Universität für Bodenkultur Wien

Biomolecular Technology of Proteins

Department für Biotechnologie

Departmentleiter:

Ao.Univ.Prof. Dipl.-Ing. Dr.nat.techn. Florian Rüker

Betreuer:

Ao.Univ.Prof. Dipl.-Ing. Dr.nat.techn. Nicole Borth

Assoc. Prof. Dr. Johannes Grillari

Providing a sound basis for microRNA engineering in CHO cells

Dissertation

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Dipl.-Ing. Gerald Klanert,

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Kurzfassung

Chinesische Hamster Ovarien (CHO) Zellen sind die Zellfabrik der Wahl für die Produktion von komplexen, menschenähnlichen therapeutischen Proteinen. Viel Aufwand wurde aufgebracht, um diese Zellen zu optimieren, insbesondere durch gentechnische Veränderungen. Eine neue Methode ist die Verwendung von microRNAs (miRNAs), um Zellen in Richtung gewünschter Phänotypen zu lenken. Seitdem die erste genomische Sequenz von CHO Zellen im Jahr 2011 veröffentlicht wurde, wurden Bemühungen unternommen, um miRNAs im CHO-Genom zu identifizieren und zu annotieren. Wie es bei anderen Spezies gezeigt worden ist, sind miRNAs oft in chromosomaler Nähe zueinander (miRNA Cluster) angeordnet, und werden gemeinsam transkribiert und reguliert.

Der Schwerpunkt dieser Arbeit war es, die neu gewonnenen Sequenzinformationen zu verwenden, um endogene miRNA Cluster zu vervielfältigen und zu überexprimieren, und ihre Wirksamkeit zu chimären zu vergleichen, welche in CHO-Zellen in der prä-genomischen Sequenz Ära Stand der Technik waren. Außerdem, um miRNAs, welche mit dem Zellwachstum verbunden sind, zu identifizieren, wurden die miRNA Expressionsprofile von mehreren CHO-Zelllinien in verschiedenen Kultiviergefäßen und Wachstumsphasen analysiert und es zeigte sich, dass mehrere miRNAs eine konstante Korrelation zur Wachstumsrate aufweisen.

Abstract

Chinese Hamster Ovary (CHO) cells are the cell factory of choice when producing complex human-like therapeutic proteins. Lots of effort has been taken to optimize these cells, especially by using genetic engineering. A novel method is the use of microRNAs (miRNAs) to drive cells towards desired phenotypes. When the first genomic sequence of CHO cells became publicly available in 2011, effort has been taken to identify and annotate miRNAs in the CHO genome. As it has been shown in other species, miRNAs are often located in close chromosomal proximity to each other (miRNA cluster), and are transcribed and regulated jointly.

The focus of this thesis was to use the newly gained sequence information to amplify and overexpress endogenous miRNA cluster, and compare their effectivity to chimeric ones, which were state-of the art in CHO cells in the pre-genomic sequence era. In addition, to identify miRNAs connected to cell growth, miRNA expression profiles of several CHO cell lines in different cultivation vessels and growth phases were analyzed and indicated several miRNAs constantly correlating to the growth rate.

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1. Rahmenschrift zur kumulativen Dissertation

1.1. CHO cells and their biotechnological value

Since the isolation of CHO (Chinese Hamster Ovary) cells from the Chinese hamster around 60 years ago[1], they have been used extensively in radiation and cytogenetic studies. The first approval of a recombinant protein (tissue plasminogen activator) produced in CHO cells for clinical studies by the FDA indicated their value for industrial applications[2]. Up to date, around one third of all approved biopharmaceutical products are produced in these cells[3]. The extensive use of CHO cells for therapeutic protein production is the result of several advantages over other mammalian cell lines. Correct folding and human-like posttranslational modifications are major advantages of proteins produced in CHO. Furthermore, these cells adapt easily to altered culture conditions and are of simple maintenance, which makes them even more attractive for industrial use. CHO cells are also resistant to many human-pathogenic viruses, simply because many genes important for viral entry and recognition are absent in the genome or lacking expression[4]. These characteristics lead to their extensive application in recombinant protein production processes.

In spite of the above mentioned qualities, their product yield is low, especially when compared to microbial cell factories, which contributes to the high expenses for biopharmaceuticals. Media optimization, process design and cell line engineering strategies were implemented to increase the volumetric productivity[5]. Still, further improvements are necessary to continue to meet the requirements of the highly competitive market[3]. Especially cell line engineering by genetic manipulation has become more attractive in the last years, since the first genomic sequence of CHO became publicly available in 2011[4,6]. These strategies focus mainly on improving cellular phenotypes to gain higher specific productivity, faster growth and improved culture

longevity[5]. Directed manipulation of CHO cells can either be accomplished by genome editing[7], or overexpression of coding[5] or non-coding genes, especially miRNAs (microRNAs)[8,9].

1.2. miRNAs in CHO cells

miRNAs are evolutionarily conserved small, non-coding RNAs with a length of ~20 nucleotides. The sequence information is encoded in either intergenic or intronic regions in the genome, and is often clustered in close chromosomal proximity[10]. Many clustered miRNAs are found to be cooperatively regulated and are transcribed polycistronically[11]. The primary transcript (pri-miRNA), which bears one or more miRNAs, is processed to form precursor miRNAs (pre-miRNA or mir), consisting of a single miRNA hairpin[12]. After being exported and further processed to a miRNA duplex, formed by the five prime (5p) and the three prime (3p) mature miRNA (miR)[13,14], one of the two strands preferentially serves as a template[15] to guide the RNA-induced silencing complex to mRNAs which contain (mostly imperfect) complementary regions to the miRNA, leading to either translational repression[16] or destabilization[17], thus forming an additional layer of post-transcriptional gene regulation. miRNAs share many similarities to transcription factors, as single miRNAs are able to target many different mRNAs, and mRNA often contain target sites for more than one miRNA[18].

Though miRNAs were discovered in 1993[19], it took until 2007 for the first study observing miRNAs in CHO cells to be published[20]. Up-to-date, the sequences of 256 expressed endogenous pre-miRNAs encoding 378 mature miRNAs were identified and localized in the genome of CHO cells [21,22,23,24,25], providing a solid basis for miRNA research in CHO cells.

1.3. miRNAs indicate and drive cellular phenotypes

Ever since miRNA research in CHO cells started, the abundance of distinct miRNAs or patterns were associated with observed phenotypes. miRNA profiles specific for certain cellular phenotypes and culture conditions were revealed by next-generation sequencing[22,23,26] and microarrays[20,27,28,29,30,31,32,33], and exploited to identify miRNAs with the ability to drive cellular phenotypes upon manipulation of their expression (also termed engimiRs)[26,27,29,34]. In addition, high-throughput screening approaches identified further engimiRs[35,36], expanding the knowledge for this prospering cell line engineering strategy.

1.4. Endogenous sequences improve effectivity in miRNA engineering

miRNAs are often located in close chromosomal proximity at the same strand, which indicates polycistronical transcription[10]. As clustered miRNAs often execute related biological functions[37], it is of interest to study the impact of clustered miRNAs on CHO cells by manipulating the expression of a whole miRNA cluster. For this, we chose the well-characterized oncogenic miR-221/222 cluster[38,39,40] and the pro-apoptotic miR-15b/16-2 cluster[41,42,43], which were all found to be expressed in CHO[23].

Two methods were applied for cloning of the respective miRNAs into the pcDNA6.2-GW/emGFP-miR vector. Chimeric miRNAs were constructed, consisting only of the endogenous mature miRNA sequences linked by modified loop and flanking regions of mmu-miR-155[34,44], and combined into clusters by concatenation. This method was the only possible one before the genomic sequence became public. After the CHO genome became available, it was possible to map previously identified miRNAs to their respective genomic regions[25]. This information was used to amplify and insert the genomic regions, including the endogenous miRNA cluster, into the same vector system.

Both miRNA clusters generated by the two methods were transiently transfected into CHO cells, and the mature miRNA levels (miR-15b-5p, miR-16-5p, miR-221-3p and miR-222-3p) were quantified 48h post transfection using the Taqman qPCR assay. While none of the mature miRNA levels was induced upon transfection with the chimeric cluster, both endogenous clusters were able to elevate the mature miRNA levels 2-3 fold, indicating better transcript processing. EmGFP expression analysis indicated adequate transfection efficiencies and pri-mir-221 levels of the endogenous construct were in concordance with the miR-221-3p levels. But the pri-miR-221 level of the chimeric construct was highly elevated, indicating a bottleneck in primary miRNA processing. RNA secondary structure prediction reveals differences in the hairpin-structures, which might be responsible for the inefficient processing of the chimeric miRNAs[45]. This demonstrates the importance of using endogenous sequences for miRNA cluster expression, relying on genomic sequence availability.

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Technical Report

Endogenous microRNA clusters outperform chimeric sequence clusters in Chinese hamster ovary cells

Gerald Klanert[1,2], Vaibhav Jadhav[1], Konstantina Chanoumidou[1], Johannes Grillari[1], Nicole Borth[1,2] and Matthias Hackl[1]

[1] Department of Biotechnology, Boku University Vienna, Austria
[2] ACIB GmbH, Austrian Centre of Industrial Biotechnology, Graz, Austria

MicroRNAs (miRNAs) are small non-coding RNAs (~22 nucleotides) which regulate gene expression by silencing mRNA translation. MiRNAs are transcribed as long primary transcripts, which are enzymatically processed by Drosha/Dgcr8, in the nucleus, and by Dicer in the cytoplasm, into mature miRNAs. The importance of miRNAs for coordinated gene expression is commonly accepted. Consequentially, there is a growing interest in the application of miRNAs to improve phenotypes of mammalian cell factories such as Chinese hamster ovary (CHO) cells. Few studies have reported the targeted over-expression of miRNAs in CHO cells using vector-based systems. These approaches were hampered by limited sequence availability, and required the design of "chimeric" miRNA genes, consisting of the mature CHO miRNA sequence encompassed by murine flanking and loop sequences. Here we show that the substitution of chimeric sequences with CHO-specific sequences for expression of miRNA clusters yields significantly higher expression levels of the mature miRNA in the case of miR–221/222 and miR–15b/16. Our data suggest that the Drosha/Dgcr8-mediated excision from primary transcripts is reduced for chimeric miRNA sequences compared to the endogenous sequence. Overall, this study provides important guidelines for the targeted over-expression of clustered miRNAs in CHO cells.

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1 Introduction

The advantage of Chinese hamster ovary (CHO) cells over microbial production systems is that they can produce proteins with human-like post translational modifications [1]. Yet the space/time yield of recombinant proteins produced in CHO cells is at least ten–fold lower when compared to microbial hosts [2]. Different bioprocess [3–5] and medium optimizations [2, 6, 7] were developed and implemented to overcome this drawback. Another approach has been to directly improve the host cell by genetically engineering cellular functions such as apoptosis [8–11], productivity [12–14], and metabolism [15–17]. Given the wealth of published data in this field, the references given above are illustrative of the strategies employed, but not an exhaustive survey of the literature. In this context, microRNAs (miRNAs) are increasingly considered as promising tools for CHO cell line development as they were shown to be essential regulators of cellular functions that support cell cycle progression and protein expression (for example [18–20]) .

The biogenesis of this class of small non-coding RNAs, with a length of approximately 22 nucleotides, is a complex multi-step process that relies on coordinated

Correspondence: Dr. Matthias Hackl, Department of Biotechnology, Boku University Vienna, Muthgasse 18, 1190 Vienna, Austria
E-mail: matthias.hackl@boku.ac.at

Abbreviations: 3'UTR, 3' untranslated region; **CHO**, Chinese hamster ovary; **CMV**, cytomegalovirus; **emGFP**, emerald green fluorescent protein; **miRNA/miR**, microRNA; **pri-miRNA/pri-miR**, primary microRNA; **pre-miRNA**, precursor microRNA; **RT-–qPCR**, quantitative real time – polymerase chain reaction; **RISC**, RNA inducing silencing complex; **shRNA**, short hairpin RNA

action of several enzymes and RNA binding proteins. First, primary miRNA transcripts (pri-miRNAs), which are called miRNA clusters when they give rise to more than one mature miRNA, are long single-stranded RNA molecules that are usually generated by RNA polymerase II or occasionally by RNA polymerase III. Pri-miRNAs from intergenic regions are processed by the Drosha/DGCR8 protein complex, which cleaves the RNA to form 50–70 nt long RNAs exhibiting a characteristic RNA-secondary structure consisting of a dsRNA region connected by a short loop sequence. These intermediate forms of miRNAs are termed precursor-miRNAs (pre-miRNAs), but are often referred to as "hairpins" or "stem-loops". The hairpins are exported into the cytoplasm where the RNase-III enzyme Dicer catalyses the production of two largely complementary mature miRNAs that form a duplex. One or sometimes even both strands are selectively incorporated into the RISC complex and used as guides to scan for mRNAs with complementary sequences. Once a target is bound to the protein-miRNA complex, it is either degraded or translationally repressed [20–24]. Despite the small size and principal ease of over-expression of miRNAs, their biogenesis mechanism is complex, requiring well characterized tools to achieve stable over-expression [25, 26] or knockdown in mammalian cells [27, 28].

With respect to CHO cells, the identification and annotation of the miRNA transcriptome [29, 30] allowed the use of mature endogenous miRNA sequences (CHO-sequences in contrast to orthologous sequences from human, mouse, or rat) to study their biological effect. These gain-of-function studies employed either transfection of synthetic mature miRNA mimics [31], or plasmid encoded pre-miRNAs, that were pieced together from mature CHO miRNAs and ectopic flanking and loop sequences from mouse ("artificial chimeric miRNA construct") [32]. These gain-of-function studies needed no information on the genomic location or hairpin structure of miRNAs and could be rapidly performed using DNA synthesis. As this technology had been developed for construction of short hairpin (shRNA) for gene knockdown in a variety of cellular systems, its use for miRNA engineering in CHO was an obvious choice [26]. Soon after the publication of the CHO genome in 2011 [33], pre-miRNA sequences and the respective genomic loci were published [34], making it possible to amplify and clone endogenous pri–miRNAs and to use them for cell line engineering ("endogenous miRNA construct").

In the following study we compare both constructs for the expression of two different miRNA clusters, miR-15b-16 and miR-221-222. Our data clearly indicate that endogenous miRNA constructs are better suited for expression of miRNA clusters than artificial constructs.

2 Material and methods

2.1 Cell culture

A previously described recombinant serum- and L-glutamine-free suspension production cell line CHO DUKXB11 EpoFc 14F2 [35, 36] was cultivated in CD CHO medium (Gibco®, Carlsbad, CA, USA) supplemented with 0.19 µM Methotrexate and 0.2% Anti-Clumping Agent (Gibco) in a shaker-incubator at 37°C, 7% CO_2 and 140 rpm.

2.2 Genomic DNA isolation

gDNA was isolated using the DNeasy® Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. In brief, 5×10^6 cells were harvested and resuspended in DPBS no calcium, no magnesium (PAA, Austria) including proteinase K. Buffer AL was added and the samples were incubated at 56°C for 10 minutes. Ethanol was added and the suspension was filtered through the DNeasy mini spin column by centrifugation. After washing with Buffer AW1 and AW2, the membrane was dried and the DNA was resuspended by the addition of 200 µL Buffer AE followed by a centrifugation step. The quality and quantity of the gDNA were determined by UV-VIS spectrophotometry (Nanodrop ND–1000 spectrophotometer, Thermo Scientific Inc., Waltham, MA, USA).

2.3 Cloning of miRNA cluster expression plasmids

The chimeric miR-15b/16-2 and miR-221/222 clusters were created by concatenation of miRNA expression plasmids with artificial miRNA constructs (Fig. 1) as previously described [26, 32]. In short, the chimeric miRNAs, consisting of the mature CHO miRNA sequences with restriction sites on either end, and an optimized murine loop sequence (Supporting information, Table 1), were cloned into the 3' untranslated region (3'UTR) of emerald green fluorescent protein (emGFP) located in the pcDNA6.2–GW/EmGFP–miR vector (BLOCK–iT™ Pol II miR RNAi Expression Vector Kit, Invitrogen Inc., Carlsbad, CA, USA), already containing artificial flanking regions. One of the two corresponding chimeric cluster miRNAs was cut out, including the artificial flanking regions, and inserted into the plasmid with the other chimeric cluster miRNA for artificial cluster generation (Fig. 1) according to the manufacturer's instructions.

For endogenous miRNA-cluster construct generation, the relevant gDNA regions were amplified by polymerase chain reaction (PCR) using primers located in the flanking regions at least 20 bp from the outermost miRNAs of each cluster (Fig. 1A, Supporting information, Table S1). The resulting PCR products were cloned into the same region of the pcDNA6–GW/EmGFP–miR vector (Fig. 1B), and the accuracy of the insertion and the sequence were confirmed by conventional sequencing.

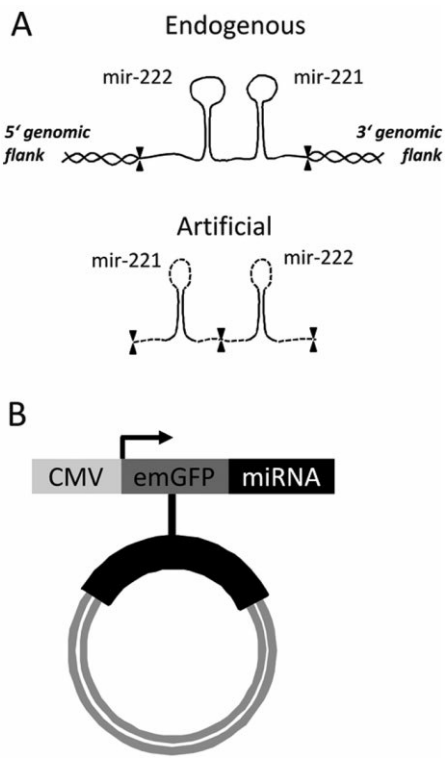


Figure 1. Schematic representation of endogenous and artificial constructs for over-expression of microRNA clusters. **(A)** Endogenous mir-221/222 was PCR amplified from CHO-K1 genome, using primers 70 nt up and downstream of the genomic location. Primers contained restriction sites, which were used for cloning the sequence into a pcDNA 6.2 expression vector containing emGFP. Artificial constructs of ~60 nucleotides are composed of CHO-specific mature miRNA sequences (solid lines) as well as the flanking and loop sequences of mir-155 (dotted lines). Artificial mir-221 and mir-222 were synthesized individually and cloned into the pcDNA 6.2 vector using restriction sites as indicated by black arrows. **(B)** A schematic of the pcDNA 6.2 expression vector used in this study, with CMV-controlled emGFP expression and microRNA cloning site contained in the 3'UTR of emGFP.

2.4 Transfection

Nucleofection was performed using the Amaxa Nucleofector I/program H–14 and the Amaxa cell line nucleofector kit V (Lonza Group Ltd., Switzerland). 10^7 cells in exponential growth phase were harvested and resuspended in 82 µL of Cell Nucleofection Solution V supplemented with 18 µL supplement I and 10 µg of the respective endotoxin-free plasmid. The same plasmid without insert was used as negative control. The solution mixtures were transferred into a cuvette and nucleofected. After transfection, 2 mL of pre-warmed media was added to the cuvette and the whole solution was transferred into a 125-mL shaking flask (Corning®, Life Sciences, Tewksbury, MA, USA) containing 58 mL of pre-warmed media. Immediately after the transfer, the cells were divided into 2 × 30 mL aliquots generating two technical replicates. Cells were incubated for 2 hours at 37°C, 7% CO_2 and humidified air without shaking for recovery. Subsequently, culture flasks were transferred into the shaking incubator at 37°C, humidified air containing 7% CO_2 and constant shaking at 140 rpm.

2.5 RNA isolation

Total RNA samples were collected, using TRI® reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol, 48 and 96 hours after transfection. In brief, up to 5×10^6 viable cells were harvested, resuspended and homogenized in 0.5 mL of TRI® reagent. 0.1 mL of chloroform was added and the mixtures were centrifuged at 4°C for phase separation. The upper, aqueous phases were mixed with isopropanol and centrifuged for RNA precipitation and pelleting. The pellets were washed with 75% ethanol and then air-dried. After re-suspension in 25 µL of nuclease free water, the quantity and quality were determined by the NanoDrop ND–1000 Spectrophotometer (Thermo Scientific). Only RNA samples with a 260/280 and a 260/230 ratio of 2.0–2.1 and 1.8–2.2, respectively, were used.

2.6 Flow cytometry

Cells were analyzed 48 hours after transfection using the Gallios Cytometer (Beckman Coulter Inc., Brea, CA, USA). A forward/side scatter plot was used to discriminate the living from the dead cells. At least 1×10^4 cells were excited by a 488 nm argon laser and the emitted signals were collected by a 525/40 BP filter.

2.7 Quantitation of mature miRNA levels

Mature miRNA levels were determined by quantitative real-time PCR (RT-qPCR) using the TaqMan® MicroRNA Assays (Applied Biosystems, Carlsbad, CA, USA). In general, cDNA was generated out of 10 ng total RNA in 10 µL reaction volumes via the TaqMan® MicroRNA reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. The kit includes the Multiscribe™ Reverse Transcriptase and a specific reverse-transcription primer against each miRNA. The 10 µL RT-qPCR mix consisted of the generated cDNA, the TaqMan® Universal PCR Master Mix (Applied Biosystems) and the respective 20× TaqMan MicroRNA Assay (Applied Biosystems, TM000390, TM000391, TM000524, TM000525, TM002271). Quadruplets of each cDNA sample were used for the PCR, performed on the Rotor-Gene-Q (QIAGEN). The expression levels of each mature miRNA relative to the cgr-miR-185-5p [32], an endogenous control, were determined using the $2^{-\Delta\Delta CT}$ method [37]. Average fold differences in the transcript levels were determined by comparison against the negative control transfection.

2.8 Quantitation of primary miRNA transcripts and GFP

800 ng of DNase I (Fermentas, Waltham, MA, USA) treated total RNA of each sample were denatured for 2 minutes at 72°C and then put on ice. cDNA was generated by the DyNAmo cDNA Synthesis Kit (Thermo Scientific, Pittsburgh PA), consisting of the M-MuLV RNase H^+ reverse transcriptase and random hexamer primers. The resulting cDNAs were diluted 1:3 and each sample was analyzed in quadruplicate RT-qPCR reactions in 10 µL with SensiMix SYBR Hi–ROX Polymerase (Bioline, UK) according to the manufacturer's protocol. Primers for the chimeric pri-miR-221 were designed to overlap the mature miRNA and the artificial flanking region of the vector. Primers for the endogenous pri-miR were designed in an analogous fashion, overlapping the stem-loop and the respective flanking regions. The RT-qPCR was performed on the Rotor-Gene Q (QIAGEN) and the transcript levels of the pri-miRNAs and of GFP relative to GAPDH were determined using the $2^{-\Delta\Delta CT}$ method. Average fold differences in the transcript levels are calculated via comparison to the negative control transfection.

