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MAR-Mediated Dystrophin Expression in Mesoangioblasts for Duchenne Muscular Dystrophy Cell Therapy

Ruthger W van Zwieten[1], Stefano Majocchi[1], Pavithra Iyer[1], Yves Dussere[1], Stefania Puttini[1], Francesco Saverio Tedesco[2], Giulio Cossu[3] and Nicolas Mermod[1]*

[1]*Laboratory of Molecular Biotechnology, Center for Biotechnology UNIL-EPFL and Institute of Biotechnology, University of Lausanne, 1015 Lausanne, Switzerland*
[2]*Department of Cell and Developmental Biology, University College London, WC1E 6DE London, UK*
[3]*Institute of Inflammation and Repair, University of Manchester M13 9PL Manchester, UK*

Abstract

A cornerstone of autologous cell therapy for Duchenne muscular dystrophy is the engineering of suitable cells to express dystrophin in a stable fashion upon differentiation to muscle fibers. Most viral transduction methods are typically restricted to the expression of truncated recombinant dystrophin derivatives and by the risk of insertional mutagenesis, while non-viral vectors often suffer from inefficient transfer, expression and/or silencing. Here we addressed such limitations by using plasmid vectors containing nuclear matrix attachment regions (MAR). Using *in vitro* transfection and intra muscular transplantation in nude and immunosuppressed mdx mice, we show that clones of mesoangioblast skeletal muscle progenitors can be generated to mediate stable expression from MAR-containing vectors, while maintaining their ability to differentiate *in vitro* and *in vivo* and to express dystrophin after transplantation in dystrophic mouse muscles. We conclude that the incorporation of MARs into plasmid vectors may improve non-viral plasmid-based cell therapy feasibility.

Keywords: Matrix attachment regions (MAR); Dystrophin; Insertional mutagenesis; Duchenne muscular dystrophy

Introduction

Duchenne muscular dystrophy is an X-linked progressive muscular wasting disease that affects skeletal and cardiac muscles, and for which there is currently no cure. It is caused by mutations in the dystrophin gene, resulting in the lack or reduction of the protein. This deficit leads to a disruption of the dystroglycan complex and destabilization of the sarcolemma, resulting in progressive muscle wasting [1]. Promising experimental approaches that aim to restore the dystrophin complex at the sarcolemmal membrane include i) exon skipping by pharmacological strategies, ii) systemic gene therapy and iii) cell therapy [2,3]. Gene therapy approaches aim to engineer vectors that efficiently transduce myofibers with a dystrophin expression cassette, whereas cell therapy approaches aim to deliver the dystrophin transgene to the myofiber by stem/progenitor cells, while preferably replenishing the satellite cell pool with genetically corrected or complemented autologous cells. In this study, we assessed a novel approach to express full-length dystrophin in a cell therapy setting.

Besides myoblasts, multiple myogenic stem/progenitor cells were described for potential use in cell therapy. Unlike myoblasts that require high-density injection and are limited to superficial muscles [4], mesoangioblasts can cross the blood vessel wall and home into damaged muscle after intra-arterial delivery [5], thus providing an approach towards systemic cell therapy [6-9]. Mesoangioblasts were isolated from mice, dogs and humans, and they maintain their differentiation potential upon *in vitro* culture. Although aneuploidy and transformation may be observed upon prolonged culture of murine cells, human and canine mesoangioblasts maintained a normal karyotype and did not escape eventual senescence in long-term cultures [10,11].

The therapeutic potential of wild-type mesoangioblasts injected intra-arterially was shown in α-sarcoglycan null mice and dystrophic dogs, leading to dramatic improvement of muscle morphology and function [9,10,12]. In contrast, the therapeutic efficacy of transplantations with viral vector-transduced dystrophic mesoangioblasts was modest. This may result in part from the transcriptional silencing of the transgene by epigenetic effects [13]. In addition, limitations of typical viral vector cargo size necessitated the development of shorter, Becker-like dystrophins, which may have reduced therapeutic effects [14]. Despite significant advances in viral vector engineering, safety concerns remain regarding genotoxic effects and potential malignant cell transformation, because of the tropism of some viral vectors for cellular genes [15]. Use of non-viral vectors or gene correction may be promising alternative approaches. However, their efficiency remains generally low, and gene correction is also limited by the breadth of mutations that affect dystrophic patients [16]. Therefore, expression of truncated dystrophin derivatives from viral vectors is still mostly used in experimental cell therapy approaches.

Strategies that allow the introduction of a functional copy of the full-length dystrophin coding sequence into autologous cells might thus be beneficial. Current efforts are thus increasingly focused on the isolation of single clones and on the characterization of the genomic integration locus to reduce the risk of adverse effects. Here, we investigated a novel non-viral transgene introduction approach as an alternative to viral vector transduction, assessing whether the stable transfection of nuclear matrix attachment regions (MAR)-containing plasmids may allow the engineering of primary muscle precursor cells. A recent genome-mining effort yielded potent human MARs (hMARs) that enhance and stabilize transgene expression in cultured cells as well as in murine muscles [17]. hMAR elements were shown to promote plasmid integration in the host cell genome by homologous recombination-related mechanisms, to increase transgene transcription and to oppose epigenetic silencing effects in cultured rodent and human cells [17-21].

