Residual laminin-binding activity and enhanced dystroglycan glycosylation by LARGE in novel model mice to dystroglycanopathy

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Hypoglycosylation and reduced laminin-binding activity of α-dystroglycan are common characteristics of dystroglycanopathy, which is a group of congenital and limb-girdle muscular dystrophies. Fukuyama-type congenital muscular dystrophy (FCMD), caused by a mutation in the fukutin gene, is a severe form of dystroglycanopathy. A retrotransposonal insertion in fukutin is seen in almost all cases of FCMD. To better understand the molecular pathogenesis of dystroglycanopathies and to explore therapeutic strategies, we generated knock-in mice carrying the retrotransposonal insertion in the mouse fukutin ortholog. Knock-in mice exhibited hypoglycosylated α-dystroglycan; however, no signs of muscular dystrophy were observed. More sensitive methods detected minor levels of intact α-dystroglycan, and solid-phase assays determined laminin binding levels to be ~50% of normal. In contrast, intact α-dystroglycan is undetectable in the dystrophic Large*myd mouse, and laminin-binding activity is markedly reduced. These data indicate that a small amount of intact α-dystroglycan is sufficient to maintain muscle cell integrity in knock-in mice, suggesting that the treatment of dystroglycanopathies might not require the full recovery of glycosylation. To examine whether glycosylation defects can be restored in vivo, we performed mouse gene transfer experiments. Transfer of fukutin into knock-in mice restored glycosylation of α-dystroglycan. In addition, transfer of LARGE produced laminin-binding forms of α-dystroglycan in both knock-in mice and the POMGnT1 mutant mouse, which is another model of dystroglycanopathy. Overall, these data suggest that even

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partial restoration of α-dystroglycan glycosylation and laminin-binding activity by replacing or augmenting glycosylation-related genes might effectively deter dystroglycanopathy progression and thus provide therapeutic benefits.

INTRODUCTION

Dystroglycanopathy is a group of congenital and limb-girdle muscular dystrophies that includes Walker–Warburg syndrome (WWS), muscle-eye-brain (MEB) disease, Fukuyama-type congenital muscular dystrophy (FCMD), congenital muscular dystrophy 1C/D (1,2) and limb-girdle muscular dystrophy (LGMD) 2I/K/M/N (3–6). Hypoglycosylation of α-dystroglycan is a hallmark of these disorders. So far, six genes (POMT1, POMT2, POMGnT1, fukutin, FKRP and LARGE) have been implicated in dystroglycanopathies and all are thought to be involved in glycosylation of α-dystroglycan. POMGnT1 and the POMT1/2 complexes are known to have glycosyltransferase activities that place O-mannosyl sugar chains on α-dystroglycan (7,8). The exact functions of fukutin, FKRP and LARGE are still unknown.

α-Dystroglycan (α-DG) is a receptor for laminin in the basement membrane and is anchored on the plasma membrane through non-covalent interaction with a transmembrane-type β-DG (9). α- and β-DG are encoded by a single mRNA that is cleaved into two subunits during post-translational maturation. O-glycosylation of α-DG is required for ligand-binding activity. Although the exact binding epitope for ligand is still unknown, one unique O-mannosyl glycan [Neu5Ac(α2–3)Gal(β1–4)GlcNAc(β1–2)Man-Ser/Thr] (10) appears to be involved in ligand binding among extensive and heterogeneous groups of O-linked sugar chains. β-DG interacts with dystrophin, which in turn binds to actin filaments. The DG complex spans the plasma membrane, connecting the basement membrane to the actin cytoskeleton and presumably conferring mechanical stability to muscle cells during muscle contraction.

In Japan, FCMD is the most common congenital muscular dystrophy and, following Duchenne muscular dystrophy, is the second most common childhood muscular dystrophy. An autosomal recessive disorder, FCMD is characterized by severe muscular dystrophy, abnormal neuronal migration associated with mental retardation and epilepsy and, frequently, eye abnormalities (11). A recent study revealed aberrant neuromuscular junction formation and delayed muscle terminal maturation in FCMD, suggesting that a maturational delay of muscle fibers underlies the etiology of FCMD (12). Through positional cloning we identified fukutin, the gene responsible for FCMD (13). The predominant mutation in FCMD was identified as a 3 kb SINE-VNTR-Alu (SVA) retrotransposon insertion into the 3′UTR of fukutin. In Japan, 70–80% of FCMD patients are homozygous for this retrotransposon insertion. Compound heterozygosity, exhibiting both a retrotransposon mutation and a point mutation, is sometimes seen and generally exhibits more severe pathologies (13–15). Only a few cases with non-founder mutations (homozygous for point mutations) have been reported outside of Japan (5,16–19).

