Transcriptional effects of transfection: the potential for misinterpretation of gene expression data generated from transiently transfected cells

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Transfection is used to introduce a gene of interest into a cell. To interpret the downstream results, understanding which effects are the true biological responses to the gene and which, if any, are off-target effects can be difficult. In order to discriminate true biological effects from off-target effects, we transfected a breast cancer cell line, MCF7, with a vector encoding either a reporter gene or the identical vector without the reporter gene insert. Both resulted in similar numbers of differentially expressed transcripts, suggesting that very few of the responses were directly due to the introduction of the reporter gene. We postulate that many differentially expressed transcripts are the result of the introduction of foreign DNA, as the biological processes associated with these genes are primarily associated with an immune response to a viral infection. Interestingly, different transfection reagents resulted in >10-fold difference in the number of differentially expressed transcripts. This suggests the importance of testing multiple reagents and selecting the best transfection reagent along with the appropriate vector within the context of the experimental model system to ensure that the majority of the observed responses are biological effects of the gene of interest and not based on a particular transfection process used.

Introduction

The insertion of exogenous DNA or RNA into a cell through the process of transfection is an important, well-established tool for molecular and cellular biologists. The application of transfection as a research tool includes the production of the protein encoded by an exogenous DNA that is either transiently maintained in the nucleus or has been integrated into the genome of the host cell, resulting in the production of a stable cell line. Other uses include: (*i*) introduction of short interfering RNA (siRNA) or short hairpin RNA (shRNA) to examine the impact of the decreased expression of a particular gene, (*ii*) studying the effects of a constitutively active or dominant negative enzyme construct, and (*iii*) identifying the subcellular localization of a protein by using a construct encoding the gene of interest fused to the coding region for a marker such as green fluorescent protein (GFP).

The two most common approaches for transfecting mammalian and insect cells involve either the use of chemical reagents (usually, liposome-based) or the application of an electrical current (electroporation), which is typically used for cells grown in suspension or that are difficult to transfect using chemical reagents. Unfortunately, many transfection reagents and electroporation have considerable cytotoxic effects on the cells. Furthermore, it has long been recognized that different transfection reagents cause different levels of cytotoxicity in different cell lines. These effects are often directly attributable to the transfection reagents themselves and not the nucleic acid of interest that is being introduced to the cell.

Additionally, published literature suggests that there are a variety of negative effects resulting from cryptic or 'poison' sequences within the plasmid vector itself (1). This raises the question of what other effects might be a direct result of the transfection event itself or other sites in the vector and not due to the presence of the exogenous RNA or DNA selected for study. Clearly, understanding these off-target effects is important to the proper interpretation of results generated using transfected cells. Initial studies have been done that indicate these changes can be identified at the level of transcription (www.roche-applied-science.com/PROD_INF/BIOCHEMI/no4_06/pdf/22.pdf).

For these reasons, we chose the common model cell line MCF7 to identify the effects of different transfection reagents. To pinpoint potential off-target transcriptional effects, we performed microarray-based gene expression profiling assays on cells that had been transfected with four different commercially available transfection reagents at three different time points. The microarray data was used to determine genes and biological processes that were consistently modified in the presence of only the gene of interest, or as a result of exposure to a given transfection reagent. Our findings indicate that transfection-mediated transcriptional effects need to be fully appreciated by researchers within their own model systems. This information might be used to prevent the misinterpretation of results generated when studying a specific gene of interest.

Materials and methods

Transfection

MCF7 (ATCC HTB-22) cells were obtained directly from ATCC (Manassas, VA, USA) and maintained under stringent standard operating procedures to prevent

