Neurology India

Free full text at
www.neurologyindia.com and www.bioline.org.br/ni

March, 2008 | CONTENTS | Vol. 56 Issue 1

Editorial

View and Review

Original Articles

Neurology India

Free full text at
www.neurologyindia.com and www.bioline.org.br/ni

March, 2008 | CONTENTS | Vol. 56 Issue 1

Case Reports

Letters to Editor

Neurology India

Free full text at
www.neurologyindia.com and www.bioline.org.br/ni

March, 2008 | CONTENTS | Vol. 56 Issue 1

Original Article

Expression of truncated dystrophin cDNAs mediated by a lentiviral vector

Sun Shunchang, Chen Haitao, Chen Weidong[1], He Jingbo, Peng Yunsheng

Department of Medical Laboratory Science, Shenzhen Baoan Hospital, Southern Medical University, Shenzhen, Guangdong, China,
[1]Department of Clinical Laboratory, Shenzhen Hospital, Peking University, Shenzhen, Guangdong, China

Background: The success of Duchenne muscular dystrophy gene therapy requires promising tools for gene delivery and mini-gene cassettes that can express therapeutic levels of a functional protein. **Aims:** To explore the expression feasibility of truncated dystrophin cDNAs mediated by a lentiviral vector derived from feline immunodeficiency virus. **Materials and Methods:** Three truncated dystrophin cDNAs were constructed by PCR cloning, then these cDNAs were inserted into lentiviral vectors. Recombinant lentiviruses were generated by transient transfection of lentiviral vector constructs into 293Ad^{5+} cells. Cultured myoblasts were then infected with recombinant lentiviruses. Expression of truncated dystrophin cDNAs was detected by Western blot analysis. **Results:** Mediated by lentiviral vectors, three cDNAs constructed by PCR cloning expressed relative truncated dystrophins in cultured myoblasts. **Conclusions:** Truncated dystrophin cDNAs can express themselves successfully mediated by feline immunodeficiency virus vectors. It offers the possibility of an approach utilizing truncated dystrophin cDNAs and lentiviral vectors toward gene therapy of Duchenne muscular dystrophy.

Key words: Duchenne muscular dystrophy, dystrophin, gene transfer, lentivirus, vector

Duchenne muscular dystrophy (DMD) is a severe life-threatening X-linked disorder which affects one of every 3,500 males born and is characterized by a progressive muscle degeneration and weakness.[1] The symptoms of the disease usually occur before three years of age, the patients are wheelchair bound by their early teens and death is normally in their early twenties. Duchenne muscular dystrophy is caused by an absence of dystrophin that has a critical role in both force transduction out of myofibers and in sarcolemmal membrane stabilization during muscle contraction.[2] Because of the lack of effective treatment for DMD, gene therapy has been actively explored. However, the dystrophin gene with a cDNA of 14 kb is too large to be packaged for common viral vectors. The selection of a safe and efficient vector is also important for gene therapy of DMD. Wild-type feline immunodeficiency virus (FIV) is a lentivirus that causes an immunodeficiency disease in cats, but despite prevalent exposure, does not result in infection or disease in humans.[3] Feline immunodeficiency virus vectors were constructed from FIV; preparation of FIV vectors has been described previously.[4] Based on these observations, we designed three truncated dystrophin cDNAs as therapeutic genes, delivered them into myoblasts by FIV vectors and explored the expression feasibility of truncated dystrophin cDNAs mediated by lentiviral vectors.

