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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

Table 1
Transfection reagents tested for small dsRNA delivery in 12 well plates (1 mL culture volume).

Transfection reagent	Supplier	Quantity of transfection reagent	RNA concentration	Time for complex formation
TransPass™ D2 (+V enhancer)	NEB, Ipswich, MA, USA Cat No. M2554S (M2561S)	2 μL (+1 μL)	50 nM	20 min
siPORT™ Neo FX™	Life Technologies, Carlsbad, CA, USA Cat No. AM4511	2 μL	50 nM (microscopy) 15 nM (functional studies)	10 min
ScreenFect® A	InCella, Eggenstein-Leopoldshafen, Germany Cat. No. S-3001	2 μL	50 nM (microscopy) 15 nM (functional studies)	25 min
HiPerFect®	Qiagen Cat. No. 301702	2 μL	50 nM	10 min
FreeStyle™ MAX	Life Technologies Cat. No. 16447100	2 μL	50 nM	20 min
GeneJuice®	Merck Millipore, Darmstadt, Germany Cat. No. 70967	2 μL	50 nM	15 min
Lipofectamine® RNAiMAX	Life Technologies Cat. No. 13778150	2 μL	50 nM	5 min
siRNA Prime	PAA, Pasching, Austria Cat. No. Q051-042	8 μL	50 nM	15 min
Fecturin™	Polyplus, Illkirch, France Ca No. 109-001	2 μL	50 nM	30 min
jetPEI™	Polyplus Ca No. 101-10	2 μL	50 nM	20 min
Linear 25 kDa PEI	Polysciences, Eppelheim, Germany Cat. No. 23966-2	2 μg	50 nM	10 min
PEI Max	Polysciences Cat. No. 24765-2	2 μg	50 nM	10 min
Branched 1.2 kDa PEI	Polysciences Cat. No. 06088-100	2 μg	50 nM (microscopy) 15 nM (functional studies)	10 min
Branched 1.8 kDa PEI	Polysciences Cat. No. 06089-100	2 μg	50 nM	10 min
Branched 10 kDa PEI	Polysciences Cat. No. 19850-100	2 μg	50 nM	10 min

(Miltenyi Biotech, Bergisch-Gladbach, Germany) equipped with a violet (405 nm), blue (488 nm) and red (635 nm) excitation laser. Toward this end, 100 nM of the Calcein-AM viability dye (Life Technologies) were used and transfection efficiency was determined by analyzing viable cells for Alexa Fluor®647 fluorescence.

2.4. Reverse transcription and real-time PCR (RT-PCR)

Total RNA (including small RNAs <200 bp) was isolated using the miRNeasy mini Kit (Qiagen) according to the protocol provided by the manufacturer. RNA concentration and purity was determined by UV-spectrometry using a NanoDrop® spectrophotometer (Thermo Scientific). The mean ± SD of the 260/280 nm ratios was 2.1 ± 0.1. Complementary DNA (cDNA) was synthesized from 400 to 1000 ng RNA using either miScript II RT Kit (Qiagen) or Maxima First Strand cDNA Synthesis Kit (Fermentas, St.Leon-Rot, Germany), respectively, depending on the subsequent analysis of mature miRNA or mRNA transcripts. RT-PCR was performed with 10^{-1} diluted cDNA using the miScript SYBR green PCR kit (Qiagen) for detection of mature miRNAs on a LightCycler® 480 (Roche Diagnostics). For gene expression analysis Roche UPL probes (Roche Diagnostics) were applied in combination with gene-specific primer sets. Hence, the following gene-specific primers were used: mature miR-1 FW, 5′-TGAATGTAAAGAAGTATGTAT-3′; the miScript Universal Primer (Qiagen) served as reverse primer for mature miR-1; U6 FW, 5′-CTCGCTTCGGCAGCACA-3′; U6 reverse (RV), 5′-AACGCTTCACGAATTTGCGT-3′; 18S rRNA FW, 5′-GCAATTATTCCCCATGAACG-3′; 18S rRNA RV, 5′-GGGACTT-AATCAACGCAAGC-3′; *twf-1* FW, 5′-GCAACAAGAGCGACTCTGAA-3′; *twf-1* RV, 5′-TCCATGTAATGATACGTCCTCCT-3′; *SEAP* FW, 5′-CTCCAACATGGACATTGACG-3′; *SEAP* RV, 5′-CCCATGCGAAACATGTACTTT-3′. Roche UPL probes No. 48 (*18S*), No. 10 (*twf-1*), and No. 39 (*SEAP*) were used for detection and in combination with 2x LightCycler® 480 Probes Master Mix (Roche Diagnostics). Relative gene expression differences were calculated by applying the comparative C(T) method (Schmittgen and Livak, 2008).

3. Results

3.1. Transfection reagent screen for efficient delivery of short dsRNAs

A screen of potential transfection reagents (Table 1) for small RNA duplex intermediates, such as miRNA mimics or siRNAs, was performed with CHO DG44 cells cultivated in ProCHO5 production medium. Cells were transfected with commercially available, RISC-independent, DY-547-labeled siGLO® ribonucleic acids using either different PEIs or various commercially available transfection reagents. Qualitative analyses for positively transfected cells were performed 24 h post transfection by fluorescence microscopy (Fig. 1). Cells transfected with siGLO® using BPEI 1.2k showed high levels of red fluorescent dsRNA as discrete spots either close to or co-localized with the DAPI-stained nuclei (Fig. 1A). In contrast, most cells which were transfected with ScreenFect®A (Fig. 1M) exhibited a strong siGLO®-fluorescence spread all over the cell. Using Lipofectamine®RNAiMAX, a few cells were also found to be fluorescent, but similar to BPEI 1.2k many fluorescent precipitates could also be observed in the surrounding area of the cells (Fig. 1O). Microscopic images of cells transfected with the other reagents tested revealed either none or only a weak fluorescence (Fig. 1B–L and 1N). Appropriate brightfield images of transfected CHO cells shown in Fig. 1 are provided as Supplementary Figure S1.

