Therapeutic Potential of Dermal Cells Following Transplantation and In Vivo Myogenic Conversion in Dystrophic Muscle

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Abstract

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Cell-based therapies have the potential to contribute to functional muscle regeneration in Duchenne muscular dystrophy (DMD) and other muscle disorders. Various cell types have been studied for their ability to fulfill this requirement, with reports of high engraftment after transplantation into muscle. However, studies rarely demonstrate improvements in whole muscle function, and very few have characterized cell populations that are amenable to autologous transplantation. To this end, we have characterized the in vitro and in vivo potential of dermal fibroblasts in the *mdx*^{4cv} mouse model of DMD. Fibroblasts were isolated from transgenic mice carrying a minidystrophin gene, transduced with a lentiviral vector carrying tamoxifen-inducible MyoD, and transplanted into muscles of *mdx*^{4cv} mice to model ex vivo gene therapy for DMD. Treatment of host mice with tamoxifen drove conversion of transduced fibroblasts into myogenic cells that fused into muscle fibers that subsequently expressed high levels of minidystrophin. Transplantation of various cell doses revealed a limit for reliable engraftment of up to 1 x 10^6 cells in single injections. In vivo converted dermal fibroblasts engrafted similarly to primary myoblasts, with up to 30% of the host muscle expressing minidystrophin. We found no evidence

of fibrosis or structural abnormalities following in vivo conversion of dermal fibroblasts. However, these muscles showed no improvement in force development or protection from contraction-induced injury. We hypothesized that engraftment was below a threshold for improvement of contractile properties in dystrophic muscle, and therefore tested whether a cocktail of pro-survival factors could enhance engraftment. A quantitative PCR-based method allowing rapid screening for these cells in host tissue revealed significant early cell death, and a nearly 3-fold increase in engraftment when cells were injected intramuscularly with the pro-survival cocktail. Histologic analysis showed increased dystrophin-positive fiber number, area engrafted, and spread of engrafted cells with the pro-survival cocktail. Furthermore, engraftment of cells with pro-survival cocktail led to modest but statistically significant protection from contraction-induced injury over *mdx*^{4cv} controls. The clinical relevance of this strategy lies in the transplantation of autologous cells that can survive and form functional muscle fibers without prior muscle irradiation or toxin treatment in immunocompetent hosts.

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DEDICATION

I dedicate this dissertation to my husband Indika, our beautiful daughter Eden,

my father Vyrn, and my mother Diane.

You have made everything possible.

Chapter 1

Background and Introduction

Muscular Dystrophy

Muscular dystrophy is a class of inherited disorders characterized by muscle weakness and wasting. Over 40 forms of muscular dystrophy have been identified, based on underlying genetic and molecular etiology, clinical manifestation and prognosis (Emery, 2001). Duchenne muscular dystrophy (DMD, MIM 310200) is the most common lethal genetic disorder of children, affecting ~1 in 3500 newborn males (Emery and Muntoni, 2003). The second and third most common are myotonic dystrophy (DM1) and facioscapulohumeral muscular dystrophy (FSHD) (Tawil et al., 1998; Wheeler et al., 2007).

The molecular pathology of various muscular dystrophies is diverse because of the heterogeneity of the defective proteins involved. Understanding these proteins and their interactions will be a crucial aspect of the search for therapeutic targets. Many muscular dystrophies result from defects in muscle-membrane-associated proteins that help maintain the structural integrity of muscle fibers (Bansal and Campbell, 2004; Davies and Nowak, 2006; Gee et al., 1998). DMD is caused by mutations in the dystrophin gene (official symbol *DMD* for human and *Dmd* for mouse), most of which result in translational frameshifts and/or failure to express a functional protein (Monaco et al., 1988). The four major domains of dystrophin (N-terminal, central rod, cysteine-rich and C-terminal) mediate a link between the subsarcolemmal cytoskeleton and a complex series of protein–protein interactions at the sarcolemma (Abmayr and Chamberlain, 2006) (**Figure 1.1**). The N-terminal and central rod domains interact with filamentous actin. The rod domain contains 24 repeats that are homologous to those in spectrin,

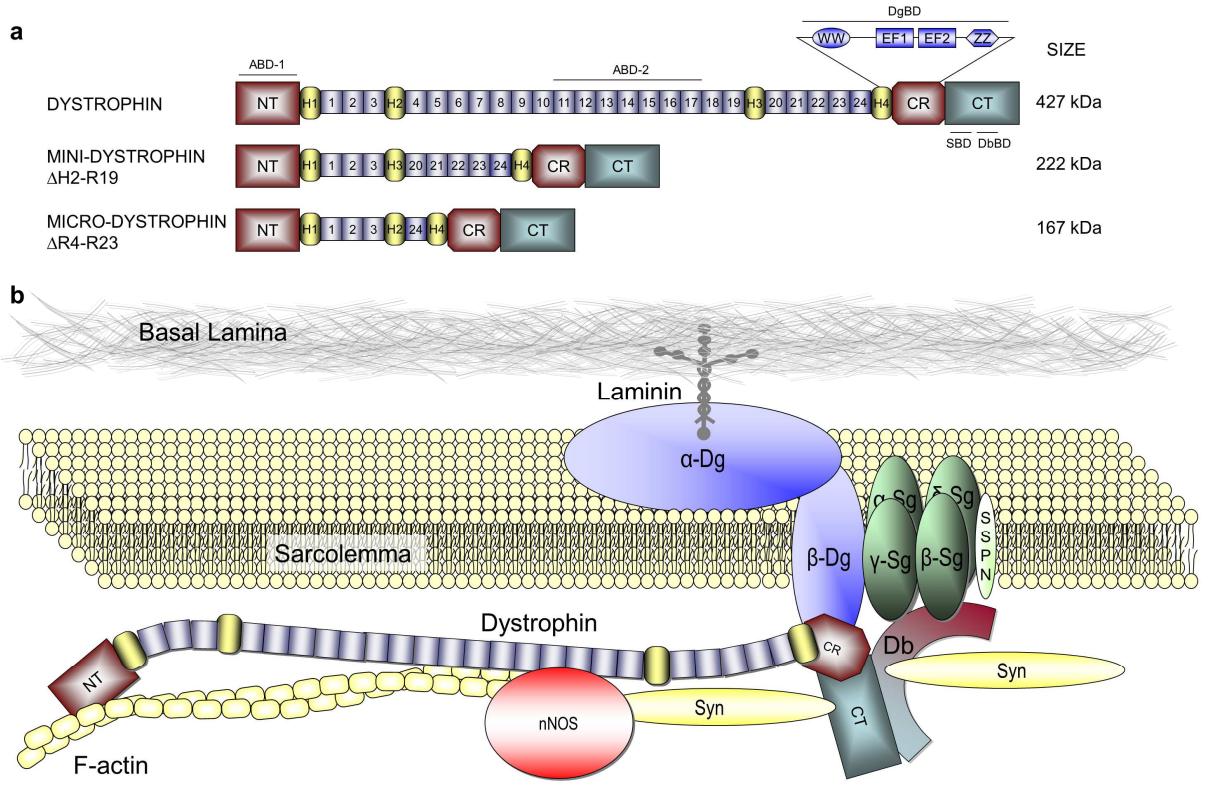


Figure 1.1 Dystrophin and the dystrophin–glycoprotein complex. (a) Comparison of the structural domains in full-length dystrophin with those of highly functional miniaturized versions, which have been engineered in response to the limited packaging capacity of delivery vectors for gene therapy. Full-length dystrophin contains four major domains: an N-terminal (NT) cytoskeletal-binding domain, a rod domain composed of 24 spectrin-like repeats (1–24) and four hinge regions (H1–H4), a cysteine-rich (CR) and a C-terminal (CT) domain. The two known actin-binding domains (ABD-1 and ABD-2) are within the N-terminal and rod domains, respectively. The dystroglycan-binding domain (DgBD) is composed of a WW motif (in hinge 4) connected to two EF-hand-like motifs and a ZZ domain (the two EF-hand and ZZ regions comprise the so-called cysteine-rich 'CR' domain). Towards the C-terminus is a syntrophin-binding domain (SBD) and a dystrobrevin-binding domain (DbBD). Minidystrophin and microdystrophin retain most of the necessary regions for the signaling and structural roles of dystrophin, as well as the ability to assemble members of the dystrophin–glycoprotein complex (DGC) at the plasma membrane. **(b)** Important interactions among members of the DGC, many of which are defective in various muscular dystrophies. Within striated muscle fibers, dystrophin binds to cytoskeletal proteins such as filamentous actin (F-actin) at its N-terminal domain. The rod domain encodes a second actin-binding domain in spectrin-like repeats 11-17, and repeats 16-17 also participate in binding to neuronal nitric oxide synthase (nNOS). Although localization of nNOS has been found to require the presence of both repeats 16-17 and syntrophin, the precise three-dimensional structure of the dystrophin-nNOS complex is speculative (Lai et al., 2009). The DgBD anchors β-dystroglycan to dystrophin, and might help to assemble other proteins. The CT domain binds to and localizes members of the syntrophin (Syn) and dystrobrevin (Db) protein families. Studies suggest that up to four syntrophins could attach to the DGC at any one time, two to dystrophin and two to dystrobrevin (Newey et al., 2000). At present it is unclear how many of these syntrophins are attached to nNOS, as it has been shown that syntrophin can also bind to other proteins, such as sodium channels and aquaporin-4 (Adams et al., 2001). In addition to dystrophin, dystrobrevin is known to bind to another member of the DGC, which has yet to be identified. Abbreviations: Dg, dystroglycan; Sg, sarcoglycan; SSPN, sarcospan.

providing a flexible and elastic region connecting the end domains that are crucial for dystrophin function. The third and fourth are the cysteine-rich and C-terminal domains, which contain many of the protein-interaction domains essential for signaling and assembly of the dystrophin–glycoprotein complex (DGC) (**Figure 1.1)**. The most critical binding site in dystrophin is the dystroglycan-binding domain (DgBD), which is made up of a WW domain (Huang et al., 2000) at the end of the rod domain and the adjacent cysteine-rich domain. Inactivation of the DgBD renders dystrophin non-functional (Abmayr and Chamberlain, 2006).

Dystrophin is thought to have a primarily structural role, linking the cytoskeleton to the extracellular matrix via the DGC (**Figure 1.1b**) (Ervasti and Campbell, 1993; Yoshida and Ozawa, 1990). This linkage transduces the forces of contraction to the extracellular matrix to protect myofibers from contraction-induced injury (Petrof et al., 1993). The absence of dystrophin results in membrane instability and repeated tears in the sarcolemma with calcium entry into the muscle cell (Batchelor and Winder, 2006; Turner et al., 1988). Stretch-activated calcium channels might have a role in this process, because their blockade reduces membrane permeability and loss of force in dystrophic muscle following eccentric contractions (Whitehead et al., 2006). The resulting cascade of events forces muscle fibers to undergo cycles of degeneration and regeneration until repair capacity is no longer sufficient, and muscle fibers are replaced by adipose and fibrous connective tissue (Emery and Muntoni, 2003). In Becker muscular dystrophy (BMD), mutations typically maintain the mRNA reading frame but lead to reduced expression, or expression of smaller forms of dystrophin in striated muscle (Baumbach et al., 1989; Monaco et al., 1988). Mutations in other components of the DGC result in a number of other muscular dystrophies. For example sarcoglycanopathy results in several limb-girdle

muscular dystrophies, and integrin or laminin deficiencies result in congenital muscular dystrophies (Campbell, 1995; Ozawa et al., 1998).

Despite tremendous effort and major advances in our understanding of the molecular basis for the muscular dystrophies, no cure has been found. Symptom management and prolonging mobility are therefore the primary aims of clinical interventions (Chamberlain and Rando, 2006; Manzur et al., 2009). Many hopes rest on recent advances in gene and cell therapies to prevent muscle degeneration and potentially reverse dystrophy-related damage. The goal of gene therapy is to deliver a functional copy of the gene, or repair the damaged gene, such that it produces sufficient product to halt the dystrophic phenotype. Methods of gene delivery include both viral and nonviral vectors. Current cell therapy strategies involve transplantation of stem or progenitor cells with skeletal myogenic potential that can fuse with existing myofibers or form new ones. To avoid the host-versus-graft immune response, a patient's own cells that have been corrected for the genetic defect could be used (ex vivo gene therapy). Upon transplantation, these cells would ideally engraft and regenerate the muscle, as well as repopulate the muscle stem cell (satellite cell) niche.

Mammalian preclinical testing often uses the X-linked muscular dystrophy *Dmd*^{mdx} mouse model of DMD (hereafter *mdx*), or the canine *cxmd* model (Banks et al., 2008a; Ng et al., 2012). I will mainly discuss therapies with respect to models of DMD; however, many approaches are applicable to a wide range of muscle and genetic disorders.

Gene Therapy for Muscular Dystrophies

Approaches

Gene therapy approaches for treatment of DMD must either develop methods to deliver dystrophin, or repair the locus within a patient's genome (**Figure 1.2**). As dystrophin encodes a very large 14 kb mRNA (the gene itself spans about 2.1 Mb), therapeutic delivery is a significant challenge (Chamberlain and Caskey, 1990; Chamberlain, 2002). However the observation of large genomic deletions in some very mildly affected BMD patients (England, 1990; Matsumura et al., 1994) prompted construction of highly functional 'mini' and 'micro' versions of dystrophin to facilitate gene transfer using viral vectors, such as adeno-associated virus, that have a limited carrying capacity (Harper et al., 2002; Phelps et al., 1995; Sakamoto, 2002; Wang et al., 2000). Internally-deleted dystrophins, illustrated in **Figure 1.1a**, retain their N-terminal actin-binding and C-terminal dystroglycan-binding domains, which are thought to contain most of the necessary regions for dystrophin's role in signaling, structural support and assembly of dystrophin-associated proteins at the cell membrane (Harper et al., 2002; Ishikawa-Sakurai et al., 2004) (**Figure 1.1b**). Although inclusion of actin- and dystroglycan-binding domains is crucial, meticulous design of the deletions in the rod domain is also essential for maintaining functionality and rescuing the dystrophic phenotype (Harper et al., 2002).

It has long been considered in the field of gene therapy that expression of a delivered transgene not normally expressed in a host could evoke an immune response. Transgenes delivered via adeno-associated viral vectors undergo MHC class I processing and surface presentation (Brockstedt et al., 1999), and can therefore be detected by immune effector cells. Immunological studies indicate that dystrophin could act as a neoantigen (Gilchrist et al., 2002; Huard et al., 1992; Mendell et al., 2010a; Ohtsuka et al., 1998), either through transduced cell

surface presentation or release from degenerating muscle fibers and contact with antigen presenting cells (Mays and Wilson, 2011; Wells et al., 2002). $CD4^+$ and $CD8^+$ T cell responses to truncated dystrophin were observed in a subset of patients following intramuscular delivery of recombinant adeno-associated virus in a recent clinical trial, as well as the unexpected discovery of dystrophin-specific T cells prior to vector administration (Mendell et al., 2010b). Delivery route can also affect the immune response (Brockstedt et al., 1999), and intravascular administration, in addition to the advantage of reaching all striated muscle (Gregorevic et al., 2004a), appears to reduce transgene immunogenicity compared to intramuscular delivery (Mingozzi and High, 2011; Rodino-Klapac et al., 2010; Toromanoff et al., 2010). Blocking more than one pathway involved in activation of a cytotoxic T cell response against transgene-carrying cells encourages tolerance and has shown promise for viral vector-mediated delivery to muscle (Adriouch et al., 2011).

A possible alternative is gene therapy using utrophin, a highly similar protein to dystrophin in both structure and properties (Tinsley et al., 1992). Utrophin has been suggested to have a comparable functional role in muscle, with the potential to compensate for dystrophin absence (Matsumura et al., 1992; Winder et al., 1995). Although it is primarily expressed at the neuromuscular and myotendinous junctions in adult muscle (Khurana et al., 1991; Nguyen, 1991), it has been speculated that elevated expression of utrophin in some DMD patients partially compensates for lack of dystrophin (Mizuno et al., 1993; Weir et al., 2004).

Delivering or upregulating endogenous utrophin are therefore potential therapies for DMD. Full-length utrophin transferred via adenoviral vector ameliorated the dystrophic phenotype in the limb muscles of *mdx* mice (Deol et al., 2007). In addition, miniutrophins significantly improve the pathophysiology of dystrophic *mdx* and dystrophin$^{-/-}$ and utrophin$^{-/-}$

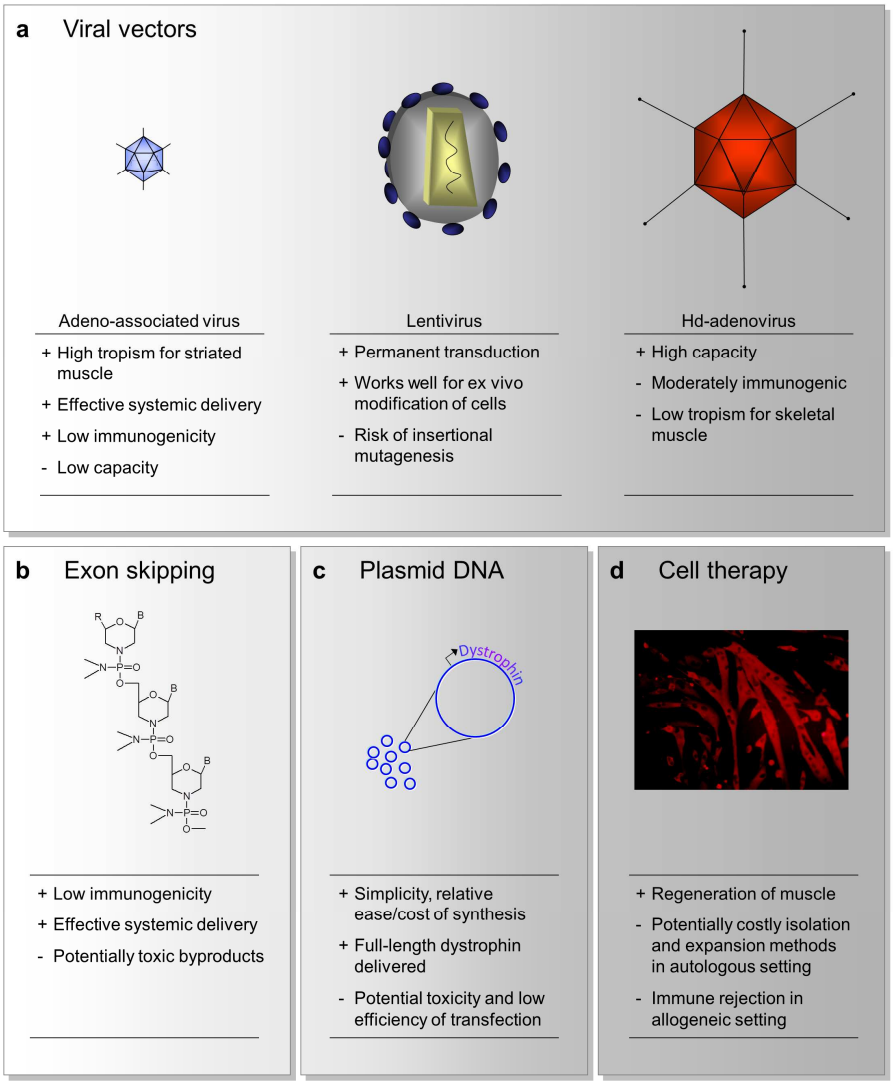


Figure 1.2 Strategies for treating the muscular dystrophies. Major advantages and limitations of viral vectors, exon skipping, plasmid DNA and cell therapies. Each virus-based delivery system **(a)** has particular strengths, such as high striated muscle tropism (adeno-associated virus), ability to integrate into the host genome (lentivirus), and packaging capacity (hd-adenovirus). However, viral systems must carefully address safety concerns such as insertional mutagenesis, where provirus integration into the genome might alter the structure or expression of nearby genes, and immunogenicity of capsid proteins. Exon skipping **(b)** is a promising new approach in which mutations can be bypassed using antisense oligonucleotides of various chemistries that modify splicing of pre-mRNAs. Shown is the phosphorodiamidate morpholino oligomer (PMO), which incorporates morpholine rings linked by phosphorodiamidate groups instead of the ribose rings linked by phosphodiester groups found in RNA. R, cell-penetrating moiety; B, RNA nucleobases. **(c)** Plasmid DNA is a straightforward approach and can potentially deliver full-length dystrophin. Current studies are focused on lowering toxicity of transfection reagents as well as improving delivery efficiency. **(d)** Cell-based therapies might be able to regenerate muscle by replacing muscle fibers lost during the course of progressive muscle-wasting conditions such as DMD. Transplanted cells derived from the patient (autologous setting) require genetic modification to include a functional copy of the defective gene, whereas donor cells (allogeneic setting) are at risk for immune rejection. Image shows differentiating muscle cells in culture, with immunofluorescent staining for myosin heavy chain (red). Most therapies must be adapted for systemic delivery and targeted specifically to striated muscle using muscle-specific promoters to regulate expression of the delivered transgene. Hd, helper-dependent.

(*mdx:utrn*$^{-/-}$) double-knockout transgenic animals, as well as dystrophic dogs (Cerletti, 2003; Deconinck, 1997; Tinsley, 1996). Recently, microutrophins have been shown to alleviate a wide range of histopathological features of the *mdx:utrn*$^{-/-}$ model when delivered systemically using recombinant adeno-associated viral vectors (Odom et al., 2008). Assessment of muscle morphology, mass, fiber size, and contractile properties suggested that microutrophin might work similarly to microdystrophin. Other studies demonstrate amelioration of dystrophy after delivery of truncated utrophins to muscles of *mdx* mice (Sonnemann et al., 2009; Tinsley, 1996). Additionally, Lin et al. (2012) suggest that microutrophin's actin-binding domains may more effectively interact with actin, a connection that is critical during muscle contraction, compared to the actin-binding domains in internally-deleted dystrophins. While utrophin itself may not be immunologically novel, it should be noted that internal deletions still create novel peptide junctions and therefore the possibility of an immune response. Overall, these results support the potential of delivering truncated utrophin to dystrophic muscle as a way of ameliorating pathology, without eliciting a cellular immune response against exogenous dystrophin.

Adeno-associated virus

Use of recombinant vectors derived from adeno-associated virus (rAAV) is among the most promising methods for delivery of genes to striated muscle (Seto et al., 2012). The wild-type virus is a nonpathogenic single-stranded DNA parvovirus that requires a helper virus to replicate (Atchison et al., 1965; Muzyczka and Berns, 2001). The recombinant form can be produced at high titers in the absence of helper virus, carrying a desired transgene, and it can infect both dividing and nondividing cells (Podsakoff et al., 1994). Although its small size and range of target tissues facilitates dissemination, the limited packaging capacity (less than 5 kb)

precludes delivery of full-length dystrophin. However rAAV has been used to successfully deliver microdystrophin systemically to all striated muscle (Gregorevic et al., 2004b).

At least nine AAV serotypes have been identified in primates, referred to as AAV1-AAV9 (Gao et al., 2004). The different serotypes display various tropisms in vivo (Chao et al., 2000; Duan et al., 2001a; Gregorevic et al., 2004b; Grimm et al., 2003; Halbert et al., 2001). rAAV genomes persist as nonintegrated episomes following infection of cells (Duan et al., 1998; Schnepp et al., 2009), except at very high doses, where low levels of integration have been found in cultured cells, liver and muscle (Chamberlain et al., 2004; Inagaki et al., 2007). Stable gene expression following rAAV injection into muscle has been reported for up to 2 years in mice and more than 7 years in dogs and rhesus monkeys (Herzog et al., 1999; Manno, 2003). However, the mostly episomal AAV in muscle will almost certainly be lost over an extended period of time because of natural muscle turnover during exercise.

