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Cleavage of β-dystroglycan occurs in sarcoglycan-deficient skeletal muscle without MMP-2 and MMP-9

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ABSTRACT

Background: The dystroglycan complex consists of two subunits: extracellular α-dystroglycan and membrane-spanning β-dystroglycan, which provide a tight link between the extracellular matrix and the intracellular cytoskeleton. Previous studies showed that 43 kDa β-dystroglycan is proteolytically cleaved into the 30 kDa fragment by matrix metalloproteinases (MMPs) in various non-muscle tissues, whereas it is protected from cleavage in muscles by the sarcoglycan complex which resides close to the dystroglycan complex. It is noteworthy that cleaved β-dystroglycan is detected in muscles from patients with sarcoglycanopathy, sarcoglycan-deficient muscular dystrophy. *In vitro* assays using protease inhibitors suggest that both MMP-2 and MMP-9 contribute to the cleavage of β-dystroglycan. However, this has remained uninvestigated *in vivo*.
Methods: We generated triple-knockout (TKO) mice targeting MMP-2, MMP-9 and γ-sarcoglycan to examine the status of β-dystroglycan cleavage in the absence of the candidate matrix metalloproteinases in sarcoglycan-deficient muscles.
Results: Unexpectedly, β-dystroglycan was cleaved in muscles from TKO mice. Muscle pathology was not ameliorated but worsened in TKO mice compared with γ-sarcoglycan single-knockout mice. The gene expression of MMP-14 was up-regulated in TKO mice as well as in γ-sarcoglycan knockout mice. *In vitro* assay showed MMP-14 is capable to cleave β-dystroglycan.
Conclusions: Double-targeting of MMP-2 and MMP-9 cannot prevent cleavage of β-dystroglycan in sarcoglycanopathy. Thus, matrix metalloproteinases contributing to β-dystroglycan cleavage are redundant, and MMP-14 could participate in the pathogenesis of sarcoglycanopathy.

1. Introduction

The dystroglycan complex is a plasma membrane protein complex composed of α- and β-dystroglycan (α-and β-DG) that is encoded by a single gene *DAG1* [1]. After post-translational cleavage, a peripheral membrane protein, α-DG, binds to laminin in the basal lamina, and an integral membrane protein, β-DG, anchors α-DG to the cell surface at its N-terminus and binds to the intracellular cytoskeletal protein dystrophin at its C-terminus [2]. Thus, the dystroglycan complex constructs a tight link between the basal lamina and the cytoskeleton.

In skeletal muscles, the sarcoglycan complex composed of four subunits; namely, α-, β-, γ-, and δ-sarcoglycan (SG) resides close to the dystroglycan complex [3,4]. The sarcoglycan complex binds the intracellular actin cytoskeleton to the extracellular matrix and

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stabilizes the dystrophin and dystroglycan complex [4]. A deficiency of any sarcoglycan subunit by gene alteration in humans leads to a complete loss of the sarcoglycan complex, resulting in autosomal recessive limb-girdle muscular dystrophy (LGMD) 2C, 2D, 2E, and 2F [4]. The precise molecular mechanisms by which loss of the sarcoglycan complex leads to muscular dystrophy remain unknown.

Cleavage of β-DG was first found as a 30 kDa fragment in crude skeletal muscle homogenates from a biopsied specimen in a patient with γ-SG-deficient LGMD2C [5]. The monoclonal antibody raised against the C-terminus of β-DG (43DAG/8D5) recognizes the cleaved 30 kDa β-DG as well as 43 kDa full-length β-DG. The 30 kDa β-DG has been proved to be derived from neither alternative splicing nor expression of other dystroglycan isoforms [6]. Matsumura et al. further clarified that cleavage of β-DG occurs at the N-terminal extracellular domain of β-DG in several non-muscle cultured cells and tissues where the sarcoglycan complex is absent [7]. They found that addition of specific inhibitors of MMP-2 and MMP-9 to the culture media decreases cleavage of β-DG in these sarcoglycan-absent cells, and proposed that both MMP-2 and MMP-9 are the candidate enzymes cleaving β-DG. In fact, recombinant MMP-2 and/or MMP-9 are able to cleave recombinant β-DG in a time-dependent manner [8]. These findings raise the intriguing possibility that the sarcoglycan complex masks the cleavage site of β-DG and that gene disruption of the sarcoglycan subunits leads to unmasking the cleavage site and cleavage of β-DG, resulting in muscular dystrophy due to disintegration of the tight link between the basal lamina and the intracellular cytoskeleton [7].

