Contents

Preface

Strikingly fast technological progress in genomic sequencing and genetic engineering spawns numerous novel strategies for so-called gene therapy, and also accelerates the innovative realization of its applications. A one-step gene therapy is envisioned as one of the potential revolutionizing treatments for curing or preventing inherited and acquired life threatening diseases caused by genetic deficiencies or abnormalities. Clearly, an eventual success of gene therapy would require a targeted delivery of a functional therapeutic gene to target cells.

In general, gene delivery systems are divided into three approaches; i.e., viral, chemical-nonviral and physical-nonviral methods. Despite the fact that viral and chemical-nonviral gene delivery systems are being mostly developed, their clinical applications are hampered by well-known adverse effects and use restrictions such as immunogenicity, random integration in the human genome, difficulties in handling and large-scale production, and limited length of the target genes [1-3]. Therefore, development of an ideal *in vivo* gene delivery system with high efficiency, low side effects and few use limitations becomes a crucially determinant factor for future practical application of gene therapy. Initial success of physical gene delivery system began from local injection of naked DNA [4]. Furthermore, several vascular injection systems for naked DNA transfection in combination with pressure, large volume [5, 6], electricity [7, 8] and ultrasound [9, 10] have been reported. Modest transgene expression can be achieved by these techniques, but several drawbacks such as severe tissue injury make human clinical application infeasible and thus hinder the practical implementation [11-15].

Liver suction-mediated naked plasmid DNA transfection is a reported technique in which a direct suction mediated transfection of liver and kidney can induce efficient transgene expression in each organ [16]. Moreover, this suction microdevice is able to be mounted to the head of endoscope, used in minimally invasive endoscopic surgery, what could be practical in future clinical applications. However, this suction technique is necessary to be optimized in terms of efficiency and expression levels of transgene as well as liver damage. In this study, I set up a computer system with pressure sensor, which makes precise control of treatment including suction pressure and pressure waveform possible (Chapter I). Further, I investigated precise cellular delivery and

long-term transgene expression of therapeutic gene. I could confirm that naked plasmid DNA was transfered mainly into hepatocytes and sustained transgene expression was observed by liver suction-mediated naked plasmid DNA transfection with the so-called CpG free vector as described in Chapter II. Moreover, for the first time this technique was applied for curing disease model (Chapter II-3). My results provide useful information for the realization of *in vivo* gene therapy through the optimization of liver suction-mediated gene delivery system.

Chapter I

Gene transfection efficiency and safety evaluation of liver suction-mediated naked plasmid DNA delivery system

Section 1: *In vivo* suction-mediated transfection of naked plasmid DNA to the liver using a pressure-controlled computer system

I-1-1 Introduction

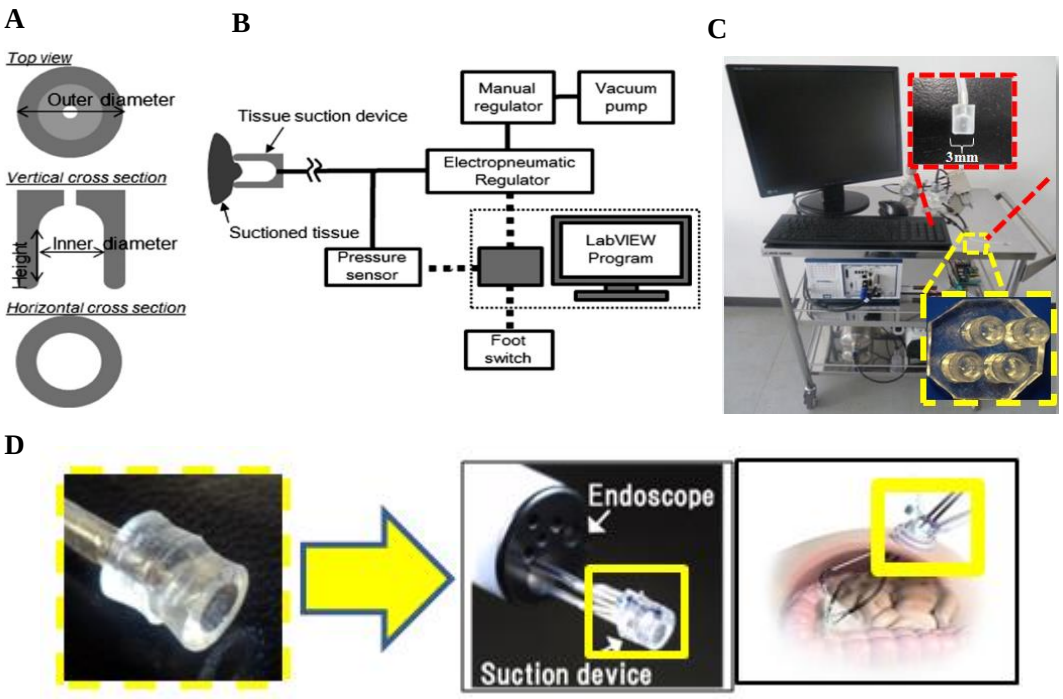
The functions of the liver are numerous i.e., intermediary metabolism and synthesis of serum proteins, working closely with nearly every system and process in human body. The hepatic parenchymal cells (hepatocytes, PC) are responsive to a broad range of hormones and growth factors that stimulate or inhibit their proliferation [17, 18]. Given various anatomical properties and cellular functions in the production of most soluble proteins, the liver is a highly relevant target organ for gene therapy as the secreting platform for therapeutic protein [19-21].

