Satellite cells from dystrophic muscle retain regenerative capacity

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Abstract

Duchenne muscular dystrophy is an inherited disorder that is characterized by progressive skeletal muscle weakness and wasting, with a failure of muscle maintenance/repair mediated by satellite cells (muscle stem cells). The function of skeletal muscle stem cells resident in dystrophic muscle may be perturbed by being in an increasing pathogenic environment, coupled with constant demands for repairing muscle. To investigate the contribution of satellite cell exhaustion to this process, we tested the functionality of satellite cells isolated from the mdx mouse model of Duchenne muscular dystrophy. We found that satellite cells derived from young mdx mice contributed efficiently to muscle regeneration within our in vivo mouse model. To then test the effects of long-term residence in a dystrophic environment, satellite cells were isolated from aged mdx muscle. Surprisingly, they were as functional as those derived from young or aged wild type donors. Removing satellite cells from a dystrophic milieu reveals that their regenerative capacity remains both intact and similar to satellite cells derived from healthy muscle, indicating that the host environment is critical for controlling satellite cell function.

Introduction

Skeletal muscle maintenance, repair, and regeneration are mediated by skeletal muscle stem cells. Although there are several cell types resident in skeletal muscle that can contribute to these processes under certain circumstances (Dellavalle et al., 2011; Meng et al., 2011), the principal skeletal muscle stem cell is the satellite cell, located underneath the basal lamina of a myofiber (Mauro, 1961; Relaix and Zammit, 2012). Satellite cells are normally mitotically quiescent, but can be activated to produce myoblast progeny that will differentiate to repair muscle. In healthy muscle, repair is normally a remarkably efficient process. However, it is likely that satellite cell function is compromised in muscular dystrophies, inherited disorders in which there is a loss of muscle structure and function, leading to weakness and disability (Emery, 2002; Morgan and Zammit, 2010).

In Duchenne muscular dystrophy (DMD), the dystrophin (DMD) gene is mutated, leading to a loss of dystrophin...
protein. In healthy skeletal muscle, dystrophin is present beneath the basal lamina of muscle myofibers and interacts with other members of the dystrophin-associated protein complex (DAPC) to maintain muscle structure and function. It also has a signaling role, including mechanotransduction of forces and localization of signaling proteins within muscle myofibers (Emery, 2002). The absence of dystrophin renders a myofiber prone to damage by mechanical stress, leading to necrosis. Although muscle regeneration occurs, the regenerating myofibers still lack dystrophin and consequently undergo further cycles of degeneration and regeneration, which eventually completely fails, with the muscle tissue becoming substituted by fibrotic/adipose/connective tissue and unable to generate sufficient force (Webster and Blau, 1990). As dystrophin protein is part of the force transduction apparatus of a muscle fiber, it should not be expressed in satellite cells until after they undergo myogenic differentiation (Hoffman et al, 1987). Thus, the lack of dystrophin in DMD will have only an indirect effect on satellite cell function, as it leads to chronic fiber necrosis and consequent activation, proliferation and then differentiation of nearby satellite cells in an increasing hostile dystrophic microenvironment (Morgan and Zammit 2010).

The mdx mouse is a naturally-occurring genetic and biochemical homologue of DMD and has been widely used as an experimental model. Although mdx muscles retain their capacity to regenerate throughout life, certain muscle in old mdx mouse, including diaphragm (Stedman et al, 1991), soleus and plantaris muscles (Pastoret and Sebille, 1993), accurately model DMD, exhibiting muscle fiber loss and severe pathological features such as fat infiltration and extensive fibrosis (Pastoret and Sebille, 1995; Wineinger et al, 1998).

In DMD, satellite cell function may be indirectly affected, through constant recruitment to muscle repair and regeneration and so their regenerative capacity may become exhausted by the progression of the dystrophy with time. This may then synergise with the increasing hostile microenvironment of the dystrophic muscle to prevent effective repair (Morgan and Zammit 2010). We hypothesise that long-term residence within a dystrophic muscle environment has a deleterious effect on satellite cell function. We therefore tested specifically the regenerative potential of satellite cells derived from the dystrophin-deficient mdx mouse model of DMD at different ages.

