Human mesenchymal stem cells ectopically expressing full-length dystrophin can complement Duchenne muscular dystrophy myotubes by cell fusion

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Received September 12, 2005; Revised and Accepted November 25, 2005

Duchenne muscular dystrophy (DMD) is the most prevalent inheritable muscle disease. It is caused by mutations in the ~2.5-megabase dystrophin (Dys) encoding gene. Therapeutic attempts at DMD have relied on injection of allogeneic Dys-positive myoblasts. The immune rejection of these cells and their limited availability have prompted the search for alternative therapies and sources of myogenic cells. Stem cell-based gene therapy aims to restore tissue function by the transplantation of gene-corrected autologous cells. It depends on (i) the capacity of stem cells to participate in tissue regeneration and (ii) the efficient genetic correction of defective autologous stem cells. We explored the potential of bone marrow-derived human mesenchymal stem cells (hMSCs) genetically modified with the full-length Dys-coding sequence to engage in myogenesis. By tagging hMSCs with enhanced green fluorescent protein (EGFP) or the membrane dye PKH26, we demonstrated that they could participate in myotube formation when cultured together with differentiating human myoblasts. Experiments performed with EGFP-marked hMSCs and DsRed-labeled DMD myoblasts revealed that the EGFP-positive DMD myotubes were also DsRed-positive indicating that hMSCs participate in human myogenesis through cellular fusion. Finally, we showed that hMSCs transduced with a tropism-modified high-capacity hybrid viral vector encoding full-length Dys could complement the genetic defect of DMD myotubes.

INTRODUCTION

The 427 kDa muscle-specific isoform of dystrophin (Dys) bridges the intracellular cytoskeleton of myofibers with a sarcolemma-embedded protein cluster named the Dys-associated glycoprotein complex (DGC) (1). The DGC is in turn linked with the extracellular matrix surrounding muscle fibers. In Dys-defective muscle, this macromolecular continuum is interrupted leading to the breakdown of the sarcolemma by mechanical forces. The discovery that Duchenne muscular dystrophy (DMD) is caused by mutations in the X-linked dystrophin gene (Dys) opened perspectives to find a cure for this lethal muscle-wasting disease. Three main therapeutic approaches are being pursued. These consist of (i) introducing (e.g. via viral or non-viral vectors) or repairing (e.g. via exon skipping or premature stop codon suppression) the genetic message, (ii) transplanting Dys-positive cells (e.g. via allogeneic myoblast transplantation) or (iii) modulating synthesis of an endogenous gene product (e.g. via up-regulation of utrophin gene expression). Perspectives to ameliorate or, ideally, cure DMD will increase by diversifying the therapeutic options. As each strategy has its own set of pros and cons, a way to enrich the portfolio of potential treatments consists of combining positive aspects from different principles. In this context, it will be highly valuable to integrate cell- and gene-based therapies to allow transplantation...
of the patient’s own cells following their genetic correction. To this end, however, further research into cells with myogenic potential and improved gene delivery systems is warranted.

Satellite cells and their myoblast progeny have the intrinsic capacity to repair multinucleated myofibers and constitute a natural reservoir of muscle precursors. In DMD patients, these Dys-defective cells are eventually exhausted because of the continuous cycles of degeneration and regeneration. Therefore, allogeneic Dys-positive myoblasts have been and remain at the forefront of cell-based DMD therapy (2). However, these cells are not without shortcomings. For instance, donor myoblasts are difficult to obtain in large quantities and integrate poorly in the recipient’s muscles. Hence, alternative stem or progenitor cells with myogenic capacity are being investigated (3). To many of those involved, adult stem cells either from muscle or non-muscle origin are preferred over embryonic stem cells because of ethical and safety reasons. Among adult stem cells, human mesenchymal stem cells (hMSCs) found in the bone marrow (BM) as well as in other tissues are attracting much attention as they can differentiate into bone, stroma, cartilage, ligament/tendon and fat. Moreover, hMSCs are easy to harvest and can be expanded ex vivo to clinically relevant numbers while retaining their normal karyotype and differentiation capacity (4–6). Experiments using xenogeneic models showed that hMSCs can also give rise to muscle cells in vitro and in vivo (7–10). In this study, we extended these observations to a context involving human Dys-defective muscle cells and demonstrated that the participation of BM-derived hMSCs in human myogenesis occurs through cellular fusion.

