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Exosome-mediated improvement in membrane integrity and muscle function in dystrophic mice

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Duchenne muscular dystrophy (DMD) is a devastating genetic disorder that leads to compromised cellular membranes, caused by the absence of membrane-bound dystrophin protein. Muscle membrane leakage results in disrupted intracellular homeostasis, protein degradation, and muscle wasting. Improving muscle membrane integrity may delay disease progression and extend the lifespan of DMD patients. Here, we demonstrate that exosomes, membranous extracellular vesicles, can elicit functional improvements in dystrophic mice by improving muscle membrane integrity. Systemic administration of exosomes from different sources induced phenotypic rescue and mitigated pathological progression in dystrophic mice without detectable toxicity. Improved membrane integrity conferred by exosomes inhibited intracellular calcium influx and calcium-dependent activation of calpain proteases, preventing the degradation of the destabilized dystrophin-associated protein complex. We show that exosomes, particularly myotube-derived exosomes, induced functional improvements and alleviated muscle deterioration by stabilizing damaged muscle membrane in dystrophic mice. Our findings suggest that exosomes may have therapeutic implications for DMD and other diseases with compromised membranes.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a life-threatening muscular disorder that shows progressive muscle deterioration, caused by the deficiency of functional membrane-associated dystrophin, a protein essential for maintaining the structural integrity of muscle cell membrane.[1] Although a number of different therapeutic approaches have been under intensive investigation, including strategies to restore partially functional dystrophin expression such as gene replacement, exon skipping, stop codon read-through, and genome-editing therapies, or to improve muscle function by targeting different pathological aspects involved in DMD,[2] there is no one-size-fits-all treatment available in the clinic.

The absence of dystrophin leads to the destabilization and mislocalization of dystrophin-associated protein complex (DAPC), a large macromolecular complex ensuring membrane stability,[3] and results in severely compromised membrane integrity.[4] Loss of muscle membrane barrier function triggers a cascade of deleterious events, including abnormally elevated intracellular calcium influx and activation of proteasome signaling pathway, which leads to disrupted intracellular homeostasis and progressive muscle cell death.[5] Therefore, disrupted membrane integrity is a primary defect caused by the lack of functional dystrophin protein and also a potential therapeutic target for DMD.[5] Approaches to improve the integrity and stability of muscle membrane have been under intense scrutiny, with membrane-stabilizing copolymers such as poloxamer 188 (P188) leading the way (https://clinicaltrials.gov/ct2/show/NCT03558958). P188 has been investigated in *mdx* mice and golden retrievers with MD comprehensively, but the results in stabilizing skeletal and cardiac muscle membrane were variable and controversial.[6–9] Therefore, other strategies that can improve muscle membrane integrity should be explored.

Exosomes are membrane-bound nano-sized vesicles and serve as intercellular messengers in physiological and pathological conditions.[10] Recently, exosomes have been used as regenerative medicine[11] or harnessed as drug-delivery vehicles in different disease models including DMD, as exosomes intrinsically have favorable

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qualities such as enrichment in functional moieties, low or no immunogenicity, biocompatibility, and relatively longer circulation than liposomes.[12–14] Notably, exosomes, as membranous vesicles, can be readily taken up by recipient cells via membrane fusion or endocytosis.[15] Exosomes derived from human mesenchymal stromal cells (MSCs) were shown to alleviate fibrosis *in vitro* and in quadriceps of *mdx* mice via local intramuscular injection.[16] However, the systemic therapeutic potential of exosomes in treating DMD remains to be determined. Here, we demonstrate that repeated systemic administration of exosomes from different sources, particularly from murine myotubes, elicited functional rescue and mitigated muscle pathological progression in *mdx* mice without detectable toxicity. Further studies revealed that exosomes exerted their protective effects by improving membrane integrity and resulting in partial blockade of intracellular calcium influx and calcium-dependent protease activation, and prevention of DAPC degradation. Our findings prove the feasibility of exosomes as a viable supplementary therapeutic approach for DMD.

RESULTS

EXO_{MSC} improve muscle function and pathologies in *mdx* mice

Given the benefits associated with local intramuscular injection of exosomes derived from human MSCs (EXO_{MSC}) in dystrophic quadriceps,[16] we wished to determine whether EXO_{MSC} were beneficial in *mdx* mice systemically. EXO_{MSC}, characterized by sauce-cup shapes with an average diameter of 100 nm and the expression of exosomal marker proteins (Figures S1A–S1C), were injected into adult *mdx* mice at the dose of 150 μg twice per week (3 days apart) for 3 weeks intraperitoneally (i.p.). The choice of dosing route is based on i.p. administration having a more disperse distribution than intravenous injection,[17] while the dose and administration interval were adopted from our previous study and other studies in *mdx* mice.[18–20] Strikingly, a significant force recovery was observed in EXO_{MSC}-treated *mdx* mice compared to age-matched untreated *mdx* controls (Figure 1A). Levels of serum creatine kinase (CK), an enzyme, is usually elevated in DMD patients' blood due to muscle membrane leakage,[21] which is also markedly declined (Figure 1B). To examine whether the species has any direct impact on the therapeutic efficacy of exosomes, we administered exosomes derived from murine bone marrow MSCs into *mdx* mice i.p. under identical conditions. The results showed similar therapeutic effects achieved in *mdx* mice treated with exosomes derived from murine MSCs compared to human MSCs under identical conditions, evidenced by grip strength (Figure S2A) and serum CK levels (Figure S2B), indicating that the potency of exosomes is species independent. Interestingly, although no dystrophin was detected, β-dystroglycan and α-sarcoglycan, key components of DAPC that are usually mislocalized in the absence of dystrophin,[22] were correctly relocalized to sarcolemma in EXO_{MSC}-treated muscles (Figure 1C), suggesting that EXO_{MSC} facilitate the relocalization of DAPC via a dystrophin-independent manner. Muscle fibrosis and inflammation were also ameliorated, demonstrated by reduced collagen staining and fewer macrophages, monocytes predominantly present in dystrophic muscles,[23] in diaphragm and triceps of EXO_{MSC}-treated *mdx* mice compared to untreated *mdx* controls (Figures 1D–1G). These results indicate that systemic administration of EXO_{MSC} improves muscle function and pathologies in *mdx* mice.

EXOser exert protection on dystrophic muscles

To determine whether the protective effect conferred by EXO_{MSC} is a common trait of exosomes, we tested exosomes from other sources. As serum exosomes (EXOser) derived from healthy individuals or close relations of the patients are considered more clinically acceptable and can be deployed more quickly, we administered EXOser derived from murine serum, confirmed by transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) (Figures S3A and S3B), into adult *mdx* mice under identical dosing regimens. EXOser likewise elicited marked functional improvements with significantly increased grip strength (Figure 2A) and muscle endurance (Figure 2B) and reduced levels of serum CK (Figure 2C) compared to untreated *mdx* controls. Improved myoarchitecture was similarly observed as evidenced by correct relocalization of DAPC to sarcolemma in the absence of dystrophin (Figure 2D), reduced collagen staining, and fewer macrophages (Figures 2E–2H) in the diaphragm and triceps of EXOser-treated *mdx* mice compared to untreated *mdx* controls. Unsurprisingly, systemic administration of murine dendritic cell (DC)-derived exosomes (DEX) (Figures S4A–S4C) elicited similar beneficial effects under identical conditions (Figures S4D–S4F). In contrast, exosome-free supernatants derived from murine DCs did not improve myoarchitecture or alleviate fibrosis and inflammation in *mdx* mice under identical conditions (Figures S4D–S4F), indicating that the protective effect is exosome specific.

