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Disease Prevention and Alleviation by Human Myoblast Transplantation

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Abstract

Myoblast implantation is a unique, patented technology of muscle regeneration being tested in Phase III clinical trials of muscular dystrophy, ischemic cardiomyopathy, Phase II trial of cancer, and Phase I trial of Type II diabetes. Differentiated and committed, myoblasts are not stem cells. Implanted myoblasts fuse spontaneously among themselves, replenishing genetically normal myofibers. They also fuse with genetically abnormal myofibers of muscular dystrophy, cardiomyopathy, or Type II diabetes, transferring their nuclei containing the normal human genome to provide stable, long-term expression of the missing gene products. They develop to become cardiomyocytes in the infracted myocardium. Myoblasts transduced with $VEGF_{165}$ allow concomitant regeneration of blood capillaries and myofibers. They are potent biologics for treating heart failure, ischemic cardiomyopathy, diabetic ischemia, erectile dysfunction, and baldness. Myoblasts, because of their small size, spindle shape, and resilience, can grow within wrinkles and on skin surfaces, thus enhancing the color, luster and texture of the skin "plated" with them. They can be injected subcutaneously as a cellular filler to reduce wrinkles. Intramuscular injection of myoblasts can augment the size, shape, consistency, tone and strength of muscle groups, improving the lines, contours and vitality to sculpt a youthful appearance. This highly promising technology has great social economic values in treating hereditary, fatal and debilitating disease conditions.

Keywords

Human Gene Therapy, Myoblasts, Muscular Dystrophies, Heart Failure, Ischemic Cardiomyopathy, Type II Diabetes, Anti-Aging, Cosmetology, Muscle Regeneration and Repair

1. Introduction

Grandstands over the Human Genome Project (HGP) [1]-[6] and somatic gene therapies [7]-[12] in the last two

decades fueled enthusiasm that most human diseases will eventually be cured with molecular medicine [13] [14]. The global market for gene therapy is forecasted to reach US$794 million by 2017, and will grow further as driven by the human genome project and the increasing incidence of cancer and other critical diseases [15].

In face of debilitation and death of patients without effective remedy, the FDA, EMA and SFDA have been under pressure since year 1990 to approve initiation of various gene therapy clinical trials. The U.S. National Institutes of Health registers 3253 gene therapy studies worldwide, enrolling tens of thousands of subjects, mostly dying from incurable diseases such as cancer, autoimmune diseases (AIDS), and viral infections.

Despite huge efforts and expenses spanning nearly a generation of molecular genetics research, only three products have gained marketing authorization:

Gendicine® authorized in China in 2003: Ad-p53 gene therapy for head and neck squamous cell carcinoma (HNSCC) [16].

Oncorine® authorized in China in 2005: Recombinant human adenovirus Type 5 injection for nasopharyngeal carcinoma combined with chemotherapy [17].

Glybera® authorized in Holland in 2013: Functional lipoprotein lipase (LPL) gene packaged in an adeno-associated virus, corrects a rare genetic deficiency of LPL, a protein that helps breakdown fats [18].

All three products are of adenoviral origin and are based on single gene transduction. Together they provide therapies to less than 0.01% of the sick population.

2. Gene Therapy in USA

The US FDA has not authorized any gene therapy product because it has not been convinced about a positive benefit/risk ratio of any gene therapy medicinal products. Whereas viral vectors are impregnated with risks [19]-[23], non-viral transduction efficiency using liposomes or nanoparticles are significantly reduced. Typically, product development is slow because of numerous clinical failures, high compliance standards and stringent regulatory surveillance.

The viral related death of Jesse Gelsinger and participants of other trials in 1999 [24] compelled the FDA to re-evaluate gene therapy regulations, setting back the development of all gene therapy programs that were then regulated by the Center for Biologics Evaluation and Research (CBER). One of these programs was the human genome therapy using myoblasts to treat the X-linked Duchenne muscular dystrophy (DMD) [25] [26]. This Human Myoblast Genome Therapy (HMGT), also called Myoblast Transfer Therapy (MTT), had previously shown significant safety and efficacy to merit the FDA granting of fast-track on a multi-center Phase III clinical trial, and allowance to charge following annual reviews of progress for four consecutive years of Phase II/III clinical trials [27]. Ironically, HMGT/MTT did not have any viral involvement and had previously demonstrated complete safety record.

3. What Constitute Genetic Diseases?

Genetic diseases were formerly believed to have resulted from DNA defect of a single locus with 100% heritability as exhibited in Phenylketonuria (PKU), Sickle-cell anemia, Adenosine deaminase (ADA) deficiency and others. Although these genetic ailments constitute less than 2% of all human diseases and affect less than 1% of sick people, far more currently incurable diseases are the result of inadequate genetic predisposition and/or haphazard interactions between multiple genes.

With the advent of diagnostics using molecular genetics, we now consider genetic diseases as those that have a genetic component with or without 100% heritability. Accordingly, the top killers of mankind: cardiovascular diseases, Type II diabetes, cystic fibrosis, cancer and aging fall within the definition of genetic diseases. Together, these fatal and debilitating diseases account for more than 80% of human death. Published reports indicated that the HMGT/MTT platform has applications to treating most if not all of these diseases, and it is the goal of this review to provide the essence.

4. Two Kinds of Gene Therapies

Gene therapy encompasses interventions that involve deliberate alteration of the genetic material of living cells to prevent or to treat diseases [28]. This FDA definition places two major technologies into the field of gene therapy:

1) Single gene transduction

In September 1990, a clinical trial was started using retroviral-mediated transfer of the adenosine deaminase (ADA) gene into the T cells of a 4-year-old girl with severe combined immunodeficiency (SCID) [8]. Although integrated vector and ADA gene expression persisted, the subject had to take regular medication throughout the two years of gene treatment. It was concluded that this single gene therapy was safe and effective to treating patients with this very rare form of disease [11]. William French Anderson [29] of the National Institutes of Health claimed to be the first person ever to succeed in gene therapy [30] and became widely acclaimed as the "Father of Gene Therapy" [31] [32].

