*Regular Article*

# Determining Transgene Expression Characteristics Using a Suction Device with Multiple Hole Adjusting a Left Lateral Lobe of the Mouse Liver

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**We developed a tissue suction-mediated transfection method (suction method) as a relatively reliable and less invasive technique for *in vivo* transfection. In this study, we determined hepatic transgene expression characteristics in the mouse liver, using a suction device, collecting information relevant to gene therapy and gene functional analysis by the liver suction method. To achieve high transgene expression levels, we developed a suction device with four holes (multiple hole device) and applied it to the larger portion of the left lateral lobe of the mouse liver. Hepatic transfection with physical stimuli was potentially controlled by activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-*κ*B). We examined the spatial distribution of transgene expression in the suctioned lobe by 2-dimensional imaging with histochemical staining and 3-dimensional multicolor deep imaging with tissue clearing methods. Through monitoring spatial distribution of transgene expression, the liver suction method was used to efficiently transfect extravascular hepatocytes in the suction-deformable upper lobe of the liver. Moreover, long-term transgene expression, at least 14 d, was achieved with the liver suction method when cytosine-phosphate-guanine (CpG)-free plasmid DNA was applied.**

**Key words** gene delivery; naked plasmid DNA; liver; hepatocyte

The liver is a vital organ for removing harmful substances, metabolizing drugs and maintaining colloid osmotic pressure. With time, chronic hepatitis progresses to cirrhosis and hepatoma. Chronic liver diseases often advance in symptomatic severity, even when there is a high degree of liver function. In addition, hepatoma, as an advanced state of liver disease, often relapses in the many cases involving underlying liver disorders. Therefore, it is important to treat cirrhosis as a precancerous state.

For treating intractable liver diseases, *in vivo* transfection methods using viral and non-viral vectors were developed. Among them, methods involving intravenous injection of naked plasmid DNA (pDNA) are relatively easy to perform. Although hepatic transgene expression after intravenous injection of naked pDNAs is low,[1)] various investigators, including our group, reported that *in vivo* naked pDNA transfection methods, when combined with physical stimuli, led to higher transgene expression levels in the liver. The types of physical stimuli tested include hydrodynamics,[2–7)] mechanical massage,[8,9)] pressure[10–12)] and suction.[13–16)]

We described a liver suction-mediated transfection method (liver suction method), using a suction device with a single hole (single hole device) fabricated from polydimethylsiloxane. We believe that suction pressure waveform can be controlled by using a pressure-controlled computer system and the tissue suction device can be uploaded onto the head of an endoscope.[13,14)] Therefore, the suction method could be applicable as a less invasive and certain transfection method in the clinic using the new medical device. This procedure achieved transfection into the upper region of the liver, around the suctioned area, when the upper and lower sites of the liver were compared.[13)] Therefore, we hypothesized that transgene expression was induced by deformation resulting from the liver suction. Moreover, Nishikawa *et al.*[17)] reported that hydrodynamic stimuli activated transcriptional factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-*κ*B) and activator protein 1 (AP-1). We subsequently demonstrated that pressure[12)] and suction of the kidney[16)] also transiently activated these transcriptional factors.

To obtain information useful for gene functional analysis and gene therapy for cirrhosis, using the liver suction method, measurements of hepatic transgene expression characteristics, including controlling factors and spatial distribution, will be needed. Although AP-1 and NF-*κ*B might influence transgene expression in response to certain physical stimuli, effects on these transcriptional factors in the suctioned liver were not previously evaluated. In addition, the spatial distribution of transgene expression was not known, though we reported that the liver suction method enabled transfection of, broadly, the upper region. Similarly, we did not previously identify the cells important for transgene expression in the suctioned liver. Also important, sustained transgene expression for sufficient time periods will be essential for therapeutic efficacy. In addition, because changes, such as protein expression, caused by tissue deformation during suction can depend on the areas suctioned, these changes might not be detectable if the suctioned areas are too small. Finally, relatively high transgene expression levels would be needed for successful gene therapy of cirrhosis.