3 Results and discussion

3.1 Over-expression of chimeric and endogenous miRNA clusters after transient transfection

In the absence of a genomic CHO reference sequence we initially generated artificial chimeric miRNA constructs to express miRNAs in CHO cells (Fig. 1). These constructs consist of CHO-specific mature miRNAs and mmu-miR-155 loop and flanking regions that have been reported to yield high miRNA expression [26]. In order to assess the function of miRNA clusters, which are polycistronic primary miRNA transcripts that give rise to two or more mature miRNAs, we constructed two artificial miRNA cluster expression constructs (miR-15b and miR-16; miR-221 and miR-222) by sequence concatenation, as outlined in material and methods. An empty vector was used as negative control that consisted of the same expression cassette with cytomegalovirus (CMV) promoter, emGFP, but no miRNA insert in the emGFP 3' untranslated region (3'UTR). Each construct was transfected into a recombinant CHO cell line producing an Epo-Fc fusion protein (erythropoietin fused to the FC domain of immunoglobulin A) in three independent replicates. From each transfection cells were split into two batch cultures. Transfection efficiency was estimated from the portion of emGFP expressing cells 48 h after transfection (Supporting information, Fig. S1), which was previously determined to be the time point when cells reach maximum transient gene expression [32]. At this time point, 92 ± 7% of cells were GFP-positive.

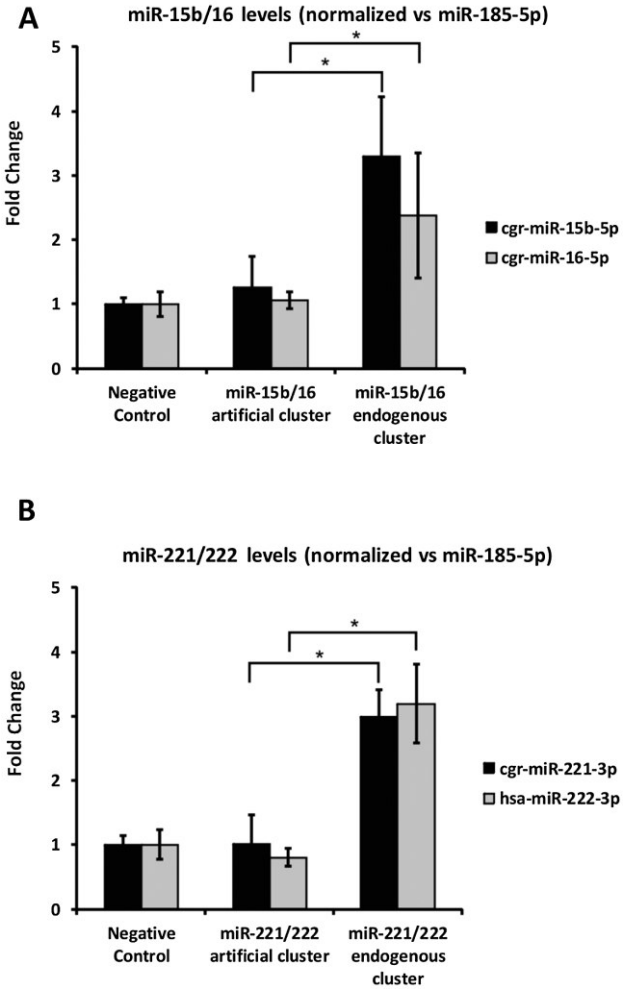
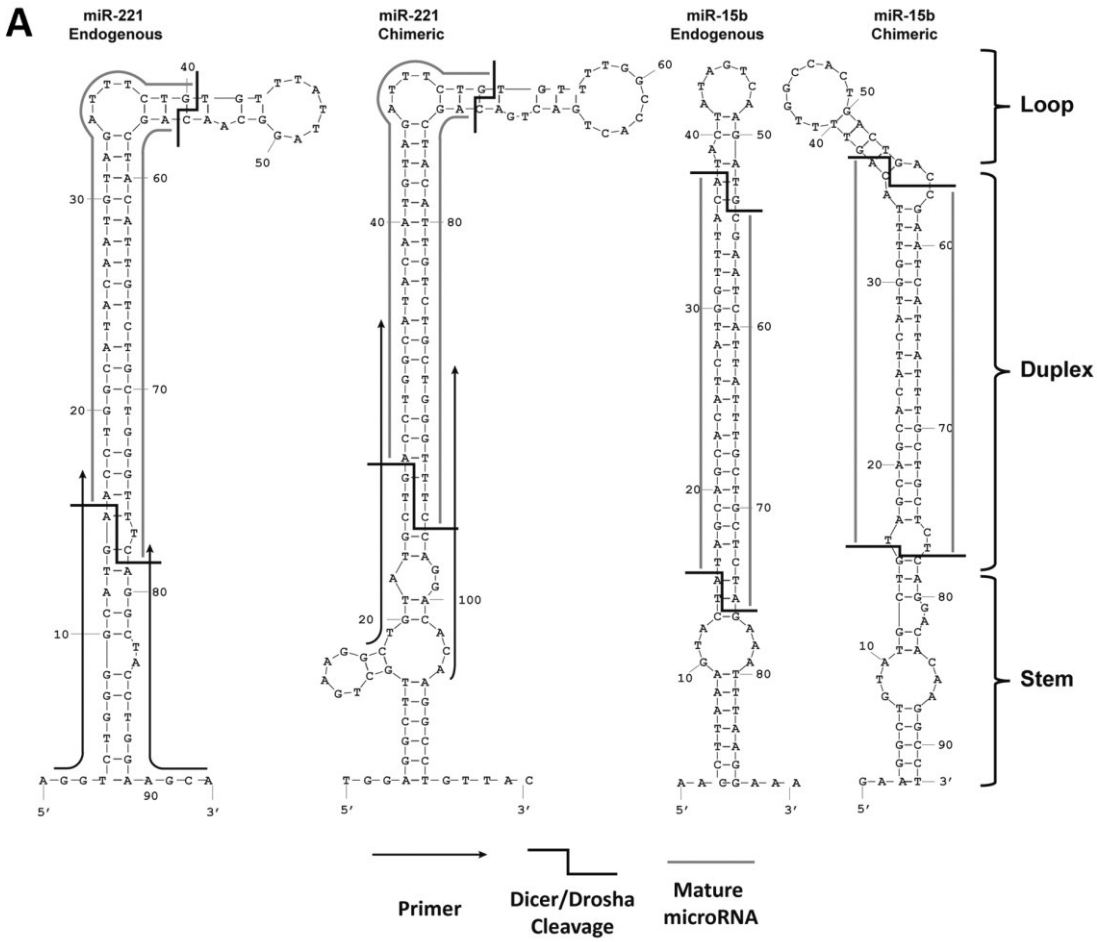


Figure 2. RT-qPCR analysis of mature miRNA levels. Fold changes in mature miRNA levels are shown relative to the negative control (mean ± standard deviation of three individual transfections). miR-185-5p was used as reference miRNA to assess miRNA over-expression after transfection of artificial and endogenous miRNA expression constructs. *$p<0.05$ (Student's t-test). **(A)** miR-15b/16-2 constructs. **(B)** miR-221/222 constructs.

The transcript levels of mature miRNA were analysed by RT-qPCR for each of the miRNAs of the two clusters (miR-15b-5p, miR-16-5p, mir-221-3p and mir-222-3p) and normalized against miR-185-5p as a stably expressed control [32]. During cDNA synthesis miRNA-specific looped RT-primers, which specifically reverse transcribe a single mature miRNA, were used to ensure amplification of mature miRNAs only. Compared to the empty vector control, the transcript levels of the mature miRNAs of the chimeric cluster constructs were not increased (Fig. 2).

Based on these results, we investigated whether the expression of miRNA clusters could be improved using the complete CHO sequence. Therefore endogenous miR-221/222 and the miR-15b/16-2 clusters were amplified from genomic DNA and cloned into the 3'UTR of the same



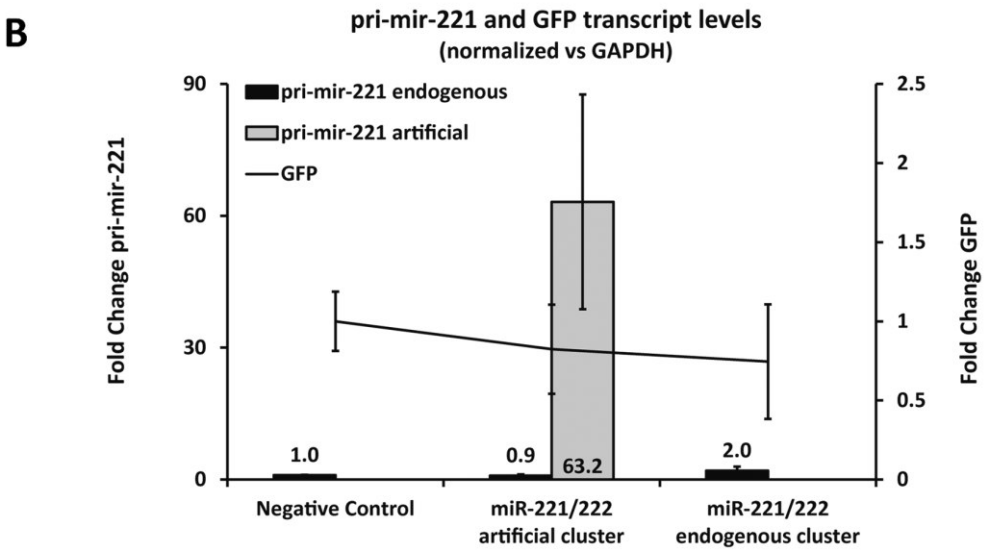


Figure 3. Analysis of pri-miRNA folding and transcription. **(A)** Illustration of putative secondary structures for both artificial and endogenous mir–221 and mir-15b using Quikfold [38, http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold] with energy rules for RNA(3.0) and default settings. The location of primers used from amplifying the respective pri–miRNAs, the mature miR–sequences, and the cleavage sites (Drosha/Dgcr8 at the stem/duplex interface, Dicer at the duplex/loop interface) are indicated. **(B)** pri-mir-221 and GFP transcription levels two days post transfection analyzed by RT-qPCR, normalized against GAPDH and related to the negative control (mean ± standard deviation of three individual transfections).

vector that was used for the chimeric constructs (Fig. 1). The same transfection procedure as for the chimeric clusters were performed and resulted in significant 2.3 to 3.3-fold over-expression of all mature miRNAs of these clusters (Fig. 2).

3.2 Identification of bottleneck of chimeric miRNA biogenesis

Since emGFP expression suggested adequate transfection efficiencies and transcription rates (Supporting information, Fig. S1), and therefore availability of primary microRNA transcripts, the lack of miRNA over-expression from chimeric miRNA clusters could be due to inefficient processing in the nucleus by Drosha/Dgcr8 or in the cytosol by Dicer. To evaluate this possibility, primers were designed to amplify the primary mir-221 transcripts derived from both the endogenous and the chimeric miR-221/222 cluster (Supporting information, Table 1). These primers were designed individually for each construct, and were located at the border between mature miRNA and the flanking region (Fig. 3A, Supporting information, Table 1). RT-qPCR analysis of pri-miRNA levels after transfection of the endogenous expression construct showed a 2-fold increase in endogenous pri-miRNA levels relative to the empty vector control (Fig. 3B). This result is in line with the ~3-fold increase observed for mature miRNA levels. However, following transfection of artifical mir-221/222 constructs, a strong (above 50-fold) increase in artificial pri-miRNA was detected when compared to the endogenous pri-miR-221 levels of the empty vector control (see Fig. 3B). This result suggests that the transcription of the chimeric miRNA clusters works well. However, possibly due to misfolding of the resulting hairpins (Fig. 3A) or to the artificial cluster sequence, the pri-miRNA transcripts are not processed and accumulate in the nucleus.

4 Concluding remarks

Originally, the chimeric cloning approach for vector-based miRNA expression that was used in this study was developed and tested for the stable over-expression of mouse miRNAs and shRNAs [26]. For this purpose the method is widely in use. Later, this system was adapted for use in CHO cells for single miRNAs, which yielded relatively low levels of over-expression for various mature miRNAs, ranging from 1.2 to 2.3-fold [32], depending on the overall expression level. The application of the same cloning strategy for expression of miRNA clusters in this study did not result in elevated mature miRNA levels. From our present results it appears that these constructs are not properly processed compared to constructs containing the endogenous cluster sequence amplified from gDNA. Analysis of the primary miRNA transcript level using RT-qPCR showed an enrichment of these transcripts for the chimeric constructs, suggesting that the murine flanking regions used in this study result in structural changes that cannot be efficiently processed by Drosha/DGCR8 in the nuclear processing step. Hackl et al. [34] have previously shown that while the mature miRNAs are highly conserved between human, mouse, rat, and the Chinese hamster, the homology of the hairpin sequences is much lower. In this context our results indicate that the precise secondary structure of miRNAs and, even more importantly, miRNA clusters has important implications for their processing and biogenesis. While for miRNAs and natural miRNA clusters the problem can easily be overcome using the species-specific genomic sequences for engineering purposes, it is not as easily resolved in the design of shRNAs or for construction of artificial clusters consisting of multiple miRNAs that do not naturally occur in a cluster. Here careful design of the artificial sequences taking into consideration the expected folding, especially the drosha and dicer cut sites, may be required.

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1.5. Expanding the number of phenotype-related miRNAs

Several miRNA profiles were previously linked to certain phenotypes in CHO cells, but only one study directly connected miRNA expression to the growth rate[31]. As this study focused on interclonal changes, we wanted to expand the knowledge of growth-connected miRNAs by a more general approach that included CHO cells from different lineages and grown under a variety of culture conditions.

For this purpose, previously published miRNA microarrays[33] as well as other in-house available miRNA profiles of a wide range of CHO cell lines were combined, and the resulting data was processed together. After the array data was updated to the miRBase version 21[46] to remove falsely annotated miRNAs, samples were clustered based on their miRNA profiles. Clustering revealed that certain phenotypes can be associated to global miRNA expression patterns (growth phase, serum-free adapted DUKXB11).

After that, the expression of each miRNA was correlated to the growth rate of all, the serum-free, the exponential, or the combination of serum-free and exponential samples, and growth-correlating miRNAs were identified by applying a Pearson or a Spearman correlation coefficient cutoff of > |0.6|. Most growth-correlating miRNAs were identified when samples of all growth phases were included, which spanned the highest range of growth rates. Twelve miRNAs were found to be consistently growth-related in all sample sets, indicating an important role in growth.

Two of the positively growth-correlating miRNAs, miR-222-3p and miR-23a-3p, are found frequently upregulated in tumor tissues, where they exhibit growth-promoting functions. The other positively correlating miRNAs (miR-300, miR-647, miR-706, miR-3613, miR-4288, miR-4317 and miR-669a-3-3p) are less well characterized. All three negatively growth-correlating miRNAs (miR-29a-3p, miR-29b-3p and miR-29c-3p)

belong to the same miRNA family and are also found clustered (mir-29a to mir-29b-1 and mir-29b-2 to mir-29c) at the genomic level. This miRNA family is known for its tumor-suppressive function.

Many of the here identified miRNAs were already connected to certain phenotypes in CHO cells, but none was directly linked to growth rate, indicating further regulatory potential of the here identified miRNAs.

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A signature of 12 microRNAs is robustly associated with growth rate in a variety of CHO cell lines

Gerald Klanert[a,b], Vaibhav Jadhav[a], Vinoth Shanmukam[a], Andreas Diendorfer[a], Michael Karbiener[c], Marcel Scheideler[e], Juan Hernández Bort[a], Johannes Grillari[b], Matthias Hackl[d,**], Nicole Borth[a,b,*]

[a] *Austrian Centre of Industrial Biotechnology, Austria*
[b] *University of Natural Resources and Life Sciences, Austria*
[c] *Graz University of Technology, Austria*
[d] *TAmirNA GmbH, Austria*
[e] *Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany*

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ABSTRACT

As Chinese Hamster Ovary (CHO) cells are the cell line of choice for the production of human-like recombinant proteins, there is interest in genetic optimization of host cell lines to overcome certain limitations in their growth rate and protein secretion. At the same time, a detailed understanding of these processes could be used to advantage by identification of marker transcripts that characterize states of performance.

In this context, microRNAs (miRNAs) that exhibit a robust correlation to the growth rate of CHO cells were determined by analyzing miRNA expression profiles in a comprehensive collection of 46 samples including CHO-K1, CHO-S and CHO-DUKXB11, which were adapted to various culture conditions, and analyzed in different growth stages using microarrays. By applying Spearman or Pearson correlation coefficient criteria of > |0.6|, miRNAs with high correlation to the overall growth, or growth rates observed in exponential, serum-free, and serum-free exponential phase were identified. An overlap of twelve miRNAs common for all sample sets was revealed, with nine positively and three negatively correlating miRNAs.

The here identified panel of miRNAs can help to understand growth regulation in CHO cells and contains putative engineering targets as well as biomarkers for cell lines with advantageous growth characteristics.

1. Introduction

Chinese Hamster Ovary (CHO) cell lines are the most frequently used mammalian cell factory for the production of therapeutic proteins (Walsh, 2014). Enhancement of the phenotype of these cells towards faster growth, higher productivity, better product quality and increased robustness is the major goal in bioprocess and cell line engineering and therefore highly desired. Media optimization (Kishishita et al., 2015; Xu et al., 2014), process improvement (Trummer et al., 2006; Lu et al., 2013) and genetic engineering were used to achieve the above mentioned objectives. The latter was mostly conducted by directly altering gene expression of a small number of protein-coding genes, known to be related to a certain pathway (Jeon et al., 2011; Lee et al., 2013). More recently, additional engineering candidates were revealed by different "-omics" technologies (Lin et al., 2015; Courtes et al., 2013).

Another promising genetic engineering approach of CHO cells, which is based on post-transcriptional regulation by miRNAs, gained interest in the last years (Jadhav et al., 2013). The advantages of those short (21–24 nucleotides), non-coding RNAs (Finnegan and Pasquinelli, 2012) are that one miRNA can target hundreds of genes without burdening the translational machinery (Hackl et al., 2012a). MicroRNAs are encoded in intergenic and intronic regions, and are often located in close chromosomal proximity (Altuvia et al., 2005). According to miRBase version 21 (v21) (Kozomara and Griffiths-Jones, 2014), 31% of *Mus musculus*, 25% of *Homo sapiens*, and 54% of *Rattus norvegicus* annotated miRNAs are found in an

* Corresponding author at: Austrian Centre of Industrial Biotechnology, Austria.
** Corresponding author.
E-mail addresses: gerald.klanert@boku.ac.at (G. Klanert), vaibhav.jadhav@boku.ac.at (V. Jadhav), vinoth.shanmukam@boku.ac.at (V. Shanmukam), andreas.diendorfer@boku.ac.at (A. Diendorfer), michael.karbiener@medunigraz.at (M. Karbiener), marcel.scheideler@helmholtz-muenchen.de (M. Scheideler), juan.hernandez.bort@baxalta.com (J.H. Bort), johannes.grillari@boku.ac.at (J. Grillari), matthias.hackl@tamirna.com (M. Hackl), nicole.borth@boku.ac.at (N. Borth).

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inter-miRNA distance below 10 kilobases (kb), and are considered to be clustered. Individual or cooperative transcription, occurring for clustered miRNAs (Baskerville and Bartel, 2005), forms a long, primary transcript (pri-miRNA), which is processed in the nucleus by the microprocessor complex to release a stem-loop with a length of ~65 nucleotides, called precursor miRNA (pre-miRNA or mir) (Lee et al., 2003). After export into the cytoplasm by Exportin-5 (Yi et al., 2003), the stem-loop is recognized and processed by Dicer (Hutvágner et al., 2001) to produce a RNA duplex, consisting of the 5p and the 3p mature microRNA (miR) strands. While one is preferentially incorporated into the miRISC (microRNA-loaded RNA-induced silencing complex) (Bergauer et al., 2009), the other strand is usually, but not always, degraded (Krol et al., 2010). The miRISC recognizes target mRNAs, which bear mostly imperfect complementary regions in the three prime untranslated region (3′ UTR) to the miRNA, guiding translational repression (Zeng et al., 2002) or mRNA cleavage (Zeng et al., 2003).

Identification (Gammell et al., 2007; Kantardjieff et al., 2009; Johnson et al., 2011; Hackl et al., 2011), localization (Hackl et al., 2012b) and expansion (Diendorfer et al., 2015) of the CHO miRNome and method development (Jadhav et al., 2012; Fischer et al., 2013; Klanert et al., 2014) to alter miRNA expression have provided a sound basis for miRNA research and application in CHO cells. Several miRNAs were reported to drive CHO cells to desired phenotypes. These so-called engimiRs were able to enhance productivity (Barron et al., 2011; Sanchez et al., 2014; Strotbek et al., 2013; Loh et al., 2014; Fischer et al., 2014; Jadhav et al., 2014; Kelly et al., 2015), growth (Jadhav et al., 2012; Sanchez et al., 2014) or increase apoptosis-resistance (Druz et al., 2011, 2013). MiRNAs for engineering were selected the same way as protein-coding genes, based on previously published literature, 'omics'-studies or high-throughput screenings. The latter is able to test the effect of the whole miRNome and identifies all miRNAs related to the phenotypes observed (Strotbek et al., 2013; Fischer et al., 2014, 2015). Since the method is very cost- and labor-intense, normally only one cell line in one condition is tested, and the best candidates are selected for further testing in other cell lines. Profiling of CHO miRNA expression using either next-generation sequencing (Hackl et al., 2011; Johnson et al., 2011; Hammond et al., 2012; Loh et al., 2014) or arrays (Gammell et al., 2007; Barron et al., 2011; Lin et al., 2011; Druz et al., 2011; Bort et al., 2012; Clarke et al., 2012; Maccani et al., 2014; Hackl et al., 2014) revealed differentially expressed miRNAs for specific conditions and phenotypes, but the conducted studies were often focused on a small set of cell lines.

Here we present the results from a comprehensive approach to identify growth associated miRNAs by profiling miRNA expression in CHO-K1, CHO-DUKXB11 and CHO-S cell lines, among them serum, serum-free, and L-Glutamine (L-Gln)-free adapted host, recombinant and engineered cells, grown in different cultivation systems. MiRNA expression was characterized in different growth phases by microarray analysis. This comprehensive set of miRNA expression data allowed us to identify growth-correlating miRNAs valid for a wide variety of different CHO cell lines and culture conditions. These miRNAs will be good candidates to test for their potential as engimiRs, to favorably alter cell growth in biotechnological processes, but also as 'diagnostic' tools to identify cell lines and clones with high growth potential and to indicate changes in growth during process development.

2. Material and methods

2.1. Sample ID generation

Sample IDs were generated, based upon following structure: [cell line]-[cultivation method]-[L-Gln supplementation]-[serum supplementation]-[host/producer]-[engineered (optional)]-[biological replicate (optional)]-[RNA sampling timepoint]-[technical replicate]-[array-number]. The [cell line] represents either the CHO-K1 (K1), CHO-S (S), or the CHO-DUKXB11 (DUKXB11) cell line, whereas the [cultivation method] implies whether cells were cultivated statically for adherent (ADH) growth, or either shaken (SHAKE) or in a fermenter (FERM) for suspension cultures. The [L-Gln supplementation] depicts the amount of L-glutamine (0/4/8) in mM added, and the [serum supplementation] indicates whether cells were cultivated in the presence of 5% fetal calf serum (FCS) or serum-free (SF). The [host/producer] points out whether the cells function as host cell line (HOST) or produce an Erythropoietin-Fc fusion protein (PROD). The [engineered (optional)] is only depicted at samples which harbor a vector overexpressing miR-17 (M17) or an empty vector control (MNC). For Fig. 1 and Supplementary Table 1, [biological replicate] indicates the biological replicate by an additional number (1–4), if more than one biological replicate was conducted, and also the timepoint of RNA sampling (D1-D9) is included via [RNA sampling timepoint]. In addition, Fig. 1B also contains [technical replicate], depicting the technical replicate (R1 or R2) used for hybridization. Also, in Fig. 1B, if more than one microarray was performed per technical replicate, it is indicated via [array-number] by an additional number (1–3).