EXOmyo elicit phenotypic rescue without detectable toxicity in *mdx* mice

Next, we wondered whether exosomes from myogenic origins will offer greater efficacy in dystrophic muscles, as these carry myogenic mRNAs and proteins.[12] To test this notion, we harvested exosomes from murine differentiated myotubes (EXOmyo) (Figures S5A–S5C), which were enriched in more functional moieties than exosomes derived from myoblasts[24,25] and evaluated in *mdx* mice under identical conditions. Remarkably, a significant muscle force recovery to levels comparable to wild-type controls was achieved in EXOmyo-treated *mdx* mice compared to untreated *mdx* controls (Figure 3A). Also, EXOmyo elicited greater levels of force recovery (Figure S6A) and serum CK reduction (Figure S6B) than EXO_{MSC} and EXOser. Improved myoarchitecture was also observed, reflected by profoundly reduced serum CK levels (Figure 3B), relocalization of DAPC (Figure 3C), decreased collagen staining, and fewer macrophages (Figures 3D–3G). Notably, a marginal decrease in serum CK-MB, an enzyme, is primarily found in the heart and released into the blood during cardiac damage,[26] and was found in EXOmyo-treated *mdx* mice compared to untreated *mdx* controls (Figure S7). More EXOmyo accumulated in the diaphragm than in other muscles, although the majority of EXOmyo was found in the liver and kidney (Figures 3H and 3I) when EXOmyo were injected i.p.. In contrast, EXOmyo were primarily found in the liver of *mdx* mice when injected

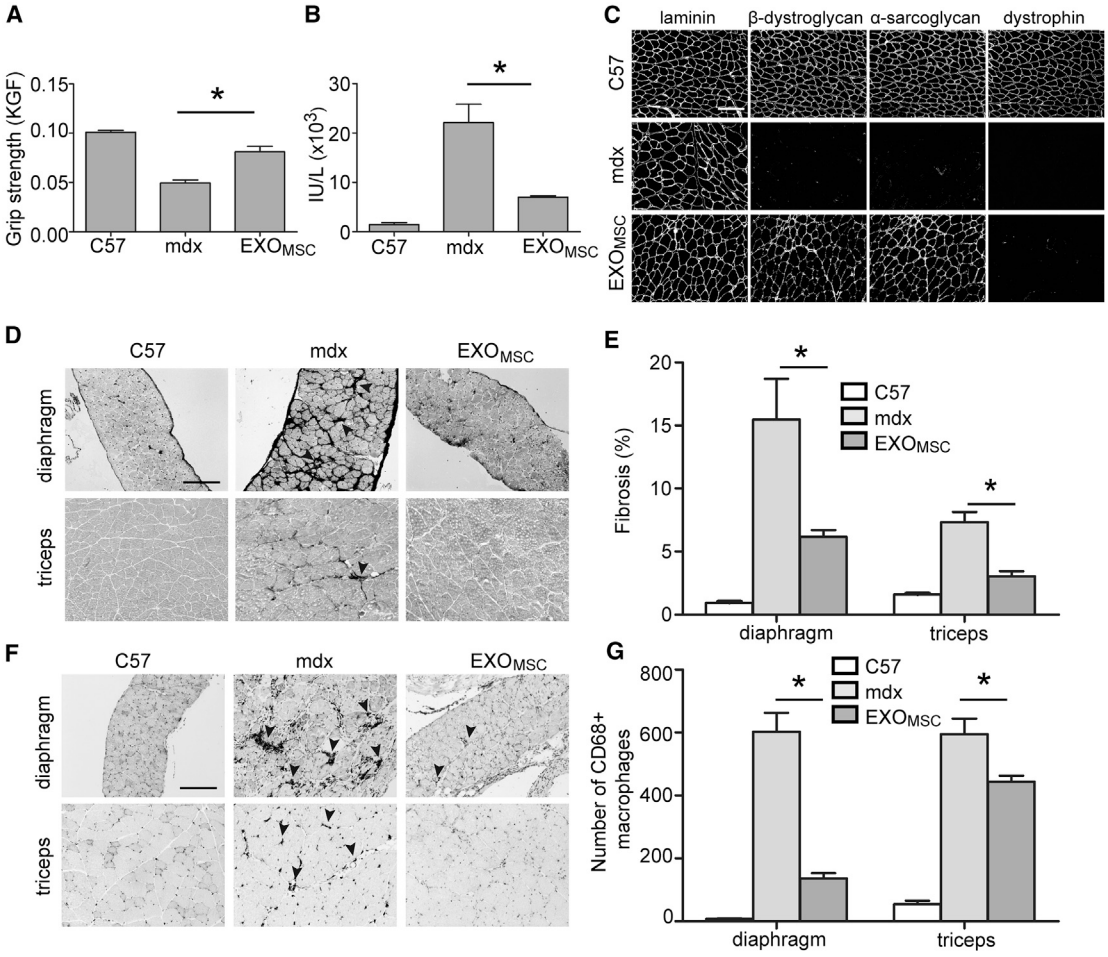

Figure 1. Functional and pathological assessments of muscles from *mdx* mice treated with EXO_{MSC}

EXO_{MSC} were injected into adult *mdx* mice intraperitoneally (i.p.) at the dose of 150 μg twice per week 3 days apart for 3 weeks and muscles were harvested 3 days after last injection. (A) Muscle function was assessed to determine the physical improvement with grip strength test in C57BL/6 (n = 3), treated (n = 3), and untreated *mdx* controls (n = 3) (*$p < 0.05$; 1 way-ANOVA post hoc Student-Newman-Keuls test). The same number of mice was used for each group for other assays unless otherwise specified. (B) Assessment of serum creatine kinase (CK) levels in treated *mdx* mice (n = 3, *$p < 0.05$; 1-way ANOVA on ranks). (C) Relocalization of DAPC components in treated *mdx* mice to assess dystrophin function and recovery of normal myoarchitecture (scale bar, 100 μm). Triceps was used as a representative muscle and the same for the rest unless otherwise specified (n = 3). (D and E) Masson trichrome staining (D) and quantification (E) of fibrotic areas for diaphragm and triceps from treated *mdx* mice (scale bar, 100 μm) (n = 3, *$p < 0.05$; 1 way-ANOVA post hoc Student-Newman-Keuls test). Arrowheads point to the collagen-positive areas. (F and G) Immunohistochemistry (F) and quantification (G) of $CD68^+$ macrophages in diaphragm and triceps from treated *mdx* mice (scale bar, 100 μm) (n = 3, *$p < 0.05$; 1 way-ANOVA post hoc Student-Newman-Keuls test). Arrowheads point to $CD68^+$ macrophages. The data were presented as mean values ± SEMs.

