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Hydrodynamic Injection for Developing NASH Model

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Abstract

The hydrodynamic tail vein injection (HTVi) is a technique that is used to deliver plasmid genes into live mice or rats. The HTVi leads to the in vivo transfection of exogenous DNA primarily in the liver, serving as a reliable approach of establishing animal models for the study of liver diseases. The nonalcoholic steatohepatitis (NASH) is liver inflammation and damage resulting from an accumulation of fat in the liver. With the rising prevalence of obesity worldwide, NASH is becoming an increasingly common health problem. The pathogenesis of NASH is a multi-step process involving complicated pathways. The molecular mechanisms of NASH remain poorly understood. Here, we describe the use of HTVi to establish animal models for the study of NASH.

Keywords

Hydrodynamic tail vein injection; Nonalcoholic steatohepatitis; Mouse; Rat

1 Introduction

The nonalcoholic steatohepatitis (NASH) is liver inflammation and damage resulting from an accumulation of fat in the liver. NASH was first defined in 1980 by Ludwig et al. among patients who have fat build up in the livers without excessive alcohol use [1]. Subsequently, both the pathological and clinical features of NASH have been defined. The consensus of NASH pathophysiological change is the inflammatory insult which occurs in the fatty liver. Increased free fatty acids and hepatic lipid peroxidation as well as the presence of peripheral insulin resistance are associated with NASH patients [2]. Because of the rising prevalence of obesity worldwide, NASH is becoming an increasingly common health issue [3]. However, the underlying mechanisms of NASH development are complicated and remain unclear.

During the past decades, genetic studies have revealed crucial signaling pathways involved in nonalcoholic fatty liver disease (NAFLD) and NASH [4, 5]. Recently, high-throughput genomic studies, including microarrays, array-based comparative genomic hybridization, and deep sequencing, in combination with bioinformatics and other computational biological approaches, have identified many genes that are deregulated along NAFLD and NASH development [6]. However, the functional contributions of these candidate genes to NASH development have yet to be investigated in preclinical studies. In this regard, the application of cell lines and in vitro studies is not optimal. This is largely due to the fact that once in culture, hepatocytes rapidly de-differentiate into fibroblast-like cells. In addition, the inflammatory features of NASH are produced via interaction between hepatocytes and

inflammatory cells, including liver-resident macrophages (known as Kupffer cells), natural killer cells, and dendritic cells within the liver [7]. It is difficult to recapitulate these sophisticated cell–cell interactions using in vitro systems. In contrast, the animal models, especially mouse and rat NASH models, are beneficial to recapitulate the pathophysiological changes which are observed in the patients. Moreover, the use of animal models is critical in exploring therapeutic approaches.

