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Prox1 gene transfer combined with voluntary exercise improves dystrophic muscle fragility in Mdx mice

Running head: Prox1 improves fragility of exercised Mdx muscle

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Abstract

Background. Voluntary exercise can improve skeletal muscle fragility, i.e. higher susceptibility to contraction induced-injury, as shown by a greater force drop following lengthening contractions, in the dystrophic Mdx mice as compared to healthy mice with dystrophin. This beneficial effect is related to the activation of the calcineurin activation. Unfortunately, voluntary running only partly rescued fragility, so it would be interesting to combined the effects of exercise, for example, with those of others treatments activating the calcineurin pathway and promoting slow and more oxidative fibres. This is of particular interest because slow muscle fibres are apparently less affected and genetic or pharmacological treatments promoting slow and more oxidative fibres are been shown to be beneficial in the Mdx mice.

Methods. Here, we tested whether voluntary exercise (1 month of running in a wheel) combined with Prospero-related homeobox factor 1 gene (*Prox1*) transfer would better improve functional dystrophic features in Mdx mice as compared to the voluntary exercise single approach. *Prox1* is known to promote the promotion of slow contractile gene program in healthy muscle.

Results. We found that *Prox1* transfer promoted slower molecular and functional contractile features in both voluntary exercised and sedentary Mdx mice. However, it improved fragility only in exercised Mdx mice. Moreover, *Prox1* transfer reduced absolute maximal force production by causing reduction in muscle weight in both exercised and sedentary Mdx mice.

Conclusion. Our results indicate that the beneficial effects of voluntary exercise and *Prox1* transfer on fragility are additive in Mdx mice.

Background

Dystrophin deficiency results in Duchenne muscular dystrophy (DMD), the most common lethal inherited muscle disease in boys. Dystrophin is a costameric protein that plays a role in force transmission and sarcolemma stability in skeletal muscle (1–3). Muscle of dystrophin deficient mice exhibits two important functional dystrophic features. The first is weakness, i.e. reduced specific maximal force (absolute maximal force generated relative to muscle cross-sectional area or weight), whereas absolute maximal force was unchanged because muscle is hypertrophied in the “classic” Mdx DMD model (C57BL/10ScSn-*Dmd* Mdx/J)(4–6), or decreased in the “new” D2.B10 DMD model (D2-*Mdx*) (7). The second is fragility, i.e., fast and low oxidative muscle in Mdx mice, but not slow and high oxidative muscle, is susceptible to damage caused by lengthening (eccentric) contractions, which cause an immediate marked force drop following lengthening contractions (7–12).

Interestingly, chronic muscular exercise can improve these functional dystrophic features in Mdx mice (13–15). In particular, voluntary wheel running (1 week to 1 year) can increase specific or absolute maximal forces in hindlimb muscle of Mdx mice (5,16–21). It was also reported that voluntary running improves (reduces) fragility in Mdx mouse fast muscle (21,22), whereas physical inactivity (disuse) aggravates it (21). Different signalling pathways are activated by exercise in healthy muscle, leading to beneficial muscle remodelling, i.e. cellular and molecular muscle adaptations, such as the promotion of slower and more oxidative muscle fibres (23–26). Numerous studies support the notion that the muscle remodelling induced by chronic exercise is partly dependent on the activation of the

calcineurin pathway in healthy muscle (25–30). Recently, we found that the improved fragility induced by voluntary running in Mdx mice was related to calcineurin pathway activation, and changes in the program of genes involved in slower contractile features of muscle fibre (22). Unfortunately, voluntary running only partly rescued fragility (21,22), so it would be interesting to combine the effects of exercise, for example, with those of other treatments targeting the calcineurin pathway.

While voluntary exercise offers potential therapeutic benefit, additional adjunct therapies could further improve functional dystrophic features. In the recent years, genetic or pharmacological treatments promoting slower and more oxidative fibres have been shown to be beneficial in the Mdx mice. In fact, several studies support the idea that activation of the AMPK, calcineurin, E2F1, ERR γ , IGF1, SIRT1 and PGC1 signalling pathways alleviates some of the dystrophic features in Mdx muscle (31–50). For example, genetic activation of calcineurin pathway improves fragility in fast muscle of the Mdx mouse, but decreases maximal force production, thus, aggravating weakness (47). Recently, it was demonstrated that the loss of Prospero-related homeobox factor 1 (*Prox1*) represses the expression of slow contractile genes in healthy fast muscle, whereas its overexpression via *Prox1* transfer has the opposite effect and downregulates the fast contractile genes (51,52). In particular, the inactivation of *Prox1* reduces the expression of the slowest myosin heavy chain *Myh7* in fast healthy muscle, without affecting oxidative capacity (succinate dehydrogenase staining) and absolute maximal force (52). It was found that *Prox1*, that is more expressed in slow fibres, is involved in the activation of the NFAT/calcineurin pathway and several of its downstream genes involved in the promotion of slow gene program, and is a regulator of satellite cell differentiation (51). However, it is not yet known (i) whether *Prox1* transfer is also beneficial

for functional dystrophic features, and in particular (ii) whether voluntary running and *Prox1* transfer have additive beneficial effects.

