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Toxicogenomics of Nonviral Cationic Gene Delivery Nanosystems

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1. Introduction

To date, both viral and nonviral vectors have been exploited for delivery of gene-based therapies to target cells/tissues. Despite high efficiency of the viral vectors (e.g., retroviruses and adenoviruses), these vectors appear to be immunogenic and potentially harmful when used in clinical gene therapy protocols (Ferber, 2001b). Besides, the preparation and purification of the viral vectors appear to be laborious, cost-prohibitive and not amenable to industrial-scale manufacture. Nonviral vectors such as cationic lipids (CLs) and cationic polymers (CPs) have been categorized as advanced materials and their low immunogenicity, lack of pathogenicity, and ease of pharmacologic production continue to make them attractive alternatives to viral vectors (Medina-Kauwe et al., 2005). However, these vectors may also suffer from relatively low levels of gene transfer compared to viruses. Thus, the drive to advance these vectors continues resulting in considerable progresses in improved transfection efficiency. Nonviral vectors (in particular cationic gene delivery systems) are able to bind and enter the target cells, however they yield low gene expression. No substantial information is available on interactions of these vectors with cellular biomolecules. Since these medicaments tend to act at genomic levels, thus understanding the genomic impacts of the nonviral vectors may help develop more efficient gene delivery systems. Nonetheless, this needs recruitment of high throughput screening methodologies.

To date, exploitation of the "omics" concepts (e.g., genomics, proteomics and metabolomics) is going to change the face of pharmacotherapy towards significantly more advanced and efficient pharmaceuticals (e.g., gene based nanomedicines) with minimal adverse consequences (Aardema & MacGregor, 2002). Enormous efforts have also been devoted for application of the global gene expression profiling in pharmacologic and toxicological investigations. The gene expression profiling technology has been primarily exploited for identification of underlying mechanisms for toxicity of pharmaceuticals and their genomic signatures, by which the safety liabilities can be determined and manifestations of undesired genotoxicity can be prohibited (Suter et al., 2004; Yang et al., 2004).

This methodology can be successfully used for the discovery and development of any chemicals and pharmaceuticals including gene delivery nanosystems. The main focus of the current book chapter is to provide some useful information about "genocompatibility" and

"toxicogenomics" of the nonviral vectors using global gene expression profiling techniques i.e. DNA microarray.

2. Gene therapy challenges and dilemmas

The principle of gene therapy possesses undeniable therapeutic advantages over the conventional therapeutic modalities that are basically dependent upon exploitation of small molecules or biological pharmaceuticals. These advantages are: 1) specific or selective treatment of diseased cells/tissue, 2) minimal adverse consequences, 3) correction of the genetic cause of a disease, and 4) long-term treatment after single application (Rubanyi, 2001). Basically, to silence/suppress a target gene or to correct a genetic defect, the gene-based therapeutics such as oligodeoxynuleutides (ODNs), plasmid DNA, ribozymes, DNAzymes or short interfering RNA (siRNA) need to be shuttled to the target site. Delivery of gene-based therapeutics has been also advanced by development and implementation of various strategies, including: biological (e.g., viral vectors), physical (e.g., microinjection and electroporation, gene gun, ultrasound, and hydrodynamic delivery), and chemical (e.g., non-viral vectors) approaches. However, gene transfer into various target cells still faces major obstacles including poor delivery efficiency, cellular toxicity, immunogenicity and oncogenicity, as well as short-term transgenic expression and poor expression levels.

The first clinical test of gene therapy was accomplished a decade ago with the transfer of the missing "adenosine deaminase" gene into lymphocytes isolated from patients with severe combined immune-deficiency syndrome (i.e., as ex-vivo gene therapy approach). However, despite the early promising prophecy on the high effectiveness of gene therapy, the existing clinical experience indicate insufficient therapeutic efficacy coupled with increasing safety concerns and ethical issues (Verma & Somia, 1997). In some cases, aptamer-based genomedicines (e.g., Pegaptanib sodium, Macugen™) have been successfully utilized for treatment of the age related macular degeneration (Barar et al., 2008). Gendicine™ is an adenoviral p53-based gene medicine that was approved by the Chinese FDA in 2003 for treatment of head and neck cancer, while Advexin™ (a similar gene therapy approach from Introgen) was turned down by the US FDA in 2008. In fact, the death of Jesse Gelsinger in a gene therapy experiment in 1999 imposed a significant setback to gene therapy research in the United States, however many scientists aimed to resolve problems associated with the gene therapy strategies. In 2006, an international group of scientists announced the successful use of gene therapy to treat two adult patients for a disease affecting myeloid cells (Ott et al., 2006). Also in 2007, the world's first gene therapy trial for inherited retinal disease was announced for treatment of Leber's congenital amaurosis which is a inherited blinding disease caused by mutations in the RPE65 gene (Maguire et al., 2008). It should be evoked that the performance and pathogenicity of viral vectors (e.g., retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses) and nonviral vectors have been evaluated in animal models. Promising results form the basis for clinical trials to treat genetic disorders and acquired diseases, however vector development/advancement remains a seminal concern for improved gene therapy technologies (Verma & Weitzman, 2005). Fundamentally, an ideal gene delivery method should protect the transgene against degradation, transport the transgene into the cytoplasm and then nucleus of target cells with little undesired detrimental effects (Gao et al., 2007).

Results obtained from *in vitro* studies have revealed that treatment of cells with antisense oligonucleotides (As-ODNs) for a period of only a few hours can bestow the desired effects

of As-ODNs, while animal experiments demand repeated administration through multiple injections for prolonged exposure to As-ODNs. Despite promising results of some in vivo studies with free As-ODNs, improved delivery systems are essential to increase the efficacy of As-ODNs and to reduce its amount and frequency of administration (Hughes et al., 2001). Successful delivery of desired genes are important for both *ex vivo*, where cells undergo gene therapy in culture prior to implantation into the patient, or *in vivo* gene therapy where nucleic acids are administered directly to the patient to attain the desired gene change. Preferably, in either approach, only the therapy-intended gene expression changes should occur. However, this is not always the case, for example viral vectors are known to be efficient delivery systems for nucleic acids but can also induce immunogenic responses (Audouy et al., 2002; Ferber, 2001a; Ferber, 2001b). Hence, several nonviral gene delivery nanosystems such as cationic polymer- or lipid-based formulations have been developed for nucleic acid delivery. These cationic nanostructures can readily condense DNA into complexes and form polyplexes/lipoplexes to be used for *ex vivo* and *in vivo* gene therapy.

Although the CPs/CLs can principally enhance the delivery and improve the biological end-point of genomic-therapeutics, they often exert cytotoxicity depending on delivery system and target cell/tissue (Pedroso de Lima et al., 2001). Thus, both transfection efficiency evaluation and safety assessment are essential for gene transfer with these gene therapy vectors. A number of factors may affect the efficacy and safety of nonviral vector-mediated gene transfer; in particular their structural properties and type of target cells and tissue. It should be noticed that as various target cells may display different responses, the transfection efficacy and safety of vectors should be carefully optimized upon types of target cells and target organs. Once transfection accomplished, specific attention should be given to the genotoxicity potentials of gene-based medicines. Surprisingly, no substantial information is available about the genomic signature of the cationic delivery systems. We have previously investigated the potential of the commercially available nonviral vectors (e.g., Polyamidoamine (PAMAM) dendrimers such as Polyfect™ (PF) and Superfect™ (SF)) and lipids (e.g., Lipofectin™ (LF) and Oligofectamine™ (OF)) on global gene expression within human epithelial A431 and A549 cells by exploiting the cDNA microarray technology (Barar et al., 2009; Omidi et al., 2003; Omidi et al., 2005a; Omidi et al., 2005b; Omidi et al., 2008). These investigations revealed occurrence of inadvertent nonspecific gene expression changes within target cells upon treatments with these cationic gene delivery nanosystems. These findings led us to screen series of lipid- or polymer-based non-viral vectors for their toxicogenomic and genomic toxicity potentials in target cells.

Fig. 1 represents schematic illustrations of polymer/lipid based micro/nano systems used for delivery of genes/drugs.

3. Cellular trafficking and toxicity of polycationic nanostructures

For achievement of an efficient systemic delivery of gene-based nanomedicines, various factors appear to play crucial role, including: 1) the physicochemical characteristics of the gene-based therapies, 2) the effects of biological environment, 3) the functionality of membranes and barriers, and 4) the biological impacts of cellular microenvironment.

