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Viral vector-mediated gene transfer in fundamental and applied cardiovascular research

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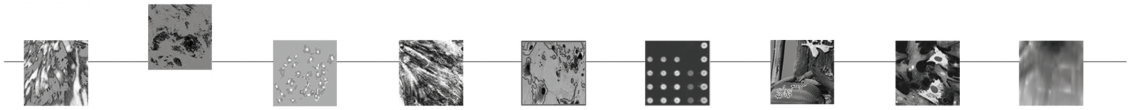


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Chapter 2

Genetic Complementation of Human Muscle Cells via Directed Stem Cell Fusion

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Abstract

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Duchenne muscular dystrophy (DMD) is caused by mutations in the X chromosome-linked *DMD* gene, which encodes the sarcolemma-stabilizing protein-dystrophin. Initial attempts at DMD therapy deployed muscle progenitor cells from healthy donors. The utilization of these cells is, however, hampered by their immunogenicity, while those from DMD patients are scarce and display limited *ex vivo* replication. Nonmuscle cells with myogenic capacity may offer valuable alternatives especially if, to allow autologous transplantation, they are amenable to genetic intervention. As a paradigm for therapeutic gene transfer by heterotypic cell fusion we are investigating whether human mesenchymal stem cells (hMSCs) can serve as donors of recombinant *DMD* genes for recipient human muscle cells. Here, we show that forced *MyoD* expression in hMSCs greatly increases their tendency to participate in human myotube formation turning them into improved DNA delivery vehicles. Efficient loading of hMSCs with recombinant *DMD* was achieved through a new tropism-modified high-capacity adenoviral (hcAd) vector directing striated muscle-specific synthesis of full-length dystrophin. This study introduces the principle of genetic complementation of gene-defective cells via directed cell fusion and provides an initial framework to test whether transient *MyoD* synthesis in autologous, gene-corrected hMSCs increases their potential for treating DMD and, possibly, other muscular dystrophies.

2.1 Introduction

Cell therapies are gaining much attention due to the perceived potential of these new treatment modalities to cure a wide variety of inherited illnesses. However, before non-hematopoietic cell therapies can enter the mainstream of medical practice, much research has still to be performed at both the fundamental and technological level. It is generally recognized that one of the main limitations in the development of cell-based therapies concerns the lack of methods that can reproducibly and quantitatively derive therapeutic cell types from cells that are either uncommitted to a particular fate or possess a phenotype different from the desired one. Thus, a great deal of effort is being put into devising procedures that can direct robust differentiation of target cells into cell types useful for the repair of specific tissues/organs.

Duchenne muscular dystrophy (DMD) afflicts ~1 in every 3,500 boys, making this lethal muscle-wasting disease the most prevalent hereditary muscle disorder. Strategies that aim at DMD therapy include (i) transplanting cells with myoregenerative capacity from healthy donors, (ii) restoring dystrophin synthesis from defective *DMD* transcripts by premature stop codon suppression or exon skipping, (iii) modulating the expression of nondefective endogenous genes such as upregulation of *utrophin* or downregulation of *myostatin*, and (iv) adding recombinant *DMD* genes via viral or nonviral gene transfer.^{1,2} The integration of gene- and cell-based approaches in DMD therapy increases the possibility of treating patients with their own cells. However, making autologous cell therapy for DMD viable requires much more research so as to establish (i) easily accessible cells with myoregenerative capacity, (ii) efficient gene carriers that can accommodate full-length, and thus fully functional, recombinant *DMD* genes, preferably under the control of striated muscle gene-specific promoters, and (iii) methods to disseminate the therapeutic cells throughout the musculature.

After birth, satellite cells and their descendants, referred to as myoblasts, constitute the natural reservoirs of muscle stem and progenitor cells, respectively. Accordingly, transplantation of allogeneic myoblasts has been at the forefront of DMD therapy research.³ Avoiding rejection of these cells requires strong and continuous immunosuppression,⁴ which increases the patient's risk of infections and is associated with nephrotoxicity. The deployment of gene-corrected autologous DMD myoblasts should obviate the need for immunosuppression but is hampered by the scarcity of functional satellite cells in DMD patients and their limited *ex vivo* expansion capacity.⁵ It is therefore important to investigate the myogenic potential of alternative cell types. Human mesenchymal stem cells (hMSCs) in particular are gaining much attention as therapeutic agents because they can easily be obtained from bone marrow or adipose tissue and can be greatly expanded *ex vivo*. Moreover, hMSCs can readily differentiate into specialized mesodermal cell types such as osteocytes, adipocytes, and chondrocytes. In addition, *in vitro* and *in vivo* experiments have demonstrated that at least a small fraction of these cells has myoregenerative capacity.⁶⁻⁹ We recently elaborated on these findings by showing that genetically modified hMSCs expressing recombinant full-length *DMD* genes can fuse with dystrophin-negative human muscle cells.¹⁰ Unfortunately, mosaic DMD myotubes derived from these heterotypic cell fusion events were rare indicating that few hMSCs display intrinsic myogenic fusion capacity.

In this study, we investigated whether (i) forced MyoD expression in hMSCs increases their proficiency to merge with DMD muscle cells and (ii) MyoD-stimulated hMSCs can be used as cellular vehicles for the delivery of recombinant genes encoding full-length dystrophin (Dys^{FL}).

2.2 Materials and methods

2.2.1 Cells

The hMSCs and human myoblasts used in this study were isolated from healthy individuals and DMD patients, respectively. The isolation, culture, and characterization of these cells have been detailed before.¹⁰⁻¹² The hMSCs were isolated from orthopedic surgery remnants from a healthy 38-year-old female. For all experiments, hMSCs of low passage number (<8) were employed. The human myoblasts used in this study were immortalized with *Bmi-1* and *TERT*.¹² Cultures of these diploid cells do not contain fibroblasts and show no obvious differences in myogenic differentiation capacity in comparison to primary myoblast cultures.

2.2.2 Viral vectors

The vesicular stomatitis virus G protein-pseudotyped self-inactivating human immunodeficiency virus type 1-based vectors CMVPRES (hereafter referred to as LV.eGFP),¹³ LV.hMyoD.eGFP, LV.mMyoD::ER^{T2}.eGFP, and LV.DsRed were produced in 293T cells with the aid of the packaging plasmids psPAX2 (Addgene, Cambridge, MA) and pLP/VSVG (Invitrogen, Breda, The Netherlands) as previously described.¹⁴ Lentivirus vector particles were purified by filtration through 0.45- μ m pore-sized cellulose acetate filters (Pall, Mijdrecht, The Netherlands) and centrifugation through 20% (wt/vol) sucrose (Merck, Nottingham, UK) cushions.¹⁴ The generation and purification of the Dys^{FL}-encoding and capsid-modified hcAd vector hcAd.F50.hrGFP.Dys^{FL} was carried out essentially as described for other hcAd-based vector systems using Per.tTA.Cre76 as producer cells¹⁵ and Ad.floxed^{1/2}.F50 as helper vector.¹⁶ The use of the latter reagent together with shuttle plasmid phcAd.Dys^{FL} yields Dys^{FL}-encoding hcAd particles whose fibers consist of knob and apical shaft domains from human Ad serotype 50 (species B) instead of the prototypic serotype 5 (species C). The resulting change in cell tropism overcomes the absence of the Coxsackie B virus and adenovirus receptor moiety on the surface of hMSCs allowing efficient gene transfer into these cells.^{10,11} Recombinant DNA techniques were performed using established methods. The complete nucleotide sequences of the shuttle plasmids used to generate LV.hMyoD.eGFP, LV.mMyoD::ER^{T2}.eGFP, LV.DsRed, and hcAd.F50.hrGFP.Dys^{FL} have been deposited in GenBank under accession numbers EU048697, EU048696, EU048695, and EU048698, respectively. The gene transfer activity of all vector stocks was determined by end-point titration on HeLa indicator cells using flow cytometric analysis of reporter gene expression as read-out. Consequently, vector titers are expressed in HeLa cell-transducing units per ml.

