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Human Adipose-Derived Mesenchymal Stromal Cells Injected Systemically Into GRMD Dogs Without Immunosuppression Are Able to Reach the Host Muscle and Express Human Dystrophin

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Duchenne muscular dystrophy (DMD), a lethal X-linked disorder, is the most common and severe form of muscular dystrophies, affecting 1 in 3,500 male births. Mutations in the DMD gene lead to the absence of muscle dystrophin and a progressive degeneration of skeletal muscle. The possibility to treat DMD through cell therapy has been widely investigated. We have previously shown that human adipose-derived stromal cells (hASCs) injected systemically in *SJL* mice are able to reach and engraft in the host muscle, express human muscle proteins, and ameliorate the functional performance of injected animals without any immunosuppression. However, before starting clinical trials in humans many questions still need to be addressed in preclinical studies, in particular in larger animal models, when available. The best animal model to address these questions is the golden retriever muscular dystrophy (GRMD) dog that reproduces the full spectrum of human DMD. Affected animals carry a mutation that predicts a premature termination codon in exon 8 and a peptide that is 5% the size of normal dystrophin. These dogs present clinical signs within the first weeks and most of them do not survive beyond age two. Here we show the results of local and intravenous injections of hASCs into GRMD dogs, without immunosuppression. We observed that hASCs injected systemically into the dog cephalic vein are able to reach, engraft, and express human dystrophin in the host GRMD dystrophic muscle up to 6 months after transplantation. Most importantly, we demonstrated that injecting a huge quantity of human mesenchymal cells in a large-animal model, without immunosuppression, is a safe procedure, which may have important applications for future therapy in patients with different forms of muscular dystrophies.

Key words: Human multipotent mesenchymal stromal cells; Xenotransplantation; Muscular dystrophy; Therapy

INTRODUCTION

Progressive muscular dystrophies (PMDs) are a clinically and genetically heterogeneous group of disorders caused by the deficiency or abnormal muscle proteins, resulting in progressive degeneration and loss of skeletal muscle function. As effective treatments for these diseases are still unavailable, they have been widely investigated as possible candidates for stem cell therapy. Among the different forms, the most frequent and severe form of muscular dystrophy is Duchenne muscular dystrophy (DMD), a recessive lethal X-linked disease. The onset is around 3–5 years of age and the clinical course is severe and progressive. Affected boys usually are confined to a wheelchair by 10–12 years of age and without assisted ventilation death due to respiratory or cardiac failure occurs usually before the third decade (6).

The golden retriever muscular dystrophy (GRMD) dogs are the closest animal model to DMD. Affected animals carry a frameshift point mutation that causes the skipping of exon 7 and a premature stop codon, resulting in the absence of dystrophin in their muscles. GRMD dogs and DMD patients have many phenotypic and biochemical similarities, including early progressive muscle degeneration and atrophy, fibrosis, contractures, and elevated serum creatine kinase levels (20). However, differently from the DMD patients, the GRMD dogs show difficulties in swallowing while the loss of ambulation is uncommon. They also show a great clinical variability. Death may occur within the first weeks but is most

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frequent around 1 or 2 years of age as a result of failure of respiratory or cardiac muscles (25).

Adult skeletal muscle is capable of regeneration by activating a population of muscle cells precursors (19). However, the continuous and gradual muscle degeneration in PMDs leads to a depletion of satellite cells and, consequently, the capability to restore the skeletal muscle is lost (9,13). One therapeutic approach to treat tentatively PMDs is to use stem cells as precursors to regenerate muscle fibers or compensate for the defective protein expression.

In the first preclinical trial involving stem cells and GRMD dogs, bone marrow hematopoietic stem cells were transplanted from normal litter mates to immunosuppressed GRMD dogs, but dystrophin expression was not restored (3). Subsequently, Sampaolesi et al. (18) reported that the delivery of normal dog mesoangioblasts to the muscle of dystrophic dogs after intra-arterial injection resulted in the restoration of dystrophin expression. The mesoangioblasts show similarities with human adipose-derived stem cells in size, cell surface protein analysis, proliferation, and differentiation capacity. All transplanted dogs were maintained on steroids as standard treatment and received immunosuppressant drugs, which makes it difficult to evaluate clinical results, since it is known that immunosuppressive and anti-inflammatory drugs can ameliorate the phenotype in muscular dystrophy patients (2). Our group had previously analyzed the result of early systemic delivery of human dental pulp stem cells in GRMD dogs but very few dystrophin-positive-labeled fibers were found in just one injected dog (11).

An abundant and accessible source of stem cells is adipose tissue. Adipose-derived stromal cells (ASCs) have the ability to differentiate into skeletal muscle when in contact with dystrophic muscle cells in vitro (26) and in vivo (4,17,22). Rodriguez et al. (17) reported that local injections of human ASCs (hASCs) into the muscle of *mdx* mice (which lack dystrophin), restored dystrophin expression in the area near to the injection site. However, the *mdx* mice have no evident muscular weakness and therefore are not a good model to assess potential functional effects of stem cell therapy. We have previously shown that hASCs injected systemically in *SJL* mice, a murine model for Limb-Girdle muscular dystrophy type 2B (LGMD2B), were able to reach the host muscle, engraft, express human muscle proteins, and ameliorate their functional performance (23). These results led us to investigate if the hASCs have a comparable behavior in the closest animal model to human DMD, the GRMD dog.