Satellite cells isolated from young mdx mice were transplanted into a permissive host muscle environment (pre-irradiated muscles of mdx nude mice) (Boldrin et al, 2012; Boldrin et al, 2009; Collins et al, 2005; Neal et al, 2012). Surprisingly, satellite cells from young mdx muscles were able to contribute efficiently to muscle regeneration. We next isolated satellite cells from aged mdx mice to test their capacity to regenerate muscle after long-term residence in a dystrophic environment and found that they too were able to regenerate muscle as efficiently as satellite cells derived from young or aged wild type donors. Our data imply that the impaired muscle regeneration observed in this model of DMD arises mainly from the pathological environment, rather than from endogenous defects in the regenerative capacity of satellite cells.

Materials and methods

Donor satellite cell preparation and grafting

Mice were bred and experimental procedures were carried out in the Biological Services Unit of Institute of Child Health, University College London, and in the Biological Services Unit of Kings College London, in accordance with the Animals (Scientific Procedures) Act 1986.

Donor mice were obtained by breeding either homozygote 3F-nlacZ-2E mice—whose myonuclei express β-gal (Kelly et al, 1995), or heterozygote Myf5gnlacZ/+ mice—that have nlaZ encoding nuclear-localizing β-gal targeted to the Myf5 locus (Tajbakhsh et al, 1996) that identifies the majority of satellite cells (Beauchamp et al, 2000)—with mdx and C57Bl/10 mice. Within muscles grafted with satellite cells derived from 3F-nlacZ-2E mice, β-gal identifies myonuclei of donor origin, whereas in muscles grafted with satellite cells derived from Myf5gnlacZ/+ mice, β-gal marks satellite cells of donor origin in regenerated muscles (Boldrin et al, 2012; Boldrin et al, 2009; Collins et al, 2005).

Satellite cells were isolated from extensor digitorum longus (EDL) muscles of young (2–3 months old) or aged (15 months old for mdx × Myf5gnlacZ/+ and age matched Myf5gnlacZ/+ control mice) donor mice as previously described (Boldrin et al, 2009; Collins and Zammit, 2009). Briefly, the EDL muscles were extracted from tendon to tendon and were then digested in 2% collagenase type I (Sigma)/Dulbecco’s modified Eagles medium (DMEM; Gibco) at 35 °C for 70 min. Muscles were serially washed to separate muscle fibers from cell contaminants and debris. Myofibers were counted and triturated with a 19-gauge needle, to allow release of satellite cells. Satellite cells were separated from debris and contracted myofibers using a 40 μm cell strainer and the resulting cell suspension centrifuged. Cells were then suspended in the desired volume and placed immediately on ice before injection (Boldrin 2013). Approximately 400 satellite cells were grafted into each pre-irradiated tibialis anterior (TA) muscle of 3 week old mdx nude mice, as described previously (Boldrin et al, 2009).

In vivo assay of donor satellite cell functionality

To investigate functionality of grafted satellite cells, host TA muscles were injected with 10 μl of notechis scutatus notexin (10 μg/ml) (Latoxan, France) (Harris, 2003) three weeks after donor cell grafting. Muscles were analyzed a week after notexin injection (Boldrin et al, 2012; Boldrin et al, 2009; Collins et al, 2005; Gross and Morgan, 1999).

Analysis of grafted muscles

TA muscles were analyzed 4 weeks after grafting (Boldrin et al, 2009). Some muscles grafted with mdx × Myf5gnlacZ/+ satellite cells derived from young donor mice (Fig. 2) were fixed in 4% paraformaldehyde for 15 min and then X-gal stained to capture images of the whole TA muscles. All grafted muscles were mounted in gum tradacanth and frozen.
Single fiber immunohistochemistry

Single myofibers isolated from mdx × 3F-nlacZ-2E and mdx × Myf5nlacZ/× donor mice were stained with X-gal and DAPI to determine expression of the reporter gene. To investigate the phenotype of Myf5nlacZ/× satellite cells derived from old mdx × Myf5nlacZ/× and Myf5nlacZ/× mice, single EDL myofibers were isolated from 19 month old mdx × Myf5nlacZ/× male mice, n = 3) and a 19 month old Myf5nlacZ/× male mouse. At least 20 myofibers per muscle were fixed in 4% paraformaldehyde at T0 (time of isolation) and equivalent numbers were kept in plating medium (10% horse serum (Gibco), DMEM (Gibco), 0.5% chick embryo extract, 4 mL L-glutamine (Sigma), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma)) for 24 h (T24). After fixation, myofibers were permeabilized with 0.5% Triton X-100 (Sigma), blocked with 10% goat serum and incubated overnight at 4 °C with primary antibodies: Pax7 (DHSB, mouse monoclonal), MyoD (Santa Cruz, rabbit polyclonal), MyoD (Mouse monoclonal, DAKO). Myofibers were then washed in PBS and incubated with the appropriate Alexa-Fluor secondary antibody. Nuclei were counterstained with DAPI (Sigma) (Moyle and Zammit 2014).