MEB disease is a severe autosomal recessive disease, similar to FCMD, characterized by congenital muscular dystrophy, ocular abnormalities and brain malformation. The gene responsible for MEB is POMGnT1, which encodes protein O-linked mannose β1,2-N-acetylglucosaminyltransferase 1 (7). In both FCMD and MEB disease, α-DG glycosylation and laminin-binding activity are severely disrupted (20). The Large<sup>myd</sup> mouse, a spontaneous mutant, has been used as a model for dystroglycanopathy. As is the case with human dystroglycanopathies, α-DG in Large<sup>myd</sup> mice is hypoglycosylated and shows reduced ligand-binding activity (20,21). Positional cloning in this model identified a disease-causing mutation in the Large gene (22), which encodes a protein with a transmembrane domain followed by a coiled-coil domain and two DxD-containing putative catalytic domains (23). LARGE mutations are also seen in human dystroglycanopathy (24). Although the exact function of the LARGE protein is not fully understood, it has been shown to produce hyperglycosylated α-DG in culture cells and mice (25,26). In addition, physical interaction between LARGE and α-DG is an essential step in acquiring ligand-binding activities of α-DG (25). Therefore, it is believed that LARGE plays a post-translational role in modulating both α-DG glycosylation and its functional expression.

To further investigate molecular pathogenesis and to explore therapeutic strategies for dystroglycanopathy, we generated several model mice for FCMD. We first generated mice with a targeted fukutin disruption, but this model showed embryonic lethality (27). We also generated chimeric fukutin mice by injecting homozygous targeted (fukutin<sup>−/−</sup>) ES cells into blastocysts (28). Mice with high chimerism showed dystrophic skeletal muscle; however, the variability of chimerism among individuals, and with growth, limits this experimental approach. Therefore, we generated a transgenic knock-in mouse model carrying the retrotransposonal insertion in fukutin. Our data revealed that even a small amount of intact α-DG is sufficient to maintain skeletal muscle function, and suggest that increasing the expression of glycosylation-related genes, which could be accomplished through various approaches, can be a therapeutic strategy for preventing or slowing progression of a broad range of dystroglycanopathies.

RESULTS

Generation of model mice for FCMD

To generate a transgenic knock-in mouse carrying the retrotransposon insertion, we replaced mouse fukutin exon 10 with a FCMD patient’s exon 10, engineered to contain the retrotransposon insertion using a site-directed DNA integration technique. Exon 10 encodes amino acids from Tyr-392 to the C-terminal end and the 3′UTR. We also generated another transgene containing a normal human exon 10. The terms Hn (human normal; Fig. 1A, no. 6) and Hp (human patient; Fig. 1A, no. 7) refer to transgenes containing the normal human exon 10 and the patient’s exon 10, respectively.
Recombination was confirmed using Southern blot analysis of genomic DNA from ES cells (data not shown). Targeted ES cell clones were injected into blastocysts to obtain chimeric mice. Germline transmission of the knock-in allele was established via Southern blot analysis of mouse genomic DNA (Fig. 1B). Germline-competent heterozygous mice were in turn mated to generate homozygous mutants (Hn/Hn and Hp/Hp) (Fig. 2A, nos 3 and 4). RT–PCR showed a dramatic reduction of fukutin mRNA transcript levels in Hp/Hp mice (Fig. 1C). Through quantitative PCR, we determined that Hp/Hp mice express fukutin transcript at 5–10% of normal levels (data not shown). We consider Hp/Hp mice to be models for most FCMD cases that are homozygous for the retrotransposal insertion. Human patients who are compound heterozygous for the insertion and a nonsense fukutin mutation generally show more severe pathology than those who are homozygous for the insertion (14). Therefore, we crossed Hp/Hp mice with transgenic mice carrying a neo cassette disruption of one fukutin allele (fukutin\(^{+/−}\)) (27) to create a compound heterozygous line. The Hp/+- mice in this line represent retrotransposon carriers (Fig. 2A, no. 5) and the Hp/− mice represent compound heterozygotes (Fig. 2A, no. 6).