Materials and Methods

Construction of truncated dystrophin cDNAs

We constructed three truncated dystrophin cDNAs by the PCR cloning method using Pfu polymerase (Promega) and human dystrophin cDNA (GenBank NM 004006) as the template. The sequences of the primers and other oligonucleotides used in the construction of the cDNAs are shown in Table 1. As depicted in Figure 1 mini-dystrophin △4047 contains nucleotides 1-1668 (N terminus, hinge 1 and rods 1 and 2), 8059-10227 (rods 22, 23 and 24, hinge 4 and CR domain) and 10849-11058 (6-heptad repeat and the last 12 amino acids of dystrophin). Similarly, mini-dystrophin △4188 contains nucleotides 1-1668, 7270-7410 (hinge 3), 8059-10227 and 10849-11058. Finally, mini-dystrophin △4371 contains nucleotides 1-1992 (N terminus, hinge 1 and rods 1, 2 and 3), 8059-10227 and 10849-11058.[5]

Mini-dystrophin △4047 was generated as follows. Three PCRs were independently performed using human dystrophin cDNA as the template and primers F1/R1a, F2a/R2 or F4/R4. Subsequently, a mixture of the last two resulting PCR products was used as the template for the second round PCR with primers F2a /R4. Finally, a mixture of two resulting PCR products obtained from primers F1/R1a and F2a/R4 was used as the template for

Chen Haitao
Shenzhen Baoan Hospital, Southern Medical University, 118 Longjing Er Road, Baoan, Shenzhen, Guangdong, 518101, China. E-mail: bayyyz@yahoo.com.cn

Table 1: DNA sequences of synthetic oligonucleotides used for construction of truncated dystrophin cDNAs

Primer	Primer sequences (5'→3')	Sequence position
F1	ccggaattcatgctttggtgggaagaagtag	1~22
R1a	gtaatctatgagtgtcttgtaaaagaacccagcggtc	8071~8059, 1668~1645
R1b	ctaggtcaggctggtcttgtaaaagaacccagcggtc	7282~7270, 1668~1645
R1c	gtaatctatgagtctgtgctgtactcttttcaagttt	8071~8059, 1992~1969
F2a	tcttttacaagacactcatagattactgcaacagttc	1656~1668, 8059~8082
F2b	gttggaggtacctactcatagattactgcaacagttc	7398~7410, 8059~8082
F2c	gagtacagcacagactcatagattactgcaacagttc	1980~1992, 8059~8082
R2	acctctgtagagagggagtttccatgttgtc	10861~10849, 10227~10210
F3	tcttttacaagaccagcctgacctagctcctg	1656~1668, 7270~7288
R3	gtaatctatgagtaggtacctccaacatcaaggaag	8071~8059, 7410~7388
F4	catggaaactccctctctacagaggtccgacagc	10215~10227, 10849~10869
R4	atagtttagcggccgcctacattgtgtcctctctcattg	11058~11036

Underlines correspond to the recognition sequence of *Eco*RI and *Not*I

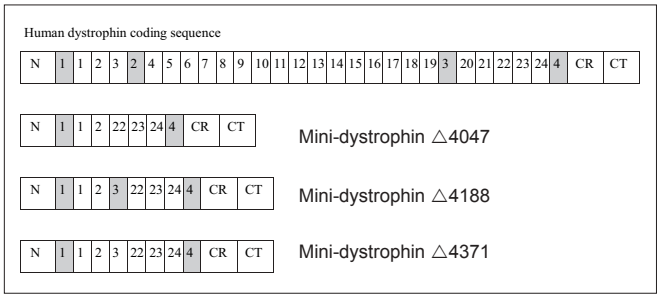

Figure 1: Diagrammatic representation of the coding regions of full-length human dystrophin and mini-dystrophin constructs
Dystrophin has four major domains: the N-terminal domain (N), the central rod domain, the cysteine-rich domain (CR) and the C-terminal domain (CT). The central rod domain contains 24 rod repeats and 4 hinges (grey boxes).

the third round PCR with primers F1/R4.

Mini-dystrophin △4188 was generated as follows. Four PCRs were independently performed using human dystrophin cDNA as the template and primers F1/R1c, F3/R3, F2c/R2 or F4/R4. Subsequently, a mixture of the first two resulting PCR products was used as the template for the second round PCR with primers F1/R3, a mixture of the last two resulting PCR products was used as the template for the other second round PCR with primers F2c/R4. Finally, a mixture of two resulting PCR products amplified from primers F1/R3 and F2c/R4 was used as the template for the third round PCR with primers F1/R4.

