表 1 心機能評価（吸引 10 日後）

		コントロール群	sham手術群	吸引群
tail-cuff法	収縮期血圧 (mmHg)	108.0 ± 4.0	104.0 ± 4.2	106.7 ± 2.3
	心拍数 (/min)	704.5 ± 22.5	712.0 ± 2.1	688.0 ± 35.3
心臓超音波検査	左室拡張末期径 (mm)	2.05 ± 0.05	2.10 ± 0.06	1.97 ± 0.09
	左室収縮末期径 (mm)	0.80 ± 0.10	0.93 ± 0.13	0.83 ± 0.09
	内径短縮率 (%)	62.0 ± 5.0	54.0 ± 5.51	59.0 ± 2.52
	駆出率 (%)	94.0 ± 2.00	89.67 ± 3.84	92.67 ± 1.33
	壁厚 (mm)	0.85 ± 0.00	0.78 ± 0.03	0.85 ± 0.03
	心拍数 (/min)	634.0 ± 13.0	679.0 ± 18.4	684.0 ± 11.9
	体重 (g)	18.9 ± 1.7	20.1 ± 0.9	18.8 ± 0.1
	心重量(mg)	100.0 ± 2.8	93.5 ± 1.9	99.1 ± 1.6
	肺重量 (mg)	12.8 ± 1.3	12.7 ± 1.6	12.7 ± 4.6
	心体重比	5.34 ± 0.63	4.66 ± 0.11	5.27 ± 0.07
	肺体重比	6.79 ± 0.54	6.32 ± 0.25	6.75 ± 0.22

表 2 心機能評価（吸引約 3 か月後）

		コントロール群	sham手術群	吸引群
tail-cuff法	収縮期血圧 (mmHg)	99.7 ± 3.1	101.5 ± 2.9	101.5 ± 2.0
	心拍数 (/min)	623.7 ± 24.1	600.5 ± 24.1	627.0 ± 20.8
心臓超音波検査	左室拡張末期径 (mm)	2.98 ± 0.14	3.06 ± 0.13	2.95 ± 0.11
	左室収縮末期径 (mm)	1.54 ± 0.14	1.49 ± 0.17	1.37 ± 0.11
	内径短縮率 (%)	48.5 ± 2.2	51.9 ± 3.9	53.9 ± 2.3
	壁厚 (mm)	0.90 ± 0.01	0.88 ± 0.01	0.89 ± 0.02
	心拍数 (/min)	744.4 ± 10.1	711.3 ± 23.0	707.2 ± 32.5

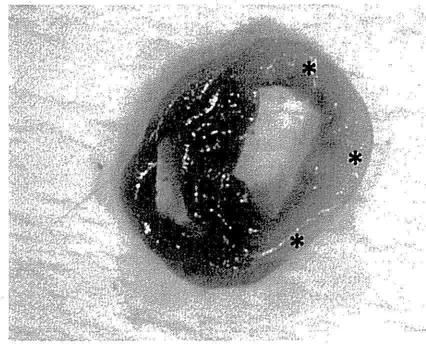

図 34 心筋梗塞モデルマウスの心臓の断面写真

C-3-7. 病態心臓への吸引圧法の適用

最後に、心筋梗塞モデルマウスの心臓に対して、吸引圧法を行い、梗塞部位周辺への核酸導入が可能であるかどうかを調べた。ルシフェラーゼ発現プラスミドDNAを投与後、心筋梗塞モデルマウスの心臓の虚血部位を吸引デバイスで吸引した。その結果、ルシフェラーゼ発現量は、健常マウスを用いた場合と同等であった（図35）。このことから、吸引圧法は虚血部位を持つ心臓へも適用可能であることが明らかになった。

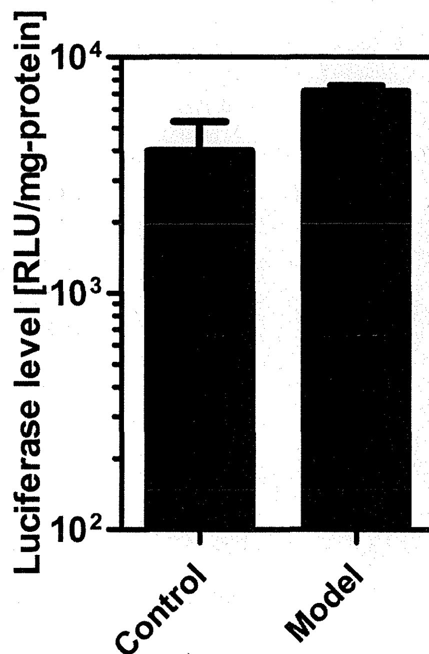

図 35 心筋梗塞モデルマウス心臓に対する吸引圧法

D. 考察

D-1. 吸引圧制御システムを用いた吸引圧法の利点

本研究ではまず吸引圧法の最適化を行うために吸引圧制御システムを開発した。このシステムでは、吸引圧の大きさや吸引圧の波形を自在に設定、制御することが可能であった。システムはフットスイッチを使って作動するため、作業者は両手を自由に使うことが可能であった。これまでの吸引圧法では、手動で吸引圧を発生させていたため、最小到達圧力の正確な制御や吸引圧波形の制御が出来なかった。これに対して本研究では、吸引圧制御システムを構築することで、最小到達圧力や波形の制御が可能になり、吸引圧法の最適条件を見出すことに成功した。このようなシステムを用いることで、将来的には再現性が良い高精度で安全な吸引圧法を、広範な研究施設や医療機関で実施することが可能になると考えられる。

D-2. 臓器ごとの最適吸引条件の違い

本研究では、肝臓、腎臓、心臓を対象に、吸引圧法の最適化を進めた。導入核酸発現量に対する最小到達圧力の影響を調べ、肝臓では－5 kPa、腎臓では－30 kPa、心臓では－75 kPaで吸引すると良いことがわかった。
また発現量に対する波形の影響を調べたが、波形への感受性は臓器により異なることがわかった。肝臓では、発現量が圧力供給時間や圧力維持時間によって変化したが、腎臓や心臓では違いが観察されなかった。この違いの原因は明らかではないが、臓器の形や硬さといったマクロな違いや組織内部構造といったミクロな違いがその要因ではないかと考えている。今後、この要因が明らかにすることで吸引圧法のメカニズム解明が進み、疾患治療応用への展開が進むと考えられる。

D-3. 吸引圧法によるオリゴ核酸送達

本研究では、核酸医薬品を用いた腎疾患、心疾患動物の治療を目指したが、腎臓や心臓において核酸医薬品のモデルとして用いたsiRNAにより、遺伝子発現が抑制されなかった。これまでの研究で、肝臓においては外因性、内因性の遺伝子の発現量を、吸引圧法によるsiRNAの送達で達成しており、本研究において、肝臓で効果があったsiRNAと同じ配列のsiRNAを用いたが、標的遺伝子の発現量を抑制することが出来なかった。
本研究では、疾患モデル動物の治療を行うことを優先し、遺伝子治療薬であるプラスミドDNAを用いて疾患モデル動物の治療応用へと研究を展開した。このため、なぜ腎臓、心臓でsiRNAによる遺伝子発現抑制効果が観察されなかったのかは現在のところ不明であり、この点は、今後の検討課題の一つであると考えられる。

D-4. 腎疾患、心疾患治療に向けた今後の課題

本研究では、腎疾患モデル動物としてUUOマウス、UUOラットを作製した。また心疾患モデルマウスとして、心筋梗塞モデルマウスを作製した。これらの疾患モデル動物に対して吸引圧法を行い、プラスミドDNAを送達可能であることを示したことは本研究の大きな成果の一つである。特に腎臓においては、すでに治療用タンパク質としてhBMP7に注目し、これを発現するプラスミドDNAを作製し、健常マウスの腎臓に吸引圧法により導入可能であることを示した。今後、作製したUUOマウス、ラットの病態腎臓に対して吸引圧法を適用し、治療効果を調べる予定である。

E. 結論

本研究では、吸引圧法を用いた腎・心疾患治療の実現を目指して研究を進めた。まず吸引圧制御システムの開発を行い、それを用いて腎臓、心臓に対する吸引圧法の最適化を行った。さらに腎疾患・心疾患モデル動物に対する吸引圧法の適用を行い、吸引圧法による疾患治療実現の可能性を示唆する結果を得るに至った。

F.　研究発表

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Highlighted paper selected by Editor-in-Chief

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4. 著書
なし

G.　知的財産権の出願・登録状況（予定を含む。）

1. 特許取得
METHOD FOR OPERATING A DEVICE FOR DELIVERING A SUBSTANCE TO BE INTRODUCED, AND METHOD FOR DELIVERING A SUBSTANCE TO BE INTRODUCED
Inventor：Kazunori Shimizu, Satoshi Konishi, Shigeru Kawakami, Mitsuru Hashida
PCT/JP2011/062102
WO 2012/056756
米国出願番号：13/881, 304
欧州出願：11835898.5

2. 実用新案登録
なし

3. その他
なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shimizu, K., Kawakami, S., Hayashi, K., Kinoshita, H., Kuwahara, K., Nakao, K., Hashida, M., Konishi, S.	In vivo site-specific transfection of naked plasmid DNA and siRNAs in mice by using a tissue suction device.	PLoS ONE	7(7)	e41319	2012
Shimizu, K., Zhang, G., Kawakami, S., Taniguchi, Y., Hayashi, K., Hashida, M., Konishi, S.	Liver suction-mediated transfection in mice using a pressure-controlled computer system.	Biological Pharmaceutical Bulletin	37(4)	569-575	2014

In vivo Site-Specific Transfection of Naked Plasmid DNA and siRNAs in Mice by Using a Tissue Suction Device

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Abstract

We have developed an *in vivo* transfection method for naked plasmid DNA (pDNA) and siRNA in mice by using a tissue suction device. The target tissue was suctioned by a device made of polydimethylsiloxane (PDMS) following the intravenous injection of naked pDNA or siRNA. Transfection of pDNA encoding luciferase was achieved by the suction of the kidney, liver, spleen, and heart, but not the duodenum, skeletal muscle, or stomach. Luciferase expression was specifically observed at the suctioned region of the tissue, and the highest luciferase expression was detected at the surface of the tissue (0.12±0.03 ng/mg protein in mice liver). Luciferase expression levels in the whole liver increased linearly with an increase in the number of times the liver was suctioned. Transfection of siRNA targeting glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene significantly suppressed the expression of GAPDH mRNA in the liver. Histological analysis shows that severe damage was not observed in the suctioned livers. Since the suction device can be mounted onto the head of the endoscope, this method is a minimally invasive. These results indicate that the *in vivo* transfection method developed in this study will be a viable approach for biological research and therapies using nucleic acids.