cross-contamination. Cells were plated 1 day before transfection at a density of 400,000 cells/well in a 2-mL volume in 6-well plates. MCF7 cells were grown in EMEM (Cat. no. 11090081; Invitrogen, Casrlsbad, CA, USA) supplemented with 10% FBS (Cat. no. SH30070.03; Hyclone Laboratories, Logan, UT, USA), glutamine (Cat. no. 25030081; Invitrogen), non-essential amino acids (Cat. no. 11140050; Invitrogen), sodium pyruvate (Cat. no. 11360070; Invitrogen), and human recombinant insulin (Cat. no. 11376497001; Roche Applied Science, Indianapolis, IN, USA). Transfections with all reagents were performed according to the manufacturer's protocols which included performing optimization experiments to determine the most ideal transfection conditions for each reagent. In instances in which several parameters were found to be optimal for a given transfection reagent (with regard to the ratio of the volume of transfection reagent and the mass of exogenous DNA), different ratios were tested in some experiments. Table 1 lists the reagents and amounts used in the experiments performed for microarray analysis. Reagents were tested in two to four separate experiments for the 48-h time point; shorter time points were tested in fewer experiments. Four to twelve independent transfections were done in each experiment for use in microarray analysis. Two different vectors were used within the context of this project. The pM1-SEAP is a vector expressing secreted alkaline phosphatase (SEAP) and was used in all four experiments. The pM1-MT is a control vector containing an identical backbone to pM1-SEAP, but without the SEAP insert, which was used in two experiments. Vectors were diluted in OPTIMEM I Reduced Serum Medium (Invitrogen) or buffer supplied with reagent. SEAP was selected for these studies as it is secreted and has minimal effects on the biological processes within a cell (https://www.roche-applied-science.com/PROD_INF/BIOCHEMI/no4_04/PDF/p09.pdf).

Quantification of transfection efficiency

At 24 and 48 h post transfection, an aliquot of the media from the transfected cells was removed, diluted (1:400) and measured for SEAP activity according to the pack insert of the chemiluminescent SEAP Reporter Gene Assay Kit (Roche Applied Science).

RNA extraction and gene expression profiling

At 8, 24, and 48 h post transfection, the culture media was removed from each of

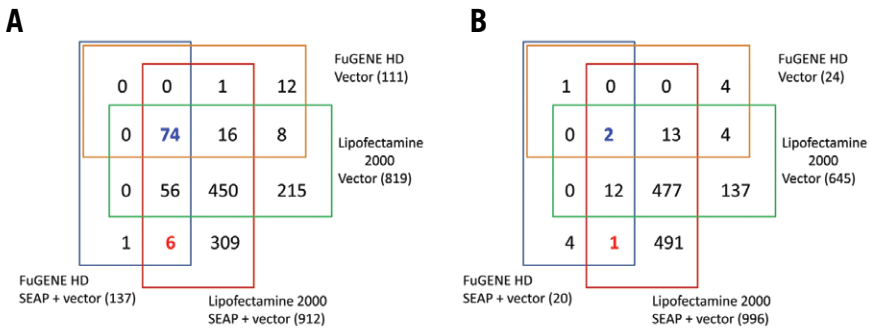

Figure 1. Overlap of differentially expressed transcripts at 48 h post-transfection resulting from a comparison of each of four different samples [two different transfection reagents (FuGENE HD and Lipofectamine 2000) and two different DNA vectors (control vector and SEAP-containing) vector]. Differentially expressed transcripts were either (A) up-regulated or (B) down-regulated as compared with untransfected control cells. Transcripts (up-regulated, $n = 74$; down-regulated, $n = 2$) found to be differentially expressed in all four transfection samples are indicated by the bolded blue font, while transcripts (up-regulated, $n = 6$; down-regulated, $n = 1$) found to be differentially expressed only in the presence of SEAP with both transfection reagents are indicated by the bolded red font. The parenthetical number after the label denotes the number of differentially expressed transcripts for that comparison at 48 h post-transfection. "Vector" denotes the control vector lacking SEAP, while "SEAP + vector" indicates the identical vector backbone containing a SEAP insert in the multiple cloning site.

Table 1. Amounts of Reagents and DNA Used in Transfection of Cells Used in Microarray Experiments

Transfection reagent	Manufacturer	Amount of transfection reagent; amount of exogenous DNA per well	Number of experiments using pM1-SEAP	Number of experiments using pM1-MT
Effectene	Qiagen	8 µL; 0.8 µg	2	Not tested
Lipofectamine 2000	Invitrogen	12 µL; 4 µg	3	2
Lipofectamine LTX and PLUS Reagent	Invitrogen	9.4 µL; 3.75 µg	1	Not tested
		7.5 µL; 2.5 µg	1	
FuGENE HD	Roche Applied Science	10 µL; 2 µg	1	1
		7 µL; 2 µg	3	1
Untransfected control cells	NA	0 µL; 0 µg	4	2

The Effectene complex also contained 6.4 µL of the Enhancer. The LTX-Plus complex contained 3.75 or 2.5 µL of the Plus reagent. NA, not applicable.

