

# Impaired viability of muscle precursor cells in muscular dystrophy with glycosylation defects and amelioration of its severe phenotype by limited gene expression

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**A group of muscular dystrophies, dystroglycanopathy is caused by abnormalities in post-translational modifications of dystroglycan (DG). To understand better the pathophysiological roles of DG modification and to establish effective clinical treatment for dystroglycanopathy, we here generated two distinct conditional knock-out (cKO) mice for *fukutin*, the first dystroglycanopathy gene identified for Fukuyama congenital muscular dystrophy. The first dystroglycanopathy model—myofiber-selective *fukutin*-cKO [muscle creatine kinase (MCK)-*fukutin*-cKO] mice—showed mild muscular dystrophy. Forced exercise experiments in presymptomatic MCK-*fukutin*-cKO mice revealed that myofiber membrane fragility triggered disease manifestation. The second dystroglycanopathy model—muscle precursor cell (MPC)-selective cKO (*Myf5*-*fukutin*-cKO) mice—exhibited more severe phenotypes of muscular dystrophy. Using an isolated MPC culture system, we demonstrated, for the first time, that defects in the *fukutin*-dependent modification of DG lead to impairment of MPC proliferation, differentiation and muscle regeneration. These results suggest that impaired MPC viability contributes to the pathology of dystroglycanopathy. Since our data suggested that frequent cycles of myofiber degeneration/regeneration accelerate substantial and/or functional loss of MPC, we expected that protection from disease-triggering myofiber degeneration provides therapeutic effects even in mouse models with MPC defects; therefore, we restored *fukutin* expression in myofibers. Adeno-associated virus (AAV)-mediated rescue of *fukutin* expression that was limited in myofibers successfully ameliorated the severe pathology even after disease progression. In addition, compared with other gene therapy studies, considerably low AAV titers were associated with therapeutic effects. Together, our findings indicated that *fukutin*-deficient dystroglycanopathy is a regeneration-defective disorder, and gene therapy is a feasible treatment for the wide range of dystroglycanopathy even after disease progression.**

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## INTRODUCTION

Dystroglycanopathy includes Walker–Warburg syndrome, muscle–eye–brain disease, Fukuyama congenital muscular dystrophy (FCMD) and several forms of congenital and limb–girdle muscular dystrophies (1). Dystroglycanopathy is indicated by a wide variety of clinical symptoms; the most severe end of the clinical spectrum is characterized by congenital muscular dystrophy with severe structural brain and eye abnormalities, whereas the mildest end presents in adult life with limb–girdle muscular dystrophy without brain or eye involvement (1). FCMD is the first dystroglycanopathy to be reported (2,3), and it is the second most common childhood muscular dystrophy in Japan. The founder mutation, a SINE-VNTR-*Alu* retrotransposon insertion in the 3′ noncoding region of *fukutin*, causes abnormal splicing that leads to the production of non-functional proteins in FCMD (4,5). FCMD is characterized by severe congenital muscular dystrophy, abnormal neuronal migration associated with mental retardation and epilepsy and, frequently, eye abnormalities. It often results in early death before the age of 20 (6). Several point mutations in *fukutin* have also been reported to be associated with dystroglycanopathy in Japan and other countries (7,8).

More than 10 genes [protein *O*-mannosyltransferase 1 (*POMT1*), protein *O*-mannosyltransferase 2 (*POMT2*), protein *O*-linked mannose  $\beta$ -1,2-*N*-acetylglucosaminyltransferase 1 (*POMGNT1*), *fukutin*, fukutin-related protein (*FKRP*), *LARGE*, dolichol-phosphate-mannose synthase (*DPM2* and *DPM3*), isoprenoid synthase domain containing (*ISPD*) gene, glycosyltransferase-like domain containing 2 (*GTDC2*) gene and  $\beta$ -1,3-*N*-acetylglucosaminyltransferase 1 (*B3GNT1*)], implicated in dystroglycanopathies, have been shown or expected to be involved in the glycosylation pathway of  $\alpha$ -dystroglycan ( $\alpha$ -DG) (1,9,10). *POMGNT1* and the *POMT1/2* complex possess glycosyltransferase activities and can directly synthesize *O*-mannosyl sugar chains on  $\alpha$ -DG (11,12). *Fukutin*, *FKRP* and *LARGE* are involved in a novel phosphodiester-linked modification, namely, a post-phosphoryl modification, of *O*-mannose on  $\alpha$ -DG (13,14). Recently, it has been shown that *LARGE* can act as a bifunctional glycosyltransferase with both xylosyltransferase and glucuronyltransferase activities (15). The DG gene *DAG1* encodes both  $\alpha$ - and  $\beta$ -DG, which is post-translationally cleaved into the two subunits (16).  $\alpha$ -DG is a highly glycosylated protein and serves as the receptor subunit for extracellular proteins such as laminins, perlecan, agrin, neuexin and pikachurin (9,17). *O*-mannosyl glycosylation and the post-phosphoryl modification are required for the ligand-binding activities of  $\alpha$ -DG (3,13). Hypoglycosylation and reduced ligand-binding activity of  $\alpha$ -DG are common characteristics of dystroglycanopathy.  $\alpha$ -DG is anchored to the plasma membrane through non-covalent interactions with the transmembrane subunit  $\beta$ -DG.  $\beta$ -DG intracellularly interacts with dystrophin, whose mutations lead to Duchenne/Becker muscular dystrophy, and dystrophin, in turn, binds to actin filaments. This molecular linkage, created by laminin-DG-dystrophin-actin filaments, is thought to provide mechanical stability to the plasma membrane of the muscle fiber; thus, disruption of this linkage is considered a key pathological event in several forms of muscular dystrophy. In FCMD skeletal

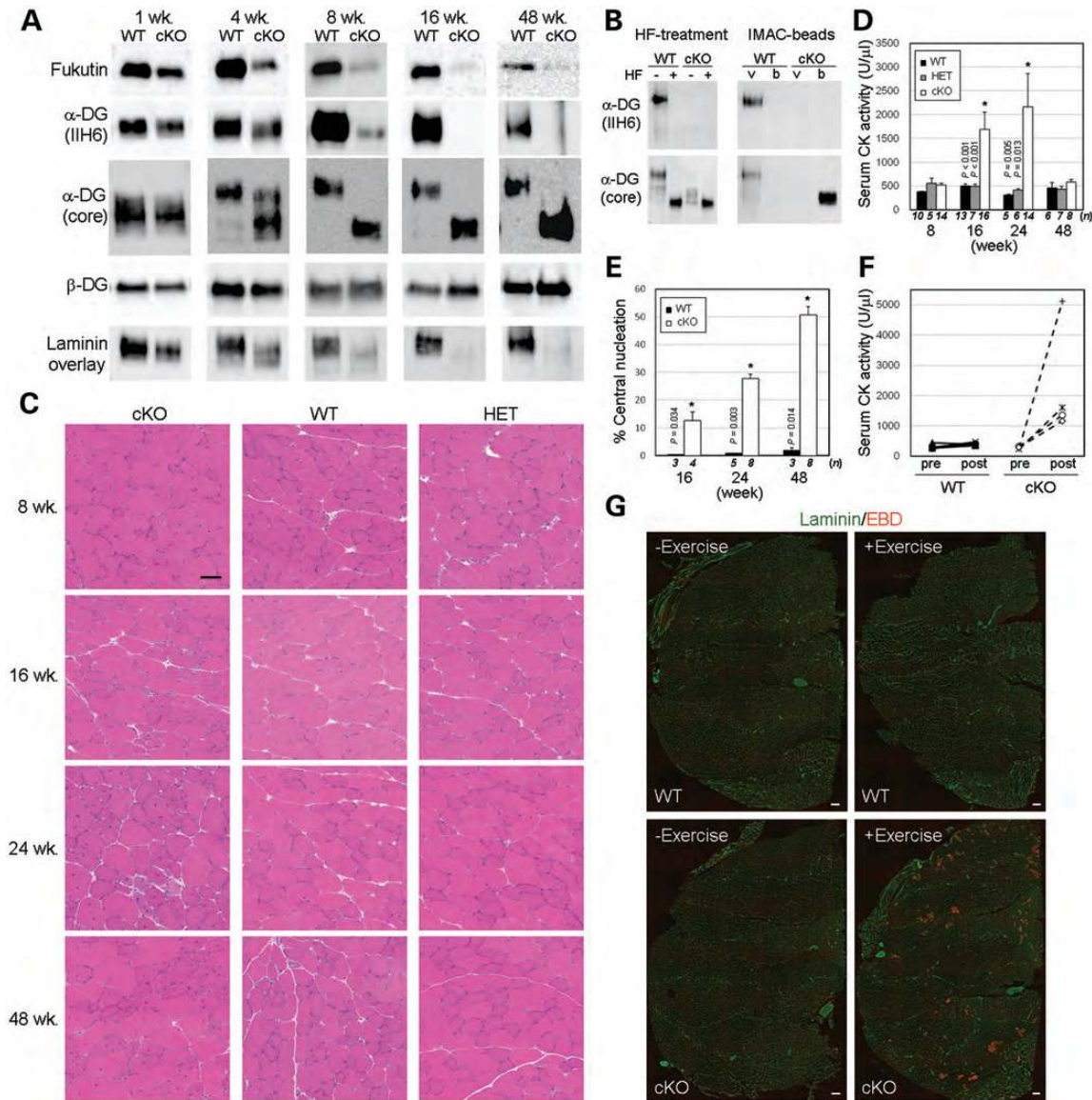
muscles, in addition to dystrophic muscular changes, there are certain characteristics such as intensive connective tissue infiltration and the presence of predominant small-sized fibers from the early infantile stage (6). Furthermore, aberrant neuromuscular junctions and delayed muscle fiber maturation have been implicated in the pathology of FCMD (18). FCMD also shows central nervous system involvement. Together, these data suggest that more complex and unknown physiological roles of  $\alpha$ -DG modification underlie the skeletal muscle pathology of dystroglycanopathy.

