

Eighth French-Japanese Workshop
on Muscular Dystrophies

“From pathophysiology to biotherapies”

July 3 – 4, 2009

Institut de Myologie

Hôpital de la Salpêtrière 47 Boulevard de l'Hôpital 75651 Paris Cedex

Eighth French-Japanese Workshop
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ABSTRACTS

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JULY 3, 2009 Morning

PW1 interstitial cells (PICs): a non-satellite cell source of myogenic progenitors

David A. Sassoon, Alice Pannérec, Katherine J. Mitchell, Nathalie Didier, Vanessa Besson, Ara Parlakian, Ludovic Arandel, Edgar Gomes, Bruno Cadot, and Giovanna Marazzi.

Myology Group, INSERM and Paris University

Non-satellite cells participate in muscle regeneration, however their precise anatomical location and level of contribution is unclear. PW1 is expressed in satellite cells (SCs) and a sub-population of interstitial cells, termed PICs (PW1 interstitial cells), which do not express other known lineage markers. PICs can be FACs-isolated from muscle using stem cell-surface markers to obtain a population distinct from SCs. PICs generate smooth and skeletal muscle in vitro and skeletal muscle in vivo. Most notably, PICs are highly myogenic in vivo and also generate numerous PICs after a single round of regeneration following injection into damaged muscle. In the presence of SCs, PICs convert readily to the skeletal myoblasts (PW1+/Pax7+/MyoD+), and fuse with primary satellite cells and myotubes. Pax7 mutant PICs cannot generate skeletal muscle whereas Pax7 mutant SCs show pronounced myogenicity revealing that PICs require Pax7 to enter the skeletal muscle lineage. We observe that PICs are not derived from a Pax3 lineage using a Pax3^{Cre} x Rosa^{lacZ} cross, however, PICs initiate Pax3 expression upon skeletal muscle conversion. Affymetrix-based profile comparisons of PICs and SCs reveal that PICs express genes involved in embryonic development as well as many stem cell-related markers. In addition, several growth factors and their receptors are differentially expressed including many members of the TGF/BMP/fst and Wnt pathway. A PW1 reporter line has been generated which reveals that PW1 not only marks progenitors in skeletal muscle, but labels stem cells in a wide variety of adult tissues suggesting a common regulatory pathway in stem cells.

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Making muscle from induced pluripotent stem (iPS) cells

Yuko Suzuki, Norio Motohashi, Erika Yada, Makoto Segawa, Wang Bo, Chika Harano, Satoru Masuda, Mikiharu Yoshida, Shin'ichi Takeda

Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan.

Duchenne muscular dystrophy (DMD) is a devastating muscle disorder caused by mutations of the dystrophin gene. Patient-specific induced pluripotent stem (iPS) cells are a potential cell source for cell therapy for DMD. As a first step toward iPS cell-based therapy for DMD, we generated iPS cells from 12-day-old, 6-week-old, or 6-month-old *mdx* mice by retroviral transduction of *Sox2*, *Klf4*, *Oct3/4* and *c-Myc*. We also generated *mini-dystrophin*-expressing *mdx* iPS lines. All *mdx*-derived iPS lines were indistinguishable from mouse embryonic stem (ES) cells in morphology, growth, gene expression, and differentiation potential *in vitro* and *in vivo*. Next, we tried to define the culture conditions that promote myogenic differentiation of ES cells or iPS cells, using Myf5-GFP ES cells and Pax3-GFP iPS cells. After formation of embryoid bodies (EBs) by a hanging drop method, the EB cells were cultured on Matrigel-coated dishes, and SM/C-2.6-positive cells were collected using a cell sorter. More than 50% of these cells were positive for MyoD, but their proliferative activity and muscle differentiation were limited *in vitro*. We next screened a chemical library to find novel compounds that promote differentiation of ES cells/ iPS cells into paraxial mesoderm or augment *Myf5-GFP* or *Pax3-GFP* expression in differentiating ES cells and iPS cells. We identified several molecules that increased the number of PDGF-R (+) Flk-1(-) cells (presumptive paraxial mesoderm), and *GFP* (*Myf5*)-positive cells. Our findings would help not only develop methods to induce myogenic cells from iPS cells, but also provide mechanistic insights into myogenesis in the early stages of development.

Regulation of embryonic muscle progenitor cells and satellite cells development

Sonia Alonso-Martin, Frederic Aurade, Keren Bismuth, Ted Chang, Andrew Ho, Jessica Morais, Anne RoCHAT and Frédéric Relaix.

Mouse Molecular Genetics team, UMR-S 787 -INSERM -UPMC-Paris VI -Institut de Myologie, Faculté de Médecine Pitié-Salpêtrière, 105 bd de l'Hôpital, 75634 -Paris Cedex 13, France. Tel (+33) 140 778 125. Fax (+33) 153 600 802

Nearly all skeletal muscle cells are derived from Pax3/7-expressing muscle progenitor cells, both during development and in the adult. We are interested in the molecular and cellular mechanisms regulating these muscle progenitor cells. Using mouse molecular genetics, we will show that establishment of the skeletal muscle during development is a highly regulated process with critical cellular interaction required. Around birth, fetal muscle progenitor cells adopt a satellite cell position, becoming embedded within the basal lamina in close contact to the muscle fibers. Importantly, in addition to this morphological change, the emerging satellite cells enter quiescence, a molecular state poorly characterized *in vivo*. During post-natal growth or in response to injury or disruption of the basal lamina, a subset of the satellite cells become activated, proliferate and either fuse to form multinucleated myotubes or reestablish a residual pool of quiescent satellite cells that have the capability of supporting additional rounds of growth/regeneration. Little is known on the molecular pathways involved in the emergence of post-natal muscle progenitors in vertebrates, as well as the genetic changes associated with the cellular and stemness capacity of these cells. We have previously generated a GFP-targeted *Pax3* mouse line where GFP labels the muscle progenitor cells. Using these mice, we have initiated a large-scale gene profiling throughout development and after birth using FACS sorting of fluorescent muscle progenitor cells combined with microarray analysis. We will present the result of this analysis and show that important molecular changes take place during maturation and transition of the skeletal muscle progenitor cells to post-natal satellite cells. In addition, we will present the identification and characterization of new factors potentially involved in satellite cells specification, determination and differentiation.