Evading an immune response will be critical for safe vector administration and robust transgene expression in the gene therapy setting. While rAAV has minimal immunogenicity compared with other vectors such as adenovirus (Zaiss et al., 2002), AAV capsid proteins have the potential to incite immunotoxicity in target tissues, thereby reducing or eliminating transgene expression. Initial studies in immunocompetent mice found no cellular immune responses to viral capsid proteins or the transgene (Fisher et al., 1997; Kessler et al., 1996; Xiao et al., 1996), with robust and long-term expression of the delivered transgene, in contrast with immune clearance of transduced cells following adenoviral delivery (Jooss et al., 1998). Phase 1 clinical trials using rAAV2 also reported relative safety, with no adverse events after intramuscular injections, and pre-existing antibodies to the serotype having little effect on myofiber

transduction (Manno, 2003). However, it has since become clear that in many settings the immune response can strongly diminish the efficacy of AAV-mediated gene delivery.

Recent studies have found cytotoxic T cell and humoral immune responses to the viral capsid of various AAV serotypes in murine as well as canine models, nonhuman primates, and humans (Chirmule et al., 2000; Halbert et al., 1998; Jiang et al., 2006; Manning et al., 1998; Manno et al., 2006; Mingozzi and High, 2007; Mingozzi et al., 2007; Rivière et al., 2006; Wang et al., 2007a). In addition, the magnitude of muscle-specific capsid T cell response in humans appears dose-dependent (Mingozzi and High, 2011). Transient immune suppression might avoid a cytotoxic T cell response by allowing time for clearance of viral capsids from transduced cells and professional antigen presenting cells. It may also prevent activation of $CD4^+$ T cells involved in stimulating the development of neutralizing antibodies to capsid proteins (Reis e Sousa et al., 1997). The presence of neutralizing antibodies to capsid, whether from prior administration or natural infection (Boutin et al., 2010), can diminish transgene expression, though in some cases may not entirely prevent transduction (Arnett et al., 2011; Brockstedt et al., 1999; Chirmule et al., 2000; Fisher et al., 1997; Jiang et al., 2006; Manning et al., 1998; Moskalenko et al., 2000; Rivière et al., 2006). Several studies have shown that transient immune suppression during the initial vector administration effectively allows readministration in murine and canine models as well as nonhuman primates. Methods include blocking T cell activation with antibodies that interfere with immune priming (Chirmule et al., 2000; Halbert et al., 1998; Manning et al., 1998) and chemical suppressants such as cyclosporine, tacrolimus, and mycophenolate mofetil (Jiang et al., 2006; Wang et al., 2007b).

These methods may prevent formation of neutralizing antibodies, but additional strategies are required to evade pre-existing immunity. One example is the use of alternative serotypes for

readministration (Manning et al., 1998; Rivière et al., 2006). Furthermore, capsid engineering has generated useful variants through rational design, for example to avoid immunogenic epitopes, and directed evolution, where diverse libraries can be screened for immunological advantages (Bartel et al., 2012; Maheshri et al., 2006; Moskalenko et al., 2000; Perabo et al., 2006). Other approaches for evading pre-existing immunity include the application of biomaterials to shield antibody recognition of capsid proteins and plasmapheresis to reduce the presence of neutralizing factors (Bartel et al., 2011; Monteilhet et al., 2011).

The many strategies under development to reduce immunotoxicity improve the clinical potential of rAAV-mediated gene therapy. Considering the complexity and wide range of immune responses to AAV-mediated gene delivery observed within and between species (Gao et al., 2009; Wang et al., 2011), translational studies in nonhuman primates and humans that include immunomodulation should undergo careful design and evaluation. Efficient targeting of vectors to muscle should further limit the immune response. For example, using AAV serotypes with natural tropism for muscle (Gregorevic et al., 2004a; Inagaki et al., 2006a; Wang et al., 2005a; Yue et al., 2008a) lowers off-target transduction, while muscle specific promoters reduce ectopic expression in cells that can be involved in T cell priming (Cordier et al., 2001; Jooss et al., 1998; Koo et al., 2011; Mays and Wilson, 2011; Wang et al., 2007b; Yuasa et al., 2002). Another recent strategy for restricting transgene expression to desired tissues is to use microRNAs to target the transgene itself via post-transcriptional silencing in off-target tissues (Brown et al., 2007; Gentner et al., 2009). MicroRNAs can be incorporated into the 3’UTRs of transgene cassettes and have effectively reduced off-target transgene expression in the liver (Geisler et al., 2011; Qiao et al., 2011). This is an important finding considering that systemic

delivery will likely be required, where a range of tissues become exposed to the therapeutic transgene.

Systemic delivery of rAAV will be a critical aspect of administration, given the need to target all striated muscle, including the heart and diaphragm, for an effective DMD therapy. Targeting skeletal muscle alone will strain the dystrophic heart and aggravate cardiomyopathy (Townsend et al., 2008). A major breakthrough for MD gene therapy came with the discovery that several serotypes of AAV could transduce muscle tissue body-wide following intravascular delivery. Initial studies of systemic delivery used vectors derived from AAV6 (Gregorevic et al., 2004b), but subsequent studies have also had success with AAV1, AAV8 and AAV9 (Inagaki et al., 2006b; Mah et al., 2005; Wang et al., 2005b). Feasibility of the systemic approach coupled with early intervention is validated by successful transduction of all skeletal muscle in newborn dogs in the absence of immune suppression (Yue et al., 2008b).

The intimate association between myofibers and capillaries allows close contact between extravasating vectors and the surface of muscle cells. Coupled with an inherent tropism for muscle cells, some AAV serotypes readily transduce muscle following intravascular delivery. The mechanisms responsible for vector extravasation remain unclear, and few cell surface receptors have been identified for AAV1, AAV6, AAV8 or AAV9. Also, little is known of the intracellular events following vector uptake by myofibers that enable vector decapsidation and gene expression (Schultz and Chamberlain, 2008). Identification of these mechanisms could well lead to modified delivery protocols to enhance body-wide muscle transduction using lower vector doses.

Split-vector approaches for full-length dystrophin delivery by rAAVs

Restoration of the major functions of dystrophin is key to alleviating deficits in DMD, and truncated dystrophins lose some functionality (Banks et al., 2007; Harper et al., 2002). For example, although most mini and microdystrophins restore the components of the DGC to the sarcolemma, only recently has a truncation been found that supports sarcolemmal localization of neuronal nitric oxide synthase (nNOS) (Judge et al., 2006; Lai et al., 2009; Wells et al., 2003). nNOS is involved in production of the signaling molecule nitric oxide (NO), which is important for maintaining vasodilation and adequate blood flow in skeletal muscle during activity (Kobayashi et al., 2008; Thomas et al., 1998). Although reconstitution of dystrophin as represented in the internally truncated forms might be the crucial factor for stabilizing muscle fibers, delivery of mini or microdystrophins that fail to localize nNOS could lead to a relatively mild BMD phenotype, with the associated susceptibility to fatigue. It is also unknown whether other properties of myofibers are affected by large deletions in dystrophin, such as the structure of the myotendinous junctions (Banks et al., 2008b; Banks et al., 2009). It is therefore unclear whether some of the milder BMD phenotypes that result from truncated dystrophins would benefit from delivery of a designer micro- or minidystrophin, or whether an alternative therapy would be necessary to relieve symptoms.

Since rAAV packaging capacity is limited to less than 5 kb, microdystrophin is the only dystrophin variant small enough to package into a single vector. However, one possible strategy to deliver full-length dystrophin is to package fragments of the cDNA into separate vectors. Transduction of target tissues with more than one vector, each carrying a dystrophin cDNA fragment, could allow production of larger dystrophins if the parts of each vector could be brought together (Nakai et al., 2000; Sun et al., 2000; Yan et al., 2000). Two approaches for

achieving this goal are showing promise in vivo. In the first, portions of introns carrying appropriate splicing signals are incorporated into the gene fragments, such that the cellular RNA splicing mechanisms can be harnessed to reconstruct a larger gene from two smaller halves (Duan et al., 2001b; Lai et al., 2006). Another approach is to package partially overlapping fragments into the different vectors, such that a larger dystrophin expression cassette can be reconstructed via homologous recombination in transduced tissues (Duan et al., 2001b; Halbert et al., 2002; Odom et al., 2011). A recently developed hybrid system outperforms splicing and homologous recombination split-vector systems alone, and improves efficiency of minidystrophin delivery to muscle (Ghosh et al., 2007). This system incorporates a highly recombinogenic portion of the alkaline phosphatase (AP) gene into two half-transgene cassettes and additional splicing signals between each AP region and transgene half. Thus reconstruction of an intact transgene can occur via recombination of AP and subsequent splicing, or recombination between viral inverted terminal repeats and splicing.

Lentivirus

Retrovirus-derived lentiviral vectors can be used to stably integrate a transgene such as dystrophin into the genome of target cells (Li et al., 2005). However, integration into the host genome could cause insertional mutagenesis, resulting in activation of nearby proto-oncogenes or inactivation of tumor suppressor genes (Beard et al., 2007; Ciuffi et al., 2006; Hacein-Bey-Abina et al., 2003). Self-inactivating (SIN) lentiviral vectors partially address this issue, as viral promoter and enhancer elements are removed prior to integration into the host genome (Miyoshi et al., 1998; Zufferey et al., 1998). However, further studies have found that specific deletions in SIN vectors may not entirely suppress promoter activity (Logan et al., 2004), and this strategy does not address disruptions at integration sites.

Unlike vectors derived from onco-retroviruses such as Moloney murine leukemia virus, which are commonly used for gene transfer (Barquinero et al., 2004), lentiviral vectors can infect a wide range of dividing and nondividing cells, including hepatocytes, skeletal and cardiac muscle cells, and neurons (Bonci et al., 2003; Kafri et al., 1997; Naldini et al., 1996). Unlike adenoviral vectors (discussed in the next section), lentiviral vectors have low immunogenicity; however, the delivered transgene is still a potential neoantigen (Annoni et al., 2007). Lentiviral vectors have a larger carrying capacity (~9 kb) than rAAVs, and have been used to stably transduce myogenic cells with minidystrophin (Kimura et al., 2010; Li et al., 2005), whereas rAAVs can only deliver the smaller microdystrophin (Harper et al., 2002).

Permanent gene transfer by lentiviral vectors is especially advantageous for DMD treatment. Patient satellite cells within skeletal muscle are an ideal target, and lentiviral reconstitution of dystrophin into the satellite cell niche in vivo would provide a source of dystrophin during ongoing cycles of regeneration. Lentiviral vectors are also a useful tool for transducing autologously derived cells with dystrophin and other genes ex vivo before expansion and transplantation (Bachrach et al., 2006; Dellavalle et al., 2007).

Adenovirus

Vectors derived from adenovirus are attractive for gene transfer because of their large carrying capacity, high titer production and ability to infect nondividing cells. Successive modifications have improved carrying capacity, reduced immunogenicity and prolonged transgene expression. The latest generation of adenovirus vectors are helper-dependent or ‘gutted’ (hdAd) and are entirely devoid of viral genes. Thus they require a helper virus to supply genes needed for vector replication and packaging during vector production (Chen et al., 1997; Clemens et al., 1996; Kumar-Singh and Chamberlain, 1996). The presence of a helper virus

renders production technically challenging, and additional purification steps are required to remove it from the final vector preparations (Hartigan-O'Connor et al., 2002). HdAd has been used to stably transduce muscle with dystrophin and has improved muscle function in *mdx* mice (DelloRusso et al., 2002; Gilbert et al., 2003).

Despite improvements, many hurdles to effective hdAd-vector-based therapy remain. Adenovirus vectors are five times larger than AAVs, reducing their ability to exit capillaries and infect muscle (Su et al., 2005). Also, most intravascularly administered adenovirus vectors are taken up by the liver (Gao et al., 1996). Coupled with low transduction efficiency in adult skeletal muscle, systemic therapeutic administration could require extremely high doses of hdAd (Acsadi et al., 1994; DelloRusso et al., 2002). Such high doses resulted in acute inflammation and a cytotoxic T cell response – which mediate lethal acute toxicity – in nonhuman primates (Brunetti-Pierri et al., 2004; Muruve et al., 1999; Zoltick et al., 2001) and one patient (Raper et al., 2003). As with AAVs, delivered transgenes exist as episomes, which might be lost over time and require repeated doses. Readministration with the same serotype is problematic given the development of neutralizing antibodies to both the hdAd vector and the transgene (Gilchrist et al., 2002; Morral et al., 1999). Use of hdAd vectors has not progressed to clinical trials for these reasons, and much of the current work is focused on overcoming the immunological issues.

Nonviral gene transfer

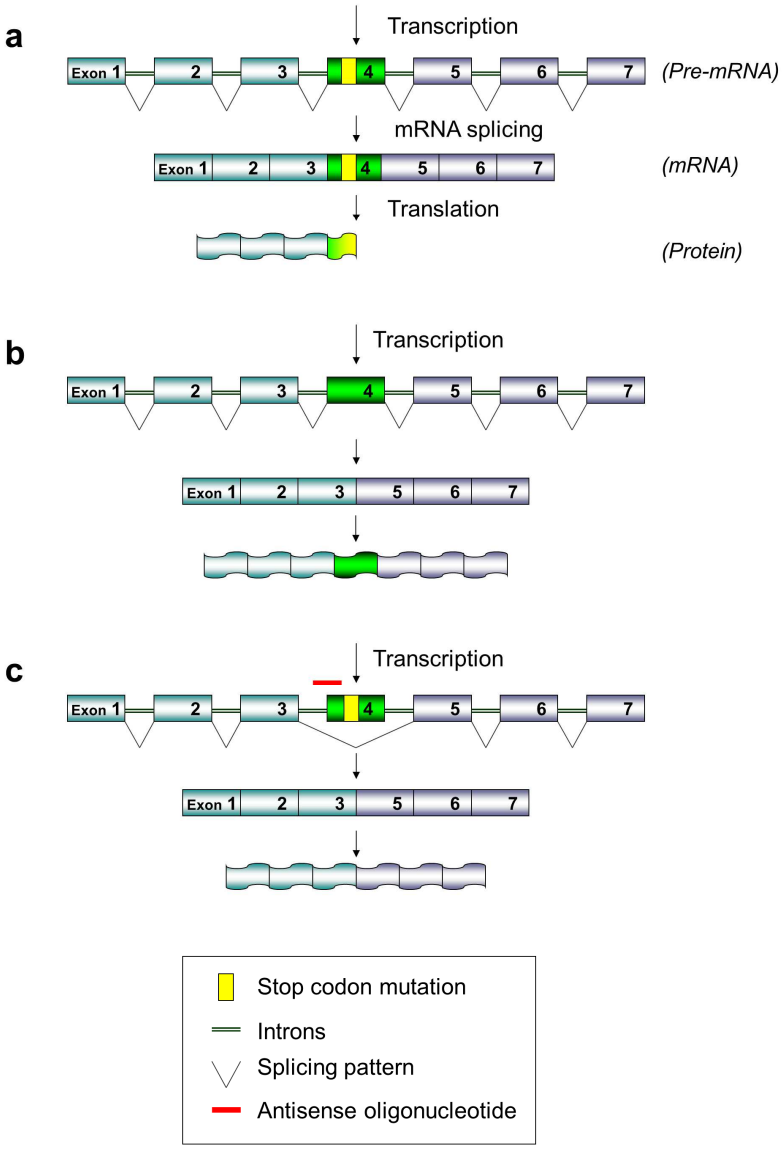
Nonviral gene transfer methods involve administration of plasmid DNA (pDNA) (Wolff et al., 1990), usually in complex with synthetic compounds. Although the approach is conceptually straightforward, less expensive compared to viral vector production, and avoids the inherent risks of viral vector administration, preclinical testing has not yet demonstrated a feasible approach for system-wide treatment of DMD. Upon intramuscular and intravascular

injection, naked dystrophin pDNA has not been shown to express in more than 5% of target myofibers (Zhang et al., 2004). Brief blood flow occlusion achieves targeting of the diaphragm and improves transgene expression in limb muscles following intravascular administration (Hagstrom et al., 2004; Zhang et al., 2004). Use of cationic polymers and lipids also improves in vivo tissue transfection after systemic administration (Richard et al., 2005). However, the possibility of acute toxicity of plasmid DNA-cationic polymer/lipid complexes must be resolved, especially considering potentially high dose systemic administration (Chollet et al., 2002; Trubetskoy et al., 2003). The size of the plasmids encoding full-length dystrophin may also impede DNA transfer through the vasculature (Richard et al., 2005; Zhang et al., 2004). While additional methods have improved transfection of striated muscle, clinically safe and efficient body-wide dystrophin expression in both skeletal and cardiac muscle has not yet been achieved. Still, a recent Phase 1 trial testing naked dystrophin pDNA by intramuscular injection resulted in low levels dystrophin expression (Romero et al., 2004), giving hope that development of a safe method to boost gene transfer will lead to a new DMD therapy.

Exon skipping

Exogenous delivery of dystrophin has shown promise for treatment of DMD, but limitations, such as vector capacity and delivery, potential immune response and appropriate transgene expression, still must be overcome. In the various forms of muscular dystrophy as well as other genetic diseases, mutations give rise to premature stop codons, and splice-site mutations, or deletions/duplications can shift the reading frame of a transcript or cause aberrant splicing, resulting in little or no functional protein.

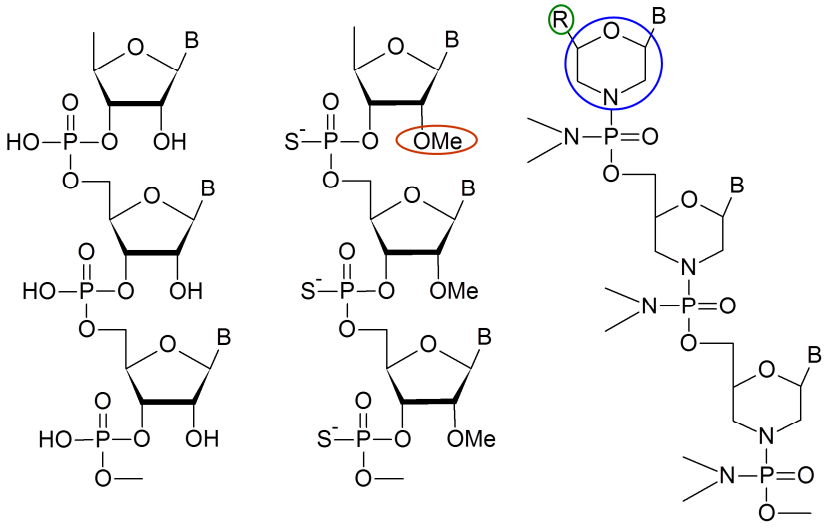
Exon skipping addresses these disorders at the post-transcriptional level, using the process of transcript maturation to remove problematic exons (**Figure 1.3**). Sites within the


a
Transcription
Exon 1
2
3
4
5
6
7
(Pre-mRNA)
mRNA splicing
(mRNA)
Translation
(Protein)
b
Transcription
c
Transcription
Stop codon mutation
Introns
Splicing pattern
Antisense oligonucleotide

newly synthesized pre-mRNA that signal the end of an exon are blocked, and the whole exon containing the stop codon or frameshift mutation, along with its flanking introns, are thereby removed. In the case of dystrophin, a multiexon deletion in the rod domain was found in mild and asymptomatic BMD (Monaco et al., 1988). Thus exons in this domain with a stop codon or frameshift mutation can be removed using exon skipping. Translation then generates an in-frame, albeit internally truncated, gene product that retains many critical functions. Studies of rare, dystrophin-positive 'revertant' fibers in some DMD patients first indicated that this might be a feasible therapeutic approach (Sherratt et al., 1993). The revertant fibers contained a restored dystrophin reading frame, although it remains unclear whether this was a result of secondary mutations or spontaneous alternative splicing (Partridge and Lu, 2008).

Synthetic antisense oligonucleotides (AONs) were developed for specific exons within dystrophin that can block transcript splicing sites in vitro (Dominski and Kole, 1993; Pramono, 1996; Takeshima et al., 1995). Most of the early work on AONs for therapeutic benefit was in the form of 2'-O-methyl-phosphorothioate AONs (2'OMe-AONs) (**Figure 1.4**), which are more stable than nonmethylated RNA (Shibahara et al., 1989). 2'OMe-AONs restore the reading frame of dystrophin in primary muscle cells from *mdx* mice (Dunckley et al., 1998). This was also achieved in cells from *cxmd* dogs (McClorey et al., 2006), human cells derived from DMD patients (van Deutekom, 2001) and in vivo, in *mdx* mice by intramuscular administration (Mann et al., 2001). Other muscular disorders have also successfully used exon skipping to correct abnormal gene expression. In myotonic dystrophy, AONs have been used to correct aberrant splicing in the chloride channel 1 gene (*CLCN1*) pre-mRNA and the associated myotonia (Wheeler et al., 2007), demonstrating the broad applications of this tool.

	RNA	2'OMe-AON	PMO
Ring linkage:	Phosphodiester	Phosphorothioate	Phosphorodiamidate
Modifications:		O-Methylation	CPM, Morpholine rings



AON chemistry

AON modifications have undergone many iterations, resulting in various chemistries affecting efficiency and delivery to cells in vitro and in vivo (Summerton and Weller, 1997). Morpholino oligomers show promise for in vivo applications (Yokota et al., 2009). Antisense morpholino oligomers, commonly called PMOs (for phosphorodiamidate morpholino oligomers), resemble 2'OMe-AONs, but have several key differences, as shown in **Figure 1.4**. PMOs incorporate morpholine rings in place of the ribose sugar rings of RNA and are nonionic, which avoids nonspecific electrostatic interactions within the cell. Replacement of the phosphodiester linkage with phosphorodiamidate imparts resistance to nuclease degradation (Summerton and Weller, 1997). Because PMOs are non-ionic, and therefore inefficient at penetrating cell membranes (Sazani et al., 2002), they are commonly conjugated to cell-penetrating cationic moieties (**Figure 1.4**) (Lebleu et al., 2008; Wu et al., 2009). Peptide nucleic acids represent another type of chemistry developed and tested in vivo (Ivanova et al., 2008). Aside from sequence specificity and efficient exon skipping, clinical considerations for AON chemistries must include the potential for toxicity or induction of a host immune response, biostability, penetration into striated muscle cells, and the cost of large-scale synthesis for systemic delivery.

AON administration

System-wide targeting of morpholino oligomers to skeletal muscle was initially achieved in the *mdx* mouse with no observed immune response or toxicity, but also without targeting of the heart (Alter et al., 2006; Fletcher et al., 2007) – outcomes observed previously with 2'OMe-AONs (Lu et al., 2005). Recently, groups have successfully targeted skeletal muscle

system-wide, as well as the heart in the *mdx* mouse, upon incorporation of peptide or chemical moieties to facilitate cell uptake (Aoki et al., 2012; Jearawiriyapaisarn et al., 2008; Wu et al., 2008; Wu et al., 2009).

