Development of liver suction-mediated naked plasmid DNA delivery system for *in vivo* gene therapy

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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, kidney, spleen and heart 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 transgene efficiency 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

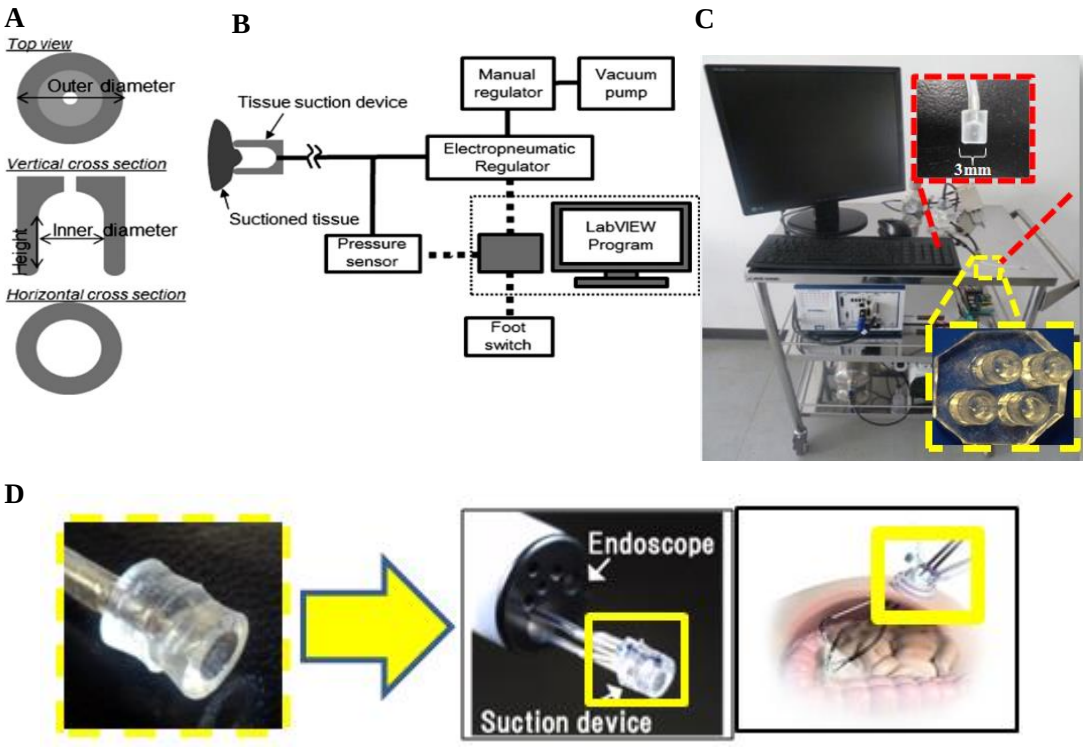
The functions of the liver are numerous i.e., intermediary metabolism and synthesis of serum proteins, working closely with nearly every system and process in human body. The hepatic parenchymal cells (hepatocytes, PC) are responsive to a broad range of hormones and growth factors that stimulate or inhibit 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, spleen and heart 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 **(Scheme.1)**.



Scheme.1 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)**.

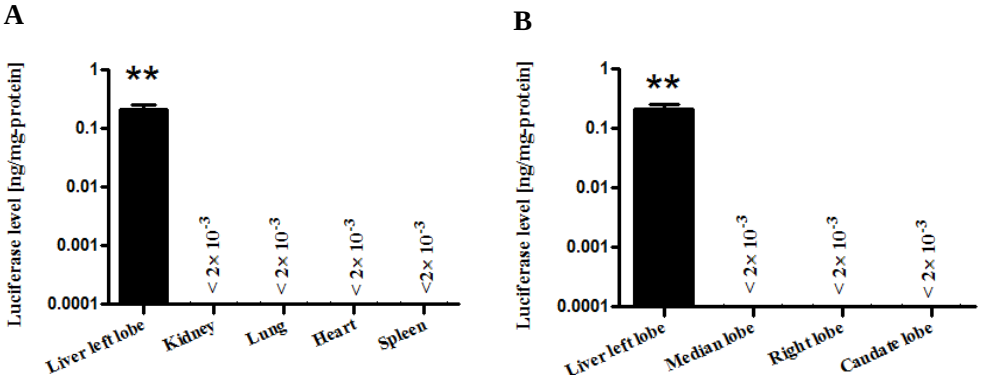


Fig.1 *In vivo* transfection of plasmid DNA by liver suction-mediated transfection method.

A) Luciferase levels in various organs, and **B)** various lobes of liver. Six hours after intravenous injection of 100 μg 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].

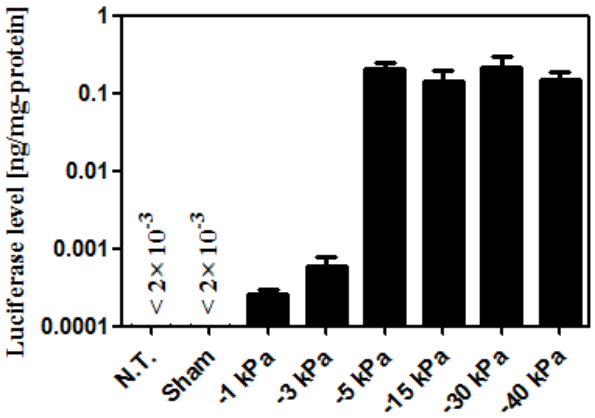


Fig.2 Effect of the magnitude of the suction pressure on the transgene expression level.
Each value represents mean±S.D. (n=4)

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.

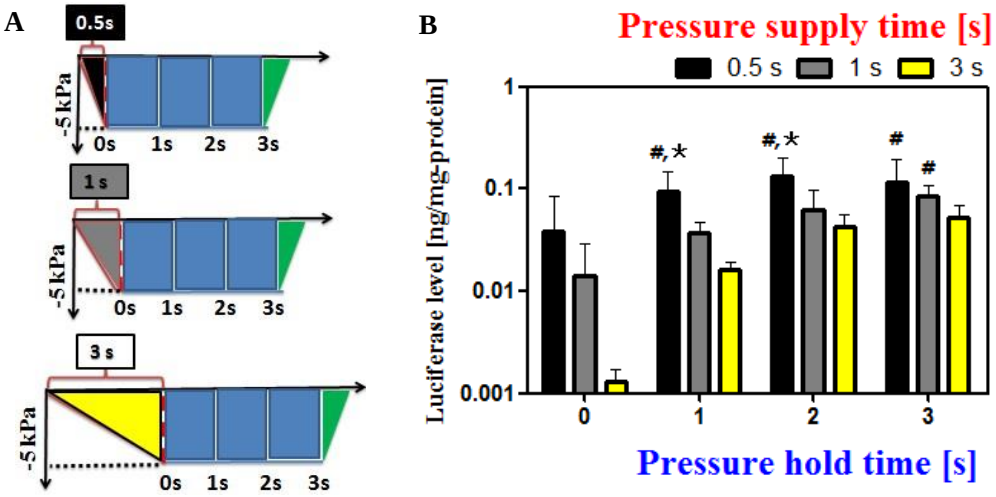


Fig 3. Effect of the suction pressure waveform on the transgene expression level.

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 100 μg 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.

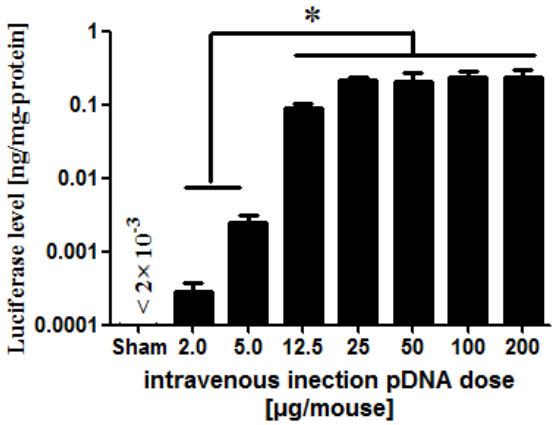


Fig.4 Effect of administration dose of naked plasmid DNA on transgene luciferase expression. Various amounts of naked plasmid DNA (pCMV-Luc) in 200 μl saline were intravenous injected to each mouse and liver suction treatment was immediately applied on left lobe of liver under the condition (-5 kPa suction 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 composition of plasmid DNA solution on transgene luciferase expression

Dextrose solution, 0.9% NaCl solution, PB, PBS (pH 7.4), and water were evaluated as injection solutions for intravenous pDNA administration. The composition of each solution is shown in **Table1**. Dextrose solution is a typical nonionic solution and NaCl solution, PB and PBS are typical ionic solutions. Water was used as a simple solvent. The experimental conditions were set as -5 kPa suction pressure, and 0.5 s - 3 s - 0.5 s pressure waveform which gave maximum transgene expression level in Fig. 3.A. At six hours after liver suction-mediated transfection, the mice were killed and samples from each group were collected for luciferase activity measurement. **Fig.5** shows the effect of the different pDNA solutions on transgene expression. Among dextrose, PB, PBS, and water groups, no significant differences were observed in transgene expression level, possibly due to the dilution of pDNA solutions by blood. In the case of NaCl solution of pDNA administration, transgene expression was slightly higher than for the other solutions.

Table 1. Solution composition of pDNA

Solution	Composition
Dextrose	0.5%, 1%, 5%, 25%, 50% dextrose (w/v)
NaCl	0.09%, 0.9% NaCl (w/v)
PB	Phosphate buffer of 0.15M NaH_2PO_4/0.15M Na_2HPO_4 pH 5.4, 6.4, 7.4, 8.4, 9.2
PBS	Equal mixture (v/v) of PB (pH7.4) and 0.9% NaCl
Water	Distilled H_2O

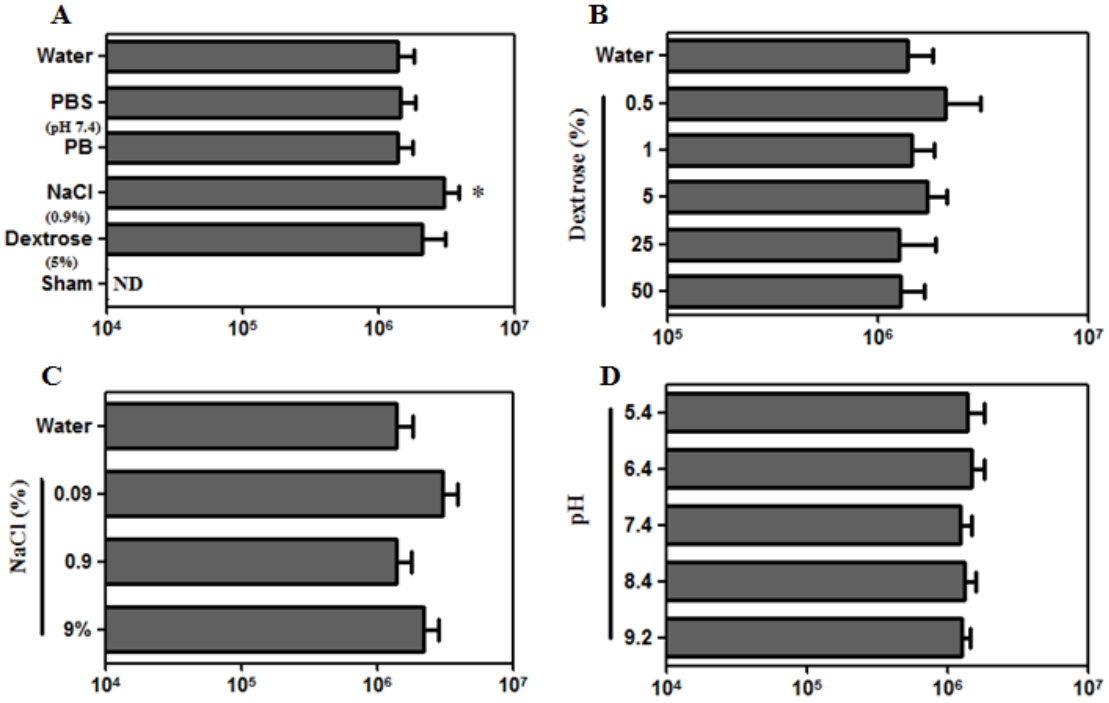


Fig.5 Effect of type of administration plasmid DNA solution on transgene luciferase expression.

