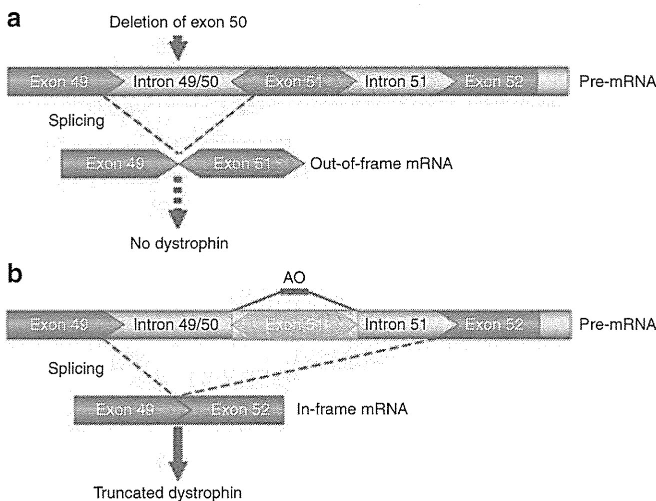

Figure 2 Example of exon skipping in Duchenne muscular dystrophy (DMD) patient who has a deletion of exon 50. (**a**) The absence of exon 50 in the dystrophin gene leads to an out-of-frame mRNA creating a premature stop codon in exon 51, thus aborting dystrophin synthesis during translation. (**b**) Using an antisense oligonucleotides (AO) targeting exon 51, this exon is skipped during splicing. This restores the open reading frame of the transcript and allows the synthesis of an internally deleted dystrophin. Modified from Van Deutekom *et al.*[65]

parallel, 2OMPs were designed to target the exon 23 of the mouse *DMD* gene since the nonsense mutation of *mdx* mouse is localized in this exon. Intramuscular administration of an AO targeting the exon 23 donor splice-site in these mice induced the restoration of dystrophin (without the exon 23) in the treated muscles.[61] These AOs were also intravascularly injected in *mdx* mice. Treated mice showed dystrophin restoration in many muscles.[62] However, low levels of dystrophin restoration were detectable in the heart.[63] A study demonstrated that repeated 2OMP injections increased the AO efficiency without increasing its toxicity.[62] A subcutaneous 2OMP injection has also been tested and this type of injection showed better pharmacokinetics and pharmacodynamics than intramuscular or intravenous injections.[64]

After these positive results in the *mdx* mouse model, a clinical trial on four DMD patients with the PRO051/GSK2402968 (2OMP targeting exon 51) was done. The muscle injected with 0.8 mg of this 2OMP showed 64–97% dystrophin-positive fibers (not corrected for positive muscle fibers in saline-injected contralateral muscle) with a level of dystrophin expression between 17 and 35%.[65] No adverse effects were found in the treated muscles. A phase I/II clinical trial, in which this same AO was injected subcutaneously, was recently completed and showed that this AO was well tolerated in all patients and that novel dystrophin expression was detected in each treated patient in a dose dependent manner.[66] A phase III study has started with this AO on DMD patients.[67]

Despite the fact that long-term toxicity studies in animal models with 2OMP are lacking, this approach seems promising.

Phosphorodiamidate morpholino oligomer: Similar to 2OMPs, PMOs (commonly referred to as morpholinos) are obtained by modifying the classic synthesis of oligonucleotides. Their ribose is replaced by a morpholine ring and the oxygen present in the phosphodiester link (the one that is not negatively charged) is replaced by a nitrogen atom. These modifications allow morpholinos to be biologically stable[68] and have antisense properties.[69]

Exon 23 of the mouse *DMD* gene was the first target of morpholinos. Restoration of dystrophin was observed in the treated *mdx* mouse muscles when morpholinos were intramuscularly injected[70] and in many muscles when intravenously[71] or intraperitoneally injected.[72] A partial restoration of dystrophin in the heart of *mdx* mice was also shown but the morpholino dose used was 50 times superior to the one used to treat skeletal muscles.[72] Recent studies of long-term repeated systemic treatment of *mdx* mice over a year with naked PMO at doses of 5 and 50 mg/kg have shown significant improvement in pathology and complete normalization of locomotor behavior without signs of renal or hepatic toxicity.[73] A morpholino designed to restore dystrophin expression in dystrophic (golden retriever muscular dystrophy) dogs was also synthesized and intravenously injected in these dogs. Five months later, treated dogs showed about 25% dystrophin-positive fibers throughout the body with a global improvement in muscle pathology in PMO-treated dogs compared to pretreated and untreated control dogs.[74] No significant signs of toxicity were found.

To enhance the cellular uptake of PMOs, they can be conjugated to peptides or other conjugates. The delivery of a morpholino conjugated with a dendrimeric octaguanidine (Vivo-Morpholino) was efficient to induce dystrophin expression in *mdx* mouse muscles.[75] Indeed, repeated injections at biweekly intervals achieved near 100% dystrophin-positive fibers in many skeletal muscles without eliciting a detectable immune response; the dystrophin restoration in the cardiac muscle reached up to 40%. PMOs conjugated with arginine-rich cell-penetrating peptides,[76] called pPMOs, also produced excellent restoration of dystrophin expression in *mdx* mice.[77,78] A pPMO targeting exon 23 was applied as well in utrophin$^{-/-}$ *mdx* mice by intraperitoneal injection. Whereas untreated animals typically died by 15 weeks of age, treated animals showed few signs of weakness, improved histopathology and appeared essentially normal at 1 year of age.[79] A muscle-targeting heptapeptide (MSP) fused to an arginine-rich cell-penetrating peptide (B-peptide) and conjugated to a PMO, called B-MSP-PMO, was also shown to be efficient for restoring dystrophin in *mdx* muscles.[80] Indeed, using an intravenous dose of 6 mg/kg of B-MSP-PMO administered biweekly over the course of 12 weeks, the dystrophin expression was found at a level of 100% in several muscles except for the heart. These pPMO seem well tolerated in *mdx* mice. Indeed, a pPMO targeting the exon 23 of the mouse *DMD* gene exhibited no toxic effects in kidneys at either 20 mg/kg weekly injection to the wild-type mice for 6 weeks or 30 mg/kg biweekly injection to *mdx* mice for 3 months. However, the same peptide conjugated to the PMO targeted to human exon 50 (AVI-5038) was found to cause mild tubular degeneration in the kidneys of nonhuman primates at 9 mg/kg weekly injections for 4 weeks.[81]

To target more dystrophin mutations occurring in DMD patients, other exons such as the exon 51 in *mdx*52 mice were targeted.[82] In addition, it is possible to remove in-frame exons from the dystrophin pre-mRNA and induce specific internally deleted dystrophin by using AOs. This has been done for exons 19/20 and 52/53 in wild-type mice.[83]

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After these positive results in animal models, a clinical trial in seven DMD patients was undertaken to skip exon 51 and thus to restore the reading frame of their dystrophin mRNA using unmodified morpholinos. The morpholino (AVI-4658) was intramuscularly injected and biopsies were taken 3–4 weeks later. Two patients were treated with a low dose of this morpholino (0.09 mg) and five patients with a higher dose (0.9 mg). Only the patients receiving the higher dose produced dystrophin although exon skipping was observed in all patients by reverse transcriptase PCR. In the five patients receiving the higher dose, the muscles injected with the AO showed 44–79% dystrophin-positive fibers (corrected for positive fibers in saline-injected contralateral muscle) with a level of dystrophin expression between 22 and 32%.[84] No signs of toxicity were observed. After these encouraging results, a systemically delivered morpholino phase Ib/II clinical trial was undertaken. According to a press release from AVI Biopharma (Bothell, WA),[85] 19 DMD patients were enrolled in six dose cohorts (0.5, 1, 2, 4, 10, or 20 mg/kg) and treated during 12 weeks by weekly intravenous infusion. Some patients expressed dystrophin-positive fibers; those treated with the higher doses of morpholino had more uniform and widespread dystrophin-positive fiber distribution than patients who received lower doses. The morpholino was well tolerated in all patients. A phase II clinical trial is currently in preparation to evaluate higher weekly doses of AVI-4658 (50 and 100 mg/kg).[85]

Although pPMO seems to cause some toxicity in nonhuman primates, there are other ways to modify the peptide conjugate, which are hopefully less toxic, to allow clinical development for DMD patients.

Modification of the DMD gene with meganucleases or zinc finger nucleases. A new alternative treatment for DMD relies on the restoration of the dystrophin reading frame by inducing a micro-deletion or a micro-insertion in the *DMD* gene.[86] This can be done by inducing double strand breaks at the end of the exon, which precedes a deletion, or at the beginning of an exon, which follows a deletion. These double strand breaks can be induced with specially engineered meganucleases or zinc finger nucleases. They are spontaneously repaired by a process called nonhomologous end-joining, which introduces a micro-insertion or a micro-deletion. Alternatively, double strand breaks can be repaired by homologous recombination by providing a donor plasmid containing the coding sequence that is deleted in the patient's genome.

Other approaches

Myostatin. A potential therapeutic method to improve muscle strength is to block myostatin. Myostatin is a member of the transforming growth factor-β family implicated in muscle size regulation. Indeed, in the myostatin gene knockout mouse, robust muscular hypertrophy and hyperplasia are observed.[87] Antibodies against myostatin were produced and intraperitoneally injected in *mdx* mice. The treated mice showed muscular hypertrophy, muscle strength increase, and histological improvement.[88] There are also other methods to block the myostatin pathway such as the use of follistatin[89] or of myostatin propeptide.[90] Another approach is to directly mutate the myostatin receptor, the activin type-II receptor[91] or to inject a soluble form of this receptor.[92] All these approaches led to improvements of the treated mouse phenotype similar to that observed in myostatin$^{-/-}$ mice. Recently, the use of destructive exon skipping of the myostatin pre-mRNA induced by 2OMP and PMO has been described to induce skeletal muscle hypertrophy, which along with dystrophin exon skipping (see above) may thus provide a potential combined antisense strategy to simultaneously reactivate dystrophin expression and increase muscle bulk.[93] In a recent clinical trial, the use of an antibody against myostatin (MYO-029) was undertaken. Although the antibody was well tolerated, no muscle strength improvements were detected perhaps due to a lower dose of antibody.[94] Other clinical trials with myostatin inhibitors are currently undertaken by at least four biotechnology and pharmaceutical companies.[95]

Utrophin. Utrophin shares 80% sequence identity with dystrophin and is expressed in the muscles during embryonic development.[96] However, in adult myofibers, it is located only at the neuromuscular junction and at the myotendinous junctions. Utrophin is over-expressed in muscle fibers of dystrophic mice and of DMD patients.[97,98] Since it has sequence homology with dystrophin, it was suggested that its upregulation could slow down DMD development. When its expression is increased three- to fourfold in transgenic *mdx* mice, their phenotype is similar to wild-type mice.[99] Therefore, an increase of the utrophin expression may be a potential therapy to improve DMD patients. The injection of heregulin in *mdx* mice increased utrophin expression by two to threefold and led to histological improvements.[100] The injection of L-arginine or nitric oxide also allowed utrophin upregulation in *mdx* mice.[101] Recently, the intraperitoneal injection of a TAT-utrophin protein in *mdx* mice increased their muscle strength.[102] A drug developed by Summit PLC (C110/BM195) to upregulate the utrophin expression was carried out by BioMarin pharmaceuticals in a phase I clinical trial with normal individuals. No adverse effects were reported but the pharmokinetics of the drug did not allow them to continue the development of this drug. Summit PLC is currently working on a new formulation, which may improve the pharmokinetics. Further investigation in increasing utrophin expression is required since the molecules tested so far in *mdx* mice did not increase utrophin expression sufficiently to completely suppress the symptoms due to the dystrophin deficiency in *mdx* mice.[103,104] Moreover, utrophin does not seem to anchor nitric oxide synthase at the sarcolemma like dystrophin does, thus leading to a premature muscle ischemia.[105] However, the levels of utrophin upregulation may be sufficient to alleviate most of the DMD symptoms.