2.2.3 Establishment of cocultures of eGFP-labeled hMSCs and DsRed-marked DMD myoblasts

LV.DsRed was applied to DMD myoblasts in wells of 24-well culture plates at a multiplicity of infection of 30 HeLa cell-transducing units per cell. The 4-hour transduction procedure was carried out in 500 μ l of GM supplemented with 20 μ g/ml diethylaminoethyl-dextran sulfate (GE Healthcare, Leiderdorp, The Netherlands). In parallel, hMSCs were transduced with LV.eGFP or with LV.hMyoD.eGFP as described in the Supplementary Materials and Methods, Supplementary Table S1. Three days later the cells were trypsinized and expanded in 25-cm² cell culture flasks (Greiner, Alphen aan den Rijn, The Netherlands). Two weeks after infection, 1.6×10^5 DsRed-marked DMD myoblasts were seeded in wells of 6-well culture plates alone or together with 4×10^4 hMSCs transduced either with LV.eGFP or with LV.hMyoD.eGFP. Four days later, the GM was replaced by DM. After incubation for 5 days in DM, the cocultures were analyzed by direct fluorescence microscopy.

2.2.4 Immunophenotyping

The surface antigen profile of human myoblasts, unmodified hMSCs and LV.mMyoD::ERT².eGFP-transduced hMSCs was determined by flow cytometry essentially as described before.^{11,17} All antibodies used were directly coupled to phycoerythrin and were purchased from Bioconnect, Huissen, The Netherlands (CD105), BD Biosciences (Breda, The Netherlands) (CD13, CD44, CD49e, CD73, and CD184) or Sanquin (Amsterdam, The Netherlands) (CD10, CD56, CD62P, and CD71). Isotype-matched, phycoerythrin-conjugated antibody controls from BD Biosciences were used to determine background fluorescence.

2.2.5 Analysis of skeletal muscle gene expression by IFM

hMSCs were transduced in triplicate with LV.eGFP or LV.hMyoD.eGFP as mentioned earlier and cultured for 16 days in GM, after which time, they were either exposed to DM or kept in GM for another 25 days. IFM was carried out with antibodies directed against desmin, skMHC-II, dystrophin, and MyoG as specified below.

2.2.6 Light microscopy and immunostainings

Phase-contrast microscopy, direct fluorescence microscopy, and IFM were performed with an Olympus IX51 inverse fluorescence microscope (Olympus, Zoeterwoude, The Netherlands). Images were captured by a ColorView II Peltier-cooled charge-coupled device camera (Olympus, Zoeterwoude, The Netherlands) and were archived using AnaLYSIS software (Soft Imaging Systems, Zoeterwoude, The Netherlands). In addition, direct fluorescence microscopy and IFM images of cells grown on glass cover slips were acquired by using a conventional Leica DM 5500 B fluorescence microscope (Leica, Rijswijk, The Netherlands) together with a CoolSnap K4 cooled charge-coupled device camera (Roper Scientific, Evry Cedex, France). The same specimens were also analyzed with a confocal Leica TCS SL laser scanning fluorescence microscope. Cells

were fixed with phosphate-buffered 4.0% (wt/vol) formaldehyde solution (pH 6.8-7.2; J.T. Baker; Deventer, The Netherlands) at room temperature for 30 minutes. After fixation, the cells were washed three times with phosphate-buffered saline (PBS) containing 10 mmol/l glycine (PBSG) and permeabilized with 0.1% (vol/vol) Triton X-100 (Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS for 5 minutes at room temperature. Following three washes with PBSG, aspecific antibody-binding sites were blocked by incubating the cells for at least 2 hours at room temperature with PBSG containing 5% 0.22- μ m filter-sterilized fetal bovine serum. Primary antibodies specific for desmin (clone D33; Dako, Heverlee, Belgium), skMHC-II (clone MY-32; Sigma-Aldrich, Zwijndrecht, The Netherlands), dystrophin (clone Dy8/6C5; Novocastra, Norwell, MA), MyoD (clone 5.8A; Dako), MyoG (clone F5D; Dako), and β -dystroglycan (clone 43DAG1/8D5; Novocastra, Norwell, MA) were diluted 1:50, 1:200, 1:20, 1:50, 1:50, and 1:40, respectively, in PBSG containing 5% 0.22- μ m filter-sterilized fetal bovine serum. The cells were incubated overnight with the primary antibodies at 4°C. Excess primary antibody was removed by three 5-minute washes with PBSG and the cells were labeled for 3-4 hours at 4°C with highly cross-absorbed, affinity-purified, AlexaFluor568-conjugated goat anti-mouse immunoglobulin G (Invitrogen, Breda, The Netherlands), diluted 1:500 in PBSG containing 5% 0.22- μ m filter-sterilized fetal bovine serum. The cells were again washed three times at room temperature with PBSG and their nuclei were stained by incubating for 10 minutes with 10 μ g/ml Hoechst 33342 (Invitrogen Breda, The Netherlands) in PBS, followed by four additional washes with PBS.

2.2.7 Analysis of mMyoD::ER^{T2} activity by IFM, heterotypic cell fusion assays and flow cytometry

After transduction with LV.mMyoD::ER^{T2}.eGFP at a multiplicity of infection of 5 HeLa cell-transducing units per cell, hMSCs were expanded for 2 weeks in GM. Parallel cultures of uninfected hMSCs served as controls. Next, the cells were kept for another 5 days in GM with or without 250 nmol/l of the synthetic drug 4-OHT (Sigma-Aldrich, Zwijndrecht, The Netherlands). Finally, uninfected and LV.mMyoD::ER^{T2}.eGFP-infected hMSCs were exposed for 72 hours to DM $\pm$ 250 nmol/l 4-OHT. Thus for a total of 8 days, cultures were either treated or not treated with 4-OHT. The effect of 4-OHT on the hMSCs was evaluated through desmin and skMHC-II immunostainings.

Twenty-nine days after infection, LV.mMyoD::ER^{T2}.eGFP-transduced hMSCs kept in GM were either exposed or not exposed to 4-OHT for an additional period of 8 days. Subsequently, 1×10^4 of untreated or 4-OHT-treated hMSCs derived from three independent cultures each were mixed with 5×10^4 DMD myoblasts. After 5 days in GM with or without 250 nmol/l 4-OHT, the cells were kept in 4-OHT-containing and drug-free DM, respectively. The frequency of mosaic myotubes in the presence and in the absence of 4-OHT was determined through fluorescence microscopy after the regular myoblast differentiation period. Finally, aliquots of hMSCs exposed to 4-OHT or kept in its absence, were subjected to flow cytometric analysis to determine cell-surface levels of CD10 and CD56.

2.2.8 Statistical analysis

Mean, SD, and SEM were computed using GraphPad Prism software (version 4.03). Student's *t*-test was used to compare data sets. $P < 0.05$ were considered significant.

