Myoblast-mediated gene transfer and its repeated applications were tested for achieving a long-term stable systemic production of human factor IX (hFIX) at a therapeutic level in SCID mice. Primary skeletal myoblasts were stably transfected with a hFIX expression plasmid vector, pDLMe4βA-hIXm1, which contains a hFIX minigene under the control of a β-actin promoter with muscle creatine kinase enhancers. Myotubes derived from the myoblasts produced 1,750 ng hFIX/10^6 cells/24 hours in culture. hFIX secretion by the myoblasts and thereof derived myotubes were equally efficient, and myotubes were shown to have a sufficient secretory capacity to handle a substantially elevated production of hFIX. After intramuscular injection of 5, 10, and 20 × 10^6 myoblasts, SCID mice stably produced hFIX into the systemic circulation proportional to the number of implanted cells, and the expression levels were maintained for at least up to 10 months (end of the experiment). Additional cell injections administered to animals that originally received 10 × 10^6 cells approximately 2 months later elevated the systemic hFIX levels to an average of 182 ± 21 ng/mL, a therapeutic level, which persisted for at least 8 months (end of the experiment). These results indicate that long-term, stable systemic production of hFIX at therapeutic levels can be achieved by repeated application of myoblast-mediated gene transfer.

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actin promoter (βA) and four copies of muscle creatine kinase gene enhancer (Me4). Plasmid vectors pLIXSN (Fig 1) and pBAG contain hFIX cDNA and a bacterial β-galactosidase (β-gal) gene, respectively, in retroviral vector frames under the transcriptional control of the long-terminal repeat (LTR), and a neomycin resistance gene (Neo) under the control of SV40 promoter.\textsuperscript{24,25} pSV2Neo is a Neo expression vector\textsuperscript{26} and pCH110 is a β-gal expression vector.\textsuperscript{27} Mice primary skeletal myoblasts were isolated from muscles of SCID mice and grown in growth medium containing Dulbecco’s modified essential medium (DMEM), 20% fetal calf serum (FCS), 0.5% chicken embryo extract (GIBCO/BRL), and antibiotics (streptomycin and penicillin).\textsuperscript{21,23} hFIX assay medium was composed of the growth medium supplemented with BaSO\textsubscript{4}-treated FCS (20%) and Vitamin K1 (10 µg/mL).\textsuperscript{2,23} Differentiation medium was the same as the hFIX assay medium except that FCS was replaced with BaSO\textsubscript{4}-treated horse serum (2%). In all experiments, culture medium was collected every 24 hours for quantifying hFIX by enzyme-linked immunosorbent assay (ELISA) as previously described,\textsuperscript{21,23} and replaced with fresh medium. Changing the differentiation of myoblasts to myotubes was induced by medium containing the differentiation medium for 3 days. All cells were kept at 37°C in a humidized incubator under 5% CO\textsubscript{2}.

*Transient expression assay of hFIX expression vectors in muscle cells.* Transient expression assays were carried out using LipofectAMINE-mediated cell transfection as previously described.\textsuperscript{23} Myoblasts at a density of 2 × 10\textsuperscript{5} per well in 6-well plates were transfected with 2 µg of a hFIX expression vector and 0.2 µg of pCH110 DNA. Myoblasts in one well from each group were collected at the end of day 2 and were used for determining cell number, β-gal activity, and total cellular protein content, which were used for normalizing the transfection efficiency as previously described.\textsuperscript{21} Cell number at the time of switching to the differentiation medium was approximately 2 × 10\textsuperscript{6}/well at 80% to 90% confluency.

*Stable transfection of myoblasts and hFIX expression assay.* Primary myoblasts at a density of approximately 1 × 10\textsuperscript{6} per 10-cm dish were cotransfected with 10 µg pdMLMe4/AbiXm1 and 0.8 µg pSV2Neo using LipofectAMINE as described above, and subjected to G418 selection (1 mg/mL). Primary myoblasts transfected with LIXSN and BAG retrovirus were prepared as previously described.\textsuperscript{23} BAG was used as a background control, and LIXSN was used as a hFIX expression reference control because the cells carrying LIXSN were well characterized in our previous studies for their in vitro and in vivo hFIX expression and served for assessing any improvements in hFIX expression with the new vector. hFIX expression levels were determined by plating myoblasts stably transfected with pdMLMe4/AbiXm1, or myoblasts transfected with LIXSN or BAG retrovirus at a density of about 4 × 10\textsuperscript{5} cells per well in 6-well plates. hFIX protein produced into the medium was quantified by ELISA, and its activity was determined by one-stage clotting assay.\textsuperscript{25}