GENE THERAPY

Since the first clinical trial of gene therapy in 1990,[106] there has been a strong interest for this therapeutic approach. However in 1999, a major setback occurred due to the death of a patient treated with an adenovirus for ornithine transcarbamylase deficiency.[107] This death is believed to have been triggered by a severe innate immune response to the adenoviral vector. In 2002, another death occurred in a clinical trial for severe combined immunodeficiency with the use of a retrovirus where one of the treated

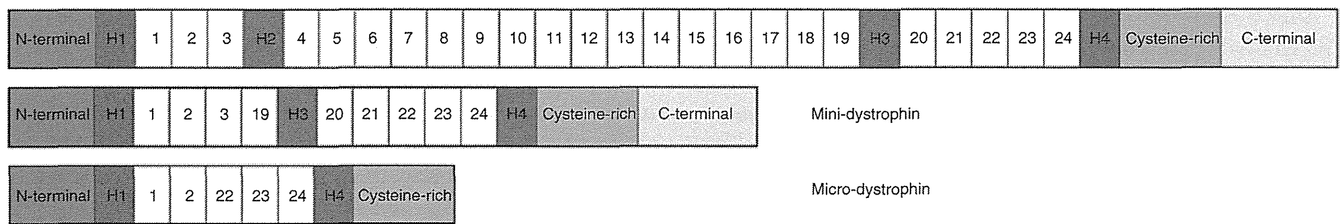

Figure 3 Dystrophin versions. The full-length dystrophin cDNA (11 kb) is represented at the top. The middle schema represents an example of a mini-dystrophin cDNA with an H2-R18 deletion; the approximate size of mini-dystrophins is about 6 kb. The bottom representation is a schema of a micro-dystrophin cDNA (around 4 kb) with an R3-R21 and C-terminal deletion.

patients died due to the activation of an oncogene.[108] However, the fatality rate of gene therapy is still much lower than that of the standard bone marrow transplantation treatment for severe combined immunodeficiency patients.[109] Moreover, >45 patients have now been treated via gene therapy, resulting in one death and >40 cures. Gene therapy is thus an appealing approach to cure many hereditary diseases such as DMD.

Gene therapy in DMD consists of the introduction of a functional copy of the *DMD* gene in muscle fibers with the aim of restoring muscle function including force generation and resistance to muscle contraction induced damage. The concept of dystrophin internally deleted genes that would fit the packaging capacity of small viral vectors came from clinical observations that some BMD patients with internally deleted dystrophins could maintain ambulation for many decades. This gave rise to the concept of mini-dystrophin (mDYS) or micro-dystrophin (μDys). Gene therapy is divided in two distinct categories: those using viral vectors to transfer the gene are referred to as "viral gene therapy" and those employing naked DNA as "nonviral gene therapy".

Internally deleted dystrophin genes

In gene therapy, the transgenes generally contain complementary DNA (cDNA) corresponding only to coding regions of a gene, *i.e.*, exons without introns. The dystrophin cDNA size is about 11 kb and is called full-length dystrophin (FLDYS). Apart from this FLDYS, several mDYS and μDys internally deleted versions exist (**Figure 3**). Indeed, a BMD patient with a deletion of the exons 17–48 in the *DMD* gene was reported to have only a mild dystrophic phenotype.[30] The missing region was located in the spectrin-like repeats of the rod domain resulting in an internally deleted dystrophin with only eight of these repeats instead of 24. The corresponding transgene was thus constructed[110] and other, even smaller, truncated versions were designed subsequently.[111,112] These constructions were called mDYS, or μDys when the C-terminal part is also missing.

Several transgenic mice expressing these internally deleted dystrophins were generated and analyzed[112–115]; all these mice showed the restoration of the DGC. The simple fact of restoring the DGC improves the muscle histology as well as the reduced leukocyte infiltration and the decreased number of centro-nucleated muscle fibers. The muscle strength is also increased but does not reach wild-type levels. However, the observed improvements vary depending on which exons are deleted. The use of internally deleted dystrophins is attractive but the best phenotypic restorations are still obtained with the use of FLDYS.

Viral gene therapy

Different viral vectors could be used for DMD gene therapy. Adenoviral vectors show poor efficiency in adult animal models compared to newborns. Moreover, the use of adenoviral vectors is complicated since half of the human population already has neutralizing antibodies against the adenoviral capsid and also tends to be far more immunogenic than adeno-associated viral vectors (AAV) and retroviral vectors. Due to these limitations, only AAV and lentiviral vectors are described below.

AAV. There are many different AAV, *i.e.*, >100 different sequences are available. Some of the differences lead to different serotypes. The serotypes 1, 2, 6, 8, and 9 are more frequently used for muscle gene therapy. The AAV vector is the only efficient vector for local or systemic delivery to the skeletal muscle and heart[116,117] but its packaging capacity limits the size of the dystrophin transgene.

AAV1[118] and AAV2[111] carrying transgenes encoding for μDYS were injected in *mdx* mouse muscles with success. Indeed, up to 80% dystrophin-positive fibers were found in the treated muscles. These AAV injections also restored the DGC. The results on the *mdx* mouse model being conclusive, experiments using AAV vectors were done in larger animal models. AAV6 and AAV8 coding for μDYS were injected in the dog model. Although dystrophin expression was observed, cytotoxic immune response against the viral capsid was detected,[119,120] which has also been observed for other transgenes delivered by AAV vectors in the dog model.[121] The AAV vector was also tested in nonhuman primates. Five months after the intramuscular injection of an AAV8 coding for μDYS, the transgene expression reached 80% in the treated muscle but this percentage decreased to 40% when the animal already had pre-existing antibodies against the AAV.[122] In small rodent studies, AAV vectors rarely cause cellular immune responses against either the capsid proteins or the transgene products. But in large animal and human studies, variable immunological outcomes have been observed.

Recently, a clinical trial was undertaken on six DMD patients with an AAV vector coding for a functional μDYS. Of the six treated patients, two showed pre-existing T-cells recognizing the rare dystrophin-positive revertant fibers that presented peptide epitopes deemed by the host as nonself. This was detected in ELISpots of peripheral blood mononuclear cells before and after intramuscular injection of the AAV.[123] Another patient had T-cells recognizing an epitope that encoded the transgene product but absent in the revertant fibers. Although the clinical trial was safe and muscle biopsies from the gene vector-treated arms and the contralateral control arms showed no difference in lymphocytes infiltration,

these intriguing findings strongly suggest that additional work is required to determine how many patients have T-cells to dystrophin epitopes and whether those T-cells will prevent successful gene therapy in DMD. In addition, choices of AAV vector serotypes and promoters may also make an impact on the clinical outcome.

Exon skipping was also investigated in combination with AAV vectors. AAV1 coding for the U7 snRNA or U1 small nuclear RNA (snRNA) genes modified to target the mouse dystrophin exon 23 were injected in *mdx* mice. The expression of the internally deleted dystrophin was observed up to 3 months following the injection of an AAV1 coding for the U7 snRNA[124] and for at least 1 year and half with an AAV1 coding for the U1 snRNA.[125] These results are encouraging but this approach has to be further investigated in larger animals such as nonhuman primates or dogs.

AAV vectors were also used to interfere with the myostatin pathway. An AAV vector coding for the myostatin propeptide, a myostatin inhibitor, was designed and injected in *mdx* mice. Muscle hypertrophy leading to phenotypic improvements was observed in the treated mice.[126] Dogs were also treated with the same vector. Unfortunately, few parameters were studied in this experiment and only the hypertrophy of some muscles was noted.[127] In contrast to the other dog studies using AAVs coding for μDYS, no immune responses against the AAV capsid were observed in this study. In the mouse, a recent experiment used an AAV coding for the activin type-II receptor to block the myostatin pathway. The effects of this AAV injection were similar to those observed in the mouse following the injection of the purified activin type-II receptor alone.[128]

The results obtained with AAV vectors are interesting for the development of a DMD therapy. Nevertheless long-term studies of the transgene expression and the immune response against the capsid will be required before this can be considered as potential treatment for DMD.

Lentivirus. The lentivirus encapsidation size is limited to carry the mDYS. Thus, a lentiviral vector carrying this internally deleted *DMD* gene was intramuscularly injected in adult and newborn *mdx* mouse muscles. The best results were obtained in younger mice where 65% of muscle fibers expressed the transgene.[129] In addition, better strength and protection against contraction induced injury were observed in the treated muscles. The lentivirus injection also transduced satellite cells.[130] Despite favorable results in small animals, no studies are available for larger animal models. Moreover, the random integration of lentiviral vectors, according to the target tissues and the enhancers used in a construct, predisposes to induction of tumors (insertional mutagenesis) even though they have not been observed to date in the described experiments.

Lentivirus can also be used to genetically modify cells, which can be transplanted or injected in animal models or eventually in patients. This technique is called *ex vivo* gene therapy. A lentiviral vector coding for μDYS was used to integrate this gene in the genome of side population cells, which were then intravenously injected in *mdx* mice. Only 1% of muscle fibers expressed the transgene in the treated muscles,[131] though this percentage was increased to 5% when these cells were intra-arterially injected.[132] Dystrophic dog mesoangioblasts were also transduced with a lentiviral vector coding for the human μDYS and intra-arterially injected in the same dogs.[133] The treated dogs showed good expression of human μDYS but two of the three treated dogs died of pneumonia during the experiment. The cause of this death was not explained by the investigators but the accumulation of the injected cells in the lungs could be involved in this mortality. Other cell types such as human and nonhuman primate myoblasts were transduced with human μDYS and transplanted with success in immunodeficient mouse and in nonhuman primate muscles respectively.[134] A lentiviral vector coding for dog μDYS was also used to transduce human and dystrophic dog myoblasts. Subsequently, these cells were transplanted in mouse muscles and transgene-positive fibers were observed in the treated muscles.[135]

In addition to the possibility of delivering an internally deleted dystrophin, the lentiviral vector may be used to induce exon skipping as well. A lentiviral vector coding for the U7 snRNA gene modified to induce the skipping of human dystrophin exon 51 was designed. Myoblasts of DMD patients having a deletion of exons 49 and 50 were transduced with this lentivirus and transplanted in immunodeficient mouse muscles. One month later, the expression of internally deleted dystrophin (without the exons 49–51) was detected in the treated muscles.[134] This approach was also used successfully with $AC133^+$ cells.[136]

The use of lentiviral vector is promising for DMD but its efficacy and the risk of tumorigenicity from cells transduced by direct injection of a lentiviral vector or by *ex vivo* genetic modification need to be evaluated in clinical trials.

Nonviral gene therapy

Nonviral gene therapy allows the introduction of a transgene into a tissue without using a viral vector. Thus, the main advantage of this method is to avoid any immune response due to viral capsids or other viral proteins. There are also no limitations concerning the transgene size but the transfection efficiency of nonviral gene therapy is progressively reduced with the increasing plasmid size.