Proliferative arrest of human muscle precursors in control and dystrophic cells

Vincent Mouly¹, Anne Bigot, Kamel Mamchaoui, Soraya Chaouch, Denis Furling, Woody Wright², Gillian Butler-Browne

¹UMR S 974, Thérapies des maladies du muscle strié, 105 bd de l'hôpital, 75013Paris, France ²Dept of Cell Biology, UT Southwestern Medical Center, Dallas, Texas, USA.

The proliferative capacity of human satellite cells, as that of other human somatic cells, is limited by telomere shortening, which represent a mitotic clock. Although this limit is usually not reached during a normal lifespan, this is not the case for patients suffering from muscular dystrophies, where the limit in proliferation may participate to the evolution of the dystrophic phenotype. Our group has studied how this limit is controlled in human myoblasts isolated from both normal and pathological biopsies. We show that although the telomere dependent mitotic clock is active in all these cells, there is an additional activation of the p16 stress pathway in vitro, probably due to the culture conditions, although this pathway is inactive in human fibroblasts in culture. By neutralising both of these pathways we have now been able to isolate immortal cell lines from both normal and pathological human myoblasts, which maintain their differentiation potential both in vitro and in vivo. We have evidence that the p16 stress pathway can be involved in pathological situations where a premature proliferative arrest of the muscle precursors is triggered, such as in myotonic dystrophy (DM1). The group is currently studying if this stress response may also be involved in other muscle pathologies, and how proliferative arrest, premature or not, may impair muscle differentiation. The expression of genes involved in triggering muscle differentiation has been compared between young and senescent cells, and deregulations have been observed. Consequences for both muscle pathology and muscle ageing in humans will be discussed.

Roles of Meltrin beta/ADAM19 in Formation of Neuromuscular Junction

Atsuko Sehara-Fujisawa

Department of Growth Regulation, Institute for Frontier Medical Sciences, Kyoto University Kawahara-cho 53, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan Tel: +81-75-751-3826, FAX: +81-75-751-4642, asehara@frontier.kyotou.ac.jp

Development and regeneration require various kinds of intercellular signaling and adhesion molecules. Our research has been focused on regulatory mechanisms of such cell-cell interactions. Numerous intercellular signaling molecules are generated as membrane-anchored proteins, and they are subjected to proteolytic processing to liberate their extracellular domains (ectodomain shedding). Evidence suggests that ADAM (A Disintegrin And Metalloprotease) family proteases are involved in the ectodomain shedding of various membrane proteins. We previously showed that Meltrin beta (ADAM19) plays a role in ectodomain shedding of neuregulin/ acetylcholine receptor inducing activity *in vitro*. I report here that Meltrin beta participates in the formation of the neuromuscular junction (NMJ) formation. Development of NMJ is initiated by the formation of postsynaptic specializations in the central zones of muscles, followed by the arrival of motor nerve terminals opposite the postsynaptic regions. The post- and presynaptic components are then stabilized and modified to form mature synapses. By the analyses of Meltrin beta knockout mice, we found that the zone of acetylcholine receptor mRNA distribution was broader, and excess sprouting of motor nerve terminals was more prominent in *meltrin beta*-deficient than in wild-type embryonic diaphragms. A microarray analysis revealed that the preferential distribution of *ephrin-A5* mRNA in the synaptic region of muscles was aberrant in the *meltrin beta*-deficient muscles. A similar perturbed formation of the NMJ was found in *ephrin-A5* knockout mice, suggesting that stable localization of the axon terminals onto the postsynaptic sites in muscles requires *ephrin-A5* as well as Meltrin beta. Meltrin beta and EphA4 interacted with each other in developing motor neurons, and both of these proteins localized in the NMJ. Coexpression of Meltrin beta and EphA4 strongly blocked endocytosis of *ephrin-A5*-EphA4 complexes without requiring the protease activity of Meltrin beta. We propose that Meltrin beta stabilizes the interaction between *ephrin-A5* and EphA by regulating endocytosis of the *ephrin-A5*-EphA complex negatively, which would contribute to the formation of NMJ.

Selenoprotein N is dynamically expressed during mouse development and detected early in muscle precursors

Castets Perrine^{1,2,†}, Maugendre Svetlana^{1,2,†}, Gartieux Corine^{1,2,§}, Rederstorff Mathieu³, Lescure Alain³, Krol Alain³, Tajbakhsh Shahragim⁴, Allamand Valérie^{1,2,§}, Guicheney Pascale^{1,2,†}

¹ Inserm, U582, Institut de Myologie, Paris, France.

² Université Pierre et Marie Curie-Paris 6, UMR-S582, Institut de Myologie, IFR14, Paris, France.

³ UPR 9002, CNRS, IBMC, Strasbourg, France.

⁴ CNRS, URA 2578, Pasteur Institute, Paris, France.

Current affiliations: † Inserm, UMR-S956, F-75013 Paris, France ; § Inserm, UMRS974, Institut de Myologie, CNRS UMR7215, UPMC Univ Paris 06, F-75013 Paris, France.

In humans, mutations in the *SEPN1* gene, encoding selenoprotein N (SeIN), are involved in early onset recessive neuromuscular disorders, referred to as *SEPN1* related myopathies. The mechanisms behind these pathologies are poorly understood since the function of SeIN remains elusive. However, previous results obtained in humans and more recently in zebrafish pointed to a potential role for SeIN during embryogenesis. Using qRT-PCR, Western blot and whole mount *in situ* hybridization, we characterized in detail the spatio-temporal expression pattern of the murine *Sepn1* gene during development, focusing particularly on skeletal muscles. In whole embryos, *Sepn1* transcripts were detected as early as E5.5, with expression levels peaking at E12.5, and then strongly decreasing until birth. In isolated tissues, only mild transcriptional variations were observed during development, whereas a striking reduction of the protein expression was detected during the perinatal period. Furthermore, we demonstrated that *Sepn1* is expressed early in somites and restricted to the myotome, the subectodermal mesenchyme and the dorsal root ganglia at mid-gestation stages. Interestingly, *Sepn1* deficiency did not alter somitogenesis in embryos, suggesting that SeIN is dispensable for these processes in mouse.

