Universität für Bodenkultur.
University of Natural Resources and Life Sciences.

Department für Biotechnologie
Department of Biotechnology

Institut für Angewandte Mikrobiologie
Institute of Applied Microbiology

Vorstand: Ao. Univ. Prof. Dr. Florian Rüker
Head of the Department

Betreuer: Priv. Doz. Dr. Johannes Grillari
Supervisor

Ao. Univ. Prof. Dr. Nicole Borth

MicroRNA engineering in Chinese hamster ovary (CHO) cells: its biological effects on cell growth and productivity.

Dissertation
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Vaibhav Jadhav, M.Sc

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Dedicated to my parents
and
My wonderful wife

“कर्मण्येवाधिकारस्ते मा फलेषु कदाचन

मा कर्मफलहेतुर्भूर्मा ते सङ्गोऽस्त्वकर्मणि”

Meaning

“Do your duty and be detached from its outcome, do not be driven by the end product, enjoy the process of getting there”

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Thank you,

Vaibhav Jadhav

Kurzfassung

MikroRNAs (miRNAs) sind kurze regulatorische RNA-moleküle, welche durch spezifische Bindung die Genexpression in höheren Organismen kontrollieren können. Mit einer Länge von nur etwa 22 Nukleotiden sind sie dennoch maßgeblich an der Steuerung komplexer zellulärer Vorgänge beteiligt, wie zum Beispiel Zellstoffwechsel, -differenzierung und -entwicklung. Aus der Erforschung der biologischen Funktionen von microRNAs innerhalb der letzten 10 Jahren haben sich zahlreiche Anwendungen für diese Moleküle ergeben, wie zum Beispiel als Biomarker für die Diagnostik oder als therapeutische Wirkstoffe. Seit kurzem wird in Fachkreisen auch die Anwendung von microRNAs für die gentechnische Optimierung von Chinese hamster ovary (CHO) Zellen diskutiert („microRNA Engineering“). Erste Studien zeigen, dass microRNAs für die Steigerung der Produktivität von biopharmazeutischen Prozessen verwendet werden könnten, indem das Wachstum oder die Proteinsekretion von Zellfabriken wie CHO verbessert wird. Durch die Vernetzung von CHO–omics Datensätzen können in CHO-Zellen Effekte des „microRNA Engineerings“ durch einen systematischen Ansatz und auf breiter Basis untersucht werden und um mögliche neue Anwendungen erweitert werden.

In der vorliegenden Doktorarbeit wurde daher insbesondere auf die Entwicklung von molekularbiologischen Werkzeugen und Methoden für die Erforschung von microRNAs in CHO Zellen - unter Berücksichtigung der vorhandenen -omics Datensätze – eingegangen. Weiters wurde miR-17 als potenzieller Kandidat für die Verbesserung der Produktivität in CHO Zellen identifiziert und charakterisiert. Die in dieser Arbeit generierten Daten und Methoden finden nun Anwendung als Plattform für die Verbesserung von Wachstum und Produktivität für die Produktion rekombinanter Proteine in einer der wichtigsten biopharmazeutischen Zellfabriken, den CHO Zellen.

Abstract

MicroRNAs have evolved as a powerful genetic regulatory system to control gene expression in higher eukaryotes. These regulatory RNAs are about 22 nucleotides in length and since their discovery they have been shown to regulate complex molecular pathways from development to metabolism. In the last decade these molecules have generated eminent interest for possible applications as biomarkers and therapeutics. Recently miRNAs have gained lot of attention in Chinese Hamster Ovary (CHO) cells engineering, where first reports show their potential for application in the production of biopharmaceuticals. In combination with CHO -omics data sets, the effect of engineered miRNAs can be further explored in an elaborate and systematic manner to broaden the scope of their application. Therefore, this doctoral thesis was focused on the development of tools and methods for miRNA research in CHO cells based on available -omics data sets. Furthermore, we identified miR-17 to potentially improve productivity in CHO cells. These newly developed tools and methods can be utilized as a platform to enhance growth and recombinant protein production of CHO cells, which are one of the most important biopharmaceutical cell factories.

Keywords: Chinese Hamster Ovary (CHO) cells, microRNA, Cell engineering, miR-17.

1.1 CHO cells in therapeutic protein production.

Chinese Hamster Ovary (CHO) cells were first isolated in the late 1950's and extensively used in radiation and cytogenetic research [1]. These fundamental research studies produced various immortalized mutant CHO cells [2-4], which were then used as hosts for recombinant therapeutic protein production. Human tissue plasminogen activator (tPA) was the first therapeutic produced from CHO cells approved for treatment in humans [5]. These discoveries and advances in biotechnology paved the way for rapid development of the therapeutic protein production industry [6,7]. The current market value estimate has risen to nearly $120 billion per year. Today, CHO cells are the predominant cell factory, as approx. 70% of all therapeutic proteins are produced in CHO cells which have become one of the most important workhorses for therapeutic protein expression at industrial scale [8-10].

The distinctive characteristics of CHO cells, such as ease of genetic manipulation, powerful gene amplification systems and the ability to grow in suspension in serum-free medium are preferred by the industry [11]. Additionally, therapeutics produced in CHO cells are considered safe for use in humans because of their low immunogenicity due to similar glycosylation [12]. Safety is also considered to be high due to low infectability of CHO cells by viruses, and the species boundary of pathogens. There has been a continuous challenge to improve CHO cells with respect to enhancing space-time yields, viable cell densities, and stress resistance which are contributing to the high cost of recombinant therapeutics. The notion of affordable therapeutics (biosimilars) and the increasing number of approved products pose further challenges on existing health care systems. The development of cost-effective medicines by establishing high-producer CHO cells in bioreactors is therefore of high interest to society [13].

These challenges can be overcome by genetic engineering of CHO cells by modulating one or more components of molecular pathways. Several cell engineering strategies have been proposed to improve the characteristics of CHO cells, for instance to increase the integral of viable cell density (IVCC) and/or to improve specific productivity of recombinant protein [14]. Conventionally this is done by introduction of key regulators of important cellular pathways such as cell cycle, translation, secretion and folding [15-19]. However, these strategies were only in part successful and resulted in highly clone-specific and heterogeneous phenotypes [20]. These drawbacks limited their applicability as universal and robust cell engineering tools; nevertheless these approaches provided first methods to be employed in the development of genetically engineered CHO cells. Recent advances of various -omics technologies, such as genomics, transcriptomics, proteomics, and metabolomics, have been applied to understand the regulation of gene networks at different physiological levels [21-23]. The introduction of -omics platforms to CHO cell research can shift classical single gene engineering to global network control by manipulating multiple genes for robust CHO cell phenotypes. The first results from these new technologies are already emerging for advances in cell engineering, clone selection, culture media development and quality control of the recombinant protein [24,25].

1.2 MicroRNOmics of CHO host and production cell lines.

One of the most attractive engineering targets as global regulators are microRNAs (miRNA) [26 - 28], these are small non-coding RNA molecules that control gene expression at the post-transcription level to fine tune molecular pathways. Completely processed mature miRNA functions by binding to sites on the 3'-UTR of mRNA with either complete or partial complementarity, followed by incorporation into a large multimeric RNA-induced silencing complex (RISC). The complementarity of the binding to the target determines the mechanism of action: if miRNA-mRNA interaction has perfect complementarity, the target is cleaved and rapidly degraded. In case of

partial complementarity, translation is blocked and the blocked mRNA-miRNA–RISC complex is stored in so-called “P-bodies” for later release or nonsense-mediated mRNA decay [29,30]. Studies from a broad range of organisms, from plants to mammals, showed that these tiny RNA molecules can orchestrate multiple gene networks simultaneously regulating complex phenotypes similar to transcription factors [31]. This rapid development in miRNA biology, including miRNAs functions in mammalian cells in relation to functional phenotypes has provided the motivation to introduce them as a promising strategy for CHO cell-engineering (see publication in Appendix 2.1 C, Ref [32]).

First applications of miRNAs in CHO cell engineering have been reported during the last years. For example, overexpression of miR-7 induces growth arrest, a behavior observed during temperature shift leading to enhanced productivity [33]. In another case, nutrient depleted culture condition lead to upregulation of the miR-297-669 cluster. mmu-miR-466h, a member of this cluster, was found to be targeting anti-apoptotic genes like Caspase-3/7. Inhibition of miR-446h resulted in increased cell viability and decreased Caspase-3/7 activity, suggesting a role for miR-446h as a pro-apoptotic miRNA [34]. These findings strongly support the importance of miRNAs in cell line engineering and cell line development, emphasizing their potential to improve bioprocessing for therapeutic protein production.

Another avenue growing quite rapidly is the utilization of -omics tools, such as next-generation sequencing, microarray and high throughput qPCR arrays, to describe possible functional associations of miRNAs in various biological pathways and phenotypes in CHO cells [35-41] (see publication in Appendix 2.2 E, Ref [36]). These data not only allow to identify and quantitatively measure miRNA expression but also to explore the role of miRNA as potential engineering targets and also to utilize them as biomarkers for bioprocess conditions such as growth, productivity, nutrient depletion and temperature shift. These studies suggest that hundreds of miRNAs are expressed in different host and producer cell lines which could be potential engineering targets.

However, an integrative approach is required for targeted knowledge based cell-engineering and for functional analysis of such large numbers of miRNAs.

In view of these rapid developments, this doctoral thesis set forth to explore the following goals in order to develop a general platform for knowledge based cell-engineering of CHO cells.

a) To integrate omics data sets and associate them to phenotypes from both expression data and literature.

b) To develop an efficient and robust genetic screening method, to analyze candidate miRNAs.

c) To establish miRNA engineered CHO cells for their functional analysis in future biotechnological applications.

The developed tools and methods can be utilized with the ultimate objective of enhancing growth and recombinant protein production and of improving the bio-industrially relevant characteristics of CHO cells, which are one of the most important biopharmaceutical cell factories.

1.3 Identification of potential CHO-engimiRs associated with growth and protein production phenotypes.

The knowledge of miRNAs and their regulation in mammalian systems is led by the development of methods such as expression profiling (miRNA microarray) and quantitative PCR. These methods have enabled to quantitatively measure expression and to identify differentially regulated miRNAs in different physiological situations [36, 41]. Further advances were made by utilizing next-generation-sequencing technologies, which enable not only the de novo identification of sequences, but also the quantitative measurement of their expression [35]. The application of these technologies to identify miRNAs involved in a variety of bioprocess relevant conditions such as

cell proliferation, apoptosis, dynamic effects during batch culture, hypothermic growth and high and low productivity is an active area of research [36, 41] (See publication in Appendix 2.2 E, Ref [36]). To this end, we analyzed expression profiles of mRNA and miRNA during batch cultures. The interaction between mRNA and miRNA at specific culture stages provided a global view of dynamic changes in processes that are controlled by these interactions.