Citation: Shimizu K, Kawakami S, Hayashi K, Kinoshita H, Kuwahara K, et al. (2012) *In vivo* Site-Specific Transfection of Naked Plasmid DNA and siRNAs in Mice by Using a Tissue Suction Device. PLoS ONE 7(7): e41319. doi:10.1371/journal.pone.0041319

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Introduction

In the post-genomic era, increased importance has been placed on the development of *in vivo* transfection techniques that can be used for biological research or gene therapy. Many *in vivo* transfection methods have been developed using recombinant viral vectors or non-viral carriers such as cationic liposomes and polymers [1–4]. However, transfection methods for naked nucleic acids, including plasmid DNA (pDNA) or siRNA, have many advantages, including convenient preparation, ease of handling, and lack of toxicity associated with the transfection agents. Therefore, this is largely considered to be the simplest and safest method [5].

Liu *et al.* reported that non-invasive gene delivery to the liver was performed by a mechanical massage around the abdomen after intravenous injection of naked pDNA in mice [6,7]. Inspired by their study, our group found that direct pressure to the kidneys, spleen, and liver induces the transfection of naked nucleic acids, which we termed as tissue pressure-mediated transfection [8–10]. This method has been used with naked pDNA, siRNA, and microRNA [8–11], and the miR-200 family of microRNAs introduced by renal pressure-mediated transfection ameliorated renal tubulointerstitial fibrosis in mice [11]. Further, we previously reported that the secretion of pro-inflammatory cytokines was not observed under the experimental conditions for transfection and the degree of direct pressure applied to the target tissue is one of the key factors for controlling the expression levels of the transfected pDNA [9].

In tissue pressure-mediated transfection, 2 objects were used to apply direct pressure effectively to the target tissue; the first is used to directly press the target tissue, and the other is used to support the pressed tissue. Examples include the index finger and the thumb [8,11], a syringe-modified pressure controlling device and a spatula [9,10], and a pneumatic balloon actuator and a renal case [12]. We have used these objects to perform tissue pressure-mediated transfection in small animals such as mice and rats. However, considering future clinical use, we sought to develop an easier method using a simpler device that required minimally invasive treatment. Previously, we developed a micro-pneumatic suction device for medical diagnosis and operation [13]. The simple suction device was used to fix medical Micro Electro Mechanical Systems (MEMS) such as temperature sensors or micropumps on the surface of pulsating target tissues. The tissue suction device, made of polydimethylsiloxane (PDMS), suctions a tissue surface by applying negative pressure. Although the suctioned part of the tissue was deformed temporarily, *in vivo* experiments revealed that the damage was negligible [13]. Furthermore, it has been demonstrated that the suction device

can be mounted to the head of the endoscope, allowing for increased use in a clinical setting [13].

Our previous results using the pneumatic balloon actuator and the renal case suggested that tissue deformation following the tissue pressure would be a key factor for the transfection efficiency of naked pDNA [12]. This prompted us to investigate whether a tissue suction device could be used for *in vivo* site-specific transfection of naked pDNA or siRNA in mice. In this study, transfection of mouse kidney, liver, spleen, heart, duodenum, skeletal muscle, and stomach were evaluated after tissue suction by a PDMS device following intravenous injection of naked pDNA or siRNA. This is our initial study concerning *in vivo* site-specific transfection using a tissue suction device.

Results

Naked pDNA Transfection by Tissue Suction

To prove our hypothesis, mouse livers were transfected with pCMV-Luc by using the tissue suction devices (Fig. 1). The liver surface of the anesthetized mice was suctioned once immediately after intravenous injection of pCMV-Luc, and *in vivo* imaging of luciferase activity was performed 6 h after the suction. As shown in Fig. 2A, luciferase expression was detected at the region where the liver had been suctioned. *Ex vivo* imaging of the suctioned liver clearly shows that the luciferase was expressed at the site of tissue suction (Fig. 2B and 2C). The tissue suction device was also used to transfect pDNA into mouse kidneys. The right kidney of the anesthetized mouse was suctioned by the suction device just after pCMV-Luc injection, and the luciferase levels in various tissues were investigated. As shown in Fig. 2D, luciferase expression was specifically found in the suctioned right kidney. Therefore, as we expected, the transfection of naked pDNA was possible by using the tissue suction device.

Investigation of Applicable Tissues of pDNA Transfection by Tissue Suction

Next, we investigated the extended application of the naked pDNA transfection by tissue suction. Luciferase activity of the tissues, including the kidney, liver, heart, spleen, duodenum, muscle, and stomach, were examined 6 h after the mice had received an intravenous injection of pCMV-Luc, immediately followed by suction of each tissue. As shown in Fig. 3, high luciferase gene expression was obtained in the right kidney, heart, spleen, and liver. The expression levels were approximately 0.014 ng/mg protein for the right kidney, 0.006 ng/mg protein for the heart, 0.002 ng/mg protein for the spleen, and 0.001 ng/mg protein for the liver. In contrast, the luciferase levels of the duodenum, muscle, and stomach were less than 2×10^{-5} ng/mg protein.

Distribution of Transgene Activities

The distribution of luciferase gene expression around the suctioned region of the liver was investigated. The liver surface of the anesthetized mice was suctioned once just after intravenous injection of pCMV-Luc. Six hours after suction, the tissue was separated into 4 parts as shown in Fig. 4A, and the luciferase level of each part was independently examined (Fig. 4A). The highest luciferase gene expression was obtained from Part I, and this level was set at 100% (Fig. 4B). In contrast, the relative luciferase expression levels were approximately 3.2% for Part II, 3.4% for part III, and 1.5% for Part IV. Subsequently, the luciferase expression induced by tissue suction was compared to that of other methodologies such as the hydrodynamic method and the liver pressure-mediated transfection (Fig. 4C). For transfection by tissue suction, the luciferase level at Part I was determined to be 0.12±0.03 ng/mg protein. For the hydrodynamic method, the luciferase level reached a higher level of 6.29±5.98 ng/mg protein. For the liver pressure-mediated transfection method, a luciferase level of 0.14±0.12 ng/mg protein was obtained at the treated part, and this was not significantly different from the results obtained by tissue suction. Furthermore, the distribution of the liver cells transfected by tissue suction was investigated at the cellular level using pCMV-green fluorescent protein (GFP). A large number of GFP-expressing cells were observed at the top surface of Part I (Fig. 4D).

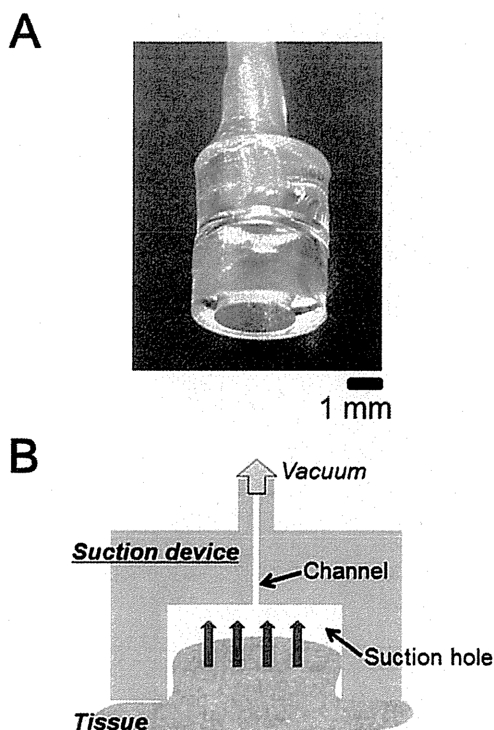

Figure 1. Tissue suction by using the device. A) Representative photograph of the tissue suction device (type II). B) Schematic illustration of *in vivo* transfection by tissue suction. The surface of the target tissues was suctioned by the suction device just after intravenous injection of naked nucleic acids.
doi:10.1371/journal.pone.0041319.g001

Effects of the Number of Tissue Suctions on Luciferase Expression

We investigated the effects of the number of tissue suctions on pDNA expression. First, 2 different parts of the tissue were simultaneously suctioned by using 2 tissue suction devices just after intravenous injection of pCMV-Luc. *In vivo* imaging of luciferase activity was then performed 6 h after the suction. As shown in Fig. 5A and 5B, luciferase was expressed at the 2 different regions where the liver was simultaneously suctioned. Next, a different part of the tissue was serially suctioned. Since luciferase expression levels did not decrease if the liver was suctioned after 180 s following pCMV-Luc injection (Fig. 5C), the liver surface was suctioned 1, 3, or 7 times within 180 s. As shown in Fig. 5D, the luciferase level in the whole liver increased linearly ($R^2 = 0.9414$), with an increase in the suction number and reached approximately 0.02 ng/mg protein.