In the current study, we generated triple-knockout (TKO) mice targeting MMP-2, MMP-9 and γ-SG and examined the status of β-DG cleavage and skeletal muscle pathology in comparison with γ-SG knockout LGMD2C model mice [9].

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Recombinant DNA Experiments Safety Committee (#16-12) and Animal Research Committee (#16-031) of Kawasaki Medical School. Generation and characterization of γ-SG knockout mice and MMP-2 knockout mice have been reported [9–11]. MMP-9 knockout mice (Jax stock No. 004104) [12] were purchased from The Jackson Laboratory. Double-knockout (DKO) mice deficient in both MMP-2 and MMP-9 were generated by crossing single-knockout mice deficient in MMP-2 with single-knockout mice deficient in MMP-9. TKO mice deficient in γ-SG, MMP-2, and MMP-9 were generated and maintained by heterozygous mating with DKO and γ-SG knockout mice. These mice were maintained at 22 °C under a 12:12 h light/dark cycle with free access to water and standard laboratory food (CE-2, CLEA Japan, Fuji, Shizuoka, Japan). Water and food intake of the mice was monitored daily. Muscles were isolated following euthanasia under sevoflurane-induced anesthesia.

2.2. Vital staining of damaged myofibers

Membrane-impermeable Evans Blue Dye (EBD), 20 mg/mL in phosphate-buffered saline (PBS), was intraperitoneally injected into the mice (0.15 mg/10 g body weight) to detect damaged myofibers. Skeletal muscles were sectioned, and fluorescent images were captured under a fully monitored inverse microscope.

2.3. Immunoblot analyses

Freshly isolated skeletal muscle tissues were homogenized in 10 vol (w/v) of a phosphate buffer containing 20 mM sodium pyrophosphate, 20 mM sodium phosphate, 1 mM $MgCl_2$, 0.3 mM sucrose, 0.5 mM EDTA, 0.83 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride. The crude skeletal muscle homogenates were denatured and size-fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinyl difluoride membrane. The membrane was blocked with 5% milk in a TBST buffer containing 20 mM Tris-HCl, pH7.4, 138 mM NaCl with 0.1% Tween 20, and incubated overnight at 4 °C with antibodies against γ-SG (clone 35DAG/21B5; NCL-γ-SARC, Leica Biosystems, Wetzlar, Germany), α-SG (clone Ad1/20A6; NCL-α-SARC, Leica Biosystems), β-DG (clone 43DAG1/8D5; NCL-β-DG, Leica Biosystems), dystrophin (clone Dy8/6C5; NCL-Dys2, Leica Biosystems), and α-tubulin (clone DM1A; T9026, Sigma-Aldrich, MO, USA). After washing with TBST buffer, the membrane was incubated with an antibody against mouse monoclonal IgG. Immunoreactive bands were visualized with ECL Western Lightening R Ultra (NEL112001EA, PerkinElmer, Waltham, MA, USA).

2.4. Immunohistochemical analyses

Unfixed skeletal muscle was snap frozen in liquid-nitrogen-cooled isopentane, sectioned on a cryostat, and melted directly onto a glass slide. Sections were post-fixed with 1% freshly depolymerized paraformaldehyde in PBS at room temperature for 15 min. After blocking with the blocking agent of the MOM kit (FMK-2201, Vector laboratories, Burlingame, CA, USA), the sections were incubated with antibodies against γ-SG (clone 35DAG/21B5; NCL-γ-SARC, Leica Biosystems), α-SG (clone Ad1/20A6; NCL-α-SARC, Leica Biosystems), β-DG (clone 43DAG1/8D5; NCL-β-DG, Leica Biosystems), dystrophin (clone Dy8/6C5; NCL-Dys2, Leica Biosystems), and caveolin-3 (ALX-210-241, Enzo Life Sciences, NY, USA).