Next, we sought to exploit this process to rescue synthesis of full length (FL) and thus fully functional Dys in DMD muscle cells. As gene carrier, we used dual high-capacity (hc) adenovirus (Ad)—adeno-associated virus (AAV) hybrid vectors (11). Because hMSCs are virtually devoid of the Coxsackie B virus and Ad receptor (CAR), we endowed these vectors with fiber domains from the CAR-independent Ad serotype 50 (Ad50). This genetic retargeting maneuver allowed effective delivery and expression of the FL Dys coding sequence (DysFL) in hMSCs. The resulting genetically modified cells could restore FL dystrophin (DysFL) synthesis in human Dys-defective muscle cells. Overall, these results provide a first proof of concept for the treatment of DMD using genetically engineered hMSCs.

**RESULTS**

**Characterization of BM-derived hMSCs**

hMSCs are defined on the basis of cell surface antigens and in vitro differentiation assays. In particular, hMSCs express certain non-hematopoietic markers and can differentiate along the adipocytic, osteocytic and chondrocytic lineages depending on specific cultures conditions (4–6). The mononuclear adherent BM cells employed in the experiments described here fulfill the defining criteria of hMSCs. First, they can differentiate into adipocytes, osteoblasts (12) and chondrocytes (data not shown). Secondly, the surface antigen profile of these cells, as determined by flow cytometry, matched the immunophenotype ascribed to hMSCs. Specifically, they expressed very high levels of the hyaluro-

![Figure 1. Immunophenotyping of BM-derived hMSCs. Presented are histograms of flow cytometry data of a representative hMSC sample stained with antibodies directed against the surface antigens indicated above each graph. Y-axis, number of events; X-axis, fluorescence intensity; broken lines, specific antibodies; solid lines, control antibodies.](https://academic.oup.com/hmg/article-abstract/15/2/213/596918/1839418)
A non-viral labeling technique involving the red-emitting membrane intercalating dye PKH26 was also applied. After establishment of the mixed cultures, the cells were exposed to myogenic differentiation medium (14). This triggered the DMD myoblasts to coalesce and form multinucleated structures during a 5- to 6-day period. Regardless of the labeling method used, areas of fluorescently labeled syncytia were observed in the resulting cultures (Fig. 2A and B). Controls consisting exclusively of fluorescently marked hMSCs subjected to the same experimental conditions did not contain any multinucleated structures, whereas syncytium formation in cultures of DMD myoblasts occurred to the same extent as in the mixed cultures. To confirm that the fluorescently labeled multinucleated structures (Fig. 3A and B) corresponded to differentiated myotubes, an immunofluorescence assay was performed for the myotube-specific marker fast-twitch skeletal myosin heavy chain (skMHC II) (Fig. 3C and D). The specificity of this assay was attested by the lack of signal in control samples in which the primary but not the secondary antibody was omitted (data not shown) and by the absence of staining in the mononuclear cells (i.e. hMSCs and DMD myoblasts) (Fig. 3D). Merging the EGFP- and skMHC II-specific signals clearly revealed that the pool of mature myotubes consisted of EGFP-negative and EGFP-positive structures (Fig. 3E and F). These results demonstrated that hMSCs can contribute to the generation of human myotubes in the presence of differentiating myoblasts.

**hMSCs contribute to DMD myotube formation through cellular fusion**

We next designed an experiment to test whether hMSCs contribute to the pool of human myotubes by differentiation, fusion or both (Fig. 4). An adherent human primary cell clone from umbilical cord blood (hUCB-B1) known to have limited differentiation potential due to its incapacity to originate adipocytes (data not shown) was tested in parallel. Both cell types were labeled with EGFP whereas the DMD myoblasts could be traced through their marking with a DsRed variant of the *Discosoma* sp. red fluorescent protein (Fig. 4). The results from these experiments are summarized in Fig. 5. Essential for the unambiguous interpretation of these data, the EGFP-specific signal (Fig. 5A) could not be visualized with the filter used to detect the DsRed fluorescence (Fig. 5C). Equally important, the DsRed-specific emission (Fig. 5D) was not detectable with the filter employed to visualize the EGFP fluorescence (Fig. 5B). From the direct fluorescence microscopy analysis of co-cultures of hMSCs and DMD myoblasts kept under myogenic differentiation conditions (Fig. 5E, G and I), we concluded that hMSCs participate in the generation of human myotubes through cellular fusion because EGFP-marked syncytia were also labeled with DsRed (Fig. 5I). The occurrence of doubly labeled structures in the co-cultures was dependent on the induction of myogenic differentiation (data not shown). Parallel analysis of mixed hUCB-B1 and DMD myoblast cultures (Fig. 5F, H and J) revealed that the former cells do not participate in human myotube formation at detectable levels because the EGFP-specific fluorescence remained exclusively associated with mononuclear cells (Fig. 5J). On the basis of the number of EGFP-positive hMSCs added to the cultures, we determined that the frequency of EGFP and skMHC II co-labeled myotubes was 1.62 ± 0.33% (mean ± SD, n = 4).