The purpose of the present study was to analyse the effect of *Prox1* transfer in voluntary exercised Mdx mice on hindlimb muscle weakness and fragility. We demonstrate that voluntary running and *Prox1* transfer have additive beneficial effects on fragility, without affecting specific maximal force. However, we also found that *Prox1* transfer reduced muscle weight and consequently reduced absolute maximal force in voluntary exercised Mdx mice.

Materials and Methods

Animals and voluntary running

All procedures were performed in accordance with national and European legislations and were approved by our institutional Ethics Committee “Charles Darwin” (Project # 01362.02). Mdx mice (C57BL/10ScSc-DMDMdx/J)(Mdx) and sex and age-matched wild-type control mice (C57BL/10)(C57) were used. Mice were randomly divided into different control and experimental groups. In the first set of experiment, Mdx mice at 2.5 months of age were placed (Mdx+wheel) or not (Mdx) in separate cages containing a wheel and were allowed to run 1 month ad libitum. Mdx mouse runners received *Prox1* transfer into the muscle 3 days before the initiation of voluntary exercise (Mdx+wheel+Prox1). The running distances were collected and daily running distance was 4.2 ± 0.1 km/day. In the second set of experiments, the muscle of sedentary Mdx mice received (Mdx+Prox1) or not (Mdx) *Prox1* transfer. Muscles were measured and collected 4 weeks after *Prox1* transfer.

Prox1 transfer

Adeno-associated vectors (AAV9) carrying the *Prox1* constructs (AAV-Prox1)(51) were injected in the right Tibialis anterior (TA) muscle. Titer for AAV-Prox1 was 7.1×10^{12} vector genomes (vg).ml⁻¹. Briefly, mice were anesthetized (3% isoflurane) and TA muscles of the right hindlimb were injected (30 μ l). Left TA (control muscle) was injected with saline solution only.

Muscle weakness and fragility

Muscle weakness (reduced specific maximal force) and fragility (susceptibility to contraction-induced injury) were evaluated by measuring the *in situ* TA muscle contraction in response to nerve stimulation, as described previously (53,54). Mice were anesthetized using pentobarbital (60 mg/kg, ip). Body temperature was maintained at 37°C using radiant heat. The knee and foot were fixed with pins and clamps and the distal tendon of the muscle was attached to a lever arm of a servomotor system (305B, Dual-Mode Lever, Aurora Scientific) using a silk ligature. The sciatic nerve was proximally crushed and distally stimulated by a bipolar silver electrode using supramaximal square wave pulses of 0.1 ms duration. We measured the absolute maximal force that was generated during isometric contractions in response to electrical stimulation (frequency of 75–150Hz, train of stimulation of 500ms). Absolute maximal force was determined at L0 (length at which maximal tension was obtained during the tetanus). Absolute maximal force was normalized to the muscle mass as an estimate of specific maximal force, an index of muscle weakness. The rate of force development (RFD), a marker of fibre-type, was measured when the force increased from 5 to 50% of P0.

Fragility, i.e., susceptibility to contraction-induced injury in Mdx mice was estimated from the force drop resulting from lengthening contraction-induced injury. The sciatic nerve was stimulated for 700ms (frequency of 150Hz). A maximal isometric contraction of the TA muscle was initiated during the first 500ms. Then, muscle lengthening (10% L0) at a velocity of 5.5 mm/s (0.85 fibre length/s) was imposed during the last 200 ms. All isometric contractions were made at an initial length L0. Nine lengthening contractions of the TA muscles were performed in Mdx mice, each separated by a 45-s rest period. Maximal isometric force was measured 1 min after each lengthening contraction and expressed as a

percentage of the initial maximal force. After contractile measurements, the animals were killed with cervical dislocation.

Real-time quantitative PCR (polymerase chain reaction)

Muscles (TA) were snap frozen in liquid nitrogen and stored at -80°C until use. Total RNA was isolated from TA muscles using Trizol (Invitrogen). Complementary DNA (cDNA) was then synthesized from 1 μg of total RNA using the RevertAid First Strand cDNA Synthesis kit with random hexamers, according to the manufacturer's instructions (Thermo Scientific). RT-PCR was performed on a LightCycler 480 System at the platform iGenSeq of the Institut du Cerveau et de la Moelle épinière, using LightCycler 480 SYBR Green I Master Mix (Roche, Basel, Switzerland)(53). The expression of *Hmbs* was used as reference transcript. All sequences of primers used are presented in Table 1.

