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Breaking limitations of complex culture media: Functional non-viral miRNA delivery into pharmaceutical production cell lines

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ABSTRACT

MicroRNAs (miRNAs) are promising targets for cell engineering through modulation of crucial cellular pathways. An effective introduction of miRNAs into the cell is a prerequisite to reliably study microRNA function. Previously, non-viral delivery of nucleic acids has been demonstrated to be cell type as well as culture medium dependent. Due to their importance for biopharmaceutical research and manufacturing, Chinese hamster ovary (CHO) and Cevec's Amniocyte Production (CAP) cells were used as host cell lines to investigate transfection reagents with respect to successful delivery of small non-coding RNAs (ncRNAs) and their ability to allow for biological activity of miRNAs and small interfering RNAs (siRNAs) within the cell. In the present study, we screened numerous transfection reagents for their suitability to successfully deliver miRNA mimics into CHO DG44 and CAP cells. Our investigation revealed that the determination of transfection efficiency for a given transfection reagent alone is not sufficient to draw conclusions about its ability to maintain the functionality of the miRNA. We could show that independent from high transfection rates observed for several reagents only one was suitable for efficient introduction of functional miRNA mimics into cells cultured in complex protein production media. We provide evidence for the functionality of transferred ncRNAs by demonstrating siRNA-mediated changes in protein levels and cellular phenotype as well as decreased *twinfilin-1* (*twf-1*) transcript levels by its upstream miR-1 regulator. Furthermore, the process could be shown to be scalable which has important implications for biotechnological applications.

1. Introduction

Since the discovery of the first miRNA in *Caenorhabditis elegans* almost two decades ago (Lee et al., 1993; Wightman et al., 1993), research of this class of small ncRNAs has resulted in numerous important biomedical discoveries (Esteller, 2011; Filipowicz et al., 2008; Grosshans and Slack, 2002). In the past, miRNAs have been shown to play a central role as key fine tuners of gene expression in various cellular signaling networks or cellular processes and have therefore gained increasing interest as promising targets for biotechnological applications (Barron et al., 2011b; Druz et al., 2013; Hackl et al., 2012a,b; Jadhav et al., 2013; Muller et al., 2008; Sun et al., 2010).

Careful examinations of miRNA function in mammalian cells call for a successful delivery of sufficient amounts of functional miRNA mimics or inhibitors, allowing for gain- or loss-of-function studies, respectively. For chemical introduction of plasmid DNA (pDNA) into mammalian cell lines cationic lipids (lipofection), calcium phosphate and cationic polymers (polyfection) are mostly employed. At first sight, miRNA mimics and pDNA share several common physicochemical properties, such as the negatively charged phosphodiester backbone and the double-stranded appearance. For several reasons the delivery of small ncRNA molecules into mammalian cells, however, differs substantially from the transfection of the much larger pDNA molecules which finally leads to different requirements for the delivery vehicle for double-stranded RNA (dsRNA) (Gary et al., 2007; Spagnou et al., 2004). For transfection of siRNAs, polyethylenimines (PEIs) both in linear or branched appearance and over a broad range of molecular

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weights have been successfully applied both for target validation purposes in vitro as well as for gene therapy approaches in vivo (Behr, 2012; Chen et al., 2009; Grayson et al., 2006; Lee et al., 2010; Liu et al., 2011; Tsai et al., 2011; Werth et al., 2006; Zintchenko et al., 2008). In addition, lipofection reagents initially designed for pDNA introduction into eukaryotic cells were also tested for their applicability to deliver siRNAs (Felgner et al., 1987; Yang et al., 2001). However, all these studies were limited to the functional delivery of siRNA, and none of them comprised the use of biotechnologically relevant manufacturing cell lines cultured in nutrient-rich production media as both aspects can crucially influence the process of transfection.

The most widespread expression host for the manufacturing of biopharmaceuticals are CHO cells (Chu and Robinson, 2001; Ye et al., 2009), whereas the novel versatile human expression system CAP recently gained much attention as a very promising candidate for the biopharmaceutical industry (Fischer et al., 2012; Genzel et al., 2012). Although research on miRNAs in human cells is at an advanced stage, there have been relatively few studies to date exploring the potential of miRNAs as cell line engineering tools for bioprocess applications, and very little is known to date about functional miRNA targets in CHO cells (Barron et al., 2011a; Hackl et al., 2011; Jadhav et al., 2012; Meleady et al., 2012). For further studies of miRNA function in mammalian cells a successful delivery of functional miRNA is an absolute prerequisite.

In the present study, we used CHO and CAP cell lines to examine a broad range of non-viral transfection reagents – among them different PEIs in a variety of molecular weights as well as several cationic lipids – for their ability to facilitate miRNA-mediated gene regulation. Only very few transfection reagents were capable to appropriately deliver small ncRNAs into cells grown in complex media, among those branched 1.2 kDa PEI (BPEI 1.2k) and ScreenFect®A, a cationic lipid. To assess functionality of the delivered RNAs, we established a variety of tools for CHO and CAP cells and identified *twinfilin 1* (*twf-1*) as a target gene for miR-1 in CHO. Surprisingly, subsequent analyses suggested that not all transfection reagents rendered the transfected small RNAs into a functional state in the cell. In this conjunction, although BPEI 1.2k showed transfection efficiencies greater than 90%, the polymer was not able to introduce mi- or siRNAs in a functional manner within the cell. In contrast, ScreenFect®A exclusively allowed for high transfection efficiency and similarly kept the miRNAs active within the cell leading to sufficient level of gene regulation as measured by downregulation of mRNA and protein levels, as well as induction of changes in cell phenotype. This reagent was also suitable for upscaling of cell culture volume, which has important implications for biotechnological applications and further target gene identification strategies in CHO cells.

