Pharm Res (2011) 28:694–701
DOI 10.1007/s11095-010-0338-9

EXPERT REVIEW

Hydrodynamic Gene Delivery and Its Applications in Pharmaceutical Research

Barbara Bonamassa • Li Hai • Dexi Liu

Received: 2 September 2010 / Accepted: 24 November 2010 / Published online: 30 December 2010
© Springer Science+Business Media, LLC 2010

ABSTRACT Hydrodynamic delivery has emerged as the simplest and most effective method for intracellular delivery of membrane-impermeable substances in rodents. The system employs a physical force generated by a rapid injection of large volume of solution into a blood vessel to enhance the permeability of endothelium and the plasma membrane of the parenchyma cells to allow delivery of substance into cells. The procedure was initially established for gene delivery in mice, and its applications have been extended to the delivery of proteins, oligo nucleotides, genomic DNA and RNA sequences, and small molecules. The focus of this review is on applications of hydrodynamic delivery in pharmaceutical research. Examples are provided to highlight the use of hydrodynamic delivery for study of transcriptional regulation of CYP enzymes, for establishment of animal model for viral infections, and for gene drug discovery and gene function analysis.

KEY WORDS gene therapy · hydrodynamic delivery · nonviral gene delivery · oligo nucleotides · protein drug discovery · siRNA

INTRODUCTION

Hydrodynamic delivery is one of non-viral methods developed originally for intracellular gene delivery but later found applicable to delivery of other macromolecules such as oligo nucleotides, RNA, proteins, and the compounds that are not permeable to cell membrane. The procedure involves a rapid injection of a large volume of solution into a vasculature to facilitate substance transfer into parenchyma cells. It has been used as a research tool for introducing macromolecules into cells to study their biochemical, cell biological, physiological, or/and therapeutic activity in the context of a whole animal. Excellent reviews are available in literature comparing the pros and cons of this technique to other methods of delivery (1–3). The primary focus of this review is to summarize recent progress in applying hydrodynamic delivery to pharmaceutical research. The objective of this article is to inspire the use of hydrodynamic principle to solve pharmaceutical problems.

B. Bonamassa · L. Hai · D. Liu (✉)
Department of Pharmaceutical Sciences
University of Pittsburgh School of Pharmacy
527 Salk Hall, 3501 Terrace Street,
Pittsburgh, Pennsylvania 15261, USA
e-mail: dliu@pitt.edu

A BRIEF HISTORY OF HYDRODYNAMIC GENE DELIVERY

There are three critical elements in hydrodynamic delivery: large volume, high injection speed, and a vasculature for needle placement. Experiments suggesting that intracellular delivery of nucleic acids could be achieved by injection of a large volume of solution were first reported by Budker *et al.* (4). Using plasmid carrying a reporter gene, the authors demonstrated that about 1% of the hepatocytes were transfected when 1 ml of hypertonic solution was intraportally injected into a mouse with transient occlusion of the hepatic vein. Decreasing the volume by half resulted in a 70-fold reduction in reporter gene expression. It was hypothesized based on these early observations that the hydrostatic pressure resulting from the injection of DNA solution is the driving force for DNA transfer into the hepatocytes. Further support for this hypothesis was generated when the same group of researchers showed successful gene transfer by injecting a large volume of plasmid DNA solution into the inferior vena cava (IVC) or

bile duct in mice and rats, and into the bile duct in dogs (5). However, the principle of hydrodynamic delivery conceptualizing the combined effect of large volume and rapid injection was not established until 1999, when Liu *et al.* (6) and Zhang *et al.* (7) demonstrated efficient gene delivery in mice by a rapid (5–7 s) tail vein injection of plasmid DNA in a volume equal to 8–10% of body weight. Under such experimental conditions, reporter gene expression was detected in all internal organs examined, including the lung, heart, spleen, kidney and liver, with the highest level seen in the liver. By a simple tail vein injection of 10 µg of plasmid DNA into a mouse, approximately 30–40% of transfection efficiency in the liver was achieved (6). Such delivery efficiency remains the highest ever achieved *in vivo* without using a viral carrier.

MAJOR FEATURES OF HYDRODYNAMIC GENE DELIVERY

Although hydrodynamic gene delivery is well tolerated by rodents, impacts of the procedure on animals for tail vein injection are evident. Immediately after the injection, mice become immobile and manifest labored breathing for about 5 min. Rats react more severely and may stop breathing after the injection (8). A gentle massage of the animal abdomen is usually sufficient to stimulate breathing and facilitate the recovery. These acute signs of hydrodynamic impact on cardio function occur and recede quickly (9,10). A sharp increase of intravascular pressure was also observed and coincided with liver expansion up to 240% of its original size (10). Sinusoidal expansion and disruption of fenestrae soon after hydrodynamic injection into a mouse were also documented (11). Signs of recovery are already apparent in 5 min after the procedure. The heart rate returns to normal range in 2 min (9), while the intravascular pressure (at both the IVC and portal vein) drops sharply right after the end of the injection and reaches near the basal level within 3 min (10). The expanded liver returned to its original size in 30 min (10) and the disrupted sinusoids return to their original structure and function 24–36 hr after the hydrodynamic delivery (10) and membrane pores in hepatocytes reseal in less than 2 min (9). Serum concentrations of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) reach the peak level soon after the injection and drop to normal range 72 hr post-injection (6,11). No apparent long-term complication was identifiable, even in animals receiving 8 weekly hydrodynamic injections (Ma and Liu, unpublished data).

