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The artificial gene Jazz, a transcriptional regulator of utrophin, corrects the dystrophic pathology in *mdx* mice

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The absence of the cytoskeletal protein dystrophin results in Duchenne muscular dystrophy (DMD). The utrophin protein is the best candidate for dystrophin replacement in DMD patients. To obtain therapeutic levels of utrophin expression in dystrophic muscle, we developed an alternative strategy based on the use of artificial zinc finger transcription factors (ZF ATFs). The ZF ATF 'Jazz' was recently engineered and tested *in vivo* by generating a transgenic mouse specifically expressing Jazz at the muscular level. To validate the ZF ATF technology for DMD treatment we generated a second mouse model by crossing Jazz-transgenic mice with dystrophin-deficient *mdx* mice. Here, we show that the artificial Jazz protein restores sarcolemmal integrity and prevents the development of the dystrophic disease in *mdx* mice. This exclusive animal model establishes the notion that utrophin-based therapy for DMD can be efficiently developed using ZF ATF technology and candidates Jazz as a novel therapeutic molecule for DMD therapy.

INTRODUCTION

Utrophin up-regulation represents a promising therapeutic possibility for Duchenne muscular dystrophy (DMD), a devastating X-linked recessive disease characterized by a progressive destruction of muscle tissue (1,2). The diagnostic marker of DMD is the absence of the cytoskeletal protein dystrophin, which provides stability to the sarcolemma by linking the intracellular cytoskeletal network to the extracellular matrix. In absence of dystrophin, muscle contraction mechanically stresses the plasma membrane, inducing damage to the myofibres (3,4). DMD is among the most difficult diseases to treat even though the underlying pathogenesis is well understood (3,5). At least two factors have delayed the development of effective therapeutic strategies: (i) the dystrophin gene, responsible for the disease, is the largest known gene with a 14 kb cDNA; (ii) since the body can mount an immune response against a previously absent protein, restored dystrophin expression in dystrophic muscle could itself trigger an immunological attack as a neo-antigen. Thus, up-regulating proteins expressed endogenously in dystrophic muscle and able to compensate for the absence of dystrophin could help to avoid the negative impact of the immunological reaction, providing a defined target for DMD therapy. The utrophin

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protein is the best candidate for dystrophin replacement in DMD muscle. It exhibits 80% homology with dystrophin, shares structural/functional motifs with dystrophin throughout the length of the molecule and is capable of interacting with the same proteins that dystrophin binds within the cytoskeleton network (6,7). However, despite their extensive homology, dystrophin and utrophin differ in their localization patterns and are apparently expressed in a reciprocal manner. In developing muscle, utrophin is thought to perform the function of dystrophin because it is highly expressed along the entire length of the sarcolemma, from which it disappears postnatally, when it is substituted by dystrophin (8). As a result, utrophin localizes preferentially at the myotendinous junction and neuromuscular junction in adult tissue, whereas dystrophin predominates in the sarcolemma of muscle fibres (9,10). In the absence of dystrophin, as is the case in DMD muscle, utrophin is up-regulated and redistributed to the sarcolemma but not to a level sufficient to impede the progression of muscular disease (11,12). This observation led to the hypothesis that utrophin might have a complementary as well as a protective role in dystrophic muscle. Indeed, increased expression of utrophin restores plasma membrane integrity and rescues dystrophin-deficient muscle in *mdx* mice, a model of DMD (13–16). However, as is the case for its homologue dystrophin, the particular size of the utrophin gene is a critical disadvantage for gene therapy. Therefore, studies searching for natural or synthetic small molecules that can up-regulate utrophin could accelerate the clinical translation process and hold new hope for individuals with DMD.

As an alternative to pharmacological approaches (17), we developed an innovative strategy in order to up-regulate utrophin expression levels. We engineered several artificial zinc finger-based transcription factors (ZF ATFs) that exclusively target the utrophin gene promoter (18–20).

ZF ATFs provides a unique opportunity to target a gene of interest and can be tailor-made to bind virtually any DNA sequence. Multiple natural/synthetic zinc fingers assembled in tandem generate multifinger proteins that, when fused to the appropriate effector domain, can act as transcription factors with a wide range of potential applications (21–25). Using the available 'recognition code', we engineered several ZF ATFs as transcriptional regulators of utrophin. Among these, the artificial gene 'Jazz', encoding a three-zinc finger peptide, was designed to specifically bind the nine-base pair DNA sequence 5′-GCT-GCT-GCG-3′ present in promoter 'A' of both the human and mouse utrophin genes (18). To test the function of Jazz *in vivo*, we recently generated a novel transgenic mouse expressing the artificial Jazz gene under the control of the myosin light chain (MLC1) promoter (26). We have shown that Jazz is able to selectively bind its DNA target sequence *in vivo* and successfully increase endogenous utrophin expression at the muscular level. Moreover, the over-expressed utrophin exhibits redistribution along the sarcolemma of muscle fibres. Jazz-transgenic mice (Tg-Jazz) represent the first animal model generated with a ZF ATF and validate the strategy of transcriptional targeting of endogenous genes (26).

To evaluate the therapeutic potential of the artificial Jazz gene, we generated a novel mouse model by crossing Tg-Jazz mice with dystrophin-deficient *mdx* mice. This study demonstrates the efficacy of Jazz at increasing utrophin expression in a dystrophic background and provides important evidence for its beneficial effects in muscular diseases caused by the absence of dystrophin.