Naked DNA. The simplest method to deliver a plasmid into muscle is its direct injection. Plasmids coding for μDYS and for FLDYS were injected in *mdx* mice[110]; however, the transfection efficiency was very low. Nevertheless, there is a possibility for prolonged transgene expression in muscles since muscle fibers are postmitotic. A phase I clinical trial was undertaken in 2004 on nine dystrophic patients[137] that were intramuscularly injected with a plasmid coding for human FLDYS. The three treated DMD patients just showed rare dystrophin-positive fibers. In the six treated BMD patients, the average level of dystrophin expression was slightly higher (about 3%). Although the application of naked DNA is appealing since this method is fast and the plasmids are easy to produce, the efficiency of direct intramuscular injection is currently too low to be clinically relevant. To improve gene delivery, chemical and physical methods can be used. However, due to the low effectiveness of chemical methods *in vivo*, only physical approaches are included in the present review.

Physical approach

Hydrodynamic pressure: Good expression levels were obtained following a rapid injection of a large quantity of plasmid DNA coding for luciferase or β-galactosidase.[138] This intravenous injection of a

large volume while using a tourniquet to occlude blood flow allows good dissemination of the naked DNA in muscles.[139] Indeed, the intravascular pressure induced the formation of transient pores in the endothelium of blood vessels allowing macromolecules, such as plasmids, to leak into the surrounding muscle and thereby access the muscle fibers.[140] The safety of this method was demonstrated in mice and in nonhuman primates.[139,141] The hydrodynamic limb vein injection used in *mdx* mice with a plasmid coding for FLDYS resulted in dystrophin expression in up to 20% of muscle fibers for >1 year.[142] The phenotype of the treated mice was also improved. Golden retriever muscular dystrophy dogs were also treated with this technique. The procedure appeared safe in the treated animals and enabled to obtain dystrophin expression but further work is required to determine the exact level of dystrophin expression.[143] This approach seems thus promising to introduce naked DNA in muscles.

Electroporation: A second method to improve the efficiency of muscle transfection is electroporation. The electric field used in this method enhanced the uptake of a plasmid previously injected in the muscle.[144,145] Indeed, the electric pulses permeabilized the cellular membrane, creating transient pores that facilitated the plasmid entry into the cell. However, these pores also increased calcium entry and activated proteases.[146] Therefore, it is important to select voltage settings, which allow maximal efficiency with the least amount of damage. As with the hydrodynamic pressure method, the electroporation of naked DNA in muscles resulted in transgene expression for >1 year.[147] The heart can also be treated by electroporation according to a recent research article.[148] A study showed that satellite cells can be transfected with this technique.[149] However, this study has not been confirmed. According to Schwann's equation, the threshold intensity of the applied electric field necessary to obtain membrane permeabilization is inversely proportional to the cell radius.[150] Since the radius of satellite cells is smaller than that of muscle fibers, the satellite cells and the muscle fibers cannot be electroporated simultaneously.

Since its first use in a clinical trial in 1991,[151] plasmid electroporation has proven to be safe and effective for transgene delivery to several tissues.[152–154] In the DMD context, a plasmid coding for mouse FLDYS was electroporated in *mdx* mouse muscles. The electroporated muscle fibers expressed the transgene for at least 1 month and exhibited a reduced number of centro-nucleated muscle fibers as well.[155,156] Dog FLDYS was also introduced with success in dystrophic dog muscle.[157] In this case, a specific immune response was observed in the treated dog muscle. Further studies are thus required to determine whether this immune response was against dystrophin or against the product of another transgene also present in the plasmid.

DISCUSSION

DMD is a devastating pathology leading to severe muscle weakness. This disease is due to the lack of dystrophin in smooth, cardiac, and skeletal muscles. Although there are currently no curative treatments for DMD, several therapeutic approaches are undergoing clinical evaluation such as pharmaceutical approaches and gene therapy.

Pharmaceutical approaches

The stop codon read-through is one of pharmaceutical approaches. The last clinical trial with ataluren showed that it was unable to achieve its primary outcome for improved muscle function. The long-term gentamicin clinical trials gave mixed results and showed too many toxicity issues to consider this antibiotic as a feasible approach to treat DMD patients having nonsense mutation. Moreover, stop codon read-through would only be relevant to only about 10 to 15% of DMD patients.

Exon-skipping can in theory be applied to 80% of DMD patients.[25] This method has shown its efficiency in mouse and dog models. Clinical trials using 2OMPs and morpholinos were also undertaken on DMD patients. In both cases, dystrophin expression was observed in the treated muscles and no significant adverse effects have been encountered. Only the results of intramuscular exon skipping trials have been published so far with results restricted to the site of delivery. However, the first results on the clinical trials using a morpholino (AVI-4658) or a 2OMP (PRO051/GSK2402968) systemically delivered showed good dystrophin expression.[66,85] Even though there are no long-term toxicity studies (>6 months) available on 2OMPs and morpholinos in nonhuman primate, these two compounds are promising for DMD.

Currently, no molecules upregulate utrophin expression sufficiently to restore the phenotype of dystrophic mouse models. Therefore, utrophin upregulation must be further improved before applying it in DMD.

Gene therapy

Another method to obtain a functional dystrophin is to introduce a cDNA in muscle fibers using gene therapy. The most promising viral vector to introduce a micro-dystrophin cDNA in muscle fibers is currently the AAV vector. The results obtained with this vector in mice, dogs, and nonhuman primates are good despite the fact that antibodies against the AAV capsid were sometimes found in the treated animals (humans also have pre-existing antibodies against AAV and adenovirus). However, a recent clinical trial using an AAV coding for micro-dystrophin did not demonstrate significant transgene expression in the treated DMD patient muscles. Moreover, this study detected lymphocytes reacting with dystrophin in response to transgene expression.[123]

One way to eventually avoid the potential toxicity following the dissemination of viral vectors throughout the body[158] is to transplant autologous cells, which have been genetically modified *ex vivo*. This *ex vivo* gene therapy has shown positive results in mice and nonhuman primates but is nevertheless limited by the same problems as myoblast transplantation, *i.e.*, the difficulty of reaching small muscles and the high number of injection trajectories necessary to obtain a high percentage of dystrophin-positive fibers. Exon skipping can also be induced by viral vectors carrying the U7 snRNA gene modified to target a specific exon. Since no results are yet available in large animals with this gene, the AO technology currently remains the most efficient and most frequently used method to induce exon skipping in DMD.

An alternative to *ex vivo* gene therapy is the use of naked plasmid delivered by hydrodynamic pressure or by electroporation. These two techniques have shown good efficiency to deliver dystrophin cDNA or internally deleted versions of it in mouse

model, although these physical methods are less efficient than systemic injection of viral vectors. Moreover, only a few preliminary results are available in larger animal models, such as dogs and nonhuman primates. The main limiting factor for electroporation is that at this time only a small number of muscle fibers can be treated with this technique since it requires penetration with electrodes into each muscle. The hydrodynamic method can be applied only to arm and leg muscles but not to muscles of the head and trunk.

Response to dystrophin in clinical trials

During clinical trials on DMD patients, anti-dystrophin antibodies were observed following nondystrophic myoblast transplantation[159] and dystrophin-specific T-cells were detected following the injection of AAV coding for micro-dystrophin. The presence of dystrophin-specific T-cells was also detected in one patient after treatment with gentamicin.[123] No anti-dystrophin antibodies were found in the DMD patients treated with AVI-4658 or with PRO051 but the presence of dystrophin-specific T-cells was not investigated. Apparently, there were no T-cell responses, or if there were, it was not effective enough to hamper dystrophin expression. This seems to indicate that if a therapeutic approach is effective to restore dystrophin in muscle fibers, some DMD patients may have to be under a sustained immunosuppression treatment.

Conclusion

Even though the *DMD* gene was discovered 23 years ago, there are still no curative treatments for DMD although the use of steroids and assisted ventilation have greatly improved the quality of life and extended life span by nearly 50%.[33]

When a therapeutic approach is found to restore dystrophin in the DMD patient's muscles, the problems of fat infiltration or fibrosis in the muscles will still need to be resolved, as well as the existing muscle weakness or bone deformation. An approach to improve muscle strength is to block the myostatin pathway. Indeed, myostatin inhibition leads to muscle hypertrophy and muscle strength increases in animals. The process of fat infiltration and fibrosis in DMD patient's muscles is not well understood and needs to be further investigated. The best approach will thus be to treat DMD patients when they are still young to avoid most of the consequences due to the absence of dystrophin. Moreover, all muscles (or a large proportion of them) will need to be treated to obtain a curative treatment.

SUPPLEMENTARY MATERIAL

Figure S1. AOs used in DMD.

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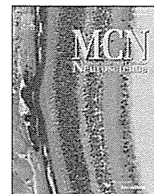

Reactive gliosis of astrocytes and Müller glial cells in retina of POMGnT1-deficient mice

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ABSTRACT

Protein O-linked mannose beta1, 2-N-acetylglucosaminyltransferase 1 (POMGnT1) is an enzyme that catalyzes the transfer of N-acetylglucosamine to O-mannose of glycoproteins. Alpha-dystroglycan, a substrate of POMGnT1, is concentrated around the blood vessels, in the outer plexiform layer (OPL), and in the inner limiting membrane (ILM) of the retina. Mutations of the *POMGnT1* gene in humans cause muscle-eye-brain (MEB) disease. Several ocular abnormalities including retinal dysplasia, ERG abnormalities, and retinal detachments have been reported in patients with MEB. We have analyzed the eyes of POMGnT1-deficient mice, generated by standard gene targeting technique, to study the retinal abnormalities. Clinical examination of adult mutant mice revealed a high incidence (81% by 12-months-of-age) of retinal detachments. Sheathing of the retinal vessels and the presence of ectopic fibrous tissues around the optic nerve head were also found. Histological examinations showed focal retinal detachment associated with GFAP immunopositivity. The ILM of the mutant mice was disrupted with ectopic cells near the disruptions. The expression of Dp71, a shorter isoform of dystrophin, was severely reduced in the ILM and around retinal blood vessels of POMGnT1-deficient mice. The expression of Dp427, Dp260, Dp140 were also reduced in the OPL of the mutant mice. Electroretinographic (ERG) analyses showed reduced a- and b-wave amplitudes. Examinations of flat mounts revealed abnormal vascular network associated with highly irregular astrocytic processes. In addition, ER-TR7-positive fibrous tissue was found closely associated with reactive astrocytes especially around the optic nerve head. Our results suggest that altered glycosylation of alpha-DG may be responsible for the reactive gliosis and reticular fibrosis in the retina, and the subsequent developments of retinal dysplasia, abnormal ERGs, and retinal detachment in the mutant mice.

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Introduction

Protein O-linked mannose beta1, 2-N-acetylglucosaminyltransferase 1 (POMGnT1) is an enzyme that catalyzes the transfer of N-acetylglucosamine to O-mannose of glycoproteins (Yoshida et al., 2001). Mutations of the *POMGnT1* gene cause muscle–eye–brain (MEB) disease, one of a family of dystroglycanopathies, in humans (Yoshida et al., 2001). The dystroglycanopathies include a group of muscular dystrophies including Walker-Warburg syndrome (WWS), Fukuyama-type congenital muscular dystrophy (FCMD), congenital muscular dystrophy (MDC) 1C/D, limb-girdle muscular dystrophy (LGMD) 2I/K/M/N, and MEB. This group of disorders is clinically characterized by various combinations of severe muscular dystrophy, mental retardation, and ocular abnormalities. To date, mutations in six known or putative glycosyltransferase genes, viz., *POMT1*, *POMT2*, *Fukutin*, *FKRP*, *LARGE*, and *POMGnT1*, have been identified to be associated with these disorders (Beltrán-Valero de Bernabé et al., 2002; van Reeuwijk et al., 2005; Kobayashi et al., 1998; Brockington et al., 2001; Longman et al., 2003). A common molecular defect for the dystroglycanopathies is the post-translational modification or hypoglycosylation of alpha-dystroglycan (alpha-DG).