Recent studies have identified new genes associated with dystroglycanopathy (19–21), and an increasing number of patients are being diagnosed with dystroglycanopathy worldwide. However, the pathogenesis of this condition is not fully understood, and no effective clinical treatment has been established. To understand the pathogenesis and establish a therapeutic strategy for dystroglycanopathy, we developed two distinct *fukutin* conditional knock-out (cKO) mice as models for dystroglycanopathy. In our study, investigation of presymptomatic *fukutin*-deficient mice provided direct evidence that fragility of the myofiber membrane triggers the pathogenesis of dystroglycanopathy. We also used an isolated muscle precursor cell (MPC) culture system to demonstrate, for the first time, that defects in the *fukutin*-dependent modification of DG lead to impairment of MPC proliferation, differentiation and muscle regeneration. We predicted that protection from disease-triggering myofiber degeneration would prevent substantial and/or functional loss of MPC, thereby providing therapeutic effects. Indeed, we demonstrate that restoration of *fukutin* expression in myofibers successfully ameliorates the severe pathology even after disease progression. These results indicate that gene therapy is a feasible treatment for dystroglycanopathy.

## RESULTS

### Generation and characterization of myofiber-selective *fukutin* cKO mice

To generate *fukutin*-cKO mice, floxed *fukutin* mice (*fukutin*<sup>lox/lox</sup>) were crossed with muscle creatine kinase (MCK)-*Cre* mice (22) or *Myf5*-*Cre* mice (23), which express the *Cre* gene with the help of the MCK promoter or *Myf5* promoter, respectively (Supplementary Material, Fig. S1). The MCK promoter is active in differentiating and differentiated muscle cells (24), and MCK expression reaches maximum levels at post-natal day 10 and remains constantly high throughout life (25). First, we analyzed the MCK-*fukutin*-cKO mice at different time points. In the skeletal muscles of these mice, we confirmed dramatic reduction in the *fukutin* protein after the age of 4 weeks (Fig. 1A). Abnormal modification of  $\alpha$ -DG is indicated by decreased molecular weight, loss of immunoreactivity against the monoclonal I1H6 antibody, which recognizes properly glycosylated  $\alpha$ -DG (3), and decreased laminin-binding activity. Abnormally modified  $\alpha$ -DG was predominant in MCK-*fukutin*-cKO mice aged >8 weeks (Fig. 1A). Loss of the post-phosphoryl modification was further confirmed by subjecting the protein to treatment with cold aqueous hydrofluoric (HF) acid, which cleaves phosphoester linkages, and to inorganic metal-affinity chromatography (IMAC), which captures monoester-linked phosphorylated compounds



**Figure 1.** Pathological characterization of MCK-fukutin-cKO mice. (A) Western blot analysis of fukutin protein expression and  $\alpha$ -DG modification in MCK-fukutin-cKO (cKO) and litter control WT skeletal muscles at different ages (1, 4, 8, 16 and 48 weeks).  $\beta$ -DG was used as a loading control. Laminin-binding activity of  $\alpha$ -DG was examined by the laminin overlay assay. (B) Post-phosphoryl modification of  $\alpha$ -DG from MCK-fukutin-cKO and control WT skeletal muscles. The absence of the post-phosphoryl modification was confirmed by HF treatment and IMAC-bead-binding assay. The void (v) and bound (b) fractions of the IMAC beads were analyzed by western blotting. (C) H&E staining of tibialis anterior muscles. Bar = 50  $\mu$ m. (D) Serum CK activity and (E) proportion of myofibers with centrally located nuclei. Data shown are mean  $\pm$  SEM for each group ( $n$  is indicated in the graph). \* $P \leq 0.05$  for both cKO versus WT and cKO versus HET (D) and cKO versus WT (E) (Mann-Whitney  $U$  test;  $P$ -values are indicated in the graph). (F) Serum CK activity before and after forced exercise. Serum CK levels of individual mice ( $n = 4$  in each genotype) were measured before (pre) and after (post) exercise. (G) Uptake of Evans blue dye into myofibers after forced exercise. MCK-fukutin-cKO and control WT mice were subjected to forced exercise (+Exercise); subsequently, the muscle sections were stained with laminin (green) for individual fibers and merged with Evans blue dye (red). Mice not subjected to exercise were used as controls (-Exercise). Bar = 200  $\mu$ m.

(13,14). The molecular weight of  $\alpha$ -DG in the skeletal muscles of control (WT) mice was dramatically reduced after HF treatment, and  $\alpha$ -DG did not bind to IMAC beads because the phosphodiester-linked modification was intact; in contrast,  $\alpha$ -DG in the skeletal muscles of MCK-fukutin-cKO mice showed little sensitivity to HF and bound to IMAC beads (Fig. 1B), indicating incomplete post-phosphoryl modification. Immunofluorescence staining with an antibody against an  $\alpha$ -DG core protein showed that  $\alpha$ -DG localized to the

sarcolemma of MCK-fukutin-cKO mice as seen in normal controls (Supplementary Material, Fig. S2A), which suggests that cellular trafficking of  $\alpha$ -DG is little affected by fukutin deficiency. These results confirmed abnormal modification of  $\alpha$ -DG in the skeletal muscles of MCK-fukutin-cKO mice.

Hematoxylin and eosin (H&E) staining revealed that 16-week-old MCK-fukutin-cKO mice showed signs of muscular dystrophy, such as myonecrosis and central nucleation (Fig. 1C). Serum creatine kinase (CK) activity in

16-week-old MCK-fukutin-cKO mice was significantly higher than that in controls (Fig. 1D). These pathological features were not observed in 8-week-old mice (Fig. 1C and D); a possible reason may be the presence of residual  $\alpha$ -DG with proper glycosylation (Supplementary Material, Fig. S2B). The population of myofibers with centrally located nuclei, an indication of repeated cycles of myofiber degeneration/regeneration, increased with age (Fig. 1E); however, more advanced pathology, such as infiltration of fat and connective tissues, was rarely observed even in 48-week-old MCK-fukutin-cKO mice (Fig. 1C).

