RESEARCH ARTICLE

REVISED 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 2; peer review: 3 not approved]

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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 still expressed in the smooth muscle of blood vessels allowing normal blood flow through vasodilation during exercise.

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

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

Keywords

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

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REVISED Amendments from Version 1

In this revised version of the article, the reviewers concerns were addressed and additional results were included (new Figure 2; the old figures are unchanged but have been re-numbered). The new results reflect additional similarities between the dystrophin deficient mdx mouse and the conditional knock-out mouse where neuregulin/ERBB (NRG/ERBB) signalling is specifically abolished in skeletal muscles. The raw data for Figure 2 is provided in Dataset 2. These similarities not only support the *in vitro* observations that NRG/ERBB signalling is required for dystrophin expression, they also argue against alternative regulatory mechanisms that would compensate *in vivo* NRG/ERBB signalling in the knock-out mouse. Additional published studies are included and discussed to explain why the NRG/ERBB dKO mice in contrast to the mdx mice do not show a dystrophic pathology and why our results suggest that the loss of dystrophin in vascular smooth muscle, resulting in aberrant vasoregulation of blood and lymph vessels, may be the cause of/be involved in, the onset of Duchenne and Becker muscular dystrophy.

See referee reports

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 phosphorylates 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, 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 synapse^{1,2}.

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. 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, transfections

Erb2/4 dKO and loxP flanked *Erb2/4* myoblasts (a kind gift from M. Courtet, and previously described¹) as well as, α -dystrobrevin KO (α -db^{-/-}) myoblasts¹³ (kind gift from B. Pawlikowski and M. Maimone (Upstate Medical University, State University of New York, Syracuse, NY) and C2C12 cells 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)). Myoblasts were transfected with expression constructs using Eugene HD (Promega, Madison, Wisconsin) according to their protocol when they reached 70% confluency. Expression constructs for GFP- α -DB1 and GFP- α -DB1-P3 were gifts from J.R. Sanes (Harvard University, Cambridge, MA) and C. Mouslim (University of Michigan, Ann Arbor, MI), respectively. Transfected myoblasts were then sorted for EGFP⁺ cells using the influx cell sorter (Becton Dickinson). To obtain more than 90% positive EGFP⁺ population, myoblasts were sorted at least twice with a cell culture phase (3–4 passages) between each sort.

Western analysis

Myotubes from 10-cm culture dishes were harvested in 600 μ l lysis buffer and protein complexes were immunoprecipitated as described previously^{1,14} 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 8% acrylamide/0.8% bis-acrylamide (Figure 1 A & B) or 6–8% gradient/0.8% bis-acrylamide (Figure 2B) 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)

Figure 1. Dystrophin, and utrophin levels in *Erbb2/4* dKO myotubes. (A and B), Western blots of immunoprecipitated DAPC 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 part of the blot 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 (C) and utrophin (D) 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.

Figure 2. Dystrobrevin 1 expression levels in *Erbb2/4* dKO myotubes and association with the DAPC. (A) qPCR data of α-dystrobrevin 1 expression in *Erbb2/4* dKO (*erbb2/4*^{-/-}) myotubes relative to C2C12 myotubes. Expression levels were normalized to ribosomal protein L8 (rL8) expression. This experiment was performed at least twice and gave similar results. (B) Western blots of control and transfected C2C12 and dystrobrevin KO (db^{-/-}) myotubes following IP of the DAPC and detection of dystrobrevin and EGFP-dystrobrevins (GFP-DB1 & GFP-DB1-P3 with 3 mutated tyrosine phosphorylation sites) with anti-α-dystrobrevin 1 antibodies. The EGFP-DB1 transfected dystrobrevin KO myotubes appears to have lost the expression construct however a repeat of the experiment showed expression of the transfected construct (see raw data). Following syntrophin detection (lower panel), the blot was stripped and used for dystrobrevin detection (upper panel). The lower panel in figure 2b (syntrophin detection) was previously published in a patent (Patent Link: [WO 2017/036852 A1](#)), but the copyright is the author's own.

monoclonal (diluted 1:400, catalog number sc-136116) were from Santa Cruz Biotechnology, Inc., mouse monoclonal anti-syntrophin 1351 (4 µl antibody/80 µl protein G beads for lysate from a 10 cm culture dish of myotubes, catalog number ab11425) from abcam, rabbit anti- α -dystrobrevin (1:1,500; a kind gift of D.J. Blake and R. Nawrotzki, University of Cardiff, Wales, UK) and rabbit anti- α -syntrophin 258 (5 µg/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 described¹⁵ and the 2^{-ΔΔCt} 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 µg 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 µl SyBR Green mix, 2.5 µl of a 3 µM solution each of forward and reverse primer, 1 µl of diluted cDNA and made up to 25 µl 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, dystrobrevin and ribosomal protein L8 (rL8) amplifications: dystrophin forward, 5'-GATGATGAACATTTGTTAATCCAGC-3' and reverse, 5'-CATATTCTGCTTGCAGATTCCTG-3'; utrophin forward, 5'-CTAAACTCCTGCGGCAGCAC-3' and reverse, 5'-GTGTCAAGTGAGTAGCTCAATGC-3', dystrobrevin (exon 22) forward, 5'-AACCCAACTTGCTGGCAGAAC-3' and reverse (exon 26), 5'-AGGCAGATGCTGAACGGATG-3' and rL8 (normalization gene) forward, 5'-ACTGGACAGTTTCGTGTACTG-3' and reverse, 5'-GCTTCACTCGAGTCTTCTTG-3'.