2.2. Cell culture

All CHO cell lines were cultivated at 37 °C, 7% CO_2 and humidified atmosphere. K1-STAT-4-FCS-HOST (CHO-K1, ECACC-CCL61) and DUKXB11-STAT-4-FCS-HOST (CHO-DUKXB11, ATCC CRL-9096) were grown in DMEM/Ham's F12 media (1:1, Merck KGaA, Darmstadt, Germany) supplied with 5% fetal calf serum (GE Healthcare, Little Chalfont, UK) and 4 mM L-Gln. DUKXB11-STAT-4-FCS-HOST cells were additionally supplied with 1x hypoxanthine/thymidine (HT) supplement (Thermo Fisher Scientific, Waltham, MA).

DUKXB11-SHAKE-4-SF-HOST (suspension and serum-free adapted CHO-DUKXB11) cells were cultivated in DMEM/Ham's F12 (1:1), supplemented with 4 mM L-Gln, 0.25% soy peptone, 0.1% Pluronic F68 (BASF, Germany), 1x protein free supplement (Polymun Scientific, Austria) and 1x HT supplement. K1-SHAKE-8-SF-HOST/K1-FERM-8-SF-HOST (suspension and serum-free adapted CHO-K1), K1-SHAKE-0-SF-HOST/K1-FERM-0-SF-HOST (suspension, serum-free and L-Gln-free adapted CHO-K1), S-SHAKE-8-SF-HOST (CHO-S), DUKXB11-SHAKE-0-SF-PROD/DUKXB11-FERM-0-SF-PROD-M17/DUKXB11-0-SF-FERM-PROD-MNC (recombinant suspension and serum-free adapted CHO-DUKXB11, producing an Erythropoietin-Fc fusion protein) were grown in CD CHO (Thermo Fisher Scientific), supplemented with 1x Anti-Clumping Agent (Thermo Fisher Scientific). K1-SHAKE-8-SF-HOST/K1-FERM-8-SF-HOST and S-SHAKE-8-SF-HOST were additionally supplied with 8 mM L-Gln. DUKXB11-SHAKE-0-SF-PROD, DUKXB11-FERM-0-SF-PROD-M17 and DUKXB11-0-SF-FERM-PROD-MNC cells were supplied with 0.19 μM methotrexate. DUKXB11-FERM-0-SF-PROD-M17 additionally harbors a vector overexpressing miR-17, while DUKXB11-FERM-0-SF-PROD-MNC harbors an empty vector control. DUKXB11-SHAKE-4-SF-HOST, K1-SHAKE-8-SF-HOST, K1-SHAKE-0-SF-HOST, S-SHAKE-8-SF-HOST and DUKXB11-SHAKE-0-SF-PROD were cultivated in shaker flasks at 140 revolutions per minute (rpm) and a 25 mm shaking diameter.

K1-FERM-8-SF-HOST, K1-FERM-0-SF-HOST, DUKXB11-FERM-0-SF-PROD-M17 and DUKXB11-FERM-0-SF-PROD-MNC were cultivated in DASGIP bioreactor system (DASGIP AG, Jülich, Germany) controlled by DASGIP Control 4.0 software with similar settings as described before (Taschwer et al., 2012). Briefly, advanced spinner vessels with 800 ml working volume, magnetic drive and

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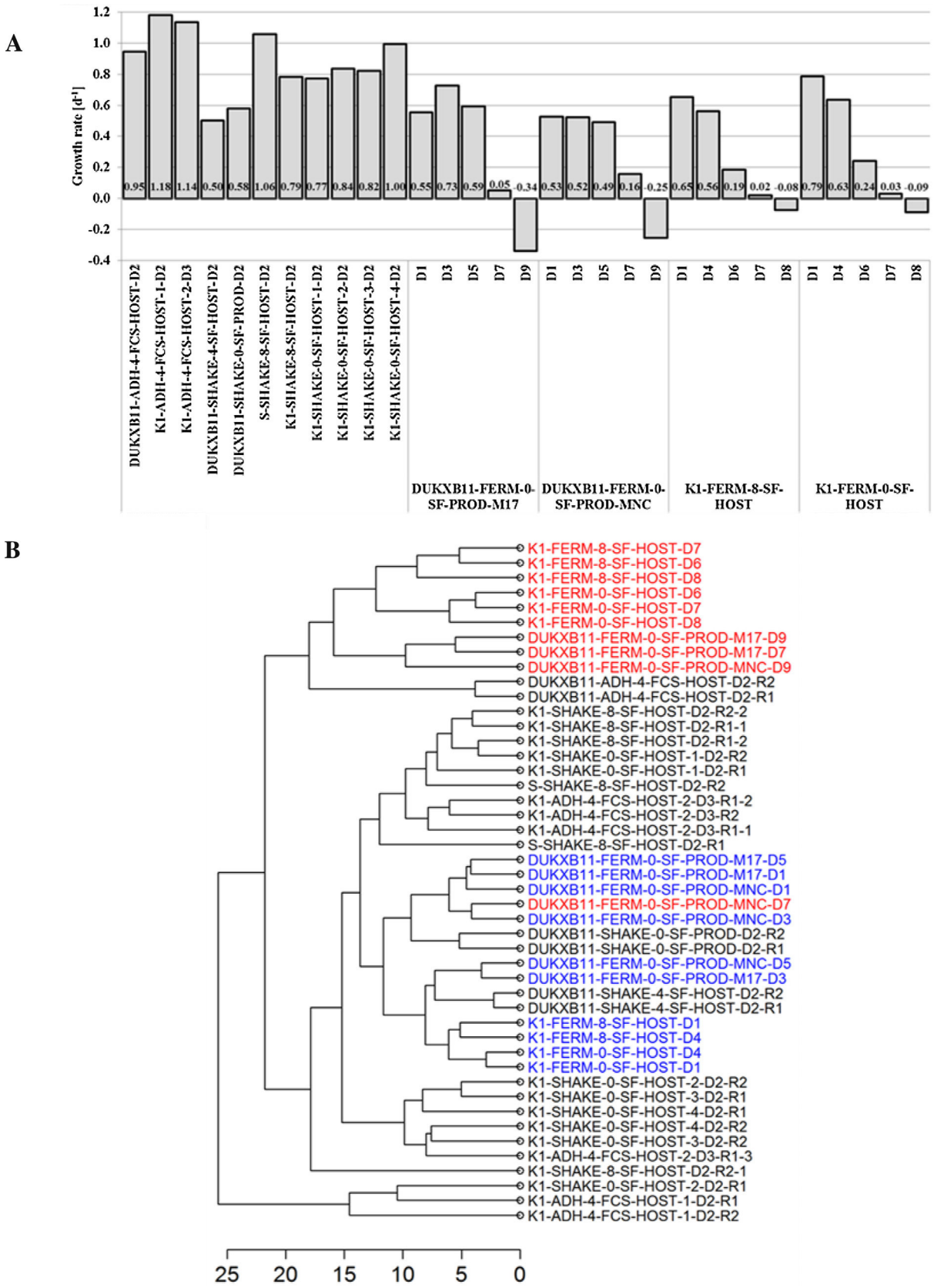

Fig. 1. Sample characteristics. (A) Growth rates of all CHO cell lines analyzed in this study at the time of RNA sampling. (B) Unsupervised sample clustering based on miRNA expression patterns. Red: Samples drawn from the fermenter in stationary/decline phase. Blue: Samples drawn from the fermenter in exponential phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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pitch blade impellers for constant stirring (80 rpm) were used for the cultivation. Temperature was maintained at 37 °C, constant 30% oxygen concentration was held via probe (Broadley James Oxyprobe) by air saturation and neutral pH (Mettler Toledo) was regulated with 0.5 M NaOH and CO_2.

2.3. RNA isolation

RNA was extracted using the phenol-chloroform extraction method. Adherent CHO cell lines were washed with PBS and detached using Trypsin. Adherent and suspension cells were collected by centrifugation and lysed using TRI reagent (Sigma-Aldrich, St. Louis, MO). RNA isolation was performed according to the manufacturer's recommendations. In brief, phase separation was conducted by the addition of chloroform, and the aqueous phase was collected. After precipitation by 2-Propanol addition and washing with 70% Ethanol, RNA pellets were air-dried, and resuspended in nuclease-free water (NFW).

2.4. RNA quality and quantity assessment

For quantity and purity estimation, absorbances at 230, 260 and 280 nm were measured using a NanoDrop ND-1000 UV–vis Spectrophotometer (Thermo Fisher Scientific). Total RNA quality was assessed on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using the RNA 6000 Nano Kit (Agilent Technologies). Only RNA samples with a RNA integrity number (RIN) > 7 were used for microarray hybridization.

2.5. MicroRNA microarray hybridization

Cross-species microRNA microarrays were generated as described previously (Bort et al., 2012) by spotting a locked nucleic acid (LNA) probe set on epoxy-coated Nexterion glass slides. The probe set consisted of 8 replicates per slide, each targeting 2367 human, mouse, rat and viral miRNAs of miRBase v16. All samples, consisting of 800 ng total RNA extracts, were blended with the Spike-in miRNA kit v2 (Exiqon, Denmark) and labelled with Hy3 by the miRCURY LNA microRNA Hi-Power labeling Kit (Exiqon) according to the manufacturer's protocol. A common reference pool (CRP) was processed equally, but labelled with Hy5. Samples were then pooled with the CRP, heat-denaturated for 2 min at 95 °C, and hybridized onto the microarrays mentioned above at 56 °C for 16 h using the TECAN HS 400 hybridization station (Tecan, Switzerland). Immediately after washing and drying, the array slides were scanned at 532 and 635 nm wavelengths at 10 μm resolution using a Roche Nimblegen MS200 scanner (Roche, Switzerland) and auto-gain settings.

2.6. Data processing

Feature extraction from the generated images was performed using GenePix 4.1 (Molecular Devices, Sunnyvale, CA). The resulting GPR-files were processed in R/Bioconductor (R Core Team, 2015) using the LIMMA package (Ritchie et al., 2015). Raw data of previously published arrays (Hackl et al., 2014) (Gene Expression Omnibus accession number: GSE52994) was compiled with the here generated data for jointly analysis. Global LOESS normalization and Normexp background correction were performed, and the $\log_2$-fold changes of the miRNAs were calculated against the common reference pool (LogFC) for each array. To be considered expressed, the average probe intensity had to exceed the average background intensity plus two times the standard deviation. The probe's validity was reviewed by updating the corresponding miRNA accessions to miRBase v21. The compiled data has been submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and can freely be downloaded and reanalyzed using the accession number GSE75830.

2.7. Growth rate determination

Culture's viable cell densities were determined by the ViCell (Beckman Coulter, Brea, CA), which uses the trypan blue exclusion method. One time point before, the time point of RNA isolation and the time point after isolation were linearly correlated with their respective ln-transformed viable cell densities to retrieve the slope of the function, which corresponds to the growth rate at RNA sampling, and the coefficient of determination. If existent, technical replicates were combined.

2.8. Probe mapping

Consensus sequences of all microRNAs assigned to a microarray probe were retrieved from miRBase v16 and mapped to annotated microRNA stem-loop sequences of *Cricetulus griseus* (*C. griseus*) of miRBase v21 and to additionally identified microRNA stem-loop sequences present in the four genome assemblies of CHO and *C. griseus* (Diendorfer et al., 2015).

2.9. Clustering

Based on the LogFC values, euclidean distance matrices were calculated and dendrograms were generated in R for all samples and for all valid microRNA probes above background.

2.10. Growth rate correlation

All valid probes above the background were correlated to the growth rates of all arrays, all serum-free samples (SF), all exponential samples (samples taken before day 6), or all exponential and serum-free samples (SF-samples taken before day 6). A Pearson correlation coefficient or a Spearman correlation coefficient > |0.6| was set as cutoff. Furthermore, a Δ(LogFC) > |1| between all samples was set as an additional criteria.

3. Results

3.1. Growth and miRNA profiling of diverse CHO cells

Growth profiles of different CHO cell cultures were collected, including adherent and suspension-adapted host and producer cell lines, cultivated under different conditions. A total of 46 miRNA profiles were generated from 31 biological samples by microarrays, and growth rates were calculated, ranging from −0.34 to 1.18 d^{-1} (see Fig. 1A and Supplementary Table 1). MicroRNA microarrays based on miRBase v16 were hybridized using a common reference design, and the resulting raw data were normalized. $\log_2$ fold changes against the common reference pool (LogFC) and average expression values (AvExp) were retrieved for all miRNA probes. Out of 2367 probes, 295 showed a signal intensity (AvExp) above background criteria (see Supplementary Table 2). The annotated miRNA IDs for these probes were updated from miRBase v16 to miRBase v21. While the majority of the probes (n = 277) were still valid in miRBase v21, some of the probe IDs (n = 18) had been disproved as mature miRNAs in miRBase v21 and were excluded from further analyses. Around 46% (n = 128) of the probe sequences perfectly matched to already annotated stem-loop sequences.

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3.2. Sample clustering indicates factors influencing global miRNA expression

Unsupervised clustering was performed using the expression values (LogFC) of all 277 expressed miRNAs in 46 samples. The Dendrogram (see Fig. 1B) was visually inspected to identify factors influencing overall miRNA expression. It was observed that all but one sample (DUKXB11-FERM-0-SF-PROD-MNC-D7) taken in stationary/death-phase cluster together, and all samples drawn from the fermenter in exponential phase (. . .-FERM-. . .-D1/D3/D4/D5) also cluster together. All samples of the producer cell line (DUKXB11-. . .-PROD-. . .) taken in exponential phase cluster together despite different cultivation methods, and share miRNA expression patterns with the serum-free and suspension-adapted host cell line (DUKXB11-SHAKE-4-SF-HOST-D2). The miRNA expression patterns of the other cell lines vary more widely, as individual samples of the same cell lines do not clearly cluster together.

3.3. Growth correlation indicates that specific miRNA expression is associated with the growth rate

In order to identify growth-correlating miRNAs, the LogFC of the miRNAs were correlated to the growth rate measured for all samples. A Pearson and a Spearman correlation were applied, and all miRNAs exceeding a correlation coefficient of |0.6| in at least one of those two correlations were considered as growth-correlating miRNAs. Further, to only include miRNAs for which the expression also changes upon growth variation, a $\Delta(\text{LogFC}) > |1|$ between all samples was set as criteria (the range of observed logFC values had to exceed 1 log-value). When all 46 arrays were considered, 43 miRNAs fulfilled the above mentioned criteria. Growth correlations for sample subsets identified 48 growth-correlating miRNAs for the serum-free samples (all sample IDs without FCS), 25 correlating miRNAs for the exponential (all sample IDs up to day 5), and 30 correlating miRNAs for serum-free exponential samples (all sample IDs without FCS up to day 5, see Fig. 2A and Supplementary Table 3). The majority of miRNAs fulfilling the criteria were positively correlated to the growth rate (~72–83%). Each data set bears a separate amount of identified miRNAs (n = 9–13, see Fig. 2B), but an overlap between all sample sets, consisting of 12 miRNAs (see Table 1) and another overlap between all samples and the serum-free samples, comprising 20 miRNAs, were identified. Fig. 3 visually depicts the changes in miRNA expression due to growth changes for the 12 miRNAs identified in all four analyses.

3.4. Expression distance matrix indicates jointed expression of some clustered growth-correlating miRNAs

In order to determine whether the here identified overlap between all sample sets, consisting of 12 growth-correlating miRNAs, are located in miRNA clusters, genomically clustered miRNAs of human, mouse and rat were retrieved from miRBase v21 based on the 10 kb chromosomal distance criterion. Out of the 12 miRNAs, 8 were found to be clustered with other miRNAs, with 5 of them also found to be clustered in CHO (see Supplementary Table 4). The expression levels of the miRNAs clustered together were visually analyzed by the dendrogram created for all miRNAs based upon their LogFC at different samples (see Supplementary Fig. 1). MiR-29b-3p, which is found twice in the genome, once clustered to miR-29a-3p, and once to miR-29c-3p, shows very good expression correlation to both of them. Both miR-29a-3p and miR-29c-3p were also identified to be growth-correlating in all sample sets. Also, miR-222-3p is in close proximity at the dendrogram to its clustered miRNA, miR-221-3p. MiR-23a-3p only correlates to miR-24-3p expression, but not to miR-27a-3p, and also miR-669a-3-3p expression only correlates to part of the expressed miRNAs found in close chromosomal proximity (miR-467c-3p, miR-669b-3p and miR-669p-3p). While miR-300 does not show any correlation to its genomically clustered miRNAs, miR-647 is the only member of its cluster which is found expressed.

4. Discussion

4.1. Global miRNA expression and evaluation

So far, one study was conducted which reported miRNA levels in CHO cells with different growth rates (Clarke et al., 2012), focusing on interclonal changes where clones were derived from the same host cell line. In order to gain a more comprehensive overview about miRNA expression changes due to growth variation, different CHO cell lines, including adherent, suspension- and serum-free adapted, L-Gln-free adapted host and recombinant cells under varying cultivating conditions and in different batch phases, were used for this study. The here performed analysis of 46 cross-species miRNA microarrays, a platform which was previously successfully used and validated by qPCR in transcriptomic experiments (Maccani et al., 2014; Hackl et al., 2014; Bort et al., 2012) revealed 295 out of 2376 probes with a signal intensity above our background criteria (see Supplementary Table 2). According to miRBase v21 (Kozomara and Griffiths-Jones, 2014), 277 probe IDs detecting distinct miRNAs are still valid. The number of expressed miRNAs agrees with previously published miRNA profiling studies in CHO, ranging from 190 (Hammond et al., 2012) to 496 (Loh et al., 2014) expressed miRNAs. More than 50% of these 277 probe sequences did not match perfectly to already annotated CHO stem-loops. The signals of those probes are most likely derived from (a) unidentified stem-loops for CHO, (b) non-miRNA RNA species, and (c) cross-hybridization. As the CHO (Hammond et al., 2011; Xu et al., 2011) and *C. griseus* (Lewis et al., 2013; Brinkrolf et al., 2013) genomes have been sequenced just recently, the assemblies are still considered draft genomes (Jakobi et al., 2014), and it is probable that not all miRNAs encoded in the CHO genome are present in the current version of those draft genomes. Due to this, all 277 miRNAs were included for further analyses to not lose any miRNAs possibly present in CHO. Another possibility is that sequences were falsely annotated as miRNAs, an issue that has been addressed before (Berezikov et al., 2010), and is attracting more and more attention (Kozomara and Griffiths-Jones, 2014; Wong and Wang, 2015). Cross-hybridization is a general technical issue of microarrays and could in future be prevented by using RNA-Seq.

4.2. Factors influencing global miRNA expression

As indicated by hierarchical clustering of sample IDs, the growth phase is the factor with the most impact on global miRNA expression, as all but one sample in stationary or death phase cluster isolated from all exponential samples (see Fig. 1B). The miRNA expression of CHO cells is known to be dynamic during batch cultures (Bort et al., 2012). Also, serum-free and suspension-adaptation seems to have an influence on the miRNA expression, as part of the FCS-supplied adherent cells cluster apart from suspension and serum-free adapted cell lines. The change in miRNA expression in CHO cells upon serum-free and suspension adaptation has also been shown before (Hackl et al., 2011). The cell line itself, the L-Gln-free adaptation, the cultivation method (shaken or in a fermenter) and miRNA engineering exhibit only a marginal effect on global miRNA expression in comparison to the above mentioned factors, as no distinct clustering pattern was observed.

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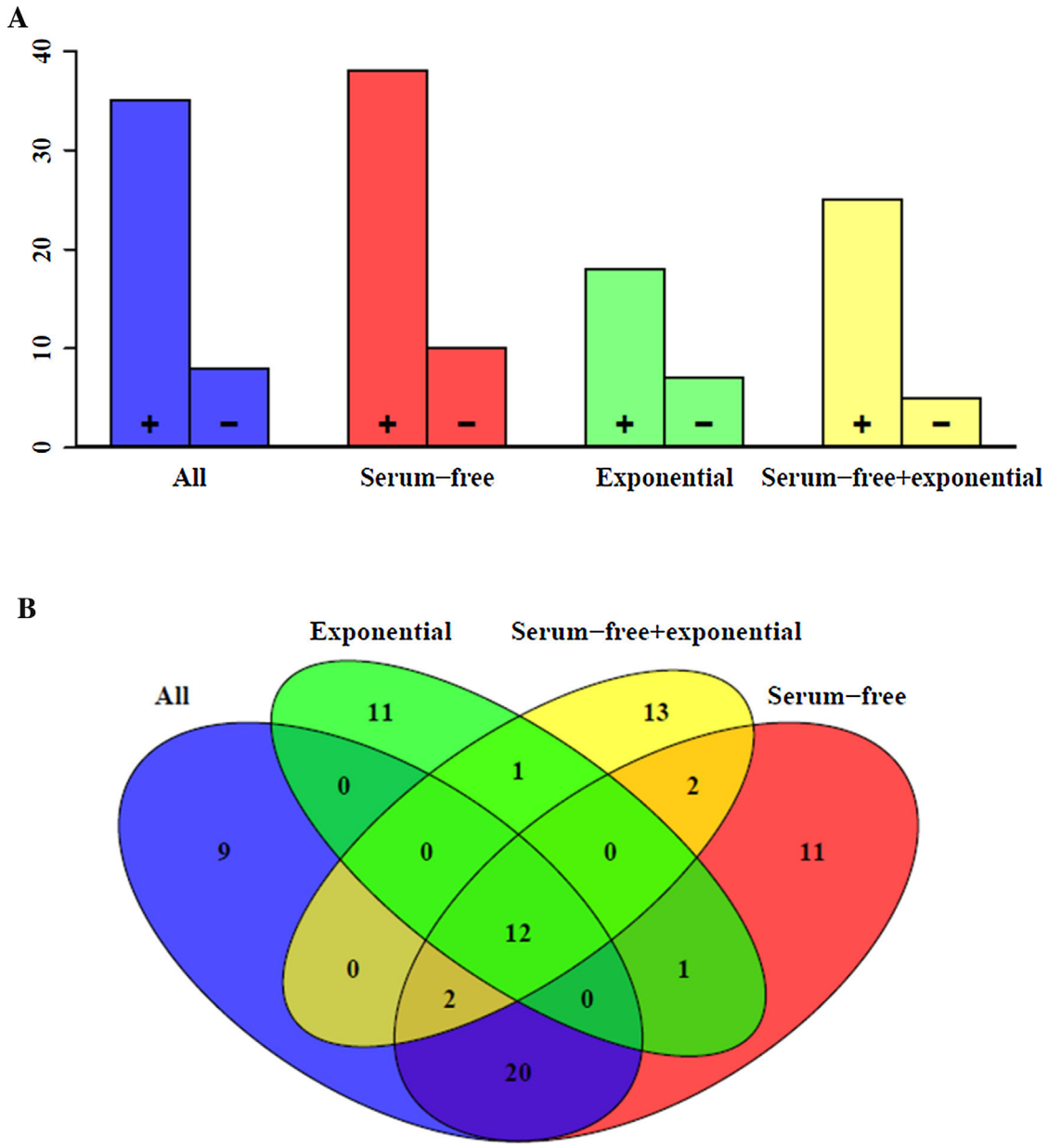

Fig. 2. Growth-correlating miRNAs. (A) Number of positively (+) and negatively (−) growth-correlating miRNAs including either all samples (All), samples of the exponential growth phase (Exponential), serum-free samples (Serum-free) or serum-free samples in exponential growth phase (Serum-free + exponential). (B) Overlap of growth-correlating miRNAs.