At experimental level, a 27-gauge needle is commonly used for injection in mice, whereas a 20- to 24-gauge needle appears appropriate for rats with different body weight. Other sized needles can also be used depending on the injection volume and speed and the type of blood vessel receiving the injection. When a local injection is performed, occlusion of the blood flow from the area of interest can make a significant difference in gene transfer efficiency (12,13). Also, the volume administered through the tail vein to rodents is usually 80–100 ml/kg (6,7,14). The volume of ~15 ml/kg has been administered to the isolated liver of the rabbit (15). Hagstrom *et al.* (12) demonstrated that the administration of permeability enhancers prior to the hydrodynamic injection can decrease the volume required for optimal gene delivery. For maximal delivery efficiency, mice should be injected as rapidly as possible, typically in 3–5 s (6). Instead, rats are typically injected at a rate of 2 ml/s using a power injector, although a hand-held syringe can be used (14). The isolated liver of the rabbit is injected at a rate of 15–20 ml/s, which can only be accomplished by a power injector (15). DNA dosage employed ranges from 0.1 to 10 mg/kg, depending on the application. An optimal dose for rats is 3 mg/kg (14) and 7 mg/kg for rabbits (15). For mice, the plasmid DNA dose range is 0.5–2.5 mg/kg, and no more than 50 µg/injection should be used, as higher amount does not appreciably improve the level of transgene product (6). While saline is the most commonly used vehicle for DNA (6), other solutions have also been employed, including Ringers solution (7) and phosphate-buffered saline (16). The highest level of gene product reported by a single hydrodynamic injection into the mouse tail vein was 500–1,000 µg per ml of serum (17–19), and 45 µg of cellular protein per gram of liver. It was shown that such level of gene expression was sufficient for restoring the function of blood coagulation in hemophilia mice (20). It is important to point out that the level of transgene expression is significantly influenced by the structure of plasmids used (different promoter and regulatory sequence, inclusion of intron sequences, etc.) and the type of transgene to be expressed. In addition, while organs including the lung, heart, spleen, kidney and liver showed significant reporter gene expression, the liver appears to be the most sensitive organ because the level of reporter gene expression in liver is at least 10,000-fold higher than that of other organs (6,7).

The mechanism of hydrodynamic delivery to liver hepatocytes has been well studied (9,10,21). Anatomically, a bolus injection of DNA solution into the tail vein induces immediate congestion in the heart, causing accumulation of the injected solution in the IVC and elevation of intravascular pressure in this venous section. Consequently, DNA solution refluxes into the liver via the hepatic vein, pushing the existing blood in the liver backward toward portal vein, thereby avoiding immediate mixing of DNA with nucleases in the blood. Once entering the liver, the dynamic force

carried by the pressurized solution expands the sinusoids, enlarges the liver fenestrae, and permeabilizes the plasma membrane of parenchyma hepatocytes to allow DNA to enter the cells. With continuous cardiac activity the body adapts to the volume load, and, over the time, homeostasis is restored.

Because of its physical nature of the hydrodynamic delivery, various molecules have been delivered to hepatocytes in the liver, including circular DNA (6,7), DNA fragments (22), bacterial artificial chromosomes (22), RNA (single- or double-stranded, synthetic or genomic) (16,23–27), oligo nucleotides (28), proteins (antibodies, enzymes) (9,29), polymers (29), and small compounds (9,11). Modified procedures for gene delivery to muscle, kidney, heart and spleen have been reported for hydrodynamic gene delivery in different animal species (for review, see 21).

HYDRODYNAMIC DELIVERY FOR ASSESSING TRANSCRIPTION REGULATION OF DRUG METABOLIZING ENZYMES

The study of drug metabolism is essential, as it is not only one of the main determinants for drug disposition, biological effects, and toxicity, but also the cause of undesirable metabolism-based drug-drug interactions upon exposure to multiple xenobiotics. Expression of CYP genes responsible for drug metabolism is subject to diverse regulatory controls that display tissue-specific, sex-specific, and developmental patterns. Certain CYP genes are constitutively expressed, and others are known to be induced by various xenobiotics. Most CYP regulation is at the transcription level, although post-transcriptional regulation is also known (30). Valuable information has been obtained about CYP regulation using cell lines or primary culture. However, results from the cell culture studies showed significant variation depending on the experimental conditions employed and the physiological conditions of the tissue samples obtained from the patients (31). This problem can be solved, at least in part, by adopting the system of hydrodynamic gene delivery. For example, reporter plasmids with a human CYP gene promoter cloned at the 5′ end of a reporter gene can be hydrodynamically injected into a mouse liver, and the level of reporter gene expression obtained in the presence or absence of inducers/suppressors will be a direct indication of promoter activity. To study the role of nuclear receptor (NR) in transcription regulation, plasmids containing the nuclear receptor gene can be co-injected into animal with the reporter plasmids before the xenobiotic inducers/suppressors are introduced into the same animal.

