Fall 12-4-2015

HIGH THROUGHPUT SCREENING OF PRIMING CANDIDATES FOR IMPACT ON NONVIRAL GENE DELIVERY

Albert L. Nguyen
University of Nebraska-Lincoln, aln15083@gmail.com

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HIGH THROUGHPUT SCREENING OF PRIMING CANDIDATES FOR IMPACT ON NONVIRAL GENE DELIVERY

By

Albert L. Nguyen

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Agricultural and Biological Systems Engineering

Under the Supervision of Professor Angela K. Pannier

Lincoln, Nebraska

November 2015
Priming, in the context of nonviral gene delivery, is the treatment of cells with a compound prior to gene transfer that enhances transfection efficiency and/or transgene expression. Essentially, it is the application of an adjuvant approach to gene delivery. Effective transfection strategies may require priming to compete with the efficiency of viral transduction in order to achieve clinically relevant efficiency and expression in vivo.

To search for priming compounds, a high throughput screen of the NIH Clinical Collection was performed using 25kDa b-PEI, an EGFP/luciferase plasmid, and HEK293T cells. The EGFP reporter was multiplexed with Hoechst 33342 and Resazurin fluorescence to measure transfection efficiency, transgene expression, proliferation, and viability of the cells in response to priming with the screened NCC compounds and PEI transfection. The screen identified dozens of compounds and several compound classes that appear to affect transfection through modulation of mitochondrial dysfunction in response to the toxicity of PEI complexes. With further investigation and development, the mechanisms by which these and other priming compounds are affecting gene transfer can be understood and applied towards the development of efficient gene delivery strategies.
ACKNOWLEDGEMENTS

There are many people to whom I owe a debt of gratitude for their role in the work and in my life while I completed this thesis research:

Jared Beyersdorf, formerly an undergraduate in the Pannier Lab, performed a preliminary screen for transfection priming compounds, the protocols from which the screening methods in thesis research were developed. He’s also a cool guy who is on to bigger and better things.

My fellow graduate students and staff in the BSE department at UNL, especially the Pannier Lab members (Sarah Plautz, Amy Mantz, Eric Farris, Andrew Hamann, and Taylor Laughlin), who were most often helpful, and happy diversions otherwise.

Drs. Mark Riley and Nicole Iverson for their comments and guidance, and patience, as committee members for my thesis and defense. Dr. Deepak Keshwani for graciously allowing me to use his fluorescence plate reader. Also, Danielle Shea, for helping me with flow cytometry that doesn’t appear in this work.

I’m most extremely grateful to my advisor, Dr. Angela Pannier, for having faith in me, and giving me a chance to complete this Master’s thesis research. I’ve learned a lot from working with you, and I hope I continue to live up to your expectations.

Finally, thanks to my family for supporting me on my wandering path. I love you so much.
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LIST OF ACRONYMS

- °C: Degrees Celsius
- ADME: Absorption, distribution, metabolism, and excretion
- ANOVA: Analysis of variance
- ASO: Antisense oligonucleotide
- ATP: Adenosine triphosphate
- BCA: Bicinchorinic acid assay
- Ca²⁺: Calcium ion
- CatL: Chloramphenicol acetyltransferase
- CMV: Cytomegalovirus
- CO₂: Carbon dioxide
- ddH₂O: Double distilled water
- DMEM: Dulbecco's modified eagle medium
- DMSO: Dimethyl sulfoxide
- DNA: Deoxyribonucleic acid
- DTS: DNA targeting sequence
- EDTA: Ethylenediaminetetraacetic acid
- EGCG: Epigallocatechin gallate
- EGFP: Enhanced green fluorescent protein
- EGFPLuc: Enhanced green fluorescent protein and luciferase fusion protein
- FACS: Fluorescence activated cell sorting
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence or Förster resonance energy transfer</td>
</tr>
<tr>
<td>GABAA</td>
<td>Gamma-Aminobutyric acid A receptor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney 293T cell line</td>
</tr>
<tr>
<td>hMSC</td>
<td>Human mesenchymal stem cell</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HSV TK</td>
<td>Herpes Simplex Virus Thymidine Kinase</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screen</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LacZ</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>LAR</td>
<td>Luciferase assay reagent</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NCC</td>
<td>NIH Clinical Collection</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PLL</td>
<td>Polylsine</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RGB</td>
<td>Red Green Blue</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RLB</td>
<td>Reporter lysis buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SARS</td>
<td>Severe acute respiratory syndrome</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small/short interfering RNA</td>
</tr>
<tr>
<td>SLO</td>
<td>Streptolysin O</td>
</tr>
<tr>
<td>SSO</td>
<td>Splice switching oligonucleotide</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TIFF</td>
<td>Tagged Image File Format</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>VC</td>
<td>Vehicle control</td>
</tr>
<tr>
<td>WST-1</td>
<td>Water-soluble Tetrazolium salt</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
</tbody>
</table>
• \( \mu \text{L} \) Microliter
• \( \mu \text{M} \) Micromolar
CHAPTER 1

Pharmacological Priming, High Throughput Screening, and Drug Repurposing, in the Context of Nonviral Gene Delivery

1.1 Introduction

The purpose of this chapter is to provide context for the research performed for this thesis. After a brief introduction to the various methods of gene delivery, the primary barriers to successful nonviral gene delivery are presented. A literature review follows, which covers nucleic acid and cationic polymer design for transfection, pharmacological priming to enhance transfection, high throughput screening methods in general and in the context of gene delivery, and drug repurposing considerations in the search for new priming compounds.

1.2 Gene delivery

Gene delivery is the transfer of exogenous nucleic acids into cells to modify cellular gene expression. Modulation of gene expression is useful for studying biological mechanisms which genes control, and can potentially enable gene therapies for treatment of genetic disorders. There are countless applications in medical research that depend on the expression or silence of genes, and gene delivery methods are the tools which must be developed to achieve these functions inside target cells.
Sickle-cell anemia is an example of a genetic disorder which can possibly be treated by gene delivery [1-3]. In sickle-cell anemia, abnormal red blood cell morphology is caused by mutation to the beta chain structure of hemoglobin, causing hemoglobin polymerization. Correcting this mutation by gene therapy might be possible in different ways. One therapy might involve delivering a combination of RNA interference and DNA plasmid encoding correct hemoglobin to hematopoietic stem cells (HSCs) for simultaneous knockdown of faulty hemoglobin expression and expression of functional hemoglobin. Other methods might involve the correction (excision and replacement) of the faulty gene in vitro of harvested autologous HSCs for re-implantation, or perhaps involve targeted genome editing of HSCs in vivo. These gene therapies would all require safe and effective gene delivery. Towards this end, various methods of gene delivery have been extensively investigated.

1.2.1 Viral gene delivery

Gene delivery can be broadly classified into viral and nonviral methods. Viral methods employ viral vectors for gene transfer, while nonviral methods accomplish gene delivery by physical or chemical means. Viruses naturally transfer their genome into host cells for replication, a process which can be adapted for exogenous gene transfer [4] by insertion of the transgene into the viral genome. The viral vector then facilitates the gene transfer into cells, a process referred to as transduction. Transduction is highly efficient in vitro and in vivo, however, it is not trivial to modify a virus to deliver a specific gene of interest to target cells [5], and transduction is limited by design constraints and safety
concerns, a few of which include: size of the transgene [6], host immune response [7], and insertional mutagenesis [8]. Insertional mutagenesis is a risk for viral methods such as adeno-associated viral vectors, which integrate their genomes into the host genome. Transgene length is also a design constraint for many vectors, for instance, adeno-associated vectors are limited to a transgene capacity of around 4.8kb. Also, the host immune response to transduction can be potentially harmful to the host, and can decrease gene transfer efficiency in initial or repeated dosing as the host develops immunity to the viral vector [9]. Research is ongoing to develop viral vectors which overcome design constraints and decrease safety risks, however poor results in clinical trials (including subject fatality [10]) have prevented viral gene delivery from being utilized clinically. Even with limitations and concerns, viral gene delivery remains the gold standard for gene transfer efficiency against which all other gene delivery methods are measured.

1.2.2 Nonviral gene delivery

To surmount issues associated with viral gene delivery, nonviral methods of gene delivery have been developed. Physical methods of nonviral gene delivery can be employed which internalize the transgene into cells past the cell membrane. Bypassing the cell membrane can be accomplished by physical methods which include electroporation, magnetofection, ballistic insertion, ultrasound, and hydrodynamic delivery [6]. Electroporation and ultrasound disrupt the cell membrane allowing for nucleic acids to enter the cell, while ballistic and magnetofection methods use accelerated particles or magnetic nanoparticles to force nucleic acids into cells. Hydrodynamic
delivery is the highest efficiency nonviral gene delivery method [6], which involves rapid injection of a large volume of fluid containing the nucleic acid into blood vessels. This injection causes a transient high pressure in the local vasculature which disrupts capillary walls and cell membranes, allowing the nucleic acids to enter parenchyma cells surrounding the capillaries [11]. While many applications for these and other physical methods have been developed in vitro and in vivo, clinical applications of physical methods of gene transfer are limited because the physical disruption of cell membranes can be damaging, and targeting gene delivery to deep tissue with these methods requires invasive measures to provide direct access.

Chemical methods of nonviral gene delivery do not rely on physical disruption of the cell membrane. Instead, certain chemical compounds are able to associate with nucleic acids to form nanoparticles that facilitate the gene transfer into cells. For human and mammalian cells, this chemical nonviral gene delivery is referred to as transfection. Transfection compounds are generally cationic, allowing them to condense the negatively charged nucleic acid into nanoparticle complexes which can associate with cell membranes and be internalized by endocytosis [6]. There are several types of cationic materials, referred to as carriers, which can associate with nucleic acids to achieve gene delivery, including various natural and synthetic lipids and polymers. These materials can offer advantages over physical and viral methods of nonviral gene delivery, in safety, cost, fabrication, and flexibility in design and application. For instance nonviral carriers are not necessarily limited like viral vectors by size of the transgene cargo or inherent immunogenicity of the viral capsid. However, compared to viral methods, chemical
methods of nonviral gene delivery all suffer from lower transfection efficiency, especially in vivo [12].

1.2.3 Barriers to nonviral gene delivery

There are many barriers to successful transfection, with each step contributing to the overall low efficiency of chemical methods of nonviral gene delivery. Systemic barriers include serum stability, clearance, and targeting [13]. Serum stability refers to the ability of carrier nanoparticles to protect nucleic acids from degradation by nucleases in blood serum, avoid dissociation in serum buffer conditions, and avoid aggregation with other complexes or serum proteins. Clearance refers to the natural removal of exogenous material by the body, by macrophages [14] and through renal and hepatic filtration [15]. Targeting is required to specifically transfect the cells which require the gene transfer, as non-specific transfection will decrease transfection efficiency at the target cells and could have unintended consequences.

At the cellular level, the barriers to efficient transfection include internalization, endosomal escape, nuclear transport, nuclear import, and transcriptional and translational regulation [13] (illustrated in Figure 1.1 below). The carrier complexes need to be able to associate with the cell membrane and be internalized by various endocytosis pathways in order to enter the cell. The complexes then need to escape the endosome to avoid degradation in a lysosome. Escape into the cytosol followed by unpacking of the complexes is sufficient for RNA delivery, as RNA function is achieved in the cytoplasm, though nucleases in the cytoplasm will degrade the delivered RNA. Transgenes encoded
into DNA require transport into the nucleus for the DNA to be transcribed for
downstream expression through translation of mRNA to protein and protein folding.
However, diffusion is not sufficient to carry the DNA through the cytoplasm to and into
the nucleus, requiring active transport and import mechanisms [16]. At each step in the
gene delivery process, there is enzymatic degradation of the delivered nucleic acids. The
rate limiting barriers and degradation in the transfection process combine to result in the
overall low efficiency of chemical methods of nonviral gene delivery.

Figure 1.1 Illustration of the cellular barriers to chemical nonviral gene delivery:
internalization, endosomal escape, nuclear transport and import, and transcriptional and
translational regulation.

1.3 Nucleic acid design for nonviral gene delivery

The exogenous nucleic acids delivered to cells can be designed and modified to
improve the gene transfer efficiency and effectiveness of the gene expression or
silencing. Certain properties can be tuned to increase their effectiveness while other properties can be optimized to aid in overcoming cellular barriers. The tunable design parameters depend on the intended application, with the nucleic acid being single or double stranded DNA or RNA. For gene silencing, interfering RNAs or oligonucleotides are used, while for transgene expression, plasmid DNAs is suitable.

Targeted reduction in expression of a gene can be accomplished through RNA interference (RNAi) or oligonucleotides. Oligonucleotides are single stranded nucleic acids that can bind complementary mRNA sequences and prevent their expression through degradation by RNase H or steric blocking of translation [17], while RNAi involves short sequences of RNA which bind and degrade target mRNA through the RNA-induced silencing complexes (RISCs) [17]. It is thought that endogenous RNAi plays roles in both gene regulation and in defense against viruses and transposons [18]. The interfering RNA in RNAi are generally approximately twenty base pairs in length, double stranded in the case of small interfering RNA (siRNA) or single stranded in the case of micro-RNA (miRNA) [17-19]. Beyond internalization and endosomal escape, the primary determinants of gene silencing effectiveness by oligonucleotides and RNAi are the specific nucleic acid sequence and the delivered nucleic acid’s degradation rate, for which chemical modifications can be made to enhance their silencing activity and specificity, and improve nuclease degradation resistance [17, 19].