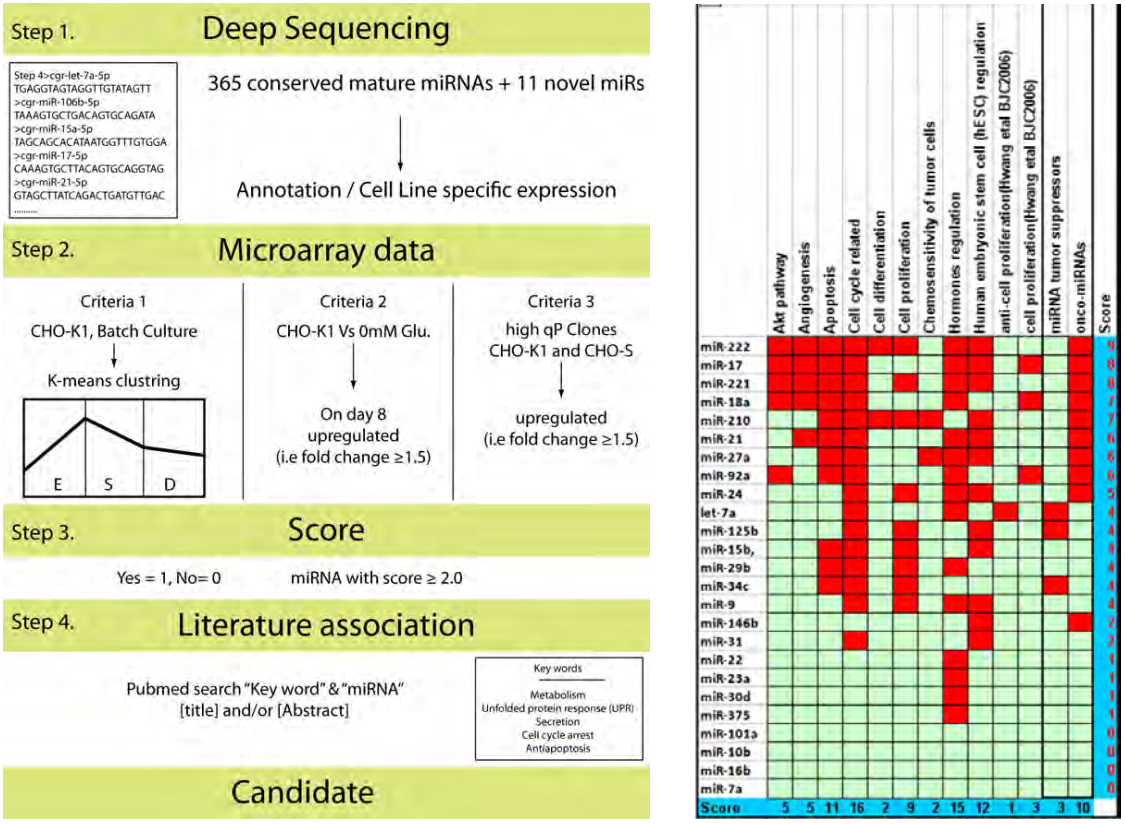

Figure 1. Identifying engimiR candidates. (A) Work flow for selection and prioritization of candidate miRNAs using omics data sets. (B) Bioinformatics for analysis of candidate miRNAs for association with related biological pathways using predicted targets.

To identify potential engimiRs candidates associated with growth and productivity, we performed a systematic analysis of miRNA expression profiles in CHO host cells (CHO-K1) with different metabolic background (see publication in Appendix 2.2 E, Ref [36]) and CHO host (CHO-K1 and

CHO-S) cells with increased productivity [42]. Microarray experiments were performed using LNA miRBase v9.2 platforms. Linear models for microarray analysis (limma) were used in R/ Bioconductor for processing array data. The processed microarray data was filtered with small RNA sequencing data [35] as reference data specific to CHO cells, as all the array experiments were performed using a cross-species miRNA array platform. These datasets were integrated to identify miRNA associated to control growth and recombinant protein production. This is done by prioritizing miRNAs with the following selection criteria (fig. 1A): (i) the miRNAs must be overexpressed in the exponential phase of the batch, and expression profiles was analyzed by K-means clustering. (ii) miRNA should be >1.5 fold up-regulated in early stationary phase (Day 8) in differential expression analysis in cells reaching higher cell densities (CHO-K1 glutamine free adapted) when compared to CHO-K1 cells. (iii) miRNAs should be >1.5 fold up-regulated in differential expression analysis in clones reaching higher productivity when compared to host cells (CHO-K1 and CHO-S: *manuscript in preparation*). With these criteria, we identified 25 (Table 1) potential candidate miRNAs for screening with respect to enhanced growth and productivity. Candidate miRNAs were further analyzed for association to biological pathways using web DIANA-mirPath [43] (fig. 1 B). These candidate *engimiRs* were tested for their effects in CHO cells.

Table 1. Candidate engimiRs				
No.	miRNA ID	mirbase accession	5p Sequence	3p Sequence
1	cgr-let-7a	MI0020368	TGAGGTAGTAGGTTGTATAGTT	CTATACAATCTACTGTCTTTCT
2	cgr-miR-101a	NA	CAGTTATCACAGTGCTGATGC	TACAGTACTGTGATAACTGAATT
3	cgr-miR-125b	MI0020386	TCCCTGAGACCCTAACTTGTGA	ACGGGTTAGGCTCTTGGGAGC
4	cgr-miR-146b	MI0020408	TGAGAACTGAATTCCATAGGCTG	TGCCCTAGGGACTCAGTTCTGGT
5	cgr-miR-15b	MI0020417	TAGCAGCACATCATGGTTTACA	CGAATCATTATTTGCTGCTCT
6	cgr-miR-16b	NA	TAGCAGCACGTAAATATTGGCG	CCAATATTATTGTGCTGCTTTA
7	cgr-miR-17	MI0020419	CAAAGTGCTTACAGTGCAGGTAG	ACTGCAGTGCAGGCACTTGTGG
8	cgr-miR-18a	MI0020433	TAAGGTGCATCTAGTGCAGATAG	ACTGCCCTAAGTGCTCCTTCTGG
9	cgr-miR-21	MI0005725	TAGCTTATCAGACTGATGTTGAC	CAACAGCAGTCGATGGGCTGTC
10	cgr-miR-210	MI0020458	AGCCACTGCCCACCGCACACTG	CTGTGCGTGTGACAGCGGCTGA
11	cgr-miR-22	MI0020463	AGTTCTTCAGTGGCAAGCTTT	AAGCTGCCAGTTGAAGAACTGT
12	cgr-miR-221	MI0020464	ACCTGGCATACAATGTAGATTTCTGT	AGCTACATTGTCTGCTGGGTTTC
13	cgr-miR-222	MI0020465	TCAGTAGCCAGTGTAGATCCTG	AGCTACATCTGGCTACTGGGTCTCT
14	cgr-miR-23a	MI0020466	GGGGTTCCTGGGGATGGGATTT	ATCACATTGCCAGGGATTTCCAAT
15	cgr-miR-24	MI0020468	GTGCCTACTGAGCTGAAACAG	TGGCTCAGTTCAGCAGGAACAGT
16	cgr-miR-27a	MI0020473	AGGGCTTAGCTGCTTGTGAGCA	TTCACAGTGGCTAAGTTCCGC
17	cgr-miR-29b	MI0020479	GCTGGTTTCATATGGTGGTTTAGA	TAGCACCATTTGAAATCAGTGTTT
18	cgr-miR-30d	MI0020487	TGTAAACATCCCCGACTGGAAGC	CTTTCAGTCGGATGTTTACAGT
19	cgr-miR-31	MI0020489	AGGCAAGATGCTGGCATAGCTG	GCTATGCCAACATATTGCCATC
20	cgr-miR-34c	MI0020507	AGGCAGTGTAGTTAGCTGATTGC	AATCACTAACCACACGGCCAGG
21	cgr-miR-92a	MI0020560	AGGCTGGGATTTGTTGCAATGCT	TATTGCACTTGTCCCGGCCTGT
22	cgr-miR-375	NA	TTTGTTCGTTCGGCTCGCGTGA	GCGACGAGCCCCTCGCACAAAC
23	cgr-miR-9	MI0020559	TCTTTGGTTATCTAGCTGTATG	ATAAAGCTAGATAACCGAAAGT
24	cgr-miR-10b	MI0020382	TACCCTGTAGATCCGAATTTGT	ACAGATTCGATTCTAGGGGAAT
25	cgr-miR-7a	MI0016998	TGGAAGACTAGTGATTTTGTTGTT	CAACAAATCACAGTCTGCCATA

1.4 Screening of biological effects of engimiRs in CHO cells.

There are currently 365 identified miRNA genes in CHO cells from small RNA sequencing and the CHO-K1 genome data [35]. In theory at least 25 miRNAs out of these might be associated to growth and productivity by applying our selection approach. To test these candidates, a fast, robust and efficient genetic screening system had to be developed. Several known strategies were available for overexpression, such as miRNA mimics and miRNA hairpins [33, 44]. Mimics and miRNA hairpins are short synthetic RNA molecules similar either to the mature miRNA or precursor miRNA sequence, which are introduced in cells to achieve target knock down. However, the disadvantage of these strategies is the limited supply of synthetic molecules, the variation in effectiveness and as a major issue, the problem of scale-up to large culture volumes. In contrast, vector based expression

of miRNAs is an alternative strategy, which allows highly reproducible overexpression levels as well as long-term target gene knockdown based on expression under the control of selected promoters of various strengths. Since promoter activity is controlled by cellular state or physiology, miRNA overexpression ranges within physiological limits, which has the advantage of reducing off-target effects [45, 46]. Another advantage of expression vectors is the introduction of selectable markers, which provides the benefits of stable cell engineering. Considering these facts, we chose a vector based overexpression system to clone and express CHO specific miRNAs. To develop miRNA expression vectors, knowledge on the genomic locations for miRNA is necessary. However, at the time of development of our method, only the mature miR-5p and miR-3p sequences were available, generated by next-generation sequencing data from our lab specific for CHO cells, but no information on adjacent sequences or the CHO genome was published. Thus we designed chimeric pre-miRNA constructs with loop and flanking sequences from mouse miR-155, using the commercially available BLOCK-iT™ Pol II miR RNAi Expression vector (Invitrogen) for cloning of mature miRNA sequences from the miRNA sequencing study (Fig. 2). The expression vectors were characterized by high and reproducible transfection efficiencies of > 80%, resulting in endogenous levels of miRNA overexpression in the range of 1.5 to 2.5 fold over a period of 4 days. The miRNA levels achieved were lower compared to transient miRNA mimic transfection, but were in an acceptable range. Nevertheless, as proof of principle experiments, miRNAs from these constructs miR-221, miR-17 and miR-21 were chosen, as they were identified to be expressed at low, medium, and high abundance in CHO cells, respectively. This selection allowed evaluation of the dynamic range of miRNA overexpression from the pcDNA6.2-GW/EmGFP-miR system against differing endogenous miRNA levels in CHO cells (See publication in Appendix 2.1 B, Ref [47]).

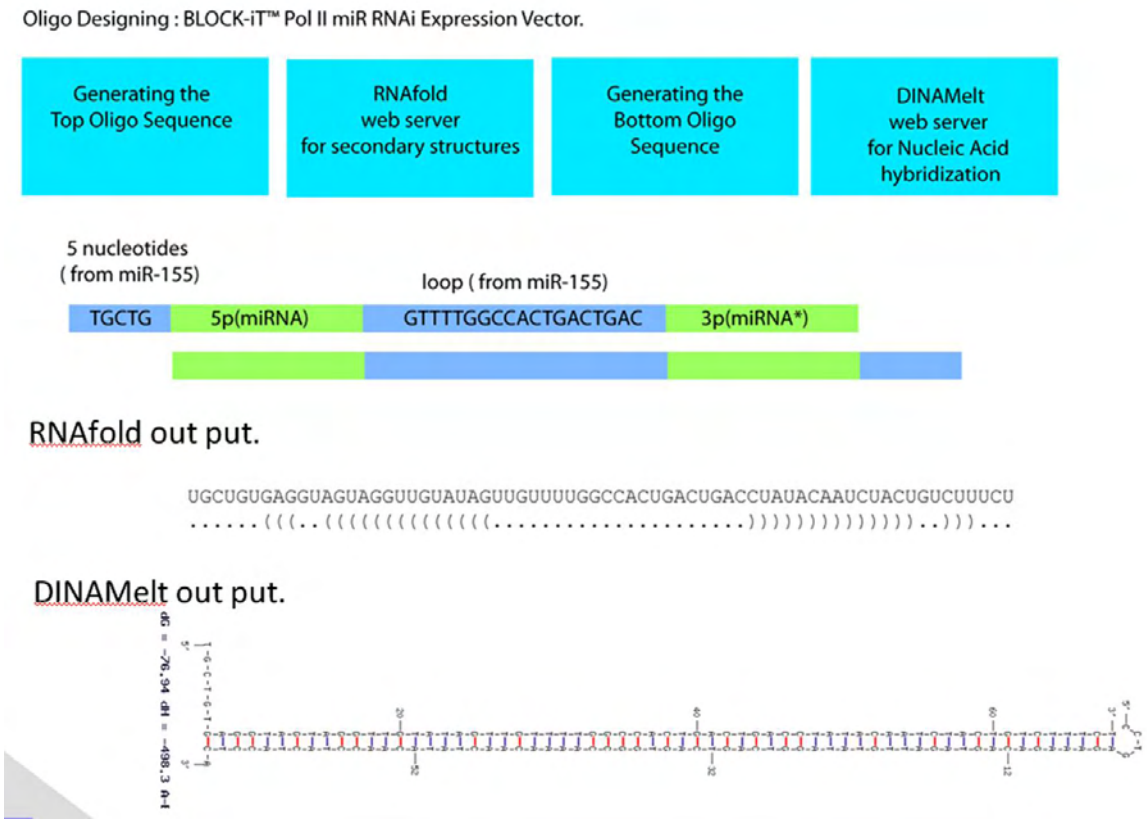

Figure 2. Scheme for design of the oligonucleotides for the artificial vector based expression system.