AON design itself must be optimized for the target sequence within a given mRNA, because of the secondary structure in single-stranded RNA and splice signal sites (Popplewell et al., 2009). Indeed, this might explain why multiple AONs, or an oligo 'cocktail', seem to be more efficient than a single AON at targeting a single site (Mitrpant et al., 2009). Oligo cocktails could also be used to achieve multi-exon skipping (Aoki et al., 2012) and thereby cover many DMD mutations with a single treatment.

Another systemic approach for AONs uses rAAV vectors for intravascular delivery. rAAVs can be used to deliver DNA encoding an AON linked to a small nuclear RNA sequence for nuclear targeting of the transcript for modification of dystrophin (De Angelis, 2002). Treatments show therapeutic improvement in the *mdx* mouse, with reduced serum creatine kinase and improved skeletal muscle function (Denti et al., 2006; Goyenvalle et al., 2004). Tropism of certain serotypes also allows targeting of the heart (Denti et al., 2006). Another advantage of rAAV vector delivery of AONs is the persistence of rAAV episomes (Schultz and Chamberlain, 2008). Longer-term in vivo AON expression from episomes would be preferred over lifelong ongoing treatments with PMOs. For rAAV AONs, however, potential immune responses as well as scalability of vector production must also be considered.

AON safety

Although it is debatable which AON chemistry will be the most effective (Heemskerk et al., 2009; Mitrpant et al., 2009; Wu et al., 2008), it must be determined whether the most

effective are actually safe for human use. The various modifications require careful investigation of potentially harmful degradation products or co-delivery agents, immune responses, and nonspecific actions. Owing to inherent resistance to degradation, some morpholinos might be especially harmful if they have low specificity for RNA targets, and at high doses these effects could be amplified (Wu et al., 2012; Yokota et al., 2009).

Recent clinical trials show the promise of this approach as well as its challenges. Phase 1 and 2 trials with AVI-4658 (Eteplirsen) and PRO051 (GSK2402968) demonstrate that AONs can restore dystrophin expression and appear well tolerated at the doses tested and for repeated dosing regimens (Cirak et al., 2011; Cirak et al., 2012; Goemans et al., 2011; Kinali et al., 2009). Dystrophin restoration was somewhat variable and dose-dependent, as was restoration of the DGC and associated signaling proteins. For example, Kinali et al. (2009) show that intramuscular delivery restores dystrophin at higher levels (up to 79%) near the needle track, emphasizing the importance of systemic delivery for distribution, and by immunostaining they show highly variable dystrophin expression levels among positive fibers, at 11-42% of controls. Systemic delivery of PRO051via subcutaneous injection resulted in dose-dependent increases in levels and the percentage of fibers expressing dystrophin, but no improvement was found in muscle force or serum creatine kinase levels (Goemans et al., 2011). Discrepancies illustrate the importance of careful dystrophin quantification (Anthony et al., 2012). Results also highlight our extremely limited understanding of how individual dystrophin positive fibers contribute to force development.

Alternatives

Stop codon read through is an alternative approach that uses drugs to bypass mutations in dystrophin that create premature nonsense codons. Gentamicin is an antibiotic that binds the 40S

ribosomal subunit and promotes introduction of an amino acid at stop codons (Palmer et al., 1979; Singh et al., 1979; Yoshizawa et al., 1998). In *mdx* mice, gentamycin drives restoration of dystrophin expression in up to 20% of muscle fibers, with some protection from contraction-induced injury (Barton-Davis et al., 1999). However, the effectiveness of this approach has since been variable in animal models and human trials, in some cases resulting in little to no new dystrophin expression (Arakawa et al., 2003; Dunant et al., 2003; Malik et al., 2010; Politano et al., 2003; Wagner et al., 2001). Ataluren (PTC124), another read-through drug that binds to the 60S ribosomal subunit, has also shown promise in *mdx* mice, resulting in around 20% dystrophin positive fibers (Welch et al., 2007). Human clinical trial results showing functional improvement have yet to be published, though the drug appears well tolerated at doses tested (Beytía et al., 2012; Hirawat et al., 2007; Mendell et al., 2012; Pichavant et al., 2011). Potential disadvantages of read-through drugs include toxicity and low specificity leading to read-through of critical regulatory stop codons, though there is some indication that read-through is context dependent and thus more likely for nonsense codons than regular stop codons (Manuvakhova et al., 2000).

Direct gene repair methods are also under development. These employ endonucleases designed to create sequence specific double strand breaks within dystrophin near mutations or deletions. Double strand breaks are then repaired through nonhomologous end-joining that introduces a small insertion or deletion, or homologous recombination with plasmids that donate an intact version of the region (Chapdelaine et al., 2010; Rousseau et al., 2011). rAAV has also been used to introduce defined sequence changes via homologous recombination to a variety of cell types (Khan et al., 2011). This technology could be a useful alternative to lentiviral vectors for ex vivo genetic correction of cells prior to transplantation. However, novel endonucleases are

potentially antigenic, and strategies employing them should incorporate methods for ensuring only transient presence in target cells.

Cell Therapy for Muscular Dystrophies

Approaches

Cell transplantation as a therapeutic tool is a promising avenue for treatment of muscular dystrophies (**Figure 1.2**). Transplanted cells must be able to fuse with existing myofibers or form new ones, and transplanted cell nuclei within those myofibers must express the missing gene product. In addition to regeneration of myofibers, a major goal of stem cell therapies is reconstitution of the satellite cell niche. Satellite cells are the primary stem cell of skeletal muscles, such that their replacement might promote future functional regeneration of the muscle.

Cell transplantation might either be allogeneic (donor-derived cells) or autologous (patient-derived cells). Although allogeneic transplantation from a wild-type donor does not require genetic manipulation to reintroduce functional dystrophin, the risk for graft rejection remains. Autologous transplantation requires genetic manipulation, potentially by using lentiviral vectors to deliver functional dystrophin. Inherent risks in using lentiviral vectors apply, but the risk of immunogenic graft rejection is much lower. With either approach, expansion and culturing of the cell population will probably be necessary, requiring careful control of conditions to preserve muscle engraftment ability. For example, Gilbert et al.(2010) demonstrate the importance of substrates in preserving muscle stem cell properties. Cells cultured on substrate elasticities similar to that of muscle retained the ability to self-renew in vitro and had more extensive contributions to regenerating muscle than cells cultured on rigid plates or substrates with other elasticities.

An additional goal will be achieving appropriate disbursement of cells. Donor transgene influence is limited by diffusion distances along engrafted fibers (Blaveri et al., 1999; Mueller et al., 2002; van Putten et al., 2012). Therefore fusion of a single donor cell carrying dystrophin with each existing muscle fiber is insufficient. Fusion of multiple donor cells along the length of each fiber is required. Likewise, if primarily new muscle fibers are formed through fusion among donor cells, those fibers require appropriate distribution, in addition to obligatory innervation (Borisov et al., 2001; Harris, 2003). Regardless of whether myofibers are of host or donor, or mixed origin, the relationship between force development and dystrophin distribution is poorly understood, but clearly important. Studies that observe partial restoration of dystrophin expression suggest that dystrophin levels must be above a particular threshold in the majority of fibers to rescue function, or a particular proportion of fibers must highly express dystrophin (Chamberlain, 1997; Sharp et al., 2011; van Putten et al., 2012). In addition, cell-based therapy studies in mice have shown variability in recovery of function, and disbursement methods are likely a factor (Darabi et al., 2012; Gang et al., 2009; Mooney and Vandenburgh, 2008; Rousseau et al., 2010). Development of consistent transplantation methods will be critical for distribution of cells within target muscles. This may include intramuscular, local or systemic routes of administration. Thorough quantification methods will further this goal by permitting reliable engraftment comparisons among conditions.

An important issue will also be identifying an adequate cell source for scalable autologous transplantation in humans. Ultimately, the most promising approach for muscular dystrophy cell therapy will be the use of accessible patient-derived myogenic precursors capable of efficient engraftment into skeletal muscle following ex vivo expansion and genetic correction. A variety of cell types have been investigated for their potential to fulfill these criteria.

Cell types

The most widely studied cell type for muscular dystrophy cell therapy is the satellite cell and cultured daughter cells known as myoblasts. Transplanted myoblasts from wild-type donors engraft into skeletal muscle (Skuk et al., 2007a), but massive cell death, limited migration from injection sites and immune rejection of allogeneic cells is observed (Gussoni et al., 1992; Gussoni et al., 1997; Huard J et al., 1992; Karpati, 1993; Mendell et al., 1995; Montarras et al., 2005; Peault et al., 2007; Skuk et al., 2007b; Tremblay, 1993), even with genetic matching (Huard et al., 1992). Isolation and autologous transplantation of satellite cells might avoid host-versus-graft immune rejection. However, in later stages of muscle degeneration, fewer myogenic progenitors can be isolated, and expansion of cells in culture significantly reduces their engraftment capacity (Fan et al., 1996; Webster and Blau, 1990). Many recent approaches aim to improve muscle precursor cell isolation with the rationale of avoiding in vitro expansion to determine therapeutic potential (Montarras et al., 2005). Although this has yielded much greater understanding of satellite cell properties and engraftment, immune responses and the cost of human and animal antibodies commonly used in these isolation techniques limit clinical applications (Tremblay and Skuk, 2008). These studies could, however, potentially lead to new discoveries on how to maintain myogenic cell populations in a particular progenitor state in vitro (Collins et al., 2005; Deasy et al., 2002; Gilbert et al., 2010), and how to increase transplantation engraftment success. A further complication of these studies is that myogenic progenitors derived from muscle are typically a heterogeneous population, displaying unique properties depending on isolation methods and culture conditions (Cornelison, 2008). It is also unclear whether satellite cells themselves might derive from a satellite cell precursor within muscle or from a circulating progenitor (Collins et al., 2005).

Muscle-derived progenitors that can engraft in muscle after intravascular delivery have also been found; this is an important step for making cell-based therapies a feasible treatment for DMD. Such progenitor cells include muscle side-population cells (Bachrach et al., 2006; Gussoni et al., 1999) and cells isolated based on expression of specific markers, such as CD34, Sca1 (Lee et al., 2000) and CD133 (Benchaouir et al., 2007; Peault et al., 2007; Torrente et al., 2007).

Progenitors derived from bone-marrow or circulation have migrated and engrafted in muscle and show modest recruitment to muscle from the circulation in mice (Bittner et al., 1999; Ferrari et al., 1998). In dogs, however, Dell'Agnola et al. (2004) found that hematopoietic stem cell transplantation does not restore detectable dystrophin expression to skeletal muscle fibers. Enrichment, expansion, and directed differentiation of subsets of circulating cells may be required for effective therapeutic applications (Dezawa et al., 2005; Torrente et al., 2004).

The mesoangioblast (Sampaolesi et al., 2003) has shown impressive levels of engraftment and functional recovery in a mouse model of limb girdle muscular dystrophy (Sampaolesi et al., 2003) and in dystrophic dogs after arterial delivery (Sampaolesi et al., 2006). Mesoangioblasts have been suggested to derive from the pericyte, which is a microvessel-associated cell type (Dellavalle et al., 2007). To fully understand the clinical relevance and limitations of mesoangioblasts or pericytes in muscular dystrophy, it will be crucial to develop reproducible isolation and expansion methods for them and to better characterize their skeletal muscle regenerative capacity (Meng et al., 2011; Tedesco et al., 2012). Furthermore, reduced numbers of resident pericytes in dystrophic muscle may limit their use in autologous cell therapies (Tedesco et al., 2012).

Conversion of alternative cell types into myogenic precursors has recently become a plausible approach. Promising cell types that can be induced into the myogenic lineage include embryonic stem (ES) cells (Barberi et al., 2007; Darabi et al., 2008), embryonic and postnatal-derived fibroblasts (Kimura et al., 2008), and induced pluripotent stem (iPS) cells derived from adult fibroblasts and other cell types (Goudenege et al., 2012; Takahashi et al., 2007; Tedesco et al., 2012; Trokovic et al., 2012; Yu et al., 2007). Although ES cells have shown promise in development of cell therapies, some mouse and human ES cell lines might be predisposed to chromosomal abnormalities that could limit their application in cell replacement therapy (Catalina et al., 2008; Rebuzzini et al., 2008). iPS cells are generated by expression of a defined set of transcription factors. Once derived, they display characteristics that are remarkably similar to ES cells, including expression of ES cell markers, teratoma formation and the ability to contribute to generation of chimeric mice following injection into a blastocyst. Use of iPS cells avoids the ethical concerns and reduces the risk of immunorejection associated with human ES cells, and has shown promising therapeutic potential as a pluripotent stem cell source (Hanna et al., 2007). Patient-specific iPS cells could be derived from highly accessible and expandable fibroblast populations. However, the process of reprogramming and induction into the myogenic lineage must be streamlined for clinical feasibility, and carefully controlled to avoid the formation of tumors and more subtle immune complications (Zhao et al., 2011).

Note to Chapter

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Chapter 2

Myogenic Potential of a Dermal Cell Population

Introduction

In the autologous cell transplantation setting, fibroblasts have shown promise in a variety of tissues, including bone, neural, cardiac and skeletal muscle (Ieda et al., 2010; Kim, 2011; Kimura et al., 2008; Phillips et al., 2007; Vierbuchen et al., 2010). Importantly, dermal fibroblasts (dFbs) are readily accessible in patients, highly expandable in vitro after a minimally invasive dermal biopsy (Elder et al., 1996; Lortal et al., 2008), and possess increased mitotic activity and resistance to differentiation into myofibroblasts compared with fibroblasts from other tissues (van den Bogaerdt et al., 2002).

Fibroblasts of various origins can undergo myogenic conversion with addition of the basic helix-loop-helix skeletal muscle-specific transcription factor MyoD (Davis et al., 1987; Gibson et al., 1995; Huard et al., 1998; Lattanzi et al., 1998). However, forced expression of MyoD can lead to premature cell growth arrest and differentiation (Crescenzi et al., 1990; Lattanzi et al., 1998), thereby reducing the engraftment capacity of donor cells into host skeletal muscle. Improved control over the timing of myogenic differentiation has been achieved by MyoD activation systems, such as tetracycline-induced MyoD expression, and by the MyoD-estrogen receptor fusion protein that is transported into myonuclei in response to estradiol (Del Bo et al., 2001; Hollenberg et al., 1993). However, these systems are not ideal for the conversion of transplanted cells in vivo (Del Bo et al., 2001)**.** Recently, the discovery of estrogen receptor mutations allowing selective binding of the drug 4-hydroxytamoxifen (4OHT) have given rise to the MyoD-ER(T) fusion protein; the 4OHT-mediated inducible system provides greatly

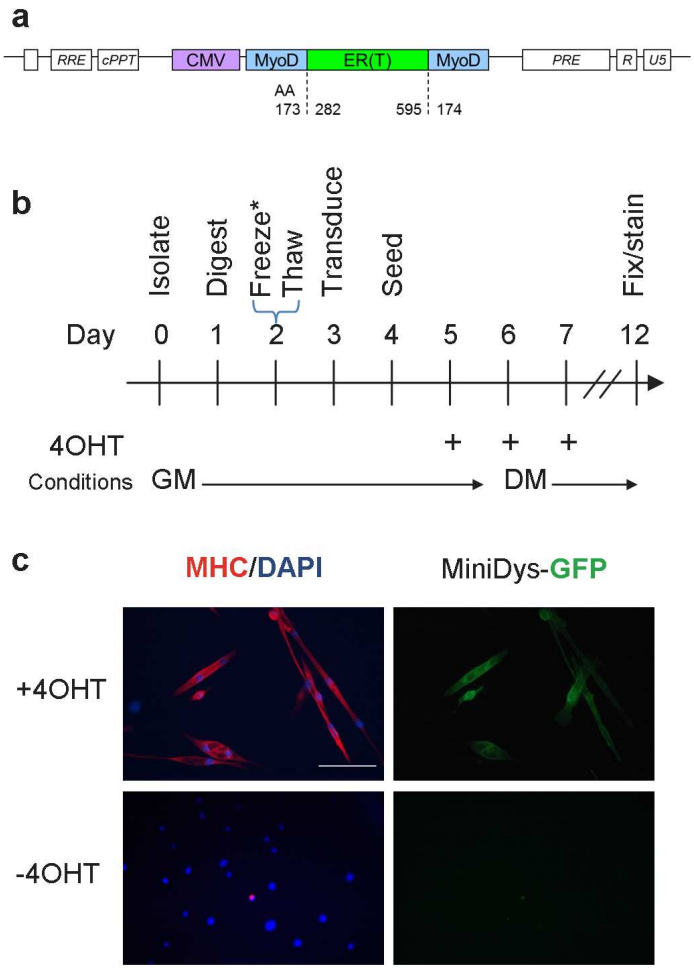
increased post-translational control of MyoD activity (Danielian et al., 1993; Goncalves et al., 2008b; Kimura et al., 2008).

The proof of principle for in vivo myogenic conversion of neonatal tail tip fibroblasts from *mdx*^{4cv} mice using the inducible MyoD-ER(T) system has been demonstrated (Kimura et al., 2008). This chapter describes the in vitro and in vivo properties of dFbs carrying a minidystrophin transgene as well as MyoD-ER(T) for inducible control of myogenic differentiation. This dFb population engrafts into dystrophic *mdx*^{4cv} muscles after expansion and in vivo myogenic conversion. Results suggest that autologous fibroblasts could serve as a robust source of myogenic cells for ex vivo cell therapy of DMD.

Results

Dermal fibroblasts efficiently convert into the myogenic lineage

To explore the myogenic conversion of dFbs isolated from *mdx*^{4cv} mice expressing minidystrophin we transduced cells isolated from whole skins of neonatal transgenic miniDys-eGFP/*mdx*^{4cv} (miniDys-GFP) (Li et al., 2006) mice with a lentiviral vector expressing MyoD-ER(T) (**Figure 2.1a**). Beginning 48 hours post-transduction, MyoD-ER(T) was activated by three daily additions of 4-hydroxytamoxifen (4OHT) to the culture media (**Figure 2.1b**). After several days, myosin heavy chain (MHC) and miniDys-GFP expressing cells were observed (**Figure 2.1c**). Converted dFbs were compared to converted 10T1/2 cells and MM14 myoblasts under conditions that promote differentiation (see Materials and Methods) (**Figure 2.2a**). A similar percentage of dFbs and 10T1/2 cells expressed MyoD, as detected by immunofluorescence, after transduction with MyoD-ER(T) (**Figure 2.2b**). Following 4OHT treatment, ~80% of dFbs became MHC$^+$, and ~50% fused into multinucleated myotubes, a roughly 2-fold greater efficiency compared to 10T1/2 cells (**Figure 2.2b**). In control MM14s, greater than 90% of differentiated cells were MHC$^+$, and ~80% fused into multinucleated myotubes. Similarly, when clones were evaluated for stable myogenic conversion, only ~30% of 10T1/2 clones were myogenic while nearly 80% of the dFb clones expressed MHC (**Figure 2.2c**). Importantly, >90% of the MHC$^+$ dFbs in high density cultures expressed the miniDys-GFP transgene (**Figure 2.1c**, **2.2d**). In the absence of 4OHT only ~0.3% of MyoD-ER(T) transduced dFbs became MHC$^+$, while cells not treated with 4OHT and not transduced displayed less than 0.1% MHC expression (**Supplemental Figure 1**). In dFbs not transduced with MyoD-ER(T), treatment with 4OHT resulted in ~0.1% MHC expression. These data illustrate the relatively high response of MyoD-ER(T)-expressing dFbs to myogenic conversion following 4OHT


a
RRE
cPPT
CMV
MyoD
ER(T)
MyoD
PRE
R
U5
AA
173
282
595
174
b
Isolate
Digest
Freeze*
Thaw
Transduce
Seed
Fix/stain
Day
0
1
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6
7
12
4OHT
+
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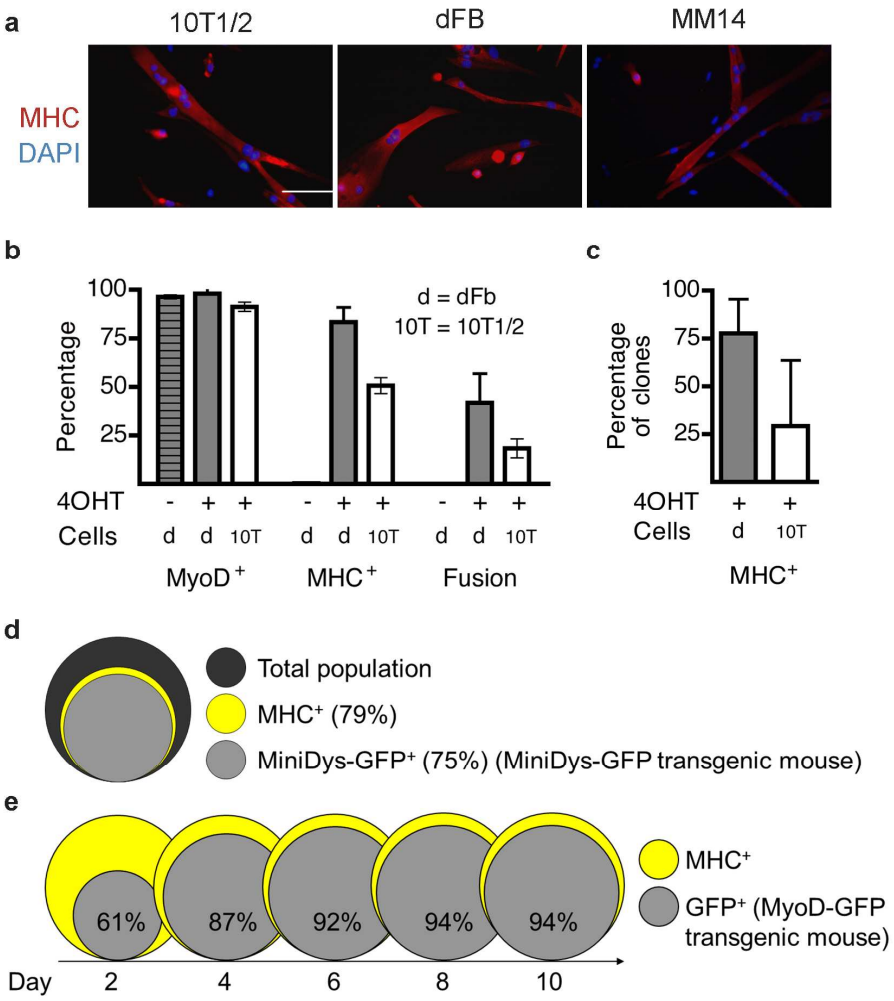
+
Conditions
GM
DM
c
MHC/DAPI
MiniDys-GFP
+4OHT
-4OHT


a
10T1/2
dFB
MM14
MHC
DAPI
b
Percentage
100
75
50
25
d = dFb
10T = 10T1/2
4OHT
Cells
MyoD+
MHC+
Fusion
c
Percentage of clones
MHC+
d
Total population
MHC+ (79%)
MiniDys-GFP+ (75%) (MiniDys-GFP transgenic mouse)
e
61%
87%
92%
94%
94%
Day
2
4
6
8
10
MHC+
GFP+ (MyoD-GFP transgenic mouse)

treatment, as well as the extremely low spontaneous myogenic conversion rates of non-transduced and MyoD-ER(T)-expressing dFbs in the absence of 4OHT.

