and astrocytes, as we performed here, from larger numbers of patients might result in the classification of sporadic AD.

To date, the clinical effectiveness of DHA treatment is still controversial (Freund-Levi et al., 2006; Quinn et al., 2010). It is of particular interest that one of two sporadic AD neurons accumulated intracellular Aβ oligomers and showed cellular phenotypes that could respond to DHA but the other did not, and this result may explain why DHA treatment was effective for some AD patients, those with the intracellular Aβ oligomer-associated type of AD, although the timing (that is, the stage of disease development) for starting the treatment would be another critical factor. These results may suggest that patient-specific iPSCs provide a chance to re-evaluate the effect of a drug that failed in AD clinical trials, depending on the selection of the patient type. In the present study, the amount of Aβ oligomers in our culture was not affected by DHA, although it would be effective for reducing cellular stresses, and reducing the oligomerization of Aβ was also presumed to be a candidate mechanism of DHA treatment (Cole and Frautschy, 2006). These results indicate that therapy with DHA would alleviate symptoms. Furthermore, the data showing that BSI treatment leads to a reduction in ROS formation at a relatively similar level (Figure 2G) in both AD and control cells might indicate an Aβ oligomer-independent effect, in addition to an Aβ oligomer-dependent effect, of BSI.

In any event, patient-specific iPSCs would provide disease pathogenesis, irrespective of the disease being in a familial or sporadic form, as well as enable the evaluation of drug and patient classification of AD.

EXPERIMENTAL PROCEDURES

Derivation of Patient-Specific Fibroblasts

Control and AD-derived human dermal fibroblasts (HDFs) were generated from explants of 3 mm dermal biopsies. After 1–2 weeks, fibroblast outgrowths from the explants were passaged.

iPSC Generation

Human complementary DNAs for reprogramming factors were transduced in HDFs with episomal vectors (*SOX2, KLF4, OCT4, L-MYC, LIN28*, and small hairpin RNA for p53). Several days after transduction, fibroblasts were harvested and replated on an SNL feeder cell layer. On the following day, the medium was changed to a primate embryonic stem cell medium (ReproCELL, Japan) supplemented with 4 ng/ml basic FGF (Wako Pure Chemicals Industries, Japan). The medium was changed every other day. iPSC colonies were picked up 30 days after transduction.

Statistical Analysis

All data are shown as mean ± SD. For comparisons of the mean between two groups, statistical analysis was performed by applying Student's t tests after confirming equality between the variances of the groups. When the variances were unequal, Mann-Whitney U tests were performed (SigmaPlot 11.2.0, Systat Software, USA). Comparisons of the mean among three groups or more were performed by one-way, two-way, or three-way analysis of variance followed by a post hoc test with the use of Student-Newman-Keuls Method (SigmaPlot 11.2.0). p values < 0.05 were considered significant.

ACCESSION NUMBERS

The Gene Expression Omnibus accession numbers for microarray data reported in this paper are GSE43326 (gene-expression comparison between control and AD clones), GSE43382 (gene-expression change along with the astroglial differentiation), and GSE43328 (gene-expression comparison of generated iPSCs).

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.01.009.

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(D) Quantitative data of (C) is shown. Each value indicated the ratio of the CellROX-stained area (an average of random 25 fields per sample) adjusted with DAPI counts. Data represent mean ± SD (n = 3 per clone). Two-way ANOVA showed significant main effects of DHA treatment ($F_{[1,32]} = 43.140$; $p < 0.001$) with a significant interaction between the APP mutation and DHA treatment ($F_{[3,32]} = 23.410$; $p < 0.001$). The DHA group in AD(APP-E693Δ) neural cells was significantly different from the other groups (**, $p < 0.005$).

(E) Real-time survival rate of control and AD neural cells with and without DHA showing cell viability. The numbers of control and AD(APP-E693Δ) neurons with Synapsin I-promoter-driven EGFP were sequentially imaged (average of 25 random fields per sample) and counted to assess the survival ratio (n = 3 per clone). Data represent mean ± SD (n = 3 per clone). In the cell-survival ratio, three-way ANOVA showed significant main effects of the APP mutation ($F_{[1,256]} = 377.611$; $p < 0.001$), DHA treatment ($F_{[1,256]} = 36.117$; $p < 0.001$), and time ($F_{[7,256]} = 65.272$; $p < 0.001$), with significant interactions between the APP mutation and DHA treatment ($F_{[1,256]} = 18.315$; $p < 0.001$), between the APP mutation and time ($F_{[7,256]} = 20.023$; $p < 0.001$), between DHA treatment and time ($F_{[7,256]} = 4.534$; $p < 0.001$), and among all three factors ($F_{[7,256]} = 5.277$; $p < 0.001$). Post hoc analysis revealed that, on day 14 and day 16, AD(APP-E693Δ) neural cells were more vulnerable in the long culture than control neural cells and that DHA treatment rescued the vulnerability (*, $p < 0.001$).

(F) Typical images of Synapsin::EGFP neurons used in real-time survival assay. The scale bar represents 50 μm.

(G) Cytotoxicity in neural culture derived from control and AD iPSCs after treatment with DHA (5 μM) for 16 days. Measured fluorescent lactate dehydrogenase (LDH) release served as a measure of cytotoxicity. Data represent mean ± SD (n = 3 per clone). Two-way ANOVA showed significant main effects of DHA treatment ($F_{[1,32]} = 16.710$; $p < 0.001$) with a significant interaction between APP-E693Δ mutation and DHA treatment ($F_{[3,32]} = 9.210$; $p < 0.005$). There was a significant difference in AD(APP-E693Δ) neural cells between the DMSO-control and DHA groups (*, $p < 0.05$).

(H) Aβ40 and Aβ42 secreted from iPSC-derived neurons into medium (extracellular Aβ) at day 16 of the long-term culture were measured at 48 hr after the last medium change. Data represent mean ± SD (n = 3 per clone).

See also Figure S4 and Table S2.