In this study, we determined hepatic transgene expression characteristics in a suctioned lobe of the mouse liver. First, we developed a suction device with four holes (multiple hole

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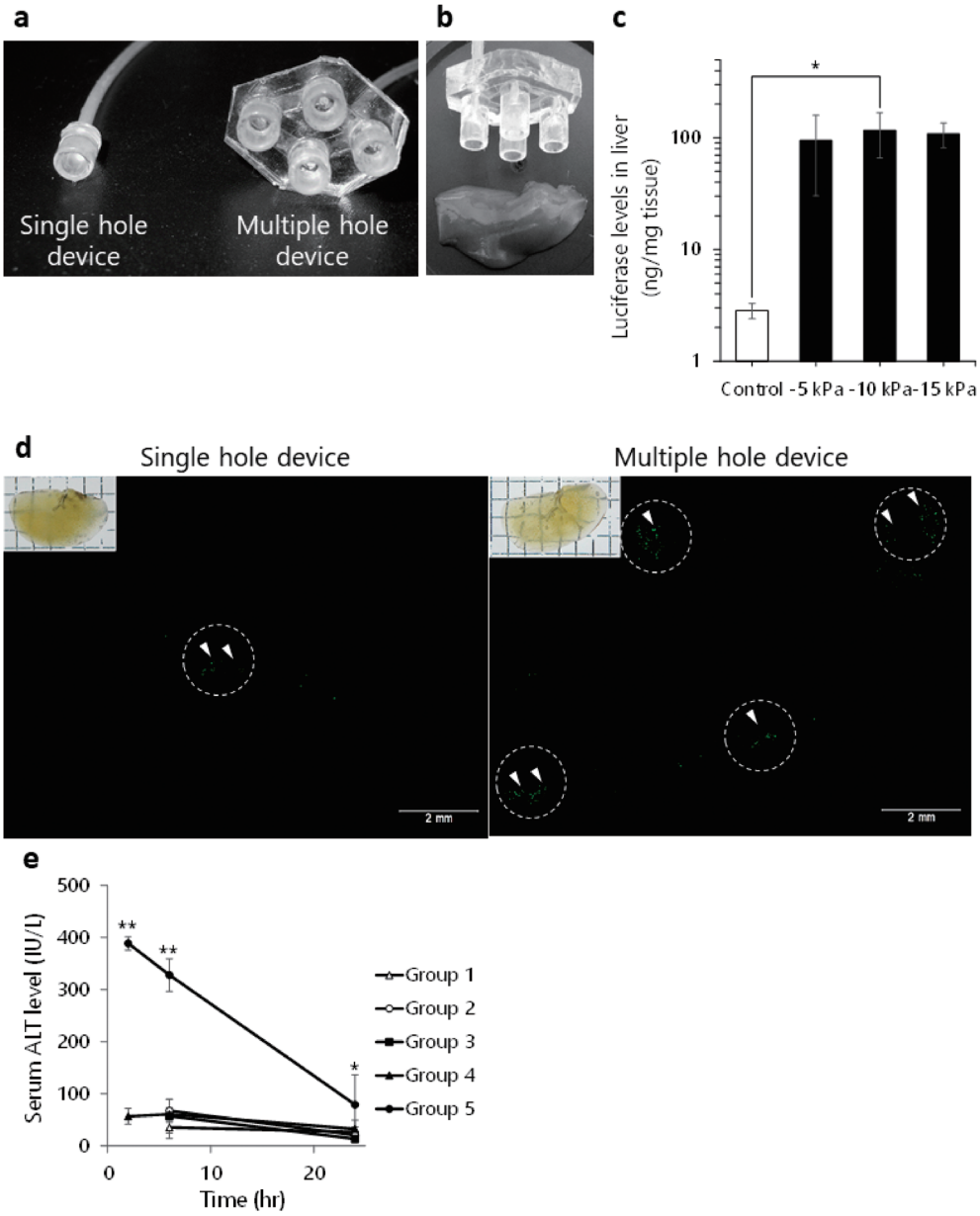


Fig. 1. Optimization of Suction Conditions in the Mouse Liver with the Newly Designed Multiple Hole Device

