termination and selectively inducing ribosomal read-through of premature, but not normal, termination codons, was recently identified using nonsense-containing reporters (Welch et al., 2007). The selectivity of PTC124 for premature termination codons, its oral bioavailability and its pharmacological properties indicate that this drug may have broad clinical potential for the treatment of a large group of genetic disorders with limited or no therapeutic options.

5.2 Modification of mRNA splicing

By inducing the skipping of specific exons during mRNA splicing, antisense compounds against exonic and intronic splicing regulatory sequences were shown to correct the open reading frame of the DMD gene and thus to restore truncated yet functional dystrophin expression *in vitro* (Takeshima et al., 1995). Intravenous infusion of an antisense phosphorothioate oligonucleotide created an in-frame *dystrophin* mRNA via exon skipping in a 10-year-old DMD patient possessing an out-of-frame exon 20 deletion of the *dystrophin* gene (Takeshima et al., 2006). Moreover, the adverse-event profile and local dystrophin-restoring effect of a single intramuscular injection of an antisense 2'-O-methyl phosphorothioate oligonucleotide, PRO051, in patients with DMD were explored (van Deutekom et al., 2007). Four patients received a dose of 0.8 mg of PRO051 in the TA muscle. Each patient showed specific skipping of exon 51 of dystrophin in 64 to 97% of myofibers, without clinically apparent adverse side effects.

The efficacy and toxicity of intravenous injection of stable morpholino phosphorodiamidate (morpholino)-induced exon skipping were tested using $CXMD_J$ dogs, and widespread rescue of dystrophin expression to therapeutic levels was observed (Yokota et al., 2009). Furthermore, a morpholino oligomer with a designed cell-penetrating peptide can efficiently target a mutated *dystrophin* exon in cardiac muscles (Wu et al., 2008).

Long-term benefits can be obtained through the use of viral vectors expressing antisense sequences against regions within *dystrophin* gene. The sustained production of dystrophin at physiological levels in entire groups of muscles as well as the correction of muscular dystrophy were achieved by treatment with exon-skipping AAV1-U7 (Goyenvalle et al., 2004).

5.3 *Ex vivo* gene therapy

Transplantation of genetically corrected autologous myogenic cells is a possible treatment for DMD. Freshly isolated satellite cells transduced with lentiviral vectors expressing micro-dystrophin were transplanted into the TA muscles of *mdx* mice, and these cells efficiently contributed to the regeneration of muscles with micro-dystrophin expression at the sarcolemma (Ikemoto et al., 2007). Mesoangioblasts are vessel-associated stem cells and might be candidates for future stem cell therapy for DMD (Sampaolesi et al., 2006). Intra-arterial delivery of wild-type canine mesoangioblasts resulted in the extensive recovery of *dystrophin* expression, normal muscle morphology and function in the GRMD. Multipotent mesenchymal stromal cells (MSCs) are less immunogenic and have the potential to differentiate and display a myogenic phenotype (Dezawa et al., 2005).

6. Future perspectives

6.1 Pharmacological Intervention

The use of a histone deacetylase (HDAC) inhibitor would likely enhance the utility of rAAV-mediated transduction strategies in the clinic (Okada et al., 2006). In contrast to adenovirus-

mediated transduction, the improved transduction with rAAV induced by the HDAC inhibitor is due to enhanced transgene expression rather than to increased viral entry. The enhanced transduction was proposed to be related to the histone-associated chromatin form of the rAAV concatemer in transduced cells. Since various HDAC inhibitors are currently being tested in clinical trials for many diseases, the use of such agents in rAAV-mediated DMD gene therapy is theoretically and practically reasonable.

6.2 Capsid modification

A DNA shuffling-based approach for developing cell type-specific vectors is an intriguing possibility to achieve altered tropism. Capsid genomes of AAV serotypes 1-9 were randomly reassembled using PCR to generate a chimeric capsid library (Li et al., 2008). A single infectious clone (chimeric-1829) containing genome fragments from AAV1, 2, 8, and 9 was isolated from an integrin minus hamster melanoma cell line previously shown to have low permissiveness to AAV.

7. Conclusion

DMD remains an untreatable genetic disease that severely limits motility and life expectancy in affected children. The systemic delivery of rAAV to transduce truncated dystrophin is predicted to ameliorate the symptoms of DMD patients in the future. To translate gene transduction technologies into clinical practice, development of an effective delivery system with improved vector constructs as well as efficient immunological modulation must be established. A novel protocol that considers all of these issues would help improve the therapeutic benefits of DMD gene therapy.

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Long-term Engraftment of Multipotent Mesenchymal Stromal Cells That Differentiate to Form Myogenic Cells in Dogs With Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is an incurable genetic disease with early mortality. Multipotent mesenchymal stromal cells (MSCs) are of interest because of their ability to differentiate to form myogenic cells *in situ*. In the present study, methods were developed to expand cultures of MSCs and to promote the myogenic differentiation of these cells, which were then used in a new approach for the treatment of DMD. MSC cultures enriched in CD271$^+$ cells grew better than CD271-depleted cultures. The transduction of CD271$^+$ MSCs with MyoD caused myogenic differentiation *in vitro* and the formation of myotubes expressing late myogenic markers. CD271$^+$ MSCs in the myogenic cell lineage transplanted into dog leukocyte antigen (DLA)-identical dogs formed clusters of muscle-like tissue. Intra-arterial injection of the CD271$^+$ MSCs resulted in engraftment at the site of the cardiotoxin (CTX)-injured muscle. Dogs affected by X-linked muscular dystrophy in Japan (CXMD$_J$) treated with an intramuscular injection of CD271$^+$ MSCs similarly developed muscle-like tissue within 8–12 weeks in the absence of immunosuppression. In the newly formed tissues, developmental myosin heavy chain (dMyHC) and dystrophin were upregulated. These findings demonstrate that a cell transplantation strategy using CD271$^+$ MSCs may offer a promising treatment approach for patients with DMD.

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INTRODUCTION

Duchenne muscular dystrophy (DMD) is a severe X-linked muscle disease in which mutations in the gene encoding the cytoskeletal protein dystrophin result in a deficiency of the dystrophin–glycoprotein complex of the sarcolemma.[1,2] The deficiency causes the progressive degeneration of striated muscle, manifesting as muscle weakness and eventual skeletal muscle atrophy.[3,4]

Cell-based therapy for DMD has the potential to restore dystrophin expression and therefore also the muscle parenchyma. The transplantation of normal stem cells, such as hematopoietic stem cells, myoblasts, and muscle-derived stem cells, has been examined as a possible treatment strategy for DMD and as a system to deliver therapeutic recombinant proteins to target muscle tissues.[5–7] Indeed, the intramuscular or intravenous injection of these cells was shown to improve the disease phenotype in the *mdx* mouse model.[8]

Bone marrow stem cells have been shown to participate in the regeneration of injured muscle.[9,10] Bone marrow mesenchymal stem cells (MSCs) are conventionally defined as adherent nonhematopoietic (NH) cells expressing several common cell-surface antigenic markers, such as CD44, CD73, CD90, and CD105, but not the hematopoietic markers CD34 and CD45.[11] After treatment with inducing agents,[12] MSCs can differentiate *in vitro* into adipocytes, chondrocytes, and osteocytes, but also into neurons, hepatocytes, pancreatic islets, and muscle.[13] In *mdx* mice, transplanted adult human MSCs were incorporated into myofibers with the subsequent restoration of dystrophin expression.[14–16] However, no therapeutic strategy using MSCs has proven successful for models of DMD in mid-sized animals, such as the dog. Moreover, while successful cell therapy in canine muscular dystrophy has been demonstrated using heterologous mesoangioblasts,[17] the development of an analogous approach for clinical use in humans has been hindered by the inability to overcome several obstacles, including poor cell survival rates, limited dissemination of injected cells, immune responses to allogeneic cells, the presence of the neotransgene product in dystrophic muscle, and the inability to specifically target the cells, such as to cardiac tissue.[18]

Clinical interest in MSCs in cell therapy applications is based on their anti-inflammatory properties and ability to release cytokines into the surrounding environment, thereby modifying the developmental fate of neighboring cells. Thus, not only can MSCs themselves be induced to differentiate along, *e.g.*, a myogenic pathway, in which they fuse with myotubes and promote the formation of new muscle fibers,[16] but in skeletal muscle, for

