HYPERACTIVE *PIGGYBAC* TRANSPOSONS EXPRESSING FULL-LENGTH HUMAN DYSTROPHIN ENABLE CORRECTION OF DYSTROPHIC MESOANGIOBLASTS

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ABSTRACT

Duchenne muscular dystrophy (DMD) is a genetic disorder characterized by the absence of the dystrophin protein at the sarcolemma. This provokes myofiber degeneration and progressive muscle wasting, ultimately resulting in significant morbidity and mortality in the afflicted patients. Currently there is no treatment that prevents or reverses disease progression. Genetically corrected stem/progenitor cells could potentially provide an effective treatment. However, commonly used viral vector technologies preclude efficient gene transfer of the full-length dystrophin cDNA. In this study, we validated a novel stem cell-based non-viral gene therapy approach for DMD, based on the use of the hyperactive *piggyBac* (*PB*) transposons. We demonstrated that this system enabled stable non-viral gene delivery and sustained expression of both full-length and truncated versions of the human dystrophin cDNA into a murine myogenic cell line and into dystrophic skeletal muscle pericyte-derived mesoangioblasts (MABs). These MABs were isolated from a Golden Retriever muscular dystrophy (GRMD) dog, a clinically relevant large animal model of DMD. The use of hyperactive *PB* transposons therefore overcomes one of the main bottlenecks in delivering large therapeutic transgenes, such as full-length dystrophin, into clinically relevant stem/progenitor cells, paving the way towards a relatively efficient and safe non-viral gene therapy approach for DMD.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common and severe form of muscular dystrophy, affecting approximately 1 in 3,500 boys (Emery, 1991). DMD is an X-linked disorder caused by mutations or deletions in the gene encoding dystrophin which is required for the assembly of the dystrophin-glycoprotein complex (Hoffman et al., 1987; Petrof et al., 1993). This complex is responsible of maintaining the integrity of the sarcolemma during muscle contraction, providing a mechanical and functional link between the cytoskeleton of the muscle fiber and the extracellular matrix. The absence of dystrophin causes DMD, a severe inheritable myopathy with its onset in the first few years of life. This pathology leads to a progressive muscle weakness, consistent with fiber degeneration, inflammation, necrosis and replacement of muscle with scar and fat tissue (Mercuri and Muntoni, 2013). Impairment of the patient's daily functional abilities rapidly results in a profound reduction in quality of life together with a shortened life expectancy, due mainly to cardiac and respiratory failure. The current standard of care involves the use of anti-inflammatory and immunosuppressive drugs (e.g. corticosteroids) that have shown to modestly improve muscle function (Bushby et al., 2010a, 2010b; Serra et al., 2012; Mercuri and Muntoni, 2015), prolonging the patient's life expectancy up to 30 years of age. Nevertheless, it is necessary to develop effective therapies that also counteract muscle degeneration in DMD patients and have a more profound impact of the patient's quality of life and life expectancy.

Several approaches are currently being pursued to address this unmet medical need aimed at restoring dystrophin expression (Benedetti et al., 2013). Exon-skipping approaches based on antisense oligonucleotides had initially been proposed as a promising strategy to correct the reading frame and restore dystrophin expression, (Aartsma-Rus, 2010; Mendell et al., 2013). However, exon skipping is only applicable to a subset of patients with specific mutations and ultimately leads to the production of a truncated dystrophin protein, similar to that found in patients affected by Becker muscular dystrophy (BMD). This a milder form of muscular dystrophy that can still cause significant disability (Emery, 2002; Helderman-van den Enden et al., 2010). Consequently, exon-skipping does not replicate and fully reconstitute all of the essential functions

of dystrophin (van den Bergen et al., 2014; Findlay et al., 2015). Despite early encouraging results, recent clinical trials in larger patient cohorts failed to show a statistically significant improvement in muscle function likely due to insufficient restoration of dystrophin expression (Lu et al., 2014).

Gene therapy for DMD is particularly challenging given the large size of the dystrophin gene (2.4 Mb) and its corresponding cDNA (14 kb) (Koenig et al., 1987; Muntoni et al., 2003). Moreover, *in vivo* gene therapy using viral vectors able to provide the full-length dystrophin cDNA (14 kb), such as helper-dependent adenoviral vectors, faces challenges related to the delivery of a therapeutic dose to the entire body musculature (Kawano et al., 2008; Guse et al., 2012). Adeno-associated viral (AAV) vectors might be considered as a good alternative for an efficient systemic delivery in the muscles, but their use is impeded by their restricted packaging capacity (Gregorevic et al., 2004, 2006; Koppanati et al., 2009; Rodino-Klapac et al., 2010). This precludes gene therapy with the full-length human dystrophin cDNA and requires truncated human dystrophin isoforms instead, that may not replicate all of the essential functions associated with full-length dystrophin. Moreover, the use of viral vectors may evoke potential immune responses against the vector and/or the gene-modified cells (Brunetti-Pierri et al., 2004; Wang et al., 2007; Mingozzi et al., 2009). Hence, there is a need to develop strategies that allow for efficient and safe delivery of the full-length dystrophin cDNA.

In the current study, we therefore validated a new gene therapy approach that aims to overcome some of these limitations, allowing stable gene delivery of the full-length human dystrophin cDNA. To achieve this goal, we explored the use of a hyperactive *piggyBac* (*PB*) transposon system, that reportedly can accommodate relatively large inserts (Wu et al., 2006; Li et al., 2011). *PB* transposons, originally identified in the cabbage looper moth *Trichoplusia ni* (Fraser et al., 1985; Cary et al., 1989), have been adapted for use in mammalian cells following codon-usage optimization and incorporation of several hyper-activating mutations (Ding et al., 2005; Yusa et al., 2011; Doherty et al., 2012). For gene therapy, an expression plasmid that encodes for the PB transposase is transiently transfected along with a donor plasmid containing the therapeutic gene, flanked by the transposon terminal repeat sequences (Di Matteo et al.,

2012). The binding of the PB transposase in the terminal repeat sequences enables transposition via a cut-and-paste mechanism. To develop a *PB* transposon-based stem cell/gene therapy approach for DMD, we choose to employ mesoangioblasts (MABs) (Minasi et al., 2002; Tonlorenzi et al., 2007; Dellavalle et al., 2011). MABs are vessel-associated stem/progenitor cells with multi-lineage mesodermal differentiation potential that have the capacity to cross the vessel wall upon intra-arterial transplantation and contribute to the regeneration of the dystrophic muscles (Sampaolesi et al., 2003, 2006; Palumbo et al., 2004; Tedesco et al., 2011). This occurs either by direct fusion with the muscle or by entering the muscle satellite cell niche (Dellavalle et al., 2011; Tedesco et al., 2011). Allogeneic MABs have been recently tested in a first-in-man phase I/II clinical trial, in DMD patients receiving immunosuppressive treatment (EudraCTno. 2011-000176-33) (Tedesco and Cossu, 2012). The use of genetically-corrected autologous MABs is preferred over allogeneic cells since it would potentially obviate immune suppression. In the current study, we provided –for the first time- proof of concept that the hyperactive *PB* transposons are well suited to genetically correct dystrophic MABs by delivering the full-length human dystrophin cDNA.

MATERIALS AND METHODS

Cell culture

C2C12 myoblasts ((91031101; Sigma-Aldrich; (Yaffe and Saxel, 1977)) were cultured, as previously described (Tonlorenzi et al., 2007), in D20 medium composed of high glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 20% Fetal Bovine Serum (FBS; Life Technologies), 2 mM L-glutamine (Life Technologies), 100 IU/ml penicillin and 100 mg/ml streptomycin (P/S; Life Technologies), at 37°C in a 5% CO_2 cell culture incubator. A biopsy from a Golden Retriever muscular dystrophy (GRMD) dog was kindly provided by Le Centre Hospitalier Universitaire Vétérinaire de l' Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation ONIRIS (Nantes-Atlantique, France). Canine mesoangioblasts (MABs) were isolated and maintained in culture, as described previously (Sampaolesi et al., 2006; Tonlorenzi et al., 2007). Briefly, a biopsy from the *vastus lateralis* muscle of a 10-month-old male dog was minced in 1mm^2 pieces. These pieces of muscle were transferred onto collagen type I-coated dishes (Sigma-Aldrich) and incubated in growth medium composed of MegaCell DMEM (Sigma-Aldrich) containing 5% FBS (Life Technologies), 0.1 mM β-mercaptoethanol (Life Technologies), 1% MEM non-essential amino acids (Life Technologies), 1% P/S, 1% L-glutamine, human basic fibroblast growth factor (5 ng/ml, Peprotech) at 37°C in a 5% CO_2, 3% O_2 cell culture incubator. After seven days, we identified small, round, refractile cells (Figure S1A) that adhered weakly to the initial outgrowth of adherent cells. This population of cells was collected and maintained in growth medium on collagen-coated flasks. In order to confirm that the identified population was composed of *bona fide* MABs, cells were characterized for the typical markers of adult dog MABs after five and ten passages (Figure S1B-E). The alkaline phosphatase expression was detected by enzymatic activity using the NBT/BCIP kit (Roche). The staining was performed on fixed cells and allowed the formation of a black/purple precipitate in the cells expressing the enzyme (Figure S1B). Moreover, their capacity to differentiate into mesodermal lineages was analyzed by assessing their intrinsic differentiation potential into multinucleated skeletal myotubes or, under appropriated stimuli, into smooth muscle

cells or adipocytes (Figure S1F,G; see specific section "*In vitro* differentiation assay"). Human skeletal muscle cells (SKM; C-12530; Promocell) were used as positive control and cultured in the same condition of GRMD MABs.