It has been widely believed that functional and/or substantial loss of DG-containing protein complexes (i.e. the dystrophin-glycoprotein complex) leads to disease-causing membrane fragility. This concept is based on results of forced exercise experiments in animals with muscular dystrophy, which led to increases in the serum CK levels and uptake of membrane-impermeable Evans blue dye by myofibers (26,27). However, these experiments were conducted in diseased animals; therefore, it remains unclear whether membrane fragility triggers disease-causing phenotype. Therefore, we subjected 10-week-old MCK-fukutin-cKO mice, which showed abnormal  $\alpha$ -DG modification but no pathology (Supplementary Material, Fig. S2C), to forced exercise. After forced exercise, serum CK levels were dramatically increased in the MCK-fukutin-cKO mice but not in the control mice (Fig. 1F). Myofibers with membrane-impermeable Evans blue dye uptake were also observed only in the exercise-administered MCK-fukutin-cKO mice (Fig. 1G). These data indicate that the plasma membrane of the muscle cells becomes weak before disease onset, providing proof-of-principle that membrane fragility triggers disease manifestation.

### Characterization of MPC-selective fukutin cKO mice

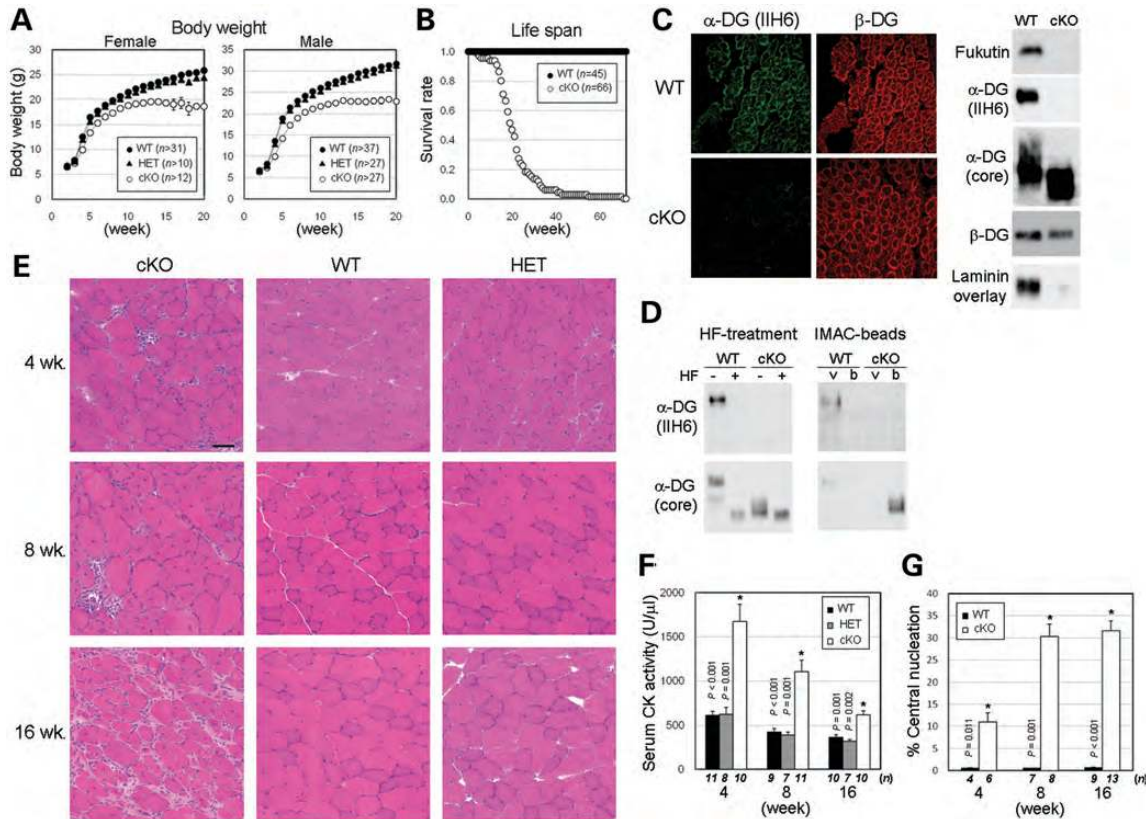
Loss of fukutin in differentiated myofibers results in only mild and slow-progressing disease-causing phenotypes. We hypothesized that fukutin-dependent modification also plays a role in MPCs that are not targeted by MCK-Cre-mediated recombination. Therefore, we generated cKO mice lacking fukutin in MPCs by crossing flox fukutin mice with Myf5-Cre knock-in mice (23) expressing Cre recombinase under the control of the endogenous Myf5 promoter (Myf5-fukutin-cKO mice; Supplementary Material, Fig. S1). It has been reported that the Myf5-Cre allele recapitulates the expression pattern of the endogenous Myf5 gene and is uniformly expressed in all proliferating myoblasts (23). The Myf5-fukutin-cKO mice grossly show little difference compared with the litter controls until  $\sim$ 2 weeks of age; thereafter, increase in body weight was significantly retarded (Fig. 2A). Most Myf5-fukutin-cKO mice died by 6 months (Fig. 2B). Reduction in fukutin protein expression and abnormal modification were confirmed by immunofluorescence, western blotting, HF treatment and IMAC-bead assay (Fig. 2C and D). As is the case of MCK-fukutin-cKO,  $\alpha$ -DG in Myf5-fukutin-cKO localizes to the sarcolemma (Supplementary Material, Fig. S2D). H&E staining revealed progressive pathological changes in Myf5-fukutin-cKO skeletal muscles (Fig. 2E). At 2 weeks, myonecrotic fibers were sparse (Supplementary Material, Fig. S2E), and at 4 weeks, in addition to myonecrotic fibers,

myofibers with centrally located nuclei were observed (Fig. 2E). Serum CK levels and the proportion of the myofibers with centrally located nuclei were significantly higher in Myf5-fukutin-cKO mice than in the controls at 4, 8 and 16 weeks (Fig. 2F and G). Sixteen-week-old Myf5-fukutin-cKO mice showed more advanced pathological changes, such as fiber size variation and fibrosis (Fig. 2E). A few specimens showed milder phenotypic changes accompanied by increases in the normally glycosylated  $\alpha$ -DG population (Supplementary Material, Fig. S2F). Overall, different phenotypes of the MCK-fukutin-cKO and Myf5-fukutin-cKO mice suggested a pathophysiological role of fukutin-dependent modification in MPCs.

### Impaired viability of MPCs in Myf5-fukutin-cKO mice

To determine the impact of fukutin deficiency on MPC activity, we isolated SM/C-2.6(+) satellite cells from young (slightly affected) and adult (diseased) Myf5-fukutin-cKO muscles and then cultured them as MPCs (i.e. myoblasts) (28). The number of isolated SM/C-2.6(+) cells tended to be less in young fukutin-deficient muscles and was significantly reduced in adults compared with the litter controls (Fig. 3A). The proliferation activity of the isolated MPCs was slightly but significantly decreased in young and severely reduced in adult Myf5-fukutin-cKO muscles (Fig. 3B). The differentiation activity of fukutin-deficient myoblasts was significantly lower than that of the control mice (Fig. 3C). Quantification of the Pax7 immunofluorescence signal, a satellite cell marker, on skeletal muscle sections also suggested decreases in the number of satellite cells in adult Myf5-fukutin-cKO mice compared with control mice (Supplementary Material, Fig. S3A). We also examined the population of activated satellite cells by Pax7/MyoD double staining on the skeletal muscle sections from Myf5-fukutin-cKO mice. The results suggested that the number of active satellite cells was reduced in 16-week-old Myf5-fukutin-cKO mice compared with that in 8-week-old Myf5-fukutin-cKO mice (Supplementary Material, Fig. S3B). These data suggest that in addition to decreases in the number of satellite cells, the activation state of satellite cells is impaired in Myf5-cKO mice as the disease progresses.

