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Bone marrow transplantation improves outcome in a mouse model of congenital muscular dystrophy

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Abstract **We examined whether pathogenesis in dystrophin-deficient (*mdx*) mice and laminin-α2-deficient (*dy*) mice is ameliorated by bone marrow transplantation (BMT). Green fluorescent protein (GFP) mice were used as donors. In *mdx* mice, BMT failed to produce any significant differences in muscle pathology, although some GFP-positive fibers with restored dystrophin expression were observed. In contrast, in the *dy* mice, BMT led to a significant increase in lifespan and an increase in growth rate, muscle strength, and respiratory function. We conclude that BMT improved outcome in *dy* mice but not *mdx* mice.**

Keywords: Bone marrow transplantation; Muscular dystrophy; *mdx* mouse; *dy* mouse; Laminin α2; Basal lamina

1. Introduction

The muscular dystrophies are groups of inherited myogenic disorders characterized by progressive muscle wasting and weakness of variable distribution and severity. Two major types of severe muscular dystrophy, Duchenne muscular dystrophy (DMD) and congenital muscular dystrophy, have been identified [1]. DMD is caused by mutations of the dystrophin gene [2]. Most cases of congenital muscular dystrophy are caused by mutations in the laminin-α2 chain (merosin) gene. This disease has been termed merosin-deficient congenital muscular dystrophy (MCMD) or MDC1A [3]. Pathogenesis of dystrophin-deficient or laminin-α2-deficient muscular dystrophy can be studied in mouse models [4]. Loss of dystrophin protein is observed in the *mdx* mouse, the mouse model of DMD [5]. The *dystrophia muscularis* (*dy*) mouse has spontaneous mutation in the *Lama2* gene encoding laminin-α2 and is used as a model of MDC1A [6,7]. Although some potential treatments including pharmacologic methods, gene therapy, and cell therapy have been tried, there are no effective therapeutic approaches for muscular dystrophy at present [8].

Bone marrow transplantation (BMT) is an established clinical procedure used to treat various human diseases. Adult bone marrow (BM) cells contain mesenchymal stem cell progenitors, which can give rise to osteocytes, chondrocytes, adipocytes, and myocytes [9,10]. BM is also a promising source of myogenic stem cells [11]. Recently, several investigators have reported that transplanted BM cells participate in the muscle regeneration process in irradiated recipient mice [12–15] or DMD patient [16]. However, analyses of these studies are often limited to histopathologic assessments.

In the present study, we examined the therapeutic effect of whole BMT on muscular dystrophy model mice by evaluating clinical phenotypes such as body weight, lifespan, muscle strength, and respiratory function as well as histopathology. We also compared the therapeutic effect of whole BMT on two distinct models of muscular dystrophy, *mdx* and *dy* mice. Our results showed that BMT improved outcome in *dy* mice but failed to affect pathology of *mdx* mice. Thus a therapeutic approach of transplanting BM cells could be considerable benefit in MDC1A.

2. Materials and methods

2.1. Mice

The C57BL/6 (wild-type) mice were purchased from Clea Japan (Tokyo, Japan). The *mdx* mice (of C57BL/10 background) were provided by Central Institute for Experimental Animals (Kanagawa, Japan). The laminin-α2-deficient *Lama2*−/− mice (*dy*) mice and the EGFP transgenic (GFP-Tg) mice with a C57BL/6 background [17] were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). All experiments involving animals were performed under the guidelines of the Institutional Animal Care and Research Advisory Committee, Kawasaki Medical School.

2.2. Bone marrow reconstitution

BM chimeras were established by following the method of Fukada et al. [15] with modifications. Briefly, adult (8-week-old) wild-type, *mdx* or *dy* mice received 9 Gy TBI (X-ray), split into two doses separated by 3 h to minimize gastrointestinal toxicity. BMT was performed according to a standard protocol described previously [18,19]. Recipient mice were injected with 5×10^6 T cell-depleted BM cells. T cell depletion of donor BM cells was performed using anti-CD90-MicroBeads and an AutoMACS system (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. No unfavorable results such as GVHD were observed in recipient mice received BM cells from GFP mice.