Table 1
Growth-correlating miRNAs.

Probe ID	Detected strand	Growth Correlation	All Samples—Correlation Coefficient	
			Pearson	Spearman
hsa-miR-222/mmu-miR-222/rno-miR-222	3p	positive	0.752	0.800
hsa-miR-23a/mmu-miR-23a/rno-miR-23a	3p	positive	0.518	0.679
hsa-miR-300	only one strand annotated	positive	0.780	0.814
hsa-miR-3613-3p	3p	positive	0.718	0.800
hsa-miR-4288	only one strand annotated	positive	0.687	0.758
hsa-miR-4317	only one strand annotated	positive	0.522	0.665
hsa-miR-647	only one strand annotated	positive	0.772	0.791
mmu-miR-669a-3-3p	3p	positive	0.694	0.696
mmu-miR-706	only one strand annotated	positive	0.825	0.868
hsa-miR-29a/mmu-miR-29a/rno-miR-29a	3p	negative	−0.748	−0.762
hsa-miR-29b/mmu-miR-29b/rno-miR-29b	3p	negative	−0.734	−0.768
hsa-miR-29c/mmu-miR-29c/rno-miR-29c	3p	negative	−0.766	−0.789

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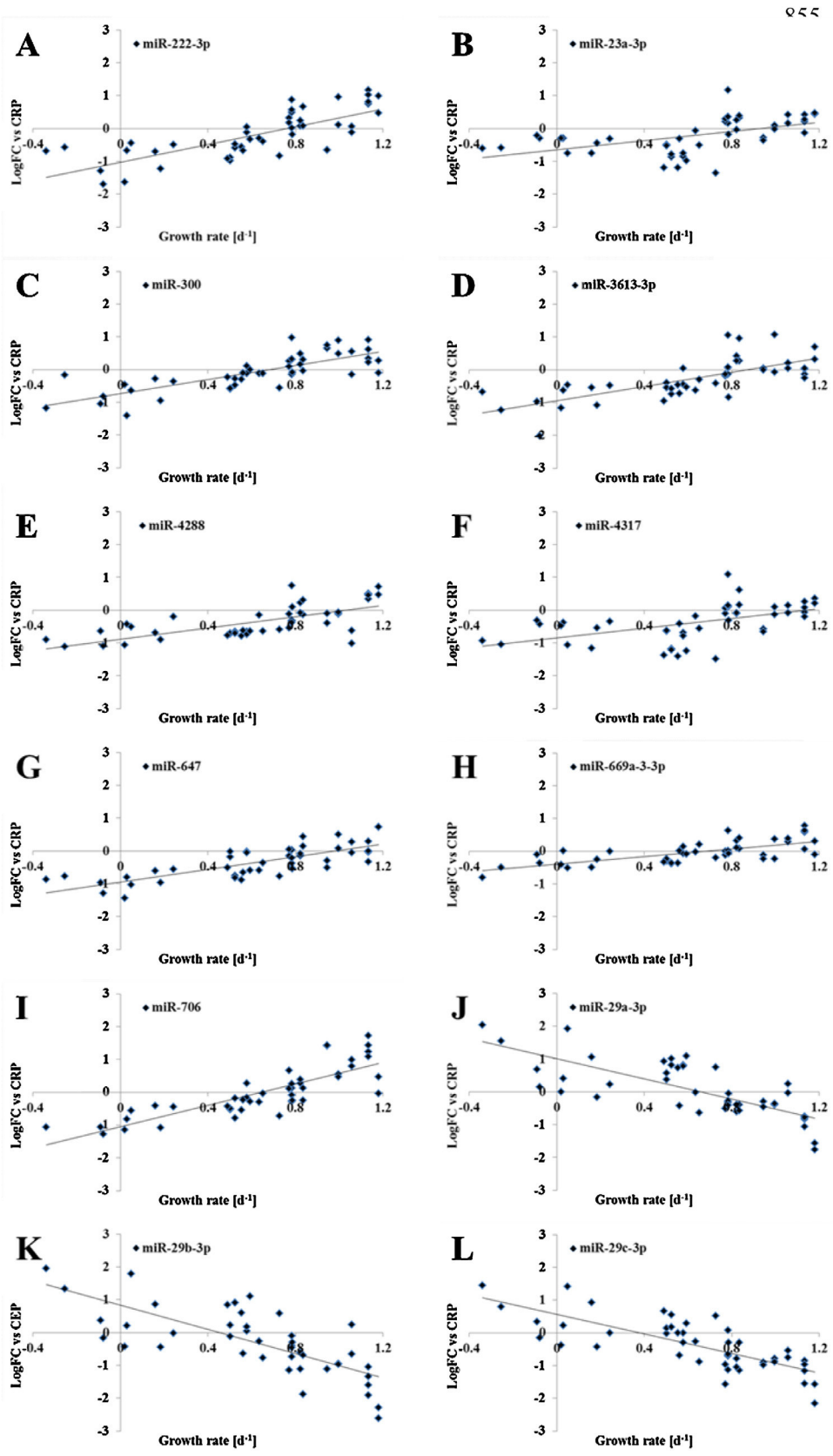

Fig. 3. MiRNA expression versus growth rate. LogFC of each sample was aligned to the growth rate for all constantly growth-correlating miRNAs. The line depicts the linear correlation. (A–I) Positively growth-correlating miRNAs. (J–L) Negatively growth-correlating miRNAs.

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4.3. Identification of growth-correlating miRNAs and their association to miRNA clusters

High throughput screening (Fischer et al., 2015) and miRNA profiling (Druz et al., 2011) (Lin et al., 2011; Bort et al., 2012; Clarke et al., 2012; Hackl et al., 2014), applied to CHO cells eased the identification of miRNAs related to the growth phenotype. To expand the repertoire of known growth-correlating miRNAs in CHO cells, we correlated the relative logarithmic expression values (derived from relating the miRNA expression levels of each sample against the CRP) to the growth rates for either all 46 samples, or sample subsets (exponential, n = 36; serum-free, n = 38; serum-free + exponential, n = 28). Most growth-correlating miRNAs were identified when samples of the stationary and of death phase were included (see Fig. 2), which spanned the highest difference in growth rates (−0.34 to 1.18 d^{-1}, see Fig. 1A). The majority of the here identified miRNAs correlated positively to the growth rate (see Fig. 2A), which is consistent with earlier findings (Hackl et al., 2014; Clarke et al., 2012). An overlap of 12 growth-correlating miRNAs was identified between all analyzed sample sets.

As miRNAs which are in close chromosomal proximity, often show clustered (i.e. polycistronic) expression, we wanted to know whether this phenomenon is also true for the 12 growth-correlating miRNAs identified before. Chromosomal locations were additionally derived from other species, as only limited information about the genomic location of miRNAs is available for CHO. Of these 12 miRNAs, 8 are found in miRNA clusters, based on their interchromosomal distance in the human, mouse and/or rat genomes, with miR-29b-3p deriving from two genomic locations. A subset of those 8 miRNAs (n = 5) is also located in miRNA clusters in CHO (see Supplementary Table 4). Chromosomal instability of CHO cells (Worton et al., 1977; Lewis et al., 2013) and differences in post-transcriptional miRNA maturation regulation of clustered miRNAs (Ryazansky et al., 2011) could lead to non-correlating expression patterns. Still, 4 of those 8 miRNAs present in clusters were found to correlate to all other cluster members, and indicate polycistronical expression in CHO (see Supplementary Fig. 1). The other four miRNAs show either partly, or no correlation to their cluster members.

4.4. Function of positively growth-correlating miRNAs

Among the positively growth-correlating microRNAs, miR-23a and miR-222-3p are the most widely researched miRNAs. MiR-222-3p is in close chromosomal proximity to miR-221-3p, to which it also shows correlated expression levels (see Supplementary Table 4 and Supplementary Fig. 1), and they belong to the same gene family. MiR-221-3p is found to be well correlated with growth rate only in the serum-free samples (see Supplementary Table 3). MiR-222-3p is mostly recognized for the growth promoting ability in tumor cells and normal tissues, as it represses known cell cycle and growth inhibitors (Gillies and Lorimer, 2007), and also impairs apoptosis by targeting pro-apoptotic genes (Terasawa et al., 2009). This 'oncomiR' is considered as biomarker for prognostic and diagnostic purposes, as it is found to be constantly upregulated in various types of cancer (He et al., 2005; Wong et al., 2010). On the other hand, a limited number of studies reported that miR-222-3p also possesses pro-apoptotic and anti-proliferative features (Felli et al., 2005; Xiao et al., 2011). The expression of the microRNA itself is induced by growth factors via the MAPK pathway (Terasawa et al., 2009), NF-kB and JUN (Galardi et al., 2011) and by oncogenes (Tsunoda et al., 2011).

Mir-23a together with mir-23b make up the mir-23 gene family. The 3p-strands of these two miRNAs show a good expression correlation between all samples (see Supplementary Fig. 1), but miR-23b-3p is found correlated to the growth rate in only two data sets (see Supplementary Table 3). Mir-23a is located in close chromosomal proximity to two other miRNAs, mir-24-2 and mir-27a. The expression levels of miR-24-3p and miR-23a-3p correlate well between all samples (see Supplementary Fig. 1), and miR-24-3p is also found to be correlating to the growth rate in one data set. However, no good correlation is found between the expression levels of miR-23a-3p and miR-27a-3p, which has been reported before (Cao et al., 2012). The 'oncomiR' miR-23a has the ability to protect cells from apoptosis by targeting pro-apoptotic genes (Ruan et al., 2012). It is also able to induce epithelial- mesenchymal transition (EMT) (Jahid et al., 2012), promote tumorigenesis (Tan et al., 2012) and alleviate oxidative stress (Zhao et al., 2014) and is discussed as biomarker, as it is found to be upregulated in different types of cancer (Yong et al., 2013; Lee et al., 2011). In spite of its oncogenic characteristics, miR-23a has also been reported to be downregulated in certain types of cancer (Koller et al., 2013; He et al., 2014b), as it can induce cellular senescence (Xishan et al., 2014), lower the migration potential (Arabanian et al., 2014), impair growth (Wang et al., 2014), and exhibit proapoptotic functions (Siegel et al., 2011). The expression of the miRNA itself is directly induced by proto-oncogenic CREB1 (Tan et al., 2012), and indirectly by STAT3 (Wang et al., 2012), both of which are found upregulated in cancer. It is also induced by HSP70 and CDK5 to prevent heat-shock induced apoptosis (Roufayel et al., 2014).

Less is known about the other positively correlating miRNAs. MiR-300, which does not show any correlation to its genomically clustered miRNAs, is a member of the mir-154 gene family and is able to repress EMT in human tumor metastasis (Haga and Phinney, 2012), elevates the ability to repair DNA damages and to relieve IR-induced G2 cell cycle arrest (He et al., 2014a). It is also found to be higher expressed in cancer cells and in less differentiated cells, upregulating their proliferation and downregulating differentiation (He et al., 2014a), and, though no direct mRNA targets are validated, it is predicted to target p53 (Bailey et al., 2010).

Likewise, miR-647, which is the only member of its miRNA cluster found expressed here, has also been predicted to target p53 (Bailey et al., 2010), and Notch signaling (Zhao et al., 2013). In addition, it has been associated with drug-resistance in tumors (Kim et al., 2014) and metastasis (Yang et al., 2013).

MiR-706 was found to inhibit vesicular stomatitis virus-induced apoptosis (Lian et al., 2010). In addition, it has also been shown that miR-706 alters Stat1 levels (Wong et al., 2014).

Nothing is known about the function of miR-3613, miR-4288, miR-4317 and miR-669a-3-3p, but miR-3613 and miR-4317 were associated with cancer (Ji et al., 2014) (Sand et al., 2012) and miR-669a-3-3p was found to correlate to some of its other miRNA cluster members.

4.5. Function of negatively growth-correlating miRNAs

All 3 miRs, which correlate negatively to the growth rate, belong to the mir-29 gene family. Mir-29b is located twice in the genome, once in close proximity to mir-29a, and once next to mir-29c. All 3 share similar expression levels between all samples (see Supplementary Fig. 1). The mir-29 gene family is mostly recognized for its tumor suppressive function by executing anti-proliferative (Fabbri et al., 2007), anti-migrational (Lang et al., 2010) and pro-apoptotic functions (Park et al., 2009) in normal and tumor cells. It is repressed in different types of cancer (Lang et al., 2010; Zhao et al., 2010). The tumor-suppressive effect is exerted by directly targeting anti-apoptotic (Xiong et al., 2010), cell cycle-promoting (Gong et al., 2014), and oncogenic genes (Ugalde et al., 2011). In addition, it is able to upregulate and activate p53, which is capable of inducing apoptosis (Park et al., 2009). The miR-29 family is found upregulated in quiescent cells (Bandyopadhyay et al., 2011), promotes differentiation (Wang et al., 2008) and is capable of inducing

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senescence in aged cells (Ugalde et al., 2011). But it is also found upregulated in certain tumor cells (Hong et al., 2014), and can have a positive effect on the migrational potential (Franceschetti et al., 2013). It controls global gene expression by targeting several genes involved in DNA (de) methylation (Fabbri et al., 2007). The family members are associated with extracellular matrix (ECM) remodeling, as they inhibit ECM gene expression (Maurer et al., 2010), target genes involved in the extracellular matrix breakdown (Ramdas et al., 2013), and negatively regulate EMT (Rostas et al., 2014). They are also involved in nutrient metabolism control, as the expression is upregulated by nutrient limitation (Liang et al., 2013), and downregulated by high glucose levels (Du et al., 2010). The miR-29 family itself is downregulated by oncogenes (Zhang et al., 2012).

4.6. Phenotypes assigned with identified growth-correlating miRNAs in CHO

Interestingly, none of the 12 miRNAs revealed by this study has been identified by the only miRNA profiling study directly related to growth in CHO cells (Clarke et al., 2012), which was focusing on miRNA expression changes due to interclonal growth rate variations. But miR-23a was found to be downregulated in CHO cells due to serum-free and suspension adaption, which is often accompanied with decreased growth (Hackl et al., 2011), and in a tissue plasminogen activator (tPA)-producing cell line when compared to its host (Hammond et al., 2012). As miR-23a, the expression of miR-222 was also reduced in a recombinant cell line producing secreted alkaline phosphatase (Hammond et al., 2012). Its expression level is highest in the exponential growth phase in CHO cells, with a reduced expression in later batch phases (Bort et al., 2012). The unexplored miR-4317 was downregulated in both recombinant cell lines mentioned above. These previous reports confirms our findings, as recombinant protein production often reduces the growth capacity. The miR-4288 was found to be higher expressed in high producer cells, which exhibited the same growth rate as low producer CHO cells due to steady state cultivation (Maccani et al., 2014), indicating an additional function for this miRNA.

The proapoptotic function of miR-29a and miR-29c has been confirmed in CHO cells (Fischer et al., 2015), while the expression of miR-29b (Bort et al., 2012) declined during later batch phases, which is contradictory to our results. The levels of miR-29b and miR-29c are also lower in tPA-producing CHO cells (Hammond et al., 2012), which could lead to higher stress capability.

5. Conclusion

To this stage, studies that reported miRNA expression profiles of CHO cells only included few cell lines and conditions or the comparison of the same cell line under different conditions. We have here reported miRNA profiles from a comprehensive transcriptomic approach, which included a variety of CHO cell types harvested under various growth conditions. Growth-correlating miRNAs for the entire sample set as well as subsets (i.e. exponential, serum-free, and exponential and serum-free CHO cells) revealed a set of 12 miRNAs with robust correlation to growth in all sample sets. This combination of 12 miRNAs includes known oncomiRs, tumor-suppressive miRNAs and seven less studied miRNAs. Eight of the 12 identified miRNAs are considered to be present in genomic miRNA cluster in human, mouse and rat, and five of those eight were also found in miRNA cluster in CHO, with four of them showing correlative expression to all other cluster members, indicating jointed expression. The here identified growth rate correlating miRNAs represent rational CHO cell line engineering targets, and thus will be further tested to evaluate their potential to regulate growth rate. In addition, they are valuable biomarkers for clone selection and bioprocess/media optimization to enhance growth performance.

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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.2016.03.022.

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1.6. Aims of the thesis

The thesis aimed to further explore the possibilities of miRNA engineering in CHO cells by (i) developing an efficient method to overexpress miRNA clusters and by (ii) identifying robustly growth-correlating miRNAs. The following experiments were performed to evaluate the miRNA cluster overexpression method, and to find growth-correlating miRNAs in CHO cells:

(i)

- Amplify CHO-specific miRNA cluster sequences, and insert into a plasmid.
- Construct chimeric clusters in the same plasmid system by concatenating previously used chimeric miRNAs.
- Compare the functionality of the endogenous miRNA clusters to the chimeric miRNA clusters by plasmid-based overexpression and expression analysis of the primary transcript, the control gene and the mature miRNA by qPCR.
- Evaluation of hairpin structures by in silico RNA secondary structure prediction.

(ii)

- miRNA profile generation by RNA sample collection, labelling with fluorescent dyes and hybridization onto cross-species miRNA microarrays.
- Updating and processing of the generated profiles together with already published array data[33] and additional in-house available profiles.
- Clustering of cell samples by miRNA expression profiles and of miRNA with similar expression over all samples to find phenotypes associated with miRNA expression and co-expressed miRNAs.
- Correlation of all samples and subsets to the growth rate, determined at the time point of RNA collection, to identify growth-correlating miRNAs.

1.7. Conclusion

With the annotation and mapping of miRNAs in CHO cells, a new cell line engineering strategy became available. Several methods were developed to investigate and manipulate the expression of miRNAs, and manipulation of miRNA expression was already used to drive CHO cells to certain phenotypes. But only little information was available on growth-enhancing miRNAs. High throughput screening revealed that only few distinct miRNAs are able to enhance the growth of CHO cells[35]. The here presented method allows the use of genomically clustered miRNAs, which often execute biologically linked functions, for cell line engineering. The alteration of a whole miRNA cluster could lead to additional or synergistic effects when compared to distinct miRNA engineering. Also, the growth-correlating miRNAs identified here by miRNA profiling provide potential engineering targets to be tested for favorable effects on growth.

A major obstacle remains the fact that the biological function of certain miRNAs strongly depends on their target interaction and the abundance of both the miRNA and the potential target mRNAs. The latter is highly diverse in various CHO cell lines and their subclones[31,32,47]and will also change during typical processes[30,48] including batch and fed-batch cultures. Until now, identified target mRNAs are derived from cross-species comparisons and computational target predictions. As many genes are already deregulated[4] in CHO cells, and prediction algorithms are constrained by the complex miRNA-mRNA binding mechanisms, there is a great need for a CHO-specific target database, based on experimentally validated target genes.

Before the implementation of the CRISPR technology for mammalian cells, CHO cells completely absent of certain miRNAs or cluster were hard to generate. With this novel technology, it is possible to achieve complete knockouts of miRNAs within a relatively

short time, opening up a new potential research field for miRNA engineering in CHO cells.

In summary, miRNA research is an established field in CHO cells, but still many questions need to be answered to fully understand their specific and individual function and to use the full potential of those small, non-coding RNAs, which gets more accessible by the use of novel technologies.

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2. Appendix

2.1. Additional thesis-relevant publications

A. Hackl M, Jadhav V, **Klanert G**, Karbiener M, Scheideler M, Grillari J, Borth N. **Analysis of microRNA transcription and post-transcriptional processing by Dicer in the context of CHO cell proliferation.** J Biotechnol. 2014 Nov 20;190:76-84. doi: 10.1016/j.jbiotec.2013.12.018. Epub 2014 Jan 28. PubMed PMID: 24486028; PubMed Central PMCID: PMC4247382.

B. Jadhav V, Hackl M, **Klanert G**, Hernandez Bort JA, Kunert R, Grillari J, Borth N. **Stable overexpression of miR-17 enhances recombinant protein production of CHO cells.** J Biotechnol. 2014 Apr 10;175:38-44. doi: 10.1016/j.jbiotec.2014.01.032. Epub 2014 Feb 8. PubMed PMID: 24518263; PubMed Central PMCID: PMC3991393.

C. Diendorfer AB, Hackl M, **Klanert G**, Jadhav V, Reithofer M, Stiefel F, Hesse F, Grillari J, Borth N. **Annotation of additional evolutionary conserved microRNAs in CHO cells from updated genomic data.** Biotechnol Bioeng. 2015 Jul;112(7):1488-93. doi: 10.1002/bit.25539. Epub 2015 Apr 8. PubMed PMID: 25689160. Epub 2014 Feb 8. PubMed PMID: 24518263; PubMed Central PMCID: PMC3991393.

2.2. Additional Appendices

D. Curriculum Vitae

Appendix A

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

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

Matthias Hackl[a], Vaibhav Jadhav[a], Gerald Klanert[b], Michael Karbiener[c], Marcel Scheideler[c], Johannes Grillari[a], Nicole Borth[a,b,*]

[a] Department of Biotechnology, BOKU – University of Natural Resources and Life Sciences, Vienna, Austria
[b] ACIB GmbH, Austrian Centre of Industrial Biotechnology, Graz, Austria
[c] RNA Biology Group, Institute for Genomics and Bioinformatics, Graz University of Technology, 8010 Graz, Austria

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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

* Corresponding author at: Department of Biotechnology, BOKU – University of Natural Resources and Life Sciences, Vienna, Austria. Tel.: +43 1 47654 6232; fax: +43 1 47654 6675.
E-mail address: nicole.borth@boku.ac.at (N. Borth).

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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

in 1 ml TRI reagent. RNA extraction using chloroform and purification were performed as described previously (Hackl et al., 2011). RNA pellets were resuspended in nuclease-free water (Life Technologies) and concentrations and purity were analyzed through absorption at 230, 260, and 280 nm using a NanoDrop spectrophotometer (Thermo Scientific).

2.3. Determination of RNA quality and small RNA concentration

In order to assess total RNA quality and the fraction of small RNAs and microRNAs, total RNA was diluted to a concentration of 100 ng/μl. Total RNA quality was estimated on a Bioanalyzer 2100 instrument using the RNA 6000 Nano Kit. SmallRNA and microRNA concentrations were measured from the same RNA aliquots using the small RNA Series II Kit according to the instructions by the manufacturer (Agilent Technologies, Santa Clara, USA).

2.4. cDNA synthesis and PCR and real-time quantitative PCR

Total RNA in various amounts ranging between 200 ng and 1 μg was used for cDNA synthesis using a M-MuLV RNase H+ reverse transcriptase supplied with the Dynamo Kit (Thermo Scientific). cDNA was diluted in nuclease-free water depending on the initial input of total RNA and directly used for end-point PCR as well as real-time quantitative PCR (qPCR). PCR analysis of human Dicer expression was performed using a Taq polymerase provided with the Phusion high-fidelity polymerase kit (Thermo Scientific) with 35 cycles of denaturation (95 °C, 15 s), annealing (58 °C, 20 s) and extension (72 °C, 20 s).

