Published in final edited form as:

Adv Drug Deliv Rev. 2010 October 30; 62(13): 1250–1256. doi:10.1016/j.addr.2010.08.005.

Non-Viral Gene Transfer as a Tool for Studying Transcription Regulation of Xenobiotic Metabolizing Enzymes

Barbara Bonamassa and Dexi Liu

Department of Pharmaceutical Sciences, University of Pittsburgh School of Pharmacy, Pittsburgh, PA15261

Abstract

Numerous xenobiotic metabolizing enzymes are regulated by nuclear receptors at transcriptional level. The challenge we currently face is to understand how a given nuclear receptor interacts with its xenobiotics, migrates into nucleus, binds to the xenobiotic response element of a target gene, and regulates transcription. Toward this end, new methods have been developed to introduce the nuclear receptor gene into appropriate cells and study its activity in activating reporter gene expression under the control of a promoter containing xenobiotic response elements. The goal of this review is to critically examine the gene transfer methods currently available. We concentrate on the gene transfer mechanism, advantages and limitations of each method when employed for nuclear receptor-mediated gene regulation studies. It is our hope that the information provided highlights the importance of gene transfer in studying the mechanisms by which our body eliminates the potentially harmful substances and maintains the homeostasis.

Keywords

Xenobiotics; nuclear receptor; transcription factor; drug metabolism; non-viral vector; gene delivery; cationic lipid; electroporation; hydrodynamic gene delivery

1. Introduction

Mammals are constantly exposed to foreign substances or xenobiotics from a large variety of sources such as dietary substances, therapeutic drugs, environmental contaminants, occupational chemicals, and even noxious molecules endogenously produced as part of physiological response to an exogenous insult. Xenobiotics are often biologically active and can do harm upon interacting with the body. The evolution of the xenobiotic response has been evolved in removing most, if not all, of the substances through one of the mechanisms called transcription regulation of xenobiotic metabolizing enzyme (XME). In essence, genes encoding a specific set of XMEs are activated by the presence of xenobiotics, resulting in production of the XMEs and removal of the substances from the body. This need-based regulation offers an advantage of xenobiotic response to be adequate to counter the chemical stimulus and to maintain chemical homeostasis.

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To whom correspondence should be addressed: Dexi Liu, PhD 527 Salk Hall Department of Pharmaceutical Sciences University of Pittsburgh School of Pharmacy 3501 Terrace Street Pittsburgh, PA 15261 Tel: (412) 648-8553 Fax: (412) 383-7436 dliu@pitt.edu.

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Variety of XMEs have been identified, including phase I, phase II enzymes [1-3] and some membrane transporters [4]. Phase-I enzymes (primarily cytochrome P450) enhance the renal clearance of xenobiotics by increasing their polarity through oxidization [5]. In humans, there are 57 genes and more than 58 pseudogenes divided into 18 families of CYPs and 43 subfamilies (<http://drnelson.utmem.edu/CytochromeP450.html>). Fifteen CYPs are involved in xenobiotic metabolism [6]. Phase-II enzymes make their contribution through conjugation reaction to extend the increase of polarity and water solubility of xenobiotics for renal removal. They are transferases belonging to the UDP-glucuronosyltransferase, glutathione S-transferase, sulfotransferases, epoxide hydrolase, and acetyltransferase superfamilies [7]. An equally important group of XMEs is membrane transporters [4] responsible for directing xenobiotics to appropriate site for metabolism. The human genome encodes 48 transporters grouped in seven families [8].

Certain XMEs are constitutively expressed and others are induced by various xenobiotics at transcriptional level. Transcription regulation of XMEs is mediated by a large family of transcription factors known as nuclear receptors (NRs) or xenobiotic activating receptors [9]. The common features of the NRs include a xenobiotic binding domain (also called ligand binding domain) and a DNA binding domain. They are generally cytoplasmic proteins but migrate into nucleus upon xenobiotic binding. Dimerization (either homodimer or heterodimer) of NRs appears essential for their binding to the response element in order to facilitate RNA polymerase binding to its promoter for transcription initiation.

