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Genetics

Long-term production and delivery of human growth hormone *in vivo*

(nonviral gene therapy/skin fibroblasts/somatotropin)

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ABSTRACT **The application of somatic cell gene therapy to large patient populations will require the development of safe and practical approaches to the generation and characterization of genetically manipulated cells. Transkaryotic implantation is a gene therapy system based on the production of clonal strains of engineered primary and secondary cells, using nonviral methods. We demonstrate here that, on implantation, these clonal cell strains stably and reproducibly deliver pharmacologic quantities of protein for the lifetime of the experimental animals.**

Efforts to develop human gene therapies have their roots in the 1950s and 1960s, when early successes with renal transplantation led to the consideration of injecting healthy cells into patients suffering from genetic diseases. For example, almost 30 years have passed since it was first speculated that organ transplantation or gene transfer might be applied to the treatment of patients afflicted with Gaucher disease (1). In the 1970s, initial clinical studies designed to test organ and tissue transplantation to treat Gaucher disease (2) and Hunter syndrome (3) were undertaken with, at best, limited success. It was in this setting that the term "gene therapy" was coined (4).

Until recently, the theoretical and experimental work on human gene therapy was centered on extremely rare genetic diseases. It has become apparent, however, that gene therapy may be appropriate in a variety of clinical settings regardless of their etiologies. Gene therapy can thus be thought of as an *in vivo* protein production and delivery system, and many diseases that are currently treated by the administration of proteins may be amenable to treatment by gene therapy. This broad definition allows a variety of common diseases to be thought of as candidates for gene therapy, including diabetes (insulin), hypercholesterolemia (low density lipoprotein receptors), various forms of anemia (erythropoietin), and cancer (cytokines and cytotoxic proteins).

We have developed an approach to the long-term administration of systemic proteins based on the isolation of somatic cells from a patient, the stable introduction of a gene of therapeutic interest into the cells, the isolation and clonal propagation of a single engineered cell, and finally, the reintroduction of the cells into the patient. This approach, termed "transkaryotic implantation" (5, 6), has several features that will be beneficial from the patient's and clinician's points of view. In particular, the approach was designed to permit detailed functional characterization of engineered cells prior to implantation, minimize genetic change to the patient, and allow pharmacologic and physiologic regulation of the secreted protein. It follows that these properties are logical extensions of a system based on generation and implantation of an autologous clone of genetically engineered cells. Furthermore, based on safety and efficacy considerations for the use of gene therapy in large patient populations, we have avoided the use of viral vectors of any type.

Previous work (5, 6) demonstrated certain properties of transkaryotic implantation in a simple animal model system. In particular, it was established that intraperitoneal injection of cells engineered to express human growth hormone (hGH) or human proinsulin resulted in systemic delivery of the relevant protein. The methodology described in these earlier studies is limited in that the use of immortalized fibroblasts in isogeneic or allogeneic hosts may result in tumor formation. In contrast, the use of nonimmortalized clonal strains of primary and secondary cells may offer significant benefits to the patient. In addition to lacking tumorigenicity, primary and secondary cells are more likely to maintain differentiated functions than their immortalized counterparts. Here, we demonstrate the long-term expression of pharmacologic quantities of hGH in a mouse model system. Expression of hGH by transkaryotic implantation of genetically engineered primary fibroblasts is stable in the circulation for the lifetime of the experimental animals, is reproducible, and causes no side effects.

MATERIALS AND METHODS

Fibroblast Transfection. Fibroblasts were isolated from the fascia underlying the dorsolateral dermis of female New Zealand White rabbits by enzymatic digestion (7). For transfection, rabbit fibroblasts were harvested by trypsinization and suspended at 10^6 cells per ml in 20 mM Hepes, pH 7.3/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM dextrose with 15 μg each of plasmids pXGH5 (8) and pcDneo (9). Electroporation (300 V, 960 μF) was performed with a Gene Pulser (Bio-Rad) and transfected colonies were selected in medium with G418 (1 mg/ml). Cell counts and mean cell volumes at confluence were determined with a Coulter Counter model ZM. Soft-agar assays were performed as described (10). Molecular biological techniques were performed by standard methods (11). Hybridization was performed at 65°C on a nylon membrane (Hybond-N; Amersham) and was followed by washing in 15 mM NaCl/15 mM sodium citrate, pH 7/0.2% SDS at 56°C.

