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Subcellular Localization of Dystrophin Isoforms in Cardiomyocytes and Phenotypic Analysis of Dystrophin-deficient Mice Reveal Cardiac Myopathy is Predominantly Caused by a Deficiency in Full-length Dystrophin

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Abstract: Duchenne muscular dystrophy (DMD) is an X-linked recessive progressive muscle degenerative disorder that causes dilated cardiomyopathy in the second decade of life in affected males. *Dystrophin*, the gene responsible for DMD, encodes full-length dystrophin and various short dystrophin isoforms. In the mouse heart, full-length dystrophin Dp427 and a short dystrophin isoform, Dp71, are expressed. In this study, we intended to clarify the functions of these dystrophin isoforms in DMD-related cardiomyopathy. We used two strains of mice: *mdx* mice, in which Dp427 was absent but Dp71 was present, and *DMD*-null mice, in which both were absent. By immunohistochemical staining and density-gradient centrifugation, we found that Dp427 was located in the cardiac sarcolemma and also at the T-tubules, whereas Dp71 was specifically located at the T-tubules. In order to determine whether T tubule-associated Dp71 was involved in DMD-related cardiac disruption, we compared the cardiac phenotypes between *DMD*-null mice and *mdx* mice. Both *DMD*-null mice and *mdx* mice exhibited severe necrosis, which was followed by fibrosis in cardiac muscle. However, we could not detect a significant difference in myocardial fibrosis between *mdx* mice and *DMD*-null mice. Based on the present results, we have shown that cardiac myopathy is caused predominantly by a deficiency of full-length dystrophin Dp427.

Key words : cardiomyopathy, dp427, dp71, dystrophin, heart

Introduction

Mutations in the *dystrophin* gene cause Duchenne or Becker's muscular dystrophy (DMD or BMD) as well as X-linked dilated cardiomyopathy [3]. The dystrophin gene has seven tissue-specific promoters, encoding at least seven protein isoforms: three 427 kDa full-length

dystrophins (Dp427c, Dp427m, and Dp427p) and four N-terminus truncated short isoforms (Dp260, Dp140, Dp116, and Dp71). Dp427m is responsible for DMD and BMD, and is expressed mainly in skeletal and cardiac muscle, while other dystrophin isoforms are detected in various non-muscle tissues, mainly in neural tissues. Dp260 is expressed in the retina, and Dp140 in expressed

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in the brain, retina, and kidney. Dp116 is expressed in adult peripheral nerves, while Dp71 is ubiquitously detected in various tissues [10].

Cardiomyopathy in association with DMD and BMD has been well documented. During clinical progression of DMD, approximately 90% of patients develop a serious impairment of cardiac function, and cardiomyopathy is the cause of death in approximately 20% of cases [15]. Pathogenesis of cardiomyopathy has been examined using the *mdx* mouse, a naturally occurring animal model for DMD [14]. The *mdx* mouse has a point mutation in exon 23 of the dystrophin gene, which forms a premature stop codon. Therefore, *mdx* mice lack the expression of full-length dystrophin, but short dystrophin isoforms are expressed normally. These mice develop late onset and progressive cardiomyopathy that have some similarities with that observed in human dystrophic patients [11]. This makes it a suitable model system to study the pathogenetic mechanism of DMD-related cardiomyopathy.

Previously, we generated a new DMD model mouse by means of deleting the entire 2.4 Mb genomic region of the *dystrophin* gene on mouse chromosome X using a Cre-loxP recombination system [8]. Thus, the *DMD*-null mice produced are a suitable experimental animal for studying the function of the short dystrophin isoform. In cardiomyocytes, the dystrophin isoforms Dp427 and Dp71 are both expressed, as contrasted with skeletal myofibers, in which Dp427 is solely present. Dp71 has been reported to play a variety of unique roles. In PC12 cells, Dp71 is involved in cell-substrate interactions by regulating adhesion to the substrate [4]. In mice spermatozoa, absence of Dp71 has been reported to cause a significant increase in abnormal flagella morphology [6]. In addition, we have reported that loss of Dp71 in olfactory ensheathing cells causes vomeronasal nerve defasciculation in the mouse olfactory system [16]. Therefore, it is plausible that Dp71 expressed in cardiomyocytes may play a unique role. However, no study has reported thus far on the function of Dp71 in cardiac muscle. Involvement of Dp71 in cardiomyopathy can be assessed by comparing cardiac phenotypes of *mdx* mice and *DMD*-null mice. In the present study, we investigated the subcellular localization of Dp427 and Dp71 in cardiomyocytes and also examined the link between Dp71 and cardiomyopathy using *mdx* and *DMD*-null mice.