RESULTS

Jazz increases utrophin expression levels in dystrophic mice

Recently, we showed that the ZF ATF Jazz expressed in transgenic mice works efficiently on its target DNA sequence at the utrophin promoter chromosomal site (26). As previously described for the transgenic approach, we used a construct containing the Jazz zinc-finger sequence fused to the transcriptional activation domain VP16 and the MLC1 promoter/enhancer region to specifically drive transgene expression in skeletal muscle. In this study, we wanted to test whether the ZF ATF Jazz is able to target utrophin gene expression in a dystrophic background, and whether its function yields therapeutic benefits in muscular disease. To this end, we generated a second mouse model by crossing Jazz-transgenic mice with dystrophic *mdx* mice (*mdx*-Jazz mice; see *M&M*) and we compared the pathological phenotype in male *mdx*-Jazz littermates. First, we determined whether utrophin up-regulation could be achieved by the transgenic expression of Jazz in dystrophic muscle. Skeletal muscle from neonatal *mdx*-Jazz mice was subjected to real-time PCR and western blot analysis in order to detect utrophin expression levels. As shown in Figure 1A, using the housekeeping gene β2-microglobulin as a normalising control, a 1.8-fold increase in utrophin transcripts was observed in Jazz-positive *mdx* mice compared with Jazz-negative *mdx*. To check whether differences in the expression of utrophin mRNA were consistent with changes at the protein level, a western blot analysis was performed using a utrophin-specific antibody. As shown in Figure 1B, a consistent over-expression of utrophin protein was observed in the skeletal muscle of Jazz-positive *mdx* mice, compared with non-transgenic littermate controls. Notably, as previously observed in the Tg-Jazz model (26), up-regulation of the utrophin promoter by the artificial transcription factor Jazz resulted in the over-expression of all utrophin protein isoforms. These data demonstrate that the ZF ATF Jazz are able to re-programme utrophin expression with high efficiency even in a dystrophic background.

Jazz improves muscle histopathology in dystrophic mice

To verify whether the Jazz transgene increases utrophin expression in dystrophic muscle to a level sufficient for therapeutic benefit, several parameters of disease were evaluated. To compare muscle morphology, the tibialis anterior (TA) from 4-week-old *mdx*-Jazz mice was stained with haematoxylin and eosin (H&E). As shown in Figure 2A and in Supplementary Material, Figure S1, Jazz-negative dystrophic muscle displayed evident extensive myofibre damage with a strong infiltration of mononuclear inflammatory cells and several necrotic foci. In addition, an ongoing degenerative/regenerative process was indicated by the presence of numerous centrally nucleated myofibres (CNFs). Conversely, Jazz-positive dystrophic mice clearly showed a dramatic

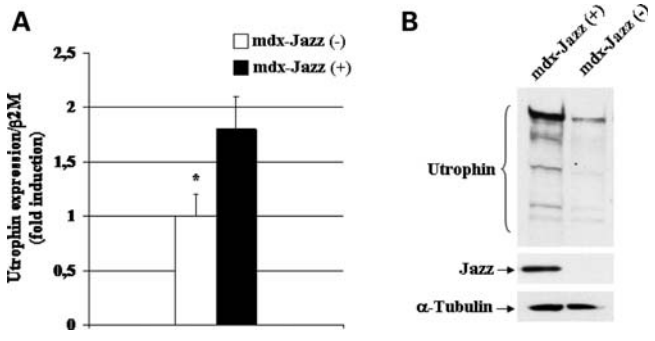

Figure 1. Jazz increases utrophin expression in *mdx* mice. (**A**) Quantification of utrophin transcripts by real-time PCR of skeletal muscle isolated from 2-day-old Jazz-positive and Jazz-negative *mdx* mice. The gene expression ratio between utrophin and β2-microglobulin (β2M) is shown as means (±SEM) from three independent experiments performed in triplicate. $^{*}P < 0.05$ indicates statistical significance by *t*-test. (**B**) Evaluation of utrophin protein levels by western blot analysis of skeletal muscle isolated from 2-day-old Jazz-negative and Jazz-positive *mdx* mice. The monoclonal antibody against utrophin shows increased utrophin levels in Jazz-positive dystrophic muscle. Blot immunoreactivity to anti-myc and anti-α-tubulin was used as a control for Jazz expression and sample loading, respectively.

reduction in muscle pathology, as displayed by better tissue architecture as well as a less pronounced inflammatory process. Then, we quantified the inflammatory infiltrates and the frequency of CNFs (Fig. 2B), and observed a reduced percentage of both parameters in Jazz-positive dystrophic muscle, compared with muscle of non-transgenic littermate controls. Consistent with a reduced number of small regenerating myofibres, the fibre cross-sectional area (CSA) of muscle isolated from Jazz-positive mice was almost 3-fold that of muscle from control mice (Supplementary Material, Fig. S2). Next, we further compared the extent of muscle damage by detection of embryonic myosin heavy chain (eMHC), an adult muscle regeneration marker (27,28). TA sections from 4-week-old *mdx*-Jazz mice were subjected to double staining with antibodies to eMHC and laminin proteins to visualize the new regenerating fibres and the extracellular matrix, respectively. We identified and measured several regenerating fibres in non-transgenic mice compared with Jazz-positive mice (Fig. 2C). In agreement with immunofluorescence data, western blot analysis of skeletal muscle from 4-week-old *mdx*-Jazz mice demonstrated that the amount of eMHC was essentially reduced in Jazz-positive mice. As expected, no expression of eMHC could be observed in dystrophic mice at the age of 2 days, as at this time the degenerative/regenerative process had not yet started. Finally, the expression of the Jazz transgene prevented the increase in serum levels of the muscle enzyme creatine kinase (CK), a marker of muscle damage (Supplementary Material, Fig. S3). Collectively, these data demonstrate that utrophin up-regulation by the artificial transcription factor Jazz effectively prevents muscle histopathology in dystrophin-deficient muscle.