Statistical analysis

Results are expressed as mean ± S.E.M. from the number of samples detailed in the figure legends. Depending on the number of groups compared, one way ANOVA or Mann-Whitney test was used for statistical analysis.

Microscopy

Fluorescence and bright-field microscopy image were captured using a Zeiss Axiophot (Carl Zeiss, UK, http://www.zeiss.co.uk) microscope and Metamorph (Metamorph Productions, UK, http://metamorphproproductions.co.uk) software. Macrosopic pictures of whole X-gal stained muscles were captured with a Leica stereomicroscope (Leica, UK, www.leica-microsystems.com).

Images were assembled into figure panels and minor adjustments to contrast and brightness were made using Adobe Photoshop CS2 (Adobe Photoshop UK, http://www.Adobe.com).

Results

mdx-derived satellite cells contribute to muscle regeneration

To investigate the contribution of satellite cells to muscle regeneration derived from dystrophin-deficient mdx mice, we grafted them into mdx nude hosts. As muscle fibers of mdx donor origin could not be identified by dystrophin expression, mdx mice were bred with 3F-nlacZ-2E transgenic mice, whose myonuclei uniformly express β-galactosidase in vitro (Beauchamp et al, 2000). This marker is effective for identifying a cluster of dystrophin + ve myofibers as being of donor, rather than of host “revertant” origin in transplantation experiments. However, it will underestimate fibers of donor origin, as when donor satellite cells derived from non-dystrophic 3F-nlacZ-2E transgenic mice are transplanted, not all myofibers expressing dystrophin within a transverse cryosection of grafted muscle may contain a β-gal +ve nucleus (Boldrin et al, 2012).

Cells isolated from young mdx × 3F-nlacZ-2E muscles were grafted into TA muscles of mdx nude host mice. Surprisingly, very few X-gal +ve nuclei were detected in muscles grafted with mdx satellite cells (4.5 ± 1.6 myofibers with at least one X-gal +ve nucleus), indicating significantly less donor-derived myofibers (P < 0.05) than those obtained from donor wild-type 3F-nlacZ-2E satellite cells (84 ± 33 myofibers with at least one X-gal +ve nucleus and 223 ± 100 dystrophin + ve myofibers) (Fig. 1A–E). It should be noted that the rare, dystrophin +ve, X-gal -ve myofibers (Fig. 2B) in these muscles grafted with donor cells derived from mdx mice are most likely host, revertant myofibers (Hoffman et al, 1990; Lu et al, 2000; Yokota et al, 2006).

To test the validity of the donor 3F-nlacZ-2E transgene as a marker of muscle in mdx, mdx × 3F-nlacZ-2E isolated EDL myofibers of donor muscles were incubated in X-gal to reveal β-galactosidase activity. This analysis revealed that not all the myonuclei in an isolated myofiber expressed 3F-nlacZ-2E (Fig. 1F), suggesting that this marker is not suitable for quantifying muscle regeneration following grafting of mdx satellite cells. It is possible that the 3F-nlacZ-2E transgene had become inhibited or inactivated during the in vivo cycles of muscle degeneration and regeneration in the donor muscle prior to its use to prepare donor cells for transplantation.

Satellite cells are more numerous in aged mdx than in aged wild-type mice and retain their ability to activate

Old mdx mouse muscles have undergone several rounds of degeneration and regeneration (Pastoret and Sebille, 1995), thus providing a good model to determine whether the
regenerative potential of their satellite cells is compromised as a result.

We first examined the extent of regeneration and hence satellite cell recruitment, in old mdx mice. Single fiber analyses and X-gal/DAPI staining revealed that all EDL myofibers derived from old donor mdx × Myf5\(^{nlacZ/+}\) were regenerated, as 100% of them were branched (Fig. 2A) and contained regions that were centrally-nucleated (Fig. 2C). By contrast, myofibers from age-matched wild type mice were unbranched, with peripherally located myonuclei (Fig. 2B and D). These branched mdx fibers (Fig. 2A) bore 18 ± 2 satellite cells per mdx × Myf5\(^{nlacZ/+}\) myofiber. We have previously shown that old Myf5\(^{nlacZ/+}\) mouse (19–22 months) have 4.4 ± 0.3 Pax7 +ve satellite cells per EDL myofiber (Boldrin et al, 2009). We analyzed a fourth old (19 month) Myf5\(^{nlacZ/+}\) mouse here and found a comparable number (4 ± 1) of satellite cells per myofiber (Table 1). Combining these current data with the aged Myf5\(^{nlacZ/+}\) mice in (Boldrin et al 2009), shows that aged mdx mice have more satellite cells per myofiber than their wild type counterparts (p < 0.0001).