Compared with viral vectors employing their natural ability to transfer gene into cells, nonviral gene delivery systems use the cellular function of endocytosis or physical force to overcome the membrane barrier of a cell to facilitate gene transfer to target cells. Physical methods do not involve any substance that could be cytotoxic or immunogenic. Due to several merits of naked plasmid DNA transfection in nonviral approaches such as easier to mass-produce, quality control, and low immunogenicity, various physical methods including needle injection, gene gun, electroporation, sonoporation, and hydrodynamic injection have attracted great interest. These methods also enable naked plasmid DNA based gene delivery to reach efficiencies close to those achieved with nonviral approaches [22, 23]. Regrettably, most of these methods mentioned above have several drawbacks, namely, severe tissue injury, and clinical impracticality due to difficulty of surgical treatment on human [24-26].

Tissue suction-mediated naked delivery system has been invented as an *in vivo* gene transfection method originally from the tissue pressure-mediated previously developed in our group [27-29]. In the case of tissue suction-mediated transfection, a direct suction treatment to the liver, kidney, and spleen can induce efficient transgene expression without any severe tissue damage using naked plasmid DNA. In this method, the target organs of the mice receive a treatment of direct suction

after intravenous injection of naked plasmid DNA [16]. In the future clinical uses, microdevice named a suction device can be mounted at the head of an endoscope, so that this system can be practically performed in a minimally invasive manner by exploiting endoscopic surgery.

In this section, I with my colleagues assembled a computer system to precisely control the suction pressure conditions such as pressure and pressure waveform, and investigated the effect of these suction conditions and animal experimental procedures on the efficiency of the liver transfection of naked pDNA in mice.



The suction-mediated transfection method using computer-controlled pressure system

A) Design of the tissue suction device. The device measures 3 mm in inner diameter, 5 mm in outer diameter, and 3 mm in height. **B)** Configuration of the suction pressure control computer system. **C)** Picture of suction-mediated system, up: single device; down: multiple device. **D)** Proposed set-up for mounting suction-device to endoscope tip for the future clinical application.

I-1-2 Results

a. Investigation on liver lobes for pDNA transfection level

Left lobe of the mouse liver was transfected with pCMV-Luc by using liver suction-mediated transfection method [16]. Luciferase expression levels of the tissues including kidney, lung, heart and spleen, and four lobes (left lobe, median lobe, right lobe and caudate lobe) of the liver were measured at 6 h after intravenous injection of pCMV-Luc and suction of liver left lobe. As shown in **Fig.1A**, high luciferase gene expression was obtained in left lobe and its expression level was approximately 0.11ng/mg protein. In contrast, the luciferase levels of other organs and other liver lobes were less than 2×10^{-3} ng/mg tissue protein **(Fig.1B)**.

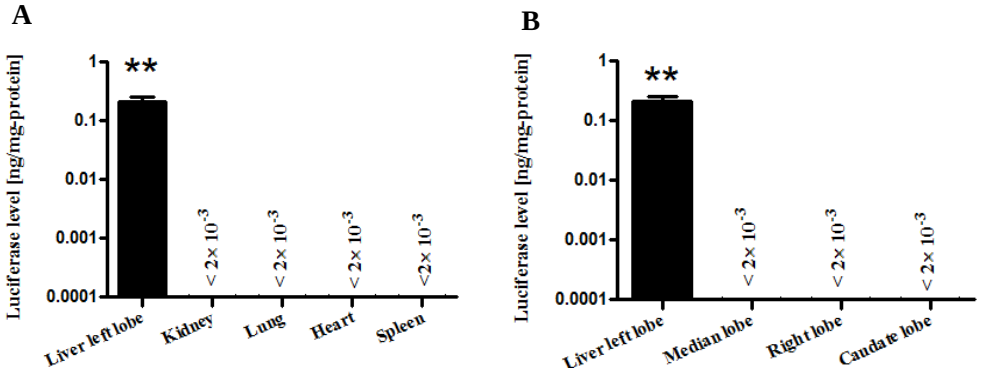


Fig.1 *In vivo* transfection of plasmid DNA by liver suction-mediated transfection method.