For quantitation of mRNA expression, specific qPCR primers that overlap exon–exon junctions or are separated by at least one intron, were designed for beta-Actin (Actb), human and Chinese hamster Dicer, Drosha, and Dgcr8 and are provided in Supporting Table S1. Primer specificity was tested by melting curve analysis. Standards for copy number determination were prepared by purification of PCR products and dilution to 10^8–10^3 copies/μl and included in each run. Quantitative PCRs were run in quadruplicates on a Rotorgene Q (Qiagen), using SYBR green fluorescent dye and a hot-start polymerase supplied with the SensiMix mastermix (Bioline) with 40 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 15 s) and elongation (72 °C, 15 s). SYBR Green fluorescence was acquired at 72 °C and 80 °C, and chosen for detection depending on the base of the melting peak.

2.5. Analysis of microRNA transcription

2.5.1. MicroRNA microarray hybridization

Cross-species microRNA microarray experiments were run as described previously (Hernández Bort et al., 2012). In brief, epoxy-coated Nexterion glass slides were spotted using the miRBase version 16.0 locked nucleic acid (LNA) probe set consisting of 2367 probes against human, mouse and rat miRNAs in 8 replicates. For hybridization, 800 ng total RNA extracts from two biological replicates of each cell line from exponential growth phase were hybridized against a common reference pool RNA from all samples. End-labeling of miRNAs was performed using the Exiqon Power Labeling Kit (Exiqon, Denmark) together with synthetic spike-in controls according to the instructions by the manufacturer. Slides were hybridized over night at 56 °C in a Tecan HS 400 hybridization station, followed by automated washing and drying with nitrogen (Tecan, Austria). Immediately after drying, arrays were scanned using the Roche Nimblegen MS200 scanner (Roche, Germany) at 10 μM resolution and auto-gain settings.

2.5.2. MicroRNA microarray data analysis

Feature extraction from high-resolution tiff-images was performed using GenePix software (Molecular Devices, Sunnyvale, CA). Background correction, normalization and statistical analysis were performed as previously described (Hackl et al., 2010), using the LIMMA package under R/Bioconductor (Smyth, 2004). *Normexp* background correction and *Global Loess* normalization were performed and $\log_2$-fold changes of miRNAs for each sample were calculated against the common reference sample and served as relative expression value for each miRNA. Pearson correlation was performed to test for positive or negative correlation of miRNA expression with specific growth rate. Normalized as well as raw microarray data have been submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and can freely be loaded and reanalyzed using the accession number GSE52994.

2.5.3. MicroRNA qPCR analysis

In order to quantify mature miRNA transcript levels as well as precursor miRNA levels, the miScript kit was used (Qiagen, Germany). Reverse transcription (RT) was performed using 300–400 ng of total RNA and "HiFlex" RT Buffer, which allows detection of both microRNA and messengerRNA. Temperature settings were chosen according to the suppliers recommendations (37 °C for 1 h, 95 °C for 5 min). cDNA was diluted 1:4 in nuclease-free water and qPCRs were run in quadruplicates using the miScript SYBR Green Kit (Qiagen, Germany) on the Rotorgene Q instrument (Qiagen, Germany): 95 °C → 15 min, 40 cycles of 94 °C → 15 s, 55 °C → 30 s, 70 °C → 30 s. SYBR Green fluorescence was measured at 70 °C and 80 °C. Commercial primer assays (Qiagen, Germany) were used for mature miRNA quantification. In-house designed primer assays were used for precursor-miRNA quantification (primer sequences are listed in Supporting Table 1).

2.6. Western blot

Protein lysates were prepared by cold lysis of 5×10^6 cells in 1× RIPA buffer for 15 min and centrifugation at 12,000 × *g* and 4 °C for 10 min. Total protein concentration was measured by BCA assay (Pierce), and equal amounts of protein were denatured in 1× LDS buffer with 1× reducing agent (Life Technologies) at 70 °C for 10 min. Samples were separated on 4–15% gradient SDS-PAGE gels (Biorad), blotted onto PVDF membrane, blocked with 3% dry milk in 1× PBS/0.1% Tween 20 (Sigma–Aldrich) and incubated with mouse anti-beta-Actin IgG (1:20,000, Sigma) or rabbit anti-Dicer IgG (1:1000, Sigma–Aldrich) at 4 °C over night. Detection was performed with the IR-Dye system on an Odyssey scanner (Licor) after incubation with anti-mouse (1:10,000) or anti-rabbit (1:5000) secondary antibodies for 60 min at room temperature. Western blot images were analyzed with ImageJ software (Abramoff et al., 2004).

3. Results

3.1. miRNA transcription in protein-free adapted suspension cell lines with low, medium, and high proliferation rates

To investigate the relationship between CHO cell proliferation rate and miRNA transcription in detail, a panel of 5 CHO cell lines that were previously adapted to serum-free growth in suspension were selected and batch cultivations were performed in duplicate in the same chemically defined media without the addition of growth-factors (Fig. 1a). The cell-specific growth rates (μ) that were achieved during exponential growth phase in batch cultivations were found to be lowest (0.43 d^{-1}) in case

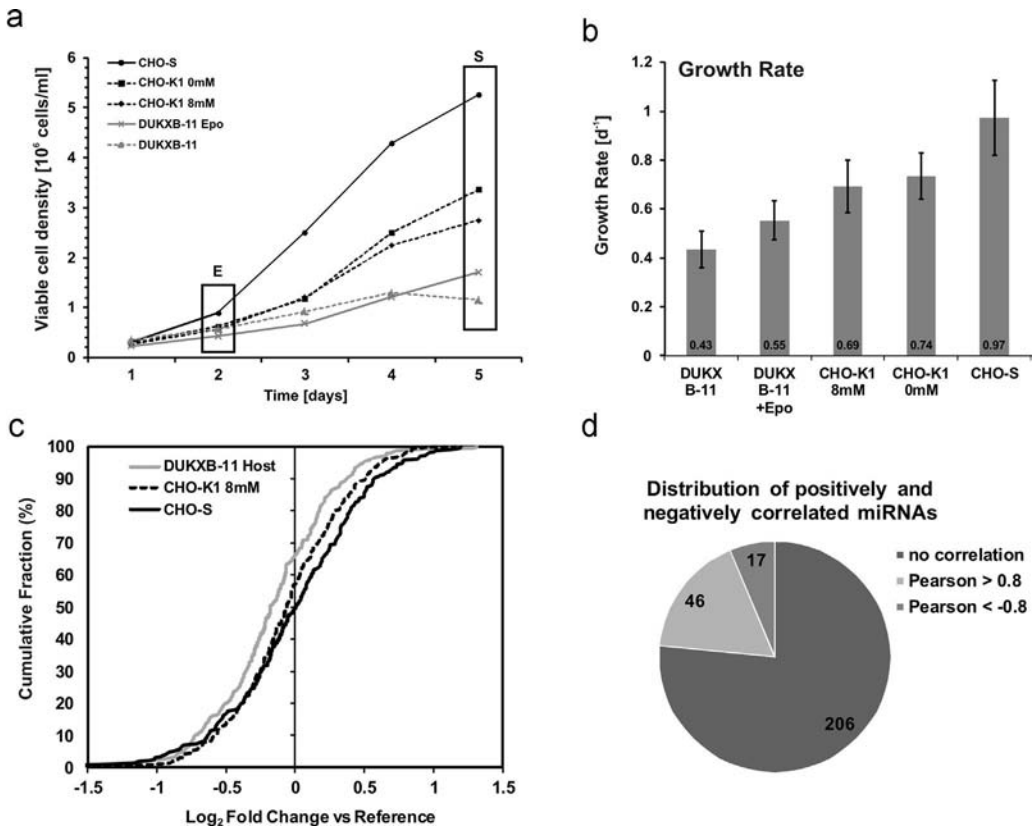

Fig. 1. Global microRNA transcription correlates with growth-rate in protein-free and suspension adapted CHO cell lines. (a) Five CHO cell lines were selected for cultivation in chemically defined media. Two individual batch cultivations were performed and samples were harvested during exponential and stationary growth phase. (b) For each cell line, the specific growth rate was calculated between day 1 and day 3. (c) RNA from exponential growth phase was used for miRNA microarray hybridization. Log_2 transformed fold changes in miRNA expression between each cell line and the common reference pool are shown (average from $n = 2$ per group). Data from DUKXB-11 Epo and CHO-K1 0 mM are not shown. (d) The relationship between miRNA transcript level and specific growth rate was analyzed by Pearson correlation. miRNAs with Pearson correlation coefficients (PCC) greater 0.8 or smaller −0.8 were filtered. Distribution of miRNAs with positive (46), negative (17) or no correlation (206) is given.

of DUKXB-11 host cells and a derived recombinant cell line expressing an Epo-Fc fusion protein (DUKXB-11 Epo, 0.55 d^{-1}). Medium μ was achieved by CHO-K1 cell lines cultivated in the presence (CHO-K1 8 mM, 0.69 d^{-1}) or absence of L-glutamine (CHO-K1 0 mM, 0.74 d^{-1}) as described previously (Bort et al., 2010). The highest specific growth rate was achieved by CHO-S cells (0.97 d^{-1}). Fig. 1b gives an overview of the average growth rates observed in three individual batch cultivations. Total RNA was isolated during exponential growth phase on day 2 and stationary growth phase on day 5. Analysis of mature miRNA levels was performed only during exponential growth phase using a previously established microarray platform (Hackl et al., 2010; Hernández Bort et al., 2012). A total of 270 miRNA probes gave signals that were significantly above the background. For these miRNAs log_2-transformed fold changes (LFC) were calculated against the common reference RNA sample and treated as relative expression values. LFC-values were ranked from low to high and plotted for three cell lines (CHO-DUKXB11, $\mu = 0.43\ d^{-1}$; CHO-K1, $\mu = 0.69\ d^{-1}$; CHO-S, $\mu = 0.97\ d^{-1}$) against the cumulative fraction (Fig. 1c). The results show an increase in miRNA transcription from the slow to fast proliferating CHO cells, which was confirmed by qPCR for selected miRNAs on the level of precursor and mature transcripts (Supporting Fig. 1). Pearson correlation coefficients (PCC) of growth rate and mature miRNA expression were calculated, and miRNAs with stringent PCC values greater 0.8 or below −0.8 were regarded as positively or negatively correlated, respectively. This resulted in a total number of 63 growth-correlating miRNAs, of which 46 (73%) exhibited a positive correlation (Fig. 1d).

3.2. Expression of Dicer, but not Drosha or Dgcr8 correlates well with cell-specific growth rate of CHO cell lines

In order to test whether increased post-transcriptional processing of miRNAs by Dicer could mediate this effect, Dicer expression was analyzed by qPCR during exponential growth phase, as well as stationary growth phase. Indeed, we observed enhanced expression in fast proliferating cells during exponential phase (Fig. 2a). However, on day 5 when proliferation has decreased due to nutrient consumption and accumulation of toxic metabolites, the difference in Dicer expression was attenuated (Fig. 2b), which is in line with the earlier report of predominant miRNA down-regulation during stationary growth phase (Hernández Bort et al., 2012). Dicer up-regulation during exponential growth phase was further evaluated by immunoblot analysis (Supporting Fig. 2a), which confirmed the strong correlation of Dicer expression and specific growth rate of 5 CHO cell lines ($PCC_{mRNA} = 0.97$, $PCC_{protein} = 0.93$, see Fig. 2b). Analogous correlation analyses for Drosha and Dgcr8 expression did not show any significant correlation (Supporting Fig. 2b and c).

These results demonstrated that specific growth rate of CHO cell lines positively correlates with a large fraction of transcribed miRNAs as well as post-transcriptional processing by Dicer. In order to investigate more closely the effect of Dicer expression on CHO cell phenotype, and especially whether the de-regulation of Dicer directly impacts cell proliferation, we conducted loss- and gain-of-function experiments by siRNA-mediated knockdown and ectopic overexpression of Dicer, respectively.

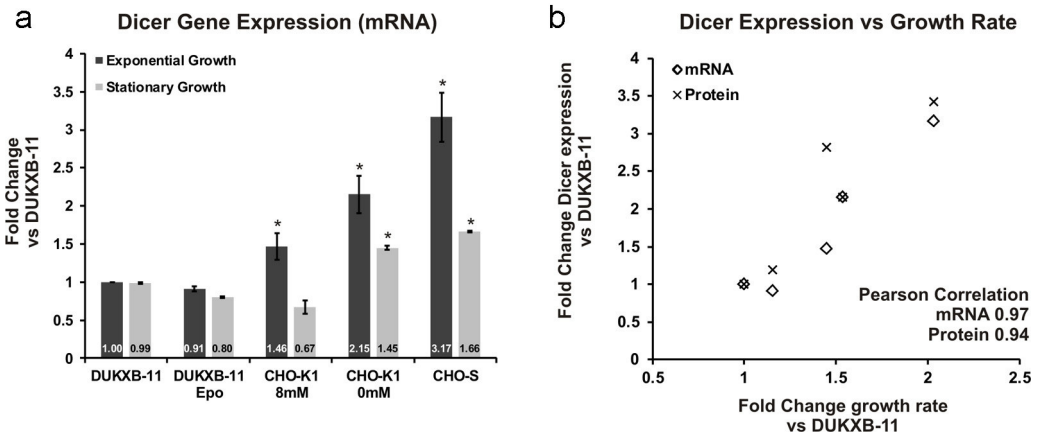

Fig. 2. Dicer expression correlates with growth-rate in protein-free and suspension adapted CHO cell lines. (a) qPCR analysis of Dicer transcript levels at two time-points during batch cultivation is shown (dark, exponential growth phase; bright, stationary growth phase). Fold changes are given relative to Dicer levels in DUKXB-11 host cells ($n = 3$ per group, Actb used as reference gene, error bars represent s.d. of mean). Significance tests were performed using pairwise Student' T-test between DUKXB-11 and each cell line. Significant differences ($p < 0.05$) are marked with an asterisk (*). (b) Scatter plot depicting the relationship between specific growth rate and Dicer expression on mRNA (rectangle) and protein (cross) level. Shown are fold changes in expression relative to DUKXB-11 host cells.

3.3. Knockdown of Dicer and consequently miRNA maturation impairs growth of CHO cell lines

First, we designed two siRNAs directed against two positions in the coding region of Dicer, which were separated by 1850 nucleotides. Two of the characterized cell lines (DUKXB-11 Epo and CHO-K1 8 mM) with medium proliferation rates were transfected using a recently optimized RNA transfection strategy for CHO cell lines (Fischer et al., 2013) and analyzed 72 h later. This time-point was chosen for analysis, since miRNA half-life is known to range between 24 h and 48 h for most miRNAs (Gantier et al., 2011). Knockdown of Dicer to 60% and 50% residual expression on mRNA level was achieved for both cell lines (Fig. 3a), which resulted in a similar reduction of the levels of 6 selected miRNAs (Fig. 3b). In terms of growth behavior, a significant reduction of viable cell densities by 20% could be observed (Fig. 3c and d), without negatively affecting cell viability. These data suggest that down-regulation of miRNA maturation due to reduced post-transcriptional processing by Dicer limits the proliferation rate of CHO cells.

3.4. Ectopic overexpression of Dicer can improve growth of CHO cell lines

In order to test whether an up-regulation of miRNA maturation by overexpression of Dicer can enhance cell proliferation, we transfected recombinant human endoribonuclease Dicer1 (NP_085124.2), which is 94% homologous to Dicer1 of CHO-K1, into DUKXB-11 host cells, as these cells exhibited the slowest proliferation rate of 0.5 d^{-1}. Stable bulk transfected cells were selected for several weeks and screened for human Dicer1 expression by PCR using a primer-pair specific to human Dicer. In order to estimate the overall expression of Dicer in these cells, a primer-pair capable of binding both human and Chinese hamster Dicer was designed (Supp. Tab. S1), and used for qPCR screening: three recombinant cell lines with 1.4-fold (E10), 2.0-fold (F4), and 5.1-fold (B10) increase in Dicer1 expression relative to the host cell line were selected for further characterization (Fig. 4a). Therefore, three independent batch cultivations were inoculated in shake flasks at a viable cell concentration of 1.5×10^5 cells/ml, and grown until viability dropped below 70% at day 9 (Fig. 4b). For E10 and F4, a moderate increase in maximum growth rate (E10, 16.8%; F4, 26.6%) and cumulative cell days (E10, 10.5%; F4, 18.4%) was observed compared to untransfected DUKXB-11 cells (Table 1). This effect also resulted in a 24 h earlier decrease of viability below the 80% threshold (Fig. 4b). Interestingly, the stable pool with highest overexpression of Dicer (B10) showed a decrease in growth performance compared to the host cell line (Tab. 2, Fig. 4b). In order to assess whether Dicer overexpression resulted in an induction of mature miRNA levels, we performed RT-qPCR analysis of 5 miRNAs that were positively (miR-1b, miR-17, miR-30a) or negatively (miR-21, miR-22) correlated to growth rate in our microarray analysis (Fig. 5a). A comparison of miRNA levels between cell lines with significant ectopic overexpression of Dicer (F4, B10) and endogenous up-regulation (CHO-K1, CHO-S) relative to DUKXB-11 host cells is shown in Fig. 5: it was found that (i) ectopic overexpression of Dicer only slightly increases the levels of three selected mature miRNA in CHO cells (Fig. 5b) when compared to the up-regulation observed between fast and slow growing cell lines (Fig. 5a) and (ii) that miRNAs with negative correlation to growth rate (miR-21, miR-22) were also upregulated.

Together, these data suggest that enhanced expression of Dicer in fast growing CHO cell lines is a response to increased microRNA transcription rather than the underlying cause of miRNA up-regulation. Nevertheless, moderate overexpression of Dicer does enhance growth performance by 15–20%, presumably due to up-regulation of growth-enhancing miRNAs. However, strong overexpression of Dicer negatively impacts growth behavior as it does not differentiate between specific growth promoting and growth inhibiting microRNAs. Therefore Dicer may be regarded as a surrogate marker for specific growth rate in CHO cells, but does not constitute a promising target for engineering the growth of CHO cell lines.

4. Discussion

This study addresses the importance of miRNA regulation in the context of CHO cell proliferation. It was found that ~75% of mature miRNA transcripts that correlate with cell-specific growth rate across several distinct CHO cell lines, are up-regulated. A similar observation was made in 2012 when Clarke et al. reported 35 positively and only 16 negatively correlated miRNAs when looking at subclones of a single CHO cell line (Clarke et al., 2012).

We therefore raised the question as to how far miRNA processing by Dicer, Drosha and Dgcr8 is relevant for this effect. We found that Dicer mRNA and protein levels – in contrast to Drosha and Dgcr8 levels – positively correlate to cell-specific growth rate during exponential growth phase. However, upon growth arrest

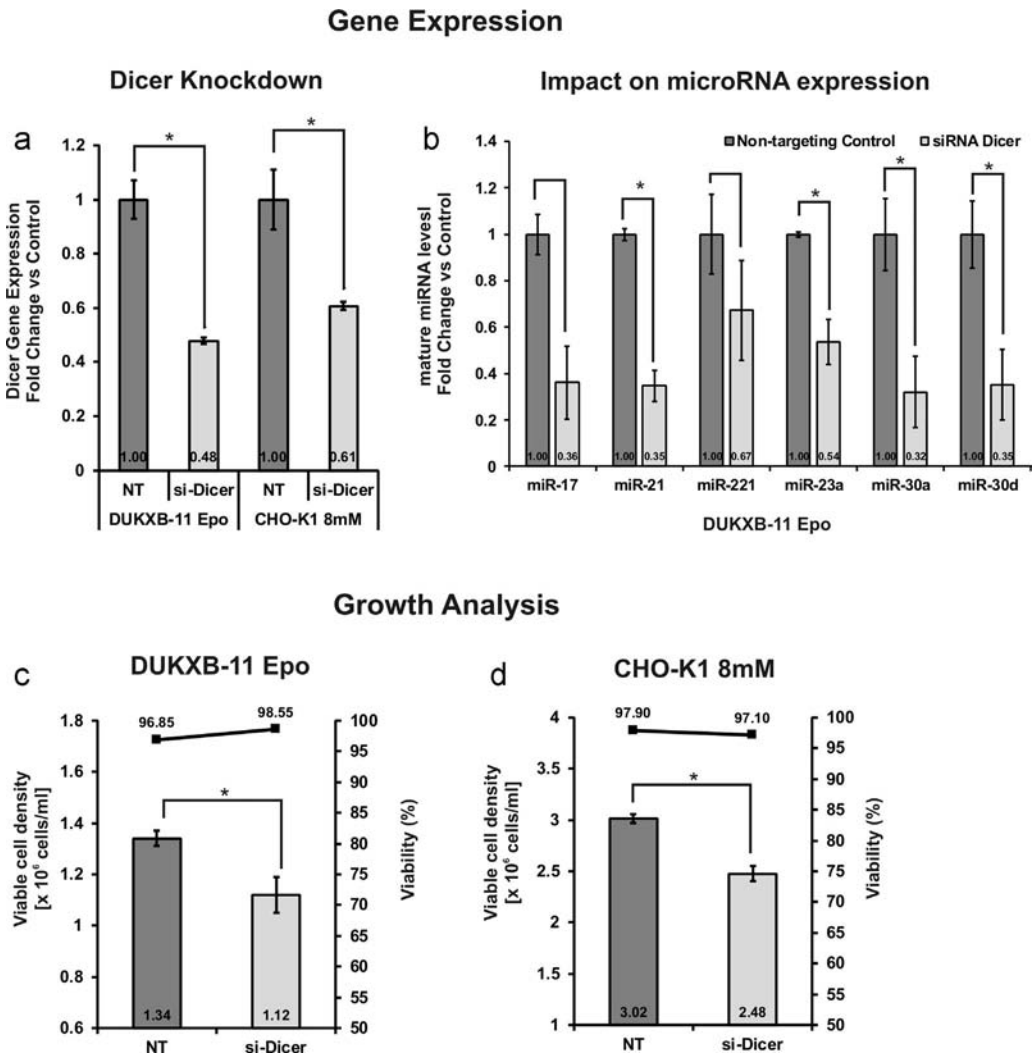

Fig. 3. Targeted transient knockdown of Dicer expression using siRNA. Transfection of 30 nM siRNAs targeting Dicer and non-targeting control (NT) was performed in CHO-K1 8 mM and DUKXB-11 Epo cells. Cells were analyzed 72 h post-transfection: Dicer transcript levels (a) were analyzed by qPCR ($n = 3$, Actb used as reference gene, error bars represent s.d. of mean). The impact of Dicer down-regulation on mature miRNA levels was analyzed in DUKXB-11 Epo cells by qPCR ($n = 3$, Actb used as reference gene, error bars represent s.d. of mean). Effect of Dicer knockdown on viable cell density (VCD) and viability of DUKXB-11 Epo (c) and CHO-K1 8 mM cells (d) is shown after 72 h ($n = 3$, error bars represent s.d. of mean). Significance tests were performed using pairwise Student' *T*-test between non-targeting control (NT) and transfected samples (siRNA). Significant differences ($p < 0.05$) are marked with an asterisk (*).