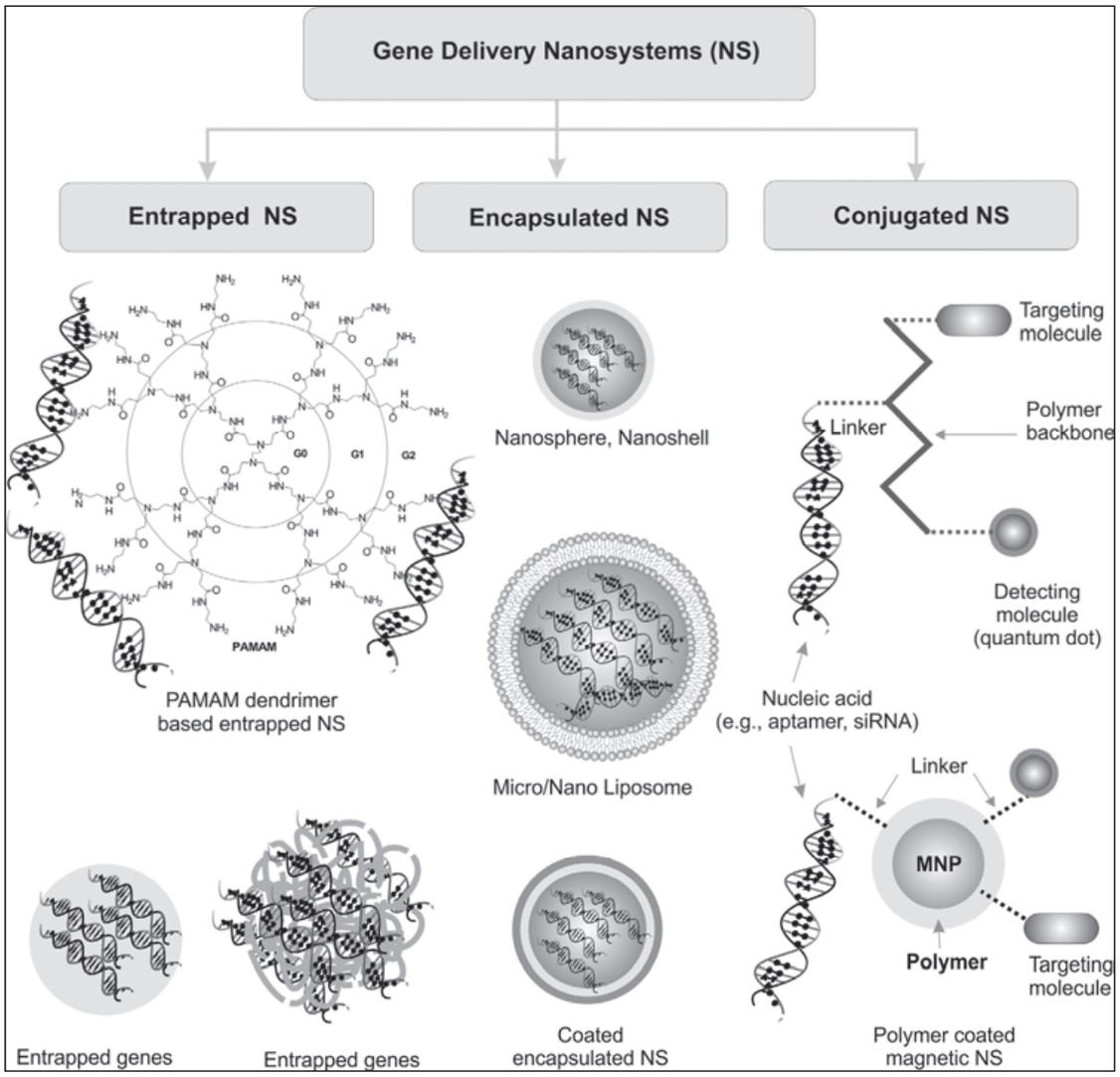

Fig. 1. Schematic representation of various polymer based gene delivery nanosystems. To prepare gene medicine nanosystems (NS) nucleic acids (e.g., antisense, siRNA, and aptamer) are generally entrapped, encapsulated or conjugated with polymers. Genes can be conjugated to magnetic nanoparticles (MNP) and quantum dots for concurrent detection and therapy.

Within the circulation system, blood cells, proteins, enzymes and serum components may bind to the genomedicines and cause instability and lowered transfection efficiency (Konopka et al., 2005). In addition, the circulating gene therapies must circumvent the immune system clearance and cross the capillary endothelial cells to reach the target cells/tissue. Once inside the target cells (normally via receptor-mediated endocytosis pathway), the genomedicine must overcome the subcellular and/or biomolecular impacts. In fact, the amphipathic sheet like lipid bilayer architecture of the biological membranes along with the integrated proteins separate cells from their environment and form the boundaries of different organelles inside the cells, at which exchange of materials among the different parts of a cell is controlled (Omidi & Gumbleton, 2005). Nonviral vectors may bind

to cells by means of one or both of two types of cell binding interaction machineries, i.e. receptor and non-receptor mediated bindings (Medina-Kauwe et al., 2005). At cellular level, trafficking of the gene-based nanomedicines is basically performed through vesicular transportation pathways, in which they may engineer their own escape from demise in the lysosome. Endocytosis of macromolecular nanomedicines occurs through various cellular pathways, including clathrin coated pits, caveolae membranes and lipid rafts (Conner & Schmid, 2003; Spang, 2008). More likely, these complexes enter cells through nonspecific exploitation of these endocytic machineries, presumably mainly involving clathrin-mediated endocytic pathway. This route initiates and stabilizes membrane curvature formation, in which the adaptor proteins bind to clathrin pits and augment the inward pull of the membrane towards the cytoplasm leading to vesicle formation (Young, 2007).

It has been evidenced that the N-1(-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammoniummethylsulphate (DOTAP) lipoplexes are internalized by cells solely via clathrin-mediated endocytosis, however PEI polyplexes were shown to be internalized both by clathrin-mediated and caveolae-mediated endocytosis (Rejman et al., 2005). Once inside the cytoplasm, DNA is released from vesicular compartment upon physicochemical properties of the genomedicine. The endosomal escape of DNA at an early stage of endocytosis is deemed to be critical for cytosolic DNA delivery and determination of overall transfection efficiency. Among CPs and CLs, fusogenic lipid dioleoylphosphatidylethanolamine (DOPE) as a helper lipid for liposome-based DNA delivery were reported to induce membrane fusion between the endosome and the liposome and result in membrane destabilization and release of DNA into the cytoplasm (Farhood et al., 1995). Such destabilization of the vesicular membrane further highlights the interaction of cationic lipids with cellular compartments. This inadvertent nonspecific interaction may be exacerbated for *in vivo* systemic gene, which requires high and potentially toxic doses of nonviral vectors. Utilization of the cell-specific ligands or antibodies were reported to lower the cytotoxicity, while facilitating tissue targeting (Rawat et al., 2007), in which the ligand choice is largely dictated by whether or not the target receptor undergoes vesicular trafficking and the endocytic pathway used by the vector is dependent upon the targeting ligand as well as cell type. The structural architecture of the gene delivery nanosystems was shown to be important from gene expression changes viewpoints (Omidi et al., 2005b), which is also largely dependent upon cell type, in particular the membrane lipid composition and membrane phase state (Kabanov, 2006). Adsorption of polycations such as poly(N -ethyl-4-vinylpyridinium) salts (PEVP) in liposomic biomembranes was shown to induce flip-flop of negatively charged lipids (e.g., cardiolipin, phosphatidylserine, and phosphatidic acid) from the inner to the outer leaflet of the liquid liposomal membrane, but not in solid membranes (Yaroslavov et al., 1994; Yaroslavov et al., 2006). Among polycations, starburst PAMAM dendrimers and PEI appeared to elicit the most dramatic increase in membrane permeability by interacting the membranous biomolecules and forming holes in lipid membranes (Hong et al., 2006; Leroueil et al., 2007). Such structures could function as gates, through which the lipid molecules can be transported across the biomembranes (Kabanov, 2006). Fig. 2 represents cytotoxicity of linear and branched PEI in A431 cells (Kafil & Omidi, 2011).

Upon differences in cell types, the polyions can bind to the cellular compartments and accordingly induce compartmentalization within certain areas of the membranes and inadvertently trigger various signaling paths. Furthermore, nanoscaled defects were shown to be induced by PAMAM dendrimers through removing lipid from the fluid domains at a

significantly greater rate than for the gel domains (Erickson et al., 2008). This reinforces a possibility of compartmentalization of synthetic polymers within different membrane domains as well as a differential effect of polymers on functional systems in the membranes that consecutively provoke inadvertent cytoplasmic/nucleic consequences directly and indirectly via secondary messengers such as G proteins.

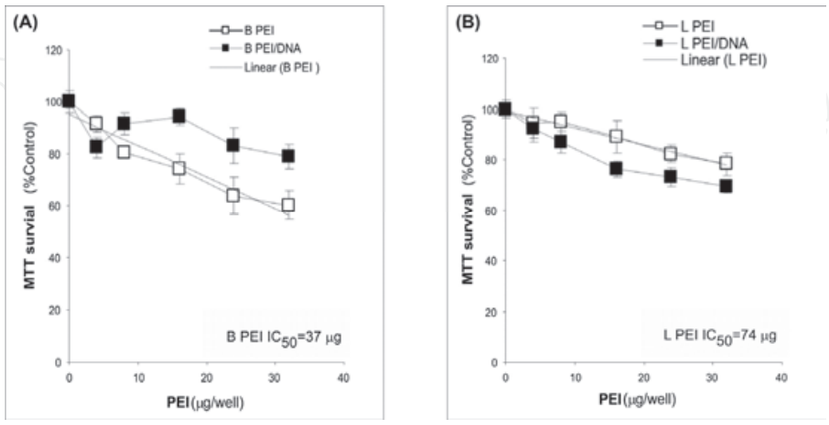

Fig. 2. Cytotoxicity of polyethylenimine (25 kDa) polymers in A431 cells evaluated by MTT assay. A) Cytotoxicity of B PEI with IC_{50}=37 µg. B) Cytotoxicity of LPEI with IC_{50}=74 µg. BPEI: Branched polyethylenimine; LPEI: linear polyethylenimine; adapted with permission from (Kafil & Omidi, 2011).

