

**PW 2:
DMD – Molecular and cell
therapy**

PW2-018	<p><u>ABUNDANCE OF CIRCULATING PROGENITORS WITH MYO-ENDOTHELIAL POTENTIAL CORRELATES WITH A MILD PHENOTYPE IN PATIENTS AFFECTED BY DUCHENNE MUSCULAR DYSTROPHY</u></p> <p>FARINI A¹, BELICCHI M¹, MEREGALLI M¹, MARCHESI C¹, LOPA R², PORRETTI L², PAROLINI D¹, D'ANGELO MG³, BRESOLIN N¹, COSSU G⁴, TORRENTE Y¹</p> <p>(1) 1Stem cell Laboratory, Department of Neurological Science, Fondazione IRCCS Ospedale Maggiore Policlinico, Centro Dino Ferrari, University of Milan, Milan, ITALY. (2) 2Centro Trasfusionale e di Immunologia dei Trapianti, Fondazione IRCCS Ospedale Maggiore Policlinico, Milan, ITALY. (3) 3IRCCS E. Medea, La Nostra Famiglia, Bosisio Parini, Bosisio Parini, ITALY. (4) 4Stem Cell Research Institute, San Raffaele Hospital, Milan, ITALY.</p>
	<p>Although the natural history of patients with Duchenne Muscular dystrophy (DMD) is characterized by a progressive impairment of muscle function leading to death for cardio-pulmonary failure, there is a clinical variability in these patients regarding age of onset, patterns of skeletal muscle involvement, heart damage, and rate of progression. Most therapeutic strategies for DMD have been palliative rather than curative. Experimental treatments in DMD are difficult due the absence of reliable biomarkers that could be prognostic of the progression of the disease or response to the treatment. Recent works from several laboratories support the idea that increased circulating endothelial progenitors predict cardiovascular and vascular diseases. We hypothesized that the levels of circulating stem cells expressing the CD133 antigen which possess myo/endothelial potential would predict the progression of DMD. The count of circulating CD133+ stem cells was similar in DMD patients and healthy subjects. We found a subpopulation of CD133+ stem cells also expressing the CXCR4 receptor but not CD34 that was significantly higher in DMD patients compared with healthy controls and positively correlated with the clinical score. DMD patients exhibiting mild phenotype have higher levels of this subpopulation of circulating CD133+ stem cells than patients exhibiting severe phenotype. Linear regression analysis showed a direct correlation between the levels of these cells and the clinical condition of the DMD patients. The circulating AC133+CXCR4+CD34- cells isolated from DMD and healthy subjects express early myogenic and endothelial markers in vitro and differentiate into muscle and endothelial cells in vivo after their transplantation into scid/mdx dystrophic mice. Based on these data we believe that the levels of a subpopulation of circulating CD133+ stem cells in DMD patients may be a promising new prognostic clinical marker of the progression of the disease with practical significance to allow any beneficial effect in future clinical trials.</p>

PW2-019	<p><u>EX VIVO EXPANSION OF HUMAN CIRCULATING CD133+ PROGENITOR CELLS: PROMISING TOOL FOR CELL-BASED THERAPEUTIC APPROACHES IN MUSCULAR DYSTROPHY</u></p> <p>BELICCHI M¹, MEREGALLI M¹, RAZINI P¹, CATTANEO A², FARINI A¹, IACCHETTI E³, PORRETTI L², MILANI P³, BRESOLIN N¹, TORRENTE Y¹</p> <p>(1) Stem Cell Laboratory, department of neurological Sciences, Fondazione IRCCS Ospedale Maggiore Policlinico, Centro Dino Ferrari, University of Milan, Milan, ITALY. (2) Centro Trasfusionale e di Immunologia dei Trapianti, Fondazione IRCCS Ospedale Maggiore Policlinico, Milan, ITALY. (3) Centro Interdisciplinare Materiali e Interfacce Nanostrutturati (CIMAINA), Dipartimento di Fisica, Università di Milan, Milan, ITALY.</p>
	<p>The use of stem cells in regenerative medicine and cell-based therapies offers immense potential in diseases which have currently no treatment such as Duchenne muscular dystrophy. A limitation to the use of CD133+ for a therapeutic application is the relatively low number of cells that can be recovered from peripheral blood mononuclear cells. The goal of ex vivo expansion is to induce proliferation of blood derived (circulating) CD133+ cells while maintaining their primary functional characteristic. In this work we explored an alternative methods for the expansion of blood-derived stem cells we investigated the interactions between CD133+ cells and smooth surfaces of titanium (TiO₂). Our results indicate that cluster-assembled nanostructured TiO₂ is biocompatible surface for cell culturing directly supporting normal growth and proliferation of hematopoietic stem cells. In these experiments we also identified a cocktail of cytokines SCF, bFGF, EGF, VEGF, LIF, TEPA, IL6 which supported the growth of blood CD133+ stem cells. In this condition the cells can be expanded for more than 50 and we observed no indication of replicative senescence or significant changes in cellular division time. The proliferating cells still had the capacity to form hematopoietic and endothelial colonies in semisolid media and differentiate into myogenic cells. Human circulating CD133+ cells were also cultured at 5-percent or 20-percent oxygen in liquid culture in presence of the better cocktail of cytokines and we analysed and compared their expansion capacity and their vitality. The total number of cells increased 6-fold at 5-percent oxygen and could result in a better maintenance of the balance between primitive progenitor cell renewal and clonogenic progenitor expansion, thus representing a tool of remarkable therapeutic interest.</p>