**FCMD model mice exhibit hypoglycosylation of α-DG**

To characterize the biochemical properties of α-DG in the knock-in mice, we prepared skeletal muscle samples enriched for α-DG with wheat germ agglutinin (WGA) beads, which is able to bind nearly all the DG in the muscle sample (20,29). These preparations were analyzed using western blot analysis with goat polyclonal antibodies against α-DG core protein (AP-074G-C) and the monoclonal antibody IIH6. IIH6 recognizes glycosylated epitopes on α-DG, and hypoglycosylation results in the absence of epitopes for the antibody (20).
A small amount of intact \( \alpha \)-DG prevents muscular dystrophy

We examined hematoxylin and eosin (H&E) stained sections of the quadriceps, gastrocnemius, tibialis anterior, soleus, ilioptos and diaphragm muscles in Hp/+ and Hp/- mice. H&E staining revealed no clear difference between Hp/+ and Hp/- mice. Histopathological features of muscular dystrophy, such as centrally located nuclei, tissue fibrosis and fatty infiltration were not observed in 10-week-old FCMD models Hp/+ (Fig. 3A) and Hp/Hp mice (data not shown). Although FCMD onset in humans occurs at or near birth, we also examined older mice to determine whether onset in Hp/- mice was delayed. Even in older mice (>1 year old), we observed no signs of muscular dystrophy (Fig. 3B). There was no obvious change in the expression level of laminin \( \alpha 2 \) chain, which is the major ligand of \( \alpha \)-DG in the skeletal muscle (Supplementary Material, Fig. S1).

Both hypoglycosylated and IIH6-positive intact \( \alpha \)-DG proteins were detected in Hp/Hp and Hp/- mouse brains (Supplementary Material, Fig. S2). As is the case with skeletal muscle, Hp/- mice contained more hypoglycosylated \( \alpha \)-DG. Apparent brain histological abnormality was hardly detected in Hp/- mice; only a few mice showed a very small ectopic cluster of neurons migrating into the marginal zone. We also analyzed \( \alpha \)-DG in heart, liver, and lung from Hp/- mice, and found that the levels of hypoglycosylation and laminin-binding activity vary between the tissues (less affected in heart and liver) (Supplementary Material, Figs S2 and S3).

To analyze potential weakness in muscle cell membrane integrity, which may not be detectable in housed mice by H&E staining, Hp/- mice were subjected to treadmill exercise followed by the measurement of Evans blue dye (EBD) incorporation into muscle fibers. EBD is a membrane-impermeant molecule that binds to serum albumin and is physically restricted from fibers unless the skeletal muscle membrane is damaged (30). Even after exercising to exhaustion, Hp/- mice showed no EBD uptake in muscle cells (data not shown).
Reduction of laminin-binding activity due to hypoglycosylation of α-DG is thought to be the main cause of dystroglycanopathy. Therefore, we hypothesized that the minimal levels of intact α-DG species observed in Hp/− mice are sufficient to maintain linkage to laminin and prevent disease progression. To test this hypothesis, we compared the laminin-binding activity in Hp/− mice with that in Large myd (myd/myd) mice, which represent another dystroglycanopathy model with a muscular dystrophy phenotype (21). H&E analysis confirmed signs of muscular dystrophy (centrally located nuclei and fiber size variation) in myd/myd mice, but not in Hp/− mice (Fig. 4A and B). In contrast with Hp/− mice, western blot analysis of α-DG core protein in myd/myd mice revealed no intact size (~150 kDa) of α-DG species (Fig. 4C and D), indicating that almost all α-DG is hypoglycosylated in myd/myd mice. The laminin-binding activity of α-DG in Hp/− and myd/myd mice was measured using a quantitative solid-phase laminin-binding assay and compared with litter controls (Hp/+ and myd/+ mice, respectively) (Fig. 4E and F). Laminin-binding activity was ~50% of normal in Hp/− mice but less than 5% of normal in myd/myd mice. The solid-phase binding analysis shows no obvious difference between wild-type and Hp/+ mice. These data demonstrate that levels of glycosylation (indicated by IIH6 immunoreactivity and the presence of ~150 kDa α-DG) influence laminin-binding activity and indicate that only a small amount of IIH6-reactive α-DG is required to maintain skeletal muscle function.