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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 β-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 β-1,3-*N*-acetylglucosaminyltransferase 1 (*B3GNT1*)], implicated in dystroglycanopathies, have been shown or expected to be involved in the glycosylation pathway of α-dystroglycan (α-DG) (1,9,10). POMGnT1 and the POMT1/2 complex possess glycosyltransferase activities and can directly synthesize *O*-mannosyl sugar chains on α-DG (11,12). Fukutin, FKRP and LARGE are involved in a novel phosphodiester-linked modification, namely, a post-phosphoryl modification, of *O*-mannose on α-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 α- and β-DG, which is post-translationally cleaved into the two subunits (16). α-DG is a highly glycosylated protein and serves as the receptor subunit for extracellular proteins such as laminins, perlecan, agrin, neurexin and pikachurin (9,17). *O*-mannosyl glycosylation and the post-phosphoryl modification are required for the ligand-binding activities of α-DG (3,13). Hypoglycosylation and reduced ligand-binding activity of α-DG are common characteristics of dystroglycanopathy. α-DG is anchored to the plasma membrane through non-covalent interactions with the transmembrane subunit β-DG. β-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 α-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, flox fukutin mice (*fukutin*$^{lox/lox}$) 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 α-DG is indicated by decreased molecular weight, loss of immunoreactivity against the monoclonal IIH6 antibody, which recognizes properly glycosylated α-DG (3), and decreased laminin-binding activity. Abnormally modified α-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

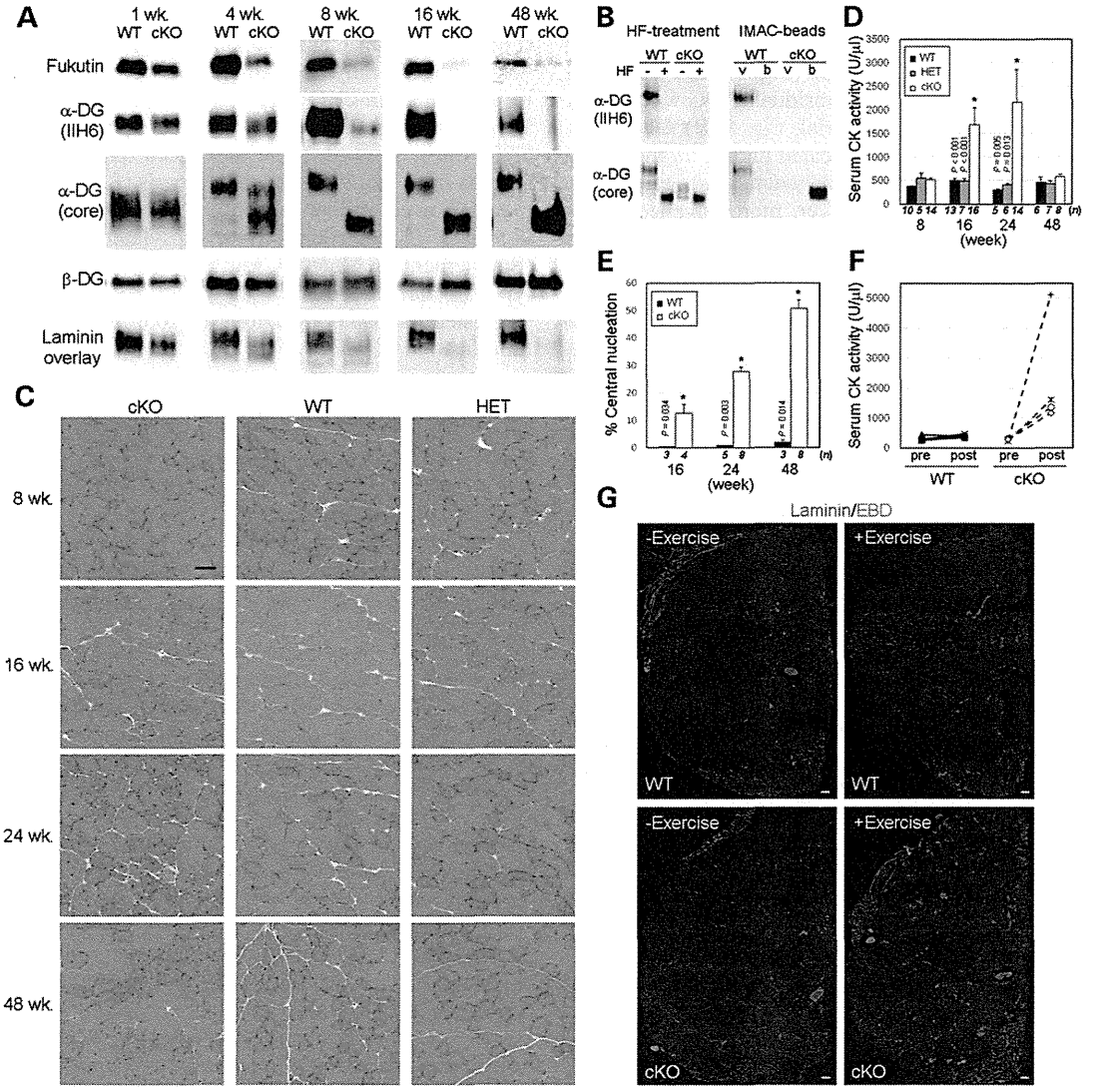

Figure 1. Pathological characterization of MCK-fukutin-cKO mice. (**A**) Western blot analysis of fukutin protein expression and α-DG modification in MCK-fukutin-cKO (cKO) and litter control WT skeletal muscles at different ages (1, 4, 8, 16 and 48 weeks). β-DG was used as a loading control. Laminin-binding activity of α-DG was examined by the laminin overlay assay. (**B**) Post-phosphoryl modification of α-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 μm. (**D**) Serum CK activity and (**E**) proportion of myofibers with centrally located nuclei. Data shown are mean ± 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 μm.