Table 2. Transcripts Differentially Expressed at 48 h in SEAP-expressing Cells

Gene symbol	Gene decription	Directionality
ALPPL2	alkaline phosphatase, placental-like 2	Up-regulated
ALPP	alkaline phosphatase, placental (Regan isozyme)	Up-regulated
FLJ37078	nac17 g11.x1 NCI_CGAP_Brn23 Homo sapiens cDNA clone IMAGE:3277652 3′, mRNA sequence.	Up-regulated
C20orf148	chromosome 20 open reading frame 148	Up-regulated
TRIM56	tripartite motif-containing 56	Up-regulated
SCOTIN	Scotin	Up-regulated
NBPF3	neuroblastoma breakpoint family, member 3	Down-regulated

Transcripts differentially expressed at 48 h in SEAP-expressing cells using both Lipofectamine 2000 and FuGENE HD Transfection Reagents. The 7 transcripts in Figure 1 that were unique to cells transfected by the SEAP encoded vector compared with the control (lacking SEAP) vector were identified.

the wells and the entire plate was frozen at -80°C until further processing. RNA was isolated from the frozen dishes by adding RLT lysis buffer (Qiagen, Valencia, CA, USA) to each well and then processed using RNeasy Mini Spin Columns (Qiagen) following the manufacturer's recommended procedure. The quantity and purity of the extracted RNA was evaluated using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and its integrity measured using an Agilent Bioanalyzer (model no. 2100; Agilent Technologies, Santa Clara, CA, USA). For microarray hybridizations, 1 µg total RNA from each sample was labeled using the GeneChip One-Cycle Target Labeling kit (Affymetrix, Inc., Santa Clara, CA, USA) following the manufacturer's protocol to prepare complementary RNA (cRNA). The amount and quality of the cRNA was assessed using a NanoDrop ND-1000 spectrophotometer and an Agilent Bioanalyzer. The cRNA was then fragmented and hybridized to the Human U133 Plus 2.0 Array for analysis of >47,000 transcripts (Affymetrix, Inc., Santa Clara, CA, USA) for 17 h, prior to washing and scanning. Data was extracted from scanned images using GeneChip Operating Software (Affymetrix, Inc.).

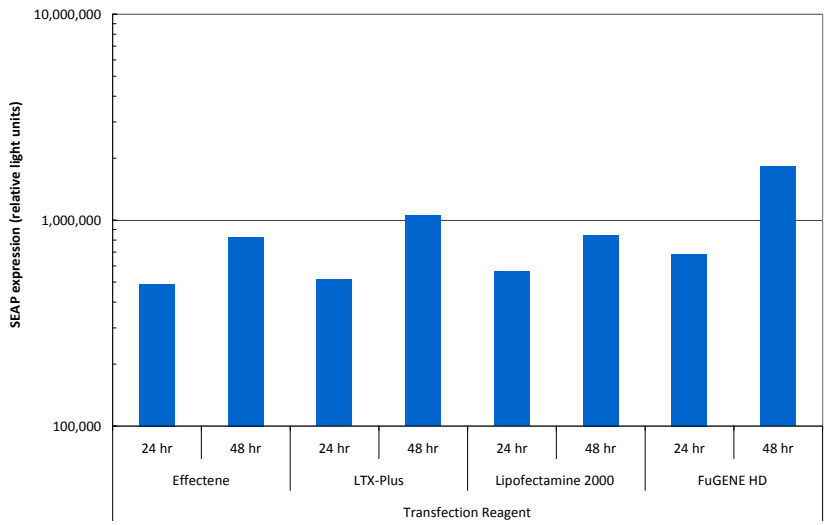

Figure 2. SEAP expression following transfection using different reagents at two different time points. MCF7 cells were transfected with SEAP using four different reagents, and total SEAP expression was determined at 24 and 48 h post-transfection. Results from one experiment are shown; similar results were found in replicate experiments.