intravenously under identical conditions (Figure S8), confirming that i.p. injection results in more dispersed distribution of exosomes than intravenous injection in *mdx* mice. Commensurate with muscle pathological improvements, levels of serum aspartate transaminase (AST) and alanine aminotransferase (ALT), which are usually elevated in DMD patients,[27] were significantly decreased (Figure 3J), whereas no change was found in the levels of circulatory alkaline phosphatase (ALP), blood urea nitrogen (BUN) and uric acid (UA) (Figures 3J and 3K), functional parameters of the liver and kidney,[28,29] respectively, in EXOmyo-treated *mdx* mice compared to untreated *mdx* controls. These results demonstrate the therapeutic potency and tolerability of EXOmyo in *mdx* mice.

EXOmyo prevent the degradation of DAPC in dystrophic muscles

As upregulation of utrophin, a protein usually elevated in DMD,[30] was suggested to be one of the mechanisms accounting for the functionality of EXO_{MSC} in the previous study,[16] we examined the level of utrophin in EXOmyo-treated muscles. However, only a marginal increase was seen in the diaphragm of EXOmyo-treated *mdx* mice (Figures 4A and 4B). Similarly, a slightly greater level of utrophin expression was detected in the diaphragm of EXO_{MSC}-treated *mdx* mice compared to untreated *mdx* controls (Figures S9A and S9B). This minor elevation of utrophin cannot explain the profound therapeutic effects conferred by exosomes. As relocalization of DAPC

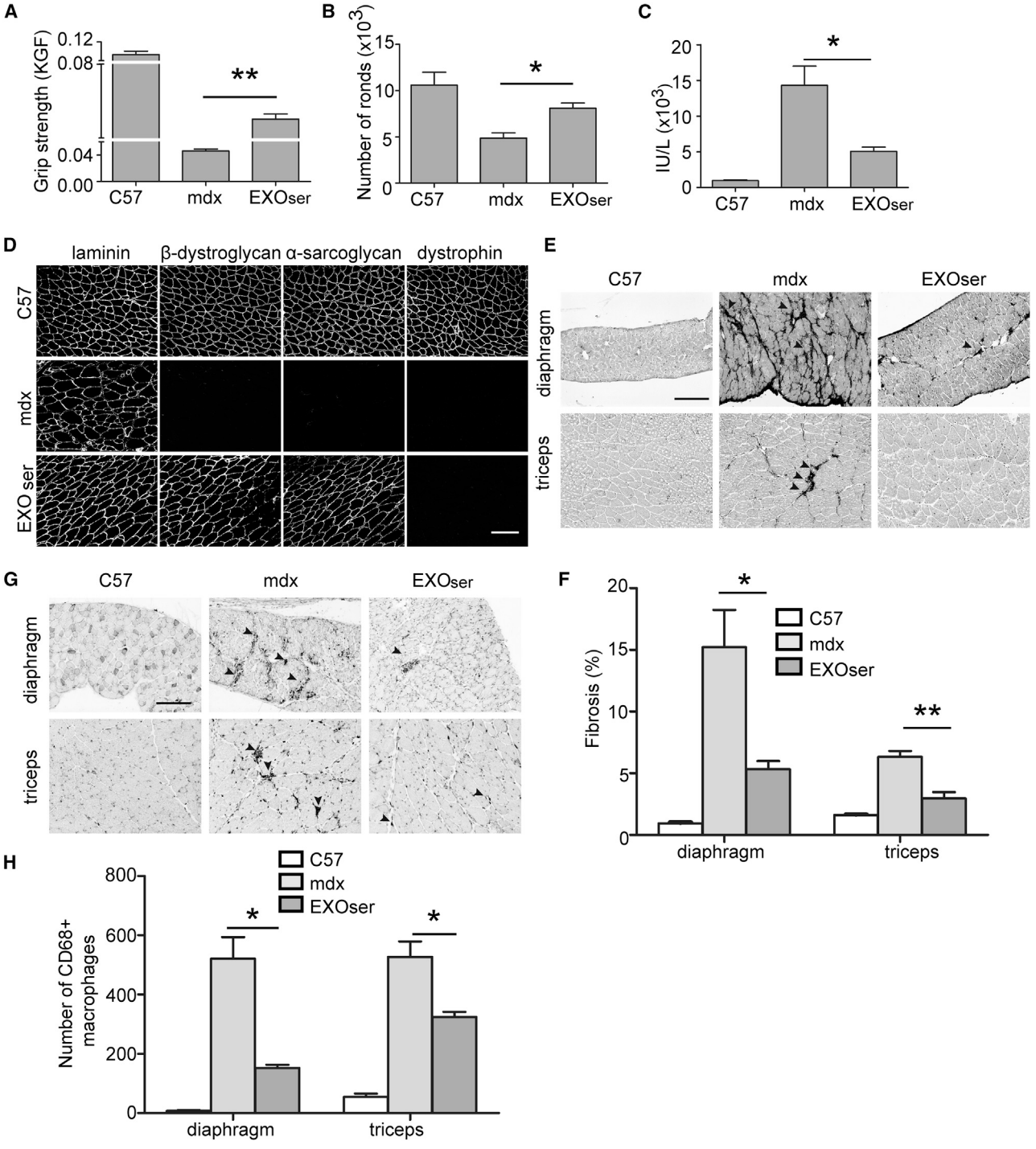

Figure 2. Systemic evaluation of EXOser in adult *mdx* mice

EXOser were injected into adult *mdx* mice i.p. at the dose of 150 μg twice per week 3 days apart for 3 weeks and muscles were harvested 3 days after last injection. (A) Muscle function was assessed to determine the physical improvement with grip strength test in C57BL/6 (n = 3), treated (n = 3), and untreated *mdx* controls (n = 3) (**$p < 0.001$; 1 way-ANOVA post hoc Student-Newman-Keuls test). The same number of mice was used for each group for other assays unless otherwise specified. (B) Measurement of muscle endurance with the running wheel test (n = 3, *$p < 0.05$; 1 way-ANOVA post hoc Student-Newman-Keuls test). (C) Assessment of serum CK levels in *mdx* mice treated with EXO_{MSC} (n = 3, **$p < 0.001$; 1-way ANOVA on ranks). (D) Relocalization of DAPC components in treated *mdx* mice to assess dystrophin function and recovery of

