Chapter 1

Nucleic Acid-Based Therapy: Development of a Nonviral-Based Delivery Approach

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Additional information is available at the end of the chapter

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Abstract

Gene therapy returns to the center stage of medicine to treat patients with diseases that are unable to be cured with the conventional therapeutic strategies. This development is due to various reasons, including vector development and significant achievement in next-generation sequencing. Among the various methodologies of gene therapy, nucleic acid-based therapy has been considered to be promising in various diseases. The development of delivery methods to target cells *in vivo*, however, remains critical. These include viral vector-based and nonviral vector-based gene delivery methods as well as physical approaches such as hydrodynamic gene delivery (HGD). HGD is a simple and effective *in vivo* gene transfer method for the functional analysis of therapeutic genes and regulatory elements in small animals. Moreover, this chapter outlines the principle of HGD, gene expression studies in rodents, and recent advances in clinical application of HGD and provides future perspectives in developing a safe and efficient method for nucleic acid-based therapy.

Keywords: nucleic acid-based therapy, nonviral delivery, hydrodynamic gene delivery, site-specificity, computer-controlled injection, human gene therapy

1. Introduction

In 1990, first human gene therapy was conducted, targeting adenosine deaminase deficiency *via* retrovirus-mediated delivery system [1]. Since then, the number of clinical trials has gradually increased, and approximately 2600 trials have been globally undertaken or approved until November 2017 [2]. Most trials (75%) utilized a viral vector as a delivery tool of gene. Viral vector-based delivery resulted in a high level of gene expression for a long period;

however, carcinogenesis and lethal immune reaction were reported [3–5]. Numerous researchers have been attempting to overcome these serious obstacles to enable safe and efficient therapy. For this purpose, the improvement of viral vector has been extensively studied in the last decade, and in addition, nonviral vector-based gene delivery method has developed with great promise. As expected, it resulted in less antigenicity and less chance of integration into the human genome than viral vector; therefore, it can be regarded as a biologically safer method than viral vector-based gene delivery method. However, the period of transgene expression tends to be limited.

This chapter focuses on nonviral vector-based delivery method, which could be used for the nucleic acid-based therapy. In these methods, a transgene is not integrated into the host genome; hence, gene expression is transient. Because temporal transgene expression is applied to promising technologies, such as generation of iPS cells and gene editing by CRISPR/Cas9, nonviral vector-based gene delivery may play a big role in future medicine.

The last section of this chapter outlines the recent progress in the HGD, which enables the highest level of delivery efficiency among nonviral vector-based approaches and the clinical application utilizing the well-established method of catheter insertion into the vessels in the multiple organs.

2. Nonviral approaches for nucleic acid transfer

This section focuses on gene delivery methods using nonviral vector-based approach. Nucleic acids loaded in artificial or natural cargos or in naked condition are transferred to target cells. The characteristics of various gene deliveries are briefly described in **Table 1**.

2.1. Liposome-based approach

Lipofection, a cationic lipid-mediated approach, is widely used in numerous *in vitro* and *in vivo* studies. The first study reporting lipofection was published in 1987 [6]. Molecules comprising hydrophilic head, linker, and hydrophobic anchor form a spherical structure. The positively charged hydrophilic head plays a role in condensing the negatively charged DNAs. It also helps in establishing an electrostatic interaction with the negatively charged cell membrane. As a result, it promotes the cellular uptake of DNA-loaded liposome (lipoplex), endosomal escape, and subsequent release of the condensed DNAs into the cytoplasm. On the contrary, the hydrophobic anchor protects DNAs from degradation by nucleases. Liposome is a popular carrier to deliver even large-sized transgene; it is easy to prepare and modify and is utilized in numerous laboratories worldwide. Nevertheless, there are several drawbacks for its use in gene therapy. It has difficulty in achieving therapeutic level of transgene expression, shows no tropism to desired cells, and exhibits a short life span. Furthermore, the positively charged head has cell toxicity. An inflammatory response occurs when unmethylated CpG DNA is transported, which is one of the obstacles that need to be addressed. Various strategies to achieve high level of safety and efficiency, such as introduction and improvement of polyethylene

