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Analysis of microRNA transcription and post-transcriptional processing by Dicer in the context of CHO cell proliferation

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ABSTRACT

CHO cells are the mammalian cell line of choice for recombinant production of therapeutic proteins. However, their low rate of proliferation limits obtainable space-time yields due to inefficient biomass accumulation. We set out to correlate microRNA transcription to cell-specific growth-rate by microarray analysis of 5 CHO suspension cell lines with low to high specific growth rates. Global microRNA expression analysis and Pearson correlation studies showed that mature microRNA transcript levels are predominately up-regulated in a state of fast proliferation (46 positively correlated, 17 negatively correlated). To further validate this observation, the expression of three genes that are central to microRNA biogenesis (Dicer, Drosha and Dgcr8) was analyzed. The expression of Dicer, which mediates the final step in microRNA maturation, was found to be strongly correlated to growth rate. Accordingly, knockdown of Dicer impaired cell growth by reducing growth-correlating microRNA transcripts. Moderate ectopic overexpression of Dicer positively affected cell growth, while strong overexpression impaired growth, presumably due to the concomitant increase of microRNAs that inhibit cell growth. Our data therefore suggest that Dicer dependent microRNAs regulate CHO cell proliferation and that Dicer could serve as a potential surrogate marker for cellular proliferation.

1. Introduction

Recombinant expression of therapeutic proteins in Chinese hamster ovary (CHO) cells has a long history (Hacker et al., 2009; Jostock and Knopf, 2012), due to the ease of cultivation of CHO cells in suspension and protein-free media, the availability of tools for clone selection and gene amplification and due to various safety aspects (reviewed by Wurm, 2004). Collaborative effort has recently been put into their characterization in terms of genome (Brinkrolf et al., 2013; Lewis et al., 2013; Xu et al., 2011), cDNA (Becker et al., 2011; Rupp et al., 2014) and non-coding RNA sequencing projects (Hackl et al., 2012b, 2011; Johnson et al., 2011) as well as characterization of the CHO proteome (Baycin-Hizal et al., 2012; Meleady et al., 2012a) and metabolome (Martínez et al., 2013). These data are essential for understanding and eventually also predicting and adapting CHO cell phenotypes to the requirements of modern bioprocesses.

One approach to increase yields from mammalian bioprocesses is to increase the viable cell number by reducing the rate of apoptosis. Therefore, multiple cell engineering strategies were developed to increase apoptosis resistance of CHO cells by overexpression of endogenous (Han et al., 2011) or evolved anti-apoptotic proteins of the Bcl-family (Majors et al., 2012). Sophisticated transcriptomic, proteomic and metabolomic approaches identified bottlenecks in the energy metabolism of CHO cells that prevent efficient growth and/or protein production (Chong et al., 2010; Doolan et al., 2010). These limitations might be overcome by engineering the expression of single genes, however, the alteration of entire gene networks seems most promising, but at the same time most difficult. In order to meet the challenge of manipulating entire gene networks without burdening the translational machinery of a cell factory, non-coding RNAs, and especially microRNAs (miRNAs) constitute a promising alternative (Hackl et al., 2012a; Jadhav et al., 2013). To this date, miRNAs in CHO cells were

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identified to regulate growth (Jadhav et al., 2012), stress resistance (Druz et al., 2011) or specific productivity (Barron et al., 2011) by repressing the expression of hundreds of target genes (Meleady et al., 2012b). In fact, across all cell biological disciplines these small (18–24 nt) RNAs have been widely recognized as central regulators of cellular phenotype (Kosik, 2010), with potential applications beyond cell engineering as therapeutic targets (Rooij et al., 2012) or diagnostic markers of disease (Velu et al., 2012). miRNAs are transcribed mostly from RNA Polymerase II promoters in the genome, or excised from intronic regions of mRNA primary transcripts (Carthew and Sontheimer, 2009). These primary miRNA transcripts (pri-miRNAs) consist of a stem-loop structure flanked by single-stranded RNA regions and are subject to two sequential maturation steps: in the nucleus the "microprocessor complex" formed by Drosha and Dgcr8 binds pri-miRNAs and cleaves off a ~50–80 nt long precursor-miRNA (pre-miRNAs) structure containing the RNA stem-loop (Gregory et al., 2004). Export into the cytoplasm occurs via Exportin-5 and results in the association of pre-miRNAs with Dicer, a ~230 kDa protein of the helicase family consisting of two RNase-III domains as well as RNA binding, helicase and protein interaction domains (Soifer et al., 2008; Takeshita et al., 2007). Dicer cleavage sets free a ~22 nt miRNA duplex, from which the guide miRNA is selected and incorporated into a large protein complex called RISC (RNA-induced silencing complex). miRNAs select their targets by imperfect base-pairing to recognition sites present in 3′UTRs or coding regions of messenger RNA (mRNA). The relative position of miRNA:mRNA interaction and the type of Argonaute protein incorporated in the miRNA-RISC decides whether translational repression or mRNA destabilization and degradation will occur (Carthew and Sontheimer, 2009). The imperfect nature of miRNA:target interaction allows single miRNAs to repress the expression of hundreds of different mRNAs, depending on target mRNA availability as well as interaction site accessibility (Arvey et al., 2010), thus attributing miRNAs an important role in the global regulation of gene expression similar to transcription factors (Hobert, 2008).