A) Comparison between different types of of pDNA solution, **B) Dextrose** solutions with different dextrose concentrations, **C)** NaCl solution with different NaCl concentrations, **D)** and pH of PB on transgene expression levels. pDNA solution (50 μg/200 μl) was i.v. injected in to each mouse, immediately treated by liver suction on the left lobe of liver under same condition (-5 kPa suction pressure, 0.5 s -3 s- 0.5 s pressure waveform). Six hour after liver suction-mediated transfection the mice(n=4)were killed and left lobes from each group were collected for luciferase activity analysis. Each value represents mean±S.D.

f. Effect of intravenous infusing duration on luciferase expression

Assuming that the intravenous infusing duration of plasmid DNA solution should have some effects on transgene expression level, equal amount of 200 μl volume per mouse of pCMV-Luc plasmid solution containing 50 μg were injected at indicated infusing duration from 3, 15, 60 to 120 s. As shown in **Fig.6**, the relatively long duration of infusion usually resulted in a higher level of transgene expression. The level of luciferase in the left lobe of mice receiving the plasmid DNA solutionin15 s was around 120 fold higher than 60 s injection group, and moreover, approximately 180 fold higher than 120 s injection group.

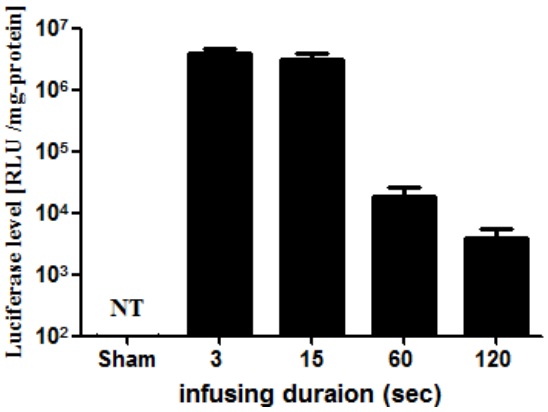


Fig 6. Effect of intravenous infusing duration on luciferase expression.

Mice (18-20g) were intravenously injected with 50 μg of pCMV-Luc plasmid in 200 μl saline. The injection time varied from 5 to 120 s followed by immediate liver suction treatment on left lobe of liver under the 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 of each group were collected for luciferase activity analysis. Each value represents mean ± S.D. (n=4).

g. 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.7**, in the case of suctioning on the left lobe at +0, +30, +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.

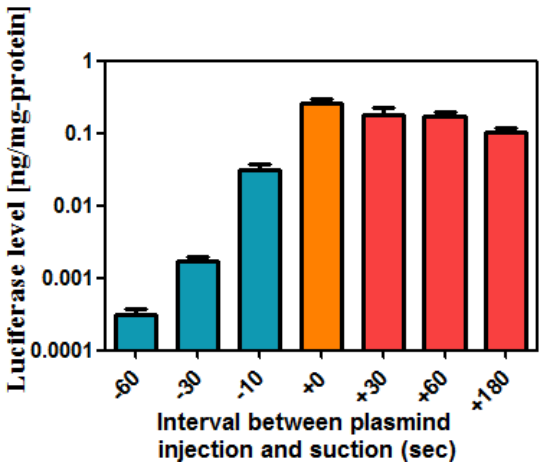


Fig.7 Effect of time interval between pDNA injection and liver suction on transgene luciferase expression. The time point of the suction treatment time varied from 60 s before (-) to 180 s after (+) 100 μg pCMV-Luc injection. The suction was carried on the left lobe of the liver with the treatment condition 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).

h. 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.8**, 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.9A)**. 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.9B)**. 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. 9C)**.

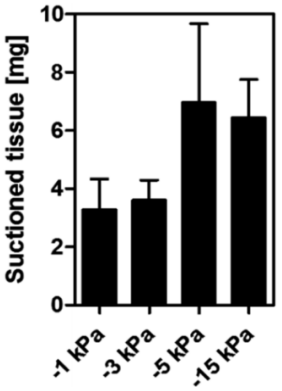


Fig 8. Volume of the suctioned liver tissue in the hole of the suction device.
Volume of the suctioned liver tissue when the mice liver was subjected to suction pressures of −1,−3,−5 and −15 kPa using the suction device. Each value represents mean ± S.D. (n=3)

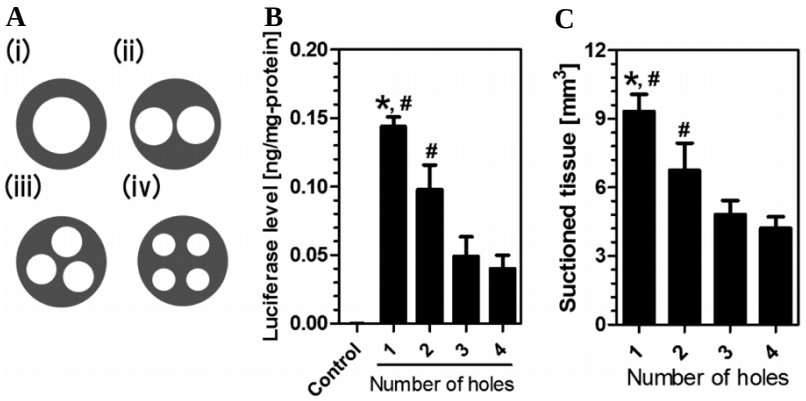


Fig.9 Relationship between the degree of tissue deformation and the transgene expression level.

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. Each value represents mean ± S.D. (n=3)

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.8**,

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.9A)** and confirmed that larger deforming action volumes obtained by a device with single hole by suction got relatively higher transgene expression **(Fig.9B-C)**. These results support the hypothesis that the tissue deformation is a key parameter influencing the efficiency of the tissue suction method.

On the other hand, considering animal experimental procedures, several factors including plasmid administration dose, composition of pDNA solution, intravenous injection rate and interval time between pDNA injection and liver suction were examined. 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.10**, 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.

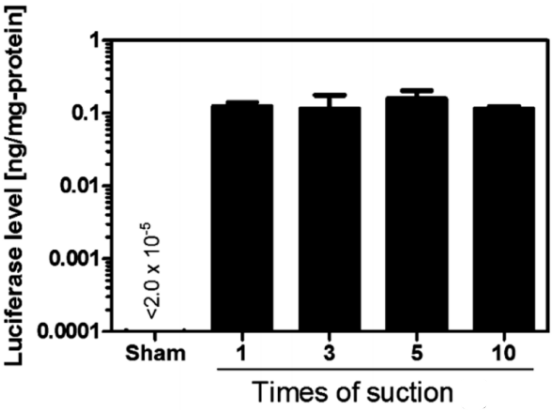


Fig.10 Effects of repetition numbers of liver suctions.

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.11**, 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.

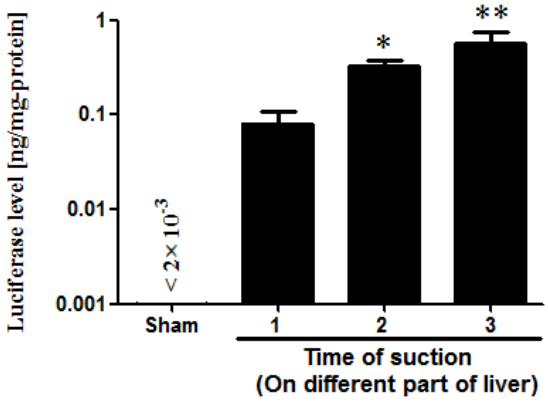


Fig.11 Effect of area of suction on luciferase expression.

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

c. Effect of multiple suction device on luciferase expression

In consideration of feasibility of performing liver suction-mediated transfection, I constructed four types of new device, as shown in **Fig.12A**, the diameters of each suction-pore on the device are same 3 mm. From number 1 to number 4 device, the increasing number of pores on the device are able to provide from 1 to 4-fold attachment of suction on liver surface. As shown in **Fig.12B**, I

got identical results that along with increased attachment of liver surface from number 1 device to number 4 device, transgene luciferase expression was relatively enhanced. These results suggest that using multiple 4-pore suction device also enhanced the transfection level for liver suction-mediated method in mice.

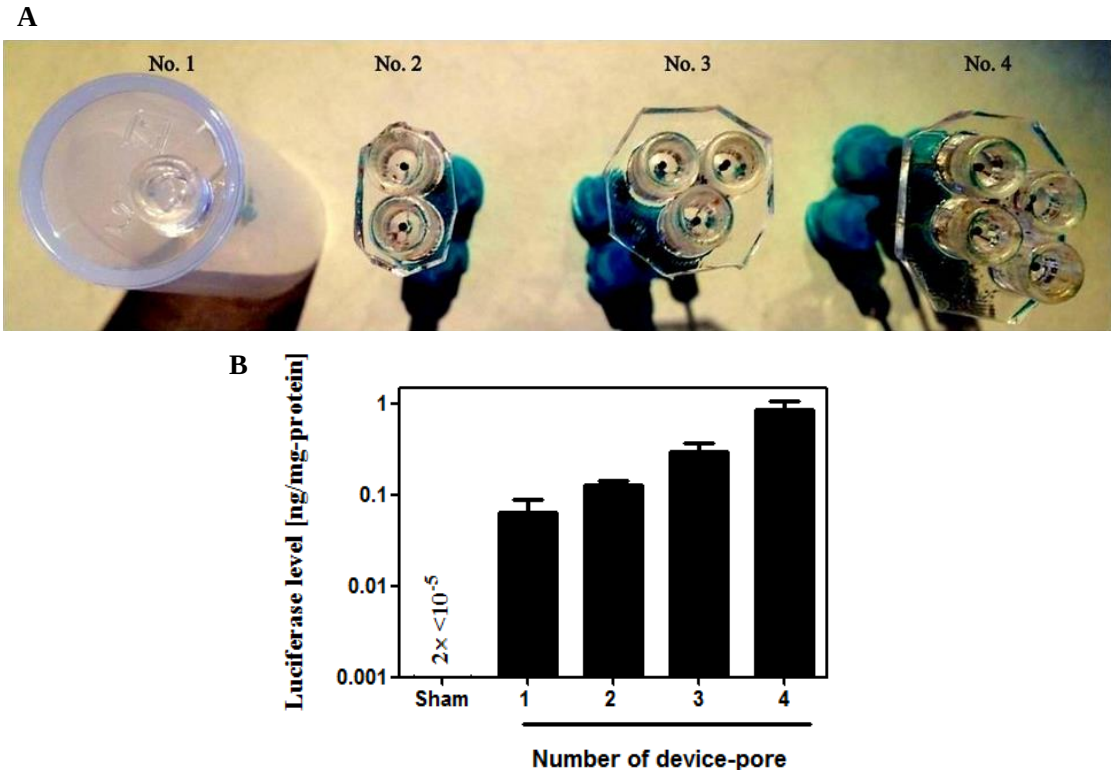


Fig.12 Effect of multiple suction device on luciferase expression.