Transposase and transposon constructs

The *piggyBac* (*PB*) transposase constructs encoding the native *PB* transposase (*mPB*) or the hyperactive *PB* transposase (*hyPB*) were described previously (Di Matteo et al., 2014). An identical expression plasmid devoid of the *PB* transposase gene (denoted as "empty") was used as control (Figure 1). The terminal inverted repeats (IRs) of the *PB* transposon constructs correspond to the wild-type IRs (Li et al., 2001, 2005). The codon-optimized full-length human dystrophin cDNA and the truncated variants (previously described in (Athanasopoulos et al., 2011)) were cloned into the *PB* transposon plasmids. Cloning details are available upon request. The human dystrophin cDNAs were driven by the synthetic muscle-specific promoter *SPc5-12* (Li et al., 1999). The transgene of the copepod GFP (Lonza) was used as reporter protein, driven by *SPc5-12* or the constitutive phosphoglycerate kinase 1 (*Pgk*) promoter. The simian virus 40 late polyadenylation (*SV40pA*) signal was cloned down-stream each transgene of the *PB* transposon constructs.

Transfection

C2C12 myoblasts and GRMD MABs were electroporated with the Amaxa Nucleofector® II (Lonza) by using respectively the Cell Line Nucleofector™ Kit V, program B-32 and the Human MSC Nucleofector™ Kit, program U-23. Transfections were optimized to obtain high efficiencies with low levels of toxicity by modifying the amount of plasmids used and the ratio transposase: transposon. Briefly, cells were trypsinized, washed in PBS (Life Technologies) and counted; $1x10^6$ cells were electroporated and subsequently seeded in a single well of a 6-well plate. The day after the medium was replaced with fresh medium. Transfection efficiency was assessed calculating the percentage of GFP+ cells at 24, 48 and 72 hours post-electroporation (EP) by fluorescence microscopy and fluorescence activated cells sorter (FACSCanto™ flow cytometer,

Becton Dickinson); analyses were performed with FACSDiva software. Transfected C2C12 cells or GRMD MABs were therefore trypsinized, washed in PBS, counted and resuspended into PBS supplemented with 1% FBS and 2mM EDTA (Life Technologies). Where indicated, GFP+ C2C12 cells or GRMD MABs were enriched 4-7 days post-EP by FACS sorting (BD FACSAria™, Becton Dickinson). Transfected cells were monitored for 28-30 days post-EP to assess transposition efficiency.

Transduction

GRMD MABs were transduced with a lentiviral vector containing a tamoxifen-inducible MyoD-ER expression cassette (Kimura et al., 2008; Tedesco et al., 2012; Gerli et al., 2014; Maffioletti et al., 2015) to induce myogenic differentiation at late passages (i.e. passage 17). Lentiviral vector titration was calculated based upon the p24 assay (Sigma-Aldrich). Working concentrations were determined by assessing myogenic differentiation efficiency at increasing multiplicities of infection (MOI). Briefly, $14x10^5$ cells were transduced with 1, 5 or 50 MOI of MyoD-ER lentiviral vector in 1 ml of culture medium and incubated for 12 hours at 37°C in a 5% CO_2, 3% O_2 cell culture incubator. Media was subsequently removed, cells were washed with PBS and maintained in culture for 2-3 passages, after which the *in vitro* differentiation assay into skeletal muscle was performed (see specific section below).

***In vitro* differentiation assays**

The ability of C2C12 cells, GRMD MABs, SKM cells to differentiate into skeletal muscle was assessed *in vitro* by exposing the cells upon reaching 80% of confluence to a specific differentiation medium composed of DMEM supplemented with 2% horse serum (Euroclone), 1% P/S, 1% L-glutamine for 4 to 10 days (Tonlorenzi et al., 2007). C2C12 cells were cultured and induced to differentiate on a plastic supporting substrate while GRMD MABs and SKM cells were seeded onto a matrigel (BD Biosciences) coated plastic substrate. When cells were transduced with the tamoxifen-inducible MyoD-ER lentiviral vector, 1 μM 4-hydroxy-tamoxifen (Sigma-Aldrich) was added in the growth medium that was replaced 24 hours later by differentiation

medium supplemented with 1 µM 4-hydroxy-tamoxifen. Half of the medium was replaced with fresh differentiation medium every other day. Immunofluorescence (IF) staining for myosin heavy chain (MyHC) was performed to confirm multinucleated myotubes (see specific section for "IF staining").

Differentiation of GRMD MABs into smooth muscle-like cells was induced by treating the cells for 10 days with differentiation medium supplemented with 5 ng/ml transforming growth factor β1 (Sigma-Aldrich) (Ross et al., 2006). Smooth muscle differentiation was confirmed by IF staining for α-smooth muscle actin (αSMA; see specific section for "IF staining"). The Mesenchymal Stem Cell Adipogenesis Kit (Millipore) was used to coax differentiation of MABs into adipocytes, according to the manufacturer's instructions. Adipogenesis induction medium was replaced with maintenance medium every 2-3 days for 21 days. Oil Red O staining of lipid vacuoles was performed to detect mature adipocytes after 21 days.

Flow cytometry

Expression of CD44, CD34 and CD45 markers on GRMD MABs was assessed after incubating the cells with specific anti-dog fluorochrome-conjugated monoclonal antibodies for 1 hour at 4°C (anti-CD44-APC, FAB5449A; R&D Systems), (anti-CD34-PE, 559369; BD Biosciences), (anti-CD45-RPE, MCA1042PE; AbD Serotec). Cells were subsequently washed with PBS and fixed in 2% paraformaldehyde (Sigma-Aldrich). Cytofluorimetric analysis was performed using a FACSCanto™ flow cytometer and FACSDiva software. At least 10,000 events were acquired for each sample.

Immunofluorescence staining

In vitro differentiated C2C12 cells or GRMD MABs were washed with PBS (Sigma-Aldrich), fixed with 4% paraformaldehyde (Sigma-Aldrich) at room temperature (RT) for 10 minutes and permeabilized with PBS containing 0.2% Triton X-100 (Sigma-Aldrich) and 1% BSA (Sigma-Aldrich) at RT for 30 minutes. As a blocking solution, 10% donkey serum (Sigma-Aldrich) was used at RT for 30 minutes to reduce secondary antibody background signal. The cells were

subsequently incubated overnight at 4°C with the following primary antibodies: rabbit anti-turboGFP (AB513; Evrogen), mouse anti-dystrophin (NCL-DYS1, NCL-DYS2, NCL-DYS3; Novocastra), mouse anti-myosin heavy chain (MyHC MF20; Developmental Studies Hybridoma Bank, USA), rabbit anti-myosin (476126; Calbiochem), mouse anti-myogenin (F5D; Developmental Studies Hybridoma Bank, USA), rabbit anti-MyoD (M-318; Santa Cruz), mouse anti-sarcomeric α-actinin (ab9465; Abcam), mouse anti-α smooth muscle actin (A2547; Sigma-Aldrich). After incubation, cells were washed with PBS and then incubated with the appropriate 488, 546, 594 or 647-fluorochrome conjugated secondary antibodies (Life Technologies) together with Hoechst 33342 for nucleic acid staining (B2261; Sigma-Aldrich) for 1 hour at RT in PBS containing 0.2% Triton X-100. After three successive washings with PBS, slides were mounted using fluorescent mounting medium (Dako) and examined by fluorescence microscopy (DMI6000B, Leica, Germany). Images were analyzed using ImageJ software (NIH). The monoclonal antibody MF20 developed by Donald Fischman was obtained from DSHB developed under the auspices of the NICHD and mainteined by the University of Iowa, Department of Biology, Iowa City, IA 52242.

RNA analysis

Total RNA was extracted and reversed transcribed using a cDNA synthesis kit (SuperScript® III First-Strand synthesis system for RT-PCR kit, Life Technologies). cDNA was then amplified on a Step-One Real-Time PCR System (Applied Biosystems; Life technologies) with SYBR green PCR Master mix (Applied Biosystem). For the characterization of GRMD MABs, amplification was performed with an annealing temperature of 60°C for 33 cycles by using the primers in Supplementary data, Table S1. Amplicons were then resolved by electrophoresis on a 2% agarose gel. Transgene mRNA levels were determined by quantitative RT-PCR (qRT-PCR) using the primers in Supplementary data, Table S2. Data were normalized to the GAPDH housekeeping gene or to the MyHC gene specific for the skeletal muscle differentiation, and the expression levels of the selected transgene was calculated using a standard ΔΔCt method (Livak and Schmittgen, 2001).