**Fukutin gene transfer restores glycosylation of α-DG in knock-in mice**

Our data strongly suggest that even partial restoration of α-DG glycosylation is effective in reducing disease severity in...
dystroglycanopathy. To examine whether glycosylation defects can be recovered in vivo, a recombinant fukutin adenovirus was injected into the hind limb muscle of 3-day-old Hp/− and litter control Hp/+ mice. Following 4 weeks of injections, α-DG enriched samples were prepared using WGA beads and analyzed for glycosylation and laminin-binding activity. Western blot analysis with anti-α-DG core protein antibodies revealed that fukutin gene transfer into Hp/− mice reduced hypoglycosylated α-DG (−90 kDa) and increased levels of the normal-sized α-DG species (−150 kDa) (Fig. 5A, lanes 3 and 4). IIH6 reactivity and laminin-binding activity also increased following fukutin gene transfer into Hp/− mice (Fig. 5B and C, lanes 3 and 4). No obvious changes were observed in Hp/+ mice after the gene transfer (Fig. 5C, lanes 1 and 2). These results demonstrate that fukutin gene transfer can correct biochemical abnormalities of α-DG in fukutin-deficient skeletal muscle, and support that fukutin protein is involved in glycosylation of α-DG.

Large gene transfer produces laminin-binding forms of α-DG in dystroglycanopathy models

Hypoglycosylation leading to dystroglycanopathies is caused by mutations in six known genes (fukutin, POMGnT1, POMT1, POMT2, FKRP and LARGE) and other, unidentified genes. In an effort to bypass the need for identification of disease-causing genes in developing therapies (e.g. gene transfer), we further explored a unique feature of LARGE. LARGE has been demonstrated to induce α-DG hyperglycosylation, which is detected by IIH6 as a broad band detected at 150–300 kDa via SDS gel electrophoresis. This band shows increased ligand-binding activity in samples from genetically distinct diseases showing defective α-DG glycosylation (FCMD, MEB and WWS) (26).

We examined whether adenoviral LARGE gene transfer into Hp/− skeletal muscle induces hyperglycosylation and increases laminin-binding activity of α-DG. Immunofluorescence analysis of untreated control muscles revealed weaker IIH6 reactivity in Hp/− than in Hp/+ (Fig. 6A, −LARGE). Muscle sections subjected to gene transfer showed increased α-DG glycosylation in transduced areas, as indicated by eGFP expression in both Hp/− and Hp/+ mice (Fig. 6A, +LARGE). We also examined adenovirus-injected and non-injected contralateral leg muscles using western blot analysis with antibodies against α-DG core protein and IIH6. These experiments showed that the LARGE gene transfer increased IIH6 reactivity at −150 kDa in the Hp/− muscle and produced a broad band with a molecular weight of 150–250 kDa in both Hp/− and Hp/+ muscles (Fig. 6B). Anti-α-DG core protein antibodies poorly recognized a higher molecular weight α-DG species (Fig. 6C), which is consistent with previous reports (26). Following the LARGE gene transfer, levels of hypoglycosylated α-DG species decreased (Fig. 6C, lanes 3 and 4). These data indicate that

Figure 5. Fukutin gene transfer rescues the glycosylation abnormality in Hp/− mice. Hp/+ or Hp−/− pups were injected with adenovirus encoding wild-type human fukutin in one leg (+) and with saline in the contralateral leg (−). Calf muscle was analyzed using western blot with antibodies against core α-DG protein (A) and glycosylated α-DG (B) and using a laminin overlay assay (C). Transfer of fukutin produced increases in α-DG molecular weight, IIH6 reactivity and laminin binding activity in Hp/−/− mice.

Figure 6. LARGE gene transfer produces functionally glycosylated α-DG in Hp/− mice. Hp/+ or Hp−/− pups were injected with an adenovirus encoding LARGE in one leg (+) and with saline in the contralateral leg (−). Calf muscle was analyzed using IIH6 immunofluorescence (A). GFP fluorescence represents muscle fibers successfully transduced by the adenoviral vectors. WGA preparations were analyzed using western blots with antibodies against glycosylated α-DG (B), α-DG core protein (C) and using a laminin overlay assay (D). The western blot for β-DG shows comparable amounts of DG proteins in each lane. Images with longer-exposures better indicate the presence of hyperglycosylated α-DG (arrowheads). These results show that the transfer of LARGE increases IIH6 reactivity and laminin-binding activity in Hp/− mice.
LARGE-induced glycosylation occurs on hypoglycosylated α-DG species. The IIH6-positive broad-molecular-weight band was able to bind laminin in both Hp/− and Hp/+ skeletal muscle samples (Fig. 6D, lanes 2 and 4). These data indicate that LARGE can increase laminin-binding forms of α-DG in fukutin-deficient skeletal muscle.