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Abbreviations: BMT, bone marrow transplantation; DMD, Duchenne muscular dystrophy; MDC1A, congenital muscular dystrophy type 1A; SpO_2, arterial hemoglobin saturation

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2.3. Histology and immunohistochemistry

Cryosections of diaphragm muscle were prepared as described previously [20]. For immunohistochemical analysis, sections were immunostained with a rabbit polyclonal antibody against GFP (MBL, Nagoya, Japan) or a monoclonal antibody against C-terminus of dystrophin (NCL-DYS2; Novocastra, Newcastle, United Kingdom), laminin-α2 (merosin) (clone 4H8-2; Sigma–Aldrich, St. Louis, MO, USA) followed by fluorescein isothiocyanate-conjugated secondary antibodies according to the MOM procedure (Vector Laboratories, Burlingame, CA, USA). The slides were mounted with VECTASHIELD plus DAPI (Vector Laboratories). The fluorescence images were recorded photographically using a microscope (Nikon, Tokyo, Japan) and analyzed with Lumina Vision software (Mitani Corporation, Fukui, Japan).

2.4. Grip strength test and pulse oximetry

Peak grip strength (g) was measured using an MK-380S automated grip strength meter (Muromachi Kikai, Tokyo, Japan) as described previously [21]. Arterial hemoglobin saturation (SpO_2) was measured with Masimo SET (Masimo Corp., Irvine, CA, USA) [22].

2.5. Statistics

Statistical analysis was performed on paired observations using Bonferoni's test after one-way ANOVA.

3. Results

3.1. BMT promotes survival and growth of laminin-α2-deficient mice

We first examined whether BMT affects lifespan in the *mdx* mice and *dy* mice. Kaplan–Meier survival curves revealed that a large percentage of control *dy* mice died around the first 20 weeks after birth (Fig. 1), whereas *dy* mice that received BMT survived up to 40 weeks, or almost double the lifespan of control *dy* mice. In *mdx* mice, we kept both groups of mice up to 2 years and found no difference for lifespan between BMT and control groups. These results indicate that BMT eliminated early death of *dy* mice.

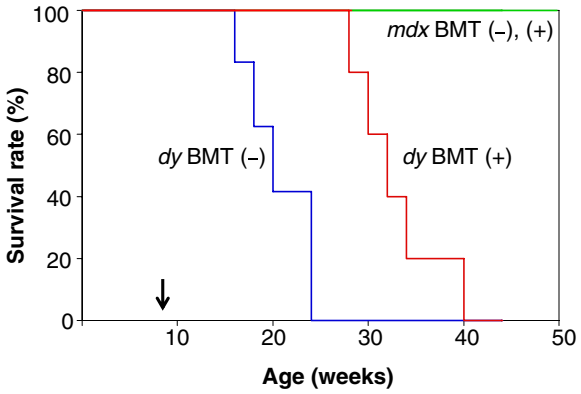


Fig. 1. Kaplan–Meier survival curves for BMT or non-treated control groups of each model of mice ($n = 10$, each). Arrow indicates 8 weeks of age at the time of BMT was performed. BMT (−), control group; BMT (+), treated group.

We further examined progressive changes in body weight of these models. BMT did not affect the growth rate of *mdx* mice in both males and females (Fig. 2A). The *dy* mice that received BMT lost weight right after BMT but gained weight more quickly and grew significantly larger than non-treated littermates (Fig. 2B). This tendency was found in both males and females. Thus, in addition to increasing lifespan, BMT improved the growth of *dy* mice.

