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Decreased mAKAP, ryanodine receptor, and SERCA2a gene expression in *mdx* hearts

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Abstract

Duchenne muscular dystrophy (DMD) is a common genetic disease resulting from mutations in the dystrophin gene. The lack of dystrophin function as a cytoskeletal protein leads to abnormal intracellular Ca^{2+} homeostasis, the actual source and functional consequences of which remain obscure. The *mdx* mouse, a mouse model of DMD, revealed alterations in contractile properties that are likely due to defective Ca^{2+} handling. However, the exact mechanisms of the Ca^{2+} handling defect are unclear. We performed suppressive subtractive hybridization to isolate differentially expressed genes between 5-month-old *mdx* and control mice. We observed a decrease in muscle A-kinase anchoring protein (mAKAP) in the *mdx* hearts. We noticed not only down-regulation of mAKAP mRNA but also decreased mRNA level of the molecules involved in Ca^{2+} handling and excitation–contraction (E–C) coupling in the sarcoplasmic reticulum (SR), the cardiac ryanodine receptor, and the sarcoplasmic reticulum Ca^{2+} ATPase. Therefore, dystrophin deficiency may cause an impairment of SR Ca^{2+} homeostasis and E–C coupling in *mdx* hearts, in part, by decreased gene expression of molecules involved in SR Ca^{2+} handling.

Keywords: Duchenne muscular dystrophy; *mdx* mice; Muscle A-kinase anchoring protein; Ryanodine receptor; Sarcoplasmic reticulum Ca^{2+} ATPase

Duchenne muscular dystrophy (DMD) is an inherited X-linked neuromuscular disorder that leads to severe skeletal muscle wasting followed by premature death in early adulthood [1]. The *mdx* is a mouse model of DMD. To date, the *mdx* mouse has been the most widely utilized animal model of DMD. This model carries a loss-of-function mutation in dystrophin, a component of the membrane-associated dystrophin–glycoprotein complex [2,3]. Dystrophin is located at the muscle sarcolemma in a membrane-spanning protein complex that connects the cytoskeleton to the basal lamina. Although the precise function of dystrophin is unknown, the lack of protein causes membrane destabilization of the cytoskeleton and also causes an abnormal influx of Ca^{2+}. How this increased entry of Ca^{2+} affects the local concentration of Ca^{2+} in subcellular compartments and whether this process is involved in the development of the disease are still unclear [4]. A vast majority of researches have focused on skeletal muscle dystrophy however only a few studies have investigated the alteration in cardiac muscle function. Previous studies have suggested that the old *mdx* might be a better model for DMD-associated cardiac disorders as shown by many investigators, suggesting that myocardial lesions were readily demonstrable after two to eight months of age [5–8]. Another study showed that *mdx* mice have profound alterations in contractile properties which may be due to defective Ca^{2+} homeostasis [9]. How dystrophin deficiency affects contractility remains poorly elucidated.

In the present study, we investigated the different gene expressions between the 5-month-old *mdx* and control hearts by using the suppressive subtractive hybridization method. We observed decreased gene expression of mAKAP, which is independent of cardiac hypertrophy seen in 5-month-old *mdx* hearts. Moreover,

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we also noticed down-regulation of the cardiac ryanodine receptor (RyR2) and sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2a) in *mdx* hearts. The expression of FKBP12.6 and phospholamban remained unchanged in *mdx* mice as compared to control mice.

Materials and methods

RNA isolation. Total RNA was isolated from C57BL/10mdx (*mdx*) and C57BL/10ScSn (control) mice hearts by Isogen (Pikkagen, Japan). Poly(A)$^+$ RNA was purified from 5-month-old heart total RNA using the Fast track 2.0 mRNA isolation kit (Invitrogen) according to the manufacturer's protocol.

