

# Transient Depletion of Kupffer Cells Leads to Enhanced Transgene Expression in Rat Liver Following Retrograde Intrahepatic Infusion of Plasmid DNA and DNA Nanoparticles

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## Abstract

In this report, we have demonstrated that by temporarily removing Kupffer cells (KCs), the transgene expression levels mediated by retrograde intrahepatic infusion (RII) of plasmid DNA, polyethylenimine-DNA, and chitosan nanoparticles were enhanced by 1,927-, 131-, and 23,450-fold, respectively, in comparison with the respective groups without KC removal. KC removal also led to significantly prolonged transgene expression in the liver that received all three carriers. This increased transgene expression was correlated with significantly reduced serum tumor necrosis factor- $\alpha$  level as an indicator for KC activation. These results suggest that KC activation is a significant contributing factor to the lowered transgene expression by polycation-DNA nanoparticles delivered by RII. More importantly, the combination of RII and transient removal of KCs may be adopted as an effective approach to achieving high and persistent transgene expression in the liver mediated by nonviral nanoparticles.

## Introduction

FOR LIVER-TARGETED GENE DELIVERY, Kupffer cells (KCs) present a major challenge because they can mediate significant sequestration of viral or nonviral nanoparticles. More importantly, KCs as a key component of the innate immune system in the liver also contribute to nonspecific inflammation response following uptake of these nanoparticles or naked plasmid DNA. Several studies have demonstrated that systemic administration of adenoviral (Ad) vectors can activate KCs and lead to rapid clearance of Ad vectors, strong suppression of transgene expression, and liver toxicity (Lieber *et al.*, 1997; Wolff *et al.*, 1997; Alemany *et al.*, 2000; Tao *et al.*, 2001; Y. Zhang *et al.*, 2001). Recently, it has been found that nonviral vectors, such as lipoplexes (liposome/plasmid DNA complexes), can also activate KCs and induce high levels of inflammatory cytokines (Sakurai *et al.*, 2002; Zhang *et al.*, 2005).

Retrograde intrahepatic infusion (RII) has recently been developed as a more effective administration route for the liver-targeted delivery of a variety of vectors, including adenovirus, retrovirus, lipoplexes, and polyplexes, to the liver (Yang *et al.*, 1993; Sullivan *et al.*, 1997; De Godoy *et al.*, 1999; Uehara *et al.*, 1999; Otsuka *et al.*, 2000; X.H. Zhang *et al.*, 2001, 2003; Tominaga *et al.*, 2004; Chen *et al.*, 2005). Compared

with two other commonly used administration routes for liver-targeted gene delivery, *i.e.*, intravenous and intraportal infusion, RII provides a more direct access to hepatocytes in liver parenchyma through bile canaliculi (Bircher, 1999). In addition, gene vectors delivered through the bile duct will reduce the nonspecific uptake by KCs, due to the absence of KCs in the biliary system (Bircher, 1999). However, a recent study indicated that the canalicular tight junctions may become permeable to large protein molecules or even 10-nm gold nanoparticles during RII (Hu *et al.*, 2005). Gold nanoparticles infused through bile duct were found also in sinusoidal endothelial cells and KCs. This is likely dependent on the infusion parameters. Similarly, we also observed colocalization of polyethylenimine (PEI)-DNA nanoparticles with KCs after RII in our previous study (Dai *et al.*, 2006). These findings suggest that a fraction of gene delivery vectors administered through RII may cross canalicular tight junctions and the space of Disse. The entry of gene delivery vectors into the blood circulation will possibly lead to the activation of KCs.