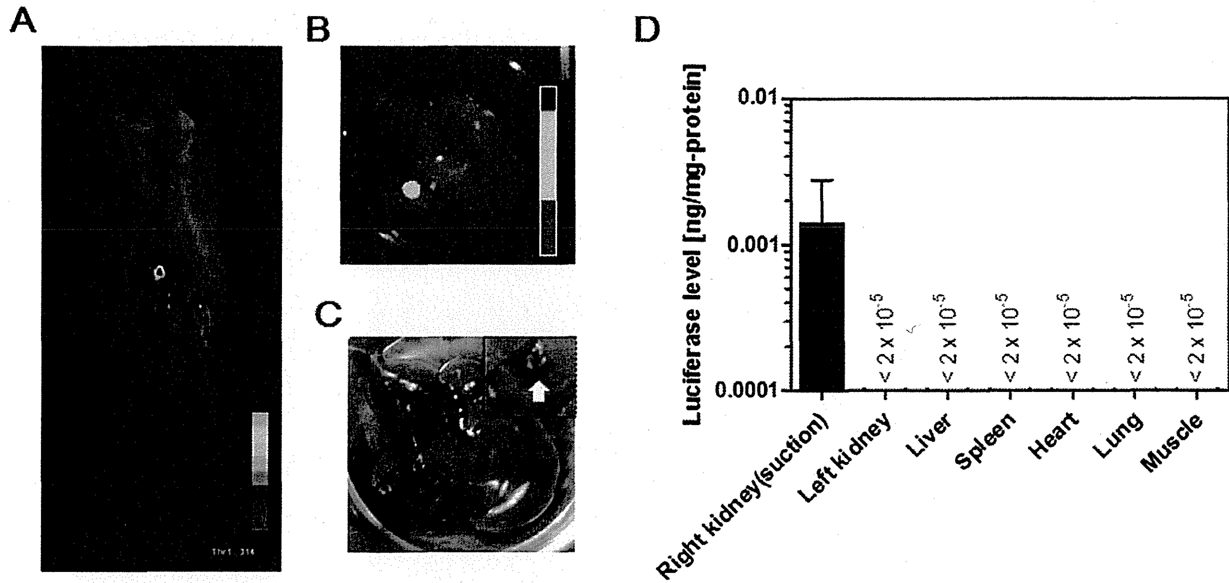

Figure 2. *In vivo* transfection of naked pDNA by tissue suction. A) *In vivo* imaging of luciferase activity in a mouse liver that was suctioned once by the type I device just after intravenous injection of pCMV-Luc. B) *Ex vivo* imaging of luciferase activity in the liver suctioned by the type I device. C) Bright field image of (B). D) Luciferase levels of various tissues. The right kidney in mice was suctioned once by the type III device. Each value represents means + SD (n =4). All mice were alive at the end of the experiment.
doi:10.1371/journal.pone.0041319.g002

siRNA Transfection of the Liver by Tissue Suction

To evaluate the potential for naked oligonucleotide delivery of the transfection by tissue suction, we demonstrated silencing of an endogenous gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [14,15]. Transfection of GAPDH siRNA was performed on mouse livers by using the tissue suction device. The liver surface of the anesthetized mice was suctioned once just after the administration of GAPDH siRNA, and the GAPDH mRNA expression of suctioned part of the liver (Part I in Fig. 4A) was investigated 24 h after suction. As shown in Fig. 6, the GAPDH mRNA expression in the liver was significantly reduced, with about 61% suppression. In contrast, when the scramble siRNA were administered, no marked suppression of GAPDH mRNA level was observed (Fig. 6).

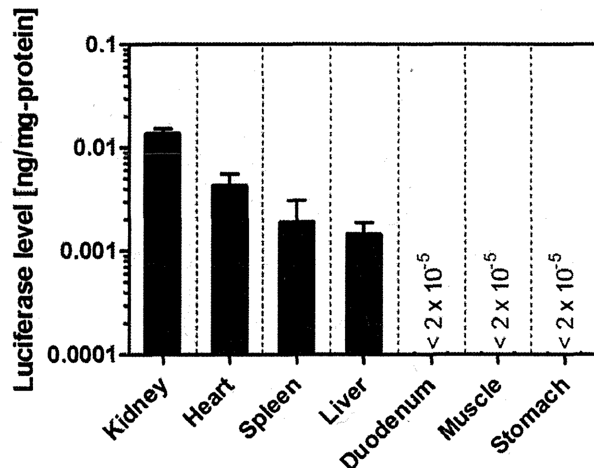

Figure 3. *In vivo* transfection to various tissues by tissue suction. *In vivo* transfection by tissue suction was applied to various tissues (including the kidney, heart, spleen, liver, duodenum, muscle, and stomach). A type III device was used for the muscle and stomach. A type IV device was used for the kidney, heart, spleen, liver, and duodenum. Each value represents means + SD (n =3 [the kidney, spleen, and muscle], n =4 [the liver, duodenum, and stomach], or n =5 [the heart]). All mice were alive at the end of the experiment.
doi:10.1371/journal.pone.0041319.g003

Effects of Tissue Suction on Hepatic Toxicity

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the serum were examined to determine the occurrence of liver toxicity after treatment with the tissue suction device (Fig. 7A and 7B). pCMV-Luc was intravenously injected, and the liver surface was suctioned once by the device. After 0, 6, 24, and 48 h, ALT and AST activities in the serum were measured. ALT activity at 6 and 24 h and AST activity at 6 h in the mice treated with the tissue suction device were significantly higher than those in mice with the sham operation ($p<0.05$); however, the activities returned to normal levels within 48 h (Fig. 7A and 7B). We also performed HE staining of the liver sections to examine damage that may be caused by liver suction. However, we did not observe any severe damage to the suctioned livers following transfection (Fig. 7C).

Discussion

In the present study, it was shown that *in vivo* transfection of naked nucleic acids such as pDNA and siRNA can be achieved by tissue suction. Previously, positive pressure had been used to directly press the target tissue to induce transfection [8–12]. The present study demonstrates the first use of negative pressure to induce transfection. The negative pressure supplied by the tissue suction device deformed the target tissue and induced transfection. Transfection by negative pressure has 2 main advantages over

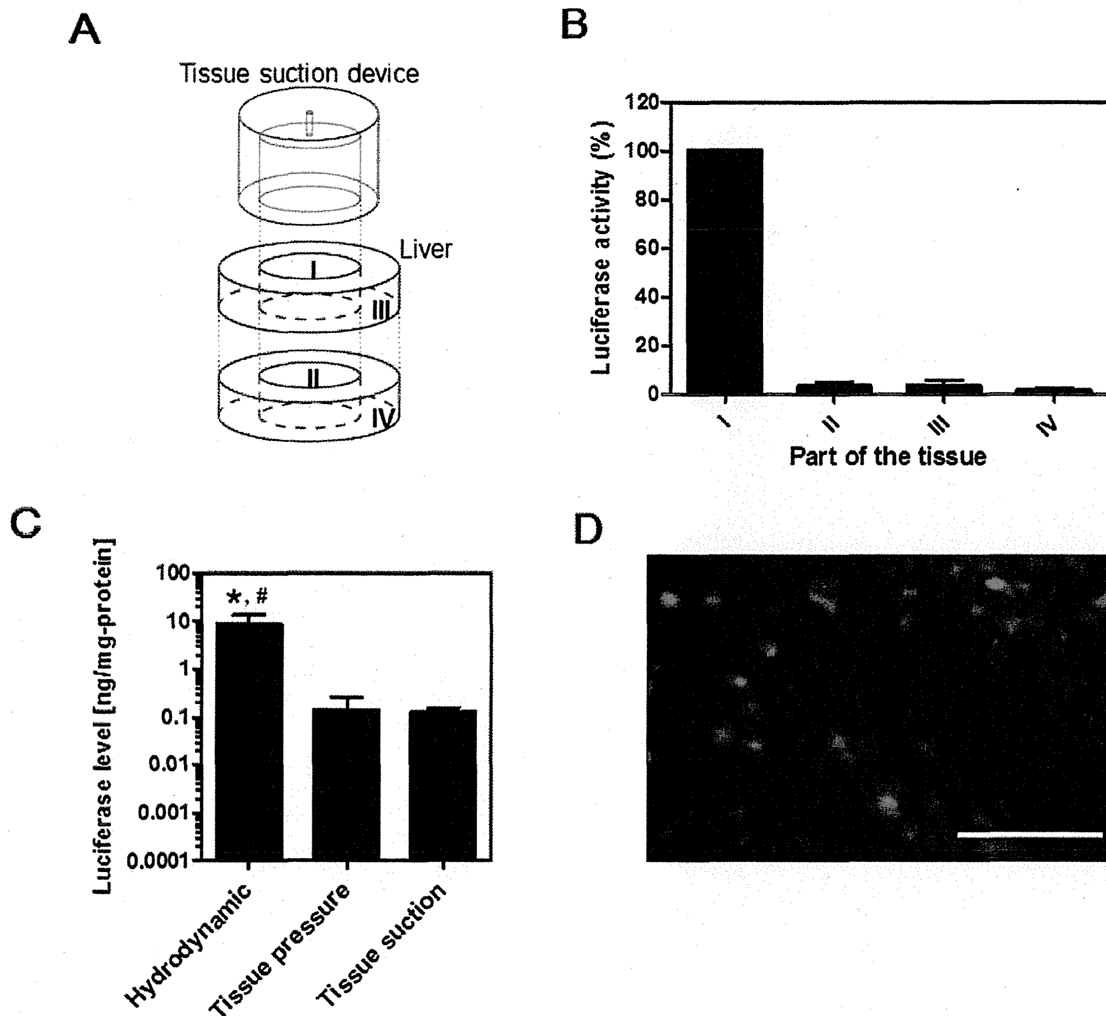

Figure 4. Luciferase gene expression around the suctioned region of the liver. A) Schematic illustration of the experiment. The liver was suctioned by the type III device, and the liver was cut into 4 parts (I to IV). B) Luciferase levels in each of the 4 parts. The values were normalized to levels from Part I (n = 4). C) The luciferase level of *in vivo* transfection by tissue suction was compared with that by hydrodynamic method and tissue pressure-mediated transfection. *$p<0.01$ versus tissue pressure. #$p<0.01$ versus tissue suction. Each value represents means + SD (n = 3 [hydrodynamic method], n = 8 [tissue pressure], or n = 5 [tissue suction]). D) Imaging of the top surface of Part I transfected with pCMV-GFP. Scale bar, 200 µm. All mice were alive at the end of the experiment.
doi:10.1371/journal.pone.0041319.g004

positive pressure: (i) it is easier to fix the relative position between the target tissue and the device with negative pressure [13,16] and (ii) the tissue suction devices are simpler than the combinations of 2 objects used to press tissues in the positive pressure studies [8–12]. Therefore, our findings suggest that it is now possible to perform tissue pressure-mediated *in vivo* transfection with greater ease than previously reported. Moreover, since the tissue suction devices are small enough to be mounted to the head of the endoscope, it is possible to perform transfections less invasively than before. Thus, we have successfully innovated a useful procedure for tissue pressure-mediated transfection employing tissue suction.