An advantage of RNAi and oligonucleotide nucleic acid delivery for gene silencing over nucleic acid delivery for transgene expression is that RNAi and oligonucleotides do not require translocation through the crowded cytoplasm to the nucleus and nuclear import, as plasmid DNA (pDNA) does. These requirements are
additional barriers to efficient transfection that must be overcome to achieve successful transgene expression. After endosomal escape, pDNA can remain complexed or unpack from the nanoparticles. Depending on the carrier material, both packed and unpacked pDNA have been observed in the nuclei of transfected cells [20]. The transport of pDNA to the nucleus is not by passive diffusion, but through active transport along cytoskeletal elements [21, 22].

Size and sequence of the pDNA influence the rate of transport and import into the nucleus, degradation, and transgene expression. Size of the pDNA has been shown to affect overall transfection efficiency and transgene expression, with pDNA of shorter length being more effective, however it is uncertain whether this is due to more efficient nuclear transport and import [23]. pDNA sequence has also been shown to affect coiling structure of the nucleic acid, with supercoiled pDNA being optimum for transfection [23]. Bacterial elements in the pDNA sequence can also cause an immune response within the cell. For instance, un-methylated CPG sequences are common in bacterial genomes, but not in mammalian DNA [24]. Toll-like receptors (TLRs) recognize bacterial elements like CPG sequences in pDNA and evoke an immune response at the cellular level that prevents sustained transgene expression by silencing of the transgene on the pDNA or elimination of the transfected cell [24]. To avoid this, CPG sequences can be minimized in plasmid design either by removal or methylation. For instance, “minicircles”, plasmids which don’t contain a bacterial origin or antibiotic resistance cassettes, can be used to remove all bacterial elements from the plasmid for more sustained expression of the transgene [24].
pDNA sequence has also been shown to influence the rate of active transport to the nucleus and nuclear import. Transcription factor binding sites on pDNA promoter and enhancer regions have been implicated in protein-DNA interactions, between transcription factors and pDNA, that shuttle pDNA within the cytoplasm along microtubules [16]. The nucleotide sequences that bind pDNA to transcription factors for transport are referred to as DNA targeting sequences (DTS) [16]. Transcription factors which translocate to the nucleus from the cytoplasm have nuclear localization signals (NLS) in their peptide sequence that facilitate import of transcription factors into the nucleus by importin proteins through Ran nuclear transport protein and the nuclear pore complex [16]. This nuclear import of transcription factors that possess NLS and are capable of binding pDNA at DTS may be the means by which pDNA enter the nucleus. Both cytomegalovirus (CMV) promoter and simian virus 40 (SV40) enhancer regions are known DTS that are commonly incorporated into plasmid designs and mediate nuclear transport and import through transcription factor binding in the cytoplasm followed by active transport along microtubules to the nucleus [16]. Though strong promoter regions in pDNA can compensate for low nuclear plasmid count [25], rendering optimizations of pDNA of limited value in vitro, low internalization rates in vivo will require such optimization in any nonviral gene delivery strategy which hopes to match viral transduction efficiencies and achieve sustained transgene expression.
1.4 Polyethylenimine carrier design in nonviral gene delivery

In addition to the properties of the nucleic acid cargo which generally determine the efficiency of a transfection strategy after endosomal escape, carrier properties and optimization are the most important determinants in the efficiency of a chemical nonviral gene delivery strategy in terms of facilitating delivery to and into cells. Nonviral gene delivery with cationic polymers was first demonstrated with polylysine (PLL) in 1987 [6], whose structure is illustrated in Figure 1.2 below. Its cationic nature allowed for the condensation of nucleic acids into nanoparticles which achieve gene delivery at low efficiency [26].

![Figure 1.2 Polylysine (PLL) [27]](image)

The discovery of transfection with polyethylenimine (PEI), another cationic polymer, followed in 1995, with PEI demonstrating the ability to successfully transfect in vitro and in vivo in multiple cell types [28]. The chemical structure of the PEI polymer is shown in Figure 1.3 below.

Cationic PEI also forms complexes with nucleic acids by electrostatic interactions, condensing with nucleic acids to form nanoparticles. PEI nanoparticles protect nucleic acids from degradation by nucleases and facilitate gene transfer into cells. In contrast with PLL, PEI has a higher cationic charge density that enables more efficient transfection, though at the cost of increased toxicity [6].
PEI chains have an amine group every three atoms along the polymer chain, with two carbon spacing between amine groups. Substitution of secondary amines with tertiary amine groups in a PEI chain will introduce branching into the polymer structure. The cationic nature of PEI arises from the protonation of the densely packed secondary and tertiary amine groups in aqueous conditions, as amine groups are weak bases. These positively charged ammonium groups are able to electrostatically associate with negatively charged nucleic acids to form the transfection competent nanoparticle complexes.

The cationic nature of PEI not only condenses nucleic acids into nanoparticles for protection, but also allows for association of nucleic acids with the cell membrane. Nucleic acids are negatively charged due to their phosphate groups, which are naturally electrostatically repelled from the negative charge of the phosphate groups in the phospholipid bilayer of the cell membrane. In order to enter the cell, the negative charge of nucleic acids must be neutralized. Cationic PEI not only neutralizes the electrostatic repulsion of nucleic acids with the cell membrane, the net charge of PEI complexes is positive, achieving electrostatic attraction to the cell membrane, allowing PEI complexes to associate with the cell membrane for endocytosis.

Endocytosis of PEI complexes occurs through clathrin and/or caveolar pathways, depending on cell type, and differing between branched and linear PEI variants [30].
Syndecan-dependent transport mechanisms involving filopodia have also been implicated in the cell membrane association and internalization process of nanoparticle complexes [31]. Endocytosis results in invagination of complexes which have associated with the cell membrane into the cell, in vesicles called endosomes. PEI is able to escape endosomes at a higher rate than PLL (PLL designs often incorporate modification to include moieties such as chloroquine to enhance endosomal escape [12]), for reasons which again relate to the high density of amine groups in the PEI structure. The exact mechanisms of PEI endosomal escape are still debated, as it is difficult to definitively observe events which occur in endosomes, but the combination of osmotic swelling of the endosome from the “proton sponge” effect, polymer swelling, and polymer endosomal membrane association and disruption, are thought to be involved [32].

The proton sponge effect is explained by the increased affinity for protons of the amine groups in PEI under acidic conditions. Endosomal pH is approximately 5, which increases the protonation degree of PEI to 40%, versus 20% at normal physiological buffered pH of 7.4 [33]. The high proton affinity of PEI in the endosome causes an influx of protons into the endosome, leading to an influx of chloride ions as well. The overall influx of ions into the endosome causes an osmotic pressure which swells the endosome, contributing to its rupture [32]. In addition to the proton sponge effect, the increasingly protonated PEI experiences more and more electrostatic repulsion between its own ammonium groups, which leads to swelling of the polymer volume that also disrupts the endosome membrane [32]. The highly protonated and positively charged PEI complexes also associate with the negatively charged endosome membrane causing further disruption [34]. The combination of proton sponge endosome swelling, polymer
swelling, and electrostatic interaction with the endosome membrane results in rupture of
the endosome which allows PEI complexes to escape into the cytosol. After endosomal
escape, pDNA unpacking decreases the impact of PEI properties on the remaining steps
in the transfection process, with the properties of the pDNA becoming the predominant
determinants of subsequent successful transgene expression, though, some intact
complexes have been observed localized in the nucleus [20], and PEI can have toxic
effects in the cell [35].

1.4.1 Inefficiency and toxicity of PEI transfection

General optimization of PEI transfection is accomplished through tuning of the
N/P ratio. N/P is the ratio of amine groups in the PEI polymer to phosphate groups in the
nucleic acid, determining the overall size and charge of the nanoparticle complexes. The
size and charge properties of these PEI nucleic acid complexes are the primary
determinants of their ability to circulate in serum, be internalized into the cell, and escape
the endosome, and thus are the primary determinants of transfection efficiency [28, 32,
36]. It should be noted that higher N/P ratios have been shown to add free PEI that do
not contribute to nanoparticle formation and instead remain free [37]. This free PEI has
been shown to enhance overall transfection efficiency, for unknown reasons. Optimized
PEI transfection achieves efficiency of 50-80% in vitro depending on cell type [32], type
of PEI, plasmid, and cell type, compared to the 100% efficiency achieved by optimized
viral transduction in vitro [38].
PEI transfection efficiency of 50-80% in vitro could possibly translate to clinically relevant transfection efficiency in vivo if systemic barriers to transfection in vivo can be minimized and sustained transgene expression achieved [12]. PEI can be functionalized with ligands that allow for targeting of specific cell types through, ligand-receptor binding, in order to increase effective transfection efficiency in vivo [32]. PEI can also be modified with polyethylene glycol (PEG) or other moieties to form a hydrophilic corona around the core PEI-nucleic acid nanoparticle, decreasing toxicity and improving degradation resistance by shielding the complexes from interacting with proteins, enzymes, and macrophages, increasing circulation time of the complexes in vivo [32]. However, PEGylation has been shown to decrease the ability of complexes to associate with cells and affect both the association and unpacking of the nucleic acid and the carrier, reducing overall transfection efficiency, so improvements in circulation and degradation resistance through PEGylation are achieved at a cost [32]. Bioresponsive linkages of PEG in transfection complexes are being investigated as a strategy to employ PEGylation while retaining optimized transfection efficiency, through shedding of the PEG shield at the cell membrane, in the endosome, or in the cytoplasm [34].

Another tradeoff in PEI design relates to its toxicity. PEI has been shown to cause mitochondrial dysfunction through disruption of the mitochondrial membrane that can result in oxidative stress and apoptosis [39]. Paradoxically, PEI designs which are more effectively able to overcome barriers to transfection also tend to be more toxic to the transfected cell. For instance, high molecular weight (MW) and branched PEI complexes have been demonstrated to transfect at higher efficiencies compared to low MW and linear PEI complexes [40, 41], but also exhibit higher toxicity. Attempts to
resolve this dilemma include bioresponsive linkages of low MW PEI into higher MW PEI-based polymers that will degrade in the cell into less toxic lower MW constituents [40].

In the context of gene therapy clinical trials up to the year 2012, PEI and cationic polymer strategies in general have not been highly utilized in comparison to viral vectors and physical methods of naked DNA delivery [42]. Viral vectors achieve the highest targeted sustained gene transfer efficiency in vivo while naked DNA methods are less efficient and effective, yet arguably have the best and most predictable safety profile. Nonviral gene delivery strategies continue to be improved, but need to demonstrate improved efficiency and safety profiles that are competitive with or exceed both viral and physical methods of naked DNA gene delivery, respectively, to warrant vetting in clinical trials.

1.5 Pharmacological priming as a tool for enhancing transfection

Constraints on carrier and nucleic acid design limit the extent to which carriers and nucleic acids can be modified, and their properties tuned, towards higher transfection efficiency and effectiveness in nonviral gene delivery. It may be necessary to employ additional methods to modulate the cellular response to transfection in order to achieve clinically relevant transfection efficiency and transgene expression in vivo. One approach is to pharmacologically prime cells with chemical compounds before, during, or after delivering carrier nucleic acid complexes to cells in order to improve some aspect of the gene transfer process. This approach has been demonstrated to have merit in some
studies which are discussed below. It appears that priming enhancement of transfection efficiency and transgene expression can be through direct modulation of the barriers to gene delivery, or indirect through modulation of the cellular response to transfection in terms of toxicity and altered gene expression.

1.5.1 Priming compounds that enhance internalization and endosomal escape

Streptolysin O (SLO) is a bacterial endotoxin that forms pores in cell membranes, permeabilizing the membrane and allowing diffusion of molecules of up to 100kDa across and into cells [43]. With low doses of SLO treatment, toxicity to cells can be minimized, and the pores in the cell membrane can be resealed by treatment with a low concentration of Ca$^{2+}$ ions. After permeabilization and resealing with this low dose SLO and Ca$^{2+}$ method, cells demonstrate internalization of external molecules as well as measures of recovery to normal phenotype and cellular processes [43]. This technique has been used in delivering both proteins [43] and nucleic acid [44] into cells in vitro.

Chloroquine is an antimalarial compound that has been demonstrated to enhance nonviral gene delivery in transfections across several nanoparticle carrier formulations [45]. A primary mechanism by which chloroquine priming enhances gene delivery is through buffering of endosomes, aiding complexes in escaping the endosome and avoiding lysosomal degradation. The buffering capability of chloroquine is attributed to its behavior as a weak base [45], similar to the proton sponge effect seen in PEI amine groups. The observation that chloroquine priming does not improve PEI transfection has been part of the evidence that PEI itself buffers endosomes through the proton sponge
effect to escape the endosomal compartment. Chloroquine priming does dramatically enhance PLL transfection [46], which does not have the innate ability to escape the endosome that PEI has. It has been shown through structure-function correlation studies [45] that chloroquine may also enhance transfection efficiency through aiding in unpacking of the nucleic acids from the carrier nanoparticle complex and possibly electrostatically interacting with nucleic acids in a way that alters intracellular processing or degradation.