*Southern blot analyses.* Genomic DNA prepared from cells was digested with KpnI or BamHI, and subjected to Southern blot analysis using \textsuperscript{32}P labeled hFIX cDNA as a hybridization probe.\textsuperscript{25} The transgene band intensity was quantified with PhosphorImager (Molecular Dynamics). The filter was then stripped of hybridization probe. The filter was exposed to X-ray films, and radioactivity of RNA bands on the filter were quantified by a PhosphorImager (Molecular Dynamics). The filter was then stripped of the hFIX probe and rehybridized with \textsuperscript{32}P labeled 18S ribosomal RNA cDNA to confirm equal RNA loading to the lanes.

*Intramuscular implantation of myoblasts.* Myoblasts carrying hFIX or β-gal expression vectors were harvested and washed as described,\textsuperscript{21,23} and resuspended in serum-free DMEM supplemented with 1 µg/mL basic fibroblast growth factor (bFGF) (R&D systems) at 50 or 100 × 10\textsuperscript{5} cells/mL. Cells were then injected into the limb muscles of SCID mice through a 30 G needle at five or ten different sites of 10 µL per site.\textsuperscript{23} At various time points after cell injection, blood samples (0.1 to 0.3 mL) were collected by orbital sinus bleeding under brief anesthesia with inhalation of methoxyflurane (Pitman-Moore) and plasma samples were prepared for ELISA. At various time points, animals were killed by deep anesthesia with 10% chloral hydrate and the muscle tissue which received cell implantation were subjected to Northern blot analysis using hFIX cDNA labeled with \textsuperscript{32}P as the hybridization probe. The filter was exposed to X-ray films, and radioactivity of RNA bands on the filter were quantified by a PhosphorImager (Molecular Dynamics). The filter was then stripped of the hFIX probe and rehybridized with \textsuperscript{32}P labeled 18S ribosomal RNA cDNA to confirm equal RNA loading to the lanes.

*Immunohistochemical analysis.* Tissue sections were stained for hFIX using a murine anti-hFIX monoclonal antibody, AHIX-5041 (Haematologic Technologies, Inc), and H&E staining and immunohistochemical staining. Myoblasts isolated from the muscle tissue which received cell implantation were subjected to G418 selection and used for in vitro hFIX expression assays as previously described.\textsuperscript{23}

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**Fig 1.** hFIX expression vectors. pLIXSN contains a hFIX cDNA driven by long terminal repeat (LTR) promoter, pdMLMe4/AbiXm1 contains a hFIX minigene (AhIXm1) under the transcriptional control of βA-actin promoter (βA) and four copies of MCK enhancer (Me4). I to VIII indicates eight exons of hFIX gene. SVNeo indicates a neomycin resistant gene under the control of SV40 immediate early promoter. X in LTR indicates the deleted enhancer region. Thin lines with arrow show the predicted mRNA species; and zigzag line indicates the portion (hFIX intron) to be spliced.
RESULTS

Transient and stable expression of hFIX by muscle cells in vitro. In vitro hFIX production levels of myoblasts transiently transfected with pdLMe4βAhIXm1 were 6- to 10-fold higher than those transfected with pLIXSN (Fig 2A). Myotubes carrying pdLMe4βAhIXm1 secreted hFIX at a rate of 212 ± 21 ng/10⁶ cells/24 hours on day 6, a 15-fold higher level than that of myotubes with pLIXSN (14 ± 5 ng/10⁶ cells/24 h). This was consistent with the presence of the differentiated muscle cell-specific enhancer in pdLMe4βAhIXm1, but not in pLIXSN.

Myoblasts stably transfected with pdLMe4βAhIXm1 were prepared by cotransfection with pSV2Neo followed by G418 selection. Fifty-three myoblast colonies picked were individually expanded and their medium samples were sub-

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myoblasts stably carrying BAG, LIXSN, or pdLMe4βA-hIXm1. Animals in groups 1 and 2 were injected with 20 × 10⁶ myoblasts transduced with BAG or LIXSN retroviral vectors. Animals in groups 3, 4, and 5 were injected with 5, 10, and 20 × 10⁶ myoblasts with pdLMe4βAhIXm1, respectively. As assayed 26 days after the first cell injection, animals in groups 2, 3, and 4 produced hFIX at average levels of 23.1, 27.1, and 58.5 ng/mL serum, respectively. Animals in group 5 gave the highest hFIX production (94.1 ng/mL serum on average), 4- to 5-fold higher than those in group 2, which received myoblasts carrying LIXSN. These results were remarkably consistent with the in vitro observations (Fig 2B). hFIX expression levels in groups 3, 4, and 5 were approximately proportional to cell numbers injected (Fig 6). Stable hFIX production at levels of approximately 12, 18, and 60 ng/mL serum were observed for groups 2, 3, and 5, respectively, at least up to 10 months (end of observation).