To track myogenic conversion over time and the activation of MyoD expression in converting cells**,** we isolated dFbs from MyoD-GFP mice. As GFP expression in these mice is driven by MyoD regulatory elements (see Materials and Methods), we used GFP as a surrogate for endogenous MyoD activation. This dFb population was then transduced with the MyoD-ER(T) lentivirus and converted into myogenic cells. Differentiation was monitored for 10 days after initiating 4OHT treatment. By day 4, nearly 90% of the MHC^+ cells were also GFP^+, coincident with maximum culture density and peak conversion (**Figure 2.2e**). These phenotypes persisted for at least 10 days even though 4OHT treatment ceased on day 2. Since MyoD is known to have a short half-life (Thayer et al., 1989), these data support that sustained 4OHT treatment and MyoD-ER(T) activity are not required to maintain myogenic differentiation. Consistent with previous studies demonstrating MyoD auto-activation, presumably MyoD-ER(T) activates endogenous MyoD and a self-perpetuating myogenic program (Berkes and Tapscott, 2005; Thayer et al., 1989; Zingg et al., 1994).

To determine the minimum MOI of the MyoD-ER(T) lentivirus required to obtain optimal transduction and conversion in dFbs, cultures were transduced with various MOIs of MyoD-ER(T) lentivirus and separately transfected with a CK8e-luciferase plasmid as a means of detecting converted cells (see Materials and Methods). Normalized luciferase activity increased linearly for MOIs 1-10 (**Supplemental Figure 2**). At an MOI of 10, >90% of the cells immunostained positive for MyoD (**Figure 2.2b**), and qPCR indicated a population average of at least one MyoD-ER(T) lentiviral integration event per cell (**Supplemental Figure 3**). Transductions with MOIs between 10 and 100 led to similar levels of luciferase activity with

maximal expression levels attained between MOIs of 10-20, and decreasing levels at an MOI of 200. At MOIs of 100 and 200, I also noted that cell proliferation slowed dramatically compared with lower MOIs. Thus lentiviral transduction with an MOI of 10 is sufficient for expression of the MyoD-ER(T) and myogenic conversion.

Conditions affecting myogenic conversion

We additionally tested whether conditions that promote differentiation of myogenic cells also promote conversion of dFbs. Self-depletion of mitogens from the medium during conversion resulted in a higher percentage of MHC^+ cells than if cells were maintained in 2% FBS via medium change every two days. Concurrent treatment (through day 6 in **Figure 2.1b**) of 4OHT and 10 ng/ml bFGF (once per day), a known mitogen for both myoblasts and fibroblasts, resulted in at least twice the percentage of differentiated MHC^+ cells in dFb and 10T1/2 cells, but only when cultures were also periodically refed with medium containing 2% FBS (**Supplemental Figure 4**).

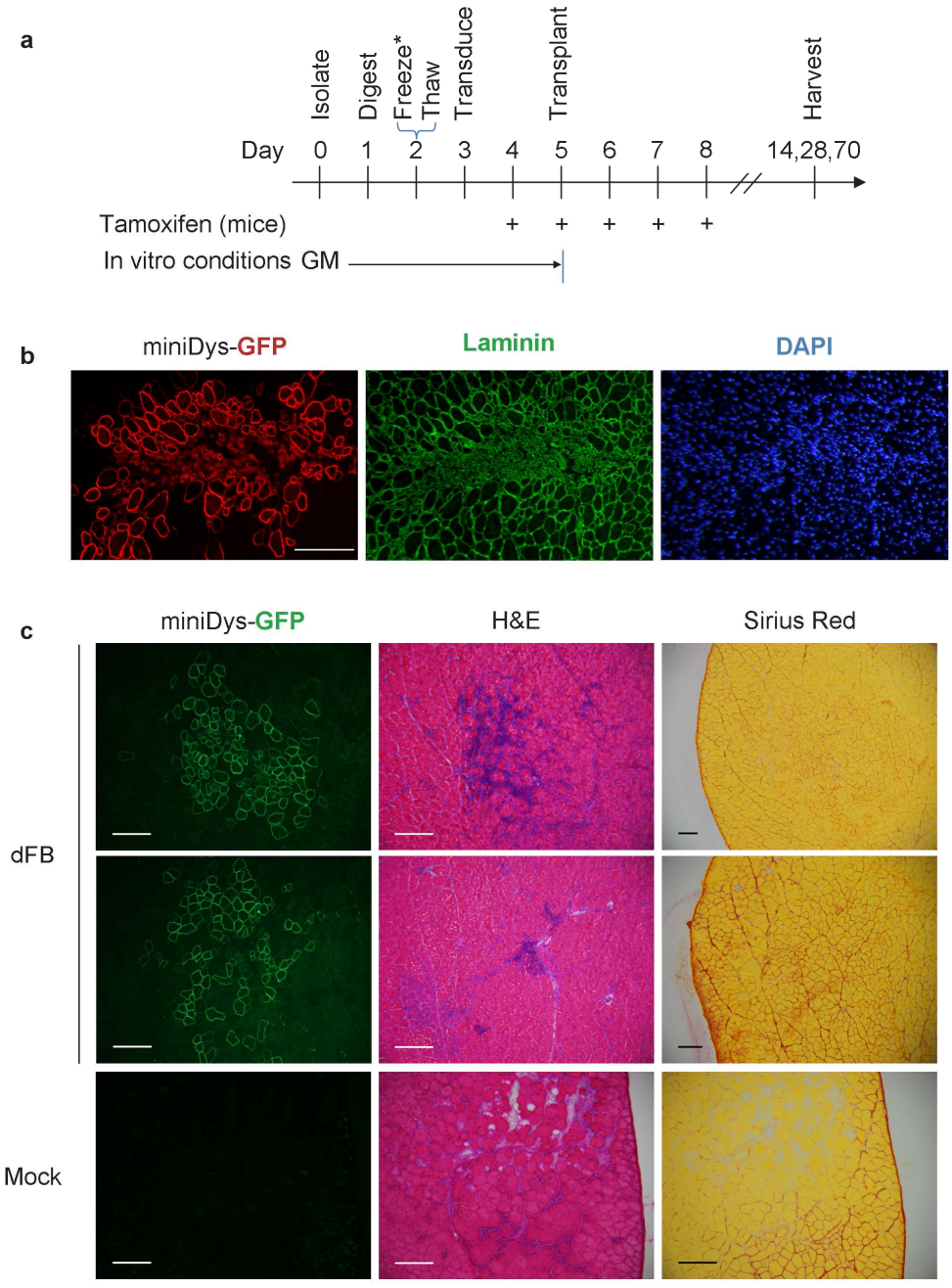
Since it is known that cell density and fusion into myotubes can influence myogenic differentiation of various cell types (Bhutani et al., 2010; Blau et al., 1983; Clegg and Hauschka, 1987; Pomerantz et al., 2009; Salvatori et al., 1995; Terranova et al., 2006), we explored these influences on dFb differentiation. We started with a seeding density that limited cell-cell contact and then increased cell density over a 20-fold range, and found that this range did not significantly affect myogenic conversion, and no relationship was found between local density of cells and the percentage conversion in a particular field (**Supplemental Figure 5**). Similar results were obtained with 10T1/2 cells (**Supplemental Figure 6**). This does not exclude the possibility of soluble factors subtly influencing reprogramming at these cell densities, but it suggests that conversion is not favored by direct cell contact.

Donor miniDys-GFP cells are not rejected in a syngeneic setting

Since the potential therapeutic strategy being investigated in this model system involves expression of an immunologically novel protein, we next asked whether donor cells carrying the miniDys-GFP transgene might be rejected by the *mdx*^{4cv} hosts. To avoid any potential effect of tamoxifen treatment on immune system function, we transplanted 5 x 10^5 mononuclear cells from whole muscle isolated from miniDys-GFP mice into tibialis anterior (TA) muscles of *mdx*^{4cv} hosts. TAs were harvested at 8 or 16 weeks and the total number of GFP$^+$ fibers were counted in multiple cross-sections throughout each transplanted muscle. For both time points GFP$^+$ fibers were retained, and the average number did not differ significantly between 8 and 16 weeks (**Supplemental Figure 7**). These data indicate that expression of the transgene was stable, and that miniDys-GFP$^+$ fibers were not cleared by the immune system of *mdx*^{4cv} hosts.

Transplantation of dermal fibroblasts does not lead to fibrosis

Given the known role of fibroblasts in formation of fibrotic lesions in dystrophic muscle (Bulfield et al., 1984; Natarajan et al., 2010; Serrano and Muñoz-Cánoves, 2010; Vidal et al., 2008) we examined whether transplantation of dFbs resulted in ectopic collagen deposition or structural abnormalities after in vivo myogenic conversion (**Figure 2.3a**). **Figure 2.3b** shows a typical cross-section with engrafted muscle fibers 4 weeks after transplantation and in vivo conversion of 5 x 10^5 miniDys-GFP donor dFbs into *mdx*^{4cv} TA muscles. Examination by hematoxylin and eosin staining revealed no gross abnormalities in muscle morphology with only occasional foci of mononuclear cells near engraftment sites (**Figure 2.3c**). These mononuclear cells may represent immune cell infiltration, a common feature in *mdx*^{4cv} mice (Hartigan-O'Connor et al., 2001), as similar concentrations of mononuclear cells were observed at locations outside the engraftment site in both injected and control sections. Importantly, no collagen


a
Isolate
Digest
Freeze*
Thaw
Transduce
Transplant
Harvest
Day 0 1 2 3 4 5 6 7 8 14,28,70
Tamoxifen (mice)
+ + + + +
In vitro conditions GM
b
miniDys-GFP
Laminin
DAPI
c
miniDys-GFP
H&E
Sirius Red
dFB
Mock

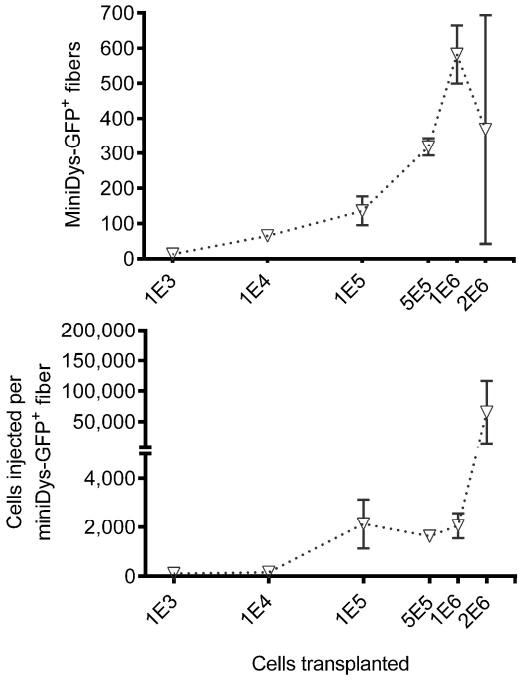
accumulation beyond control levels was detected at engraftment sites by Sirius red staining (**Figure 2.3c**).

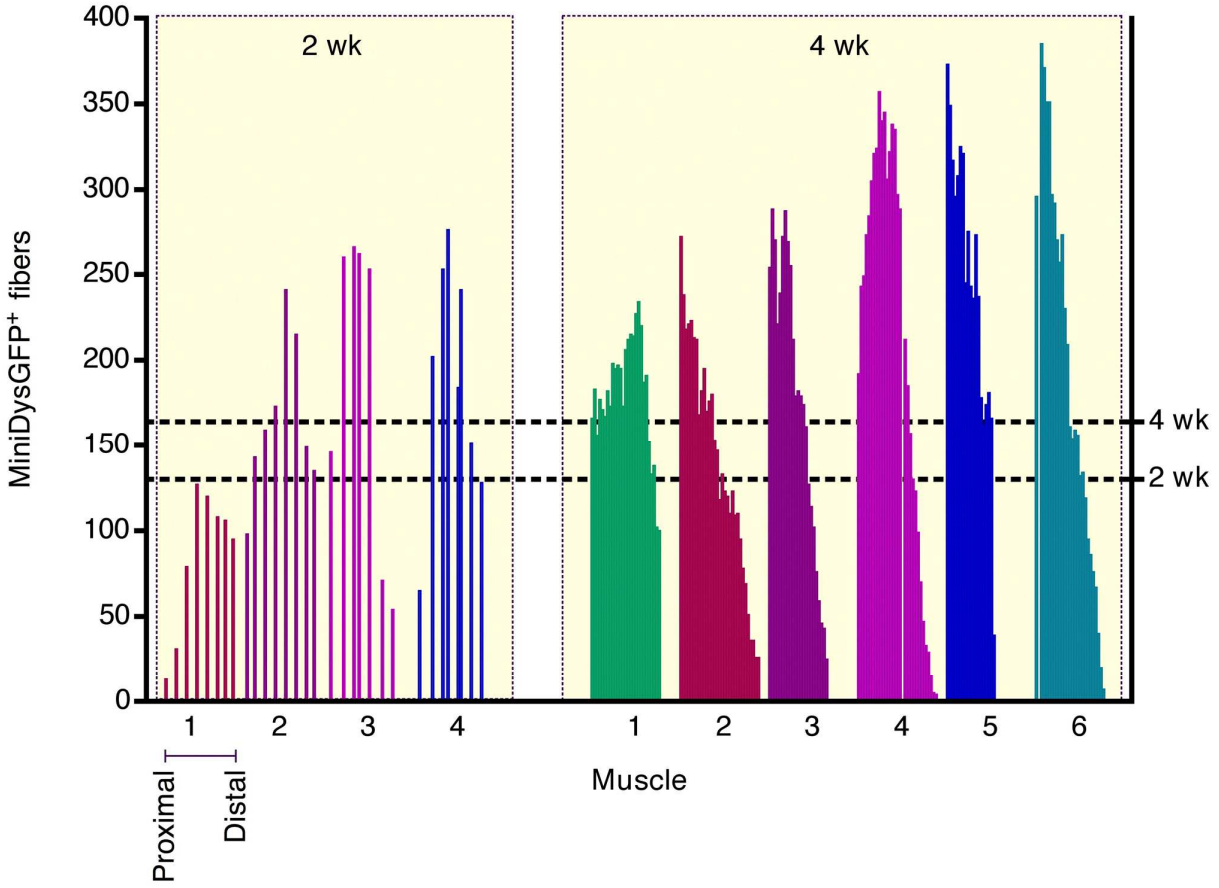
Engraftment vs. donor dFb cell dose

The efficiency of dFb cell engraftment in conjunction with in vivo conversion was determined by injecting graded doses of miniDys-GFP donor-derived dFbs into *mdx*^{4cv} TA muscles, with the in vivo conversion scheme shown in **Figure 2.3a**. Muscles were harvested 4 weeks after transplantation and total GFP$^+$ fibers from the single cross section containing the maximum number of GFP$^+$ fibers were analyzed (**Figure 2.4a**). The results indicate more GFP$^+$ fibers at higher dFb doses for up to 1 x 10^6 cells per transplant. The number of injected cells required to generate GFP$^+$ fibers attained a plateau level between doses of 1 x 10^5 to 1 x 10^6 cells, and then became orders of magnitude less efficient at 2 x 10^6 cells (**Figure 2.4b**).

Engrafted fiber distribution in host muscles

We next sought to quantify the extent of engraftment that could be obtained by dFb transplantation. For these studies 5 x 10^5 dFbs from miniDys-GFP donors were transplanted into recipient TA muscles as previously described (**Figure 2.5a**). At 2 and 4 weeks post-transplantation, injected muscles were harvested and cross sections were evaluated at approximately 0.5 mm and 0.1 mm intervals, respectively, throughout the muscle length (**Supplemental Figure 8)**. Each section was directly imaged for GFP$^+$ fibers, and these were counted and plotted corresponding to the respective proximal-to-distal location of each section (**Figure 2.5**). TAs harvested at both time points exhibited longitudinal distributions of miniDys-GFP$^+$ fibers, but at 4 weeks the average positive fiber numbers were ~20% higher compared with 2 weeks. In all sections tested, less than 10% of the total muscle cross-sectional area was


MiniDys-GFP+ fibers
700
600
500
400
300
200
100
0
1E3
1E4
1E5
5E5
1E6
2E6
Cells injected per miniDys-GFP+ fiber
200,000
150,000
100,000
50,000
4,000
2,000
0
1E3
1E4
1E5
5E5
1E6
2E6
Cells transplanted

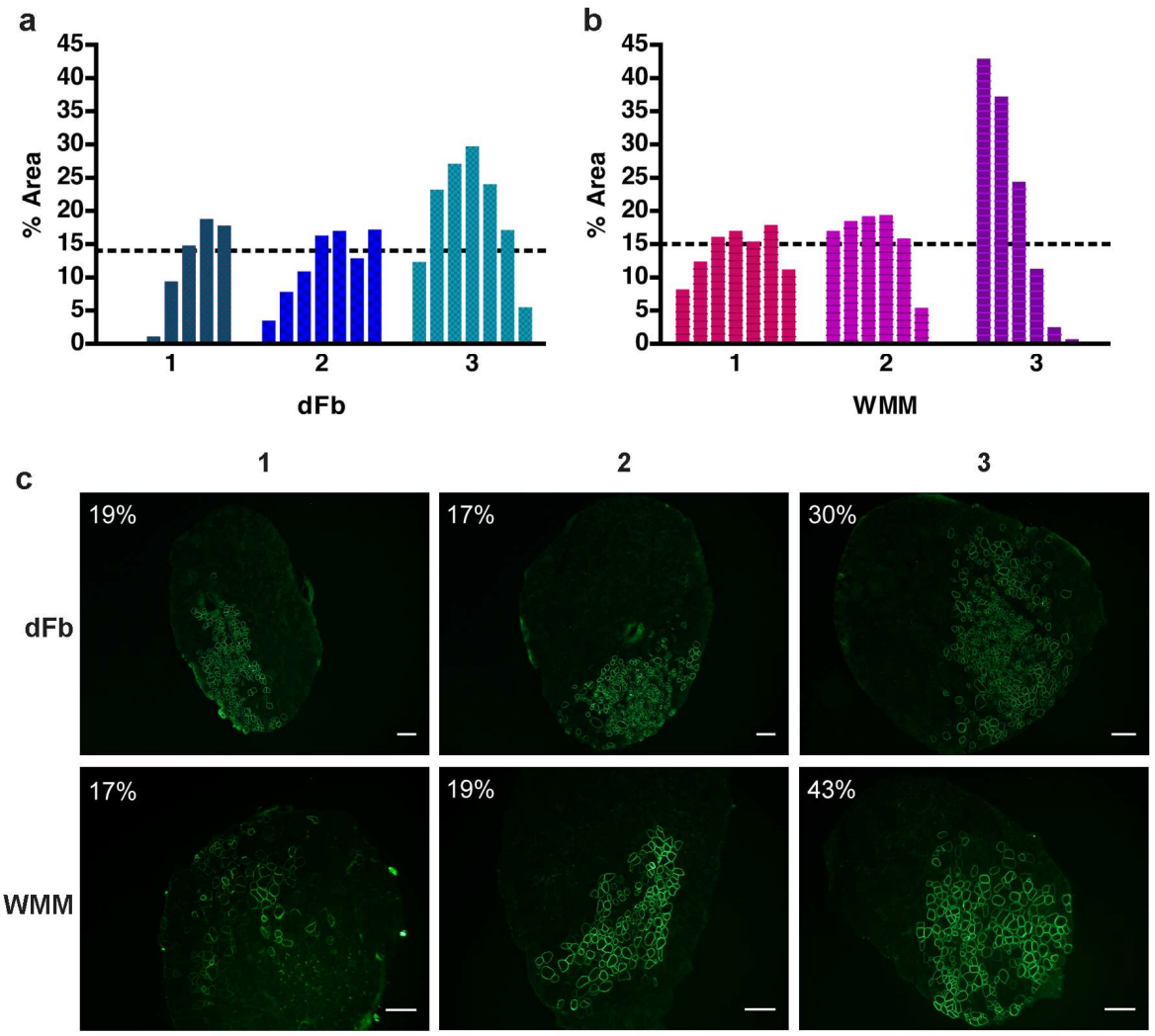

400
350
300
250
200
150
100
50
0
MiniDysGFP+ fibers
2 wk
4 wk
4 wk
2 wk
1
2
3
4
1
2
3
4
5
6
Muscle
Proximal
Distal

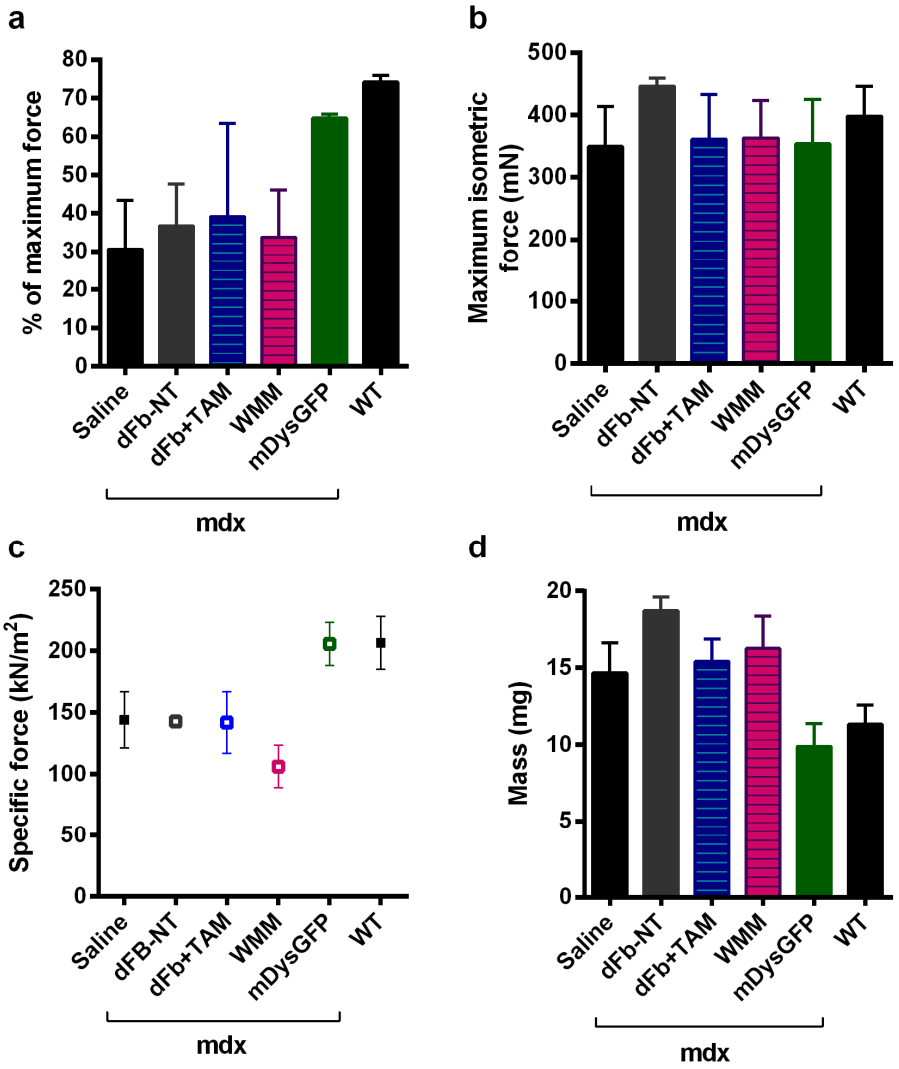
engrafted (**Supplemental Figure 9**). The percentage of engrafted area in each transplanted TA muscle was determined by comparing the engrafted area to the total muscle cross-sectional area.