Only a minority of mdx satellite cells expressed MyoD protein at T0, similar to wild type cells (3% and 7% respectively), indicating that the majority of mdx satellite cells was quiescent. When cultured under conditions designed to activate fiber-associated satellite cells (Zammit et al, 2004), we observed that all mdx-derived satellite cells retained their capability to activate similarly to wild type cells, with 100% of them expressing Pax7 and MyoD after 24 h (Table 1). These data indicate that the activation status of mdx and wild type satellite cells was similar at the time of transplantation.

Transplanted mdx satellite cells reconstitute the satellite cell pool comparably to wild-type satellite cells

As a marker of satellite cells of donor mdx origin, we instead used the targeted Myf5 locus, as Myf5\(^{nlacZ/+}\) identifies the majority of satellite cells, but not myonuclei, in mature myofibers (Beauchamp et al, 2000). Satellite cells isolated from young mdx × Myf5\(^{nlacZ/+}\) muscle gave rise to satellite cells expressing the donor satellite cell marker Myf5\(^{nlacZ/+}\) 4 weeks after transplantation into TA muscles of mdx nude recipient mice, as shown by X-gal staining to reveal β-galactosidase activity (Fig. 3A, C). Whole muscle preparations had many satellite cells of donor origin throughout the muscle (Fig. 3A, C), indicating that young transplanted mdx × Myf5\(^{nlacZ/+}\) satellite cells gave rise to satellite cells to a similar extent as wild type transplanted satellite cells derived from sex and age-matched Myf5\(^{nlacZ/+}\) donors (Fig. 3B, D).

Quantification of the number of X-gal positive nuclei underneath the basal lamina of myofibers in representative transverse sections (Fig. 3E and F) confirmed that there was no significant difference between the number of Myf5\(^{nlacZ/+}\) satellite cells derived from mdx or wild type donor mice (28 ± 10 and 16 ± 5 respectively) (Fig. 3G).

Although Myf5\(^{nlacZ/+}\) is transiently expressed in the centrally-located myonuclei of recently-regenerated mouse muscle fibers (Collins et al, 2005), we analyzed our grafted muscles 4 weeks after cell transplantation, when repair/regeneration derived from donor cells would be completed. Cells expressing Myf5\(^{nlacZ/+}\) within these grafts are therefore predominantly, if not exclusively, satellite cells of donor origin. This was confirmed by the combination of X-gal and laminin immunostaining, showing that the Myf5\(^{nlacZ/+}\) cells are in the satellite cell position, i.e. beneath the basal lamina and at the periphery of the myofiber (Fig. 3E and F) rather than in the center of the myofiber, the characteristic location of a myonucleus in a regenarated myofiber.

**Satellite cells derived from aged mdx muscles are as regenerative in vivo as their wild type counterparts**

To explore the in vivo functionality of satellite cells derived from aged mdx × Myf5\(^{nlacZ/+}\) mice compared to aged (or young) Myf5\(^{nlacZ/+}\) mice, satellite cells were grafted into TA muscles of recipient mdx nude mice. Three weeks after transplantation, regenerated muscles were injected with notexin, which destroys myofibers, but spares satellite cells (Harris, 2003), to test whether donor-derived cells had retained the ability to contribute to muscle regeneration. Muscles were analyzed 7 days later by X-gal staining and immunostaining, as this is the time at which mouse myofibers that have regenerated in response to notexin are expressing neonatal myosin heavy chain (MyHC) (Gross and Morgan, 1999). Neonatal MyHC marks newly-regenerated, but not mature, myofibers, permitting ready quantification of the notexin-induced regenerative response. In addition, myonuclei of donor origin will still express Myf5\(^{nlacZ/+}\) a week after the fiber had begun to regenerate (Collins et al, 2005), as this becomes down-regulated only after fiber maturation. Thus, a newly-regenerated myofiber of donor origin expressing neonatal MyHC, whose myonuclei contain β-galactosidase, is evidence that satellite cells of donor origin are functional (Boldrin et al, 2012; Boldrin et al, 2009). We found no difference in the number of newly regenerated myofibers of donor origin (Fig. 4A–I) derived from aged mdx × Myf5\(^{nlacZ/+}\) satellite cells (93 ± 16) compared to young (85 ± 32) or aged Myf5\(^{nlacZ/+}\) (65 ± 32) satellite cells.