We next examined *in vivo* regeneration capability of the Myf5-fukutin-cKO muscles after cardiotoxin (CTX)-induced muscle degeneration. After 14 days of the CTX challenge in adult mice ( $\sim$ 3 months old), we observed that the proportion of small myofibers was strikingly higher in Myf5-fukutin-cKO mice than in the controls (Fig. 3D and E). In some cases, the CTX-injected Myf5-fukutin-cKO muscles showed severe atrophic changes compared with the contralateral saline-injected ones (Supplementary Material, Fig. S3C). In younger ( $\sim$ 4 weeks old) Myf5-fukutin-cKO mice, after 14 days of the CTX challenge, no obvious histological difference was noted compared with the controls (Supplementary Material, Fig. S3D); however, after 5 days of the CTX challenge, the proportion of smaller regenerating fibers (that are embryonic myosin-positive) was higher in Myf5-fukutin-cKO muscles than in the controls (Supplementary Material, Fig. S3E and F). These minor impairments in the regeneration of younger Myf5-fukutin-cKO skeletal muscles are consistent with the *in vitro* results. Overall, our data showed that fukutin-dependent



**Figure 2.** Pathological characterization of Myf5-fukutin-cKO mice. (A) Temporal changes in body weight. Data shown are mean  $\pm$  SEM for each group ( $n$  is indicated in the graph). (B) Survival curve of Myf5-fukutin cKO mice. (C) Immunofluorescence and western blot analyses of fukutin expression and  $\alpha$ -DG modification. Skeletal muscles of new born and 1-week-old Myf5-fukutin-cKO and control WT mice were used for immunofluorescence and western blotting, respectively.  $\beta$ -DG was used as a control. Laminin-binding activity of  $\alpha$ -DG was examined by the laminin overlay assay. (D) Post-phosphoryl modification of  $\alpha$ -DG from Myf5-fukutin-cKO and control WT skeletal muscles. The absence of post-phosphoryl modification was tested by HF treatment and IMAC-bead-binding assay. The void (v) and bound (b) fractions of the IMAC beads were analyzed by western blotting. (E) H&E staining of tibialis anterior muscles. Bar = 50  $\mu$ m. (F) Serum creatin kinase activity and (G) proportion of myofibers with centrally located nuclei. Data shown are mean  $\pm$  SEM for each group ( $n$  is indicated in the graph). \* $P \leq 0.05$  for both cKO versus WT and cKO versus HET (F) and cKO versus WT (G) (Mann–Whitney  $U$  test;  $P$ -values are indicated in the graph).

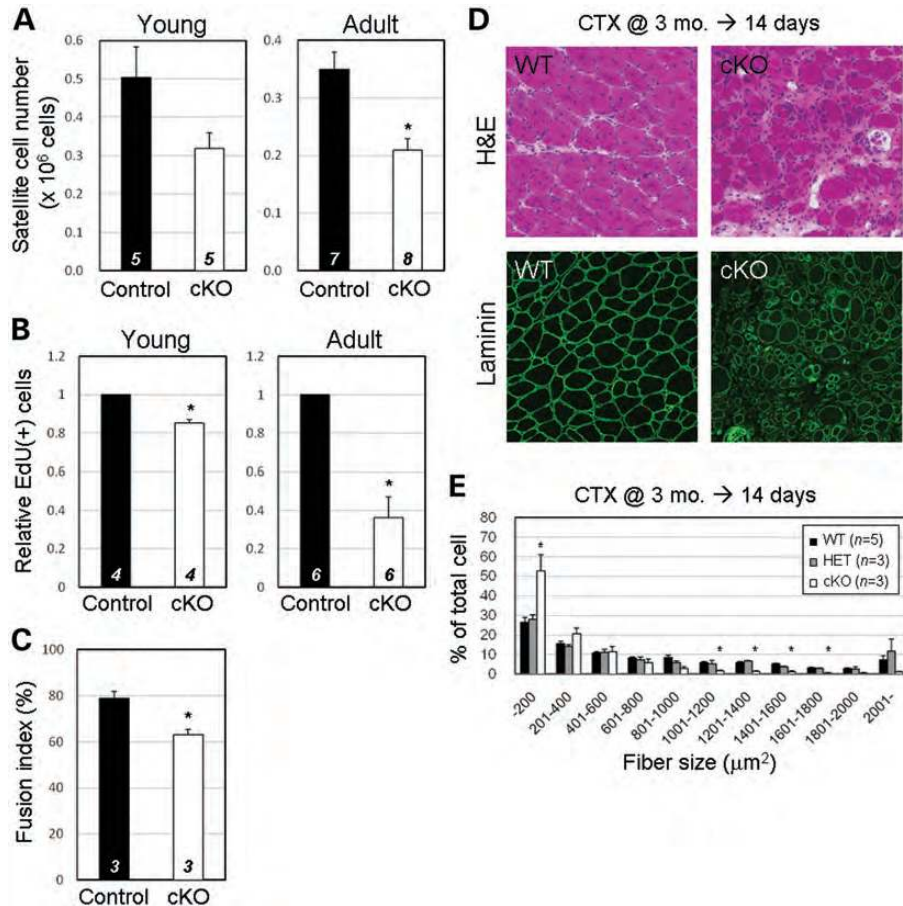
modification plays important roles in maintaining MPC viability, and consequently, muscle regeneration capability, suggesting that these defects may contribute to the severe phenotype of dystroglycanopathy.

#### Amelioration of the severe pathology by limited fukutin rescue in myofibers

Our pathological analysis of the two distinct fukutin-cKO mice suggested that membrane fragility triggers disease manifestation and that impaired MPC viability is related to disease progression and severity of dystroglycanopathy. These findings indicate that a therapeutic strategy must involve prevention of myofiber membrane weakness and/or rescue of substantial loss and dysfunction of MPCs. In addition, dystroglycanopathy is usually diagnosed after disease manifestation, and thus, treatments should be effective even after disease progression. Since our data suggested that frequent cycles of myofiber degeneration/regeneration accelerate substantial and/or functional loss of MPC, we expected that protection from disease-triggering myofiber degeneration provides therapeutic effects even in mouse models with MPC defects. In this

study, to prevent disease-causing myofiber degeneration, we examined whether rescue of fukutin expression that is limited in myofibers is therapeutically beneficial in Myf5-fukutin-cKO mice. Therefore, we constructed recombinant AAV9 (AAV, adeno-associated virus) vectors containing the mouse *fukutin* cDNA under the MCK promoter (AAV9-MCK-*fukutin*).

We first administered intramuscular injections of AAV9-MCK-*fukutin* to 1-week-old (i.e. before disease manifestation) or 8-week-old (i.e. after disease manifestation) Myf5-fukutin-cKO mice; then, we examined the therapeutic effects after 2 months. In both cases, fukutin protein expression was higher in the AAV-injected Myf5-fukutin-cKO muscles than in the control WT muscles (endogenous fukutin protein in muscle lysates is below detectable levels) (Fig. 4A and E). IIH6-positive  $\alpha$ -DG was restored, indicating functional rescue of *fukutin* gene expression even in adult cases (Fig. 4A, B, E and F). Histological and quantitative analyses showed that gene transfer at 1 week prevented disease manifestation (Fig. 4C and D). When gene transfer was challenged in 8-week-old mice, H&E staining showed milder phenotype in AAV-treated Myf5-fukutin-cKO muscles than



