Proliferation Induced by Keratinocyte Growth Factor Enhances In Vivo Retroviral-mediated Gene Transfer to Mouse Hepatocytes

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Abstract

Retroviral gene transfer to liver without prior injury has not yet been accomplished. We hypothesized that recombinant human keratinocyte growth factor would stimulate proliferation of hepatocytes and allow for efficient in vivo gene transfer with high titer murine Moloney retroviral vectors. This report shows that 48 h after intravenous injection of keratinocyte growth factor, hepatocyte proliferation increased approximately 40-fold compared to non-stimulated livers. When keratinocyte growth factor treatment was followed by intravenous injection of high titer (1×10^8 colony forming units/ml) retrovirus coding for the Escherichia Coli β-galactosidase gene, there was a 600-fold increase in β-galactosidase expression, with 2% of hepatocytes transduced. Thus, by exploiting the mitogenic properties of keratinocyte growth factor, retrovirus-mediated gene transfer to liver may be accomplished in vivo without the use of partial hepatectomy or pretreatment with other toxins to induce hepatocyte cell division. (*J. Clin. Invest.* 1996. 98:2683–2687.) Key words: gene therapy • KGF • retrovirus • liver • gene transfer • hepatocytes

Introduction

Gene transfer to hepatocytes as an approach to the treatment of inherited diseases has been accomplished with viral and non-viral techniques. Gene transfer with recombinant adenoviruses is efficient but transient, due in part to a robust immune response after vector delivery (1–4). Transient gene transfer to hepatocytes has also been achieved with asialoglycoprotein or lipid complexed to DNA (5, 6). Recently, successful gene transfer to liver has been reported with retroviral vectors in rodents, rabbits, and humans (7–12). Only a fraction of the total number of hepatocytes can be targeted by recombinant retroviral vectors, however, since the spontaneous rate of hepatocyte proliferation is low (0.05 and 0.005%) (13) and dividing cells are required for nuclear entry and integration of murine Moloney leukemia based retroviruses. For these reasons, Moloney leukemia based retroviral gene transfer to liver has largely been accomplished using invasive approaches such as ex vivo transduction (7, 9, 12) or 70% hepatectomy followed by in vivo infusion of retrovirus (8, 10, 14–16). More recent studies have shown that induction of hepatocyte regeneration by prior administration of toxins (17) or toxic gene products (18) allows for retroviral mediated gene transfer.

We initiated studies to address the problems of low vector titer and low levels of hepatocyte proliferation. We hypothesized that direct retroviral mediated gene transfer to liver could be accomplished by combining growth factor pretreatment with high titer retroviral vectors. Support for our hypothesis comes from studies by others showing that mitogenic polypeptide growth factors including EGF, hepatocyte growth factor, and acidic fibroblast growth factor stimulate hepatocyte proliferation in vitro and are important in regeneration after injury or partial hepatectomy (19–24). Intravenous administration of keratinocyte growth factor (KGF;FGF-7) to rats also stimulates hepatocyte proliferation in non-injured liver in vivo (25). Our results show that recombinant keratinocyte growth factor (rKGF)[1] is also a potent mitogen for murine hepatocytes. More importantly, this mitogenic response allows for direct in vivo transduction of 2% of hepatocytes with high titer retroviral vectors.

Methods

Reagents. Endotoxin free recombinant human keratinocyte growth factor was provided by Amgen Inc. (Thousand Oaks, CA). Total bilirubin and ALT/AST kits were from Sigma Chemical Corp (St. Louis, MO). High titer retrovirus expressing *Escherichia coli* β-galactosidase (DA-βgal) was prepared by Chiron Viagene, Inc. (San Diego, CA) (International patent publication numbers WO 95/10601 and WO 92/05266). 10 liters of crude vector supernatants were collected and pooled at 4°C, filtered through a 0.45 μm filter (Millipore, Bedford, MA) then ultrafiltered in a tangential flow concentration apparatus (Millipore) with a nominal pore of 300,000 mol wt to remove most media components. The concentrate was dialyzed in the same apparatus, loaded on a Sepharose 4B size exclusion column and the vector collected from the void volume. The void volume was pooled and formulated to give a final concentration of 25 mM tromethamine pH 7.4, 60 mM NaC1, 1 mg/ml arginine, 5 mg/ml HSA, and 50 mg/ml lactose. Yields were typically between 2%–30% of

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1. *Abbreviations used in this paper:* βgal, β-galactosidase; BrdU, 5′-bromo-2′-deoxyuridine and 5-fluore-2′-deoxyuridine (10:1); DA-βgal, high titer retrovirus expressing E. coli β-galactosidase; LTR, long terminal repeat; rKGF, recombinant keratinocyte growth factor.

starting material with a 10- to 50-fold-concentration. Vector preparations were shown to be free of replication competent retrovirus by hygromycin marker rescue assay of vector, or by *Mus dunni*/producer cell co-cultivations as described (26). The colony forming units (CFU/ml) were determined by dilution series titration on the human fibrosarcoma cell line HT 1080.

