

# Myoblast transfer as a platform technology of gene therapy

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## Summary

**Myoblasts divide profusely, and fuse during muscle regeneration, interiorizing MHC-I antigens and inserting myonuclei with the normal genome into muscles of genetically deficient recipients, where any replacement gene can be stably integrated and naturally expressed. Myoblasts are the natural source and vehicle for many gene therapies. Myoblast transfer therapy is completing US FDA Phase II clinical trials for Duchenne muscular dystrophy.**

## I. Introduction

The National Institute of Standards and Technology has recently announced that tissue engineering will likely be the key to treating genetic diseases and degenerative disorders that accounted for 50% of the \$1+ trillion U.S. health care cost in 1995 (Schwartz, 1997; Langer and Vacanti, 1993; Nerem and Sambanis, 1995).

Among the many programs of tissue engineering, gene therapy has been hailed as the medicine of the 21<sup>st</sup> century. Despite the nearly universal belief that gene therapy will ultimately allow the treatment of currently incurable diseases and conditions, its potential remains largely unfulfilled (Hillman et al., 1996). Only when a safe and effective gene delivery technology has been proven in humans can the full potential of gene therapy be realized.

To date, over 3000 subjects worldwide have received gene therapies among the 280+ protocols approved. Data indicate that no single vector will serve all systems. In examining gene transfer methods mediated by particle bombardment (Jiao et al., 1993; Sautter et al., 1991), liposomes (Stewart et al., 1992; Ray and Gage, 1992),

calcium phosphate precipitation (Ray and Gage, 1992; Albert and Tremblay, 1992), and electroporation (Ray and Gage, 1992; Albert and Tremblay, 1992; Puchalski and Fahl, 1992), one can conclude that transduction efficiency is extremely low and variable. The level of transgene expression depends on the promoter strength in a particular cell type. Only liposomes, together with retroviruses, adenoviruses, adeno-associated viruses and myoblasts have been used in clinical trials.

## A. Vectors

### 1. Liposomes

Cationic liposome/DNA complexes gain cellular entry via receptor-mediated endocytosis (Stewart et al., 1992; Trubetsky et al., 1992). Assuming the transgene escapes digestion by the endosome, it has no built-in mechanism to get across the nuclear membrane and is therefore non-integrative. The minimal and transient expression of the transgene is the result of random targeting, integration, and regulation. Liposomes have the advantage of being non toxic and can therefore be used in large quantities and repeatedly (Brenner, 1995).

## 2. Viruses

The viral vectors were the first to gain widespread scientific applications. Notable was "the first federally approved gene therapy protocol, for correction of adenosine deaminase (ADA) deficiency, began on 14 September 1990" (Anderson, 1990, 1992, 1995).

Retroviral vectors can transduce dividing cells with integration into host DNA. They integrate randomly and may cause mutation and cell death. They exhibit no toxicity. Although they can house larger transgenes than adenoviruses and adeno-associated viruses, the capacity is less than 10 kb. They are unstable in primate complement and cannot be targeted to specific cell types *in vivo* (Brenner, 1995; Cornetta et al., 1991).

Adeno-associated viruses and adenoviruses have shown considerable promise and are widely used. They can accommodate a broad range of genetically modified genes; are efficiently taken up by non-dividing cells *in vivo*; do not integrate into chromosomal DNA, thus reducing the risk of insertional mutagenesis; and are amenable to redirected tissue targeting (Morsey and Caskey, 1997).

All viruses can cause harm when they revert to wild type and become replication-competent (Brenner, 1995; Coutelle et al., 1994; Curiel et al., 1996). Dose-dependent inflammation occurred after nasal (Knowles et al., 1995) or lung (Crystal et al., 1994) administration of the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA conjugated with adenoviral vectors. The low efficacy, if any, is what one would have expected of pioneering studies. However, the risk to benefit ratio cannot be ignored. Also viruses produce antigens. When exposed to the host immune system, through leakage, secretion or cell damage, these antigens trigger immune reactions against the transduced cells. Certain viral elements are also toxic. These three inherent problems post almost insurmountable difficulties that prohibit the safe and efficacious clinical use of viral vectors at the present except for terminal cases. To raise caution, the FDA has mandated viral vector validation of every batch to be used on humans.

## 3. Plasmids

Single gene manipulation, often exercised *ex vivo*, has been used *in vivo*. Recombinant genes by themselves were shown to have been taken up and expressed in murine skeletal myofibers (Wolff et al., 1990; Ascadi et al., 1991; Davis et al., 1993) and cardiac myocytes (Leinwand and Leiden, 1991) following intramuscular injections. Gene expression is invariably low despite different delivery conditions and methods (Wolff et al.,

1991). This approach lacks basis and evidence of gene integration and regulation.

## 4. Combinations

A more logical approach is to include viral or cellular transcriptional regulatory sequences to effect expression. In the prophylactic treatment of hemophilia A, a retroviral factor-VIII cDNA conjugate was used to induce secretion of the blood-clotting factor in athymic mice from transduced human skin fibroblasts implanted (Hoeben, 1995). Both adenoviral (Smith et al., 1993) and Herpes Simplex virus-derived (Miyanojara et al., 1992) vectors have similarly been used for *in vivo* transfer of factor IX cDNA to the liver. Although therapeutic levels of factor IX were obtained, the expression decayed in a few weeks, possibly due to immune response and gene inactivation (St. Louis and Verma, 1988).