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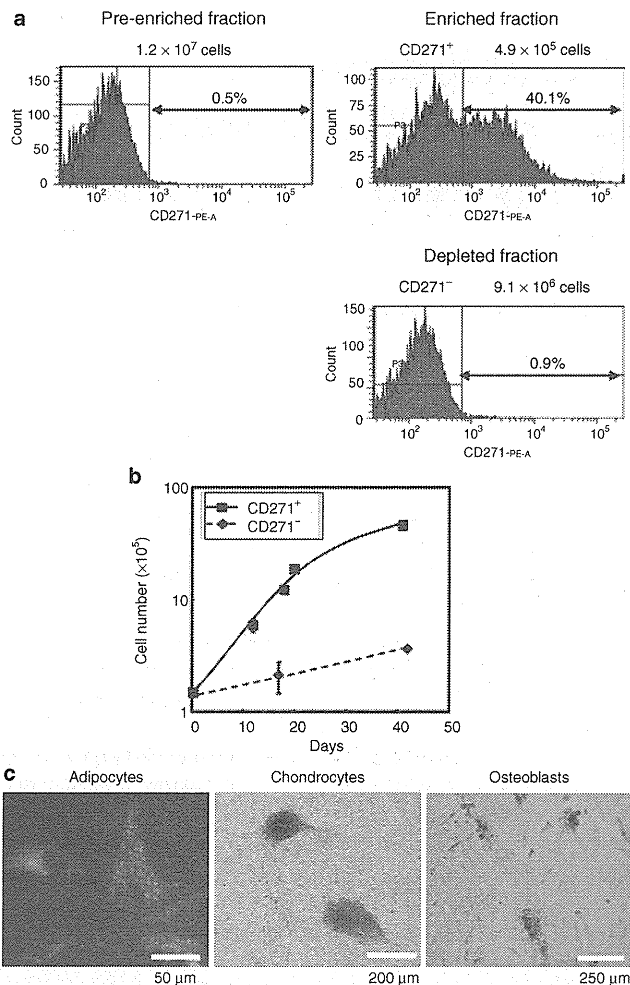

Figure 1 Isolation and expansion of canine CD271$^+$ mesenchymal stromal cells (MSCs) using immunomagnetic isolation. (a) CD271$^+$ cells from bone marrow mononuclear cells were enriched by immunomagnetic isolation using CD271 microbeads. Flow cytometric analysis of the pre-enriched cells and the CD271-enriched (+) and -depleted (−) fractions after separation. The percentages of CD271$^+$ cells are indicated. **(b)** Growth curve of CD271$^+$ and CD271$^-$ cells derived from wild-type dogs. **(c)** The differentiation of CD271$^+$ MSCs obtained from wild-type dogs into adipocytes, chondrocytes, and osteoblasts. Differentiation was confirmed *in vitro* using Nile red staining after culture in adipogenic medium (left panel), toluidine blue staining after chondrogenic induction (middle panel), and alkaline phosphatase activity measurements after osteogenic induction (right panel).

example, they can induce the myogenic differentiation of neighboring satellite cells in the interstitial tissue.[19] Nonetheless, the transplantation of cells already induced to differentiate along the pathway of interest would seem to offer a more direct and readily controllable therapeutic approach than conventional strategies that rely on the innate properties of MSCs to target undifferentiated cell populations. Here, we demonstrate that CD271-enriched (+) MSCs isolated from bone marrow showed improved growth expansion in culture and, as evidenced by MyoD expression, myogenic conversion. CD271 is a marker of progenitor cells and a specific marker for bone-marrow–derived MSCs.[20] Our study describes the development of CD271$^+$ MSC expanded cultures, the myogenic differentiation of these cells, and the methods used to achieve their delivery and effective cell transplantation. Successful engraftment was achieved with CD271-enriched (+) MSCs injected by either the intramuscular or the systemic, intra-arterial route in littermate donor-recipient pairs of dogs with dog leukocyte antigen (DLA) identity[21] (wild-type dogs) as well as in a Beagle with canine X-linked muscular dystrophy in Japan ($CXMD_J$),[22] in which a splice-acceptor site mutation in intron 6 of the *DMD* gene leads to the failed expression of dystrophin.[23] This is the first report of a protocol for the preparation of dog MSCs and the successful long-term engraftment of CD271$^+$ MSCs with a myogenic lineage. This cell-based therapeutic strategy merits further investigation for clinical use in DMD patients.

RESULTS

Isolation and characterization of CD271$^+$ MSCs from canine bone marrow

Canine bone marrow cells were isolated from biopsy specimens from donor Beagle dogs. Since bone marrow MSCs are difficult to expand in culture, they were enriched for CD271$^+$ cells using an immunomagnetic column system (**Figure 1a**) to obtain a subpopulation of cells with better growth potential, which are thus more readily expandable for use in transplantation (**Figure 1b**). Both CD271$^+$ and CD271$^-$ MSCs demonstrated a fibroblast-like morphology after monolayer growth expansion *in vitro* (data not shown). Flow cytometry analysis showed that CD44$^+$ and CD90$^+$ cells were present in the CD271$^+$ fraction, whereas CD45$^+$ cells were limited to the CD271$^-$ fraction (**Supplementary Figure S1a**). We then tested whether CD271$^+$ MSCs were able to differentiate into adipogenic, chondrogenic, and osteogenic cell lineages (**Figure 1c**). Adipogenesis was evidenced by the accumulation of neutral lipid vacuoles that stained with Nile red. Characteristic morphological changes and toluidine blue staining were indicative of chondrogenesis. Osteogenesis was confirmed by an increase in alkaline phosphatase activity. Whereas the CD271 expression on MSCs decreased after 4 weeks in culture (**Supplementary Figure S1b**), these cells retained their ability to differentiate into adipocytes, chondrocytes, and osteoblasts. Similar results were also obtained for dystrophic CD271$^+$ MSCs (**Supplementary Figure S2**), which suggested that CD271-enriched canine MSCs are capable of growth expansion as well as differentiation into multiple cell lineages.

MyoD-mediated myogenic differentiation of canine CD271$^+$ MSCs

CD271$^+$ MSCs from wild-type or DMD dogs were tested for myogenic conversion following their *in vitro* transduction with MyoD, which was transiently expressed from an adenovirus vector (Ad-MyoD). Efficient adenovirus infection requires coxsackie-adenovirus receptor and α(v) integrin. The efficiency of adenovirus-mediated transgene expression[24] is improved by increasing coxsackie-adenovirus receptor expression, which is achieved by treating the transduced cells with the histone deacetylase inhibitor FK228 24 hours before infection.[24] As shown in **Figure 2a**, this resulted in a dose-dependent enhancement of infection, as confirmed in CD271$^+$ MSCs transduced with an adenovirus vector expressing enhanced green fluorescent

