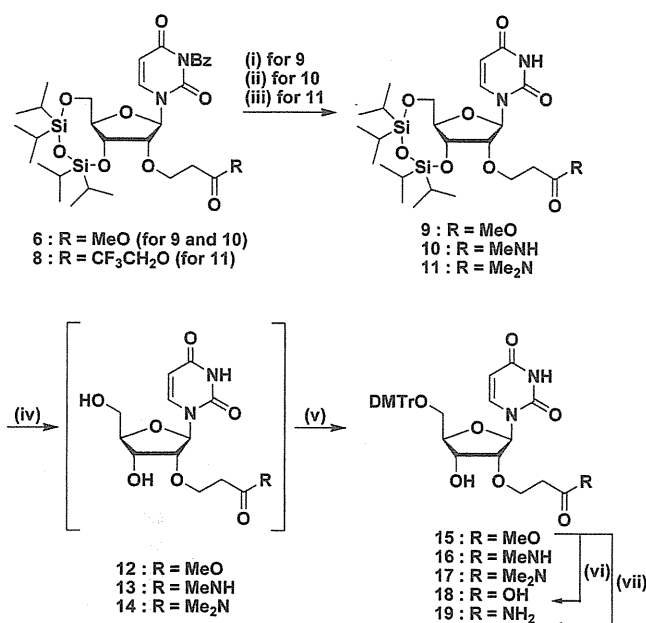


Table 1. Chemical Properties of Compound 15–17

conditions <sup>a</sup>	compd 15	compd 16	compd 17
1 M NaOH	hydrolysis within 5 min	stable for 24 h	hydrolysis in 18 h
28% NH <sub>4</sub> OH	amidation within 5 min	stable for 24 h	stable for 24 h
1 M TBAF/THF	elimination within 5 min	stable for 24 h	elimination <sup>b</sup>

<sup>a</sup> All reactions were done at room temperature. <sup>b</sup>  $t_{1/2}$  = 2 h. The reaction was completed at 40 °C for 2 h.

Scheme 2. Preparation of Compound 15–19<sup>a</sup>

<sup>a</sup> Conditions: (i) *n*-PrNH<sub>2</sub>, THF, rt, 4 h, yield: 9 (71%); (ii) 40% MeNH<sub>2</sub> in MeOH, rt, 4 h, yield: 10 (66%); (iii) 2 M Me<sub>2</sub>NH in THF, rt, 2 h, yield: 11 (81%); (iv) Et<sub>3</sub>N·3HF, THF, rt; (v) DMTrCl, pyridine, rt, yield: 15 (90%, 2 steps), 16 (51%, 2 steps), 17 (70%, 2 steps); (vi) 0.1 M NaOHaq, rt, 30 min, yield: 18 (65%); (vii) 28% NH<sub>4</sub>OH, rt, 30 min, yield: 19 (99%).

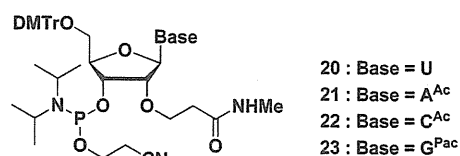
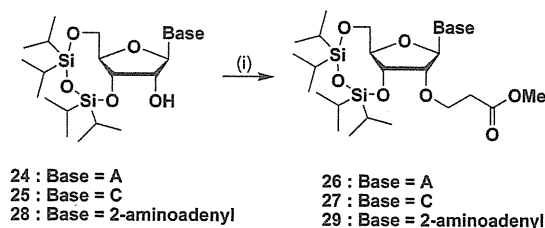
with 1 M TBAF in THF. In our previous study, we reported that the cyanoethyl group was removed from 2'-*O*-cyanoethyluridine by using this reagent.<sup>18</sup>

The monoalkyl amide 16 was very stable under all tested conditions. In addition, it was also stable under the conditions of 28% NH<sub>4</sub>OH at 55 °C for 5 h, which are the conditions for the oligonucleotide synthesis in this study. The amide proton of the MCE group was supposed to be dissociated faster than the  $\alpha$ -proton under basic conditions, so that the dissociated species suppressed further deprotonation at the  $\alpha$ -position.

The *N,N*-dialkyl amide 17 demonstrated the most unique properties. The DMCE group was stable under ammonia treatment but could be removed by treatment with 1 M TBAF.

Table 1 shows that the MCE group is the most stable among the three modified groups. Therefore, it is tolerable in the standard RNA synthesis. This result led us to synthesize the 2'-*O*-MCE-ribonucleoside 3'-phosphoramidite derivatives 20–23, as shown in Figure 2.

**Synthesis of 2'-*O*-MCE-Ribonucleoside 3'-*O*-Phosphoramidite Derivatives 20–23.** For the synthesis of 2'-*O*-MCE-ribonucleoside 3'-phosphoramidite derivatives, we applied the Michael addition to the *N*-protected cytidine, adenosine,

Figure 2. The structures of 2'-*O*-MCE-RNA-phosphoramidites 20–23.Scheme 3. Selective Oxa-Michael Reactions to Compounds 26, 27, and 29<sup>a</sup>

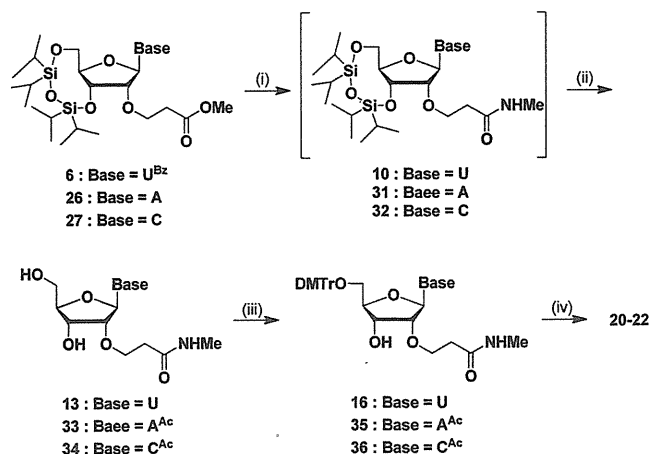
<sup>a</sup> Conditions: (i) Cs<sub>2</sub>CO<sub>3</sub>, methyl acrylate, *t*-BuOH, rt, 6–18 h, yield: 26 (72%), 27 (75%), 29 (79%).

and guanosine derivatives such as 6-*N*-benzoyl-3',5'-(1,1,3,3-tetraisopropylidisilane-1,3-diyl)adenosine,<sup>20</sup> 4-*N*-benzoyl-3',5'-(1,1,3,3-tetraisopropylidisilane-1,3-diyl)cytidine,<sup>20</sup> and 2-*N*-phenoxyacetyl-3',5'-(1,1,3,3-tetraisopropylidisilane-1,3-diyl)guanosine.<sup>21</sup> However, these reactions gave complex mixtures in all cases. These results indicated that there were side reactions on the *N*-protected base moieties that had amido protons.

Several research groups have reported the selective alkylation of the 2'-hydroxyl group of ribonucleosides, such as adenosine and 2-aminoadenosine, with alkylating reagents under basic conditions.<sup>22</sup> On the basis of these results, we studied whether a Michael reaction would be available in the presence of *N*-free nucleobases.

The results are shown in Scheme 3. We found that the *N*-unmasked adenosine derivative 24 underwent smooth regioselective Michael additions with methyl acrylate to give the desired 2'-*O*-MOCE-adenosine derivative 26 in 72% yield. Similar conditions were also tested on *N*-unmasked cytidine derivative 25. Alkylation on the nucleobase moiety was observed as time passed at room temperature, but Michael addition to the 2'-hydroxyl group was much faster than the side reaction so that the desired 2'-*O*-MOCE-cytidine derivative 27 was selectively obtained in 75% yield.

The reaction of 3',5'-*O*-TIPDS-guanosine<sup>20</sup> was also tested under Cs<sub>2</sub>CO<sub>3</sub> conditions, but the reaction resulted in formation of a complex mixture. The fully base-protected species, i.e., 6-*O*-diphenylcarbonyl-2-*N*-dimethylaminomethylene-3',5'-*O*-TIPDS-guanosine,<sup>23</sup> was also examined, but the rate of Michael reaction was very slow. The dimethylaminomethylene (dmf) and diphenylcarbonyl (dpc) group were gradually degraded so that the reaction

Scheme 4. Synthesis of Phosphoramidites 20–22<sup>a</sup>

<sup>a</sup> Conditions: (i) 40% MeNH<sub>2</sub> in MeOH, EtOH, rt; (ii) AcCl, pyridine, rt (for 31 and 32); (c) Et<sub>3</sub>N·3HF, Et<sub>3</sub>N, THF, rt, yield: 13 (73%, 2 steps), 33 (60%, 3 steps), 34 (53%, 3 steps); (iii) DMTrCl, pyridine, rt, yield: 35 (73%) and 36 (52%); (iv) 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphordiamidite, diisopropylammonium 1*H*-tetrazolide, MeCN, rt, yield: 20 (60%), 21 (76%), 22 (48%).

gave a complex mixture. Finally, based on the successful results using the *N*-free adenosine and cytidine derivatives 24 and 25, we chose 3',5'-*O*-TIPDS-2-aminoadenosine 28<sup>24</sup> as a precursor for the synthesis of 2'-*O*-MCE-guanosine derivative 30 (see Scheme 5) since the 2-aminoadenine moiety can be converted to the guanosine one.<sup>24</sup> Consequently, it was found the reaction of *N*-unmasked 2-aminoadenosine derivative 28 with methyl acrylate in *t*-BuOH gave the desired 2'-*O*-MOCE-2-aminoadenosine product 29 in 78% yield without base modification.

The imino proton of uridine ( $pK_a = 9.25$ )<sup>25</sup> is apparently more acidic than the amino protons of adenosine or cytidine (probably more than that of aniline ( $pK_a = 30.6$ )<sup>26</sup>) and the 2'-OH proton ( $pK_a = 12.14$ – $12.86$ ).<sup>27</sup> Therefore, it was considered that the imino proton of uridine would be easily dissociated by cesium carbonate as a base so that, as compared to the nondissociated species, the dissociated one became more reactive toward the Michael acceptors. In contrast to the dissociation property of the uracil base, the amino protons of adenosine and cytidine could not be dissociated by CsCO<sub>3</sub>. Therefore, it was expected that this property was dependent on the inherent nucleophilicity of the nucleobases when a Michael reaction competitively occurs on the unprotected nucleobase sites toward a CsCO<sub>3</sub>-mediated dissociated 2'-hydroxyl group.

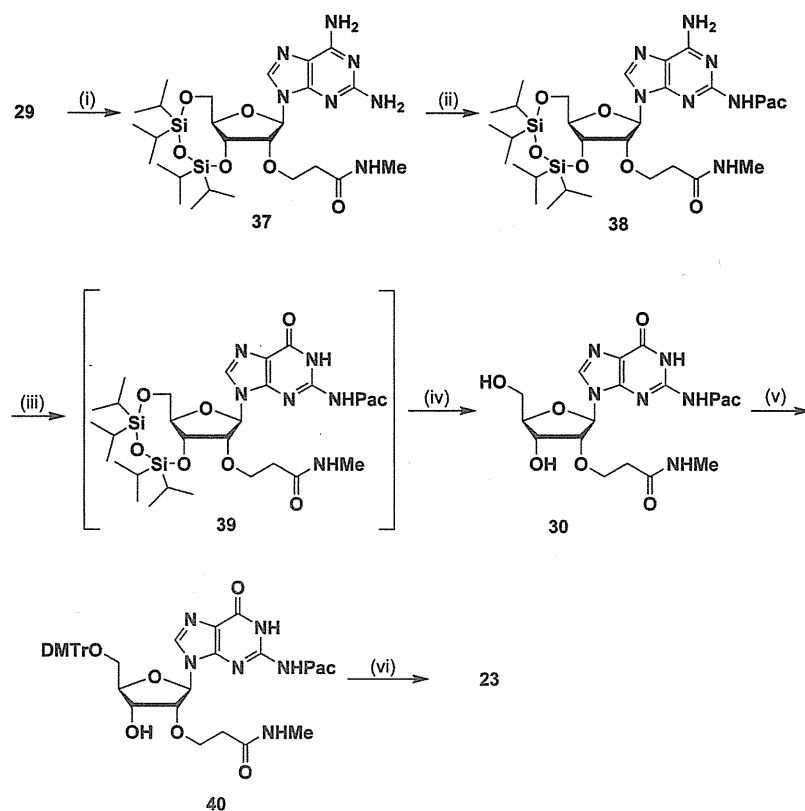
Scheme 4 shows the synthesis of phosphoramidite compounds 20–22. We converted the 2'-*O*-MOCE derivatives 6, 26, and 27 to the *N*-free 2'-*O*-MCE-uridine derivative 10, adenosine derivative 31, and cytidine derivative 32 via treatment with methylamine. Under these conditions, the *N*<sup>3</sup>-benzoyl group of the uridine derivative 6 was simultaneously removed to give the 2'-*O*-MCE disilylated derivative 10, which in turn was allowed to react with Et<sub>3</sub>N·3HF in THF to give 2'-*O*-MCE-uridine (13) in 73% yield. In the case of cytidine derivative 27, a trace amount of the  $\beta$ -elimination product 25 was formed by methylamine treatment. The *N*-free intermediates 31 and 32 thus obtained from 26 and 27 were treated in situ with acetyl chloride in pyridine followed by Et<sub>3</sub>N·3HF in THF to give the 2'-*O*-MCE *N*-acetyl adenosine (33) and cytidine (34) in 51% and 53% yields, respectively. 2'-*O*-MCE *N*-acetyl derivatives

33 and cytidine derivative 34 were treated with DMTrCl to give the 5'-*O*-protected adenosine derivative 35 and cytidine derivative 36 in 73% and 52% yields, respectively. The 3'-phosphitylation of 5'-*O*-DMTr derivatives 16, 35, and 36 with 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphordiamidite<sup>28</sup> in the presence of diisopropylammonium 1*H*-tetrazolide gave the phosphoramidite uridine derivatives 20, adenosine derivative 21, and cytidine derivative 22 in 66%, 76%, and 48% yields, respectively.

For the guanosine 3'-phosphoramidite building block 23, we succeeded in synthesizing this compound via a six-step reaction from the 2-aminoadenosine derivative 29, as shown in Scheme 5. The treatment of 2'-*O*-MOCE derivative 29 with methylamine yielded the 2'-*O*-MCE derivative compound 37 in good yield (77%). In this reaction, the selective conversion of 29 to 37 occurred without the  $\beta$ -elimination of the MCE group. We attempted the selective acylation of the 2-amino group of compound 37 by using isobutyryl chloride and phenoxyacetyl chloride. The latter gave a better result, and the 2-*N*-phenoxyacetyl derivative 38 could be easily obtained by reprecipitation. The deamination of compound 38 with NaNO<sub>2</sub> in the presence of AcOH gave the desired guanosine derivative 39. Since we observed the partial elimination of the TIPDS group during this reaction, we treated the resulting product 39 with Et<sub>3</sub>N·3HF in THF in situ to give the 3',5'-unprotected product 30 in an overall yield of 71%. The usual dimethoxytritylation of compound 30 followed by the 3'-phosphitylation of the resulting 5'-protected compound 40 gave the guanosine 3'-*O*-phosphoramidite derivative 23 in a good yield (80%).

**Synthesis of 2'-*O*-MCE/2'-*O*-Me-Chimeric Oligonucleotides.** 2'-*O*-MCE-oligonucleotides and chimeric oligonucleotides partially modified with 2'-*O*-MCE-ribonucleosides were synthesized by using the ribonucleoside 3'-phosphoramidite building blocks 20–23, according to the standard phosphoramidite<sup>40,41</sup> procedure. We carried out the synthesis of these modified oligonucleotide using an ABI-392 DNA/RNA synthesizer and CPG resins. Either 5-benzylthio-1*H*-tetrazole (BTT)<sup>29</sup> or 5-[bis(3,5-trifluoromethyl)phenyl]-1*H*-tetrazole<sup>30</sup> was used as an activator. We used BTT for the synthesis of ORNs 1–5 (Table 2). BTT was also used for the synthesis of ORN 6; however, the coupling reaction with compound 23 did not proceed well. Compound 23 was less reactive than the other phosphoramidites 20–22. Therefore, we tested several activators to obtain an optimized coupling reaction using compound 23 at room temperature for 10 min. When 1*H*-tetrazole or BTT was used as the activator, the coupling efficiency was lower than 10%. On the other hand, the use of 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole gave a much better result with more than 90% coupling efficiency, and thereby, 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole was the best choice for our reagent. The release of oligonucleotides from the resin and simultaneous deprotection of the protecting groups were carried out by using 28% NH<sub>4</sub>OH at 55 °C for 5 h. In all cases, we obtained the desired products as the main peaks. The side products derived from  $\beta$ -elimination and transamidation were not observed. 3-[(Dimethylaminomethylidene)amino]-3*H*-1,2,4-dithiazole-5-thione (DDTT)<sup>31</sup> was used as a sulfurization agent for the synthesis of ORNs 10 and 12. We characterized the synthesized oligonucleotides by MALDI-TOF.