Dystroglycan (DG) is encoded by a single gene and is cleaved into two proteins, alpha-DG and beta-DG, by post-translational processing (Ibraghimov-Beskrovnaya et al., 1992). Alpha-DG is a heavily glycosylated glycoprotein and is a central component of the dystrophin glycoprotein complex (DGC). A major function of the DGC is to link cytoskeletal actin to the basal lamina which maintains the structural integrity of skeletal muscles (Ervasti and Campbell, 1993). Mutations in the components of DGC cause various forms of muscular dystrophies (Straub and Campbell, 1997).

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DG and other components of DGC are widely expressed in the CNS and the retina (Henry and Campbell, 1999; Blake and Kröger, 2000). Alpha-DG functions as a cell surface receptor for laminin, perlecan, agrin, neurexin, and pikachurin in a variety of tissues (Ervasti and Campbell, 1993; Gee et al., 1994; Peng et al., 1998; Sugita et al., 2001; Sato et al., 2008).

In the retina, alpha-DG is concentrated in the inner limiting membrane (ILM), around blood vessels, and in the outer plexiform layer (OPL; Blake and Kröger, 2000). The alpha-DG in the ILM is concentrated at the endfeet of the Müller cells, and that in the blood vessels in the perivasular astrocytes (Montanaro et al., 1995; Claudepierre et al., 1999). The alpha-DG in the OPL is localized around the site of expression of ribbon synapses of rod and cone photoreceptor terminals (Ueda et al., 1995; Montanaro et al., 1995).

Abnormal electroretinograms (ERGs) have been recorded from patients with MEB disease (Pihko et al., 1995; Fahnehjelm et al., 2001), and have frequently been recorded from individuals with Duchenne and Becker muscular dystrophies (Pillers et al., 1999). The findings in several mouse models with disruption of dystrophin, *Large*^{vls} and fukutin indicated that DGC is associated with the normal physiology of the retina (Pillers et al., 1995; Kameya et al., 1997; Lee et al., 2005; Takeda et al., 2003). Abnormal ERGs in mice with a targeted disruption of pikachurin, an extracellular ligand of alpha-dystroglycan at ribbon synapses, also support the idea that DGC must be present in the OPL for normal retinal physiology (Sato et al., 2008).

It was recently shown that inactivation of glial specific dystroglycan, located in the endfeet of Müller cells and perivascular astrocytes, led to a reduction of the b-wave of the ERG. This suggested that glial specific dystroglycan also plays an important role in the normal physiology of the retina (Satz et al., 2009).

Patients with MEB have a variety of ocular abnormalities including myopia, glaucoma, anterior chamber malformation, microphthalmia, buphthalmus, nystagmus, strabismus, cataract, chorioretinal atrophy, retinal dysplasia, and retinal detachment (Cormand et al., 2001; Mercuri et al., 2009). The purpose of this study was to determine the effect of altered glycosylation of alpha-DG caused by inactivation of POMGnT1 in the retina. To accomplish this, we studied mice with targeted disruption of the *POMGnT1* gene. We shall show that POMGnT1-deficient mice have a high incidence of retinal detachment with reactive gliosis of the Müller glial cells and the perivascular astrocytes. Our results suggest that altered glycosylation of alpha-DG may be responsible for the retinal dysplasia, abnormal ERGs, and retinal detachment in humans with MEB.

Results

Retinal detachment in POMGnT1-deficient mice

At 6-weeks-of-age, the retinal vessels of all POMGnT1-deficient mice were tortuous but none of the mice had a retinal detachment (Fig. 1B). These mutant mice also had fibrous tissue over the retina especially around the optic nerve head (Fig. 1B). Fibrous tissues were not observed in any of the wild type mice.

By 6-months-of-age, the retinal vessels were sheathed, and focal and extensive retinal detachments were present in the areas of the sheathed vessels (Fig. 1C). The retinal detachment of some of the mice covered

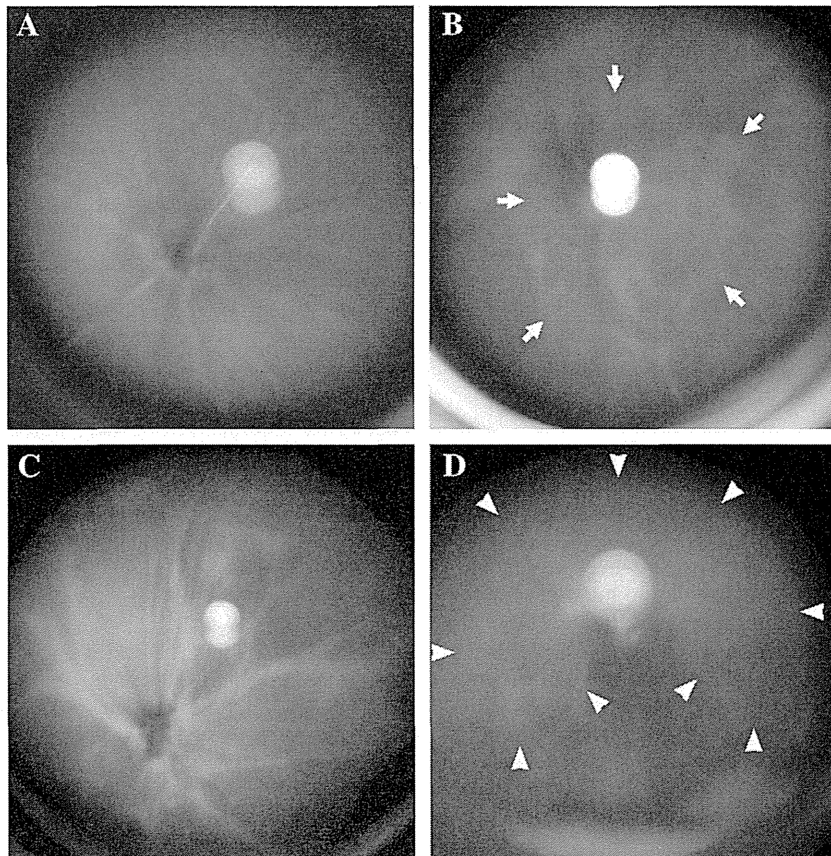

Fig. 1. Fundus photograph of wild-type and POMGnT1-deficient mice. A. Six-week-old wild type mouse. B. Retinal vessel tortuosity can be seen in this 6-week-old POMGnT1-deficient mouse. Arrows point to ectopic fibrous tissue surrounding the optic nerve head. C. Six-month-old POMGnT1-deficient mouse showing sheathing of the retinal vessels. D. Retinal detachment (arrowheads) in a 6-month-old POMGnT1-deficient mouse.

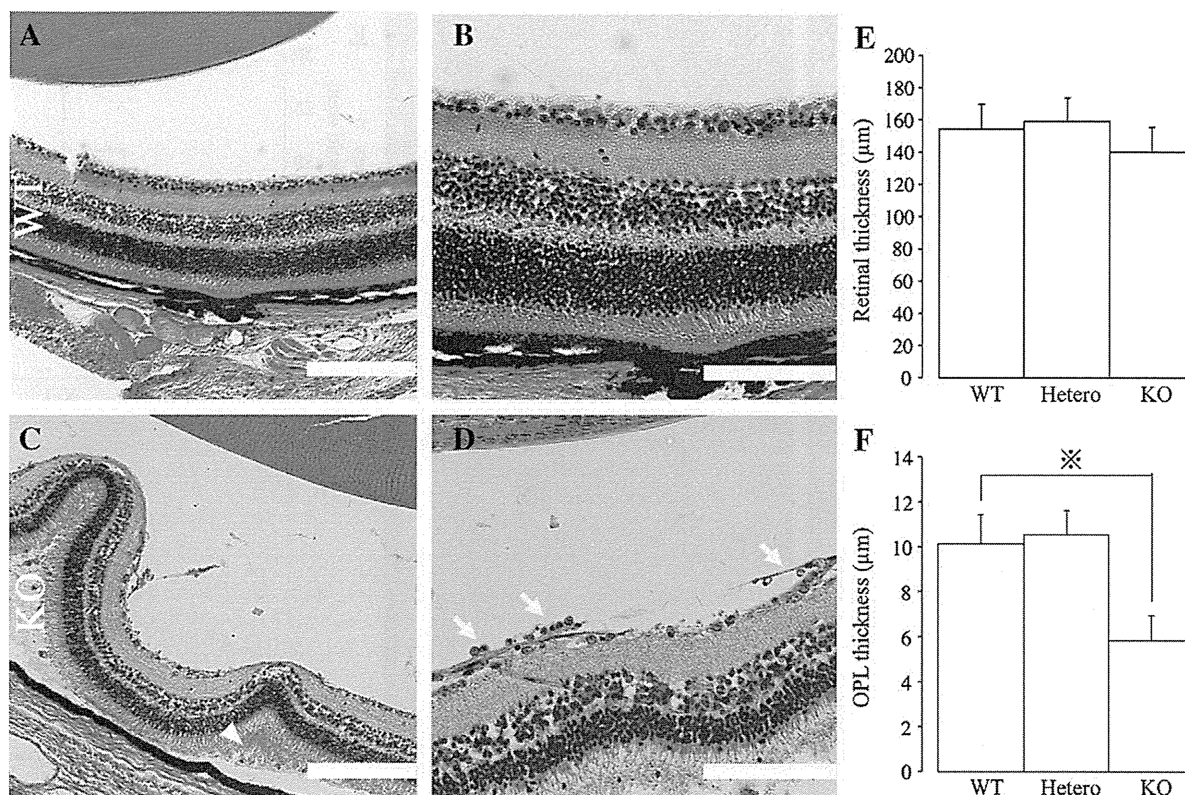

Fig. 2. Histological observations of the retina of wild-type and POMGnT1-deficient mice. A. and B. Sections of 15-week-old wild-type retinas stained with hematoxylin and eosin. C. and D. Sections of 15-week-old POMGnT1-deficient retinas stained with hematoxylin and eosin. In (C), focal retinal detachments can be seen in several regions. There are some connective tissue-like materials beneath the detached retina (arrowhead), indicating the retinal detachments are not embedding or sectioning artifacts. In (D), ectopic cells and vitreal fibroplasia can be seen on the ILM of POMGnT1-deficient retina (arrows). Scale bars represent 200 μm in A, C and 100 μm in B, D E and F. Quantification of the retinal thickness-measurements of wild-type, Heterozygous and Homozygous POMGnT1-deficient mice. Animals used for this study ranged from 18-week-old to 28-week-old. The average of each 5 animals is plotted. There was no significant difference in the whole retinal thickness (E), but significant thinning of the OPL was observed in POMGnT1-deficient mice compared to wild-type mice (10.12 ± 1.34 μm [SD] vs. 5.84 ± 1.13 μm; P = 0.045) (F).

more than a quadrant of the retina (Fig. 1D). By 12-months-of-age, 13 of 16 mutant eyes (81%) showed obvious retinal detachments.