1.5.2 Glucocorticoid priming enhances transfection

Glucocorticoid priming has been demonstrated to enhance viral and nonviral gene delivery across multiple cell types [47-49]. Recent work has shown that human mesenchymal stem cells (hMSCs), a primary cell type that is typically difficult to transfect, showed increases in transfection efficiency and transgene expression of up to 10-fold across multiple donors, with dexamethasone (a synthetic glucocorticoid) priming prior to transfection [49]. qRT-PCR of cytoplasmic and nuclear fractions isolated by gradient centrifugation subcellular fractionation showed that glucocorticoid priming increased total cellular and nuclear uptake of delivered plasmid in transfected cells [49]. The specific mechanisms of this enhancement remain unclear, but preliminary data suggest that activation of cytosolic glucocorticoid receptors by glucocorticoid priming may improve internalization, nuclear translocation, and nuclear import of complexes [49].
1.5.3 Genes implicated in successful gene delivery are priming targets for enhancing transfection

In studies by Martin and Plautz et al [50-52], enhancement of transfection efficiency and transgene expression were demonstrated by pharmacological priming with activators and inhibitors of genes which had been identified as differentially expressed in successfully versus unsuccessfully transfected HEK293T cells. At various time points, fluorescence activated cell sorting (FACS) was used to sort cells which had been treated with polymer or lipid pDNA complexes encoding for enhanced green fluorescent protein (EGFP). Microarray analysis was performed on sorted and control cell populations to create gene expression profiles for successfully transfected, unsuccessfully transfected, and control cells which had not been treated with carrier pDNA complexes. Network analysis was then performed on the gene expression profiles to determine differentially expressed genes between the populations at each time point. Table 1.1 below shows an abbreviated list of genes identified by these studies as being differentially expressed in successfully versus unsuccessfully transfected HEK293T cells in PEI transfection.

Table 1.1: Differential gene expression between HEK293T cell populations sorted by successful and unsuccessful EGFP expression (adapted from [50])

<table>
<thead>
<tr>
<th>Gene</th>
<th>Role</th>
<th>Differential Expression (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAP1A</td>
<td>Cell adhesion</td>
<td>12.58</td>
</tr>
<tr>
<td>CHORDC1</td>
<td>Heat shock</td>
<td>10.38</td>
</tr>
<tr>
<td>NEB</td>
<td>Cytoskeletal dynamics</td>
<td>7.81</td>
</tr>
<tr>
<td>WDR78</td>
<td>Lipid rafts</td>
<td>6.96</td>
</tr>
<tr>
<td>PGAP1</td>
<td>Lipid rafts</td>
<td>6.33</td>
</tr>
<tr>
<td>ATF3</td>
<td>Cell stress pathways (NF-Kb, JNK/SAPK)</td>
<td>5.34</td>
</tr>
<tr>
<td>IREB2</td>
<td>Oxidative stress</td>
<td>5.3</td>
</tr>
</tbody>
</table>
Furthermore, pharmacological activation or inhibition of these differentially expressed genes was shown to enhance or decrease transgene expression, demonstrating that genomic targets can be modulated by priming to enhance nonviral gene delivery. The results of these FACS/microarray studies also suggest that in addition to overcoming the primary barriers to transfection, nonviral gene delivery strategies may need to modulate the overall cellular response to transfection, including toxicity that causes cell stress and genomic effects, to achieve higher transfection efficiency and more sustained transgene expression.

1.6 High throughput screening of gene delivery

The examples of pharmacological priming described above suggest that priming may be an effective means for improving transfection in vitro. Broad applicability of transfection priming to different carriers, nucleic acids, and cell types has not been fully established, but a next step would be to search for other compounds which might have priming effects, a drug discovery endeavor. While drug discovery has historically been a matter of educated trial and error, the modern medical research and pharmaceutical industry has established high throughput screening methods to implement these searches in higher volume to accelerate the process [53].

Broadly, high throughput screens are based on the idea that multiple treatment conditions can be tested in parallel against a single target to determine which of the treatments have desired effects on the target. In high throughput screens for drug discovery, the treatments are large arrays of drug molecules in various stages of
development tested against cellular targets for which the drugs could be further
developed. These cellular targets can be bioactive molecules such as signal transduction
receptors [54] and antibodies [55], or live cultured cells [56, 57].

An example application of high throughput screening for identification of novel
drugs is the identification of antiviral compounds. A recent study performed a high
throughput screen to identify broad spectrum antiviral molecules against zoonotic viruses
(SARS coronavirus and Ebola, Nipah, and Hendra viruses) [58]. The approach was to
search for compounds that inhibited CatL cleavage of viral glycoproteins, a process
required by SARS, Ebola, Nipa, and Hendra viruses, without inhibiting the CatL enzyme
itself (to preserve endogenous host functionality of the enzyme). Representative peptide
sequences for each virus were synthesized and labeled with both a light quencher and
light emitter for a FRET signal which reported cleavage of the peptide. The initial screen
of 5,000 small molecules revealed compounds that inhibited CatL cleavage of SARS
peptide, the top 50 of which were rescreened for broad spectrum inhibition of CatL
cleavage of Ebola, Nipa, and Hendra peptides, while minimizing inhibition of CatL
cleavage of host peptides. The screen was successful in identifying a molecule that was
an effective broad spectrum antiviral against all four zoonotic viruses.

High throughput screening methods have also been applied to genome-scale gene
expression through siRNA library screens [59] and DNA microarray screens [60]. In
DNA microarray screens, nucleic acid probe sequences representative of the entire
human genome are tested for complementary hybridization against mRNA samples to
determine any and all gene expression associated with the sample. This approach was
utilized as described in 1.5.3 to identify differentially expressed genes in the human
genome of successfully versus unsuccessfully transfected HEK293T cells, with the samples being the mRNA extracted from the FACS sorted cell populations [50-52]. These samples were tested in high throughput for hybridization against tens of thousands of probes in parallel, with each probe corresponding to a unique gene in the human genome, producing a comprehensive dataset identifying differentially expressed genes in successful polymer and lipid mediated gene delivery, providing a holistic context for nonviral gene delivery strategies to be developed which account for the total cellular response to transfection.

High throughput RNAi screens with genome-scale siRNA libraries are similar to DNA microarrays in that they examine gene expression at the genome wide level; however siRNA screens search for genes and proteins which are involved in a process by knocking down gene expression [59], in contrast to DNA microarrays which identify genes that are expressed through mRNA in a tested sample. For example, a recent genome wide siRNA high throughput screen revealed genes which are involved in regulation of interleukin-8 secretion, revealing new information about the mechanisms which contribute to the inflammation of the intestine in Crohn’s disease [61]. The DNA microarray studies in 1.5.3 identified gene pathways involved in successful transfection followed by secondary modulation of these genes pharmacologically; a similar yet different differential gene expression screen can be performed from an RNAi silencing approach that combines identification of genes involved in gene delivery and modulating those identified genes directly through mRNA silencing by siRNA into one step, instead of a two-step process which involves indirect pharmacological modulation of the gene. This also implies that siRNA and not only pharmacological compounds are capable of
priming effects on gene delivery, and should be considered potential candidates as priming agents as well.

There are other ways high throughput screens can be specifically applied to the application of gene delivery as a design tool. For example, high throughput screens have been performed for optimization of transfection with lipid and polymer carrier complex formulations. In these screens, different variations of a carriers design are tested in high throughput transfection of cell arrays to reveal candidates whose design optimally improves their efficacy in transfection. For example, a high throughput screen tested 144 variants of low MW linear and branched PEI (423-Da and 1.8kDa) biodegradably crosslinked together with oligo-acrylate esters, under the premise that a high effective MW PEI-based polymer can retain optimum transfection efficiency while also being degradable to lower MW constituents to minimize toxicity [62], as has been demonstrated previously [40]. The screen successfully identified vectors which exhibited potency twice as high as 22-kDa linear PEI, while being less toxic.

The reporter proteins used to measure transgene expression in high throughput screens of gene delivery can generally be assayed by colorimetric or luminescent reactions, or fluorescence imaging. Fluorescence is easily multiplexed, allowing for multiple fluorophores of different excitation and emission wavelengths to be imaged simultaneously. Multiplexing multiple readouts in a screen is generally referred to as high content screening. A recent study performed a high content screen of different PEI formulations and was able to localize rhodamine labeled PEI within cells by fluorescence microscope imaging, multiplexing rhodamine labeled PEI, GFP reporter protein, and Hoechst DNA stain, and demonstrating the utility of a high content screening approach in
the context of nonviral gene delivery [63]. Multiplexing of fluorophores allows for multiple readouts from a single screen, allowing for simultaneous measurement of not only transgene expression, but other measurements as well, such as cell viability and proliferation. A similar multiplexed fluorescence readout high throughput and high content screening approach was utilized in the research of this thesis which is presented in Chapter 2.

Finally, to the extent of the literature search for this thesis, two high throughput screens have been applied to the search for compounds which enhance gene delivery, specifically priming adjuvants for delivery of oligonucleotides as well as viral transduction. The most recent of these studies was the high throughput screen for compounds that enhanced antisense and splice switching oligonucleotide (ASO and SSO) delivery and activity [64]. ASO selectively degrade target mRNA in the cytoplasm, while SSO modulate splicing of pre-mRNA in the nucleus [65]. The primary hits were 3-deazapteridine analogs which, in vitro, showed enhancement of activity of both ASO and SSO, with modest toxicity, in the low micromolar range.

The second of these high throughput screens for priming agents, was a high throughput screen for priming of viral transduction, where siRNA and chemical compounds were screened for priming of transduction efficiency and transgene expression, identifying both siRNA sequences and chemical probes which prime viral transduction [66, 67]. Priming compounds which enhanced transduction included antioxidants, tyrosine kinase inhibitors, nucleoside analogs, alkylating agents, metal chelators, and cell cycle arrestors [66]. The most potent siRNA sequences identified in
enhancing transduction contained consensus sequences which were concluded to modulate the interferon pathway response to viral infection [67].

The successful identification of priming compounds for viral transduction and oligonucleotide delivery by high throughput screens suggests that it should be possible to identify compounds that enhance pDNA delivery using nonviral methods by the high throughput screening methods performed in this thesis research, described in Chapter 2.

1.7 Drug repurposing

In addition to using high throughput screening methods to search for priming compounds, a drug repurposing approach was also taken in this thesis research to increase the expected bioavailability and biocompatibility of potential priming candidates identified by the screen, with the objective being to find priming compounds that are already clinically accepted, like the glucocorticoids are [49]. For this purpose, the NIH Clinical Collection was used [68]. This collection of 725 compounds is provided by the NIH for drug repurposing research, and the goal of this thesis research was to identify potential clinically approved compounds from this collection for repurposing to priming of nonviral gene delivery. High throughput screens of the NCC have already been performed to repurpose compounds for antiviral [69, 70], anti-cancer [71], and other applications [72].

High throughput methods accelerate the process of drug discovery, but pharmaceutical development costs remain high, an estimated $800,000,000 [73] to bring a drug to market. In the specific application of drug discovery to transfection priming
compounds, this expense may be justified in the future, if priming adjuvants become widely adopted as an integral aspect of effective gene therapy, but presently, preliminary research into transfection priming should seek compounds that have a low development cost and are known to be biocompatible and bioavailable.

There are various metrics that are generally used to predict suitability of compounds as pharmaceutical compounds. ADME (absorption, distribution, metabolism, and excretion) is an acronym which describes some of the main processes a drug encounters within an organism [74]. Additional considerations are toxicity and liberation, which describes the release of drug from delivery vehicle. With respect to drug properties, Lipinsky’s rule of five suggests guidelines that increase the chances a compound will have desirable behavior in vivo [75]: 1) Molecular weight less than 500 Daltons. 2) Less than five hydrogen donors. 3) Less than 10 hydrogen acceptors. 4) octanol-water partition coefficient (logP) less than five. It is not trivial to design a drug that has a specific target effect while also optimizing these parameters and properties, one reason for the extensive cost and time of drug development.

![Graphs](image)

Figure 1.4 Drug-like properties of the NIH Clinical Collection [68]
From Figure 1.4 shown above, the drug-like properties of NCC compounds are already well defined and can be seen to adhere to Lapinsky’s rule of five, making the NCC an ideal starting point for a high throughput screen to discover priming compounds for nonviral gene delivery.

1.8 Thesis objectives

In summary of this chapter, the rationale for a high-throughput and high-content screen of clinically approved compounds for priming of nonviral gene delivery has been presented. Priming is an emerging tool for improving transfection outcomes, with potential to be incorporated into overall efficient nonviral gene delivery strategies through understanding and modulation of the barriers to transfection as well as the total cellular response to transfection, and a high throughput fluorescence imaging screen of the NCC is an ideal method of discovering additional priming agents. Chapter 2 will describe and discuss in detail the design, implementation, and results of this screen, while Chapter 3 will elaborate on future work which the screen suggests.
CHAPTER 2

High Throughput Screening of Priming Candidates for Impact on Nonviral Gene Delivery

2.1 Introduction

Gene delivery is the delivery of exogenous DNA into cells, and can be accomplished by viral and nonviral methods, however viral methods have safety concerns and design constraints [5], while nonviral methods lack efficiency [12]. Attempts to improve nonviral gene delivery strategies have, to date, generally hinged on the careful design of carriers and nucleic acid cargo [24, 76], however, their optimization may not alone be sufficient to achieve clinically relevant transfection efficiency, transgene expression, or gene silencing. This lack of efficiency may be due to a host of cellular pathways associated with both successful and unsuccessful nonviral delivery [50, 51], and these pathways may have more to do with the overall cellular response to being transfected, than the ability of carrier complexes to overcome the barriers to transfection, or the ability of delivered nucleic acids to have their desired effects. It could be possible to further optimize carriers and nucleic acids to induce an appropriate cell response and avoid an undesirable cell response, in addition to fulfilling their current design criteria; however, further modifications to intricate carriers adds complexity to a challenge that is already fairly intractable. Modulating these cellular response pathways by other means, perhaps pharmacologically [50, 51, 77] or through substrate interactions [78, 79], may
decrease the design burden on the transfection complexes and allow effective nonviral gene delivery strategies to be developed.