We further investigated whether LARGE gene transfer induced hyperglycosylation and produced laminin-binding forms of α-DG species in another dystroglycanopathy model, the POMGnT1-disrupted mouse (POMGnT1+/−) (Miyagoe-Suzuki et al., manuscript in preparation). Western blot analysis using α-DG core protein antibodies showed a reduction of α-DG molecular weight to 60–90 kDa in POMGnT1−/− mice (Fig. 7C, lane 4). Little IIH6 reactivity was detected via immunofluorescence (Fig. 7A) and western blot (Fig. 7B, lane 4) analysis. These data indicate hypoglycosylation of α-DG in POMGnT1−/− mice. Accordingly, laminin-binding activity was significantly reduced in POMGnT1−/− mice compared with POMGnT1+/− or POMGnT1+/+ littersmates (Fig. 7D, lanes 2, 4 and 6). The minor laminin binding protein (~80-100 kDa, lane 4) detected only in POMGnT1−/− is unidentified; however, similar laminin binding was also observed in POMGnT1-deficient MEB patients (20). A solid-phase binding assay also showed minor levels of laminin-binding activity in POMGnT1−/− (Supplementary Material, Fig. S4). For all genotypes, adenoviral LARGE gene transfer increased IIH6 reactivity in transduced areas indicated by eGFP expression (Fig. 7A, +/+,

+/−, and −/−). Western blot analysis using IIH6 showed that LARGE gene transfer also induced hyperglycosylation of α-DG in all genotypes, as indicated by broad bands with molecular weights from 150 to >250 kDa (Fig. 7B). After the gene transfer, the POMGnT1−/− skeletal muscle showed only hyperglycosylated IIH6-positive species, while the POMGnT1+/− and the POMGnT1+/+ muscles showed both hyperglycosylated and the original 150 kDa IIH6-positive species. Overlay assays showed that the laminin-binding epitope was produced on hyperglycosylated α-DG (Fig. 7D). These data support the idea that LARGE is an effective target for increasing or restoring laminin-binding activity of α-DG in dystroglycanopathy.

**DISCUSSION**

We have used several approaches to generate FCMD model animals. Fukutin-null mice result in embryonic lethality (27). Fukutin-chimera mice derived from ES cells targeted for both fukutin alleles (28) develop muscular dystrophy, but are inappropriate therapeutic study models because (i) they show wide variation in disease severity, and (ii) muscle cell fusion events during growth and regeneration can alter the population of fukutin-null cells. Therefore, we decided to introduce the disease-causing retrotransposon into the mouse fukutin gene to mimic the most prevalent form of human FCMD. In these knock-in Hp/Hp and Hp+/− mice, we detected hypoglycosylated α-DG, as is seen in FCMD patients (20,31), so we consider them to be novel models for FCMD.

Spontaneous Large<sup>myd</sup> and Large<sup>vls</sup> mice (21,32) and genetically engineered POMGnT1-deficient mice (33) have been reported as dystroglycanopathy models. Because these models mimic null mutations such as nonsense and frameshift mutations, they do not necessarily represent human diseases caused by missense mutations. Our knock-in mice with the retrotransposon fukutin insertion are the first dystroglycanopathy model that carries a human disease-causing mutation. Such models are needed to explain the molecular pathogenesis of diseases, to determine the function of responsible genes and to screen drugs that correct specific defects (34).

Although these mice genetically and biochemically represent features of fukutin-deficient muscular dystrophies, histological analysis has revealed no signs of muscular dystrophy. In typical cases of FCMD, normal-sized α-DG with IIH6-reactivity is barely detected, and laminin-binding activity is dramatically reduced (20). Comparing Hp/− mice with Large<sup>myd</sup> mice led us to reason that the remaining intact α-DG and laminin-binding activity in Hp/− mice might be sufficient to prevent disease progression. In the future, it would be important to elucidate the threshold level of glycosylation required to avoid a phenotype by using a model system that can control glycosylation levels in vivo. In Hp/− mice, residual laminin-binding is detected from DG species with slightly lower molecular weight (<150 kDa) (Fig. 2F), whereas this is not the case for human patients even with retained laminin binding (35). The difference suggests that mice may have additional laminin-binding epitopes, which are less susceptible to fukutin defects. Alternatively, other factors may compensate for...
reduced laminin-binding to α-DG. For example, it has been suggested that integrin α7, another laminin receptor in skeletal muscle, may account for the difference in clinical severity between mice and humans with dystrophin- or the DGC-defects (36,37). Clarifying the factors involved would be necessary for a better understanding of pathomechanism, which could promote identification of novel therapeutic targets.

