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The Impact of Transfection Mediated Toxicity -

Gene Expression and Cytotoxicity Analysis of Transfection Reagents

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Introduction

While plasmid DNA delivery is a widely used method to study cellular functions of proteins of interest, studies to identify nontoxic gene delivery reagents are limited. With the advent of high-information content technologies, especially RT-qPCR array, it is now possible to identify the gene expression response to a particular cellular insult. This improvement, coupled with the observation that virtually all toxic responses are accompanied by changes in gene expression, suggests that gene expression analysis has the potential to refine the identification of minimal-toxicity transfection reagent where phenotypic responses such as altered morphology is not immediately evident. Consequently, we conducted an integrative study to explore the conventional toxicological endpoints and to identify the minimal-transcriptomic effects of *Trans*IT®-LT1, *Trans*IT®-2020 and Lipofectamine® 2000 Transfection Reagents using quantitative reverse transcriptase PCR (RT-qPCR) array and pathway analysis software.

Results

Effect of Transfection on Cell Morphology and Viability

To evaluate a role of transfection-related toxicity, time course and dose-dependent experiments were conducted. Hela cells were treated with various concentrations of pDNA/transfection reagent complexes for up to 24 hours. Transfection toxicity was then evaluated by morphology (Figure. 1) and by Lactate Dehydrogenase (LDH) leakage assays (Figure. 2 B). No change in cellular morphology was observed after 8 hour treatment of the cells with complex of pDNA and any transfection reagents (Figure. 1 A). However treatment of the cells with the complex of pDNA and Lipofectamine® 2000 for 24 hours caused substantial morphological changes as some cells were shrunken and many were detached

from the bottom of the culture dish (Figure. 1 B). No such changes in morphology were evident in the cells that were treated with either pDNA/*Trans*IT®-LT1 or pDNA/*Trans*IT®-2020 complexes (Figure. 1 B). The morphological changes associated with Lipofectamine 2000 treatment is also accompanied by massive cell loss as evidenced in lower total RNA yields from these samples (Figure. 2 A). Similar results were also obtained with the LDH leakage assay. As shown in Figure 2 B, treatment with the complex of luciferase encoding pDNA and Lipofectamine 2000 at various doses caused 20–55% loss of viability in Hela cells, whereas no significant loss of viability (0–10%) was found in cells that were treated with either pDNA/*Trans*IT-LT1 or pDNA/*Trans*IT -2020 complexes. Moreover, the luciferase expression level from the cells that were transfected with Lipofectamine 2000 was also reduced compared to the cells that have been transfected with either *Trans*IT-LT1 or *Trans*IT-2020.