A) Luciferase levels in various organs, and **B)** various lobes of liver. Six hours after intravenous injection of 100μg pCMV-Luc followed by liver suction-mediated transfection treatment on left lobe of liver, the mice were killed and samples from each group were collected for luciferase activity analysis. **p< 0.05 versus other organs and lobes. Each value represents mean±S.D. (n=4).

b. Effect of suction pressure magnitude on luciferase expression level

To elucidate whether the magnitude of the suction pressure affects the expression level of transfected naked pDNA with the developed pressure controlled system. The magnitudes of the suction pressures were set to −1, −3, −5, −15, −30 and −40 kPa. The pressure supply time, pressure hold time, and pressure release time was set to 1, 3, and 1 s, respectively (1 s – 3 s – 1 s). As shown in **Fig.2** the luciferase expression level increased as the magnitude of the suction pressure decreased, reaching a constant level at less than −5 kPa. Thus, the expression levels of transfected pCMV-Luc were controlled by the magnitude of the suction pressure. It was previously reported that the expression levels of pDNA transfected using the tissue pressure-mediated method with the positive pressure; 0.59 N/cm^2 (0.59 kPa) was sufficient to achieve efficient and reproducible pDNA transfection for the kidney and spleen in mice [27].

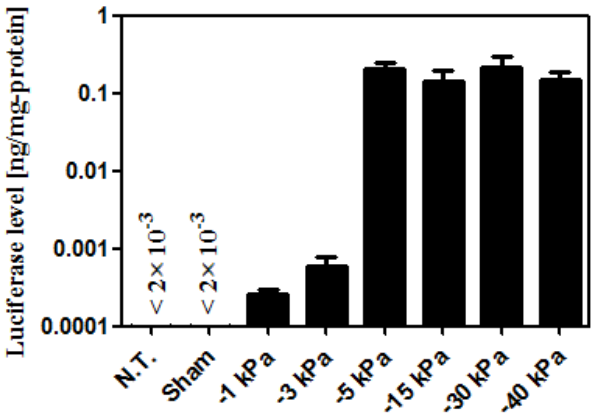


Fig.2 Effect of the magnitude of the suction pressure on the transgene expression level.
Each value represents mean±S.D. (n=4)

c. Effect of suction pressure waveform on luciferase expression level

Fig.3 shows the effects of the suction pressure waveform on the luciferase expression level of pDNA transfected by liver suction method. The magnitude of the suction pressure was set to −5 kPa. The pressure supply time and the pressure hold time were both varied as shown in **Fig.3A**. Relatively higher luciferase levels were observed when the pressure supply time was set to 0.5 s in all of individual groups treated under same pressure hold times. Statistically significant differences were observed between 0.5 and 3 s supply time in the 1 - , 2 -, 3 - s pressure hold time groups. The luciferase levels at 1, 2, and 3 s hold time groups were significantly higher than that at 0 s. From these results, a shorter pressure supply time and a longer hold time were proved to increase the transgene expression after suction-mediated transfection on left lobe of liver.

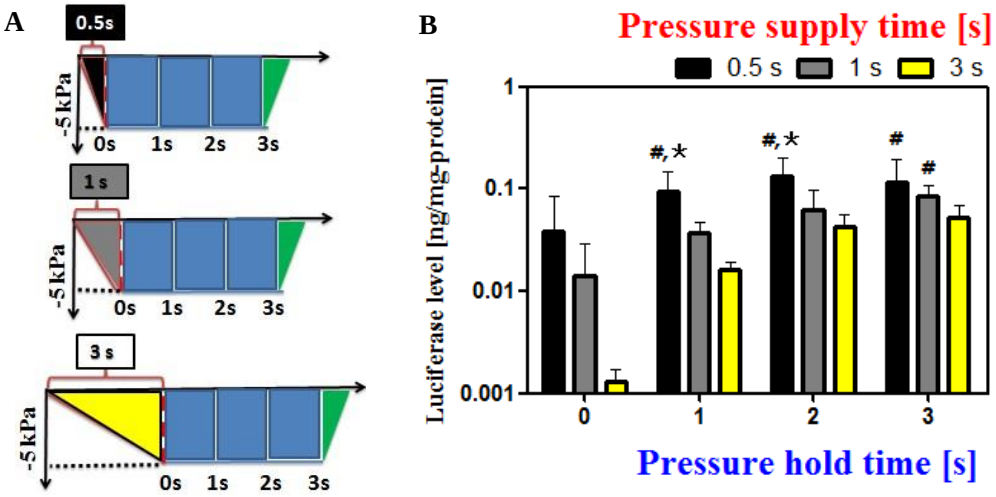


Fig 3. Effectof the suction pressure waveform on the transgene expression level.

