Stem Cell Biology and Regenerative Medicine

Martin K. Childers *Editor*

Regenerative Medicine for Degenerative Muscle Diseases

Humana Press

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Preface

Regenerative medicine—an emerging multidisciplinary field composed of clinicians, bioengineers, pharmacologists, surgeons, and others—seeks to develop new ways of repairing and replacing cells, tissues, and organs. A great example of regenerative medicine technology was pioneered by Anthony Atala, MD, who pioneered the world's first urinary bladder made from the patient's own cells and grown in a laboratory before being implanted into the patient. This process of regenerative medicine differs radically from conventional medicine by not only treating symptoms but by recreating damaged tissues and organs in the human body. Unthinkable only a few years ago, clinicians and scientists are rapidly exploiting nature's secrets to replace damaged tissue or to stimulate the body's own natural repair mechanisms to heal previously irreparable tissues or organs. For degenerative muscle diseases, this is a tough order, since skeletal muscle comprises the largest tissue type in the human body. Not only is skeletal muscle the largest "organ" in the body, but most degenerative diseases, such as the muscular dystrophies, are caused by rare inherited genetic mutations. So in this case, regenerative technologies must overcome two very difficult problems. The first problem is to create enough "medicine" to treat the entire volume of skeletal muscle in a human body, and the second is to replace defective genes. Both problems may seem insurmountable at first glance, but the history of medical progress, particularly in the field of genetics and gene therapy, tells us otherwise.

In this book, the first three chapters are dedicated to an overarching view of how genetics and regenerative technologies can synergistically work together to target skeletal muscle genetic diseases. The next five chapters explore how an understanding of cellular biology (particularly stem cell biology and cellular reprogramming) proffers alternative methods to gene replacement. Chapters 8 and 9 examine new ways to look at our conventional approaches (rehabilitation medicine and nutrition) when combined with regenerative technologies. And the final chapters introduce the concepts of "micro-RNAs" and how fish and dog models can be used to enhance our understanding of these devastating and often fatal diseases of skeletal muscle.

Altogether, these new concepts, technologies, and approaches should give patients, families, and doctors cutting-edge information about incredible regenerative advances already under way in laboratories and clinics worldwide.

Seattle, WA, USA Martin K. Childers

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Martin K. (Casey) Childers, D.O., Ph.D. is a professor in the Department of Rehabilitation Medicine and Investigator at the Institute for Stem Cell & Regenerative Medicine, University of Washington. He is a graduate of Seattle Pacific University (B.A., Music Performance), Western University (D.O., Medicine, Osteopathic), and The University of Missouri (Ph.D., Physiology & Pharmacology; residency, Rehabilitation Medicine). The Childers' laboratory works in two areas of investigation. In a series of preclinical studies, they address the hurdles required for systemic gene replacement delivery for patients with X-Linked Myotubular Myopathy (XLMTM). In other studies, they use a "disease-in-a-dish" approach with induced pluripotent stem (iPS) cells to study heart disease in patients with Duchenne muscular dystrophy (DMD). Dr. Childers' clinical medicine practice at the University of Washington Medical Center is dedicated to serve patients with neuromuscular diseases.

Chapter 1
Regenerative Medicine Approaches to Degenerative Muscle Diseases

Martin K. Childers and Zejing Wang

1.1 Background

1.1.1 What Is Regenerative Medicine?

An innovative interdisciplinary scientific field, termed *regenerative medicine*, focuses on new approaches to repairing and replacing cells, tissues, and organs and may involve the use of gene therapy. Derived from the fields of biomedical engineering, developmental biology, nanotechnology, physiology, molecular and cellular biology, and surgery, regenerative medicine holds new promise for previously incurable conditions. This emerging multidisciplinary field is rapidly bringing advances in cell therapy, bioengineering, and surgery to transform health care for those currently underserved by transplantation medicine.

Potentially, any disease or condition (acquired or genetic) that results in damaged, failing, or malfunctioning tissue may be amenable to regenerative medicine technologies. These technologies fall into two general categories: (1) a focus on growing tissues and organs in vitro (i.e., tissue engineering) and subsequently implanting them into the patient and (2) a focus to fully leverage the host's regenerative in vivo capacity using cellular and/or gene therapies.

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Regenerative medicine can be defined as the "process of creating living, functional tissues to repair or replace tissue or organ function lost due to age, disease, damage, or congenital defects" [1]. This new field holds the promise of regenerating damaged tissues and organs in the body by either replacing damaged tissue or by stimulating the body's own repair mechanisms to heal previously irreparable tissues or organs. As regenerative medicine becomes integrated into medical practice, new approaches will address the root cause of disease and offer prospects of tissue repair previously not possible.

