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Sustained Transgene Expression in Rat Kidney with Naked Plasmid DNA and PCR-Amplified DNA Fragments

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Recently, we developed a kidney-targeted gene transfer technique, in which naked DNA was injected into the renal vein while the renal vein and artery were clamped. Kidney-targeted DNA transfer with only the renal vein clamped is an important modification that may permit less invasive catheter-based gene transfer in future clinical applications. The preparation of PCR-amplified DNA fragments is less time-consuming than that of naked plasmid DNA. We examined rat erythropoietin (Epo) plasmid, pCAGGS-Epo, or PCR-amplified DNA fragment, fCAGGS-Epo, transfer into the rat kidney with only the renal vein clamped. The Epo level peaked at week 3 and then was sustained for 24 weeks, which resulted in significant erythropoiesis. This modified technique, allowing long-term expression of both PCR-amplified DNA fragments and naked plasmid DNA, could potentially be used for catheter-based gene transfer in humans, and could help determine the physiological functions of putative genes.

Key words: CAG promoter, catheter-based gene transfer, hydrodynamics-based transfection, interstitial fibroblast, kidney, naked plasmid DNA, PCR-amplified DNA fragment, renal vein injection.

Abbreviations: CAG promoter, cytomegalovirus immediate-early enhancer/chicken β-actin hybrid promoter; Epo, erythropoietin; PTC, peritubular capillaries.

Kidney-targeted gene transfer is potentially very important for broadening our understanding of renal disease processes and for revolutionizing the treatment of renal diseases. Recently, we demonstrated that retrograde injection into the renal vein with clamping of the renal vein and artery is useful for transferring naked DNA into renal interstitial fibroblasts near the peritubular capillaries (PTC), which comprise a network of interstitial vessels that connects veins at every cortical level and plays a major role in maintaining renal function and hemodynamics (*1*).

We considered how to improve our technique, *i.e.* to make it simpler, and less invasive, and to minimize tissue damage. We wondered if the profile of vascular pressure along the intra-renal vasculature and the sharp vascular resistance gradient between the efferent arterioles and the PTC (*2*) would substitute well for the blockade caused by clamping of the renal artery. We tested this idea by examining whether or not our technique could efficiently transfer a gene of interest without a clamp on the renal artery.

To make the technique simpler, we exploited the ease of preparing PCR-amplified DNA fragments. Hofman *et al.* (*3*) recently developed an efficient method of *in vivo* gene transfer involving PCR-amplified DNA fragments in mice via rapid tail vein injection of a large volume of a DNA solution, the "hydrodynamics-based procedure." However, in their study, the reporter gene expression was transient, lasting only 1 week. Chen *et al.* (*4*) demonstrated stable, long-term transgene expression *in vivo* after transfecting a linear plasmid DNA digested with restriction enzymes into mouse liver. The preparation of linear plasmid DNA requires several more procedures than naked plasmid DNA preparation. Therefore, we next examined whether the retrograde injection of PCR-amplified DNA fragments into the renal vein could lead to long-term gene expression in normal rats.

MATERIALS AND METHODS

Plasmid DNA—We constructed plasmid pCAGGS-Epo (*5*) by inserting the rat Epo cDNA into the *Xho*I site of the pCAGGS expression vector, which has the CAG (cytomegalovirus immediate-early enhancer/chicken β-actin hybrid) promoter (*6*), and grew the plasmid in *Escherichia coli* DH5α cells (Toyobo, Osaka, Japan). The plasmid was isolated using a Qiagen EndoFree plasmid Giga kit (Qiagen, Hilden, Germany) as described (*5*). The quantity and quality of plasmids were determined by measuring the optical density at 260 and 280 nm. Immediately before injection, the DNA was diluted in Ringer's solu-

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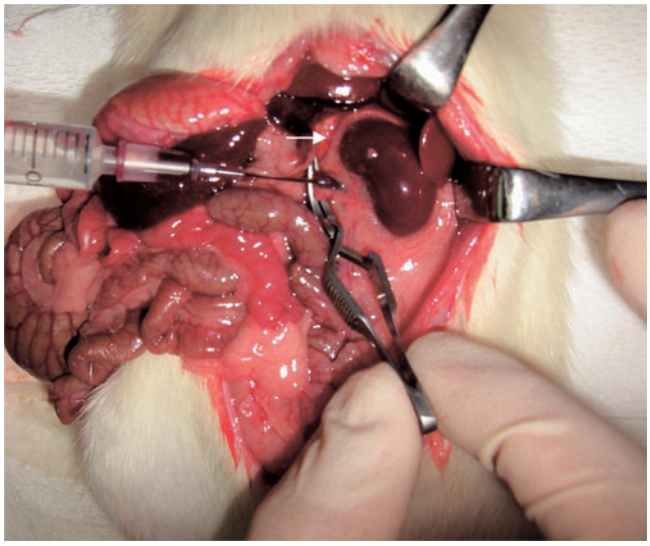

Fig. 1. **Rat kidney after clamping of the left renal vein with angled-type Diethrich bulldog clamps (Muromachi Kikai) without occlusion of the adrenal vein (arrow).** Retrograde renal vein injection was performed through a 24-gauge SURFLO® I.V. catheter (Terumo) connected to a 2.5-ml capacity syringe.

tion (Ohtsuka, Tokushima, Japan). We used the empty pCAGGS plasmid as a control.

PCR-Amplified Linear DNA Fragments—We designed a primer set to amplify a 2,994-bp region of plasmid pCAGGS-Epo, which contains the CAG promoter, an intron, the rat Epo cDNA, and a rabbit β-globin polyadenylation signal (*7*), as follows: CAGGS-Epo Sense-1, 5′-GTC GAC ATT GAT TAT TGA CTA GTT ATT AAT AGT AAT CAA T-3′ (G, nucleotide 1 of pCAGGS-Epo and of pCAGGS); CAGGS-Epo Antisense-2994, 5′-AAG CTT GGG CTG CAG GTC GAG GGA TCT TCA TAA GAG AAG A-3′ (A, nucleotide 2994 of pCAGGS-Epo, nucleotide 2280 of pCAGGS). We performed PCR using a PCRx enhancer solution (Gibco BRL, Rockville, MD). To amplify the targeted region of pCAGGS-Epo by PCR, we used 25 ng of plasmid template linearized by *Sca*I digestion, 50 pmol of each primer, 10 μl of dNTPs (2.5 mM each), 3.75 units of TaKaRa LA *Taq* DNA polymerase (Takara, Shiga, Japan), and 5 μl of 10× PCRx enhancer solution. The PCR protocol consisted of 1 cycle of 1 min at 94°C, then 30 cycles of 20 s at 98°C, 30 s at 60°C, and 3 min at 72°C, and finally extension for 10 min at 72°C. The resulting PCR-amplified DNA fragment (fCAGGS-Epo) was purified using a QIAquick PCR purification kit (Qiagen). A 2,280-bp PCR-amplified DNA fragment of pCAGGS (fCAGGS; GC content 60.2%), which contained the entire fCAGGS-Epo region except for the rat Epo cDNA, was similarly created and purified using these protocols.

