2011

Microarray Analysis of Gene Expression Profiles in Cells Transfected With Nonviral Vectors

Sarah A. Plautz
University of Nebraska-Lincoln, sarah.plautz@unl.edu

Gina Boanca
University of Nebraska-Lincoln

Jean-Jack M. Riethoven
University of Nebraska-Lincoln, jeanjack@unl.edu

Angela K. Pannier
University of Nebraska-Lincoln, apannier2@unl.edu

Microarray Analysis of Gene Expression Profiles in Cells Transfected With Nonviral Vectors

Sarah A Plautz[1], Gina Boanca[1], Jean-Jack M Riethoven[2] and Angela K Pannier[1]

[1]Department of Biological Systems Engineering, University of Nebraska-Lincoln, Lincoln, Nebraska, USA; [2]Bioinformatics Core Research Facility, University of Nebraska-Lincoln, Lincoln, Nebraska, USA

Inefficient gene delivery is a critical factor limiting the use of nonviral methods in therapeutic applications including gene therapy and tissue engineering. There have been few efforts to understand or engineer the molecular signaling pathways that dictate the efficacy of gene transfer. Microarray analysis was used to determine endogenous gene expression profiles modulated during nonviral gene transfer. Nonviral DNA lipoplexes were delivered to HEK 293T cells. Flow cytometry was used to isolate a population of transfected cells. Expression patterns were compared between transfected and nontransfected samples, which revealed three genes that were significantly upregulated in transfected cells, including *RAP1A*, a GTPase implicated in integrin-mediated cell adhesion, and *HSP70B'*, a stress-inducible gene that may be important for maintaining cell viability. Furthermore, *RAP1A* was also significantly upregulated in untransfected cells that were exposed to lipoplexes but that had not expressed the transgene as compared to control, untreated cells. Transfection in the presence of activators of upregulated genes was enhanced, demonstrating the principle of altering endogenous gene expression profiles to enhance transfection. With a greater understanding of signaling pathways involved in gene delivery, more efficient nonviral delivery schemes capitalizing on endogenous factors can be developed to advance therapeutic applications.

Received 9 February 2011; accepted 9 May 2011; published online 9 August 2011. doi:10.1038/mt.2011.161

INTRODUCTION

Gene delivery approaches provide a mechanism to directly alter gene expression within a cell population, with tremendous potential in basic science (*e.g.*, to study gene expression), therapeutics (*e.g.*, gene therapy to correct genetic deficiencies or treat acquired disease), and tissue engineering (*e.g.*, to present factors in scaffolds that guide tissue formation). Nonviral delivery techniques are less efficient than viral systems, but offer the advantages of low toxicity and immunogenicity, lack of pathogenicity, and ease of production with greater control and flexibility, making these vectors attractive alternatives to viruses. Typical nonviral gene delivery systems involve the ionic/electrostatic complexation of DNA with cationic polymers (polyplexes)[1] or lipids (lipoplexes).[2] Complexation protects DNA against degradation by nucleases and serum components and enhances cellular uptake by reducing the effective size of DNA and promoting interactions between positively charged DNA complexes and the negatively charged cellular membrane. These complexation agents also can facilitate intracellular trafficking, while dissociating from the DNA to allow expression.[3,4] However, even with complexation strategies as described above, both extracellular[5] and intracellular[6] barriers exist that prevent efficient nonviral gene transfer. Extracellular barriers to gene delivery include mass transport limitations, cytotoxicity, degradation, and aggregation,[5] as well as cell targeting. Once a complex reaches a target cell, intracellular barriers to gene transfer include cell binding, cell entry, release from endosomal compartments, cytosolic transit, nuclear entry,[7] and subsequently expression of the transgene.[6]

To date, most efforts to understand and improve the efficiency of nonviral gene delivery have focused on altering the physicochemical properties of delivery systems and developing new delivery strategies, focusing on the physical and chemical characteristics of the cationic vector[8–10] or examining the effect of complexing agents on subcellular localization/distribution,[11] intracellular trafficking and pharmacokinetics,[12–14] and transfection efficiency of DNA complexes, providing only a fragmented understanding of that process because of the differences in techniques and study variables, without regard to the underlying cellular states or signaling pathways. Even with these efforts, nonviral gene delivery remains inefficient and its mechanisms largely unknown because of the complexity of the process. The lack of direct information on the endogenous gene expression profiles and intracellular signaling pathways that might be controlling transfection efficiency limits the optimal design of delivery vectors.

Microarray analysis has been performed to investigate the toxicogenomics of nonviral vectors used for gene delivery.[15–18] However in these previous studies, the objective was to treat the cells with cationic polymer or lipid (often in the absence of complexation with DNA) to observe the associated gene expression profiles and cellular responses, in particular cytotoxicity. In these studies, there was no isolation of transfected cells from untransfected, rather cell populations treated with vector alone or vector complexed with DNA were compared to untreated conditions. In most studies,

Correspondence: *Angela K Pannier, Department of Biological Systems Engineering, University of Nebraska-Lincoln, 231 LW Chase Hall, Lincoln, Nebraska 68583-0726, USA. E-mail: apannier2@unl.edu*

apoptosis was observed and confirmed with gene expression profiles, which should not be unexpected as nonviral vectors are typically more toxic when not complexed with DNA.[19]

While these previous studies have used microarray analysis to explore global gene expression profiles for pharmacological and toxicological studies of lipids and polymers used in nonviral gene delivery, there have been few efforts to understand the molecular signaling pathways that dictate efficient gene transfer. In this report, gene expression profiles of an isolated population of successfully transfected cells were investigated using microarray analysis and related to cellular mechanisms that might be responsible for successful gene transfer. Upregulation of two identified genes was confirmed with transfection studies performed in the presence and absence of activators of these genes. With a greater understanding of key signaling pathways involved in gene delivery afforded by the analysis reported here, the mechanisms that render cells responsive to DNA transfer will be determined, which can be used to develop more efficient nonviral delivery schemes.

