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RESEARCH ARTICLE

The root cause of Duchenne muscular dystrophy is the lack of dystrophin in smooth muscle of blood vessels rather than in skeletal muscle per se [version 1; peer review: 2 not approved]

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V1 First published: 20 Aug 2018, 7:1321
<https://doi.org/10.12688/f1000research.15889.1>
 Latest published: 04 Dec 2018, 7:1321
<https://doi.org/10.12688/f1000research.15889.2>

Abstract

Background: The dystrophin protein is part of the dystrophin associated protein complex (DAPC) linking the intracellular actin cytoskeleton to the extracellular matrix. Mutations in the dystrophin gene cause Duchenne and Becker muscular dystrophy (D/BMD). Neuronal nitric oxide synthase associates with dystrophin in the DAPC to generate the vasodilator nitric oxide (NO). Systemic dystrophin deficiency, such as in D/BMD, results in muscle ischemia, injury and fatigue during exercise as dystrophin is lacking, affecting NO production and hence vasodilation. The role of neuregulin 1 (NRG) signaling through the epidermal growth factor family of receptors ERBB2 and ERBB4 in skeletal muscle has been controversial, but it was shown to phosphorylate α -dystrobrevin 1 (α -DB1), a component of the DAPC. The aim of this investigation was to determine whether NRG signaling had a functional role in muscular dystrophy.

Methods: Primary myoblasts (muscle cells) were isolated from conditional knock-out mice containing lox P flanked ERBB2 and ERBB4 receptors, immortalized and exposed to CRE recombinase to obtain *Erb2/4* double knock-out (dKO) myoblasts where NRG signaling would be eliminated. Myotubes, the *in vitro* equivalent of muscle fibers, formed by fusion of the lox P flanked *Erb2/4* myoblasts as well as the *Erb2/4* dKO myoblasts were then used to identify changes in dystrophin expression.

Results: Elimination of NRG signaling resulted in the absence of dystrophin demonstrating that it is essential for dystrophin expression. However, unlike the DMD mouse model mdx, with systemic dystrophin deficiency, lack of dystrophin in skeletal muscles of *Erb2/4* dKO mice did not result in muscular dystrophy. In these mice, ERBB2/4, and thus dystrophin, is expressed in the smooth muscle of blood vessels allowing normal blood flow through vasodilation during exercise.

Conclusions: Dystrophin deficiency in smooth muscle of blood vessels, rather than in skeletal muscle, is the main cause of disease

Open Peer Review

Approval Status **XXX**

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version 2 (revision) 04 Dec 2018		view	view
version 1 20 Aug 2018	view	view	

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Any reports and responses or comments on the article can be found at the end of the article.

progression in DMD.

Keywords

Dystrophin, DAPC, Duchenne muscular dystrophy, Smooth muscle, Blood vessels, Neuregulin, ERBB2/4, HER2/4

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Competing interests: The author declares that the figure and corresponding data reported in this paper was used as part of the information for filing a patent application on ERBB4 receptor modulators based on the discovery that NRG signaling through ERBB2/4, via the cleaved ERBB4 intracellular domain, stimulated dystrophin expression.

Grant information: This work was supported by funds from the University of Basel, Basel, Switzerland.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Gajendran N. **The root cause of Duchenne muscular dystrophy is the lack of dystrophin in smooth muscle of blood vessels rather than in skeletal muscle per se** [version 1; peer review: 2 not approved] F1000Research 2018, 7:1321 <https://doi.org/10.12688/f1000research.15889.1>

First published: 20 Aug 2018, 7:1321 <https://doi.org/10.12688/f1000research.15889.1>

Introduction

Signaling from neuregulin 1 (NRG), through its epidermal growth factor (EGF) family of receptors ERBB1-4, has major functions in several organs such as heart, breast, and nervous system including central and peripheral synapses. The role of NRG signaling in skeletal muscle has been controversial. To investigate signaling events in muscle fibers, myotubes formed by fusion of myoblasts, are routinely used as the *in vitro* equivalent of muscle fibers. We already reported that in myotubes, formed from C2C12 myoblasts, NRG signaling through ERBB2/4 heterodimeric receptors phosphorylated α -dystrobrevin 1 (α -DB1)¹, one of the components of the dystrophin associated protein complex (DAPC). DAPC links the intracellular actin cytoskeleton to the extracellular matrix and is thereby thought to provide structural stability during muscular activity. DAPC, apart from containing dystrophin which is a 427 kDa protein, consists of several other proteins such as α - and β -dystrobrevins, dystroglycans, sarcoglycans, sarcospan, syntrophins, and laminins. At the neuromuscular synapse, the DAPC is also formed with utrophin, also a 427 kDa protein, instead of dystrophin. The phosphorylation of α -DB1 through NRG/ERBB signaling stabilized acetylcholine receptors (AChRs) at the neuromuscular synapse². Duchenne and Becker muscular dystrophy (D/BMD) patients have mutation(s) in the dystrophin gene, resulting in the expression of a truncated dystrophin protein³⁻⁶. Taken together, the main body of research on DMD argues for the lack of dystrophin in skeletal muscle as the cause for DMD.

In mice, apart from muscular dystrophy, absence of dystrophin causes neuromuscular junction (NMJ) fragmentation⁷ similar to the NMJ fragmentation associated with a loss of NRG/ERBB signaling¹. Lack of dystrophin, besides causing muscular dystrophies, results in cardiomyopathy⁸ and is also responsible for several disease states in the brain⁶. The importance of NRG signaling for normal cardiac development in mice was firmly established by the fact that ablation of NRG, ERBB4, or ERBB2 resulted in premature death during midgestation⁹⁻¹¹. In cardiac muscle NRG/ERBB4 signaling is sufficient for cardiomyocyte proliferation and repair of heart injury¹², but knowledge of the detailed signaling mechanisms and the target proteins through which this was achieved are lacking. The aim of this investigation was to identify the function of NRG/ERBB signaling in muscle and, as it phosphorylated α -DB1 in the DAPC complex, determine if it had a functional role in muscular dystrophy by identifying downstream signaling targets.

Methods

Cell culture, cell lines

erbb2/4 dKO and loxP flanked *erbb2/4* myoblasts (a kind gift from M. Courtet, and previously described¹) were cultured on laminin-coated dishes (Roche) and upon reaching 70–80% confluency, were allowed to form myotubes by changing to differentiation media (2% horse serum, 1% penicillin/streptomycin (Sigma-Aldrich), DMEM (Sigma-Aldrich)).