Histological examination of 15-week-old mutant mice showed focal retinal detachments (Fig. 2C). Ectopic cells and vitreal fibroplasia were found on the inner limiting membrane (ILM; Fig. 2D) as was found in Large^{vls} mice (Lee et al., 2005). There were no significant difference in the whole retinal thickness, but significant thinning of the OPL was observed in POMGnT1-deficient mice compared to wild-type mice (Figs. 2E and F). This fibroplasia was immunopositive for glial fibrillary acidic protein (GFAP; Figs. 3C and D). Because GFAP expression is a reliable early marker of reactive gliosis of the astrocytes and Müller glial cells (Lewis and Fisher, 2003), an up-regulation of GFAP staining of the ILM and the radial immunoreactivity in the mutant mice indicated the development of reactive gliosis of the Müller cells. Quantitative analysis revealed that GFAP immunoreactivity was significantly elevated in the retina of POMGnT1-deficient mice (Fig. 3E). To confirm if elevated immunoreactivity of GFAP is truly associated with gliosis, we have also characterized the retina of POMGnT1-deficient mice using anti-vimentin antibody that is other cellular marker associated with gliosis. The vimentin immunoreactivity of POMGnT1-deficient mice along with ILM and radial morphology of Müller cells are also upregulated compared to that of wild-type mice (Figs. 4A and B). We have applied another cellular markers to characterize the different cell types in the anatomical regions. Immunoreactivity for both anti-syntaxin antibody and anti-PKC antibody, cellular markers for amacrine cells and bipolar cells, revealed no obvious differences between POMGnT1-deficient and wild-type mice (Figs. 4C–F). These data indicate that the up-regulation of GFAP and vimentin immunoreactivity is specifically associated with gliosis of Müller cells of the POMGnT1-deficient mice in the region.

Electroretinographic findings of POMGnT1-deficient mice

To examine the function of the retina of POMGnT1-deficient mice, ERGs were recorded from 4-month-old wild type and POMGnT1-deficient mice. The mixed rod–cone ERGs recorded from POMGnT1-deficient mice at higher stimulus intensities had a negative waveform with the amplitude of the b-wave smaller than that of the a-wave (Fig. 5A). Amplitudes of the a-wave and b-wave of mixed rod–cone ERGs obtained from POMGnT1-deficient mice are reduced significantly compared to wild-type mice (Figs. 5B and C). The amplitudes of the scotopic b-wave elicited by lower stimulus intensities were also reduced in POMGnT1-deficient mice (Fig. 5D). The amplitude of the b-wave of photopic ERGs of POMGnT1-deficient mice was also reduced and significantly smaller than that of wild-type mice (Fig. 5E).

Expression of alpha-DG and dystrophin in retina of POMGnT1-deficient mice

To confirm that the retina of POMGnT1-deficient mice completely lacked POMGnT1 enzyme activity, we used a monoclonal antibody, VIA4-1, that reacts against the sugar moiety of alpha-DG. Our findings showed that VIA4-1 immunoreactivity was present on the ILM, around blood vessels, and in the OPL of the retina of the wild type mice but was completely absent in the POMGnT1-deficient mice (Figs. 6A and B). It has been clearly shown that at the OPL there are three DMD gene products, Dp427 (or full length dystrophin), Dp260 and Dp140 and at

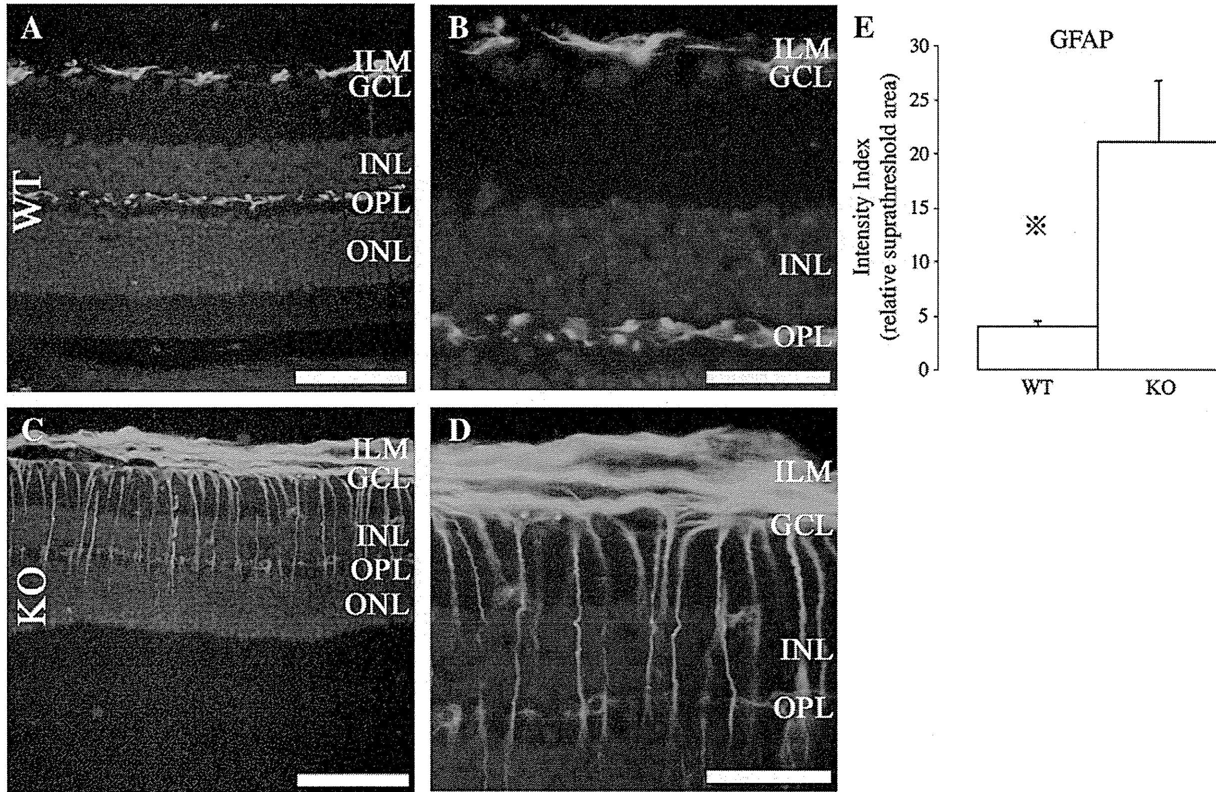

Fig. 3. GFAP staining of paraffin sections from wild-type and POMGnT1-deficient mice. A. and B. Sections of 18-week-old wild-type retinas with GFAP staining. C. and D. Sections of 18-week-old POMGnT1-deficient retinas. There was an up-regulation of GFAP staining in the ILM. Scale bars represent 100 μm in A, C and 50 μm in B, D and E. Quantification of GFAP immunoreactivity in paraffin sections from wild-type and POMGnT1-deficient mice. Animals used for this study ranged from 18-week-old to 28-week-old. The average of each 3 animals is plotted. Intensity Index for GFAP immunoreactivity was significantly elevated in the retina of POMGnT1-deficient mice compared to wild-type mice (3.90 ± 0.54 [SD] vs. 20.61 ± 5.65; P = 0.003).

the Müller cells only Dp71 is found (Dalloz et al., 2003 and Fort et al., 2008). The expression of Dp71 in the ILM and around retinal blood vessels of POMGnT1-deficient mice was severely reduced (Fig. 6D). The expression of Dp427, Dp260, Dp140 were also reduced in the OPL of the mutant mice (Fig. 6D).

Glial proliferation closely associated with ER-TR7-positive fibrous tissue around abnormal retinal vessels in POMGnT1-deficient mice

Two mouse models with aberrant glycosylation of alpha-DG have been reported to have reactive gliosis with an up-regulation of GFAP expression in the retina and the brain. One of these is the *Large*^{vls} mice, which has a disorganization of astrocytic processes in the retina (Lee et al., 2005). The second mutant is the POMGnT1-disrupted mouse, which has reactive gliosis in the cerebral cortex (Yang et al., 2007). To further characterize the glial proliferation and associated vascular abnormalities in the retina of our POMGnT1-deficient mice, we examined flat mounts of the retina of 4-month-old wild type and POMGnT1-deficient mice.

In flat mounts of POMGnT1-deficient mice retina, the GFAP staining of astrocytes was highly irregular especially around the retinal vasculature (Figs. 7E and K). Retinal vascular staining derived from perfusion of FITC-dextran showed disorganization of the normal pattern of the vascular networks (Figs. 7D and J). Double staining for GFAP and FITC-dextran showed that abnormal retinal vasculature was highly co-localized with the irregular astrocytic processes (Figs. 7F and L). Quantitative analysis for GFAP immunoreactivity and vascularization stained by FITC dextran perfusion showed significant elevation of intensity index in both central and peripheral retina of POMGnT1-deficient mice compared to wild-type mice (Fig. 8).

In POMGnT1-deficient mice, it was also reported that most of the GFAP-positive reactive astrocytes in the brain were in close contact with ectopic fibroblasts, suggesting that they were induced by the fibroblasts (Yang et al., 2007). To confirm that our POMGnT1-deficient mice also showed fibrosis associated with the GFAP-positive reactive astrocytes in the retina, we examined flat mounts of 4-month-old wild type and POMGnT1-deficient mice using the ER-TR7 antibody. Although the antigen of the ER-TR7 antibody has not been fully characterized, it is known to detect an antigen present in and produced by reticular fibroblasts. Reticular fibers are synthesized by a family of collagen proteins, and the fibers are made by reticular fibroblasts.

In the flat mount preparations, ER-TR7-positive fibrous tissue was found closely associated with GFAP-positive reactive astrocytes especially around the optic nerve head (Figs. 9D–F). An up-regulation of ER-TR7-positive fibrous tissue was also found in the peripheral retina associated with the irregular retinal vasculature (Figs. 9J–L).

Discussion

Patients with MEB show a variety of ocular abnormalities, and at least six cases with retinal detachment have been reported (Cormand et al., 2001; Matsumoto et al., 2005; Godfrey et al., 2007; Demir et al., 2009). Although an involvement of the retina has been frequently described in MEB patients, many patients without any retinal abnormalities have also been reported (Mercuri et al., 2009). A broader phenotypic spectrum was reported for MEB disease worldwide, and no consistent genotype–phenotype correlation has been established (Hehr et al., 2007). Thus, Finnish patients homozygous for the founder mutation showed a wide variation in their phenotype (Diesen et al., 2004).