In this study we evaluated plasmid vectors containing distinct MAR

***Corresponding author:** Nicolas Mermod, Laboratory for Molecular Biotechnology, Station 6, EPFL, 1015 Lausanne, Switzerland, Tel: +41 21 693 6151; E-mail: nicolas.mermod@unil.ch

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elements to promote transgene expression in mesoangioblasts, in an attempt to improve non-viral engineering of muscle progenitors for cell therapy. We show that MARs increase stable transfection efficiency up to 10-fold. Clones generated with MAR-containing vectors retained their ability to differentiate *in vitro* and *in vivo* while sustaining transgene expression. This indicates that transfection-based cell therapy approaches may be improved with MAR element-containing non-viral vectors.

Materials and Methods

Cell culture

MDX mesoangioblasts cultures were established and cultured in Dulbecco's modified eagle medium (DMEM, Gibco) containing 20% FBS as described previously, and provided by G Cossu, Milan [22]. C2C12 mouse myoblasts were maintained in DMEM (Gibco) plus 10% FBS. All culturing was done in a humidified 37°C/5% CO_2 incubator. Differentiation was induced by the co-culture of C2C12 cells and mesoangioblasts at a 1:4 ratio in DMEM 20% for 24h, after which the culture medium was changed to DMEM plus 2% horse serum. Myotube formation was confirmed by immunofluorescence for α-actinin using a mouse polyclonal antibody (Sigma). eGFP expression levels of differentiated and undifferentiated mesoangioblasts were recorded by fluorescence microscopy.

DNA constructs and transfection

The construction of MAR-containing eGFP expression vectors was as previously described [17]. pMDA (full length mouse dystrophin cDNA driven by the muscle creatine kinase promoter) was kindly provided by JS Chamberlain [23]. Plasmids were amplified in DH5α bacteria and purified using a plasmid maxiprep kit (Genomed). Transfections were done with Lipofectamine 2000 (Invitrogen), Fugene 6 (Roche), Fugene HD (Roche), while electroporations were performed using the Nucleofector (Amaxa) or the Neon electroporator (Invitrogen) following the manufacturers' instructions. For equimolar transfections of various eGFP-expressing constructs, pUC18 was added to maintain an equal amount of total DNA. Cells were plated 27 h before transfection to allow an appropriate timing of the transfection with the cell cycle, as adapted from [17]. At time of transfection cell confluency was 80%, and 4.5 µl of Fugene HD was added together with 2 µg of plasmid DNA. For stable clone isolation, the cells were placed after 48 h post transfection in DMEM supplemented with 20% FBS and 2.5 µl/ml puromycin dihydrochloride (Sigma). After 20-30 days, eGFP expressing clones were isolated by mechanical dislodging of the colony with a sterile pipet tip. Transfection efficacy was measured by fluorescence acquisition for eGFP using a fluorescence-activated cell sorter (FACSCalibur, BD biosciences). 100,000 events were counted per given cell population. Statistical analyses were performed with the Student's t test. The described lentivirally transduced mesoangioblast population was generated as described previously [6,9].

Plasmid rescue for genomic integration locus DNA sequencing

We extracted total genomic DNA from cells with the Blood and Tissue kit (QIAGEN), following the manufacturer's instructions. After digestion of 2 µg of genomic DNA with a unique restriction site cutter, BamHI (NEB), we took 1/10 of digested DNA and ligated with 15 µl T4DNA ligase (NEB) in 500 µl at 16°C over-night. The ligation was dialysed against water, precipitated and resuspended in 4 µl, of which 1 µl was used to transform 20 µl of electrocompetent DH10B cells (Invitrogen).

In vivo transplantation assay

5 x 10^5 cells were suspended in 30 µl phosphate buffered saline (PBS) and injected intramuscularly in the Tibialis anterior of 5 wk old CD-1 nude (Charles river), C57Bl6 or *mdx*^{5cv} mice using a 29G 'Myjector' syringe (Terumo), while the contralateral muscle was injected with PBS alone. 3 mice were transplanted for each cell clone. During the procedures, the mice were anaesthetized by intra-peritoneal injection with xylazine/ketamine. Mice were sacrificed by cervical dislocation at day 9, day 40 or 3 months post injection, and muscles were collected and frozen in liquid nitrogen cooled isopentane (Sigma). All precautions were taken to reduce animal suffering, and the procedures were approved by the Service de la consommation et des Affaires vétérinaires of the Canton of Vaud (Lausanne, Switzerland). Immunofluorescence for eGFP was performed using a rabbit polyclonal antibody (Invitrogen) on paraformaldehyde 4% fixed sections. Dystrophin staining was performed on non-fixed 10 µm TA sections using mouse monoclonal antibodies NCL dys1 and NCL dys2 from Novocastra (Leica).

Fluorescence *in situ* hybridization

Mesangioblast clones were exposed for 2 hours to colcemid (Invitrogen) to block cell division in metaphase. After harvesting, cells were exposed to a hypotonic shock with 37.5 mM KCl for 20 minutes, fixed with 25% acetic acid and 75% methanol and spread onto superfrost microscope slides. Hybridization probes were prepared using a nick translation DNA labeling system (Enzo Life Sciences) and Orange 552 dUTP (Enzo Life Sciences) according to the manufacturer's instructions. The probe targeting eGFP was generated from the eGFP expressing vector devoid of MAR whereas the dystrophin probe was derived from the pMDA vector. Precipitated probes were resuspended in hybridization buffer (2x SSC, 50% formamide, 10% Dextran Sulfate), denaturated for 10 min at 75°C, cooled down on ice and finally pre-warmed at 37°C. Before applying the probes, slides were washed in PBS, denaturated in denaturation buffer (2x SSC, 70% formamide at 75°C), dehydrated through ethanol series performed at room temperature (70%, 85%, 100%) and air-dried. Hybridization occurred overnight at 37°C. Slides were first washed for 90 seconds with 0.4x SSC and 0.3% NP-40 at 72°C followed by a 1 minute wash in 2x SSC and 0.1% NP-40 at room temperature. Metaphases were counterstained with Vectashield Mounting Medium with DAPI (Vector Labs) and observed using a 100X oil immersion objective on an Axio Vert Inverted microscope (Carl Zeiss).