It has been demonstrated that temporary and partial removal of KCs with clodronate liposomes can effectively enhance the level and duration of gene expression in the liver after systemic delivery of Ad viruses (Wolff *et al.*, 1997;

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Schiedner *et al.*, 2003; Van Til *et al.*, 2005). In an effort to understand the role of KCs in nanoparticle-mediated gene delivery through RII, we compared the effect of transient and partial depletion of KCs on the transfection efficiencies of naked DNA, chitosan-DNA nanoparticles, and PEI-DNA nanoparticles when delivered through RII. We also investigated the effect of this treatment on the duration of transgene expression and the serum tumor necrosis factor (TNF)- $\alpha$  level as an indicator of KC activation.

## Materials and Methods

### Preparation of PEI-DNA and chitosan-DNA nanoparticles

Polyethylenimine [branched PEI, average molecular weight (MW) of 25 kDa] was purchased from Sigma-Aldrich (St. Louis, MO). PEI was purified by dialysis against water (dialysis tubing with MWCO 3500; Pierce, Rockford, IL) for 3 days and then lyophilized. Chitosan C390 (MW 390 kDa, deacetylation degree 83.5%) was kindly provided by Vanson Halosource (Redmond, WA). Plasmid pVR1255 is a 6.4-kb pcDNA encoding luciferase driven by human cytomegalovirus promoter (a gift from Dr. Carl J. Wheeler, Vicral Inc., San Diego, CA). The plasmid was amplified in *Escherichia coli* DH5 $\alpha$  and purified by a Qiagen Giga plasmid purification kit (endotoxin free; Qiagen, Hilden, Germany). To prepare chitosan-DNA nanoparticles, a chitosan solution (0.02% in 5 mM sodium acetate buffer, pH 5.5) and a DNA solution (100  $\mu$ g/ml in 5 mM sodium acetate solution) at an N/P ratio of 3 (N/P ratio is defined as the molar ratio of chitosan nitrogen to DNA phosphate) were preheated to 50–55°C separately. An equal volume of both solutions was quickly mixed while vortexing. PEI-DNA nanoparticles were prepared by mixing PEI solution (133  $\mu$ g/ml in 5% glucose) with an equal volume of DNA solution (100  $\mu$ g/ml in 5% glucose) at an N/P ratio of 10. Both chitosan-DNA and PEI-DNA nanoparticles were incubated at room temperature for 30 min before characterization and transfection. The particle size and zeta potential of chitosan-DNA and PEI-DNA nanoparticles were measured on a Zetasizer 3000 (Malvern Instruments, Malvern, Worcestershire, UK).

### Transient depletion of KCs and RII of PEI-DNA, chitosan-DNA nanoparticles, and naked DNA

Clodronate liposomes were prepared as described (Van Rooijen and Sanders, 1994) and injected intravenously to rats at a dose of 2  $\mu$ l/g of body weight at 48 hr prior to RII of nanoparticles and naked DNA. Wistar rats (male, 200–250 g) were randomly assigned to groups of 15–17 rats. Animals were laparotomized under general anesthesia, and the liver was isolated from the surrounding tissue. A 33G needle was inserted into the common bile duct, and a tie was used to secure the needle. Nanoparticles and naked DNA were administered at the dose equivalent to 200  $\mu$ g of plasmid (0.8 mg/kg of body weight) in 4 ml of medium into the common bile duct over 20 min (0.2 ml/min) using a syringe pump. A tie was then placed around the bile duct between the liver and the point of infusion to prevent back flow, and the needle was withdrawn. After 30 min, all ties were removed. The needle hole in the bile duct might require stitches with 10-O nylon suture (Ethicon, Somerville, NJ) to

prevent bile leakage, whenever necessary. Rats were kept on normal diet. On days 3, 5, and 7, rats from each group were sacrificed. Major organs (liver, heart, lung, spleen, and kidney) were harvested and stored at  $-80^{\circ}\text{C}$  for analysis. Each liver was divided into four sections composed of median, left, right, and caudate lobes. Two milliliters of lysis buffer (0.1% Triton X-100, 2 mM EDTA, and 0.1 M Tris-HCl, pH 7.8) per gram of tissue was used for each sample, and the tissue was homogenized and subjected to two freeze-thaw cycles. The homogenates were centrifuged at 14,000 rpm for 10 min. Luciferase activity in the homogenate was measured for 10 sec on a luminometer and converted to the mass of luciferase expressed per gram of tissue using a standard curve generated in parallel on the same luminometer. Student's *t* test was used to compare the difference between selected groups. Differences were considered statistically significant when  $p < 0.05$ .

