**MYOBLAST MEDIATED GENE TRANSFER TO SKELETAL MUSCLE BASED ON NON-VIRAL VECTORS**

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**Introduction:** Duchenne Muscular Dystrophy is a progressive muscle weakness that is characterized by a lack of dystrophin expression in the sarcosome of muscle fibers. Two major approaches have been investigated to deliver dystrophin in dystrophic muscle: myoblast transplantation and gene therapy. Myoblast transplantation has been hindered by the immune rejection problems, as well as the low spreading and poor survival of the injected myoblasts.1-3 Gene therapy based on viral vectors has been limited by the viral cytotoxicity, the immune rejection problems, as well as the low spreading and poor survival of the injected viral transduction of muscle fibers.4-6 Even though ex vivo gene transfer can be utilized as a gene delivery mechanism, the possibility exists that virally infected cells can present viral antigens and be immune-rejected by the host.7 Indeed, we have observed that myoblast-mediated ex vivo gene transfer of genes to skeletal muscle of mice only results in a short-term expression of the transgene; CD4- and CD8-activated lymphocytes have been detected in the injected muscle at 25 days post-injection.7 We developed a new approach based on the use of non-viral vectors through the ex vivo approach to deliver genes in skeletal muscles. We have observed that non-virally transfected myoblasts can be used to deliver β-galactosidase and dystrophin in mdx mouse muscle. Although the number of dystrophin positive myofibers decreases over time, this approach allows the delivery of dystrophin, which can persist up to 90 days in the injected mdx muscle. The use of myoblast mediated ex vivo gene transfer of non-viral vectors may help to circumvent limitations facing the application of cell and gene therapy to skeletal muscle, because it will allow efficient gene transfer to mature skeletal muscle.

Furthermore, the use of plasmid DNA will reduce the immune rejection problems.

**Methods:** The policies and procedures of the animal laboratory are in accordance with those detailed by the USA Department of Health and Human Services. The research protocols used for these experiments were approved by the Animal Research and Care Committee (ARCC) at the authors' institution. Services. The research protocols used for these experiments were approved by the Animal Research and Care Committee (ARCC) at the authors' institution. The ARCC's approval ensures adherence to the USA Department of Health and Human Services guidelines for animal research. Services. The research protocols used for these experiments were approved by the ARCC, which ensures compliance with animal welfare regulations. Services. The research protocols used for these experiments were approved by the ARCC, which guarantees the ethical treatment of animals.

**Preparation of the plasmid DNA.** The plasmid carrying dystrophin, which was used in this experiment, was constructed on a pBluescript plasmid backbone and the mouse cDNA full-length dystrophin expression was driven by the MCK promoter and neomycin-resistant gene under the human cytoskeletal glutamin (HCMV) promoter. The β-galactosidase expressing plasmid (pBluescript) used carried the β-galactosidase gene under the MCK promoter and the neomycin-resistant gene under the HCMV promoter. Ex vivo approach based on plasmid DNA. We established the cell and gene therapy condition of ex vivo gene transfer in primary and immortalized mdx mouse myoblasts. The transfected myoblasts were selected under G418-supplemented media. The pure population of transfected myoblasts was evaluated for their capacity to regenerate muscle fibers in vivo by myoblast-mediated ex vivo gene transfer approach. Newborn mdx mice were used as primary myoblast donors. The myoblasts were purified by the preplate technique, and the population of muscle-derived cells displaying over 90% desmin positive cells was used for this experiment.8 Three-week-old mice (period of massive muscle degeneration) were used as hosts for transplantation. Preparation of myoblasts. Different populations of transfected mdx myoblasts were used for these experiments. Primary mdx myoblast cultures were transfected with two different plasmids (plasmid encoding for β-galactosidase and full-length dystrophin expression). Forty eight hours post-transfection, the different groups of transfected myoblasts were selected under neomycin resistance (G418) for 1 additional week until confluence; and intramuscularly injected as a pellet with a Drummond syringe. At different time points post-injection, the animals were sacrificed and the injected muscles assayed for LacZ staining and dystrophin expression (immunohistochemistry). The β-galactosidase expression was monitored by histochemistry and β-galactosidase assay (ONPG). The myoblasts were injected at several sites into the injected muscle to achieve an efficient distribution of the cells to determine whether the transfected myoblasts can fuse and deliver dystrophin and β-galactosidase into the mdx injected muscle.

**Results:** First, myoblasts were isolated from the tibialis anterior of mdx muscle. The cells were subsequently transfected with a plasmid encoding for the β-galactosidase reporter gene under the control of the human cytomegalovirus promoter, the dystrophin under the control of the CAG promoter, and the neomycin resistance gene under the control of the PGK promoter. The cells following the transfection were selected under G418, with the resistant colonies selected and expanded to obtain large quantities of transfected myoblasts. The selected population of myoblasts expressing the neomycin resistance gene, dystrophin, and β-galactosidase was injected in the gastrocnemius muscle of the mdx mouse.

The selected colonies expressing the β-galactosidase reporter gene were expanded (Figure #1a) and the resulting cells were capable to differentiate into myotubes which expressed dystrophin (Figure #1b). The transplantation of these transfected cells in the mdx muscle resulted in the production of β-galactosidase and dystrophin expressing myofibers at 5 days post-injection (Figure #2). Many dystrophin and β-galactosidase positive fibers were found in the injected dystrophic muscle at 5 and 15 days post-injection. A major decrease in the number of dystrophin positive myofibers was found at 30 days post-injection, which persisted for up to 90 days post-injection (Figure #3). The number of dystrophin positive myofibers was monitored at different time points.

The detection of CD4 and CD8 activated lymphocytes in the injected muscle at 15, 30, 45, 60, 75, and 90 days following injection suggests that the transplantation of the transfected myoblasts triggered an immune response. The number of activated lymphocytes was greatly reduced after 30 days following injection, suggesting that the immune response decreased within graft maturation. The antigenic nature of the immune response remains unknown, but the β-galactosidase has been found immunogenic in many experiments and represents a good candidate antigen for this immune response.

**Discussion:** Gene transfer to skeletal muscle has been hindered by two major limitations: the immune rejection problems related to viral infection and the inability of viral vectors to transduce mature myofibers. The use of myoblast mediated ex vivo gene transfer of non-viral vectors allows an efficient gene transfer to mature skeletal muscle. The use of non-viral vectors for dystrophin gene transfer is hindered by the immune rejection problems. Although a major decrease in transduced myofibers occurs in the first 30 days post-injection, the remaining transduced myofibers seem capable of persisting for at least 90 days post-injection. The ability of the myoblast mediated ex vivo gene transfer of non viral vectors to deliver marker and therapeutic genes to skeletal muscle may open new avenues for the treatment of many other tissues of the musculoskeletal system.