After transfection, cells were analyzed for growth and productivity over a 4-day period. Even from this small set of four miRNAs, the overexpression of miR-17, one of the members of the oncogenic miR-17-92a cluster, gave proof of principle that this method enables the identification of miRNA engineering candidates as its overexpression increased the speed of cell proliferation without negatively impacting specific productivity. This method can be readily applied for medium-throughput screening of microRNAs, or can be adapted to miR-sponge, siRNA, or mRNA overexpression for detailed functional characterization. As the same procedure can be applied to different production cell lines, the protocol can also be used to test for individual, cell line specific

responses to microRNAs. Thus our system represents a general platform to functionally screen candidates for rational cell factory design (See publication in Appendix 2.1 B, Ref [47]).

This screening platform was further used to test the effect on growth and protein production of 10 miRNAs out of the 25 candidate engimiRs (Table.1). Selected miRNAs were transfected and their influence on growth and recombinant protein production was recorded (fig. 3). Out of 10 miRNAs, only miR-17 had a positive influence on the growth rate over a period of 4 days, increasing μ by 23 (±5) %, (fig. 3A), which is consistent with our previous results [27]. The remaining miRNAs had no or little positive impact on growth rate compared to the transfection of an empty vector control. However, after 4 days we observed that miR-210, miR-101a and miR-23a overexpression had significantly reduced protein production, while let-7a and miR-17 showed a positive effect (fig. 3B), with miR-17 being the only miRNA that gave a significant, if minor, increase of 13% (±4.2) in protein titer. MiR-210, miR-101a and miR-23a might be good candidates for stable knock down to enhance CHO productivity by using a similar approach as used for improving apoptosis resistance and recombinant protein production in CHO cells [48].

MiR-17 is natively transcribed as a part of the miR-17-92a cluster, which comprises three families of miRNAs: miR-17/20a/18a, miR-19a/19b and miR-92a. The miRNAs produced by the miR-17-92a cluster are associated with malignancy and have been implicated in several human cancer types [49, 50]. Interestingly, another miRNA of this cluster, miR-92a, was found to be differentially regulated in serum-free and non-adherent growth of CHO cells relative to cells grown in serum-containing media [35, 36]. These results suggest that miR-17 might be the most promising candidate for stable overexpression in order to improve growth and recombinant protein production in CHO cells. While miR-17 and miR-92a were synthesized and cloned as described [47], overexpression of the entire miR-17-92a cluster was achieved using the human cluster whose

mature microRNA sequences are homologous to CHO, since CHO genomic sequences were still unavailable at that time.

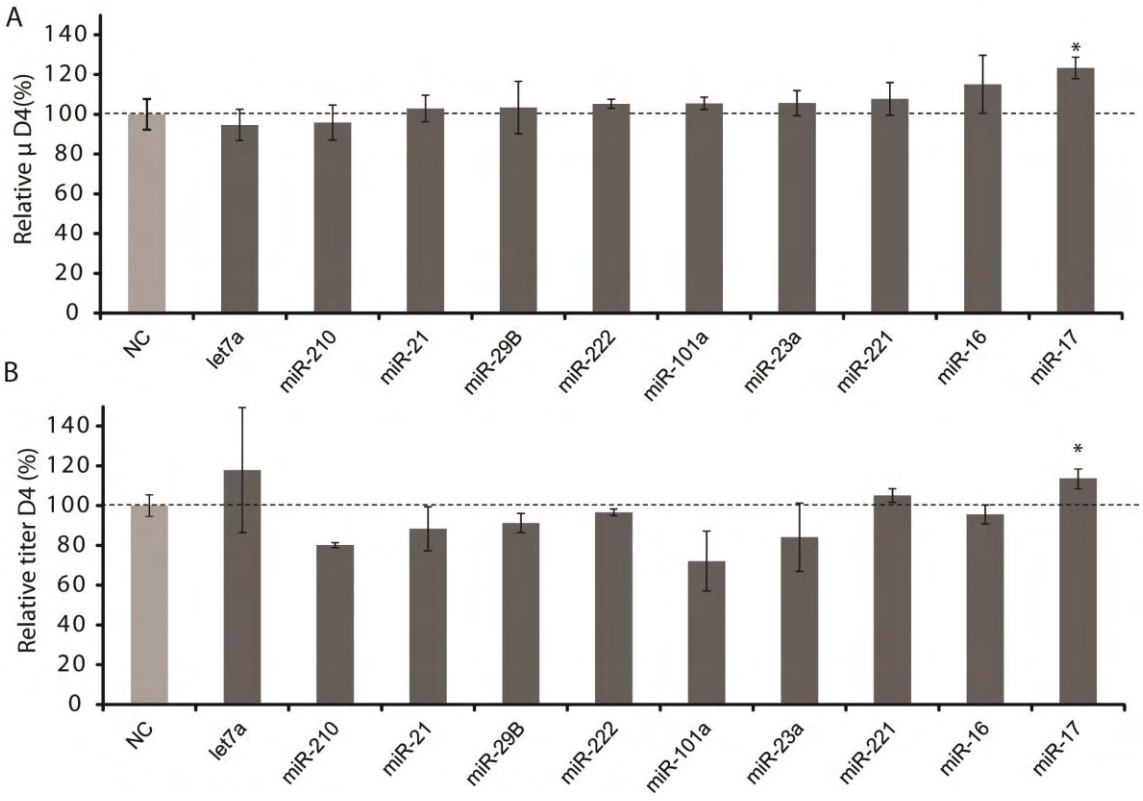

Figure 3. Effect of overexpression of selected miRNAs on proliferation rate and productivity in CHO-EpoFc cells. (A) Effect of miRNA overexpression on the growth rate is represented by the average growth rate (μ) calculated for 4 days of transient miRNA overexpression relative to the negative control transfection (NC). (B) Similarly, the effect of miRNA overexpression on recombinant protein production is represented by the EpoFc titer on day 4 after transfection. The data are based on three independent transfections with two technical replicates for each transfection. Error bar represents ± standard deviation (s.d.). *$P < 0.05$ to NC.

1.5 Stable engineering of miR-17 enhances protein production in CHO cells.

We have previously explored the effect of transient miRNA overexpression on growth and productivity of a recombinant EpoFc producing (EpoFc-CHO) model cell line and observed a positive effect of transient miR-17 overexpression on CHO cell growth without negatively affecting cell specific productivity [47]. 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. Moreover, the advantage of vector-based miRNA screening is that the same vectors can be used to generate stable miRNA overexpressing CHO cells within a short span of time. Thus, we generated stable miR-17, miR-92a and miR-17-92a cluster expressing pools by applying the mammalian selection marker gene present on the vector in recombinant EpoFc producing CHO cells. Such pools were preferred to clonal populations in order to reduce the bias by the inherent clonal variation observed in CHO cells [51]. Our results show that the positive effects seen in transient screening 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. Stable miR-17 overexpression confirmed a minor increase in growth rate, while at the same time resulted in 3 fold increased 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. The stable overexpression of miR-17 significantly enhanced productivity without any loss in growth, due to the result of slight improvement in specific growth rate in early exponential phase (See publication in Appendix 2.1 D, Ref [52]).

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. This parallel induction of both cell-specific growth rate and productivity is unique and requires more detailed investigation to understand the molecular mechanisms involved. This has not yet been explored in the present work and is currently underway as an extension of this work. A dissection of the detailed mechanisms of this effect of both enhanced growth and productivity will be valuable knowledge to understand the physiological role of this miR-17 and can be applied in a more refined strategy to engineering CHO cells for industrial applications. In any case, these results confirm that transient testing of miRNA effects enables time-efficient pre-selection of promising candidates for stable miRNA engineering of CHO cells for a further detailed analysis of their long term effects.

1.6 Identification of miRNA genome loci and applications in overexpression.

So far in our study, we showed applications of miRNAs to control complex cellular phenotypes such as growth and protein production. However, until recently the lack of genomic sequence information limited the overexpression strategy necessitating the use of heterologous mouse sequences for design of expression vectors for ectopic expression of miRNA. Even though this strategy was successful in the identification of miR-17 as an enhancer of recombinant protein production in CHO cells, we nevertheless observed low expression levels for engineered miRNAs, particularly in case of expression of miRNA clusters (i.e. expression of multiple miRNAs from a single transcript). With the publication of the genomic sequence and other relevant data sets for CHO cells since 2011, the doors were opened for an improved understanding of CHO cell physiology

and for the development of the necessary tools for novel, CHO specific engineering strategies [35, 53-56].

To this end, we developed computational tools to identify CHO genomic locations of miRNAs based on the available mature (415) miRNA sequences [35] and two independent CHO-K1 genomes [55, 56]. The developed computational pipeline was utilized to characterize 365 CHO mature miRNAs sequences and to assign them to a distinct genomic locus (See publication in Appendix 2.1 A, Ref [57]). This first comprehensive data set on genomic miRNA loci in CHO cells is now available at the public repository http://www.mirbase.org/. Furthermore, current data on the endogenous miRNA genomic sequences of CHO cells can be employed to improve existing tools to overexpress or to delete miRNAs, for identification of miRNA promoters and to improve quantifications methods.

In a constant effort to improve our overexpression strategy, we designed and compared chimeric sequences with CHO endogenous sequences for overexpression of miRNA clusters. Indeed, miRNA clusters with endogenous sequences yielded significantly higher expression levels of the mature miRNA (see publication in Appendix 2.2 F, Ref [57]). This data highlights the importance of miRNA endogenous sequence and genomic loci information for the targeted overexpression of clustered miRNAs in CHO cells [58], providing guidelines for next generation vector design.

1.7 Conclusion and future perspectives

The current thesis work provides details on the applicability and advancement of miRNA based cell engineering for production of biopharmaceuticals in CHO cells. Highlights of this study range from identification of potential engineering candidates to developing functional screening assays to successful stable engineering of cell lines. Furthermore, miR-17 was identified as an engimiR that is capable of enhancing both recombinant protein production and growth.

Engineering of CHO cells for enhanced phenotypes such as cell growth, apoptosis & death resistance, culture stress (oxidative, shear and osmotic) resistance, productivity and protein expression requires precise control and coordination of several physiological functions. miRNAs regulate post-transcriptional expression of mRNAs, thereby acting as global regulators targeting several mRNAs targets in a way similar to transcription factors [31]. As a new engineering targets, miRNAs can play a pivotal role in control of cellular phenotypes (see publication in Appendix 2.1 C, Ref [32]), which could also allow for better prediction of cell behavior in an industrial process.

Compared to other published studies on engineering of miRNAs in CHO cells [33, 34], the unique feature of miR-17 overexpressing cells is that both growth and productivity are enhanced, while in other cases typically it is either the one or the other.