2.3 Results

2.3.1 MyoD induces expression of skeletal muscle genes in hMSCs

Forced expression of a recombinant gene encoding the basic helix-loop-helix myogenic transcription factor MyoD, also known as Myf3, has been shown to activate skeletal muscle genes in various differentiated nonmuscle cell types.^{18,19} To investigate whether MyoD also upregulates expression of skeletal muscle genes in undifferentiated hMSCs, they were transduced with the bicistronic human immunodeficiency virus type 1-based vector LV.hMyoD.eGFP, which encodes human MyoD (hMyoD) and enhanced green fluorescent protein (eGFP), or with LV.eGFP, which only directs the synthesis of eGFP (Figure 1).

hMyoD was readily detected by immunofluorescence microscopy (IFM) to be exclusively found in the nuclei of LV.hMyoD.eGFP-treated hMSCs (Figure 2a). Next, through reverse transcription-polymerase chain reaction analysis, we searched for potentially hMyoD-responsive genes by targeting the RNA of the *desmin*, *Mef2C*, *MyoG*, *Myf5*, and *Myf6* genes. Figure 2b shows that *desmin* transcripts are already present in hMSCs transduced with LV.eGFP. *Desmin* gene expression is, nonetheless, upregulated after LV.hMyoD.eGFP infection. A much more striking induction was observed for *MyoG* and *Myf6* (Figure 2b). *Mef2C* transcripts, on the contrary, were not noticeably upregulated by *hMyoD* gene transfer since they were present in both hMSC populations at similar levels. Finally, *Myf5* messenger RNA was detected neither in MyoD-negative nor in MyoD-positive hMSCs (Figure 2b). We also tested the effect of ectopic *hMyoD* expression in hMSCs at the protein level. To this end, we performed desmin, fast-twitch skeletal myosin heavy chain-II (skMHC-II), dystrophin, and MyoG IFM on hMSCs transduced with LV.hMyoD.eGFP or LV.eGFP and cultured in growth medium (GM) or differentiation medium (DM) (Figure 3).

With the exception of dystrophin, which could not be detected regardless of the experimental setup used, the frequency of desmin-, skMHC-II-, or MyoG-positive cells was much higher in LV.hMyoD.eGFP-transduced hMSCs than in LV.eGFP-infected cells (Figure 3a). In fact, skMHC-II and MyoG were only detected in hMSCs transduced with LV.hMyoD.eGFP and not in cells infected with the control vector (Figure 3a). Quantification of desmin-, skMHC-II-, or MyoG-positive hMSCs revealed that myogenic induction was highest in cells exposed simultaneously to LV.hMyoD.eGFP and DM (Figure 3b). Moreover, LV.hMyoD.eGFP-treated cultures contained cells with a more slender morphology than those exposed to LV.eGFP and sporadically displayed syncytia with up to three nuclei (Figure 3a; white arrowheads). Thus, forced *hMyoD* expression can, at least to some extent, orchestrate the myogenic conversion of hMSCs.

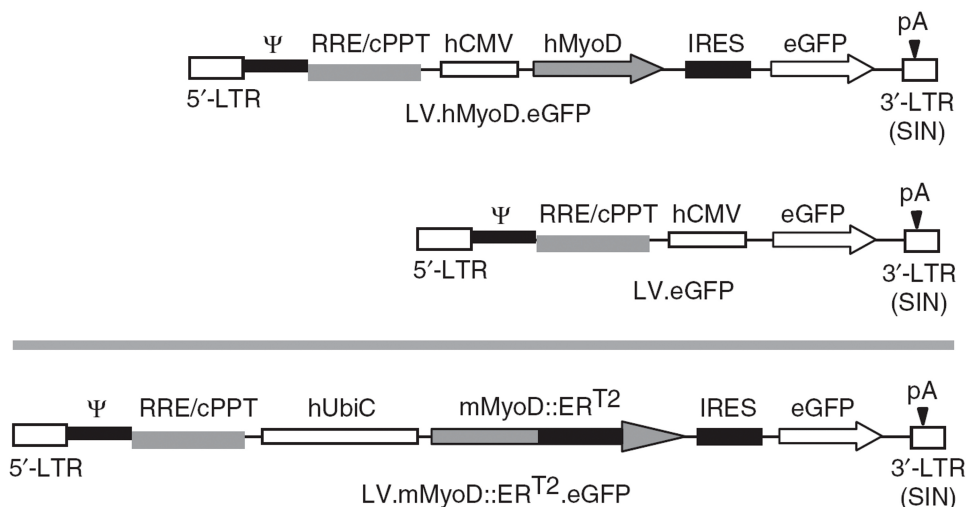


Figure 2.1: Genome structure of LV.hMyoD.eGFP, LV.eGFP and LV.mMyoD::ER^{T2}.eGFP. LV.hMyoD.eGFP encodes the human basic helix-loop-helix myogenic transcription factor MyoD (hMyoD) and, through the IRES, also directs the synthesis of eGFP. Expression of *hMyoD* and *eGFP* is driven by the human cytomegalovirus immediate-early gene promoter (hCMV). LV.mMyoD::ER^{T2}.eGFP encodes eGFP and a conditionally active version of murine MyoD (mMyoD) designated mMyoD::ER^{T2}. The *mMyoD::ER^{T2}* gene codes for a fusion product of mMyoD and a modified version of the ligand-binding domain of human estrogen receptor 1 named ER^{T2}. The superscript T2 denotes the presence of two point mutations in comparison to the original *mMyoD::ER* gene³⁶ that permits interaction of the fusion product with the synthetic hormone 4-hydroxytamoxifen (4-OHT) and not with natural estrogens. The ER^{T2} sequence in *mMyoD::ER^{T2}* is located between the coding regions for the bHLH and activation domains of mMyoD (solid box within gray arrow). *mMyoD::ER^{T2}* and *eGFP* expression are under the control of the human *ubiquitin C* gene promoter (hUbiC). In the absence of 4-OHT mMyoD::ER^{T2} is localized predominantly in the cytoplasm, while in its presence the protein is transported to the nucleus to exert its biological effects. ψ , packaging signal; eGFP, enhanced green fluorescent protein; IRES, encephalomyocarditis virus internal ribosomal entry site; LTR, long-terminal repeat; pA, polyadenylation signal; RRE/cPPT, Rev-responsive element and central polypurine tract; SIN, self-inactivating LTR (LTR with a deletion in the U3 region).

