Symposium: Review Article

Human myoblast genome therapy

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Abstract Human Myoblast Genome Therapy (HMGT) is a platform technology of cell transplantation, nuclear transfer, and tissue engineering. Unlike stem cells, myoblasts are differentiated, immature cells destined to become muscles. Myoblasts cultured from satellite cells of adult muscle biopsies survive, develop, and function to revitalize degenerative muscles upon transplantation. Injection injury activates regeneration of host myofibers that fuse with the engrafted myoblasts, sharing their nuclei in a common gene pool of the syncytium. Thus, through nuclear transfer and complementation, the normal human genome can be transferred into muscles of patients with genetic disorders to achieve phenotype repair or disease prevention. Myoblasts are safe and efficient gene transfer vehicles endogenous to muscles that constitute 50% of body weight. Results of over 280 HMGT procedures on Duchenne Muscular Dystrophy (DMD) subjects in the past 15 years demonstrated absolute safety. Myoblast-injected DMD muscles showed improved histology. Strength increase at 18 months post-operatively averaged 123%. In another application of HMGT on ischemic cardiomyopathy, the first human myoblast transfer into porcine myocardium revealed that it was safe and effective. Clinical trials on approximately 220 severe cardiomyopathy patients in 15 countries showed a <10% mortality. Most subjects received autologous cells implanted on the epicardial surface during coronary artery bypass graft, or injected on the endomyocardial surface percutaneously through guiding catheters. Significant increases in left ventricular ejection fraction, wall thickness, and wall motion have been reported, with reduction in perfusion defective areas, angina, and shortness of breath. As a new modality of treatment for disease in the skeletal muscle or myocardium, HMGT emerged as safe and effective. Large randomized multi-center trials are under way to confirm these preliminary results. The future of HMGT is bright and exciting. (J Geriatr Cardiol 2006;3:135-51.)

Key Words heart regeneration; human genome therapy; myoblasts

Introduction

This review is about the landmark development of a biomedical platform technology called Human Myoblast Genome Therapy (HMGT), known previously as Myoblast Transfer Therapy (MTT). The procedure involves cell transplantation, nuclear transfer, and tissue engineering to correct genetic defects of skeletal muscle and to strengthen degenerated and weak myocardium.

This is the most up-to-date review on HMGT, with critical evaluation of both basic experiments and clinical trials. The conceptual approaches and technological developments are discussed. State-of-the-art methodology and results of clinical trials are described in detail, along with future perspectives. The work traverses the fields of genetics, neurology, pediatrics, cardiology, endocrinology, immunology, surgery, and developmental biology.

Basic concept: HMGT at the cellular level

The cell is the basic unit of all living organs. It is the infinitely small entity of which life is made. Even at this most advanced level of modern science, a living cell has still not been synthesized from nonliving ingredients such as DNA, ions, and biochemicals. Culture is the only method of cell replication and multiplication in vitro; however, the cardiac muscle cells (cardiomyocytes) could not multiply significantly in culture. They undergo no more than three to five divisions, yielding an insufficient number of myogenic cells. To the contrary, with proper techniques, normal
immature skeletal muscle cells called myoblasts can be cultured and multiplied in abundant quantity.¹

Myoblasts

Mammalian skeletal muscles are derived from the mesodermal germ layer in the embryo. In human beings, the mesoderm first appears at 20 days after fertilization.² Concomitant is the appearance of somites. Within the somites are uncommitted mitotic stem cells capable of giving rise to muscle, bone, cartilage, blood, lymphatic, fat, and connective tissues (Fig. 1). The commitment to being myogenic occurs early since myoblasts are found in the limb buds at 26th day of gestation. Such lineage determination is influenced by embryonic induction and irreversible gene expression.³

<table>
<thead>
<tr>
<th>Pluripotent</th>
<th>Differentiated</th>
<th>Early</th>
<th>Mature</th>
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<tbody>
<tr>
<td>Stem cells</td>
<td>Myoblasts → Myotubes → Muscle fibers</td>
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<td>transfer</td>
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<td>Fibroblasts</td>
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<td>Chondrocytes</td>
<td>Cartilage</td>
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Fig. 1. Pluripotent stem cells in the mesenchyme exhibit uncontrolled differentiation to become differentiated cells such as myoblasts, cardiomyocytes, fibroblasts, adipocytes, chondrocytes, and osteoblasts. The benefit vs risk ratio of using myoblasts over stem cells in treating heart failure is greater. MTT = myoblast transfer therapy.

Figure 2 shows the different characteristics of the myoblasts as they develop. Beginning as small spheres of about 12 μm in diameter, myoblasts grow best on collagen, which resembles the extracellular matrix in vivo. Under proper culture condition, transformation into spindle-shaped cells occurs within three days. Then they can fuse spontaneously among themselves and develop to become multinucleated myotubes. In the human fetus, close confinement within the somites ensures that the myoblasts are always at a state of confluence. The latter is a prerequisite for cell fusion. Cell division is an intrinsic property of myoblasts as evident by myoblast proliferation in serum-free culture media. However, proliferation in large quantity requires neurotrophic elements and growth factors.⁴,⁵ It is known that myoblasts do not fuse when they are in the S, G₂, M, or even the early G₁ phases of the cell cycle. Division synchronization would appear to be a less important factor in myoblast fusion, considering that myoblast doubling time in vitro is approximately 20 hours, and that myoblasts within a somite are essentially replicating clones.

Natural cell fusion

Reduction in serum or chick embryo extract concentration precipitates cell fusion in vitro. Cell fusion occurs only after the myoblasts have undergone considerable cycles of division and are withdrawn from the mitotic cycle.⁶ The decision to fuse in vivo is genetically programmed, and appears to occur without any neural contact or influence. It involves structural gene transcription and RNA synthesis, especially in the formation of the receptors on the myoblast surface that are responsible for cell recognition, cell adhesion, and membrane restructuring.⁷ The end products are multinucleated myotubes, each of which is formed from fusion of 200 or more myoblasts.