The establishment of a safe and efficient in vivo gene delivery method is indispensable for the study of molecular biological features of NASH and the development of gene therapies. Recently, hydrodynamic tail vein injection (HTVi) is introduced to the liver field and has evolved rapidly [8]. The HTVi delivers massive plasmid DNA into the body in a short duration, giving a transient but high in vivo gene expression especially in the liver of mice and rats [9]. The main targets of hydrodynamic transfection through tail vein are parenchymal cells (mainly hepatocytes) since parenchymal cells and capillary endothelium are closely associated in the liver. This anatomical feature provides the possibility that plasmid DNA enters the parenchyma cells rapidly through the breached endothelial barrier, with the help of hydrodynamic forces (Fig. 1a). The standard procedure of HTVi requires a fast (5–9 s) tail vein injection of a large volume of plasmid DNA-saline solution. The total volume of dosing solution for an ideal transfection efficiency is equivalent to 10% of the body weight [10, 11]. With such a large volume of DNA solution entering directly into inferior vena cava, the myocardial fibers stretch overwhelmingly, resulting in a temporary dysfunction of the cardiac system. Consequently, the DNA solution is driven into the hepatic vein in retrograde direction due to the sharp increase of blood pressure across the liver, leading to the DNA transfection into the hepatocytes (Fig. 1b) [12]. Liver has been shown to be the major organ which uptakes the plasmid DNA in the body, with up to ~40% hepatocytes being transfected after HTVi. In contrast, transfection efficiency in all other organs is lower than 0.1% of that of the liver [13]. Therefore, this transfection technology appears to be rather specific for the liver. In addition to DNA, HTVi enables delivery of other components such as RNA, proteins, synthetic small compounds, and even viruses in vivo [14]. While HTVi has been used to deliver plasmid(s) into the liver, one issue has been that plasmid(s) would be degraded and within 1 week, no plasmid(s) could be detected in the liver. To solve this issue, sleeping beauty (SB) transposase-based gene editing was combined with HTVi (Fig. 2a). Specifically, combinational injection of pCMV-SB transposase expressing plasmids and transposons with gene of interest in pT3 backbone under a mammalian expression promoter, such as EF1α (pT3-EF1α) or CAGGS (pT3-CAGGS) through hydrodynamic tail vein injection delivers plasmids into hepatocytes. The SB transposases subsequently “cut” and “paste” the inserted gene, resulting in random integration into genomic DNA of the hepatocytes. HTVi mediated somatic genomic integration of target genes with a mammalian expression promoter can remain expressing in the liver stably and longitudinally. It is worth to note that gene under CMV promoter could be silenced in a few days after HTVi in the hepatocytes, and such pT3-CMV construct is not suitable for this technology [15].

Recently, there have been several reports about the applications and further improvement of this technology in the research of NASH. It has been demonstrated that genetic depletion of phosphatase and tensin homolog (*Pten*) in mice leads to hepatic steatosis and NASH.

Using HTVi in combination with the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas technology (Fig. 2b), Yu et al. [16] have faithfully established a rat recapitulating NASH features, suggesting the potentiality of applying HTVi in the study of NASH. Here, we introduce the materials and methods of HTVi as an approach for the study of NASH.

2 Materials

2.1 Preparation of Plasmid-Saline Solution

1. The NanoDrop™ UV-Vis spectrophotometer for nucleic acid concentration measurement.
2. 0.9% Saline solution (Sterile).
3. 0.22-μm Syringe filters (Sterile, PVDF).
4. 20-ml Syringe (BD Luer-Lok™ Disposable Syringes).
5. 50-ml Conical centrifuge tubes (Sterile).

2.2 Hydrodynamic Tail Vein Injection

1. 25G × 5/8 Needle (BD PrecisionGlide™ needles).
2. 26G × 5/8 Sub-Q Needle (BD PrecisionGlide™ needles).
3. 3-ml Syringe (BD Luer-Lok™ Disposable Syringes).
4. Red Tube Tailveiner® Restrainer (TV-150 Tailveiner, Braintree scientific, INC).
5. 300 Watts (or more) Red-ray lamp.
6. Alcohol prep pad.

3 Methods

3.1 Preparation of Plasmid-Saline Solution

1. Before the preparation of the plasmid-saline mixtures, purify the endotoxin-free plasmid DNAs from bacterial cells by following the protocol of a commercially available endotoxin-free plasmid purification kit (*see* Note 1).
2. Use endotoxin-free plastic ware and handle DNA with care to ensure that endotoxin is not re-introduced into the DNA sample after the removal step (*see* Note 2).
3. Measure the concentration (C) of plasmid DNA by determining its absorbance at 260 nm using a micro-volume UV spectrophotometer. Since the use of pure DNA (260/280 ratio of 1.8) for HTVi is highly desirable, avoid using a DNA sample with a 260/280 ratio below 1.7.
4. Determine the total amount of plasmid solution (plasmid DNA and saline solution).

a. Weigh and record the body weight of the mice before the preparation of plasmid solution.

