Utrophin Up-Regulation by an Artificial Transcription Factor in Transgenic Mice

Elisabetta Mattei1,2, Nicoletta Corbi3, Maria Grazia Di Certo1,4, Georgios Strimpakos1, Cinzia Severini1, Annalisa Onori3, Agata Desantis3,4, Valentina Libri5, Serena Buontempo1, Aristide Floridi6,5, Maurizio Fanciulli2,5, Dilair Baban6, Kay E. Davies6, Claudio Passananti2,3

1Istituto di Neurobiologia e Medicina Molecolare, Consiglio Nazionale delle Ricerche, European Brain Research Institute, Rome, Italy, 2Italian Association for Cancer Research, Roman Oncogenomic Center, Rome, Italy, 3Istituto di Biologia e Patologia Molecolari, Consiglio Nazionale delle Ricerche, Regina Elena Cancer Institute, Rome, Italy, 4Department of Experimental Medicine, University of L’Aquila, L’Aquila, Italy, 5Laboratory B, Regina Elena Cancer Institute, Rome, Italy, 6Department of Physiology, Anatomy and Genetics, Medical Research Council Functional Genetics Unit, University of Oxford, Oxford, United Kingdom

INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is a severe muscle degenerative disease, due to absence of dystrophin. There is currently no effective treatment for DMD. Our aim is to up-regulate the expression level of the dystrophin related gene utrophin in DMD, complementing in this way the lack of dystrophin functions. To this end we designed and engineered several synthetic zinc finger based transcription factors. In particular, we have previously shown that the artificial three zinc finger protein named Jazz, fused with the appropriate effector domain, is able to drive the transcription of a test gene from the utrophin promoter “A”. Here we report on the characterization of Vp16-Jazz-transgenic mice that specifically over-express the utrophin gene at the muscular level. A Chromatin Immunoprecipitation assay (ChIP) demonstrated the effective access/binding of the Jazz protein to active chromatin in mouse muscle and Vp16-Jazz was shown to be able to up-regulate endogenous utrophin gene expression by immunohistochemistry, western blot analyses and real-time PCR. To our knowledge, this is the first example of a transgenic mouse expressing an artificial gene coding for a zinc finger based transcription factor. The achievement of Vp16-Jazz transgenic mice validates the strategy of transcriptional targeting of endogenous genes and could represent an exclusive animal model for use in drug discovery and therapeutics.

Citation: Mattei E, Corbi N, Di Certo MG, Strimpakos G, Severini C, et al (2007) Utrophin Up-Regulation by an Artificial Transcription Factor in Transgenic Mice. PLoS ONE 2(8): e774. doi:10.1371/journal.pone.0000774

Academic Editor: Lin Mei, Medical College of Georgia, United States of America

Received May 8, 2007; Accepted July 23, 2007; Published August 22, 2007

Copyright: © 2007 Mattei et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Telethon A160, Regional Grant-Lazio (AIRC), FIRB project “Epigenetica e Cromatina” and M.U.R. ex 60%. Dr. Maria Grazia Di Certo is a recipient of a fellowship supported by Regione Lazio fundings for “Sviluppo della Ricerca sul Cervello”. Dr. Dilair Baban and Prof. Kay E. Davies are supported by the Medical Research Council UK.

Competing Interests: The authors have declared that no competing interests exist.

* To whom correspondence should be addressed. E-mail: passananti@ifo.it
fusing Jazz with the appropriate effector domains, are able to drive the transcription of a test gene from both natural and synthetic utrophin promoters [17].

Here, we show that the ZF ATF “Vp16-Jazz” expressed in transgenic mice under the control of the myosin light chain (MLC1) promoter powerfully up-regulates its target gene “utrophin” at the chromosomal site. To our knowledge, this is the first example of a transgenic mouse expressing an artificial zinc finger transcription factor working on its endogenous target gene.

RESULTS
Structure of the artificial Vp16-Jazz gene
We have previously shown that the chimeric proteins, obtained by fusing the Jazz zinc-finger gene with the appropriate transcriptional effector domain are able to drive the transcription of a test gene from both natural and synthetic utrophin promoters [17]. Now, we have engineered a novel Jazz-fusion protein named “Vp16-Jazz” in order to optimize its expression in transgenic mouse skeletal muscle. The Vp16-Jazz construct (Fig. 1A) contains the Myosin Light Chain (MLC) promoter/enhancer regions, an intron region derived from the Simian virus SV40, the strong transcriptional activation domain Vp16 from herpes simplex virus, the epitope tag myc and a nuclear localization signal (NLS) derived from SV40 early genes. The Vp16-Jazz protein specifically binds the 9 base pairs DNA sequence 5’-GCT-GCT-GCG-3’ present in the promoter region of both the human and mouse utrophin genes [32] (Fig. 1B).

