P.19.7
Muscle wasting and repair after injury can be potentially modulated by autologous growth factors combined with a TGF-β antagonist
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Injured skeletal muscle repairs spontaneously via regeneration; however, this process is often incomplete because of fibrotic tissue formation. Growth factors from platelet-rich plasma (PRP) with proven effects in tendonous and ligamentous healing have not yet been studied in skeletal muscles mainly due to the concern that exogenous TGF-β application could lead to even greater fibrosis development in an injured muscle. Therefore, only some TGF-β antagonists like decorin have shown their positive role in muscle repair so far. In our study, we investigated the effects of PRP in combination with TGF-β antagonist decorin in skeletal muscle regeneration.

A novel human myoblast cell culture, defined as CD56 (NCAM)+ developed in our laboratory, was used for evaluation of potential bioactivity of PRP and decorin. The influence on the cells mitochondrial activity, expression of TGF-β was studied in parallel with cell proliferation. Further we have studied the ability of the therapeutic agents to influence the differential cascade of dormant myoblasts towards fully differentiated myotubes by monitoring step wise activation of single nuclear factors like MyoD and Myogenin via multicolor flow cytometry.

Our results clearly showed that PRP and decorin treated myoblasts have a significant increase in the mitochondrial activity and in the cell proliferation rate as compared to non-treated control cells. At the same time lower expression of TGF-β and MSTN was evident in PRP treated myoblasts, although PRP itself contains some amount of TGF-β. Further, significant increase in desmin and myogenin positive cells was detected together with MyoD down-regulation.

Despite concerns about promoting fibrosis development, PRP inhibits the TGF-β and MSTN activity to even greater extent than decorin alone. We can conclude that PRP can be a highly potential therapeutic agent for skeletal muscle regeneration and repair, especially if in combination with a TGF-β antagonist.

http://dx.doi:10.1016/j.nmd.2013.06.688

P.19.8
The effect of water-soluble fullerene with different number of hydroxyl groups in muscle regeneration process of experimental murine skeletal muscle
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Muscular dystrophy is a hereditary disease, which cause severe muscle weakness and atrophy in clinically, and skeletal muscle degeneration and necrosis in pathology. In recent years, reducing oxidative stress is considered as one of the new treatment strategy in muscular dystrophy. Fullerenes are a good candidate to detoxify and absorb free radical. We have already revealed that fullerene has a function to promote the regeneration of skeletal muscle. To evaluate the effectiveness of different type of water-soluble fullerenes, we chronologically evaluate the histology in mice tibial muscles during a cycle of regeneration induced by cardiotoxin injection.

We used three types of water-soluble fullerene, 44 hydroxyl fullerene, fullerene with PVP, and fullerene with hydrophilic cyclodextrin. Tibial muscles of C57BL10 mice (4–5 weeks old) were injected with cardiotoxin with 3 different types of fullerene or without fullerene. The injected muscles were removed and stained H&E on 1, 3, 5, 7, 14, and 28 days after the injection. Western-blotting was performed to evaluate expression of muscle proteins, such as dystrophin, desmin, and nNOS.

Average diameter of regenerate muscles 28 days after injection, fullerene group was bigger than those of cardiotoxin group. There was no difference of average diameter with 3 types of fullerene. Muscle protein expression in co-administered fullerene group, dystrophin, desmin, and nNOS was observed in the earlier stage by Western blot than those of cardiotoxin group. During the experiment, there is no significant and critical change was observed.

We revealed that 44 hydroxyl fullerene, fullerene with PVP, and fullerene with hydrophilic cyclodextrin has the same function to promote the regeneration of skeletal muscle in this experiment. We believe that water-soluble fullerene reduce oxidative stress and can be applied in the treatment of muscular dystrophy.

http://dx.doi:10.1016/j.nmd.2013.06.689

P.19.9
Gz12 signaling is required for skeletal muscle regeneration and for satellite cell differentiation
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We have previously shown that expression of a constitutively active mutant of the α subunit of the heterotrimeric Gzα2 protein, Gzα2 (Q205L), is sufficient to induce skeletal muscle hypertrophy, myoblast differentiation and to accelerate muscle regeneration. To elucidate the requirement of Gzα2 in skeletal muscle growth and regeneration we have analyzed mice genetically deficient in Gzα2. We show that Gzα2 KO mice display decreased lean body mass, reduced muscle size and impaired skeletal muscle regeneration after cardiotoxin-induced injury. In addition, Gzα2 expression is increased during skeletal muscle regeneration after cardiotoxin injection. ShRNA-mediated knock down of Gzα2 in satellite cells leads to reduced expression of the activation markers Pax3 and Pax7, as well as defective satellite cell proliferation, fusion and differentiation ex vivo. The impaired differentiation is consistent with the observation that the myogenic regulatory factors MyoD and Myf5 are down-regulated upon knock down of Gzα2. Interestingly, the expression of miR-1 and miR-206, two microRNAs that have been shown to regulate satellite cell proliferation and differentiation, is upregulated by a constitutively active mutant of Gzα2. These findings provide evidence for the requirement of Gzα2 in satellite cell differentiation ex vivo and for skeletal muscle regeneration in vivo.

