Dental pulp stem cells can improve muscle dysfunction in animal models of Duchenne muscular dystrophy

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Abstract

Background: Duchenne muscular dystrophy (DMD) is an inherited progressive disorder that causes skeletal and cardiac muscle deterioration with chronic inflammation. Dental pulp stem cells (DPSCs) are attractive candidates for cell-based strategies for DMD because of their immunosuppressive properties. Therefore, we hypothesized that systemic treatment with DPSCs might show therapeutic benefits as an anti-inflammatory therapy.

Methods: To investigate the potential benefits of DPSC transplantation for DMD, we examined disease progression in a DMD animal model, mdx mice, by comparing them with different systemic treatment conditions. The DPSC-treated model, a canine X-linked muscular dystrophy model in Japan (CXMDJ), which has a severe phenotype similar to that of DMD patients, also underwent comprehensive analysis, including histopathological findings, muscle function, and locomotor activity.

Results: We demonstrated a therapeutic strategy for long-term functional recovery in DMD using repeated DPSC administration. DPSC-treated mdx mice and CXMDJ showed no serious adverse events. MRI findings and muscle histology suggested that DPSC treatment downregulated severe inflammation in DMD muscles and demonstrated a milder phenotype after DPSC treatment. DPSC-treated models showed increased recovery in grip-hand strength and improved tetanic force and home cage activity. Interestingly, maintenance of long-term running capability and stabilized cardiac function was also observed in 1-year-old DPSC-treated CXMDJ.

Conclusions: We developed a novel strategy for the safe and effective transplantation of DPSCs for DMD recovery, which included repeated systemic injection to regulate inflammation at a young age. This is the first report on the efficacy of a systemic DPSC treatment, from which we can propose that DPSCs may play an important role in delaying the DMD disease phenotype.

Keywords: Dental pulp stem cells, Duchenne muscular dystrophy, Anti-inflammatory therapy
**Background**

Duchenne muscular dystrophy (DMD) is a progressive and fatal X-linked recessive inherited skeletal and cardiac muscle disorder. It is the most common muscular dystrophy, affecting 1 in 3500 male births [1]. The dystrophin-glycoprotein complex deficiency of the sarcolemma results from mutations in the dystrophin gene and causes progressive degeneration/regeneration cycles in the striated muscle, manifesting as muscle weakness and eventual skeletal muscle atrophy [2, 3]. DMD is a primary degenerative myopathy with a necrotizing phase with secondary inflammation. Consequently, steroids are widely used to improve muscle strength in DMD patients [4–6]. However, the beneficial effects of steroid therapy, including glucocorticoid administration, vary from patient to patient, and administration of these compounds may have side effects. In principle, severe inflammation regulation in muscle tissues can prolong the duration of therapeutic effects.

Multipotent mesenchymal stromal cells (MSCs) express several common cell-surface antigenic markers, such as CD44, CD73, CD90, and CD105, and low levels of major histocompatibility complex class I molecules, without expressing hematopoietic markers CD34 or CD45 [7]. Although originally identified in the bone marrow [7], MSCs can be extracted from numerous tissues including adipose [8], peripheral blood [9], cord blood [10], and amnion [11]. Dental pulp stem cells (DPSCs) obtained from the deciduous tooth tissue are a less invasive cell source that shows multipotency [12], as well as high proliferative and immunosuppressive activities [13]. DPSCs can also inhibit the proliferation of phytohemagglutinin-stimulated T cells more strongly than BM-MSCs [14].

The main purpose of DMD treatment is to recover motor function by restoring the expression of dystrophin and to delay pathological progression by suppressing inflammation. Cell-based therapy for DMD has the potential to restore dystrophin expression and restore the muscle parenchyma using hematopoietic stem cells, myoblasts, muscle-derived stem cells, and mesangioblasts in DMD model mice or Golden retriever muscular dystrophy (GRMD) [15–18]. We have also reported that BM-MSCs could be implanted into the injured muscles of canine X-linked muscular dystrophy in Japan (CXMD1), a beagle-based dog colony [19], and successfully used for long-term engraftment in the myogenic lineage [20]. However, improvement of whole-body muscle function and long-term therapeutic effects have not been sufficiently demonstrated by cell therapy.

Our clinical interest in DPSCs for therapeutic cellular applications is based on their anti-inflammatory properties. Therefore, systemic DPSC administration is expected to benefit the immune-modulatory effect in dystrophic muscles and has the capacity to ameliorate progressive DMD. Here, we evaluated the efficacy and safety of systemic DPSC treatment for DMD using animal models. CXMD1 shows affected temporalis and limb muscles at a young age, which are comparable to those observed in patients [21–23]. Through experiments using mdx mouse and CXMD1 models, we demonstrate a therapeutic strategy for long-term functional recovery in DMD using repeated DPSC administration.

**Materials and methods**

**Animals**

C57BL/6-background mdx mice, which show phenotypes similar to C57BL/10 mdx mice [24, 25], were a generous gift from Dr. T. Sasaoka (National Institute for Basic Biology). C57BL/6 (WT) mice were purchased from Nihon CLEA (Tokyo, Japan). All experiments using mice were performed in accordance with the guidelines approved by the Nippon Medical School and National Center of Neurology and Psychiatry (NCNP) Animal Ethics Committees. Beagle-based CXMD1 colony dogs were bred and housed at NCNP [21]. CXMD1 used for cell transplantation and healthy Beagle dogs as controls were cared for and treated in accordance with the guidelines approved by the Ethics Committee for the Treatment of Laboratory Animals at NCNP.