Imaging

Microphotographs of eGFP autofluorescence, DAPI and secondary antibodies conjugated with Alexa fluor 546 were made with an Observer A.1 equipped with an Axiocam (Zeiss) using the Axiovision software.

Results

Optimizing transfection of primary murine mesoangioblasts

A panel of human and animal MAR elements was tested for their effect on the establishment of stable cell clones and for transgene expression level and stability using murine mesoangioblasts (Table 1). The MAR elements were inserted upstream of the SV40 promoter and of the eGFP coding sequence. As primary progenitor cells are often difficult to transfect, we first tested several transfection reagents and assessed the transient eGFP fluorescence levels in primary mesoangioblasts that were obtained from the commonly used mdx-5Cv mouse model for Duchenne muscular dystrophy. Transient transfection with Fugene HD was most efficient, with 14.5% and 9.3% of eGFP positive cells with the

MAR name	Species of origin	MAR size (bp)
Control genomic DNA	Human Chr. 1	2275
hMAR 1-6	Human Chr. 1	4618
hMAR 1-42	Human Chr. 1	4660
hMAR 1-68	Human Chr. 1	3643
hMAR X-29	Human Chr. X	3343
cLys MAR	Chicken Chr. 1	2827
Mouse MAR S4	Mouse Chr. 1	5457

These MAR elements were previously described in references [6,9].

Table 1: Overview of MAR elements used in this study.

MAR-devoid and hMAR X-29-containing plasmids, respectively, while other reagents yielded lower transfection efficacies, as measured by FACS for GFP autofluorescence 48 h after transfection. As transfections were carried out with equimolar amounts of plasmid, the lower eGFP obtained from the hMAR X-29 MAR construct likely resulted from the larger plasmid size and thereby reduced transfection efficacy. Electroporation also proved to be a very efficient transient transfection method, yielding up to 20% eGFP expressing cells after 48 h (Figure 1).

Transfection of primary murine mesoangioblasts by various approaches

Transfection was done according to manufacturers instructions using optimized amounts of DNA. The percentage of eGFP positive cells was recorded 48h after transfection by FACS cytofluorometry (A) or fluorescence microscopy (representative fields shown, panel B). Scale bars indicate 200 µm. FACS profiles of eGFP expression obtained from the hMAR X-29-containing vector of from a control construct devoid of any insert (no MAR), as determined 48 h after transfection with lipofectamine 2000 (C) or Fugene HD (D). Cells were transfected with equimolar amounts of either the no MAR eGFP or the hMAR X-29 eGFP plasmid.

Effect of MARs on stable transfection efficacy of primary murine mesoangioblasts

The MAR-eGFP or control vectors were then co-transfected with a plasmid bearing the puromycin-resistance gene and puromycin was added to the transfected cell pools for up to 30 days. This selection period allowed for the formation of colonies of antibiotic resistant cells of sufficient size to be picked manually and individually. Despite the low initial number of eGFP positive cells at 48 h, the vector containing hMAR X-29 showed a statistically significant 10-fold increase of stable eGFP-expressing clones as compared to the MAR devoid construct, and up to a ~ 60x increase when compared to the vector whose MAR element was substituted by a genomic fragment of comparable size but without MAR activity (Figure 2A). Overall, 1.8 x 10^5 transfected cells yielded on average 17 eGFP-expressing clones when using hMAR X-29. While electroporation yielded the highest efficacy of transient eGFP expression, the subsequent selection of cells mediating sustained transgene expression under antibiotic selection yielded no stable clones from the two electroporation devices tested (Figure 1A). Therefore, subsequent transfections performed to generate stable clones relied on the Fugene HD reagent.

With hMAR X-29, 82 ± 17 % of the obtained clones expressed eGFP, which was significantly higher than the 26 ± 14 % of expressing clones obtained with the MAR-devoid construct ($p<0.05$, student's t-test), and the 11 ± 19% recovered for the construct containing a non-MAR control genomic fragment ($p<0.01$; See S1 Table for a summary of all clones). hMAR 1-68 also significantly increased stable transfection efficacy by ~ 6x as compared to the no MAR control ($p<0.05$). Other human MARs, namely hMAR 1-6 and 1-42 also showed a positive trend for stable transfection efficacy. The chicken lysozyme (cLys) MAR was the only tested element that did not show a detectable effect (Figure 2A).