Jazz corrects muscle function in dystrophic mice

Dystrophin-deficient muscle is characterized by a severe deficit in contractile force and marked susceptibility to contraction-induced injury (29,30). In order to verify whether Jazz expression improves the mechanical responses in dystrophic muscle, we measured the contractile activity of muscles from 4-month-old *mdx*-Jazz mice. Isolated diaphragm and extensor digitorum longus (EDL) preparations from both hind limbs of the animals were subjected to *in vitro* physiological assessment of muscle force, using variable voltages until the supramaximal value was reached. As shown in Figure 3A, both the diaphragm and EDL muscles exhibited a linear relationship between muscle force and applied voltage. However, muscle preparations from Jazz-positive mice showed a significant increase in muscle strength compared with muscle preparations from non-transgenic littermate controls. Notably, the differences in mechanical performance were more striking for EDL than for the diaphragm; this is consistent with the notion that EDL, a fast-twitch muscle, tends to be more affected by the absence of dystrophin (16,31). At the end of the force test, we assessed contraction-induced injury of the sarcolemma by staining each muscle with Procion Orange dye. Uptake of this fluorescent dye into individual fibres is an index of membrane integrity loss. As shown in Figure 3B top, fluorescence microscopy of both diaphragm and EDL cross-sections showed extensive uptake of the dye into non-transgenic muscles compared with Jazz-positive muscles. Quantification of the percentage of dye-positive area in each section confirmed the increased ability of Jazz-positive muscles to exclude the dye from stressed fibres (Fig. 3B bottom). As observed in the force test, the EDL muscle in dystrophic mice was more susceptible to contraction-induced damage than was the diaphragm. Altogether, these data provide physiological evidence for recovery in both contractile force and sarcolemmal integrity in *mdx* mice expressing the artificial gene Jazz.

In addition to *in vitro* testing, we assessed the effects of Jazz on the force production of dystrophic muscles *in vivo*. Different groups of mice (WT, *mdx* and both Jazz-negative and Jazz-positive *mdx* mice), at 3 or 12 months of age, were subjected to forced physical exercise on an accelerating treadmill. The exercise was repeated once a week for four consecutive weeks and the running time was recorded in each session. As shown in Figure 3C, the WT mice were able to run for at least 30 min before reaching exhaustion, regardless of the age of the mice. This endurance performance remained almost constant for the four consecutive trials. Compared with WT mice, *mdx* mice showed a significantly reduced running time, which progressively decreased through the successive trials, with a cumulative endurance of about 15 min before reaching exhaustion. Jazz-negative *mdx* mice behaved similarly to *mdx* mice and, although they showed a cumulative endurance somewhat better than *mdx* mice, they also showed progressive worsening over the consecutive trials. It should be noted that the slightly higher value of cumulative endurance in Jazz-negative *mdx* compared with *mdx* mice was probably due to the different genetic background of these two mouse models. Indeed, *mdx* mice have a C57BL/6J background whereas *mdx*-Jazz mice have a mixed C57BL/6JxDBA background. Usually, C57BL/6J mice show significantly lower performance in treadmill exercises than do other strains, including DBA mice (32). The up-regulation of utrophin achieved by the

