats in DM1-1 and DM1-2, respectively (Fig. 9A), which suggests mosaicism of the repeat length in somatic cells. Furthermore, RNA-FISH analysis using a Cy3-labeled $(CAG)_7$ probe showed the presence of typical intranuclear RNA foci (Fig. 9B-C).

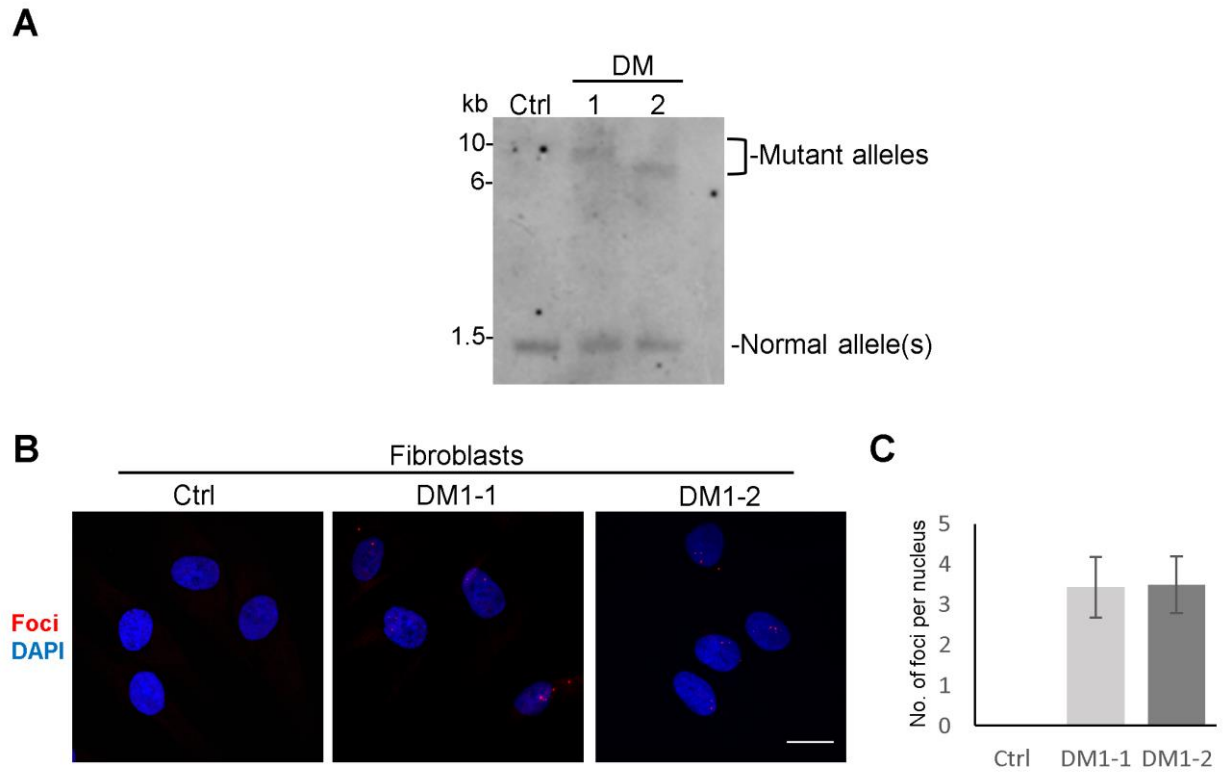

Figure 9. Molecular characterization of DM1 patient-derived fibroblasts. (A) Southern blot analysis using a digoxigenin-labeled probe binding to the 3' UTR of the *DMPK* gene to determine the length of CTG repeats in fibroblast samples from two DM1 patients (referred as DM1-1 and DM1-2). Fibroblasts from an unaffected individual were used as control. The DM1-1 sample showed an expansion of about 2,000 CTG repeats whereas the DM1-2

contained about 1,500 CTG repeats. **(B)** Representative RNA-FISH images show foci only in fibroblasts from DM1-1 and DM1-2 patients. A Cy3-labeled $(CAG)_7$ probe was used to detect the foci (in red) and DAPI to stain nuclei. Maximum projection of the Z sections is shown using confocal microscopy. Scale bar is 20 μm. **(C)** Bar graph shows respective quantification of foci (from B), represented as average number of foci per nucleus in 150 cells. Bars indicate S.D. from three independent experiments.

We then reprogrammed DM1-1 and DM1-2 fibroblasts using the Sendai virus transduction approach. Three weeks after transduction, iPS cell colonies showing typical embryonic stem cell-like colony morphology were picked and expanded for 10 passages (Fig. 10A). For each patient-specific iPS cell line, three clones were selected for pluripotency characterization. Gene expression analysis demonstrated that DM1 iPS cells display expression levels of the endogenous pluripotency factors *OCT3/4*, *SOX2* and *NANOG* similar to control ES cells (Fig. 10B). RNA levels for the reprogramming Sendai virus (SeV) were detected only as a faint band on day 7 after transduction, but were absent in expanded iPS cells, as expected for this non-integrating vector. Expression of the endogenous pluripotency factors was confirmed at the protein level (Fig. 10C). The pluripotent capabilities of DM1-1 and DM1-2 iPS cells were validated by their ability to develop teratomas upon their subcutaneous injection into immunodeficient mice (Fig. 10D). Importantly, no karyotypic abnormalities were found in generated DM1 iPS cells (Fig. 10E).