The pertinent literature offers a few noteworthy examples of the application of the hydrodynamics-based procedure to studies of transcription regulation of CYP enzymes. For instance, Al-Dosari *et al.* (32) demonstrated the transcription regulation of human CYP2C9 gene by human PXR in mice. The use of different deletion construct of plasmids also allowed the authors to locate the element responsible for PXR-xenobiotic-mediated regulation within the promoter of CYP2C9 gene (32). The hydrodynamic delivery has also been employed to establish a real-time assay that measures the induced transcription of luciferase genes under the control of 5′-flanking region of a human CYP gene (33). By means of quantitative whole body imaging of bioluminiescence generated by luciferase in transfected animals, Schuetz *et al.* (33) were able to measure the effects on transcription as it occurred when hydrodynamically transfected mice were administered with pharmaceuticals known to induce the human CYP3A4 and multi-drug resistance 1 (MDR1) gene expression. A similar approach was used by Tirona *et al.* (34) and Zhang *et al.* (35) to elucidate the transcription regulation of the human CYP3A4 gene by hydrodynamic co-injection of human NRs in mice and by Merrell *et al.* (36) to study the induction of human CYP2B6 gene by the chemopreventive chemical oltipraz. These examples not only provide evidence that this system easily offers the possibility to set up the most informative biological conditions to study drug metabolism, but it also substantially reduces the time and cost required for gene expression and regulation analysis.

A related application of the hydrodynamics-based procedure concerns the study of NR subcellular distribution. Analysis of factors controlling the subcellular compartmentalization and movement of NRs within cells, especially nuclear/cytoplasmic shuffling, is vital for understanding NR action as transcription regulators of CYP genes. Immunohistochemical analysis is frequently employed to analyze the *in vivo* distribution of endogenously expressed NRs. However, this approach suffers from the cross-reaction of antibodies against NR isoforms that differ by a few amino acid residues. As a consequence, the existence of identical antibody epitopes shared between multiple NR isoforms may mask the subcellular localization of the specific NR in study. Additional complications arise when *in vivo* analysis of mutant variant is desired because it requires the time-consuming and laborious process to generate transgenic animals. These compounding factors usually are in favour of cell culture-based systems, which, however, suffer from the lack of a complex physiological interplay between different cell types and tissues in the context of a whole organism. This is especially true for proteins whose action can be modulated not only by drugs but also by endogenous ligands potentially produced elsewhere within the body. The use of the hydrodynamics-based procedure can easily overcome these daunting drawbacks. In fact, the combined use of confocal microscopy and delivery of fluorescently labelled

NR that can be rapidly visualized, processed and analysed within a short time after the injection allows for the study of the subcellular distribution of NRs and for dissecting the structural features that govern their subcellular localization in a living organism. So far, this approach has been used to generate important insights into the constitutive androstane receptor (CAR) signalling pathway (37–39). It is important to point out that the functions of nuclear receptors appear to be animal species-dependent. The animal model selected has to match with the nuclear receptor to be studied.

Hydrodynamics-based procedure can also be used to express human CYP gene(s) in mice or rats to verify or predict the pattern of drug metabolism in clinic. To certain degrees, the system proposed here is more likely to generate a humanized organ in a whole animal (e.g. liver by hydrodynamic tail vein injection) with the exception that it is much faster and highly flexible on the type of human CYP gene(s) one wishes to study. In principle, such system is applicable to various toxicological studies as well. For instance, the hydrodynamic tail vein injection of a plasmid vector containing the constitutively activated Signal Transducer and Activator of Transcription 3 (STAT3) protein has allowed Meng *et al.* (40) to elucidate the role of STAT3 in mediating the effects of the Farnesoid X receptor (FXR) in liver repair in mice treated with CCl_4.

HYDRODYNAMIC DELIVERY FOR ESTABLISHING ANIMAL MODEL FOR VIRAL RESEARCH

Diseases caused by viral infection represent a major type of diseases that could have severe consequences if not properly treated. Although vaccination is very effective in preventing many viral infections, the method for effective treatment of viral diseases such as hepatitis or AIDS is not available. Among the factors limiting the progress in viral research, one is the lack of a convenient animal model that people can work on. For example, hepatitis B virus (HBV) is a human hepatotropic DNA virus that causes severe morbidity and mortality. Approximately two billion people worldwide have been infected by HBV (41). Despite significant effort in vaccine development to reduce the risk of HBV transmission, an estimated 378 million people (or 6% of the world population) are chronically infected (42) and at risk of leading to end-stage liver disease or hepatocellular carcinoma. Chimpanzees appear to be the only type of animal susceptible to HBV infection (43,44). However, its large size, high cost, and ethical considerations severely restrict their usage. The primary reason that HBV cannot be studied in rodents and other animals is because HBV cannot infect these animals due to lack of an appropriate receptor on their liver cells. To solve this problem, Yang *et al.* (45) hydrodynamically injected the HBV genome into a mouse to bypass the requirement of membrane receptor and force an entry (infection) of HBV DNA into the mouse hepatocytes. They showed that viral particles were produced in the hydrodynamically transfected mice and virus persisted for at least 81 days in these animals. Similar results were also obtained by hydrodynamic injection of plasmid DNA containing a 1.5-fold overlength of HBV genome (46). Huang *et al.* (47) demonstrated that about 40% of the HBV-injected mice carry surface antigen for more than 6 months, and HBV replication intermediates, transcripts and proteins for up to 1 year. The hydrodynamics-based procedure has also been used to generate mouse models for chronic infection of HCV (48,49) and HDV (23). In principle, the same strategy can be used to generate viral infection in mouse for any human viruses to facilitate viral research and antiviral drug development. Similarly, any other disease model can also be created in rodents by expressing the disease-causing gene(s) using hydrodynamic delivery of plasmids carrying the disease causing gene(s).