Changing the cultivation medium from ProCHO5 to DMEM/F12 led to an increase in the number of siGLO®-positive cells for most transfection reagents tested except for GeneJuice®, linear 25 kDa

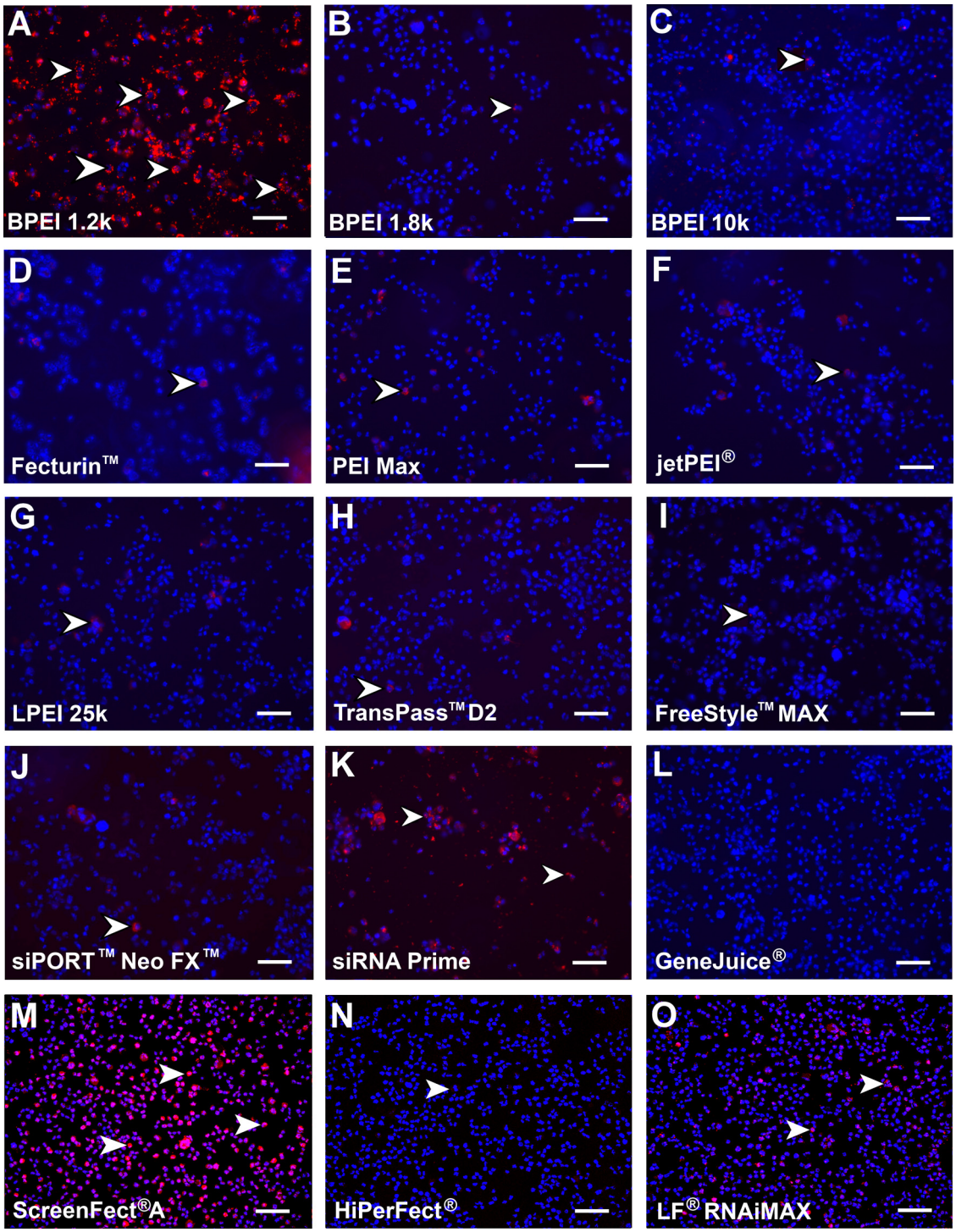

Fig. 1. Fluorescent micrographs of paraformaldehyde-fixed CHO DG44 cells transiently transfected with fluorescent siGLO® RNA molecules (red) in ProCHO5 culture medium using following transfection reagents: (A) BPEI 1.2k, (B) BPEI 1.8k, (C) BPEI 10k, (D) Fecturin™, (E) PEI Max, (F) jetPEI™, (G) LPEI 25k, (H) TransPass™ D2 (+V), (I) FreeStyle™MAX, (J) siPORT™ Neo FX™, (K) siRNA Prime, (L) GeneJuice®, (M) ScreenFect®A, (N) HiPerFect®, (O) Lipofectamine®RNAiMAX. Cell nuclei were counterstained with DAPI (blue). Arrows indicate siGLO® RNA. Images were obtained at 20× magnification. Scale bars indicate 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

PEI and PEI Max (Fig. 2). Occurrence of fluorescent precipitates as described previously for BPEI 1.2k and Lipofectamine®RNAiMAX was additionally observed in cultures transfected with BPEI 1.8k, BPEI 10k and siPORT™ Neo FX™.

To confirm the successful intracellular RNA uptake mediated by BPEI 1.2k and ScreenFect®A, we analyzed siGLO® transfected CHO cells by confocal laser scanning microscopy. CHO DG44 cells were transfected in ProCHO5 culture medium and actin cytoskeleton as well as cell nuclei were additionally counterstained to indicate cellular boundaries. Representative confocal micrographs depicted in Fig. 3 revealed that siGLO® was efficiently transferred to the inner part of the cells by both transfection reagents. However, in contrast to BPEI 1.2k most of the ScreenFect®A transfected cells exhibited a diffusely distributed fluorescent signal throughout the

