rated into the injected muscle by differentiating into multinucleated myotubes in vivo.

The studies described in this report suggest that genetically engineered myoblasts may represent a useful delivery system for recombinant proteins in vivo. These cells can produce large amounts of secreted recombinant proteins. They can be stably introduced into muscle by simple intramuscular injection, allowing their secreted protein products to gain access to the circulation. The finding that the technique can be used to produce detectable levels of hGH is especially encouraging given the short half-life of hGH (less than 20 min) (*16*) as compared with those of other serum proteins. When taken together with previous reports demonstrating that primary human myoblasts can be readily isolated, expanded in vitro, and reinjected into muscle (*7, 9*), these data suggest that such a system may be adaptable to human therapy.

A number of questions must be answered before myoblast therapy can be considered for humans. First, more needs to be learned about the longevity of gene expression in this system. In addition, it remains to be determined if this system can be used to produce physiological levels of circulating proteins in large animals. Studies with primary myoblasts in larger mammals should answer many of these questions and allow an accurate assessment of the usefulness of this approach for the treatment of human disease.

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Systemic Delivery of Human Growth Hormone by Injection of Genetically Engineered Myoblasts

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A recombinant gene encoding human growth hormone (hGH) was stably introduced into cultured myoblasts with a retroviral vector. After injection of genetically engineered myoblasts into mouse muscle, hGH could be detected in serum for 3 months. The fate of injected myoblasts was assessed by coinfecting the cells with two retroviral vectors, one encoding hGH and the other encoding β-galactosidase from *Escherichia coli*. These results provide evidence that myoblasts, which can fuse into preexisting multinucleated myofibers that are vascularized and innervated, may be advantageous as vehicles for systemic delivery of recombinant proteins.

MAJOR ADVANCES HAVE BEEN made in the design of vectors that allow cells to express and secrete recombinant proteins at high concentrations in vitro. Yet, a general problem encountered with a number of cell types is that expression is not sustained after implantation of genetically engineered cells in vivo (*1–3*). Properties unique to myoblasts appear to enhance the long-term survival and function of these cells after implantation. Myoblasts have the unusual capability of crossing basal lamina (*4*). As a result, they contribute progeny to multiple, multinucleated myofibers in the course of normal postnatal mouse development (*5*). In addition, myoblasts injected into genetically deficient *mdx* mice fuse into the muscle fibers of the host and provide a missing endogenous muscle gene product, the intracellular protein dystrophin (*6–8*). Thus, injected myoblasts, unlike other cell types, become incorporated into a preexisting structure, which is sustained by neuronal activity and in contact with the circulation.

To examine whether myoblasts could deliver nonmuscle gene products to the circulation, we selected hGH for study. Sensitive assays distinguish mouse from human hormones and hGH has a short half-life of 4 min in serum (*9*), providing a stringent test for continuous production, secretion, and access to the circulation over time. To introduce the hGH gene into myoblasts, we used the MFG retroviral vector (*10*). The efficiency of stably introducing DNA into human myoblasts afforded by this approach is at least three orders of magnitude greater than by stable transfection of plasmids (*11*). A second retroviral vector, α-SGC, was used to introduce the *E. coli lacZ* gene which encodes β-galactosidase (β-gal) into the same myoblasts, providing an independent marker of implanted cell number and location. Recombinant retroviruses were generated in the ψ-CRIP line that produces replication-incompetent virus with an amphotropic host range, allowing infection of mouse and human cells. Cells of the C2C12 mouse myogenic line were infected with a

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mixture of the hGH and β-gal retroviruses. The efficiency of infection was high: 13 of 14 clones (93%) randomly selected from the pool expressed and secreted hGH at levels ranging from 1400 to 4600 ng per 10^6 cells per day; 4 of the 13 hGH-positive clones (30%) also expressed β-gal (*12*). The average rate of secretion for the entire myoblast pool was 4000 ng per 10^6 cells per day, on a par with the most effective cell types secreting recombinant hGH in vitro described to date (*13*).

Because some retroviral vectors cease to be expressed after cell differentiation or implantation (*14, 15*), we examined whether transduced myoblasts continued to secrete hGH upon myotube formation in vitro. By several criteria hGH-expressing myoblasts differentiated (Fig. 1, A and B). The cells fused to form multinucleated myotubes. Northern (RNA) analysis revealed the typical increase in transcripts for muscle α actin, myosin heavy chain, and myosin light chain. Differentiation was accompanied by a change in the location of the Golgi apparatus in which hGH accumulated before secretion; in myoblasts it was polar, and in myotubes it was characteristically circumnuclear (*16*). Despite these changes in differentiated state, the hGH secretion rate was not altered and averaged 4300 ng per 10^6 cells per day for the pool. Thus, although muscle is not generally considered to be a secretory tissue, both proliferating myoblasts and differentiated myotubes are capable of secreting hGH into the culture medium.