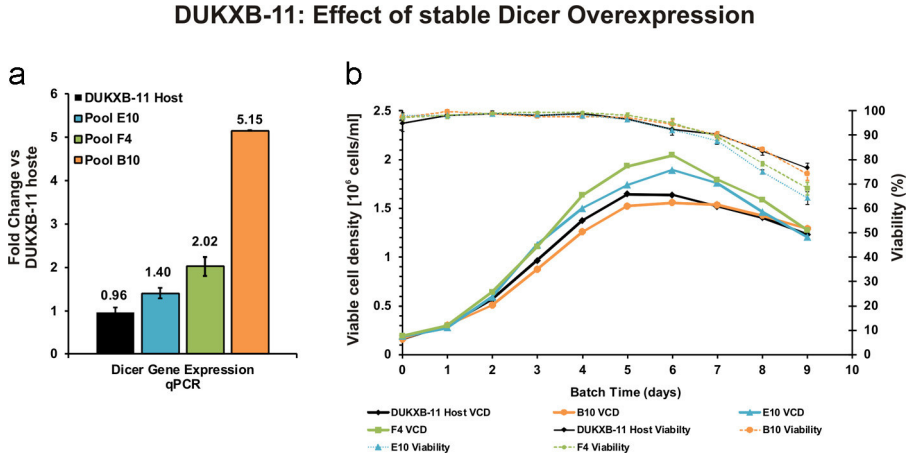

Fig. 4. Engineering Dicer expression in CHO DUKXB-11 cells affects growth behavior. (a) Overexpression of Dicer in three stable bulk cell lines compared to DUKXB-11 host cells was analyzed on day 2 during three individual batch cultivations by qPCR ($n = 3$, Actb used as reference gene, error bars represent s.d. of mean). (b) Three independent batch cultivations of all cell lines were performed. Viable cell densities (VCD) and viability were analyzed every 24 h.

Table 1
Growth characteristics of Dicer1 overexpressing CHO cell lines.

	DUKXB-11 HOST	POOL Dicer+ E10	POOL Dicer+ F4	POOL Dicer+ B10
Average μ_{0-3} [d^{-1}] (%)	0.61 ± 0.01 (100)	0.60 ± 0.06 (98.7)	0.59 ± 0.03 (96.7)	0.58 ± 0.11 (96.1)
Maximum μ_{1-2} [d^{-1}] (%)	0.65 ± 0.05 (100)	0.75 ± 0.05 (116.8)	0.82 ± 0.04 (126.6*)	0.52 ± 0.02 (81.1*)
Average IVC_8 [10^6 cells days] (%)	7.25 ± 0.65 (100)	8.02 ± 0.34 (110.5)	8.59 ± 0.45 (118.4*)	8.30 ± 0.30 (95.2)

* Student' *T*-test: $p < 0.05$.

during stationary growth phase Dicer is overall downregulated and the difference in Dicer levels between fast and slow growing cell lines is insignificant.

Other studies have reported up-regulation of the entire miRNA protein machinery consisting of Argonaute, Dicer and Drosha along tumor progression – and thus faster growth rates – of serous ovarian carcinoma cells (Vaksman et al., 2012). Furthermore, in endothelial cells the removal of serum was shown to increase cellular sensitivity to apoptosis via the down-regulation of Dicer expression (Asada et al., 2008).

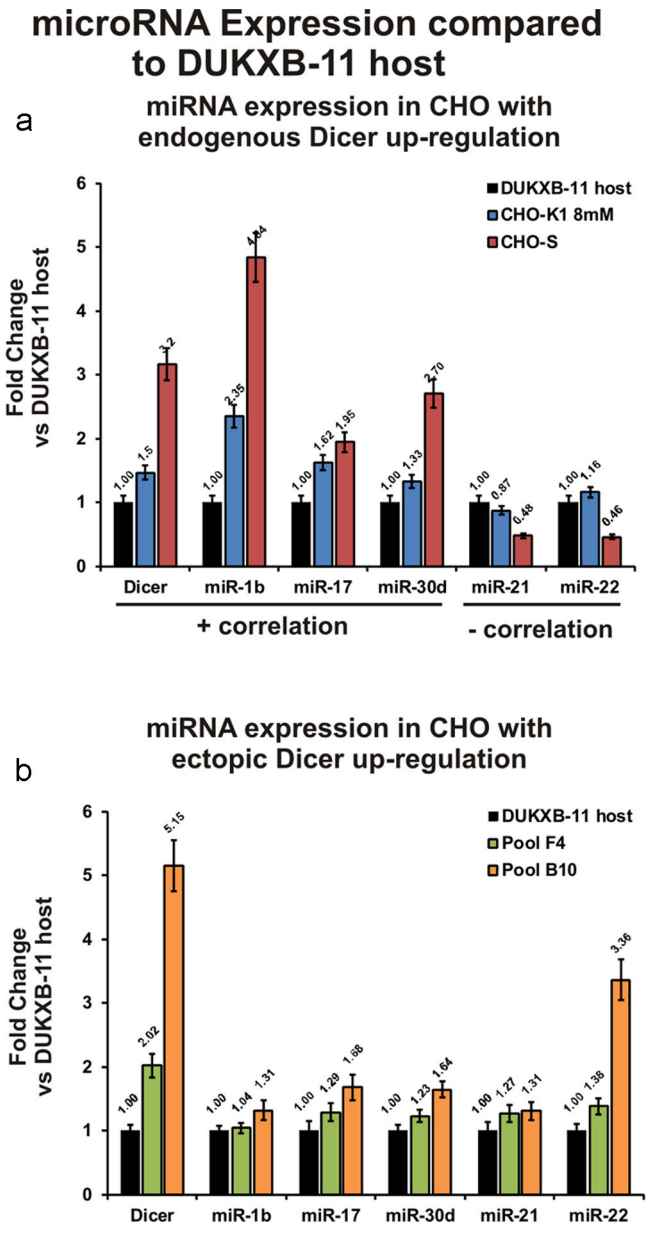

Fig. 5. Comparison of miRNA transcription in cell lines with endogenous and ectopic up-regulation of Dicer. DUKXB-11 host cells were chosen as reference for the comparison of miRNA expression in response to endogenous (a) and ectopic (b) Dicer up-regulation. (a) Microarray fold changes for 5 selected miRNAs (three positively and two negatively correlated to growth rate) and qPCR fold change of Dicer expression in CHO-K1 and CHO-S cells relative to DUKXB-11 cells. (b) qPCR fold changes for Dicer and the same miRNAs in two pool cell lines exhibiting moderate and strong ectopic overexpression of human Dicer ($n = 3$, Actb used as reference gene, error bars represent s.d. of mean).

In order to test whether Dicer expression is causally related to growth rate, transient down-regulation of Dicer expression, and in consequence miRNA maturation was performed and indeed significantly decreased the growth rate of CHO cells. To further confirm this relationship, we investigated whether an increase in miRNA maturation by ectopic overexpression of Dicer could improve growth. Therefore, three independent stable pools with Dicer overexpression levels between 1.5 and 5-fold were generated. In batch cultivations these three cell lines show that moderate overexpression of Dicer indeed enhances cell proliferation slightly (~20%), while more than 5-fold overexpression negatively affected growth performance. In order to investigate the effect of Dicer overexpression, qPCR analysis of selected miRNAs was performed. We observed that ectopic up-regulation of Dicer moderately increased the levels of miRNAs with positive correlation to growth. However, the degree of up-regulation was well below the induction observed for the same miRNAs between fast and slow growing cell lines. In addition, 5-fold induction of Dicer expression also resulted in significant up-regulation of mature miRNAs with negative correlation to growth. This could explain the inhibitory effect of strong Dicer overexpression on growth, and indicates that Dicer is not an ideal engineering target.

Overall it seems that up-regulation of specific miRNAs supports high proliferation rates in CHO cell lines. Simultaneous up-regulation of Dicer seems to be necessary to allow rapid maturation of pre-miRNAs into mature miRNAs, but does itself not mediate growth stimulation. The weaker induction of Drosha and Dgrcr8 could be due to the fact that miRNAs derived from intronic regions can bypass Drosha/Dgcr8 cleavage (Ruby et al., 2007). Therefore, this work establishes Dicer as a potential surrogate marker for growth rate in CHO cells, but not as a promising target for engineering proliferation. For this purpose, it will be worthwhile to test the biological function of those miRNAs exhibiting strong negative or positive correlation to growth rate, such as miR-7 or miR-17, for which respective data already exists (Barron et al., 2011; Jadhav et al., 2012).

Conflict of interest

The authors declare no conflicts of interest.

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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.2013.12.018.

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Appendix B

Stable overexpression of miR-17 enhances recombinant protein production of CHO cells.

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Stable overexpression of miR-17 enhances recombinant protein production of CHO cells

Vaibhav Jadhav[a], Matthias Hackl[a], Gerald Klanert[a,b], Juan A. Hernandez Bort[a,b], Renate Kunert[a], Johannes Grillari[a], Nicole Borth[a,b,*]

[a] Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria
[b] Austrian Centre of Industrial Biotechnology GmbH, Graz, Austria

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ABSTRACT

miRNAs negatively regulate gene expression at post-transcriptional level, and consequently play an important role in the control of many cellular pathways. The use of miRNAs to engineer Chinese hamster ovary (CHO) cells is an emerging strategy to improve recombinant protein production. Here, we describe the effect of transient and stable miRNA overexpression on CHO cell phenotype. Using an established transient miRNA screening protocol, the effects of miR-17, miR-92a and cluster miR17-92a on CHO growth and protein productivity were studied and followed by analysis of cell pools with stable overexpression of these miRNAs. CHO cells stably engineered with miR-17 exhibited both enhanced growth performance and a 2-fold increase in specific productivity, which resulted in a 3-fold overall increase in EpoFc titer. While further studies of miRNA–mRNA interactions will be necessary to understand the molecular basis of this effect, these data provide valuable evidence for miR-17 as a cell engineering target to enhance CHO cell productivity.

1. Introduction

Recombinant production of therapeutic proteins has surpassed the 100 billion $ per year market volume and plays an important role in the global economy as well as in advanced medical care (Aggarwal, 2012; Brower, 2005). Today, a significant fraction of therapeutic proteins is produced in Chinese hamster ovary (CHO) cells due to their regulatory approval, biosafety compliance and ability to produce proteins with human-like glycosylation patterns. CHO cells inherently have high somatic genetic instability, which generates large clonal variation, a property that is commonly harnessed for screening, selection and development of production clones for expression of recombinant proteins (Hacker et al., 2009; Jayapal et al., 2007).

So far, several successful genetic engineering approaches have been used to enhance CHO cell performance in relation to apoptosis or autophagy, cell cycle (growth and proliferation), protein secretion and production, unfolded protein response (UPR) and metabolic engineering (Arden and Betenbaugh, 2006; Baker et al., 2001; Lee et al., 2013; Peng and Fussenegger, 2009; Wang et al., 2012; Wlaschin and Hu, 2007). In addition to engineering the expression of protein-coding genes, miRNAs have recently emerged as a tool to modify the phenotype of CHO cells (Barron et al., 2011b). RNAs are small non-coding RNA molecules, which negatively regulate gene expression at the post-transcriptional level. Similar to protein coding genes, transcription of most microRNAs is controlled by RNA polymerase II promoters. The longer primary transcripts undergo cleavage in the nucleus to form a hairpin like structure termed precursor microRNA (pre-miRNA) of 50–70 nucleotides length. Further, the pre-miRNA is exported from the nucleus to the cytoplasm where it is incorporated into a multimeric protein complex consisting of Dicer, Ago1–4 and others, and cleaved again to give rise to the miRNA induced silencing complex (miRISC) encompassing a 17–22 nt long mature miRNA. Subsequent sequence specific targeting of the miRISC toward the 3′-UTR of a target mRNA results either in the inhibition of mRNA translation or mRNA cleavage (Graves and Zeng, 2012; Havens et al., 2012). In recent years miRNA research has enhanced our understanding of gene expression control and lead to a better view of cellular physiology in both the normal as well as in a pathological

* Corresponding author at: Muthgasse 18, 1190 Vienna, Austria.
Tel.: +43 1 47654 6232; fax: +43 1 47654 6675.
E-mail address: nicole.borth@boku.ac.at (N. Borth).

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state of a cell (Cui et al., 2006; Grillari et al., 2010; Ha, 2011). Evidence is accumulating for the great importance of small non-coding RNAs like piRNAs (Gerstl et al., 2013) and miRNAs in CHO cells for many fundamental biological processes including cell division and metabolism (Hatziapostolou et al., 2013), thus drawing the interest of cell engineers to this class of RNA molecules (Barron et al., 2011b; Hackl et al., 2012a; Jadhav et al., 2013; Muller et al., 2008).

To date, around 350 CHO miRNAs were sequenced (Hackl et al., 2011) and annotated (Hackl et al., 2012b). In view of the biotechnological use of miRNAs, the correlation of miRNA transcription to distinctive cellular phenotypes such as fast proliferation (Bort et al., 2012; Clarke et al., 2012) or stress response (Druz et al., 2012) was shown. In a recent study, the general importance of miRNA expression and the miRNA processing machinery in CHO cells was described (Hackl et al., 2014). These studies provide the basis for the development of CHO cell lines with improved phenotypes through engineered miRNA expression.

We have previously established a method for screening the effect of miRNA overexpression on growth and productivity of a recombinant EpoFc producing (EpoFc-CHO) model cell line (Jadhav et al., 2012) and observed a positive effect of transient miR-17 overexpression on CHO cell growth without negatively affecting cell specific productivity. A question remaining open with transient overexpression was whether such short term effects of increased miRNA expression remain valid in a cell line that continuously and stably overexpresses a miRNA. Here we therefore tested how the transient effects of the entire miR-17–92a cluster, miR-17, or miR-92a alone are consistent in stable overexpressing cell lines and how these growth enhancing miRNAs differ in their effects when overexpressed in recombinant EpoFc-CHO cell pools. Stable miR-17 overexpression confirmed a minor increase in growth rate, while at the same time resulted in 3-fold increase in EpoFc titers compared to controls. Overexpression of the entire miR-17–92a cluster resulted in no change in growth rate, but a reduced productivity, while miR-92a overexpression reduced growth and increased productivity. These results confirm that while transient over-expression is well able to identify engimiRs for stable miRNA engineering of CHO cells, it is still necessary to generate stable overexpressing cell lines for a detailed analysis of their effects. Our results also show that miR-17 is one of the so far few miRNAs demonstrated to boost productivity.

2. Materials and methods

2.1. Cell line and media

The recombinant CHO-EpoFc cell line was established as previously described (Lattenmayer et al., 2007) and was later adapted to growth in serum-free and L-glutamine free CD CHO medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 0.096 μM MTX and 1 ml anti clumping agent (Gibco, Invitrogen, Carlsbad, CA, USA) per 500 ml medium (Taschwer et al., 2012). Cell cultures were cultivated in conical flasks with a working volume of 30 ml at 37 °C in a humidified atmosphere containing 7% carbon dioxide and constant shaking at 140 rpm.

2.2. Cloning miRNA expression plasmids

miRNA expression plasmids for miR-17 and miR-92a were developed as previously explained (Jadhav et al., 2012) by using sequences from miRbase: miR17 = MI0020419, miR-92a = MI0020560 (Kozomara and Griffiths-Jones, 2011). Briefly, chimeric CHO pre-miRNAs were cloned into pcDNA™6.2-GW/±EmGFP-miR vector (Invitrogen Inc., USA) in the 3′ untranslated (3′ UTR) region of the emerald green fluorescent protein (EmGFP) following the instruction manual. The miR17-92a cluster was cloned by PCR amplifying the Human genomic region (chr13: 92002682 + 92003780) containing the hsa-miR-17–92a cluster using primers FP: GGATCCCTAAATGGACCTCATATCTTTGAG and RP: GAATTCGAAAACAAGACAAGATGTATTTACAC and cloned into BamHI and EcoRI sites of pcDNA™6.2-GW/±EmGFP-miR vector. Sequences of all clones were confirmed using standard sequencing method.

2.3. Transient screening and generation of stable pools

The transient screening for candidate miRNA expression plasmids was carried out by standard protocol as previously described (Jadhav et al., 2012). Briefly, 1×10^7 cells in exponential growth phase were transfected with 10 μg of pcDNA6.2-EmGFP-miR plasmid or pcDNA6.2-EmGFP-negative endo-free plasmid using the Nucleofector II (Lonza, Basel, Switzerland) and Nucleofector Kit V/program H-14. Post-transfection cells were seeded into 60 ml of pre-warmed culture medium in a conical flask. Cells were allowed to recover from electroporation in a static incubator at 37 °C for 2–3 h and then transferred to a humidified CO_2 incubator with constant shaking at 140 rpm. Data for growth and productivity were recorded for the next four days.

For generation of pools with stable miRNA overexpression, cells were transferred 24 h post-transfection to 96-well culture plates containing 10,000 cells/well in selection media with 10 μg/ml Blasticidin-S (InvivoGen, California, USA). Cells were maintained by adding 50% fresh medium every 3–4 days for 4 weeks. Subsequently, surviving clones were expanded to 12-well plates for another 4 weeks with selection pressure and final pools were picked based on the GFP expression profiles. Most of the selected pools represented heterogeneous populations according to GFP expression. Thus, secondary selection was done without antibiotics selection pressure, but by sorting cells for homogenous GFP positive populations, followed by the generation of master cell banks that were used for further characterization and analysis.

2.4. Cell sorting and flow cytometry analysis

Cell sorting was done for viable and GFP positive cells in bulk using a FACS Vantage™ (Becton Dickinson, Franklin Lakes, NJ, USA), equipped with a Sort enhancement module. A combination of a SSC-H/FSC-H gates and GFP positive sorting gates was set using non-transfected cells. GFP positive cells were detected with a 488 nm argon ion laser and fluorescence was measured using a BP filter at 530/30 nm. Sorted cells (100,000) were collected in a 6-well plate with 3 ml pre-warmed culture medium supplemented with penicillin–streptomycin 1× concentration. After sorting, an aliquot of the sorted cells was run on the BD FACSCANTO to check the purity of the populations twice weekly for all pools for two weeks. Another round of sorting was done based on GFP expression once weekly for two weeks. Final pools deposited were homogenous and stable for GFP expression under normal culture conditions. All GFP measurements were performed using a BD FACS CANTO (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer. A minimum of 10,000 events were analyzed at excitation wavelength of 488 nm using a 530/30 BP filter for collection of the emitted signal. Untransfected cells were used as negative control for gating of GFP-positive cells. Data were analyzed using the BD FACS DIVA software.

2.5. RNA isolation and quantitative real-time PCR

$1–5 \times 10^6$ cells were harvested and homogenized by vortexing in 1 ml Trizol reagent (Invitrogen, Carlsbad CA, USA) followed by 5-min incubation at room temperature and stored at −80 °C until used. Total RNA was isolated using the chloroform

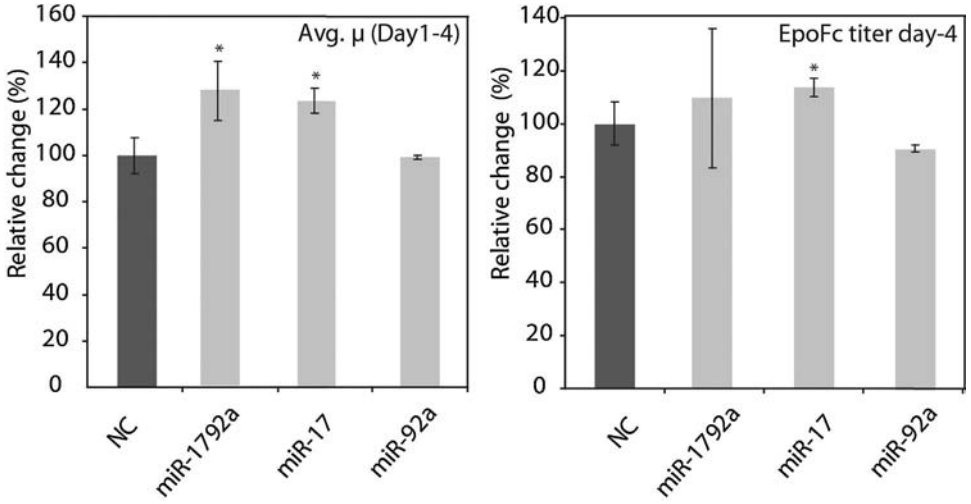

Fig. 1. Effect of transient overexpression of miRNAs on growth rate and recombinant protein titers: (A) effect of miRNA overexpression on the growth rate is represented by the average growth rate (μ) calculated until day 4 of transient miRNA over-expression relative to the negative control transfection (NC). (B) Similarly, the effect on recombinant protein production is represented by the EpoFc titer on day 4 post-transfection. The data are presented as mean (±standard deviation; SD) of three independent experiments with two technical replicates each. $^{*}P < 0.05$ to NC.

protocol provided by the manufacturer. In the final step RNA pellets were resuspended in 30 μl of nuclease free H_2O. Absorbance at 230, 260 and 280 nm was measured using the ND-1000 spectrophotometer (NanoDrop Technologies, DE, USA) to calculate RNA quality and quantities. miRNA expression was measured using a TaqMan miRNA quantitative PCR assay from Applied Biosystems (Foster City, CA) that has been previously described (Cheng et al., 2005). Briefly, cDNA was made with 10 ng total RNA in 10 μl reactions using MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and specific TaqMan® primers for each miRNA (TaqMan® miRNA Assay ID's, MIR92a: ID-00431, MIR17: ID-002308, MIR185: ID-002271). The cycle parameters for the RT reaction are 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and hold at 4 °C. The PCR mix consists of the RT product, TaqMan® 2× Universal PCR Master Mix and the appropriate 5× MicroRNA Assay Mix. The PCRs were run on the Corbett Rotorgene rotorcycler (Qiagen, Germany) including 4 technical replicates per sample. The expression of each miRNA relative to miR-185 (endogenous control) was determined using the $\Delta\Delta C_t$ method. Average fold differences were calculated by normalizing the relative expression (ΔC_t values) to that in the negative control transfection. Average fold differences between batches are presented.

2.6. Analysis of growth and productivity

After selection of stable miRNA overexpressing pools their performance in batch culture was tested in small scale conical flasks. These batches were run in duplicate and repeated twice. Exponentially growing CHO-EpoFc cells were seeded in 60 ml of pre-warmed culture medium at a density of 2×10^5 cells/ml. The daily measurements of cell viability and viable cell density were performed using a ViCell analyzer (Beckman Coulter, USA) based on the trypan-blue dye exclusion method. Total cell counts were analyzed using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA) after isolation of nuclei in 0.1 M citric acid (2% Triton X-100) for at least 30 min. Growth data were further analyzed to calculate specific growth rates using the formula $\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1)$ where X_1 and X_2 is viable cell number at the respective time points (t_1 and t_2). The EpoFc concentration was quantified by ELISA as described previously (Jadhav et al., 2012) using an ELISA reader (Sunrise, TECAN) at 492 nm and 620 nm as reference wavelength and the software Magellan according to the instruction manual. Specific productivity was calculated as $qP = (C_{p,n} - C_{p,1})/\mathrm{CD}$ where C_p is recombinant protein concentration at time n and CD is cumulative cell density, which is calculated as $\mathrm{CD} = \sum(X_{i-1} - X_i)/\mu$, where X_i is integral viable cell density and μ is growth rate.

2.7. EpoFc gene copy number determination using real-time Q-PCR

Gene copy number determinations were made by quantitative PCR (Q-PCR). EpoFc PCR was performed with specific primers and as reference Bcl-2 was used, to develop a standard curve with data points ranging from 10^3 to 10^8 copies. Primers used for PCR were Bcl-2 FP: TTCAGCTCAAACTGGGCTTT, Bcl-2 RP: AACTTGAGCGGCTCCCTAAT, EpoFc FP: CATGGGGGTGCACGAATGTC, EpoFc RP: CAAGCTGCAGTGTTCAGCAC. The Q-PCR runs were done using the Corbett Rotorgene rotorcycler (Qiagen, Germany) system, amplification reactions (20 μl) were performed in 4 technical replicates per sample with 20 ng of input genomic DNA, 1 μl of each primer and 2 μl of 5× SensiMixPlus SYBR master mix. PCR parameters were as follows: an initial 10 min-denaturation step at 95 °C followed by 40 cycles of 15 s at 95 °C, 10 s at 65 °C and 15 s at 72 °C. Specificity of the primers was verified by a melting curve analysis of the PCR products with a temperature gradient of 0.2 °C/s from 68 °C to 98 °C. Copy number variation of EpoFc was determined as a ratio of the estimated copies of EpoFc gene to that of the reference gene, Bcl2.