A) Schematic illustration of three suction pressure waveform patterns. The pressure supply times including 0.5, 1, 3 s are colored black, gray and yellow; pressure hold time is colored blue, and pressure release time is colored green. The indicated proportion increases from 1 to 3 fold along with pressure hold time from 0 to 3 s. **B)** Six hours after 100μg pCMV-Luc administration by liver suction-mediated transfection method on left lobe of liver under indicated pressure suction waveform by – 5 kPa suction pressure, the mice were killed and samples from each group were collected for luciferase activity analysis. Each value represents mean±S.D. (n=4) $^{*}P<0.05$ 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.

d. Effect of administration dose of naked plasmid DNA on transgene luciferase expression

Effect of administration dose of naked plasmid DNA on the level of gene expression was also evaluated. As shown in **Fig.4**, the levels of luciferase protein at 8 h after liver suction-mediated treatment were detected in left lobe of mice livers when the injected amount of plasmid DNA was as low as 2.0 μg per mouse. The luciferase expression level increased with increasing the injected amount of plasmid DNA and reached a maximum level at approximately 25 μg pCMV-Luc plasmid DNA per mouse up to a dose of 200 μg.

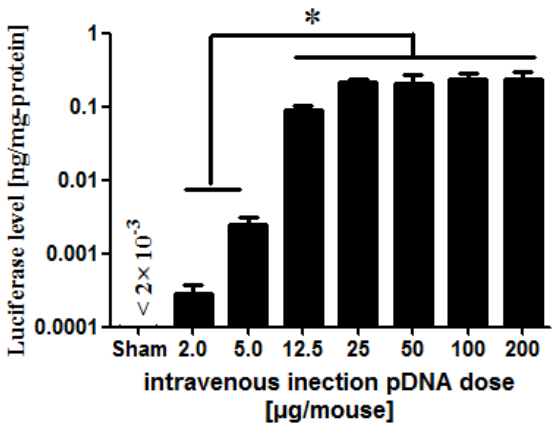


Fig.4 Effect of administration dose of naked plasmid DNA on transgene luciferase expression. Various amounts of naked plasmid DNA (pCMV-Luc) in 200 μl saline were intravenous injected to each mouse and liver suction treatment was immediately applied on left lobe of liver under the condition (5 kPa suction pressure, 0.5 s – 3 s- 0.5 s pressure waveform). Six hours after liver suction-mediated transfection, the mice were killed and left lobes from each group were collected for luciferase activity analysis. $^{*}P<0.05$ versus 2.0 and 5.0 μg of administration dose of pDNA. Each value represents mean ± S.D. (n=4).

e. Effect of time interval between pDNA injection and liver suction on transgene expression

The liver suction treatment may create transient membrane defect/destabilization in liver cells, which cause the formation of permeabilized state and allow the passage of pDNA. In order to obtain information about the cellular uptake of plasmid DNA caused by the liver suction-mediated transfection, I examined the effect of this time interval. Left lobe of liver was suctioned at different time points, from 60 s before to 180 s after intravenous administration of pCMV-Luc plasmid DNA into mice. As shown in **Fig.5**, in the case of suctioning on the left lobe at +0, +60, +180 s after plasmid DNA injection, the luciferase levels of the left lobe were 0.1~0.75 ng/mg protein. In contrast, the luciferase levels for the time points of 60 s and 30 s before injection were only 0.0005 and 0.003 ng/mg protein, respectively. On the other hand, the luciferase level in the case of suction treatment at 10 s before injection was approximately 0.065 ng/mg protein.