Results

NRG/Erbb signaling is required for dystrophin expression

We previously reported that there were two isoforms of α -DB1 associated with the DAPC, a 75 kDa and an 89 kDa protein¹. These two α -DB1 proteins correspond to the previously reported proteins between 66-97 kDa with the smaller protein containing more phosphotyrosine¹⁶. We also demonstrated that ablation of ERBB2/4 receptors resulted in a lack of phosphorylation of the 75 kDa protein¹ and that lower levels of this α -DB1 isoform associated with the DAPC compared to the 89 kDa isoform. However in myotubes with intact ERBB2/4 receptors, such as in C2C12 myotubes, we did not observe such a difference in the levels of these two isoforms¹. In the absence of NRG/Erbb signalling we also observed AChR cluster fragmentation both *in vitro* in myotubes and *in vivo* in muscle fibers. Similar observations

were made in mdx mice deficient in dystrophin. Mdx mice have a reduction in all the dystrobrevin isoforms at the sarcolemma¹⁷, and thus in the DAPC, as well as AChR cluster fragmentation⁷ in muscle compared to wild-type mice. These observations suggested that a lack or reduced level of dystrophin may have caused the reduction in the 75 kDa α -DB1 protein associated with the DAPC, and AChR cluster fragmentation, in the *Erbb2/4* dKO myotubes implying 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 containing loxP flanked *Erbb2/4* genes, before transfection with Cre recombinase, were used. Both, ERBB2 and ERBB4 receptors were ablated in skeletal muscle to eliminate NRG signaling¹, because ERBB4 receptors, apart from forming heterodimers, can also form homodimers and ERBB2 can heterodimerize with ERBB3¹⁸. Furthermore, as cultured myotubes secrete NRG¹⁹, the external addition of NRG was not necessary, as demonstrated for phosphorylation of α -dystrobrevin 1 by NRG/ERBB¹ signaling.

In three independent experiments (Figure 1A) each using myotubes formed from a different myoblast clone with loxP-flanked *Erbb2/4* genes, dystrophin was clearly detected (lanes 1–3). However dystrophin was not detected in *Erbb2/4* dKO myotubes (lanes 4–6) where ERBB2/4 receptors were ablated after Cre mediated recombination of loxP flanked *Erbb2/4* genes. Utrophin on the other hand was detected in myotubes with and without ERBB2/4 receptors (Figure 1B) demonstrating that NRG/ERBB signaling selectively regulates dystrophin expression (Figure 3).

The estimation by qPCR of dystrophin and utrophin mRNA levels (Figure 1C, D) confirm that not only the protein but also dystrophin mRNA expression is absent in *Erbb2/4* dKO (shown in Figure 1 as *erbb2/4*^{-/-}) myotubes. 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 rL8, used to normalize expression of dystrophin and utrophin, was above threshold at about 22 cycles in *Erbb2/4* 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 absent in *Erbb2/4* dKO myotubes. The level cannot be estimated. This is because the level of dystrophin in C2C12 was relative to that in *Erbb2/4* 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 *Erbb2/4* dKO myotubes was intact. Utrophin expression was reduced to only less than half the amount (Figure 1D) in *Erbb2/4* dKO myotubes compared to C2C12 myotubes which may be due to the lack of NRG signaling since NRG was reported to stimulate utrophin expression to some extent²⁰. Myotubes formed from C2C12 myoblasts were used as a control for qPCR instead of myotubes containing loxP flanked *Erbb2/4* genes (used for the western

Figure 3. 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.

blots in [Figure 1A, B](#)) as the loxP-flanked *ErbB4* gene²¹ is a hypomorph due to the insertion of the neo selection cassette and therefore levels of dystrophin mRNA may have been affected (NRG signaling through ERBB2/4 stimulate dystrophin expression and reduced *ErbB4* expression may have affected this). This is not a problem for dystrophin protein detection (not estimation) in immunoprecipitated samples from myotubes containing loxP flanked *ErbB2/4* genes. qPCR on *ErbB2/4* dKO myotubes 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. However, the *ErbB2/4* dKO mice did not show dystrophic symptoms¹.

Due to the presence of tissues and cells other than skeletal muscle and myoblasts, such as vascular smooth muscles (VSM) and satellite cells, in muscle lysates and in primary myoblast purifications from *ErbB2/4* dKO mice, it is not possible to resolve the lack of dystrophin in skeletal muscle in these mice. In particular satellite cells were shown to express a high level of dystrophin²² and will still express dystrophin in *ErbB2/4* dKO mice since the HSA promoter driving Cre recombinase expression is not active²³, 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 fibers²². Satellite cells, however, appear to have a limited potential to regenerate skeletal muscle²³ and hence it would not fully explain the lack of dystrophic symptoms in *ErbB2/4* dKO mice.

Association of α -DB1 with the DAPC is not dependent on its phosphorylation state

We previously demonstrated that phosphorylation of the 75 kDa α -DB1 by NRG/ErbB signaling stabilized AChR at the NMJ¹ and that in *ErbB2/4* dKO myotubes we detected on western blots, after immunoprecipitation (IP) of the DAPC, lower amounts of the 75 kDa α -DB1 protein compared to the

89 kDa protein. The 89 kDa protein was also recognized by the anti- α -DB1 antibody but did not show phosphorylation. However in IP performed on C2C12 myotubes, we detected similar amounts of the 75 kDa and 89 kDa proteins on western blots¹. Hence this raises the possibility that the AChR cluster fragmentation observed *in vitro* and *in vivo* when ERBB2/4 receptors are lacking was due not only to a lack of phosphorylation of α -DB1 but also due to the low amounts of the 75 kDa α -DB1 associated with the DAPC. Since *ErbB2/4* deletion causes a lack of dystrophin ([Figure 1](#)), this low amount of α -DB1 protein associated with the DAPC could be due to: i) the lack of dystrophin as levels of α -DB1 is reduced at the sarcolemma in mdx mice¹⁷; 2) due to downregulation of the 75 kDa α -DB1 expression, if NRG/ErbB signaling normally increased its expression; 3) due to the lack of phosphorylation of the 75 kDa α -DB1 since phosphorylation may have stabilized its association with the DAPC.

To resolve this question, qPCR was performed on cDNA from *ErbB2/4* dKO myotubes and control C2C12 myotubes using primers that would detect α -DB1 message based on an mRNA sequence (NCBI Ref. NM_207650.3) that would express a dystrobrevin protein with a calculated molecular weight of 76.817 kDa (NCBI Ref. NP_997533.1). Both, *ErbB2/4* dKO and C2C12 myotubes expressed similar amounts of α -DB1 ([Figure 2A](#)). For both *ErbB2/4* dKO and C2C12 samples α -DB1 expression was above threshold at about 28 cycles whereas detection of rL8, used to normalize expression of α -DB1, was above threshold at about 22 cycles. As in [Figure 1](#), myotubes formed from C2C12 myoblasts were used as a control instead of myotubes containing loxP flanked *ErbB2/4* genes as the loxP-flanked *ErbB4* gene²¹ is a hypomorph. Hence the lower amounts of the 75 kDa α -DB1 associated with the DAPC in *ErbB2/4* dKO myotubes is not due to lower expression levels of α -DB1.

