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Stable microRNA expression enhances therapeutic antibody productivity of Chinese hamster ovary cells ☆

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ABSTRACT

MicroRNAs (miRNAs) are short non-coding RNAs that post-transcriptionally regulate the expression of different target genes and, thus, enable engineered gene networks to achieve complex phenotypic changes in mammalian cells. We hypothesized that exploiting this feature of miRNAs could improve therapeutic protein production processes by increasing viable cell densities and/or productivity of the mammalian cells used for manufacturing. To identify miRNAs that increase the productivity of producer cells, we performed a genome wide functional miRNA screen by transient transfection of Chinese hamster ovary (CHO) cells stably expressing an IgG1 antibody (CHO-IgG1). Using this approach, we identified nine human miRNAs that improved the productivities not only of the CHO-IgG1 cells but also of CHO cells expressing recombinant human serum albumin (HSA), demonstrating that the miRNAs act in a product-independent manner. We selected two miRNAs (miR-557 and miR-1287) positively impacting the viable cell density and the specific productivity, respectively, and then stably co-expressed them in IgG1 expressing CHO cells. In these cells, higher IgG1 titers were observed in fed-batch cultures whilst product quality was conserved, demonstrating that miRNA-based cell line engineering provides an attractive approach toward the genetic optimization of CHO producer cells for industrial applications.

1. Introduction

CHO cells are the most widely used mammalian cell line for the manufacture of recombinant protein therapeutics. These cells can easily be adapted to growth in suspension under serum-free conditions and engineered to stably secrete recombinant proteins of interest that are correctly processed and post-translationally modified (Jayapal et al., 2007; Wurm, 2004). Despite optimization of the bioprocess, manufacturing of biologics for therapeutic use is expensive, and therefore, the industry seeks means to lower costs. In recent years, cell line engineering efforts have improved the productivity of CHO cells. These approaches included the stable expression of genes that impact cell performance at different levels by, for example, increasing cell proliferation (Dreesen and Fussenegger, 2011), protecting from apoptosis (Becker et al., 2010; Lim et al., 2006), or enhancing their secretory capacity (Becker et al., 2008; Florin et al., 2009; Ku et al., 2008; Le Fourn et al., 2013; Peng and Fussenegger, 2009; Peng et al., 2010; Tigges and Fussenegger, 2006).

Switching the phenotype of a cell from a low to a high producer state may be difficult to achieve by a single genetic alteration. Here, the class of RNA regulatory molecules, so-called microRNAs or miRNAs, are promising as they can regulate entire networks of genes and thus contribute to cell fate determination (Bartel, 2009; Fabian et al., 2010). miRNAs are small non-coding RNAs between 19 and 25 nucleotides in length that control the expression of target mRNAs at the post-transcriptional level. Because miRNAs bind to imperfect sequence matches within the 3′ UTR of target mRNAs, a single miRNA generally has multiple mRNA targets. miRNA binding results in translational repression and/or degradation of the target mRNA, thereby fine-tuning protein expression (Bartel, 2009; Fabian et al., 2010). Imposing no extra burden on the cell's translational machinery is an additional advantage of using miRNAs in cell line engineering (Hackl et al., 2012a).

miRNAs have been well characterized in the context of development and cell transformation, where the expression of specific

Abbreviations: miRNA, microRNA; CHO, Chinese hamster ovary; CGE, capillary gel electrophoresis; FAM, 6-FAM (6-carboxyfluorescein); HSA, human serum albumin; hsa-miR, *Homo sapiens*-microRNA

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miRNAs correlates with different cell lineages, cancer types or tumor progression stages (Iorio and Croce, 2012; Lee and Ambros, 2001; Reinhart et al., 2000). In CHO cells, several miRNA profiling studies have also revealed that miRNAs are differentially expressed in response to specific culture conditions, such as nutrient depletion (Druz et al., 2012) or temperature shift (Barron et al., 2011; Gammell et al., 2007), and during the different growth phases of batch cultivation (Hernandez Bort et al., 2012). While this has led to the identification of distinct miRNA expression patterns, these data alone cannot predict which miRNA is functionally involved in a specific response and should be manipulated to confer an advantage to CHO cells in the bioprocess.

To identify potential miRNAs for CHO cell line engineering, we performed a functional genome-wide screen using the titer of a therapeutic antibody model protein in the cell culture supernatant as a read-out. By transient transfection of an IgG1 producing CHO cell line (CHO-IgG1) with a human miRNA library, we were able to identify nine miRNAs that improved the titer and the specific productivity of CHO-IgG1 cells. These miRNAs also increased the productivity of CHO cells stably secreting recombinant HSA. We further demonstrated that the combined stable expression of two miRNAs selected from the CHO-IgG1 cell screening gave rise to IgG1 producing cell pools with increased productivity in fed batch cultures. To our knowledge this is the first report describing the improved performance of CHO cells by stable miRNA expression in a cell culture format that closely mimics the production process of a therapeutic protein.