Rats—Eight-week-old male Wistar rats were purchased from Charles-River Japan Inc. (Tokyo, Japan), and were used for gene transfer.

The DNA Injection Techniques—We diluted the DNA in Ringer's solution. We anesthetized the rats with diethyl ether, performed an incision in the median section of the abdomen, and then immediately before injecting the DNA solution, clamped the left renal vein with angled-type Diethrich bulldog clamps (Muromachi Kikai, Tokyo, Japan) without occlusion of the adrenal vein. Using a 24-gauge SURFLO® I.V. catheter (Terumo, Tokyo, Japan), we injected the solution containing naked plasmid DNA or the PCR-amplified DNA fragment solution into the vein and re-established the blood flow immediately after the injection (Fig. 1). No incubation was performed in this procedure. Hemostasis was seen at the injected site after applying pressure for 10 s.

Total Tissue RNA Extraction and RT-PCR—On day 1 and at 24 weeks after the injection, we sacrificed rats that had received 100 μg of pCAGGS-Epo, 10 μg of fCAGGS-Epo or 7.6 μg of fCAGGS under general anesthesia, and then harvested both kidneys, the brain, heart, lungs, liver, spleen, muscle, skin, and testes. We isolated total RNA from the tissue samples using Isogen (Nippon Gene, Tokyo, Japan). We detected Epo mRNA or glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA by RT-PCR as described previously (*1*), using the following specific primers: Epo backward primer, 5′-GCCCAGAGGAATCAGTAGCA-3′; Epo forward primer, 5′-TCTGACTGACCGCGTTACTC-3′; G3PDH backward primer, 5′-TCCACCACCCTGTTGCTGTA-3′; G3PDH forward primer, 5′-ACCACAGTCCATGCCATCAC-3′. We designed the Epo forward primer to hybridize with the sequence immediately downstream of the transcriptional start site of the CAG promoter. There is an intron between the CAG promoter and the Epo cDNA in pCAGGS-Epo or fCAGGS-Epo. The primer set for detection of the Epo mRNA was designed to encompass intronic sequences, allowing us to distinguish any possible PCR products from contaminating transgene or genomic DNA. The primer set for detecting G3PDH mRNA was also designed to span introns. We analyzed the RT-PCR products by electrophoresis on a 4% agarose gel. The lengths of the expected products were 170 bp for Epo mRNA and 452 bp for G3PDH mRNA.

Quantitative RT-PCR Analysis—At one day and 24 weeks after the injection, we sacrificed rats that had received 10 μg of fCAGGS-Epo or 18.4 μg of pCAGGS-Epo, and then harvested the injected kidneys. The total RNA of the kidney samples was isolated using Isogen (Nippon Gene), and used for the synthesis of first-strand cDNA with Moloney Murine Leukemia Virus reverse transcriptase (RT; Gibco BRL, Rockville, MD) and random hexamers (Promega, Madison, WI). The RT product was amplified by PCR with *Taq* DNA polymerase (Promega), and Epo backward and forward primers. The PCR product was directly inserted into the pGEM-T Easy Vector (Promega), to create pGEM-T-Epo. This plasmid was grown in *E. coli* DH5α cells and prepared using a QIAprep Spin Miniprep kit (Qiagen) for use as the external standard. To determine the levels of transgene-derived Epo mRNA in major organs, we performed quantitative real-time PCR analysis using a LightCycler Quick system 330 (Roche Diagnostics, Mannheim, Germany). The PCR was performed with LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the manufacturer's protocol and using the primer pairs described above. The PCR reaction consisted of 95°C for 10 min, and then 95°C briefly, 62°C for 10 s, and 72°C for 13 s with a transition rate of 20°C/s

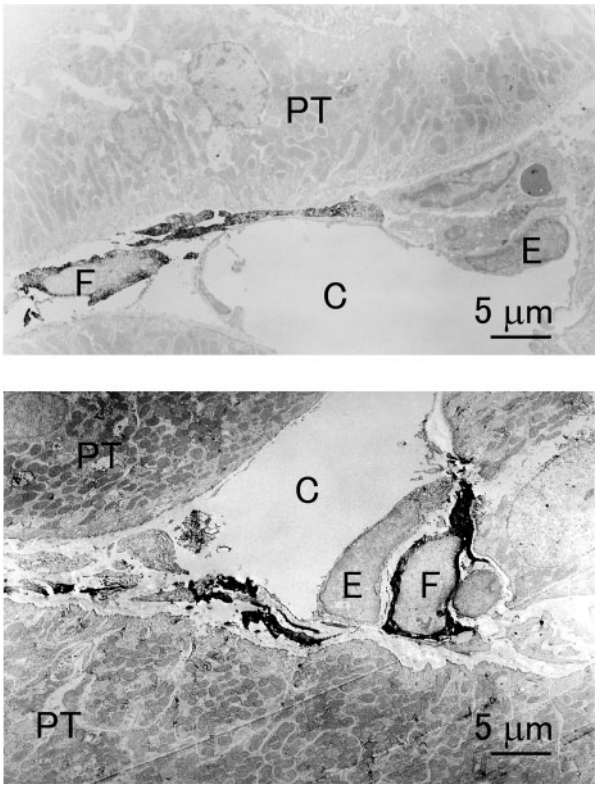

Fig. 2. **Immunoelectron microscopic analysis of ultrathin sections of rat kidneys at 1 day after injection of pCAGGS-lacZ (original magnification, ×4,000) (a) and fCAGGS-lacZ (original magnification, ×5,000) (b).** DAB products are distributed throughout the cytoplasm of the fibroblasts. Fibroblasts have long cytoplasmic processes and make close contact with the cytoplasm of endothelial cells. C, capillary lumen; E, endothelial cell; F, fibroblast; PT, proximal tubule.

between temperature plateaus, for a total of 40 cycles. The data were quantified using the LightCycler analysis software (version 3, Roche Diagnostic). A standard curve for the Epo plasmid was obtained, the standard error being <0.001. The results were expressed initially as the number of target molecules/2 μl cDNA. We confirmed the RT-PCR products by 4% agarose gel electrophoresis.