Animals. All animal procedures had prior institutional approval. 8–12 wk-old C57B1/6 males were used in all experiments and were purchased from Jackson Laboratories (Bar Harbor, ME). Intravenous injections of rKGF and retrovirus were done via tail vein without anesthesia. rKGF was injected intravenously at doses of 5 μg/g of body weight. Retrovirus was administered in 100-μl aliquots and had a titer of 1×10^8 (see Fig. 2) or 5×10^7 CFU/ml (see Fig. 3). At the time of death, mice were anesthetized with an overdose of ketamine/xylazine and perfused through the left ventricle with 2% paraformaldehyde in PBS or 10% formalin. Injection strategies were as follows: to test the effects of rKGF, animals were injected with rKGF once (day −1) or for two consecutive days (days −1 and 0) and animals were killed starting 2 d after the first injection (day 1). Retroviral gene transfer with DA-βgal was done on day 0 and +1, with rKGF injections given both 24 h before and simultaneous to the first retrovirus injection. Mice were killed at the indicated times and the number of blue hepatocytes/mm^2 determined as described below.

Tissue analyses. Identification of proliferating hepatocytes. Mice were injected with 10 μl/g body weight i.p. of 5′-bromo-2′-deoxyuridine and 5-fluore-2′-deoxyuridine (10:1) (BrdU labeling reagent; Zymed, San Francisco, CA) 2 h before death. Animals were perfused with normal saline and tissues were fixed with 10% formalin for 16 h. 5 μm thick paraffin embedded sections were stained with a monoclonal antibody against BrdU conjugated with biotin (Zymed) and an avidin secondary antibody conjugated with fluorescein (Sigma Chemical Co.). Slides were evaluated using a Leitz DM RBE fluorescent photomicroscope from Leica (Wetzlar, Germany). Proliferating hepatocytes from random fields (× 100) were counted from four non-adjacent regions for each section, with eight non-consecutive sections per animal counted. Differences in proliferation between rKGF treated groups and PBS controls were analyzed by one-way analysis of variance followed by Bonferonni *t*-test at 0.05 significance level. The combined average of day 1 and day 3 values for controls (PBS treated) was used in the comparisons with rKGF treatment.

X-gal staining. Some mice injected with retrovirus were perfused with 2% paraformaldehyde in PBS and fixed overnight. Livers were blocked and stained for 4 h at 37°C with 40 mg/ml of X-gal from Gold Biotechnology Inc. (St. Louis, MO) using previously described techniques (28) and photographed using a Leica WildMZ8 stereomicroscope. After en bloc staining, tissues were frozen in O.C.T. (Miles, Inc., Elkhart, IN) and 10 μm sections placed onto slides and counterstained in neutral red for photomicroscopy. The number of transduced hepatocytes/mm^2 of tissue were counted for each experimental group. This value was compared to the number of hepatocytes that can occupy 1 mm^2 of tissue, based on a mean hepatocyte diameter of 25 μm and that hepatocytes account for 65% of the total liver volume (15, 27). Statistical evaluation of the differences in numbers of transduced hepatocytes between the rKGF-treated groups and the control groups (no rKGF) were determined using a 1 tail 2 sample *t*-test with unequal variance of 0.05 significance level.

Another animal injected with retrovirus was subjected to a partial lobectomy 5 mo after treatment. The hepatocytes were dispersed with collagenase and plated onto 100 mm tissue culture dishes (18). 4 h after plating, hepatocytes were fixed with 0.05% gluteraldehyde, rinsed in PBS, and stained in X-gal solution for 2 h. Random fields from 4 separate platings were counted for mean percent positive hepatocytes. 4 mo later (9 mo postinjection of rKGF and retrovirus), the animal was killed and hepatocytes dispersed and counted as described.