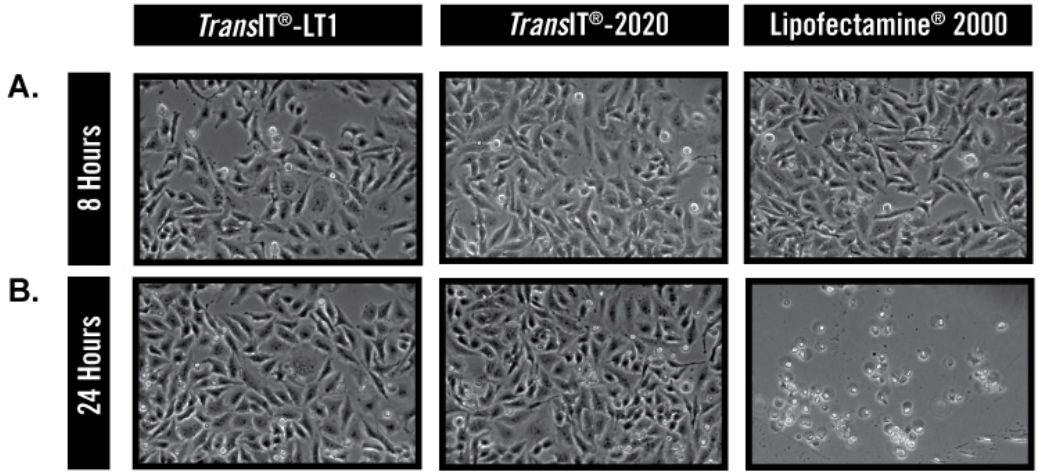

Figure 1. Minimal Morphological Changes are observed with *Trans*IT®-LT1 and *Trans*IT®-2020 Transfection Reagents Compared to Lipofectamine® 2000 at 24 Hours. HeLa cells were transfected with a GFP encoding plasmid DNA using either *Trans*IT®-LT1, *Trans*IT®-2020 or Lipofectamine® 2000 at reagent-to-DNA ratios of 3:1 for all reagents. Phase contrast and GFP images were taken in the same field of view at 8 (A) and 24 (B) hours post-transfection. Cells transfected with *Trans*IT®-LT1, *Trans*IT®-2020 remain healthy after 24 hours while maintaining high transfection efficiency, whereas cells transfected with Lipofectamine® 2000 display significant cytotoxicity after 24 hours. Representative data from two independent experiments is shown.

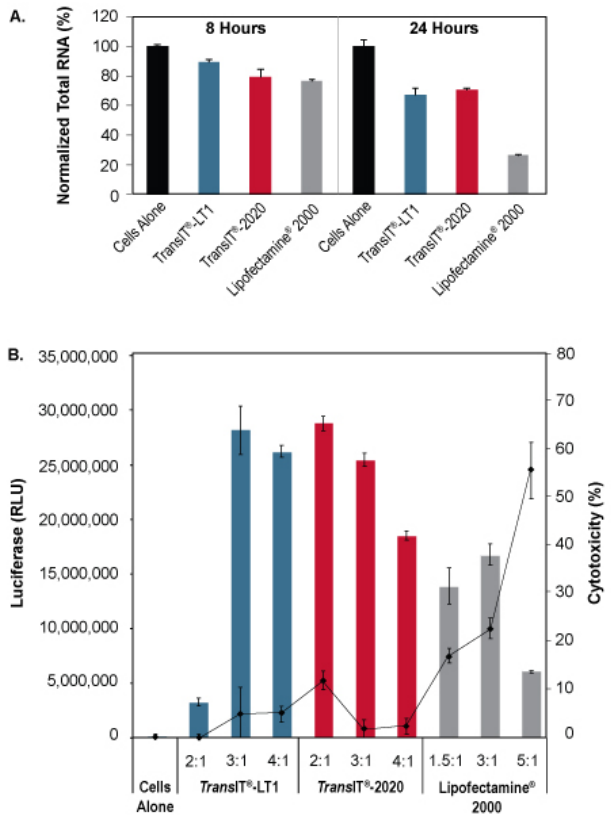

Figure 2. *Trans*IT® Broad Spectrum Reagents Balance High Efficiency Delivery with Low Toxicity. (A) RNA was harvested from HeLa cells that were transfected with a non-coding plasmid and *Trans*IT®-LT1, *Trans*IT®-2020 or Lipofectamine®2000 at 8 and 24 hours. Total RNA levels were decreased in samples transfected with Lipofectamine® 2000 for 24 hours. (B) HeLa cells were transfected with luciferase encoding plasmid DNA using either *Trans*IT®-LT1, *Trans*IT®-2020 or Lipofectamine® 2000 for 24 hours. Transfection was measured by luciferase activity using a conventional assay. Cytotoxicity was assessed by quantifying the LDH released from the cytosol of damaged cells compared to cells alone. Higher transfection efficiency and lower cytotoxicity was observed in cells transfected with *Trans*IT®-LT1 and *Trans*IT®-2020 at optimal ratios compared to cells transfected with Lipofectamine® 2000. Representative data from two independent experiments is shown.

Effect of Transfection on Gene Expression

In response to environmental changes, cells alter their gene expression profile. To investigate the role of transfection induced cytotoxicity on gene expression, a non-coding plasmid was transfected into Hela cells with either *Trans*IT-LT1, *Trans*IT-2020, or Lipofectamine 2000 at the ratio of 3:1 which is the optimal dose determined by the previous experiment (Figure. 2 B). Quantitative RT-PCR -based measurements of mRNA levels was used to analyze the expression of 84 key genes involved in cytotoxicity at 8 and 24 hour post transfection. The result was normalized with that were treated only with pDNA. Alteration in gene expression was assessed using a 2-fold increase/decrease cutoff and the probability value of less than 0.05. Expression of 9 and 25 transcripts was significantly altered by pDNA/Lipofectamine 2000 treatment at 8 and 24 hour respectively whereas fewer genes, 3 and 9, were affected by treating the cells with pDNA/*Trans*IT-LT1 or pDNA/*Trans*IT-2020 complexes respectively

(Figure. 3 A-D). Results are presented as Venn diagram displaying the commonality of transcripts with 2-fold or greater changes in expression between the cells transfected with each reagent for 8 and 24 hours (Figure. 3 C and D). Notably, most of the transcripts that were differentially regulated by transfecting the cells with either pDNA/*Trans*IT-LT1 or pDNA/*Trans*IT-2020 for both 8 and 24 hours were also differentially regulated with pDNA/Lipofectamine 2000 treatment (Figure. 3 C and D).