Myotubes

Myotubes, with the many nuclei within the syncytiun, are differentiated cells capable of producing large quantities of contractile proteins and related molecules. These proteins include, among others, myosin, actin, tropomyosin, and myoglobin. The contractile proteins are structured into filaments as the myotube develops, and are packaged into basic contracting units called sarcomeres. Hundreds of sarcomeres are aligned to form a myofibril many of which comprise a myofiber. Each sarcomere is made up of myosin and actin filaments linked together by cross-bridges. Upon excitation-contraction coupling, millions of cross-bridges are formed, sliding the actin filaments toward the center of each myosin filament, and resulting in contraction.⁷ Myotubes exhibit sarcomeres and immunostain positively for myosin and actin; they contract spontaneously in vitro. Mature
myotubes will undergo degeneration if there is no trophic support.

Myotube maturation

The myotubes will not develop into a myofiber unless innervated by a motor axon or supported with growth factors. With the arrival of the axonal terminal, more contractile proteins are deposited, and the sarcoplasmic reticulum becomes better developed. The centrally located nuclei migrate peripherally. Numerous structural proteins and glycoproteins are synthesized and deposited during the transition from myotube to myofiber. Among these is dystrophin, a surface membrane protein which is not present in the myofibers of Duchenne muscular dystrophy subjects due to a genetic defect.

Satellite cells

A built-in regenerative measure resides with the satellite cells that are myoblast reserves in adult skeletal muscle fibers. These are mononucleated cells located between the basal lamina and the plasma membrane. Satellite cells are the major source of myoblasts during regeneration. The undifferentiated satellite cells are mobilized upon muscle injury to multiply and become myoblasts that eventually fuse to form myotubes.

It is estimated that 11% of all nuclei within the basement membrane of normal muscle fibers in the gastrocnemius and soleus muscles of the rat belonged to satellite cells, and that only 6% of the nuclei remained viable 8 to 10 h after mincing. This 6% fraction would undergo mitosis before fusion. When expressed as a percentage of the total number of myonuclei in a muscle fiber, satellite cell contribution becomes less with age.

Basic concept: HMGT at the tissue level

The goals of HMGT or any modality of treatment for diseases of the skeletal muscle and myocardium on the tissue level is to develop vessel (angiogenesis, vasculogenesis, and arteriogenesis) and skeletal or myogenic cells (myogenesis). However, at the end, angiogenesis or myogenesis has to improve the clinical function as measured by left ventricle ejection fraction or higher level of exercise tolerance. Without concrete proof of robust angiogenesis, vasculogenesis, or myogenesis, it is hard to explain the success or failure of any treatment modality on degenerative skeletal muscle disease or cardiomyopathy.

Therapeutic angiogenesis

Therapeutic angiogenesis aims to alleviate tissue ischemia by supplementing the intrinsic process of angiogenesis. It exploits the natural process for enhanced neovascularization. In addition to the administration of angiogenic growth factors or naked DNA, viral and non-viral vector constructs carrying angiogenic genes have been used. A new approach utilizes cell mediated gene transfer. Endothelial progenitor cells and stem cells from bone marrow and embryos have been transduced to secrete angiogenic factors to achieve therapeutic angiogenesis in ischemic heart disease.

Angiogenesis/Arteriogenesis/Vasculogenesis

Angiogenesis and vasculogenesis are primarily responsible for development of the vascular system in the embryo. Whereas vasculogenesis involves angioblasts for the in situ new vessel development, angiogenesis occurs by sprouting of the already present primitive vasculature using previously differentiated cells. Another phenomenon contributing to the development of collateral vessels is arteriogenesis through remodeling of the existing arterioles. Angiogenesis is an integral part of many physiological and pathological processes, and it is controlled by various factors that induce or inhibit blood vessel formation. Disruption of the natural balance between the pro- and anti-angiogenic factors results in pathological angiogenesis with abnormal blood vessel formation.

Myogenesis

The transplantation of transduced myoblasts into the ischemic myocardium is like installing a bioreactor to produce biologically active angiogenic factors in a localized and sustained fashion. Concomitantly, the development of these myogenic cells can provide new contractile elements to restore myocardial function. An example of angiomyogenesis in a porcine heart model of myocardial infarction using human myoblasts transduced with vascular endothelial growth factor-165 (VEGF165) or angiopoietin-1 (Ang-1) was highlighted again by Ye et al. in this issue of the Journal of Geriatric Cardiology.

Most previous studies relied on a single growth factor or its gene administration. Angiogenesis involves interplay between numerous growth factors, their receptors, and intercellular signals. The synergistic interaction in vivo between VEGF165 and Ang-1 to produce functional and leak-resistant neovascularization support the suitability of their combined administration. Combining the synergistic effect between VEGF165 and Ang-1, together with autologous myoblasts, has the additional advantage of inducing angiomyogenesis for treating ischemic limbs in the rabbit. Unlike the monocistronic vectors, bicistronic vector provides a less cumbersome delivery option by simplifying the construct design and enabling delivery of the two transgenes in a single administration.

Our human myoblasts transduced by adenoviral bicistronic vector that expressed both VEGF165 and Ang-1 significantly increased neovascularization in the ischemic porcine myocardium (Fig.3). Double fluorescent immunostaining of tissue sections for von Willebrand factor (vWF) VIII and smooth muscle actin (SMA) revealed that a large proportion of the vessels observed were coated with a smooth muscle layer. This observation is in agreement with previous studies that show the role of Ang-1 in development of mature, stable, non-leaky vessels. This suggests that increased blood vessel density was genu-
inily the result of treatment with VEGF165/Ang-1 transduced myoblasts.