Suppressive subtractive hybridization. Reverse transcription of poly(A)$^+$ RNA and generation of subtracted libraries were performed using the PCR select cDNA subtraction kit (Clontech) according to the manufacturer's protocol. We referred to cDNA (control) that contains specific (differentially expressed) transcripts as "testers," and the reference cDNA (*mdx*) as "drivers." Briefly, first-strand cDNA synthesis was carried out with the template of 2 μg poly(A)$^+$ RNA from *mdx* and control hearts by using an AMV reverse transcriptase. The first-strand cDNA was immediately subjected to the second-strand synthesis with T4 DNA polymerase. The synthesis was terminated by 4 μl of 20× EDTA/glycogen cDNA. The samples were then purified twice with 100 μl of a phenol:chloroform:isoamyl alcohol (25:24:1) mixture followed by extraction with chloroform:isoamyl alcohol (24:1) and ethanol precipitation. The blunt-end of the driver was generated by overnight *Rsa*I enzyme digestion and the digested products were purified as mentioned above. Once the preparation of the driver cDNA was completed, the tester populations were then created by ligation with different adaptors but the driver cDNA has no adaptor. *Rsa*I enzyme digestion and adaptor ligation efficiency assay was done in advance hybridization. An excess of driver was added to each tester cDNA during the 12 h first hybridization followed by the addition of the denatured driver to further enrich the differentially expressed cDNA. The cDNA was then subjected to PCR amplification. PCR amplification was optimized using a Perkin–Elmer GeneAmp PCR system 2400. To extend the adaptors, the cDNA was incubated at 75 °C for 5 min following preheating at 94 °C for 25 s before the first round of 30 amplification cycles (94 °C for 10 s, 66 °C for 30 s, and 72 °C for 1.5 min). Nested PCR was performed using the following condition: 94 °C for 1 min and 94 °C, 10 s; 68 °C, 30 s; 72 °C, 1.5 min for 15 cycles. All amplifications were carried out using *Taq* DNA polymerase. The PCR products were resolved by electrophoresis using 2% agarose gels.

cDNA cloning and screening. The secondary PCR products were subcloned into pT7Blue-Vector (Novagen). These clones were subjected to PCR amplification to determine the cDNA insert. For arraying subtracted clones, identical blots were prepared using the PCR-Select Differential Screening kit (Clontech) according to the manufacturer's protocol. Three hundred randomly selected white bacterial colonies of the subtracted cDNA libraries were incubated at 37 °C by shaking for 2 h and then subjected to PCR amplification to determine the cDNA insert sizes. One microliter of each bacterial culture was subjected to 23 amplification cycles (95 °C for 30 s and 68 °C for 3 min). Subsequently, 1.5 μl of the denatured PCR products was transferred to the two-nylon membrane for the cDNA dot blot analysis preparation. The adaptors were removed from the ends of the cDNA by *Rsa*I, *Sma*I, and *Eag*I enzymes and used as probes for dot blot analysis. The probes were then labeled with [^{32}P]dCTP (Amersham–Pharmacia). Quantitative analysis of the dot blot hybridization signal was measured using a FUJIX Bas2000 Image Scanner EWS software.

Northern blot analysis. Total RNA from control and *mdx* or hypertrophied hearts was resolved on 1.2% agarose–formaldehyde gels and transferred to the positively charged Nylon membranes (Boehringer–Mannheim). PCR products from inserts of selected clones were used as probes and randomly labeled with [^{32}P]dCTP. Pre-hybridization and hybridization were performed at 68 °C for 1 h using a QuikHyb solution (Stratagene). The membranes were then washed with 2× SSC and 0.1% SDS for 2 × 15 min at room temperature and twice at 15 min each with 0.1% SSC and 0.1% SDS at 60 °C. Quantitative analysis of signals was done as described above. The hybridization signals were normalized to a housekeeping gene, GAPDH, to compensate for unequal loading.

3′ *and* 5′ *RACE.* The 5′ end of the cDNA was cloned using the 5′ RACE system (Life Technologies) against mouse heart total RNA. The first strand cDNA was synthesized with SuperScript reverse transcriptase (Life Technologies) by using a specific primer (5′-CATG TCAGCTCTGTAAA-3′). An oligo(dC) anchor was added to the 3′ end of the first-strand cDNA with terminal deoxynucleotidyltransferase. The first round of PCR was performed as recommended by the manufacturer with a specific 3′ primer (5′-CATCTTGCAGGTCTCT CTATTT-3′) and a 5′ anchor primer. The product of this PCR was subjected to second amplification using a nested specific 3′ primer (5′-G GCTTTCTCTCTTTTCACACATGTA-3′). The PCR products were subsequently subcloned into a pT7 vector and sequenced. 3′ RACE was carried out using the 3′ RACE system for rapid amplification (Life Technologies), according to the manufacturer's protocol, with a specific 5′ primer (5′-TGACTTCAGATCCATTTCAACCT-3′) and a nested specific 5′ primer (5′-GACTGAATGTTATACAGGCAGAA-3′). All primer sequences were designed according to the sequences of the WT17 (mAKAP) clone obtained from the subtracted libraries.