http://dx.doi:10.1016/j.nmd.2013.06.690

DYSTROPHINOPATHY IMMUNITY, FIBROSIS AND GENE THERAPY

P.20.1
Optimised dystrophin mini-constructs for gene delivery
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The aim of our project is to obtain an optimal dystrophin construct that will be lentivirally-transduced into skeletal muscle stem cells derived from Duchenne Muscular Dystrophy (DMD) patients. These stem cells
would restore functional dystrophin expression following their transplantation into dystrophin-deficient skeletal muscles. To this end, we have cloned a number of different dystrophin truncated forms ranging from 4.2 kb to 8.4 kb cDNA, encoding various components of the dystrophin molecule that may be used in a lentiviral system focusing on elongated transcript length and selected dystrophin domains. The constructs were assessed using in vivo and in vitro tests for functionality, such as membrane stability following osmotic shock as measured by creatine kinase release in myotubes and electroporation into mdx muscle. The construction, cloning strategy and characterisation of the constructs as well as first functional results will be presented. We are aware of challenges to gene transfer approaches and know that further long-term experiments are required to assess the potential of this strategy, but this project may bring us closer to a robust and reproducible system for testing functionality of both engineered and naturally occurring dystrophin mutants.

http://dx.doi.org/10.1016/j.jnmd.2013.06.691

P.20.2
Restoration of dystrophin expression after skipping of single and double exon DMD duplications in patient-derived cell lines using antisense oligonucleotide and AAV-U7snRNA approaches

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Exon skipping strategies in Duchenne muscular dystrophy (DMD) have largely been directed toward altering splicing of exons flanking out-of-frame deletions or excluding exons carrying a point mutation in order to restore an open mRNA reading frame. Translation of such a transcript results in an internally truncated yet partially functional dystrophin protein, typically associated with the milder Becker muscular dystrophy (BMD). We sought to apply exon skipping to duplication mutations of one or two exons, making use of the inherently limited efficiency of exon skipping in vivo to generate significant amounts of wild-type, full-length DMD mRNA.

We developed an improved lentiviral-delivered tet-inducible MyoD construct and used it to generate stably-infected fibroblast cell lines from patients with a variety of singly (exons 2, 12, 18, 21, and 44) or doubly (exons 3-4, and 8-9) duplicated exons. Treatment of each cell line with doxycycline results in transdifferentiation into the myogenic lineage and expression of the DMD gene. Using a variety of 2’O-Me antisense oligonucleotides, significant skipping can be induced for each duplication, leading to a wild-type transcript as a major mRNA product. Furthermore, for two duplications – exon 2 (the most common single exon duplication) and the tandem duplication of exons 8-9 – we have developed AAV-U7snRNA vectors; after efficient transduction of these into the corresponding cell line, differentiation into myotubes results in restoration of dystrophin protein expression.

These results confirm that this cell model is a reliable system to assess restoration of dystrophin expression, and suggest that exon skipping if a feasible approach to therapy for single or double exon duplication mutations that can result in expression of full-length protein, suggesting that personalized exon skipping may be expected to be highly beneficial for a subset of DMD patients.

http://dx.doi.org/10.1016/j.jnmd.2013.06.692

P.20.3
Targeting fibrosis and inflammation in Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy is the most frequent genetic muscle disease worldwide affecting ~1:5000 male births. It is caused by a defective DMD gene, which leads to reduced and defective dystrophin protein expression. The constant breakdown of fibres leads to focal necrosis, myophagocytosis and a considerable influx of inflammatory cells into the muscle tissue, which is followed by increasing endomysial fibrosis. Both, inflammation and fibrosis as well as a putative relation are not yet understood immunologically. Fibrosis directly correlates with adverse outcome and early loss of ambulation. We have studied how inflammation is linked to fibrosis in DMD, with an emphasis on the communication between fibroblasts, macrophages and the immune response, especially Th2-immunity, at different time points of disease and identified target molecules involved in this process. Classically activated M1 macrophages exhibit a pro-inflammatory phenotype, while alternatively activated (M2) macrophages are profibrotic and are therefore thought to exhibit a ‘repair phenotype' but may be deleterious at certain stages of the disease in DMD. We have analysed muscle biopsies derived from patients suffering from DMD, which were obtained at different time-points after onset of disease. The immune response was studied on the protein and on the mRNA levels. Depending on the time-point and disease activity, the immune response showed a Th1-M1 or Th2-M2 phenotype respectively. Increasing fibrosis was associated with an M2 polarized immune milieu. The results of this study may provide a basis for the development of a specifically targeted and putatively time-dependent immune intervention in DMD patients, based on the immune profile of their muscle biopsy specimen, which can be systematically and individually assessed. This approach may provide a useful additional therapeutic intervention in addition to modern gene-therapeutic approaches.

http://dx.doi.org/10.1016/j.jnmd.2013.06.693

P20.4
Antithetic role of miR-21 and miR-29 in the progression of fibrosis in Duchenne muscular dystrophy

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Muscle fibrosis that progressively replaces muscle fibers in severe muscular dystrophies and chronic myopathies is characterized by excessive deposition of collagen and other extracellular matrix (ECM) proteins. Recent data indicate a major involvement of microRNAs in regulation of pro- and anti-fibrotic genes. In particular, persistent over-expression of miR-21 perturbs tissue repair and contributes to tissue fibrosis; furthermore, induction of miR-21 expression by TGF-beta 1 treatment indicates that it may mediate TGF-beta 1 fibrogenic activity. In fibrotic diseases reduced miR-29 expression correlates with increased expression of collagens and ECM-related genes, while in skeletal muscle it acts as pro-myo-genic factor during muscle cell differentiation, suggesting a major role of miR-29 in ECM deposition and remodelling and in muscle regeneration.

To define the role of miR-21 and miR-29 in Duchenne muscle dystrophy (DMD), we evaluated their expression in DMD muscles and in muscle-derived fibroblasts and myoblasts. Our results show significant increase of miR-21 expression in DMD fibroblasts, more marked after TGF-beta 1 treatment, and significant reduction of miR-29a and miR-29c expression in myoblasts. In DMD fibroblasts we also observed changes in miR-21 target genes including Pten and Sprouty-1, that were significantly reduced and collagen I and VI that were significantly increased. In DMD fibro-