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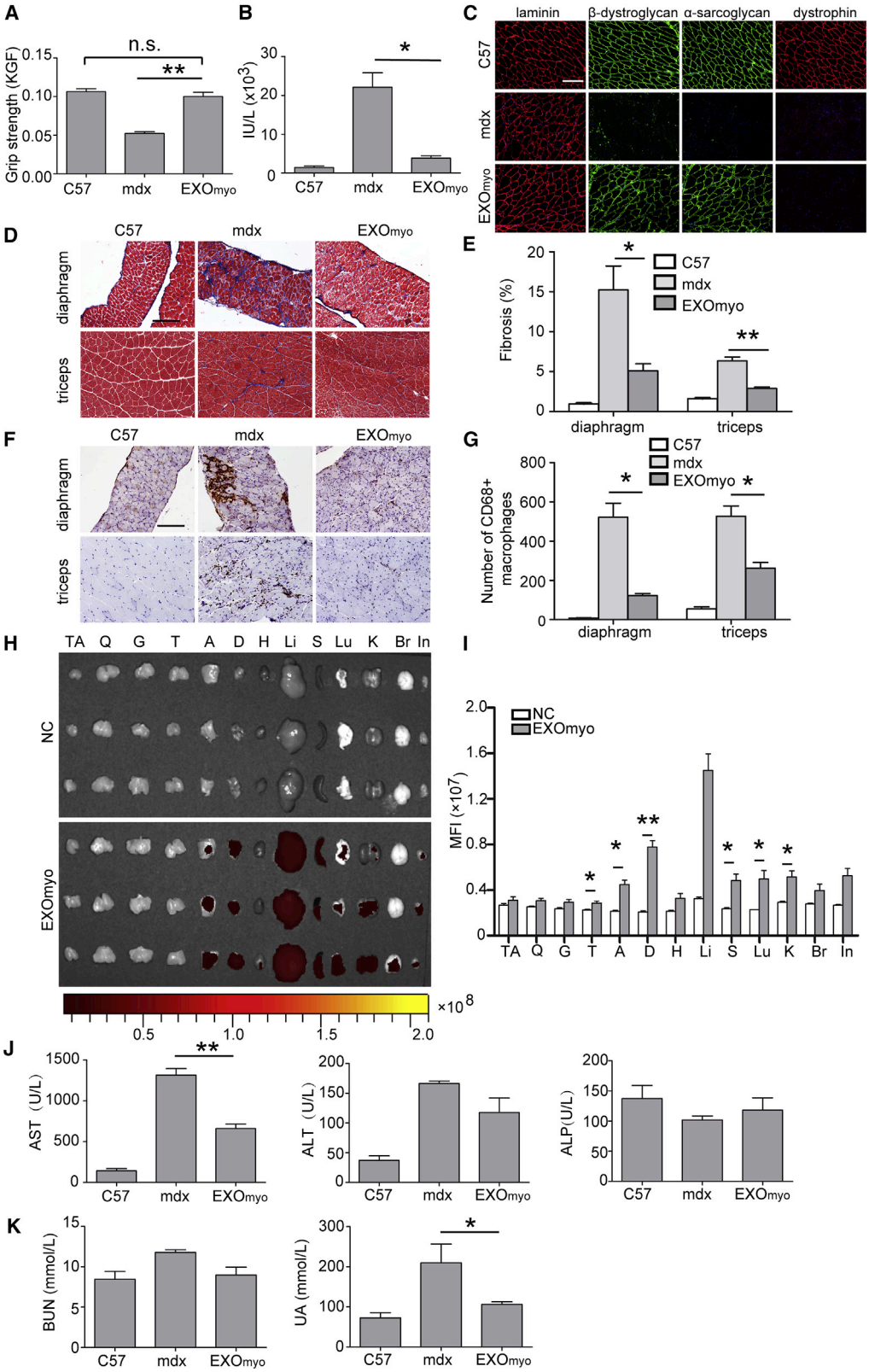
to the membrane was generally observed in muscles treated with exosomes of different sources and the elevation of DAPC is supposed to contribute to relocalization,[31] we examined the expression of DAPC in the EXOmyo-treated diaphragm, the most severely damaged muscle during disease progression,[32] and triceps, the muscle mainly responsible for the forelimb strength test (https://www.treat-nmd.org). Levels of β-dystroglycan and α-sarcoglycan protein significantly rose in muscles from EXOmyo-treated *mdx* mice compared to untreated *mdx* controls (Figures 4C and 4D). Strikingly, levels of β-dystroglycan and α-sarcoglycan proteins were also significantly elevated in the hearts of EXOmyo-treated *mdx* mice compared to untreated *mdx* controls (Figures S9C and S9D), implying that EXOmyo may exert beneficial effects on dystrophic hearts. However, examination of β-dystroglycan and α-sarcoglycan mRNA revealed no change between EXOmyo-treated and untreated *mdx* mice (Figure 4E), indicating that EXOmyo do not have any impact on the transcription. A similar pattern was observed in EXO_{MSC}-treated muscles (Figures S9E and S9F). Concordantly, levels of α-dystroglycan and β-sarcoglycan, other membrane-associated components in DAPC,[22] were also significantly elevated in EXOmyo- and EXO_{MSC}-treated *mdx* muscles compared to untreated *mdx* controls (Figures S9G and S9H). Notably, a significant increase in the level of neuronal nitric oxide synthase (nNOS), another DAPC component reduced in DMD patients,[33] was observed in the quadriceps of EXOmyo-treated *mdx* mice compared to untreated *mdx* controls (Figure S9I), indicating that exosomes enabled relocalization of dystrophin-binding proteins. However, there was no elevation in dysferlin, a transmembrane protein stabilized or upregulated in DMD patients,[34] in the diaphragm and triceps of EXOmyo- and EXO_{MSC}-treated *mdx* mice compared to untreated *mdx* controls (Figures 4F, 4G, and S9J). As DAPC proteins are known to undergo rapid degradation within dystrophic muscles,[22] these results suggest that exosomes prevent the degradation of DAPC rather than upregulate the expression.

EXOmyo reduce calpain activity and cytosolic calcium by stabilizing membrane in dystrophic muscles