In the myogenesis study, cultured myoblasts derived from satellite cells of human rectus femoris biopsies were transduced with a retroviral vector carrying Lac-Z reporter gene, and about 75% of the myonuclei were successfully transduced. Trypan blue stain revealed >95% cell viability immediately before injection.

**Terminology**

The terms “Myoblast Transfer” and “MTT” were coined in a workshop held in Perth, Western Australia, in 1989. Subsequently, in the same year, the First International Conference on Myoblast Transfer Therapy was held in New York City. In both meetings, pioneer researchers of the new, innovative modality of treatment from the US and abroad voiced their confidence that MTT would be the most logical approach in the development of treatment in the skeletal muscle disease.

![Fig3. Polar views on PET and SPECT tomograms of the left ventricular myocardium before and at 3 and 6 months after myoblast allograft for the 63 y.o. patient. Before surgery (A) 35% perfusion defect during exercise and (B) 20% at rest, (C) significant decrease of systolic thickening and (D) preserved metabolism are indicative of scarred and hibernated myocardium. After surgery (A) 14% perfusion defect during exercise and (B) 5% at rest, (C) decrease in systolic thickening is less, and perfusion in the posterior wall (arrow) has been retrieved. (D) Metabolism is preserved and shows no change.](image)

**Production of myoblast: culture and multiplication**

Human myoblasts were manufactured according to the investigators’ standard of procedure (U.S. Patent No. 5,130,141). This method yielded a purity of 99% by human desmin immunostaining (Fig. 4). From 2g human muscle biopsy, 50 billion pure myoblasts were produced routinely in our Good Manufacture Practice (GMP) facilities in about 45 days.

![Fig4. Human desmin immunostained for myoblast purity. (A) Positive control of leiomyosarcoma, staining brown. (B) Negative control of human skin fibroblasts. (C) Pure human myoblasts immunostained with desmin. (D) Pure human myoblasts in culture.](image)

Until now, the term “MTT” has been used in the scientific literature interchangeably for animal and human studies. However, in human studies, a more appropriate term is “Human Myoblast Genome Therapy” or “HMGT” because of concomitant cell and genome therapy.

**Clinical applications of HMGT**

**HMGT for degenerative diseases of the skeletal muscle**

Muscle degeneration and weakness is the common pathway and hallmark of muscular dystrophies, heart diseases, type 2 diabetes, and aging. These diseases and conditions are characterized by dysfunction and death of muscle cells. HMGT has been developed to repair abnormal cells and to replace dead cells of degenerative skeletal muscles. Since myoblasts are native to skeletal muscles, Duchenne Muscular Dystrophy (DMD) was selected to be
the first human disease evaluated for HMGT in our initial clinical trials.

**Duchenne muscular dystrophy** The natural history of DMD depicts a skeletal muscle degeneration and loss of strength,27 to begin at three years or younger, and continuing throughout the course of the disease. Degeneration is more severe in the proximal and anti-gravitational muscles than in the distal ones, and proximal muscle weakness in the lower body is responsible for the Gowers’ sign used in physical diagnosis. Debilitating and fatal, DMD affects 1 in 3300 live male births,29 and is the second most common lethal hereditary disease in humans.29 DMD individuals usually lose 50% of the strength in their leg muscles by age 9. They are wheelchair-bound by age 12, and three-quarters die before age 20. Pneumonia usually is the immediate cause of death, with underlying respiratory muscle degeneration. Cardiomyopathic symptoms develop in mid adolescence30 in about 10% of the DMD population. By age 18, all DMD individuals develop cardiomyopathy,21 but cardiac failure is seldom the primary cause of death.27

**Pathological basis of Duchenne muscular dystrophy** Dystrophin is a cytoskeletal membrane protein similar to β-spectrin and α-actinin.8,21 Dystrophin is a large structural protein associated with glycoproteins in the inner surface of the plasma membrane of normal myotubes or myofibers. The dystrophin/glycoproteins complex provides support to the cell membrane cytoskeleton. Dystrophin is absent in DMD, and is reduced in Becker muscular dystrophy (BMD) due to various gene defects.31 The absence of dystrophin causes a disruption of the sarcolemmal linkage, with loss of the glycoproteins, especially during muscle contraction, and results in cell necrosis and muscle weakness in DMD. Whereas dystrophin serves as a good genetic/biochemical marker, more pertinent are the technical questions of how to replace dystrophin, and how to increase muscle contractility.

**Genetic manipulation in duchenne muscular dystrophy** At first gene therapy was tried in DMD; however, the results were not as satisfactory as one might hope. The explanation is that expression of a foreign gene requires appropriate integration and regulation involving numerous cofactors, many of which are transient and unidentified during embryonic development. This is especially true in the deposit of structural proteins such as dystrophin, rather than in the secretion of angiogenic factors, enzymes, and hormones. That is why single gene transduction cannot be effective for complex diseases such as DMD and diabetic cardiomyopathy.

In gene therapy, genes only transmit biological characteristics and are not primary functioning units like cells of the body. Like drug therapy, dystrophin up-regulation cannot replace degenerated cells. At best, it can only repair degenerating cells. In a 12-year-old DMD boy, over 70% of the muscle cells of the quadriceps have been lost; it is unlikely that dystrophin replacement in the remaining cells alone will bring forth a cure or a treatment, whatever the delivery system of dystrophin may be. Similarly, in a 60-year-old cardiac patient whose over 50% of the cardiomyocytes in the left ventricle are dead from myocardial infarction, it is not possible to cure his or her cardiomyopathy with injection of angiogenic gene or angiogenic factor. What are needed in both cases are new myogenic cells to deposit contractile proteins.

Besides lacking dystrophin, muscle cells of DMD patients are deprived of other dystrophin-related proteins and glycoproteins. Since the absence of these latter proteins can cause dystrophy by themselves,34 single gene replacement will not be effective in repairing the defective cells except for the very young patients in whom cell degeneration has not begun. Retroviral infection of mature myofibers with conjugated DNA or factor(s) to up-regulate dystrophin production seems inadequate in view of low efficiency, high risk, and instability of transduction. There is no better method than HMGT to replenish these proteins through natural integration of the normal genome.