Vp16-Jazz expression in transgenic lines
Using the above described Vp16-Jazz construct, several F0 transgenic mice founders were obtained. The expression of the transgene was analyzed by western blot on F1 positive mice using a monoclonal anti-myc antibody. Different levels of Vp16-Jazz expression were detected in skeletal muscle in several independent families. Figure 2A reports the level of Vp16-Jazz in transgenic families tg9 and tg11, that presented optimal transgene expression.

In order to verify the specificity of transgene expression in families tg9 and tg11, total protein extracts from heart and brain were analyzed in western blot. As shown in figure 2B the Vp16-Jazz is absent in brain, strongly present in skeletal muscle and slightly present in heart only in family tg9. Other tissues were also analysed, which confirmed muscle specific expression of Vp16-Jazz (data not shown).

Vp16-Jazz and utrophin up-regulation
In order to characterize the biological activity of the Vp16-Jazz protein in transgenic mice chromatin immunoprecipitation experiments (ChIP) were performed in muscle to verify whether the Vp16-Jazz transcription factor binds its DNA target in vivo in the context of the utrophin promoter chromatin infrastructure. In both tg9 (Fig. 3A) and tg11 (data not shown) Vp16-Jazz is able to bind efficiently and specifically to its DNA target sequence (5’-GCT-GCT-GCG-3’) at the endogenous utrophin promoter chromosomal site. In the ChIP experiment dystrophin promoter/enhancer region was used as further specificity control (Fig. 3A, bottom).

Figure 1. Vp16-Jazz and its DNA target sequence. A: Schematic representation of the Vp16-Jazz gene in the pMex-vector, used to generate transgenic mice. The 9 base pair long Vp16-Jazz DNA target sequence is indicated. B: The nucleotide sequence of the mouse utrophin promoter A. The Vp16-Jazz DNA target sequence is indicated in bold characters and underlined. The main transcription factor binding sites present in this promoter region are indicated.
doi:10.1371/journal.pone.0000774.g001
Next, we determined whether endogenous utrophin expression is effectively up-regulated by the artificial Vp16-Jazz protein. To this end, we performed a quantitative analysis of utrophin mRNA levels by real-time PCR. As shown in figure 3B, about 3 to 4 fold increase of utrophin expression was observed in transgenic mice compared to the control wild type (wt) mice. As shown, similar values were obtained using two different normalizing housekeeping genes (β2-microglobulin and β-glucuronidase).

To determine whether changes in the expression of utrophin mRNA were consistent with changes in the protein level a western

Figure 2. Muscle specific expression of Vp16-Jazz in transgenic mice. A: Western Blot analysis of total proteins extracted from the skeletal muscle of wild type (wt) and transgenic mice derived from two different founders (tg9 and tg41). The expression of Vp16-Jazz transgene was monitored by the anti-myc monoclonal 9E10 antibody. Detection of α-tubulin was used to normalize the amount of proteins. B: Western Blot analysis of total proteins extracted from the skeletal muscle, heart and brain of transgenic mice from families tg9 and tg41.

doi:10.1371/journal.pone.0000774.g002

Figure 3. Vp16-Jazz and utrophin up-regulation. A: Vp16-Jazz chromatin immunoprecipitation, performed in skeletal muscle derived from wt mice and transgenic mice (family tg9) using myc monoclonal antibody/protein G-agarose beads or protein G-agarose beads as a control (no-Ab). Immunoprecipitates from each sample were analyzed for the presence of utrophin promoter by PCR. A sample representing linear amplification of the total input chromatin (input) was included (lane 1). As control, samples from transgenic mice were also tested for the presence of dystrophin promoter sequence. B: Real-time PCR analysis of the utrophin gene expression rate in Vp16-Jazz transgenic mice (tg9 and tg41) and control wt mice. The gene expression ratio between utrophin and β-glucuronidase (GUS) and β2-microglobulin (β2M) is shown as means±S.D. from three independent experiments performed in triplicate. C: Western blot of total protein extracts derived from skeletal muscle and heart from wt mice and Vp16-Jazz transgenic mice (tg9 and tg41) incubated with monoclonal antibody against utrophin. The same membrane was incubated with anti-α-tubulin monoclonal antibody for loading normalization. D: Relative utrophin expression of wt and transgenic mice (tg9 and tg41) was determined by densitometric analysis. E: Total protein extracts from skeletal muscle of wt and transgenic mice (tg9 and tg41) were subjected to immunoblotting to detect the expression levels of the dystrophin and α-sarcoglycan proteins. The anti-α-tubulin and anti-myc antibodies were used to normalize the protein content and to test the Vp16-Jazz transgene expression respectively.

doi:10.1371/journal.pone.0000774.g003
blot analysis was performed using the anti-utrophin antibody. As shown in figures 3C and 3D, a consistent up-regulation of utrophin protein levels in skeletal muscle is observed in both transgenic families, tg9 and tg41, in comparison to wild type control animals. Notably, as expected, the up-regulation from the promoter region produces an increase of all the utrophin protein isoforms in the transgenic lines so far analyzed. Then we demonstrated that in both transgenic families, tg9 and tg41, and in wild type control mice dystrophin and alpha-sarcoglycan protein levels remain invariable in skeletal muscle (Fig. 3E).