To improve the percentage area engrafted and establish a system conducive to whole muscle functional evaluation, 5 x 10^5 dFbs from miniDys-GFP donors were injected into the much smaller extensor digitorum longus (EDL) muscle. Ten weeks after transplantation, injected EDL muscles contained cross sections in which 10 to ~30% of the muscle cross sectional area was occupied by miniDys-GFP$^+$ fibers, and the average percent engrafted area across all sections of all muscles was ~14% (**Figure 2.6a).** Importantly, the persistence of miniDys-GFP expression 10 weeks after dFb injection implies relatively long-term survival of muscle fibers engrafted by donor cells. Comparable engraftment was observed when 5 x 10^5 whole muscle mononuclear cells (which include satellite cells) were transplanted into EDL muscles (**Figure 2.6b**). The individual sections with the highest percentage area engrafted are shown for each transplanted EDL and cell type (**Figure 2.6c**).

Contractile properties after cell transplantation

We next tested whether transplantation of dFbs or WMM cells (both derived from miniDys-GFP donors) into dystrophic muscle improves force development or protects whole muscle from injury. Ten weeks after transplantation, EDL muscles were dissected and subjected to in vitro force measurements and an eccentric contraction protocol (see Materials and Methods). We note a slight trend toward protection from contraction-induced injury in the dFb+TAM condition, but no significant differences were observed (**Figure 2.7**a). We found no improvement in maximum isometric force or specific force (**Figure 2.7b, c**). The masses were equivalent in all injected *mdx*^{4cv} muscles (**Figure 2.7d**).


a
% Area
45
40
35
30
25
20
15
10
5
0
1
2
3
dFb
b
% Area
45
40
35
30
25
20
15
10
5
0
1
2
3
WMM
c
1
2
3
dFb
WMM
19%
17%
30%
17%
19%
43%


a
% of maximum force
80
70
60
50
40
30
20
10
0
Saline
dFb-NT
dFb+TAM
WMM
mDysGFP
WT
mdx
b
Maximum isometric force (mN)
500
400
300
200
100
0
Saline
dFb-NT
dFb+TAM
WMM
mDysGFP
WT
mdx
c
Specific force (kN/m2)
250
200
150
100
50
0
Saline
dFB-NT
dFb+TAM
WMM
mDysGFP
WT
mdx
d
Mass (mg)
20
15
10
5
0
Saline
dFb-NT
dFb+TAM
WMM
mDysGFP
WT
mdx

Discussion

Here, we investigated the therapeutic potential of using autologous dermal fibroblasts for treating dystrophic skeletal muscle. The model system involved the in vitro expansion of dFbs from miniDys-GFP transgenic mice, transduction with a lentiviral vector containing a constitutively expressed MyoD-ER(T) cDNA, injection of the transduced dFbs into host *mdx*^{4cv} TA or EDL muscles, and subsequent in vivo conversion of the transplanted dFbs via intraperitoneal injections of tamoxifen.

One clinical component of autologous cell-based transplantation DMD therapy not directly tested in the present study was the ex vivo transduction of donor dFbs with a dystrophin expression cassette. Rather, we used myogenically convertible *mdx*^{4cv} dFbs that already carried a miniDys-GFP transgene, in order to focus attention on the myogenic conversion aspect of the protocol without confounding the multiple variables pertinent to this component with additional variables involved in the simultaneous transduction of dFbs with a muscle-specific minidystrophin expression cassette. The technical parameters of this essential portion of the treatment protocol are being investigated in on-going studies. However, dual lentiviral transduction was previously demonstrated in tail tip fibroblasts with microdystrophin-GFP and MyoD-ER(T) (Kimura et al., 2008).

Importantly, the present study avoided pretreatment of recipient muscles with toxins and irradiation, two common protocols that initiate extensive regeneration and can eliminate host satellite cells (Harris, 2003; Morgan et al., 1990) as neither of these procedures would be applicable in a clinical DMD setting. Additionally, myogenically convertible donor dFbs were transplanted into immunocompetent, syngeneic mice in order to mimic the autologous immune system setting that would be encountered in DMD treatment.

A key aspect of the current studies involved establishing that dFbs carrying MyoD-ER(T) could efficiently convert into myogenic cells in vivo following tamoxifen treatment. We first confirmed that in the presence of 4OHT, MyoD-ER(T) efficiently converts dFbs into the myogenic lineage in vitro, as measured by the percentage of nuclei in MHC^+ cells following differentiation. Lentiviral delivery of MyoD-ER(T) at MOI 10 - 20 is effective for myogenic conversion, and additional vectors, while possibly maximizing MHC^+ cells, are unnecessary. Additionally, minimizing the essential vector number decreases the probability of insertional mutagenesis and reduces the potential for premature differentiation due to MyoD overexpression. During conversion, cells expressed myogenic genes and became responsive to factors known to influence differentiation of myoblasts, such as mitogen deprivation. Additionally, the increase in percentage of MHC^+ cells with addition of bFGF in vitro suggests that FGF treatment differentially affects the converting population, possibly due to enhanced survival, selective mitogenic stimulation, or other signaling effects (Seed and Hauschka, 1988; Tortorella et al., 2001). The dFb conversion process does not appear to depend on cell contact and fusion (**Supplemental Figure 5**). Thus in the transplantation setting, where a variety of cell types and environmental variables are present, dFbs should have no specific requirement for close contact with myogenic or other converting cells to convert into the myogenic lineage.

Our studies suggest that the optimal cell transplantation range for dFbs injected longitudinally into the TA muscles of an adult mouse is between 1×10^5 and 1×10^6 cells in 20 ul (**Figure 2.4**). Interestingly, these cell numbers/concentrations are similar to what has been reported for myoblast transplantations into even much larger non-human primate muscles, where 1×10^6 cells or greater injected at a single site resulted in increasing proportions of central necrosis (Skuk et al., 2007b). While the contribution of transplanted dFbs to dystrophin-positive

fibers indicates their in vivo tamoxifen-mediated conversion into the myogenic lineage, an unknown proportion of cells may remain as fibroblasts. Potentially low in vivo conversion efficiency could interfere with whole muscle function or lead to fibrosis; however, histological examination of dFb-engrafted sites indicated no gross morphological abnormalities or lesions in the recipient dystrophic muscle beyond that which is characteristic for the dystrophic host (**Figure 2.3**). The absence of ectopic connective tissue in the current study is in contrast to observations in a pioneering MyoD-converted fibroblast study (Huard et al., 1998). This may be due to the earlier study's inclusion of notexin injection and irradiation of host limbs prior to transplantation, and to the injection of the dFbs into many regions rather than as a single dispersed cell injection.

When the distribution of engrafted fibers was observed in TA muscles 2 and 4 weeks after transplantation, the number of engrafted fibers was slightly higher at 4 weeks (**Figure 2.5**). This may be due to increased stability of mature engrafted fibers or to further contribution from differentiating converted cells during the intervening two weeks. Analogous studies of EDL muscles 10 weeks after single longitudinal intramuscular injections indicated engraftment levels approaching 30% of the total area in cross sections through the best transplants (**Figure 2.6**). The percent engraftment data and relatively restricted area occupied by miniDys-GFP^{+} fibers following the in vivo conversion and differentiation of transplanted dFbs (**Figure 2.6**), also suggests that dFbs, like myoblasts, do not migrate throughout the muscle following their injection. The greater area of engraftment in EDL compared to TA muscles, coupled with the longitudinal distribution of engrafted cells throughout the entire muscle, suggests that EDLs may provide a better platform for whole muscle physiology studies in this setting.

Comparison of whole muscle EDL function following transplantation of WMM or in vivo-converted dFb reveals that the cells do not protect against contraction induced injury (**Figure 2.7**). We also observe no improvement in maximum isometric force, specific force, or mass among injected dystrophic muscles compared to wild type and transgenic mice carrying functional dystrophin. Surprisingly, the specific force for WMM transplanted EDLs was slightly lower than the other injected muscles. The reason for this finding is unclear, though we note that this is a heterogeneous population for which myoblasts or myogenic progenitors are not selected or sorted.

During engraftment, donor cells likely contribute to existing fibers and form de novo fibers during the healing and regeneration process. Thus compared to non-cell gene transfer methods, cell-based therapy alone may require more time to achieve optimal transgene expression and structural maturity of muscle fibers. We find that whole muscle function of donor transgenic miniDys-GFP mice is similar to that of wild type mice, therefore the donor transgene is capable of proper expression and localization (**Figure 2.7**). However, unlike *mdx*^{4cv} host mice, miniDys-GFP transgenic mice have not been continually affected by dystrophy. Thus we cannot rule out a potential ceiling for functional benefit in *mdx*^{4cv} mice due to permanent changes in dystrophic muscle. In addition, gross structural characteristics of dFb and WMM engrafted muscles were highly similar and showed no engraftment site lesions in TA or EDL muscles (**Figure 2.3**). It is unlikely that unconverted dFb interfere, as muscles do not show a decline in muscle function in non-tamoxifen treated controls. We consistently observed, however, regions of very small miniDys-GFP^{+} fibers after transplantation of either cell population (**Figure 2.3b**) that could indicate fiber immaturity or branching that affects function (Chan et al., 2007; Lovering et al., 2009b). Furthermore, if miniDys-GFP^{+} fibers are the result of fusion among

donor cells only, there is the possibility that they lack innervation, which can affect maturity or survival (Borisov et al., 2001; Harris, 2003). In our EDL contractile assay, this could obscure improvements that resulted from successful transgene delivery. Additional studies with longer time points may provide insight on the fate of such fibers.

The most compelling explanation for our physiology results is that minidystrophin contributed by donor cells, regardless of origin, does not reach enough of the muscle to improve force or protect from injury. Transplantation of myogenically converted dFbs and WMM cells each achieved an average of 14% engraftment across EDL muscles. Previous studies using transgenic animal models, virus-mediated gene transfer, or exon skipping support that a certain minimum number of fibers (20%) must have moderate levels of dystrophin protein, or that the majority of fibers must have dystrophin levels at a minimum of 20% of wild type levels to confer benefit to whole muscle (Chamberlain, 1997; Sharp et al., 2011; van Putten et al., 2012).

Thus I wanted to test the hypothesis that functional benefit should be provided if 20% of the total muscle area is engrafted by fibers highly expressing dystrophin delivered via dFbs. Furthermore, accurate quantitation of engraftment is critical for predicting whether it is realistic to expect functional recovery. Even when regions of high fiber number and high area of engraftment are achieved, these experiments show that they are insufficient for therapeutic benefit. Fiber number and area analysis with consistent sampling throughout a transplanted muscle is necessary (**Figure 2.5**, **2.6**). In addition, it may be important to achieve the highest levels of transgene expression at sites that experience the highest stress during force development (Lovering et al., 2009a).

Materials and Methods

Primary cell isolation

Dermal cell populations were isolated from whole skins of 1-3 day old neonatal mice. Two to four pups were anesthetized on ice before cervical dislocation, transferred to a sterile environment, immersed in Betadine solution (Purdue Products L.P., Stamford, CT) twice for 2 minutes each, rinsed in sterile deionized water, immersed in 70% ethanol twice for 2 minutes, then immersed in sterile phosphate-buffered saline (pH 7.2) (Gibco, Grand Island, NY), with penicillin/streptomycin (Gibco) (PBS-P/S) until dissection. The dermal skin layer was isolated as described (Lichti et al., 2008). Briefly, limbs and tail were removed and the epidermis and dermis were carefully detached from the remaining tissue. Blood vessels or fat adhering to these layers was removed. Skins were then carefully floated dermis side down in 0.25% Trypsin-EDTA at 4°C overnight (~16 hours). The next day, skins were laid epidermis side down and dermis was lifted away from epidermis with fine forceps, rinsed with PBS-P/S, minced lightly and digested in 0.2% collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ), 1.2 U dispase II (Worthington), and PBS-P/S in 5 ml total for 45-75 minutes at 37°C, with trituration by 10 ml serological pipette every 15 minutes. Cells were centrifuged at 300 x g for 5 minutes, resuspended in growth medium, which consisted of DMEM (Gibco) with 10% fetal bovine serum (Thermo Scientific/HyClone, Logan, UT), 1% P/S, and 2 mM L-glutamine (Gibco), and plated out at sub-confluent density in growth medium with 10 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems, Inc., Minneapolis, MN. Cells were trypsinized and cryopreserved in growth medium with 10% DMSO 24-48 hours later. This heterogeneous dermal cell population, referred to here as dermal fibroblasts (dFbs), was used for all in vitro and in vivo conversion experiments. Extremely low spontaneous myogenic conversion rates were

found for non-transduced and MyoD-ER(T)-expressing dFbs in the absence of 4OHT (**Supplemental Figure 1**).

Whole muscle mononuclear cells were isolated from 6 week old miniDys-GFP transgenic mouse hindlimb and diaphragm muscles as described previously (Li et al., 2006). Briefly, muscles were dissected out and the majority of visible connective tissue removed, followed by mincing and 1 hour digestion at 37°C in PBS-P/S with 0.2% collagenase IV and 1.2 U dispase II, with trituration by 10 ml serological pipette every 15 minutes. Tissue debris was filtered using 70 and 40 µm cell strainers and cells were centrifuged and resuspended in 154 mM NH_4Cl red blood cell lysis buffer for 5 minutes at room temperature, followed by addition of PBS-P/S to a total volume of 30 ml. Cells were centrifuged again and resuspended in Ham's F10 medium (Gibco) with 15% horse serum (Thermo Scientific/Hyclone), 1 mM $CaCl_2$, 1% P/S and 0.5 µg/ml bFGF, counted, adjusted to 25,000 cells/ul and kept on ice for 30 minutes to 3 hours until transplantation.

Drug preparation and dosage

Tamoxifen (Sigma-Aldrich, St. Louis, MO) was equilibrated to room temperature, then 65°C preheated corn oil (Sigma-Aldrich) was added to the dry stock bottle at 100 mg/ml, and the bottle was then alternately heated to 56°C and vortexed frequently for several hours or until most of the crystals were dissolved. The solution was then transferred and diluted to 40 mg/ml in preheated corn oil, and the heating/vortexing procedure was repeated until no crystals were visible. The tamoxifen preparation was then filter sterilized and aliquoted for storage at -20°C. Working solutions were prepared by diluting aliquots to 20 mg/ml in sterile corn oil, and all mice were treated with a dose of 100 mg/kg per day by intraperitoneal (IP) injection for 5 days, beginning one day prior to cell transplantation. Vehicle-treated mice were IP injected with an

equal volume of sterile corn oil. For in vitro studies stock 4OHT (Sigma-Aldrich), the active form of the drug tamoxifen, was diluted to desired concentrations with 95% ethanol.

Construct and lentivirus preparation

The inducible MyoD construct (Kimura et al., 2008) included mouse MyoD and mouse ER(T) sequences driven by the cytomegalovirus (CMV) promoter (GenBank #AF369966.1) within a lentiviral vector backbone (Barry et al., 2001) as shown in **Figure 2.1a.** All recombinant DNA work was performed following guidelines for biosafety and containment measures provided by the University of Washington Institutional Biosafety Committee and the National Institutes of Health.

VSVG pseudotyped self-inactivating (SIN) lentiviruses were generated as described (Li et al., 2005). Briefly, MyoD-ER(T) in a lentiviral transfer plasmid was co-transfected, along with three viral plasmids, by calcium phosphate precipitation into approximately 80% confluent human embryonic kidney 293D cells on 150 mm plates. Growth medium was refreshed 16 hours after transfection. Cells were cultured for an additional 48 hours and supernatant was collected. Viral supernatant was centrifuged at 400 x g for 10 minutes at 4°C to remove cell debris, filtered once with a 0.45 um SFCA filter unit, once with a 0.20 um SFCA filter unit, then concentrated by centrifugation at 50,000 x g in a Beckman L8-70M ultracentrifuge for 2 hours at 4°C. Supernatant was aspirated and virus gently resuspended in small volumes of PBS. Titering was carried out by transduction of NIH-3T3 cells with serial dilutions of vector preparations. After culturing for one week, proviral integration was evaluated by TaqMan real-time PCR (Applied Biosystems, Foster City, CA) of a virally packaged portion of the lentiviral transfer plasmid, and normalized to genomic DNA copies of the low density lipoprotein receptor to determine infectious units per ml of concentrated virus.

Culture conditions

Growth medium (GM) for fibroblasts was DMEM, 10% FBS, 1% P/S, and 2 mM L-glutamine. GM was supplemented with 10 ng/ml bFGF once per day. Differentiation medium (DM) was equivalent to GM except with 2% FBS. Dermal cells were thawed and expanded in GM with 10 ng/ml bFGF for one day prior to transduction with the lentiviral vector carrying MyoD-ER(T). Cells were transduced at a multiplicity of infection (MOI) of 10, or 10 infectious units per cell unless otherwise stated, in the presence of 8 ug/ml polybrene in 1 ml GM for 10 minutes, followed by plating of the cell/virus solution at 30 - 60% confluency in 10 ml GM per 150 cm plate. Transduction at other MOIs was performed under identical conditions. Transduced cells were either plated for conversion experiments the next day or transplanted within five days so that cells were used for myogenic conversion within nine growth days of initial isolation (**Figure 2.1**, **2.3a**). For in vitro myogenic conversion of fibroblasts, unless otherwise specified, cells were plated at a density of 20,000 cells per well in 6-well plates, and the next day treated with 4OHT at 2 uM or the same volume of 95% ethanol in growth medium.

10T1/2 mouse embryonic fibroblasts and MM14 mouse myoblast (Linkhart et al., 1980) cell lines were used for comparison to dFb conversion. 10T1/2 cells were treated with a conversion protocol identical to that used with dFbs, and MM14 myoblasts were grown in identical growth medium to whole muscle mononuclear cells, including 5 ng/ml bFGF, then differentiated by allowing nutrients in cultures to self-deplete as a positive control for dFb in vitro differentiation.

Mice

Several different transgenic mouse lines were used in these studies. The *mdx*^{4cv} mice have a point mutation resulting in a stop codon in exon 53 of the dystrophin gene, on the C57Bl/6

background (Chapman et al., 1989), and were used as hosts in all transplantation experiments. MiniDys-GFP/*mdx*^{4cv} mice (miniDys-GFP) contain a minidystrophin-eGFP fusion protein cDNA driven by human alpha-skeletal actin regulatory elements on the *mdx*^{4cv} background (Li et al., 2006). I bred these mice to homozygosity to guarantee maximal transgene expression in donor cells, and ensure that dFb populations (containing pooled cells from whole litters) were isolated from all transgene positive pups. Homozygosity was confirmed by test crossings to wild type mice across multiple generations. The MyoD-GFP mice contain a GFP cDNA driven by a 24 kb regulatory sequence upstream of the MyoD gene, on an enriched FVB background (a kind gift from Z. Yablonka-Reuveni, University of Washington) (Kirillova et al., 2007). Mice were developed in the laboratory of D. Goldhamer, using the same approach as in Chen et al. (2001) for the MyoD-LacZ mouse. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Transplantations

Cells were transplanted by open skin intramuscular injection into tibialis anterior (TA) or extensor digitorum longus (EDL) muscles in a single injection along the length of each muscle. dFbs were prepared for transplantation by dilution to desired concentrations in identical medium to the whole muscle mononuclear cells, kept on ice for 30 minutes to 3 hours, and aspirated into a 25 ul Gastight Hamilton syringe equipped with a 32 gauge needle. By hand, needles were inserted into the distal ends of each muscle, about 2 mm from the tendon, and pushed longitudinally through the muscle to about 3 mm from the proximal tendon. Injections were performed concurrently with needle withdrawal at a rate of about 2 ul/second. One or both legs were injected with cells, and control legs were injected with saline. Mice were anesthetized with inhaled 1-5% isoflurane in oxygen and treated with standard post-operative care.

Cell and tissue processing and analysis

Antibodies used for immunofluorescence included mouse anti-myosin heavy chain (MF20, Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-GFP (A-11122, Invitrogen/Molecular Probes, Grand Island, NY), rabbit anti-MyoD (M-318, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and rat anti-laminin (MAB 1914, Millipore/Chemicon, Billerica, MA For in vitro studies, cells were fixed for 10 minutes in 2% paraformaldehyde with 1.5% sucrose at room temperature prior to staining. For transplantation studies, muscles were dissected out and frozen in Optimal Cutting Temperature Compound by floating on liquid nitrogen cooled isopentane. Two 20 µm thick transverse cryosections were mounted on glass slides every 100 µm throughout each transplanted muscle (**Supplemental Figure 8**). Immunofluorescence was performed on unfixed sections, and hematoxylin and eosin and Picrosirius Red staining were performed on methanol and unfixed sections, respectively. GFP^+ fibers were counted following direct imaging of GFP after confirmation of colocalization of the direct GFP signal with the signal detected by immunofluorescence using the anti-GFP antibody. Percentage area calculations for EDL transplantations were performed using Image J software. The area of the engrafted site was obtained using the software's region of interest feature to outline the outer edges of the region that contained GFP^+ fibers, and inputting scale information into the software. The same procedure was used to determine total area, by outlining the entire EDL cross section. The outlined engrafted area values were then divided by the outlined total area values to determine the percentage engrafted area. Note that EDLs with an individual average across all sections of <10% area were considered negative and excluded from this comparison.

Luciferase assay

A firefly luciferase cDNA driven by modified enhancer and promoter regulatory elements from the mouse muscle creatine kinase gene (CK8e) was subcloned into the polylinker of a self-inactivating lentiviral vector backbone (Barry et al., 2001; Li et al., 2005) and used for all luciferase experiments. The CK8e regulatory cassette is expressed only in differentiated skeletal and cardiac muscle cells (Q.G. Nguyen and S.D. Hauschka et al., manuscript in preparation). dFbs were transduced with the lentiviral vector carrying the MyoD-ER(T) cDNA as described in the conversion conditions section, transfected with the CK8e-luciferase cassette by calcium phosphate precipitation, and converted into the myogenic lineage as in **Figure 2.1b**. Cells were lysed using Cell Culture Lysis Buffer for luciferase assays or Reporter Lysis Buffer for protein quantitation (Promega, Madison, WI Lysates were evaluated using the Promega Luciferase Assay System and a Victor^3 V plate reader, with automatic pump dispensing of Luciferase Assay Reagent (Promega). The Thermo Scientific Pierce Coomassie Plus Assay Reagent was used for total protein quantification on the Victor^3 V.

Physiology

Mice were anesthetized with an initial dose of 300 mg/kg Avertin, followed with additional doses necessary to maintain deep anesthesia for the duration of the procedure. Contractile properties were measured as described (Li et al., 2006). Briefly, proximal and distal EDL tendons were secured with 5-0 silk suture in situ, after which intact muscles were dissected and one tendon was tied to the lever arm of a servomotor and one to a force transducer for whole muscle physiology. Muscles were maintained in a 25°C bath of buffered mammalian Ringer's solution (121 mM NaCl, 5 mM KCl, 50 µM $MgCl_2 \cdot 6H_20$, 40 µM NaH_2PO_4, 24 mM $NaHCO_3$, 1.8 mM $CaCl_2 \cdot 2H_20$, 5.5 mM glucose), bubbled with 95% O_2/5% CO_2. One test tetanus at low

frequency (100 Hz) was performed after securing the muscle onto the rig to check viability and suture knot stability. Muscle length was adjusted to optimal length (L_o) for force development. Muscles were stimulated with a pulse duration of 2 ms, and a voltage that produced maximum twitch force. A stimulation frequency of 180 Hz for EDL muscles, with 300 ms duration, gave the maximum isometric tetanic force (P_o). The susceptibility of muscles to contraction-induced injury was assessed by six lengthening contractions. The muscles were set at L_o, activated maximally, and then stretched through a strain of 30% of L_o at a velocity of 1 fiber length/s and then returned at the same velocity to L_o, allowed a 10 s recovery period, then exposed to subsequent stretches of 30% each. Next, muscles were removed from the bath, trimmed to remove sutures and tendons, weighed, and frozen in liquid nitrogen cooled isopentane. Cross-sectional area (CSA, cm^2) was calculated based on the measurements of optimal muscle length (mm), muscle mass (mg), a muscle density of 1.06 g/cm^2 and a fiber length to optimal length ratio of 0.44. The specific P_o (kN/m^2) was determined by dividing P_o (kN) by calculated CSA (m^2). The force deficit produced by the lengthening contraction protocol was assessed by expressing the P_o (mN) measured after the each lengthening contraction as a percentage of the P_o before injury.