A) Four different tissue suction devices with different numbers of pore. (diameter of single pore is 3 mm) **B)** Transgene expression level in the suctioned liver after using a different suction device. Each value represents mean ± S.D. (n=4 or 5).

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.10)**. Second, I found that increasing area of suction treatment can enhance transgene expression under one time injection of naked pDNA **(Fig.11)**. 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. Furthermore, I developed a

new type of device named multiple suction device with four pores. By the means of multiple device, approximate 8-fold increase in transgene expression level was obtained **(Fig.12)**. Data from such experiments could allow researchers to effectively perform transfection by using the multiple suction on mice liver.

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

I-3-1 Introduction

The past decade significant progress has been made in the major three gene delivery systems: (1) viral vector based; (2) nonviral vector based; (3) physical methods [47]. However, we are still far from the ideal gene delivery system suitable for clinical application. Safety issue in gene therapy, one of the most directly and real-life points, has been the hottest public concern. While viral vectors are highly effective and have been used in a few clinical trials, the intrinsic property of viral genome and proteins in stimulating an immune response remains as the largest hurdle [48-51]. In nonviral vector based gene delivery, variety of natural and synthetic compounds have been used to reduce the possibility of their pro-inflammatory production [52, 53], but the effectiveness of nonviral gene delivery remains lower than that of viral vectors in order of magnitude [54]. As results, currently nonviral vectors are generally considered unacceptable for clinical use. Physical hybrid system used for gene delivery is relatively new at present. Physical techniques of gene delivery have demonstrated their potential to transfer DNA into target tissue, but the problems of tissue damage or invasive surgical treatment are big challenges for physical gene delivery system [55].

With the involvement of new technology and computer systems, and understanding of biological systems, overcoming technical problems of current methods is highly feasible. One of the most promising features of gene delivery for gene therapy is in hand of those who are able to combine the principle of cell biology, engineering, and computer science. In this section, to answer these questions, I examined liver injury index such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the serum to determine the occurrence of liver damage after liver suction-mediated treatment with different type of suction device. it is also important to consider the proinflammatory cytokine production as a significant side effect caused by the *in vivo* nucleic acid transfection. Here I evaluated proinflammatory cytokine concentrations in serum including TNF-α, and interleukin (IL)-6, and performed histological examination on infiltration of inflammatory cells.

I-3-2 Results

a. Effect of suction pressure magnitude on liver injury

The liver specific enzymes, ALT and AST, are commonly used as standard indexes of liver injury. It has been reported that slight increase of serum ALT and AST levels occurred after tissue pressure and tissue suction treatments [16, 29]. In my study, we assembled a suction pressure controlled system to perform suction with accurate suction pressure, to investigate suction pressure effects on liver injury. As shown in **Fig.13**, the following magnitudes of the suction pressure were employed in this experiment: -5, -15, -30 and -40 kPa. Both ALT and AST activities in serum were measured at 6 h after liver suction treatment. ALT activities at -5, -15, -30 kPa were not significantly different from that at 0 kPa (sham treatment), whereas the activity at -40 kPa was significantly higher than that at 0 and -5 kPa. 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. When the liver suction was performed at -5 kPa, an increase in ALT activity was not observed and AST activity was significantly lower than those of higher pressure groups (-15, -30, -40 kPa). Taking results above together, -5 kPa suction pressure was selected as optimal condition for other experiments in this study.

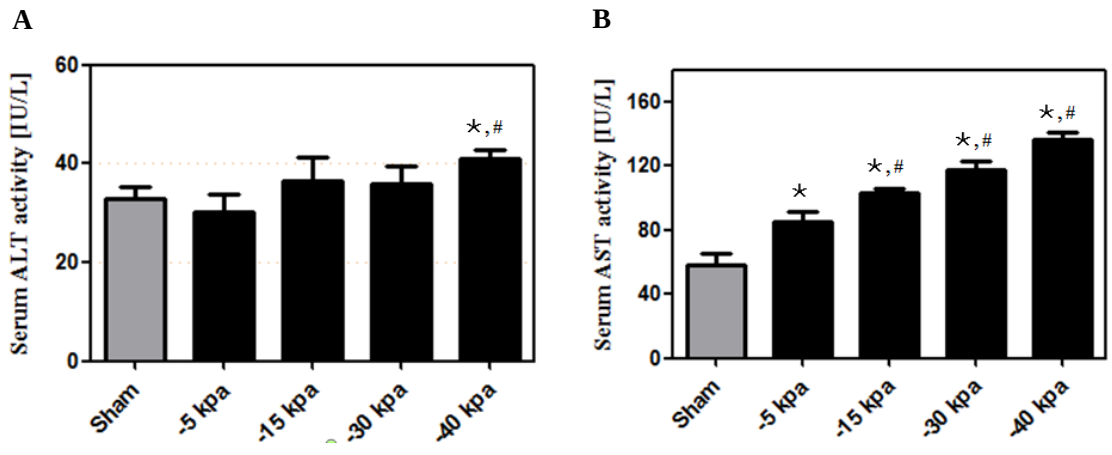


Fig.13 Effects of the minimum magnitude of the suction pressure on liver injury.

A) ALT and **B)** AST activities in serum 6 h after the liver suction treatment. Each value represents mean ± S.D. (n=4 or 5). $^{*}p < 0.05$ *versus* Sham group. $^{\#}p < 0.05$ *versus* -5 kPa.

b. Effect of softness of suction devices on liver injury

To examine whether the softness of device could have an effect on liver injury by suction treatment, we molded three types of suction devices in an increasing degree of hardness by adjusting material component of polydimethylsiloxane (PDMS) polymer and curing agent platinum-based catalyst in weight ratios including 10:1, 15:1 and 20:1 [56]. **Fig.14A-B** shows the effect of softness of suction device on liver injury as indicated by serum AST and ALT levels by employing increasing suction pressure of -5 kPa and -40 kPa. Both ALT and AST activities in serum were measured at 6 h after the liver suction. Both ALT and AST activities at -5 kPa were not significantly different between soft and hard devices. Whereas, under higher -40 kPa suction pressure, significantly lower liver injury was observed using a soft device compared to hard device. In addition, I also examined the luciferase transfection levels under -5 kPa and -30 kPa suction treatment with soft and hard devices (**Fig.14C)**, but no significant differences between soft and hard device were observed.

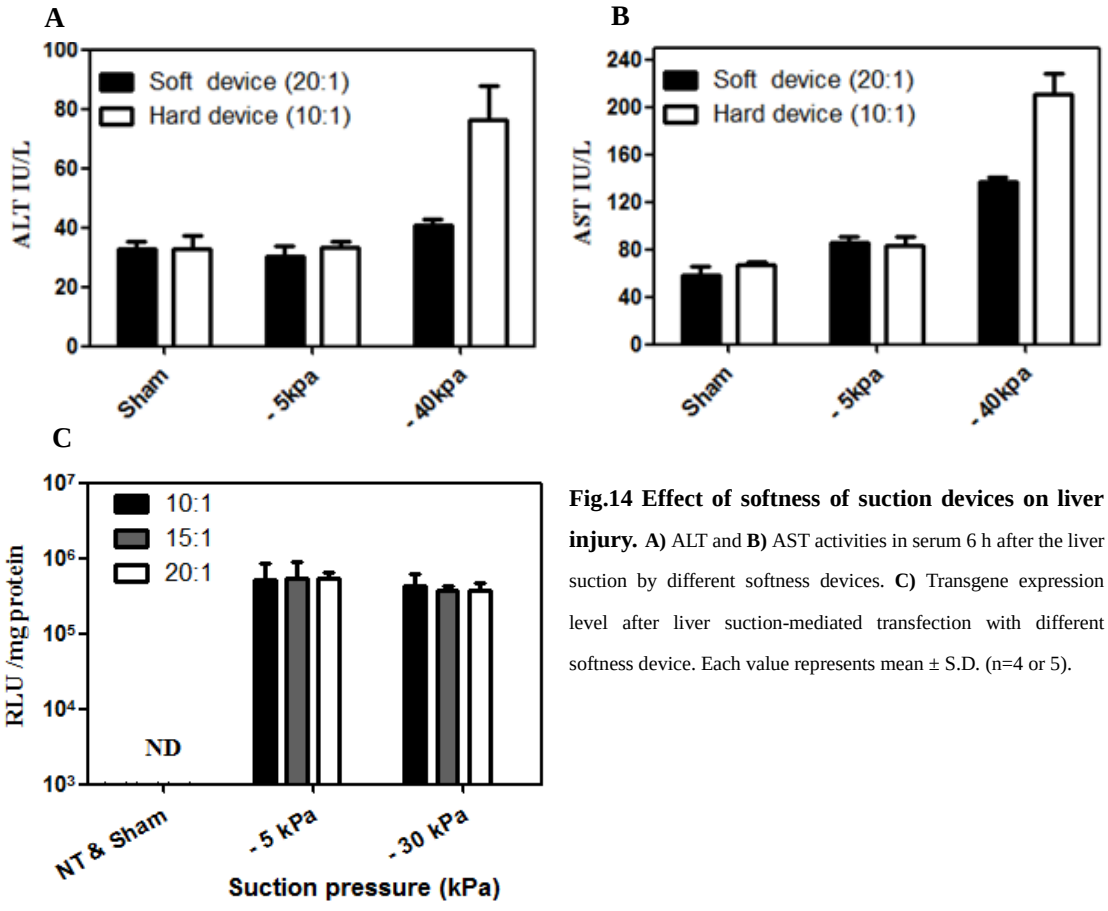


Fig.14 Effect of softness of suction devices on liver injury. A) ALT and **B)** AST activities in serum 6 h after the liver suction by different softness devices. **C)** Transgene expression level after liver suction-mediated transfection with different softness device. Each value represents mean ± S.D. (n=4 or 5).

c. Time-course of serum transaminase activities

It has been reported that the hydrodynamic-based procedure caused transient liver damage with high serum ALT and AST levels, which return to normal in a few days [5, 6]. On the other hand, liver injury which occurred under liver suction-mediated transfection also showed a recovery within 24 h [16]. I assumed that, similar to the case mentioned above, transiently increasing levels of ALT and AST were attributed to release from hepatocytes when the cellular membrane was transiently rendered permeable by physical treatment on liver. **Fig.15A-B** shows the serum concentration profiles of ALT and AST activities detected at 6 h after suction treatment using four different types of suction device mentioned in **Fig.12A**. Along with enlarging suctioning area on liver surface by various devices form 1-pore to 4-pore devices, serum ALT and ALT activity level were slightly higher in 4-pore device group than 1 pore device group. However, even the relatively high levels of ALT and AST activities in 4-pore device treat groups returned to normal level as control group with intravenous injection of saline within 48 h **Fig.15 C-D.**