RESULTS

Microarray analysis was used to identify how endogenous gene expression profiles are modulated during nonviral gene transfer. Nonviral DNA complexes (composed of cationic lipids complexed with plasmid DNA encoding for green fluorescent protein, GFP) were delivered to HEK 293T human embryonic kidney epithelial cells, a widely used cell line in transfection experiments (**Figure 1**). Transfection conditions were optimized for both high transfection efficiency and low cytotoxicity (**Supplementary Figure S1**). Flow cytometry was then used to sort GFP$^+$ cells from GFP$^-$ cells, allowing isolation of a population of transfected cells and a population of cells that had been exposed to complexes, but not transfected, respectively (**Figure 1** and **Supplementary Figure S2**). Expression patterns were compared between GFP$^+$ and GFP$^-$ samples (six versus three replicates), which revealed three genes that were statistically differentially expressed between the two samples (twofold or greater change in expression, false discovery rate (FDR) adjusted $P < 0.05$, **Table 1**). These three genes that were substantially upregulated in the GFP$^+$ (transfected cells) as compared to GFP$^-$ (untransfected cells), include *RAP1A*, a GTPase implicated in a variety of cell behaviors including integrin-mediated cell adhesion; *HSP70B′*, a stress-inducible gene that encodes for a heat shock protein that may be important for maintaining cell viability; and *PVT1*, a non-protein coding oncogene.

Expression patterns were compared between GFP$^-$ and Control samples (three versus six replicates) to determine genes which may be upregulated in cells that have been exposed to DNA complexes, but that were not expressing GFP at the time when cells were sorted and RNA was isolated. *RAP1A* was the only gene to be upregulated, with a substantial fold change in expression (FDR adjusted $P < 0.05$, **Table 2**).

Finally, expression patterns were compared between GFP$^+$ and Control samples (six replicates each), which revealed 19 genes

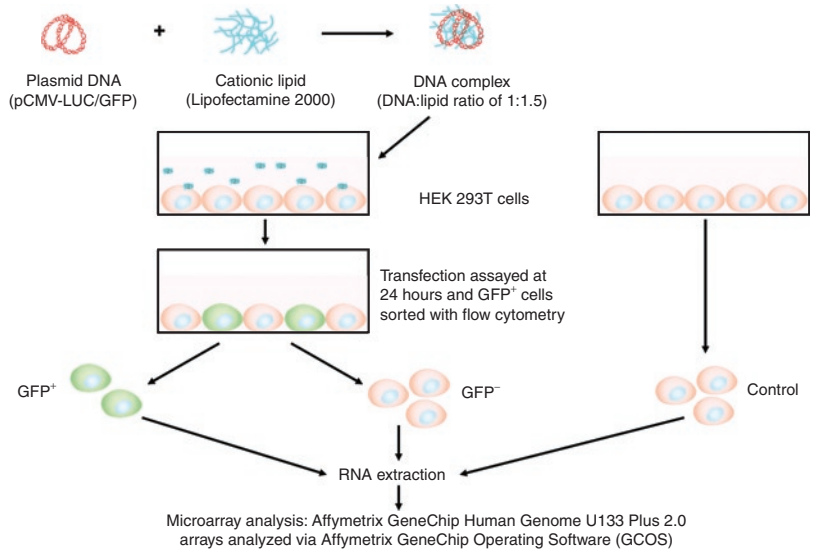

Figure 1 Schematic of experimental design. Cell transfection, sorting of transfected cells (GFP$^+$) from untransfected cells (GFP$^-$) cells using flow cytometry, as well as Control cell population, RNA extraction and microarray analysis used to generate gene expression profiles.

Table 1 Genes upregulated in transfected cells (GFP$^+$) as compared to untransfected cells (GFP$^-$)

Representative public ID	Gene symbol	Gene title	Fold change
NM_002155/X51757	***HSPA6***	Heat shock 70 kd protein 6 (HSP70B′)	10.332/2.966[a]
AB051846	***RAP1A***	RAP1A, member of RAS oncogene family	9.172
BG200951	***PVT1***	Pvt1 oncogene (non-protein coding)	2.507

Abbreviation: GFP, green fluorescent protein.
Upregulation greater than twofold and FDR adjusted $P < 0.05$.
[a]Two fold changes are reported as this gene is located at two distinct probes on microarray.

Table 2 Genes upregulated in untransfected cells (GFP⁻) as compared to Control cells

Representative public ID	Gene symbol	Gene title	Fold change
AB051846	***RAP1A***	RAP1A, member of RAS oncogene family	59.162
AB051846	***RAP1A***	RAP1A, member of RAS oncogene family	110.187

Abbreviation: GFP, green fluorescent protein.
Upregulation greater than twofold and FDR adjusted $P < 0.05$.

Table 3 Genes upregulated in transfected cells (GFP⁺) as compared to Control cells

Representative public ID	Gene symbol	Gene title	Fold change
AB051846	*RAP1A*	RAP1A, member of RAS oncogene Family	542.631
AB051846	*RAP1A*	RAP1A, member of RAS oncogene family	1016.865
NM_002155/X51757	*HSPA6*	Heat shock 70 kd protein 6 (HSP70B′)	12.001/3.773[a]
X65232/AA284829	*ZNF79*	Zinc finger protein 79	2.261/3.026[a]
NM_001674/AB066566	*ATF3*	Activating transcription factor 3	3.775/3.328[a]
NM_002394	*SLC3A2*	Solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	2.121
BG538800	*ZNF473*	Zinc finger protein 473	2.004
BG200951	*PVT1*	Pvt1 oncogene (non-protein coding)	2.514
BE788667	*PRR14*	Proline rich 14	2.772
BC000487/NM_012230	*POMZP3 /// ZP3*	POM (POM121 homolog, rat) and ZP3 fusion /// zona pellucida glycoprotein 3 (sperm receptor)	2.532/2.664[a]
NM_000107	*DDB2*	Damage-specific DNA binding protein 2, 48 kd	2.521
NM_006145/BG537255	*DNAJB1*	DnaJ (Hsp40) homolog, subfamily B, member 1	2.2/2.293[a]
BC002426	*PEA15*	Phosphoprotein enriched in astrocytes 15	2.047
AF003934	*GDF15 /// LOC100292463*	Growth differentiation factor 15 /// similar to growth differentiation factor 15	2.576
U83981/NM_014330	*PPP1R15A*	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	2.003/2.291[a]
NM_002133	*HMOX1*	Heme oxygenase (decycling)1	3.179
NM_001613	*ACTA2*	Actin, α-2, smooth muscle, aorta	2.116
AW592227	*KHDRBS1*	KH domain containing, RNA binding, signal transduction associated 1	2.015
BF340083	*SLC1A4*	Solute carrier family 1(glutamate/neutral amino acid transporter), member 4	2.554