Reverse transcription-PCR and probe preparation. First strand cDNA synthesis was carried out with 3 μg total RNA of mouse/rat heart using SuperScript reverse transcriptase II (Life Technologies), as recommended by the manufacturer. The cDNA products were used as template for PCR. For further confirmation whether the 5′ RACE product was part of the mouse mAKAP, a forward primer (5′-GGGT TTGGTGGATGGTATAGAAA-3′) corresponding to nucleotides 8378–8400 of rat mAKAP and a reverse primer (5′-CCTGATCAAC TTGGTGTTAGTGTTAA-3′) corresponding to the original unknown sequence wt17 were used for RT-PCR. The PCR product was subcloned into a pT7 vector and sequenced using the ABI PRISM 310 genetic analyzer sequencer. The putative mouse mAKAP clone was used as a probe and other cDNA probes were prepared using primers as shown in Table 1. All of the expected PCR products were subcloned and sequenced for confirmation before using them as Northern blot probes.

cDNA purification and sequencing. cDNA was purified from overnight bacterial cultures using the QIAprep Spin Miniprep Kit (Qiagen). DNA sequencing was performed using the ABI PRISM 310 genetic analyzer sequencer (Applied Biosystems) and the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems). The cDNA sequences were then subjected to BLAST homology search to compare them with sequences present in the GenBank.

Cultured cardiomyocytes. Cultured cardiomyocytes were prepared from 2- to 3-day-old neonatal rat hearts at 4.5×10^6 cells per 10 cm dish as described [10]. After 1 day in culture, one plate was harvested and the others were changed to a serum free medium and allowed to stay overnight. The following day, the cells were washed three times with PBS and then left in a serum free medium for 1 day with or without 100 μM phenylephrine or 10^{-7} M endothelin-1 (ET-1) in order to induce a hypertrophic response.

Results

Identification of differentially expressed genes in 5-month-old mdx hearts

To generate an equalized cDNA library enriched in clones of genes that were differentially expressed in *mdx* and control hearts, we used the suppressive subtractive

Table 1
Primer pairs used to detect gene expression

Name	Sequences	Corresponding to nucleotides
mAKAP-F	ACCGCATTTGAACTCTCCGAC	800–820 of rat mAKAP
mAKAP-R	AATCTGTTTCTGGTCTCATCGTGG	1766–1743 of rat mAKAP
SERCA2a-F	CCGATGACAATGGCACTTTCT	2161–2181 of rat SERCA2a
SERCA2a-R	CTCCAGTATTGCAGGCTCCAG	3194–3174 of canis SERCA2a, nt 16th change T to C
PLB-F	GACTTGTCTTCCTCTTTTGT	781–800 of rat PLB
PLB-R	TCACAGAAGCATCACAATGA	972–953 of rat PLB
RyR2-F	GGAGAACTCAGAGACCAACAA	22–24 of mouse RyR2
RyR2-R	CTGGTCTTCATACTGTTC	306–288 of mouse RyR2
FKBP12.6-F	GGGCGAAGGCGAGGATGAGATCCAGTT	3–23 of rat FKBP12.6
FKBP12.6-R	GGCCACGCTCCAGAGCGTCTGATGGAA	318–298 of rat FKBP12.6
PDE4D3-F	CTAATTTGCAAGATCGCGCACCCA	323–346 of rat PDE4D3
PDE4D3-R	CCTGGTTGCCAGACCGACTCATTT	577–554 of rat PDE4D3

SERCA2a, sarcoplasmic reticulum ATPase-2a; PLB, phospholamban; RyR2, cardiac ryanodine receptor; FKBP12.6, FK 506-binding protein; PDE4D3, rolipram-inhibited cAMP-specific phosphodiesterase PDE4D3 isoform.

hybridization (SSH) and the T/A cloning technique as described in Materials and methods. Screening of 290 inserts by dot blot analysis revealed that 30 clones hybridized with the control-specific probe but not with *mdx*-specific probes. The sequence analyses of the 30 clones revealed that they corresponded to 22 different cDNAs. A total of 20 cDNAs were identical or highly homologous to a known sequence whereas two cDNAs did not match with any known sequence available in public databases (data not shown).