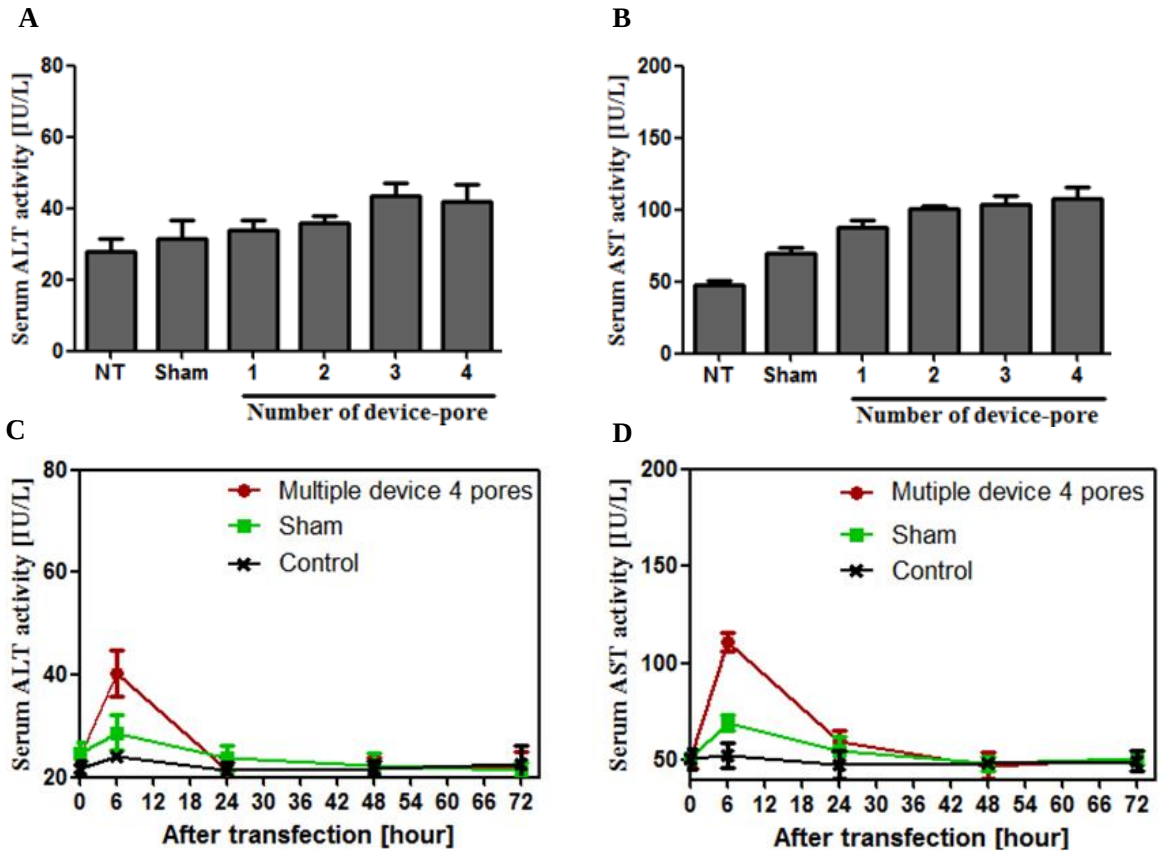


Fig.15 Effects of multiple suction device on liver injury.

A) ALT and **B)** AST activities in serum 6 h later the liver suction by different devices with indicated number of pores from 1 to 4. **C)** ALT and **D)** AST in serum was measured at 0, 6, 24, 48 and 72 h after liver suction transfection. Each value represents mean ± S.D. (n=4 or 5).

d. Evaluation of proinflammatory cytokine production induced by liver suction transfection

To evaluate proinflammatory cytokine production induced by liver suction-mediated transfection, the TNF-α and IL-6 concentrations in serum were determined by ELISA in a time dependent manner after 50 μg of pCMV-Luc administration by liver suction-mediated transfection. Cationic liposome-mediated transfection was performed as a positive control. As shown in **Fig.16**, TNF-α and IL-6 in serum exhibited no significant changes, compared with nontreatment group and sham group, at 3, 6, 12 and 24 h time points.

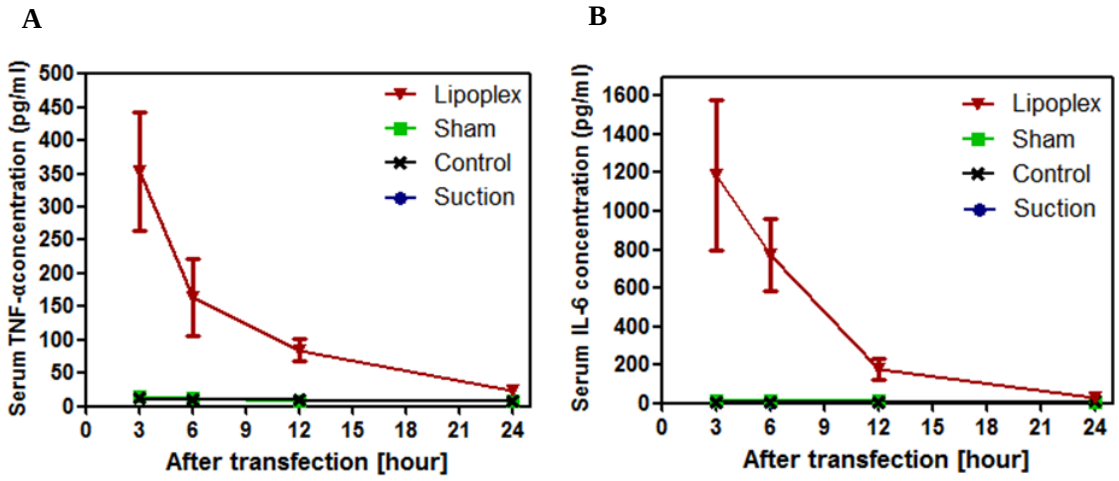


Fig.16 Evaluation of serum proinflammatory cytokine concentrations induced by liver suction-mediated transfection. A) TNF-α, and **B)** IL-6 concentrations in serum 3, 6, 12 and 24 h after administration of 50μg of pCMV-Luc. Solid triangles, administration of 30 μg of pCMV-Luc by cationic liposome-mediated transfection; solid squares, sham groups injected with pCMV-Luc by intravenous administration without suction treatment; crosswire non-treatment group; solid circle liver suction-mediated transfection. Each value represents mean ± S.D. (n=4 or 5).

e. Histological observation of mice liver after plasmid DNA administration by various transfection methods

In the left lobe of mice liver of sham group, the basic features of hepatocytes, portal triads and vasculature appeared within normal limit. The liver treated by suction-mediated transfection did not show any pathological alternations much as inflammation, necrosis and fibrosis at 3 h and 24 h. **Fig.17B**. In contrast, the liver of mice having cationic liposome based transfection mice showed massive infiltration of mononuclear cells at 3 h and 24 h after the treatment in **Fig.17C**.

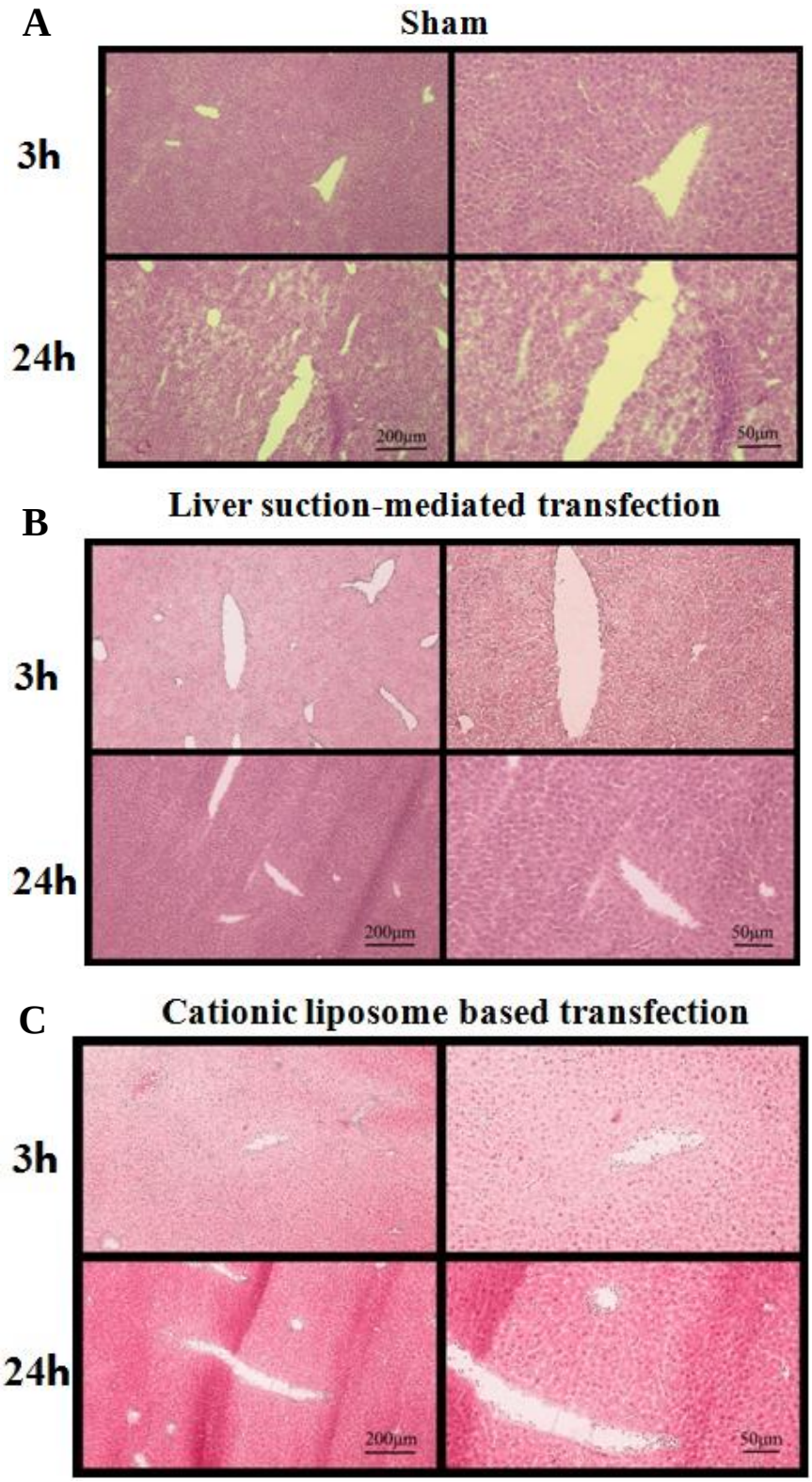


Fig.17 Histological examination of mice liver after plasmid DNA administration by liver suction-mediated and cationic liposome methods.

H&E staining of the liver sections at 3 and 24 h after 50 μg pCMV-Luc administration by liver suction-mediated transfection and 30 μg pCMV-Luc cationic liposome based transfection methods. Representative images were captured from 3 groups (scale bar: 200 μm and 50 μm). **A)** Sham group: normal lobular architecture and cell structure; **B)** Suction-mediated transfection group: few numbers of mononuclear cells around can be seen around central vein; **C)** Cationic liposome based transfection group: Lipoplex induced a moderate increase in inflammatory cell numbers.