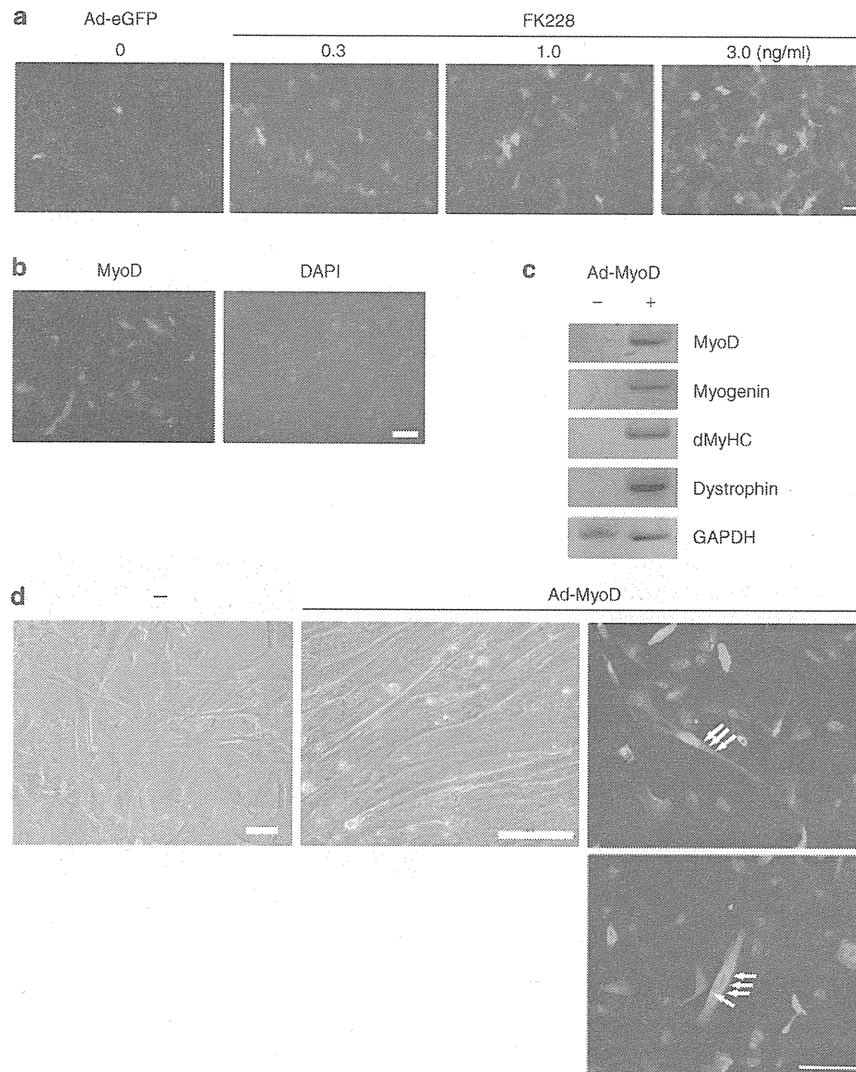

Figure 2 MyoD induces the myogenic differentiation of CD271$^+$ mesenchymal stromal cells (MSCs). (a) Canine CD271$^+$ MSCs were pretreated with FK228 (0.3, 1.0, or 3.0 ng/ml) and then transduced with adenovirus vector expressing enhanced green fluorescent protein (Ad-eGFP). After 7 days of incubation, eGFP$^+$ cells were detected. Bar = 200 μm. (b) CD271$^+$ MSCs were transduced with Ad-MyoD (multiplicity of infection (MOI) = 50), cultured for 3 days and examined for immunofluorescence after staining for MyoD (red); nuclei of the corresponding cells were labeled with 4′, 6′-diamidino-2-phenylindole (DAPI). Bar = 100 μm. (c) CD271$^+$ MSCs transduced with control Ad-null or Ad-MyoD were cultured for 14 days and then analyzed by reverse transcriptase (RT)-PCR using primer-specific MyoD, myogenin, developmental myosin heavy chain (dMyHC), dystrophin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (d) Bright-field images show control Ad-null (left panel) or Ad-MyoD-treated wild-type CD271$^+$ MSCs (middle panel). Images of cells stained for α-actinin immunofluorescence (green) were compared with those of DAPI-stained nuclei (blue) from the same microscopic field (right panels, arrow). Bar = 100 μm.

protein (Ad-eGFP). Indeed, 2 days after Ad-MyoD transduction of CD271$^+$ MSCs in the presence of FK228, >90% of the CD271$^+$ MSCs expressed MyoD in the nucleus, as shown with an anti-MyoD antibody (**Figure 2b**), whereas no MyoD signal was observed in the cells transduced with the control Ad-eGFP vector (data not shown). Several markers of myogenesis, such as myogenin, developmental myosin heavy chain (dMyHC) and dystrophin, were detected in the MyoD-transduced CD271$^+$ MSCs using reverse transcriptase-PCR (**Figure 2c**). After 14 days of culture in differentiation medium, multinucleated myotube formation was noted. These results demonstrated that Ad-MyoD–transduced CD271$^+$ MSCs had undergone myogenic differentiation (**Figure 2d**), suggesting that MyoD is able to initiate myogenesis of CD271$^+$ MSCs.

Transplantation of CD271$^+$ MSCs into DLA-identical dogs by intramuscular injection

To test whether the MyoD-transduced CD271$^+$ MSCs contributed to muscle regeneration, allogeneic CD271$^+$ MSCs were transplanted into DLA-identical donor-recipient pairs of dogs, described in **Table 1**. The donor was a wild-type Beagle and the recipients were wild-type or DMD phenotype $CXMD_J$ littermates. Transplantation was carried out as follows: DLA-identical wild-type dogs were allogeneically transplanted with wild-type CD271$^+$

Table 1 Summary of transplantation experiments

Dog ID	Sex	Age[a]	BW[b]	Transgene	Route[c]	Muscle	Cell number	Engraftment of MSCs	Immunosuppression (mg/kg/day)	Experimental period
3403MN	M	2	7.6	GFP	i.m.	ECU	1×10^6	+	–	10 days
3404MN	M	5	8.5	GFP	i.m.	TA, ECR	2×10^6, 1×10^6	+	–	10 days
3403MN	M	6	9.5	Luciferase	i.m.	TA	2×10^6	+	MMF (30), CSP (25)	4 weeks
3407MA	M	6	8.7	GFP	i.m.	ECR	2×10^6	+	CSP (25)[d]	4 weeks
3407MA	M	9	10.4	Luciferase	i.m.	TA	$4 \times 10^6 \times 2$	++	–	12 weeks
4203FC	F	0.5	0.5	GFP	i.a.	TA	2×10^6	–	–	1 week
3405MN	M	16	11.0	GFP	i.a.	TA	5×10^6	+	MMF (10), CSP (8.3)	4 weeks
5606MN	M	2	4.0	GFP	i.a.	TA	1×10^6	++	MMF (10), CSP (8.3)	8 weeks

Abbreviations: BW, body weight; CSP, cyclosporine; F, female; GFP, green fluorescent protein; M, male; MMF, mycophenolate mofetil; MSC, mesenchymal stromal cell.
[a]Age at injection (months). [b]BW at injection (kg). [c]MSCs were administered by intramuscular (i.m.) or intra-arterial (i.a.) injection; the latter was preceded by transient avascularization. MSC-positive fibers: –, not detected; ±, <10; +, >10; ++, >100. [d]Before transplantation, cyclosporine administration was terminated because of the side effects.

MSCs transduced with MyoD and luciferase into the cardiotoxin (CTX)-injured tibialis anterior (TA) muscle (2×10^6 cells/injection). Eight weeks after transplantation, CD271$^+$ MSC clusters were observed at the injection site of the muscle, as determined using fluorescence microscopy and anti-luciferase antibody. In addition, CD271$^+$ MSCs gave rise to dystrophin-positive fibers (21/4,872 fibers, 0.4%) in the CTX-injured TA muscle (**Figure 3**).

Widespread engraftment of CD271$^+$ MSCs by intra-arterial injection

Next, we examined whether systemic arterial injection of CD271$^+$ MSCs resulted in their ultimate distribution in muscle. As described in **Materials and Methods**, the dogs used in these experiments were administered immunosuppressants, which did not cause any side effects either during or after treatment. The TA muscles of three wild-type dogs were treated with CTX. Five days later, 5×10^8 CD271$^+$ MSCs transduced with MyoD and eGFP were injected into the dogs' femoral artery. For more effective cell engraftment, prior to the injection the artery was treated with papaverine, a clinically used blood vessel relaxant that increases blood flow to the heart and throughout the body. Eight weeks after the transplantation, eGFP$^+$ myofibers (7/216 fibers, 3.2%) derived from the donor were distributed in the TA muscle (**Figure 4a**) and in the heart (**Supplementary Figure S3**). Most of these eGFP$^+$ fibers expressed dMyHC, which is normally transiently re-expressed in regenerated muscle after injury or in response to disorders involving skeletal muscle.[25] This result suggested that the muscle fibers had regenerated following injury. PCR analysis of *eGFP* gene expression in various dog tissues detected donor cells at the injected hindlimb, the right TA and right extensor digitorum longus muscles, and in the heart, but not in the diaphragm, liver, kidney, or lung (**Figure 4b**). In addition, eGFP expression was higher in dogs whose femoral arteries were pretreated with papaverine hydrochloride than in dogs not treated with the drug. Neither ectopic tissue formation nor tumor development occurred after transplantation. There was no increase in acute-phase C-reactive protein levels in the recipient dogs during the experiments, suggesting the absence of a severe inflammatory response (data not shown). Other biochemical tests confirmed normal liver and kidney function and that there was no reaction to the intra-arterial injection of CD271$^+$ MSCs (data not shown). These