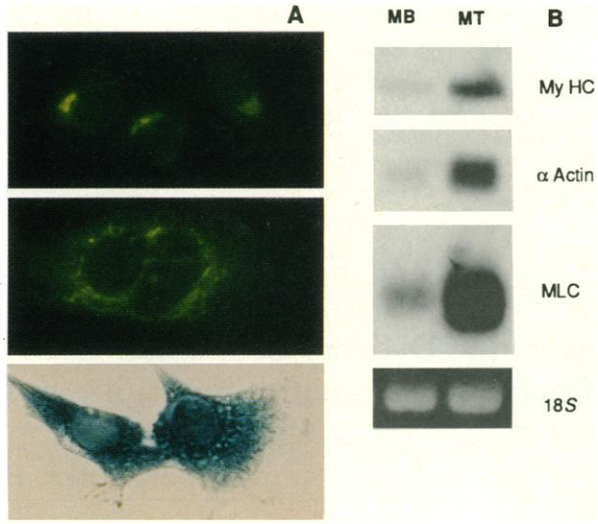

Fig. 1. Characterization of hGH- and *lacZ*-transduced myoblasts in vitro. (**A**) Single cell analysis: hGH in undifferentiated myoblasts (top) and differentiated myotubes (middle) is present in the Golgi apparatus (immunofluorescence). A replicate plate shows that the myoblasts also express β-gal (bottom, bright field). The hGH was detected after fixation of myoblasts with 1% paraformaldehyde in phosphate-buffered saline (PBS) permeabilization with −20°C methanol, two washes with 0.25% Triton X-100 in tris-buffered saline, and incubation with rabbit antibody to hGH (1:10) (Accurate Corporation), followed by fluorescein isothiocyanate (FITC)–conjugated goat antibody to rabbit immunoglobulin G (IgG) (1:100) (Cappel, Durham, North Carolina). The β-gal was detected [as described (*12*)]. Cells were photographed with a Zeiss Axiophot microscope (×350). (**B**) Differentiation properties of myoblasts are not altered by high levels of hGH expression as shown by Northern (RNA) analysis of total RNA isolated from a pool of genetically engineered proliferating myoblasts at low density (MB) and 3 days later from replicate cultures of postmitotic multinucleated myotubes (MT). Each sample (5 μg) was separated by electrophoresis, transferred to nitrocellulose, and assayed for muscle-specific mRNAs encoding α actin, myosin light chain (MLC), and myosin heavy chain (MyHC). Ethidium bromide staining of the same gel reveals equivalent amounts of ribosomal RNA (18*S*). ^{32}P-labeled probes were prepared by random priming, blots were washed at high stringency (0.1× saline sodium citrate at 65°C), and autoradiograms were exposed for 2 hours (α actin) or 18 hours (MLC and MyHC). RNA isolation, probes, and random priming were as described (*34*).

To determine whether transduced myoblasts could effectively deliver hGH in vivo, we injected the highest hGH-producing clone (clone 6), which also expressed β-gal, into the hind limbs of 12 mice. After an initial decline, serum hGH levels plateaued and persisted at approximately 0.5 ng/ml for 35 days (Fig. 2A). This level was clearly above that of control animals. In a second series of experiments, we injected the entire pool of transduced myoblasts into the hind limbs of 24 mice to determine whether cells that had not been selected by clonal analysis could effectively deliver hGH to the circulation. Again, after an initial decline, hGH levels persisted in serum at 1.0 ng/ml for 30 days (Fig. 2B). At 85 days, serum hGH levels had risen to 16.1 ng/ml (Fig. 2C). These results show that hGH encoded by the MFG retrovirus can be continuously produced and secreted by myoblasts implanted into muscle tissue in vivo.