Blood Analyses—Under general anesthesia, blood samples (2.0 ml) were obtained from the heart according to the method of Ohwada (*8*). Serum rat Epo levels were determined using a Recombigen EPO kit (Iatron, Chiba, Japan), which involves a radioimmunoassay with a rabbit polyclonal antibody against Epoetin α. This kit exhibits a linear range of measurement between 3.0 and 200 mU/ml of human Epo with a detection threshold of 3.0 mU/ml. Hematocrit, reticulocytes, and serum creatinine were measured as described (*5*, *9*).

Immunoelectron Microscopy—To determine the transgene expression site at the ultrastructural level, we examined the kidneys injected with 100 μg of pCAGGS-lacZ or 70 μg of fCAGGS-lacZ. One day after the injection, rats were placed under general anesthesia with diethyl ether, and then perfused through the aorta with 50 ml PBS followed by 50 ml periodate-lysine-paraformaldehyde using a gravity-feed system. The kidneys were further fixed with periodate-lysine-paraformaldehyde for 4 h at 4°C, treated with 10% sucrose in PBS for 1 h, embedded in Tissue-Tek O.C.T. compound (Sakura Finetechnical), and then frozen in hexane at –80°C. Sections (4-μm thick) were cut with a cryostat and incubated with rabbit polyclonal anti–*E. coli* β-galactosidase antibodies (1:800 dilution, Biogenesis) for 30 min at room temperature. After being washed in PBS, the sections were incubated with goat anti–rabbit Ig EnVision+ Peroxidase Rabbit (1: 10 dilution. DAKO, Carpinteria, CA) for 1 h. The sections were then washed in PBS and incubated with 0.02% diaminobenzidine (DAB) in 0.05 M Tris buffer (pH 7.6) containing 0.01% H_2O_2 for 3 min. After being washed in PBS, the sections were fixed with 2.5% glutaraldehyde for 3 min at room temperature. The sections were then washed in PBS, post-fixed with 1% OsO_4 in 0.1 M phosphate buffer (pH 7.2) for 3 min, washed in distilled water, dehydrated in a graded ethanol series, and flat-embedded in Epok 812 (Oken, Tokyo, Japan), which is equivalent to Epon. After the Epok 812 had polymerized, we examined the sections by light microscopy, and clipped out selected fields and then cut them with an ultramicrotome into ultrathin (100 nm) sections, which were then examined for DAB-stained sites under an H-600A electron microscope (Hitachi, Ibaragi, Japan).

Kidney Histology—We harvested kidneys at 5 h, on days 1 and 7, and at 24 weeks after the naked plasmid DNA injection, fixed them in 10% buffered formaldehyde, embedded them in paraffin, and then processed them for routine light microscopy. We then stained 5-μm sections with periodic acid-Schiff (PAS) for detection of possible tissue injury due to the gene transfer procedure.

Kidney Function—We assessed whether the kidneys could function normally immediately after the renal vein injection of 0.5 ml of 100 μg of pCAGGS-Epo or pCAGGS. To examine the function of an injected kidney, we needed to remove the uninjected one, because the uninjected kidney might have been able to compensate for functional problems and thus have skewed our data. Therefore, we injected the left kidney and nephrectomized the right one. Normal, unoperated rats and uninephrectomized ones without DNA injection served as controls. We measured the serum creatinine level, which is an endogenous marker for kidney function.

Statistical Analysis—The data are presented as mean values ± standard deviation of the mean. We analyzed all data using the StatView statistical program for Macintosh (SAS, Cary, NC), and evaluated the statistical significance with the unpaired t test. P values of <0.05 were considered statistically significant.

RESULTS

Localization of pCAGGS-lacZ or fCAGGS-lacZ Gene Expression—The transgene-expressing cells were examined by immunoelectron microscopy (Fig. 2a), and identified based on the morphology of renal interstitial cells (*10*) as follows: fibroblasts extend long cytoplasmic processes and adhere via pedicle-like attachments to the basement membranes of the tubules and PTC, macrophages are round in shape and have a prominent lysosomal apparatus, dendritic cells lack these attachments to the basement membranes, and pericytes are found in intimate contact with the PTC and are clearly distinguished from