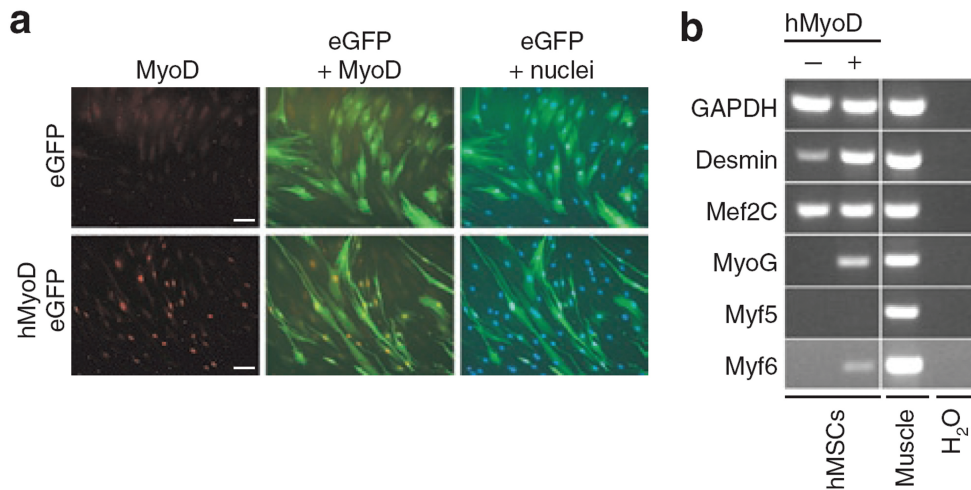


Figure 2.2: Analysis of human mesenchymal stem cells (hMSCs) transduced with LV.eGFP or LV.hMyoD.eGFP by fluorescence microscopy and reverse transcription-polymerase chain reaction (RT-PCR). (a) MyoD immunostaining (left and middle panels) and eGFP direct fluorescence microscopy (middle and right panels) of hMSCs transduced with LV.eGFP (upper panels) or with LV.hMyoD.eGFP (lower panels). The red human MyoD (hMyoD)-specific signal was combined with the green eGFP-specific fluorescence in the middle panels whereas in the right panels the eGFP-specific signal was overlaid with the blue fluorescence derived from hMSC nuclei labeled with the fluorochrome Hoechst 33342. Bars = 200 μ m. (b) RT-PCR analysis of hMyoD-reprogrammed hMSCs. Total cellular RNA from LV.eGFP- or LV.hMyoD.eGFP-transduced hMSCs maintained in growth medium (GM) was extracted 10 days after infection, reverse-transcribed and PCR amplified. Targeted transcripts are specified on the left. Skeletal muscle RNA from a human adult and water served as positive and negative controls, respectively. eGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

2.3.2 MyoD-transduced hMSCs have superior myogenic fusion capacity

We hypothesized that after MyoD-dependent reprogramming, hMSCs would become better fusion partners for neighboring DMD myoblasts undergoing differentiation. To test this hypothesis, we deployed an *ex vivo* system based on cocultures of hMSCs and DMD myoblasts.¹⁰ Before being mixed with DMD myoblasts, hMSCs were eGFP-labeled via infection with LV.hMyoD.eGFP or LV.eGFP. After the cocultures had been established, the cells were exposed to DM. Differentiated myotubes were detected by skMHC-II immunostaining (Figure 4a).

Merging of the eGFP- and skMHC-specific signals derived from cocultures initiated with MyoD-negative hMSCs revealed a few areas containing colabeled mature myotubes (data not shown), whereas in those containing hMyoD-positive hMSCs, multinucleated structures coexpressing eGFP and skMHC-II were ubiquitous (Figure 4a). Quantification of colabeled syncytia showed that ectopic hMyoD expression in hMSCs leads to a large increase in the frequency of mosaic myotubes (Figure 4b).

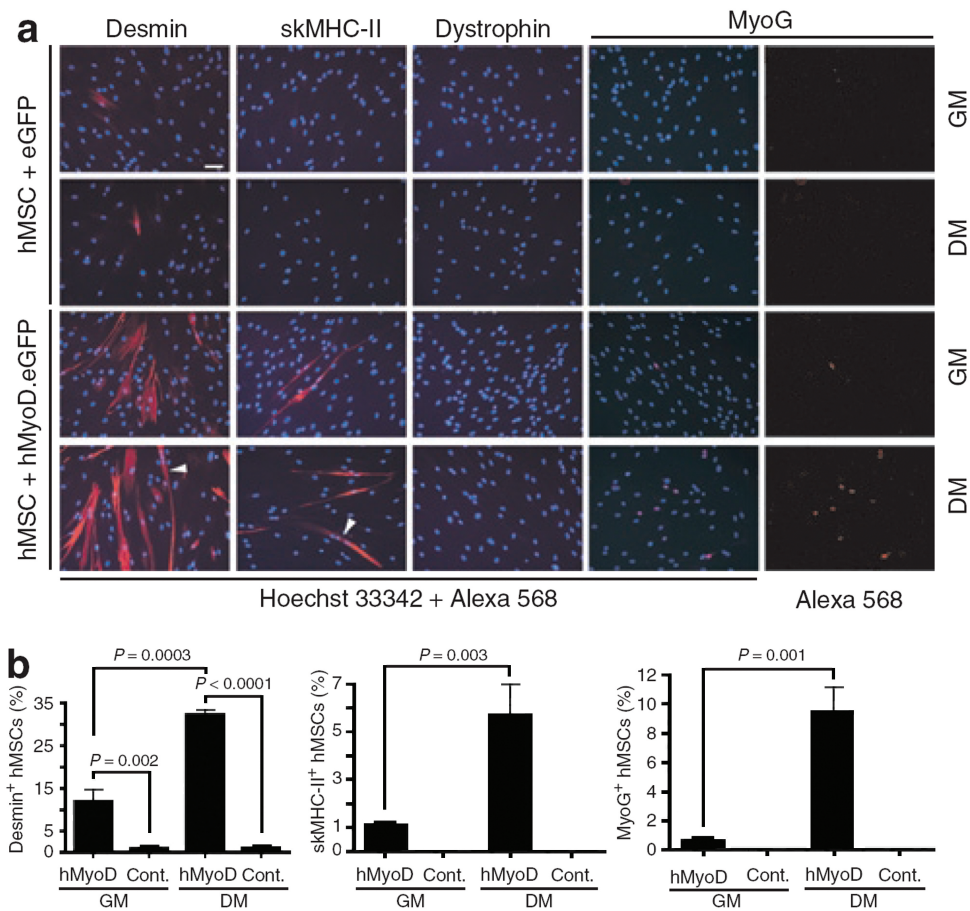


Figure 2.3: Immunodetection and quantification of human mesenchymal stem cells (hMSCs) synthesizing skeletal muscle proteins following human MyoD (hMyoD) gene delivery. (a) Analysis of hMyoD-directed differentiation of hMSCs by desmin, skeletal myosin heavy chain-II (skMHC-II), dystrophin and MyoG immunofluorescence microscopy. hMSCs were transduced in triplicate with LV.eGFP (upper two rows) or LV.hMyoD.eGFP (lower two rows). After a 16-day culture period the cells were either kept in growth medium (GM) or were exposed to differentiation medium (DM) for another 25 days. Nuclei were visualized with the aid of Hoechst 33342 counterstaining. (b) Frequency of hMSCs expressing desmin, skMHC-II, or MyoG in the cell cultures described in a was determined by counting cells positive for each marker protein present in randomly selected fields. Cumulative data are expressed as means $\pm$ SD. Relevant P values are also indicated. hMyoD, LV.hMyoD.eGFP-transduced hMSCs; Cont., LV.eGFP-transduced hMSCs. eGFP, enhanced green fluorescent protein.

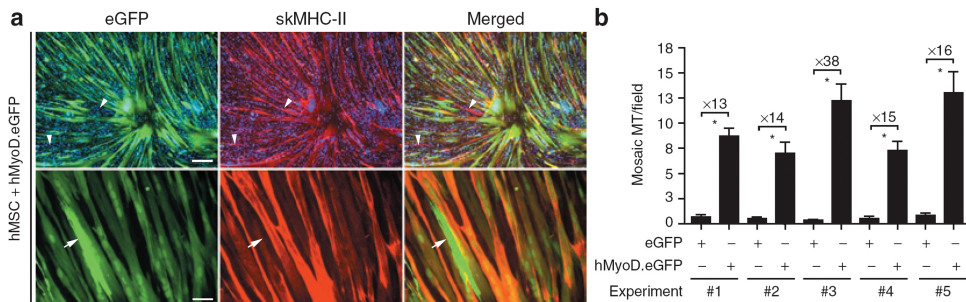


Figure 2.4: Light microscopy analysis of cocultures of Duchenne muscular dystrophy (DMD) myoblasts and enhanced GFP (eGFP)-tagged human mesenchymal stem cells (hMSCs). (a) eGFP direct fluorescence microscopy (left panels) and skeletal myosin heavy chain-II (skMHC-II) immunofluorescence microscopy (middle panels), 4 days after induction of differentiation. Arrowheads (upper panels) point at two examples of mature myotubes without a detectable contribution by hMSCs. Arrows (lower panels) point to an eGFP-positive hMSC among eGFP- and skMHC-II-labeled myotubes. The presence of skMHC-II in the eGFP-labeled syncytia as observed by overlaying both signals (right panels) indicates that they represent mature mosaic myotubes (MTs). The blue fluorescence in the upper panels corresponds to nuclei labeled with Hoechst 33342. The scale bars in the upper and lower panels correspond to 500 and 100 μm , respectively. (b) Frequency of eGFP- and skMHC-II-labeled MTs present in randomly selected fields of cocultures of DMD myoblasts and LV.hMyoD.eGFP- or LV.eGFP-transduced hMSCs from five independent experiments. Graph shows mean $\pm$ SEM from groups of a minimum of 25 and a maximum of 42 randomly selected fields. * $P < 0.0001$ versus LV.eGFP-transduced hMSC controls. The fold increase in mosaic MTs resulting from constitutive hMyoD expression in the hMSCs is indicated above each pair of bars. hMyoD, human MyoD.