**Hybridization Properties of 2'-*O*-MCE-Oligonucleotides.** We measured the  $T_m$  values of the duplexes of 2'-*O*-MCE-oligonucleotides with the cDNA and RNA strands in 10 mM sodium phosphate buffer (pH 7.0) in the presence of 100 mM

Scheme 5. Synthesis of Phosphoramidite 23<sup>a</sup>

<sup>a</sup> Conditions: (i) MeNH<sub>2</sub> in MeOH, EtOH, rt, yield: 37 (77%); (ii) phenoxyacetyl chloride, pyridine, -10 °C, yield: 38 (73%); (iii) NaNO<sub>2</sub>, AcOH, H<sub>2</sub>O, rt; (iv) Et<sub>3</sub>N·3HF, Et<sub>3</sub>N, THF, rt, yield: 30 (71%); (v) DMTrCl, pyridine, rt, yield: 40 (75%); (vi) 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite, diisopropylammonium 1*H*-tetrazolide, MeCN, rt, yield: 23 (80%).

Table 2. Synthesized Oligonucleotides Used in This Study and Mass Data

entry	sequence <sup>a</sup>	backbone <sup>b</sup>	calcd mass	obsd mass
ORN 1	5'-UUU UUU UUU UUU-3'	PO	4630.0	4628.8
ORN 2	5'-(2'-O-CE)-UUU UUU UUU UUU-3'	PO	4236.4	4238.4
ORN 3	5'-(2'-O-Me)-UUU UUU UUU UUU-3'	PO	3779.5	3779.5
ORN 4	5'-AAA AAA AAA AAA-3'	PO	4908.8	4908.3
ORN 5	5'-(2'-O-Me)-CGU AGA CUA UCU C-3'	PO	4241.7	4240.3
ORN 6	5'-(2'-O-Me)-CGU AGA CUA UCU C-3'	PO	4315.8	4314.8
ORN 7	5'-(2'-O-Me)-CGU AGA CUA UCU C-3'	PO	4315.8	4315.2
ORN 8	5'-(2'-O-Me)-CGU AGA CUA UCU C-3'	PO	4315.8	4315.3
ORN 9	5'-(2'-O-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3'	PO	10019.8	10025.9
ORN 10	5'-(2'-O-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3'	PS	10483.2	10492.2
ORN 11	5'-(2'-O-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3'	PO	10446.0	10453.6
ORN 12	5'-(2'-O-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3'	PS	10909.4	10915.1

<sup>a</sup> B: 2'-O-MCE-RNA. <sup>b</sup> PO, PS means phosphate backbone, and phosphorothioate backbone, respectively.

NaCl and 0.1 mM EDTA. These results are summarized in Table 3. The data were compared with those of natural RNAs and 2'-O-methyl RNAs. When 2'-O-MCE-U was incorporated into all U's of U<sub>12</sub> (ORN 1, entry 2), the *T<sub>m</sub>* value of the duplex of 2'-O-MCE-U<sub>12</sub> with the complementary RNA strand was 12 °C higher than that of the duplex derived from natural RNA (entry 1). The  $\Delta T_m$  value was 1 °C per modification. The degree of this *T<sub>m</sub>* increase was the same as that of 2'-O-Me-U<sub>12</sub>.<sup>17</sup> As opposed to this result, when we incorporated 2'-O-MCE-A into all A's of

A<sub>12</sub> (entry 4), we did not observe such a significant *T<sub>m</sub>* increase. Next, the effect of one point 2'-O-MCE-ribonucleoside modification on the thermostabilities of the duplexes was examined. We incorporated 2'-O-MCE-G, A, and C into the fifth G (ORN 6, entry 6), sixth A (ORN 7, entry 7), and seventh C (ORN 8, entry 8), respectively, of 5'-(2'-O-Me)-CGUAGACUAUCUC-3' (ORN 5, entry 5). The differences in *T<sub>m</sub>* between the modified and unmodified duplexes were -2, +1, and 0 °C for the complementary RNA strand, respectively. Meanwhile, the  $\Delta T_m$

Table 3. Hybridization Affinity of 2'-O-MCE-Oligonucleotides

entry	sequence	$T_m$ (°C) vs RNA <sup>a</sup>	$T_m$ (°C) vs DNA <sup>a</sup>
1	5'-UUU UUU UUU UUU-3'	14	9
2	5'-UUU UUU UUU UUU-3' (ORN 1)	26	n.d.
3	5'-AAA AAA AAA AAA-3'	14	n.d.
4	5'-AAA AAA AAA AAA-3' (ORN 4)	15	n.d.
5	5'-(2'-O-Me)-CGU AGA CUA UCU C-3' (ORN 5)	67	49
6	5'-(2'-O-Me)-CGU AGA CUA UCU C-3' (ORN 6)	65	45
7	5'-(2'-O-Me)-CGU AGA CUA UCU C-3' (ORN 7)	68	48
8	5'-(2'-O-Me)-CGU AGA CUA UCU C-3' (ORN 8)	67	48
9	5'-(2'-O-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3' (PO backbone, ORN 9)	85	74
10	5'-(2'-O-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3' (PS backbone, ORN 10)	81	69
11	5'-(2'-O-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3' (PO backbone, ORN 11)	85	71
12	5'-(2'-O-Me)-CUC CAA CAG CAA AGA AGA UGG CAU UUC UAG-3' (PS backbone, ORN 12)	81	73

<sup>a</sup>  $T_m$  values were measured in 2  $\mu$ M oligonucleotide, 10 mM sodium phosphate buffer (pH 7.0), 100 mM NaCl, and 0.1 mM EDTA.

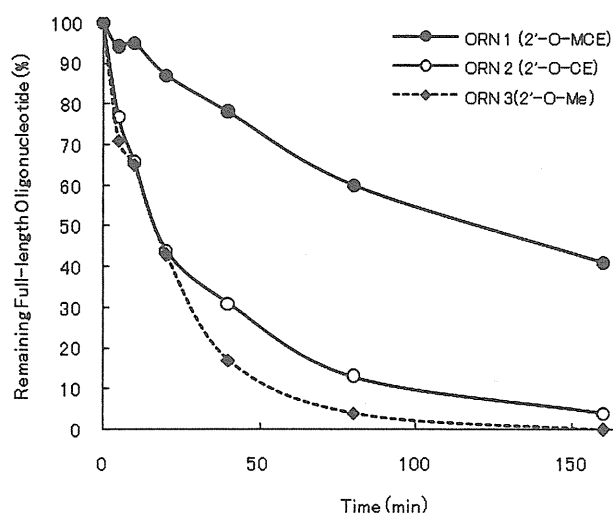


Figure 3. Time courses of degradation of 2'-O-MCE, 2'-O-CE, and 2'-O-methyl oligonucleotides by snake venom phosphodiesterase.

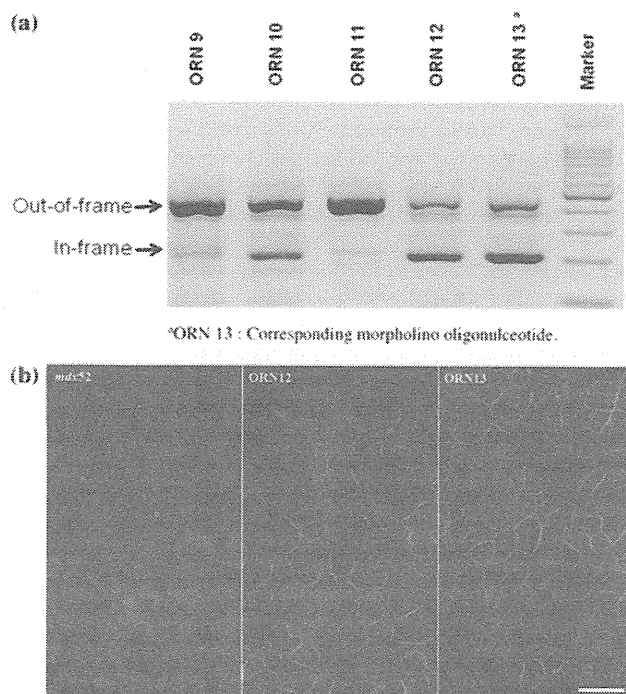
values of the same 2'-O-MCE oligonucleotides for the cDNA strand were  $-4$ ,  $-1$ , and  $-1$  °C, respectively. The 2'-O-MCE oligonucleotides exhibited stronger binding affinities for RNAs than DNAs, as shown in Table 3 similar to the 2'-O-methyl RNAs. Six-point modification of ORNs 9 and 10 with 2'-O-MCE-U showed these modified ORNs 11 and 12 could maintain the original binding affinity for the complementary RNA and DNA strands, as shown in entries 9–12. This property should be suitable for oligonucleotide therapeutics targeting RNAs.

**Nuclease Resistance of 2'-O-MCE RNA.** We examined the 3'-exonuclease resistance of 2'-O-MCE oligonucleotides using snake venom phosphodiesterase (SVPD).<sup>32</sup> The 2'-O-MCE-oligonucleotide ORN 1 was subjected to the digestion reaction using SVPD. The time course of the remaining full-length ORN 1 was measured by RP-HPLC, as shown in Figure 3. The 3'-exonuclease resistance properties of 2'-O-CE oligonucleotide (ORN 2) and 2'-O-Me oligonucleotide (ORN 3) were also studied to compare with that of ORN 1. As a result, the 3'-exonuclease resistance of ORN 1 was much higher than those of ORN 2 and ORN 3. Although  $t_{1/2}$  of ORN 1 was about 120 min, it was six times larger than those of ORN 2 and ORN 3.

**Exon-Skipping Activity of 2'-O-MCE RNAs in the Splicing of the Pre-mRNA of Mouse Dystrophin.** Because the  $T_m$

analysis and nuclease resistance assay of 2'-O-MCE oligonucleotides described above demonstrated their potential availability for oligonucleotide therapy targeting specific RNAs, we examined the biological activities of the exon skipping of pre-mRNA of mouse dystrophin.<sup>33</sup> Exon skipping is currently the most promising approach to the oligonucleotide therapy of Duchenne muscular dystrophy.<sup>34</sup> Because antisense oligonucleotides for exon skipping must be delivered to the cellular nucleus in order to modulate the splicing of pre-mRNA, they must possess sufficient stabilities against nucleases in blood and cytoplasm. Once antisense oligonucleotides are delivered into the nucleus successfully, they must hybridize with the targeting pre-mRNA selectively. It was reported that phosphorothioate (PS) oligonucleotides with nuclease resistance<sup>35</sup> were easily delivered into the nucleus once they permeated the cells.<sup>36</sup> We considered that 2'-O-MCE-oligonucleotides with phosphorothioate backbones would improve the efficacy of exon skipping by adding further nuclease resistance to the phosphorothioate 2'-O-methyl-oligonucleotides and improve the binding toward the complementary RNA strands. Recently, 2'-O-methyl-PS-oligonucleotides and morpholino oligonucleotides have been proven to be effective for exon skipping, as exemplified by successful *mdx* mice *in vivo* experiments. Therefore, we synthesized a 2'-O-MCE-PS-oligonucleotide of ORN 12 that corresponded to a 30 base sequence (mB30) reported by Arechevala-Gomez et al.<sup>37</sup> The mB30 sequence was reported to be effective for exon skipping when incorporated into not only 2'-O-methyloligonucleotides with phosphorothioate linkages but also morpholino oligonucleotides. We incorporated 2'-O-MCE-U into the U residues of 2'-O-methyl-phosphorothioate ORN 10. There are a total of 6 uridine residues in mB30, and they are mainly localized at the 3'-end of the strand. To evaluate the influence of the phosphorothioate backbone, we also synthesized phosphate derivatives ORNs 9 and 11. We purchased the antisense morpholino ORN 13 from Gene Tools.

ORNs 9–13 were administered to the exon 52-deleted *mdx* mouse (*mdx52*) by intramuscular injection.<sup>38</sup> We evaluated the efficacy of exon skipping by the RT-PCR of the total RNAs as described previously.<sup>39</sup> Figure 4 displays the results. The experiments were performed two times. As shown in part a, six-point substitution of 2'-O-MCE-U (ORN 12, exon-skipping efficacy = 62%) dramatically enhanced the exon-skipping efficacy compared with that of the 2'-O-methyl-PS oligonucleotides (ORN 10, exon-skipping efficacy = 34%). Its efficacy was almost the



**Figure 4.** (a) One canonical example of the detection of antisense-mediated exon-skipping in *mdx52* mice by reverse transcriptase polymerase chain reaction (RT-PCR). (b) Immunohistochemical staining of dystrophin in TA muscle of untreated and treated *mdx52* mice (ORN 12 and ORN 13). There was no expression of dystrophin around the rim of any muscle fibers from untreated *mdx52* mice. In contrast, restoration of dystrophin around the rim of muscle fibers was observed in both muscles from treated *mdx52* by ORN12 and ORN 13. Dystrophin was detected with with dystrophin C-terminal antibody (*Dys-2*). Bar = 100 μm.

same as that of the corresponding morpholino nucleic acid (ORN 13, exon-skipping efficacy = 65%). We also evaluated the efficacy of ORN 12 by immunohistochemistry with dystrophin C-terminal antibody (*Dys-2*) as shown in part b. The number of dystrophin positive fibers was almost the same as that which resulted from ORN 13. These results indicated that the incorporation of 2'-O-MCE-Us into antisense RNA was effective for exon skipping.

## CONCLUSIONS

In conclusion, we have developed an effective method for the synthesis of 2'-O-alkoxycarbonyl-ribonucleosides via Michael addition by using acrylate esters. We easily converted the MOCE group to the chemically stable MCE group by aminolysis. The amide group of the MCE group was not detrimental to the hybridization and enhanced metabolic stability. The phosphorothioate derivative of a 2'-O-Me-oligonucleotide incorporating six 2'-O-MCE-Us with a base sequence of mB30 proved to exhibit effective exon skipping in the splicing of the pre-mRNA of mouse dystrophin.

## EXPERIMENTAL SECTION

**Materials and Methods.** All reagents and solvents obtained from commercial suppliers were used without further purification. All reactions were carried out under argon atmosphere in oven-dried glassware. NMR chemical shifts are given in parts per million (ppm); *J* values are given in hertz (Hz). <sup>1</sup>H and <sup>13</sup>C spectra were internally referenced to the

appropriate residual undeuterated solvent. <sup>31</sup>P NMR was externally referenced with 85% H<sub>3</sub>PO<sub>4</sub>. Column chromatography was performed with silica gel C-200. Reversed phase HPLC on a C18 column was performed using a linear gradient of acetonitrile in 0.1 M ammonium acetate buffer. Anion-exchange HPLC was performed using a linear gradient of 0–100% solution A (20% CH<sub>3</sub>CN in 0.5 M KH<sub>2</sub>PO<sub>4</sub>) in solution B (20% CH<sub>3</sub>CN in 0.005 M KH<sub>2</sub>PO<sub>4</sub>). Thin-layer chromatography was performed using 60F-254 (0.25 mm). MALDI-TOF was measured in reflectron positive mode. The matrix condition was 3-hydroxypicolinic acid (HPA) 50 mg/mL in MeCN:H<sub>2</sub>O (1:1, v/v), diammonium hydrogen citrate (AHC) 100 mg/mL in H<sub>2</sub>O.

**N<sup>3</sup>-Benzoyl-2'-O-(2-methoxycarbonylethyl)-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)uridine (6).** To a solution of N<sup>3</sup>-benzoyl-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)uridine (5) (1.80 g, 3.03 mmol) in *t*-BuOH (15 mL) were added methyl acrylate (5.40 mL, 60.2 mmol) and cesium carbonate (489 mg, 1.39 mmol). The solution was stirred at ambient temperature for 4 h. The solution was extracted with EtOAc and saturated NH<sub>4</sub>Cl. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/EtOAc (9:1, v/v) gave compound 6 (1.47 g, 2.17 mmol, 71%) as a white foam: <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 8.00 (d, *J* = 8.0 Hz, 1H), 7.94–7.48 (m, 5H), 5.77 (d, *J* = 8.0 Hz, 1H), 5.75 (s, 1H), 4.27–4.04 (m, 6H), 4.00 (dd, *J* = 2.5, 13.5 Hz, 1H), 3.88 (d, *J* = 4.0 Hz, 1H), 3.62 (s, 3H), 2.60–2.58 (m, 2H), 1.11–0.97 (m, 28H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 171.7, 168.9, 162.3, 149.1, 139.3, 135.4, 131.4, 130.7, 129.3, 101.6, 89.2, 82.6, 81.9, 68.2, 66.7, 59.5, 51.7, 35.2, 17.6, 17.4, 17.3, 17.1, 17.0, 13.6, 13.2, 13.0, 12.6. HRMS *m/z* calcd for C<sub>32</sub>H<sub>49</sub>N<sub>2</sub>O<sub>10</sub>Si<sub>2</sub> [M + H]<sup>+</sup> 677.2926, found 677.2931.

**N<sup>3</sup>-Benzoyl-2'-O-(2-ethoxycarbonylethyl)-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)uridine (7).** To a solution of compound 5 (120 mg, 0.20 mmol) in *t*-BuOH (1 mL) were added cesium carbonate (65 mg, 0.20 mmol) and ethyl acrylate (0.43 mL, 4.0 mmol). The solution was vigorously stirred at ambient temperature for 18 h. The solution was extracted with EtOAc and saturated NH<sub>4</sub>Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/EtOAc (5:1, v/v) gave compound 7 (83 mg, 0.12 mmol, 60%): <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.00 (d, *J* = 8.0 Hz, 1H), 7.93 (d, *J* = 7.5 Hz, 2H), 7.65 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.50 (dd, *J* = 7.5, 7.5 Hz, 1H), 5.77 (d, *J* = 8.0 Hz, 1H), 5.75 (s, 1H), 4.09–3.88 (m, 9H), 2.65–2.55 (m, 2H), 1.09–0.85 (m, 31H). HRMS *m/z* calcd for C<sub>33</sub>H<sub>51</sub>N<sub>2</sub>O<sub>10</sub>Si<sub>2</sub> [M + H]<sup>+</sup> 691.3078, found 691.2997.