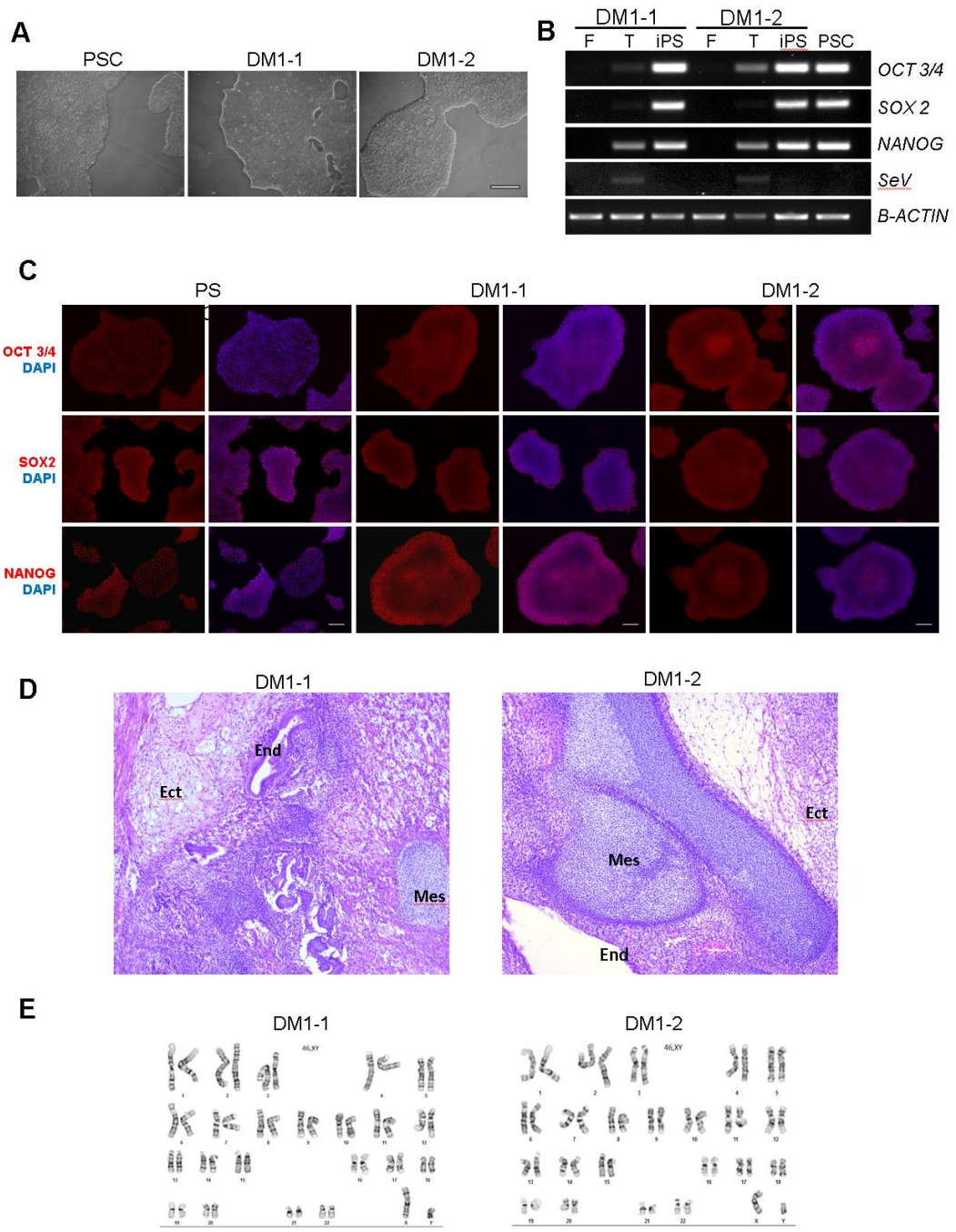

Figure 10. Characterization of reprogrammed DM1-iPS cell lines. (A) Representation of the typical colony morphology of DM1-1 and DM1-2 iPS cells compared to a control iPS cell line. (B) RT-PCR analysis for pluripotency genes OCT3/4, SOX2, and NANOG, as well as for the Sendai virus used for reprogramming (SeV) in DM1-1 and DM1-2 fibroblasts (F), fibroblasts on D7 after transduction (T) and iPS cell colonies after 10 passages. (C) Representative images show immunostaining for pluripotency markers OCT3/4, SOX2 and NANOG (red) in DM1-1 and DM1-2 iPS cells. Scale bar is 200 μm. (D) Hematoxylin-eosin staining of the teratoma induced by subcutaneous injection of DM1-1 and DM1-2 iPS cells in immunodeficient mice. (E) Karyotype analysis of DM1-1 and DM1-2 iPS cells.

Using RNA FISH, we confirmed the expression of the intranuclear RNA foci in both DM1 iPS cell lines (Fig. 11A-B).