Regulation studies of XME are mostly performed on human hepatocytes because they retain the expression of both phase I and II enzymes for several days in culture [10,11] and are capable of generating a metabolic profile similar to that in patients. However, attention has been shifted to established cell lines due to that human cells are scarce, phenotypically unstable, and highly variable in liver samples obtained from different donors. The major advantages of liver cell lines, compared to primary culture of human hepatocytes, are that they are readily available and easy to maintain, and they grow continuously with almost unlimited life span with a rather stable phenotype. A major drawback of cell line, however, is that these cells express biotransformation activities to a very limited extent (*i.e.* only a few XMEs) and even if they do so, levels are very low in comparison to normal liver cells [12]. Moreover, the isoenzymes expressed by these cells are not often found in normal liver cells. For instance, in HepG2 cells which are among the most widely used human hepatoma cell lines, lack of CYP3A4 mRNA and CYP3A activity has been reported [13,14]. It was postulated that the lack of metabolizing enzyme expression in hepatoma is the consequence of an altered expression of key regulatory transcription factors, *e.g.* C/EBP α , C/EBP β , HNF1 α , HNF3 α , *etc.* [15]. Alternatively, low concentrations of NR, transcription factors, and co-activators (*e.g.* SRC-2) could also occur [16].

Rodent represents the commonly used *in vivo* experimental model for XME studies. Their short life-span, proclivity for reproduction, known genetic background, genetic uniformity and well-defined physiological parameters are the most desirable characteristics. Although it is believed that drug-metabolizing enzyme modulation is regulated in humans in the same fashion as in animals, significant species differences are known in response to xenobiotics both qualitatively and quantitatively [17]. For instance, omeprazole is a CYP1A2 inducer in humans but not in rats and mice [18-20]. 3-Methylcholanthrene is a strong human CYP1A2 inducer but a weak modulator of the same rodent isoform [21]. While 2,3,7,8-tetrachlorodibenzodioxin (TCDD) induces mainly CYP1A2 in human hepatocytes, it affects CYP1A1 in rat hepatocytes [22]. In addition, rifampicin is known to be a potent inducer and dexamethasone a moderate inducer of human CYP3A4, whereas pregnenolone-16 α -carbonitrile (PCN) has little effect. On the contrary, rifampicin isn't an effective CYP3A inducer in rats but PCN is [23,24].

Numerous molecular biology strategies have been put into action to overcome the metabolic incompetence of human hepatoma cell lines and the species differences in commonly used animal models. Among those, transient expression upon gene transfer or transfection is nowadays widely used to address, unravel and elucidate many biological effects triggered or controlled by xenobiotics [25-38]. This approach offers significant advantages. Transient gene expression through gene transfer is fast, easy and cheap to perform. Not only expression plasmids but also reporter plasmids as well as small interfering RNA (siRNA) can be delivered to target cells *in vitro* and *in vivo*. Contrary to traditional transgenic approaches, multiple transgenes can be introduced into target cells, reproducing at least in part the complex interplay of signals (*e.g.* cross-talks) which characterizes transcriptional regulation of XMEs.

In general, assessment of transcriptional regulation of XME genes is complicated because regulation rarely occurs as a consequence of an exposure to one single chemical agent. Other agents potentially implicated in the process include cytokines, anti-inflammatory substances, and steroid hormones [39]. While gene knockout animals have been useful in identifying a specific NR involved in transcriptional regulation of a particular XME gene, more detailed study in determining the function of key amino acid residues in ligand and DNA binding domains cannot be conveniently conducted by the transgenic approach. As discussed below, gene transfer methods to introduce plasmid carrying nuclear receptor gene or its mutated forms, and a reporter plasmid driven by the promoter of a XME gene into cells have become extremely useful system for studying transcription regulation of XME genes.

The primary objective of this review is to summarize recent progress in applying nonviral methods of gene transfer to studies aiming at elucidation of NR mediated transcription regulation of XME genes.

2. Methods of Nonviral Gene Transfer

Naked DNA cannot pass through cell membrane because of its large molecular weight and lack of adequate transporters on cell surface. Both physical and chemical approaches have been used to overcome the membrane barrier and facilitate intracellular DNA transfer [40]. More commonly explored physical methods include needle injection, electroporation and particle bombardment [41]. Chemical approaches employ natural or synthetic compounds to facilitate DNA internalization through endocytosis pathway [40]. The following sections are a brief summary of each of the nonviral gene transfer methods that can be employed for studies of transcription regulation of XME genes.