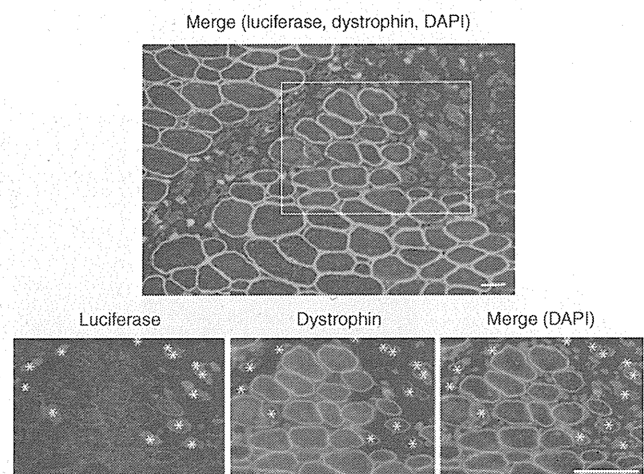

Figure 3 Engraftment of CD271$^+$ mesenchymal stromal cells (MSCs) after intramuscular injection into dog leukocyte antigen (DLA)-identical dogs. CD271$^+$ MSCs obtained from DLA-identical wild-type dogs were transduced with Ad-MyoD and RV-Luc and then injected into cardiotoxin (CTX)-treated tibialis anterior (TA) muscle. Eight weeks after transplantation, the muscles were biopsied. Microscopy cross-sections of the muscles were prepared and then analyzed by immunofluorescence staining with luciferase (red), dystrophin (green), and 4′, 6′-diamidino-2-phenylindole (DAPI) (blue), which revealed luciferase and dystrophin double-positive muscle fibers (arrow and *). High-magnification images (lower panels) were from the boxed region in the upper panel.

results indicate that the intra-arterially injected CD271$^+$ MSCs are capable of long-term survival and widespread tissue engraftment, without adverse consequences for the transplanted animal. Furthermore, successful engraftment of CD271$^+$ MSCs in DLA-identical dogs was achieved not only by the intramuscular but also by the intra-arterial route of injection.

Long-term engraftment and myogenic lineage of CD271$^+$ MSCs in dystrophic dogs without immunosuppression

Based on the encouraging results obtained in wild-type dogs, we asked whether the same results, i.e., MyoD expression, could be obtained in DMD dogs. In addition, due to the well-known side

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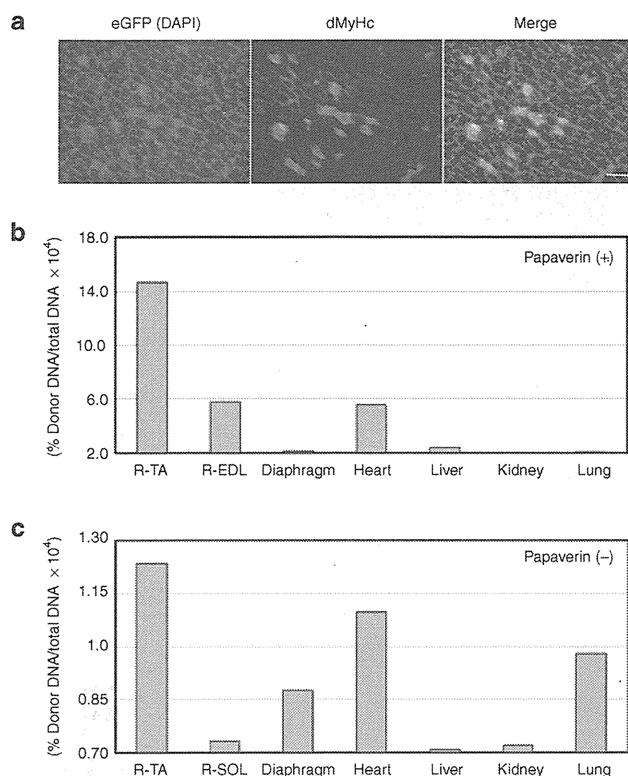

Figure 4 Survival and distribution of CD271$^+$ mesenchymal stromal cells (MSCs) after intra-arterial injection in dog leukocyte antigen (DLA)-identical dogs. CD271$^+$ MSCs obtained from DLA-identical wild-type dogs were transduced with Ad-MyoD and a retroviral vector expressing enhanced green fluorescent protein (eGFP). The femoral artery was transiently avascularized using a tourniquet and then injected with the transduced CD271$^+$ MSCs. (**a**) Cryosections from recipient cardiotoxin (CTX)-treated tibialis anterior (TA) muscle 8 weeks after injection with CD271$^+$ MSCs were stained using antibodies specific for eGFP (red) or developmental myosin heavy chain (dMyHC) (green), and the nuclear stain 4′, 6′-diamidino-2-phenylindole (DAPI) (blue). Bar = 100 µm. (**b**) Two independent experiments were performed in DLA-identical dogs. Cell-transplanted right tibialis anterior (TA) muscle (R-TA), right extensor digitorum longus muscle (R-EDL), right soleus muscle (R-SOL), diaphragm, heart, liver, kidney, and lung were analyzed by reverse transcriptase (RT)-PCR using primers specific for eGFP.

effects associated with the use of immunosuppressants (cyclosporine and mycophenolate mofetil), we decided to transplant the cells into DMD dogs without immunosuppression. CD271$^+$ MSCs transduced with luciferase and MyoD (4×10^6 cells) were injected into the TA muscles of $CXMD_J$ in the absence of immunosuppression. Eight weeks later, numerous clusters of luciferase-positive cells (188/9,408 fibers, 2.0%) with several centrally located nuclei were detected in the TA muscle by immunohistochemistry (**Figure 5a**). The mean diameters of the luciferase-positive cells (8 weeks after transplantation, 33.6 ± 10.7 µm; 12 weeks after transplantation, 47.6 ± 12.0 µm) were smaller than those of the untreated muscle fibers (95.4 ± 23.3 µm). Twelve weeks later, the diameters of the luciferase-positive cells had increased and were larger than observed 8 weeks after transplantation (**Figure 5b**).

To identify whether the smaller cells found in the treated muscles were of a myogenic lineage, dMyHC expression was analyzed. Merged images showed that the luciferase-positive fibers (95% positivity) also expressed the skeletal muscle isoform of dMyHC (**Figure 5a**). Reverse transcriptase-PCR analysis and immunohistochemical experiments demonstrated the expression of myogenin, dMyHC, and dystrophin (**Figure 5c**). Dystrophin expression was also confirmed in wild-type donor-derived cells at the engrafted site, although the number of dystrophin-positive cells was relatively small (**Figure 5d**).

Hematoxylin and eosin staining of the CD271$^+$ MSCs-injected muscles showed cellular infiltrates around degenerating fibers (**Figure 5a**). The infiltrates were also detected using antibodies directed against CD8 and CD11b, indicative of lymphocytes and macrophages, respectively. As shown in **Figure 5e**, small CD8$^+$ and CD11$^+$ T cells accumulated close to the luciferase-positive myofibers. Twelve weeks after engraftment, these accumulations decreased in size compared to those observed in muscle biopsied 8 weeks after transplantation (**Supplementary Figure S4**). Immunoblot analysis failed to detect anti-dystrophin antibodies (**Supplementary Figure S5** and **Supplementary Materials and Methods**), implying that they were not produced in the recipient dogs. Dog sera were also tested for the presence of neutralizing antibody to adenovirus type 5.[26] Transplanted dogs initially negative for neutralizing antibody levels remained negative, with titers <1:4. Mean serum creatine kinase, and C-reactive protein also did not increase during the experiment.

These results demonstrated that transplanted CD271$^+$ MSCs expressing MyoD were converted to myogenic cells and fused with existing muscle fibers, and then formed myotubes in muscle tissue. Moreover, engraftment, differentiation, and the long-term survival of myogenic cells were accomplished by the transplantation of CD271$^+$ MSCs into dystrophic skeletal muscle in the absence of immunosuppression.

