GENERAL ARTICLE

Mineralocorticoid receptor antagonists improve membrane integrity independent of muscle force in muscular dystrophy

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Abstract

Mineralocorticoid receptor (MR) drugs have been used clinically for decades to treat cardiovascular diseases. MR antagonists not only show preclinical efficacy for heart in Duchenne muscular dystrophy (DMD) models but also improve skeletal muscle force and muscle membrane integrity. The mechanisms of action of MR antagonists in skeletal muscles are entirely unknown. Since MR are present in many cell types in the muscle microenvironment, it is critical to define cell-intrinsic functions in each cell type to ultimately optimize antagonist efficacy for use in the widest variety of diseases. We generated a new conditional knockout of MR in myofibers and quantified cell-intrinsic mechanistic effects on functional and histological parameters in a DMD mouse model. Skeletal muscle MR deficiency led to improved respiratory muscle force generation and less deleterious fibrosis but did not reproduce MR antagonist efficacy on membrane susceptibility to induced damage. Surprisingly, acute application of MR antagonist to muscles led to improvements in membrane integrity after injury independent of myofiber MR. These data demonstrate that MR antagonists are efficacious to dystrophic skeletal muscles through both myofiber intrinsic effects on muscle force and downstream fibrosis and extrinsic functions on membrane stability. MR antagonists may therefore be applicable for treating more general muscle weakness and possibly other conditions that result from cell injuries.

Introduction

Mineralocorticoid receptor (MR) antagonists are Food and Drug Administration-approved drugs that have a long history of safety and efficacy for treating heart failure. These drugs block activation of the MR by the endogenous mineralocorticoid

agonist aldosterone and prevent these steroid hormone receptors from translocating to the nucleus and regulating gene transcription (1). Chronic over-activation of MR by the natural hormone aldosterone is known to exacerbate cell damage in cardiovascular diseases and lead to fibrosis (2,3). Angiotensin-converting enzyme inhibitors act upstream of the MR to inhibit

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aldosterone production and have been shown to delay cardiac dysfunction in Duchenne muscular dystrophy (DMD) patients (4,5). We have repeatedly demonstrated that treatment with an MR antagonist plus an angiotensin-converting enzyme inhibitor results in therapeutic benefits on both heart and skeletal muscles in mouse models of DMD, although a role for MR in skeletal muscles has not been previously investigated (6–8).

DMD is a degenerative disease of striated muscles caused by a loss of function in the dystrophin protein. Dystrophin anchors the dystrophin-associated glycoprotein complex at striated muscle membranes. Without this complex, the muscle membrane is fragile and easily ruptures when forces act upon it (9). Continual damage of skeletal muscle membranes results in rounds of myofiber degeneration, immune cell infiltration, fibroblast proliferation, extracellular matrix deposition and regeneration (10–12). After many rounds of the damage response, skeletal muscles lose regenerative potential and DMD patients suffer from severe skeletal muscle weakness (13).

DMD patients typically become dependent on a wheelchair by their early teens, require ventilatory support in their late teens and develop cardiomyopathy by the age of 20 years (14–16). The average lifespan of DMD patients is currently only 25 years (17). Our team has already translated the efficacy of MR antagonists observed for DMD cardiomyopathy from mouse models to a published 1-year, double-blind, placebo-controlled clinical trial in non-ambulatory DMD patients where significant efficacy on cardiac outcomes was demonstrated (8,18).

The observed preclinical efficacy of MR antagonists on dystrophic skeletal muscle function and pathology was a surprise, given that MR had never been identified in skeletal muscles (7,19). We have now demonstrated that the MR is present in normal and dystrophic skeletal muscles, that the MR functions in regulation of gene expression in normal human myotubes and that MR antagonist treatment of dystrophic mice leads to conserved *in vivo* gene expression changes (19,20). We have also shown that MR antagonists prevent ongoing dystrophic muscle damage *in vivo* and *in vitro*, supporting these drugs act at an early stage of the pathogenic process (6,7,20). We have previously demonstrated that inflammatory cells present in damaged muscles contain high levels of the enzyme required for aldosterone synthesis and that local levels of aldosterone are increased in dystrophic muscles and therefore may contribute to chronic muscle damage (21).

We investigated the myofiber MR in muscular dystrophy because it is the cell type within skeletal muscle that produces muscle force and has a fragile muscle membrane in DMD. We tested the hypothesis that conditional knockout of the MR in the myofibers of *mdx* muscular dystrophy mice would reduce ongoing muscle damage, immune cell and fibroblast infiltration and fibrosis and improve skeletal muscle strength. Information about the role of MR in skeletal muscle will provide the basis for modulating MR as a therapeutic target for a wide range of muscle pathologies.

Results

Validation of MR conditional knockout model and analysis of dystrophinopathy modifiers

To investigate the function of the myofiber MR in dystrophinopathy, we generated a mouse model with a conditional knockout of the myofiber MR (MRcko) using an MR floxed allele and Cre recombinase under the regulation of the muscle creatine kinase

promoter (MCK-Cre). We validated excision of the MR locus with polymerase chain reaction (PCR) of DNA isolated from skeletal muscles of MRcko mice. The MR excision PCR product indicating a null allele was only present in striated muscles of mice that had the MCK-Cre transgene (Fig. 1A). The MR null allele PCR product resulted from excision of the floxed allele by Cre recombinase. There was strong excision in 69 out of the 71 tested mice. Since many cell types express MR in the muscle microenvironment, including immune cells, endothelial cells and fibroblasts, and MR protein has low stability, we next tested the result of MR excision through MR expression in myofibers (22–29). We isolated single myofibers and performed quantitative PCR (qPCR) to detect levels of MR transcripts (Fig. 1B; 30,31). Greatly reduced expression of MR in the MRcko-isolated single myofibers compared with Cre– littermates further supports efficient knockout ($P = 0.0003$).

There was no observed difference in muscle histology, fiber-type, weight or glucose tolerance in MRcko mice on a wild-type background compared to Cre– mice at baseline (data not shown). We then crossed the MRcko mice onto an *mdx* dystrophin-deficient mouse background to generate MR flox/flox, MCK-Cre+, *mdx* (MRcko/*mdx*) experimental mice and MR flox/flox, *mdx* (Cre–/*mdx*) littermate controls. We validated that there was excision of the MR floxed allele in diaphragm, tibialis anterior and flexor digitorum brevis skeletal muscles of MRcko/*mdx* mice (Supplementary Material, Fig. S1).

To determine if the MRcko/*mdx* mice on the mixed C57BL6/C57BL10 background had known modifiers of muscular dystrophy segregating in the mouse liters, we tested our mice for previously identified annexin A6 (*Anxa6*) and latent-transforming growth factor β -binding protein 4 (*LTBP4*) muscular dystrophy modifiers (32,33). All tested mice including C57BL/10 wild-type mice had the *Anxa6* susceptible allele (Fig. 1C). These data suggest that only the susceptible allele of *Anxa6* was present in MRcko/*mdx* mice (Fig. 1C). DNA isolated from a SV129 mouse had the protective *Anxa6* allele as expected. All of the mice tested had the *LTBP4* protective allele as expected, since the susceptible allele of *LTBP4* has only been reported in the DBA/2J strain (Fig. 1D; 32,34). These data show that all strains involved in generating the experimental mice have the same alleles of these modifier genes.