To find out if phosphorylation of the 75 kDa α -DB1 stabilized its association with the DAPC when dystrophin was present,

GFP- α -DB1 and GFP- α -DB1-P3, where the three tyrosine phosphorylation sites were mutated², were expressed in dystrobrevin KO (lacking dystrobrevin)¹³ and in C2C12 (expressing dystrobrevin) myotubes. Both, dystrobrevin with (GFP- α -DB1) or without (GFP- α -DB1-P3) tyrosine phosphorylation sites associated with the DAPC irrespective of whether endogenous dystrobrevin was present as in C2C12 myotubes or absent as in dystrobrevin KO myotubes (Figure 2B) as they could be pulled down with IP of the DAPC. Hence association of the 75 kDa α -DB1 to the DAPC is independent of its phosphorylation state. Interestingly, the 89 kDa α -DB1 isoform was not observed in these cultures but the 75 kDa α -DB1 isoform was consistently observed. In this particular experiment, the dystrobrevin KO (db^{-/-} in Figure 2B) myotubes transfected with GFP- α -DB1 appears to have lost its expression construct during culture (Figure 2B) and the band observed is most probably from spill over from the adjacent well. However, in a repeat of this experiment (see raw data), expression of the transfected constructs was observed in all transfected myotubes. Control myotubes were not transfected and hence GFP- α -DB1 expression was not detected, and as expected in the dystrobrevin KO myotubes, endogenous dystrobrevin expression was not detected, on western blots.

This implies that the AChR cluster fragmentation observed *in vitro* in *ErbB2/4* dKO myotubes and *in vivo* in skeletal muscle¹ may be due to a combination of the reduced amounts of the 75 kDa α -DB1 associating with the DAPC and due to a lack of phosphorylation (of the reduced amount of the 75 kDa α -DB1) as NRG/ErbB signaling, that stimulates dystrophin expression and phosphorylates α -DB1, is lacking. Hence the lack of NRG/ErbB signaling causing AChR cluster fragmentation as observed *in vitro* in myotubes, is not compensated for by another signaling pathway *in vivo* as this AChR cluster fragmentation is also observed *in vivo*¹. The reduced association of the 75 kDa α -DB1 to the DAPC is most likely due to a lack of dystrophin since in mdx mice, where NRG/ERBB signaling is present, and therefore α -DB1 associated with the DAPC would be phosphorylated, less dystrobrevin is found at the sarcolemma in muscle and AChR cluster fragmentation was observed¹⁷. Therefore abolishing NRG/ErbB signaling in myotubes and skeletal muscle results in fragmentation of AChR clusters, most likely due to a lack of dystrophin and as a consequence reduced association of the 75 kDa α -DB1 (which would not be phosphorylated), with the DAPC.

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

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Dataset 2. Raw data for figure 2

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Discussion

We previously reported that in *HSA-Cre/ErbB2/4* dKO mice, loss of NRG signaling in skeletal muscles led to a lack of α -DB1

phosphorylation and its reduced association with the DAPC, resulting in AChR cluster fragmentation¹. The current study shows abolishing NRG/ErbB signaling in myotubes results in the loss of dystrophin expression and that the reduced association of α -DB1 with the DAPC is independent of its phosphorylation state.

Abolishment of NRG/ERBB signaling in skeletal muscle results in the specific loss of dystrophin and this is supported by: 1) *ErbB2/4* dKO myotubes fail to express dystrophin whereas utrophin is still expressed; 2) Loss of NRG/ERBB signaling abolishes phosphorylation of the 75 kDa α -DB1 and results in lower amounts of the 75 kDa α -DB1 associated with the DAPC¹. Similarly in dystrophin deficient mdx mice less dystrobrevin isoforms are detected at the sarcolemma¹⁷; 3) The reduced association of α -DB1 with the DAPC is not dependent on the phosphorylation state of α -DB1 (Figure 2B) and hence is not due to a lack of NRG/ErbB mediated phosphorylation of α -DB1 in *ErbB2/4* dKO myotubes, but more likely due to the loss of dystrophin; 4) The reduced association of α -DB1 with the DAPC is not due to downregulation of α -DB1 expression (Figure 2A) as a consequence of the loss of NRG/ErbB signaling; 5) Loss of NRG/ERBB signaling *in vitro* and *in vivo* results in AChR cluster fragmentation¹, implying that the loss of NRG/ERBB signaling is not compensated for *in vivo* by another signaling pathway.

Reduced localization of dystrobrevin at the sarcolemma and AChR cluster fragmentation due to a lack of dystrophin are also seen in dystrophin deficient mdx mice. However in contrast to mdx mice, *ErbB2/4* dKO mice do not show dystrophic symptoms²⁴. In these mice²⁴, muscle fibers were examined extensively and centralized nuclei, a hallmark of DMD^{25,26} were not observed. Also atrophic fibers could not be observed in these mice (supplementary information of reference 24)²⁴. Finally, sustained muscle strength was also not affected in the *ErbB2/4* dKO mice and neither did they have a myasthenic condition²⁴. However these mice showed a hind limb extension reflex (personal observation) which was also reported for the *HSA-Cre/ErbB2* KO mouse²⁷. The loxP flanked *ErbB2* mouse is the same mouse that was used in breeding to finally generate the *HSA-Cre/ErbB2/4* dKO mouse²⁴. The abnormal muscle spindle formation reported for the *HSA-Cre/ErbB2* mouse was not looked at in the *HSA-Cre/ErbB2/4* dKO mouse probably because it had already been described for the *HSA-Cre/ErbB2* KO mouse.

The reason why *ErbB2/4* dKO mice do not show a dystrophic muscle phenotype as seen in mdx mice, despite the absence of dystrophin, could be due to the specificity of the promoter that drives the expression of Cre recombinase in these conditional KO mice. In both, *HSA-Cre/ErbB2* mice²⁷ and *HSA-Cre/ErbB2/4* dKO mice²⁴ used in this study, Cre recombinase expression is driven by the HSA promoter that is active in the striated muscles, skeletal and heart muscle but not in vascular smooth muscle (VSM)^{17,28,29}. Hence, ERBB2 receptors would not be ablated and dystrophin will still be expressed in VSM, in contrast to skeletal muscles in these mice.