HYDRODYNAMIC DELIVERY FOR GENE DRUG DISCOVERY

The advantage for the use of hydrodynamic gene delivery to study therapeutic functions of a gene is obvious. A candidate gene in plasmid or DNA fragment with regulatory sequence for its expression can be hydrodynamically injected into an animal (mouse or rat) carrying a target disease. The therapeutic effect of the gene product (protein or RNA) on disease progression or regression is then evaluated with time. Similar to a conventional drug study, repeated gene transfer, different amount of DNA, and different regiment can be used to maximize the therapeutic outcome. Compared to conventional biotech approach that relies on purified protein for therapeutic assessment, hydrodynamics-based protocol makes the liver the manufacture site for therapeutic proteins, eliminating the steps and cost for protein production and characterization. In addition, since therapeutic evaluation is directly conducted in animals, no issues about formulation development and bioavailability need to be addressed.

Numerous examples are available demonstrating the effectiveness of hydrodynamic delivery for identifying gene (s) with therapeutic activity. Although many of the studies focused on genetic diseases, the underlying principle is the same, i.e. therapeutic activity of a gene sequence can be evaluated in a diseased animal by hydrodynamic injection of the gene sequence properly oriented in an expression vector. Nakamura *et al.* (50) demonstrated that the hydrodynamics-based transfection of the plasmid pKSCX-α-Gal A, carrying a human alpha-galactosidase A gene,

partially reduces systemic accumulation of globotriaosylceramide in Fabry mice. Hydrodynamic injection of plasmid carrying FIX gene into hemophilia mice resulted in sufficient FIX protein and cure of disease (20). Chen and Woo demonstrated a complete cure of phenylketonuria (PKU) in PKU mice by hydrodynamic injection of plasmid containing phenylalanine hydroxylase gene (51). Additional examples include the use of hydrodynamic gene delivery to demonstrate therapeutic effect of the human growth hormone gene in hypophysectomized mice (52); arylsulfatase A and formylglycine-generating enzyme genes for metachromatic leukodystrophy (53); α-L-iduronidase gene for mucopolysaccharidosis I disease (54); short-chain acyl-CoA dehydrogenase gene for short chain acyl-CoA dehydrogenase deficiency (55); α-1 antitrypsin gene for treatment of alpha 1 deficiency (17,19); insulin gene for diabetes (56); hepatocyte growth factor gene for diabetic nephropathy (57); uncoupling protein 1 gene (58) and leptin or ciliary neurotrophic factor genes (59) for obesity; interleukin (IL)-22-immunoglobulin (60), IL-13-immunoglobulin (61) and IL-1 receptor antagonist-immunoglobulin (62) fusion genes for myocarditis; IL-10 gene for glomerulonephritis (63); paraoxonase gene for reducing organophosphate toxicity (64); endo-beta-galactosidase C (65) and cytotoxic T-lymphocyte antigen 4-immunoglobulin (66) genes for prolonging the survival of xenotransplantation; eritropoietin gene for hypoxic-ischemia encephalopathy (67); nitric oxide synthase-2 gene for liver regeneration (68); and cytokine genes for cancer treatment (69–82).

In addition, hydrodynamic gene delivery has also been employed to define gene functions in whole animal. For example, the hepatocyte growth factor receptor proto-oncogene MET, which encodes a tyrosine protein kinase, is believed to be involved in development of hepatocellular adenomas. Its oncolytical activity was demonstrated when a MET plasmid was co-injected into mouse with plasmid carrying *ΔN90-CTHNB1* (constitutively active version of β-catenin) or *DNHNF1α* (a dominant-negative hepatocyte nuclear factor 1α version of TCF1) gene. Approximately 74% of injected mice developed hepatocellular adenomas, compared to none in animals injected with *MET*, *ΔN90-CTHNB1* or *DNHNF1α* alone (83).

Practically, any genomic sequence (coding and non-coding) or its mutated form can be cloned and studied for its function in the liver of either a mouse or rat employing the technique of hydrodynamic tail vein injection.

CONCLUSIONS AND FUTURE PERSPECTIVES

The goal of any delivery method is to transfer molecules to desired cells and tissues with sufficient efficiency and minimal toxicity. In this regard, the hydrodynamics-based delivery system has established itself as the leading strategy to perform *in vivo* studies. In fact, its transient and minimal toxicity together with unprecedented delivery efficiency have made the hydrodynamics-based transfection the most desirable procedure to study the function of coding or non-coding sequences in whole animals. Also, its simplicity and flexibility offer great convenience for the *in vivo* intracellular delivery of DNA, RNA, proteins or other membrane-impermeable compounds. For gene delivery studies, the use of tissue-specific promoter to drive transgene expression could limit the site of gene expression to a selected tissue/organ. Although similar to many physical methods, such as microinjection, particle bombardment and electroporation, the hydrodynamic delivery is actually different from these device-based methods because it doesn't require any sophisticated equipment. Other than a syringe, saline and the test substance, no other additional materials are needed. Since its establishment in 1999, an ever-increasing number of laboratories have thus chosen and adapted this method for their studies in rodents requiring intracellular delivery of membrane-impermeable substances. In this context, it is worth noting that the first demonstration of effective gene knockdown by small interfering RNA *in vivo* was conducted by using the hydrodynamics-based delivery (27).