The roots of regenerative medicine draw from the field of transplantation medicine that together with implantable medical devices have profoundly altered the trajectory of patients suffering from chronic and end-stage organ failure [2]. Indeed, the transplantation field has become so successful that the demand for organs now vastly exceeds the supply of donors. The US Department of Health and Human Services Organ Procurement and Transplantation Network cites the current US waiting list for all organs at 122,439 patients as of May 1, 2014 [3]. Although organ transplantation has become standard medical practice, in the United States more than 28,000 patients received transplanted organs in 2009 [4]. But, the gap between available organ donation and recipients is rapidly widening. Recipients, although fortunate to receive donor organs, are placed at risk for tissue rejection and must receive lifelong immunosuppressive drug therapy. Transplant complications plague certain tissues. For example, compared with recipients of other solid organ transplants [5–8], lung transplant recipients experienced the highest rates of rehospitalization for transplant complications: 43.7 per 100 patients in the first year [9]. Together, these data indicate that transplantation needs far exceed the supply, and even when transplanted organs are received, patients face lifelong challenges of tissue rejection.

The emerging field of regenerative medicine was created, in part, to address this unmet medical need for organ and tissue replacement. The multidisciplinary field of regenerative medicine involves close collaboration between clinicians and bench scientists to generate new tissues, not from human donors, but from the patient's own body. Regenerative technologies are aimed at growing organs and tissues in the laboratory to safely implant them into patients. This can potentially solve the problem of the shortage of organs available for donation and also address the problem of organ transplant rejection if the organ's cells are derived from the patient's own tissue or cells. Indeed, the world's first successful laboratory-grown organ (a urinary bladder) was generated from the patient's own cells, shaped by a laboratory bioreactor, and subsequently surgically implanted back into the patient [10–15]. In 2006, Atala and colleagues created engineered bladder tissues by seeding autologous urothelial and muscle cells onto collagen–polyglycolic acid scaffolds. After 7 weeks of culture in laboratory bioreactors, the autologous engineered bladders were used in a surgical reconstruction for seven myelomeningocele patients. The laboratory-grown bladders were implanted either with or without an omental wrap. Long-term follow-up in patients treated by this regenerative technique demonstrated improvement in bladder leak point pressure, volume, and compliance. This proof-of-concept technology began a new era in an emerging field that is rapidly gaining traction around the world.

A few examples of regenerative medicine technologies already tested in patients are presented in Table 1.1. A wide array of engineered organs (urinary bladder [10–15], trachea [17, 18], cornea [20, 22, 23]) or tissues (skin [16, 24–26], cartilage [19, 27–30], muscle [21, 31, 32]) are developed to the point of clinical applications. Many other regenerative technologies are under development in preclinical stages prior to human trials. For example, a breakthrough reported in the journal *Nature* from the laboratory of Charles Murry, MD, PhD described the use of human embryonic stem cells (hESCs) to regenerate damaged heart tissue in nonhuman primates [33]. Investigators injected one billion heart muscle cells derived from hESCs into the infarcted muscle of pigtail macaques. This was ten times more of these types of cells than researchers have ever been able to previously generate. This report demonstrated that hESCs can be grown, differentiated into cardiomyocytes, and cryopreserved at a scale sufficient to treat a large-animal model of myocardial infarction. The group found that over subsequent weeks, the stem cell-derived heart muscle cells infiltrated into the damaged heart tissue, then matured, assembled into muscle fibers, and began to beat in synchrony with the macaque heart cells. After 3 months, the cells appeared to have fully integrated into the macaque heart. Future efforts by this group will work to reduce the risk of arrhythmias (seen in the monkeys after stem cell transplantation), perhaps by using more electrically mature stem cells. They also will investigate if the stem cells strengthen the contractile strength of the recipient hearts.

1.1.2 Degenerative Muscle Diseases Are Rare, but Rare Diseases Are Common

The majority of degenerative muscle diseases are inherited genetic diseases passed from one generation to the next. These genetic degenerative diseases of muscle are rare, but altogether, rare diseases are common. In fact, one in ten Americans has a rare disease [34]. If all of the individuals with rare diseases were to move to an unpopulated continent—like Greenland—it would instantly become the third most populated country in the world. Therefore, "rare" diseases make up a large group of collected disorders that are not rare, but rather they are common among our population.

Table 1.1 Regenerative medicine technologies reported in patients

Engineered organ or tissue	Types of cells used	Scaffold	References
Skin	Epithelial or none	Complex; multiple in use	[16]
Urinary bladder	Urothelial, muscle	Collagen; collagen/polyglycolic acid	[10]
Trachea	Epithelial, chondrocytes	Cadaver trachea	[17, 18]
Cartilage	Mesenchymal stem cells, others	None	[19]
Cornea	Limbal stem cells	Amniotic membrane	[20]
Muscle	None	Porcine intestine submucosa	[21]

1.2 What Disorders Lead to Chronic Muscle Degeneration?

1.2.1 Myopathies

The term, myopathy, simply means muscle disease and is derived from two Greek terms, "myo" (muscle) and "pathos" (suffering). A myopathy implies that the primary defect is within the muscle, as opposed to a peripheral nerve or within the central nervous system. Chronic myopathies are generally inherited (familial), progressive, and clinically distinguished by the age of onset (congenital versus adult). In contrast, acquired myopathies result from different disease processes including endocrine, inflammatory, paraneoplastic, infectious, drug- and toxin-induced, critical illness, and metabolic disorders.