Gene therapy with viral vectors has been developing rapidly, but judging from the results of cystic fibrosis and brain tumor clinical trials, it is still a young discipline (Rosenfeld and Collins, 1996; Alton and Geddes, 1994). Since the main thrust of this chapter is on myoblast transfer therapy (MTT), additional details of non-myoblastic single gene manipulations can be found in the books entitled "Gene Therapy - A Primer for Physicians" (Culver, 1996) "Somatic Gene Therapy" (Chang, 1994) and "Gene Therapy for Neoplastic Diseases" (Huber and Lazo, 1994).

## 5. Myoblasts

Although genetic ailments constitute less than 2% of all human diseases, far more currently incurable diseases are the result of inadequate genetic predisposition and/or haphazard interactions between multiple genes. Symptoms precipitate when a regulatory or a structural protein is either missing or malfunctional. Without knowing these defect(s) or how they can be corrected, tissue engineering will favor genome replacement rather than single gene(s) replacement. The cell knows more than we do.

Furthermore, for a gene therapy to be effective and efficient, transgene expression requires appropriate targeting into a specific cell type, integration onto a specific site on a specific chromosome, and regulation by factors that are the products of other genes. This chain of events involves numerous cofactors many of which are produced transiently during embryonic development but not in adulthood. This is where the approach of single gene manipulation is conceptually inadequate because it cannot provide these cofactors. In complex systems, one hardly knows what they are. Again only transfer of the whole normal genome can allow the orderly provision of these cofactors necessary for the transgene expression.

Finally secondary degenerative changes often accompany the primary protein defect. Additional structural and/or regulatory protein(s) are lost (**Fig. 1**). Even if single gene manipulation replaces the primary protein deficit, transduced cells still degenerate because of the secondary changes. These latter proteins can only be replaced by re-transcribing the normal genome inserted.

Myoblasts are muscle-building cells endogenous to the human body. Contained within the nucleus of each human

myoblast is the normal genome with over 100,000 normal genes that determine cell normality and cell characteristics. Less than 10% of the gene actions is known. Myoblasts is the only somatic cell type in the body capable of natural cell fusion. Through this process, they insert their nuclei, and therefore all of the normal genes, into multinucleated myofibers of the host to effect genetic repair (**Fig. 2**).

**Fig. 1.** Diagram of some of the known genetic factors in DMD muscle cells that differ from normal muscle cells. These include genes for membrane structural proteins that are decreased or absent in DMD, dystrophin (DIN), dystrophin-related-proteins (DRP) and dystrophin-associated glycoproteins (DAG), genes for enzymes elevated in serum levels of DMD patients, creatine phosphokinase (CPK), aldolase (ALD) and aspartate transaminase (AST), and genes for mitochondrial (Mito) differences.

**Fig. 2.** Immunocytochemical localization of donor (stained, white arrowheads) and host (unstained, dark arrowheads) nuclei in longitudinal muscle sections. A and B are normal and dystrophic controls, respectively. C is from a dystrophic muscle 18 months after normal myoblast injection. A mosaic fiber (M) is demonstrated by the presence of both stained and unstained nuclei.

The transfer of genetic material and information occurs *in vivo*, with the myoblasts serving as the source and the vehicle to effect gene transfer.

Myoblasts are the only cells that divide extensively (Law et al., 1997a), migrate (Law et al., 1992), fuse naturally to form syncytia (Law et al., 1992), interiorizing major histocompatibility complex class I (MHC-1) antigens after fusion (Daar et al., 1984; Appleyard et al., 1985), and develop up to 50% of human body weight. Myoblast recipients need no more than two months of immunosuppression after MTT because mature myotubes and myofibers do not exhibit MHC-1 antigens (Daar et al., 1984; Appleyard et al., 1985). These combined properties render myoblasts superior for gene transfer. Being endogenous cells, myoblasts do not produce the adverse reactions of viral vectors.

## II. Myoblast Transfer Therapy (MTT) technology

MTT is a platform technology of gene therapy and tissue engineering. The procedure consists of culturing large quantities of myoblasts from muscle biopsies of genetically normal human donors. Cultured myoblasts are injected into patient's muscles while the patient is under general anesthesia. An immunosuppressant is administered following the procedure to minimize donor cell rejection.

The injection injury activates regeneration of host myofibers, allowing them to fuse with the injected myoblasts, thus forming genetically mosaic multinucleated myofibers (**Fig. 2**) (Law et al., 1988a,b, Chen et al., 1992). In addition, injected myoblasts fuse among themselves, forming genetically normal myofibers (Law et al., 1988a,b; Chen et al., 1992). Thus, MTT delivers the normal nuclei, the genetic software and hardware in total, into muscles of the genetically defective host, where the critical transgene is naturally and stably integrated, regulated and expressed.

Since the fusion process is a natural occurrence, there should not be any problem with specificities of integration, complementation, regulation, and expression of the normal genome inserted. It is not necessary to know which gene(s) is responsible for the defect. Abnormal gene identification is time-consuming and expensive. Furthermore, the injection of normal myoblasts directly into the host muscle eliminates any uncertainty of tissue targeting. Natural transcription of the normal genome within the donor nuclei following MTT ensures orderly replacement of any protein deficiency resulted from single gene defects or from haphazard polygenic interactions, much of which is unknown.

## III. Muscular dystrophies: the testing ground

Muscular dystrophies are genetic diseases of progressive muscle degeneration. Debilitating and fatal, these hereditary degenerative diseases deprive their sufferers of a normal quality of life and life span. Duchenne muscular dystrophy (**DMD**) confines boys to wheelchairs by age 12 and claims their lives by 20. Second in prevalence only to cystic fibrosis, DMD afflicts one in every 3300 male births worldwide (Emery, 1991).