**a** Two suction devices: (left) the conventional single hole device, (right) the multiple hole device, modified for the left lateral lobe of the mouse liver. The size of this multiple hole device was 17 mm wide and 22 mm long. **b** Suctioned image. **c** Effects on suction pressure conditions with the multiple hole device. Luciferase activities were measured at 6 h after transfection with pCMV-Luc (Control group, $n$=3; suction groups, $n$=7). The control group received pCMV-Luc with no suction. **d** Comparison of transgene expression regions resulting from use of the two suction devices. Mice were perfused and fixed at 24 h after transfection with pZsGreen1-N1 and the liver was cleared with CUBIC reagents. ZsGreen1 expression was observed by the tile scan mode. Magnification: 10×. Green: ZsGreen1. Dashed line with open circle: Suction hole formed. **e** Assessment of hepatic toxicity, based on serum ALT levels, after treatments. Serum ALT levels were measured in the same mice at 2, 6 and 24 h after treatment. Mice were divided into the following 5 groups: group 1, open surgery only; group 2, open surgery and liver suction; group 3, open surgery and intravenous (i.v.) administration; group 4, open surgery, i.v. administration and liver suction (liver suction group); and group 5, hydrodynamic-based delivery ($n$=4-5). Mice were administered pCMV-Luc, except for groups 1 and 2. Each bar represents the mean±standard deviation (S.D.) (ANOVA and Tukey's test, *$p$<0.05, **$p$<0.001).

device), with width and length of 17 and 22 mm, respectively, and applied it to a left lateral lobe of the liver in mice. Following development of this multiple hole device, we optimized suction conditions to minimize invasiveness. Then, we evaluated activation of AP-1 and NF-$\kappa$B, as controlling factors for transgene expression, in response to deformation by liver suction. We also examined the spatial distribution of transgene expression in the suctioned lobes. We developed 3-dimensional multicolor deep imaging, applied with a combination of the tissue clearing methods, with observation by confocal laser microscopy.[16,18,19] This 3-dimensional multicolor deep imaging was then applied to analyze the spatial distribution of transgene expression in the suctioned liver. We evaluated the selectivity and depth of transgene expression using CUBIC reagents[20] that have high sensitivity for transgene expression.[18] Then, we identified the cells expressing the transgene by 5-bromo-4-chloro-3-indolyl $\beta$-D-galactopyranoside (X-gal) staining of frozen liver sections. We recently proposed that it would be possible to evaluate cells and distribution of transgene expression in the kidney while maintaining its organizational structure.[16] In the present study, we observed spatial distributions from relative positions in the suctioned liver using Scale SQ (0) reagents,[21] allowing staining of cell membranes and blood vessels. To address the necessity that

sustained transgene expression results from each surgical procedure, we evaluated long-term transgene expression after a single transfection of a cytosine-phosphate-guanine (CpG)-free pDNA.

## MATERIALS AND METHODS

**pDNA** We used the following pDNAs: pCMV-Luciferase (pCMV-Luc), pDNAs for Mercury Pathway Profiling Luciferase system 1 (pTAL-Luc, pAP-1-Luc and pNF-$\kappa$B-Luc), pZsGreen1-N1, pCpGfree-LacZ, and pCpGfree-Lucia. These pDNAs were purchased as previously reported.[16,22]

**Animals** ICR female mice (5 weeks old) were from CLEA Japan, Inc. (Shizuoka, Japan). The mice were housed in an air-conditioned room with free access to drinking water and a standard laboratory diet. All animal experiments were performed in accordance with the Guide for Animal Experimentation of Nagasaki University.

**Tissue Suction Device for the Liver Suction Method** The tissue suction devices, the single hole and multiple hole devices, are shown in Fig. 1a. The multiple hole device was newly constructed, as described previously,[13] with dimensions of 17 mm by 22 mm to fit the left lateral lobe (approximately 30 mm) (Fig. 1b). Additionally, we used a simplified tissue suction system, applying the minimum magnitude of suction pressure.[16]

***In Vivo* Transfection by the Liver Suction Method** After laparotomy anesthetized as previously described,[16] 100 $\mu$g of pDNA dissolved in 200 $\mu$L of saline was injected *via* the tail vein and the left lateral lobes were immediately suctioned for 5 s. We defined the left lateral lobe as the "suctioned lobe" and an anterior lobe as the "not suctioned lobe."

**Blood Sampling** Blood samples were collected *via* the tail vein in conscious mice and centrifuged at 2000×***g*** for 5 min at room temperature. The supernatants, serum samples, were retained.

**Luciferase Assay** Luciferase activity was assayed in the tissues as described previously[22] using a luminometer (MiniLumat LB9506, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Assays were performed on samples from the left lateral lobe of the liver or the left kidney. Lucia, indicating the secretory luciferase, was evaluated in serum from the same mice over time, as described previously.[16]

**Hepatic Toxicity by the Liver Suction Method** To evaluate hepatic toxicity by the liver suction method, alanine aminotransferase (ALT) in serum was measured by the Transaminase C-II test Wako® from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). We prepared samples from 5 groups: group 1, open surgery only; group 2, open surgery and liver suction; group 3, open surgery and intravenous (i.v.) administration; group 4, open surgery, i.v. administration and liver suction (liver suction group); group 5, hydrodynamic-based delivery. Group 5 was included as a positive control because hydrodynamic-based delivery caused transient liver damage. At 2 h after treatments, the serum ALT levels in only groups 4 and 5 were measured. At 6 and 24 h after treatments, the serum ALT levels of all groups were measured.