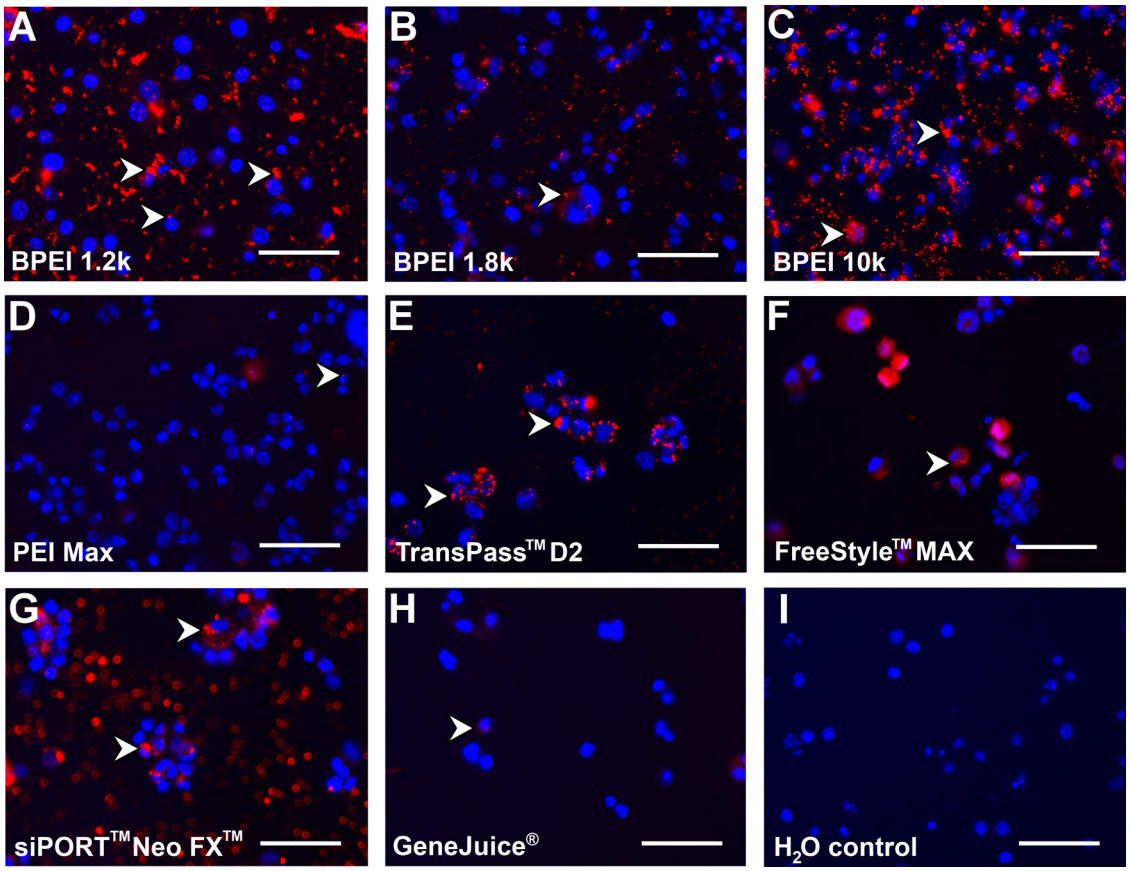

Fig. 2. Fluorescent micrographs of paraformaldehyde-fixed CHO DG44 cells transiently transfected with fluorescent siGLO® RNA molecules (red) in DMEM/F12 culture medium using following transfection reagents: (A) BPEI 1.2k, (B) BPEI 1.8k, (C) BPEI 10k, (D) PEI Max, (E) TransPass™ D2 (+V), (F) FreeStyle™MAX, (G) siPORT™ Neo FX™, (H) GeneJuice®, (I) H_2O (Control). Cell nuclei were counterstained with DAPI (blue). Arrows indicate siGLO® RNA. Images were obtained at 40× magnification. Scale bars indicate 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

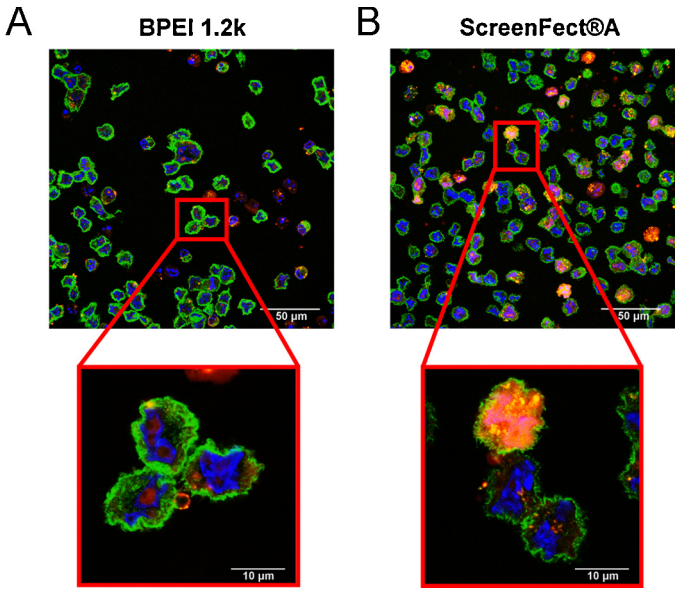

Fig. 3. Confocal laser scanning micrographs of CHO DG44 cells transfected with siGLO® (red) using (A) BPEI 1.2k or (B) ScreenFect®A as delivery vehicle. Cells were transfected in ProCHO5 production medium and paraformaldehyde-fixed 24 h post transfection and cell nuclei (blue) as well as actin cytoskeleton (green) was counterstained using DAPI and Alexa Fluor®488 labeled phalloidin, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

whole cytoplasm as early as 24 h post transfection arguing for a rapid and successful endosomal escape of the siGLO® RNA.

For quantitative analysis of transfection efficiency, BPEI 1.2k and ScreenFect®A transfected cells were analyzed by flow cytometry. CHO DG44 cells as well as a second production cell line of human origin, CAP, were transfected with an Alexa Fluor® 647-labeled, scrambled siRNA (AF647-siRNA). CHO and CAP cells were transfected in ProCHO5 and PEM production medium, respectively. Analyses of viable (Calcein-AM-positive) and Alexa Fluor® 647-positive cells were conducted 24 h post transfection which yielded very high transfection efficiencies of up to >95% in CHO and 93% in CAP cells (Fig. 4). The results identified BPEI 1.2k and ScreenFect®A as potential transfection reagents to deliver small ncRNAs in production cell lines grown in complex media.

3.2. *Transfection reagents influence the biological function of miRNAs*

One of the most important properties of a suitable miRNA transfection vehicle is the ability to render miRNAs functional upon delivery into the cell. To test functional delivery, the reduction of miRNA target mRNA levels can be analyzed. Since there are no miRNA targets identified for CHO cells so far, we searched for a suitable model miRNA. In human and mouse, miR-1 has been shown to bind to the 3′-UTR of the *twf-1* (also known as *PTK-9*) transcript and induce mRNA degradation (Li et al., 2010; Lim et al., 2005). Changes in mRNA levels could be investigated via qRT-PCR. In the recently published CHO genome we identified two putative miR-1 direct miRNA:mRNA binding sites in the