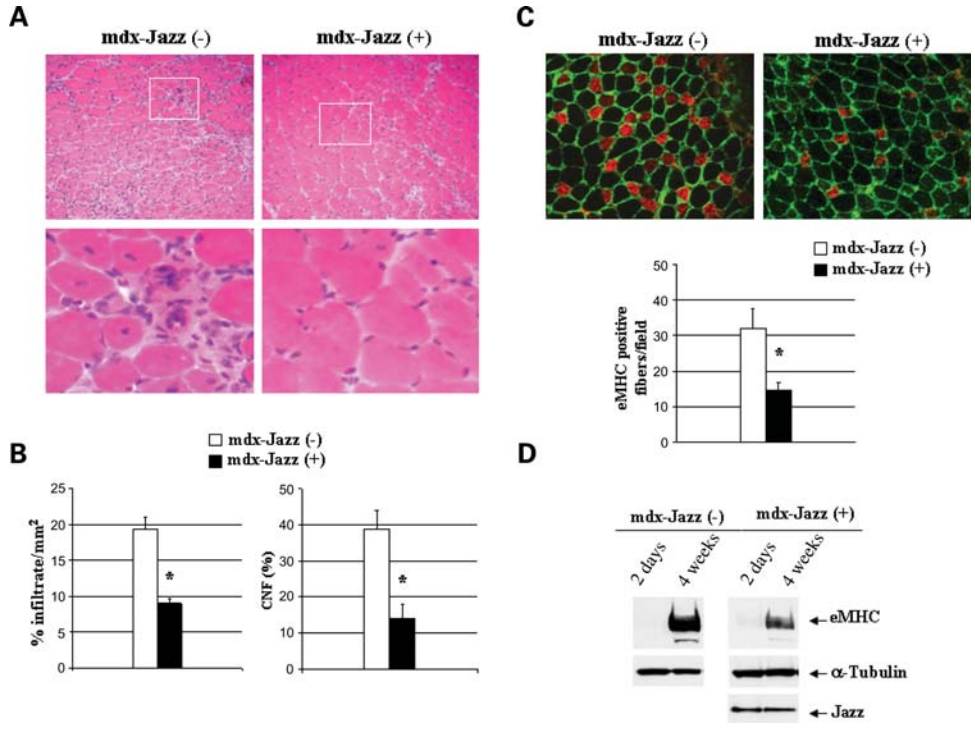

Figure 2. Morphological improvement of dystrophic muscle in Jazz-positive mice. (**A**) H&E staining of TA muscle from 4-week-old *mdx* mice showing the reduction in degeneration, necrotic foci and inflammatory cells in Jazz-positive myofibres. High magnifications of inset boxes are shown below. (Representative sections of six mice examined in each group). (**B**) Quantification of inflammatory infiltrates and central nucleation (CNF) on H&E stained sections of TA muscle from 4-week-old Jazz-positive and Jazz-negative *mdx* mice [Six mice were analysed in each group. The number of CNFs was obtained by normalising to the number of total myofibres per cross-sectional area (CSA), and at least 200 myofibres for section were counted]. $^{*}P < 0.05$ indicates statistical significance by *t*-test. (**C**) Top: Immunohistochemistry of TA muscle isolated from Jazz-positive and Jazz-negative *mdx* mice at the age of four weeks ($n = 6$ per group). Regenerating fibres are visualized by staining with anti-embryonic MHC (eMHC) monoclonal antibody (red). The extracellular matrix is counterstained with anti-laminin polyclonal antibody (green). Bottom: Graph shows the quantification of eMHC-positive myofibres. $^{*}P < 0.05$ indicates statistical significance by *t*-test. (**D**) eMHC detection by western blot of protein extracts of skeletal muscle derived from Jazz-positive and Jazz-negative *mdx* mice at the age of 2 days (no muscle regeneration) and 4 weeks (muscle regeneration period). Blot immunoreactivity to anti-myc and anti α-tubulin was used as a control for Jazz expression and sample loading, respectively.

expression of Jazz fully counteracted the deleterious effects of the dystrophic pathology, resulting in a significantly enhanced endurance performance in Jazz-positive mice, independent of the age of the mice. Finally, to demonstrate that the differences in treadmill running performance between mice groups did not depend on their spontaneous activity, the same mice tested for accelerating treadmill running were also tested for spontaneous motor activity and no significant differences between mice groups were observed (Supplementary Material, Fig. S4).

Furthermore, Jazz expression improved *mdx* sarcolemmal integrity during exercise, as shown by reduced *in vivo* Evan's blue dye uptake (Supplementary Material, Fig. S5).

These results demonstrate the full functional recovery of skeletal muscle in *mdx* mice expressing the ZF ATF Jazz and provide important evidence for the therapeutic potential of this artificial gene in muscular dystrophy treatment.

Expression profiling of Jazz-positive *mdx* versus Jazz-negative *mdx* mice

Using microarray analysis, we examined the gene expression profile of muscle from Jazz-positive *mdx* mice compared with Jazz-negative mice (4 months of age). No global wide-range genome perturbation was observed (fewer than 100 genes are differentially expressed considering a cut-off of 1.3-fold change). Significantly, genes that showed altered expression are mainly involved in muscle development/regeneration and inflammatory/immune response (Fig. 4 and Supplementary Material, Fig. S6). Ingenuity Pathways Analysis software allowed to retrieve data for graphic visualization of differentially expressed genes (DEG) between Jazz-positive *mdx* and Jazz-negative *mdx*, organized in an interactive network related to inflammatory pathways (Fig. 4B). In addition, to validate our microarray data, eight genes among most relevant DEG were analysed by Real-time PCR (Supplementary Material, Fig. S7). Utrophin up-regulation achieved by our artificial zinc finger protein induces a global down-regulation of inflammatory genes in Jazz-positive *mdx* mice. Among Jazz-positive *mdx*, down-regulated inflammatory genes are particularly relevant: (i) Mpeg1, a gene expressed by macrophages and an index of elevated macrophage infiltration into muscle, (ii) Lgals 3, a serine protease inhibitor up-regulated during a series of cellular events including inflammation, (iii) Gpnmb, a serine protease inhibitor that acts as a feedback regulator of pro-inflammatory responses, (iv) Mmp12, a member of a cluster of matrix metalloproteinase genes, strongly up-regulated in major inflammatory