In addition to the exploration of miRNA function by overexpression, knockdown and target validation studies, studies of miRNA biosynthesis and the regulation of this multistep process have been conducted (Davis-Dusenbery and Hata, 2010; Krol et al., 2010). It is known that the maturation of specific pri-miRNAs by Drosha is dependent on the binding of proteins, for example p53 which induces the biosynthesis of selected growth-suppressive miRNAs (Suzuki et al., 2009). Unlike Drosha activity, which generally requires binding of auxiliary proteins, Dicer is constitutively active which is mirrored in low detectable levels of pre-miRNAs compared to pri-miRNAs or mature miRNAs (Lee et al., 2008). Rather, regulation of miRNA biosynthesis at the Dicer step depends on the inhibition of Dicer activity, or on the de-regulation of Dicer expression, which have been observed during organism development (Rybak et al., 2008), disease progression (Coley et al., 2010; Han et al., 2010) or even in vitro cultivation (Asada et al., 2008; Hwang et al., 2009). As a consequence, mature miRNA levels are subject to change on a global scale under these conditions, thus broadly affecting gene expression.

To our best knowledge, no study has addressed the biological effect of deregulated miRNA biogenesis in CHO cells. Based on miRNA microarray data from five CHO suspension cell lines with slow to high proliferation rates, we observed a global increase in miRNA transcripts along an increase in growth rate. In order to test whether this shift in miRNA transcript levels is assisted or caused by enhanced miRNA transcription or maturation, expression analyses of Dicer, Drosha and Dgcr8 were performed, as well as functional analysis of Dicer by performing loss- and gain-of-function experiments.

2. Material and methods

2.1. Cell culture

2.1.1. Cell maintenance

Suspension and serum-free adapted CHO-DUKXB-11 cells were grown in DMEM:Ham' F12 (1:1) supplemented with 4 mM L-glutamine and protein-free additives without growth-factors (CHO-DUKXB-11). All other cell lines were cultivated in CD CHO media (Life Technologies) supplemented with 8 mM L-glutamine (CHO-K1-8 mM and CHO-S) or without (CHO-K1-0 mM) and 1:500 anti-clumping agent (Life Technologies). Recombinant CHO-DUKXB-11 cells expressing an erythropoietin-Fc fusion protein were grown in suspension in CD CHO media with 0.019 μM methotrexate and without L-glutamine supplementation (Taschwer et al., 2011). No defined growth factors such as Insulin or IGF were used as additives in this study.

All cell lines were cultivated in suspension in Erlenmeyer shake flasks in 50 ml volume at 140 rpm in a shaking incubator (Kuhner, Switzerland) in a humidified atmosphere (90%) conditioned with 7% CO_2.

2.1.2. Generation of stable Dicer overexpressing pools

CHO-DUKXB-11 host cells (10^7 cells in total) were transfected by nucleofection (LONZA) with 10 μg of recombinant human Dicer plasmid (Genecopoeia, GC-H0470) containing the open reading frame of human Dicer (NM_030621.2 and NP_085124.2) under a CMV promoter and neomycin resistance gene. Post-transfection, cells were seeded at a concentration of 3.0×10^5 cells/ml in 30 ml media and maintained at 37 °C with humidified air, 7% CO_2, and constant shaking at 140 rpm for 24 h. At this point, selection media containing 800 μg/ml G418 (Invivogen, San Diego, USA) was added, and cells were transferred to a 96 well plate at a concentration of 10,000 cells/well. Throughout selection, media was replaced every 3–4 days, and wells with growing cells were expanded to 12-well plates after 4 weeks of selection. At this stage individual wells containing stable growing CHO pools were tested for human Dicer1 incorporation and expression by PCR amplification from genomic DNA (gDNA) and copied DNA (cDNA) using specific primers (Supporting Table S1) and Western blot as described below (2.5).

2.1.3. siRNA mediated knockdown of Dicer

For targeted knockdown of Dicer expression in CHO cells, two 21 nt long siRNAs were designed based on the NCBI reference sequence NM_001244269.1: siRNA#1 target site: GAGTGGTAGCTCTCATTTGCT; siRNA#2 target site: TAACCTGGAGCGGCTTGAGAT. All siRNAs were custom synthesized at 25 nm scale (Qiagen, Germany). For transfection, both siRNAs were pooled at equimolar concentration. As control, a non-targeting RNA duplex was designed (GUGUAACACGUCUAUACGCCCA) and custom synthesized (Biomers, Germany). Small RNAs were transfected at 30 nM concentration in three replicates in 6-well plate format. ScreenfectA (Incella, Germany) was used for lipid/RNA complex formation according to the provided protocol. Cells were seeded at 3.5×10^5 cells/ml in 2.5 ml, before complexed siRNAs were added to each well. Cultivation was performed at 37 °C in humidified air with 7% CO_2 and constant shaking at 60 rpm. After 72 h cells were harvested for RNA isolation and cell density/viability measurements.

2.2. RNA Isolation

Isolation of total RNA was performed using phenol–chloroform extraction from Trizol lysed CHO cell pellets. In brief, CHO suspension cells were lysed in 1 ml TRI reagent (Sigma–Aldrich) and stored at −80 °C or processed immediately. Adherent CHO cell lines were detached from the surface by trypsinization, PBS-washed and lysed

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