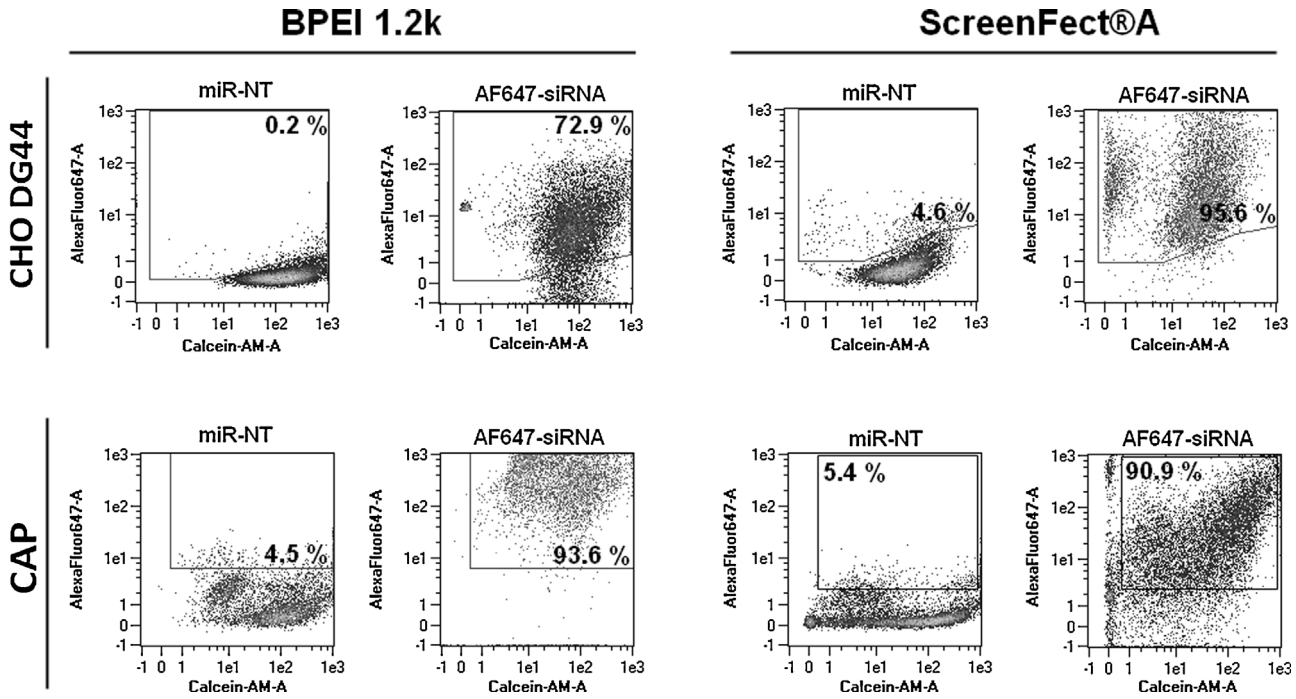

Fig. 4. Determination of transfection efficiency of CHO DG44 (upper panel) and CAP cells (lower panel) after transient transfection with either Alexa Fluor®647 labeled scrambled siRNA or non-labeled scrambled siRNA. Cells were transfected either using BPEI 1.2k (left) or ScreenFect®A (right) and analyzed for Calcein-AM positive (viable) and Alexa Fluor®647-positive cells 24 h post transfection via flow cytometry. CHO DG44 cells were transfected in ProCHO5 and CAP cells in PEM culture medium.

3′-UTR of the *twf-1* gene showing perfect homology to the human and mouse orthologs (Fig. 5A). miR-1 was therefore chosen as a model miRNA to test transfection reagents for their ability to deliver functional miRNA mimics into CHO cells.miR-1 mimics were transfected into CHO DG44 cells grown in ProCHO5 culture medium using either BPEI 1.2k or ScreenFect®A as delivery vehicle. After transfection the cells were incubated for 48 h until total RNA (including small RNAs <200 bp) was isolated and duplicate samples were pooled followed by reverse transcription reactions. To assess the successful introduction of miR-1 mimics, we used qRT-PCR to measure mature miR-1 levels relative to control samples of cells transfected with non-targeting miRNA (miR-NT). Fold-changes are depicted in Fig. 5B and illustrate that CHO cells transfected with ScreenFect®A showed a 29.0-fold (±0.9 SD; $n = 2$) increase in mature miR-1 levels whereas no significant increase could be observed in cultures transfected with BPEI 1.2k. This was rather surprising since the BPEI 1.2k polymer initially showed very promising results during fluorescence microscopy and flow cytometry.

To assess if the transferred miR-1 was able to downregulate mRNA levels, we quantified *twf-1* mRNA from the same cDNA samples. Transient introduction of miR-1 into CHO DG44 cells mediated by ScreenFect®A led to a functional downregulation of the *twf-1* gene of 80.8 ± 5.8% compared to control cells transfected with miR-NT (Fig. 5C). However, no significant changes in *twf-1* mRNA levels could be achieved after transfection with BPEI 1.2k. Notably, control transfections of miR-1 using the widely applied siPORT™ Neo FX™ reagent were additionally conducted in a basal DMEM/F12 medium since it was not applicable in ProCHO5 production medium (Fig. 1J). Although the siPORT™ Neo FX™ reagent attained a 42.0-fold (±14.1 SD; $n = 2$) increase in mature miR-1 concentrations, no significant reduction in *twf-1* mRNA levels could be observed (data not shown). These results reveal that the delivery of functional miRNA mimics is highly dependent on the transfection agent. In our study, only ScreenFect®A was able to deliver functional miRNAs into production cell lines grown in complex media.

3.3. Knockdown of protein levels highlights applicability of ScreenFect®A

Small ncRNAs are known to be able to downregulate both mRNA and protein levels. To evaluate, if ScreenFect®A was also able to downregulate protein levels by delivered small ncRNAs in pharmaceutical production cell lines, CHO-SEAP and CAP-SEAP cell lines stably expressing the human placental secreted alkaline phosphatase (SEAP) were transfected with an *anti*-SEAP siRNA. Transfections were again performed as previously described employing ScreenFect®A, and SEAP mRNA as well as protein levels were determined 48 h post transfection (Fig. 6). Quantification of SEAP mRNA was performed by qRT-PCR and SEAP protein activity was determined by chemiluminescence using a SEAP reporter gene assay. Transfection of *anti*-SEAP siRNA in CHO-SEAP cells resulted in a 47.9 ± 3.5% knockdown of SEAP mRNA level leading to a 46.2 ± 9.9% decrease in SEAP protein levels. Likewise, in CAP-SEAP cells ScreenFect®A facilitated a 56.6 ± 7.3% downregulation of SEAP mRNA which yielded a 36.5 ± 1.3% reduction in SEAP protein levels.

3.4. Transfection of small dsRNAs induces a phenotype change and is scalable

To show scalability of the transient transfection of small dsRNAs using ScreenFect®A, CHO-SEAP and CAP-SEAP suspension cells were transiently transfected with either species-specific Cell Death Control siRNA, *anti*-SEAP siRNA or non-targeting siRNA (NT siRNA) as control. Viable cell density (VCD) and cell viability was assessed over time during 6 days post transfection.

