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OVEREXPRESSION OF MIR-7 HAS AN IMPACT ON HEK 293 GROWTH AND RECOMBINANT PROTEIN PRODUCTIVITY

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Abstract

By regulating gene expression at the posttranscriptional level, microRNAs (miRNAs) play an important role in control of different cellular signaling pathways including proliferation, death and cell productivity. The use of miRNAs for modification of mammalian cells – recombinant protein producers – is a novel approach for increasing their productivity. Herein data are presented on the impact of transient and stable mrRNA-7 overexpression on HEK 293 cell growth and recombinant protein productivity. The data demonstrate that miRNA-7 could be potentially used in the HEK 293 cell line to generate producer cells with an improved product yield.

Keywords: microRNAs, soluble Her2, soluble TNF-R1

Introduction

Active development of biopharmaceutics, accompanied by an ever growing number of new biological substances and biosimilars, necessitates creation of cell lines of high productivity. The use of miRNAs is one of the recently tested strategies in improving the producer cells productivity. miRNAs have been shown to be able to regulate multiple cellular processes including proliferation and cell productivity. Inasmuch as a single miRNA affects the expression of several genes concurrently, miRNA transfection of mammalian cells, producers of recombinant proteins, can prove to be more effective than transfection by a single gene. Lately a number of papers have been published which describe the change in cell productivity caused by the action of miRNAs. For example, Fischer et al. [1–3] discovered enhancing recombinant protein expression by miR-30 and miR-2861, targeting the uniquitin E3 ligase S-phase kinase-associated protein 2 and HDAC5, respectively. Strotbek et al. [4] demonstrated a positive effect of miR-557 and miR-1287 on the viable cell density and the specific protein productivity. Jadnev et al. [5] studied the effect of miR-17, miR-92a and cluster miR17-92a on the growth and protein productivity of producer cells. The authors showed that miR-92a overexpression reduced the growth and insignificantly increased the recombinant protein productivity. miR-17 overexpression leads to an enhanced growth and 2-fold increase in the specific protein productivity. The known target of miR-17 is TBC1D2/Armus plays an essential role in membrane trafficking [6], and therefore it is likely that miR-17 could enhance protein secretion. Some authors investigated the effect of miR-7 overexpression on cell growth and productivity [7–10]. Overexpression of miR-7 resulted in slowing down the growth and increasing recombinant protein production. The observed effects of miR-7 can be explained, at least, in part by up-regulation of proteins involved in protein folding and secretion [8]. It should be noted that the major part of such studies, including the above mentioned, were performed on Chinese hamster ovary cells (CHO) [11-13]. However, other cell cultures, specifically human embryonic kidney (HEK-293) cells are also used for generation of therapeutically significant recombinant proteins. But at present only single studies devoted to examination of the influence of miRNAs on the productivity of cell cultures other than CHO are available. For example, it has been demonstrated that miR-15 and let-7b enhance the productivity of HEK 293 by inhibiting the expression of CDK6 and cyclin E1 [14]. Therefore it remains unclear whether it is valid to propagate the data on the effect of either miRNAs on the productivity of recombinant

proteins, obtained on CHO cells, also on other cell cultures used in biopharmaceutic production. Taking into account the high evolutionary conservatism of many miRNA sequences, in particular miR-7 [15], we have verified the effect of transient and stable overexpression of miRNA-7, studied in detail on CHO cells, on the HEK 293 cell growth and recombinant protein productivity. We have shown that miRNA-7 overexpression enhances recombinant protein secretion in HEK 293 cells and evidently can be considered as a useful tool for increasing the productivity of various mammalian cell cultures.

Materials and Methods

Cell lines and transfection

HEK 293 cells were transfected with an expression vector encoding human soluble Her2 (sHer2) or soluble TNF-R1 (sTNF-R1). cDNA fragment sHer2 was amplified by PCR using the pHER2WT plasmid as a template. The pHER2 WT preparation was a gift from Mien-Chie Hung (Addgene plasmid #16257) [16]. The fragment, coding sTNF-R1, was amplified using RT-PCR with RNA isolated from peripheral blood leucocytes. Then the fragments were integrated in the Signal pIgplus (Ingenius) vector. Cell selection was carried out with 10 μg/ml Zeocin (Invitrogen) for 2 weeks followed by limited dilution cloning. After 6 passages in culture, the cells with the highest expression level of sHer2 or sTNF-R1 were identified as HEK 293-H2 and HEK 293-TR, respectively, and stored in DMEM/F-12 medium, 05% FBS.