3.2. BMT dose not significantly alter pathology in mdx muscle

We then questioned weather BMT improves muscle pathology of *mdx* mice. We examined diaphragm muscle, known to

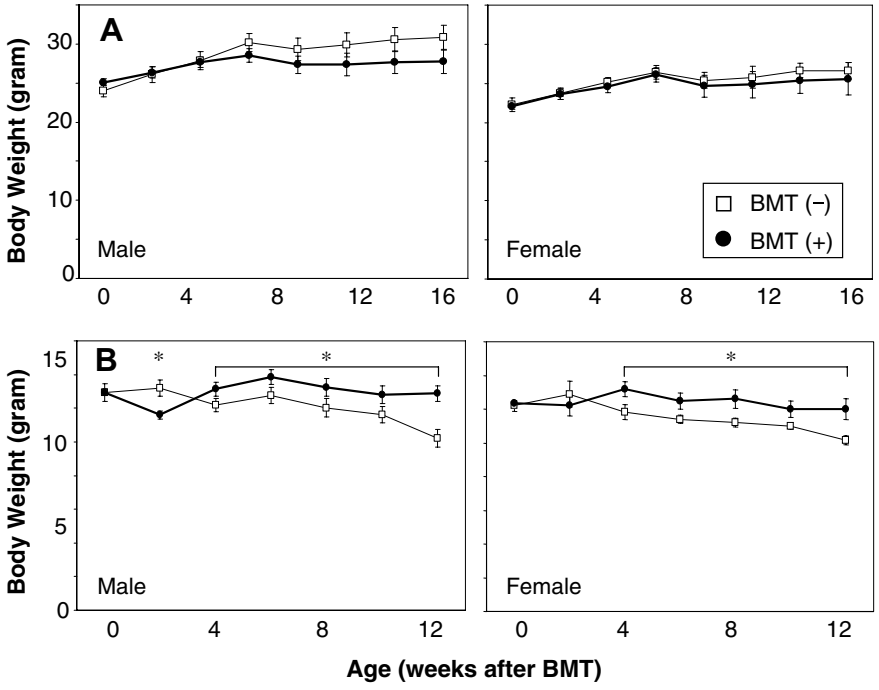


Fig. 2. (A) Growth curves for *mdx* mice between the control and BMT groups in males or females up to 16 weeks after BMT ($n = 5$, each). (B) Growth curves for *dy* mice until 12 weeks after BMT ($n = 5$, each). Data are expressed as means ± S.D. $^{*}P < 0.05$, Bonferoni's test after one-way ANOVA. BMT (−), control group; BMT (+), treated group.

the most severely damaged muscle in *mdx* mice [23]. H&E staining of diaphragm muscles both in BMT and control groups showed characteristic of dystrophic change including fiber size variability, central nucleus, fibrosis and fatty replacement. There is no detectable difference in gross pathology between both groups (data not shown). Next, diaphragm muscle sections of wild-type, control, and BMT group were stained for GFP and dystrophin immunoreactivity and DAPI (Fig. 3). We successfully obtained GFP-positive muscle fiber generation in irradiated BM chimeras. The frequency of GFP-positive fibers in diaphragm muscle was 4.9 ± 2.2% of total muscle fibers ($n = 3$). The frequency of dystrophin-positive fibers in diaphragm muscle was 0.9 ± 0.1% ($n = 3$). Merge images of *mdx* mice that underwent BMT revealed few GFP-positive fibers with dystrophin expression restored. Although some GFP-positive BM-derived skeletal muscles were observed, dystrophin expression was limited in *mdx* mice. These results indicate that muscle pathology was not significantly improved by BMT in *mdx* mice.

3.3. BMT improves pathology and significantly restores laminin-α2 expression in dy muscle

We next examined muscle pathology of *dy* mice. H&E staining of diaphragm muscles of *dy* mice in control group showed degenerative changes and atrophy in comparison with wild-type mice. Although degenerative changes are still observed, thickness of diaphragm muscles of *dy* mice is restored close to that of wild-type mice after BMT (Fig. 4A). We immunostained muscle sections of wild-type and *dy* mice (both control and BMT groups) with GFP and laminin-α2 antibodies and DAPI (Fig. 4B). The frequency of GFP-positive fibers in diaphragm muscle was 61.7 ± 5.9% of total muscle fibers ($n = 3$). The frequency of laminin-α2-positive fibers in diaphragm muscle was 82.6 ± 3.9% ($n = 3$). The merge image showed a significant number of GFP-positive fibers with laminin-α2 expression. Of particular importance, laminin-α2 expression was restored not only in GFP-positive fibers but also in GFP-negative fibers. In contrast to *mdx* mice, BM cells engrafted into skeletal muscle of *dy* mice with robust GFP-positive cells and laminin-α2 expression was significantly restored.

