Stable Integration of an mdx Skeletal Muscle Cell Line into Dystrophic (mdx) Skeletal Muscle: Evidence for Stem Cell Status

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Abstract
We have previously described a method for the derivation of long term cultures of undifferentiated myoblasts from the skeletal muscle of dystrophic (mdx) mice (J. Smith and P. N. Schofield, Exp. Cell Res., 210: 98–93, 1994). We now show that a clonal mdx-derived skeletal muscle cell line labeled with a retrovirus conferring β-galactosidase activity and G418 resistance (PD50A) is capable of incorporation into mdx skeletal muscle myofibers for up to 14 months with no incidence of tumor formation. After a lag period of 5 days, injected PD50A cells disperse throughout the injected tibialis anterior muscle and take up satellite cell positions on the perimeter of myofiber bundles. PD50A cells begin to incorporate into fused muscle syncytium as early as 8 weeks after injection and persist for at least 14 months. We have rederived myoblasts expressing β-galactosidase from PD50A-injected muscles 12 months after injection, demonstrating that a reserve of mononuclear proliferation-competent PD50A cells are present in host muscle up to a year after their original introduction. These data support the contention that myoblasts derived by this culture method are functionally representative of a class of skeletal muscle “stem cells” and thus have potential both as agents for cellular therapy of intragenic diseases such as Duchenne muscular dystrophy as well as being a useful tool for the further investigation of normal muscle development.

Introduction
Cell therapy, by means of transplanting precursor or stem cells, has been suggested as a treatment for a variety of genetic diseases including Duchenne muscular dystrophy (1, 2). A prerequisite of such an approach must be the availability of stem or precursor cells appropriate to the disease target. Duchenne muscular dystrophy (3), which is primarily a pathology of skeletal muscle, has been identified as an important candidate for such an approach (4) because of its relative frequency (1 of 3500 male live births) and high rate of new incidence (5). Progress in this field has, however, been restricted because of the lack of an appropriate muscle stem cell culture system. To date the majority of myogenic cells used in muscle repopulation experiments (both mouse and human) have been derived either from bulk primary cultures (1, 2, 6), where reproducibility and cell numbers are a problem, or from established clonal muscle cell lines such as C2 or F8B (7, 8). Without exception, the established lines previously reported to incorporate into fused muscle are tumorigenic and generate rhabdomyosarcomas in the injected muscle within the first 6 months after implantation. More recently, Morgan et al. (9) have reported the use of primary muscle cultures derived from transgenic mice carrying a thermolabile SV40 T-antigen gene (10). Expression of this gene at permissive temperatures immortalizes cells in culture, but as they differentiate in vivo, this approach can produce large numbers of myogenic cells but is probably inappropriate for therapeutic use in humans.

An additional problem that needs to be addressed is the extent of incorporation of grafted muscle cells into the target muscle fibers. Without irradiation (11), toxins (8, 12), or mechanical induction of muscle damage, incorporation rates of injected cells into differentiated skeletal muscle are generally poor, even in mice and with proportionally (to muscle size) huge numbers of cells being injected (107 cells/muscle, Ref. 2; 5 × 105 cells per muscle, Ref. 9). The few experiments using human cells injected into human muscle (6, 13) resulted in disappointingly low incorporation levels of injected (dystrophin-positive) myoblasts, and these seemed to be largely confined to the area of injection. In addition, either immunosuppression, needle-assisted muscle damage, or both have been used in these studies. Injection of cells with stem cell characteristics would obviate the need for the injection of large numbers of cells into pre-damaged muscles because they would be expected to migrate to take up appropriate positions within the injected muscle and to undergo repeated divisions. This would enable a long-term therapeutic approach because the stem cell population would be expected to retain a small pool of undifferentiated cells, which would differentiate as required during fiber turnover. An important additional benefit of such an approach would be that true stem cells do not form tumors in an appropriate environment.

We have developed a method of deriving long-term primary cultures (14) from the skeletal muscles of both normal and dystrophic mice, which retain the features of the original “satellite” cell. Using previously validated principals of stem cell culture (15, 16), we have been able to readily derive...
clonal cell lines from such cultures without subjecting the cells to the extreme selective pressure used previously for the isolation of skeletal muscle myoblasts (17). Using β-galactosidase tagging, we have been able to demonstrate the apparent stem cell properties of one of these cell lines in vivo.