Method	Functional component	Advantages	Disadvantages
Lipids	Cationic lipids	High efficiency *in vitro* Ease to prepare	Low efficiency *in vivo* Acute immune response
Polymers	Cationic polymers	Highly effective *in vitro* Ease to prepare	Toxic to cells Acute immune response
Exosomes	Natural or modified exosomes	Less toxic (Insufficient data)	Low efficiency? (Insufficient data)
Needle injection	Mechanic force	Simple	Low efficiency Expression limited to needle track
Gene gun	Pressure	Good efficiency	Limited to target area Need surgical procedure for internal organ
Electroporation	Electric pulse	High efficiency	Tissue damage Limited target area Need surgical procedure for internal organ
Sonoporation	Ultrasound	Site specific	Low efficiency Tissue damage
Magnetofection	Magnetic field	Site specific	Low efficiency Limited target area Need surgical procedure for internal organ
Hydrodynamic delivery	Hydrodynamic pressure	Simple High efficiency Site specific	Need catheter insertion technique in large animals

Table 1. Characteristics of nonviral gene delivery method.

glycol [7] and cell-specific targeting ligand on the surface of the liposome, have been extensively studied. Development of a promising linker also improves stability, biodegradability, and transfection efficiency and reduces cytotoxicity [8]. Lipofection has been utilized in 4.4% of clinical trials worldwide [2]. The results of human gene therapy for cystic fibrosis in clinical trials of phase I/IIa and IIb have been reported in the UK [9, 10]. Patients had cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations and suffered from hypofunction of CFTR in multiple organs. Because secretory fluid becomes viscous, the patient may experience repeated respiratory infection and, finally, respiratory failure. CFTR gene was nebulized as lipoplex every 28 days for 1 year for significant stabilization of lung function [9, 10]. In 2016, other clinical trials for genitourinary cancers and solid tumors reportedly used the truncated forms of the RB gene and p53 gene with docetaxel, respectively [11, 12].

2.2. Polymer-based approach

Cationic polymer is an artificially synthesized vehicle, and various types of polymer have been studied. DNA condensed in cationic polymer (polyplex) acquires tolerance to enzymatic degradation, which results in stability in the blood. Cellular uptake is via receptor-mediated endocytosis, which leads to a high level of transfection activity. Clinical trials using this approach for cystic fibrosis and ocular degenerative disease have been reported [13, 14]. Nevertheless, the stability of polyplex and persistent positive charge leads to high cytotoxicity.

Because cationic polymer is easy to prepare and improve, various constructs, such as polyethylenimine, polyamidoamine, polyallylamine, chitosan, dendrimers, cationic proteins, and peptides, have been studied to overcome the obstacles.

2.3. Lipopolyplex-based approach

Lipopolyplex comprises polycation (cationic polymer or peptide) and condensed DNA with lipid shell and is divided into diverse categories according to the combination and ternary structure. Its advantages are of both lipoplex and polyplex, that is, more efficient transfection and less cytotoxicity. Previous study [15] and reviews [16, 17] have described the strategy, variety, and preparation of lipopolyplex.

2.4. Exosome-based approach

Exosome is a kind of extracellular vesicle secreted by various cells. It comprises a lipid bilayer with several surface antigens derived from the parent cell. DNA, mRNA, miRNA, and protein can be included in the lipid bilayer. Moreover, exosome is known to have organ and cell tropism; however, the mechanism is not completely clarified. This indicates that exosome plays a role in intercellular communication. Cancer cells as well as healthy cells secrete exosome. Integrin included in exosome reportedly determines organ tropism for metastasis. Exosome from metastatic lung tumor of breast cancer induced lung metastasis of breast cancer, which originally had metastatic ability only to the bone [18]. An attempt to utilize cancer-derived exosome for cancer therapy was also reported, wherein the cancer-derived exosome was used as a natural carrier of CRISPR/Cas9 plasmids. Compared to epithelial cell-derived exosome, cancer-derived exosome with CRISPR/Cas9 plasmids selectively accumulated in cancer cells, suppressed PARP-1 gene expression, and achieved induction of apoptosis [19]. Recently, many researchers have been studying exosome as delivery system for cancer therapy. Surface antigens of exosomes are known to be modified directly and genetically. The exosomes from leukemia cells, marrow stromal cells, adipose-derived mesenchymal stem cells, breast cancer cells, and kidney cells including siRNA and miRNA were reported to be used for colorectal tumor, glioma, hepatocellular carcinoma, breast cancer, and chronic myelogenous leukemia [20–24]. Although the exosome-based approach has been seen as a new and promising method of gene delivery, it is rather obvious that further understandings of the mechanisms and structures as well as improvement in exosomes' preparation are necessary to achieve the high level of efficiency and safety needed for clinical application.

