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# Implanted myoblasts not only fuse with myofibers but also survive as muscle precursor cells

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## SUMMARY

**Intramuscular implanted myoblasts can fuse with existing myofibers. Here we report that implanted primary myoblasts marked with retroviral transgenes can also persist as muscle precursor cells. These cells can be recovered as viable myoblasts from muscles of recipent mice even months after myoblast implantation, and they can fully resume expression of the transgenes in culture. Upon re-implantation into muscles, they again not only fuse with existing myofibers, but also survive as muscle precursor cells in the tissue. These reserve myogenic cells should be able to contribute to host myofibers in muscle regeneration when the recombinant myofibers are damaged, providing an additional mechanism to maintain a persistent expression of transgenes delivered by myoblast-mediated gene transfer.**

Key words: gene transfer, muscle, retrovirus, factor IX

## INTRODUCTION

Myoblasts are muscle precursor cells originally derived from mesodermal stem cell population (Blau et al., 1985; Mazanet and Franzini-Armstrong, 1986). During muscle development, myoblasts proliferate and fuse with each other to form multinucleated myofibers. At a later stage of myogenesis, a small population of myoblasts take a position between the maturating myofibers and basal lamina, and they become reserve stem cells, called satellite cells. Satellite cells are quiescent in DNA synthesis and do not divide. When muscle injury or damage occurs, satellite cells are activated to proliferate and fuse to form new muscle fibers in a process of muscle regeneration (Mazanet and Franzini-Armstrong, 1986).

Myoblasts prepared from skeletal muscles can be grown to a large number in tissue culture (Yaffe and Saxel, 1977; Blau and Webster, 1981; Webster et al., 1988). Implantation of cultured myoblasts into skeletal muscles can result in myogenic fusion with existing host myofibers (for review, see Partridge, 1991). Myoblast transfer therapy for Duchenne muscular dystrophy has been extensively tested in both a murine model and humans (Law et al., 1988, 1990; Partridge et al., 1989; Gussoni et al., 1992; Huard et al., 1992). More recently, myoblast-mediated gene transfer has been utilized to deliver recombinant proteins into the systemic circulation (Yao and Kurachi, 1992; Barr and Leiden, 1991; Dhawan et al., 1991; Dai et al., 1992). After injection into skeletal muscles, genetically engineered mouse myoblasts efficiently fused with host myofibers and products of transgenes were transported into the systemic circulation.

At present it is not known whether implanted myoblasts can also survive as muscle precursor cells. In the present study, we have examined the possibility of long-term survival of implanted myoblasts as muscle precursor cells by utilizing recombinant retrovirus-transduced primary myoblasts. The results obtained demonstrate that a small population of the implanted myoblasts can survive for a long period of time as quiescent muscle precursor cells.

## MATERIALS AND METHODS

### Isolation and culture of primary myoblasts

Primary skeletal myoblasts were isolated from a male NIH III nude mouse (4 weeks of age) according to Yaffe and Saxel (1977) with modifications. Briefly, hind limb muscles (about 0.5-1.0 g) were dissected free from skin, fat and other connective tissues, and minced into pieces of about 1 $mm^3$. Cells were dissociated in 20 ml of 0.25% trypsin, 1 mM EDTA at 37°C for 2 h and filtered through an 85 μm nylon mesh filter (Spectrum, Houston). Dissociated single cells were washed in phosphate-buffered saline (PBS, GIBCO/BRL) and plated in gelatin-coated culture plates in growth medium (DMEM containing 20% fetal calf serum, 2% chick embryo extract (GIBCO/BRL), streptomycin and penicillin) and incubated at 37°C in 5% $CO_2$. Myoblast growth was observed starting at day 6 with about 10% contaminating fibroblast population. To obtain a purified myoblast population, 10 isolated myoblast colonies were picked and pooled. Primary myoblasts were characterized, based on typical cell morphology, myotube formation in vitro, and myosin fluorescent immunostaining. Differentiation of myoblasts to myotubes was induced by exposing confluent myoblast culture to DMEM containing 2% horse serum for 48 h.

## Retrovirus infection, implantation and recovery of myoblasts

Primary myoblasts at passage 4 were infected with retrovirus vectors LIXSN or BAG in the presence of polybrene at 8 μg/ml at 37°C overnight as previously described (Yao and Kurachi, 1992). LIXSN and BAG carry a neomycin-resistance gene as a selection marker, in addition to human factor IX cDNA or *lacZ* gene, respectively. After exposure to retrovirus medium, primary myoblasts were fed with growth medium for one day and then passaged with 0.05% trypsin into growth medium containing 1 mg/ml of G418. G418-resistant myoblasts (about 200 colonies) were pooled and expanded for myoblast implantation.