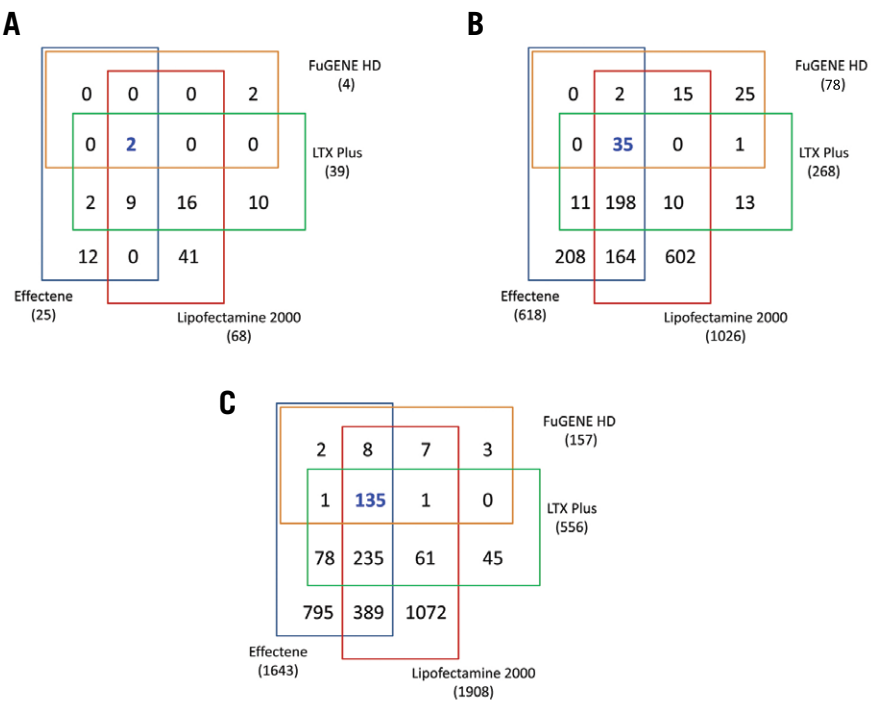

Figure 3. Overlap of differentially expressed transcripts resulting from different transfection reagents at three different time points. Transcripts differentially expressed in cells transfected with pM1-SEAP vector, as compared with the corresponding untransfected control, at (A) 8 h, (B) 24 h, and (C) 48 h post-transfection. Transcripts found to be differentially expressed in the presence of all four transfection reagents are indicated by the bolded blue font. The parenthetical number after the label denotes the total number of differentially expressed transcripts for that reagent.

Data analysis

Gene expression data was loaded into the Rosetta Resolver Gene Expression Analysis System version 7.1.0.1.11 (Rosetta Biosoftware, Seattle, WA, USA) using the Rosetta error model for gene expression analysis (2). An error-weighted average of biological replicate samples was calculated using Rosetta Resolver and then ratio comparisons were constructed in which the transfected samples for a given transfection reagent at a specific time point was in the numerator and the untransfected control was in the denominator. To identify differentially expressed transcripts, the following criteria were employed: absolute fold-change greater than 1.3, log ratio *p*-value less than 0.001, and a $\log_{10}$ intensity greater than -0.5. Gene ontology enrichment analysis was performed using High-Throughput GoMiner (3,4).

Results and discussion

When transfecting cells with a particular gene of interest, several different aspects of the experiment can result in changes to transcript levels. The gene of interest, once expressed, may impact transcript abundance for other genes besides itself. These are the desired, biologically relevant changes that are the focus of the experiment. However, the backbone of the vector containing the gene of interest as well as the selected transfection reagent may also impact transcript levels. These transcript changes are not relevant to the biology of the gene of interest and may mask mechanisms underlying the action of the gene of interest and/or complicate the downstream data analysis and interpretation. For this reason, we refer to transcript changes associated with experimental conditions that are not related to the action of the gene of interest as off-target effects.

Differential expression comparing two vectors and two reagents

To identify potential off-target transcriptional effects, MCF7 cells were transfected with either a control vector or the same vector in which the gene for SEAP had been inserted in the multiple cloning site. This was performed using two different transfection reagents either FuGENE HD Transfection Reagent (Roche Applied Science) or Lipofectamine 2000 Transfection Reagent (Invitrogen). Gene expression profiling was performed on each well of transfected cells and untransfected cells. The results from each transfected sample were compared with the untransfected control to identify differentially expressed transcripts as shown in Figure 1 and Tables 2 and 3. A complete list of all transcripts differentially expressed with either vector is provided in Supplementary Table 1.