Pharmacological treatment of cells prior to transfection, i.e. priming, can enhance or knockdown transfection efficiency and gene expression. For instance, pharmacological modulation of genes found to be differentially expressed in, successfully versus unsuccessfully transfected HEK293T cells, resulted in up to five fold increases and five-fold decreases in transfection [50, 51]. Another example of priming is work which demonstrated that glucocorticoid priming markedly improved gene delivery to hMSCs in vitro, a cell line which is difficult to transfect, enhancing transfection over 10-fold across five human donors [77]. These results suggest that priming is a tool that should be investigated for enhancement of nonviral gene delivery.

To efficiently search for compounds which may have priming effects on transfection, an obvious starting point is to screen clinically approved compounds. Previous clinical approval of a compound suggests acceptable biocompatibility and bioavailability of the compound in vivo, which would be primary criteria for incorporating priming into the design of gene delivery applications. If clinically approved compounds can be repurposed for priming, the drug discovery and development costs of viable priming compounds could be dramatically decreased.

For this purpose, the NIH provides the NIH Clinical Collection (NCC) [68], a set of 725 clinically approved compounds to be screened for repurposing. Several studies have successfully performed high throughput screens of the NCC to repurpose the drugs for their potential antiviral [69, 70], regenerative [72], chemotherapeutic [71], and other [80-82] properties. High throughput screening of gene delivery has been attempted,
mostly for optimization of polymer [62, 83], lipid [84], and peptide [85] carriers. Perhaps most relevantly to this thesis, a high throughput screen has been conducted for priming of viral transduction with siRNA and chemical compounds [66, 67], which identified several siRNA sequences and types of compounds (antioxidants, metal chelators, alkylating agents, nucleoside analogs, cell cycle arrestors) to have priming effects on viral transduction. The research in this thesis attempts a similar screen, though of clinically approved compounds from the NCC, for priming of nonviral gene delivery, with the goals being to identify an assortment of priming adjuvants for transfection, and to improve our understanding of the biology of transfection through investigation of their priming mechanisms.

The specific methods used in screening for enhancement of gene delivery can vary, but generally, cell arrays are transfected with bolus and/or substrate mediated delivery of DNA complexes [86]. Priming screens involve treatment of these cell arrays with compounds for a certain amount of time before transfection with complexes delivering plasmids encoding for common reporter genes like β-galactosidase (LacZ), luciferase (Luc), and green fluorescent protein (GFP), which are often used as markers for expression of co-transfected genes. Quantification of LacZ and Luc expression are colorimetric and luminescence assays, respectively, while GFP can be quantified by fluorescence measurement. A key benefit of using a fluorescent reporter for gene expression is the ability to easily multiplex fluorophores, like Hoechst and propidium iodide, for multiple readouts. Because of this, high throughput screens that utilize fluorescent reporters are generally also high content imaging screens [63, 72, 85], which can reveal information on cell viability, cell proliferation, cell cycle, cell morphology and
more, depending on the fluorescent markers and image processing methods used, valuable information to consider for assessing the various responses a cell may have to priming and transfection in addition to overall transfection efficiency and transgene expression.

We hypothesized that application of a high content fluorescence imaging methodology to high throughput screening of clinical compounds for priming of nonviral gene delivery would reveal drugs, or even classes of drugs, which modulate transfection efficiency and/or transgene expression. We chose to use 25 kDa branched polyethylenimine (PEI) as our polymer carrier because it achieves moderate, reliable, and cheap transfection, with room to increase or decrease in efficiency and gene expression, depending on the priming effect observed. Another reason was that 25 kDa branched PEI was also tested as a transfection carrier in the previously discussed transfection gene expression profiling experiments [50, 51], the data from which we intend to perform network analysis upon in combination with the data from this screen.

In testing our hypothesis, we hope to validate the screen methods and demonstrate that transfection priming is a promising undeveloped tool for improving transfection, identifying clinical compounds and drug classes from the NCC whose priming effects have potential to be incorporated into efficient nonviral gene delivery strategies through understanding and modulation of the cellular response to transfection.
2.2 Materials

2.2.1 HEK293T cells

HEK293 are an existing mammalian cell line created from human embryonic kidney cells of an aborted embryo, which were immortalized by adenovirus type 5 transformation [87]. The HEK293T variant of this cell line was further transformed to express the simian virus 40 (SV40) T antigen, which is a viral transcription factor for gene sequences that contain the SV40 promoter region, a reason HEK293T cells are commonly used for both transient and stable recombinant protein expression [88]. Plasmids encoding genes with CMV and SV40 promoters are highly expressed in these cells upon successful transfection, making them an ideal cell line for studies of nonviral gene delivery [89].

Figure 2.1. Phase contrast microscope image of cultured HEK293T cells.
In this screen, HEK293T cells were cultured in Falcon T25 flasks (Fisher Scientific) in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies) completed with 10% fetal bovine serum (Life Technologies), 1% Penicillin/Streptomycin (Life Technologies), and 1% sodium pyruvate (Life Technologies). Cells were incubated at 37 °C, 5% CO₂, and passaged using 1 mM ethylenediaminetetraacetic acid in PBS (EDTA) (SigmaAldrich) at 75%-85% confluence approximately every 48 hours, with passage number ranging from 15 to 35 over the course of the screen.

2.2.2 pEGFPLuc plasmid

![pEGFPLuc plasmid map](image)

Figure 2.2. pEGFPLuc plasmid map [90].

The pEGFPLuc plasmid encodes for a fusion protein of enhanced green fluorescent protein (EGFP) and luciferase. EGFP is a red-shifted variant of wild-type green fluorescent protein (excitation/emission maxima at 488nm/507nm), while is an enzyme that catalyzes a luminescent reaction of luciferin substrate [90]. Both are common reporters for transfection efficiency, with this fusion providing a co-transfection
marker that is measurable with both fluorescence and luminescence quantification methods. The EGFPLuc sequence is flanked by a human cytomegalovirus (CMV) promoter and an SV40 polyadenylation tail, with the plasmid backbone also containing an SV40 origin [90], for expression in mammalian cells, especially those expressing SV40 T antigen, as the HEK293T cells used in this screen do [87].

Neomycin and kanamycin resistance are also encoded in this plasmid, with a bacterial promoter, SV40 promoter, and HSV TK polyadenylation tail, allowing for propagation and selection in E. coli, by treatment with kanamycin, as well as selection of stably transfected mammalian cells, by treatment with G418 (Geneticin) [90]. For this screen, pEGFPLuc (Clontech) was propagated in E. coli, selected by kanamycin treatment, isolated from the E. coli using Qiagen Giga Prep (Qiagen), and prepared in endotoxin-free TE buffer at 1mg/mL, with purity assured by NanoDrop absorbance ratios at 260/280 and 260/230 of approximately 1.8 and 2.0-2.2, respectively [91], and subsequently stored in frozen aliquots at -20 °C. A single 1 mL aliquot was sufficient for all transfections in this screen.

2.2.3 Polyethylenimine (PEI)

PEI is a cationic polymer carrier for nonviral gene delivery that serves as a gold-standard to which newly developed carriers are often compared, as it reliably overcomes the primary barriers to transfection of cultured mammalian cells: internalization, endosomal escape, and nuclear transport [92]. In this screen, the commonly utilized 25 kDa branched PEI (SigmaAldrich) variant was used, prepared at 1 mg/mL, dissolved at
80 °C, in 0.1 M sodium bicarbonate, in ddH20, at pH 8.2. The 2 mL aliquots were frozen and stored at -80°C, with a new aliquot thawed for use weekly and kept at 4 °C. Aliquots were vortexed for 30 seconds before use in forming complexes with plasmid (as described in Methods 2.3.4) for delivery to HEK293T cells.

2.2.4 NIH Clinical Collection

Restated simply, the primary objective of this screen was to identify, with high throughput methods, existing clinical compounds that prime cultured cells for nonviral gene delivery. The particular collection of pharmacological compounds screened was the NCC from the NIH Small Molecule Repository [68], which is provided to researchers endeavoring to repurpose clinically approved pharmaceutical compounds. The 725 compounds of the NCC were received in ten, 96-well plates, with 10 μL of each compound (10 mM) in dimethyl sulfoxide (DMSO). Each plate was empty in the 16 wells of columns 1 and 12 (See Figure 2.3 below), with eight of the plates populated with one compound per well in the 80 remaining wells, and the two other plates populated with approximately 40 compounds. The 2 μL aliquots of each compound were prepared in four 96-well plates per original NCC plate, and kept frozen at -20 °C to be thawed before use.

Figure 2.3. NIH NCC 96-well plate, compounds in columns 2 through 11.
2.2.5 Hoechst 33342 DNA stain

Hoechst 33342 fluorescent dye was used to stain cell nuclei and enable cell count of fluorescence microscope images at low magnification and resolution. Hoechst stained nuclei require minimal image processing to automate a cell count for each image. Hoechst 33342 is a DNA intercalating dye with excitation/emission maxima at 355nm/465nm when bound to DNA, which is permeable to the cell membranes of live cells, and is sufficiently non-toxic to allow for endpoint live cell imaging [93].

In addition to cell count and proliferation, Hoechst 33342 also provides information on cell cycle and cell death processes, as Hoechst staining kinetics depend on chromatin packing, amount of DNA in the cell (dependent on cell cycle), and permeability of the cell and nuclear membranes [94]. The integrity of cell and nuclear membranes is impaired in apoptotic and necrotic cells. Hoechst 33342 stock solution was prepared from lyophilized powder (Sigma Aldrich), dissolved in ddH2O at a concentration of 10 mg/mL, and stored at 4 °C.

![Hoechst structure](image-url) Figure 2.4. Hoechst structure [95].
2.2.6 Resazurin metabolic indicator

Resazurin is a reagent that is commonly used to measure proliferation and viability of cultured cells. Resazurin solutions are a nonfluorescent blue, but become reduced to pink resofurin in the presence of metabolic activity of cells, which fluoresces with excitation/emission maxima at 530nm/590nm [96]. Unlike MTT assays, resazurin does not require cell lysis, allowing for repeated viability measurements over time in some applications, such as lymphocyte proliferation [97], though long term incubation with resazurin has been shown to have some cytotoxic effects, recommending its use as an endpoint assay [96]. Compared to WST-1 colorimetric assays (which also do not require cell lysis) resazurin is much lower in cost, and its measurement by fluorescence allows for multiplexing in live cell imaging applications by fluorescence plate reader measurement. Stock resazurin solution was prepared from lyophilized resazurin sodium salt powder (SigmaAldrich), dissolved in PBS at a concentration of 10 mg/mL, and stored at 4 °C.

![Figure 2.5. Resazurin (left) and Resofurin (right) [98].](image-url)
2.2.7 Rationale for fluorophore selection

For multiplexing applications, the excitation and emission spectra of the fluorophores must be sufficiently separated to not have cross talk between the signals. Figure 2.6 below shows the excitation and emission spectra for Hoechst 33342, EGFP, and Resazurin, the fluorophores used in this screen.

![Fluorescence spectra of Hoechst 33342, EGFP, and Resazurin](image)

There were several fluorescent markers that could have been used for each measurement (in this screen, gene expression, cell enumeration, and viability), each with different excitation and emission wavelengths. Other considerations included quantum yield, sensitivity to photobleaching, permeability to the cell membrane, toxicity, etc. EGFP was chosen for its convenient availability in our lab as a fusion protein with luciferase, which adds a potential chemiluminescent measure of gene expression to the screen. EGFP left the blue and red emission wavelengths open for cell counting and viability dyes. Dyes that enable cell counting come in many forms, from nuclei stains to cytoskeletal and membrane stains. Cytoskeletal and membrane stains generally require
the fusion of fluorophores to antibodies of the proteins found in those features, while
nuclei stains naturally and specifically target and bind DNA. For this reason, the blue
nuclei stain Hoechst 33342 was chosen, as well as for its permeability to live cell
membranes. For viability measurements from the remaining red emission wavelengths,
there were several options. Higher permeability of certain DNA dyes into unhealthy cells
with compromised membranes compared to healthy cells with intact cell membranes is
one method of differentially staining cells for viability, with such dyes including
ethidium bromide and propidium iodide. Another type of viability measuring dyes is
metabolic indicators, which interact with the reducing environment of the cell to become
fluorescent, an example of which would be resazurin. One benefit of resazurin in
comparison to ethidium bromide and propidium iodide is that it is very inexpensive in its
unbranded form. One thing to note is that resazurin cannot be imaged only inside cells,
because its reduced form resofurin is also permeable to the cell membrane and does not
remain localized within cells, diffusing back out into surrounding media. This does not
preclude it from use as a measurement of cell viability though, and in the end, resazurin’s
metabolic measurement, similar to WST-1 and MTT assays, was deemed more sensitive
to potential priming toxicity than permeability of dyes to the cell membrane.
2.3 Methods

2.3.1 Screening schedule

![Screen timeline]

Figure 2.7. Screen timeline

The screen of the 725 clinically approved compounds in the ten 96-well plates of the NIH NCC, for priming of PEI/pEGFPLuc transfection of HEK293T cells, was accomplished over the course of one month. In brief, cells were seeded in 96-well plates, primed 17 hours after seeding, transfected one hour after priming, and imaged (as described below) 48 hours after transfection. For each NCC plate, four 96-well plates were...
total, the cell handling portion of the screen took approximately 120 hours to complete over the one month period.

2.3.2 Seedling HEK293T cells for the screen

Each T25 flask at typical confluence (75-85%) provided enough cells to seed two full 96-well plates at a seeding density of 8,000 cells per well and seeding volume of 80 μL per well. With each well having a surface area of 0.32 cm², the seeding density was 25,000 cells/cm². This approximate seeding density was previously optimized in a preliminary screen that assessed PEI transfection efficiency in HEK293T cells by LacZ expression 48 hours after transfection.