I-3-3 Discussion

To utilize the full clinical potential of gene therapy, it is important to consider the tissue damage caused by transfection procedures. In this section, I carried out both biochemical and histological assays to assess the liver injury by transfection using liver suction-mediated method. In the beginning, I examined the effects of liver suction on the hepatic damage for different magnitudes of suction pressure. As shown in **Fig.13**, both serum ALT and AST activities increased with a decrease in the magnitude of the suction pressure and the AST activity at -5 kPa was significantly higher than at 0 kPa. In case of multiple suction device groups, slight increase of ALT and AST activities in serum was observed at 6 h after suction treatment. However, both ALT and AST activities returned to normal levels within 24 h. Considering these results, the liver suction method with -5 kPa was proved to be the most feasible as it showed lowest hepatic injury in mice among tested. In previous study, our laboratory reported that both ALT and AST activities at 6 h after liver suction without control of suction pressure were significantly higher than the activities with 0 kPa, and the activities returned to normal level at 48 h [16]. In contrast, when the liver suction was performed at -5 kPa in this study, an increase in ALT activity was rarely observed and the increased AST activity quickly returned to normal level within 24 h. Consequently, the liver injury by the suction method was reduced by using the suction pressure control system.

The induction of proinflammatory cytokine is a serious side effect caused by various *in vivo* nucleic acid transfection. Several groups have reported that proinflammatory cytokine production, including TNF-α, IL-6, IL-12 and IFN-γ, is induced mostly by Kupffer cell Toll-like receptor 9-mediated recognition of the bacterial CpG motif in plasmid DNA backbone [57]. Suppression of cytokine production is considered as a pressing need to be improved for future clinical application. In this section, I demonstrated that liver suction-mediated transfection methods induced few inflammatory responses, including TNF-α, IL-6 production compared to sham groups **(Fig.16)**. Histological examination (**Fig.17)** corroborates the conclusion. Comparison with intravenous administration of cationic lipoplex group which resulted in heavy cell infiltration, the suction transfection of pCMV-Luc plasmid and sham groups, showed only few cells infiltrating around the portal tract.

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 as shown in **Fig.14**. When the suction pressure was over -40 kPa, soft device showed less liver damage than hard device. I believe that suction-mediated transfection method will be able to be abroad applied in transfection of other tissues with the development of engineering technology.

Chapter II

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

Section 1: Mechanisms of suction-mediated hepatic gene transfection

II-1-1 Introduction

The first challenge of *in vivo* gene delivery is how to bring the transgene across the extracellular barrier. Injection of naked plasmid DNA is regarded as one of the promising gene delivery methods due to its safety and simplicity compared with viral and non-viral carrier method. However, before DNA reaches the target cells, three major obstacles should be overcome. (1) Destruction of pDNA by serum nucleases [58, 59], opsonization, and inflammatory responses [60]. (2) Numerous host systems which can significantly impair gene delivery, resulting in sequestration of naked pDNA by first pass organs. For instance, after intravenous injection of pDNA, pDNAs firstly face primarily endothelial cells in which plasmid DNA is degraded as an exogenous material, resulting in no transgene expression [61, 62]. (3) Barriers including the extracellular matrix which can greatly affect traffic of vectors in and between tissues and into their target cells [63-65]. Similar to the tissue pressure-mediated transfection method studied in our laboratory for various organs, I hypothesized the enlargement of the fenestrae of endothelial cells should be underling mechanism by which naked pDNA are able to overcome sinusoidal walls and go on to hepatocytes. However it is not the case for liver, mice sinusoidal is leakier in the liver. On the other hand, getting DNA into cell is not the end, because the plasmid DNA must still navigate through the cytoplasm to reach the nucleus and then gain access to the nuclear compartment in order to be expressed. Some groups found that remarkable gap of transfection efficiency arises from differences in nuclear transcription between adenoviral and nonviral vectors in lipoplex based transfection in vitro [66-68].

In Chapter I, liver suction-mediated transfection was established successfully. In this section, I confirm that compared with nonparenchymal cells, the hepatocytes were mainly transfected by suction-mediated transfection. In addition I examined the intracellular effect of transcriptional level on transfection efficiency of plasmid DNA by suction-mediated transfection.

II-1-2 Results

a. Evaluation of cellular transfection in liver by suction-mediated transfection *in vivo*

The left lobe liver cells of mice treated by liver suction-mediated transfection of pCMV-Luc plasmid DNA were separated into parenchymal cells (PC) and nonparenchymal cells (NPC) by collagenase perfusion [69]. Suction treatments were performed by 1-pore device and 4-pore device. As shown in **Fig.18A,** in both of 1-pore and 4-pore groups, the luciferase expression levels in PC were significantly higher than NPC groups. The ratio of the luciferase expression in PC and NPC (PC/NPC) on a cell-number basis was calculated to be 14~25 fold, indicating that plasmid DNA was preferentially transfected to hepatocytes by liver suction-mediated transfection. **Fig.18B** shows the purity of >97% and cell viabilities 24 h after separation by collagenase perfusion under light microscopy.

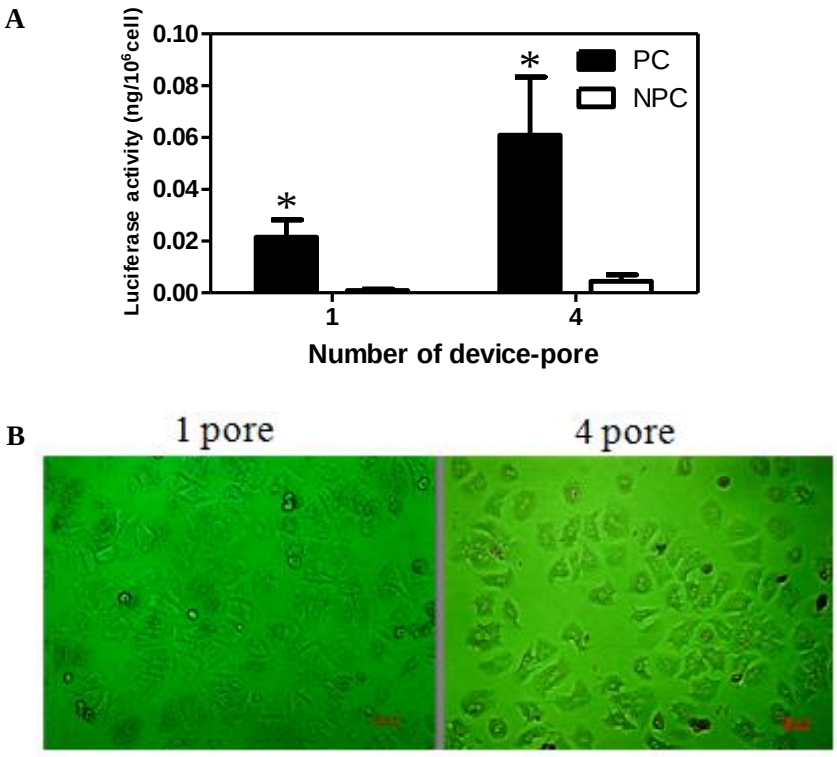


Fig.18 Cellular transfection after liver suction-mediated transfection.

A) Transfected luciferase expression level after suction-mediated transfection of pCMV-Luc in liver parenchymal cell (PC) and nonparenchymal (NPC). After digestion of the left lobe of liver by collagenase, PC and NPC were separated by different centrifugation, and the luciferase activity in each cell fraction were counted as described in material and methods. **B)** After separation of liver cells, PC cell were primary cultured for 24 h, and the cell purification was reconfirmed, by observation of non NPC cell under light microscopy. PC cells purity > 97%. Each value represents mean ± S.D. (n=3 or 4). $^{*}p < 0.05$ *versus* NPC groups.

b. Effect of the transcription factors on luciferase expression

To examine the effect of transcription factors on luciferase expression by suction-mediated transfection, firstly I selected the pathway profiling luciferase system as a means to assess cell signaling pathway activation *in vivo*. As shown in methods and materials part, various plasmid vectors (pTAL-Luc, pAP-l-Luc, pNFkB-Luc, pCRE-Luc and pSRE-Luc) containing different binding sequences for transcription factors were tested. Mice treated by suction-mediated transfection with each pAP-1-Luc, or pNFκB-Luc showed significantly higher luciferase expression levels compared with a control plasmid, pTAL-Luc injected mice. Mice injected with other plasmids showed much lower luciferase expression level, suggesting a lower contribution of CRE and SRE binding site to this event **(Fig.19)**.

In addition, I examined the changes in the mRNA expression of endogenous genes (c-fos and c-jun), at 1, 3 and 6 h after suction-mediated transfection. **Fig.20** shows the time course of mRNA expression in left lobe of mice liver after suction-mediated transfection of pCMV-Luc plasmid. The levels of c-fos and c-jun mRNAs were considerably increased soon after treatment, about 37.5- and 8.7-fold increases were observed for c-fos and c-jun, respectively. The increase in mRNA expression was 18% that of liver after hydrodynamics-based transfection that has been reported to induce similar transcriptional factor activation [77]. Then, the levels of both mRNAs fell and reached normal levels at 6 h.

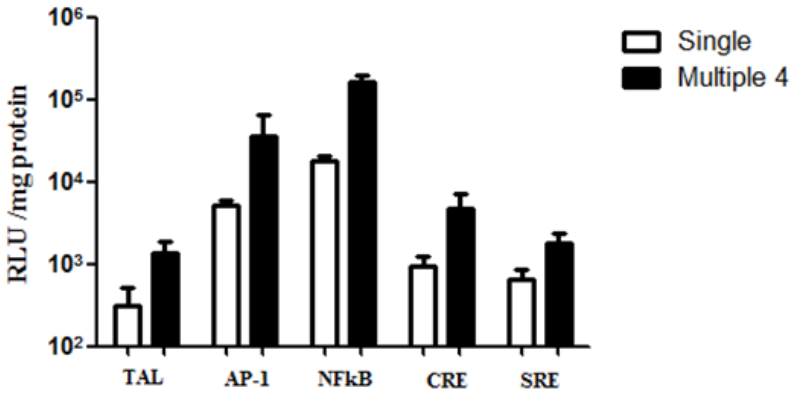


Fig.19 Effect of the transcription factors on gene expression levels by the liver suction-mediated transfection method. At 8 h after transfection of 50 μg pTAL-Luc, pAP-l-Luc, pNFkB-Luc, pCRE-Luc, and pSRE-Luc by liver suction-mediated transfection method. Each value represents mean ± S.D. (n=3 or 4).