Interestingly an *ErbB2* conditional knock-out mouse with Cre expression driven by the muscle creatine kinase (MCK)

promoter, *MCK-Cre/Erbb2* did show impaired muscle regeneration and a requirement of ERBB2 for survival of muscle spindles and myoblasts³⁰. As MCK is expressed in both, skeletal muscle and VSM³¹, the MCK promoter would be active in these tissues. Hence in mice where Cre recombinase expression is driven by the MCK promoter, ERBB2 would be ablated in both skeletal muscle and VSM, resulting in a loss of dystrophin expression in both muscle types, explaining the different histopathology with *HSA-Cre/Erbb2/4* dKO mice.

In the *HSA-Cre/Erbb2/4* dKO mice, ERBB2/4 receptors and dystrophin in smooth muscle of blood vessels would still be expressed, as Cre recombinase would not be expressed in VSM, allowing the formation of a normal functional DAPC. Hence VSM in these mice still allows for increased blood flow to skeletal and cardiac muscle during exercise. In healthy VSM, neuronal nitric oxide synthase (nNOS) associates with dystrophin and generates nitric oxide (NO) that signals to soluble guanylate cyclase, generating cyclic guanosine 3',5'-monophosphate (cGMP) in VSM, causing vasodilation enabling exercise-induced increase of blood flow and thereby preventing muscle ischemia³². It cannot be excluded from the results presented here, that dystrophin expression in endothelial cells also plays a role in vasodilation during exercise, assuming that the HSA promoter used to drive Cre recombinase expression to ablate ERBB2/4 receptors is not active in those cells in *HSA-Cre/Erbb2/4* dKO mice.

The absence of obvious dystrophic pathology in *HSA-Cre/Erbb2/4* dKO mice despite the lack of dystrophin in skeletal muscle 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 exercise³². This hypothesis is consistent with published data describing the effects of phosphodiesterase type 5 (PDE5) inhibitors, which interfere with breakdown of NO by PDE5 and thereby prolong the half-life of cGMP, the target of NO³³. Treatment with PDE5 inhibitors alleviated the dystrophic phenotype in mdx mice³² and also in DMD patients. PDE5 inhibition with either tadalafil or sildenafil treatment in Duchenne muscular dystrophy boys restored normal blood vessel function and blood flow during exercise³³. However, a phase 3 randomized trial of tadalafil for DMD did not slow down the decline in ambulatory ability in boys with DMD³⁴. The authors suggested that the boys in the trial may not have engaged in sufficient daily ambulation, something they could not keep track of due to the large number of patients enrolled and geographical locations of the study. Tadalafil regulates blood flow through its target NO-cGMP signaling in skeletal muscle, only when muscles are active. Since smooth muscle cells are also lining the lymph vessels, ambulation would also help lymphatic drainage and in patients where ambulation is limited, lymph drainage massage may be beneficial for skeletal muscle health.

The observation that absence of dystrophin in skeletal muscle does not result in a dystrophic phenotype is supported by a study where siRNA mediated silencing of dystrophin expression in the muscles of adult mice resulted in a clear absence of dystrophin

in skeletal muscle without any of the histopathology characteristics observed in mdx mice³⁵. Because in this study adult mice were used, the authors suggested that the dystrophic pathology normally 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 recombinase expression is active on embryonic day 9.5 (E9.5), when dystrophin would also start to be expressed³⁶, which would argue against the dystrophic pathology being developmentally regulated.

Taken together, the following studies support the conclusion that the lack of dystrophin in VSM is the root cause of DMD: 1) Skeletal muscle specific *HSA-Cre/Erbb2/4* dKO mice, based on the results described here, lack dystrophin in skeletal muscle but would still express dystrophin in VSM and do not show a dystrophic pathology²⁴; 2) *HSA-Cre/Erbb2* KO²⁷ mice show a similar pathology to the *HSA-Cre/Erbb2/4* dKO mice; 3) *MCK-Cre/Erbb2* mice³⁰ where NRG/ErBB signaling and hence dystrophin expression is ablated in both, skeletal muscle and VSM (as MCK promoter is active in both), suffered from impaired muscle regeneration; 4) Loss of dystrophin in skeletal muscle fibers through RNAi knock down did not result in an overt dystrophic pathology³⁵; 5) expression of dystrophin in VSM, even at significantly lower levels compared to wild type (wt) controls, improved aberrant vasoregulation in mdx mice³⁷. Taken together, all these studies support the notion that aberrant vasoregulation, when dystrophin is lacking in VSM, is likely the cause of the onset of DMD and that lack of dystrophin in skeletal muscle exacerbates the dystrophic pathology.

There have been numerous attempts to restore dystrophin in skeletal muscle that ameliorated the dystrophic pathology to some extent, but none have led to an applicable therapy. As the focus of most therapies is to restore skeletal, and sometimes also cardiac muscle, it was not of concern whether VSM would or would not be targeted during therapy. In a study where dystrophin overexpression in transgenic mdx mice eliminated dystrophic symptoms³⁸, the MCK promoter was used to drive dystrophin expression and hence dystrophin would have been expressed in both, skeletal muscle and VSM. Gene therapy using Rous sarcoma virus (RSV) or cytomegalovirus (CMV) promoters³⁹ would also result in the expression of a functional dystrophin in surrounding VSM after intra-muscular (IM) injection or particle bombardment⁴⁰. In a Phase I study of gene therapy for Duchenne/Becker muscular dystrophy (D/BDM) the CMV promoter was also used⁴¹ which would also restore dystrophin expression in VSM. Systemic delivery of antisense oligoribonucleotides⁴², or restoration of dystrophin expression by (intravenous) stem cell transplantation⁴³, would also result in dystrophin restoration in VSM.

Similarly, even though *in vivo* rescue of the dystrophic phenotype in dystrophin deficient mice and dogs focused on delivery of the therapeutic to skeletal muscle (see example below), these therapies would also target VSM. Furthermore, simply expressing dystrophin in skeletal muscle fibers lacking dystrophin would restore the dystrophin associated protein complex (DAPC) but it does not imply impaired skeletal muscle regeneration and

function is restored. The VSM should be analysed to determine whether dystrophin may also have been expressed through leaking of the therapeutic into VSM, 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 dystrophy⁴⁴. However, 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, 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 indicating a broad distribution. Therefore, from what is known so far, it is neither possible to exclude the role of VSM in the onset of DMD, nor to conclude that expression of dystrophin in skeletal muscle only, is sufficient for the rescue of the dystrophic phenotype in DMD.