1.2.2 Acquired Myopathies

A common acquired myopathy results from administration of anti-inflammatory glucocorticoids (anti-inflammatory steroids) [35]. Referred to as "glucocorticoid-induced myopathy," administration of high doses of glucocorticoids in animals results in both a decrease in muscle mass and also muscle weakness. In patients, limb and respiratory muscle weakness in pulmonary disease can be attributed to glucocorticoid use. Limb muscle weakness has also been observed in patients with Cushing's syndrome who exhibit high levels of endogenous glucocorticoids [36]. Chronic glucocorticoid administration causes atrophy by a decrease in the rate of protein synthesis and increase in the rate of protein breakdown. Interestingly, atrophy is selective for type II (fast-twitch) muscle fibers with little effect on type I (slow-twitch) fibers [37]. The mechanism of such fiber specificity might be related to higher glucocorticoid receptor expression in type II fibers [35]. In patients, short-term glucocorticoid excess blunts the insulin-induced protein anabolism, suggesting that muscle loss occurs most likely through inhibition of the anabolic response to insulin [38].

1.2.3 Congenital Myopathies

Defined by childhood onset, congenital myopathies make up a wide spectrum of inherited muscle disorders. As an example, a form of congenital myopathy, termed centronuclear myopathy (CNM), has variable features that are most often present in childhood [39] and follow a progressive clinical course. Infants may present as "floppy" and hypotonic and many succumb to respiratory failure. The typical features of a CNM include proximal muscle weakness, atrophy, hypotonia, and elongated facies with a high-arched palate in young children [39].

Because of the overlap between clinicopathological features associated with individual gene mutations in CMs, classification of these disorders has been based on the appearance of abnormal structures on muscle biopsies examined under the light microscope. Further classification of CMs was based on the appearance of centrally located nuclei (central nuclear myopathies), rod-like structures, and other distinguishing features of organelles. Thus, "structured" CMs include central core disease (CCD), multi-minicore disease (MMD), centronuclear myopathies, nemaline myopathies, actin aggregate myopathy, desminopathy, and hyaline body myopathy. In contrast, "unstructured" CMs include congenital fiber-type disproportion (CFTD), a nonprogressive childhood neuromuscular disorder without prominent accumulation of abnormal structures visible under the light microscope. While these characteristic features provide some distinguishing characteristics that help differentiate congenital myopathies from metabolic myopathies and muscular dystrophies, such structural features are not informative about the underlying pathophysiology or genetic mutations associated with each disease.

Genetic abnormalities in the CNMs give rise to characteristic pathological changes within the sarcomere, the contractile apparatus of the myofiber. Examples of such changes include aberrant calcium handling and alterations of the normal excitation–contraction coupling machinery leading to inefficient muscle contraction. In contrast to muscular dystrophies where recurring cycles of myofiber degeneration and regeneration occur, in congenital myopathies, the myofibers do not undergo recurring degeneration/regeneration. Replacement of contractile tissues with noncontractile connective tissue or fat in muscular dystrophies or accumulation of glycogen depots in metabolic myopathies is usually not a typical feature of congenital myopathies.

1.2.4 Muscular Dystrophies

The muscular dystrophies are yet another collection of myopathies generally distinguished by ongoing muscle fiber degeneration with incomplete regeneration. The most common example is Duchenne muscular dystrophy (DMD). This progressive, lethal, X-linked disease of skeletal and cardiac muscle affects nearly 1 in 3500 males born each year in the United States. DMD is caused by mutation of the dystrophin gene (2.4 megabases—the largest known gene) that, together with its location at Xp21, provides a vulnerable target for new mutations. The cardiac and skeletal muscles of DMD patients are deficient in the dystrophin gene product, a 427-kD protein found primarily in the outer cell membrane in cardiac and skeletal muscle [1]. Without dystrophin in the outer membrane, the muscle fiber is particularly vulnerable to damage from normal daily activities [2]. As a result, damaged DMD muscle fibers eventually succumb to injury [3]. Normally, muscle damage is repaired by resident muscle stem cells (satellite cells) [4]. However, continuous cycles of damage eventually overwhelm the capacity for regeneration, potentially

due to impaired ability of muscle satellite cells [5]. To address this progressive and ultimately fatal degeneration in DMD muscles, intense research efforts are aimed at tilting the balance in favor of regeneration. Stem cell transplantation therapy may offer one approach to enhance the regenerative ability of damaged and degenerating muscle cells in patients with DMD.