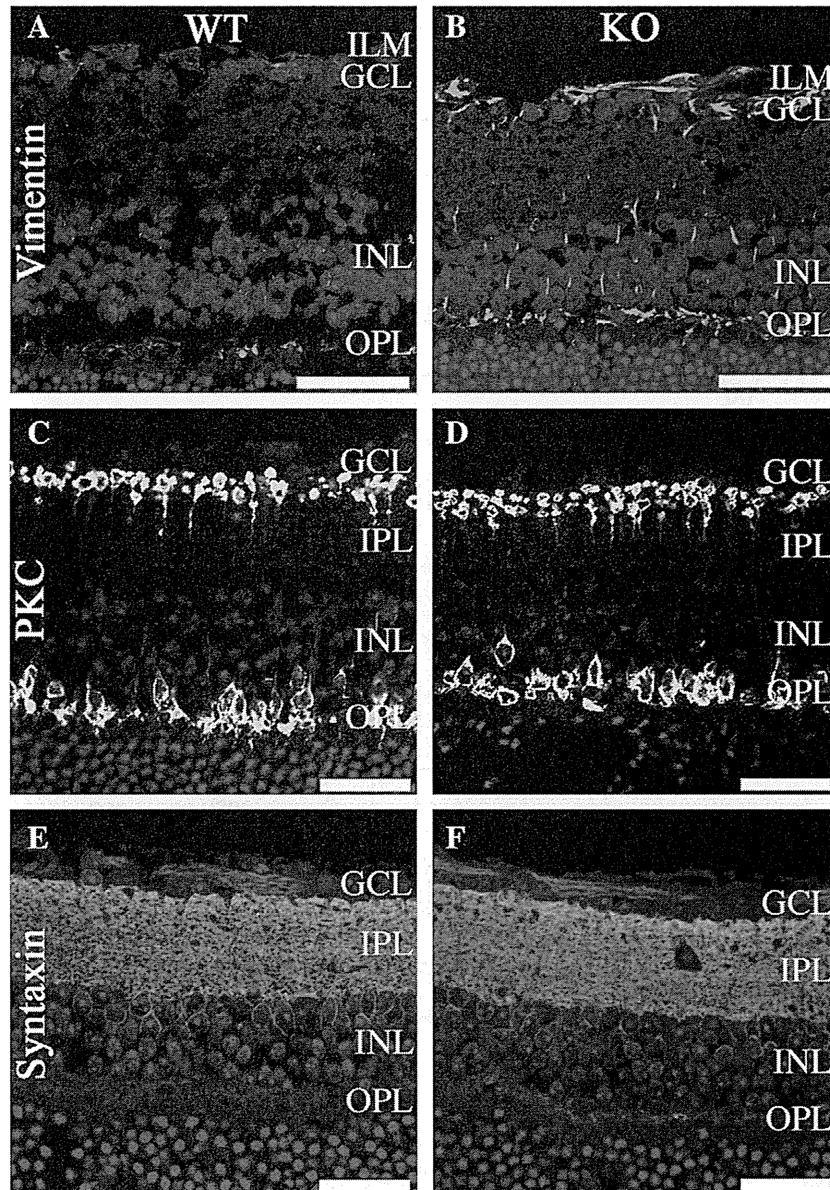

Fig. 4. Immunohistochemical observations of several markers for different cell types of the regions from 18-week-old wild-type and 20-week-old POMGnT1-deficient mice. A. and B. Sections of retina labeled with anti-vimentin antibody. The vimentin immunoreactivity of POMGnT1-deficient mice along with ILM and radial morphology of Müller cells were upregulated compared to that of wild-type mice. C. and D. Sections of retina labeled with anti-PKC antibody for bipolar cells. There were no significant differences between the retina of wild-type and POMGnT1-deficient mice. E. and F. Sections of retina labeled with anti-Syntaxin antibody for amacrine cells. There were no significant differences between the retina of wild-type and POMGnT1-deficient mice. Scale bars represent 50 μm in A–F.

Two mouse models with mutations of the *POMGnT1* gene have been reported including our model (Liu et al., 2006 and Hu et al., 2010; Miyagoe-Suzuki et al., 2009). These two mouse models have different phenotypes and different location of the POMGnT1 gene mutation. Our model was produced by standard gene targeting techniques with disruption of exon 18, while the other model was generated by gene trapping with a retroviral vector inserted into exon 2 of the *POMGnT1* gene. Our model has a milder muscle phenotype and a lower survival rate than the other model, but had frequent retinal detachment which was not described in the other model. Homozygous mutants of our mouse model are sterile, and homozygous mutants are obtained by heterozygote matings. However, the homozygous offsprings have a very low survival rate (Miyagoe-Suzuki et al., 2009).

A spectrum of retinal abnormalities was observed in our POMGnT1-deficient mice, although all of these mice had the same *POMGnT1* gene mutation. These findings are consistent with earlier hypotheses that factors other than the activity of *POMGnT1* gene, e.g., environmental factors, play a role in determining the severity of the mutation (Diesen et al., 2004; Matsumoto et al., 2005).

Abnormal ERGs are found in patients and mice with a mutation in the DGC component (Pillers et al., 1993; Pihko et al., 1995; Kameya et al., 1997). These findings suggest an involvement of the DGC localized in the OPL for signal transduction at the ribbon synapse of photoreceptor terminals. Mice with targeted disruption with pikachurin gene support this hypothesis. The reduced ERG b-waves in pikachurin-deficient mice suggest an involvement of DGC and pikachurin in retinal signal transduction at the ribbon synapses of photoreceptors (Sato et al., 2008). Recently, mice with a conditional deletion of dystroglycan in the CNS were generated by Satz et al. These mice showed that an inactivation of the glial specific dystroglycan located in the glial endfeet of Müller cells and perivascular astrocytes was sufficient to reduce the amplitude of the ERG b-wave (Satz et al., 2009).

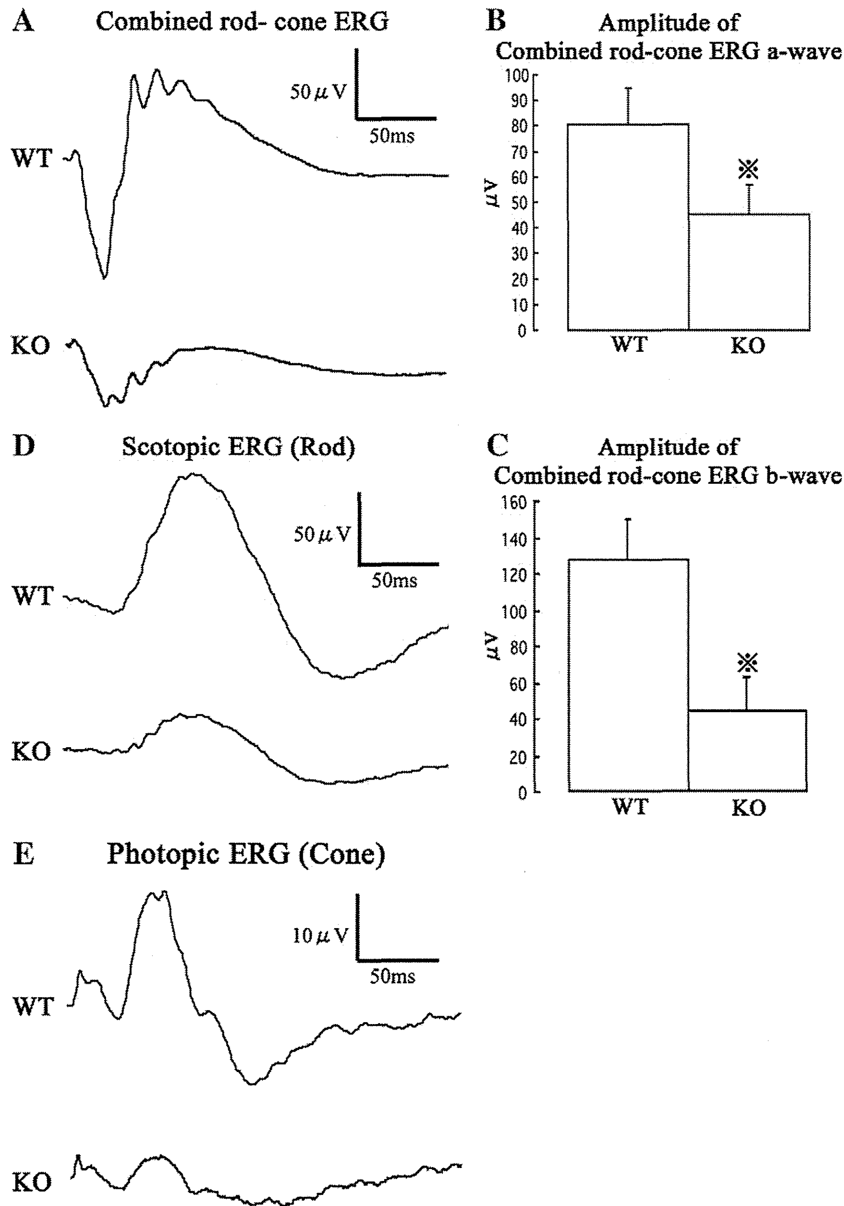

Fig. 5. Electroretinograms of wild-type and POMGnT1-deficient mice. Animals used for this study ranged from 11-week-old to 19-week-old. A. Combined rod–cone ERGs obtained from POMGnT1-deficient mouse showing negative-type ERG with decreased amplitude of a-wave and b-wave. B. and C. Amplitudes of the a-wave and b-wave of combined rod–cone ERGs obtained from POMGnT1-deficient mice are reduced significantly compared to wild-type mice. The average of each 3 animals is plotted. D. and E. Amplitudes of the b-wave of scotopic and photopic ERGs obtained from POMGnT1-deficient mice are also decreased compared to wild-type mice.

The abnormal ERGs of POMGnT1-deficient mice could be explained by several mechanisms. They could arise from an inactivation of glial specific alpha-DG, or disturbed Pikachurin-DG interaction at the ribbon synapse, or dysfunction of retina caused by vascular network abnormalities, or the retinal detachments.

POMGnT1-deficient mice showed decreased expression of dystrophin in the ILM similar to that observed in the mice with conditional deletion of dystroglycan in the CNS, suggesting inactivation or dysfunction of alpha-DG caused by hypogycosylation.

Pikachurin is necessary for the apposition of presynaptic and postsynaptic terminals in the photoreceptor ribbon synapses because Pikachurin is an extracellular ligand of alpha-dystroglycan at ribbon synapses (Sato et al., 2008). A recent study using the same POMGnT1-deficient model clearly showed that the degree of pikachurin immnoreactivity in the ribbon synapse of the mutant mice is reduced (Kanagawa et al., 2010). Because a proper localization of pikachurin at the ribbon synapse supported by functionally mature DG plays important roles in the physiology of the retina, reduced expression of pikachurin in the mutant mice caused by disturbed pikacurin-DG interaction could be one of the cause of abnormal ERG of POMGnT1-deficient mice.

The vascular networks in the mutant retina were grossly disorganized associated with GFAP-positive irregular astrocytic processes compared to that of wild-type mice. The dysfunction of the mutant retina caused by the disorganization of retinal vascular network might be one of the causes of abnormal ERGs of mutant mice.

We have obtained the ERG data from the mice without gross retinal detachment, because retinal detachment is generally known to cause abnormal ERGs. However, we cannot rule out the possibility that these mice had shallow and focal retinal detachment associated with sheathed retinal vessels caused by the reactive gliosis.

In the retina, reactive gliosis can result from retinal injury and disease, including retinal trauma, choroidal neovascularization, retinal detachment, and diabetic retinopathy (MacLaren, 1996; Caicedo et al.,

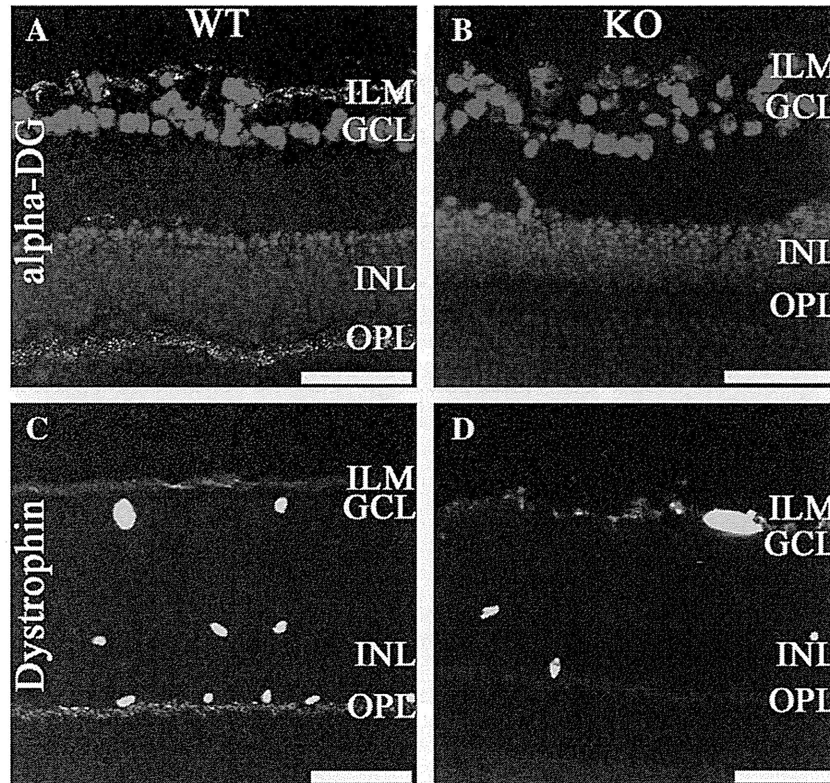

Fig. 6. Immunohistochemical observations of the dystrophin–glycoprotein complex in the retina from 18-week-old wild-type and 20-week-old POMGnT1-deficient mice. A. Sections of wild-type retina labeled with the VIA4-1 antibody for alpha-dystroglycan. VIA4-1 staining was found in ILM, around blood vessels, and in the OPL. B. VIA4-1 immunoreactivity was completely absent in POMGnT1-deficient retina. C. Sections of wild-type retinas labeled with the MAB1694 antibody for Dystrophin. Expression of Dystrophin can be seen in ILM, around blood vessels, and in the OPL. D. In the retina of POMGnT1-deficient mice, expression of Dystrophin was reduced in ILM, around the blood vessels and OPL. Scale bars represent 50 μm in A–D.

