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REVIEW ARTICLE

Nucleic-acid based gene therapeutics: delivery challenges and modular design of nonviral gene carriers and expression cassettes to overcome intracellular barriers for sustained targeted expression

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Abstract
The delivery of nucleic acid molecules into cells to alter physiological functions at the genetic level is a powerful approach to treat a wide range of inherited and acquired disorders. Biocompatible materials such as cationic polymers, lipids, and peptides are being explored as safer alternatives to viral gene carriers. However, the comparatively low efficiency of nonviral carriers currently hampers their translation into clinical settings. Controlling the size and stability of carrier/nucleic acid complexes is one of the primary hurdles as the physicochemical properties of the complexes can define the uptake pathways, which dictate intracellular routing, endosomal processing, and nucleocytoplasmic transport. In addition to nuclear import, subnuclear trafficking, posttranscriptional events, and immune responses can further limit transfection efficiency. Chemical moieties, reactive linkers or signal peptide have been conjugated to carriers to prevent aggregation, induce membrane destabilization and localize to subcellular compartments. Genetic elements can be inserted into the expression cassette to facilitate nuclear targeting, delimit expression to targeted tissue, and modulate transgene expression. The modular option afforded by both gene carriers and expression cassettes provides a two-tier multicomponent delivery system that can be optimized for targeted gene delivery in a variety of settings.

Keywords: Gene delivery, non-viral, gene carriers, nucleic acid, RNAi, plasmid, sustained-release system, targeted drug delivery, transgene, Immune response

Introduction

The ability to alter or transform cellular physiology via the delivery of exogenous nucleic acid molecules to cells has been a common research tool in the laboratory for decades to study gene functions. The therapeutic potential of this approach was not fully realized due to lack of reliable and practical methods to transfer and express recombinant DNA in mammalian cells. By the 1980s, the concept of gene therapeutics has moved from the bench side to the bedside, when a series of clinical trials demonstrated therapeutic efficacy from the transplantation of virally transduced cells (Rosenberg et al., 1993; Blaese

et al., 1993). Gene therapy quickly became an intensely investigated field with the promising potential to devise treatment not only for genetic diseases but also for a wide range of disorders including metabolic disorders, infectious diseases, chronic illnesses and cancer. The power of gene therapy is derived from the ability to manipulate cell physiology at genetic and epigenetic levels, accessing molecular processes that are previously unreachable by conventional pharmacological means. This allows particular pathways and factors to be targeted with unparalleled specificity, thereby greatly improving the efficacy in therapy and dramatically reducing side effects

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commonly associated with wide spectrum pharmacological compound.

As is common to all drug development processes, delivery is the foremost challenge for gene therapeutics. The large molecular weight and anionic charges prohibits nucleic acid molecules from entering the cell via passive diffusion across the negatively charged lipid bilayer of the plasma membrane, and thus calls for a facilitated uptake process. This challenge was initially met by engineering disarmed retroviruses, whose virulence factors that enable viral replication have been removed from the viral genome and replaced with the nucleic acid sequences coding for a protein with therapeutic potential (Eglitis et al., 1988). Clinical translation of recombinant virus-based gene delivery vectors demonstrated promising result, with several trials reporting long-term remission of symptoms in patients suffering from difficult to treat genetic diseases such as severe combined immune deficiency (Blaese et al., 1993). However, the initial success of the gene therapy trials came into question when a few of the patients developed significant reactions to the administered vector. In one instance, patients developed leukemia-like symptoms, which was later determined to be the result of random vector integration at sensitive genomic sites, which transformed nearby genes into oncogenes (Hacein-Bey-Abina et al., 2003). In another trial, an acute inflammatory response was mounted against viral coat proteins (Marshall, 1999), which lead to massive tissue damage that eventually resulted in death of the patient. These tragic conclusions prompted the community to reexamine the way viral safety is being evaluated and subsequently spurred a shift in focus toward finding alternative nonviral gene transfer methods.

The development of synthetic nonviral gene delivery systems has been met with various technical and biological challenges. The essential features of gene carriers includes the ability to (i) condense the negatively charged nucleic acid molecule into a compact size with an overall positive charge that are conducive to interaction with the plasma membrane and subsequent cellular uptake, (ii) protect of genetic cargo from degradation by extracellular and intracellular nucleases, (iii) circumnavigate intracellular compartments to unload the cargo in the targeted subcellular domain, and (iv) minimize off-target associated toxicity, which includes genotoxicity, immunogenicity and cytoxicity. Several types of cationic polymers and lipids have been explored for this purpose with varying levels of transfection efficiencies (Midoux et al., 2008; Putnam, 2006; Park et al., 2006; Wasungu & Hoekstra, 2006). While these biocompatible materials meet some of the requirements of a gene carrier, they do not yet have the comprehensive capability to overcome the intracellular barriers that viruses have naturally evolved to evade. As such, nonviral gene delivery systems at present are comparatively inefficient for clinical application with respect to viral vectors. Furthermore, while researches into nonviral carriers are largely driven by the

promise of their theoretical safety profiles, the clinical data on their biodistribution and metabolism is limited. Thus, the promise of a safer gene delivery system in clinical application remains to be fulfilled. Finally, unlike conventional pharmacological compounds, nucleic acids are delivered as a prodrug, where the activity, instructed by nucleic acid sequences, would depend on the physiology of the cell carrying out those instructions. An inherent disconnect between the pharmacokinetics of the nucleic acid complexes and the kinetics of the expressed transgene product is natural. Determining the correlation between delivery efficiencies and therapeutic efficacy would necessary involve retooling of existing methods.

Over the last 2 decades, strategies to improve nonviral gene delivery have largely borrowed approaches from conventional drug design, such as conjugate derivatization and liposome encapsulation. But unlike pharmaceutical compounds, nucleic acids can be modified through genetic recombination to insert functional elements that can self-modulate its own activities ranging from target specificity, bioavailability, intracellular trafficking, to regulated expression and sustained protein production, all without affecting the integrity or competency of the nucleic acid. In this review, we will examine the types of genetic modification currently employed in the field of gene therapy, the major physicochemical and biological barriers complicating delivery and expression, and highlight some of the most promising solutions to overcome those barriers.

Types of genetic modifications

Gene-based therapy is most commonly associated with gene augmentation or gene replacement therapy, where the deficient gene product is supplied with a functional version. Our expanding knowledge of molecular genetics has broadened avenues of gene therapy to include gene inhibition, editing, and repair (Figure 1). While the mode of genetic modifications differs in their effects on gene of interest, the biological outcome is largely dependent on the identity of the targeted gene. These modifications are not mutually exclusive in their application, and can work in parallel or in concert to achieve the same therapeutic outcome.

Gene augmentation

In gene augmentation therapy (GAT), the aim of the treatment is to enhance the amount of protein product by delivering exogenous nucleic acid molecules containing instruction for the expression of the deficient protein. This type of therapy is typically applied to correct monogenic loss-of-function mutations that underlay many metabolic and physiological disorders, which are conventionally treated with protein-based enzyme or hormone supplement (Figure 1a). Beyond inherited or chronic metabolic disorders, GAT has been applied to cancer therapy via *de novo* expression of suicide genes that have the ability to induce cell death following its own

expression and can range from apoptosis inducer, such as caspases (Xie et al., 2001; Carlotti et al., 2005) to enzymes that converts prodrugs to cytotoxic compound (Mullen, 1994). In addition, the augmented expression of tumor antigen epitope in cancer cells has been demonstrated to enhance its immunogenicity and anti-tumor activity of cytotoxic T cell (He et al., 2003). Evidently, the clinical utility of GAT is diverse and is highly dependent on the activity of the expressed protein, but in all cases, the objective is to get transgene production from the expression construct.

The most common nucleic acid for GAT is bacterial-derived, mammalian expression plasmid DNA (pDNA). These DNA molecules typically range in sizes from 3 to 10 kilobases and contain coding sequences for the gene of interest, promoters, enhancers, and polyadenylation sites, which are crucial to the expression and posttranscriptional processing of the transgene mRNA. Beyond these mammalian elements, the remainder of the molecules is occupied by bacterial sequences required for replication, partition and selection of the pDNA during clonal expansion of the molecule from a bacterial host. pDNA is a popular choice for GAT owing to well-established methods that allow convenient insertion and removal of sequences to modularize the performance and activity of the vector with genetic elements. Alternatively, expression cassettes can be constructed *in vitro* by PCR-based oligonucleotide extension or synthetically via nucleotide polymerization; the latter method is primarily used to optimize codons for the expression of transgene in nonnative species. As can be seen, these expression cassettes come in various molecular weights, conformation and topologies. We have previously shown that polymeric gene carriers do not discern between these structural differences, and can promote the condensation and uptake of both linear and circular pDNA with equal efficiencies (Hsu & Uludağ, 2008). However, transfection efficiencies between the different conformations of DNA molecules vary significantly, hinting intracellular processes, rather than physicochemical properties, to be predominantly involved as rate-limiting steps. Regardless of DNA topography, one of the major limitations with DNA-based expression cassettes is its requirement for nuclear import in order to access the transcription machinery. An alternative approach to bypassing the need for nuclear entry is through the delivery of mRNA instead of pDNA, which can be directly translated in the cytoplasm. A significant drawback of this approach is that RNA molecules are highly susceptible to degradation by extracellular and intracellular nucleases. However, recent advances in both *in vitro* transcription and chemical modification have significantly improved the stability and synthesis scale of RNA that is feasible for gene therapy (Tavernier et al., 2011).

Beyond pDNA, development of artificial chromosomes (AC) has gained significant momentum toward clinical readiness. The major advantage of AC is their ability to be replicated and maintained autonomously as an episome, which allows transgene expression to be sustained in subsequent generations. The capacity for the size of the transgene in AC is also much greater than any vector system currently available, which can prove to be ideal for treating diseases such as Duchenne muscular dystrophy (DMD), where the relative expression of multiple tissue-specific isoform of the dystrophin gene needs to be preserved and precisely regulated, and thus require the entire locus for dystrophin be introduced to the cell (Muntoni et al., 2003). Despite its advantages, clinical utility of ACs is hampered by challenges in their construction, purification, in addition to delivery.

Gene knockdown

Gene knockdown refers to the downregulation of gene expression at either the transcriptional or translational levels. This is typically applied to reverse the deleterious effects caused by the abnormal expression of a mutated protein, an oncogene or a virulence factor (Figure 1b). Gene knockdown in mammalian cells can be mediated through a number of natural processes. RNA interference (RNAi) is a posttranscriptional silencing pathway that uses short stretches of double-stranded RNA (dsRNA) molecules as inducers. The dsRNA are cleaved by a RNase III-like protein called the "Dicer protein", to yield shorter 21–23 nt molecules with 2-nucleotide 3′-overhang at both ends, known as small interfering RNA (siRNA); siRNA are then bound to RNA-induced silencing complexes (RISC) where the sense strand is cleaved, allowing the antisense strand to guide the complex through a homology-dependent base pairing through a degradation pathway, thereby preventing the translation of the mRNA into protein (Preall & Sontheimer, 2005). RNAi can be induced by exogenously delivered dsRNA (siRNA), whose activity is located in the cytosol. The silencing activity, however, is transient as degradation consumes the siRNA. Alternatively, pDNA containing either the RNA Pol II or Pol III promoter have been used to drive the transcription of small hairpin RNA (shRNA), an intermediate in the RNA processing pathway and a precursor of siRNA, for more sustained silencing activity (Pardridge, 2007). However, nuclear import is required for the transcription of shRNA and may therefore limit its silencing efficiency.