Myofiber MR knockout shows functional benefits on dystrophic respiratory muscle force and fatigue

Since the clinical deficiencies in respiratory muscle force lead to the respiratory insufficiency requiring ventilation in DMD patients and diaphragm force is improved in MR antagonist-treated dystrophic mice, we tested whether myofiber MR knockout had a direct benefit on diaphragm function (7). MRcko/*mdx* mice at 12 weeks of age showed a significant improvement ($P = 0.0247$) in diaphragm-specific force (144.7 ± 6.9 mN/mm²) compared with Cre–/*mdx* littermates (118.1 ± 8.0 mN/mm²; Fig. 2A). MRcko/*mdx* diaphragm-specific force reached 78% of the specific force of wild-type controls (185.9 ± 17.2 mN/mm²) measured separately as an independent cohort, consistent with the ~80% of normal wild-type force obtained with MR antagonists in 20-week-old dystrophic mice (6,7). The Cre–/*mdx* diaphragm-specific force was 64% of the specific force in the wild-type controls. A force–frequency protocol showed a significant improvement throughout the entire frequency range between MRcko/*mdx* compared to Cre–/*mdx* (Fig. 2B), as well as a small left-ward shift in sensitivity between MRcko/*mdx*

Figure 1. Validation of conditional knockout of MR in myofibers with muscle creatine kinase-driven Cre recombinase. (A) PCR for excised null MR allele and floxed allele. The excision is specific to skeletal muscle and only occurs when the Cre transgene is present. Tissues from a representative Cre- and MRcko mouse are shown. (B) qPCR for levels of MR expression in isolated extensor digitorum longus single myofibers further validates conditional knockout of MR in myofibers. Four Cre- and four MRcko mice were analyzed ($P = 0.0003$). (C and D) Analysis of known modifiers of muscular dystrophy in mice showed that only the annexin A6 (Anxa6) susceptible allele and only the protective latent-transforming growth factor β -binding protein 4 (LTBP4) allele were present in the experimental mice. (C) The table shows that all mice tested on the C57BL/10 and C57BL/6 backgrounds, but not the SV129 background, had the Anxa6 susceptible allele. (D) LTBP4 genotyping shows that all of the tested mice have the protective LTBP4 allele. *** $P \leq 0.001$ compared to Cre-.

and Cre-/mdx (Fig. 2C). Although the initial force drop during the first fatigue protocol was virtually identical ($P = 0.9971$) for Cre-/mdx ($50\% \pm 3\%$) compared with MRcko/mdx ($50\% \pm 3\%$), there was a significant improvement ($P = 0.0034$) in MRcko/mdx diaphragm absolute recovery from fatigue (23.7 ± 2.4 mN/mm²) compared to Cre-/mdx (13.9 ± 1.6 mN/mm²; Fig. 2D) as assessed by the improvement in force after a 15-min rest after the initial fatigue protocol. The diaphragm relative recovery showed a trend of improvement ($P = 0.0595$) for MRcko/mdx (29.1 ± 2.2 mN/mm²) compared to Cre-/mdx (23.4 ± 1.8 mN/mm²; Fig. 2E).

To determine whether there was a long-term effect on overall muscle force throughout the body, forelimb grip strength measurements were performed on 1-year-old MRcko/mdx and Cre-/mdx littermates. Maximal grip strength was significantly higher ($P = 0.0471$) in MRcko/mdx (1.24 ± 0.04 N) than in Cre-/mdx littermates (1.12 ± 0.04 N). However, normalized grip strength was not significantly different between ($P = 0.3815$) groups (39.6 ± 1.5 mN/g for MRcko/mdx versus 37.7 ± 1.5 mN/g for Cre-/mdx; Fig. 2F). Upon further analysis, the increased total forelimb grip strength could be explained by significantly larger body mass ($P = 0.0501$) in MRcko/mdx (27.8 ± 0.9 g) than in Cre-/mdx (25.8 ± 0.5 g) female mice only.

Myofiber MR knockout temporally reduces ongoing damage in dystrophic quadriceps

In order to evaluate if MRcko/mdx mice had a reduction in ongoing myofiber degeneration previously observed with MR antagonist treatment, we quantified the number of damaged myofibers containing serum IgG in several representative skeletal muscles and time points throughout the evolution of pathogenesis (6,7,35). To determine whether the observed increases in muscle force may be explained by an increased number of viable myofibers in MRcko/mdx diaphragms, we first quantified the amount of ongoing damage in diaphragms. There was no difference in the number of damaged myofibers per area in diaphragms from 4-, 8- or 12-week-old mice, or percent of IgG infiltration in diaphragms from 52-week-old MRcko/mdx and Cre-/mdx mice (Fig. 3A, C and D).

We next analyzed quadriceps and tibialis anterior muscles because these muscles are important for daily functions in patients like standing and walking, and we have previously observed reduced ongoing damage in these muscles after MR antagonist treatment of dystrophic mice. There was a reduction ($P = 0.0324$) in the amount of ongoing damage as indicated by IgG positive myofibers per area in MRcko/mdx

Figure 2. Diaphragm force and grip strength of MRcko/mdx and Cre-/mdx littermates. (A) Diaphragm-specific forces at 180 Hz were higher in MRcko/mdx mice compared with Cre-/mdx mice ($n = 9$ Cre-/mdx and $n = 8$ MRcko/mdx). As a reference for diaphragm physiology parameters, data from independently measured MRcko mice on a wild-type background ($n = 8$) are shown. (B) Diaphragm absolute force-frequency showed an increased force throughout the entire frequency range for MRcko/mdx compared to Cre-/mdx, and a small left-ward shift in sensitivity for MRcko/mdx relative force-frequency (C). (D) Absolute diaphragm recovery showed a significant improvement in MRcko/mdx compared with Cre-/mdx, and the normalized diaphragm recovery had a trend of an increase in diaphragm function ($P = 0.0595$) in MRcko/mdx mice (E). (F) There was no difference in normalized forelimb grip strength at 1 year of age (25 Cre-/mdx and 20 MRcko/mdx mice) Dia: diaphragm. * $P \leq 0.05$ MRcko/mdx compared to Cre-/mdx; ** $P \leq 0.01$ MRcko/mdx compared to Cre-/mdx; *** $P \leq 0.001$ MRcko/mdx compared to Cre-/mdx.

($10.1 \pm 1.9/\text{mm}^2$) quadriceps at 8 weeks of age compared to Cre-/mdx ($24.7 \pm 6.0/\text{mm}^2$; Fig. 3A and C). However, there was no difference in the amount of ongoing damage in quadriceps at 4, 12 or 52 weeks of age (Fig. 3A and C). There was no difference in the amount of ongoing damage in tibialis anterior at 4 or 8 weeks of age (Fig. 3A and C).

We then quantified the percentage of myofibers containing centrally located nuclei, indicative of cumulative damage. Fibers that have regenerated in the past several months have a nucleus in the center of the myofiber instead of the periphery (36,37). There was no difference in the percentage of fibers with centralized nuclei between MRcko/mdx and Cre-/mdx littermates at 8 or 12 weeks of age in quadriceps or 12 weeks of age in diaphragm (Fig. 3B).