Results

Isolation of β-Galactosidase Expressing mdx Skeletal Myoblast Cell Lines. Clonal myoblast cell lines were derived from established passaged primary stocks (dfd13) of undifferentiated, but fusion-competent, mdx skeletal myoblasts as described elsewhere (14). To retain the proliferative capacity of these cell lines, we cloned them in conditioned medium prepared from passaged primary mdx myoblasts supplemented with 20% FCS. The culture medium was depleted for calcium (70 μM CaCl₂) to inhibit myoblast fusion and to reduce the likelihood of selecting for fusion-impaired clones. Myoblasts that have been cultured in this way retain both the morphological and antigenic properties of undifferentiated skeletal muscle cells. When cultured at reduced densities, SMS cells become bipolar and line up end-to-end, prior to fusion, to form a multinucleate syncitium. At this stage, the cells begin to express skeletal muscle-specific myosin (Fig. 1A). Both passaged primary mdx cultures and clonally derived myoblast (SMS) lines were uniformly non-staining with anti-dystrophin (18) antibodies (Fig. 1B) and positive for an antibody (19) directed against the dystrophin-related protein, also known as utrophin (Fig. 1, C–D).

To select clones stably expressing β-galactosidase, we infected subconfluent cultures of putative clones with 50% virus containing medium taken from pIRV producer cells (pec/pIRV) in the presence of Polybrene. Transient expression of β-galactosidase was observed in pIRV-infected myoblasts fixed at 36 h after infection, and these cells retained their immunogenicity for the skeletal muscle intermediate filament desmin. Remaining cultures were placed into G418 selection at 36 h and cultured for 2 weeks until noninfected cells began to die. Surviving colonies were observed in all dishes except control (0% virus) cultures. Cultures were maintained in G418 selection until resistant colonies were large enough (20–50 cells) for picking off with a drawn-glass pipette. At this stage, several colonies per dish were picked off and expanded further. Six of these cultures were subject to a further round of clonal selection before they were expanded and characterized. One of these was PD50A. All of the lines thus derived expressed cytoplasmic β-galactosid-
ase protein as assessed by histochemistry; PD50A was selected for injection experiments because of its uniform and intense cytoplasmic staining (Fig. 2B) for β-galactosidase. Morphologically, these cells were indistinguishable from the mdx passaged primary cultures from which they were derived. To determine the stability of β-galactosidase retrovirus integration into the PD50A line, we tested supernatant from these cells on the mouse fibroblast cell lines NIH-3T3TK− and NR6, as well as the parent myoblast cell line Dfd13, at a range of concentrations up to 100% and in the presence of Polybrene. In no experiment did we find either blue cells or surviving colonies under G418 selection indicative of the presence of virus. We thus conclude that β-galactosidase integration in the PD50A cell line was stable and that helper virus was not present.

Examination of metaphase spreads of PD50A revealed that these cells were essentially diploid with a stable modal chromosomal number of 40. Closer examination (Fig. 2B) revealed that there was a small amount of additional chromosomal material on two of the smaller acrocentric chromosomes. This may be due to either a reduplication of the smaller chromosomal material or may represent additional satellite material. The karyotype presented in Fig. 2 has been stable in this line for the time during which it has been used experimentally, i.e., karyotypes of cells of early passage PD50A do not differ from those of later passage cultures.

Survival of PD50A Myoblasts in mdx TA3: 0–8 weeks.
To determine the effect of graft size on survival of PD50A cells in vivo and to determine the minimum number of cells required to obtain reasonable incorporation of PD50A into muscle, we injected three different concentrations (400, 4000, and 40 000 per 10 μl) of PD50A cells into mouse TA. To avoid potential complications with the immune system, we used mdx mice for all of these injection experiments. Two mice (one male and one female) from each of these three injection groups were killed 5 days after injection, and both right and left TAs were subject to complete serial section and β-galactosidase histochemistry. In no cases did we observe blue staining in the sections from the contralateral (right TA) muscles. Examination of the injected (left TA) muscles (Fig. 3) revealed that grafts remained largely intact at the injection site after 5 days with little dispersal observed, although cells were apparently beginning to disperse in the larger graft (Fig. 3D). The majority of cells appeared to be unfused at this stage (Fig. 3, E–G). The size of the blue staining area of cells was roughly related to the number of cells originally injected (Fig. 3, compare A–C), and counting the number of blue staining cells present in serial sections of the muscle of the two mice injected with 400 cells per TA (Fig. 3C) yielded a mean number of 398 ± 17 (SD), which suggested that virtually all of the PD50A cells injected (at least at this concentration) had survived almost 1 week after injection or that division had kept pace with cell death. This finding suggests the absence of extensive in vivo selection against cells of this cloned cell line, which may occur in cell injection experiments using primary cultures or uncloned cell lines.