Analysis of cell density and viability revealed a strong decrease in the number and percentage of viable cells in both CHO-SEAP and CAP-SEAP cells transfected with Cell Death Control siRNA as compared to NT siRNA transfected cells (Fig. 7A). *Anti*-SEAP siRNA transfected CHO-SEAP cells showed a similar growth behavior until day three post transfection, but reached

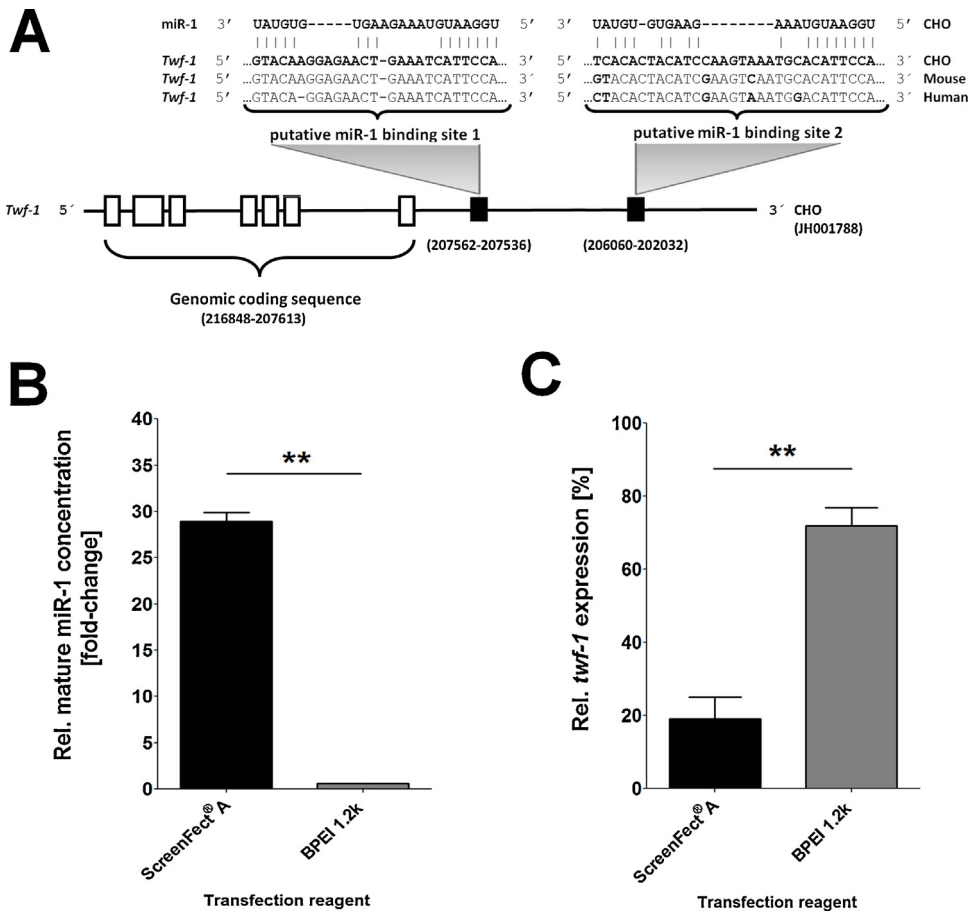

Fig. 5. (A) Two putative miR-1 binding sites are present within the 3′-UTR of the *twinfilin-1* (*twf-1*) gene and are highly conserved between *C.griseus* (CHO) and human or mouse. (B) Relative cellular abundance of mature miR-1a-3p or (C) *twf-1* mRNA in CHO DG44 cells transfected with miR-1a-3p mimics using either ScreenFect®A or BPEI 1.2k, as compared to endogenous miR-1a-3p levels in miR-NT transfected control cells. Total RNA was isolated 48 h post transfection employing the miRNeasy Mini Kit (Qiagen). Isolated RNA samples of triplicate transfections were pooled and qRT-PCR reactions were performed in technical replicates ($n = 3$) indicated by error bars. Mature miR-1a-3p levels are expressed relative to U6 snRNA, whereas *twf-1* mRNA levels are normalized to the levels of 18S rRNA. For statistical analysis a one-way ANOVA was applied ($^{**}p < 0.01$).

a slightly lower maximal cell density compared to control cells whereas growth behavior of *anti*-SEAP siRNA transfected CAP-SEAP cells was highly similar to the NT siRNA transfected control cells.

Transfection efficiency and cell viability were additionally determined by means of Alexa Fluor®647- and Calcein-AM-fluorescence intensity using flow cytometry on day 3 post transfection (Fig. 7B). More than 80% of both CHO-SEAP and CAP-SEAP cells were found to be Alexa Fluor®647-positive indicating high transfection efficiencies even in agitated cultures. The slightly lower percentages of AF647-positive cells compared to previous experiments (Fig. 4) were probably caused by agitation of cultures and the use of only 10 nM of AF647-siRNA spiked into each of the effector siRNAs. A substantial decrease in cell viability could be confirmed for both cell lines by an increased number of Calcein-AM-negative cells in cultures transfected with the Cell Death Control siRNAs.

Furthermore, significantly lower specific SEAP productivities were determined for cells transfected with *anti*-SEAP siRNA (Fig. 7C) resulting in lower SEAP titer during 6 days batch cultivation (Fig. 7D). These observations highlight the applicability of the ScreenFect®A reagent to facilitate functional delivery of small ncRNAs in various scales, different modes of agitation and in conjunction with different pharmaceutical production cell lines grown in complex culture media.

4. Discussion

MicroRNAs are key regulators of gene expression in mammalian cells and thus have been extensively investigated in clinical and basic research (Aschrafi et al., 2008; Bartel, 2009; Esteller, 2011; Frankel et al., 2008; Luo et al., 2012). However, miRNAs still represent a neglected player regarding cell engineering strategies and only a few studies addressed this topic so far, although miRNAs could be used as powerful regulators of cellular phenotypes (Barron et al., 2011a; Druz et al., 2011, 2013; Jadhav et al., 2013; Muller et al., 2008).

During recent years a multitude of commercially available transfection reagents based on cationic lipids or polymers have been designed for introduction of small ncRNAs into cells and tissues as a result of emerging RNA interference (RNAi) applications. However, the suitability of the different transfection reagents is specifically determined by the cell type employed and in conjunction with a compatible cultivation medium (Bertschinger et al., 2006; Eberhardy et al., 2009; Geisse and Fux, 2009; Schlaeger and Christensen, 1999). For the production of biopharmaceuticals CHO cells have been one of the most frequently used hosts for decades. They comprise several advantages such as growth in suspension culture to very high cell densities and the resistance to viral infection (Chu and Robinson, 2001; Hackl et al., 2012a; Birch and Onakunle, 2005). The ProCHO5 medium was developed