Abbreviation: GFP, green fluorescent protein.
Upregulation greater than twofold and FDR adjusted $P < 0.05$.
[a]Two fold changes are reported as this gene is located at two distinct probes on microarray.

that were statistically differentially expressed between the two samples (twofold or greater change in expression, FDR adjusted $P < 0.05$, **Table 3**), including the three genes identified as upregulated between GFP⁺ and GFP⁻ (*RAP1A*, *HSP70B′*, and *PVT1*), as expected. In addition to *RAP1A*, there were other genes upregulated in GFP⁺ cells with a link to integrin activation and cell adhesion (**Table 3**), including two members of the solute carrier family of proteins, solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2 (*LOC442497/SLC3A2/CD98*) and solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 (*SLC1A4*). In addition to upregulation of *HSP70B′*, the DnaJ (Hsp40) homolog, subfamily B, member 1 gene was also shown to be upregulated in GFP⁺ cells as compared to Control cells (**Table 3**), and like *HSP70B′*, *Hsp40* plays a role in a variety of cell functions related to cell stress. Other upregulated genes of interest include several potentially involved in apoptosis, including the activating transcription factor 3 (*ATF3*), a transcription factor that may activate or repress transcription to allow cells to adapt to various responses, including apoptosis; the damage-specific DNA binding protein 2 (*DDB2*), which facilitates the cellular response to DNA damage; the phosphoprotein enriched in astrocytes 15 gene (*PEA15*), which is a death effector domain-containing protein, and thus could be implicated in apoptosis; and the protein phosphatase 1, regulatory (inhibitor) subunit 15A gene (*PPP1R15A*), which is a member of a group of genes whose transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents. The actin, α-2 (*ACTA2*) gene, a component of the cytoskeleton, was also upregulated.

As *RAP1A* and *HSP70B′* were both approximately tenfold upregulated in GFP⁺ cells as compared to GFP⁻ cells, as well as substantially upregulated in GFP⁺ cells as compared to Control cells (and *RAP1A* was also upregulated in GFP⁻ as compared to Control cells) their upregulation was confirmed by quantitative real-time PCR, probing for expression of both *RAP1A* and *HSP70B′* in GFP⁺, GFP⁻, and Control cells (**Figure 2**). These two genes were also further studied in transfection experiments, in the presence and absence of pharmacological activators of these two

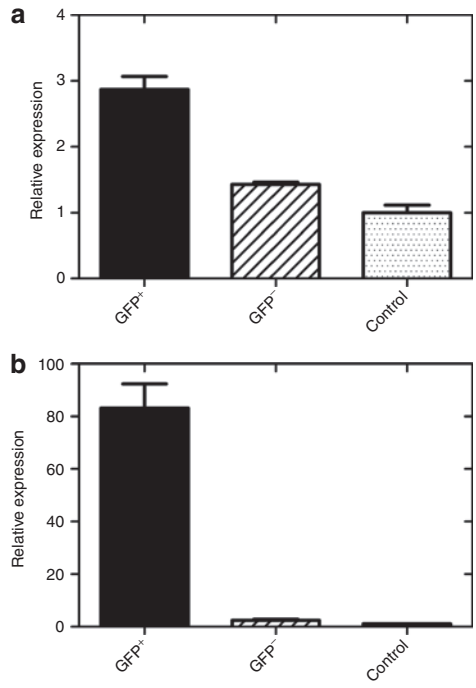

Figure 2 Verification of gene expression profiles. Real-time PCR was performed to verify microarray results for (**a**) *RAP1A* and (**b**) *HSP70B′*. Data normalized to 18S endogenous control and reported as mean with SEM.

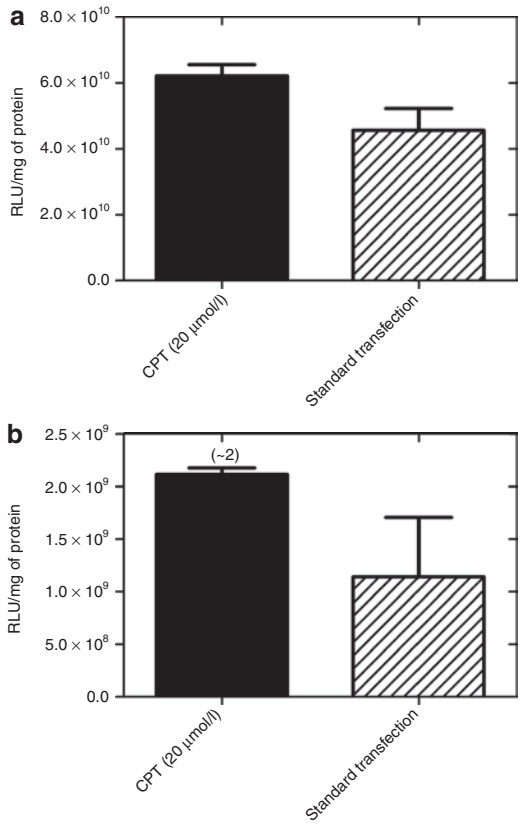

Figure 3 *RAP1A* activation and DNA delivery. Transfection in (**a**) HEK 293T and (**b**) NIH/3T3 cells in with activator of Rap1a (CPT), compared to standard transfection conditions with vehicle. Transfection is reported as relative light units (RLU) per protein amount, represented as mean with SEM. Number in parentheses represents fold changes over standard transfection.

genes to determine if their modulation prior to DNA complex delivery might affect transfection efficiency. These transfection studies were performed in HEK 293T cells and NIH/3T3 mouse fibroblast cells. The fibroblast cells were used to determine if the genes identified as upregulated in HEK 293T cells might be global regulators of gene transfer (*i.e.*, affect transfection profiles in another cell type). For either cell type, cells were seeded, treatments were applied for a period of time, and then media was removed and replaced with fresh media containing DNA-lipid complexes. For *RAP1A*, 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate sodium salt (8CPT-2Me-cAMP, referred to as CPT), a cAMP analog that has been shown to specifically activate EPAC in a variety of cell lines, including HEK 293 cells, was added.[20] EPAC is a cAMP-activated guanine-nucleotide exchange factor and thus regulator of RAP1A.[20] For HSP70B′, the gene was activated by placing cells at 42.5 °C for 1 hour and allowing them to recover at 37 °C for 6 hours,[21] a technique which has been shown to upregulate HSP70B′ expression levels in human epithelial cell lines. Standard transfection in the presence of vehicle alone was used as a control. Activation of RAP1A and HSP70B′ in both HEK 293T and NIH/3T3 cells (**Figures 3** and **4**) resulted in approximately twofold enhancement of transfection.