Northern blot analysis demonstrated that 5 of the 22 clones were significantly lower in the *mdx* heart than in the control. In the present study, we focused on the wt17 clone that hybridized with a 8.8 kb band. This transcript was 57% lower in *mdx* than in control hearts. Computer database searches of wt17 revealed that no significant similarity was found with any known sequences. In an attempt to identify this unknown clone, we performed 5′ RACE. We obtained a 900 bp fragment that revealed 93% identity to a part of the 3′ noncoding region of the rat muscle anchoring A-kinase protein (mAKAP) cDNA. Therefore, we conclude that the wt17 clone is the mouse counterpart of the rat mAKAP (Fig. 1A).

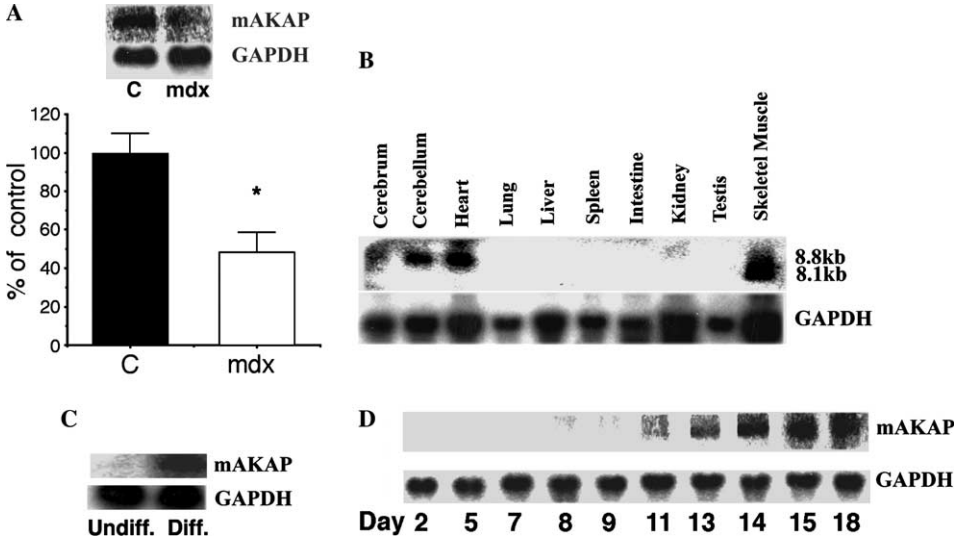

Fig. 1. mAKAP gene expression in the heart. (A) Decreased mAKAP mRNA in *mdx* hearts. Northern blot analysis of mAKAP in 5-month-old *mdx* and control hearts. Upper panel: representative northern blots; lower panel: normalized quantitative analysis of mAKAP levels. The hybridization signals are presented as ratio of mAKAP to GAPDH mRNA, the control was normalized to 100%. The bar graph shows the mean ± SEM from three independent samples, $*p < 0.001$ vs. control. (B) Tissue distribution of mAKAP. Northern blot analysis of tissue distribution shows that mAKAP is expressed exclusively in the cerebellum, heart, and skeletal muscles. (C) mAKAP is expressed in differentiated cardiomyocytes. Cultured cardiomyocytes were prepared as described in Materials and methods. mAKAP is expressed at the day 3 of cultured cardiomyocytes when cardiomyocytes exhibited complete differentiation (diff). (D) mAKAP is expressed after day 13 in SD rat hearts corresponding to a decline in differentiating cardiomyocytes progressively reaching nearly zero [13]. Ten micrograms of total RNA from the hearts or cultured cardiomyocytes was used for Northern blot analysis. The hybridization signals were normalized to a housekeeping gene, GAPDH.

Mouse mAKAP is expressed in the cerebellum, heart, and skeletal muscles

We next investigated the tissue distribution of the putative mouse mAKAP. Northern blot analysis demonstrated that this gene was expressed exclusively in the cerebellum, heart, and skeletal muscles as shown in Fig. 1B. The transcript in skeletal muscle was slightly lower in size than those of the cerebellum and the heart. Our observation was compatible with a previous report showing the tissue distribution of rat mAKAP at the protein level [11].

mAKAP is expressed in differentiated cardiomyocytes

In order to further characterize mAKAP gene expression regulation, we examined mAKAP expression in cultured cardiomyocytes. mAKAP was expressed in day 3 cultures when cardiomyocytes showed a differentiated phenotype. Mono- or binucleate differentiated cardiomyocytes have been reported to undergo myofibrillar organization and to exhibit spontaneous contractions [12]. However, we did not see any signal in day 1 cultures when the neonatal ventriculocytes reverted to the undifferentiated phenotype (Fig. 1C). Moreover, we examined mAKAP expression in 2–18-day-old Sprague–Dawley rat hearts. The mAKAP signals significantly appeared on 13-day-old rat hearts and were increased by 15 days of age (Fig. 1D) when differentiating cardiomyocytes of rats declined progressively reaching nearly zero [13]. Thus, these observations suggested that mAKAP was expressed in terminally differentiated cardiomyocytes.