Materials and Methods

Mice

C57BL/6 and *mdx* mice (genetic background C57BL/10) were purchased from CLEA Japan, Inc. (Tokyo, Japan). The colony of *DMD*-null mice (genetic background: C57BL6/CBA hybrid) was maintained according to a previously described method [8]. The Institutional Animal Care and Use Committee of Kitasato University approved all experimental protocols.

Histology

Hematoxylin and eosin staining was conducted as described previously [8].

Masson trichrome staining was conducted on paraffin sections (7 μ m) using Accustain trichrome stain and Weigert's iron hematoxylin set (Sigma-Aldrich, Tokyo, Japan).

Isolation of cardiomyocytes

Single cardiomyocytes were prepared according to the partially modified method of Shioya [13] with some modifications. Isolated cells were plated on four-well chambers coated with poly-L lysine. For immunostaining, the cells were quickly fixed with 1% (w/v) paraformaldehyde and stained according to standard protocols.

Immunofluorescence procedures

Immunofluorescence procedures were essentially the same as those previously reported [16].

Western blot analysis

Tissues were extracted with Tris buffer containing 67.5 mM Tris-HCl (pH 6.8), 15% (w/v) sodium dodecyl sulfate, 20% (v/v) glycerol, 50 mM dithiothreitol, and protease inhibitor cocktail (Complete®, Roche, Tokyo, Japan). The soluble fraction after centrifugation for 15 min at 13,000 g (RT) was used according to a previously described method [16]. Primary antibodies against dystrophin (ab15277, Abcam, Tokyo, Japan), β -dystroglycan (Novocastra, Wetzlar, Germany), α 1-syntrophin (AG-17,1; Sigma-Aldrich), neuronal nitric oxide synthase (nNOS; BD), vinculin (Sigma-Aldrich), utrophin (H-300, SantaCruz Biotechnology, Texas, U.S.A.), and GAPDH (3H12, MBL, Nagoya, Aichi, Japan) were used.

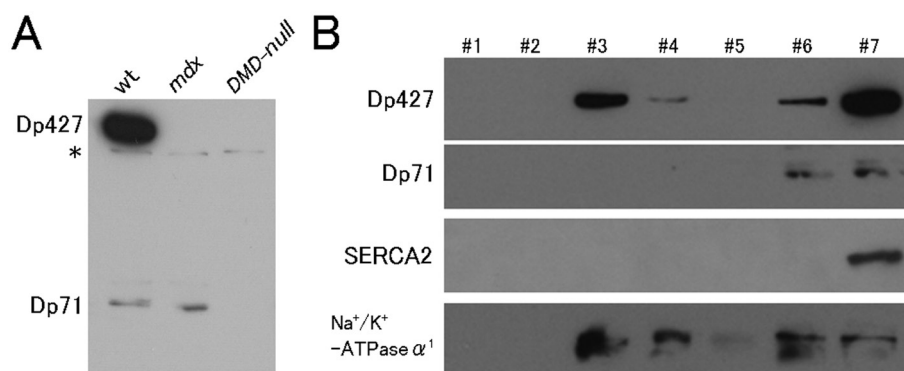


Fig. 1. Western blot analysis. A: Immunoblots of heart extracts prepared from wt, *mdx*, and *DMD*-null mice. Note: Both Dp427 and Dp71 were present in the hearts of wt mice, only Dp71 was present in the *mdx* mice, and neither was present in the *DMD*-null mice. The asterisk indicates a nonspecific signal. B: Immunoblots of subcellular fractions after separating heart homogenates from wt mice by iodixanol density-gradient centrifugation. Note: Dp427 was present in the cardiac sarcolemmal (#3, 4) and intracellular membrane fractions (#6, 7), while Dp71 was present in intracellular membrane fractions (#6, 7).