We characterized the expression pattern of *Sepn1* during mammalian embryogenesis and we demonstrated that its differential expression is most likely dependent on major post-transcriptional regulations. Overall, our data strongly suggest a potential role for selenoprotein N from mid-gestation stages to the perinatal period.

JULY 3, 2009 Afternoon

Identification of a Novel Disease Gene for Hereditary Cardiomyopathy

Takuro Arimura, Akinori Kimura

Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University

Idiopathic cardiomyopathy (ICM) is the primary heart muscle disorder caused by functional abnormalities of cardiomyocytes and the major cause of cardiac sudden death and/or progressive heart failure. There are several clinical subtypes of ICM such as hypertrophic cardiomyopathy (HCM) characterized by ventricular hypertrophy with diastolic dysfunction, dilated cardiomyopathy (DCM) showing ventricular dilation with systolic dysfunction, and restrictive cardiomyopathy (RCM). Although the etiology of ICM has not been fully elucidated, a part of the patients with ICM have apparent family histories of the disease consistent with autosomal dominant genetic trait, and recent genetic analyses have revealed that ICM could be caused by mutations in the genes encoding for components of cardiac sarcomere and/or Z/I-bands including titin/connectin. However, disease-causing mutations can be found in about half of the ICM patients, suggesting that other disease-causing genes remain to be identified. To further explore the ICM-causing mutations, we investigated *ANKRD1* encoding for cardiac ankyrin repeat protein (CARP) that is Z/I-bands component interacting with N2A domain of titin/connectin and N-terminal domain of myopalladin. Three *ANKRD1* missense mutations, Pro52Ala, Thr123Met and Ile280Val, were found in the HCM patients. We also searched for mutations in the N2A domain of titin/connectin and identified two novel HCM-associated mutations, Arg850His and Arg860Gln. As the functional alteration due to the mutations, the HCM-associated *ANKRD1* mutations significantly increased the binding to myopalladin and N2A domain of titin/connectin, and HCM-associated mutations in the N2A domain of titin/connectin also significantly increased the binding to CARP. Myc-tagged CARP showed that the mutations resulted in abnormal nuclear and/or perinuclear localization of CARP in mature cardiomyocytes. These observations suggested that the assembly and/or binding of CARP with myopalladin and titin/connectin would be required for the maintenance of cardiac function since its abnormality was associated with hereditary cardiomyopathy.

Mutations of *FHL1* gene cause Emery-Dreifuss muscular dystrophy

Lucie Gueneau^{1,2}, Anne T. Bertrand^{1,2}, Jean-Philippe Jais³, Mustafa A. Salih⁴, Tanya Stojkovic⁵, Manfred Wehnert⁶, Maria Hoeltzenbein⁶, Simone Spüler⁷, Shinji Saitoh⁸, Annie Verschuere⁹, Christine Tranchant¹⁰, Maud Beuvin^{1,2}, Emmanuelle Lacène^{5,12}, Norma B. Romero^{1,2,5,12}, Simon Heath¹¹, Diana Zelenika¹¹, Thomas Voit^{1,2,5,12}, Bruno Eymard⁵, Rabah Ben Yaou^{1,2,12}, Gisèle Bonne^{1,2,13}

¹ Inserm, U974, Paris, F-75013, France.

² Université Pierre et Marie Curie-Paris6, UMR-S974, CNRS, UMR7215, Institut de Myologie, IFR14, Paris, F-75013, France.

³ Université Paris Descartes, EA 4067, Faculté de Médecine, Biostatistique et Informatique Médicale, GH Necker Enfants-Malades, Paris, F-75015, France.

⁴ Division of Pediatric Neurology, College of Medicine, King Saud University, Riyadh, Saudi Arabia.

⁵ AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Centre de référence des maladies rares neuromusculaires, Paris, F-75013, France.

⁶ Institute of Human Genetics, Greifswald, D-17487, Germany.

⁷ Muscle Research Unit, Experimental and Clinical Research Center, Charité University Medicine Berlin, D-13125, Germany.

⁸ Hokkaido University Graduate School of Medicine, Department of Pediatrics, Sapporo, 060-8638, Japan.

⁹ AP-HM, Hôpital de la Timone, Service de neurologie et maladies neurologiques, Marseille, F-13000, France.

¹⁰ Service de Neurologie, Hôpitaux universitaires, Strasbourg, F-67000, France

¹¹ Centre National de Génotypage, Evry, F-9100, France.

¹² Association Institut de myologie, Unité de Morphologie Neuromusculaire, Groupe hospitalier Pitié-Salpêtrière, Paris, F-75013, France.

¹³ AP-HP, Groupe Hospitalier Pitié-Salpêtrière, U.F. Cardiogénétique et Myogénétique, Service de Biochimie Métabolique, Paris, F-75013, France.

Emery-Dreifuss Muscular Dystrophy (EDMD) is a rare disorder characterized by early joint contractures, muscular dystrophy, cardiac involvement with conduction defects and arrhythmias. So far only 30% of EDMD cases are genetically elucidated and associated with *LMNA* or *EMD* gene mutations, suggesting the existence of additional major genes. By whole genome scan, we identified linkage to the Xq26.3 locus containing the *FHL1* gene in three informative families belonging to our *LMNA*- and *EMD*-negative cohort. Analysis of *FHL1* gene identified 7 new mutations in the distal exons of *FHL1* in these families and in 3 additional ones plus one isolated case, which differently affect the three *FHL1* protein isoforms: two missense mutations affecting highly conserved cysteines, one abolishing the normal

termination codon, and for the first time, four out-of-frame insertions or deletions. The predominant phenotype was characterized by myopathy with scapulo-peroneal and/or axial distribution, joint contractures, and associated with a peculiar cardiac disease characterized by conduction defects, arrhythmias and hypertrophic cardiomyopathy in all index cases of the 7 family. Heterozygous female carriers were

either asymptomatic or had cardiac disease sometimes associated with myopathy. Interestingly, isolated cardiac disease was also observed in some male relatives. Expression and functional studies demonstrated that the FHL1 proteins were severely reduced in all tested patients and this was associated with a severe delay in myotube formation in the two patients where myoblasts were available. In conclusion, *FHL1* should be considered as a new gene associated to the X-linked EDMD phenotype.