Fischer et al. (2003) monitored cytotoxicity of various polycationic gene delivery systems in L929 mouse fibroblasts using MTT assay and the release of the cytosolic enzyme lactate dehydrogenase (LDH). They showed a pattern for cellular toxicity as follow, poly(ethylenimine)=poly(L-lysine)>poly(diallyl-dimethyl-ammonium chloride)>diethylaminoethyl-dextran>poly(vinyl pyridinium bromide)>Starburst dendrimer>cationized albumin>native albumin. These researchers, interestingly, confirmed the molecular weight and the cationic charge density of the polycations as key parameters for the interaction with the cell membranes and accordingly the cell damage (Fischer et al., 2003). Besides, interaction of dendrimers with erythrocyte membrane proteins was shown to trigger echinocytosis (Domanski et al., 2004), while the cationic liposomes are less cytotoxic than dendrimers. The toxicity by CLs appeared to be dependent upon the type of cationic lipid macromolecule, concentration, molecular weight and the presence of DNA, where complexation of the polycations with DNA resulted in reduced tissue damage. However, Gebhart et al. (2001) showed increased cytotoxicity in the cos-7 cells upon complexation of various polymers with DNA (Gebhart & Kabanov, 2001).

Filion et al. (1997) have performed an important body of work by evaluating the toxicity of liposomes, formulated with various cationic lipids, towards murine macrophages and T lymphocytes and the human monocyte-like U937 cell line. They reported occurrence of pronounced toxicity by cationic liposomes formulated from DOPE and cationic lipids based on diacyltrimethylammonium propane (dioleoyl-, dimyristoyl-, dipalmitoyl-, disteroyl-: DOTAP, DMTAP, DPTAP, DSTAP) or dimethyldioctadecylammonium bromide (DDAB) in the phagocytic cells (macrophages and U937 cells), but not within non-phagocytic T lymphocytes. They also showed the rank order of toxicity as follows: DOPE/DDAB > DOPE/DOTAP > DOPE/DMTAP > DOPE/DPTAP > DOPE/DSTAP.

Once complexed with nucleic acid (e.g., antisense oligonucleotide or plasmid vector), lipoplexes revealed marginally reduced toxicity towards macrophages (Filion & Phillips, 1997b). Furthermore, since cationic lipids display intrinsic anti-inflammatory activity, they should be cautiously utilized as a gene delivery system to transfer nucleic acids for gene therapy *in vivo*.

DNA microarray technology has advanced and accelerated the identification process for mechanistic toxicology to illuminate genomic aspects of toxicology that could consequently postulate early effect within targets cells/tissues upon exposure to the toxicants (de et al., 2004). Recently, an interesting study was performed to compare different commercially available cationic liposome–DNA lipoplexes (Masotti et al., 2009), and it was reported that the lipoplex size and cationic lipid to DNA ratio are the two main parameters affecting the transfection efficiency of lipoplexes. The lipofection efficiency was determined mainly by lipoplex size, but not by the extent of lipoplex–cell interactions including binding, uptake or fusion. In the presence or absence of serum, lipoplex size was found to be a major factor determining lipofection efficiency. These researchers concluded that, by controlling lipoplex size, an efficient lipid delivery system may be achieved for *in vitro* and *in vivo* gene therapy.

Florea et al. (2002) evaluated PEIs with different molecular weights for their efficiency in transfecting undifferentiated COS-1 and well-differentiated human submucosal airway epithelial Calu-3 cells and showed that transfection efficiency was dependent upon the cell types, but not molecular weights. These researchers reported that gene transfer by PEI was 3 orders of magnitude more effective in COS-1 than in Calu-3 cells, perhaps because of secretion of mucins by Calu-3 cells (Florea et al., 2002). However, the larger molecular weights of PEI were also shown to yield the highest transfection efficiency in EA.hy 926 cell line derived from a fusion of the human A549 cell line with human umbilical vein endothelial cells, HUVEC (Godbey et al., 2001). Two types of cytotoxicities in process of PEI -mediated cell transfection have been reported: 1) an immediate toxicity associated with free PEI, 2) a delayed toxicity associated with cellular processing of PEI/DNA complexes (Godbey et al., 1999; Godbey et al., 2001). The immediate toxicity seems to occur upon interaction of the free PEIs with negatively charged serum proteins (e.g., albumin) and red blood cells (cytotoxic effects), while the delayed toxicity by PEI/DNA complex appeared to be closely related to the release of DNA (genomic effects). In cell culture, free PEI interacts with cellular components and inhibits normal cellular process. It causes several changes to cells, which include cell shrinking, reduced number of mitoses and vacuolization of the cytoplasm. We have observed significant genotoxicity impacts induced by PEI in A431 cells (Kafil & Omidi, 2011) and xenografted mice (our unpublished data).

Toxicity impacts of nanostructured materials have been recently reviewed (Nel et al., 2006), while many aspects of this issue (in particular at genomics/protemics levels) still remains unresolved. As a result, necessity of analysis of toxicogenomics of the nanoscaled advanced biomaterials is very clear. It will direct us towards development of safe pharmaceutical formulations with maximal efficiency and wide therapeutic index yet displaying minimal toxicity profiles since the conventional assessment of toxicity solely provide preliminary information with little devotion to the global genomic/proteomic impacts (Hollins et al., 2007; Kabanov et al., 2005; Kabanov, 2006; Omidi et al., 2005a). If this is the case, then the gene and drug delivery paradigms are going to stumble upon new era to deal with "functionalized excipients".

4. Genocompatibility and toxicogenomics of polycationic nanostructures

To pursue the genomic impacts of any gene based medicine, it is necessary to exploit high throughput screening methodologies (e.g., DNA microarray) for evaluation of global gene changes induced by the gene medicine or any other chemicals/compounds. Such genome based impact could be termed as "genotoxicity" or "toxicogenomics".

The DNA microarray technology combines standard molecular techniques with high-throughput screening to monitor the expression of up to ~40000 genes, which may provide a means for toxicity prediction prior to classical toxicological endpoints such as histopathology or clinical chemistry (Goldsmith & Dhanasekaran, 2004). In gene silencing experiments, such approach may allow a genomic characterization of delivery systems leading to identification of possible incompatibilities with intended target genes or biological effects of the gene based medicine. This may allow screening of compatible or useful delivery systems early in drug development that could subsequently save time and money in pre-clinical and clinical studies (Fielden & Kolaja, 2006; Lettieri, 2006).

Cytotoxicity and genotoxicity potentials of CPs and CLs are going to be well acknowledged, and accordingly these cationic nanosystems should undergo a rigorous genocompatibility evaluation prior to *in vitro* and *in vivo* exploitation (Kabanov, 2006; Omidi et al., 2005a). These systems alone or in combination with biologically active molecules (e.g., siRNA, antisense, aptamer) are able to alter cell signaling and biological responses in cells and organisms, emerging a cluster of genomic and post genomic consequences. In general, toxic responses to these kinds of nanomaterials are deemed to be very profound, in which various signaling pathways such as oxidative stress, immune responses and apoptosis pathways may be involved in response to generation of reactive oxygen species in the membranes (Kabanov, 2006). Cationic liposomes, irrespective of complexation with DNA, can downregulate the synthesis of pro-inflammatory mediators such as nitric oxide (NO) and tumor necrosis factor-alpha (TNF-alpha) in lipopolysaccharide (LPS)/interferon-gamma (IFN-gamma)-activated macrophages (Filion & Phillips, 1997a; Filion & Phillips, 1997b). Under the oxidative stress, cells may undergo the Nrf-2 signaling or the pro-inflammatory signaling cascades such as mitogen-activated protein kinase (MAPK) and nuclear factor kB (NFkB) cascades and eventually a programmed cell death may occur (Kabanov, 2006). Certain proteins such as protein kinase C (PKC) may also be affected detrimentally by cationic amphiphiles (Aberle et al., 1998), which function as PKC inhibitors and may inevitably result in inadvertent toxicity. It seems that the cationic amphiphiles with steroid backbones can exert more potent inhibitors of PKC than their straight-chain analogues, resulting in greater toxic impacts (Bottega & Epand, 1992). Polycations such as PEI formulated with plasmid DNA and administered to mouse lungs was reported to activate the p38 pathway involved in endocytosis, phagocytosis and hydrogen peroxide production. The observed *in vitro* and *in vivo* toxicity of such PEI polyplex formulations appeared to link to a general stress reaction, inflammatory responses, cell cycle regulation and DNA damage repair (Regnstrom et al., 2006). To obtain a complete image, it is essential to recruit high throughput screening methods such as DNA microarray.

5. DNA microarray technology

Practically, in the exploring stage, the expression of ~40,000 gene spots and replicates can be simultaneously analyzed on a couple of glass array in a single experiment by means of

microarray technology. However, for accomplishment of a significant correlation between the gene expression profiles and their functionality expression, it is important to implement substantial complementary investigations to verify the results at the molecular level and as a result extend our understanding of gene expression patterns and molecular pathways.

Microarray technology can be exploited to attain a wealth of data that can be used to develop a more complete understanding of gene expression, which can be used for transcriptional regulation and interactions as well as functional genomics. Despite its successful *in vitro* cell-based implementation, application of this technology for *in vivo* investigations is deemed to be more sophisticated because of complexity of cytotoxicity and genotoxicity studies, which can be confounded by a number of variables such as type of target organ, effect of pharmacokinetics and/or pharmacodynamics parameters (Lobenhofer et al., 2001). Since its advent and application in life sciences, microarray has been widely applied for molecular/biological studies. In fact, a large number of indexed articles in various data banks (e.g., MEDLINE/PubMed) highlight the importance of microarray technology in post-genomics era.