β-galactosidase enzyme activity assay. The Galactolight Plus kit from Tropix, Inc. (Bedford, MA) was used to quantitatively evaluate β-galactosidase activity. 100 mg (wet weight) of liver was homogenized and endogenous β-galactosidase activity inactivated for 60 min at 48°C. Light units were normalized to microgram of protein. Significant differences were determined by *t*-test with Bonferonni correction for multiple comparisons with a significance level of 0.05.

PCR for retrovirus sequences. Two primers specific for the retroviral LTR (5′-CCCTGTGCCTTATTTGAACTAACC-3′) and gag sequences (5′-CCTACCAGAACCACATATCCCTCC-3′) were used to identify the presence of integrated viral sequences within spleen, liver, lung, testis, intestine, kidney, heart, and muscle. Tissues were extracted from mice 5 d after DA-βgal/rKGF treatment and 200 ng of DNA were amplified using standard conditions for Taq polymerase (Boehringer Mannheim) with 40 μM of each primer.

Results

To test the effect of rKGF in mouse liver, 5 μg per gram of body weight of rKGF or the same volume of PBS was injected

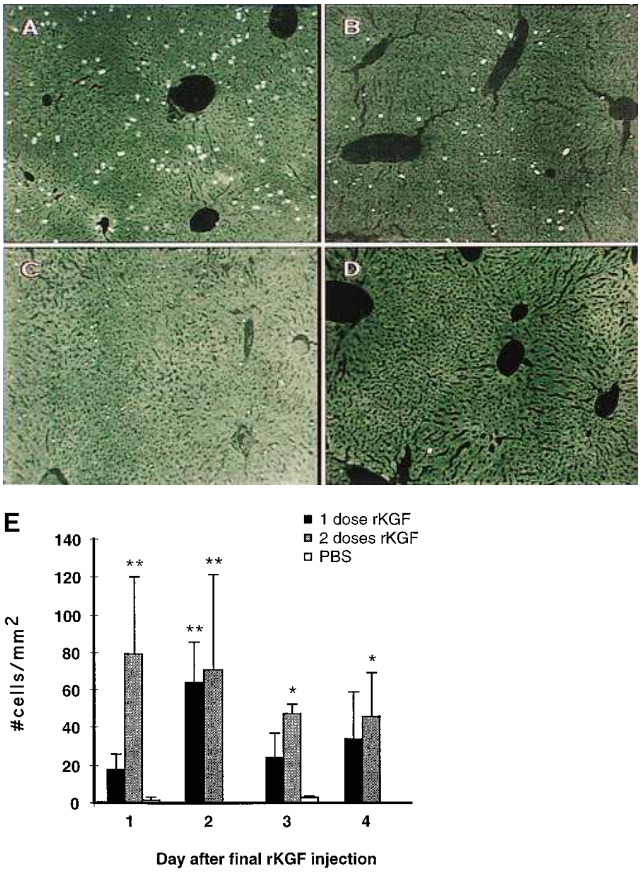

Figure 1. Immunohistochemical detection and quantitation of cellular proliferation in mouse hepatocytes after in vivo rKGF administration. (*a–c*) Mice were treated with rKGF for two consecutive days. 2, 3, and 4 d later (*a–c*, respectively) mice were given BrdU intraperitoneally before sacrifice. Fixed, paraffin embedded tissue sections were stained by IHC for BrdU and analyzed by fluorescence microscopy. (*d*) a representative photomicrograph from the vehicle-only control group. All photomicrographs were taken at × 100. (*e*) treatment with rKGF stimulates hepatocyte proliferation that peaks two days after the initial dose. rKGF was administered once or on two consecutive days, animals killed on the day indicated, and the number of BrdU positive hepatocytes/mm^2 in tissue sections counted. Control animals received sham injections of vehicle (PBS). Bars represent the mean±average deviation. Asterisks indicate statistically significant differences compared to PBS treated mice (*, $P < 0.05$; ‡, $P < 0.005$).