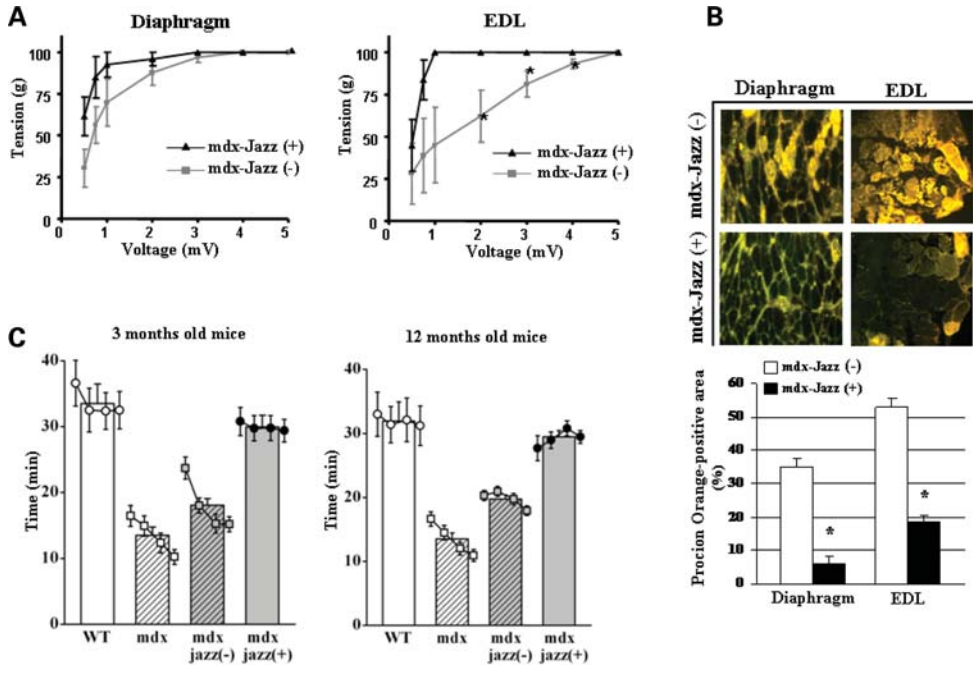

Figure 3. Jazz corrects muscle function in dystrophic mice. (**A**) Diaphragms and EDL muscles isolated from Jazz-positive and Jazz-negative *mdx* mice were electrically stimulated *in vitro* to elicit force and contractile activity ($n = 4$ per group). The relationship between contractile response (g) and stimulation frequency (voltage) in muscle preparations demonstrates a significant enhancement of muscles strength in Jazz-positive *mdx* mice. (**B**) Procion Orange dye uptake in sections of diaphragm and EDL muscles after the force test. Top: Representative images demonstrating the increased ability of Jazz-positive *mdx* mice to exclude dye from stretched fibres. Bottom: Graph shows the mean ($\pm$SEM) area of dye-positive fibres, expressed as the percentage of the total CSA of muscle sections. $^{*}P < 0.05$ indicates statistical significance by *t*-test. (**C**) Mean running time during four treadmill trials with exhaustive exercise protocol. Different groups of WT, *mdx*, Jazz-negative *mdx* and Jazz-positive *mdx* mice were tested at either 3 or 12 months of age. For each strain/age group, lines indicate the mean duration of each trial, and columns indicate the cumulative mean time over the consecutive trials. Number of animals is $n = 10$ for each strain/age group. The *mdx* and Jazz-negative *mdx* mice had a shorter time to exhaustion than did healthy WT mice. The Jazz-positive *mdx* mice showed a full recovery of exercise performance. Statistical analysis with ANOVA for repeated measures, conducted separately at 3 months (3M) and at 12 months (12M), showed a significant main effect for mice groups (3M: $F_{3,36} = 25.765$, $P < 0.0001$; 12M: $F_{3,36} = 26.979$, $P < 0.0001$), for treadmill trials (3M: $F_{3,108} = 24.579$, $P < 0.0001$; 12M: $F_{3,108} = 2.904$, $P = 0.0381$) and for interaction between mice groups and treadmill trials (3M: $F_{9,108} = 2.940$, $P = 0.0037$; 12M: $F_{9,108} = 3.635$, $P = 0.0005$). *Post hoc* comparisons with WT mice showed significant differences in endurance levels between WT and *mdx* or Jazz-negative *mdx* mice, between Jazz-positive *mdx* mice and *mdx* or Jazz-negative *mdx* mice, but not between WT and Jazz-positive *mdx* mice ($P < 0.05$, Tukey–Kramer).

response induced by macrophage-secreted factors and (v) C3ar1, the receptor of the proteolytic cleavage product of complement component 3 (C3a), a potent anaphylatoxin that induces the production of inflammatory mediators including histamine.

In contrast, Serpina3, a member of the cysteine or serine protease inhibitor family, involved in the control of calcium-activated proteases in a pro-inflammatory response, is up-regulated in Jazz-positive *mdx* mice. This incongruence was also reported in *mdx* mice expressing high level of utrophin, using an approach different from ours (33). Moreover, in Jazz-positive *mdx* mice, there is a significant up-regulation of the pyruvate dehydrogenase kinase Pdk4, a key gene in the carbohydrate metabolic process. Considering that Pdk4, which is an index of adipose tissue replacement of functional muscle tissue associated with muscle weakness and atrophy, is down-regulated in *mdx* mice, an increasing in the levels of this protein in our transgenic mice is in agreement with an induced dystrophic rescue.