Animal Studies. Prior to implantation, mice were anesthetized by intraperitoneal injection of Avertin [solution of 2% (wt/vol) 2,2,2-tribromoethanol and 2% (vol/vol) 2-methyl-2-butanol] at a dose of 0.0175 ml/g of body weight. For blood drawings (12), animals were anesthetized with a methoxyflurane inhalant (Metofane; Pitman-Moore, Washington Crossing, NJ). Serum hGH levels were measured with a commercially available kit (Nichols Institute, San Juan Cap-

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Abbreviations: cpd, cumulative population doubling(s); hGH, human growth hormone.

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istrano, CA). Mouse growth hormone is not detectable with this assay.

RESULTS

Development of a Model System for Implantation of Genetically Engineered Primary Cells. Based on earlier studies, we estimate that 100 million to 1 billion genetically engineered cells would need to be implanted into an adult to achieve a therapeutic level of a given protein in the circulation. To derive this quantity of cells from a single transfected cell, the clonal population must undergo ≈30 cumulative population doublings (cpd). One of the best-characterized cell types capable of this degree of *in vitro* growth is the human fibroblast (13). Primary and secondary human fibroblasts have additional properties that make them attractive for a gene therapy system: they are highly refractory to spontaneous transformation (14–16), they are readily obtained (by punch biopsy) and reimplanted (by subcutaneous injection), their numbers show little fluctuation *in vivo* [with the notable exceptions of growth and wound healing (17)], and finally, they are ubiquitous throughout the major organ systems of the body.

Neither human nor mouse cells are ideally suited for an animal model system for transkaryotic gene therapy. Human primary and secondary fibroblasts have one major limitation in this regard; following xenogeneic implantation, the cells do not generally show long-term survival, even in immunocompromised animals (18). Murine primary and secondary fibroblasts also are inappropriate in this system because they undergo spontaneous transformation following ≈15 cpd *in vitro* (19), well short of the 30 cpd required. These considerations led to a search for a non-human, non-mouse mammalian fibroblast that would not undergo early transformation *in vitro* yet would be capable of long-term, nontumorigenic survival following implantation in a nude mouse model system. The nude mouse was selected as the host animal because a genetically homogeneous immunocompromised mouse strain allows for the generation of a large body of data on the reproducibility and safety (particularly tumorigenicity) of the system.

Primary and secondary rabbit fibroblasts were evaluated in this context by first determining their susceptibility to spontaneous early transformation, here defined as occurring prior to the attainment of 30 cpd in culture. Fibroblasts were isolated from skin samples obtained from New Zealand White rabbits by enzymatic digestion and grown under standard conditions for human and mouse primary fibroblasts (7). Unlike mouse fibroblasts, the rabbit cells did not appear to experience crisis or transformation based on observation by light microscopy. More importantly, over the course of 30 cpd, cell volume increased, saturation density decreased, and mean doubling time increased (Table 1), all characteristic behaviors of untransformed, senescing cells (20–22). Similarly, rabbit fibroblasts were unable to form colonies in soft agar, a reliable predictor of *in vivo* tumorigenicity (23) (see Table 1 legend). Furthermore, the analysis of over 30 untransfected and transfected clonal strains of rabbit fibroblasts in nude mice has shown no indication of tumorigenicity of the implanted cells (see below).