DISCUSSION

To develop a new strategy for the cell-based therapy of DMD, we examined methods for the *in vitro* expansion and myogenic differentiation of CD271$^+$ MSCs, using these cells in the allogeneic transplantation of dogs. The enrichment procedure yielded a large population of canine CD271$^+$ MSCs that could be induced to differentiate into myogenic cells following the transient expression of MyoD. Long-term engraftment of CD271$^+$ MSCs within muscle tissues was successfully accomplished by either intramuscular or intra-arterial injection of myogenic cells into DLA-identical dogs.

The effective therapeutic use of MSCs requires their relatively simple large-scale production and a method to induce their differentiation along a myogenic lineage. CD271 provides a marker for the enrichment of non-HSCs from bone marrow aspirates.[20,26] Canine MSCs were obtained from the bone marrow of wild-type or $CXMD_J$ in amounts sufficient for transplantation experiments by enriching the subpopulation of CD271$^+$ cells, as previously reported for humans,[27] under conditions in which the capacity of these cells for multipotent differentiation was maintained. We hypothesized that the CD271-enriched fraction of MSCs would promote allogeneic as well as autologous cells transplantation. CD271 is also known as the low-affinity nerve growth factor receptor/p75, which plays a key role in satellite cell function.[28] Nerve growth factor acts through its low-affinity CD271

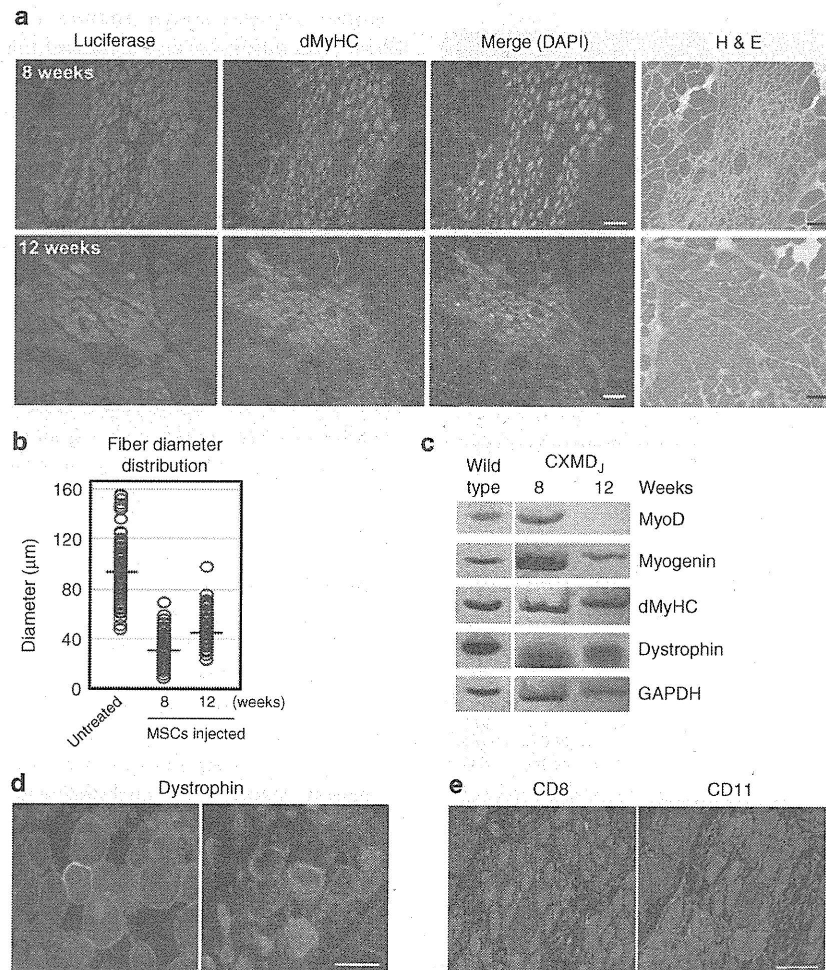

Figure 5 Successful long-term engraftment and myogenic differentiation of CD271$^+$ mesenchymal stromal cells (MSCs) in dog leukocyte antigen (DLA)-identical dystrophic dogs. CD271$^+$ MSCs obtained from DLA-identical wild-type dog were transduced with Ad-MyoD and RV-Luc and then injected into the left and right tibialis anterior (TA) muscles of the nonimmunosuppressed recipient CXMD$_J$ dog. (**a**) Cryosections from the recipient muscle at 8 and 12 weeks after CD271$^+$ MSCs injection were stained using antibodies specific for luciferase (red) and the late myogenic marker developmental myosin heavy chain (dMyHC) (green), or the nuclear stain 4′, 6′-diamidino-2-phenylindole (DAPI) (blue); the images were then merged. Hematoxylin and eosin (H&E) staining of the same field is shown for comparison (right panels). Bar = 100 μm. (**b**) The mean values ± SD of the diameters of CD271$^+$ MSC$^+$ fibers in the recipient muscle 8 and 12 weeks after injection. (**c**) Cell-transplanted muscles were analyzed by reverse transcriptase (RT)-PCR using primers specific for MyoD, myogenin, dMyHC, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and dystrophin. (**d**) Immunofluorescence staining of dystrophin (red) and corresponding DAPI staining are shown. (**e**) Merged images of cells stained for luciferase (red), CD8$^+$ or CD11$^+$ (green), and DAPI (blue) 8 weeks after transplantation.

receptor to induce a developmentally regulated signaling pathway necessary for myogenic differentiation and muscle repair *in vivo*. Therefore, it was presumed that CD271 would enhance the *in vivo* myogenic differentiation of MSCs to form muscle fibers.

Recombinant adenovirus transduction of MSCs usually occurs with low efficiency, which limits its utility. Therefore, improved adenovirus-mediated transduction *in vitro* was achieved by pretreating CD271$^+$ MSCs with the histone deacetylase inhibitor (FK228),[29] which resulted in the efficient expression of MyoD and, subsequently, myogenic differentiation of the cells.

Other agents that induce the differentiation of MSCs, such as 5-azacytidine[30] and Notch I intracellular domain,[16] are known to be injurious to primary cultured cells. Here, we showed that MyoD expression triggers the myogenic differentiation of CD271$^+$ MSCs. These cells maintained their multipotent characteristics, including their myogenic potential, and were successfully used for transplantation in dogs. Furthermore, MyoD transduction of CD271$^+$ MSCs was shown to cause their myogenic differentiation with the induced expression of the late myogenic markers myogenin, dMyHC and dystrophin, although multinucleated myotube formation was less efficient. These results demonstrate that MyoD expression is able to trigger myogenic differentiation of CD271$^+$ MSCs and that, after cell transplantation in muscle tissue, MyoD-transduced MSCs fuse with myoblasts to form the final differentiated myofibers.

MyoD has also been shown to trigger myogenic conversion in other cell types.[31] For example, telomerase gene transfer was used to transduce MyoD into immortalized fibroblasts, which were then engrafted into regenerating muscle, demonstrating that dystrophin rescue is achieved *ex vivo* as well as *in vivo*.[32] However,

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the potential for ectopic tissue formation and cell tumorigenicity remains a concern.