As confirmation of the utility of Myf5\(^{nlacZ/+}\) as a marker of myonuclei in newly-regenerated myofibers of donor origin, we used dystrophin as a second marker of muscle fibers of wild-type donor origin. Analysis revealed that all dystrophin positive myofibers were newly regenerated as shown by neonatal MyHC immunostaining (Fig. 4F and I).

**Discussion**

Duchenne muscular dystrophy (DMD) is a chronic and debilitating genetic disorder in which muscle regeneration fails to compensate for the loss of muscle tissue (Emery, 2002).

It has been suggested that inadequate muscle regeneration in muscular dystrophies may be due to loss of satellite cells, which after many rounds of muscle degeneration and regeneration, become ‘exhausted’ (Morgan and Zammit, 2010). In particular, in mdx muscles, the myogenic activity of satellite cells has been reported to be lost with age (Smythe et al, 2008) and the “stem cell” fraction of satellite cells appears to be depleted (Heslop et al, 2000). However, in contrast to those findings, we show that satellite cells...
Figure 1  A–D: Donor-derived muscle formation in host mice (n = 6), whose right TA muscles were grafted with ~400 mdx × 3F-nlaZ-2E donor satellite cells (donors cells pooled from 3 × 2 month old male mice) and left TA with ~400 3F-nlacZ-2E (donor cells combined from 3 × age-matched male mice) donor satellite cells. Representative cryosections stained with X-gal (A and C); serial sections stained with dystrophin (dys, red) revealing a few revertant myofibers (B) and several donor-derived myofibers (D) respectively in muscles grafted with mdx × 3F-nlaZ-2E or 3F-nlacZ-2E donor satellite cells. Nuclei in (B) and (D) were counterstained with DAPI. Scale bar = 100 μm. E: Quantification of dystrophin positive myofibers and myofibers containing at least one X-gal +ve nucleus. F: X-gal stained myofiber isolated from mdx × 3F-nlacZ-2E EDL muscles. Scale bar = 100 μm.
isolated from *mdx* mouse muscles have a similar regenerative capacity and ability to give rise to functional satellite cells as wild type satellite cells, when transplanted into a permissive host muscle environment.

In contrast to DMD patients, skeletal muscles of *mdx* mice retain their ability to regenerate throughout life. Muscle fiber degeneration/regeneration peaks at 3 weeks of age and continues throughout life, albeit with less intensity (Muntoni et al, 1993; Pastoret and Sebille, 1995). Evidence of ongoing muscle degeneration and regeneration can be found in old *mdx* mouse muscles (Chamberlain et al, 2007), with myofibers progressively lost and pathological features becoming more severe with age (Blavas and Carlson, 1991; Pastoret and Sebille, 1993; Pastoret and Sebille, 1995; Pichavant and Pavlath, 2014). It was therefore intriguing to explore the regenerative potential of satellite cells derived from old, rather than young, *mdx* mice, as it could be hypothesised that their capacity to regenerate skeletal muscle would decrease with increasing age. Surprisingly, once transplanted into muscles of young host mice, the contribution of satellite cells derived from aged *mdx* donors to muscle regeneration, as a result of the combination of their engraftment and response to widespread myofiber destruction (notexin injection), was comparable to old and young wild type satellite cells.

We also showed that in the old *mdx × Myf5lacZ/−* mouse, whose muscle pathology is exacerbated by age, satellite cell number per myofiber is not reduced compared to their wild type counterparts (Boldrin et al, 2009). In fact, there were more than 4 fold more satellite cells per myofiber, and although old *mdx* myofibers are branched, they do not have 4 fold the volume of equivalent wild type ones. This is in contrast to a recent paper that showed that satellite cell number/EDL myofiber was elevated in 6 month old C57Bl/10 *mdx* mice (approximately 18 Pax7+ cells/fiber) and reduced thereafter (approximately 5 cells/fiber at 24 months of age) (Jiang et al, 2014). This difference could be due to the genetic background and/or on the sex of the mice analyzed, as male and female adult mouse EDL myofibers bear different number of satellite cells (Neal et al, 2012). However, satellite cell number is also elevated in DMD patients relative to controls (Bankole et al, 2013; Kottlors and Kirschner, 2010). Unlike our findings in *mdx*, reduction of satellite cell number is a hallmark of some other mouse models of muscular dystrophies, e.g. the *lmna−/−* mouse.