Myofiber MR temporally regulates myofiber size in quadriceps

To determine if MR regulates myofiber growth in dystrophic muscles, we next measured myofiber size. We used the semi-automatic muscle analysis using segmentation of histology (SMASH) program to detect only the centrally nucleated myofibers in skeletal muscles (38). MRcko/mdx quadriceps myofibers were overall smaller at 8 weeks of age indicated by a higher percentage ($P = 0.0357$) of medium-sized myofibers from 800 to 1200 μm^2 ($10.4 \pm 0.3\%$) compared with Cre-/mdx ($6.9 \pm 0.3\%$), and a lower percentage ($P = 0.0157$) of large-sized myofibers from 3500 to 5700 μm^2 ($2.7 \pm 0.6\%$) than Cre-/mdx ($5.5 \pm 0.8\%$; Fig. 4). By 12 weeks of age, the MRcko/mdx mice

only had a slightly higher percentage ($P = 0.0220$) of large-sized myofibers from 4400 to 4900 μm^2 ($1.9 \pm 0.4\%$) compared to Cre-/mdx ($1.2 \pm 0.2\%$; Fig. 4). These data suggest that myofiber MR regulates myofiber size temporally.

After we determined that myofibers are smaller in MRcko/mdx quadriceps at 8 weeks of age, we wanted to evaluate myofiber size in MR antagonist-treated quadriceps to see if MR antagonists also improve dystrophinopathy in skeletal muscle by regulating myofiber size. There was no difference in myofiber size in 20-week-old dystrophic mice that were treated with MR antagonists versus untreated (Fig. 4; 6). To evaluate if the difference in myofiber size was specific to quadriceps muscle, we measured myofiber size in 12-week-old MRcko/mdx diaphragm. There were no differences in myofiber sizes in MRcko/mdx diaphragm versus Cre-/mdx, further supporting that the improved specific force in diaphragm at this age was not due to changes in the architecture of muscle fibers (Fig. 4).

Since we observed that MRcko/mdx mice have smaller myofibers, we measured the levels of myogenic transcripts to determine whether a particular transcript might be regulating myofiber growth in MRcko/mdx quadriceps at 8 weeks of age. There was no difference in the level of myostatin (negative regulator of myofiber growth), myogenin (aids muscle precursor cells in fusing with myofibers), MyoD (myogenic determination factor) or insulin-like growth factor 1 (positive regulator of myofiber growth) in MRcko/mdx 8-week-old quadriceps (Fig. 5; 39–41). These data suggest that these myogenic transcripts are likely not directly responsible for the observed difference in myofiber size.

Figure 3. Myofiber MR knockout reduces ongoing damage at 8 weeks of age in dystrophic quadriceps. (A) Representative confocal images of IgG- and laminin-stained quadriceps and diaphragm tissues. (B) Dot plots showing percent centrally nucleated fibers for 8-week-old ($n = 8$ Cre-/mdx and $n = 8$ MRcko/mdx) and 12-week-old ($n = 7$ Cre-/mdx and $n = 8$ MRcko/mdx) quadriceps and 12-week-old ($n = 5$ Cre-/mdx and $n = 5$ MRcko/mdx) diaphragms. (C) Dot plots showing number of IgG positive fibers per total muscle area for 4-week-old ($n = 10$ Cre-/mdx and $n = 15$ MRcko/mdx), 8-week-old ($n = 15$ Cre-/mdx and $n = 14$ MRcko/mdx) and 12-week-old ($n = 7$ Cre-/mdx and $n = 8$ MRcko/mdx) quadriceps; 4-week-old ($n = 10$ Cre-/mdx and $n = 15$ MRcko/mdx) and 8-week-old ($n = 15$ Cre-/mdx and $n = 15$ MRcko/mdx) tibialis anterior; and 4-week-old ($n = 8$ Cre-/mdx and $n = 11$ MRcko/mdx) and 12-week-old ($n = 5$ Cre-/mdx and $n = 5$ MRcko/mdx) diaphragm. (D) The 52-week-old diaphragm samples were analyzed for percent of IgG infiltration including interstitial infiltration of serum proteins in fibrotic regions ($n = 10$ Cre-/mdx and $n = 10$ MRcko/mdx). Quad: quadriceps, Dia: diaphragm and TA: tibialis anterior. * $P \leq 0.05$ compared to Cre-/mdx.

Myofiber MR regulates collagen deposition and conformation in skeletal muscles

Since immune cells are capable of producing the enzyme that makes the MR endogenous hormone aldosterone and immune cells regulate the skeletal muscle damage response in DMD patients, we investigated if myofiber MR regulates immune cell infiltration into dystrophic skeletal muscle. We performed immunofluorescence on skeletal muscle sections with the CD11b leukocyte marker that is high on monocytes and macrophages (42). Fibrosis is also a key pathological feature of DMD patient skeletal muscle and excessive fibrosis impairs regeneration of skeletal muscle (43). Fibroblasts produce extracellular matrix that over time results in fibrosis, so we also stained the skeletal muscle sections with the fibroblast intermediate filament marker vimentin (44,45). There was no difference between MRcko/mdx and Cre-/mdx littermates in the

percentage of area infiltrated by CD11b and vimentin positive cells in quadriceps or tibialis anterior muscles from 8-week-old mice or diaphragms from 1-year-old mice (Fig. 6). There was no difference in CD11b infiltration alone in 8-week-old quadriceps or 1-year-old diaphragm, but there was a trend of a modest decrease in the percentage ($P = 0.0592$) of CD11b infiltration alone in 8 weeks tibialis anterior for MRcko/mdx ($1.0\% \pm 1.2\%$) versus Cre-/mdx ($1.7\% \pm 0.3\%$; Fig. 6).

The trend of a decrease in CD11b infiltrated area in 8-week-old tibialis anterior led us to evaluate the amount of M1 or pro-inflammatory macrophage infiltration in 8-week-old tibialis anterior with the CD68 marker (46). We saw no difference in the amount of muscle area infiltrated by CD68 positive cells into 8 weeks of age tibialis anterior (Fig. 6). These data suggest that either the myofiber MR is not working directly through immune cell signaling or immune cells have an effect on disease progression earlier than 8 weeks of age in mice.

Figure 4. Quantification of myofiber sizes in MRcko/*mdx* and MR antagonist treated dystrophic mice. (A) Confocal representative images of laminin- and DAPI-stained muscle sections of quadriceps from 8-week-old ($n = 8$ Cre-/*mdx* and $n = 8$ MRcko/*mdx*) and 12-week-old ($n = 7$ Cre-/*mdx* and $n = 8$ MRcko/*mdx*) mice, diaphragm from 12-week-old ($n = 5$ Cre-/*mdx* and $n = 5$ MRcko/*mdx*) mice and quadriceps from 20-week-old *mdx:utrn*^{+/-} ($n = 5$ untreated *mdx:utrn*^{+/-} and 5 MR antagonist treated *mdx:utrn*^{+/-}) treated with lisinopril and spironolactone. Fiber sizes are significantly different in quadriceps of MRcko/*mdx* mice at 8 weeks of age, but not for any other time point or tissue. The distribution is broken down into (B) line graph of small myofibers, 75–600 μm²; (C) line graph of medium myofibers, 600–3000 μm²; and (D) line graph of large myofibers, 3000–5700 μm². For myofiber size analysis the following numbers of centrally nucleated myofibers were analyzed for each group and age: 3604 ± 482 Cre-/*mdx* and 3593 ± 549 MRcko/*mdx* in 8-week-old quadriceps; 3928 ± 572 Cre-/*mdx* and 5332 ± 534 MRcko/*mdx* in 12-week-old quadriceps; 2353 ± 272 Cre-/*mdx* and 2526 ± 374 MRcko/*mdx* in 12-week-old diaphragm; and 5530 ± 700 untreated *mdx:utrn*^{+/-} and 4156 ± 746 MR antagonist treated *mdx:utrn*^{+/-} mice. Quad: quadriceps, Dia: diaphragm, and * $P \leq 0.05$ compared to Cre-/*mdx*.