**Skeletal muscle histological analysis**

The histological analysis of the tibialis anterior (TA) stained with the utrophin antibody revealed a significant increase and consequent re-localization along the sarcolemma of utrophin protein in the transgenic mice tg9 (Fig. 4B) and tg41 (data not shown) compared to wt control animals (Fig. 4A). In order to further investigate utrophin re-localization, we stained TA with both anti-utrophin antibody and with Alexa Fluor 488 labeled α-bungarotoxin to visualize the acetylcholine receptors (AChR) at the neuromuscular junction. As shown in figure 4C the utrophin overexpression in transgenic mice tg9 causes a remarkable re-localization of utrophin protein along the sarcolemma in addition to its canonical localization at the synaptic regions.

**Mechanical response of isolated muscles**

The contractile activity of muscles from two month old Vp16-Jazz transgenic mice, and wt control animals was measured. Isolated diaphragm and extensor digitorum longus (EDL) preparations of both hind limbs were examined *in vivo* by physiological assessment of muscle force. As shown in figure 4D, both diaphragm and EDL muscles from transgenic mice show a slight, but constant increase in muscle strength as compared to wild type littermates. The increase in muscle strength was similar between the tg9 and tg41 families.

**Microarray analysis**

To test the wide range genome perturbation induced *in vivo* by the artificial zinc finger transcription factor Vp16-Jazz and consequent utrophin over-expression, microarray analysis was performed,

![Figure 4. Effects of Vp16-Jazz in transgenic mice.](image-url)
comparing the gene expression profiles of the Vp16-Jazz transgenic tg9 and tg14 mice versus control wt mice. Figure 4E represents scatter plots of the natural log transformed expression values of all genes (22690) on the array. Differentially expressed genes (DEGs) found in families 9 and 41 versus control wt mice are colour coded with lines on the graph set at 2-fold differential expression.

MATERIALS AND METHODS

Constructs

The Jazz gene fused to a nuclear localization signal region and the Vp16 activation domain from the herpes simplex virus was subcloned into the pMEX mammalian vector containing the MLC1 promoter/enhancer [33].

Generation of Jazz transgenic mice

The DNA fragment from pMEX-Jazz containing the MLC1 promoter/enhancer and the Jazz-TF as described above was purified using a GeneClean Kit (Q-BIOgene, MP Biomedicals, Morgan Irvine, CA) and resuspended in injection buffer (10 mM Tris pH 7.5, 0.1 mM EDTA, 30 mM NaCl) at a concentration of 2 ng/μl.

BDF1 (C57Black6×DBA) mice were purchased from Charles River (Calco, Italy), housed under conventional, controlled standard conditions and sacrificed by carbon dioxide asphyxiation.

Transgenic mice were generated by microinjecting the transgenic DNA into the pronuclei of fertilized eggs derived from BDF1×BDF1 mating, following previously described standard procedures [34]. The transgenic lines were maintained by crossing founders (F0) and F1 homozygous mice with wild type BDF1 partners. F1 and F2 transgenic mice and their wild type littermates of age comprised between 2 and 3 months were used in all experiments.

Mice Genotyping

Transgene integration in F0 (founders) and transmission in F1/F2 animals was checked by polymerase chain reaction (PCR) analysis. Genomic DNA was isolated from the tail following standard procedures. Briefly, tails were incubated overnight at 53°C in 0.5 ml of tail solution (50 mM Tris-HCl, pH 8, 100 mM EDTA, 1% SDS, 100 mM NaCl) supplemented with 50 μg of Proteinase K (PCR grade, Roche). DNA was extracted using phenol/chloroform, precipitated with isopropanol, and dissolved in TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.5 ml of reaction buffer containing 1 mM PMSF and a proteinase inhibitor cocktail (Complete™, Roche Diagnostics GmbH Mannheim, Germany), using a homogenizer (7 mm, OMNI International GLH). Homogenates were boiled for 10 minutes and clarified by centrifugation for 20 minutes at 12000×g. The Quant-iT Protein Assay Kit was used to measure protein concentration by a Qubit™ fluorometer (Invitrogen), according to the manufacturer’s instructions. 25 μg of protein extract was electrophoresed through SDS-polyacrylamide gel (SDS-PAGE) under reducing conditions and transferred onto nitrocellulose membranes (Schleicher&Schuell). Blots were probed with a dual immuno-staining with appropriate primary antibody and horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology Inc. Danvers, MA USA). The immunoreactive bands were visualized by chemiluminescence (ECL plus; GE Healthcare Amersham Buckinghamshire, UK), according to the manufacturer’s instructions.