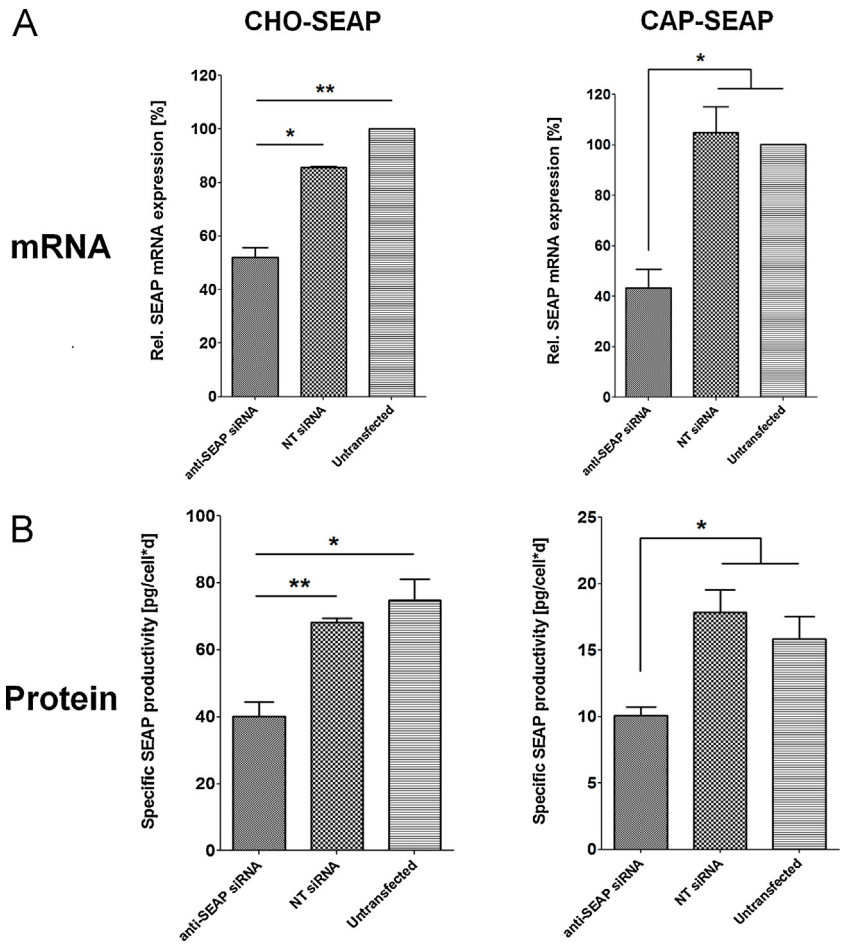

Fig. 6. (A) Relative SEAP mRNA levels in CHO-SEAP (left) and CAP-SEAP (right) cells after transfection of anti-SEAP siRNA or non-targeting siRNA (NT siRNA) using ScreenFect® A, as compared to SEAP mRNA levels in untransfected control cells. Total RNA was isolated 48 h post transfection employing the RNeasy Mini Kit (Qiagen). Isolated RNA samples of triplicate transfections were pooled and qRT-PCR reactions were performed in technical replicates ($n = 3$) indicated by error bars. SEAP mRNA levels are normalized to the levels of 18S rRNA. (B) Specific SEAP productivity of CHO-SEAP (left) and CAP-SEAP (right) cells after introduction of either anti-SEAP siRNA or NT siRNA using ScreenFect® A and compared to untransfected control cells. SEAP protein levels were calculated for viable cell density 48 h post transfection. Error bars represent the SD of three independent transfections. For statistical analysis an unpaired two-tailed *t*-test was applied ($^{*}p < 0.05$; $^{**}p < 0.01$).

for optimized growth and production of recombinant proteins using CHO cells and has been widely employed (Link et al., 2004; Rajendra et al., 2011; Ye et al., 2009). However, high transfection rates of DNA into CHO cell lines facilitated by cationic lipids or polymers have been generally described to be both sophisticated and culture medium dependent (Derouazi et al., 2004; Geisse, 2009; Godbey et al., 1999; Reisinger et al., 2009; Thompson et al., 2012). Recently a novel versatile production cell system derived from human amniocytes named Cevec's Amniocyte Production cell line has gained much attention in the biopharmaceutical community (Fischer et al., 2012; Genzel et al., 2012; Schiedner et al., 2008, 2000). Recombinant therapeutics exhibiting fully human posttranslational modifications while produced in a non-tumor originated and ethically accepted human cell line are desired by regulatory authorities. Up to now, there are no studies available addressing a direct introduction of miRNAs into CAP or CAP-T cells and only very few refer to CHO cells (Barron et al., 2011a; Meleady et al., 2012).

The aim of the present study was to identify a non-viral transfection reagent capable of efficiently delivering active miRNA mimics into pharmaceutical production cell lines grown in complex culture media optimized for high-level recombinant protein production.

The employed transfection reagent screen clearly supports the assumption that a successful transfection of small ncRNAs into CHO and CAP cells is indeed highly dependent on the combination of transfection reagent and cultivation medium. The recently developed and commercialized cationic lipid ScreenFect®A outperformed all other transfection reagents tested. ScreenFect®A was exclusively able to confer efficient downregulation of the selected target gene *twf-1* by transfected miR-1 mimics. Only very recently the CHO genome and transcriptome (*Cricetulus griseus*) was sequenced (Becker et al., 2011; Xu et al., 2011) and miRNA expression profiles as well as miRNA sequences expressed in CHO-K1 cells have become publicly available (Hackl et al., 2011, 2012b; Hernandez Bort et al., 2012). MiR-1 was selected as model miRNA since it has been previously reported to lower *twf-1* mRNA levels in human and mouse (Li et al., 2010; Lim et al., 2005). Decreasing mRNA levels in CHO cells were detected directly via qRT-PCR 2 days after miR-1 introduction. Furthermore, mouse/human miR-1 shows high sequence identity to the *cgr*-miR-1, in fact, the *cgr*-miR-1 sequence is nearly 100% homologous to the miR-1 derived from mouse and human except for one single nucleotide at position 17 (G → A). Moreover, two putative miR-1 binding sites within the 3′-UTR of the *twf-1* mRNA predicted from CHO genome scaffold data (GenBank: NW_003615367.1./transcript XM_003512136) were identified (Fig. 5A). Our data suggest that miR-1 regulates *twinfilin-1* in CHO cells as well, providing evidence for a potential direct target gene for miR-1 in Chinese hamster ovary cells. As shown with human and mouse cell lines (Li et al., 2010; Lim et al.,