Central to this technology is the use of viruses as vectors to deliver normal copies of the faulty or missing gene into a particular cell type of a patient, hoping that the therapeutic gene will be expressed to produce a structural or regulatory protein, thereby alleviating the disease symptom(s) [20] [21]. Single gene transduction using adenoviral and retroviral vectors accounted for about one-third of gene therapy studies.

Retroviral vectors exhibit no toxicity and integrate randomly into host DNA of dividing cells, and may cause mutation and cell death. 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 a specific cell type in vivo [24].

Adeno-associated viruses and adenoviruses 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 [33].

All viruses can cause harm when they revert to wild type and become replication-competent [19] [34] [35]. Dose-dependent inflammation occurred after nasal [36] or lung [20] administration of the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA conjugated with adenoviral vectors. Viruses, even "fully deleted or helper-dependent", produce antigens. When exposed to the host immune system, through leakage, secretion or cell damage, these antigens trigger immune reactions against the transduced cells. Jesse Gelsinger and many others were believed to have died as a result of a severe immune reaction to the adenovirus vector used [24]. 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 except for terminal cases. To raise caution, the FDA has mandated viral vector validation of every batch to be used on humans.

Non-viral approaches using lipofection, nanoparticles and plasmids (naked RNA) account for about 40% of gene therapy studies. Cationic liposome/DNA complexes gain cellular entry via receptor-mediated endocytosis [37] [38]. Assuming the transgene escapes digestion by lysozyme within endosomes, 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 and nanoparticles have the advantage of being non-toxic and can therefore be used in large quantities and repeatedly [19] [23], especially in large organs such as muscles [23] [39] [40].

Recombinant genes by themselves, plasmids or naked DNA, were shown to have been taken up and expressed in murine skeletal myofibers [41]-[43], and cardiac myocytes [44] following intramuscular injections. Gene expression is insignificantly low despite different delivery conditions and methods [45]. This approach lacks basis and evidence of gene integration and regulation.

Single gene transduction deficiencies

There are numerous deficiencies associated with the use of the single gene transduction technology. Much of the hurdles that were 18 years ago [12] [19] [20] remain unresolved today [18]. These are:

a) In hereditary degenerative diseases, gene defects cause cells to degenerate and die with time. An effective treatment must not only repair degenerating cells, but replace dead cells with live ones. Single gene transduction cannot replenish live cells. There is very limited evidence that it repairs degenerating cells. The technology appears at best applicable to rare diseases involving single regulatory protein deficit but not to diseases involving cell death, because it has no provision to replenish live cells.
b) Single gene transduction cannot repair multiple gene defects such as in Type II diabetes, cancers and hereditary cardiomyopathies.
c) Viral vectors have limited capacity to accommodate large DNA fragments such as the dystrophin gene the anomaly of which is responsible for Duchenne and Becker muscular dystrophies.
d) Gene expression lasts usually for less than a month and therefore incapable to treat genetic diseases like cystic fibrosis that need a continual supply of the therapeutic protein throughout the patient's lifetime.

e) The host immune system often mounts severe reaction against foreign antigens originated from viral vectors, transcriptional regulatory sequences and transgene products. This is a significant safety issue. Every infected cell is a potential target for immune assault.
f) Plasmids are often digested by lysozymes and rendered ineffective. There are no built-in mechanisms for gene integration, regulation and expression.
g) For a gene therapy to be effective and efficient, transgene expression requires precise 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 transduction is conceptually inadequate because it cannot provide these cofactors. In complex systems, one hardly knows what they are. Only transfer of the whole normal genome can allow the orderly provision of these cofactors necessary for the transgene expression [22].
h) In diseases such as DMD or cystic fibrosis, secondary degenerative changes often accompany the primary protein deficit. Additional structural and/or regulatory protein(s) are lost. Even if single gene transduction 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 complete normal genome inserted [22].

How single gene transduction fares?

Without resolving the above inherent deficiencies, and considering its high risks/benefits ratio for the patients, this kind of gene therapy should only be reserved for terminal cases. It is erroneous to believe that gene therapy is at hand with the discovery of the human genome sequence. Too many people have died believing that the shots of genes into their blood stream would cure them. A revisit of the basics may help.

The cell is the origin of all life. Contained within its nucleus are more than 30,000 genes that determine cell normality and cell characteristics. The genes are composed of deoxyribonucleic acids (DNA) that are spatially and temporally switched on and off during development to produce more than 100,000 different transcripts of ribonucleic acid (RNA). The transcriptional events occur inside the nucleus and require the nuclear matrix and/or the chromatin to operate efficiently. These regulatory events are poorly understood but invariably involve polygenic interactions.

Whereas the basic sequence of the human genome has been determined, exactly how the genome functions will take many decades of further research. Scientists do not know the spatial and temporal interactions of the RNA transcripts within the cell nucleus and know little of their modes of action. Numerous methods have yet to be developed to determine the diverse functions of some 30,000 genes and more techniques have to be refined to effect gene regulation and expression. It is through this knowledge that molecular genetics may one day provide rational approaches to gene therapy.

Today, the analysis of DNA/RNA variations and gene expression are used mainly in diagnostics, while gene therapy success through single gene transduction has been rare. With genetic diagnostics, physicians may identify their patients' genetic predispositions earlier and help patients take steps to minimize damages. Genetic information can also be used by pharmaceutical and biotech companies to develop therapeutics.

2) Human genome therapy

An alternative perspective is that a genetically abnormal cell degenerates due to the lack of the normal genome. In hereditary degenerative diseases such as muscular dystrophies, hereditary cardiomyopathies, and Type II diabetes, the much-needed normal genome can be incorporated into the genetically abnormal myofibers. This is achieved by taking a muscle biopsy from a normal donor, isolating and proliferating in culture the regenerative satellite cells, now called "myoblasts" as they are in culture, and injecting the normal myoblasts into the genetically abnormal muscles. This cell transplant procedure is called Myoblast Transfer Therapy (MTT) or Human Myoblast Genome Therapy (HMGT).