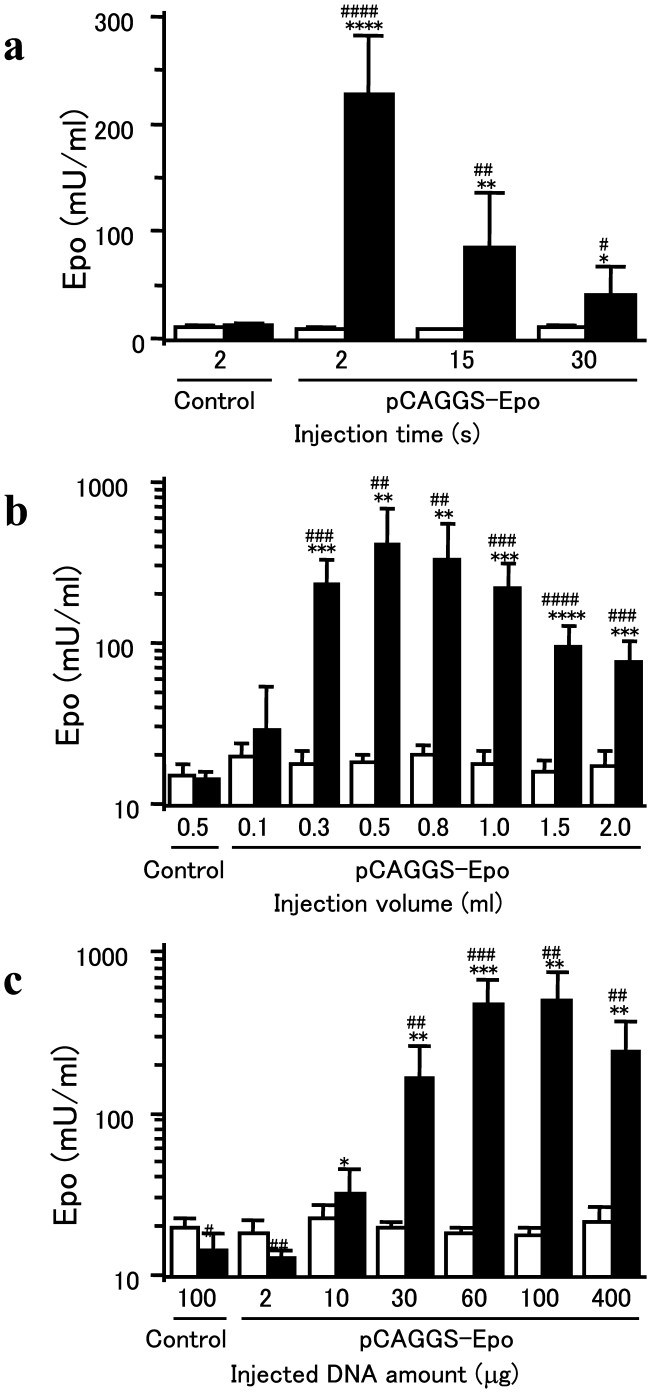

Fig. 3. **The effect of injection characteristics on the serum Epo level.** Serum Epo was measured 1 week before (open squares) and 3 weeks after (solid squares) naked plasmid DNA injection. The effect of the injection time (a) or volume (b) on the serum Epo level was examined using 100 μg of pCAGGS-Epo or pCAGGS. $n = 4$ in each group. (c) The effect on the serum Epo level of varying amounts of injected DNA. $n = 5$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ for comparisons with control rats (comparisons were made at each time point), $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$ for differences between pre- and postinjection Epo levels within each group.

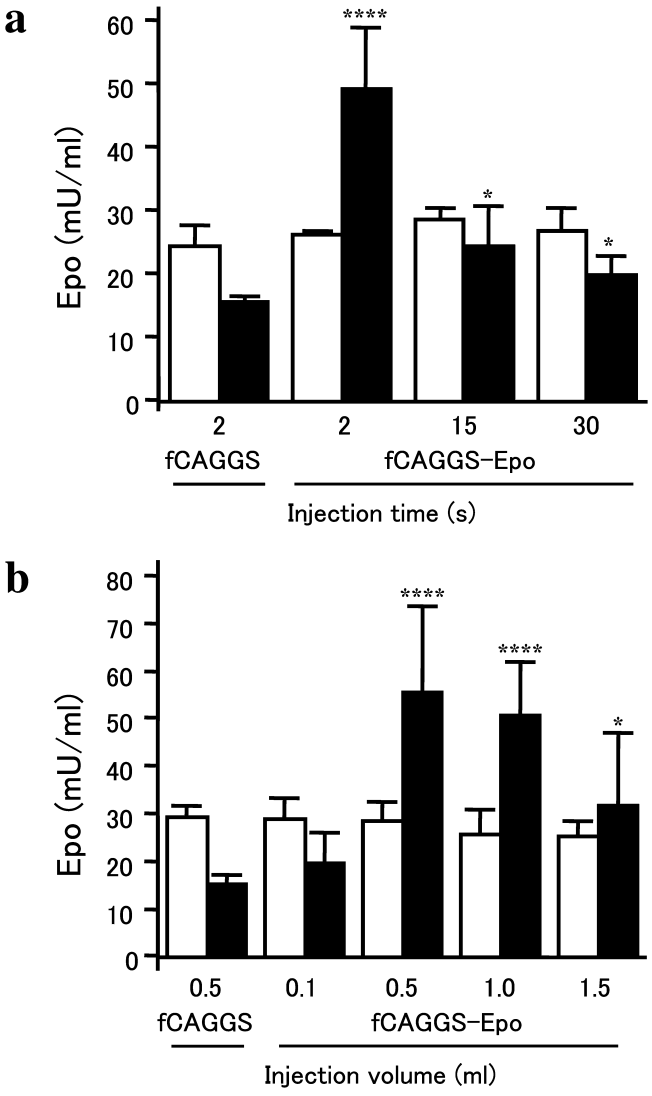

Fig. 4. **The effect of injection characteristics on the serum Epo level.** Serum Epo was measured 1 week before (open squares) and 3 weeks after (solid squares) PCR-amplified DNA injection. The effect of the injection time (a) or volume (b) on the serum Epo level was examined using 5 pmol of either fCAGGS-Epo (10 μg) or fCAGGS (7.6 μg); $n = 4$ in each group (a), $n = 6$–8 in each group (b). $^{*}P < 0.05$, and $^{****}P < 0.0001$ for comparisons with control rats (comparisons were made at each time point).

the fibroblasts by the basal lamina continuously surrounding the pericytes. We detected the DAB product exclusively throughout the cytoplasm of fibroblasts near the PTC. The PCR-amplified DNA fragment, 70 μg of fCAGGS-lacZ (19 pmol), was also expressed in the interstitial fibroblasts near the PTC, like naked plasmid DNA (Fig. 2b). The fibroblasts extended long cytoplasmic processes, and made close contact with the cytoplasm of the PTC endothelial cells (Fig. 2, a and b) and the proximal tubules (Fig. 2, a and b).

Effects of Injection Characteristics on Gene Transfer Efficiency—In the subsequent experiments, we used a rat Epo expression plasmid vector, pCAGGS-Epo. We evaluated the effects of varying the injection time (2–30 s) and volume (0.1–2.0 ml) on the efficiency of gene transfer,

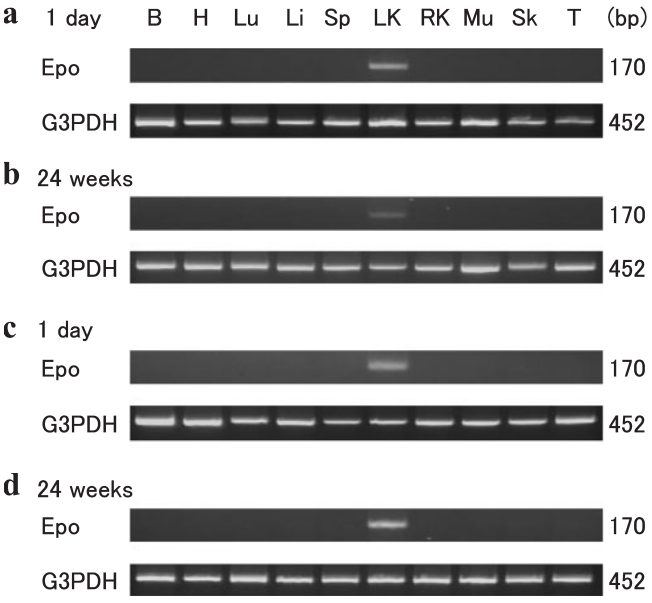

Fig. 5. **RT-PCR analysis of Epo mRNA and control G3PDH mRNA in major organs.** One day (a) or 24 weeks (b) after pCAGGS-Epo (100 μg) injection. One day (c) or 24 weeks (d) after 5 pmol fCAGGS-Epo (10 μg) injection. B, brain; H, heart; Lu, lung; Li, liver; Sp, spleen; LK, left kidney; RK, right kidney; Mu, muscle; Sk, skin; T, testis.