The potential long-term deleterious effects of constitutive and high-level *MyoD* expression on myotubes or myofibers have not been thoroughly investigated. We therefore generated the bicistronic human immunodeficiency virus type 1-based vector LV.mMyoD::ER^{T2}.eGFP encoding eGFP and a version of murine *MyoD* (designated mMyoD::ER^{T2}) whose transactivating activity is controlled by the clinically applicable small-molecule drug 4-hydroxytamoxifen (4-OHT) (Figure 1). The functionality of the mMyoD::ER^{T2} construction was tested by transducing hMSCs with LV.mMyoD::ER^{T2}.eGFP and culturing the modified cells in the presence or absence of 250 nmol/l 4-OHT. IFM results depicted in Figure 5a reveal a clear 4-OHT-dependent upregulation of desmin and skMHC-II levels in cells exposed to LV.mMyoD::ER^{T2}.eGFP.

To further establish the functionality of the 4-OHT-controlled mMyoD::ER^{T2} fusion protein, we performed flow cytometric analysis of LV.mMyoD::ER^{T2}.eGFP-transduced hMSCs that had been cultured in medium with or without 250 nmol/l 4-OHT. For the purpose of comparison, we first screened human myoblasts and hMSCs by flow cytometry with a panel of antibodies to identify molecules differentially displayed on the surface of these two cell types. The resulting histograms revealed that CD10 (also known as CALLA) and CD56 (also known as NCAM) were abundantly decorating the surface of myoblasts (Figure 5b) while being very scarce on the plasma membrane of hMSCs (data not shown).

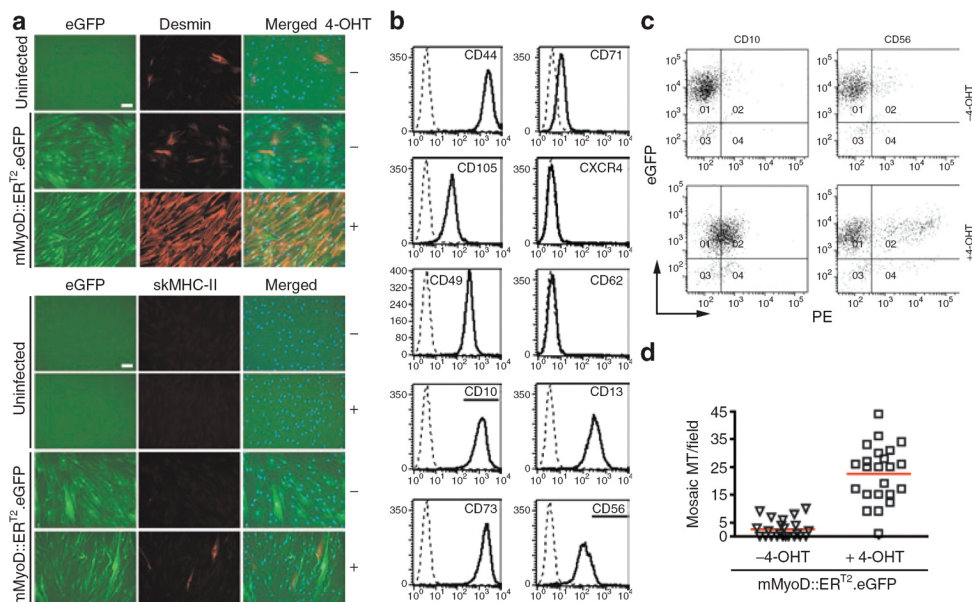


Figure 2.5: Effects of LV.mMyoD::ERT2.eGFP on human mesenchymal stem cells (hMSCs) cultured in the presence or absence of 4-hydroxytamoxifen (4-OHT). (a) Desmin and skeletal myosin heavy chain-II (skMHC-II) immunofluorescence microscopy of uninfected or LV.mMyoD::ERT^{T2}.eGFP-transduced hMSCs exposed (+) or not exposed (-) to 250 nmol/l 4-OHT. Bars = 200 μ m. (b) Flow cytometric analysis of human myoblasts identifying CD10 and CD56 (underlined) as antigens differentially expressed on the surface of hMSCs and human myoblasts. Targeted antigens are specified in the corresponding histograms (representing 10^4 events each). γ -Axis, cell counts; x -axis, phycoerythrin (PE) fluorescence. Broken and solid lines, control and specific antibodies, respectively. (c) Dot plot representation of CD10 and CD56 expression on the surface of LV.mMyoD::ERT^{T2}.eGFP-modified hMSCs cultured in the absence (-) or presence (+) of 250 nmol/l 4-OHT. γ -Axis, eGFP fluorescence; x -axis, PE fluorescence. (d) Frequency of mosaic myotubes (MTs) present in randomly selected fields ($n = 23$) of mature cocultures of Duchenne muscular dystrophy (DMD) myoblasts and LV.mMyoD::ERT^{T2}.eGFP-modified hMSCs maintained in medium without (-) or with (+) 250 nmol/l 4-OHT. eGFP, enhanced green fluorescent protein; ER^{T2}, 4-OHT-responsive variant of the ligand-binding domain of human estrogen receptor; mMyoD, murine MyoD.

consistent with a previous report.¹⁰ At this point, it is worth noting that, CD56 and CD10 have both been implicated in homotypic myoblast adhesion and fusion.^{20,21} Significantly, flow cytometric analysis showed a clear 4-OHT-dependent increase of CD10 and CD56 on the surface of LV.mMyoD::ER^{T2}.eGFP-transduced hMSCs (Figure 5c). Interestingly, these two factors were upregulated to different extents. While most MyoD-stimulated hMSCs expressed CD10, the corresponding cell-surface levels were relatively low. A much smaller fraction of MyoD-reprogrammed hMSCs expressed CD56 on their surface but the amount of this protein on a per cell basis was higher (Figure 5c). Regardless, one can conclude that mMyoD::ER^{T2} induces the expression on the surface of hMSCs of molecules present on the plasma membrane of bona fide muscle cells. Collectively, these data indicate that mMyoD::ER^{T2} exhibits myogenic reprogramming activity and is 4-OHT-responsive. Next, cocultures of DMD myoblasts and LV.mMyoD::ER^{T2}.eGFP-modified hMSCs were established and incubated with or without 4-OHT. After the regular differentiation period, the frequency of mosaic myotubes was on average much higher in cocultures exposed to 4-OHT than in those that did not receive the drug (Figure 5d). These data are consistent with the results obtained with the constitutively active form of hMyoD.