Western blotting

Myotubes from 10-cm culture dishes were harvested in 600 μ l lysis buffer and protein complexes were immunoprecipitated as

described previously^{1,13} with modifications. In brief, myotubes harvested in ice-cold lysis buffer (10 mM Na₃PO₄, pH 7.8, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, protease inhibitor mixture (Roche), and phosphatase inhibitors Pic1 and Pic2 (Sigma-Aldrich)) were homogenized in a Dounce homogenizer and incubated for 3 h at 4°C with protein G-coupled mouse monoclonal syntrophin antibody 1351 (4 μ l/80 μ l protein G beads, Abcam, catalog number ab11425). Beads were then washed in lysis buffer containing protease inhibitors, but without Triton X-100, resuspended in 3 x SDS loading buffer (150 mM Tris-HCl [pH 6.8], 300 mM dithiothreitol [added just before use], 6% SDS, 0.3% bromophenol blue, and 30% glycerol), and denatured (94°C, 3 min) before loading on an 6–8% gradient/0.8% bis-acrylamide SDS-PAGE gels buffered with Tris-glycine.

Gels were transferred onto PVDF membranes (Millipore) and subject to ECL (Thermo Fisher Scientific) development after incubation with primary and secondary antibodies. BSA (3%) was used as a blocking reagent. The following primary antibodies were used: rabbit anti-dystrophin (H300) polyclonal (diluted 1:400, catalog number sc-15376) and mouse anti-utrophin (55) monoclonal (diluted 1:400, catalog number sc-136116) were from Santa Cruz Biotechnology, Inc., mouse monoclonal anti-syntrophin 1351 (4 μ l antibody/80 μ l protein G beads for lysate from a 10 cm culture dish of myotubes, catalog number ab11425) from abcam and rabbit anti- α -syntrophin 259 (5 μ g/ml for Westerns; a kind gift from Stanly C. Froehner and Marvin Adams, University of Washington, Seattle, WA). Goat anti-mouse IgG-HRP (catalog number sc-2005) and goat anti-rabbit IgG-HRP (catalog number sc-2004) secondary antibodies (Santa Cruz Biotechnology, Inc.) were used at a 1:5,000 dilution.

RNA isolation and qPCR

RNA isolation and qPCR were performed as previously described¹⁴ and the 2^{- $\Delta\Delta$ Ct} method was used to analyze relative changes in gene expression. RNA from myotube cultures was isolated with TRIzol (Invitrogen) according to their protocol. DNase I (Promega) treatment and reverse transcription was performed on 1 μ g total RNA with random primers and superscript reverse transcriptase from Invitrogen according to their protocol. cDNA was diluted 1:5 before use in qPCR, which was performed with SyBR Green mix (Applied Biosystems) using the Applied Biosystems StepOne machine with two-step PCR (60°C, 1 min and 95°C 15 s) for 40 cycles using the standard program. The quantitative PCR mix was prepared as follows: 12.5 μ l SyBR Green mix, 2.5 μ l of a 3 μ M solution each of forward and reverse primer, 1 μ l of diluted cDNA and made up to 25 μ l total volume with sterile water. Each sample for real time PCR was done in triplicate and the mean of the resulting three values were taken. The following primers, designed to recognize exons with at least one intron in between for each primer pair, were used for dystrophin, utrophin, and rL8 amplifications: dystrophin forward, 5'-GATGATGAACATTTGTTAATCCAGC-3' and reverse, 5'-CATATTCTGCTTGCAGATTCCTG-3'; utrophin forward, 5'-CTAAACTCCTGCGGCAGCAC-3' and reverse, 5'-GTGTCAAGTGAGTAGCTCAATGC-3' and rL8 (normalization gene) forward, 5'-ACTGGACAGTTCGTGTACTG-3' and reverse, 5'-GCTTCACTCGAGTCTTCTTG-3'.

Results

We reported previously that on western blots following immunoprecipitation, there were two isoforms of α -dystrobrevin1 associated with DAPC, a 75 kDa and 89 kDa protein¹. We also demonstrated that ablation of ERBB2/4 receptors resulted in a lack of phosphorylation of the 75 kDa protein¹. In addition, the amount of the 75 kDa protein detected on western blots, compared to the 89 kDa protein, was reduced in *Erbb2/4* dKO myotubes. However in myotubes with intact ERBB2/4 receptors, such as in C2C12 myotubes, it was the other way around¹ i.e. less 89 kDa protein. As absence of dystrophin in the DMD mouse model mdx, also resulted in a reduction in the 75 kDa protein compared to wild-type mice¹⁵, it was possible that the reduction in the 75 kDa protein in the *Erbb2/4* dKO myotubes was due to a reduction in dystrophin. Furthermore the AChR fragmentation observed in mdx mice⁷, paralleled those seen in *Erbb2/4* dKO mice¹ raising the possibility that another reason for the observed destabilization of the AChR cluster in *Erbb2/4* dKO myotubes could be due to the reduced levels of dystrophin in these myotubes. These observations taken

together suggest that one of the targets of NRG/ERBB signaling is dystrophin. To investigate this, *Erbb2/4* dKO myotubes derived from immortalized *Erbb2/4* dKO myoblasts¹ and myotubes formed from myoblasts, before transfection of myoblasts with Cre recombinase, containing loxP flanked *Erbb2/4* genes were used. Both, ERBB2 and ERBB4 receptors were ablated to eliminate NRG signaling¹ through these receptors in muscle, because ERBB4 receptors, apart from forming heterodimers, can also form homodimers and ERBB2 can heterodimerize with ERBB3¹⁶. Furthermore, as cultured myotubes secrete NRG¹⁷, the external addition of NRG was not necessary, as demonstrated for phosphorylation of α -dystrobrevin 1 by NRG/ERBB¹.

Three independent experiments (Figure 1A) each using myotubes formed from a different myoblast clone containing loxP-flanked *Erbb2/4* genes, clearly detected dystrophin (lanes 1–3). However dystrophin was not detected in *Erbb2/4* dKO myotubes (lanes 4–6) where ERBB2/4 receptors were ablated after CRE recombination of loxP flanked *Erbb2/4* genes. Utrophin on the other hand was detected in myotubes with and without

Figure 1. Dystrophin, and utrophin levels in *Erbb2/4* dKO myotubes. (A and B), Western blots of immunoprecipitated proteins from myotubes with loxP flanked exons of *Erbb2* and *Erbb4* gene (lanes 1 to 3) and cre mediated knock-out of *Erbb2* and *Erbb4* genes (lanes 4 to 6) detected with dystrophin (A) and utrophin (B) antibodies. Lanes 1 to 3 and 4 to 6 each represent the same experiment performed independently and loaded on the same gel. The lower panel shows detection of syntrophin that served as a loading control. Immunoglobulin G (IgG) detected is the syntrophin antibody used for the immunoprecipitation. As the western blot in (A) was stripped of antibodies and used in (B), the loading control in (B) applies to both A and B. (C and D) qPCR data of dystrophin and utrophin levels in C2C12 myotubes, relative to *Erbb2/4* dKO (*Erbb2/4*^{-/-}) myotubes. Expression levels were normalized to ribosomal protein L8 (rL8) expression. This experiment was performed at least twice with similar results. This figure was previously published in a patent (Patent Link: [WO 2017/036852 A1](#)), but the copyright is the author's own.