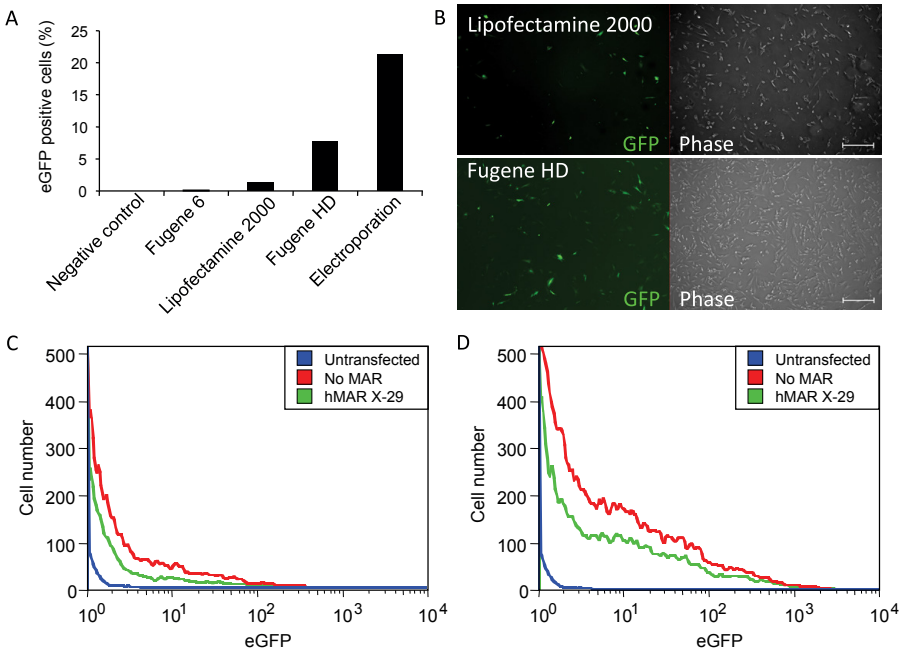

Figure 1: Transfection of primary murine mesoangioblasts by various approaches. Transfection was done according to manufacturer's instructions using optimized amounts of DNA. The percentage of eGFP positive cells was recorded 48 h after transfection by FACS cytofluorometry (A) or fluorescence microscopy (representative fields shown, panel B). Scale bars indicate 200 µm. FACS profiles of eGFP expression obtained from the hMAR X-29-containing vector of from a control construct devoid of any insert (no MAR), as determined 48 h after transfection with lipofectamine 2000 (C) or Fugene HD (D) Cells were transfected with equimolar amounts of either the no MAR eGFP or the hMAR X-29 eGFP plasmid.

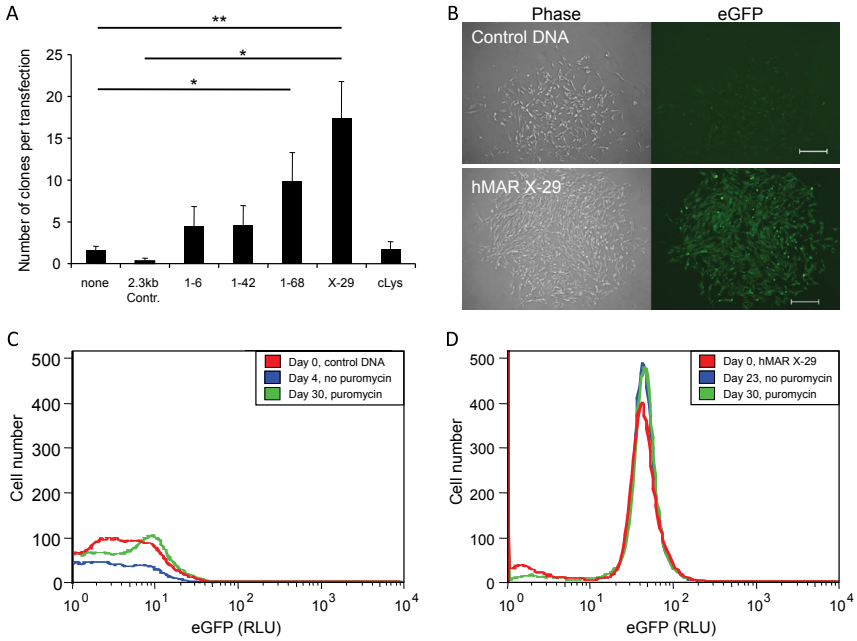

Figure 2: Effect of MARs on mesoangioblast stable transfection efficacy.
(A) Mesoangioblasts were transfected with equimolar amounts of eGFP plasmid with or without a MAR insert, as indicated, together with the pSVpuro antibiotic resistance plasmid, and selection with 2.5 μg/ml of puromycin was initiated 48 h later. Resistant eGFP positive colonies were quantified after 30 days of selection. Statistical significance was determined from at least three independent experiments by unpaired student's t-test (*: $p<0.05$, **: $p<0.01$). (B) Primary colony morphology of clones without and with the MAR (clones noMAR 2.3Kb.A and hMAR X-29.J, respectively, as described in the supplementary Table S1). hMAR X29.J has an increased RFU of 910% as compared to the noMAR 2.3Kb.A control. These clones generated without (C) or with (D) the MAR displaying the highest expression were picked after 20-30 days of antibiotic selection, and they were further cultured with or without antibiotic selection. eGFP expression levels were assessed by cytofluorometry at different time-points, where day 0 refers to the day of antibiotic removal.

Overall, the maximum level of eGFP fluorescence of individual colonies was higher in presence of hMAR X-29 than for clones generated without a MAR (Figure 2B and Table S1). Analysis of the mean fluorescence of the 4 highest expressing clones generated with hMAR X-29 or without a MAR yielded 39 and 10.5 relative fluorescence units (RFU), respectively. Clones displaying the highest eGFP fluorescence were isolated from the transfections performed with or without the hMAR X-29, and they were subsequently expanded without antibiotic selection pressure. eGFP expression was rapidly lost in the isolated clone generated using the vector containing the control genomic DNA, whereas it was stable for the hMAR X-29-containing clone (Figure 2C and 2D). While selection allowed the maintenance of eGFP expression in the control MAR-devoid cells after 30 days of culture, the selection pressure had no noticeable effect on transgene expression in the hMAR X-29-containing clone, indicating a lack of detectable silencing over this time-period in presence of the MAR.