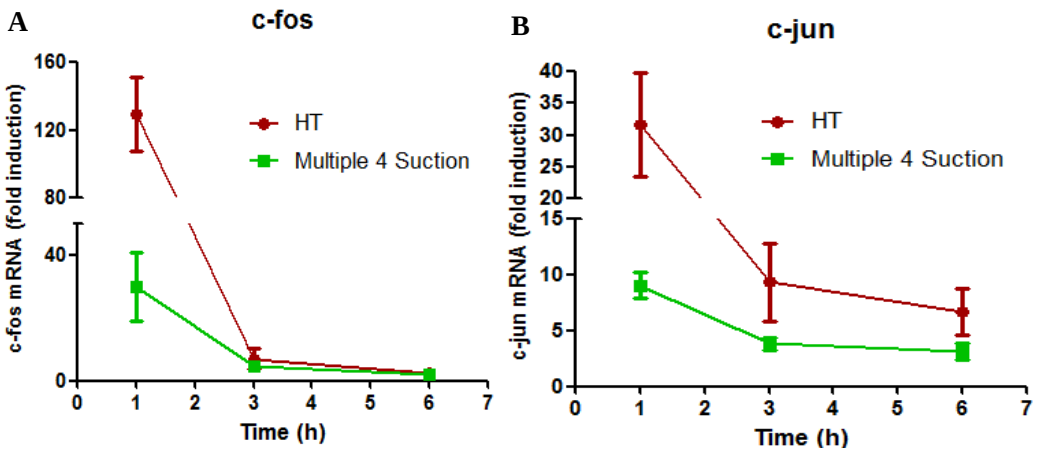


Fig.20 Induction of activator protein-1 (AP-1) production by liver suction-mediated transfection method. **A)** c-fos and **B)** c-jun mRNA levels after 50 μg pCMV-Luc administration by (square) liver suction-mediated method and 10 μg pCMV-Luc administration by (circle) by hydrodynamics-based transfection (HT). The levels were normalized using the mRNA level of gapdh in each sample. Then the x-fold induction was calculated using the mRNA levels between treated and control mice. Each value represents mean ± S.D. (n=3 or 4)

II-1-3 Discussion

Intravascular delivery of gene is considered as an attractive approach because the plasmids with the targeted gene can be disseminated throughout the tissue, and as a result more cells can get transfected compared to local transfection methods. However, in case of naked pDNA delivery system, due to its inability to enter cells and to its susceptibility to enzymatic degradation caused by nucleases in blood, many efforts have been made to overcome plasma barrier for naked pDNA transfer. Efficient transgene expression in hepatocytes can be obtained following delivery of naked pDNA combined with physical force, which changes physiological profile, such as permeability of cell membrane in case of hydrodynamic transfection of naked pDNA [70, 71]. Based on these researches, our laboratory established pressure-mediated *in vivo* transfection method to successful transfection in the liver and kidney, which increase the permeability of the cell membrane is one of underling mechanism.

In this section, first I demonstrate that naked pDNAs were mainly uptake and transfected in hepatocytes (PC) (**Fig.18A)**. PC transgene expression level was 16.5-fold greater than NPC expression level. This phenomenon suggests that after intravenous injection of naked pDNAs, they pass through sinusoidal wall subsequently reaching to hepatocytes, and then is taken up by hepatocytes to get successful transfection. Furthermore, there was a 3.85-fold increase of transgene expression levels treated by 4-pore device compared with 1-pore device **(Fig.18A)**, suggesting that enlargement of suction treatment area enhanced transgene expression level.

About translocation of naked plasmid DNA, once in the hepatocyte cytoplasm, plasmid DNAs do not stay naked for long, as the negatively charged nucleic acid are quickly coated with various cytoplasmic protein and cationic materials, at least some of these species are sequence-specific DNA-binding proteins [72]. Dean et al reported that plasmids containing promoter or enhancer sequences with binding sites for transcription factors are able to productively move in the cytoplasm [73]. In addition, transcriptional factors are also reported to participate in transcription process of pDNA [74]. Furthermore, reactivation of silenced transgene expression by administration of histone deacetylase [75, 76] or stimulation with physical force [77, 78] has been reported both in vitro and vivo. In this study, as shown in **Fig.19**, significantly higher luciferase gene expression was observed in suction-mediated transfection of pAP-1-Luc and pNFκB-Luc in the liver compared with pTAL-Luc transfection group due to no binding sites for transcriptional factors. Moreover the activation of transcriptional factor, c-fos and c-jun which are component proteins of AP-1, was observed at 1 h after liver suction-mediated transfection, as their mRNA level increased rapidly and about 37.5- and 9.5 folds, respectively **(Fig.20)**. Peaking at 1 h, the levels of both mRNA fell and reached almost normal level at 6h. In the same way, the expressed luciferase expression levels peaked at about 12 h and then decreased gradually to background levels after 1 week following suction-mediated transfection of pCMV-Luc containing binding elements for AP-1 and NFκB [79]. Together with the time lag between c-fos and c-jun expression, AP-1 mediated gene expression, and the half-life of luciferase (about 3 h), these results support the view that liver suction-mediated transfection activates the transcriptional factors, resulting in a high levels of gene expression.

In conclusion, efficient gene delivery and targeted expression to hepatocytes was achieved by liver suction-mediated transfection. Moreover, I demonstrated that liver suction-mediated transfection method activated the transcriptional factors such as c-fos and c-jun in short time. These findings shed a light on *in vivo* transgene expression based on naked plasmid DNA.

Section 2: Long-term gene expression using CpG free based plasmid DNA vector

II-2-1 Introduction

An efficient and safe gene therapy should offer both an appropriate level and desired duration of expression. Depending on both the therapeutic strategy and the disease, the long-term transgene expression must be kept efficiently. However, many gene delivery systems are proved to be less effective as the transgene expression is transient in both viral and nonviral based approaches [80, 81]. Most clinical gene transfer systems use viral vectors, such as retroviruses, adenoviruses, and others. Despite effective and stable transduction, there are concerns about risks such as deleteriously infectious form or activation of oncogenes [82-84]. Moreover, viral vectors like adenovirus are known to stimulate severe innate and adaptive immune responses and to induce cellular and humoral responses to the transgene product, resulting in failure to provide long-term gene expression [85-87]. On the other hand, for nonviral vectors in gene therapy, one of the major obstacles are the short duration of transgene expression. Standard plasmid vectors composed of a transgene expression cassette and bacterial backbone DNA are able to express a high level of transgene product in short term, but the transgene product usually declines to low or undetectable level within a period of days even if the transfected cell and vector DNA are not lost [88].

In last decade, several research groups presented an interlined strategy based on the fact that cumulative reductions in CpG dinucleotides within pDNA lead to decrease level of acute toxicity and substantially improve the durability of therapeutic transgene expression in mice. In this context, MIDGE vector [89, 90], minicircle vector [91] and already being on the market, so-called CpG free vector [92-94] are available. In Chapter I the liver suction-mediated transfection has been shown not to elicit any immune response and liver injury. In addition to the hepatocytes-target transgene efficiency, I speculated that sustainable transgene expression can be established by combination of suction-mediated transfection method with CpG free vector based plasmid DNA. I examined the expressing period for reporter luciferase and therapeutic interleukin-22 by two types of device such as 1-pore and 4-pore device. Here, I demonstrate that intravenous injection of CpG free based plasmid DNA followed by liver suction-mediated transfection was found to be a promising approach for sustainable gene transfection in liver.

II-2-2 Results

a. Time-course of transgene luciferase expression in the liver after suction-mediated transfection of CpG free vector based plasmid

To examine the effect of CpG motif in the plasmid vector, I compared the expression profiles of two luciferase-expression plasmid vectors, pCMV-Luc and CpG free vector based pCpGfree-Luc**.** pCpGfree-Luc and pCMV-Luc were individually injected to mice in equimolar concentration, and suction was applied on left lobe of mice livers immediately. After that the obtaining transgene expression levels are shown in **Fig.21**, where the transgene expression from pCMV-Luc is slightly higher than from pCpGfree-Luc within 1 day thereafter. However, pCMV-Luc showed a typical decline to undetectable background level after 7days. In contrast, the mice transfected with pCpGfree-Luc with 1-pore device group, sustained transgene luciferase expression in the liver over the period of 40days. Moreover, in 4-pore device group showed sustained transgene luciferase expression was detected up to 60 days.

In detectable period of luciferase expression in both pCpGfree-Luc and pCMV-Luc transfected groups, 4-pore device groups showed significantly higher luciferase expression level in compassion with 1-pore device group. These results indicate a trend in that transgene expression by suction-mediated transfection of pCpGfree vector prolonged in comparison with CpG containing vectors.

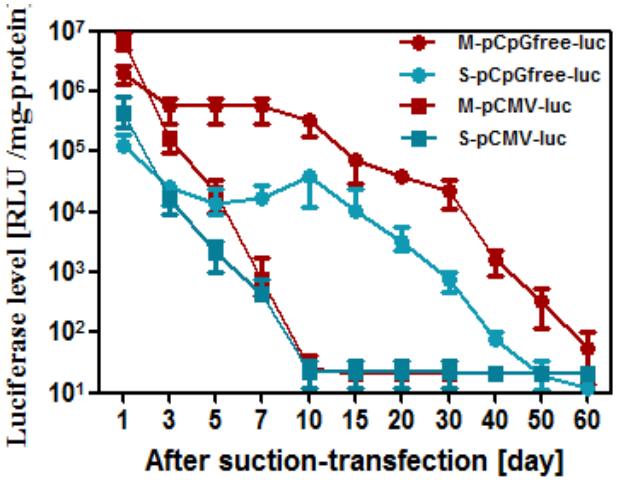


Fig.21 Comparison of CpG free based hEF1-promoter driven (pCpGfree-luc) with CpG-inclusing, CMV-promoter (pCMV-luc) luciferase expression in mice liver. Plasmids were transfected by liver suction-mediated transfection using (S) 1-pore or (M) 4-pore device. Each value represents mean ± S.D. (n=3 or 4)

b. Time-course of transgene interleukin-22 expression in the liver after suction-mediated transfection of CpG free vector based plasmid

Elimination of transfected cells by apoptosis or immune cells is one of the major reasons for transgene silencing, due to induction of cell-mediated immune response to the transgene product which can be perceived as "foreign" by the host's immune system [95-97]. I investigated whether transgene product such as secreted interleukin-22 (IL-22) could achieve sustainable expression by liver suction-mediated transfection combination with CpG free vector based plasmid.

Methodology of both plasmid construction and *in vivo* mice transfection are similar to experiments described in II-2-3-a. I compared the expression profiles of two IL-22-expression plasmid vectors, pCMV-IL22 and CpG free vector based pCpGfree-IL22. Equimolar amounts of either CpGfree-IL22 or pCMV-IL22 were injected and suction was applied to left lobe of mice livers immediately. The obtaining expression-over-time graph is shown in **Fig.22.** In both serum and liver, the transgene expression of pCMV-IL22 is slightly higher than pCpGfree-IL22 during the 1 day. Afterwards pCMV-IL22 showed a significant decline to undetectable background level in 3~5 days. In contrast, the mice transfected with pCpGfree-IL22 demonstrated sustained transgene IL-22 expressions in serum and liver were up to 35 days.

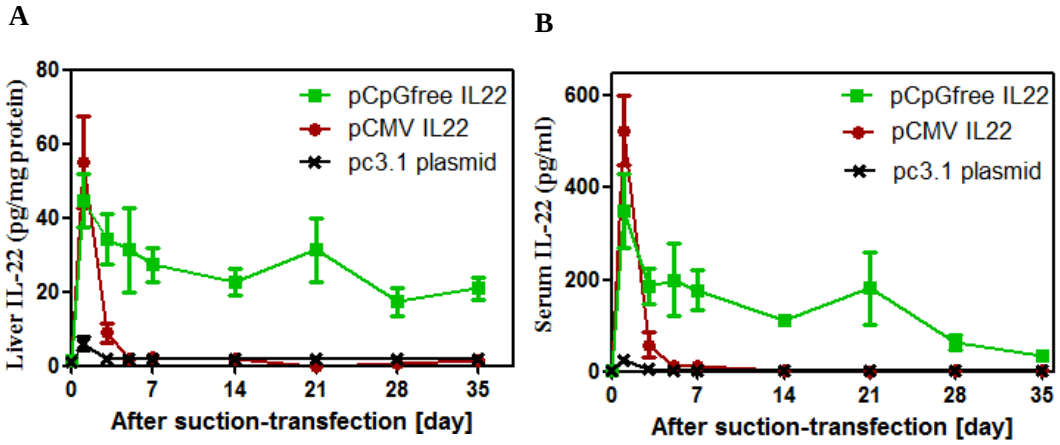


Fig.22 Long-term expression of interleukin-22 from CpG free based vector.