Since calpain, a protease, was markedly activated due to elevated levels of intracellular calcium in dystrophic muscles,[35] we wanted to investigate whether the degradation of DAPC was primarily affected by the calcium-dependent calpain pathway. Thus, we repressed calpain activation with PD150606, a calpain inhibitor binding to full-length calpain 2 and the protease core of calpain 1,[36] in tibialis anterior (TA) muscles of *mdx* mice by local intramuscular injection. As expected, calpain activity was largely abolished with PD150606, as revealed by a significant decline in fluorescence intensity (Figure S10A). Concordantly, a significant increase in the levels of β-dystroglycan and α-sarcoglycan was found in PD150606-treated TA muscles compared to untreated *mdx* controls (Figures 5A and 5B), a level comparable to EXOmyo; however, there was no change in the levels of β-dystroglycan and α-sarcoglycan when 26S proteasome activation was suppressed with bortezomib (Figures S10B and S10C), an inhibitor for ATP-dependent 26S proteasome,[37,38] demonstrating that calcium-dependent calpain activation directly mediates DAPC degradation. Correspondingly, a significant reduction in calpain activity was observed in muscles from EXOmyo-treated *mdx* mice compared to untreated *mdx* controls, as demonstrated by decreased fluorescence intensity (Figure 5C). This is in spite of the levels of calpain 1 and 2, two main isoforms of calplain functionally involved in calcium-dependent proteolysis in dystrophic muscles,[39,40] remaining unchanged (Figures S10D and S10E). This suggests that EXOmyo inhibit calpain activity but do not alter the expression. To examine whether EXOmyo-mediated calpain inhibition can be attributed to decreased intracellular calcium influx, we adopted an *in vitro* model system.[24] Differentiated *mdx* myotubes were treated with EXOmyo for 48 h, allowing sufficient uptake of EXOmyo[24] before the introduction of Fluo-4 AM, a green fluorescent calcium indicator.[41] Strikingly, levels of cytosolic calcium dramatically declined in the presence of EXOmyo reflected by a significant reduction in the fluorescence intensity of Fluo-4 AM compared to untreated *mdx* myotubes (Figures 5D and 5E), indicating that EXOmyo reduced the levels of intracellular calcium.

As leaky membranes contributed to elevated intracellular calcium in dystrophic muscle[42] and exosomes can enter cells via membrane fusion,[43] we postulated that exosomes may exert their protective effects by direct membrane stabilization in dystrophic muscles. To validate this assumption, we i.p. administered EXOmyo into *mdx* mice at single dose of 150 μg followed by the systemic introduction of Evans blue dye (EBD), an *in vivo* marker for myofiber membrane permeability,[44] to the same mice 2 h later, to allow sufficient circulation of exosomes in body-wide tissues.[13,45] As predicted, significantly reduced amounts of EBD were found in the diaphragm and triceps of EXOmyo-treated *mdx* mice 24 h after the introduction of EBD, compared to untreated *mdx* controls (Figures 5F and 5G). To exclude the possibility that the improved membrane integrity is due to elevated levels of DAPC, we examined the expression of DAPC in the triceps of *mdx* mice treated with EXOmyo 2 h after injection. There was no elevation in the levels of β-dystroglycan and α-sarcoglycan in the triceps of *mdx* mice treated with a single injection of EXOmyo at 2 h compared to untreated *mdx* controls (Figure S11), confirming the direct effect of EXOmyo on membrane stabilization. Concordantly, intramuscular injection of liposomes of similar sizes (Figure S12A) into the TA muscles of *mdx* mice resulted in the significant elevation of β-dystroglycan and α-sarcoglycan compared to untreated *mdx* controls (Figures S12B and 12C), further strengthening the conclusion that exosomes are primarily responsible for membrane stabilization. To examine whether

normal myoarchitecture (scale bar, 100 μm) (n = 3). (E and F) Masson trichrome staining (E) and quantification (F) of fibrotic areas for diaphragm and triceps from treated *mdx* mice (scale bar, 100 μm) (n = 3, *p < 0.05; 1 way-ANOVA on ranks). Arrowheads point to the collagen-positive areas. (G and H) Immunohistochemistry (G) and quantification (H) of $CD68^+$ macrophages in diaphragm and triceps from treated *mdx* mice (scale bar, 100 μm) (n = 3, *p < 0.05; 1 way-ANOVA on ranks). Arrowheads point to $CD68^+$ macrophages. The data were presented as mean values ± SEMs.



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EXOmyo can induce a long-lasting effect, we administered EXOmyo at a dose of 150 μg twice per week for 1 week, followed by the systemic injection of EBD 2 h after the last injection, and examined the membrane integrity 24 h after EBD injection. Interestingly, there was no significant difference in the EBD^+ area between single injection and double injections of EXOmyo (Figures S13A and 13B), although levels of β-dystroglycan and α-sarcoglycan were significantly increased in the triceps of *mdx* mice treated with double injections of EXOmyo compared to single injection (Figures S13C and 13D), indicating the long-lasting biological effects of EXOmyo. Corroborating with *in vivo* EBD data, EXOmyo also reduced the release of lactate dehydrogenase (LDH), an enzyme that leaks out of membrane-damaged cells,[46] in the cell culture supernatant of damaged differentiated *mdx* myotubes compared to untreated controls (Figure 5H), strengthening the conclusion that EXOmyo improve muscle membrane integrity. Correspondingly, co-localization of EXOmyo with the sarcolemma of *mdx* myotubes (Figure 5I) and the quadriceps and triceps of *mdx* mice treated with EXOmyo i.p. (Figure S14) is further evidence that EXOmyo stabilize membrane by membrane fusion. These data suggest that EXOmyo improve membrane integrity and thus inhibit intracellular calcium influx and calcium-dependent calpain activation, resulting in the prevention of a cascade of detrimental damages to dystrophic muscles.

DISCUSSION

Exosomes from human cardiosphere-derived cells (CDCs) have shown therapeutic potential as regenerative medicine in DMD[47,48]; however, CDCs are specialized cardiac progenitors,[48] which are time-consuming and highly technical for isolation, purification, and maintenance. Clinically, the long derivation time for CDCs can affect production costs and lead time for therapeutics. In contrast, MSCs are more commonly available and clinically applicable. Although an earlier local intramuscular study showed promising results with human MSC-derived exosomes in *mdx* mice,[16] the systemic potential remains to be determined. Here, we demonstrate that repeated systemic administration of exosomes derived from human MSCs, murine serum, DCs, and myotubes restored muscle function,myoarchitecture and prevented pathological progression in *mdx* mice without detectable toxicity. These exosomes improved membrane integrity and thus inhibited intracellular calcium influx and calcium-dependent calpain activation. In turn, this resulted in the stabilization of DAPC and prevented associated muscle damage (Figure 6). A synergistic effect would be expected when exosomes are used in combination with exon skipping or genome editing therapies. Our findings demonstrate the general clinical applicability of exosomes as a systemic treatment modality for DMD and thus provide a viable therapeutic option for DMD.

The major limitation of exosomes as a membrane blocker for DMD treatment is that this approach is not a cure and requires lifelong chronic administration. However, the advantages of serum exosomes, particularly those from healthy donors and close relations of the patients, are low or non-immunogenic, clinically accessible, and can be fast-tracked to the clinic. The abundance of exosomes in serum[49] and a large volume of serum that can be withdrawn regularly make serum exosomes an invaluable treatment option for DMD patients. If large amounts are required, then it may be plausible to tap into apheresis as a source of plasma exosomes, which should behave similarly to serum exosomes, although this has yet to be established. Also, the prolonged therapeutic effects and half-life of injected exosomes *in vivo* were demonstrated in DMD mice and other diseases models,[47,50,51] supporting the possibility of exosomes as a treatment for DMD. In the best scenario, this treatment can be effectively used to manage the disease in a way similar to insulin for type 1 diabetic patients.[52] To maximize the beneficial effect, one could envision that intervention can be started as early as the diagnosis is confirmed to preserve muscle integrity and function. If successful, then this approach may improve the quality and span of life for afflicted DMD patients while they await an effective cure. However, given that the body weight of DMD boys (50 kg on average) is ~2,000 times heavier than mice (25 g on average), the scalability of exosome production may be another issue to be handled before clinical deployment. In addition, based on the promising results from i.p. injection of exosomes in *mdx* mice, we would like to explore the possibility of more clinically available delivery routes such as intravenous or subcutaneous injection and other dosing regimens in *mdx* mice in future studies.