**Real Time PCR Analysis** The mRNA levels of *c-fos* and *c-jun*, as components of the AP-1 complex, and of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control were monitored using real-time PCR analysis, as previously described.[12] The *c-fos* and *c-jun* mRNA levels were normalized based on GAPDH mRNA levels. Total RNA was extracted using the FastGene™ RNA Basic Kit from Nippon Genetics Co., Ltd. (Tokyo, Japan).

**Tissue Clearing Methods** Mice were fixed with 4% paraformaldehyde at 24 h after transfection with pZsGreen1-N1, as described previously.[16,18,19] The fixed organs were treated using CUBIC[20] and Scale SQ (0)[21] reagents. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) from Sigma-Aldrich Japan Co. (Tokyo, Japan). The cleared organs were observed with a confocal microscope (LSM710 system). Z-Stack images were obtained as previously described.[18]

**Blood Vessel Staining** To observe spatial distributions relative to blood vessels, using the Scale SQ (0) reagents, blood vessels were stained with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI) solution be-

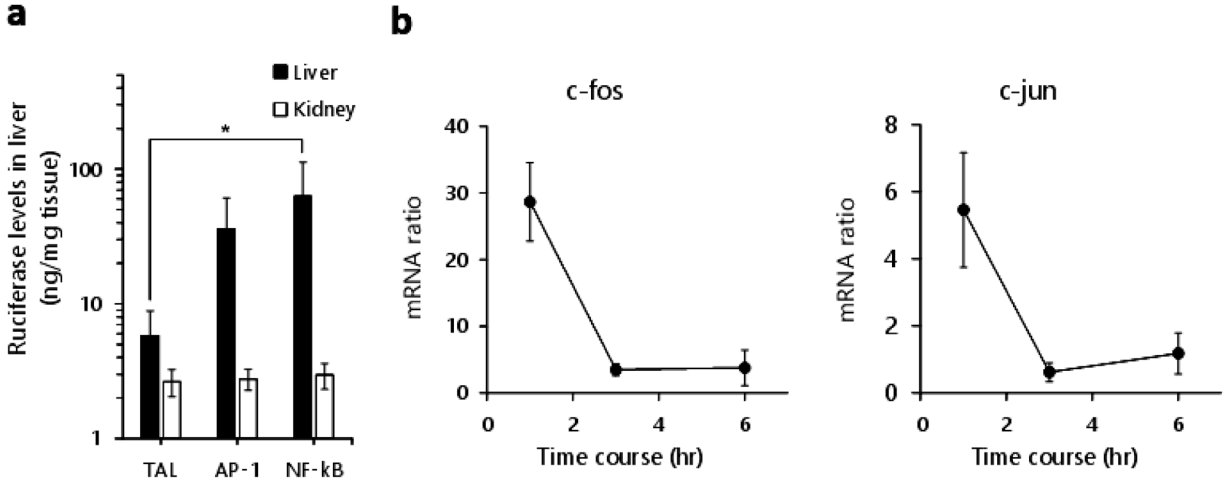


Fig. 2. Activation of Transcriptional Factors by Liver Suction

**a** Bioluminescence system for *in vivo* evaluation. Mice were transfected with each pDNA for the Mercury Pathway Profiling Luciferase system 1. At 6 h after transfection, binding activities of AP-1 and NF-$\kappa$B in the liver and kidney were measured by the luciferase assay. pTAL-Luc without a binding region of transcriptional factors served as a negative control. TAL, pTAL-Luc; AP-1, pAP-1-Luc; NF-$\kappa$B, pNF-$\kappa$B-Luc administration groups. **b** *c-fos* and *c-jun* mRNA levels in the suctioned lobe, measured at 1, 3 and 6 h after transfection with pCMV-Luc by real-time PCR. The *c-fos* and *c-jun* mRNA levels were normalized to those for GAPDH, used as a housekeeping gene. Each bar is the mean±S.D. ($n$=3). (ANOVA and Tukey's test, *$p$<0.05).