Table 1. Growth properties of untransfected rabbit fibroblasts

cpd	Cell volume, fl	Saturation density, cells per cm^2	Doubling time, hr
8–10	2100 ± 180	106,000 ± 12,500	31.4 ± 2.1
18–21	2640 ± 210	82,800 ± 13,200	ND
29–31	3330 ± 320	68,100 ± 13,100	60.8 ± 13.8

At intervals of ≈10 cpd in culture, rabbit fibroblasts were subjected to growth studies. Cell volumes and saturation densities are presented as the mean ± SE obtained from six independent rabbit fibroblast cultures. Doubling times (± SE) were calculated for four independent rabbit fibroblast cultures and are presented as average growth rate over a 4-day period. The ability of rabbit fibroblasts to grow in soft agar was assessed; even when scoring for colonies used a cutoff of ≈32 cells, colony formation by normal rabbit fibroblasts was undetectable (<0.001%).

Stable Transfection of Primary Rabbit Fibroblasts. To generate cells stably producing hGH, rabbit fibroblasts were cotransfected with plasmids pcDneo (9) and pXGH5 (8). pXGH5 is a pUC12-based plasmid containing 5′ mouse metallothionein I gene sequences fused to the hGH gene. The plasmid directs the expression of hGH in both transiently transfected cells (8) and transgenic mice (24). For rabbit fibroblasts, transfection efficiency averaged ≈1 stable transformant per 10^3 treated cells and ranged from 10^{-2} to 10^{-4}.

Approximately 20% of the clones secreted hGH into the medium. Expressing clones were expanded and propagated for *in vitro* characterization as well as implantation. The highest-expressing cell strain generated in these initial experiments, RF917, produced ≈40 μg of hGH per 10^6 cells per day; this level of hGH production was stable throughout the cells' lifetime *in vitro* (data not shown). Strain RF917 appears to have incorporated approximately two copies of each of plasmids pXGH5 and pcDneo per haploid genome (Fig. 1).

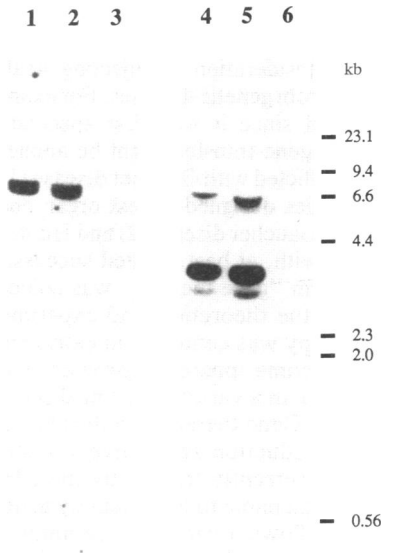

FIG. 1. Stability of integrated hGH and neomycin-resistance (*neo*) gene sequences. Genomic integration patterns of plasmids pXGH5 and pcDneo were analyzed by Southern hybridization in RF917 cells at 32.6 and 45 cpd. Cellular DNA was digested with *Eco*RI and electrophoresed in a 0.7% agarose gel. Lanes 1 and 4, RF917 DNA at 32.6 cpd; lanes 2 and 5, RF917 DNA at 45 cpd; lanes 3 and 6, control DNA (untransfected rabbit fibroblasts). Lanes 1–3 were hybridized with a ^{32}P-labeled 0.8-kb *Pst* I fragment containing the *neo* gene (9) and lanes 4–6 with a ^{32}P-labeled hGH cDNA probe (25). The *neo* probe reveals a doublet at 6.8 and 6.6 kb (lanes 1 and 2). If the entire 4.4-kb pcDneo plasmid was integrated, this pattern suggests that the cells contain two independent integrations. The hGH cDNA probe (lanes 4 and 5) reveals a major band at 4.0 kb with minor bands at approximately 6.6 and 2.0 kb. The major band corresponds to an internal 4.0-kb fragment from plasmid pXGH5 (8), containing the hGH gene and the mouse metallothionein I (mMT-I) promoter. The minor bands most likely represent the integration of pXGH5 by crossing-over within hGH gene sequences, generating two fragments with homology to the hGH cDNA probe. Reconstruction studies are consistent with the presence of approximately two copies of the mMT-I/hGH fusion gene per haploid genome. The endogenous rabbit growth hormone gene shows no hybridization with the hGH cDNA (lane 6).