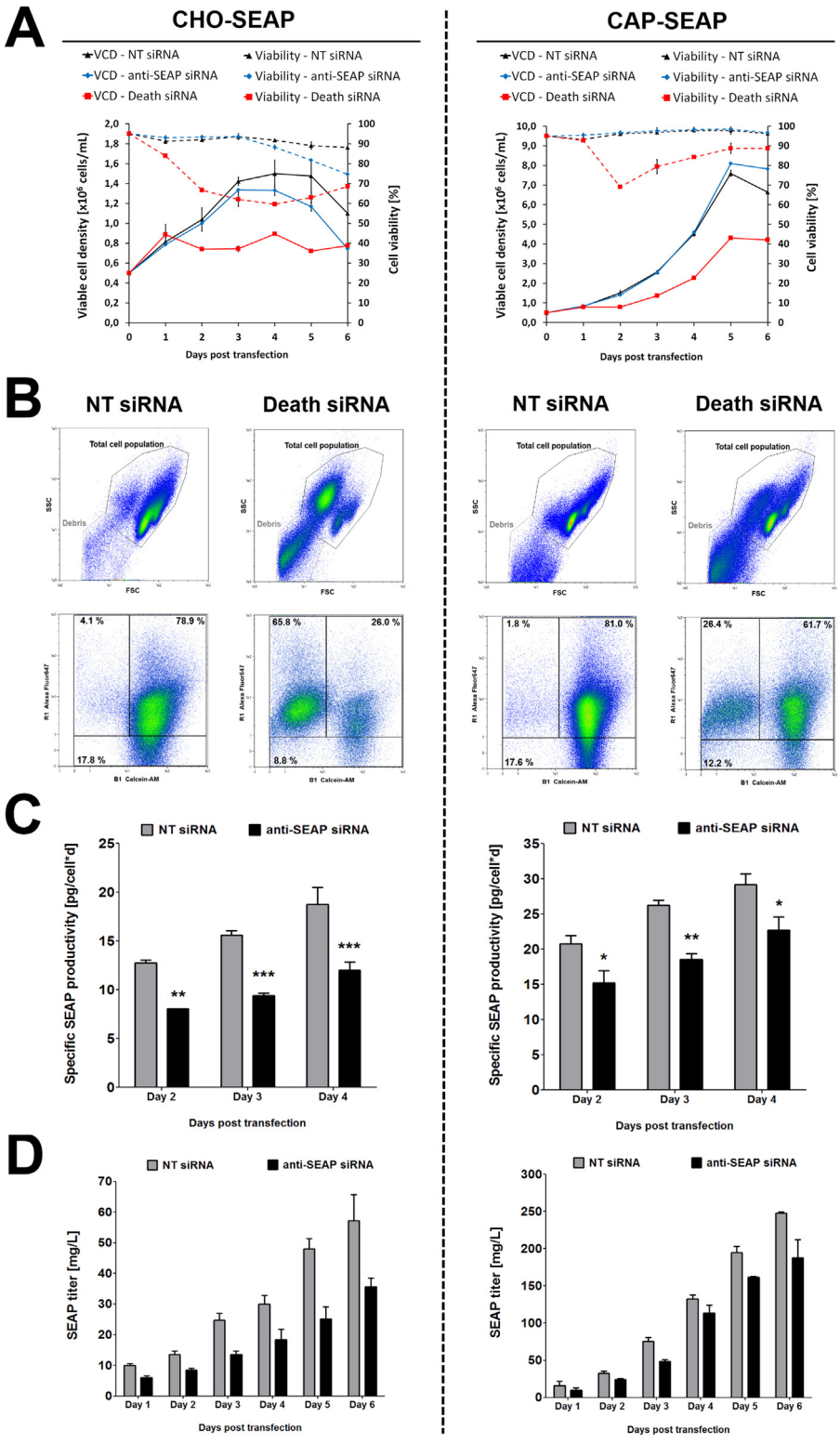

Fig. 7. Large scale siRNA transfection using ScreenFect®A in CHO-SEAP and CAP-SEAP cells. (A) Time-resolved determination of viable cell density (VCD) and cell viability of CHO-SEAP (left) and CAP-SEAP cells (right) following introduction of either Cell Death Control siRNA (red squares), *anti*-SEAP siRNA (blue diamonds) or NT siRNA (black triangles) as control. Cell density and viability were analyzed using a CEDEX XS cell counter (Roche Diagnostics) for a period of 6 consecutive days. Error bars represent the SD of two independent measurements. (B) Determination of transfection efficiency (Alexa Fluor®647) and cell viability (Calcein-AM) of CHO-SEAP (left) and CAP-SEAP cells (right) via flow cytometry 72 h following large scale siRNA transfection. Upper density blots illustrate forward/side scatter analyses whereas lower density blots show AlexaFluor®647- against Calcein-AM-fluorescence. 10 nM Alexa Fluor®647 labeled NT siRNA (AF647-siRNA) was spiked into each culture to follow transfection efficiency. (C) Specific SEAP productivity of *anti*-SEAP- (black bars) or NT siRNA-transfected (gray bars) CHO-SEAP and CAP-SEAP cells on days 2, 3 and 4 post transfection. Quantification of SEAP concentration was performed in technical duplicates ($n = 2$) indicated by error bars and calculated for viable cell density. For statistical analysis a two-way ANOVA was applied (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (D) SEAP titer of CHO-SEAP and CAP-SEAP cells either transfected with *anti*-SEAP (black bars) or NT siRNA (gray bars). SEAP protein concentrations were determined in the culture supernatant on each day during cultivation. Error bars indicate the SD of $n = 2$ technical replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2005) miR-1 mediated downregulation of *twf-1* can be useful as a positive control for validation of miRNA transfection even in CHO cells and can be quickly assessed via qRT-PCR.

In order to demonstrate full functionality as well as scalability of ScreenFect®A-mediated small ncRNA delivery, downregulation of protein levels by *anti*-SEAP siRNAs were analyzed and significant reduction of specific SEAP productivity could be observed (Figs. 6 and 7). The moderate knockdown of SEAP protein levels may depend on the overexpression of the SEAP transgene being under the control of a strong human cytomegalovirus (hCMV) immediate-early promoter. However, the aim of future applications of a scaled ncRNA transfection will be the targeting of endogenous genes being expressed at much lower levels. Such a targeting of endogenous genes was addressed by the introduction of Cell Death Control siRNAs and influences on cellular phenotypes by these transferred siRNAs were shown in both production cell lines by lethal knockdowns (Fig. 7A and B). The demonstration of efficient mRNA knockdown, reduction of protein levels and phenotype changes, ultimately paves the way for a successful functional introduction of miRNAs into cell lines grown in complex culture media.

The exact route, however, of the ScreenFect®A/miRNA complexes to the cytoplasm and its dissociation from each other still needs to be elucidated. Regarding the fluorescent micrographs depicted in Fig. 3B, the route of small dsRNA molecules into the cytoplasm of the cell mediated by ScreenFect®A appears to be similar to pDNA although the physicochemical nature of pDNA and miRNA differs substantially (Gary et al., 2007). Generally, cationic lipid molecules consist of a hydrophilic head group connected to a hydrophobic tail and bind to negatively charged miRNA mimics leading to the formation of lipoplexes still featuring a surplus of positive charges (Felgner et al., 1987; Ma et al., 2007). These complexes interact with the cell surface and transfer the RNA to the cytoplasm either via a fusion with the cell membrane or through endocytosis followed by endosomal escape (Delgado et al., 2011; Duan et al., 2009; Kamiya et al., 2002; Nichols, 2003; Wasungu and Hoekstra, 2006; Zhou and Huang, 1994). Notably, the complexed miRNA mimics appear to be rapidly released to the cytoplasm indicated by the early blurring effect observed for the siGLO® RNA (Fig. 3B). Once the miRNA mimics have successfully been transferred to the cytoplasm they can eventually be incorporated into the miRISC complex, which is then recruited by the miRNA to its target mRNA (Gurtan and Sharp, 2013; Krol et al., 2010; Pasquinelli, 2012). A schematic overview on how the introduction of miRNA mimics into mammalian cells mediated by ScreenFect®A might occur is illustrated in Fig. 8.