Histology

Transverse serial sections (8 μm) of TA muscles were obtained using a cryostat, in the mid-belly region. Some of sections were processed for histological analysis according to standard protocols (succinic dehydrogenase, SDH). Other sections were processed for immunohistochemistry as described previously (55). For determination of muscle fibre diameter, fibre expressing myosin heavy chain (MHC) frozen unfixed sections were blocked 1h in phosphate buffer saline plus 2% bovine serum albumin, 2% sheep serum. Sections were then incubated overnight with primary antibodies against, laminin (Sigma, France), MHC-2a (clone SC-71, Developmental Studies Hybridoma Bank, University of Iowa), MHC-2b (clone BF-F3, Developmental Studies Hybridoma Bank), MHC-1 (clone BA-D5,

Developmental Studies Hybridoma Bank). After washes in PBS, sections were incubated 1 h with secondary antibodies (Alexa Fluor, Invitrogen). Slides were finally mounted in Fluoromont (Southern Biotech). Images were captured using a digital camera (Hamamatsu ORCA-AG) attached to a motorized fluorescence microscope (Zeiss AxioImager.Z1), and morphometric analyses were made using the software ImageJ. MHC-2x fibres were identified as fibres that do not express MHC-2b, MHC-2a or MHC-1. The numbers of fibre type expressing MHC-1, MHC-2x and MHC-2a per muscle cross-section were determined. Data presented correspond to pure MHC-1, MHC-2x and MHC-2a expressing fibres (without mixed MHC coexpression).

Statistical analysis

Groups were statistically compared using Student T-test, and variance analysis (1 or 2 ways). If necessary, subsequent Bonferroni post-hoc test was also performed. For groups that did not pass tests of normality and equal variance, non-parametric tests were used (Kruskal Wallis and Wilcoxon). Values are means $\pm$ SEM.

Results

1-*Prox1* transfer in voluntary exercised Mdx mice promotes slower contractile features

We first determined whether *Prox1* transfer increased slower contractile features in voluntary exercised Mdx mice. *Prox1* transfer into the TA muscle markedly increased the expression of ***Prox1*** (x 37.0) in voluntary exercised Mdx TA muscle (Mdx+wheel+*Prox1*) as compared to voluntary exercised Mdx TA muscle (Mdx+wheel)($p < 0.05$)(Figure 1A), as assessed by qPCR analysis. We also found that the expressions of ***Myh7*** coding for MHC-1 (x 15.1) and ***Tnni1*** coding for troponin I slow (x 1.6) were increased in Mdx+wheel+*Prox1* muscle as compared to Mdx+wheel muscle, whereas that of ***Myh4*** coding for MHC-2b was reduced (x 0.6)(Figure 1B)($p < 0.05$). Since TA muscle was composed mainly of MHC-2b expressing fibres (56), we wanted to determine whether *Prox1* transfer increased the number of MHC-1, MHC-2a and MHC-2x expressing fibres. Using immunohistological analyses, we found that these changes at the mRNA level were not yet translated into alterations in the numbers per muscle cross-section of MHC-1, MHC-2a and MHC-2x expressing fibres since they were not different between Mdx+wheel+*Prox1* muscle and Mdx+wheel muscle (Figure 1C). Moreover, there was no difference between Mdx+wheel+*Prox1* and Mdx+wheel muscles in the expression of a marker of oxidative capacity, ***Sdh***, a gene encoding a complex of the mitochondrial respiratory chain (Figure 1B). In line, histological analyses revealed that the percentage of the cross-sectional muscle area occupied by weak succinic dehydrogenase staining (SDH) staining was not different between Mdx+wheel+*Prox1* muscle and Mdx+wheel muscle (Figure 1D). In situ isometric TA muscle force production in response to nerve stimulation was also performed and indicated that the **rate of force development**, a marker of the contractile functional phenotype, was decreased (x 0.5) in

Mdx+wheel+Prox1 muscle as compared to Mdx+wheel muscle ($p < 0.05$)(Figure 1E). These data demonstrate that intramuscular delivery of AAV-*Prox1* induced a substantial fast to slower contractile transition in the TA muscle of voluntary exercised Mdx mice, in line with previous studies using not-exercised healthy muscle (51,52).

Similarly to voluntary exercised Mdx mice, *Prox1* transfert in sedentary Mdx muscle (Mdx+Prox1) increased the expression of ***Prox1*** (x 27.3)(Figure 1A)($p < 0.05$), ***Myh7*** (x 6.2) and ***Tnni1*** (x 2.4)($p < 0.05$), and reduced that one of ***Myh4*** (x 0.7)(Figure 1B)($p < 0.05$) as compared to sedentary Mdx muscle (Mdx mice), without changes in the numbers per muscle cross-section of MHC-1, MHC-2a and MHC-2x expressing fibres per muscle cross-section (Figure 1F). Moreover, it also reduced the rate of force development (x 0.6)(Figure 1H)($p < 0.05$). In contrast to voluntary exercised Mdx muscle, *Prox1* transfer reduced the expression of *Sdha* (x 0.8)(Figure 1B)($p < 0.05$) and weak SDH staining ($p < 0.05$)(Figure 1G).