Mini-dystrophin △4371 was generated as follows. Three PCRs were independently performed using human dystrophin cDNA as the template and primers F1/R1b, F2b/R2 or F4/R4. Subsequently, a mixture of the last two resulting PCR products was used as the template for the second round PCR with primers F2b/R4. Finally, a mixture of two resulting PCR products obtained from primers F1/R1b and F2b/R4 was used as the template for the third round PCR with primers F1/R4.

The above constructs were made by PCR cloning in three steps using human dystrophin cDNA, which incorporates deletions of coding region. Therefore, three protein coding sequences are precisely spliced together in-frame. Mini-dystrophin △4047, mini-dystrophin △4188 and mini-dystrophin △4371 fragments were digested using flanking 5' *Eco*RI and 3' *Not*I sites. Fragments digested then were cloned into a FIV transfer vector plasmid digested with *Eco*RI and *Not*I, respectively, to generate vector constructs FIV-△4047, FIV-△4188 and FIV-△4371.

Cell culture

Human embryonic kidney 293 cells, human embryonic lung fibroblast cells and C2C12 cells were obtained from the China Center for Type Culture Collection. They were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum, 2 mM of glutamine (Invitrogen).

Transfection and recombinant feline immunodeficiency virus production

293 cells were plated at the density of 6×10^5 cells in six-well plates. The next day, they were transfected with packaging plasmid (pCPR△Env): transfer vector: vesicular stomatitis virus envelope plasmid (pCI-VSVG) at a 1:2:1 rate by PolyFect Transfection Reagent (QIAGEN) according to the manufacturer's recommendations. Medium was only changed at 30 h posttransfection. At 42-44 h posttransfection, plates were moved to 32°C. At 48 h posttransfection, viruses were collected and cleared via centrifugation at 1500 rpm for 5 min and by filtration through a low-protein-binding 45-μm filter.[6] For titer experiments, 100 μl of viral supernatant was used in a total volume of 1 ml to infect 1.5×10^5 human embryonic lung fibroblast cells. The viral titers of the recombinant FIV particles were determined by the cytopathic effects. Titers were calculated in viral supernatant by the Reed-Muench method.[7]

Infection

Four to five hundred microliters of recombinant FIV was used to infect 0.5×10^6 C2C12 cells. C2C12 cells were infected with recombinant FIVs at a multiplicity of infection (MOI) of 100 p.f.u./cell. All infections were performed in six-well plates in the presence of 5 μg/ml Polybrene (Sigma), which were centrifuged for 90 min

at 2500 rpm immediately following addition of viruses. Plates were then incubated for 24 h at 32°C prior to having their media changed to fresh, virus-free media. Cells were harvested after three days and were washed three times with PBS to remove residual FIV.[4]

Western blotting

Lysates from C2C12 cells were obtained in lysis buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 14 mM β-mercaptoethanol, 10 mM NaF, 1 mM sodium orthovanadate, 0.2 mM PMSF, 10 μg/ml Leupeptin and 1% Triton).[8] 20 μg proteins were separated on a 4-10% SDS polyacrylamide gradient gel and transferred onto nitrocellulose Hybond membrane (Amersham Bioscience). Protein transfer was confirmed by Coomassie blue R250 stain. Membranes were blocked in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat milk powder and primary and secondary antibodies were diluted in the same solution. Blots were probed with NCL-DYS2 (1:50 dilution, Novocastra Laboratories), a monoclonal antibody recognizing an epitope in the C terminus of dystrophin. Anti-mouse secondary antibody (1:1000 dilution, Jackson Laboratories) linked to peroxidase was used for ECL immunodetection (Amersham Bioscience) according to the manufacturer's recommendations. Visualization of specific bands was obtained by exposure of blots to film.