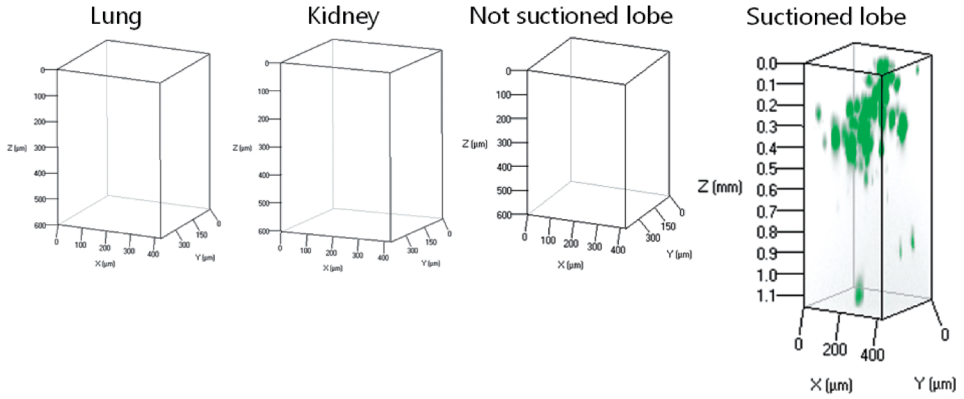


Fig. 3. 3-Dimensional Imaging of Transgene Expression in Various Tissues, Including Lung, Kidney and Liver, with CUBIC Reagents

Fixed tissues were cleared with the CUBIC reagents. Magnification: 20×. Green: ZsGreen1.

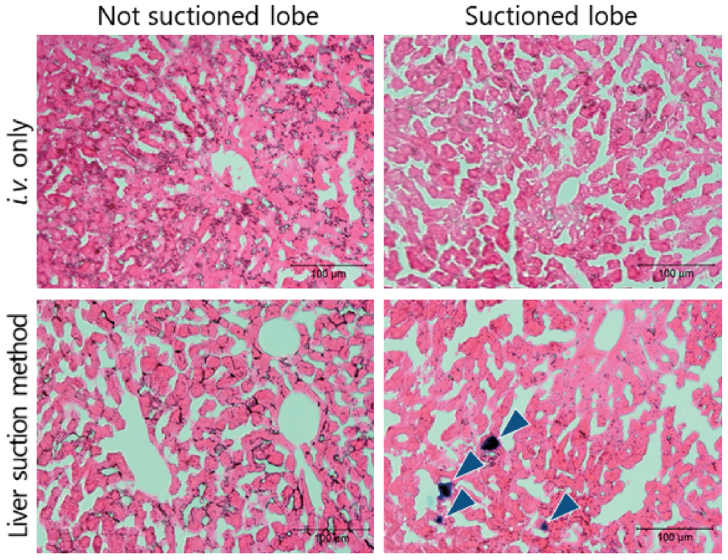


Fig. 4. Identification of Cells in the Liver Expressing Transgene by Histochemical Staining

The 10-$\mu$m frozen liver sections were stained with X-gal and counterstained with eosin. Magnification: 200×. Arrows indicate blue LacZ-stained cells.

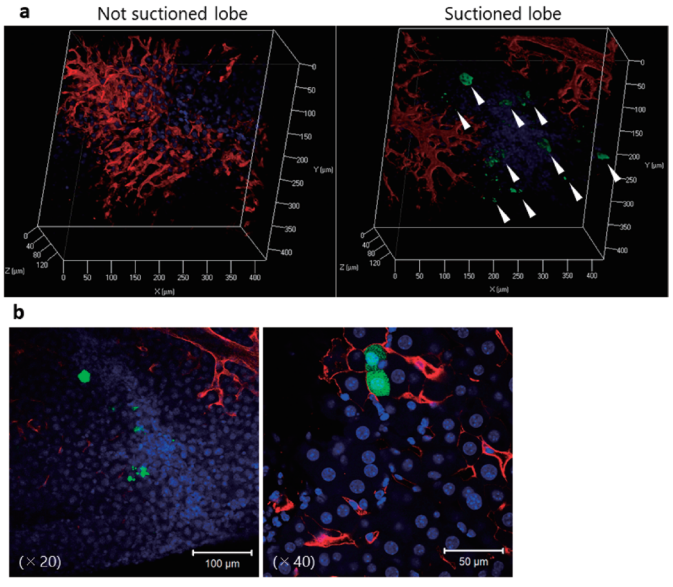


Fig. 5. Evaluation of Relative Positions by Scale SQ (0) Reagents

**a** 3-Dimensional imaging in "suctioned" and "not suctioned" lobes. Arrows indicate ZsGreen1-N1 expression cells. **b** 2-Dimensional imaging in slices of the suctioned lobe. Fixed liver was cleared with Scale SQ (0) reagents. Magnification: 20× and 40× oil. Green: Zsgreen1; red: blood vessels (DiI); blue: nuclei (DAPI).