Statistical analyses

Differences between samples evaluated by unpaired *t*-test and other analyses were done using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla, CA. All error values are plotted as one standard deviation (SD) and significance set at $\alpha = 0.05$ unless stated otherwise. Prism software calculated $s_{y.x}$ according to the equation: $s_{y.x} = \sqrt{\frac{SS}{df}}$, where *SS* is the sum of squares of the distances of the curves from the points and df is the degrees of freedom of

the fit, which is the number of data points (N) minus the number of parameters fit. Since linear regression was used here, df = N-2.

Note to Chapter

Portions of this chapter were adapted and/or reproduced from: Muir LA, Nguyen QG, Hauschka SD, Chamberlain JS. Engraftment potential of a dermal cell population following in vivo myogenic conversion in dystrophic skeletal muscle *(under revision for Molecular Therapy)*.

Chapter 3

Enhanced Cell Survival and Therapeutic Potential

Introduction

The major goals of the work described here were to test whether dFbs are a viable population of cells for engraftment into dystrophic muscle, restoration of dystrophin, and improvement of whole muscle function. However, engraftment levels in previous experiments were not sufficient to determine whether dFbs can improve contractility or protect from contraction-induced injury (Chapter 2). I therefore wanted to find a method to improve engraftment and subsequently to test whether a higher dFb-contributed dystrophin-positive area provides functional benefit to dystrophic muscles.

A variety of factors have contributed to low engraftment of transplanted donor myoblasts in humans. One of the most immediate issues is poor donor cell survival. The inflammatory and ischemic micro-environment following transplantation likely leads to rapid necrosis and apoptosis for donor cells (Huard et al., 1992; Skuk and Tremblay, 2003; Skuk et al., 2007b). Indeed, studies in animal models in a variety of tissues indicate that the majority of transplanted cells die within 24 hours (Bliss et al., 2007; Guérette et al., 1997; Robey et al., 2008; Snyder et al., 2010; Suzuki et al., 2004; Terrovitis et al., 2008). In principle, preserving donor cells in this early time window should improve engraftment and maximize therapeutic efficacy for injection of a given cell number. In addition, using autologous cell populations such as dFbs in humans would avoid immunological incompatibility between donor and host. The experiments described here therefore mimic the autologous setting with syngeneic donor miniDys-GFP and host *mdx*^{4cv} mice.

An effective method for preventing rapid cell death may be supplying factors that combat necrosis and apoptosis in the injectate (Laflamme et al., 2007). In addition, pre-conditioning that tolerizes cells to stressors encountered during transplantation may promote cell survival (Bartoszuk-Bruzzone et al., 2012; Laflamme et al., 2005; Nakagawa et al., 2005; Skuk et al., 2007b). Since injected cells receive multiple signals that promote cell death, addressing a single pathway may not adequately protect cells (Hill et al., 2006a; Mendell et al., 1995). Thus a cocktail of multiple pro-survival and anti-death components was injected with cells to test whether it effectively promoted survival following transplantation into dystrophic muscle. Components of this pro-survival cocktail (PSC) included Matrigel to prevent anoikis (Zvibel et al., 2002), cyclosporine A (CsA) to inhibit cell death by blocking the mitochondrial permeability transition pore (Baines, 2005; Mott et al., 2004; Nakagawa et al., 2005), a Bcl-XL cell-permeant peptide to inhibit mitochondrial death pathways (Cao, 2002), the pan-caspase inhibitor ZVAD (Montolio et al., 2005), IGF-1 to activate Akt (Davis, 2006), and Pinacidil to open K_{ATP} channels and tolerize cells to ischemic stress (Ardehali et al., 2005). Cells were additionally heat-shocked (Laflamme et al., 2005) one day prior to transplantation and mice were treated with sub-immunosuppressive doses of CsA.

In order to rapidly screen transplanted muscles for differences in cell survival, I developed a qPCR-based method of detecting transplanted donor dFbs in host tissue. The method detected a portion of the lentiviral backbone that integrated with the MyoD-ER(T) cassette in donor cell genomes. I found a nearly 3-fold increase in engrafted cell number when PSC was added to the cells prior to intramuscular injection. Subsequent histological analysis of injected muscles confirmed these results and showed increased fiber number, area engrafted, and migration with PSC. Whole muscle physiology of muscles injected with PSC-treated dFbs

showed modest but significant protection from contraction-induced injury over saline-injected muscles of *mdx*^{4cv} controls.

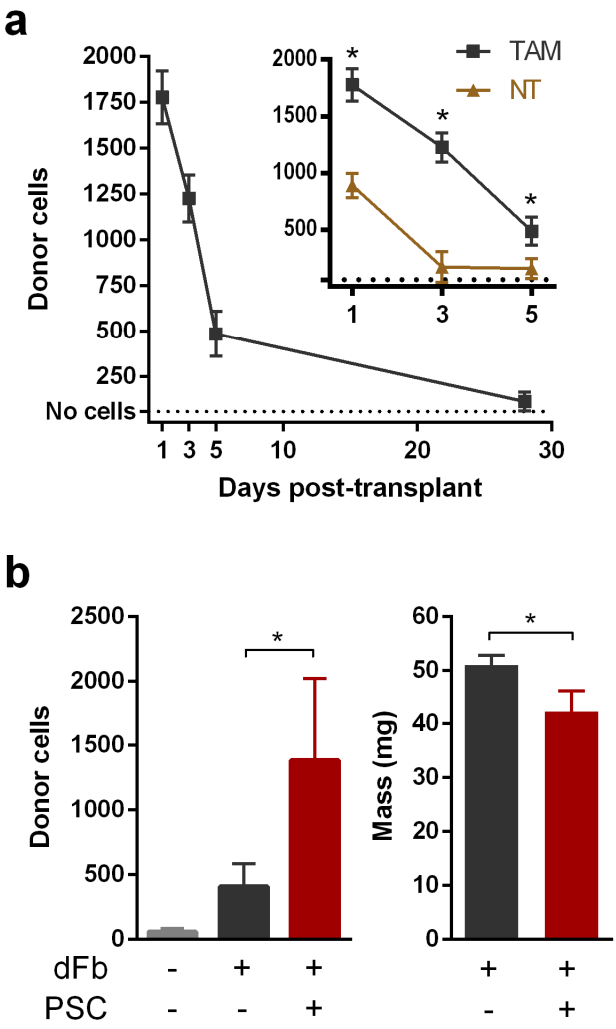
Results

Rapid death of transplanted dFbs is attenuated by PSC

Rapid detection of transplanted dFbs was achieved through whole muscle homogenization and qPCR-based screening of host tissue, using probes and primers that detect a universal integrated sequence of the lentiviral backbone that flanks the MyoD-ER(T) expression cassette. Initial qPCR experiments on transplanted dFbs confirmed a population average of at least one integration per cell (see Materials and Methods). Following transplantation, dFbs were detected in host TA muscles in mice either not treated (NT) or treated with tamoxifen (TAM) to induce conversion into the myogenic lineage (**Figure 3.1a**). Cell numbers rapidly declined in the first few days, with 73% of the cells lost between days 1 and 5 and 94% of the cells lost by day 28. I found an even more rapid loss of non-converted dFbs for the NT cohort, with roughly half as many dFbs detected on day 1 compared with the TAM cohort (**Figure 3.1a, inset**). NT muscles lost 82% of dFbs between days 1 and 5 and had a slightly higher rate of loss than muscles exposed to tamoxifen. Quantitative PCR on dFb-transplanted TA muscles revealed that addition of PSC increased the number of donor cells present at 1 week by about 3-fold (**Figure 3.1b**). Muscle mass was also lower in the PSC cohort, consistent with therapeutic benefit in dystrophic *mdx*^{4cv} host muscles (**Figure 3.1c**).

PSC improves engraftment in dystrophic muscle

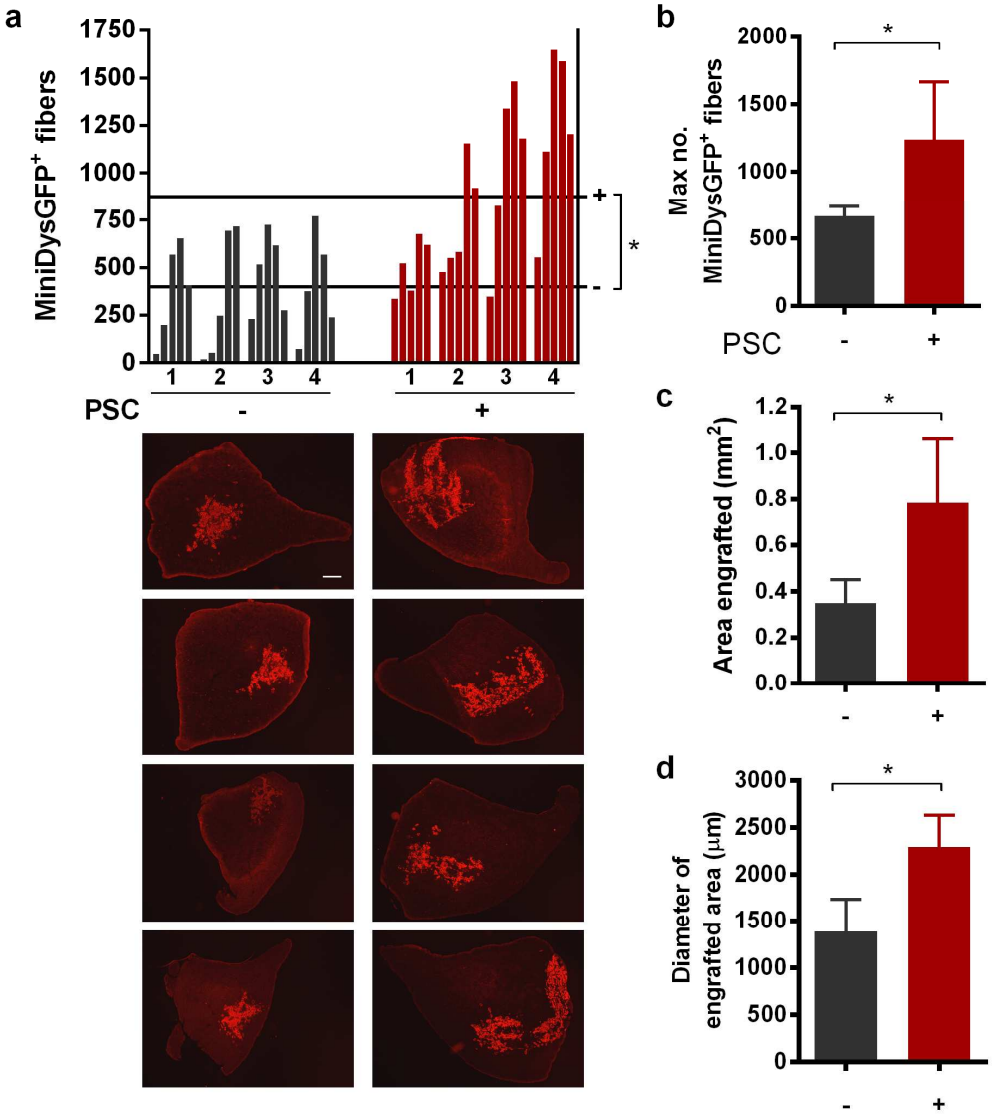
To confirm the qPCR results, dFb-transplanted TAs with and without PSC were harvested one week after transplantation, cryosectioned, and stained for the donor miniDys-GFP transgene. I quantified engraftment in five sections taken at equal intervals throughout regions of the transplanted muscles that spanned the highest engrafted area. The PSC group had significantly higher average miniDys-GFP^{+} fibers across all sections of all muscles,

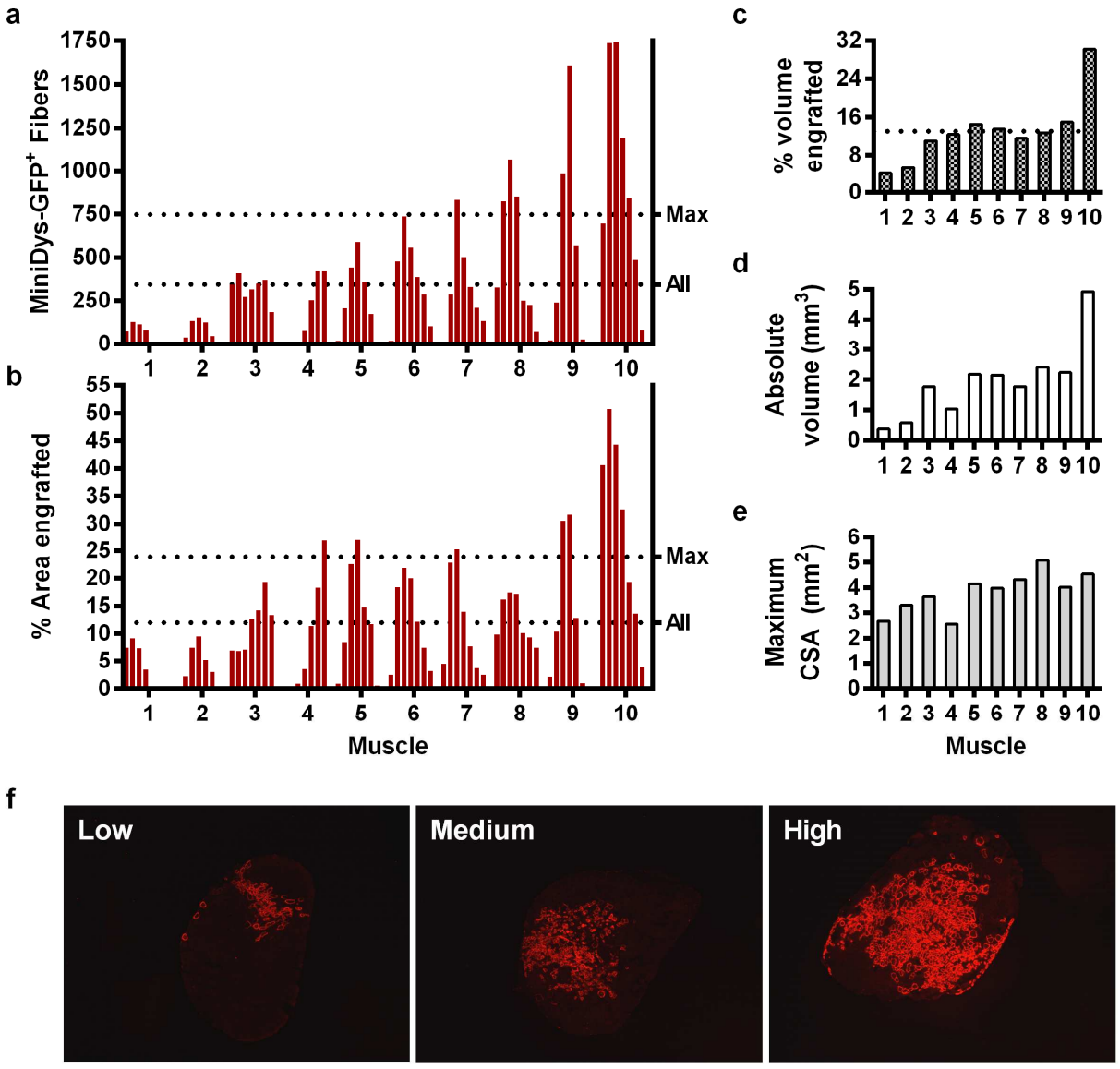

a
Donor cells
2000
1750
1500
1250
1000
750
500
250
No cells
1 3 5 10 20 30
Days post-transplant
TAM
NT
*
b
Donor cells
2500
2000
1500
1000
500
0
dFb - + +
PSC - - +
Mass (mg)
60
50
40
30
20
10
0
+ +
- +

higher miniDys-GFP$^+$ fibers in maximally engrafted sections, and higher average area engrafted (**Figure 3.2a-c**). In addition, higher engraftment persisted over longer distances longitudinally in the PSC group, as the first and fifth sections flanking the highest engrafted section were significantly higher (**Figure 3.2a** and **Supplemental Figure 10**). Finally, I found that the diameter across engraftment sites was greater for the PSC group (**Figure 3.2d**). Panels in **Figure 3.2a** are images of the maximally engrafted sections for cells transplanted with and without PSC.

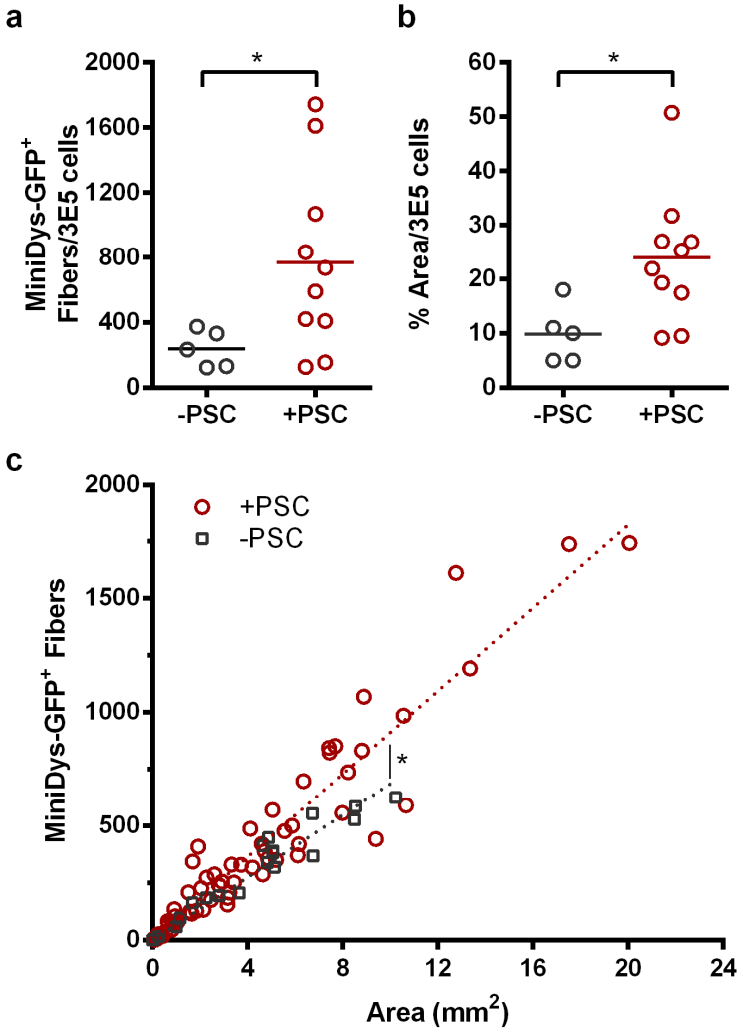
As a model for whole muscle functional testing, EDLs were transplanted with dFbs in the presence of PSC (**Figure 3.3**). Seven sections at equal intervals that flanked the highest engrafted section for each muscle were evaluated for miniDys-GFP$^+$ fibers and the percentage area engrafted using GFP immunofluorescence (**Figure 3.3a, b**). In an effort to further assess whole muscle engraftment, I estimated absolute and percentage volume engrafted using the average areas engrafted and the longitudinal distance engrafted for each muscle (**Figure 3.3c, d**). This analysis revealed an average 14% EDL volume engrafted (**Figure 3.3c**). **Figure 3.3e** shows the cross-sectional area at the mid-belly of each muscle and **Figure 3.3f** shows examples of low, medium, and highly engrafted EDLs.

Consistent with TA data, including PSC with dFb transplantations into EDLs significantly improves the fiber number and percentage area engrafted per cell number injected compared to transplantations without PSC (**Figure 3.4a, b**). These graphs also illustrate the engraftment variability across transplanted EDLs. When the number of miniDys-GFP$^+$ fibers vs. percentage area of engraftment was plotted, the slope of the regression line for the PSC cohort was significantly greater than the cohort without PSC (**Figure 3.4c**). This suggests that for a given engrafted area, more transgene-positive fibers were present when cells were injected with PSC. The effect appears more pronounced for highly engrafted areas. Several other measures


a
MiniDysGFP+ fibers
1750
1500
1250
1000
750
500
250
0
1 2 3 4 1 2 3 4
PSC - +
+
-
*
b
Max no. MiniDysGFP+ fibers
2000
1500
1000
500
0
*
PSC - +
c
Area engrafted (mm2)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
- +
d
Diameter of engrafted area (μm)
3000
2500
2000
1500
1000
500
0
*
- +


a
MiniDys-GFP+ Fibers
1750
1500
1250
1000
750
500
250
0
Max
All
1 2 3 4 5 6 7 8 9 10
b
% Area engrafted
55
50
45
40
35
30
25
20
15
10
5
0
Max
All
1 2 3 4 5 6 7 8 9 10
Muscle
c
% volume engrafted
32
24
16
8
0
1 2 3 4 5 6 7 8 9 10
d
Absolute volume (mm3)
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10
e
Maximum CSA (mm2)
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10
Muscle

f
Low
Medium
High


a
b
c
MiniDys-GFP+ Fibers/3E5 cells
% Area/3E5 cells
-PSC
+PSC
MiniDys-GFP+ Fibers
Area (mm2)
*

also show that engraftment improved in the PSC condition, including an increase in the number of muscles with an average area engrafted above 5%, the number containing a single section with engraftment area above 20%, and higher peak engraftment in the maximum engrafted section for each cohort (**Table 3.1**).