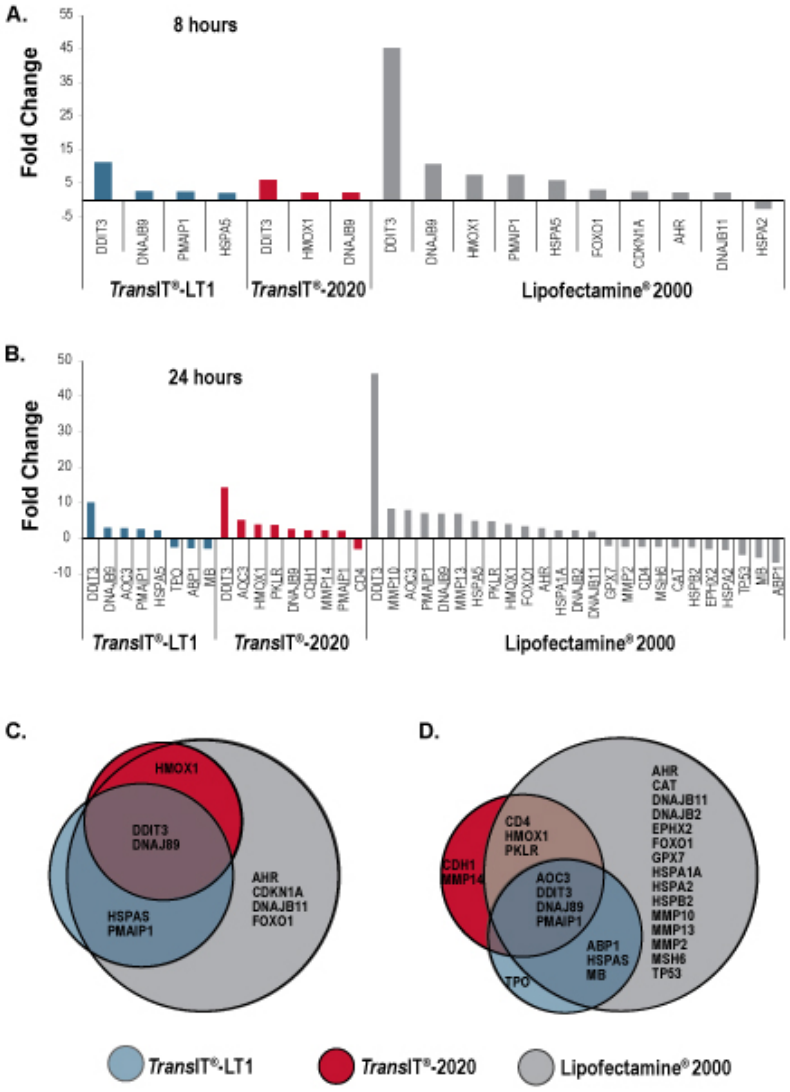

Figure 3. Mirus *Trans*IT® Transfection Reagents Minimize the Stress Response in Transfected HeLa Cells. Stress-related gene expression changes were determined by RT-qPCR from total RNA samples harvested from HeLa cells that were transfected with a non-coding plasmid and *Trans*IT®-LT1, *Trans*IT®-2020 or Lipofectamine®2000 at 8 (A) and 24 (B) hours. Eighty-four genes were analyzed using the Human Stress Response 96 StellARray™ (Lonza). Bar graphs (A and B) and Venn diagrams (C and D) display the gene expression alterations. At both time points, the number and magnitude of stress-related gene expression changes were lower when cells were transfected with *Trans*IT®-LT1 or *Trans*IT®-2020 than when cells were transfected with Lipofectamine® 2000. Representative data from two independent experiments is shown.