Despite the fact that similar therapeutic efficacy was achieved with exosomes from different sources, EXOmyo were shown to confer greater protective effects on dystrophic muscles than other exosomes. We speculated that the benefits associated with EXOmyo could be attributed to the combined effects of membrane stabilization and

Figure 3. EXOmyo improved muscle function and pathologies in *mdx* mice without detectable toxicities

EXOmyo were injected into adult *mdx* mice i.p. at the dose of 150 μg twice per week 3 days apart for 3 weeks and muscles were harvested 3 days after last injection. (A) Muscle function was assessed to determine physical improvement with the grip strength test in C57BL/6 (n = 3), treated (n = 3), and untreated *mdx* controls (n = 3) (*$p < 0.05$; 1 way-ANOVA post hoc Student-Newman-Keuls test). n.s., not significant. The same number of mice was used for each group for other assays unless otherwise specified. (B) Assessment of serum CK levels in treated *mdx* mice (n = 3, **$p < 0.001$; 1 way-ANOVA post hoc Student-Newman-Keuls test). (C) Re-localization of DAPC components in treated *mdx* mice to assess dystrophin function and recovery of normal myoarchitecture (scale bar, 100 μm) (n = 3). (D and E) Masson trichrome staining (D) and quantification (E) of fibrotic areas for diaphragm and triceps from treated *mdx* mice (scale bar, 100 μm) (n = 3, *$p < 0.05$, **$p < 0.001$; 1 way-ANOVA on ranks). (F and G) Immunohistochemistry (F) and quantification (G) of $CD68^+$ macrophages in diaphragm and triceps from *mdx* mice treated with EXOmyo (scale bar, 100 μm) (n = 3, *$p < 0.05$; 1 way-ANOVA on ranks). (H and I) Tissue distribution (H) and quantification (I) of DiR-labeled EXOmyo in *mdx* mice. DiR-labeled EXOmyo (150 μg) was injected into *mdx* mice i.p., and body-wide tissues were harvested 1 h after single injection (n = 3, *$p < 0.05$, **$p < 0.001$; 2-tailed t test). TA, tibialis anterior; Q, quadriceps; G, gastrocnemius; T, triceps; A, abdominal; D, diaphragm; H, heart; Li, liver; S, spleen; Lu, lung; K, kidney; Br, brain; and In, intestine. Measurement of serum AST, ALT, and ALP (J) or BUN and UA (K) from treated *mdx* mice to reflect liver or kidney functions (n = 3, *$p < 0.05$, **$p < 0.001$; 1 way-ANOVA post hoc Student-Newman-Keuls test). The data were presented as mean values ± SEMs.

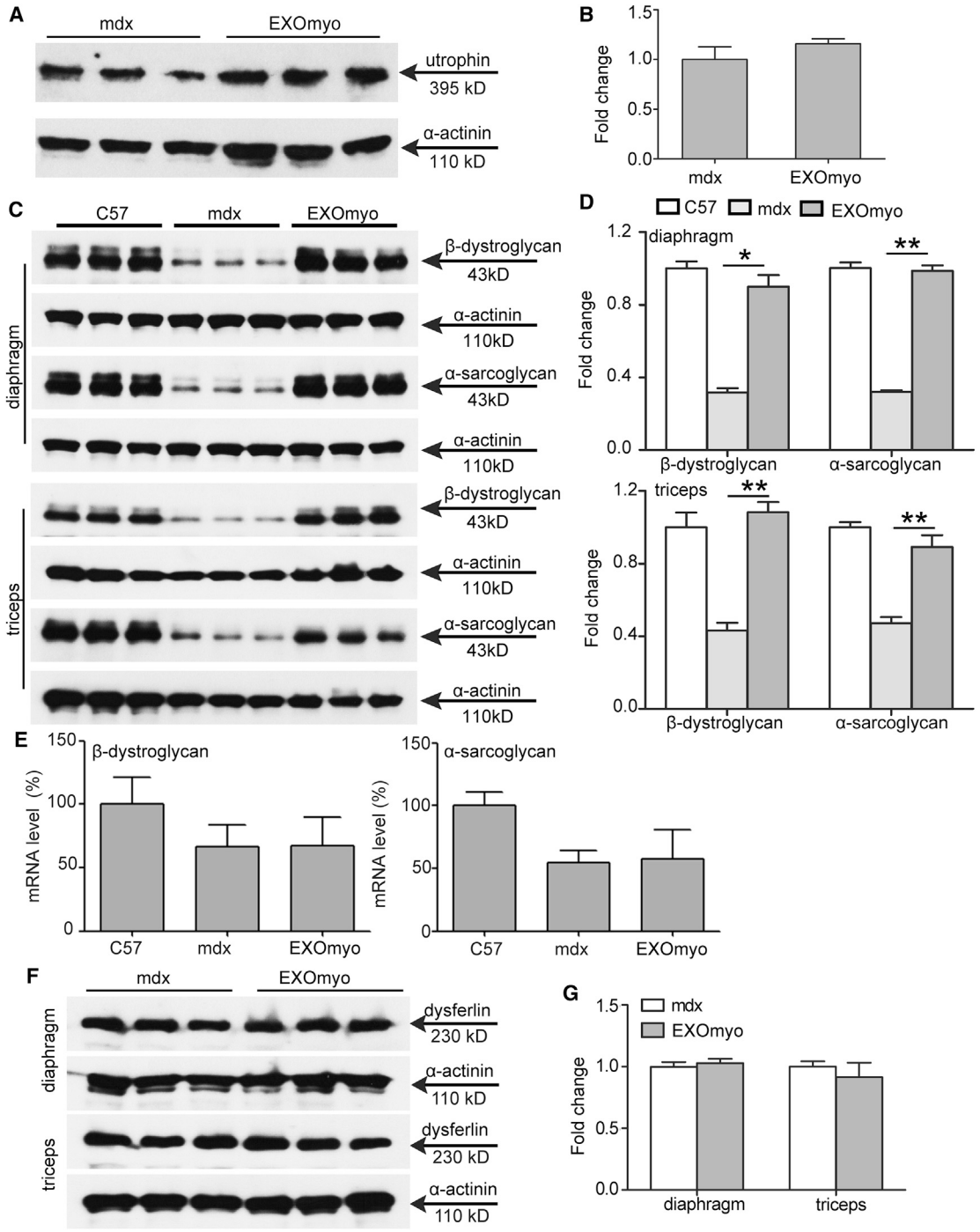

Figure 4. Systemic effects of EXOmyo on membrane-associated proteins in dystrophic muscles