**N<sup>3</sup>-Benzoyl-2'-O-[2-[(2,2,2-trifluoroethoxy)carbonyl]ethyl]-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)uridine (8).** To a solution of compound 5 (9.00 g, 15.23 mmol) in *t*-BuOH (75 mL) were added 2,2,2-trifluoroethyl acrylate (30.2 g, 304.67 mmol) and cesium carbonate (4.96 g, 15.23 mmol). The solution was stirred at ambient temperature for 2 h. Then the solution was extracted with EtOAc and saturated NH<sub>4</sub>Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica. Elution with hexane/EtOAc (6:1, v/v) gave compound 8 (8.29 g, 11.12 mmol, 73%): <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 8.02 (d, *J* = 7.5 Hz, 2H), 7.85 (d, *J* = 8.0 Hz, 1H), 7.78 (dd, *J* = 7.0 Hz, 2H), 7.60 (dd, *J* = 7.5, 2 Hz), 5.82 (d, *J* = 8.0, 1 Hz), 5.64 (s, 1H), 4.66 (dd, *J* = 9.0, 13.0 Hz, 2H), 4.28–4.25 (m, 1H), 4.19–4.17 (m, 2H), 3.99–3.93 (m, 4H), 2.72 (dd, *J* = 6.0, 2 Hz), 1.05–0.94 (m, 27H); <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>) δ 169.5, 169.4, 161.9, 148.6, 140.1, 135.5, 131.1, 130.3, 129.4, 100.6, 89.0, 81.2, 68.4, 65.9, 59.7, 59.5, 59.4, 34.3, 17.3, 17.2, 17.1, 17.0, 16.9, 16.84, 16.82, 16.7, 12.8, 12.3, 12.2, 11.9. HRMS *m/z* calcd for C<sub>33</sub>H<sub>48</sub>F<sub>3</sub>N<sub>2</sub>O<sub>10</sub>Si<sub>2</sub> [M + H]<sup>+</sup> 745.2798, found 745.2781.

**2'-O-(2-Methoxycarbonylethyl)-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)uridine (9).** To a solution of compound 6

(1.71 g, 2.53 mmol) in anhydrous THF (20 mL) was added *n*-PrNH<sub>2</sub> (1.5 mL, 18.0 mmol). The solution was vigorously stirred for 4 h. The solution was evaporated under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/EtOAc (1:4, v/v) gave compound 9 (1.03 g, 1.79 mmol, 71%) as a white foam: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 9.49 (s, 1H), 7.89 (d, *J* = 5.0 Hz, 1H), 5.73 (s, 1H), 5.67 (d, *J* = 5.0 Hz, 1H), 4.24–4.09 (m, 6H), 3.96–3.84 (m, 1H), 3.84 (s, 1H), 2.66–2.65 (m, 1H), 1.09–0.92 (m, 28H). HRMS *m/z* calcd for C<sub>25</sub>H<sub>45</sub>N<sub>2</sub>O<sub>9</sub>Si<sub>2</sub> [M + H]<sup>+</sup> 573.2663, found 573.2619.

**2'-O-[2-(*N*-Methylcarbamoyl)ethyl]-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)uridine (10).** To a solution of compound 5 (1.80 g, 3.03 mmol) in *t*-BuOH (15 mL) were added cesium carbonate (489 mg, 1.39 mmol) and methyl acrylate (5.4 mL, 60.2 mmol). The solution was vigorously stirred at ambient temperature for 4 h. The suspension was diluted with EtOAc, and extracted with EtOAc and saturated NaHCO<sub>3</sub> aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was treated with 40% MeNH<sub>2</sub> in MeOH (30 mL) at ambient temperature for 2 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with hexane/EtOAc (1:1, v/v) gave compound 10 (1.14 g, 66%): <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 9.90 (br, 1H), 7.91 (d, *J* = 8.0 Hz, 1H), 6.90 (br, 1H), 5.73–5.71 (m, 2H), 4.29–3.85 (7H, m), 2.81–2.79 (m, 3H), 2.63–2.47 (m, 2H). HRMS *m/z* calcd for C<sub>25</sub>H<sub>46</sub>N<sub>3</sub>O<sub>8</sub>Si<sub>2</sub> [M + H]<sup>+</sup> 572.2823, found 572.2826.

**2'-O-[2-(*N,N*-Dimethylcarbamoyl)ethyl]3',5'-O-(tetraisopropylidisiloxane-1,3-diyl)uridine (11).** To a solution of compound 8 (240 mg, 0.32 mmol) in THF (3 mL) was added 2 M Me<sub>2</sub>NH (2 mL). The solution was stirred for 2 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 5% MeOH in CHCl<sub>3</sub> gave compound 11 (152 mg, 0.26 mmol, 81%): <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 9.56 (br, 1H), 7.85 (d, *J* = 5.0 Hz, 1H), 5.74 (s, 1H), 5.65 (d, *J* = 5.0 Hz, 1H), 4.19–4.06 (m, 5H), 3.92 (m, 1H), 3.85 (s, 1H), 3.03 (s, 3H), 2.91 (s, 3H), 1.01–0.90 (m, 28H). HRMS *m/z* C<sub>26</sub>H<sub>48</sub>N<sub>3</sub>O<sub>8</sub>Si<sub>2</sub> [M + H]<sup>+</sup> 586.2980, found 586.2921.

**2'-O-[2-(*N*-Methylcarbamoyl)ethyl]uridine (13).** A solution of compound 10 (563 mg, 0.98 mmol) in dry THF (10 mL) was treated with triethylamine trihydrofluoride (570 μL, 3.50 mmol) and triethylamine (253 μL, 1.80 mmol) at ambient temperature for 1 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 10% MeOH in CHCl<sub>3</sub> gave compound 13 (250 mg, 0.75 mmol, 76%): <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O) δ 5.95 (d, *J* = 4.5 Hz, 1H), 5.92 (d, *J* = 8.5 Hz, 1H), 4.33 (t, *J* = 5.5 Hz, 1H), 4.17–4.12 (m, 2H), 3.98–3.88 (m, 3H), 3.79 (dd, *J* = 4.5, 13.0, 1H), 2.72–2.68 (m, 3H), 2.57–2.53 (m, 2H); <sup>13</sup>C NMR (125 MHz, D<sub>2</sub>O) δ 175.0, 166.7, 152.0, 142.3, 102.9, 88.2, 85.1, 81.8, 68.9, 67.2, 61.1, 36.5, 26.4. HRMS *m/z* calcd for C<sub>13</sub>H<sub>20</sub>N<sub>3</sub>O<sub>7</sub> [M + H]<sup>+</sup> 330.1301, found 330.1307.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-[2-(methoxycarbonyl)ethyl]uridine (15).** To a solution of compound 9 (780 mg, 1.37 mmol) in anhydrous THF (13 mL) was added Et<sub>3</sub>N·3HF (780 μL, 4.77 mmol). The solution was stirred at ambient temperature for 2 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 10% MeOH in CHCl<sub>3</sub> gave compound 12 as an intermediate, which was coevaporated three times with dry pyridine and finally dissolved in dry pyridine (13 mL). To the solution was added DMTrCl (510 mg, 1.50 mmol). The solution was stirred vigorously for 4 h. Then the solution was extracted with EtOAc and brine. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with EtOAc containing 0.5% Et<sub>3</sub>N gave compound 15 (780 mg, 1.23 mmol, 90%): <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.78 (br, 1H), 7.95 (d, *J* = 8.0 Hz, 1H), 7.28–7.21 (m, 9H), 6.82–6.80 (m, 4H), 5.91 (d, *J* = 1.5 Hz, 1H), 5.26 (d, *J* = 8.0 Hz, 1H), 4.49–4.45 (m, 1H), 4.04–3.92 (m, 4H), 3.76 (s, 6H), 3.68 (s, 3H), 3.50–3.45 (m,

3H), 2.70–2.56 (m, 2H). HRMS *m/z* calcd for C<sub>34</sub>H<sub>36</sub>N<sub>2</sub>NaO<sub>10</sub> [M + Na]<sup>+</sup> 655.2268, found 655.2269.

**(4,4'-Dimethoxytrityl)-2'-O-[2-(*N*-methylcarbamoyl)ethyl]uridine (16).** To a solution of compound 13 (553 mg, 1.67 mmol) in dry pyridine (15 mL) was added DMTrCl (622 mg, 1.84 mmol). The solution was stirred at ambient temperature for 4 h. The solution was diluted with EtOAc and washed with saturated NaHCO<sub>3</sub> aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 2% MeOH in CHCl<sub>3</sub> containing 0.5% triethylamine gave compound 16 (740 mg, 1.17 mmol, 70%) as a white foam: <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 7.95 (d, *J* = 8.0 Hz, 1H), 7.39–7.22 (9H, m), 6.86–6.83 (m, 4H), 5.93 (d, *J* = 3.0 Hz, 1H), 5.86 (br, 1H), 5.23 (d, *J* = 8.0 Hz, 1H), 3.97–3.90 (m, 2H), 3.79 (s, 3H), 3.54–3.48 (m, 2H), 2.81 (m, 3H), 2.61–2.43 (m, 2H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 172.2, 163.6, 158.8, 150.8, 144.5, 140.3, 135.5, 135.3, 130.3, 130.2, 128.1, 127.3, 113.4, 102.4, 87.9, 87.2, 83.7, 82.9, 69.2, 66.6, 62.1, 55.4, 35.9, 26.5. HRMS *m/z* calcd for C<sub>34</sub>H<sub>37</sub>N<sub>3</sub>NaO<sub>9</sub> [M + Na]<sup>+</sup> 654.2427, found 654.2446.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-[2-(*N,N*-dimethylcarbamoyl)ethyl]uridine (17).** To a solution of compound 11 (3.61 g, 6.18 mmol) in THF (60 mL) was added Et<sub>3</sub>N·3HF (3.4 mL, 21.6 mmol). The solution was vigorously stirred for 4 h. The solution was evaporated under reduced pressure. The residue was chromatographed on silica gel. Elution with 10% MeOH in CHCl<sub>3</sub> gave compound 14 as an intermediate, which was coevaporated three times with anhydrous pyridine three times. The residue was dissolved in anhydrous pyridine (60 mL). To the solution was added DMTrCl (2.30 g, 6.80 mmol). The solution was vigorously stirred overnight. The solution was evaporated under reduced pressure. The residue was chromatographed on silica gel. Elution with 5% MeOH in CHCl<sub>3</sub> containing 0.5% Et<sub>3</sub>N gave compound 17 (2.78 g, 4.32 mmol, 70%): <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.72 (br, 1H), 7.92 (d, *J* = 8.0 Hz, 1H), 7.29–7.21 (m, 9H), 6.84–6.82 (m, 4H), 5.96 (d, *J* = 3.5 Hz, 1H), 5.36 (d, *J* = 3.5 Hz, 1H), 5.28 (d, *J* = 8.0 Hz, 1H), 4.59–4.56 (m, 1H), 4.11–4.07 (m, 2H), 4.00–3.98 (m, 1H), 3.90–3.87 (m, 1H), 3.79 (s, 6H), 3.54–3.44 (m, 2H), 3.00 (s, 3H), 2.96 (s, 3H), 2.78–2.75 (m, 1H), 2.45–2.41 (m, 1H). HRMS *m/z* calcd for C<sub>35</sub>H<sub>39</sub>N<sub>3</sub>NaO<sub>9</sub> [M + Na]<sup>+</sup> 668.2584, found 668.2582.

**Triethylamine Salt of 5'-O-(4,4'-Dimethoxytrityl)-2'-O-(2-carboxyethyl)uridine (18).** To a solution of compound 15 (10 mg, 0.015 mmol) in pyridine (1 mL) was added 0.1 M NaOH (1 mL). The solution was stirred at ambient temperature for 30 min. The solution was extracted with CHCl<sub>3</sub> and 10% citric acid in H<sub>2</sub>O. To the organic solution was added Et<sub>3</sub>N (1 mL). The organic layer was dried over sodium sulfate and concentrated under reduced pressure to give compound 18 (7 mg, 0.0097 mmol, 65%): <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz) δ 8.08–7.16 (m, 14H), 6.83 (d, *J* = 10.0 Hz, 1H), 6.00 (d, *J* = 5.0 Hz, 1H), 5.31 (d, *J* = 10.0 Hz, 1H), 4.52–4.53 (m, 1H) 4.10–3.92 (m, 5H), 3.78 (s, 6H), 3.64–3.63 (m, 2H), 3.12–3.08 (m, 6H), 2.67–2.51 (m, 2H), 1.29–1.27 (m, 9H). HRMS *m/z* calcd for C<sub>33</sub>H<sub>34</sub>N<sub>2</sub>NaO<sub>10</sub> [M + Na]<sup>+</sup> 641.2111, found 641.2174.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-(2-carbamoylethyl)uridine (19).** To a solution of compound 15 (10 mg, 0.015 mmol) in pyridine (1 mL) was added 28% NH<sub>4</sub>OH (1 mL). The solution was stirred at ambient temperature for 30 min. The solution was concentrated under reduced pressure to give compound 19 (10 mg, 0.015 mmol, 99%): <sup>1</sup>H NMR (CD<sub>3</sub>CN, 500 MHz) δ 7.79 (d, *J* = 8.0 Hz, 1H), 7.53–7.34 (m, 11H), 6.98–6.96 (m, 11H), 6.50 (br, 1H), 5.99 (br, 1H), 5.89 (d, *J* = 4.0 Hz, 1H), 5.53 (s, 1H), 5.38 (d, *J* = 8.0 Hz, 1H), 4.48–4.46 (m, 1H), 4.08–4.05 (m, 2H), 3.92–3.86 (m, 2H), 3.50 (s, 6H), 3.41–3.39 (m, 2H), 2.60–2.51 (m, 2H); <sup>13</sup>C NMR (CD<sub>3</sub>CN, 125 MHz) δ 174.9, 163.9, 159.7, 159.7, 151.4, 145.8, 141.1, 136.6, 136.4, 131.0, 131.0, 129.0, 128.9, 127.9, 118.3, 114.1, 114.1, 102.5, 88.3, 87.5, 84.2, 82.8, 70.0, 66.9, 63.4, 55.9, 35.7. HRMS *m/z* C<sub>33</sub>H<sub>35</sub>N<sub>3</sub>NaO<sub>9</sub> [M + Na]<sup>+</sup> 640.2271, found 640.2257.



**2'-O-[2-(Methoxycarbonyl)ethyl]-3',5'-O-(1,1,3,3-tetraiso-propyldisiloxane-1,3-diyl)adenosine (26).** A solution of 3',5'-O-(1,1,3,3-tetraiso-propyldisiloxane-1,3-diyl)adenosine (24) (10.0 g, 19.6 mmol) in *t*-BuOH (100 mL) was treated with cesium carbonate (6.4 g, 19.6 mmol) at ambient temperature for 30 min. To the suspension was added methyl acrylate (35.5 mL, 392 mmol). The suspension was vigorously stirred for 9 h. The suspension was extracted with EtOAc and saturated NH<sub>4</sub>Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 0.5% MeOH in CHCl<sub>3</sub> gave compound 26 (8.40 g, 14.1 mmol, 72%): <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 8.19 (s, 1H), 8.07 (s, 1H), 7.35–7.25 (br, 2H), 5.92 (s, 1H), 4.33 (dd, *J* = 4.0 Hz, 6.5 Hz, 1H), 4.47 (d, *J* = 4.0 Hz, 1H), 4.04–4.02 (m, 3H), 3.92–3.90 (m, 3H), 2.65–2.55 (m, 2H), 1.06–0.95 (m, 28 H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 171.3, 156.0, 152.5, 148.5, 139.2, 119.2, 87.5, 81.3, 80.6, 70.0, 66.5, 60.3, 51.2, 34.7, 17.2, 17.14, 17.10, 17.0, 16.9, 16.8, 16.7, 12.7, 12.3, 12.1, 12.1. HRMS *m/z* calcd for C<sub>26</sub>H<sub>45</sub>N<sub>5</sub>NaO<sub>7</sub>Si<sub>2</sub> [M + Na]<sup>+</sup> 618.2758, found 618.2772.

**2'-O-[2-(Methoxycarbonyl)ethyl]-3',5'-O-(1,1,3,3-tetraiso-propyldisiloxane-1,3-diyl)cytidine (27).** To the solution of 3',5'-O-(1,1,3,3-tetraiso-propyldisiloxane-1,3-diyl)cytidine (25) (5.0 g, 10.3 mmol) in *t*-BuOH (50 mL) was added cesium carbonate (3.33 g, 10.3 mmol). The suspension was stirred at ambient temperature for 30 min. To the suspension was added methyl acrylate (22.5 mL, 392 mmol). The suspension was vigorously stirred for 6 h. The suspension was extracted with EtOAc and saturated NH<sub>4</sub>Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 2% MeOH in CHCl<sub>3</sub> gave compound 27 (4.41 g, 7.73 mmol, 75%): <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 7.68 (d, *J* = 7.5 Hz, 1H), 7.19 (br, 1H), 5.68 (d, *J* = 7.5 Hz, 1H), 5.59 (s, 1H), 4.16–4.12 (m, 2H), 4.01–3.83 (m, 5H), 3.58 (s, 3H), 2.63–2.60 (t, *J* = 6.5 Hz, 2H), 1.06–0.92 (m, 28H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 172.1, 166.3, 155.8, 140.8, 93.9, 89.4, 82.4, 81.4, 68.1, 66.5, 59.7, 51.7, 35.2, 17.6, 17.6, 17.4, 17.4, 17.2, 17.14, 17.11, 17.0, 13.5, 13.1, 13.0, 12.7. HRMS *m/z* calcd for C<sub>25</sub>H<sub>46</sub>N<sub>3</sub>O<sub>8</sub>Si<sub>2</sub> [M + H]<sup>+</sup> 572.2832, found 572.2738.