An additional three animals (one from each group) were killed at 2 months after injection of PD50A cells. By 8 weeks after injection, blue-staining (PD50A) cells were dispersed throughout the injected TA, and some appeared to be incorporated into fused muscle fibers (Fig. 3H). Blue-staining cells were also observed in satellite cell positions and adjacent to muscle fibers (Fig. 3H). In muscles that had been injected with the higher concentrations of cells (4,000 and 40,000), we also noted a small bolus of undispersed cells (Fig. 3H), which appeared to be the remnants of the original injection. These small foci of cells were only observed at this time point and were not associated with any signs of inflammatory changes. These foci were not associated with increased levels of PD50A incorporation into adjoining muscle fibers. No other differences were observed between animals injected with different numbers of cells.

To further characterize the distribution of PD50A within mdx TA at this time point, we counterstained some sections with hematoxylin to illustrate the position of nuclei. Examination of these sections enabled the confirmation of the presence of nucleated, single cells situated in satellite positions around the periphery of mature skeletal muscle fibers with β-galactosidase-positive cytoplasmic staining and of

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3 The abbreviations used are: TA, tibialis anterior; mGM, myoblast growth medium; DAB, 3,3′-diaminobenzidine; DAPI, 4,6-diamidino-2-phenylindole.
newly fused β-galactosidase-positive myofibers present within mature myofiber bundles (Fig. 4A).

**Long-Term Incorporation.** The remaining four injected mice were killed at 10 months (n = 2), 12 months (n = 1), and 14 months (n = 1) and autopsied. We found no evidence of tumor formation or other graft-related pathology in any of these mice. Examination of the left TA of injected animals at both 10 and 14 months after injection of PD50A revealed the extensive presence of β-galactosidase-positive cells in these muscles; these were well dispersed throughout the entire TA. PD50A cells appeared to be integrating into fused muscle and freely mixing with myofibers already present. Labeling paraformaldehyde-fixed sections with antibodies to β-galactosidase (Fig. 4B) confirmed this observation. Using this technique in combination with H&E staining of these sections clearly demonstrated that uninucleate β-galactosidase-expressing cells were also present in injected muscles (Fig. 4C), these single-nucleate, β-galactosidase-positive cells appeared to be located in the satellite cell position around the periphery of muscle fiber bundles but within the perimysium. No staining was observed in muscle from the contralateral legs of these animals stained with anti-β-galactosidase antibody (Fig. 4D).

**Recovery of PD50A from Injected Muscle.** To unequivocally demonstrate the presence of single proliferating PD50A myoblasts in injected mdx TA, we have cultured mdx TA from mice injected 12 months previously with PD50A cells. Both injected and noninjected muscles were cultured and readily yielded primary myoblasts, which were then stained for β-galactosidase (Fig. 4E). Approximately 2–5% of myoblasts from cell cultures from the left (PD50A)-injected TA stained positively for β-galactosidase, whereas no such staining was observed in cultures derived from the right (saline-injected) TA. Some of these cells were then placed into G418 selection to isolate a cell line (PDR).
Table 1 Repopulation of/mdx TA with PD50A muscle stem cells

<p>| Time after | No. of cells | Gender | No. of fibers | % positive fibers (± SD) |</p>
<table>
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<th>injection (mo)</th>
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<tr>
<td>1</td>
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<td>Female</td>
<td>414</td>
<td>31 ± 17</td>
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<td>40,000</td>
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<td>29 ± 12</td>
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<tr>
<td>2</td>
<td>400</td>
<td>Female</td>
<td>1131</td>
<td>71 ± 31</td>
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<tr>
<td>10</td>
<td>4,000</td>
<td>Female</td>
<td>254</td>
<td>52 ± 18</td>
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<tr>
<td>10</td>
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<td>34 ± 18</td>
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<td>14*</td>
<td>4,000</td>
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<td>1071</td>
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* Assessed in muscle stained with anti-β-galactosidase antibody. The remaining data were obtained from muscle stained histochemically for β-galactosidase.