ERBB2/4 receptors (Figure 1B) demonstrating that NRG/ERBB signaling selectively regulates dystrophin expression (Figure 2).

The qPCR estimation of dystrophin and utrophin levels (Figure 1C, D) confirmed that dystrophin expression is absent in *Erb2/4* dKO (shown in Figure 1 as *erbb2/4^{-/-}*) myotubes, as there was no detectable mRNA and signals in qPCR were only observed above threshold at about 34 cycles which is essentially detection of non-specific amplification or background signal, whereas detection of ribosomal protein L8, used to normalize expression of dystrophin and utrophin, was above threshold at about 22 cycles in *Erb2/4* dKO and C2C12 samples. Detection of dystrophin mRNA in C2C12 cells by qPCR confirmed that the primers used to amplify dystrophin functioned. It is only possible to conclude that dystrophin is present in C2C12 and the level cannot be estimated since the level of dystrophin in C2C12 was relative to that in *Erb2/4* dKO myotubes, for which essentially background non-specific values were obtained in qPCR due to the absence of dystrophin mRNA. Utrophin detection (Figure 1D), using the same RNA/cDNA preparation used for dystrophin detection, confirmed that the cDNA preparation from *Erb2/4* dKO myotubes used for dystrophin detection was intact. Utrophin expression was reduced to only less than half the amount (Figure 1D) in *Erb2/4* dKO myotubes compared to C2C12 myotubes which may be due to the lack of NRG signaling since NRG stimulates utrophin expression to some extent¹⁸. Myotubes formed from C2C12 myoblasts were used as a control for qPCR instead of myotubes containing loxP flanked *Erb2/4* genes (used for the western blots in Figure 1A, B) as the loxP-flanked *Erb2/4* gene¹⁹ is a hypomorph due to the insertion of the neo selection cassette. Hence C2C12 myotubes were used instead of *Erb2/4* dKO myotubes to exclude the possibility that levels of dystrophin mRNA may have been affected (NRG signals through ERBB2/4 to stimulate dystrophin expression and reduced *Erb2/4* expression may have affected this). This is not a problem for dystrophin protein detection (not estimation) in

immunoprecipitated samples from myotubes containing loxP flanked *Erb2/4* genes. qPCR on *Erb2/4* dKO confirmed the absence of dystrophin expression (Figure 1C) as observed on the western blot (Figure 1A). Hence NRG/ERBB signaling is necessary for dystrophin expression.

Dataset 1. Uncropped western blot images and raw Ct values from qPCR

<http://dx.doi.org/10.5256/f1000research.15889.d214628>

Discussion

Even though dystrophin is lacking in skeletal muscles of *Erb2/4* dKO mice, they do not show dystrophic symptoms²⁰. The promoter used for CRE expression in generating *Erb2/4* dKO mice, the human skeletal actin promoter (HSA), is expressed in the striated muscles, skeletal and heart muscle^{15,21,22}. Therefore ERBB2/4 and dystrophin levels in smooth muscle of blood vessels would not be affected, as CRE is not expressed in smooth muscle of *Erb2/4* dKO mice, allowing the formation of a normal functional DAPC. Hence smooth muscle of blood vessels in these mice allows for increased blood flow to skeletal and cardiac muscle during exercise. This is because neuronal nitric oxide synthase (nNOS) associating with dystrophin and generating nitric oxide (NO) that signals to soluble guanylate cyclase, generating cyclic guanosine 3',5'-monophosphate (cGMP) in smooth muscle of blood vessels, causes vasodilation enabling exercise-induced increase of blood flow and thereby prevents muscle ischemia²³. Thus the absence of obvious dystrophic symptoms in *Erb2/4* dKO skeletal muscle where there is a lack of dystrophin strongly suggests that the main cause of muscular dystrophy is not the lack of dystrophin in skeletal muscle per se but systemic lack of functional dystrophin, especially in smooth muscle of blood vessels, resulting in impaired sympatholysis and muscle ischemia during exercise²³. This hypothesis is consistent with published data using phosphodiesterase type 5 (PDE5) inhibitors, which interfere

NRG/ERBB2/4 (HER2/4) signaling targets dystrophin expression

Figure 2. Schematic drawing of neuregulin (NRG) signaling to stimulate dystrophin expression. Under normal circumstances, NRG signaling through ERBB2/4 (HER2/4) receptors stimulates dystrophin expression, allowing the formation of a normal dystrophin-associated protein complex (DAPC). If either the dystrophin gene contains mutations or NRG signaling is blocked, then in the absence of functional dystrophin, a normal DAPC is not formed, resulting in various disease states such as dilated cardiomyopathy, Duchenne/Becker muscular dystrophy (D/BMD), and neuromuscular synapse instability.

with breakdown of NO by PDE5 and thereby prolong the half-life of cGMP, the target of NO²⁴. This was demonstrated in mdx mice where PDE5 inhibition alleviates the dystrophic phenotype²³ and also in DMD patients where PDE5 inhibition with either tadalafil or sildenafil treatment in Duchenne muscular dystrophy boys restored normal blood vessel function and blood flow during exercise²⁴. Thus the muscle has an extensive vasculature to provide more oxygenated blood through vasodilation the absence of which would result in muscle ischemia, injury and fatigue during exercise.

We previously reported that the blockade of NRG signaling through ERBB2/4 receptors prevented phosphorylation of α -dystrobrevin1 and hence affected NMJ stability¹. However ablation of ERBB2/4 receptors, and thus elimination of NRG signaling through them, results in a lack of dystrophin expression (Figure 1). Thus NRG/ERBB signaling maintains NMJ stability through at least two pathways, one where it phosphorylates α -dystrobrevin 1^{1,2} and the other where it stimulates dystrophin expression and thereby allowing the formation of a DAPC that stabilizes acetylcholine receptors (Figure 2).