2.5. Northern blot analyses

Reverse transcription-polymerase chain reaction (RT-PCR) products of MMP-14 (nt 1245–1690), MMP-15 (nt 1553–1950), and 18S ribosome (nt 550–1189) of mouse origin were subcloned into TA vector pCRII-TOPO (45-0640, Invitrogen, Carlsbad, CA, USA). The digested insert was extracted from agarose gel with a QIAquick Gel Extraction Kit (28706, QIAGEN, Hilden, Germany). Each cDNA product was labeled with [α-^{32}P]dCTP with a Megaprime DNA labeling system (RPN1607, GE Healthcare, Chicago, IL, USA). Ten micrograms of total RNA from mouse skeletal muscle was separated on 0.7% agarose gel containing 7% formaldehyde. The gel was then transferred onto the Hybond-N+ membrane (GE Healthcare Life Sciences, London, UK). The cDNA product was hybridized to the total RNA on the membrane at 42 °C overnight, and autoradiography was performed with a BAS-MS 2040 Image Plate (FujiFilm, Tokyo, Japan). The signal intensity of each transcript was measured by an FLA-3000 Image Analyzer (FujiFilm).

2.6. Proteolysis of the extracellular domain of recombinant β-DG

A glutathione S-transferase (GST) fusion protein of the extracellular domain of β-DG (β-DGext), corresponding to amino acid residues 654–750 of human origin, was expressed in BL21

Escherichia coli using a pGEX2TK-β-DGext expression vector [13]. Forty nanograms per microliter of GST-β-DGext (37 kDa) was incubated at 37 °C with the crude muscle homogenate in a buffer containing 50 mM Tris-HCl, pH7.4, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35, and 2 mM 4-aminophenylmercuric acetate [14], without or with a specific inhibitor of MMP-2/MMP-9, Inhibitor II (444249, Merck KGaA, Darmstadt, Germany), or a broad-spectrum inhibitor of MMPs, GM6001 [15,16]. GST-β-DGext was also incubated with 4 ng/μL of active human MMP-2 (PF023, Merck KGaA), MMP-9 (909-mm, R&D systems, Minneapolis, MN), or MMP-14 (475935, Merck KGaA). The resulting reaction mixture was size-fractionated by SDS-PAGE and immunoblotted with anti-GST-HRP conjugate (RPN1236, GE health care).

2.7. Statistical analysis

Paired observations were statistically analyzed by one-way analysis of variance followed by Bonferroni's test. P values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Cleavage of β-DG occurs in TKO mice as well as in γ-SG knockout mice

We generated and examined mice with six distinct genotypes: wild-type (WT), MMP-2 knockout (k/o MMP-2), MMP-9 knockout (k/o MMP-9), MMP-2/MMP-9 DKO, MMP-2/MMP-9/γ-SG TKO and γ-SG knockout (k/o γ-SG) (Supplementary Fig. 1). Skeletal muscles from these mice were homogenized and analyzed by immunoblot. As shown in Fig. 1A, the γ-SG and α-SG proteins were lost in γ-SG knockout and TKO mice, respectively. Unexpectedly, cleavage of β-DG occurred in the TKO mice as well as in the γ-SG knockout mice (Fig. 1A and B).

3.2. Muscle pathology in TKO mice is not ameliorated compared with γ-SG knockout mice

The gross appearance of the quadriceps femoris muscle from γ-SG knockout mice showed hypertrophy and enhanced vital staining with EBD, which represents disruption of the sarcolemma and necrosis of myofibers (Fig. 2A, Supplementary Fig. 2). Hematoxylin and eosin staining of the quadriceps femoris muscle showed variability in myofiber size in γ-SG knockout mice. TKO mouse quadriceps femoris muscles showed enhanced vital staining with EBD, with profound variability in myofiber size. Consistently, diaphragm showed more severe dystrophic pathology with necrosis and fibrosis in TKO mice (Fig. 2B).

3.3. Gene expression of MMPs in muscles deficient in the sarcoglycan complex

To address the question of why MMP-2/MMP-9 DKO cannot prevent the cleavage of β-DG in TKO mice muscles, we examined the gene expression of MMPs in muscles from mice with six different genotypes. Previous studies showed that MMP-14 (MT1-MMP), a member of membrane-type MMPs has similar enzymatic properties to MMP-2 and MMP-9 [7,11]. Compared to the WT mice, MMP-14 was significantly up-regulated in muscles from γ-SG knockout mice and TKO mice, whereas MMP-15 (MT2-MMP) was unchanged in muscles from mice with six different genotypes (Fig. 3A). We next examined the expression of MMPs in β-SG knockout mice and *mdx* mice in which the sarcoglycan complex is secondarily deficient due to the loss of dystrophin. The gene expression of MMP-2, MMP-9, and MMP-14 was consistently up-regulated in these sarcoglycan-deficient mice as well as γ-SG knockout mice (Fig. 3B).