Extent of Incorporation. To assess the efficiency of PD50A incorporation into /mdx TA, we have counted the number of positive and nonpositive fibers present in nonadjacent sections of injected muscle (Table 1). Nonadjacent sections were counted for two reasons: (a) to maintain consistency with previous reports of such estimates (20), which also used this strategy, and (b) to minimize the number of fibers counted more than once and to ensure a representative spread throughout the muscle. PD50A positive cells were found in all of the injected muscles examined (13 animals in total) and in all of the animals maintained for more than 5 days (7 in total). The percentage of positive fibers varied from 20 to 71% with a mean incorporation of 39.6 ± 15.7%. However, the level of incorporation and evenness of distribution were greater in animals sacrificed at 10 months onward (see Table 1), suggesting a gradual colonization of muscle over time, whereas in animals sacrificed at 2 months there was considerable unevenness in the distribution of PD50A-positive fibers. Neither dispersal nor colonization frequency of PD50A cells in TA muscles appeared to be significantly influenced by the gender of the animal injected. To our surprise, we also found that the extent of colonization in those muscles injected with 400 cells was comparable to that seen in muscles injected with 4,000 or 40,000 cells. We found no β-galactosidase incorporation in adjacent muscles or in the muscles of the contralateral leg control, which was examined carefully in every animal. Furthermore, NIH 3T3T K- or Dfd13 cells incubated with either blood samples from injected animals taken at sacrifice or with supernatant taken from rederived (PDR) cell cultures were killed by G418 and did not stain positively for β-galactosidase. These data also demonstrate the absence of systemic or local free β-galactosidase retrovirus and, therefore, helper virus, in PD50A-injected mice.

Discussion

We injected 13 animals (6 males and 7 females) with PD50A myoblasts. All of these animals had blue-staining muscle fibers and single cells located on the periphery of fiber bundles. Retention of unfused marked cells has been reported.
Integration of Skeletal Muscle Stem Cells

previously as a rare event following injection of immortalized cells (9) and also after injection of uncloned bulk primary cell cultures (2), where clonal selection might be expected. None of our injected animals developed tumors, despite maintaining four of these animals for more than 10 months. This is in direct contrast to injections of C2 cells (the best studied of injectable muscle cell lines) which, in common with other myogenic cell lines (8), will form sarcomas as early as 2 weeks after their injection into mouse skeletal muscle (7) with virtually 100% frequency. We suggest that the nontumorigenic nature of PD50A cells is likely to be a result of their close relationship to muscle satellite or "stem" cells, as demonstrated by their normal karyotype, positive staining for utrophin, and the presence of single, uninucleate PD50A cells located around the periphery of muscle fiber bundles in muscles injected many months previously.

We have demonstrated that in vivo PD50A is able to contribute both to differentiated myofibers and the mononuclear stem cell population over 14 months. Proliferation competent cells can be recovered from the muscle after this period of time, indicating the functional stem cell status of this clone. An important feature of these experiments was the lag period observed between PD50A injection and dispersal and fusion of the injected cells. The gender of the animals did not appear to affect the percentage of incorporation of PD50A cells, and the injection of 400 cells was found to be comparable to the injection of $4 \times 10^6$ or $4 \times 10^5$ cells in inducing widespread incorporation of PD50A into muscle fibers. This observation is potentially of great importance to future therapeutic strategies and suggests that PD50A cells may be autoregulated in vivo.