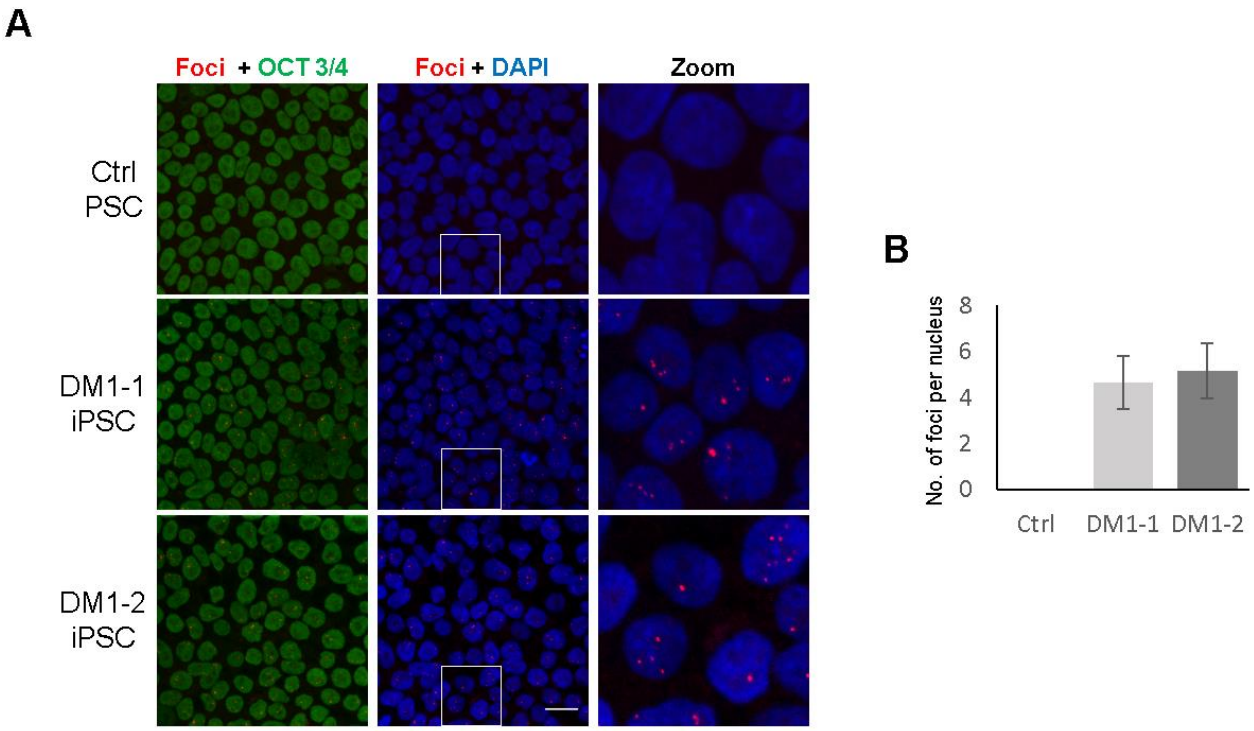

Figure 11. Molecular characterization of DM1 patient-derived fibroblasts and reprogrammed DM1 iPS cells. (A) Representative image of RNA-FISH (red) coupled with immunostaining for the pluripotency marker Oct 3/4 (green) in DM1 iPS cells and control pluripotent stem cells (PSC). Maximum projection of the Z sections is shown by confocal microscopy. Scale bar is 20 μm. (B) Bar graph shows respective quantification of foci (from A), represented as average number of foci per nucleus in 150 cells. Bars indicate S.D. from three independent experiments.

2.2 Differentiation of DM1 iPS cells into skeletal myogenic progenitors

Next, we differentiated DM1-1 and DM1-2 iPS cells towards the myogenic lineage using the doxycycline-inducible PAX7 system (iPAX7), as we have previously described [39]. To confirm whether DM1 iPS cell-derived patient-specific myogenic progenitors represent a valid model to study DM1-related features *in vitro*, we assessed the molecular phenotype of the disease in these cells. RNA FISH analysis revealed the presence of intranuclear RNA foci in both DM1-1 and

DM1-2 myogenic progenitors (Fig. 12A). The average number of RNA foci per nucleus was higher in DM1 myogenic progenitors than in fibroblasts and iPS cells for both patient-specific cell lines (Fig. 12B). As discussed above, another key molecular feature of DM1 is the intranuclear sequestration of MBNL1 by the RNA foci. To test whether DM1 iPS cell-derived myogenic progenitors recapitulate this process, DM1-1- and DM1-2 iPS cell-derived myogenic progenitors were evaluated for MBNL1 expression. We performed RNA FISH followed by immunostaining with an antibody against MBNL1 in the myogenic progenitors. In control myogenic progenitors, we observed a diffused distribution of MBNL1 throughout the nucleus and cytoplasm, whereas DM1-1 and DM1-2 cells showed a pattern of staining that co-localized with the RNA foci, confirming MBNL1 sequestration in DM1 myogenic progenitors (Fig. 12C). We also observed RNA foci in the cytoplasm of these cells, in agreement with previous studies involving other affected DM1 cell types [56,57].