HMGT provides concomitantly new healthy cells to replace dead cells and the normal human genome to repair the genetic defects of surviving impaired cells. In addition to providing the genetic platform for normal phenotype expression, HMGT can also be used to up-regulate individual gene expression for angiogenesis. Thus, HMGT is conceptually the best possible treatment for DMD and cardiomyopathy patients.

**Therapeutic mechanism of HMGT** HMGT is defined as a platform technology of cell transplantation, nuclear transfer, and tissue engineering developed to treat diseases associated with muscle degeneration and weakness. The procedure involves isolation and culturing of satellite cells derived from muscle biopsies of genetically normal donors. It is customary to refer to myoblast reserve cells in adult muscles as satellite cells, and satellite cells in culture as myoblasts. Cell culture mass-produces the myoblasts and removes the antigenic leukocytes, adipocytes, and fibroblasts. These donor myoblasts are then injected or surgically implanted into a number of foci in the degenerative muscles where host satellite cells and regenerating myofibers are lacking.

During transplant, mechanical injury by the injecting needle activates regeneration of the host myofibers that fuse with the injected myoblasts, sharing their nuclei in a common gene pool of the myofiber syncytium. Cell fusion is a spontaneous occurrence during myogenesis35 and muscle regeneration.36,37 Through nuclear transfer and complementation, the normal human genome is transferred into muscle cells of genetically ill patients to achieve phenotype repair.36-41 In addition, myoblasts fuse among themselves to form new myofibres to replace those that are undergoing degeneration. The procedure leads to the formation of a genetically mosaic muscle.38

The idea of using myoblasts as donor cells in HMGT is to provide the genetic blueprint for activation through the regeneration process. In addition, although skeletal and cardiac muscles are capable of regeneration, there are not enough regenerative myogenic cells on site to fully compensate for major muscle damage. HMGT provides new
myogenic cells through myoblast culture and transplantation. It replaces lost cells and repairs degenerative cells.

The spontaneous cell fusion process inherent in myogenesis and muscle regeneration is utilized to incorporate the normal nuclei into dystrophic muscle cells. 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 genes inserted. It is not necessary to know which gene(s) are responsible for the defect. Furthermore, the injection of normal myoblasts directly into the dystrophic muscle eliminates the uncertainty of tissue targeting with conventional gene therapy. By virtue of incorporating full complements of normal genes into genetically abnormal cells to achieve repair, HMGT is a gene therapy, or better still, a genome therapy.

Through genetic complementation, the inserted normal genes encode one or more missing protein(s) to supplement the metabolism or development of the heterokaryons, thereby sustaining the normal integrity of these genetically transformed cells. In this respect, HMGT differs from bone marrow grafting, which is based strictly on the replacement strategy where cell fusion and genetic complementation do not occur. HMGT is the first therapy that has demonstrated significant improvement in cell genetics and phenotypes of live mammals.

**Safety and efficacy of HMGT** A phase I HMGT clinical trial was started in 1990 for patients with DMD. There was no side effects. Correction of the DMD genetic defect was evidenced when dystrophin was found in the myoblast-injected DMD muscle.

Two months later, Anderson announced the beginning of the single gene manipulation clinical trial on an adenosine deaminase-deficient patient. In addition to fulfilling their primary muscle-building mission, the myoblasts served as both source and transfer vehicles of normal genes to correct the gene defects of DMD.

Six years after the first HMGT, dystrophin was still found in the myoblast-injected muscle, while there was none in the sham-injected muscle. Six years is the longest period of time for which any gene therapy had sustained positive results. Despite cyclosporine withdrawal at 3 months after HMGT, 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-I surface antigens. Not only has the result demonstrated HMGT overall safety and efficacy in this single case, it also shows stability in the integration, regulation, and expression of the dystrophin gene. The presence of dystrophin in the myoblast-injected, but not the sham-injected muscle, provided unequivocal evidence of the survival and development of donor myoblasts in the myoblast-injected muscle.

Once injected, the myoblasts are subjected to scavenger hunt by macrophages for up to three weeks, because myoblasts exhibit MHC-1 surface antigens that become absent after cell fusion. The latter occurs between one to three weeks after myoblast injection. An allowance in the number of injected myoblasts has to be made to satisfy the unavoidable scavenger process. Although myoblast loss can be minimized by down-regulating macrophage activity, such additional compromise of the host immune system may lead to higher risk of infection, since HMGT subjects are already on immunosuppres-ants.

Beginning with 8 million myoblasts into a small foot muscle, 5 billion cells were injected into 22 leg muscles, and then 25 billion cells into 64 body muscles. The latter two protocols were under FDA Phase II and Phase III review. With over 280 procedures having been conducted to date, the safety and efficacy of the HMGT procedure has been proven. There have been no severe adverse reactions (Table 1).

**Table 1. Recent clinical trails of HMGT**

<table>
<thead>
<tr>
<th>Phase</th>
<th>Myoblasts</th>
<th>No. of muscles</th>
<th>No. of Subjects</th>
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<tr>
<td>I</td>
<td>8 million</td>
<td>1</td>
<td>11</td>
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<td>I/II</td>
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<td>22</td>
<td>32</td>
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<tr>
<td>II</td>
<td>25 billion</td>
<td>64</td>
<td>40</td>
</tr>
<tr>
<td>II/III</td>
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<td>82</td>
<td>197</td>
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**HMGT for cardiomyopathy**

Cardiomyopathy (heart muscle degeneration) is the leading cause of debilitation and death in humans. It is the end result of a common pathway for congenital and acquired cardiomyopathies, myocardial infarction, or end stage coronary artery disease.