As with any hereditary degenerative disease, DMD treatment will require repairing degenerating cells and replenishing dead cells. MTT is unique in treating the muscular dystrophies in that it transfers the normal genome to repair degenerative myofibers and it provides normal cells to replenish degenerated myofibers. As such, MTT is a combined cell/gene therapy. Potentially, not only can MTT prevent further weakening, it can also increase muscle strength.

Like murine dystrophy, DMD serves as a disease model to test MTT as a cell/gene therapy in treating hereditary degenerative diseases. MTT is being developed to repair degenerating cells and to replenish degenerated cells of the muscles in all of the neuromuscular diseases affecting over one million people worldwide. In a broad sense MTT is tested for its feasibility, safety, and efficacy to integrate the normal human genome into genetically abnormal patients.

Since MTT incorporates all of the normal genes into the dystrophic myofibers to repair them, it should exert similar effects regardless of which gene is abnormal or which protein is missing. Accordingly, MTT should be as beneficial to the murine dystrophies showing laminin  $\alpha 2$  mutation in the *dy* and *dy*<sup>2J</sup> phenotypes (Sunada et al., 1995) as DMD showing dystrophin deletion (Hoffman et al., 1987), given adjustments from mouse to human.

## IV. Animal experiments

To develop a treatment we need to know the pathogenesis of the disease. By comparing the electric (Law and Atwood, 1972; Law et al., 1976) and ultrastructural properties (Mokri and Engel, 1975; Law et al., 1983) of normal vs. dystrophic myofibers, the genetic defects in muscular dystrophy were established to result from membrane deterioration and dysfunction. Using a normal/dystrophic parabiotic mice model with cross-reinnervation of muscles, it was demonstrated that the dystrophic nervous system would support normal muscle development (Law et al., 1976; Saito et al., 1983). Without such knowledge, it would be imprudent to attempt strengthening dystrophic muscles with normal myogenic cell transfer.

Earlier developmental work of MTT consisted of two approaches that were disparate but complementary. These are the demonstration of safety and efficacy of transferring normal myogenic cells into the  $dy^{2J}dy^{2J}$  dystrophic mice (Law, 1978; Law and Yap, 1979; Law, 1982) and the examination of the developmental fate of donor cells in normal mice (Partridge et al., 1978; Watt, 1982; Watt et al., 1982). The  $dy^{2J}dy^{2J}$  dystrophic mice share a common gene defect of laminin  $\alpha 2$  mutation with congenital muscular dystrophy, the most severe form of human dystrophies (Sunada et al., 1995).

It was not until 1989 that a study of MTT on mdx mice was first published (Partridge et al., 1989; Karpati et al., 1989). The majority of evidence in support of MTT safety and efficacy is derived from previous studies using the  $dy^{2J}dy^{2J}$  mice (Law et al., 1988a,b; Chen et al., 1992; Law, 1978; Law and Yap, 1979; Law, 1982; Law et al., 1990b,d).

This was at a time when neither the golden retriever muscular dystrophy (GRMD) nor the xmd canine dystrophy was known. Dystrophic dogs are available to a few laboratories that have not produced any significant results with MTT (Kornegay et al., 1992).

Central to MTT is the correlation of genetic and phenotypic improvement at the cellular and at the whole muscle levels. These studies play an essential role in the elucidation of the mechanisms by which MTT exerts its beneficial effects on dystrophic muscles (Law et al., 1978; Law and Yap, 1979; Law, 1982; Law et al., 1988a,b; Chen et al., 1992; Partridge et al., 1989; Karpati et al., 1989; Law et al., 1990b,d).

The demonstration that cultured cells survived, developed and functioned *in vivo* after implantation into an organ of a genetically abnormal mammal bridges the gap between *in vitro* and *in vivo* cell biology. This was first achieved with myoblast transfer (Law et al., 1988a,b).

The foremost study in adult *dystrophic* mice was aimed at producing mosaic muscles containing normal, dystrophic and mosaic myofibers from the *normal* and *dystrophic* minced muscle mixes (Law, 1978). It focused on incorporating the "missing" gene and its product(s) into genetically defective cells through cell transplantation and natural cell fusion, the result of which is strengthened dystrophic muscles (Law, 1978) having a gene defect similar to human congenital muscular dystrophy (Sunada et al., 1995). The result contradicts the study of Partridge and Sloper (1977) who concluded, in transplanting normal minced muscles into normal hosts, that little or none of the regenerates was of donor origin. Eventually, fusion between host and donor myogenic cells of normal genotypes using skeletal muscle grafts were demonstrated with genotype marker (Partridge et al., 1978). Although this latter study did not involve dystrophic animals, it was

inferred that MTT was a distinct development with potential applicability to hereditary myopathies.

In a later study, muscles of newborn normal mice were grafted into recipient soleus muscles of dystrophic mice. Results obtained 6 months after the grafting indicated that the grafts survived, developed, and functioned in the dystrophic environment. The regenerates had larger cross-sectional areas and more muscle fibers than the contralateral dystrophic solei. MTT increased the mean twitch tension of adult dystrophic muscles to that of the normal (Law and Yap, 1979). The concept of replenishing lost cells and repairing degenerative cells through the production of genetic mosaicism using MTT was firmly established (Law and Yap, 1979).

An important finding was that myoblasts cultured from muscle biopsies of adult normal rats could survive and develop in the original donor after implantation (Jones, 1979). MTT with cultured myoblasts became the logical development since myoblasts do not require neuronal and capillary connections to survive and develop, and since myoblasts can fuse to effect genetic repair.