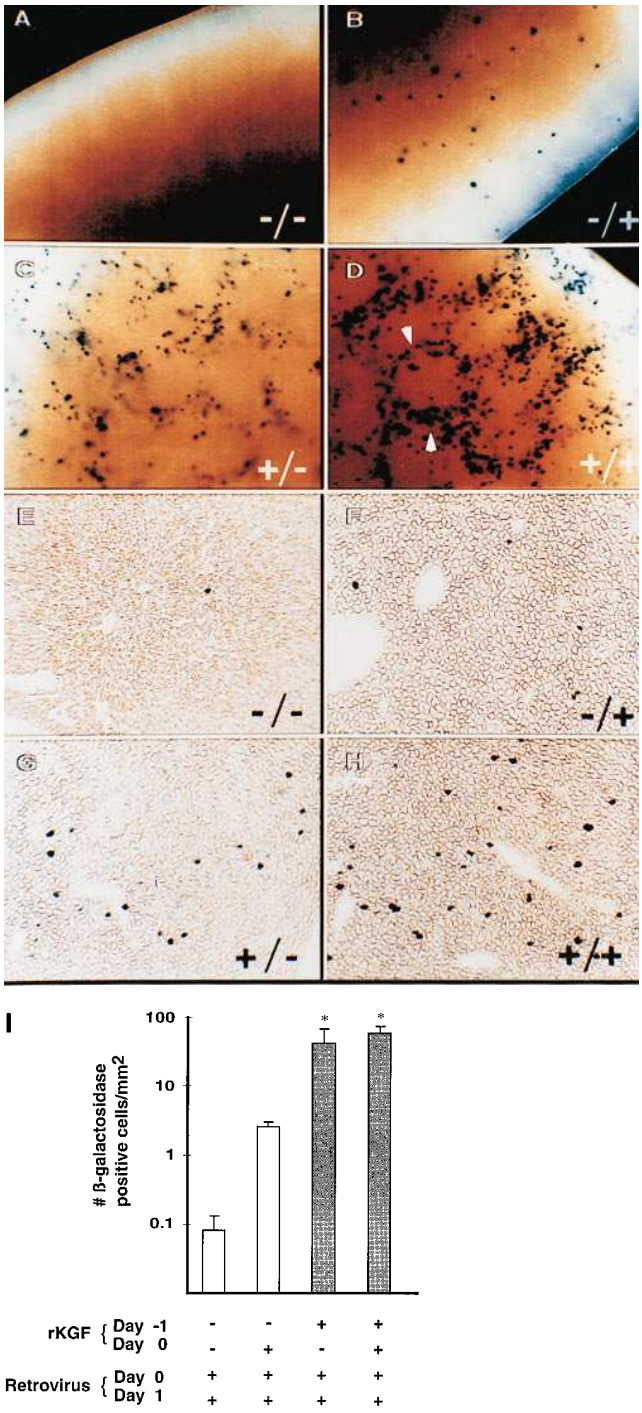

Figure 2. Pre-treatment with rKGF enhances retroviral mediated gene transfer of lacZ. (*a–d*) photomicrographs of X-gal stained lobes from animals treated with DA-βgal (*a*) or rKGF and DA-βgal (*b–d*). (*e–h*) representative sections from *a–d*, respectively. All animals were given two single doses of DA-βgal (100 μl/dose) on consecutive days (days 0 and 1). The +/− signs in the lower right of *a–h* correspond to rKGF treatment 24 h before (day -1) and/or in conjunction (day 0) with the first dose of DA-βgal, respectively. For example, the −/+ for *b* and *f* indicate that rKGF was given only on day 0, the same time as the first retrovirus injection. Arrow heads indicate periportal region. (*i*) rKGF 24 h before DA-βgal treatment significantly increases the number of X-gal positive hepatocytes/mm^2 400–600 fold (*; $P < 0.05$).

into the tail veins of C57B1/6 mice. This dose was previously shown to induce hepatocyte proliferation in rats (25). Mice were injected once or twice with rKGF and cohorts of three were killed daily from 1–4 d after the final rKGF injection. The mitogenic response to rKGF was assayed by BrdU immunohistochemistry (Fig. 1, *a–c*). The number of proliferating hepatocytes was notably above background (Fig. 1 *d*). Increases in the number of proliferating hepatocytes were noted acutely (1 d) with a subsequent decline to background levels by 2 wk (data not shown).

Quantitative analysis of the number of proliferating hepatocytes shows a peak response 2 d after the initial dose of rKGF whether the mice received 1 or 2 doses of rKGF (Fig. 1 *e*). The number of proliferating hepatocytes 2 d after the final dose of rKGF was significantly higher than in non-stimulated controls ($P < 0.005$; Fig. 1 *e*). However, there was no significant difference in the extent of proliferation between these two groups (79.3±40 vs 63.5±21.6 positive hepatocytes/mm^2). The livers of all animals appeared grossly and microscopically normal. Also, we did not detect any change in bilirubin or ALT/AST ratios after rKGF treatment (data not shown).