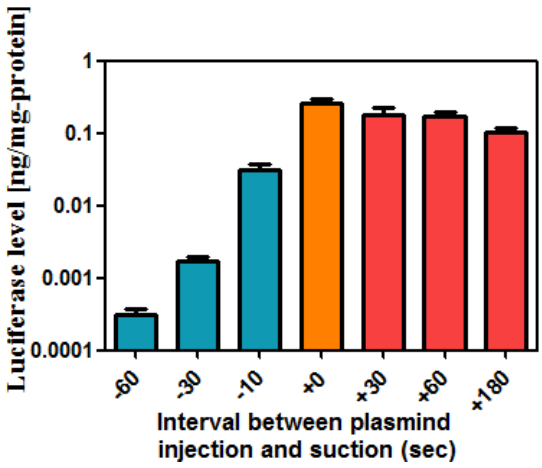


Fig.5 Effect of time interval between pDNA injection and liver suction on transgene luciferase expression. The time point of the suction treatment time varied from 60 s before (-) to 180 s after (+) 100 μg pCMV-Luc injection. The suction was carried on the left lobe of the liver with the treatment condition of 5 kPa suction pressure, and 0.5 s - 3 s - 0.5 s pressure waveform). Six hours after liver suction-mediated transfection the mice were killed and left lobes from each group were collected for luciferase activity analysis. Each value represents mean ± S.D. (n=4 or 5).

f. Effect of tissue deformation induced by liver suction on luciferase expression

To prove the hypothesis that deformation of the liver induced by suction treat is the major factor of suction-mediated transfection, I measured the volume change of the suctioned liver tissue in the hole of the suction device at suction pressures of −1, −3, −5 and −15 kPa. As shown in **Fig.6**, the difference was observed in the suctioned volume between at pressures of −3 and −5 kPa, but was not significant. Furthermore, I controlled the tissue deformation using four different types of tissue suction devices having the same hold area and investigated the effects of hole area on the transgene expression. The used devices had different numbers of holes but their total dimensions were equivalent **(Fig.7A)**. Even though an equivalent pressure of -5 kPa was supplied to the device, the highest luciferase level was detected for the one hole-device and the second highest level was observed for the two holes-device **(Fig.7B)**. Similarly, among the four types of devices, the enlarged volume of the tissue having suction with one hole-device was the largest and that suction treatment with two holes-device was the second largest **(Fig. 7C)**.

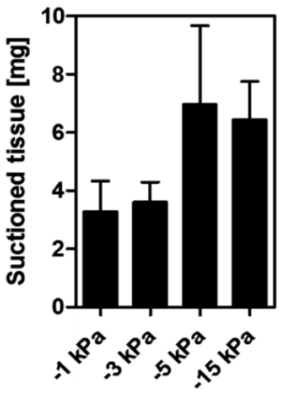


Fig 6. Volume of the suctioned liver tissue in the hole of the suction device. Volume of the suctioned liver tissue when the mice liver was subjected to suction pressures of −1,−3,−5 and −15 kPa using the suction device. Each value represents mean ± S.D. (n=3)

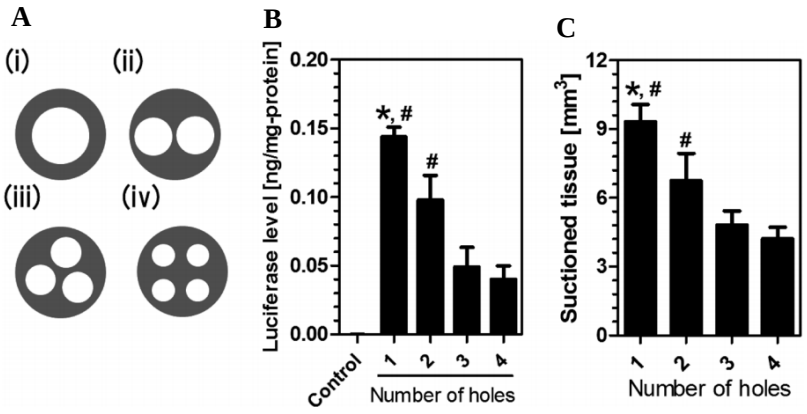


Fig.7 Relationship between the degree of tissue deformation and the transgene expression level.

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 area of the holes. **B)** Transgene expression level in the suctioned liver treated with different suction device. The maximum 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 liver 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.

I-1-3 Discussion

Site-specific suction-mediated transfection of naked plasmid DNA method has been developed based on the experience on previous tissue pressure-mediated transfection method [27-30]. The negative pressure supplied by the tissue suction device deformed the target tissue and induced transfection **(Fig.1)**. Transfection by negative pressure suction has these major advantages over positive pressure method; it is easier to fix the appropriate position between target tissue and the device with negative pressure [31], the tissue suction devices are simpler than the tissue pressure-mediated transfection system [27-30], and more precise control of loading area is possible. Therefore, suction-mediated *in vivo* transfection shows greater ease than pressure-mediated method. Moreover, since the suction devices are small enough to be mounted to the end of endoscope, it is possible to perform transfection with less invasive injury.