We made the tissue suction devices in several sizes (Table 1) and suctioned the tissue surface until the inner space of the suction devices was filled with deformed tissue. In this study, we selected the device type according to tissue size or surgical procedure. For the smaller tissues including the spleen and duodenum, we used the device with the smaller inner diameter (type IV). The type IV device was also used to suction the heart due to limited space between the ribs. We could use all the types of suction devices listed in Table 1 for larger tissues including the liver and found that they could induce pDNA transfection. However, our preliminary data showed that device inner diameter and height affected transfection efficiency (Fig. S1). Furthermore, hemorrhage was occasionally observed around the suctioned parts of the tissue (around part III in Fig. 4A) when the target tissue was excessively deformed by high negative pressure suction. Thus, further studies should be conducted to optimize the size of the tissue suction device and the amount of negative pressure applied to the target tissues. Data from such experiments could allow researchers 'to effectively perform the tissue suction method without causing toxicity or damage to the target tissues.

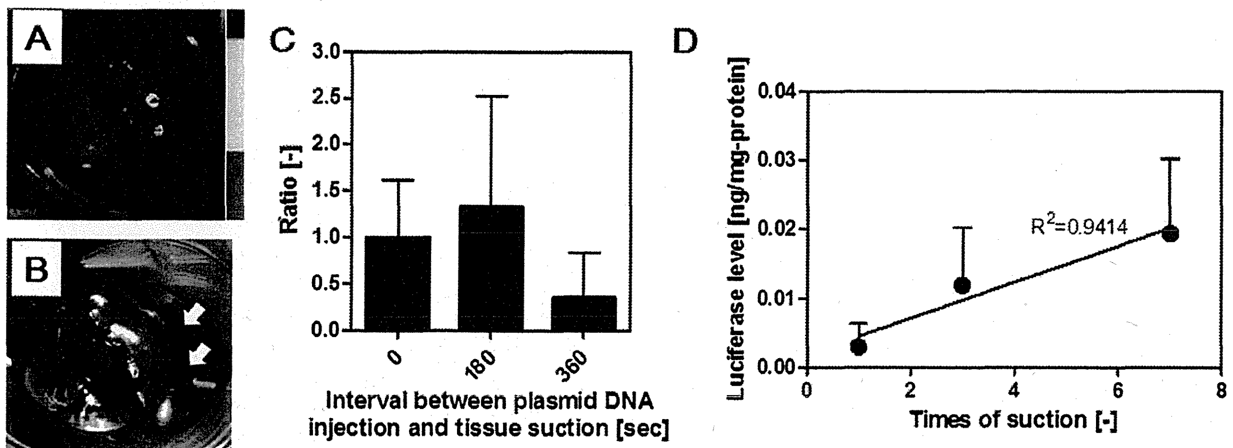

Figure 5. Effects of the number of tissue suctions on luciferase levels. A) *Ex vivo* imaging of luciferase activity in the liver simultaneously suctioned at 2 different parts. Two type III devices were used. B) Bright field image of (A). C) Effects of interval between pCMV-Luc injection and tissue suction on the luciferase levels in liver. The liver was suctioned by type III devices. D) Effects of the number of tissue suctions on luciferase levels were investigated. Liver were serially suctioned 1, 3, and 7 times by using the type III device within 180 s of pCMV-Luc injection. All mice were alive at the end of the experiment.
doi:10.1371/journal.pone.0041319.g005

In vivo transfection by tissue suction achieved site-specific gene transfection in the applied organs (Figs. 2, 4, and 5). In contrast, conventional methods, including recombinant viral vectors and non-viral carriers, have difficulty in selectively inducing transfection within specific target tissues. However, selective hydrodynamic gene delivery of naked pDNA to the liver segments by using balloon catheters was recently performed [17,18]. The catheter-based method is a promising technique for clinical use, but requires specialized skills and may be difficult to apply to relatively small animals. In contrast, transfection by tissue suction is easy to perform and can be applied to both large and small animals. Transfection can also be induced at a specific site of the tissue; therefore, it represents a unique technology for performing selective *in vivo* transfection.

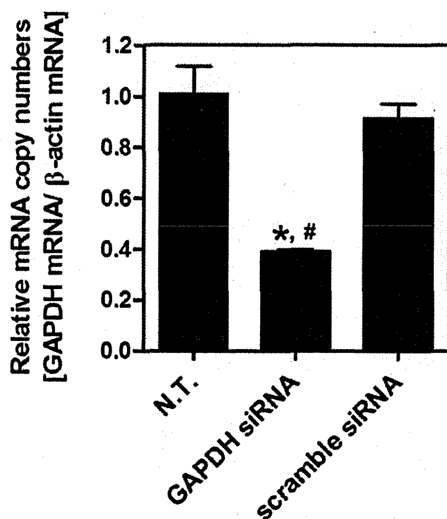

Figure 6. siRNA transfection to the liver by tissue suction. GAPDH siRNA and scramble siRNA were transfected by using type II device. mRNA expression of GAPDH after 24 h of transfection was measured. Each value represents means + SD (n = 3 [N.T.], n = 4 [GAPDH siRNA and scramble siRNA]). There was a statistically significant difference between 3 groups (ANOVA; F = 99.72, $p<0.0001$). Post-hoc analysis (Bonferroni's test) was performed. *$p<0.001$ versus N.T. #$p<0.001$ versus scramble siRNA. All mice were alive at the end of the experiment.
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Transfection by tissue suction can control the amount of transgene expressed in the target tissue (Fig. 5); our experiments demonstrated that expression levels of the transfected gene in the whole liver increased linearly with an increase in the number of liver suctions (Fig. 5D). Therefore, it is expected that the concentration of the expressed protein in the serum can also be controlled by using the pDNA that encodes secretory proteins. We believe that this ability to control is especially valuable when transfecting genes that may have toxicity at high concentrations.

On the other hand, it is one of the strategies that uses pDNA to encode non-secretory protein or the oligonucleotides for silencing gene expression. For this strategy, it is important to consider the number of transfected cells within the suctioned part of the tissue. In the present study, naked pCMV-GFP was transfected to the liver via tissue suction, and a large number of GFP-expressing cells were observed at the top surface of the suctioned part of the liver (Fig. 4D). Moreover, silencing of the expression of an endogenous gene, GAPDH, within the suctioned part of the liver was demonstrated by transfection of GAPDH siRNA (Fig. 6). The earlier study showed that the renal pressure-mediated transfection method could transfect the microRNA precursor into many tubular cells in the kidney and ameliorate renal tubulointerstitial fibrosis [11]. Thus, it is expected that transfection by tissue suction is applicable for expressing non-secretory protein using pDNA or silencing gene expression using siRNA or microRNA. Nonetheless, a detailed consideration should be performed for further applications of this method.

We found that transfection by tissue suction was effective in the kidney, liver, spleen, and heart, although not the duodenum, muscle, or stomach (Fig. 3). In the previous tissue pressure-mediated transfection study, however, it was reported that the transfection of naked pDNA was possible in the kidney, liver, and spleen, but not the heart [9]. This may be attributed to the fact