To avoid immune-mediated cell rejection, DLA-identical dogs were transplanted with allogeneic CD271$^+$ MSCs. These MyoD-transduced CD271$^+$ MSCs were able to efficiently engraft and then develop along a myogenic cell lineage in the muscle tissue of the recipient dog. The use of allogeneic cell types generally requires long-term immunosuppression to prevent immune-mediated rejection of the transplanted cells.[33,34] However, in this study, the long-term engraftment of CD271$^+$ MSCs was successful whether or not the DLA-identical dogs were treated with immunosuppressants after transplantation. Although immunohistochemistry revealed several inflammatory infiltrates, mainly containing macrophages, in the recipient muscle of the CXMD$_J$, the majority of CD271$^+$ MSCs migrated into only transient, mild inflammatory foci. The infiltrations were observed at 8 weeks after transplantation but were less abundant after 12 weeks. Our results contrast with those obtained following the transplantation of heterologous mesoangioblasts in golden retrievers with muscular dystrophy, in which the use of immunosuppressants, such as cyclosporine and rapamycin, was required for dystrophin expression.[17] In another study, the intramuscular injection of bone marrow cells in littermate recipients rendered tolerant to the cells did not lead to the production of new myofibers.[35]

The successful induction of dystrophin expression achieved in our experiments may have been due to the anti-inflammatory and immunosuppressive properties of MSCs, which inhibit T-cell proliferation. Mechanistically, MSCs are known to inhibit tumor necrosis factor-α secretion and to promote interleukin-10 secretion, which together may affect the maturation state and functional properties of dendritic cells and skew the immune response towards an anti-inflammatory/tolerant phenotype.[36] Alternatively, the presence of MSCs in an inflammatory microenvironment inhibits interferon-γ secretion from T_H1 and NK cells and increases interleukin-4 secretion from T_H2 cells, thereby promoting a T_H1–T_H2 shift and thus an increase in the proportion of regulatory T-cells. However, once MSCs differentiate into skeletal muscle, they may lose their immunosuppressive properties. Nonetheless, the differentiated cells might be invisible to the host's immune system because of their low-level expression of HLA class I. This would allow the long-term engraftment of CD271$^+$ MSCs without rejection by the DLA-identical CXMD$_J$ recipient following immunosuppressant-free transplantation.

The safety and utility of MSCs underlines their potential clinical benefits. Indeed, the infusion of MSCs has been shown to relieve immune-mediated complications, as reported in patients with severe acute graft versus host disease (GVHD).[37] However, in a phase III trial, a single infusion of MSCs at the time of transplantation did not prevent the development of GVHD in major histocompatibility complex-mismatched donor-recipient pairs. There was also no significant difference in the incidence of GVHD between patients receiving MSCs and the controls.[38] Therefore, further studies aimed at improving the immunosuppressive function of MSCs are needed. In a combined approach, a DMD patient was treated with MSC-like endometrial regenerative cells, CD34 umbilical cord blood, and mixed lymphocyte reaction-matched positive cells, which resulted in improvements in muscular strength and clinical respiratory function.[19] Although these improvements were ascribed to the anti-inflammatory properties of MSCs, our cell transplantation strategy with subsequent myogenic differentiation offers a more direct form of therapy and thus may well be more effective.

In the transplanted CXMD$_J$, most of the CD271$^+$ MSC-derived muscle fibers expressed dMyHC and myogenin. Indeed, MSCs can fuse with dystrophic muscle and produce trophic factors that augment the activity of endogenous myosatellite cells.[19,39] Because myogenin is deeply associated with myotube differentiation, our result suggested that the engrafted cells were committed to differentiate, and some of them were even dystrophin-positive. The infiltration of CD8$^+$ and CD11$^+$ T cells might explain the low number of dystrophin-positive fibers; however, it may be that dystrophin expression and fiber formation by MSCs-derived myotubes is a long-term event. Indeed, dystrophin expression was induced in MSC-like dental pulp stem cells one year after their transplantation in golden retrievers with muscular dystrophy littermates, whereas only a small number of fibers were detected 3 months after transplantation.[40] Thus, our method requires continued studies to determine the efficiency of post-transplantation dystrophin expression by MyoD-transduced CD271$^+$ MSCs. Future studies might also include the modification of the immunosuppressive function of MSCs to further improve engraftment efficiency and differentiation.

The low efficiency of widespread engraftment and differentiation of intramuscularly injected cells is clearly inadequate for the effective treatment of large muscles. To overcome this problem, we devised a strategy in which the intra-arterial injection of cells allowed the widespread engraftment of the CD271$^+$ MSCs in CTX-injured muscle and in the heart. It therefore seems likely that MSCs selectively accumulate in injured muscles,[41] perhaps in response to various chemokine signals, such as stromal cell-derived factor 1 or macrophage/monocyte chemotactic protein-3, both of which are associated with the migration of MSCs into injured tissues.[42] To our knowledge, this is the first report demonstrating the potential for systemic delivery of MSCs to injured tissues in dogs.

In conclusion, the work described herein offers a method to isolate and expand canine bone-marrow–derived CD271$^+$ MSCs *in vitro*. Since MSCs have anti-inflammatory properties, long-term engraftment in dog muscle was facilitated by the intramuscular or intra-arterial injection of CD271$^+$ MSCs into DLA-identical dogs, which obviated the need for immunosuppression. Finally, MyoD expression was able to direct the myogenic differentiation of CD271$^+$ MSCs in dog muscle. Further development of this approach is needed to obtain fully differentiated adult muscle cells from undifferentiated fetal cells.

In addition to their ability to regenerate muscle tissue, MSCs can be used to produce vectors for efficient gene delivery to target tissues.[43,44] For example, MSCs producing recombinant adeno-associated virus (rAAV) can effectively express the gene products at sites of inflammation. In fact, it was also reported that rAAV2 or rAAV6 delivery to the striated muscles of a nonimmunosuppressed canine recipient resulted in insufficient transgene expression but strong immune responses,[45] although Gregorevic *et al.* reported that the administration of rAAV carrying the microdystrophin

gene restored specific muscle force and extended the lifespan of dystrophic mice.[46] Also, dystrophin-specific T cells were detected after the treatment of DMD patients with functional rAAV that encoded dystrophin.[47] MSCs producing rAAV-microdystrophin would be beneficial in autologous cell transplantation for DMD treatment if the immune reactions were inhibited by specifically using immunosuppressive CD271$^+$ MSCs for transduction.

MATERIALS AND METHODS

Production and vector transduction. The canine MyoD genome or eGFP complementary DNA was inserted into the adenoviral vector AVC2.null DNA-protein complex through homologous recombination using an In-Fusion Dry-Down PCR cloning kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions.[48] The resultant recombinant adenovirus vectors (Ad-cMyoD, Ad-eGFP, and Ad-null) were propagated in 293 cells and purified by two-tier CsCl gradient centrifugation. Viral titers were determined based on the 50% tissue culture infective dose ($TCID_{50}$). To generate the vesicular stomatitis virus-glycoprotein (VSV-G)-pseudotyped retroviral vector encoding firefly luciferase (RV-Luc) or eGFP (RV-eGFP), HEK-293 cells were transduced with pDNLuc or pDNeGFP, pGag-pol[49] and pVSV-G.[49] The culture supernatant was collected 48 hours later and centrifuged to enrich the recombinant retrovirus, which was further purified using an ion-exchange procedure.[44] MSCs were transduced with RV-eGFP or RV-Luc and Ad-cMyoD or control Ad-null at a multiplicity of infection of 10 or 50.

Animals. All animals were housed in the National Center of Neurology and Psychiatry (Tokyo, Japan). DLA-identical littermate donor/recipient pairs (n = 6) were matched at DLA class II (DRB-1, DQA-1, DQB-1). Male and female wild-type dogs, 2–16-months-old, were obtained from the Beagle-based $CXMD_J$ breeding colony at the National Center of Neurology and Psychiatry (Tokyo, Japan) and used as recipients or donors for cell transplantation. Age-matched $CXMD_J$ were used as donors for cell transplantation. All of the animals were cared for and treated in accordance with the guidelines approved by the Ethics Committee for the Treatment of Laboratory Animals at National Center of Neurology and Psychiatry which has adopted the three fundamental principles of replacement, reduction, and refinement.

Bone marrow aspiration and isolation of canine MSCs. Donor wild-type dogs were anesthetized with thiopental and isoflurane, and then 1.0 ml of bone marrow fluid was obtained from each humerus by aspiration with a syringe containing 2 ml of RPMI-1640 culture medium (Invitrogen, Carlsbad, CA) with 16 mmol EDTA-2Na. Mononuclear cells were isolated by density centrifugation using Histopaque-1077 (Sigma-Aldrich, St Louis, MO). CD271$^+$ MSCs were enriched and cultivated by using the MSC Research Tool Box-CD271 (LNGFR) containing CD271 (LNGFR)-PE and Anti-PE Micro Beads for cell separation (Miltenyi Biotec, Bergisch Gladbach, Germany). The percentage of CD271$^+$ cells in the enriched and depleted cell fractions was assessed by flow cytometry (FACS Canto II; BD Biosciences, San Jose, CA). Each of the cell fractions was cultured with NH expansion medium (Miltenyi Biotec) supplemented with 100 U penicillin/ml and 100 μg streptomycin (Sigma-Aldrich)/ml and cultured at 37 °C in a humidified 5% CO_2-containing atmosphere.