Fig. 3 shows a schematic illustration of step-wise processes of the DNA microarray technology.

Technically, DNA microarray can be generated in two different types including printing pre-synthesized cDNAs (500–2000 bp) or synthesizing short oligonucleotides (20–50 bases) onto glass microscope slides, in which gene spots include either fully sequenced genes of known function or collections of partially sequenced cDNA derived from expressed sequence tags (ESTs) corresponding to the messenger RNAs of unknown genes. For example in practice, one may compare two different cells/tissues from untreated (UT) versus treated (T). For gene expression profiling, normally total RNA is extracted from the untreated and treated samples. Using an indirect labeling methodology, they are converted to labeled cDNA (e.g., with aminoallyle-dUTP). The aminoallyle-dUTP-cDNA is then labeled with cyanine dye (e.g., Cy3 or Cy5). The Cy3 and Cy5 labeled aminoallyle-dUTP-cDNA from UT and T samples are hybridized on a single glass array, which is subjected to several washing steps, scanning with an appropriate scanner (e.g., using RS Reloaded™, TECAN, Switzerland) and data mining (e.g., using GeneMath™ software; Applied Maths, Sint-Martens-Lathem, Belgium); for detailed information reader is directed to see (Hegde et al., 2000; Omidi et al., 2005b; Omidi et al., 2008).

For microarray analysis, significantly upregulated and/or downregulated genes can be identified using traditional method (gene expression changes with a fixed cutoff threshold usually in 2 fold) to infer significance differences (i.e., the so called “fold change method”). The resultant data are normally presented as scatter plots of treated (T) versus untreated (UT) control. To reach this stage, data need to undergo a number of processes called as “transformation” and “normalization” to minimize the experimental erroneousness (i.e., the so called “data mining”). Since a scatter plot of T versus UT genes would cluster along a straight line, normalization of this type of data is equivalent to calculating the best-fit slope using regression techniques and adjusting the intensities so that the calculated slope is one. In many experiments, the intensities are nonlinear, and local regression techniques are more suitable, such as Locally WEighted Scatterplot Smoothing (LOWESS) regression (Berger et al., 2004; Chen et al., 2003).

In our studies, we have successfully exploited both approaches to study the impacts of the nonviral vectors (CPs and CLs based formulation) on global gene expression experiments. To get the significant alterations in gene expression, we rejected the arrays showing non-

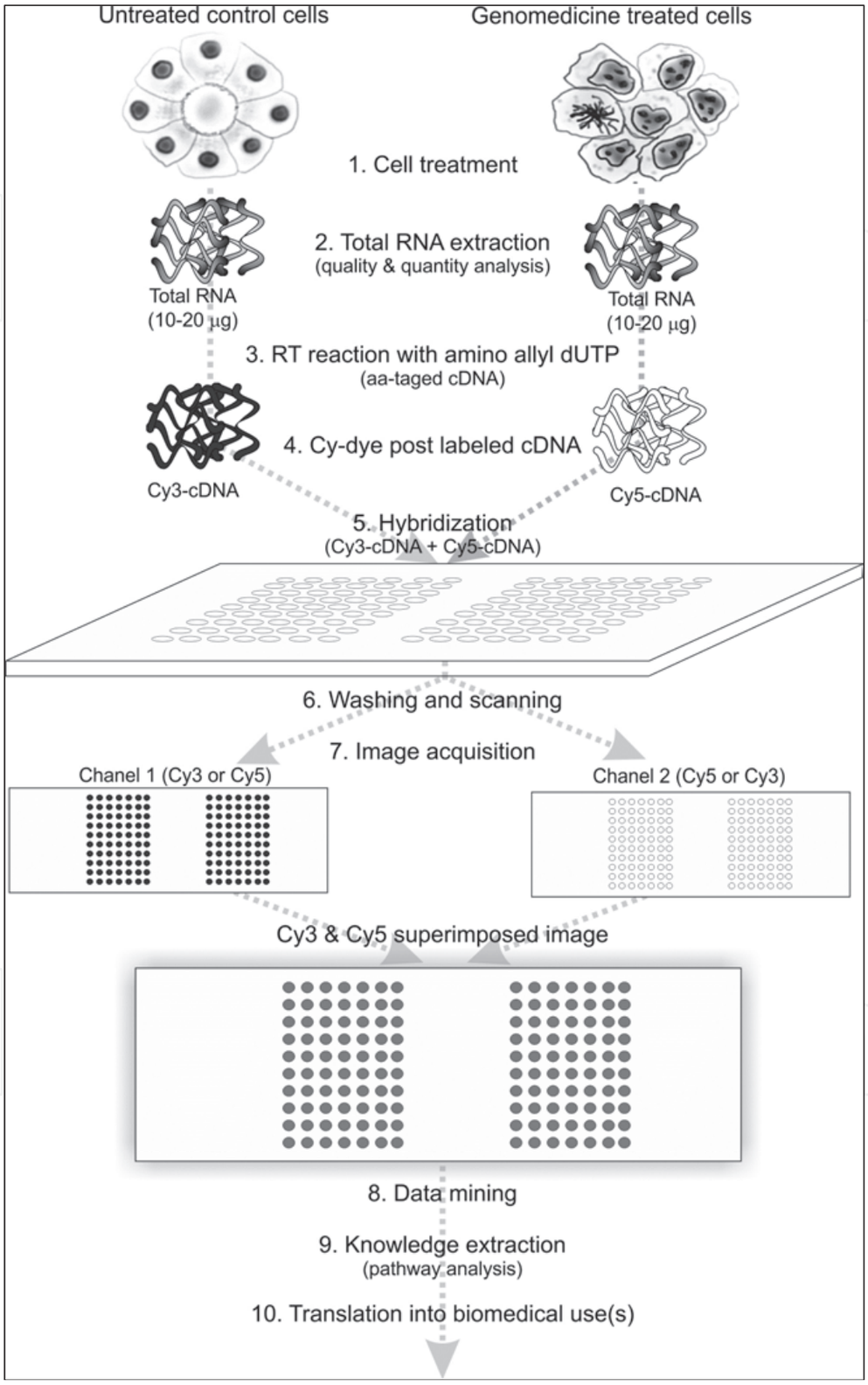

Fig. 3. Schematic illustration of step-wise process of DNA microarray methodology.

equal intensity or variable intensity of control gene spots in replicates on the same slide or between slides in dye-flipping experiments (Hollins et al., 2007; Omidi et al., 2003; Omidi et al., 2005b; Omidi et al., 2008). Data for each gene were typically reported as an "expression ratio" or as the base 2 logarithm (log_2) of the expression ratio of T to UT control. Genes were assumed to be up regulated or downregulated if they revealed an expression ratio of >2 and <0.5 (or >1 and <-1 for log_2 transformed data), respectively.

Based on our findings, the starburst PAMAM dendrimer alone or as complexed with DNA can elicit inadvertent gene expression changes. We also found that the linear and branched PEI (25 kDa) are able to induce gene expression changes in A431 cells, as shown in Fig. 4 (our unpublished data).

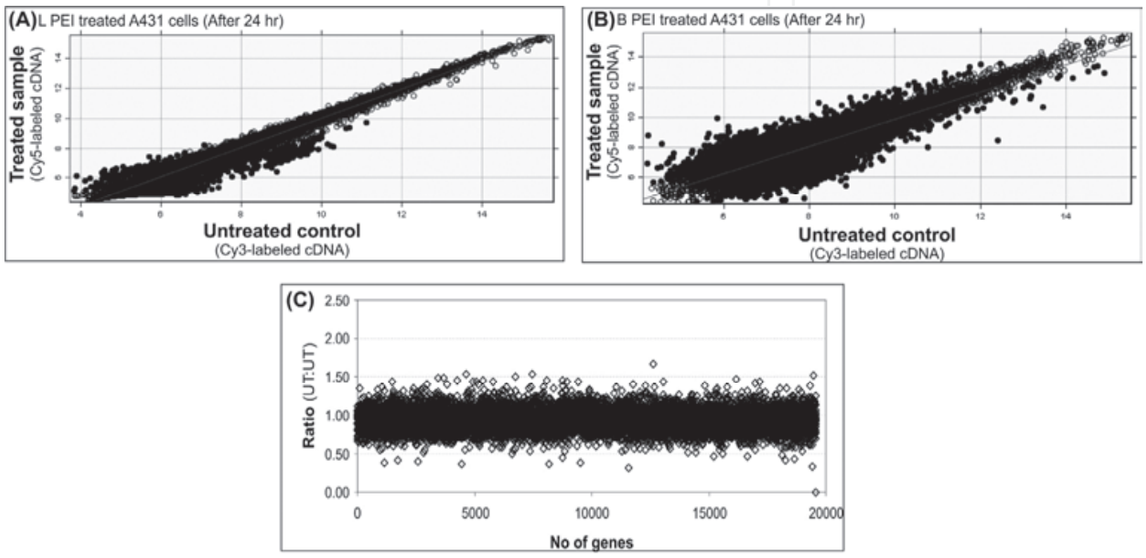

Fig. 4. Scatter plots of gene expression changes induced by cationic linear (A) and branched (B) PEI (25 kDa) in A431 cells. Data represent Log2 transformed gene expression values for large arrays housing 20000 genes. Above 2-fold change in expression of treated to untreated is indicated by bold circles and unchanged genes by unfilled circles. Panel C represents gene expression changes ratio between untreated A431 cells from different experiment. BPEI: branched polyethylenimine; LPEI: linear polyethylenimine (our unpublished data produced by Omidi et al.).