In vitro and *in vivo* differentiation of clonal primary murine mesoangioblasts

We next tested whether transfected mesoangioblasts retained their myogenic differentiation potential. Thus, we co-cultured the mesoangioblasts with the C2C12 murine myoblast cell line under myogenic differentiation conditions, to induce the *in vitro* co-differentiation and fusion of these cells into myotubes. Most clones generated with the MAR yielded eGFP-expressing myotubes in such assay, indicating that the mdx mesoangioblast cells had maintained their ability to fuse with differentiating myotubes, and that differentiation was not accompanied by the silencing of the transgene (S1 Table and Figure S1). In contrast, eGFP expression was low prior to differentiation in the cells generated without the MAR, and it became nearly undetectable after myotube differentiation (Figure S2 and data not shown).

Overall, we were unable to obtain a clone devoid of the MAR that would express sufficient eGFP levels for detection in subsequent *in vivo* differentiation assays with this approach. Thus, we adapted an iterative transfection procedure phased on the cell division cycle, as described previously for CHO cells, in which the cells were transfected a second time 27 h after the first transfection [19]. One of the resulting clones had an expression level close to those of hMAR X-29-containing clones (Figure 3A and Table S1).

When comparing the eGFP expression levels of stably transfected clones to a lentiviral vector-transduced polyclonal population by cytofluorometry, the maximum eGFP fluorescence levels were quite similar between the hMAR-containing clone and the transduced population (Figure 3A).

When the clone transfected twice with the MAR-devoid construct was assessed in the differentiation assay, eGFP expression could be detected. However expression was low in comparison to that elicited by the hMAR-containing clone, indicating an expression stabilizing effect from the hMAR (Figure 3B). Fusion of mesoangioblasts generated with the hMAR allowed significant levels of transgene expression in myotubes, despite the fact the latter are formed by an excess of non-expressing C2C12 cells. Proper myogenic differentiation of the clonal mesoangioblast populations was confirmed by α-actinin immunofluorescent staining, indicating that the fusion of transfected mesoangioblasts had not impaired myotube formation. The transfection and selection protocol had virtually no negative effect on the differentiation potential of the mesoangioblasts, as 29 out of 30 tested eGFP-expressing clones gave rise to eGFP-positive myotubes (Table S1).

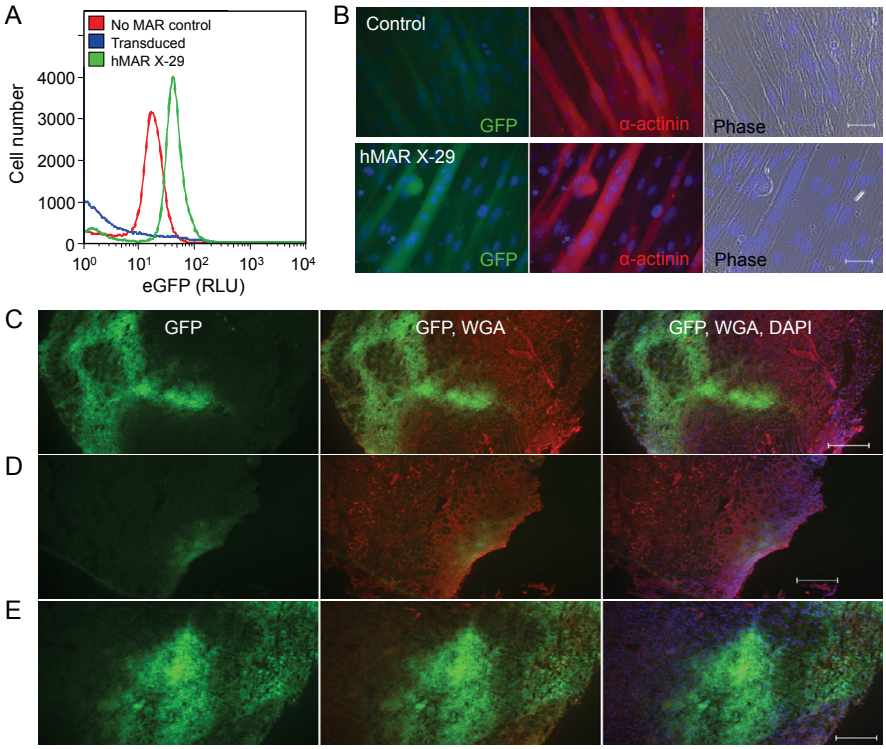

Figure 3: *In vitro* and *in vivo* differentiation of clonal eGFP-expressing mesoangioblasts.
(A) Comparison of the eGFP expression levels from clones obtained from an iterative transfection of transfected clones of a MAR-devoid eGFP expression vector (clone noMAR.B), from a single transfection of the hMAR X-29-containing vector (clone X-29.J), or from a polyclonal population of mesoangioblasts transduced with an eGFP-expressing lentiviral vector. The cytofluorometric profiles for eGFP fluorescence were monitored for each population. (B) Differentiation of the noMAR.B and hMAR X-29.J cell clones after a co-culture with C2C12 cells for 7 days in differentiation medium. eGFP fluorescence is shown in green, while the α-actinin immunofluorescence performed to determine myogenic differentiation and DAPI nuclear staining are displayed in red or blue, respectively. Scale bars indicate 50 μm. (C-E) *In vivo* transplantation of eGFP-expressing mesoangioblasts clones with hMAR X-29 (clone X29.J, panel C) or without a MAR (noMAR.B, panel D), and of a lentiviral vector-transduced mesoangioblast polyclonal population without a MAR. A single intramuscular injection with 5 x 10^5 cells was done in the TA of 5 wk old CD-1 nude mice, and muscles were isolated for immunostaining at day 9 after injection. Brightness and contrast were increased for better visibility of the muscle fibers delineation for the red channel (WGA) of the enlargement panels (C, D and E).