**Cell preparation**

Pluripotent stem cell-enriched human dental pulp-derived cells (hDPSCs) were provided by JCR Pharmaceuticals (Hyogo, Japan). The cells were cultured in Dulbecco’s modified Eagle medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% antibiotic-antimycotic solution (FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) at 37°C in an atmosphere containing 5% CO₂.

**Systemic delivery procedure**

Systemic delivery of hDPSCs into mdx mice was conducted using four injections with an interval of 1 week between injection doses of 1.0 × 10⁶ cells (high dose) or 5.0 × 10⁵ cells (low dose) starting at 4–5 weeks of age (body weight (BW) > 10 g). Age-matched mice were used as controls for the experiments. The experiments using CXMD1 were performed using hDPSC administration in the acute phase at 2–3 months of age (Table 1). Polaramine (chlorpheniramine maleate, 0.15 mg/kg, MSD) pre-treated CXMD1 (three subjects) were intravenously injected with hDPSCs (4.0 × 10⁵ cells/kg/week) at a rate of 1 mL/min. Four injections at weekly intervals were performed as the first course, followed by four injections carried out as the second course after 8–13 weeks.
Littermate CXMD\(_1\) were untreated controls that were injected with saline at identical time intervals.

**Biodistribution of hDPSCs**

DNA extractions were performed on tissue suspensions using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) and quantified using a spectrophotometer (NanoDrop; Thermos Fisher Scientific). Real-time quantitative PCR was performed using the DNA Master SYBR Green I kit (Roche Diagnostics, Basel, Switzerland) and primers for the human Alu site. The primer sequences used were as follows: 5′-GTCAGGAGATCGAGACCATCCC-3′ (forward) and 5′-TCCTGCCTCAGCCTCCCAAG-3′ (reverse). PCR conditions were as follows: 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, 68 °C for 30 s, and then 72 °C for 30 s. Standards were generated by adding 10-fold serial dilutions of hDPSCs to determine the concentration of hDPSCs in genomic DNA.

**Blood tests**

Hematological and serum biochemical testing were performed using a semiautomatic hematology analyzer (Sysmex Hematology Analyzer F-820; Sysmex, Hyogo, Japan). Serum alkaline phosphatase (ALP), aspartate transferase (AST), and blood urea nitrogen (BUN) levels were measured using an automated analyzer (DRICHEM3506; Fuji Film Medical, Tokyo, Japan). C-reactive protein (CRP) levels were measured using a colormetric assay with an FDC3500 clinical biochemistry analyzer.

**ELISA**

The serum IL-6 levels were determined using a Quantikine ELISA mouse kit (R&D Systems, Minneapolis, MN). A canine IL-6 immunoassay (R&D Systems) was carried out according to the manufacturer’s recommendations.

**Grip strength**

Forelimb grip strength was measured using a grip strength meter (MK-380 M; Muromachi Kikai Co., Ltd., Tokyo, Japan) as previously described [26]. Five trials were performed with a resting period of 5 s between trials. The average tension force (g) was calculated from 3 highest measurements for each group of mice.

**Analysis of locomotor activity**

Physiological mouse activity was analyzed in each cage with a computerized wheel system (dual activity monitor system, SHINFACTORY Co., Ltd., Fukuoka, Japan) by counting the number of wheel revolutions during each 5 min interval using ACTIMO-DATA II software [27]. The activity of dogs was monitored and counted using an infrared sensor system (Supermex, Muromachi Kikai) as previously described [28]. The average daily locomotor activity shown by the dogs over 5 days and nights (12 h light/dark cycles) was calculated. We also compared the 15-m running time of CXMD\(_1\) during the experimental period. The running speed was averaged from four measurements. To determine the acceleration parameter, we used portable wireless hybrid sensors (TSND121; ATR-Promotions Inc., Kyoto, Japan) on the thoracic and lumbar regions of the dogs, as described previously [29]. The acceleration magnitude (AM) was calculated from the three acceleration vectors (\(Ax\), \(Ay\), \(Az\)) as the square root of the sum of the three-axial values (\(AM = \sqrt{Ax^2 + Ay^2 + Az^2}\)) [30] and was averaged for each trial. The relative components of the AM along the three axes (%) were calculated by dividing the absolute values of each axis by the AM [31], and these components that were averaged in each trial were calculated as acceleration ratios (\(Ax\) ratio, \(Ay\) ratio, \(Az\) ratio).

**Magnetic resonance imaging**

Images of the T2-weighted and fat-saturated T2-weighted series were obtained in CXMD\(_1\) anesthetized animals with an inhalational mixture of 2–3% isoflurane and oxygen according to a method described previously [32] with constant monitoring of heart rate and oxygen saturation. We examined the crus muscles of the lower limbs using a superconducting 3.0-Tesla MRI device (MAGNETOM Trio; Siemens Medical Solutions, Germany).