Perhaps the greatest potential for the hydrodynamic delivery procedure is to reduce the cost associated with research and discovery of protein drugs. This is noteworthy especially in the field of pharmaceutical research, considering that on average every new drug requires 12–15 years to reach the patient and costs $400–650 million (84). In most cases, a large amount of proteins has to be prepared and characterized *in vitro* before *in vivo* evaluation can be conducted. When the hydrodynamics-based gene delivery is employed, there is no need to go through the difficult and time-consuming process of using transgenic animals or less reliable cell-based systems for protein production. In fact, the hydrodynamic procedure allows to draw reliable conclusions under the most informative setting, i.e. in a whole animal where all of the physiological processes are recapitulated. Also, no expensive devices or specialized expertise, which might not be available to most investigators, is required. As discussed in the previous sections, the elucidation of NR pathways and study of drug metabolism under humanized conditions, the establishment of human disease models in animals, the unravelling of gene function by delivery of siRNA and discovery of gene drug are some of the pharmaceutical research-related fields which are already benefiting from the advantages of the hydrodynamic delivery.

Despite the fact that the physical nature of intrahepatic gene delivery following hydrodynamic injection has been firmly established, many other aspects of the procedure still

have to be explored. For example, gene transfer into cells in organs other than the liver needs to be elucidated, as there is evidence that the hydrodynamic delivery is applicable not only to fenestrated but also continuous capillaries (7,12). In addition, feasibility to perform the hydrodynamic procedure in large animals has been demonstrated using an image-guided hydrodynamic procedure using a computer-controlled injection device (85). It is foreseeable that in the near future, the pharmaceutical research will take great advantage of the efficiency of the hydrodynamic gene delivery procedure not only for drug discovery but also for treatment of human diseases.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants RO1EB007357 and RO1HL098295. B. Bonamassa is recipient of the Rotary Foundation Ambassadorial Scholarship for the 2009/2010 academic year.

REFERENCES

1. Gao X, Kim KS, Liu D. Nonviral gene delivery: what we know and what is next. AAPS J. 2007;9:E92–104.
2. Herweijer H, Wolff JA. Gene therapy progress and prospects: hydrodynamic gene delivery. Gene Ther. 2007;14:99–107.
3. Kobayashi N, Nishikawa M, Takakura Y. The hydrodynamics-based procedure for controlling the pharmacokinetics of gene medicines at whole body, organ and cellular levels. Adv Drug Deliv Rev. 2005;57:713–31.
4. Budker V, Zhang G, Knechtle S, Wolff JA. Naked DNA delivered intraportally expresses efficiently in hepatocytes. Gene Ther. 1996;3:593–8.
5. Zhang G, Vargo D, Budker V, Armstrong N, Knechtle S, Wolff JA. Expression of naked plasmid DNA injected into the afferent and efferent vessels of rodent and dog livers. Hum Gene Ther. 1997;8:1763–72.
6. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 1999;6:1258–66.
7. Zhang G, Budker V, Wolff JA. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. Hum Gene Ther. 1999;10:1735–7.
8. Hodges BL, Scheule RK. Hydrodynamic delivery of DNA. Expert Opin Biol Ther. 2003;3:911–8.
9. Zhang G, Gao X, Song YK, Vollmer R, Stolz DB, Gasiorowski JZ, *et al.* Hydroporation as the mechanism of hydrodynamic delivery. Gene Ther. 2004;11:675–82.
10. Suda T, Gao X, Stolz DB, Liu D. Structural impact of hydrodynamic injection on mouse liver. Gene Ther. 2007;14:129–37.
11. Kobayashi N, Nishikawa M, Hirata K, Takakura Y. Hydrodynamics-based procedure involves transient hyperpermeability in the hepatic cellular membrane: implication of a nonspecific process in efficient intracellular gene delivery. J Gene Med. 2004;6:584–92.
12. Hagstrom JE, Hegge J, Zhang G, Noble M, Budker V, Lewis DL, *et al.* A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs. Mol Ther. 2004;10:386–98.
13. Zhang G, Budker V, Williams P, Subbotin V, Wolff JA. Efficient expression of naked DNA delivered intraarterially to limb muscles of nonhuman primates. Hum Gene Ther. 2001;12:427–38.
14. Maruyama H, Higuchi N, Nishikawa Y, Kameda S, Iino N, Kazama JJ, *et al.* High-level expression of naked DNA delivered to rat liver via tail vein injection. J Gene Med. 2002;4:333–41.
15. Eastman SJ, Baskin KM, Hodges BL, Chu G, Gates A, Dreusicke R, *et al.* Development of catheter-based procedures for transducing the isolated rabbit liver with plasmid DNA. Hum Gene Ther. 2002;13:2065–77.
16. McCaffrey AP, Ohashi K, Meuse L, Shen S, Lancaster AM, Lukavsky PJ, *et al.* Determinants of hepatitis C translational initiation *in vitro*, in cultured cells and mice. Mol Ther. 2002;5:676–84.
17. Zhang G, Song YK, Liu D. Long-term expression of human alpha1-antitrypsin gene in mouse liver achieved by intravenous administration of plasmid DNA using a hydrodynamics-based procedure. Gene Ther. 2000;7:1344–9.
18. Stoll SM, Sclimenti CR, Baba EJ, Meuse L, Kay MA, Calos MP. Epstein-Barr virus/human vector provides high-level, long-term expression of alpha1-antitrypsin in mice. Mol Ther. 2001;4:122–9.
19. Alino SF, Crespo A, Dasi F. Long-term therapeutic levels of human alpha-1 antitrypsin in plasma after hydrodynamic injection of nonviral DNA. Gene Ther. 2003;10:1672–9.
20. Miao CH, Thompson AR, Loeb K, Ye X. Long-term and therapeutic-level hepatic gene expression of human factor IX after naked plasmid transfer *in vivo*. Mol Ther. 2001;3:947–57.
21. Suda T, Liu D. Hydrodynamic gene delivery: its principles and applications. Mol Ther. 2007;15:2063–9.
22. Magin-Lachmann C, Kotzamanis G, D'Aiuto L, Cooke H, Huxley C, Wagner E. *In vitro* and *in vivo* delivery of intact BAC DNA—comparison of different methods. J Gene Med. 2004;6:195–209.
23. Chang J, Sigal LJ, Lerro A, Taylor J. Replication of the human hepatitis delta virus genome is initiated in mouse hepatocytes following intravenous injection of naked DNA or RNA sequences. J Virol. 2001;75:3469–73.
24. Giladi H, Ketzinel-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E. Small interfering RNA inhibits hepatitis B virus replication in mice. Mol Ther. 2003;8:769–76.
25. Kobayashi N, Matsui Y, Kawase A, Hirata K, Miyagishi M, Taira K, *et al.* Vector-based *in vivo* RNA interference: dose- and time-dependent suppression of transgene expression. J Pharmacol Exp Ther. 2004;308:688–93.
26. Layzer JM, McCaffrey AP, Tanner AK, Huang Z, Kay MA, Sullenger BA. *In vivo* activity of nuclease-resistant siRNAs. RNA. 2004;10:766–71.
27. McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA. RNA interference in adult mice. Nature. 2002;418:38–9.
28. McCaffrey AP, Meuse L, Karimi M, Contag CH, Kay MA. A potent and specific morpholino antisense inhibitor of hepatitis C translation in mice. Hepatology. 2003;38:503–8.
29. Kobayashi N, Kuramoto T, Yamaoka K, Hashida M, Takakura Y. Hepatic uptake and gene expression mechanisms following intravenous administration of plasmid DNA by conventional and hydrodynamics-based procedures. J Pharmacol Exp Ther. 2001;297:853–60.
30. Nakata K, Tanaka Y, Nakano T, Adachi T, Tanaka H, Kaminuma T, *et al.* Nuclear receptor-mediated transcriptional regulation in Phase I, II, and III xenobiotic metabolizing systems. Drug Metab Pharmacokinet. 2006;21:437–57.
31. Donato MT, Lahoz A, Castell JV, Gómez-Lechón MJ. Cell lines: a tool for *in vitro* drug metabolism studies. Curr Drug Metab. 2008;9:1–11.