Through endomyocardial injections of cultured skeletal myoblasts, which spontaneously transfer their nuclei into cardiomyocytes, myogenic regeneration was initiated. Some injected cells develop to become cardiomyocytes. Some fuse among themselves to form new myofibers, depositing contractile filaments to improve myocardial contractility.

**Pathological basis of cardiomyopathy** The pathological process of cardiomyopathy cascades with cardiomyocyte membrane leakage, uncontrolled Ca²⁺ influx, mitochondrial ATP shutdown, inability to exude Ca²⁺ through the cell surface and to reabsorb Ca²⁺ into the sarcoplasmic reticulum, myofibrillar hypertrophy, and disarrangement. Apoptosis then ensues and fibroblasts proliferate. The myocardium, which was once populated by live cardiomyocytes with proteinaceous contractile filaments such as myosin, actin, troponin, and tropomyosin, is now partially occupied by fibroblast scars that are incapable of electric conduction or mechanical contraction. These scars continue to exert a negative effect on contractility and compliance of the ventricle despite remodeling.

**Natural myocardial repair** Ultimately the degenerative process in the myocardium results in loss of live cardiomyocytes, contractile filaments, and contractility. The degenerative myocardium also transmits biochemical signals to recruit stem cells from the stroma and the bone marrow in an attempt to repair the damage. This process could be
augmented with stem cell transplants. Being pluripotent, embryonic or adult stem cells exhibit uncontrolled differentiation into various lineages to produce bone, cartilage, fat, connective tissue, skeletal and heart muscles (Fig. 5). Because fibroblast growth factor level is elevated in the degenerative heart, much of the recruited stem cells differentiate to become fibroblasts instead of cardiomyocytes, thus depositing fibrous scars and not contractile filaments. Despite the claimed success of transmyocardial revascularization using laser, angiogenic factors, and genes, the damaged myocardium really needs additional live myogenic cells to deposit contractile filaments or to the angiogenic factors to encourage angiomyogenesis.

Animal studies have shown that engrafted myoblasts form myotubes in the myocardium and eventually mature to become myofibers with contractile capability. These results have been confirmed in humans. They regenerate acquire a fatigue-resistant slow-twitch muscle phenotype which is more suited to perform the cardiac work load. Transplantation of myoblasts produces significant functional improvement in the ventricles. There have been contradicting reports on the differentiation of myoblasts into cardiomyocyte-like cells with intercalated discs. It is also questionable if the myogenic cells developed from the injected myoblasts make electromechanical connections with host cardiomyocytes through gap junctions.

HMG1 in animal models

Case report: Myoblast transplant to the endocardium through the percutaneous approach. The first successful endovascular transfer of human myoblasts into the porcine myocardium was reported in 2000. The first successful endovascular transfer of human myoblasts into the porcine myocardium was reported in 2000. Following the International Animal Care Guidelines, a 100-lb juvenile female pig was anesthetized. Vascular access was obtained via the right femoral artery cutdown. A catheter was advanced into the left ventricle under fluoroscopy. Endomyocardial mapping was performed with the NOGA system (Biosense Webster Inc, Johnson and Johnson, USA). Approximately 10^9 human myoblasts were injected through a needle timed to protrude into the myocardium for no more than 6 mm from the tip of the catheter. Twenty injections were made at different locations within 40 minutes, with different volumes of 0.1, 0.2, 0.3, 0.5, 1.0 ml, and cell concentration of 10^6/ml (Fig. 5A). Heart rate, electrocardiogram, and temperature were continuously monitored. Other than transient short runs of ventricular ectopy, the pig remained in stable condition throughout the injection time. Vital dye staining of the myoblasts before versus after the procedure showed no significant difference in cell viability. Cell passage through the injection catheter showed less than 5% of cell death.

At the completion of the procedure, the pig was sacrificed and the heart processed for histological examination. Transmyocardial perforation was not observed. Numerous prominent round and mononucleated human myoblasts were found widely and evenly distributed throughout the apex and the lateral wall of the pig's left ventricle where the myoblasts were injected (Fig. 5B). This first human myoblast transfer into the porcine heart model revealed that it was safe to administer one billion myoblasts at 10^6/ml through the Myostar catheter of the NOGA system (Biosense Webster Inc, Johnson and Johnson, USA). It was also determined that 0.3 ml to 0.5 ml would be the optimal volume per injection.
Case report: Myoblast transplant to the epicardium in an open chest approach  
A porcine heart model of chronic ischemia was produced by clamping an atherosclerotic ring around the left circumflex artery. Four weeks later, the heart was exposed by left thoracotomy. Twenty injections (0.25 ml each) containing 300 million myoblasts, or 5 ml total volume of basal DMEM as control, were injected on the epicardium. Left ventricular function was assessed by MIBI-Tc⁹⁹⁹ SPECT scanning one week before procedure to confirm the location and the size of the myocardial infarction, and at 6 weeks after myoblast injection. Animals were maintained on cyclosporine at 5 mg/kg body weight from 15 days before, until 6 weeks after cell transplantation. The animals were euthanized at 6 weeks to 7 months post-operatively, and the hearts were processed for histological, immunocytochemical, and ultrastructural studies.

Histological examination of myoblast-injected myocardium showed cardiomyocytes containing Lac-Z positive nuclei (of donor origin) after 12 weeks (Fig. 6A). More than 80% of the Lac-Z positive cardiomyocytes immunostained positively for human myosin heavy chain (Fig. 6B). The human genome was integrated (Lac-Z labelled nuclei) and expressed (myosin immunostain) in the porcine myocardium.

Donor myoblasts developed to cardiomyocytes that are characterized by having four to five nuclei each. The control heart without myoblast injection did not show Lac-Z positive myonuclei or human myosin (Fig. 6C). Triple stain of myoblast-injected myocardia demonstrated multinucleated heterokaryons containing human and porcine nuclei with expression of human myosin (Fig. 7). Electron microscopy demonstrated human myotubes and skeletal myofibers with satellite cells in the porcine myocardium (Fig. 8).