2-*Prox1* transfer in voluntary exercised Mdx mice further improves muscle fragility

An **immediate force drop** was observed following lengthening contractions in Mdx muscle ($p < 0.05$)(Figures 2A). There was no such force drop following 9 lengthening contractions in C57 muscle with dystrophin (22,54). This result indicated the higher susceptibility to contraction-induced injury in Mdx muscle, i.e. fragility. In line with previous published results (21,22), the force drop following lengthening contractions in Mdx+wheel muscle was reduced as compared to Mdx muscle ($p < 0.05$)(Figure 2A). Interestingly, *Prox1* transfer in voluntary exercised Mdx muscle further reduced the force drop following lengthening contractions ($p < 0.05$)(Figure 2A). In fact, the force drop following the 6th and 9th

lengthening contractions was lower in Mdx+wheel+Prox1 muscle as compared to Mdx+wheel muscle ($p < 0.05$)(Figure 2A), indicating that *Prox1* transfer improved fragility in voluntary exercised Mdx muscle. In contrast to voluntary exercised Mdx muscle, the force drop following lengthening contractions was not significantly reduced by *Prox1* transfer in sedentary Mdx muscle since there was no significant difference between Mdx+Prox1 muscle and Mdx muscle (Figure 2B).

The increased expression of slow contractile genes (*Myh7* and *Tnni1*) and the reduced expression of fast contractile gene (*Myh4*) described above can explained at least in part, the improved fragility in Mdx+wheel+Prox1 muscle since in the recent years, genetic or pharmacological treatments promoting slow fibre gene program are been shown to improve fragility in the Mdx mice. In addition, we tested the possibility that *Prox1* transfer also improved fragility via the modifications of the expression of genes coding membrane ions channels since fragility in Mdx mice was also related to excitability and membrane ions channels (22,54). Several genes coding ion membrane channels interacting with dystrophin are involved in muscle excitability, such as *Scn4a*, *Cacna1s*, *Slc8a1*, *Trpc1* and *chrna1* (57–59). We found that the expression of *Trpc1* encoding for transient receptor potential cation channel subfamily C member 1 (x 2.1) was increased in Mdx+wheel+Prox1 muscle as compared to Mdx+wheel muscle ($p < 0.05$)(Figure 2C). No difference between Mdx+wheel+Prox1 and Mdx+wheel muscles was observed concerning the expression of *Scn4a*, *Cacna1s*, *Slc8a1* and *Chrna1* (Figure 2C). Similarly to voluntary exercised Mdx muscle, *Prox1* transfer in sedentary Mdx muscle increased *Trpc1* expression (Figure 2C)($p < 0.05$), but to lesser extent (x 1.4). In contrast to voluntary exercised Mdx muscle, it altered the expression of *Chrna1* in sedentary Mdx muscle (Figure 2C)($p < 0.05$).

Increased NADPH oxidase 2 (NOX2) activity was also related to fragility in Mdx mice (60–62). Thus, we determined whether the reduced force drop following lengthening contractions induced by *Prox1* transfer was associated to change in NOX2 pathway. We found no change in the expression of *PrxII*, *Gp91phox*, *P47phox* and *Rac1* (Figure 2D) in Mdx+wheel+Prox1 muscle as compared to Mdx+wheel muscle (Figure 2D). Similarly to voluntary exercised Mdx muscle, *Prox1* transfer in sedentary Mdx muscle did not altered the expression of these genes.

Since genetic interventions that constitutively induce high levels of utrophin, a homolog of dystrophin, improve the force drop following lengthening contractions in Mdx mice (63,64), we determined whether *Prox1* transfer increased *Utrn* expression. We found that the expression of *Utrn* was not increased in Mdx+wheel+Prox1 muscle as compared to Mdx+wheel muscle, whereas that one of ***Des***, a gene coding for intermediate filament, desmin, increased (x 1.2) in Mdx+wheel muscle ($p < 0.05$)(Figure 2E). Both the expressions of *Utrn* and ***Des*** (Figure 2E) did not change in response to *Prox1* transfer in sedentary Mdx muscle.

Thus, the improved TA muscle fragility induced by *Prox1* transfer in voluntary exercised mice was associated with the modification of expression of several genes involved in different aspects of muscle function and structure (*Myh7*, *Myh4*, *Tnni*, *Trpc1* and *Des*).