(13,14). The molecular weight of α-DG in the skeletal muscles of control (WT) mice was dramatically reduced after HF treatment, and α-DG did not bind to IMAC beads because the phosphodiester-linked modification was intact; in contrast, α-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 α-DG core protein showed that α-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 α-DG is little affected by fukutin deficiency. These results confirmed abnormal modification of α-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 α-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 α-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 ~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, α-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 α-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-fuktuin-cKO muscles after cardiotoxin (CTX)-induced muscle degeneration. After 14 days of the CTX challenge in adult mice (~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 (~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

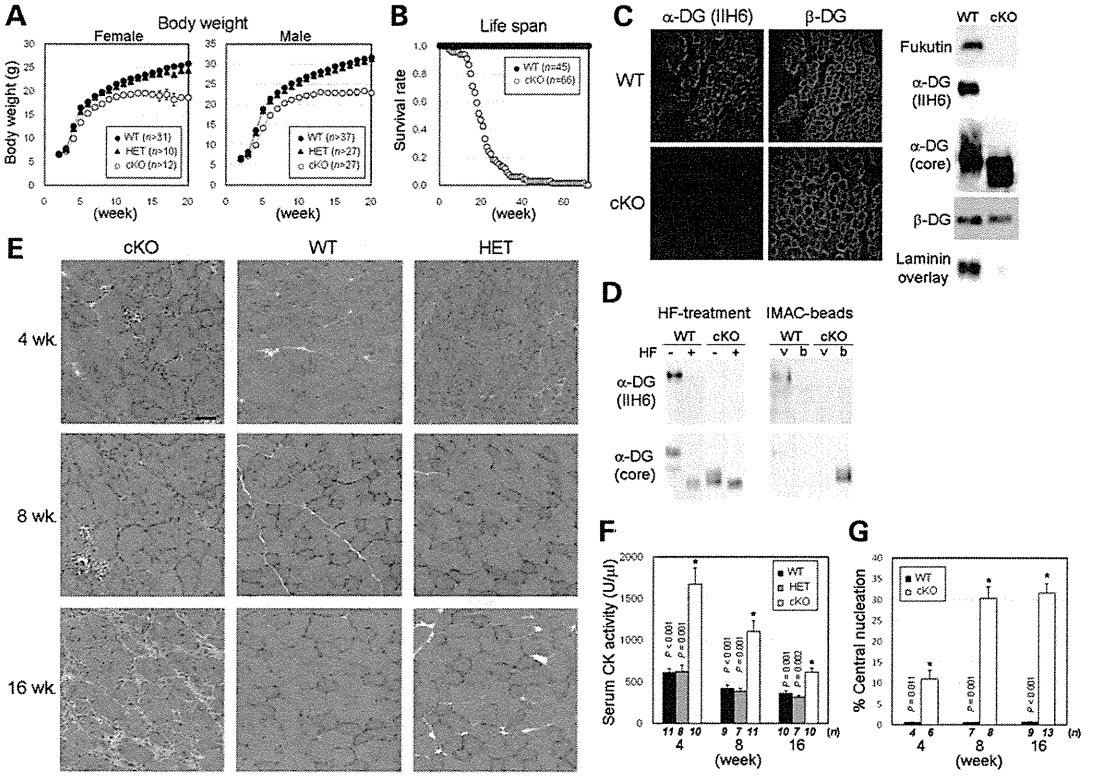

Figure 2. Pathological characterization of Myf5-fukutin-cKO mice. (**A**) Temporal changes in body weight. Data shown are mean ± SEM for each group (*n* is indicated in the graph). (**B**) Survival curve of Myf5-fukutin cKO mice. (**C**) Immunofluorescence and western blot analyses of fukutin expression and α-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. β-DG was used as a control. Laminin-binding activity of α-DG was examined by the laminin overlay assay. (**D**) Post-phosphoryl modification of α-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 μm. (**F**) Serum creatin kinase activity and (**G**) proportion of myofibers with centrally located nuclei. Data shown are mean ± 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 α-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

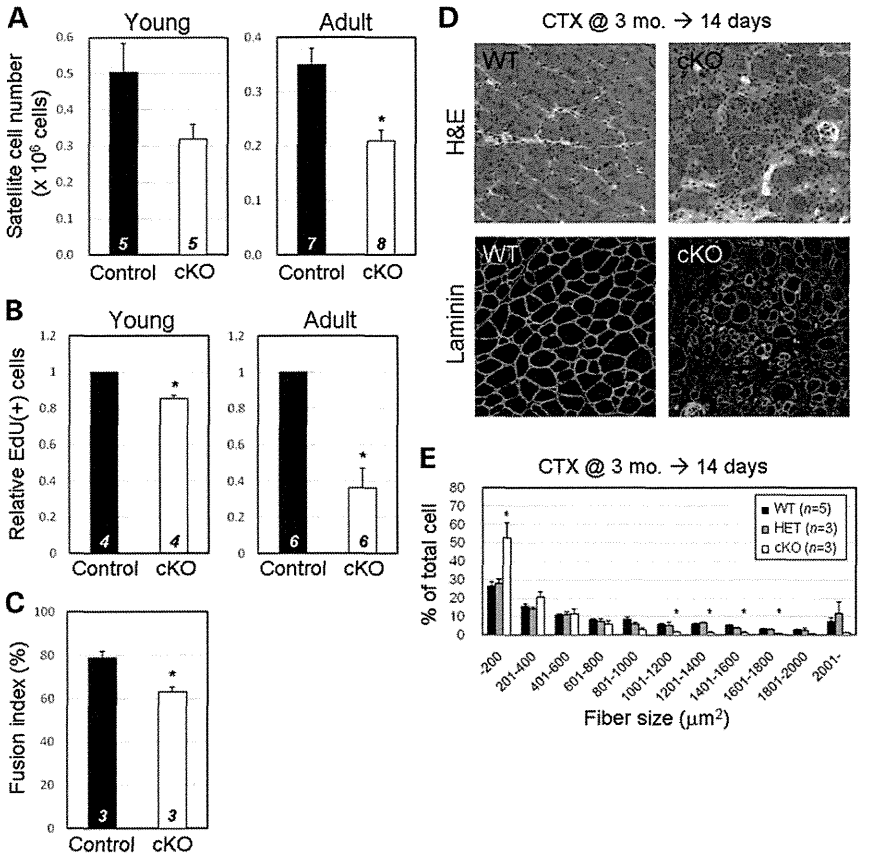

Figure 3. Impaired MPC activities and muscle regeneration in Myf5-fukutin-cKO mice. (**A**) The number of isolated satellite cells from young (2 weeks old) and adult (4 months old) Myf5-fukutin-cKO mice. $P = 0.117$ for young, and $P = 0.005$ for adult (Mann–Whitney U test). (**B**) Proliferation activity 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 ± 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 ± 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 α-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 IIH6 immunoreactivity (the proportion of IIH6-positive to laminin-positive fibers) between the cases of AAV9-MCK-*fukutin* (77.4 ± 3.8%, $n = 5$) and AAV9-CMV-*fukutin* (74.8 ± 3.6%,