Three myogenesis mechanisms were elucidated as proof of concept with 50 human/porcine xenografts using cyclosporine as immunosuppressant. Some myoblasts developed to become cardiomyocytes. Others transferred their nuclei into host cardiomyocytes through natural cell fusion. Others formed skeletal myofibers with satellite cells. De novo production of contractile filaments augmented heart contractility (Fig. 9).
Laser nuclear capture together with single nucleus reverse transcription polymerase chain reaction (RT-PCR) was performed to delineate host and donor nuclei. In situ hybridization using fluorescent DNA probes specific for human chromosome 22 and chromosomes 1 and 10 for pig were used to demonstrate nuclear mosaicism (Fig. 10). The implantation of human myoblasts derived from young males into female pigs provided additional demonstration of donor cell survival and development in the host muscles.70 Discontinuation of cyclosporine after 6 weeks prompted no xenograft rejection for up to 30 weeks. There was a transient elevation of the porcine anti-human myoblast antibodies at one week after the xenograft (Fig. 11). The antibody level subsided at the second week after HMGRT, indicating that no more than two weeks of cyclosporine immunosuppression would be necessary for human/pig xenografts. Modification of the cyclosporine administration protocol will be implemented accordingly in the future.

**HMGRT in humans**

**Case report: Myoblast autograft transplant** In May 2000, Philippe Menasche led his team in the Hospital Bichat in Paris to perform the world’s first myoblast transfer into the myocardium of a 72-year-old patient with ischemic heart disease. Donor cells survived, developed, and still functioned in the host myocardium at the 17.5 month follow-up. Significant improvement in ventricular function was reported in this and subsequent cases.91,101 The study led the way to evaluating HMGRT in Phase I and Phase II clinical trials in 15 countries around the world to date.

Two years later, in 2002, a 55-year-old man suffering from acute myocardial infarction (AMI) received cGMP-produced pure myoblasts as an adjunct to off-pump CABG by Professor Eugene Sim of the National University Hospital of Singapore.

Coronary angiography showed an 80% lesion in the mid-left anterior descending artery, 70% occlusion of the proximal first diagonal branch, and 50% stenosis of the circumflex artery. The mid-right coronary artery was occluded with good collateral supply from the left coronary artery. Echocardiography revealed akinesis of the apex, anterior wall, and septum of the left ventricle. The LV ejection fraction was 31%. Tomographic Tc99m tetrofosmin scan 25 days after AMI revealed a large partially reversible defect on the anterior wall and a moderate-sized reversible defect on the inferior wall. After being qualified for inclusion criteria, signing the informed consent, and receiving approval from the hospital ethics committee, the patient was selected for
myocardial cell transplantation adjunct to CABG.

Approximately 2.5 g of rectus femoris muscle biopsy was taken under local anesthesia from the patient and cultured under cGMP for 32 days in order to produce one billion cells. Myoblast purity of >99% was ascertained by positive immunostain for human desmin.

Under direct vision and stabilization with the Octopus III tissue stabiliser, 4.65×10⁹ autologous myoblasts in 3 ml serum were injected into the epicardium using a 27-gauge needle. Twenty-five injections were made on the anterior wall, near the apex, on the posterior wall, in and around the infarcted areas. These injections of 0.1 ml to 0.2 ml each were given at half- to one-minute intervals. The patient recovered well from the operation. Serum creatine kinase was 222 µg/L and the MB fraction was 6 µg/L at 3 hours post-operatively, and 385 µg/L and 6 µg/L, respectively, at 12 hours. Postoperative 24-hour Holter monitoring revealed no arrhythmia. The patient was discharged on the eighth post-operative day. His EKG has shown no arrhythmia. He has shown good effort tolerance, no dyspnea or angina, and a significant increase in ejection fraction at 3 weeks post-operatively. This case study suggests that myoblast transplant in the epicardium during open heart surgery was safe and effective.

Case report: Myoblast allograft transplant The feasibility and safety of myoblast allograft was assessed by injecting 1.1 and 1.2 billion myoblasts respectively in the infarcted myocardium of two men, aged 63 and 49. Both patient received 2-month cyclosporine immunosuppression. Donor myoblasts were manufactured in compliance with current cGMP and ISO 9001 conditions. About 2.18 g of muscle biopsy was taken under local anesthesia from the rectus femoris of a 20-year-old pathogen-free male volunteer, after he had met all the muscle donor criteria and signed the informed consent. At harvest, the culture yielded 3.64×10⁶ myoblasts that were 98.3% pure by positive desmin immunostain. It was 91.5% viable according to vital dye exclusion tests. The cells were potent in myogenecity in that myotubes comprised more than 99% of the culture in fusion medium. Throughout the culture and for the final injectates, the myoblasts were free of endotoxin and mycoplasma, and were negative for sterility (14 day test) and gram stain (absence of gram positive or negative bacteria) according to certified laboratory analyses.

Both patients enrolled after being qualified for inclusion/exclusion criteria, signing the informed consents, and receiving institutional and ethics committee approval. They had coronary artery disease, history of AMI, angina Functional Class IV (CCS), and hypertension class II (risk 4). Positron emission tomography (PET) with ¹⁸F-FDG revealed scarred myocardium in the septum, apex, and anterior/posterior wall of the left ventricle. Echocardiography showed regional akinesis/hypokinesis. The LVEF was 41% and 38%, respectively. Single-photon emission computed tomography (SPECT) with 30 mCi ⁹⁹mTc-tetrofosmin showed large reversible and irreversible areas (Fig. 12). Coronary angiography revealed severe stenosis in both left and right coronary artery systems.