Biological Pathways Affected by Transfection

Biological themes underlying the expression pattern from the cells that were transfected with various reagents for 24 hours were identified based on the overrepresentation of predefined groups of transcripts (Figure. 3 B and D). As shown in Figure. 4A, the most overrepresented canonical pathways include pathways associated with oxidative stress response and xenobiotic metabolism. The negative log of the *p* values for the probability of obtaining these genes associated with the given pathways by random chance is given at the top, whereas the bar line in the bottom graph is derived from the ratio of the number of genes on our list associated with a given pathway divided by the total number of genes that make up that pathway. Among the genes involved in oxidative stress response, CHOPS (DDIT3) mRNA expression was increased approximately 50-fold in the cells that were transfected with pDNA/Lipofectamine 2000, whereas the expression of up to 15-fold was observed the cells that have been transfected with either pDNA/*Trans*IT-LT1 or pDNA/*Trans*IT-2020 (Figure. 3 A and B). Based on pathway analysis, the most significant biological function affected in response to transfecting the cells with Lipofectamine 2000 is related to unfold protein and DNA damage response (Figure. 4 B). This pathway includes deregulated genes coding for heat shock proteins; heat shock 70kDa protein 5 (HSPA5), heat shock 70kDa protein 1 (HSPA1A), DnaJ homolog, subfamily B, member 9 (DNAJB9) and DNA damage and cell cycle regulation; cyclin-dependent kinase inhibitor 1A (CDKN1A) and tumor protein 53 (TP53).

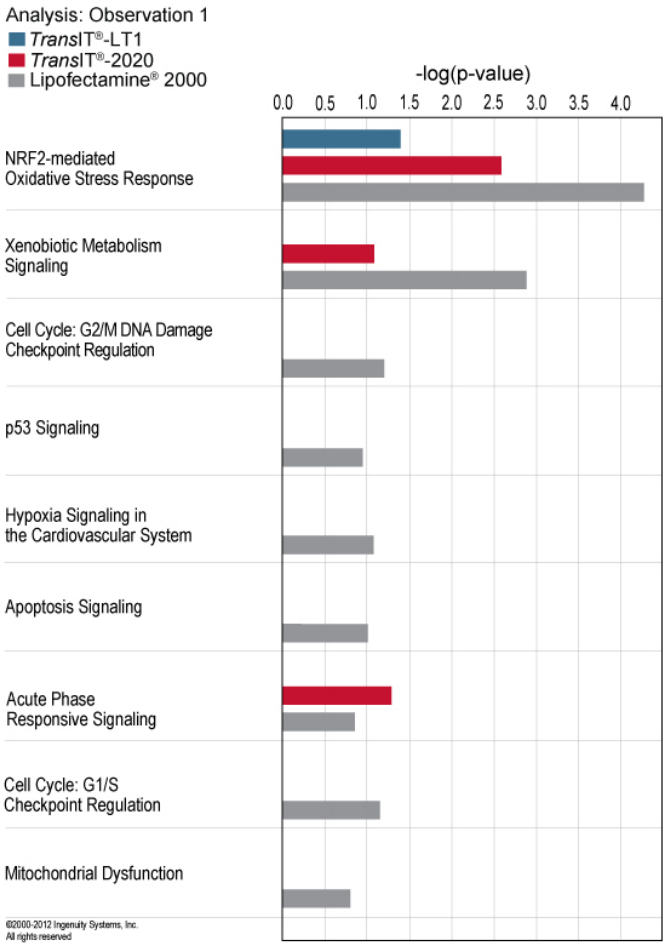

Figure 4. Transfections with *Trans*IT®-LT1 or *Trans*IT®-2020 Affect Fewer Core Pathways than Transfections with Lipofectamine® 2000. Canonical pathway enrichment analysis shows the primary biological processes impacted in HeLa cells that were transfected with *Trans*IT®-LT1, *Trans*IT®-2020 or Lipofectamine® 2000 at 24 hours. The negative log of the p values is the probability of obtaining these genes associated with the given pathways by random chance. The bar line at the bottom of each graph is derived from the ratio of the number of genes on our list associated with a given pathway divided by the total number of genes that make up that pathway. Pathway analysis was performed using Pathway Analysis (Ingenuity Systems). Representative data from two independent experiments is shown.