3.4. BMT improved muscle strength in dy mice

We measured the peak force of grip strength of mice. The grip strength of the *dy* mice that underwent BMT was significantly stronger ($P < 0.05$) than the *dy* control mice at 12 weeks after BMT (Fig. 5B). On the other hand, there were no substantial differences between the two groups of *mdx* mice (Fig. 5A). The ratio of grip strength per body weight revealed that BM transplanted *dy* mice had restored the ratio close to that of wild-type and *mdx* mice (Fig. 5C).

3.5. BMT improved respiratory function of dy mice

The SpO_2 measurements of *mdx* mice revealed no significant differences between control and BMT groups (Fig. 6A). In contrast, *dy* mice underwent BMT retained higher oxygen

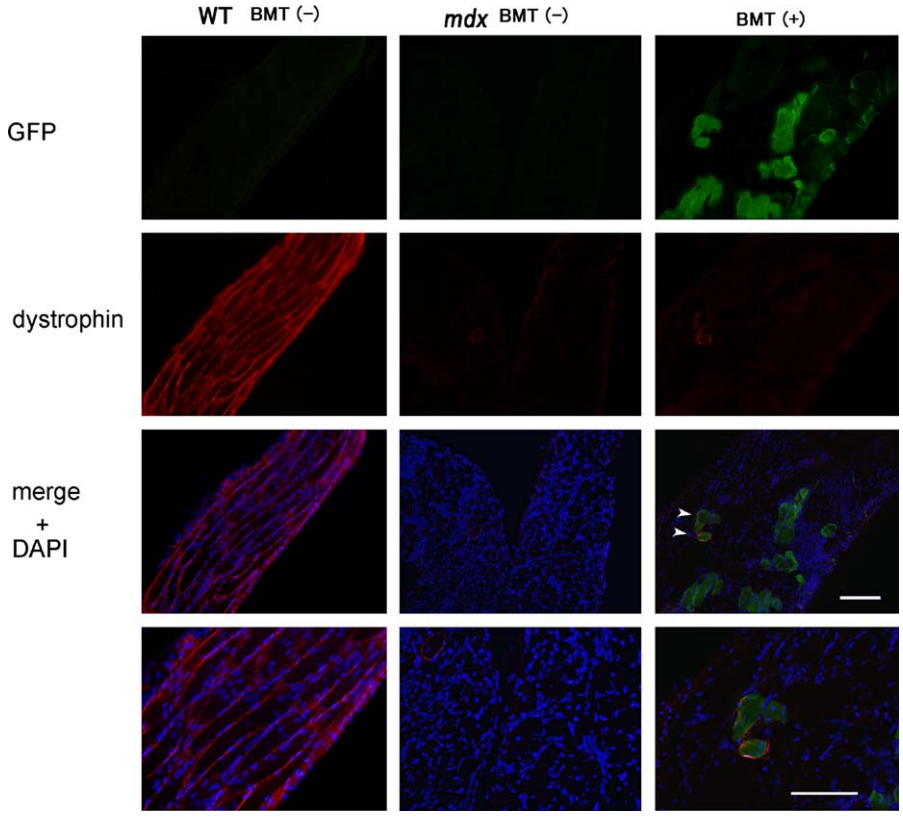


Fig. 3. Immunohistochemistry of diaphragm muscle sections of wild-type (WT-control, left column), control (*mdx*-control, middle column) or BMT group (*mdx*-BMT, right column). Merge image of *mdx*-BMT revealed that some GFP-positive fibers with dystrophin expression were restored (arrow heads). Bottom row is higher magnification of merge image. Bars: 100 μm. BMT (−), control group; BMT (+), treated group.