A) liver and **B)** serum IL-22 protein levels after equimoler IL-22 expressing pDNA administration by liver suction-mediated method. **(Square)** pCpGfree-IL22: CpG free based vector, hEF1-promoter driven with CpG-inclusion; **(Triangle)** CpG-inclusion, CMV-promoter; **(Crosswire)** nude plasmid DNA, pc3.1 vector. Plasmids were transfected by liver suction-mediated transfection using 4-pore type of device. Each value represents mean ± S.D. (n=3 or 4)

II-2-3 Discussion

Reduction in the numbers of CpG motifs is extensively used as an effective mean for prolonging the duration of transgene expression. Usually, gene therapy vectors contain numerous unmethylated CpG motifs, which upon introduction into a mammalian host interact with host's toll-like receptor-9 (TLR-9) to stimulate immune responses [98, 99]. These responses induce innate, proinflammatory immune responses, which would decrease the therapeutic effect and limit the practical application of the vectors. Upon transduction of cells, the presence of CpG motifs within a gene therapy vector may have two disadvantages: (1) Induction of proinflammatory cytokine responses through TLR engagement; (2) the chronic suppression of transgene expression coordinated by methy-CpG binding protein. Therefore, one could predict that removal of CpGs from the plasmid DNA would prevent the recognition of the pDNA as exogenous materials, and consequently solve these two major problems. Several groups reported that physical gene delivery techniques could be combined with rational vector design to further improve the long-term expression of plasmid DNA mediated transgene expression *in vivo*, such as in case of electroporation [100] and hydrodynamic-based injection [101, 102].

In this study, I employed CpG free vector as a tool to test whether combination with liver suction-mediated transfection can improve the long-term sustainable transgene expression in the liver. As shown in **Fig.21**, comparing with CpG containing pCMV vector whose expression declined rapidly within 5 days, expression with the CpG-free vector slowly decreased in 50 days. Considering immune response to transgene products, additionally I examined the mice IL-22 expression from pDNA based on CpG free and pCMV vectors. As shown in **Fig.22A-B**, the transgene IL-22 proteins were obtained sustainably in liver and serum more than 35 days, and the decline was not observed in pCpGfree-IL22 suction-mediated transfection group after 7 days.

The present study provides evidence that sustainable transgene expression in liver can be obtained by liver suction-mediated transfection method in combination with CpG free vectors.

Section 3: Therapeutic effect of suction-mediated IL-22 gene delivery on acute hepatitis mice models

II-3-1 Introduction

Various biological activities of cytokines played an essential role in cell proliferation, differentiation and mobility, as well as in defense against pathogens and tumours. Due to their natural role as immune modulators, over the three past decades, along with development of cytokine immunotherapy, many cytokines have been identified as a promising therapeutic candidate for the treatment of a number of infectious diseases [103, 104]. However two properties shared by most cytokines are thought to be a crucial role in the solvement of treatment-associated adverse effects. (1) Cytokines are pleiotropic, that is to say that they are able to influence more than a single cell type. In nature, some cytokines can stimulate cell types that mediate opposing biological effects. (2) Cytokines have a short serum half-life, the rapid disappearance of therapeutic cytokines leads to high dose administration to achieve their therapeutic effect [105, 106]. While effectively enhancing therapeutic efficacy, high dose exacerbate pleiotropic activities that manifest as adverse effects in clinical application [107, 108]. In spite of molecular engineering approach for cytokine with prolonged half-life, enhanced specificity or localization activity is therefore required to enhance the pharmacological properties of these proteins [109].

In this section, I try to solve these two points mentioned above. IL-22 was selected as a therapeutic protein showing more targetable than other cytokines. IL-22 is unique among the interleukins because it does not directly regulate the function of immune cells [110, 111]. Moreover, IL-22 targets cells at outer-body barriers, such as the skin and tissue of the digestive and respiratory systems, as well as cells of liver, kidney and pancreas. In addition, IL-22 protects its target cells against damage, by inhibiting their differentiation and/or increasing their proliferation. These effects are not shared by other cytokines [112, 113].

Numerous in vitro and *in vivo* mouse studies have shown that IL-22 has a strong effect against hepatocyte damage. The protective role of IL-22 in acute hepatitis was confirmed using IL-22-deficient mice which are highly susceptible to concanavalin A (Con A) - induced liver injury, and IL-22 overexpressing mice which are highly resistant to Con A mediated liver injury [114, 115]. Furthermore, IL-22 application ameliorated liver injury, fatty liver, and hepatic oxidative

stress in mouse models of acute and chronic alcohol-induced liver damage [116]. However, most of the animal studies were intended via circulation of IL-22 to cure different diseases.

Particularly worth mentioning is, in the aspect of gene therapy targeted hepatocyte, hydrodynamic-based injection might immediately obtain surplus level of transgene IL-22 which leads to side effect among various physical gene delivery systems. In consideration of clinical infeasibility of hydrodynamic-based procedure and transnormal level of IL-22, based on Chapter I, I assumed that liver suction-mediated transfection is able to cure Con A-induced hepatitis mice model in a controllable pattern of transgene expression level of IL-22.

II-3-2 Results

a. Protection of Con A-induced liver injury by suction-mediated transfection of IL-22

As results shown, liver transgene expression of IL-22 at one day after pDNA administration was found that in Chapter II-2 both of liver suction-mediated based transfection of pCMV-IL22 and pCpGfree-IL22. To further confirm the therapeutic effect of IL-22, I examined its protective effect against Con A-induced acute liver injury in mice. Mice were transfected with equimolar amount of pCMV-IL-22 and pCpGfree-IL22 by liver suction-mediated transfection 1 day before intravenous injection of 10 and 15μg/kg Con A per mice. Eight hours later, liver injury was quantified by measuring collected serum enzyme activities of AST. As shown in **Fig.23**, administration of 10 and 15 μg/kg of Con A caused significant elevations in serum AST level activity at 8 h post injection in mice treated with nude pc3.1 pDNA and sham groups. Serum AST levels were markedly lower in mice treated with the pCMV-IL22 and pCpGfree IL-22.

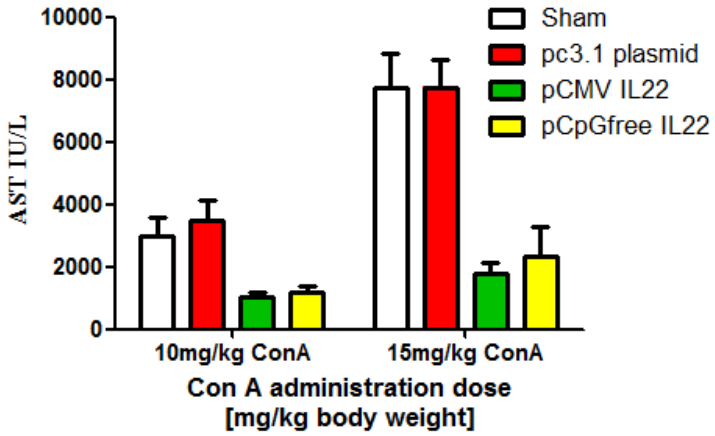


Fig.23 Protection of mice against Con A-induced liver injury by liver suction-mediated transfection of IL-22 pDNA. Balb (6 weeks old) mice were treated by liver suction-mediated transfection of IL-22 expression plasmid, followed 1 days later by intravenous injection of Con A. Mice were then sacrificed 8 h post Con A injection, and serum was collected for determination of AST levels. Each value represents mean ± S.D (n=3 or 4)

b. Survival rate of hepatitis mice having suction-mediated transfection of IL-22

The survival time of the mice with the Con A-induced liver injury was significantly prolonged by liver suction-mediated transfection of IL-22 expressing plasmid DNA (pCMV-IL22 and pCpGfree-IL22) compared with nude pc3.1 DNA treated mice. When mice had intravenous injection of 15 μg/kg per mouse of Con A, they began to die at 6 to 12 h after administration. In total 50% of mice having nude pc3.1 DNA treatment group were dead within 12 h, and 75% of the mice died within 24 h. In contrast, transgene expression of IL-22 dramatically improved the survival of ConA-treated mice. Furthermore, this protective effect was higher in pCpGfree-IL22 group. **(Fig.24)**

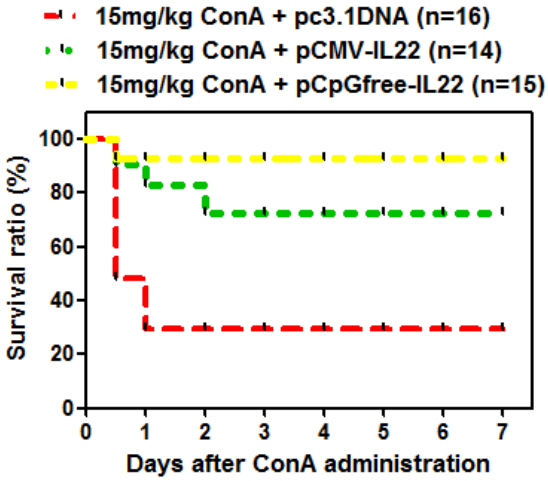


Fig.24 Effect of IL-22 expressed by liver suction-mediated transfection of pDNA on the survival of mice with ConA-induced liver injury. Balb (6 weeks old) mice were treated by liver suction-mediated transfection of IL-22 expression plasmid, followed 1 days later by intravenous injection of Con A. Kaplan-Meier curve shows the survival rates of each group from day 0 to day 7.

II-3-3 Discussion

Fulminant hepatitis resulted from several conditions, such as autoimmune hepatitis, alcohol consumption, and chronic or acute viral hepatitis infection, remains a significant cause of morbidity and mortality internationally [117, 118]. However, the underlying pathogenic mechanisms are still not fully understood. A high percentage of patients with chronic hepatitis C is still resistant to existing antiviral therapies, such as conventional interferon therapy [119]. Therefore, a novel therapeutic strategy is needed to help to ameliorate or prevent fulminant hepatitis.

The possibility of transferring functional genes to liver cells opens an entirely new horizon in the therapy of liver diseases. In this section, I provided experiments relating to plasmid based gene therapy with IL-22 against Con A induced fulminant hepatitis model.

Several reports have indicated that hydrodynamic-based method is applicable for transfection of naked pDNA encoding therapeutic proteins such as IL-6, IFN-α, and IL-22. As far as the side effect of cytokine therapy is concerned, IL-22 shows more potential due to its unique nature such as no affect on immune cells and specific targeting abilities [110, 111]. In this section, as shown in **Fig.23-24**, liver suction-mediated transfection of two type of IL-22 expressing plasmid DNA (pCpGfree-IL22 and pCMV-IL22) are proved to be effective in preventing liver injury induced by Con A administration [114].