Transduced myoblasts ($5\times10^6$ to $1\times10^7$ cells) were injected into hind limb muscles of 4- to 6-week-old male NIH III nude mice ($n$=4) or SCID mice ($n$=2) as previously described (Yao and Kurachi, 1992). Briefly, cultured myoblasts were harvested with trypsin, washed twice with PBS and then resuspended in serum-free DMEM at about $5\times10^7$ or $1\times10^8$ cells/ml. Mice were anesthetized by intraperitoneal injection of 0.4 ml 2% Avertin. Muscles on the posterior side of each hind leg were exposed under surgical condition and injected with 0.1 ml of myoblast suspension at 10-20 sites through a 30 G needle. The incisions were closed with autoclips, and mice were allowed to recover in a pathogen-free room.

At various time points, muscles containing the cell-injected sites were removed and subjected to myoblast isolation as described above. The dissociated single muscle cells were seeded in 8-10 culture dishes (10 cm), which were divided into two groups for determining total myoblast colonies and neomycin-resistant or β-galactosidase-positive myoblast colonies. Neomycin-resistant myoblasts were selected in medium containing 1 mg/ml of G418 for 10-14 days. Resistant myoblasts were then pooled and used for further characterization including Southern blot analysis, factor IX expression, β-galactosidase activity, myotube formation and isozyme analysis.

## Southern blot analysis

Genomic DNA samples of myoblasts (10 μg) were subjected to Southern blot analysis as described (Yao and Kurachi, 1992). Human factor IX cDNA radiolabeled with $^{32}P$ to a specific activity of $5\times10^8$ c.p.m./μg was used as a probe.

## Immunocytochemistry

Primary myoblasts were characterized by immunofluorescence staining for myosin in myotubes as follows. Myoblasts were seeded in wells of Lab-Tek (Nunc) chambers and induced to form myotubes in 2% horse serum/DMEM for 2 days. Cells were then fixed in 4% paraformaldehyde in PBS at room temperature for 10 min, followed by permeabilization in cold acetone (−20°C) for 30 s. After blocking in 0.25% BSA in PBS for 30 min at room temperature, cells were incubated with mouse monoclonal anti-myosin heavy chain antibody (Sigma), biotinylated anti-mouse IgG, and streptavidin-Texas Red conjugates. The slides were mounted and visualized under a Nikon fluorescence microscope.

## Histochemistry for β-galactosidase

Cultured myoblasts and myotubes were fixed in 4% paraformaldehyde at room temperature for 10 min, followed by β-galactosidase staining. Whole muscles were fixed overnight in the above fixative at 4°C and stained for β-galactosidase. Fixed and stained tissue samples were then imbedded in glycol methacrylate, sectioned at 8 μm and counterstained with hematoxylin for 10 min at room temperature. For cryostat sections, freshly dissected muscle samples were imbedded in OCT medium, immediately frozen in isopentane/liquid nitrogen, and sectioned at 20 μm. Sections were then fixed in 4% paraformaldehyde for 10 min and stained for β-galactosidase activity. β-Galactosidase staining was carried out by incubating fixed samples at 37°C overnight in 2.45 mM 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal, Boehringer