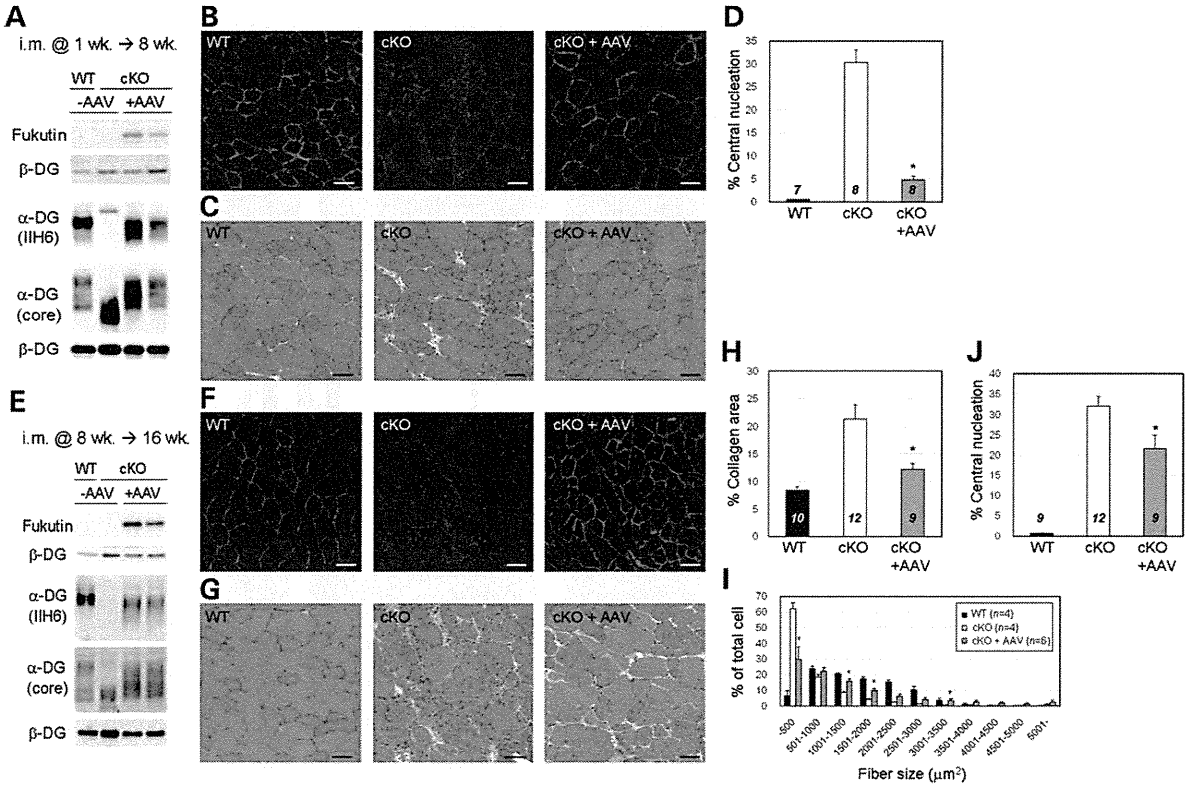

Figure 4. Gene transfer of *fukutin* via AAV9 intramuscular injection into Myf5-fukutin-cKO mice. AAV9-MCK-*fukutin* was administered to 1-week-old (**A**–**D**) and 8-week-old (**E**–**J**) Myf5-fukutin-cKO mice via intramuscular injection. Two months after the gene transfer, recovery of fukutin protein and α-DG glycosylation was confirmed by western blotting (A and E) and IIH6-immunofluorescence staining (B and F) (bar = 50 μm). For fukutin protein expression and α-DG modification, total lysate and wheat germ agglutinin-enriched fractions, respectively, were subjected to western blotting. In both cases, β-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 ± 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 α-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 α-DG, a small amount of intact α-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 α-DG glycosylation status after 16 and 48 weeks. We detected increased levels of fukutin expression and IIH6-positive α-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 α-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