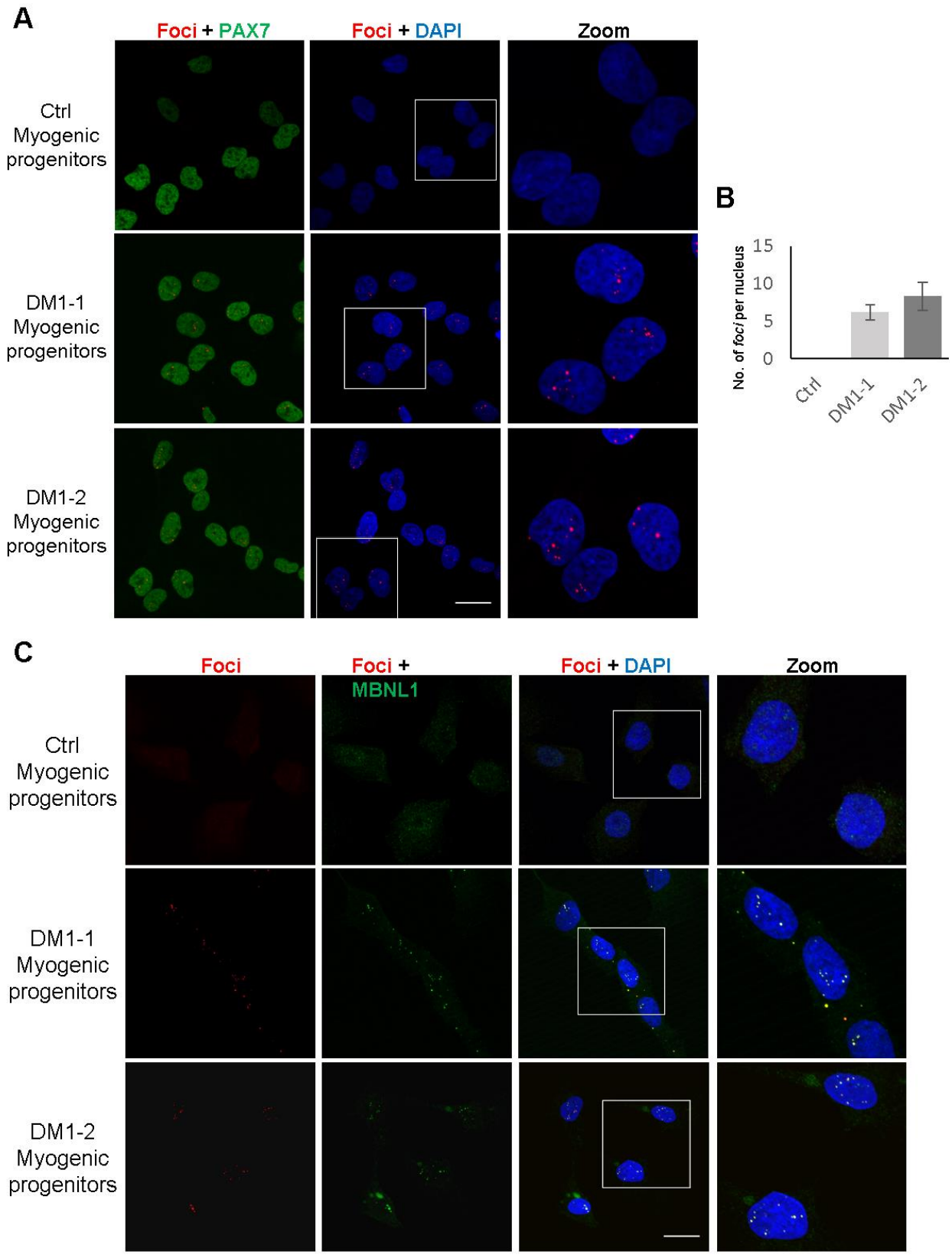

Figure 12. Characterization of DM1 myogenic progenitors. (A) Representative images show RNA-FISH of foci (red) co-stained with the myogenic transcription factor PAX7 (green) in myogenic progenitors derived from control

and DM1 iPS cells using confocal microscopy. Maximum projection of the Z sections is shown. Scale bar is 20 μm. (B) Bar graph shows respective quantification of foci (from A), represented as average number of *foci* per nuclei in 150 cells. Bar represents S.D. from three independent experiments. (C) Representative images show RNA-FISH of foci (red) co-stained with the splicing factor MBNL1 (green) in myogenic progenitors derived from control and DM1 iPS cells. Mid Z section is shown. Scale bar is 20 μm.

2.3 Terminal differentiation of DM1 iPS cells-derived myogenic progenitors into myotubes

DM1-1 and DM1-2 iPS cell-derived myogenic progenitors were subsequently differentiated into myotubes expressing myosin heavy chain (MYHC), a marker of myogenic terminal differentiation, by culturing them to confluency, and then switching to a low nutrient medium (Figure 13).

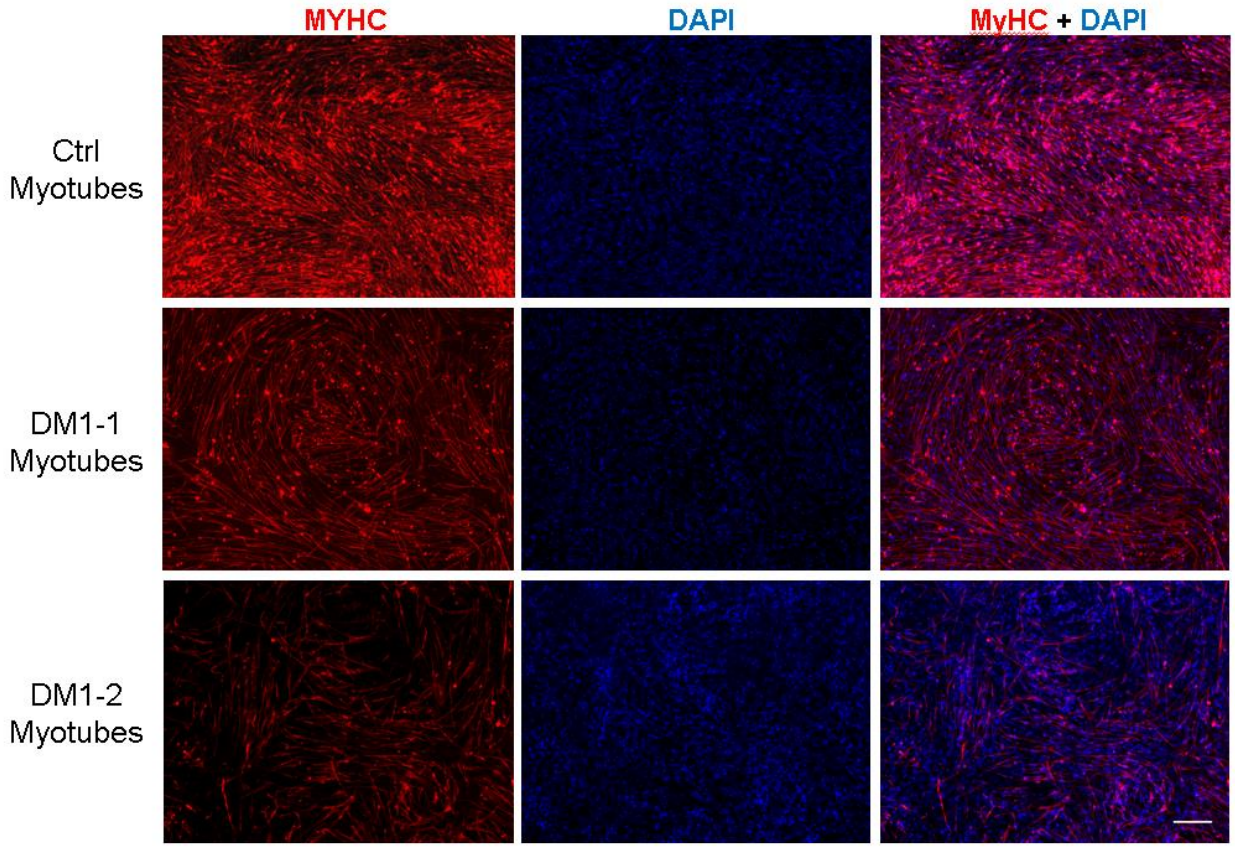

Figure 13. Terminal myogenic differentiation of DM1 iPS cell-derived myogenic progenitors into myotubes. Representative images show immunostaining for MYHC (red), a marker of myogenic terminal differentiation in myotubes derived from control and DM1 myogenic progenitors after 5 days of differentiation. DAPI stains nuclei. Scale bar is 200 μm.

RNA-FISH analysis of DM1-1 and DM1-2 myotubes showed the presence of intranuclear RNA foci (Fig. 14A). The number of foci in the myotubes was higher compared to the cell stages previously evaluated, particularly for DM1-2, in which we consistently found about 14 foci per nucleus (Fig. 14B). Next, we analyzed the distribution of MBNL1 in DM1 myotubes and observed co-localization between MBNL1 and the RNA foci, therefore confirming the sequestration of this splicing factor (Fig. 14C).