To monitor the fate of implanted cells, we used β-gal, a marker that is independent of the expression of the secreted protein, hGH. We used a highly sensitive fluorogenic assay of β-gal activity (*17*) to estimate cell number by comparing values for injected muscles with those obtained for a known number of transduced myoblasts in culture (Table 1). Although this extrapolation is not precise, since it assumes that expression of β-gal in vivo and in vitro is similar, both in experiments with the clone and the pool, the estimated number of myoblasts required to deliver 0.5 to 1.0 ng of hGH per milliliter to the serum approximated 4×10^6. For the clone and pool, respectively, the relative levels of β-gal activity paralleled values for serum hGH at different time points. These results suggest that the hGH concentration in serum at any given time is determined

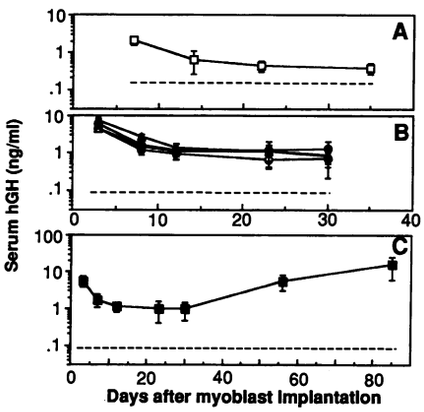

Fig. 2. Persistent expression of hGH by virus-transduced myoblasts implanted in mouse muscle in vivo. (**A**) A single myoblast clone (Fig. 2A) was implanted into 12 mice, and serum hGH levels were monitored for 35 days by collection of tail blood for radioimmunoassay (RIA) (*12*). Each point represents the mean ± SD for three to nine mice. The dashed line represents the mean ± SD (0.16 ± 0.09 ng/ml) from six control mice (uninjected or injected with cells expressing only the *lacZ* gene.) Myoblasts were cultured [as described (*35*)]. For injection, cells were trypsinized, washed twice, and suspended in PBS at 4°C to prevent clumping. Approximately 1×10^7 myoblasts were delivered in a total of 100 μl via ten injections throughout the hind limbs of anesthetized mice at 3 to 4 weeks of age. The mice used in these experiments differed from C2C12 in the expression of minor histocompatibility antigens described elsewhere (*25*) and received cyclosporine A (75 μg per gram of body weight, intraperitoneally) daily. Animals were maintained in accordance with the guidelines of Stanford University. (**B**) A pool of transduced myoblasts (Fig. 2B) was implanted into 24 C3H mice (four groups of six) as described in (A). Greater than 90% of the cells expressed and secreted hGH and 30% expressed β-gal, determined by clonal analysis in vitro. Each point represents the mean ± SD for two to six mice. The dashed line is the mean ± SD (0.08 ± 0.08 ng/ml) for serum samples from five uninjected control mice. Mice used in these experiments were syngeneic with C2C12 cells and were not immunosuppressed with cyclosporine A (*25*). The weight of injected mice, monitored weekly, increased at the same rate as control mice in both (A) and (B). (**C**) Serum hGH was monitored in mice described in (B) for a period of 85 days after myoblast implantation. Data from all four groups were pooled; each point represents the mean ± SD for 4 to 24 mice.

primarily by implanted cell number.

The location of β-gal–labeled cells was determined at time intervals after implantation by histochemical analysis of serially sectioned hind limbs. During the first few days, multiple small-diameter fibers were observed that are probably derived from implanted myoblasts that had fused to one another. By 10 days, many β-gal–labeled fibers had a large diameter typical of the surrounding tissue, suggesting that implanted cells fused into and contributed to preexisting myofibers (Fig. 3). Immunohistochemical analysis revealed that these myofibers produced hGH (*18*). In general, the morphology of individual fibers and the architecture of the tissue as a whole were not greatly altered during the period of stable production of hGH.

Injected myoblasts of the C2C12 cell line appear to have three fates after implantation into mice: some are lost, some become integrated into fibers, and some proliferate. Mice analyzed at late time points, days 56 and 85 after myoblast injection, had elevated serum hGH levels of 5.8 ± 2.6 and 16.1 ± 10.1 ng/ml, respectively, and an abundance of mononucleated myoblast clusters. Proliferation appears to be a characteristic of the permanent C2C12 mouse myogenic cell line that is manifested months after implantation (*19*); it was not observed several months after primary mouse (*7, 8*) or primary human myoblasts (*20*) were implanted in mice.

Table 1. β-gal activity correlates with serum hGH and provides an estimate of cell number.