2.1 Needle injection

Needle injection method consists of the direct injection of a DNA or RNA solution into an individual cell or a tissue. The mechanism of intracellular delivery appears to involve the break of cell membrane. For tissue gene transfer, penetration of a sharp needle into the tissue allows DNA solution to enter the damaged cells on the needle track before the damaged plasma membranes reseal.

This technique is not widely used in studies of transcription regulation. However, a few papers reported the use of this method to study *Xenopus laevis* pathways. For example, Watson and Torres [42] injected recombinant plasmids containing the coding sequence of the human estrogen receptor (ER) into *Xenopus* oocytes to check the ability of the NR to drive vitellogenin gene expression. Similarly, the role of retinoid signaling in neuron pattern development in *Xenopus* was investigated by microinjection of constitutively active or dominant negative retinoid acid receptor (RAR) expression plasmid *in vivo*, using intact embryos [43]. Moreover, by microinjecting thyroid hormone receptors (TRs) and 9-cis

retinoic acid receptors (RXR) individually or together into *Xenopus* developing embryos, the RXR's critical role for developmental function of TRs was demonstrated [44].

A modified needle injection procedure, called hydrodynamic gene delivery, was reported by Liu *et al.* [45] and Zhang *et al.* [46]. The method involves a rapid injection of a large volume of DNA solution via the mouse tail vein to facilitate gene transfer *in vivo*. Hydrodynamic delivery of genetic material into parenchyma cells takes advantage of the mechanism of hydroporation, which is induced by the hydrodynamic pressure generated by the rapid injection of an isotonic solution of plasmid DNA into a blood vessel [47]. Mechanistically, a rapid injection of a large volume of DNA solution into the tail vein of a mouse results in a transient cardiac congestion and retrograde flow of the injected solution into the liver. The intravascular pressure in the liver sinusoids enlarges hepatic fenestrae and generates pores on the plasma membrane of parenchyma hepatocytes. Consequently, DNA molecules diffuse into cells and are trapped inside when the membrane pores close [48]. To date, the hydrodynamics-based procedure is the most effective non-viral method for *in vivo* gene delivery in rodents. In a mouse model, the optimal condition involves a rapid injection (5-8s) of a volume equal to 8-10% of body weight through the tail vein [45]. Approximately 30-40% of the hepatocytes are transfected by a single mouse tail vein injection of less than 50 μ g of plasmid DNA [45] with a transient and reversible impact on liver structure and functions [48]. Because of its simplicity, high efficiency and reproducibility, hydrodynamic delivery has become a routine method for delivery of DNA, siRNA, proteins, small molecules and even viral vectors into the hepatocytes *in vivo* [49]. Since its development in 1999, this procedure has been used widely for gene expression, gene knockdown, function analysis of genetic elements and for establishing disease model in research animals.

A few papers are available in literature reporting the use of hydrodynamic delivery to study transcriptional regulation of phase I enzymes [50-55]. Among these, a couple of studies focused on NR mediated transcription regulation. For example, Al-Dosari *et al* [53] demonstrated the transcriptional regulation of CYP2C9 by mouse constitutive androstane receptor (CAR) and human pregnane X receptor (PXR) through the hydrodynamic co-transfection of NR expression plasmids and reporter gene controlled by 5' regulatory sequence of CYP2C9 gene. De Souza *et al* [55] reported both molecular and phenotypic evidence in support of successful delivery of siRNA and knockdown of peroxisome proliferation activated receptor alpha (PPAR α) in mice.

2.2 Electroporation

Electroporation was first utilized for gene transfer to mammalian cells by Neumann *et al.* [56] and has been extensively studied in recent years as an effective nonviral method for gene delivery not only *in vitro* but also *in vivo* [57]. As suggested by its name, gene transfer by this technique is achieved through electric pulses. Three processes seem to be involved: DNA distribution, membrane permeabilization and DNA electrophoresis [58]. After systemic or local injection of DNA solution, a high-voltage pulse permeabilizes cell membranes for few milliseconds while the following low-voltage pulses drive the DNA into cells through the transient membrane pores generated by the electric pulses.