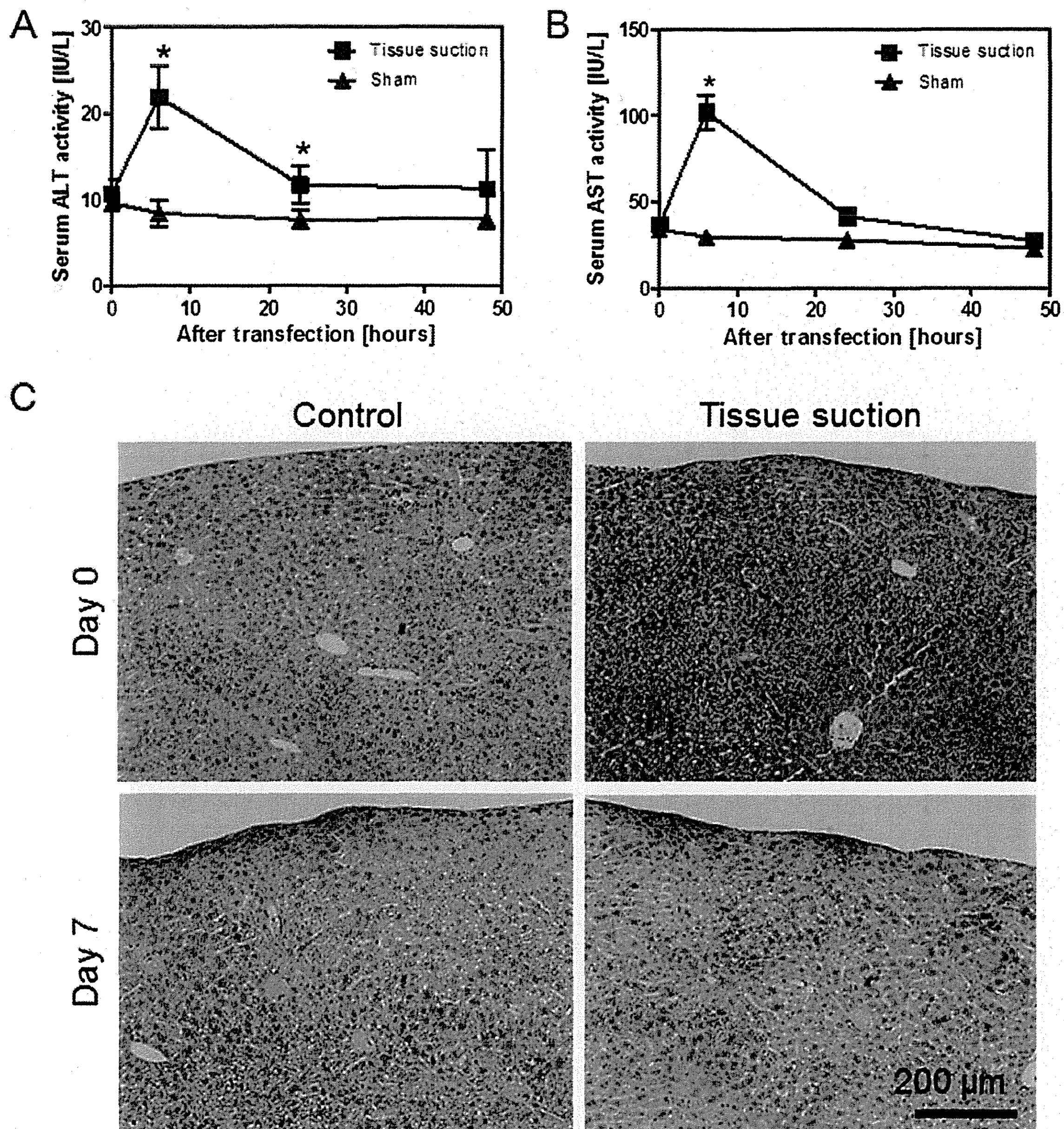

Figure 7. Effects of tissue suction on hepatic toxicity. A) Alanine aminotransferase (ALT) in serum. ALT activity was measured at 0, 6, 24, and 48 h after transfection. Type III device was used. Each value represents mean ± SD (n = 3 [sham operation], or n = 4 [tissue suction]). *p<0.05 versus sham operation. B) Aspartate aminotransferase (AST) in serum. AST activity was measured at 0, 6, 24, and 48 h after transfection. Type III device was used. Each value represents mean ± SD (n = 3 [sham operation], or n = 4 [tissue suction]). *p<0.05 versus sham operation. C) HE staining of the liver section. The suctioned part of the liver (Part I in Fig. 4A) was sampled at 0 and 7 days after tissue suction. Type III device was used. All mice were alive at the end of the experiment.
doi:10.1371/journal.pone.0041319.g007

that the heart was not directly pressed for heart pressure-mediated transfection; the abdomen and chest of the mice were held between the thumb and the index and middle fingers of both hands, and pressed without exposure of the tissues [9]. In the present study, the heart was suctioned directly by using the tissue suction device and we found that naked pDNA transfection could be achieved in the heart. This is the first study that demonstrates *in vivo* transfection by tissue suction in the heart. These findings indicate that heart gene therapy is a promising application of transfection by tissue suction. So far, several methods that can induce transfection of naked pDNA into cardiac tissue have been reported. Direct myocardial injection of naked pDNA has been used for gene therapy for heart failure [19,20]; retrograde injection of naked pDNA in the coronary sinus by catheterization was used for whole cardiac gene transfer [21], and *in vivo* electroporation was conducted on rat and porcine hearts [22,23]. As shown in Fig. 4C, the transfection efficiency by tissue suction in the liver was equal to that of the liver pressure-mediated transfection method.

Table 1. Tissue suction devices used in this study.

Type	Inner diameter [mm]	Outer diameter [mm]	Height [mm]	Applied tissues
I	4	8	3	Liver
II	4	6	3	Liver, kidney
III	3	5	3	Liver, kidney, muscle, stomach
IV	2	3	3	Liver, kidney, heart, spleen, duodenum

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We previously compared the efficiency of the kidney pressure-mediated transfection method with that of other transfection methods–renal parenchymal injection and a combination method with electroporation in the kidney–and reported almost equal efficiency [8]. Considering these results, we expected both the heart suction method and the other methods to be simple and promising techniques for heart gene therapy [19–23].

For potential clinical use, it is important to consider the toxicity of transfection by tissue suction on the treated tissues. In the present study, we performed both biochemical and histological assays to assess the toxicity of transfection by tissue suction of the liver (Fig. 7). The ALT and AST activities were transiently increased and returned to normal levels within a few days (Fig. 7A and 7B), which is in good agreement with the time-course profiles of ALT and AST activities of the hydrodynamic method [24,25]. It was also reported previously that renal pressure-mediated transfection did not induce pro-inflammatory cytokines in the serum and did not affect renal function [8,9]. Considering these results, we hypothesized that tissue pressure-mediated transfection, regardless of suction or pressure, is generally safe *in vivo*. Nevertheless, further investigation about the safety of tissue suction methods is warranted.

In conclusion, the present study investigated the feasibility of an *in vivo* transfection method by using a tissue suction device for the transfection of naked pDNA or siRNA in mice, and demonstrated that this method would be an effective approach in biological research and therapies using nucleic acids.

Materials and Methods

pDNA, siRNAs, and Mice

pDNA-encoding complementary luciferase DNA (pCMV-Luc) [26] and emerald GFP (pCMV-GFP; pcDNA6.2-EmGFP, Life Technologies, Carlsbad, CA) were used. They were driven by the CMV immediate-early promoter. The amplification, isolation, and purification of pDNA were performed as described previously [26]. siRNAs (21 mer) with 3′-dTdT overhangs were chemically synthesized by SIGMA Aldrich Japan (Hokkaido, Japan), and siRNA sequences are shown as follows: mouse GAPDH siRNA, 5′-CAA GAG AGG CCC UAU CCC AdTdT-3′ (sense) and 5′-UGG GAU AGG GCC UCU CUU GdTdT-3′ (antisense); scrambled siRNA, 5′-CGC AAC UAC CGA UGC GAA CdTdT-3′ (sense) and 5′-GUU CGC AUC GGU AGU UGC GdTdT-3′ (antisense). ICR mice (female, 5 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the U.S. National Institutes of Health (Bethesda, MD) and the Guidelines for Animal Experiments of Kyoto University (Kyoto, Japan). The study protocols permission numbers 2010–47, 2011–39, and 2012–50 were approved by the Animal Research Committee, Kyoto University.

Fabrication of Tissue Suction Devices

A tissue suction device was fabricated as described previously, with some modifications [13]. Briefly, a polydimethylsiloxane (PDMS) (10:1) solution was poured into a plastic dish and cured at 75°C for 2 h. The PDMS slab was punched out using a disposable biopsy punch (Kai Industries Co., Ltd., Gifu, Japan), and ring-shaped PDMS structures were fabricated. Then, the ring-shaped structures were bonded to disc-shaped PDMS structure with the same diameter, and the disc-shaped part was punched out to connect a silicone tube with an outer diameter of 1 mm used for supplying negative pressure. Various suction device specifications such as size and shape can be designed depending on the requirements [13]. Four sizes of the device structure were used in the present study (Fig. 1 and Table 1).

pDNA and siRNA Transfection Using Tissue Suction Devices

In a typical case of pDNA transfection using tissue suction devices, the mice were anesthetized with isoflurane, and the target tissue was minimally exposed. pCMV-Luc (100 μg in 200 μL of saline) was intravenously injected into the mice, and the target tissue was suctioned by the device with small amounts of negative pressure (Fig. 1). The tissue was suctioned until the inner space of the device was filled with the deformed tissue. In case of the kidney, liver, spleen, stomach, and duodenum, the target tissue was slightly exposed by a midline incision, and the target part of the tissue was suctioned by the device. In case of the heart, anesthetized mice were ventilated by a respirator (SN-480-7; Shinano, Tokyo, Japan) during the treatment. The left costal cartilage (the fourth rib) was removed to minimally expose the left ventricle, and the ventricle was suctioned by the device. In case of muscle, the dermis of the left hind leg was cut to expose the femoral muscle, and the muscle was suctioned by the device.

In siRNA transfection experiments, anesthetized mice were intravenously injected with GAPDH siRNA or scramble siRNA (50 μg in 200 μL saline), immediately followed by small negative pressure-mediated suctioning of the liver by the suction device.

pDNA Transfection by the Hydrodynamic Method

The hydrodynamic method was performed as described previously [24]. Five micrograms of pCMV-Luc in 1600 μL of saline was intravenously injected within 5 s.

pDNA Transfection by the Liver Pressure-mediated Transfection Method

The liver pressure-mediated transfection method was performed as previously described [9]. The applied pressure was controlled at around 0.5 N/cm^2 by using the pressure control devices, namely, a syringe-like device and a spatula.

Luciferase activity Assay and Imaging

Gene expression levels were determined by luciferase assay after 6 h of tissue suction as described previously [26]. Imaging of luciferase activity was performed following a previously described method [8].

Imaging of the GFP-expressing Cells

pCMV-GFP (500 μg in 200 μL of saline) was intravenously injected into the mice and the livers were suctioned using the type III device. After 6 hours, the mice were sacrificed and the surfaces of the target liver parts were observed under an inverted fluorescence microscope (BZ-8100, Keyence, Osaka, Japan).

Quantitative Real-time RT-PCR

Total RNA was isolated from the cells and organs using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Reverse transcription of mRNA was carried out using PrimeScript® RT reagent Kit (Takara Bio, Shiga, Japan). The detection of complementary DNA (GAPDH and β-actin) was conducted using real-time PCR using SYBR® Premix Ex Taq (Takara Bio) and a Lightcycler Quick System 350S (Roche Diagnostics, Indianapolis, IN). Primers for GAPDH and β-actin cDNA were synthesized by Invitrogen as follows: GAPDH, 5′-CTC ACT CAA GAT TGT CAG CAA TG-3′ (forward) and 5′-GGC AGT GAT GGC ATG GAC TGT-3′ (reverse); β-actin, 5′-GTT CTA CAA ATG TGG CTG AGG ACT T-3′ (forward) and 5′-TTG GGA GGG TGA GGG ACT T-3′ (reverse). mRNA copy number was calculated for each sample from the standard curve using the thermal-cycler software ('Arithmetic Fit Point analysis' for the Lightcycler). Results were expressed as relative copy number calculated relative to β-actin mRNA (GAPDH mRNA copy number/β-actin mRNA copy number).

Measurement of the Transaminase Activity in Serum

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the serum were determined as described previously [27]. Briefly, the serum was collected from the transfected mice 0, 6, 24 and 48 h after transfection. Transaminase CII-Test Wako kit (Wako Pure Chemical Industries, Tokyo, Japan) was used according to the manufacturer's instructions.

Hematoxylin and Eosin Staining

The mice livers were harvested at 0 and 7 days after the suction and fixed in 10% neutral buffered formalin. Four-micrometer thick paraffin sections were stained with hematoxylin and eosin (HE). The histology of the liver sections was microscopically examined.

Statistical Analysis

Prism 5 software (Graphpad Software, La Jolla, CA, USA) was used. Statistical significance was determined by two-tailed t-test or one-way analysis of variance (ANOVA), followed by Bonferroni's test.