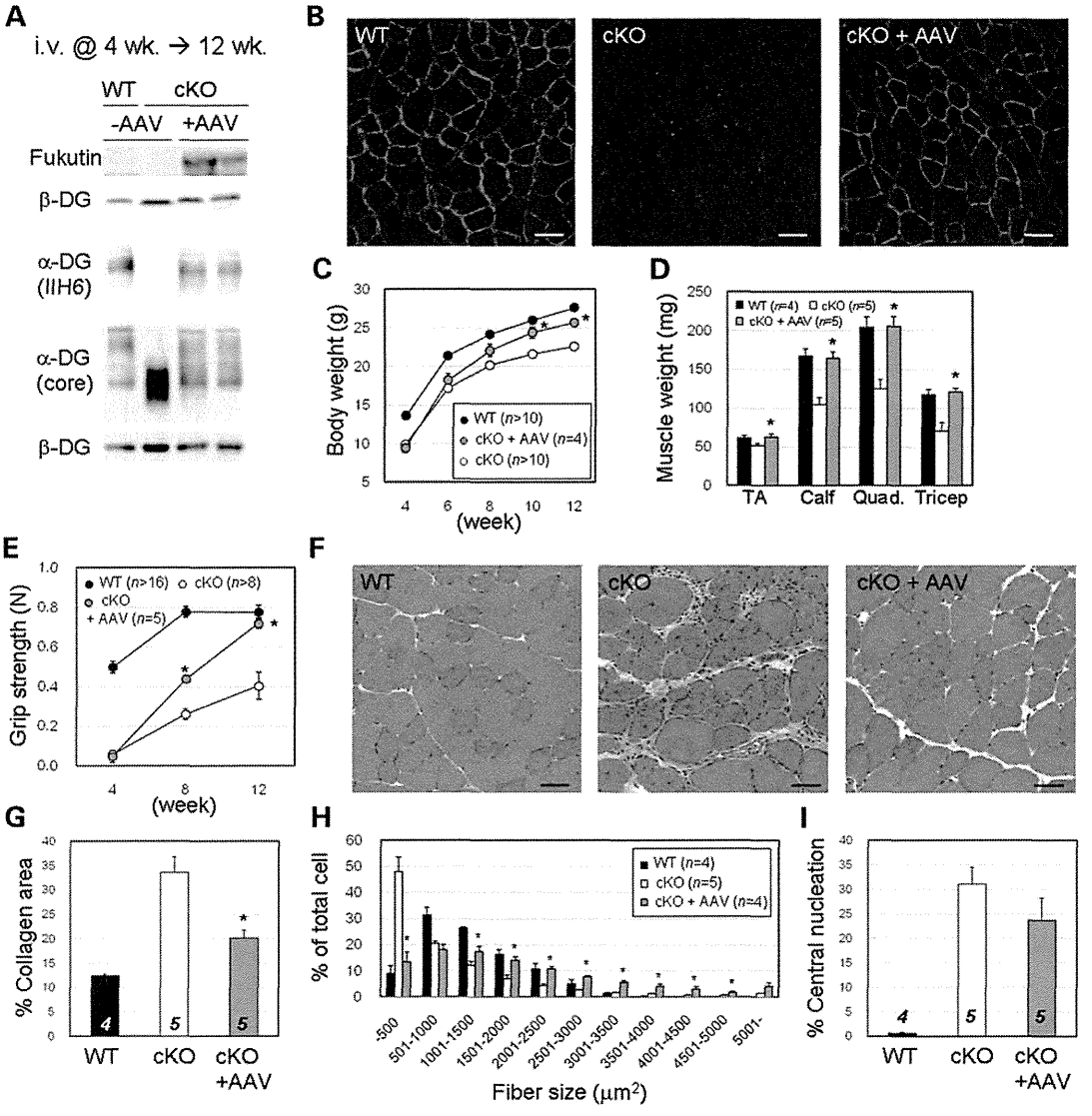

Figure 5. Systemic gene transfer of *fukutin* into Myf5-fukutin-cKO mice. AAV9-MCK-*fukutin* was administered to 4-week-old Myf5-fukutin-cKO mice via tail vein injection. Two months after the gene transfer, the skeletal muscles were analyzed and compared with non-treated Myf5-fukutin-cKO muscles. Recovery of fukutin protein and α-DG modification was confirmed by western blotting (**A**) and immunofluorescence (**B**) (bar = 50 μm). For fukutin protein expression and α-DG modification, total lysate and wheat germ agglutinin-enriched fractions, respectively, were subjected to western blotting. In both cases, β-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 μm), 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 ± 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., quadricep.

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 α-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 α-DG (31) and that myoblasts/MPCs also express properly glycosylated α-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 α-DG glycosylation may affect survival signaling regulated by α-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 α-DG glycosylation in myofibers affects disease progression and severity. For example, the absence of α-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 α-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 Flp 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 ($fukutin^{lox/+}$). The heterozygous flox mice were intercrossed to obtain homozygous flox mice ($fukutin^{lox/lox}$).

MCK-Cre mice (22) [$MCK\text{-}Cre^{Tg}(+)$, backcrossed for at least 10 generations to C57BL/6] and Myf5-Cre knock-in mice (23) [$Myf5\text{-}Cre^{KI}(+)$] were obtained from The Jackson Laboratory. Myf5-Cre mice were backcrossed for more than six generations to C57BL/6 before crossing with $fukutin^{lox/lox}$ mice. The heterozygous $fukutin^{lox/+}$ carrying MCK-Cre [$fukutin^{lox/+}$:$MCK\text{-}Cre^{Tg}(+)$] or Myf5-Cre [$fukutin^{lox/+}$:$Myf5\text{-}Cre^{KI}(+)$] were then bred with $fukutin^{lox/lox}$ mice to obtain cKO mice. Using this breeding strategy, we obtained the following four genotypes (Supplementary Material, Fig. S1C): for MCK-fukutin-cKO line—[$fukutin^{lox/lox}$:$MCK\text{-}Cre^{Tg}(-)$] (used as WT control), [$fukutin^{lox/lox}$:$MCK\text{-}Cre^{Tg}(+)$] (used as cKO), [$fukutin^{lox/+}$:$MCK\text{-}Cre^{Tg}(+)$] (used as heterozygous control, HET) and [$fukutin^{lox/+}$:$MCK\text{-}Cre^{Tg}(-)$]; and for the Myf5-fukutin-cKO line—[$fukutin^{lox/lox}$:$Myf5\text{-}Cre^{KI}(-)$] (used as WT), [$fukutin^{lox/lox}$:$Myf5\text{-}Cre^{KI}(+)$] (used as cKO), [$fukutin^{lox/+}$:$Myf5\text{-}Cre^{KI}(+)$] (used as HET) and [$fukutin^{lox/+}$:$Myf5\text{-}Cre^{KI}(-)$]. Alternatively, we crossed cKO mice with $fukutin^{lox/lox}$ 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 β-DG (Novacastra); mouse monoclonal antibody IIH6 against glycosylated α-DG (Millipore); goat polyclonal antibody against the C-terminal domain of the α-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 α-DG core protein (3D7-7) was generated using the recombinant α-DG-Fc fusion protein (46); hybridoma clones were selected for reactivity to the C-terminal domain of the α-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 μm 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 ≤ 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 $3-5 \times 10^6$ or $3-9 \times 10^6$ 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

(Ptprc, 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 μg/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 μg/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 μM; purified from the venom of the snake *Naja nigricollis*; Latoxan) was injected intramuscularly (for young mice, 30 μl to the tibialis anterior and 70 μl to the calf; for adult mice, 50 μl to the tibialis anterior and 100 μl 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, $0.8–1.6 \times 10^9$ vector genome in saline solution; at 1 week to the calf or at 8 weeks to the tibialis anterior, $2–4 \times 10^9$ vector genome; and at 8 weeks to the calf, $4–8 \times 10^9$ 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 μl/10 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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ORIGINAL ARTICLE

Mutations in *COQ2* in Familial and Sporadic Multiple-System Atrophy

The Multiple-System Atrophy Research Collaboration

ABSTRACT

BACKGROUND

Multiple-system atrophy is an intractable neurodegenerative disease characterized by autonomic failure in addition to various combinations of parkinsonism, cerebellar ataxia, and pyramidal dysfunction. Although multiple-system atrophy is widely considered to be a nongenetic disorder, we previously identified multiplex families with this disease, which indicates the involvement of genetic components.