DISCUSSION

Recently, the fields of "toxicogenomics" and "polymer genomics" have begun to investigate pharmacogenomic responses to synthetic polymers and nanomaterials used to deliver genes or drugs into cells, with a focus on cell toxicity and associated gene expression changes that control the cellular response to various drugs and genes.[22] Most studies utilize gene expression profiles obtained by microarray technology, which allows for the accurate representation of the cellular response to delivery vectors.[18] For instance, treatment of human A431 epithelial cells with cationic lipids altered expression of 27 genes involved in cell proliferation, differentiation, and apoptosis,[15] confirmed by apoptosis assays. However, in that study cells were merely treated with the cationic lipid without complexation first with DNA, and thus the results correlate to properties of the vector itself, and not transfection. Transcriptional effects of lipid-mediated transfection were explored in MCF-7 cells, and gene expression profiling revealed that the transfection reagents caused off-target effects and these effects were not attributed to expression of the transgene itself, but like in most reported studies, transfected cells were not isolated prior to RNA extraction for analysis.[23] However, several genes and gene families identified in that study correlate with genes shown to be differentially expressed in this current study, identified between GFP^+ and control samples (**Table 3**), including *ATF3*, *HMOX1*, *PPP1R15A*, and *DNAJB1*, as well as members of the heat shock family of proteins and the solute carrier gene family. Cationic polymers have also been examined for their toxicogenomic effect on cells, including polyethylenimine[16] and dendrimers.[17] In these studies, there was no isolation of transfected cells from untransfected, rather just treated versus untreated using polymer alone or polymers complexed with DNA. Gene expression changed

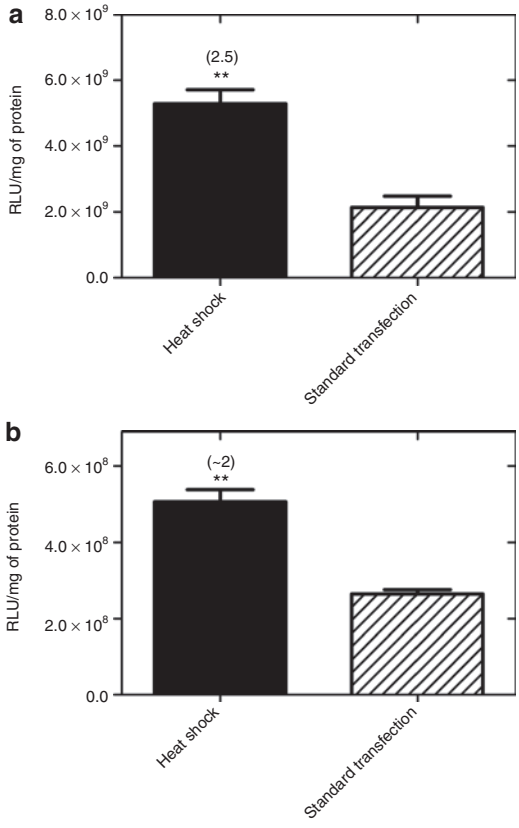

Figure 4 HSP70B′ activation and DNA delivery. Transfection in (**a**) HEK 293T and (**b**) NIH/3T3 cells that were heat-shocked compared to standard transfection conditions. Transfection is reported as relative light units (RLUs) per protein amount, represented as mean with SEM. Number in parentheses represents fold changes over standard transfection. (**P <0.01).

in ~10% of the genes[16] and cationic-dendrimer–induced gene changes in cells were increased upon complexation with small interfering RNA molecules,[17] suggesting the presence of the dendrimer might help determine the level of gene silencing by small interfering RNAs. Microarray analysis was also used to study local tissue response after delivery of a polymeric gene delivery system *in vivo*.[18] Specifically, the toxicological profile of polyethylenimine, chitosan, and their complexes with DNA was determined in the mouse lung after administration of these complexes. Genes involved in stress reactions were induced by polyethylenimine, but also genes that stabilized the reporter protein luciferase and other proteins, but again, profiles were not determined on pure populations of transfected cells. These previous studies highlight not only the importance of understanding cellular responses to gene delivery agents, but also the importance of studying an isolated population of transfected cells to identify genes that may be responsible for successful gene transfer.

Upregulation of genes involved in integrin activation and cell adhesion

In this current study, gene expression profiles of successfully transfected cells provide molecular evidence for some findings in the literature on nonviral delivery mechanisms, including the role of integrin activation and cell adhesion. The *RAP1A* gene was determined to be upregulated in transfected cells (GFP^+) as compared to untransfected cells (GFP^-) (**Table 1**), as well as upregulated in untransfected cells (GFP^-) as compared to control cells (**Table 2**), and highly upregulated in transfected cells (GFP^+) as compared to control cells (**Table 3**). As stated above, *RAP1A* is a Ras-like GTPase implicated in growth modulation, differentiation, and integrin-mediated cell adhesion.[24] RAP1A-mediated adhesion and spreading has been demonstrated in several cell types, including fibroblasts[25] and endothelial cells.[26] RAP1A appears to modulate cell adhesion and spreading through the Rho family of GTPases, with RAC1 required downstream of RAP1A for cell spreading. Overexpression of RAP1A has been shown to activate integrins and inhibition of RAP1A inhibits integrins,[27] which has been shown to block cell adhesion to various substrates. The overexpression of *RAP1A* in GFP^+ cells indicates that it may play a role in nonviral gene delivery; furthermore its upregulation in GFP^- cells suggests that *RAP1A* expression may also be important for internalization of DNA complexes.