3-*Prox1* transfer in voluntary exercised Mdx mice reduced absolute maximal force and induces atrophy

Prox1 transfer combined to voluntary running and voluntary running alone did not affect specific maximal force before lengthening contractions (Figure 3A). However, absolute maximal force was reduced in Mdx+wheel+Prox1 muscle (x 0.6) as compared to Mdx+wheel muscle (Figure 3B)($p < 0.05$), indicating that *Prox1* transfer induced muscle weakness in voluntary exercised Mdx muscle. Similarly to voluntary exercised Mdx muscle, *Prox1* transfer in sedentary Mdx muscle reduced absolute maximal force (x 0.7)(Figure 3C)($p < 0.05$), without affecting specific maximal force (Figure 3D).

The reduced absolute maximal force was due to a lower muscle weight (x 0.7) in Mdx+wheel+Prox1 muscle as compared to Mdx+wheel muscle (Figures 3E)($p < 0.05$). Similarly to voluntary exercised Mdx muscle, *Prox1* transfer in sedentary Mdx muscle reduced muscle weight (x 0.8), although not significantly ($p = 0.08$)(Figure 3F).

Numerous genes encoding proteins are involved in muscle growth and maintenance (65–70). The ubiquitin-proteasome system plays a key role in triggering muscle atrophy when the expressions of *Murfl* and *Myfbox* are increased. Quantitative real-time PCR revealed that the expressions of these genes were not increased in Mdx+wheel+Prox1 muscle as compared to Mdx+wheel muscle (Figure 3G). We then analysed another atrophic mechanism, autophagy, which involves a battery of genes including *Lc3* which could contribute to the degradation of muscle proteins (67). We did not find any change in *Lc3* expression in Mdx+wheel+Prox1 muscle (Figure 3G). Similarly, *Gadd45*, *Hdac4*, *Fnl4*, *Redd1*, *Redd2*, *Mstn*, *Fst*, *Igf1*, and *Smox* genes also did not seem to participate to the atrophic state of Mdx+wheel+Prox1 muscle (Figure 3G). For example, *Mstn*, the negative regulator of muscle growth, was down-regulated in Mdx+wheel+Prox1 muscle as compared to Mdx+wheel muscle (Figure 3G)($p < 0.05$). Similarly to voluntary exercised Mdx muscle,

Prox1 transfer in sedentary Mdx muscle decreased the expression of *Mstn* (Figure 4C)($p < 0.05$). In contrast to voluntary exercised Mdx muscle, *Prox1* transfer decreased the expression of *Mafbox*, *Reed2* and *smox*, whereas it increased the expression *Gadd45*, *Fn14*, and *Fst* in Mdx+Prox1 muscle (Figure 3G)($p < 0.05$).

Thus, the muscle weakness induced by Prox1 transfer in voluntary exercised Mdx muscle was explained by a reduced weight and associated with the modification of the expression of *Mstn*.

Discussion

***Prox1* transfer only improves fragility in voluntary exercised Mdx mice**

The present study confirms our previous studies (21,22) showing that voluntary exercise alleviates the fragility, a major dystrophic functional feature, in fast anterior crural muscles (TA and extensor digitorum longus) of Mdx mice, such as *Dmd* based preclinical therapy (54). For the first time, we demonstrate that *Prox1* transfer have additive effect on fragility in voluntary exercised Mdx mice. Moreover, the muscle was protected from damaging muscle contractions by *Prox1* transfer only when Mdx mice performed voluntary exercise. This **improved fragility** in exercised Mdx mice treated with *Prox1* transfer might be very interesting if it is assumed that fragility causes the exhaustion of the muscle stem cells during successive degeneration/repair cycles (71). *Prox1* transfer might reduce the progressive muscle wasting in exercised dystrophic muscle because of the promotion of less fragile fibres.

We found that this beneficial effect of *Prox1* transfer in exercised Mdx mice is **related to the promotion of slower contractile features**, but not to a more oxidative phenotype. This observed relationship between improved fragility and slower contractile features was supported by the 2 following points. First, slow muscle is less fragile than fast muscle in Mdx mice (9–11). Second, exercise and pharmacological or genetic activation of signalling pathways, such as calcineurin, PPAR- β , PGC1- α , and AMPK, promoting a slower and more oxidative gene program, improve fragility in Mdx mice (21,22,38,46,47,72–74). It was previously demonstrated that *Prox1* promotes slower fibres by activating the NFAT-calcineurin pathway (51), a signalling pathway playing an important role in fibre type

specification (27,75). In particular, *Prox1* induces nuclear translocation of NFATC1 (51), reflecting the activation of the NFAT-calcineurin pathway (75).