Regulation of mAKAP gene expression: decreased in mdx and increased by phenylephrine treatment

A previous study has suggested that mAKAP may serve as the scaffold for a cAMP and calcium ion-sensitive signaling complex [14]. Consequently, we expected that regulation of mAKAP gene expression was associated with intracellular Ca^{2+} and cAMP levels or related with cardiac hypertrophy since the 5-month-old *mdx* heart also exhibited hypertrophic features [15]. To dissect the two possibilities, we checked the mAKAP mRNA level in hypertrophy-induced cardiomyocytes. ET-1 treated cardiomyocytes showed no significant difference in the mAKAP mRNA level (Fig. 2A) even if we noticed the hypertrophic feature of ET-1-treated

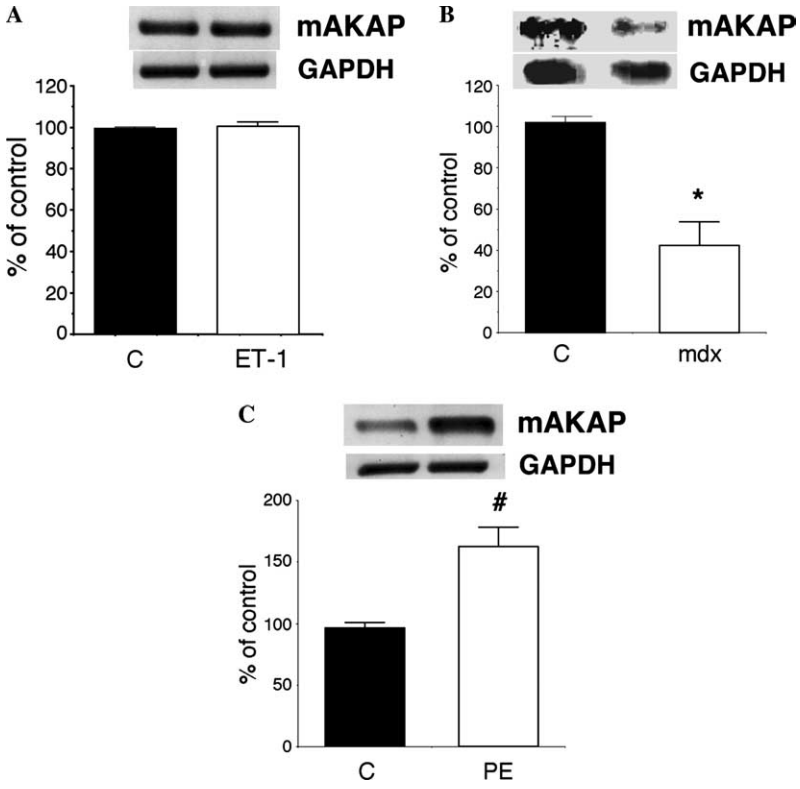

Fig. 2. Regulation of mAKAP expression. Northern blot analysis of mAKAP in ET-1 or PE treated cardiomyocytes and 16-month-old *mdx* hearts. Endothelin-1 or phenylephrine was added for 1 day after overnight incubation in a serum free medium of day 2 cultured cardiomyocytes as described in Materials and methods. (A) There is no change in mAKAP expression of endothelin-1 (ET-1) induced hypertrophy. (B) mAKAP is significantly lower in 16-month-old *mdx* as compared to the control littermate. (C) Phenylephrine (PE) treatment resulted in an increase of mAKAP in cardiomyocytes as compared to the control. Upper panel: representative Northern blots; lower panel: normalized mRNA level (control = 100%). mRNA results are presented as ratios of mAKAP mRNA to GAPDH mRNA. Data are presented as means ± SE from three independent samples. $^{*}p < 0.001$ vs. control, $^{\#}p < 0.005$ vs. control.

cardiomyocytes. Furthermore, in the hearts of 16-month-old *mdx* mice, which are known to have abnormal Ca^{2+} homeostasis, mAKAP mRNA was significantly decreased as compared to the control although there was no observed hypertrophic phenotype (Fig. 2B). Next, we treated cultured cardiomyocytes with phenylephrine to induce hypertrophy and increase cAMP level. Phenylephrine treated cardiomyocytes resulted in up regulation of mAKAP mRNA as compared to the control (Fig. 2C). These results suggested that mAKAP was decreased in *mdx* hearts and increased by phenylephrine.