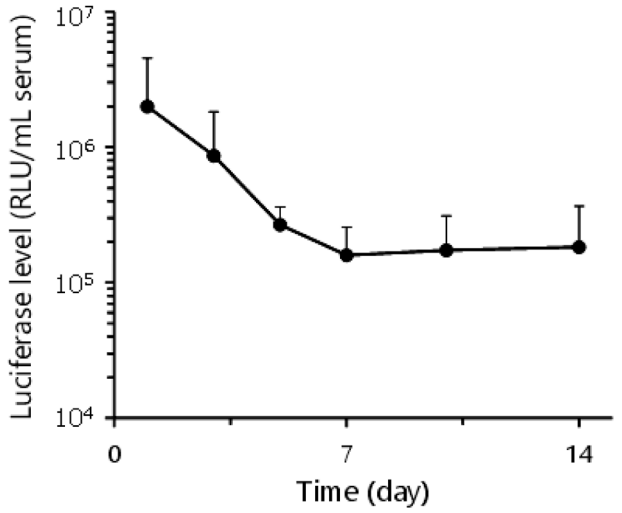


Fig. 6. Sustained Transgene Expression after Single Administration of CpG-free pDNA

Temporal changes in secretory luciferase levels were measured in serum from the same mice at 1, 3, 5, 7, 10 and 14 d after a single transfection with pCpGfree-Lucia. The plot shows mean+S.D. ($n$=5). (ANOVA and Tukey's test).

fore fixation.[16]

**Histochemical Staining** To identify cells expressing transgenes in the suctioned liver, frozen liver sections were visualized by X-gal staining. At 24 h after transfection with pCpGfree-LacZ, mice were perfused and fixed with 0.5% glutaraldehyde, as previously described.[16] Frozen sections were counterstained with eosin.

**Statistical Analysis** Significant difference between groups was determined by ANOVA and Tukey's test. $p<0.05$ was considered significant.

## RESULTS

**Optimization of Suction Conditions for the Murine Liver** We determined the minimum magnitude of pressure for suctioning the murine liver with the multiple hole device (Fig. 1c). We found that acceptable luciferase activities were observed at a minimum magnitude of under −5 kPa. The luciferase activities resulting from use of the multiple hole device were about 3.81 times higher than those obtained using the single hole device (Supplementary Fig. S1). We compared regions of transgene expression for each type of suction device using CUBIC reagents under low magnification, 10× (Fig. 1d). When ZsGreen1 expression regions were compared, transfection by the single hole device resulted in one transfected region while the multiple hole device produced four transfected regions. To confirm effectiveness of the less invasive suction conditions, the time course of elevated serum ALT levels, indicating the degree of liver damage, was evaluated (Fig. 1e). No detectable liver damage resulted from liver suction. Hydrodynamic-based delivery, used as a positive control, produced elevated ALT levels.

**Effects of Suction Stimuli on the Factors Controlling Transgene Expression in the Liver** To evaluate the influence of the suction stimuli on transgene expression, we analyzed activation of AP-1 and NF-$\kappa$B after liver suction. The DNA binding activities of AP-1 and NF-$\kappa$B were increased at 6 h after suction (Fig. 2a). Consistent with this, *c-fos* and *c-jun* mRNA levels were increased at only 1 h after treatment (Fig. 2b).

**Spatial Distribution of Transgene Expression in Tissues** The spatial distribution of ZsGreen1 expression in tissues was evaluated using a tissue clearing method (Figs. 3, 5). To observe lobe selectivity and depth dimension, we used the CUBIC reagents with high transparency (Fig. 3). We observed the spatial distribution of ZsGreen1 expression in tissue samples under high magnification, 20×. Comparing ZsGreen1 expression in various tissues, including the lung, kidney and liver, we observed expression only in the suctioned lobe. Based on depth dimension, ZsGreen1 expression was observed primarily in the upper part of the tissue, to 0.5 mm in depth. In addition, ZsGreen1 expression was detected with the CUBIC reagents at an observable depth of 1.175 mm.