**2'-O-[2-(Methoxycarbonyl)ethyl]-3',5'-O-(1,1,3,3-tetraiso-propyldisiloxane-1,3-diyl)-2-aminoadenosine (29).** To a solution of 3',5'-O-(1,1,3,3-tetraiso-propyldisiloxane-1,3-diyl)-2-aminoadenosine (28) (11.0 g, 21.0 mmol) in *t*-BuOH (110 mL) were added cesium carbonate (7.03 g, 21.6 mmol) and methyl acrylate (39.1 mL, 431 mmol). The solution was stirred at ambient temperature for 18 h. The suspension was extracted with CHCl<sub>3</sub> and saturated NH<sub>4</sub>Cl aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was dissolved in CHCl<sub>3</sub> and absorbed with silica gel. The silica gel was subjected to a column. Elution with 3% MeOH in CHCl<sub>3</sub> gave compound 29 (10.1 g, 16.5 mmol, 79%): <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 7.74 (s, 1H), 6.82–6.78 (br, 2H), 5.75 (s, 1H), 5.72 (s, 2H), 4.56 (dd, *J* = 5.0, 10 Hz, 1H), 4.30 (d, *J* = 5.0 Hz, 1H), 4.06–4.02 (m, 2H), 3.93–3.88 (m, 3H), 3.55 (s, 3H), 2.63–2.60 (m, 2H), 1.08–0.94 (m, 28H); <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>) δ 171.3, 160.3, 156.1, 151.0, 134.5, 113.3, 86.3, 81.2, 80.5, 69.9, 66.3, 60.2, 51.3, 34.7, 17.3, 17.2, 17.1, 17.0, 16.9, 16.87, 16.80, 12.8, 12.4, 12.2, 12.1. HRMS *m/z* calcd for C<sub>26</sub>H<sub>47</sub>N<sub>6</sub>O<sub>7</sub>Si<sub>2</sub> [M + H]<sup>+</sup> 611.3039, found 611.3038.

**6-N-Acetyl-2'-O-[2-(*N*-methylcarbamoyl)ethyl]adenosine (33).** To a solution of compound 26 (4.70 g, 7.90 mmol) in EtOH (90 mL) was added 40% MeNH<sub>2</sub> in MeOH (90 mL). The solution was stirred at ambient temperature for 18 h. Then the solution was concentrated under reduced pressure. The residue was coevaporated three times with dry pyridine and finally dissolved in dry pyridine (90 mL). To the solution was added acetyl chloride (790 μL, 11.1 mmol). The solution was vigorously stirred for 6 h. The solution was diluted with EtOAc, and then extracted with EtOAc and saturated

NaHCO<sub>3</sub> aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure to give compound 31 as an intermediate. The residue was coevaporated with toluene and dissolved in dry THF. To the solution were added triethylamine and triethylamine trihydrofluoride. The solution was vigorously stirred for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel without aqueous workup. Elution with 3.5% MeOH in CHCl<sub>3</sub> gave compound 33 (1.87 g, 4.74 mmol, 60%): <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 10.75–10.65 (br, 1H), 8.69 (s, 1H), 8.65 (s, 1H), 7.83 (d, *J* = 4.5 Hz, 1H), 6.06 (d, *J* = 6.0 Hz, 1H), 5.34 (d, *J* = 3.5 Hz, 1H), 5.18 (dd, *J* = 5.0 Hz), 4.57 (dd, *J* = 6.0 Hz, 1H), 4.41 (d, *J* = 3.0 Hz, 1H), 3.99 (d, *J* = 3.0 Hz, 1H), 3.08–3.76 (m, 1H), 3.69–3.33 (m, 3H), 2.51–2.52 (d, *J* = 4.5 Hz, 3H), 2.33–2.30 (m, 2H), 2.25 (s, 3H); <sup>13</sup>C NMR (125 MHz, DMSO-*d*<sub>6</sub>) δ 170.8, 168.8, 151.7, 151.6, 149.6, 142.7, 123.7, 86.1, 85.6, 80.9, 68.9, 65.6, 61.2, 35.2, 25.4, 24.3. HRMS *m/z* calcd for C<sub>16</sub>H<sub>22</sub>N<sub>6</sub>NaO<sub>6</sub> [M + Na]<sup>+</sup> 417.1499, found 417.1498.

**4-N-Acetyl-2'-O-[2-(*N*-methylcarbamoyl)ethyl]cytidine (34).** Compound 27 (2.90 g, 5.08 mmol) was treated with 40% MeNH<sub>2</sub> in MeOH (50 mL) at ambient temperature for 6 h. The solution was concentrated under reduced pressure and coevaporated three times with dry pyridine. The residue was dissolved with dry pyridine (50 mL), and to the solution was added acetyl chloride (398 μL, 5.58 mmol). The solution was vigorously stirred for 4 h. The solution was concentrated under reduced pressure, and coevaporated with toluene to give compound 32 as an intermediate. The residue was dissolved in THF, and to the solution were added TEA (1.25 mL, 8.63 mmol) and TEA·3HF (2.92 mL, 17.77 mmol). The solution was vigorously stirred for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 7% MeOH in CHCl<sub>3</sub> gave compound 34 (994 mg, 2.68 mmol, 53%) as white foam: <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 10.88 (s, 1H), 8.43 (d, *J* = 7.5 Hz, 1H), 7.84 (d, *J* = 4.0 Hz, 1H), 7.18 (d, *J* = 7.5 Hz, 1H), 5.80 (d, *J* = 2.5 Hz, 1H), 5.21 (dd, *J* = 5.0 Hz, 1H), 5.17 (d, *J* = 6.0 Hz, 1H), 4.08 (dd, *J* = 4.0, 6.0 Hz, 1H), 3.86–3.39 (m, 6H), 2.56 (d, *J* = 4.0 Hz, 1H), 2.39–2.35 (m, 2H), 2.09 (s, 3H). HRMS *m/z* calcd for C<sub>15</sub>H<sub>22</sub>N<sub>4</sub>O<sub>7</sub> [M + H]<sup>+</sup> 371.1556, found 371.1516.

**6-N-Acetyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-[2-(*N*-methylcarbamoyl)ethyl]adenosine (35).** To a solution of compound 33 (1.50 g, 3.80 mmol) in dry pyridine was added DMTrCl (1.05 g, 3.24 mmol). The solution was stirred at ambient temperature for 5 h. The solution was extracted with CHCl<sub>3</sub> and brine. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 3% MeOH in CHCl<sub>3</sub> containing 0.5% Et<sub>3</sub>N gave compound 35 (1.95 g, 2.80 mmol, 73%): <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ 10.70 (br, 1H), 8.57 (s, 1H), 8.56 (s, 1H), 7.83–7.82 (m, 1H), 7.33–7.17 (m, 9H), 6.84–6.80 (m, 4H), 6.09 (d, *J* = 5.0 Hz, 1H), 5.38 (d, *J* = 5.0 Hz, 1H), 4.71–4.69 (m, 1H), 4.49–4.47 (m, 1H), 4.09–4.08 (m, 1H), 3.82–3.67 (m, 8H), 3.21–3.16 (m, 2H), 2.50 (s, 3H), 2.33–2.25 (m, 2H), 2.11 (s, 3H). HRMS *m/z* calcd for C<sub>37</sub>H<sub>41</sub>N<sub>6</sub>O<sub>8</sub> [M + H]<sup>+</sup> 697.2986, found 697.2946.

**4-N-Acetyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-[2-(*N*-methylcarbamoyl)ethyl]cytidine (36).** To a solution of compound 34 (374 mg, 1.00 mmol) in dry pyridine (6 mL) was added DMTrCl (244 mg, 0.72 mmol). The solution was stirred at ambient temperature for 5 h. The solution was extracted with CHCl<sub>3</sub> and brine. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 6% MeOH in CHCl<sub>3</sub> containing 0.5% Et<sub>3</sub>N gave compound 36 (350 mg, 0.52 mmol, 52%): <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 9.49 (br, 1H), 8.48 (d, *J* = 7.5 Hz, 1H), 7.41–7.23 (m, 9H), 6.85–6.83 (m, 4H), 6.52 (br, 1H), 5.89 (s, 1H), 4.49–4.45 (m, 2H), 4.15–3.92 (m, 4H), 3.79 (s, 3H), 3.79 (s, 3H), 3.54–3.54 (m, 2H), 2.76 (d, 3H),

2.62–2.39 (m, 2H), 2.18 (s, 3H). HRMS  $m/z$  calcd for  $C_{36}H_{40}N_4NaO_9$  [ $M + Na$ ] $^+$  695.2693, found 695.2694.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-[2-(*N*-methylcarbamoyl)ethyl]uridine-3-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) (20).** To a solution of compound 16 (490 mg, 0.77 mmol) in dry MeCN (2 mL) were added diisopropylammonium 1*H*-tetrazolidine (101 mg, 1.19 mmol) and 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphordiamidite (358 mg, 1.19 mmol). The solution was stirred under argon atmosphere at ambient temperature for 8 h. The solution was diluted with EtOAc and washed with saturated  $NaHCO_3$  aq. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 1% MeOH in  $CHCl_3$  containing 0.5% triethylamine gave compound 20 (384 mg, 0.46 mmol, 60%) as a white foam:  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.09–8.01 (m, 1H), 7.47–7.27 (m, 9H), 6.86–6.82 (m, 4H), 6.49 (br, 1H), 5.90 (m, 1H), 5.28–5.21 (m, 1H), 4.61–4.48 (m, 1H), 4.25–3.86 (m, 4H), 3.80–3.79 (m, 1H), 3.75–3.43 (m, 6H), 2.80–2.77 (m, 3H), 2.64–2.42 (m, 4H), 1.29–1.03 (m, 12H);  $^{31}P$  NMR (125 MHz,  $CDCl_3$ )  $\delta$  151.32, 150.48. HRMS  $m/z$  calcd for  $C_{43}H_{55}N_5O_{10}P$  [ $M + H$ ] $^+$  832.3687, found 832.3650.

**6-*N*-Acetyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-[2-(*N*-methylcarbamoyl)ethyl]adenosine-3'-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) (21).** To a solution of compound 35 (1.00 g, 1.44 mmol) in dry MeCN (150 mL) were added 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphordiamidite (920  $\mu$ L, 2.87 mmol) and diisopropylammonium 1*H*-tetrazolidine (147 mg, 0.86 mmol) at ambient temperature for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 3% MeOH in  $CHCl_3$  gave compound 21 (980 mg, 1.09 mmol, 76%):  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.63–8.61 (m, 2H), 8.26–8.22 (m, 1H), 7.42–7.21 (m, 9H), 6.82–6.79 (m, 4H), 6.41–6.38 (m, 1H), 6.16–6.15 (m, 1H), 4.69–4.59 (m, 2H), 4.40–4.33 (m, 1H), 4.04–3.97 (m, 1H), 3.90–3.85 (m, 2H), 3.79–3.62 (m, 6H), 3.60–3.52 (s, 4H), 3.39–3.35 (m, 1H);  $^{31}P$  NMR (125 MHz,  $CDCl_3$ )  $\delta$  151.34, 151.30. HRMS  $m/z$  calcd for  $C_{46}H_{58}N_8O_9P$  [ $M + H$ ] $^+$  897.4064, found 897.4022.

**4-*N*-Acetyl-5'-O-(4,4'-dimethoxytrityl)-2'-O-[2-(*N*-methylcarbamoyl)ethyl]cytidine-3'-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite) (22).** To a solution of compound 36 (480 mg, 0.71 mmol) in dry MeCN (2 mL) were added diisopropylammonium 1*H*-tetrazolidine (92 mg, 0.54 mmol) and 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphordiamidite (343  $\mu$ L, 1.07 mmol). The solution was stirred at ambient temperature for 10 h. The solution was extracted with  $CHCl_3$  and brine. The organic layer was dried, collected over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 1.5% MeOH in  $CHCl_3$  containing 0.5%  $Et_3N$  gave compound 22 (300 mg, 0.34 mmol, 48%):  $^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  9.70–9.67 (m, 1H), 8.63–8.56 (m, 1H), 7.34–6.86 (m, 11H), 5.93 (s, 1H), 4.57–4.48 (m, 1H), 4.30–4.02 (m, 4H), 3.84–3.83 (m, 6H), 3.73–3.46 (m, 6H), 2.81–2.62 (m, 4H), 2.43–2.37 (m, 2H), 2.26 (s, 3H), 1.27–1.03 (m, 12H);  $^{31}P$  NMR (125 MHz,  $CDCl_3$ )  $\delta$  151.43, 150.23. HRMS  $m/z$  calcd for  $C_{45}H_{58}N_6O_{10}P$  [ $M + H$ ] $^+$  873.3952, found 872.3911.

**2'-O-[2-(*N*-Methylcarbamoyl)ethyl]-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)-2-aminoadenosine (37).** To a solution of compound 29 (1.0 g, 1.64 mmol) in EtOH (8 mL) was added 40% methylamine in MeOH (8 mL) at ambient temperature for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 4% MeOH in  $CHCl_3$  gave compound 37 (770 mg, 1.26 mmol, 77%):  $^1H$  NMR (500 MHz,  $DMSO-d_6$ )  $\delta$  7.76–7.74 (m, 2H), 6.82–6.76 (br, 2H), 5.75 (s, 1H), 5.73 (s, 2H), 4.54 (dd,  $J = 5.0, 8.0$  Hz, 1H), 4.27 (d,  $J = 5.0$  Hz, 1H), 4.06–3.98 (m, 2H), 3.94–3.90 (m, 2H), 3.87–3.82 (m, 1H), 2.53 (d,  $J = 4.0$  Hz, 3H), 2.37 (dd,  $J = 6$  Hz, 2H), 1.04–1.02 (m, 28H). HRMS  $m/z$  calcd for  $C_{26}H_{48}N_7O_6Si_2$  [ $M + H$ ] $^+$  610.3198, found 610.3183.

**2'-O-[2-(*N*-Methylcarbamoyl)ethyl]-2-*N*-phenoxyacetyl-3',5'-O-(1,1,3,3-tetraisopropylidisiloxane-1,3-diyl)-2-aminoadenosine (38).** To a solution of compound 37 (500 mg, 0.82 mmol) in dry pyridine (20 mL) at  $-10$  °C was dropwise added phenoxyacetyl chloride (124  $\mu$ L, 0.90 mmol) over 5 min. The solution was vigorously stirred at  $-10$  °C for 2 h and then at ambient temperature for 1 h. The solution was diluted with  $CHCl_3$  and extracted with brine. The organic layer was collected, dried over sodium sulfate, and concentrated under reduced pressure. Elution with 5% MeOH in  $CHCl_3$  gave compound 38 (442 mg, 0.60 mmol, 73%):  $^1H$  NMR (500 MHz,  $DMSO-d_6$ )  $\delta$  10.00 (s, 1H), 8.04 (s, 1H), 7.68 (d,  $J = 4.0$  Hz, 1H), 7.50–7.38 (br, 2H), 7.29–7.26 (m, 2H), 6.37–6.91 (m, 3H), 5.88 (s, 1H), 5.02 (s, 2H), 4.53 (dd,  $J = 5.0, 8$  Hz, 1H), 4.38 (d,  $J = 8.0$  Hz, 1H), 4.11–4.08 (m, 1H), 4.03–3.86 (m, 4H), 2.49 (m, 3H), 2.33 (dd,  $J = 7.0, 7.0$  Hz, 2H), 1.04–0.93 (m, 28H);  $^{13}C$  NMR (125 MHz,  $DMSO-d_6$ )  $\delta$  170.1, 158.0, 156.1, 152.5, 149.4, 137.2, 129.4, 120.8, 116.1, 114.5, 86.8, 81.0, 80.9, 69.7, 67.4, 67.1, 60.4, 40.0, 36.1, 25.3, 17.3, 17.16, 17.12, 17.0, 16.9, 16.8, 12.7, 12.3, 12.1, 12.0. HRMS  $m/z$  calcd for  $C_{34}H_{53}N_7NaO_8Si_2$  [ $M + Na$ ] $^+$  766.3392, found 766.3400.