To definitely demonstrate that the eGFP-labeled myotubes arose through heterotypic cell fusion between hMyoD-stimulated hMSCs and DMD myoblasts and were not the result of homotypic cell fusion of MyoD-reprogrammed hMSCs under coculture conditions, LV.hMyoD.eGFP-transduced hMSCs were mixed with DMD myoblasts that had been labeled with a variant of the *Discosoma* sp. red fluorescent protein (DsRed).²² After the regular differentiation period, direct fluorescence microscopy was performed. Most areas in control cultures initiated with hMyoD-negative and eGFP-positive hMSCs together with DsRed-marked DMD myoblasts did not contain eGFP- or double-labeled myotubes (Figure 6, left column). In a few discrete regions, however, doubly marked myotubes could clearly be discerned in addition to the abundant DsRed-labeled syncytia (data not shown). On the contrary, the cocultures initiated with hMyoD- and eGFP-positive hMSCs together with DsRed-labeled DMD myoblasts contained many multinucleated structures expressing both *eGFP* and *DsRed* but again no large myotubes containing only the eGFP label were observed (Figure 6, right column). Co-localization of both markers in hybrid myotubes was confirmed by confocal microscopy. We hence conclude that, as previously shown for naïve hMSCs,¹⁰ MyoD-stimulated hMSCs participate in myogenesis predominantly via heterotypic cell fusion originating as end-products mosaic myotubes.

2.3.3 Recombinant DMD gene delivery into DMD myotubes by MyoD-transduced hMSCs

Taken together, the results described earlier demonstrate that forced *MyoD* expression in hMSCs (i) induces expression of skeletal muscle genes in these cells and (ii) increases significantly their recruitment by differentiating human myoblasts. These findings provide a rationale for exploiting MyoD-reprogrammed hMSCs as delivery vehicles of genes/proteins into diseased skeletal muscle. Toward this end, we investigated the use of *MyoD*-transduced hMSCs as carriers of full-length recombinant *DMD* (Dys^{FL}) genes into dystrophin-defective human muscle cells. Additionally, we studied whether Dys^{FL}-transduced hMSCs produce sufficient dystrophin to restore the proper localization

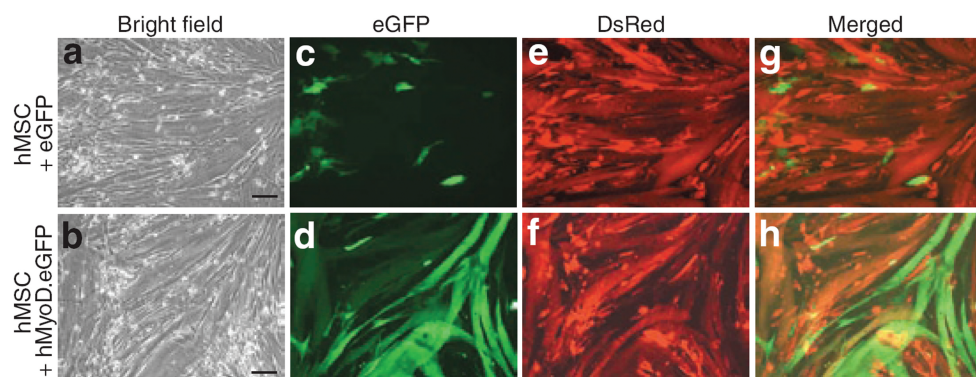


Figure 2.6: Live cell imaging of mature cultures of *Discosoma* sp. red fluorescent protein (DsRed)-tagged Duchenne muscular dystrophy (DMD) myoblasts and enhanced green fluorescent protein (eGFP)-labeled human mesenchymal stem cells (hMSCs). (a) and (b) Phase-contrast microscopy, (c) and (d) direct eGFP, and (e) and (f) DsRed fluorescence microscopy of DsRed-tagged DMD myoblasts mixed with human MyoD (hMyoD)-negative (left panels) or hMyoD-positive (right panels) eGFP-marked hMSCs 5 days after the addition of differentiation medium. The eGFP- and DsRed-specific signals were combined in (g) and (h). Bars = 200 μm .

of β -dystroglycan along the plasma membrane of mosaic myotubes. For this purpose, hMSCs were transduced with LV.hMyoD.eGFP or LV.eGFP alone or in combination with hcAd.F50.hrGFP.Dys^{FL}. hcAd.F50.hrGFP.Dys^{FL} is a tropism-modified high-capacity adenoviral (hcAd) vector in which Dys^{FL} expression is driven by a 5.2-kilobase fragment of the human *muscle creatine kinase* gene promoter. The use of the human *muscle creatine kinase* gene promoter leads to the synthesis of Dys^{FL} in differentiated myotubes but not in hMSCs, which may be important given the possible adverse effects of Dys^{FL} misexpression on mononuclear cells.²³ In addition, hcAd.F50.hrGFP.Dys^{FL} particles have their fiber domains tailored to by-pass the absence of the human Ad species C receptor (Coxsackie B virus and adenovirus receptor) on the surface of hMSCs.^{10,11} Analysis of the four different cultures by IFM revealed that dystrophin-positive myotubes were detected exclusively in mixed cultures initiated with LV.hMyoD.eGFP- and hcAd.F50.hrGFP.Dys^{FL}-transduced hMSCs (Figure 7a and data not shown). These results indicate that the endogenous *DMD loci* present in the nuclei of control and of MyoD-stimulated hMSCs do not give rise to detectable amounts of Dys^{FL} after their incorporation into DMD myotubes. While eGFP was evenly distributed over entire myotubes, dystrophin was often lining the plasma membrane of the syncytia suggesting its proper localization (see arrows in Figure 7a). Consistently, preferential membrane association of β -dystroglycan, a type I trans-membrane component of the dystrophin-associated glycoprotein complex, was observed in mosaic (see arrows in Figure 7b) but much less so in nonmosaic myotubes (see arrowheads in Figure 7b). In addition, the β -dystroglycan-specific staining was stronger in mosaic myotubes than in their nonmosaic counterparts (Figure 7b). These results indicate that introduction of Dys^{FL} into DMD myotubes by heterotypic cell fusion can rescue the dystrophin-associated glycoprotein complex at its normal location as observed after

more conventional genetic interventions such as those involving direct delivery and repair of recombinant and endogenous *DMD* messages, respectively.^{1,2}

2.4 Discussion

Forced *MyoD* expression induces skeletal myogenesis in various differentiated cell types of nonmuscle origin.^{18,19} In this study we report that undifferentiated hMSCs also acquire properties of muscle cells in response to ectopic *MyoD* expression. However, the differentiation of these cells along the myogenic lineage was found to be incomplete as demonstrated by IFM, reverse transcription-polymerase chain reaction analysis, and flow cytometry. Previous research has indicated that different cell types respond differently to forced *MyoD* expression.^{19,24-26} Myogenic conversion varies from undetectable *e.g.*, hepatocytes²⁴ to robust (*e.g.*, fibroblasts).²⁴⁻²⁶ Interestingly, ectopic *MyoD* expression causes some nonmuscle cell types to acquire an elongated shape without concomitant formation of extensive syncytia.^{19,25} These results are reminiscent of our data on monotypic hMSC cultures exposed to MyoD. In other cell types, however, forced MyoD synthesis triggers the formation of large myotubes.^{19,26} For instance, Ad vector-mediated delivery of *MyoD* into fetal dermal fibroblasts led to extensive homotypic cell fusion.²⁶ In our study, only a fraction of the *MyoD*-transduced hMSCs obtained properties of skeletal muscle cells suggesting the presence of negative regulators and/or the absence of positive regulators of myogenesis in the refractory cells. It is known, for example, that *Mef2C* and *Twist* can interact with MyoD-E proteins heterodimers to enhance and repress myogenesis, respectively.^{27,28} In this regard it is of note that *Mef2C* transcripts were detected by reverse transcription-polymerase chain reaction in both naïve and hMyoD-transduced hMSC cultures suggesting that (i) they are only present in the MyoD-responsive hMSC fraction, (ii) they are nonfunctional in the MyoD-insensitive cells, (iii) *Mef2C* activity is suppressed in the refractory cells, or (iv) *Mef2C* is not limiting.^{27,28}