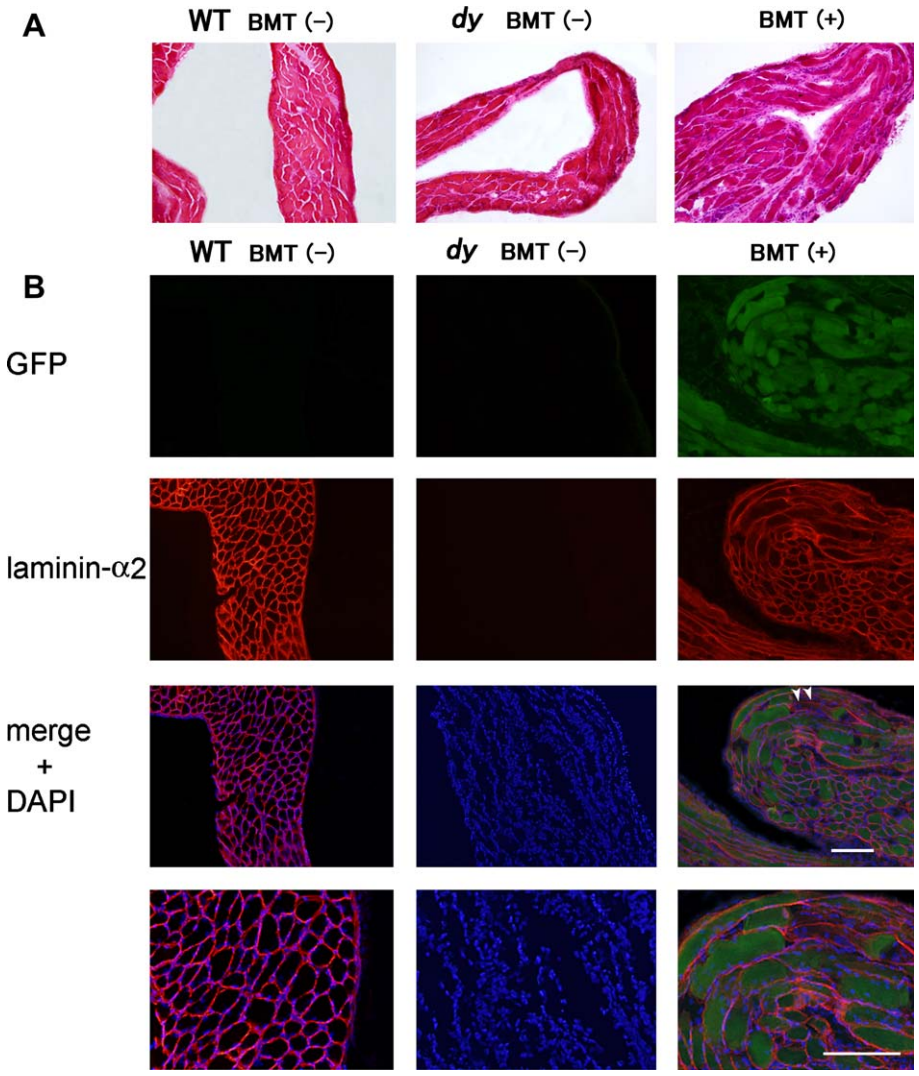


Fig. 4. BMT improved histopathology of *dy* muscles. (A) H&E staining of diaphragm muscle sections of wild-type (WT-control, left column), control (*dy*-control, middle column), or BMT group (*dy*-BMT, right column). (B) Immunohistochemistry of diaphragm muscle sections of wild-type (WT-control, left column), control (*dy*-control, middle column), or BMT group (*dy*-BMT, right column). Note that merge image of *dy*-BMT revealed a significant number of GFP-positive fibers with laminin-α2 expression. Of particular importance, laminin-α2 expression was restored not only in GFP-positive fibers but also in GFP-negative fibers (arrow heads). Bottom row is higher magnification of merge image. Bars: 100 μm. BMT (−), control group; BMT (+), treated group.

saturation and control *dy* mice showed decreased hypoxia at 20 weeks of age (12 weeks after BMT) (Fig. 6B).