NRG/Erbb signaling also induces cardiomyocyte proliferation and repairs heart injury¹² and is essential for normal cardiac development^{9–11}, whereas dystrophin deficiency does not impair cardiac development but does result in dilated cardiomyopathy (DCM)⁸. Since ERBB4 can heterodimerize with ERBB2 and a function blocking ERBB2 antibody treatment results in DCM in mice²¹ and DCM in cancer patients with a function blocking HER2 antibody treatment²⁵, this is consistent with NRG/ERBB signaling stimulating dystrophin expression, since DMD patients and mdx mice, both lacking functional dystrophin, develop DCM (Figure 2). Hence the data presented here provides a mechanism for the reported beneficial effects of ERBB4 in repairing heart injury whereby NRG/ERBB2/4 signaling stimulates dystrophin expression. Thus NRG/ERBB carries out different functions in cardiac development and maintenance through different signaling targets, in the latter case through regulating dystrophin expression.

Increasing NRG signaling through ERBB2/4, especially in the smooth muscle of blood vessels, could be a way to increase truncated dystrophin expression in D/BMD patients. This would ameliorate dystrophic symptoms in those patients where the mutation in dystrophin does not affect association of nNOS²⁶ and thereby enabling normal blood flow during exercise. Furthermore, as dystrophin is also present in all regions of the brain, being most abundant in the cerebellum²⁷, and since NRG/ERBB signaling regulates dystrophin expression, levels of dystrophin could be the underlying cause of some of the disease states such as schizophrenia, associated with NRG/ERBB function in the brain.

Conclusions

NRG signaling through ERBB2/4 receptors is necessary for stimulation of dystrophin expression. However, when ERBB2/4 receptors are lacking in skeletal muscle but expressed in smooth muscle, mice do not exhibit dystrophic symptoms demonstrating that lack of dystrophin expression in smooth muscle is the root cause of the onset of D/BMD.

Data availability

Dataset 1. Uncropped western blot images and raw Ct values from qPCR. DOI: <https://doi.org/10.5256/f1000research.15889.d214628>²⁸.

Competing interests

The author declares that the figure and corresponding data reported in this paper was used as part of the information for filing a patent application on ERBB4 receptor modulators based on the discovery that NRG signaling through ERBB2/4, via the cleaved ERBB4 intracellular domain, stimulated dystrophin expression.

Grant information

This work was supported by funds from the University of Basel, Basel, Switzerland.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Current Peer Review Status:

Version 1

Reviewer Report 29 October 2018

<https://doi.org/10.5256/f1000research.17347.r39666>

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This manuscript suggests the possibility that the lack of dystrophin expression in cell types different than myofibers is the cause of Duchenne muscular dystrophy (DMD). In particular smooth muscle cells are proposed to be the cells responsible for the disease.

The idea that DMD is not necessarily due to a lack of dystrophin in skeletal muscle fibres is interesting but of course it challenges a plethora of previous studies and needs to be demonstrated properly. The main issue with this study is that the claims are not supported by results. At the end of the Introduction it is stated that "The aim of this investigation was to identify the function of NRG/ERBB signalling in muscle and, as it phosphorylated α -DB1 in the DAPC complex, determine if it had a functional role in muscular dystrophy by identifying downstream signaling targets...". Clearly the manuscript fails to show this as only a very short dataset is provided.

To support the claim of the title the authors should provide evidences that ... "Even though dystrophin is lacking in skeletal muscles of *Erb2/4* dKO mice, they do not show dystrophic symptoms"... This crucial point is mentioned in Discussion by citing the ref paper #20 which anyway does not include any information about a lack of dystrophin in muscles.

The suggestion that the lack of dystrophin in smooth muscle cells (or blood vessel cells) is causing the disease is speculative and it is not supported by the data provided. A solid set of data demonstrating that the dystrophin expressed in smooth muscle cells (or other blood vessel cells) only (ie not in myofibres) is sufficient to rescue the pathology should be provided. Clearly a proper set of experiments in vivo is needed to support the authors' claim.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Muscular dystrophy, gene therapy

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 01 Nov 2018

Nadesan Gajendran, University of Basel, Basel, Switzerland

αI thank the reviewer for reading the article and for giving comments/suggestions.

The reviewer refers to previous studies that are challenged by this article. This comment was also made by reviewer 1 to which I have responded in detail below referring to published studies. Briefly, although several of the previous studies to restore dystrophin expression targeted skeletal muscle, dystrophin or mini dystrophin would also be expressed in vascular smooth muscle (VSM) due to the delivery route, promoter used for expression of dystrophin or gene editing components, or the vehicle used for delivery and hence it is neither possible to conclude that restoring dystrophin expression in skeletal muscle is sufficient to rescue the dystrophic pathology nor to exclude the role of VSM in the onset of dystrophic symptoms. Furthermore, studies that reported restoring dystrophin expression in skeletal muscle will result in the dystrophin associated protein complex (DAPC) to be restored and enable signalling from this complex to be re-established, but this does not mean that on the long term the aberrant vasoregulation due to a lack of dystrophin in VSM will not cause again the onset of dystrophy in skeletal muscle.

As the reviewer is aware, the current article concludes that the root cause of DMD is the lack of dystrophin in VSM but it does not claim that restoring dystrophin expression in VSM will restore the dystrophic pathology in skeletal muscle to the normal state although it may ameliorate it. If lack of dystrophin in VSM is responsible for the onset of DMD as the current article concludes, then from what is known so far, the lack of dystrophin in skeletal muscle

may exacerbate the dystrophic pathology caused by aberrant vasoregulation, possibly due to structural defects as well as the interruption of signalling events from the dystrophin associated protein complex (DAPC). This hypothesis is supported by a published study where they demonstrated that satellite cells from mdx mice retained their regenerative capacity and that the host environment was critical for satellite cell function¹. Hence if the host environment is in a dystrophic state (onset of which may have been due to aberrant vasoregulation), such as in mdx skeletal muscle, impaired muscle regeneration is observed. Thus the current article suggests that treatment strategies should (also) target VSM for any long term benefit for DMD patients.

The reviewer comments that the manuscript (article) fails to show if NRG/ERBB signaling had a functional role in muscular dystrophy by identifying downstream signalling targets as written at the end of the introduction. Clearly the results in Fig. 1 shows that dystrophin is a signalling target of NRG/ERBB and the fact that, based on HSA promoter activity, ERBB2/4 dKO mice do not show dystrophic symptoms (explained below) is due to ERBB2/4 and hence also dystrophin still being expressed in VSM (and possibly endothelial cells). Thus NRG/ErbB signalling is essential for dystrophin expression, and the systemic lack of dystrophin causes muscular dystrophy.