To further understand the interplay between the myofiber MR and fibrosis, we quantified the degree to which the collagen was packaged by visualizing picosirius red staining of collagen with polarized light and the amount of total collagen infiltration (47). Quadriceps muscle from 8-week-old MRcko/*mdx* had a lower ($P = 0.0061$) percentage ($29.2\% \pm 2.5\%$) of red, tightly packaged collagen relative to total collagen compared to Cre-/*mdx* ($39.4\% \pm 1.8\%$; Fig. 7A and B). There was no difference in percent total collagen infiltration relative to the

whole tissue area in 8-week-old quadriceps. Diaphragms from 1-year-old MRcko/*mdx* mice had a lower ($P = 0.0403$) percentage ($30.2\% \pm 1.8\%$) of total collagen infiltration relative to the whole tissue area compared to Cre-/*mdx* ($37.7\% \pm 2.8\%$; Fig. 7A and B). However, there was no difference in the amount of fibronectin infiltration into 1-year-old diaphragm between MRcko/*mdx* and Cre-/*mdx* littermates (Fig. 7C). These data suggest that myofiber MR regulates collagen deposition and conformation into skeletal muscles, but not all components of fibrosis are affected.

Figure 5. Myogenic transcript levels in quadriceps from 8-week-old MRcko/mdx mice. Dot plot showing four representative genes involved in muscle growth and differentiation assessed in MRcko/mdx ($n = 6$) and Cre-/mdx ($n = 4$) littermates. Beta-actin levels were used as a normalization control. IGF-1: insulin-like growth factor 1.

Spironolactone has an MR-independent protective effect on muscle membrane integrity

MR antagonism has previously been shown to prevent force drop from initial contraction after eccentric contraction of *extensor digitorum longus* limb muscles, suggesting its ability to help prevent contraction-induced injury. To assess whether this function is intrinsic to myofiber MR signaling, we measured *extensor digitorum longus* force and force drop after an eccentric contraction protocol in MRcko/mdx and Cre-/mdx at 12 weeks of age. Similar to previous reports in young mdx mice, we observed no differences between the two groups or differences from an independently measured MRcko wild-type cohort in specific force generated by *extensor digitorum longus* muscles (Fig. 8A; 6,48–50). The eccentric contraction protocol also showed no significant differences between MRcko/mdx and Cre-/mdx, suggesting that myofiber MR function does not directly contribute to prevention of contraction induced injury (Fig. 8B).

To further investigate membrane integrity in MRcko mice, we used a laser injury assay in *flexor digitorum brevis* muscle bundles. We previously showed that MR antagonist treatment for 1 month in dystrophic mice stabilized muscle membrane integrity after being subjected to laser-induced sarcolemmal membrane damage (20). An infrared multiphoton laser produced equivalent amounts of localized damage to the sarcolemmal membrane of intact muscle fibers from MRcko/mdx and Cre-/mdx littermates as determined by the extent of entry of FM 4-64, a lipophilic dye that does not fluoresce in aqueous solution, but will fluoresce intensely upon cell entry (20,51). Values were equivalent to those previously observed for dystrophic mice for both MRcko/mdx and Cre-/mdx groups (Fig. 9; 20). These data suggest that the absence of MR signaling in muscle fibers is not able to reproduce the membrane stability effects of MR antagonists in dystrophic muscles, consistent with the results from the *extensor digitorum longus* eccentric contraction assays.

To resolve the discrepancy between MR antagonism and MR myofiber knockout on myofiber membrane stabilization, we tested whether acute application of the MR antagonist spironolactone was able to improve dystrophic sarcolemma integrity. Addition of spironolactone for 5 min to *flexor digitorum*

brevis muscle bundles *ex vivo* resulted in significant improvements in membrane integrity after laser injury compared to the muscle bundles from the same mice that were treated with ethanol vehicle for both MRcko/mdx ($P = 0.0076$) and Cre-/mdx ($P = 0.0052$) mice (Fig. 9). Notably, the improvements in muscle membrane integrity after spironolactone treatment in MRcko/mdx and Cre-/mdx muscles were approximately at the same level of local damage previously observed in untreated wild-type *flexor digitorum brevis* muscle bundles, supporting a myofiber MR-independent effect of MR antagonists on improving myofiber membrane integrity (20).

Discussion

DMD skeletal muscles have fragile myofiber membranes, severe weakness, impaired regeneration and stiffening fibrosis. In the current study, we define contributions of myofiber MR in dystrophic muscles on regulation of muscle force, myofiber size and fibrosis. Myofiber MR knockout recapitulates the improvements on basal myofiber damage, but not on membrane integrity after stress previously observed with MR antagonist treatment (6,7). Surprisingly, we show for the first time that the MR antagonist spironolactone stabilizes myofiber membranes in an MR-independent manner, which may have implications for treating a wide variety of disorders that involve cell membrane damage and necrotic cell death.

Both diaphragm-specific force and resistance to fatigue were improved in 12-week-old MRcko/mdx mice compared to Cre-/mdx littermates. This exciting finding suggests that MR signaling intrinsic to the myofiber can sustain muscle force even in the presence of muscular dystrophy. The ability to inhibit MR signaling as a means to improve muscle weakness caused by other genetic and acquired conditions will need to be investigated. Prolonged mechanical ventilation leads to loss of contractile force and atrophy of the diaphragm muscle, or ventilator-induced diaphragm dysfunction, that greatly prolongs the time patients stay in intensive care units and correlates to patient morbidity and mortality (52,53). Steroid treatment regimens may exacerbate the loss of diaphragm function for patients with ventilator-induced diaphragm dysfunction (53,54). Testing the role of MR signaling in respiratory weakness may provide new therapeutics for these severe and costly conditions.

Diaphragm muscles from 1-year-old MRcko/mdx mice had less total collagen infiltration relative to the whole tissue area. However, there was no difference in the amount of fibronectin infiltration into 1-year-old diaphragm. Taken together, these data indicate that the myofiber MR regulates collagen deposition and conformation into skeletal muscles and that other extracellular matrix components are less affected. Less collagen infiltration means that the diaphragm is less stiff and can likely function better at late stages in dystrophinopathy. There was no difference in forelimb grip strength at 1 year of age. This is not entirely surprising since we have shown that MR antagonist treatment appears to have a temporary effect on overall body strength that is no longer present at 1 year (50). However, total grip strength in female MRcko/mdx mice was higher due to increased body mass. The question of whether MR knockout in muscle leads to increased body weight and overall improved muscle force throughout lifespan in non-dystrophic female mice will need to be further investigated in the future.