2.4 Reversal of DM1 molecular phenotype by antisense oligonucleotide treatment of DM1 iPS cells-derived myotubes

The generation of DM1 iPS cell-derived myotubes provides the possibility of using these cells as an alternative to primary myoblasts for drug screening purposes. Therefore, we evaluated the effectiveness of treating DM1-1 and DM1-2 myotubes with 2'-OMePT(CAG)7 antisense oligonucleotides (ASO), which have been shown to abolish the RNA foci in myotubes differentiated from primary myoblasts [24]. One day after ASO treatment, we observed a significant reduction in the number of nuclei containing RNA foci in DM1-1 and DM1-2 myotubes (Fig. 15A-B). Subsequently, we assessed whether this reduction was sufficient to rescue the mis-splicing of *BIN1* exon 11, which has been related to myopathy, T tubule alterations, and was also found to be among the genes with higher splicing disruption in DM1[19,58]. We observed that upon ASO treatment of DM1-1 and DM1-2 myotubes, there was a significant rescue in the inclusion of *BIN1* exon 11, which correlated with the reduction of RNA foci (Fig. 15C-D).

Of note, ASO treatment had no effect on the ability of DM1-1 and DM1-2 myogenic progenitors to differentiate into myotubes (Fig. 16A-B).

Overall, our results demonstrate that myogenic progenitors and myotubes differentiated from patient-specific DM1 iPS cells through the iPAX7-EB protocol display the key molecular features of DM1, such as intranuclear RNA foci, MBNL1 sequestration and subsequent splicing disruption. Hence, these cells represent a myogenic model that can be used as an alternative to primary myoblasts for studying the disease pathogenesis and/or drug screening purposes.

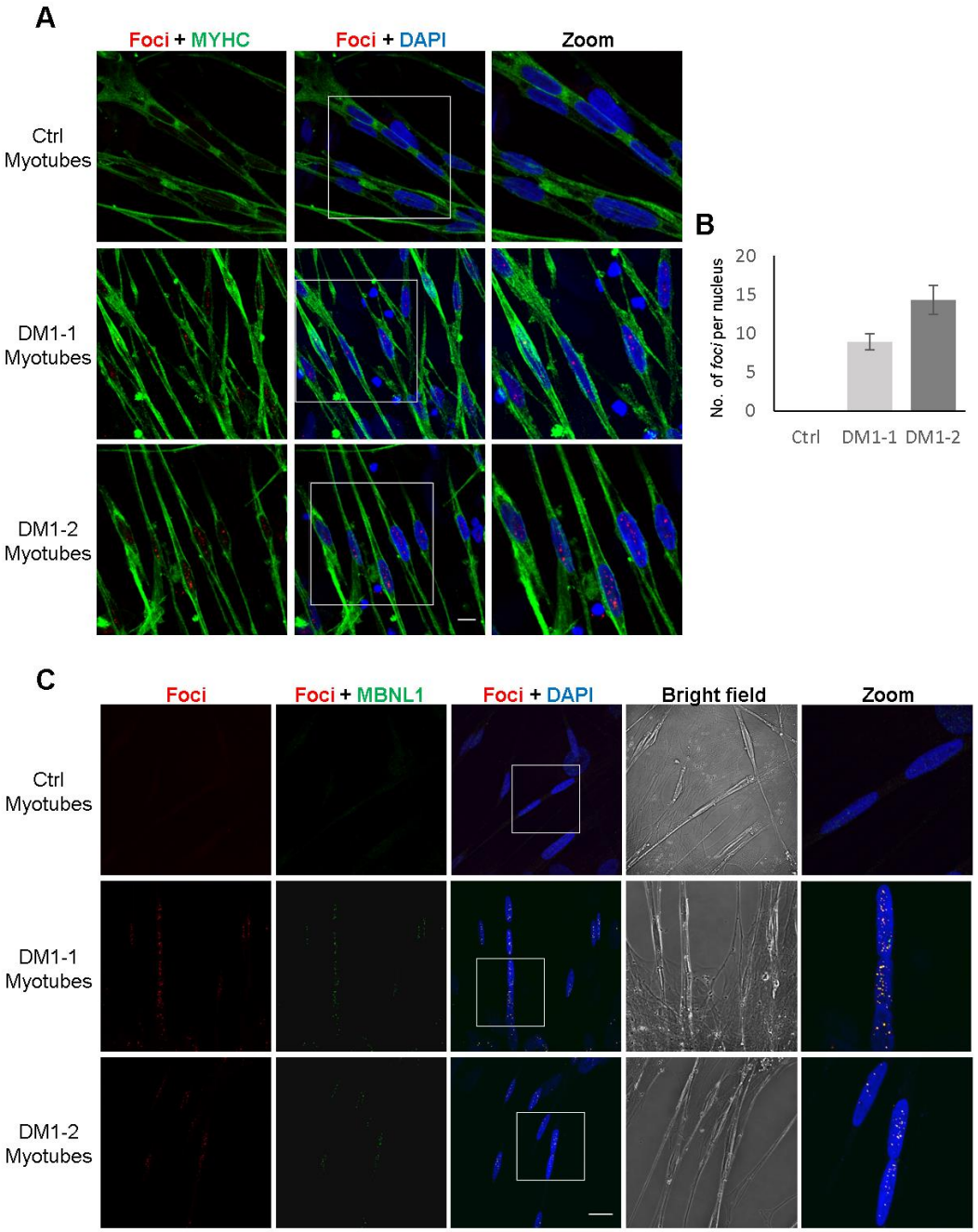

Figure 14. Terminal differentiation of DM1 patient-specific iPS cell-derived myogenic progenitors into myotubes. (A) Representative images show RNA-FISH of foci (red) co-stained with MYHC (green) in control- and DM1-derived myotubes. Confocal microscopy shows maximum projection of the Z sections. Scale bar is 10 μm. (B) Bar graph shows respective quantification of foci (from A), represented as average number of *foci* per nuclei in 150 cells. Bar represents S.D. from three independent experiments. (C) Representative image of RNA-FISH (red) coupled with immunostaining of the splicing factor MBNL1 (green) in Control, DM1-1 or DM1-2 myotubes, analyzed by confocal microscopy. Mid Z section is shown. Scale bar is 20 μm.