### Table 1: Summary of transplantation experiments

| Dog ID  | Sex | Age\(^a\) | BW\(^b\) | Cell      | Cell number | Interval                        | Injection number |
|---------|-----|-----------|----------|-----------|-------------|---------------------------------|-----------------|
| 12202MA | M   | 2         | 3.2      | –         | –           | –                               | –               |
| 12205MA | M   | 2         | 4.1      | hDPSCs    | 4.0 × 10\(^6\) | 1 week (1st and 2nd cool)      | 8               |
| 13201MA | M   | 3         | 3.6      | hDPSCs    | 4.0 × 10\(^6\) | 1 week (1st and 2nd cool)      | 8               |
| 13303MA | M   | 3         | 3.8      | –         | –           | –                               | –               |
| 13304MA | M   | 3         | 4.0      | hDPSCs    | 4.0 × 10\(^6\) | 1 week (1st and 2nd cool)      | 8               |
| 14102MA | M   | 3         | 3.3      | –         | –           | –                               | –               |

\(^a\)Age at injection (months)

\(^b\)BW body weight at first injection (kg)
Echocardiography

Echocardiographic images of unanesthetized dogs were obtained using a Vivid S6 Dimensions (GE Healthcare Japan, Tokyo, Japan) probe equipped with a linear array ultrasound transducer (i13L) transmitting at 10 MHz as described previously [34]. The ejection fraction (EF) (%) was calculated using M-mode parameters based on multiple measurements.

Statistical analysis

Data are presented as mean ± S.D. Differences between two groups were assessed using unpaired two-tailed t tests. Multiple comparisons between three or more groups were performed using one-way or two-way ANOVA. Statistical significance is defined as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Statistical significance was calculated using Excel (Microsoft) and GraphPad Prism 8.

Results

Systemic injection of human DPSCs (hDPSCs) into dystrophic mice

*Mdx* mice received a single dose of hDPSCs (high dose, 1.0 × 10⁶ cells, or low dose, 5.0 × 10⁵ cells) or repeated administration of high- and low-dose hDPSCs via the tail vein (Fig. 1a). None of the hDPSC-treated *mdx* mice showed any significant effect on body weight (BW) during the experiments (Fig. 1b). Grip strength in *mdx* mice showed significant restoration after repeated administration of high-dose hDPSC (Fig. 1c, Table S1, *mdx* vs. repeated high dose of hDPSC-*mdx*, P = 0.0002; WT vs. repeated high dose of hDPSC-*mdx*, P = 0.995). However, grip strength did not improve in mice administered a single high-dose or repeat low-dose injections. The grip strength of high-dose hDPSC-treated *mdx* mice was not significantly different from that of 1-year-old *mdx* mice or WT mice (Figure S1A).

We examined the progressive resistance during wheel running in *mdx* mice. The hDPSCs-treated *mdx* mice had improved running speed compared to *mdx* mice (Fig. 1d, Table S2, *mdx* vs. repeated high dose of hDPSC-*mdx*, P = 0.007; WT vs. repeated high dose of hDPSC-*mdx*, P = 0.642; *mdx* vs. single high dose of hDPSC-*mdx*, P = 0.664; *mdx* vs. low dose of hDPSC-*mdx*, P = 0.019) and had a daily running distance similar to WT mice (Fig. 1e, Table S2, *mdx* vs. repeated high dose of hDPSC-*mdx*, P = 0.0069; WT vs. high dose of hDPSC-*mdx*, P = 0.214; *mdx* vs. single high dose of hDPSC-*mdx*, P = 0.07; *mdx* vs. low dose of hDPSC-*mdx*, P = 0.015). Surprisingly, there was a difference in running speed between the repeated treatment and untreated groups at 1 year of age (Fig. 1f, *mdx* vs. repeated high dose of hDPSC-*mdx*, P = 0.022), although their daily running distance was not significantly different (Figure S1B and Table S2, P = 0.24).

The cross-section of the tibialis anterior (TA) muscle of *mdx* mice showed smaller (regenerating fibers) and larger (hypertrophic fibers) fiber diameter in the dystrophic muscles, centrally nucleated fibers (CNFs), spread muscle interstitium, and cell infiltration interspersed in the muscle interstitium (Fig. 2a–d). The histopathological findings observed in the repeatedly hDPSC-treated *mdx* mice included limited muscle interstitium, nuclear infiltration (Fig. 2a, b, d), and reduced frequency of larger fiber areas (Fig. 2c), but not in a dose-dependent manner. We also observed that the
**Fig. 1** (See legend on next page.)

(A) Repeat injection

- **hDPSCs**
  - High-dose (1.0x10^6 cells/mouse)
  - Low-dose (5.0x10^5 cells/mouse)

(B) BW

(C) Normalized grip strength

(D) Maximum running speed

(E) Daily running distance

(F) Maximum running speed

**Legend**

- WT
- mdx
- hDPSCs-mdx (high-dose)
- hDPSCs-mdx (low-dose)

**Notes**

- *P < 0.05
- **P < 0.01
- ***P < 0.001
- ns: non-significant
CNFs in dystrophic muscle, which are indicative of regenerated myofibers following degeneration, were reduced in the hDPSC-treated mdx mice with a repeated high dose (Fig. 2e), suggesting that degeneration was regulated in the hDPSC-treated muscle. When we examined the distribution of hPDSCs by human-specific Alu-PCR, 1 week after the transplantation, many cells accumulated in the lung, and some survived in the skeletal muscle (Fig. 2f), but these were detected for only a short period of time.