b. Calculate the number of mice to prepare (n) by dividing the total body weight (t-BW) to 20 g per mice, as the optimal amounts of dosage are generally provided as mice with body weight of 20 g. Considering the filter process will lose some plasmid solution and proper loading of the syringes will require some extra injection mixtures, it is recommended to prepare one additional mouse per group. The eventual number of mice (N) to prepare will be $n + 1$ (Table 1).

c. Calculate the total volume of plasmid-saline solution to prepare (t-V) by multiplying 2 ml with the eventual no. of mice to prepare (N). However, the actual volume of plasmid solution (V) used in HTVi for each mouse is 10% of its body weight (Table 2). For example, if the mouse body weight is 22 g, the volume of plasmid solution for HTVi should be 2.2 ml.

d. Calculate the total amount of plasmid DNA and saline solution to prepare (Table 3). The optimal amount of plasmid DNA for HTVi is based on the study design and it may need pilot experiments for optimization. When determining the amount of DNA required, assume injection of one additional mouse per group.

5. Dilute the plasmid DNA with saline solution to the final volume and mix well by shaking upside down (*see* Note 3).

6. Filter the plasmid-saline solution to a new 50 ml conical tube by filtering with 20 ml Luer-Lok™ syringes and 0.22-μl filter. Be careful not to contaminate with other plasmids when preparing more than one plasmid mixture. Always mark the name and plasmid information on the tube.

7. Let plasmid-saline solution stay at room temperature for 30–60 min.

3.2 Preparation of Syringes

1. For each mouse, load a sterile, 3 ml, Luer-Lok™ syringe with the plasmid-saline solution using a sterile, 25G × 5/8 needle (0.5 mm × 16 mm). Adjust the volume of the plasmid-saline solution as calculated based on the mouse body weight. Tap air bubbles out and eject the excess plasmid-saline solution to the 50-ml conical tube.

2. Before injection, change the needle to a sterile, 26G × 5/8 Sub-Q needle (0.45 mm × 16 mm) and fill the needle with plasmid-saline solution without introducing air bubbles. If necessary, needles may be re-capped using the one-handed technique.

3.3 HTVi

1. Place one cage of mice under an overhead heater (i.e., red-ray lamp) to warm the mice for 2–3 min to dilate the blood vessels. Do not leave the heat lamp too close

to the mouse cage and be careful not to overheat the mice. Alternatively, animal may be warmed by placing the animal in a commercially available warming box, brass restrainer or by using a warm water circulating pad placed under the cage. These are the safest and most effective ways to warm rodents.

2. Anesthesia is not required for HTVi.

3. Place the mouse in an immobilized Red Tube Tailveiner® Restrainer (Fig. 3a) and adjust the mouse to a position where the lateral caudal vein can be comfortably visualized and injected (Fig. 3b). Rodents sometimes spin in the restrainer; be sure to confirm the location of the lateral tail vein before performing injections. Be careful not to restrain the mouse too tight and always observe the animal breathing. If any abnormal sign is noticed, release the mouse and allow it to rest. The duration of the restraint should be kept to a minimum.

4. Disinfect the tail of the mouse with an alcohol prep pad.

5. Grasp the tail at mid-length or at the distal (further down the tail) end. The index and middle fingers of the non-dominant hand are placed around the tail above where the needle will be inserted (digital pressure will act as a tourniquet). The lower part of the tail is held between the thumb and ring finger below the injection site. Put slight tension on the tail by applying pressure with both sets of fingers.

6. Insert needle into vein at about a 30° angle. Needle should enter the vein at a shallow depth, keeping syringe and needle parallel to tail. Release pressure to the proximal fingers before administering the agent into the vein. No resistance should be felt when depressing the plunger. Avoid moving the needle (*see* Note 4).

7. The full volume of the plasmid-saline solution should be injected through the tail vein in one continuous motion in 5–9 s (*see* Note 5).

8. Remove needle from vein and stop the bleeding by applying gentle pressure to the tail with a tissue. Discard unshielded needle and syringes in the sharps' container.