PW2-020	<p><u>ESC DIFFERENTIATION IN CARDIAC PRECURSORS FOR DMD CARDIOMYOPATHY CELL THERAPY</u> NIVET AL¹, PLANCHERON A¹, LUSTREMAN C¹, PESCHANSKI M¹, MONVILLE C¹ (1) Inserm UEVE UMR 861, I-Stem, AFM, Evry, FRANCE.</p>
<p>To contact the author:: anivet@istem.genethon.fr.</p>	<p>Duchenne muscular dystrophy (DMD) is a genetic disease with an X-linked recessive pattern of inheritance. It affects one in 3,500 boys at birth. Muscular dystrophies are caused by mutation of the same gene encoding for the dystrophin. This protein is expressed in the skeletal muscle, the heart and the digestive system and is essential to the maintenance of cellular architecture. Thus, a defect of the dystrophin involves the rupture of this bond and causes a weakness of the muscle cell membrane. In the late-stage disease, the majority of DMD patients develop a dilated cardiomyopathy. For the moment, there is no curative treatment available. Embryonic stem (ES) cells, which can divide indefinitely and give rise to many cellular types including cardiomyocytes, represent a very promising tool for a cell therapy approach of the cardiac pathology in this disease. However, defining the appropriate cell population to transplant is still in question.</p> <p>The aim of this project is to obtain an homogeneous and self-renewable population of cardiac precursors from embryonic stem cells, based on two types of approaches: (i) co-cultures with visceral endoderm-like (END-2) cells or new-born rat heart fibroblasts which are potentially able to induce cardiac differentiation and (ii) using growth factors that promote cardiac differentiation (BMP2, Wnt3a).</p> <p>The preliminary results showed that addition of Wnt3a during the first three days of differentiation of mESc increase the proportion of contracting myocytes. Moreover, we have established co-culture of hESc with new-born rat heart fibroblasts and results are encouraging to follow through these studies.</p>