**Effective delivery and expression of the DysFL in hMSCs by retargeted dual hcAd/AAV hybrid vectors**

The CAR is the primary attachment molecule for human species C Ads such as Ad serotype 5 (Ad5) (15,16). As yet,
recombinant versions of this virus constitute the vast majority of Ad-based vectors. Although robust gene transfer into many different cell types can be achieved with these agents, they have limited efficacy in the virtually CAR-negative hMSCs (Fig. 1) (12,17). Others and we have demonstrated that retargeting first-generation, early region 1 (E1)-deleted, Ad5 vectors by exchanging Ad5 fibers for those from CAR-independent human species B members, such as serotypes 35 and 50, greatly enhances gene transfer into hMSCs (12,17). On the basis of these results, we have altered the tropism of dual hcAd/AAV hybrid vectors (11) by using a helper Ad vector (i.e. Ad.floxed\textsuperscript{C}.F50) that codes for chimeric fibers in which most of the shaft and the complete knob domain from Ad5 is substituted by homologous sequences from Ad50. Use of this helper in combination with a hybrid vector shuttle plasmid containing the two independent expression units Dys\textsuperscript{FL} and DsRed resulted in the production of vector particles equipped with chimeric fibers (dual hcAd/AAV.F50 hybrid vectors) and encoding both DysFL and DsRed (Fig. 6A). The DsRed allowed us to monitor and accurately quantify by direct fluorescence microscopy (Fig. 6B, left panel) and flow cytometry (Fig. 6B, right panel), dual hcAd/AAV.F50 hybrid vector-mediated gene transfer into hMSCs. At 7 days post-infection (p.i.), high-level and widespread DsRed expression was detected in hMSCs exposed to dual hcAd/AAV.F50 hybrid vectors whereas mock-treated cultures did not exhibit significant red fluorescence (Fig. 6B, left panel). Flow cytometry analysis at 7 days p.i. showed a clear dose-dependent increase in the frequency of DsRed-positive cells and revealed that a multiplicity of infection (MOI) of 30 is sufficient to label the vast majority of hMSCs. Parallel cultures of mock- and vector-treated hMSCs were subjected to direct fluorescence microscopy to investigate the route by which hMSCs contribute to the generation of human myotubes. Co-cultures of EGFP-labeled hMSCs or hUCB-B1s and DsRed-marked DMD myoblasts were exposed to myogenic differentiation conditions and the resulting cultures were analyzed by direct fluorescence microscopy. EGFP-positive and DsRed-negative syncytia indicate the differentiation of hMSCs or hUCB-B1s into human myotubes without the involvement of muscle cells (green). The participation of hMSCs or hUCB-B1s in the generation of human myotubes through cellular fusion yields syncytia co-labeled with both reporter proteins (yellow). DsRed-positive and EGFP-negative syncytia arise by conventional myogenesis involving myoblast blending (red), that is, through myoblast–myoblast or myoblast–myotube fusion.
Genetic complementation of DMD myotubes by forced expression of the DysFL in hMSCs