In addition, it is possible that *Prox1* transfer improves fragility in voluntary exercised Mdx mice by a **preserved excitability**, as voluntary exercise and *Dmd* based therapy (22,54). In our experiments, reduced excitability, i.e. plasmalemma electrical dysfunction leading to defective generation and propagation of muscle potential action, largely contributes to the immediate force drop following lengthening contractions in Mdx mice (22,54). It remains to be determined whether the **upregulation of the membrane ion stretch-activated channel *Trpc1*** induced by *Prox1* transfer in voluntary exercised Mdx mice contributes to this improvement. Higher level of TRPC1 or activity of stretch-activated channels are generally associated with a worst dystrophic phenotype and fragility (8,76,77). In line with the present study, it was previously reported that the improved TA muscle excitability and fragility induced by voluntary exercise and calcineurin pathway activation were also related to change in the expression of genes encoding membrane ion channels (22).

Previous studies suggest that increased NOX2 activity is related to fragility in Mdx mice (60–62). However, our results show that *Prox1* transfer in exercised Mdx muscle does not reduce the expression of *Nox2* subunits (*Gp91phox*, *P47phox* and *Rac1*), which are shown to produce an elevated level of ROS in Mdx mice (62). Moreover, we found no increased expression of the gene encoding the antioxidant enzyme *PrxII*, whose overexpression improves fragility in Mdx mice (61). Concerning *Des* it is not impossible that the increased expression of *Des* in exercised Mdx mice in response to *Prox1* transfer contributes to the improvement of the fragility, since desmin plays a structural role and participate to lateral force transmission (2).

This beneficial effect of *Prox1* overexpression on fragility in exercised Mdx mice does not appear to be related to the reduced muscle weight that we observed, since it can be associated with an aggravation of fragility (21) whereas increased muscle weight can have the opposite effect (38,73,78).

Of note, *Prox1* transfer alone does not significantly improve fragility in sedentary Mdx mice. The difference cannot be attributed to the fact that *Prox1* was not highly overexpressed in sedentary Mdx mice treated with *Prox1* transfer. Moreover, the differential changes induced by *Prox1* transfer in exercised and sedentary Mdx mice were rather small (rate of maximal force development, *Myh4*, *Tnni1*, *Des*, etc.), except the modifications concerning *Myh7* (x 15.1 versus x 6.2) and *Trpc1* (x 2.1 versus x 1.4). Thus, our study interestingly suggest that voluntary exercise can potentiate a possible gene-based therapy, at least in the preclinical field.

***Prox1* transfer reduces maximal force production in both exercised and sedentary Mdx mice**

Although *Prox1* transfer improves fragility in voluntary exercised Mdx mice, we found that it has a detrimental effect on maximal force production (-33%). This latter effect was caused by a reduced muscle weight that was likely explained by decreased muscle fibre diameter since we found no increased numbers of fibres expressing MHC-1, MHC-2a and MHC-2x, that are smaller than the fibres expressing MHC-2b (56) whereas the total number of fibres was likely not altered by *Prox1* transfer. Our result suggesting that *Prox1* is a factor influencing muscle growth and maintenance in Mdx mice is another interesting finding. In

line, several genetic or pharmacological treatments promoting slower and more oxidative fibres are been shown to induce atrophy in Mdx mice (46,47,50). Unexpectedly, we found that the atrophic state in response to *Prox1* transfer is associated to downregulation of *Mstn*, a negative regulator of muscle growth (79,80), without other transcriptional changes regarding several well-known atrophic processes.

Conclusion

Our results indicate that *Prox1* transfer promotes a slower molecular and functional contractile features in both exercised and sedentary Mdx mice, as previously shown in sedentary healthy mice (51). Combined to voluntary exercise, *Prox1* transfer further ameliorates fragility, whereas the single *Prox1* transfer approach failed to induce a significant effect on fragility. This beneficial effect on fragility is related to the activation of the calcineurin pathway promoting slower molecular and functional contractile features and associated with *Trpc1* upregulation. However, *Prox1* overexpression also reduces maximal force production by decreasing muscle weight, despite reduced expression of *Mstn*. Thus, although *Prox1* transfer could aggravate muscle weakness, it can have the potential to reduce the occurrence of degeneration-regeneration cycles and consequently to stop the progression of the disease in exercised dystrophic muscle. Is this knowledge could be exploited for therapeutic advantage?

List of abbreviations

AAV9: Adeno-associated vectors

AMPK : Adenosin monophosphate-activated protein kinase

C57: C57BL/10 mice

DMD: Duchenne muscular dystrophy

E2F1: E2F transcription Factor 1

ERR γ : estrogen-related receptor gamma

MHC: myosin heavy chain

NFAT: Nuclear factor of activated T-cells

NOX2: NADPH oxidase 2

IGF1: insulin-like growth factor-1

IP: intraperitoneal

PCR: polymerase chain reaction

PGC1: peroxisome proliferator-activated receptor gamma coactivator 1-alpha

P0: Absolute maximal force

Prox1: Prospero-related homeobox factor 1 gene

RFD: rate of force development

SDH: succinic dehydrogenase

sP0 : specific maximal force

SIRT1: Sirtuin 1

TA: tibialis anterior

TRPC1: transient receptor potential cation channel subfamily C member 1

Ethics approval and consent to participate

All procedures were performed in accordance with national and European legislations and were approved by our institutional Ethics Committee “Charles Darwin” (Project # 01362.02). No humans were used in the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing interests

The authors declare that they have no competing interests.