Published data on DMD, together with the data presented in this article, suggests that the lack of dystrophin in skeletal muscle exacerbates the onset of dystrophy caused by a lack of increased blood flow during exercise. This is also supported by an observation in mdx muscles that only some fibers, and not all, in skeletal muscle initially show dystrophic symptoms⁴⁵ 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 in wt mice, improved aberrant vasoregulation³⁷. 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.*)⁴⁶. The exacerbation of dystrophic symptoms in skeletal muscle, apart from structural defects, may also be as a result of disruption of signaling events from the DAPC due to the absence of dystrophin.

We previously reported that the blockade of NRG signaling through ERBB2/4 receptors prevented phosphorylation of α -dystrobrevin1 and hence affected NMJ stability¹. In the current study it is shown that ablation of ERBB2/4 receptors, and thus elimination of NRG signaling, 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 thereby allowing the formation

of a functional DAPC that stabilizes acetylcholine receptors (Figure 3).

If the absence of NRG/ERBB signaling, and as a consequence absence of dystrophin in skeletal muscle and lack of phosphorylation of α -DB1, 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 skeletal muscles of *ErbB2/4* dKO mice. However there is probably no compensatory signal for NRG/ERBB *in vivo* as AChR cluster fragmentation is observed *in vivo* as well. 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 formation²⁷, dilated cardiomyopathy (DCM) following Herceptin (antibody against HER2/ERBB2) treatment⁴⁷, DCM in conditional *ErbB2*^{28,48} or *ErbB4*⁴⁹ KO mice, AChR cluster fragmentation (also observed in mdx mice) in muscle specific *ErbB2/4* dKO mice¹, and the embryonic lethality observed in *ErbB2*¹¹ or *ErbB4*¹⁰ KO mice. Based on what is known so far, NRG and its ERBB receptors appear to exert their function in a spatio-temporal fashion in development and maintenance (in adults).

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. ERBB4 can heterodimerize with ERBB2 and a function blocking ERBB2/HER2 antibody treatment results in DCM in mice²⁸ and in cancer patients⁴⁷, consistent with our observations that NRG/ERBB signaling is required for dystrophin expression since DMD patients and mdx mice lacking functional dystrophin, develop DCM (Figure 3). Hence the data presented here suggests that a target protein for the reported beneficial effects of signalling through ERBB4 in repairing heart injury is dystrophin whose expression is regulated through NRG/ERBB2/4 signaling. Thus NRG/ERBB signaling carries out different functions, through different signaling targets, in cardiac development and maintenance, with the latter being carried out through regulating dystrophin expression.

Although normal blood flow is important for prevention of the onset of dystrophic symptoms, the general consensus is that exercise would cause damage to skeletal muscle and in the absence of dystrophin, muscle regeneration would be impaired. However, the *ErbB2/4* dKO mice lacking dystrophin in skeletal muscle only, do not show any obvious dystrophic pathology which challenges this general consensus.

There are two studies that may give an explanation as to why a dystrophic pathology is not observed in skeletal muscles when dystrophin is lacking as in *HSA-Cre/ErbB2/4* dKO mice. It has been demonstrated that the host environment is critical for controlling the function of satellite cells⁵⁰. Hence normal dystrophin expression in VSM may prevent or delay the onset of dystrophy in skeletal muscle and thereby maintain a host environment that is permissive to muscle regeneration by

satellite cells. Alternatively muscle regeneration in response to injury could be mediated by myofibers derived from circulating mature myeloid cells (blood cells) that migrate to skeletal muscle in response to inflammatory cues⁵¹. Hence, upon muscle damage in *HSA-Cre/Erbb2/4* dKO mice where dystrophin is absent only in skeletal muscle, satellite cells and/or circulating myeloid cells could help regenerate mature myofibers underscoring the importance of the vasculature and increased blood flow during exercise.

Although current therapy for Duchenne patients is largely focused on skeletal muscle, the present study suggests 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. Dystrophin deficient mdx mice show a milder dystrophic pathology and have a shorter life span compared to humans. It thus needs to be determined whether in humans, restoring dystrophin in VSM (and endothelial cells) and thereby a functional DAPC, is sufficient to ameliorate the dystrophic pathology.

The current study also suggests that increasing NRG signaling through ERBB2/4 receptors, 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 nNOS⁵² and thereby enabling normal blood flow during exercise. Apart from its implications for muscular dystrophy, the demonstration that NRG/ERBB signaling stimulates dystrophin expression should help to improve treatment regimens with the anti-HER2 antibody, Herceptin/Trastuzumab, to prevent cardiomyopathy. Furthermore, as dystrophin is also present in all regions of the brain, being most

abundant in the cerebellum⁵³, and since NRG/ERBB signaling regulates dystrophin expression, changes in functional dystrophin levels could be the underlying cause of some of the disease states such as schizophrenia, associated with abnormal NRG/ERBB signaling 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 only, mice do not exhibit dystrophic symptoms supporting the notion that lack of dystrophin expression in smooth muscle of blood vessels is the root cause of the onset of D/BMD.

Data availability

F1000Research: Dataset 1. Uncropped western blot images and raw Ct values from qPCR. DOI: <https://dx.doi.org/10.5256/f1000research.15889.d214628>⁵⁴.

F1000Research: Dataset 2. Raw data for figure 2. DOI: <https://doi.org/10.5256/f1000research.15889.d226797>⁵⁵

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As much as the manuscript is compellingly written and there certainly is a good possibility that loss of dystrophin in vascular cells might contribute to DMD, there is no direct experimental evidence to support the notion that VSMC dystrophin loss is the root cause of DMD - as claimed in the title. To make such a claim, experiments involving VSMC rescue of dystrophin in mdx mice or other versions of mdx mice needs to be performed. Targeting of ERRb2/4 in VSMC is also not performed. Furthermore, myotubes isolated from mice may not be a system complex enough to address this question. As much as utrophin expression is measured, compensation by some other structural proteins is not considered. As such the intact utrophin levels might be sufficient to maintain differentiation of myotubes. Overall, it is premature to claim that VSMC dystrophin deficiency in SMC is a root cause of DMD without direct experimental evidence.

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?

Yes

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: Skeletal muscle, signaling,. transcription, nuclear receptor signaling and muscle-linked diseases such as DMD, diabetes, vascular regression/PVD.