Immunohistochemistry

Tibialis anterior (TA) muscles from transgenic and littermate control mice were fixed for 2 hours in 4% formaldehyde and cryoprotected in phosphate buffer (PBS)-33% sucrose before storage at −20°C. Transversal sections (8 μm thick) were obtained by cryostate at −20°C (LEICA, CM1900UV) and placed onto polyvinyl-coated microscope slides (Menzel Gmbh&Co). Sections were permeabilized in PBS-0.2% NP-40 for 15 minutes and blocked in 5% goat serum. Slides were incubated with anti-utrophin monoclonal antibody (Novocasra, NCL-DRP2; 1:20 dilution) for 1 hour, washed thoroughly and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes; 1:500 dilution) for 40 minutes. Nuclei were detected by co-staining sections with Hoechst 33258 (1 μg/ml) (Sigma) for 1 minute. All incubation steps were carried out at room temperature. Stained specimens were examined by conventional epifluorescence microscopy (Olympus BX51; Tokyo, Japan). Images were captured using a digital camera at 40× magnification and merged using the IAS2000 software.

Unfixed transversal sections (8 μm thick) from TA of wt and transgenic mice were stained with the α-Bungarotoxin Alexa Fluor 488 conjugated (Molecular Probes), a specific ligand of Acetylcholine Receptor (ACHR), for 40 minutes at 37°C. Stained specimens were first examined by epifluorescence microscopy and then fixed in 4% formaldehyde to perform the utrophin staining. The Zenon Mouse IgG Labelling Kit (Molecular Probes) was used to directly conjugate the anti-utrophin monoclonal antibody with the Alexa Fluor 594, according to the manufacturer’s instructions.

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 RNA yield was quantified by a Qubit™ fluorometer using the Quant-iT assay kit (Invitrogen).

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

2 μg of total RNA was reverse transcribed using oligo (dT) 12-18 primers and SUPERSCRIPT II (Invitrogen) in a final 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). To obtain the utrophin gene expression rate the amount of
target gene was normalized to that of the two housekeeping genes β2-microglobulin (β2M) and β-glucuronidase (GUS) to correct for the differences in the quantity of the cDNA present in the PCR reactions. Primers and probes for the target gene (UTRN) and for housekeeping genes were purchased as TaqMan Gene Expression Assays (AB). The PCR mixtures containing the cDNA template, the TaqMan Universal PCR master mix (AB), and the primers/probes in a final volume of 25 μl were analyzed in triplicate, using the following conditions: incubation at 50°C for 2 minutes, denaturation at 95°C for 10 minutes and then 40 cycles of amplification at 95°C for 1 second and 60°C for 1 minute. For each gene amplification, a standard curve was generated using serial dilutions (200, 40, 8, 1.6 and 0.32 ng) of cDNA from mouse wild type (negative control). The results were analyzed using Applied Biosystems analysis software. The data are expressed as the ratio between UTRN and β2M or GUS mRNA expression. A minimal number of 9 mice were analyzed for each category.

**Chromatin Immunoprecipitation (ChIP) Assay**

For one ChIP assay reaction, 100 mg of skeletal muscle from transgenic and littermate control mice was chopped into small pieces and cross-linked in 1% formaldehyde at 37°C for 15 minutes. After wash with PBS, tissues were homogenized (7 mm, OMNI International GLH) in lysis buffer (1%SDS, 1% TritonX-100, 10 mM EDTA 50 mM Tris-Hcl pH 8.1) supplemented with a protease inhibitor cocktail (Complete™, Roche), and incubated for 60 minutes at 8°C. Samples were sonicated to generate DNA fragments with an average length of about 600 bp, diluted 10 fold in lysis buffer and centrifuged for 10 minutes at 13,000 rpm at 8°C. Supernatants were pre-cleared with Protein G-Agarose (Invitrogen) absorbed with sheared salmon sperm DNA diluted 10 fold in lysis buffer and centrifuged for 10 minutes at 20°C. A “no-antibody” immunoprecipitation was performed with Protein G-Agarose/sSS-DNA for a further 20 minutes. A “no-antibody” immunoprecipitation was performed as a negative control. All samples were diluted with 1% SDS, 0.1 M NaHCO3 and the cross-links were reverted by overnight incubation with 0.2 M NaCl at 65°C. Proteins were digested with Proteinase K (Roche) and DNA was purified by phenol/chloroform extraction. The final DNA was subjected to PCR in 25 μl of reaction buffer containing 0.8 mM dNTPs, 2.5 units of Taq DNA polymerase (Invitrogen) and 1 μM of the primers specific for the mouse utrophin or dystrophin promoter respectively, as designed as follows:

- m-utro forward: 5′-GCACGCGACGACTTGGTCCGGGAT-TC-3′, m-utro reverse: 5′-CTTTGTTCTCCGGGGAGACGAGTGC-3′.
- m-dystro forward: 5′-CTGTGGGACGCTTAAGGGTTGTTCGCCA-3′, m-dystro reverse: 5′-CATGTGCGCCCGAGTTTATTCGTCG-3′.