Also important is the finding that even a small amount of IIH6-immunoreactivity of α-DG is sufficient to maintain skeletal muscle function. This concept is supported by milder cases of human patients with fukutin mutations (35). Murakami et al. have described reduced but detectable IIH6-reactivity and intact α-DG in patients who are compound heterozygous for the fukutin retrotransposon insertion and a missense mutation (R179T or Q358P). These individuals showed minimal dystrophic features and normal intelligence. Laminin-binding activity is also retained in all cases. These findings provide further evidence that the disease severity of fukutin-deficient muscular dystrophy is related to the ratio of normal glycosylation to hypoglycosylation.

Such correlation has been observed in other dystroglycanopathies. LGMD2I patients at the severe end of the clinical spectrum tend to show the greatest reduction in α-DG glycosylation, while those at the milder end tend to have relatively well-preserved α-DG glycosylation (38). Most known missense mutations in POMGnTI disrupt POMGnT enzyme activity, causing hypoglycosylation of α-DG and a severe congenital muscular dystrophy phenotype (39,40). Clement et al. (6) have reported a patient with a milder LGMD phenotype who carries a novel homozygous missense mutation in POMGnTI. Studies of this patient’s fibroblasts showed an altered kinetic profile but intact enzyme activity, explaining the relatively mild phenotype. Furthermore, a recent systematic and large-scale study of genotype–phenotype correlation in dystroglycanopathy revealed a wide spectrum of clinical severity in specific disease-causing genes (18). A broad correlation between the amount of depleted glycosylated epitope and phenotypic severity was described, though not systematically quantified. A more recent study reported a few cases with less correlation between clinical course and α-DG immunolabeling (41). We propose that, in addition to immunolabeling, combination of western blotting and laminin binding assays will be necessary for further advances in both clinical and basic biomedical research.

The present study strongly suggests that full recovery of α-DG glycosylation is not always necessary; partial restoration of α-DG glycosylation might be enough to prevent or slow disease progression. The simplest way to restore α-DG glycosylation in dystroglycanopathies would be by replacing a defective gene with the normal version. In many cases, though, the disease-causing gene is not known. A recent study revealed that most patients with a dystroglycanopathy harbor mutations in novel genes (18). To increase amounts of glycosylated α-DG with laminin-binding activity regardless of the responsible gene, we took advantage of the observation that overexpression of LARGE can produce hyperglycosylated α-DG with increased laminin-binding activity in cells from genetically distinct dystroglycanopathies (26). LARGE-induced hyperglycosylation of α-DG has also been observed in both CHO glycosylation mutants showing defective transfer of sialic acid, galactose or fucose to glycoconjugates and in a mutant that is unable to synthesize O-mannose glycan (42). Such a ‘super-effect’ of LARGE on α-DG glycosylation has been observed in vitro, but no in vivo study has been reported except in Large<sup>myd</sup> mice (26). Gene transfer of LARGE into Large<sup>myd</sup> mice essentially replaces the defective gene with the normal version of the gene. Our results provide the first in vivo evidence that LARGE gene transfer can bypass the glycosylation defects of α-DG in models other than the Large<sup>myd</sup> mice. These results support the idea that glycotherapies aimed at modulating LARGE may be a therapeutic option for many α-DG glycosylation-deficient muscular dystrophies.

Overall, our biochemical, histological and gene transfer experiments using novel model mice with disease-causing mutations support the efficacy of glycotherapy in dystroglycanopathies. The models developed here will be powerful in understanding the pathomechanism of FCMD and other related diseases.