4. Discussion

We found that BMT led to no significant improvements in muscle pathology of *mdx* mice. This result is consistent with recent reports [12–15]. We also found there was some discrepancy between GFP and dystrophin expressions in *mdx* mice that received BMT. This discrepancy is similar to that of a recent report describing that up to 5% of total muscle fibers expressed GFP, whereas dystrophin restoration after BMT was always <1% of total muscle fibers [24]. We also demonstrated that BMT failed to affect lifespan, growth rate, grip strength, and respiratory function of *mdx* mice. Taken together, BMT is unlikely to significantly ameliorate pathogenesis in dystrophin-deficient muscle.

In contrast to *mdx* mice, BMT led to a significant increase in lifespan and an increased growth rate of *dy* mice. Diaphragm muscle pathology of *dy* mice was also improved by BMT. Of particular significance is that laminin-α2, which is deficient in *dy* mice, was restored not only in GFP-positive myofibers but also GFP-negative myofibers. Additionally, BMT improved muscle strength and respiratory function of *dy* mice. This is the first report to demonstrate that BMT improves clinical symptoms of a mouse model of muscular dystrophy. Our study indicates that muscular dystrophy due to loss of laminin-α2 can be significantly ameliorated by BMT. Thus BMT could be an effective therapy for laminin-α2-deficient muscular dystrophy.

The reason why BMT was effective in *dy* mice but not *mdx* mice remains to be clarified. Two possible mechanisms to correct dystrophic pathology in *dy* mice by BMT are considered. First, the circulating BM-derived cells more easily fuse into host dystrophic cells through disrupted basal lamina in