The conditional HSA-CRE/ErbB2 KO mouse² (is the same ErbB2 lox P flanked mouse used in breeding to generate the HSA-CRE/ErbB2/4 dKO mouse) shows similar pathology to the HSA-CRE/ErbB2/4 dKO mouse. However when dystrophin is lacking in skeletal muscle and VSM as in the conditional MCK-CRE/ErbB2 KO mouse³ where CRE expression is driven by the muscle creatine kinase promoter, it shows impaired muscle regeneration similar to the mdx mouse that lacks dystrophin and hence would be expected to show a more severe pathology compared to the MCK-CRE/ErbB2 KO mouse.

The reviewer commented that only a very short dataset is provided. Studies supporting the conclusion of the current article that the lack of dystrophin in VSM is the root cause of DMD are:

- 1) Ablation of ErbB2/4 receptors results in a lack of dystrophin in myotubes;
- 2) Skeletal muscle specific HSA-CRE/ErbB2/4 dKO mice that would lack dystrophin in skeletal muscle but express dystrophin in VSM do not show a dystrophic pathology⁴;
- 3) HSA-CRE/ErbB2 KO² mice used in breeding to generate HSA-CRE/ErbB2/4 dKO show a similar pathology to the latter;
- 4) MCK-CRE/ErbB2 mice³ that would lack dystrophin in skeletal muscle and VSM (as MCK promoter is active in both), show impaired muscle regeneration;
- 5) Lack of dystrophin in skeletal muscle fibers through RNAi knock down did not show an overt dystrophic pathology⁵;
- 6) expression of dystrophin in VSM, even at significantly lower levels compared to wild type controls, improved aberrant vasoregulation in mdx mice⁶;
- 7) Taken together, the current article and several published studies involving rescue of dystrophic muscle would suggest, that aberrant vasoregulation is the cause of the onset of DMD (current article) and that lack of dystrophin in skeletal muscle exacerbates the dystrophic pathology.

Other data are available in the publication of the patent filing⁷ (patent link: [WO 2017/036852 A1](#), link also in legend to Fig. 2) reporting that signalling (stimulation/upregulation) from NRG/ERBB is most likely mediated by the intracellular domain of ERBB4 (4ICD) whereas signalling from α -dystrobrevin 1 (α -DB1) downregulates dystrophin expression since genetic deletion of dystrobrevin results in increased dystrophin and ERBB4 expression with most of the ERBB4 being cleaved to generate 4ICD. Data in the published patent filing also show that restoring α -DB1 in DB KO myotubes downregulates dystrophin expression to levels found in myotubes formed from C2C12 cells. These data, although clearly demonstrate signalling events occur from the DAPC, are not necessary to support the conclusion in the current article and hence were not included.

The reviewer comments that the ref provided (ref. number 20) in the article does not include any information about a lack of dystrophin in muscles. As I wrote in my comment to the report of reviewer 1, the supplemental data for that paper includes information that reports the absence of centralized nuclei and lack of atrophic fibers thus there was no indication to look for dystrophin expression. The paper⁴ also reports that sustained muscle strength was not affected nor was a myasthenic condition observed. These mice did show a hind limb extension reflex which was not reported in the paper. As I also wrote to in my comment to the report of reviewer 1, it was not possible to resolve dystrophin expression (nor phosphorylation of α -DB1⁸) in vivo or in muscle lysates of ErbB2/4 dKO mice for the reasons explained in my comment to the report of reviewer 1 below.

The reviewer also comments that "...data demonstrating that the dystrophin expressed in smooth muscle cells (or other blood vessel cells) only (i.e. not in myofibers) is sufficient to rescue the pathology should be provided. This experiment has been published whereby dystrophin was expressed in VSM that improved aberrant vasoregulation in mdx mice even though the amount of dystrophin was significantly lower⁶ than that in wild type control mice. Although the current article concludes that lack of dystrophin in VSM is the root cause (onset) of DMD, this does not necessarily mean that expressing dystrophin in VSM when the skeletal muscle is already in a dystrophic state (due to systemic dystrophin deficiency) would rescue the dystrophic phenotype. Thus experiments designed to express full length dystrophin in VSM only at embryonic day 9.5 (when dystrophin is normally expressed) is not feasible given the size of the dystrophin cDNA and complicated by the fact that expression levels, even of a mini dystrophin, may not be sufficient. Hence an alternative experiment to obtain a situation in vivo where dystrophin is expressed in VSM as well as in other cells and tissues but not in skeletal muscle is to prevent NRG signalling by ErbB2/4 dKO and hence dystrophin expression in skeletal muscle i.e. the HSA-CRE/ErbB2/4 dKO mouse mentioned in the current study.

The loss of dystrophin when ErbB2/4 receptors are ablated by CRE expression driven by the HSA promoter in skeletal muscle does not result in dystrophic symptoms (when dystrophin is expressed in VSM and other cells and tissues) is consistent with published observations whereby RNAi knock-down of dystrophin in skeletal muscles did not show an overt dystrophic pathology⁵.

I would like to invite the reviewer to read my comments below to reviewer 1 as several of

the concerns raised were similar. As I will submit a revised version that would address the concerns raised by reviewer 1 (report and comment) and reviewer 2, if the reviewer has any additional concerns that is communicated as a comment to this response, I would try and address those as well.

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Competing Interests: No competing interests were disclosed.

Reviewer Response 05 Nov 2018

Alberto Malerba, Royal Holloway, University of London, Egham, UK

I thank the author for replying to my concerns. As I mentioned when revising the manuscript, I do consider interesting the concept that non-skeletal muscle cells have a role in DMD. This is plausible and, as the author states, there are several published studies showing that cells other than myofibres have a role in DMD but the general consensus is that the main issue is a lack of dystrophin in myofibres and that the lack of dystrophin in other cell types exacerbate the disease (which is pretty much the opposite of the author's claim). For example the paper Ito et al 2006 concludes that "these data suggest that dystrophin in VSMCs may play an important role in the local autocrine regulation of α -adrenergic constriction, and that the loss of this regulatory mechanism may exacerbate muscle fiber necrosis".

However, I am not criticizing the claim of the current manuscript per se, my only concern is that this claim is not supported by experimental evidences (as also observed by the other reviewer). The author mentioned several publications supporting the conclusions of this manuscript but, clearly, the manuscript must sustain the claim with solid original data.