In the previous study on tissue suction-mediated transfection, manual control of pressure with the tissue suction device by using a syringe in which suction was adopted, the inner space of the device was filled with the deformed tissue [16]. Therefore, it remains unclear how the suction pressure conditions affect the results of the tissue suction method. To perform the tissue suction method safely and accurately transfection at research facilities and medical institutions, the suction method must be carried out under the well controlled condition. Therefore, in this study, firstly I assembled a computer system to control the suction pressure and investigate the effects of the suction conditions on the efficiency of the liver transfection of naked pDNA in mice. Using the

suction pressure control computer system is shown in introduction part, the minimum magnitude of the suction pressure and controllable pressure waveform constituted by pressure supply time, pressure hold time, and the pressure release time were input in to an original Labview program to define the suction pressure waveform. After that, the effects of the indicated suction pressure **(Fig.2)** and pressure waveform **(Fig.3)**, these results indicated that suction pressure -5 kPa with suction pressure waveform 0.5 s -3 s -3 s pressure pattern were the optimal suction condition. Additionally, in order to explain the underlying mechanism by which suction pressure effects on hepatic transgene expression, I hypothesized that the significant factors of the tissue suction method is not only the pressure, but also the resulted extent of deformation of the tissue. To prove this hypothesis, I measured the volume of enlarged volume of hepatic tissue in the hole of suction device as an indication of deformation of the tissue due to suction pressure. As shown in **Fig.6**, slight differences of tissue volume by suction between -3 and –5 kPa was observed. Furthermore, I controlled the extent of tissue deformation using four types of device with the same hole area **(Fig.7A)** and confirmed that larger deforming action volumes obtained by a device with single hole by suction got relatively higher transgene expression **(Fig.7B-C)**. These results support the hypothesis that the tissue deformation is a key parameter influencing the efficiency of the tissue suction method. Regarding plasmid administration dose for the future therapeutic use, relatively low plasmid DNA dose gives potential to save cost. My results show that relatively lower pDNA administration dose of 12.5 μg per mouse **(Fig.4)** was able to achieve efficient transgene expression. As known, the nonviral gene delivery systems of pDNA targeting liver *in vivo*, normally need 30~50 μg per mouse pDNA dose for injection [32, 33]. In addition, physical method of naked pDNA transfection targeting tissue such liver, kidney and lung needs more than 25 μg per mouse dose of naked pDNA injection [34]. Therefore liver suction-mediated method has a merit of low pDNA dose compared to other current pDNA delivery systems.

In conclusion, these results provide evidence that effective and accurate hepatic transfection can be obtained by the computer controlled suction system. This technology may therefore prove to be applicable in various aspects of gene therapy.

Section 2: Enhancement of suction-mediated hepatic gene transfection using new multiple suction device

I-2-1 Introduction

The liver is a very important organ that supports almost every organ in the body in some facet and defects in the liver cause a serious threat to life. Therefore, this is another reason why scientists have their interests in developing gene therapy that targets to hepatocytes and developing regenerative research for curing an injured liver. In recent decades, development of physical naked transfection for gene therapy becomes attractive *in vivo* and in vitro due to several advantages over viral-based vectors, i.e., simplicity of construction, ease of large-scale production, cost effectiveness, less toxicity, nonimmunogenicity. In addition, the introduced exogenous genes do not be integrated into the host genome [35-38]. However, the relatively low transgene efficiency in physical gene transfection compared with most clinically used viral based gene transfer methods, which limits the application of naked DNA transfer system [39].

In laboratory animal studies, the gene transfer efficiency in the liver by injection with adenoviral vectors is 80%; while in plasmid-based delivery, it is only 10% to 15% [40, 41]. Liu and colleagues developed a novel method to transfect hepatocytes with naked plasmid DNA *in vivo*, in which approximately 40% hepatocytes obtained resultful transfection [5] .Like in other *in vivo* gene transfection techniques, the strategy of increase in the number of transfected cells has been considered as one of the main approaches of enhancing transfection efficiency in one-step treatment. Liu, et al employed a rapid intravenous injection of large amount of solution containing naked plasmid DNA so-called hydrodynamics-based gene delivery. Their technology appears to be simple for the efficient transfection of hepatocytes, but unfortunately only a few improvements for this technology have been made to date [42, 43].