METHODS

In combination with linkage analysis, we performed whole-genome sequencing of a sample obtained from a member of a multiplex family in whom multiple-system atrophy had been diagnosed on autopsy. We also performed mutational analysis of samples from members of five other multiplex families and from a Japanese series (363 patients and two sets of controls, one of 520 persons and one of 2383 persons), a European series (223 patients and 315 controls), and a North American series (172 patients and 294 controls). On the basis of these analyses, we used a yeast complementation assay and measured enzyme activity of parahydroxybenzoate-polyprenyl transferase. This enzyme is encoded by the gene *COQ2* and is essential for the biosynthesis of coenzyme Q_{10}. Levels of coenzyme Q_{10} in lymphoblastoid cells and brain tissue were measured on high-performance liquid chromatography.

RESULTS

We identified a homozygous mutation (M128V-V393A/M128V-V393A) and compound heterozygous mutations (R387X/V393A) in *COQ2* in two multiplex families. Furthermore, we found that a common variant (V393A) and multiple rare variants in *COQ2*, all of which are functionally impaired, are associated with sporadic multiple-system atrophy. The V393A variant was exclusively observed in the Japanese population.

CONCLUSIONS

Functionally impaired variants of *COQ2* were associated with an increased risk of multiple-system atrophy in multiplex families and patients with sporadic disease, providing evidence of a role of impaired COQ2 activities in the pathogenesis of this disease. (Funded by the Japan Society for the Promotion of Science and others.)

The members of the Multiple-System Atrophy Research Collaboration are listed in the Appendix. Address reprint requests to Dr. Shoji Tsuji, Department of Neurology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan, or at tsuji@m.u-tokyo.ac.jp.

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Multiple-system atrophy is a progressive neurodegenerative disease that is clinically characterized by autonomic failure in addition to various combinations of parkinsonism, cerebellar ataxia, and pyramidal dysfunction. The term multiple-system atrophy was introduced in 1969 to encompass the disease entities of olivopontocerebellar ataxia, striatonigral degeneration, and the Shy–Drager syndrome, on the basis of neuropathological findings in these disorders.[1] Multiple-system atrophy is characterized by the development of cytoplasmic aggregates of α-synuclein, primarily in oligodendroglia.[2-7] However, the pathogenic mechanisms underlying this disease remain unknown, making it difficult to develop effective therapies.

The disorder is classified into two subtypes: subtype C, characterized predominantly by cerebellar ataxia, and subtype P, characterized predominantly by parkinsonism.[8] Among patients with multiple-system atrophy, subtype C has been reported to be more prevalent than subtype P in the Japanese population (65 to 67% vs. 33 to 35%),[9,10] whereas subtype P has been reported to be more prevalent than subtype C in Europe (63% vs. 34%)[11] and North America (60% vs. 13%, with 27% of cases unclassified).[12] Although multiple-system atrophy has been defined as a nongenetic disorder until recently, several multiplex families with the disease have been described, indicating that strong genetic factors confer susceptibility to the disease.[13-15]

METHODS

PATIENTS AND MULTIPLEX FAMILIES

Patients with multiple-system atrophy were enrolled in the study on the basis of research protocols that were approved by the institutional review board at each participating center. Written informed consent was obtained from all participants.

The diagnosis of multiple-system atrophy was made on the basis of the current consensus criteria for the disease.[8] Four Japanese families (Families 1 through 4, whose members have been described previously[13]) and two additional Japanese families (Family 8 and Family 12) were enrolled in this study (Fig. 1). In Family 1, the parents were first-degree cousins, which is consistent with autosomal recessive inheritance. The clinical features of these families are summarized in Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org.

Autopsy findings for Participants II-4[13] and II-8 in Family 1 and Participant II-6 in Family 8 showed widespread and abundant cytoplasmic aggregates of α-synuclein, primarily in oligodendroglia, in association with neurodegeneration in striatonigral and olivopontocerebellar structures. These findings confirmed the diagnosis of multiple-system atrophy.

PATIENTS WITH SPORADIC DISEASE AND CONTROLS

As with the multiplex families, the diagnosis of sporadic multiple-system atrophy was made on the basis of the current consensus criteria.[8] A total of 363 patients with multiple-system atrophy and 520 controls were included in the Japanese series, 223 patients and 315 controls in the European series, and 172 patients and 294 controls in the North American series (persons of European or Hispanic descent living in North America) (Text S2 and Table S2 in the Supplementary Appendix). Ancestry was determined by self-report on a multiple-choice questionnaire. We also enrolled an independent series of 2383 Japanese controls.

ASSOCIATION WITH OTHER NEURODEGENERATIVE DISEASES

To determine the specificity of the association between variants in candidate genes and multiple-system atrophy, we enrolled 2728 Japanese patients with Alzheimer's disease, 659 with Parkinson's disease, and 634 with amyotrophic lateral sclerosis (ALS). Their demographic characteristics are provided in Text S2 in the Supplementary Appendix.

LINKAGE ANALYSIS AND WHOLE-GENOME SEQUENCING

We performed parametric and nonparametric linkage analyses using Affymetrix SNP 6.0 arrays and software for linkage analysis.[16,17] The genomic DNA from Participant II-4 in Family 1 was subjected to four runs in an Illumina Genome Analyzer IIx (100-bp-long paired ends). We used BWA software[18] and SAMtools sequence-alignment mapping[19] with the default settings for alignment and variation detection against the human reference genome (National Center for Biotechnology Information build 36 [also known as hg18]).

