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Engraftment of embryonic stem cell-derived myogenic progenitors in a dominant model of muscular dystrophy

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Abstract

Muscular dystrophies (MD) consist of a genetically heterogeneous group of disorders, recessive or dominant, characterized by progressive skeletal muscle weakening. To date, no effective treatment is available. Experimental strategies pursuing muscle regeneration through the transplantation of stem cell preparations have brought hope to patients affected by this disorder. Efficacy has been demonstrated in recessive MD models through contribution of wild-type nuclei to the muscle fiber heterokaryon, however to date, there has been no study investigating the efficacy of a cell therapy in a dominant model of MD. We have recently demonstrated that Pax3-induced embryonic stem (ES) cell-derived myogenic progenitors are able to engraft and improve muscle function in mdx mice, a recessive mouse model for Duchenne MD. To assess whether this therapeutic effect can be extended to a dominant type of muscle disorder, here we transplanted these cells into FRG1 transgenic mice, a dominant model that has been associated with Facioscapulohumeral muscular dystrophy. Our results show that Pax3-induced ES-derived myogenic progenitors are capable of significant engraftment after intramuscular or systemic transplantation into Frg1 mice. Analyses of contractile parameters revealed functional improvement in treated muscles of male mice, but not females, which are less severely affected. This study is the first to use Frg1 transgenic mice to assess muscle regeneration as well as to support the use of a cell-based therapy for autosomal dominant types of MD.

Keywords

Embryonic stem cells; Pax3; muscle differentiation; dominant disease; muscular dystrophy; FRG1 mice; transplantation

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Muscular dystrophies are a heterogeneous group of inherited neuromuscular disorders, including X-linked recessive as in Duchenne MD, autosomal recessive as in limb-girdle MD type 2, or autosomal dominant as in Facioscapulohumeral MD, myotonic dystrophy and limb-girdle MD type 1 (Emery, 2002). The concept of a cell-based therapy to promote muscle regeneration, in particular in recessive types of MD, originated with the observation of the intrinsic ability of myofibers to fuse to each other (Watt, et al., 1982, Watt, et al., 1984). Thus, functional correction could be obtained by generating hybrid muscle fibers, where the donor nuclei provide the missing gene product. Based on this premise, several investigators have assessed the ability of adult myoblasts to treat Duchenne MD, which is characterized by the lack of dystrophin. Although initial results in *mdx* mice were encouraging (Brussee, et al., 1999, Gussoni, et al., 1997, Partridge, et al., 1989), early clinical trials failed due especially to the poor survival and limited migratory ability of injected myoblasts (Mendell, et al., 1995, Partridge, et al., 1989, Tremblay, et al., 1993, Vilquin, 2005). The lesson learned from these early studies is that a more primitive cell population endowed with self-renewal and differential potential would be preferable for therapeutic applications. Accordingly, a number of studies involving different sources of stem cells, including muscle satellite cells (Cerletti, et al., 2008, Collins, et al., 2005, Montarras, et al., 2005), vessel derived stem cells (mesoangioblasts) (Sampaolesi, et al., 2006, Sampaolesi, et al., 2003), and embryonic stem cells (Darabi, et al., 2008) have provided better outcomes with wider distribution of transplanted cells, higher levels of engraftment, and importantly, improvement in the contractile properties of muscles from transplanted dystrophic mice. Because all these studies were performed in recessive forms of MD, it is unknown to what extent dominant forms of MD could benefit from stem cell-based therapy. Since it takes fewer nuclei to add back a missing product than it takes to dilute out a deleterious product, the bar is expected to be higher for effective treatment of a dominant disease, however this will depend on the specific disease mechanism.

Here we investigated whether Pax3-induced ES-derived myogenic progenitors, a cell population able to promote functional skeletal muscle regeneration in *mdx* mice (Darabi, et al., 2008), would produce engraftment and ameliorate the phenotype in a mouse model for a dominant neuromuscular disorder. For these studies, we have chosen mice overexpressing FRG1, which have been proposed as a mouse model for FSHD (Gabellini, et al., 2006). Transgenic mice overexpressing *FRG1* (medium and high expressers) (Gabellini, et al., 2006) develop a myopathy and phenotypic abnormalities that are typical of MD, including abnormal spinal curvature, skeletal muscle atrophy, increased variability in fiber size, fiber necrosis, centrally located nuclei and fibrosis (Deconinck, et al., 1997, Gabellini, et al., 2006, Grady, et al., 1997). Although there is some controversy in the field as to the relationship of this pathophysiology to human FSHD, for the purposes of this study, the salient feature is that it is dominantly inherited.