Here we show for the first time the results obtained with systemic transplantation of hASCs, with no immunosuppression, in four affected male littermate GRMD dogs, with a protocol comparable to the previously used in the *SJL* mice model (23). With this study we aimed to assess if hASCs are able to migrate, engraft, and differentiate into muscle cells expressing human dystrophin in a large-animal model. We also analyzed the efficiency of local injections compared to multiple systemic injections and how long we could detect human cells after the last injection.

MATERIALS AND METHODS

All experiments were approved by the research ethics committee of the Biosciences Institute, University of São Paulo. All human samples were obtained after written informed consent from the donors. All researches were carried out in the Human Genome Research Center, and the GRMD Genocão (Genedog) kennel at the Biosciences Institute, University of São Paulo.

hASC Isolation and Expansion

Human adipose tissue was obtained from elective liposuction procedures. Cells were isolated using methods previously described (26). Briefly, the unprocessed lipoaspirate was washed extensively with equal volumes of phosphate-buffered saline (PBS) containing antibiotics (100 U/ml of penicillin and 100 g/ml of streptomycin; Gibco) and then dissociated with 0.075% collagenase (Sigma). Enzyme activity was neutralized with Dulbecco's modified Eagle's media-high glucose (DMEM-HG; Gibco) containing 10% fetal bovine serum (FBS; Gibco). The infranatant was centrifuged at $1,200 \times g$ for 5 min to pellet the cells. The cells from the pellet stromal vascular fraction (SVF) were filtered to remove debris and seeded in tissue culture plates (NUNC) at 1,000–3,500 cells/cm^2 in DMEM-LG (low glucose) 10% FBS. Cultures were washed with PBS 24–48 h after plating to remove unattached cells and fed with fresh media.

The cultures were maintained at 37°C with 5% CO_2 in growth media (GM: DMEM-LG/10% FBS). When they achieved about 70% confluence, the cells were trypsinized (0.025%, TrypLE Express; Gibco) and plated at a density of 5,000/cm^2. Cultures were passaged repeatedly after achieving a density of 70–80% until passage 4. The remaining cells were cryopreserved in cryopreservation media (10% dimethyl sulfoxide, 10% DMEM-LG, 80% FBS), frozen at −80°C in isopropanol-jacked closed container, and stored in liquid nitrogen the next day.

Flow Cytometry

The flow cytometry was performed on Guava Easy Cyte System (Guava Technologies) using a blue laser (488 nm). Cells were pelleted, resuspended in PBS at concentration of 10^5 cells/ml, and stained with saturating

concentration of antibodies. Cells were incubated in the dark for 45 min at room temperature. After incubation, cells were washed three times with PBS and resuspended in 0.25 ml of cold PBS. Cell viability was accessed with Guava ViaCount reagent (Guava Technologies).

hASCs were incubated with the following primary antibodies: human leukocyte antigen–DR-phycoerythrin (HLA-DR-PE), HLA-ABC-fluorescein isothiocyanate (FITC), cluster of differentiation 13 (CD13)-PE, CD29-phycoerythrin cyanine 5 (PECy5), CD31-PE, CD34-peridinin chlorophyll protein complex (PerCP), CD44-FITC, CD45-FITC, CD73, CD90-PE, CD105, and CD117-PE (Becton Dickinson). Unconjugated markers were reacted with anti-mouse PE secondary antibody (Guava Technologies).

Flow cytometer gates were set using unstained cells. Cells were gated by forward scatter to eliminate debris. To eliminate the possible autofluorescence of ASCs, we removed the contribution of unstained cells in the measurement channel. A minimum of 10,000 events was counted for each analysis.

Multilineage Differentiation

Before injections, the cells were analyzed for their capacity to differentiate toward adipogenic, osteogenic, chondrogenic, and myogenic lineages as described in Zuk et al. (26).

Animals

All animals were housed and cared for in the University of São Paulo and genotyped at birth as previously described (10). GRMD dogs were identified by microchips. Animal care and experiments were performed in accordance with animal research ethics committee of the Biosciences Institute, University of São Paulo. A total of seven dogs were used in this study.

Systemic Transplantation

Four 2-month-old male dogs were transplanted and one carrier female was maintained as normal control. To minimize the effects of variability previously observed in GRMD dogs we used animals from the same litter (Fig. 1). Each animal was injected in the cephalic vein with 5×10^7 cells kg^{-1} in 0.1 ml of Hank's buffered salt solution (HBSS). Four animals were injected for 6 months, weekly in the first month and then monthly, receiving a total of nine injections. The cells were injected right after their preparation. One dog (II-8) died at 4 months old, 1 week after the sixth injection, and another (II-11) at 7 months old, 1 week after the eighth injection. The two dogs that are still alive are currently 34 months old.

Local Transplantation

Two female 2-year-old affected dogs, from another litter, received a single injection of 10^7 cells in 0.1 ml of HBSS into the right hind limb biceps femoralis.

Muscle Biopsies

Biceps femoralis biopsy samples were taken from all dogs after hASCs transplantation.

In dogs systemically injected, the muscle biopsies were taken before and after the injected period; DNA and muscle dystrophin were analyzed in autopsied muscle while in the two dogs that are still alive, Yuan and Dolar, muscle biopsies were taken before the hASC transplantation, 2 months, 6 months, and 1 year after the last injection. Samples of biceps femoralis from a normal and GRMD dogs of a comparable age were cryoprotected and frozen in liquid nitrogen and used as controls.