Author contributions

AK and AF conceived, coordinated and designed the study.

AM, ML, CD, AF performed animal experiments.

AM, CD, ML and AF performed and analyzed skeletal muscle function measurements.

AM and CL performed gene expression analyses, with the contribution of OA and AK.

AM performed and analyzed histology measurements, with the contribution of OA.

AM, OA, DF, AK and AF wrote and reviewed the manuscript.

All authors reviewed the results and approved the final version of the manuscript.

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Table 1. Sequences of primers used.

Gene	Forward	Reverse
House keeping gene		
<i>Hmbs</i>	5'- AGGTCCCTGTTTCAGCAAGAA -3'	5'- TGGGCTCCTCTTGGAATGTT -3'
Genes of interest		
<i>Cacna1</i>	5'-CCTCATCAGCAAGAAGCAGG-3'	5'-TATGACAGACAGACCCTGGC-3'
<i>Chrna1</i>	5'- TGGTCTTTGTCATTGCGTCC -3'	5'- GATAAAAACCTTCCGCACCCA -3'
<i>Des</i>	5'- GTCCTCACTGCCTCCTGAAG-3'	5'- AGCATGAAGACCACAAAGGG-3'
<i>Fn14</i>	5'- AGGGGCTATAATGCCACTCC -3'	5'- GGGAGATGGTTGTTTCCGTG -3'
<i>Fst</i>	5'- CGAGTGTGCCATGAAGGAAG -3'	5'- GGTCTTCCTCCTCCTCCTCT -3'
<i>Gadd45</i>	5'- GGTGACGAACCCACATTCAT -3'	5'- GATTAATCACGGGCACCCAC -3'
<i>Gp91phox</i>	5'-TCACATCCTCTACCAAAACC-3'	5'-CCTTTATTTTCCCCATTCT-3'
<i>Hdac4</i>	5'- AAGTAGCTGAGAGACGGAGC -3'	5'- GCATGCGGAGTCTGTAACAT -3'
<i>Igf1</i>	5'-ACAAGCCCACAGGCTATGGCTC -3'	5'-AGTCTCCTCAGATCACAGCTCCG -3'
<i>Lc3</i> <i>Map1lc3a</i>	5'- CATGAGCGAGTTGGTCAAGA -3'	5'- CCATGCTGTGCTGGTTGA -3'
<i>Atrogin1</i> <i>Fbxo32</i>	5'-TCACAGCTCACATCCCTGAG-3'	5'- TCAGCCTCTGCATGATGTTC-3'
<i>Mstn</i>	5'- GCTACCACGGAAACAATCAT-3'	5'-CAATACTCTGCCAAATACCA-3'
<i>Murf1</i>	5'-TGAGGTGCCTACTTGCTCCT-3'	5'-GTGGACTTTTCCAGCTGCTC-3'
<i>Myh2</i>	5'-AAGCGAAGAGTAAGGCTGTC-3'	5'-GTGATTGCTTGCAAAGGAAC-3'
<i>Myh4</i>	5'-ACAAGCTGCGGGTGAAGAGC-3'	5'-CAGGACAGTGACAAAGAACG-3'
<i>Myh7</i>	5'-AGGTGTGCTCTCCAGAATGG-3'	5'-CAGCGGCTTGATCTTGAAGT-3'
<i>P47phox</i>	5'-AGAACAGAGTCATCCCACAC-3'	5'-GCTACGTTATTCTTGCCATC-3'
<i>PrxII</i>	5'-GGTTTGGGCCACGCATAAAA-3'	5'-GCCATGACTGCGTGAGCAAG-3'
<i>Prox1</i>	5'-GCTACCCCAGCTCCAACATGCT-3'	5'-TGATGGCTTGACGCGCATACTTCT-3'
<i>Rac1</i>	5'-GTAAACCTGCCTGCTCATCA-3'	5'-GAGAGGGGACGCAATCTGT-3'
<i>Redd1</i> <i>Ddit4</i>	5'- ACTACTGACCTGTTCGAGGC -3'	5'- TCAAGTGTCTGAAGATCCCGA -3'
<i>Redd2</i> <i>Ddit4l</i>	5'- GTGCAGCCCCATCAAAACATA -3'	5'- GAAGCCATGCTCTTGTCCTG -3'