2.5. Needle injection

Direct injection to the tissue is the simplest approach for the physical delivery of nucleic acid. The first report for delivery to muscle was published in 1990 [25]. Needle injection was expanded to the skin [26], heart muscle [27], liver [28], and tumor [29]. Currently, microneedle is studied as a minimally invasive delivery for skin disease and vaccination [30, 31]. Microneedles are arrays of 25–2000-μm long needles [32]; on the basis of the delivery mechanism,

they are divided into solid, coated, and dissolving types [31]. In a mouse study, siRNA delivery is reported to be effective for skin conditions with aberrant gene expression, such as alopecia, allergic skin diseases, hyperpigmentation, psoriasis, skin cancer, and congenital pachyonychia [33].

2.6. Gene gun

Gene gun is known as microprojectile bombardment, and the first study reporting its use was published in 1987 [34]. At first, this method was developed for gene delivery into plant cells. A bullet with the microparticles containing DNA is shot to a target cell, and gene delivery is achieved. On the basis of the principle of obtaining a driving force, a gene gun is divided into three major groups: powder gene gun [34], high-voltage electric gene gun [35], and gas gene gun [36]. The driving force moves the microparticles containing DNA toward a target tissue and penetrates the cell membrane. Because delivery efficiency and cell damage are two sides of the same coin, appropriate operating pressure is required. A phase I clinical study was performed to treat melanoma using *IL-12* gene [37]. Although an attempt of combining delivery with microneedles reportedly enhanced the penetration depths of microparticles [38], gene gun may be more appropriate for delivery to the skin, such as for vaccination.

2.7. Sonoporation, electroporation, and magnetofection

Sonoporation, using ultrasound [39, 40], and electroporation, using electric pulse [41], increase the permeability of cell membrane for cellular uptake of nucleic acid. Magnetofection utilizes magnetic field to enable microparticles with nucleic acid to pass through the cell membrane [42]. These methods are used in combination with other methods, such as lipofection, to protect nucleic acid against degradation by nucleases. To increase gene delivery efficiency of sonoporation, microbubbles were shown to be effective [43] and applied for delivery to cancer cells [44, 45] and the central nervous system [46, 47]. Clinical trials in phases I and II have been reported for the treatment of melanoma [48–50] and solid tumors [51].

2.8. Hydrodynamic gene delivery (HGD)

HGD is one of the simplest methods for gene transfer. The efficiency of HGD is the highest among nonviral vector-based delivery methods, and its physical force to deliver the gene into the cells relies on a high level of flow rate and volume of the injected solution. Since the first published reports in 1999 [52, 53], many researchers have utilized this methodology for gene transfer in animal experiments, particularly in rodent studies. For its application in human, safety and efficacy of this approach have been extensively studied and improved. To date, various types of nucleic acid have been delivered by this approach in rodents as well as pigs [54–57], dogs [58, 59], and rhesus monkeys [60, 61]. Functional analyses of therapeutic gene were reported in nonalcoholic steatohepatitis [62], hepatitis B and C [63], fulminant hepatitis [64, 65], liver fibrosis [66, 67], liver regeneration [68], Fabry's disease [64], and colon cancer [69]. The next section describes its principle and progress in human gene therapy.

3. Principle and progress of hydrodynamic gene delivery toward human gene therapy

3.1. Principle, efficiency, and safety of hydrodynamic gene delivery

HGD is achieved by the quick injection of a large amount of naked nucleic acid solution into the vein. In case of a rodent, the solution is injected from the tail vein. The most important step of successful gene delivery is a precise insertion of an injection needle into the tail vein. The details of technical tips are described in **Figure 1**. The quick injection can transiently increase an intravenous pressure. Mechanical force by rapid increase in venous pressure allows nucleic acid to pass through the cell membrane into the cytoplasm and nucleus.