For utrophin promoter/enhancer amplification PCR reactions were carried out at 94°C for 45 seconds, 66°C for 30 seconds and 72°C for 30 seconds, for a total of 33 cycles. For dystrophin promoter/enhancer PCR reactions were carried out at 94°C for 45 seconds, 62°C for 30 seconds and 72°C for 30 seconds, for a total of 33 cycles.

**Mechanical response of isolated muscles**

Contractile activity of muscles from two month old Vp16-Jazz male mice and non-transgenic male littermates, was examined in vitro by physiological assessment of the muscle force of isolated diaphragm and extensor digitorum longus (EDL) preparations of both hind limbs. The muscle preparations were suspended in a 20 ml bath of oxygenated Krebs solution, maintained at 37°C, stretched to a tension of 1.0 g and allowed to equilibrate for 30–60 minutes, changing the superfusion buffer every 15–20 min. The diaphragm and EDL muscles were directly stimulated using single stimulations (Electric Stimulatore Digit 3T, Lace Elettronica, Pisa, Italy), carried out with rectilinear pulses of 0.5 msec duration at 0.05–0.2 Hz, using variable voltage, until the supramaximal voltage was reached. The mechanical activity of the muscle was recorded isotonically (7006 isometric transducer) by a strain-gauge transducer and displayed on a recording micro-dynamometer (Unitrecord 7050, Basile, Milano, Italy).

**Microarray**

Total RNA was extracted using Trizol (Invitrogen Corporation, Carlsbad, Ca) and further purified using the RNAeasy mini kit (Qiagen GmbH, Germany) according to the manufacturer’s instructions. RNA was quantified using NanoDrop ND-1000 and RNA quality was checked using the Agilent bioanalyzer 2100 (Agilent Technologies Santa Clara, CA). A total of 12 high density oligonucleotide mouse MOE430A arrays (Affymetrix Toronto, Canada) were used in this study. All the procedures and hybridization were performed according to the Genechip expression technical manual (Affymetrix) as previously reported [35]. Briefly, 5 μg of RNA was converted to double-stranded cDNA with Superscript II (Invitrogen) using a 17-mer (dT17) primer (Affymetrix), followed by in vitro transcription to generate biotin-labelled cRNA probes. Fragmented cRNA (15 μg) was used in 300 μl hybridization cocktail containing spiked controls (Affymetrix), 0.1 mg/ml Herring Sperm DNA (Promega Madison, WI, USA), and 0.5 mg/ml acetylated BSA (Invitrogen). A 200 μl aliquot of this hybridization cocktail was used on each chip which was incubated at 45°C for 16 hours rotating at 60 rpm. Following hybridization, the arrays were processed using a Genechip Fluidics Station 400 according to the recommended protocols (EukGEWSv2.4, Affymetrix) of double-staining and post-hybridizations washes. Fluorescent images were captured using gene Array Scanner 2500 (Affymetrix). All the experiments were designed and the information was compiled in compliance with MIAME [36] guide lines to ensure that the microarray data can be correctly interpreted and independently verified.

**Microarray data analysis**

Gene transcript levels were determined from data image files using algorithms in Gene Chip Operating Software (GCOS1.2, Affymetrix). Global scaling was performed to compare genes from chip to chip thus the average signal intensity of the arrays was set to the same target intensity (TGT = 100). The key assumption of the global scaling strategy is that there are few changes in gene expression among the arrays being analyzed. All the data quality controls were carried out and met the Affymetrix quality assessment guidelines. Data analysis was performed using Data Mining Tool (DMT 3.1, Affymetrix) and GeneSpring 7.2 (Silicon Genetics). Robust Multi-Array Average (RMA) expression measurement was also used with the help of probe sequence (GC content) information (GCRMA) [37] as implemented in BioConductor R statistics (www.biocductor.org). Cell Intensity files were processed into expression values for all the 22,620 probe sets (transcripts) on each of these arrays following the normalization step. Differentially expressed genes (DEG) were selected if they passed the Welch t-test, parametric test, with variance not assumed...
to be equal, p<0.05 and at least 2-fold changes between any two of the age matched genotypes used in this study.

DISCUSSION

Over the past few years, novel designed zinc fingers with modified functions led to a variety of applications [29,31]. ZF ATFs permit to alter expression profiles of genes related to disease, with the aim of treating pathologies like cancer and genetic diseases [38–40]. Our laboratory has been focused on creating several novel transcription factors based on modified Cys2-His2 zinc finger motifs fused to positive or negative regulatory effector domains [17–18,41–42]. In particular, we engineered ZF ATFs with the aim of up-regulating the utrophin gene, a possible substitute for dystrophin in DMD patients. Many research groups are approaching functional recovery of dystrophic muscle with different strategies [43–46]. One of the main problem emerged to perform any dystrophin gene therapy consists in the extremely large size of its mRNAs, that exceeds the incapsulation limits of many viral vectors. Recently, the last generation of gutted adenovirus appears to be able to package very large cDNAs including dystrophin [47]. In addition dystrophin minigenes, isolated from patients affected by Becker Muscular Dystrophy can partially circumvent the problem of dystrophin mRNA large size.