2. Material and methods

2.1. miRNAs, siRNAs, DNA oligos, primers, and probes

The human microRNA library (CS-001010 mimic microRNA library, lot 09167, ThermoFisher scientific, Waltham, MA, USA), miRNAs (see Table S1) and the mimic miRNA negative control #1 (CN-001000-01) were obtained from Dharmacon. The siRNAs siLacZ-FAM (5′-FAM-GCGGCUGCCGGAAUUUACCTT-3′), siLC (specific siRNA targeting the light chain of the IgG1) and siHSA (5′-AUUCCAGAAUGCGCUAUUATT-3′) were purchased from MWG (Ebersberg, Germany). Taqman® microRNA Assays included reverse transcription primers (for hsa-miR-557: RT001525, for hsa-miR-1287: RT002828 and for RNU6B: RT001093), qPCR primers and Taqman probes (for hsa-miR-557: TM001525, for hsa-miR-1287: TM002828 and for RNU6B: TM001093) and were ordered from Life Technologies (Darmstadt, Germany). DNA oligos for cloning were from Biomers (Ulm, Germany) and are listed in Table S2.

2.2. Cell culture

CHO-DG44 cells (Urlaub et al., 1986) stably secreting human serum albumin (CHO-HSA) or a monoclonal IgG1 antibody (CHO-IgG1) and stable transfectants thereof were cultivated in suspension in a BI proprietary, serum-free media (Boehringer-Ingelheim, Biberach, Germany) supplemented with 400 nM methotrexate (MTX, Sigma-Aldrich, Taufkirchen, Germany) and 500 μg/mL G418 (Life Technologies). Seed stock cultures were sub-cultivated every 2–3 days with seeding densities of $2–3 \times 10^5$ cells/mL, respectively. Cells were grown in T-flasks (Greiner, Frickenhausen, Germany) in humidified incubators at 37 °C and 5% CO_2. The cell concentration and viability was determined by trypan blue (Sigma-Aldrich) exclusion using a Neubauer counting chamber. MDA-MB468 cells (CLS, Eppelheim, Germany) were grown at 37 °C and 5% CO_2 in humidified incubators in DMEM/F-12 medium (Life Technologies) supplemented with 10% fetal calf serum (FCS, PAN Biotech, Aidenbach, Germany). Cells were sub-cultured every 3 to 4 days.

2.3. Fed-batch cultivation

A 3×10^5 cells/mL cells were seeded into 125 mL shake flasks (Corning, Wiesbaden, Germany) in 30 mL of BI-proprietary production medium (Boehringer-Ingelheim) without antibiotics or MTX. The cultures were agitated at 120 rpm at 37 °C and 5% CO_2 in a minitron incubator (Infors, Einsbach, Germany). On day 3, CO_2 was reduced to 2%. BI-proprietary feed solution (Boehringer-Ingelheim) was added daily and the pH was adjusted to pH 7.0 using $NaCO_3$. At concentrations below 2 g/L, glucose (#G8769, Sigma-Aldrich) was adjusted to 4 g/L. Cell densities and viabilities were determined by trypan blue exclusion using an automated counting chamber TC10 (Biorad, Munich, Germany). Cumulative specific productivity was calculated by dividing the product concentration at the respective day by the "integral of viable cells" (IVC).

2.4. Transient miRNA screen

CHO-IgG1 cells were transfected with 1 μM RNA via nucleofection one day after passaging (4×10^5 cells/sample) in SG Cell Line 96-well Nucleofector™ Kit solution (#V4SC3096, Lonza, Verviers, Belgium) using the Amaxa 96-well Shuttle Device (Lonza) and program 96-DT-133 according to the manufacturer´s instructions. Subsequently, 12.5% of the cells were seeded in 100 μL medium into a well of a 96-well U-bottom plate (Greiner). In total, four plates were prepared. One day after transfection, the volume of the medium was doubled by addition of fresh medium without antibiotics or MTX. Supernatants were collected on days 1–4 post transfection by centrifugation of one of the 96-well plates ($290 \times g$, 5 min). Supernatants were transferred into a new 96-well plate and stored at −20 °C. A FAM-coupled non-targeting siRNA (siLacZ-FAM) and mock-transfected cells were used as negative controls. Transfection efficiency was determined by flow cytometry analysis (Cytomics FC-500, Beckman Coulter, Krefeld, Germany) of siLacZ-FAM transfected cells. The siLacZ-FAM, siLC and a mock control were included on each screening plate in duplicate. Two independent biological replicates of the screen were performed. For statistical analyses, the antibody concentration of each sample was first normalized to the median antibody concentration of the respective screening plate, referred to as the fold change. Then, the mean fold change of the two independent biological replicates was calculated. *p*-Values were determined by comparing antibody concentrations of the two biological replicates to the antibody concentrations of all siLacZ and mock controls using Student's *t*-test (two-tailed, unpaired).

2.5. Validation screen

CHO-IgG1 and CHO-HSA cells were transfected with miRNAs via nucleofection as described above and seeded into 12-well plates (Greiner). Cell densities and viability were determined by trypan blue exclusion using a CEDEX cell quantification system (Roche, Mannheim, Germany). Product concentrations in the supernatant were measured by ELISA. siLacZ-FAM, and siLC or siHSA served as controls. Statistical analysis of antibody concentrations and specific productivities obtained on day 1–4 was performed using a two-way ANOVA followed by a Bonferroni post-test. For the co-transfection of hsa-miR-557 and hsa-miR-1287, 0.5 μM of each miRNA was used, and the amount of total RNA in the single control transfections was adjusted to 1 μM by adding mimic miRNA negative control #1.