32. Al-Dosari MS, Knapp JE, Liu D. Activation of human CYP2C9 promoter and regulation by CAR and PXR in mouse liver. Mol Pharm. 2006;3:322–8.
33. Schuetz E, Lan L, Yasuda K, Kim R, Kocarek TA, Schuetz J, *et al*. Development of a real-time *in vivo* transcription assay: application reveals pregnane X receptor-mediated induction of CYP3A4 by cancer chemotherapeutic agents. Mol Pharmacol. 2002;62:439–45.
34. Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, *et al.* The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. Nat Med. 2003;9:220–4.
35. Zhang W, Purchio A, Chen K, Burns SM, Contag CH, Contag PR. *In vivo* activation of the human CYP3A4 promoter in mouse liver and regulation by pregnane X receptors. Biochem Pharmacol. 2003;65:1889–96.
36. Merrell MD, Jackson JP, Augustine LM, Fisher CD, Slitt AL, Maher JM, *et al.* The Nrf2 activator oltipraz also activates the constitutive androstane receptor. Drug Metab Dispos. 2008;36:1716–21.
37. Zelko I, Sueyoshi T, Kawamoto T, Moore R, Negishi M. The peptide near the C terminus regulates receptor CAR nuclear translocation induced by xenochemicals in mouse liver. Mol Cell Biol. 2001;21:2838–46.
38. Sueyoshi T, Moore R, Pascussi JM, Negishi M. Direct expression of fluorescent protein-tagged nuclear receptor CAR in mouse liver. Methods Enzymol. 2002;357:205–13.
39. Sueyoshi T, Moore R, Sugatani J, Matsumura Y, Negishi M. PPP1R16A, the membrane subunit of protein phosphatase 1beta, signals nuclear translocation of the nuclear receptor constitutive active/androstane receptor. Mol Pharmacol. 2008;73:1113–21.
40. Meng Z, Wang Y, Wang L, Jin W, Liu N, Pan H, *et al.* FXR regulates liver repair after CCl4-induced toxic injury. Mol Endocrinol. 2010;24:886–97.
41. Shepard CW, Simard EP, Finelli L, Fiore AE, Bell BP. Hepatitis B virus infection: epidemiology and vaccination. Epidemiol Rev. 2006;28:112–25.
42. Zanetti AR, Van Damme P, Shouval D. The global impact of vaccination against hepatitis B: A historical overview. Vaccine. 2008;26:6266–73.
43. Bertoni R, Sette A, Sidney J, Guidotti LG, Shapiro M, Purcell R, *et al.* Human class I supertypes and CTL repertoires extend to chimpanzees. J Immunol. 1998;161:4447–55.
44. Will H, Cattaneo R, Koch HG, Darai G, Schaller H, Schellekens H, *et al.* Cloned HBV DNA causes hepatitis in chimpanzees. Nature. 1982;299:740–2.
45. Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. Proc Natl Acad Sci USA. 2002;99:13825–30.
46. Suzuki T, Takehara T, Ohkawa K, Ishida H, Jinushi M, Miyagi T, *et al.* Intravenous injection of naked plasmid DNA encoding hepatitis B virus (HBV) produces HBV and induces humoral immune response in mice. Biochem Biophys Res Commun. 2003;300:784–8.
47. Huang LR, Wu HL, Chen PJ, Chen DS. An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. Proc Natl Acad Sci USA. 2006;103:17862–7.
48. Wang Q, Contag CH, Ilves H, Johnston BH, Kaspar RL. Small hairpin RNAs efficiently inhibit hepatitis C IRES–mediated gene expression in human tissue culture cells and a mouse model. Mol Ther. 2005;12:562–8.
49. Kim SI, Shin D, Lee H, Ahn B, Yoon Y, Kim M. Targeted delivery of siRNA against hepatitis C virus by apolipoprotein A-I-bound cationic liposomes. J Hepat. 2009;50:479–88.
50. Nakamura G, Maruyama H, Ishii S, Shimotori M, Kameda S, Kono T, *et al.* Naked plasmid DNA-based alpha-galactosidase A gene transfer partially reduces systemic accumulation of globotriaosylceramide in Fabry mice. Mol Biotechnol. 2008;38:109–19.