measuring the serum Epo levels 1 week before and 3 weeks after injecting 100 μg of naked DNA. We obtained maximal Epo expression when the DNA solution was injected within 2 s (Fig. 3a). Good Epo expression was seen with a wide range of volumes (0.3–1.0 ml), and was highest with a volume of 0.5 ml (Fig. 3b). During the injection, the kidneys showed slight swelling.

We next evaluated the effect of varying the amount of DNA injected using a 2-s injection time and a volume of 0.5 ml. We observed a dose-response relationship between the serum Epo level and the amount of injected DNA up to 100 μg, and saw substantial levels of Epo gene expression with only 10 μg of DNA (Fig. 3c).

We evaluated the effects of varying the injection time (2–30 s) and volume (0.1–1.5 ml) on the efficiency of gene transfer, measuring the serum Epo levels 1 week before and 3 weeks after injecting 5 pmol of each PCR-amplified DNA fragment: fCAGGS-Epo (10 μg) or fCAGGS (7.6 μg). We obtained maximal Epo expression when the DNA solution was injected within 2 s (Fig. 4a), and with a volume of 0.5 ml (Fig. 4b).

RT-PCR Analysis of the Transgene-Derived Epo mRNA in Major Organs—It was important to determine whether the naked plasmid DNA or PCR-amplified DNA fragment was expressed exclusively in the injected kidneys. We detected the transgene-derived Epo mRNA by RT-PCR only in the left kidneys that have received injections of pCAGGS-Epo (Fig. 5a) or fCAGGS-Epo (Fig. 5c), *i.e.*, not in any other organs. We did not detect the transgene-derived Epo mRNA in the animals that received injections of pCAGGS or fCAGGS (data not shown), although the control G3PDH mRNA was detected in all the injected kidneys. We obtained similar results on PCR performed to detect the Epo transgene (data not shown),

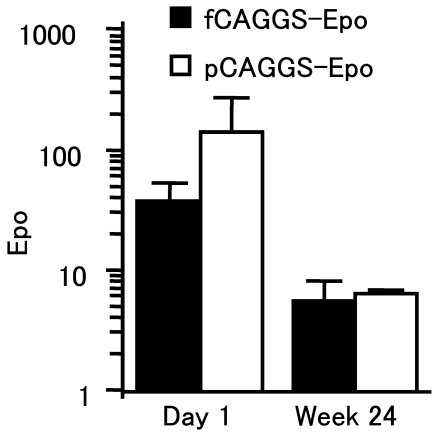

Fig. 6. **Epo mRNA expression in the kidneys at 1 day and 24 weeks after 5 pmol fCAGGS-Epo (10 μg) or pCAGGS-Epo (18.4 μg) injection.** The levels of Epo mRNA were determined by real-time PCR analysis. $n = 3$ at each time point.

and on RT-PCR performed to detect the transgene-derived Epo mRNA (Fig. 5, b and d) at 24 weeks after injection. Thus, the transgene expression of pCAGGS-Epo injected retrogradely via the renal vein was confined to the left kidney on day 1 after the injection and the expression was maintained for more than 24 weeks.

Quantitative Real-Time PCR Analysis of Epo mRNA in Kidneys—It was also important to determine whether the level of Epo gene expression of fCAGGS-Epo was different from that of pCAGGS-Epo. We detected the transgene-derived Epo mRNA on quantitative real-time PCR in the left kidneys of rats that had received an injection of 5 pmol of fCAGGS-Epo (10 μg) or pCAGGS-Epo (18.4 μg). Although the level of Epo in the pCAGGS-Epo group on day 1 seemed higher than that in the fCAGGS-Epo group (more than 3-fold), as shown in Fig. 6, the level of Epo gene expression in the kidneys were not statistically different from each other at any time point in the experiment. Thus, the transgene expression level achieved with fCAGGS-Epo injection via the renal vein was not statistically different from that achieved with pCAGGS-Epo injection.

Time Course after pCAGGS-Epo or fCAGGS-Epo Injection—We examined the time course of Epo expression when the amount of naked plasmid DNA injected was varied using a 2-s injection time and a volume of 0.5 ml. Rats were assigned to six groups, as shown in Table 1.

After an injection of 100 μg of pCAGGS-Epo, the serum Epo level peaked at 507.7 ± 333.8 mU/ml at week 3, and gradually decreased to 198.8 ± 151.7 mU/ml at week 24 (Fig. 7a). Until week 24, a similar pattern was obtained with smaller doses of pCAGGS-Epo: 10, 30, or 60 μg.

Table 1. **Six rat groups.**

Group	Gene	Dose (μg)	*n*
pCAGGS-Epo 100 μg rats	pCAGGS-Epo	100	3
pCAGGS-Epo 60 μg rats	pCAGGS-Ep	60	4
pCAGGS-Epo 30 μg rats	pCAGGS-Epo	30	4
pCAGGS-Epo 10 μg rats	pCAGGS-Epo	10	4
pCAGGS-Epo 2 μg rats	pCAGGS-Epo	2	4
pCAGGS rats	pCAGGS	100	4