Identification of a novel gene for muscular dystrophy with secondary caveolin deficiency

Yukiko K. Hayashi

National Institute of Neuroscience, NCNP, Tokyo, Japan

Caveolae are specific invaginations of the plasma membrane characterized by the presence of the protein caveolin. So far, three caveolin family members have been identified. Caveolin-1 and -2 are co-expressed in many cell types such as endothelial cells, smooth muscle cells, fibroblasts, and adipocytes, and form a hetero-oligomeric complex. In contrast, caveolin-3 is expressed exclusively in skeletal and cardiac muscles. Deficiency of caveolin-3 due to *CAV3* gene mutations is known to cause muscular dystrophy. We found 5 non-consanguineous Japanese patients whose muscle showed caveolin-3 deficiency but without *CAV3* mutation. The patients commonly showed mild muscle weakness, muscle hypertrophy, muscle mounding, and high CKemia. Importantly, all 5 patients were also diagnosed as congenital generalized lipodystrophy. Muscle biopsy showed chronic dystrophic changes with markedly reduced caveolar structure. We identified mutations in a novel gene in all the patients.

X-linked myopathy with excessive autophagy : phenotype-genotype correlations in the French families

M. Fardeau, B. Eymard, F. Chapon, P. Laforêt, N. Romero, K. Claeys, B. Minassian

After 12 years of hard work, the X-linked myopathy with excessive autophagy (XMEA) was deciphered at the gene and molecular level by B. Minassian and his group (N. Ramachandran et al., 2009). Soon after the description of this disease by Kalimo et al. (1988), we had the opportunity to analyze a large French family of Italian ascent, which was very soon included in the clinical material studied in the Finnish-Canadian Group, and contributed to restrict the initial interval of 0.58 Mb on Xq28 (Minassian et al., 2002). Moreover, in the biopsies from this family it was shown that a C5b-9 membrane attack complex was present on the surface of injured muscle fibers (Villanova et al., 1995). From that time on we could analyze five new cases of XMEA, belonging to three families, in our Institute. Clinical, pathological, and biological data of these cases will be presented. They confirm and somewhat extend the usual phenotype of XMEA. For two of these families the gene mutations in the VMA21 gene have been determined.

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“Necklace” fibers in MTM1-associated myopathies, a helpful morphological marker

Jorge A. Bevilacqua^{1,3,6}, Marc Bitoun^{1,2}, Valérie Biancalana⁴, Anders Oldfors⁵, Kristl G. Claeys^{6,7}, Pascal Laforêt^{2,7}, Bruno Eymard^{2,7}, Pascale Guicheney^{2,8}, Michel Fardeau^{2,6}, Norma B. Romero^{1,2,6,7}

1Inserm U974, Institut de Myologie, Paris, F-75013, France

2UPMC Université Pierre et Marie Curie-Paris 6, UMR S582, IFR14, Paris, F-75013, France.

3Departamento de Neurología y Neurocirugía, Hospital Clínico Universidad de Chile and Instituto de Ciencias Biomédicas Universidad de Chile, Santiago, Chile

4Laboratoire Diagnostic Génétique, Faculté de Médecine -CHRU Strasbourg France

5Department of Pathology, Sahlgrenska University Hospital, Goteborg, Sweden

6Unité de Morphologie Neuromusculaire, Association Institut de Myologie (AIM), Groupe Hospitalier Pitié-Salpêtrière, Paris, F-75013, France

7Centre de Références ; AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Paris, F-75013, France.

8Inserm UMR S956, Groupe Hospitalier Pitié-Salpêtrière, Paris, F-75013, France.

Mutations in the gene encoding the phosphoinositide phosphatase myotubularin 1 protein (MTM1) are usually associated with severe neonatal X-linked myotubular myopathy (XLMTM). However, mutations in MTM1 have also been recognized as the underlying cause of “atypical” forms of XLMTM.

At the Institute of Myology were reviewed systematically a large series of more than one hundred of CNM patients; among the CNM patients in whom DNM2 mutations were excluded (about 50% of cases), structural alterations within muscular fibres helped to find out different groups of CNM patients. Amongst these we identified a cohort of four patients presenting with a peculiar histological alteration that resembled a necklace (“necklace fibers”). We analyzed further the clinical and morphological features and performed a screening of the genes involved in CNM. Muscle biopsies in all four patients demonstrated 4–20% of fibers with internalized nuclei aligned in a basophilic ring (necklace) at 3 µm beneath the sarcolemma. Ultrastructurally, such necklaces consisted of myofibrils of smaller diameter, in oblique orientation, surrounded by mitochondria, sarcoplasmic reticulum and glycogen granules. In the four patients (three women and one man), myopathy developed in early childhood but was slowly progressive. All had mutations in the MTM1 gene. Two mutations have previously been reported (p.E404K and p.R241Q), while two are novel; a c.205_206delinsAACT frameshift change in exon 4 and a c.1234A>G mutation in exon 11 leading to an abnormal splicing and the deletion of nine amino acids in the catalytic domain of MTM1. Necklace fibers were not seen in the other recognized forms of CNM (i.e. DNM2- or BIN1-related CNM), nor in newborn males with classical XLMTM. Although a resemblance of necklace fibers with the previously described “trilaminar fibers” has been recently pointed out, we demonstrated that necklace-fiber-myopathy and trilaminar myopathy are different, both morphologically and clinically. We therefore conclude that the presence of necklace fibers is helpful as a marker to direct genetic analysis to MTM1 in CNM, and emphasises the role of morphological criteria to orientate molecular analysis.