Southern hybridization analysis indicates the locus of integration is stable from 32.6 to 45 cpd and is grossly intact. At 33.7 cpd, just prior to implantation, cell volume was ≈3700 fl, saturation density was ≈6.5 × 10^4 cells per cm^2, and doubling time was 60.9 hr. By these criteria, the transfected, hGH-secreting cells resembled untransfected rabbit fibroblasts (see Table 1). Similarly, RF917 cells were unable to form colonies in soft agar.

Subcapsular Implantation of hGH-Producing Fibroblasts. The renal capsule was chosen as the implantation site, to allow viewing of the cells following their introduction into the animal. Approximately 3 million RF917 cells were introduced under the left renal capsule of each of ten Swiss nude (*nu/nu*) mice. The animals were initially bled at weekly intervals and later at semi-monthly and monthly intervals for assay of serum hGH levels. Sera from untreated mice and from mice implanted with untransfected fibroblasts contained no detectable hGH. In contrast, the experimental animals were found to express hGH in their sera, and this expression continued for the lifetime of the animals (Fig. 2*A*). The longest-surviving animals expressed hGH in their serum for over 500 days.

The mice experienced no side effects from treatment and exhibited a normal life expectancy compared with control mice. Most importantly, the implantation of the cells was not associated with either local or metastatic tumor formation as judged by gross and histologic organ evaluation.

To assess the reproducibility of the level and duration of hGH expression in transkaryotic implantation, RF917 cells stored in liquid nitrogen for ≈6 months were thawed, cultured, and implanted into a group of 14 nude mice as described above. The serum hGH expression profile from these mice was quite similar to that of the initial experiment (Fig. 2*B*, ▲). In both groups, average hGH expression fluctuated within a relatively narrow range (approximately 1–3 ng/ml in the first group and 1.5–4.5 ng/ml in the second) for most of the duration of the experiments.

In an attempt to alter the serum concentration of hGH in treated mice, a strain of rabbit fibroblasts secreting 4 μg of