## Results

Before RII, we characterized the size of PEI-DNA nanoparticles and chitosan-DNA nanoparticles by dynamic light scattering. In the presence of 5% (w/v) glucose solution, PEI-DNA nanoparticles and chitosan-DNA nanoparticles showed an average particle size of  $90.6 \pm 2.0$  nm and  $243.1 \pm 2.9$  nm (Fig. 1), respectively. After incubation of these nanoparticles with isotonic (0.15 M) NaCl solution for 30 min, the particle size of PEI-DNA nanoparticles increased drastically to  $277 \pm 6.4$  nm. On the other hand, chitosan-DNA nanoparticles appeared more stable in NaCl solution: the average particle size increased only by about 27 nm after 30 min of incubation.

Three days after RII, chitosan-DNA nanoparticles mediated a luciferase expression of 33  $\mu$ g/g of tissue in the liver of the rats that received no pretreatment. PEI-DNA nanoparticles were less efficient than chitosan-DNA nanoparticles in the liver, and the luciferase expression was about 17-fold lower than that by chitosan-DNA nanoparticles on day 3. It is surprising that naked DNA given by RII also gave a transient luciferase expression (0.62  $\mu$ g/g of tissue) in the liver. In stark contrast, after clodronate liposome treatment prior to bile duct infusion, luciferase expression in the liver on day 3