PW2-021	<p><u>DYSTROPHIN RESCUE IN DUCHENNE MUSCULAR DYSTROPHY FIBROBLAST-DERIVED MYOBLASTS.</u> AVRIL-DELPLANQUE A¹, MERCIER S², DAOUD F², NUSBAUM P³, LETURCQ F³, KAPLAN JC³, DREYFUS P¹, CHELLY J², GARCIA L¹ (1) UMR S 787, Inserm/UPMC-Paris 6, Institut de Myologie, paris, FRANCE. (2) Département de Génétique et Développement, UMR 8104, Université Paris Descartes/CNRS, Institut Cochin, paris, FRANCE. (3) Laboratoire de Biochimie et Génétique Moléculaire, Hôpital Cochin, paris, FRANCE.</p>
To contact the author:: aurelie.avril@chups.jussieu.fr.	<p>Duchenne Muscular Dystrophy (DMD) is the most severe form of dystrophinopathy, in which nul mutations in the <i>DMD</i> gene (mostly frameshifting deletions, and nonsense point mutations) result in the complete absence of dystrophin. A milder phenotype of the disease, Becker Muscular Dystrophy (BMD), generally arises from in-frame deletions allowing the synthesis of a shorter but still quasi-functional protein. Based on these observations, therapeutic approaches to restore the reading frame by exon skipping have been developed. We and others demonstrated the use of exon skipping to restore the expression of a fully functional quasi-dystrophin. In our experiments, the targeted pre mRNA sequences are carried by an engineered U7 small nuclear RNA which is delivered to the myoblasts by a lentiviral vector. When getting patients muscle biopsies is unavailable or impossible, we used skin fibroblasts (CD90+, CD56-) easily obtained. After having cultured control fibroblasts, they are transfected with a lentivirus carrying MyoD under the tet on promoter to induce myogenic differentiation (63% after 3 days are CD56+). Control fibroblast-derived myoblasts expressed myogenic protein e.g. myogenin, desmin and dystrophin.</p> <p>Using DMD patients fibroblast-derived myoblasts, we obtained the similar myogenic differentiation but no dystrophin. On these cells, using the same U7 lentivirus, skipping either exon 45 or exon 51 restored a dystrophin open reading frame mRNA detected by nested RT PCR and a quasi dystrophin detected by Western Blot.</p> <p>Conclusions: We validated efficient target sequences to successfully skip exon 45 or 51 in the dystrophin pre messenger RNA. Moreover, we showed that the exon skipping method worked on skin biopsies fibroblast-derived myoblasts, providing an alternative to muscular biopsy.</p>

PW2-022	<p>MUSCLE FUNCTION RECOVERY IN DYSTROPHIC DOG AFTER EXON SKIPPING GENE THERAPY</p> <p>VULIN A¹, BARTHÉLÉMY I², DREYFUS P¹, BLOT S², GARCIA L¹ (1) Association Institut de Myologie - UMR787, Paris, FRANCE. (2) Laboratoire de Neurobiologie ENVA, Maisons-Alfort, FRANCE.</p>
<p>To contact the author:: adeline.vulin@chups.jus sieu.fr.</p>	<p>Duchenne Muscular Dystrophy (DMD) is an X-linked recessive disorder due to mutations in the gene that encodes dystrophin. Most of these mutations consist in large genomic deletions, although their extent is not directly correlated with the severity of the phenotype. Out-of-frame deletions lead to abortion of translation, dystrophin deficiency and severe DMD phenotypes, while internal deletions that produce in frame mRNAs leading to shorter proteins are responsible for a milder myopathy known as Becker Muscular Dystrophy (BMD). About 80% of the out-of-frame mutations could be theoretically rescued after restoring the translational frame by using exon skipping strategies. Here we used gene transfer in a large animal model of DMD, the Golden Retriever Muscular Dystrophy (GRMD) dog, to achieve the precise skipping of multiple exons spaced over 125,000 bp of the dystrophin pre-mRNA and the re-expression of a functional protein. This led to sustained correction of the dystrophic phenotype in extended muscle areas and muscle strength recovery. Exon skipping was obtained with U7snRNAs (U7smOPT) carrying antisense sequences designed to mask determinants of exon 6 and 8 definition. These U7smOPT were introduced into skeletal muscle fibres by using Adeno Associated Viral (AAV2/1) vectors. After two months, levels of dystrophin were almost normal in transduced fibres. Histological examination revealed that the dystrophin glycoprotein complex was restored and that spontaneous muscle damages were stopped. Muscle architecture was fully corrected and fibres displayed the hallmarks of mature and functional units. Muscle force reflecting fibers functional integrity were improved. Our study documents for the first time the recovery of dystrophin at the scale of entire limbs in a large animal and thus represents a critical milestone for the development of clinical trials in human patients.</p>