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 08 Apr 2019

Nadesan Gajendran, University of Basel, Basel, Switzerland

I thank the reviewer for having reviewed the article and for giving comments.

The reviewer's main concern is that there is no direct evidence that VSMC dystrophin loss is the root cause of DMD. The reviewer suggests performing an experiment involving restoration of dystrophin expression in VSMC in mdx mice or specifically ablating ERBB2/4 in VSMC in WT mice. As I suggested in my response to reviewer 2's comments, it may be better to knock-out dystrophin in skeletal muscle only using the HSA promoter (whose tissue specific expression is rather well characterized) thereby preserving dystrophin expression in all other tissues including VSM and thus preserving oxygenated blood and lymph flow to and from skeletal muscles. In the experiment suggested for specifically ablating ERBB2/4 in VSMC, one would not necessarily see dystrophic symptoms since the results from the current article suggest that lack of dystrophin in VSM is the root cause of DMD, i.e. the onset of dystrophic symptoms when there is systemic dystrophin deficiency (including lack of functional dystrophin in both skeletal muscle and VSM).

I did not fully understand the point regarding utrophin or other structural proteins compensating, structurally, for lack of dystrophin. As reported in the article and consistent with a published report¹, utrophin expression is reduced to some extent when ERBB2/4 receptors are ablated. Furthermore, whatever compensation may be provided by other structural proteins does not prevent AChR cluster fragmentation neither in vitro nor in vivo² due to a lack of dystrophin.

The scope of the current article is to provide evidence of a new concept/discovery, that dystrophin expression in muscles cells is dependent on NRG/ERBB signaling, and based on these experiments as well as previous published observation is the resulting conclusion that absence of dystrophin in VSM has a greater impact i.e. causes the onset of DMD than its absence in skeletal muscles only. Publication of this hypothesis is important as it will allow research groups who already have animal models that can be used to rapidly generate skeletal muscle specific dystrophin knock-out mice as described above, to substantiate (or refute) the results in the current article.

In the current article I demonstrated that NRG/ERBB signaling is essential for dystrophin

expression in myotubes. We previously demonstrated acetylcholine receptor (AChR) cluster fragmentation² in ERBB2/4 dKO myotubes and also (in vivo) in muscle fibers isolated from the (same) HSA-CRE/ERBB2/4 dKO mouse. Similarly AChR cluster fragmentation has been reported for the dystrophin deficient mdx mouse³. We had reported that AChR cluster fragmentation was due to a lack of phosphorylated alpha dystrobrevin 1 (α -DB1). However, we had also reported that in the absence of NRG/ERBB signaling there were reduced levels of the 75 kDa α -DB1 protein (that gets phosphorylated) in the dystrophin associated protein complex (DAPC)². Similarly, in dystrophin deficient mdx mice there is a reduced amount of dystrobrevin at the sarcolemma⁴. I have demonstrated in the current article that the reduced amount of the 75 kDa α -DB1 is due to a reduced association of it with the DAPC when dystrophin is lacking and not due to downregulation of gene expression. Hence our previous observation on AChR cluster fragmentation² is most likely due to a lack of dystrophin in skeletal muscles (in ERBB2/4 dKO mice) and not due to a lack of phosphorylation of the 75 kDa α -DB1 since the amount of α -DB1 available for phosphorylation, is reduced. This is similar to the situation in mdx mice.

It cannot be excluded that the AChR cluster fragmentation we observed² in skeletal muscles (in ERBB2/4 dKO mice) is a combination of reduced α -DB1 levels and absence of its phosphorylation. However, in view of the fact that there is no detectable dystrophin expression (protein nor messenger RNA, Figure 1 A–C) in the current article) in the absence of signaling through NRG/ERBB, the AChR cluster fragmentation we observed is most likely due to a lack of dystrophin rather than to a lack of phosphorylation of the reduced amount of the 75 kDa α -DB1. This is supported by published observations in mdx mice where the lack of dystrophin also results in a reduction in the amount of dystrobrevin associated with the sarcolemma and AChR cluster fragmentation, despite the presence of NRG/ERBB signaling that could phosphorylate (the reduced amount of) α -DB1. The observations in mdx mice substantiate the observations presented in the current manuscript, supporting the notion that the reduced amount of α -DB1, even if it can be phosphorylated as in the mdx mouse, is not sufficient to prevent AChR cluster fragmentation. Together the data strongly suggest that in ERBB2/4 dKO mice, the cause of the AChR cluster fragmentation we observed² is due to a lack of dystrophin protein in skeletal muscle rather than due to a lack of phosphorylation of α -DB1. The observed AChR cluster fragmentation in vitro in Erbb2/4 dKO myotubes, due to a lack of dystrophin, is also observed in vivo suggesting that the lack of dystrophin expression is not compensated for in vivo.

The main discovery in our previous paper² is that signaling through NRG/ERBB results in the phosphorylation of α -DB1 and as it had already been reported that phosphorylation of α -DB1 stabilizes AChR clusters at the NMJ⁵, our discovery provided the missing link i.e. that ERBB2/4 receptors phosphorylated α -DB1. My contribution to that manuscript was not only to demonstrate NRG/ERBB signaling phosphorylated α -DB1 but also that α -DB1 in myotubes appears to be very rapidly phosphorylated and dephosphorylated in WT muscles which may be important for further downstream signaling mechanisms and/or in postsynaptic responses during neurotransmission in addition to, as already reported, stabilizing AChR clusters at the NMJ (this is my opinion and not necessarily that of the other co-authors on that paper).

In conclusion, even though dystrophin is lacking in skeletal muscles of ERBB2/4 dKO mice

these mice do not show dystrophic symptoms suggesting that the VSM in these mice, which still express dystrophin because the HSA promoter used to ablate dystrophin in these mice is not active in VSM, enable increased blood flow and lymph clearance, especially during exercise. The results presented in this article, together with published observations, support the notion that lack of dystrophin in skeletal muscle only does not result in DMD but rather that the lack of dystrophin in both, skeletal muscle and VSM results in DMD. Hence the root cause of DMD is the lack of dystrophin in VSM.