In conclusion, my study demonstrates that liver suction-mediated transfection of therapeutic protein IL-22 for treatment of fulminant hepatitis would be a valuable approach for future liver-target gene therapy.

Summary

In this thesis, I tried to develop and optimize the *in vivo* liver suction-mediated transfection method towards realization of *in vivo* gene therapy by focusing on; (1) development of liver suction-mediated transfection to perform transgene under precisely controllable conditions to increase safety and transgene efficiency by computer controlled system, (2) optimizing the suction device to increase transgene expression, (3) exploration of extracellular and intracellular underlying mechanisms of liver suction, (4) investigation of long-term transgene expression through combining CpG free based vector with suction-mediated transfection, (5) confirmation of therapeutic effect of hepatic transfection of IL-22 on Con A-induced hepatitis model using suction-mediated approach. The following conclusions were obtained.

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

First, I assembled a computer system to control the suction pressure and pressure waveform precisely. Under the conditions of -5 kPa suction pressure and 0.5 s - 3 s - 0.5 s pressure waveform, the peak level of transgene expression and minimal liver injury were achieved. Second, the parameters included in animal experiment procedure were confirmed, on such as administration dose of pDNA (12.5 μg per mouse), injection solution for pDNA solution, intravenous injection rate (with 3 s), interval time between pDNA injection and tissue suction (3 min later after injection of pDNA). Furthermore, I found that the deformed volume of liver is the key factor affecting transgene expression related to suction pressure. Thirdly 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*.

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.

In conclusion, the liver suction-mediated transfection system can be performed in a minimally invasive and precisely controllable manner by computer controlled system. In addition, I found that hepatocytes were mainly transfected in mice liver. Finally, I demonstrated the effective treatment on acute hepatitis mice model via liver suction-mediated transfection of secreting IL-22 protein. These findings provide useful information for the realization of *in vivo* gene therapy by optimization of physical gene delivery systems.

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Finally, my sincere and profound gratitude is extended to my beloved parents, old sister and brother for their infinite love, understanding, moral and financial support throughout my studying abroad as well as affectionate encouragement throughout my life.

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)[120]. 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[121]. The procured PDMS (20:1) was cured at 75℃ 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).

Measurement of the Transaminase Activity in Serum

At indicated time points, blood was collected from the inferior vena cava of mice. The blood samples were incubated at 4 ℃ for 4 h to allow clotting and then centrifuged at 8000 × g for 20 min to obtain serum samples. Alanine aminotransferase (ALT) andaspartate aminotransferase (AST) activities in the serum were determined using Transaminase CII-Test Wako Kit (Wako Pure Chemical Industries, Tokyo, Japan) according to manufacturer's instructions.

Measurement of proinflammatory cytokine concentrations in serum

(1) Preparation of liposome: Cationic liposome and lipoplexe were prepared as reported previously [53]. Briefly, mixtures of 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP) and cholesterol were dissolved in chloroform at a molar ratio of 1:1, vacuum-desiccated, and resuspended in sterile 5% dextrose. The suspension was sonicated for 3 min, and the resulting liposomes were extruded five times through a 220 nm polycarbonate filter. Then, lipoplexes were formed by adding an equal volume of pDNA in 5% dextrose to the liposomes at various mixing ratios (-:+) and incubated at room temperature for 30 min. The particle size and zeta potential of the lipoplexes were measured using a zeta potential analyzer (Zetasizer Nano ZS, Malvern Instruments Ltd.,Worcestershire, UK)

(2) Preparation of lipoplexes: Cationic liposome-mediated plasmidDNA transfection was carried out as a positive control. DOTAP-cholesterol liposomes (molar ratio, 1:1). Lipoplex was prepared by adding an equal volume of cationic liposomes to pCMV-Luc in 5% dextrose at a charge ratio (-/+) of 1.0:3.1 and at a pCMV-Luc dose of 30mg and then stored at room temperature for 30 min. Mice were intravenously injected with prepared lipoplex.

(3) Measurement of proinflammatory cytokine: Serum samples were obtained as described above. Serum samples were immediately stored at -80℃ until enzyme-linked immunosorbent assay (ELISA) was carried out. TNF-α and IL-6 concentrations in serum and tissue lysate were determined by ELISA Ready-SET-Go! (eBioscience, San Diego, CA).

Hematoxylin and eosin staining

Mice were euthanized by cutting the vena cava, and the liver was gently infused with 2 ml of saline through the portal vein to remove the remaining blood. The liver was then fixed in 4 % paraformaldehyde in PBS, embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE). The stained sections were examined using a microscope (Biozero BZ-8000, KEYENCE, Osaka, Japan).

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.

Chapter II

Plasmid DNA

pCpG-mcs and pUNO1-miL22 vectors were purchased from Invivogene (San Diego,DA, USA). The interleukin-22 (IL-22)-expressing plasmid, pCpGfree-IL22 and pCMV-IL22 were constructed respectively, were constructed by inserting murine IL-22 cDNA into the DpnI site of the pCpG-mcs vector, and the EcoRV site of pcDNA3.0. The SfoI/NheI IL-22 cDNA fragment from pUNO1-miL22 vector was introduced into the pCpG-mcs and pcDNA3.0 vector. The luciferase (Luc)-expressing plasmids were constructed by subcloning the Hind III/XbaI luciferase cDNA fragment into the DpnI site of pCpG-mcs vector. pCMV-IL22 and pCMV-Luc were amplified in the Escherichia coli strain DH5α as described in Chapter I, whereas pCpG-mcs, pCpGfree-IL22

and pCpGfree-Luc were amplified in the E. coli strain GT115. Isolation and purification of pDNAs were carried out using QIAGEN Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany)[120]. Characteristics of used pDNAs are shown in **Appendix I**. Pathway profiling Luciferase system: pTAL-Luc, pAP-l-Luc, pNFkB-Luc, pCRE-Luc and pSRE-Luc; No.631911 were purchased from Clontech. Characteristics of the used pDNAs are summarized in **Appendix II**. Plasmid isolation and purification were performed as described in Chapter I.

Animals

ICR mice (female, 5 weeks old) and Balb mice (female, 6 weeks old) were purchased from Japan SLC Inc, (Shizoka, Japan). The liver suction-mediated transfection were performed as in Chapter I

Isolation of liver PC and NPC after liver suction-mediated transfection

At 8 h after pDNA administration and the suction-mediated transfection on left lobe of liver, the liver cells were separated into parenchymal cell (PC) and nonparenchymal cells (NPC) by liver collagenase perfusion method as reported [69]. In brief, the liver was perfused with HEPES buffer (pH 7.4) containing collagenase, and the dispersed cells were separated into PC and NPC fractions by differential centrifugation. These cell fractions underwent luciferase assay for naked pCMV-Luc transfection.

Evaluation of the c-fos and c-jun mRNA level by RT-PCR

Mice were euthanized and total RNA was extracted from the left lobe of liver samples using a Gen Elute^{TM} Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, Co., Ltd., St. Louis, MO, U.S.A.). Subsequently, reverse transcription was performed using a Prime-script RT reagent Kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. For a quantitative analysis of mRNA expression, real-time PCR was carried out with total cDNA using a Light-Cycler instrument (Roche Di-agnostics, Basle, Switzerland).The oligodeoxynucleotide primers used for amplification were as follows: c-fos , for-ward 5'-CCAGTCAAGAGCATCAGCAA-3', reverse 5'-AAGTAGTGCAGCCCGGAGTA-3'; c-jun, forward 5'-TC-CCCTATCGACATGGAGTC-3', reverse 5'-TGAGTTGGC-ACCCACTGTTA-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward5'-TCTCCTGCGACTTCAACA-3', reverse 5'-GCTGTAGCCGTATTCATTGT-3'. Amplification products were detected online via intercalation of the fluorescent dye SYBR green (SYBR Premix Ex*Taq*; TakaraBio, Inc., Otsu, Japan). The cycling conditions were as follows:

initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 20 s. Gene specific fluorescence was measured at 60 °C.

ELISA assay of IL-22 expression in the serum and liver

At the indicated time points after suction-mediated transfection of IL-22 expressing plasmid DNA (pCMV-IL22 and pCpGfree-IL22), serum was collected as described in Chapter I. After blood withdrawal, the left lobe of liver was harvest quickly and homogenized in 10 ml/g liver of lysis buffer (0.1 M Tris, 0.05 % TritonX-100, 2 mM EDTA, pH7.8). The homogenates were centrifuged at 13000 × g for 10 min at 4 °C. The obtained supernatant and serum were used for measurement the intrahepatic and serum concentration of IL-22 protein with ELISA Kit (ELISA MAX^{TM} Deluxe Sets, MouseIL-22; Biolegend, San Diego, CA, USA).

Induction of Con A-induced liver injury

ConA (Type IV, No. C2010,Sigma) was dissolved in pyrogen-free PBS and intravenously injected into Balb/c mice (6 weeks old, female) through the tail vein at a dose of 10 and 15 μg/kg [122]. Serum samples were obtained at 8 h after Con A injection. Serum ALT/AST activities were measured as described in Chapter I to confirming successful induction of liver in injury mice model.

Luciferase Assay

Protocol was same as described in Chapter I.

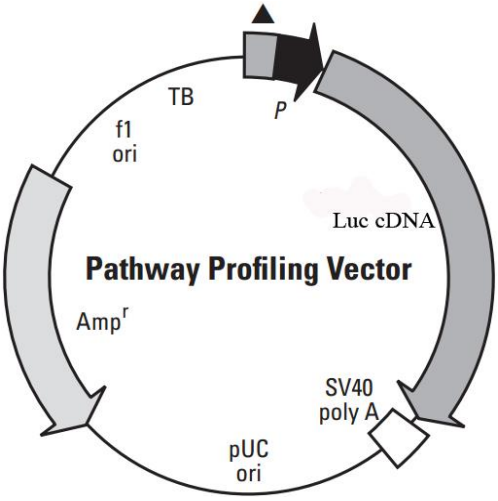
Statistical analysis

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. Survival time of mice was on a Kaplan-meier survival plot followed by a Logrank (Mntel-Cox) test.

AppendixI. Characteristics of the plasmid DNAs used in this study

Plasmid	Size (kbp)	Number of CpG	Enhancer	Promoter	cDNA
pCMV-Luc	7.1	846	hCMV	hCMV	Firefly
pCpG-mcs	3	0	hCMV	hEF1	None
pCpGfree-	4.7	194	hCMV	hEF1	Firefly
pCMV-IL22	6	652	hCMV	hCMV	Interleukin-22
pCpGfree-	3.7	0	hCMV	hEF1	Interleukin-22

Appendix II. Pathway of profiling luciferase system



▲ cis-acting Enhancer Element
No **(TAL)**
Activator protein-1 **(AP-1)**
Nuclear factor κB **(NFκB)**
cAMP response element **(CRE)**
Serum response element **(SRE)**

Appendix II. Structures of plasmid DNAs of pathway of profiling luciferase system.

TATA like promoter-driven plasmid DNAs encoding the complementary DNA luciferase with an enhancer region involving various transcription factors; no (pTAL-Luc, activator protein-1 (pAP-1-Luc), nuclear factor κB (pNFκB-Luc), cAMP response element (pCRE-Luc), and serum response element (pSRE-Luc)

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