Transgene integration site determination in transfected stable mesoangioblast clones

We next wished to determine the number of transgene integration sites by fluorescent *in situ* hybridization (FISH). Aneuploidy was found in metaphase spreads of parental cells and of the transfected clones, as expected from the long-term culture of the mouse mesoangioblasts. Similar chromosome numbers were found in the clonal populations and in the parental cells prior to transfection, indicating that the transfection procedure did not cause chromosomal abnormalities *per se* (Figure S2A). A single integration site was found in all tested clones, although the genomic integration locus varied from clone to clone (Figure 4). While a unique integration locus is advantageous in terms of safety when compared to vectors yielding multiple integration sites like retroviral vectors, we nevertheless wished to determine if the genomic integration site may be identified in individual clones, so as to assess the potential insertional mutagenesis of cellular genes. Thus, we next attempted to characterize the genomic integration site of clones generated with MAR-eGFP plasmids.

Genome-integrated vectors were released by digesting total genomic DNA with a restriction enzyme that cleaves the plasmid once and fragments were circularized by ligation. Plasmids were rescued by bacterial transformation and sequence determination of the genomic flanking DNA region of the plasmid rescued from the hX29.J clone showed transgene integration into an intronic region of solute carrier family 12 member 8 gene, on chromosome 16. As this and other related genes have not been linked to oncogenesis, we predicted that the hX29.J clone would not give rise to tumors *in vivo*. Although we were unsuccessful in determining the transgene integration sites for other clones due to the low intrinsic success rate of the protocol, no safety issues related to mutagenesis arose as no tumorigenesis was observed during subsequent mouse experiments.

Following the validation of the eGFP-expressing clones for *in vitro* differentiation and for the lack of potentially oncogenic mutagenic event elicited by plasmid genomic integration, we investigated the *in vivo* differentiation potential of the hMAR X-29.J and noMAR.B clones. 5 x 10^5 cells were injected intra-muscularly into the Tibialis anterior (TA) of 5-week old CD-1 nude mice. Cells from the lentiviral vector-transduced population were similarly injected as a control. Both transfected clones differentiated into eGFP-positive myofibers after transplantation (Figure 3C). In agreement with *in vitro* differentiation results, the hMAR X-29.J transplanted TA resulted in fibers displaying higher eGFP levels than the muscle transplanted with the MAR-devoid clone (Figure 3D).

Transfection and differentiation of primary murine mesoangioblasts with dystrophin

We then proceeded to generate dystrophin-expressing clones from

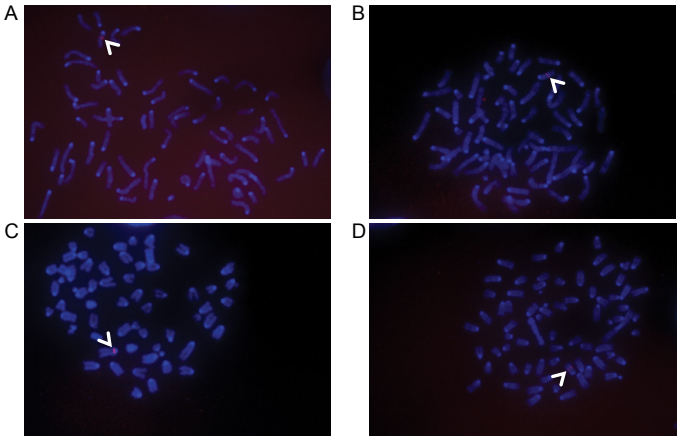

Figure 4: Clones derived from plasmid transfection have single genomic integration sites.
Representative FISH images of clones used for *in vivo* transplantation studies. Arrows indicate the transfected plasmid integration sites. Inlays are enlargements of transgene-bearing chromosomes. (A) eGFP clone X29.J. (B) eGFP clone noMAR.B. (C) eGFP clone S4.B. (D) eGFP clone 1-6.E.