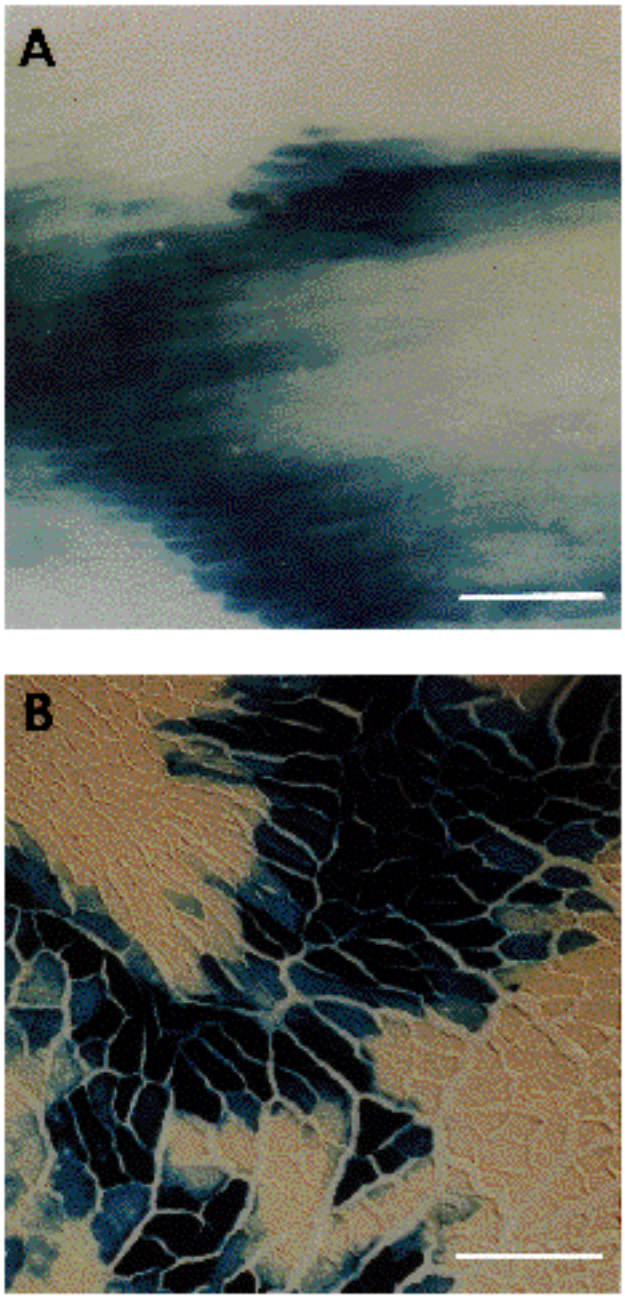


**Fig. 1.** Genetically modified primary myoblasts can efficiently fuse with existing myofibers after intramuscular implantation. Primary myoblasts were transduced with retrovirus vector BAG and about $5\times10^6$ cells were injected into muscles of each hind limb of 6-week-old NIH III mice. (A) Hind limb muscles removed 35 days after myoblast implantation were stained for β-galactosidase activity and photographed under a Nikon stereo microscope. (B) Hind limb muscles removed 18 days after injection of BAG-transduced myoblasts were frozen in isopentane, sectioned at 20 μm, fixed and stained for β-galactosidase activity. Photomicroscopy was performed on a Nikon Diaphot microscope with Nomarski optics. β-Galactosidase activity is shown as dark blue. Bars: A, 800 μm; B, 160 μm.

Mannheim), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM $MgCl_2$ in PBS.

### Mobilization assay for helper virus production

The presence of helper viruses was investigated by cultivation of C2C12 myoblasts with the filtered culture medium from transduced primary myoblasts in the presence of polybrene (8 μg/ml) as described (Danos and Mulligan, 1988; Nabel et al., 1990). A cultivation of C2C12 cells for 2 consecutive days was followed by selection with G418 or staining in X-gal solution for β-galactosidase activity.

### Test of cellular tumorigenicity

The tumorigenicity of recovered myoblasts was tested by subcutaneous injection of the cells in NIH III nude mice. Cultured myoblasts and mouse melanoma cells (BL-6) (Poste et al., 1980) were harvested and resuspended in PBS at $1\times10^7$ or $1\times10^8$ cells/ml. The cells in 0.1 ml aliquots were deposited subcutaneously at a single site on bilateral sides of the abdomen. Mice were checked for tumor growth twice a week by both visual inspection and manual palpation.

### Glucose phosphate isomerase (GPI) isoenzyme electrophoresis

Cell typing for GPI isozymes (EC 5.3.1.9) by electrophoretic analysis was adapted from the method of Rogers and O'Day (1983). Myoblasts were harvested from one 10 cm subconfluent plate and resuspended in 0.2 ml distilled water, followed by repeated freezing and thawing between liquid nitrogen and a 37°C water bath, 3 times. The cell extracts were then centrifuged for 10 min in a microfuge and the supernatant was collected. Aliquots (5 μl) of cell extracts were electrophoresed in a 5.5% polyacrylamide gel in 10 mM phosphate buffer (pH 7.0) at 150 V for 5 h. The acrylamide gel was then overlaid with a staining gel containing 0.6% agarose, 1.3 mM fructose 6-phosphate, 0.6 mM NAD (β-nicotinamide adenine dinucleotide), 0.27 mM NADP (β-nicotinamide adenine dinucleotide phosphate), 0.6 unit/ml glucose-6-phosphate dehydrogenase, 0.65 mM phenazine methosulfate, 0.25 mM nitro blue tetrazolium, 0.5 mM MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide), 20 mM $MgCl_2$ in 80 mM Tris-HCl buffer (pH 7.2). The gels were incubated in dark at 37°C for 1 h and the agarose staining gel was fixed in 40% methanol and 10% acetic acid for 5 min and dried under vacuum.