51. Chen L, Woo SL. Complete and persistent phenotypic correction of phenylketonuria in mice by site-specific genome integration of murine phenylalanine hydroxylase cDNA. Proc Natl Acad Sci USA. 2005;102:15581–6.
52. Sondergaard M, Dagnaes-Hansen F, Flyvbjerg A, Jensen TG. Normalization of growth in hypophysectomized mice using hydrodynamic transfer of the human growth hormone gene. Am J Physiol Endocrinol Metab. 2003;285:E427–32.
53. Takakusaki Y, Hisayasu S, Hirai Y, Shimada T. Coexpression of formylglycine-generating enzyme is essential for synthesis and secretion of functional arylsulfatase A in a mouse model of metachromatic leukodystrophy. Hum Gene Ther. 2005;16:929–36.
54. Camassola M, Braga LM, Delgado-Canedo A, Dalberto TP, Matte U, Burin M. Nonviral *in vivo* gene transfer in the mucopolysaccharidosis I murine model. J Inherit Metab Dis. 2005;28:1035–43.
55. Holm DA, Dagnaes-Hansen F, Simonsen H, Gregersen N, Bolund L, Jensen TG. Expression of short-chain acyl-CoA dehydrogenase (SCAD) proteins in the liver of SCAD deficient mice after hydrodynamic gene transfer. Mol Genet Metab. 2003;78:250–8.
56. He CX, Shi D, Wu WJ, Ding YF, Feng DM, Lu B. Insulin expression in livers of diabetic mice mediated by hydrodynamics-based administration. World J Gastroenterol. 2004;10:567–72.
57. Dai C, Yang J, Bastacky S, Xia J, Li Y, Liu Y. Intravenous administration of hepatocyte growth factor gene ameliorates diabetic nephropathy in mice. J Am Soc Nephrol. 2004;15:2637–47.
58. Gonzalez Muniesa P, Milagro FI, Campion J, Martinez JA. Reduction in energy efficiency induced by expression of the uncoupling protein, UCP1, in mouse liver mitochondria. Int J Mol Med. 2006;17:591–7.
59. Jiang J, Yamato E, Miyazaki J. Long-term control of food intake and body weight by hydrodynamics-based delivery of plasmid DNA encoding leptin or CNTF. J Gene Med. 2003;5:977–83.
60. Chang H, Hanawa H, Liu H, Yoshida T, Hayashi M, Watanabe R. Hydrodynamic-based delivery of an interleukin-22-Ig fusion gene ameliorates experimental autoimmune myocarditis in rats. J Immunol. 2006;177:3635–43.
61. Elnaggar R, Hanawa H, Liu H, Yoshida T, Hayashi M, Watanabe R. The effect of hydrodynamics-based delivery of an IL-13-Ig fusion gene for experimental autoimmune myocarditis in rats and its possible mechanism. Eur J Immunol. 2005;35:1995–2005.
62. Liu H, Hanawa H, Yoshida T, Elnaggar R, Hayashi M, Watanabe R. Effect of hydrodynamics-based gene delivery of plasmid DNA encoding interleukin-1 receptor antagonist-Ig for treatment of rat autoimmune myocarditis: possible mechanism for lymphocytes and noncardiac cells. Circulation. 2005;111:1593–600.
63. Higuchi N, Maruyama H, Kuroda T, Kameda S, Iino N, Kawachi H. Hydrodynamics-based delivery of the viral interleukin-10 gene suppresses experimental crescentic glomerulonephritis in Wistar-Kyoto rats. Gene Ther. 2003;10:1297–310.
64. Fu AL, Wang YX, Sun MJ. Naked DNA prevents soman intoxication. Biochem Biophys Res Commun. 2005;328:901–5.
65. Miki Y, Maruyama S, Liu D, Kobayashi T, Sato F, Shimizu H. *In vivo* gene transfer of endo-beta-galactosidase C removes alphaGal antigen on erythrocytes and endothelial cells of the organs. Xenotransplantation. 2004;11:444–51.
66. Inoue S, Hakamata Y, Kaneko M, Kobayashi E. Gene therapy for organ grafts using rapid injection of naked DNA: application to the rat liver. Transplantation. 2004;77:997–1003.