Mice experiments were carried out according to protocols approved by the University of Texas Southwestern Medical Center and University of Minnesota Institutional Animal Care and Use Committee which meet US National Institutes of Health guidelines for the humane care of animals. A total of 24 FRG1-medium mice at 12 weeks of age were used for these experiments. All mice were treated with an immunosuppressive agent (tacrolimus; Sigma) to prevent rejection of nonisogenic cells at a daily dose of 5mg/kg intraperitoneally (IP) from the day before cell injection until the time of euthanasia (30 days).

Myogenic progenitors from GFP-labeled Pax3-induced ES-derived cells were obtained as previously described (Darabi, et al., 2008). Briefly, GFP⁺ iPax3 ES cells were differentiated as embryoid bodies (EBs) using the hanging drop method. EBs were subjected to Pax3 induction (doxycycline was added to the EB culture medium) from day 2 to day 5 of EB differentiation, at which point early myogenic progenitors were purified by FACS using antibodies to PDGF α R and Flk-1. PDGF α R⁺Flk-1⁻ cells were expanded in the same medium

containing doxycycline for 7-10 days and then transplanted into FRG1 transgenic mice. In the first series of experiments, female FRG1 mice were transplanted with Pax3-induced ES-derived myogenic progenitors through intramuscular (i.m.; n=4) and intravenous (i.v.; n=4) injections. Twenty-four hours before transplantation, 15 μ l of cardiotoxin (10 μ M, Sigma) was injected into the tibialis anterior (TA) muscle of each mouse (both legs) to induce muscle injury. In the intramuscular group, mice received 1×10^6 cells by direct intramuscular injection into the left TA muscle whereas the right TA muscle received only PBS (control). In the systemic i.v. group, mice received 5×10^5 cells through tail vein injection while the control group (n=4) received PBS. After 30 days, muscles were harvested and analyzed by immunofluorescence to assess engraftment by the presence of GFP (donor cells) and MHC, a marker of terminal muscle differentiation. In a second set of experiments, female and male FRG1 mice were transplanted with Pax3-induced ES-derived myogenic progenitors through intramuscular injection (a total of 6 mice in each group), as described above, and muscle function was assessed 30 days following the transplantation. Muscle cryosections were also evaluated for engraftment.

Muscles were frozen in isopentane cooled in liquid nitrogen. Tissue cryosections (6-8 mm) were fixed with cold acetone for 5 min., permeabilized with 0.5% Triton X-100 (Sigma) for 20 min., blocked with 10% goat serum (or 3% BSA) for 1 hr, and then incubated with primary antibodies: anti-MHC (1:20 overnight 4°C; Developmental Studies Hybridoma Bank) and chicken anti-GFP (1:500, 1hr RT; Abcam). For secondary staining, appropriate secondary alexa fluor antibodies were used (Invitrogen).

As observed in Figure 1, whereas PBS-injected FRG1 mice were negative for GFP (Fig. 1A), transplantation of Pax3-induced ES-derived myogenic progenitors, independent of the route of administration, resulted in substantial engraftment as evidenced by the presence of GFP⁺MHC⁺ myofibers (Fig. 1B-E). In the case of intramuscular injection, transplanted cells migrated and engrafted well around the site of injection (Fig. 1B-D). As observed previously (Darabi, et al., 2008), these cells were able to home to the muscle following their systemic injection (Fig. 1E). TA muscles from these mice contained about 14.5 ± 1.07 % of GFP⁺ myofibers.

The measurement of muscle contractile properties was performed as previously described (Darabi, et al., 2008). Interestingly we observed gender differences in regard to the contractile ability of TA muscles from FRG1 mice. Whereas male FRG1 mice showed significant reduction in their contractility ability when compared to the B/6 control group, female mice demonstrated no functional deficit (Fig. 1F-G). Accordingly, cell transplantation had no effect in this latter group, while the transplantation of Pax3-induced ES-derived myogenic progenitors in male FRG1 resulted in improvement of maximal isometric tetanic and specific force (Fig. 1F-G). It is important to note that engraftment levels were similar among female and male groups (17.8 ± 3 % vs. 18.4 ± 2.4 % of GFP⁺ myofibers, respectively). CSA and weight were not affected by cell transplantation (Fig. 1H-I). According to our previous results, while systemic delivery of ES-derived myogenic progenitors into mdx mice reduced these parameters close to wild-type mice (Darabi, et al., 2008), intramuscular transplantation did not affect CSA and weight ((Darabi, et al., 2008) (supplementary Fig. 5), as observed here. However it is important to note that mdx and FRG1 mice present quite distinct phenotypes. While mdx mice are hypertrophic due to the multiple rounds of degeneration and regeneration, FRG1 mice are atrophic.