HEK 293-H2 and HEK 293-TR cells were transfected with psiRNA-h7SKneo bearing the sequence hsa-miR-7-5p (MIMAT0000252) using a TurboFect Transfection reagent (ThermoFisher Scientific). Briefly, psiRNA-h7SKneo-mir7 (20 μg) were mixed with the TurboFect Transfection reagent and added directly onto cells (2×10^5 cells per ml) in the medium. The medium was harvested at 96 h post-transfection, and the concentrations of the secreted products were determined. Transiently transfected cells were rinsed with phosphate-buffered saline (PBS) and used to isolate RNA.

Stable miR-7 expressing cells were generated by incubation in selection media with 800 μg/ml G418 (InvivoGen) in 24 h post-transfection. Cells were maintained by adding fresh medium every 4 days for 3 weeks and then limited dilution cloning was performed. After other 3 weeks with selection pressure 8 stable pools of HEK 293-H2-mir7 cells as well as 8 stable pools of HEK 293-TR-mir7 were chosen for the further analysis.

Quantitative RT-PCR

The concentration of miRNAs in the cells was determined using a “TaqMan™ Advanced miRNA Assays” set (ThermoFisher Scientific). To this end, RNA was isolated using a “MagMAX™ mirVana™ Total RNA Isolation Kit” (ThermoFisher Scientific). Then cDNA was synthesized using a “TaqMan™ Advanced miRNA cDNA Synthesis Kit” (ThermoFisher Scientific). The obtained cDNA was amplified on a CFX 96 Real-Time System instrument, and the results were analyzed by the Bio-Rad CFX Manager 2.0 software. The data were normalized to the levels of endogenous U6 RNA.

Cell growth and protein productivity analysis

The number of cells and viability were estimated with a haemocytometer and the trypan-blue dye exclusion method, respectively. The concentrations of the secreted products were determined from the culture supernatants using sandwich ELISA assays. Briefly, 96-well microtiter plates (Nunc MaxiSorp, ThermoFisher Scientific) were coated with 0.33 μg/well anti-Her2 monoclonal antibody (ITEB, RAS, RF) to detect soluble Her2. Affinity purified soluble Her2 was used as a standard. The samples were applied in serially three-fold dilutions, and sHer2 captured from the cultural supernatant was incubated with 0.3 μg/well biotinilated Herceptin (Hoffman La Roche) with the following incubation together with streptavidin and peroxidase conjugate (Vector Laboratories, Inc., USA). Staining was conducted using o-phenylenediamine and H2O2. The absorption was measured at 490 nm with a microplate reader (Bio-Rad Lab., USA). The concentration of soluble TNF-R1 was determined using an Abcam’s Human sTNF-R1 ELISA kit (Abcam, USA) according to the manufacturer’s instructions.

The specific product secretion rate $\boldsymbol{q_p}$ (pg cell^{-1}) was calculated according to the equation 1.

$$q_p = (C_p \times D)/CN, \quad (1)$$

where C_p (μg×mL^{-1}) is the protein concentration, D is the dilution rate, and CN is the viable cell number.

Results

Effects of transient miR-7 overexpression on cell growth and recombinant protein production

It was shown earlier [8, 17 - 25] that miR-7 is highly conserved from annelids to humans, and its targets are well studied. Moreover, miR-7 overexpression in CHO cells caused enhancement of recombinant protein production [7]. Thereby we decided to test

miR-7 as a promising tool for increasing recombinant protein secretion in HEK 293 cells.

We transfected sHer2- and sTNF-R1-expressing HEK 293 cells transiently with the miR-7-encoding vector to investigate how its expression would affect the cell behavior. The empty vector was used as a control for non-specific effects of the transfection procedure. The transfection efficiency was confirmed by qRT-PCR. It turned out that HEK 293-H2-mir7 and HEK 293-TR-mir7 showed a slight decrease in the growth rate over a 96 h culture period when compared to the cells transfected by the empty vector (data not shown). However, miR-7 overexpression resulted in increasing productivity of both proteins (sHer-2 and sTNF-R1) on the average by 15–20% (Figure 1). These results encourage us to test the impact of this miRNA in the case of stable overexpression in HEK 293-H2 and HEK 293-TR cells.