Showing that in vitro “Ablation of ErbB2/4 receptors results in a lack of dystrophin in myotubes” is simply not enough to persuade the readers that the lack of dystrophin in smooth muscle cells is “The root cause of Duchenne muscular dystrophy”. The manuscript should stand on its own and observations from other published studies supporting the conclusion are valuable but should not replace original data.

I would be pleased to see a revised version that addresses these concerns, but I advise the author to include on it solid in vivo data to support its claims.

Competing Interests: I have no competing interests

Reviewer Report 20 September 2018

<https://doi.org/10.5256/f1000research.17347.r38333>

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Kay E. Davies

Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UK

This paper concludes that the root cause of DMD is the lack of dystrophin in smooth muscle blood vessels. The only result to support this is their use of ERBB2/ERBB4 knock out myotubes which are negative for dystrophin. In the abstract under results they imply that there are data from the double mutant mice but results are not presented. There is just one analysis in myotubes showing dystrophin absence. In vivo, this signalling could well be compensated for and dystrophin may be expressed. In the original article on these mice (ref 20; Esther et al¹), this signalling is compensated for by agrin in vivo at the NMJ. No comments are made about dystrophin being absent in the muscle of these animals nor their muscle phenotype .

Thus this report does not contain sufficient evidence for the conclusion which is based on one blot from a cell line.

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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: muscular dystrophy

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 24 Sep 2018

Nadesan Gajendran, University of Basel, Basel, Switzerland

I thank the reviewer for taking the time to review the article and for the comments.

I realise that the role of neuregulin in muscle, especially in transcription of synapse specific genes and NMJ formation has been controversial. The reviewer suggests that in vivo NRG/ErbB signalling could be compensated by another signalling pathway (when ERBB2/4 receptors are ablated) to stimulate dystrophin expression¹ since, the reviewer comments, agrin compensates for this (NRG signalling through ERBB2/4) at the NMJ². However, in the article that is referred to by the reviewer and that describes the *ErbB2/4* dKO mice², the aim was to determine if neuregulin (NRG) signaling through ERBB2/4 receptors in muscle was required for synapse specific transcription and NMJ formation. That Agrin plays an important role in acetylcholine receptor clustering and NMJ formation was demonstrated by the fact that mice carrying a mutation in the agrin gene lacked NMJs³. Thus the original article on the *ErbB2/4* dKO mice concluded that NRG was not required for synapse-specific gene transcription at the NMJ and that development and maintenance of neuromuscular synapses were only marginally affected. Hence, the article does not conclude that NRG signalling is compensated for by Agrin, but rather that NRG is not required for synapse specific gene expression and NMJ formation. In addition, unlike NRG/ERBB signalling stimulating dystrophin expression in muscle¹, transcription of synapse specific genes and the formation of the NMJ involves reciprocal interactions between muscle fibers and motor neurons.

We subsequently demonstrated that neuromuscular synapses being marginally affected in the *ErbB2/4* dKO mice was due to a lack of phosphorylation of alpha-dystrobrevin 1 (α -DB1)⁴.

To demonstrate phosphorylation of α -DB1 by signalling through ERBB2/4 receptors, I used *ErbB2/4* dKO myotubes (as I did in this article) as well as myotubes formed from C2C12 and primary myotubes formed from myoblasts from wild type (wt) mice (Schmidt et al. Fig. 7 a & b)⁴. We observed phosphorylation of α -DB1 in samples prepared from myotubes formed from freshly purified primary myoblasts from *ErbB 2/4* dKO mice. This phosphorylation of α -DB1 could be blocked by ERBB inhibitors (Schmidt et al. Fig. 7a)⁴ demonstrating that: (i) signalling through ERBB2/4 receptors was responsible for phosphorylation of α -DB1, (ii) the observed phosphorylation of α -DB1 in myotubes formed from *ErbB2/4* dKO primary myoblasts was likely caused by the presence of contaminating cells in the myoblast preparation. Contaminating cells in the myoblast preparation is obviously lost after extended culture periods as confirmed by the lack of phosphorylation of α -DB1 in *ErbB2/4* dKO myotubes in the absence of any ERBB inhibitor (Schmidt et al. Fig. 7c)⁴ For the same reason I used the *ErbB2/4* dKO myotubes instead of muscle tissue, to demonstrate a lack of dystrophin expression¹.

Once we discovered that signalling through ERBB2/4 receptors phosphorylated α -DB1 (Schmidt et al. Fig. 7 a-c)⁴ we realised that this explained the fragmentation of acetylcholine receptor (AChR) clusters at the neuromuscular synapse in *ErbB2/4* dKO mice. This was subsequently confirmed by experiments in cultured myotubes (wild type and α -DB1 KO) as well as with and without α -DB1 expression constructs (Schmidt et al. Fig. 6, results were not presented in order of discovery)⁴. We could not show absence of α -DB1 phosphorylation in vivo in the muscles of *ErbB2/4* dKO mice for the same reasons mentioned above.

Due to the presence of tissues other than skeletal muscle in muscle lysates and cells other than myoblasts in primary myoblast purifications from *ErbB2/4* dKO mice, it was not possible to resolve either the lack of phosphorylation of α -DB1 or the lack of dystrophin in the absence of ERBB2/4 signaling in the dKOs. In particular satellite cells present in muscle lysates (and in primary myoblast purifications) were shown to express a high level of dystrophin⁵ and will still express dystrophin in *ErbB2/4* dKO mice since the HSA promoter driving CRE expression is not active⁶, and hence ERBB2/4 receptors would not be ablated in these cells. The juxtaposition of dystrophin expression in satellite cells and in myofibers makes it very difficult to distinguish dystrophin expression in satellite cells from that in muscle fibers⁵. It may well be possible that researchers who may have looked for the dystrophin expression in muscle specific *ErbB2* and/or *ErbB4* KO may have run into this problem and not observed a lack of dystrophin in muscle or muscle sections. Satellite cells, however, appear to have a limited potential to regenerate skeletal muscle⁶ and hence would not fully explain the lack of dystrophic symptoms in *ErbB2/4* dKO mice.

Dystrophin and utrophin were detected in loxP-flanked *ErbB2/4* myotubes (Gajendran et al., Fig 1.)¹ formed from myoblasts, even after the extended culture period to generate them, and it was only after transient CRE transfection and ablation of *ErbB2/4* receptors (confirmation of recombination checked by PCR) dystrophin expression was not observed but utrophin was, in different clones. Understandably, it was not possible to either demonstrate a lack of phosphorylation of α -DB1 or the lack of dystrophin expression when using lysates of total muscle tissues due to the presence of other tissues and cells, including the smooth muscle of blood vessels and satellite cells where *ErbB2/4* receptors were not

ablated (the HSA promoter driving CRE expression is not active there). In addition to dystrophin detection, any other component of the DAPC present in vivo in the muscle that we wanted to detect in myotubes such as utrophin, α -dystrobrevin, and α -syntrophin were also detected on western blots following immunoprecipitation of the dystrophin associated protein complex (DAPC).