These microarray data, in agreement with the results discussed above, confirm the notion that ZF ATF Jazz efficiently ameliorates the pathophysiology in *mdx* mice.

DISCUSSION

Strategies to re-programme endogenous gene expression directly at the DNA level are highly appealing and offer promising prospects in medical research. ZF ATFs, designed *ad hoc* to alter the expression profile of genes related to diseases, provide a valuable platform for unlimited therapeutic applications (24,25).

Over the past years, our group has focused on engineering several novel ZF ATFs with the aim of up-regulating the utrophin gene, a functional substitute for dystrophin in DMD patients (18–20). It is widely accepted that utrophin up-regulation could be an attractive alternative to dystrophin-based therapies (1,2,34). As utrophin is not a neo-antigen, it is unlikely to induce immune rejection. Since ubiquitous over-expression of utrophin had no deleterious consequences, a strict control of the therapeutic effect would not be necessary (35). In addition, a 2-fold up-regulation of utrophin is sufficient to alleviate dystrophic muscle pathology, as demonstrated by transgenic approaches (15). Compared with other strategies that have been proposed to increase utrophin levels in muscle (13–15,36–38), the possibility to up-regulate

A

Gene	Fold Change Jazz+/Jazz-	Gene	Fold Change Jazz+/Jazz-
Fmod	-4,55	Stat3	1,33
Spp1	-4,41	Pdk4	1,34
Mmp12	-3,38	Glul	1,35
Padi2	-2,58	Steap4	1,38
Gpnmb	-2,29	Myf6	1,40
Cd68	-2,17	Xirp1	1,43
Thbs4	-2,08	Zbtb16	1,54
Itgb1bp3	-2,05	Abra	1,55
Mpeg1	-1,92	Dub2a	1,62
Aspn	-1,85	Scd1	1,69
Cybb	-1,82	Slc25a25	1,73
Itgb2	-1,80	Mt1	1,76
Zcrb1	-1,80	Retnla	1,77
Lyzs	-1,79	Serpina3	1,79
Mup2	-1,78	Nfil3	1,83
C3ar1	-1,75	Mt2	1,84
Ctss	-1,75	Hp	1,94
Lgals3	-1,73	Ppp1r3c	1,96
Hexb	-1,69	Lrg1	2,00
Anpep	-1,65	Csrp3	2,00
Postn	-1,62	Rrad	2,32

B

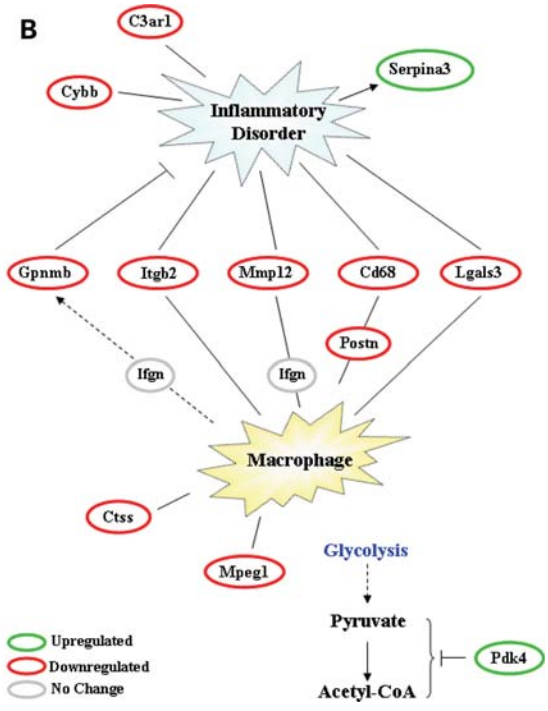

Figure 4. Microarray analysis. (**A**) A representative list of differentially expressed genes (DEG) in the skeletal muscle of Jazz-positive *mdx* versus Jazz-negative *mdx* mice (Jazz+/Jazz−; $n = 3$ per group), obtained by array hybridization. These genes represent a subset of those reported in Supplementary Material, Figure S6. Expression value calculation was performed using the DNA-Chip Analyzer (44) and the lower confidence bounds of fold changes were conservative estimates of the real fold changes (minimum fold change 1.33). Up-regulated genes are coloured in green and down-regulated genes are coloured in red. Fold changes are indicated. (**B**) Graphical representation of main genes differentially expressed between Jazz-positive *mdx* and Jazz-negative *mdx* skeletal muscle. Molecular role and functional signalling were elaborated and organized in interactive relationships derived from Ingenuity Pathways Analysis Ingenuity Systems (see Materials and Methods). Network analysis shows alterations in inflammatory response and cellular metabolism. Signals are shown and colour-coded green for up-regulated genes in Jazz-positive *mdx* versus Jazz-negative *mdx* mice, although down-regulated genes are coloured in red; genes whose expression was not modified are shown in gray.

the expression of the endogenous utrophin gene with ZF ATFs offers several advantages. ZF ATFs are active at very low concentrations, they are very small and they have the advantage of being within viral cloning capacities (39). In addition, ZF ATFs appear to be promising therapeutic molecules since they belong to the large family of zinc finger proteins characterized by low immunogenicity. Induction of endogenous gene expression by ZF ATFs results in the production of all different isoforms of the target gene products. This is a crucial feature because expressing all splice variants of a gene in the correct ratio could be essential to achieve the whole cascade of gene regulatory events, as demonstrated in a mouse model of angiogenesis (40). Importantly, we have previously reported that a synthetic transcription factor introduced *in vivo* is well tolerated and safe for the health of the transgenic animal throughout its life, and that it works accurately without significant non-specific global transcriptional effects (26). Therefore, the next step was to validate the feasibility of using ZF ATF technology in gene therapy with a transgenic approach.