The overlap of these lists of transcripts was examined to identify if the transcript changes could be explained entirely by the gene of interest. Figure 1 clearly illustrates that a small number (up-regulated, $n = 6$, red font in Figure 1A; down-regulated, $n = 1$, red font in Figure 1B) were found to be differentially expressed in the presence of SEAP with both transfection reagents, which is consistent with the expectation that SEAP (commonly used as a reporter construct) has very little biological activity within the cell. The seven probes that were found to be differentially expressed only in SEAP-expressing cells after 48 h are listed in Table 2. The two probes present on the microarray that correspond to SEAP are included in this list. Three of the probes correspond to sequences that are not currently annotated to known genes, but do not have sequence similarity to SEAP (data not shown). In addition, only two genes of known function were found to be consistently regulated in the presence of SEAP: SCOTIN and NBPF3.

Several transcripts (up-regulated, $n = 74$, blue font in Figure 1A; down-regulated, $n = 2$, blue font in Figure 1B) were found to be differentially expressed in all four transfected samples. As the only commonalities in all four samples are the vector backbone and the presence of a lipid-based transfection reagent, these changes likely reflect true off-target effects. These probes were mapped to 59 different genes. A Gene Ontology enrichment analysis was performed using GoMiner to identify the biological themes that were enriched in this group of genes. Table 3 details the top seven most significantly enriched categories.

Interestingly, the seven categories affected (Table 3) suggest that the common biological effect across all transfections was the cellular response to the introduction of foreign DNA, similar to a viral infection or intrinsic cellular immune response. The response to viruses might be predicted as the host MCF7 cell is responding to foreign DNA. We did not determine if these responses were the result of "poison sequences" reported in plasmids over a quarter of a century ago (1), "demon sequences" (5), or plasmid methylation and CpG motifs known to induce an immune response (6–8).

In addition to the transcripts that were differentially expressed by both reagents, there were a large number of transcripts that were changed in the presence of Lipofectamine 2000, regardless of the presence or absence of the gene of interest (up-regulated, $n = 450$, Figure 1A; down-regulated, $n = 477$, Figure 1B). This was not observed with the FuGENE HD Transfection Reagent (up-regulated, $n = 0$, Figure 1A; down-regulated, $n = 1$,

Table 3. Top Seven Enriched Gene Ontology (GO) Categories: SEAP-containing or Control Vectors

GO category	Total genes	Changed genes	$\log_{10}$ enrichment *p*-value
GO:0009607 Response to biotic stimulus	220	17	-17.4
GO:0009615 Response to virus	92	13	-16.7
GO:0051707 Response to other organism	164	13	-13.3
GO:0051704 Multi-organism process	249	13	-11.0
GO:0050896 Response to stimulus	1873	24	-7.6
GO:0006955 Immune response	542	12	-6.0
GO:0002376 Immune system process	656	12	-5.1

Top seven enriched Gene Ontology categories for transcripts found to be differentially expressed at 48 h in all transfections regardless of the exogenous DNA (control vector or vector containing SEAP). The first column indicates the name of the biological process (GO category), the second column details the total number of genes in this category that are represented on the microarray, the third column details how many of the 59 genes are associated with this category, and the fourth column provides an enrichment *p*-value for that category.

Table 4. Differentially Expressed Transcripts

Transfection reagent	Directionality	Time post-transfection 8 h	24 h	48 h
Effectene	Up-regulated	19	430	810
	Down-regulated	6	188	833
	Total	**25**	**618**	**1643**
Lipofectamine 2000	Up-regulated	68	613	912
	Down-regulated	0	413	996
	Total	**68**	**1026**	**1908**
LTX with Plus	Up-regulated	38	226	302
	Down-regulated	1	42	254
	Total	**39**	**268**	**556**
FuGENE HD	Up-regulated	4	40	137
	Down-regulated	0	38	20
	Total	**4**	**78**	**157**

Number of differentially expressed transcripts at three different time points following transfection with pM1-SEAP using four different transfection reagents as compared with untransfected cells.

Figure 1B), indicating that transfection reagents differ greatly in the extent to which they cause off-target effects.