## RESULTS

### Genetically modified primary myoblasts efficiently fuse with existing myofibers after intramuscular implantation

In the present study, primary myoblasts were obtained from NIH III nude mice. These myoblasts could proliferate in culture with a doubling time of 14.8 h and could be readily differentiated in culture to spontaneously contracting myotubes, which were characterized for myosin expression by immunofluorescence staining (data not shown). After transduction with recombinant retrovirus LIXSN or BAG containing a human factor IX cDNA or a bacterial β-galactosidase gene, respectively, primary myoblasts could express transgenes at high levels similar to those of C2C12 cells (Yao and Kurachi, 1992). Myotubes differentiated from these retrovirus-marked myoblasts continued expressing the transgenes. Primary myoblasts transduced with LIXSN produced human factor IX into culture media at a rate of 700 ng/$10^6$ cells per day.

When primary myoblasts transduced with BAG ($5\times10^6$ cells) were injected into quadriceps femoris muscles of each hind limb of four syngenic inbred NIH III nude mice (6 weeks old), myofibers at or near the cell-injected sites produced β-galactosidase as assayed between day 7 and day 46 (Fig. 1A). Histological analyses of frozen muscle sections showed that injected primary myoblasts fused with the host myofibers, which in turn expressed recombinant β-galactosidase (Fig. 1B).

### A small population of implanted myoblasts can survive without differentiation for a long period of time

To test the possibility that some of the implanted myoblasts can survive without fusing with myofibers, we carried out a series of cell recovery studies after implanting retrovirus-marked myoblasts into skeletal muscles. Primary myoblasts transduced with LIXSN or BAG retroviruses were implanted into muscles of hind limbs of NIH III nude mice and SCID mice. At various time points, the hind limb muscles were removed and primary myoblasts were isolated. Retrovirally marked primary myoblasts were selected in media containing G418. From all the animals that were assayed between 46 days and 5 months post-cell implantation, about 1 to 2% of the myoblast colonies in culture were isolated as G418-resistant or β-galactosidase-expressing cells (Table 1). These surviving unfused myoblasts were not significantly proliferating as evidenced by the constant ratio of retrovirus-marked myoblasts (G418 resistant) versus endogenous primary myoblasts of recipient mice at various time points post-implantation. These recovered myoblasts have the integrated retroviral vector sequence without obvious rearrangement, as shown by Southern blot for LIXSN (Fig 2). Furthermore, recovered myoblasts retained a morphological appearance identical to that of the retrovirally infected primary myoblasts used for implantation (Fig. 3A). These myoblasts could normally fuse with each other and form multinucleated contractile myotubes that could continue expressing the transgene (Fig. 3B).

Low levels of serum human factor IX (5-25 ng/ml) were detected for about 3 weeks in these mice, which received LIXSN-transduced myoblasts after cell implantation (Yao and Kurachi, data not shown). Human factor IX (6 ng/ml) was also detected in the muscle homogenates of these mice even after 5 months post-implantation. The LIXSN-marked myoblasts that recovered at various time points between 2 and 5 months post-cell-implantation could continue expressing human factor IX in culture at the same level as that of their parent myoblasts (Fig. 4). These results indicate that the transferred human factor IX gene was stably retained in the reserve myoblasts and was actively expressed in recovered myoblasts.

### Recovered myoblasts retain myogenic potential in vivo

In order to test the myogenic capacity in vivo, recovered BAG-marked myoblasts (about $5\times10^6$ cells/hind leg) were re-implanted into quadriceps femoris muscles of two NIH III nude mice. Muscles at the injected sites, removed after

**Table 1. Recovery of surviving myoblasts from muscles implanted with retrovirally transduced primary myoblasts**

| Recipient mice | Marking retroviruses | Culture of surviving myoblasts: Days post-implantation | Neomycin-resistant myoblasts (%)* | Expression of transgenes |
|---|---|---|---|---|
| NIH III | LIXSN | 72 | + (1.04) | Factor IX |
| NIH III | LIXSN | 162 | + (1.8) | Factor IX |
| SCID | LIXSN | 50 | + | ND‡ |
| SCID | LIXSN | 120 | + | ND |
| NIH III | BAG | 46 | + | β-gal |
| NIH III† | BAG | 38 | + (2.2) | β-gal |

Primary myoblasts obtained from a NIH III mouse were transduced with retroviral vectors LIXSN or BAG, and implanted into hind limb muscles of recipient mice. After various days as shown, hind limb muscles were removed and used for myoblast isolation. Myoblasts were cultured in growth medium with or without G418 (1 mg/ml). Expression of transgenes for human factor IX or β-galactosidase (β-gal) was determined by ELISA or X-gal staining, respectively, as described in Materials and Methods.