Transposon copy number

The number of copies of *PB* transposon integrated per cell (diploid genome) was calculated by performing a qPCR on genomic DNA (DNeasy Blood & Tissue Kit, Qiagen) by using primers specific for the 5' inverted repeat region (5'IR) of the *PB* transposon, as previously described (Di Matteo et al., 2014). The standard curve was determined by a known copy number of the corresponding *PB* transposon plasmid.

Statistical analysis

Data were presented as mean and standard deviation (SD) or standard error of the mean (SEM). Comparison between groups was performed by using Student's t-test assuming two-tailed distribution and equal variances or One-way ANOVA (Bonferroni's multiple comparison test).

RESULTS

Generation of *PB* transposon vectors encoding for human dystrophin

We generated *PB* transposons designed to express the full-length or truncated versions of a codon-optimized human dystrophin cDNAs (Athanasopoulos et al., 2011) from the synthetic muscle-specific promoter SPc5-12 (360 bp) (Li et al., 1999; Koo et al., 2011; Rincon et al., 2014) (Figure 1). SV40 polyadenylation sites were incorporated as transcription termination signals in all the expression cassettes. Due to the large cargo that *PB* transposons could accommodate (Ding et al., 2005; Li et al., 2011), we generated a large-size transposon named *PB-SPc-DYS-Pgk-GFP* encoding for both the full-length human dystrophin cDNA (*huDYSco*; size: 11.2 kb) driven by the synthetic SPc5-12 promoter, and a GFP reporter gene driven by the constitutive phophoglycerokinase (*Pgk*) promoter (transposon size: 17 kb) (Figure 1A). *PB-SPc-MD1* (Figure 1B) and *PB-SPc-MD2* (Figure 1C) transposons encoded respectively for two different codon usage-optimized micro-dystrophin cDNAs: *huMD1co* (size: 3.6 kb) and *huMD2co* (size: 4 kb) whereby the C-terminal domain was deleted in *huMD1co* (Koo et al., 2011) (transposon size: 8.2 kb, 8.6 kb, respectively). As controls, *PB-SPc-GFP* (Figure 1D) and *PB-Pgk-GFP* (Figure 1E) transposons carried only the copepod GFP sequence under the control of the SPc5-12 or the constitutive *Pgk* promoter (transposon size: 5.2 kb, 5.4 kb, respectively). The transposase plasmids included the *mPB* expression plasmid, encoding the murine codon-optimized, non-hyperactive *PB* transposase (Figure 1F) and the *hyPB* expression plasmid encoding the murine codon-optimized, hyperactive *PB* transposase (Figure 1G). An “empty” plasmid that contained an identical expression cassette but devoid of transposase (Figure 1H) was employed as control (Di Matteo et al., 2014).

Expression of human micro-dystrophins after *PB*-mediated transposition in the C2C12 murine myoblasts

To test the functionality of the *PB* transposons, we co-transfected C2C12 cells, a murine myoblast cell line (Yaffe and Saxel, 1977), by electroporation with the *PB-SPc-GFP* transposon and the *mPB* transposase-encoding construct (ratio of 1.26 pmol transposase DNA: 3.48 pmol transposon DNA). In parallel, *PB-SPc-MD1* and *PB-SPc-MD2* transposons were electroporated under the same conditions. To determine the transfection efficiency, FACS analysis was performed 48 hours post-electroporation (EP), showing 26±4% GFP+ cells which remained relatively stable (24±4% GFP+ cells at 72 hours post-EP; not statistically significant) (Figure 2A, left panel). As control, the "empty" expression plasmid- devoid of *mPB* transposase gene- was electroporated with the *PB-SPc-GFP* transposon resulting initially in comparable transfection efficiencies at 48 hours (17±1% GFP+ cells). However, in contrast to when the *mPB* expression vector was employed, the percentage of GFP+ transfected cells gradually declined at 72 hours post-EP, consistent with the loss of non-integrated *PB-SPc-GFP* plasmids in the rapidly dividing C2C12 cells (9±1% GFP+ cells; $p \leq 0.01$). Similarly, the mean fluorescent intensity (MFI) was sustained when the *PB-Spc-GFP* transposon was cotransfected with *mPB* and significantly different ($p \leq 0.001$) from the controls that resulted in a concomitant decline in MFI, consistent with the lack of genomic integration in the absence of *mPB* transposase (Figure 2A, right panel). These FACS data were confirmed by live cell imaging (Figure 2B).

Subsequently, we assessed whether C2C12 cells transposed with the *PB* transposons retained their ability to differentiate into skeletal muscle and whether expression of the GFP and microdystrophins MD1 and MD2, encoded by the transposons, was sustained upon differentiation. Four days after incubation with the differentiation medium (corresponding to 10 days post-EP), multinucleated myotubes were observed. The transcription levels of GFP (Figure 2C) and the human microdystrophins *MD1* and *MD2* (Figure 2E) were detectable by qRT-PCR and showed a significantly increased expression upon differentiation ($p \leq 0.001$). This confirmed the muscle-specificity of the synthetic SPc5-12 promoter, consistent with its up-regulation upon myogenic differentiation. The transcript levels of the microdystrophins *MD1* and *MD2* in the

differentiated C2C12 cells were comparable to the level of human dystrophin transcripts in differentiated human skeletal muscle (SKM) cells (Figure 2E). In contrast, we were not able to detect any GFP expression from differentiated C2C12 cells that were co-transfected with the "empty" plasmid and the *PB-SPc-GFP* transposon. This indicates that transposition was a prerequisite to ensure stable GFP transcript levels. Immunofluorescence (IF) staining independently confirmed GFP (Figure 2D) and human microdystrophin MD1 and MD2 expression (Figure 2F) in the myosin heavy chain (MyHC) positive myotubes, derived from the transposed C2C12 cells. The percentage of myotubes positive for GFP, MD1 or MD2 was determined by counting the nuclei within these myotubes divided by the total number of nuclei within the MyHC positive myotubes (68±6% for GFP; 65±2% for MD1 and 66±2 for MD2, respectively). Collectively, these results demonstrate that the *PB* transposon system is well suited to achieve sustained muscle-specific expression of human micro-dystrophins upon transposition in differentiated myogenic cells.

Increased transposition in the C2C12 murine myoblasts with the hyperactive *PB* transposase

Different research groups, including ours, have shown that the *hyPB* transposase resulted in increased transposition as compared to the *mPB* transposase (Yusa et al., 2011; Di Matteo et al., 2014). In line with these results, we therefore assessed the transposition efficiency of *hyPB* transposase in a head-to-head comparison with the *mPB* transposase. The *mPB* or *hyPB*-encoding expression constructs were therefore electroporated with the large-size *PB-SPc-DYS-Pgk-GFP* (17 kb) transposon. Controls were transfected with the "empty" expression plasmid devoid of any *PB* transposase and the *PB-SPc-DYS-Pgk-GFP* transposon.

Transfected C2C12 myoblasts (0.32 pmol transposase DNA: 0.87 pmol transposon DNA) expressing GFP were enriched by FACS sorting at 4 days post-EP and monitored at different time points by FACS analysis and fluorescence microscopy (Figure 3A). At 30 days post sorting, cells transfected with the *hyPB* transposase showed a statistically significant ($p \leq 0.001$) higher percentage of GFP (78±3% GFP+ cells) compared to when the *mPB* was employed (63±3%

GFP+ cells). This was consistent with a statistically significant difference in MFI ($p \leq 0.001$) detected by FACS when the *hyPB* was used compared to *mPB* (Figure 3B).

We subsequently assessed whether C2C12 cells transposed with the *hyPB* transposase and the large-size transposon *PB-SPc-DYS-Pgk-GFP* expressed the full-length dystrophin and retained their ability to differentiate into skeletal muscle. The transcription levels of full-length human dystrophin were detectable by qRT-PCR and showed a significant increase in expression in C2C12 cells differentiated in myotubes compared to when the cells were retained in a proliferative, non-differentiated state ($p \leq 0.001$) (Figure 3C). This is consistent with our previous results (Figure 2C,E) and confirms the muscle-specificity of the synthetic *SPc5-12* promoter and its up-regulation upon myogenic differentiation. The expression of the full-length human dystrophin expression was confirmed by IF in multinucleated myotubes positive for GFP (Figure 4A) and positive for myosin (Figure 4B). We were not able to detect any dystrophin or GFP expression in differentiated C2C12 cells co-transfected with the "empty" plasmid and the *PB-SPc-DYS-Pgk-GFP* transposon, confirming the need for transposition to obtain stable dystrophin or GFP expression.

Collectively, these results justify the use of hyperactive *PB* transposase to achieve sustained expression of full-length dystrophin in differentiated myogenic cells.