Subcellular fractionation

Plasma membranes and intercellular membranes of cardiomyocytes were separated using the method described by Biao Lei *et al.* [9], with some modifications. Homogenizing buffer (pH7.4) containing 0.1 M sucrose, 10 mM EDTA, 46 mM KCl, 100 mM Tris-HCl, and Complete® protease inhibitor cocktail (Roche Diagnostics, Tokyo, Japan) was used.

Results

Subcellular localization of dystrophin isoforms in cardiomyocytes

Western blot analysis confirmed that Dp427 and Dp71 were present in the cardiac muscle of wild-type (wt) mice, whereas Dp71 was only present in *mdx* mice. None of the isoforms were present in *DMD*-null mice (Fig. 1A). To examine subcellular localization of the dystrophin isoforms, immunostaining using an anti-dystrophin antibody was performed in ventricular cardiomyocytes isolated from wt, *mdx*, and *DMD*-null mice (Fig. 2A). In wt mice, dystrophin localized in the sarcolemma and also in the horizontally striped cytoplasmic area of the cytoplasm. The intense sarcolemmal immunostaining observed in wt mice was not found in *mdx* mice or *DMD*-null mice. Horizontally striated immunostaining in wt cardiomyocytes overlapped with that of the T-tubule marker vinculin, suggesting that cytoplasmic dystrophin was associated with T-tubules. Importantly, *mdx* cardio-

myocytes exhibited a similar striated T-tubule-associated pattern. Because Dp71 was the only dystrophin isoform expressed in *mdx* cardiomyocytes, Dp71 was suggested to localize in T-tubules. The same results were obtained when immunostaining was conducted on frozen sections of the heart from wt, *mdx*, and *DMD*-null mice (Fig. 2B).

In order to know whether localization of Dp427 was restricted to the cardiac sarcolemma, we performed subcellular fractionation of wt cardiac homogenates. Using the discontinuous iodixanol density-gradient centrifugation method, the cardiac sarcolemmal and intracellular membranes were separated and used for Western blotting to check for the presence of dystrophin isoforms. As shown in Fig. 1B, we detected immunoreactivity for Dp427 and Dp71 in the intracellular membrane fraction, indicating that both Dp427 and Dp71 are associated with T-tubules.

Influence of loss of Dp71 in cardiomyocytes

As shown in Fig. 2, the immunostaining pattern for vinculin in cardiomyocytes of *DMD*-null mice was essentially the same as that of *mdx* mice, suggesting that the structure of T-tubules was unaffected by the absence of Dp71. Moreover, no apparent difference could be observed in the ultrastructure of T-tubules between *mdx* mice and *DMD*-null mice (Supplemental Fig. 1: refer to J-STAGE at <https://www.jstage.jst.go.jp/browse/expanim>). The amount of dystrophin-associated proteins in the