Results

Construction of mini-dystrophin genes

To explore the feasibility of using FIV vectors for DMD gene therapy, we have designed three mini-dystrophin genes that were based on human dystrophin cDNA. These mini-dystrophin genes are small enough to be packaged into FIV vectors and yet retain the essential functions that should protect muscle from the pathological symptoms. We confirmed the sizes of the three mini-dystrophin genes by gel electropheresis (data not shown).

Production of recombinant FIV

Three FIV vector constructs are shown in Figure 2. A three-plasmid expression system was designed for the production of pseudotyped FIV particles by transient cotransfection. The system consists of a FIV packaging construct, a FIV vector construct and a plasmid encoding the surface glycoprotein of VSV-G.[9] The viral titers of the recombinant FIV particles were approximately 1.1×10^8 to 1.6×10^8 viral particles per ml obtained in human embryonic lung fibroblast cells transduced with 293 cell supernatants.

Expression of truncated dystrophin cDNAs

Western blot analysis showed that three truncated

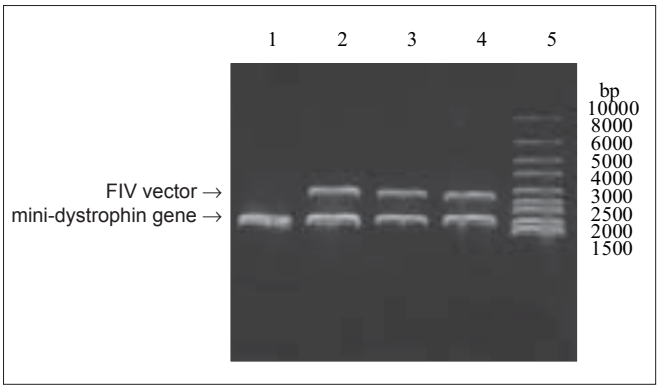

Figure 2: The FIV vectors were digested with *Eco*RI plus *Not*I. Lane 1: transfer vector digested with *Eco*RI plus *Not*I, lane 2: FIV-△4047 vector digested with *Eco*RI plus *Not*I, lane 3: FIV-△4188 vector digested with *Eco*RI plus *NotI*, lane 4: FIV-△4371 vector digested with *EcoRI* plus *Not*I, and lane 5: MassRuler DNA ladder

dystrophin cDNAs constructed by PCR cloning expressed relative truncated dystrophins in cultured myoblasts. The expression of mini-dystrophin △4047 and mini-dystrophin △4188 was considerably higher than that of mini-dystrophin △4371 mediated by FIV vectors, though myoblasts were infected with the same amount of recombinant FIV particles [Figure 3].

Discussion

The native dystrophin gene measures 2.4Mb, while the full-length dystrophin cDNA is about 14 kb.[10] The product of dystrophin gene is a 427 kDa cytoskeletal protein that is situated at the inner surface of the sarcolemma.[11] The dystrophin protein has four major structural domains: N-terminal, central rod, cysteine-rich and C-terminal domains. The central rod domain contains 24 triple-helix rod repeats and four hinges, which account for 80% of the total dystrophin length.[12]

There is currently no effective treatment for DMD, although gene therapy could be an attractive approach to the disease. The tremendous size of the dystrophin gene and mRNA are formidable obstacles to the development of gene therapy. Based on these observations, we designed three truncated dystrophin cDNAs with consecutive deletions in the rod domain and examined their expression effects in cultured myoblasts mediated by lentiviral vectors.