Finally, building on the previously described cell fusion phenomenon and on the efficient ectopic expression of the DysFL in hMSCs after dual hcAd-AAV.F50 hybrid vector-mediated gene delivery, we tested whether the resulting hMSCs could rescue DysFL synthesis in human Dys-defective myotubes. For this purpose, dual hcAd-AAV.F50 hybrid vectors encoding either DsRed and DysFL or a fusion product between EGFP and DysFL (eDYS) were used. These constructions allowed discrimination between endogenous and recombinant DysFL in fixed and living cells. As a control, hMSCs were also labeled with EGFP through Ad5F50 transduction. Like before, mock- or vector-treated cells were incubated together with DMD myoblasts and, after a myotube differentiation period of 6 days, direct fluorescence microscopy and immunofluorescence microscopy were performed on these cultures. The former assay was used to detect DsRed and eDYS, whereas the latter was deployed to identify DysFL-positive cells (Fig. 7). Nuclei were identified by phase-contrast microscopy (Fig. 7A, C, E, G and I) or by Hoechst staining and direct fluorescence microscopy (Fig. 7Q–S). Myotubes in co-cultures initiated with mock-transduced hMSCs exhibited no noticeable fluorescence in the assays used to detect eDYS (Fig. 7B), DsRed (Fig. 7K) and DysFL (Fig. 7N). As previously observed, mixed cultures containing EGFP-labeled hMSCs (Fig. 7D) also harbored EGFP-positive myotubes (Fig. 7F). However, Dys was not detected in these myotubes (data not shown). These results suggested that myogenic reprogramming of hMSC nuclei either did not occur or was not enough to induce the accumulation of endogenous Dys above the background level of our assay.

In co-cultures of DMD myoblasts and hMSCs expressing the DysFL in frame with EGFP (Fig. 7H), the presence of the corresponding gene product in DMD myotubes was demonstrated by direct fluorescence microscopy (Fig. 7J). Although EGFP was located both in the nucleus and in the cytoplasm (Fig. 7D and F), it was excluded from the nucleus when fused to DysFL (Fig. 7H and J). In addition, proper localization of eDYS along the plasma membrane could be discerned (arrow in Fig. 7J). Finally, the feasibility of exploiting forced expression of the recombinant DysFL in hMSCs to complement the genetic defect of DMD myotubes was independently confirmed by the co-detection of DsRed (Fig. 7L and M) and Dys (Fig. 7O and P) in co-cultures started with hMSCs transduced by the two-gene-containing hybrid vector (Fig. 7U and V).

DISCUSSION

The full, non-canonical, multi-lineage potential of adult stem cells and the pathways by which such potential is materialized

**Figure 6.** (A) Genome structure of the dual hcAd/AAV.F50 hybrid vector encoding both the reporter protein DsRed and the therapeutic protein DysFL. The vector genome consists of a tail-to-tail dimer of double-stranded DNA. Shaded boxes, 5′ Ad5 sequence encompassing the origin of replication and packaging signal; solid circles, AAV p5 integration efficiency element; arrowheads named SV, SV40 polyadenylation signal (pA); green boxes, the DysFL; solid boxes with broken arrows, UbiC promoter; arrowheads designated βG, rabbit B-globin pA; red boxes, DsRed ORF; open boxes with broken arrows, EF1α promoter; solid oval, AAV ITR sequences. (B) Light microscopy analysis at 7 days p.i. of mock-infected hMSCs (mock) and of hMSCs infected with the dual hcAd/AAV.F50 hybrid vector depicted in (A) at an MOI of 30 HTU/cell (30) and flow cytometry analysis at 7 days p.i. of hMSCs infected with different doses of the same vector (n = 3). (C) DsRed-direct fluorescence microscopy (upper row) and Dys immunofluorescence microscopy (middle row) at 7 days p.i. of hMSCs that were either mock-infected (mock) or transduced with the DsRed- and DysFL-encoding dual hcAd/AAV.F50 hybrid vector at the dosages specified (i.e. 10, 30 and 100 HTU/cell). To the merged DsRed- and Dys-specific signals the blue fluorescence derived from the nuclei-specific dye Hoechst 33342 was added (bottom row).
(i.e. transdifferentiation or fusion) have been the sources of heated debate in recent years (18). Beyond their scientific aspects, these matters are also relevant for the therapeutic use of these undifferentiated cells. For instance, the exploitation of fusion-derived heterokaryons or transdifferentiated cells is, intuitively, not equally graded in light of a probable higher genetic instability of the former entities. It is reasonable to argue, however, that the hypothetical risks associated with stem cell fusion are less of an issue in repairing inherently multinucleated muscle fibers.