Table 1 Number of quiescent (Pax7+) and activated (Pax7+/MyoD+) satellite cells on myofibers isolated from *mdx × Myf5lacZ/−* (17 month old males, *n* = 3) and *Myf5lacZ/−* (19 month old male, *n* = 1) EDL muscles. Percentages of fibers with satellite cells expressing Pax7 or Pax7 and MyoD are also presented. At least 20 fibers were analyzed from each mouse at T0 and T24.

|                | Pax7       | Pax7/MyoD |
|----------------|------------|-----------|
| **Old Myf5lacZ/−** |            |           |
| T0             | 3.9 (±0.5) | 0.3 (±0.15) |
|                | 93%        | 7%        |
| T24            | 0          | 3.2 (±0.29) |
|                | 100%       | 100%      |
| **Old mdx × Myf5lacZ/−** |        |          |
| T0             | 17.46 (±1.47) | 0.5 (±0.16) |
|                | 97%        | 3%        |
| T24            | 0          | 14.56 (±1.48) |
|                | 100%       | 100%      |
whose satellite cell content is decreased compared to wild type mice (Gnocchi et al., 2011).

The vast majority of satellite cells from old mdx mice did not express MyoD, indicating that they are quiescent, as found with wild type satellite cells. The Large<sup>mdx</sup> mouse model of dystroglycanopathy, in which there are defects in the glycosylation of α-dystroglycan (a component of the dystrophin-associated glycoprotein complex) (Muntoni et al., 2004), has significantly more satellite cells than wild type control muscles, at 2–3 months of age (Ross et al., 2012). Unlike mdx, however, significantly more satellite cells in Large<sup>mdx</sup> mouse muscles are activated (expressing MyoD) or differentiating (expressing myogenin) (Ross et al., 2012). Earlier differentiation has also been observed for mdx

Figure 3  A–D: Representative images of X-gal stained whole muscles (n = 8) grafted with either ~400 mdx × Myf5<sup>lacZ</sup>/+ (isolated from a 2 month old male mouse) (A, magnified in C) or ~400 Myf5<sup>lacZ</sup>/+ (isolated from a 2 month old male mouse) donor satellite cells (B, magnified in D). A, B: 2× magnification; C–D: 5× magnification. E, F: X-gal stained nuclei on cryosections immunostained for laminin. Scale bar = 100 μm. G: Numbers of donor-derived satellite cells per section.
Figure 4  Representative transverse sections from 12 mice (1-12), whose right TA muscles were grafted with ~400 satellite cells derived from old $mdx \times Myf5^{lacZ/+}$ mice (donor cells pooled from 2 × 15 month old males) (A, A', B, B'). Of the same 12 mice, 7 left TA muscles (1-7) were engrafted with ~400 old $Myf5^{lacZ/+}$ (15 month old male, $n = 1$) (D, D', E, E') and the remaining 5 left TA (8-12) muscles were engrafted with ~400 young $mdx \times Myf5^{lacZ/+}$ (3 month old male, $n = 1$) (G, G', H, H') mice. Sections were either stained with X-gal (A A', D, D', G, G') or immunostained for neonatal MyHC (nMyHC, green) and dystrophin (dys, red) (B, B', E, E', H, H'). Boxed area in A, B, D, E, G and H shown at higher magnification in A', B', D', E', G', H'. Scale bar = 100 μm. (C, F, I) depict numbers of myofibers with at least one X-gal +ve nucleus (blue, of donor origin) and neonatal MyHC (green) (i.e. myofibers of donor origin and newly regenerated). (F, I) Where $Myf5^{lacZ/+}$ were transplanted, dystrophin was also quantified. Nuclei in B, E, H and B', E', H' were counterstained with DAPI.
satellite cells (from 8 to 11 week old mice) expanded in vitro, compared to satellite cells derived from control, wild type mice (Yablonka-Reuveni and Anderson, 2006).

Factors within the host dystrophic environment that may be detrimental to effective muscle regeneration include the inflammatory milieu and/or fibrotic environment (Abou-Khalil et al., 2010; Boldrin et al., 2009; Emery, 2002; Mann et al., 2011). For effective donor-derived muscle regeneration, the host muscle environment has to be modulated (Boldrin et al., 2012; Boldrin et al., 2009; Brimah et al., 2004; Morgan et al., 2002). In the in vivo model that we use to assay donor satellite cell function, the recipient muscles had been pre-irradiated in order to create an environment permissive to donor-derived muscle regeneration (Boldrin et al., 2009; Collins et al., 2005), by reducing competition with endogenous satellite cells and preserving the host satellite cell niche (Boldrin et al., 2012). Modifications of the host muscle environment are indeed critical to permit effective muscle regeneration (Boldrin et al., 2009; Collins et al., 2005). For example, it has recently been shown that miR-29 expression is down-regulated in muscles of mdx mice; by restoring its expression, the dystrophic pathology improves, as regeneration is promoted and fibrogenesis inhibited (Wang et al., 2012).