In limb muscles, there was less ongoing myofiber damage in MRcko/mdx quadriceps at 8 weeks of age. It appears that conditional knockout of the myofiber MR is able to temporally reduce the basal muscle damage from baseline muscle force

Figure 6. Immune cell infiltration into MRcko/mdx skeletal muscles. (A) Confocal images of CD11b-stained immune cells (green), vimentin-stained fibroblasts (red) and merged images with DAPI-stained nuclei (blue) of tibialis anterior muscles from 8-week-old MRcko/mdx and Cre-/mdx mice. (B) CD68 pro-inflammatory macrophage marker (green) and merged representative images containing DAPI of tibialis anterior from 8-week-old mice. (C) Composites of entire transverse sections of tibialis anterior from 8-week-old mice stained for immune cells, fibroblasts and DAPI or (D) for M1 macrophages and DAPI. (E) Dot plots showing percentages of muscle area containing immune cell and fibroblast staining together in tibialis anterior (15 Cre-/mdx and 15 MRcko/mdx) or quadriceps (8 Cre-/mdx and 8 MRcko/mdx) from 8-week-old mice and diaphragm from 52-week-old mice (10 Cre-/mdx and 10 MRcko/mdx), immune cells alone in 8-week-old tibialis anterior (15 Cre-/mdx and 15 MRcko/mdx) or 8-week-old quadriceps (8 Cre-/mdx and 8 MRcko/mdx) or M1 macrophages in 8-week-old tibialis anterior (8 Cre-/mdx and 8 MRcko/mdx). TA: tibialis anterior, Quad: quadriceps and Dia: diaphragm.

in a similar manner to MR antagonist treatment of dystrophic skeletal muscle (6,7). MRcko/mdx quadriceps myofibers at 8 weeks-of-age were overall smaller than the Cre-/mdx quadriceps supporting that genetic removal of myofiber MR may alter the kinetics of the degeneration-regeneration cycle. There were no differences in the level of combined CD11b positive leukocyte and vimentin positive fibroblast infiltration into skeletal muscles. A limitation of this experiment is that vimentin is not specific to fibroblasts and is also expressed in

vascular smooth muscle cells (45). However, when only the level of CD11b leukocyte infiltration was quantified, we saw a trend of a modest decrease in immune cell infiltration into 8-week-old tibialis anterior. CD11b is not only a marker for leukocytes, but also highest on monocytes and macrophages. M1 macrophages have been implicated in the progression of dystrophinopathy, so we further analyzed MRcko/mdx muscles for infiltration of the CD68 pro-inflammatory macrophage marker (46). We detected no gross differences between MRcko/mdx and Cre-/mdx mice

Figure 7. Quantification of collagen infiltration and packaging. (A) Representative images of picrosirius red staining under brightfield to quantify total collagen and polarized light to quantify loosely packaged collagen in green and tightly packaged collagen in red. Scale bar, 100 μ m. (B) Dot plots of percent muscle area containing collagen infiltration and percent of total collagen representing loose and tight packaging. For 8-week-old quadriceps, total collagen and collagen packaging Cre-/mdx ($n = 7$) and MRcko/mdx ($n = 7$) mice were quantified, and for 1-year-old diaphragm Cre-/mdx ($n = 8$) and MRcko/mdx ($n = 8$) mice were quantified. (C) Representative images and quantified percent area of infiltration of fibronectin staining of diaphragm sections from 1-year-old mice (scale bar, 200 μ m; 16 Cre-/mdx and 15 MRcko/mdx). Quad: quadriceps and Dia: diaphragm. * $P \leq 0.05$ compared to Cre-/mdx, ** $P \leq 0.01$ compared to Cre-/mdx.

in the area of infiltration of CD68 positive cells into 8-week-old *tibialis anterior*. These data suggest that the myofiber MR is not necessary for signaling immune cells to damaged muscle. However, more quantitative and complex cell sorting methods will need to be employed in the future to more carefully assess whether immune cell populations are altered in dystrophic muscles after MR genetic or pharmacological inhibition.

Despite there being no difference in immune response and the known contribution of the immune response on downstream fibrosis, MRcko/mdx 8-week-old quadriceps muscles did show a lower percentage of tightly packaged collagen relative to total collagen compared to Cre-/mdx. Tightly packaged collagen networks can create barriers that block the migration of muscle precursor cells to the site of damage, which inhibits muscle regeneration (47). Less tightly packaged collagen supports that the MRcko/mdx 8-week-old quadriceps muscles were

less stiff and had more potential to regenerate. We have previously reported that MR agonist treatment of cultured myotubes increases expression of collagen type 11, a regulator of collagen fibril assembly and cell migration, and collagen type 8, a regulator of fibroblast migration and enhancer of collagen types 1 and 3 secretion (55–60). It is possible that reduced levels of these transcripts from myofibers may have had an impact on the infiltration of collagen into MRcko/mdx skeletal muscle, independent of immune cell signaling.

The results from myofiber MR knockout mice help shape our understanding of MR signaling processes and have differentiated between effects of MR antagonists on other cell types in the muscle microenvironment, with potential feedback on reducing myofiber damage and improving muscle force, and direct muscle effects. The data in this study demonstrate that improved force generation in the diaphragm is intrinsic to MR

Figure 8. Extensor digitorum longus force measurements in MRcko/mdx mice. (A) Extensor digitorum longus specific force at 150 Hz ($n = 9$ Cre-/mdx, and 8 MRcko/mdx mice). As a reference for extensor digitorum longus physiology parameters, data from independently measured MRcko ($n = 8$) mice are shown. (B) Graph of fraction of extensor digitorum longus force from initial contraction with 6 eccentric contractions at 150 Hz for 450 ms with a 3% stretch for the final 200 ms of the contraction. There was a 5 minute rest between each eccentric contraction except between the fifth and sixth stimulation where there was a 15 minute rest to differentiate between membrane damage and muscle fatigue.

Figure 9. Membrane integrity measurements of MRcko/mdx muscles before and after MR antagonist treatment. (A) Laser injury induced membrane damage measured by FM 4-64 dye fluorescence in flexor digitorum brevis muscle bundles after either ethanol vehicle or spironolactone treatment for 5 min. (B) Quantified area under the curve for the FM 4-64 dye fluorescence. Each flexor digitorum brevis muscle bundle was analyzed under both treatment conditions ($n = 7$ Cre-/mdx and $n = 7$ MRcko/mdx mice). Spirono: spironolactone. * $P \leq 0.05$ compared to indicated group, ** $P \leq 0.01$ compared to indicated group.

signaling in the myofiber. Therefore, it is possible that MR over-activation only contributes to dystrophic disease pathology at earlier stages of the disease before increased fibrosis dampens some of the signaling from immune cell aldosterone production to the myofiber MR. Since mdx mice have less ongoing muscle inflammation than DMD patients, MR antagonism may still be more beneficial in patients.

In contrast to MR antagonist drug treatment (6,7,21), conditional knockout of myofiber MR was unable to prevent stress-induced damage from eccentric contraction protocols in extensor digitorum longus muscles. To further evaluate the role of the myofiber MR in muscle membrane damage response, we investigated membrane repair after laser injury in flexor digitorum brevis muscle bundles. There was no difference in the amount of FM 4-64 dye entry into the muscle bundles after laser injury between MRcko/mdx and Cre-/mdx. However,

a 5 minute short term spironolactone treatment of isolated muscles improved membrane integrity in both MRcko/mdx and Cre-/mdx muscles, demonstrating that the protective effect of spironolactone is independent of myofiber MR expression. The MR independent action of spironolactone on muscle membrane integrity is in contrast to glucocorticoid in vivo treatment 1 day preceding laser injury of flexor digitorum brevis muscle bundles, resulting in increased membrane integrity through upregulation of annexins A1 and A6 (61). Since we are reporting for the first time that C57BL/10 mice have the Anxa6 susceptible allele, it is possible that this allele prevents genomic effects downstream from myofiber MR knockout to act on enhancing membrane repair in these animals (33). The original mdx mouse was identified on a C57BL/10 background, so the Anxa6 susceptible allele may have facilitated its discovery (62).

Aldosterone, the physiological MR agonist, has documented non-genomic effects on secondary messengers such as cAMP, diacylglycerol, inositol triphosphate, protein kinases C and D, mitogen-activated protein kinases and ions like sodium, calcium and potassium within seconds to minutes (63,64). The role of secondary messengers and ions in rapid protection of the muscle membrane in spironolactone treatment will need to be further evaluated. Spironolactone is a very hydrophobic molecule, so it could feasibly insert into the muscle membrane and increase stability (65,66). It is possible that spironolactone improves membrane integrity in many cell types. Future elucidation of this possible mechanism may support the potential of spironolactone to protect tissues from many forms of injury.