As determined by IFM, forced *MyoD* expression in hMSCs did not suffice to induce discernable endogenous dystrophin synthesis. This situation applied to both single hMSCs as well as to hMSCs incorporated in myotubes. The lack of detectable amounts of dystrophin in MyoD-treated hMSCs is intriguing considering that after fusion with myotubes these cells are embedded in an environment supporting the skeletal muscle phenotype. Muscle differentiation is a temporally regulated process with certain gene products such as desmin accumulating early on, with others, including the dystrophin-associated glycoprotein complex components dystrophin and sarcoglycans, emerging later. A hypothesis consistent with our data is that the timeframe of the experiments might have been insufficient to induce significant expression of the very large *DMD* gene (cultured myotubes typically detach from their support 6-7 days after the initiation of myogenic differentiation). Alternatively, due to the partial genetic reprogramming of hMSCs, nonmuscle nuclei within myotubes may continue to express dominant-negative regulators of myogenesis such as those that block endogenous *DMD* gene expression by epigenetic mechanisms. This hypothesis might also explain the absence even long after transplantation of significant δ -sarcoglycan and dystrophin derived from wild-type bone marrow stem cell nuclei integrated into the myofibers of δ -sarcoglycan- and dystrophin-negative mice, respectively,

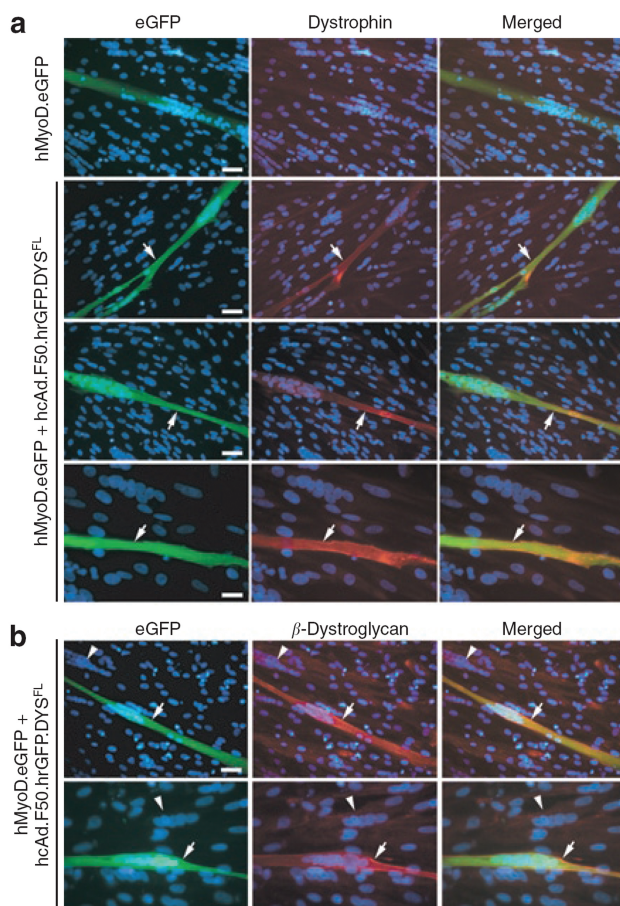


Figure 2.7: Genetic complementation of Duchenne muscular dystrophy (DMD) myotubes by *hMyoD*- and *Dys*^{FL}-expressing human mesenchymal stem cells (hMSCs). (a) hMSCs were transduced with LV.hMyoD.eGFP alone (upper row) or in combination with hcAd.F50.hrGFP.Dys^{FL} (lower three rows) and mixed with DMD myoblasts. After a 5-day differentiation period the cells were subjected to eGFP direct fluorescence microscopy (left column) and dystrophin immunofluorescence microscopy (IFM) (middle column). Signals derived from both analyses are combined in the right panels. Arrows point to myotubes displaying plasma membrane-associated dystrophin-specific staining. Bars = 100 μ m (upper three rows) and 50 μ m (lowest row). (b) hMSCs were transduced with LV.hMyoD.eGFP and hcAd.F50.hrGFP.Dys^{FL} and mixed with DMD myoblasts. After a 5-day differentiation period the cells were subjected to eGFP direct fluorescence microscopy (left column) and β -dystroglycan IFM (middle column). Signals derived from both analyses are combined in the right panels. Arrows point at myotubes displaying plasma membrane-associated β -dystroglycan-specific staining. Arrowheads indicate nonmosaic DMD myotubes showing diffuse β -dystroglycan labeling. Bars = 100 μ m (upper row) and 50 μ m (lower row). eGFP, enhanced green fluorescent protein; hMyoD, human MyoD.

even after long time post-transplantation.^{29,30}

Our results show that, cell-autonomous full-fledged differentiation of nonmuscle cells into bona fide muscle progenitor cells is not necessary in order for heterotypic cell fusion to occur. Instead, what one needs is the activation of the myogenic program to the extent that it endows the nonmuscle cells with factors required for their fusion with neighboring muscle cells. The set of molecules involved in cell adhesion/fusion in hMyoD-reprogrammed hMSCs, although incomplete for significant homotypic cell fusion to occur, can apparently be complemented in the presence of myoblasts undergoing canonical myogenic differentiation. Interestingly, we found that MyoD induced the expression on the surface of hMSCs of two membrane proteins (*i.e.*, CD10 and CD56) that have previously been reported to be engaged in myoblast differentiation, adhesion, and fusion.^{21,22}

By combining the use of MyoD-reprogrammed hMSCs with tropism-modified and Dys^{FL}-encoding hcAd vectors we accomplished the genetic rescue of DMD muscle cells without having to rely on epigenetic remodeling and subsequent activation of endogenous *DMD* loci in donor cell nuclei. Sufficient amounts of recombinant dystrophin can be introduced into DMD myotubes through heterotypic fusion with Dys^{FL}-expressing hMSCs to rescue β -dystroglycan at its normal location along the plasma membrane. Moreover, although dependent on the further development of safe and efficient hc vectors and despite the fact that recombinant dystrophin neoantigens may pose concerns, introducing Dys^{FL} into hMSCs has the distinct advantage of allowing transplantation of readily obtainable autologous cells. To minimize as much as possible the immunogenicity of the graft, noninternalized vector particles should be thoroughly removed and the transduced hMSCs might be cultured in the patient's own serum. Additional measures may entail the use of vectors directing Dys^{FL} expression from a late striated muscle gene-specific promoter. Regarding the neoantigenicity of recombinant dystrophin, it is relevant to mention that the occurrence in many DMD patients of so-called "revertant fibers"³¹ might aid in creating tolerance to the exogenous protein. In this context, it is also worth mentioning that hMSCs have been shown to display immunosuppressive properties.³² To the best of our knowledge this study demonstrates for the first time the directed differentiation of gene-corrected cells as a means of delivering therapeutic foreign DNA into another cell type through cell-cell fusion. These results provide a rationale for incorporating *MyoD* and Dys^{FL} expression units into a single vector particle. Such a vector might also hold value for direct DysFL transfer to dystrophic muscle since a substantial fraction of this tissue is fibrotic and thus composed of potentially MyoD-reprogrammable nonmuscle cells. However, for either application (*i.e.*, *ex vivo* or *in vivo* gene transfer) proper spatial and temporal control over the expression of *MyoD* is fundamental for harmonious skeletal muscle tissue development and repair. For instance, *MyoD* expression can trigger unscheduled cell cycle withdrawal.³³ Interestingly, within the time frame of our experiments we did not detect overt differences in the proliferation rate between LV.eGFP- and LV.hMyoD.eGFP-transduced hMSC cultures as determined by cell counts (data not shown). Until now, we do not know whether this is a consequence of (i) cell-specific factors, (ii) the relatively short time the cells were kept in GM or, (iii) MyoD levels. Clearly, further experiments directly probing cell cycle status endpoints are warranted to establish the impact of MyoD expression on the progression of hMSCs through mitosis. Moreover, although previous results clearly established that MyoD-null myoblasts have impaired dif-