Altogether, our data supported the conclusion that short-term amelioration of the DMD phenotype was observed in all groups of hDPSC-treated mdx mice. Among them, the mice repeatedly treated with high-dose hDPSCs showed long-term and remarkable beneficial effects on the DMD phenotype.

Safe systemic transplantation of hDPSCs into CXMDJ
We next investigated the possibility of long-term benefits in hDPSC-treated animals using dog models. We started with the administration of hDPSCs in CXMDJ with the DMD phenotype in the acute phase, when the disease signs were already observable (n = 3 per group, Fig. 3a, Table 1). Eight systemic injections of 4 × 10⁶ cells/kg were performed on three CXMDJ dogs (12205MA, 13201MA, 13304MA) with two courses of weekly injections for 4 weeks (Table 1). After each injection, we carefully monitored the activity, heart rate, respiratory rate, and appearance of any abnormal signs. During development, hDPSC-treated CXMDJ showed good growth and no severe weight loss due to continuous administration (Fig. 3b). No obvious abnormalities related to hepato-renal damage or anemia due to systemic administration in all hDPSC-treated CXMDJ were noted in blood tests, which included the determination of ALP, AST, BUN levels, and CRP levels (Fig. 3c, Figure S2A).

Spontaneous locomotor activity measured using an infrared sensor system showed a largely reduced mobility of CXMDJ with aging [28]. In contrast, hDPSC-treated CXMDJ maintained activity for longer periods compared with untreated dogs until they turned 1-year-old (Fig. 3d), suggesting that no serious adverse effects in hDPSC-treated CXMDJ.

Regulatory effects of hDPSC treatment on inflammation in CXMDJ
During the experiments, serum IL-6 and TNF-α levels in CXMDJ did not increase over the normal range after hDPSC-treatment, whereas an increase was transiently detected in the untreated CXMDJ (Fig. 4a, Figure S2B). To address the regulation of progressive inflammation, the intensity of T2-signals on MRI was measured, which is characteristic of necrosis/edema and inflammatory lesions in CXMDJ. When comparing the quantitative changes of higher T2-signals (4–6 sites) in hindlimb muscles between 2 and 7 months of age, these signals were significantly reduced in the hDPSC-treated CXMDJ (Fig. 4b, c, [13201MA, 13303MA], Figure S3 [12205MA], and Table S3). These findings indicate that hDPSC treatment can enhance the regulation of inflammatory lesions in dystrophic muscles.

Structural stability of the skeletal muscle of hDPSC-treated CXMDJ
To investigate the pathological changes in hDPSC-treated muscle, we examined cross-sections of DMD muscles. Dystrophic phenotypes, including nuclear infiltration and spread of muscle fiber interstitium, were downregulated in the skeletal muscle of hDPSC-treated CXMDJ (Fig. 5a, Figure S4). Although dystrophic muscles also displayed a high myofiber size variability due to a higher number of smaller fibers and the occurrence of hypertrophic fibers, the fiber size distribution in the TA muscles shifted toward a lower number of both smaller and hypertrophic fibers in the case of hDPSC-DMD (Fig. 5b, P = 0.0425, Figure S5). Immunostaining analysis showed significantly decreased accumulation of IgG, a marker for damaged myofiber [32], in the skeletal muscle of hDPSC-treated CXMDJ (Fig. 5c). Although the muscle tissue from CXMDJ also showed a number of developmental myosin heavy chain (dMyHC)-positive fibers, which are not usually observed in the muscles of normal dogs [35], there were dMyHC-positive fibers only in limited areas within the tissue of hDPSC-treated CXMDJ, suggesting that systemic hDPSC treatment can improve the dystrophic phenotype. By Alu-PCR analysis, we also confirmed that circulating transplanted hDPSCs were not detectable in blood within 48 h after injection.
Fig. 2 (See legend on next page.)
(Figure S2C). Seven weeks after treatment, the retention of hDPCs was confirmed in parts of the skeletal muscle, such as the TA and extensor digitorum longus muscle and cardiac muscle (left ventricular, LV), but not detectable in the lung and diaphragm of recipient dogs (Fig. 5d).

**Improved locomotor activity in hDPC-treated CXMDj**

The CXMDj model displays progressive clinical impairment with a rapid decline in the walking ability of dogs with progressive weakness, abnormal stiff limbs, and short strides [21, 29]. hDPC-treated CXMDj showed continued stabilization of clinical status characterized by a higher clinical score maintained up to the age of 12 months (Figure S5), reflecting reduced fatigability, decreased limb stiffness intensity, and less severe ankyloses [36], as described in our previous reports [21, 28, 29, 37]. Indeed, the home cage physiological activity of hDPC-CXMDj during the daytime (9213 ± 2871 counts) was higher than that of control DMD (4645 ± 1839 counts, P = 0.0075) in 12-month-old dogs (Fig. 3d), even though it was still significantly different from that of normal dogs (41,746 ± 6241 counts, P < 0.0001). Video data showed an increased mobility of hDPC-CXMDj compared to untreated CXMDj in the cage based on jumping and playfulness (Supporting Information, movie S1). These observations encouraged us to investigate whether hDPC could increase the locomotor activity of CXMDj. We monitored the 15 m running speed of CXMDj to determine motor function and confirmed that CXMDj had a slower speed according to their progressive phenotype (Fig. 6a, Supporting Information, movie S2, 3). Meanwhile, hDPC-treated CXMDj maintained their running speed and were active for more than 12 months (vs. control DMD, P < 0.00001; vs. normal, P < 0.00005). We also measured multiple acceleration parameters, which severely decrease with age in dystrophic dogs compared to normal dogs, as we have previously reported [29]. When using the acceleration parameter to evaluate motor function, acceleration magnitudes (AM) were not significantly different between untreated and hDPC-treated CXMDj in either the thoracic or lumbar region (Figure S7 and Table S4). Interestingly, the higher AM (>10,000 mG) maintenance ratio was rarely reached in CXMDj, but was observed more frequently in hDPC-treated CXMDj (Fig. 6b).