Materials and Methods

MRcko/mdx mice and muscular dystrophy modifier genotyping

All protocols were approved by the Institutional Animal Care and Use Committee of The Ohio State University, are in compliance with the laws of The United States of America and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. MR floxed mice on a C57BL6/NCrL background were crossed to hemizygous muscle creatine kinase-driven Cre– recombinase transgene (MCK-Cre) mice from Jackson Laboratory (Bar Harbor, ME) on a C57BL/6 background (006475) to make a myofiber specific conditional knockout in both male and female mice (28,67–69). MCK-Cre also expresses in the heart, although at relatively low levels compared to skeletal muscle. However, since cardiomyocyte-specific MR conditional knockout mice have already been investigated and 1-year-old *mdx* mice do not have readily quantifiable cardiomyopathy, we restricted our analysis in this study to skeletal muscle (22,50,69). To study the absence of the MR in the context of muscular dystrophy, MRcko mice were crossed with *mdx* mice on a C57BL/10 background for two generations to make MRcko/*mdx* and Cre–/*mdx* littermates.

The mice were genotyped with primers for the Cre transgene and the MR floxed allele (Table 1). The flox sites increase the distance between the complementary regions for the floxed allele, so the MR wild-type and MR floxed alleles can be differentiated with the same primer set (28,68). To validate excision of the floxed exon of the MR locus specifically in skeletal muscle, we performed PCR on genomic DNA. The MR excision PCR product was only present in muscles when mice had the MCK-Cre transgene (69 out of 71 samples tested had efficient knockout of the MR). Presence of Cre recombinase results in excision of the MR floxed allele that makes the MR null allele (Table 1). All PCR was run on a ProFlex PCR System (ThermoFisher Scientific, Waltham, MA). To validate that the conditional knockout was specific to myofibers, we isolated *extensor digitorum longus* single myofibers from mice (*n* = 4 Cre– [2M, 2F], and MRcko [2M, 2F]) as previously described (30,31). Briefly, *extensor digitorum longus* muscles were dissected and digested in 0.2% collagenase type 1 solution in Dulbecco's modified Eagle's medium (DMEM) at 37°C until the myofibers were loose but not over-digested, 60 min. DMEM solution was pipetted onto the muscle bulk to mechanically dislodge the myofibers. About 25 single myofibers per mouse were pipetted under an inverted microscope and placed into a tube. Myofiber RNA was isolated from each mouse individually with TRIzol (ThermoFisher, 15596018), DNase-treated, reverse-transcribed

into cDNA and analyzed with qPCR as previously described (Table 1; 19).

To validate that there were not known modifiers of muscular dystrophy segregating in litters producing confounding variables in our results, we genotyped for the *Anxa6* and *LTBP4* modifiers. *Anxa6*-genotyping PCR reaction was performed (Table 1). The PCR product was sequenced to determine the presence of a single nucleotide polymorphism in Annexin A6 (*Anxa6*) that corresponds to a truncated protein with loss of function (33). Purified products were sequenced at The Ohio State Nucleic Acid/Microarray Shared Resource using the forward and reverse primers to analyze for the presence of an SNP in affected mice that have an A where unaffected mice have a G. The sequences were aligned to NCBI BLAST to determine if the 5'-GAGTATTTTGCAGAAAGGCTGTTC-3' (affected or *Anxa6*) or 5'-GAGTATTTTGCAGAAAGGCTGTTC-3' (unaffected *Anxa6*) allele was present (*n* = 4 MR floxed mouse breeders on a C57BL/6 background [4M], *n* = 6 MR floxed littermates on a mixed C57BL6; C57BL10 *mdx* background [3M, 3F], *n* = 2 *mdx* C57BL/10 [2M], *n* = 1 wild-type C57BL/10 [M] and *n* = 1 wild-type SV129 [M]). *LTBP4*-genotyping PCR reaction run on a 3% agarose gel to determine if a 295 bp (unaffected) or a 259 bp (affected) product was present (*n* = 6 MR floxed littermates on a mixed C57BL6; C57BL10 *mdx* background [3M, 3F], *n* = 2 *mdx* C57BL/10 [2M], *n* = 1 wild-type C57BL/10 [M] and *n* = 1 wild-type SV129 [M]) (Table 1; 32).

Physiology measurements

For skeletal muscle physiology, mice were analyzed at 12 weeks of age (*n* = 9 Cre–/*mdx* [5M, 4F] and *n* = 8 MRcko/*mdx* [5M, 3F]). MRcko mice on a wild-type background were measured as a separate cohort (*n* = 8 MRcko [4M, 4F]). For diaphragm force measurements, two linear strips with a width of 2.5–3.5 mm were carefully cut from the center of each diaphragm. The muscle strips were stretched to optimal length using twitch contractions as previously described (6,50). The muscles were then rested for 10 min. The diaphragm strips were subjected to tetanic contractions of 250 ms at the physiological temperature (37°C) at 20, 50, 80, 120, 150 and 180 Hz with 2 min of rest between stimulations. For most mice, two strips were measured and averaged for analysis. Five minutes after the final tetanic force measurement a diaphragm fatigue protocol was started. The diaphragm strip was pulse-train stimulated at 100 Hz for 250 ms of each second 66 times (6,50,70). After 15 min of rest the protocol was repeated. Fatigue was assessed as the percentage of force drop from the beginning to the end of the first fatigue protocol. Diaphragm absolute recovery was measured by the change in the force/cross sectional area for the beginning of the second contraction regimen minus the end of the first contraction regimen. Relative recovery is found by dividing absolute recovery by the initial force/cross sectional area. Force recordings and analyses were both done using custom-made LabView (National Instruments) programs. All data were analyzed by individuals blinded to the genotypes of the mice.

For *extensor digitorum longus* muscle physiology measurements, the muscle from each leg was visualized under a dissection microscope. A suture was tied to each tendon and the muscle was removed from the leg (50). Each *extensor digitorum longus* muscle from the mouse was stretched to the optimal length using twitch contractions as previously described in an oxygenated muscle bath of continuously cycled Krebs–Henseleit

Table 1. PCR primers

Intended product	Primers	Product size (bp)
Cre transgene	Cre-For 5'-ATGTCCAATTTACTGACCG-3', Cre-Rev 5'-CGCCGCATAACCAGTGAAAC-3'	356
MR wild-type or floxed allele	MR flox-7 5'-CTGGAGATCTGAACTCCAGGCT-3', MR flox-10 5'-TAGAAACACTTCGTAAGTAGAGCT-3'	292 (wild-type) or 335 (floxed)
MR null allele	MRflox-7 5'-CTGGAGATCTGAACTCCAGGCT-3', MRflox-8 5'-CCTAGAGTTCTGAGCTGCTGA-3'	390
MR for qPCR	MR_qPCR_For 5'-GAGTTTCCTTTCCGCTGTCA-3' MR_qPCR_Rev 5'-GCCTCATCTCCACACACCAA-3'	189
Anxa6	Anxa6-For 5'-GGAGCAAGTTAGTGAGTTAACCTGTTG-3' Anxa6-Rev 5'-GCAGCAGGATTCGCCATGATCTCTG-3'	507, sequence for mutation
LTBP4	LTBP4-For 5'-ATTCGCCGCAACCAACCTCTGATTCCTATGG-3' LTBP4-Rev 5'-TCTTTACCAGGCTTTCTGCCTACTCGTCG-3'	295 (unaffected) or 259 (affected)

Primers for genotyping, excision and quantitative PCR are listed together with their predicted product size.

solution at 30°C (6,50). After 10 min, each *extensor digitorum longus* muscle was subject to a tetanus contraction with 150 Hz for 250 ms. After a 5 min rest period, the *extensor digitorum longus* muscles were subject to six eccentric contractions with 150 Hz for 450 ms with a 3% stretch for the final 200 ms of the contraction. There was a 5 min rest between each eccentric contraction except between the fifth and sixth stimulation where there was a 15 min rest. After the measurements the *extensor digitorum longus* muscles were weighed and the forces were expressed per unit of cross-sectional area. Analysis was done by averaging values of each *extensor digitorum longus* muscle.