Among various organs, the liver can achieve the highest level of gene expression because of the presence of the specific structure fenestra. Fenestra is a small window in the sinusoidal vessel, and hepatocytes are partly exposed to the blood stream. In other words, hepatocytes can be directly affected by intravascular pressure. A rapid stream of hydrodynamic injection can wash out the blood in the sinusoid vessel transiently and thoroughly, and nucleic acid can reach the hepatocytes without degeneration by nucleases. A high intravascular pressure

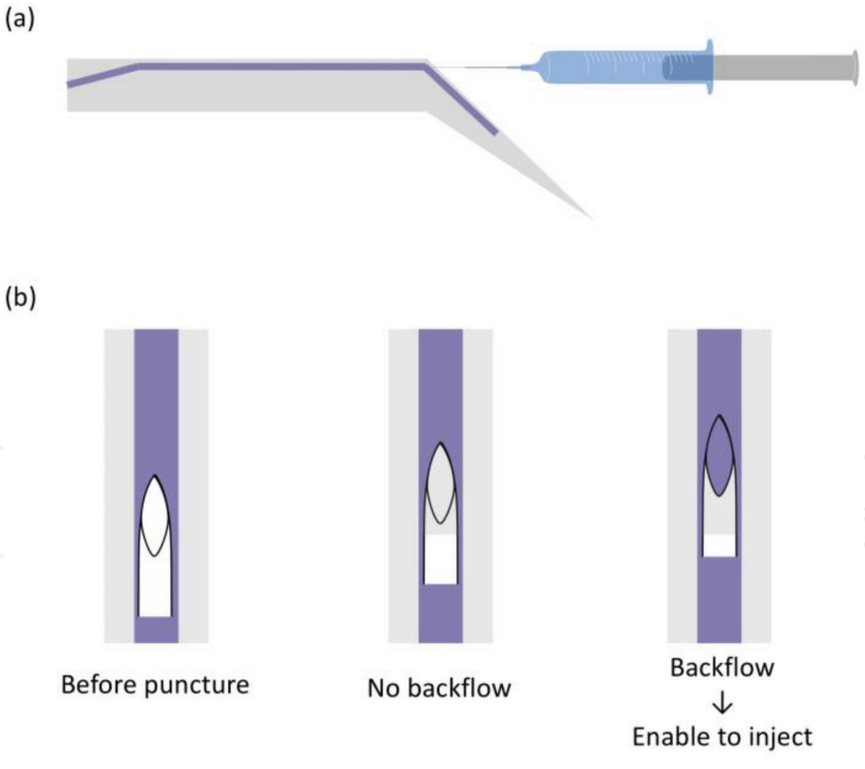

Figure 1. Technical details of the tail vein injection in a mouse. (a) When inserting a needle tip, the tail vein and needle shaft should be at the same angle. The puncture can be performed from the top of the tail curve. (b) If a needle tip successfully enters the tail vein, backflow of the blood is visible on the needle tip. Once the backflow is confirmed, a needle tip can be further inserted to the proximal side of the tail vein.

