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March, 2008 C	ONTENTS	Vol. 56 Iss	ue 1
Editorial			
'Aqualisation' of neuraxis: Wondrous neu	raqua CSE 1		
Manu Kothari, Atul Goel			1
View and Review			
Organization of neurology services in Ind	ia: Unmet needs and the way forward		
Mandaville Gourie-Devi			4
Original Articles			
Endoscopic management of brain absces	SSES		
Yad Ram Yadav, Mallika Sinha, Neha, Vijay	' Parihar		13
Pattern of cerebellar perfusion on single A clinical and computed tomography cor	photon emission computed tomography in sub relation	ocortical hemato	oma:
Jayantee Kalita, Usha K. Misra, Prasen Ra	njan, P. K. Pradhan		17
Imaging features in Hirayama disease			
Hemant A. Sonwalkar, Rakesh S. Shah, Fir Sukalyan Purkayastha	osh K. Khan, Arun K. Gupta, Narendra K. Bodhey, Sur	rjith Vottath,	22
Delayed habituation in Behcet's disease			
Sefa Gulturk, Melih Akyol, Hulusi Kececi, S	edat Ozcelik, Ziynet Cınar, Ayse Demirkazık		27
Erythrocyte indicators of oxidative chang	ges in patients with graded traumatic head inju	ry	
Chandrika D. Nayak, Dinesh M. Nayak, Ani	naswamy Raja, Anjali Rao		31
Repeat gamma knife radiosurgery for rec	current or refractory trigeminal neuralgia		
Liang Wang, Zhen-wei Zhao, Huai-zhou Qi	n, Wen-tao Li, Hua Zhang, Jian-hai Zong,		
Jian-Ping Deng, Guo-dong Gao			36
Taste dysfunction in vestibular schwanne	omas		
Rabi Narayan Sahu, Sanjay Behari, Vimal I	K. Agarwal, Pramod J. Giri, Vijendra K. Jain		42
Surgical management of traumatic intrac	pranial pseudoaneurysms: A report of 12 cases		
Xiang Wang, Jin-Xiu Chen, Chao You, Min	He		47
Expression of truncated dystrophin cDNA	As mediated by a lentiviral vector		
Sun Shunchang, Chen Haitao, Chen Weido	ong, He Jingbo, Peng Yunsheng		52
Gamma knife radiosurgery for glomus iu	gulare tumors: Therapeutic advantages of mini	malism in the sk	ull base
Manish S. Sharma, A. Gupta, S. S. Kale, D	. Agrawal, A. K. Mahapatra and B. S. Sharma		57

Neurology India

March, 2008

CONTENTS

Vol. 56 Issue 1

Case Reports

Subarachnoid hemosiderin deposition after subarachnoid hemorrhage on T2*-wei with the location of disturbed cerebrospinal fluid flow on computed tomography c	ghted MRI correlates isternography	
Yoshifumi Horita, Toshio Imaizumi, Yuji Hashimoto, Jun Niwa		62
Anesthesia management of awake craniotomy performed under asleep-awake-asl laryngeal mask airway: Report of two cases	eep technique using	
Gadhinglajkar Shrinivas Vitthal, Rupa Sreedhar, Mathew Abraham		65
High cervical C3-4 'disc' compression associated with basilar invagination		
Atul Goel		68
Short-lasting unilateral neuralgiform headache with conjunctival injection and tea to antiepileptic dual therapy	ring: Response	
Ravi Gupta, Manjeet S. Bhatia		71
Correlation of autism with temporal tubers in tuberous sclerosis complex		
Kavitha Kothur, Munni Ray, Prahbhjot Malhi		74
Non-traumatic carotid dissection and stroke associated with anti-phospholipid an Report of a case and review of the literature	tibody syndrome:	
Benzi M. Kluger, Richard L. Hughes, C. Alan Anderson, Kathryn L. Hassell		77
Osteoma of anterior cranial fossa complicated by intracranial mucocele with emplits radiological diagnosis	hasis on	
Jinhu Ye, Hui Sun, Xin Li, Jianping Dai		79
Vasospasm after transsphenoidal pituitary surgery: A case report and review of th	e literature	
Manish Kumar Kasliwal, Ravinder Srivastava, Sumit Sinha, Shashank S. Kale, Bhawani S.	Sharma	81
Chondromyxoid fibroma of the seventh cervical vertebra		
Ashish Jonathan, Vedantam Rajshekhar, Geeta Chacko		84
Acute progressive midbrain hemorrhage after topical ocular cyclopentolate admin	istration	
Tarkan Calisaneller, Ozgur Ozdemir, Erkin Sonmez, Nur Altinors		88
Letters to Editor		

Digital subtraction angiography laboratory with inbuilt CT (DynaCT): Application during intracranial anurysm embolization 90 Concomitant tuberculous and pyogenic cerbellar abscess in a patient with pulmonary tuberculosis 91 Drug complianceafter stroke andmyocardial infarction: Is complementary medicine an issue? 93