Supporting Information

Figure S1 Effects of the size of the tissue suction devices on luciferase levels. *In vivo* transfection by tissue suction was applied to the right kidney by using type II and IV device. *$p<0.05$ versus type IV device (n = 6 [type II], n = 3 [type IV]). All mice were alive at the end of the experiment.
(TIF)

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Author Contributions

Conceived and designed the experiments: Kazunori Shimizu, Shigeru Kawakami, Kouji Hayashi, Hideyuki Kinoshita, Koichiro Kuwahara, Kazuwa Nakao, Mitsuru Hashida, Satoshi Konishi. Performed the experiments: Kazunori Shimizu, Shigeru Kawakami, Kouji Hayashi, Hideyuki Kinoshita. Analyzed the data: Kazunori Shimizu, Shigeru Kawakami, Kouji Hayashi. Contributed reagents/materials/analysis tools: Wrote the manuscript: Kazunori Shimizu, Shigeru Kawakami, Kouji Hayashi.

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Highlighted Paper selected by Editor-in-Chief

Liver Suction-Mediated Transfection in Mice Using a Pressure-Controlled Computer System

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We previously developed an *in vivo* tissue suction-mediated transfection method (denoted as the tissue suction method) for naked nucleic acids, such as plasmid DNA (pDNA) and small interfering RNA (siRNA), in mice. However, it remains unclear whether the suction pressure conditions affect the results of this method. Therefore, in the present study, we assembled a computer system to control the suction pressure and investigate the effects of the suction pressure conditions on the efficiency of the liver suction transfection of naked pDNA that encodes luciferase in mice. Using the developed system, we examined the effects of the minimum magnitude of the suction pressure, suction pressure waveform, and suction times of the luciferase expression level in mice livers. We determined that the liver suction method at −5 kPa was not only effective but also caused the lowest hepatic toxicity in mice. Additionally, the results indicated that the suction pressure waveform affects the luciferase expression levels, and a single period of suction on the targeted portion of the liver is sufficient for transfection. Thus, the developed system is useful for performing the tissue suction method with high accuracy and safety.

Key words gene delivery; mechanical stimulus; computer system; naked DNA; tissue suction

It is important to develop *in vivo* transfection methods that can be used for biological research and therapies that use nucleic acids. *In vivo* transfection methods using recombinant viral vectors or nonviral drug delivery system (DDS) carriers, such as cationic polymers and liposomes, have been investigated widely[1–4]; however, the methods that deliver naked nucleic acids using physical stimuli are considered the most straightforward because of low toxicities associated with the transfection agents, convenient preparation, ease of handling, and so on.[5–7]

We have reported a tissue pressure-mediated transfection method (denoted as the tissue pressure method) in which a direct pressure to the kidneys, spleen, and liver can induce efficient gene transfection using naked nucleic acids into each organ.[8–10] In this method, the target organs of the mice are pressed directly after intravenous injection (i.v.) of the naked nucleic acids. Previous studies demonstrated that the degree of pressure could be used to control the expression levels of the transfected plasmid DNA (pDNA).[9–11] Regarding kidneys in mice, renal dysfunction[8] and secretion of proinflammatory cytokines[9] were not observed under the experimental conditions of the tissue pressure method. Recently, Oba *et al.* transfected the miR-200 family of microRNA to mice kidneys *via* the tissue pressure method and reported that that transfection ameliorated renal tubulointerstitial fibrosis in the mice.[12] Pons *et al.* used the tissue pressure method to transfect pDNA encoding heat shock protein 70 (HSP70) to rat kidneys and reported that the immune reactivity to HSP70 expressed in the kidney is cause of salt-sensitive hypertension.[13] Therefore, the tissue pressure method is beneficial for biological research using small laboratory animals, such as mice and rats. However, the method must be improved for application in humans in the future.

Considering the potential clinical uses, we have developed an *in vivo* tissue suction-mediated transfection method (denoted as the tissue suction method).[14] Its procedure is different from the tissue pressure method: the target site of the tissue is supplied with a suction pressure from tissue suction microdevices after the i.v. administration of naked nucleic acids in mice. Because the microdevices can be mounted at the head of an endoscope,[15] this method can be performed in a minimally invasive manner by exploiting endoscopic surgery. Transfection was achieved by the suction of the mouse kidney, liver, spleen, and heart; severe toxicity and tissue damage were not observed in the mouse liver suctioned with the appropriate methodology.[14]

In the previous study, we performed the tissue suction method with uncontrolled suction pressure,[14] that is, we supplied a negative pressure with the tissue suction device manually by using a syringe until the inner space of the device was filled with the deformed tissue. It remains unclear whether the suction pressure conditions affect the results of the tissue suction method. To perform the tissue suction method safely and accurately at research facilities and medical institutions, the suction method must be performed with a controlled suction pressure. Therefore, in the present study, we assembled a computer system to control the suction pressure and investigate

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the effects of the suction pressure conditions on the efficiency of the liver suction transfection of naked pDNA in mice.

MATERIALS AND METHODS

Fabrication of Tissue Suction Devices The tissue suction devices were manufactured *via* a polydimethylsiloxane (PDMS) replica molding process. The molds were fabricated with a 3D printing system (Objet Geometries Ltd., Rehovot, Israel) and coated with Parylene C (Specialty Coating Systems, Inc., Indianapolis, IN, U.S.A.) as described in a previous report.[16] The precured PDMS (20 : 1) was cured at 75°C for 12 h in the molds and the cured PDMS was peeled off and cut into individual devices. Then, an individual device was punched out using a disposable biopsy punch (Kai Industries Co., Ltd., Gifu, Japan), and a silicone tube with an outer diameter of 2 mm was connected to the device. The tube was used to supply the negative pressure. Unless mentioned otherwise, the device sizes had an inner diameter of 3 mm, an outer diameter of 5 mm, and a height of 3 mm (Fig. 1A).

Suction Pressure Control Computer System Figure 1B shows the block diagram of the suction pressure control system. The negative pressure was generated by a vacuum pump. An electropneumatic regulator (ITV0090; SMC Corp., Tokyo, Japan) was controlled by a PC with specially designed LabVIEW software (National Instrument, Austin, TX, U.S.A.). The LabVIEW software was developed to record the actual suction pressure monitored by the pressure sensor (Sensez Corp., Tokyo, Japan). The suction pressure waveform was defined as a trapezoidal pulse, as shown in Fig. 1C.

pDNA and Mice The pDNA was amplified, isolated, and purified as described in a previous study.[17] pDNA encoding complementary luciferase DNA that was driven by a cytomegalovirus (CMV) immediate-early promoter was employed (pCMV-Luc).[17] Five-week-old ICR mice (female) were purchased from Japan SLC Inc. (Shizuoka, Japan). All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted and promulgated by the U.S. National Institute of Health (Bethesda, MD, U.S.A.). The study protocol permission numbers 2012-50 were approved by the Animal Research Committee, Kyoto University, Japan.

***In Vivo* Transfection by Tissue Suction** The mice were anesthetized with isoflurane, ensuring minimal exposure of the liver. Thereafter, 100 μg of pCMV-Luc dissolved in 200 μL of saline was injected intravenously into the mice. The targeted portion of the left liver lobe was suctioned using the device with a negative pressure, which was controlled by the suction pressure control system. Six hours after tissue suction, the left lobe was harvested and the gene expression levels were determined by the luciferase assay, as previously described.[10,14,17]

Transaminase Activity in Serum Serum was collected from the mice after 6, 24, and 48 h of the liver suction. The alanine transaminase (ALT) and aspartate transaminase (AST) activities in the serum were measured, as described previously.[18]

Statistical Analysis Prism 5 software (Graphpad Software, La Jolla, CA, U.S.A.) was used for statistical analysis.

RESULTS AND DISCUSSION

Development of Suction Pressure Control Computer System To perform the tissue suction method under controlled suction pressure conditions, we set up a suction pressure control computer system (Fig. 1B). The minimum magnitude of the suction pressure (a in Fig. 1C), pressure supply time (b), pressure hold time (c), and pressure release time (d) were input to an original LabVIEW program to define the suction pressure waveform. Because the controlled suction pressure is generated as defined in the program by turning on a foot switch, the operators can use both hands to perform the tissue suction method. The actual suction pressure waveform supplied to the tissue is monitored by a pressure sensor.

Figure 2 shows the results of the operation conditions. First, we investigated the controllability of the minimum magnitude of the suction pressure, which was set to −1, −3, −5, −15, and −30 kPa. The pressure supply time, pressure hold time, and pressure release time were set to 1, 3, and 1 s, respectively (1-3-1). As shown in Fig. 2A, the minimum magnitude of the actual suction pressure, which was monitored by the sen-

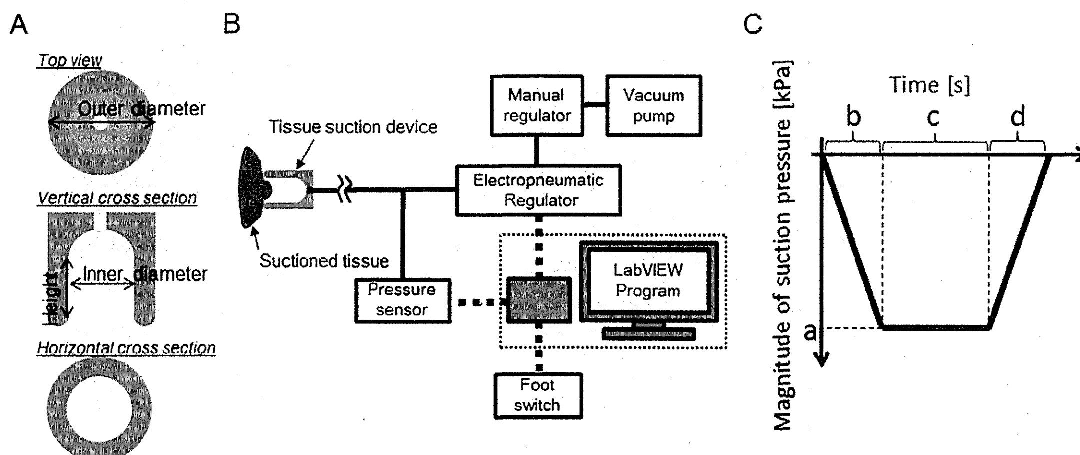

Fig. 1. The Tissue Suction Method Using the Suction Pressure Control System

(A) Design of the tissue suction device. The device sizes had an inner diameter of 3 mm, an outer diameter of 5 mm, and a height of 3 mm. (B) Configuration of the suction pressure control computer system. (C) Suction pressure waveform: a, minimum magnitude of the suction pressure; b, pressure supply time; c, pressure hold time; d, pressure release time.