It is important to differentiate between progenitor cells determined to form specific muscle fiber isoforms and committed muscle stem cells with a wider potential range of fates. The experiments of Hughes and Blau (21) demonstrate that there may be several uninucleate cell types in neonatal and adolescent rodent skeletal muscle of which only some will have characteristics of pluripotential stem cells; the remainder are determined to form specific fiber isoforms. Within the context of adult or neonatal muscle, these populations are termed respectively "muscle stem cells" or "myoblasts." This distinction may go some way to explaining the wide variation in repopulation efficiencies observed in many experiments using primary cultured muscle cells or partially differentiated myoblast or myogenic cell lines (1, 2, 22, 23). We have also recently identified a potential further complication of cell replacement studies, that of increased programmed cell death or apoptosis (24). Programmed cell death is up-regulated in dystrophic (mdx) skeletal muscle and is under the control of specific peptide growth factors. This phenomenon is reflected in the stem cell cultures and cell lines derived from these muscles, suggesting that it is the survival and proliferation of this group of cells that is perturbed in dystrophic muscle. Consequently, further investigation of the control of this process in vivo would be of importance in establishing the significance of these data for future therapeutic strategies.

Although repopulation of muscle with wild-type or genetically repaired myogenic cells seems to be the most powerful approach, injection of cells into either human or mdx dystrophic muscle have to date resulted in very poor colonization (25). The induction of muscle damage by X-irradiation facilitates repopulation, which has lead to the suggestion that poor results from the injection of unlesioned muscle are related to the state of the host muscle rather than the intrinsic capacity of the injected cells to act as stem cells. Our data suggests that it is the intrinsic stem cell status of the grafted cells that determines the efficiency of colonization. Similar considerations have been important in the isolation of stem cells and committed progenitors in the immune system (15).

The dispersal of PD50A cells from the graft site is much slower than that reported for the injection of primary muscle cells or established cell lines (2, 9), and many previous experiments have been carried out over a much shorter time course, the end point often determined by the time taken to form tumors (8). It is apparent from our data that complete dispersal and incorporation of injected stem cells into muscle can take weeks to months. Earlier experiments may have therefore underestimated the potential extent of repopulation of injected cell grafts. However, to date, we have not observed migration of cells from the injected muscle (TA) to underlying or adjacent tissues such as the extensor digitorum longus, as reported by Watt et al. (26). We have shown, however, that after an 8–12-week lag period, PD50A cells can disperse very efficiently throughout the target muscle and are able to contribute stably to host muscle fibers for more than one-half of the life span of the mdx mouse. Furthermore, it is clear that once injected, these cells will persist without pathological consequences for some considerable time. It is likely that this persistence is due to the ability of these cells to remain in an undifferentiated, uninucleate state within the injected muscle.

The retention of proliferative capacity in the absence of transformation of cells derived under these conditions has assisted enormously in estimating the repopulation ability of PD50A and enabled much longer term experiments to be carried out than previously. Although PD50A and similar clones are, therefore, a powerful tool for transferring genes into dystrophic muscle, these data also have useful implications for the derivation of cell lines for human therapeutic program and the time course over which such program should be extended. These studies also provide insight into the behavior of normal adult muscle stem cells in vivo, which may have important implications for tumors of skeletal muscle origin.

Materials and Methods

Isolation of PD50A. mdx skeletal explant cultures were established and maintained as described previously (14). A long term (uncloned) passaged primary culture (designated df13) was derived from the skeletal muscle of 5-week-old mdx mice by this method and was frozen at passage 11. Myoblast cultures were maintained in mGM [a 50:50 mix of DMEM and Ham's F-12 medium supplemented with 20% (v/v) batch tested, heat-inactivated FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin]. To maintain survival and an undifferentiated phenotype when cultured at low densities ($10^5$–$10^6$ cells/cm²), myoblasts were grown in a 50:50 mix of mGM and myoblast conditioned medium (see Ref. 14 for further details). To introduce the β-galactosidase tag to PD50A cells, we infected cells with pHR (27, 28). pHR is a replication-defective retrovirus that carries the genes for both β-galactosidase and G418 resistance,
driven from an internal promoter dependent of the retroviral transcription machinery. Therefore, there is no requirement to carry a nuclear localization construct; therefore, all of the β-galactosidase activity detected in a cell line will be cytoplasmic rather than nuclear.