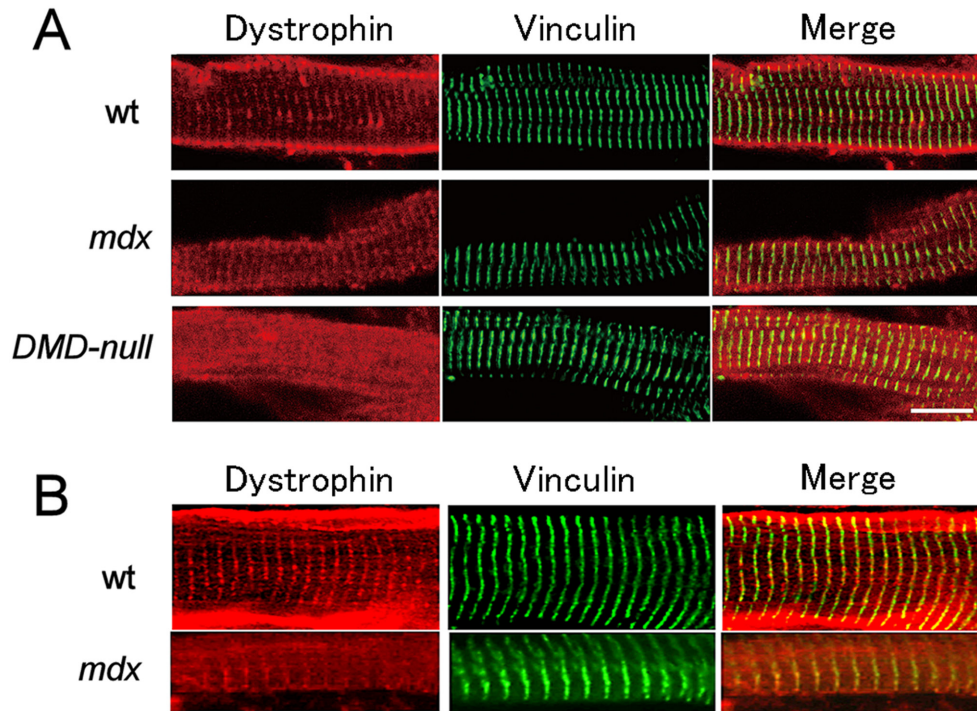


Fig. 2. Immunofluorescence images of cardiomyocytes labeled with anti-dystrophin (red) and anti-vinculin (green) antibodies. **A:** Cardiomyocytes isolated from either wt mice, *mdx* mice, or *DMD*-null mice. **B:** Longitudinal cryosections from either wt mice, *mdx* mice.

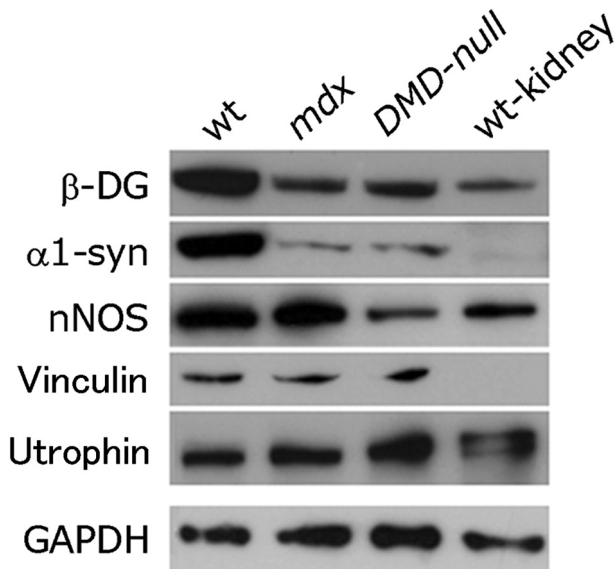


Fig. 3. Western blot analysis. Immunoblots for dystrophin-related proteins in heart extracts. β -DG, β -dystroglycan; α 1-syn, α 1-syntrophin.

heart was examined by Western blot analysis. As shown in Fig. 3, the amounts of β -dystroglycan and α 1-syntrophin were equally low in *mdx* and *DMD*-null mice

compared with wt mice. However, nNOS levels in the *DMD*-null mouse heart were lower than in the *mdx* mouse, suggesting that Dp71 may play some role in nNOS regulation. Additionally, we also immunohistochemically examined the expressions of some of the ion channels that have been known to locate in T-tubules and shown to be important for excitation–contraction coupling. The ion channels examined were Nav1.5, Kir2.1, and Cav1.2. All of them were found to locate at T-tubules as judged by their overlapped anti-vinculin immunostaining. No difference could be detected between *DMD*-null mice and *mdx* mice in the expression patterns of these ion channels (data not shown). Therefore, absence of Dp71 may have little influence on the expression of ion-channels located in T-tubules.