1.3 Can Human Cells Be Transplanted to Regenerate Damaged Muscles?

Diseased tissue may be regenerated in vivo by transplantation of healthy cells that are able to replicate extensively. Progenitor and stem cells have this intrinsic ability and are used in regenerative medicine to enhance or restore damaged tissue. According to data from the Centers for Disease Control, as many as one million Americans will die every year from disease that, in the future, may be treatable with tissues derived from stem cells [40]. Diseases that might benefit from stem cell-based therapies included diabetes, heart disease, cerebrovascular disease, liver and renal failure, spinal cord injuries, and Parkinson's disease. In the following section, classes of cells available for regenerative techniques and research advances using these cells in selected clinical conditions are discussed.

1.3.1 Adult Stem Cells

Adult stem cells are defined as any stem cell population that corresponds to a point in development subsequent to the inner cell mass (ICM), or possibly the slightly later epiblast, of embryos at the blastocyst stage, prior to gastrulation. Those from the ICM are called embryonic stem (ES) cells. Adult stem cells are relatively abundant during fetal development and persist in tissues throughout adult life. Much of our current understanding of stem and progenitor cell biology derives from studies of a particular class of adult stem cells, namely, those of the hematopoietic (blood forming) system [41]. In fact, the therapeutic use of adult stem cells dates back to the first bone marrow transplant in 1956 [42]. Early suggestions supporting the existence of cells that are capable of reconstituting the blood system came from experience with persons exposed to lethal doses of radiation during World War II. In the early 1960s, James Till and Ernest McCulloch in Toronto, Canada, found evidence for a specific subpopulation of cells in bone marrow that could restore hematopoiesis after transplantation into irradiated mice. In the spleens of recipient animals, they observed large colonies and showed that these arose as clones from individual stem cells in the donor marrow that both could replicate to generate more stem cells and could give rise to multiple types of mature, differentiated blood cells [43]. These two characteristics, termed self-renewal and multipotency, remain generally accepted as the defining features of stem cells.

1.3.2 Embryonic Stem Cells

In contrast to adult stem cells, embryonic stem (ES) cells show essentially unlimited capacity for self-renewal in culture and should be able to give rise to any of the more than 200 cell types in the body (with the exception of certain extraembryonic cell types). ES cells have great therapeutic potential, but there are also significant barriers that must be overcome for their implementation in the clinic. ES cells are pluripotent, that is, they have the ability to form tissue representing all three embryonic germ layers—the ectoderm, mesoderm, and endoderm. Thus, the ES cell has the intrinsic ability to give rise to daughter cells that can form virtually any tissue in the body. This was demonstrated convincingly by the finding that mouse ES cells injected into developing embryos can contribute to all adult cell types, including germ cells [44]. Human ES cells have been induced to generate representatives of all three germ layers in culture and are widely assumed to have the same degree of pluripotency as mouse ES cells.

The great plasticity of ES cells can also represent a drawback to their use, because it may prove more difficult to induce them exclusively to yield a single desired cell type than when starting with lineage-committed adult stem cells. However, significant progress has been made in the directed production of many specialized cells, or at least lineage-specific progenitors, from mouse and human ES cells [45] [46]. Examples include cells of neuronal, epidermal, cardiac myocytic, hematopoietic, endothelial, hepatic, endocrine pancreatic, and germ cell lineages. Another important problem that must be overcome is that undifferentiated ES cells form teratoma tumors [47, 48]. Therefore, for clinical use, it will be essential to differentiate the cells quantitatively before implantation and to rigorously exclude the presence of residual stem cells. Progress is being made in the derivation of human ES cells in the absence of xenogeneic feeder cells and animal proteins that may pose risks for clinical application.

The derivation and certain potential uses of human ES cell lines have engendered ethical debate, especially because embryos, though donated for research, are destroyed to isolate the inner cell mass. Technological advances may offer at least a partial resolution to the ethical controversy. For example, human ES lines can be developed from single-cell biopsy at the eight-cell stage of development, without compromising the viability of the embryo, similar to a widely used procedure for prenatal genetic diagnosis [49, 50]. The development of ES-like cell lines by reprogramming of normal adult cells ultimately may put the debate to rest by providing pluripotent cells entirely without the use of donated embryos or oocytes.

1.3.3 Cellular Reprogramming

Reprogramming is a technique that involves dedifferentiation of adult somatic cells to produce patient-specific pluripotent stem cells without the use of embryos. Cells generated by reprogramming are theoretically identical to somatic cells and would not be rejected by the donor. This method also avoids nuclear transfer into oocytes.

Takahashi and Yamanaka were the first to report that mouse embryonic fibroblasts (MEFs) and adult mouse fibroblasts can be reprogrammed into an induced pluripotent state (IPS) [51]. They identified four key genes required to bestow embryonic stem cell-like properties in fibroblasts. Mouse embryonic fibroblasts and adult fibroblasts were cotransduced with retroviral vectors, each carrying Oct3/4, Sox2, c-Myc, and Klf4. Reprogrammed cells were selected via drug resistance. In this case, a downstream gene of Oct4, Fbx15, was replaced with a drug resistance gene via homologous recombination. The resultant IPS cells possessed the immortal growth characteristics of self-renewing ES cells, expressed genes specific for ES cells, and generated embryoid bodies in vitro and teratomas in vivo. When the IPS cells were injected into mouse blastocysts, they contributed to a variety of diverse cell types, demonstrating their developmental potential. Although IPS cells selected by *Fbx15* were pluripotent, they were not identical to ES cells. Unlike ES cells, chimeras of IPS cells did not result in full-term pregnancies. Gene expression profiles of the IPS cells showed that they possessed a distinct gene expression signature compared to ES cells. The epigenetic state of the IPS cells was somewhere between their somatic origins and fully reprogrammed ES cells, suggesting that the reprogramming was incomplete.