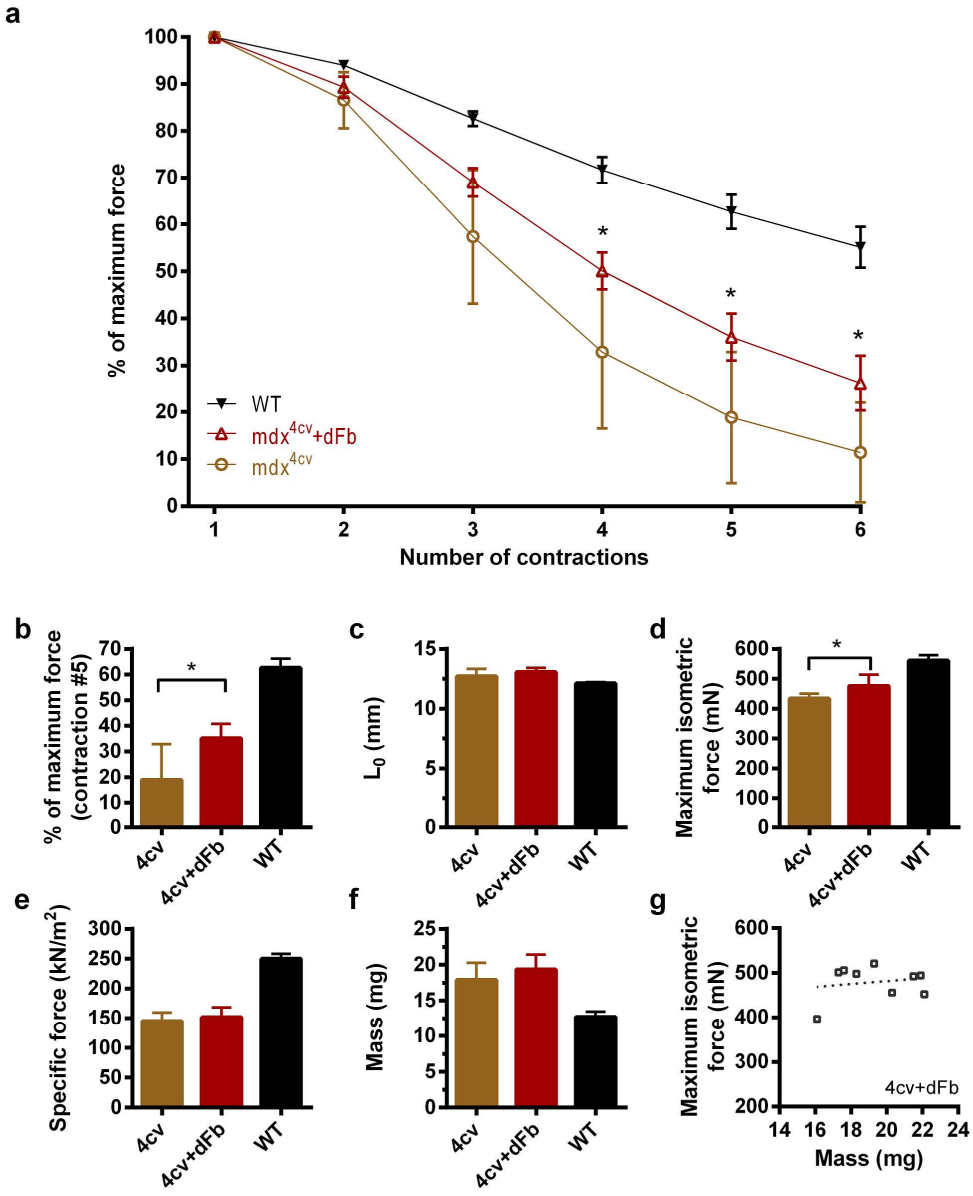
Interestingly, for transplanted dFb with PSC, I found that highly engrafted areas had a positive non-linear correlation with cross-sectional area (**Supplemental Figure 11a**). When the maximum CSAs for each muscle in transplanted cohorts were averaged, transplanted dFb with and without PSC had much higher CSAs than control, saline injected host muscles (**Supplemental Figure 11b**). A similar trend was observed for muscles engrafted with whole muscle mononuclear cells.

Moderate improvement in whole muscle function after dFb transplantation

Whole transplanted EDLs were tested in vitro to determine whether transplanted dFbs improved physiological measures in dystrophic muscle. **Figure 3.5a** shows the percentage of total force remaining after six lengthening contractions designed to induce injury. dFb transplanted muscles had modest but statistically significantly higher force remaining from contractions 4-6, compared to *mdx*^{4cv} controls (**Figure 3.5a, b**). Transplanted and control muscles were no different in length at optimal force production and thus underwent equivalent stretching (**Figure 3.5c**). When compared with age-matched *mdx*^{4cv} controls, dFb transplanted muscles had significantly higher maximum isometric force (**Figure 3.5d**). I found no significant differences in specific force and mass (**Figure 3.5e, f**). If the trend in increased mass accounts for the increase in isometric force, a significant increase in mass might also be expected, but there was no significant correlation between mass and isometric force (**Figure 3.5g**).

Table 3.1 Additional measures demonstrate improved engraftment for EDLs transplanted with dFbs with PSC. Columns show dFb-transplanted muscles without (NT) and with pro-survival cocktail (+PSC). Percentages and counts where applicable are given for three measures of engraftment. The percentage increase in engraftment (Δ) for the +PSC cohort compared to the NT cohort is given.

Measure	**NT**		**+PSC**		Δ
	%	No.	%	No.	
No. of muscles with average area engrafted above 5%	56%	5/9	80%	8/10	+ 25%
No. of muscles with peak engraftment above 20% (single section)	20%	1/5	60%	6/10	+ 40%
Peak engraftment in cohort (single section)	30%	NA	51%	NA	+ 21%


a
% of maximum force
Number of contractions
WT
mdx4cv+dFb
mdx4cv
b
% of maximum force (contraction #5)
c
L0 (mm)
d
Maximum isometric force (mN)
e
Specific force (kN/m2)
f
Mass (mg)
g
Maximum isometric force (mN)
Mass (mg)
4cv
4cv+dFb
WT

Discussion

Rapid loss of the majority of dFbs is consistent with other studies that show rapid loss of myoblasts following transplantation (Beauchamp et al., 1999; Fan et al., 1996; Guérette et al., 1997; Huard et al., 1994; Qu et al., 1998). This suggests that engraftment is limited by the host muscle environment and injection methods, and the first 24 hours are a critical window for cell survival. Additionally, the more rapid loss of non-converting dFbs compared to myogenically converting dFbs points to higher sensitivity of non-converting cells to transplantation stress, a feature also noted among different myogenic cell populations (Qu et al., 1998). These data support the hypothesis that unconverted dFbs are quickly cleared from the muscle and are unlikely to interfere with muscle repair, in agreement with the histology data showing no increase in fibrosis (Chapter 2). Furthermore, the difference in survival between the two dFb populations indicates that changes to the converting dFb occur rapidly and only when dFbs were injected into mice treated with tamoxifen. Thus the conversion process itself appears to promote cell survival, possibly through activation of growth or differentiation pathways associated with the myogenic program.

When included in dFb injectates, PSC clearly improved cell survival and engraftment. Higher cell numbers in the PSC condition by qPCR at one week post-transplantation predicted the subsequent improvement in engraftment in TA and EDL muscles. The initial qPCR screen showed that the PSC boosted cell survival beyond the effect of heat shock alone, indicating that use of multiple measures to combat apoptosis and necrosis is likely more effective than a single approach. The influence of PSC on proliferation of transplanted dFbs and on host muscle itself could also contribute to higher engraftment, given the known physiological role of factors such as bFGF and IGF-1 in the growth, regeneration, and differentiation of skeletal muscle (Allen and

Boxhorn, 1989; Cornelison et al., 2001; Kinoshita et al., 1995; Seed and Hauschka, 1988; Ten Broek et al., 2010).

EDL engraftment of myogenically converting dFbs with PSC provides a model system for investigating changes in force development in dystrophic muscle. My evaluation of whole EDL contractile properties revealed slightly higher maximum isometric force in dFb transplanted muscles, with no change in specific force or mass. These data, coupled with protection from contraction-induced injury, suggests that dFbs are capable of improving function in dystrophic muscle. Other studies have collected similar data after transplantation of myogenic cells, though improvements are not always observed (Cerletti et al., 2008; Darabi et al., 2008; Darabi et al., 2012; Mueller et al., 2002; Rousseau et al., 2010; Tedesco et al., 2012).

Discrepancies among studies that test muscle function after cell transplantation may be attributed to cell origin, insufficient engraftment, high engraftment variability, and the specific methods used to assess contractile properties. Whole muscle studies are appropriate here, since force transduction is a whole muscle event that depends on sound single fiber structure as well as all appropriate lateral and longitudinal extracellular connections (Chamberlain, 1997; Phelps et al., 1995; Rafael et al., 1994). These features, as well as proximity among engrafted fibers, may be a critical part of improving dystrophic muscle function and protecting from contraction-induced injury. All of this depends on appropriate expression and localization of the structural and signaling proteins, such as components of the DGC, that mediate force development and connectivity among myofibers (**Figure 1.1b**). Thus while single engrafted myofiber isolation and physiology measures provide valuable information about fiber structure and expressed transgene localization (Tedesco et al., 2012), they cannot be used in lieu of whole muscle analysis to assess functional improvement.

High engraftment variability within cohorts is common. Thus careful evaluation of engraftment sites is necessary for quantifying differences between cohorts, for instance those with different transplantation conditions. Consistent engraftment quantification measures also help to determine whether it is realistic to expect functional improvement. Note that improved contractile properties have been reported despite a low percentage of fibers engrafted, with no definitive mechanism (Darabi et al., 2012). Clearly many unknown variables contribute to functional improvement in dystrophic muscle. Collecting additional engraftment metrics in this case might greatly improve understanding of how engrafted fibers contribute to force development.

In the studies described in Chapters 2 and 3, I quantified both fiber number and area engrafted, with sampling of sections throughout the muscle at a distance of greater than half its length, to sufficiently capture lateral and longitudinal characteristics of whole muscle engraftment. Use of more than one measure additionally checks data corroboration between measures and allows valid comparisons of engraftment between muscles. For example, I frequently observed very small fibers in engrafted muscles. This is captured in the ratio of fiber number to area engrafted, approximated by the slope of the linear regression line derived from a scatter plot of these values (**Figure 3.4c**). In a few locations, partially transverse fibers were noted, though the large majority of fibers were in the same orientation as transgene negative fibers, and should not affect the fiber size ratio stated above. Differences between slopes in plots of transplantations with and without PSC suggest a fundamental difference in cell behavior during engraftment, as in **Figure 3.2a**. This effect was more pronounced in the PSC condition for more highly engrafted areas. This could mean that the PSC not only promoted dFb survival, but de novo fiber formation as well.

Variability in fiber sizes across sections within a muscle, or between muscles can be quantitatively evaluated by assessing, again in a plot of fiber size vs. area, the relationship of the data to the linear regression line. Analysis of the parameter $s_{y.x}$, which is the standard deviation of the vertical distances of the data points from the line given in y-axis units (GraphPad Prism, see Materials and Methods), reveals considerably higher scatter of the data around the best fit line for muscles injected with dFbs with PSC, compared to dFbs alone (+PSC $s_{y.x}$ = 124, -PSC $s_{y.x}$ = 53). In other words, the size and number of fibers for a given engrafted area was more variable for the PSC condition than without PSC. This provides an additional metric with which to assess transplanted muscles in a setting of high engraftment variability.

Reasons for the correlation of higher CSAs with higher engraftment are unclear (**Supplemental Figure 11a**). One possibility is that regions of high CSA, such as the muscle midbelly, accommodate a higher injection volume without the cell suspension leaking out, and therefore more cells engraft in that area. However, very slight increases in CSA are unlikely to cause very large changes in engraftment. In addition, as shown in **Supplemental Figure 11b**, cell-injected muscles have consistently higher maximum CSA per muscle compared to age-matched saline-injected controls. Thus changes were not likely due to initial differences in muscle sizes between cohorts (n = 4-10 muscles per group), and not due to needle injury-induced regeneration. This suggests that cells promote a slight increase in CSA at higher engraftment levels, possibly due to increased total fiber number.

Numerous additional methods have been investigated for their potential to promote survival and engraftment of cells during transplantation. Injected cell suspensions rely on perfusion from existing vasculature, so it follows that implantation of large cell masses into muscle lowers viability (Chapter 2) and results in necrotic central regions (Skuk et al., 2007b).

Using fewer cells per injection improves survival, while additional injections restore total cell number and circumvent migration issues (Pellegrini and Beilharz, 2011; Skuk et al., 2007a). Alternatively pro-migratory factors could be used to aid distribution of cells (Hill et al., 2006b; Lafreniere et al., 2009). Other approaches for reducing cell death related to oxidative stress include promoting activation of HIF-1, application of H_2S, exposing cells to physiological rather than atmospheric oxygen levels in culture systems, or conditioning with antioxidants (Bartoszuk-Bruzzone et al., 2012; Budde and Roth, 2010; Drowley et al., 2010; Semenza, 2004; Wu et al., 2005). Factors that stimulate angiogenesis could similarly combat hypoxia-induced death and promote skeletal muscle repair (Bouchentouf et al., 2008).

It is also important to consider the longevity of pro-survival effects when using cell populations that have pluripotent origins (Cunningham et al., 2012), or have the potential to be transformed through previous genetic manipulations. For example, rAAV2-mediated expression of IGF-1 in myoblasts improves cell survival and stimulates angiogenesis (Subramanian et al., 2009). While effective in its immediate goal, this approach is not necessarily desirable in cells intended for gene replacement therapies. Alternatives include co-transplantation of additional cells or bio-scaffolds for supply of paracrine factors that affect survival, muscle repair, or angiogenesis (Lesault et al., 2012; Saif et al., 2010; Sonnet et al., 2006). However, prolonged expression of modifying factors could have unforseen consequences for transplanted or resident cells. Karvinen et al. (2011), for example, show that long-term rAAV2 mediated expression of VEGF promotes abnormal angiogenesis and fibrosis in rabbit skeletal muscle.

It will be important to specifically consider modulation of interactions between donor cells and the host muscle environment or resident cells when addressing donor cell viability. For instance, preventing interactions between resident natural killer cells and donor cells during early

engraftment improves survival (Guérette et al., 1997; Laumonier et al., 2012). Targeting fibrotic lesions in the dystrophic muscle environment via signaling molecules or extracellular matrix remodeling agents could further improve donor cell competence (Cohn et al., 2007; El Fahime et al., 2000; Emery and Muntoni, 2003; Zhou and Lu, 2010).

In addition, it is likely that donor cells interact, and even compete, with resident stem cells in host muscle. Treatments affecting satellite cells and their niche, such as irradiation, toxins, or other injury methods generally improve donor cell engraftment (Boldrin et al., 2012; Gross et al., 1999; Harris, 2003; Heslop et al., 2000). These studies reveal important donor-host interactions and potential targets for future treatments, though direct application of such techniques is precluded in humans (Boldrin et al., 2012). Furthermore, intact resident populations may be necessary for robust skeletal muscle repair, especially in patients with advanced muscular dystrophy.

Many of the required factors for a multi-pathway pro-survival approach could be adapted for use in a cell injectate in human clinical applications. Substrates to combat anoikis of injected cells could be produced from decellularization of autologous tissue biopsies (Turner and Badylak, 2012). For example, extracellular matrix from a dermal tissue biopsy could be extracted and processed to provide a substrate for co-injection with transplanted cells. A variety of other substrates could also be derived from xenogeneic or synthetic origins, while keeping in mind specifications, such as elasticity and binding sites, that preserve donor cell regenerative properties (Discher et al., 2009; Fernandes et al., 2012; Gilbert et al., 2010; Mooney and Vandenburgh, 2008; Turner and Badylak, 2012).

Materials and Methods

Mice

See Materials and Methods, Chapter 2.

Drug preparation and dosage

Tamoxifen (Sigma-Aldrich, St. Louis, MO, www.sigmaaldrich.com) was equilibrated to room temperature, then 65°C preheated corn oil (Sigma-Aldrich) was added to the dry stock bottle at 100 mg/ml, and the bottle was then alternately heated to 56°C and vortexed frequently for several hours or until most of the crystals were dissolved. The solution was then transferred and diluted to 40 mg/ml in preheated corn oil, and the heating/vortexing procedure was repeated until no crystals were visible. The tamoxifen preparation was then filter sterilized and aliquoted for storage at -20°C. Working solutions were prepared by diluting aliquots to 20 mg/ml in sterile corn oil, and all mice were treated with a dose of 100 mg/kg per day by intraperitoneal (IP) injection for 5 days, beginning one day prior to cell transplantation.

Culture conditions

Media formulations were as follows. Growth medium (GM) for dermal cells was DMEM + 10% FBS + 1% P/S + 2 mM L-glutamine, with 10 ng/ml bFGF supplemented once per day. For transplantations, dermal cells were thawed (day -3) and expanded in GM for one day, then transduced (day -2) with the lentiviral vector carrying MyoD-ER(T) at a multiplicity of infection (MOI) of 10, or 10 infectious units per cell unless otherwise stated, in the presence of 8 ug/ml polybrene in 1 ml GM for 10 minutes. The cell/virus solution was then plated at 30 - 60% confluency in 10 ml GM per 15 cm plate. The following day (day -1) the pro-survival cocktail group cells were heat shocked with pre-warmed GM and kept at 43°C for 30 minutes, after

which the GM was refreshed and supplemented with 200 nM cyclosporine A (Bedford Laboratories). For initial qPCR experiments, cells in both groups (with and without pro-survival cocktail) were heat shocked. Cells were trypsinized (0.05%) on the day of transplantation (day 0) and resuspended in either pro-survival cocktail or Ham's F10 medium + 15% horse serum + 1 + mM $CaCl_2$ + 1% P/S.

Pro-survival cocktail

The following factors are based on the pro-survival cocktail tested by Laflamme et al. (2007) in cardiac tissue. Injectate consisted of 50% vol/vol growth factor-reduced Matrigel, 100 nM cyclosporine A, 50 nM Bcl-XL (Calbiochem), 100 uM ZVAD (benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethyl ketone, Calbiochem), 100 ng/ml insulin-like growth factor-1 (IGF-1, Peprotech), 50 uM Pinacidil (Sigma), 25 ng/ml basic fibroblast growth factor (bFGF, R & D Systems). Additionally, mice receiving cells with pro-survival cocktail were treated with a sub-immunosuppresive dose (5 mg/kg) of cyclosporine A beginning one day prior and ending 7 days after transplantation (Aharoni et al., 2005; Batiuk et al., 1996). For initial qPCR experiments, mice receiving cells without pro-survival cocktail were given a single 5 mg/kg dose of cyclosporine A one day prior to cell transplantation.

Transplantations

Cells were transplanted by open skin intramuscular injection into tibialis anterior (TA) or extensor digitorum longus (EDL) muscles in a single injection along the length of each muscle. dFbs were prepared for transplantation by dilution to desired concentrations in Ham's F10 medium (Gibco) with 15% horse serum (Thermo Scientific/Hyclone), 1 mM $CaCl_2$, 1% P/S and 0.5 µg/ml bFGF, kept on ice for 30 minutes to 3 hours, and aspirated into a 25 µl Gastight Hamilton syringe equipped with a 32 gauge needle. By hand, needles were inserted into the

distal ends of each muscle, about 2 mm from the tendon, and pushed longitudinally through the muscle to about 3 mm from the proximal tendon. Injections were performed concurrently with needle withdrawal at a rate of about 2 µl per second. Mice were anesthetized with inhaled 1-5% isoflurane in oxygen and treated with standard post-operative care.

Tissue processing

Transplanted muscles for quantitative real-time PCR were harvested and processed using the DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD) according to the manual with the following adaptations. We used 2-3 columns per muscle, adhering to a maximum of 20 mg per column. Muscles were digested overnight at 37°C and DNA extracted the following day into a final pooled volume of 800 µl per muscle, eluting twice in 65°C prewarmed elution buffer per column. This volume therefore served as a normalization parameter for host muscle mass.

Transplanted muscles for sectioning were harvested and frozen in optimal cutting temperature compound in liquid nitrogen cooled isopentane as in Chapter 2. Cryosections were obtained for analysis every 100 um throughout each muscle.

Quantitative real-time PCR

A 5 ul sample of final processed eluate for each muscle was used in triplicate for TaqMan quantitative real-time PCR (Applied Biosystems, Foster City, CA) to quantify the number of donor cells present per volume of tissue-derived eluate. The number of donor cells was determined by detecting a virally packaged portion of the lentiviral transfer plasmid lentiviral copies (LV2 probe and primer set, Sastry et al., 2002) in the tissue eluate. These values were normalized to the calculated cell population average of lentiviral copies per cell (LV2 and genomic low-density lipoprotein receptor (LDLR) probe and primer sets), determined through a separate qPCR on DNA extracted from a reserved portion of the transplanted cells. The TaqMan

probe, forward, and reverse primers were respectively as follows: LV2, 5'-FAM-AGCTCTCTCGACGCAGGACTCGGC-TAMRA, 5'-ACCTGAAAGCGAAAGGGAAAC, 5'-CACCCATCTCTCTCCTTCTAGCC; LDLR, 5'-FAM-ATGCCAGGATGGCAAGTGCATCTCC-TAMRA, 5'-CGTGCTCCCAGGATGACTTC, 5'-CTCCATCACACACAAACTGCG.

Physiology

See Materials and Methods, Chapter 2.

Statistical analyses

See Materials and Methods, Chapter 2.

Chapter 4

Conclusions

Applications and Impact

MyoD-ER(T) system

Kimura et al. (2008) demonstrated proof of principle for the potential therapeutic utility of transplanting tail tip-derived fibroblasts that can be converted in vivo into myogenic cells using tamoxifen-induced in vivo activation of MyoD. The tail tip fibroblasts were derived from *mdx*^{4cv} mice and transduced with a lentivirus carrying MyoD-ER(T) and a lentivirus carrying microdystrophin-GFP. An advantage of the approach of Kimura et al. and the experiments detailed in this dissertation is that it allows post-translational control of MyoD transcriptional activity, since constitutively expressed MyoD-ER(T) accumulates in the cytoplasm but is excluded from the nucleus by the ER(T) domain. This prevents premature myogenic differentiation and facilitates rapid myogenic conversion following tamoxifen treatment (Crescenzi et al., 1990; Del Bo et al., 2001; Lattanzi et al., 1998). Additionally, since MyoD-ER(T) activates expression of the endogenous MyoD gene as well as many other myogenic genes (**Figure 2.2**, Seed and Hauschka, 1988; Tapscott, 2005), converted cells maintain their myogenic properties following the withdrawal of tamoxifen. Furthermore, tamoxifen has prior approval for use in humans.

An exciting direction for the MyoD-ER(T) system is its potential to reprogram resident skeletal muscle fibroblasts or other cell types in situ into the myogenic lineage following local or systemic gene delivery (Gregorevic et al., 2004b; Murry et al., 1996). Direct conversion in situ is

precedented in pancreatic tissue, where exocrine cells have been reprogrammed into insulin-producing beta-cells (Zhou et al., 2008). This strategy may work not only for dystrophic muscle, but for a pathological states such as rhabdomyosarcomas (Yang et al., 2009). This inducible system can also be applied to other cell types that have been successfully converted with MyoD (Choi et al., 1990; Goudenege et al., 2009; Weintraub et al., 1991). In addition, it might be a useful tool for exerting temporal control over MyoD activity while exploring its potential influence on cell specification in pluripotent and multipotent progenitors at different stages of differentiation (Goudenege et al., 2012).

dFbs in cell-based therapy

As shown in the present study, tamoxifen-induced MyoD-mediated myogenic conversion works particularly well with dermal fibroblasts, and dFbs are of therapeutic interest because they are safely accessible from patients and relatively easy to expand and transduce in vitro (Elder et al., 1996; Lortal et al., 2008). Direct conversion of dFbs to muscle cells is not, however, without therapeutic drawbacks. The major concern with this approach is the possibility of lentiviral-induced insertional mutagenesis due to random integration of the vectors, as they are presently constructed, in dFb genomes. While the alternative of using patient-derived induced pluripotent stem cells (iPSCs) may also be an attractive option (Hanna et al., 2007; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007), this strategy involves similar risks of insertional mutagenesis, as well as the possible reduction of immune tolerance due to iPSC changes during the isolation and expansion process (Robinton and Daley, 2012; Zhao et al., 2011). In addition, direct reprogramming of fibroblasts into a variety of other lineages, including neurons and cardiomyocytes, has been successful without a pluripotent intermediate (Ieda et al., 2010; Vierbuchen et al., 2010). Further investigation and direct in vivo

comparisons may help determine whether myogenic cells derived from various reprogramming strategies have equivalent properties and engraftment potential.