The siRNA molecules can be considered as a substrate for the enzymatic processes in gene silencing. As with protein engineering, chemical constituents on the oligonucleotides can be "re-engineered" to alter this lock-and-key binding interaction. Indeed, a growing repertoires of chemical modifications have been applied to siRNA molecules in an effort to improve their efficacy, potency, serum stability, specificity, and delivery as well as modulating their immunogenicity and minimizing off-target effect. These include modifications to the sugar moieties (Kraynack and Baker, 2006; Choung et al., 2006), phosphate linkages (Amarzguioui et al., 2003; Harborth et al., 2003), (Hall et al., 2004; Prakash et al., 2006),
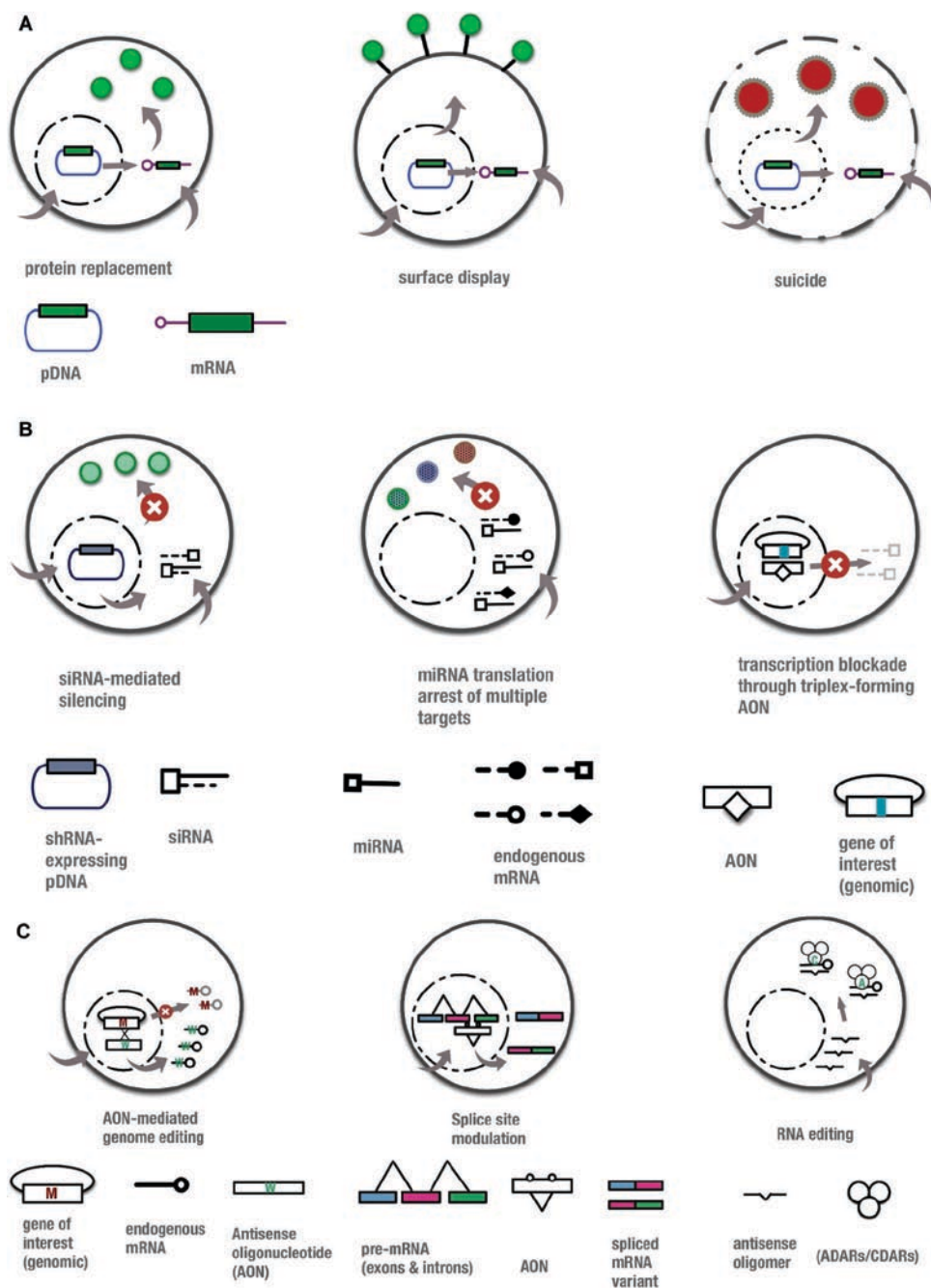
Figure 1. Schematic representations of different modalities of gene modifications. (a) In gene augmentation, an expression cassette can be delivered as plasmid DNA (pDNA) where it is transcribed in the nucleus, or as mRNA, where it is directly translated in the cytoplasm. The gene of interest can code for a protein that include, but not limited to, a growth factor in intracellular signaling, a ligand for surface display or secretion, or an enzyme that cleaves prodrugs into toxic compounds in suicide therapy. (b) In gene knockdown, RNAi is facilitated by introduction of either shRNA-expressing pDNA or siRNA to inhibit the translation of a target protein. miRNA-mediated gene silencing can target multiple genes in a transcriptome by translational blockade or induction of target mRNA degradation. Anti-sense oligonucleotide (AON) can also block gene expression by complementary binding to its target site in the genome, blocking transcription via a triplex structure. (c) Gene editing can be facilitated by delivery of oligonucleotides or small DNA fragments to promote mismatch repair or site-specific recombination to remove the deleterious mutation from the genome. Alternatively, gene repair can be undertaken at the RNA level through splice site modulation during mRNA processing or through nucleotide modification to induce base transition in RNA editing.

nitrogenous bases (Xia et al., 2006; Hornung et al., 2006), duplex architecture (Holen et al., 2003; Kim et al., 2005; Abe et al., 2007), and the overhang/termini (Morrissey et al., 2005a; Morrissey et al., 2005b; Kraynack and Baker, 2006). For detailed discussion on the design, architecture and modifications of siRNA, the reader is referred to

excellent reviews elsewhere (Watts et al., 2008; Aartsma-Rus et al., 2009; Gaglione and Messere, 2010).

RNAi can also be achieved through microRNA (miRNA). Endogenously transcribed miRNA is a type of noncoding double stranded RNA used to regulate complex expression of network gene networks involved

in various cellular processes. Processed mature miRNA range from 21 nt to 24 nt and share similar structural features to siRNA. miRNA share partial complementary sequences to their target mRNA, and suppress gene expression by a noncleavage dependent degradation pathway or by steric blockade of mRNA translation (Shi, 2003; Davidson & McCray, 2011). While siRNA is designed to target specific gene, miRNA can bind to multiple mRNA targets, ranging up to the thousands of genes and thus have the potential to regulate complex network of pathways that dictate multifaceted cellular responses.

Gene knockdown can further be mediated through an RNAi-independent mechanism involving antisense oligonucleotides (As-ODN). As-ODN are 18–21 nt single-stranded DNA molecules that share complementary sequences to its target gene (Patil et al., 2005; Aartsma-Rus et al., 2009). Hybridization of the As-ODN to target sequences inhibits gene expression via several mechanisms including the following: (i) As-ODN can enter the nucleus and bind to the target loci in the genome to form a triple helix structure with chromosomal DNA to arrest transcription by physical blockade. (ii) As-ODN can interfere with mRNA maturation by destabilizing pre-mRNA processing in the nucleus, attenuating downstream translation. (iii) Alternatively, As-ODN can bind to the target mRNA and inhibit translation through either an occupancy-based mechanism or through the activation of RNase H mediated degradation of the target mRNA (Crooke, 1998; 1999; Chan et al., 2006).

Similar to siRNA, As-ODNs are chemically modified to enhance their efficacy and stability while warding off attacks from nucleases. The first and second generations of As-ODN involved modification to the sugar ribose by replacing the 2′OH with sulfur or alkyl groups to yield phosphorothioate (PS-ODN), and 2-O-methyl, (2′OMe) or 2′-O-methoxyethyl (2′MOE) modifications. While these modified ODNs exhibited enhanced stability, it also resulted in reduced binding affinity (Eckstein, 2000), and led to higher cellular toxicity, including stimulation of immune responses. (Levin, 1999; Crooke, 2000). In the case of 2′ OMe and 2′MOe modified ODN, RNase H-mediate target mRNA cleavage was no longer supported, reducing its overall efficacy. The third generation of As-ODN builds on top of the previous generations of modifiers but completely replaces the furanose ring with new chemical moieties to yield nucleotide analogs. Notable analogues include peptide nucleic acid (PNA; Nielsen, 2004), lock nucleic acid (LNA; Petersen and Wengel, 2003) and morpholino phosphoroamidates (Kurreck, 2003; Gleave and Monia, 2005). These nucleotide analogues do not support RNase H-mediated cleavage of target mRNA and thus exert their antigene activity primarily through steric hindrance to arrest expression at either the transcription or translation stage. The new generation of chemical moieties and stereoisomers improve upon the previous generation by further enhancing binding affinity, target specificity and nuclease resistance. For detailed review

of chemically modified As-ODN, the discussion is continued elsewhere (Chen et al., 2005; Chan et al., 2006; Bell and Micklefield, 2009).

Gene repair
GAT alone may not be sufficient to reverse pathological conditions due to a number of inherent limitations with the approach. First, the promoters used to drive the transgene expression are typically ubiquitous or constitutively active, which do not provide the fine level of quantitative expression that is appropriate to reestablish physiological equilibrium. Second, a single gene locus may produce multiple isoforms of the same protein through splice site modulation (Muntoni et al., 2003)—a typical pDNA expression cassette used in GAT may only encode one of the variants and therefore cannot provide all the isoforms needed. Finally, in instances a dominant mutant protein is causing a detrimental effect on the cell, simply augmenting the wild type copies of the protein does not counter the abnormal activity of the mutant protein (Oren, 1992). By extension, suppressing the expression of dominant mutants via RNAi does not provide functioning protein required to reconstitute normal cellular activity. Thus, a combination of gene augmentation and gene knockdown is likely necessary. While gene replacement is the ideal corrective approach, achieving stable and accurate genome integration remains a technical challenge at present; the risk of ectopic integration often outweighs the therapeutic benefits. For this reason, there is great interest in gene repair as a method for restoring wild type functions in dominant negative mutations. Repair can be implemented at the mRNA level or at the genome level by editing out miscoded sequences through nucleotide base transition, splice site modulation, mismatch repair using antisense oligomers, or oligonucleotide-mediated genome editing (Figure 1c).

RNA editing via base transition is based on the premise that nucleotide can be covalently modified to change its complementary base pairing properties. For example, the deamination of adenosine and cytosine result in inosine and uracil, respectively. Inosine have base-pairing properties similar to guanosine, and pairs with cytosine, thus A to I editing leads to base transition from A to G. Similarly, uracil behaves like adenosine such that C-to-U editing results in transition from C to A. RNA editing is initiated by binding of antisense oligomer to the mutated target mRNA to form double-stranded structures. The RNA duplex structures are recognized by adenosine deaminase acting on RNA (ADARs) and cytosine deaminase acting on RNA (CDARs) to catalyze the base modifications. RNA editing is a naturally occurring event utilized by cells for posttranscriptional processing (Watanabe & Sullenger, 2000). While RNA editing is limited to A-to-G and C-to-U base transition, ADAR can act on not only pre-mRNA, but viral RNA and noncoding miRNA as well (Nishikura, 2006; Samuel, 2001). Thus, it has therapeutic potential in wide range of diseases (Gallo & Galardi, 2008; Maas, 2010).