EXOmyo were i.p. administered into adult *mdx* mice at the dose of 150 μg twice per week 3 days apart for 3 weeks. (A and B) Western blot to examine the expression of utrophin (A) and quantitative analysis (B) in triceps from EXOmyo-treated *mdx* mice (n = 3). Total protein (100 μg) from muscle lysates was loaded and α-actinin was used as a loading control. (C and D) Western blot to examine the expression of β-dystroglycan and α-sarcoglycan (C) and quantitative analysis (D) in diaphragm and triceps from EXOmyo-treated *mdx* mice (n = 3, *$p < 0.05$, **$p < 0.001$; 1-way ANOVA on ranks). Total protein 40 μg from muscle lysates was loaded. (E) Quantitative RT-PCR analysis of β-dystroglycan and α-sarcoglycan gene expression in triceps from *mdx* mice treated with EXOmyo (n = 5). (F and G) Western blot to examine the expression of dysferlin (F) and quantitative analysis (G) in diaphragm and triceps from EXOmyo-treated *mdx* mice (n = 3). Total protein 40 μg from muscle lysates was loaded. The data were presented as mean values ± SEMs.

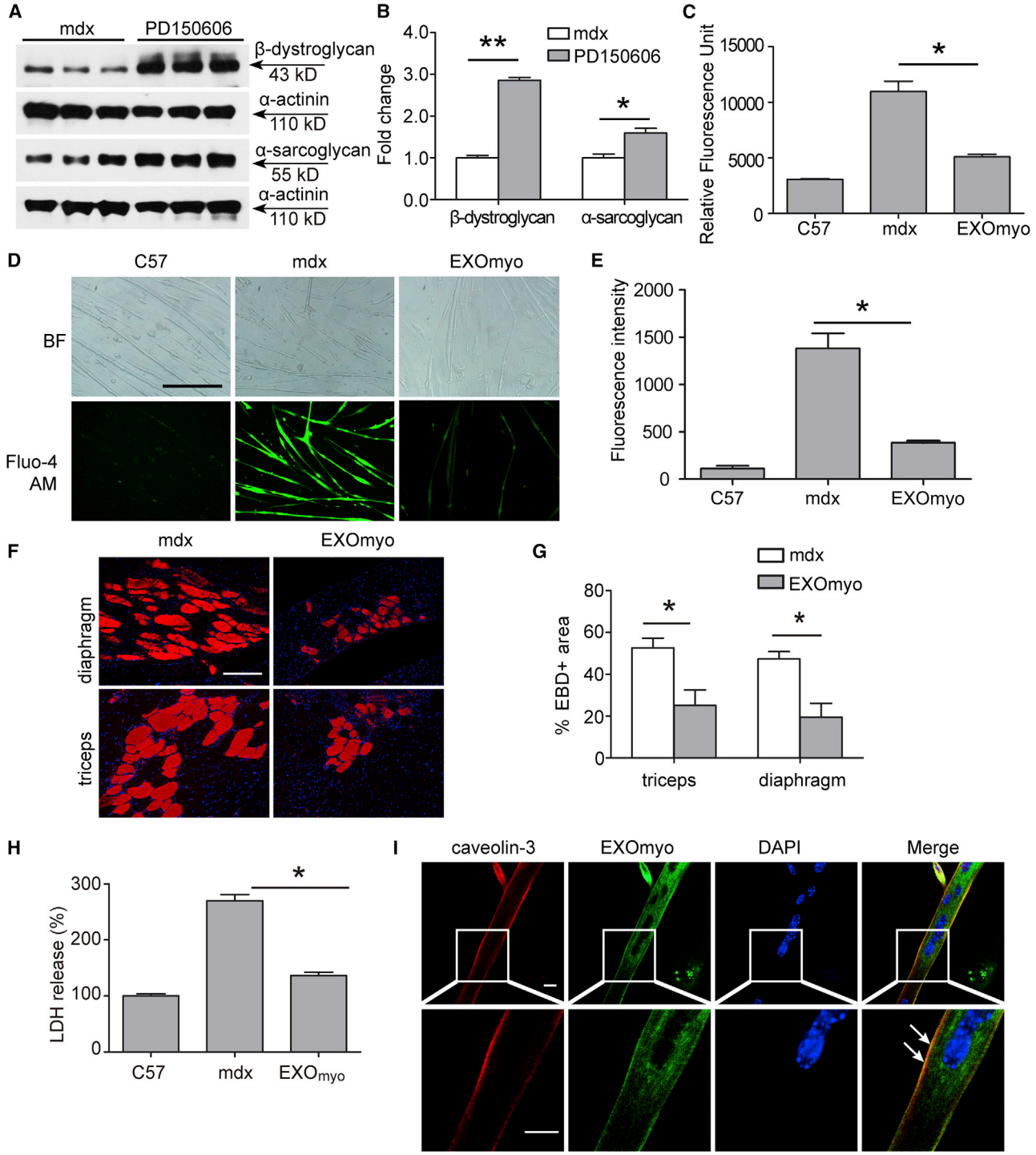

Figure 5. EXOmyo improved membrane integrity and inhibited calpain activity and intracellular calcium influx in dystrophic muscles *in vivo* and *in vitro*

(A and B) Western blot to examine the expression of β-dystroglycan and α-sarcoglycan (A) and quantitative analysis (B) in TA muscles of *mdx* mice treated with PD150606 intramuscularly (n = 3, *p < 0.05, **p < 0.001; 2-tailed t test). Total protein 40 μg from muscle lysates was loaded and α-actinin was used as a loading control. (C) Evaluation of calpain activity in triceps of *mdx* mice treated with EXOmyo (n = 3, *p < 0.05; 1-way ANOVA post hoc Student-Newman-Keuls test). (D) Fluorescence microscopic images showing the Ca^{2+} influx in EXOmyo-treated *mdx* myotubes (scale bar, 100 μm). BF, bright field. (E) Quantitative analysis of fluorescence intensity in *mdx* myotubes treated with EXOmyo (n = 3, *p < 0.05; 1-way ANOVA on ranks). (F and G) Examination of EBD^+ myofibers (F) and quantitative analysis (G) in diaphragm and triceps from *mdx* mice

(legend continued on next page)