In addition to *RAP1A*, there were other genes upregulated in transfected cells (GFP^+) as compared to Control cells with a link to integrin activation and cell adhesion (**Table 3**), including *LOC442497/SLC3A2/CD98* and *SLC1A4*. In particular, *SLC3A2* (CD98) is a type II membrane glycoprotein expressed in all cell types, with the exception of platelets, and is a major contributor to the integrin-dependent activation of RAC.[28] CD98 has been shown to promote activity of $\alpha_5\beta_1$ integrin in fibroblast cultures and cells deficient in this gene are markedly defective in integrin-dependent cell spreading and migration.[29]

The upregulation of genes involved in integrin activation and cell adhesion explain studies that link the presence of extracellular matrix molecules to nonviral gene transfer. The addition of fibronectin to lipoplexes, for example, enhanced transfection efficiency in prostate tumor cells,[30] most likely by exposing the integrin binding site in fibronectin available upon fibronectin binding to lipoplexes, and thus enhancing the association of the DNA complexes with the cells. Fibronectin or collagen I addition to DNA calcium phosphate particles resulted in high transgene expression in mammalian cells, presumably since these particles could be recognized by the integrin receptors for subsequent internalization.[31] Extracellular matrix molecules have also been shown to enhance transfection in substrate-mediated gene delivery strategies,[32–35] where plasmid DNA or DNA complexes are immobilized to a surface or biomaterial that supports cell adhesion, thereby placing the DNA directly in the cellular microenvironment,[36] most likely by promoting cell adhesion and the DNA polyplex internalization process.

Upregulation of genes involved in stress response (heat shock)

The heat shock 70 kd protein 6 gene (HSPA6) was found to be upregulated in transfected cells (GFP^+) as compared to untransfected cells (GFP^-) (**Table 1**). The heat shock protein 70 family is a group of chaperones involved in protein folding, stabilization, and shuttling functions through the cell. Members of this family can be induced by various cellular stresses.[37] *HSP70B′*, a member of the family, is strictly inducible, having no detectable levels of expression in most cells,[38] and may be important for maintaining cell viability[37] and cytoprotection.[21] In addition, the DnaJ (Hsp40)

homolog, subfamily B, member 1 gene was also shown to be upregulated in transfected cells (GFP^+) as compared to Control cells (**Table 3**), and like *HSP70B′*, plays a role in a variety of cell functions and has been shown to enhance nuclear localization of viral particles.[39]

The upregulation of genes involved in stress response indicate that a cell's ability to respond to stress could control transfection efficiency. Thus, expression of the *HSP70B′* gene may be important for maintaining cell viability[37] during transfection. A related member of the family, *hsp70*, was also shown to be upregulated in human A431 epithelial cells in response to treatment with cationic lipids.[15] In addition to protecting the cells from the stress of treatment with DNA complexes, HSP70B′ and DnaJ have also been shown to be involved in nuclear import of viral particles[39,40] and thus could potentially be involved with nuclear entry of nonviral gene delivery complexes, in particular those with plasmids containing viral promoters, thereby improving transfection levels.

Upregulation of genes involved in apoptosis

Several genes with potential roles in apoptosis were found to be upregulated in transfected cells (GFP^+) as compared to Control cells (**Table 3**), including the activating transcription factor 3 (*ATF3*) gene. ATF3 induction may activate or repress transcription to allow cells to adapt to various stresses and stimuli, including induction of apoptosis.[41] Typically expressed in low levels, *ATF3* is a stress-inducible gene.[42] While *ATF3* has been shown to be a key regulator in cell stress response, whether its expression is a protective response or if it is part of the cellular response that leads to detrimental outcomes seems to be dependent on the cell type and nature of the stimulus. These dual roles have now led to the characterization of *ATF3* as an "adaptive response" gene that participates in cellular processes to adapt to extra- or intracellular changes.[41] In addition to *ATF3*, other genes with putative roles in apoptosis were identified as upregulated in GFP^+ cells as compared to Control cells (**Table 3**), including *DDB2*, *PEA15*, and *PPP1R15A*. Given the toxicity of lipid-DNA complexes, it is not surprising that some apoptotic genes were revealed in the studies reported here. These results further highlight the need for less toxic nonviral delivery systems.

Transfection in presence of activators of Rap1a and HSP70B′

While the major objective of this paper was to examine gene expression profiles of transfected cells to identify candidate genes for future studies into the molecular mechanisms of gene delivery, initial transfection experiments, in the presence and absence of activators of *RAP1A* and *HSP70B′*, further implicate the role of these genes in nonviral gene delivery and demonstrate an approach to enhancing transfection through priming of the cells through molecular factors, without any changes to the cationic vector. Most attempts to alter transfection profiles have focused on modification of vectors, as opposed to molecular modification of cells. While treatment with CPT and heat shock, reported here, were likely not specific modulators of *RAP1A* and *HSP70B′* gene expression, respectively, these data serve to demonstrate the principle of altering endogenous gene expression profiles to enhance transfection, and suggest that a cell's ability to respond to stress and adhesion factors may determine the efficacy of gene transfer. Furthermore, given that activation strategies employed here were likely not specific to RAP1A or HSP70B′, it might be anticipated that molecular approaches to modulate these genes prior to DNA delivery could result in an even greater enhancement in transfection.

The use of gene delivery in therapeutic applications, including gene therapy to treat genetic deficiencies or tissue engineering matrices for the treatment of organ loss and failure, has remained limited due to challenges with current delivery systems. The lack of direct information on the endogenous gene expression profiles and intracellular signaling pathways that might be controlling transfection efficiency limits the optimal design of delivery vectors. This study is the first to examine the effect of transfection on basal cell signaling, to understand the molecular basis for efficient gene transfer, and to then in turn use that information to reveal as methods to prime cells for delivery. Further work is needed to assess the effects of different delivery agents, different cell types, earlier time points, and molecular interventions on gene expression profiles of transfected cells. However, with a greater understanding of endogenous gene expression profiles of transfected cells, more efficient nonviral delivery schemes capitalizing on endogenous factors can be developed to advance therapeutic applications.