These results were improved significantly by Wernig and Jaenisch [52]. Fibroblasts were infected with retroviral vectors and selected for the activation of endogenous *Oct4* or *Nanog* genes. Results from this study showed that DNA methylation, gene expression profiles, and chromatic state of the reprogrammed cells were similar to those of ES cells. Teratomas induced by these cells contained differentiated cell types representing all three embryonic germ layers. Most importantly, the reprogrammed cells from this experiment were able to form viable chimeras and contribute to the germ line-like ES cells, suggesting that these IPS cells were completely reprogrammed.

Reprogramming by transduction of defined factors can be done with human cells [53, 54] Yamanaka's group began by optimizing the transduction efficiencies of human dermal fibroblasts (HDF) and determined that the introduction of a mouse receptor for retroviruses into HDF cells using a lentivirus improved the transduction efficiency from 20 % to 60 %. Yamanaka then showed that retrovirus-mediated transfection of *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* generates human IPS cells that are similar to hES cells in terms of morphology, proliferation, gene expression, surface markers, and teratoma formation. In contrast, Thompson's group showed that retroviral transduction of *OCT4*, *SOX2*, *NANOG*, and *LIN28* could generate pluripotent stem cells without introducing any oncogenes (c-MYC). Both studies showed that human IPS cells were similar but not identical to hES cells. Another concern is that these IPS cells contain three to six retroviral integrations (one for each factor) that may increase the risk of tumorigenesis.

These studies used retroviral transduction to induce reprogramming of somatic cells into a pluripotent state. Okita et al. studied the tumor formation in chimeric mice generated from Nanog-IPS cells and found 20 % of the offspring developed tumors due to the retroviral expression of c-Myc (33). An alternative approach would be to use a transient expression method, such as adenovirus-mediated system,

since both Meissner and Okita showed strong silencing of the viral-controlled transcripts in IPS cells (33, 34, 34). This indicates that they are only required for the induction, not the maintenance, of pluripotency. Another concern is the use of transgenic donor cells for reprogrammed cells in the mouse studies. In both mouse studies, IPS cells were isolated by selecting for the activation of a drug-resistant gene inserted into endogenous *Fbx15*, *Oct3/4*, or *Nanog*. The use of genetically modified donors hinders its clinical applicability for humans.

More recently, the development of direct reprogramming technology allows for the direct conversion from one cell type into another without reverting back into a stem cell state. Indeed, direct reprogramming has proven sufficient in yielding a diverse range of cell types from fibroblasts, including neurons, cardiomyocytes, endothelial cells, hematopoietic stem/progenitor cells, and hepatocytes [55]. Direct reprogramming studies [56–58] indicate that cell fate plasticity is much wider than previously anticipated and that direct reprogramming may offer a new system to study the mechanisms underlying cell fate decisions during development and also open up yet another possible regenerative medicine approach for muscle disease.

1.4 What Is Gene Therapy and When Will It Be Used in Clinical Practice?

Gene therapy—the process of introducing foreign genomic materials into host cells to elicit therapeutic benefit—became available for clinical practice on November 2, 2012, when Glybera (alipogene tiparvovec) became the first gene therapy in the Western world to receive market approval for patients with lipoprotein lipase (LPL) deficiency, a rare genetic disease previously without effective treatment [59, 60]. Since 1989, gene therapy clinical trials have been undertaken in 31 countries with more than 1800 human trials ongoing, completed, or approved worldwide [61]. Many of these trials target rare "orphan diseases." The Orphan Drug Act of 1983 defined an orphan product as a drug intended to treat a condition affecting fewer than 200,000 persons in the United States or a drug that would not be expected to be profitable within 7 years following FDA approval [62]. Orphan disease designation allows a sponsor to apply for market protection for the product following approval. 2700 orphan drug designations and more than 400 approvals associated with these designations were approved as of 2012 [62]. While most gene therapy trials have addressed cancer or cardiovascular disease, a significant number of gene therapy trials have targeted rare monogenic (single-gene) diseases (Table 1.2) [61]. These groundbreaking therapies involve the insertion of DNA sequences that encode functional, therapeutic genes into patients to replace mutated dysfunctional genes causing disease. In the case of Glybera, a DNA sequence encodes a therapeutic LPL gene packaged within a vector, in this case an adeno-associated virus (AAV). The recombinant AAV is injected into a patient harboring a mutant disease-causing LPL gene. The injected AAV is capable of shuttling the replacement DNA sequence from inside the vector to the cells of the targeted tissue. Once inside, the patient's