Here, we have shown that the transgenic expression of the artificial gene Jazz, designed to specifically increase utrophin expression at the muscular level, corrects the dystrophic phenotype in *mdx* mice. First, real-time PCR and western blot analysis revealed that Jazz efficiently targets utrophin gene expression also in a dystrophic background. Utrophin mRNA was up-regulated 1.8-fold in neonatal Jazz-positive *mdx* mice compared with non-transgenic littermate controls, and this difference was consistent with changes observed at the protein level. These results were extremely encouraging in two respects: (i) a 1.8-fold increase in utrophin transcripts is consistent with the level of utrophin predicted to be sufficient to alleviate the dystrophic phenotype (15); (ii) utrophin up-regulation induced in *mdx* mice within a few days after birth is most effective in preventing the onset of muscular necrosis due to membrane fragility (16). Indeed, the dystrophic pathology in Jazz-positive *mdx* mice was strongly reduced, as assessed by morphological and physiological criteria. Morphologically, muscle from Jazz-positive *mdx* mice showed a significant improvement in fibres organization, associated with a strong reduction in both muscle necrosis and cellular infiltrate. Significantly, the frequency of CNFs, a parameter commonly used to monitor the efficiency of gene therapy trials in *mdx* mice (15), was strongly reduced in Jazz-positive muscle. The decreased number of small regenerating fibres was consistent with the increase in fibre diameter, as quantified in Jazz-positive *mdx* mice compared with non-transgenic littermate controls. Evaluation of embryonic MHC expression in skeletal muscle from *mdx*-Jazz mice further confirmed the beneficial effects of Jazz in preventing myofibres damage. Importantly, we clearly showed that the transgenic expression of ZF ATF Jazz in dystrophic

backgrounds not only improves muscle histopathology, but also fully corrects muscle function. Using *in vitro* testing methods, we showed that Jazz improves both muscle contractile force and sarcolemmal integrity in transgenic *mdx* mice. During a forced physical exercise on an accelerating treadmill, Jazz-positive *mdx* mice exhibited better exercise performance than Jazz-negative *mdx* and, surprisingly, they reached endurance levels similar to those recorded in healthy wild-type mice. Importantly, the enhanced performance of Jazz-positive mice was maintained throughout the life of the animals. Finally, microarray analysis showed that expression of the designed transcription factor Jazz in *mdx* mouse muscle adjusts the global gene expression profile to one that results in reduced pathophysiology.

In conclusion, to our knowledge, the transgenic *mdx*-Jazz mouse represents the first dystrophin-deficient model expressing an artificial transcription factor targeted to an endogenous gene. This exclusive animal model has been useful to establish the 'concept' that utrophin-based therapy can be efficiently developed using ZF ATF technology and will provide a useful tool in drug discovery and therapeutics, from which experimental results could be translated to DMD clinical trials. Importantly, this model offers the opportunity to test the synergic combination of the utrophin-based therapy with other approaches proposed to ameliorate the dystrophic phenotype, in particular those shown to increase myofibre size (41). Moreover, the results presented here, as well as those from a recently published work (42), encourage the combination of ZF ATF technology with the strategy of adeno-associated virus (AAV)-mediated gene delivery. We thought that using both a transgenic strategy and AAV-based gene therapy would provide very useful indications on the validity of ZF ATFs, thus accelerating the clinical translation of novel therapeutic treatments for DMD.

MATERIALS AND METHODS

Animal care and genotyping

Transgenic Jazz mice (Tg-Jazz) were generated as described previously (26) and the colony was maintained by crossing transgenic heterozygous Jazz mice with wild-type BDF1 (C57Black6 × DBA) partners. Jazz positive male mice were then routinely crossbred with *mdx* female mice to generate Jazz-positive *mdx* and Jazz-negative *mdx* male mice in the same litter. Both Tg-Jazz and *mdx*-Jazz mice were genotypized as reported previously (26).

Preparation of total RNA

Total RNA was isolated from skeletal muscle by homogenization of tissue in Trizol reagent (Invitrogen), according to the manufacturer's instructions. The quality of the RNA was assayed by the Agilent 2100 Bioanalyzer, using the RNA 6000 Nano Assay Kit (Agilent Technologies).

Real-time PCR analysis

Two microgram of total RNA was reverse transcribed using oligo (dT) 12–18 primers and SUPERSCRIPT II (Invitrogen) in a volume of 20 μl at 42°C for 50 minutes. Real-time PCR assays were performed in a 96-well format using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). To obtain the utrophin gene expression rate the amount of target gene was normalized to that of the housekeeping gene β2-microglobulin (β2M). Primers and probes for the target gene (UTRN) and for housekeeping gene were purchased as TaqMan Gene Expression Assays (AB). PCR reaction was performed as we previously described (26). The results were analysed using Applied Biosystems analysis software. The data are expressed as the ratio between UTRN and β2M mRNA expression. A minimal number of six mice were analysed for each category.