DHPR α 1 S subunit controls skeletal muscle mass and morphogenesis

France Piétri-Rouxel¹, Christel Gentil¹; Dominique Baas²; Etienne Mouisel¹, Christophe Hourdé¹, Arnaud Ferry^{1,3}, Alban Vignaud¹, Thomas Voit and Luis Garcia¹

¹Inserm UMRS 974 / UPMC / CNRS UMR7215 /Institut de Myologie, 105 boulevard de l'Hôpital 75013 Paris, France ²UMR 5239 CNRS/ENS/Université Lyon 1 46, allée d'Italie 69 364 Lyon cedex 07, France ³ Université Paris Descartes, Paris, F-75006 France

Skeletal muscle is a complex tissue which requires a correct balance of myotrophic factors and a functional innervation with normal excitation-contraction coupling and muscle activity to maintain mass and function. L-type Ca^{2+} channels have been reported to be voltage-gated sensors possibly linked to transcriptional activity and thus controlling differentiation. In skeletal muscle, the 1 S protein (DHPR) functions both as the L-type Ca^{2+} channel and the voltage sensor for excitation-contraction coupling. We have combined optimized U7snRNA exon skipping and gene transfer to achieve long-lasting knockdown of the 1 S in adult muscle by disrupting its mRNA reading frame. This led to a prominent atrophy involving dislocation of nNOS, FoxO3A activation, up-regulation of autophagy-related genes and autophagosome formation, while innervation was not affected and muscle activity persisted. Our study demonstrates, for the first time, a morphogenetic role for the 1 S subunit in adult skeletal muscle.

Abnormal glycosylation and muscular dystrophies-pathomechanism and therapeutic strategy

Tatsushi Toda

Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Japan

Hypoglycosylation and reduced laminin-binding activity of alpha-dystroglycan are common characteristics of dystroglycanopathy, which is a group of congenital and limb-girdle muscular dystrophies. Fukuyama-type congenital muscular dystrophy (FCMD), caused by a mutation in the fukutin gene, is a severe form of dystroglycanopathy. A retrotransposal insertion in fukutin is seen in almost all cases of FCMD. To better understand the molecular pathogenesis of dystroglycanopathies and to explore therapeutic strategies, we generated knock-in mice carrying the retrotransposal insertion in the mouse fukutin ortholog. Knock-in mice exhibited hypoglycosylated alpha-dystroglycan; however, no signs of muscular dystrophy were observed. More sensitive methods detected minor levels of intact alpha-dystroglycan, and solid-phase assays determined laminin binding levels to be approximately 50% of normal. In contrast, intact alpha-dystroglycan is undetectable in the dystrophic Large(myd) mouse, and laminin-binding activity is markedly reduced. These data indicate that a small amount of intact alpha-dystroglycan is sufficient to maintain muscle cell integrity in knock-in mice, suggesting that the treatment of dystroglycanopathies might not require the full recovery of glycosylation. To examine whether glycosylation defects can be restored in vivo, we performed mouse gene transfer experiments. Transfer of fukutin into knock-in mice restored glycosylation of alpha-dystroglycan. In addition, transfer of LARGE produced laminin-binding forms of alpha-dystroglycan in both knock-in mice and the POMGnT1 mutant mouse, which is another model of dystroglycanopathy. Overall, these data suggest that even partial restoration of alpha-dystroglycan glycosylation and laminin-binding activity by replacing or augmenting glycosylation-related genes might effectively prevent dystroglycanopathy progression and thus provide therapeutic benefits.

New gene therapy perspectives for motor neuron diseases

Sandra Duque, Thibaut Marais, Nicolas Chatauret, and Martine Barkats.

Inserm UMR974, Institut de Myologie.

Motor neuron (MN) diseases are neurodegenerative diseases characterized by the selective degeneration of MN in the spinal cord, the brainstem and/or the motor cortex. There is no treatment for these disorders and failure of the classical pharmacology led the scientific community to develop new therapeutic strategies based on gene transfer technology using viral vectors. Intramuscular injection of viral gene vectors and subsequent axonal retrograde transport to MNs has been successfully used in animal models of MN diseases but remains questionable for clinical application. We have developed alternative gene therapy strategies for MN diseases based on either local (brain) or systemic administration of recombinant adeno-associated vectors (rAAV). First, we showed that injection of a rAAV expressing a reporter gene into the motor cortex or the red nucleus of C57Bl6 mice allowed the production of the synthesized protein into the whole spinal cord following anterograde transport along the descending axonal pathways to the spinal cord. Moreover, we demonstrated the therapeutic potential of this method following gene transfer of the Vascular Endothelial Growth Factor (VEGF) in transgenic mice overexpressing the human copper/zinc superoxide dismutase carrying the G93A mutation (SOD1^{G93A} mice), a mouse model of amyotrophic lateral sclerosis (ALS). The second approach that we developed is based on intravenous administration of self-complementary AAV9 vectors. We demonstrated, for the first time, that this new serotype/genome based rAAV was able to transduce neural cells (including motor neurons) after intravenous injection in adult mice, despite of the presence of the blood brain barrier. Intravenous MN transduction was achieved in adults without pharmacological disruption of the BBB and transgene expression lasted at least 5 months. The therapeutic potential of this gene therapy strategy is now evaluated following intravenous injection of scAAV9 encoding the human SMN1 ("Survival of Motor Neuron") in a mouse model of amyotrophic lateral sclerosis (SMA). Our results highlight the potential of AAV vectors for efficient and durable MN gene transfer in adult animals, offering new hopes for gene therapy of MND.

JULY 4, 2009 Morning

Prophylactic treatment with sialic acid metabolites precludes the development of the myopathic phenotype in the DMRV/hIBM mouse model

Satoru Noguchi, May Christine V. Malicdan, Yukiko K. Hayashi, Ikuya Nonaka, Ichizo Nishino

Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), Japan

Distal myopathy with rimmed vacuoles (DMRV), also called hereditary inclusion body myopathy (hIBM), is an autosomal recessive debilitating disorder affecting young adults with the age of onset ranging from 15 years to late thirties. The disease is characterized clinically by preferential involvement of tibialis anterior and hamstring muscles and relative sparing of quadriceps, and pathologically by the presence of rimmed vacuoles, which are seen as clusters of autophagic vacuoles on electron microscopy, in addition to scattered atrophic fibers and muscle degeneration. Up to this time, treatment for this myopathy remains elusive. DMRV/hIBM is caused by missense mutations in *GNE* gene that encodes the essential enzyme in sialic acid biosynthesis. In the serum and skeletal muscles of patients, we have noted a reduction in sialic acid levels. We recently generated a model mouse for DMRV/hIBM that expressed human *GNE* with the missense mutation D176V, but lacks the endogenous mouse *GNE*. This DMRV/hIBM model exhibited hyposialylation in serum and various organs which predated the skeletal muscle weakness, atrophy, rimmed vacuole formation, and deposition of amyloid and various proteins within the myofibers, supporting the theory of hyposialylation in the pathomechanism of DMRV/hIBM. Because we have previously demonstrated that the sialylation status in patients' fibroblasts and myotubes can be recovered by metabolites of *GNE in vitro*, we hypothesized that similar metabolites may treat the disease. In my talk, I will show our results on preclinical trial for DMRV/hIBM using sialic acid metabolites in mouse model.