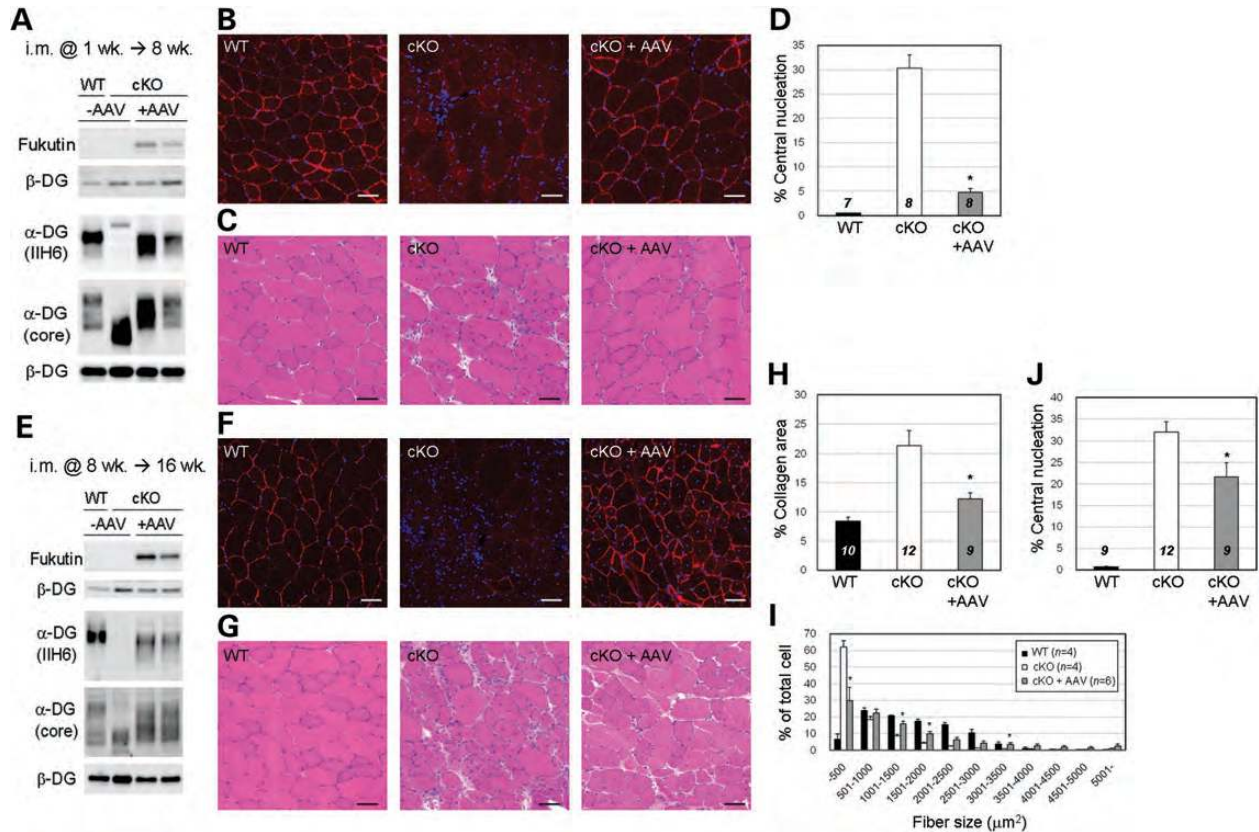
**Figure 3.** Impaired MPC activities and muscle regeneration in Myf5-fukutin-cKO mice. (A) The number of isolated satellite cells from young (2 weeks old) and adult (4 months old) Myf5-fukutin-cKO mice.  $P = 0.117$  for young, and  $P = 0.005$  for adult (Mann–Whitney  $U$  test). (B) Proliferation activity of the isolated MPCs. MPCs from young or adult Myf5-fukutin-cKO muscles were cultured for 2 days, and 5-ethynyl-2'-deoxyuridine-positive (EdU+) cells were counted.  $P = 0.014$  for young, and  $P = 0.002$  for adult (Mann–Whitney  $U$  test). (C) Differentiation activity of the isolated MPCs. MPCs from young Myf5-fukutin-cKO muscles were cultured in growth media for 2 days and then in differentiation media for 2 days. The cells were fixed, and multinucleated myotubes were counted.  $P = 0.05$  (Mann–Whitney  $U$  test). For (A)–(C), data shown are mean  $\pm$  SEM for each group ( $n$  is indicated in the graph). \* $P \leq 0.05$  compared with litter controls (Mann–Whitney  $U$  test). (D) Regeneration after CTX-induced muscle degeneration. CTX was injected into the tibialis anterior muscles of 3-month-old Myf5-fukutin-cKO and control mice (WT and HET). After 14 days, the muscle sections were analyzed by H&E staining and immunofluorescence staining with laminin. (E) Quantitative analysis for myofiber size variation after the CTX challenge. Data shown are mean  $\pm$  SEM for each group ( $n$  is indicated in the graph). \* $P \leq 0.05$  for both cKO versus WT and cKO versus HET (Mann–Whitney  $U$  test).

in non-treated ones (Fig. 4G). Quantitatively, connective tissue infiltration and prevalence of small fibers were significantly reduced (Fig. 4H and I), whereas a substantial number of myofibers with central nucleation was still present after the gene transfer (Fig. 4J).

Next, we examined systemic delivery of the *fukutin* gene via tail vein injection into 4-week-old Myf5-fukutin-cKO mice with early-stage muscular dystrophy, primarily because diagnosis occurs during this stage in humans. After 2 months of the injection, we confirmed fukutin protein expression and recovery of  $\alpha$ -DG modification in the treated Myf5-fukutin-cKO mice (Fig. 5A and B). After gene transfer, body and muscle weight were restored (Fig. 5C and D), and grip strength was dramatically improved, indicating recovery of muscle physiological function (Fig. 5E). H&E staining (Fig. 5F) and quantitative analyses of connective tissue infiltration (Fig. 5G) and fiber size variation (Fig. 5H) showed amelioration of muscle pathology; however, there were still a few necrotic fibers

and a substantial proportion of myofibers with centrally located nuclei (Fig. 5I). Similar therapeutic effects were also obtained in other muscles (Supplementary Material, Fig. S4). Our results show that limited recovery of fukutin expression in myofibers, even after disease progression, can successfully ameliorate the severe phenotype of Myf5-fukutin-cKO mice.

We also constructed recombinant AAV9 vectors containing the mouse *fukutin* cDNA under the CMV promoter (AAV9-CMV-*fukutin*), which is commonly used for driving the expression of transgenes in a wide range of cell types. Two months after tail-vein injection in 4-week-old Myf5-fukutin-cKO mice, we observed therapeutic effects similar to those observed in AAV9-MCK-*fukutin*-treated Myf5-fukutin-cKO mice (Supplementary Material, Fig. S5). There was no obvious difference in the efficiency for the recovery of IHH6 immunoreactivity (the proportion of IHH6-positive to laminin-positive fibers) between the cases of AAV9-MCK-*fukutin* ( $77.4 \pm 3.8\%$ ,  $n = 5$ ) and AAV9-CMV-*fukutin* ( $74.8 \pm 3.6\%$ ,



**Figure 4.** Gene transfer of *fukutin* via AAV9 intramuscular injection into Myf5-fukutin-cKO mice. AAV9-MCK-*fukutin* was administered to 1-week-old (A–D) and 8-week-old (E–J) Myf5-fukutin-cKO mice via intramuscular injection. Two months after the gene transfer, recovery of fukutin protein and  $\alpha$ -DG glycosylation was confirmed by western blotting (A and E) and IIH6-immunofluorescence staining (B and F) (bar = 50  $\mu$ m). For fukutin protein expression and  $\alpha$ -DG modification, total lysate and wheat germ agglutinin-enriched fractions, respectively, were subjected to western blotting. In both cases,  $\beta$ -DG was used as a loading control. Western blotting results for two AAV-treated mice are shown. Therapeutic effects were quantitatively evaluated in terms of the proportion of myofibers with centrally located nuclei (D;  $P = 0.001$ , J;  $P = 0.023$ ), connective tissue infiltration (H;  $P = 0.016$ ) and fiber size variation (I;  $*P \leq 0.05$ ). Data shown are mean  $\pm$  SEM for each group ( $n$  is indicated in the graph).  $*P \leq 0.05$  compared with non-treated cKO mice (Mann–Whitney  $U$  test).