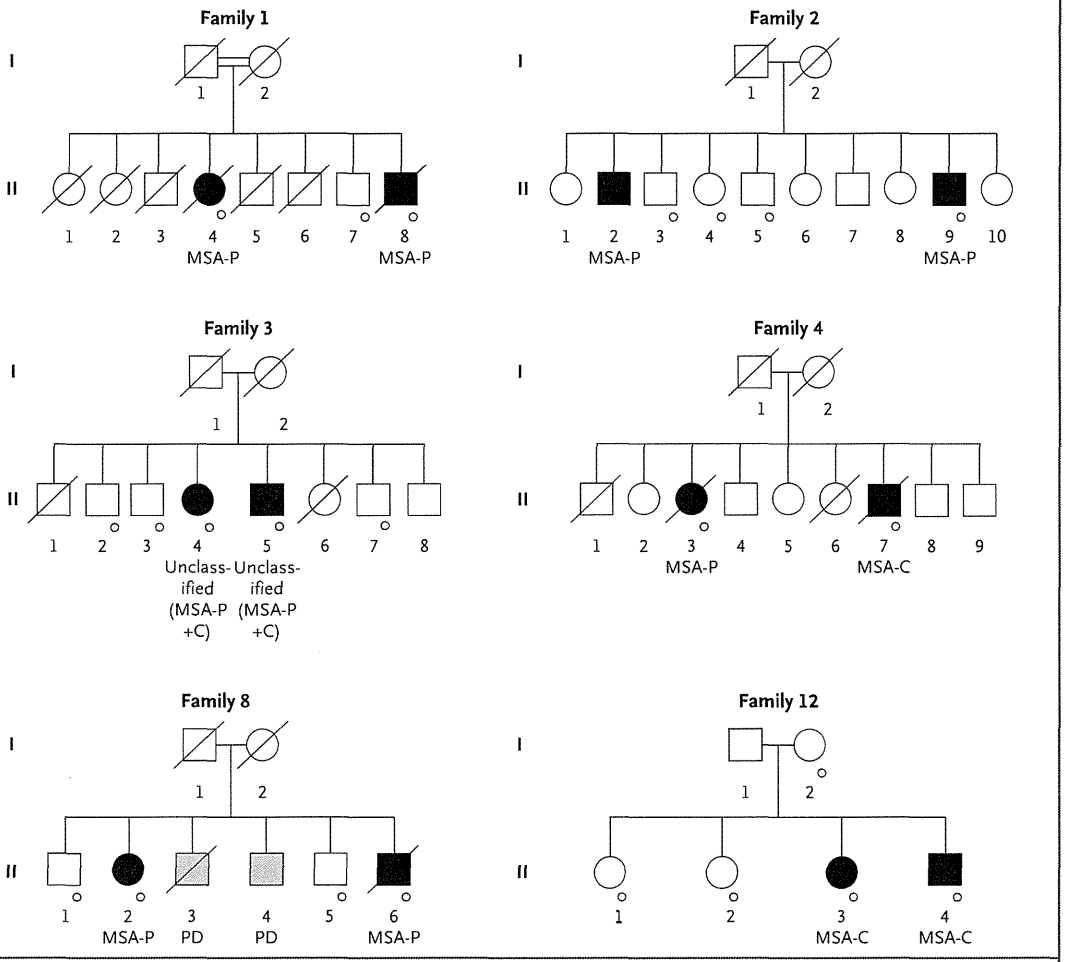

Figure 1. Pedigrees of Six Multiplex Families with Multiple-System Atrophy.

The affected siblings in Family 1 were born to consanguineous parents (first cousins).[13] In this family, the two patients with multiple-system atrophy (Participants II-4 and II-8) also had retinitis pigmentosa, which was not present in the other siblings. The diagnosis of definite multiple-system atrophy in three patients (Participants II-4 and II-8 in Family 1 and II-6 in Family 8) was confirmed at autopsy. In Family 8, two siblings (Participants II-3 and II-4) of the affected family members had Parkinson's disease (PD). In Family 1, in which homozygous M128V-V393A mutations in *COQ2* were identified, the parents (Participants I-1 and I-2), who were obligate carriers of the mutation, showed no overt signs of parkinsonism, cerebellar ataxia, or autonomic dysfunction, according to family report. In Family 12, in whom compound heterozygous R387X/V393A mutations were identified, Participants I-1 and I-2 (obligate carriers of the mutations) and the heterozygous carrier (Participant II-2) showed no overt signs of parkinsonism, cerebellar ataxia, or autonomic dysfunction on examination by a neurologist. Squares represent male family members, circles female family members, black symbols family members with multiple-system atrophy, gray symbols family members with Parkinson's disease, open symbols unaffected family members, slashes deceased family members, and small circles family members for whom genomic DNA samples were available. MSA-C denotes multiple-system atrophy of the cerebellar type, MSA-P multiple-system atrophy with predominant parkinsonism, and unclassified MSA-P+C similarly predominant parkinsonian and cerebellar signs.

ANALYSIS OF *COQ2* AND OTHER GENES ASSOCIATED WITH COENZYME Q_{10}

On the basis of linkage analysis and whole-genome sequencing, we sequenced *COQ2* and the other 11 genes involved in the biosynthetic pathway for coenzyme Q_{10} (*PDSS1*, *PDSS2*, *COQ3*, *COQ4*, *COQ5*, *COQ6*, *COQ7*, *ADCK3*, *COQ9*, *COQ10A*, and *COQ10B*), using the Sanger method (Table S3 in the Supplementary Appendix).

We prepared samples of mutant human *COQ2* complementary DNA (cDNA) by means of site-directed mutagenesis (Table S4 in the Supple-

mentary Appendix). A yeast *coq2*-null mutant, the BY4741Δ*coq2* strain, was transformed with pAUR123 (Takara Bio) containing the nonmutated or mutated human *COQ2* cDNA. We measured the growth rate in a medium with a nonfermentable carbon source by monitoring the optical density of a sample measured at a wavelength of 600 nm (OD_{600}). We used mitochondrial fractions prepared from lymphoblastoid cell lines with the QProteome Mitochondria Isolation Kit (Qiagen) as the enzyme source. COQ2 activity (Enzyme Commission number, 2.5.1.39) was assayed as described previously.[20]

COENZYME Q_{10} LEVEL IN TISSUES

Using high-performance liquid chromatography, we measured levels of coenzyme Q_{10} (ubiquinone-10 and ubiquinol-10) and free (unesterified) cholesterol in lymphoblastoid cell lines established from 152 patients with multiple-system atrophy and 76 controls and in cerebellum samples obtained on autopsy from 3 patients with multiple-system atrophy and 3 controls.[21]

STATISTICAL ANALYSIS

All results are presented as means and standard deviations. We used Student's t-test to evaluate the significance of differences in the mean age at disease onset between carriers and noncarriers of the *COQ2* mutation. We used Fisher's exact test to calculate the significance of the difference in allele frequencies between carriers and noncarriers, with contingency tables and standard methods used to compute odds ratios and corresponding 95% confidence intervals. We used the Kruskal–Wallis test, followed by the Steel test, to perform an analysis of variance. All statistical tests were two-sided, and a P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

LINKAGE ANALYSIS OF FAMILIAL DISEASE

Parametric linkage analysis of the six family pedigrees revealed no single locus showing a linkage compatible with autosomal recessive inheritance. However, in the parametric linkage analysis allowing for heterogeneity, we detected several loci showing positive scores for heterogeneity logarithm of the odds (HLOD), indicating that more than one locus was involved in the different multiplex families (Fig. S1B in the Supplementary Appendix). In particular, two regions on chromosome 4 showed the highest HLOD scores, exceeding 2.0. Results of nonparametric linkage analysis (Fig. S1C in the Supplementary Appendix) were consistent with those of parametric linkage analysis allowing for heterogeneity. Parametric linkage analysis of chromosome 4 in individual pedigrees revealed positive LOD scores in an overlapping region in four families (Family 1, Family 2, Family 4, and Family 12), with Family 1 having the highest LOD score of 1.93 (72.795 to 89.616 Mb) (Fig. S1A and S2A in the Supplementary Appendix). Thus, we selected Family 1 to undergo whole-genome sequencing.