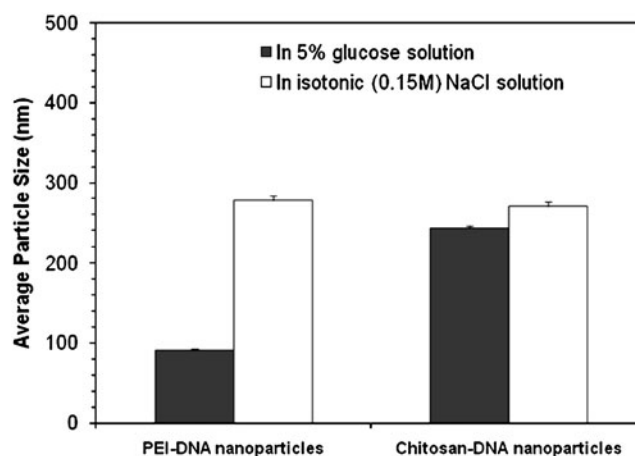
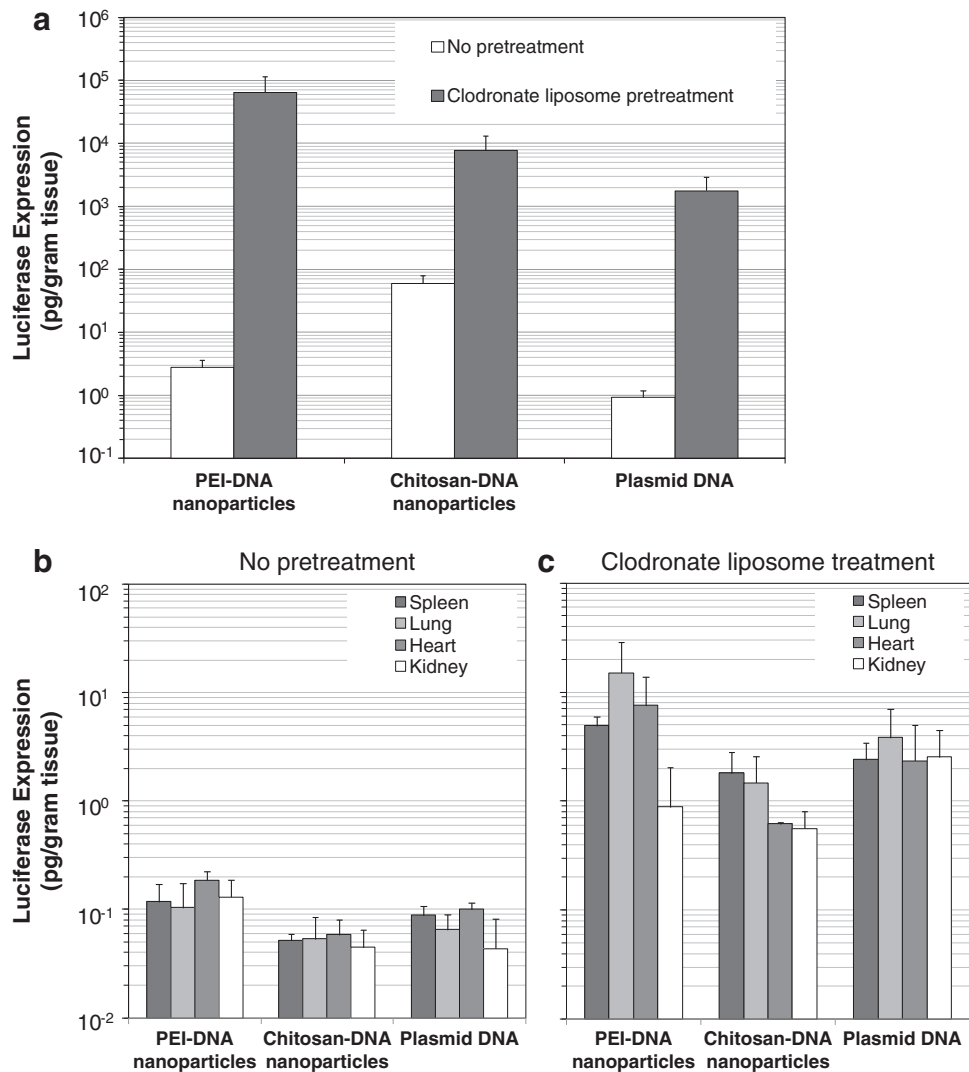


FIG. 1. Particle size of PEI-DNA nanoparticles (N/P = 10) and chitosan-DNA nanoparticles (N/P = 3) in 5% glucose solution and in isotonic (0.15 M) NaCl solution.



**FIG. 2.** Effect of KC depletion on transfection efficiency of different carriers in the liver (a) and in other organs without clodronate liposome treatment (b) and with clodronate liposome treatment following RII (c). Luciferase expression was analyzed on day 3. Each bar represents the mean ± SD (*n* = 3).

mediated by both nanoparticles and naked DNA was significantly increased. As shown in Fig. 2a, the biggest increase (23,477-fold) in transgene expression was observed for PEI-DNA nanoparticles. For chitosan-DNA nanoparticles and naked DNA, the transfection efficiency in the liver was also enhanced by 131- and 1,927-fold, respectively. This significant enhancement in transfection efficiency after partial KC depletion indicated that KCs may be involved in the uptake of polymer-DNA nanoparticles or naked DNA and play an important role in regulating transgene expression mediated by these nanoparticles. It is interesting that, for all three groups, transgene expression in other organs, particularly in the lung and heart, also increased by 20- to 125-fold (Fig. 2c) as a result of KC depletion, even though luciferase activity detected in the liver still accounted for more than 95% of the total expression. These results support the hypothesis that nanoparticles can cross canalicular tight junctions and sinusoidal fenestrae and enter the blood circulation under this infusion condition. Therefore, without KC depletion, uptake of nanoparticles and naked DNA by KCs and KC activation may occur, leading to the reduced transgene expression.