**2'-O-[2-(*N*-Methylcarbamoyl)ethyl]-2-*N*-phenoxyacetylguanosine (30).** To a stirred solution of compound 38 (1.00 g, 1.34 mmol) in AcOH (100 mL) were added  $H_2O$  (40 mL) and  $NaNO_2$  (752 mg, 10.75 mmol). After 30 min another portion of  $NaNO_2$  (752 mg, 10.75 mmol) was added. The solution was stirred at ambient temperature for 48 h. The solution was concentrated under reduced pressure to give a yellow syrup. The syrup was extracted once with brine and three times with saturated  $NaHCO_3$  aq. The organic layer was dried, collected over sodium sulfate, and concentrated under reduced pressure to give compound 39 as an intermediate. The residue was coevaporated with dry pyridine. The residue was dissolved in anhydrous THF (130 mL). To the solution were added triethylamine (340  $\mu$ L) and triethylamine trihydrofluoride (650  $\mu$ L). The solution was stirred vigorously at ambient temperature for 6 h. The solution was concentrated under reduced pressure. The residue was extracted with  $H_2O$  and  $CHCl_3$ . After several minutes, white precipitates appeared in the aqueous layer. The aqueous layer was concentrated to reduce its volume. The precipitate was collected by filtration and dried. Finally, it was dried by vacuum and gentle heating to give compound 30 (490 mg, 0.97 mmol, 71%):  $^1H$  NMR (500 MHz,  $DMSO-d_6$ )  $\delta$  11.84 (s, 1H), 11.78 (s, 1H), 8.29 (s, 1H), 7.89 (d,  $J = 4.0$  Hz, 1H), 7.32–7.29 (m, 2H), 6.99–6.97 (m, 3H), 5.87 (d,  $J = 6.5$  Hz, 1H), 5.30 (d,  $J = 3.5$  Hz, 1H, diminished with  $D_2O$ ), 5.09 (dd,  $J = 5.5, 5.5$  Hz, 1H, diminished with  $D_2O$ ), 4.86 (s, 2H), 4.44–4.42 (m, 1H), 4.38–4.37 (m, 1H), 3.95 (d,  $J = 2.0$  Hz, 1H), 3.79–3.76 (m, 1H), 3.66–3.55 (m, 3H), 2.54 (d,  $J = 4.0$  Hz, 3H), 2.33 (s, 2H);  $^{13}C$  NMR (125 MHz,  $DMSO-d_6$ )  $\delta$  171.05, 171.01, 157.6, 154.8, 148.7, 147.4, 137.8, 129.5, 121.3, 120.3, 114.5, 86.1, 84.5, 81.2, 68.9, 66.2, 65.4, 61.3, 35.1, 25.0. HRMS  $m/z$  calcd for  $C_{22}H_{26}N_6NaO_8$  [ $M + Na$ ] $^+$  525.1710, found 525.1715.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-[2-(*N*-methylcarbamoyl)ethyl]-2-*N*-phenoxyacetylguanosine (40).** To a solution of compound 30 (330 mg, 0.66 mmol) in pyridine (6 mL) was added  $DMTrCl$  (244 mg, 0.72 mmol). The solution was stirred at ambient temperature for 3 h. The solution was diluted with  $CHCl_3$  and extracted with saturated  $NaHCO_3$  aq. The organic layer was dried, collected over sodium sulfate, and concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with TEA/MeOH/ $CHCl_3$  (0.5:2:98, v/v/v) gave compound 40 (401 mg, 0.50 mmol, 75%):  $^1H$  NMR (500 MHz,  $DMSO-d_6$ )  $\delta$  11.95–11.85 (m, 2H), 8.13 (s, 1H), 7.89 (d,  $J = 4.5$  Hz, 1H), 7.36–7.21 (m, 11H), 7.00–6.97 (m, 3H), 6.85–6.82 (m, 4H), 5.92 (d,  $J = 5.5$  Hz, 1H), 5.36 (d,  $J = 4.5$  Hz, 1H, diminished with  $D_2O$ ), 4.86 (dd,  $J = 15.5, 22.5$  Hz, 2H), 4.50 (dd,  $J = 5.5, 10.0$  Hz, 1H), 4.39–4.36 (m, 1H), 4.05–4.04 (m, 1H), 3.84–3.82 (m, 1H), 3.76–3.68 (m, 7H), 3.30–3.27 (m, 1H), 3.17–3.15 (m, 1H), 2.55 (d,  $J = 4.5$  Hz, 3H), 2.38–2.34 (m, 2H);  $^{13}C$  NMR (125 MHz,  $DMSO-d_6$ )  $\delta$  172.42, 170.78, 158.49, 156.97, 148.39, 144.44, 137.31, 135.53,



135.48, 130.00, 129.61, 128.08, 127.80, 126.88, 122.16, 121.26, 114.64, 114.53, 113.12, 86.50, 84.19, 82.24, 69.67, 66.94, 66.18, 63.34, 60.33, 55.12, 45.98, 35.61, 26.22, 14.12, 9.03. HRMS  $m/z$  calcd for  $C_{43}H_{45}N_6O_{10}$   $[M + H]^+$  805.3119, found 805.3112.

**5'-O-(4,4'-Dimethoxytrityl)-2'-O-[2-(*N*-methylcarbamoyl)-ethyl]-2-*N*-phenoxyacetylguanosine-3'-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) (23).** To a solution of compound 40 (500 mg, 0.62 mmol) in dry MeCN (50 mL) were added 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphordiamidite (477  $\mu$ L, 1.49 mmol) and diisopropylammonium 1*H*-tetrazolide (64 mg, 0.37 mmol). The solution was stirred at ambient temperature for 18 h. The solution was concentrated under reduced pressure. The residue was chromatographed on silica gel. Elution with 2% MeOH in  $CHCl_3$  gave compound 23 (498 mg, 0.49 mmol, 80%):  $^1H$  NMR (500 MHz, DMSO- $d_6$ )  $\delta$  7.91–7.87 (m, 1H), 7.47–7.26 (m, 13H), 7.23–7.20 (m, 1H), 7.07–7.04 (m, 1H), 6.99–6.95 (m, 2H), 6.83–6.80 (m, 2H), 6.16–6.00 (m, 1H), 5.99–5.93 (m, 1H), 4.74–4.70 (m, 2H), 4.46–3.84 (m, 3H), 3.78–3.77 (m, 6H), 3.67–3.32 (m, 4H), 2.74–2.73 (m, 3H), 2.66–2.35 (m, 5H), 1.29–1.00 (m, 12H). HRMS  $m/z$  calcd for  $C_{52}H_{61}N_8O_{11}PNa$   $[M + Na]^+$  1027.4095, found 1027.4098.

**Oligonucleotide Synthesis.** RNA oligonucleotides (Table 2) were synthesized on an Applied Biosystems 392 oligonucleotide synthesizer on a 1  $\mu$ mol scale, using a 0.1 M solution each of 20, 21, 22, and 23 with commercially available 5'-O-(4,4'-dimethoxytrityl)-2'-O-methyl-3'-O-(2-cyanoethyl-*N,N*-diisopropylphosphoramidite monomers of uridine, 4-*N*-benzoylcytidine, 6-*N*-benzoyladenine (ABz), and 2-*N*-isobutyrylguanosine (GiBu). A 0.25 M solution of 5-ethiothio-1*H*-tetrazole in dry acetonitrile or a 0.25 M solution of 5-(bis-3,5-trifluoromethylphenyl)-1*H*-tetrazole in dry acetonitrile was used as the activator for the coupling reaction. Coupling time was set to be 10 min. [(Dimethylaminomethylidene)amino]-3*H*-1,2,4-dithiazole-3-thione (DDTT)<sup>42</sup> was used as the sulfurization agent for the phosphorothioate synthesis. After completion of the synthesis, the release of 2'-O-modified oligonucleotides from the support and deprotection of all protecting groups were simultaneously carried out in  $NH_4OH$  (1 mL) at ambient temperature for 2 h, and then CPG was filtrated. The filtrate was heated at 55 °C for 5 h. The filtrate was concentrated under reduced pressure and then subjected to C-18 cartridge purification. Each product was purified by using anion-exchange chromatography.

**$T_m$  Experiments.** Each oligonucleotide (1.0 nmol) was dissolved in 10 mM sodium phosphate buffer (pH 7.0, containing 150 mM NaCl and 0.1 mM EDTA) (500  $\mu$ L) to arrange the final concentration of each oligonucleotide to be 2  $\mu$ M. The solution was separated into quartz cells (10 mm) and incubated at 85 °C. After 10 min, the solution was cooled to 5 °C at a rate of annealing and melting, the absorption at 260 nm was recorded and used to draw UV-melting curves.  $T_m$  values were calculated as the temperature that gave the maximum of the first derivative of the UV-melting curve.

**Nuclease Resistance Assay.** Each oligonucleotide (10.0 nmol) was dissolved in Tris-HCl buffer (at pH 8.5, 72 mM NaCl, 14 mM  $MgCl_2$  at 37 °C) (990  $\mu$ L). To the solution was added snake venom phosphodiesterase ( $5 \times 10^{-4}$  unit/mL) (10  $\mu$ L) to arrange the final concentration of each oligonucleotide to be 10  $\mu$ M. Aliquots of the reaction solution were removed at indicated times, quenched by heating at 100 °C for 2 min. The mixture was analyzed by anion exchange HPLC.

**Delivery Method.** Eight weeks old *mdx52* mice were used. As antisense oligonucleotides against exon 51 of dystrophin gene, ORNs 9–12 were used. Each antisense oligonucleotide (20  $\mu$ g) was dissolved in saline. Ten micrograms of antisense oligonucleotide was injected into each tibialis anterior muscle with lipofectamine 2000. Two weeks after injection, mice were sacrificed and muscles were dissected. Total RNA was extracted from frozen tissue and 50 ng of total RNA was used for one-step RT-PCR according to the manufacturer's instructions. The

primer sequences were Ex50F 5'-TTTACTTCGGGAGCTGAGGA-3' and Ex53R 5'-ACCTGTTCGGCTTCTTCCTT-3' for amplification of cDNA from exons 50–53. The PCR conditions were 95 °C for 4 min, then 35 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 7 min. The intensity of PCR bands was analyzed by using ImageJ software (<http://rsbweb.nih.gov/ij/>), and skipping efficiency was calculated by using the following formula [(the intensity of skipped band)/(the intensity of skipped band + the intensity of unskippedband)], after the resulting PCR bands were extracted using a gel extraction kit.

**Immunohistochemistry.** Eight micrometer cryosections were cut from flash-frozen muscle, then placed on poly-L-lysine-coated slides and air-dried. The sections were stained with monoclonal mouse antibody Dys-2 against C-terminus of cardiac muscular dystrophin from normal mouse used as a secondary antibody. 4',6-Diamidino-2-phenylindole containing a mounting agent was used for nuclear counterstaining.

## ■ ASSOCIATED CONTENT

Supporting Information.  $^1H$  NMR,  $^{13}C$  NMR, and  $^{31}P$  NMR charts. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ■ REFERENCES

- (1) Jackson, A. L.; Burchard, J.; Leake, D.; Reynolds, A.; Schelter, J.; Guo, J.; Johnson, J. M.; Lim, L.; Karpilow, J.; Nichols, K.; Marshall, W.; Khvorova, A.; Linsley, P. S. *RNA* 2006, 12, 1197–1205.
- (2) Manoharan, M. *Antisense Nucleic Acid Drug Dev.* 2002, 12, 103–128.
- (3) Ge, Q.; Dallas, A.; Ilves, H.; Shoresten, J.; Behlke, M. A.; Johnston, B. H. *RNA* 2010, 16, 118–130.
- (4) Burmeister, P. E.; Lewis, S. D.; Silva, R. F.; Preiss, J. R.; Horwitz, L. R.; Pendergrast, P. S.; McCauley, T. G.; Kurz, J. C.; Epstein, D. M.; Wilson, C.; Keefe, A. D. *Chem. Biol.* 2005, 12, 25–33.
- (5) Inoue, H.; Hayase, Y.; Imura, A.; Iwai, S.; Miura, K.; Ohtsuka, E. *Nucleic Acids Res.* 1987, 15, 6131–48.
- (6) Yu, R. Z.; Geary, R. S.; Monteith, D. K.; Matson, J.; Truong, L.; Fitchett, J.; Levin, A. A. *J. Pharm. Sci.* 2004, 93, 48–59.
- (7) Griffey, R. H.; Monia, B. P.; Cummins, L. L.; Freier, S.; Greig, M. J.; Guinasso, C. J.; Lesnik, E.; Manalili, S. M.; Mohan, V.; Owens, S.; Ross, B. R.; Sasmor, H.; Wanczewicz, E.; Weiler, K.; Wheeler, P. D.; Cook, P. D. *J. Med. Chem.* 1996, 39, 5100–5109.
- (8) Prakash, T. P.; Kawasaki, A. M.; Wanczewicz, E. V.; Shen, L.; Monia, B. P.; Ross, B. S.; Bhat, B.; Manoharan, M. *J. Med. Chem.* 2008, 51, 2766–2776.
- (9) Bumcrot, D.; Manoharan, M.; Koteliansky, V.; Sah, D. W. Y. *Nat. Chem. Biol.* 2006, 2, 711–719.
- (10) Geary, R. S.; Watanabe, T. A.; Truong, L.; Freier, S.; Lesnik, E. A.; Sioufi, N. B.; Sasmor, H.; Manoharan, M.; Levin, A. A. *J. Pharmacol. Exp. Ther.* 2001, 296, 890–897.

- (11) Freier, S. M.; Altmann, K.-H. *Nucleic Acids Res.* 1997, 15, 4429–43.
- (12) Manoharan, M.; Tivel, K. L.; Cook, P. D. *Tetrahedron Lett.* 1995, 36, 3651–3654.
- (13) Sakamoto, T.; Kobori, A.; Murakami, A. *Bioorg. Med. Chem. Lett.* 2008, 18, 2590–2593.
- (14) Winkler, J.; Gilbert, M.; Kocourkova, A.; Stessl, M.; Noe, C. R. *ChemMedChem* 2008, 3, 102–110.
- (15) (a) Ross, B. S.; Springer, R. H.; Tortorici, Z.; Dimock, S. *Nucleosides Nucleotides* 1997, 16, 1641–1643. (b) Oeda, Y.; Iijima, Y.; Taguchi, H.; Ohkubo, A.; Seio, K.; Sekine, M. *Org. Lett.* 2009, 11, 5582–5585.
- (16) Sivets, G. G. *Nucleosides Nucleotides Nucleic Acids* 2007, 26, 1237–1240.
- (17) Saneyoshi, H.; Seio, K.; Sekine, M. *J. Org. Chem.* 2005, 70, 10453–10460.
- (18) Saneyoshi, H.; Ando, K.; Seio, K.; Sekine, M. *Tetrahedron* 2007, 63, 11195–11203.
- (19) Sekine, M. *J. Org. Chem.* 1989, 54, 2321–2326.
- (20) (a) Urata, H.; Hara, H.; Hirata, Y.; Ohmoto, N.; Akagi, M. *Nucleic Acids Symp. Ser.* 1985, 16, 165–168. (b) Markiewicz, W. T. *J. Chem. Res., Miniprint* 1979, 173.
- (21) Pon, R. T.; Yu, S.; Prabhavalkar, T.; Mishra, T.; Kulkarni, B.; Sanghvi, Y. S. *Nucleosides Nucleotides Nucleic Acids* 2005, 24, 777–781.
- (22) Robins, M. J.; Hansske, F.; Bernier, S. E. *Can. J. Chem.* 1981, 59, 3360–3364.
- (23) Grotli, M.; Douglas, M.; Beijer, B.; Garcia, G.; Eritja, R.; Sproat, B. *J. Chem. Soc., Perkin Trans. 1* 1997, 18, 2779–2788.
- (24) Beigelman, L.; Haeberli, P.; Sweedler, D.; Karpeisky, A. *Tetrahedron* 2000, 56, 1047–1057.
- (25) Sanger, W. *Principles of Nucleic Acid Structure*; Springer: New York, 1984.
- (26) Bordwell, F. G.; Algrim, D.; Vanier, N. R. *J. Org. Chem.* 1977, 42, 1817–1819.
- (27) Velikyan, I.; Acharya, S.; Trifonova, A.; Foeldes, A.; Chattopadhyaya, J. *J. Am. Chem. Soc.* 2001, 123, 2893–2894.
- (28) Nielsen, J.; Taagaard, M.; Marugg, J. E.; van Boom, J. H.; Dahl, O. *Nucleic Acids Res.* 1986, 14, 7391–7403.
- (29) Wu, X.; Pitsch, S. *Nucleic Acids Res.* 1998, 26, 4315–4323.
- (30) Leuck, M.; Wolter, A. *PCT Int. Appl.* 2006, WO 2006/116476 A1 20061102.
- (31) Overhoff, M.; Sczakiel, G. *EMBO Rep* 2005, 6, 1176–1181.
- (32) Shaw, J.-P.; Kent, K.; Bird, J.; Fishback, J.; Froehler, B. *Nucleic Acids Res.* 1991, 19, 747–750.
- (33) Aartsma-Rus, A.; van Ommen, G.-J. B. *RNA* 2007, 13, 1609–1624.
- (34) Lu, Q. L.; Rabinowitz, A.; Chen, Y. C.; Yokota, T.; Yin, H.; Alter, J.; Jadoon, A.; Bou-Gharios, G.; Partridge, T. *Proc. Natl. Acad. Sci. U.S.A.* 2005, 102, 198–203.
- (35) Crooke, S. T.; Graham, M. J.; Zuckerman, J. E.; Brooks, D.; Conklin, B. S.; Cummins, L. L.; Greig, M. J.; Guinosso, C. J.; Kornbrust, D.; Manoharan, M.; Sasmor, H. M.; Schleich, T.; Tivel, K. L.; Griffey, R. H. *J. Pharmacol. Exp. Ther.* 1996, 277, 923–937.
- (36) Marcusson, E. G.; Bhat, B.; Manoharan, M.; Bennett, C. F.; Dean, N. M. *Nucleic Acids Res.* 1998, 26, 2016–2023.
- (37) Arechavala-Gomez, V.; Graham, I. R.; Popplewell, L. J.; Adams, A. M.; Aartsma-Rus, A.; Kinali, M.; Morgan, J. E.; Van Deutekom, J. C.; Wilton, S. D.; Dickson, G.; Muntoni, F. *Hum. Gene Ther.* 2007, 18, 798–810.
- (38) Ciccarone, V.; Chu, Y.; Schifferli, K.; Pichet, J.-P.; Hawley-Nelson, P.; Evans, K.; Roy, L.; Bennett, S. *FOCUS* 1999, 21, 2.
- (39) Araki, E.; Nakamura, K.; Nakao, K.; Kameya, S.; Kobayashi, O.; Nonaka, I.; Kobayashi, T.; Katsuki, M. *Biochem. Biophys. Res. Commun.* 1997, 238, 492–497.
- (40) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* 1981, 22, 1859–1862.
- (41) Nielsen, J.; Taagaard, M.; Marugg, J. E.; van Boom, J. H.; Dahl, O. *Nucleic Acids Res.* 1986, 14, 7391–7403.
- (42) Overhoff, M.; Sczakiel, G. *EMBO Rep.* 2005, 6, 1176–1181.