In the two female dogs that received local injections into their right hind limb muscle biopsies from both hind limb biceps femoralis were taken 1 month after being injected, the left hind limb muscle sample was used as noninjected control.

Human DNA Analysis

The DNA was obtained using DNeasy Blood & Tissue Kit (Qiagen). The presence of human DNA in the host samples were evaluated as described in Pelz et al. (16). Centromeric region of human chromosome 7 and the dog dystrophin gene was amplified by PCR (35 cycles, annealing at 59°C). Primers sequences were as follows: human chromosome 7 sense: AGCGATTTGA GGACAATTGC and antisense: CCACCTGAAAATG CCACAGC; dog dystrophin gene (10): sense: CTTAA GGAATGATGGGCATGGG and antisense: ATGCAT AGTTTCTCTATCATGC. The PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Nonsaturated digital images were obtained using an ImageQuant imaging system (GE HealthCare).

Immunofluorescence (IF) and Histology

Muscle samples were frozen in liquid N_2. Sections (7 μm) were used for routine histology and immunohistochemistry. Hematoxylin and eosin stain was carried out for morphology studies. Each sample was analyzed by two different and independent persons in a blind test. Muscle cryosections were fixed in 4% paraformaldehyde in PBS for 20 min at 4°C, permeabilized in 0.05% Triton X-100 in PBS for 5 min. Nonspecific binding was blocked with 10% FBS in PBS for 1 h at room temperature. Muscle sections were incubated with primary antibody overnight at 4°C and with secondary antibody for 1 h at room temperature. The following primary antibodies were used: anti-human-dystrophin Mandys1062C6

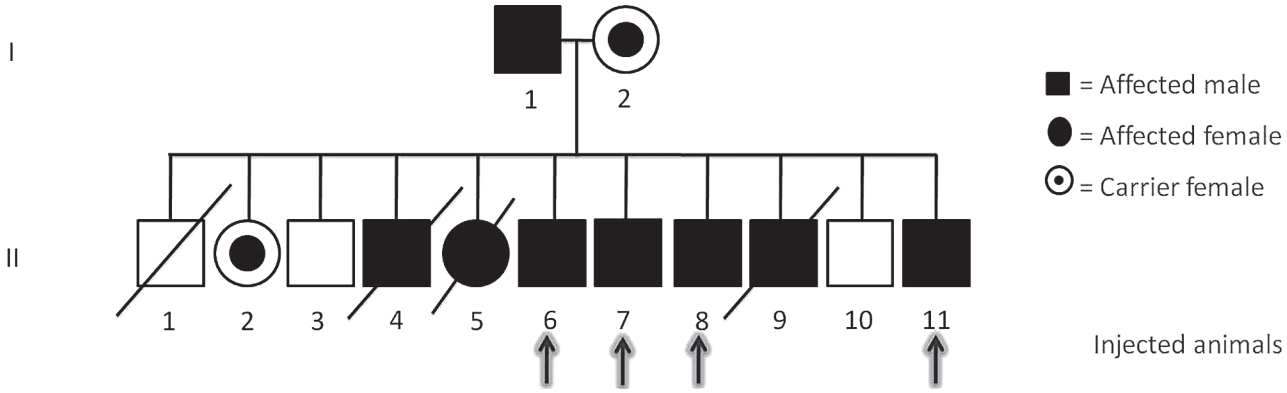

Figure 1. Pedigree of the systemic injected dogs.

(1:100), kindly provided by Dr. Glenn E. Morris at the Center for Inherited Neuromuscular Diseases, Oswestry, Shropshire, UK; anti human-nuclei (1:100; Chemicon) combined with rabbit anti-mouse IgG secondary antibody Cy3-conjugated (1:200; Chemicon) or FITC-conjugated (1:100; Chemicon). We visualized nuclei with 4′,6′-diamidino-2-phenylindole (DAPI; Sigma). The fluorescence signal was examined in Axiovert 200 (Carl Zeiss) and in AxioImager Z1 (Carl Zeiss).

RNA Isolation and RT-PCR

Total RNA was harvested from the human cell-transplanted dog muscle biopsies using Tryzol (Invitrogen) following the manufacturer's instructions. The RNA was treated with DNase (Invitrogen). A total of 1 μg of total RNA was reverse-transcribed with SuperScript™ III First-Strand Synthesis System (Invitrogen). All amplifications were performed in an MJ Research PTC-200 thermocycler (MJ Research) for 30 cycles after the initial 2-min denaturation at 94°C. Primer sequences for human genes (except for dog β-actin) were as follows: myogenin: sense CAGCTCCCTCAACCAGGAG and antisense: CGCTGTGAGAGCTGCATTC; myogenic factor 5 (Myf5) sense: TTTGACAGCATCTACTGTC CTGA and antisense: GAGGTGATCCGGTCCACT ATGT; β-actin: sense: AAGATGACCCAGATCATGT TCG and antisense: GGAGTCCATCACGATGCCAGT. The PCR products were analyzed by electrophoresis of 5 μl aliquots in 2% agarose gel, and the amplicons were visualized by ethidium bromide staining.