3.4. MMP-14 redundantly cleaves β-DG

We examined the cleavage activity in muscle homogenates from TKO mice. The recombinant extracellular domain of β-DG (β-DGext) was cleaved by the muscle homogenates as the enzymatic source. Addition of a specific inhibitor of MMP-2 and MMP-9, or a broad-spectrum inhibitor of MMPs [15,16] both significantly decreased cleavage activity of β-DGext in the muscle homogenates (Fig. 4A). Thus, MMPs other than MMP-2 and MMP-9 may redundantly cleave the extracellular domain of β-DG *in vivo*. Since MMP-14 was up-regulated in muscles from TKO mice, we examined the cleavage activity of β-DGext by MMP-14. Recombinant MMP-14, as well as MMP-2 or MMP-9, cleaved β-DGext. Thus, MMP-14 can redundantly cleave β-DG *in vivo* (Fig. 4B).

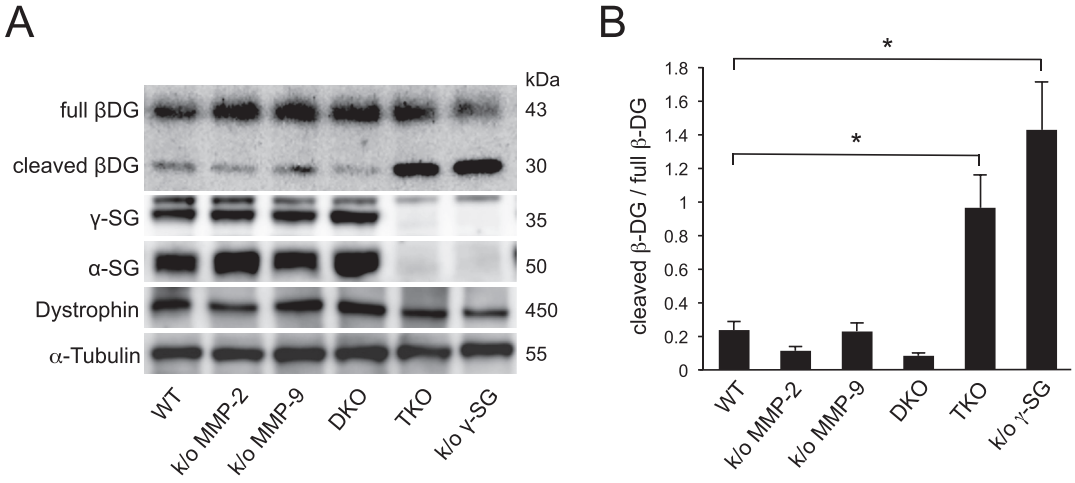

Fig. 1. Cleavage of β-DG occurs in muscles from TKO mice.
A. Ten micrograms of crude muscle homogenate from 24-week-old mice with indicated genotypes was size-fractionated by SDS-PAGE. Immunoblot analysis of β-DG was performed using 43DAG/8D5 mouse monoclonal antibody raised against the C-terminal β-DG. γ-DG: γ-sarcoglycan, α-DG: α-sarcoglycan. B. Ratios of cleaved β-DG (30 kDa)/full β-DG (43 kDa) are shown. Data are expressed as means ± SD ($n = 5$). *$P < 0.05$.