## ■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published to the Web on March 23, 2011, with errors in schemes 1 and 3. These errors were fixed when the paper was published to the Web on March 29, 2011.

# Identification of Muscle-Specific MicroRNAs in Serum of Muscular Dystrophy Animal Models: Promising Novel Blood-Based Markers for Muscular Dystrophy

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## Abstract

Duchenne muscular dystrophy (DMD) is a lethal X-linked disorder caused by mutations in the *dystrophin* gene, which encodes a cytoskeletal protein, dystrophin. Creatine kinase (CK) is generally used as a blood-based biomarker for muscular disease including DMD, but it is not always reliable since it is easily affected by stress to the body, such as exercise. Therefore, more reliable biomarkers of muscular dystrophy have long been desired. MicroRNAs (miRNAs) are small, ~22 nucleotide, noncoding RNAs which play important roles in the regulation of gene expression at the post-transcriptional level. Recently, it has been reported that miRNAs exist in blood. In this study, we hypothesized that the expression levels of specific serum circulating miRNAs may be useful to monitor the pathological progression of muscular diseases, and therefore explored the possibility of these miRNAs as new biomarkers for muscular diseases. To confirm this hypothesis, we quantified the expression levels of miRNAs in serum of the dystrophin-deficient muscular dystrophy mouse model, *mdx*, and the canine X-linked muscular dystrophy in Japan dog model (CXMD<sub>J</sub>), by real-time PCR. We found that the serum levels of several muscle-specific miRNAs (miR-1, miR-133a and miR-206) are increased in both *mdx* and CXMD<sub>J</sub>. Interestingly, unlike CK levels, expression levels of these miRNAs in *mdx* serum are little influenced by exercise using treadmill. These results suggest that serum miRNAs are useful and reliable biomarkers for muscular dystrophy.

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## Introduction

Duchenne muscular dystrophy (DMD) is a lethal X-linked disorder caused by mutations in the *dystrophin* gene, which encodes a cytoskeletal protein, dystrophin[1]. The absence of dystrophin results in progressive degeneration of skeletal and cardiac muscle with fibrotic tissue replacement, fatty infiltration, and subsequent early death by respiratory or heart failure[2,3]. Creatine kinase (CK) is an enzyme related to energy metabolism present in various types of cells[4]. CK is commonly used as a blood-based biomarker for muscular dystrophy to evaluate the level of muscle damage and necrosis, and the efficacy of potential therapies, but it is not always reliable since it is easily affected by stress to the body, such as exercise[5,6,7]. Other markers for muscular dystrophy, such as myoglobin, aldolase or lactate dehydrogenase, also have the same problem. Therefore, more reliable biomarkers of muscular dystrophy have long been desired.

MicroRNAs (miRNAs) are small, ~22 nucleotide, noncoding RNAs which play important roles in the regulation of gene expression at the post-transcriptional level[8]. Recently, it has been reported that specific miRNAs in blood are promising biomarkers

for cancer, liver injury and heart failure [9,10,11]. These studies showed that the levels of specific circulating miRNAs are associated with the development of these pathological processes. It has also been reported that miRNAs are released from cells through an exosomal-mediated pathway[12], suggesting that circulating miRNAs are packaged in exosomes, which protects them from RNases.

We hypothesized that the expression levels of specific serum circulating miRNAs may be useful to monitor the pathological progression of muscular diseases, and therefore explored the possibility of these miRNAs as new biomarkers for muscular diseases. Here, we demonstrate that the serum levels of several muscle-specific miRNAs are increased in the dystrophin-deficient muscular dystrophy mouse model, *mdx*, as well as the canine X-linked muscular dystrophy in Japan dog model (CXMD<sub>J</sub>) [13,14,15]. These results suggest that serum miRNAs are useful as markers for muscular dystrophy.

## Results

To explore the possibility of miRNA as a biomarker for DMD, we quantified the expression levels of several miRNAs in the serum

of *mdx* by real-time PCR. The expression levels of miRNAs are indicated as either cycle threshold (Ct) (Figure 1a) or fold expression compared to wild-type (Figure S1). The Ct values of the ubiquitously expressed miR-16, brain-rich miR-132 [16] and small nucleolar RNA 202 (sno202) did not show any significant differences between wild-type and *mdx* serum (Figure 1a). In contrast, muscle-specific miR-1, -133a and -206 [17,18,19] were significantly increased in *mdx* (Figure 1a). The expression levels of these miRNAs in *mdx* were 10- to 100-fold higher than in wild-type controls (Figure S1). In Figure 1, the data are shown without normalization by an internal control RNA. Although small nuclear RNA U6, sno202 and ubiquitously expressed miRNA, such as miR-16, are often used as an internal control for miRNA analysis, there is currently no consensus for a serum internal control miRNA for real-time PCR analysis. Indeed, we examined the expression of U6 but found it was undetectable in serum (data not shown), and sno202 and miR-16 revealed no significant difference between wild-type and *mdx* (Figure 1a and Figure S1). In addition, miR-16 was more abundant than sno202 in serum (Figure 1a). Therefore, we employed miR-16 as the internal control for normalization of muscle-specific miRNAs in serum in the subsequent studies.

We also confirmed the accuracy of miR-16 as an internal control by using exogenous miRNA (spiked-in miRNA). *C. elegans* miRNA-39 (cel-miR-39) was used as a spiked-in miRNA because of the lack of sequence homology to mouse miRNAs. Synthetic cel-miR-39 was spiked into serum after the addition of denaturing solution including RNase inhibitors. Then, miRNAs were isolated and the levels of cel-miR-39, miR-16, -1, -133a and -206 were determined by real-time PCR (Figure S2). In three-repeated experiments, the quantities of cel-miR-39 and miR-16 showed similar levels each time (Figure S2a). Furthermore, the expression levels of miR-1, -133a and -206 were calculated by normalization with cel-miR-39 or miR-16, individually (Figure S2b). The expression levels of miR-1, -133a and -206 were highly elevated in *mdx*, and the results were consistent between normalization with cel-miR-39 and miR-16.

It is conceivable that leakage or secretion from skeletal muscle fibers is the major cause of the increase in muscle-specific miRNAs in serum, but there remains the possibility that these miRNAs are excessively expressed in dystrophic skeletal muscle, which then influences serum expression levels. To investigate this possibility, we examined the expression level of these miRNAs in the skeletal muscle (soleus: Sol, tibialis anterior: TA and diaphragm: DIA) of *mdx* (Figure S3). Levels of ubiquitously expressed miR-16 were not different among the muscles examined, but miR-1 and miR-133a were significantly decreased in Sol and TA of *mdx*, although the differences are less than 2-fold. On the other hand, miR-206 was significantly increased in *mdx* TA and DIA, but not in Sol, and this increase of miR-206 in some *mdx* muscles could be related to a previously reported role for miR-206 in muscle regeneration [20]. Since miR-1 and -133a levels were highly elevated in *mdx* serum, although they were not increased in *mdx* skeletal muscle, suggests that the increase of muscle-specific miRNAs in *mdx* serum is caused by an increase in leakage or secretion of miRNAs from muscle.

Since it is very important to investigate whether muscle-specific miRNA levels are affected by exercise like as CK, we compared CK and miRNA levels in mice serum after exercise using a treadmill. Both CK and miRNA were increased after the treadmill exercise (Figure 1b, left, normalized to wild-type control), however miRNAs appeared to be less affected. When the increase in miRNAs were corrected by the data before exercise in each group (Figure 1b, right, normalized by each control), CK showed

almost a 60-fold increase after exercise, whereas the change of muscle-specific miRNA levels was less than 10-fold.

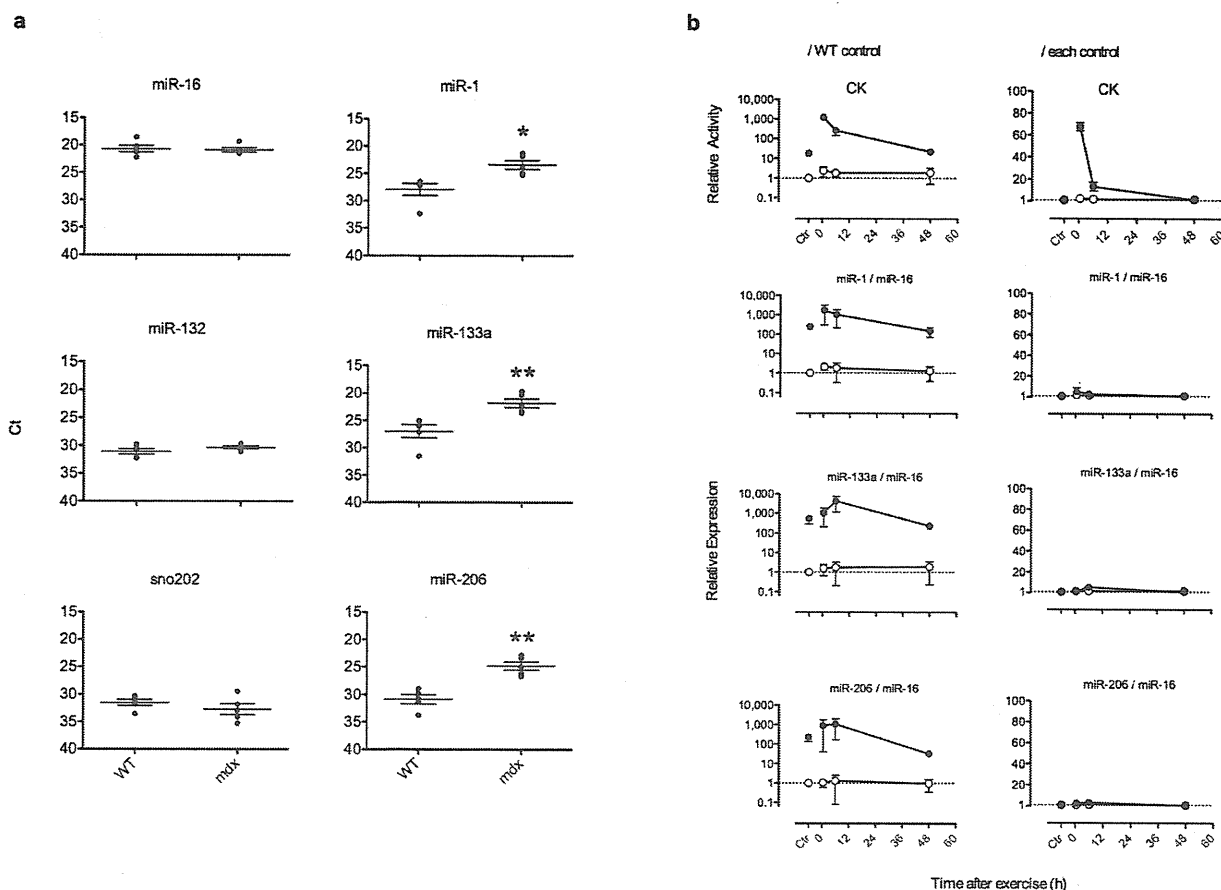
CXMD<sub>J</sub> is a well characterized dog model of DMD, which shows severe and progressive symptoms [13,14,15]. We therefore analyzed the expression levels of miRNAs in normal, carrier (females possessing a mutant *dystrophin* gene on one of two X-chromosomes) and CXMD<sub>J</sub> dog serum at various ages. The Ct value of these miRNAs in CXMD<sub>J</sub> was significantly smaller than age-matched controls (Figure S4). Relative expression levels corrected by miR-16 are shown in Figure 2. These miRNAs are apparently able to distinguish CXMD<sub>J</sub> from age-matched normal dogs. Shimatsu *et al.* previously reported that the CK concentration of CXMD<sub>J</sub> dogs do not increase with age [21]. Thus, our results of these miRNA levels are consistent with the CK levels in this model.

Our data indicate that the levels of miR-1, -133a and -206 relative to miR-16 are increased in the serum of two animal models of muscular dystrophy, *mdx* and CXMD<sub>J</sub>. It is very intriguing that serum miRNA were less affected by stress, such as exercise, compared with CK. In conclusion, muscle-specific miRNAs in serum may be useful biological markers for muscular dystrophy which are more reliable than CK, and further investigations are required to clarify the molecular mechanisms by which miRNAs are released from the inside of cells into serum.

## Discussion

Recently, several studies have reported that miRNAs in serum are promising biomarkers for diseases, such as cancers, liver injury or heart failure [9,10,11]. CK is commonly used as a biomarker of muscular diseases to evaluate the level of muscle damage and necrosis, and the efficacy of potential therapies, but it is not always reliable since it is easily affected by stress to the body, such as exercise [5,6,7]. Therefore, more reliable biomarkers of muscular dystrophy have long been desired. We hence investigated whether serum miRNAs are useful for monitoring the pathological condition of muscular diseases. In this report, we demonstrate that the serum levels of several muscle-specific miRNAs are increased in two dystrophin-deficient muscular dystrophy animal models. Importantly, we show that the levels of these miRNAs are much less affected by stress to the body compared with CK levels.

To investigate the mechanism of the increase of miRNA expression, we also examined the expression level of miR-1, miR-133a and miR-206 in the skeletal muscle of *mdx* (Figure S3). miR-1 and -133a were significantly decreased in Sol and TA of *mdx*. On the other hand, miR-206 was significantly increased in TA and DIA of *mdx*. Our results suggest that the increase of muscle-specific miRNAs in the serum of these DMD models is caused by an increase in leakage or secretion of miRNAs from muscle, and not by the change of expression in skeletal muscle. However, it is not yet clear whether the increase of these miRNAs is caused by leakage or secretion from muscle. It is conceivable that leakage from skeletal muscle fibers is the major cause of the increase in muscle-specific miRNAs in *mdx* serum, but it is hard to explain why these miRNAs were not degraded by RNase. Mitchell *et al.* [10] showed that synthetic miRNAs are immediately degraded in serum even though endogenous circulating miRNAs are stably expressed in serum. To explain these results, they suggested that miRNAs are released from cells through an exosomal-mediated pathway. If circulating miRNAs are secreted by an exosomal-mediated pathway, it is possible that dystrophin is involved in the regulation of exosome secretion and a lack of dystrophin results in increased miRNA release. However, further investigation is required to clarify the contribution of dystrophin in exosome secretion.



**Figure 1. Elevation of muscle-specific miRNA levels in mdx mouse serum.** (a) Expression levels of miRNAs in 8-week old male wild-type and mdx serum. Ct was determined by real-time PCR. In these graphs, the longer bars on each plot indicate the mean, and the shorter bars indicate  $\pm$  SEM, n=5. Asterisk (\*) indicates a significant difference (\*, P<0.05; \*\*, P<0.01, two-tailed Student's t-test.). The actual P value for each test was P=0.797 (miR-16), 0.222 (miR-132), 0.344 (sno202), 0.011 (miR-1), 0.007 (miR-133a) and 0.001 (miR-206). (b) CK and miRNA expression levels in wild-type and mdx serum after treadmill exercise. Running on the treadmill was continued for 20 min. About 100  $\mu$ l of blood was collected from the tail vein at 0.5, 6 and 48 h after the exercise. Six days before the test, blood was collected as a control. Expression levels were normalized to the wild-type control (left) or each individual control (right). Data are presented as mean  $\pm$  SEM, n=3.  $\circ$ : wild-type;  $\bullet$ : mdx. doi:10.1371/journal.pone.0018388.g001

The change of miRNA expression levels in skeletal muscle of mdx in this report is consistent with previous reports [20,22]. It is intriguing that TA muscle of denervated mice also showed an increase in miR-206 and a decrease in miR-1 and -133a [23]. Yuasa *et al.* [20] also showed that miR-206 expression was increased after cardiotoxin-induced muscle regeneration and that miR-206 contributes to muscle regeneration. Interestingly, it has been showed that the expression levels of miR-206 in DMD patients are not increased [24] or that the increase is not as large as in mdx [22]. Although mdx mice are deficient in dystrophin, they do not show lethality unlike in humans. Increased miR-206 expression levels in mdx therefore contribute to the different phenotype between humans and mice. In addition, Williams *et al.* [23] showed that expression of miR-206 delays disease progression and promotes regeneration of neuromuscular synapses in amyotrophic lateral sclerosis (ALS) model mice. Taken together, these results indicate that gene therapy using miR-206 may be a useful treatment for muscular diseases.