Multipotent hMSCs constitute promising cellular substrates to regenerate tissues that are damaged because of injury or disease by autologous cell-based therapy. The use of genetically modified hMSCs may expand or enhance the potential of these cells. There is a need, however, to (i) better characterize and determine the differentiation capabilities of hMSC subpopulations and (ii) develop gene carriers that can safely and efficiently transduce these cells. Recently, it was shown that hMSCs from cystic fibrosis patients can be genetically corrected via retrovirus vector-mediated CFTR transfer and that almost 10% of hMSCs acquired an epithelial cell phenotype in an ex vivo model of human airway epithelium. No cell fusion was observed in this study (19). We instead found that up to 2% of hMSCs when cultured together with differentiating DMD myoblasts were involved in the formation of DMD myotubes via cellular fusion. This level of hMSC contribution to human myotube generation is similar to that observed in an ex vivo xenogeneic system based on co-cultures of hMSCs and murine C2C12 myoblasts (9). Whether hMSCs directly fuse with DMD muscle cells or first acquire a muscle progenitor phenotype remains to be investigated. Currently, we also do not know whether cellular fusion of hMSCs with muscle cells takes place during the process of syncytium formation or, alternatively, occurs with preformed myotubes. Live cell-imaging techniques may shed some light on this matter.

The fact that a clearly measurable but relatively low frequency of hMSCs contributes to myotube formation may be related with the heterogenous nature of current hMSC preparations. This heterogeneity, most likely, stem from the way these cells are isolated, that is, not by virtue of specific markers but solely on the basis of adherence to plastic supports. The present ex vivo model may constitute a valuable and relatively fast assay to test different hMSC fractions for their myogenic capacity and to evaluate factors that enhance DMD myotube rescue. In this respect, it will be interesting to exploit the large coding capacity of dual hcAd/AAV.F50 hybrid vectors for the co-expression in hMSCs of the DysFL and genes encoding myogenic factors.

Finally, we probed the feasibility of using gene-modified hMSCs for autologous cell therapy of DMD. To this end, we equipped dual hcAd/AAV hybrid vectors encoding DysFL with fiber domains from the CAR-independent Ad50 to bypass the CAR deficiency of hMSCs. These retargeted hc vectors allowed very efficient delivery and expression of the DysFL in hMSCs. Subsequently, we showed that hMSCs ectopically expressing the DysFL can complement the genetic defect of DMD myoblasts. We did not find Dys in hMSCs (Fig. 6C). Interestingly, Dys was also not detected in mosaic DMD myotubes containing wild-type hMSCs that had not been transduced with DysFL-encoding dual

Figure 7. Analysis of co-cultures of DMD myoblasts and unlabeled hMSCs or hMSCs labeled with EGFP, eDYS or DsRed and DysFL at 6 days after induction of myogenic differentiation. Phase-contrast microscopy (A, C, E, G and I) and direct fluorescence microscopy (B, D, F, H and J) of (A and B) unlabeled, (E and F) EGFP-marked or (I and J) eDYS-labeled DMD myotubes and of (C and D) EGFP- or (G and H) eDys-labeled hMSCs. Arrowheads mark the positions of nuclei. Arrows in micrographs I and J indicate the DMD myotube surface and the corresponding plasma membrane-associated eDYS staining, respectively. DsRed-direct fluorescence microscopy (K–M), Dys immuno-fluorescence microscopy (N–P) and Hoechst direct fluorescence microscopy (Q–S) analysis of (K, N, Q, T) one DsRed- and Dys-positive DMD myotube and of (L, M, O, P, R, S, U and V) two DMD myotubes expressing DsRed and DysFL. The DsRed-, Dys- and Hoechst-specific signals were combined in micrographs (T–V). Magnification: ×400.
hcAd/AAV.F50 hybrid vectors. This indicates either no or very modest induction of endogenous Dys synthesis despite the embedding of hMSC nuclei in a muscle cell environment sustained by the action of, among others, myogenic transcription factors. Dys levels increase dramatically during the late stages of the differentiation cascade that originates mature myofibers (20). The possibility remains that myogenic reprogramming of hMSC nuclei within a myotube might have only been partial, that is, early but not late skeletal muscle-specific genes were activated. In this context, it will be interesting to investigate whether the expression of skeletal muscle-specific genes can be induced/enhanced by the treatment of hMSCs with drugs known to modulate the activity of chromatin remodeling factors (e.g. histone deacetylase inhibitors) and/or by forced expression in these cells of genes encoding myogenic regulatory proteins (e.g. MyoD1 and Mrf4). These maneuvers may render skeletal muscle-specific cis-acting control elements located in hMSC genomes more susceptible to their cognate trans-acting factors. Integration of non-muscle cell nuclei in muscle syncytia without the subsequent synthesis of a DGC component has been described earlier. Side population cells from BM of wild-type mice when transplanted into Δ-sarcoglycan-null recipients were shown to be incorporated into myofibers without giving rise to appreciable Δ-sarcoglycan accumulation (21). In contrast, human stem cell populations with characteristics similar to those used in this study but isolated from synovial membrane (8) or adipose tissue (22) gave rise to Dys-positive myofibers after transplantation into the Dys-defective mdx mice. A myriad of factors may have been responsible for the different outcomes of these experiments. What seems to be plain, however, is that to be of any practical value, once fused with DMD muscle cells, unmodified hMSCs have to undergo some level of epigenetic remodeling before Dys synthesis can ensue. Our approach of introducing recombinant DysFL into hMSCs bypasses the need for nuclear reprogramming and, in addition, should allow the use of the patient’s own stem cells, thus minimizing the risk of immunological graft rejection.