A convenient way to obtain normal myoblasts in mice is through dissection of limb-bud mesenchyme of day-12 embryos. Dissected mesenchyme was surgically implanted into the solei of  $dy^{2J}dy^{2J}$  mice. Host and donors were histocompatible. Contralateral solei served as controls. Six to seven months postoperatively, the myoblast-implanted solei exhibited greater cross-sectional area, total fiber number, better cell structure, and twitch and tetanus tensions than their contralateral controls (Law, 1982).

The incorporation and fusion of allogeneic muscle precursor cells *in vivo* were further explored using normal mice (Watt, 1982). The implants consisted of minced muscle mixes or newborn muscles (Watt et al., 1982; Watt et al., 1984; Morgan et al., 1988). It was confirmed that donor cells survived and developed in the host muscles, using electrophoretic analyses of glucose phosphate isomerases (GPI), the genetic markers to identify hosts vs. donor cells.

The use of cultured myoblasts with dystrophic mice eventually appeared. In the first study, primary myoblast cultures from limb-bud explants of normal mouse embryos were injected into the soleus muscles of histocompatible dystrophic hosts (Law et al., 1988,b). In the second study, clones of normal myoblasts were injected into the leg and intercostal muscles of histoincompatible hosts with cyclosporine-A (CsA) as a host immunosuppressant (Law et al., 1988a). Using GPI as genotype markers, donor myoblasts were shown to have fused among themselves, developing into normal myofibers. They also fused with dystrophic host myogenic cells to form mosaic myofibers of normal phenotype (Law et al., 1988a,b; Law et al., 1990a,c). These two mechanisms of genetic complementation were shown to be responsible for

improvement in muscle genetics, structure, function and animal behavior of the test dystrophic mice (Law et al., 1988a,b; Law, 1978; Law and Yap, 1979; Law, 1982; Law et al., 1990b,d). Prolongation of the life-spans of the myoblast-injected dystrophic mice was demonstrated (Law et al., 1990b,d). The improvement persisted despite CsA withdrawal.

Morgan *et al.* (1988) reported the synthesis of trace amounts of phosphorylase kinase (**PhK**) in about 5% of the myoblast-injected muscles of the PhK-deficient mice. Although there have been frequent claims of supplying normal muscle precursor cells to alleviate hereditary myopathies, no evidence of any structural or functional improvement after transplantation was presented.

With the discovery that the absence of the gene product dystrophin is the cause of DMD (Hoffman et al., 1987) and mdx mouse dystrophy, a new biochemical marker became available to demonstrate MTT efficacy (Partridge et al., 1989; Karpati et al., 1989; Chen et al., 1992). With implantation of cultured normal myoblasts into muscles of immunosuppressed mdx mice, MTT was shown to convert mdx myofibers from dystrophin-negative to -positive (Partridge et al., 1989; Karpati et al., 1989). The study demonstrates biochemical improvements in the mdx mouse model, an additional evidence to confirm the efficacy of MTT.

Given the use of inbred mice that afford histocompatible MTT, the reality is that fully matched human donors and dystrophic recipients are rarely available. MTT would thus necessitate the inclusion of host immunosuppression to facilitate myoblast survival after transfer. Cyclosporine (Cy) is the most widely documented immunosuppressant in transplantation studies (Kahan and Bach, 1988). Availability of FK506 in the late 80's was limited (Starzl et al., 1991). Typically, host mice were primed 1 week with CsA injected subcutaneously every day at 50 mg/kg body weight before receiving myoblasts. The same CsA treatment continued for 6 months after MTT (Law et al., 1988b).

Aside from donor cell survival in an immunologically hostile host, cell fusion is the key to strengthening dystrophic muscles with MTT. To improve the fusion rate between host and donor cells, various injection methods aimed at wide dissemination of donor myoblasts were tested and compared. The goal was to achieve maximum cell fusion with the least number of injections.

The results indicate that delivery of myoblasts is best conducted by diagonal placement of needle into the host muscle with ejaculation of the myoblasts as the needle is withdrawn. This method of myoblast injection yields even and wide distribution of donor myoblasts with a high rate of cell fusion. Myoblasts injected perpendicular to myofiber orientation are partially distributed. Myoblasts injected longitudinally through the core of the muscles and parallel to the myofibers are poorly distributed (Law et al.,

1994b). Thus myoblast injection method regulates cell distribution and fusion.

## V. Clinical trials

Gene therapy encompasses interventions that involve deliberate alteration of the genetic material of living cells to prevent or to treat diseases (Kessler et al., 1993). According to this FDA definition, the first MTT on a DMD boy on February 15, 1990 marked the first clinical trial on human gene therapy (Hooper, 1990). In addition to fulfilling their primary muscle-building mission, the myoblasts served as the source and the transfer vehicles of normal genes to correct the gene defects of DMD. The protocol was approved by four institutional review boards (Law, et al., 1990c). Subjects and parents gave informed consents.

The safety and efficacy of MTT was assessed by injecting the left extensor digitorum brevis (EDB) muscle of a 9-yr-old DMD boy with about  $8 \times 10^6$  myoblasts. Donor myoblasts were cloned from satellite cells derived from a 1 g rectus femoris biopsy of the normal, adoptive father. Cyclosporine was administered for three months at a dose of 5-7 mg/kg body weight divided into two daily oral doses.

Donor myoblasts survived, developed, and produced dystrophin in myofibers biopsied from the myoblast-injected EDB 92 days later. Dystrophin was not found in the contralateral sham-injected muscle. This first case suggested that MTT offered a safe and effective means for alleviating biochemical deficit(s) inherent in muscles of DMD (Law et al., 1990a).