Differentiation of MSCs. The differentiation of CD271$^+$ MSCs into adipocytes, chondrocytes, and osteoblasts was analyzed *in vitro* after 2 or 4 weeks of culture in NH differentiation medium. To confirm adipogenesis and chondrogenesis, cells cultured for 4 weeks in differentiation medium in 24-well plates (IWAKI, Tokyo, Japan) were fixed in 1% formaldehyde-phosphate-buffered saline (PBS) for 1 hour at 4 °C, permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes, and then washed twice with PBS. The cell monolayers were incubated with 100 ng Nile red (Invitrogen)/μl for 1 hour or toluidine blue for 30 minutes at room temperature, and washed twice with PBS. For osteogenesis, cells that had been cultured for 4 weeks in differentiation medium in 24-well plates were fixed in cold methanol for 5 minutes at −20 °C and then washed twice with dH_2O. The cell monolayers were incubated with 2 ml of alkaline phosphatase BCIP/NBT (Sigma-Aldrich) for 10 minutes at room temperature and washed twice with PBS.

Myogenic differentiation of MSCs. CD271$^+$ MSCs (5.0×10^5–1.0×10^6 cells/well) plated on collagen-coated coverslips (IWAKI) in 24-well plates were exposed to 3.3 ng of the histone deacetylase inhibitor FK228 (Astellas Pharma, Tokyo, Japan) per ml of NH expansion medium for 7 hours at 37 °C. CD271$^+$ MSCs obtained from wild-type dogs were pretreated with FK228 and then cultured for 2 days with the adenovirus vectors (Ad-cMyoD or Ad-eGFP, multiplicity of infection = 25, ref. 50). These cells were washed and maintained in NH expansion medium or DMEM (Invitrogen) containing 2% horse serum, 100 U penicillin/ml, and 100 μg streptomycin/ml for 7–10 days, with a medium change every 3 days.

Characterization of DLA genotyping. DNA from blood sampled from the wild-type, carrier and $CXMD_J$ was extracted using the DNeasy extraction kit (Qiagen, Crawley, UK). DLA class II typing was performed by means of PCR and sequencing for DLA-DRB1, DQA-1, and DQB-1 alleles.[21] DNA sequence-based typing was used to characterize the dogs.

MSCs transplantation experiments. Allogeneic MSCs were transplanted on day 0 of the experiment. Five days earlier, muscle degeneration and regeneration cycles were induced in the TA muscle of wild-type dogs by the injection of 10.5 nmol CTX (Sigma-Aldrich)/kg. Stable MSC transformants obtained from the DLA-identical recipient wild-type dog were established using RV-Luc expressing luciferase. MSCs were transduced with Ad-MyoD (multiplicity of infection = 50); 2 days later, these cells were injected into the extensor carpi ulnaris and TA muscles. Recipient dogs were given 25 or 8.3 mg oral cyclosporine (Sandimmune; Novartis, E. Hannover, NJ)/kg body weight daily from day 5 until the end of the experiments, and 30 or 10 mg mycophenolate mofetil (CellCept; Roche Laboratories, Nutley, NJ)/kg body weight daily from day 0 until the end of the experiments.[50] The same MSCs were also injected into the extensor carpi ulnaris (1×10^6 cells diluted in 1 ml PBS) and TA (2 or 4×10^6 cells diluted in 1 ml PBS) muscles of nonimmunosuppressed $CXMD_J$. The injected muscles were then biopsied at 10 days and 4, 8, and 12 weeks after transplantation, or the animals were sacrificed at the end of the experiment. MyoD and eGFP-transduced MSCs (5×10^6 cells) were also administered into the femoral artery. Five days after the injection of CTX into the TA muscle of recipient wild-type dogs, the femoral artery was subjected to transient avascularization using a tourniquet, followed by the injection of papaverine hydrochloride (0.44 mg/kg). Serum creatine kinase, C-reactive protein, and cyclosporine concentrations were estimated 1 week after initial treatment and at the time of necropsy. The transplanted muscles were biopsied at 2, 4, or 8 weeks after injection. For biopsy and necropsy, the individual muscle was dissected tendon-to-tendon, divided into several pieces and immediately frozen in liquid nitrogen-cooled isopentane. At least 30 sections from the blocks were analyzed. The dogs underwent periodic veterinary examinations during the experiments. Hematological and serum biochemical testing was performed using a semiautomated hematology analyzer (Sysmex Hematology Analyzer F-820; Sysmex, Hyogo, Japan). Serum alanine aminotransferase, alkaline phosphatase, and creatine kinase, C-reactive protein levels were measured using an automated analyzer (DRI-CHEM3506; Fujifilm, Tokyo, Japan).

Histopathology and immunohistochemistry. The biopsied muscles were fixed in 15% neutralized formaldehyde-PBS. Transverse cryosections (10-μm thick) prepared from the MSCs-injected muscles were stained with hematoxylin and eosin using standard procedures. For immunohistochemistry, cells cultured on glass coverslips in 24-well plates were fixed

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in 1% formaldehyde-PBS for 1 hour at 4°C. Thick cryosections (8-μm thick) prepared from MSCs-injected muscles tissues were fixed in 1% paraformaldehyde-PBS for 30 minutes at 4°C. Cell monolayers and tissue sections were permeabilized for 5 minutes in PBS containing 0.1% Triton X-100 and then blocked with 3% bovine serum albumin in PBS. The following antibodies were used for antigen detection at 1:50–1:100 dilutions of rabbit anti-MyoD antibodies (Santa Cruz Biotechnology, Delaware, CA): mouse anti-actinin antibodies (EA-53; Sigma-Aldrich), rabbit anti-firefly luciferase antibodies (ab21176; Abcam, Cambridge, UK), chicken anti-GFP antibodies (AB16901; Millipore, Billerica, MA), mouse anti-dMyHC antibodies (NCL-MHCd; Leica, Heidelberg, Germany), or mouse antidystrophin (NCL-DYS3; Leica). All antibodies were diluted with 0.5% bovine serum albumin in PBS and incubated with the cells or tissue sections for either 1 hour at room temperature or overnight at 4°C. The cells or tissue sections were washed with PBS and then incubated with Alexa 488- or Alexa 568-conjugated anti-mouse IgG antibodies (Invitrogen), Alexa 488- or Alexa 568-conjugated anti-rabbit IgG antibodies (Invitrogen), or Cy3.5-conjugated anti-chicken IgY (IgG) antibodies (ab6962; Abcam) at a 1:500 dilution for 1 hour at 4°C. Coverslips or glass slides were washed with PBS and mounted in Vectashield (Vector Laboratories, Burlingame, CA) with 4′, 6′-diamidino-2-phenylindole. Immunofluorescence was visualized using an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

Reverse transcriptase-PCR. Total RNA (1 μg) from cultured MSCs or muscle samples disrupted in a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) was isolated using the RNeasy Micro kit (Qiagen). First-strand complementary DNA was synthesized using a Super Script III First-Strand Synthesis System for reverse transcriptase-PCR (Invitrogen). From the 20 μl complementary DNA reaction volume, 0.5–2 μl were used for each PCR assay. The primers used in this study were as follows: developmental myosin heavy chain: forward, 5′-gcatcgagtggaccttcattgac-3′, and reverse, 5′-acagtctcattgagagggt ccttg-3′; myogenin: forward, 5′-acgagcggactgagctcagcc-3′, and reverse, 5′-ggtagcggaggtcccgctcctcctggttgag-3′; MyoD: forward, 5′-atggagcttcta tcgccgccac-3′, and reverse, 5′-aggcctcattcactttgctcag-3′; dystrophin: forward, 5′-gattctcctgagctgggtccgac-3′, and reverse, 5′-gccttggcaacatttcca-cttcctg-3′; and eGFP: forward, 5′-gtgagcaagggcgaggag-3′, and reverse, 5′-gtggtgcagatgaacttcagg -3′. As an internal control, a primer set of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase or TATA-binding protein was used. complementary DNA was amplified using an Ampli Taq Gold 360 Master (Applied Biosystems, Tokyo, Japan) and primers, with a total of 40 cycles. PCR products were resolved by agarose gel electrophoresis. The eGFP gene was amplified using SYBR Premix Ex TaqII (Perfect Real Time; Takara Bio) for 60 cycles. Quantitative PCR was carried out using SYBR green detection of PCR products in real time with the MyiQ single-color detection system (Bio-Rad, Hercules, CA).