PW2-023	<p><u>SYSTEMIC RESTORATION OF DYSTROPHIN EXPRESSION IN DMD BY PEPTIDE-CONJUGATED ANTISENSE OLIGONUCLEOTIDES</u> YIN H¹, SEOW Y¹, MOULTON HM², IVERSEN PL², BOUTILIER JK², WOOD MJA¹ (1) Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford, UNITED-KINGDOM. (2) AVI BioPharma Inc, Corvallis, USA.</p>
To contact the author:: matthew.wood@dpag.ox.ac.uk.	<p>Duchene Muscular Dystrophy (DMD) is a severe muscle disorder caused by mutations in the dystrophin gene. The efficacy of antisense oligonucleotide (AO)-mediated exon skipping for the restoration of dystrophin has been established in animal models and in DMD patients. However there remain significant limitations to this therapeutic approach due to the lack of effective systemic AO delivery to target tissues of muscle and heart. Here we investigate systemic tissue-specific AO delivery by testing AOs directly conjugated to cell penetrating peptides (CPPs) alone or in combination with tissue-specific homing peptides (e.g. muscle-specific peptide, MSP). Morpholino (PMO) chemistry AOs were directly conjugated to CPPs alone or in combination with homing peptides and evaluated in mdx mice following systemic delivery. Effective exon skipping and dystrophin expression were induced in body-wide skeletal muscles at extremely low AO doses of 3mg/kg and also in heart. This is the first time that targeted AO delivery to muscle and successful body-wide restoration of dystrophin expression have been achieved at such low AO doses. In parallel we also report the discovery and characterization of a novel delivery formulation which facilitates AO uptake in muscle. A series of studies have shown that this delivery formulation enhances the delivery of AOs of different chemistries (e.g. 2-OMeRNA, PNA and PMO), depends on the activity of specific muscle membrane transporters, and that it induces significant restoration of dystrophin expression in muscle compared with commonly used delivery formulations. In summary, we report data demonstrating the potential of tissue-specific homing peptides, CPPs and novel delivery formulations for the targeted restoration of dystrophin in DMD.</p>

PW2-024	<p>DIRECT COMPARISON OF 2'O-METHYL AND PMO AONS FOR EXON SKIPPING IN DMD HEEMSKERK H¹, DE WINTER C¹, DE KIMPE S², VAN KUIK-ROMEIJN P², HEUVELMANS N², PLATENBURG G², VAN OMMEN G-J¹, VAN DEUTEKOM J², AARTSMA-RUS A¹ (1) DMD Genetic Therapy Group, Department of Human Genetics, Leiden University Medical Center, Leiden, THE NETHERLANDS. (2) Prosensa B.V., Leiden, THE NETHERLANDS.</p>
To contact the author:: j.a.heemskerk@lumc.nl.	<p>Antisense-mediated exon skipping is a potential treatment for Duchenne muscular dystrophy (DMD). Using antisense oligonucleotides (AONs) the disrupted DMD reading frame is restored, allowing the generation of partially functional dystrophin and conversion of a severe Duchenne into a milder Becker muscular dystrophy phenotype. This strategy has been studied in cultured patient cells and in mouse and dog models. Further, clinical proof of concept was obtained through an exploratory study in which four DMD patients received a local dose of a 2'O-methyl phosphorothioate (2OMePS) AON targeting exon 51. Recent studies in <i>mdx</i> mice show relatively high exon 23 skipping efficiencies with another type of AON chemistry, morpholino AONs (PMOs). We here directly compared both chemistries and assessed the effects of length (PMOs are typically 25 nucleotides and 2OMePS 20 nucleotides), cellular uptake and sequence. The study included short 2OMePS, long 2OMePS and PMO AONs targeting mouse exon 23 and human exons 44, 45, 46 and 51. Exon 23 AONs were tested intramuscularly and intravenously in <i>mdx</i> mice, human AONs only intramuscularly in the hDMD mouse, which has an integrated full-length copy of the human <i>DMD</i> gene. For mouse exon 23, PMO was confirmed to be more efficient than 2OMePS, however for the human exons PMO and 2OMePS skipping efficiencies were more comparable, which suggests sequence-dependence. Notably two mismatches rendered 2OMePS but not PMO AONs ineffective, implying PMOs may be less sequence-specific. Increasing the length of 2OMePS AONs enhanced skipping efficiencies of human exon 45, but decreased efficiency for mouse exon 23. After intravenous administration, exon skipping and novel protein was shown in the heart for the first time with both AON chemistries. Further, PMO showed lower intramuscular concentrations with higher exon 23 skipping levels compared to 2OMePS AONs. Possibly, the charged 2OMePS is sequestered in the interstitial space.</p>

PW2-025

**MODULATION OF SMALL MUTATIONS IN DYSTROPHIN “SKIPPABLE” EXONS:
IN VITRO STUDIES TO IDENTIFY THE OPTIMAL PS-AONS**

SPITALI P¹, FABRIS M¹, FALZARANO S¹, SABATELLI P², BOVOLENTA M¹, NERI M¹, MARTONI E¹, TUFFERY-GIRAUD S³, CLAUSTRES M³, CUISSET J⁴, GUALANDI F¹, RIMESSI P¹, FERLINI A¹

(1) Molecular Genetics - Section of Medical Genetics, Ferrara, ITALY. (2) IGM-CNR, IOR, Bologna, ITALY. (3) Genetique Moleculaire - Institut Universitaire de Recherche Clinique et CHU, Montpellier, FRANCE. (4) CHRU, Lille, FRANCE.