A pioneering work (Anderson, 1990; see also Brenner, 1995; Karlsson, 1991) is often considered as the "first human gene therapy"; correction of the ADA deficiency study began on September 14, 1990 (Anderson, 1990), two months after the MTT correction of the DMD gene defect was published (Law et al., 1990a). In the ADA protocol, T cells from a patient with a severe combined immunodeficiency disorder (SCID) were transduced with functional ADA genes *ex vivo* and returned to the patient after expansion through culture. In the MTT protocol, primary culture of myoblasts derived from a muscle biopsy of a normal donor was injected into a muscle of the DMD subject to produce *in vivo* nuclear complementation. Both gene therapies utilize cell transplantation to treat diseases.

However, it is pointed out that the ADA protocol involved genetic modification and correction of the patients T cells with the *adenosine deaminase* gene whereas in the DMD protocol normal donor cells were used which were not genetically modified *ex vivo*.

Six years after the foremost MTT, dystrophin was found in the myoblast-injected muscle but not in the sham-injected muscle (**Figure 3**, Law, 1997). Six years is the

longest period through which any gene therapy has sustained positive results. Despite cyclosporine withdrawal at 3 months after MTT, myofibers expressing foreign dystrophin were not rejected. This is because dystrophin is located in the inner surface of the plasma membrane, and because mature myofibers do not exhibit MHC-1 surface antigens. Not only has the result demonstrated MTT overall safety and efficacy in this single case, it also shows stability in the integration, regulation and expression of the inserted dystrophin gene. The presence of dystrophin in the myoblast-injected but not in the sham-injected muscle provided unequivocal evidence of the survival and development of donor myoblasts in the myoblast-injected muscle.

In a randomized double-blind study involving three subjects, myoblast-injected EDBs showed increases in tensions whereas sham-injected EDBs showed reductions (Law et al., 1991a,b). Both immunocytochemical staining and immunoblot revealed dystrophin in the myoblast-injected EDBs. Dystrophic characteristics such as fiber splitting, central nucleation, phagocytic necrosis, variation in fiber shape and size, and infiltration of fat and connective tissues were less frequently observed in these muscles. Sham-injected EDBs exhibited significant structural and functional degeneration and no dystrophin. Throughout the study, there was no sign of erythema, swelling or tenderness at the injection sites. Serial laboratory evaluation including electrolytes, creatinine, and urea did not reveal any significant changes before or after MTT.

To reconcile these positive results with less convincing ones (Gussoni et al., 1992; Huard et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Miller et al., 1992; Morandi et al., 1995; Tremblay et al., 1993), several issues need to be addressed. To begin with, the use of large quantities of pure live myoblasts is a pre-requisite of successful MTT. Except for one study (Law et al., 1992), there is no published pictorial evidence to substantiate the purity, myogenicity and viability of the injected myoblasts as claimed.

Myoblast cultures are usually contaminated with fibroblast overgrowth. MTT with such impure culture could lead to deposition of connective tissues rather than myofiber production. Culturing 50 billion pure human myoblasts for MTT from two grams of muscle biopsy has only been reported by our team (Law et al., 1997a). Other teams work at ranges of hundreds of millions of myoblasts.

In most studies (Gussoni et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Miller et al., 1992; Morandi et al., 1995) myoblasts were transported frozen, chilled for over two hours from the site of harvest before being injected. Since myoblasts have a high metabolic rate, they could not have survived for two hours without significant nutrients, oxygen and proper pH, being closely packed in

saline within a vial for transport. Determination of cell viability before MTT were not conducted in these studies. Our myoblasts were injected into the subject within minutes of harvest, at the same location without transport.

MTT studies that reported failure (Gussoni et al., 1992; Huard et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Miller et al., 1992; Morandi et al., 1995; Tremblay et al., 1993) subscribed to the fallacy of making 55 to 330 injections into a muscle the size of an egg, traumatizing indiscriminately the underlying nerves, muscle, and vasculature. These injection traumas boosted macrophage access and host immune responses (Guerette et al., 1995). They also induced fibrosis (Chen et al., 1988). Surviving myoblasts fused within three weeks in small mouse muscles (Chen et al., 1992). A nerve with multiple trauma could not regenerate soon enough through scar and connective tissues to innervate the newly-formed myotubes in a large human dystrophic muscle. Stabilization of muscle contractile properties in a similar situation is achieved by 60 days in the rat, and functional return is incomplete (Carlson, 1983). Non-innervated myotubes died within one week. Whatever few myotubes that developed in the unsuccessful MTT studies could not compensate for the traumatized myofibers.

In the study yielding positive results, 5 to  $8 \times 10^8$  pure myoblasts were delivered with eight injections into the biceps brachii without nerve injury (Law et al., 1994a, 1997a). Contrarily, in another study, 55 sites, each 5 mm apart, distributed in 11 rows and 5 columns, were injected throughout the depth of each biceps of 5- to 9- year old boys (Mendell et al., 1995). This was repeated monthly for six months. Axonal sprouts, myotubes and neuromuscular junctions that take six weeks to mature (Fex and Jirmanova, 1969) were repeatedly traumatized by a total of 330 injections until the biceps, with or without myoblast/cyclosporine, were irreversibly damaged or destroyed. The result: no functional difference between myoblast- and sham-injected muscles (Mendell et al., 1995).

Once injected, the myoblasts are subjected to scavenger hunt by macrophages for up to three weeks. This is because myoblasts exhibit MHC-1 surface antigens (Friedlander and Fischman, 1979; Fang et al., 1994) that become absent after cell fusion. The latter occurs between one to three weeks after myoblast injection (Chen et al., 1992). An allowance in the number of injected myoblasts has to be made to satisfy the unavoidable scavenger process. As reflected in the small numbers of myoblasts injected in unsuccessful studies, it appears that either such allowance was not considered or that the teams were not able to produce larger quantities of pure myoblasts. Although myoblast loss can be minimized by down-regulating macrophage activity (Guerette et al., 1997), such additional compromise of the host immune

system may lead to higher risk of infection, since MTT subjects are already taking immunosuppressants.