Western blot analysis

All frozen tissues were processed in lysis buffer (2% SDS, 5 mM EDTA) supplemented with a proteinase inhibitor cocktail (Complete TM, Roche Diagnostics GmbH Mannheim, Germany), using a homogenizer (7 mm, OMNI International GLH). Homogenates were boiled for 10 min and clarified by centrifugation for 10 min at 12 000*g*. Twenty-five microgram of protein extract was electrophoresed through NuPAGE 3-8% Tris-Acetate gel (Invitrogen), according to the manufacturer's instructions. The antibodies used are listed in the Supplementary Material.

Histological analysis

TA muscles from transgenic and littermate control mice were dissected, embedded in Tissue-Tek O.C.T. medium (Sakura Finetechnical, NL) and rapidly frozen in liquid nitrogen-cooled isopentane. Transversal sections (9 μm thick) were obtained by cryostate at −20°C (LEICA, CM1850UV), placed onto polysine-coated microscope slides (Menzel Gmbh&Co) and fixed with formaldehyde 4% in PBS for 10 min. Sections were stained with hematoxylin and eosin (H&E; Roth, Germany) following the manufacture's instructions. The entire cross-section, taken at midbelly, was analysed by a microscope (Olympus BX51; Tokyo, Japan). Images were captured using a digital camera at ×40 magnification.

Immunohistochemistry

TA cross sections (9 μm thick) were obtained as above described. Sections were fixed with formaldehyde 4% in PBS for 10 minutes, permeabilized in PBS-Triton 0.25% for 10 min and blocked in PBS-BSA 1% for 60 min. Slides were incubated with appropriate antibodies. A detailed description of this method is provided in the Supplementary Material. Stained specimens were analysed by conventional epifluorescence microscope (Olympus BX51; Tokyo, Japan). Images were captured using a digital camera at ×40 magnification and merged using the IAS2000 software.

Mechanical response of isolated muscles

Diaphragm and EDL were isolated from both hind limbs of 4-month-old transgenic and littermate control mice. Muscle

preparations and mechanical activity recording were carried out as we previously described (26). At the end of the tension recordings, EDL and diaphragm muscles were subjected to a period of repetitive stimulation. Contractions of the muscles were elicited by trains of stimuli at a frequency of 40 Hz for 250 ms every second for 3 min. Following this procedure, able to obtain the muscle fatigue (43), tissues were removed from the chamber and subjected to Procion Orange staining.

Procion orange uptake

After the electrophysiological tests diaphragm and EDL muscles from each animal were incubated in 0.2% Procion Orange dye (Sigma-Aldrich) in PBS for 45 min at 30°C. Muscles were washed three times with PBS for 5 min, embedded in Tissue-Tek O.C.T. medium (Sakura Finetechnical, NL) and rapidly frozen in liquid nitrogen-cooled isopentane. Cross sections (9 μm thick) were obtained as above described. Sections were viewed under a fluorescent microscope (Olympus BX51; Tokyo, Japan) at ×20 magnification through a filter, with the excitation wavelength range set at 450–490 nm and the emission range set at 505–520 nm. The entire cross-section, taken at midbelly, was analysed for each muscle. Colour images were captured using a digital camera and processed using the IAS2000 software.

Assays of mice performances by treadmill running

Exercise studies were performed on five-lane motorized treadmill equipped with an electronic control unit [Treadmill Model LE8710, PanLab, Cornella (BCN), Spain], and an electric shock grid at one end of the treadmill. A detailed description of this method is provided in the Supplementary Material.

Microarrays analysis

Expression profiling has been carried out by using the Mouse Gene 1.0 ST arrays (Affymetrix), according to the manufacturer's instructions. Briefly, the Affymetrix GeneChip® Whole Transcript Sense Target Labelling Assay has been used to amplify and reverse-transcribed total RNA, and to biotinylate sense-strand DNA targets. Arrays has been hybridized with the labelled-target hybridization cocktail by rotation in the Affymetrix Gene Chip hybridization oven at 45°C for 16 h, washed in the Affymetrix GeneChip Fluidics station FS 450, and scanned by Affymetrix Gene Chip scanner 3000 7G system.

Array normalization, expression value calculation was performed using DNA-Chip Analyzer (www.dchip.org; 44). The Invariant Set Normalization method (44) was used to normalize arrays at probe cell level to make them comparable, and the model-based method (45) was used for probe-selection and computing expression values. These expression levels were attached with standard errors as measurement accuracy, which were subsequently used to compute 90% confidence intervals of fold changes in two-group comparisons (45). The lower confidence bounds of fold changes were conservative estimate of the real fold changes. Genes with increased or decreased expression after treatments by more than 1.3-fold (lower confidence bound) were selected for further study. The 'Jazz network' was generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). The network is a graphical representation of the molecular relationships between genes/gene products. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by the literature, or by information stored in the Ingenuity Pathways Knowledge Base.

Data analysis

All values of biochemical, morphological and behavioural tests are expressed as mean ± SEM; *n* represent the numbers of individuals in each experiment. Two way ANOVAs for repeated measures were used to analyse the effects of treadmill running on different mouse models. *Post hoc* comparisons were carried out using Tukey–Kramer test. Statistical significance was assessed by Student's *t*-test. Differences were considered significant at $P < 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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