**Improvement in skeletal muscle and cardiac dysfunction**

Finally, we investigated whether repeated systemic administration of hDPCs would lead to long-term improvement of dystrophic muscle function. An instantaneous force by torque evaluation was used to assess skeletal muscle function. The tetanic force on the CXMDj hindlimbs was 51.0 ± 12.3% (3.12 ± 1.0 N m/s; P < 0.0001) compared to normal dogs (6.12 ± 0.49 N m/s), while all hDPC-treated CXMDj (4.96 ± 1.24 N m/s) showed significantly stronger torque values (81.0 ± 12.8% of normal dogs, P = 0.042) compared to untreated CXMDj (P = 0.0039) as described in Fig. 6b. With regard to cardiac function, CXMDj shows progressive cardiac dysfunction, which is similar to DMD patients presenting with dilated cardiomyopathy [22, 38]. Echocardiography showed that LV function was maintained, with higher levels of EF in hDPC-treated CXMDj (mean ± SD, 67.3 ± 0.53%) than that in control DMD (60.5 ± 3.2%, P = 0.001), and comparable to that in normal dogs (69.6 ± 4.7%, Fig. 6c).

Altogether, these observations consistently indicate that repeated systemic hDPC treatment in DMD animals can improve the dystrophic phenotype by maintaining muscle function.

**Discussion**

Here, we investigated and proposed a protocol for safe and effective stem cell transplantation aimed at the functional recovery of skeletal muscles. A comprehensive analysis was performed during experiments performed by administering hDPCs in animal models of DMD. Results from both mdx mice and CXMDj models showed that disease progression slowed down after repeated rounds of hDPC treatment to induce long-term effects.

Short-term amelioration of locomotor function was also observed in all groups of hDPC-treated mdx mice. However, the dose-dependent effects of hDPC on physiological activity, grip strength, running speed, and longer running distances suggest that sufficient amelioration would require repeated high-dose administration...
Fig. 3 (See legend on next page.)
of cells (Fig. 1). The maintained running ability up to 1 year of age in mice repeatedly treated with high-dose hDPSCs in the acute DMD phase also supports the conclusion that the effects are long-term. Since transplanted cells are temporary, but accumulate in muscle tissues, it is generally considered that hDPSCs play a protective role against inflammation in the dystrophic muscle. In our study, this was supported by the histopathological appearance of the hDPSC-treated muscle, with findings such as reduced areas of nucleic infiltration (Fig. 2).

In our experiments using dog models, repeated systemic hDPSC injections into the CXMD1 were safe and caused no severe side effects without the need for immunosuppression (Fig. 3). Since hDPSCs share characteristics with clinically used BM-MSCs that lack HLA-DR expression, these cells are not likely to be subjected to immunological attack in the recipient body.

The repeated use of hDPSCs in the CXMD1 prevented severe inflammation with an IL-6 and TNF-α surge, as validated by cell infiltration that was much more localized, and attenuation of T2 signals in muscles on MRI (Fig. 4, Figure S2B). Higher concentrations of circulating IL-6, IL-1, and TNF-α have also been reported in DMD compared to that in healthy subjects [39]. These facts indicate that hDPSCs have an immune-modulatory effect in DMD and may attenuate the histopathological changes that lead to dysfunction in dystrophic muscles. Histopathological appearance improvements after hDPSC administration indicate the functional recovery of dystrophic muscle (Fig. 5). Importantly, the home cage activity and running function of hDPSC-treated CXMD1 were maintained until they reached 1 year of age (Figs. 3d and 6a). The therapeutic effects of hDPSCs are considered to be more effective in the long-term maintenance of running function, a capacity that diminishes with age in the disease, rather than contributing to recovery. It appears that hDPSC treatment at a young age could alleviate the DMD phenotype by preserving the whole-body muscle function.

Our results included an acceleration parameter to determine the instantaneous running ability of CXMD1 (Fig. 6b). Since there is a large difference in the evaluation of running speeds among individuals, and there are issues for some DMD patients in walking for even 6 min, introducing the acceleration parameter into the evaluation of running ability could be applied to assess outcomes in clinical trials for hereditary neuromuscular disorders, including DMD.

Significantly stronger isometric torque values in hDPSC-treated CXMD1 clearly demonstrate that the progressive loss in limb muscle strength is ameliorated by repeated hDPSC treatment (Fig. 6c). Echocardiography showed that decreased EF in CXMD1 due to progressive cardiac dysfunction [22, 38] was rescued in the hDPSC-treated CXMD1 (Fig. 6d), suggesting that hDPSC treatment improved not only limb muscle strength but also cardiac muscle function. Since DMD patients sometimes exhibit dilated cardiomyopathy, the DPSC therapies presented in this study may be promising for maintaining cardiac function.