To test the feasibility of repeat cell implantation, animals in group 4 were injected with additional doses of cells on day 43 (10 × 10⁶, left leg muscle), day 52 (10 × 10⁶, right leg muscle), and day 59 (20 × 10⁶, both forearm muscles). Systemic hFIX levels in these animals were elevated to a range of 160 to 210 ng/mL serum, again approximately proportional to the implanted cell numbers, and stayed stable for an additional 8 months (end of the observation) (Fig 6). This range was approximately 2.5-fold higher than that of group 5, and approximately 15-fold higher than that of group 2 (cells with LIXSN).

Two hundred days after the first cell injection, primary myoblasts were obtained in culture from the muscle tissues of a representative animal in group 4. After G418 selection, cells showed a hFIX expression level equivalent to that of the original unimplanted, parent cells (Fig 7). Myoblasts isolated from another animal in group 1 3 months after implantation also gave similar results (data not shown). These results were consistent with our previous observations, demonstrating that a fraction of implanted cells can acquire the satellite cell status, and that little inactivation of LTR or Me4βA promoter took place in vivo. Although it is not likely, the possibility of partial inactivation of these promoters in vivo and regaining of their full activities in vitro cannot be denied. Immunohistochemical analysis of muscle tissue sections prepared from mice in groups 4 and 5 after 200 to 300 days of the original cell implantation also confirmed active hFIX production by the implanted cells (Fig 8, A and B).

**Characterization of tumor.** About 6 months post-cell implantation, one of the group 4 animals developed a tumor in the right leg muscle where cells were injected. The left leg muscle injected with the same batch and number of cells was normal and did not grow any tumor. This animal did not show any unusually elevated level of hFIX expression.

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**Fig 4.** Relative levels of intracellular and secreted hFIX of myotubes in comparison with those of myoblasts. Arrow indicates the time point of switching to the differentiation medium. Results shown are averages of two independent experiments with observed ranges shown in vertical bars. hFIX expressed by myoblasts at day 2 are 7.7 ± 0.1 ng/10⁶ cells and 593.2 ± 8.9 ng/10⁶ cells/24 hours for intracellular and secreted hFIX, respectively. hFIX expressed by myoblasts at day 2 are 6.2 ± 2.1 ng/10⁶ cells and 581.6 ± 74.9 ng/10⁶ cells/24 hours for intracellular hFIX and secreted hFIX, respectively. Intradie hFIX levels of myotubes were normalized to those of myoblasts (day 2), which were defined as 1 as shown on the Y-axis.

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**Fig 5.** Northern blot analysis of hFIX expressed by myoblasts and myotubes. (A) Total cellular RNAs prepared from cells on day 2 (myoblasts) and day 5 (myotubes) in Fig 4 were subjected to Northern blot analysis. Lanes 1 and 2: myoblasts (day 2) and myotubes (day 5) carrying LIXSN, respectively. Lanes 3 and 4: myoblasts (day 2) and myotubes (day 5) carrying pdLMe4βAhIXm1, respectively. Three major bands are seen in lane 4; they are transcription products from LTR and Me4βA, respectively, corresponding to the predicted mRNA size shown in Fig 1. Lane 5: total RNAs prepared from untransfected myotubes. Numbers on the right indicate the RNA molecular size markers and arrows on the left indicate the major hFIX mRNA bands. (B) Hybridization of the same filter membrane with 18S ribosome RNA probe after the dehybridization of the hFIX probe as RNA loading controls.
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Fig 6. hFIX levels in the systemic circulation of SCID mice after intramuscular implantation of myoblasts carrying pdLMe4βAhIXm1 or LIXSN. The background ELISA signals obtained from group 1 (control group) mice, which were injected with $2 \times 10^7$ myoblasts transduced with BAG, were subtracted from the observed levels of other groups. Group 2 mice were injected with $2 \times 10^7$ myoblasts transduced with LIXSN. On day 0, group 3, group 4, and group 5 animals were injected with 5, 10, and $20 \times 10^6$ myoblasts stably transfected with pdLMe4βAhIXm1, respectively. Mice in group 4 were injected with additional cell doses ($1, 1$, and $2 \times 10^7$ cells, respectively) on days 43, 52, and 59 as shown with arrows. Results from individual animals in each group were shown.