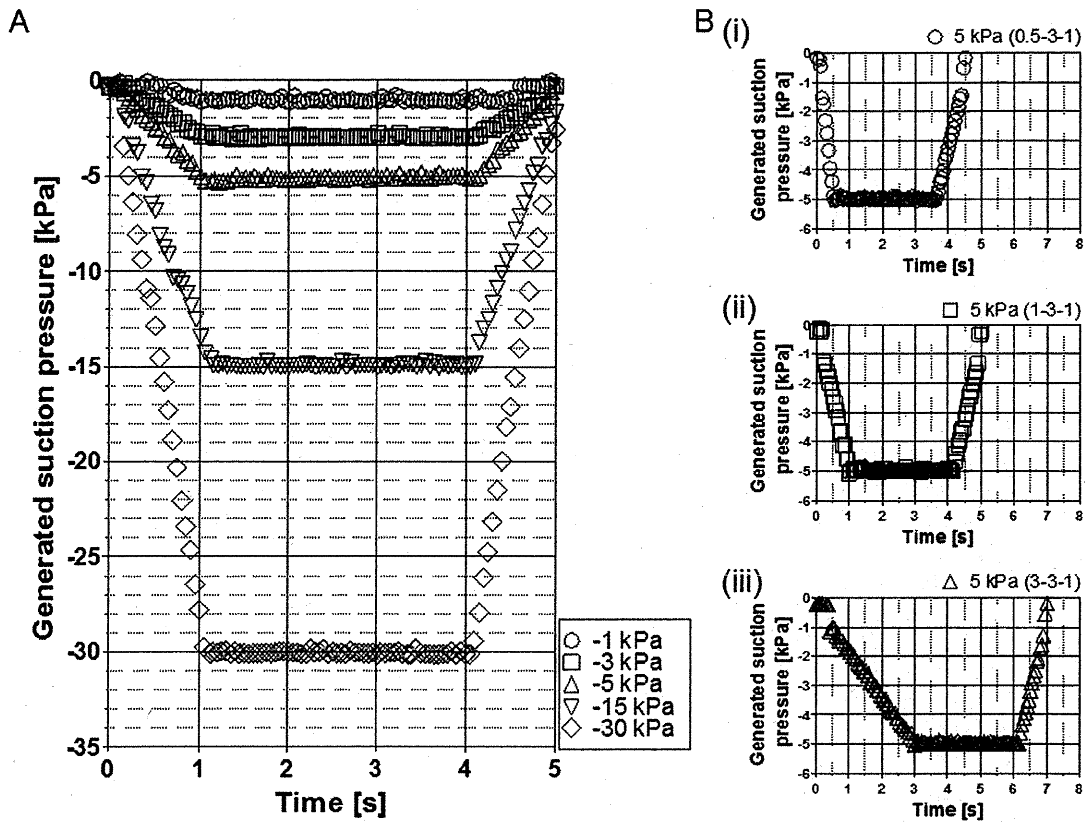

Fig. 2. Operation Conditions of the Developed System

(A) Minimum magnitudes of the suction pressure of −1, −3, −5, −15, and −30 kPa. (B) Pressure supply times of (i) 0.5 s, (ii) 1 s, and (iii) 3 s.

sor, was similar to the input minimum magnitude of suction pressure. Secondly, we investigated the controllability of the pressure supply time, which was set to 0.5, 1, and 3 s (b-3-1, where b=0.5, 1, 3 s). For this case, the minimum magnitude of the suction pressure was set to −5 kPa. As shown in Fig. 2B, the actual pressure supply time and suction pressure waveform, which were monitored by the sensor, were similar to the programmed pressure supply time and the waveform that was input to the program, respectively. Thus, the suction pressure could be controlled by using the developed system.

Effects of Suction Pressure Magnitude on Luciferase Level in Mice Livers We elucidated whether the magnitude of the suction pressure affects the expression level of transfected naked pDNA when using the developed system. The minimum magnitudes of the suction pressure were set to −1, −3, −5, −15, −30, and −40 kPa. The pressure supply time, pressure hold time, and pressure release times were set to 1, 3, and 1 s, respectively (1-3-1). As shown in Fig. 3, the luciferase level increased as the minimum magnitude of the suction pressure decreased, reaching a constant level at less than −5 kPa. Thus, the expression levels of transfected pDNA are controlled by the minimum magnitude of the suction pressure. Similarly, we previously reported that the expression levels of pDNA transfected using the tissue pressure method are controlled by the magnitude of the positive pressure; 0.59 N/cm^2 (5.9 kPa) was sufficient to achieve efficient and highly reproducible pDNA transfection using the tissue pressure method for kidneys and spleens in mice.[9] Although the absolute values of the positive and negative pressures are similar, it is

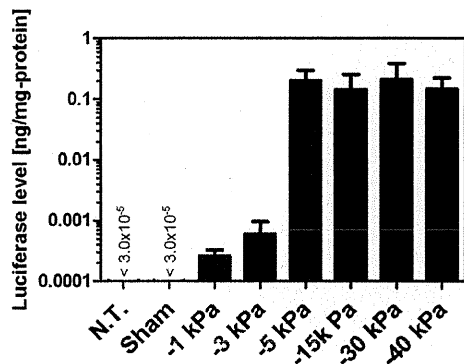

Fig. 3. Effects of the Minimum Magnitude of the Suction Pressure on the Transgene Expression Level

Each value represents mean±S.D. (n=4).

still unknown whether the similarity is significant because the studies were performed on different target tissues. This will be clarified in the future.

Next, we examined the effects of liver suction on the hepatic toxicity for different minimum magnitudes of suction pressure. Considering the results in Fig. 3, the following effective magnitudes of the suction pressure were employed in this experiment: −5, −15, −30, and −40 kPa. Both ALT and AST activities in the serum were measured 6 h after the liver suction. The ALT activities at −5, −15, and −30 kPa were not significantly different from that at 0 kPa (Sham), whereas the activity at −40 kPa was significantly higher than that at

0 and −5 kPa (Fig. 4A). The AST activities increased with a decrease in the minimum magnitude of the suction pressure and the activity at −5 kPa was significantly higher than that at 0 kPa (Fig. 4B). However, the AST activities returned to normal levels within 24 h (−5 kPa, 56.7±9.6 U/L; Sham, 52.7±7.6 U/L). Considering these results, the liver suction method at −5 kPa was the most effective and had the lowest hepatic toxicity in mice.

In the previous study, we reported that both ALT and AST activities 6 h after liver suction with an uncontrolled suction pressure were significantly higher than the activities in mice with 0 kPa, and the activities returned to normal levels within 48 h.[14] In contrast, in the present study, when the liver suction method in mice was performed at −5 kPa, an increase in ALT activity was not observed and the increased AST activity quickly returned to normal levels within 24 h. Therefore, the toxicity of the liver suction method was reduced by using the suction pressure control system.

Effects of Suction Pressure Waveform on Luciferase Level in Mice Livers Next, we investigated the effects of the suction pressure waveform on the expression level of pDNA transfected by liver suction. The minimum magnitude of the suction pressure was set to −5 kPa. The pressure supply time and the pressure hold time were both varied. High luciferase levels were measured when the pressure supply time was set to 0.5 s at all the pressure hold times, and statistically significant differences were observed between 0.5 and 3 s for both the 1- and 2-s pressure hold times. The luciferase levels at 1, 2, and 3 s were significantly higher than that at 0 s when the pressure supply time was 0.5 s (Fig. 5), and a constant luciferase level was maintained when the hold time was 30 s (data not shown).

A shorter pressure supply time increased the transgene expression in the suctioned mice livers (Fig. 5). In a previous study, we reported that the pDNA permeability of the plasma membranes in the pressed tissues transiently increased.[10] This phenomenon may be related to the increased transgene expression owing to the shorter pressure supply time observed in the present study. In the field of neural injury, the responses of cells and tissues to mechanical loading have been investigated[19] and *in vivo*[20] and *in vitro*[21] studies reported that the mechanical deformations of cells and tissues form transient survivable pores on the plasma membrane, resulting in the transient increase of permeability. More recently, *in vitro* experimental models of neural injuries showed an increase in the permeability with respect to the strain rate; a higher strain rate effectively alters the membrane permeability.[22,23] Thus, similar tendencies may occur for these different phenomena. However, further experiments should be performed to explain the phenomenon of cell membrane permeability in the field of neural injury and studies regarding the tissue suction method.

There is possibility that a pressure supply time of less than 0.5 s may increase the luciferase levels (Fig. 5). However, the tissue suction method could not be performed with a pressure supply time of less than 0.5 s because our pneumatic system could not control times lower than 0.5 s. Thus, the suction pressure control system can be further modified to include a rapid response speed, such as the use of hydraulic pressure systems. Nonetheless, in the present study, we determined that the pressure waveform affects the efficiency of the tissue suction method in mice livers and the most effective suction method was performed with a suction pressure waveform of 0.5-1-1 when the minimum magnitude of the suction pressure was −5 kPa.