On the other hand, utrophin has the fundamental advantage of being a protein present in DMD patients and thus being well tolerated by the immune system of the host. Our strategy is to re-program the expression of endogenous utrophin by ZF ATFs. We previously demonstrated that the three zinc finger protein Jazz is capable of binding and activating transcription from the utrophin promoter “A” [17]. Then, in order to obtain utrophin up-regulation in an animal model system, we designed and engineered a transgenic construct, named Vp16-Jazz, containing the Jazz zinc finger sequence under the control of the muscle specific promoter of the myosin light chain. Here, we report that the transgenic mouse model expresses Vp16-Jazz exclusively in skeletal muscle, with the exception of a faint expression of the transgene in the cardiac muscle of family tg9.

Once the chromatin immunoprecipitation experiments demonstrated that Vp16-Jazz was able to bind efficiently and specifically in vivo the DNA target sequence at the endogenous utrophin promoter, Vp16-Jazz ability to re-program expression of utrophin was investigated. The increase of utrophin mRNA ranged from 3 to 4 fold in mice from different transgenic families as compared with their non-transgenic littersmates. The changes in the expression of utrophin mRNA were consistent with the increase in the utrophin protein level demonstrated by western blot analysis and by immunohistochemistry in the skeletal muscle of transgenic lines. Moreover, western blot analysis revealed, as expected, that the up-regulation of the endogenous promoter region mimics the body's natural regulatory mechanism, resulting in the production of several different utrophin isoforms in the transgenic lines analyzed so far. Interestingly, the tg9 that showed leaky expression of the transgene in cardiac muscle also displays an enhanced level of utrophin in the heart. This finding, not only confirms the efficiency of the artificial Vp16-Jazz in up-regulating utrophin expression, but also could become useful in future studies considering that utrophin deficiency worsens cardiac contractile dysfunction present in dystrophin-deficient mdx mice [40].

Moreover Vp16-Jazz specific expression has a promising positive effect on muscular strength as demonstrated by in vivo analysis of the mechanical response of isolated muscles. The different contractile responses to electric stimulation between diaphragm and EDL can be easily ascribed to the different nature and fiber composition of these two muscle [49–50].

Next, the wide range genome perturbation induced in vivo by Vp16-Jazz and by the consequent utrophin over-expression was tested by microarray analysis. The analysis of the gene expression profiles of the transgenic tg9 and tg1 mice versus control wt mice revealed an irrelevant global transcriptional effect. On the other hand, microarray analysis doesn’t allow to distinguish between the transcriptional effects due to Vp16-Jazz transgene and the effects on global transcription due to the overexpression of the target gene utrophin.

In conclusion, the main results of our work were: i) the demonstration that a synthetic transcription factor introduced in vivo is well tolerated and safe for the health of the transgenic animal both during development and in adult life; ii) Vp16-Jazz was able to recognize specifically its DNA target sequence on the utrophin promoter and to up-regulate utrophin in muscle fibers without significant non-specific global transcriptional effects; iii) Vp16-Jazz specific expression has a promising positive effect on muscular strength as demonstrated by in vivo analysis of the mechanical response of isolated muscles. Thus the transgenic mouse described here represents the first example of an animal that could be useful to validate the use of ZF ATF technology for human gene therapy.

In future work, these Vp16-Jazz transgenic mice will be cross-bred with mdx mice. The achievement of Vp16-Jazz and Vp16-Jazz/mdx mice represents an useful animal model for use in drug discovery and therapeutics.

ACKNOWLEDGMENTS

We thank Leila Tifaa for the excellent technical assistance and we thank Dr. Kosa G. Ruscitì for her precious assistance.

Author Contributions

Conceived and designed the experiments: CP KD. Performed the experiments: CP EM NC MD GS AO AD VL. SB DB. Analyzed the data: CP EM NC MD GS DB KD. Contributed reagents/materials/analysis tools: AF MF. Wrote the paper: CP EM NC MD.