Decreased mAKAP coincides with down regulation of RyR2 and SERCA2a in 5-month-old mdx hearts

The intracellular Ca^{2+} homeostasis disturbance found in *mdx* led us to hypothesize that this condition affects the gene expression of SR Ca^{2+} handling proteins. We therefore examined the expression of SR proteins in 5-month-old *mdx* hearts. First, we checked the expression of RyR2 and FKBP 12.6, the important molecules for SR Ca^{2+} release. The RyR2 mRNA level was reduced to 52% in *mdx* as compared to the control littermate (Fig. 3A). We detected no difference in FKBP12.6 expression of the 5-month-old *mdx* and control hearts (Fig. 3B). Subsequently, we examined the gene expression of the SR Ca^{2+} pump proteins, SERCA2a, and phospholamban. SERCA2a expression exhibited a 75% reduction in the 5-month-old *mdx* but no difference in the expression of phospholamban (Figs. 3C and D). Furthermore, we investigated whether decreased mAKAP mRNA in *mdx* affects the cAMP equilibrium system by examination of the PDE4D3 mRNA level. There was no significant change in the gene expression of PDE4D3 for both *mdx* and control hearts (Fig. 3E).

Discussion

The regulation of mAKAP gene expression decreased in mdx hearts

In the present study, we demonstrate that the gene expression of mAKAP, a muscle specific A-kinase anchoring protein localized at the sarcoplasmic reticulum (SR), is significantly lower in the 5-month-old *mdx*