Inspired by their studies, our group found that direct pressing on the surface of liver kidneys, and spleen, induces the transfection of naked nucleic acids and we termed it as tissue pressure-mediated transfection [44]. This method has been applied for naked pDNA, siRNA, and microRNA, and the miR-200 family of microRNAs introduced by the renal pressure-mediated transfection successfully ameliorated renal tubulointerstitial fibrosis in mice [44]. Pons et al. used the tissue pressure-mediated method to transfect pDNA encoding heat shock protein 70 (HSP70)

to rat kidneys and reported that the immune reactivity to HSP70 in the kidney is cause of salt-sensitive hypertension [45]. Further, we have reported that the secretion of pro-inflammatory cytokines was not observed under the experimental conditions in transfection. 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 [29, 30]. Based on this key factor, a novel site specific transfection method for naked plasmid DNA was invented as mentioned in **section 1**, and named liver suction-mediated transfection, and consequently, effectively gene transfection was observed. Although, the suction part of the liver was deformed temporarily, the observation of transient pDNA transfection in liver further encouraged us to investigate progressive approaches to enhance transgene efficiencies.

In this section, I examined whether the repetition of tissue suctions on the same part of the liver increases the expression level of pDNA. In addition, I also examined whether the increase in the area of tissue suctions on the different part of the liver elevates the expression level of pDNA. Based on these results, I designed a new suction device named multiple suction device, in consideration of feasibilities of the treatment, using which remarkable enhancement of the level of transgene expression was obtained in the left lobe of liver.

I-2-2 Results

a. Effect of the repetition numbers of suction on luciferase expression

To examine whether the repeated number of tissue suctions on the same part of the liver increases the expression of the pDNA. A serially repeated suction treatment on the same part of liver including 1, 3, 5, 10 times was employed immediately after i.v injection of pCMV-Luc pDNA. Using the previously described suction pressure control system, I could perform suction treatments on the same part of the liver repeatedly under same conditions. As shown in **Fig.8**, the number of liver suctions did not alter the luciferase levels of the liver. These results suggest that a single suction on the targeted portion of the liver is sufficient for the liver suction-mediated method in mice.

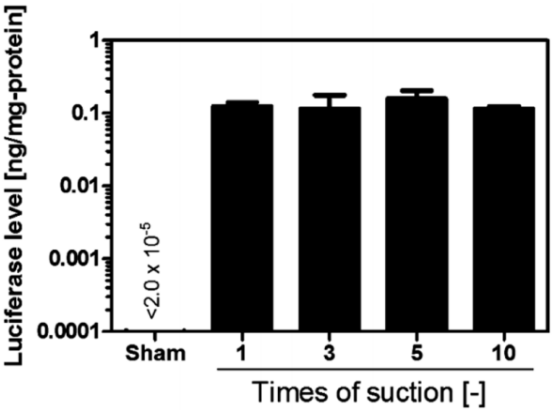


Fig.8 Effects of repetition numbers of liver suctions.

The same part of the liver was suctioned serially under same condition (5 kPa suction pressure, 0.5 s - 3 s - 0.5 s pressure waveform). Six hour liver suction-mediated transfection the mice were killed and left lobes from each group were collected for luciferase activity analysis. Each value represents mean ± S.D. (n=4 or 5)

b. Effect of area of suction on luciferase expression

To examine whether increasing area of liver suctions through suctioning on different the same part of the liver increases the expression of the pDNA. A serially separately suction treatment on the different part of the liver including 1, 2, and 3 times was employed immediately after i.v injection of pCMV-Luc pDNA. As shown in **Fig.9**, along with increasing the number of liver suctions, the enlarged surface area of liver enhanced the luciferase levels of the liver. These results suggest that several individual suctions on the different portion of the liver enhanced the transfection level for liver suction-mediated method in mice.

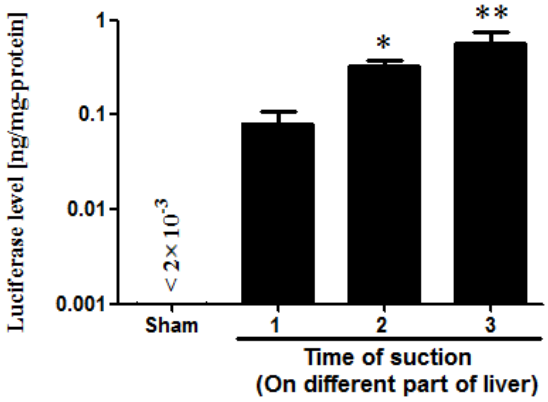


Fig.9 Effect of area of suction on luciferase expression.