For a more reliable extrapolation of miRNA function in CHO cells, miRNA: mRNA target databases specifically designed for CHO are required. For specific cases like miR-17, the analysis of the transcriptome as well as the proteome of miR-17-engineered CHO cells could provide insight on the effects of miRNA on protein expression during each stage of the batch culture, while methods like TAP-tar [59] will aid to identify direct miRNA:mRNA interactions in the absence of target databases. Such an approach was recently taken by Clarke et al., where integrated analysis was performed with respect to microRNA, mRNA and protein expression in a set of clones with variable growth rate [60]. In total, 35 miRNAs were identified to be up-regulated with increased growth, and 16 miRNAs that were down-regulated. By combining this information with mRNA and protein expression data, certain biological processes such as mRNA processing and protein synthesis were found to be relevant for enhanced proliferation. Only by looking at multiple layers of regulation and effect can a complete picture of the molecular mechanisms of miRNA control be obtained.

In summary, the present work along with contemporary studies [61, 62] provides the basis for advancement of utilizing miRNAs as CHO cell engineering tools, thus emerging as a novel cell engineering strategy. However, miRNAs are by no means the only interesting non-coding RNAs that could be used for cell line optimization, particularly for improvement of genomic stability and epigenetic control. An example are piwi-interacting RNA (piRNA) that were also identified in CHO cells [63]. This type of small non-coding RNAs have been associated with epigenetic and post-transcriptional gene silencing mechanisms [64]. No publication so far exists on the expression of long non-coding RNAs or other classes of RNAs in CHO. Taken together, non-coding RNA molecules hold great promise to enable exciting new approaches in optimising CHO cell factories by avoiding translational burdening for robust industrial purposes. The methods and tools developed in the present work can be further applied in this direction.

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2.0 Appendix – Thesis relevant Publications

2.1. List of First-Author Publications

A. Computational Identification of microRNA Gene Loci and Precursor microRNA Sequences in CHO Cell Lines

Matthias Hackl, **Vaibhav Jadhav**, Tobias Jakobi, Oliver Rupp, Karina Brinkrolf, Alexander Goesmann, Alfred Pühler, Thomas Noll, Nicole Borth and Johannes Grillari. *Journal of Biotechnology* 158, no. 3 (2012): 151-155.

B. A Screening Method to Assess Biological Effects of microRNA Overexpression in Chinese Hamster Ovary Cells.

Vaibhav Jadhav, Matthias Hackl, Juan A Hernandez Bort, Matthias Wieser, Eva Harreither, Renate Kunert, Nicole Borth, and Johannes Grillari. *Biotechnology and Bioengineering* 109, no. 6 (2012): 1376-1385.

C. CHO microRNA Engineering Is Growing Up: Recent Successes and Future Challenges.

Vaibhav Jadhav, Matthias Hackl, Aliaksandr Druz, Smriti Shridhar, Cheng-Yu Chung, Kelley M Heffner, David P Kreil, Mike Betenbaugh, Joseph Shiloach, and Niall Barron, Johannes Grillari, and Nicole Borth. *Biotechnology Advances* 31, no. 8 (2013): 1501-1513.

D. Stable Overexpression of mir-17 Enhances Recombinant Protein Production of CHO Cells.

Vaibhav Jadhav, Matthias Hackl, Gerald Klanert, Juan A Hernandez Bort, Renate Kunert, Johannes Grillari and Nicole Borth. *Journal of Biotechnology*, (2014):175C:38-44.

2.2. List of Co-Authored Publications.

E. Dynamic mRNA and miRNA Profiling of CHO-K1 Suspension Cell Cultures.

Juan A Hernández Bort, Matthias Hackl, Helga Höflmayer, **Vaibhav Jadhav**, Eva Harreither, Niraj Kumar, Wolfgang Ernst, Johannes Grillari, and Nicole Borth. *Biotechnology Journal* 7, no. 4 (2012): 500-515.

F. Endogenous microRNA Clusters Outperform Chimeric Sequence Clusters in Chinese Hamster Ovary Cells.

Gerald Klanert, **Vaibhav Jadhav**, Konstantina Chanoumidou, Johannes Grillari, Nicole Borth, and Matthias Hackl. *Biotechnology Journal*, (2013).

G. Analysis of microRNA Transcription and Post-Transcriptional Processing by Dicer in the Context of CHO Cell Proliferation.

Matthias Hackl, **Vaibhav Jadhav**, Gerald Klanert, Michael Karbiener, Marcel Scheideler, Johannes Grillari, and Nicole Borth. *Journal of Biotechnology*, (2014). Jan 28. pii:S0168-1656(14)00037-6.

Appendix A

Computational Identification of microRNA Gene Loci and Precursor microRNA Sequences in CHO Cell Lines.

Journal of Biotechnology 158 (2012) 151–155

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Short communication

Computational identification of microRNA gene loci and precursor microRNA sequences in CHO cell lines

Matthias Hackl[a,1], Vaibhav Jadhav[a,1], Tobias Jakobi[b], Oliver Rupp[b], Karina Brinkrolf[b], Alexander Goesmann[b], Alfred Pühler[b], Thomas Noll[c], Nicole Borth[a,d], Johannes Grillari[a,*]

[a] Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria
[b] Centrum für Biotechnologie, Universität Bielefeld, 33594 Bielefeld, Germany
[c] AG Zellkulturtechnik, Technische Fakultät, Universität Bielefeld, 33549 Bielefeld, Germany
[d] ACIB GmbH, Austrian Centre of Industrial Biotechnology, Graz, Austria

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ABSTRACT

MicroRNAs (miRNAs) have recently entered Chinese hamster ovary (CHO) cell culture technology, due to their severe impact on the regulation of cellular phenotypes. Applications of miRNAs that are envisioned range from biomarkers of favorable phenotypes to cell engineering targets. These applications, however, require a profound knowledge of miRNA sequences and their genomic organization, which exceeds the currently available information of ~400 conserved mature CHO miRNA sequences. Based on these recently published sequences and two independent CHO-K1 genome assemblies, this publication describes the computational identification of CHO miRNA genomic loci. Using BLAST alignment, 415 previously reported CHO miRNAs were mapped to the reference genomes, and subsequently assigned to a distinct genomic miRNA locus. Sequences of the respective precursor-miRNAs were extracted from both reference genomes, folded *in silico* to verify correct structures and cross-compared. In the end, 212 genomic loci and pre-miRNA sequences representing 319 expressed mature miRNAs (approximately 50% of miRNAs represented matching pairs of 5′ and 3′ miRNAs) were submitted to the miRBase miRNA repository. As a proof-of-principle for the usability of the published genomic loci, four likely polycistronic miRNA cluster were chosen for PCR amplification using CHO-K1 and DHFR (-) genomic DNA. Overall, these data on the genomic context of miRNA expression in CHO will simplify the development of tools employing stable overexpression or deletion of miRNAs, allow the identification of miRNA promoters and improve detection methods such as microarrays.

Chinese hamster ovary (CHO) cells are currently the first choice mammalian cell line for the production of complex therapeutic proteins requiring proper folding and post-translational modifications, creating an annual revenue exceeding 100 billion USD (Mudhar, 2006). With the publication of a CHO-K1 draft genome (Xu et al., 2011), as well as thorough analysis of the CHO mRNA transcriptome (Becker et al., 2011), the basis for genomic characterization of CHO cells has been set and will allow the development of novel tools to rationally design CHO cells as bioindustrial work horses.

Therefore, microRNAs (miRNAs) have been discussed as promising tools for CHO cell characterization as well as engineering (Barron et al., 2011). This family of small non-coding RNAs, which by now encompasses more than 1000 sequences for mouse and human (Griffiths-Jones, 2010), acts by negative regulation of gene expression due to post-transcriptional repression of mRNA translation (Hüttenhofer and Schattner, 2006). The ~22 nt long mature miRNAs that catalyze this repression are the result of enzymatic processing of a primary RNA-Polymerase II miRNA transcript: in the nucleus, RNase III Drosha together with Dgcr8 cleave a ~70 nt long single-stranded RNA referred to as precursor miRNA (pre-miR) or miRNA hairpin/stem-loop due to its characteristic secondary structure (Gregory et al., 2004). Pre-miRNAs are exported into the cytoplasm where cleavage of the loop by the RNase Dicer generates a duplex of two ~22 nt long mature miRNAs (Takeshita et al., 2007). The partial sequence complementarity underlying the miRNA:mRNA interaction, allows single miRNAs to bind up to 100 distinct mRNAs (Selbach et al., 2008), thus potentially orchestrating the expression of whole gene networks similar to transcription factors. This range in target regulation achieved by individual miRNAs is mirrored in their biological relevance, which includes control of cellular proliferation and energy metabolism as well as stress resistance and cell death (Müller et al., 2008).

* Corresponding author. Tel.: +43 1 47654 6230.
E-mail address: johannes.grillari@boku.ac.at (J. Grillari).
[1] These authors contributed equally.

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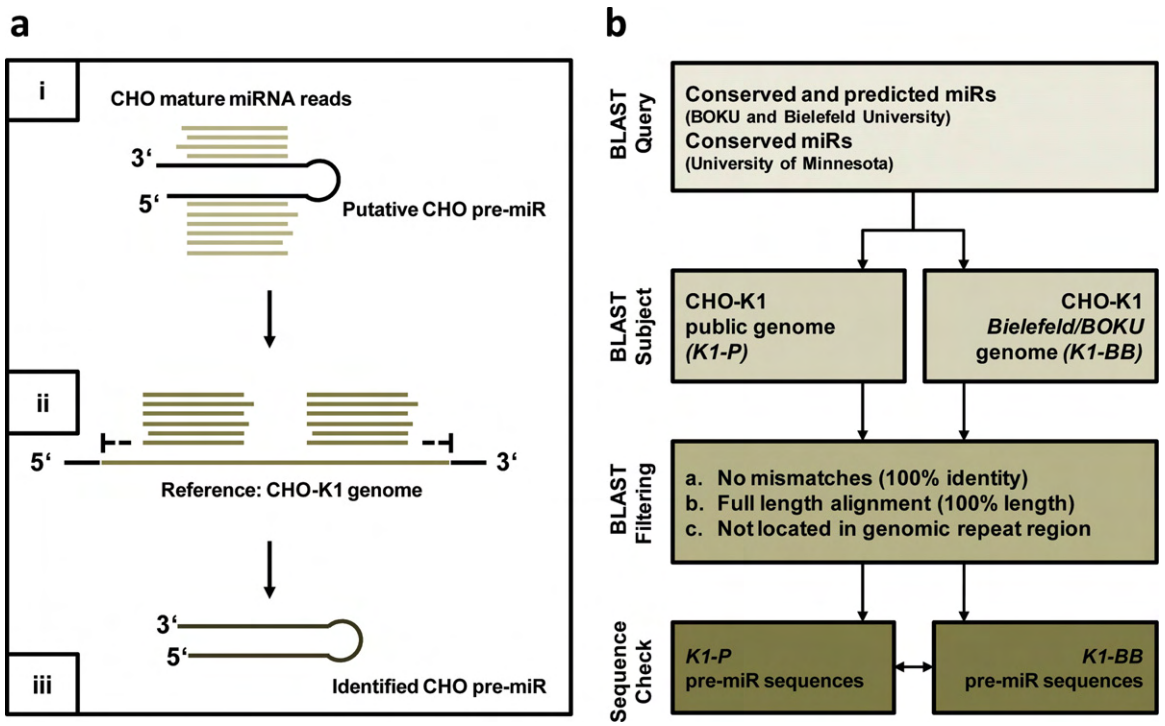

Fig. 1. Strategy for identification of CHO pre-miR sequences from genomic references. (a) Schematic outline of identification strategy. (b) Flow chart illustrating the sequence identification strategy in detail: all currently published CHO mature miRNA sequences were BLAST-aligned to two independent CHO-K1 genomic reference sequence assemblies (*K1-P and K1-BB*). BLAST results were filtered for alignments with zero mismatches (100% identity) and alignment lengths equal to the mature miRNA length (100% length). Additionally, miRNAs mapping to genomic repeat regions were removed. From the remaining genomic loci, the respective pre-miRNA sequences were extracted independently from both genomic references and cross-checked.

