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T.P.22

Novel antisense GNE myopathy therapy using NMR 1H imaging and 31P spectroscopy


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Replacement therapy of dystrophinopathies has begun with promising results both in animals and in patients. Non-invasive quantitative tools are needed to evaluate its potential benefits or side effects but also to determine the optimal protocol. Fifteen GRMD dogs were treated by unilateral high-pressure high-volume injection into the cephalic vein with a rAAV8-U7-ES6-ESE8 solution. They were divided into 6 groups according to the dose of viral particles and the volume injected. The lowest dose-lower volume combination was skipped. Two dogs were injected with saline only. After high dose injection, some muscles displayed up to 80% of dystrophin positive fibers. Three months after injection, we evaluated both forearms with spectroscopy at 4T and NMR imaging at 3T. Comparisons between injected and non-injected arm were blinded. All indices of mock dogs were within the reference range of the GRMD population. Both spectroscopy and imaging indices showed differences between the two arms at the highest dose irrespective of volume injected and at the intermediate dose-high volume combination. The putative identification of the treated arm proved systematically correct. Among the spectroscopy indices, Pi/PCr, PCr/ATP and PDE/(Pi + PCr) were the most sensitive and 31P NMR showed changes proportional to the number of AAV particles injected. With regard to imaging, 31 indices were evaluated in the extensor carpi radialis and flexor carpi ulnaris. The most relevant were: muscle heterogeneity in T2w images, the T1w/T2w signal ratio (SR), the T2w/PDw SR and the maximal relative signal enhancement after injection of 0.5 mmol/kg gadoteric acid. When high or intermediate doses were injected, indices actually decreased towards the normal range in both arms, with a reduction that was more substantial in the muscles of the injected side. This study demonstrated NMR ability to detect changes in dystrophic muscle structure and metabolism in response to exon-skipping therapy.

We have tested different types of polymeric cationic core-shell nanoparticles (NPs) for delivering 2-O-methyl-phosphorothioate antisense oligoribonucleotides (AONs), in mdx mice. Both T1 and ZM2 NP bind and convey AONs: intraperitoneal (IP) injections of low doses (52.5 mg/kg) of NP-AON complex restored dystrophin protein synthesis in skeletal and cardiac muscles, allowing protein localization in up to 40% of muscle fibers with skipping level up to 20%. We have tested in vivo (mdx) the tissue biodistribution and elimination timing of NPs by Odyssey, using an infrared dye conjugated ZM2 NP. Administration mode was both IP and oral. Elimination by feces was up to 80% after 22 days of a single injection treatment in mdx treated by intraperitoneal doses, and close to 100% in those treated orally after 72 h of a single dose treatment. Evaluation of NP biodistribution, measured by Odyssey, in organs/tissues cryosections of sacrificed mdx revealed an intense positivity of labeled NP in muscles, heart, intestine, and all organs. We also demonstrated that NP-AON formulations pass the gastric barrier and induce dystrophin rescue in the intestinal smooth muscles as well as in the diaphragm. However the intense positivity at biodistribution of NPs in the heart (and skeletal muscles) conflicts with the relatively low efficacy of the compound in terms of skipping and dystrophin rescue. We are therefore performing ELISA assay to dose the antisense in the treated mice muscles in order to define the release kinetic of the AON from NPs. This is crucial in order to further proceed with other NP studies.

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T.P.24

Correction of the GNE Myopathy M712T founder mutation by trans-splicing

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GNE Myopathy is a rare neuromuscular worldwide autosomal recessive disease, which is very common among Persian Jews. The disorder results from mutations in the gene UDP N-Acetylgalactosamine 2-epimerase/N-Acetylmannosaminidase kinase (GNE). To date, over 60 different mutations in GNE have been reported to cause the disease worldwide, but a...
single homozygous mutation, M712T, has been identified in all patients of Persian descent, 10 amino acids before the end of the protein. The same mutation was found in other Jews and non-Jews families in the Middle East therefore we term it the Middle East mutation. The mechanism leading from GNE mutations to the myopathy phenotype is not yet understood. Since the disease is recessive, it most likely results from the lack of wild type GNE protein; therefore the production of wild type protein in patients might alleviate the disease phenotype. Trans-splicing is a phenomenon where splicing between two different pre-mRNA molecules occurs. This mechanism can be used to address mutated gene products and correct transcripts defects on the transcript itself, by altering the cis-splicing process but conserving the original endogenous regulation of the gene. Based on an Adeno Associated Virus platform we have generated a series of transsplicing vectors designed to transsplice the last exon of GNE which carries the most frequent mutation occurring in GNE myopathy. Transfection and injection of these vectors result in transspliced transcripts both in mouse and human cells. Furthermore, human transsplicing vectors are able to partially correct the M712T mutation in patients derived muscle cells. The goal of this project is to use an AAV based trans-splicing vector as a gene therapy tool to overcome the GNE myopathy mutation M712T.