Analysis of cardiac muscle phenotype between mdx mice and DMD-null mice

The body and heart weights of wt mice, *mdx* mice and *DMD*-null mice are shown in Fig. 4A. At 10–12 months of age, the heart weight of *DMD*-null mice was lower than that of *mdx* mice. Representative images of histological sections from 12-month-old wt, *mdx*, and *DMD*-

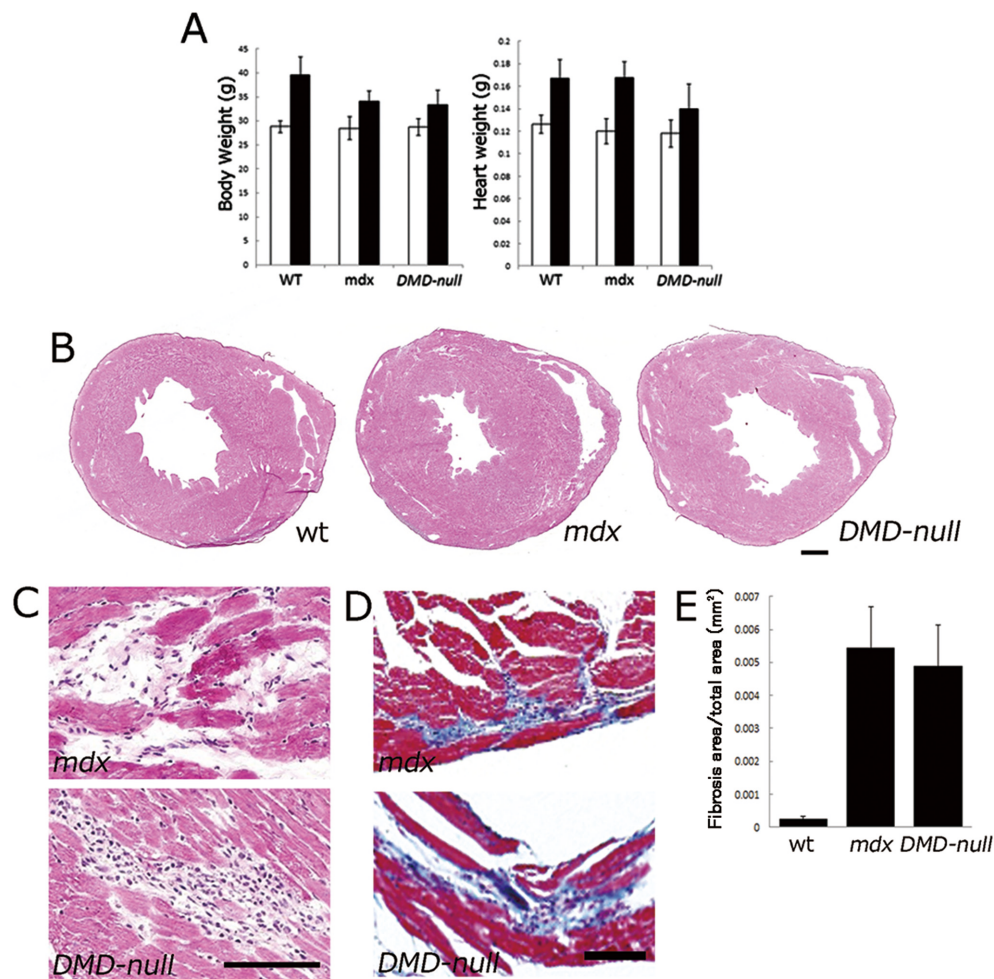


Fig. 4. Phenotype analyses of cardiac muscle in *mdx* mice and *DMD*-null mice. **A:** Body weight (left panel) and heart weight (right panel) at 3 months (white bar) and 10–12 months (black bar) of age. **B, C:** Histological sections with hematoxylin-eosin staining. **D:** Histological sections with Masson's trichrome staining. **E:** Graphic depiction of the ratio of connective tissue to normal myocardium in 12-month-old *mdx*, *DMD*-null mice and wt mice. The total area of blue-stained collagen was determined by digital image analysis. Note: No significant difference in necrotic area was observed between *mdx* and *DMD*-null mice ($P=0.55$).