Two studies have so far addressed the identification and annotation of CHO miRNAs, and independently reported the expression of 350 (Johnson et al., 2011) and 365 (Hackl et al., 2011) mature miRNAs, but have not identified the respective genomic loci or pre-miRNA sequences. This information is, however, necessary for (i) mimicking endogenous miRNA expression, since pre-miRNA secondary structures can be target to regulation of miRNA stability (Michlewski et al., 2008), for (ii) understanding transcriptional regulation of specific miRNAs, as well as for (iii) phylogenetic analyses.

Based on the alignment of a combined set of previously reported mature miRNA sequences against two independent CHO-K1 genome reference sequences, we here report the identification of miRNA gene loci and extraction of the respective pre-miRNA sequences from both genomic references, followed by cross-comparison of the derived sequences (Fig. 1). In detail, the employed strategy used two public datasets containing sequences of mature CHO miRNAs with expression levels detectable by next-generation sequencing (Johnson et al., 2011; Hackl et al., 2011). Both datasets were downloaded, reduced by redundant isomiR sequences as well as recently reported non-coding RNAs in miRBase version 18.0 (Griffiths-Jones, 2010), and then merged into one dataset containing 415 miRNAs of which 22 were putative novel miRNA sequences. These sequences were further used as “query” (given in Supplementary Data 1) for BLAST alignment against two distinct CHO genome references using blastn with nucleotide mismatch penalty −2, and nucleotide match reward +1. The first reference consisted of the recently published CHO-K1 sequence (Xu et al., 2011) hereafter referred to as “*K1-P*” (for “public”) and the second reference being a low coverage, so far unpublished CHO-K1 genome assembly by Bielefeld University and BOKU University referred to as “*K1-BB*” (Table 1). In brief, the K1-BB genome (ATCC CCL-61) was sequenced on an Illumina Genome Analyser IIx in a 2 × 125 bp sequencing run on six lanes according to the manufacturer’s manuals. Sequencing resulted in 411 million reads and 51 Gbp which leads to an estimated genome coverage of 17-fold considering a genome size of 3 Gbp. Assembly of the sequence data was performed with velvet 1.0.4 resulting in 11.4 million contigs that can be downloaded at ftp://ftp.cebitec.uni-bielefeld.de/pub/supplements/2011/Hackl_JBiotech/.

Following filtering of BLAST alignments (Fig. 1), a total of 365 out of 415 distinct mature miRNAs could be mapped to either genomic reference. In detail, 353 distinct mature miRNAs gave a perfect BLAST hit against the *K1-P* reference, while 330 miRNAs could be aligned to the *K1-BB* reference with an overlap of 318 miRNAs, shown as Venn diagram in Fig. 2a (Hulsen et al., 2008). While the majority of miRNAs exhibited a single exact match in the reference genome, some miRNAs exhibited two or more exact matches (Fig. 2b). This might have biological reasons, since duplications of miRNA genes are known to result in 100% identical paralogous sequences present in other parts of the genome (Gardner et al., 2009). Alternatively, the observed multiple hits could be a consequence of incomplete assembly of the genomic references. This would explain the reduction in multiple perfect matches from the incompletely assembled 2.9 Gbp *K1-BB* genome to the almost completely assembled 2.45 Gbp *K1-P* genome from 28% to 16% of the aligned miRNAs (Fig. 2b). Nevertheless, 15 miRNAs exhibited more

Table 1
Genome references for identification of CHO pre-miR sequences.

	K1-P	K1-BB
Genome size (Gbp)	2.40	2.98
Contigs	109,151	11,400,490
Average contig length	21,986	261
Median contig length	503	124.5
x Coverage	95	17.1

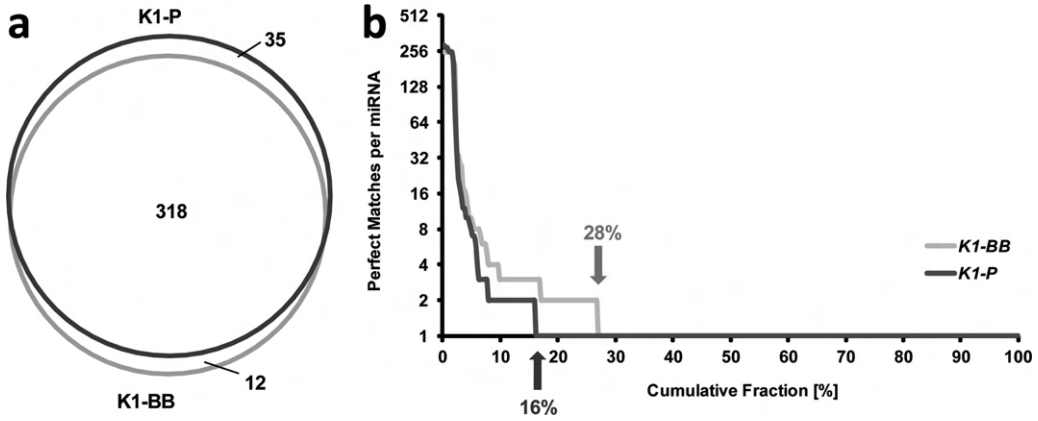

Fig. 2. BLAST alignment of mature miRNAs to two different genomic reference sequences. (a) Size-adjusted Venn diagram indicating that 318 mature miRNAs were aligned to both reference genomes, while 35 and 12 mature miRNAs could only be aligned to *K1-P* or *K1-BB*, respectively. (b) The cumulative fraction of BLAST-aligned miRNAs is plotted against the number of perfect genomic matches identified; for each miRNA; 16% and 28% of miRNAs could be perfectly aligned to two or more genomic locations in the *K1-P* (black) or *K1-BB* (gray) genomic reference sequence.

than 10 and up to 250 perfect matches (Supplementary Table 1), which indicates that these are repeat derived small RNAs rather than canonical miRNAs. Hence, these miRNAs were removed from the BLAST results and not considered for further analysis as well as submission to miRBase.

In the next step, the genomic locations of BLAST aligned mature miRNAs were analyzed in detail to identify the respective pre-miRNA sequences (Fig. 3a): genomic locations where two miRNAs could be aligned in close proximity indicate miRNA genes from which two mature miRNAs – corresponding to the 5′ and 3′ miRNA – are produced. Other genomic loci were mapped by only one miRNA, suggesting the expression of just one active mature miRNA, which is either derived from the 5′ or 3′ arm of the hairpin. Approximately 50% of the genomic loci were identified by alignment of both 5′ and 3′ mature miRNAs (Fig. 3b). For these genomic loci the pre-miRNA sequence lengths was estimated as the length from the 5′ miRNA start to the 3′ miRNA end. The resulting sequence lengths were plotted against the cumulative fraction of the number of pre-miRNAs, showing that the majority (>95%) of hairpins exhibited a length between 50 and 70 bases (Fig. 3c).

Since it has been shown that Drosha cleavage is dependent on the hairpin loop rather than consensus sequences in the flanking regions, the precise start and stop sites of a pre-miRNA are difficult to determine (Zeng et al., 2005). Therefore, an arbitrary distance of 10 bases upstream the 5′ miRNA and 10 bases downstream the 3′ miRNA was included as "buffer" during sequence extraction from the genomic references (Fig. 3a). Based on the observation that most pre-miRNA sequences ranged between 50 and 70 bases, an

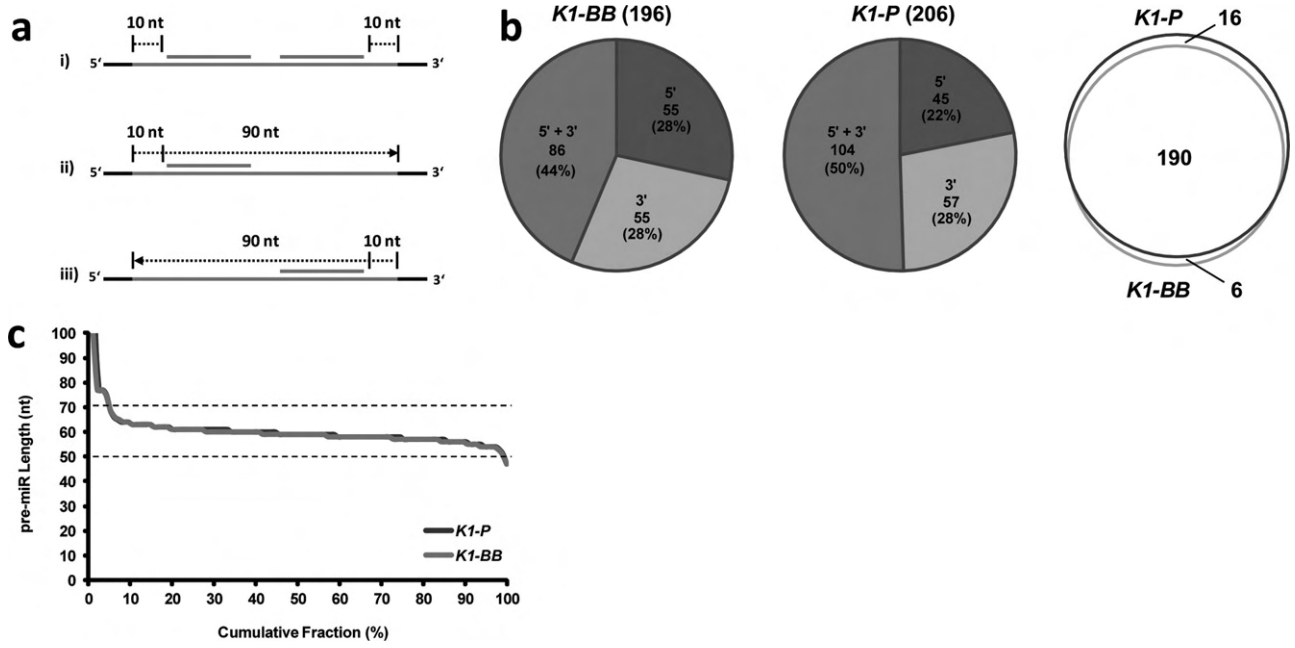

Fig. 3. Characterization of CHO pre-miRNA sequences. (a) Scheme representing the strategy for pre-miRNA sequence extraction from a genomic locus mapped by either one or two mature miRNAs: (i) a buffer of 10 bases up- and downstream the mature miRNAs was taken in case both hairpin-arms were mapped. (ii) and (iii) For genomic positions aligned by a single miRNA a total pre-miRNA of 100 bases was extracted, starting 10 bases upstream a 5′ miRNA match or 10 bases downstream a 3′ match. (b) Distribution of CHO miRNA loci identified by alignment of either 5′ or 3′ mature miRNAs or both is shown. Venn overlap of miRNA genomic loci as identified independently in each CHO-K1 genomic reference sequence. (c) For pre-miRNA genomic loci mapped at both the 5′ and 3′ miRNA hairpin-arm, length of the pre-miRNA was calculated as the distance between the start of the 5′ miRNA alignment and the end of the 3′ miRNA alignment. Cumulative fraction of pre-miRNAs is plotted against the pre-miRNA length, showing that for most pre-miRs length ranged between 50 and 70 bases.

arbitrary sequence length of 100 bases was defined for pre-miRNAs with only one expressed miRNA detected (*i.e.* only one hairpin-arm mapped by a mature miRNA), including a buffer of 10 bases upstream or downstream the miRNA start site (Fig. 3a). The important information whether a single match represented a 5′ or 3′ miRNA was derived from orthologous pre-miRNAs (mainly human, mouse or rat) in miRBase. In order to verify sequence correctness, all CHO pre-miRNA sequences were folded *in silico* using the DINAMelt webserver that is based on the mfold++ software (Markham and Zuker, 2005). Manual curation of all folding resulted in the removal of 7 putative novel CHO pre-miRs that did not resemble structures of canonical miRNAs with a complementary stemloop and 3′ overhangs, while all of the conserved CHO pre-miRs (209 sequences) as well as three novel pre-miRs passed manual curation. The respective 212 RNA secondary structures are provided as Supplementary Data 2. Table 3 exemplarily gives the pre-miRNA sequences of all 6 miRNAs belonging to the miR-17-92 cluster, which were identified in close proximity on one genomic scaffold.