We next tested the hypothesis that rKGF induced hepatocyte proliferation would increase the efficiency of retroviral gene transfer to the liver by intravenous injection when compared to non-stimulated controls. The retrovirus used in these studies and in those described below is DA-βgal, an amphotrophic enveloped retrovirus expressing β-galactosidase off the viral long terminal repeat (LTR). This virus was purifed and concentrated to high titer (1–3 × 10^8 CFU/ml) as described in Methods (26, 28).

Gene transfer was initially assessed using histochemical methods. Eight mice were injected with retrovirus on two consecutive days. Some mice received rKGF while others did not, as summarized in the legend to Fig. 2. The mice were killed 21 d after the second dose of retrovirus and the livers stained with X-gal en bloc to detect β-galactosidase expression. Gross examination of liver lobes revealed that rKGF enhanced gene transfer (Fig. 2), and that injection of rKGF 1 d before DA-βgal was most effective (Fig. 2 *c* and *d*). Gene transfer was most evident in the periportal regions of the liver acini (Fig. 2 *c* and *d*).

X-gal positive hepatocytes from mice treated with rKGF and DA-βgal retrovirus were not found in clones greater than two hepatocytes, with all lobes transduced to an equal amount. In most instances single, positive cells were noted. This staining pattern is different than the focal gene transfer seen after partial hepatectomy where the liver mass is rapidly regenerated from the remaining hepatocytes (29). Serial sections stained for glycogen demonstrated that nearly all positive cells were glycogen containing hepatocytes (> 98%; not shown).

A 600-fold increase in the number of β-galactosidase expressing hepatocytes was seen in mice that received rKGF 24 h before DA-βgal (47.5 hepatocytes/mm^2±5.68) relative to controls (0.08 hepatocytes/mm^2±0.05; $P < 0.05$; Fig. 2 *i*). This striking difference was also reflected in total numbers of transduced hepatocytes; direct administration of high titer retrovirus alone resulted in very low levels of gene transfer (< 0.001%) while DA-βgal gene transfer to rKGF treated animals resulted in transduction of 2% of all hepatocytes under optimal conditions.

In rodents, liver mass returns to normal size by 3 wk after injection of rKGF (25). We assayed enzyme activity after rKGF treatment to determine the relative stability of trans-

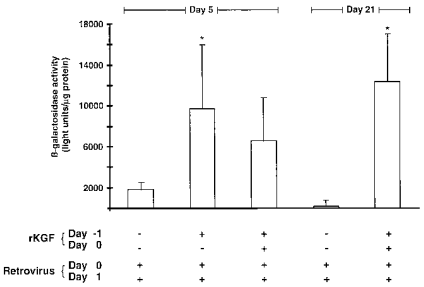

Figure 3. Expression of β-galactosidase in liver is stable after delivery of rKGF and DA-βgal. β-galactosidase activity at 5 and 21 d after the second injection of DA-βgal. Mice treated with DA-βgal alone had significantly lower levels of enzyme activity than mice given rKGF and DA-βgal at day 5 ($1 \times$ KGF, $P < 0.05$) and day 21 ($2 \times$ KGF, $P < 0.001$) post-retrovirus treatment. Samples are corrected for background levels of β-galactosidase activity (neither rKGF nor DA-βgal).

gene expression as the liver weight normalizes. There was no significant change in levels of β-galactosidase activity from 5 to 21 d for a given treatment group (Fig. 3). We also noted that although the number of apoptotic cells increased after rKGF injections, they were localized to the mid-lobular region (data not shown). Thus, hepatocellular loss after rKGF treatment should not greatly affect the number of transduced cells, since X-gal positive hepatocytes are located in the peri-portal region. Indeed, the number of X-gal positive hepatocytes in sections from mice sacrificed from 5 to 21 d post gene transfer did not differ (not shown). The slight increase in β-galactosidase activity over the course of the experiment (Fig. 3) is probably a result of accumulation of this stable enzyme.