REFERENCES

1. Voisin V, de la Porte S (2004) Therapeutic strategies for Duchenne and Becker muscular dystrophies. Int Rev Cytol 240: 1–30.
2. Miura P, Jasmin BJ (2006) Utrophin upregulation for treating Duchenne or Becker muscular dystrophy: how close are we? Trends Mol Med. 5: 122–9.
3. Nowak KJ, Davies KE (2004) Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. EMBO Rep 5(9): 872–6.
4. Blake DJ, Weir A, Newey SE, Davies KE (2002) Function and genetics of dystrophin and dystrophin-related proteins in muscle. Physiol Rev 82: 291–329.
5. Davies KE, Nowak KJ (2005) Molecular mechanisms of muscular dystrophies: old and new players. Nat Rev Mol Cell Biol 7(10): 762–73.
6. Ohlendieck K, Ervasti JM, Matsumura K, Kahl SD, Leveille CJ, Campbell K (1991) Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. Nature 351: 790–6.
7. Granovski AO, Karpas G, Jasmin BJ (1999) Discordant expression of utrophin and its transcript in human and mouse skeletal muscles. J. Neuropathol Exp Neurol 58: 235–44.
8. Finley JM, Potter AC, Phelps SR, Fisher R, Trickett JI, et al. (1996) Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature 384: 549–53.
9. Campbell KP, Croasbie RH (1996) Muscle-specific expression of dystrophin transgenes in mdx mice. Nature 384: 349–53.
10. Davies KE, Nowak KJ (2006) Utrophin upregulation for treating Duchenne or Becker muscular dystrophies: how close are we? Trends Mol Med 5: 122-9.
11. Blake DJ, Jasmin BJ (2006) Utrophin upregulation for treating Duchenne or Becker muscular dystrophy: how close are we? Trends Mol Med 5: 122-9.
12. Leveille CJ, Campbell K (1991) Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. Nature 351: 790-96.
13. Granovski AO, Jasmin BJ (1999) Discordant expression of utrophin and its transcript in human and mouse skeletal muscles. J. Neuropathol Exp Neurol 58: 235-44.
14. Finley JM, Potter AC, Phelps SR, Fisher R, Trickett JI, et al. (1996) Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature 384: 549-53.
15. Campbell KP, Croasbie RH (1996) Muscle-specific expression of dystrophin transgenes in mdx mice. Nature 384: 349-53.
Mice with an Artificial Gene