**Identification of Transgene-Expressing Cells in Suctioned Liver** To identify transgene-expressing cells, frozen liver sections were stained for $\beta$-galactosidase activity (Fig. 4). To clarify the hepatic structures, the sections were counterstained with eosin. $\beta$-Galactosidase activity was observed only in the suctioned lobe in mice undergoing the liver suction method. Although $\beta$-galactosidase expressing cells are normally bluish green, they stained darker blue in our study. We next observed the relative spatial distribution of transgene expression in the tissues by multicolor deep imaging with the Scale SQ (0) reagents and DiI staining (Fig. 5). Zsgreen1 expression was detected only in the suctioned lobe (Fig. 5a), in agreement with results summarized in Figs. 3 and 4. In addition, Zsgreen1 was expressed in extravascular hepatocytes, as shown by 3-dimensional imaging (Fig. 5a) and 2-dimensional slices (Fig. 5b) of the suctioned lobe.

**Sustained Transgene Expression after Single Transfection** Sequential transgene expression of Lucia was monitored in a time-dependent manner in serum from the same mice (Fig. 6). Lucia expression was sustained for up to 14 d after a single transfection of pCpGfree-Lucia.

## DISCUSSION

The aim of this study was to determine hepatic transgene expression characteristics in the suctioned livers of mice. Previously, we developed a tissue suction-mediated transfection method (suction method) in various mouse tissues using a single hole device with following hole dimensions: inner diameter 3 mm, outer diameter 5 mm and height 3 mm.[13–16] This tissue suction device can be loaded onto the head of an endoscope, making the tissue suction method potentially applicable in the clinic, using a novel medical device. Because we previously reported that transgene expression levels were dependent on the number of suctions,[13] transgene expression would be expected to increase in proportion to the number of holes in the suction device. Taking this into consideration, we developed a multiple hole device with four holes to achieve higher transgene expression levels in the liver than those obtained with the single hole device (Fig. 1a). The whole liver could be suctioned using a multiple hole device (Fig. 1b). Using this multiple hole device, we evaluated the influence of suction pressure on the liver, finding that transgene expression was achieved at −5 kPa (Fig. 1c), with similar expression levels at −10 and −15 kPa. Although there is still little information about suction pressure, our findings with the multiple hole device were consistent with our previous report.[14] Therefore,

for subsequent experiments, we selected −10 kPa for stable transgene expression. Next, we clarified the region of transgene expression in the whole suctioned lobe (Fig. 1d), confirming that expression was higher at the suctioning sites of the liver. Under our suction conditions, using a multiple hole device, there was no increase in serum ALT levels (Fig. 1e).

Determining the factors controlling transgene expression is important to understand the mechanism of applying the liver suction method to gene therapy and gene function analysis. The transcription factors AP-1 and NF-$\kappa$B are important for high transgene expression and their activation was reported in response to physical stimuli such as hydrodynamics,[17] pressure[12] and kidney suction.[16] There were, however, no such reports on activation of transcription factors with the liver suction method. Therefore, we estimated activation of AP-1 and NF-$\kappa$B in suctioned liver by a luciferase assay (Fig. 2a), finding that they were activated in only the suctioned liver. Then, we assessed time-dependent changes in *c-fos* and *c-jun* mRNA levels, because these are components of the AP-1 complex (Fig. 2b). The mRNA levels of *c-fos* and *c-jun* were transiently increased after liver suction, in agreement with previously reported effects of hydrodynamics,[17] pressure[12] and kidney suction.[16] These observations strongly suggested that AP-1 and NF-$\kappa$B are controlling factors for hepatic transgene expression during implementation of the liver suction method.

Previously, we reported that the liver suction method transfected into approximately the upper 1.5 mm of the suctioned areas,[13] but the detailed spatial transgene distribution was not clarified. In the present study, we analyzed the spatial distribution characteristics of transgene expression in the suctioned lobe using tissue clearing methods and confocal microscopy.[16,18,19] Because tissue clearing by the high-sensitivity CUBIC reagents detected slight transgene expression in multiple tissues,[18] we focused on the depth dimension of transgene expression with these reagents (Fig. 3). We successfully clarified spatial distribution of transgene expression in the suctioned liver, observing specificity for the suctioned lobe. Regarding the depth dimension, we determined that the liver suction method frequently produced transfection into the upper part of the suctioned region, up to 0.5 mm in depth. We attributed this to the deformation produced by liver suction. These transgene expression characteristics were in accord with our previous report using a luciferase assay.[13] The results supported our mechanistic hypothesis that deformation produced by suctioning tissue is an important factor for transfection by the suction method.