If the absence of NRG/ErbB2/4 signaling, and as a consequence absence of dystrophin in skeletal muscle, could be compensated in vivo by another mechanism, even possibly through another ligand other than NRG1, then this compensation mechanism would be independent of ERBB2/4 receptors as they are ablated in the skeletal muscles in these mice.

From experiments using α -DB1 KO myotubes in combination with α -DB1 expression constructs, it also became clear that signaling from α -DB1 downregulates dystrophin expression but not utrophin expression (results available at [WO 2017/036852 A1](#) under documents "09.03.2017 Initial Publication with ISR (A1 10/2017)")⁷. Thus opposing signals from α -DB1 (downregulation) and NRG/ERBB2/4 (upregulation) regulates dystrophin expression. This regulation appears to be mediated by the cleaved intracellular domain of ERBB4 (4ICD) since increased dystrophin levels in dystrobrevin KO myotubes are accompanied by a strong upregulation of ERBB4 expression most of which is cleaved to generate 4ICD (see figures at [WO 2017/036852 A1](#))⁷.

Together with a wealth of published information, the data presented in this article, in my opinion, is essential and sufficient to conclude that expression of dystrophin is stimulated by NRG/ERBB signalling. It follows that in *ErbB2/4* dKO mice that did not show dystrophic symptoms (see below), the absence of dystrophin in skeletal muscle but not in smooth muscle of blood vessels, clearly argues that the root cause of DMD is due to the lack of dystrophin in the smooth muscle of blood vessels. Naturally it is possible that dystrophin expression in the endothelial cells, between the smooth muscle and the lumen of blood vessels, in *ErbB2/4* dKO mice also plays a role in vasodilation during exercise, assuming that the HSA promoter used to ablate ERBB2/4 receptors is not active in these cells.

Published data on DMD, together with the data presented in this article, would suggest that the lack of dystrophin in skeletal muscle exacerbates the onset of dystrophy caused by a lack of increased blood flow during exercise. This observation is also supported by a very early observation in mdx muscles that only some fibers, and not all, in skeletal muscle initially show dystrophic symptoms⁸ and also by the fact that expressing the full length dystrophin in smooth muscles in mdx mice, even though it was expressed at significantly lower levels than the level of dystrophin found wild type mice, improved aberrant vasoregulation⁹. The fact that skeletal muscle in those mice still showed some atrophy could be due to insufficient dystrophin expression in the smooth muscle causing dystrophic symptoms as a consequence of reduced blood flow during exercise and exacerbated due to the lack of dystrophin in skeletal muscle (Reviewed in Ennen JP et al.)¹⁰. The exacerbation of dystrophic symptoms in skeletal muscle, apart from structural defects, may also be as a result of disruption of signalling events from the DAPC due to the absence of dystrophin and this is further supported by the observation that α -DB KO results in increased dystrophin and ERBB4 expression in myotubes⁷.

As far as could be determined from the literature, there are no compensatory signals in vivo that can ameliorate the effects caused by deficient ERBB2 or ERBB4 signalling such as defective muscle spindle formation¹¹, dilated cardiomyopathy (DCM) following Herceptin (antibody against HER2/ErbB2) treatment¹², DCM in conditional ErbB2^{13,14} or ErbB4¹⁵ KO mice, AChR cluster disintegration (also observed in mdx mice) in muscle specific ErbB2/4 dKO mice⁴, and the embryonic lethality observed in ErbB2 or ErbB4 KO mice^{16,17}. NRG and its ERBB receptors appear to exert their function in a spatio-temporal fashion in development and maintenance (in adults).

The reviewer is correct that no comments were made about dystrophin in the ErbB2/4 dKO mice². However in ErbB2/4 dKO mice nuclei in muscle fibers were examined extensively (see figures in supplemental data of that publication)² and centralized nuclei, a hallmark of DMD^{18,19} were not observed. It was also reported in the supporting online text (supplemental data)² that atrophic fibers could not be observed. Hence, based on these observations, there was no indication to look for dystrophin expression. It was also mentioned in the article that sustained muscle strength was not affected and that the *ErbB2/4* dKO mice did not have a myasthenic condition. However these mice showed a hind limb extension reflex which was not reported in the article.

I trust that I have addressed the reviewers concern sufficiently, and if the reviewer would be willing to review a revised version of this article, I would welcome the opportunity to submit one that would include some of the explanations/clarifications presented here.

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Competing Interests: No competing interests were disclosed.

Reviewer Response (F1000Research Advisory Board Member) 27 Sep 2018

Dame Kay Davies, University of Oxford, Oxford, UK

The author has responded with some reasonable arguments but the issue with the paper is lack of evidence. No new evidence is provided. In vivo rescue of the dystrophic phenotype in dystrophic deficient mice and dogs by delivery to skeletal muscle is a compelling argument for the role of dystrophin in skeletal muscle. If there is a role in smooth muscle, then this needs to be shown by in vivo experiments not a cell line.

Competing Interests: none

Author Response 01 Oct 2018

Nadesan Gajendran, University of Basel, Basel, Switzerland

I thank the reviewer for responding to my comments.

Although the reviewer does not question the discovery described in the manuscript, that NRG signalling is required for dystrophin expression as demonstrated in myotube cultures, concerns remain regarding in vivo evidence to confirm that the root cause of muscular dystrophy is due to the lack of dystrophin in smooth muscle of blood vessels rather than in skeletal muscle.

The reviewer's concern is understandable given the vast number of publications that focused on the lack of dystrophin in skeletal muscle as the cause of muscular dystrophy. These reports are indeed in contrast to our observation that ErbB2/4 dKO mice don't show dystrophic symptoms¹. In these mice, NRG signalling, and as a consequence also dystrophin expression (based on our in vitro results/ new findings), is abolished in skeletal muscles but not in vascular smooth muscle (VSM).

Ideally, to address the reviewers concern, a conditional transgenic mouse where either ERBB2 (or ERBB4) is knocked out in both skeletal and VSM should be examined to have a situation where dystrophin would be lacking in both.

On the other hand it would also be good to demonstrate that dystrophin absence in skeletal muscle does not cause dystrophic symptoms, confirming our observations on the ErbB2/4 dKO mouse.

