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Co-expression of phenylalanine hydroxylase and tetrahydrobiopterin-cofactor genes by using polycistronic rAAV vectors for PKU gene therapy

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Background: Phenylketonuria (PKU) is an autosomal recessive disorder caused by deficiency of the hepatic phenylalanine hydroxylase (PAH) that clears plasma phenylalanine by conversion to tyrosine using molecular oxygen and tetrahydrobiopterin (BH4) as cofactor. Biosynthesis of BH4 requires the enzymes GTP cyclohydrolase I (GTP-CH), 6-pyruvoyltetrahydropterin synthase (PTPS), and sepiapterin reductase, only the latter being ubiquitously and constitutively expressed.
Objective: Hyperphenylalaninemia is a metabolic disease which can potentially be corrected by heterologous gene expression and degradation of phenylalanine in another organ than liver. However, for efficient degradation of phenylalanine in for instance muscle or blood tissues, that do not produce sufficient BH4 cofactor, heterologous expression of PAH together with at least two BH4 biosynthetic genes is required.
Methods: We generated various polycistronic gene expression cassettes by the use of different 'internal ribosome entry sites' (IRES) between the mouse cDNAs for PAH, PTPS and/or GTPCH. Expression from the CMV promoter with or without the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was tested. The level of expression from AAV-vector plasmids was verified in a first step by transient lipofectamine-transfection into COS-1 cells, followed by assaying specific enzyme activities.
Results: Whereas untransfected COS-1 cells do not express PAH (<0.4 mU/mg), GTPCH, and PTPS (<0.05 microU/mg), we found expression from single rAAV-plasmids between 1-10 mU/mg for PAH, 39-190 microU/mg for GTPCH, and 64-116 microU/mg for PTPS. Thus, expression in at least this cell line was found to be significant, with various expression levels from the different constructs. Production of high-titer rAAV and tests in a mouse muscle cell line are under way. Conclusion: Muscle or blood as heterologous candidate tissues that do not produce sufficient BH4 to support PAH activity can now be tested for potential efficacy of phenylalanine clearance with these rAAV-vectors by direct injection into either skeletal muscle or tail vein of a PKU mouse model.

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The use of small inteference RNA technology for the identification of differentially expressed genes in duchenne muscular dystrophy: possible targets for gene therapy

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Duchenne Muscular Dystrophy (DMD) is part of a group of genetically heterogenous muscular dystrophies that is characterized by progressive weakness and wasting of skeletal muscle. Loss of myofibers is caused by dystrophin deficiency, a protein responsible for myofiber maintenance and integrity. Dystrophin forms a link between the cytoskeleton and the membrane-spanning dystrophin-associated glycoprotein complex (DAPC). Proteins which form part of the DAPC have been found to be mutated in other inherited forms of muscular dystrophy like limb girdle muscular dystrophy. The applications of gene therapy protocols for DMD still presents many daunting challenges which is partly due to intrinsic features of the dystrophin gene. Hence, improvement of the understanding of the underlying primary molecular events leading to a dystrophic pathology might pave the way for the discovery of new starting points. Here we present an approach to use siRNA in an *in vitro* system to study the events occurring early in muscle cell development due to dystrophin deficiency. siRNA has been proven to be a powerful technology to study molecular effects due to a knock down of singular genes. Zyxin, a gene involved in focal adhesion, was used as a control to test the suitability of C2C12 cells for siRNA studies and optimize conditions. In addition time courses of primary mouse muscle cultures were characterized with a subset of markers for their suitability for siRNA studies. The establishment of a siRNA protocol for primary muscle cell cultures represents a flexible and efficient strategy to study the effects of deficiency of varies genes leading to muscular dystrophy and will lead to the identification of numerous targets for the development of alternative gene therapy strategies.

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Feline immunodeficiency virus (FIV)-based lentiviral vectors for therapeutic correction of ornithine transcarbamylase deficiency (OTCD)

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Background: FIV-based lentiviral vectors have been shown by us to stably and efficiently transduce hepatocellular carcinoma cell lines, *in vitro*, as well as murine hepatocytes, *in vivo*. These vectors undergo integration into the host cell genome and therefore lead to prolonged gene expression. We hypothesized that transduction of hepatocytes, with FIV-based lentiviral vectors may provide sufficient levels of transgene expression for phenotypic correction of liver enzyme deficiencies. Orninthine transcarbamylase (OTC) is the second enzyme in the mammalian urea cycle and OTC deficiency (OTCD) is the most common inborn error of urea synthesis in humans. Approximately half of affected males develop marked elevations of ammonia leading to coma in the first week of life. Sustained hyperammonemia caused by OTCD leads to significant cognitive deficits. Current therapy for OTCD involves dietary nitrogen restriction combined with the stimulation of alternate pathways of waste nitrogen excretion. The only other available treatment is liver transplant.
Objective: In the present study, we investigated the possibility of using FIV-based lentiviral vectors containing the OTC sequence for delivery to liver cells.
Methods: OTCD murine hepatoma cells (BWTG3) and transformed murine hepatocytes (BNL1ME) were transduced with VSV-G pseudotyped FIV-based vectors encoding for murine OTC (mOTC) driven by a liver-specific promoter.
Results: PCR analysis revealed the presence of vector sequences in genomic DNA extracted from transduced cells. The presence of OTC RNA was observed by dot blot analysis using an mOTC cDNA probe. Transgene expression and enzyme activity were monitored by histochemistry, which revealed the intracellular location of the enzyme. Analyses included untransduced control cells, which demonstrated the absence of vector sequence DNA, RNA, and transgene expression. Conclusion: The use of FIV-based lentiviral vectors for correction of OTCD may be a novel paradigm for therapy of other liver enzyme deficiencies.