10. Rafael JA, Tinley JM, Potter AC, Deconinck AE, Davies KE (1998) Skeletal muscle-specific expression of a utrophin transgene rescues utrophin-dystrophin deficient mice. Nat Genet 19: 79–82.
11. Tinley J, Deconinck N, Fisher R, Kahn D, Phelps S, et al. (1998) Expression of full-length utrophin prevents muscular dystrophy in mdx mice. Nat Med 4: 1441–4.
12. Wakefield PM, Tinley JM, Wood MJ, Gilbert R, Karpati G, et al. (2000) Prevention of the dystrophic phenotype in dystrophin/utrophin-deficient muscle following adenovirus-mediated transfer of a diminin myogen. Gene Ther 7: 201–4.
13. Squire S, Raymakers JM, Vandebrule G, Potter A, Tinley J, et al. (2002) Prevention of pathology in mdx mice by expression of utrophin: analysis using an inducible transgenic expression system. Hum Mol Genet 11: 3333–44.
14. Khurana TS, Davies KE (2003) Pharmacological strategies for muscular dystrophy. Nat Rev. Drug Discov 2: 379–90.
15. Weir AF, Morgan JE, Davies KE (2004) A-utrophin up-regulation in mdx skeletal muscle is independent of regeneration. Neuro muscul Disord 14: 19–23.
16. Stockley MA, ChakkalakaI JV, Bradford A, Miura P, De Repentigny Y, et al. (2005). 1.3 kb promoter fragment confers spatial and temporal expression of utrophin A mRNA in mouse skeletal muscle fibers. Neuro muscul Disord 15(6): 437–49.
17. Corbi N, Libri V, Fanciulli M, Tinley JM, Davies KE, Passananti (2000) The artificial zinc finger coding gene ‘Jazz’ binds the utrophin promoter and activates transcription. Gene Ther 7: 1076–83.
18. Onori A, Desantis A, Buontempo S, Di Cerio MG, Fanciulli M, et al. (2007) The artificial four zinc finger protein Bagly binds human utrophin promoter A as at the endogenous chromosomal site and activates transcription. Biochim Biophys Acta 1775(3): 358–365.
19. Beltran A, Liu Y, Parikh S, Temple B, Blancafort P (2006) Interrogating genomes with combinatorial transcription factor libraries: asking zinc finger questions. Assay Drug Dev Technol 4: 317–31.
20. Lee DK, Seol W, Kim JS (2003) Custom DNA-binding proteins and artificial transcription factors. Curr Top Med Chem 3: 645–657.
21. Jamieson AC, Miller JC, Pabo CO (2005) Drug discovery with engineered zinc-finger proteins. Nat. Rev. Drug Discov 2: 361–368.
22. Choo Y, Klag A (1994) Toward a code for the interactions of zinc fingers with DNA: selection of randomized fingers displayed on phage. Proc Natl Acad Sci U S A 91: 11163–7.
23. Choo Y, Klag A (1994) Selection of DNA binding sites for zinc fingers using rationally randomized DNA reveals coded interactions. Proc Natl Acad Sci U S A 91: 11168–72.
24. Choo Y, Klag A (1997) Physical basis of a protein-DNA recognition code. Curr Opin Struct Biol 7: 117–25.
25. Segal DJ, Dreier B, Beertli R, Barbas CF III (1999) Toward controlling gene expression at will: selection and design of zinc finger domains recognizing each of the 5'-GNN-3' DNA target sequences. Proc Natl Acad Sci U S A 96: 2758–63.
26. Dreier B, Segal DJ, Barbas CF III (2000) Insights into the molecular recognition of the 5'-GNN-3' family of DNA sequences by zinc finger domains. J Mol Biol 303: 498–502.
27. Dreier B, Beertli RR, Segal DJ, Flippin JD, Barbas CF III (2001) Development of zinc finger domains for recognition of the 5'-ANN-3' family of DNA sequences and their use in the construction of artificial transcription factors. J Biol Chem 276: 29466–78.
28. Pabo CO, Prisacar E, Grant R (2001) Design and selection of novel Cys2His2 zinc finger proteins. Annu Rev Biochem 70: 313–40.
29. Blancafort P, Segal DJ, Barbas CF III (2004) Designing transcription factor architectures for drug discovery. Mol Pharmacol 66: 1361–71.
30. Dreier B, Fuller RP, Segal DJ, Lund CV, Blancafort P, et al. (2005) Development of zinc finger domains for recognition of the 5'-CGNN-3' family DNA sequences and their use in the construction of artificial transcription factors. J Biol Chem 280: 35588–97.
31. Corbi N, Libri V, Onori A, Passananti C (2004) Synthetic zinc finger peptides: old and novel applications. Biochim Biophys Acta 1682: 428–36.
32. Dennis CL, Tinley JM, Deconinck AE, Davies KE (1996) Molecular and functional analysis of the utrophin promoter. Nucleic Acids Res 24: 1646–52.
33. Musaró A, McCallagh K, Paul A, Houghton L, Dobrovolsky G, et al. (2001) Localized Igf-1 transcription expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 27 (2): 195–200.
34. Nagy A, et al. (2003) “Manipulating the Mouse Embryo”-Third edition-Cold Spring Harbor Laboratory Press.
35. O’Rourke D, Bahan D, Demidova M, Mott R, Hodgkin J (2006) Genomic clusters, putative recognition molecules, and antimicrobial genes are induced by infection of C. elegans with M. nematophilum. Genome research 16 (8): 1005–16.
36. Buzza A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, et al. (2001) Minimum information about a microarray experiment (MIAME)-toward standards for microarray data. Nature genetics 29 (4): 365–71.
37. Belotai BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19 (2): 183–93.
38. Pandolfi PP (2003) Transcription therapy for cancer. Oncogene 22: 3116–3127.
39. Blancafort P, Chen EI, Gonzalez B, Bergquist S, Zijlstra A, et al. (2005) Genetic reprogramming of tumor cells by zinc finger transcription factors. Proc Natl Acad Sci U S A 102: 11176–21.
40. Papworth M, Koliasinska P, Minczuk M (2006) Designer zinc-finger proteins and their applications. Gene 366: 27–38.
41. Corbi N, Perez M, Maione R, Passananti C (1997) Synthesis of a new zinc finger peptide; comparison of its ‘code’ deduced and ‘CASTing’ derived binding sites. FEBS Lett 417: 71–4.
42. Libri V, Onori A, Fanciulli M, Passananti C, Corbi N (2004) The artificial zinc finger protein Blues binds the enhancer of the fibroblast growth factor FGF-4 and represses transcription. FEBS Lett 560: 75–80.
43. Cossu G, Sampaolesi M (2004) New therapies for muscular dystrophy: cautious optimism. Trends Mol Med 10: 516–20.
44. Sampaolesi M, Blot S, D’Antona G, Granger N, Tonnlerozi R, et al. (2006) Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs. Nature 444 (7119): 574–9.
45. Minetti GC, Colusii C, Adami R, Serra C, Mozzetta C, et al. (2006) Functional and morphological recovery of dystrophic muscles in mice treated with deacetylase inhibitors. Nat Med 1210: 1147–50.
46. Odorn GL, Gregorevic P, Chamberlain J S (2007) Vira-mediated gene therapy for the muscular dystrophies: successes, limitations and recent advances. Biochem Biophys Acta 1772(2): 243–62.
47. Matecki S, Dudley KW, Dvanganlil G, Gilbert R, Nalhantoglu J, et al. (2004) Therapeutic gene transfer to dystrophic diaphragm by an adenoviral vector deleted of all viral genes. Am J Physiol Lung Cell Mol Physiol. 287(3): L569–76.
48. Janssen PM, Hiranandani N, Mays TA, Rafael-Fortney JA (2005) Urophin deficiency worsens cardiac contractile dysfunction present in dystrophin-deficient mdx mice. Am J Physiol Heart Circ Physiol 289(6): H2373–8.
49. Gramolini AO, Belanger G, Thompson JM, Chakkalakal JV, Jasmin BJ (2001) Increased expression of utrophin in a slow vs. a fast muscle involves posttranscriptional events. Am J Physiol Cell Physiol. 281(4): C1300–9.
50. Petros BJ, Shragger JB, Stedman HH, Kelly AM, Sweeney HL (1994) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. Proc Natl Acad Sci U S A 15: 90 (8): 3710–4.