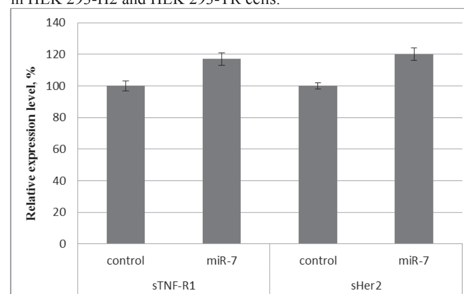

Figure 1. miR-7 overexpression results in increasing the productivity of both sHer2 and sTNF-R1 proteins. Relative levels of sTNF-R1 and sHer2 expression after transient transfection by empty vectors psiRNA-h7SKneo (control) or psiRNA-hSKneo-mir7 (miR-7) are shown. Protein productivity was determined from the culture supernatants using the sandwich ELISA assay. Expression levels in control experiments were accepted to be 100%. Data are expressed as the mean (% of the control) ± SD of three independent experiments.

Effects of stable miR-7 overexpression on cell growth and recombinant protein production

The main advantage of vector-based systems is that the same vectors can be directly used to generate both transient and stable miRNA overexpressing cells. Thus, the cells obtained at the stage of transient transfection were subjected to selection for several weeks in the medium containing an antibiotic to generate stable miR-7 overexpressing cells. The level of mir-7 expression was checked in eight pools of each of the obtained cell lines HEK 293-H2-mir7 and HEK 293-TR-mir7. To estimate the growth and recombinant protein production, three pools from each line with approximately the same enhanced level of mir-7 expression were chosen. Overexpression of mir-7 causes a decrease in the growth of both HEK 293-H2-mir7 and HEK 293-TR-mir7 cells. However, if in the case of CHO cells, upregulation of miR-7 lead to an 8-fold decrease in cell growth during 96 h, as shown earlier [7], the slowing down of the growth of HEK 293-H2-mir7 and HEK 293-TR-mir7 cells was no more than 30% as compared to HEK 293-H2 and HEK 293-TR cells. At the same time, the concentration of recombinant proteins in supernatants of mir-7 overexpression cells was higher than in HEK 293-H2 and HEK 293-TR cells (Figure 2). Therewith, if for HEK 293-TR cells this increase in specific productivity did not exceed 20%, HEK 293-H2 cells produced a twice larger amount of soluble Her2 than HEK 293-H2 cells. Therefore, upregulation of mir-7 in HEK 293 cells apparently enhances the recombinant protein production. Moreover, as concerns the requirements to the production cell lines, deceleration of the cell growth rate with high productivity can be considered as a beneficial property, providing a possibility for a more prolonged culturing of producer cells and thereby a higher yield of specific proteins in the course of one cycle of culturing in a bioreactor.

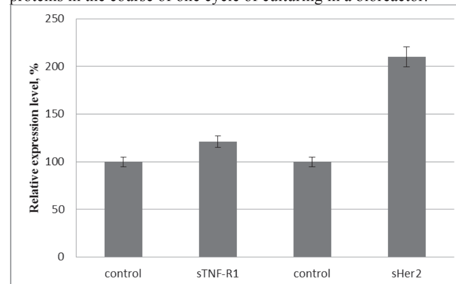

Figure 2. Relative levels of sTNF-R1 and sHer2 expression in stably transfected HEK 293-TR-mir7 and HEK 293-H2-mir7 cells are shown. Protein productivity was determined from the culture supernatants using the sandwich ELISA assay. Expression levels in control experiments (HEK 293-H2 and HEK 293-TR cells) were accepted to be 100%. Data are expressed as the mean (% of the control) ± SD of three independent experiments.

Conclusions

The results of our experiments have allowed us to make the following conclusions.

(1) miR-7 can be considered as a cell engineering tool for enhancing the recombinant protein production of HEK293 cells.

(2) As compared to the transient transfected cells, stably transfected cells are the most adequate model for analysis of the effects of microRNAs.

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