This delivery system could virtually be used in any tissue or cell suspension where a pair of electrodes can be appropriately located. Because of its simplicity and efficiency, electroporation has become a common means for *in vitro* gene transfer in bacterial, yeast, plants and animal cells, and for *in vivo* gene transfer as well. This method was used to examine whether introduction and expression of PPAR γ gene could trans-differentiate skeletal muscle satellite cells to adipocytes *in vivo* [59]. By using a newly developed electroporation method Sauma *et al* [60] studied the effect of fatty acids on the activity of PPAR γ response elements in primary human adipocytes. The same authors have also studied

the PPAR γ activity *in vitro* using a reporter assay [61]. Liao *et al* [62] electroporated siRNAs directed against PPAR γ RNA sequence into 3T3-L1 adipocytes to suppress PPAR γ gene expression and elucidate the role of the NR in adipogenesis and insulin signaling. Fujishiro *et al* [63] analyzed the tissue-specific and PPAR α -dependent expression of the fatty acid binding protein gene in mouse liver. Kawana *et al* [64] electroporated HeLa cells using a β -galactosidase/hPXR fusion protein expression vector to unravel the molecular mechanisms of PXR nuclear translocation and CYP3A4 transactivation. The functional role of the orphan chicken ovalbumin upstream promoter transcription factors (COUP-TF) in differentiation of preplate layer cells was studied by ectopically expressing COUP-TF in the outer most layer of the E12.5 cortex after electroporation of organotypic cultures [65].

Electroporation-based DNA delivery into the glucocorticoid-resistant leukaemia T-cell clone ICR-27 allowed to elucidate the apoptotic-like mechanism induced by glucocorticoid receptor (GR) fragment 465* [66]. Hirose *et al* [67] elucidated the functional impairment of GR in steroid-sensitive Shionogi carcinoma 115 cells by electroporation gene transfer. Long duration electroporation allowed higher level expression of GR in mammalian cell lines than standard electro-mediated gene transfer [68]. In addition, a 23-component hydroxystilbene library was screened for estrogenic and anti-estrogenic activity using a cell-based bioassay that measured ER-mediated transcription of a reporter gene using electroporation delivery to ERC1 cells [69].

In vivo electrotransfer of siRNAs specific for the NR Nur 77 into mouse tibialis cranialis muscle clarified the role of the NR itself in the lipolysis process of skeletal muscle [70]. Li *et al* [71] highlighted the role of the orphan human homologue of the *Drosophila* tailless gene (TLX) in neuron development by *in utero* electroporation of TLX siRNA-expression vector in mice. The role of the same NR and its downstream Pax 2 signaling cascade in vertebrate eye development was assessed by *in vivo* electroporation of chicken embryo [72].

2.3 Gene gun

Gene gun-based gene transfer, also known as method of particle bombardment or ballistic DNA transfer [73], is achieved by propelling DNA coated fine particles (~ 1 to 3 μ m) directly against cells or surgically exposed tissues using a device with pressured helium as the driving force. The force created by the compressed gas is able to accelerate the coated gold or tungsten particles to high speed with sufficient momentum to penetrate physical barrier of plasma membrane of a cell both *in vitro* and *in vivo* [74]. In the field of transcription regulation of XMEs, this technique has been widely used to unravel the role and effects of the nuclear RAR and its isoforms on the regenerating amphibian limb [75-77]. A helium gene gun was also used to investigate the molecular mechanism of human transcription regulation of angiotensin gene by COUP-TF in mouse liver [78]. Tanigawa *et al* [79] refined the ability of the synthetic steroid RU24858 to transactivate the glucocorticoid responsive element *in vivo* on mouse abdominal skin. It was also shown that CAR and PXR can transactivate both CYP2B1 or CYP3A1[80].