The less successful MTT teams focused on immunosuppression to prevent T-lymphocyte proliferation and antibody production without overcoming the primary hurdle of providing enough pure and live myoblasts. A basic study indicates that cyclophosphamide did not permit myoblast engraftment in the mouse (Vilquin et al., 1995), and a MTT clinical trial was conducted without success using cyclophosphamide immunosuppression (Karpati et al., 1993). Cyclosporine (Law et al., 1990a) and potentially FK506 (Kinoshita et al., 1994) remain the immunosuppressants of choice for MTT. Results could have been more positive if either was employed in the study of Tremblay *et al.* (Huard et al., 1992; Tremblay et al., 1993).

All of these single muscle MTT studies had begun before the FDA established policies and regulations for cell/gene therapies. Our studies are the only ones that received permission for an investigational new drug application (IND) on MTT for treatment of multiple muscles. As a cell/gene therapy, all American MTT clinical trials must come under FDA purview.

Beginning with 8 million myoblasts into a small foot muscle, our team proceeded to test 5 billion cells into 22 leg muscles, 25 billion cells into 64 body muscles, and

now 50 billion cells into 82 muscles (**Table 1**). With over 150 procedures having been conducted, the complete safety of the MTT procedure has been proven. There have been no adverse reactions or side effects.

**Table 1.** Dose escalation protocols of MTT and the number of subjects receiving such procedures.

**Fig. 3.** Immunocytochemical demonstration of dystrophin in DMD muscles 6 yr after MTT. Dystrophin absent in sham-injected EDB muscle (**A,C**), but present in the contralateral myoblast-injected muscle (**B,D**). Dystrophin was immunocytochemically localized at the sarcolemma (arrows). Dystrophin demonstrated at low (**E**) and high (**F**) magnification in normal control muscle. Cross-section; bar = 100 $\mu$ m.

**Fig. 4.** Dystrophin immunocytochemistry showing the presence of dystrophin in (A) normal control and in (C,E,G) muscle biopsy specimens of three subjects. Dystrophin is absent in (B) Duchenne's muscular dystrophy control and in (D,F,H) contralateral biopsy specimens from the same subjects.

**Fig. 5. (A,C,E)** Three dystrophin-positive muscle biopsy specimens exhibit less dystrophic characteristics than the contralateral dystrophin-negative biopsy specimens **(B,D,F)**. Dystrophic characteristics include increases in fat and connective tissue, fiber splitting, central nucleation, round and oval fibers.

**The five billion myoblast cell protocol.**

The 5-billion myoblast MTT protocol was tested in 32 DMD boys aged 6-14 yr. Through 48 injections, 5 billion myoblasts were transferred into 22 major muscles in both lower limbs under general anesthesia. Only four donors were histocompatible with their recipients. All subjects

took cyclosporine for six months after MTT. More than 88% of the injected ankle plantar flexor muscles showed either increase in strength or no further deterioration at 9 months after MTT (Law et al., 1992, 1993).

**Fig. 6.** Dose-dependent responses to MTT of plantar flexion with greater increase in maximum isometric force using the 50-billion MTT protocol than with the 25-billion MTT protocol. Both protocols show efficacy in strengthening the plantar flexion when compared to the natural history control.

**Table 2.** Percentage increases over a one-year natural history control in the maximum isometric force of the plantar flexor muscles at 3, 6, 9, 12, and 15 months after the administration of the 25-billion MTT protocol or the 50-billion MTT protocol.

### **The 25 billion myoblast cell protocol.**

Under FDA purview, MTT is completing Phase II clinical trials on DMD. The whole body trial (WBT) consisted of injecting 25 billion myoblasts in two MTT procedures separated by 3 to 9 mo. Each procedure delivered up to 200 injections or 12.5 billion myoblasts to either 28 muscles in the upper body (UBT) or to 36 muscles in the lower body (LBT). A randomized double-blind portion of the study was conducted on the biceps brachii or quadriceps. Subjects took oral cyclosporine for 3 months after each MTT. One infantile facioscapulohumeral dystrophy and 40 DMD boys aged 6 to 16 received WBT in the past 36 months with no adverse reaction.

Nine months after MTT immunocytochemical evidence of dystrophin were demonstrated in 18 of the 20 DMD subjects biopsied (**Fig. 4**). Dystrophin positive sections showed less dystrophic characteristics than dystrophin-negative ones (**Fig. 5**). Forced vital capacity increased by 33.3% and maximum voluntary ventilation increased by 28% at 12 months after UBT (Law et al., 1997a).

Plantar flexion showed an increase of 45% in maximum isometric contraction force in 12 months in the DMD subjects when compared to the natural deterioration (**Fig. 6, Table 2**). Behavioral improvements in running, balancing, climbing stairs and playing ball were noted (Law et al., 1995; Law et al., 1996; Law et al., 1997a,c,d). Notable was a 16-yr-old DMD subject who continued to walk without assistance and capable of driving an automobile by himself.

### **50 Billion myoblast cell protocol.**

The current study involves a one time injection of 50 billion myoblasts into 82 muscles with 179 skin punctures, approved by the FDA for subjects with DMD, Becker MD and Limb-girdle MD (Law et al., 1997d). Twenty-nine subjects who underwent this protocol have experienced no adverse reaction.