Splice site modulation for alternative exon skipping is based on the principle that As-ODN targeted to splice site regulatory regions of pre-mRNA could sterically block aberrant splicing and restore normal processing of the transcript (Kole & Sazani, 2001). The As-ODN used in this instance is similar to the ones for gene knockdown, except it has been resequenced and chemically modified to improve its binding affinity and bioavailability. The third generation of As-ODNs (e.g. LNA, PNA, morpholinos, 2′O-methyl phosphorothiate, ethylene-bridged nucleic acid) with synthetic nucleotide analogues serve as ideal splice site modulators since they no longer act as substrate for RNase H and exhibit enhanced resistance against nuclease attacks (Kurreck, 2003; Karkare and Bhatnagar, 2006; Wilson and Keefe, 2006). The limitation to RNA splice site modulation is that the mutation still exists in the genome and thus repeated administration is required to sustain therapeutic benefits. In contrast, gene editing at the genome level provides a long-term solution to gene correction and is based on the observation that DNA fragments with homologous sequences to a target locus can induce site-specific recombination or single-base mismatch repair (Igoucheva et al., 2001). Various types of DNA molecules ranging from triplex-forming oligonucleotides (Vasquez et al., 2000), RNA/DNA hybrid oligonucleotides (Wu et al., 2001) and small DNA fragments (Goncz & Gruenert, 2000) have been applied to genome editing in model diseases.

One can see both the simplicity and complexity of nucleic-acid based therapy in the prevailing discussion. The simplicity comes from the fact that structurally and chemically identical nucleic acid molecules can impart and modulate a wide array of activities—As-ODN can be used for gene editing, RNA repair, and gene knockdown; pDNA can be adapted to transcribe protein-encoding mRNA, or transcript-modulating shRNA. The complexity and challenge then arise in accurately identifying the molecular targets, specifying their activities through sequences and delivering the molecules to appropriate subcellular compartments in which the activity is to be carried out.

Barriers to nucleic acid based therapeutics

Transfection pathway employed with nonviral carriers is a multistep process that involves cell-surface binding, internalization, intracellular trafficking and, if appropriate, expression of the nucleic acids. Efforts to improve transfection efficiency have primarily focused on conjugate chemistry and carrier modification. Despite a large depository of novel carrier systems in the literature, only a limited subset has successfully translated to a setting appropriate for clinical testing (e.g. functionality in primary cells or in animal models). Several barriers were identified along the transfection pathway; however, identification of dominant rate-limiting steps has been difficult to reconcile due to the ubiquity of the carriers

and cell types used in the investigations. The distinction among rate-limiting steps is a systemic process aimed at simplifying the understanding of the events taking place. In practice, it is likely that no such line exists between each barrier and that the transfection pathway exists as one integral nonlinear process. The list of barriers will likely differ for each type of carrier, delivery platform (*ex vivo* vs. *in vivo*), the types of nucleic acid cargo (DNA vs. RNA) and the types of genetic modification intended (expression vs. inhibition vs. repair). Below, we will focus our discussion on *ex vivo* transfection using cationic gene carriers for pDNA delivery to explore the overall transfection pathway that encompasses barriers common to most transfections.

Overview of transfection pathway

To facilitate delivery of genetic materials, carriers such as cationic polymers are first mixed with nucleic acid molecules in solution where the two species spontaneously bind to each other to assemble into positively charged submicron particles. These complexes, termed polyplexes or lipoplexes, can then be administered to the cell where they physically interact with the negatively charged plasma membrane and/or specific ligands on the cell surface. Cell binding induces an energy-dependent endocytosis whereby engulfed materials are enclosed in a membrane-bound vesicle called an "endosome" (Khalil et al., 2006); release from the endosome to the cytosolic domain is facilitated by the carrier through membrane destabilization. Following endosome escape, the nucleic acids navigate through the cytosolic milieu and traverse across the nuclear envelope into the nucleus where the pDNA may access the transcription machinery for expression. After transcription, the transgene mRNA is processed and exported out of the nucleus, into the cytosol, and where it is translated by the ribosome to generate the protein product (Figure 2).

Physicochemical properties and cell type dictate uptake pathways

The pDNA-carrier complexes are characterized by size, zeta potential, morphology, chemical composition, spatial features, stability and polydispersity, which altogether define the physicochemical properties of the ensemble of complexes, and are used to predict carrier efficiency (Park et al., 2006; Mintzer & Simanek, 2009). Prepared complexes are rarely homogeneous in size, charge, and stability (Sharma et al., 2005). Our previous studies have demonstrated that polyethyleneimine (PEI) polyplexes range in sizes that exhibit a bell shaped distribution when measured in a low serum transfection medium (Figure 3a and Figure 3b; Hsu et al., 2011). These sizes may further change over time and especially upon interfacing medium with different solute concentration, ionic strength, and pH, as well as when encountering other charged molecules, such as those in the serum or on the cell-surface (Wightman et al., 2001). The size distributions would present the particles as a mixture of different

species of molecules, which means the uptake of particles would likely proceed through multiple pathways.

There are four mechanistically distinct endocytic pathways known: (i) clathrin-mediated, (ii) caveolae-dependent, (iii) macropinocytosis, and (iv) clathrin/caveolae-independent endocytosis (Khalil et al., 2006; Zhou et al., 2004; Medina-Kauwe et al., 2005). These pathways differ in the size of the formed vesicles, the coat protein embedded in the endosome, which aids in subsequent sorting of the internalized molecule, and preference for the size of particles taken up. Clathrin-mediated endocytosis generally internalize particles that are <200 nm (Takei and Haucke, 2001; Rejman et al., 2004; Ehrlich et al., 2004; Cureton et al., 2009), while particles between 200 and 500 nm are preferentially taken up via the caveolae-mediated pathway (Rejman et al., 2004). This size preferences comes somewhat as a surprise considering that the reported size of caveolar vesicles are much smaller (50–60 nm) (Conner and Schmid, 2003). However, others have shown that large viruses, such as Newcastle disease virus (~300 nm) and the respiratory syntial virus (~250 nm), are taken up via the caveolar endocytosis as well (Werling et al., 1999; Cantín et al., 2007). Therefore, the size of the cargo does not appear to be restricted by the size of the vesicles and vesicles may changes in size to accommodate the cargo. Larger particles beyond the 500 nm are predominantly taken up by clathrin-and-caveolae independent endoyctosis, such as macropinocytosis or even phagocytosis, in specialized phagocytic cells such as macrophages (Khalil et al., 2006; Kopatz et al., 2004; Vercauteren et al., 2011; Grosse et al., 2005). Thus, each of the uptake pathways is specialized for a particular size range. Since prepared complexes typically contain particles with different sizes, cellular uptake of particles is likely going to be a heterogeneous process involving multiple endocytic pathways contributing simultaneously in varying proportions.

Caveolae-mediated pathway plays an important role in cellular homeostasis and cargo transport, most notably in the transcytosis of serum protein across the epithelial layer, in intracellular trafficking of cholesterol, and regulation of specific signaling cascade (Anderson, 1998; Razani et al., 2002). The flask shape and organization of caveolae is conferred by caveolin, which is a class of cholesterol-binding protein, inserted as a loop into the leaflet of the plasmid membrane (Harris et al., 2002).

Cell physiology can dictate the qualitative and quantitative nature of receptors embedded on the cell surface. The expression of the receptors is dynamically regulated by the metabolic requirements of the cells, which are defined by cell type (e.g. epithelial or skeletal), cell lineage (e.g. pluripotent stem cells, mesenchymal stem cells, erythrocytes) and cell cycle (e.g. senescent vs. dividing). In the case of epithelial cells, for example, up to 20% of the receptors for endocytosis are contributed by caveolae (Maxfield & McGraw, 2004). As such, the predominant endocytic pathways for a given molecule will differ among cells, and the proportion contributed by each
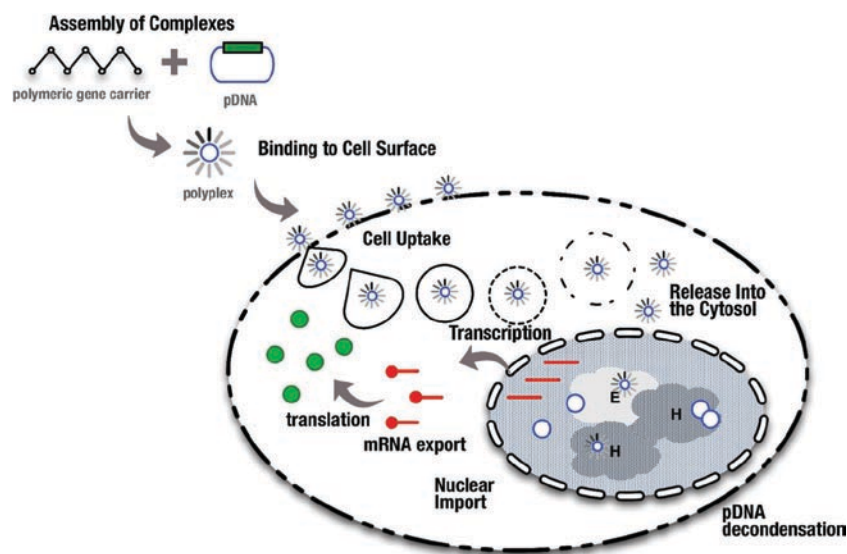

Figure 2. Schematic overview of the transfection pathway. The initial step involves assembly of complexes between the gene carrier and the nucleic acid molecules. Complexes must be stable during delivery and exhibit uniformity in size distribution to better control internalization pathway. In the above figure, pDNA is condensed into sub-micron particles with an overall positive surface charge. This allows binding to the negatively charged cell surface and promote subsequent uptake via endocytosis. Release into the cytosol is facilitated by destabilization of endosome membrane with pH-responsive components of the gene carrier (i.e. proton sponge or endosomolytic peptide). Alternative, fusogenic peptide or pore-forming lipids can facilitate endosomal escape by fusion with the membrane. Once in cytosolic domain, the pDNA must be imported into the nucleus for transcription. This can be mediated by movement along the cytoskeleton network, or actively imported by importins through signal peptide or nuclear DNA targeting sequences. Transcriptional activity of the transgene is favored by intranuclear disposition within the euchromatin domain, as well as efficient de-condensation from the gene carrier. Long-term expression of the transgene will require replication and nuclear retention of the pDNA as well as avoiding the transgene clearance activity of the immune response.
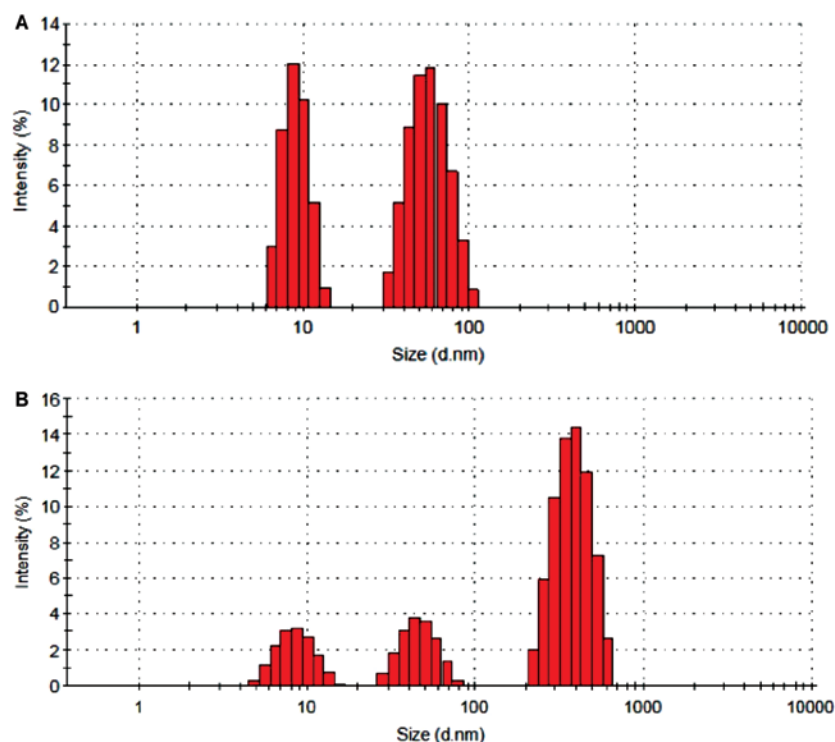
Figure 3. Intensity histograms of particle sizes in obtained with (a) OptiMEM with 1% FBS (no pDNA complexes) and (b) PEI25/pDNA polyplex at polymer-to-DNA weight ratio of 2.5 at a pDNA concentration of 2 μg/mL, in OptiMEM + 1% FBS. The two distributions at approximately 10 and 80 nm in (a) are interpreted as signals from serum proteins. The additional third distribution in (b) is interpreted as the PEI25 polyplexes. Note that while polyplex particles have an average size of ~150 nm, the size distribution is significantly heterogeneous.

of the endocytic pathways will be cell type dependent (Traub, 2009). Indeed, Douglas et al. showed that, the predominant endocytic pathways for internalizing alginate/chitosan polyplexes differ among 293T, COS7 and CHO cells. In the case of 293T, clathrin-dependent endocytosis was the major pathway, as clathrin inhibition led to a greater reduction in both complex internalization and transfection efficiency than caveolae inhibition. In contrast, transfection efficiency in COS7 cells was substantially reduced by caveolae inhibitors, whereas clathrin inhibitors had a minor impact. Furthermore, clathrin inhibitors had no effect on complex internationalization in CHO cells while cavaeloe inhibitors resulted in ~76% reduction, suggesting the presence of additional pathways in the uptake of complexes. Similarly, transfection of HUH-7 cells with linear PEI polyplexes involved both clathrin-mediated (70%) and cavaelae-mediated (30%) endocytosis. The author further demonstrated pathway asymmetry in the uptake of particles with different sizes; smaller polyplexes were routed to both the clathrin and cavaelae-mediate uptake pathway, while large particles proceeded through auxiliary pathways that were not interfered by clathrin or cavaelae inhibitors (Gersdorff et al., 2006). Taken together, these recent findings point to an uptake process in which the predominant endocytic pathways would dependent on both the physicochemical properties of the complexes and the physiology of the cell.