Figure 3: Western blot of truncated dystrophins in cultured myoblasts. Lane 1: myoblasts infected with recombinant FIV (FIV-△4047), lane 2: myoblasts infected with recombinant FIV (FIV-△4188), lane 3: myoblasts infected with recombinant FIV (FIV-△4371), and lane 4: myoblasts

The previous study using an adenovirus vector-mediated gene transfer indicated that mini-dystrophins with N-terminal actin-binding and C-terminal domains were stably expressed at the sarcolemma. These mini-dystrophins with at least four rod repeats and two hinges can ameliorate the dystrophic phenotypes.[13] We constructed three mini-dystrophin genes, their length are 4047 bp, 4188 bp and 4371 bp, respectively. Mini-dystrophin △4047 construct has five rod repeats and two hinges. Mini-dystrophin △4188 construct has six rod repeats and two hinges. Mini-dystrophin △4371 construct has five rod repeats and three hinges. Three mini-dystrophin constructs expressed successfully truncated dystrophins in myoblasts mediated by FIV vectors, although mini-dystrophin △4371 construct was less strongly expressed than the other two constructs. The differential expression of mini-dystrophin constructs may be affected by their sizes. The low expression of mini-dystrophin △4371 construct could be caused by its larger size. All three mini-dystrophin constructs can be candidate therapy genes for DMD.

Gene transfer remains one of the main challenges for gene therapy. Viral vectors could be useful to deliver genes into tissue because of a high efficiency and long-term gene expression. Both human immunodeficiency virus (HIV) and adeno-associated virus (AAV) vectors have been used for mini-dystrophin gene delivery to the muscle cells due to their ability to integrate into the host genome, and to infect nondividing cells. *In vivo* targeting of muscle progenitor cells with a pseudotyped HIV vector encoding the mini-dystrophin, restored dystrophin expression and provided functional correction in skeletal muscle of *mdx* mice.[14] There have been no direct reports on insertional mutagenesis by lentiviral vectors, however, the employment of HIV-based vectors in clinical trials is controversial mainly due to the lethal nature of the virus.[15] An AAV has never been associated with any human disease and has the combined advantages of high-efficiency gene transfer, persistent transgene expression and low immunogenicity.[16] The adenovirus has been the vector of choice to deliver dystrophin constructs to the muscle of *mdx* mice, the mouse model of DMD, but the expression of dystrophin was transient. Adenovirus is not integrated into the host cell genome and it is highly immunogenic, both contribute to the transient expression.[17] Nonprimate lentiviral vectors based on FIV and equine infectious anemia virus (EIAV) hold great promise as gene delivery vehicles for the treatment of a wide variety of diseases. Like HIV, FIV and EIAV can integrate into the genome of non-dividing cells, resulting in long-term transgene expression. Currently, the EIAV vector is one of the most attractive gene delivery systems with respect to neuronal tropism.[18] The packaging size of FIV vectors is smaller than 8.0 kb. Considering the potential safety, FIV provides a potential tool for human gene transfer purposes.[19]

In summary, our data demonstrate the effectiveness of FIV vectors for ex gene transfer of mini-dystrophin constructs to myoblasts. Our findings indicate that FIV vectors may be useful gene delivery vehicles for the gene therapy of DMD. Our next goal is to transfer these mini-dystrophin constructs by FIV vectors into skeletal muscles of *mdx* mice to test its therapeutic effects.

Acknowledgment

We thank G.P. Nolan (Stanford University Medical Center) for the FIV vector plasmid and pCPR△Env packaging plasmid, as well as the pCI-VSVG envelope plasmid.