For the 22 DMD subjects aged 5 to 16, there was a significant increase in the maximum isometric force generated by the plantar flexor muscles at 3, 6, and 9 months after MTT (**Fig. 6, Table 2**).

This functional improvement is more pronounced with the 50-billion MTT than with the 25-billion MTT, indicating that it is dose-dependent. Thus, in the 25-billion MTT, 800 million myoblasts were injected into the plantar flexors, producing a mean 61% increase in force at 15-months after MTT. With the 50 billion MTT, 50% more myoblasts were injected, projecting a 10% greater increase in force at 15 months after MTT (**Fig. 6, Table 2**).

**Fig. 7.** Serum creatine kinase (CK) level of DMD subjects increased before MTT and decreased after MTT.

**Fig. 8.** Serum aspartate aminotransferase (AST) level of DMD subjects increased before MTT and decreased after MTT.

Elevated serum creatine kinase (CK) has traditionally been used to diagnose muscle degeneration, notable in DMD (Heyck et al., 1966). The 22 DMD subjects, mean ages 10.7-yr-old and, median age 9.9 yr-old, showed a 19.3% increase in serum CK within 3 months before MTT (**Fig. 7**). This trend was reversed after MTT, and the serum CK declined at a steady rate of 48.7% over 12 months. This result provides strong evidence that MTT repairs muscle cell membrane leakage of enzymes. This contention is further substantiated by similar findings with another muscle enzyme AST, aspartate aminotransferase (**Fig. 8**).

The breakthrough came when a 29-yr-old Becker MD (BMD) subject began to walk, with his hands being held, beginning at 2.5 months after the 50-billion MTT. He had previously been diagnosed repeatedly with BMD. He had been non-ambulatory and required the use of a wheelchair for over four years as documented in his medical record. He began walking with assistance a total of eight steps at 3 months after MTT. This ability increased with time, now reaching 60 steps at eight months after MTT. He began to stand and walk with his crutches at four months after MTT (**Fig. 9**).

## VI. Future perspectives

As an universal gene transfer vehicle with which the entire human genome can be integrated into patient's

**Fig. 9.** First muscular dystrophy subject ever to walk after wheelchair bound for years.  
(A). The 29-yr-old BMD subject had been

muscles, myoblasts have shown promise in studies of the following diseases:

**Cardiomyopathy.** Labeled cultured myoblasts engrafted and formed structures resembling desmosomes, intercalated discs, fascia adherens junctions, and gap junctions in myocardia of dogs (Chiu et al., 1995), rats (Murry et al., 1996) and mice (Robinson et al., 1996) when MTT was delivered intramuscularly (Chiu et al., 1995; Murry et al., 1996) or intraarterially (Robinson et al., 1996). Donor muscle regenerates exhibited cardiac-like properties such as central nucleation (Chiu et al., 1995), fatigue resistance, slow twitching, and were capable of twitch and tetanus contractions when stimulated (Murry et al., 1996). Similar results were obtained when cardiomyocytes were injected in dystrophic mice and dogs (Koh et al., 1995), rats (Li et al., 1996) and swine (Van Meter et al., 1995). These findings, together with established MTT safety, pave the way to MTT clinical trial in treating myocardial degeneration and dysfunction.

**Insulin-resistant diabetes mellitus.** Commonly known as Type II diabetes, this disease is genetically predisposed and afflicts 90% of the diabetic population. Virtually all identical siblings of these patients develop the disease, and the genetic defect can be traced to the GLUT4 gene deletion. The major sequela of insulin resistance is decrease muscle uptake of glucose, due to the moderate decrease in insulin receptors on muscle cell surface. Conceptually MTT can add genetically normal myofibers with normal insulin receptors. It can also genetically repair the patients' myofibers and produce normal insulin receptors on the heterokaryons. Basic research is need to test this hypothesis on diabetic rats.

wheelchair-bound  
for over 4 years.

**(B,C,D,E)** He  
began to walk with  
his hands held at  
2.5 months after the  
50-billion MTT.

**(F)** At 4  
months after MTT,  
he was able to walk  
on crutches for  
about 20 steps.

**Bone/cartilage degeneration.** During embryonic development, mesenchymal progenitor cells differentiate into myoblasts, osteoblasts, chondrocytes and adipocytes under controls of various regulatory factors. Ectopic bone formation in muscle has been achieved through implantation of bone morphogenetic protein (BMP). BMP-2 was shown to convert the differentiation pathway of clonal myoblasts into the osteoblast lineage (Katagiri et al., 1994). This opens new ways to treat conditions of bone degeneration such as the degeneration of tooth pulp, hip, bone/joint, and long bone fractures. Given the ability to mass-produce myoblasts that can be transformed into osteoblasts, and potentially chondrocytes, the difficulty of proliferating osteoblasts and chondrocytes can be overcome. Cultured autologous chondrocytes can be used to repair deep cartilage defects in the femorotibial articular surface of the human knee joint (Brittberg et al., 1994).

The use of normal or transduced myoblasts as the source and vehicles for gene delivery has found application in the potential treatments of restenosis (Morishita et al., 1995), soft tissue deformities (Teboul et al., 1995), hemophilias (Dai et al., 1992; Yao et al., 1994), anemia (Hamamori et al., 1994), muscle trauma (Almeddine et al., 1994), human growth hormone deficiency (Barr and Leiden, 1991) and allograft rejection (Lau et al., 1996). MTT has produced a new frontier in medicine.

## **VII. Our vision**

MTT implementation can benefit from the development of the following programs (Law, 1994):

**Controlled cell fusion.** It will be useful to be able to control, initiate or facilitate cell fusion once myoblasts are injected. This is to minimize loss of myoblasts from

macrophages whose presence is unavoidable if the patient is to have some immune protection.