I address below the reviewers comments.

In vivo evidence for a role for dystrophin in smooth muscle:

Mice where ErbB2 is conditionally knocked out in skeletal muscle, HSA-CRE/ErbB2, following CRE expression driven by the HSA promoter² show a similar phenotype as ErbB2/4 dKO¹ mice mentioned in this article (in both mouse strains the same HSA promoter is used to express CRE). As the HSA promoter is not active in smooth muscle, ERBB2 receptors would not be ablated and dystrophin will still be expressed in VSM, in contrast to skeletal muscles in these mice. The loxP flanked ErbB2 mouse is the same mouse that was used in breeding with an ERBB4 conditional KO mouse and an HSA-CRE transgenic to finally generate the ErbB2/4 dKO mouse¹. The abnormal muscle spindle formation reported for the HSA-CRE/ErbB2 mouse was not looked at in the ErbB2/4 dKO mouse probably because it had already been described in the HSA-CRE/ErbB2 KO mouse. In addition the study describing the ErbB2/4 dKO mouse was focused on the role of NRG/ERBB signaling in synapse specific transcription and NMJ formation.

Interestingly an ErbB2 conditional knock-out mouse with CRE expression driven by the muscle creatine kinase (MCK) promoter, MCK-CRE/ErbB2 was made that was different from the one mentioned above as it showed impaired muscle regeneration and a requirement of ERBB2 for survival of muscle spindles and myoblasts³. As VSM express both the brain and muscle isoforms of creatine kinase⁴, the MCK promoter would be active in VSM and hence ERBB2 would be ablated resulting in a loss of dystrophin expression in VSM in these mice, explaining the different histopathology characters between the two ErbB2 KO mice that differ in the promoter driving CRE expression. The muscle phenotype described for the MCK-CRE/ErbB2 KO mouse was not observed in the HSA-CRE/ERBB2 KO mouse where the HSA promoter is not active in VSM, and hence ErbB2 would not be ablated and therefore dystrophin would be expressed. MCK-CRE/ERBB2 KO mouse primary myoblasts lacking ErbB2, were reported to undergo extensive apoptosis when differentiating into myofibers³, an abnormality that I did not observe that when using ErbB2/4 dKO myotubes⁵.

In another study, siRNA mediated silencing of dystrophin expression in the muscles of adult

mice was meticulously analysed⁶. The authors concluded that in spite of the clear absence of dystrophin in the skeletal muscle, they did not observe any of the histopathology characters observed in mdx mice⁶. This paper describes silencing of dystrophin expression in skeletal muscles of adult mice, which results in a delay before the existing dystrophin is depleted, and the authors suggest that the dystrophic pathology observed in dystrophin deficiency may be developmentally regulated. However in the ERBB2/4 dKO mice, dystrophin would be ablated early during development in skeletal muscles since the HSA promoter driving CRE expression is active on E9.5, when dystrophin would also start to be expressed⁷, which would argue against the dystrophic pathology being developmentally regulated.

In vivo rescue of the dystrophic phenotype in mice and dogs may be due to delivery of the therapeutic (construct, anti-sense oligoribonucleotides, gene editing components) into skeletal and vascular smooth muscle

There have been numerous attempts to express dystrophin in skeletal muscle that ameliorated the dystrophic pathology to some extent, but none have led to applicable therapy. As the focus of most therapies are to restore skeletal, and sometimes also cardiac muscle it was not of concern whether VSM would or would not be targeted during therapy. Gene therapy using Rous sarcoma virus (RSV) or cytomegalovirus (CMV) promoters⁸ would also result in the expression of dystrophin, or smaller versions of it, in surrounding VSM after intra-muscular (IM) injection or particle bombardment⁹. In a Phase I study of gene therapy for Duchenne/Becker muscular dystrophy (D/BDM) the CMV promoter was also used¹⁰ and hence also here dystrophin would be restored in VSM. Systemic delivery of antisense oligoribonucleotides¹¹, or restoration of dystrophin expression by (intravenous) stem cell transplantation¹², would also result in dystrophin restoration in VSM.

Although in vivo rescue of the dystrophic phenotype in dystrophin deficient mice and dogs focused on delivery of the construct to skeletal muscle, it would also target VSM and hence it is neither possible to exclude the role of VSM nor to conclude that delivery to skeletal muscle is sufficient, to rescue the dystrophic phenotype. Furthermore, simply expressing dystrophin in skeletal muscle fibers lacking dystrophin would restore the dystrophin associated protein complex (DAPC) without saying much about whether muscle function is restored. If indeed muscle function is restored, then it should be looked at whether dystrophin may also have been expressed in VSM through leaking of the therapeutic into those tissues, sometimes through the circulation especially when using viral based therapies as described below.

Gene editing of mutated dystrophin using adeno-associated viruses (AAV) to deliver CRISPR components is a promising therapeutic strategy and has recently been demonstrated to function in a canine model of Duchenne muscular dystrophy¹³. But it still needs to be determined if the gene editing will be sustainable over long term. In this study an AAV serotype 9 that shows tropism for heart and skeletal muscle was used to deliver the CRISPR components. To drive the expression of Cas9 (one of the gene editing components), a muscle specific creatine kinase regulatory cassette was used that should also result in gene editing of dystrophin in VSM especially when the viruses are injected intravenously for systemic delivery. In a second experiment in the same study, 6 weeks following delivery through direct injection into muscles, dystrophin expression and assembly of the DAPC in

dystrophic muscles was observed. Dystrophin expression in contralateral muscles that were not injected was also observed and it was attributed to leakage of AAV9 into circulation.

In contrast to the siRNA silencing of dystrophin mentioned earlier, which did not affect muscle function, a very high amount of virus carrying the gene editing components was used in the case of CRISPR mediated gene editing in the canine, which will increase the likelihood for some of it leaking into the circulation and transducing other tissues, including VSM.

Given that therapy for Duchenne patients is largely focused on skeletal muscle, this article would suggest that development of therapeutics should (also) focus on VSM (and possibly also vascular endothelial cells) for any long term therapeutic benefit for patients with Duchenne and Becker muscular dystrophy. In addition the demonstration that NRG/ERBB signalling stimulates dystrophin expression would help to improve treatment regimens with Herceptin/Trastuzumab to ameliorate cardiomyopathy. Finally, this article suggests that investigation of changes in functional dystrophin levels as a possible cause of disease states associated with NRG/ERBB signalling is warranted.

I trust I have addressed the concerns the reviewer raised.

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Competing Interests: No competing interests were disclosed.

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