[Image of vWF VIII, Smooth muscle actin, merged, and Ang-1 myoblast group]
The subjects took two oral doses of cyclosporine totaling 5 to 7 mg/kg body weight per day, beginning at five days before grafting, weaning at half-doses in the last two weeks, and off cyclosporine at eight weeks after grafting. Whole blood trough level of cyclosporine was monitored every three days at 23 hours after the morning dose. Doses were adjusted to maintain the level at about 250 ng/ml.

In 2003, Academician Leo A. Bockeria of the Russian Academy of Medical Sciences was the world’s first to inject autologic myoblasts into heart of patients. The subjects underwent bypass grafting immediately prior to myoblast implantation. They received 18/19 injections totaling 1.1/1.2 billion myoblasts, respectively. Cells were injected at 100×10⁶/ml. The 0.5 ml injections were made under direct vision between the infarcted and viable myocardium, and into the scarred tissue.

The subjects recovered from general anesthesia without rash or fever. Holter EKG monitor registered sporadic ventricular arrhythmias and ventricular extrasystoles that were eventually eliminated with amiodarone treatment. Both patients were discharged in good condition after 3 weeks without uncontrolled hypertension or renal failure.

During follow-up, despite cyclosporine discontinuation at 2 months postoperatively, no sign of rejection was observed. At 3-, 6-, and 9-month follow-up, both subjects were in stable condition at Class I-II (CCS). Echocardiography showed significant increases in LVEF, respectively without local akinetic/hypokinetic regions. SPECT with 30 mCi ⁹⁹mTc-tetrofosmin confirmed similar LVEF increases and demonstrated reduction of perfusion defects during stress and rest (Fig. 3). ¹⁸FDG accumulation was homogenous throughout the myocardium, and glucose metabolism changes were not revealed.

**Advantages of myoblast allografts**

Human myoblast allografts may provide an alternative therapy for heart muscle degeneration, with virtually unlimited cell availability. There are many advantages of using myoblast allograft rather than autograft in the treatment of heart diseases (Table 2).

| Cells are readily available from cell banks in allografts, autograft patients have to be biopsied and wait about one month for their own myoblasts to grow and reach one billion in number. In acute myocardial infarction, the immediate transplant of allograft may prevent fibroblast infiltration and eventual scar formation. | Patients with infectious diseases such as AIDS can be treated with allografts without fear of contamination of the cell culture system. Furthermore, heart patients with genetic diseases such as the muscular dystrophies can be treated with genetically normal allografts.

Survival without any sign of rejection after cyclosporine withdrawal confirmed our report on safety in the human/porcine cardiac xenografts. Cyclosporine immunosuppression for 3 months allowed allogeneic myoblasts to survive, develop, and function in muscle of a DMD patient, expressing the therapeutic protein dystrophin at 3 months to 6 years after myoblast transfer. Myoblast allograft has been demonstrated to have survived and developed up to 17.5 months after implantation in an ischaemic heart patient. The myoblast purity of this study was determined using CD56, an antibody which reacts with stem cells, neurons, and fibroblasts rather than with myoblasts. CD56 antibody yields false data on myoblast purity.

More than 220 patients have received myoblasts worldwide since the initial procedure of June 2000. Mortality has been less than 10%. If future myoblast allografts prove to be safe and efficacious in treating cardiomyopathy, the 2-month immunosuppression, when compared to life-long immunosuppression in heart transplant patients, will greatly improve the quality of life of cardiac patients. Moreover, myoblast allografts can significantly reduce medical cost, considering that quality control and analysis tests on one lot of 50 billion myoblasts are much less costly than the same tests on 50 lots of one billion myoblasts. Future allograft studies are warranted.

The future of HMGT lies with myoblast allograft, 90% delivered endovascularly and 10% epicardially in adjunct to CABG. Myoblasts will likely be transduced with VEGF.
Ang-1, TGF-β, or similar factors to allow concomitant angiomyogenesis. Our ongoing clinical trial is based on unequivocal evidence of cGMP-produced pure human myoblasts and proof of concept for HMGT. Human myoblasts survived and integrated into porcine ischemic myocardium, allowing concomitant cell therapy and gene therapy to produce angiomyogenesis. Whereas the newly formed myofibers harbor satellite cells an impart regenerative capacity to the myocardium, the genetic transformation of cardiomyocytes in vivo to become regenerative heterokaryons through myoblast genome transfer constitutes the ultimate myocardial repair. The regenerative heart also contains trans-differentiated cardiomyocytes of myoblastic origin. In all three scenarios, new contractile filaments are deposited to improve heart contractility. The latter can be translated into improvement in the quality of life of cardiac patients.

It can be concluded that pure myoblasts transduced with Ang-1 and VEGF, when injected intramyocardially, are potential therapeutic transgene vehicles for concurrent angiogenesis and myogenesis for treatment of ischemic cardiomyopathy. Immunosuppression using cyclosporine for six weeks is effective for long-term survival of xenografts or allografts. The feasibility and preliminary safety/efficacy observed in the world’s first human myoblast allografts leads the way to developing a low-cost, easy-to-use treatment for heart failure and prevention of heart attacks, with virtually unlimited cell availability and short-term immunosuppression.

Future perspectives

Heart muscle genes

The development of CardioChip allows early diagnosis of cardiovascular diseases using 10,368 expressed sequence tags (ESTs). Heart muscle genes can be identified to provide the template for early diagnosis (Fig.13). Subjects so identified can have muscle biopsy taken before any symptom occurs. Myoblasts can be processed and deposited in a cell bank for future transduced HMGT to prevent sudden heart attack.