Through natural cell fusion, which is inherent in myogenesis and muscle regeneration, donor myoblasts insert their nuclei that contain full complements of normal genes into the genetically abnormal myofibers, forming multinucleated heterokaryons. The donor nuclei integrate spontaneously and transcribe the missing RNA(s) to effect genetic complementation repair [46]-[48] [59].

Only transfer of the normal nuclei, carrying the genomic software and the chromosomal hardware, will allow the orderly provision of various co-factors necessary for the regulation and the expression of the transgenes [22]. Natural transcription of the normal genome within the donor nuclei following HMGT/MTT ensures orderly replacement of any protein deficiency resulting from single gene defects or from haphazard polygenic interactions,

much of which is unknown. This differs significantly from single gene transduction, effected through viral or non-viral vectors, in that the transgene may find no transcriptional factors/co-factors in the adult environment for its regulation and expression. Many of these co-factors are the products of other genes that are only operative in early development.

HMGT/MTT is a platform technology of cell transplantation, nuclear transfer, gene therapy and tissue engineering. It is the only human genome therapy in existence, and will remain so until another modality is discovered to deliver the human genome into the defective cells of a genetically ill patient. Myoblast is the only somatic cell type that has the ability of natural cell fusion. HMGT/MTT is uniquely suited to treat hereditary muscle degeneration and weakness through nuclear transfer or genome transfer. Myoblasts cultured from muscle biopsy survive, develop and function, after transplantation in animal studies and clinical trials, to revitalize degenerative organs in patients with heart failure, ischemic cardiomyopathy, Type II diabetes, muscular dystrophies, aging dysfunction and disfigurement. When donor myoblasts fuse among themselves after HMGT/MTT, they form new muscle fibers to repopulate the degenerative organ, depositing contractile filaments to augment its function. Thus, as a cell therapy, HMGT/MTT applies not only to all forms of skeletal muscle degeneration, but to heart muscle degeneration, body-building, anti-aging and soft tissue augmentation [22]. HMGT/MTT replenishes live cells through cell therapy, and genetically repairs degenerating myofibers through genome therapy.

5. World's First Human Gene Therapy

First conducted in February 1990 and published on 14 July 1990, HMGT/MTT truly is the world's first human gene therapy [46]. Through natural cell fusion, which is inherent in myogenesis and muscle regeneration, donor myoblasts insert full complements of normal genes into DMD dystrophic muscle cells to produce dystrophin, a structural protein that is not produced in DMD muscles due to the genetic defect. The transfer of genetic material and information occurs in vivo, with the myoblasts serving as the source and the vehicle of gene transfer.

This first case suggests that HMGT/MTT offers a safe and effective means to replenish biochemical deficit(s) in muscles of hereditary diseases [46]. The report stated that, "it does not matter which gene is abnormal and which protein is missing, MTT has potential application for many hereditary muscle diseases". If so, one would expect that MTT could replenish the structural and regulatory protein deficits in Type II diabetes via improvement of genetic transcriptional pathways as described later in this article. Indeed, HMGT/MTT alters gene expression profiles of insulin signaling pathway and mitochondrial biogenesis and function in skeletal muscles of diabetic KK Cg-A^y/J mice [47] [48] and reduces blood glucose in Type II diabetes patients [49].

6. HMGT/MTT Corrects Gene Defects in DMD Clinical Trials

HMGT/MTT is the first genetic treatment to have produced any functional improvement in humans, through incorporation of normal genes into genetically defective cells and through incorporation of genetically normal cells into the genetically abnormal organs [25] [46] [50]-[53]. It provides stable foreign gene expression and its effect is long lasting [54]. Many DMD subjects treated 16 to 20 years ago in the Cell Therapy Research Foundation are still alive; some are now 32 to 40 years of age (**Figure 1**). The US-FDA approved 50-billion MTT Phase II/III clinical trials on DMD [27] demonstrated:

1) Dystrophin,
2) 70% more myofibers and histological improvement,
3) 123% increase in contractile force at 18 months post-operatively,
4) 39 % decrease in serum CPK,
5) 19 % increase in forced vital capacity at 9 months post-operatively,
6) Clinical improvement in 75% of all subjects,
7) Life prolongation.

Allograft immunogenicity is minimal as demonstrated in clinical trials with 280 patients having muscular dystrophies [22] and two patients with ischemic cardiomyopathy [55]. Donor myoblasts fused among themselves or with host myofibers within two weeks, losing their MHC-1 surface antigens in the fusion process [56]. Daily use of cyclosporine (Cy) at 5 to 7 mg/kg body weight for no more than one month was all that was necessary to prevent allograft rejection [56]. This is highly significant for treating genetic diseases such as muscular dystrophies and Type II diabetes where allografts are must, and for genetically pre-disposed heart failure where

Figure 1. Myoblast transfer therapy extended the lifespan of six DMD patients, among others, up to 40 years of age when some of their DMD uncles died at the age of 18 to 20. For the first time in human history has this been achieved and being reported. It adds tremendous scientific values to the safety and efficacy of the therapy. These patients were treated 16 to 20 years ago in the Cell Therapy Research Foundation, USA.

allografts necessitate only three weeks of immunosuppressant.

The safety and efficacy of HMGT/MTT was published before the human genome was sequenced. The development of HMGT/MTT is completely independent of the Human Genome Project which has yet to perform its treatment claims. It appears that in treating genetic diseases with muscle defects, HMGT/MTT completely by-passed the necessity of the Human Genome Project and Single Gene Transduction. HMGT/MTT is an independent development in biomedical technology.

7. HMGT/MTT in Heart Disease Clinical Trials

Heart muscle degeneration is the leading cause of debilitation and death in humans. Atherosclerosis, ischemic cardiomyopathy and heart failure are genetic predisposed [57] [58]. These are multi-factorial and polygenic diseases with significant polymorphism. It will be an unsurmountable task to identify the various gene defects and to design gene therapies towards treatment, not to mention that such designs do not replenish myocardial cells that had degenerated previously, without which the damaged myocardium cannot regain its function.