Experiment	Time after implantation (days)	Serum hGH* (ng/ml)	Cell estimate† (×10⁶)
Pool	12	1.1 ± 0.5	2.8 ± 0.3
	30	1.0 ± 0.6	3.5 ± 0.5
Clone	14	0.8 ± 0.4	5.7 ± 1.5
	35	0.4 ± 0.1	4.0 ± 1.1

*Serum hGH (mean ± SD) was assayed [as described (*12*)]. †Cell number: a standard curve was generated from known numbers of transduced myoblasts (either clone 6 for experiments in Fig. 3A, or the entire myoblast pool for experiments in Fig. 3B) and cell number (mean ± SEM) was estimated from β-gal activity (*17*) in injected muscles. As few as 25 *lacZ* transduced cells could be detected. All assays were performed in duplicate. Extracts of muscle tissue were prepared by sonication (five 10-s pulses at 4°C) in 0.1 M sodium phosphate, pH 7.0, containing protease inhibitors [90 μg/ml phenylmethylsulfonyl fluoride (PMSF) aprotinin (0.2 units/ml), 0.1 mM leupeptin]. Aliquots of tissue extract containing 10 to 20 μg of protein were diluted 100- to 1000-fold in 0.1 M sodium phosphate, pH 7.0, 0.1% Triton X-100, 0.1 M β-mercaptoethanol. The substrate (MUG; Sigma) was added to a final concentration of 0.66 mM. After 30 min at room temperature, the reaction was stopped by the addition of glycine-EDTA, pH 11.2, to a final concentration of 120 mM glycine, 6 mM EDTA, and fluorescence generated by β-gal activity measured in a Fluoroskan microtiter plate reader (Flow Labs) with 355-nm excitation and 460-nm emission wavelengths. Control tissue samples from uninjected animals were consistently at least 30-fold below assays of injected animals.

Our experiments suggest that myoblasts may have advantages over other cell types (*13–15, 21–24*) as vehicles for long-term delivery of secreted recombinant gene products. There was no evidence of tumor formation after implantation of primary human cells (*20*).

Myoblast implantation was successful in syngeneic mice (Fig. 2B) but required continuous immunosuppression in mice mismatched at minor antigens (Fig. 2A) (*25*). Although direct DNA injection circumvents the immune response and has led to remarkable persistence of gene expression in muscle, the efficiency of this method is now too low to be useful for systemic delivery of therapeutic proteins (*26*). Thus, strategies for achieving continuous immunosuppression that are less toxic than those that involve cyclosporine A need to be tested (*27*) and methods for genetically engineering universal donor myoblasts that may evade immune surveillance explored (*28*).

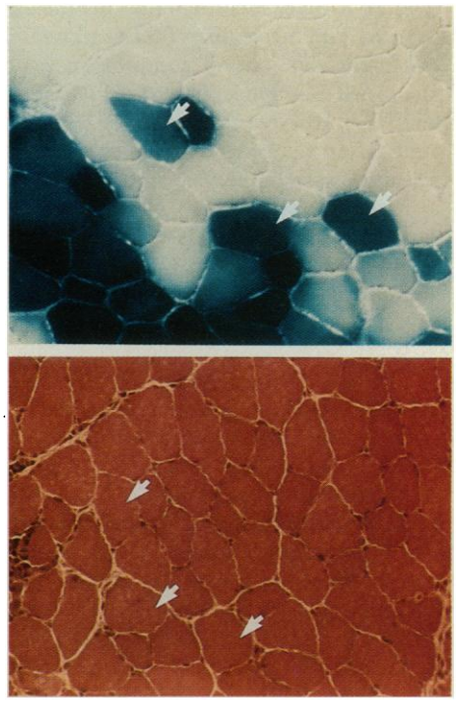

Fig. 3. Genetically engineered myoblasts can contribute to existing muscle fibers. Transverse sections of mouse legs 12 days after myoblast implantation show that β-gal–labeled myoblasts (top) have fused into large-diameter myofibers (bottom). Lower hind limbs were frozen in melting isopentane, serially sectioned, and fixed and stained for β-gal activity [as described (*12*)] or with hematoxylin and eosin to reveal muscle architecture. Muscle fibers were monitored across multiple serial sections; arrows indicate the same three muscle fibers in two sections approximately 50 μm apart. Sections were mounted in gelvatol-PBS and photographed with Nomarski optics with a Zeiss Axiophot microscope. Sections from uninjected control mice showed no blue reaction product (×175).