Uptake pathway dictates sorting and release of the cargo

The implication of the heterogeneity in endocytic pathways is that not all complexes internalized will contribute to transfection—some pathways may be transfection conducive while others lead to transgene inactivation. Clathrin-mediated endocytosis has consistently demonstrated to be conducive to transgene expression (Douglas et al., 2006; Gersdorff et al., 2006; van der Aa et al., 2007), though this may in part be dependent on carrier used and its mechanism of endosome disruption.

Clathrin-mediated endocytosis is thought to be involved in a number of cellular processes including intercellular communication, modulating signal transduction by regulating the expression of cell-surface protein receptors, and recycling/sorting of activated receptors (Conner and Schmid, 2003). During classical clathrin-mediated endocytosis, internalized vesicles are first uncoated and routed to sorting endosome by interacting with other vesicles through membrane-embedded signal molecules. They are subsequently recycled to either cell periphery or trafficked to the endo-lysosomal pathway for degradation (Maxfield & McGraw, 2004). In the latter case, sorting endosomes are directed to late endosomes, which undergo gradual acidification as proton pumps transport H^+ into the lumen, and eventually fuse with lysosomes to degrade the engulfed material through hydrolytic enzyme

digestion. The acidic environment inside lysosome is thought to be undesirable for internalized complexes as it leads to breakdown of the pDNA. However, routing to the endo-lysosomal pathway might be essential for endosomal escape and subsequent transgene expression (Gersdorff et al., 2006; Douglas et al., 2008). The abundance of amine groups on cationic polymers such as PEI is thought to act as a "proton-sponge" by absorbing protons to buffer the charge accumulation. The excessive proton and chloride inside the vesicle lead to an increase in osmotic pressure, which swell and ultimately ruptures the vesicles, releasing the complexes into the cytosol (Boussif et al., 1995). In contrast, caveolin-dependent macropinocytosis, and phagocytosis leads to formation of macropinosome and phagosome, respectively, which are not known to contain the membrane-embedded signal molecules necessary to interact with other vesicles for sorting and processing (Khalil et al., 2006; Medina-Kauwe et al., 2005). Instead, these vesicles often result in complexes sequestered in cytosol or possibly get exocytosed (Douglas, 2008). The lack of acidic environment in nonendosomolytic pathway deprives the opportunity for pH-responsive carriers to promote membrane destabilization required to escape the endosome. Furthermore, it has been shown that microsphere beads with diameters of <200 nm were internalized preferentially through the clathrin-dependent endocytosis and were directed to the late endosomal/lysosomal compartments. Larger ~500 nm beads, however, entered cells predominantly through caveolin-mediated pathway and did not co-localize with endolysosomal markers (Rejman et al., 2004). Thus, it can be inferred that pH-responsive cationic carriers that form <200 nm particles (e.g. particles formulated with 25 kDa PEI) would give higher transfection efficiency than polymers that inherently form larger particles (e.g. particles formed with 2 kDa PEI), since the smaller polyplexes can take advantage of the pH changing environment to promote endosome escape, while the larger polyplexes become inactivated in nonacidic compartments. Conversely, pore-forming lipids and fusogenic peptides, which promote endosome escape via fusion with the vesicular membrane, do not require acidic compartment for endosomal disruption and may escape more efficiently in neutral nondegradative environment. In that case, the ideal particle size may be well above >200 nm, allowing them to be taken up by nonclathrin mediated uptake pathway. The desirable endocytic pathway will thus depend on the carrier as the chemical and structural features will determine both the mechanism of endosome disruption and size of the particles. It should be noted that vesicle sorting may not be exclusively limited to one pathway since in some cell lines, vesicles can engage in pathway crosstalk whereby neutral caveosomal vesicles can fuse with acidic endosomes (Pelkmans et al., 2004). Regardless of the endocytic uptake pathways, the optimal transfection protocol would likely involve tuning the size of the particles with respect to cell type such that the complexes are internalized through the transgene conducive pathway.

Nuclear import

Once exogenous pDNA gets released into the cytosol, it must translocate across the nuclear membrane to access the main transcription machinery. The nuclear membrane is a double lipid bilayer that acts as a physical barrier to separate genomic materials from the cytoplasm. Bidirectional transport in and out of the nucleus is a tightly regulated process facilitated through a series of aqueous channels called the nuclear pore complex (NPC) that are embedded in the nuclear envelope. The NPC core structure is comprised of multiple units of nucleoporins that are arranged in an eight-fold rotational symmetry perpendicular to the membrane, forming a cylinder with hollow center. In its relaxed state, the cylinder has a diameter of 10 nm, which allows passive diffusion of molecules <60 kDa, such as ions, metabolites and some of the smaller proteins across the channel. The core diameter may further dilate or reshape dynamically to accommodate transport of larger molecules during active transport (Akey, 1990; Kiseleva et al., 1998). The transport of larger molecules like proteins, RNA and DNA across the NPC is mediated through an energy-dependent process that generally involves the recognition of specific signal recognition motifs on the substrate by soluble transport receptors (Grünwald et al., 2011; Rodriguez et al., 2004). For RNA, transport signal is provided by adaptor proteins, which bind to mRNA to form a RNA-protein complex called the messenger ribonucleoprotein. For proteins, signal peptides termed the nuclear localization sequences (NLS) and nuclear export sequences (NES), are synthesized as part of the protein presequence, which may be cleaved following transport or become buried (through either conformational changes or sequestered by binding to a repressor) as a mechanism to regulate its activity (Lei & Silver, 2002). These evolutionarily conserved mechanisms of nuclear transport may provide viable strategies to artificially promote the import of pDNA into the nucleus.

Particle sizes of carrier-pDNA complexes are typically >100 nm, which are well above the NPC size cut-off. Without signal peptide or adaptor protein, entry into the nucleus would need to be facilitated by the carrier. The capability of gene carrier to mediate entry into the nucleus appears to vary among carriers—some are able to promote nuclear uptake as a complex (Itaka et al., 2004; Bieber et al., 2002; Brunner et al., 2002), while others may dissociate in the cytosol, leaving pDNA to traverse across the nucleocytoplasmic pathway on its own (de Semir et al., 2002; Feng et al., 2006). The mechanism for gene carrier-assisted nuclear uptake is not very well described. Some carriers can directly facilitate entry by fusion with the nuclear membrane whereby a flip-flop mechanism allows complex to translocate to the nucleoplasm side of the nuclear envelope (Akita et al., 2007). Others may

indirectly gain entry by riding along the cytoskeleton network, which spans the cytoplasm and extends into the nucleus (Wagstaff & Jans, 2009). Still, complexes may be permitted to enter the nucleus passively, provided that the spatial diameter can be condensed down to a size that is below the NPC molecular weight cut-off.

For carriers with limited nuclear delivery capability, import can still take place indirectly through processes associated with mitotic events. It is now widely regarded that transfection efficiency directly correlates with cell division and cell growth (Grosse et al., 2006; Brunner et al., 2002; Männistö et al., 2005). The strong correlation has been attributed to the mitotic phase (M-phase) of the cell cycle, whereby transient breakdown of nuclear membrane temporarily removes the physical barrier, to allow pDNA to interact and associate with nuclear components. Following reassembly of the nuclear envelope, nuclear factors-associated pDNA become opportunistically incorporated into the nucleoplasm (S Brunner et al., 2000; Mortimer et al., 1999; Tseng et al., 1999). However, a recent study suggested that transfection efficiency is also correlated to the synthesis phase (S-phase) of the cell cycle. S-phase of the cell cycle is characterized by replication of the genome, which is accompanied by synthesis of intranuclear histone proteins. The relative increase in the intranuclear concentration of cationic histones prior to cell division may facilitate dissociation and decondensation of pDNA from complexes through ionic exchange. Binding of histones to pDNA may further promote the formation of a nucleosome structure that enhance the transcription efficiency from pDNA templates (Akita et al., 2007). Though this correlation may be specific to lipoplexes, it is nevertheless conceivable that the heightened global transcriptional activity during the late S-phase could also increase the number of transcription initiation from the pDNA. In any case, it is likely that both M- and S-phases are correlated to better nuclear uptake and more frequent transgene expression, which would translate to higher transfection efficiencies.

Although the nuclear membrane remains a physical barrier for pDNA entry into nucleus, it is certainly not the final barrier; subnuclear trafficking and posttranscriptional events pose as major limiting steps as well. However, the postnuclear events at present would appear stochastic at best, due to limited data on the kinetic in this part of the transfection pathway.

Subnuclear trafficking and post transcriptional events

Perhaps the least understood aspect of nonviral gene delivery is intranuclear trafficking of pDNA. The nucleus is a highly dynamic structure organized into compartments and domains that are closely associated with specific gene regulatory functions and transcriptional activity (Hendzel et al., 2001; Jackson, 2003). It is widely known that genes are defined into chromatin domains with euchromatin being transcriptionally active and heterochromatin being transcriptionally repressed. Studies on lymphoid cells showed that inactivation of transgene

expression correlates with its relocation to a heterochromatic nuclear site (Baxter et al., 2002), suggesting the expression status of a gene can be strongly influenced by its nuclear localization. Indeed, comparative studies to evaluate the subnuclear localization of nonvirally delivered pDNA and virally transduced adenovirus genomic DNA showed that the latter is preferentially localized with the euchromatin while pDNA were found primarily in the heterochromatin sites (Hama et al., 2007). The ability for adenovirus to traffic to transcriptional active domains are modulated by both *cis* and *trans* factors. *Trans*-acting viral core proteins such as protein V, protein VII and mu, complex with adenoviral DNA to facilitate entry into the nucleus; these core proteins can interact with nuclear subdomains (e.g. PML body, nucleoli and the nuclear matrix) to exchange chromatin remodeling factors, loosening the chromatin structure within the transcriptional region of the genome to facilitate binding by transcription factors (Tim W R Lee et al., 2003; Lee et al., 2004; Matthews, 2001). *Cis*-acting sequence elements or structural features on the adenoviral DNA are then bound by endogenous transcription factors or chromatin remodeling proteins to maintain the viral genome within euchromatin domains (de Jong et al., 2002; Matsumoto et al., 1989). Similarly, Shaheen et al. found that the more efficient polycationic gene carrier containing 46 dimethylaminoethyl-modified polyrotaxane (46DMAE-ss-PRX), which formed a tighter condensed particle, was found to decondense and co-localize preferentially with euchromatin. In contrast, the less efficient sibling polymer 16DMAE-ss-PRX were found decondensed in the heterochromatins, suggesting that the condensation/decondensation efficiency in heterochromatin/euchromatin can be modulated by the number of cationic moieties per carrier (Shaheen et al., 2011). Aside from these "euchromatin induction mechanism", the chromatin status of the exogenous pDNA may also be influenced by the nuclear uptake pathway. It has been suggested that complexes entering the nucleus through the NPC may be able to access the euchromatin more efficiently than those entering via other non-NPC entry ways such as flip-flop fusion (Hama et al., 2007). Thus, analogous to the dependence of transfection efficiency on the uptake pathways, transgene expression efficiency may be closely tied with the mechanism of nuclear import.