It is noteworthy that there is no direct evidence to support the current “general consensus” that lack of dystrophin in skeletal muscle is the cause of DMD. DMD is a pathological state resulting from a systemic dystrophin deficiency. Thus in D/BMD, both skeletal muscle and VSM lack fully functional dystrophin. As far as can be determined from the current literature, there has been no study published that supports the notion that lack of dystrophin in skeletal muscle only causes DMD. In contrast, the findings in the current article argue against the current “general consensus” and instead suggest that although lack of dystrophin in skeletal muscle does not cause DMD, it is a prerequisite for the onset of DMD when dystrophin is also lacking in VSM. This does not mean that restoring dystrophin expression in VSM will rescue the dystrophic state in skeletal muscles in mdx mice that are already dystrophic (it may, but this will need to be determined experimentally). Our working hypothesis does predict that when dystrophin would be expressed early in VSM, hence when it would be expressed during normal developmental stages such as in WT mice, it would prevent or delay the onset of the dystrophic state (in mice where dystrophin is lacking in skeletal muscle only).

Therapeutic attempts have been reported to ameliorate the dystrophic phenotype in dystrophin deficient muscles but only when strategies were used that result in the expression of dystrophin or variants of it in both skeletal muscle and VSM (discussed in detail in the current article), which is in line with the observations in the current study. In view of the current article and lack of direct experimental evidence demonstrating DMD when dystrophin is lacking in skeletal muscles only, the general consensus may need to be revised i.e. that DMD is caused by a lack of dystrophin in both skeletal muscle and VSM (at least) and that lack of dystrophin in skeletal muscles only may not be sufficient to result in DMD. This is an important consideration for long lasting therapy as mentioned in the discussion of the current article.

Not only structural components (such as dystrophin) within the muscle fibers are essential to keep skeletal muscles healthy and prevent dystrophy during electrical stimuli/exercise, but also increased blood flow (to provide sufficient nutrients and oxygen to the muscle tissue) as well as a properly functioning lymphatic system (to remove resulting waste products). It is thus not surprising that even though dystrophin is lacking in skeletal muscles, such as in the Erbb2/4 dKO mice, the onset of dystrophic symptoms is only observed during exercise when blood flow (and subsequent lymph drainage) is affected as a consequence of the systemic absence of dystrophin in both skeletal muscle and VSM (compounded by inflammation/immune system involvement) such as in the case of the mdx mice or DMD patients.

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Competing Interests: No competing interests were disclosed.

Reviewer Report 10 December 2018

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Alberto Malerba

Centres of Gene and Cell therapy and Biomedical sciences, School of Biological Sciences, Royal Holloway, University of London, Egham, UK

I thank the author for providing a revised version of the manuscript. The text is clearer and the discussion includes more comments. However, the data show no major improvement and the statement that DMD is caused essentially by loss of dystrophin in vascular smooth muscle cells, while the lack of protein in myofibres only exacerbates the disease, is not supported by experimental data. This claim challenges hundreds of previous studies and should be supported by solid data. Just as an example Wasala *et al.* recently showed that a transgenic mouse expressing a minidystrophin gene (Δ H2-R15) driven by the HSA promoter (that induces protein expression only in myofibres) substantially prevented muscular dystrophy in skeletal muscle ¹. Once the minigene was removed from the skeletal muscles of adult mice, the pathology developed. This is a strong indication that muscular dystrophy depends primarily on dystrophin expression in myofibres. Of course this does not exclude a role for the missing dystrophin in other cells that could exacerbate the disease.

The author failed to provide solid data to support the claim of the manuscript. They state multiple times that the HSA-Cre/Erbb2/4 dKO does not express dystrophin in myofibres, but this has never been shown previously, neither in this manuscript. They should have demonstrated at least that:

1. In the skeletal muscle of such mouse model (cell culture is not sufficient as a model) myofibres do not express dystrophin while vascular cells do. There are many systems to do so (eg immunostaining in muscle sections is the easiest).
2. In those muscles only vascular cells (but in particular which ones?) express dystrophin. Again, there are standard methodologies to check this as immunostaining, cell sorting and staining etc.

Without these data the manuscript is interesting to read, but remains mostly speculative.

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

No

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?

No

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

No

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

No

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 17 Dec 2018

Nadesan Gajendran, University of Basel, Basel, Switzerland

I thank the reviewer for reviewing the revised version of the article.

The current article¹ supports the notion that the root cause of Duchenne muscular dystrophy (DMD) is a lack of dystrophin in vascular smooth muscle (VSM). Thus it addresses

what causes the onset of dystrophic symptoms in patients suffering from DMD where there is systemic dystrophin deficiency including in skeletal muscle. This is of importance since it suggests that therapeutic attempts should (also) target VSM, which is not always the focus of current therapies.

Loss of dystrophin expression as a consequence of abolishing neuregulin (NRG) signalling is directly demonstrated in *ErbB2/4* dKO myotubes. Our results, together with previous published observations² suggest that the absence of dystrophin in vivo in skeletal muscle in HSA-CRE/*ErbB2/4* dKO mice is responsible for the observed fragmentation of acetyl choline receptor (AChR) clusters in these mice, similar to that observed in muscles of dystrophin deficient *mdx* mice. As in *mdx* mice^{3,4} (in reference 4, figure 4B, less dystrobrevin is detected in microsomal preparations from *mdx*), less α -dystrobrevin1 (α -DB1) is associated with the DAPC in *ErbB2/4* dKO myotubes strongly suggesting that this is a direct consequence of the absence of dystrophin. Additional results in the current manuscript also show that the AChR cluster fragmentation observed in vitro in *ErbB2/4* dKO myotubes and in vivo in both HSA-CRE/*ErbB2/4* dKO and *mdx* mice is likely caused by a loss of dystrophin which results in the reduced association of α -DB1 with the DAPC² and this reduced association is not due to a lack of phosphorylation of α -DB1¹.

Thus we observed that loss of NRG/ERBB signalling results in a loss of dystrophin expression, affecting normal DAPC assembly and AChR cluster formation similarly to that observed in dystrophin deficient *mdx* mice. However, in contrast to *mdx* mice, impaired muscle generation is only seen in **MCK**-CRE/*ErbB2* KO mice, where NRG/ERBB signalling and hence dystrophin expression is abolished in both skeletal muscle and VSM, but not in **HSA**-CRE/*ErbB2* KO or **HSA**-CRE/*ErbB2/4* dKO mice⁵ where NRG/ERBB signalling is only abolished in skeletal muscles since the HSA promoter is not active in VSM⁶.