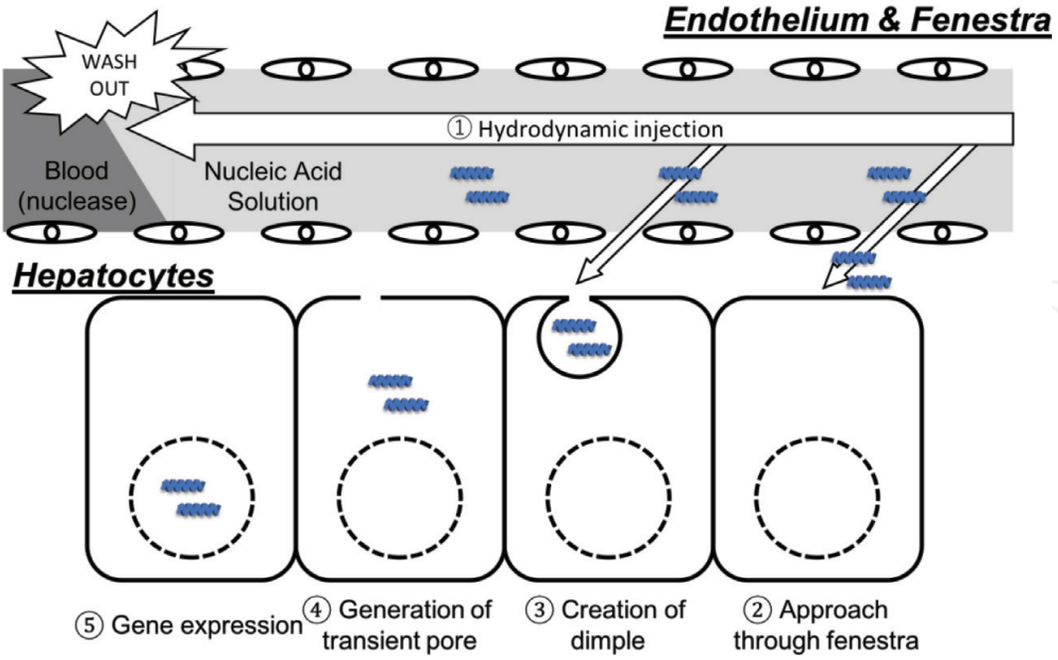

Figure 2. Scheme of hydrodynamic gene delivery. The hepatocyte partly faces to the blood stream via the fenestra in the sinusoidal structure. A rapid stream of hydrodynamic injection has the blood in the sinusoid washed out transiently, and the nucleic acid can be delivered into hepatocytes without being degraded by nucleases. A high intravascular pressure makes dimples on the surface of hepatocyte, and finally generates transient pores. Nucleic acid is pushed into the hepatocyte through the transient pores.

creates dimples on the surface of the hepatocyte and finally generates transient small pores. The nucleic acid is pushed into the hepatocyte through the transient pores **(Figure 2)**. Moreover, it was clarified that the pores naturally reduce and disappear in 24–48 h [70]. Although serum transaminase shows transient increase after a hydrodynamic injection, these values return to the background level within a short period. Considering the short life time of transaminase, an increase in serum transaminase is speculated to be caused by leakage from the transient pores. If the intravascular pressure is kept within an adequate range, this change in hepatocyte is reversible and does not result in apoptosis and necrosis; therefore, acute liver failure is not a concern.

To apply this method into the clinic, the modification of the original procedure is essential as in mouse studies, hydrodynamic injection is performed via the tail vein. Looking back to the original method, in detail, naked DNA solution equivalent to 10% of the body weight (BW) is injected for 5–7 s via the tail vein. The details of hydrodynamics during the injection have been reported using contrast medium under fluoroscopic imaging and cone-beam computed tomography (CT) [71]. Briefly, the injected solution is led to the inferior vena cava (IVC) and then flowed back to the hepatic veins. The retrograde flow passes through the sinusoid vessel into the portal vein. Given that contrast medium transiently stayed in the liver after the injection, the flow generated transient pores on the surface of the hepatocyte while passing through the sinusoid vessel. Because of the filling of sinusoidal and interstitial space by the

solution and transfer of nucleic acid into the hepatocyte, the volume of the liver reportedly increased by 165% compared to the preinjected condition.

The efficiency of transfer was indicated by microscopic images. Transgene expression was observed in approximately 20–40% of hepatocytes. Wide distribution of transgene expression in the liver can achieve therapeutic level of transgene expression [72]. In a rat model with bile duct ligation, hydrodynamic delivery of MMP13 gene indicated prophylactic effect on liver fibrosis [67]. Given its simplicity, safety, and efficiency, HGD has been utilized in numerous rodent studies [63, 65, 66, 73, 74]. HGD can be also applied to various organs other than the liver, such as the kidneys [75], muscle [61], and pancreas [76].