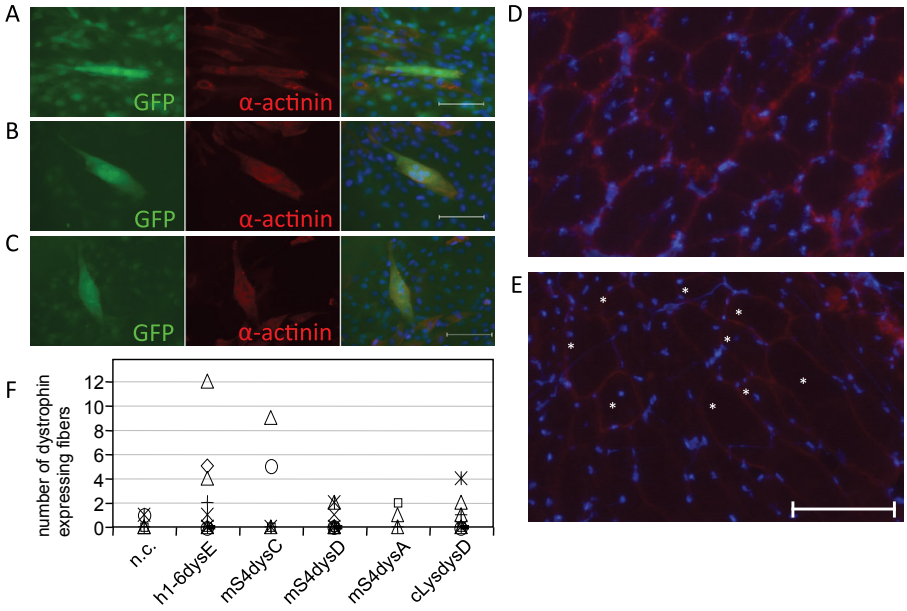

Figure 5: Differentiation and *in vivo* dystrophin expression from transfected mesoangioblast stable cell clones.
In vitro differentiation assays were performed on all selected clones, and the eGFP expression, α-actinin staining and an overlay are shown at day 7 after a co-culture with C2C12 cells in the co-differentiation assay conditions. A representative clone for each of the expression construct is shown, namely clone h1-6dys.E (panel A), cLysdys.D (panel B) and mS4dys.C (panel C). Scale bars represent 100 µm. (D) Dystrophin staining on cross-section of TA from a 5 wk old C57Bl6 mouse (positive control, panel D) and cross-section of a TA from a 5 wk old mdx mouse 1 month after a single intra-muscular injection with 5 x 10^5 cells of the S4dys.D clone (panel E). Mdx mice were immunosuppressed by daily injections of FK506 before sacrifice, and dystrophin-positive fibers were counted in distal and proximal transversal sections of each TA muscle. The scale bar represents 50 µm. (F) Dystrophin positive fibers were counted for the negative control (n=6) and after transplantation of cells from the h1-6dys.E (n=12), mS4dys.C (n=6), mS4dys.D (n=12), mS4dys.A (n=4), or cLysdys.D (n=10) clones.

the mdx mesoangioblasts by co-transfecting the puromycin selection gene with the full-length murine dystrophin cDNA placed under the control of the muscle creatine kinase promoter. Various MAR-eGFP expression plasmids were co-transfected at equimolar amounts to supplement the dystrophin sequence with the MAR elements in trans, as previous evidence indicated that distinct plasmids co-integrate at the same genomic locus upon co-transfection [17]. No clone was obtained without a MAR, despite good initial transfection efficacies, and we did not obtain eGFP-expressing clones with hMAR X-29 either. This could be attributed to the fact that in presence of hMAR X-29, the muscle specific MCK promoter displayed a leaky expression in non-differentiated cells (Figure S3), which leads to cellular toxicity effects due to ectopic dystrophin expression. However, relatively less potent MARs such as the cLys and h1-6 MAR generated 1 and 3 clones, respectively. Transfection with a mouse MAR termed S4 yielded three additional clones. Consistently with the results from the earlier eGFP-expressing clones, all dystrophin vector-transfected clones yielded a single transgene integration locus (Figure S2B and S2C). In addition, all these clones were found to co-differentiate with C2C12 cells to yield eGFP-expressing myotubes *in vitro* (Figure 5A-5C). These five clones were transplanted in the TA muscles of 5-week old mdx mice, which were immunosuppressed with FK506 until sacrifice to prevent rejection

of the allogeneic cells. The number of dystrophin positive fibers was then counted 1 month after transplantation in immunostaining studies of proximal and distal cross-sections of the TA of all mice. Overall, the dystrophin expression levels of injected mdx TA muscles were lower than those of wild-type myofibers, but clusters of dystrophin expressing fibers could be observed in muscles transplanted with clones S4dys.D and 1-6dys.E along with centrally located nuclei indicative of muscle fiber regeneration (Figure 5D and 5E). No such cluster of dystrophin-expressing cells was detected from the transplantation of the other three clones or from the muscles of non-transplanted animals (Figure 5F). Overall, we concluded that some of the transfected clones were capable of dystrophin expression after transplantation into the muscle of dystrophic mdx mice.

Discussion

Non-viral cell-based therapy has often been limited by gene transfer and maintenance, and thus by the lack of cell populations mediating stable and consistent expression. Here we showed that stable clones could be obtained from the transfection of adult stem cells propagated *in vitro*. This involved the development of methods allowing antibiotic selection, the mechanical isolation of single clones, and the subsequent expansion in tissue culture in conditions that prevent anoikis, an often-noted problem when cultivating isolated primary cells. Although mesoangioblasts significantly slowed cell cycling when cultured at low density, and a minority of cells showed signs of spontaneous differentiation, i.e. a flattened morphology of multiple nucleated syncytia, most cells did not exit the cell cycle and expanded to form clonal populations expressing the transgene at homogeneous levels. Conversely, another limitation of muscle progenitor cells is a loss of differentiation potential resulting from contact inhibition, when allowing the stable clones to form colonies. However, clones grown according to this protocol were consistently able to form eGFP-expressing myotubes *in vitro*, and the inclusion of MARs in the expression vector had no negative effect on differentiation.