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T.P.25
Development of the exon exchange method for repair of mutant nebulin transcripts
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The nebulin gene (NEB) has 183 exons encoding transcripts up to 26 kb in length. Mutations found in NEB are dispersed throughout the gene, i.e. no mutational hot spots are evident. Mutations cause autosomal recessive nemaline myopathy, distal nebulin myopathy and core-rod myopathy, for which no therapy is available. The size of NEB limits the options of gene therapy development. Thus, our research has focused on methods correcting the mutation carrying transcripts. Targeted exon exchange requires the spliceosome provided by the cell in addition to the target pre-mRNA and pre-transsplicing molecules (PTMs) both produced from expression vectors transfected into C2C12 mouse myoblasts. We have developed wild type and mutant NEB minigenes for production of target pre-mRNA and PTMs for internal exon exchange of one or several NEB exons at a time. We have previously obtained successful results from our 3′ exon exchange and first internal exon exchange experiments and continue to develop the internal exon exchange technique further. NEB introns are usually short and binding of PTM can interfere with exon recognition. We have designed PTMs targeted to larger introns on both sides of an area of two exons close to each other to overcome this problem. Exon exchange is assessed by RT-PCR and sequencing and at the protein level by Western blotting and immunofluorescence staining. Our preliminary results from co-transfection of mutant minigenes and PTMs indicate successful, but weak trans-splicing at the RNA level. Immunofluorescence staining of co-transfected C2C12 cells show protein production in some cells, which may indicate successful trans-splicing, but the results need to be confirmed by western blotting. Being able to exchange several exons at a time from the 183-exon-containing NEB at the RNA level would be an advantage in therapy as the same therapy molecule would be useful for patients with mutations in any of the exons in the exchanged area.

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T.P.26
Low dystrophin levels increase survival and improve pathology and motor function in dystrophin/utrophin double knockout mice
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In Duchenne Muscular Dystrophy (DMD) patients muscle fibers are susceptible to exercise-induced injury due to absence of functional dystrophin. No cure is available, but in the last decade major progress has been made in the challenge to restore dystrophin expression in DMD patients. It is unknown how much dystrophin is needed to slow or prevent disease progression. To elucidate this, we generated mdx-Xist—/—mice in which skew X-inactivation results in expression of variable, low dystrophin levels in a utrophin negative background. These mice (n = 20) underwent a 12 week functional test regime after which histopathology was assessed. Dystrophin levels of 3–10% already significantly improved performance of two and four limb hanging wire tests and histopathology, while 10–17% further normalized this towards wild type. For improvement in grip strength higher dystrophin levels are needed. Most striking was the effect of already very modest dystrophin levels in maintenance of basic muscle function and protection against death from overall weakness. Whereas mdx—/— mice did not live beyond 12 weeks, 62% of the mice expressing 3–10% dystrophin and all mice expressing 10–17% dystrophin survived 16 weeks. A survival study in 42 mdx-Xist—/— mice assessing skeletal muscle function and histopathology showed a median survival extension to 26 weeks in mice with 3–10% dystrophin, while mice with 10–30% lived even longer. Biomarkers, skeletal muscle and heart function, and histopathology were significantly improved in mice with 3–10% dystrophin and further improvement was achieved with 10–30% dystrophin. These results suggest that even very low dystrophin levels already may have beneficial effects, and that survival and improvement of endurance efforts may be amongst the early effects of treatment. This underscores the urgency to develop better clinical readouts for the non-ambulatory phase.

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T.P.27
Characterization of the modular domains of dysferlin for gene transfer
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Dysferlin, a transmembrane protein involved in muscle membrane repair and T-tubule homeostasis is composed by several domains including seven C2 domains. Supported by the identification of specific protein partners for some of these domains, independent function of each domain has been proposed without a clear demonstration until now. Since the identification of a partially functional, naturally occurring “mini-dysferlin” protein composed of the last two C2 and C-terminal trans-membrane domains (Krahn et al., 2010), we decided to better characterize the modular properties of dysferlin. In order to test several combinations of dysferlin domains to obtain the most functional construct we employed a “mini-gene” transfer approach based on AAV vectors. Features of the native dysferlin and notably topological and phylogenetic studies were conserved in the design of six different “mid-dysferlins”. We first analyzed the expression and stability of our constructs in cellular models and we found that, even if proteins show different levels of expression, their stability are not affected. Then, using micro-patterned support of culture (CYTOO technology), we showed that all our constructs are correctly addressed