Western Blot (WB) Analysis

Muscle sample proteins were extracted through treatment with a buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 60 mM octylglucoside. Samples were centrifuged at $13{,}000 \times g$ for 10 min to remove insoluble debris. Soluble proteins were resolved by 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes (Hybond; Amersham). All membranes were stained with Ponceau (Sigma) to evaluate the amount of loaded proteins. Blots were blocked for 1 h in Tris-buffered saline Tween (TBST) containing 5% powdered skim milk and reacted overnight with the following primary antibody: anti human-dystrophin Mandys1062C6 (1:100). Blots were incubated 1 h with secondary antibodies. Immunoreactive bands were detected with ECL chemiluminescence detection system (GE Healthcare).

Quantitative analysis of human dystrophin at the host muscle was performed by using the ImageJ software (http://rsb.info.nih.gov/ij/), using myosin at the Ponceau staining as a protein loading control.

RESULTS

Characterization of hASCs

hASCs were previously characterized (23) by flow cytometry for the expression of 12 cell surface proteins (HLA-DR, HLA-ABC, CD13, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, and CD117). Cell viability was above 96% by Guava ViaCount reagent (Guava Technologies).

At passage 4, hASCs did not express either endothelial markers [CD31-platelet/endothelial cell adhesion molecule (PECAM1)] or hematopoietic markers (CD34, CD45, and CD117-c-kit). The majority of hASCs expressed high levels of CD13, CD44, adhesion markers [CD29-integrin-β1, CD90-thymocyte differentiation antigen-1 (Thy-1)], and mesenchymal stem cell marker CD73 (ecto-5′-nucleotidase). Expression of some markers, such as CD105 (endoglin), was variable among the donors. hASCs were negative for HLA-class II (HLA-DR), but positive for HLA-class I (HLA-ABC) (data not shown).

The plasticity of hASCs was assessed 3 weeks after lineage induction (23). Myogenic, adipogenic, chondrogenic, and osteogenic differentiation was demonstrated

by the expression of myogenic markers (myosin and desmin), lipid vacuoles, mucopolysaccharide-rich extracellular matrix, and calcium deposits, respectively. These results confirmed the mesenchymal nature of the isolated cells as well as their multipotent potential (data not shown).

hASC Capacity to Reach and Engraft at the Host Muscle

In order to assess the potential of hASCs to reach and colonize the host muscle we injected undifferentiated hASCs, previously characterized by flow cytometry and differentiation potential (23), into the cephalic vein of four dogs. One uninjected GRMD carrier female from the same litter was analyzed as age-matched normal control. Two dogs (Peso, II-8 and Real, II-11) were euthanized due to hiatal hernia. Peso (II-8) died at 4 months old, 1 week after the sixth injection, and Real (II-11) died at 7 months old, 1 week after the eighth injection. The two dogs that are still alive are currently 34 months old. In order to compare the results of systemic injections versus local delivery we injected two homozygous affected GRMD females from another litter at the biceps femoralis.

The PCR method as previously reported by Pelz et al. (16) was used to evaluate the presence of human cells in the biceps femoralis of the systemically and locally injected animals. All systemically injected animals showed human DNA in the biceps femoralis (Fig. 2A). However we did not find human DNA in any of the locally injected muscles (Fig. 2B).

Immunofluorescence analysis with anti-human nuclei antibody also showed a few positive labeled nuclei in systemically injected animals (Fig. 2C).

Expression of Human Muscle Proteins in the Host Muscle

To explore the expression of human muscle proteins after the engraftment of hASCs we analyzed the presence of mRNAs of the following human muscle proteins: myogenin, Myf5, and dystrophin, at the host muscle, by RT-PCR. Human muscle proteins were found in muscle samples of all injected animals (Fig. 3A).

To evaluate the presence of human dystrophin in GRMD host muscles we used a specific human-dystrophin antibody (21). No human DNA was found at the injected muscles following local injections, and as expected, no positive dystrophin bands were observed by WB as well (Fig. 3B). Human dystrophin expression, which was found until 6 months after the last injection, varied from 11% to 19% in the two systemically injected animals that were submitted to the entire protocol of nine injections. Two months after the last injection, dystrophin expression, when compared to a human normal muscle sample, was: 11% in Dolar (II-6) and 16% in Yuan (II-7); and six months after the last injection: 15% in Dolar (II-6) and 19% in Yuan (II-7). No dystrophin was found 1 year after the last injection (Fig. 3C). At the necropsied muscles, Peso (II-8), which received only six injections, showed 6% expression of human dystrophin; Real (II-11), which received eight injections, showed 15% expression of human dystrophin in its necropsied muscle (Fig. 3D), both compared to a normal muscle dystrophin expression.

A modest number of labeled fibers by immunofluorescence (IF) analysis in the injected animals using the anti-human dystrophin antibody was also observed, which are compatible with the WB results (Fig. 3E).

Histopathological Analysis

Histopathological analysis of skeletal muscles from injected dogs showed a typical dystrophic pattern including size variation among individual muscle fibers, fiber splitting, small regenerated basophilic fibers, numerous fibers with centrally located myonuclei, and significant connective tissue replacement (Fig. 4).

DISCUSSION

The role of mesenchymal stem cells (MSCs) in clinical applications for progressive muscular dystrophies (PMD) is still controversial. According to some authors these cells would mediate tissue repair through their multilineage differentiation potential, enabling them to replace damaged cells. Others suggest that this mechanism is unlikely and believe that in response to tissue injury, MSCs home to the site of damage and enhance repair through the production of trophic factors. These include growth factors, cytokines, and antioxidants, some of which provide the basis for their capacity to modulate immune responses (7).