Cardiomyocytes do not multiply significantly because the human telomeric DNA repeats in these terminally differentiated cells are minimal. Without significant mitotic activity, surviving cardiomyocytes cannot provide enough new cells to generate the contractile filaments necessary to sustain normal heart contractility.

Through endomyocardial injections of cultured skeletal myoblasts, three mechanisms of myogenesis were elucidated as proof of concept with 50 human/porcine xenografts using cyclosporine as immunosuppressant [55] [59] [60]. Some myoblasts developed to become cardiomyocytes. Others transferred their nuclei into host cardiomyocytes through natural cell fusion. As yet others formed skeletal myofibers with satellite cells. De novo production of contractile filaments augmented heart contractility [61] [62]. This latter can be translated into the improvement in the quality of life of heart patients and in the prevention of heart attacks.

There was a transient elevation of the porcine anti-human-myoblast antibodies at one week after the xenograft [56] [63]. The antibody level subsided at the second week after HMGT/MTT, indicating that no more than two weeks of cyclosporine immunosuppression would be necessary for human/pig xenografts or for human allografts.

When compared with a heart transplant, myoblast allograft eliminates the use of lifelong immune-suppressants, which is the major cause of infection and death of heart transplant patients. Myoblast transplant, either autograft

or allograft, is much less invasive, and tissue availability is not an issue. At a fraction of the cost of a heart transplant, it also promises a reduction in health costs.

To date, approximately 300 subjects with chronic myocardial infarction have received essentially autologous myoblasts via catheter delivery, or epicardial injection following coronary artery bypass grafting (CABG). Conclusions drawn from Phase I, Phase II and early Phase III clinical trials from multi-centers over 15 countries [27] [63]-[80] were:

1) Myoblast implantation with catheter or surgery following CABG is feasible, safe and efficacious.

2) It improves left ventricular ejection fraction (LVEF), perfusion, viability, kinesis, wall thickness, diastolic stiffness and stroke volume of the infarcted myocardium, and 6-minute walk distance.

3) It regenerates the scarred myocardium in ischemic cardiomyopathy.

4) It offers a potential treatment for end-stage heart disease.

The 35% to 45% relative increases in LVEF at one year after HMGT/MTT reported independently by several teams [55] [68] [70]-[79] are highly significant. This has never been achieved with any pharmaceutic or therapeutic modality in the treatment of ischemic cardiomyopathy and heart failure. Such significant increases in LVEF would most likely improve the quality of life and extend the lifespan of the patients. Undoubtedly, HMGT/MTT is the most promising treatment for heart diseases in the horizon [27] [70] [71].

Review on HMGT/MTT in treating muscular dystrophies and heart diseases had previously been published in detail [27]. The current article focuses on mechanisms of HMGT/MTT in treating Type II diabetes.

8. HMGT/MTT Corrects Gene Defects in Type II Diabetes

8.1. Human Study

Type II diabetes, also called non-insulin-dependent diabetes mellitus (NIDDM), can be traced to the genetic defects of the glucose transporter 4 (GLUT4) and the insulin-regulated aminopeptidase (IRAP) genomes [81]-[85]. Such genetic defect is manifested in reduced GSVs (GLUT4 storage vesicles) exocytosis and endocytosis trafficking, resulting in significant reduction in uptake of blood glucose into muscle fibers and adipose tissue where 75% of the body's glucose metabolism normally occurs. In Type II diabetics, normal or even elevated levels of plasma insulin would not elicit normal glucose uptake and high blood sugar persists.

Previous reports [22] [27] demonstrated that the injected myoblasts fused spontaneously with host skeletal muscle fibers, transferring their nuclei that carried the complete normal human genome to replenish normal copies of all aberrant genes, presumably in Type-II diabetes just as it replenished the dystrophin gene in DMD or BMD (Becker Muscular Dystrophy) to achieve genetic complementation repair. Other genetically normal donor myoblasts fuse among themselves to form 70% more new myofibers that undoubtedly exhibit normal GLUT-4/IRAP and GSV activities. The two proven mechanisms of HMGT/MTT from the muscular dystrophy studies led us to formulate a feasibility/safety study in two Type-II diabetic human subjects in year 2004 with Institutional Review Board (IRB) approval [49].

Human myoblasts were manufactured according to in-house SOPs. Cell production was in compliance with current Good Manufacturing Practice (cGMP) and International Organization for Standardization (ISO) 9001 standards. About 2 g of muscle biopsy was isolated under local anesthesia from a 20-year-old, pathogen-free, male volunteer after he had met muscle donor criteria. Initial dissociation isolated approximately 10,000 satellite cells that constituted the primary culture for myoblasts.

The culture yielded 47.4×10^9 myoblasts that were 100% pure by positive desmin immunostain, and 92.8% viable according to vital dye exclusion tests. The cells were potent in myogenicity in that numerous myotubes were observed within four days in a fusion medium. Throughout the culture and for the final injectates, the myoblasts were free of endotoxin (<1.0 EU/ml) and mycoplasma, and negative for sterility (14-day test) and gram stain (absence of gram positive or negative bacteria) according to certified laboratory analyses.

Patient 1 was 42 years old, 157 cm tall, and weighed 68 kg. Patient 2 was 36 years old, 158 cm tall, and also weighed 68 kg. Patients showed about two-year-history of Type II diabetes and hypertension but were otherwise normal in heart, lung, kidney, and liver function without obesity. The laboratory report revealed tests results for syphilis, hepatitis B surface antigens, antibodies to HIV and hepatitis C virus to be negative.

Both patients had previously been enrolled as clinical trial subjects after qualifying for inclusion/exclusion criteria, and signing patients' informed consents with institutional approval. The subjects took two oral doses of cyclosporine totaling 5 - 7 mg/kg body weight per day, beginning two days before grafting, weaning at half-do-

sage in the last two weeks, and off cyclosporine at eight weeks after grafting.

The whole blood trough level of cyclosporine was monitored every two weeks. Doses were adjusted in an attempt to maintain the level at about 250 ng/ml.