Such an increase was invariably observed, when the implanted primary cells were transformed and proliferated in vivo. Cells isolated from the tumor biopsy expressed only a background level of hFIX (less than 10 ng/10^6/24 hours in culture), and they did not survive the G418 selection. Cells isolated from the other leg of the same animal, where the same batch and number of cells were also injected with no subsequent tumor development, survived the G418 selection well. The selected cells expressed hFIX at a comparable level with that of the unimplanted parent cells (Fig 7). Southern blot analysis detected substantially lowered level (23%) of the factor IX transgene in the genomic DNA prepared from the tumor tissue in comparison with that of the other leg where no tumor developed (Fig 9). The lowered transgene level is presumably due to the presence of a massive tumor tissue interspersed with a lesser amount of the normal muscle cells carrying the transgene in the muscle tissue removed for various analyses (Fig 8C). Masson trichrome stain (Fig 8D) and electron microscopic studies (data not shown) identified that this tumor is a fibrosarcoma. These results probably determined that the tumor is not derived from the implanted myoblasts.

DISCUSSION

In recent years, the promising potential of myoblast-mediated gene transfer for systemic production of therapeutic proteins has been reported by multiple groups including ours.\textsuperscript{3,5-8} In most of these studies, retroviral expression vectors combined with single dose cell implantation were used. In the present study, we have successfully demonstrated that the nonviral myoblast-mediated gene transfer and its repeat application can be used to achieve a long-term stable production of hFIX into the systemic circulation in mice at a therapeutic level. The systemic level achieved in this feasibility study is equivalent to 4%-5% of the normal hFIX level, sufficient to change a clinically severe hemophilia B to a mild condition.

pdLMe4βAhIXm1, one of our refined hFIX expression vectors,\textsuperscript{23} was used as a plasmid vector in the present study. In transient assays, this vector, which contains a muscle specific transcription control unit, can express approximately 15-fold higher hFIX than LIXSN (our first generation retroviral vector) in myotubes (Fig 2A). Myotubes derived from $1 \times 10^6$ myoblasts stably transfected with this vector produced as high as 1,750 ng of hFIX per 24 hours, approximately 4-fold higher than those of the LIXSN transduced myotubes (Fig 2B). This is a relatively small increase in comparison to that observed in the transient expression assays (~15-fold) (Fig 2A). This difference cannot be simply explained by the transgene copy number, because cells trans-
Fig 8. Immunohistochemical analyses of sections of muscle tissue and a spontaneous tumor. (A) Tissue section of muscle with no cell injection. (B) Tissue section of muscle received myoblasts carrying pdLMe4/\(\beta\)-AhIXm1. Tissue sections in panels A and B were stained with hFIX monoclonal antibody followed by counter-staining with hematoxylin (\(\times 630\)). Brownish-red color indicates the presence of hFIX. (C) Section of the tumor from a group 4 mouse stained with H & E (\(\times 500\)). The fibrosarcoma cells are small and spindle-shaped; one large muscle fiber is on the left. (D) Tumor section stained with Masson trichrome shows small spindled cells of fibrosarcoma and the collagen produced by the neoplasm in blue color; several muscle fibers are red or purple-red in color and large in size (\(\times 500\)).

Animals injected with various numbers of myoblasts carrying pdLMe4/\(\beta\)-AhIXm1 or LIXSN persistently produced hFIX into the circulation for at least 10 months (end of the experiment) (Fig 6). In addition, hFIX levels in the systemic circulation were roughly proportional to the cell numbers injected. Importantly, this repeat cell implantation scheme works well, thus enabling us to achieve much higher, truly therapeutic levels of stable hFIX production into the circulation (Fig 6, group 4). The elevated hFIX levels were again approximately proportional to the total cell number injected. Rando and Blau also reported that, after intramuscular injection of myoblasts transduced with the \(\beta\)-gal expression retroviral vector, the expression levels of \(\beta\)-gal are proportional to the injected cell numbers in a given range. The cell numbers as well as cell concentration used by Rando and Blau, however, were much smaller than those used in the present study. The hFIX levels in the systemic circulation reported here were 10- to 15-fold higher than the stable levels we previously achieved in mice, and much higher than those (approximately 10 ng/mL of canine FIX in mice) reported by Dai et al, who also used an approach of primary myoblast-mediated gene transfer with retroviral vectors. Kay et al reported a long-term stable canine FIX expression in dogs by in vivo delivery of FIX retrovirus through the portal vein, but only at low levels (<10 ng/mL plasma). Although we must be cautious in direct comparison of our results with those obtained by others because of the different experimental conditions used, the stable systemic hFIX level we have achieved and the possibility of its further elevation by repeated therapies are important.