These results demonstrate that myoblasts genetically engineered with retroviruses can be used to deliver recombinant human proteins such as growth hormone to the circulation over at least a 3-month period. This type of delivery system may be useful in the treatment of children with growth hormone deficiency (*29*) and in counteracting the decrease in muscle mass and increase in adipose tissue typical of aging in adults (*30*). From the data presented here, we estimate that approximately 10^6 pooled myoblasts are capable of producing and secreting 4 μg of hGH per day in vitro and upon implantation in mice maintain a steady-state serum level of greater than 1 ng/ml (Fig. 2 and Table 1). An adult man produces approximately 400 μg hGH per day, a production rate that results in a mean serum concentration over 24 hours of 1.8 ng per ml (*31*). Thus, in theory, the implantation of approximately 10^8 myoblasts could provide therapeutic serum levels of hGH in humans. In a phase 1 clinical trial of myoblast transfer in Duchenne muscular dystrophy patients, we have delivered 10^8 human myoblasts to a small region of a single muscle (*32*). In the course of this trial, we have shown that human myoblasts can be purified and expanded to large numbers, as predicted (*33*). Taken together, these findings suggest that somatic cell therapy using myoblasts may have application in the delivery to the circulation of a number of recombinant proteins such as hormones, coagulation factors, and anticancer agents for the treatment of inherited and acquired diseases.

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produced by transfection of the ψ-CRIP amphotropic packaging line were used to infect myoblasts. Supernatants from MFG and α-SGC producer cells were filtered (0.45 μm), mixed, and added with polybrene at 8 μg/ml to C2C12 mouse myoblasts for 12 hours, then replaced with fresh viral supernatant for an additional 12 hours. Transduced cells were tested for production of helper virus by L. Cohen (Somatix Therapy Corporation), who used the *his* mobilization assay [O. Danos and R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6460 (1988)]. Using ecotropic Moloney murine leukemia virus as the control for replication competent virus, we determined the sensitivity of the assay to be 1 particle per 2.0 ml of culture supernatant. All of the test samples registered negative.
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Regulation of Phagocyte Oxygen Radical Production by the GTP-Binding Protein Rac 2

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A major action of the microbicidal system of human neutrophils is the formation of superoxide anion (O_2^-) by a multicomponent oxidase that transfers electrons from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to molecular oxygen. The mechanism of assembly and activation of the oxidase from its cytosolic and membrane-bound components is unknown, but may require the activity of a guanosine 5′-triphosphate (GTP)-binding component. A cytosolic GTP-binding protein (G_{ox}) that regulates the NADPH oxidase of neutrophils was identified. G_{ox} was purified and shown to augment the rate of O_2^- production in a cell-free oxidase activation system. Sequence analysis of peptide fragments from G_{ox} identified it as Rac 2, a member of the Ras superfamily of GTP-binding proteins. Antibody to a peptide derived from the COOH-terminus of Rac 2 inhibited O_2^- generation in a concentration-dependent manner. These results suggest that Rac 2 is a regulatory component of the human neutrophil NADPH oxidase, and provide new insights into the mechanism by which this oxygen radical–generating system is regulated.

HUMAN NEUTROPHILS AND OTHER phagocytic cells undergo a respiratory burst in which superoxide anion (O_2^-) and its derivatives (H_2O_2, HOCl, HO·) are produced as a means of destroying ingested microorganisms. The significance of the respiratory burst in host defense is made evident by the recurrent and life-threatening infections that occur in patients with chronic granulomatous disease (CGD) in whose phagocytes the burst does not occur. It is known that CGD results from genetic defects in any one of the four known protein components of the NADPH oxidase enzyme responsible for generating O_2^- (*1*). Studies with various cell-free oxidase activation systems (*2*) and cellular material from CGD patients have helped to determine the identity and function of the components of the NADPH oxidase. The oxidase is composed of membrane-bound proteins that include cytochrome b_{558} and possibly a 45-kD flavoprotein, as well as cytosolic components, of which two have been well characterized: $p47_{[phox]}$ and $p67_{[phox]}$ (*3*). The NADPH oxidase is activated, at least in part, by the association of these components into a membrane-bound complex that can transfer electrons from NADPH to molecular oxygen, generating O_2^- (*3*).

A GTP-binding protein (*5*) appears to regulate NADPH oxidase activity. GTP is required for oxidase activation in cell-free systems and GDP analogs inhibit this activation (*4*). Also, inhibition of protein isoprenylation decreases the rate of O_2^- generation (*5*). Post-translational modifications that attach 15- or 20-carbon isoprenoid moieties are a common feature of GTP-binding proteins of the Ras superfamily, suggesting that a member of this family may be involved in oxidase activation. Members

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