As the myoblasts are injected intramuscularly into the extracellular matrix, injection trauma causes the release of basic fibroblast growth factor (bFGF) and large chondroitin-6-sulfate proteoglycan (LC6SP). These latter growth factors stimulate myoblast proliferation. Unfortunately, they also stimulate the proliferation of fibroblasts that are already present in increased amount in the dystrophic muscle. That is why it is necessary to inject as pure as possible fractions of myoblasts in MTT without contaminating fibroblasts.

Controlled cell fusion can be achieved by artificially increasing the concentration of LC6SP over the endogenous level. In addition, insulin or insulin-like growth factor I (IGF-1) may facilitate the developmental process, resulting in the formation of myotubes soon after myoblast injection. Enhanced fusion of myoblasts into myotubes had been achieved with the use of PDO98059 (Coalicant et al., 1997) and ED2+ macrophages conditioned medium (Massimino et al., 1997). The use of these compounds in the cell culture medium and in the injection medium will likely lead to greater MTT success.

**Superior cell lines.** These cell lines should be highly myogenic, nontumorigenic, nonantigenic, and will develop very strong muscles. The superior cell lines will bypass the use of immunosuppressant, and will provide a ready access for patients who do not have a donor. A unique property of myoblasts is their loss of MHC-I antigens soon after they fuse. The immunosuppression period depends on how soon the myoblasts lose their MHC-I antigens after MTT. Even more ideal is the establishment of a myoblast cell line in which MHC-I antigens are absent. In human myoblasts cultured from normal muscle biopsies, some 91.7% of the myoblasts reacted with MHC-I MAb (monoclonal antibodies). The remaining 8.3% of the myoblasts, that were negative for MHC-I antigen

expression were successfully separated by cytofluorometry. The lack of MHC-I antigens on these latter myoblasts may enhance survival of these myoblasts in recipients after MTT (Fang et al., 1994).

**Automated cell processors.** The great demand for normal and transduced myoblasts, the labor intensiveness and high cost of cell culturing, harvesting and packaging, and the fallibility of human imprecision will soon necessitate the invention and development of automated cell processors capable of producing huge quantities of viable, sterile, genetically well-defined and functionally demonstrated biologics.

This invention will be one of the most important offspring of modern day computer science, mechanical engineering and cytogenetics. The intakes will be for biopsies of various human tissues. The computer will be programmed to process tissue(s), with precision controls in time, space, proportions of culture ingredients and apparatus maneuvers. Cell conditions can be monitored at any time during the process and flexibility is built-in to allow changes. Different protocols can be programmed into the software for culture, controlled cell fusion, harvest and package. The outputs supply injectable cells ready for cell therapy or shipment. The cell processor will be self-contained in a sterile enclosure large enough to house the hardware in which cells are cultured and manipulated.

**Transport medium.** A transport medium that can sustain the survival and myogenicity of myoblasts in package for up to four days will allow the cell packages to be delivered to remote points of utilization around the world. **Fig 10** shows the effectiveness of such a medium developed in our foundation. Fifty billion myoblasts can be shipped at 4° C for four days with 90% viability.

**Cell banks.** The automated cell processors will constitute only a part of the cell banks. The current thought is to obtain donor muscle biopsies from young adults aged 8 to 22 to feed the inputs. Each donor has to undergo a battery of tests that are time-consuming and expensive. From the test results and from the donor's physical conditions, one can determine if the donor cells

are genetically defective or infected with viruses and/or bacteria.

Human fetal tissues can potentially provide greater supplies of cells. However, aside from ethical issues surrounding abortion, it is difficult to determine the genetic normality of the cells. Muscle primordia of fetus derived from *in vitro* fertilization of genetically well-defined background may be an alternative. Sperm and ova can be recovered from healthy individuals that are known for their muscle strength and mass. *In vitro* fertilization will be followed by embryo culture to a specific developmental stage (day 26 to day 56 gestation) of the embryos. The muscle primordia that are rich in myoblasts can then be dissected out to feed the automated cell processors.

## **VIII. Conclusion**

This chapter describes the landmark development of the first gene therapy study in humans. Through natural cell fusion, myoblasts transfer the human genome into dystrophic muscle cells to effect phenotype repair. The innovative cell transplantation procedure also revitalizes the degenerative organ by providing living cells of normal genotype to replenish cell loss. The result is potentially a new form of medicine. The conceptual approaches of single gene transfer and myoblast transfer toward treatment of hereditary degenerative diseases are compared.

As more scientists continue to recognize myoblasts as a stable source of genes and a safe and efficient gene transfer vehicle, MTT application will extend far beyond the treatment of neuromuscular diseases. This chapter provides insights to guide future development of MTT in battling against genetic and acquired diseases that presently have only diagnoses but no treatment.

## **Acknowledgment**

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**Fig. 10.** Transport medium effectiveness as demonstrated by myoblast survival and myotube formation. (A). Myoblasts before a 50-billion MTT showing 99% viability using the vital stain erythrocin B, 1% at pH 7.23. (B). Myoblast left-over from a 50-billion MTT maintained in the transport medium for 4 days at 4° C and stained with erythrocin B. The sample showed 90% viability. (C). Cells in B were put back into culture for 2 days before feeding fusion medium. (D). Cells in C in fusion medium for 1 day, showing myoblast fusion (arrow). (E). Cells in C in fusion medium for 2 days, showing myotubes (arrow). (F). Cells in C in fusion medium for 5 days, showing extensive myogenic capability in myotube formation (arrows).

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