**MATERIALS AND METHODS**

Generation of model mice

A targeting vector containing the retrotransposon insertion of human FCMD patients was generated using a site-directed DNA integration technique (43). Briefly, lox71 and TK-loxP-neo pA fragments (44) were inserted 5’ and 3’ to exon 10 of mouse fukutin (Fig. 1A, no. 2). To excise a floxed exon into mouse embryonic stem (ES) cells. Meanwhile, lox66 and TK-loxP fragments were inserted into an ES cell line containing normal human exon 10 and mutant exon 10 of human fukutin, with or without a retrotransposon insertion (Fig. 1A, nos 4 and 5). Each construct was co-transfected with a Cre-expressing vector into ES cells that constitutively express the Δexon10 construct, to obtain recombinant knock-in alleles (Fig. 1A, nos 6 and 7). The transgenic alleles containing normal human exon 10 and mutant exon 10 were named Hn (representing ‘human normal’) and Hp (representing ‘human patient’), respectively. Targeted ES cell clones were injected into blastocysts, and germline-competent heterozygous mice were in turn mated to generate homozygous mutants.

Genotyping of each transgene was performed using PCR with the following primers: FCMDKIF1, GAAACTCTGC-CATGACACCTC: HNC440R, ACCAGCTTTAATTGCCA-GAAG: Wild R2, GAAGCCAACTGTGTACCACAC. The FCMDKIF1 and HNC440R, and FCMDKIF1 and Wild R2 primer pairs yielded bands of ~800 bp (knock-in allele) and ~1100 bp (wild-type allele), respectively. Genotyping of a fukutin allele disruption by a neo replacement (fukutin null) was described previously (45). The primers for fukutin RT–PCR are AGGGATGGGGCTGTAAGACT and GTGCCATTTTGGGCAAGTTTT.

C57BL/6 mice were obtained from Japan SLC, Inc., and Large<sup>myd</sup> mice were obtained from The Jackson Laboratory. Mice were maintained in accordance with the animal care guidelines of Otsuka Pharmaceutical Co. Ltd. and Osaka University.
Antibodies

Antibodies used in western blots and immunofluorescence were as follows: mouse monoclonal antibody 8DS against β-DG (Novacastra); mouse monoclonal antibody IIH6 against α-DG (Upstate); and polyclonal anti-laminin (Sigma). We generated goat polyclonal antibodies against α-DG core protein using GST fusion proteins containing the N- or C-terminal domains of mouse α-DG. Antisera (074G) were affinity-purified using an α-DG–Fe fusion protein expressed in HEK293 cells. The purified antibody was named AP-074G-C.

Dystroglycan preparation and western blotting

DG was enriched from solubilized skeletal muscle as previously described (20,29). Briefly, 100 mg of muscle was solubilized in 1 ml of Tris-buffered saline (TBS) containing 1% Triton X-100 and protease inhibitors (Funakoshi). The solubilized fraction was incubated with 30 μl of WGA–agarose beads (Vector Labs) at 4°C for 16 h. Beads were washed three times in 1 ml TBS containing 0.1% Triton X-100 and protease inhibitors. The beads were then either directly boiled for 5 min in SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer (western blot and laminin overlay) or eluted with 300 μl TBS containing 0.1% Triton X-100, protease inhibitors and 300 mM N-acetylglucosamine (solid-phase binding assay). Proteins were separated using 7.5% or 10% SDS–PAGE. Gels were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). Blots were probed with DG antibodies and then developed with horseradish peroxidase (HRP)-enhanced chemiluminescence (Supersignal West Pico, Pierce; or ECL Plus, GE Healthcare).

Immunofluorescence and histological analysis

Cryosections (7 μm) were prepared and analyzed using immunofluorescence or H&E staining. Sections were stained for 2 min in hematoxylin, 1 min in eosin and then dehydrated with ethanol and xylenes. For immunofluorescence staining with IIH6, sections were treated with cold ethanol/acetone (1:1) for 1 min, blocked with 5% goat serum in MOM Mouse Ig Block Reagent (Vector Laboratories) at room temperature for 1 h and then incubated with primary antibodies diluted in MOM Diluent (Vector Laboratories) overnight at 4°C. The slides were washed with PBS and incubated with Alexa Fluor 488-conjugated antimouse IgM antibody (Molecular Probes) at room temperature for 30 min. For GFP detection, sections were washed with 4% paraformaldehyde in PBS for 10 min, washed with PBS three times and then mounted. Permount® (Fisher Scientific) and TISSU MOUNT® (Shiraimatsu Kiikai) were used for H&E staining and immunofluorescence, respectively. Sections were observed under fluorescence microscopy (Leica DMR, Leica Microsystems). For EBD uptake, mice were exercised on a treadmill (MK-680S, Muromachi Kikai) as described (34).