In the case of arrays with thousands of spots, one needs to employ the "feature reduction" or "dimension reduction" to find the minimum number of the features (i.e., genes or maybe even the conditions) that can best describe the data and the classification using statistical methods such as principal component analysis (PCA), correspondence analysis (CA), multi-dimensional scaling (MDS), and cluster analysis, reader is directed to see the following citation (Hegde et al., 2000; Quackenbush, 2001; Quackenbush, 2002). Of the dimension reduction methods, PCA is the most widely used method as a tool in exploratory data analysis, which involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. PCA ignores the dimensions in which data do not vary significantly and it is closely related to factor analysis.

6. Pathway analysis for functional genomics and gene ontology

To understand the functions of the genomic changes, one needs to implement appropriate methods on knowledge extraction from DNA microarray data. Such aim can be performed by means of "pathway analysis" (PA), which should be towards functional enrichment for establishing networks between genes. In fact, understanding the expression dynamics of gene networks helps us infer innate complexities and phenomenological networks among genes. Likewise, studying the regulation patterns of genes in groups, using clustering and classification methods may help us understand different pathways in the cell, their functions, regulations and the way one component in the system affects the other one. For pathway analysis, one of the most widely used methods is comparing the gene list to a pathway which gives a *p* value as a result. Basically, such scoring enrichment methods compare a list of the genes to that of a pathway and count the hits, so that the greater the number of the hits, the greater the score and the enrichment (Curtis et al., 2005). GenMAPP is an open source package that allows users to visualize microarray and proteomics data in the context of biological pathways (freely available at http://www.genmapp.org/). It represents biological pathways in a special file format called 'MAPPs' which are independent of the gene expression data. It is used to group genes by any organizing principle (e.g., apoptosis pathways). In addition, the gene set enrichment analysis (GSEA) is a novel method that uses all the data on the microarray in the order of expression, determining whether a priori defined set of genes shows statistically significant, concordant differences between two biological states such as phenotypes (Subramanian et al., 2005). In 2003, Hosack et al. developed a powerful software named, "the Expression Analysis Systematic Explorer" (EASE), which is customizable software for rapid biological interpretation of gene lists resulted by "omics" technology such as toxicogenomics, proteomics, or other high-throughput genomic data, in particular DNA microarray gene expression profiles. In fact, the biological themes returned by EASE recapitulate manually determined themes in previously published gene lists and are robust to varying methods of normalization, intensity calculation and statistical selection of genes (Hosack et al., 2003). We have largely exploited EASE to rapidly searching the Genbank in order to find the functional 'themes' in our microarray experiments. We have found various functional themes for the upregulated or downregulated genes induced by CLs in human epithelial cells, mainly: signal transducer activity, catalytic activity, response to external stimulus, cell growth and/or maintenance, cell cycle, response to biotic stimulus, regulation of programmed cell death, humoral immune response, cellular defense response, positive regulation of biosynthesis, negative regulation of cell proliferation, regulation of interferon-gamma biosynthesis, transcription factor binding, DNA repair, regulation of nucleocytoplasmic transport, apoptosis, apoptosis inhibitor activity, positive regulation of apoptosis, nuclease activity, transcriptional elongation regulator activity, regulation of caspase activation, response to oxidative stress, DNA damage response, and cell-mediated immune response (Omidi et al., 2005a).

As a secondary goal of array experiments it necessitates to look for groups of genes that behave similarly across a series of treatments (i.e. clustering analysis). There are a number of methodologies for clustering that can be employed upon experimental and statistical objectives; for clustering methods see citations (Azuaje, 2003; Sturn et al., 2002; Yang et al., 2001). In our studies on toxicogenomics of gene delivery systems, we have used softwares such as GeneSight™ or GeneMath™gene expression to present data as a single linkage

Hierarchical clustering plot**.** The algorithm used subjects the expression intensity ratio of treated versus untreated samples to single-linkage Hierarchical clustering (by means of Euclidean distance metric) analyses in order to arrange each gene with its related group members exhibiting a similar ratio of change in expression. We have shown that some overexpressed - or underexpressed genes display not only a similar pattern of expression but also a related cellular functionality and themes (e.g. apoptotic related genes) (Omidi et al., 2003; Omidi et al., 2005a). Such Hierarchical clustering maybe considered as a "genomic signature" of any chemical.

Taken all these facts together, surprisingly, still little information is available upon specific genomic effects elicited by chemicals within various cells/tissues despite implementing the "omics" technology for discovery of intrinsic genomic signature of chemicals/compounds in various targets. As a result, extensive investigations are yet to be performed to get sufficient information on genetic-signature of chemical and pharmaceuticals in target cells/tissues. Accordingly, many individuals and some organizations have attempted to accomplish such aim. For example, the Comparative Toxicogenomics Database (CTD) is a useful platform providing insights into complex chemical–gene and protein interaction networks (http://ctd.mdibl.org/about) that can be used for successfully advancement of novel pharmaceuticals.

7. Genomic impacts of cationic lipids

To date, cationic lipids have been the most widely used delivery system for delivery of nucleic acids both *in vitro* and *in vivo*. For example, Lipofectin™ is the 1:1 mixture of DOTMA and DOPE. It is the first cationic lipid formulation that was received widespread attention. We found that cationic liposomes such as LF and OF, at concentrations routinely used to obtain efficient delivery of gene based medicines, were able to induce gene expression changes in human epithelial A431 cells (Table 1). Such alterations in gene expressions appeared to be largely dependent upon the physicochemical characteristics of the lipid, wherein OF elicited greater gene expression than LF, i.e., up to 16% of the genes studied (Omidi et al., 2003). We speculate that the surface charge may play a key role in terms of such genotoxicity. In these cells, we witnessed that the affected genes were functionally involved in various cellular processes such as cell proliferation, differentiation and apoptosis. The upregulated or downregulated genes include some important genes such as bcl-2-related protein a1 (BCL2A1), caspase 8 isoform c (CASP8), heat shock protein 70 (HSP70) and 60 (HSP60), annexin a2 (ANXA2), and tubulin beta 5 (TUBB5) (Omidi et al., 2003). Up regulation of caspase-8 clearly impart activation of procaspases and caspases that may provoke activity of a series of apoptotic signaling cascades such as electron carrier protein cytochrome C, adaptor protein Apaf-1, Bcl-2 family, p53 and various transcription factors (Kanduc et al., 2002). Given that the heat shock protein 70 acts as an inhibitor of apoptosis (Li et al., 2000), it's upregulation by OF in A431 cells is deemed to be a cellular compensatory or defense response. We assume that cells recognize the xenobiotics upon their biological properties. To examine such concept, we compared OF genotoxicities within two epithelial cell lines (i.e., A431 and A549 cells).

In A549 cells, the genomic impacts were intriguingly dissimilar compared to that of A431 cells (Table 1). Further, we observed some commonalities in gene expression modulation between two different cell lines (Omidi et al., 2008). Upon EASE analyses, the changes in gene expression fell into a number of various functional genomic ontologies. For example,

the upregulated genes by OF nanoliposomes included the genes involved in apoptosis, oxidative stress and external/biotic stimulus (e.g., IL9R, DUSP1, CSK, CSE1L); while the downregulated genes were related to the cell growth and/or cell maintenance, cell proliferation and apoptosis (e.g., SEP6, PSMA4).

Gene ID (Accession No.)	Gene description	LF-A431	OF-A431	OF-A549
NM_004417	Dual specificity phosphatase 1; DUSP1	—	+	+
NM_033356	Caspase 8, isoform c; CASP8	NC	+	NC
NM_002467	V-mycmyelocytomatosis viral oncogene homolog (avian); MYC	NC	+	NC
NM_004049	Bcl2-related protein a1; BCL2A1	NC	+	NC
NM_003195	Transcription elongation factor a (sii), 2; TCEA2	NC	+	NC
NM_001983	Excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence); ERCC1	NC	+	NC
NM_004094	Eukaryotic translation initiation factor 2, subunit 1 (alpha, 35kd); EIF2S1	NC	+	NC
NM_000994	Rbosomal protein l32; RPL32	NC	+	NC
NM_001274	Chk1 checkpoint homolog (s. pombe); CHEK1	NC	+	NC
NM_002849	Protein tyrosine phosphatase, receptor type, r; PTPRR	NC	+	NC
NM_002156	Heat shock 60kd protein 1 (chaperonin); HSPD1	NC	+	+
NM_002957	Retinoid x receptor, alpha; RXRA	NC	+	NC
NM_001242	Cd27 antigen; TNFRSF7	NC	+	NC
NM_006083	Red protein; IK	NC	+	NC
L12723	Heat shock protein 70; HSP70	NC	+	+
NM_004383	C-src tyrosine kinase; CSK	NC	+	+
NM_004635	Mitogen-activated protein kinase-activated protein kinase 3; MAPKAPK3	NC	+	NC
NM_005546	Il2-inducible t-cell kinase; ITK	NC	+	NC
NM_006235	Pou domain, class 2, associating factor 1; POU2AF1	NC	+	NC
NM_002623	Prefoldin 4; PFDN4	NC	NC	NC
NM_001316	Cse1 chromosome segregation 1-like (yeast); CSE1L	NC	NC	+
NM_002953	Ribosomal protein s6 kinase, 90kd, polypeptide 1; RPS6KA1	NC	NC	NC
NM_000660	Transforming growth factor, beta 1; TGFB1	NC	NC	NC
NM_000043	Apoptosis (apo-1) antigen 1; TNFRSF6	NC	NC	NC
NM_001961	Eukaryotic translation elongation factor 2; EEF2	NC	NC	NC
NM_001786	Cell division cycle 2 protein, isoform 1; CDC2	NC	NC	NC
NM_021103	Thymosin beta, TMSB10	NC	NC	NC
NM_004315	N-acylsphingosineamidohydrolase (acidceramidase);	—	NC	NC