SUSCEPTIBILITY GENE IN FAMILIAL DISEASE

Whole-genome sequencing of a sample obtained from Participant II-4, one of two affected members of Family 1, generated 187.5 Gb of short reads, with an average coverage of 58× and 3,492,429 single-nucleotide variants (SNVs) or insertions or deletions. We winnowed the 3,492,429 variants down to 4 by selecting SNVs that were located in the candidate regions defined on linkage analysis in Family 1 (regions with the highest LOD score spanning approximately 80 Mb in total), that were located in exons or splice sites, that were predicted to cause amino acid changes or changes in pre-messenger RNA splicing, and that were not registered in the database of single-nucleotide polymorphisms, build 130 (dbSNP130), indicating that the variants are extremely rare in the general population (Fig. S2B in the Supplementary Appendix). Each of these 4 SNVs is predicted to result in an amino acid substitution: K707R (c.2120A→G) in *SHROOM3* (NCBI Reference Sequence, NM_020859.3), M128V (c.382A→G) and V393A (c.1178T→C) in *COQ2* (NCBI Reference Sequence, NM_015697.7), and R231G (c.691A→G) in *SCEL* (NCBI Reference Sequence, NM_144777.2).

In the 180 Japanese control samples, we did not observe the SNV encoding the M128V variant but did observe SNVs encoding K707R in *SHROOM3*, V393A in *COQ2*, and R231G in *SCEL*, which were present on 3, 5, and 98 of 360 alleles, respectively. We therefore considered the SNP encoding M128V in *COQ2*, which encodes parahydroxybenzoate-polyprenyl transferase, an enzyme involved in the biosynthesis of coenzyme Q_{10}, as a candidate variant in conferring susceptibility to familial multiple-system atrophy.

Cosegregation analysis of samples from Family 1 revealed that the two affected family mem-

bers, Participants II-4 and II-8, carried the homozygous M128V-V393A variant in *COQ2*, and the unaffected sibling who was tested (Participant II-7) did not carry this variant (Fig. S2C in the Supplementary Appendix). Mutational analysis of *COQ2* in Family 12 revealed heterozygous mutations consisting of nonsense (R387X, c.1159C→T) and missense (V393A) variants in both affected siblings (Participants II-3 and II-4). Their mother (Participant I-2) was heterozygous for V393A, one unaffected sibling (Participant II-1) lacked this variant, and the other unaffected sibling (Participant II-2) was heterozygous for R387X. R387X was not observed in the 180 Japanese controls.

We did not detect variants of *COQ2* in the other four families (Families 2, 3, 4, and 8). Because *COQ2* encodes an enzyme essential for the biosynthesis of coenzyme Q_{10}, we further sequenced the other 11 genes in the biosynthetic pathway for coenzyme Q_{10} (*PDSS1*, *PDSS2*, *COQ3*, *COQ4*, *COQ5*, *COQ6*, *COQ7*, *ADCK3*, *COQ9*, *COQ10A*, and *COQ10B*) in the remaining four families and in a previously described multiplex family[14] but did not observe variants that cosegregated with disease.

COQ2 VARIANTS AND SPORADIC DISEASE

To investigate the involvement of *COQ2* variants in sporadic multiple-system atrophy, we extended the mutational analysis of *COQ2* to a Japanese series consisting of 363 patients with multiple-system atrophy and 520 controls. A common *COQ2* variant (rs6818847, predicted to result in an amino acid substitution, V66L (c.196G→T) with allele frequencies of 0.10 and 0.12 in the Japanese patients with multiple-system atrophy and controls, respectively, was not included in further analysis. Four patients with multiple-system atrophy carried two variants simultaneously (one carried an I147T [c.440T→C] and a nonmutated [NM] allele at codon 147 and V393A/NM at codon 393, one had R387Q [c.1160G→A]/NM at codon 387 and V393A/NM at codon 393, and two had V393A/V393A), whereas none of the controls had two variants of *COQ2* (Table 1). Sequencing of the subcloned mutated alleles confirmed that R387Q/V393A was present in a compound heterozygous state. We were unable to determine the phase of I147T/V393A, because the distance between I147T and V393A was too large to be amplified by means of polymerase-chain-reaction (PCR) assay in a single fragment, and samples

Table 1. *COQ2* Variants Found in Patients with Sporadic Multiple-System Atrophy in Japanese, European, and North American Series, as Compared with Controls.*

Genotype	Japanese Series		European Series		North American Series	
	Patients (N=363)	Controls (N=520)	Patients (N=223)	Controls (N=315)	Patients (N=172)	Controls (N=294)
P72L/NM	0	1	0	0	0	0
F79L/NM	0	0	1	0	0	0
P99H†/NM	0	0	0	0	1	0
S107T†/NM	0	0	1	0	0	0
R119H†/NM	0	0	0	0	0	1
I147T‡/V343A§	1	0	0	0	0	0
P157S†/NM	1	0	0	0	0	0
S163F†/NM	1	0	0	0	0	0
T317A‡/NM	0	0	1	0	0	0
S347C‡/NM	0	0	1	0	0	0
N386H/NM	0	1	0	0	0	0
R387Q†/V393A§	1	0	0	0	0	0
V393A§/NM	29	17	0	0	0	0
V393A§/V393A§	2	0	0	0	0	0

* NM denotes nonmutated.
† This variant was deemed to be severely deleterious on yeast complementation assay.
‡ This variant was deemed to be mildly deleterious on yeast complementation assay.
§ This variant had decreased COQ2 activity on enzyme assay.