*Percentage of G418-resistant myoblasts per total isolated myoblasts.

†Recovered G418-resistant myoblasts were re-implanted into this NIH III recipient mouse.

‡ND, not determined.

18 and 35 days, expressed detectable level of β-galactosidase (Fig. 3C). Histological sections of these muscle samples clearly showed β-galactosidase-positive myofibers, indicating that recovered myoblasts are able to fuse with existing myofibers (Fig. 3D). Retrovirus-marked myoblasts were again isolated from these mice and grown in culture (Table 1). These results clearly indicate that the surviving retrovirus-marked myoblasts represent a reserve population of muscle precursor cells that are able to participate in muscle regeneration.

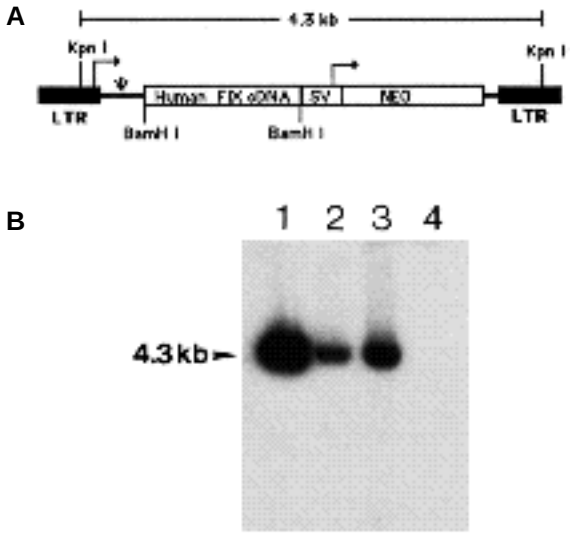


**Fig. 2.** DNA blot analysis of retrovirally marked myoblasts that were recovered from implanted muscles. (A) Structure of retrovirus vector LIXSN. Restriction sites are shown by vertical bars. The *Kpn*I restriction fragment size is shown above. LTR, long terminal repeat. (B) DNA blot analysis of mouse primary myoblasts transduced with retrovirus vector LIXSN. Retrovirus-marked myoblasts were transplanted into hind limb muscles of NIH III mice. After 72 days, retrovirus-labelled myoblasts were recovered in tissue culture. Ten micrograms of genomic DNA prepared from uninjected LIXSN-transduced myoblasts (lane 2), recovered myoblasts (lane 3) and uninfected myoblasts (lane 4) were digested with *Kpn*I and analyzed for proviral sequences using a *Bam*HI fragment of human factor IX cDNA as a probe. Lane 1, plasmid DNA of LIXSN digested with *Kpn*I.

## Implanted myoblasts were not tumorigenic

When implanted into mouse skeletal muscles, C2C12 cells, an established myoblast cell line, proliferate and eventually grow into tumors (Dhawan et al., 1991; data not shown). The possibility of implanted primary myoblasts being immortalized or transformed during in vitro manipulation was tested. In all 12 nude mice receiving intramuscular implantation of BAG- or LIXSN-marked primary myoblasts, no tumor was found by visual examination, by manual palpation or dissection of whole mice between 2 weeks and 5 months after cell implantation. We have also tested the cellular tumorigenicity of recovered myoblasts by subcutaneously injecting them into seven nude mice. Four of these mice also received an inoculation of BL-6 melanoma cells (Poste et al., 1980) as a positive tumor cell control on the opposite lateral side of the abdomen. All animals ($n$=4) inoculated with BL-6 cells ($5\times10^5$ to $2\times10^6$ cells) developed large tumors (1-2 cm in diameter) at the injected sites within 2 to 3 weeks, while no tumors were observed on the opposite side, which received an injection of recovered myoblasts ($1\times10^6$ to $1\times10^7$ cells). In the other three mice injected only with recovered myoblasts at higher cell doses ($1\times10^7$/site), no tumor was observed over 4 months after injection. On the basis of these results, we conclude that the survived myoblasts in muscle tissue were not derived from neoplastic myoblasts that might have been generated in vitro or in vivo.

## Recovered retrovirus-marked myoblasts are not of host origin

Our identification of the originally implanted myoblasts was based on retroviral marking of these cells. It is possible that marked myoblasts are derived from host endogenous satellite cells due to a transduction with possibly contaminating helper viruses. A mobilization assay (Danos and Mulligan, 1988), however, showed no detectable helper viruses in the retrovirus-marked primary myoblast preparation.