Comparison of pre-miRNA sequences derived from two CHO-K1 genomic references (*K1-P* and *K1-BB*) gave four sequences with either one or two mismatches, of which only mir-486 harbored a potential single-nucleotide polymorphism (SNP) within a mature miRNA (Supplementary Table 2). In the other cases, SNPs were identified in the hairpin-loop (mir-324 and mir-486) or regions flanking mature miRNAs to the 5′ (mir-1956) or 3′ end (mir-542). Conservation of CHO pre-miRNA sequences was estimated for mir-17-92 by calculating ClustalW alignments (Thompson et al., 1994) to the respective sequences from *Mus musculus*; the results indicate high conservation for mir-18a and mir-19b, while several mismatches were found between mouse and CHO hairpin-loops of mir-17, mir-20a, and mir-92a, respectively (Fig. 4a). Supplementary Data 3 gives the sequences of all 212 miRNA hairpins, as they were extracted from the *K1-P* and *K1-BB* genomic reference as well as the respective genomic location. To show that the here provided information can easily be applied to amplify and clone CHO miRNAs, four distinct clusters of miRNAs were chosen for PCR amplification using primers designed based on the *K1-P* genomic reference (Supplementary Table 2). Genomic DNA isolated

Table 2
Number of aligned miRNAs, unique genomic loci and precursor-miRNA sequences.

	K1-P	K1-BB
miRNAs mapped to genome (100% ID, 100% length)	353	330
miRNAs mapped to genomic repeat regions	14	15
miRNAs used for identification of genomic loci and pre-miRNAs	339	315
High confidence genomic miRNA loci[a]	206	196
pre-miRNA sequences submitted to miRBase[b]	206	6

[a] After removal of loci that give rise to incorrectly folded pre-miRs.
[b] In total 212 pre-miRNA sequences submitted to miRBase.

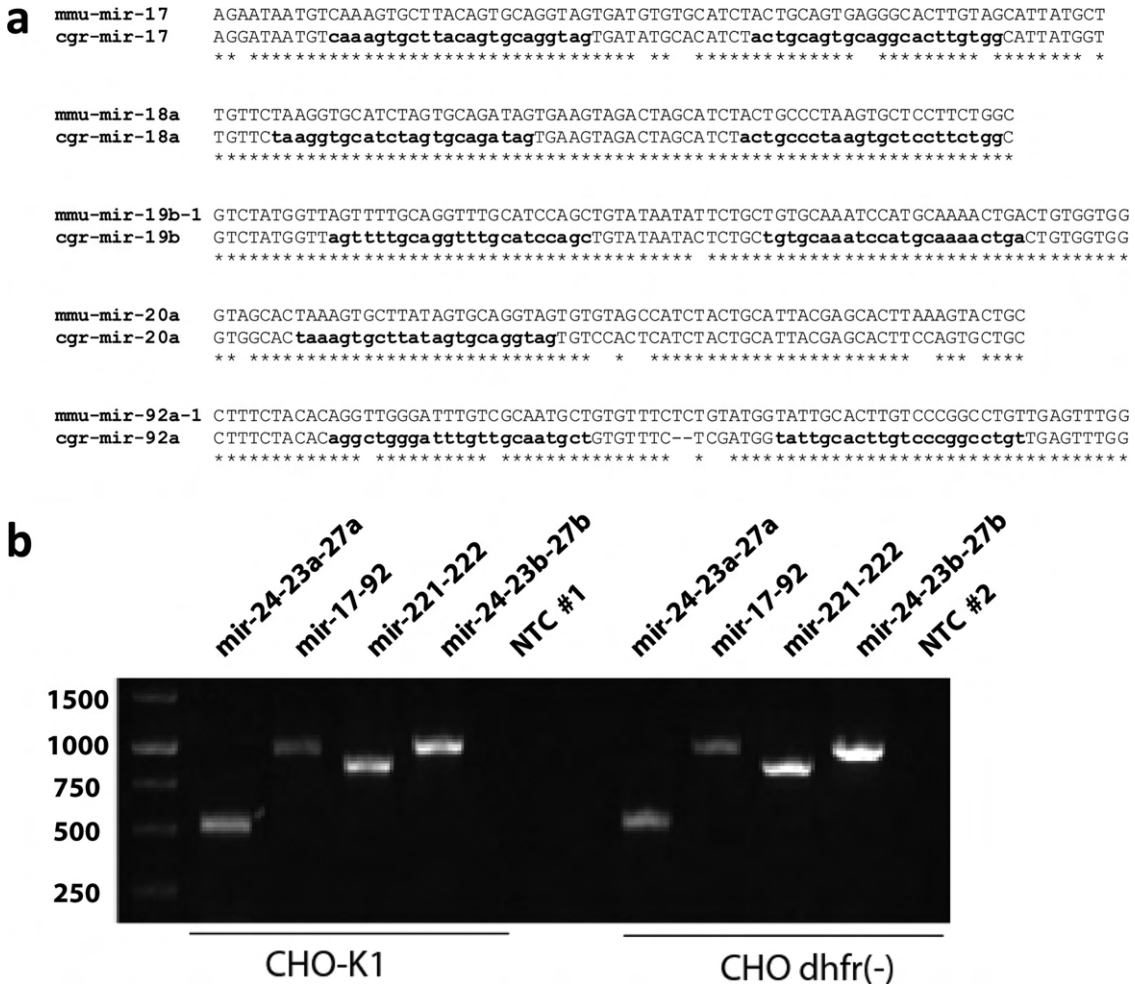

Fig. 4. Sequence characterization of CHO pre-miRNAs. (a) Conservation CHO (cgr) mir-17-92 pre-miRNAs in respect to *Mus musculus (mmu)*; *, sequence matches; - sequence deletions. (b) PCR amplification of miRNA clusters: PCR amplification of miRNA clusters using genomic DNA from CHO-K1 and CHO dhfr (-) cells. Lanes 1–4 and 6–9 showing specific amplification for miR-24-23a (1/6), miR-17-92a, miR-221-222 and miR-24-23b. Lanes 5 and 10 no template control PCR.

Table 3
miR-17-92 pre-miRNA sequences.

>cgr-mir-17_scaffold_gi|344163086|gb|JH001979.1|_REV
AGGATAATGTcaaagtgcttacagtgcaggtagTGATATGCACATCTactgcagtgcaggcacttgtggCATTATGGT
>cgr-mir-18a_scaffold_gi|344163086|gb|JH001979.1|_REV
CTTTTTGTTCtaaggtgcatctagtgcagatagTGAAGTAGACTAGCATCTactgccctaagtgctccttctggCATAAGAAG
>cgr-mir-19a_scaffold_gi|344163086|gb|JH001979.1|_REV
GCAGCCCTCTGTTAGTTTTGCATACTTGCACTACAAGAAGAATGCAGTtgtgcaaatctatgcaaaactgaTGGTGGCCT
>cgr-mir-19b_scaffold_gi|344163086|gb|JH001979.1|_REV
GTCTATGGTTagttttgcaggtttgcatccagcTGTATAATACTCTGCtgtgcaaatccatgcaaaactgaCTGTGGTGG
>cgr-mir-20a_scaffold_gi|344163086|gb|JH001979.1|_REV
TCTGTGGCACtaaagtgcttatagtgcaggtagTGTCCACTCATCTACTGCATTACGAGCACTTCCAGTGCTGCCAGCTGGAGAGCCCCAGCCTCGCTCG
>cgr-mir-92a_scaffold_gi|344163086|gb|JH001979.1|_REV
CTTTCTACACaggctgggatttgttgcaatgctGTGTTTCTCGATGGtattgcacttgtcccggcctgtTGAGTTTGG

Lower case letters indicate mature miRNAs; upper case letters indicate 5′ and 3′ flanking regions as well as loop regions.

from adherent CHO-K1 and DHFR (-) cell lines cultivated at 37 °C at 7% atmospheric CO_2, served as template for the PCR reaction that gave specific bands at the expected size (Fig. 4b).

Overall, these data demonstrate a successful identification of the genomic location of 365 out of 415 (88%) expressed mature miRNA sequences. After exclusion of 15 miRNAs due to multiple alignments to genomic repeat regions, 350 miRNAs remained for annotation of genomic loci based on miRNA alignment patterns. After manual verification of miRNA-like RNA secondary structures, a total of 212 miRNA loci as well as the respective pre-miRNA sequences were identified with high confidence (Table 2), cross-checked to confirm correctness of sequences, and provided as Supplementary Data to this publication. In addition all sequences were submitted to the miRBase database (Griffiths-Jones, 2010) for assignment of miRBase accession numbers (Supplementary Data 3).

This data can now be used to establish CHO specific tools for miRNA overexpression as engineering strategy using endogenous pre-miRNA sequences, which do show differences in nucleotide sequence compared to mouse homologs (Fig. 4b). In addition, the development of knockout strategies to specifically reduce miRNA expression will benefit from these data, and finally, knowledge of the genomic loci also allows amplification and cloning of polycistronic miRNA clusters that are likely to have a stronger influence on CHO cell phenotypes upon overexpression compared to single miRNAs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2012.01.019.

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Appendix B

A Screening Method to Assess Biological Effects of microRNA Overexpression in Chinese Hamster Ovary Cells.

ARTICLE

BIOTECHNOLOGY AND BIOENGINEERING

A Screening Method to Assess Biological Effects of MicroRNA Overexpression in Chinese Hamster Ovary Cells

Vaibhav Jadhav,[1] Matthias Hackl,[1] Juan A. Hernandez Bort,[1,2] Matthias Wieser,[2] Eva Harreither,[1] Renate Kunert,[1] Nicole Borth,[1,2] Johannes Grillari[1]

[1]Department of Biotechnology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria; telephone +43-1-47654-6230; fax: +43-1-47654-6675; e-mail: johannes.grillari@boku.ac.at

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

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ABSTRACT: MicroRNAs (miRNAs) are a novel class of short non-coding RNAs, which negatively regulate target gene expression at post-transcriptional level. They mediate an important layer of control in the global regulation of gene networks, controlling a broad range of physiological as well as patho-physiological pathways including development, cancer, metabolism, proliferation, and stress resistance. So far, more than 365 miRNA genes have been identified in CHO cells. The functional analysis of the physiological effect of such large numbers of miRNAs, however, requires an efficient functional screening method. In the current study, we therefore established and evaluated a protocol to perform miRNA overexpression and to screen their effect on bio-industrially relevant phenotypes, such as growth, viability and productivity, using a recombinant, Epo-Fc producing CHO cell line. For protocol optimization, four CHO miRNAs (cgr-miR-17, cgr-miR-221, cgr-miR-21, and cgr-miR-210) were cloned into small hairpin vectors including a GFP cassette and transfected. After transfection cells were analyzed for growth and productivity over a 4-day period. Even from this small set of four miRNAs, the overexpression of miR-17, one of the members of the oncogenic miR-17-92 cluster, gave proof of principle that this method enables the identification of miRNA engineering candidates as its overexpression increased the speed of cell proliferation without negatively impacting specific productivity. The here presented method is applicable for medium-throughput screening for microRNA, miR-sponge, siRNA, or mRNA overexpression along with detailed functional characterization using the same experimental set up. As the same procedure can be applied to different production cell lines, the protocol can also be used to test for individual, cell line specific responses to microRNAs. Thus our system represents a general platform to functionally screen candidates for rational cell factory design.