Maximal forelimb grip strength was measured at 1 year of age in MRcko/*mdx* (*n* = 20 [10M, 10F]) and Cre-/*mdx* (*n* = 25 [11M, 14F]) mice by an individual blinded to the genotypes of the mice. Initially the mice were subjected to two trainings. The investigator held the mice by the tail and allowed it to grip the bar of a force transducer (Chatillon, Largo, FL #DFE2-002) with both forepaws grasping in an overhand grip and pulled the tail of the mouse rapidly so that it would let go of the bar. This was repeated five times per training. The same individual performed all of the measurements to limit variability. For the measurements, the investigator repeated the procedure five times and watched to make sure that the mouse had an overhand grip and did not bite the bar or thrash while the measurement was taken. The highest force produced of the five pulls was taken and divided by the mouse's weight measured the same day to obtain grip strength normalized to body weight (50).

Immunofluorescence and collagen staining

Any section containing an excess of longitudinal skeletal muscle was excluded from analysis. Mouse skeletal muscles were frozen in liquid nitrogen-cooled isopentane in optimal cutting temperature compound and sectioned at a thickness of 8 µm. For quantification of ongoing damage, sections were incubated with 1:500 laminin-2 alpha 2 chain (rat anti-mouse monoclonal Sigma-Aldrich, St. Louis, MO #L0663) primary antibody and then incubated with 1:200 Cy3-conjugated goat anti-rat IgG (Jackson Immuno Research Laboratories, West Grove, PA #112-165-167) along with 1:200 Alexa Fluor R 488 conjugated goat anti-mouse IgG (Invitrogen #A11029) secondary antibodies. The 4- (*n* = 10 Cre-/*mdx* [6M, 4F] and *n* = 15 MRcko/*mdx* [8M, 7F]), 8- (*n* = 15 Cre-/*mdx* [4M, 11F] and *n* = 14 MRcko/*mdx* [8M, 6F]) and 12- (*n* = 7 Cre-/*mdx* [4M, 3F] and *n* = 8 MRcko/*mdx* [5M, 3F]) week-old quadriceps and 4- (*n* = 8 Cre-/*mdx* [4M, 4F] and *n* = 11 MRcko/*mdx* [6M,

5F]), 12- (*n* = 5 Cre-/*mdx* [3M, 2F] and *n* = 5 MRcko/*mdx* [3M, 2F]), 52- (*n* = 10 Cre-/*mdx* [6M, 4F] and *n* = 10 MRcko/*mdx* [6M, 4F]) week-old diaphragm and 4- (*n* = 10 Cre-/*mdx* [6M, 4F] and *n* = 15 MRcko/*mdx* [8M, 7F]) and 8- (*n* = 15 Cre-/*mdx* [4M, 11F] and *n* = 15 MRcko/*mdx* [8M, 7F]) week-old tibialis anterior were analyzed. To measure the size of the centrally nucleated myofibers sections stained for ongoing damage were used except the green IgG channel was removed so that only the red laminin myofiber outline and DAPI stained nuclei were visualized in 8- (*n* = 8 Cre-/*mdx* [4M, 4F] and *n* = 8 MRcko/*mdx* [4M, 4F]) and 12- (*n* = 7 Cre-/*mdx* [4M, 3F] and *n* = 8 MRcko/*mdx* [5M, 3F]) week-old quadriceps and 12- (*n* = 5 Cre-/*mdx* [3M, 2F] and *n* = 5 MRcko/*mdx* [3M, 2F]) week-old diaphragm. For the leukocyte and fibroblast infiltration analysis, the sections were incubated with the 1:50 CD11b (rat anti-mouse monoclonal BD Pharmingen, San Jose, CA #550282) and 1:600 vimentin antibody (rabbit anti-mouse polyclonal Abcam, Cambridge, MA #ab45939) primary antibodies and then probed with 1:200 Alexa Fluor R 488 conjugated chicken anti-rat antibody (Life Technologies, Carlsbad, CA A-21470) and 1:200 Alexa Fluor R 555 conjugated goat anti-rabbit antibody (Invitrogen, Waltham, MA #A21429) secondary in 8-week-old tibialis anterior (*n* = 15 Cre-/*mdx* [4M, 11F] and *n* = 15 MRcko/*mdx* [8M, 7F]), 8-week-old quadriceps (*n* = 8 Cre-/*mdx* [4M, 4F] and *n* = 8 MRcko/*mdx* [4M, 4F]) and 52-week-old diaphragm (*n* = 10 Cre-/*mdx* [6M, 4F] and *n* = 10 MRcko/*mdx* [6M, 4F]). For the CD11b quantitation alone, the red vimentin channel was removed so that only the green CD11b channel was visualized in 8-week-old tibialis anterior (*n* = 15 Cre-/*mdx* [4M, 11F] and *n* = 15 MRcko/*mdx* [8M, 7F]) and 8-week-old quadriceps (*n* = 8 Cre-/*mdx* [4M, 4F] and *n* = 8 MRcko/*mdx* [4M, 4F]). For CD68 infiltration quantitation, the sections were incubated with 1:200 CD68 antibody (rat anti-mouse monoclonal BioRad, Hercules, CA #MCA1957) and then incubated with 1:200 Alexa Fluor R 488 conjugated chicken anti-rat antibody (Life Technologies A-21470) in 8-week-old tibialis anterior (*n* = 8 Cre-/*mdx* [4M, 4F] and *n* = 8 MRcko/*mdx* [4M, 4F]). For the fibronectin infiltration analysis, the sections were incubated with 1:40 fibronectin antibody (rabbit anti-mouse polyclonal Abcam #23750) and then incubated with 1:200 Alexa Fluor R 555 conjugated goat anti-rabbit antibody (Invitrogen #A21429) in 1-year-old diaphragm (*n* = 16 Cre-/*mdx* [8M, 8F] and *n* = 15 MRcko/*mdx* [8M, 7F]). All sections were mounted with Vectashield mounting medium with 2 ng/µL of DAPI to visualize nuclei.

The IgG and CD11b and Vimentin sections were imaged on a Nikon A1Rsi Resonant scanning inverted confocal system with

the 20× objective with the resonant scanner, 4× averaging, a pinhole of 13.1 AU and perfect focus to make seamless composites. Representative images were taken on a Nikon A1Rsi Resonant scanning inverted confocal system with the 20× objective with the galvano scanner, a pinhole of 1.2 AU and perfect focus. The low and high levels for each channel were optimized to brighten and increase contrast between staining and the background on Nikon Elements AR and then applied consistently between samples. The only non-linear modification used was on the DAPI channel, and then, it was only implemented when there were dark pockets of the tissue that were not evenly stained for nuclei. The most an image was non-linearly brightened with the gamma was from 1 to 0.75. The diaphragms stained for fibronectin were imaged on a Nikon (Melville, NY) Eclipse 800 microscope with the 10× objective with a SPOT RT slider digital camera software and composited using Adobe (San Jose, CA) Photoshop (CS6). For collagen analysis, sections were stained with picosirius red (24901-500; Polysciences) according to the manufacturer's recommendations (47). The sections were imaged with the 4× objective with either brightfield for total collagen infiltration images or polarized light for collagen packaging images with a Zeiss Axioskop Widefield LM microscope and QImaging QCapture Pro software.