Strong evidence that further supports the recovery of originally implanted myoblasts was obtained by analysis for glucose-6-phosphate isomerase (GPI) isozymes. Primary myoblasts transduced with LIXSN were derived from NIH III nude mice that are homozygous for GPI slow type (GPI-$1^b$ in Fig. 5). These myoblasts were injected into muscles

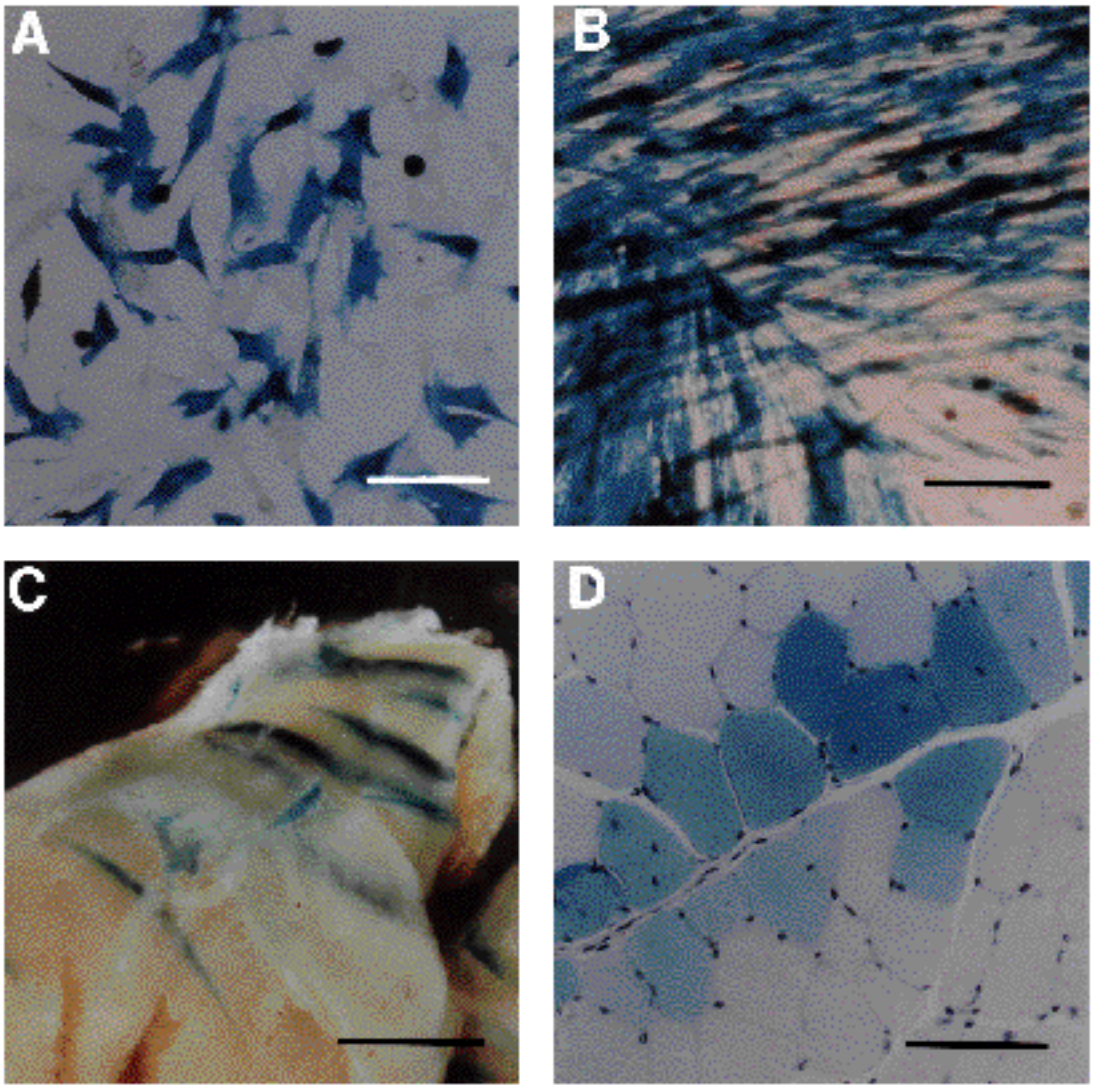


**Fig. 3.** Characterization of recovered BAG-transduced myoblasts for expression of β-galactosidase and myogenic capacity in vitro and in vivo. (A) BAG-transduced myoblasts recovered in culture 42 days after injection into skeletal muscles. (B) Myotubes derived from the recovered myoblasts shown in A. (C) Mouse quadriceps removed 16 days after re-implantation of recovered BAG-transduced myoblasts were stained in X-gal solution and photographed on a stereo microscope. (D) The stained muscles shown in C were embedded in glycol methacrylate, sectioned at 8 μm and counterstained with hemotoxylin to show myofibers and myonuclei. Dark blue in all panels represents β-galactosidase activity. Bars: A, 100 μm; B, 200 μm; C, 1 mm; D, 80 μm.