Gender differences in skeletal muscle have been demonstrated with energy metabolism, hormonal status, and muscle fiber types. For instance male skeletal muscles generally contain more fast fibers than female muscles (Clark, et al., 2003, Glenmark, et al., 2004). Conversely, during repeated contractions, female muscles are generally more fatigue resistant and recover

faster (Clark, et al., 2003, Glenmark, et al., 2004). It has been recently shown in FRG1 mice that fast fibers are preferentially involved in the pathology (D'Antona, et al., 2007). Accordingly, staining for slow and fast isoforms of MHC in male and female FRG1 mice showed gender specific differences (Fig. 1J). Female FRG1 mice contained significantly more slow myofibers than males, as evidenced by NADH tetrazolium reductase staining (Paljärvi and Naukkarinen, 1990), while immunohistochemistry for a fast isoform of MHC (Havenith, et al., 1990) revealed a lower frequency of fast myofibers (Fig. 1J). This may explain, at least in part, the results observed here. Consistently, female FRG1 mice presented much less fibrosis than their male counterparts (Fig. 1K), as evidenced by Mason's Trichrome staining, which corroborates their milder phenotype. It is important to note that cell transplantation did not affect fibrosis or the ratio of fast and slow myofibers in male or female FRG1 mice (data not shown). Moreover, it has recently been pointed out that gender also affects stem-cell mediated skeletal muscle regeneration, with female muscle-derived stem cells (MDSCs) having higher potential for muscle regeneration than their male counterparts (Deasy, et al., 2007). Although these studies were performed in mdx mice through transplantation experiments, one could hypothesize that resident MDSCs in female FRG1 mice are better able to respond to the damage caused by overexpression of FRG1.

In conclusion, our results demonstrate for the first time the feasibility of a cell therapy approach using a dominant model of muscular dystrophy. A simple mechanistic explanation for our results would be that transplantation of ES-derived myogenic progenitors dilute out an unwanted protein from hybrid myofibers, in this case, FRG1. Another possibility is that the proliferative capacity of FRG1-overexpressing myogenic progenitors may be compromised (Brian Kennedy and Steve Hauschka, personal communication). In this case, our transplanted donor cells may be capable of generating *de novo* myofibers with very little or no host contribution. Regardless of the exact mechanism, these findings are extremely relevant since to date, there has been no indication that a stem cell treatment could be effective in patients presenting a dominant type of muscular dystrophy, and thus provide support for the future application of ES/iPS cell therapies.

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Figure 1. Engraftment of Pax3-induced ES-derived myogenic progenitor into dystrophic muscles of FRG1 mice

Staining for MHC (Red) and GFP (Green) in cross-sections of TA muscles from (A)FRG1 control mice, which present no signal for GFP, and (B-C) GFP⁺ myofibers in female and male FRG1 mice treated with Pax3-induced ES-derived ES cells, respectively. (D-E) Longitudinal sections of FRG1 mice that had received cell transplantation via IM and IV, respectively. Compared to the intramuscular method, the transplantation pattern following IV injection is more diffuse across the muscle. (F-H) Contractile properties of TA muscles in FRG1 mice. Values shown are the results of experiments involving IM transplantations in female and male mice $\pm$ SEM (six animals per group). Values for C57BL/6 (B/6) TA muscles are shown as

reference (n=6). (F) Representative example of force tracing in TA muscles from CTX-injured FRG1 mice transplanted with *Pax3*-induced PDGF α R⁺Flk-1⁻ EB derived cells. Red and blue lines show force tracing from muscles that had received cell transplantation or PBS (control), respectively. Green line represents force tracing from wild-type controls (B/6). (G) Effect of cell transplantation on specific (sF_0 : F_0 normalized to CSA) force. * $P < 0.05$ treated vs. untreated muscles. (H-I) Average CSA and weight of analyzed muscles, respectively. (J) Staining and quantification of fast and slow myofibers in male and female untreated FRG1 mice. Left upper panel: PFA fixed muscle sections were stained with a monoclonal anti-skeletal myosin (fast) antibody (clone MY-32, Sigma). Followed by an anti-mouse Ig peroxidase reagent, and DAB substrate subsequently. Stained fibers (type II) were quantified using NIH image J software. Left lower panel: NADH tetrazolium reductase staining was used to demonstrate the activity of the mitochondrial electron transport chain in slow oxidative (type I) fibers. The oxidative capacity of fibers was assessed by the relative intensity of the colorimetric assay. Stained sections were viewed by light microscopy and the percentage of high oxidative (dark stained) fibers (Type I) were calculated using NIH ImageJ Software. Right panel: Graph representing the quantification of fast and slow myofibers in male and female FRG1 mice. A total of 4 male and 4 female FRG1 mice were assessed. For each mouse, 5 sections were used for quantification. *** $P < 0.001$ female compared to male fast fibers; +++ $P < 0.001$ female compared to male slow fibers. (K) Staining for fibrosis in male and female untreated FRG1 mice. PFA fixed muscle sections were stained with Mason's Trichrome staining. Fibrosis (collagen deposition) is observed in blue.