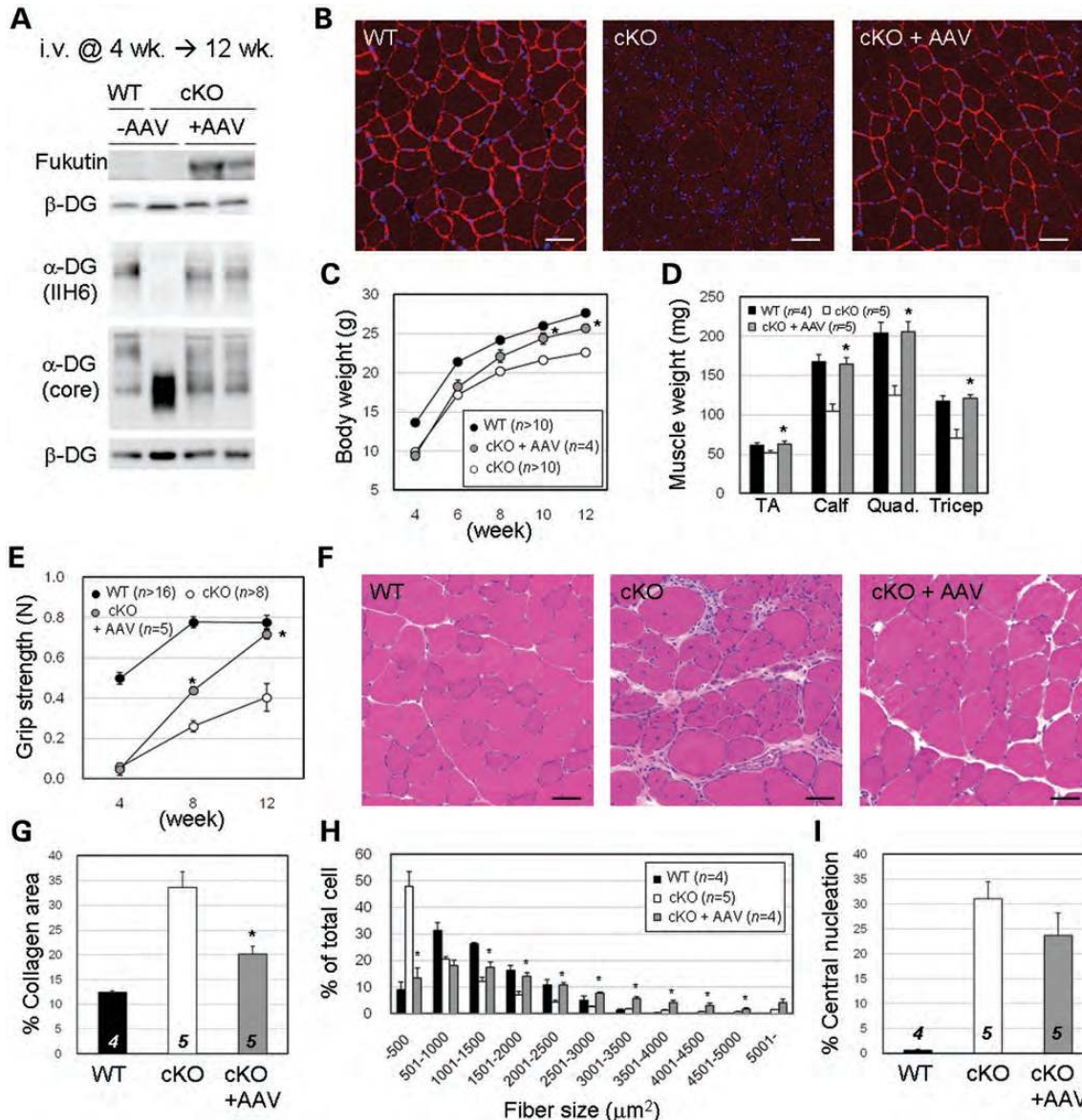
$n = 5$ ). In addition, the population of myofibers with centrally located nuclei was significantly improved (Supplementary Material, Fig. S5I). We also examined the effects of intraperitoneal injections of AAV9-CMV-*fukutin* in 1-week-old Myf5-fukutin-cKO mice. Two months after the injections, we observed partial recovery of  $\alpha$ -DG glycosylation and amelioration of the pathology compared with that observed in non-treated Myf5-fukutin-cKO mice (Supplementary Material, Fig. S6A–D).

The predominant mutation in FCMD is a retrotransposon insertion (4). We previously generated a transgenic knock-in mouse model carrying this insertion (29). The knock-in Hp/– mice represent compound heterozygotes for the insertion and a nonsense *fukutin* mutation. Although Hp/– mice show abnormal glycosylation of  $\alpha$ -DG, a small amount of intact  $\alpha$ -DG is also present; this prevents muscular dystrophy (29). We administered intraperitoneal injections of AAV9-CMV-*fukutin* to 1-week-old Hp/– mice and examined  $\alpha$ -DG glycosylation status after 16 and 48 weeks. We detected increased levels of fukutin expression and IIH6-positive  $\alpha$ -DG in the AAV-treated Hp/– skeletal muscles even 48 weeks after the gene transfer (Supplementary Material, Fig. S6E). These data suggest that the transferred *fukutin* gene persists

in correcting abnormal glycosylation of  $\alpha$ -DG for a considerable length of time.

## DISCUSSION

In this study, we developed and analyzed two distinct *fukutin* cKO mice to understand the pathogenesis and to establish a therapeutic strategy for dystroglycanopathy. Our data showed that MPC-selective Myf5-fukutin-cKO mice exhibited more severe phenotypes of muscular dystrophy than myofiber-selective MCK-fukutin-cKO mice. Very recently, Campbell and colleagues also generated *fukutin* cKO mice, using Myf5-Cre and MCK-Cre mice (30). Pathological analysis of our Myf5-fukutin-cKO and MCK-fukutin-cKO mice showed results that were mostly consistent with those reported by Campbell and colleagues: increased serum CK levels in both cKO lines; milder phenotypes of MCK-fukutin-cKO than Myf5-fukutin-cKO; and decreases in grip strength, body mass and longevity of Myf5-fukutin-cKO mice. Our study includes further detailed histopathological characterization of the disease onset and progression in both lines. More importantly, our present study clarifies features of dystroglycanopathy that



**Figure 5.** Systemic gene transfer of *fukutin* into Myf5-fukutin-cKO mice. AAV9-MCK-*fukutin* was administered to 4-week-old Myf5-fukutin-cKO mice via tail vein injection. Two months after the gene transfer, the skeletal muscles were analyzed and compared with non-treated Myf5-fukutin-cKO muscles. Recovery of fukutin protein and  $\alpha$ -DG modification was confirmed by western blotting (A) and immunofluorescence (B) (bar = 50  $\mu$ m). For fukutin protein expression and  $\alpha$ -DG modification, total lysate and wheat germ agglutinin-enriched fractions, respectively, were subjected to western blotting. In both cases,  $\beta$ -DG was used as a loading control. Therapeutic effects were evaluated by body weight change (C;  $P = 0.016$  and  $0.01$  for 10 and 12 weeks, respectively) and muscle weight change (D;  $P = 0.047$  for tibialis anterior, and  $0.014$  for other muscles), grip strength (E;  $P = 0.007$  and  $0.003$  for 8 and 12 weeks, respectively), H&E staining (F) (bar = 50  $\mu$ m), connective tissue infiltration estimated by quantification of collagen I immunoreactive areas (G;  $P = 0.028$ ), fiber size variation (H;  $*P \leq 0.05$ ) and central nucleation (I). Data from the tibialis anterior muscle are shown (F–I). Histopathology (F) and quantitative analyses (G and H) indicate amelioration of disease severity after the gene transfer. Data shown are mean  $\pm$  SEM for each group ( $n$  is indicated in the graph).  $*P \leq 0.05$  compared with the non-treated cKO mice (Mann–Whitney  $U$  test). TA, tibialis anterior; Quad., quadriceps.

enhance our understanding of its pathogenesis—specifically, the trigger initiating disease pathogenesis and the biological mechanism underlying the severe phenotype.

Our study provides direct evidence using presymptomatic fukutin-deficient mice that myofiber membrane fragility triggers disease manifestation. Although histological data in both studies suggested regeneration delay or impairment in Myf5-fukutin-cKO skeletal muscles, our study provides the first direct evidence for impaired MPC activity and viability

using the isolated myoblast culture system. We observed that the proliferation and differentiation activities of the isolated MPCs from little-affected young Myf5-fukutin-cKO muscles were decreased, which may suggest that fukutin-dependent modification of  $\alpha$ -DG plays a role in MPCs. Furthermore, our data showed that MPC proliferation and muscle regeneration deteriorate more severely as the disease progresses. The mechanisms underlying the decreases in the number of isolated satellite cells and MPC proliferation are



currently unknown. It has been suggested that satellite cells express properly glycosylated  $\alpha$ -DG (31) and that myoblasts/MPCs also express properly glycosylated  $\alpha$ -DG although its signals are relatively weak compared with those of myotubes (32,33). The basement membrane of skeletal muscle contains DG ligands, laminins and perlecan. It is well established that interactions between the extracellular matrix and cell-surface receptors are involved in cell survival signaling (34). Since it has been proposed that DG-ligand interactions are also involved in cellular signaling mechanisms such as survival and apoptosis pathways (35,36), it is possible that loss of  $\alpha$ -DG glycosylation may affect survival signaling regulated by  $\alpha$ -DG-basement membrane interaction. In addition, since the Myf5-fukutin-cKO myofibers showed an earlier reduction of fukutin compared with those of MCK-fukutin-cKO, there is a possibility that earlier loss of  $\alpha$ -DG glycosylation in myofibers affects disease progression and severity. For example, the absence of  $\alpha$ -DG glycosylation during postnatal/juvenile muscle growth and development may have a high impact on muscle degeneration and/or dystrophic pathology in later stages.