<p>To contact the author:: pietro.spitali@unife.it.</p>	<p>Exon skipping by antisense oligonucleotides (AONs) represents a promising tool successfully used for reframing dystrophin as demonstrated in a recent pilot trial in DMD patients. Since all the effort has been focused on favourable exons skipping in the commonest deletion mutations, we focused our research on the identification of optimal AONs for modulating small mutations. These account for 20-30% of all reported mutations and, if occurring within skippable exons, are eligible for AONs modulation. Among 50 patients characterised by us and carrying small mutations in the dystrophin gene we have selected 5 patients (c.1132_1135dup in exon 10, c.1912delC in exon 16, c.3447_3448delinsTT in exon 26, c.4565delT in exon 33, c.4780delTins37 in exon 34) with mutations occurring in skippable exons, and we have designed for each exon one AON on the wild type sequence and one on the mutated sequence. In order to avoid the usage of patients' cells we have set up an <i>in vitro</i> cell-free splicing assay for testing the designed AONs. We have therefore validated all the AONs designed on MyoD transformed patients' fibroblasts. Our experiments in cells resulted both in a re-framing of the dystrophin transcript lacking the skipped mutated exon and in a protein product. Although both wild type and mutated AONs were able to induce a specific exon-skipping, their efficiency varied if measured by qRT-PCR in patients' cells and densitometry analysis on cell free splicing assay. In conclusion, we have developed a cell-free splicing assay able to reproduce the splicing of dystrophin exons with private small mutations useful to search for optimal antisense without using patients' material. These data highlight the complexity in identifying the optimal AON for exons with private small mutations with possible implications on therapeutic designing.</p>
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<p>PW2-026</p>	<p><u>ANTISENSE OLIGOMER (AO) INDUCED EXON SKIPPING IN THE MDX4CV MOUSE MODEL</u> MITRPANT C¹, FLETCHER S¹, WILTON S¹ (1) Australian Neuromuscular Research Institute (ANRI), Perth, AUSTRALIA.</p>
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Duchenne muscular dystrophy (DMD), a relentless progressive muscular dystrophy is caused by protein truncating mutations in the dystrophin gene that result in the absence of functional dystrophin. Loss of dystrophin leads to irreparable membrane damage, thus promoting calcium ion influx and cell death.

Antisense Oligomer (AO) induced exon skipping is a molecular intervention whereby AOs are targeted to motifs involving in pre-mRNA splicing. This has been used to induce specific exon removal and by-pass the disease-causing gene lesion in the *mdx* mouse model of muscular dystrophy. We are investigating exon skipping in the B6Ros.Cg-Dmd^{mdx-4Cv}/J (*mdx* 4^{Cv}) muscular dystrophy mouse, which carries a nonsense mutation in exon 53 of the dystrophin gene. To restore the reading frame, both exons 52 and 53 must be excised from the mature dystrophin gene transcript to by-pass the primary gene lesion and maintain the reading frame. 2'-O-Methyl AOs, on a phosphorothioate backbone have been designed to mask these exons from the splicing machinery and lead to their excision. Initial AO combinations induced skipping of exons 52/53 and restored some protein expression. However the predominant transcript was missing only exon 53 as determined by RNA studies. We designed additional AOs to enhance exon skipping of both exons 52 and 53.

We highlight the importance of AO design to enhance efficiency of single and multi-exon removal.