Isolation and characterization of adult skeletal muscle pericyte-derived stem/progenitor cells from the Golden Retriever muscular dystrophy (GRMD) dog model

Adult pericyte-derived stem/progenitor cells or mesoangioblasts (MABs) were isolated from the muscle of a Golden Retriever Muscular Dystrophy dog, as previously described (Sampaolesi et al., 2006; Tonlorenzi et al., 2007). Small, round, refractile and poorly adherent cells were visible with the initial outgrowth of adherent cells (Figure S1A). This population of cells was collected, kept in culture and characterized for the typical markers of adult skeletal muscle MABs, as they expressed pericyte markers such as alkaline phosphatase (AP), CD44, NG2, PDGFRβ and did not express the endothelial markers CD34, the hematopoietic marker CD45, nor the satellite cell marker CD56 (Tonlorenzi et al., 2007; Dellavalle et al., 2011). AP+ cells were detected in the

cultured cell population (Figure S1B). The presence of the CD44 marker and the absence of CD34 and CD45 markers were assessed by FACS analyses, showing the typical profile of this subset of adult pericytes (Figure S1C). In order to confirm that the identified cell population was composed of *bona fide* MABs, further markers were subsequently analyzed by semi-quantitative RT-PCR on the cultured cells (at passage P5 and P10). A heterogeneous bulk cell population directly derived from the GRMD muscle biopsy (passage P1) was used as a positive control. As expected (Figure S1D,E), the transcript levels of *PAX7*, *MYF5*, *CD56* and desmin, that are typically associated with satellite cells, decreased significantly in both the cultured cells at P5 and P10 ($p \leq 0.01$, $p \leq 0.001$). In contrast, the transcript levels of *NG2* and *PDGFRβ* that are typical markers of pericytes, remained stable, as was the case in the bulk population. Finally, the isolated cell population was capable to spontaneously differentiate *in vitro* into skeletal myotubes or into mesodermal lineages upon appropriated stimulation (Figure S1F,G). Collectively, this gene expression profile and functional analyses confirmed that the GRMD cell isolated were *bona fide* mesoangioblasts.

Genetic correction of dystrophic mesoangioblasts by delivery of the full-length human dystrophin in *PB* transposon system

We subsequently optimized the transfection conditions of the dystrophic primary GRMD MABs. The GRMD MABs were transfected by electroporation with the *hyPB* transposase-encoding construct (0.32 pmol DNA) and the *PB-Pgk-GFP* transposon (0.87 pmol DNA) showing a transfection efficiency of 44±1% GFP+ cells at 7 days post-EP (Figure 5A). The transfected cells were sorted and GFP expression was maintained, resulting in 88±3% GFP+ cells at 28 days post sorting (Figure 5B). In contrast, in the absence of the *hyPB* transposase, the percentage of GFP+ cells gradually declined resulting in only background levels of GFP, indistinguishable from non-transfected control GRMD MABs (Figure 5B). This indicates that the sustained GFP expression in the transfected MABs could be ascribed to and is dependent upon *bona fide* transposition. Based on these encouraging results, we subsequently transfected the GRMD MABs by electroporation with the same molar ratios of *hyPB* transposase expression construct (0.32 pmol DNA) and the

large-size transposon *PB-SPc-DYS-Pgk-GFP* (0.87 pmol DNA), encoding both the full-length human dystrophin and the GFP. This resulted in a transfection efficiency of 3±1% GFP+ cells at 7 days post-EP (Figure 5A). The difference in transfection efficiency between the equimolar doses of *PB-SPc-DYS-Pgk-GFP* and *PB-Pgk-GFP* transposons reflected the different sizes of the plasmids, as previously reported (Sharma et al., 2013). Though doubling the *hyPB* transposase and transposon doses, and maintaining the transposase: transposon ratio, further increased the percentage of GFP+ cells (Fig. S2), the viability of the transfected cells concomitantly declined ($p \leq 0.05$). During the 28 days post sorting, GFP expression was monitored by FACS analysis and fluorescence microscopy (Figure 5B). Most importantly, GRMD MABs transfected with the *PB-SPc-DYS-Pgk-GFP* transposon exhibited stable expression of 50±5% GFP+ cells, consistent with stable transposition ($p \leq 0.001$). The transposon copies per diploid genome quantified by qPCR corresponded to 0.5±0.03 copies for the condition *hyPB* + *PB-SPc-DYS-Pgk-GFP* and 1.7±0.04 copies for *hyPB* + *PB-PgkGFP* (Figure 5C). GRMD MABs that were transfected with the transposon but without the transposase harbored no detectable integrated transposons, indistinguishable from non-transfected control cells.

To assess if the integrated *PB-SPc-DYS-Pgk-GFP* transposon could result in sustained expression of the full-length human dystrophin, transposed GRMD MABs were subsequently induced to differentiate *in vitro* into myocytes/myotubes after tamoxifen-induced overexpression of MyoD-ER (Sampaolesi et al., 2006; Kimura et al., 2008). The transcript levels of the full-length human dystrophin were successfully detected by qRT-PCR (Figure 5D, $p \leq 0.05$). The co-localized expression of the full-length human dystrophin and GFP (Figure 6A) mediated by the *PB* transposon *PB-SPc-DYS-Pgk-GFP* was confirmed by IF in differentiated cells. The expression of the full-length human dystrophin was confirmed in myocytes/myotubes positive for myosin (Figure 6B). In contrast, we were not able to detect any dystrophin or GFP expression from the control GRMD MABs co-transfected with the *PB-SPc-DYS-Pgk-GFP* transposons and an “empty” plasmid that does not encode any transposase (Figure 5D, Figure 6A,B). Similarly, sustained GFP transcript levels were also detected by qRT-PCR in GRMD MABs than had undergone transposition after cotransfection with *hyPB* and *PB-Pgk-GFP* (Figure 5D, $p \leq 0.001$). GFP

expression was confirmed by IF in differentiated cells (Figure S3). In the absence of the hyPB transposase, GFP expression was not sustained, confirming the need for transposition to enable stable integration and transgene expression (Figure S3).

Taken together, these findings demonstrate unequivocally that it is possible to genetically correct dystrophic adult mesoangioblasts by using a *PB*-transposon based platform to deliver the full-length human dystrophin.

DISCUSSION

In this study, we demonstrated that the *piggyBac (PB)* transposon platform could be used to obtain stable expression of a therapeutically relevant, large-sized transgene such as human dystrophin, leading to the correction of dystrophic myogenic stem/progenitor cells derived from a dystrophic large animal model. Recent studies have tried to explore the use of transposons to investigate therapeutic strategies in muscular dystrophies, but they were limited to the use of reporter or marker genes (Muses et al., 2011a; Quattrocelli et al., 2011; Cassano et al., 2012; Ley et al., 2014), truncated versions of either dystrophin or utrophin (Muses et al., 2011b; Filareto et al., 2013) or conditionally immortalized muscle cell-derived cell-lines (Muses et al., 2011a, 2011b), which cannot be used clinically. To our knowledge, this is the first report that establishes the use of *PB* transposons encoding for the full-length human dystrophin enabling correction of dystrophic primary cells. This represents a proof of concept study towards the development of a novel stem cell-based gene therapy approach for Duchenne muscular dystrophy with potentially broad implications for the field at large.

The main advantage of *PB* transposons is that they efficiently integrate their cargo into the target cell genome. This property enables robust stable gene expression in both human and mouse cells, *ex vivo* or *in vivo* (reviewed by (VandenDriessche et al., 2009; Di Matteo et al., 2012), (Ding et al., 2005; Nakazawa et al., 2009; Yusa et al., 2011; Doherty et al., 2012; Di Matteo et al., 2014)). This attribute is particularly relevant for stable genetic modification of stem/progenitor cells, given their intrinsic self-renewal and differentiation potential (Belay et al., 2011). As we observed in this study, *PB* transposons efficiently provided sustained dystrophin expression in MABs and their differentiated progeny. In contrast, only transient or no expression was observed in the absence of any transposase-encoding construct. This indicates that *bona fide* transposition was a prerequisite for a robust dystrophin expression in dystrophic MABs. It is known, indeed, that transfection of conventional plasmids results in a very low stable integration

frequency (typically 1 stable integrant per 10^5 transfected cells) (VandenDriessche et al., 2009), that consequently hampers potential clinical applications.

Another advantage of the *PB* transposons is that they enabled stable delivery of the full-length human dystrophin cDNA (14 kb). This exceeds the packaging capacity of γ-retroviral and lentiviral vectors (< 10 kb) and AAV vectors (5 kb). Consequently, this obviates concerns associated with the use of truncated dystrophins that may not replicate all of the necessary functions of its wild-type counterpart (Sampaolesi et al., 2006). Moreover, expression of full-length human dystrophin is broadly applicable to all patients suffering from DMD, regardless of the underlying genetic defect in the dystrophin locus. This is in contrast with other approaches that are restricted in their application to correcting specific mutations of the gene, such as exon-skipping strategies (Arechavala-Gomeza et al., 2012; Echigoya and Yokota, 2014; Lu et al., 2014) or the use of engineered nucleases (Li et al., 2015; Ousterout et al., 2015a, 2015b). The ability of PB transposons to deliver such a relatively large transgene is consistent with previous reports indicating that PB transposons can mediate the transfer of large genetic cargos up to 100 kb (Ding et al., 2005; Li et al., 2011). The availability of *PB* transposons encoding either full-length or truncated dystrophins characterized in this study may pave the way towards future comprehensive structure-function and comparative studies. This study has broader implications for the use of other hyperactive transposon systems, such as *Sleeping Beauty* for gene therapy of DMD (Mates et al., 2009; Belay et al., 2010; Grabundzija et al., 2010;).