Perspectives of improvement with bezafibrate of metabolic myopathies due to fatty acid oxidation defects

Pascal Laforêt¹, John Vissing², Anthony Béhin¹, Bruno Eymard¹, Christine VianeySaban³

¹Centre de Référence de pathologie neuromusculaire Paris-Est, Groupe Hospitalier Pitié-Salpêtrière, Assistance Publique-Hôpitaux de Paris, Paris, France

²Neuromuscular clinic and research unit, Department of neurology 2082 Rigshospitalet, university of Copenhagen, Blegdamsvej 9, dk-2100, Copenhagen, Denmark.

³Centre de Référence des Maladies Héréditaires du Métabolisme, and INSERM U820, Centre de Biologie et de Pathologie Est, Hospices Civils de Lyon, 69677 Bron cedex, France

Carnitine palmitoyltransferase 2 (CPT2) and very-long chain Acyl-CoA dehydrogenase (VLCAD) deficiencies are the two most common inherited disorders of mitochondrial fatty acid oxidation (FAO) in adults. Studies of fuel utilization in subjects with CPT2 and VLCAD deficiencies, with stable isotopes during exercise, have shown that in vivo oxidation of LCFA was severely impaired during prolonged, low-intensity exercise. These findings indicated that residual CPT2 and VLCAD activities are sufficient to maintain normal oxidation of fat at rest, but that fat oxidation rate cannot increase above basal level during exercise (Orngreen et al., 2004; 2005).

Recently PPAR α receptors were identified as potential targets for pharmacological therapy of CPT2 and VLCAD deficiencies. PPAR α is a transcription factor, belonging to the superfamily of steroid-thyroid hormone receptors, which is able to modify CPT2 and VLCAD gene expression. The research group of J. Bastin hypothesized that the hypolipidemic drug bezafibrate, acting as an activator of PPAR α , might stimulate FAO in CPT2 and VLCAD-deficient cell lines. More recently, Djouadi et al. (2005) demonstrated that addition of bezafibrate in the culture medium induced a dose-dependent increase in palmitate oxidation capacities in fibroblasts from patients with the myopathic form of VLCAD deficiency, but not from severely affected patients. The above data provided the first evidence for a possible pharmacological effect of PPAR agonists on FAO defects in humans. In the last two years, a pilot clinical trial assessing the potential beneficial effects of bezafibrate on the muscular form of CPT2 deficiency has been performed in collaboration between Pitié-Salpêtrière and Necker hospitals. Six adult patients with CPT2-deficiency received a daily 400 mg dose of bezafibrate during 6 months. Clinical tolerance of the treatment was excellent, and muscular symptoms improved in 5/6 patients with a decrease in myalgia intensity and duration. LCFAO in isolated muscle mitochondria was strongly induced in 6/6 patients, and this effect was shown to result from drug-induced upregulation of CPT2 mRNA and protein levels (Bonfont et al. 2009). We propose to evaluate in a near future the effect of bezafibrate on metabolism during exercise in adult patients affected with CPT2 or VLCAD deficiencies. This study will be a randomized double blind, placebo-controlled crossover trial. The trial will be conducted in two centers: Myology Institute, Pitié-Salpêtrière hospital and neuromuscular research unit, Rigshospitalet, university of Copenhagen, in Denmark. Since exercise test on cycle ergometer after stable isotopes infusion is probably the most reliable way to assess in vivo fat oxidation, this technique will be utilized as the major outcome for this study.

Protein anchoring therapy for endplate acetylcholinesterase deficiency

Kinji Ohno¹, Mikako Ito¹, Yumi Suzuki¹, Takashi Okada², Takayasu Fukudome³, Toshiro Yoshimura⁴, Shin'ichi Takeda², Eric Krejci⁵

¹Division of Neurogenetics, Nagoya Univ, Nagoya, Japan ²Dept of Molecular Therapy, National Institute of Neuroscience, NCNP, Tokyo, Japan ³Dept of Neurology, Nagasaki Medical Center of Neurology, Nagasaki, Japan ⁴Dept of Occupational Therapy, Nagasaki Univ, Nagasaki, Japan ⁵INSERM U686, Paris, France

Congenital endplate acetylcholinesterase (AChE) deficiency is caused by mutations in *COLQ* encoding collagen Q (ColQ). ColQ makes a triple helical structure and anchors the AChE catalytic subunit to the synaptic basal lamina in the form of asymmetric AChE. There has been no rational therapy for this disorder. Here we exploited AAV8 (adeno-associated virus serotype 8), which has tropism for skeletal muscle, carrying the human *COLQ* gene (rAAV8-*COLQ*) to anchor asymmetric AChE to the synaptic basal lamina of *Colq*-knockout mice. We injected $\sim 2 \times 10^{12}$ vector genomes into the tail vein of *Colq*-knockout mice at postnatal weeks 3 to 4. In 3 weeks after injection, motor symptoms recovered to the level of the wild-type littermates, which lasted at least 17 months after injection without a decline. Cytochemical and immunohistochemical analyses of skeletal muscles disclosed AChE and ColQ expression at all the neuromuscular junctions (NMJs). Sedimentation profile of asymmetric AChE in skeletal muscles was indistinguishable from that of the wild-type littermates. The amount of asymmetric AChE in skeletal muscles was $\sim 70\%$ of the wild-type littermates. Analysis of miniature endplate potential (MEPP) revealed shortened decay time constants and reduced MEPP amplitudes. A decrement of compound muscle action potentials (CMAPs) in response to 2-Hz nerve stimulations was also improved. On electron micrographs, treated mice exhibited normal NMJs. In order to examine if the excreted asymmetric AChE was indeed delivered to remote muscle fibers, we intramuscularly administered AAV8-*COLQ* to the hindlimbs. We expectedly observed expression of asymmetric AChE at the NMJs of the forelimbs. These observations readily explain why the motor functions of the intravenously treated mice were restored almost to the normal level, even though *COLQ* should have been introduced into a limited number of skeletal muscle fibers. Our current studies imply feasibility of the "protein anchoring therapy" in which a recombinant extracellular matrix molecule expressed in a limited number of cells can be delivered to the target organ throughout the whole body by exploiting its tissue-targeting signal of the recombinant molecule itself.