3.2. Improvement of a hydrodynamic injection for larger animals

Based on efficiency and safety in rodents, HGD has been improved extensively and can be potentially applied in humans **(Figure 3)**. Two major obstacles that should be overcome are poor site specificity and very large injection volume. HGD with adequate range of intravascular pressure, a key factor for efficient and safe delivery, is facile to achieve by a manual injection in mice. On the contrary, in larger animals, such as rabbits, pigs, dogs, and nonhuman primates, controlling intravascular pressure is difficult because of a large amount of injection

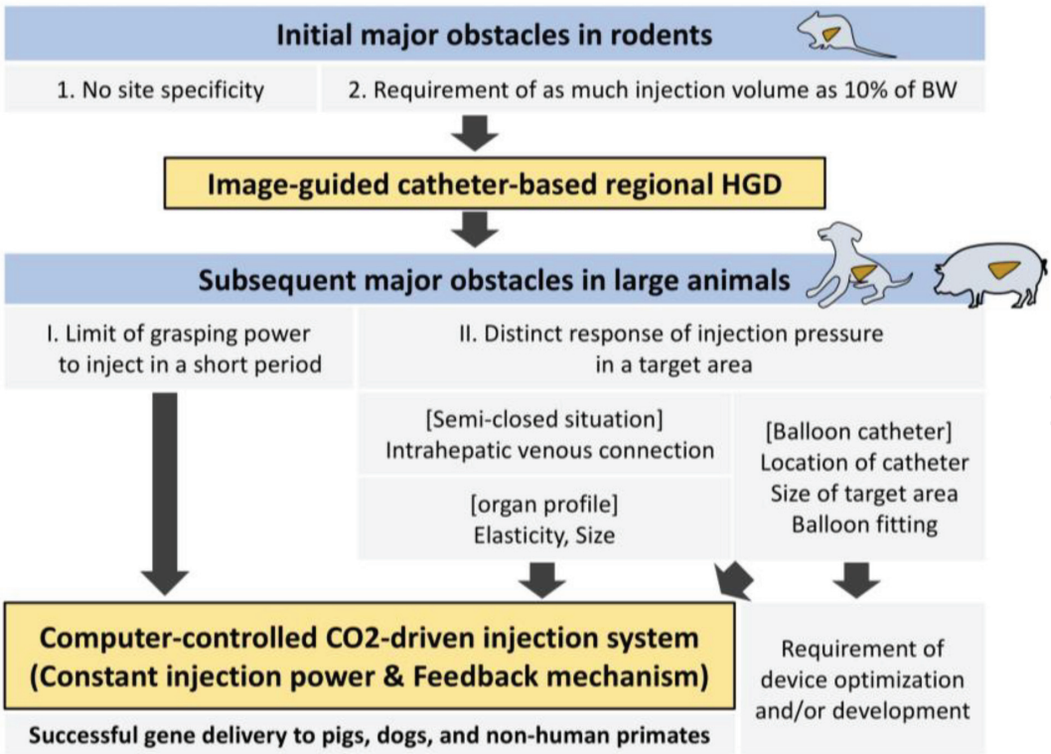

Figure 3. Improvements of hydrodynamic gene delivery toward human gene therapy.

volume per second. Several studies have tried to resolve these problems using catheter technique. A balloon catheter is inserted from the jugular vein into the hepatic vein under X-ray guidance, which is often performed in clinic [56]. When the catheter is placed in the hepatic vein, the balloon on its tip is inflated, which causes venous occlusion to prevent leakage of DNA solution from the hepatic vein to the IVC. This technique targeting each lobe of the liver can reduce injection volume per one procedure to <1% BW, maintaining efficiency of gene delivery.

During the establishment of catheter technique, another important problem arises, that is, distinct response of injection pressure in a targeted area. Precise control of intravascular pressure is essential to achieve efficient and safe gene delivery **(Figure 4)**. Inconsistent intravascular pressure caused by leakage of DNA solution to the adjacent area, which results from physiological connections of intrahepatic vessels and tissue elasticity, is highly possible, and the leakage volume can be also associated with intravascular pressure during injection. To achieve precise control of intravascular pressure, a computer-controlled injector with feedback mechanism has been developed [54]. Although the initial version of the injector utilized CO_2 as its driving force, the current version adopts electric motor for pursuit of more accurate control [58, 77] (**Figure 5**). This injection system leads to reproducible results of efficiency. Not only efficiency but also safety is confirmed in various aspects, such as blood test, electrocardiogram, hemodynamic CT study, laparoscopic observation, and histologic assessment [56, 78, 79] (**Figure 6**).