To passage, cells were rinsed with phosphate buffered saline (PBS) (Life Technologies), detached from the flask surface by incubation in 400 μL of 1 mM EDTA in PBS for five minutes at 37 °C, then diluted to a total cell suspension volume of 4.3 mL in HEK293T media, with 1 mL of this volume used for passaging to a new flask, and the remaining approximately 3.3 mL available to seed plates for the screen.

Each well was seeded with 80 μL of diluted cell suspension, requiring a minimum of approximately 15 mL dilute cell suspension for two full 96-well plates, however, an excess dilute cell suspension of 25 mL was used, in an attempt minimize variation in seeding density due to volume change as the suspension is used. At typical cell counts, between approximately 1.5-2 mL of the cell suspension was diluted to 25 mL for the desired 10⁵ cells per mL concentration. This 25 mL suspension was split into four volumes of approximately 6.25 mL, with two of these volumes being used to seed each
plate. Before seeding, each volume was inverted to mix, then transferred to an autoclaved fluid reservoir for an eight channel pipette, where they were then tritraped to mix further. From these well mixed suspensions, an eight channel pipette was used to seed. Six rows were seeded with every 6.25 mL volume, at 80 μL of cell suspension per well, to seed all wells of two 96-well plates. These plates were briefly examined under phase contrast to ensure the seeding density was uniform, and then transferred to an incubator (37 °C, 5% CO₂) for the cells to adhere over 17 hours before priming and transfection.

2.3.3 Priming HEK293T cells with NCC compounds 17 hours after seeding

From each stock NCC plate, four aliquot plates were made, containing 2 μL of each compound per well at 10mM in DMSO. This screen required one aliquot plate from each stock NCC plate to complete the screen of every compound in the collection at both 5 μM and 50 μM. These concentrations are relative to the 141 μL total liquid volume added to the wells in seeding, priming, and transfecting. The following descriptions are of the priming compound preparations of one aliquot plate of the NCC collection, which were repeated for all ten of the NCC plates. To prepare the compounds from an aliquot plate for delivery to cells, the plate was thawed for 15-30 minutes at room temperature prior to dilution in HEK293T media, while the HEK293T media was concurrently warmed to 37 °C in a water bath. As vehicle controls, 2 μL DMSO was added to each of the empty wells of columns 1 and 12 of the aliquot plate (Figure 2.4 for reference). To dilute the aliquot plate for the 50 μM priming concentrations, and equivalent 1.4%
DMSO (v/v) in the vehicle control wells, 111.48 μL of warmed HEK293T media was added to each well of the aliquot plate, triturating to mix well. Approximately 17 hours after seeding two plates with HEK293T cells, 40μL of each compound of the aliquot plate were gently delivered by eight channel pipette into corresponding wells of both seeded plates. The priming volumes were pipetted with the tips angled to gently dispense against the side of the wells and minimally disturb the cells in the wells. The primed plates were then incubated for one hour at 37 °C, 5% CO₂ before transfection. Priming two plates requires 80 μL of each 50 μM compound, so there was approximately 31.5 μL remaining of the original 113.48 μL of each compound in the aliquot plate after priming both seeded plates. The remainder of the 50 μM concentrations in the aliquot plate was frozen, to be thawed and diluted for priming at 5 μM within 24 to 48 hours. To prepare the 5 μM priming concentrations, 12 μL of the thawed remainder of the 50 μM concentrations were diluted with 108 μL of warmed HEK293T media, triturating to mix well, before delivering 40μL of each compound to corresponding wells in two seeded plates. The primed plates were then incubated for one hour at 37 °C, 5% CO₂ before transfection.

2.3.4 Transfection of HEK293T cells one hour after priming with NCC compounds

DNA/PEI complexes were formed in Tris-buffered saline (TBS). Previously, a preliminary screening experiment determined that 0.17 μg of pEGFPLuc, complexed with 0.34 μg of 25 kDa branched PEI at N/P ratio of 15, and delivered in a 21 μL TBS volume to each well, was an optimum formulation for this screen format. For
transfection of two full 96-well plates, this formulation amounted to four pairs of 1.5mL microcentrifuge tubes consisting of 20.4 μL of 1 mg/mL 25 kDa branched PEI, in 471.91 μL of TBS, to be mixed into 10.2 μL of 1 mg/mL pEGFPLuc, in 728.26 μL of TBS. The total volume ratio of DNA to PEI tubes was 60:40. To form the complexes, the PEI tubes were pipetted into the pEGFPLuc tubes, triturated five times, vortexed for 10 seconds, and then incubated at room temperature for 15 minutes. After the incubation, the complexes were combined into an autoclaved fluid reservoir to be dispensed at 21 μL per well into all wells of both plates, by eight channel pipette, after one hour of incubation with priming compounds.

2.3.5 Staining HEK293T cells 48 hours after PEI transfection

After a 48 hour incubation following transfection, at 37 °C, 5% CO₂, the primed and transfected HEK293T plates were stained with Hoechst and Resazurin fluorescent dyes to enable subsequent nuclei counts and viability assessments, respectively, multiplexed with the EGFP fluorescent reporter for transgene expression. The staining solution consisted of 38.5 μL of 10 mg/mL Hoechst 33342 stock solution, and 22 μL of 10 mg/mL Resazurin stock solution, in 22 mL of warmed Fluorobrite DMEM (ThermoFisher). Instead of aspirating the wells before rinsing and staining, the plates were “flicked” upside down several times over a container which caught the ejected liquid. Flicking two plates was much faster than aspirating each well, did not noticeably detach cells from the plate bottoms, and left small volumes of liquid in each well, preventing bare air exposure of the cells. Plates were flicked and rinsed with 100 μL of
PBS per well, and flicked again before 100 μL of the staining solution was added to each well. The plates were then incubated for 30 minutes at 37 °C, 5% CO₂. Care to avoid exposure of the dyes to light was taken throughout the staining process, to prevent photobleaching.

2.3.6 Fluorescence microscope imaging of screen plates

After incubation with the staining solution, plates were imaged using a DMI3000B manual inverted microscope (Leica) with DFC340FX digital camera (Leica), EL-6000 mercury halide lamp (Leica) for fluorescence excitation, and LAS software V4.0 (Leica) for digital image viewing and capture. Filters for excitation and detection at the EGFP and Hoechst wavelengths (488nm/509nm and 355nm/465nm, respectively) were used to take grey scale images of their fluorescence, in addition to phase contrast images. Images were taken with a 5x objective (HCX FL PLAN 5X/0.12), center well. Cells were manually focused in phase contrast, with the focus unchanged for the EGFP image, and unchanged or with minor focus adjust for the Hoechst image to bring into focus as many nuclei as possible. Phase contrast images were captured at 473 μs exposure (gain 1x, gamma 1.00), while EGFP and Hoechst fluorescence images were captured at 108.6 ms exposure (gain 2.0x, gamma 1.15). Consistent fluorescence excitation lamp intensity (second highest setting) was used for all EGFP and Hoechst images in the screen. Keeping those settings consistent allowed for comparison of image intensities between wells in the same plate. Using these settings was also much faster than auto-exposure by the software, allowing for faster overall imaging times, which
averaged to about 45 minutes per plate (acquiring phase contrast, EGFP, and Hoechst images for every well) and 288 images per plate. Wells were imaged down each column from row A to row H, from column 1 through column 12. Images were captured at 1600x1200 pixels in 8-bit TIFF image format. Figure 2.8 below shows plate montages of EGFP and Hoechst fluorescence images acquired from a typical plate in the screen.

![Image: EGFP and Hoechst Images](image-url)

Figure 2.8. EGFP and Hoechst images acquired from a typical plate in the screen, stitched together to form montages which resemble the 96 well plate format.

Figure 2.9(a) is a phase contrast microscope image of a typical vehicle control well in this screen, taken with a 5x objective, center well. Figure 2.9(b,c) shows the fluorescence microscope images of Hoechst and EGFP taken of the same well. These fluorescence images were pseudo-colored and overlaid by merging the images into RGB color format to provide a combined visual representation of transfection efficiency, transgene expression, and cell count, in one image, as shown in Figure 2.9(d).
Figure 2.9. Microscope images of a vehicle control well, in phase contrast (a) as well as EGFP (c) and Hoechst (d) fluorescence. The fluorescence images were also pseudo-colored and overlaid (d).

Initially, a primary concern was whether the cells would remain viable enough for the length of time required to manually acquire all images of a plate outside controlled temperature and carbon dioxide regulation. The most obvious hindrance encountered in imaging for this length of time without environmental control was the water condensation on the lids of the plates, which caused uneven illumination and shadows between wells, shown in Figure 2.10 below.
Phase images are difficult to process with uneven illumination and shadows due to their bright-field background illumination. To normalize these variations between all wells would require the development of image processing algorithms beyond the current scope of this screen, so phase images were acquired in the screening process but not processed. There is potential for future processing of these images to reveal information about overall cell morphological changes due to priming and transfection.

Fluorescence images did not have the same uneven background signal, and were more easily processed for intensity and count measurements. The important acquisition parameters were excitation intensity, detector exposure time, image location within the well, and focus. The lamp and detector parameters were kept consistent for the entire screen, while image location within wells was kept as consistently center of the well as possible by, in phase contrast, aligning and centering the image area on the brightest area of the well, which is almost always the center of the well. The cells were also brought into focus while viewing in phase contrast.

Figure 2.10. Phase contrast image montage that demonstrates shadowing due to condensation on the plate lid.
The nearly hour long exposure of the plate to room temperature and unregulated CO₂ did not noticeably affect the ability to capture representative images and quantify GFP or Hoechst fluorescence. Cells were not seen to detach from the well surface. The Fluorobrite DMEM imaging media was used due to its compatibility with live cell imaging, lacking phenol red that could interfere with the fluorescence measurements, and including glucose and sodium bicarbonate. The manufacturer’s website also claims a higher signal to noise compared to PBS or other media formulations. The Fluorobrite DMEM was used as is, without supplementing with FBS or L-glutamine. It is important to note that imaging without temperature and carbon dioxide regulation was accepted for this endpoint imaging of cumulative transfection efficiency, gene expression, and cell number and viability after 48 hours of incubation post-transfection, not for more transient and delicate processes or with the intention to return these cells to the incubator for further culture, in which cases, environmental control would be required.

2.3.7 Fluorescence plate reader measurement

In addition to microscope imaging, EGFP, Hoechst, and Resazurin fluorescence was measured by a Synergy H1 plate reader (BioTek), with excitation/emission settings of 475nm/509nm, 355nm/464nm, and 545nm/590nm, and gain settings of 100, 50, and 50, respectively. Probe height was re-calibrated for each of these measurements for each plate by determining the probe heights that yielded maximum signal in a selected control well, and using those setting for the entire plate. Gain and probe height were selected such that the signal was strong enough to be detected but also not saturating the detector.
The excitation wavelength used for EGFP was shifted away from the 488nm maximum down to 475nm, as the plate reader required the excitation wavelength to be sufficiently far from the emission wavelength. At the EGFP, Hoechst, and Resazurin wavelengths, nine measurements were taken per well in a three by three array equally spaced within the well, from which mean intensities was calculated. The plate reader was used to quantify the amount of EGFP (transgene expression reporter), Hoechst (nuclei stain), and Resazurin (viability measure) in every well, to measure the effects each drug had on transfection and cell viability. Hoechst was used as nuclei marker for cell counting by microscope image processing, but plate reader measurements were also acquired, as normalized Hoechst intensity may indicate cell cycle and cell death processes. Figure 2.10 below shows an example of these data for a typical plate of the screen, with heat map color shading to ease visual interpretation of the data.

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Figure 2.11. Typical plate reader measurements of EGFP, Hoechst, and Resazurin fluorescence. Darker shades indicate more intense fluorescence signal.
2.3.8 Comparison of microscope image and plate reader measurements

For a comparison of plate reader and microscope images, see Figure 2.11 below. The well outlined in yellow immediately stands out as having an approximately two fold higher of EGFP than any other well in both the plate reader measurement and EGFP images. Very low values in these measurements generally corresponded to wells having few cells due to toxicity of the priming condition, though in some cases, like the well outlined in red, there was very low EGFP expression with normal or high Hoechst and Resazurin readings, indicating normal or enhanced proliferation/viability.

![Figure 2.12. Comparison of plate reader and fluorescence microscope images showing qualitative similarity in the observed fluorescence by both methods.](image)

The primary limitation to the quantification of fluorescence through microscope images was the limited viewing area. The culture area of a 96-well plate is approximately 32 cm², while at lowest magnification (5x) the image area is approximately 5.9 mm², so images capture approximately 18% of the well area. Cells were always more densely
packed in the central area of the well, so there was always more than 18% of the cells in the well in the images, up to 100%, seeming to depend on the toxicity of the priming compound. That being said, a significant portion of each well’s cell population was generally outside the image area. The options for measuring the fluorescence from these cells were either taking images at multiple locations per well, or measuring with a fluorescence plate reader in an array scan. The microscope imaging time of a full plate with three images per well (phase contrast, EGFP, and Hoechst, center well) was already 45 minutes, so it was hard to justify adding more locations and time per well without environmental and CO₂ control, unless there was no other option. A 3x3 array scan covers approximately 24 mm² of the well, 75% of the well area, and about four times the area of the microscope image.