In summary, we found that satellite cells from mdx mice retain their capacity to contribute to muscle regeneration within pre-irradiated muscles of mdx nude host mice. This is evidence that, when removed from the pathological donor muscle environment and placed within a permissive milieu, satellite cells retain their muscle stem cell properties and contribute effectively to muscle regeneration. Therefore, we conclude that the dystrophic muscle environment is deleteriously affecting satellite cell-derived muscle regeneration. This suggests that dystrophic muscle could be modified to improve either endogenous muscle regeneration, or for therapy involving engraftment of stem cells.

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References

Abou-Khalil, R., Mounier, R., Chazaud, B., 2010. Regulation of myogenic stem cell behavior by vessel cells: the “menage a trois” of satellite cells, periendothelial cells and endothelial cells. Cell Cycle 9, 892–896.

Bankole, L.C., Feasson, L., Pensot, E., Kadi, F., 2013. Fibre type-specific satellite cell content in two models of muscle disease. Histopathology 63, 826–832.

Beauchamp, J.R., Heslop, L., Yu, D.S., Tajbakhsh, S., Kelly, R.G., Wernig, A., Buckingham, M.E., Partridge, T.A., Zammit, P.S., 2000. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J. Cell Biol. 151, 1221–1234.

Blalivas, M., Carlson, B.M., 1991. Muscle fiber branching—difference between grafts in old and young rats. Mech. Ageing Dev. 60, 43–53.

Boldrin, L., Zammit, P.S., Muntoni, F., Morgan, J.E., 2009. Mature adult dystrophic mouse muscle environment does not impede efficient engrafted satellite cell regeneration and self-renewal. Stem Cells 27, 2478–2487.

Boldrin, L., Neal, A., Zammit, P.S., Muntoni, F., Morgan, J.E., 2012. Donor satellite cell engraftment is significantly augmented when the host niche is preserved and endogenous satellite cells are incapacitated. Stem Cells 30, 1971–1984.

Boldrin, L., Morgan, J.E., 2013. Modulation of the host skeletal muscle niche for donor satellite cell grafting. Methods Mol. Biol. 1035, 179–190.

Brimah, K., Ehhardt, J., Mouly, V., Butler-Browne, G.S., Partridge, T.A., Morgan, J.E., 2004. Human muscle precursor cell regeneration in the mouse host is enhanced by growth factors. Hum. Gene Ther. 15, 1109–1124.

Chamberlain, J.S., Metzger, J., Reyes, M., Townsend, D., Faulkner, J.A., 2007. Dystrophin-deficient mdx mice display a reduced life span and are susceptible to spontaneous rhabdomyosarcoma. FASEB J. 21, 2195–2204.

Collins, C.A., Zammit, P.S., 2009. Isolation and grafting of single muscle fibres. Methods Mol. Biol. 482, 319–330.

Collins, C.A., Olsen, I., Zammit, P.S., Heslop, L., Petrie, A., Partridge, T.A., Morgan, J.E., 2005. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. Cell 122, 289–301.

Delavalle, A., Maroli, G., Cavallero, D., Azzoni, E., Innocenzi, A., Perani, L., Antonini, S., Sambasivan, R., Brunelli, S., Tajbakhsh, S., Cassu, G., 2011. Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells. Nat. Commun. 2, 499.

Emery, A.E., 2002. The muscular dystrophies. Lancet 359, 687–695.

Gnocchi, V.F., Scharner, J., Huang, Z., Brady, K., Lee, J.S., White, R.B., Morgan, J.E., Sun, Y.B., Ellis, J.A., Zammit, P.S., 2011. Uncordinated transcription and compromised muscle function in the lnma-null mouse model of Emery–Emery–Dreyfus muscular dystrophy. PLoS ONE 6, e16651.

Gross, J.G., Morgan, J.E., 1999. Muscle precursor cells injected into irradiated mdx mouse muscle persist after serial injury. Muscle Nerve 22, 174–185.

Harris, J.B., 2003. Myotrophic phospholipases A2 and the regeneration of skeletal muscles. Toxicicon 42, 933–945.