Derivation of engraftable myogenic precursors from murine ES/iPS cells

Tomonari Awaya^{1,6}, Hsi Chang¹, Yuta Mizuno¹, Akira Niwa^{1,6}, Toru Iwasa¹, Katsutsugu Umeda¹, So-ichiro Fukada², Hiroshi Yamamoto², Norio Motohashi³, Yuko-Miyagoe-Suzuki³, Shin'ichi Takeda³, Shinya Yamanaka^{4,5,6}, Tatsutoshi Nakahata^{1,6}, Toshio Heike¹

¹ Department of Pediatrics, Graduate School of Medicine, Kyoto University, Kyoto, Japan

² Department of Immunology, Graduate School of Pharmaceutical Science, Osaka University, Osaka, Japan ³ Department of Molecular Therapy, National Institution of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan ⁴ Department of Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan ⁵ CREST, Japan Science and Technology Agency, Kawaguchi, Japan ⁶ Center for iPS Cell Research and Application, iCeMS, Kyoto University, Kyoto, Japan

Duchenne muscular dystrophy (DMD), caused by mutations in the X-linked dystrophin gene, is a progressive, lethal muscle disorder with no effective cure despite extensive research efforts in the field. In recent years, many different myogenic cells originating from adult tissues have been reported. However, the establishment of a reliable cell source is required for clinical application. Embryonic stem (ES) cells and the recently established induced pluripotent stem (iPS) cells are totipotent stem cells that are infinitely expandable and capable of differentiating into all types of somatic cells. In this study, we established a novel protocol to derive myogenic precursors from murine ES (mES) cells with a monoclonal antibody SM/C-2.6 that recognizes quiescent satellite cells. SM/C-2.6-positive cells are highly myogenic and efficiently differentiate into myofibers both in vitro and in vivo. Furthermore, the transplanted cells demonstrated extensive muscle regeneration activity in a second injury model without cell transplantation as well as long-term engraftment up to 24 weeks. Both these results indicated that the transplanted cells act as muscle stem cells as well as myogenic precursors. Next, we investigated the myogenic capacity of murine iPS (miPS) cells using the same protocol. miPS cells exhibit essentially the same myogenic potential as mES cells both in vitro and in vivo and also act as muscle stem cells. Our data suggest that iPS cells are a new attractive cell source for cell-based therapies.

Myostatin Blockade Therapy for Muscular Dystrophy

Yoshihide Sunada¹, Yutaka Ohsawa¹, Tadashi Okada¹, Kunihiro Tsuchida², and Sumihare Noji³

¹ Division of Neurology, Department of Internal Medicine, Kawasaki Medical School 84-9 Matsushima, Kurashiki-City, Okayama 701-0192 Japan ² Division for Therapies against Intractable Diseases, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake-City, Aichi 470-1192 Japan

³ Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, 2-1 Minami-Jyosanjima-cho, Tokushima-City, Tokushima 770-8506 Japan

Myostatin, a muscle-specific member of the TGF- β superfamily, negatively regulates skeletal muscle mass. Thus, it has been considered to be a therapeutic target of muscular dystrophy. Although an anti-myostatin antibody ameliorated dystrophic pathology and muscle strength in *mdx* mice, precise molecular mechanisms underlying the phenotypic improvement remain unknown. Our group has demonstrated that caveolin-3, the muscle-specific caveolin isoform implicated in limb-girdle muscular dystrophy 1C, suppresses activation of the type I myostatin receptor, thereby inhibiting subsequent intracellular myostatin signaling. In addition we found that myostatin signaling was enhanced in a mouse model of LGMD1C due to caveolin-3 deficiency. Overexpression of myostatin propeptide (also called prodomain) that is a potent inhibitor of myostatin activation restored muscle atrophy and exercise disability in LGMD1C model mice. Our results suggest that the myostatin blockade therapy may be effective for certain types of muscular dystrophy in which enhanced myostatin signaling is involved. We are currently taking various approaches to establish the myostatin blockade therapy, including a follistatin derivative or a peptide inhibitor for myostatin activation, a small-molecule inhibitor of the type I myostatin receptor, and anti-myostatin siRNA. Among these strategies, a small-molecule compound that inhibits the serine/threonine receptor kinase showed a significant therapeutic effect on LGMD1C model mice by oral administration lasting for ten weeks. In addition, systemic injection of anti-myostatin siRNA mixed with atelocollagen as a carrier particle significantly increased muscle mass both in wild-type and *mdx* mice. Application of these therapeutics for the treatment of patients with muscular dystrophy needs further evaluation of safety and specification of the target disease types among various muscular dystrophies.

Advances towards biotherapies for muscular dystrophies based on myostatin blockade

Helge Amthor, Christophe Hourdé, Etienne Mouisel, Julie Dumonceaux, Alban Vignaud, Arnaud Ferry and Luis Garcia

INSERM UMR S 974 / CNRS UMR 7215 and INSERM UMR S 787, Université Pierre et Marie Curie, Institut de Myologie, 75013, Paris, France

Absence or blockade of myostatin induces excessive skeletal muscle growth. During prenatal development, myostatin regulates proliferation and differentiation of muscle precursors and genetic deletion of myostatin in mice results in a moderate increase in muscle fibre numbers at birth. However, the overwhelming effect on muscle growth in lack of myostatin results from muscle hypertrophy. Interestingly, such hypertrophy involves no input from satellite cells and myofibres contained no more myonuclei and satellite cells despite a massive fibre hypertrophy. Moreover, hypertrophy of dystrophic muscle arising from myostatin blockade was achieved without any apparent enhancement of contribution of myonuclei from satellite cells. These results predict that any benefits of myostatin blockade in chronic myopathies are unlikely to impose any extra stress on the satellite cells. Muscle growth stimulation can also be brought about by blockade of myostatin at adult mouse stages such as using a gene therapy approach (overexpressing of the myostatin propeptide) or using a pharmacological approach (administration of a soluble activin IIb receptor). Importantly, postnatally induced muscle growth is associated with an increase in muscle force. However, lack of myostatin results in a profound muscle fibre type conversion towards a glycolytic phenotype. Such changes in oxidative properties are associated with a decreased fatigue resistance and decreased exercise capacities. Currently, we are exploring the effect of myostatin blockade in combination with dystrophin exon skipping in mdx mouse, the mouse model of Duchenne muscular dystrophy. First results of this ongoing work will be presented. Furthermore, we currently begin a preclinical trial using myostatin blockade on GRMD dogs.