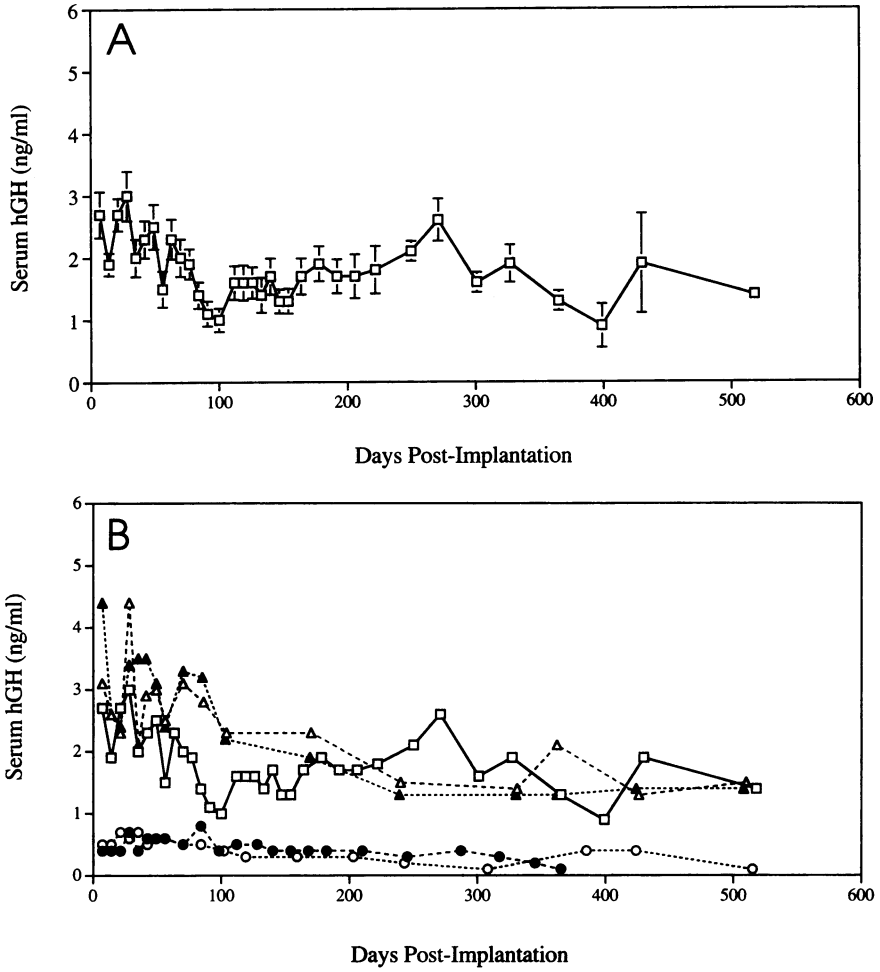

FIG. 2. *In vivo* delivery of hGH in immunodeficient mice. (*A*) Stability of serum hGH over time in mice implanted with RF917 cells. Approximately 3 × 10^6 cells were implanted under the renal capsule of 10 Swiss *nu/nu* mice (Taconic Farms; approximate age at implantation was 12 weeks). The mice were bled at weekly intervals for 5 months, at which point the bleeding intervals were lengthened to limit mortality due to bleeding of older animals. Each point represents the mean ± SE of serum hGH assays of surviving mice; n = 10 animals at day 7 after implantation, n = 9 at day 84, n = 7 at day 206, n = 6 at day 250, n = 5 at day 365, n = 2 at day 399, and n = 1 at day 518. (*B*) Reproducibility of transkaryotic implantation of two strains of rabbit fibroblasts. Approximately 3 × 10^6 cells were implanted under the mouse renal capsule. □, Same group as shown in *A*; ▲, repeat of the experiment shown in *A* but initiated with 14 Swiss *nu/nu* mice; △, data obtained from the subcapsular implantation of RF917 cells implanted into 14 *scid/scid* mice; ● and ○, subcapsular implantation of RF403 into two groups of 10 and 15 Swiss *nu/nu* mice, respectively. In addition, a group of 20 Swiss *nu/nu* mice were implanted with 3 × 10^6 cells of clonal, untransfected rabbit fibroblasts. No hGH (<0.05 ng/ml) was detectable in the sera of these control animals. Survival data: ▲ group, n = 14 at day 7, n = 13 at day 14, n = 12 at day 28, n = 11 at day 239, n = 6 at day 330, n = 3 at day 424, and n = 1 at day 508; △ group, n = 14 at day 7, n = 13 at day 170, n = 12 at day 331, n = 9 at day 426, and n = 3 at day 510; ● group, n = 10 at day 154, n = 9 at day 168, n = 8 at day 210, n = 7 at day 287, n = 6 at day 317, and n = 2 at day 365; ○ group, n = 15 at day 84, n = 14 at day 102, n = 12 at day 243, n = 11 at day 308, and n = 8 at day 424.

hGH per 10^6 cells per day (RF403) was implanted under the renal capsule in two groups (10 and 15 mice) of Swiss nude (*nu/nu*) mice. For most of the duration of the experiments, serum hGH expression was centered about 0.4 ng/ml (Fig. 2*B*, circles). Finally, RF917 cells were implanted under the renal capsule of 14 *scid/scid* (severe combined immunodeficiency) mice, and the pattern of hGH expression in these animals (Fig. 2*B*, △) was virtually indistinguishable from that obtained using Swiss *nu/nu* mice. None of these animals experienced side effects.