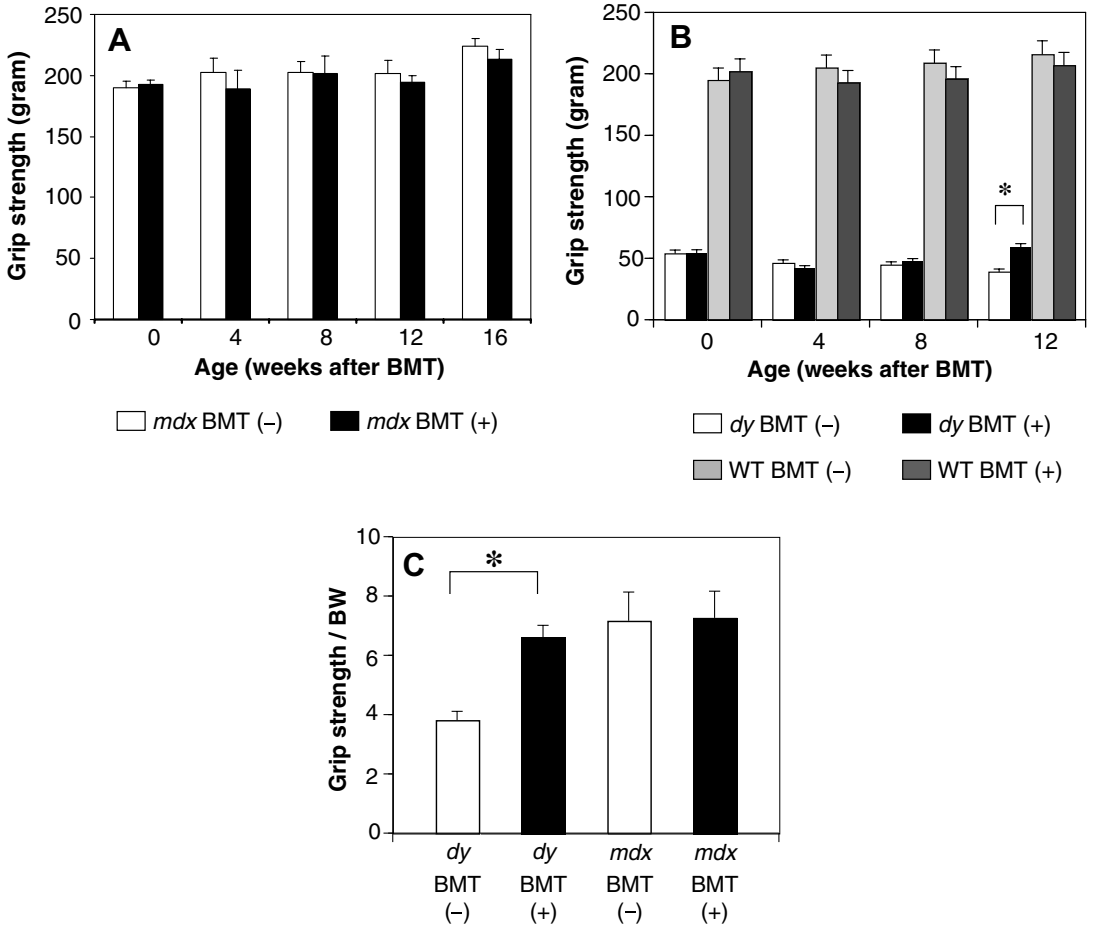
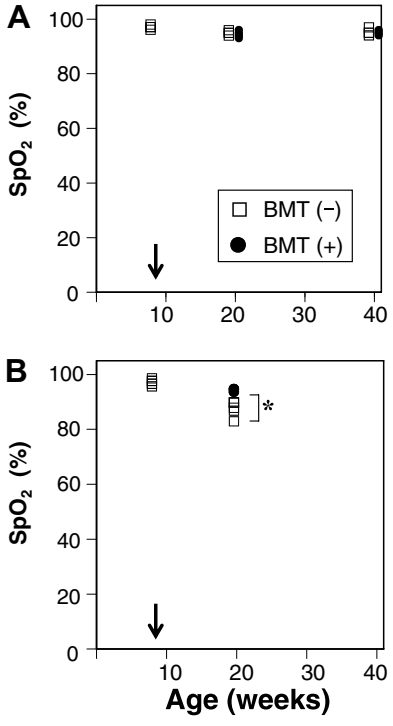


Fig. 5. Peak force measurement (g) of grip strength. (A) *mdx* mice before, 4, 8, 12, and 16 weeks after BMT ($n = 7$, each). (B) *dy* mice before, 4, 8, and 12 weeks after BMT ($n = 7$, each). Note that the grip strength of *dy*-BMT mice was significantly stronger than *dy*-control mice at 12 weeks after BMT. (C) Representation of grip strength per body weight of each group. Data are expressed as means ± S.D. $^{*}P < 0.05$, Bonferoni's test after one-way ANOVA. BMT (−), control group; BMT (+), treated group.



dy skeletal muscles. Second, laminin-α2 molecules produced by BM-derived cells diffuse in the vicinity to form normal laminin-2 networks even in GFP-negative myofibers. In contrast, in *mdx* mice, the basal lamina is so well-preserved that foreign BM-derived cells could not integrate into recipient cells.

In addition to MDC1A, BMT could be effective for other types of dystrophy including Fukuyama congenital muscular dystrophy, muscle-eye-brain disease, and Walker–Warburg syndrome in which partial laminin-α2 deficiency and disruption of basal lamina are commonly observed [25].

In conclusion, on the basis of our results, BMT may be more successful in the treatment of muscle diseases such as MDC1A than DMD.

Fig. 6. SpO_2 measurements. (A) *mdx* mice before and 12 and 32 weeks after BMT ($n = 5$, each). (B) *dy* mice before BMT and 12 weeks after BMT. Note that SpO_2 of *dy*-BMT group retained higher oxygen saturation whereas the *dy*-control group showed a decrease in hypoxia ($n = 5$, each). Arrow indicates 8 weeks of age at the time of BMT was performed. BMT (−), control group; BMT (+), treated group.

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