Myoblasts were harvested and processed under biological safety cabinets (Class 100) inside a cleanroom. Having been washed thoroughly, they were suspended in the injection medium. Quality assurance/quality control processes ensued, and the final quality control release test forms were issued for each of the two subjects. The myoblasts were then carried in syringes within sterile enclosures into two surgical suites for simultaneous implantation into both subjects. These were the world's first cases of allogeneic myoblasts being injected into Type II diabetic patients.

The patients received 132 injections each and 24/23.4 billion myoblasts, respectively. The two hour procedure was performed with the patients under general anesthesia. Cells were injected at 50×10^6/ml and the injections were made under direct vision into 54 major muscle groups of each subject. The patients were transferred to the intensive care unit, where they recovered from the anesthesia and routine monitors were administered. The subjects recovered from the general anesthesia without rash or fever, and both patients were discharged at 48 hours post-operatively.

Most pertinent to the specific goal of this study was that, despite cyclosporine discontinuation at two months postoperatively, no sign of rejection was observed. Cyclosporine is known to increase plasma glucose. The patients appeared to have good general health before and after MTT. Plasma glucose and insulin levels did not show significant difference before versus after 24-billion HMGT.

This was a feasibility and safety study and was not designed to test efficacy. This pioneering feasibility/safety study of myoblast allografts into the skeletal muscles of Type II diabetic patients led the way in developing a genetic treatment for the disease [49]. The subjects were weaned off cyclosporine. The procedure was shown to be safe for both subjects.

A potential genetic treatment of the disease involves HMGT/ MTT similar to the 50-billion myoblast protocol used to treat muscular dystrophy. It consists of culturing genetically normal, immature muscle cells called myoblasts, derived originally from a 2 g skeletal muscle biopsy from a healthy, young, male donor free of blood-borne pathogens, and injecting these allogeneic myoblasts with host serum at approximately 10^8 cells/mL into 80 major muscles of a diabetic patient. A distinct improvement of the technology will be the use of host serum as the carrier solution which enhances the survival and development of the myoblast allograft. Cyclosporine will be used for 2 to 3 weeks as an immunosuppressant.

8.2. Animal Studies

Mice with muscle-specific Glut-4 knockout were insulin resistant and glucose intolerant from an early age [84] [86]. Muscle specific LKB1 (a serine/threonine kinase that is a negative regulator of insulin sensitivity) knockout increased insulin sensitivity and improved glucose homeostasis [87]. These studies indicated that defects in skeletal muscle insulin-stimulated glucose transport were key factors in insulin resistance and Type 2 diabetes mellitus [88]. Ye *et al.* demonstrated that HMGT/MTT attenuated hyperglycaemia and hyperinsulinemia, and improved glucose tolerance in a mouse model (KK) of type 2 diabetes mellitus (47) as follows.

KK Cg-Ay/J mice, aged 12 - 14 weeks, after an initial intraperitoneal glucose tolerance test (GTT) were divided into three groups: KK control group receiving basal medium (M199); KK myoblast group receiving 3×10^7 human myoblasts; KK fibroblast group receiving 3×10^7 human fibroblasts. Non-diabetic C57BL mice were used as an additional normal control and also had HMGT/MTT. All animals were treated with cyclosporine for 6 weeks.

Immunohistochemistry studies at 12 weeks showed extensive integration of human myoblast nuclei into mouse muscle fibers. Repeat GTT showed a significant decrease in glucose concentrations in the KK myoblast group compared to the KK control and KK fibroblast groups. The KK myoblast group also had reduced mean HbA1c, cholesterol, insulin, triacylglycerol, and increased adiponectin compared with the KK control and KK fibroblast groups. C57BL mice showed no change in glucose homeostasis after HMGT/MTT.

Ma *et al.* demonstrated for the first time that HMGT/MTT resulted in a change of gene transcripts in multiple (50) genes involved in the insulin signaling pathway, and in the mitochondrial biogenesis and function of the skeletal muscles of KK mice. Methods of human myoblast implantation and immunosuppression follow that of Ye *et al.* [47]. Hind limb muscles were harvested and used for study of gene expression profiling.

As in Ye *et al.* [47], extensive integration of donor myoblast nuclei into host muscle fibers was demonstrated

at 12 weeks after HMGT/MTT (**Figure 2**). Glucose tolerance test showed a significant decrease of blood glucose in the mice of KK myoblast group compared to the KK control and fibroblast groups (**Figure 3**). Transcriptional patterns of 23 genes in the insulin signaling pathway showed upregulation and downregulation in KK