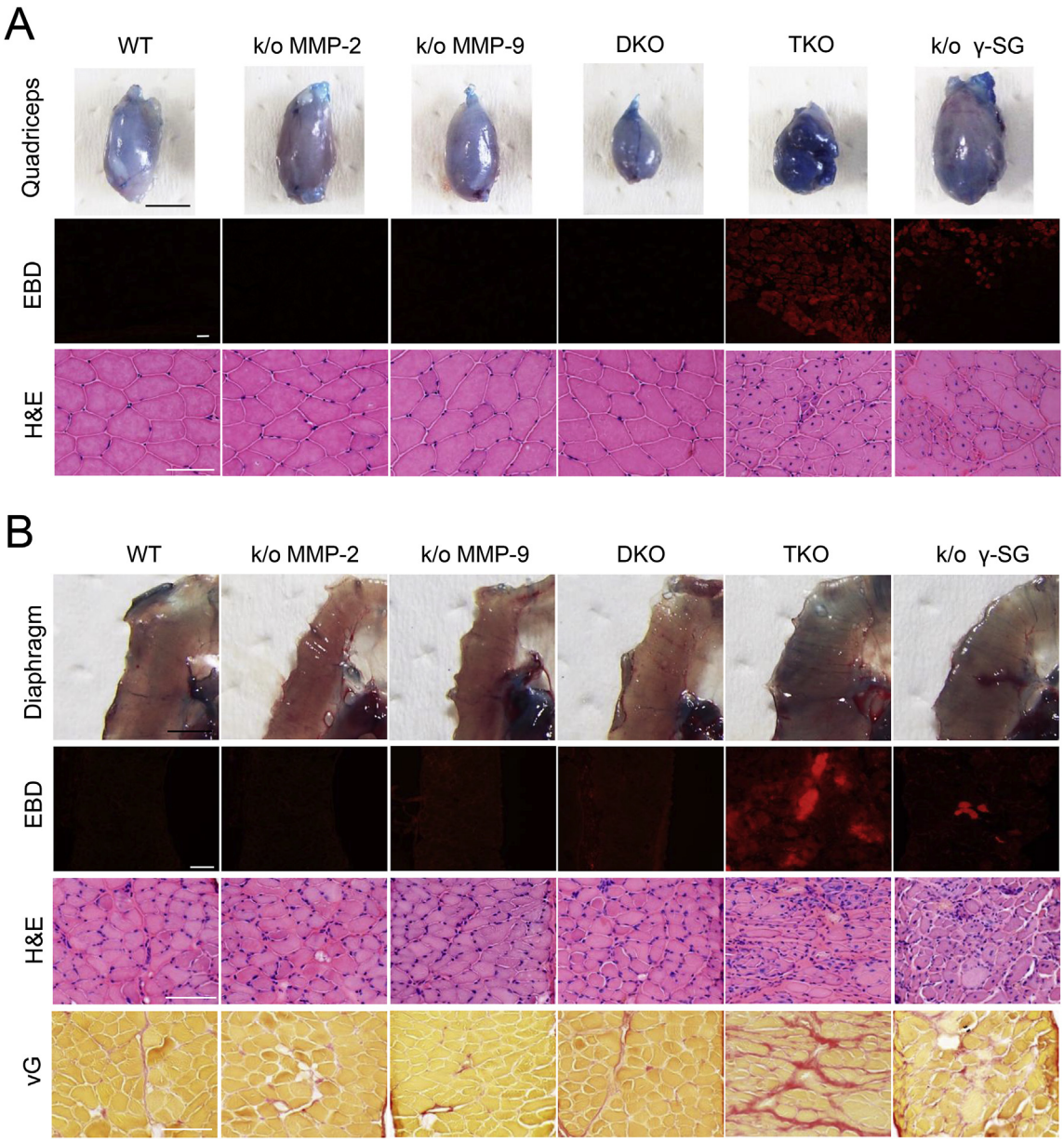

Fig. 2. Sarcolemmal damage and dystrophic pathology in muscles of TKO mice were not ameliorated, but worsened compared with those from γ-SG knockout mice.
Vital staining with EBD and histological appearance in quadriceps femoris muscle (A) or in diaphragm (B) from 24-week-old mice with indicated genotypes. Upper: gross appearance of muscles stained by EBD (blue, Scale bar: 5 mm); middle: fluorescent image of EBD (red, Scale bar: 100 μm); lower: H&E (A) or H&E and van Gieson (vG) staining (B, Scale bar: 100 μm). EBD staining indicates sarcolemmal damage and myofiber necrosis. vG staining represents collagen fibers (red).

4. Discussion

The dystroglycan complex provides the tight connection between the extracellular matrix and the cytoskeleton, especially in skeletal muscles. Disruption of this connection in muscle cells is thought to have a deleterious effect on muscle integrity, leading to various types of muscular dystrophy. At present, there are three conceivable mechanisms by which the disruption of the dystroglycan complex leads to muscular dystrophies. First, the defective glycosylation of α-DG by gene mutations of several glycosyltransferases, including *POMT1*, *POMT2*, *POMGNT1*, *FKTN*, *FKRP*, and *LARGE*, disturbs the binding of α-DG to laminin in the basal lamina and causes congenital muscular dystrophies [17,18]. Second, heterozygous or homozygous mutations in the *DAG1*, which secondarily induce the defective glycosylation of α-DG, cause muscular dystrophies (limb-girdle muscular dystrophy-dystroglycanopathy type C9: MDDGC9) [19,20]. Third, loss of the sarcoglycan complex by its gene disruption, causes LGMD 2C, 2D, 2E, and 2F, due to β-DG cleavage as proposed by Matsumura et al. [7]. His group and others demonstrated cleavage of β-DG in its extracellular domain *in vitro* by recombinant MMP-2 and/or MMP-9 [8,21–24]. However, *in vivo* cleavage of β-DG by MMP-2 or MMP-9 has not been demonstrated [4].