It has also been reported that transplantation of hDPSCs in the GRMD model improved muscle pathology, resulting in limited dystrophin expression [40]. Since hDPSCs can differentiate into the myogenic lineage only with very low efficiency without the use of agents, including the demethylating agent 5-aza-2-deoxycytidine [41], our study indicates that the benefits obtained from hDPSCs depend on their function as anti-inflammatory agents, and not by direct contribution to tissue repair. We examined the possibility of dystrophin expression derived from hDPSCs in the skeletal muscle but did not confirm the presence of dystrophin mRNA by reverse transcription PCR (Figure S8). In fact, the restoration of dystrophin protein levels is the major target for the treatment of DMD patients. In contrast, the benefit of DPSCs in this study is likely dependent on their role as a systemic anti-inflammatory agent and not their differentiation directly promoting muscle fiber regeneration. The present innovation is a therapeutic approach utilizing the inflammation-regulating ability of MSCs. Since stem cells other than MSCs do not exhibit such ability, this function can be expected to be novel.

We previously provided evidence that severe phenotypes in IL-10 knockout mdx mice, such as increased M1-macrophage infiltration, high inflammatory factor levels, and progressive cardiopulmonary dysfunction, show a predisposition toward inflammation [42]. Glucocorticoids are widely used in patients to interrupt and improve muscle strength during early stages, which may also act directly on muscle fibers by stabilizing the
sarcolemma [43, 44]. However, this is frequently associated with severe side effects. Several anti-inflammatory therapies reportedly have beneficial effects on DMD phenotypes [45, 46]. TNF-α blockers, such as infliximab, have been investigated using mdx mice as an anti-inflammatory agent for DMD [45, 47]. Proteasome inhibitors, such as bortezomib, have been shown to block NF-κB activation, improve the appearance of muscle

Fig. 4 Regulation of inflammation associated with the DMD phenotype. a Serum levels of IL-6 from dogs quantified using ELISA during the experimental period. b Cross-sectional magnetic resonance images (MRI) in the lower leg muscles of untreated (control DMD) and hDPSC-treated CXMD (hDPSC-DMD). T2-weighted imaging was comparable in the lower legs: (R, right side; L, left side, left/right asymmetry) of CXMD, (untreated 13303MA and hDPSC-treated 13201MA and 13304MA). c Quantitative changes of higher T2-signals (signal-to-noise ratio, SNR) in the hindlimb muscles on CXMD, shown in MRI data (b). Relative SNR was calculated from the highest signals in each hindlimb of 2-month-old compared to 7-month-old dogs. Data are represented as mean ± SD and statistical differences compared to control DMD (*P < 0.05), t test.
fibers, and reduce both connective tissue deposition and inflammatory cell infiltration in GRMD. Moreover, treatment with an adeno-associated virus vector encoding a short hairpin RNA (shRNA) that specifically targets NF-κB ameliorated muscle pathologies in mdx mice [48]. Therefore, our hDPSC transplantation strategy has the potential to be used in the form of combined therapy with steroid or other immune-modulating treatments.
Comparison of human MSCs derived from different tissues revealed no differences in cell morphology or expression of surface markers typical of mesenchymal stem cells [49]. Many therapeutic approaches have been developed using MSCs derived from bone marrow (BM) [50, 51], adipocytes [52], and placenta [53]. In this study, we first demonstrated the long-term therapeutic effects of systemic administration of DPSCs. hDPSCs showed immunoregulatory properties similar to those of BM-MSCs in terms of the cellular proliferation inhibition of both CD4+ and CD8+ activated T cells, and increased IL-10 and prostaglandin E2 production compared to BM-MSCs [54]. In addition, hDPSCs proliferate much faster than those from human BM-MSCs, e.g., when the yields of hDPSCs were $1.2 \times 10^6$ cells, BM-MSCs were $6.0 \times 10^5$ cells at passage 3 [55]. Under serum/xeno-free, good manufacturing practice-compliant (GMP) conditions, DPSCs showed shorter doubling times compared to BM-
MSCs and maintained long-term “stemness” [56]. Comparing hDPSCs-cultivation in xeno-free serum and FBS medium, population doublings showed an initial linear trend, but a statistically significantly lower number of cumulative doublings in xeno-free serum versus FBS medium was detected by passage 6 [57]. Based on the safety evaluation of MSC expansion, a consistent decrease in telomere length was found in both DPSC and BM-MSC cultures under GMP conditions [56]. These findings indicate that DPSCs are a promising cell source for transplantation, at least for expansion under the GMP level; however, there is still a need for the development of qualified protocols for clinical-grade expansion of oral MSCs.

While the gene expression profiles of BM-MSCs, adipocyte-MSCs, and umbilical cord tissue-derived MSCs were similar, DPSCs differed in relative pancreatic and duodenal homeobox 1 (PDX1) and Sox2 gene expression and had higher expression of E-cadherin and lower expression of Snail associated with tissue reparative functions in the epithelial-mesenchymal transition [49]. Furthermore, we confirmed that expression of the chemokine, stromal-derived factor-1(SDF-1/CXCL12), from hDPSCs was upregulated in response to TNF-α stimulation (Figure S9). SDF-1 and growth factors might enhance DPSC retention by altering the microenvironment.