To evaluate the fate of the implanted cells, a series of subcapsular implantations identical to those described above was performed. At various intervals after implantation, two mice were sacrificed and the left kidneys were removed for assessment. The renal capsules of untreated mice were sparsely vascularized, but within 1 month of implantation of fibroblasts, an increase in vascularization was noted. The degree of vascularization plateaued at ≈4 months after implantation and remained constant thereafter (Fig. 3). This vascularization was observed with hGH-secreting rabbit fibroblasts as well as with untransfected rabbit fibroblasts. Mock (saline)-implanted kidneys showed no significant neovascularization (data not shown).

DISCUSSION

We have demonstrated the stable transfection of primary rabbit fibroblasts and the generation of clonal secondary strains expressing large quantities of hGH. Following implantation, these genetically engineered strains directed the expression of pharmacologic levels of hGH in the sera of nude mice. *In vivo* expression persisted at stable levels for the lifetime of the animals, was reproducible, and could be controlled based on the production levels of the cells chosen for implantation. These results were obtained with the notable absence of side effects. To our knowledge, this is the first report of lifetime expression in any gene therapy system, regardless of methodology.

The untransfected and transfected rabbit fibroblasts utilized in these studies did not appear to be transformed as judged by both *in vitro* (soft agar) and *in vivo* (*nu/nu* and *scid/scid* mice) assays. In addition, the stability of the locus of integration and hGH expression, the gradual decrease in saturation density, and the increase in cell volume and doubling time are all characteristic of untransformed cells. These data strongly suggest that the processes of transfection, selection, and *in vitro* expansion have no major effect on the growth properties of rabbit fibroblasts.

The long-term synthesis of stable levels of serum hGH following implantation suggests that cell number remains approximately constant over time. It seems reasonable that the implanted cells should not increase in number; fibroblasts in tissue do not exhibit significant increases in cell number with the exceptions of wound healing and somatic growth (17). Similarly, the implanted cells should not decrease in number; since fibroblasts are present in all tissue types in the body (including the renal capsule and parenchyma), the implanted cells are likely to encounter a supportive local environment (and in immunocompromised mice, the rabbit cells are protected from rejection). In any case, the long-term survival and function of the transfected fibroblasts following implantation is likely to be due in part to the ability of the cells to induce vascularization. Neovascularization of primary fibroblast implants has also been noted in the work of Kawaja *et al.* (26).

There are several advantages to the transkaryotic implantation of primary and secondary fibroblasts. The cells are capable of high levels of expression of therapeutic proteins and compare favorably to expression in immortalized cell lines (perhaps because immortalized cells are fuel inefficient; ref. 27). In addition, human primary and secondary fibroblasts are resistant to spontaneous transformation *in vitro*. Finally, the cells can be removed from the site of implantation (particularly if a subcutaneous route is utilized), a property that may be beneficial with respect to safety (if the cells behave unexpectedly) and efficacy (if the treatment is intended to be transient).

Fibroblasts have been utilized in other gene therapy studies. Palmer *et al.* (28) implanted heterogeneous populations of retrovirally infected primary rat skin fibroblasts into syngeneic rats and observed expression of the therapeutic protein for ≈2 weeks. The authors suggested that this short-lived expression may have been due to irreversible inhibition of expression of DNA sequences present within the retroviral vector. Another group (29) obtained similar results in mouse model systems, and Scharfmann *et al.* (30) suggested that the observed inactivation of proviral expression could be solved by the choice of appropriate promoter sequences in the viral vector.

The major advantage of retrovirally mediated gene therapy is the high efficiency of transduction of the target cells. For cell types that are incapable of significant growth and characterization *in vitro*, retroviral infection and viral infection in general remain viable approaches to gene transfer. For cells that are capable of significant growth *in vitro*, however, the ability to select stably transfected cells reduces the need for extremely high-efficiency transduction; as demonstrated here, even a transfection efficiency of 10^{-3} is at least 3 orders of magnitude higher than is required for a practical gene therapy system. The use of a physical method for the introduction of DNA sequences into primary cells followed by clonal isolation of appropriately engineered strains should in theory reduce the risks of mutagenesis (as all cells within the clonal population have the same integration pattern) and recombination (with endogenous proviruses) that limit the retroviral system (31). In addition, physical methods of