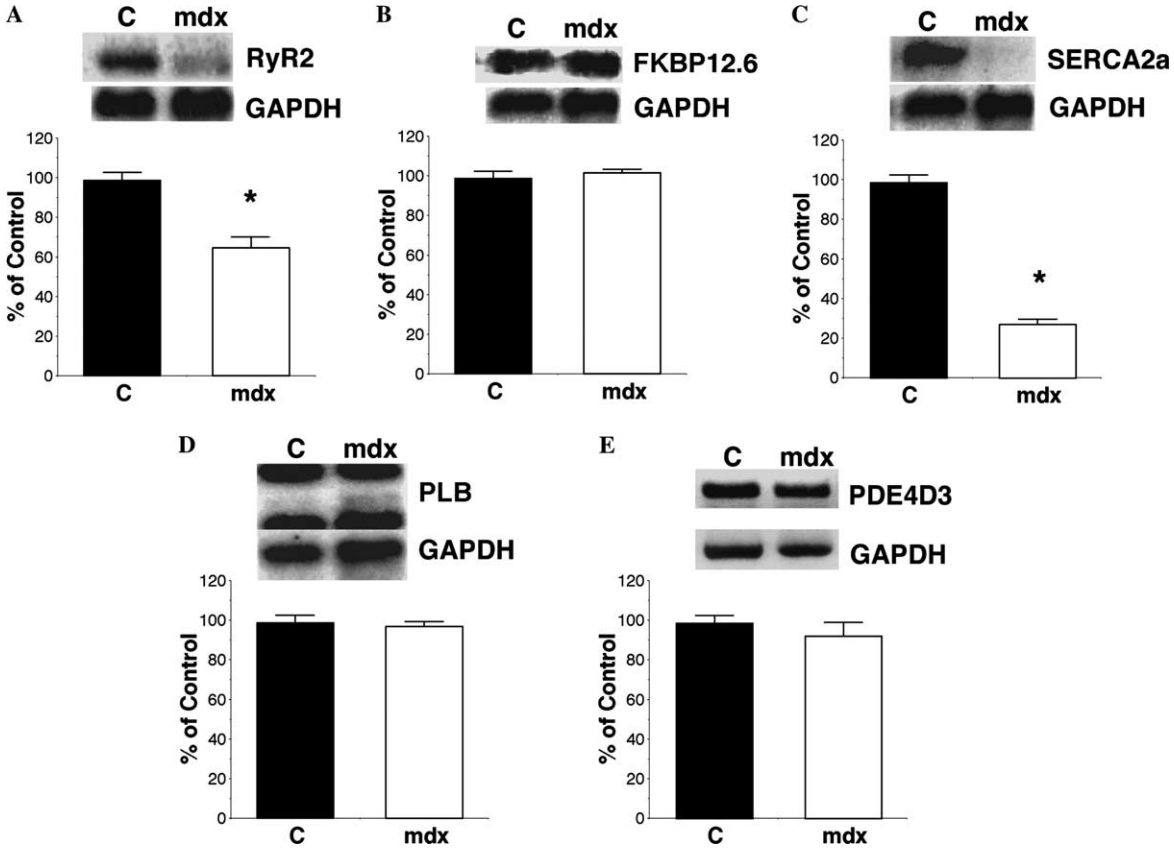

Fig. 3. Northern blot analysis of RyR2, FKBP12.6, SERCA2a, phospholamban, and PDE4D3 in *mdx* and control mice. RyR2 (A) and SERCA2a (C) expressions are significantly decreased in the 5-month-old *mdx* heart as compared to the littermate control. FKBP12.6 (B) and phospholamban (D) levels are unchanged in *mdx*. PDE4D3 (E) expression is comparable between the *mdx* and the control. Mouse phospholamban mRNAs were represented by two major form of transcripts approximately 2.8 kb and approximately 0.7 kb. Upper panel: representative Northern blots; lower panel: quantitative analysis of sarcoplasmic reticulum (SR) Ca^{2+} handling and PDE4D3 mRNA levels in the control and *mdx* hearts. The signals are normalized to the mRNA level of the respective controls (F), $*p < 0.001$ vs. control.

heart. We noticed not only a decrease in mAKAP mRNA but also in its binding protein, RyR2. RyR2 is a Ca^{2+} release channel removing Ca^{2+} from the SR to the cytosol leading to cardiac muscle contraction. Decreased gene expression of the cardiac Ca^{2+} release channel accompanies down-regulation of the cardiac SR Ca^{2+} pump, SERCA2a, which removes cytosolic Ca^{2+} into the SR leading to cardiac muscle relaxation.

Mageney et al. [15] reported that at 5 months of age, the *mdx* heart was significantly heavier than the wild type littermate the hypertrophic phenotype of which was confirmed by histological studies. Another study showed that mouse hearts lacking dystrophin appeared to have hypertrophied by 6 months of age and this was reflected by the decreasing S/R wave ratio in the *mdx* mouse ECG [16]. We therefore checked the mAKAP mRNA level in hypertrophy-induced cardiomyocytes. Our results suggested that the decrease in mAKAP in *mdx* hearts is independent of cardiac hypertrophy. On the contrary, the regulation of mAKAP gene expression may be associated with intracellular Ca^{2+} and cAMP levels. ET-1 treated cardiomyocytes did not alter the mAKAP mRNA level even if we observed the hypertrophic feature whereas the 16-month-old *mdx* heart, with altered intracellular Ca^{2+} homeostasis, showed a decrease in mAKAP mRNA and did not have a hypertrophic phenotype. These results suggest that the intracellular Ca^{2+} level in *mdx* hearts may affect decreased mAKAP gene expression. However, the molecular mechanism of decreased mAKAP mRNA in *mdx* mice remains to be elucidated. When we treated cardiomyocytes with a cAMP inducible agent, phenylephrine, mAKAP mRNA was increased significantly as compared to the control. Accordingly, we sought the possible mechanism of the different effects of ET-1 and phenylephrine on mAKAP expression. One possible mechanism of increased mAKAP expression, like other AKAPs, is through cAMP signaling [17]. A previous finding suggested that ET-1 treatment did not exhibit any effect on the cAMP level in ventricular cardiomyocytes [18] whereas phenylephrine-induced cardiomyocytes resulted in increased cAMP intracellularly and in up regulation of mAKAP expression [19]. Sequence analysis of the human mAKAP 5′ flanking region (GenBank Accession No. NT_026437) revealed an existence of tgatgtca sequences at bp −137 to −130 (relative to the atg start site) similar to the cAMP-responsive element binding protein (CREB) consensus sequences (TGACG TMA). Therefore, cAMP-induced transcription of mAKAP may be directly or indirectly through the CREB binding site of the mAKAP promoter region.