MATERIALS AND METHODS

Cell culture. HEK 293T cells (ATCC, Manassas, VA), a human embryonic epithelial kidney cell line that is widely used for transfection studies, were cultured in T-75 flasks in Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA) containing 4.5 g/l glucose, supplemented with 10% fetal bovine serum (Gibco), 2 mmol/l L-glutamine (Gibco), and 100 units/ml of penicillin (Gibco) and maintained at 37 °C in a humidified 5% CO_2 atmosphere. For seeding, cells were dissociated with 1 mmol/l EDTA and counted using a hemocytometer and trypan blue staining for viable cells. Cells were then seeded at a density of 3.4×10^6 cells/flask into T-75 flasks for transfection, in order to generate enough transfected cells to be collected through sorting (see below) for RNA extraction. Additional flasks were seeded for control conditions. NIH/3T3 mouse fibroblast cells (ATCC) were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% calf serum (Colorado Serum Company, Denver, CO) and 1% penicillin-streptomycin (Gibco). For transfection studies in the presence/absence of activators, cells were seeded into wells of a 48-well plate (HEK 293T: 22,800 cells/well and NIH/3T3: 15,000 cells/well) 18 hours prior to activator treatment and subsequent transfection.

Transfection for microarray analysis. Plasmid pEGFP-LUC, which encodes for both the enhanced green fluorescent protein (EGFP) and firefly luciferase protein (LUC) under the direction of a CMV promoter (Clontech, Mountain View, CA), and was used for all transfection experiments. Plasmids were purified from bacteria culture using Qiagen (Valencia, CA) reagents and stored in Tris–EDTA buffer solution (10 mmol/l Tris, 1 mmol/l EDTA, pH 7.4) at −20 °C. DNA complexes were formed with Lipofectamine 2000 (LF2000; Invitrogen), following manufacturer's instructions. Briefly, DNA complexes were formed at a DNA:lipid ratio of 1:1.5 (μg of DNA to μl of LF2000) in serum-free, Opti-MEM media (Invitrogen) by adding transfection reagent diluted in media dropwise to DNA in media, mixing by gentle pipetting, and incubating for 20 minutes. After an 18-hour period to allow HEK 293T cells to adhere (seeded into flasks as described above), DNA complexes (11.25 μg DNA per flask) were added into the media above the cells. Note, these complex conditions (lipid to DNA ratio and DNA amount) were selected based on

an exhaustive optimization of transfection conditions in which ratio and DNA amounts were compared to (i) transfection efficiency (percentage of EGFP-positive cells assayed by fluorescence microscopy using Leica DMI 3000B, Bannockburn, IL); and (ii) cell viability and morphology (analyzed through phase microscopy using Leica DMI 3000B) (**Supplementary Figure S1**). Thus, the above complexation condition represents that with the highest transfection efficiency levels (~40%) and lowest cytotoxicity (**Supplementary Figure S1a,d,e**) after 24 hours. Transfection experiments for microarray analysis were performed in triplicate on three separate days, with some GFP^+ and control conditions performed up to six times.

Cell sorting and RNA extraction. Transfected cells (GFP^+) were isolated from untransfected cells (GFP^-) 24 hours after addition of the DNA complexes using flow cytometry (**Figure 1**). Briefly, cells were removed from the flask substrate, counted, and concentrated for subsequent sorting, using a B-D FACSVantage SE three-laser, high speed cell sorter in the University of Nebraska-Lincoln's Center for Biotechnology Flow Cytometry Core Facility. Cells were sorted based on forward and side scattered gate in the presence or absence of fluorescence (**Supplementary Figure S2**). A separate set of flasks of cells that were never exposed to DNA complexes were also dissociated, counted, and sorted through flow cytometry. These cells are referred to as the Control cells. After obtaining pure populations of transfected (GFP^+) cells, untransfected (GFP^-) cells, and control cells, total RNA was extracted using TRIzol reagent following the manufacturer's instructions (Invitrogen) and further purified using Qiagen RNeasy column (Qiagen) with a final dilution in DEPC-treated water. All RNA samples were quality assessed on a RNA 6000 Nano LabChip using Agilent BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Microarray analysis. Fifteen Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays (Affymetrix, Santa Clara, CA) were used for the analysis of expression of 39,000 human genes in GFP^+ (six arrays for six separate transfection studies), GFP^- (three arrays for triplicate studies on untransfected cells that were exposed to DNA complexes), and Control (six arrays for six separate control conditions) HEK 293T cells. All microarray analysis, as described, was performed at University of Nebraska-Lincoln's Center for Biotechnology Genomics and Bioinformatics Core Research Facilities. Five to ten micrograms of total RNA was used to synthesize cDNA using an Affymetrix One-Cycle cDNA Synthesis Kit according to the manufacturer's instructions. All sample preparations followed prescribed protocols (Affymetrix Genechip Expression Analysis Technical manual). Hybridization was performed at 45 °C overnight on the Affymetrix Human Genome Array, stained with streptavidin-phycoerythrin conjugate on an Affymetrix Fluidics Station 450, which was followed by scanning with the GeneChip Scanner 3000 7G (Affymetrix). Affymetrix GeneChip Operating Software (GCOS v1.3) was used for washing, scanning, and basic data analysis. This included calculation of absolute values and normalization of the data with respect to internal standards. These microarray data were then normalized using methods described by Wu *et al.*, 2004.[43] Differentially expressed genes between replicates of each of the three conditions (GFP^+, GFP^-, and Control) were determined using the linear models for microarrays package in R/Bioconductor, with Benjamini-Hochberg adjusted *P* values.[44] Genes that were twofold up- or downregulated and had a False Discovery Rate adjusted *P* value of 0.05 or less were deemed to be significant. The gene expression data has been deposited at the NCBI Gene Expression Omnibus with series number GSE20615.

Quantitative real-time PCR. Quantitative real-time PCR was used to confirm upregulation of the two genes, *RAP1A* and *HSP70B′*. For these studies, cells were transfected in T-75 flasks, sorted using flow cytometry and RNA extracted, as described above. Total RNA (1 µg)was treated with DNase I (Promega, Madison, WI) to remove any genomic DNA and reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega) and random primers (Promega). Gene specific primers were designed for *RAP1A*, 5′-TGGATACTGCAGGGACAGAGCAAT-3′ (forward), 5′-ACATCTTCC GTGTCCTTAACCCGT-3′ (reverse) and *HSP70B′*, 5′-TGCAAGAGGAA AGCCTTAGGGACA-3′ (forward), 5′-ACAGATTTGCTCCAGCTCCCT CTT-3′ (reverse). Real-time PCR was performed using Sybr Green and an ABI 7300 System (Applied Biosystems, Carlsbad, CA). Data was normalized to an endogenous control, 18S, using the following primers 5′-CGGCTACCACAT CCAAGGAA-3′ (forward), 5′-GCTGGAATTACCGCGGCT-3′ (reverse).