As for other muscular dystrophy models with defects in the dystrophin–glycoprotein complex, impaired muscle regeneration has also been reported in *MORE-DG null* mice, in which the DG gene (*Dag1*) is ablated in all cells in the embryo (31), and in older (>1 year) dystrophin-deficient mdx and sarcoglycan-deficient mice (31). The regeneration defects in our Myf5-fukutin-cKO mice appeared at a relatively young age (~3 months), but at this age, Myf5-fukutin-cKO mice already show severe dystrophic pathology. The pathological environment may interfere with efficient muscle regeneration, resulting in decreased regeneration activity as the disease progresses (37). A very recent study also suggested that alterations to the basal lamina microenvironment perturb regeneration potential in dystroglycanopathy (38). Moreover, our data suggest that multiple cycles of degeneration/regeneration may also affect MPC viability, which is consistent with a previous study showing that the progressive exhaustion of functional muscle satellite cells is associated with severe dystrophic phenotype (39). It appears that these disease environments and impaired MPC viability caused by loss of fukutin-dependent modification additively deteriorate regeneration activity, eventually leading to severe and rapid progressive pathology. Together, we conclude that defects in MPC activity contribute to the severe pathology of dystroglycanopathy and propose that dystroglycanopathy is a regeneration-defective disorder.

We observed that some muscle specimens from 16-week-old Myf5-fukutin-cKO mice showed mild phenotype, which was consequently supported by the presence of functionally glycosylated  $\alpha$ -DG. Beedle *et al.* (30) also reported that fukutin deletion resulted in moderate to severe muscular dystrophy using Myf5-Cre mice. Because phenotypic variation in our Myf5-fukutin-cKO colony was rarely seen before 12 weeks of age, the variation may be secondary to disease progression. The less phenotypic variation in our colonies could also be due to the number of backcross on C57BL/6 (backcross: more than seven). We speculate that during frequent cycles of muscle degeneration/regeneration, Myf5-independent or less-expressed myogenic cells (40) may be

activated and differentiated into myofibers in which the *fukutin* gene escaped Cre-mediated recombination.

Many cases of dystroglycanopathy show the most severe skeletal muscle phenotype, and the severe/typical dystroglycanopathy patients end their short lives without ever standing or walking. Although an increasing number of patients are being diagnosed with dystroglycanopathy worldwide, there have been no therapeutic studies on dystroglycanopathy models after the disease progresses. In this study, for the first time, we succeeded in ameliorating the disease severity in dystroglycanopathy mouse models based on the pathomechanism. It is of importance that limited rescue of fukutin protein in myofibers of Myf5-fukutin-cKO muscles, which have MPC defects, ameliorated the severe phenotype. These data suggest that even after functional and/or substantial loss of MPC occurs, prevention of disease-causing defects in myofibers is a probable therapeutic strategy for muscular dystrophy. Moreover, it is noteworthy that therapeutic effects of the exogenous *fukutin* gene were achieved with relatively lower AAV titers than those used in other gene therapies for structural proteins such as dystrophin and sarcoglycans (41–43). Our results showed that titers that were ~2 orders of magnitude less than those required in previous studies were sufficient to produce a therapeutic effect in Myf5-fukutin-cKO mice. This is consistent with our previous study, which suggested that only a little amount of fukutin is necessary to prevent muscular dystrophy (29). Most dystroglycanopathy genes are identified as glycosyltransferases (11,12,15) or hypothesized to have enzyme-like properties, suggesting that a small amount of exogenous gene would be sufficient for producing therapeutic effects. A small dose of AAV vectors could lower the chances of adverse effects such as immune responses in human (44). In addition, the cDNA sizes of fukutin as well as other dystroglycanopathy genes are suitable for AAV vectors. Taken together, we propose that gene transfer is a promising therapeutic strategy for the amelioration of the severe skeletal muscle pathology of dystroglycanopathy. Human dystroglycanopathy is frequently accompanied by brain and, often, cardiac disorders (1,45). The efficacy of AAV delivery to these affected tissues, timing of administration and therapeutic effects in other fukutin-cKO models should be examined in the future. Although therapeutic interventions that rescue the developmental defects of dystroglycanopathy (such as anomalies in brain structure) are difficult at present, amelioration of the muscle phenotype would be highly beneficial to patients and their families. For example, such treatment might improve patients' physical abilities and postpone the need for respiratory interventions until much later in the course of the disease. Increased physical activity could positively influence both mental development and social interactions. Overall, this study may facilitate future clinical translational research in the field of dystroglycanopathy treatment.

## MATERIALS AND METHODS

### Generation of fukutin cKO mice

Construction of the targeted allele, establishment of targeted embryonic stem (ES) cells and generation of the chimera

and F1 mice were carried out by Unitech Co. (Kashiwa, Japan). Briefly, exon 2 of mouse *fukutin* was flanked by two loxP sequences (Supplementary Material, Fig. S1A). An F1p recognition target-flanked neo-cassette was inserted upstream of exon 2. The targeting vector was electroporated into C57BL/6 mouse ES cells. Positive clones were selected, and homologous recombination was confirmed by Southern blotting (Supplementary Material, Fig. S1B). The targeted ES cells were injected into blastocysts (BALB/c), and then, chimera mice were bred with C57BL/6 mice to generate founder mice. The founder mice were crossed with the FLPe transgenic mice, producing heterozygous flox mice without the neo-cassette (*fukutin<sup>lox/+</sup>*). The heterozygous flox mice were intercrossed to obtain homozygous flox mice (*fukutin<sup>lox/lox</sup>*).

MCK-Cre mice (22) [*MCK-Cre<sup>Tg</sup>(+)*], backcrossed for at least 10 generations to C57BL/6] and Myf5-Cre knock-in mice (23) [*Myf5-Cre<sup>KI</sup>(+)*] were obtained from The Jackson Laboratory. Myf5-Cre mice were backcrossed for more than six generations to C57BL/6 before crossing with *fukutin<sup>lox/+</sup>* mice. The heterozygous *fukutin<sup>lox/+</sup>* carrying MCK-Cre [*fukutin<sup>lox/+</sup>:MCK-Cre<sup>Tg</sup>(+)*] or Myf5-Cre [*fukutin<sup>lox/+</sup>:Myf5-Cre<sup>KI</sup>(+)*] were then bred with *fukutin<sup>lox/lox</sup>* mice to obtain cKO mice. Using this breeding strategy, we obtained the following four genotypes (Supplementary Material, Fig. S1C): for MCK-fukutin-cKO line—[*fukutin<sup>lox/lox</sup>:MCK-Cre<sup>Tg</sup>(-)*] (used as WT control), [*fukutin<sup>lox/lox</sup>:MCK-Cre<sup>Tg</sup>(+)*] (used as cKO), [*fukutin<sup>lox/+</sup>:MCK-Cre<sup>Tg</sup>(+)*] (used as heterozygous control, HET) and [*fukutin<sup>lox/+</sup>:MCK-Cre<sup>Tg</sup>(-)*]; and for the Myf5-fukutin-cKO line—[*fukutin<sup>lox/lox</sup>:Myf5-Cre<sup>KI</sup>(-)*] (used as WT), [*fukutin<sup>lox/lox</sup>:Myf5-Cre<sup>KI</sup>(+)*] (used as cKO), [*fukutin<sup>lox/+</sup>:Myf5-Cre<sup>KI</sup>(+)*] (used as HET) and [*fukutin<sup>lox/+</sup>:Myf5-Cre<sup>KI</sup>(-)*]. Alternatively, we crossed cKO mice with *fukutin<sup>lox/lox</sup>* mice to obtain two genotypes: WT and cKO (Supplementary Material, Fig. S1C). Genotyping was performed using PCR (Supplementary Material, Fig. S1D). Primer sequences and PCR conditions are available on request. Mice were maintained in accordance with the animal care guidelines of Unitech Co. Ltd., Osaka University and Kobe University.