Gene ID (Accession No.)	Gene description	LF-A431	OF-A431	OF-A549
	ASAH			
NM_002026	Fibronectin 1, isoform 1 preproprotein; FN1	—	NC	NC
NM_001238	Cyclin e1, isoform 1; CCNE1	NC	NC	NC
NM_002186	Interleukin 9 receptor; IL9R	NC	NC	+
NM_002945	Replicationprotein a1 (70kd); RPA1	NC	NC	NC
NM_003875	Guanine monophosphate synthetase; GMPS	—	NC	NC
NM_000887	Integrinalpha x precursor; ITGAX	NC	NC	+
NM_000075	Cyclin-dependent kinase 4, isoform 1; CDK4	NC	NC	—
NM_032959	Dna directed rna polymerase ii polypeptide j, isoform b; POLR2J	NC	NC	+
NM_000970	Ribosomal protein l6; RPL6	—	NC	NC
NM_005319	H1 histone family, member 2; H1F2	NC	NC	NC
NM_002592	Proliferating cell nuclear antigen; PCNA	—	NC	NC
NM_020300	Microsomal glutathione s-transferase 1; MGST1	NC	NC	NC
NM_021065	H2a histone family, member g; H2AFG	NC	—	NC
NM_004832	Glutathione-s-transferase like; GSTTLP28	NC	—	NC
NM_006087	Tubulin, beta, 5; TUBB5	NC	—	NC
NM_002789	Proteasome (prosome, macropain) subunit, alpha type, 4; PSMA4	NC	—	—
NM_005566	Ldha	NC	—	NC
NM_015129	Septin 6; SEP6	NC	—	—
NM_004039	Annexin a2; ANXA2	NC	—	NC

Table 1. Gene expression changes induced by cationic liposomes in A431 and A549 cells. LF: Lipofectin™ ; OF: Oligofectamine™; NC: no changes; +: upregulation; —: downregulation; adapted with permission (Barar et al., 2009).

For example, among the genes upregulated by OF in A549 cells (but not A431 cells), the IL9R gene encodes IL9 receptor protein which is a cytokine receptor that specifically mediates the biological effects of IL9. The ligand binding of this receptor leads to the activation of various JAK kinases and STAT proteins, which connect to different biologic responses, in particular some genetic studies, suggested an association of this gene with the development of asthma (Gaga et al., 2007).

The heat shock proteins 60 and 70 as well as c-src tyrosine kinase (CSK) were observed to be upregulated in both cell lines (Table 1). Of these, the heat shock proteins family of molecular chaperones appears to act in protein folding, translocation, and assembly into complexes; while CSK is mainly involved in protein-tyrosine kinase activity as well as protein metabolism and modifications. Once looked at the overlapped activities of these genes, we found that they are cooperating mostly to activate the binding activity - we speculate that these genes somehow are collaborating perhaps in terms of protein folding and binding.

Since liposomal formulations are being explored for pulmonary drug/gene delivery, and thus their ability to activate IL9R should be assessed when used clinically for lung gene

therapy. The CSK along with some other genes were upregulated in A549 cells treated with cationic lipids similar to what we observed previously in A431 cells (Omidi et al., 2003) and is mainly involved in cell growth and/or cell maintenance. The SEP6 and PSMA4 were downregulated genes by OF in both cell lines. The SEP6 gene is a member of the septin family of GTPases. Members of this family are required for cytokinesis. One version of pediatric acute myeloid leukemia is the result of a reciprocal translocation between chromosomes 11 and X, with the breakpoint associated with the genes encoding the mixed-lineage leukemia and septin 2 proteins. This gene encodes four transcript variants encoding three distinct isoforms. An additional transcript variant has been identified, but its biological validity has not been determined. The PSMA4 is a multicatalytic proteinase complex with a highly ordered ring-shaped 20S core structure. They are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in a non-lysosomal pathway.

Because of the gene expression commonalities and distinctions between the two cell lines, we conceptualized that these cells may respond to the cationic lipid "OF" differently upon their cellular characteristics. These cells appeared to undergo somewhat adaptation upon exposure to xenobiotics, as a result of which they could dynamically respond as expressing/activating related cellular elements for recognition and internalization of the cationic lipid. Of interest, we found that the genotoxicity elicited by the cationic lipid nanosystems were largely dependent upon the structural architecture and/or physicochemical properties of the cationic lipid since no extensive overlap was observed in the gene expression profile induced by either LF or OF in A431 cells. Besides, the responsiveness of the target cells to the lipids could be different since the transfection efficiency is significantly depended upon the target cells and lipids used. Likewise, Filion and Phillips (1997) reported high toxicity rate elicited by some cationic lipids in phagocytic cells such as macrophages and U937 cells, but not in non-phagocytic T lymphocytes.

Taken all these findings together, it seems that for attaining detailed characterization of the toxicogenomics of these lipid delivery systems (based on their molecular structure), the gene expression patterns/profiles need to be determined in different cell types perhaps with known cell surface architecture.

8. Genomic impacts of cationic polymers

Despite plethora of investigations on application of polymers in drug/gene delivery, surprisingly, little attention has been devoted about possible biofunction of polymer *per se* in particular genomic effects. Many researchers have now consensus upon functionalities of polymers, and accordingly new domains of polymer science such as "polymer genomics", "polymer genocompatibly" and "polymer genotoxicity" have been arisen. To examine the polymer genocompatibly concept, we have previously reported that starburst PAMAM dendrimers (i.e., PF and SF) as well as polypropylene imine (PPI) dendrimers (e.g., DAB8 and DAB16) can inadvertently induce alterations in gene expression (Hollins et al., 2007; Omidi et al., 2005b). These dendrimers have been successfully exploited for delivery of gene based medicines. Of these dendrimers, we have previously shown dramatic alteration in gene expression induced by DAB16 dendrimer in A431 and A549 cells (Omidi et al., 2005b).

Table 2 represents the gene expression changes by DAB polymers in A431 and A549 cells. Of the altered genes in A431 cells, some are related to cell defense and response to stress (e.g., ALOX5, TNFRSF7) and apoptosis (e.g., TNFRSF7). In A549 cells, some of the altered genes

Gene ID (Accession No.)	Description	A431 cells		A549 cells	
		DAB8	DAB16	DAB16	DAB16:DNA
NM_006716	Activator of s phase kinase; ASK	NC	–	NC	NC
NM_000034	Aldolase a; ALDOα	NC	NC	NC	NC
NM_004039	Annexin a2; ANXα2	NC	+	NC	NC
NM_000698	Arachidonate 5-lipoxygenase; ALOX5	NC	–	NC	NC
NM_004049	Bcl2-related protein a1; BCL2α1	NC	NC	NC	+
NM_000591	Cd14 antigen precursor; CD14	NC	NC	+	NC
NM_001242	Cd27 antigen; TNFRSF7	NC	–	NC	NC
NM_001786	Cell division cycle 2 protein, isoform 1; CDC2	NC	NC	–	NC
NM_003467	Chemokine (c-x-c motif), receptor 4 (fusin); CXCR4	NC	NC	NC	–
NM_001274	Chk1 checkpoint homolog (s. pombe); CHEK1	NC	NC	NC	–
NM_004383	C-src tyrosine kinase; CSK	NC	NC	NC	–
NM_003914	Cyclin a1; CCNα1	NC	NC	+	NC
NM_001239	Cyclin h; CCNH	NC	NC	–	NC
NM_000075	Cyclin-dependent kinase 4, isoform 1; CDK4	NC	+	NC	NC
NM_001801	Cysteine dioxygenase, type i; CDO1	NC	NC	NC	–
NM_004417	Dual specificity phosphatase 1; DUSP1	NC	–	NC	+
NM_003875	Guanine monophosphate synthetase; GMPS	NC	–	NC	+
NM_021065	H2a histone family, member g; H2AFG	NC	+	NC	NC
NM_002156	Heat shock 60kd protein 1 (chaperonin); HSPD1	NC	–	NC	NC
NM_000879	Interleukin 5 (colony-stimulating factor, eosinophil); IL5	NC	NC	NC	–
NM_002186	Interleukin 9 receptor; IL9R	NC	NC	+	NC
NM_002358	Mad2-like 1; MAD2L1	NC	NC	NC	+
NM_002424	Matrix metalloproteinase 8 preproprotein; MMP8	NC	NC	NC	–
NM_000245	Met proto-oncogene precursor; Met	+	NC	–	NC
NM_004315	N-acylsphingosine amidohydrolase (acid ceramidase); ASAH	NC	NC	NC	+
NM_006235	Pou domain, class 2, associating factor 1; POU2AF1	NC	NC	NC	–
NM_000946	Primase, polypeptide 1 (49kd); PRIM1	NC	NC	NC	+
NM_002592	Proliferating cell nuclear antigen; PCNA	NC	NC	–	NC
NM_000532	Propionyl coenzyme a carboxylase, beta polypeptide; PCCβ	NC	–	NC	NC
NM_002789	Proteasome (prosome, macropain)	NC	+	NC	NC