It was reported that methods employing hydrodynamics[2,5–7] or mechanical massage[8,9] caused transfection of hepatocytes. Pressure on the vascular wall was generated by hydrodynamics[23] and mechanical massage,[9] transiently permeabilizing cell membranes within a few minutes and leading to pDNA uptake into the hepatocytes. However, the cells expressing transgene and their spatial distribution in the suctioned lobe of the liver were not yet identified. To address this, we performed X-gal staining of frozen liver sections (Fig. 4), with further delineation of hepatic structures by counterstaining with eosin. Results suggested that liver suction caused transfection of hepatocytes. This result coincided with those reported for hydrodynamics[2,5–7] and mechanical massage.[8,9] Next, we applied 3-dimensional multicolor deep imaging, with tissue clearing and cell membrane staining,[16] to obtain serial information about the spatial distribution characteristics of cells expressing transgene in the suctioned lobe (Fig. 5). To observe relative organizational structure, blood vessels, nuclei and transgene expression were simultaneously visualized. The results of 3-dimensional imaging (Fig. 5a) and 2-dimensional slices (Fig. 5b) indicated that cells expressing transgene were extravascular hepatocytes. This finding was consistent with observations in the liver sections (Fig. 4). Taking these and previous results into consideration, transfection methods employing physical stimuli such as hydrodynamics, mechanical massage and tissue suction can produce selective transfection of hepatocytes, among the various liver cell types.

For gene therapy, sustained transgene expression for a sufficient time period is necessary to produce pharmacological effects. In attempts to achieve long-term expression, various *in vivo* transfection methods using viral and non-viral vectors were investigated. While *in vivo* transfection methods using viral vectors can lead to expression for over 3 years, side effects such as leukemia induced by insertion into the genome of host cells were reported.[24] Conversely, transgene expression by intravascular injection of naked pDNAs, as a non-viral vector, are temporary, though do not involve insertion into the genome of host cells and have no side effects. Therefore, investigators attempted to obtain sustained transgene expression by repetitive administration and/or changes in plasmid vectors. Because the liver suction method requires a surgical procedure for suction of the liver surface, it is not suitable for repetitive administration. Thus, to prolong transgene expression by the liver suction method, we focused on changing the plasmid vectors. We investigated CpG-free pDNAs without an unmethylated CpG DNA motif to prolong transgene expression. Because CpG-free pDNAs cannot cause immunostimulation of the Toll-like receptor mediated pathway against unmethylated CpG DNA motifs,[25] transfection with CpG-free pDNAs can lead to long-term transgene expression. In the liver, long-term expression of CpG-free pDNAs by hydrodynamic-based delivery was reported,[26,27] but its sustained transgene expression by the liver suction method was not yet examined. Thus, we delivered a CpG-free pDNA loading secretory protein, applying the liver suction method, to promote long-term transgene expression in mice. pCMV-Luc transgene expression in the suctioned lobe decreased linearly over 3 d (Supplementary Fig. S2), in agreement with our previous study using the liver pressure method.[12] In contrast, transgene expression in the serum was sustained for at least 14 d when the liver suction method was combined with CpG-free pDNAs in the livers of normal mice (Fig. 6). This suggested that CpG-free pDNAs, introduced by the liver suction method, can result in long-term transgene expression in the mouse liver.

## CONCLUSION

We determined hepatic transgene expression characteristics using a suction device with multiple holes, applied to the left lateral lobe of the mouse liver. To achieve higher transgene expression levels in the suctioned lobe, we developed a multiple hole device. Regarding hepatic transgene expression characteristics, AP-1 and NF-$\kappa$B were implicated as factors controlling transfection by physical stimuli, including liver suction. Liver suction specifically and efficiently transfected

the extravascular hepatocytes, especially at depths of 0.5 mm in the upper part of the suctioned lobe, because of deformation *via* tissue suction. Moreover, we found that long-term transgene expression was obtained in the liver by delivering CpG-free pDNAs by the liver suction method.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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