## Antibodies

Antibodies used in western blots and immunofluorescence were as follows: mouse monoclonal antibody 8D5 against  $\beta$ -DG (Novacastra); mouse monoclonal antibody IIH6 against glycosylated  $\alpha$ -DG (Millipore); goat polyclonal antibody against the C-terminal domain of the  $\alpha$ -DG polypeptide (AP-074G-C) (29); goat polyclonal anti-fukutin antibody (106G2) and rabbit polyclonal anti-fukutin antibody (RY213) (5); rat anti-laminin antibody 4H8-2 (Alexis Biochemicals); rabbit polyclonal anti-collagen I antibody (AbD Serotec); and mouse monoclonal anti-embryonic myosin antibody (The Developmental Studies Hybridoma Bank, University of Iowa). A rat monoclonal antibody against the  $\alpha$ -DG core protein (3D7-7) was generated using the recombinant  $\alpha$ -DG-Fc fusion protein (46); hybridoma clones were selected for reactivity to the C-terminal domain of the  $\alpha$ -DG polypeptide. To reduce high background staining of IIH6 in severely affected skeletal muscle sections, commercial IIH6 was

labeled with biotin. The IIH6-IgM fractions were prepared from ascites, using protein L-beads (Pierce) and then biotinylated (EZ-Link Micro Sulfo-NHS-Biotinylation Kit; Pierce) according to the manufacturer's instructions.

## Preparation of fukutin and DG

Endogenous fukutin was enriched by immunoprecipitation using polyclonal goat anti-fukutin antibody (106G2) from the skeletal muscle lysates. The immunoprecipitated materials were subjected to western blotting using polyclonal rabbit anti-fukutin antibody (RY213). DG from solubilized skeletal muscle was enriched with wheat germ agglutinin-agarose beads (Vector Laboratories) as previously described (29).

## Histological and immunofluorescence analyses

For histological and immunofluorescence staining, cryosections (7  $\mu$ m thick) were prepared. For H&E staining, sections were stained for 2 min in hematoxylin, 1 min in eosin, and then dehydrated with ethanol and xylene. The slides are washed with 0.5% glacial acetic acid, dehydrated and then mounted. For immunofluorescence analysis, sections were treated with cold ethanol/acetic acid (1:1) for 1 min, blocked with 5% goat serum in MOM mouse Ig blocking reagent (Vector Laboratories) at room temperature for 1 h, and then incubated overnight with primary antibodies diluted in MOM diluent (Vector Laboratories) at 4°C. The slides were washed with phosphate-buffered saline (PBS) and incubated with Alexa Fluor 488-conjugated or Alexa Fluor 555-conjugated secondary antibodies (Molecular Probes) at room temperature for 30 min. Sections were observed by fluorescence microscopy (Leica DMR, Leica Microsystems and BZ9000, Keyence). Quantification of the number of Pax7-positive cells and the population of Pax7/MyoD-double-positive cells was performed as previously described (47).

## Quantitative and statistical analysis

For quantitative evaluation of muscle pathology, the proportion of myofibers with centrally located nuclei in at least 1000 fibers for each individual was counted. For the evaluation of connective tissue infiltration, the immunofluorescence signal of collagen I was quantitatively measured using the ImageJ software. For the assessment of myofiber size variation, areas of individual myofibers on transverse sections were measured using the ImageJ software. Data represent means with SEM, and *P*-values  $\leq 0.05$  were considered statistically significant (Mann–Whitney *U* test).

## Preparation and culture of MPCs

Mononuclear cells from uninjured limb muscles were prepared using 0.2% collagenase type II (Worthington Biochemical) as previously described (28,48). Approximately  $3\text{--}5 \times 10^6$  or  $3\text{--}9 \times 10^6$  mononuclear cells from young mice (2-week-old) or adult mice (4-month-old), respectively, were subjected to MPC isolation experiments. Mononuclear cells derived from the skeletal muscles were stained with FITC-conjugated anti-CD31 (Pecam1, Mouse Genome Informatics), anti-CD45

(Ptpcr, Mouse Genome Informatics), phycoerythrin-conjugated anti-Sca1 (Ly6a, Mouse Genome Informatics) and biotinylated SM/C-2.6 antibodies (28). Cells were then incubated with 1:400 streptavidin–allophycocyanin (BD Biosciences) on ice for 30 min and resuspended in PBS containing 2% fetal calf serum (FCS) and 2  $\mu\text{g/ml}$  propidium iodide (PI). Cell sorting was performed using an FACS Aria II flow cytometer (BD Immunocytometry Systems). Debris and dead cells were excluded by forward scatter, side scatter and PI gating. Data were collected using the FACSDiva software (BD Biosciences). Myogenic cells from the regenerating muscles were also highly enriched in the SM/C-2.6(+) CD31(–) CD45(–) Sca1(–) cell fraction.

Freshly isolated myogenic cells were cultured in a growth medium of high-glucose Dulbecco's modified Eagle's medium (DMEM-HG; Sigma-Aldrich) containing 20% FCS, 2.5 ng/ml basic fibroblast growth factor (FGF2; PeproTech) and penicillin (100 U/ml)–streptomycin (100  $\mu\text{g/ml}$ ) (Gibco BRL) on culture dishes coated with Matrigel (BD Biosciences). Differentiation was induced in a differentiation medium containing DMEM-HG, 5% horse serum and penicillin–streptomycin for 3–4 days. Quantitative analysis for cell proliferation was performed as described previously (47). Fusion index was estimated as the ratio of nuclei in the myotubes to all the nuclei in more than four independent microscopy fields.

### CTX experiments

CTX (30  $\mu\text{M}$ ; purified from the venom of the snake *Naja nigricollis*; Latoxan) was injected intramuscularly (for young mice, 30  $\mu\text{l}$  to the tibialis anterior and 70  $\mu\text{l}$  to the calf; for adult mice, 50  $\mu\text{l}$  to the tibialis anterior and 100  $\mu\text{l}$  to the calf). Mock injections used only saline solution. The injected muscles were examined 5 or 14 days after the injection. Five days after the injection, areas of individual embryonic myosin-positive fibers were measured (>300 fibers randomly chosen from 5–10 regions per toxin-challenged muscle) in each genotype. Fourteen days after the injection, fiber size variation was quantitatively evaluated by measuring individual laminin-positive fibers (>300 fibers) in each genotype.

### AAV gene transfer

To generate *fukutin*-encoding AAV9 vector, the complete open reading frame of mouse *fukutin* gene was cloned into the pAAV-IRES-hrGFP vector (49). The recombinant *fukutin*-encoding AAV9 vector was produced as described previously (49). AAV vectors were injected intramuscularly into the calf and tibialis anterior (at 1 week to the tibialis anterior,  $0.8\text{--}1.6 \times 10^9$  vector genome in saline solution; at 1 week to the calf or at 8 weeks to the tibialis anterior,  $2\text{--}4 \times 10^9$  vector genome; and at 8 weeks to the calf,  $4\text{--}8 \times 10^9$  vector genome). For tail vein injections and intraperitoneal injections,  $\sim 2 \times 10^{10}$  and  $\sim 1 \times 10^{10}$  vector genome was used, respectively.

### Miscellaneous

For western blotting, the proteins were separated using 4–15% linear gradient SDS–PAGE (Bio-Rad). Gels were transferred

to polyvinylidene fluoride membrane (Millipore). Blots were developed by horseradish peroxidase-enhanced chemiluminescence (Supersignal West Pico, Pierce; or ECL Plus, GE Healthcare). Laminin-binding activity was determined by the laminin overlay assay as described previously (29). Serum CK activity was measured using the CPK kit (WAKO). For Evans blue dye uptake, Evans blue dye (10 mg/ml in saline) was intraperitoneally injected (100  $\mu\text{l}/10\text{ g}$  of body weight). After 5 h, the mice were made to exercise on a downhill (15°) treadmill for 60 min (MK-680S, Muromachi Kikai). Twenty-four hours after the exercise, frozen tissue samples were prepared. Serum was prepared before 24 h and after 2 h of the exercise. Grip strength was measured for 10 consecutive trials for each mouse, using a strength meter (Ohara Ika Sangyo Co. Ltd, Tokyo), and 20% of the top and the bottom values were excluded to obtain the mean value.

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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