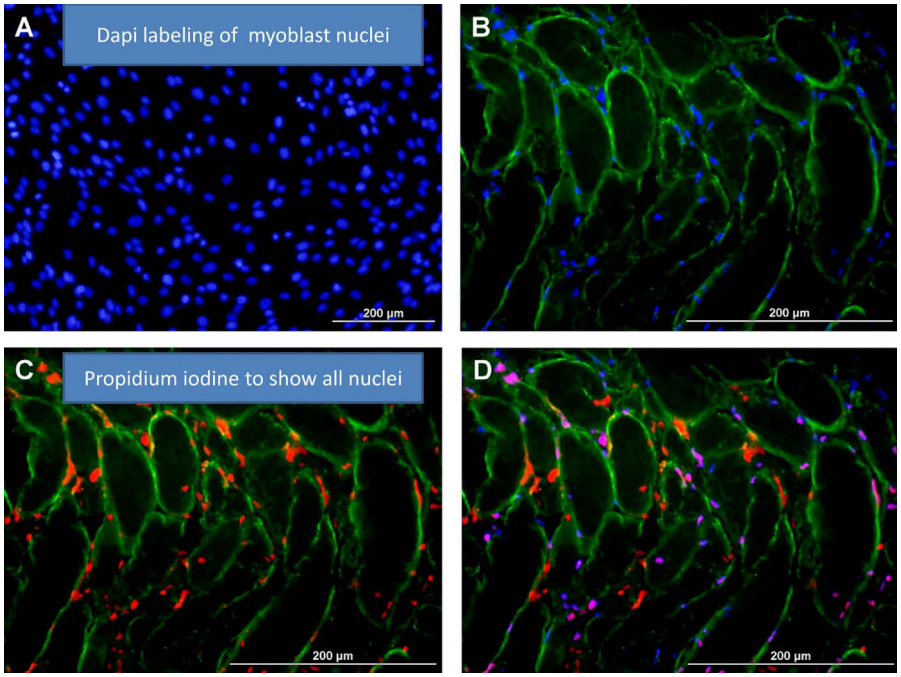

Figure 2. Human myoblast nuclei integrated into diabetic mouse muscle fibers to exercise genetic complementation repair at 12 weeks after HMGT/MTT. (A) DAPI labeling of human myoblast nuclei showing 100% labeling efficiency. (B) Integration of DAPI+ human myoblast nuclei into diabetic mouse muscle fibers immunostained also for dystrophin to show fiber boundary. (C) The same tissue was counter-stained with propidium iodine to show all nuclei. (D) Overlay pictures (B) and (C) to show genetically mosaic muscle fibers containing normal (human in blue and purple) and diabetic (KK mouse in orange) nuclei (Bar = 200 µm).

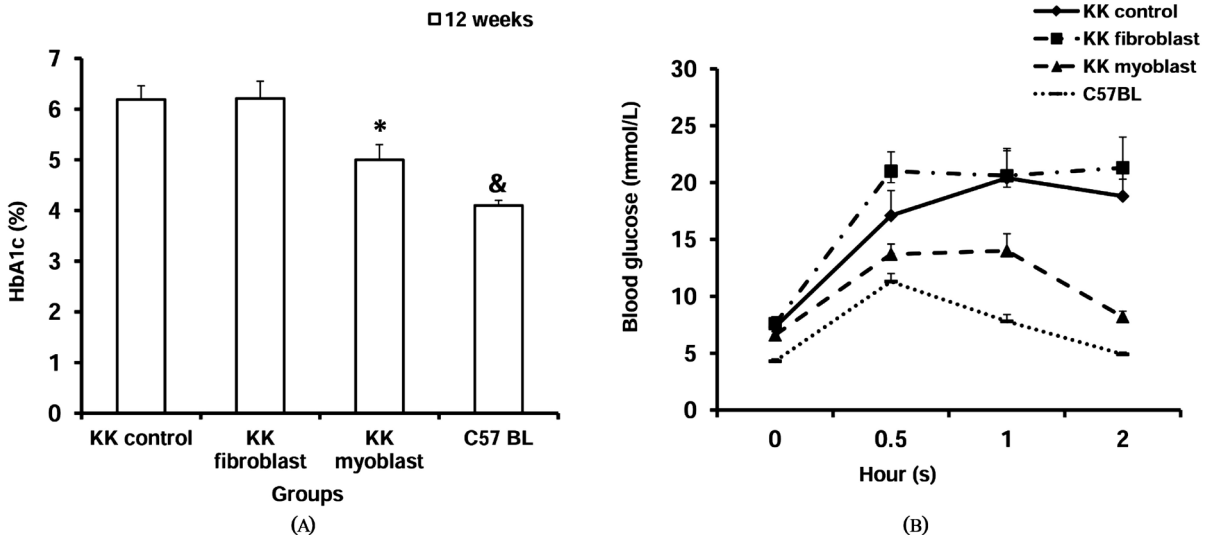

Figure 3. Glucose homeostasis of mouse at 12 weeks after treatment. (A) HbA1c of KK myoblast group was significantly reduced as compared with KK control and KK fibroblast. However, it was still significantly higher than that of C57BL group, which served as a normal control. (B) Glucose Tolerance Test (GTT) showed that KK myoblast group had significantly reduced plasma glucose concentration during GTT and similar to that of C57BL group (*vs. KK control and fibroblast $p < 0.05$, & vs. any KK group: $p < 0.05$ by ANOVA).

myoblast group as compared with KK control group, KK fibroblast group, and C57BL group (**Table 1**). In addition, transcriptional patterns of 27 genes in mitochondrial biogenesis and function also demonstrated alterations in KK myoblast group as compared with KK control group, KK fibroblast group, and C57BL group (**Table 2**). These upregulation and downregulation in transcriptional pattern levels of the 50 genes after HMGT/MTT represent genetic repair toward attenuating hyperglycaemia and hyperinsulinemia, and improving glucose tolerance in the mouse model (KK) of Type-II diabetes mellitus.

Table 1. Comparison of insulin signaling pathway gene transcript levels in the KK hSkM, KK control, and KK fibroblast groups at fasting state.

Genes	Accession no.	Categories	Fold change		
			A	B	C
Acaca	NM_133360	Target genes for SREBP1	2.06↑	2.87↑	0.94
*Acox*1	NM_015729	Lipid metabolism, target genes for PPAR*γ*	11.23↑	1.09	1.84
*Aebp*1	NM_009636	Transcription factors and regulators	9.06↑	2.01↑	1.71
Braf	NM_139294	MAPK pathway	2.5↑	0.84	2.25↑
Cebpa	NM_007678	Cell growth and differentiation protein metabolism Transcription factors and regulators	2.64↑	1.47	1.36
Cfd	NM_013459	Target genes for PPAR*γ*	2.27↑	4.29↑	1
*Frap*1	NM_020009	PI-3 kinase pathway protein metabolism	11↑	1.25	2.81↑
*Frs*2	NM_177798	Cell growth and differentiation insulin receptor-associated proteins protein metabolism	4.2↑	0.91	0.79
*Gab*1	NM_021356	Insulin receptor-associated proteins MAPK pathway protein metabolism	5.62↑	0.7	0.86
Gpd-1	NM_010271	Carbohydrate metabolism target genes for PPAR*γ*	9.38↑	2.41↑	2.51↑
*Hras*1	NM_008284	Cell growth and differentiation MAPK pathway Protein metabolism	3.03↑	1.05	1.92
*Igf*2	NM_010514	Cell growth and differentiation	19.7↑	1.92	3.51↑
*Irs*2	NM_001081212	Cell growth and differentiation insulin receptor-associated proteins	2.48↑	1.23	1.09
Jun	NM_010591	Cell growth and differentiation Primary target genes for insulin signaling Protein metabolism Transcription factors and regulators	2.93↑	2.01↑	1.05
Leptin	NM_008493	Lipid metabolism Carbohydrate metabolism Protein metabolism Primary target genes for insulin signaling Cell growth and differentiation	2.03↑	1.95	30.3↑
Pparg	NM_011146	Target genes for PPAR*γ* Cell growth and differentiation Transcription factors and regulators	0.46↓	3.32↑	0.59
*Ppp*1*ca*	NM_031868	Carbohydrate metabolism Insulin receptor-associated proteins Protein metabolism	4.59↑	1.54	2.08↑
Ptpn1	NM_011201	Insulin receptor-associated proteins protein metabolism	2.62↑	4.66↑	1.65
*Raf*1	NM_029780	Cell growth and differentiation protein metabolism, transcription factors and regulators	2.51↑	0.97	0.80
*Shc*1	NM_011368	Cell growth and differentiation insulin receptor-associated proteins lipid metabolism protein metabolism mapk pathway	3.53↑	1.11	1.64
Glut-1	NM_011400	Carbohydrate metabolism	2.25↑	0.98	0.48↓
*Rps*6*ka*1	NM_009097	MAPK pathway, PI-3 kinase pathway protein metabolism	2.58↑	1.56	1.31
*UCP*1	NM_009463	MAPK pathway, PI-3 kinase pathway protein metabolism	8.4↑	4.76↑	4.44↑