Gene ID (Accession No.)	Description	A431 cells		A549 cells	
		DAB8	DAB16	DAB16	DAB16:DNA
	subunit, alpha type, 4; PSMα4				
NM_002796	Proteasome (prosome, macropain) subunit, beta type, 4; PSMβ4	NC	NC	–	NC
NM_002737	Protein kinase c, alpha; PRKCα	NC	NC	NC	–
NM_006083	Red protein; IK	NC	–	NC	NC
NM_002914	Replication factor c (activator 1) 2 (40kd); RFC2	NC	NC	NC	–
NM_002947	Replicationprotein a3 (14kd); RPα3	NC	–	NC	NC
NM_002957	Retinoid x receptor, alpha; RXRα	NC	NC	+	NC
NM_007209	Ribosomal protein l35; RPL35	NC	NC	NC	+
NM_033301	Ribosomal protein l8; RPL8	NC	–	NC	NC
NM_003139	Signal recognition particle receptor ('docking protein'); SRPR	NC	NC	NC	+
NM_003072	Swi/snf related, matrix associated regulator of chromatin, SMARCA4	NC	–	NC	–
NM_003236	Transforming growth factor, alpha; TGFα	NC	NC	+	NC
NM_000660	Transforming growth factor, beta 1; TGFβ1	NC	NC	NC	+
NM_003292	Translocated promoter region (to activated met oncogene); TPR	NC	NC	+	NC
NM_006087	Tubulin, beta, 5; TUBβ5	NC	+	NC	NC
NM_003299	Tumor rejection antigen (gp96) 1; TRA1	NC	NC	+	NC
NM_006826	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide; YWHAQ	NC	–	NC	NC

Table 2. Gene expression changes by DAB polymers in A431 and A549 cells. NC: no changes; +/-: up/down regulation; adapted with permission from (Omidi et al., 2005b).

were in association with cell defense, DNA repair/damage and apoptosis (e.g., CCNH; ERCC1; PCNAM, CD14).

With a particular interest on toxicogenomic of the DBA16:DNA nanoparticles in A549 cells, expression changes (upregulation/downregulation) were found for some important genes (i.e., TGFβ1,BCL2α1, IL5, CXCR4 and PCKα). Of these, TGFβ1 is a member of a super-family of multifunctional cytokines that regulate cell proliferation, differentiation, and apoptosis (Chiarugi et al., 1997; Haufel et al., 1999), while the BCL2 protein family is involved in a wide variety of cellular activities that also act as anti- and pro-apoptotic regulators. The protein encoded by BCL2 is able to reduce the release of pro-apoptotic cytochrome c from mitochondria and block caspase activation which is the main apoptosis pathway. Further, this gene is a direct transcription target of NF-KAPPAβ in response to inflammatory mediators, and has been shown to be upregulated by different extracellular signals, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), CD40, phorbol ester and

inflammatory cytokine TNF and IL1, which suggests a cyto-protective function that is essential for lymphocyte activation as well as cell survival; reader is directed to see following citations (May et al., 1994; Ruvolo et al., 2001). The upregulation of TGFβ1 and BCL2α conceivably imply incitement of apoptosis in A549 cells upon treatments with DAB16:DNA polyplexes.

It was also found that the altered genes induced by PF, DAB16 and OF in A431 cells shows some commonalities and differences in pattern, presumably due to their positive charge and structural architecture. In A431 cells, treated with either DAB8 or DAB16 resulted in ~13% and ~7% similar and opposite patterns of gene expression changes, respectively. For example, BCL2α1 which acts as anti- and pro-apoptotic regulator was largely affected by DAB16 compared to DAB8. This could be due to higher surface charge and/or interaction capacity of DAB16. Similar pattern was seen for proteasomeα4, but Met proto-oncogene revealed opposite pattern. Once DAB16 was tested in different cell line (i.e., A549 cells), similar and opposite patterns of gene expression changes were ~11% and ~9%, respectively. Intriguingly, upregulation of some important genes (e.g., IL9R, TGFα) was seen solely in A549 cells, but not in A431 cells. It can be speculated that A549 cells can show greater response than A431 cells. Hence, these dendrimers could potentially affect cell growth and immune response of cells by altering the expression of some related genes at doses which did not distinctly modify cell viability (Table 2). It should be also evoked that the identity of the genes whose expression was significantly altered (i.e. the "gene signature" of the delivery system) was markedly different in the two cell lines, despite the similar expression of the majority of the genes (80%) that remained unaffected (Akhtar & Benter, 2007).

Table 3 shows the gene expression of some selected genes induced by branched and linear polyethylenimine (BPEI and LPEI, respectively) in A431 cells. These data solely present the upregulated and downregulated genes, similarly induced by these cationic polymers, while there are a large number of genes showed opposite pattern (data not shown). Based on these results, it was found that the alterations in gene expression by BPEI were significantly greater than LPEI. We contemplate that this could be because of the greater interaction of BPEI with subcellular biomolecules.

To examine the late effect of BPEI in target cells, we evaluated gene expression pattern of caspases genes in A431 cells as a time series approache (i.e., immediately after transfection, 24 h and 48 h after transfection). Fig. 5 represents the gene expression profile of selected caspase pathway genes in A431 cells treated with BPEI, showing significant impacts of BPEI even 48 h after treatment. Of these genes, as previously mentioned, caspase 8 play a key role in apoptosis.

These findings directed us to examine some other cationic polymers such as PAMAM and PEI. Upon our examination on SF and PF, we found that PF induced gene expression changes much greater than SF. This could be due to differences in dendrimers architecture. Significant decrease in gene expression changes were observed upon PF complexation with a DNA at the supplier recommended ratio of 10:1 (w/w) of PF:DNA. Reduced in number, but not in nature and magnitude, of expressed genes were observed upon PF:DNA complexation. In treated A431 cells with cationic dendrimer PF or cationic lipid OF, opposite and similar patterns of gene expression changes were 20% and 16%, respectively (Barar et al., 2009).

Function	Gene ID	T/UT ratio		
		BPEI	LPEI	
gi:407955 - membrane-associated protein hem-1	M58285	4.10	1.95	+
gi:7106883 - HSPC247	AF151081	2.74	1.98	+
gi:13569894 - diaphanous homolog 3; DIAPH3	NM_030932	2.23	2.44	+
gi:14010613 - methylmalonyl-coa epimerase	AF364547	2.13	2.21	+
gi:14248538 - STONIN2	AF255309	2.10	2.13	+
gi:188560 - prepro-mullerian inhibiting substance	K03474	2.10	2.69	+
gi:285915 - epimorphin	D14582	2.07	3.17	+
gi:7109206 - four alpha helix cytokine; ZCYTO10	AF224266	1.99	2.00	+
gi:558098 - protein kinase c-theta; PRKCT	L01087	1.97	1.93	+
gi:9843747 - putative pyroglutamyl-peptidase i; PGPEP1	AJ278828	1.93	2.82	+
gi:22041589 - similar to data source:sptr, source key:q9h4b3, evidence:iss~homolog to mucolipidin~putative; loc255231	XM70908	0.58	0.25	-
gi:14588660 - histidase; hal	AB042217	0.57	0.27	-
gi:10439114 - homo sapiens cdna: flj22644 fis, clone hsi07088; unnamed protein product.	AK026297	0.53	0.26	-
gi:10944321 - myozenin; MYOZ	AF240633	0.53	0.26	-
gi:2613124 - small cell vasopressin subtype 1b receptor	AF030512	0.52	0.26	-
gi:20278870 - delta 4 progesterone receptor; pr	AB084248	0.46	0.26	-
gi:7020101 - cdna clone unnamed protein product	AK000183	0.45	0.27	-
gi:7209599 - melatonin 1b receptor	AB033598	0.44	0.26	-
gi:307425 - nerve terminal protein; SNAP	L19760	0.43	0.25	-
gi:18182679 - nkg2d	AF461811	0.41	0.25	-
gi:347133 - succinate dehydrogenase flavoprotein subunit; SDH	L21936	0.39	0.23	-
gi:2738815 - p2y1 receptor; p2yr1	AF018284	0.28	0.26	-
gi:21928730 - seven transmembrane helix receptor	AB065731	0.26	0.27	-
gi:3088552 - cystatin-related epididymal spermatogenic protein; cres	AF059244	0.24	0.24	-
gi:22048232 - similar to riken cdna 2610027o18; KIAA1393	XM_050793	0.21	0.22	-

Table 3. Gene expression changes of selected genes induced by branched and linear polyethylenimine (BPEI and LPEI, respectively) in A431 cells (our unpublished data produced by Omidi et al.). +/-: up/down regulation