Decreased mAKAP in mdx hearts may affect PKA-dependent RyR2 phosphorylation

Northern blot analysis suggested that mAKAP was expressed in differentiated cardiomyocytes, these results were compatible with the previous studies showing mAKAP expression in multiple subcellular compartments of differentiated cardiomyocytes [11,20], suggesting that this protein physiologically functions in multiple subcellular compartments after complete differentiation of cardiomyocytes. mAKAP is a member of the AKAP family which provides a molecular framework that orients cAMP-dependent kinase (PKA) and phosphatases through their interaction with the specific targeted substrates [21,22]. The previous studies have shown that mAKAP binds to RyR2 and to the Na^+/Ca^{2+} exchanger, NCX1, the important proteins for Ca^{2+} homeostasis of cardiomyocytes [14,23,24]. Northern blot analysis of RyR2 revealed that RyR2 gene expression is also developmentally regulated (data not shown), arguing that mAKAP and RyR2 not only bind each other but are also similarly regulated by cardiomyocyte differentiation. Accordingly, mAKAP function may be related to the cAMP-regulated intracellular Ca^{2+} in differentiated cardiomyocytes.

Adrenergic agonists or stress-induced activation of the sympathetic nervous system stimulate cAMP production and activate cAMP-dependent protein kinase (PKA)-mediated phosphorylation of RyR2 leading to the channel activation [25,26]. PKA-mediated phosphorylation of RyR is regulated by mAKAP which resulted in efflux of Ca^{2+} through the RyR. Conversely, a loss of function of mAKAP significantly reduced PKA-dependent RyR phosphorylation [27]. Therefore, decreased mAKAP in the *mdx* heart may disturb adrenergic agonists or stress-induced activation of PKA-dependent RyR2 phosphorylation and Ca^{2+} release from the SR. However, the functional consequences of decreased mAKAP mRNA in *mdx* hearts, whether it possesses beneficial or deleterious effects on the cardiac function, remain to be determined.

Decreased RyR2 and SERCA2a may affect cardiac contractility in the mdx heart

The present study, for the first time, demonstrates that down regulation of mAKAP coincides with the ryanodine receptor (RyR2) and SERCA2a in the 5-month-old *mdx* heart. The previous studies have suggested that reduced RyR2 mRNA level may result in reduced RyR2 protein level [28,29]. In *mdx* hearts, RyR2 function may be disturbed not only at the level of transcription as shown by decreased RyR2 mRNA but also at the level of phosphorylation. mAKAP, which is decreased in *mdx*, is necessary for PKA induced RyR phosphorylation [27]. Defective RyR2 phosphorylation is already known to result in a defect of Ca^{2+} release whereas a defect in Ca^{2+} release could impair contractility or contribute to diastolic depolarizations [30]. Moreover, another body of evidence suggested that the filling state of the SR, which also determined the force of

contraction, is triggered mainly by Ca^{2+} release from the SR [31]. In the 5-month-old *mdx* heart, there was a significant down regulation of SERCA2a mRNA. The decrease of SERCA2a mRNA level was more apparent than RyR2 reduction. Other studies revealed that the decrease in the SERCA2a mRNA level is accompanied by a decrease in SERCA2a protein and SR Ca^{2+} ATPase activity [32–34], whereas Ca^{2+} ATPase activity in the SR contributes to about 70–80% of the Ca^{2+} flux during diastole and a defect in Ca^{2+} removal could also impair relaxation [30,31]. Accordingly, down regulation of RyR2 and SERCA2a mRNA in *mdx* hearts may result in altered relaxation, force of contraction, and Ca^{2+} pump function observed in the *mdx* myotube and heart [35,36] as also suggested by other studies showing that the twitch half-relaxation time was greatly prolonged in *mdx* atria together with a prolongation of the QT interval in *mdx* mice [37,38]. Intracellular Ca^{2+} overload may be a possible mechanism mediating the decrease in SR gene expression as shown by previous reports [39,40]. However, the exact mechanisms of decreased SR gene expression in *mdx* remain to be elucidated. Our observation suggested that FKBP12.6, a regulatory subunit that stabilizes RyR2 channel function and phospholamban, the integral membrane protein that interacts with and reversibly inhibits the activity of SERCA2a, were unchanged. We also did not observe a change in PDE3D4, a cAMP-specific phosphodiesterase, suggesting that the gene expression of the cAMP-modulator molecule at the perinuclear membrane was not affected in *mdx* mice.

Taken together, dystrophin deficiency may affect the cardiac contractile function of the *mdx* heart through effects on SR function, in part, by down regulation of SR molecules at the transcriptional level.

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