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KEYWORDS: microRNA; Chinese hamster ovary cells; functional screening; miR-17; engimiR

Introduction

The global demand for recombinant therapeutic proteins is ever increasing in the biopharmaceutical industry (Walsh, 2010). To date, Chinese hamster ovary (CHO) cells are the most extensively used mammalian cell factory for the production of therapeutic proteins due to their ability to produce human-similar glycosylated and folded proteins in accordance with the required safety standards (Jayapal et al., 2007). Over the last decade, several strategies have been developed with the aim to improve growth and recombinant protein production characteristics as well as to shorten the timelines for cell line development (DeMaria et al., 2007; Omasa et al., 2010). The most commonly pursued strategies include optimization of media components and vector design and high-throughput screening to identify highly productive cells (Codamo et al., 2011; Gaillet et al., 2010; Xing et al., 2011). Additionally, the targeted engineering of biological pathways such as transcription, translation, signaling and growth by overexpression of mRNAs, siRNAs and lately also of microRNAs (miRNAs) has been proposed (Barron et al., 2011a; Dreesen and Fussenegger, 2011; Druz et al., 2011; Lim et al., 2006; Muller et al., 2008; Zhou et al., 2011).

MiRNA mediated regulation has recently emerged as a key regulatory layer controlling gene activity in the cell

Vaibhav Jadhav and Matthias Hackl contributed equally to this work.

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(Ambros, 2004; Ambros and Lee, 2004). MiRNAs are short non-coding RNAs, which negatively regulate the expression of genes or entire gene networks at the post-transcriptional level (Bartel, 2004; Bartel, 2009). So far, more than 1,000 miRNA genes have been identified in mammalian genomes, which are transcribed and processed in the nucleus to give rise to hairpin loop structured single-stranded RNAs (~70 nt) called precursor-miRNA (pre-miRNA). After transport into the cytoplasm, the pre-miRNA is enzymatically cleaved by Dicer to generate a single stranded ~22 nt mature miRNA, which is further incorporated into the miRNA induced silencing complex (miRISC) (Cullen, 2004). The mature miRNA in the RISC complex is an active molecule, which can bind to 3′-untranslated regions (3′-UTR) of target mRNAs with full or partial base complementarity, thus inhibiting protein production by repression of mRNA translation or by inducing mRNA degradation (Subramanyam and Blelloch, 2011). The partial base complementary nature of a miRNA to its target allows miRNAs to repress large numbers of different mRNAs simultaneously, thereby potentially modulating whole biological pathways (Bartel, 2009; Lindow, 2011). Large numbers of studies have shown that miRNAs along with their mRNA target binding sites are conserved across species, thus emerging as an independent layer of gene regulation, controlling many fundamental biological processes including cell proliferation, differentiation, development, metabolism and apoptosis (Carleton et al., 2007; Cohen, 2010; Fatica et al., 2006; Fernandez-Hernando et al., 2011; Friedman et al., 2009; Krutzfeldt and Stoffel, 2006; Sayed and Abdellatif, 2011; Subramanian and Steer, 2010).

In addition to miRNAs being intensively studied in the context of human development and disease, they have recently emerged as promising molecules in the field of cell line engineering and cell line development (Barron et al., 2011b; Muller et al., 2008). The first report pointing towards a possible application of miRNAs in cell engineering, in order to regulate cell growth and proliferation and to enhance productivity, used overexpression of miR-7 mimics in CHO cells (Barron et al., 2011a). This was shown to significantly decrease cell growth, while at the same time specific productivity was increased, thus mimicking cell phenotypes observed upon temperature shift. Similarly, it was shown that the miR-297-669 cluster is up-regulated in cells exposed to nutrient depletion (Druz et al., 2011). As one of the up-regulated members of the miR297-669 cluster, mmu-miR-466h was found to target several anti-apoptotic genes, therefore potentially contributing to the onset of apoptosis upon nutrient depletion. Indeed, the study could confirm the pro-apoptotic effect of miR-446h in CHO by inhibiting its activity, which resulted in increased cell viability and decreased caspase-3/7 activity. These findings strongly support the potential role of miRNA engineering for cell line development and optimization. We here propose the term engimiR to be used to describe the engineering of miRNA expression for phenotypic modification of cells, similarly to the term oncomiRs, which is used to describe miRNAs that are involved in the development of cancer (Folini et al., 2010; Woods et al., 2007).

The application of next generation sequencing technologies together with novel approaches to miRNA identification and annotation have shown that there are 365 highly conserved miRNAs expressed in CHO cells (Hackl et al., 2011). In addition, several studies have reported the successful use of cross-species microarray and qPCR platforms to measure miRNA expression in CHO cells (Bort et al., 2011; Gammell et al., 2007). Their findings strongly support an important role for miRNAs as tools in cell line engineering and cell line development. Therefore, an efficient functional screening method is required to identify those miRNAs that positively influence their bio-industrially relevant characteristics.

Here, we describe a reliable and rapid functional screening method employing transient overexpression of individual miRNAs to assess their effect on growth and productivity in a recombinant CHO cell line. For evaluation of this functional screening system, four miRNA candidates, miR-17, miR-221, miR-210, and miR-21, were tested. One criterion for this selection was that these four miRNAs are expressed at different levels, representing the wide range of miRNA expression levels observed in CHO cells. MiR-17 is a member of the miR-17-92 cluster, an oncogenic cluster shown to be amplified in several human cancers (Grillari et al., 2010; Wang and Xu, 2011; Yang et al., 2010). Similarly, upregulation of miR-221 and miR-21 recently has been described in several types of human tumors and miR-210 is overexpressed in late stages of lung cancer (Pineau et al., 2010; Puisségur et al., 2011; Shah and Calin, 2011; Si et al., 2007), suggesting important roles for these miRNAs in cellular proliferation. In our recent studies of the dynamics in mRNA and microRNA expression during batch-cultivation of CHO-K1 cells, we found that miR-17-92 cluster expression correlates with growth kinetics and that miR-221 and miR-210 are highly expressed during exponential phase which points towards a role of these miRNAs in controlling a high proliferative state of CHO cells (Bort et al., 2011). Using the functional screening method we were able to demonstrate that indeed miR-17 acts as a growth enhancing miRNA in CHO-EpoFc cells: transient overexpression significantly increased growth rate by 15% without loss in cell specific productivity, resulting in an increase in final titer. In summary, the here developed method is a cost-effective and reliable tool for overexpression and functional analysis of miRNAs in CHO cells, which can be rapidly customized and adapted to test large numbers of miRNAs for cell engineering applications.

Materials and Methods

Cloning miRNA Expression Plasmids

miRNA expression plasmids of cgr-miR-17, cgr-miR-221, cgr-miR-210, and cgr-miR-21 were created by annealing

self-complementary oligonucleotides encompassing the mature miR-5p and miR-3p sequences and loop sequences from miR-155 (Chung et al., 2006). Cloning of chimeric CHO pre-miRNAs was performed using BLOCK-iT^{TM} Pol II miR expression system (Invitrogen Inc., Carlsbad, CA), which is based on the pcDNA^{TM}6.2-GW/±EmGFP-miR vector, where pre-miRNAs are inserted in the 3′ untranslated (3′UTR) region of the Emerald Green Fluorescent Protein (EmGFP), thus allowing simple tracking of miRNA expression. The self-complementary oligonucleotides were annealed and cloned into the pcDNA6.2-GW/EmGFP-miR vector following instruction manual. Integrity of cloned sequences was confirmed by conventional sequencing.

Cell Culture

The recombinant CHO-EpoFc suspension cell line adapted to growth without glutamine used in this study has been described previously (Lattenmayer et al., 2007). Cells were cultivated in CD CHO medium (Gibco, Invitrogen, Carlsbad, CA) and supplemented with 0.19 μM Methotrexate (MTX) and Anti-Clumping Agent (Gibco). Cultures were cultivated in shaking conical flasks with a working volume of 30 mL at 37°C in a humidified atmosphere containing 7% carbon dioxide and with constant shaking at 140 rpm.

Nucleofections and Batch Cultures

All nucleofections were performed using the Nucleofector II (Lonza, Basel, Switzerland) and Nucleofector Kit V/program H-14. Exponentially growing CHO-EpoFc cells were harvested and counted. A cell pellet consisting of 1×10^7 cells was resuspended in 100 μL Nucleofector Solution (including supplement) plus DNA. 10 μg of endofree pcDNA6.2-EmGFP-miR plasmids and pcDNA6.2-EmGFP-negative plasmids were transfected. For the mock control, nucleofection was carried out using Nucleofector Solution (including supplement) without plasmids. The mixtures were transferred to a cuvette and immediately nucleofected. Following nucleofection, 2 mL of pre-warmed cell culture medium were added to the cuvette and the whole suspension was transferred into 60 mL of pre-warmed culture medium. The cell suspension was split into 2×30 mL conical flasks to yield two technical replicates. The conical flasks were incubated for 2–3 h at 37°C in a humidified incubator containing 7% carbon dioxide for recovery. Batch cultures were started by transferring conical flasks into a shaking incubator at 37°C in a humidified atmosphere containing 7% carbon dioxide and with constant shaking at 140 rpm. For each miRNA, three independent batch cultures were run. Each batch was run for 4 days.

Flow Cytometry

All emGFP measurements were performed using a FACS Canto flow cytometer (BD Biosciences, San Jose, CA). A minimum of 10,000 events were collected and analyzed at excitation wavelength of 488 nm using a 530/30 BP filter for collection of the emitted signal. Non-transfected cells were used for gating of GFP positive cells. Data were analyzed using the BD FACS DIVA software.

Growth Measurements

The effect of miRNA overexpression during batch cultures was determined by daily measurements of cell viability and viable cell density on a Vi-Cell analyzer (Beckman Coulter Inc., Fullerton, CA) based on the trypan-blue dye exclusion method. Growth data were further analyzed to calculate specific growth rates using Equation (1), where X_1 and X_2 are the viable cell number at the respective time points (t_1 and t_2)

$$\mu = \frac{\ln x_2 - \ln x_1}{t_2 - t_1} \tag{1}$$

Measurement of Productivity

The EpoFc concentration was quantified by ELISA. Polyclonal goat anti human IgG (γ-chain specific) antibodies (Sigma-Aldrich, St. Louis, MO, # I-7883) were used for coating overnight at 4°C. The coated plates were blocked with Tween 20 and 1% bovine serum albumin (BSA). An Fc standard and the supernatant of the culture samples were serially diluted with washing buffer and loaded onto the coated plates. A horseradish peroxidase conjugated polyclonal goat anti-human IgG (γ-chain specific) antibody (Zymed Laboratory, South San Francisco, # 62-8420) was used for detection of EpoFc. The labeling reagent consisted of 100 μL OPD (Ortho-phenylenediamine dihydrochloride, Fluka, Switzerland) and 6 μL H_2O_2 in 10 mL dyeing buffer. 2.5 M sulfuric acid was used as stop solution. The product titer was measured using an ELISA reader (Sunrise, Tecan, Männedorf, Switzerland) at 492 nm and 620 nm as reference wavelength and the analysis was done using Magellan software according to the instruction manual. Specific productivity was calculated according to Equations (2) and (3), where q_P is cell specific productivity, C_p is the product concentration on day n and CD is the viable cell integral

$$q_P = \frac{C_{pu} - C_{p1}}{CD} \tag{2}$$

$$CD = \sum^{n} \frac{X_{i+1} - X_i}{\mu} \tag{3}$$

RNA Isolation

Samples for RNA isolation were taken from each batch culture replicate at days 2 and $4.1–5 \times 10^6$ cells were harvested and homogenized in 1 mL Trizol reagent (Invitrogen) followed by 5 min incubation at room temperature and storage at 80°C until used. Total RNA was extracted using chloroform, RNA pellets were washed with 70% ethanol and after drying resuspended in 30 μL of RNase free H_2O. Absorbance at 230, 260, and 280 nm was measured using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and total RNA quality and quantity were calculated. Only total RNA extracts with absorbance ratio for 260/280 and 260/230 in the range 1.9–2.0 and 1.9–2.2, respectively, were considered for further miRNA quantitative real-time PCR.