Neurology India

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

March, 2008 CONTENTS Vol. 56 Issue 1

	Multiple intracranial developmental venous anomalies associated with complex orbitofac vascular malformation	ial	93	
	Nitrofurantoin-induced peripheral neuropathy:A lesson to be re-learnt		94	
	Posterior longitudinal ligament cyst as a rare cause of lumbosacral radiculopathy with po leg raising test	sitive straight	96	
	Aqueductal stenosis caused by an atypical course of a deep collector vein draining bilater developmental venous anomalies	ral cerebellar	97	
	Recovery of increased signal intensity of the cervical cord on magnetic resonance imagin for spontaneous spinal epidural hematoma causing hemiparesis	g after surgery	98	
	Simultaneous thalamic and cerebellar hypertensive hemorrhages		100	
Neuroimages				
	MRI and MRA in spontaneous intracranial arterial dissection			

Referees List - 2007	000???	
Instructions to Authors	106	
Forthcoming Events	105	
Susceptibility weighted imaging in holohemispheric venous angioma with cerebral hemiatrophy Sivaraman Somasundaram, Chandrasekharan Kesavadas, Bejoy Thomas	104	
Shunt catheter migration into pulmonary arteries Miikka Korja, Matti K. Karvonen, Arto Haapanen, Reijo J. Marttila	103	
MRI and MRA in spontaneous intracranial arterial dissection S. Raghavendra, Sanjeev V. Thomas, Krishnamoorthy Thamburaj, Bejoy Thomas	102	

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Expression of truncated dystrophin cDNAs mediated by a lentiviral vector

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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 293Ad5+ 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 $\triangle 4047$ 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 $\triangle 4188$ contains nucleotides 1-1668, 7270-7410 (hinge 3), 8059-10227 and 10849-11058. Finally, mini-dystrophin $\triangle 4371$ contains nucleotides 1-1992 (N terminus, hinge 1 and rods 1, 2 and 3), 8059-10227 and 10849-11058.^[5]

Mini-dystrophin $\triangle 4047$ 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

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Table 1: DNA sequences of synthetic oligonucleotides used for construction of truncated dystrophin cDNAs			
Primer sequences (5'→3')	Sequence position		
ccggaattcatgctttggtgggaagaagtag	1~22		
gtaatctatgagtgtcttgtaaaagaacccagcggtc	8071~8059, 1668~1645		
ctaggtcaggctggtcttgtaaaagaacccagcggtc	7282~7270, 1668~1645		
gtaatctatgagtctgtgctgtactcttttcaagttt	8071~8059, 1992~1969		
tcttttacaagacactcatagattactgcaacagttc	1656~1668, 8059~8082		
gttggaggtacctactcatagattactgcaacagttc	7398~7410, 8059~8082		
gagtacagcacagactcatagattactgcaacagttc	1980~1992, 8059~8082		
acctctgtagagagggagtttccatgttgtc	10861~10849, 10227~10210		
tcttttacaagaccagcctgacctagctcctg	1656~1668, 7270~7288		
gtaatctatgagtaggtacctccaacatcaaggaag	8071~8059, 7410~7388		
catggaaactccctctctacagaggtccgacagc	10215~10227, 10849~10869		
atagtttagcggccgcctacattgtgtcctctctcattg	11058~11036		
	Table 1: DNA sequences of synthetic oligonucleotides used for construction of the sequences (5'-3') Primer sequences (5'-3') coggaattcatgotttggggaagaagtag gtaatctatgagtgtcttgtaaaagaacccagcggtc ctaggtcaggctggtcttgtaaaagaacccagcggtc gtaatctatgagtcgtgtgtgtactcttttcaagttt tcttttacaagacactcatagattactgcaacagttc gagtacagcacagactcatagattactgcaacagttc gagtacagcacagactcatagattactgcaacagttc gagtacagcacagactgactgtgtgt tcttttacaagaccagcctgacctagttgtc gagtacagcacagactgaccagactgactagt acctctgtagagagggagtttccatgttgtc tcttttacaagaccagcctgacctagcagag gtaatctatgagtagtagtagtagtagtagtagtagtagt		

Underlines correspond to the recognition sequence of EcoRI and Notl



Figure 1: Diagrammatic representation of the coding regions of fulllength 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 $\triangle 4188$ 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 $\triangle 4371$ 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 $\triangle 4047$, mini-dystrophin \triangle 4188 and mini-dystrophin \triangle 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- \triangle 4047, FIV- \triangle 4188 and FIV- \triangle 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 minidystrophin 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 *Not*I, lane 4: FIV-△4371 vector digested with *Eco*RI 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 $\triangle 4047$ and mini-dystrophin $\triangle 4188$ was considerably higher than that of mini-dystrophin $\triangle 4371$ 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, cysteinerich 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 vectormediated gene transfer indicated that mini-dystrophins with N-terminal actin-binding and C-terminal domains were stably expressed at the sarcolemma. These minidystrophins 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 $\triangle 4371$ 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 longterm 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.

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