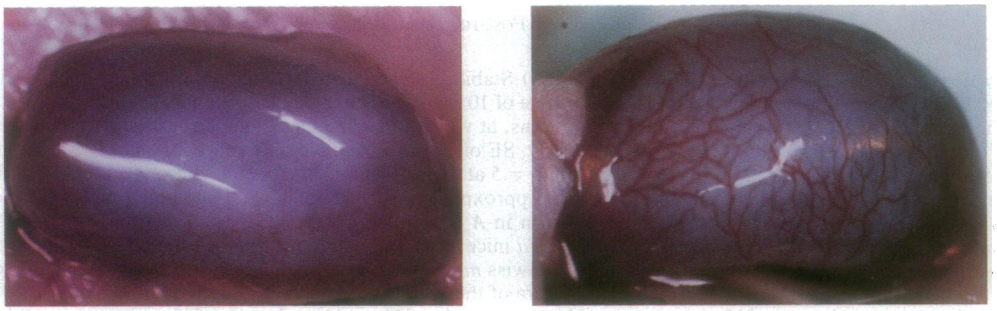

FIG. 3. Vascularization of the renal capsule following implantation of transfected rabbit fibroblasts. (*Left*) Renal capsule 35 days following implantation of 3×10^6 RF917 cells. The implanted cells were clearly visualized and early neovascularization can be seen. (*Right*) Renal capsule 515 days following implantation of 3×10^6 RF917 cells. Note the extensive vascularization of the capsule.

transfection have no constraints on the amount of exogenous genetic material that can be incorporated into recombinant retroviruses; the ability to introduce large genes or large numbers of genes into the target cell may prove useful in the engineering of complex regulatory or processing pathways.

Though the data presented here strongly support the use of clones of stably transfected fibroblasts to achieve *in vivo* protein production and delivery, the system as described has one major limitation at present. For the treatment of certain diseases, the system must be modified to allow regulated protein synthesis and secretion. We believe that there are inherent advantages in attempting to engineer such physiologic and pharmacologic control in clonal strains derived from a single genetically manipulated cell and amenable to detailed functional characterization prior to implantation. While the development of a truly regulatable system would offer maximal clinical utility for the treatment of many disorders, there are many diseases that can be adequately treated (and perhaps cured) by the long-term delivery of therapeutic proteins at steady-state levels.

Short stature caused by growth hormone deficiency may be a candidate for treatment by steady-state delivery of hGH by gene therapy. Serum hGH concentrations in healthy children generally are <5 ng/ml in resting or fasting subjects and increase to 10 ng/ml or higher on exercise (32, 33). The hGH concentrations we have achieved in our animal model system certainly fall within the physiologic range—in fact, when it is considered that the half-life of hGH is 4 min in mice (34), compared with 20 min in humans (35), it may require substantially fewer cells to treat a patient than would be expected based on considerations of body mass alone. Although progress with human cells must be made before such experiments are attempted, steady-state delivery of hGH via gene therapy may offer enhanced efficacy as compared to standard hGH delivery (which requires frequent large doses of protein to overcome hGH's short half-life). For the treatment of short stature, we believe it will be possible to perform a punch biopsy on a patient; isolate, transfect, and clone fibroblasts; and reintroduce appropriately characterized cells into the patient, ideally via subcutaneous injection.

The long-term delivery of therapeutic proteins may have a significant impact on the treatment of a variety of common diseases, including diabetes, hypercholesterolemia, and osteoporosis. As the genetic contribution and molecular biology of other chronic debilitating diseases are dissected, they too may become candidates for transkaryotic implantation. The application of gene therapy to common diseases that are not imminently life-threatening will require the development of safe, effective, and flexible systems that can be practically applied to large numbers of patients. We believe that transkaryotic implantation has the potential to become such a system.

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