We previously demonstrated that in the presence of AAV Rep proteins, dual hcAd/AAV hybrid vector DNA is inserted into a specific locus on human chromosome 19 (11). Here, we deployed a capsid-modified, CAR-independent version of the dual hcAd/AAV hybrid vector system to achieve efficient gene transfer of the DysFL into CAR-negative hMSCs. One of our future goals is to introduce both AAV Rep activities and tropism-modified dual hcAd/AAV hybrid vector DNA into hMSCs. This will allow us to (i) isolate relatively few hMSCs, (ii) insert therapeutic DNA into a defined region of their genome to limit the risk of insertional oncogenesis and (iii) expand the stably transduced cells to obtain a large pool of autologous cells for transplantation purposes. The initial testing of the myoregenerative capacity of gene-modified hMSCs can be performed in immunodeficient animals (e.g. non-obese diabetic/severe-combined immunodeficient and nude/mdx mice) in which muscle regeneration is induced by chemical or physical means. In conclusion, the ease with which hMSCs can be harvested, expanded ex vivo and transduced by dual hcAd/AAV.F50 hybrid vectors, turns them into a credible gene transfer vehicle for DMD if other important issues such as the permanent genetic modification of these cells and their dissemination and functional integration into life-threatening muscles have been addressed.

MATERIALS AND METHODS

Cells

The isolation, culture and differentiation of hMSCs from BM have been specified elsewhere (12). These cells were further characterized by determining their surface antigen profile (Fig. 1). The adherent hUCB-B1 cells were isolated from human umbilical cord blood by the same procedure as hMSCs from BM. Cultures were initiated in DMEM with 30% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen) and 10−7 M dexamethason (Sigma-Aldrich). During subsequent culturing dexamethason was omitted from the medium. The human myoblasts were obtained from a DMD patient and were immortalized with Bmi-1 and TERT by lentivirus vector-mediated transduction (14). These cells preserve a stable diploid karyotype and retain their differentiation capacity upon serial passage. Importantly, the corresponding cultures lack fibroblasts. In contrast, preparations of primary myoblasts are often contaminated with fibroblasts, which tend to overgrow the muscle progenitor cells and may confound the interpretation of experimental results. Their human origin together with the features mentioned earlier makes these Dys-defective human myoblasts an excellent system to test strategies aiming at the rescue of Dys synthesis in a non-xenogeneic context.

DMD myoblasts and hMSCs were seeded separately or together (1:1) at a density of 2–5 × 10^4 cells per 10 cm² in wells of six-well plates (Greiner). These cultures were incubated in standard culture medium for 4–5 days after which DMD myotube differentiation was induced by feeding the cells differentiation medium (14). Large multinucleated myotubes were obtained 5–6 days later.

Immunophenotyping

The surface antigen profile of BM-derived hMSCs was determined by flow cytometry essentially as described earlier (12,23).

Labeling of hMSCs with PKH26

hMSCs were stained with PKH26 (Sigma-Aldrich) at a dilution of 1:250 following the manufacturer’s protocol.

Microscopy and immunostainings

Phase-contrast microscopy, direct fluorescence microscopy and immunofluorescence microscopy were performed with an Olympus IX51 inverse fluorescence microscope. Images were captured by a ColorView II Peltier-cooled CCD camera and were archived using AnalySIS software (Soft-Imaging Systems). Cells were processed for Dys and skMHC II immunostainings as previously described (11) except that the fixation time was extended from 15 to 30 min and 0.1 instead of 1% (v/v) Triton X-100 was applied for permeabilization. Dys was detected using the
carboxy terminus-specific monoclonal antibody NCL-DYS2 (Novocastra) together with the Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes) at 40- and 500-fold dilutions, respectively. The anti-skMHC II antibody (clone MY-32, Sigma-Aldrich) was used in combination with the Alexa Fluor 568 goat anti-mouse secondary antibody (Molecular Probes) at dilutions of 1:200 and 1:500, respectively.