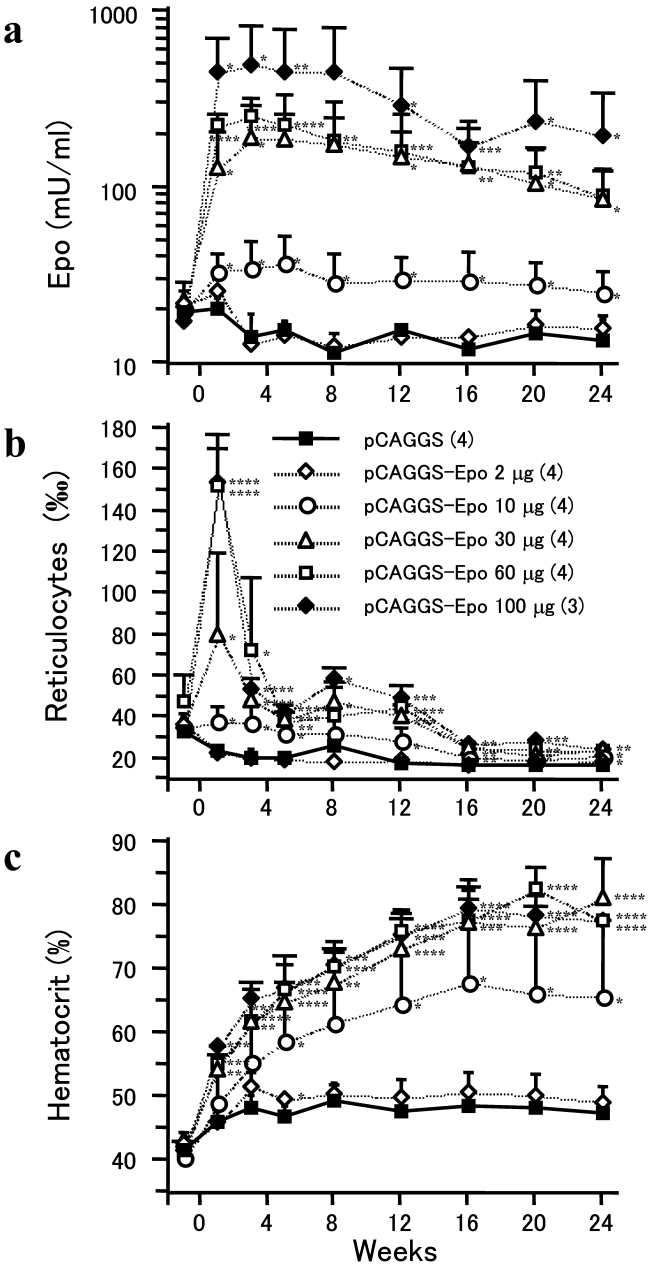

Fig. 7. **Time course after pCAGGS-Epo transfer by retrograde renal vein injection.** $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ for comparisons between pCAGGS-Epo and pCAGGS rats (comparisons were made at each time point). $n = 3$ in each group. (a) Serum Epo levels. (b) Reticulocyte counts. (c) Hematocrit. Error bars are not shown for some data points because of low variability.

Transgene-derived Epo secretion caused reticulocytosis (Fig. 7b). The hematocrit level was much higher in rats injected with 10 μg of pCAGGS-Epo than in pCAGGS-injected rats, for at least 24 weeks, which was the last time point examined (Fig. 7c). Thus, pCAGGS-Epo transfer allowed continuous production of biologically active Epo, resulting in significant elevation of the reticulocyte and hematocrit levels in dose-dependent manners.

We also examined the time course of Epo expression when the amount of PCR-amplified DNA fragment injected was varied using a 2-s injection time and a volume of 0.5 ml. Rats were assigned to six groups, as shown in Table 2.

Table 2. **Six rat groups.**

Group	Gene	Dose		*n*
		μg	pmol	
fCAGGS-Epo 10 μg rats	fCAGGS-Epo	10	5	4
fCAGGS-Epo 5 μg rats	fCAGGS-Epo	5	2.5	4
fCAGGS-Epo 3 μg rats	fCAGGS-Epo	3	1.5	4
fCAGGS-Epo 1 μg rats	fCAGGS-Epo	1	0.5	4
pCAGGS-Epo rats	pCAGGS-Epo	18.4	5	4
fCAGGS rats	fCAGGS	7.6	5	4

After an injection of 10 μg of fCAGGS-Epo, the serum Epo level peaked at 76.0 ± 22.8 mU/ml at week 3, and gradually decreased to 45.0 ± 13.6 mU/ml at week 24 (Fig. 8a). The level of Epo gene expression in fCAGGS-Epo 10 μg rats was not statistically different from that in pCAGGS-Epo rats. Until week 24, a similar pattern was obtained with 3 or 5 μg of fCAGGS-Epo. The transgene-derived Epo secretion caused reticulocytosis (Fig. 8b). The hematocrit level was much higher in the rats that had received 3 μg of pCAGGS-Epo than in the fCAGGS-Epo–injected rats, for at least 24 weeks, which was the last time point examined (Fig. 8c). Thus, fCAGGS-Epo transfer allowed continuous production of biologically active Epo, as in the case of pCAGGS-Epo, resulting in significant elevation of the reticulocyte and hematocrit levels in dose-dependent manners.

The Injected Kidney Function in Rats from Which the Uninjected Kidney Was Removed—Rats were assigned to four groups: normal, unoperated rats (Control rats, $n = 5$), uninephrectomized rats that did not receive a DNA injection (Uninephrectomy rats, $n = 5$), rats treated with 100 μg of pCAGGS (pCAGGS + Uninephrectomy rats; $n = 5$), and ones treated with 100 μg of pCAGGS-Epo (pCAGGS-Epo + Uninephrectomy rats; $n = 4$). The serum creatinine level increased with growth, and this increase was significantly enhanced by uninephrectomy. However, the serum creatinine levels in each group were within normal range. The serum creatinine levels were not significantly different among the uninephrectomized groups at any time point, irrespective of DNA injection (Fig. 9). All the rats survived surgery and the injection procedure, and appeared normal. These results demonstrated that the injected kidney could function normally after gene transfer via retrograde renal vein injection.

DISCUSSION

The present results demonstrate that kidney-targeted gene transfer can be achieved by retrograde injection into the renal vein of PCR-amplified DNA fragments as well as naked plasmid DNA when only the renal vein is clamped.