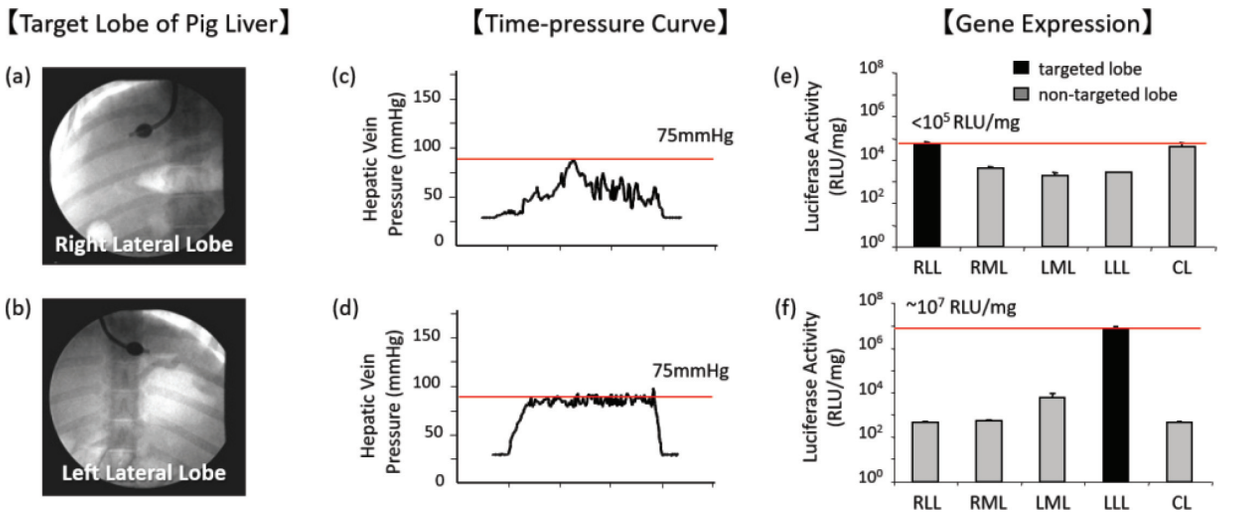

Figure 4. Relationship between time-pressure curve and transgene expression on site-specific delivery to a large animal. (a and b) HGD was performed to right and left lateral lobes of the pig liver. (c and d) Both injections achieved 75 mmHg of a peak intravascular pressure. (e and f) Gene expressions after the injections of (c) and (d) are shown in (e) and (f), respectively. This figure is partly reused and modified with updated information from Figures 3, 5, and 6 in [56] with their permission. RLL, right lateral lobe; RML, right medial lobe; LML, left medial lobe; LLL, left lateral lobe; CL, caudate lobe.

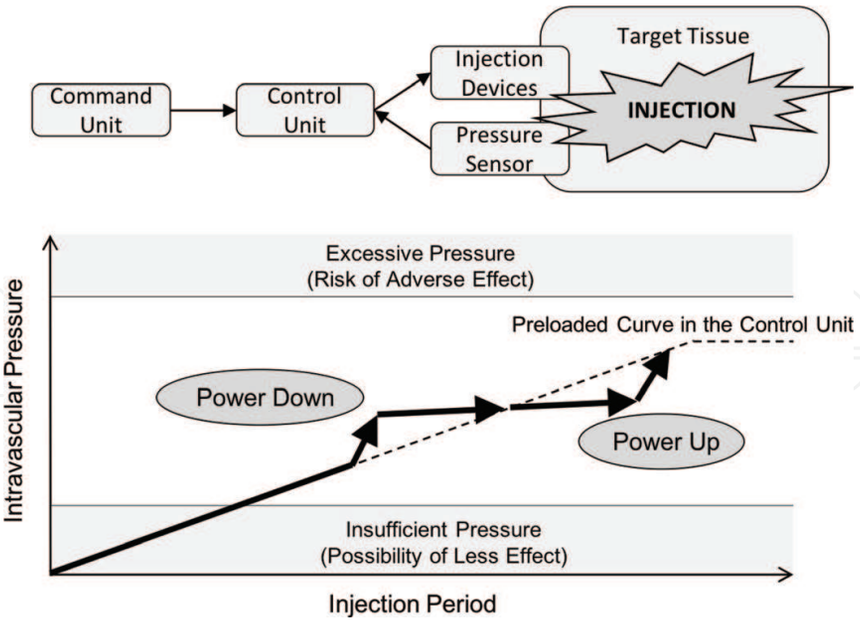

Figure 5. Scheme of the computer-controlled hydrodynamic injection system. Prior to an injection, a user selects appropriate time-pressure pattern for delivery and preload the data to the command unit. The command unit transmits the data to the control unit, which modulates electric power based on the feedback information of an intravascular pressure during the injection from the pressure sensor placed at the peripheral vein of a target area.