In the current study, we demonstrated that target disruption of both MMP-2 and MMP-9 failed to prevent β-DG cleavage in γ-SG-deficient skeletal muscles. Together with the results from MMP inhibitor assays of recombinant β-DG cleavage, using muscle homogenates from TKO mice as the enzymatic source, cleavage

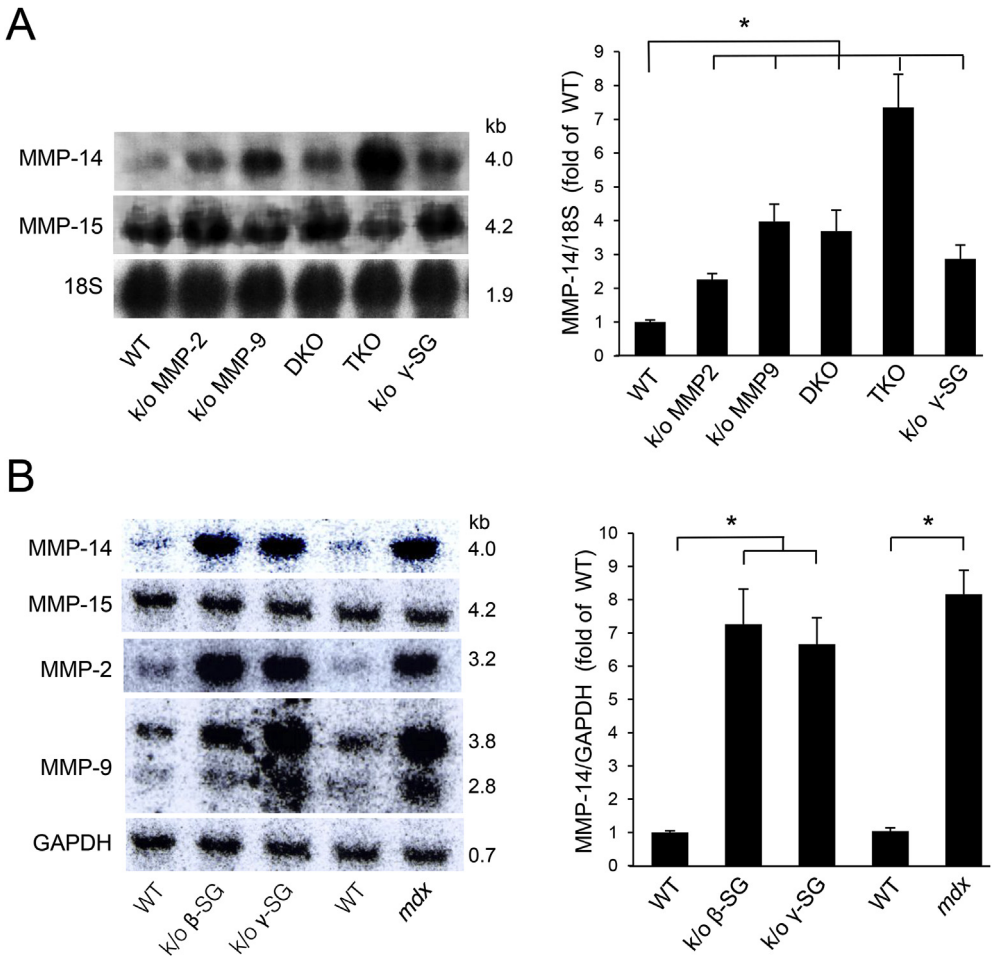

Fig. 3. Gene expression of MMPs in muscles of the TKO mice and the mice deficient of the sarcoglycan complex.
A. Northern blot analysis of MMPs in muscles of 24-week-old mice with indicated genotypes (left). Quantification of MMP-14/18S ratio by densitometric analysis (right). Values are represented as fold increase with respect to wild-type mice. B. Northern blot analysis of MMPs in muscles of β-SG knockout, γ-SG knockout, and *mdx* mice (left). Quantification of MMP-14/18S ratio by densitometric analysis (right). Data are expressed as means ± SD ($n = 5$). $^*P < 0.05$.

activity of β-DG is attributable not only to MMP-2/MMP-9 but also to other MMP-like enzymes which are suppressed by a specific inhibitor of MMP-2 and MMP-9, or a broad-spectrum inhibitor of MMPs. Among these candidate MMPs, the current study revealed that MMP-14 was commonly up-regulated in muscles from mice with loss of the sarcoglycan complex and was capable to cleave β-DG *in vitro*.