The site of gene transfer was interstitial fibroblasts near the PTC, as seen with our previous method that included clamping of the renal artery (*1*). These findings support the idea that the profile of vascular pressure along the intrarenal vasculature, and the sharp vascular resistance gradient between the efferent arterioles and the PTC (*2*) could block the retrograde stream of the

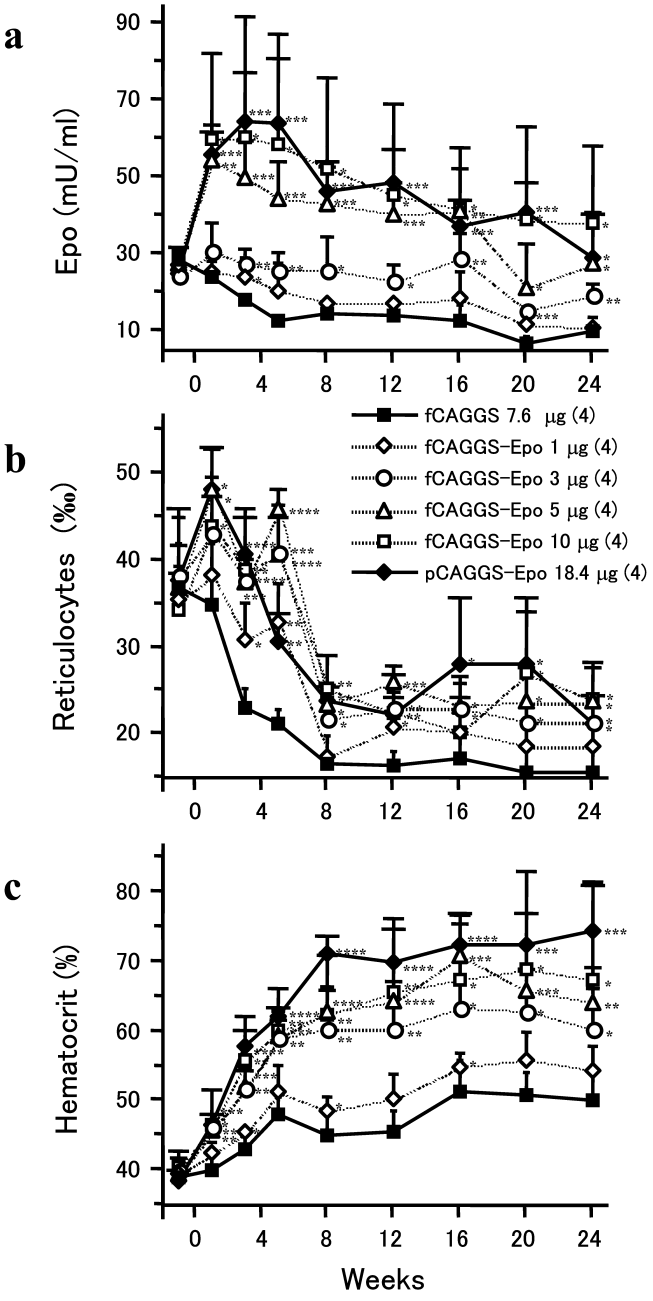

Fig. 8. **Time course after fCAGGS-Epo transfer by retrograde renal vein injection.** *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for comparisons with fCAGGS rats (comparisons were made at each time point). n = 4 in each group. (a) Serum Epo levels. (b) Reticulocyte counts. (c) Hematocrit. Error bars are not shown for some data points because of low variability.

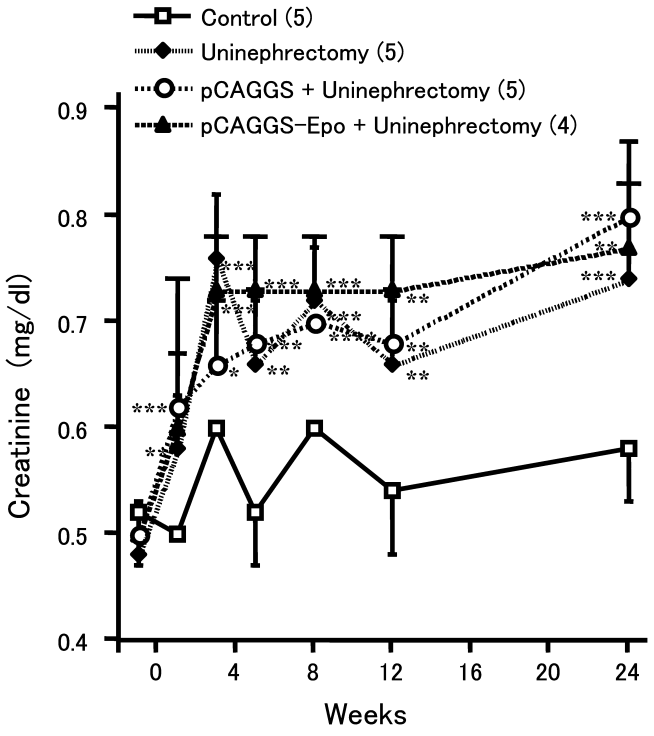

Fig. 9. **Functional analysis of the injected kidneys. Normal, unoperated rats (control rats, open squares).** Uninephrectomized rats without DNA injection (uninephrectomy rats, solid diamonds). Uninephrectomized rats with injection of pCAGGS (pCAGGS + uninephrectomy rats, open circles) or of pCAGGS-Epo (pCAGGS-Epo + uninephrectomy rats, solid triangles). n = 4 or 5 in each group. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for comparisons with normal rats (comparisons were made at each time point).

injected DNA solution, independent of clamping of the renal artery. The vascular resistance gradient directed the DNA solution stream to the PTC. The PTC wall consists of an extremely thin endothelium. Fifty percent of the PTC endothelium is fenestrated, and thus it is highly permeable to water and small solutes (*2*). We speculate that Ringer's solution and the DNA easily pass through the PTC endothelium under the large hydrostatic pressure. We believe these characteristics of the PTC endothelium could account for the gene transfer into the fibroblasts nearby, and the prevention of wall rupture due to the large hydrostatic pressure caused by the retrograde stream of injected DNA solution.

The current study was partly prompted by concerns about how our previous technique could be applied to vascular catheter-based gene therapy in humans. Direct application would have required the insertion of two balloon catheters to block the blood stream, one in the renal artery and the other in the renal vein, into which the gene solution would be injected. Hemostasis of an artery, *e.g.*, the femoral artery, subsequent to catheterization, moreover, is more difficult than hemostasis of a vein. In the present study, therefore, we have simplified the technique for catheter-based gene therapy and for more noninvasive performance. We believe that our modified technique, in which only the renal vein is clamped, will permit sufficient efficiency for kidney-targeted gene transfer to be used clinically. We are currently developing a novel balloon catheter for gene therapy in dogs.