The binding of SDF-1 to both CXCR4 and CXCR7 is responsible for the production of paracrine mediators, including VEGF, β-FGF-1, and HGF, which exert mitogenic, anti-apoptotic, pro-angiogenic, and anti-inflammatory effects [58]. Comparing to other MSCs, DPSCs have an extensive trophic secretomes, which include NGF, BDNF, NT-3, GDNF, VEGF, and PDGF, and express greater amounts of NGF, BDNF, and NT-3, which promote axon/neurite regeneration [59, 60]. The neuroprotective/pro-regenerative effects are significantly greater in DPSC transplanted animals compared to BM-MSC-treated ones and are correlated with a more favorable neurotrophic secretome by DPSC [60]. Based on this, we surmise that tissue repair mechanisms by DPSC may be associated with DMD treatment. Furthermore, MSCs derived from different sources are transcriptomically different from each other, although they share basic characteristics. This transcriptomic difference is also important in terms of the diversity of the secretions such as miRNA. For example, miR-199a-5p is known to be increased in exosomes of DMD patients, or miR-24, which is involved in myogenic differentiation [61]. Future therapeutic studies using various tissue-derived MSCs will allow for the selection of optimal cell sources.

As a possible source for cell therapy, hDPSCs have been investigated for their potential in treating various degenerative diseases such as Alzheimer’s disease, myocardial infarction, bone defects, and corneal reconstruction [62]. In the case of experimental spinal cord injury, stroke, and Parkinson’s disease models, hDPSC transplantation has been demonstrated as a promising treatment for improving functional outcome [63]. Here, we show for the first time the long-lasting restorative effect in DMD animal models produced by systemic DPSC injection that did not result in discernible side effects. Hopefully, this approach can be considered a safe and effective therapeutic measure against DMD. Although further studies are still needed to ascertain the clinical usage and to elucidate molecular mechanisms, since repeated treatments are required to prevent the DMD phenotype, hDPSC treatment can be considered a promising DMD therapy.

Conclusion

DPSCs have potential as therapeutics, since similar types of bone marrow-derived MSCs have been reported to show immunosuppressive properties. This report investigated the therapeutic effects of DPSCs using animal models of DMD. Our study demonstrates that DMD phenotypes, such as pathological inflammation and motor dysfunction, were significantly improved by repeated systemic injections of DPSCs. This study provides valuable insights into MSC cell therapy in DMD for potential clinical applications.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13287-020-02099-3.

Additional file 1: Movie S1. Activity of control and hDPSC-treated CXMDJ. Additional file 2: Movie S2. Movies showing 15 m running analysis in CXMDJ. Movies of 15 m running in untreated CXMDJ, littermates (control CXMDJ, 12202MA). Additional file 3: Movie S3. Movies showing 15 m running analysis in CXMDJ. Movies of 15 m running in hDPSC-treated CXMDJ, (hDPSCs-CXMJD, 12205MA). Additional file 4: Figure S1. Grip strength and daily running distance in aged mice. Figure S2. Blood levels of hDPSCs after injection. Figure S3. MRI of the lower leg muscle of CXMDJ. Figure S4. H&E staining of hDPSC-treated skeletal muscle. Figure S5. Muscle fiber distribution from skeletal muscle of CXMDJ. Figure S6. Clinical follow-up of CXMDJ after hDPSC transplantation. Figure S7. Multiple parameters of acceleration measured by 15 m of running. Figure S8. Reverse transcription PCR of human specific dystrophin expression. Figure S9. Cytokine and chemokine expression in hDPSCs. Table S1. Normalized grip strength in mice. Table S2. Locomotor activity in mice. Table S3. Quantitative changes of higher T2-signals in hindlimb muscles.

Abbreviations

ALP: Alkaline phosphatase; AM: Acceleration magnitude; AST: Aspartate transferase; BDNF: Brain-derived neurotrophic factor; BUN: Blood urea nitrogen; BM-MSCs: Bone marrow-derived multipotent mesenchymal stromal cells; BW: Body weight; β-FGF-1: β-Fibroblast growth factor-1; CD: Cluster of differentiation; CNFs: Centrally nucleated fibers; CRP: C-reactive protein; CXCR: C-X-C Chemokine receptor; CXMDJ: Canine X-linked muscular dystrophy model in Japan; dMyHC: Developmental myosin heavy chain; DAPI: 4, 6-Diamidino-2-phenylindole; DMD: Duchenne muscular dystrophy.
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
Animal experiments using MSCs were conducted in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Nippon Medical School (27-199) and National Center of Neurology and Psychiatry (NCNP) Animal Ethics Committees (20120111, 20150504, and 19-30-06).

Consent for publication
Not applicable.