Two different authors reported that ASCs are able to differentiate in vivo when injected directly into the muscle of the *mdx* mouse (4,17). According to Rodriguez et al. (17), local injections of hASCs into immunocompetent *mdx* mouse muscle results in a huge dystrophin expression. Subsequently, we reported that hASCs are not rejected when transferred systematically to the *SJL* mice without any immunosuppression. These cells were able to fuse with the host muscle cells, express human skeletal muscle proteins and improve the motor ability of affected animals. Although the *SJL* mice have a mild phenotype we observed functional improvement of the injected animals after 6 months of in vivo treatment, which was encouraging (23).

A significant obstacle in designing cell therapy for PMDs is the necessity to reach the entire body musculature, a problem that cannot be easily overcome unless systemic cell delivery methods of a large number of

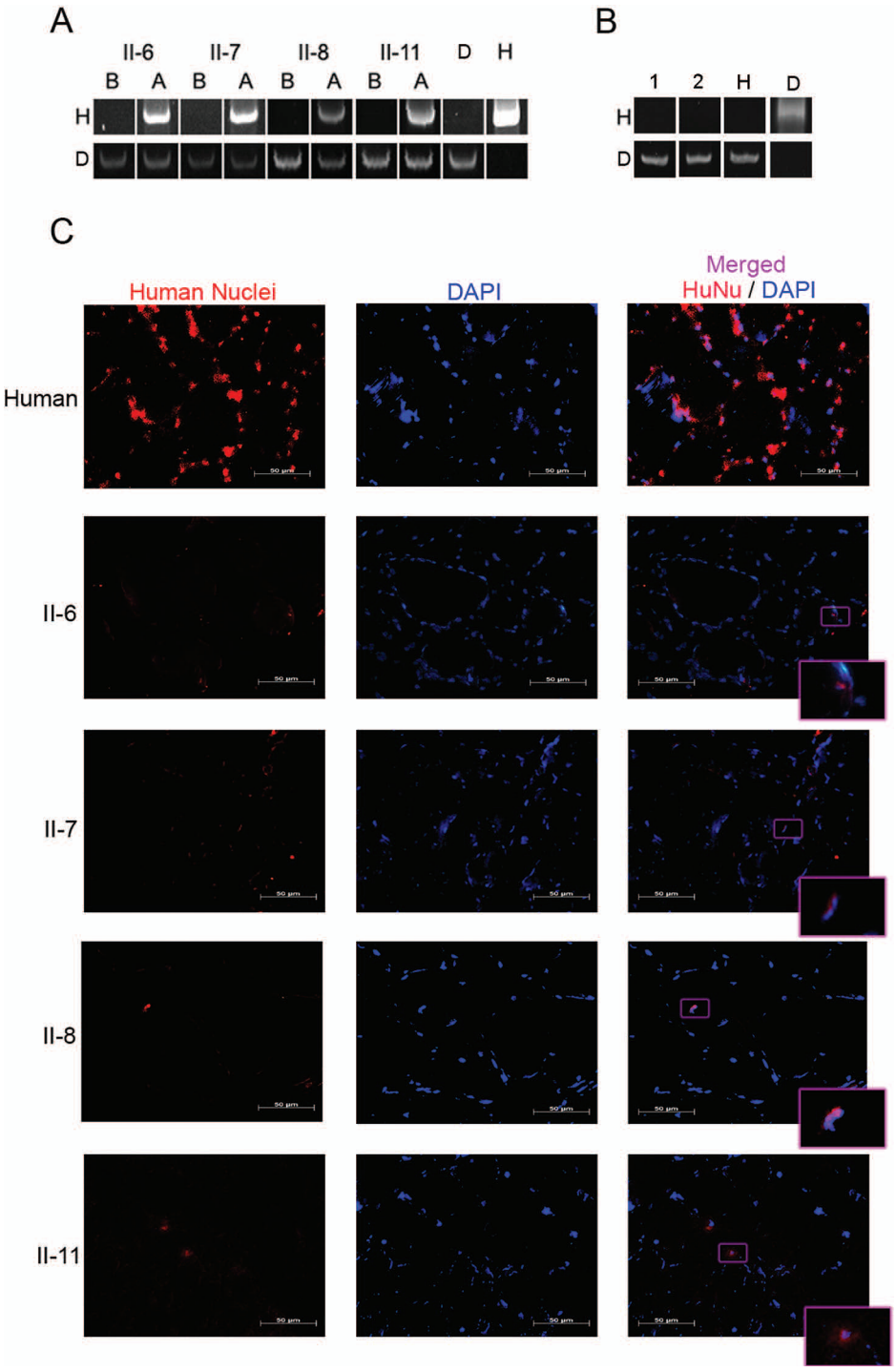

Figure 2. Engraftment of human adipose-derived stromal cells (hASCs) into muscle of golden retriever muscular dystrophy (GRMD) dogs. Polymerase chain reaction analysis for human chromosome 7 alpha-satellite sequences (H) and canine dystrophin gene (D) of biopsied muscle DNA samples. (A) Muscle samples of systemically injected animals B—before and A—after the injections; II-6 and II-8 samples were taken 2 months after the last injection; II-7 and II-11 died before this period. Samples shown are form necropsied muscle: H—human DNA; D—canine DNA. (B) Muscle samples of locally injected animals. Samples: 1 and 2, locally injected females; H, human DNA; D, canine DNA. (C) Immunofluorescence analysis shows scattered human cells into the systemically injected animals muscle (necropsied and 2M) identified by the anti-human nuclei antibody MAB1281. Preparations were counterstained with 4′,6-diamidino-2phenylindole (DAPI). Insets in merged figures (12× main figure) show details of human nuclei. Images were acquired with the same exposure time. Scale bars: 50 μm.