![Diagram](image.png)

Figure 2.13. Microscope imaging and plate reader measurement areas, demonstrating the larger area coverage of the plate reader measurement but lower resolution.
The plate reader was able to measure fluorescence of EGFP, Hoechst, and Resazurin wavelengths, in 3x3 array scans per well, in approximately 15 minutes total per plate, about three times faster than microscope imaging of phase contrast, EGFP, and Hoechst. The primary disadvantage of a plate reader measurement is that it only reads an array of intensities, rather than capturing a high resolution image that can be processed to extract more information, such as total cell counts and transfected cell counts, which were essential for normalization in this screen. For these reasons, fluorescence microscope images and fluorescence plate reader measurements were both acquired for each plate. Both of these measures were used in the hit selection and scoring process, considered individually and as part of overall transfection, proliferation, and viability scores.

2.3.9 Cell lysis

After imaging and plate reader measurements, cells were lysed for storage and potential future measurements of the lysate luciferase content. For lysis of two full 96-well plates, 22 mL of 1x Reporter Lysis buffer (RLB) was sufficient, and was prepared by dilution of 4.4 mL of 5x Reporter Lysis Buffer (Promega) in 17.6 mL of ddH2O. Each plate was flicked and rinsed with 100 μL PBS per well, with 100 μL of 1x RLB then delivered to each well, after which the plates were incubated at room temperature for 5-10 minutes before freezing and storage at -80 °C.
2.3.10 Image processing

Macros in ImageJ (NIH), a Java based image processing program, were used to automate the mean fluorescence intensity measurements and maxima counts for the processing of the 11,520 images acquired over the course of the screen. For each well, EGFP and Hoechst images were processed for average grey value, as the mean intensity EGFP and Hoechst fluorescence measurements, while local maxima were also identified to obtain counts of Hoechst stained nuclei and of EGFP expressing cells. See Figure 2.14 below for a representation of the image processing for each Hoechst and EGFP image for intensity and count, and Appendix B for the ImageJ macros used for the image processing in this screen.

![Image Processing](image.png)

Figure 2.14. Hoechst nuclei and transfected cell counting by local maxima detection.
Average grey values of the Hoechst and EGFP images were determined using the “Measure” plugin in ImageJ. Figure 2.14 shows total cells and transfected cells counted by the “Find Maxima” plugin in ImageJ. The local maxima in the Hoechst and EGFP images were determined, point selected, and counted. The noise tolerance of the “Find Maxima” plugin was adjusted to optimize detection and minimize misidentification of nuclei and EGFP cells. These measurements were automated by macros in ImageJ.

All microscope images were stitched together into image arrays to ease visual inspection of the 96-well format, such as in Figure 2.15 below. Similar image montages were created for every plate in the screen, for phase contrast, EGFP, Hoechst, and pseudo-color overlay images. The processed image data were also compiled into 8x12 spreadsheet arrays, the same format as the plate reader measurements. The image array and image data in Figure 2.15 below, are from the same plate as the plate reader data shown in Figure 2.12, and show similar results.

![Figure 2.15. Image array aside image data processed from the images.](image)
The yellow box outlines a well which, by visual inspection, obviously had a relatively high amount of EGFP, reflected in the relatively high EGFP image mean intensity measurement, but did not have a relatively high image EGFP count, implying that the higher amount of EGFP in that well was due to an increase in EGFP expression in transfected cells rather than a higher number of transfected cells, a useful distinction to make when interpreting the data to explain the effects the various compounds might be having on gene delivery. Similarly, the well outlined in red appears to have exhibited a large knockdown of both EGFP amount and transfected cell count, while exhibiting a high total cell count.

2.3.11 Control filtering

Data were transferred to Excel (Microsoft) and Prism (Graphpad) for data processing and statistical analysis. As seen in figure 2.16 below, there was variation in the Hoechst count of the transfected vehicle control wells in columns 1 and 12, which is due to variance in the initial seeding density. Low initial seeding density in a well increases the number of transfection complexes available per cell, increasing transfection efficiency as well as toxicity in that well relative to other transfected vehicle controls wells. To prevent potential unfair comparisons from control well transfection and toxicity bias due to initial seeding density which might result in discarding true priming candidates screened, as seen in Figure 2.16 below, transfected vehicle control wells which had total cell counts less than 95% of the mean of the vehicles controls as a whole for that plate were discarded from consideration for calculations of fold changes.
Normalized fold change measurements

After filtering the controls, the image data for each well were normalized by their associated Hoechst total cell count or transfected cell count and their fold changes determined relative to the filtered vehicle control wells in columns one and twelve, as seen in Figure 2.17 below. For example, the yellow outlined well was calculated to have a fold change increase in EGFP intensity per cell of 2.2, with no increase in transfection efficiency (% EGFP+) and a 0.7 fold decrease in cell count, while the red outlined well displayed a fold change decrease in both EGFP intensity per cell (0.18 fold) and percentage of cells expressing EGFP (.0003 fold), while exhibiting a fold increase in cell count (1.12 fold).
2.3.13 T-test and toxicity filter of normalized fold change measurements

Data acquired for each well included: image measurements of EGFP intensity and count, and Hoechst intensity and count, as well as plate reader measurements of EGFP, Hoechst, and Resazurin intensities. Each compound was tested in duplicate at both 5 and 50 μM concentrations. The fold changes of each measurement for each well relative to the filtered average vehicle control fold changes were then calculated, after which, the duplicate fold changes for each well were then grouped for unpaired two-tailed t-tests, assuming unequal variances, against the grouped filtered vehicle controls, to obtain a p-value for the measurements of each compound.
Figure 2.18. p-value (top right) and Hoechst count fold-change (bottom right) versus EGFP image intensity fold-change. It can be seen from this figure that compounds which appear to have high fold changes are often artifacts of wells which had extremely low cell counts.

These t-tests were not performed to assess significance, instead, the t-tests were used as an initial filter in the hit selection process, to throw out compounds whose effect size caused their $-\log(p$-values) to be lower than the plate wide $-\log(average\ of\ p$-values) for sample wells. Figure 2.18 (top right) shows this filtering, with the compounds with $-\log(p$-values) less than $-\log(p_{avg})$ discarded from further consideration. From the red and yellow arrows pointing at their corresponding compounds outlined in red and yellow on the image montage, it is obvious that a slight decrease in p-value threshold might have caused these wells to be mis-identified as false negatives, even though they clearly
deserve further investigation simply from visual inspection. This t-test filtering was performed for all EGFP, Hoechst, and Resazurin measurements.

After the t-test filters, the cytotoxicity filters were implemented. The first simply eliminates compounds which had normalized Hoechst count fold change decreases of less than 0.2, which were automatically discarded as extremely toxic, corresponding in Figure 2.18 (bottom right) to the red region, and the almost completely empty wells seen in the images of Figure 2.18 (left). From the remaining compounds, the average Hoechst count fold change was determined, including the blue and white regions of Figure 2.18 (bottom right), from which a minimum proliferation filter was implemented, one standard deviation below the average Hoechst count fold change of the remaining tested compounds in the plate, shown as the blue shaded region. From the yellow and red arrows, it can be seen that both yellow and red outlined wells survive the toxicity filters for further consideration.

2.3.14 Scoring and hit selection

The next step in the hit selection process was to create a combined average fold change score for transfection, proliferation, and viability measurements of each compound tested. The transfection (EGFP) score was determined by averaging the fold change in transfection efficiency with the four normalized fold changes of EGFP intensity. The proliferation score was determined similarly, by averaging the four normalized fold changes of Hoechst intensity. Lastly, the viability score was determined by averaging the two normalized fold changes in Resazurin intensity.
The highest and lowest transfection scoring compounds, up to ten each from each plate, were selected as potential hits, from which the preliminary hits for the screen were chosen, by inspection, based on transfection efficiency fold changes, transgene expression fold changes, and Hoechst count fold change in the screen data and images. Using Prism (GraphPad), one way ANOVA comparisons (Tukey’s post-test) were performed between EGFP transgene expression fold changes of the hit compounds and the plate vehicle controls. From these ANOVAs statistical significance of the priming fold change effect over the vehicle was determined. An example of these statistical analysis is demonstrated below in Figure 2.19, which shows how the fold changes for the duplicates of both concentrations relate to the transfected vehicle control wells. The well outline in yellow is Ketorolac, a nonsteroidal anti-inflammatory drug that showed significant fold change increases at both 5 and 50 μM concentrations, while the well outlined in red is Epigallocatechin gallate that showed significant fold change decrease in transfection at both 5 and 50 μM concentrations as well.

Figure 2.19. One-way ANOVAs for statistical analysis of duplicate normalized fold changes at 5 and 50μM
2.3.15 Preliminary screen verification

For a preliminary verification of the significant increases and decreases in transgene expression due to priming by the hit compounds identified by the screen, and to explore whether additional information could be revealed by luciferase and bicinechinonic acid (LUC/BCA) assay analysis of the screen lysate, compounds that were identified by the screen to significantly affect transfection (three compounds that increase transfection, and three compounds that decrease transfection) were tested in triplicate, in 48 well plate format, and assessed by LUC/BCA assay. The same seeding density, priming concentrations, and transfection conditions were used as in the screen, with volumes and amounts scaled from the 96-well to 48-well format by the same factor as the change in well growth surface area (0.32cm$^2$ to 0.75cm$^2$)

To quantify luciferase expression in cell lysate, 20 μL lysate samples were pipetted into 100 μL aliquots of LAR reagent (Promega), vortexed for 10 seconds, and measured by luminometer (Turner Designs). To quantify total protein concentration in cell lysate by BCA assay, 50 μL of lysate samples were pipetted into 100 μL BCA working reagent (50:1 BCA Reagent A:B) (Pierce), incubated for 30 minutes at 60 °C, then measured for absorbance at 562nm by spectrophotometer. (Beckman Coulter) For statistics, one way ANOVA’s with Tukey’s post-test were performed in Prism between the tested conditions and the transfected vehicle control.
2.4 Results and Discussion

Preliminary hit selection has been performed for four of the ten plates in the NCC thus far, producing 38 hits from 285 NCC compounds. Listed in tables in Appendix A are the top performers in terms of transfection score from the four NCC plates, demonstrating priming increases and decreases at 5 both 50 μM (Tables A.1 through A.4). To be clear, the entire NCC has been screened, data acquired and processed, simply the hit selection has not been performed on data from six of the plates. The EGFP Max/Min FC columns in Tables A.1 through A.4 denote the maximum or minimum average EGFP fold change in transfection measurements with priming by the NCC compound (in terms of plate reader and microscope image measurements, normalized by either total cell count, or transfected cell count).

From these lists, compounds were grouped to identify drug classes that prime transfection, identifying the 38 hits. The identified drug classes are represented in Table 2.1 below. This grouping was done by inspection, considering transgene expression and transfection efficiency fold changes, Hoechst count fold changes, and literature annotations of drugs class and known target receptors and pathways.

Table 2.1 Preliminary hit compound grouping by drug class.

<table>
<thead>
<tr>
<th>Compound Class</th>
<th># of hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSAID</td>
<td>8</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>7</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>7</td>
</tr>
<tr>
<td>Antifungal</td>
<td>4</td>
</tr>
<tr>
<td>Antihistamine</td>
<td>3</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>3</td>
</tr>
<tr>
<td>Stillbenoid</td>
<td>2</td>
</tr>
<tr>
<td>GABA modulators</td>
<td>2</td>
</tr>
<tr>
<td>Proton pump inhibitors</td>
<td>2</td>
</tr>
</tbody>
</table>
Statistical analysis of the hits in these drug classes was conducted by one way ANOVAs versus transfected vehicle controls, with Tukey’s post-test. The observed priming effects of the drug classes outlined in red in Table 2.1 above are discussed below.

2.4.1 Antibiotics decrease PEI transfection of HEK293T cells

Antibiotics appeared to generally knockdown transfection at the higher 50μM concentration, holding true for different classes of antibiotic, bactericidal and bacteriostatic, from cephalosporins, tetracyclines, to quinolones and fluoroquinolones. The generality of the effect over several different antibiotic classes and higher concentration at which the effect generally occurred seems to implicate a whole cell
response to priming by antibiotics that knocks down transfection, rather than an interaction with a very specific mechanism or pathway.

From the literature, it seems that the primary off target effects of antibiotics in mammalian host cells are associated with mitochondria [100]. In the clinic, chronic antibiotic treatments have been observed to cause ototoxicity, nephrotoxicity, tendinopathy, and various other side effects [101]. The endosymbiotic theory which describes the origin of mitochondria suggest that they were prokaryotes that became incorporated into eukaryotes, so it is hypothesized that antibiotics target mitochondria due to their similarities to bacteria, causing mitochondrial dysfunction and production of reactive oxygen species. Induction of an oxidative stress response could explain the knockdown effect on priming which antibiotics seem to have on transfection.

2.4.2 Stilbenoid and flavonoid antioxidants increase PEI transfection of HEK293T cells

Antioxidants were observed to have significant priming effects in increasing transfection efficiency and transgene expression. Resveratrol is a natural phenol,
specifically a stilbenoid, with antioxidant properties that can be found in the skin of grapes [102]. There was toxicity associated with 50 μM and 5 μM resveratrol priming, with 50 μM killing most cells in the well, however 5 μM toxicity was moderate, and resulted in one of the highest overall increases to normalized EGFP expression observed in the entire screening process, with a 3-fold increase in EGFP expression. Piceid is a glucoside modification of resveratrol, which is also found naturally in plants. This modification seems to blunt the effect of the priming increase on transfection compared to resveratrol, while also causing less toxicity. Like antibiotics, antioxidants affect mitochondria, however in a manner that generally protects or improves their function [102]. Resveratrol has also been shown to rescue mitochondrial dysfunction and modulate autophagy [103], which could have beneficial effects in the context of cytotoxic effects of PEI transfection [39]. Rescued mitochondria could regain normal function, while autophagy inhibits apoptosis.