Myoblasts as the universal gene transfer

Using myoblasts as gene transfer vehicles dates as early as 1978. In mammals, myoblasts are the only cell type which divides extensively, migrates, fuses naturally to form syncytia, loses MHC-1 antigens soon after fusion, and develops to occupy 50% of the body weight in humans. These combined properties render myoblasts ideal for gene transfer. Natural transduction of normal nuclei ensures orderly replacement of dystrophin and related proteins at the cellular level in DMD. This ideal gene transfer procedure is unique to muscle. After all, only myoblasts can fuse and only muscle fibers are multinucleated in the human body. By harnessing these intrinsic properties, HMGT transfers all normal genes to achieve genetic repair. Since donor myoblasts also fuse among themselves to form normal fibers in HMGT, the muscles benefit from the addition of genetically normal cells as well. This differs significantly from the gene therapy format in which single copies of the down-sized dystrophin gene are transduced as viral conjugates into the mature dystrophic myofibers in which many proteins, both structural and regulatory, are lost. Multiple gene insertion is necessary to produce the lost proteins for the development of a cure or treatment. More gene insertion is needed to produce the cofactors to regulate and express these lost proteins in order to repair the degenerating cell.

Controlled cell fusion

It will be useful to be able to control, initiate, or facilitate cell fusion once myoblasts are injected. Myoblasts fuse readily at low serum concentration in culture. The process is more complex in the in vivo situation.

As the myoblasts are injected intramuscularly into the extracellular matrix (ECM), 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 amounts in the dystrophic skeletal muscle or the infarcted myocardium. That is why it is necessary to inject fractions of myoblasts that are as pure as possible, 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-1 (IGF-1) may facilitate the developmental process, resulting in the formation of myotubes soon after myoblast injection. The use of bFGF, LC6SP, and IGF-1 at optimal concentrations in the cell culture medium and in the injection medium is to examined in future studies.
Proliferative cardiomyocytes

Whereas HMGT results in the formation of genetic mosaicism with gene transfer occurring in vivo, the production of heterokaryons in vitro has immense medical application. This can be achieved by controlled cell fusion with myoblasts. This original program of research relates to the in vitro transfer of normal nuclei with all of their normal genes from donor myoblasts into the genetically normal and/or abnormal cells, e.g., the cardiomyocytes. The ability to replace degenerated cardiomyocytes and replenish the myocardium with new healthy cells will have immense impact on end-stage ischemic cardiomyopathy, when there is a great shortage of hearts available for transplantation.

Normal cardiomyocytes exhibit very limited ability to proliferate in vivo or in vitro. The myocardial damage in infarction or in hereditary cardiomyopathy cannot be repaired by cardiomyocytes through regeneration. The integration of the skeletal muscle cell characteristic of mitosis will enable the heterokaryotic cardiomyocytes to proliferate in vitro and in vivo.

Controlled cell fusion between normal myoblasts and normal cardiomyocytes may result in heterokaryons exhibiting the characteristics of both parental myogenic cell types. Clones can be selected based on their abilities to undergo mitosis in vitro, to develop desmosomes and gap junctions, and to contract strongly in synchrony after cell transplantation.

These genetically superior cells can then be delivered through catheter pathways after mapping of the injured sites. With the ability to grow large quantities of these cardiomyocytes, the correction of structural, electrical, and contractile abnormalities in cardiomyopathy can be tested first in dystrophic and cardiomyopathic hamsters, and if safe and effective, in humans.

The genetic transfer of the mitotic property of myoblasts onto cardiomyocytes with in vitro controlled cell fusion enables the resulting heterokaryotic cardiomyocytes to multiply, yielding enough number of cells for the cell transplant to be effective.

The use of myoblasts as gene transfer vehicles does not stop with myogenic cells. Myoblasts have been genetically modified to provide systemic delivery of human growth hormone, and may in time provide long-term secretion of insulin for diabetes and various hormones and/or factors. Myoblasts transduced to secrete opioid derivatives may be used to treat pain, depression, and addictions.

**VEGF** modulation

The imbalance between supply and demand for oxygen in the hypoperfused myocardium up-regulates the expression of pro-angiogenic factors and their receptors. VEGF is the major angiogenic factor involved in physiological and pathological angiogenesis. The neovascularization is mainly achieved by endothelial cell proliferation triggered through VEGF receptors, especially the VEGF receptor-2. VEGF induces nitric oxide production and cGMP accumulation in cultured endothelial cells through the activation of endothelial nitric oxide synthetase (eNOS). The important functions of these two mediators are vasodilation, inhibition of smooth muscle proliferation, anti-platelet accumulation, and inhibition of leukocyte adhesion, leading to vascular protection. Some of the factors stimulate angiogenesis through the induction of VEGF.

Automated cell processor

The great demand for normal myoblasts, myotubes, and young muscles, 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, of which myogenic cells are an example.

This invention will be one of the most important off-spring of modern computer science, mechanical engineering, and cytogenetics. The intakes will be from 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 should be self-contained in a sterile enclosure large enough to house the hardware in which cells are cultured and manipulated. Various cell types can be manufactured depending on the software used.

The automated cell processor should replace the current bulky inefficient culture equipment and elaborate manpower. Its installation into clinics, hospitals, and medical centers should allow customized medicine for individual patients by providing autologous HMGT. Fresh cells would be nearby, while cell costs would be significantly reduced.

**HMGT is now and forever**

Health is the sum of well-being from all body cells. In hereditary degenerative skeletal diseases, sick cells need to be repaired while dead cells need to be replaced.

For cardiomyopathy patients, at this present time, cell culture is the only way to generate new, live cells that are capable of surviving, developing, and functioning in the
myocardium after transplantation, replacing degenerated and lost cells.

Myoblasts are the only cells in the body capable of natural cell fusion. The latter allows the transfer of all the normal genes into degenerating cells to achieve phenotypic repair through complementation. Initial evidence has shown that HMGT is able to transfer angiogenic genes and achieve angiomyogenesis. Even though HMGT is still in its early development phase, there is no other strategies or modalities that have had equal success.

As myoblasts are endogenous to the human body, they will be proved to be safe and efficient as universal gene transfer vehicles. Since a foreign gene always exerts its effect on a cell, cell therapy will always be the common pathway to health. After all, cells are the basis from which life is made.

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