of SCID mice that are homozygous for GPI fast type (GPI-$1^a$). The muscles of recipient SCID mice were removed for myoblast isolation 50 days after cell implantation. Recovered myoblasts cultured with or without G418 were analyzed for their GPI isozyme types by electrophoresis. Results indicate that all neomycin-resistant myoblasts had the same type of GPI isozyme as that of NIH III donor myoblasts, clearly demonstrating that the recovered neomycin-resistant myoblasts were the originally implanted myoblasts (Fig. 5).

## DISCUSSION

Myoblast transfer therapy has been extensively tested in MDX mice as well as in Duchenne muscular dystrophy patients (Partridge, 1991; Gussoni et al., 1992; Huard et al., 1992). More recently, great feasibility has been shown for utilization of myoblasts as an efficient gene delivery vehicle in developing somatic gene therapies for various disorders that need systemic deliveries of the transgene products (Barr and Leiden, 1991; Dhawan et al., 1991; Yao and Kurachi, 1992; Dai et al., 1992). It has been well established that implanted normal or genetically modified skeletal myoblasts can efficiently fuse with existing host myofibers and contribute their genetic information to the mosaic myofibers generated (Partridge, 1991; Yao and Kurachi, 1992; Barr and Leiden, 1991; Dhawan et al., 1991).

However, the possibility of long-term survival as quiescent muscle precursor cells has never been demonstrated. In the present study, we have demonstrated that a small population of the implanted myoblasts persist in muscle

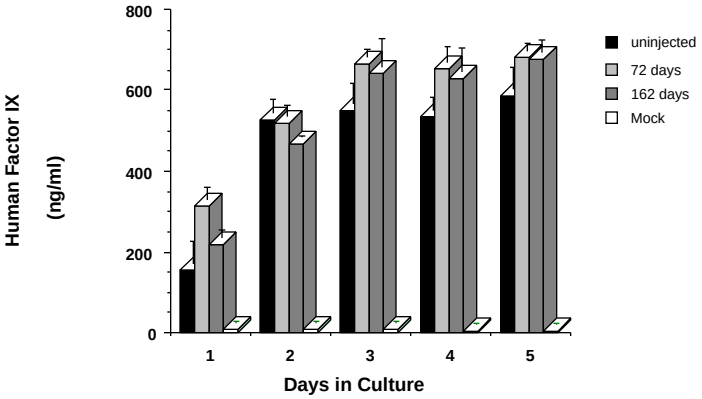


**Fig. 4.** Production of human factor IX by the uninjected LIXSN-transduced primary myoblasts, and myoblasts recovered 72 days and 162 days after injection into skeletal muscles. Uninfected parent myoblasts were used as a mock control. About $1\times10^6$ myoblasts were plated in each 60-mm gelatin-coated dish with 4 ml of growth medium containing 10 μg/ml vitamin K1. Culture medium was collected and replaced every 24 h for determination of human factor IX protein by ELISA as described (Yao and Kurachi, 1992). It was noticed that more than 90% of myoblasts had fused into myotubes after 2 days in culture. Standard deviations of assay value are indicated ($n$=6).

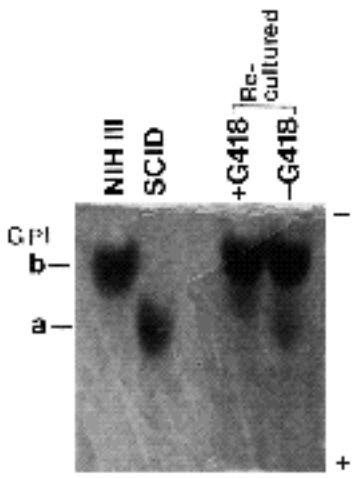


**Fig. 5.** Isozyme electrophoresis for GPI in recovered myoblasts. Primary myoblasts from a NIH III mouse were transduced with retroviral vector LIXSN, and injected into hind limb muscles of SCID mice. After 52 days, injected muscles were removed for myoblast re-culture. Aliquots of cell lysates from donor NIH III myoblasts (NIH III), host SCID myoblasts (SCID) and re-cultured myoblasts derived from injected muscles with or without G418 were electrophoresed in a polyacrylamide gel for GPI isozyme typing. a, homozygous for GPI fast type; b, homozygous for GPI slow type.