9. Remove mouse from the restrainer and place mouse into a recovery cage placed on top of a heating pad (bedding should be at 37–38 °C). If the injection room is cold, this step is critical for mouse survival. Hold onto the tail of the mouse until bleeding stops completely.

10. Observe the mouse for 1 h following hydrodynamic DNA delivery. An initial period of panting and immobility is normal due to the temporary arrhythmia caused by HGD but make sure that the mouse shows signs of recovery in approximately 5 min. If the breathing of the mouse becomes exceedingly shallow, gently massage the abdomen of the mouse to facilitate breathing. Note that rate of recovery may be slightly influenced by the mouse strain used.

11. Return mouse to housing cage and ensure that mouse has an abundant supply of food and water.

4 Notes

1. For Maxi prep, the following kit is the best: Plasmid Maxiprep Kit: GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit.
2. Avoid freeze-thawing plasmids too many times.
3. Freshly prepared plasmid solution for tail injection on the same day is better.
4. Usually keep the slope of the needle up when tail injection.
5. It is convenient for the HTVi when the needle slope is in parallel with the outside part of the syringe.

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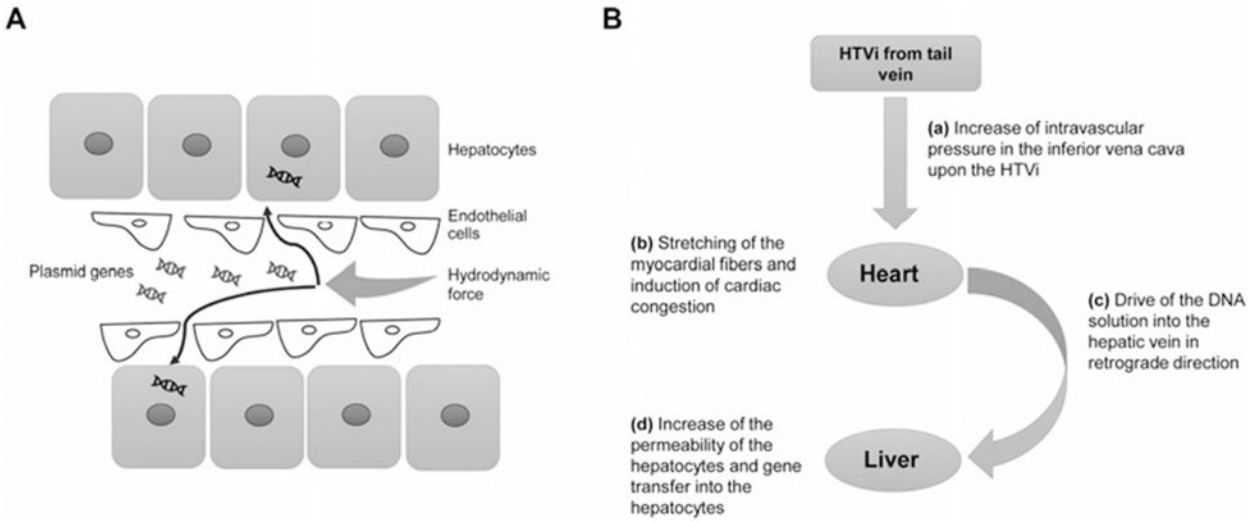

Fig. 1.

Principles of hydrodynamic tail vein injection (HTVi) for the study of liver diseases. (**a**) Hydrodynamic transfection uses a hydrodynamic force produced by the pressurized injection of a large volume of DNA solution into the blood vessel, which permeabilizes the capillary endothelium and generates pores in the plasma membrane of the surrounding hepatocytes. DNA has access to the intracellular compartment through these pores. Subsequently, the pores of the plasma membrane close, trapping the DNA inside the hepatocytes. (**b**) The HTVi delivers a large volume of DNA solution into the inferior vena cava, leading to the increased intravascular pressure. Subsequently, the myocardial fibers stretch over the optimal length for contraction, induce cardiac congestion, and drive the injected solution into the liver in retrograde. Consequently, liver is the organ with the major uptake of plasmid DNA in the body, and hepatocytes can be transfected after hydrodynamic tail vein injection