Histological quantification

Histological quantification of immunofluorescence sections was done by an individual blinded to the genotype of the mice. For the IgG quantification, the blinded individual counted the number of IgG-stained fibers surrounded by a red laminin ring (6). For CD11b and Vimentin quantification, a blinded individual quantified the amount of infiltration with Adobe Photoshop (CS6) in a green CD11b and red vimentin whole mouse *tibialis anterior* or quadriceps section by first removing all of the area without tissue very finely with the paint bucket tool. Then, the green CD11b, red vimentin or yellow merge of the two colors were marked blue. Percent infiltration was calculated by the number of blue pixels divided by the total pixels in the section (35). This quantification was also performed with CD11b only in whole mouse *tibialis anterior*, quadriceps and diaphragm sections. A similar method was used to quantify fibronectin infiltration in diaphragm sections.

For the collagen infiltration data, the area of picosirius red-stained collagen was quantified relative to the total muscle area by a blinded individual as previously described in 8-week-old quadriceps ($n = 7$ Cre-/*mdx* [4M, 3F] and $n = 7$ MRcko/*mdx* [4M, 3F]) and 52-week-old diaphragm ($n = 8$ Cre-/*mdx* [4M, 4F] and $n = 8$ MRcko/*mdx* [2M, 6F]; 47). For analysis of collagen network compaction, polarized images of the picosirius red-stained sections were analyzed and the amount of loosely (green) packaged and densely (red) packaged collagen were quantified relative to the total collagen area with the red-green-blue quantification feature in NIH (Bethesda, MD) ImageJ by a blinded individual as previously described (47).

Quantification of myofiber size and percent centrally nucleated myofibers

To quantify the size of only the myofibers, sections were stained for laminin and mounted in DAPI as above. MRcko/*mdx* and Cre-/*mdx* littermate control 8-week-old quadriceps ($n = 8$ Cre-/*mdx* [4M, 4F] and $n = 8$ MRcko/*mdx* [4M, 4F]), 12-week-old quadriceps ($n = 7$ Cre-/*mdx* [4M, 3F] and $n = 8$ MRcko/*mdx* [5M, 3F]) and diaphragm ($n = 5$ Cre-/*mdx* [3M, 2F], and 5

MRcko/*mdx* [3M, 2F]), and lisinopril (132 mg/L) and spironolactone (250 mg/L) treated to provide approximate dosages of 20 and 37.5 mg/kg per day, respectively, from 4 to 20 weeks of age in male *mdx:utrn*^{+/-} dystrophin deficient and utrophin haploinsufficient muscular dystrophy mouse model quadriceps ($n = 5$ untreated *mdx:utrn*^{+/-} [5M] and $n = 5$ MR antagonist treated *mdx:utrn*^{+/-} [5M]) sections were analyzed (6). The sections were imaged as described above with the Nikon A1Rsi Resonant scanning inverted confocal system.

The composites were analyzed with a MATLAB plug-in called SMASH (38). The settings used were 1.24 $\mu\text{m}/\text{pixel}$, red channel for fiber outline, blue channel for nuclei, segmentation smoothing factor of 5–65 depending on the intensity of the staining, nuclear smoothing factor of 5, minimum fiber area of 75 μm^2 , maximum fiber area of 5700 μm^2 , maximum eccentricity of 0.95, minimum convexity of 0.8, nuclear distance from the border of 10 μm and minimum nuclear size of 5 μm^2 . Once the data were generated, we used Microsoft Excel to further include all the small fibers from 75–550 μm^2 in area with the other centrally nucleated fibers that SMASH counted because SMASH was unable to detect these small myofibers as centrally nucleated since it determines centrally nucleated fibers by the distance of the nucleus from the edge of the fiber (38). The myofibers were then binned into 100 μm^2 intervals and the data were expressed as the percentage of total counted regenerated fibers within a given 100 μm^2 bin from 75 to 5700 μm^2 with STAT Plus LE an Excel plug-in (71).

Laser injury

MRcko/*mdx* and Cre-/*mdx* littermates ($n = 7$ Cre-/*mdx* [2M, 5F] and $n = 7$ MRcko/*mdx* [3M, 4F]) were analyzed for muscle membrane integrity using a laser injury assay. *Flexor digitorum brevis* muscle bundles were surgically isolated from mice and adhered to a 35 mm glass bottom imaging dish (MatTek Corp, Ashland, MA) with commercially available liquid bandage (New Skin Liquid Bandage, Moberg Pharma, Cedar Knolls, NJ). Tyrode's solution supplemented with 2 mM Ca^{2+} and 10.5 μM FM 4-64 dye (Thermo Fisher Scientific, Waltham, MA) was added to the glass bottom microwell of each dish to cover FDB muscle bundles (300 μL) as previously described (20). Each muscle bundle was analyzed separately in 0.2% ethanol vehicle and 250 mg/l spironolactone (Sigma S3378) in 0.2% ethanol conditions allowing each mouse to serve as its own control. Both treatments were incubated at room temperature for 5 min before the laser injury began. Laser injury assays were conducted using a FluoView FV1000 MPE multiphoton confocal laser scanning microscope (Olympus, Melville, NY) equipped with a DeepSee MaiTai titanium-sapphire laser and 25 × 1.05 numerical aperture water immersion objective. A 157 nm² region of interest was designated at the muscle membrane edge as the site of injury to ensure fluorescence measurements remained within the focal plane. Images were acquired every 3 s for 20 frames; 3 frames before injury, followed by a 5 s infrared laser irradiation at 18% maximal power and 17 frames following injury. Membrane damage was quantified by measuring fluorescence intensity at the site of injury (ΔF) for each frame. Background fluorescence (F_0) was measured using a region of interest of the same area immediately adjacent to the injury site allowing for bleaching correction. Membrane resealing kinetics are represented as a curve corresponding to the change in fluorescence intensity over time using the formula $(\Delta F - F_0)/F_0$ calculated for each time point (20,51). Total dye uptake after

injury was quantified by calculating the area under the curve for MRcko/mdx and Cre-/mdx muscle fibers in both the vehicle control and spironolactone treated conditions.

Statistical analysis

All data were included for statistical calculations. All data with two groups were analyzed using Student's t-test assuming equal variance. Summary values are presented as mean $\pm$ SE. Sex was analyzed as a co-variant in all analysis. There was no difference by sex in the parameters unless it was mentioned in the results. The laser injury data were analyzed using a one-way analysis of variance followed by Tukey's multiple comparisons test. Two-tailed $P \leq 0.05$ was accepted as significant.

Supplementary Material

The supplemental material includes excision PCR of MR in quadriceps where the MR null allele is present in MRcko quadriceps but not in Cre quadriceps similar to Figure 1. Additionally, there is excision of MR in MRcko/mdx diaphragm, tibialis anterior and flexor digitorum brevis but not in Cre-/mdx mouse tissues.

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Conflict of Interest statement. The authors declare no competing financial interests.

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