PW2-027	<p><u>IDENTIFICATION AND CHARACTERIZATION OF SMALL MOLECULES FOR THE TREATMENT OF DUCHENNE MUSCULAR DYSTROPHY</u> ACHARJEE S¹, FRIESEN W¹, TOMIZAWA Y¹, BAI AZITOV R¹, LEE S¹, NADARAJAN T¹, MOON YC¹, SWEENEY L², WELCH EM¹ (1) PTC Therapeutics, South Plainsfield, USA. (2) University of Pennsylvania, Philadelphia, USA.</p>
To contact the author:: sacharjee@ptcbio.com.	<p>PTC Therapeutics, Inc. (PTC) and Parent Project Muscular Dystrophy (PPMD) are collaborating to discover new drugs to treat Duchenne muscular dystrophy (DMD). Several targets were selected to enter the drug discovery program based on functional validation from animal studies. The targets selected for high throughput screening (HTS) included: utrophin (UTRN), muscle-specific insulin-like growth factor (mIGF1), and $\alpha 7$ integrin (ITGA7). Using a proprietary drug discovery platform technology, referred to as GEMS (Gene Expression Modulation by Small-molecules), we sought to identify small molecules that upregulate the production of these protein targets to identify potential treatments for DMD. Constructs containing the firefly luciferase (fLuc) reporter gene flanked by the 5' and 3' untranslated regions (UTR) specific for each of the targets were stably transfected in human muscle (RD) or kidney (293H) cells and used in HTS. We identified hits that demonstrate concentration dependent activities in cell-based reporter assays and in assays that measure protein levels. Further, a number of molecules exhibit good pharmacological properties (e. g., low cytotoxicity and microsomal metabolic stability). Presently, we are focused on optimizing the activity, potency and pharmacological properties of 2 chemical scaffolds for the mIGF1 program which exhibit up to 5 fold upregulation of mIGF1 and demonstrate structure-activity relationships. For UTRN and ITGA7, we have identified 2 chemical scaffolds for each target and are in the process of establishing structure-activity relationships for these chemical classes. The ultimate goal of this drug discovery and development effort is to identify small molecules that can specifically modulate the production of a number of proteins that can be used as monotherapy or as part of a combination therapy to treat Duchenne muscular dystrophy.</p>

PW2-028	<p>RHOA INDUCES EXON SKIPPING AND DYSTROPHIN RE-EXPRESSION IN MDX MICE. DURAND S¹, POMIÈS P², FABBRIZIO E¹, LEJEUNE F¹, BONET-KERRACHE A² (1) Institut de génétique Moléculaire, Montpellier, FRANCE. (2) Centre de Recherche en Biochimie Macromoléculaire, Montpellier, FRANCE.</p>
To contact the author:: armelle.bonet@crbm.cnrs.fr.	<p>Duchenne muscular dystrophy (DMD) is a severe X-linked muscle degenerative disease caused by mutations or deletions in the dystrophin gene leading to absence of the protein. The members of the Rho family of small GTPases are molecular switches that control a wide variety of signal transduction pathways linked to the regulation of the actin cytoskeleton, cell polarity, microtubule dynamics, membrane transport pathways and transcription factor activity.</p> <p>Here, we show that in <i>mdx</i> mice (which possess a stop codon within exon 23 of dystrophin and, thus, do not produce the protein), exogenous expression of activated RhoA in muscle fibers induces dystrophin reexpression. Full length dystrophin could be detected with different antibodies at the sarcolemma of fibers in which RhoA was electro-transferred and this re-expression led to re-appearance of proteins of the DGC complex. Moreover electroporated fibers presented 2 times more peripheral nucleated fibers than controls and consequently, a reduction in the severity of the dystrophic phenotype.</p> <p>We then demonstrate that RhoA-induced re-expression of dystrophin is not due to an activation of ribosomal read-through by RhoA. Moreover, we show that RhoA can activate the dystrophin promoter and inhibit NMD (Nonsense Mediated mRNA Decay) thus helping to stabilize dystrophin mRNA. Finally, we reveal by PCR amplification that RhoA induces the exon skipping of exon 23 in the dystrophin gene of <i>mdx</i> mice, allowing the translation of dystrophin mRNA and re-expression of the protein.</p> <p>In this study we demonstrate that in <i>mdx</i> mice dystrophin re-expression following exogenous expression of active form of RhoA is due to skipping of dystrophin exon 23. This finding suggests that RhoA can regulate exon skipping in the dystrophin gene. Understanding the underlying mechanism will be important for the discovery of new therapeutic targets able to lead to or to amplify exon skipping in DMD patients.</p>