However, large-size DNA plasmids adversely impact on the transfection efficiency (Sharma et al., 2013). In accordance with these previous findings, we showed that the use of large transgenes (e.g. full-length human dystrophin cDNA as opposed to truncated *MD1* and *MD2* human microdystrophin cDNAs) and/or increasing DNA concentrations resulted in a net decrease in transfection efficiency. This may be due, at least in part, by the intrinsic property of the DNA plasmids such as the presence of unmethylated CpG motifs in the backbone sequences that might impair cell viability, possibly involving Toll-like receptor (TLR) pathways (Krieg et al., 1995;

Sharma et al., 2013). Nevertheless, the surviving transfected cells could be easily enriched by FACS sorting. Moreover, we demonstrated that the use of the hyperactive *PB* transposase (Yusa et al., 2011) compared to the native *PB* transposase (Cadiñanos and Bradley, 2007) can boost the efficiency and stable expression of large-size transposons, allowing the use of lower amounts of DNA plasmids, and consequently reducing overall DNA toxicity in the transfected cells.

The efficiency of *PB* transposon-mediated delivery of the full-length human dystrophin cDNA compares favorably with alternative approaches that allowed for expression of the wild-type dystrophin protein, such as the human artificial chromosome (HAC; (Kouprina et al., 2014)). This vector is recognized as an endogenous chromosome within the host cells and is stably maintained throughout subsequent cell divisions obviating the need for genomic integration. The entire human dystrophin genomic locus (2.4 Mb), including the native promoter and regulatory elements, has been accomodated into the HAC (Hoshiya et al., 2009; Kazuki et al., 2010) and transferred in dystrophic murine mesoangioblasts and DMD patient specific human iPSC-derived mesoangioblast-like cells (HIDEMs) (Tedesco et al., 2011, 2012). This approach resulted in the full-length human dystrophin expression in the genetically corrected cells. Nevertheless, the generation of such a relatively complex vector like the HAC, together with the intrinsic low efficiency of microcell-mediated chromosome transfer (approximately $1.2x\ 10^{-5}$) required to introduce the HACs into the target cells, render the application of this approach particularly challenging. In contrast, the generation of *PB* transposons eases the manufacturing constrains as opposed to when HACs or viral vectors are used. This may ultimately facilitate the clinical translation of the *PB* transposon vectors.

Though the genomic integration of the *PB* transposons is required for sustained expression in dividing and differentiating stem/progenitor cells, it could raise some concerns related to the risk of insertional oncogenesis by potentially activating oncogenes that are in the vicinity of the integration sites (Biasco et al., 2012). *PB* transposons exhibit a biased integration pattern into genes and their regulatory regions (~ 50% into RefSeq genes) (Wilson et al., 2007; Galvan et al.,

2009; Huang et al., 2010; Meir et al., 2011). Nevertheless, in comparison to γ-retroviral and lentiviral vectors, *PB* transposons show lower integration frequency into or within 50 kb of the transcriptional start sites of known proto-oncogenes (Galvan et al., 2009). This risk of insertional oncogenesis could be minimized further by retargeting *PB*-mediated integration into potential 'safe harbor' loci (Wilson and George, 2010; Kettlun et al., 2011). However, it is reassuring that the *PB* transposon copy number detected in the present study fell within an acceptable and relatively safe range of genomic integrations per cell (i.e. 0.5-1.7 copies per diploid genome), in accordance with the European medicines agency guidelines (http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000410.jsp&mid=WC0b01ac058002958d).

One of the challenges of using primary MABs is their limited lifespan that may potentially complicate the large-scale expansion for clinical use (Tonlorenzi et al., 2007). This likely reflected normal cellular processes that gradually contribute to cellular senescence (Holliday, 2014). Nevertheless, this did not preclude clinical translation since allogeneic MABs have recently been tested in a first-in-man phase I/II clinical trial, in DMD patients (EudraCTno. 2011-000176-33) (Tedesco and Cossu, 2012). In addition, the PB transposon technology is potentially amenable to use in alternative adult myogenic stem cell sources distinct from MABs (Marg et al., 2014; Costamagna et al., 2015). Ultimately, the use of iPS-derived muscle-like cells, that have unlimited expansion potential, may represent an attractive alternative, provided they can safely and efficiently be converted into transplantable myogenic cells (Tedesco et al., 2012; Filareto et al., 2013; Gerli et al., 2014; Maffioletti et al., 2015; Loperfido et al., 2015).

In conclusion, this study shows the first evidence of *PB*-mediated human full-length dystrophin expression in dystrophic MABs for the treatment DMD. Further characterizations *in vivo* are needed upon transplantation of the *PB* transposon-modified MABs in the GRMD model, to ultimately justify a clinical study in patients suffering from DMD.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

Aartsma-Rus, A. (2010). Antisense-mediated modulation of splicing: therapeutic implications for Duchenne muscular dystrophy. RNA Biol. *7*, 453–461.

Arechavala-Gomeza, V., Anthony, K., Morgan, J., and Muntoni, F. (2012). Antisense oligonucleotide-mediated exon skipping for Duchenne muscular dystrophy: progress and challenges. Curr. Gene Ther. *12*, 152–160.

Athanasopoulos, T., Foster, H., Foster, K., and Dickson, G. (2011). Codon optimization of the microdystrophin gene for Duchene muscular dystrophy gene therapy. Methods Mol. Biol. *709*, 21–37.

Belay, E., Dastidar, S., VandenDriessche, T., and Chuah, M.K.L. (2011). Transposon-mediated gene transfer into adult and induced pluripotent stem cells. Curr. Gene Ther. *11*, 406–413.

Benedetti, S., Hoshiya, H., and Tedesco, F.S. (2013). Repair or replace? Exploiting novel gene and cell therapy strategies for muscular dystrophies. FEBS J. *280*, 4263–4280.

Biasco, L., Baricordi, C., and Aiuti, A. (2012). Retroviral integrations in gene therapy trials. Mol. Ther. *20*, 709–716.

Brunetti-Pierri, N., Palmer, D.J., Beaudet, A.L., Carey, K.D., Finegold, M., and Ng, P. (2004). Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. Hum. Gene Ther. *15*, 35–46.

Bushby, K., Finkel, R., Birnkrant, D.J., Case, L.E., Clemens, P.R., Cripe, L., Kaul, A., Kinnett, K., McDonald, C., Pandya, S., et al. (2010a). Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. Lancet Neurol. *9*, 77–93.

Bushby, K., Finkel, R., Birnkrant, D.J., Case, L.E., Clemens, P.R., Cripe, L., Kaul, A., Kinnett, K., McDonald, C., Pandya, S., et al. (2010b). Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. Lancet Neurol. *9*, 177–189.

Cadiñanos, J., and Bradley, A. (2007). Generation of an inducible and optimized piggyBac transposon system. Nucleic Acids Res. *35*, e87.

Cary, L.C., Goebel, M., Corsaro, B.G., Wang, H.G., Rosen, E., and Fraser, M.J. (1989). Transposon mutagenesis of baculoviruses: analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. Virology *172*, 156–169.

Cassano, M., Berardi, E., Crippa, S., Toelen, J., Barthelemy, I., Micheletti, R., Chuah, M., Vandendriessche, T., Debyser, Z., Blot, S., et al. (2012). Alteration of cardiac progenitor cell potency in GRMD dogs. Cell Transplant. *21*, 1945–1967.

Costamagna, D., Berardi, E., Ceccarelli, G., and Sampaolesi, M. (2015). Adult Stem Cells and Skeletal Muscle Regeneration. Curr. Gene Ther.

Dellavalle, A., Maroli, G., Covarello, D., Azzoni, E., Innocenzi, A., Perani, L., Antonini, S., Sambasivan, R., Brunelli, S., Tajbakhsh, S., et al. (2011). Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. Nat. Commun. *2*, 499.

Di Matteo, M., Belay, E., Chuah, M.K., and Vandendriessche, T. (2012). Recent developments in transposon-mediated gene therapy. Expert Opin. Biol. Ther. *12*, 841–858.

Di Matteo, M., Samara-Kuko, E., Ward, N.J., Waddington, S.N., McVey, J.H., Chuah, M.K.L., and VandenDriessche, T. (2014). Hyperactive piggyBac transposons for sustained and robust liver-targeted gene therapy. Mol. Ther. *22*, 1614–1624.

Ding, S., Wu, X., Li, G., Han, M., Zhuang, Y., and Xu, T. (2005). Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. Cell *122*, 473–483.

Doherty, J.E., Huye, L.E., Yusa, K., Zhou, L., Craig, N.L., and Wilson, M.H. (2012). Hyperactive piggyBac gene transfer in human cells and in vivo. Hum. Gene Ther. *23*, 311–320.

Echigoya, Y., and Yokota, T. (2014). Skipping multiple exons of dystrophin transcripts using cocktail antisense oligonucleotides. Nucleic Acid Ther. *24*, 57–68.

Emery, A.E. (1991). Population frequencies of inherited neuromuscular diseases--a world survey. Neuromuscul. Disord. NMD *1*, 19–29.