Competing interests
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References
1. Hadlett JS, Sanoudou D, Kho AT, Bennett RR, Greenberg SA, Kohane IS, Beggs AH, Kunkel LM. Gene expression comparison of biopsies from Duchenne muscular dystrophy (DMD) and normal skeletal muscle. Proc Natl Acad Sci U S A. 2002;99:15000–5.
2. Campbell KP. Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. Cell. 1995;80:75–9.
3. Ervasti JM, Brunning R, Kunzler T, Jichi F, Manzur AY. Corticosteroids for the treatment of Duchenne muscular dystrophy. Cochrane Database Syst Rev. 2016;13:CD003725.
4. Matsuno A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophase activation and polarization. Trends Immunol. 2004;25:677–86.
5. Dosemeci AT, Ozgun Dosemeci IB, Kirmaz C, Sariboyaci AE, Unal Halbutogullari ZS, Ozel C, Kaozo E. The paracrine immunomodulatory interactions between the human dental pulp derived mesenchymal stem cells and CD4 T cell subsets. Cell Immunol. 2016;310:108–15.
6. Camargo FD, Green R, Capetanaki Y, Jackson KA, Goodell MA. Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates. Nat Med. 2003;9:1520–7.
7. Lee-Pullen TF, Bennett AL, Beharir MW, Grounds MD, Sammels LM. Superior survival and proliferation after transplantation of myoblasts obtained from adult mice compared with neonatal mice. Transplantation. 2004;78:172–8.
8. Quenneville SP, Chapdelaine P, Rousseau J, Tremblay JP. Dystrophin expression in host muscle following transplantation of muscle precursor cells modified with the phiC31 integrase. Gene Ther. 2007;14:514–20.
9. Sampaiolesi M, Blot S, D’Antona G, Granger N, Tonlorenzi R, Innocenzi A, Zanettino AC, Paton S, Ambrosi MG, Gronthos S. Multipotent human adipose-derived stem cells exhibit a perivascular phenotype in vitro and in vivo. J Cell Physiol. 2008;214:431–21.
10. He Q, Wang E, Li G. Concise review: multipotent mesenchymal stromal cells in blood. Stem Cells. 2007;25:669–77.
11. Oh W, Kim DS, Yang YS, Lee JK. Immunological properties of umbilical cord blood-derived mesenchymal stromal cells. Cell Immunol. 2008;251:116–23.
12. Tsai MS, Lee JY, Chang YJ, Hwang SM. Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. Hum Reprod. 2004;19:1450–6.
13. Zhang W, Balboomers XF, Van Kuppevelt TH, Daamen WF, Van Damme PA, Bian Z, Janssen JA. In vivo evaluation of human dental pulp stem cells differentiated towards multiple lineages. Tissue Eng Regen Med. 2008;5:117–25.
14. Jo YY, Lee HJ, Kook SY, Chung HW, Park JY, Chung JH, Chung YH, Kim ES, Yang HC, Chung PH. Isolation and characterization of postnatal stem cells from human dental tissues. Tissue Eng. 2007;13:767–73.
15. Ozdemir AT, Ozgul Ozdemir IB, Kirmaz C, Sariboyaci AE, Unal Halbutogullari ZS, Ozel C, Kaozo E. The paracrine immunomodulatory interactions between the human dental pulp derived mesenchymal stem cells and CD4 T cell subsets. Cell Immunol. 2016;310:108–15.
16. Lee-Pullen TF, Bennett AL, Beharir MW, Grounds MD, Sammels LM. Superior survival and proliferation after transplantation of myoblasts obtained from adult mice compared with neonatal mice. Transplantation. 2004;78:172–8.
17. Quenneville SP, Chapdelaine P, Rousseau J, Tremblay JP. Dystrophin expression in host muscle following transplantation of muscle precursor cells modified with the phiC31 integrase. Gene Ther. 2007;14:514–20.
18. Sampaiolesi M, Blot S, D’Antona G, Granger N, Tonlorenzi R, Innocenzi A, Mogno P, Thibaud JL, Galvez BG, Barthulemy I, Perini L, Mantero S, Guttinger M, Parasara O, Rinaldi C, Cusella De Angelis MG, Torrente Y, Bordignon C, Bottinelli R, Cossu G. Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. Nature. 2006;445:74–9.
19. Valentine BA, Winand NJ, Pradhan D, Moise NS, de Lahunta A, Koregay JN, Cooper BJ. Canine X-linked muscular dystrophy as an animal model of Duchenne muscular dystrophy: a review. Am J Med Genet. 1992;42:352–6.
58. Liu H, Liu S, Li Y, Wang X, Xue W, Ge G, Luo X. The role of SDF-1-CXCR4/CXCR7 axis in the therapeutic effects of hypoxia-preconditioned mesenchymal stem cells for renal ischemia/reperfusion injury. Plos One. 2012;7:e34608.

59. Sakai K, Yamamoto A, Matsubara K, Nakamura S, Naruse M, Yamagata M, Sakamoto K, Tauchi R, Wakao N, Imagama S, Hibi H, Kadomatsu K, Ishiguro N, Ueda M. Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. J Clin Invest. 2012;122:80–90.

60. Mead B, Berry M, Logan A, Scott RA, Leadbeater W, Scheven BA. Stem cell treatment of degenerative eye disease. Stem Cell Res. 2015;14:243–57.

61. Sun E, Karaz E. Can Wharton jelly derived or adipose tissue derived mesenchymal stem cell can be a treatment option for duchenne muscular dystrophy? Answers as transcriptomic aspect. Am J Stem Cells. 2020;9:57–67.

62. Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res. 2009;88:792–806.

63. Yamada Y, Nakamura-Yamada S, Kusano K, Baba S. Clinical potential and current progress of dental pulp stem cells for various systemic diseases in regenerative medicine: a concise review. Int J Mol Sci. 2019;20:1132–48.

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