Transfection in presence of gene activators. Transfections were performed in the presence of activators of two identified genes, *RAP1A* and *HSP70B′*. For either gene, treatments were applied for a period of time, then media was removed and replaced with fresh media containing DNA-lipid complexes formed at 1:1.5 ratio (0.15 µg DNA per well), as described above. For *RAP1A*, 8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate sodium salt (8CPT-2Me-cAMP; Tocris Bioscience, Ellisville, MO) was added at 20 µmol/l[20] for 15 minutes, a concentration demonstrated to activate RAP1A without cytotoxicity (data not shown). For *HSP70B′*, the gene was activated by placing cells at 42.5 °C for 1 hour, allowing them to recover at 37 °C for 6 hours,[21] and then viability was monitored before proceeding with transfection. Standard transfection in the presence of vehicle alone was used as a control. After 24 hours, transfection levels were quantified by measuring the luciferase activity using the Luciferase Assay System (Promega), for which cells were lysed and assayed for enzymatic activity using a luminometer (Turner Designs, Sunnyvale, CA), and luciferase activity (RLUs) was normalized to the total protein amount determined with the BCA protein assay (Pierce, Rockford, IL). Each transfection experiment was performed in triplicate wells on duplicate days. Statistical analysis was performed using Prism software (GraphPad Prism 5, LaJolla, CA). Comparative analyses were completed using student *t*-test at a 95% confidence level. Mean values with SEM are reported.

SUPPLEMENTARY MATERIAL

Figure S1. Optimization of transfection efficiency.
Figure S2. Sorting of transfected cells.

ACKNOWLEDGMENTS

Support for this research was provided in part by funds from the American Heart Association, the University of Nebraska Foundation (Layman Funds), the Nebraska Research Initiative, NSF EPSCoR First Award, USDA CSREES-Nebraska (NEB-21–146), and NFS EPSCoR EPS-0701892 (to JR). Flow cytometry was performed at the Flow Cytometry Core Facility (Dr Kuszynski, flow cytometry was made possible by NIH grant number P20 RR15635 from the COBRE Program of the National Center for Research Resources) and the microarray analysis was performed at the Genomics Core Facility (Dr Xia), both at University of Nebraska-Lincoln's Center for Biotechnology. We thank Tim Martin for careful review of this manuscript.

REFERENCES

1. Godbey, WT, Wu, KK and Mikos, AG (1999). Poly(ethylenimine) and its role in gene delivery. *J Control Release* **60**: 149–160.
2. Tranchant, I, Thompson, B, Nicolazzi, C, Mignet, N and Scherman, D (2004). Physicochemical optimisation of plasmid delivery by cationic lipids. *J Gene Med* **6 Suppl 1**: S24–S35.
3. Varga, CM, Hong, K and Lauffenburger, DA (2001). Quantitative analysis of synthetic gene delivery vector design properties. *Mol Ther* **4**: 438–446.
4. Niidome, T and Huang, L (2002). Gene therapy progress and prospects: nonviral vectors. *Gene Ther* **9**: 1647–1652.
5. Hagstrom, JE (2000). Self-assembling complexes for *in vivo* gene delivery. *Curr Opin Mol Ther* **2**: 143–149.
6. Nishikawa, M and Huang, L (2001). Nonviral vectors in the new millennium: delivery barriers in gene transfer. *Hum Gene Ther* **12**: 861–870.
7. Medina-Kauwe, LK, Xie, J and Hamm-Alvarez, S (2005). Intracellular trafficking of nonviral vectors. *Gene Ther* **12**: 1734–1751.
8. Blessing, T, Kursa, M, Holzhauser, R, Kircheis, R and Wagner, E (2001). Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. *Bioconjug Chem* **12**: 529–537.
9. Ogris, M, Steinlein, P, Carotta, S, Brunner, S and Wagner, E (2001). DNA/polyethylenimine transfection particles: influence of ligands, polymer size, and PEGylation on internalization and gene expression. *AAPS PharmSci* **3**: E21.