67. Wang CH, Liang CL, Huang LT, Liu JK, Hung PH, Sun A. Single intravenous injection of naked plasmid DNA encoding erythropoietin provides neuroprotection in hypoxia-ischemia rats. Biochem Biophys Res Commun. 2004;314:1064–71.
68. Zeini M, Hortelano S, Traves PG, Gomez Valades AG, Pujol A, Perales JC. Assessment of a dual regulatory role for NO in liver regeneration after partial hepatectomy: protection against apoptosis and retardation of hepatocyte proliferation. FASEB J. 2005;19:995–7.
69. Barnett FH, Scharer-Schuksz M, Wood M, Yu X, Wagner TE, Friedlander M. Intra-arterial delivery of endostatin gene to brain tumors prolongs survival and alters tumor vessel ultrastructure. Gene Ther. 2004;11:1283–9.
70. Tada M, Hatano E, Taura K, Nitta T, Koizumi N, Ikai I. High volume hydrodynamic injection of plasmid DNA via the hepatic artery results in a high level of gene expression in rat hepatocellular carcinoma induced by diethylnitrosamine. J Gene Med. 2006;8:1018–26.
71. Kobayashi N, Kuramoto T, Chen S, Watanabe Y, Takakura Y. Therapeutic effect of intravenous interferon gene delivery with naked plasmid DNA in murine metastasis models. Mol Ther. 2002;6:737–44.
72. Yazawa H, Murakami T, Li HM, Back T, Kurosaka K, Suzuki Y. Hydrodynamics-based gene delivery of naked DNA encoding fetal liver kinase-1 gene effectively suppresses the growth of pre-existing tumors. Cancer Gene Ther. 2006;13:993–1001.
73. Sato A, Ohtsuki M, Hata M, Kobayashi E, Murakami T. Antitumor activity of IFN-lambda in murine tumor models. J Immunol. 2006;176:7686–94.
74. Kim KS, Park YS. Antitumor effects of angiostatin K1-3 and endostatin genes coadministered by the hydrodynamics-based transfection method. Oncol Res. 2005;15:343–50.
75. Takehara T, Uemura A, Tatsumi T, Suzuki T, Kimura R, Shiotani A. Natural killer cell-mediated ablation of metastatic liver tumors by hydrodynamic injection of IFNalpha gene to mice. Int J Cancer. 2007;120:1252–60.
76. Yonenaga Y, Mori A, Fujimoto A, Nagayama S, Tachibana T, Onodera H. The administration of naked plasmid DNA into the liver induces antitumor innate immunity in a murine liver metastasis model. J Gene Med. 2007;9:299–307.
77. Kitajima M, Tsuyama Y, Miyano-Kurosaki N, Takaku H. Antitumor effect of intravenous TNFalpha gene delivery naked plasmid DNA using a hydrodynamics-based procedure. Nucleosides Nucleotides Nucleic Acids. 2005;24:647–50.
78. Chen HW, Lee YP, Chung YF, Shih YV, Tsai JP, Tao MH. Inducing long-term survival with lasting anti-tumor immunity in treating B cell lymphoma by a combined dendritic cell-based and hydrodynamic plasmid-encoding IL-12 gene therapy. Int Immunol. 2003;15:427–35.
79. Wang G, Tschoi M, Spolski R, Lou Y, Ozaki K, Feng C. *In vivo* antitumor activity of interleukin 21 mediated by natural killer cells. Cancer Res. 2003;63:9016–22.
80. Wen J, Matsumoto K, Taniura N, Tomioka D, Nakamura T. Hepatic gene expression of NK4, an HGF-antagonist/angiogenesis inhibitor, suppresses liver metastasis and invasive growth of colon cancer in mice. Cancer Gene Ther. 2004;11:419–30.
81. Ortaldo JR, Winkler-Pickett RT, Bere EW, Watanabe Jr M, Murphy MJ, Wiltrout RH. *In vivo* hydrodynamic delivery of cDNA encoding IL-2: rapid, sustained redistribution, activation of mouse NK cells, and therapeutic potential in the absence of NKT cells. J Immunol. 2005;175:693–9.
82. Neal ZC, Bates MK, Albertini MR, Herweijer H. Hydrodynamic limb vein delivery of a xenogeneic DNA cancer vaccine effectively induces antitumor immunity. Mol Ther. 2007;15:422–30.
83. Tward AD, Jones KD, Yant S, Cheung ST, Fan ST, Chen X, *et al*. Distinct pathways of genomic progression to benign and malignant tumors of the liver. Proc Natl Acad Sci USA. 2007;104:14771–6.
84. Collins MA, Shaw I, Billington DC. Driving drug discovery and patient therapy via the encapsulation and fusion of knowledge. Drug Des Discov. 1999;16:181–94.
85. Suda T, Suda K, Liu D. Computer-assisted hydrodynamic gene delivery. Mol Ther. 2008;16:1098–104.