Fig 9. Southern blot analysis of the genomic DNAs prepared from the leg muscle tissues of tumor-bearing animal. Aliquots of the total genomic DNA samples (10 \(\mu\)g) prepared from the leg tissues of a tumor-bearing animal were digested with BamHI, and subjected to Southern blot analysis (see the Materials and Methods). The unique human factor IX transgene bands detected (2.9 kb in size as marked on the right side) were quantified with PhosphorImager. Lane 1, DNA from the right hind leg with tumor; lane 2, DNA from the left leg with no tumor; lane 3, control DNA of primary myoblasts transduced with BAG (\(\beta\)-galactosidase retrovirus).

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Based on the two-compartment distribution kinetics,\textsuperscript{16,32} we have estimated that the plasma hFIX concentration level obtained for the group 4 animals is less than 5\% of the theoretical steady-state level that could be expected with the total $5 \times 10^7$ cells injected. Here we assume that all cells can produce hFIX in vivo as efficiently as in vitro. Although it is a rough estimation, this suggests that most implanted cells are not efficiently contributing to hFIX production in vivo. This may be due to (1) the death of a large percentage of the implanted cells without successful fusion with existing myofiber cells or among themselves to form new myofiber cells, (2) the in vivo environment is substantially different from that in the culture and the transgene cannot be expressed as efficiently as in the cultured cells, (3) a combination of these possibilities, or (4) other yet unknown mechanisms. Generally low survival of nongenetically modified myoblasts implanted in humans (the above second possibility) was also reported by multiple groups\textsuperscript{33,34} suggesting that the myoblast implantation procedure and cell fusion processes are at least two critical steps that need further careful investigation for improving this gene transfer approach.

After 6 months of cell injection, one of the group 4 animals developed a tumor in the right hind leg muscle. The left hind leg muscle, which also received the same batch and number of genetically modified cells, was normal and did not grow any tumor. A series of biochemical and immunohistochemical analyses in addition to the lack of increased production in vivo of the recombinant FIX indicated that this tumor was fibrosarcoma, and not directly derived from the implanted cells. At this stage of the study, however, we should not disregard a possibility that the tumor might have been derived from the implanted cells due to an unknown mechanism. Other possible explanations for its development may include a random tumorigenesis incidence in the immune-deficient SCID mice, possible tumorigenic stimulation in the local tissue due to the damage by multiple needle stabbings or any other unforeseen mechanisms. The implanted cells were mixed with basic FGF just before their implantation to increase the hFIX expression in vivo.\textsuperscript{7} This effect is presumably due to a brief stimulation of proliferation or possibly augmentation of migration and/or invasion through basal lamina of the injected myoblast.\textsuperscript{35,36} Although no tumorigenic potential of bFGF has been reported, its angiogenic activity is well established,\textsuperscript{36,37} suggesting a possibility that once tumorigenesis is triggered due to unknown mechanism(s), it may support subsequent tumor growth. In our experimental conditions, however, bFGF should be cleared from the injection sites within minutes, making it unlikely for bFGF to be directly responsible for such an aberration(s). It has been reported that after subcutaneous implantation in athymic mice, C2 myoblasts (established cells), but not cloned primary myoblasts, develop tumors and also that intramuscularly injected C2 cells can proliferate in vivo.\textsuperscript{34} In addition, extensive human trials of myoblast implantation therapy for Duchenne muscular dystrophy (DMD) have strongly supported the general safety of myoblast implantation.\textsuperscript{33,34} Roman et al\textsuperscript{38} reported that C57BL/J6 mice intramuscularly implanted with either C2C12 cells or primary myoblasts, which are transduced with recombinant retroviruses, invariably developed rhabdomyosarcomas after 60 days of cell implantation. Unfortunately, the investigators provided neither information regarding the potential contamination of replication-competent retrovirus in the viral stock used nor other explanations for the tumor development with the primary myoblasts.

The present study has demonstrated the feasibility to refine the myoblast-mediated gene transfer method in achieving both the long-term systemic production of therapeutic level recombinant FIX and improved safety. The results warrant further intensive studies on its safety aspects and improvement of the overall FIX systemic production, before its clinical application.

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Persistent Systemic Production of Human Factor IX in Mice by Skeletal Myoblast-Mediated Gene Transfer: Feasibility of Repeat Application to Obtain Therapeutic Levels

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