To validate the hypothesis that entry of nanoparticles or plasmid DNA into blood circulation can lead to the activa-

tion of KCs, we analyzed the serum TNF- $\alpha$  levels of both KC-depleted rats and normal rats on day 3 after RII of nanoparticles or naked DNA, because the activation of KCs by lipoplexes is usually associated with the production of a high level of TNF- $\alpha$ , one of the major proinflammatory cytokines (Sakurai *et al.*, 2002; Kako *et al.*, 2008). As shown in Fig. 3, both nanoparticles and naked DNA infused through the bile duct induced a high level of TNF- $\alpha$  from 2.4 to 5.6 ng/ml, as compared with a background level of <10 pg/ml. This result indicated that polymer-DNA nanoparticles and naked DNA administered through the bile duct did induce a strong innate immune response. Consistent with this finding, after depletion of KCs, serum TNF- $\alpha$  levels dropped 20-, 21-, and 13-fold in the rats infused with chitosan-DNA nanoparticles, PEI-DNA nanoparticles, and naked DNA, respectively. The significant reduction in the levels of TNF- $\alpha$  after the depletion of KCs suggested that innate immune response after bile duct infusion is very likely to be a result of KC activation by polymer-DNA nanoparticles or naked DNA.

In addition to the effect of boosting transfection efficiency, the depletion of KCs also was found to help prolong transgene expression in the liver. Without pretreatment, PEI-DNA nanoparticles and chitosan-DNA nanoparticles mediated

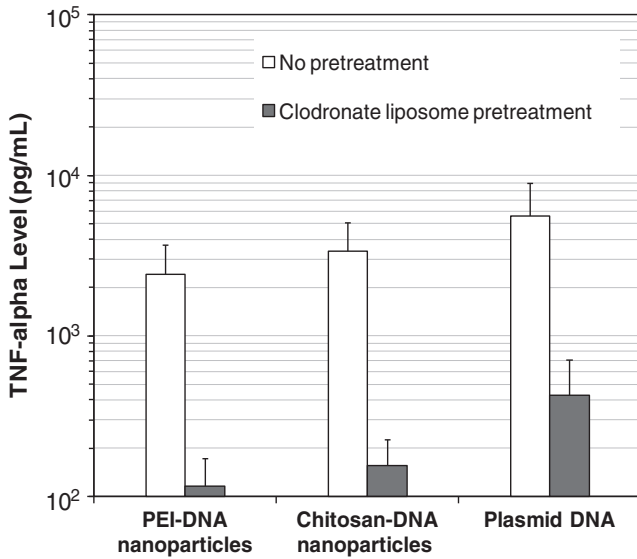


FIG. 3. Serum level of TNF- $\alpha$  after RII of PEI-DNA nanoparticles, chitosan-DNA nanoparticles, and naked DNA with or without clodronate liposome pretreatment on day 3 ( $n = 3$ ).

similar levels of transgene expression in the liver (6 and 11 pg/g of tissue, respectively) on day 7. No detectable transgene expression was observed in the liver transfected with plasmid DNA. However, after treatment with clodronate liposomes, significant enhancement of transgene expression was observed in the liver for all three groups (Fig. 4). The biggest boost (428-fold) in transgene expression on day 7 was found in the rats transfected with plasmid DNA. The transgene expression levels in the rats transfected with chitosan-DNA nanoparticles and PEI-DNA nanoparticles were also increased by 6- and 38-fold. On day 14, in

untreated rats, PEI-DNA nanoparticles and chitosan-DNA nanoparticles still mediated detectable transgene expression in the liver, but plasmid DNA did not lead to any detectable gene expression. It is interesting that, although the transfection efficiency of chitosan-DNA nanoparticles dropped another 18-fold from day 7 to day 14, the transfection efficiency of PEI-DNA nanoparticles remained almost unchanged. Similar to the results on day 7, treatment with clodronate liposomes led to 10- and 27-fold enhanced transgene expression in the liver transfected with chitosan-DNA nanoparticles and plasmid DNA. However, the transfection efficiency of PEI-DNA nanoparticles was not improved by the treatment on day 14.