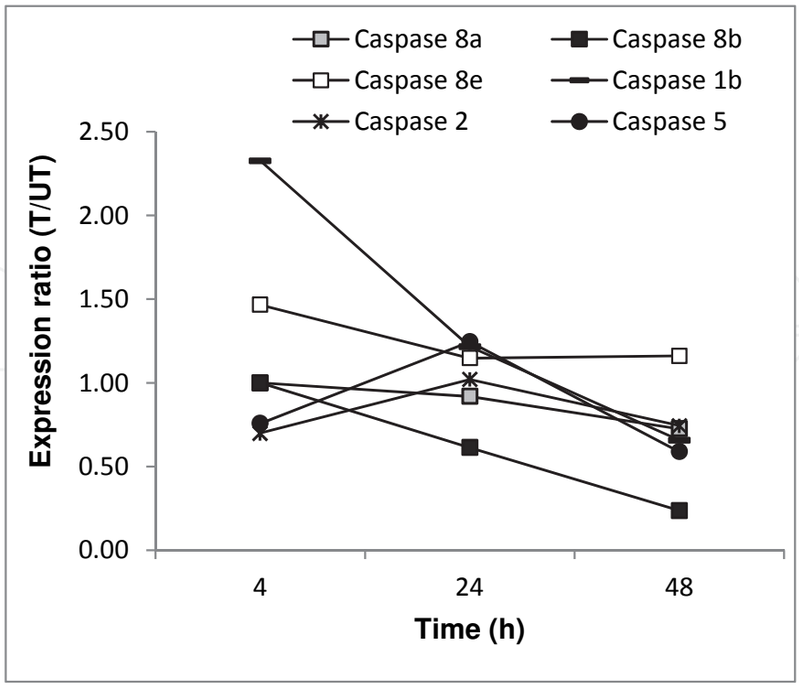

Fig. 5. Gene expression ratio of selected caspase pathway genes in A431 cells treated with BPEI after 4, 24 and 48 h (our unpublished data produced by Omidi et al.).

Likewise, Pluronic® block copolymers were shown to cause various functional alterations in cells through interacting with cellular biomolecules and thus affecting various cellular functions such as mitochondrial respiration, ATP synthesis, activity of drug efflux transporters, apoptotic signal transduction, and transcriptional activation of gene expression both *in vitro* and *in vivo* (Batrakova & Kabanov, 2008). This polymer is able to enhance expression of reporter genes under the control of cytomegalovirus promoter and NF-KB response element in stably and transiently transfected mouse fibroblasts and myoblasts *in vitro*. It has been shown that these block copolymers are able to act as biological response modifying agents through upregulating the transcription of genes via activation of selected signaling pathways such as NF-KB (Sriadibhatla et al., 2006).

Furthermore, Pluronic® P85 (P85) was reported to promote transport of the pDNA to the nucleus in cells transiently transfected with DNA/PEI polyplex (Kabanov, 2006). It has also been successfully exploited for DNA vaccine delivery, however some investigations revealed that P85 simultaneously increase transgene expression and activate immunity, in which P85 alone and P85:DNA complexes were shown to increase the systemic expansion of CD11c+ (DC), and local expansion of CD11c+, CD14+ (macrophages) and CD49b+ (natural killer) cell populations. DNA/P85 polyplex can also increase maturation of local DC (CD11c+ CD86+, CD11c+ CD80 +, and CD11c+ CD40+ (Gaymalov et al., 2009). Thus, the activation of immunogenes in the antigen-presenting cells by P85:DNA complexes can highlight new insights for these kinds of polymers.

In addition, Pluronic® can cause some alterations in HSP68 expression, suggesting that this polymer may affect stress-related pathways or there is a cross-talk between the stress and other pathways activated by the copolymer (Sriadibhatla et al., 2006). These results are in accord with what we have observed for some other cationic polymers or lipids. Pluronic®

(a mixture of Pluronic L61 and F127; also called as SP1017) has been reported to deliver plasmid DNA in skeletal and cardiac muscle, as well as in solid tumors. Unlike other polycations, Pluronic® does not bind and condense the nucleic acids, it does not protect DNA from degradation or facilitate transport of the DNA into the cell and its effects involve transcriptional activation of gene expression (Kabanov, 2006). The effect of Pluronic® was reported to be related to the activation of gene expression by activating the NF-κB and p53 signaling pathways, in which pro-apoptotic AP-l gene that is frequently regulated by the NF-κB system, was not responsive. This, perhaps, indicates that Pluronic-mediated influence on transcription is selective and it is not a result of a general nonspecific activation of immune defense system such as NO-mediated burst (Kabanov, 2006). Nonetheless, to ensure about this supposition, it is essential to recruit global gene expression screening methods such as microarray technology as we have witnessed dramatic alterations in gene expression *in vitro* and *in vivo* upon treatment with different polymers using microarray technology. Kabanov's group has reported that Pluronic block copolymers interact with biomembranes and induce gene expressions through mechanisms that differ from the delivery of the DNA into the cell. They also questioned whether upregulation of expression of genes delivered into cells can also take place by other nonviral polymer-based gene delivery systems? We have observed that various polymers, in particular polycations, are able to alter gene expressions related to immune response and cell defense (Barar et al., 2009; Hollins et al., 2007; Omidi et al., 2008).

It appears that the cytotoxicity of nonviral vectors is largely dependent upon the cationic nature of the vector, which attains different level to different structural architecture. For cationic lipid, the cytotoxic effects are mainly determined by the structure of its hydrophilic group (Prokop & Davidson, 2008), e.g. the quaternary ammonium amphiphiles are more toxic than their tertiary amine counterparts. Such toxicity (due to positive charge of the head group) can be reduced by importing a heterocyclic ring such as imidazolium or pyridinium.

The biodegradability potential of the advanced nanobiomaterials are also determined their toxicity. For example, poly(lactic-co-glycolic acid) nanoparticles elicit very low level of cytotoxicity and toxicogenomic compared to cationic polymers, but not the modified PLGA-grafted poly(L-lysine) nanosystems (Omidi & Davaran, 2011).

Surprisingly, the effect of hydrophobic chain on toxicity has not been adequately addressed to date even though it is deemed that the hydrophobic moieties may disrupt the integrity of lipid bilayer. Like cationic lipid, cationic polymers with acid-labile linkage can be rapidly degraded and less toxic. It has been reported that the toxicity of polymers (e.g., PEI, PLL or dendrimers) increases with high molecular weight (Bieber & Elsasser, 2001). Polymers synthesized by linking low molecular weight with acid-labile show low toxicity (Li et al., 2004). The creation of amphiphilic cationic polymer based on PEI or PLL, by linking PEG or other groups, reduces toxicity without compromising the gene delivery efficiency (Zhang et al., 2008).

Upon our observations the biodegradable cationic polymers (e.g., polysaccharides) which display high degree of biodegradability possess low toxicity, thus we speculate that they may be extensively used for *in vivo* transfection in the future. Further, high transfection efficiency and low toxicity can be obtained by the addition of co-lipids or co-polymers (PEGylation). Water soluble lipopolymer, to combine the advantages of both cationic polymer and liposome, seems to be our next approach for optimized gene transfer. Besides, adding cell-specific biomolecules (e.g. aptamer, peptide ligands, antibodies or nanobodies)

to gene transfer vectors potentially improve the specific problem by permitting lower and safer vector doses while facilitating tissue targeting.

9. Concluding remarks

Synthetic lipids or polymers used for gene delivery may impose selective "phenotypic effects" in cells by affecting cell signaling involved in various biological functions such as cell defense, inflammation, differentiation, proliferation and apoptosis. It is believed that these effects result basically from their interactions with cell membranes, intracellular organelles and subcellular biomolecules, as a result the target cells can respond to these effects phenotypically or genotypically. In some cases, these effects can be relatively benign as they do not induce sever cytotoxic effects, while in the case of nonviral cationic vectors it is not the case since the interaction of the polycationic gene delivery nanosystems with target cells is significantly greater than non-cationic polymers. It is now deemed that one unifying property of polycationic gene delivery nanosystems is their potential to interact with cellular/subcellular biomolecules, upon which profound changes in various cell processes may occur. From this standpoint, it becomes clear that these polycations are able to penetrate into cells and reach different critical subcellular targets and induce inventible biological functions, for which the nanoscaled range of sizes is an important factor. Different cell types as biological targets may response differently, and even modify the activities of such nanomaterials. While the genome-based therapeutics (e.g., oligonucleotides and gene silencing siRNAs) have already been lined up for clinical trials (up to 1700 trials), our knowledge is lacking upon genomic signature of such gene based medicines. As concluding statement, it is suggested that the inadvertent intrinsic genomic signature of nonviral delivery systems should be assessed and taken into consideration for a gene therapy trial since gene silencing/stimulation experiments are to target a specific gene while the gene delivery system may potentially mask or interfere with the desired genotype and/or phenotype end-point of gene therapy. The upregulation or downregulation of genes induced by gene delivery systems or any other drug carriers and excipients appears to instigate a new directionality such as "functional excipients". But, this approach simply represents the gene expression changes which are solely based on intensities of expressed genes for various signaling pathways, while we should look for ways to correlate such gene expression intensities with functional genomics.

10. References

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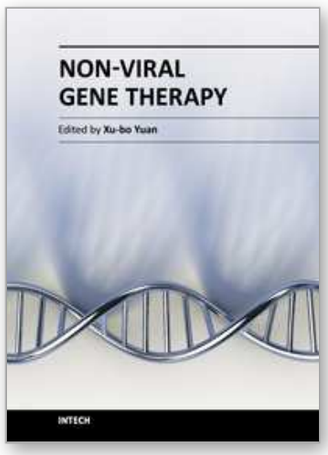

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