It is generally accepted that prior to being transcribed, pDNA must dissociate from the gene carrier and decondense into a transcriptionally favorable conformation (Schaffer et al., 2000). Studies comparing the transcription efficiency between lipoplexes and adenovirus demonstrated that DNA decondensation accounts for the difference in efficiency between the two gene delivery systems (Hama et al., 2006; 2007). Quantitative relationship between dissociation and expression activity were demonstrated by an *in vitro* transcription assay whereby low molecular weight cationic polymers, which dissociate better than high molecular weight polymers, supported higher transgene expression (Schaffer et al., 2000).

Similarly, lipoplexes formed with higher charge ratios resulted in lower transfection efficiency, despite exhibiting higher uptake and endosomal escape, hinting on the possibility that stronger compaction reduce dissociation of DNA for transcriptional access (Yasunori Saito et al., 2006; Ahmad et al., 2005). In the case of PEI, however, transcription from pDNA polyplexes appears uninhibited in cell-free system (Bieber et al., 2002), but *in vitro* transcription were inefficient at low and high N/P ratio, where particles tend to aggregate or be surrounded by excess polymers (Honoré et al., 2005). In short, transcription factors may play a tertiary role in the dissociation of complexes, but the releasing activity may be dependent on the properties of the particles.

The transcribed transgene mRNA needs to be processed and exported out of the nucleus. Presumably the export of transgene mRNA is not a rate-limiting step as it is naturally processed along with the endogenous transcripts, though data on this is currently limiting. However, free carriers may interact with RNA species, including the transgene mRNA, and compromise their utility downstream. Free carriers is a byproduct of dissociated complexes, which could be found in either the cytoplasm or in the nucleus, potentially leaving a trail of free cationic residues for RNA interactions. Some studies showed that lipoplexes dissociated following escape from the endosome, while cationic polymers traveled with the pDNA as polyplexes into the nucleus and was dissociated through competitive interaction with endogenous biomacromolecules (Pollard et al., 1998; Mui et al., 2000; Schaffer et al., 2000). Comparative evaluation of postnuclear events between Lipofectamine and adenovirus showed that translation of the transgene mRNA is inhibited in lipofection as a result of carrier interaction with mRNA species and accounts for the discrepancy in efficiency between viral and nonviral carriers (Hama et al., 2007). Data on this for other carriers is limited, so that the impact of excess carriers inside the cells is not known. Regardless, a method to anchor or sequester dissociated carriers (e.g. biodegradable carriers) from binding to endogenous nucleic acids may enhance transfection efficiency as well as preserving cell viability.

Immune responses to nucleic acid and complexes

While initial excitement surrounding nonviral carriers stem from their superior safety profile compare to viral vectors, it is now widely known that systemic injection of lipoplexes and polyplexes induce innate immune response and cause tissue damage (Loisel et al., 2001; S Li, Wu, et al., 1999b; Gautam et al., 2001; Sakurai et al., 2007; 2008). Immunogenicity of complexes is attributed to bacterial sequences on the pDNA backbone. These unmethylated CpG dinucleotide islands, which are typically present in much higher frequency in microbial genome, not only pose severe safety issues, including the dissemination of gene encoding the antibiotic selection factor and activation of cryptic expression signals (Gill et al., 2009), but can also be recognized as pathogen-associated

molecular patterns (PAMM) in mammalian cells. The CpG-based PAMM can interact with toll-like receptors (TLR) to trigger a signal cascade that leads to the activation of the innate immune response (Kumar et al., 2009). Out of the 10 TLR identified to-date, TLR3, TLR7, TLR8 and TLR9 are embedded in the endosomal membrane. These TLRs are specialized in recognizing pathogen-associated nucleic acids, such as dsRNA, ssRNA, dsDNA, and CpG-DNA (Kawai & Akira, 2008). TLR9 has been identified to be the receptor primarily responsible for detecting and triggering an immune response against CpG pDNA since systemic administration of lipoplexes in TLR9 deficient mice exhibited significantly lower production of proinflammatory cytokines (Hongmei Zhao et al., 2004). Efforts to mask or remove PAMM by methylation of the CpG dinucleotides or excision of the bacterial vector backbone resulted in significant reduction in proinflammatory cytokine (Whitmore et al., 1999; Reyes-Sandoval & Ertl, 2004; Hyde et al., 2008). However, methylation alone is not sufficient to attenuate immune response (Cornélie et al., 2004), suggesting that other aspects of the nucleic acids (e.g. sequence, base modification, structural conformation) or even other cytosolic TLR-independent DNA sensors, such as the DLM-1/Z-DNA binding protein 1, can act in parallel to trigger inflammatory reactions (Ishii et al., 2006; Takaoka et al., 2007). It should be noted that sequential administration of pDNA and liposome carrier resulted in dramatic reduction in immune response in comparison to lipoplexes (Tan et al., 2001), suggesting the assembly of carrier and DNA into a complex may render higher immunogenic properties than either of the components alone. Furthermore, comparative evaluation between different PEI polyplexes and lipoplexes showed that the former formulation resulted in dramatically reduced cytokine production, hinting carrier composition may play a factor its immunogenicity. Strategies to evade the immune response would involve removal or conformational shielding of immunogenic components, or redirecting complex uptake to avoid detection by intracellular sensors.

Limited duration of transgene expression

The practical consequences of innate immune response to CpG DNA are not limited to tissue damage, but extend to the persistence of transgene expression. TLR-triggered activation of innate immune responses results in the induction of interferon responses and renders cells in an antiviral state. Antiviral responses can include activities that range from inhibition of transcription (Masataka Suzuki et al., 2010), inactivation of translation initiation factor (Sadler & Williams, 2008), induction of apoptosis (Li et al., 2006), nuclease-mediated degradation of exogenous nucleic acids, inhibition of intracellular trafficking, and base modification to the transgene DNA to induce point mutation in the viral genome (Aguiar & Peterlin, 2008). Furthermore, immunomodulation mediated by the interferon response can initiate a feedback

loop that enhances the sensitivity of pathogen recognition and activate the adaptive immune response, which includes lymphocyte infiltration (Fensterl & Sen, 2009) that ultimately leads to the killing and systemic clearance of transfected cells.

Transgene expression can also be diminished over time due to lack of a mechanism to faithfully maintain plasmid copy number, especially in replicating cells. Typical mammalian expression cassette lacks sequence elements necessary for the replication and partitioning of pDNA among divided cells. As a consequence, the intracellular pDNA concentration will gradually decreases as cells multiply, eventually falling below the minimum that is sufficient to obtain expression. Furthermore, dissociated pDNA no longer protected by gene carrier is susceptible to degradation by intracellular nucleases. Any remaining pDNA left in the nucleus are subsequently subjected to epigenetic silencing through chromatinization or base methylation (Riu et al., 2007; Recillas-Targa, 2006), repressing and attenuating transcription activity from the plasmid (Riu et al., 2007). It was suggested that pDNA backbone acts as a focal point for heterochromatinization via the binding of histone proteins, which then spreads into the transcription unit in the plasmid, repressing the expression (ZY Chen et al., 2008). Thus, sustaining the expression of transgene would necessarily involve sequence elements that can promote the replication and nuclear retention of the plasmid, maintenance in a euchromatin state and reduction in immunological reactivity.

Strategies to improve transfection efficiencies

Strategies to improve the delivery of nucleic acid therapeutics can be divided into two thrusts: (i) those based on carrier design to control packaging, intracellular uptake/trafficking and release of the nucleic acid cargo, and (ii) those based on the design of nucleic acid cargo to mediate trafficking and expression of the transgene.

Stabilizing particles to facilitate uptake via transgene conducive pathway

A major hurdle prohibiting the efficient uptake of pDNA is the instability of complexes prior to exposure to cells. Complexes typically have an overall positive charge on the particle surface and thus may invite binding from other charged species (Yang & L Huang, 1998; S Li, Tseng, et al., 1999a; Wheeler et al., 1999). These charge-charge interactions can arise during delivery, leading to premature dissociation of complexes, heterologous aggregation between particles and proteins, or homologous aggregation between complexes. Aggregation among complexes is supposedly driven by thermodynamically induced shielding of hydrophobic pockets within the complexes (Sharma et al., 2005). The result is formation of larger particles, which are less efficiently taken up by the cell, leading to a uptake pathway that may lead to the cytosolic sequestration of the complexes.

Preparation conditions aimed at reducing intermolecular interactions are could favor the assembly of more uniform particles. Parameters such as mixing order of complex components, speed of mixing, ionic strength of solution, concentration of complexes, temperature, and pH are all factors that can be implemented to control aggregation (Sharma et al., 2005; Ikonen et al., 2008). For example, gradual dropwise polymer addition to dilute nucleic acid solution results in smaller and more uniform particle sizes. Likewise, subsequent dilution of the complexes in larger volume combined with low temperature storage can slow down rate of aggregation. Acidic pH can increase the protonation of complex, leading to greater electrostatic repulsion among particles. Finally, enhancing the viscosity of complex solutions (e.g. by glycerol) can reduce the kinetic movement of molecules to reduce intermolecular interaction (Schaffer et al., 2000; Sharma et al., 2005). Homologous aggregation among particles has been attributed to the uncharged regions on particles, which promotes intermolecular interaction between particles to mask hydrophobic patches. Measures to counter hydrophobic interaction involves the addition of stabilizing agent, such as surfactants or sugars (Yu & Thomas J Anchordoquy, 2009; Marty et al., 2009; Ikonen et al., 2008). Optimized preparation methods and stabilizing agent may facilitate the formation of stable particles in storage, but these measures do not guarantee stability during delivery. That is, the physicochemical properties of the complexes may further change upon addition to transfection media or may interact with blood components *in vivo*. Perhaps the most widely cited approach to stabilize particles is the use of PEGylated carriers to sterically stabilize complexes' surfaces and shield complexes from blood components (Harvie et al., 2000) (Sun & Zhang, 2010). PEGylated liposomes have been a common pharmaceutical practice for many years and this approach have been adapted to stabilize cationic complexes (Huang et al., 2010a; Huang et al., 2010b; Luo et al., 2010; Germershaus et al., 2008; Glodde et al., 2006). However, shielding surface charges may also reduce binding to cell surface, effectively reducing uptake efficiency (Deshpande et al., 2004). PEGylation can also compromise the H$^+$ buffering activity that is necessary to induce osmotic swelling inside the endosome (Remaut et al., 2007). Careful titration of different molecular weights of PEG chains may be necessary to balance the cost and benefit effect between particle stability and gene delivery efficiency (Sun & Zhang, 2010). However, a method to coat a layer of surfactant stabilizer that can be reversibly "ejected" upon binding to the cell surface may present be a more realistic approach to both the storage and delivery of gene formulation.