10. Liang, E, Rosenblatt, MN, Ajmani, PS and Hughes, JA (2000). Biodegradable pH-sensitive surfactants (BPS) in liposome-mediated nucleic acid cellular uptake and distribution. *Eur J Pharm Sci* **11**: 199–205.
11. Hashimoto, M, Morimoto, M, Saimoto, H, Shigemasa, Y and Sato, T (2006). Lactosylated chitosan for DNA delivery into hepatocytes: the effect of lactosylation on the physicochemical properties and intracellular trafficking of pDNA/chitosan complexes. *Bioconjug Chem* **17**: 309–316.
12. Oh, YK, Suh, D, Kim, JM, Choi, HG, Shin, K and Ko, JJ (2002). Polyethylenimine-mediated cellular uptake, nucleus trafficking and expression of cytokine plasmid DNA. *Gene Ther* **9**: 1627–1632.
13. Suh, J, Dawson, M and Hanes, J (2005). Real-time multiple-particle tracking: applications to drug and gene delivery. *Adv Drug Deliv Rev* **57**: 63–78.
14. Suh, J, Wirtz, D and Hanes, J (2003). Efficient active transport of gene nanocarriers to the cell nucleus. *Proc Natl Acad Sci USA* **100**: 3878–3882.
15. Omidi, Y, Hollins, AJ, Benboubetra, M, Drayton, R, Benter, IF and Akhtar, S (2003). Toxicogenomics of non-viral vectors for gene therapy: a microarray study of lipofectin- and oligofectamine-induced gene expression changes in human epithelial cells. *J Drug Target* **11**: 311–323.
16. Akhtar, S and Benter, I (2007). Toxicogenomics of non-viral drug delivery systems for RNAi: potential impact on siRNA-mediated gene silencing activity and specificity. *Adv Drug Deliv Rev* **59**: 164–182.
17. Hollins, AJ, Omidi, Y, Benter, IF and Akhtar, S (2007). Toxicogenomics of drug delivery systems: Exploiting delivery system-induced changes in target gene expression to enhance siRNA activity. *J Drug Target* **15**: 83–88.
18. Regnström, K, Ragnarsson, EG, Fryknäs, M, Köping-Höggård, M and Artursson, P (2006). Gene expression profiles in mouse lung tissue after administration of two cationic polymers used for nonviral gene delivery. *Pharm Res* **23**: 475–482.
19. Brazeau, GA, Attia, S, Poxon, S and Hughes, JA (1998). *In vitro* myotoxicity of selected cationic macromolecules used in non-viral gene delivery. *Pharm Res* **15**: 680–684.
20. Enserink, JM, Christensen, AE, de Rooij, J, van Triest, M, Schwede, F, Genieser, HG *et al.* (2002). A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat Cell Biol* **4**: 901–906.
21. Noonan, E, Giardina, C and Hightower, L (2008). Hsp70B' and Hsp72 form a complex in stressed human colon cells and each contributes to cytoprotection. *Exp Cell Res* **314**: 2468–2476.
22. Kabanov, AV (2006). Polymer genomics: an insight into pharmacology and toxicology of nanomedicines. *Adv Drug Deliv Rev* **58**: 1597–1621.
23. Jacobsen, L, Calvin, S and Lobenhofer, E (2009). Transcriptional effects of transfection: the potential for misinterpretation of gene expression data generated from transiently transfected cells. *BioTechniques* **47**: 617–624.
24. Bos, JL, de Rooij, J and Reedquist, KA (2001). Rap1 signalling: adhering to new models. *Nat Rev Mol Cell Biol* **2**: 369–377.
25. Lee, H, Gaughan, JP and Tsygankov, AY (2008). c-Cbl facilitates cytoskeletal effects in v-Abl transformed fibroblast through Rac1- and Rap1-mediated signaling. *Int J Biochem Cell Biol* **40**: 1930–1943.
26. Carmona, G, Göttig, S, Orlandi, A, Scheele, J, Bäuerle, T, Jugold, M *et al.* (2009). Role of the small GTPase Rap1 for integrin activity regulation in endothelial cells and angiogenesis. *Blood* **113**: 488–497.
27. Bos, JL, de Bruyn, K, Enserink, J, Kuiperij, B, Rangarajan, S, Rehmann, H *et al.* (2003). The role of Rap1 in integrin-mediated cell adhesion. *Biochem Soc Trans* **31**(Pt 1): 83–86.
28. Etienne-Manneville, S and Hall, A (2002). Rho GTPases in cell biology. *Nature* **420**: 629–635.
29. Feral, CC, Nishiya, N, Fenczik, CA, Stuhlmann, H, Slepak, M and Ginsberg, MH (2005). CD98hc (SLC3A2) mediates integrin signaling. *Proc Natl Acad Sci USA* **102**: 355–360.
30. Hattori, Y and Maitani, Y (2007). DNA/Lipid complex incorporated with fibronectin to cell adhesion enhances transfection efficiency in prostate cancer cells and xenografts. *Biol Pharm Bull* **30**: 603–607.
31. Chowdhury, EH, Nagaoka, M, Ogiwara, K, Zohra, FT, Kutsuzawa, K, Tada, S *et al.* (2005). Integrin-supported fast rate intracellular delivery of plasmid DNA by extracellular matrix protein embedded calcium phosphate complexes. *Biochemistry* **44**: 12273–12278.
32. Yoshikawa, T, Uchimura, E, Kishi, M, Funeriu, DP, Miyake, M and Miyake, J (2004). Transfection microarray of human mesenchymal stem cells and on-chip siRNA gene knockdown. *J Control Release* **96**: 227–232.
33. Bengali, Z, Pannier, AK, Segura, T, Anderson, BC, Jang, JH, Mustoe, TA *et al.* (2005). Gene delivery through cell culture substrate adsorbed DNA complexes. *Biotechnol Bioeng* **90**: 290–302.
34. Bengali, Z, Rea, JC, Gibly, RF and Shea, LD (2009). Efficacy of immobilized polyplexes and lipoplexes for substrate-mediated gene delivery. *Biotechnol Bioeng* **102**: 1679–1691.
35. Bengali, Z, Rea, JC and Shea, LD (2007). Gene expression and internalization following vector adsorption to immobilized proteins: dependence on protein identity and density. *J Gene Med* **9**: 668–678.
36. Pannier, AK and Shea, LD (2004). Controlled release systems for DNA delivery. *Mol Ther* **10**: 19–26.
37. Noonan, EJ, Place, RF, Giardina, C and Hightower, LE (2007). Hsp70B' regulation and function. *Cell Stress Chaperones* **12**: 393–402.
38. Leung, TK, Rajendran, MY, Monfries, C, Hall, C and Lim, L (1990). The human heat-shock protein family. Expression of a novel heat-inducible HSP70 (HSP70B') and isolation of its cDNA and genomic DNA. *Biochem J* **267**: 125–132.
39. Cheng, X, Belshan, M and Ratner, L (2008). Hsp40 facilitates nuclear import of the human immunodeficiency virus type 2 Vpx-mediated preintegration complex. *J Virol* **82**: 1229–1237.
40. Agostini, I, Popov, S, Li, J, Dubrovsky, L, Hao, T and Bukrinsky, M (2000). Heat-shock protein 70 can replace viral protein R of HIV-1 during nuclear import of the viral preintegration complex. *Exp Cell Res* **259**: 398–403.
41. Lu, D, Chen, J and Hai, T (2007). The regulation of ATF3 gene expression by mitogen-activated protein kinases. *Biochem J* **401**: 559–567.
42. Hai, T, Wolfgang, CD, Marsee, DK, Allen, AE and Sivaprasad, U (1999). ATF3 and stress responses. *Gene Expr* **7**: 321–335.
43. Wu, Z, Irizarry, R, Gentleman, R, Martinez-Murillo, F and Spencer, F (2004). A model based background adjustment for oligonucleotide expression arrays. *J Amer Stat Assoc* **99**: 909–917.
44. Benjamini, Y and Hochberg, Y (1995). Controlling the False Discovery Rate – a practical and powerful approach to multiple testing. *J R Statist Soc B* **57**: 289–300.