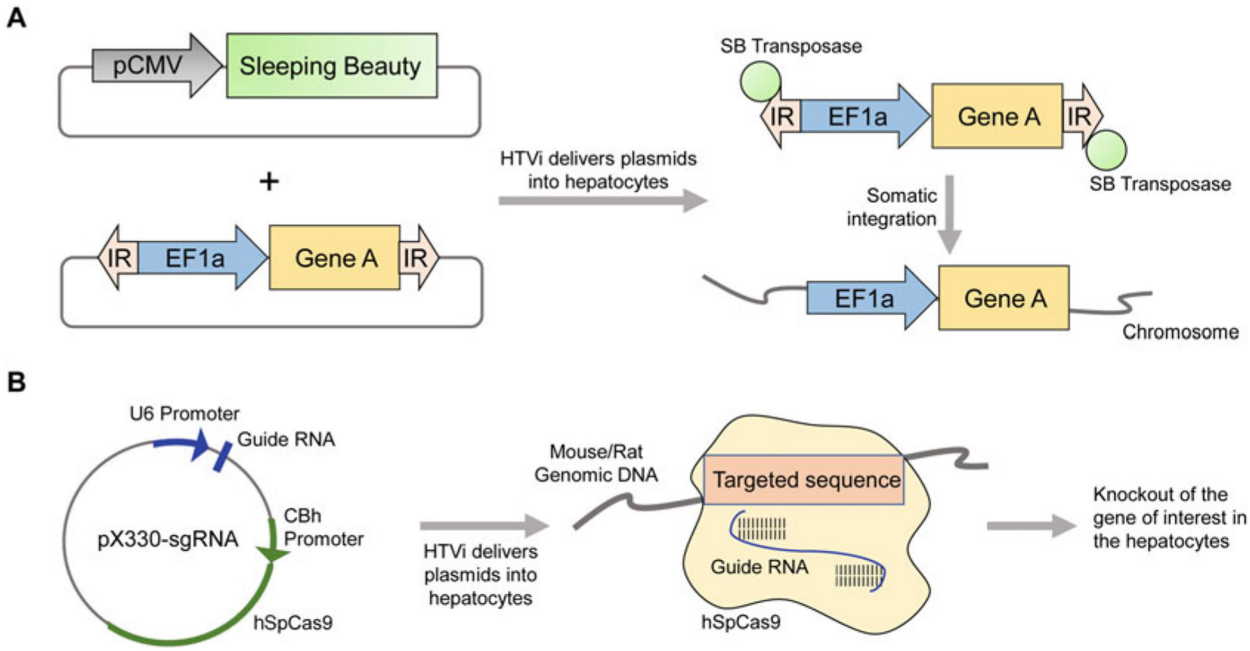

Fig. 2.

Working mode of sleeping beauty transposase and CRISPR/Cas9-mediated gene editing in the hepatocytes. (**a**) Combinational injection of pCMV-SB transposase expressing plasmids and transposons with gene of interest in pT3-EF1a backbone through hydrodynamic tail vein injection delivers plasmids into hepatocytes. The SB transposase subsequently “cut” and “paste” the insert and randomly integrates into genomic DNA of the hepatocytes. The gene with EF1a (or CAGGS, PGK) promoter can be constitutively expressed in the hepatocytes while the gene with CMV promoter could be silenced in a few days after HTVi in the hepatocytes. (**b**) Schematic diagram of pX330-based vector with U6 promoter driving sgRNA and expression of Cas9 (hSpCas9) to induce gene knock out in hepatocytes