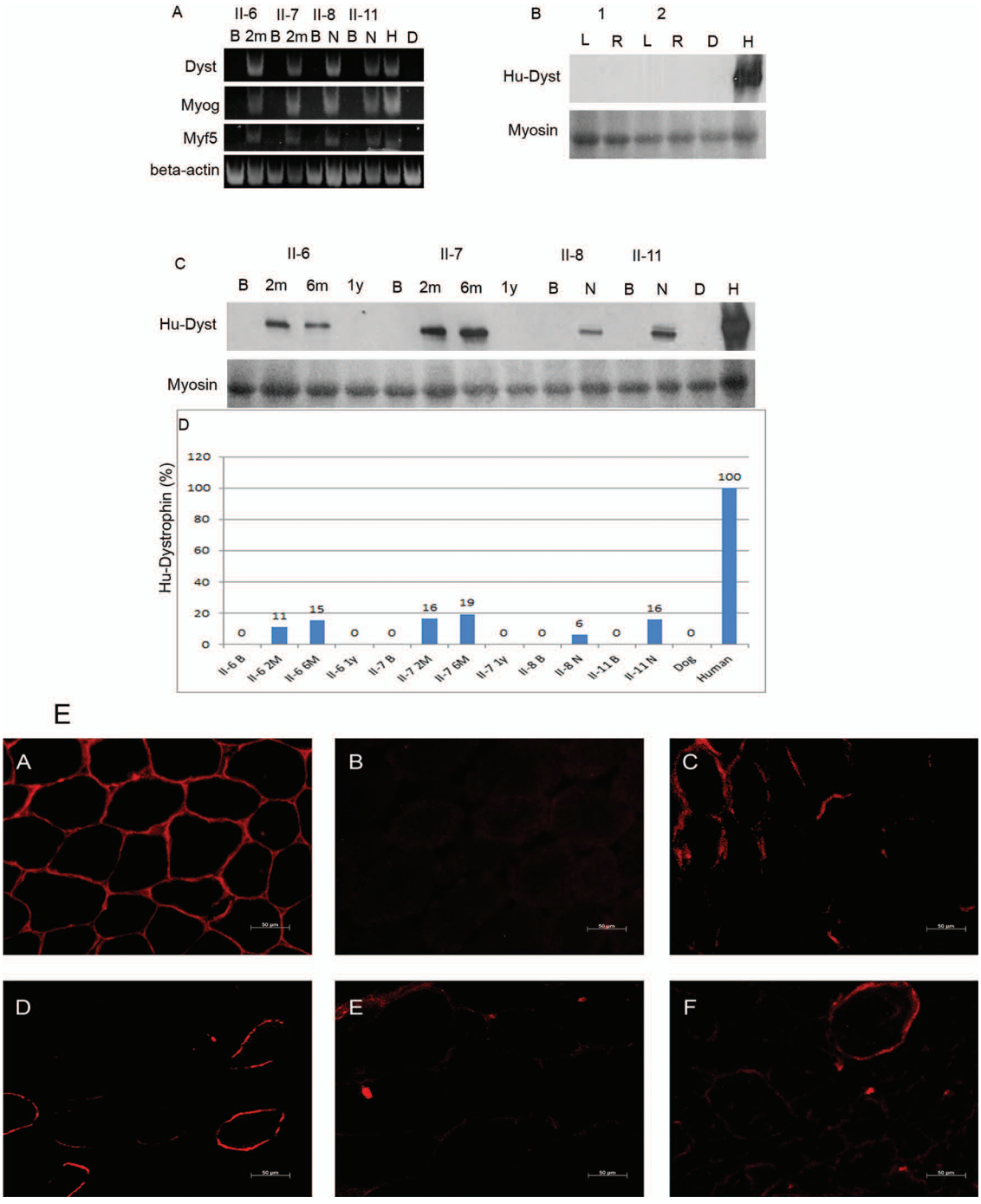

Figure 3. Expression of human muscle protein at the muscles of injected animals. (A) Myogenin, myogenic factor 5 (Myf5), and dystrophin gene expression in the muscle samples of injected dogs. (B) Western blot (WB) of muscle samples from locally injected animals against human dystrophin. (C) Western blot of muscle samples from systemically injected animals against human dystrophin. (D) Quantification of human-dystrophin dog muscle WB samples compared to a normal human muscle sample. Protein samples are the following: B, before; 2m, 2 months after the last injection; 6 m, 6 months after the last injection; 1 y, 1 year after the last injection; N, necropsied muscle; D, normal canine muscle protein; H, human muscle protein; 1 and 2, locally injected females, L, noninjected left hind limb biceps femoralis; R, injected right hind limb biceps femoralis. Myog, myogenin; Hu-Dyst, specific anti-human-dystrophin (Mandys 106 2C6 antibody); myosin, myosin heavy chain band detected in the Ponceau S prestained blot, for the evaluation of loaded muscle proteins. (E) Immunofluorescence analysis using specific anti-human dystrophin antibody of systemic injected animal muscles (necropsied and 2M) and normal human and normal dog muscles as controls; A: human normal muscle, B: canine normal muscle, C: II-6, D: II-7, E: II-8, F: II-11. Scale bars: 50 μm.