To infect mdx myoblasts with the pIRV retrovirus, dfd13 cells were plated out in 60-mm tissue culture dishes at a density of 5 × 10^5 cells/cm² and allowed to attach overnight. This plating regime yielded subconfluent cultures of dfd13 myoblasts. Preincubation with Polybrene to enhance viral infectivity was carried out on these cultures because prolonged treatment of myoblast cultures with Polybrene was found to be detrimental to their survival. At the same time, subconfluent cultures of the pIRV virus producer cell line (pec/pIRV, a gift from Dr. Rosal Beddington, National Institute of Medical Research, Mill Hill, London, United Kingdom) were incubated overnight in mGM containing (3 μg/ml) Polybrene to encourage shedding of virus. The following morning medium was collected from Polybrene-treated pec/pIRV cultures, and this was then filtered to remove cell debris. Filtered viral supernatant was then added to subconfluent cultures of dfd13 myoblasts at the following concentrations: 0, 25, 50, 75, and 100% viral supernatant made up to 100% with mGM supplemented with (3 μg/ml) Polybrene. Plates were then incubated at 37°C/5% CO₂ for 2 h, when they were refed with mGM without Polybrene. After an additional 24 h incubation, medium was replaced with G418 selection medium for 2 weeks until sizable colonies of G418-resistant cells had formed in the dishes and the remaining cells had been shed. G418 selection medium was 50% mGM and 50% myoblast conditioned medium with 800 μg/ml G418. G418 was replenished every 2 days during selection. Colonies were then picked off the dish using a drawn Pasteur pipette and expanded in myoblast conditioned medium. These cultures were then subject to a further round of clonal selection in G418-containing medium. One colony per original infection culture was expanded until sufficient cells were available for freezing. A series of β-galactosidase-expressing cell lines were derived by this method. In all of them, the β-galactosidase was found to be cytoplasmic. One of these cell lines (PDS50A) was used in vivo experiments and was maintained in culture from frozen stocks and used between passages 4 and 16. To avoid cell culture adaptation and transformation or senescence, no attempts were made to use PDS50A at passage numbers beyond 20. All cell lines used were found to be free of Mycoplasma contamination. The assay for Mycoplasma was conducted regularly in house as described previously (29).

β-Galactosidase Histochromy. Glutaraldehyde-fixed sections or cells (prepared as described below) were washed briefly in buffered detergent [2 mM MgCl₂, 0.001% (w/v) sodium deoxycholate, and 0.002% (v/v) NP40 in PBS] before overnight incubation in reaction mixture (0.2 mg/ml 5-bromo-4-chloro-3-indolyl–β–D-galactopyranoside (Sigma Chemical Co.), 1.6 mg/ml potassium ferricyanide, and 2.1 mg/ml potassium ferrocyanide made up in buffered detergent as above). After air drying, slides were mounted in DePeX for microscopy. Slides were either counterstained with hematoxylin for visualization of nuclei or with Metanil Yellow (1% in aqueous solution; Sigma).

Host Mice. C57Bl/10 (mdx) mouse stocks maintained in house and generated from a nucleus of animals generously provided by the IAPGR (Roslin Research Institute, Roslin, Edinburgh) were used as hosts for injections of PD50A. Seven female and six male mdx mice, 8 weeks of age, were anesthetized with Avertin (0.1 ml stock solution per 10 g of body weight) and received injections i.m. into the TA of the left leg with a sterile 10-μl solution of PDS50A in PBS using a microinjection pump delivering 10 μl in 8 min. The contralateral (right) leg was used as a control. Animals were divided into three injection groups on the basis of the number of cells injected: male and female animals were distributed evenly among the three groups, with the extra female being placed in group 2: group 1 (n = 4) injected with 400 cells/leg TA; group 2 (n = 5) injected with 4,000 cells/leg TA; and group 3 (n = 4) injected with 40,000 cells/leg TA. Five days after injection, one male and one female animal from each group (n = 6) were killed, and both right and left TAs were removed. Frozen sections (8 μm) were prepared from snap-frozen tissue dissected from injected and contralateral mdx TAs. Frozen transverse sections were made of the entire muscle and placed onto gelatin-subbed slides within 24 h of removal from the animal. Sections were then fixed in 0.5% (v/v) glutaraldehyde in PBS and subjected to β-galactosidase histochromy as described above. Remaining animals were sacrificed at 2 months (n = 3) and 10 months (n = 2) after injection and were subjected to the same procedure. At 12 months, the TA muscles of one of the remaining two animals was placed into tissue culture as described below, whereas the other was fixed at 14 months by paraformaldehyde (4%, w/v) cardiac perfusion as described previously (24) for a detailed histological examination of its muscles. Blood samples were taken from 10-, 12-, and 14-month animals to exclude the generation of systemic viremia caused by recombination of the pIRV retrovirus with endogenous retroviral genomes or helper virus effects.