SUPPLEMENTARY MATERIAL

Figure S1. Flow cytometry analysis of fresh or cultured CD271$^+$ and CD271$^-$ cells.
Figure S2. Characterization of CD271$^+$ MSCs derived from CXMD$_J$.
Figure S3. Immunofluorescence staining of intra-arterially injected CD271$^+$ MSCs engrafted in the heart of a recipient dog.
Figure S4. Immunofluorescence staining of lymphocytes from muscle injected with CD271$^+$ MSCs.
Figure S5. Immunoblotting for the detection of dystrophin antibody.
Materials and Methods.

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RESEARCH ARTICLE

Long-term functional adeno-associated virus-microdystrophin expression in the dystrophic *CXMDj* dog

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Abstract

Background Duchenne muscular dystrophy (DMD) is a severe, inherited, muscle-wasting disorder caused by mutations in the dystrophin gene. Preclinical studies of adeno-associated virus gene therapy for DMD have been described in mouse and dog models of this disease. However, low and transient expression of microdystrophin in dystrophic dogs and a lack of long-term microdystrophin expression associated with a $CD8^+$ T-cell response in DMD patients suggests that the development of improved microdystrophin genes and delivery strategies is essential for successful clinical trials in DMD patients.

Methods We have previously shown the efficiency of mRNA sequence optimization of mouse microdystrophin in ameliorating the pathology of dystrophic *mdx* mice. In the present study, we generated adeno-associated virus (AAV)2/8 vectors expressing an mRNA sequence-optimized canine microdystrophin under the control of a muscle-specific promoter and injected intramuscularly into a single canine X-linked muscular dystrophy (*CXMDj*) dog.

Results Expression of stable and high levels of microdystrophin was observed along with an association of the dystrophin-associated protein complex in intramuscularly injected muscles of a *CXMDj* dog for at least 8 weeks without immune responses. Treated muscles were highly protected from dystrophic damage, with reduced levels of myofiber permeability and central nucleation.

Conclusions The data obtained in the present study suggest that the use of canine-specific and mRNA sequence-optimized microdystrophin genes in conjunction with a muscle-specific promoter results in high and stable levels of microdystrophin expression in a canine model of DMD. This approach will potentially allow the reduction of dosage and contribute towards the development of a safe and effective AAV gene therapy clinical trial protocol for DMD. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords AAV vector; CXMDj; DMD; dystrophic dog; microdystrophin

Introduction

Duchenne muscular dystrophy (DMD) is a severe progressive muscle wasting disease caused by mutations in the dystrophin gene [1,2]. A lack of dystrophin protein decreases the integrity of skeletal myofibers, resulting in increased calcium influx and myofiber necrosis [3]. To prevent muscle degeneration in DMD, gene transfer leading to approximately 30% of the normal expression

levels of dystrophin protein is likely to be required [4]. Therefore, very efficient gene transfer of dystrophin is essential if muscle function is to be restored.

Adeno-associated virus (AAV) vectors are promising candidates for efficient gene transfer into skeletal and cardiac muscle and have been extensively investigated for dystrophin gene transfer [5–8]. However, a number of hurdles exist for the use of AAV vectors in DMD gene therapy, including the packaging capacity of AAV vectors [9] and immunogenicity to the vector and/or transgene [10,11]. To overcome the first hurdle, a number of studies have developed partially deleted, but highly functional microdystrophin genes (3.7–4.5 kb) [5,7,12,13]. These microdystrophin genes can be packaged successfully inside AAV vectors, which can transduce skeletal muscle at high efficiency to rescue the dystrophin-deficient phenotype in animal models of DMD. However, current limitations of a number of microdystrophins in fully restoring muscle function in dystrophic dogs suggest that the development of a novel microdystrophin with improved functionality may be necessary before implementing DMD clinical trials [10,11,14,15].

Second, AAV-mediated gene delivery to muscles of large animal models and DMD patients has been shown to elicit immune responses against the AAV capsid protein or transgene products. Strong immune responses to AAV capsid have been exhibited after intramuscular injection of AAV2 or AAV6 vectors carrying various transgenes in dogs [10], in contrast to successful gene delivery in mouse models [16,17]. After AAV2 encoding β-galactosidase delivery into dog muscles, an immune response against transgene led to the elimination of the transduced myofibers [11]. Recently, Mendell *et al.* [18] reported a Phase I clinical trial on the delivery of an AAV2.5 vector encoding a functional microdystrophin transgene to skeletal muscle in six patients with DMD. Unfortunately, in their study, using a constitutive cytomegalovirus (CMV) promoter and a non-mRNA sequence-optimized version of microdystrophin, there was limited transgene expression and minimal observed clinical benefit, which was postulated to mainly be a result of immune-mediated response fiber elimination. It was suggested that the potential for T-cell immunity to self and nonself dystrophin epitopes should be considered when designing and monitoring experimental therapies for this disease. The present study, which was performed in the *CXMDj* canine model of the disease, takes into account these considerations and suggestions by introducing a muscle-restricted species-specific and mRNA sequence-optimized version of microdystrophin.

We have previously shown that an mRNA sequence optimization of microdystrophin increased the gene expression by up to 30-fold compared to a non-mRNA sequence-optimized microdystrophin in *mdx* mice [13]. In the present study, we aimed to investigate whether the application of a canine-specific and mRNA sequence optimized microdystrophin could improve gene transfer efficiency, leading to extensive muscle transgene expression, by using relatively low titers of AAV vectors in the *CXMDj* dog model. Eight weeks after a single intramuscular injection of AAV2/8 carrying an mRNA sequence-optimized canine microdystrophin gene, transduced muscles showed strong widespread sarcolemmal expression of dystrophin and restoration of dystrophin protein complex (DPC) association at the muscle fibers with a relatively low dose of virus administration. These results demonstrate that species-specific, mRNA sequence-optimized microdystrophin vectors can generate high transgene expression levels and restore normal muscle pathology in a muscular dystrophy dog model without any apparent immune destruction of transduced muscle fibers.

Materials and methods

Construction of AAVITR2-based canine-specific, mRNA sequence-optimized microdystrophin plasmids

The construction of canine-specific, mRNA sequence-optimized cMD1 cDNAs incorporated deletions of rod domains 4–23 and exon 71–78 of the CT domain of dystrophin, containing the last three amino acids of exon 79 of dystrophin followed by three stop codons and incorporating the SV40 poly adenylation site. A murine analog has been recently described [13]. cDNA sequences were modified to include a consensus Kozak sequence. An mRNA sequence was optimized based on transfer RNA frequencies in humans and GC content was increased to promote RNA stability. mRNA sequence optimization of microdystrophin (GENEART, Regensburg, Germany) resulted in the GC content being increased from 48% to 61% in canine microdystrophin and 23.6% of codons being modified in microdystrophin (Figure S1). The size of cMD1 gene cDNA is 3609 bp and the flanking inverted terminal repeat (ITR)-containing transgene cassette size of these vectors is 4427 bp, which corresponds to 94.6% of the 4682 bp of wild-type-AAV2 genome length. We have also introduced a modified optimal Kozak sequence changed from TCAAAATGC to CCACCATGC to improve the initiation of the translation; additionally, 5′- and 3′-untranslated regions of the dystrophin gene were removed to decrease the flanking ITR size of the microdystrophin cassette. Expression was regulated by the muscle-specific synthetic promoter (SPc5-12) [19]. Next, the construct was digested from pGA4 and subcloned into a pAAVITR-based plasmid, which contains the 360 bp muscle-synthetic SPc5-12 promoter, an SV40 polyadenylation signal and the flanking AAV serotype 2 inverted terminal repeats (ITRs).

AAV vector production and titration

The pAAV vector was cotransfected with an AAV8 chimeric helper plasmid encoding the AAV2 rep gene and the AAV8 cap gene (p5E18-VD2/8), and an adenovirus helper

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