Table 1.2 Single-gene disorders reported in gene therapy clinical trials

Adrenoleukodystrophy
α-1 antitrypsin deficiency
Becker muscular dystrophy
β-thalassemia
Canavan disease
Chronic granulomatous disease
Cystic fibrosis
Duchenne muscular dystrophy
Fabry disease
Familial adenomatous polyposis
Familial hypercholesterolemia
Fanconi anemia
Galactosialidosis
Gaucher's disease
Gyrate atrophy
Hemophilia A and B
Hurler syndrome
Hunter syndrome
Huntington's chorea
Junctional epidermolysis bullosa
Late-infantile neuronal ceroid lipofuscinosis
Leukocyte adherence deficiency
Limb-girdle muscular dystrophy
Lipoprotein lipase deficiency[a]
Mucopolysaccharidosis type VII
Ornithine transcarbamylase deficiency
Pompe disease
Purine nucleoside phosphorylase deficiency
Recessive dystrophic epidermolysis bullosa
Sickle cell disease
Severe combined immunodeficiency
Tay–Sachs disease
Wiskott–Aldrich syndrome

[a]Glybera approved in Europe

own cellular machinery works to transcribe the new replacement DNA sequence to produce the therapeutic protein to treat the patient's disease. Besides the success of Glybera, other notable examples of gene therapy successes are highlighted in clinical trials undertaken in rare genetic childhood diseases such as X-linked severe combined immunodeficiency (SCID-X1) and Leber congenital amaurosis (LCA). In the first example, SCID-X1 results in recurrent and often fatal infections caused by genetic deficiency in cellular and humoral immunity. Long-term follow-up of

nine SCID-X1 boys treated in a French adenovirus-mediated gene therapy trial reported eight survivors after nearly 10 years [63]. In the second example, LCA leads to congenital blindness caused by mutations in a retinal gene that causes progressive loss of vision; young patients become completely blind by adulthood. Three independent gene therapy trials for LCA patients have been initiated, and follow-up results indicate improvement in vision for up to 2 years with no serious adverse events [64–66]. These and other gene therapy successes have been offset by serious, and rarely fatal, adverse events that lead to early clinical trial "holds." The most famous case was the death of Jesse Gelsinger in 2000, who was the 19th patient enrolled in a gene therapy trial of a deficiency of ornithine transcarbamylase (OTCD) [67]. Despite these early setbacks, the field of gene therapy continues to move forward at an extraordinary pace.

1.5 Will Gene Replacement Therapy Be Useful for Inherited Muscle Diseases like Muscular Dystrophy?

Most gene therapy clinical trials have targeted genes involved in cancer [61] with fewer trials initiated in monogenic diseases, such as Duchenne muscular dystrophy (DMD). Muscular dystrophies make up only a small percentage of the single-gene, or monogenic, diseases (Table 1.2), notably Becker, Duchenne, and limb-girdle muscular dystrophy. Pompe disease, among others listed in Table 1.2, is not considered a muscular dystrophy even though skeletal muscles of affected patients grow progressively weaker due to accumulation of abnormal proteins within the muscle's contractile tissue. Therapeutic approaches at replacing defective genes in monogenic diseases of muscle, like DMD, include the use of recombinant viral vectors engineered to target specific tissues. In the 1960s, the discovery of naturally occurring adeno-associated virus (AAV) isolates led to the clinical application of recombinant AAV vectors with early successes in clinical trials [68]. AAV vectors are attractive for clinical use because AAVs are not associated with human disease [60]; the virus persists in the infected host for years, and a large "toolkit" of AAV vectors is available as clinical gene therapy delivery tools [69]. In addition to muscle diseases, AAV vectors have been used in clinical gene therapy trials targeted to the liver for the treatment of hemophilia B, to the lung for treatment of cystic fibrosis, to the brain for treatment of Parkinson's, Batten's, and Canavan's disease, to joints for treatment of rheumatoid arthritis, and to the eye for treatment of LCA [68].

In recent years, AAV-mediated gene replacement has rapidly moved from preclinical studies to clinical trials due to encouraging results from animal models. Major challenges surrounding this strategy include (1) effective delivery methods to target muscles throughout the body, including diaphragm and cardiac muscle, and (2) host immune responses to the therapeutic vector [70]. The latter challenge was encountered in a double-blinded, randomized, controlled phase I trial of limb-girdle muscular dystrophy [71, 72], AAV1-MCK. Human alpha-sarcoglycan (SGCA) was

injected locally into the "extensor digitorum brevis" muscle in three patients, and sustained transgene expression was observed in two out three patients after 6 months. Humoral and cellular immune responses to the AAV capsid proteins were detected in the patient who failed to show expression. Another phase I trial on DMD from Mendell et al. [73] also raised the potential of cellular immune responses to either self or nonself dystrophin epitopes. In the study, AAV vectors carrying a truncated but functional dystrophin gene under the control of a CMV promoter were injected into the bicep of six DMD patients. None of the patients displayed transgene expression with four out of six patients showing detectable T cell responses against the transgene product. Two of the patients had dystrophin-specific T cell responses before the treatment. Taken together, these trials are informative to emphasize the importance of prescreening patients for preexisting immune responses to both AAV capsid proteins and transgene product and also to develop strategies to circumvent immune responses, such as using a transient course of immunosuppression, shown to be effective in a dog model of DMD [74, 75].