Effects of Tissue Suction Times on Luciferase Level in Mice Livers In the previous study, we performed the liver

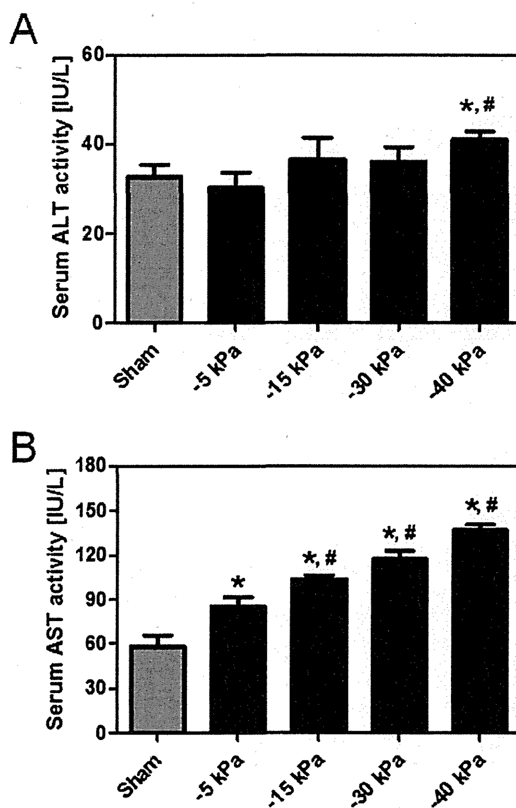

Fig. 4. Effects of the Minimum Magnitude of the Suction Pressure on Hepatic Toxicity

(A) ALT and (B) AST activities in serum 6 h after the liver suction. Each value represents mean±S.D. (n=4). One-way ANOVA, followed by Bonferroni's test was used to compare among groups. *p<0.05 *versus* Sham operation. #p<0.05 *versus* −5 kPa.

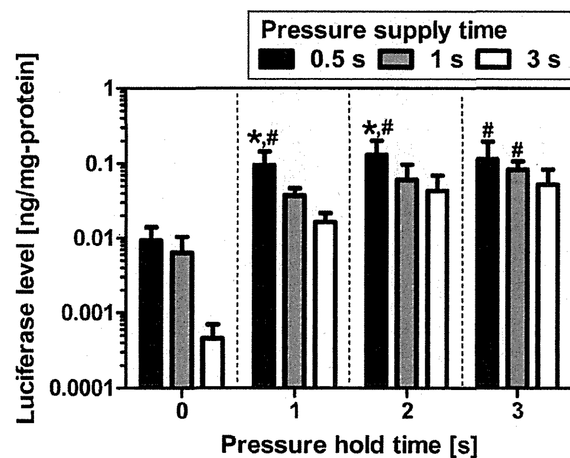

Fig. 5. Effects of the Suction Pressure Waveform on the Transgene Expression Level

Each value represents mean±S.D. (n=4). Two-way ANOVA, followed by Bonferroni's test was used to compare among groups. *p<0.05 *versus* 3 s of the pressure supply time for the same pressure hold time. #p<0.05 *versus* 0 s of the pressure hold time for the same pressure supply time.

suction method by subsequently or serially suctioning different parts of the liver and reported that the transgene expression levels increased with an increase in the tissue suction number for both cases.[14] In contrast, in the present study, we examined whether the number of tissue suctions on the same part of the liver increases the expression levels of the pDNA. We suctioned the same part of the mice liver 1, 3, 5, or 10 times immediately after i.v. injection of the pDNA solution. Because of the developed suction pressure control system, we were able to perform the tissue suction method on the same part repeatedly with the same conditions. As shown in Fig. 6, the number of liver suctions did not alter the luciferase levels of the liver. This result suggests that a single suction on the targeted portion of the tissue is sufficient for the liver suction method in mice.

Effect of Tissue Deformation on Luciferase Level in Mice Livers The minimum magnitude of the suction pressure affected the transgene expression in the liver suction method (Fig. 3). Similarly, the results of the previous study showed that the maximum magnitude of the positive pressure affected the transgene expression of mice kidneys and spleens in the tissue pressure method.[9] Because both the positive and suction pressures induced transfection in the target tissue, we hypothesized that one of the significant factors of the tissue pressure/suction methods is not the pressure, but the deformation of the tissue. To prove this hypothesis, we measured the volume of the suctioned hepatic tissue in the hole of the suction device for suction pressures of −1, −3, −5, and −15 kPa. As shown in Fig. 7, a difference was observed in the suctioned volume at the pressures of −3 and −5 kPa, but did not reach significance. A difference in transgene expression levels also existed between the pressures of −3 and −5 kPa using the tissue suction method (Fig. 3).

Furthermore, we controlled the tissue deformation using four different tissue suction devices and investigated the effects of those devices on the transgene expression. The devices had different numbers of holes but their total dimensions were equivalent (Fig. 8A). In the liver suction method, even though an equivalent pressure of −5 kPa was supplied to the device, the highest luciferase level was detected for the device with one large hole and the second highest level was observed

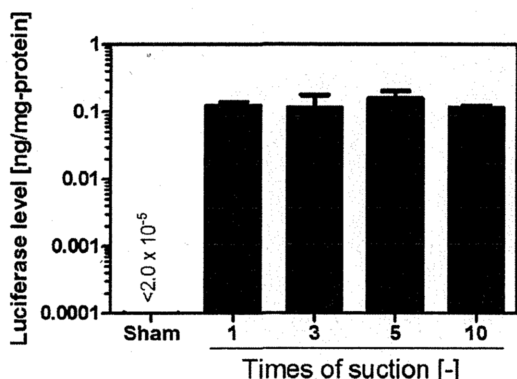

Fig. 6. Effects of the Number of Liver Suctions

The same part of the liver was suctioned serially. Each value represents a mean±S.D. (n=3).

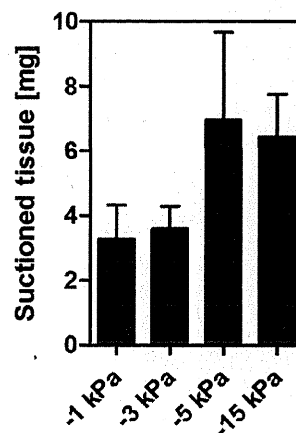

Fig. 7. Volume of the Suctioned Liver Tissue in the Hole of the Suction Device

Volume of the suctioned liver tissue when the mice liver was subject to suction pressures of −1, −3, −5, and −15 kPa using the suction device. Each value represents mean±S.D. (n=3).

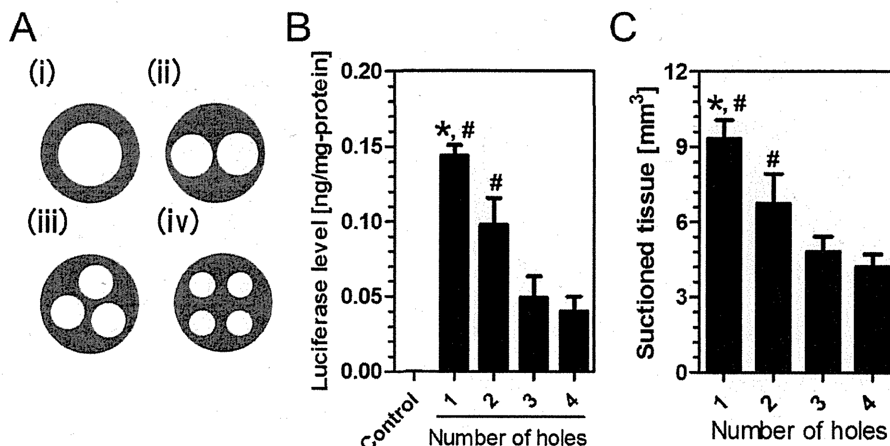

Fig. 8. Relationship between the Degree of Tissue Deformation and the Transgene Expression Level

(A) Horizontal cross section of the four different tissue suction devices with different numbers of holes. The device with one hole had an inner diameter of 3 mm, an outer diameter of 6 mm, and a height of 3 mm. The four devices had the same total dimensions of the holes. (B) Transgene expression level in the suctioned liver after using a different suction device. The minimum magnitude of the suction pressure was −5 kPa and the pressure waveform was 0.5-1-1. $^{*}p<0.05$ *versus* 2, 3, and 4 holes. $^{\#}p<0.05$ *versus* 3 and 4 holes. (C) Suctioned tissue volumes when the hepatic tissue was suctioned by using devices with different number of holes at the pressure of −5 kPa. $^{*}p<0.05$ *versus* 2, 3, and 4 holes. $^{\#}p<0.05$ *versus* 3 and 4 holes.

for the device with two holes (Fig. 8B). Similarly, among the volumes of tissue suctioned with the four devices, the volume of the tissue suctioned with the device with one large hole was the largest and that suctioned with the device with two holes was the second largest (Fig. 8C). These results support our hypothesis that the tissue deformation is a key parameter affecting the tissue pressure/suction method. Thus, the process of the tissue suction method has been clarified in part by using the developed suction pressure control system.

Potential Applications of the Liver Suction Method The tissue suction method can be performed in a minimally invasive manner by exploiting endoscopic surgery, because tissue suction devices can be mounted at the head of an endoscope.[15] The present study shows that the tissue suction method using a suction pressure control system can be accurately and safely applied for *in vivo* transfection of naked nucleic acids. These results suggest that the tissue suction method has the potential for use in clinical applications in the future.

One of the features of the tissue suction method is the site-specificity: this method enables a simple and precise site-specific transfection to the target part of the moving tissues, because the negative pressure easily fixes the position of the tissue relative to the device.[14] In addition, the transgene expression levels were increased by suctioning different parts of the tissues.[14] Therefore, we can control the transgene expression level in the suctioned tissue while avoiding transfection into the non-target parts of the tissue.

To date, treatment with the tissue suction method has not been investigated. Transfection of naked plasmid DNA encoding hepatocyte growth factor (HGF), which is a secretory protein, has been reported to prevent endotoxin-induced lethal fulminant hepatic failure, leading to dramatically enhanced survival in mice.[24] Therefore, the use of nucleic acid-based drugs that alter the expression of secretory factors in combination with the tissue suction method might be a good treatment strategy. In the future, we will investigate the appropriate combination of diseases and nucleic acid-based drugs for which the treatment with the tissue suction method could work efficiently and effectively.

CONCLUSION

We developed a pressure-controlled computer system for the suction-mediated transfection of livers in mice. Our experimental results indicate that the minimum magnitude of the suction pressure and the suction pressure waveform both affected the expression levels of the pDNA transfected *via* the tissue suction method applied to livers in mice. Our developed system can perform the tissue suction-mediated transfection method with high accuracy and safety at research facilities and medical institutions.

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