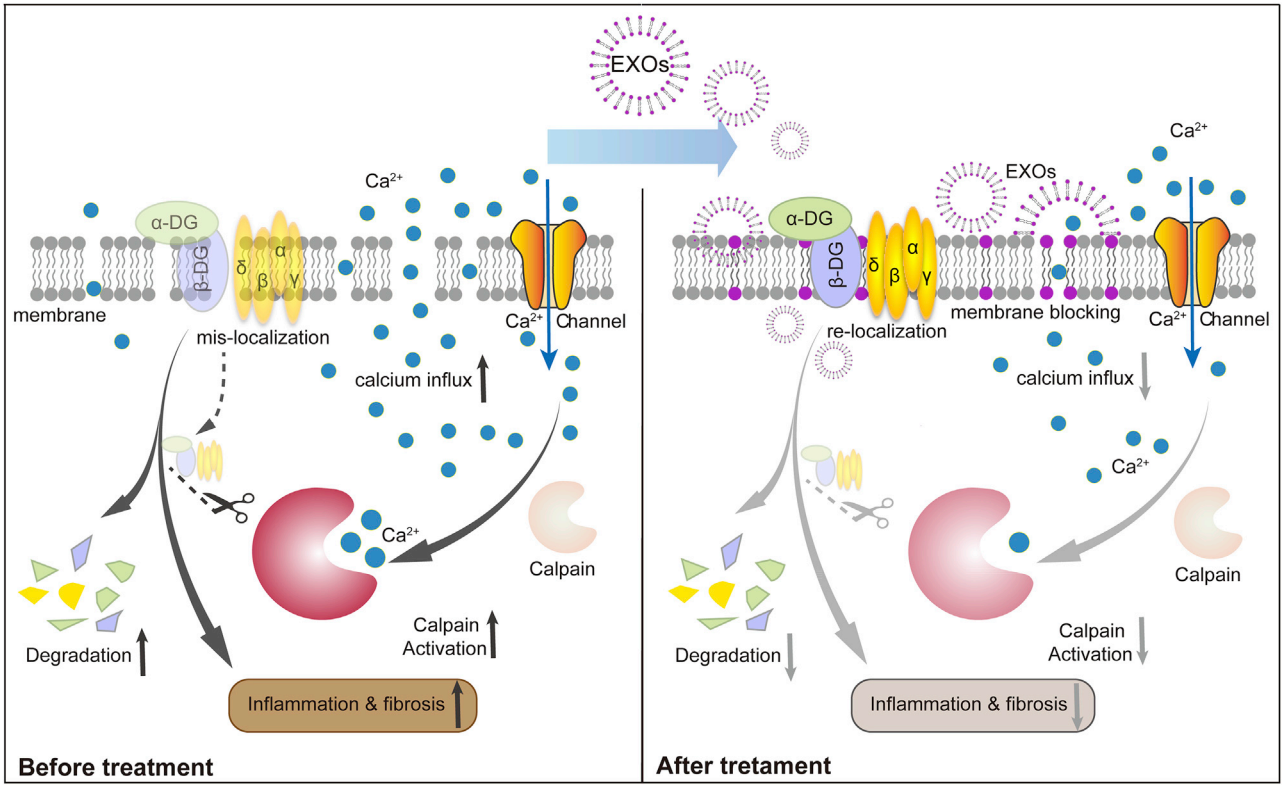

Figure 6. The possible mechanism of action underpinning exosomes' functionality in dystrophic muscles
EXOs, exosomes. Arrows, up- or downregulation.

transfer of functional molecules via endocytosis, another major pathway for exosomes uptake,[53] as demonstrated with CDC-derived exosomes.[47] Although we cannot exclude the possibility that other mechanisms of action or miRNAs/proteins laden with exosomes may also contribute to the observed biological effects, our observations in the present study support the conclusion that exosomes primarily exert their therapeutic benefits via membrane stabilization as revealed by reduced EBD staining, LDH, and co-localization of exosomes on sarcolemma.

Notably, although the membrane integrity was only marginally greater in muscles from EXOmyo-treated *mdx* mice with double injections compared to single injection as demonstrated by EBD staining, a cumulative effect on DAPC upregulation was observed in multiple injections compared to single injections, implying that membrane stabilization is transient but the triggered biological effect is long-lasting. Therefore, in our current study, we adopted a multiple dosing regimen. Nevertheless, a long-term study is warranted before the deployment to the clinic. In addition, heart function was not monitored, as adult *mdx* mice show a very mild cardiac phenotype.[54] Unexpectedly, serum CK-MB was marginally reduced and levels of β-dystroglycan and α-sarcoglycan significantly elevated in EXOmyo-treated *mdx* hearts compared to untreated *mdx* controls, suggesting that exosomes may also have beneficial effects on dystrophic hearts, which is consistent with the previous observation with exosomes from CDCs.[47] Further studies on dystrophic hearts are warranted in dystrophin and utrophin double knockout (DKO) mice, which manifest much severe cardiac dysfunction.[55]

Overall, our study provides evidence that exosomes from different origins can improve muscle function and pathologies by stabilizing muscle membrane, and thus represent a supplementary treatment option for DMD and other diseases with compromised membranes.

MATERIALS AND METHODS

Animals and injections

Adult *mdx* mice (6–8 weeks old) and age-matched C57BL/6 mice were used in all of the experiments (the number of mice per group was

treated with EXOmyo (n = 3, *p < 0.05; 2-tailed t test). Nuclei were counterstained with DAPI (blue). (H) Measurement of LDH activities in the supernatant of *mdx* myotubes treated with EXOmyo (n = 4, *p < 0.05; 1-way ANOVA post hoc Student-Newman-Keuls test). (I) Co-localization of EXOmyo with caveolin-3 in differentiated *mdx* myotube membrane (scale bar, 100 μm). PKH67-labeled EXOmyo was used (green) and nuclei were counterstained with DAPI (blue). The arrows point to the co-localization of EXOmyo with membrane protein (caveolin-3). The data were presented as mean values ± SEMs.

specified in the corresponding figure legends). All of the animal experiments were carried out in the Animal Unit, Tianjin Medical University (Tianjin, China), according to procedures authorized by the institutional ethical committee (permit no.: SYXK2019-0004). For local intramuscular injection study, PD150606 (90 μg), bortezomib (12.5 μM) (MCE, USA), or liposomes (2.1×10^8, single injection) (kindly provided by Prof. Yingsong Wang, School of Pharmacy, Tianjin Medical University) was dissolved in 40 μL saline and injected into the TA muscles of adult *mdx* mice once or twice at an interval of 3 days. Muscles were harvested 24 h after the last injection. For systemic studies, exosomes (EXO_{MSC}, EXO_{mMSC}, EXOser, EXOmyo, or DEX) in saline (150 μL) were injected i.p. into adult *mdx* mice at a dose of 150 μg twice per week at an interval of 3 days for 3 weeks. Mice were sacrificed by CO_2 inhalation 3 days after the last injection, muscles were snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C.

Data analysis

All of the data are reported as mean values ± SEMs. Statistical differences between the different treated groups were evaluated by Sigma Stat (Systat Software, Chicago, IL, USA). Both parametric and nonparametric analyses were applied as specified in the figure legends. Significance was determined based on $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.ymthe.2020.12.018.

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AUTHOR CONTRIBUTIONS

H.F.Y., L.L., and X.D. designed the project; L.L., X.D., X.G., N.R., M.G., B.Z., Y.W., W.L., and G.H. carried out the experiments; L.L., X.D., H.Y., G.H., and H.F.Y. analyzed the data; H.F.Y., L.L., and X.D. wrote the paper with the input from all of the authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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