1.6 Can Genes Be Repaired?

While AAV-mediated gene therapy is regarded as a gene replacement strategy, another exciting development, termed exon skipping, focuses on gene repair. Approximately 70 % of all DMD mutations are due to single or multiple exon deletions. Such deletions disrupt the open reading frame of dystrophin and, hence, result in a premature truncated protein. In most cases, selective removal of specific exons can restore the reading frame and produce a partially functional dystrophin protein for clinical benefit [76–79]. Antisense oligonucleotides (AOs) targeting pre-mRNA to modulate splicing have been used to induce exon skipping, and the first human phase I trials [80, 81] focused on skipping exon 51, which, if successful, would be able to correct ~13 % of DMD patients with specific deletions within exons 42–50. A therapeutic exon skipping AO, 2-O-methyl AO termed "PRO051" [81], and AVI-4658, a morpholino-conjugated AO [80] that targets an internal sequence of exon 51, were injected intramuscularly into DMD patients. AOs used in these trials were well tolerated and demonstrated successful exon skipping, and all patients demonstrated dystrophin expression to levels between 3 and 12 % and 22 and 35 % by PRO051 and AVI-4658, respectively. Following these initial clinical trials of intramuscular administration, phase I/II trials using systemic delivery of the two drugs were tested for dose, safety, and efficacy [82, 83]. Dose-dependent restoration of dystrophin expression was observed following weekly administration of the experimental compounds through abdominal subcutaneous injections in two cohorts. While no significant differences were detected in patient's walking ability following a 12-week-long treatment [82], patients treated with weekly PMO-AO (AVI-4658) [83] for 48 weeks demonstrated improvement in stabilization of the muscle and in the 6-min walking distance test (6MWT) [84]. A larger confirmatory phase III trial is in the planning for 2014. Clinical trials for skipping other exons are also underway

or in planning [76]. These "personalized gene therapy medicines" generate hope for DMD patients, with the caveat that effective therapy will need to restore dystrophin expression in both skeletal and cardiac muscle, and that treatment will need to be persistent. The future challenge for clinical development with AOs is that current forms have a short half-life, and more than 85 % of AOs are cleared from the circulation within 24 h. This short half-life requires weekly injections to maintain a therapeutic level. Long-term outcomes of these AOs are also unknown. Intramuscular administration of these agents may be limited in treating skeletal muscle, and treating the cardiac muscle will need to be addressed for this strategy to be an effective treatment for DMD-associated cardiomyopathy.

Nonsense stop codon read through is another method of gene editing and potentially benefits ~ 13 % of DMD boys with premature termination codon (PTC) mutations. Aminoglycoside antibiotics initially demonstrated the capacity to induce ribosomal read through of premature stop codon [85], but not efficient and too toxic to be used for long-term treatment. Ataluren (PTC124) was identified to be effective in this matter in *mdx* mice and subsequently tested in human DMD trials [86–88]. The drug was well tolerated in general, and the dystrophin protein was detected with treated patients showing improvement in the 6MWT. However, correlation between the level of dystrophin expression and the 6MWT remains unclear. More trials are currently under way, and the same treatment strategy could be potentially applied to other muscle diseases including spinal muscular atrophy. Progress in developing other approaches targeting repair of the muscle membrane due to lack of dystrophin is also under development for example, increasing the level of the compensatory protein utrophin [89, 90] and upregulation of glycosylation of a-dystroglycan to improve extracellular matrix attachment [91].

1.7 Can Gene Replacement Therapy Rescue Lethal Muscle Disease? Lessons Learned from Dogs

In 2008, a case report of a 5-month-old Labrador Retriever was published in a Canadian veterinary medical journal [92]. The dog presented with weakness, muscle atrophy, and histopathological changes in skeletal muscle consistent with a centronuclear myopathy. Because male littermates were similarly affected, the authors postulated that the disease was X-linked, giving rise to the possibility that the disorder could be analogous to X-linked myotubular myopathy (XLMTM). It was through the tireless and extraordinary efforts of Alison Rockett Frase that the author's research group was able to acquire a first-degree relative, a dog named "Nibs," a Labrador Retriever coming from a line of dogs with a history suspicious for XLMTM. We later discovered that Nibs harbored a canine MTM1 mutation(34), the same gene known to cause myotubular myopathy in patients [reviewed in "The Miracle of Nibs" [93]]. Mrs. Frase recalls the story of her odyssey locating and retrieving the founding carrier dog from a farmer in Canada. The following excerpt describes how a determined mother of an affected child can help shape the future of research for a disorder like myotubular myopathy:

> "In the fall of 2008, a female Labrador Retriever was discovered to carry the same gene as I do for my son's muscle disorder called mytubular myopathy (MTM). To date, this was the first MTM large animal ever discovered by researchers anywhere in the world….Nibs was a beautiful Labrador Retriever that was instrumental in giving us puppies that carry the myotubular myopathy (MTM1) gene that affects our son Joshua. I am so grateful to Nibs for the initial 12 puppy litter…Our second litter of MTM pups has been born…Knowledge gained from these animals may 1 day lead to treatments not only for MTM, but other neuromuscular diseases. It will be a miracle for our son Josh and thousands of children like him if our goals are achieved."

Joshua Frase passed away on December 24, 2010, less than 2 years after this was written. His legacy, the *Joshua Frase Foundation*, set into motion research that will, hopefully, develop the first effective treatment for this devastating disorder.

XLMTM is an orphan disease, affecting 1/50,000 live male births worldwide [94] with only supportive, palliative care available for patients [95]. This inherited muscle disease results from loss-of-function mutations in the Myotubularin 1 gene (*MTM1*) [96] that encodes the founder of a family of 3-phosphoinositide phosphatases acting on the second messengers phosphatidylinositol 3-monophosphate [PI(3)P] and phosphatidylinositol 3,5-bisphosphate [PI(3,5)P_2] [97, 98]. Although myotubularin is expressed ubiquitously, loss of this enzyme profoundly affects skeletal muscles causing hypotrophic myofibers and structural abnormalities, with associated weakness [99]. No effective therapy exists for XLMTM. Management of the disease generally consists of mechanical ventilation, gastrostomy feeding tubes, antibiotics (for respiratory infections), orthotics to prevent skeletal limb contractures, and surgical treatment to alleviate severe spinal deformities. In spite of aggressive medical care, the average life expectancy is only about 2 years, and most who survive beyond this age require mechanical ventilation.

Animal models of the disease currently exist in zebra fish, mice, and notably, in dogs [99–101]. The murine phenotype resembles human XLMTM, with similar pathology and early mortality. In a mouse knockout model of XLMTM, local delivery of the wild-type myotubularin gene (*MTM1*) via an AAV vector reversed characteristic pathological features and rescued the function of injected limb muscles [102]. Buj-Bello et al. were the first to report a gene therapy success in the *Mtm1* knockout mouse in 2008. That same year, the *Joshua Frase Foundation* provided our research group access to a female Labrador Retriever harboring an *MTM1* gene mutation that was later proven by Beggs et al. to cause a canine version of the human disease [101]. From this single founding female, our group established a canine breeding colony to study effects of the disease in dogs. Initial data revealed that affected males display a phenotype directly analogous to human XLMTM: progressive and severe muscular weakness [103] leading to the inability to walk, weak ventilatory muscles leading to respiratory impairment [104], and early death. Based upon the early experience with gene replacement in the *Mtm1* knockout mouse [102], a similar gene replacement strategy was initiated in the XLMTM dog in 2011.

For eventual gene therapy of XLMTM patients, our goal was to use a predictive large animal model (the XLMTM dog) to refine the delivery system, to assess critical safety parameters such as the potential host immune response to vector and transgene, and to optimize efficacy measurements. In collaboration with the French

nonprofit institute, Généthon [105], cohorts of *Mtm1* knockout mice were first tested for response to systemic *Mtm1* gene replacement via tail vein injection. Results indicated that a single systemic treatment with AAV-*Mtm1* sufficed for long-term (at least 1 year) survival and essentially complete amelioration of symptoms of mice with myotubularin-deficient muscles [106]. Using the same AAV vectors produced by Généthon scientists and tested in mice, our collaborative research group confirmed that local gene replacement therapy, delivered intramuscularly into the hind limb of young XLMTM dogs, reversed pathological changes in myotubularin-deficient skeletal muscles. Remarkably, the treated muscles also showed nearly normal strength at 6 weeks postinjection, compared to very weak muscles (only 20 % of normal strength) in saline-injected contralateral limbs. In subsequent experiments, intravascular administration of AAV8-*MTM1* at the same dose used in mice was well tolerated in dogs, rescued the skeletal muscle pathology and respiratory function, and prolonged life for over 1 year. Together, these initial studies demonstrated the feasibility, safety, and efficacy of gene therapy with AAV for long-term correction of muscle pathology and weakness observed in myotubularin-deficient mouse and dog models and support future clinical trials aimed at correcting this devastating disease in patients.

Conflict of Interest Statement MC is an inventor on patents related to recombinant AAV technology. MC owns stock options in a biotechnology company commercializing AAV for gene therapy applications. To the extent that the work in this manuscript increases the value of these commercial holdings, MC has a conflict of interest.

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