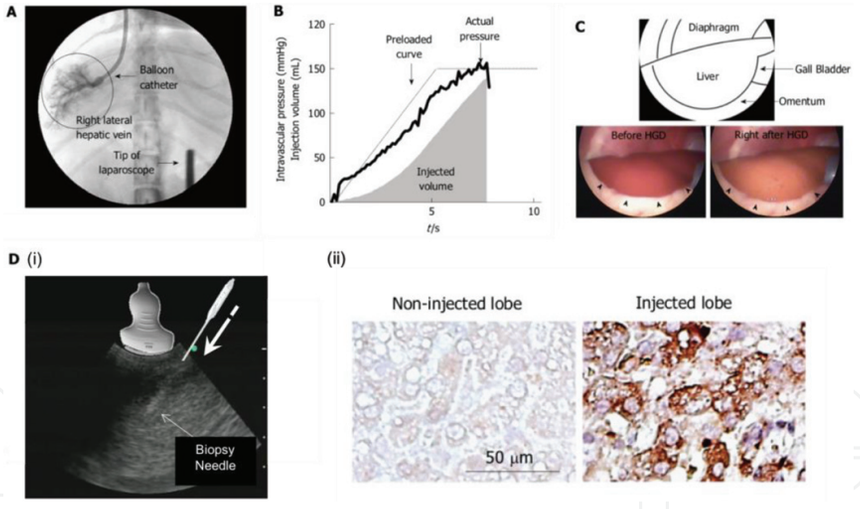

Figure 6. Image-guided, computer-controlled HGD to the dog liver. The balloon catheter was placed at the appropriate position in the hepatic veins of right lateral lobe and the occlusion of the blood flow by the balloon was confirmed by injecting a small amount of contrast medium into the hepatic vein. Then the hydrodynamic injection of naked DNA solution was performed under the real time monitoring of liver structure by the laparoscope using the computer-controlled injection system (A). (B) Time-pressure curve and the volume of injected solution recorded in the injection system. Solid and dotted lines represent actual and preloaded time-pressure curves. The gray area shows cumulative volume of injected saline (ml). (C) Laparoscopic findings of the hydrodynamically injected right lateral lobe of the dog. The injected lobe was swollen, and the injected DNA solution transiently made the liver pale. Neither destruction nor bleeding was seen on the surface of the liver (arrowheads). (D) The effect of lobe-specific hydrodynamic gene delivery of luciferase expressing plasmid. (i) Liver samples were collected by needle biopsy under the ultrasound sonography 4 days after the injection. (ii) The immunohistochemical analyses showed positively stained cells in the injected right lateral lobe. No stained cells were found in noninjected left lateral lobe. This figure is partly reused and modified with updated information from Figure 1 in [58] with their permission.

4. Conclusion

Currently, various approaches including both viral and nonviral vector-based delivery methods are studied for safe and efficient human gene therapy. They have their own properties, such as duration of gene expression, size of transgene to load, possible organs and their expected volumes in single procedure, and repeatability. Conditions to treat are also diverse. Congenital disease such as hemophilia possibly requires long-term transgene expression for decades. For *in vivo* gene editing based on CRISPR/Cas9, short-term transgene expression may be preferred, to prevent off-target effect. Therefore, the transient gene expression mediated by the nonviral vector-based delivery may have great advantages when it comes to gene editing. Among the methods, as described above, HGD may be a promising delivery approach as it is simpler and more efficient. Currently, we are modifying the original HGD method used in small animals in order to apply it into large animals to test its efficacy and safety. Metabolic and genetic diseases, which show lower level of normal functional protein, are so far good candidates for this type of procedure. Although there is evidence showing transgene expression and that the procedure was safely performed in pigs [54–57], dogs [58, 59], and baboons [60, 61], further preclinical studies are necessary prior to human therapy application.

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Conflict of interest

The authors declare no conflict of interest.

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