tissues for over 5 months without apparent proliferation. The recovered myoblasts retained their full myogenic fusion capacity. These results indicate that intramuscularly injected myoblasts can contribute to the integrity of muscles by fusing with existing myofibers or among themselves, forming a mosaic or new myofibers, and also by surviving as quiescent muscle precursor cells.

At present the mechanism by which implanted myoblasts end up as quiescent muscle precursor cells is not clear. In normal adults, satellite cells are maintained between basal lamina and plasmalemma in all skeletal muscles. Under various pathological and physical conditions such as muscle injury (Yamada et al., 1989; Mazanet and Franzini-Armstrong, 1986), satellite cells can be activated to proliferate, migrate and fuse with each other or with the existing myofibers. However, the mechanism that regulates satellite cell activity in normal or regenerating muscles is not fully understood. Several growth factors are responsible for stimulating satellite cell proliferation (White and Esser, 1989). Among them are fibroblast growth factor, which may be released from damaged heparin-rich basal lamina (Yamada et al., 1989; Bischoff, 1990b), platelet-derived growth factor released from local inflammation following an injury (Jodczyk et al., 1986; DiMario et al., 1989) and insulin-like growth factors (IGF-I and -II) (Allen and Boxhorn, 1989). Transforming growth factor-β has been reported to inhibit proliferation of myogenic cells (Allen and Boxhorn, 1989; Florini and Ewton, 1988; Bischoff, 1990a). Contact with the plasmalemma of mature myofibers, but not the basal lamina, depresses satellite cell proliferation (Bischoff, 1990a,b). This contact also inhibits the response of satellite cells to the mitogens in muscle extracts. These observations indicate that the anatomical position may be one of the critical factors for maintaining the dormancy of muscle precursor cells. It is conceivable that a small population of the implanted myoblasts may become quiescent cells by making contact with the plasmalemma of new-formed or pre-existing myofibers. Electron microscopic analysis, which would be required to prove this, is extremely difficult; e.g. to identify unambiguously a small number of reserve precursor cells derived from the implanted myoblasts in the background of endogenous satellite cells.

What fraction of implanted myoblasts can be reserved as muscle precursor cells is rather difficult to estimate. We used the endogenous muscle precursor cells isolated in culture from the muscles of recipient mice for estimating relative yields of retrovirus-marked myoblasts at various time points after cell implantation. A similar yield of about 1-2% of the total myoblasts as implanted myoblasts was obtained in independent experiments. By assuming that 1 gram of mouse leg muscles contains an average of $1\times10^6$ to $5\times10^6$ muscle precursor cells (Schmalbruch and Hellhammer, 1977) and surviving implanted myoblasts can be cultured as efficiently as endogenous myogenic cells, we estimate that approximately 0.1 to 0.5% of the implanted myoblasts ($1\times10^7$ cells) survives as muscle precursor cells.

Information obtained in the present study may also contribute to our understanding of the basic biology of myogenic stem cells. Classic stem cells can give rise to more stem cells as well as cells committed to differentiation. However, little is known about whether myoblasts activated from quiescent muscle precursor cells can also give rise to muscle precursor cells in addition to the cells that are committed to differentiate. The present studies have demonstrated that implanted primary myoblasts can both differentiate and become quiescent muscle precursor cells. This finding may explain in part how adult muscles maintain their regeneration capacity. The ability of muscle precursor cells to reproduce themselves ensures that there is no depletion of these cells after muscle damage and regeneration. In aged muscles, the numbers of muscle precursor cells is decreased (Mazanet and Franzini-Armstrong, 1986), possibly due to the increased senescence of these cells.

Muscle damage, which can result from numerous con-

ditions such as routine muscle wear, may cause a continuous loss of recombinant myofibers with transgenes in a patient receiving gene therapy by this approach. The present finding that implanted genetically modified myoblasts can survive as muscle precursor cells with a reserve potential for transgene expression further supports the rationale of using myoblast-mediated gene transfer for developing stable gene therapy approaches.

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### Note added in proof

Primary myoblasts marked with retroviral vector LIXSN were recovered after 377 days of cell implantation. The recovered cells continued producing human factor IX in culture.