null mice are shown in Fig. 4B. Focal necrotic lesions were frequently seen in *mdx* and *DMD*-null mice (Fig. 4C). Replacement of the necrotic myocardium with fibrotic tissue, assessed by Masson's trichrome staining, was usually observed in the necrotic area (Fig. 4D). Such fibrosis was increasingly frequent with age. Importantly, the tissue area of fibrosis was not significantly different between *mdx* mice and *DMD*-null mice (Fig. 4E), suggesting that cardiac disruption occurred predominantly owing to the absence of Dp427 but not Dp71.

Discussion

In the present study, we examined the subcellular distribution of the dystrophin isoforms Dp71 and Dp427 using two strains of mice: *mdx* mice and *DMD*-null mice. We showed that Dp427 was located in the cardiac sarcolemma and also at the T-tubules, whereas Dp71 was exclusively located at the T-tubules. Both *DMD*-null and *mdx* mice showed severe necrosis and fibrosis in the heart without significant differences. Based on these data, we concluded that cardiac myopathy is caused predominantly by a deficiency of full-length dystrophin Dp427.

The *mdx* mice, which develop progressive cardiomyopathy, have been widely used to study the molecular and cellular effects of dystrophin deficiency in cardiac tissues. The biochemical and physiological alterations observed in *mdx* mice have led to a number of hypotheses to explain the pathogenesis of cardiomyopathy. These include altered energetics [7], perturbation in L-type Ca^{2+} channel function [12], nitric oxide alterations [1] [18], altered function of the KATP channel complex [5], and increased oxidative damage [19]. Nevertheless, to date there is no evidence of a direct link between dystrophin and any of the aforementioned alterations in the heart.

Because Dp71 was exclusively located at the T-tubules of cardiomyocytes, Dp71 should play some role related to T-tubules. Because cardiomyopathy occurred equally in *DMD*-null and *mdx* mice, the function of Dp71 may not be linked to *DMD*-related cardiac disruption. Additionally, we found that utrophin, a protein homologous to dystrophin, was upregulated in the *DMD*-null mouse heart in our preliminary experiment. It is possible that the upregulation of utrophin may compensate for the absence of Dp71 in cardiac muscle.

As shown in Fig. 3, nNOS levels in the *DMD*-null mouse heart were lower than in the *mdx* mouse, suggesting the involvement of Dp71 in nNOS regulation. Interestingly, the decreased cardiac nNOS might affect the conduction system in dystrophic mice. According to Bia *et al.* [1], the lack of dystrophin in the *mdx* mouse and also in the dystrophin/utrophin double-deficient mouse resulted in abnormal electrocardiograms that were associated with decreased myocardial nNOS and increased iNOS activities. Furthermore, it has been reported that Purkinje-fiber degeneration occurred prior to cardiac muscle degeneration, and Purkinje-fiber degeneration might be associated with the expression abnormality of Dp71, nNOS, and utrophin [17]. Therefore, it would be particularly important to investigate the cardiac conduction system pathologically and physiologically in *DMD*-null mice.

In the *DMD*-null mice examined in the present study, expression of dystrophin was completely lost in all skeletal myofibers and cardiac muscles [8]. This complete loss of dystrophin expression contrasted sharply with that of *mdx* mice. In *mdx* skeletal muscle, low but detectable levels of full-length dystrophin have been reported to be expressed, probably due to exon skipping [2]. Although the pathology of cardiac muscle in *DMD*-null

mice was not essentially different from that of *mdx* mice, the *DMD*-null mice should be useful for studying pathological mechanisms, disease progression and therapy for cardiomyopathy due to dystrophin deficiency.

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