Laminin-binding assay

Laminin-binding activity was examined as previously reported (20) with slight modifications. Laminin overlay assays were performed on PVDF membranes using mouse Engelbreth–Holm–Swarm (EHS) laminin (Sigma). Briefly, PVDF membranes were blocked in laminin-binding buffer (LLB: 10 mM triethanolamine, 140 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, pH 7.6) containing 5% non-fat dry milk followed by incubation with 7.5 nM laminin at 4°C for 12 h in LLB with 3% BSA. Membranes were washed and incubated with anti-laminin (Sigma) at 4°C for 3 h followed by anti-rabbit IgG–HRP at room temperature for 45 min. Blots were developed by enhanced chemiluminescence (Supersignal West Pico, Pierce).

For the solid-phase binding assay, WGA eluates were diluted 1:50 in TBS and coated on polystyrene ELISA microplates (Costar) for 16 h at 4°C. Plates were washed in LBB and blocked for 2 h in 3% BSA in LBB. Mouse EHS laminin was diluted in LBB and applied for 1 h. Wells were washed with 3% BSA in LBB, incubated for 1 h with 1:10,000 anti-laminin (Sigma) followed by anti-rabbit HRP. Plates were developed with o-phenylenediamine dihydrochloride and H2O2, then reactions were stopped with 2 N H2SO4 and values obtained on a microplate reader. The data were fit to the equation 

\[ A = B_{\text{max}}x/(K_d + x), \]

where \( K_d \) is the dissociation constant, \( A \) is absorbance and \( B_{\text{max}} \) is maximal binding.

Adenoviral gene transfer

The complete open reading frame of mouse fukutin was cloned into the EcoRI site of the pKSCX-EGFP vector (46). The pKSCX-EGFP vector contains IRES-EGFP so that both the fukutin and GFP genes are expressed bicistronically under the CAG promoter. This expression cassette was digested with SwaI, and then its blunt-ended fragment was ligated into the adenoviral cosmid vector. The recombinant adenoviral vector encoding fukutin was generated using the method of Tashiro et al. (46).

Generation of the recombinant adenoviral vector encoding LARGE has been previously described (26). Amplified adenoviruses were purified using VIVAPURE ADENOPACK 100 (VIVASCIENCE).

In vivo gene transfer was performed with Hp/− and control littermate Hp/+ pups, age 2–4 d. Adenoviruses were injected percutaneously into the calf and hamstring with 1 × 108–1 × 109 particles in 10 μl of saline solution. Mock injections used saline solution only. Four weeks after injection, experimental and control contralateral leg muscles were subjected to immunofluorescence and biochemical analysis.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.
REFERENCES


SUPPLEMENTAL MATERIAL

Supplementary Figure 1

Expression of laminin α2

Quadriiceps muscles from Hp/+ or Hp/- mice were analyzed with anti-laminin α2 antibody (4H8-2). The result shows no obvious difference in the expression of laminin α2 chain.
Supplementary Figure 2

Hypoglycosylation of α-DG in Hp/- mouse brain

WGA-enriched DG from solubilized brain samples were analyzed by Western blot using antibodies against core protein and glycosylated α-DG (IIH6), and using a laminin overlay assay. The Western blot for β-DG shows comparable amounts of DG proteins in each lane. The results indicate that both intact and hypoglycosylated α-DG are present in Hp/Hp and Hp/- mouse brains.
Supplementary Figure 3
Western blotting and laminin overlay assays of Hp/- mouse heart, liver, and lung

WGA-enriched DG from solubilized heart, liver, and lung samples were analyzed by Western blot using antibodies against core protein (core) and glycosylated (IIH6) α-DG, and using a laminin overlay assay. The Western blot for β-DG shows comparable amounts of DG proteins in each lane. The results show that levels of hypoglycosylation vary between the tissues (less affected in heart and liver). It has been also reported that IIH6 reactivity and laminin-binding activity varies among tissues (1). As reported, IIH6 reactivity and laminin-binding activity of α-DG was hardly detected in liver and lung.
Supplementary Figure 4

A solid-phase laminin-binding assay of POMGnT1\textsuperscript{-/-} mice

WGA-enriched preparations from POMGnT1\textsuperscript{-/-} (open triangles), POMGnT1\textsuperscript{+/-} (filled triangles), and POMGnT1\textsuperscript{+/-} (open squares and gray line) skeletal muscles were measured for a solid-phase laminin-binding assay. The results showed minor levels of laminin-binding activity in POMGnT1\textsuperscript{-/-}.

References