Viral vectors

The production and titration of Ad5F50, an E1-deleted Ad5 vector that harbors an EGFP expression unit and displays chimeric fiber proteins consisting of basal shaft sequences from Ad5 and apical shaft and knob domains from Ad50, have been published elsewhere (13). The fiber modification allows Ad5F50 to very efficiently transduce CAR-negative hMSCs (12). DMD myoblasts were labeled with a VSV-G-pseudotyped DsRed-encoding lentivirus vector. To generate this vector, we used a derivative of the shuttle plasmid pAF2ΔSE (24) in which the simian virus 40 (SV40) promoter and enhanced yellow fluorescent protein ORF was replaced by the human elongation factor 1α gene (EF1α) promoter and the DsRed.T4 ORF (25). The hMSCs and hUCB-Bs were labeled with EGFP using the VSV-G-pseudotyped lentivirus vector CMVPRES (26). Details about lentivirus vector production are available upon request. Dual hcAd/AAV.F50 hybrid vectors encoding eDYS (27) or DsRed and DysFL (Fig. 6A) were obtained as previously described (11) except that the fiber-modified helper Ad.floxedΨ.F50 was used in place of its unmodified counterpart Ad.floxedΨ (28). Ad.floxedΨ.F50 was generated in the same manner as Ad.floxedΨ except that shuttle plasmid pAd.floxedΨ was transfected together with cosmid pWE/Ad.Afl-rlTR.pac.RFib5 instead of cosmid pWE/Ad.Afl-rlTR.pac.RFib5 (13). The lentivirus vector and hybrid vector titers, expressed in terms of HTU/cell in transducing units per ml (HTU/ml), were determined through limiting dilutions on HeLa indicator cells followed by flow cytometry (11). The shuttle plasmid to generate DsRed- and DysFL-encoding hybrid vector particles was built on pAd/AAV.DsRed (11) by inserting a human ubiquitin C gene (UbiC) promoter fragment derived from pUbcR#1 (29) into the unique NruI site preceding the DysFL. Plasmid DNA sequences are available upon request.

Transduction conditions

The lentivirus vector transductions were performed by incubating the cells with vector particles for 4 h at an MOI of 30 HTU/cell in DMEM supplemented with 10% FBS and 8 µg/ml of polybrene (Sigma-Aldrich). Prior to transduction by Ad-based vectors, 2–2.5 × 10⁶ hMSCs were seeded in wells of 24-well plates (Greiner) in standard culture medium consisting of DMEM with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Next, the cells were either mock-infected or incubated with Ad5F50 at an MOI of 30 or 50 infectious units per cell in a total volume of 250 µl. hMSCs were also transduced with dual hcAd/AAV.F50 hybrid vectors encoding either DsRed and DysFL or eDYS at 30 and three HTU/cell, respectively. After 2–3 h, the inocula were aspirated and the cells were thoroughly rinsed with large volumes of PBS to remove non-internalized vector particles. Following a 48 h incubation, the hMSCs were washed twice with PBS and incubated for 7–10 min with 0.05% trypsin solution (Invitrogen) at 37°C. The resulting cell suspensions were diluted in 15 ml of PBS and briefly centrifuged. The cell pellets were resuspended in standard culture medium and the cell numbers were determined. Aliquots of mock- and vector-treated hMSCs served also to establish the frequency of transduced hMSCs by flow cytometry. These frequencies were MOI-dependent and ranged from 64 to 95.7%.

ACKNOWLEDGEMENTS

The authors are indebted to J. Bergelson, D. Anson, B. Glick, L.-M. Houdébène, M. Nenoi, J. Seppen and J. Tremblay for donating the CAR-specific mAb and the plasmids pAF2ΔSE, pDsRed.T4.N1, pEFO, pUbCR#1, pRRLcmvgfpinspresse and pDysE, respectively. D. Trono is gratefully acknowledged for making available the lentivirus vector packaging construct psPAX2 and the immortalized DMD myoblasts. The Princes Beatrix Fonds funded part of this research (MAR04-0222).

Conflict of Interest statement. D. V. holds stock in Crucell NV which may have an interest in part of the vector systems described.

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