ferentiation capacity,³⁴ recent data indicate that their MyoD-positive counterparts suffer from decreased survival after intramuscular administration.³⁵ Therefore, the synthesis or activity of MyoD should preferably be regulated. In this regard the 4-OHT-ER^{T2} approach tested in this study might constitute an interesting strategy especially if one considers that 4-OHT is already being deployed in the clinic. Furthermore, investigation of other myogenic regulatory factors (*e.g.*, Myf5) or even muscle stem cell specification factors (*e.g.*, Pax7) may provide valuable alternatives to MyoD. In any case, therapeutic approaches based on directed cell fusion of Dys^{FL}-positive autologous nonmuscle cells, should next be evaluated in combination with strategies aiming at tackling the pressing issue of body-wide dissemination of the transplanted cells. Finally, investigation of this gene transfer principle in treating diseases that depend on or benefit from skeletal muscle produced secretory proteins, such as the blood clotting factor deficiencies hemophilia A and B, may also be considered.

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2.7 Supplementary materials and methods

2.7.1 DNA transfections

HeLa cells were seeded in wells of 24-well plates (Greiner Bio-One) at a density of $0.8\text{--}1.0 \times 10^5$ cells per well. After an overnight incubation period, each sample received 350 ng of DNA mixtures in a total volume of 50 μl of 150 mM NaCl. The DNA mixtures contained 280 ng of pGL3.MCK.Luc, 4.6 nmol of LV shuttle plasmid and 4.6 nmol of either pcDNA3.1/Hygro(+) (Invitrogen) or a human Mef2C-encoding derivative thereof. A total amount of 350 ng of DNA was obtained by adding a plasmid lacking eukaryotic promoter elements. After the addition of 2.4 μl of ExGen 500 (Fermentas), the DNA mixtures were immediately shaken vigorously for 10 s and incubated for 15 min at room temperature. The DNA-polycation complexes were then diluted 10-fold in culture medium and added onto the HeLa cell monolayers. After 20 h at 37°C in a humidified air-10% CO₂ atmosphere, the transfection medium was replaced by regular culture medium. Three days post-transfection, cell lysates were prepared for measuring luciferase activity (see main text).

2.7.2 Intracellular HA tag-specific staining and flow cytometry

To ascertain that the transduction levels of cells expressing the various TF-encoding genes were similar, we employed the LVs LV.MyoCD::HA.eGFP, LV.MyoD::HA.eGFP and LV.MyoDCD::HA.eGFP encoding HA epitope-tagged versions of MyoCD, MyoD and MyoDCD, respectively. Expression of each of these proteins in target cells was measured by flow cytometry as detailed below. Suspensions of hMSCs transduced with each of the vectors were fixed for 10 min at 37°C in 400 μl of phosphate-buffered 4% formaldehyde solution (pH 7.0-7.4; KLINIPATH) and, subsequently, chilled on ice for 1 min. After being spun down for 5 min at 400 g and 4°C, the cells were resuspended in 400 μl of ice-cold 90% methanol and incubated on ice for 30 min while being gently swirled every 5-10 min. Next, aliquots containing $5\text{--}10 \times 10^4$ cells were diluted in 4 ml of phosphate-buffered saline (PBS)-0.1% bovine serum albumin (BSA), collected by centrifugation for 5 min at 400 g and 4°C and then incubated for 1 h at room temperature with a 1:20 dilution of AlexaFluor 647-conjugated anti-HA mouse monoclonal antibody HA-Tag (clone 6E2; Cell Signaling Technology) in PBS-0.1% BSA. Parallel samples to which no antibody was added served as negative controls. The cell suspensions were gently swirled every 10-15 min. After two consecutive washing steps consisting of adding 4 ml of PBS-0.1% BSA followed by 5-min centrifugations at 400 g and 4°C, the cells were resuspended in 250 μl of PBS-0.1% BSA and kept on ice until flow cytometric analysis for eGFP- and HA-specific fluorescence.

2.7.3 Quantification of MHCK7 promoter activity in haMSCs

Thirty thousand haMSCs were incubated overnight in wells of 24-well plates with LV.PRE.pA+.Luc or LV.PRE.pA+.MHCK7.Luc at a multiplicity of infection (MOI) of 30 TU/cell. Mocktransduced haMSCs served as controls. The resulting haMSCLuc and haMSCMHCK7.Luc populations were amplified by sub-culturing. Subsequently, the cells were incubated with LV.LacZ.eGFP, LV.MyoD::HA.eGFP or LV.MyoDCD::HA.eGFP at an MOI of 25 HTU/cell and cultured in growth medium for 6 days. After another 6-day culture period in differentiation medium, cell lysates were prepared for measuring PpLuc activity. Mocktransduced haMSCs subjected to the same culture regimen served as negative control.

2.7.4 Quantification of CK8 promoter activity in human myocytes before and after differentiation

DMD myoblasts were seeded in wells of 24-well plates at a density of 2×10^5 cells per well. The following day these cells were transduced in duplicate with LVs carrying a promoterless, a human CMV promoter-driven or a CK8 promoter-based PpLuc expression unit at MOIs of 0.5, 2.0 and 8.0 TU/cell. At 48 h post-infection, the cells were rinsed once with 2 ml of PBS after which the cells in one sample of each pair of cell cultures were trypsinized and stored at -20°C whilst the cells in the other sample were exposed to differentiation medium for 4 days prior to trypsinization and storage at -20°C . After thawing and lysis of the cells, chemiluminescence measurements were performed.

2.7.5 Establishment and deployment of hfMSCCK8. μ Dys cultures

Thirty thousand hfMSCs in wells of 24-well plates were incubated overnight with LV.CK8.coh/ μ Dys at an MOI of 25 TU/cell. After expansion, the resulting hfMSCsCK8. μ Dys were seeded in wells of 24-well plates at a density of 3×10^4 cells/well and were incubated overnight with either LV.MyoD::HA.eGFP or with LV.MyoDCD::HA.eGFP at 25 HTU/cell. Unmodified hfMSCs seeded in parallel were transduced with the same dose of either of the two TF-encoding LVs. Mock-infected hfMSCs and hfMSCsCK8. μ Dys served as negative controls. Four days later, 35×10^4 cells from each of the various cell populations were seeded on glass cover slips in wells of 6-well plates (Greiner BioOne) either alone or together with 2×10^5 DMD myoblasts. After 4 days in their respective growth media, both monocultures and co-cultures were rinsed once with PBS and incubated in differentiation medium. After 4 additional days of culture, the cells were fixed and processed for dystrophin immunofluorescence microscopy as specified in the main text.