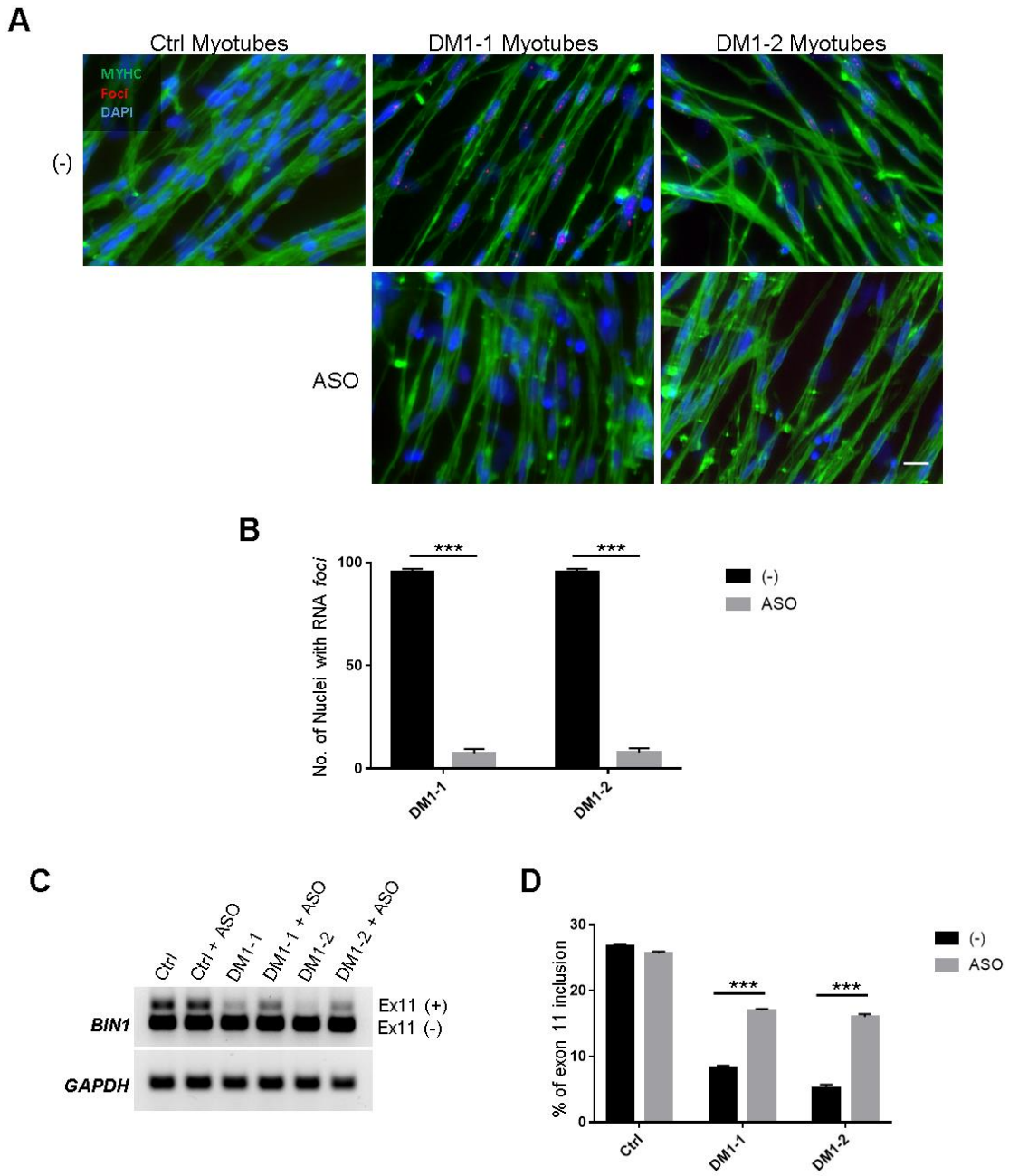

Figure 15. Antisense oligonucleotide treatment reverses the molecular phenotype of DM1 iPS cell-derived myotubes. (A) Representative images show RNA-FISH of foci (red) coupled with immunostaining of MYHC (green) following 2'-OMePT(CAG)7 antisense oligonucleotide treatment on DM1-1 or DM1-2 myotubes. Scale bar is 20 µm. (B) Bar graph represents the number of nuclei showing RNA foci in three independent experiments (n=100). Data are shown as mean ± SEM. Comparison was done using Mann-Whitney test ***p<0.001. (C) RT-PCR analysis of *BIN1* exon 11 following 2'-OMePT(CAG)7 antisense oligonucleotide treatment in Control, DM1-1 or DM1-2 differentiated myotubes. (D) Bar graph represents respective percentage of *BIN1* exon 11 inclusion (from C) from three independent replicates. Data are shown as mean ± SEM. Comparison was done using Mann-Whitney test ***p<0.001.