Figure 2.22. Flavonoid priming fold changes in transgene expression
Flavonoids are another class of plant-derived phenols [104], though the effects of their priming upon transfection varied. Epigallocatechin gallate for instance, highly knocked down transfection at both 5 and 50uM concentrations, yet Ipriflavone and Icariin both increased transfection. If these antioxidants are each modulating transfection through mitochondria and oxidative stress response [105], the variation in priming effects may indicate that the cellular stress response to transfection can be modulated to increase or decrease transfection, depending on the specific interactions with mitochondrial and oxidative pathways each compound affects.

2.4.3 GABAA allosteric modulators increase PEI transfection of HEK293T cells

Figure 2.23. GABAA modulator priming fold changes in transgene expression

Diazepam and stiripentol both demonstrated a priming increase to transfection at both 5 and 50uM. These drugs are both positive allosteric modulators of the GABAA receptor found in neurons, however, they are thought to act on different subunits [106, 107]. The priming effects seen of these GABAA modulators is curious because
neurotransmitter receptors are not typically expressed in most cell types. An interesting possibility is that the proposed adrenal cortex origin of HEK293T cells [108] could imply that the GABAA receptor, or at least some of the subunits, may be expressed in their cell membrane. Another possibility is that these drugs are acting on the translocator protein, also known as the peripheral benzodiazepine receptor, which is found in the membranes of mitochondria, and thought to be involved in steroidogenesis, and redox regulation of mitophagy [109, 110]. The latter would make sense in the context of priming the cellular stress response to transfection that many of the other hits also imply.

2.4.4 Glucocorticoids increase PEI transfection of HEK293T cells

![Graphs showing glucocorticoid priming fold changes in transgene expression](image)

Figure 2.24. Glucocorticoid priming fold changes in transgene expression

Another group of compounds that was identified by the screen to prime and increase transfection were glucocorticoids. Glucocorticoids are hormones which are involved in the body’s stress response, activating the glucocorticoid receptor, which translocates to the nucleus and acts as a transcription factor. It has been demonstrated
previously in our lab that priming with glucocorticoids in human mesenchymal stem cells increases transfection many fold [49]

The mechanism is not yet known, though it has been shown by preliminary data to be associated with cytosolic glucocorticoid receptor, not the membrane glucocorticoid receptor. Activated glucocorticoid receptors may play a role in priming transfection by assisting internalization, nuclear transport, and nuclear import of transfection complexes. Alternatively, or perhaps in conjunction, the glucocorticoid priming effects may be occurring through modulation of mitochondria and autophagy [111, 112], which would align with the possible mechanisms of priming other drug classes showed in the screen in relation to the cellular stress response to transfection.

2.4.5 Preliminary Screen Verification

Six of the compounds identified by the screen to have priming effects at 5 μM were re-tested for validation in triplicate. The validation experiment was seeded in a 48-well plate format, and LUC/BCA assays were used to assess transfection. Three of these compounds had been shown by the screen to enhance transfection, with the other three shown by the screen to decrease transfection.
From Figure 2.25, the relative levels of luciferase expression for all six compounds, normalized by total protein, indicate that the screening process was able to identify compounds that have priming effects on transfection. The yellow bars indicate compounds that increased transfection in the screen, and did the same in this validation experiment. The red bars indicate compounds that had decreased transfection in the screen. In this validation experiment, one of the compounds, epigallocatechin gallate, was consistent in knocking down transfection, but the other two compounds instead showed an apparent increase in transfection within this validation experiment.

This result is not necessarily a conflict with the screen data, as luciferase expression normalized by total protein does not providing the exact same information as EGFP intensity measurements normalized by cell count. The relative transgene protein expression levels should be consistent, but the observed differences could be attributable to the different method of normalization, if total protein does not always necessarily
correlate with total cell count. The differences could also simply be due to differences in well plate format or un-optimized scaling of the various reagents to the larger well area.

The discrepancy should be investigated to prove whether the priming effects observed of those drugs in the screen are real or a false positive. Some number of false positives are inevitable in high throughput screening. In fact, it is encouraging that four of the six identified in the screen to have priming effects, repeated those effects in the verification experiment. A visual representation is given in Figure 2.26 below, which shows the differences in EGFP expression in the priming conditions versus the transfected vehicle control. EGCG consistently demonstrated consistency in knocking down transfection, while resveratrol was one of the highest performers in the screen, and continued to demonstrate high fold change enhancement of PEI transfection. Corticosterone also repeated its enhancement of transfection, confirming its result in the screen and also providing additional evidence of the reliability of glucocorticoid priming. Noticeable in many of the compounds which had high fold change increases to transfection were increased toxicity. The toxicity may be due to the priming compound’s toxic effects, or amplification of the toxic effects of PEI, though another possibility is that the production of mass quantities of EGFP transgene is causing cytotoxicity through either EGFP toxicity [113] or the energy burden of transgene overexpression. An option to test whether the toxicity is related to overexpression is to use a weaker promoter in the pDNA sequence and/or less efficient formulation of PEI.
Figure 2.26  LUC/BCA verification images showing relative EGFP expression in cells primed with resveratrol, corticosterone, and EGCG versus transfected vehicle control treatment.

2.5  Conclusion

Altogether, the literature review of grouped screen hit compounds implies that many of the priming effects may be due to modulation of the cellular oxidative stress response to PEI transfection, in particular mitochondrial dysfunction. PEI has been shown to have cytotoxic effects through its accumulation in and interaction with mitochondrial membranes [39]. Damage to mitochondria has been implicated in the intracellular inflammatory and immune response [114, 115], and PEI has also been shown to be an effective adjuvant to stimulate an immune response [116].

Typical cellular responses to mitochondrial damage are autophagy/mitophagy, apoptosis, and necrosis. Apoptosis and autophagy are mutually inhibitory [117], with apoptosis leading to cell death, while autophagy possibly serves a protective role in PEI
cytotoxicity [118]. It seems plausible that antioxidants, in general, rescue the cell from PEI-induced mitochondrial dysfunction, thus improving transfection, while priming with other compounds, like antibiotics, may themselves induce mitochondrial dysfunction, or perhaps promote autophagy or apoptosis.

Gene expression profiling has shown that there is differential expression in the cellular oxidative stress response associated with successfully versus unsuccessfully PEI transfected cells, through ATF3, IREB2 and other gene pathways [50-52]. The screen data from this thesis support this claim, and suggest that modulating the cellular stress response by priming with clinically approved compounds prior to PEI transfection can have significant effects on overall transfection efficiency and transgene expression in vitro.

Not only did the screen identify dozens of compounds that modulate PEI transfection, the corroboration of the screen results with other work in the literature indicates that the screen was successful in reinforcing and improving understanding of pathways with which the total cellular response to nonviral gene delivery can be modulated to improve transfection, and suggests specific classes of clinically approved compounds which may be immediately useful in vitro and in vivo.
CHAPTER 3

Further Processing and Verification of the High Throughput Screen, and
Investigation of Priming Mechanisms Involving Mitochondria and Cellular Stress

3.1 Introduction

With the plenty of information gained from the screen, future work can proceed in many directions, from completing the initial hit selection process from the data of six of the ten NCC plates, to further analyzing the data already collected in the screen to extract more information. A follow up high throughput LUC/BCA screen can be performed on the lysate in cold storage, possibly with ATP quantification, to assess the initial imaging screen’s performance, providing complementary transgene expression data, as well as an additional cellular stress response measure. The screen image set can be further analyzed by incorporating more sophisticated imaging algorithms to extract additional relevant data. In the initial hit selection process, only EGFP expression and efficiency fold changes and Hoechst count fold changes were considered as criteria, so Hoechst intensity and Resazurin fold changes should also be analyzed in concert with the EGFP data to understand the cell state at the time transfection was assessed, specifically cell viability and extent of apoptotic and necrotic cell death. All of the screen measurements should be incorporated into a more sophisticated hit selection strategy that incorporates the screen data, literature information on candidate compound mechanisms and activity, and previously identified differential gene expression profiles of PEI transfection in HEK293T cells [50-52] into a network analysis approach to select priming candidates by
a method that is sensitive to trends and patterns in the data, rather than relying on inspection to catch the obvious trends. Additionally, the compounds which have been identified by the screen thus far should be investigated in detail and incorporated as priming adjuvants in new and effective gene delivery strategies.

3.2 Assessing screen performance

Preliminary analysis of the screen thus far shows that it has been successful in achieving its purpose by identifying 38 compounds, and nine different drug classes, for further investigation into priming of transfection (See Tables A.1-A.4 in Appendix A, and Table 2.1 in Chapter 2). However, a true assessment of screen performance and the hit selection methods used remains to be completed. In automated high throughput screens of siRNA, a primary screen is generally conducted without replicates and followed up with a confirmatory screen of at least triplicate [119]. In comparison, the transfection priming screen of this thesis could be thought of as a primary screen in duplicate. The addition of a duplicate to the primary screen should provide more confidence in preliminary examination of the screened compounds, but it is not enough replicates for the screen to be considered confirmatory [119].

The initial approach was to perform LUC/BCA validation of a few compounds of interest that could then be immediately investigated if confirmed. Preliminary validation of this type was performed that was generally confirmatory of the original hits, with two of the six compounds showing an opposite effect from screen observation (Figure 2.25 in Chapter 2), though as discussed previously, these differences could possibly be explained
by an un-optimized switch from 96 well to 48 well format or the differences between normalization by total cell count and by total protein. Even if all six compounds had confirmed their effects observed in the screen, six compounds out of 725 is not a sufficient sample size to assess the screen’s performance, and four out of six is actually encouraging in providing evidence that the screen results are real.

The most complete way to assess the performance of this initial primary duplicate screen would be to perform a total confirmatory screen, amounting to a repeat of the screen in triplicate. However, the cost in materials and manual effort involved in the initial screen make it unlikely that a total confirmatory screen will be performed without automation of the liquid handling and imaging processes. An alternative would be to do a partial confirmatory screen, that is, repeat the screen in triplicate for a subset of the entire collection, possibly a plate or small number of plates in the collection, from which the initial screen’s performance could be estimated.

3.3 High throughput LUC/BCA assays and ATP quantification of screen lysate for imaging screen validation and complementary measures of transfection

An alternative to a total or partial confirmatory repeat screen could be high throughput assays of LUC/BCA performed on the frozen lysate of the initial screen. This alternative measure of transfection could be used to validate the initial fluorescence imaging method. Compared to the initial LUC/BCA validation experiment (Chapter 2.4.5), there should be no issue with normalization or plate format, as the counts from the initial screen can be used for normalization and there is no need to repeat the seeding,
priming, and transfection processes, as the lysate is ready to test. The BCA assay will also incorporate total protein into the dataset as a new relevant measure of the cell response to transfection, a complementary measure of toxicity and/or viability, or an alternative measure for normalization of the screen data.

The screen was conducted with a plasmid encoding an EGFPLuc fusion protein, so LUC/BCA assays can be performed on the lysate, which has been stored at -80 °C. High-throughput luciferase assay reagent kits are offered by several manufacturers, which use a “glow” reagent formulation that reacts with luciferase for sustained luminescence, allowing multiple wells to be measured over an extended period of time without steep drop off in photons emitted due to long reaction times, which would cause erroneous variation in apparent luciferase between wells [120].

Given access to a plate luminometer, the primary barrier to performing these assays would be reagent cost. To perform LUC/BCA assays on the 40 plates in the screen, at a luciferase assay reagent cost of at least fifty cents per sample (Steady-LUC Firefly HTS Assay Kit, Biotium), material costs would be at least $3,000. With “homemade” luciferase assay reagent, the total cost could be brought down to about $1,000 total [120]. Also, a potential advantage of processing the screen lysate is the opportunity to incorporate an ATP quantification assay along with the LUC/BCA measurements. This is convenient because ATP quantification assays rely on the same luminescent reaction that is used to quantify luciferase [121], as shown in Figure 3.1 below.
In luciferase quantification, the kinetics of the luminescent reaction depend on the concentration of firefly luciferase present in the lysate [120, 121]. In ATP quantification by this same reaction, d-luciferin and luciferase are mixed at specific concentrations, and a standard curve of luminescence versus ATP concentration is measured. The relative luminescence of lysate samples treated with the same concentrations of d-luciferin and luciferase can then be compared to the standard curve to determine ATP concentration present in the lysate [120, 121]. ATP quantification can easily be multiplexed into the LUC/BCA assay, simply requiring more of the same reagents used in LUC quantification, as well as a portion of the sample lysate. Besides additional cost, the only barrier to ATP quantification would be the ability of the ATP, LUC, and BCA assays to perform accurately with smaller portions of the screen lysate sample volume.