Discussion

In this study, we have shown that both *Trans*IT-LT1 and *Trans*IT-2020 transfection reagents display minor cytotoxicity and have minimal off-target effects, which is vital in the interpretation of experimental results. Researchers may introduce additional experimental bias depending on the transfection reagent that is selected. Toxicity effects including cell morphology, viability, and deregulation in gene expression profile was evident when treating the cells with pDNA/Lipofectamine 2000 complexes for 24 hours. Remarkably, significant changes in gene expression was also observed after treating the cells with pDNA/Lipofectamine 2000 complex for 8 hour while no change in cellular morphology was evident. These studies were performed in Hela cells and they serve as a model; it can

be inferred that other cell types will display varying degrees of morphological and gene expression changes. Data obtained from gene expression analysis highlight perturbations in previously unsuspected target pathways that are either beneficial or detrimental to the cells.Our investigations illustrate the importance of selecting a transfection reagent that has minimal off-target effects, such as *Trans*IT-LT1 and *Trans*IT-2020 Transfection Reagents.

Methods

Transfection and Microscopy

Hela cells were seeded in a 12-well tissue culture plate at a density of 6×10^4 cells per well and incubated in MEM without Pyruvate, with 10% FBS for 24 hours, yielding 70 to 80% confluency prior to transfection. Cells were transfected with GFP encoding plasmid (pEGFP) using either *Trans*IT-LT1 (Mirus Bio), *Trans*IT-2020 (Mirus Bio), or Lipofectamine 2000 (Life Technologies) transfection reagent as recommended by manufacturers' protocols. Briefly, 1 µg of plasmid DNA was diluted in OPTI-MEM to a total volume of 100 µl. The solution was mixed thoroughly, and 1.5 to 5 µl of transfection reagent was added to the DNA solution. After mixing, samples were incubated for 15 min at room temperature to allow transfection complex formation. The transfection complexes were then added to the cells and incubated for 24 hours.

GFP expression was visualized using similar exposure time at 8 and 24 hour after transfection with an inverted fluorescence microscope (Axiovert 200M, Carl Zeiss) with a 20X air objective and an appropriate filter for GFP. Cell morphology was also examined under phase contrast optics. Images were processed and analyzed using ImageJ (NIH).

LDH Cytotoxicity Assays

Cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells. Hela cells were seeded in a 96-well tissue culture plate at a density of 6×10^3 cells per well and incubated in MEM without Pyruvate, with 10% FBS for 24 hours. Cells were then transfected with the complexes of Luciferase encoding pDNA (pCI luc) and transfection reagents; *Trans*IT-LT1 (Mirus Bio), *Trans*IT-2020 (Mirus Bio), and Lipofectamine 2000 (Life Technologies) according to manufacturers' protocols for 16-24 hours. At the end of treatment, the media was collected to measure LDH activity using an LDH-cytotoxicity detection kit (Roche Diagnostics) according to manufacturer's instructions.

Total RNA Isolation

Total RNA was isolated from control and treated Hela cells using RNeasy Mini kits (Qiagen) in accordance with the manufacturer's instructions. Resulting total RNA was quantified using NanoDrop ND-1000 (NanoDrop Technologies, Inc.).

cDNA Preparation and Real-Time RT-PCR Measurement of Gene Expression

Complementary DNA (cDNA) from each sample was prepared from 2ug of DNase I treated total RNA by reverse transcription with random primers (High Capacity cDNA Reverse Transcription Kit, Life Technologies). cDNA samples were analyzed with a 96-panel Human Stress Response StellARray™ qPCR Array (Lonza) on a Bio-Rad real-time RT-PCR System (Bio-Rad) using SYBR Green PCR Master Mix (Life Technologies). Data were analyzed using the Global Pattern Recognition (GPR) program (Lonza). All quantitative data for comparing gene expression between different samples were expressed as mean. Differences between groups were evaluated by the Student's *t*-test. Probability values of less than 0.05 were considered statistically significant.

Biological Pathway Analysis

Lists of differentially expressed genes were subjected to a subsequent post-analysis interrogation to find the main biological processes associated with the experimental design. Ingenuity Pathway Analysis (Ingenuity Systems) was applied to elucidate pathways associated with transfection-related gene expression changes. The data sets containing gene identifiers and corresponding fold-changes were uploaded into the web-delivered application. Canonical pathway analysis identified pathways from the IPA library that were most significant to the data sets.