↑ and ↓: significant upregulation and downregulation in the KK myoblast group compared with KK control group (A), KK fibroblast group (B), and C57BL group (C).

Table 2. Comparison of mitochondria gene transcript levels in the KK hSkM, KK control, and KK fibroblast groups at fasting state.

Genes	Accession no.	Categories	Fold change		
			A	B	C
Aifm2	NM_178058	Apoptotic genes	2.45↑	1.52	1.32
Aip	NM_016666	Mitochondrial transport targeting proteins to mitochondria mitochondrion protein import	2.33↑	1.21	0.85
Bcl2l1	NM_009743	Apoptotic genes mitochondrial transport membrane polarization & potential	13.4↑	3.68↑	3.03↑
Cox10	NM_178379	Mitochondrion protein import mitochondrial fission & fusion	2.31↑	2.52↑	1.1
Cpt1b	NM_009948	Mitochondrial transport targeting proteins to mitochondria mitochondrion protein import	16.33↑	2.06↑	1.72
Fis1	NM_025562	Mitochondrial fission & fusion	2.5↑	1.24	1.39
Grpel1	NM_024478	Mitochondrial transport targeting proteins to mitochondria mitochondrion protein import	5.39↑	1.15	1.14
Mfn1	NM_024200	Mitochondrial fission & fusion, mitochondrial localization	2.03↑	0.95	0.86
Mipep	NM_027436	Mitochondrial transport, targeting proteins to mitochondria	2.83↑	1.43	1.46
Opa1	NM_133752	Inner membrane translocation, mitochondrial localization mitochondrial fission & fusion	5.13↑	0.95	2.13↑
Rhot2	NM_145999	Mitochondrial localization	2.64↑	1.06	1.23
Sfn	NM_018754	Apoptotic genes	2.14↑	0.77	3.39↑
Slc25a15	NM_181325	Small molecule transport	2.55↑	0.86	0.91
Slc25a16	NM_175194	Small molecule transport	3.01↑	1.23	0.76
Slc25a17	NM_011399	Small molecule transport	3.16↑	0.89	1.22
Slc25a20	NM_020520	Small molecule transport	3.31↑	1.13	0.59
Slc25a22	NM_026646	Small molecule transport	3.16↑	2.01↑	2.07↑
Slc25a25	NM_146118	Small molecule transport	2.53↑	2.91↑	0.46↓
Slc25a27	NM_028711	Small molecule transport	4.26↑	0.92	1.2
Stard3	NM_021547	Mitochondrial transport	2.87↑	2.23↑	1.31
Taz	NM_181516	Inner membrane translocation	4.89↑	1.17	1.3
Timm17b	NM_011591	Inner membrane translocation	3.39↑	2.19↑	1.17
Timm22	NM_019818	Inner membrane translocation	3.46↑	1.82	1.05
Timm44	NM_011592	Inner membrane translocation	2.31↑	1.09	1.4
Tomm34	NM_025996	Outer membrane translocation	3.27↑	1.56	1.16
Tomm40	NM_016871	Outer membrane translocation	5.13↑	3.46	1.59
UCP1	NM_009463	Membrane polarization & potential mitochondrial transport	8.4↑	4.76↑	4.44↑

↑ and ↓: significant upregulation and downregulation in the KK myoblast group compared with KK control group (A), KK fibroblast group (B), and C57BL group (C).

Undoubtedly, these studies [47]-[49] provide strong evidence that HMGT/MTT mediates its beneficial effects through myoblast fusion, not only adding new normal myofibers, but replenishing normal copies of the abnormal genes upon nuclear transfer to effect genetic repair of the dystrophic [22] [46] [50]-[54], cardiomyopathic [40] [55] [56] [59] [60] [63] or diabetic myofibers [47]-[49]. Together, they demonstrated the proof-of-concept of HMGT/MTT in developing as a cell therapy and as a gene therapy for hereditary muscle diseases such as muscular dystrophies, hereditary cardiomyopathies, Type-II diabetes and others [25] [27].

9. Anti-Aging Aesthetica (AAA)

Beauty is a physical attribute that often enhances one's self-confidence, career and quality of life. The physical parameters of appearance are size, shape, tone, color, luster, texture, consistency, and density. These parameters

deteriorate in every organ according to the genetically programmed degeneration of aging. Current technologies favor using live cells to enhance these parameters of appearance [89]-[92]. The patented HMGT/MTT is at the forefront of regenerative medicine today [27] (**Table 3**).