<i>Sdha</i>	5'-TTACAAAGTGC GGGTCGATG-3'	5'-GTGTGCTTCCTCCAGTG TTC-3'
<i>Scn4a</i>	5'-GCAACCTGGTGGTCCTGAAT-3'	5'-CAGCCCCAAGAGGAAGGTTT-3'
<i>Slc8a1</i>	5'- GGAGACTGCTCGTGTGTCTA -3'	5'- TGTTGGTTGGCCTGAGAGAT -3'
<i>Smox</i>	5'-AAGTTGTGAATCCAGTGGCG-3'	5'-GTCTCCAAGCCTCACACTCT-3'
<i>Tnni1</i>	5'-ATGGAGGAGGTGGATCTGC-3'	5'-TTCAAATTTGGCCCGGCAC-3'
<i>Trpc1</i>	5'- TCTATAGATGTCTGGCCAGTCC-3'	5'- CATTTTGCAC TGACGGGCTA-3'
<i>Utrn</i>	5'- CACTATGACCCCTCCCAGTC -3'	5'- CGCTTCCTGTTGTAGAGCTG -3'

Figure legends

Figure 1. *Prox1* expression and markers of fibre type specification in voluntary exercised Mdx mice that received *Prox1* transfer into the muscle.

(A) *Prox1* expression. N = 6-11 per group

(B) Expression of genes encoding fibre type specific contractile proteins. N = 6-11 per group.

(C) Numbers per muscle cross-section of fibres expressing MHC-1, MHC-2a and MHC-2x. n = 3-5 per group.

(D) Percentage of the muscle cross-sectional areal occupied with weak SDH staining. N = 5-8 per group.

(E) Rate of force development (RFD) in Mdx+wheel+Prox1 muscle. N = 6-8 per group.

(F) Numbers per muscle cross-section of fibres expressing MHC-1, MHC-2a and MHC-2x. n = 3-5 per group.

(G) Percentage of the muscle cross-sectional areal occupied with weak SDH staining. N = 5-8 per group.

(H) Rate of force development in Mdx+Prox1 muscle. N = 5-8 per group.

Mdx+wheel+Prox1: voluntary exercised Mdx muscle that received Prox1 transfer into the muscle

Mdx+wheel: voluntary exercised Mdx muscle

Mdx+Prox1: Mdx muscle that received *Prox1* transfer into the muscle

Mdx: Mdx muscle

a : significant different from Mdx ($p < 0.05$).

b : significant different from Mdx+wheel ($p < 0.05$).

Figure 2. Fragility (susceptibility to muscle injury) and related gene expression in voluntary exercised Mdx mice that received *Prox1* transfer into the muscle.

(A) Force drop following lengthening contractions (Fragility) in Mdx+wheel+Prox1 mice.

n=6-8 per group

(B) Force drop following lengthening contractions in Mdx+Prox1 mice. n=5-8 per group.

(C) Expression of genes encoding ion channels, related to excitability. n=6-11 per group.

(D) Expression of genes, related to NADPH oxidase 2 (NOX2). n=6-11 per group.

(E) Expression of genes encoding utrophin and desmin. n=6-11 per group.

Mdx+wheel+Prox1: voluntary exercised Mdx muscle that received *Prox1* transfer into the muscle

Mdx+wheel: voluntary exercised Mdx muscle

Mdx+Prox1: Mdx muscle that received *Prox1* transfer into the muscle

Mdx: Mdx muscle

a : significant different from Mdx ($p < 0.05$).

b : significant different from Mdx+wheel ($p < 0.05$).

Figure 3. Absolute (P0) and specific (sP0) maximal forces, muscle weight and gene expression of atrophy markers in voluntary exercised Mdx mice that received *Prox1* transfer into the muscle.

(A) Specific maximal force in Mdx+wheel+Prox1 mice. n=6-8 per group

(B) Absolute maximal force in Mdx+wheel+Prox1 mice. n=6-8 per group.

(C) Absolute maximal force in Mdx+Prox1 mice. n=5-8 per group

(D) Specific maximal force in Mdx+Prox1 mice. n=5-8 per group

(E) Muscle weight in Mdx+wheel+Prox1 mice. n=6-8 per group

(F) Muscle weight in Mdx+Prox1 mice. n=5-9 per group

(G) Expression of genes related to atrophy in Mdx+Prox1 mice. n=6-8 per group.

Mdx+wheel+Prox1: voluntary exercised Mdx muscle that received *Prox1* transfer into the muscle

Mdx+wheel: voluntary exercised Mdx muscle

Mdx+Prox1: Mdx muscle that received *Prox1* transfer into the muscle

Mdx: Mdx muscle

a : significant different from Mdx ($p < 0.05$).

b : significant different from Mdx+wheel ($p < 0.05$).

Figure 1

Figure 2

Figure 3