Measurement of ATP would be complementary to the Resazurin measurements already acquired in the screen. Resazurin assays measure the overall redox state of the
cell, while measuring ATP quantifies the available energy currency of the cell [123]. Both are markers for cell viability, however, they are related but not equivalent measures, and analysis of both together might reveal subtle details of the manner in which metabolic pathways are disturbed by PEI transfection cytotoxicity and possibly rescued by some of the identified priming compounds (antioxidants, GABAA modulators, glucocorticoids, etc., see Chapter 2.4)

3.4 Normalized Hoechst intensity measurements as a marker for cell cycle and cell death

For the screen presented in Chapter 2, the Hoechst stain was used for total cell enumeration, as both a measure of toxicity and means of normalization, but more information can be extracted from the screen data through the normalized Hoechst intensity measurements. While mean image intensity of a Hoechst stain image is primarily dependent on cell count, normalizing Hoechst intensity by cell count will result in a metric that reveals permeability of the cell and nuclear membranes as well as the packing and integrity of chromatin [124, 125]. These parameters are markers for cell cycle state, as well as apoptosis and necrosis [126]. Cell cycle and cell death pathways can be associated with transfection efficiency, transgene expression, and metabolic viability, and contribute to a more complete understanding of priming effects on transfection through modulation of the total cellular response.
3.5 Phase contrast image analysis as a measurement of cell morphology

The screen image set can be further analyzed by incorporating more sophisticated image processing algorithms to extract more relevant data. As mentioned previously (Chapter 2.3.6), there is potential for the screen’s phase contrast images to be processed by algorithms that can normalize the differences in illumination and shadow, after which, total cell area can be calculated by thresholding algorithms. Normalization of cell area by total cell count would provide a metric of average cell morphology, specifically cell spreading, which could imply information of cell motility and extent of cell adhesion to the well bottom substrate. Interpretation of cell spreading as a measurement of cell motility and adhesion could allow cell spreading to serve as a proxy measurement of cell membrane features related to environmental sensing and phagocytosis, such as actin podia features and membrane ruffling, which preliminary work in our lab has indicated may play a role in transfection complex internalization (data not shown).

3.6 Image segmentation to enable determination of EGFP expression of subpopulations sorted by Hoechst stain

The image processing used in the screen described in Chapter 2 has been simple in its approach. Total image mean fluorescence intensity was processed to average fluorescence intensity per cell when normalized by cell count. A more nuanced approach would be to actually measure fluorescence intensity for each cell, allowing for population distributions to be determined. This approach would be enabled by cell segmentation,
which is simply identification of boundaries for each cell in an image. Cell segmentation image processing algorithms are fairly complex and were outside the initial scope of this screen. Such algorithms achieve segmentation by fitting contours to image features, and can be implemented by custom code, freeware such as ImageJ and CellProfiler, or commercial image cytometry software.

For this screen, the phase image of each well would be the source image from which the segmentation algorithm determines cell boundaries, so the previously described preprocessing normalization of illumination and shadows would be necessary. Segmented cell outlines can then be applied to the EGFP fluorescence images, from which EGFP intensities of each cell could be measured and used to create a histogram which captures the number of cells that are expressing EGFP at each observed intensity. From these observed probability distributions of both EGFP and Hoechst intensities, 2D histograms (scatter plots) could be created for each screened compound that allow for association of EGFP expression with different subpopulations of cells sorted by Hoechst stain. This type of information would be a more powerful data set from which to interpret priming effects of the screened compounds on PEI transfection.

3.7 Interpreting the screen data by network analysis

From the initial screen, and potential follow-up LUC/BCA/ATP screen, for each compound within the drug college, there will be a collection of measurements related to the cellular response to priming and transfection, as well as measurements of transfection efficiency and transgene expression. Beyond providing obvious candidates for
immediate investigation as priming agents, the data can be analyzed as a whole to inform our general understanding of transfection and the cellular response to transfection. For instance, a relationship between transfection priming of certain compound classes and mitochondrial dysfunction modulation is suggested by preliminary examination of the data. A rigorous network analysis of the data set should support (or deny) this correlation, while other relationships may become apparent. The network analysis should incorporate normalized transfection measurements (EGFP, Luc), normalized cellular response measurements (Hoechst, Resazurin, BCA, ATP, Phase area), keyword annotations of known targets of the screened compounds (receptors, pathways, organelles, etc.), and the gene expression profiles of successfully and unsuccessfully transfected cell populations. The software of choice for analysis of high throughput screen data is the R/Bioconductor statistical computing language and environment, which has HTS packages generally tailored for siRNA HTS and DNA microarray data. R/Bioconductor is the software which was used in previous FACS/microarray experiments in our lab, which we would like to incorporate into the network analysis for hit selection in any case, so R/Bioconductor network analysis and high throughput screening packages would be a good place to start for this bioinformatics approach to hit selection.
3.8 Verification and investigation of priming mechanisms that involve modulation of mitochondrial dysfunction and cellular stress response.

The majority of compounds identified as hits thus far from the screen seem to possibly be modulating the mitochondrial dysfunction that is caused by PEI toxicity. To verify the priming effects of these compounds, priming with these compounds should be tested at a variety of concentrations, over a variety of time points pre- and post-transfection, to determine the breadth of the priming effect and optimum priming condition. The timing information will be important in determining the window of time cells can be primed by these compounds to have their effect on mitochondrial dysfunction in a way that improves or decreases transfection. Optimized timing and dosing parameters can be determined for each compound for use in the experiments which follow that attempt to decipher the specific mechanisms of the observed priming effects.

There are methods that mitochondria can be assayed directly. Mitochondrial respirometry can be performed on isolated mitochondria to measure changes in oxygen flux due to treatment of the priming agent [127]. Certain probes are also now able to measure oxygen consumption in cultured cells (XF Extracellular Flux Analyzer, Seahorse Bioscience) [128]. Also membrane potential can be monitored to examine how redox potential across the mitochondrial membrane can change in response to PEI and priming compounds. ATP is also a good indicator of mitochondrial viability, which can be measured by luminescence [121].
Another approach would be to mimic the gene expression profiling microarray experiments performed in our lab previously [50-52], in terms of sorting the transfected and un-transfected cell populations and assaying the subpopulations for differential gene expression, in this case by qRT-PCR of genes that had been previously identified to be related to cellular stress response, such as RAP1A, ATF3, IREB2, as well as genes whose expression are known specific markers for mitochondrial dysfunction, autophagy, apoptosis, and necrosis [39, 117, 129].

In addition to the qRT-PCR measures of gene expression, the transfected and untransfected subpopulations should also be run through subcellular fractionation by gradient centrifugation in order to quantify relative amounts of PEI and plasmid in the various cell compartments. In subcellular fractionation, cells are gently lysed and centrifuged through a density gradient to separate organelles by density. With optimized subcellular fractionation protocols, nuclei, mitochondria, lysosomes, and cytoplasmic fractions can be isolated. These separated fractions can then be measured for both plasmid count and PEI concentration, with the plasmid count quantified by qRT-PCR, and the PEI likely pre-labeled by conjugation to a fluorophore for fluorescence quantification. These localization measurements in the various cellular compartments should also be determined at various time points after priming and transfection for an estimate of the transport mechanisms that are taking place for both the plasmid and PEI. The change in PEI localization within the various compartments over time should be informative, especially the amount of PEI which is found in mitochondria, in terms of the mitochondrial dysfunction modulation priming hypothesis.
Pull down methods and/or high resolution FRET imaging should also be used to identify specific protein/protein and protein/DNA interactions that may be affecting the behavior and interactions of complexes and cell machinery in response to priming. siRNA could be useful for identifying such proteomic interactions as well by knocking down specific genes/proteins and observing the resulting impact on priming. siRNA may provide more precision in modulating a gene pathway than chemical inhibitors/activators. From these experiments, screen hits should be verified by correlation of transfection fold changes, cell viability measures, and specific changes in gene expression related to cell stress response, autophagy, apoptosis, and necrosis, relative amounts of plasmid and PEI in the various compartments of the cell, as well as specific protein/protein and protein/DNA interactions, all in the context of successfully versus unsuccessfully transfected cell populations. These experiments will hopefully illuminate the specific mechanisms by which priming can rescue or mitigate mitochondrial dysfunction due to PEI toxicity.

3.9 Final thoughts

Through high throughput screening of the NIH Clinical Collection, priming compounds which impact nonviral gene delivery have been identified which reinforce and improve our understanding of transfection and provide new tools for improving the efficiency of gene transfer. With partial, preliminary hit selection from the collected data, dozens of clinically approved priming compounds have potentially been identified.
The remainder of the data should be assessed, possibly with more sophisticated image processing techniques and hit selection approaches.

Grouping of the preliminary hits has implicated modulation of the cellular stress response and mitochondrial dysfunction as important aspects of PEI transfection, so the specific mechanisms by which these drug classes modulate mitochondria to affect transfection should be investigated in detail, which will require a detailed understanding of mitochondrial physiology, cellular stress pathways, and interplay between autophagy, apoptotic, and necrotic cell death. The mechanistic relationships between PEI transfection and different priming compounds on all of these processes will require extensive literature review and experimentation. The apparent success of this study in identifying clinical compounds that prime nonviral gene delivery could also warrant a comprehensive automated screen of a larger compound library in combination with different cell types, carriers, and nucleic acids, to confirm priming as a generally applicable tool for nonviral gene delivery.

Overall, the identification of clinically approved priming compounds by this screen is promising for the potential of their future implementation as adjuvants of nonviral gene delivery in vivo and eventually in clinical gene therapy applications. Even the clinical compounds that were found to decrease transfection are also possibly important to understand as potential contraindications for patients who are depending on expression of a therapeutic gene through nonviral gene delivery.
REFERENCES


[90] Clontech. pEGFPLuc Vector Information, PR08343. PT3347-5, Catalog #6169-12000.


APPENDIX A: Tables listing NCC Compounds that prime transfection

- **FC**: Fold-change
- **EGFP Max/Min FC**: Maximum or minimum fold change in EGFP expression or transfection efficiency, plate reader or image measurement, normalized by total cell count or transfected cell count.
- **Bolded drugs** were selected as hits from these lists by fold change, toxicity, and drug grouping based on literature annotations of each drugs known mechanisms of action.

Table A.1  NCC Compounds that increase transfection at 50 μm

<table>
<thead>
<tr>
<th>EGFP Max FC</th>
<th>Hoechst Count FC</th>
<th>Drug Name</th>
<th>Grouped Drug Class</th>
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Table A.2  NCC Compounds that increase transfection at 5 μM

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</tr>
<tr>
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</tr>
<tr>
<td>AM251</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>METHOXYTRYPTAMINE</td>
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<tr>
<td>MESALAZINE</td>
<td>NSAID</td>
</tr>
<tr>
<td>SELEGILINE</td>
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</tr>
<tr>
<td>ITOPRIDE</td>
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<tr>
<td>QUETIAPIINE</td>
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<tr>
<td>CYCLOSERINE</td>
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<td>OXAPROZIN</td>
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</tr>
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# Table A.3  
NCC Compounds that decrease transfection at 50 μM

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<td>CEFDINIR</td>
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<td>CEFIXIME</td>
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<td>TAXIFOLIN</td>
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# Table A.4  
NCC Compounds that decrease transfection at 5 μM

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<td>TOSUFLOXACIN</td>
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<td>TERBINAFINE</td>
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<td>SDM25N</td>
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<td>LOXAPINE</td>
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<td>NAPROXEN</td>
<td>NSAID</td>
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<td>MALTOL</td>
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<td>CEFIXIME</td>
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<td>ISOQUERCETIN</td>
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</tr>
<tr>
<td>5-(NONYLOXY)TRYPTAMINE</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX B: ImageJ macros used for image processing

Image intensity and local maxima detection macro:

```java
start=1;
imagesperwell=3;
end=288;
path="C:\Users\anguyen2\Desktop\AN-15-40\10\5uM\2\";
i=1;
count=0;

for(x=start+1; x<=end; x=x+3)
{
    //gfp images
    open(path+"image"+x+".tif");
    run("Measure");
    run("Enhance Local Contrast (CLAHE)", "blocksize=127 histogram=256
    maximum=3 mask=*None* fast_(less_accurate)");
    run("Subtract Background...", "rolling=10");
    run("Find Maxima...", "noise=10 output=Count");
    selectWindow("image"+x+".tif");
    close();
}

for(x=start+2; x<=end; x=x+3)
{
    //hoechst images
    open(path+"image"+x+".tif");
    run("Measure");
    run("Subtract Background...", "rolling=10");
    run("Find Maxima...", "noise=10 output=Count");
    selectWindow("image"+x+".tif");
    close();
}
```
Plate montage creation macro:

```
start=1;
imagesperwell=3;
end=288;
path="C:\Users\anguyen2\Desktop\AN-15-40\10\5uM\2\";
i=1;
count=0;

//phase
for(x=start; x<=end; x=x+3)
{
    open(path+"image"+x+".tif");
}

run("Images to Stack", "name=Stack title=[] use keep");
run("Make Montage...", "columns=8 rows=12 scale=1 first=1 last=96 increment=1 border=10 font=12");

for(x=start; x<=end; x=x+3)
{
    selectWindow("image"+x+".tif");
    close();
}

selectWindow("Montage");
run("Rotate 90 Degrees Left");
run("Flip Vertically");

waitforUser;

//gfp
for(x=start+1; x<=end; x=x+3)
{
    open(path+"image"+x+".tif");
}
```
run("Images to Stack", "name=Stack title=[] use keep");
run("Make Montage...", "columns=8 rows=12 scale=1 first=1 last=96 increment=1 border=10 font=12");

for(x=start+1; x<=end; x=x+3)
{
    selectWindow("image"+x+.tif");
    close();
}

selectWindow("Montage");
run("Rotate 90 Degrees Left");
run("Flip Vertically");

waitForUser;

//hoechst
for(x=start+2; x<=end; x=x+3)
{
    open(path+"image"+x+.tif");
}

run("Images to Stack", "name=Stack title=[] use keep");
run("Make Montage...", "columns=8 rows=12 scale=1 first=1 last=96 increment=1 border=10 font=12");

for(x=start+2; x<=end; x=x+3)
{
    selectWindow("image"+x+.tif");
    close();
}

selectWindow("Montage");
run("Rotate 90 Degrees Left");
run("Flip Vertically");