9.1. Skin Cover

The polygonal skin fibroblasts are about 15 times the size of the myoblasts and produce a rough body cover. Cancers are common in skin but rare in muscle. Myoblasts, because of their small size, spindle shape, and resilience, can grow within wrinkles and on skin surfaces, thus enhancing the color, luster and texture of the skin "plated" with them [27] [90]. Biologic creams are formulated to promote cell survival, growth and development to enhance the color, luster, density and texture of the skin. Thus, a new layer of biological skin consisting of pure myoblasts can eliminate skin defects and blemishes.

9.2. Body Sculpture

Intramuscular injection of myoblasts can augment the size, shape [30], consistency, tone and strength of muscle groups, improving the lines, contours and vitality from the sculpture for a youthful appearance. The myoblast technology can be used for cosmetic enhancement such as bodybuilding, and in tissue implants for breast/buttock/facial augmentation [89].

The myoblasts can be injected intramuscularly to grow muscles, or subcutaneously as a filler. Unlike the noncellular collagen which will be absorbed in three to six months after injection, injected myoblasts are cells that will survive and last for tens of years within the host. Myoblasts are endogenous to the human body and have been proven safe in clinical trials involving over 280 muscular dystrophy patients and 300 heart patients worldwide. Myoblasts will not cause cancer like silicone, or burst and absorbed like saline or collagen implants.

9.3. Anti-Aging Angiomyogenesis

Distribution of oxygen and nutrients to the peripheral organs is significantly reduced for people aged over 45. In developing treatment for human myocardial infarction, we have grown five times more blood capillaries and muscle simultaneously using human myoblasts transduced with angiogenic factors [23] [39] [40] [61] [62]. In addition to their application to treat heart diseases, potentially these cells can be used to treat male/female impotency, baldness and to produce redder lips and pinker face because of the higher density of capillaries within layers of myogenic cells after myoblast treatment. The latter serves as a fertile ground to seed new hair follicle cells on the bald head or other body parts to give the desirable hair color, density and consistency.

10. Conclusions

HMGT/MTT mediates its effect through transfer of the normal myoblast nuclei that supply the complete human genome, in addition to just replenishing the missing gene(s) or normal copies of the aberrant gene(s). The replacement genes then transcribe to produce the necessary proteins or factors for genetic repair. Donor myoblasts also develop to supply significant large numbers of normal myofibers to combat muscle degeneration and weakness.

One can envision a variety of uses of this discovery, including that for disease treatment, disease prevention, drug discovery, and selection of superior cells and clones for therapy. It is through continual research and development that we can fully harness HMGT/MTT to relieve human suffering, to improve quality of life, and to prolong life expectancy of mankind.

Table 3. Current materials in cosmetology.

Myobasts	Long-lasting cell sculpture with live tone
Fibroblasts	Short-live; easily tumorigenic
Silicone	Bio-non-compatible; leaky; carcinogenic
Saline	No live feeling; leaky
Collagen	Absorbed within 3 to 6 months
Botox	Damages neuromuscular transmission; diminishes facial expression

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Conflict of Interest

As originator of the MTT/HMGT technology, the author holds the pioneering patent and many others related to the compositions, methods, and medical devices.

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Abbreviations

AAA = Anti-Aging Aesthetica
Ad-phVEGF$_{165}$ = Adenoviral transduced plasmid of Vascular Endothelial Factor 165 (human)
ADA = Adenosine Deaminase
AF = Ankle Plantar Flexors
AIDS = Autoimmune Deficiency Syndrome
AST = Aspartate Aminotransferase
ATCC = American Tissue Culture Collection
BMD = Becker Muscular Dystrophy
CABG = Coronary Artery Bypass Grafting
CBER = Center for Biologics Evaluation and Research
CCS = Canadian Cardiovascular Society
CD-phVEGF$_{165}$ = CD Liposome Transduced Plasmid of Vascular Endothelial Growth Factor165 (human)
CFR = Code of Federal Regulation
cGMP = Current Good Manufacture Practices
CK = Creatine Kinase
CsA = Cyclosporine A
CTRF = Cell Therapy Research Foundation
Cy = Cyclosporine
DMEM = Dulbecco's Modified Eagles Medium
DMF = Drug Master File
DNA = Deoxyribonucleic Acid
EDB = Extensor Digitorum Brevis
ELISA = Enzyme-Linked Immunosorbent Assay
EMA = European Medicine Agency
EPA = European Patent Agency
FDA = Food and Drug Administration
18FDG PET = 18F-Fluoro-Deoxy-D-Glucose
PET = Positron Emission Tomography
FG = Fluoro-Gold
FIM = First-in-Man
GLUT4 = Glucose Transporter 4
GPI = Glucose-6-Phosphate Isomerase
HCT = Heart Cell Therapy
HMGT = Human Myoblast Genome Therapy
HNSCC = Head and Neck Squamous Cell Carcinoma
HRP = Horseradish Peroxidase
IND = Investigational New Drug
ISO = International Organization for Standardization
KE = Knee Extensors
KF = Knee Flexors
LBT = Lower Body Treatment
LPL = Lipoprotein lipase
LVEF = Left Ventricular Ejection Fraction
MAGIC = Myoblast Autologous Grafting in Ischemic Cardiomyopathy
MHC-1 = Major Histocompatibility Class-1
MIBI-Tc99m = Technetium (99mTc) Sestamibi
MTT = Myoblast Transfer Therapy
NIDDM = Non-Insulin-Dependent Diabetes Mellitus
PBS = Phosphate-Buffered Saline
PCT = Patent Cooperation Treaty
PEI-phVEGF$_{165}$ = Polyethylenimine-25 Nanoparticle Transduced Plasmid of Vascular Endothelial Growth Fac-

tor 165(human)
PKU = Phenylketonuria
RT-PCR = Reverse Transcription Polymerase Chain Reaction
SCID = Severe Combined Immunodeficiency Disorder
SFDA = State Food & Drug Administration
SMT = Single Muscle Trial
SOP's = Standard Operation Procedures
SPECT = Single-Photon Emission Computed Tomography
UBT = Upper Body Treatment
USP = United States Pharmacopeia
USPTO = United States Patent Office
UTM = University of Tennessee Memphis
$VEGF_{165}$ = Vascular Endothelial Growth Factor 165 (human)
WBT = Whole Body Treatment