In contrast to the comments of the reviewer, the current article does not claim that the lack of dystrophin in VSM only would cause muscular dystrophy. Instead, it argues that functional vascular smooth muscle cells with normal dystrophin expression are essential in preventing or delaying the onset of muscular dystrophy when dystrophin is absent in skeletal muscles. This is supported by the observation that dystrophic symptoms are not observed in skeletal muscle of HSA-CRE/*ErbB2/4* dKO mice¹ that otherwise display AChR cluster fragmentation and less dystrobrevin association with the DAPC as in dystrophin deficient *mdx* myotubes, or when dystrophin expression is prevented in skeletal muscles only⁷. It is also in line with observations of improved muscle performance and reduced muscle degradation in *mdx* mice⁸ or DMD patients⁹ treated with PDE-5 inhibitors that restored normal blood vessel function and blood flow during exercise, in spite of the total absence of dystrophin.

For obvious reasons previous research has focused on the role of skeletal muscle in dystrophin deficient *mdx* mice or DMD patients since dystrophic symptoms are observed in skeletal muscle and not in VSM where absence of dystrophin results in aberrant vasoregulation. This aberrant vasoregulation is independent of whether skeletal muscles express dystrophin or not. The observation of the current study, supported by published data, suggests that muscular dystrophy in mice is only observed when dystrophin is lacking in both skeletal muscles and VSM and not in skeletal muscle only, suggesting that it is the

lack of dystrophin in VSM, resulting in aberrant vasoregulation that causes the onset of DMD when skeletal muscles lack dystrophin.

Hence, absence of dystrophin in VSM only, as is the case for example in the study by Wasala N. et al. that the reviewer refers to¹⁰, by artificially expressing dystrophin in only skeletal muscle of mdx mice (by using the HSA promoter), will not reveal this role of VSM in the onset of dystrophy observed in situations where dystrophin is absent in both skeletal muscle and VSM such as in mdx mice and DMD patients or MCK-CRE/ErbB2 KO mice that lack NRG/ERBB signalling in skeletal muscle and VSM.

Unfortunately, the paper by Wasala N. et al.¹⁰ does not report how well the expression of a mini-dystrophin gene in only skeletal muscle ameliorates muscular dystrophy in mdx mice. Skeletal muscle pathology following expression of mini dystrophin driven by the HSA promoter (in skeletal muscle) was not compared to samples from wild type (wt) or mdx control mice. Instead, the authors refer to three previously published papers^{4,11,12} that report the rescue of the dystrophic phenotype when the same mini-dystrophin is expressed using a CMV promoter, which is different from the skeletal muscle specific HSA promoter they used. Thus mini-dystrophin would be expressed in skeletal muscle and VSM in all three previous studies and obviously results in the restoration of muscle function, but it cannot be assumed that the same restoration will be obtained when dystrophin is only expressed in skeletal muscle (by using the HSA promoter). Instead, Wasala N. et al.¹⁰ only compare the skeletal muscle pathology between transgenic mice expressing the HSA-mini-dystrophin and transgenic mice where this mini-dystrophin is again removed after recombination with CRE recombinase.

Hence, when more severe dystrophic symptoms are observed by Wasala N. et al.¹⁰ when mini-dystrophin expression is abolished through CRE mediated recombination, it can not only be interpreted, as the authors have done, that the loss of mini-dystrophin in skeletal muscle causes a dystrophic state, but also that the lack of dystrophin in VSM causing aberrant vasoregulation in combination with a lack of mini-dystrophin in skeletal muscle causes a dystrophic state which is actually similar to the situation in mdx mice.

An experiment to be performed, that is beyond the scope of the current study, would be to generate a conditional dystrophin KO mouse and cross it with the HSA-CRE transgenic mouse to genetically delete dystrophin in skeletal muscles only in order to confirm our results obtained with the HSA-CRE/ErbB2/4 dKO mice. A dystrophic pathology as observed when dystrophin is absent in both skeletal muscle and VSM, as in mdx mice, would not be expected to be seen in this mouse.

The lack of an obvious dystrophic pathology of dystrophin deficient skeletal muscles in mice with functional VSM, and consequently sufficient blood and lymph flow, will allow satellite cells and circulating myeloid cells¹³ to support muscle regeneration since it has been demonstrated that satellite cells from mdx mice can regenerate muscle fibers when the host environment is not dystrophic¹⁴

Given that, unlike in humans, satellite cells are not depleted in the DMD mouse model mdx, muscle regeneration can continue during the relatively short life span of the mouse,

explaining the milder dystrophic symptoms in mdx mice as compared to DMD patients. In that regard it might even be more important for DMD patients to prevent/delay muscle degeneration by restoring dystrophin expression in VSM as well when therapeutic efforts to restore dystrophin in skeletal muscle are made.

The reviewer states that presence/absence of dystrophin should be analysed by for example immunostaining in muscle sections in the HSA-CRE/ErbB2/4 dKO mice. For the reasons mentioned in the current article (juxtaposition of satellite cells expressing a high level of dystrophin and dystrophin expression in muscle fibers¹⁵, and contaminating cells or tissues), it is practically not possible to resolve dystrophin expression in muscle sections or myotubes formed from primary myoblasts, although it was possible to observe AChR cluster fragmentation in vitro and in vivo as a consequence of the loss of dystrophin expression (demonstrated in myotubes), an observation that was also made in mdx mice. It would also not add much to the arguments presented in the current article to demonstrate dystrophin expression in VSM in these mice, since the HSA promoter is not active in VSM of HSA-CRE/ErbB2/4 dKO mice.

It is by now well accepted, perhaps considered a general consensus, that DMD is not just the consequence of the loss of structural support due to the loss of dystrophin in skeletal muscles, but rather also due to a combination of disruption of signalling events involving components of the DAPC and inflammation involving immune cells. Thus the concept that aberrant vasoregulation due to a loss of dystrophin expression in VSM, with the lymphatic system also being affected due to systemic dystrophin deficiency, is the root cause of DMD fits rather well with what is known so far. Thus a holistic approach is needed when determining the root cause of DMD and, as a consequence, treatment strategies that would be more complete and long lasting.

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Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 29 October 2018

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Alberto Malerba

Centres of Gene and Cell therapy and Biomedical sciences, School of Biological Sciences, Royal Holloway, University of London, Egham, UK

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 á-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 *ErbB2/4* 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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