Cell-specific targeting

Strategies for cell-specific targeting typically utilize receptor-mediated endocytosis through the conjugation of appropriate ligands to the carrier. Ligands such

as antibodies, transferrin, folic acid, RGD peptides, carbohydrates and lipids have been employed, which has been reviewed elsewhere (Xu et al., 2002; Bruckheimer et al., 2004; Déas et al., 2002; Harvie et al., 2003; Mamot et al., 2005; Jiang et al., 2007; Diebold et al., 1999; Meng et al., 2010; Incani et al., 2010). There may be several limitations to the use of ligand-conjugated carriers: (i) substitution of the cationic backbone can reduce binding affinity to anionic DNA, reducing the ability of the modified carrier to compact the DNA; (ii) the ligand needs to be properly displayed on the surface of the particles such that the receptor can recognize and bind to the complex; (iii) cell-specific receptors are typically presented at a lower proportion on the surface, thus, targeting to a specific subset of receptors may in effect, limit the level of nucleic acid uptake. It was estimated that at least $10^5 - 10^6$ plasmids per cell are required for transfection (Tseng et al., 1997), if the number of plasmids taken up by the limited subset of cell-specific receptors falls below this range, than subsequent nuclear delivery and transgene expression would be reduced; (iv) the actual delivery efficiency of targeted carrier might be severely diminished due to the effect of the ligand on other intracellular barrier, such as endosome escape and nuclear uptake, and (v) the ligand, particularly antibodies and peptides, can potentially be immunogenic, compromising the safety profile of a nonviral delivery system (Long Xu & Thomas Anchordoquy, 2011). While conceptually simple, ligand-based targeted delivery may enhance specificity at the expense of efficacy. Instead of targeted delivery, an alternative strategy would be to modulate target specific activity using genetic and epigenetic elements to enhance tissue-specific expression or by suppressing activity in nontargeted cells (see sections *Plasmid retention for sustained transgene expression* and *Evading silencing for sustained transgene expression*).

Endosome escape and nucleocytoplasmic trafficking

Methods to promote the release of complexes from endosomes largely rely on the composition of the carrier and its inherent reactive properties to disrupt of the enveloping membranes. This process can follow one of the following described mechanisms. (i) The flip-flop mechanism suggest that increasing acidity in the endosome lumen causes the anionic phospholipids to flip inside out, inverting the intraendosomal side of the membrane to the cytoplasmic side. The formation of charge neutral pairs between membrane and lipocarrier leads to membrane destabilization, allowing the lipoplex to penetrate into the cytoplasm, simultaneously dissociating pDNA from the cationic lipid (D Hoekstra et al., 2007; Wasungu & Hoekstra, 2006; Mui et al., 2000). (ii) In the proton sponge effect, endosomolysis is promoted through adsorption of H^+ by amine groups found on cationic polymers. Protonation induces an inflow of ions and water into the endosome lumen, leading to a gradual increase in osmotic pressure, swelling the vesicle, causing the membrane to destabilize and eventually rupture (Kichler et al., 2007; Akinc et al., 2005; Sonawane et al., 2003). (iii) For pore-forming cationic amphiphilic peptides, binding to lipid bilayer reduces the line tension in the membrane and causes the internal membrane tension to create pores in the lipid membrane, allowing the cargo to escape through the pores (Jenssen et al., 2006; Huang et al., 2004). (iv) Fusogenic peptides, on the other hand, can undergo conformational changes upon pH drop, which triggers the molecule to adopt a conformation suitable for fusion with the lipid bilayer (Marsh & Helenius, 1989). For example, haemagglutinin, a peptide from the influenza virus coat, has an anionic hydrophillic coil at physiological pH, but adopts a hydrophobic helical conformation in the acidic pH inside the endosome, which allows the helical structure to embed into the membrane (Weis et al., 1990).

In addition to the inherent endosomolytic activity of the carriers, membrane disruptive components from a number of sources have been grafted onto the carriers to further enhance cytosolic release. These functional components have been derived from viruses (Subramanian et al., 2002; Wagner et al., 1992; Lewin et al., 2000; Morris et al., 1999), bacteria (Lorenzi & Kyung-Dall Lee, 2005; Kullberg et al., 2010; Walton et al., 1999; G Saito et al., 2003), plants (Jan Sun et al., 2004; Vago et al., 2005), mammalian (or endogenous) (Foerg et al., 2005; Krauss et al., 2004; Ogris et al., 2001; Dempsey, 1990), as well as synthetic or recombinant peptides (Abes et al., 2008; Sang-Hyun Min et al., 2006; Xu-Li Wang et al., 2007; Tu & Ji-seon Kim, 2008; Lundberg et al., 2002; Magzoub et al., 2006; Asayama et al., 2004; Hatefi et al., 2006; Fernandez-Carneado et al., 2004; del Pozo-Rodríguez et al., 2009). Attachment of these endosome-disruptive components is accomplished by either covalent linkages or through attractive interactions with the complex surface. Covalent linkage provides a more secure form of transit, ensuring the endosomolytic component arrive with the carrier in the subcellular compartment. However, there is often a minimum substitution density required to sufficiently induce membrane destabilization and high degree of carrier modification may diminish the DNA binding capacity, causing the complexes to be less stable, similar to the problems faced with PEGylation and ligand-functionalization. Conjugation of functional devices could also lead to changes in the overall size of the complexes, which could in effect, redirect the uptake to a pathway that may not be allow the endosomolytic component to exert its activity. Finally, the functional component may be buried inside the core complex, rendering a spatial configuration that is suboptimal to the activity of the conjugated device. Even though endosome entrapment is widely regarded as a rate-limiting step, the arrival at this barrier may merely be a consequence of misdirected step (i.e. uptake down a transgene inactive pathway due to change in size). Some studies have demonstrated that

endosome escape was not the rate limiting step (Hama et al., 2006), and that limited nucleocytoplasmic movement might impede the gene delivery.

Movement of endosomes across the cytoplasm is facilitated by microtubules along the cytoskeleton network which extends from the plasma membrane to the microtubule organizing centre (MTOC) located in close proximity to the nucleus (Caviston & Holzbaur, 2006; Hasegawa et al., 2001; Suh et al., 2003). Movement along the MTOC appears to be bi-directional - cargo may oscillate between the perinuclear region and the cell periphery (Kulkarni et al., 2005; Kural et al., 2005). Thus, the timing of endosome release relative to movement along the microtubules may be critical to subsequent nuclear uptake. If complexes are released distal to the nucleus, diffusive mobility of large pDNA (>2 kb) may be restricted in the crowded cytoskeleton mesh, limiting nuclear uptake (Lukacs et al., 2000). If endosome escape coincides with localization around the pernuclear region, then cycle-dependent nuclear import may be enhanced. This prompts for a method to induce endosome escape that can be spatially triggered to the vicinity of the perinuclear region. Alternatively, pDNA movement can be facilitated by signal peptides (Pandey, 2010), lipids (Fukata & Fukata, 2010) or adaptor proteins for sorting, targeting and anchoring to specific subcellular compartments (Rajendran et al., 2010). We have recently demonstrated that a lipid-modified polymeric carrier exhibited enhanced trafficking to the nuclear periphery (Hsu et al., 2011), although it was not known whether the trafficking capability is an active process specific to the lipid moieties or a passive event that saw anchoring of the lipid group to nuclear membrane through hydrophobic interaction. Signal peptides are arguably the most widely used approach for subcellular trafficking. Even viruses have evolved to use specific peptide sequences to facilitate its interaction with dynein to move along the cytoskeleton network, which extends into the nucleoplasm, thereby gaining entry into the nucleus (Döhner et al., 2005; Radtke et al., 2006). Adapting endogenous mechanisms for protein import such as conjugating NLS to either the gene carrier or to the DNA vector has proven to be a viable strategy for promoting the nuclear uptake of complexes (Hébert, 2003; Cartier & Reszka, 2002; Nagasaki et al., 2003). However, the positive effect of NLS has not been consistently demonstrated among research groups (Wagstaff & Jans, 2009). The problem, which is shared by conjugated carriers, is often the lack of proper spatial presentation of the ligand to its receptor. But more critically is the fact that other cellular barriers were not simultaneously tackled, which may inadvertently undermine the benefits of NLS. In that regard, a multifunctional gene delivery system that can incorporate all of the barrier-evading moieties in a spatially coordinated order would be ideal in overcoming multiple rate-limiting steps in the transfection pathway.

Enhancing transfection efficiency using genetic and epigenetic elements

Trafficking and modulating targeting specificity through DNA sequences

Nucleocytoplasmic transport of pDNA can be indirectly facilitated by the NLS/Importin pathway through specific sequence elements called the DNA nuclear targeting sequences (DTS), which contain binding sites for ubiquitous transcriptions factors (e.g. AP1, AP2, NF-kB, Oct1, TEF-1 (Miller & Dean, 2009). While the activity of transcription factors are located in the nucleus, most reside in the cytoplasm, either as a conclusion of protein synthesis or as a mean to regulate their activities (e.g. NF-KB, NFAT, Glucocorticoid receptors). The cytosolic location of the transcription factors allows them to bind to DTS on the pDNA. Upon stimuli triggered activation of metabolic events, the associated transcription factors undergo conformation changes, exposing buried NLS peptide, allowing nuclear transport receptors to bind and shuttle the protein/pDNA complex into the nucleus (Dean, 1997; Dean et al., 2005).

DTS-mediated pDNA nuclear import can be further refined to drive tissue- or physiology-specific transgene expression. Rather than DTS that are bound by ubiquitous transcription factors, tissue-specific DTS can be employed to limit binding to tissue-specific transcription factors. Nontarget cells devoid of tissue-specific transcription factor would not be able to co-import pDNA for subsequent transcription. Indeed, DTS isolated from smooth muscle gamma actin (SMGA) promoter and surfactant promoter C (SP-C) have been shown to direct expression specifically to smooth muscle cells and type II pneumonocytes, respectively (Degiulio et al., 2010; Miller & Dean, 2008). However, tissue- or physiologically-specific pDNA import may be limited to certain cell types or physiological conditions since it requires that DNA-binding transcription factor or co-factors bearing the NLS to be regulated via cytosol sequestration prior to activation. In addition, some transcription factors may not be able to display NLS when bound to DNA, either due to steric hindrance or conformational changes and thus may not be suited to binding both DNA and the nuclear import machinery at the same time (Miller & Dean, 2008; Chan & Jans, 2001). Nevertheless, DTS provides a practical mean to shuttle pDNA into the nucleus without the aid of a gene carrier.

Perhaps the most widely used approach to limit expression in target cells is through tissue-specific promoters. Incorporating tissue-derived regulatory elements limit transcriptional activity to only targeted cells that harbor compatible transcription factors, similar to tissue-specific DTS. These tissue-specific promoters contain both proximal promoters elements and distal regulatory DNA sequences (e.g. locus control regions, enhancers, and introns) to drive targeting by enhancing tissue-specific expression and/or repressing nontissue-specific expression (Toscano et al., 2011). Naturally derived promoters

from albumin, human 1 antitrypsin (hAAT), creatine kinase, insulin, (Follenzi et al., 2002; Le et al., 1997; Gregorevic et al., 2004; Londrigan et al., 2007) as well as synthetic promoters have been constructed to enhance specificity and transgene expression in liver, muscle, and epithelium (Mount et al., 2002; Salva et al., 2007).