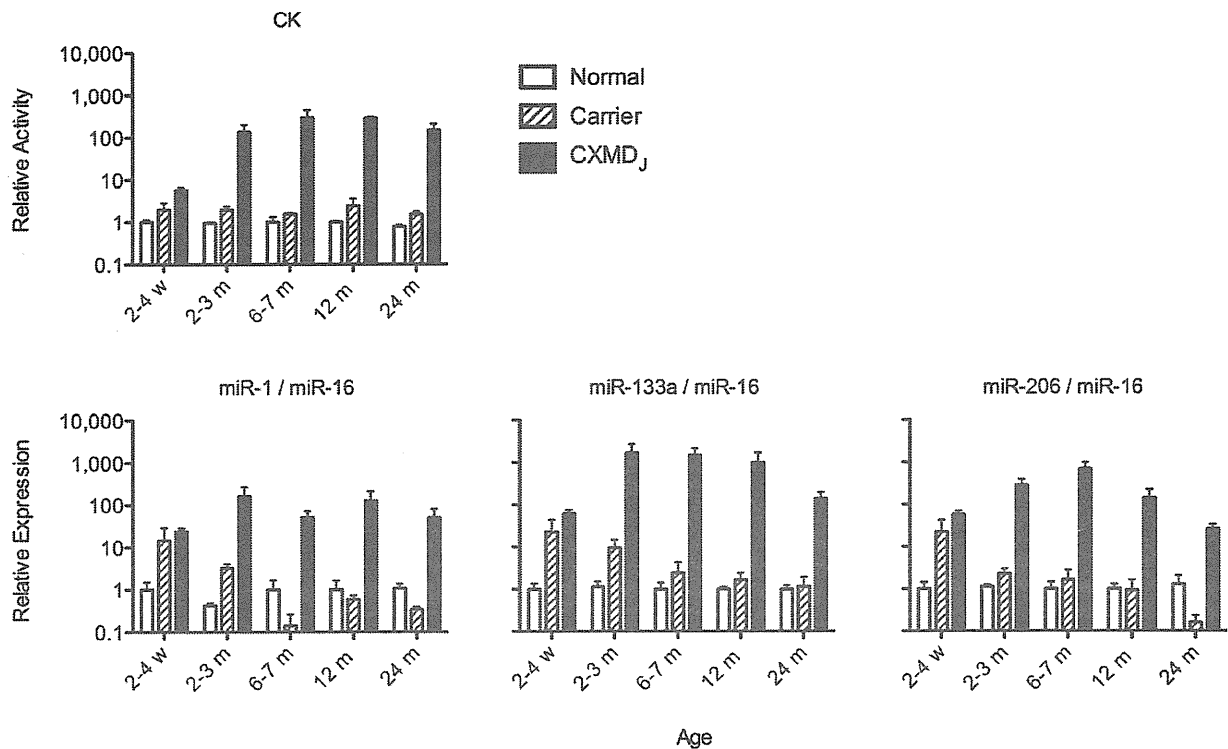
In this report, we focused on muscle-specific miRNAs and found that they are significantly increased in serum of DMD models. To investigate whether such an increase can be observed

in some myopathy models which do not have any effective diagnosis markers, we also measured these muscle-specific miRNAs in serum of steroid treated dogs. We found that serum level of miR-1, -133a and -206 were not increased in steroid treated dog did not show increase compared with non-treatment controls (data not shown). Intriguingly, Lodes *et al.* [25] performed microarray analysis with circulating miRNAs and found an increase in specific miRNAs in serum of cancer patients. Furthermore the miRNA expression patterns were able to discriminate between healthy controls and cancer patients. Such a microarray analysis may be useful for identifying diagnosis markers for muscular diseases for which effective diagnosis markers currently do not exist.

## Materials and Methods

### Ethics Statement

The dog study was approved by the Ethics Committee for the Treatment of Middle-sized Laboratory Animals of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, approval ID: 21-02 and 22-02. The mice study was



**Figure 2. Elevation of muscle-specific miRNAs in CXMD<sub>J</sub> dog serum.** CK activity and miRNA expression in the serum of normal, carrier and dystrophy dogs (CXMD<sub>J</sub>) at the indicated ages were determined. Expression levels of miR-1, miR-133a, miR-206 and miR-16 were determined by real-time PCR, and levels of each muscle-specific miRNA (miR-1, miR-133a and miR-206) was corrected by miR-16 levels. Results are indicated as relative expression to normal dogs at each age, and are presented as mean  $\pm$  SEM,  $n=3$ . w: weeks; m: months. doi:10.1371/journal.pone.0018388.g002

approved by the Ethics Committee for the Treatment of Laboratory Animals of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, approval ID: 2008011.

#### Animals and serum samples

All animals in this study were cared for and treated in accordance with the guidelines provided by the Ethics Committee for the Treatment Laboratory Animals of National Institute of Neuroscience, or the Ethics Committee for the Treatment Laboratory Middle-sized Animals of National Institute of Neuroscience. Skilled experimental animal technicians, who have special knowledge of methods to prevent unnecessary excessive pain, handled the animals and assisted in the experiments.

As Duchenne muscular dystrophy (DMD) models, the X-chromosome-linked muscular dystrophy (*mdx*) mouse and canine X-linked muscular dystrophy in Japan (CXMD<sub>J</sub>), Beagle-based medium-sized dystrophic dogs, were used in this study. In **Figure 1a**, whole body blood of male *mdx* mice ( $n=5$ ) or age-matched controls (strain C57BL/10; B10) ( $n=5$ ) at 8 weeks were collected from the abdominal vena cava under anesthesia. Blood collection after the treadmill test (**Figure 1b**), was performed from the tail vein of *mdx* ( $n=3$ ) or age-matched control ( $n=3$ ) under anesthesia. The phenotype of CXMD<sub>J</sub> has been reported previously [13,14,15]. For analysis of the serum of CXMD<sub>J</sub> dogs, mutation carrier female dogs and wild-type control dogs ( $n=3$ , at each age indicated in Figure 2), blood was collected from the subcutaneous vein of the hindlimb, and whole blood was allowed

to stand for about 1 h at room temperature before centrifugation at 1,800 g for 10 min at room temperature. The resultant serum was dispensed into a 1.5 ml cryotube and stored at  $-80^{\circ}\text{C}$  until use.

#### RNA isolation and quantification of miRNA

Total RNA, including miRNA, was extracted from 50  $\mu\text{l}$  of serum using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and finally eluted with 50  $\mu\text{l}$  of elution buffer provided by the manufacturer. Five  $\mu\text{l}$  of total RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA) and miRNA-specific stem-loop primers (part of TaqMan miRNA assay kit; Applied Biosystems). The expression levels of miRNA were quantified by real-time PCR using individual miRNA-specific primers (part of TaqMan miRNA assay kit; Applied Biosystems) with 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. There is no current consensus on the use of an internal control for real-time PCR analysis of serum miRNA. Therefore, we used fixed volumes of starting serum (50  $\mu\text{l}$ ), buffer for the elution of RNA (50  $\mu\text{l}$ ) from starting serum, and input into the RT reaction (5  $\mu\text{l}$ ) in each assay for technical consistency. Data analysis was performed by SDS 2.1 real-time PCR data analysis software (Applied Biosystems). Threshold was fixed at 0.2 in each analysis for data consistency. The similarities of linearity of primers for each target miRNA were confirmed by using a dilution series of synthetic miRNAs.



### Spiked-in miRNA experiment

We followed the protocol previously reported by Mitchell *et al.* [10] to determine endogenous miRNA levels with spiked-in miRNA. Spiked-in miRNA was designed against *C. elegans* microRNA-39 (cel-miR-39)(5'-UCACCGGGUGUAAAUCAGCUU-3'), and was synthesized by Sigma Aldrich Japan. Synthetic cel-miR-39 was spiked into serum after the addition of denaturing solution including RNase inhibitors. Isolation of total RNA, including miRNA, and quantification of the expression levels of miRNAs by real-time PCR were performed as described above.

### Creatine kinase determination

Serum creatine kinase (CK) levels were assayed with the Fuji Drychem system (Fuji Film Medical Co. Ltd, Tokyo, Japan) according to the manufacturer's instructions. Data was expressed as units per liter (U/l).

### Treadmill test

Mice were forced to run on a treadmill (MK-680S treadmill: Muromachi Kikai, Tokyo, Japan) with an inclination of 0° at 5 m/min for 5 min. Then, the speed was increased by 1 m/min every minute for a further 15 min. After the running, blood was immediately collected from the tail vein, as well as subsequently collected at the indicated times.

### Statistics

Statistical significances between groups were determined by the two-tailed t-test, or one-way ANOVA with Bonferroni post hoc test. Each analysis was performed by Prism 5 (Graphpad Software Inc., San Diego, CA, USA).

### Supporting Information

**Figure S1 miRNA expression in 8-week old male wild-type control and *mdx* serum.** Expression levels of miRNAs were determined by real-time PCR. Results are shown as relative expression, and data are presented as mean  $\pm$  SEM, n = 5. (TIFF)

**Figure S2 (a) Confirmation of the consistency of miRNA isolation from serum.** *C. elegans* miR-39 (cel-miR-39) was chemically synthesized and added to the denatured mouse serum samples. Total RNA was isolated from the mouse serum samples,

### References

- Moser H (1984) Duchenne muscular dystrophy: pathogenetic aspects and genetic prevention. *Hum Genet* 66: 17–40.
- Cullen MJ, Mastaglia FL (1980) Morphological changes in dystrophic muscle. *Br Med Bull* 36: 145–152.
- Ervasti JM, Ohlendieck K, Kahl SD, Gaver MG, Campbell KP (1990) Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* 345: 315–319.
- Ebashi S, Toyokura Y, Momoi H, Sugita H (1959) High creatine phosphokinase activity of sera of progressive muscular dystrophy. *J Biochem* 46: 103–104.
- Vassella F, Richterich R, Rossi E (1965) The Diagnostic Value of Serum Creatine Kinase in Neuromuscular and Muscular Disease. *Pediatrics* 35: 322–330.
- Florence JM, Fox PT, Planer GJ, Brooke MH (1985) Activity, creatine kinase, and myoglobin in Duchenne muscular dystrophy: a clue to etiology? *Neurology* 35: 758–761.
- Nicholson GA, Morgan GJ, Meerkin M, Strauss ER, McLeod JG (1986) The effect of aerobic exercise on serum creatine kinase activities. *Muscle Nerve* 9: 820–824.
- Chen K, Rajewsky N (2007) The evolution of gene regulation by transcription factors and microRNAs. *Nat Rev Genet* 8: 93–103.
- Ji X, Takahashi R, Hiura Y, Hirokawa G, Fukushima Y, et al. (2009) Plasma miR-208 as a Biomarker of Myocardial Injury. *Clin Chem* 55: 1944–1949.
- Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, et al. (2008) Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA* 105: 10513–10518.

and the quantity of exogenous cel-miR-39 and endogenous miR-16 were determined by real-time PCR. (b) Expression levels of miR-1, -133a and -206 in wild-type control and *mdx* serum, which were individually normalized by the cel-miR-39 spiked-in control or the endogenous control, miR-16. Results are shown as relative expression. The longer bars on each plot indicate the mean, and the shorter bars indicate  $\pm$  SEM, n = 3. (TIFF)

**Figure S3 miRNA expression in wild-type control and *mdx* muscles.** Expression levels of miR-1, -16, -133a and -206 in Soleus (Sol), tibialis anterior (TA) and diaphragm (DIA) were determined by real-time PCR. Results are shown as relative expression. sno202 was used as an internal control. Data are presented as mean  $\pm$  SEM, n = 4. Asterisk (\*) indicates a significant difference (\*, P < 0.05; P < 0.01, two-tailed Student's t-test). The actual P value for each test was P = 0.024 (miR-1) and 0.010 (miR-206) in Sol; P = 0.002 (miR-1), 0.008 (miR-133a) and < 0.001 (miR-206) in TA; P = 0.006 (miR-206) in DIA. (TIFF)

**Figure S4 Expression levels of muscle-specific miRNAs in the serum of normal, carrier and dystrophy dogs (CXMD<sub>J</sub>) at the indicated ages.** Each Ct was determined by real-time PCR. In these graphs, the longer bars on each plot indicate the mean, and the shorter bars indicate  $\pm$  SEM, n = 3. Asterisk (\*) and pound (#) indicate a significant difference (\*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001 from normal; #, P < 0.05; ##, P < 0.01; ###, P < 0.001 from carrier, one-way ANOVA with Bonferroni post hoc test). w: weeks; m: months. (TIFF)

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### Author Contributions

Conceived and designed the experiments: HM AN ST KH. Performed the experiments: HM YA NI SK. Analyzed the data: HM KH. Contributed reagents/materials/analysis tools: KY MS AN. Wrote the paper: HM.

19. McDanel TG, Smith TPL, Doumit ME, Miles JR, Coutinho LL, et al. (2009) MicroRNA transcriptome profiles during swine skeletal muscle development. *BMC Genomics* 10: 77.
20. Yuasa K, Hagiwara Y, Ando M, Nakamura A, Takeda S, et al. (2008) MicroRNA-206 is highly expressed in newly formed muscle fibers: implications regarding potential for muscle regeneration and maturation in muscular dystrophy. *Cell Struct Funct* 33: 163–169.
21. Shimatsu Y, Yoshimura M, Yuasa K, Urasawa N, Tomohiro M, et al. (2005) Major clinical and histopathological characteristics of canine X-linked muscular dystrophy in Japan, CXMDJ. *Acta Myol* 24: 145–154.
22. Greco S, De Simone M, Colussi C, Zaccagnini G, Fasanaro P, et al. (2009) Common micro-RNA signature in skeletal muscle damage and regeneration induced by Duchenne muscular dystrophy and acute ischemia. *FASEB J* 23: 3335–3346.
23. Williams AH, Valdez G, Moresi V, Qi X, McAnally J, et al. (2009) MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science* 326: 1549–1554.
24. Eisenberg I, Eran A, Nishino I, Moggio M, Lamperti C, et al. (2007) Distinctive patterns of microRNA expression in primary muscular disorders. *Proc Natl Acad Sci U S A* 104: 17016–17021.
25. Lodes MJ, Caraballo M, Suci D, Munro S, Kumar A, et al. (2009) Detection of cancer with serum miRNAs on an oligonucleotide microarray. *PLoS ONE* 4: e6229.

# Matrix metalloproteinase-2 ablation in dystrophin-deficient *mdx* muscles reduces angiogenesis resulting in impaired growth of regenerated muscle fibers

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Matrix metalloproteinases (MMPs) are a family of endopeptidases classified into subgroups based on substrate preference in normal physiological processes such as embryonic development and tissue remodeling, as well as in various disease processes via degradation of extracellular matrix components. Among the MMPs, MMP-9 and MMP-2 have been reported to be up-regulated in skeletal muscles in the lethal X-linked muscle disorder Duchenne muscular dystrophy (DMD), which is caused by loss of dystrophin. A recent study showed that deletion of the *MMP9* gene in *mdx*, a mouse model for DMD, improved skeletal muscle pathology and function; however, the role of MMP-2 in the dystrophin-deficient muscle is not well known. In this study, we aimed at verifying the role of MMP-2 in the dystrophin-deficient muscle by using *mdx* mice with genetic ablation of MMP-2 (*mdx/MMP-2*<sup>-/-</sup>). We found impairment of regenerated muscle fiber growth with reduction of angiogenesis in *mdx/MMP-2*<sup>-/-</sup> mice at 3 months of age. Expression of vascular endothelial growth factor-A (VEGF-A), an important angiogenesis-related factor, decreased in *mdx/MMP-2*<sup>-/-</sup> mice at 3 months of age. MMP-2 had not a critical role in the degradation of dystrophin–glycoprotein complex (DGC) components such as  $\beta$ -dystroglycan and  $\beta$ -sarcoglycan in the regeneration process of the dystrophic muscle. Accordingly, MMP-2 may be essential for growth of regenerated muscle fibers through VEGF-associated angiogenesis in the dystrophin-deficient skeletal muscle.

## INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most common severe X-linked muscular disorder, characterized by progressive muscle wasting and weakness (1). It is caused by a mutation in the *DMD* gene encoding the large cytoskeletal protein dystrophin (2). Dystrophin localizes to the sarcolemma of muscle fibers and forms a dystrophin–glycoprotein complex (DGC) with dystroglycans (DGs), sarcoglycans and syntrophin–dystrobrevin complexes, and DGC links the cytoskeletal protein actin to the basal lamina of muscle fibers (3). DGC may play a role in membrane stabilization during muscle

contraction or act as a transducer of signals from the extracellular matrix (ECM) to the muscle cytoplasm via its interactions with intracellular signaling molecules (4). The loss of dystrophin leads to a condition in which the membrane is leaky under mechanical stress, and the subsequent increase in Ca<sup>2+</sup> permeability results in activation of various proteases and alteration of the expression or function of dystrophin-associated plasma membrane proteins, such as neuronal nitric oxide synthase (nNOS), aquaporin-4 and ion channels (5). The pathology of the dystrophic muscle includes degeneration, necrosis with inflammatory cell invasion, regeneration and fibrous and fatty changes. However, the underlying

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mechanisms of ECM degradation, inflammation and fibrosis remain poorly understood.

ECM components have important roles in homeostasis and maintenance of muscle fiber functional integrity. Matrix metalloproteases (MMPs), a family of zinc-dependent endopeptidases, are key regulatory molecules in the formation, remodeling and degradation of ECM components in both physiological and pathological processes (6,7), such as tumor progression and metastasis, cerebrovascular and cardiovascular diseases and rheumatoid arthritis (8). Among the MMPs, MMP-2 (also called gelatinase A) and MMP-9 (also called gelatinase B) are involved in ECM remodeling of the skeletal muscle and in the dystrophic pathology of *mdx*, a mouse model for DMD (9). MMP-9 is produced by neutrophils and macrophages and is able to process  $\beta$ -DG, resulting in disruption of the link between the ECM and cell membrane in the dystrophin-deficient skeletal muscle from DMD patients (10–12). We previously reported that MMP-9 overexpresses in the *mdx* skeletal muscle after physical exercise (13) and may primarily be involved in the inflammatory process during muscle degeneration (14). Recently, it was reported that deletion of the *MMP9* gene in *mdx* mice reduces muscle inflammation and fibronecrosis, resulting in an improvement in muscle function (15). Based on these reports, MMP-9 may play an important role in ECM degradation in the dystrophin-deficient muscle.