Differential expression at three time points comparing four reagents

In order to more fully explore the hypothesis that transfection reagents differ in the extent to which they generate off-target effects, MCF7 cells were transfected with pM1-SEAP vector using two additional transfection reagents, Effectene (Qiagen) and Lipofectamine LTX with Plus Reagent (Invitrogen), in addition to using Lipofectamine 2000 and FuGENE HD Transfection Reagents. Samples were harvested from the transfected and corresponding untransfected control samples at three different time points: 8, 24, and 48 h post-transfection. SEAP expression was measured at the 24- and 48-h time points to demonstrate that transfections were successful and that the gene of interest had been introduced into the cells. With all reagents higher levels of SEAP expression was measured at 48 h post-transfection than at the 24-h time point as shown in Figure 2 for one experiment. (SEAP expression in untransfected cells and cells transfected with the pM1-MT (control) vector were 700–1500 relative light units).

Next, gene expression profiling was performed on each sample and the SEAP-transfected sample was compared with the untransfected control at each time point for each transfection reagent. Table 4 details the number of transcripts identified as differentially expressed at each time point. Interestingly, the number of up-regulated transcripts significantly outnumbered the number of down-regulated transcripts in most comparisons. It is clearly evident that the number of transcripts differentially expressed increased with time post-transfection and is very dependent upon the transfection reagent selected. Significantly, the FuGENE HD Transfection Reagent consistently produced the fewest total number of differentially expressed transcripts.

Within each time point, the overlap of each transfection reagent was identified as illustrated in Figure 3. Only a small subset of probes were found to be differentially expressed in the presence of SEAP with all four transfection reagents: two probes at 8 h (Figure 3A), 35 probes at 24 h (Figure 3B), and 135 probes (only eight of which were down-regulated) at 48 h (Figure 3C). The two transcripts differentially expressed by all four reagents at 8 h were directly related to alkaline phosphatase expression, the gene sequence that was transfected for expression, indicating how quickly the cell can transcribe the heterologous gene. A complete list of all differentially expressed transcripts at 8, 24, and 48 h is provided in Supplementary Table 2.

The 135 probes (Figure 3C) differentially expressed at 48 h in cells transfected by all four reagents mapped to 80 different genes. A Gene Ontology enrichment analysis was performed on this set of genes to identify the biological themes. The seven most enriched categories following transfection at 48 h by all four reagents were identical to the categories enriched in the analysis of the effects by the vector with and without the SEAP insert as discussed earlier (Table 3). The categorical ranking was also similar; the only differences were that the top two categories exchanged positions as did the fifth and sixth categories. This suggests that these biological processes are either affected by the transfection process itself (e.g., cellular responses to membrane perturbations) or the introduction of foreign DNA into the cells. As discussed previously, different vector constructs may impact different sets of genes. While this cannot be discerned from the data (as all transfections used the same vector backbone), these findings suggest the possibility that different plasmid vectors could evoke different cellular responses. As expected, the two transcripts directly related to alkaline phosphatase expression were included among the 135 probes.

Interestingly, a larger number of probes were found to be differentially expressed in three of the four transfection reagents (235, 8, 1, and 1, depending upon which transfection reagents are compared) as shown in Figure 3C. The greatest overlap (235 probes) was observed when examining the overlap of the Lipofectamine 2000, LTX Plus, and Effectene transfection reagents; these transcripts were not altered when FuGENE HD Transfection Reagent was used for the transfection. These 235 probes were mapped to 165 different genes to evaluate the biological response that was unique to three of the four reagents. A Gene Ontology enrichment analysis was performed using this set of genes. Table 5 details the top 10 most significantly enriched categories. Most of the processes detailed in this list are associated

Table 5. Top Ten Enriched Gene Ontology (GO) Categories: SEAP-containing Vectors

GO Category	Total Genes	Change Genes	$\log_{10}$ enrichment *p*-value
GO:0006412 Translation	294	17	-7.3
GO:0006414 Translational elongation	85	9	-6.2
GO:0044260 Cellular macromolecule metabolic process	2320	48	-4.8
GO:0044267 Cellular protein metabolic process	2232	46	-4.6
GO:0019538 Protein metabolic process	2363	47	-4.3
GO:0044249 Cellular biosynthetic process	717	20	-3.6
GO:0002474 Antigen Processing and Presentation of peptide Antigen via MHC Class I	11	3	-3.6
GO:0006413 Translational Initiation	49	5	-3.6
GO:0048002 Antigen processing and presentation of peptide antigen	13	3	-3.4
GO:0051352 Negative regulation of ligase activity	58	5	-3.2