For both the previous (*1*) and present kidney-targeted naked DNA transfer, the two critical parameters for successful PCR-amplified DNA fragment expression were the injection volume and the injection speed. PCR-amplified DNA fragments are apt to be degraded by exonucleases as well as endonucleases. The rapid injection of an appropriate volume of a DNA solution seems to overcome this problem (*11*), probably because the rapid injection

results in direct exposure of the PCR-amplified DNA molecules to the PTC before the DNA mixes with the blood.

Our present study demonstrated that the interstitial fibroblast is a suitable site for long-term gene expression of PCR-amplified DNA fragments as well as naked plasmid DNA. The interstitial fibroblast is a key element in the pathogenesis of renal failure. The final common pathway for many progressive renal diseases is the process of fibrosis, which involves the proliferation of renal fibroblasts and the secretion of extracellular matrix by these cells (*12*). Successful gene transfer into fibroblasts *in vivo* could be of great significance for the development of antifibrotic strategies, for further understanding the basic biological mechanisms of fibrosis (*13*), and for creating a depot for therapeutic secreted proteins. Recently, we demonstrated that a transgene delivered by retrograde injection into the renal vein using the original technique could have a physiological effect on gene delivery to the kidneys: the delivery of the 7ND gene was shown to attenuate tubulointerstitial renal injury induced by protein-overload proteinuria (*14*). We think that both the original and modified gene transfer techniques would be broadly available for gene therapy for renal diseases.

To evaluate the likelihood of kidney damage, we analyzed kidney sections from rats injected with 0.5 ml of Ringer's solution containing plasmid DNA, and compared them with sections from normal rats that had not been injected. Compared with the kidney sections from normal rats, the endothelial cells of the PTC were slightly difficult to identify in the gene-transferred kidney sections at 5 h, 1 day and 7 days after the injection of pCAGGS-Epo. We found no apparent pathological changes in the cortex, medulla or papilla in sections obtained at 5 h, 1 day or 7 days after the injection of pCAGGS-Epo. Similarly, no pathological changes were seen in sections obtained from rats at week 24 after the injection with pCAGGS-Epo. Therefore, nephrotoxicity attributable to gene transfer was not apparent either on histological or functional (Fig. 9) examination of the injected kidneys.

The preparation of the PCR-amplified DNA fragment solution is simple and less time-consuming (a matter of hours rather than days) than naked plasmid DNA preparation. However, compared with PCR-amplified DNA preparation, we can produce a large amount of naked plasmid DNA by large-scale preparation of plasmid DNA. When designing studies, we need to take these characteristics into consideration; we can use either form of DNA for gene transfer, depending on the requirements of the experiment. Our study demonstrated that PCR-amplified DNA fragments can cause the production of secretory proteins at essentially the same levels as equivalent moles of naked plasmid DNA, indicating that there is no loss in the efficiency of gene expression if PCR-amplified DNA fragments are used.

In this study, we have demonstrated that when only the renal vein was clamped, retrograde injection of naked plasmid DNA into the renal vein could allow kidney-targeted gene transfer in rats. We are designing a gene therapy for Fabry disease, involving this technique.

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REFERENCES

1. Maruyama, H., Higuchi, N., Nishikawa, Y., Hirahara, H., Iino, N., Kameda, S., Kawachi, H., Yaoita, E., Gejyo, F., and Miyazaki, J. (2002) Kidney-targeted naked DNA transfer by retrograde renal vein injection in rats. *Hum. Gene Ther.* **13**, 455–468
2. Lemley, K.V. and Kriz, W. (1994) Structure and function of the renal vasculature with clinical and functional correlations in *Renal pathology* (Tisher, C.C. and Brenner, B.M., eds.) pp. 981–1026, J.B. Lippincott, Philadelphia
3. Hofman, C.R., Dileo, J.P., Li, Z., Li, S., and Huang, L. (2001) Efficient *in vivo* gene transfer by PCR amplified fragment with reduced inflammatory activity. *Gene Ther.* **8**, 71–74
4. Chen, Z-Y., Yant, S.R., He, C-Y., Meuse, L., Shen, S., and Kay, M.A. (2001) Linear DNAs concatemerize *in vivo* and result in sustained transgene expression in mouse liver. *Mol. Ther.* **3**, 403–410
5. Maruyama, H., Sugawa, M., Moriguchi, Y., Imazeki, I., Ishikawa, Y., Ataka, K., Hasegawa, S., Ito, Y., Higuchi, N., Kazama, J.J., Gejyo, F., and Miyazaki, J. (2000) Continuous erythropoietin delivery by muscle-targeted gene transfer using *in vivo* electroporation. *Hum. Gene Ther.* **11**, 429–437
6. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199
7. Kameda, S., Maruyama, H., Higuchi, N., Nakamura, G., Iino, N., Nishikawa, Y., Miyazaki, J., and Gejyo, F. (2003) Hydrodynamics-based transfer of PCR-amplified DNA fragments into rat liver. *Biochem. Biophys. Res. Commun.* **309**, 929–936
8. Ohwada, K. (1986) Improvement of cardiac puncture in mice (in Japanese). *Exp. Anim.* **35**, 353–355
9. Maruyama, H., Ataka, K., Gejyo, F., Higuchi, N., Ito, Y., Hirahara, H., Imazeki, I., Hirata, M., Ichikawa, F., Neichi, T., Kikuchi, H., Sugawa, M., and Miyazaki, J. (2001) Long-term production of erythropoietin after electroporation-mediated transfer of plasmid DNA into the muscles of normal and uremic rats. *Gene Ther.* **8**, 461–468
10. Kaissling, B., Hegyi, I., Loffing, J., and Le Hir, M. (1996) Morphology of interstitial cells in the healthy kidney. *Anat. Embryol.* **193**, 303–318
11. Kawabata, K., Takakura, Y., and Hashida, M. (1995) The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharmaceut. Res.* **12**, 825–830
12. Kuncio, G.S., Neilson, E.G., and Haverty, T. (1991) Mechanisms of tubulointerstitial fibrosis. *Kidney Int.* **39**, 550–556
13. Clarke, H.C., Cook, H.T., and Hendry, B.M. (1999) Gene therapy for renal fibroblasts. *Nephrol. Dial. Transplant.* **14**, 1615–1617
14. Shimizu, H., Maruyama, S., Yuzawa, Y., Kato, T., Miki, Y., Morita, Y., Maruyama, H., Egashira, K., and Matsuo, S. (2003) Anti-monocyte chemoattractant protein-1 gene therapy attenuates renal injury induced by protein-overload proteinuria. *J. Amer. Soc. Nephrol.* **14**, 1496–1505