2.4 Cationic polymer-based gene transfer

Over the years, a significant number of linear or branched cationic polymers have been explored as a carrier for *in vivo* and *in vitro* gene transfer. Polyethylenimine (PEI) [81-83], polyamidoamine [84-86], and polypropylenimine dendrimers [87], polyallylamine derivatives [88], cationic dextran [89], chitosan [90-93], cationic proteins including polylysine, protamine, histones [94], and cationic peptides [95-97] are among the most studied. Cationic polymers can easily complex with anionic DNA molecules through electrostatic interaction [98]. It is the polymer-DNA complexes (known as polyplex) that are taken up by the cells via clatherin dependent endocytosis.

A proton-sponge hypothesis has been suggested to explain the mechanism of cationic polymer-based gene delivery, especially by PEI (and its derivatives) which is perhaps the most active and studied compound for DNA delivery. Most of PEI's amine groups are not fully protonated at physiological pH. They can absorb protons when the pH drops below 6.0 in the endosomal compartment, therefore blocking the endosome-lysosome transition and keeping away the polyplex from the harsh lysosomal milieu. Proton entrance into the endosome is accompanied by chloride counterions, which cause the osmotic pressure to rise and, thus, the polyplex-bearing endosomes to swell and rupture [99,100].

Several studies were carried out using PEI as a carrier to study the thyroid hormone receptor (TR)-associated functions and/or properties *in vivo* [101-106]. Koudhi *et al* [107] exploited a PEI-based over expression and gene silencing approaches *in vivo* to highlight the interplay between PPAR γ and TR β in the hypothalamus, depicting a functional role of PPAR γ in regulating thyrotropin-releasing hormone transcription. An *in vivo* and polymer-based gene delivery system was used to examine the intracellular localization of CAR in mouse liver [26].

2.5 Cationic lipid-mediated gene transfer

All cationic lipids developed so far for gene transfer share the common structural feature of having a positively charged head group (with one or multiple amine group) and hydrophobic tail consisting of either two alkyl chains or a cholesterol moiety connected by a linker [108]. Upon mixing with cationic lipids in liposomal form in aqueous solution, DNA is condensed into small quasi-stable particles (called lipoplexes) driven by electrostatic interaction. The process involves a rapid association between polycationic liposomes and polyanionic DNA through electrostatic interaction, followed by a slower lipid rearrangement process [109].

Lipoplexes are able to trigger cellular uptake and facilitate the release of DNA before reaching destructive lysosomal compartment [110]. Transfection typically requires a slight excess of cationic lipids over nucleic acids such that lipoplexes have a net positive charge on the surface. It has been proposed that after electrostatic interaction with the negatively-charged cell surface, the cationic lipoplexes, once endocytosed, initiate a destabilization of the endosome membrane that results in flip-flop of anionic lipids that are predominately located on the cytoplasmic side of the membrane [110]. The anionic lipids of the endosomes laterally diffuse into the lipoplexes and form charge-neutralized ion pairs with the cationic lipids. This displaces the plasmid DNA from the complex and permits DNA entry into the cytoplasm. Thus, lipid mixing between cationic lipids and endosomal lipids is crucial. Consequently, cationic lipids are often formulated with a helper (non-bilayer-forming) lipid to increase membrane fluidity, lipid exchange and membrane fusion [111,112]. Moreover, liposomes that contain targeting and functionalizing groups in their lipid bilayer can be tailor-made to satisfy a specific need.

The use of lipid-based formulations to carry out XME-related studies seems to be one of the top choices. In fact, papers reporting the use of lipid-mediated DNA delivery are numerous [for most recent papers, see 26-32,34-36;113-117]. Readers are encouraged to read any of these papers for specific information.

2.6 Calcium phosphate precipitation method

Calcium phosphate precipitation method was discovered by Graham and Van der Eb in 1973 [118]. It remains as one of the most commonly used nonviral methods for gene transfer *in vitro*.

The principle of calcium phosphate based transfection is simple even though the underlying mechanism has not been fully elucidated. The basic feature of this procedure includes the

mixing of HEPES-buffered saline solution containing phosphate ions with calcium chloride solution containing the DNA. When the two are combined, a fine precipitate of the positively charged calcium and the negatively charged phosphate forms, binding the DNA on its surface. The suspension of this precipitate is then added to the cells to be transfected (usually a cell culture grown in a monolayer). Transfection efficiency in some cell lines can be increased by shocking the cells with glycerol or dimethyl sulfoxide [119]. A BES-buffered system was exploited to allow the calcium phosphate-DNA precipitate to form gradually in the culture medium [119]. By a process not yet fully understood, which seems to involve endocytosis [120-122], cells take up the DNA together with the precipitate.