The 23 individual MMPs known in humans have overlapping substrate specificity, because multiple MMPs are able to cleave common extracellular matrix proteins, including collagen, laminin, proteoglycan, fibronectin, perlecan, and elastin [14,25,26]. Probably as a result of these enzymatic redundancies, mice targeting a single MMP usually show asymptomatic or nonlethal phenotypes at least up to a few weeks after birth [12]. MMP-14 knockout mice, however, have a unique phenotype characterized by dwarfism, bone malformation, and death before adulthood [27]. MMP-14/MMP-2 DKO mice die immediately after birth with respiratory failure and immature myofibers [11]. Thus, MMP-14 could have relatively specific substrate preferences and thereby may play a pivotal role to cleave β-DG *in vivo*.

Consistent to our findings, another group demonstrated that genetic knockout of MMP-2 did not prevent cleavage of β-DG *in vivo* in primary dystrophin-deficient and secondary sarcoglycan-deficient *mdx* mice with deteriorated muscle pathology [28]. In contrast, genetic knockout of MMP-9 ameliorated muscle pathology in *mdx* mice, unfortunately without analysis of β-DG cleavage [29]. In addition, administration of a MMP-2/MMP-9 inhibitor alleviated the dystrophic phenotype in *mdx* mice [29]. Future extensive studies will be required to clarify the pathological roles of MMPs including MMP-2, MMP-9 and MMP-14 in sarcoglycanopathy.

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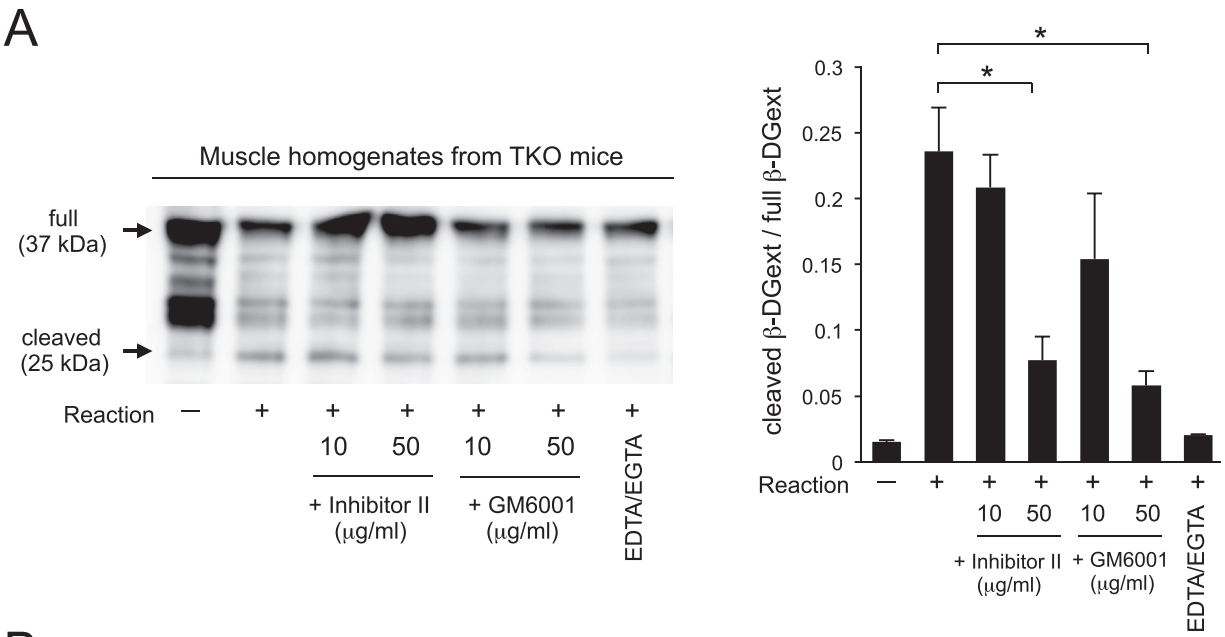