## Discussion

In this study, we administered PEI-DNA nanoparticles, chitosan-DNA nanoparticles, and plasmid DNA through RII, which has recently been shown to be an effective administration route for liver-targeted gene delivery in several animal models, including rats (X.H. Zhang *et al.*, 2001; Dai *et al.*, 2006), pigs (Yang *et al.*, 1993; Otsuka *et al.*, 2000), and primates (Sullivan *et al.*, 1997), using viral vectors (Peeters *et al.*, 1996; De Godoy *et al.*, 1999) and liposomes (Otsuka *et al.*, 2000). The exposure of hepatocytes via RII occurs through bile canaliculi, which measure 0.5–1.25  $\mu\text{m}$  (Bircher, 1999). In addition, the broad distribution of the biliary system provides great access to nearly all hepatocytes in liver parenchyma through bile canaliculi. This method also delivers nanoparticles or DNA preferentially to hepatocytes first, as opposed to KCs lining the luminal surface of the sinusoidal capillaries, when nanoparticles are delivered by intravascular infusion. It is highly likely, though, as our data suggested that, under moderate pressure and infusion volume, there is a pressure-dependent physical opening of canalicular tight junctions, which may lead to the transport of gene carriers into the space of Disse and into the bloodstream through

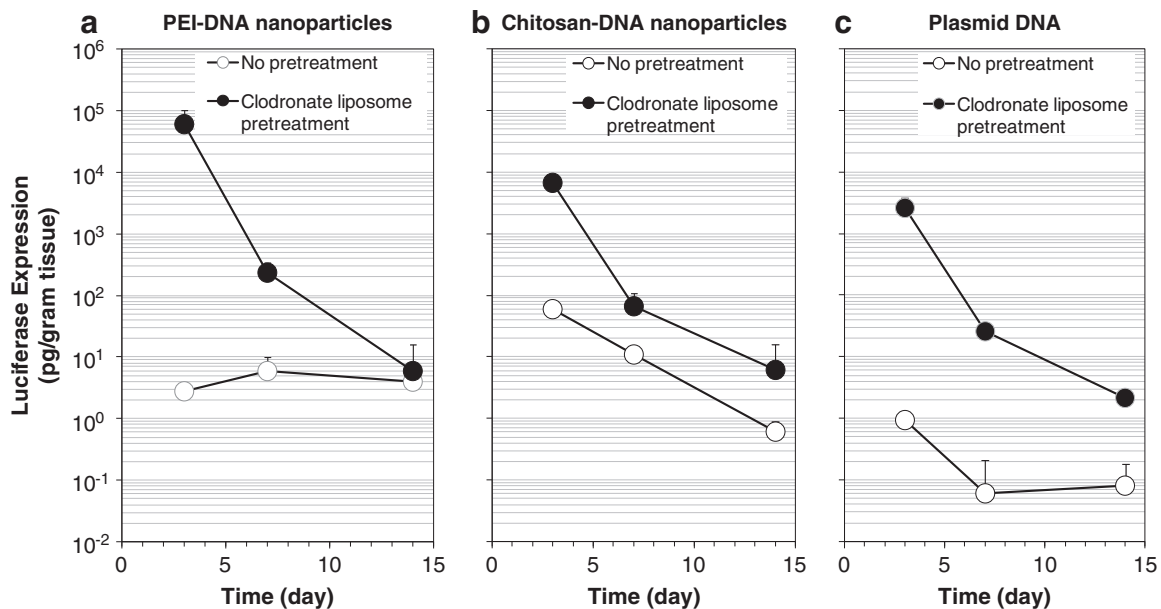


FIG. 4. Transgene expression mediated by PEI-DNA nanoparticles (a), chitosan-DNA nanoparticles (b), and plasmid DNA (c) in the liver with or without clodronate liposome pretreatment. Each data point represents the mean  $\pm$  SD ( $n = 3$ ).