2. Materials and methods

2.1. Cell culture and transfection

Suspension-adapted CHO DG44 cells (*Dhfr*$^{-/-}$) (Life Technologies, Carlsbad, CA, USA) (Urlaub and Chasin, 1980) as well as CHO-SEAP cells (IAB proprietary) were routinely grown in TubeSpin® bioreactor 50 tubes (TPP, Trasadingen, Switzerland) in ProCHO5 culture medium (Lonza, Vervier, Belgium) supplemented with 4 mM L-Glutamine, anti-clumping agent (1:1000) (Life Technologies), 100 μM sodium hypoxanthine and 16 μM thymidine (Life Technologies). CAP-SEAP cells (CEVEC Pharmaceuticals, Cologne, Germany) were grown in TubeSpin® bioreactor 50 tubes (TPP) in PEM culture medium (Life Technologies) supplemented with 4 mM L-Glutamine. Both cell lines were maintained at 37 °C, 5% CO_2 and 85% humidity with agitation at 140–160 rpm (25 mm orbit) in an orbital shaker incubator (Sartorius Stedim, Goettingen, Germany or Kuhner, Birsfelden, Switzerland) and adjusted to a final cell concentration of 0.2–0.4 × 10^6 viable cells per mL every 3–4 days. Cell density and viability were assessed using a CEDEX XS cell counter (Roche Diagnostics) by means of trypan blue exclusion. For small scale transfection, cells propagated in exponential growth phase were pelleted, resuspended in fresh culture medium and seeded in 12 well (5.0 × 10^5 cells per well; 1 mL culture volume) or 96 well (1.3 × 10^4 cells per well; 150 μL culture volume) suspension culture plates (Greiner, Frickenhausen, Germany). Transfection complexes were prepared following the instructions provided by the manufacturers or using in-house developed protocols for non-commercial reagents. The respective amounts of RNA and transfection reagent as well as incubation times are indicated in Table 1. For large scale transfection, 1.25 × 10^7 viable cells were taken up in 8.5 mL of fresh production medium and seeded in 125 mL shake flasks (Corning, Tewksbury, MA, USA) following addition of 1.5 mL lipoplexes composed of 50 nM siRNA and 30 μL ScreenFect®A. Ten nanomolar AF647-siRNA were co-transfected into all samples to follow transfection efficiency. After 4 h, 15 mL of fresh growth medium were added to the cultures to reach the final working volume and cell density of 25 mL and 0.5 × 10^6 viable cells per mL, respectively. Notably, for ncRNA transfections in agitated cultures slightly higher RNA concentrations are necessary compared to transfections in static condition. Mature miRNA mimics *mmu*-miR-1a-3p, AllStars Negative Control siRNA (with and without fluorescent Alexa Fluor®647 label), AllStars Human Cell Death Control siRNA and *anti*-SEAP siRNA were purchased from Qiagen (Qiagen, Hilden, Germany). As non-targeting miRNA *cel*-miR-67 from *C. elegans* was used (Katakowski et al., 2010; Sharma et al., 2009) and obtained from Qiagen. CHO-specific cell death control siRNAs were kindly provided by Dr. Eric Lader (Qiagen). For quantification of SEAP protein activity a SEAP Reporter Gene Assay (Roche Diagnostics) was employed according to the protocol provided by the manufacturer. Chemiluminescence was detected using a SpectraMax® M5e microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.2. Microscopic analysis

Intracellular uptake efficiency for small RNAs was followed by quantitating the abundance of DY-547-labeled scrambled RNA oligonucleotides termed siGLO® (Thermo Scientific, Langenselbold, Germany) 24 h post transfection. Transfected cells were paraformaldehyde-fixed for 30 min and counterstained for 5 min using 4′,6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich, St. Louis, MO, USA) to indicate cell nuclei. For preservation, cells were mounted in ProLong® Gold Antifade Reagent (Life Technologies). Two-dimensional images of transfected cells were captured using a Leica DMRA Fluorescence Microscope with a DFC340 FX camera (Leica, Wetzlar, Germany). For confirmation of intracellular RNA uptake, CHO DG44 were transfected with siGLO® and actin cytoskeleton as well as nuclei were counterstained with Alexa Fluor®488 labeled phalloidin (Life Technologies) and Hoechst 33342 (Sigma Aldrich), respectively. Images of fixed cells were taken by a Leica TCS SP2 AOBS Confocal Laser Scanning Microscope (Leica) equipped with the following excitation lasers: 405 (used for Hoechst33342), 458, 476, 488 (used for Alexa Fluor®488), 496, 514, 543, 561 (used for siGLO®), 594 and 633 nm.

2.3. Quantitative flow cytometry

CHO DG44 and CAP cells were transfected and analyzed for viable and Alexa Fluor®647 siRNA positive cells 24 h or 72 h following transfection. Cell density and viability were determined by quantitative flow cytometry employing a MACSQuant® Analyzer