Despite its simplicity, the reproducibility and efficiency of the calcium-phosphate precipitation method are relatively low because the appropriate window of optimal conditions seems to be very narrow. In fact, various investigators have found that the pH of the precipitate-forming solution and the culture medium, the buffer used for precipitate formation, the exact concentration of calcium and phosphate employed, the time of precipitate formation and the presence or absence of serum in the culture medium are critical factors in the efficacy of DNA transfer [123-126]. This, in turn, mirrors the wide range of transfection efficiency (0.1% to 50%) of cells [123,127,128].

Calcium phosphate precipitation method is as widely used as lipid-based DNA delivery in XME-related study [just to cite a few of these papers 129-138]. For instance, an *in vitro* human PXR activation assay was developed based on the calcium-phosphate-mediated transfection [129]. In addition, calcium phosphate mediated transfection of HEK293 and HepG2 cells was employed to study transactivation of aryl hydrocarbon receptor (AhR) [130].

3. Conclusion and future perspectives

A multitude of *in vitro* and *in vivo* studies on transcriptional regulation of xenobiotic metabolizing enzymes have been published, which showed that study of NR mediated regulation of gene expression through gene transfer is advantageous over traditional method of generating transgenic animals. Until now, many nonviral methods have been developed using either physical or chemical principle to facilitate gene transfer across cell membrane. More recent development of hydrodynamic gene delivery has made it possible to perform transfection studies in animals.

To fully exploit the potential of gene transfer for studying molecular mechanisms of transcriptional regulation of xenobiotic metabolizing enzymes, further development is required to improve transfection efficiency and minimize cellular toxicity. For *in vitro* studies, while both electroporation and cationic carrier mediated gene transfer work well for most cell lines, effective transfection of primary cells remains hard to achieve. Cell damage has been reported in transfection especially when large amount of complexes were used in order to maximize transfection efficiency. Additionally, efforts are needed to solve the problem of large experiment-to-experiment variability in gene transfer studies. Toward this end, significant efforts are seen in recent year to apply the principles of nanotechnology to solving the problems associated with current nonviral methods [139]. It is hopeful that more effective nanogene carrier will be developed.

Significant progress has been made in development of an effective method for gene transfer *in vivo*. For drug metabolism studies, the effort was motivated by the success in developing humanized mice [140-147]. It was postulated that a method that allows transfer of human genes into an animal without going through a time consuming and costly procedure for generating transgenic animal would be extremely useful not only for studies of drug metabolism but also xenobiotics induced transcriptional regulation. Among the procedure

evaluated so far including both physical and chemical methods, hydrodynamic gene delivery via a rapid tail vein injection of a large volume of plasmid containing solution into a mouse is the simplest, most convenient and effective method developed so far. With this technique, one can introduce any DNA or RNA sequences into liver hepatocytes in a mouse to study their functions. With recent development of a computer controlled injection device [148], it is also possible to perform the same set of experiments in large animals.

It should be pointed out that the power of gene transfer-based study of xenobiotic metabolizing enzymes is based on the technology of molecular biology. With PCR and RT-PCR techniques current available, the sequence information provided by genome based projects, and the computer software for primer design and sequence analysis, it is now possible for anyone with minimal training in molecular biology to clone a DNA sequence into a plasmid vector and then introduce mutation into the desirable sites. Subsequently, such sequences can be transfected into cells to study their function in regulating gene expression and/or in affecting biochemical or cellular events. Similarly, the function of each of single nucleotide polymorphism (SNP) identified in XME and NR genes can be defined and studied through nonviral gene transfer.

Acknowledgments

This work was supported in part by NIH grants R01EB007357 and RO1 HL098295. B. Bonamassa is recipient of the Rotary Foundation Ambassadorial Scholarship for 2009-2010 academic year.

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