Muscle mass regulation by myostatin-signaling related molecules

Shin-ichiro Nishimatsu¹, Shingo Tanaka¹, Hiroshi Kiyonari², Yasunori Hayashibara², Shinichi Aizawa², Yutaka Ohsawa³, Yoshihide Sunada³, and Tsutomu Nohno¹

¹ Department of Molecular and Developmental Biology, Kawasaki Medical School, Okayama Japan ² Laboratory for Animal Resources and Genetic Engineering, RIKEN Center for Developmental Biology, Kobe Japan ³ Department of Neurology, Kawasaki Medical School, Okayama Japan

Myostatin is a member of the transforming growth factor-beta family and negatively regulates skeletal muscle mass. Inhibition of myostatin signaling has been a possible therapeutic strategy to ameliorate some types of muscular dystrophy. A clinical trial using a neutralizing antibody to myostatin, MYO-029 showed that the antibody is safe but not effective for stimulating muscle growth in muscular dystrophy. More powerful inhibitors and alternatives are necessary to overcome the problem. We focus on furin and Wnt4, which are located in upstream and/or downstream of the myostatin signaling. Furin is a serine protease and cleaves pro-myostatin into mature protein. Wnt4 is a secreted protein, induced by inhibition of myostatin signaling. To examine their functions in skeletal muscle formation and regeneration, we have begun to generate mutant mice. We will present the latest results and discuss the possibilities for the future.

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Viral vector mediated dystrophin gene therapies for muscular dystrophy models

En Kimura M.D. Ph.D.¹, Masatoshi Ishizaki M.D. Ph.D.¹, Katsuhisa Uchino M.D.¹, Tomohiro Suga M.D.¹, Tatsuya Koide M.D.¹, Ryoko Kawano M.D. Ph.D.¹, Yuji Uchida M.D. Ph.D.², Yasushi Maeda M.D. Ph.D.¹, Jeff Chamberlain Ph.D.³, and Makoto Uchino M.D. Ph.D.¹

¹ Department of Neurology, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan ² Laboratory of Pharmacology, Division of Life Science, Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan ³ Departments of Neurology, Biochemistry, Medicine, University of Washington School of Medicine, Seattle, WA, USA

Duchenne muscular dystrophy (DMD) is an inherited severe muscle wasting disorder, and there is currently no effective treatment. DMD causes respiratory and/or cardiac failure and results in death at about 20 years of age. Respiratory insufficiency is currently the major problem in the management of the patients. In the *mdx* mouse, a DMD model, the pathological features of diaphragm muscle are severely affected, similar to the skeletal muscle of human patients. Although, specific force measurements of the dystrophic diaphragm have been used to estimate *mdx* mouse respiratory impairment, systemic functional assessments compared with histopathological analyses have not been demonstrated. Here we present a sensitive procedure using whole-body plethysmography to monitor respiratory parameters detected during early insufficiency in the *mdx* mouse. The results confirm that the dystrophic changes in the diaphragm lead to respiratory dysfunctions. We have also developed viral vector based gene therapy technologies: helper dependent adenoviral (HDAv), lentiviral, and adeno-associated viral (AAV) vector systems, which may target skeletal muscles including the respiratory muscles. We show here efficient gene delivery and expression in respiratory muscles, especially diaphragm. The efficient dystrophin gene replacement in *mdx* diaphragm muscle fibers results in improved respiratory function as well as histopathological improvement of the diaphragm muscles. Our data suggest that the combination of viral vector based gene therapy approaches and the total assessment system, including whole-body plethysmography, may be useful to evaluate the therapeutic approaches for respiratory impairments of neuro-muscular disease models, with a goal of developing the future clinical applications.

Exon skipping therapy toward Duchenne muscular dystrophy

Shin'ichi Takeda

Department of Molecular Therapy, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawa-higashi, Kodaira, Tokyo 187-8502, Japan

Duchenne muscular dystrophy (DMD) is caused by the lack of dystrophin at the sarcolemma. Exon skipping by antisense oligonucleotides is a novel method to restore the reading frame of the mutated *DMD* gene, and rescue dystrophin expression. We recently reported that systemic delivery of Morpholino antisense oligonucleotides targeting exon 6 and 8 of the canine *DMD* gene, efficiently recovered functional dystrophin at the sarcolemma of dystrophic dogs, and improved activities of affected dogs without serious side effects (Yokota *et al.*, *Ann Neurol*, in press). To optimize therapeutic antisense Morpholinos for more frequent mutations of the *DMD* gene, we designed 14 kinds of antisense Morpholinos targeting exon 51 of the mouse *DMD* gene, and injected them separately or in combination into the muscles of *mdx52* mice, in which exon 52 has been deleted by a gene targeting technique (Araki *et al.*, *BBRC*, 1997). A combination of two Morpholinos, targeting the acceptor and the donor splice sites of exon 51, respectively, showed an excellent restoration of sarcolemmal dystrophin in injected muscle. We, therefore, intravenously injected them into *mdx52* mice at 7 times weekly. Two weeks after the final injection, dystrophin was expressed at the sarcolemma throughout the body, with an average of about 10-50% of normal levels. This was accompanied by amelioration of dystrophic pathology, and improvement of contractile force of EDL, grip power test, and treadmill performance. Blood tests and histological examination of liver and kidney indicated no evidence of toxicity. DNA microarray analysis showed that the treatment reduced the levels of several C-C chemokine ligands in treated *mdx52* muscle to normal levels. This study provides a proof of concept for exon 51 skipping in the DMD animal model and that can be applicable up to 15% of DMD deletion patients.