The different parts of the liver were suctioned serially under same condition (5 kPa suction pressure, 0.5 s - 3 s - 0.5 s pressure waveform). Six hours after liver suction-mediated transfection, the mice were killed and left lobes from each group were collected for luciferase activity analysis. Each value represents mean ± S.D. (n=4 or 5). $^{*}p < 0.05$ *versus* one time. $^{**}p < 0.05$ *versus*1 time

I-2-3 Discussion

Several reports have presented that hepatic transgene efficiency is related to the amount of transfected somatic cells *in vivo* [5, 19, 46]. Liu et al. have shown that about 40% hepatocytes in the liver were transfection by hydrodynamic injection of low dose of naked pDNA. Inspired by their studies, I wanted to improve transgene by the means of increasing amount of transfected cells

in liver. First, I confirmed that repeated suction on the same site of liver, already single suction was able to achieve maximum transgene expression **(Fig.8)**. Second, I found that increasing area of suction treatment can enhance transgene expression under one time injection of naked pDNA **(Fig.9)**. According to my data, 3.8 - fold increase of transgene expression level occurred by triple suction on different site of liver in comparison with one time suction. Data from such experiments could allow researchers to enhance transfection levels by enlarging suction area with liver suction-mediated transfection method.

Section 3: Investigation on liver injury by suction-mediated naked plasmid DNA transfection method

First, Liver is a relatively soft tissue compared with kidney. Therefore, comparing with transfection of other organs, probably decreasing pressure less than -5 kPa is needed by suction-mediated method. I hypothesized that soft attachment to liver surface could decrease tissue injury so that I produced three devices with gradient degree of softness and tested. When the suction pressure was over -40 kPa, soft device showed less liver damage than hard device. Second, I confirmed that there was no significant induction of proinflammation by suction treatment and minimal liver injury made a recovery within 24h. Therefore, on the basis of these results, liver suction-mediated transfection method has been shown to be an effective and safe approach for the gene therapy in vivo.

Chapter II

Application of the liver suction-mediated transfection method to therapeutic genes

Hepatocytes were effectively transfected compared with nonparenchymal cells in the liver by suction treatment. It was suggested that demonstrated that the liver suction-mediated transfection activated the transcriptional factors including AP-1 and NFκB. Moreover, the level of c-fos and c-jun mRNA were increased in short time (within 1 h). Then, by means of CpG free based vector, long-term transgene expression of reporter luciferase and IL-22 protein was achieved by suction treatment. Both of these transgene proteins could be detected more than 1 month post-administration of respective naked pDNA. Finally I demonstrated the cure of hepatic injury by liver suction-mediated transfection of therapeutic IL-22 protein against Con A-induced acute hepatitis mice model.

Materials and Methods

Chapter I

Plasmid DNA

pCMV-Luc was constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI) into the polylinker of pc DNA 3.1 vector (Invitrogen, Carlsbad, CA). pCMV-fLuc was amplified in the Escherichia coli strain DH5α, isolated and purified using QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany) [47]. Characteristics of the pCMV-Luc used are shown in **Appendix I**.

Animals

ICR mice (female, 5 weeks old) and Balb/c mice (female, 6 weeks old) were purchased from Japan SLC Inc. (Shizoka, 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.).

Fabrication of Tissue Suction Devices

The tissue suction devices were manufactured via a polydimethylsiloxane (PDMS) replicamolding 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 previously[48]. The procured 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. The one single device had size with an inner diameter of 3 mm, an outer diameter of 5 mm, and a height of 3 mm.

Suction Pressure Control Computer System

The negative pressure was generated by a vacuum pump. An electro-pneumatic regulator (ITV0090; SMC Corp., Tokyo, Japan) was controlled by a personal computer 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).

***In vivo* liver suction-mediated transfection method**

The mice were anesthetized with isoflurane and had abdominal incision, ensuring minimal exposure of the liver. Thereafter, indicated amount of pCMV-Luc (12~100 μg) of 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, controlled by the suction pressure control system. Six hours after tissue suction, the left lobe was excused and the gene expression levels were determined by the luciferase assay.

Luciferase Assay

At indicated time points, the left lobe of liver was harvest and homogenized in 10 ml/g liver of lysis buffer (0.1 M Tris, 0.05 % TritonX-100, 2 mM EDTA, pH7.8), and the homogenates were centrifuged at 13000 × g for 10 min at 4 °C. Then, the supernatant was mixed with luciferase assay buffer (PicageneDual, Toyo Ink, Tokyo, Japan), and the produced chemiluminescence was measured with a luminometer (Lumat LB 9507; EG and G Berthold, Bad Wildbad, Germany).

Statistical analysis

The results were expressed as mean ± S.D. Statistical comparisons were performed by one-way ANOVA for multiple groups. Post hoc multiple comparisons used Tukey's test. $P<0.05$ was considered significant.

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