β-galactosidase expression was also monitored 5 and 9 mo after treatment with rKGF and retrovirus. In one of the animals injected with two doses of rKGF, a partial lobectomy was performed 5 mo later and primary hepatocytes isolated and cultured. X-gal histochemistry revealed that 0.94±0.17% of hepatocytes continued to express β-galactosidase (data not shown). The same animal was killed at 9 mo and the number of β-galactosidase positive hepatocytes were 0.46±0.24%. These data suggest that DA-βgal transduced hepatocytes have the capacity to express transgenic sequences for many months. Although transgene expression persisted, no nodules or microscopic abnormalities were seen at 9 mo, supporting the proliferation studies demonstrating that rKGF results in only acute mitogenic effects.

rKGF induces proliferation in multiple tissues including lung, gastrointestinal tract, and male genitourinary tract. We tested if retroviral gene transfer was occurring in these and other tissues after rKGF injection by X-gal histochemistry and PCR amplification of retroviral sequences. PCR amplification of DNA from lung, kidney, testis, heart, intestine, and muscle were negative after 30 rounds of amplification. PCR of DNA from liver and spleen was positive (data not shown), while re-amplification of the PCR product was required for a faint signal from muscle and intestine. All tissues were grossly and microscopically negative after X-gal histochemistry except for liver and spleen, with very few scattered positive cells (1–2 cells/ full cross section) noted in spleen sections (data not shown).

Discussion

Long lasting gene transfer to hepatocytes has been reported with retroviral vectors in rabbit and canine models (7–10). Furthermore, many studies have shown that expression from retroviral vectors in hepatocytes after either ex vivo or in vivo approaches persists for many months (14, 15, 30). However, low vector titers and minimal levels of spontaneous proliferation (13) necessitated partial hepatectomy (7–10, 12, 14–16), or injection of toxins (17) or transgenes which encode molecules that result in hepatocyte destruction (18). The goal of our studies was to test if growth factors could allow for noninvasive gene transfer to hepatocytes without prior injury to liver.

Our studies exploit the observation that growth factor gene expression increases after liver injury or partial hepatectomy (21, 24, 31, 32). TGFα and acidic fibroblast growth factor function by autocrine and paracrine mechanisms while hepatocyte growth factor acts only in a paracrine manner (19, 21, 32). Whether KGF acts in a paracrine or autocrine fashion in liver, or both, is not currently known (33–35). When given to rats (25) or mice, recombinant KGF at a dose of 5 μg/g stimulates hepatocyte proliferation that declines several days after single or repeated administrations. Similar to this study, there were no significant changes in serum transaminases or bilirubin after rKGF injections in rats (25).

When given on consecutive days, rKGF resulted in a level of hepatocyte proliferation that supported efficient gene transfer. We noted ~ 80 5′-bromo-2′-deoxyuridine and 5-fluore-2′-deoxyuridine B (BrdU) positive hepatocytes/field (× 100) and 40–60 β-galactosidase positive hepatocytes/field (× 100) after DA-βgal/rKGF treatment. As noted in the gross photomicrographs in Fig. 2 *c* and *d*, we have largely targeted the hepatocytes in the periportal region, with β-galactosidase expressing hepatocytes outlining the liver acini. This population of hepatocytes has the capacity to clonally expand in the liver (30). Because expression from viral LTRs can provide for long term expression (14–16, 30) and the turnover for hepatocytes is 300–400 d, the number of transduced hepatocytes may increase over time. Alternative promoters may improve expression levels and allow for tissue specific expression of transgenes (8, 14).

Counting of transduced hepatocytes showed that 1–2% of hepatocytes were transfected with only two consecutive injections of DA-βgal. This is relevant since Wilson and colleagues show that phenotypic correction of hypercholesterolemia in the Watanabe rabbit occurs with similar levels of transduction (36). If there is a selective advantage for "corrected" hepatocytes, the overall efficiency would be significantly greater (37). We have accomplished about the same level of gene transfer as 70% hepatectomy or chemical/biological ablation of hepatocytes followed by retrovirus treatment, but in a non-invasive way. Portal vein, hepatic artery injection or asanguinous infusion with this high titer retrovirus may improve efficiency and limit distribution of virus to other tissues (15, 16, 30).

These results demonstrate the feasibility of achieving efficient and stable gene transfer by sequential intravenous delivery of rKGF and high titer retrovirus. This level of gene transfer may allow for clinical improvement in clotting factor deficiencies (10), LDL receptor deficiency (36), and lysosomal storage disorders (38) and importantly, can be accomplished without prior injury. Moreover, re-dosing with rKGF or rKGF and DA-βgal can significantly increase the percentage of transduced hepatocytes (Bosch et al., manuscript in preparation),

further increasing the utility of this approach for treatment of inherited and acquired human diseases.

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