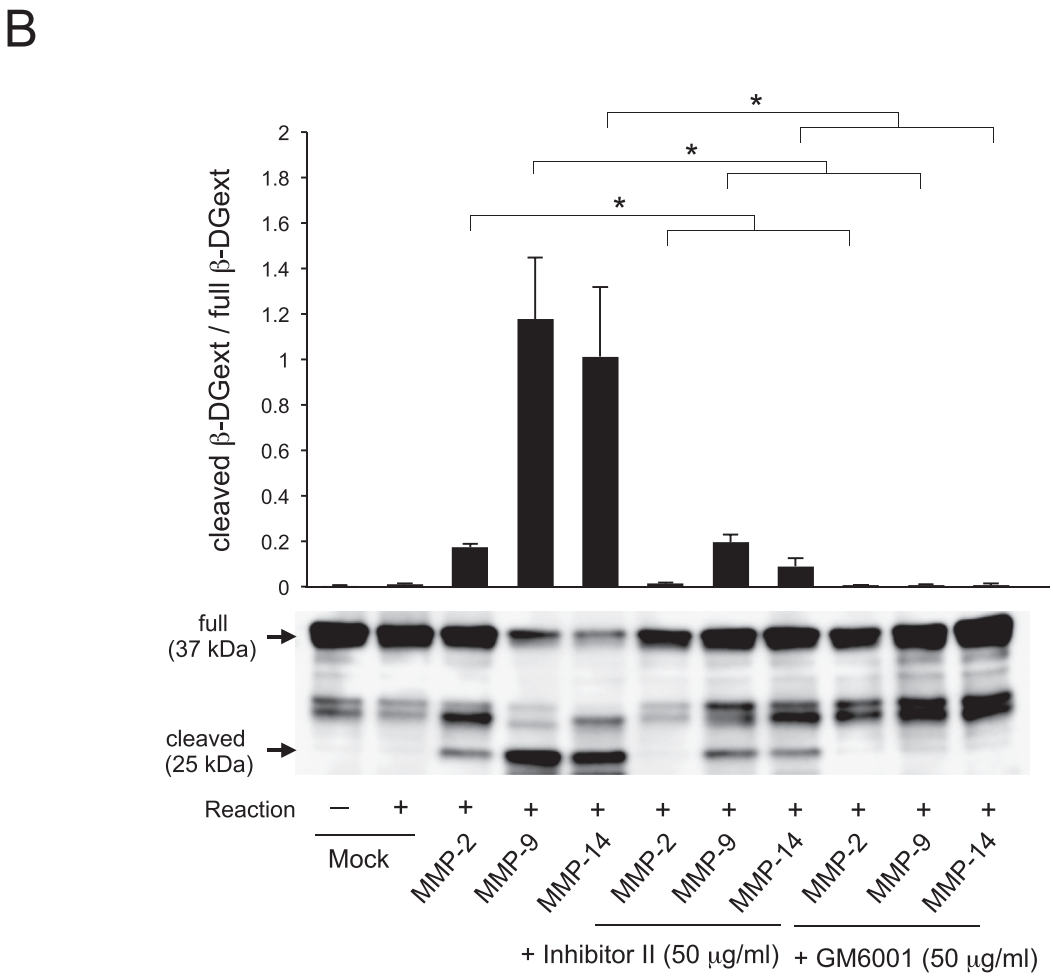

Fig. 4. Cleavage of the extracellular domain of β-DG by muscle homogenates from TKO mice and by recombinant MMP-14.
A. Cleavage of β-DG-GST fusion protein (β-DGext) by crude muscle homogenate from TKO mice in the presence or absence of a specific inhibitor of MMP-2/MMP-9, (Inhibitor II), a broad-spectrum inhibitor of MMPs (GM6001), or divalent cation chelating agents (EDTA and EGTA) suppressing all MMPs at the concentration of 2 mM β-DGext was incubated at 37 °C for 16 h with the crude muscle homogenates. Immunoblot analysis was performed using anti-GST-HRP conjugate. full (37 kDa), full β-DGext; cleaved (25 kDa), cleaved β-DGext; Reaction +: incubated for 16 h, -: incubated for 0 h. B. Cleavage of β-DGext by recombinant MMP-2, MMP-9, or MMP-14. β-DGext was incubated at 37 °C for 16 h with each MMP without or with Inhibitor II, or GM6001). Mock: incubated with the reaction buffer alone. Data are expressed as means ± SD ($n = 5$). *$P < 0.05$.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2017.08.048.

Appendix A. Supplementary data

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