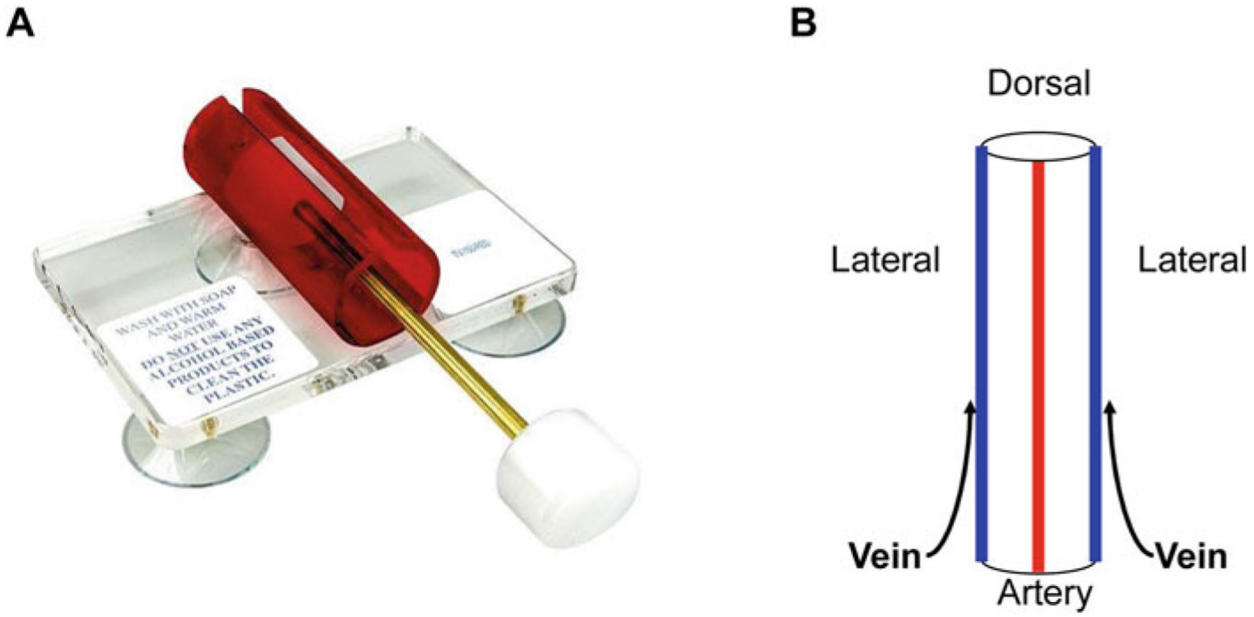

Fig. 3.

Performing hydrodynamic tail vein injection. (**a**) Representative image of an animal restrainer. (**b**) Scheme of mouse/rat tail vasculature. The tail artery (Red) locates in the middle while the two tail veins (Blue) arrange laterally from a dorsal view

Table 1

Calculation of the number of mice for preparing plasmid-saline solutions

Actual BW for each mouse (g)	A, B, C, etc.
Total BW of mice	t-BW = A + B + C + …
Number of mice to prepare	n = t-BW ÷ 20
Eventual number of mice to prepare	$N = n + 1$

Table 2

Calculation of the total volume of plasmid-saline solutions to prepare

Volume of plasmid-saline solution for each mouse	2 ml
Total volume of plasmid-saline solution to prepare (t-V)	t-V = 2 × *N* (ml)
Actual volume of plasmid-saline solution for HTVi (V)	*V* = BW × 10% (ml)

Table 3

Calculation of the volume of each component in the plasmid-saline solutions

Components	Dosage (μg)	Amount (μg)	Concentration (mg/ml)	Volume (ml)
Plasmid A	(i)	M1 = (i) × *N*	C1	V1 = M1 ÷ C1
Plasmid B	(ii)	M2 = (ii) × *N*	C2	V2 = M2 ÷ C2
Plasmid C	(iii)	M3 = (iii) × *N*	C3	V3 = M3 ÷ C3
…	…	…	…	…
Saline solution	–	–	–	Vs = t-V – (V1 + V2 + V3…)