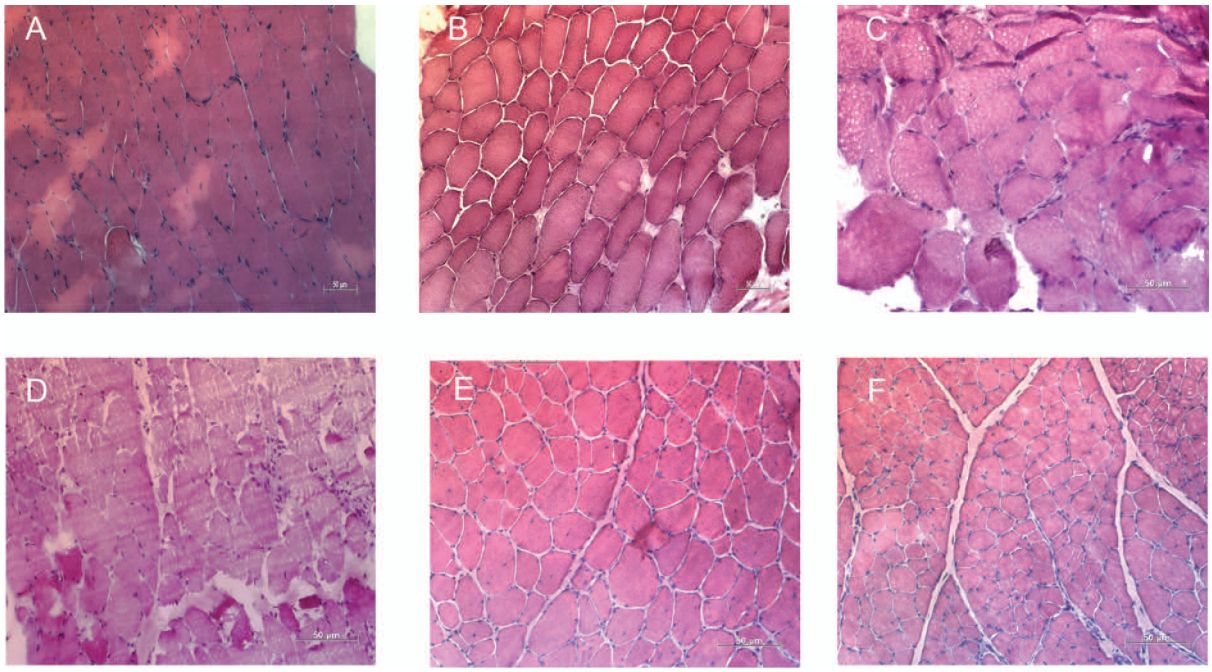

Figure 4. Histopathological analysis of skeletal muscles from injected dogs showed a typical dystrophic pattern including size variation among individual muscle fibers, fiber splitting, small regenerated basophilic fibers, numerous fibers with centrally located myonuclei and significant connective tissue replacement. (A) Human normal muscle, (B) canine normal muscle, (C) II-6, (D) II-7, (E) II-8, (F) II-11. Scale bars: 50 μm.

cells are proved to be effective. The GRMD dog, which has the weight of an 8-year-old child, is the closest animal model for DMD. They develop progressive, fatal disease strikingly similar to human condition in many aspects. Therefore, studies in GRMD dogs are more likely than those in mouse models to predict pathogenesis and treatment outcome in DMD (12). Since autologous cell therapy for DMD requires genetic modification, preclinical studies with heterologous stem cells in a larger animal model for muscular dystrophies are of utmost importance. Therefore, we have repeated the same experiment done with *SJL* mice in GRMD dogs, injecting a comparable quantity of cells based on their weight (5×10^7 cells kg^{-1}).

Here we show for the first time that a huge amount of hASCs delivered systemically into GRMD dogs is able to reach and express human dystrophin in the host muscles, without immunosuppression. Although two dogs died, of hiatal hernia, a frequent cause of death in GRMD dogs (Joe Kornegay, personal communication) most likely unrelated to the stem cell transplantation, human dystrophin was found in their necropsied muscle. In the two dogs that are alive, Yuan and Dollar, muscle biopsies were repeated 2, 6, and 12 months after the last injection. Human dystrophin was not found after 12 months but it was still present 2 and 6 months after the last injections. These dogs are currently 34 months old and healthy.

This result indicates that a huge quantity of hASCs cells can be xenotransplanted systemically, and repeatedly, in nonimmunosuppressed animals without rejection. On the other hand, in contrast to our previous observations in *mdx* mice (17), we observed that hASCs injected locally into the biceps femoralis of two other GRMD dogs, in an amount comparable to what had been injected in mice, were not found in the host muscle after 1 month. One possibility is that local injections might trigger a different reaction against injected cells than systemic delivery. Alternatively, different cell concentrations (and/or injection protocols) may be required to express dystrophin in the GRMD model, since in the case of systemic delivery the dogs received nine injections compared to only one local injection. In any case, this observation suggests that the systemic delivery of hASCs might be more effective than local delivery to reach and engraft into the host muscles, in accordance to our previous results with human dental pulp stem cells (11). It is important to point out that although we did not find human dystrophin 1 year after the last injection, the condition of the two dogs is still stable 27 months after the last injection. These results, if confirmed, suggest that biannual systemic injections would

be required for the maintenance of injected cells in the host muscles if used therapeutically.