3. Results

3.1. Effects of miRNA overexpression during transient transfection

Previously, we proposed a screening platform for plasmid based, transient miRNA overexpression to test for biological effects that might change bio-industrially important cellular characteristics. Thereby we identified miR-17 as a promising engineering target as its transient overexpression increased cell proliferation (Jadhav et al., 2012). We extended our experiment by testing whether the whole miR-17–92 cluster, or the single miR miR-92 might have similar effects (Fig. 1). While miR-17 led to a 23 (±5)% and miR-17–92 cluster to a 27 (±12)% increase of mean growth rate (μ) over the period of 4 days, miR-92a did not show significant differences

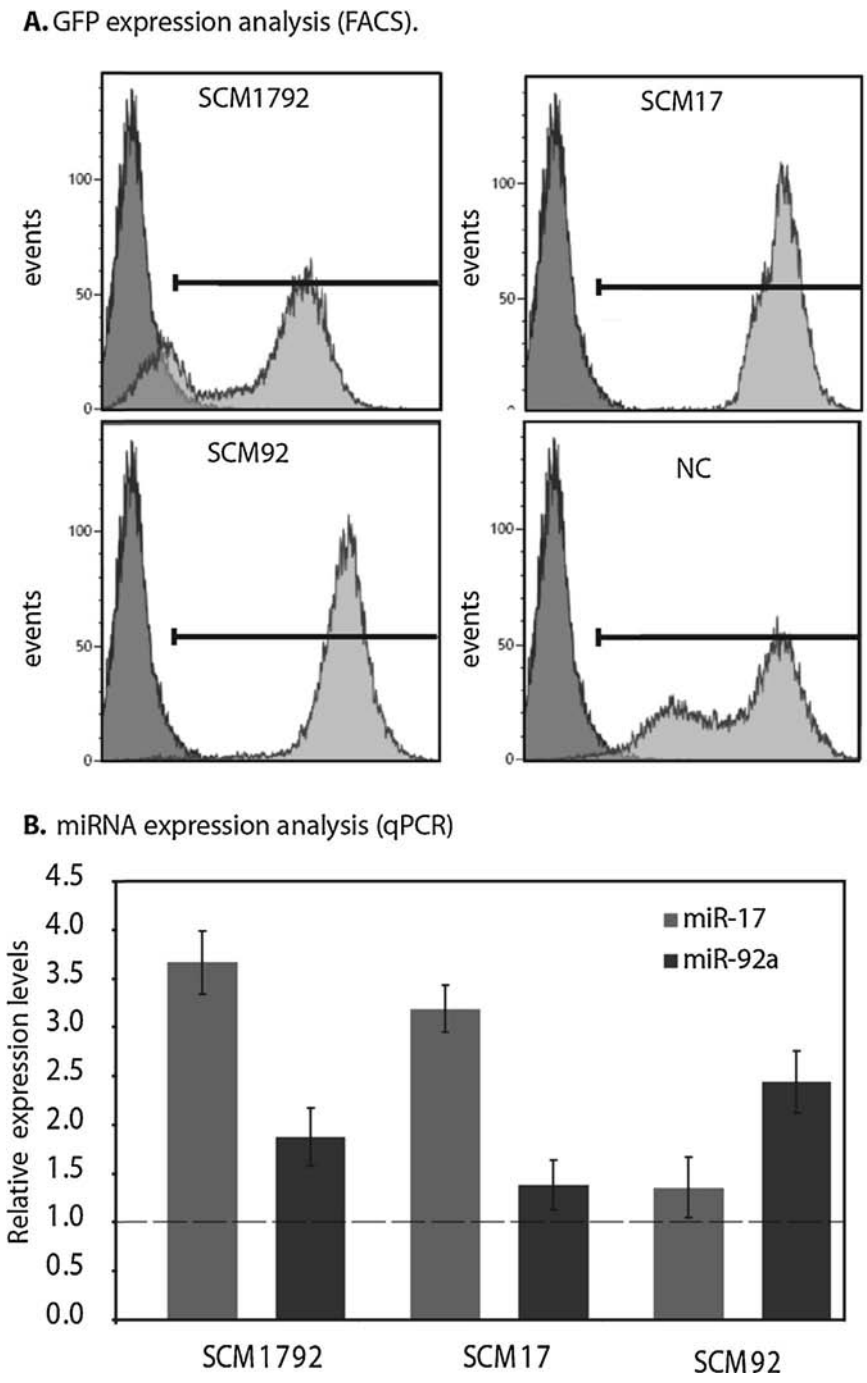

Fig. 2. Selection and characterization of stable miRNA expression clones: miRNA overexpression was driven by a constitutive CMV promoter with co-expression of GFP. (A) The panel shows GFP-expression (Fl-1H) of 4 stable-pools overexpressing the miR-17–92 cluster (SCM-1792), miR-17 (SCM-17), miR-92a (SCM-92a) and the no-miR-vector control (NC) after the entire selection process. All graphs are overlaid with fluorescence of untransfected cells. (B) qPCR was performed to assess the miRNA overexpression. The expression of miR-17 and miR-92a relative to miR-185 expression (endogenous control) was determined using the $\Delta\Delta C_t$ method. Average fold differences were calculated by normalizing the relative expression (ΔC_t values) to that of the negative control transfection.

in μ when compared to the negative control (NC). Furthermore, miR-17 showed a moderate ($13 \pm 4.7\%$), but significant difference in the EpoFc titer. These results encouraged us to test the influence of these miRNAs in stable overexpression systems.

3.2. Generation and characterization of stable miRNA overexpressing pools

The advantage of vector-based systems for functional miRNA screening is that the same vectors can immediately be used to generate stable miRNA overexpressing CHO cells. Using the mammalian selection marker gene present on the vector, stable miR-17, miR-92a and miR-17–92a cluster expressing pools were generated. Such pools were preferred to clonal populations in order to reduce the bias by the inherent clonal phenotypic variation observed in CHO cells (Pilbrough et al., 2009). Generation of stable pools was carried out in two-steps: first we performed antibiotic-selection with Blasticidin-S (10 μg/ml), followed by sorting for GFP positive cells using FACS to enrich the miRNA expressing population. GFP expressions of the final selected populations are shown in Fig. 2A.

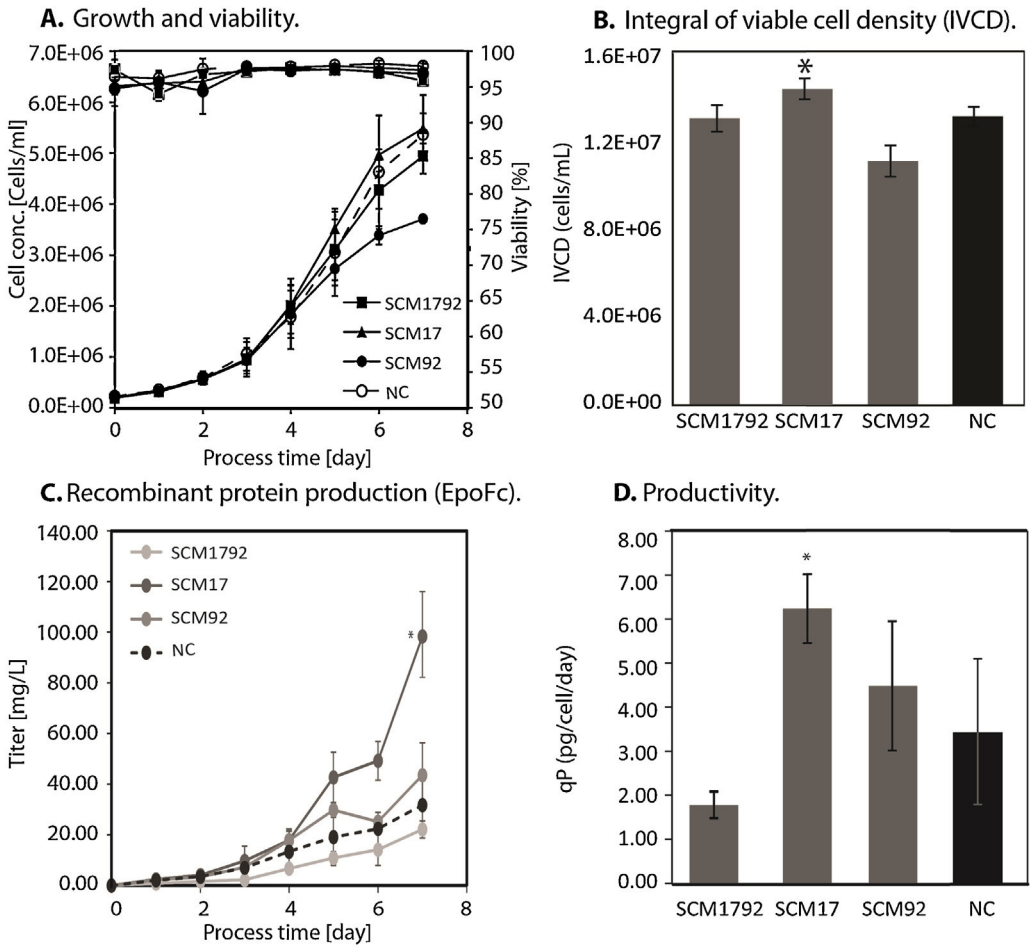

Fig. 3. Analysis of culture performance of stable miRNA overexpressing EpoFc producers in shaken batch cultures: (A) mean viable cell density (primary *y*-axis) and viability (secondary *y*-axis) of three independent batches of SCM-1792(■), SCM-17(▲), SCM-92 (●), and NC (○). (B) Integral of viable cell density (IVCD) reached until day 7. (C) EpoFc (mg/L) concentrations during the batch, quantified by ELISA. (D) Average specific productivity (*qP*) calculated from day 1 to day 7 of the batch. The data are represented as mean (±standard deviation; SD) of three independent experiments with two technical replicates each. **P* < 0.05 to NC.

SCM-17 (SCM = stable cells expressing miRNA), SCM-92a and negative control cells (NC) exhibited >90% GFP positive cells, while for the miR-17–92a cluster (SCM-1792) we could only achieve 60% GFP enrichment even after two rounds of GFP cell sorting. Since GFP negative populations reappeared soon after each round of sorting, we speculate that GFP negative cells are a consequence of destabilized GFP translation due to the simultaneous cleavage of multiple miRNAs from the 3′ UTR of GFP. To assess the miRNA overexpression in these populations, total RNA was isolated from 10^6 cells and miR-17, miR-92a and miR-185 (as reference gene) were analyzed using TaqMan real time qPCR assays. The fold changes relative to miR-185 were determined using the $\Delta\Delta C_t$ method and compared to the NC population (Fig. 2B). For SCM-1792 cells the miR-17 and miR-92a levels were found to be 3.5- and 2.0-fold increase. In case of SCM-17 cells, miR-17 was 3.2-fold overexpressed, and as expected no significant overexpression was observed for miR-92a. Vice-versa, SCM-92a exhibited only miR-92a upregulation. Thus, the GFP and miRNA expression provided proof for successful stable overexpression of specific miRNAs in these populations.

3.3. The effects of stable miRNA overexpression on cell proliferation and recombinant protein production

Stably engineered cells were cultivated in batch cultures in shaker flasks to evaluate growth, viability and recombinant EpoFc production (Fig. 3). We observed that both SCM-1792 and SCM-17 showed a minor increase in growth rate during early exponential phase when compared to the NC population as indicated by specific growth rate analysis (data not shown). While SCM-17 cells sustained this faster growth until the end of the batch and therefore showed a significant increase in cumulative cell density of 15% (Fig. 3B), SCM-1792 cells entered stationary phase earlier than the control and therefore only reached a comparable cumulative cell density relative to NC. SCM-92a cells, on the other hand, had both a reduced growth and cumulative cell density.

The EpoFc protein titers in mg/L were observed to be significantly higher for SCM-17 cells (98.3 ± 25.3), compared to SCM-1792 (22.2 ± 3.4), SCM-92 (43.5 ± 12.8) and NC (31.8 ± 11.2) (Fig. 3C). While SCM-1792 cells exhibited even weaker titers and specific productivity compared to the negative control, SCM-92a cells produced equal amounts of EpoFc. Surprisingly, miR-17 overexpression in SCM-17 cells resulted in a 3-fold increase in titer compared to NC cells and a 2-fold increase in specific productivity (*qP*, Fig. 3D). Therefore, it seems that miR-17 overexpression not only increases cell proliferation but also modulates recombinant protein production in these cells.

To confirm that this increase in protein production is due to miR-17 overexpression and not due to other confounding factors such as selection of a variant population, we checked EpoFc gene copy numbers by real-time qPCR (Fig. 4). The results show no significant

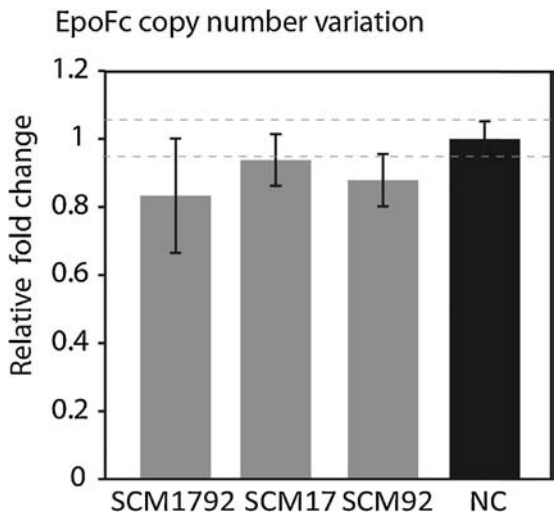

Fig. 4. EpoFc gene copy number analysis: EpoFc gene copy numbers in the miRNA overexpressing pools were analyzed using real-time Q-PCR. Shown is the relative copy number of EpoFc relative to the reference gene, normalized to the negative control. The data are represented as mean (±standard deviation; SD) of four replicates.

changes in EpoFc gene copy numbers across all four pools, thus confirming the positive effect of miR-17 overexpression on growth and especially recombinant protein production in CHO cells.

4. Discussion

In the past decade miRNA research has gained much attention in biology and found broad applications including diagnostics, therapeutics and cell engineering (Barron et al., 2011b; Bratkovic et al., 2012; Hackl et al., 2012a; Jadhav et al., 2013; Osman, 2012). Here we present results from both transient and stable overexpression of mi-17, miR-92a and the entire miR-17–92 cluster on cell proliferation and recombinant protein production in CHO cells. While transient screening is a fast approach to screen for promising candidates, one could argue that the effect of miRNA overexpression for a period of 3–5 days may be different from the effect generated by a continuous, long term increase in their transcription. Thus, the transient screening approach requires stable engineering of cells for validation of results and to ensure beneficial effects for industrial production of recombinant proteins. Our results show that the transient positive effects on growth could also be observed for the stably engineered CHO cell pools during early exponential culture, which is indeed the state that is best reflected by the transient protocol. During later culture phases, however, the effects became more divergent: while both miR92a and the cluster significantly reduced growth, resulting in a decreased (miR-92a) or comparable (miR-17–92a) IVCD relative to the control, miR-17 overexpressing cells continued to grow, resulting in an increased overall IVCD. A possible explanation for these diverging results is the fact that mRNA levels in cells during a batch culture are subject to continuous changes, as is the culture environment (Bort et al., 2012). Thus, constitutive miRNA overexpression could result in the repression of new targets as cell behavior changes during batch-cultivation and resulting in a "new" effect different to that observed during the short transient testing phase. Nevertheless, initial transient testing of miRNA effects enables time-efficient pre-selection of promising candidates, specifically if growth rate is a major target for engineering as the set-up of the transient screening best mirrors early exponential growth phase. In case other culture phases are of interest (for instance increasing productivity during stationary phase) the protocol for transient screening would have to be adapted accordingly.

Unexpectedly, stable overexpression of miR-17 not only enhanced growth, but also specific protein productivity (2-fold), resulting in a 3-fold increase in titer. This is quite remarkable in the context of cell engineering, since commonly growth and specific productivity are inversely correlated to a certain extent. This has been shown by both physical (temperature) and genetic (coding and non-coding) manipulations of CHO cells, which either reduce or arrest growth, facilitating enhanced specific productivity (Barron et al., 2011a; Fogolin et al., 2004; Kaufmann et al., 2001; Sunley and Butler, 2010; Yoon et al., 2003). Stable overexpression of miR-17, however, slightly increases growth but at the same time significantly enhances productivity. This parallel induction of both cell-specific growth rate and productivity is unique and requires a more detailed investigation of the precise interactions and effects caused by miR-17. So far, literature indicates that the six mature miRNAs derived from the cluster play a role in several hematopoietic malignancies, solid tumors and lung carcinoma (Olive et al., 2010) and in B lymphomagenesis (He et al., 2005) and that these miRNAs act as important components of the pathways that regulate many genes involved in G1/S-phase cell cycle. This would explain their significant roles during tumor development and tumor maintenance (Yang et al., 2013). It has been shown that miR-17–92 can target several other cellular pathways (e.g., Wnt, Jak/Stat signaling and TGF-β pathway) apart from cell cycle and apoptosis (Doebele et al., 2010; Mestdagh et al., 2010; Uziel et al., 2009). Further, transcriptomic analysis of CHO clones exhibiting different growth rates (Clarke et al., 2012) indicates a potential role of this cluster in growth and recombinant protein production. Importantly, miR-17 was shown to target TBC1D2/Armus, which plays an important role in membrane trafficking (Serva et al., 2012) and could therefore enhance protein secretion.

With respect to investigating miRNA function in production relevant CHO cell lines, one needs to be aware that it is highly dependent on cell type and the cell-specific transcriptome, respectively. It is therefore not clear to what extent results obtained with human or mouse (tumor) cell lines may be relevant for CHO. For a more reliable prediction of miRNA function in CHO cells, miRNA:mRNA target databases specifically designed for CHO are urgently required. This includes thorough annotation of mRNA untranslated regions (UTR) in the CHO genome database (Hammond et al., 2012). For specific cases like the presented one, the analysis of the transcriptome as well as proteome of miRNA-engineered cell lines is required to analyze direct effects of miRs on protein expression during each stage of the batch culture, while methods like TAP-tar (Nonne et al., 2010) will identify direct miRNA:mRNA interactions. Both of these approaches are currently underway.

In conclusion, this is one of the first reports describing stable overexpression of miRNAs in CHO cells and analyzing its effects on growth and recombinant protein production. Further analysis of these stable miRNA-expressing CHO cells will provide molecular insights eventually leading to refined miRNA engineering strategies for CHO cells.

Conflict of interest

JG is co-founder of Evercyte.

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Appendix C

Annotation of additional evolutionary conserved microRNAs in CHO cells from updated genomic data.

COMMUNICATION TO THE EDITOR

BIOTECHNOLOGY and BIOENGINEERING

Annotation of Additional Evolutionary Conserved microRNAs in CHO Cells From Updated Genomic Data

Andreas B. Diendorfer,[1] Matthias Hackl,[1] Gerald Klanert,[2] Vaibhav Jadhav,[1] Manuel Reithofer,[1] Fabian Stiefel,[3] Friedemann Hesse,[3] Johannes Grillari,[1] Nicole Borth[1,2]

[1]Department of Biotechnology, BOKU University of Natural Resources and Life Sciences, Vienna, Austria; telephone: +43476546803; fax: +43476546675; e-mail: andreas.diendorfer@boku.ac.at

[2]ACIB GmbH, Austrian Centre of Industrial Biotechnology, Graz, Austria

[3]Biberach University of Applied Sciences, Biberach, Germany

ABSTRACT: MicroRNAs are small non-coding RNAs that play a critical role in post-transcriptional control of gene expression. Recent publications of genomic sequencing data from the Chinese Hamster (CGR) and Chinese hamster ovary (CHO) cells provide new tools for the discovery of novel miRNAs in this important production system. Version 20 of the miRNA registry miRBase contains 307 mature miRNAs and 200 precursor sequences for CGR/CHO. We searched for evolutionary conserved miRNAs from miRBase v20 in recently published genomic data, derived from Chinese hamster and CHO cells, to further extend the list of known miRNAs. With our approach we could identify several hundred miRNA sequences in the genome. For several of these, the expression in CHO cells could be verified from multiple next-generation sequencing experiments. In addition, several hundred unexpressed miRNAs are awaiting further confirmation by testing for their transcription in different Chinese hamster tissues.

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KEYWORDS: microRNA; Chinese hamster ovary cell; next-generation sequencing

Introduction

Chinese hamster ovary (CHO) cells are important mammalian hosts for the production of biopharmaceuticals. Over the last 25 years, the optimization of growth, product quality and titer was mainly driven by the modification of media, feeding strategies, and biotechnological processes. More recently, with the availability of genomic sequences, cell engineering strategies have emerged as an alternative route to improve cell line performance (Lim et al., 2010; Xiao et al., 2014), although deeper insight into genetic, transcriptional and translational regulation is required to obtain full control over cellular metabolism (Jadhav et al., 2013; Kildegaard et al., 2013).

MicroRNAs (miRNAs) are small (18–24 nucleotides long) non-coding RNAs (ncRNA) that are transcribed as a primary transcript (pri-miRNA) by RNA polymerase II. These transcripts are cleaved in the nucleus by the RNAase Drosha, to produce a pre-miRNA with a characteristic stem-loop secondary structure and a size of about 50–80 nt. For the final miRNA sequence, pre-miRNAs are processed by the enzyme Dicer and incorporated into the miRNA-induced silencing complex (miRISC) and bind to mRNA transcripts in their 3′-UTR. Binding of the complex triggers either inhibition of translation or mRNA cleavage. By this process, miRNAs are capable of negatively regulating protein translation (Gregory et al., 2004) and therefore constitute an important layer in the post-transcriptional control of gene expression (Hobert, 2008).

In 2011, the first systematic approaches to miRNA annotation in CHO cells were reported (Hackl et al., 2011; Johnson et al., 2011). Subsequent studies were focused on providing insights into the importance of miRNAs for molecular pathways relevant to cell culture engineering, such as growth (Jadhav et al., 2014), apoptosis (Druz et al., 2011), and protein secretion (Barron et al., 2011; Loh et al., 2014).

Recent publications of genomic sequencing data from CHO cells (Xu et al., 2011) as well as that of *Cricetulus griseus* (CGR) (Brinkrolf et al., 2013; Lewis et al., 2013) now have provided a basis for refining the annotation of: (i) known expressed cgr-miRs, (ii) the identification of additional expressed miRNAs in CHO cells, and (iii) the discovery of miRNAs without current evidence for transcription in CHO cell lines.