Tissue-specific or physiologically-regulated promoters also provide levels of transgene expression that are closely matched to wild type phenotypes. This is critical for some diseases, which not only require delivery and expression of the therapeutic product in specific target cell type but also the correct pattern and the level of expression. An example of this requirement is in the Wiskott–Aldrich syndrome, an X-linked genetic disorder caused by mutation in the WAS gene. Gene therapy for WAS require precisely modulated expression of the WAS protein in the whole hematopoeitic lineage. Ectopic delivery to nonhematopoietic cells combined with WASP overexpression can interfere with cytoskeleton function, reducing cell viability and contribute to cancer cell invasion (Toscano et al., 2008; Yamaguchi & Condeelis, 2007). DNA fragments isolated from the WAS gene proximal promoter in hematopoeitic cells was shown to be sufficient to drive strong hematopoeitic-restricted expression with a concurrent reduction in deleterious effects associated with ectopic expression (Martín et al., 2005; Dupré et al., 2004). Thus, tissue-derived promoters not only promote spatially restricted expression, but concurrently provide physiologically relevant level of expression in sensitive targets. While utility of tissue- and physiological-specific promoters is preferred over constitutive viral promoters, progress in this area has been slow due to the fact the promoter must be isolated from the gene of interest, and therefore needs to be custom tailored to a particular disease. But more critically, the expression pattern of the gene of interest may be regulated by epigenetic mechanisms (i.e. chromatin positioning, posttranscriptional silencing) and thus cannot simply be isolated from the genome. Regardless, this example illustrate a method of fine-tuning transgene expression using genetic elements that is more precise than conventional pharmacokinetic measures.

In addition to tissue-specific promoter, targeted expression can be indirectly enforced by suppressing expression in nontargeted cells using miRNA-mediated gene knockdown. Given the population and distribution of endogenous miRNA varies between tissues and differentiated cell lineage, the expression of transgene can be selectively regulated by harnessing the differential pattern of miRNA profile (Toscano et al., 2011). This strategy has been implemented by incorporating a miRNA recognition elements (MREs) to the pDNA construct (Kelly & Russell, 2009); nontargeted cells which express miRNA specific to the MREs would inhibit the expression of the transgene mRNA, while targeted cells lacking the miRNA would allow transgene mRNA to be translated (Brown et al., 2006; 2007). Papapetrou et al., applied this approach to construct a lentiviral vector encoding

a chimeric antigen receptor tagged with MRE for mirR-181a. mirR181a expression is elevated in developing thymocytes but suppressed in post-thymic T cells. Utilizing this difference in intracellular mirR181a concentration, the construct was able to selectively transfect post-thymic resting and activated T cells, but not of developing T cells, to restore self-reactive TCR, which could confer anti-tumor activity for cancer immunotherapy (Papapetrou et al., 2009).

These strategies focus on targeted expression rather than targeted delivery, whereby selectivity in transfection is controlled by genetic elements. However, this approach still involves system wide delivery to all cell lines, and it could lead to nonspecific reactivity to carriers and nucleic acids. A combinatorial approach incorporating both receptor-ligand assisted delivery and tissue-delimited genetic elements in expression cassettes could work in concert to exponentially enhance the specificity of targeted gene therapy.

Enhancing the level and duration of transgene expression

Pharmacokinetics approach to enhancing transgene expression is typically achieved by increasing the intranuclear concentration of exogenous nucleic acid through optimization of delivery system, to increase the number of templates available for transcription. This approach can be technically limiting when a saturation point is reached and more intranuclear DNA does not equate to a linear return in expression. Modulating transgene expression through genetic elements provides a viable option to enhancing expression while concurrently reducing the need for high concentration of carrier/pDNA complexes. Methods to enhance transfection efficiency through genetic control elements involve the addition of positive regulators, as well as removal of inhibitory elements. Genetic elements employed in this regard include promoters (Butler & Kadonaga, 2002), enhancers (Blackwood & J T Kadonaga, 1998), locus control region (LCR; Bulger et al., 2002), scaffold/matrix attachment regions (S/MAR; Bode et al., 2000), insulators (Furlan-Magaril et al., 2011; Gomos-Klein et al., 2007) and removal of nonmammalian sequences.

Promoter strengths are dependent on two factors, namely, (i) consensus binding motif and (ii) activity and concentration of endogenous transcription factors, which can depend on the cell type as well. Promoters with a higher percentage of homology to the consensus sequences are more efficient at recruiting RNA polymerase that has a faster rate of elongation, which clears the binding sites for the next cycle of transcription complex (Brunner & Bujard, 1987). The most common constitutive promoters for mammalian expression are derived from viruses, such as cytomegalovirus (CMV), rous sarcoma virus (RSV) and simian virus (SV40). However, viral sequences can induce immune response and become attenuated in the long term (Weeratna et al., 2001). Nonviral promoters derived from human elongation

factor 1a (EF1a), human polyubiquitin C (UbC), and chicken β actin/CMV enhancer, phosphoglycerolkinase (PGK) promoter (Pringle et al., 2007; Walther & Stein, 1996) are common alternatives used in mammalian expression systems. It should be noted that the activity of the promoters is largely dependent on the cell type, which dictates the abundance of transcription factors that are compatible with those promoters (Changyu Zheng & Baum, 2005; Qin et al., 2010). Thus, the optimal promoter would dependent on the cell line and the application need (i.e. high expression vs. sustained expression) and may need to be determined empirically through screening and comparative evaluation of several types of promoters.

Enhancers are *cis*-acting regulatory elements typically incorporated into an expression construct either downstream or upstream of the promoter to increase expression by facilitating efficient recruitment of co-factors for the transcription complex. The most widely cited enhancer is derived from the CMV immediate early genes (CMV IE; Foecking & Hofstetter, 1986) as a hybrid cassette combined with a mammalian promoter (Magnusson et al., 2011). But mammalian derived enhancers such as the Apolipoprotein (ApoE), (Le et al., 1997), immunoglobulin (Laurie et al., 2007), microglobulin, and prothrombin (Yasuda et al., 2007) have all demonstrated enhancement in transgene expression. The overall performance output of an enhancer-promoter pair will likely depend on the cassette combination and the cell type (Schlabach et al., 2010).

Enhancement of posttranscriptional processing of newly synthesized transgene mRNA is another way to improve expression without modifying the carriers. The 3′-end of the expression cassette typically contains three functional sequence elements: polyadenylation (polyA) site, cleavage signal and transcription termination. The polyA tail is functionally critical for nuclear export and translation (Jackson & Standart, 1990) as well as stability of mRNA (Schambach et al., 2000). Stable transcripts have a slower turnover rate and accumulate to a higher concentration, allowing more protein to be synthesized. Several polyA sites derived from bovine growth hormone (Goodwin & Rottman, 1992), mouse β-globin (N B Pandey et al., 1990), HSV thymidine kinase gene (Schmidt et al., 1990), woodchuck post regulatory element (Zufferey et al., 1999), and SV40 early transcription unit (van den Hoff et al., 1993) have been used in mammalian expression vector. Similarly, transcription termination site is critical for termination of transcription and the dissociation of RNA polymerase (RNAP) from the DNA, minimizing promoter occlusion (Proudfoot, 1986) and enhance the rate of transcription cycle to allow RNAP to become available for a new round of transcription (Kim et al., 2003).

These *cis*-acting regulatory elements described above rely on the recruitment of endogenous *trans*-acting factors for enhanced expression; the latter may ultimately become limiting factor since it depends on cell physiology. Alternatively, both *cis* and *trans* components can be provided exogenously to create a two-step transcriptional amplification system (TSTA). TSTA utilize an expression vector containing a tissue-specific or physiologically-regulated promoter to drive the expression of a transcriptional activator, which then binds to the upstream regulatory region of a second expression construct to enhance the promoter-driven expression of the therapeutic gene (Arendt et al., 2009). The regulatory elements in the therapeutic gene construct can also be inserted into the activator construct to create a positive feedback loop where the transcriptional activator enhances its own expression (Ochiai et al., 2010). The transcriptional activator in this system is typically a recombinant fusion protein between the DNA binding domain of a transcription factor from one source and the transcriptional activation domain from another source (Ochiai et al., 2010). This allows the recombinant transcriptional activator to be modularized for adaptation to a wide range of promoters and activation level. TSTA has been shown to greatly enhance tissue-specific expression over the standalone use of tissue-specific promoter (Dzojic et al., 2007; Hattori & Maitani, 2006; Zhang et al., 2002).

Plasmid retention for sustained transgene expression

In order for exogenous nucleic acids to be mitotically stable through cell division, expression cassettes need to be capable of both replicating autonomously as an extra-chromosomal element as well as harboring a mechanism for nuclear retention. Nonintegrating episomally maintained expression cassettes can vary in size and autonomy, and ranges from self-replicating pDNA to fully functional minichromosomes (MC). Self-replicating plasmids derived from episomally maintained animal viruses such as SV40, bovine papillomavirus (BPV) and Epstein-Barr Virus (EBV) utilize both *cis*- and *trans*-acting factors as replicon and nuclear retention factors. The *cis*-acting sequence elements provide an origin of replication where it is bound by *trans*-acting protein factor, which replicates the vector through either a replicase type activity or through the recruitment of the core replication machinery (Friedrich et al., 2005; Ravnan et al., 1992; Wang & Sugden, 2005). *Trans*-acting factors can further facilitate binding to metaphase chromosome, providing a piggy-back mechanism to enhance nuclear retention and mitotic stability of the vector (Zheng et al., 2005; Ito et al., 2002). Not only are the oriP/EBNA1-based episomal vectors able to extend the expression timeframe from days to months, they also exhibit enhanced nuclear import and enhanced transgene expression (Mazda, 2002; Aliño et al., 2003; Min et al., 2003; Sclimenti et al., 2003). Despite their high level and persistence of expression in animal models, hybrid vectors with viral replicons currently have limited utility in human gene therapy due to their cell transforming and immune stimulating nature (Valls et al., 2003; Strayer & Zern, 1999; Lampela et al., 2001; Taylor et al., 2004; Humme et al., 2003).

The second class of episomally maintained expression cassettes is derived from mammalian sequences and does not require *trans*-acting factors for maintenance. Human ACs (HAC) and MCs have been applied to achieve long-term expression in a variety of settings (Katoh et al., 2004; Vanderbyl et al., 2005; Kennard, 2011; Auriche et al., 2002). HAC are constructed by a bottom-up approach where individual constituent DNA elements, such as telomere, centromere, replication origin, are retrofitted into a yeast or bacterial-based AC vector (Mejía et al., 2002; Grimes et al., 2002). MC on the other hand, are constructed by a top-down approach via the de-construction of natural chromosome by irradiation to introduce double strand break, or by telomere fragmentation to generate size-reduced derivative (Basu & Willard, 2005; Kakeda et al., 2005). The major advantage of HAC and MC over other episomal vectors is their stability throughout mitotic and meiotic events, without compromising genomic integrity. HAC also has essentially unlimited size insert capacity that can accommodate both the transgene and all of its regulatory elements to preserve wild type expression profile (Ehrhardt et al., 2008; N Suzuki et al., 2006; Carlson et al., 2007). Despite their theoretical superior features, ACs are currently limited to a few niche applications. For one, the sizes of both HAC and MC are in the range of megabase pair, which requires specialized delivery protocols such as the microcell-mediated chromosome transfer or pronuclear injection (Killary & Fournier, 1995). Even with these delivery approaches, the establishment of stably expressing cells is difficult at present. Furthermore, insertion of transgene into the AC vector require site-specific recombination, which can be technically cumbersome, making AC difficult to produce for widespread application (Werdien et al., 2001; Kuroiwa et al., 2000).