Recent reports on the recovery of dystrophin expression and functional impact of stem cell transplantation in PMD animal models have shown different results. We observed an improvement in the performance of the *SJL* mice after the transplantation of hASCs (23) but not after the transplantation of human umbilical cord mesenchymal stromal cells in the same animal model (24). Other researchers reported no functional improvement in the mdx mice after the transplantation of bone marrow mesenchymal stem cells, even after expressing dystrophin (8).

Dystrophin expression following different therapeutic approaches has been used as a marker to assess the presence of exogenous cells in the injected animals. A widespread muscle expression of an AAv9 human minidystrophin vector following intravenous injections in three neonatal dystrophin-deficient dogs was recently reported. At the end of 16 weeks, two out of three dogs showed generalized expression of minidystrophin in 15% to nearly 100% of myofibers. However, although there was a widespread muscle expression of minidystrophin, the affected dogs had pelvic limb girdle muscle atrophy and contractures, apparently associated with an early innate immune response (12). In another study, patients with frameshifting deletions in the DMD gene, who were injected with functional minidystrophin incorporated by recombinant adeno-associated virus (rAAV), showed autoreactive dystrophin-specific T cells. The authors suggest that monitoring of cellular immune responses should be a priority for any experimental therapy in PMDs (14). Different from these two studies, here the injected stem cells carried a normal length human dystrophin gene. The fact that we found a small quantity of human dystrophin, ranging from 6% to 19% through WB and weak IF staining is not surprising taking into account that the procedure involved xenotransplantation in a large-animal model. Also the observation that no human dystrophin was found after 12 months may suggest that hASCs are not replacing the satellite cell niche at the muscle, at least with the injection protocol here used.

Muscles of injected animals did not show any improvement in their histopathological pattern. Since GRMD dogs show a huge phenotypic variability, it is difficult to assess if the transplantation of hASCs had an effect on the course or survival of the injected dogs. The two dogs that died of hiatal hernia, before the end of the protocol, did not have the chance to benefit from the whole procedure. Yuan (II-7) and Dolar (II-6), currently aged 34 months, are being followed in our kennel. Dolar always had a milder phenotype, while Yuan, who was more severely affected, showed an apparent improvement after the end of the protocol. It will be extremely important to repeat this experiment in a larger number of animals that are followed for longer periods to assess if this occurred as a result of cell transplantation, a better clinical management, or due to the natural history associated with a great phenotypic variability in GRMD dogs.

Therapeutic approaches for DMD have analyzed their success based on dystrophin expression at the dystrophic muscle. However, different studies have shown that the rescue of dystrophin expression may not result in clinical improvement (8,12). In addition, it is well known that the *mdx* mice are almost asymptomatic despite the absence of muscle dystrophin. Most importantly, we have reported that a milder phenotype can occur in GRMD dogs despite the complete absence of muscle dystrophin (25), which has also been described in a 7-year-old DMD boy (5) and more recently in Labrador retriever muscular dystrophy (LRMD) dogs (Diane Shelton, personal communication). The fact that is possible to find a milder phenotype in large-animal models and humans indicates that other still unknown factors, in addition to muscle dystrophin, may play a key role in muscle recovery and function.

Muscle degeneration associated with DMD is a complex process in which inflammatory events play a major role in disease progression. hASCs are known to have an enhanced immunosuppressive capacity under inflammatory conditions, without losing their differentiation capacity (1). A growing body of evidence suggests that MSCs have the capacity to modulate immune responses via direct and indirect interactions with a broad range of cell types (7,15). These observations indicate that the success of therapeutic trials should not be based solely on the molecular analysis to assess the rescue of the defective muscle protein expression. Long-term follow-up is mandatory before reaching any conclusions. Further in vivo studies, which are currently under way, aiming to elucidate the immunologic effects of hASC transplantation and dystrophin expression will be essential before starting any therapeutic trial in DMD patients.

In short, here we show for the first time that undifferentiated hASCs are not rejected when systematically administered intravenously to the GRMD dog without immunosuppression, and are able to differentiate in the host muscle and express human dystrophin for at least 6 months after the last injection. Our results also showed that multiple injections, with intervals of no longer than 6 months, may be required to maintain the presence of exogenous dystrophin. We also observed that no human DNA or dystrophin was found in the host muscles following one local injection.

These results open new avenues for preclinical research that may have important applications for future therapy with the advantages that: a) it may be applicable

to patients affected with different forms of progressive muscular dystrophies, regardless of their specific disease-causing mutation; b) human liposuctioned fat is available in large quantities and hASCs can be easily obtained without any in vitro genetic modification or induction. Although it remains to be seen if allogenic transplantation can also be done in humans without immunosuppression, recent evidence suggests that MSCs have the property to eliminate the requirement of immunosuppressive drugs. Therefore, this simple approach reported here, if confirmed in a larger group of animals, may represent a great step toward clinical application for the future therapy of different forms of progressive muscular dystrophies.

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