The top ten enriched Gene Ontology categories for transcripts found to be differentially expressed following transfection with a SEAP-containing vector by three of the four transfection reagents (Lipofectamine 2000, Lipofectamine LTX Plus, and Effectene) at 48 h. The first column indicates the name of the biological process (GO category), the second column details the total number of genes in this category that are represented on the microarray, the third column details how many of the 165 genes are associated with this category, and the fourth column provides an enrichment *p*-value for that category.

with translation and cellular metabolic processes in addition to antigen processing and presentation.

Introduction of a gene into cells is frequently used in the field of immunology to better understand antigen processing and the molecular mechanisms underlying immune responses. Restifo et al. proposed that down-regulation of antigen processing may be one of the strategies used by tumors to escape immune surveillance (9). MCF7 cells were included among the tumor cells tested for deficiencies in antigen processing of MHC Class 1 molecules in their studies. Therefore, it was interesting to note that this pathway is affected by each of the transfection reagents that were used in our study. Table 5 shows that 3 of the 11 transcripts in this category were affected by these three reagents. We then reexamined the other eight transcripts in this pathway and found that 5 were among the 135 transcripts altered by all four reagents. Thus, among the reagents tested, FuGENE HD Transfection Reagent impacted the fewest number of transcripts implicated in the regulation of the antigen processing pathway ($n = 5$, all other transfection reagents resulted in the differential expression of eight transcripts). From this observation, we would predict that FuGENE HD Transfection Reagent would have the least impact on investigations in which the gene of interest is implicated in biological pathways associated with immune-related functions. As an example, Trgovcich and colleagues had difficulties studying viral evasion and modulation of host immune responses because the transfection reagents themselves were activating the same antiviral cellular response as was being studied (www.roche-applied-science.com/PROD_INF/BIOCHEMI/no3_06/pdf/20.pdf, and personal communication). They found FuGENE HD Transfection Reagent resulted in minimal induction of interferon-stimulating genes; thereby permitting a better understanding of the actual cellular response in the presence of viral gene sequences. Their finding is consistent with our observation of a significantly lower number of differentially expressed transcripts when using the FuGENE HD Transfection Reagent. Further studies could be done to determine if this is a specific result of the introduction of particular portions of the vector constructs used in the studies, or a more general response due to the delivery of foreign DNA.

As previously discussed, all four reagents resulted in differential expression of the same 135 transcripts, but as shown in Figure 3C, each reagent also had a unique combination of additional transcripts that were differentially expressed. Some transcripts were differentially expressed only in cells transfected by a particular reagent. Many other transcripts were found to be differentially expressed in two out of the four or three out of the four different reagents. Thus, it is clear that the gene expression observed with each tested transfection reagent is likely due to off-target effects associated with these reagents rather than introduction and subsequent expression of a specific gene.

Conclusion

Cellular transfection is a complex process that can produce both direct (intended) and indirect (unintended) results. Foreign DNA is brought into a cell and then transported to the nucleus where new mRNA is made and eventually translated into protein. The process of foreign DNA entering into the cell has the potential to cause biological responses that are unrelated to the gene encoded by the DNA.

To begin to unravel this complicated process, we used gene expression profiling to determine which genes and biologic categories were affected following transfection by different reagents. A control vector that did not contain the gene selected for expression was used to begin identification of transcripts differentially expressed that might be attributed to sequences within the vector backbone. The resulting data suggests that (*i*) many of the 135 transcripts that were found to be differentially expressed by all four transfection reagents are likely associated with the introduction of foreign DNA into cells, (*ii*) many off-target effects are specific to the transfection reagent itself, and (*iii*) only a limited number of transcripts result from the introduction and subsequent expression of a specific gene. These results suggest that transfection-mediated transcriptional effects need to be fully appreciated by researchers within their own model systems in order to prevent the misinterpretation of results generated when studying a specific gene of interest.

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