For the annotation of novel microRNAs, (Ambros et al. 2003) specified five conditions, of which a reasonable combination has to

Correspondence to: A. B. Diendorfer and N. Borth

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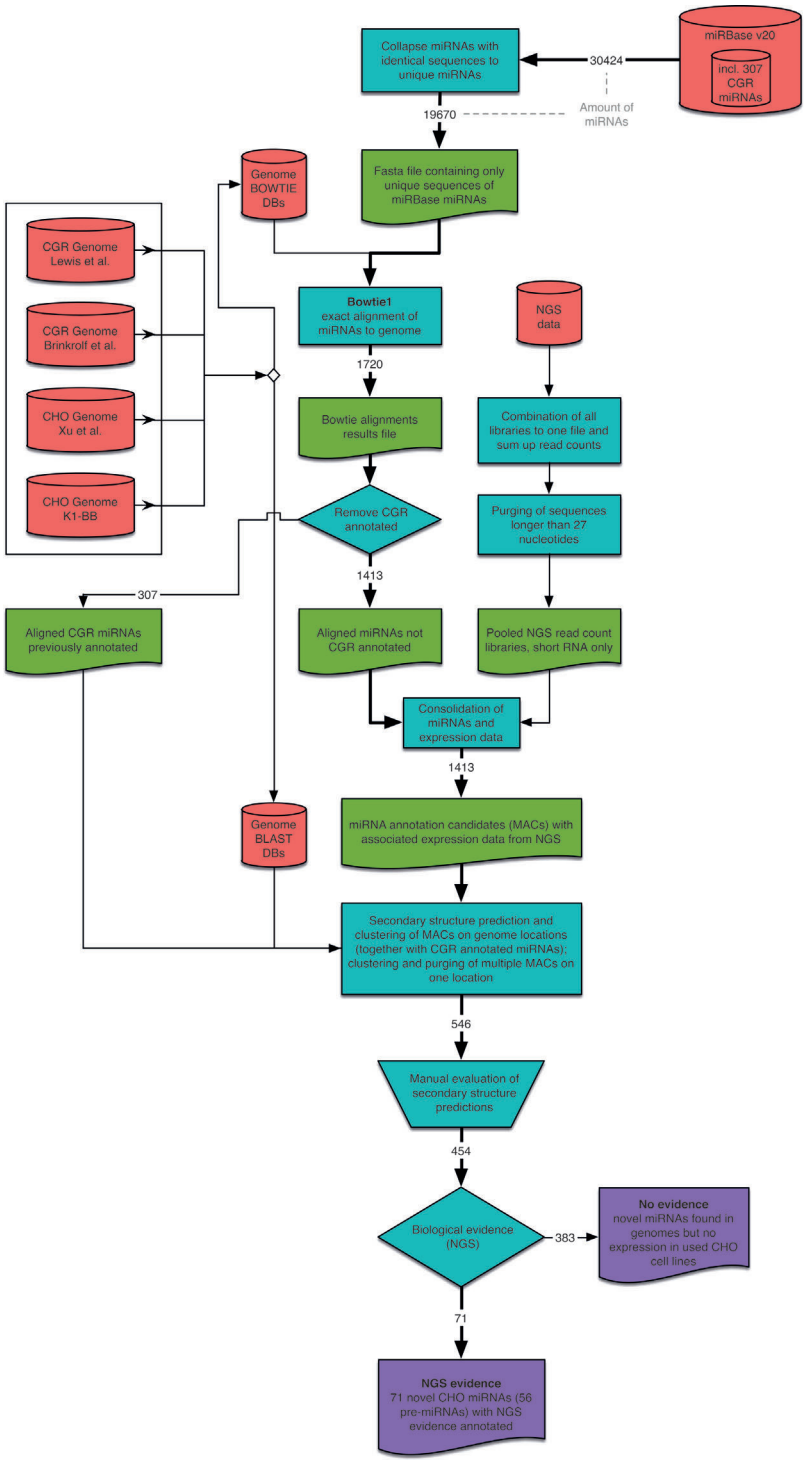

Figure 1. Bioinformatic pipeline flowchart. The main path of analysis is highlighted with a bold arrow from the top right to the bottom. Numbers on the arrows describe the amount of miRNAs processed in this step.

be met to count as valid microRNA. In this study we based our criteria on the identification of a distinct ~22 nt RNA transcript and the phylogenetic conservation of mature and pre-miRNA sequences.

To expand the list of miRNAs available as possible engineering tools, we searched for evolutionary conserved miRNA sequences from other species in four genomic datasets, identified their genomic locations and hairpin sequences, and confirmed the

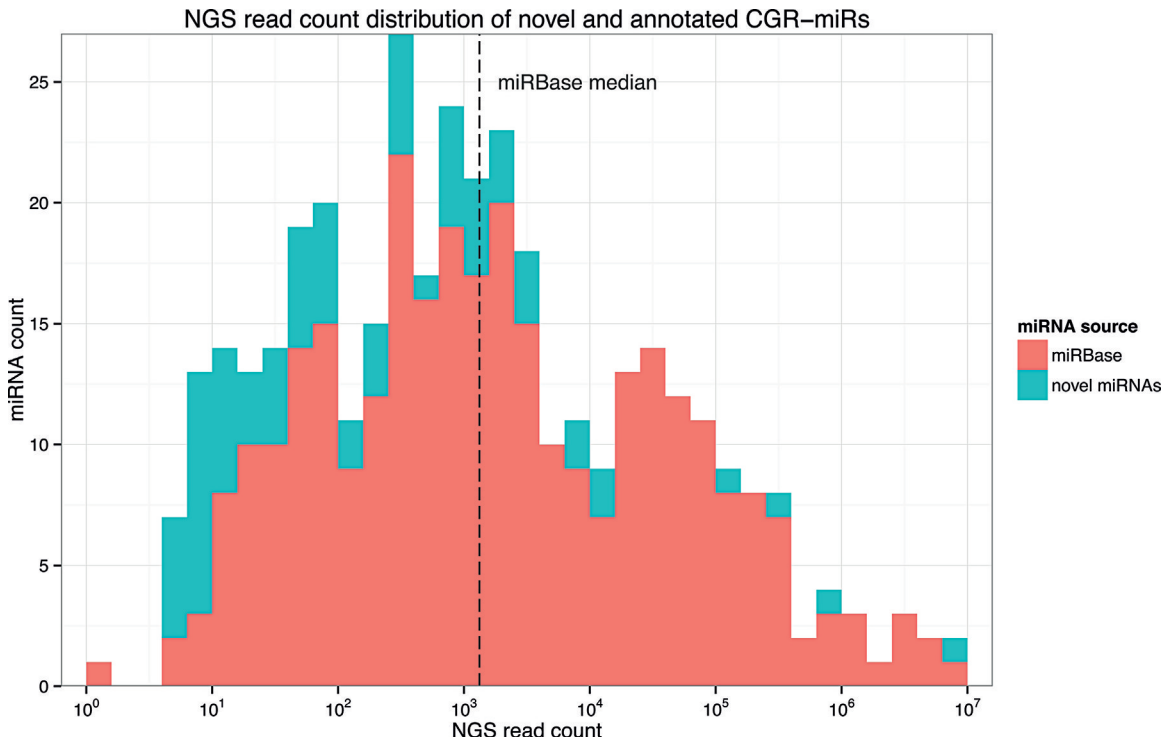

Figure 2. Read count distribution of miRBase annotated CGR and novel microRNAs showing a higher abundance of low read count microRNAs in the novel data set (77.8 % of novel miRNAs are below the miRBase median read count).

expression of some of these in CHO, using next-generation transcriptome sequencing results. This improvement and expansion of sequence and expression information of cgr-miRs will be useful for further functional investigation of miRNAs, to gain a better understanding of post-transcriptional regulation in cellular pathways, and to explore the potential function of silent CHO miRNAs, for which no expression evidence could be found yet.

miRBase Version 20 (Griffiths-Jones, 2004) contains 307 mature cgr-miRs on 200 precursors. In order to identify and annotate novel cgr-miRs, we based our search on evolutionary conservation of miRNAs by aligning all mature miRNAs to the available CGR/CHO genomic data, applying the workflow outlined in Figure 1.

Thereby, 1,720 unique mature sequences out of 19,670 miRBase v20 entries (all miRBase annotated miRNAs of any species) were aligned to at least one of the four genomes. The 307 already annotated cgr-miRNAs were excluded, leaving 1,413 miRNA sequences as candidates for novel cgr-miRNAs. Clustering of these sequences on genomic locations, to find similar, overlapping sequences that constitute only one possible new miRNA, reduced the number of sequences down to 546. These 546 candidates were

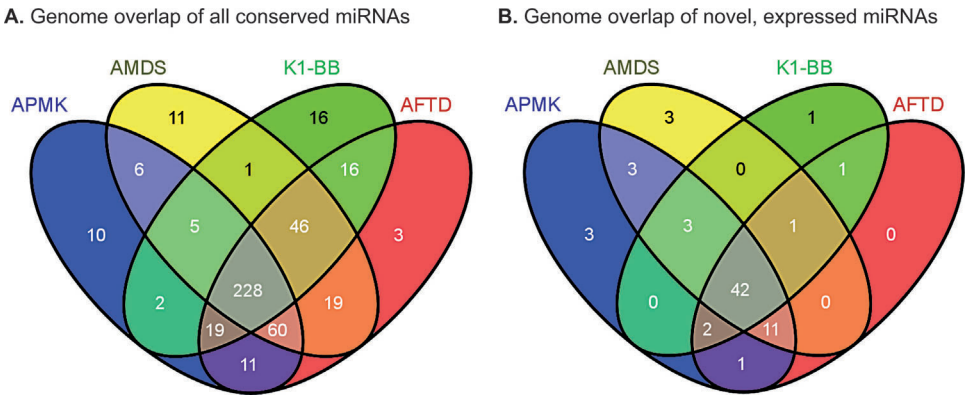

Figure 3. Venn diagrams showing the occurance of (**A**) all evolutionary conserved and (**B**) expressed, novel miRNAs in the four used genomic datasets.

then evaluated by prediction of their in silico secondary structure, whereby 454 sequences showed a pre-miRNA like secondary stem loop structure. These putative novel cgr-miRs are listed in Supplement 1.

For classification as novel cgr-miRs, the next-generation sequencing (NGS) read counts from new and existing datasets (Hackl et al., 2011) of these sequences were examined using more than five read counts as cut-off for the existence of these miRNAs. Thereby, 383 sequences did not show expression under the constraints for valid NGS signals, leaving 71 NGS confirmed miRNAs.

Four of these 71 miRNAs were present on two genomic locations (75 possible novel miRNA locations) with different hairpin sequences (highlighted in blue in Supplement 1). A set of six pairs (miRNA-5p/miRNA-3p) could be matched on the same hairpin, (highlighted in green), giving 69 pre-miRNA sequences. Thirteen of the new mature miRNA sequences were found on already annotated cgr-pre-miRNAs (see column "On hairpin with" in Supplement 1), thus complementing already annotated 5p or 3p miRNAs. Therefore, in summary, our study resulted in a total of 56 novel pre-miRNA and 71 miRNA sequences that were added to the already annotated ones, extending the hamster miRNome to a total of 378 mature sequences (+23.1%), and 256 precursors (+28.0%).

The described process presents an easy and fast pipeline for the discovery of novel miRNAs from genomic data and next-generation sequencing experiments. Recently published genomic data for *C. griseus* allowed the annotation of multiple conserved miRNAs from other species, by definition missing out on possible non-conserved miRNAs present in the Chinese hamster.

In total, 71 novel expressed mature miRNAs and 56 pre-miRNAs were added to the existing data. These novel miRNAs are mostly expressed at low levels, as their read counts were identified to be mainly below the median read count (77.8% below and 22.2% above) of already annotated cgr-miRNAs (Fig. 2). In addition, we provide information on genomic loci of 345 mature miRNAs with no evidence for expression in CHO cells, but conserved homologous sequences in related species. These may be expressed in other tissue and cell types in the Chinese hamster, however , as confirmation of expression is required, they are currently not uploaded to miRBase. The occurrence of both the evolutionary conserved and expressed newly identified miRNAs in the four genomic datasets is shown in Figure 3. In both cases, the majority of miRNAs can be found in three or all genomic datasets.

A list of validated targets for these novel miRNAs (if available) is provided in Supplement 2. We grouped the pathways into cell engineering relevant categories. A list of pathways and miRNAs that possibly influence them is given in Table I. As these include process relevant cellular properties such as growth and apoptosis, the miRNAs may be relevant engineering targets.

Taken together, the cgr-miRNAs identified here will enhance the use of miRNAs as tools for CHO cell engineering. They also pave the way for using miRNAs not transcribed in CHO cells to restore or alter biotechnologically relevant cell line characteristics using the correct hamster primary miRNA sequences for engineering. The positive effects of using endogenous in contrast to chimeric miRNA sequences was shown by Klanert et al. (2014) and highlights the importance of good and comprehensive genomic data for CHO cell engineering.

Material and Methods

Datasets

For the annotation of novel miRNAs, the mature sequences of all miRNAs available in the miRNA database (miRBase version 20 - http://www.mirbase.org; Griffiths-Jones, 2004) were used. This constitutes a set of 30,424 mature miRNAs which was merged down to 19,670 unique sequences.

These miRNAs were aligned to four genome assemblies, sequenced from CHO cells and *C. griseus* (CGR). The genomes were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and are listed with their accession IDs in Table II. In addition, the unpublished genome assembly "K1-BB" was used as described by Hackl et al. (2012).

Next-generation sequencing (NGS) data were obtained by Illumina sequencing as described by Hackl et al. (2011) and is available at the Sequence Read Archive SRA (www.ncbi.nlm.nih.gov/sra/), accession number SRA024456.1. Cell lines sequenced included CHO-DUXB11 (ATCC CRL-9096), CHO-K1 (ECACC-CCL-61), and two derivative recombinant cell lines producing a monoclonal antibody (CHO-K1) and an EpoFc fusion protein (DUXB11). Samples were taken both from cells grown in the presence of 5% FCS and after adaptation to protein free medium. To further extend the amount of cultivation conditions and cell lines we sequenced additional cell lines. CHO-DG44 (passage 13) cells were grown in a 2 L bioreactor in batch culture. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. The RNA quality was tested with an Agilent chip (Agilent Technologies, Santa Clara, California). Labelling of the samples followed the standard Illumina TrueSeq protocol for small RNA (12 barcoded samples on one lane). Sequencing of ready to load libraries was conducted by GATC, Germany on an Illumina HiSeq Analyser. The NGS data files of these experiments are available at Gene Expression Omnibus GEO (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE59838 and SRA, accession number SRP044946.

Bioinformatic Processing

Bioinformatic processing followed a pipeline of freely available tools and self-written scripts. The scripts are available upon request from the corresponding author. The pipeline is outlined in Figure 1.

The collapsed mature miRNA sequences from miRBase were aligned against each of the four genome databases using Bowtie v1.0.0 (Langmead et al., 2009) to find exact matches. This produced a list of annotated miRNAs that were present in the genomic sequences (see Supplement 1). MicroRNAs already annotated for CGR were filtered and stored in a separate file. For all other miRNAs that could be aligned to the genomes, the read counts from next-generation sequencing experiments were consolidated into one file. Next-generation sequencing read counts were summed up from different experiments to consider the different culture conditions. A read count of five reads was set as cutoff.

For these miRNA annotation candidates, the up and downstream sequences were extracted in the same length as in the originating species (assuming that not only the mature sequence is conserved,

Table I. Selected pathways possibly influenced by novel miRNAs. Data for miRNA protein interaction was derived from miRWalk (validated targets). DAVID was used to associate the proteins with pathways.

Pathway category	miRNAs
Cell cycle related	mmu-let-7a-1–3p, mmu-miR-106b-5p, mmu-miR-126–5p, mmu-miR-127–3p, mmu-miR-202–5p, mmu-miR-211–5p, mmu-miR-216b-5p, mmu-miR-24–1-5p, mmu-miR-760–3p, hsa-let-7a-3p, hsa-let-7c-5p, hsa-let-7e-3p, hsa-let-7e-5p, hsa-miR-148a-3p, hsa-miR-18b-5p, hsa-miR-192–3p, hsa-miR-20b-5p, hsa-miR-217, hsa-miR-26a-1–3p, hsa-miR-26a-2–3p, hsa-miR-449a, hsa-miR-582–5p
Apoptosis related	mmu-let-7a-1–3p, mmu-miR-106b-5p, mmu-miR-126–5p, mmu-miR-150–5p, mmu-miR-30c-1–3p, hsa-let-7a-3p, hsa-let-7c-5p, hsa-let-7e-3p, hsa-let-7e-5p, hsa-miR-192–3p, hsa-miR-30c-2–3p, hsa-miR-449a, hsa-miR-451a
Cancer related	mmu-let-7a-1–3p, mmu-miR-101a-5p, mmu-miR-106b-5p, mmu-miR-126–5p, mmu-miR-127–3p, mmu-miR-150–5p, mmu-miR-193a-5p, mmu-miR-193a-3p, mmu-miR-211–5p, mmu-miR-216b-5p, mmu-miR-24–1-5p, mmu-miR-296–5p, mmu-miR-30c-1–3p, mmu-miR-760–3p, mmu-miR-99b-3p, mmu-miR-99b-5p, hsa-let-7a-3p, hsa-let-7c-5p, hsa-let-7e-3p, hsa-let-7e-5p, hsa-miR-148a-3p, hsa-miR-18b-5p, hsa-miR-192–3p, hsa-miR-20b-5p, hsa-miR-217, hsa-miR-26a-1–3p, hsa-miR-26a-2–3p, hsa-miR-301b-3p, hsa-miR-30c-2–3p, hsa-miR-449a, hsa-miR-451a, hsa-miR-582–5p
Focal adhesion related	mmu-let-7a-1–3p, mmu-miR-101a-5p, mmu-miR-126–5p, mmu-miR-150–5p, mmu-miR-30c-1–3p, mmu-miR-99b-3p, mmu-miR-99b-5p, hsa-let-7a-3p, hsa-let-7c-5p, hsa-let-7e-3p, hsa-let-7e-5p, hsa-miR-148a-3p, hsa-miR-192–3p, hsa-miR-20b-5p, hsa-miR-217, hsa-miR-26a-1–3p, hsa-miR-26a-2–3p, hsa-miR-30c-2–3p, hsa-miR-449a, hsa-miR-451a, hsa-miR-582–5p
Jak-STAT related	mmu-let-7a-1–3p, hsa-let-7a-3p, hsa-let-7c-5p, hsa-let-7e-3p, hsa-let-7e-5p, hsa-miR-192–3p, hsa-miR-26a-1–3p, hsa-miR-451a align="center"
Cytoskeleton related	mmu-let-7a-1–3p, mmu-miR-101a-5p, mmu-miR-126–5p, mmu-miR-127–3p, mmu-miR-150–5p, mmu-miR-216b-5p, mmu-miR-24–1-5p, mmu-miR-296–5p, mmu-miR-30c-1–3p, mmu-miR-99b-3p, mmu-miR-99b-5p, hsa-let-7a-3p, hsa-let-7e-3p, hsa-miR-192–3p align="center"
MAPK related	mmu-let-7a-1–3p, mmu-miR-101a-5p, mmu-miR-106b-5p, mmu-miR-126–5p, mmu-miR-127–3p, mmu-miR-150–5p, mmu-miR-193a-5p, mmu-miR-193a-3p, mmu-miR-216b-5p, mmu-miR-296–5p, mmu-miR-30c-1–3p, mmu-miR-99b-3p, mmu-miR-99b-5p, hsa-let-7a-3p, hsa-let-7c-5p, hsa-let-7e-3p, hsa-let-7e-5p, hsa-miR-148a-3p, hsa-miR-18b-5p, hsa-miR-192–3p, hsa-miR-217, hsa-miR-26a-1–3p, hsa-miR-26a-2–3p, hsa-miR-451a align="center"

but also the precursor). For this step, a BLAST (Altschul et al., 1990) database was generated for each genome, allowing the fast retrieval of sequences from given positions in the genomes. A secondary structure prediction was performed with RNAfold v2.1.3 from the ViennaRNA package (Lorenz et al., 2011), giving a graphical representation of each folding in addition to its free energy. For each annotation candidate a results file (HTML) was generated, consolidating the secondary structure images and other information for quick evaluation in the next steps.

For most of the identified locations, more than one miRNA sequence from different species were found by clustering all aligned miRNA sequences on distinct locations in each of the four genomes. To ensure that these evolutionary conserved sequences were only considered once, these not completely identical sequences (they differ in length and/or are shifted some nucleotides in their position) were sorted for their NGS read count and the highest was kept as annotation candidate. The secondary structures were manually evaluated for common miRNA characteristics (low minimum free energy, stem-loop motif, miRNA sequence located in the stem, and no large internal loops or bulges) and are shown in Supplement 3.

To investigate possible uses of the novel miRNAs, we looked at validated miRNA-targets in the originating species (*H. sapiens* or *M. musculus*) with miRWalk (Dweep et al., 2011). The list of target proteins were clustered using the functional annotation clustering tool of DAVID (Huang et al., 2009a; Huang et al.,

Table II. Genome references and statistics used for the identification of evolutionary conserved microRNAs.

	Lewis et al. (2013)	Xu et al. (2011)	Brinkrolf et al. (2013)	K1-BB
Genome size (Gbp)	2.3	2.3	2.1	2.98
Scaffolds	7,468	109,151	28,749	11,400,490
x Coverage	89.1	130.0	70.0	17.1
Accession ID	AMDS00000000.1	AFTD00000000.1	APMK00000000.1	—
Source	CGR	CHO	CGR	CHO

2009b) and the KEGG pathway database and then grouped into seven categories (Table II).

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Appendix D

Curriculum Vitae

Wednesday, April 13, 2016

Dammstraße 24/11
1200, Vienna
0043 650 4530305
gerald.klanert@boku.ac.at

Gerald Klanert

Date of Birth: 04/07/1986
Nationality: Austria
Sex: Male
Languages: German (mother tongue), English (fluent)

Education

PhD, Biomolecular Technology of Proteins, University of Natural Resources and Life Sciences, Vienna, September 2012, ongoing

- CHO cell line with increased growth rate

Supervisor: Nicole Borth, Ao. Univ. Prof. Dipl.-Ing. Dr. nat.techn.

Master of Science, Biotechnology, University of Natural Resources and Life Sciences, Vienna, 2010 - 2012

- Enhancing Chinese Hamster Ovary Cell Phenotype: Opportunities of microRNA Engineering and Cell Sorting

Supervisor: Nicole Borth, Ao. Univ. Prof. Dipl.-Ing. Dr. nat.techn.

Bachelor of Science, Food Science and Biotechnology, University of Natural Resources and Life Sciences, Vienna, 2006 - 2010

- Identification and Evaluation of Heavy Metal Induced Genes in Salix Caprea

Electronic Engineering and Computer Science, HTL Steyr, Steyr, 2000 - 2005

Research Experience

2015 (April) – 2015 (Sep) Research stay at the NIH, Bethesda, MD, USA

A cross-species high throughput whole genome siRNA screening method was implemented for suspensional CHO cells producing and secreting a recombinant fluorescent protein.

2012 (Sep) – 2016 (June) PhD thesis

An enhanced method for miRNA cluster engineering in CHO cells was developed and growth-correlating miRNAs in CHO cells were identified by transcriptomics.

2011 (Sep) – 2012 (June) Master thesis

The effect of different miRNA constructs on a CHO producer cell line were evaluated and a new dye dilution method for heterogeneous cell lines was implemented.

2009 (Feb – Mar) Bachelor thesis

Screening of a SSH library to identifiy differentially regulated genes in Salix Caprea with and without administration of heavy metals by dot plots and BLAST-based gene identification.