Emery, A.E.H. (2002). The muscular dystrophies. Lancet *359*, 687–695.

Filareto, A., Parker, S., Darabi, R., Borges, L., Iacovino, M., Schaaf, T., Mayerhofer, T., Chamberlain, J.S., Ervasti, J.M., McIvor, R.S., et al. (2013). An ex vivo gene therapy approach to treat muscular dystrophy using inducible pluripotent stem cells. Nat. Commun. *4*, 1549.

Findlay, A.R., Wein, N., Kaminoh, Y., Taylor, L.E., Dunn, D.M., Mendell, J.R., King, W.M., Pestronk, A., Florence, J.M., Mathews, K.D., et al. (2015). Clinical phenotypes as predictors of the outcome of skipping around DMD exon 45. Ann. Neurol. *77*, 668–674.

Fraser, M.J., Brusca, J.S., Smith, G.E., and Summers, M.D. (1985). Transposon-mediated mutagenesis of a baculovirus. Virology *145*, 356–361.

Galvan, D.L., Nakazawa, Y., Kaja, A., Kettlun, C., Cooper, L.J.N., Rooney, C.M., and Wilson, M.H. (2009). Genome-wide mapping of PiggyBac transposon integrations in primary human T cells. J. Immunother. 1997 *32*, 837–844.

Gerli, M.F.M., Maffioletti, S.M., Millet, Q., and Tedesco, F.S. (2014). Transplantation of induced pluripotent stem cell-derived mesoangioblast-like myogenic progenitors in mouse models of muscle regeneration. J. Vis. Exp. JoVE e50532.

Grabundzija, I., Irgang, M., Mátés, L., Belay, E., Matrai, J., Gogol-Döring, A., Kawakami, K., Chen, W., Ruiz, P., Chuah, M.K.L., et al. (2010). Comparative analysis of transposable element vector systems in human cells. Mol. Ther. 18, 1200–1209.

Gregorevic, P., Blankinship, M.J., Allen, J.M., Crawford, R.W., Meuse, L., Miller, D.G., Russell, D.W., and Chamberlain, J.S. (2004). Systemic delivery of genes to striated muscles using adeno-associated viral vectors. Nat. Med. *10*, 828–834.

Gregorevic, P., Allen, J.M., Minami, E., Blankinship, M.J., Haraguchi, M., Meuse, L., Finn, E., Adams, M.E., Froehner, S.C., Murry, C.E., et al. (2006). rAAV6-microdystrophin preserves muscle function and extends lifespan in severely dystrophic mice. Nat. Med. *12*, 787–789.

Guse, K., Suzuki, M., Sule, G., Bertin, T.K., Tyynismaa, H., Ahola-Erkkilä, S., Palmer, D., Suomalainen, A., Ng, P., Cerullo, V., et al. (2012). Capsid-modified adenoviral vectors for improved muscle-directed gene therapy. Hum. Gene Ther. *23*, 1065–1070.

Helderman-van den Enden, A.T.J.M., Straathof, C.S.M., Aartsma-Rus, A., Dunnen, J.T. den, Verbist, B.M., Bakker, E., Verschuuren, J.J.G.M., and Ginjaar, H.B. (2010). Becker muscular dystrophy patients with deletions around exon 51; a promising outlook for exon skipping therapy in Duchenne patients. Neuromuscul. Disord. NMD *20*, 251–254.

Hoffman, E.P., Monaco, A.P., Feener, C.C., and Kunkel, L.M. (1987). Conservation of the Duchenne muscular dystrophy gene in mice and humans. Science *238*, 347–350.

Holliday, R. (2014). The commitment of human cells to senescence. Interdiscip. Top. Gerontol. *39*, 1–7.

Hoshiya, H., Kazuki, Y., Abe, S., Takiguchi, M., Kajitani, N., Watanabe, Y., Yoshino, T., Shirayoshi, Y., Higaki, K., Messina, G., et al. (2009). A highly stable and nonintegrated human

artificial chromosome (HAC) containing the 2.4 Mb entire human dystrophin gene. Mol. Ther. *17*, 309–317.

Huang, X., Guo, H., Tammana, S., Jung, Y.-C., Mellgren, E., Bassi, P., Cao, Q., Tu, Z.J., Kim, Y.C., Ekker, S.C., et al. (2010). Gene transfer efficiency and genome-wide integration profiling of Sleeping Beauty, Tol2, and piggyBac transposons in human primary T cells. Mol. Ther. *18*, 1803–1813.

Kawano, R., Ishizaki, M., Maeda, Y., Uchida, Y., Kimura, E., and Uchino, M. (2008). Transduction of full-length dystrophin to multiple skeletal muscles improves motor performance and life span in utrophin/dystrophin double knockout mice. Mol. Ther. *16*, 825–831.

Kazuki, Y., Hiratsuka, M., Takiguchi, M., Osaki, M., Kajitani, N., Hoshiya, H., Hiramatsu, K., Yoshino, T., Kazuki, K., Ishihara, C., et al. (2010). Complete genetic correction of ips cells from Duchenne muscular dystrophy. Mol. Ther. *18*, 386–393.

Kettlun, C., Galvan, D.L., George, A.L., Kaja, A., and Wilson, M.H. (2011). Manipulating piggyBac transposon chromosomal integration site selection in human cells. Mol. Ther. *19*, 1636–1644.

Kimura, E., Han, J.J., Li, S., Fall, B., Ra, J., Haraguchi, M., Tapscott, S.J., and Chamberlain, J.S. (2008). Cell-lineage regulated myogenesis for dystrophin replacement: a novel therapeutic approach for treatment of muscular dystrophy. Hum. Mol. Genet. *17*, 2507–2517.

Koenig, M., Hoffman, E.P., Bertelson, C.J., Monaco, A.P., Feener, C., and Kunkel, L.M. (1987). Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell *50*, 509–517.

Koo, T., Malerba, A., Athanasopoulos, T., Trollet, C., Boldrin, L., Ferry, A., Popplewell, L., Foster, H., Foster, K., and Dickson, G. (2011). Delivery of AAV2/9-microdystrophin genes incorporating helix 1 of the coiled-coil motif in the C-terminal domain of dystrophin improves muscle pathology

and restores the level of α1-syntrophin and α-dystrobrevin in skeletal muscles of mdx mice. Hum. Gene Ther. *22*, 1379–1388.

Koppanati, B.M., Li, J., Xiao, X., and Clemens, P.R. (2009). Systemic delivery of AAV8 in utero results in gene expression in diaphragm and limb muscle: treatment implications for muscle disorders. Gene Ther. *16*, 1130–1137.

Kouprina, N., Tomilin, A.N., Masumoto, H., Earnshaw, W.C., and Larionov, V. (2014). Human artificial chromosome-based gene delivery vectors for biomedicine and biotechnology. Expert Opin. Drug Deliv. *11*, 517–535.

Krieg, A.M., Yi, A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A., and Klinman, D.M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature *374*, 546–549.

Ley, D., Van Zwieten, R., Puttini, S., Iyer, P., Cochard, A., and Mermod, N. (2014). A PiggyBac-mediated approach for muscle gene transfer or cell therapy. Stem Cell Res. *13*, 390–403.

Li, H.L., Fujimoto, N., Sasakawa, N., Shirai, S., Ohkame, T., Sakuma, T., Tanaka, M., Amano, N., Watanabe, A., Sakurai, H., et al. (2015). Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. Stem Cell Rep. *4*, 143–154.

Li, M.A., Turner, D.J., Ning, Z., Yusa, K., Liang, Q., Eckert, S., Rad, L., Fitzgerald, T.W., Craig, N.L., and Bradley, A. (2011). Mobilization of giant piggyBac transposons in the mouse genome. Nucleic Acids Res. *39*, e148.

Li, X., Eastman, E.M., Schwartz, R.J., and Draghia-Akli, R. (1999). Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences. Nat. Biotechnol. *17*, 241–245.

Li, X., Lobo, N., Bauser, C.A., and Fraser, M.J. (2001). The minimum internal and external sequence requirements for transposition of the eukaryotic transformation vector piggyBac. Mol. Genet. Genomics MGG *266*, 190–198.

Li, X., Harrell, R.A., Handler, A.M., Beam, T., Hennessy, K., and Fraser, M.J. (2005). piggyBac internal sequences are necessary for efficient transformation of target genomes. Insect Mol. Biol. *14*, 17–30.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402–408.

Loperfido, M., Steele-Stallard, H.B., Tedesco, F.S., and VandenDriessche, T. (2015). Pluripotent Stem Cells for Gene Therapy of Degenerative Muscle Diseases. Curr. Gene Ther.

Lu, Q.-L., Cirak, S., and Partridge, T. (2014). What Can We Learn From Clinical Trials of Exon Skipping for DMD? Mol. Ther. Nucleic Acids *3*, e152.

Maffioletti, S.M., Gerli, M.F.M., Ragazzi, M., Dastidar, S., Benedetti, S., Loperfido, M., VandenDriessche, T., Chuah, M.K., and Tedesco, F.S. (2015). Efficient derivation and inducible differentiation of expandable skeletal myogenic cells from human ES and patient-specific iPS cells. Nat. Protoc. *10*, 941–958.