Immunohistochemistry. For immunostaining, parafin wax sections of paraformaldehyde (4% in PBS) fixed muscle were stained using rabbit polyclonal antibodies to β-galactosidase (1:500 dilution; Capel). As described previously (14), visualization was with DAB (Dakopatt), 1 mg/ml hydroperoxide (0.2%) after streptavidin enhancement using the Vector Elite ABC kit (Vector Laboratories). Slides prepared in this way were generally counterstained with H&E using a standard protocol. Some injected and stained muscle sections were also stained by Van Gieson's method to allow identification of the basement membrane. In frozen sections stained previously for β-galactosidase using immunohistochemistry (see above), basement membrane was identified using anti-collagen (IV) antibody (Sigma) and visualized with Vector Elite and DAB as above. Myoblasts cultured on glass chamber slides were washed in Dulbecco's PBS and fixed in formal saline (3.7% formaldehyde in PBS) for 45 min. Cells were permeabilized at room temperature in PHEM buffer (30) for 2 min and then incubated for 1 h at room temperature with one of the following primary antibodies: myosin (1:80 dilution; Sigma); anti-dystrophin (peptide IV) rabbit polyclonal antibody (18); and anti-Urokinase (LDP) rabbit polyclonal antibody (19). Both anti-dystrophin and anti-Urokinase antibodies were a gift from S. Ishura (Tokyo, Japan) and were used at 1:400 dilution. Following three PBS washes (15 min each), slides were incubated with secondary antibody (FITC conjugated goat anti-rabbit, 1:150 dilution, or tetramethylrhodamine β-isothiocyanate conjugated goat anti-rabbit, 1:150 dilution; both from Sigma) for 1 h at room temperature. After final washes in PBS, slides were incubated in DAPI (1 μg/ml) to counterstain nuclei.

Karyotyping. Cell lines were karyotyped and banded by incubation with trypsin using conventional methods as described previously (29). Mean chromosome number of PD50A was determined by counting 100 metaphase spreads, and representative spreads were photographed and enlarged to identify each chromosome.

Recruitment of PD50A from Injected Muscle. Explant cultures were established from injected and noninjected TA muscles of mdx mice as described by Smith and Schofield (14). At the first passage, cultures (designated PDR) were divided and were either stained for β-galactosidase or were placed into G418 selection using the contralateral leg cultures as controls. G418 selections were maintained in 50% conditioned medium as described above, and colonies expanded until large enough to be cloned. For β-galactosidase staining, cells were fixed in the dish in 0.5% (v/v) glutaraldehyde in PBS for 20 min.

Assays for Helper Virus. Filtered PD50A myoblast conditioned medium was assayed on mouse fibroblasts (NIH-3T3TK⁻) in the presence of Polybrene as follows. Subconfluent cultures of PD50A cells were fed with fresh mGM containing (3 μg/ml) Polybrene and incubated for 24 h; medium was then removed and filtered through a 0.2-μm-pore filter. This medium was added to monolayer NIH-3T3TK⁻ cell cultures (5 × 10⁵ per cm²) at concentrations of 0–100% (set up in triplicate) and incubated for 2–3 days. One-half of the plates were then fixed in 0.5% (v/v) glutaraldehyde and stained for β-galactosidase as described above. PD50A cells were included as a positive control for the β-galactosidase assay. This test was done three times in total. As an additional control, this assay was repeated using two other cell lines, mouse fibroblast NR6 cells and the parasitic myoblast cell line dfd13. The remaining NIH-3T3TK⁻ and dfd13 cells treated as above were subject to G418 selection to confirm the absence of functional virus. Filtered supernatant from pIRV, PD50A cells, PDR cultures, contralateral controls, and mdx mouse serum taken from PD50A-injected mice were overlaid onto mouse fibroblasts and stained for β-galactosidase (as described above) after 48 h.

References