Irrespective of the reprogrammed history of the engrafted cell population, it will be important to determine whether the myogenically converted cells participate in the formation of mosaic muscle fibers. Also, if dFbs or other donor cells generate only donor cell-derived muscle fibers, it will be especially important to investigate whether fibers become innervated, connect with myotendinous junctions, contract, and participate in force development in a coordinated fashion with the residual host muscle fibers. Such factors could influence individual de novo fiber maturity as well.

Investigating structures of engrafted fibers may provide information about the relationship between cell engraftment and force development. For example, fiber branching is common during muscle repair (Blaveri et al., 1999), and extensive branching has been shown to affect overall muscle function (Chan et al., 2007; Head, 2010; Lovering et al., 2009b). Fusion among donor cells, between donor and host cells, and between donor cells and existing host myofibers has the potential to cause improper fiber orientation, create branched fibers, or other malformations that impact function. In this work the large majority of transgene-positive fibers were correctly oriented. It is unknown whether the observed small fibers are due to fiber branching. If so, they could be a permanent structural characteristic of engrafted muscles and this could affect function. Small fibers may instead be newly formed myofibers, in which case they could mature and enlarge over time.

It will also be important to determine whether dFb engrafted muscles contain cells that are capable of regenerative responses. The high turnover of myofibers observed in dystrophic muscle will ideally be reduced in highly engrafted muscles. However, reconstitution of

progenitors will ensure a continual source of dystrophin upon natural muscle turnover, for example with exercise. In this case, readministration of cells would not be required. A progenitor state in the myogenically converted dFb population could exist in vivo, as an increase in engrafted fiber number was observed between 2 and 4 weeks (**Figure 2.5**). In addition, in vitro experiments revealed an increase in the percentage of myogenically converted dFbs (within the whole dFb population) under specific culture conditions. This suggests the presence of a proliferating myogenic population amenable to expansion given appropriate growth conditions (Chapter 2). However, analysis of these data is complicated by simultaneous myogenic conversion and differentiation.

Engraftment of cells that do not become progenitors likely still provide stabilization or protection of muscle from injury. It is unknown how long dFb-engrafted fibers last beyond the 12 weeks observed here, though presumably their rate of attrition would be lower than transgene negative fibers in the dystrophic environment. Thus in human therapies, dFbs could serve as a robust and abundant autologous cell type, capable of at least temporary stabilization of muscle. An alternative would be to use an additional cell type that could repopulate the satellite cell niche without requiring great numbers. But many challenges also still face the derivation and application of such progenitors, including the isolation of a sufficient number of cells that retain stem cell properties from an autologous source.

The work described in Chapters 2 and 3 demonstrates that dFbs are viable for gene delivery and show benefit to dystrophic muscle. Engraftment at these levels via single intramuscular injection appears to be close to a threshold for improved force development and protection from contraction induced injury (**Supplemental Figure 12**). Additional work on transplantation methods that improve engraftment efficiency may further improve function. dFbs

could also be a source of autologous cells for application to other skeletal muscle conditions, including sarcopenia, cachexia, incontinence, localized defects, or acute trauma (Carr et al., 2008; De Coppi et al., 2006; Kang et al., 2008; Lorenzi et al., 2008; Mitterberger et al., 2007; Mitterberger et al., 2008; Novara and Artibani, 2008).

Methods

The methods in these studies provide a reliable framework for screening transplantation conditions and quantifying engraftment. This is necessary in order to handle data variability observed within cohorts, and to corroborate contractility data. Here, I implemented a rapid qPCR screen for detecting lentivirally transduced donor cells in engrafted individual muscles. The screen detects a portion of the lentiviral backbone that is inserted into donor cells along with a therapeutic transgene. The method should therefore be generally useful for any cell population that is genetically modified with lentiviral vectors ex vivo. When single or very few sections of a muscle are evaluated, limited diffusion of transgenes from donor nuclei in hybrid fibers (Blaveri et al., 1999; Mueller et al., 2002; van Putten et al., 2012) may lead to skewed estimations of whole muscle engraftment. This likely contributes to the variability in engraftment reported in the literature. Sampling across muscles with more than one index improves confidence that results accurately represent whole muscle engraftment.

Future Prospects for Muscular Dystrophy Therapy

Despite current limitations, many recent developments point to the promise of viral-vector-mediated gene therapy. Increased understanding of viral transduction mechanisms have allowed generation of hybrid vectors that target desired tissues with high efficiency and long-term expression (Dickson et al., 2002; Goncalves et al., 2008a; Schultz and Chamberlain, 2008).

We envision that further tailoring with hybrid or chimeric vectors could lead to more exclusive tissue-specific tropism, thereby improving safety while retaining adequate carrying capacity for most defective genes. In addition, modulation of transgene promoters and enhancers could improve muscle-specific expression for virtually all gene-transfer methods (Salva et al., 2007). Immune responses remain a critical issue, and evasion will be an important aspect of both viral- and cell-mediated therapies (Zaldumbide and Hoeben, 2007).

A possible approach for DMD treatment using rAAV entails multisite intravascular delivery of vectors carrying microutrophin cassettes. Transient immune suppression could be used to block cellular immunity against AAV, and delivery of the microutrophin cDNA could avoid most or possibly all immune responses that occur against dystrophin. Developing optimal methods for delivery and immune suppression, as well as design of utrophin or dystrophin variants and transcriptional regulation of the cassette, are active areas of study that hold promise for clinical intervention. Recent and ongoing clinical trials focus on safety and transgene expression (Bowles et al., 2012; Mendell et al., 2010a).

Exon skipping is a rapidly developing potential therapy with several clinical trials completed or in progress (Cirak et al., 2011; Cirak et al., 2012; Kinali et al., 2009; NCT01254019 and NCT01462292 at www.clinicaltrials.gov). Further modifications to this approach might make it possible to target a large number of the mutations and deletions observed in DMD and BMD (van Deutekom et al., 2007). However, additional testing is necessary to determine optimal chemistries for the safety and feasibility of systemic delivery. Questions also remain as to the functionality of the truncated proteins that would arise from some of these induced splicing events (Harper et al., 2002).

The work described in this dissertation and numerous translational studies show the promise of cells as a gene therapy vehicle capable of restoring lost skeletal muscle tissue. Ongoing clinical trials evidence the pursuit of therapeutic benefit in human dystrophic muscle (NCT01610440 and NCT00773227). NCT01610440 is a Phase 1/2 trial investigating the safety and efficacy of injecting human umbilical cord mesenchymal stem cells into DMD patients. NCT00773227 is a Phase 2 trial in which autologous myoblasts from unaffected limb muscles are transplanted into the pharyngeal muscles of oculopharyngeal muscular dystrophy patients, aimed at determining whether contractile function can be restored. Cell therapies lag other approaches for treating muscle disorders due to their inherent complexity. Especially in the case of stem cells, we are only just beginning to understand their basic biology and how to maintain cells in a particular state that maximizes therapeutic potential (Gilbert et al., 2010).

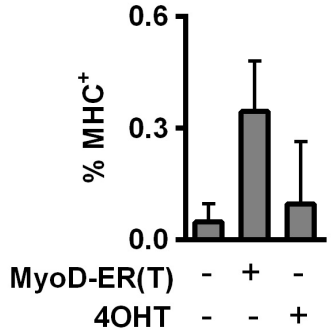
Because ongoing clinical trials for all therapies are currently strongly focused on safety, intramuscular delivery is the chosen delivery route. Local administration might be of therapeutic benefit to individual muscles or small groups of muscles; however, targeting striated muscle system-wide is required for an effective therapy. Combinations of local, regional and systemic routes of administration might be viable therapeutic options to achieve system-wide targeting of striated muscle. We should consider gene transfer, gene repair, and aspects of regenerative medicine to be complementary approaches, not at all mutually exclusive, and potentially the most effective in a wide array of skeletal muscle conditions when used in conjunction with each other. Although underestimation of challenges to gene therapy initially led to some disappointing clinical trials, there has been a recent resurgence in interest as new and exciting discoveries in the field have broken though barriers to viral, nonviral and cell-mediated therapies, bringing us a few steps closer to safe and effective treatments for the muscular dystrophies.

Note to Chapter

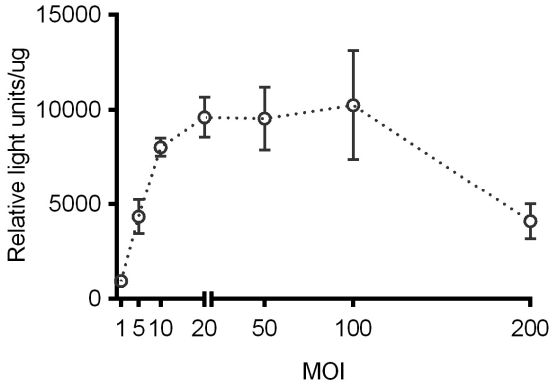
Portions of this chapter were adapted and/or reproduced from: Muir LA, Nguyen QG, Hauschka SD, Chamberlain JS. Engraftment potential of a dermal cell population following in vivo myogenic conversion in dystrophic skeletal muscle *(under revision for Molecular Therapy)*, and Muir LA, Chamberlain JS. Emerging strategies for cell and gene therapy of the muscular dystrophies. *Expert Reviews in Molecular Medicine* 2009; 11e18.

Appendix

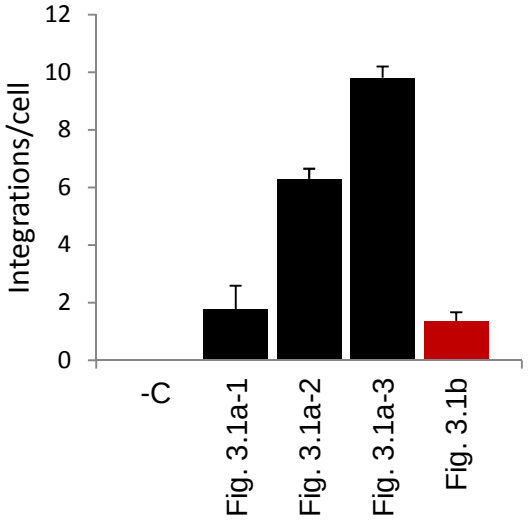
Supplemental Figures



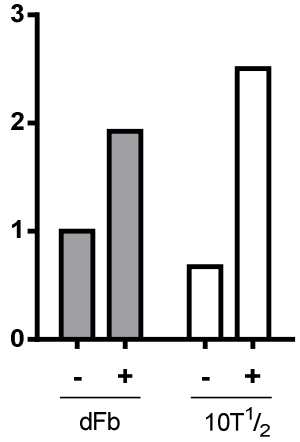
Supplemental Figure 1 dFbs exhibit very low background myogenic conversion. Without 4OHT treatment or expression of MyoD-ER(T), less than 0.1% of cells expressed MHC; without 4OHT and with expression of MyoD-ER(T), ~0.3% expressed MHC; with 4OHT treatment and without expression of MyoD-ER(T), ~0.1% of cells expressed MHC. For each condition, at least 5 fields per well across three wells were quantified. There was no statistically significant difference in background MHC expression among these controls.



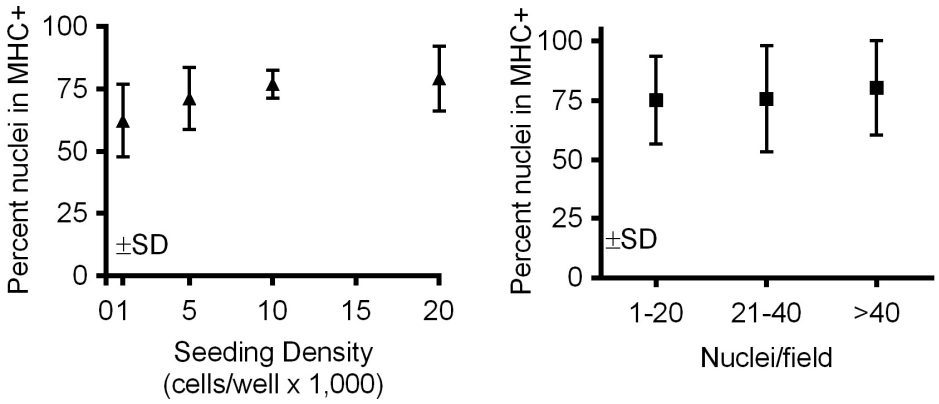
Supplemental Figure 2 Activation of a muscle-specific model therapeutic gene in myogenically converted dFbs. dFbs were transduced with the lentivirus carrying MyoD-ER(T) at indicated MOIs, then transfected with CK8e-luciferase, a model therapeutic gene construct. Data were obtained from two independent luciferase assays on two separately transfected wells for each data point and were normalized to total protein levels. Error bars are mean ± SEM.



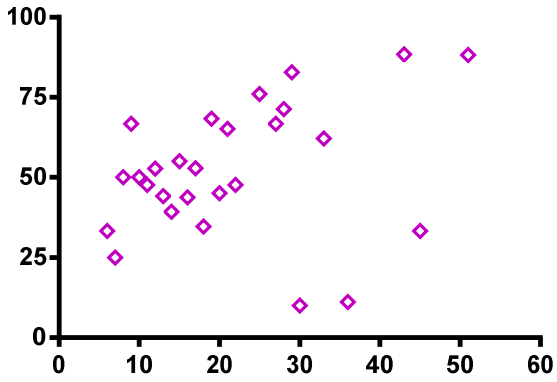
Supplemental Figure 3 qPCR-based detection of lentiviral integration events per cell. Integrations in cells that were transplanted in the cohorts used to generate the data in **Figure 3.1** were detected through qPCR for LV2, a sequence within the lentiviral transfer plasmid backbone that is integrated with the MyoD-ER(T) cassette. Integrations per cell were calculated by dividing the qPCR-derived quantity LV2 by the quantity of the low density lipoprotein receptor, known to have two copies per genome, divided by two. The negative control bar, -C, represents detection of LV2 in processed, non-transplanted *mdx*^{4cv} host muscle tissue (see Chapter 3, Materials and Methods). The remaining bars represent the data that were used to normalize the indicated parts of **Figure 3.1**, where three cohorts were used for **Figure 3.1a**. Error bars represent one standard deviation based on 2-3 qPCR replicates.



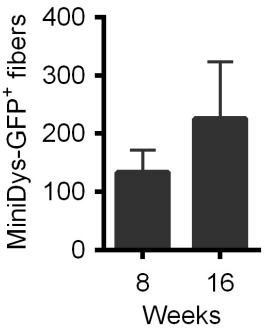
Supplemental Figure 4 The effect of bFGF treatment during in vitro myogenic conversion of two fibroblast populations on proportion of myogenic cells. dFbs and 10T1/2 cells were transduced with the lentivirus carrying MyoD-ER(T), then converted into the myogenic lineage in vitro by treating cultures with 4OHT. Treated cultures were supplemented with 10 ng/ml bFGF daily through day 6 in **Figure 2.1b**. Data for all conditions were normalized to the non-bFGF treated dFb condition. Cultures were additionally refed 2% FBS every 2-3 days and allowed to differentiate for 7-10 days. Significantly greater proportions of myogenically converted cells were found for both dFbs and 10T1/2s when treated with bFGF during initial stages of the conversion process, compared to non-treated cultures.



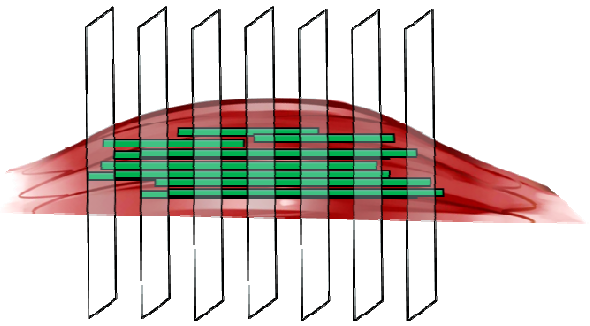
Supplemental Figure 5 Relationship between myogenic conversion of dFbs and seeding density or nuclei per field. Cells were transduced with the lentivirus carrying MyoD-ER(T), seeded on 6 well plates, and treated following the conversion scheme shown in **Figure 2.1b**. The y-axes indicate percentage of nuclei within cells that stained positive for the myogenic marker myosin heavy chain (MHC). For the seeding density graph (left), data were obtained from 2-3 wells per density, with 5-10 fields quantified per well. No statistically significant difference in percentage of MHC^+ cells was found between seeding at 1,000 cells per well vs. 20,000 cells per well. For the field density graph (right), ≥ 4 fields were grouped for each bin, and no statistically significant differences were found between bins.



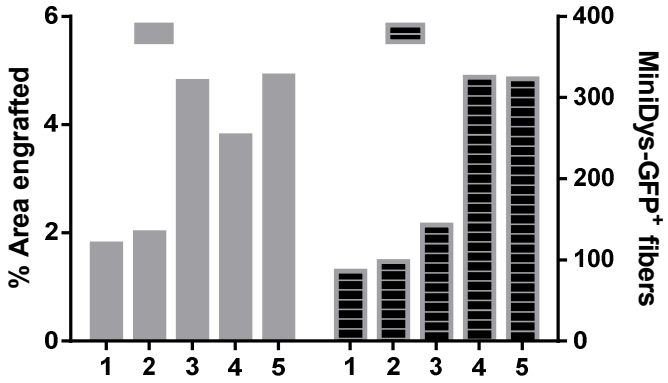
Supplemental Figure 6 Relationship between myogenic conversion of 10T1/2 cells and nuclei per field. Cells were transduced with the lentivirus carrying MyoD-ER(T) and treated following the conversion scheme shown in **Figure 2.1b**. The y-axis indicates percentage of nuclei within MHC^+ cells. No statistically significant correlation was found between cell density and myogenic conversion for this cell type.



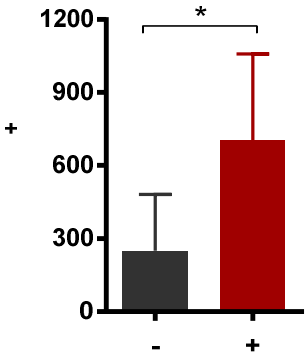
Supplemental Figure 7 No immune clearance of engrafted miniDys-GFP$^+$ muscle fibers. 5 x 10^5 whole muscle mononuclear cells isolated from miniDys-GFP donors were transplanted into TA muscles of *mdx*^{4cv} hosts. MiniDys-GFP$^+$ fibers were counted every 0.1 mm throughout each muscle following direct imaging of GFP on cross sections 8 and 16 weeks after cell transplantation. Section totals were then averaged across all sections of all muscles for each time point. An average of 136 ± 70 positive fibers were found throughout three TAs at 8 weeks, and 226 ± 165 positive fibers were found throughout four TAs at 16 weeks. The apparent difference between the two time points was not statistically significant.



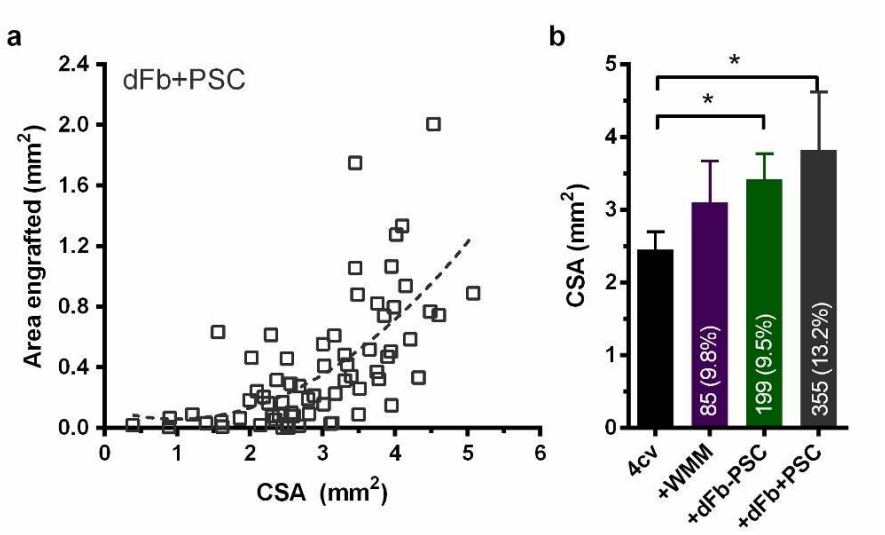
Supplemental Figure 8 Illustration of the quantification approach for an individual engrafted muscle. Cryosections were taken throughout each cell-transplanted muscle. Blue planes represent seven regions of potential sampling for engraftment analysis. This approach was used to generate engraftment bar plots in **Figures 2.5**, **2.6**, **3.2**, and **3.3**. Each plane represents a cross section, in which positive fibers or area engrafted was quantified, and a single bar within plots.



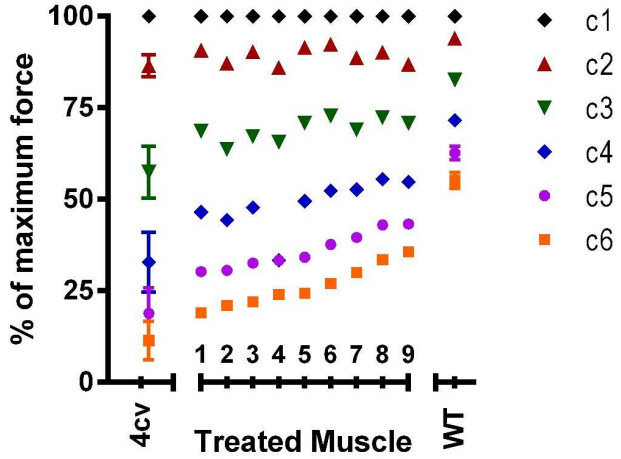
Supplemental Figure 9 Engraftment metrics in selected dFb-transplanted TA muscles. Five muscles were selected as representative examples of engraftment in TA muscles after dFb transplantation and in vivo myogenic conversion. Percentage area engrafted and the number of miniDys-GFP$^+$ fibers were quantified in multiple sections across each transplanted muscle, and the averages for each measure across each muscle are shown. In all quantified TA muscles, area was under 10% of the total cross-sectional area of the muscle, and in most cases was ≤ 5%.



Supplemental Figure 10 Longitudinal engraftment is greater for dFbs transplanted with PSC. TA muscles from the dataset in **Figure 3.2** were evaluated for amount of engraftment across a specific longitudinal distance in muscles transplanted with dFbs with and without PSC. MiniDys-GFP$^+$ fibers were counted in the two outermost sections out of the five that were sampled from the maximally engrafted region of each TA. Statistically significant differences were found, indicating greater longitudinal engraftment in the PSC cohort compared to the non-PSC cohort.



Supplemental Figure 11 Correlation of highly engrafted areas with muscle cross-sectional area. (a) EDL muscles transplanted with dFbs with PSC showed a positive non-linear correlation of area engrafted with cross-sectional area (CSA). (b) An average of the mid-belly CSAs for transplanted cohorts reveals much higher CSAs for muscles transplanted with dFbs with and without PSC, compared to control, saline injected host muscles. Muscles transplanted with WMM show a similar trend. White text within bars shows the average number of engrafted fibers across transplanted muscles in each cohort, with the average percentage engraftment in parentheses.



Supplemental Figure 12 Individual EDL muscle responses to eccentric contractions. The percentage of force remaining in each of nine transplanted muscles is shown after six consecutive eccentric contractions (c1-c6). Improvement is observed for muscles transplanted with dFbs compared to uninjected dystrophic (4cv) muscles, but after contraction 6, injury is still apparent in all transplanted muscles compared to the response of wild type (WT) control EDLs.

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VITA

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