Marg, A., Escobar, H., Gloy, S., Kufeld, M., Zacher, J., Spuler, A., Birchmeier, C., Izsvák, Z., and Spuler, S. (2014). Human satellite cells have regenerative capacity and are genetically manipulable. J. Clin. Invest. *124*, 4257–4265.

Mátés, L., Chuah, M.K.L., Belay, E., Jerchow, B., Manoj, N., Acosta-Sanchez, A., Grzela, D.P., Schmitt, A., Becker, K., Matrai, J., et al. (2009). Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. Nat. Genet. 41, 753–761.

Meir, Y.-J.J., Weirauch, M.T., Yang, H.-S., Chung, P.-C., Yu, R.K., and Wu, S.C.-Y. (2011). Genome-wide target profiling of piggyBac and Tol2 in HEK 293: pros and cons for gene discovery and gene therapy. BMC Biotechnol. *11*, 28.

Mendell, J.R., Rodino-Klapac, L.R., Sahenk, Z., Roush, K., Bird, L., Lowes, L.P., Alfano, L., Gomez, A.M., Lewis, S., Kota, J., et al. (2013). Eteplirsen for the treatment of Duchenne muscular dystrophy. Ann. Neurol. *74*, 637–647.

Mercuri, E., and Muntoni, F. (2013). Muscular dystrophies. Lancet *381*, 845–860.

Mercuri, E., and Muntoni, F. (2015). Efficacy of idebenone in Duchenne muscular dystrophy. Lancet *385*, 1704–1706.

Minasi, M.G., Riminucci, M., De Angelis, L., Borello, U., Berarducci, B., Innocenzi, A., Caprioli, A., Sirabella, D., Baiocchi, M., De Maria, R., et al. (2002). The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development *129*, 2773–2783.

Mingozzi, F., Meulenberg, J.J., Hui, D.J., Basner-Tschakarjan, E., Hasbrouck, N.C., Edmonson, S.A., Hutnick, N.A., Betts, M.R., Kastelein, J.J., Stroes, E.S., et al. (2009). AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells. Blood *114*, 2077–2086.

Muntoni, F., Torelli, S., and Ferlini, A. (2003). Dystrophin and mutations: one gene, several proteins, multiple phenotypes. Lancet Neurol. *2*, 731–740.

Muses, S., Morgan, J.E., and Wells, D.J. (2011a). A new extensively characterised conditionally immortal muscle cell-line for investigating therapeutic strategies in muscular dystrophies. PloS One *6*, e24826.

Muses, S., Morgan, J.E., and Wells, D.J. (2011b). Restoration of dystrophin expression using the Sleeping Beauty transposon. PLoS Curr. *3*, RRN1296.

Nakazawa, Y., Huye, L.E., Dotti, G., Foster, A.E., Vera, J.F., Manuri, P.R., June, C.H., Rooney, C.M., and Wilson, M.H. (2009). Optimization of the PiggyBac transposon system for the sustained genetic modification of human T lymphocytes. J. Immunother. 1997 *32*, 826–836.

Ousterout, D.G., Kabadi, A.M., Thakore, P.I., Perez-Pinera, P., Brown, M.T., Majoros, W.H., Reddy, T.E., and Gersbach, C.A. (2015a). Correction of dystrophin expression in cells from duchenne muscular dystrophy patients through genomic excision of exon 51 by zinc finger nucleases. Mol. Ther. *23*, 523–532.

Ousterout, D.G., Kabadi, A.M., Thakore, P.I., Majoros, W.H., Reddy, T.E., and Gersbach, C.A. (2015b). Multiplex CRISPR/Cas9-based genome editing for correction of dystrophin mutations that cause Duchenne muscular dystrophy. Nat. Commun. *6*, 6244.

Palumbo, R., Sampaolesi, M., De Marchis, F., Tonlorenzi, R., Colombetti, S., Mondino, A., Cossu, G., and Bianchi, M.E. (2004). Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. J. Cell Biol. *164*, 441–449.

Petrof, B.J., Shrager, J.B., Stedman, H.H., Kelly, A.M., and Sweeney, H.L. (1993). Dystrophin protects the sarcolemma from stresses developed during muscle contraction. Proc. Natl. Acad. Sci. U. S. A. *90*, 3710–3714.

Quattrocelli, M., Palazzolo, G., Floris, G., Schöffski, P., Anastasia, L., Orlacchio, A., Vandendriessche, T., Chuah, M.K.L., Cossu, G., Verfaillie, C., et al. (2011). Intrinsic cell memory reinforces myogenic commitment of pericyte-derived iPSCs. J. Pathol. *223*, 593–603.

Rincon, M.Y., Sarcar, S., Danso-Abeam, D., Keyaerts, M., Matrai, J., Samara-Kuko, E., Acosta-Sanchez, A., Athanasopoulos, T., Dickson, G., Lahoutte, T., et al. (2014). Genome-wide Computational Analysis Reveals Cardiomyocyte-specific Transcriptional Cis-regulatory Motifs That Enable Efficient Cardiac Gene Therapy. Mol. Ther. *23*, 43-52.

Rodino-Klapac, L.R., Montgomery, C.L., Bremer, W.G., Shontz, K.M., Malik, V., Davis, N., Sprinkle, S., Campbell, K.J., Sahenk, Z., Clark, K.R., et al. (2010). Persistent expression of

FLAG-tagged micro dystrophin in nonhuman primates following intramuscular and vascular delivery. Mol. Ther. J. Am. Soc. Gene Ther. *18*, 109–117.

Ross, J.J., Hong, Z., Willenbring, B., Zeng, L., Isenberg, B., Lee, E.H., Reyes, M., Keirstead, S.A., Weir, E.K., Tranquillo, R.T., et al. (2006). Cytokine-induced differentiation of multipotent adult progenitor cells into functional smooth muscle cells. J. Clin. Invest. *116*, 3139–3149.

Sampaolesi, M., Torrente, Y., Innocenzi, A., Tonlorenzi, R., Antona, G. D', Pellegrino, M.A., Barresi, R., Bresolin, N., De Angelis, M.G.C., Campbell, K.P., et al. (2003). Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts. Science *301*, 487–492.

Sampaolesi, M., Blot, S., Antona, G. D', Granger, N., Tonlorenzi, R., Innocenzi, A., Mognol, P., Thibaud, J.-L., Galvez, B.G., Barthélémy, I., et al. (2006). Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. Nature *444*, 574–579.

Serra, F., Quarta, M., Canato, M., Toniolo, L., De Arcangelis, V., Trotta, A., Spath, L., Monaco, L., Reggiani, C., and Naro, F. (2012). Inflammation in muscular dystrophy and the beneficial effects of non-steroidal anti-inflammatory drugs. Muscle Nerve *46*, 773–784.

Sharma, N., Cai, Y., Bak, R.O., Jakobsen, M.R., Schrøder, L.D., and Mikkelsen, J.G. (2013). Efficient sleeping beauty DNA transposition from DNA minicircles. Mol. Ther. Nucleic Acids *2*, e74.

Tedesco, F.S., and Cossu, G. (2012). Stem cell therapies for muscle disorders. Curr. Opin. Neurol. *25*, 597–603.

Tedesco, F.S., Hoshiya, H., Antona, G. D', Gerli, M.F.M., Messina, G., Antonini, S., Tonlorenzi, R., Benedetti, S., Berghella, L., Torrente, Y., et al. (2011). Stem cell-mediated transfer of a human artificial chromosome ameliorates muscular dystrophy. Sci. Transl. Med. *3*, 96ra78.

Tedesco, F.S., Gerli, M.F.M., Perani, L., Benedetti, S., Ungaro, F., Cassano, M., Antonini, S., Tagliafico, E., Artusi, V., Longa, E., et al. (2012). Transplantation of genetically corrected human iPSC-derived progenitors in mice with limb-girdle muscular dystrophy. Sci. Transl. Med. *4*, 140ra89.

Tonlorenzi, R., Dellavalle, A., Schnapp, E., Cossu, G., and Sampaolesi, M. (2007). Isolation and characterization of mesoangioblasts from mouse, dog, and human tissues. Curr. Protoc. Stem Cell Biol. *Chapter 2*, Unit 2B.1.

van den Bergen, J.C., Schade van Westrum, S.M., Dekker, L., van der Kooi, A.J., de Visser, M., Wokke, B.H.A., Straathof, C.S., Hulsker, M.A., Aartsma-Rus, A., Verschuuren, J.J., et al. (2014). Clinical characterisation of Becker muscular dystrophy patients predicts favourable outcome in exon-skipping therapy. J. Neurol. Neurosurg. Psychiatry *85*, 92–98.

VandenDriessche, T., Ivics, Z., Izsvák, Z., and Chuah, M.K.L. (2009). Emerging potential of transposons for gene therapy and generation of induced pluripotent stem cells. Blood *114*, 1461–1468.

Wang, Z., Kuhr, C.S., Allen, J.M., Blankinship, M., Gregorevic, P., Chamberlain, J.S., Tapscott, S.J., and Storb, R. (2007). Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. Mol. Ther. *15*, 1160–1166.