2005; Lewis et al., 1995; Mizutani et al., 1998). Secondary complications induced by the reactive gliosis of the Müller cells and astrocytes are the development of fibrosis and proliferative vitreoretinopathy (PVR; Fisher and Lewis, 2003). The POMGnT1-deficient mice showed highly reactive gliosis with strong up-regulation of GFAP expression.

Flat mount preparations of the mutant mice also showed connective tissue-like ER-TR7-positive fibrosis. The high incidence of retinal detachments in POMGnT1-deficient mice may be caused by the PVR preceding the reactive gliosis and reticular fibrosis in the perivascular astrocytes and Müller cells. In POMGnT1-deficient mice, it was shown that repetitive injury caused more fibrosis and fatty infiltration in the tibiaris anterior muscles (Miyagoe-Suzuki et al., 2009). Reactive gliosis with increased numbers of fibroblasts closely associated with capillaries in the cerebral cortex has been reported in POMGnT1-deficient mice (Yang et al., 2007). *Large*^{vls} mice with disruption of glycosyltransferase have abnormal retinal vessels with highly irregular GFAP staining similar to those observed in our mice (Lee et al., 2005). Our mutant mice also had ER-TR7-positive fibrosis associated with reactive astrocytes around both the optic nerve head and peripheral retina. These findings suggest that aberrant glycosylation of alpha-DG can cause fibroblast proliferation in the muscle, eye, and brain of these mice, and also reactive gliosis in the eye and brain.

On the other hand, mice with a complete loss of dystroglycan from the glial endfeet did not have abnormal retinal vasculature or gliosis (Satz et al., 2009). These results suggest that hypoglycosylation or incomplete glycosylation of alpha-DG rather than the absence of alpha-DG may play a role in reactive gliosis of perivascular astrocytes in the retina.

In conclusion, our findings indicate that POMGnT1-deficient mice may be a good model of human MEB. The reactive gliosis and reticular fibrosis in the perivascular astrocytes and Müller glial cells caused by hypoglycosylation or incomplete glycosylation of alpha-DG may be associated with the mechanisms of retinal dysplasia, abnormal ERG and retinal detachment in human MEB. The phenotypic variability of the mutant mice may be useful to determine other factors than POMGnT1-deficiency in determining the severity of human MEB.

Experimental methods

Experimental animals

The generation of POMGnT1-deficient mice on C57BL/6 background was described in detail by Miyagoe-Suzuki et al. (2009). For our study, normal C57BL/6J mice were used as wild type control and were purchased from CLEA Japan, Inc. The procedures used in these experiments were approved by the Animal Care and Use Committee of the Nippon Medical School and conformed to the ARVO statement for the use of animals in ophthalmic and vision research.

Clinical examination of retina

The pupils of the mice were dilated with tropicamide and phenylephrine hydrochloride for indirect ophthalmoscopy with a 90 diopter aspheric lens. Fundus photographs were taken with a Kowa GENESIS-D fundus camera (Kowa Co., Japan) for small animals using a 90 diopter aspheric lens.

Histology and immunohistochemistry

Eyes from POMGnT1-deficient and C57BL/6J mice were enucleated and fixed in SuperFix (Kurabo, Osaka, Japan) overnight. They were then embedded in paraffin, and 7 μm thickness sections were stained

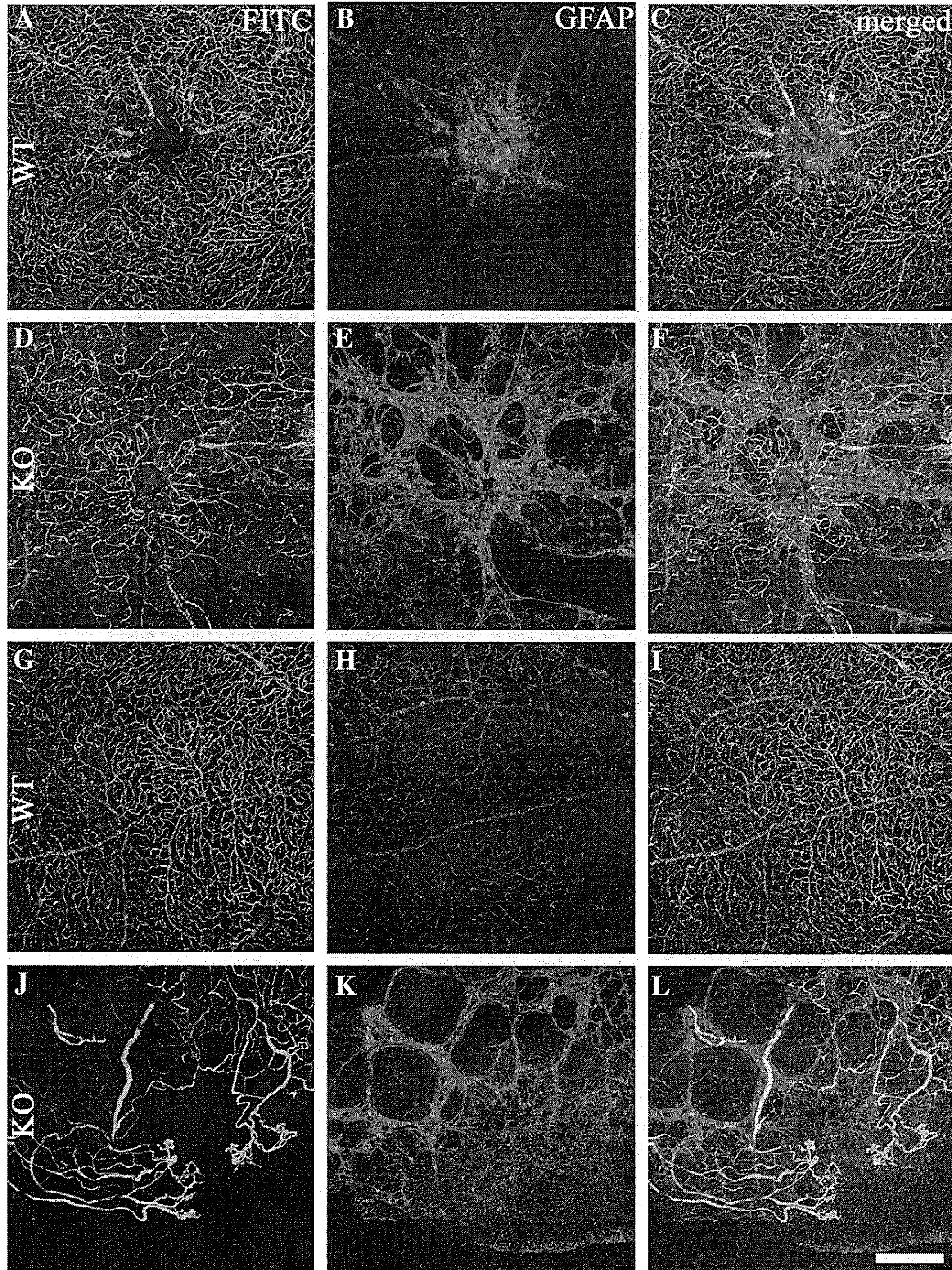

Fig. 7. Vascular network of flat mounted retinas stained by FITC-dextran perfusion and immunohistochemical staining for GFAP from wild-type and POMGnT1-deficient mice. Each animals are 10-week-old. A. D. G. and J. Flat mount retinal preparations from wild-type and POMGnT1-deficient mice perfused with FITC-dextran. B. E. H. and K. GFAP staining of astrocytes in flat mount retinal preparations from wild-type and POMGnT1-deficient mice. C. F. I. and L. Merged images are shown. Regions of central retinas (A–F) and peripheral retinas (G–L) are shown respectively. GFAP staining of astrocytes in POMGnT1-deficient retina is highly irregular especially around retinal vasculature (Eand K). Retinal vascular staining derived from perfusion of FITC-dextran showed disorganization of normal pattern of vascular network (D and J). Double staining of GFAP and FITC-dextran showed that abnormal retinal vasculature was associated with highly irregular astrocytic processes (F and L). Scale bars represent 100 μm in A–L.

with hematoxylin and eosin (H&E). For statistical analysis, measurements of retinal thickness were made at peripheral retina ~1.0–1.2 mm from the optic nerve head (Chi et al., 2010). For immunohistochemical analyses, the enucleated eyes were embedded in optimal cutting temperature compound (OCT, Miles Inc.) and frozen in liquid nitrogen before 6 μm sections were cut. After blocking the sections with 5% normal goat serum, sections were incubated overnight with rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Dako), anti-vimentin antibody (V9; Dako), anti-PKC antibody (MC5; Sigma), anti-Syntaxin antibody (HPC-1; Sigma), anti-alpha-dystroglycan antibody (VIA4-1; Upstate Biotechnology), or anti-dystrophin antibody (Dys2; Novocastra). The secondary antibodies were anti-mouse antibody conjugated with Alexa Fluor 488 (Molecular Probes) for vimentin, Syntaxin, alpha-dystroglycan, dystrophin, and anti-rabbit antibody