The third class of episomally maintained system is based on the inclusion of sequences found in the scaffold matrix attachment region (S/MAR). In higher eukaryotes, replication of the genome is tightly associated with the nuclear matrix; the onset of S-phase is often preceded by the binding of the replication origin to the nuclear scaffold or nuclear matrix (Cook, 1999). The nuclear matrix/scaffold has been implicated to function in genome organization, gene expression, and transcription regulation (Laemmli et al., 1992; Schübeler et al., 1996). S/MARs are AT-rich sequences that define the boundary of independent chromatin domain through the formation of chromatin loops (Bode et al., 2000). Based on these characteristics, S/MAR sequences have been cloned into expression cassettes to assess its role on transgene expression. The prototype vector pEPI-1, demonstrated persistence in a wide range of mammalian cell lines (Papapetrou et al., 2006; Schaarschmidt et al., 2004) and was even capable of propagating meiotically to generate transgenic animals (Manzini et al., 2006). The cassette replicates once per cell cycle during early S-phase with initiation starting at random sites throughout the cassette. Sustained reporter gene expression and long-term propagation of the cassette appears to depend exclusively on the transcription unit 5′ to the S/MAR sequence (Stehle et al., 2003; Jenke et al., 2004). Subcellular fractionation analysis determined that S/MAR-containing expression cassettes bind to the nuclear matrix by interaction with the matrix protein SAF-A and associate with chromosome during mitosis (Baiker et al., 2000; Jenke et al., 2005). Despite the improved nuclear retention and mitotic stability imparted by S/MAR sequences, transgene silencing as a result of promoter inactivation seems unavoidable in certain cells (Papapetrou et al., 2006). Furthermore, the establishment of stable clones from transfected cells is at present, inefficient, with success rate ranging from 0.5 to 5% (Ehrhardt et al., 2008). It is becoming increasingly clear that the generation of stable transgenic cell line using episomal vectors is not exclusively dependent on primary DNA sequence or particular chromatin make-up, but rather, involves a series of stochastic epigenetic events yet to be described (Jackson et al., 2006). Nevertheless, the size, safety and activity afforded by S/MAR-based vectors appear to be the most promising for facilitating long-term expression in nonviral delivery.

Evading silencing for sustained transgene expression

Removal or replacement of undesirable sequences can further contribute to long-term expression of the transgene. This involves either replacement of attenuated promoters, removal of nonexpressing bacterial derived vector backbone and/or inclusion of insulator elements to prevent chromatinization. Highly active virally derived promoters, such as the intermediate early gene promoter from CMV, are known to be subjected to epigenetic silencing. The abundance of cytosine-guanine repeats (CpG) within the promoter sequence is prone to methylation by cellular methyl transferases, which attenuate its transcriptional activity (Brooks et al., 2004). Constitutively expressed or tissue-specific promoter of mammalian origins, on the other hand, are less prone to hypermethylation and have been shown to be successful in avoiding transcriptional silencing (Magnusson et al., 2011; Wooddell et al., 2008; Gill et al., 2001; Nguyen et al., 2008).

The highly immunogenic CpG sequences (discussed in the section *Immune responses to nucleic acid and complexes*) can abolish long-term transgene expression through induction of innate and humoral responses. It can also render the pDNA in a repressed chromatin state through binding with heterochromatin-associated histones. Extending the duration of transgene expression through reduction of immune reactivity to CpG sequences can be conferred by either methylation of the nucleotides or removal of the bacterial backbone (Hodges et al., 2004; Reyes-Sandoval & Ertl, 2004; Huang et al., 2009). The bacterial derived backbone can be excised through site-specific integrase-mediated intracellular recombination technology (Mayrhofer et al., 2008; ZY Chen et al., 2003). The resulting truncated minicircle DNA showed a more

robust and persistent transgene expression than its full-length parental molecule *in vivo* (Osborn et al., 2011).

Shielding of pDNA heterochromatinization can be accomplished through incorporation of genetic insulators (ZY Chen et al., 2004). Genetic insulators are boundary elements that can act as enhancer-blocker and silencing-barrier to shield transcriptional units from being affected by neighboring regulatory elements, such as the spread of heterochromatin (Raab & Kamakaka, 2010). Its incorporation in expression cassette provides a mean to maintain the transgene in an open euchromatin state to sustain transcriptional activity (Furlan-Magaril et al., 2011; Macarthur et al., 2012). However, it is important to note that genetic insulators act as boundary elements, thus their activity can be both inductive and repressive - the directionality of insulator activity may depend on neighboring genes and nearby regulatory elements. Another genetic element that has been used in transgene construct is the recently identified locus control region (LCR). LCR are transcriptional regulators with enhancer activity relayed to genes linked in *cis* and have the ability to overcome position effect to maintain an open chromatin structure at the domain level (Li et al., 2002). Inclusion of the human β-globin LCR in an EBV-based vector was shown to extend the expression of the β-globin transgene for up to 2 months in the absence of selection (Chow et al., 2002). Furthermore, a combinatorial use of the cHS4 insulator and the α globin LCR (HS40) in retroviral vectors resulted in long-term expression of human gamma globin gene in a mouse bone marrow transduction and transplantation model, with retention rate of transfected cells increased from 2–5 to 49% (Emery et al., 2002). Numerous LCR has been discovered to date (Bonifer, 2000). However, LCR exhibit tissue-specificity and thus a universal construct may not be feasible to wide spread application, though, this may be viewed as an advantage to further maintain targeted expression in a tissue-specific manner.

Concluding remarks and perspectives

Current design of carriers and expression cassettes has been limited to a few functional elements so far, tackling one or two rate-limiting steps at a time, which may not provide as significant gain as anticipated since other inhibitory factors are still acting negatively on the process. In its fully realized form, nonviral gene delivery systems will need to evolve into multifunctional and perhaps multilayered nano-devices. A multilayer device is based on the premise that the gene carrying particle is enclosed and shielded by layers of bioresponsive elements where each layer dissembles systematically upon completing its intended function to circumnavigate intracellular barriers. The layer-by-layer assembly could involve the iterative coating with oppositely charged polyelectrolytes until layers of alternating cationic and anionic polymers are assembled. The functional devices would be embedded either in staggered orientation or interwoven within

layers (Jewell & Lynn, 2008; Leary & Prow, 2005). The outermost layer would serve to stabilize the particle from aggregation and prevent undesired adsorption/opsonization, while enabling selective adsorption to targeted cell surface for facilitated uptake. The inner layers would harbor an "endosomolytic domain" to promote the release of the core particle (Haglund et al., 2009), which would harbor the pDNA or other functional nucleic acids. The latter could provide additional trafficking and activity modulation capability to enhance the level and duration of transgene expression. This two-tiered, multicomponent drug delivery system could provide a method of targeting and activity modulation that is unmatched by conventional pharmacokinetic means.

It is becoming increasingly clear that the design of carriers as well as functional nucleic acids could be merged into one integral protocol. Nucleic acid molecules are different from the conventional pharmaceutical agents, in that they can provide both supplementary and complementary delivery and trafficking capabilities to work in concert with the gene carrier. Targeting to specific cell types can be mediated by the carrier through ligand-receptor binding, and further enhanced by genetic elements to facilitate tissue-specific expression while suppressing activity in nontargeted cells. In terms of supplementary activity, the carrier can circumnavigate the endolysosomal pathway using its membrane-disruptive components; subsequent trafficking through the nucleocytoplasmic pathway is then supplemented by nuclear targeting sequences incorporated into the DNA cassette. Where the two components work exclusively from each other is at either ends of the transfection pathway, namely extracellular and subnuclear domains. A carrier is required to package and condense the nucleic acid in order to protect and promote its uptake, which the nucleic acid does poorly on its own. On the other hand, subnuclear events such as transcriptional activity, replicative distribution, nuclear retention, and chromatin positioning of the expression system are beyond the capabilities of the carrier. Thus, incorporating genetic and epigenetic elements into the expression cassettes may overcome some of the hurdles currently limiting the advancement of nonviral gene carrier-assisted delivery systems (Figure 4).

Much of the focus on nonviral gene therapy has centered around the efficiencies of the delivery system (i.e. targeting, uptake and specificity), which is measured by the level of transgene activity. However, it is important to note that optimizing targeting and delivery efficiency are not the only means to enhance therapeutic outcome. Consider the gene therapy approach to generate induced pluripotent cells (iPSC). Initial proof-of-concept demonstrated the feasibility of generating iPSC from fibroblast via the expression of four stem cell transcription factors, Oct3/4, Sox2, Klf4 and c-Myc (OSKM; (Maherali et al., 2007; Wernig et al., 2007). The efficiency of deriving iPSC via augmented expression of transcription factors is typically low (~0.1%). Considerable efforts have gone into
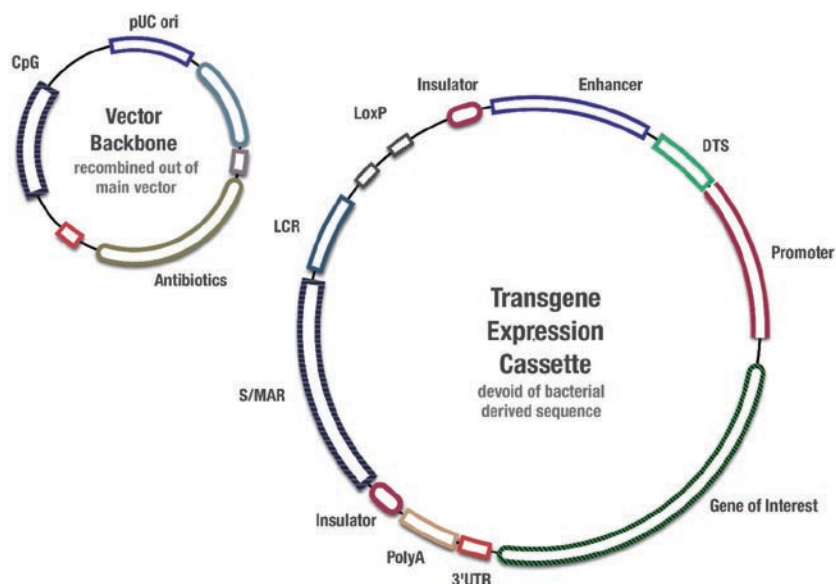
Figure 4. A hypothetical map of a long-term mammalian expression vector. In this construct, expression of the gene of interest is driven by a tissue-specific promoter with the activity complemented by an enhancer element. The promoter contains transcription factor binding sites, which can double as nuclear DNA targeting sequence (DTS) for nuclear import. Noncoding elements such as 3′-UTR and polyadenylation sites further enhance the stability of mRNA, prolonging its cytoplasmic half-life during translation. S/MAR sequences promote the replication and nuclear retention of the plasmid throughout cell division while insulator and LCR shield the transgene from heterochromatin domains. Finally, loxP or similar repeat sequences mediate the removal of bacterial derived vector backbone through site-specific recombination to minimize immune reactivity to the vector.

improving the efficiency by adjusting parameters such as delivery mode (Lee et al., 2011; Wang et al., 2011; Byrne et al., 2009), choice of starting cell type (Aasen et al., 2008), culture conditions (Marson et al., 2008; Dravid et al., 2005), and cocktail of reprogramming transcription factors (Zhao et al., 2008; Hanna et al., 2009). However, several groups have recently demonstrated that mouse and human cells can be reprogrammed by transfection with mature double-stranded miRNA (Miyoshi et al., 2011). Rather than augmenting OSKM activities to induce expression of pluripotency factors, miRNA (miR302/367 clusters) was applied to suppress molecular pathways that are involved in maintaining cells in a differentiated state, which essentially acted as antagonists to reprogramming efforts. Once these differentiation factors were removed, the cells were allowed to de-differentiated back to an embryonic-like state. This approach not only led to reprogramming of somatic cells, but the efficiency of deriving iPSC colonies was increased by more than 100-fold in comparison to OSKM (10 vs. 0.1%; Anokye-Danso et al., 2011).

This experience highlights the diversity of nucleic acid therapeutics, which readily provides alternative approaches to challenging therapeutic manipulations. In that sense, gene augmentation or gene knockdown is not limited to supplementing deficient products or removing "toxic" mediators, but as control elements that can work in parallel or in conjunction to reengineer pathways for a desired therapeutic outcome. The process of identifying alternative interventions, however, will first require a thorough understanding of the cellular and molecular processes involved. As

well, quantitatively speaking, comparative evaluation of therapeutic efficacy between different modes of genetic manipulations may not be as direct as comparing between gene delivery systems. Understanding the molecular mechanism of pathogenesis would be key to designing a gene therapy protocol with high therapeutic benefits and low ectopic effects. Ultimately, gene delivery systems may need to be optimized on a disease-by-disease basis, where both the activity and specificity of the gene carriers/nucleic acid molecules are adapted for the target cell type as well as the modality of genetic modifications.

Acknowledgement

Declaration of interest

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