References

1. Bushby KM, Hill A, Steele JG. Failure of early diagnosis in symptomatic Duchenne muscular dystrophy. Lancet 1999;353:557-8.
2. Brooke MH, Fenichel GM, Griggs RC, Mendell JR, Moxley R, Florence J, *et al*. Duchenne muscular dystrophy: Patterns of clinical progression and effects of supportive therapy. Neurology 1989;39:475-81.
3. Lin YL, Noel D, Mettling C, Reant B, Clot J, Jorgensen C, *et al*. Feline immunodeficiency virus vectors for efficient transduction of primary human synoviocytes: Application to an original model of rheumatoid arthritis. Hum Gene Ther 2004;15:588-96.
4. Curran MA, Kaiser SM, Achacoso PL, Nolan GP. Efficient transduction of nondividing cells by optimized feline immunodeficiency virus vectors. Mol Ther 2000;1:31-8.
5. Li S, Kimura E, Fall BM, Reyes M, Angello JC, Welikson R, *et al*. Stable transduction of myogenic cells with lentiviral vectors expressing a mini-dystrophin. Gene Ther 2005;12:1099-108.
6. Brooks AI, Stein CS, Hughes SM, Heth J, McCray PM Jr, Sauter SL, *et al*. Functional correction of established central nervous system deficits in an animal model of lysosomal storage disease with feline immunodeficiency virus-based vectors. Proc Natl Acad Sci USA 2002;99:6216-21.
7. Lee MS, Cohen B, Hand J, Nokes DJ. A simplified and standardized neutralization enzyme immunoassay for the quantification of measles neutralizing antibody. J Virol Methods 1999;78:209-17.
8. Roberts ML, Wells DJ, Graham IR, Fabb SA, Hill VJ, Duisit G, *et al*. Stable micro-dystrophin gene transfer using an integrating adeno-re-roviral hybrid vector ameliorates the dystrophic pathology in mdx mouse muscle. Hum Mol Genet 2002;11:1719-30.
9. Johnston JC, Gasmi M, Lim LE, Elder JH, Yee JK, Jolly DJ, *et al*. Minimum requirements for efficient transduction of dividing and nondividing cells by feline immunodeficiency virus vectors. J Virol 1999;73:4991-5000.
10. Koenig M, Hoffman EP, Bertelson CJ, Monaco AP, Feener C, Kunkel LM. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in the normal and affected individuals. Cell 1987;50:509-17.
11. Koenig M, Monaco AP, Kunkel LM. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. Cell 1988;53:219-28.
12. Hoffman EP, Brown RH, Kunkel LM. Dystrophin: The protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919-28.
13. Sakamoto M, Yuasa K, Yoshimura, Yokota T, Ikemoto T, Suzuki M, *et al*. Micro-dystrophin cDNA ameliorates dystrophic phenotypes when introduced into mdx mice as a transgene. Biochem Biophys Res Commun 2002;293:1265-72.
14. Kobinger GP, Louboutin JP, Barton ER, Sweeney HL, Wilson JM. Correction of the dystrophic phenotype by *in vivo* targeting of muscle progenitor cells. Hum Gene Ther 2003;14:1441-9.
15. Tsui LV, Kelly M, Zayek N, Rojas V, Ho K, Ge Y, *et al*. Production of

human clotting Factor IX without toxicity in mice after vascular delivery of a lentiviral vector. Nat Biotechnol 2002;20:53-7.
16. Ghosh A, Yue Y, Long C, Bostick B, Duan D. Efficient whole-body transduction with trans-splicing adeno-associated viral vectors. Mol Ther 2007;15:750-5.
17. Gilbert R, Liu A, Petrof B, Nalbantoglu J, Karpati G. Improved performance of a fully gutted adenovirus vector containing two full-length dystrophin cDNAs regulated by a strong promoter. Mol Ther 2002;6:501-9.
18. Mitrophanous K, Yoon S, Rohll J, Patil D, Wilkes F, Kim V, *et al*. Stable gene transfer to the nervous system using a non-primate lentiviral vector. Gene Ther 1999;6:1808-18.
19. Shai E, Palmon A, Panet A, Marmary Y, Sherman Y, Curran MA, *et al*. Prolonged transgene expression in murine salivary glands following non-primate lentiviral vector transduction. Mol Ther 2005;12:137-43.

Accepted on 16-06-2007
Source of Support: This study was supported by research grants from Shenzhen Municipal Bureau of Science and Technology,
Conflict of Interest: None declared.