Heslop, L., Morgan, J.E., Partridge, T.A., 2000. Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. J. Cell Sci. 113 (Pt 12), 2299–2308.

Hoffman, E.P., Knudson, C.M., Campbell, K.P., Kunkel, L.M., 1987. Subcellular fractionation of dystrophin to the triads of skeletal muscle. Nature 330, 754–758.

Hoffman, E.P., Morgan, J.E., Watkins, S.C., Partridge, T.A., 1990. Somatic reversion/suppression of the mouse mdx phenotype in vivo. J. Neurol. Sci. 99, 9–25.

Jiang, C., Wen, Y., Kuroda, K., Hannon, K., Rudnicki, M.A., Kuang, S., 2014. Notch signaling deficiency underlies age-dependent depletion of satellite cells in muscular dystrophy. Dis. Model. Mech. 7, 997–1004.

Kelly, R., Alonso, S., Tajbakhsh, S., Cassu, G., Buckingham, M., 1995. Myosin light chain 3F regulatory sequences confer regionalized cardiac and skeletal muscle expression in transgenic mice. J. Cell Biol. 129, 383–396.

Kottlors, M., Kirschner, J., 2010. Elevated satellite cell number in Duchenne muscular dystrophy. Cell Tissue Res. 340, 541–548.

Lu, Q.L., Morris, G.E., Wilton, S.D., Ly, T., Artem'yeva, O.V., Strong, P., Partridge, T.A., 2000. Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. J. Cell Biol. 148, 985–996.

Lu, Q.L., Rabinowitz, A., Chen, Y.C., Yokota, T., Yin, H., Alter, J., Jadoon, A., Bou-Gharios, G., Partridge, T., 2005. Systemic delivery of
antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. Proc. Natl. Acad. Sci. U. S. A. 102, 198–203.
Mann, C.J., Perdiguerro, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A.L., Munoz-Canoves, P., 2011. Aberrant repair and fibrosis development in skeletal muscle. Skelet. Muscle 1, 21.
Mann, C.J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A.L., Munoz-Canoves, P., 2011. Aberrant repair and fibrosis development in skeletal muscle. Skelet. Muscle 1, 21.
Mann, C.J., Perdiguero, E., Kharraz, Y., Aguilar, S., Pessina, P., Serrano, A.L., Munoz-Canoves, P., 2011. Aberrant repair and fibrosis development in skeletal muscle. Skelet. Muscle 1, 21.
Meng, J., Adkin, C.F., Xu, S.W., Muntoni, F., Morgan, J.E., 2011. Contribution of human muscle-derived cells to skeletal muscle regeneration in dystrophic host mice. PloS ONE 6, e17454.
Morgan, J.E., Zammit, P.S., 2010. Direct effects of the pathogenic mutation on satellite cell function in muscular dystrophy. Exp. Cell Res. 316, 3100–3108.
Morgan, J.E., Gross, J.G., Pagel, C.N., Beauchamp, J.R., Fassati, A., Thrasher, A.J., Di Santo, J.P., Fisher, I.B., Shiwen, X., Abraham, D.J., Partridge, T.A., 2002. Myogenic cell proliferation and generation of a reversible tumorigenic phenotype are triggered by preirradiation of the recipient site. J. Cell Biol. 157, 693–702.
Moyle, L.A., Zammit, P.S., 2014. Isolation, culture and immunostaining of skeletal muscle fibres to study myogenic progression in satellite cells, Methods Mol. Biol. 1210, 63–78.
Muntoni, F., Mateddu, A., Marchei, F., Clerk, A., Serra, G., 1993. Muscular weakness in the mdx mouse. J. Neurol. Sci. 120, 71–77.
Muntoni, F., Brockington, M., Torelli, S., Brown, S.C., 2004. Defective glycosylation in congenital muscular dystrophies. Curr. Opin. Neurol. 17, 205–209.
Neal, A., Boldrin, L., Morgan, J.E., 2012. The satellite cell in male and female and adult mouse muscle: distinct stem cells for growth and regeneration. PLoS ONE 7, e37950.
Pastoret, C., Sebille, A., 1995. mdx mice show progressive weakness and muscle deterioration with age. J. Neurol. Sci. 129, 97–105.
Pichavant, C., Pavlath, G.K., 2014. Incidence and severity of myofiber branching with regeneration and aging. Skelet. Muscle 4, 9.
Relaix, F., Zammit, P.S., 2012. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. Development 139, 2845–2856.