Considering the other tested transfection reagents, our results provide evidence that although transfection reagents like BPEI 1.2k can be highly efficacious in transferring dsRNAs into cell lines grown in complex cultivation media, they may be unable to deliver functional ncRNAs that will knockdown their target genes. Bertschinger and colleagues have shown that linear or branched PEIs have a higher affinity to RNA compared to DNA (Bertschinger et al., 2006) and it appears that the strong PEI:RNA interaction might prevent the RNA from being released into the cytoplasm once it has been taken up via endocytosis. Such a strong interaction might also even prevent the miRNA from being released during RNA isolation which could be a reason for the negative results observed for mature miR-1 quantification (Fig. 5B). Another scenario for the weak regulatory capacity might be that PEI:RNA complexes are trapped inside the endosomes due to inefficient endosomal escape. This hypothesis would be supported by differences seen in fluorescent micrographs where fluorescent RNA signals after BPEI 1.2k-mediated transfection were solely detected as discrete sphere-like structures whereas ScreenFect®A-mediated RNA delivery results in a diffusely distributed fluorescence spread throughout the whole cytoplasm (Fig. 3B).

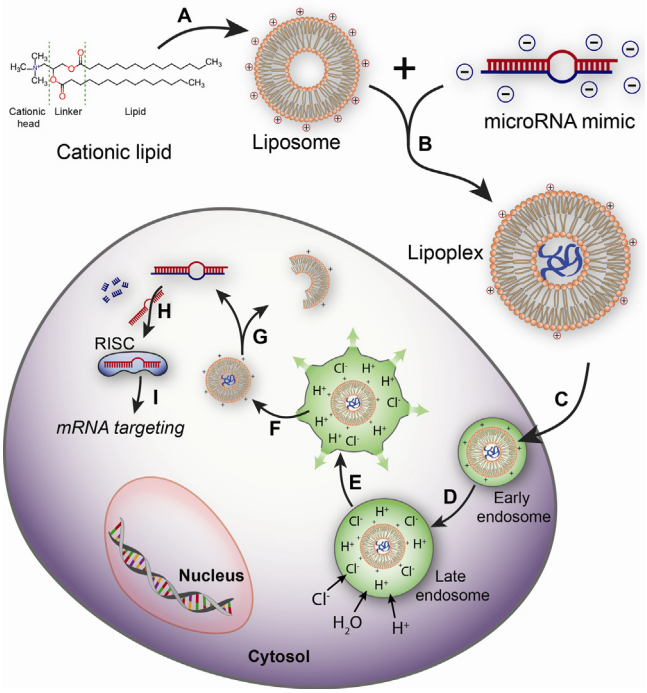

Fig. 8. Schematic overview on the ScreenFect®A-mediated microRNA transfection process. (A) ScreenFect®A as a cationic lipid can interact with anionic ribonucleic acids such as microRNA mimics (B) leading to complexes with excessive positive charges (Felgner et al., 1987; Ma et al., 2007). (C) Formed ScreenFect®A/miRNA lipoplexes can bind to negatively charged proteoglycans on the cell surface and are taken up by the cell most likely (D) via endocytosis (Duan et al., 2009; Wasungu and Hoekstra, 2006). (E) Destabilization of the endosomal membrane is then believed to cause endosomal disruption which facilitates the release of the lipoplexes to the cytoplasm (F) (Kamiya et al., 2002; Zhou and Huang, 1994). (G) After a successful dissociation from the lipid carrier, the delivered miRNA becomes activated through (H) incorporation into the RISC complex where the passenger strand of the miRNA is being removed (Krol et al., 2010). (I) Finally, the miRNA can bind and regulate their target mRNAs within the cytosol upon translational repression or mRNA decay (Gurtan and Sharp, 2013; Pasquinelli, 2012).

The fact that all the other transfection reagents failed in delivering the RNA into CHO cells cultured in ProCHO5 medium was rather surprising considering that DNA transfection into this cell type using linear PEI has been proven to be feasible (Derouazi et al., 2004; Geisse, 2009; Wulhfard et al., 2008). It appears that the cellular uptake of RNA molecules with these transfection reagents is inhibited by ingredients of the rich culture medium. An observation which was further supported after the medium was exchanged to a basal DMEM/F12 culture medium mixture leading to the recovery of most transfection reagents at least regarding the capability to deliver fluorescently labeled dsRNA molecules into CHO cells. The outcome of our studies stresses the importance of a critical and careful assessment of functionality of transfected miRNAs by confirming a specific decrease in mRNA/protein levels or phenotypic changes. Determination of high transfection efficiencies alone, e.g. via flow cytometry or fluorescence microscopy, can lead to false positive transfection reagent candidates that can entirely fail in triggering RNA interference.

Considering the applicability of ScreenFect®A as a potent delivery vehicle for small double-stranded RNAs in rich production media, this reagent may open up new horizons for miRNA research and applications in biotechnology and cell engineering. Since the ScreenFect®A reagent is capable to successfully deliver miRNAs into CHO and CAP cells in the presence of complex medium it is likely that the reagent is compatible with other (difficult-to-transfect) cell types and culture media as well. Furthermore, we demonstrated ScreenFect®A's ability to facilitate small ncRNA

transfection in shaked cultures as well as at different scales (Fig. 7). However, transfections of small ncRNAs in agitated cultures requires an increase in RNA concentration compared to static transfections since fluid dynamics might decrease the probability of cell:lipoplex interactions which could attenuate the chance of a successful uptake event. High viabilities of NT siRNA transfected control cells indicated that an increased RNA concentration of up to 50 nM in agitated transfections will not lead to off-target effects. Upscaling strategies within the optimized environment of a production medium are now becoming feasible and more cost-effective and would as well supply an increased number of cells for pull-down experiments for target mRNA identification whose success is highly dependent on the amount of starting material (Long and Lahiri, 2012; Thomson et al., 2011).

Upcoming achievements within the rather novel field of small non-coding RNAs such as miRNAs, siRNAs or piRNAs (PIWI-interacting RNAs) will certainly have the potential to drive cell line engineering strategies at industrial and academic research in the near future (Hackl et al., 2012a; Jadhav et al., 2013).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.08.027.

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