Wilson, M.H., and George, A.L. (2010). Designing and testing chimeric zinc finger transposases. Methods Mol. Biol. *649*, 353–363.

Wilson, M.H., Coates, C.J., and George, A.L. (2007). PiggyBac transposon-mediated gene transfer in human cells. Mol. Ther. *15*, 139–145.

Wu, S.C.-Y., Meir, Y.-J.J., Coates, C.J., Handler, A.M., Pelczar, P., Moisyadi, S., and Kaminski, J.M. (2006). piggyBac is a flexible and highly active transposon as compared to sleeping beauty, Tol2, and Mos1 in mammalian cells. Proc. Natl. Acad. Sci. U. S. A. *103*, 15008–15013.

Yaffe, D., and Saxel, O. (1977). Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature *270*, 725–727.

Yusa, K., Zhou, L., Li, M.A., Bradley, A., and Craig, N.L. (2011). A hyperactive piggyBac transposase for mammalian applications. Proc. Natl. Acad. Sci. U. S. A. *108*, 1531–1536.

FIGURES LEGENDS

Figure 1. Schematic representation of *PB* transposon and *PB* transposase constructs. (A) *PB-SPc-DYS-Pgk-GFP* transposon encodes for the codon optimized full-length human dystrophin cDNA (*huDYSco*; (Athanasopoulos et al., 2011)) represented as in its four major structural domains: N-terminal domain (N), a central rod domain, a cysteine-rich (CR) domain and a distal C-terminal domain (C). The central rod domain contains 24 triple-helix rod repeats and four hinges (H1–H4). The full-length human dystrophin cDNA is driven by the synthetic muscle-specific promoter SPc5-12 (Li et al., 1999). Downstream, the sequence of a copepod GFP has been cloned as a reporter driven by the *Pgk* promoter. (B,C) *PB-SPc-MD1* and *PB-SPc-MD2* transposons encode respectively for *huMD1co* and *huMD2co* sequences; these are two different codon optimized human microdystrophins that lack the original sequence of rod repeats 4-23 and differ for the presence of the C-terminal domain sequence in *MD2* (Athanasopoulos et al., 2011). This extra sequence has been shown to improve the efficiency on restoring muscle function in dystrophic mdx mice (Koo et al., 2011). Both the microdystrophins are driven by SPc5-12 promoter. (D,E) *PB-SPc-GFP* and *PB-Pgk-GFP* are transposons that accommodate the GFP sequence respectively under the SPc5-12 or the *Pgk* promoter. All the transposon constructs are flanked by wild-type inverted repeats (IR) and loxP sites (loxP). All the transgenes in the transposon constructs are followed by the simian virus 40 late polyadenylation signal (SV40pA). The transposase constructs are instead represented from (F) to (H), as already described in (Di Matteo et al., 2014).

Figure 2. *PB*-mediated transposition of *MD1* and *MD2* in C2C12 myoblasts. (A) The bar graphs show respectively the percentage of GFP+ (left) and the mean fluorescence intensity (MFI; right) of C2C12 cells at 48 and 72 hours post-electroporation (EP) with the *PB-SPc-GFP* transposon when co-transfected with the native *mPB* transposase or the “empty” plasmid (ratio of 1.26 pmol transposase DNA: 3.48 pmol transposon DNA). Untransfected cells are also shown. Shown are mean±SEM of three independent biological replicates; One-way ANOVA (Bonferroni’s

multiple comparison test); ***p≤ 0.001; **p≤ 0.01; ns: not significant. (B) Live cell imaging showing the GFP expression of the conditions described in (A) at 24, 48 and 72 hours post-EP (UNTR: untransfected; scale bar 100 µm). (C,E) The bar graphs depict the transcript levels of GFP (C) or *MD1* and *MD2* (E) detected by qRT-PCR in *PB*-transposed C2C12 myoblasts in proliferation and myotubes in differentiation. The transcript level of the human dystrophin detected in human skeletal muscle (SKM) cells was used as positive control. Values were normalized for the myosin heavy chain (MyHC) and shown as relative expression. Shown are mean±SEM of the triplicate qRT-PCR analyses performed for three independent biological replicates; One-way ANOVA (Bonferroni's multiple comparison test); ***p≤ 0.001; ns: not significant). (D,F) Immunofluorescence (IF) staining on transposed C2C12 cells upon skeletal muscle differentiation *in vitro* showing the detection of the GFP expression (D, in green) or the human microdystrophins MD1 and MD2 (F, in green) in MyHC positive myotubes (D,F in red). The nuclei were stained with Hoechst (Scale bar 100 µm. The mouse anti-human dystrophin NCL-DYS3 antibody was used).

Figure 3. Validation of the use of *hyPB* transposase in C2C12 myoblasts. (A) Graph showing the comparison between *hyPB* and *mPB* transposases when co-transfected with the large-size transposon *PB-SPc-DYS-Pgk-GFP* in C2C12 myoblasts. The GFP+ populations were sorted by FACS at 4 days post-EP and monitored for the GFP expression in the next 30 days after the sorting. The higher efficiency of the *hyPB* transposase over the *mPB* transposase was assessed at the concentration of 0,32 pmol transposase DNA: 0,87 pmol transposon DNA. Above the graph, live cell imaging showing the GFP expression in the conditions monitored at different time points (Scale bar 100 µm). (B) The graph depicts the MFI monitored during the different time points of the C2C12 cell populations described in (A). Results in (A,B) were presented as mean±SD of three independent biological replicates; One-way ANOVA (Bonferroni's multiple comparison test); ***p≤ 0.001.(C) The transcript levels of the full-length human dystrophin and the GFP were detected by qRT-PCR in C2C12 cells transposed with *hyPB* + *PB-SPc-DYS-Pgk-GFP*, while in proliferation or in differentiated myotubes (D: differentiated sample). Results were

presented as mean ±SEM of the triplicate qRT-PCR analyses performed for three independent biological replicates; One-way ANOVA (Bonferroni's multiple comparison test); ***p≤ 0.001; ns: not significant).

Figure 4. Human full-length dystrophin expression in *PB*-transposed and differentiated C2C12 myotubes. (A) C2C12 cells transposed with *PB-SPc-DYS-Pgk-GFP* transposon show a colocalized expression of GFP (in green) and full-length human dystrophin (in red) when differentiated in skeletal muscle *in vitro,* as confirmed by the myosin expression (B, in white). The nuclei were stained with Hoechst (Scale bar 100 µm. The Ab mouse anti-human dystrophin NCL-DYS3 was used).

Figure 5. Correction of GRMD MABs by the *PB*-mediated transposition of the full-length human dystrophin. (A) The bar graph shows the percentage of GFP+ GRMD MABs at the day of the sorting performed 4-7 days post-EP. GRMD MABs were co-transfected at optimized doses with *hyPB* and the large-size transposon *PB-SPc-DYS-Pgk-GFP* or the control plasmid *PB-Pgk-GFP.* Shown are mean±SEM of three independent biological replicates; One-way ANOVA (Bonferroni's multiple comparison test); ***p≤ 0.001; ns: not significant. (B) The sorted GFP+ populations were monitored at different time points for 28 days post sorting. GRMD MABs cotransfected with the "empty" plasmid and the same transposons, and untransfected cells were used as controls. Results were presented as mean±SD of three independent biological replicates; One-way ANOVA (Bonferroni's multiple comparison test); ***p≤ 0.001. Above the graph, live cell imaging showing the GFP expression of the different conditions at day 21 post sorting (Scale bar 100 µm). (C) Bar graph representing the transposon copies per diploid genome detected by qPCR in transposed GRMD MABs (*PB-SPc-DYS-Pgk-GFP* 0.5±0.03 copies/cell; *PB-Pgk-GFP* 1.7±0.04 copies/cell). Shown are mean ±SEM of the triplicate qRT-PCR analyses performed for three independent biological replicates; One-way ANOVA (Bonferroni's multiple comparison test); ***p≤ 0.001; *p≤ 0.05. (D) The transcript levels of the full-length human dystrophin (black columns) and GFP (white columns) were detected by qRT-PCR analysis in GRMD MABs

transposed with *PB-SPc-DYS-Pgk-GFP* or *PB-Pgk-GFP*. Samples are indicated with (D) when differentiated in myocytes/myotubes upon over-expression of MyoD-ER. Results were presented as mean±SEM of the triplicate qRT-PCR analyses performed for three independent biological replicates; One-way ANOVA (Bonferroni's multiple comparison test); ***p≤ 0.001; *p≤ 0.05; ns: not significant; D: differentiated sample.

Figure 6. Immunofluorescence of corrected GRMD MABs. IF pictures show the expression and co-localization of the full-length human dystrophin (A,B in red) with the GFP (A, in green), or with the myosin (B, in white) in *hyPB* + *PB-SPc-DYS-Pgk-GFP* transposed GRMD MABs upon over-expression of MyoD-ER that induces myogenic differentiation. The conditions "empty" plasmid + *PB-SPc-DYS-Pgk-GFP* and untransfected cells are also shown as negative controls The nuclei were stained with Hoechst (Scale bar 100 µm. The mouse anti-dystrophin NCL-DYS1 and NCL-DYS2 antibodies were used).