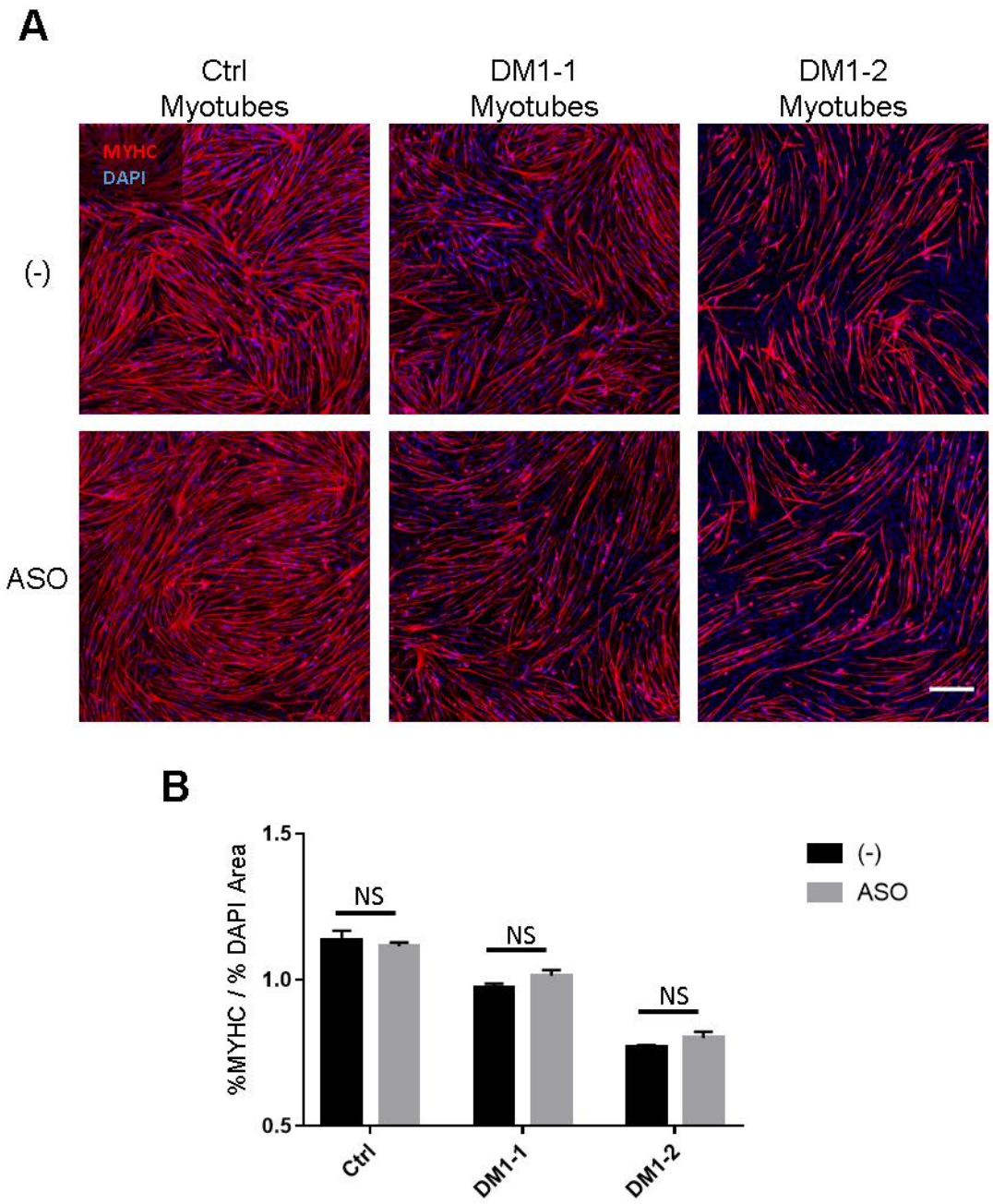

Figure 16. Antisense oligonucleotide treatment does not improve terminal differentiation of DM1-1 and DM1-2 myogenic progenitors into myotubes. (A) Representative images of MYHC immunostaining (red) of control and DM1 myotubes pretreated with antisense oligonucleotides in the myogenic progenitor stage. DAPI stains nuclei. Scale bar is 200 μm. (B) Bars graph represents the ratio of percentage of MYHC area to percentage of DAPI area of (A) from three independent replicates. Data are shown as mean ± SEM. Comparison was done using Mann-Whitney test ***p<0.001.

DISCUSSION

1. Intramuscular transplantation of non-affected hiPS cell-derived myogenic progenitors in an immunodeficient-DM1 mouse model to evaluate the effectiveness of a cell therapy approach.

Although several attempts to improve the muscle pathology in DM1 have been carried out, to date there are no effective treatments available. Therefore, there is still a need to generate novel therapeutic approaches with the potential to improve patient's quality of life. In this regard, cell-based therapy has shown promising results in the recovery of muscle function when applied to other muscular dystrophies (e.g. DMD)[59]. However, to date there are no studies addressing the feasibility of using a cell-based therapy approach to overcome the molecular hurdles associated with DM1. Transplantation of myogenic progenitors generated from hiPS cells through the EB-iPAX7 protocol in immunodeficient mice models has shown not only a successful engraftment of the injected cells in muscle fibers, but also a contribution to the pool of myogenic reserve cells[39,60]. Thus, we set out to determine whether hiPS cell-derived myogenic progenitors could improve the DM1 skeletal muscle pathology when transplanted in a DM1 mouse model. The HSA^{LR} mouse model[12] has been widely used to study the phenotype of DM1, specifically in skeletal muscle, as it resembles the main molecular features of the disease. Thus, we combined the transgene expressed in HSA^{LR} mice with the mutations of NSG mice that confer immunodeficiency, which allowed us to obtain a DM1 mouse model suitable for the transplantation of human cells. Immunodeficiency was confirmed by the ablation of T, B and NK cells revealed by FACS analysis from peripheral blood. Furthermore, expression of intranuclear RNA foci, sequestration of MBNL1 and the associated alternative splicing defects were also confirmed in NSG-HSA^{LR} mice. In line with the confirmed immunodeficiency, we observed a successful engraftment of human iPS cell-derived myogenic progenitors transplanted in tibialis anterior muscles of NSG-HSA^{LR} mice. Engraftment efficiency was similar to NSG transplanted mice. Surprisingly, we observed that nuclei derived from non-affected transplanted cells (identified as human DNA+ and human Lamin A/C+) were also positive for RNA foci, similar to NSG-HSA^{LR} endogenous nuclei. This observation was consistent regardless of the myogenic cell type transplanted (i. e. human skeletal myoblasts or mouse-derived satellite cells). Our results suggest a dynamic behavior of the RNA foci, by which these are able to transmigrate among nuclei within a muscle fiber. DM1 RNA foci