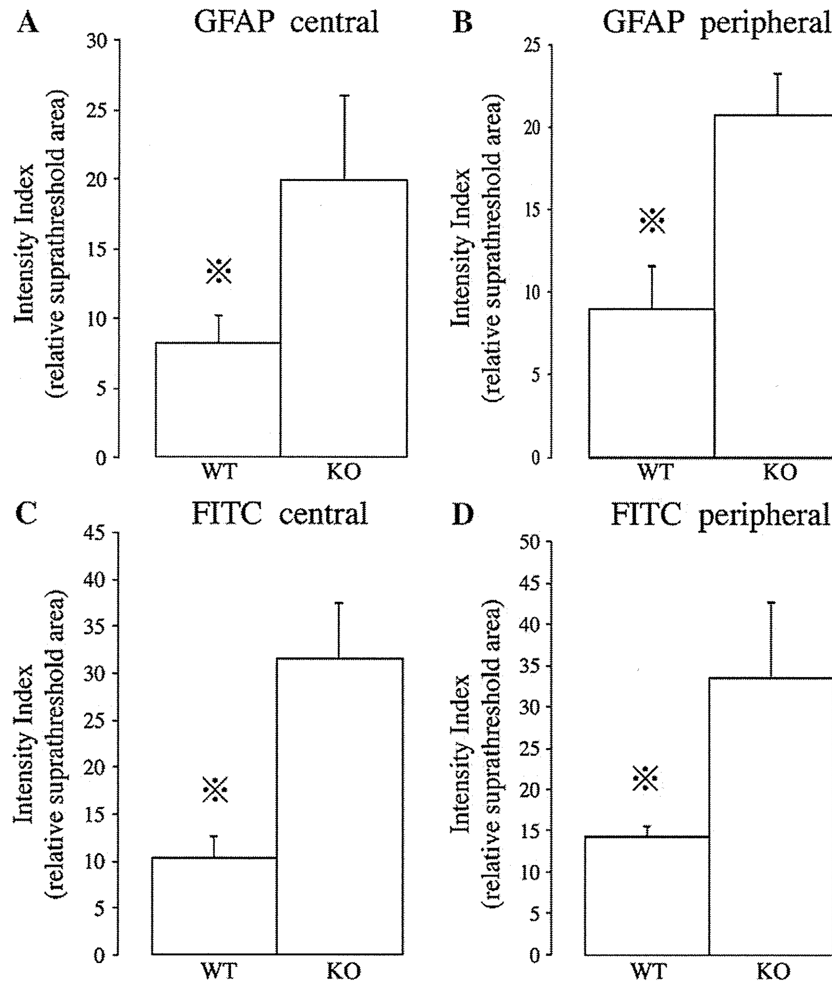

Fig. 8. Quantification of GFAP immunoreactivity and vascularization stained by FITC-dextran perfusion in flat mounted retinas from wild-type and POMGnT1-deficient mice. Animals used for this study ranged from 10-week-old to 12-week-old. The average of each 3 animals is plotted. A. and B. Intensity Index for GFAP immunoreactivity was significantly elevated in the central retina of POMGnT1-deficient mice compared to wild-type mice (8.33 ± 2.00 [SD] vs. 20.23 ± 6.22; $P = 0.003$) (A) and also in the peripheral retina of POMGnT1-deficient mice compared to wild-type mice (9.02 ± 2.54 [SD] vs. 20.70 ± 2.55; $P = 0.005$) (B). C. and D. Intensity Index for vascularization stained by FITC-dextran perfusion was significantly elevated in the central retina of POMGnT1-deficient mice compared to wild-type mice (10.50 ± 2.39 [SD] vs. 32.18 ± 6.04; $P = 0.004$) (C) and also in the peripheral retina of POMGnT1-deficient mice compared to wild-type mice (14.60 ± 1.35 [SD] vs. 34.29 ± 9.30; $P = 0.002$) (D).

conjugated with Alexa Fluor 488 (Molecular Probes) for GFAP, PKC. The immunostained sections were photographed with a confocal laser scanning microscope (TCSSP™, Leica Microsystems Japan).

Electroretinograms (ERGs)

ERGs were recorded from mice anesthesized with an intramuscular injection of ketamine (80 mg/kg) and xylazine (10 mg/kg), and the pupils were dilated with a mixture of tropicamide and phenylephrine. After overnight dark adaptation (>12 h), white light-emitting diodes embedded contact lens electrode was placed on the cornea under dim red light, this electrode was translucent to diffuse the stimulus and background lights. The stimulus intensity and duration were controlled by an electronic stimulator (LS-W; Mayo Co., Nagoya, Japan). The indifferent electrode was a needle inserted subcutaneously on the nasal bone, and a needle electrode on the neck served as the ground electrode. During recording, body temperature was kept to 33 °C with small animals heat controller (ATC-101B, Unique Medical, Tokyo, Japan).

Responses were amplified by a preamplifier (MEG-5200, Nihon Koden, Tokyo, Japan) with band pass between 1 and 300 Hz, and eight responses were recorded with the Power Lab system (AD Instruments Japan Inc., Nagoya, Japan). Stimulus intensity was calibrated by photo sensor built in LS-W. The stimulus intensity to elicit scotopic ERGs was 0.009 cds/m^2, and that to elicit the mixed rod–cone ERGs was 3.0 cds/m^2. Photopic ERGs were recorded after 10 min of light adaptation with 31.6 cd/m^2 and the photopic ERGs were elicited with a stimulus intensity of 3.0 cds/m^2. The interstimulus interval was 10 s for scotopic ERGs, 15 s for combined rod–cone ERGs, and 2 s for the photopic ERGs.

Retinal flat mounts

Anesthetized mice were perfused with 40 ml of PBS through the heart followed by 5 ml of 4% paraformaldehyde in PBS. Then, 2 ml of a mixture of fluorescein-isothiocyanate (FITC)-conjugated high-molecular-weight dextran (molecular weights: 2×10^6 and 4×10^4 Da in a proportion of 2:1 and a concentration of 10 mg/ml; Sigma, St. Louis, MO) was perfused through the heart. The eyes were enucleated and placed in 4% paraformaldehyde overnight at 4 °C. The anterior segment was removed, and four radial incisions were made in the remaining sclera-choroid-neurosensory retina complex. The isolated retinas were placed in ice-cold methanol for 15 min and transferred to PBS. After two 15 min washes in PBS at room temperature (RT), the retinas were transferred into a blocking solution of 10% fetal bovine serum (FBS) and 10% normal goat serum (NGS) for 1 h at RT. The retinas were incubated with rabbit polyclonal anti-glial fibrillary acidic protein (GFAP; Dako) or reticular fibroblasts and reticular fibers antibody (ER-TR7; Santa Cruz

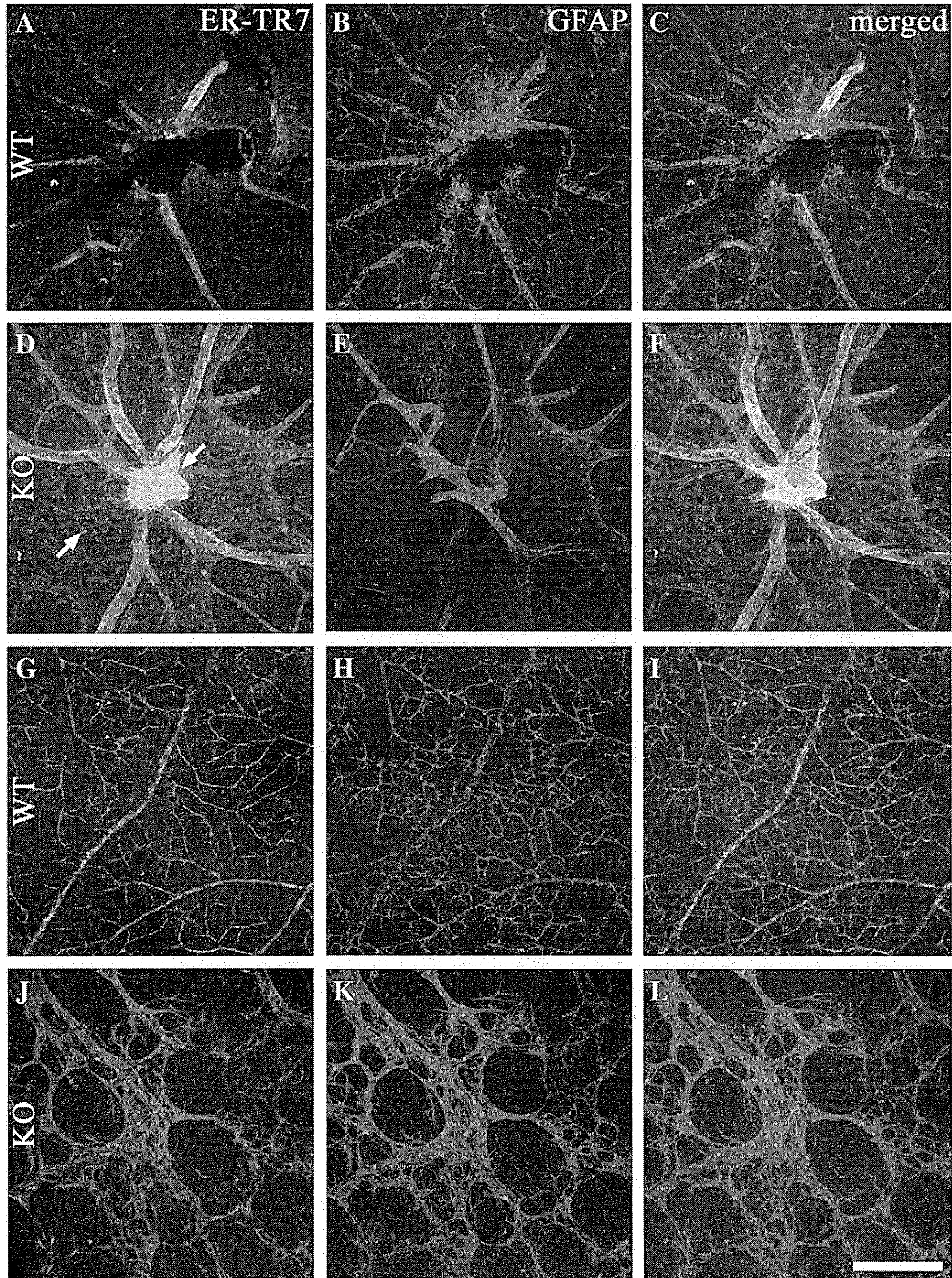

Fig. 9. Immunohistochemical staining for ER-TR7 antigen and GFAP in flat mount retinal preparations from wild-type and POMGnT1-deficient mice. Each animals are 10-week-old. A. D. G. and J. ER-TR7 staining in flat mount retinal preparations from wild-type and POMGnT1-deficient mice. B. E. H. and K. GFAP staining of astrocytes in flat mount retinal preparations from wild-type and POMGnT1-deficient mice. C. F. I. and L. Merged images are shown. Regions of central retinas (A–F) and peripheral retinas (G–L) are shown respectively. ER-TR7-positive fibrous tissues were found closely associated with reactive astrocytes around optic nerve head (D) and peripheral vasculature (J). Note that ER-TR7 and GFAP staining are not identical (arrows). Scale bars represent 100 μm in A–L.

Biotechnology) in 10% FBS and 10% NGS in PBS for 18 h at 4 °C. Retinas were incubated with anti-rabbit antibody conjugated with Alexa Fluor 568 (Molecular Probes) for GFAP, and anti-rat antibody conjugated with Alexa Fluor 488 (Molecular Probes) for ER-TR7 in 10% FBS and 10% NGS in PBS for 2.5 h at room temperature. The flat mounted retinas were photographed with a confocal laser scanning microscope system TCSSP™ (Leica).

Intensity index calculation

GFAP and FITC-dextran stained images were analyzed with ImageJ software (Version 1.44, NIH, Bethesda, MD). Each images were captured using the same camera settings for power, gain, iris aperture size. Data was obtained for the relative area of each region of interest with pixel intensity above a set threshold, and averaged across three

images for both of wild-type and mutant mice. Thus, the index of intensity is defined as the relative area of supra-threshold pixels in each region of interest averaged for both of wild-type and mutant mice (Feilchenfeld et al., 2008). All quantitation was performed with the experimenter blinded to the condition.

Statistical comparisons

All data were expressed as the mean ± standard deviation. For statistical comparisons of retinal thickness and amplitudes of a- and b-wave of ERGs, two-tailed Mann–Whitney nonparametric tests were used. For statistical comparisons of Intensity Index for GFAP immunoreactivity, unpaired t-tests were used. In all statistical comparisons, a P-value less than 0.05 was considered statistically significant.

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