sinusoidal fenestrae. A recent study has shown that large proteins and gold nanoparticles can be transported through canalicular tight junctions during RII infusion (Hu *et al.*, 2005). More importantly, colocalization of gold nanoparticles with KCs was observed. In our previous study, we have also demonstrated that some PEI-DNA nanoparticles, chitosan-DNA nanoparticles, and plasmid DNA were taken up by KCs after RII (Dai *et al.*, 2006).

On the other hand, several studies have revealed that lipoplexes (Dow *et al.*, 1999; Yew *et al.*, 1999; Kako *et al.*, 2008) encapsulating bacterially derived plasmid DNA can induce strong activation of macrophages and subsequent production of high levels of proinflammatory cytokines after intravenous administration. The activation of macrophages is mediated by the strong immunostimulatory effect of unmethylated CpG sequences present at high frequency in bacterially derived plasmid DNA (Krieg, 1999, 2000). To reduce the inflammation response induced by plasmid DNA, depletion of macrophages before systematic administration has been proven to be an effective approach to enhance gene transfection efficiency of lipoplexes (Sakurai *et al.*, 2002). Using the same strategy, we removed KCs with clodronate liposomes by tail-vein injection before RII infusion of nanoparticles or naked DNA to reduce the activation of KCs. The clodronate liposome was chosen for two reasons. First, it effectively removes more than 90% of KCs temporarily for about 6 days, and repopulation of KCs is completed within 2 weeks (Van Rooijen and Sanders, 1994). Second, it preferentially depletes KCs and removes other types of macrophages to a much lesser degree (Van Rooijen and Sanders, 1994; Van Rooijen and Van Kesteren-Hendrikx, 2003). As we expected, depletion of KCs before RII not only significantly enhanced the transfection efficiency in all major organs, but also greatly inhibited the production of TNF- $\alpha$ . These results confirmed that RII can lead to opening of the canalicular tight junction and sinusoidal leakage of the nanoparticles, and this leakage can further result in the uptake of the nanoparticles or DNA and the activation of KCs.

It is also noteworthy that the level of enhancement in transfection efficiency in the liver as a result of KC removal was carrier-dependent (Supplementary Fig. 1, available online at [www.liebertonline.com/hum](http://www.liebertonline.com/hum)). The biggest boost was found for PEI-DNA nanoparticles. In our previous study, we observed a significantly higher level of colocalization of PEI-DNA nanoparticles with KCs compared with chitosan-DNA nanoparticles and plasmid DNA (Dai *et al.*, 2006). It is also interesting to note that there was a substantially higher number of KCs observed in the rat liver that received PEI-DNA nanoparticles (Dai *et al.*, 2006). Those findings indicated that PEI-DNA nanoparticles may induce a higher level of KC activation, and thus lead to stronger inhibition of transfection efficiency in the liver, compared with the other two groups. In addition, we found that, unlike that in untreated rats, the transfection efficiency of these carriers after KC removal correlated well with that in primary hepatocyte culture (Dai *et al.*, 2006).

The method for transient depletion of KCs is an attractive approach for potential clinical application. Clodronate has been used in Europe and Canada for treating osteoporosis to increase bone mass and reduce fractures. Given by intravenous injection, the effect of clodronate liposome on KC removal can be highly specific (Van Rooijen and Van Kesteren-Hendrikx, 2002). In addition, RII can be achieved in

the clinical setting by endoscopic retrograde cholangiopancreatography. Given the drastic improvement of transgene expression by combining RII and transient depletion of KCs, this method may be adopted as a highly effective approach for liver-targeted gene delivery.

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### Author Disclosure Statement

No competing financial interests exist.

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