have been canonically reported as aggregates that are not exported from the nucleus. However, there is no convincing evidence addressing the mechanism by which they are retained in this subcellular compartment. Moreover, RNA foci have high affinity to MBNL1, a protein known to undergo a dynamic nucleus-cytoplasmic transport, making it difficult to decipher their intranuclear retention[61,62]. Few studies have been carried out to understand the RNA foci nuclear localization, from which hnRNP H has emerged as a potential candidate maintaining the RNA foci within the nucleus[63]. RNA foci have been observed in cytoplasm of different cell types, which has mainly been attributed to cell division, causing the aggregates to remain outside the nucleus after nuclear membrane dissembling[64]. However, an active export of the RNA foci has not been completely discarded[64]. Particularly in the NSG-HSA^{LR} mouse model, nuclear export of the RNA foci might be attributed to a saturation of the aggregates within the nucleus as a consequence of the high transgene expression, under the HSA promoter. Nonetheless, our results provide evidence not only of an export of the RNA foci to the cytoplasm, but also the ability of these aggregates to be imported to other nuclei. This possibility has never been addressed before. Although mechanistic studies are required to decipher the molecular dynamics of DM1 RNA foci in myofibers, our results provide new evidences of RNA foci nucleus/cytoplasm transport and opens the field for a new perspective on the molecular pathology of DM1.

2. Myogenic differentiation of DM1 patient-specific hiPS cells

Patient-derived myoblasts have been widely used for *in vitro* modeling of muscular diseases. However, expansion of muscle primary cells is limited by senescence and their terminal differentiation capabilities decrease upon passaging. Although establishing patient-specific immortalized myoblasts might overcome some of these hurdles, these have been modified to alter their cell cycle, and thus this aspect of cell physiology is abnormal. The reprogramming of human somatic cells into iPS cells by Yamanaka and colleagues[37] emerged as a promising tool to recapitulate muscle diseases in the Petri dish since these cells can be expanded indefinitely and are able to differentiate into several tissues, including skeletal muscle.

To date, several protocols have been established to promote the myogenic differentiation of iPS cells. One of the main advantages of our method based on the conditional expression of PAX7 is the generation of myogenic progenitors that can be robustly expanded *in vitro* [39], as opposed to protocols based on MYOD induction, which give rise directly to more differentiated muscle cells, and accordingly, with limited proliferation ability [65]. Furthermore, iPAX7 myogenic progenitors can be frozen/thawed by conventional methods and still efficiently differentiate into myotubes,

which allows for the generation of large stocks of cells from the same preparation for further experiments. This feature is highly relevant for a myogenic model as it makes it a suitable source for high-throughput drug screening.

In this study, we differentiated DM1 patient-specific iPS cells into the myogenic lineage [50] to determine whether these cells could recapitulate the main molecular events of DM1, and therefore be considered as a valuable alternative myogenic model of the disease. DM1 patient-specific iPS cells efficiently differentiated into myogenic progenitors able to terminally differentiate into $MYHC^{+}$ myotubes. We found DM1 iPS cell-derived myotubes to display typical expression of intranuclear RNA foci along with sequestration of MBNL1, which is the main molecular event associated with DM1 phenotype. This was further corroborated by identifying the splicing disruption of *BIN1*, a gene which mis-splicing has been related to DM1 myopathy [19]. An important aspect to be evaluated in a model is its ability to validate previously tested drugs. In this regard, DM1 iPS cell-derived myotubes treated with ASO showed a significant decrease in RNA foci along with a significant rescue of *BIN1* exon 11 splicing.

An important aspect to be taken in consideration when studying DM1 *in vitro* and in vivo is the instability of the CTG expansions as these may expand or contract depending on the cell type or upon cell passaging *in vitro* [66-69]. Two recent publications making use of DM1 iPS cells have focused on this feature, and both concluded that despite increased instability of the repeats during the reprogramming of fibroblasts into iPS cells, this is minimized upon differentiation of DM1 iPS cells into specific lineages [45,46].

Taken together, we demonstrate the efficient differentiation of two newly reprogrammed DM1 patient-specific iPS cell lines into skeletal myogenic progenitors and subsequent myotube derivatives, which faithfully recapitulate key molecular events of DM1, making them suitable for *in vitro* disease studies and drug testing.

CONCLUSIONS

DM1 is a complex disease with no cure or treatment so far. As an attempt to explore the feasibility of a cell-based therapy approach for DM1, we generated an immunodeficient/myotonic model by crossing NSG mice with HSA^{LR} mice to obtain a model suitable for human cell transplantation. We showed that NSG-HSA^{LR} mice are immunodeficient and resemble the molecular features of DM1 according to the HSA^{LR} model. Moreover, NSG-HSA^{LR} mice were successfully transplanted with unaffected human and mouse cells. Unexpectedly, we observed that intrafiber nuclei displaying markers that allowed us to recognize exogenous transplanted cells also showed intranuclear RNA *foci*, similar to the endogenous nuclei. This result was not observed in *in vitro* studies and suggests a possible transmission of RNA *foci* from the endogenous nuclei to the engrafted ones. This hypothesis remains to be further explored to define whether cell therapy can be studied as a potential therapy for DM1.

In order to obtain insights into a potential therapy for the skeletal muscle pathology, it is important to generate reliable models for the *in vitro* study of the disease. In this work, we reprogrammed DM1-patient derived fibroblasts to iPS cells and further differentiated them to the myogenic lineage. Myogenic progenitors and terminally differentiated myotubes derived from DM1 iPS cells recapitulated the molecular hallmarks of DM1 and were sensitive to ASO treatment, which implies that these cells are useful for *in vitro* disease modelling and drug screening.

PERSPECTIVES AND FUTURE WORK

It is necessary to explore deeper what is the mechanism related to the potential transmission of RNA *foci* from endogenous nuclei to the engrafted ones. As a first approach, it will be necessary to replicate our observations using a different mouse model of DM1 to confirm that the export of RNA foci is not exclusive to the transgene context of HSA^{LR} mice. If confirmed, it will be important to dissect a possible molecular mechanism related to our observations. For instance, transplantation of myogenic progenitors overexpressing a cytoplasmic form of MBNL1 will suggest whether this protein is involved in the intra-fiber migration of RNA foci as these aggregates would accumulate in the cytoplasm of the muscle fiber.

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