MMP-2 is derived from vascular endothelial cells as well as smooth muscle cells and has a role in angiogenesis, based on observation of impaired angiogenesis in an ischemic model in MMP-2 knockout mice (16). We and other researchers have also demonstrated that MMP-2 may be associated with ECM remodeling during muscle regeneration and fiber growth (9,14). Moreover, MMP-2 may be involved in myogenesis (17) and muscle regeneration (18). However, the precise role of MMP-2 has not been fully elucidated in the dystrophic muscle. To better understand the role of MMP-2 in dystrophic pathology, we generated *mdx/MMP-2<sup>-/-</sup>* mice by crossing *mdx* with MMP-2 knockout mice (MMP-2<sup>-/-</sup>) and examined the muscle pathology. Here, we demonstrate that MMP-2 ablation in *mdx* mice results in impairment of muscle fiber growth related to down-regulation of vascular endothelial growth factor-A (VEGF-A) when compared with *mdx/MMP-2<sup>+/+</sup>* mice. Moreover, expression of nNOS in 3-month-old *mdx/MMP-2<sup>-/-</sup>* mice was significantly lower than that in *mdx* mice. These results imply that MMP-2 may be required for growth of regenerated muscle fibers through angiogenesis by VEGF-A (hereafter, VEGF) in the dystrophin-deficient muscle.

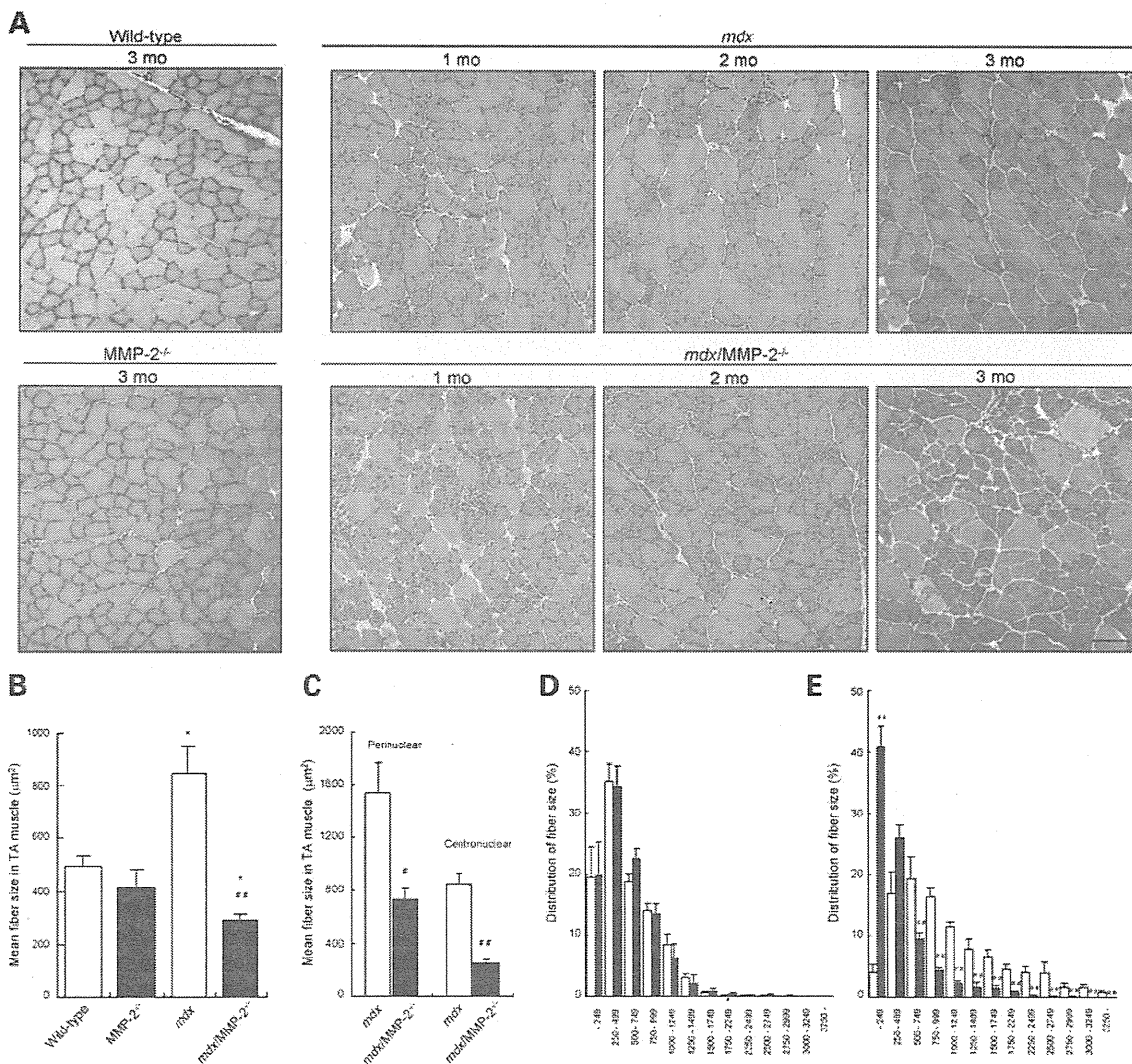
## RESULTS

### MMP-2 ablation in *mdx* mice impairs growth of regenerated muscle fibers

To elucidate the role of MMP-2 in the dystrophin-deficient muscle, we crossed MMP-2 knockout (MMP-2<sup>-/-</sup>) mice (19) with *mdx* mice with the genetic background of C57 BL/6J to generate *mdx/MMP-2<sup>-/-</sup>* mice. MMP-2 mRNA and its protein activity were absent in the skeletal muscle of both MMP-2<sup>-/-</sup> and *mdx/MMP-2<sup>-/-</sup>* mice, but not in wild-type (WT) and *mdx* mice (Supplementary Material, Fig. S1A and

C). There was no statistically significant difference in the MMP-9 mRNA level in the skeletal muscle between the groups at 1 and 3 months of age (Supplementary Material, Fig. S1B). Although we could not detect MMP-9 protein activity on gelatin zymography in mice aged 3 months (Supplementary Material, Fig. S1C), the levels of both the pro- and active forms of MMP-9 protein levels were found to be significantly increased in *mdx* and *mdx/MMP-2<sup>-/-</sup>* mice when compared with the WT and MMP-2<sup>-/-</sup> mice at both 1 and 3 months of age (Supplementary Material, Fig. S1D–F). The discrepancy between the mRNA and protein levels of MMP-9 in the dystrophic skeletal muscle is speculated to be due to post-transcriptional regulation (20). MMP-3 mRNA levels were significantly elevated in the skeletal muscle of *mdx* and *mdx/MMP-2<sup>-/-</sup>* mice at 1 and 3 months of age when compared with that of the WT mice; the level was greatly increased in *mdx/MMP-2<sup>-/-</sup>* mice at 3 months of age (Supplementary Material, Fig. S1G). The mRNA levels of tissue inhibitor of metalloproteinase (TIMP)-1, an intrinsic inhibitor of MMP-9 and -3, were significantly up-regulated in the skeletal muscle of *mdx* and *mdx/MMP-2<sup>-/-</sup>* mice when compared with that of the WT mice at 1 and 3 months of age (Supplementary Material, Fig. S1H). There was no difference in the mRNA levels of TIMP-2, an intrinsic inhibitor of MMP-2, among the mouse groups at 1 or 3 months of age (Supplementary Material, Fig. S1I). In both MMP-2<sup>-/-</sup> mice (19) and *mdx/MMP-2<sup>-/-</sup>* mice, fertility and development were glossy normal. There were no differences in body weight at 1 or 3 months of age between *mdx* and *mdx/MMP-2<sup>-/-</sup>* or between WT and MMP-2<sup>-/-</sup> mice (Supplementary Material, Fig. S2A). Muscle power, evaluated using the hanging wire test and serum creatine kinase (CPK) levels, in *mdx/MMP-2<sup>-/-</sup>* at 3 months of age was not statistically different from that of *mdx* at the same age (Supplementary Material, Fig. S2B and C).

We then examined the histopathology of the skeletal muscle from *mdx/MMP-2<sup>-/-</sup>* mice. A previous study of *mdx* mice reported muscle necrosis with infiltration of neutrophils or macrophages at around 2 weeks of age, massive muscle degeneration/necrosis at around 1 month of age and completion of muscle necrosis with substitution of many regenerated fibers at 3 months of age (21). Histopathological findings for the tibialis anterior (TA) muscle from *mdx/MMP-2<sup>-/-</sup>* mice at 1, 2 and 3 months of age were roughly comparable to those of *mdx* mice at each age (Fig. 1A). However, we found that the muscle fibers, especially the centronuclear regenerated fibers, in *mdx/MMP-2<sup>-/-</sup>* mice at 3 months of age were significantly smaller than in *mdx* mice at the same age (Fig. 1B–E). Almost all of the small regenerated fibers in *mdx/MMP-2<sup>-/-</sup>* mice at 3 months of age were type II fibers, and a similar tendency was observed in *mdx* mice at the same age (Supplementary Material, Fig. S2D). We also analyzed diaphragm, quadriceps and gastrocnemius muscles in *mdx* and *mdx/MMP-2<sup>-/-</sup>* mice at 1 and 3 months of age. Like the TA muscle, these skeletal muscles in *mdx/MMP-2<sup>-/-</sup>* mice at 3 months of age exhibited impaired growth of regenerated fibers (Supplementary Material, Fig. S3). Furthermore, we monitored the muscle regeneration process as a result of cardiotoxin injury in the skeletal muscle of MMP-2<sup>-/-</sup> mice at 6 weeks of age. The result clearly



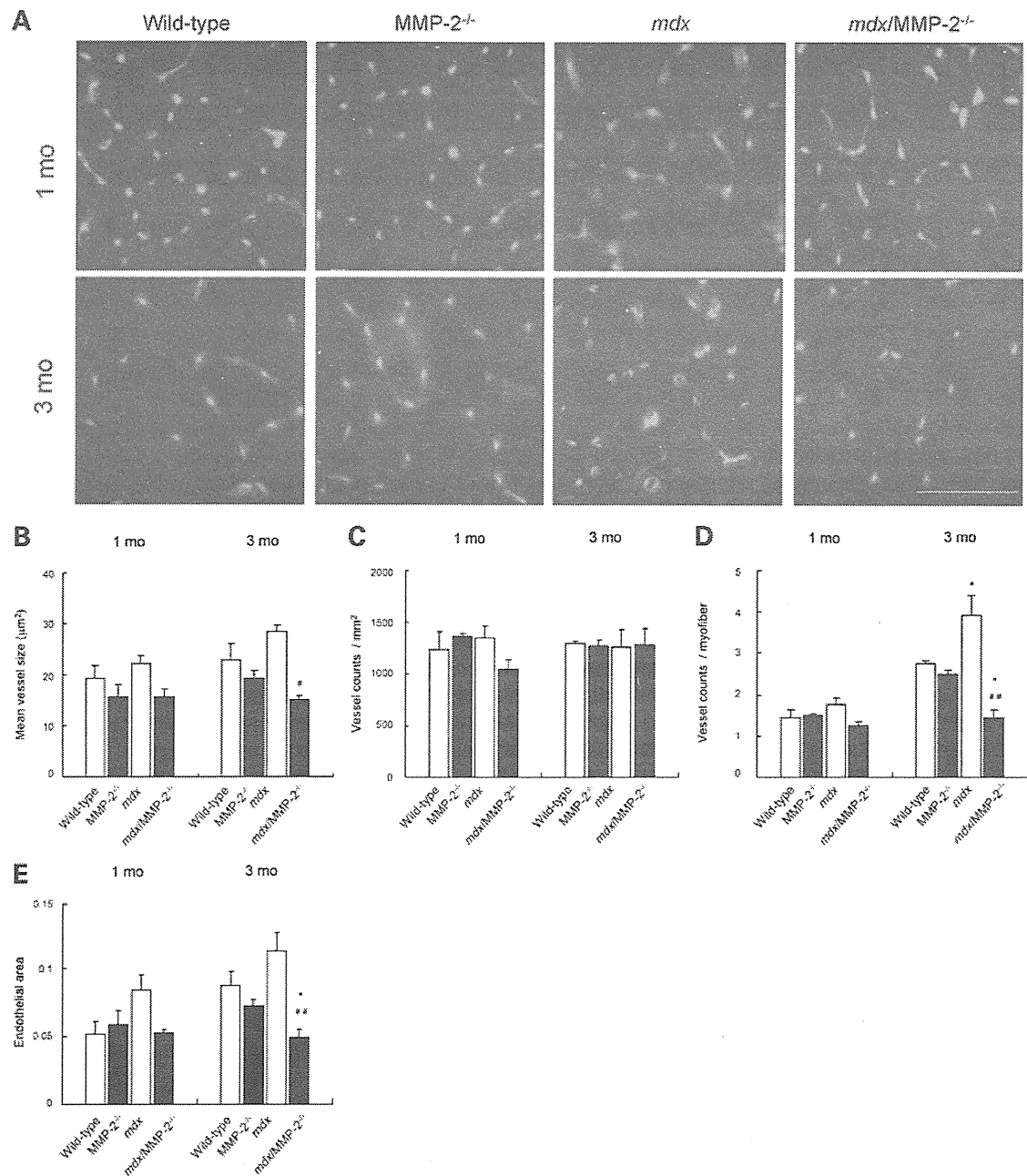
**Figure 1.** Histopathological characterization of the skeletal muscle of *mdx/MMP-2<sup>-/-</sup>* mice. (A) H&E staining of the TA muscles in *mdx* and *mdx/MMP-2<sup>-/-</sup>* mice at 1, 2 and 3 months of age. Scale bar: 100 μm. (B) The fiber size in the TA muscle at 3 months of age ( $n = 3$  for each group). Bar: mean  $\pm$  S.E.M.; \* $P < 0.05$ , significantly different from WT;  $^{##}P < 0.01$ , significantly different from MMP-2 ablation alone. (C) Comparison of perinuclear fibers and centronuclear regenerated fibers in the TA muscle at 3 months of age ( $n = 3$  for each group). Bar: mean  $\pm$  S.E.M.; # $P < 0.05$ ,  $^{###}P < 0.01$ , significantly different from MMP-2 ablation alone. (D) Distribution of fiber size of the TA muscle in WT and MMP-2<sup>-/-</sup> mice at 3 months of age. Bar: mean  $\pm$  S.E.M. (E) Distribution of fiber size of the TA muscle in *mdx* and *mdx/MMP-2<sup>-/-</sup>* mice at 3 months of age. Bar: mean  $\pm$  S.E.M.;  $^{###}P < 0.01$ , significantly different from MMP-2 ablation alone.

indicated that the centronuclear regenerating fibers 7 days after cardiotoxin injection in the skeletal muscle of MMP-2<sup>-/-</sup> mice were smaller than those of the WT (Supplementary Material, Fig. S4). These results suggest that ablation of MMP-2 may impair the growth of regenerated muscle fibers after the damage.

#### MMP-2 deficiency impairs angiogenesis in regenerated skeletal muscles of *mdx*

It has been reported that MMP-2 may be associated with angiogenesis, based on inhibition studies of MMP-2 (22,23). MMP-2 knockout mice have shown impairment of

angiogenesis in an ischemia-induced model (16) and in a tumor model (24). To determine whether angiogenesis was impaired in the skeletal muscle of *mdx/MMP-2<sup>-/-</sup>* mice, we examined vessels in the TA muscle, through immunohistochemistry using an antibody to PECAM-1: an epithelial cell marker (Fig. 2A). We found that the mean size of vessels was significantly smaller in the skeletal muscle of *mdx/MMP-2<sup>-/-</sup>* mice at 3 months of age than that of age-matched *mdx* mice (Fig. 2B). Although the number of vessels per square millimeter was not different, the vessel counts per myofiber were significantly higher in *mdx* mice at 3 months of age, but decreased in age-matched *mdx/MMP-2<sup>-/-</sup>* mice (Fig. 2C and D). We also determined the endothelial area, which is



**Figure 2.** Evaluation of angiogenesis in the skeletal muscle of *mdx/MMP-2<sup>-/-</sup>* mice. (A) Immunohistochemistry with PECAM-1, an epithelial marker, in the TA muscle at 1 and 3 months of age. Scale bar: 100 µm. (B) Mean vessel size in the TA muscle of *mdx/MMP-2<sup>-/-</sup>* mice at 1 and 3 months of age ( $n = 3$  for each group). Bar: mean  $\pm$  S.E.M.;  $^{\#}P < 0.05$ , significantly different from MMP-2 ablation alone. (C) Number of vessels per square millimeter in the TA muscle at 1 and 3 months of age ( $n = 3$  for each group). Bar: mean  $\pm$  S.E.M. (D) Number of vessels per myofiber in the TA muscle at 1 and 3 months of age ( $n = 3$  for each group). Bar: mean  $\pm$  S.E.M.;  $^*P < 0.05$ , significantly different from WT;  $^{\#\#}P < 0.01$ , significantly different from MMP-2 ablation alone. (E) Endothelial area in the TA muscle of *mdx/MMP-2<sup>-/-</sup>* mice at 1 and 3 months of age ( $n = 3$  for each group). Bar: mean  $\pm$  S.E.M.;  $^*P < 0.05$ , significantly different from WT;  $^{\#\#}P < 0.01$ , significantly different from MMP-2 ablation alone.

equivalent to vascular bed, by multiplying the size of vessels by their number in the TA muscle. The endothelial area in *mdx/MMP-2<sup>-/-</sup>* mice at 3 months of age was significantly

lower than that of the *mdx* mice at the same age (Fig. 2E). These results clearly indicate that MMP-2 ablation impairs angiogenesis in the *mdx* skeletal muscle.