We showed that several MAR elements of human origin enhanced stable transfection efficiency significantly. This effect of MARs cannot be related to a simple increase of the transfer of the DNA, as transient transfection of MAR bearing plasmids consistently yielded fewer fluorescent cells than ones devoid of MAR, even when transfecting equimolar amounts of plasmid. Thus, the effect of the MARs is rather to increase transgene genomic integration, as required to establish clones displaying stable transgene propagation and expression upon cell division. This effect of the MARs can be readily explained by the recent finding that MARs promote transgene genomic integration in cell lines by a homologous recombination-related mechanism [19]. A similar increase of the genomic integration of MAR-containing plasmids has also been observed *in vivo* upon plasmid electroporation in murine muscles [24]. Thus a more frequent establishment of mesoangioblasts cell clones is well explained by the fact that primary cells are known to have limiting recombination activity when compared to established cell lines [24], and that MAR would thus be required to increase integration by such a recombination mechanism. In this respect, it will be interesting to characterize transgene integration sequences at a large scale, to determine if MARs may promote more frequent occurrence of integration at specific types of genomic loci or of chromatin structure. Nonetheless, and despite the higher frequency, clone establishment remained relatively rare events, yielding up to 10-20 clones per 10^5 cells for the most efficient MAR-containing vectors. Therefore, isolated primary mesoangioblast populations would have to be expanded to close to a million cells in a clinical setting to yield 10-100 clones that could be stored and characterized before transplantation. Given the genomic stability of human mesoangioblasts and the maintenance of their differentiation potential upon culturing *in vitro*, this might be a feasible goal provided that senescence may be avoided during culture. This might be achieved for instance by the currently developed reversible immortalization of these human cells [25].

In addition to the effect on transgene integration, the human MARs were found to have a positive effect on transgene expression levels and stability, the most potent in this respect being hMAR X-29. In presence of hMARs, clones that expressed eGFP in mononucleated mesoangioblasts were in most cases found to maintain eGFP expression after differentiation, even in the absence of antibiotic selection pressure. Although a clone could be isolated form a MAR-devoid plasmid with a comparable expression level as those obtained from mesoangioblasts transfected with a MAR, such clones usually had lower expression or lost any detectable eGFP fluorescence upon differentiation to myotubes. This likely results from the known adoption of less permissive chromatin structures such as heterochomatin over large portions of the genome upon the differentiation of stem cells [26]. The ability of MARs to maintain a transcriptionally permissive chromatin structure, even when the chromatin structure is restricted upon myogenic differentiation, may thus explain their favorable effect on transgene expression upon myotube formation. In addition to the favorable effect of MARs on transgene expression, all clones had a single genomic integration site as is often the case for transfected cell lines, and this integration site can be mapped to increase the safety of potential transfection-based clinical protocols. This feature may be advantageous in terms of safety when compared to viral or transposable vectors that often integrate at multiple and variable number of loci.

Results from *in vivo* transplantation experiments indicated that transfected mesoangioblast clones may lead to eGFP positive myofibers upon intramuscular injection as early as 9 days after administration. In accordance with their *in vitro* differentiation properties, the hMAR-containing clone yielded clearly detectable eGFP expression *in vivo*, whereas the clone without a MAR displayed low eGFP levels. Based on these findings we concluded that human MARs like X-29 could fulfill some of the requirements of autologous muscle cell therapy. While we were able to obtain stable mesoangioblast clones that were co-transfected with a plasmid encoding full-length dystrophin driven by the muscle creatine kinase (MCK) promoter and some of the MAR-bearing eGFP plasmids, transfections with the most potent hMAR X-29-containing plasmid consistently yielded no clones. The rationale for using a muscle-specific promoter to express dystrophin stems from previous observations that the build-up of dystrophin protein expression in undifferentiated mesoangioblasts leads to toxicity effects, possibly because of the lack of concomitant expression of other components of the glycoprotein complex that dystrophin interacts with [27,28]. Thus, a likely explanation for the lack of stable clones expressing dystrophin in presence of the most potent hMAR X-29 is that the co-integration of the MAR and the dystrophin construct caused leakiness of the MCK promoter, and ectopic expression of dystrophin prior to differentiation. This interpretation is indeed supported by data showing GFP expression from the MCK promoter in presence of hMAR X-29 in undifferentiated mesoangioblast cells, which indicated an increased leakiness of the muscle promoter in presence of the most potent MAR.

Nevertheless, dystrophin cDNA-containing clones were obtained using other MAR elements like the mouse S4, the human 1-6 and the chicken lysozyme MAR, and clones obtained with these elements were capable of differentiating *in vitro* and *in vivo*. Dystrophin expressing fibers were found from the injection of the clonal mesoangioblasts. A minority of the fibers had dystrophin expression, as expected from the

previously reported relatively lower efficacy of a single intramuscular injection relative to several consecutive injections (ref. [25] and unpublished data from G.C. and collaborators), and also because potentiating protocols like the transduction with MyoD vectors or pre-injection exposure to growth factors were not used [8]. Nevertheless, our observations provide a first description of full-length dystrophin expression from the stable plasmid transfection of muscle precursor cells, followed by their incorporation in muscle fibers *in vivo*. It thereby facilitates a new strategy to pursue a possible treatment of muscular dystrophies using genetically-corrected cells.

Overall, the presented approach may be limited to the cells that maintain a normal genomic structure and differentiation potential when cultured *in vitro*, and to cloning procedures that allow the selection of transgene expressing cells. Overall, we propose that the ability to characterize the genomic structure of clonal populations after limited expansion may decrease the likelihood of adverse effects and may thus open a feasible path towards cell-based therapies involving gene transfer.

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