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Off-target responses in the HeLa proteome subsequent to transient plasmid-mediated transfection

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ABSTRACT

Transient transfection of mammalian cells with plasmid expression vectors and chemical transfection reagents is widely used to study protein transport and dynamics as well as phenotypic alterations mediated by the overexpressed protein. Despite the undisputed impact of this technique, surprisingly little is known about the cellular effects mediated by the transfection process per se. Conceivably, off-target effects could have implications upon proteins or processes being studied and understanding the molecular pathways affected would add value to the interpretation of experimental observations subsequent to cell transfection.

Here we have used a SILAC-based proteomic approach to study differentially expressed proteins after transfection of HeLa cells with ECFP vector using a commonly employed non-liposome based transfection reagent, Fugene®HD. Whereas the transfection reagent itself mediated minimal effects upon protein expression, 11 proteins were found to be significantly upregulated after transfection, all of which were associated with an interferon type I/II response. The upregulated proteins might potentially inflict major cellular processes such as RNA splicing, chromatin remodeling, post-translational protein modification and cell cycle control. The results were validated by western analysis as well as quantitative RT-PCR and this demonstrated that an essentially identical response was induced in HeLa by transfection using an empty pUC18 vector, which does not contain a mammalian virus promoter, as well as a liposome-based transfection reagent, Lipofectamine™2000. Notably, no induction of the interferon response was observed in HEK293 cells, suggesting that these cells might be preferable to HeLa to avoid undesired off-target effects in transfection studies encompassing interferon-signaling and antiviral responses.

1. Introduction

Introduction of exogenous RNA and DNA into mammalian cells by transient transfection is a widespread tool to study effects mediated by up- or downregulated expression of specific proteins. By inserting specific tags in the expressed proteins, crucial information on their localization and binding partners can also be obtained. Moreover, introduction of site-specific mutants may reveal functionally important residues in the various processes. A plethora of techniques and reagents are now available, many of which are tailored to specific cell types, and including liposome- and non-liposome based transfection reagents, viral vectors and electroporation. Some of the protocols are also easily scaled up to produce gram quantities of protein for e.g. structural analyses or for pharmacological purposes [1,2]. In a typical lab setting, commercially available chemical transfection reagents are often employed to transiently overexpress proteins, due to their ease of use and their applicability to many different cell lines. Unfortunately, some of these reagents also have cytotoxic effects on the cells [2]. Considerable effort has thus been put into developing transfection reagents with minimal cytotoxicity. In addition, introduction of exogenous DNA into cells may itself introduce off-target effects. To monitor such effects control experiments are routinely conducted in which cells transfected using an empty vector, or a vector expressing a tag only, are assumed to represent the normal state. However, such an assumption is likely not universally valid, since both the transfection reagent as well as the DNA vector may potentially mediate cellular responses that indirectly affect the biochemical processes of interest in the study. One such example was reported more than three decades ago, in which certain cryptic sequences in the pBR322 plasmid were shown to inhibit replication of SV40 in simian cells [3]. A recent study quantified expression levels of HSP10 and HSP70 to monitor stress responses and found that these markers were differentially induced when various commercial reagents were employed to transfect HeLa cells with an EGDF expression plasmid [4]. Nevertheless, surprisingly few attempts have been made to study the effects of DNA-mediated transfection per se by employing large-scale profiling approaches. One study, however, utilized mRNA profiling to study differential expression after transfection of MCF7 cells with a

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vector containing a reporter gene or the identical vector without the reporter gene insert [5]. Both DNA constructs resulted in similar number of differentially expressed transcripts, suggesting that very few responses were mediated by the reporter gene. However, >10-fold difference in the number of differentially expressed transcripts was observed when employing four different transfection reagents in the experiments. Here, the non-liposome reagent Fugene®HD yielded the lowest number of differentially expressed transcripts. In the mRNA profiling study the effect of the transfection reagents themselves was not analyzed, nor was the potential content of bacterial endotoxin in the plasmid preparations investigated. Moreover, to our knowledge potential off-target effects caused by a plasmid vector and/or a specific transfection reagent have not been previously studied at the proteome level.

Here we have employed stable isotope labeling by amino acids in cell culture (SILAC) [6] to quantify differentially expressed proteins subsequent to treatment of HeLa cells with the commonly used transfection reagent (Fugene®HD) alone, or a combination of transfection reagent and an expression vector encoding the fluorescent tag ECFP. By using stringent filtering criteria only one protein was found to be differentially expressed with transfection reagent alone, whereas addition of the ECFP expression vector mediated significant upregulation of 11 proteins. Interestingly, a marked cellular response resembling viral infection was observed, including upregulation of several interferon-regulated proteins as well as proteins involved in ubiquitin-like modification (ISGylation). These results were confirmed by western analysis as well as quantitative RT-PCR of several targets found to be upregulated in SILAC. Essentially identical results were obtained using an empty pUC18 vector as well as Lipofectamine™2000, a widely employed lipososome-based transfection reagent. Finally, we subjected an alternative cell line, human embryonic kidney HEK293 cells to the same transfection conditions as employed for HeLa. Notably, western analysis indicated no induction of the viral infection response in this cell model, suggesting that transfection-mediated off-target effects are cell-specific. In studies involving inflammatory pathways, specific viral factors or even in studies involving post-translational protein modifications by ubiquitin or ubiquitin-like modifiers, alternatives to HeLa should be considered in experiments involving plasmid-based transfection strategies.

2. Methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's mediun (DMEM) minus L-lysine and L-arginine, dialyzed fetal calf serum, lysine-^{13}C6, L-arginine-^{12}C6, L-lysine-^{12}C6 and trypsin were from Thermo Scientific. Transfection reagent Fugene®HD was from Roche, and LipofectamineTM2000 was from Life Technologies. pECFP-N1 was from Clontech and pUC18 was from Thermo Scientific. All other chemicals were from Sigma Aldrich.

2.2. SILAC quantification

HeLa cells were cultured in DMEM minus L-lysine and L-arginine with 10% dialyzed fetal calf serum, 0.03% glutamine and 0.1 mg/ml gentamicin at 5% CO_2. For labeling of heavy HeLa cells, L-lysine-^{13}C6 and L-arginine-^{12}C6 were added and for labeling of light HeLa cells L-lysine-^{12}C6 and L-arginine-^{12}C6 were added to the medium. Cells were allowed to undergo six doublings after when the incorporation efficiency was found to be >97.5% as evaluated by mass spectrometry analysis of tryptic protein extracts (described below). Heavy and light cells were then kept in continuous culture and three independent transfection experiments (biological replicates) were conducted at 5 day intervals as follows: cells were reseeded in 15 cm culture dishes and allowed to reach 60–70% confluence. Cells were then either untreated, treated with transfection reagent alone or together with plasmid DNA (empty pECFP-N1vector). Transfection reagent and plasmid DNA was premixed with water (56 μl Fugene®HD to 879 μl sterile deionized water with and without 19 μg ECFP plasmid) for 15 min at room temperature. Then 850 μl of this mixture was added to the cells and further incubated for 24 h. The cell culture media, both with and without plasmid DNA, was checked for endotoxin contamination using a QCL-1000 Chromogenic LAL-assay (Lonza). Using the LAL-assay, no endotoxin contamination from the plasmid DNA was found (data not shown).

24 h after the transfection treatment, HeLa cells were detached by trypsination and collected by centrifugation at 450 ×*g* for 5 min at 4 °C. Cells were washed twice in PBS and equal number of heavy and light (10^6 each) cells were pooled and lysed in 7 M urea, 2 M thiourea, 2.5% (*w*/*v*) CHAPS and 25 mM DTT for 30 min with shaking. Lysates were clarified by centrifugation at 16,000 ×*g* for 15 min and proteins in the soluble fractions were precipitated using chloroform/methanol [7]. Briefly, 300 μl methanol was added to 100 μl lysate, mixed, added 100 μl chloroform, mixed, added 300 μl water and mixed. The mixture was then centrifuged for 2 min at 16,000 ×*g*. The upper layer was discarded and the pellet was added 200 μl of methanol and centrifuged for 2 min at 16,000 ×*g*. The pellet was air dried and resuspended in 150 μl 50 mM ammonium bicarbonate with 1 μg trypsin (Promega) and digestion was performed overnight with shaking at 37 °C. After digestion the sample was made acidic and a C18 purification step was performed as described [8].

After desalting, the peptides were dried down in a SpeedVac centrifuge and resuspended in 0.1% formic acid. The peptides were analyzed on a LC-MS/MS platform consisting of an Easy-nLC 1000 UHPLC system (Thermo Scientific/Proxeon) interfaced with an LTQ-Orbitrap Elite hybrid mass spectrometer (Thermo Scientific) via a nanospray ESI ion source (Proxeon, Odense). Peptides were injected onto a C-18 trap column (Acclaim PepMap100, 75 μm i. d. × 2 cm, C18, 5 μm, 100 Å, Thermo Scientific) and further separated on a C-18 analytical column (Acclaim PepMap100, 75 μm i. d. × 50 cm, C18, 3 μm, 100 Å, Thermo Scientific) using a 240 min gradient from 10 to 40% CH_3CN, 0.1% formic acid at a flow rate of 250 nl/min.

Peptides eluted were analyzed on the LTQ-Orbitrap Elite hybrid mass spectrometer operating in positive ion- and data dependent acquisition (DDA) mode using the following parameters: electrospray voltage 1.9 kV, CID fragmentation with normalized collision energy 35, automatic gain control (AGC) target value of 1E6 for Orbitrap MS and 1E3 for MS/MS scans. Each MS scan (*m*/*z* 400–1600) was acquired at a resolution of 120,000 FWHM, followed by 20 MS/MS scans triggered for intensities above 500, at a maximum ion injection time of 200 ms for MS and 50 ms for MS/MS scans.

2.3. Protein identification and quantitation

Raw data files from the mass spectrometer were analyzed in Proteome Discoverer 1.4 (Thermo Scientific) using the SEQUEST HT search engine with the May 2013 version of the human protein database from UniProt [9]. The following search parameters were used: enzyme specified as trypsin with maximum two missed cleavages allowed; precursor mass tolerance was 10 Da. The isotope labeled ^{13}C6-L-lysine, N-terminal acetylation and methionine oxidation were set as dynamic modifications. The Percolator tool was used for peptide validation and a cutoff value of 0.01 for false discovery rate (FDR). Thus peptides with high confidence only were used for final protein identification. The SILAC ratios were log transformed using base 2. The median of these values from technical replicates was merged to represent a biological replicate. The missing values were imputed using a normal distribution with a shifted mean of 1.5 left and rescaled standard deviation by 0.3. This was done in order to mimic the low abundance signal [10]. A *t*-test was performed between Fugene(+) and control SILAC log transformed ratios (base 2) to obtain the *p*-values. The median values of these were calculated if the protein was quantified in at least 2 biological replicates. Only those proteins were considered for further study whose representative biological replicate value showed >1.5