Quantitative Real-Time PCR

MicroRNA expression was measured using a TaqMan miRNA quantitative PCR assay from Applied Biosystems (Foster City, CA) that has been previously described (Chen et al., 2005). Briefly, cDNA was made from 10 ng of total RNA in 10 μL reactions using MultiScribe reverse transcriptase (Applied Biosystems) and specific RT-primers for each miRNA. The cycle parameters for the RT reaction were 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and hold at 4°C. The PCR reaction mix consisted of the RT product, Taqman 2× Universal PCR Master Mix and the appropriate 20× MicroRNA Assay Mix (TM000397, TM000524, TM000512, TM002308, and TM0002271). The PCRs were run on a Rotor-Gene qPCR cycler (Qiagen, Hilden, Germany) including four technical replicates per sample. The expression of each miRNA relative to cgr-miR-185-5p (internal 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 shown.

Quantification of miR-17 Targets by Real-Time Polymerase Chain Reaction (PCR)

The mRNAs of NCOA3, MAP3K12, JAK1, CCND1, and BCL2 were quantified 48 h post-transfection of either cgr-miR-17 or negative control plasmids using quantitative real-time PCR. The cDNA was synthesized using 200–700 ng of total RNA, MMLV reverse transcriptase and random hexamer primers provided with the Dyamo cDNA Synthesis kit (Finzyme, Espoo, Finland). Each sample was incubated for 5 min at 25°C, 30 min at 37°C, and 5 min at 85°C then the cDNA was diluted 1:5 with nuclease free water. SYBR Green-based real-time PCR was performed using a Rotor-Gene 6000 apparatus (Qiagen) and SensiMix Plus SYBR (Quantace, London, UK) based on recommendations of the manufacturer. A total volume of 10 μL containing 1 μL cDNA, 9 μL SYBR mixture and target mRNA specific primers (Table I) were used. Two technical replicates of gene specific standards (ranging from 10 to 10^8 copies/μL) and four technical replicate runs for each samples were done in 40 cycles of PCR: 10 s at 95°C, 15 s at 60°C, and 25 s at 72°C. The miR-17 target mRNA expression levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control. The results of real-time PCR were analyzed by $\Delta\Delta C_t$ comparative method for observing fold changes in mRNA levels in two independent experiments. Error bars represent SD.

Results

Establishment of miRNA Expression Plasmids

Vector based overexpression of miRNAs requires complete sequence information of the miRNA precursor, which includes the loop connecting the mature 5′ and 3′ miRNA as well as flanking regions at either ends. However, in version 17.0 of miRBase (http://www.mirbase.org/) that was available at the start of this work, only two pre-miRNA

Table I. Pre-miRNA oligonucleotide sequences used for cloning.

microRNA	Oligo	Sequence (5′–3′)	Length	GC content (%)
miR-17	Forward	TGCTGCAAAGTGCmCAGTGCAGGTAGGTTTTGGCCACTGA CTGACACTGCAGTGCAGGCACTTGTGG	69	54
miR-17	Reverse	CCTGCCACAAGTGCCTGCACTGCAGTGTCAGTCAGTGG CCAAAACCTACCTGCACTGTAAGCACTTTGCAGCA	73	55
miR-21	Forward	TGCTGTAGCTTATCAGACTGATGTTGACGTTTTGGCCA CTGACTGACCAACAGCAGTCGATGGGCTGTC	69	51
miR-21	Reverse	CCTGGACAGCCCATCGACTGCTGTTGGTCAGTCAGTG GCCAAAACGTCAACATCAGTCTGATAAGCTACAGCA	73	52
miR-210	Forward	TGCTGAGCCACTGCCCACCGCACACTGGTTTTGGCCA CTGACTGACCTGTGCGTGTGACAGCGGCTGA	68	62
miR-210	Reverse	CCTGTCAGCCGCTGTCACACGCACAGGTCAGTCAGT GGCCAAAACCAGTGTGCGGTGGGCAGTGGCTCAGCA	72	63
miR-221	Forward	TGCTGACCTGGCATACAATGTAGATTTCTGTGTTTT GGCCACTGACTGACAGCTACATTGTCTGCTGGGTTTC	73	47
miR-221	Reverse	CCTGGAAACCCAGCAGACAATGTAGCTGTCAGTCAG TGGCCAAAACACAGAAATCTACATTGTATGCCAGGTCAGC	77	48

sequences (mir-21 and mir-7) are reported for Chinese hamster (*Cricetulus griseus*). For this reason we used previously published CHO specific miR-5p and miR-3p sequences and inserted them into a designed pre-miRNA sequence providing loop and flanking sequences that are based on the murine miR155 sequence resulting in a "chimeric" pre-miRNA containg CHO-specific sequences for the mature miRNA duplex.

Cloning of chimeric CHO pre-miRNAs was performed using the BLOCK-iT^{TM} Pol II miR expression system (Invitrogen Inc.), which is based on the pcDNA^{TM}6.2-GW/±EmGFP miR vector, where pre-miRNAs are inserted in the 3′ untranslated (3′UTR) region of the Emerald Green Fluorescent Protein (EmGFP), thus allowing simple tracking of miRNA expression by EmGFP co-expression. This system was successfully applied to clone chimeric CHO pre-miRNAs using synthetic self-complementary oligonucleotides sequences (Table I) encoding cgr-miR-17, cgr-miR-221, cgr-miR-21, and cgr-miR-210 miRNAs.

Assessment of miRNA Expression

As proof of principle for expression of miRNAs from these constructs miR-210, miR-221, miR-17, and miR-21 were chosen, as they were identified to be expressed at low, medium, and high abundance in CHO cells, respectively (Hackl et al., 2011). This selection allowed evaluation of the dynamic range of miRNA overexpression from the pcDNA6.2-GW/EmGFP-miR system against differing endogenous miRNA levels in CHO cells.

After transient transfection, total RNA samples were harvested after 48 h and the fold changes relative to miR-185 as endogenous control were determined as well as their basal expression levels that were calculated as normalized sequence read counts from Illumina next-generation sequencing (Hackl et al., 2011, Fig. 1a). MiR-185 was previously shown to be expressed at constant level (Bort et al., 2011; Hackl et al., 2011; Supplementary Fig. 1). For miR-221 (low abundant miRNA) the overexpression achieved at 48 h was 2.3-fold, for miR-17 (medium abundant miRNA) 1.5-fold, and for miR-21 (highly abundant miRNA) 1.3-fold. For miR-210, a low abundant miRNA, the achieved overexpression was nevertheless only 1.4-fold. In order to test whether the miRNA overexpression levels correspond to the EmGFP that is expressed from the same plasmid, we calculated the Pearson correlation of miRNA fold changes with geometric means of emGFP FL-1 fluorescence emitted from the cells as analyzed by flow cytometry. The correlation of expression emGFP with miRNA at 48 h was $r = 0.91$ with $P < 0.002$, suggesting that emGFP is a good indicator of miRNA expression (Fig. 1b).

Establishment of a Screening Protocol

Upon transient transfection with the negative control plasmid, emGFP expression was determined using flow

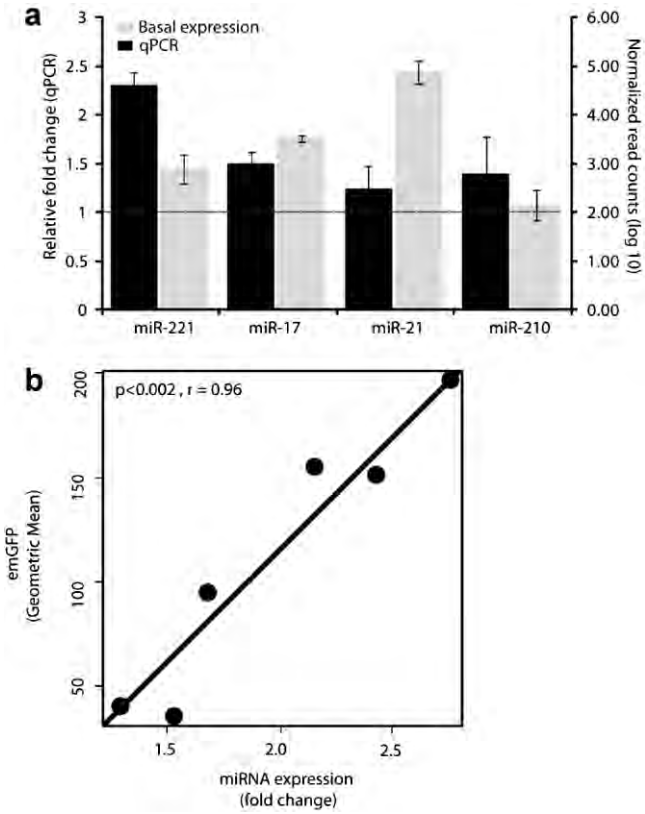

Figure 1. miRNA overexpression and corelation to GFP expression. **a**: MiRNA overexpression was quantified by real-time qPCR. On the primary axis (black bars) fold-change overexpression of miR-221, miR-17, miR-21, and miR-210 relative to the negative control (NC) plasmid are shown at 48 h post-transfection. Data was analysed by $\Delta\Delta C_t$ comparative method and normalized to miR-185 expression as endogenous control. On the secondary axis (gray bars) the basal level of endogenous miRNA expression is shown as quantified by next generation sequencing read numbers (in $\log_{10}$) across the different CHO cells used in the study (Error bars represent: SEM). **b**: Pearson correlation between the expression levels of emGFP (geometric mean of fluorescence signal intensity) and miRNA expression (fold-change relative to NC; $r = 0.96$, $P < 0.002$).

cytometry to assess the dynamics of transgene expression during subsequent batch cultivation. We observed an optimum expression of emGFP between day 2 and 3, followed by a constant decrease in emGFP intensity from day 4 to day 7 (Fig. 2). Based on these results we hypothesized that the optimal functional screening window for analysis of growth and productivity should lie between days 1 and 4. Therefore, we set up an experimental design for the functional screening as outlined in Supplementary Figure 2, using shake flasks to establish bioprocess similar conditions and enabling easy scale-up in case larger numbers of cells were required for subsequent functional analysis.

Screening for Growth Characteristics

By applying this screening strategy we evaluated the effect of miR-17, miR-221, miR-21, and miR-210 overexpression during batch cultivation for 4 days: 1×10^7 CHO-EpoFc

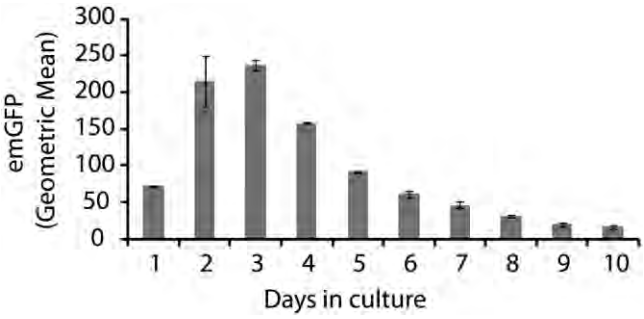

Figure 2. Transient emGFP expression during batch culture. Geometric mean of fluorescent signal intensity of 10,000 cells over a period 10 days of batch cultivation after transient transfection (error bars represent: SD).

cells from exponential growth phase were transfected and seeded into shaker flasks at a cell concentration of 1.5×10^5/mL. After a recovery period of 2–3 hours without agitation the flasks were transferred to a shaker. Transfection efficiency was determined by flow cytometry analysis of the number of emGFP-positive cells after 48 h (Fig. 3a and b). All experiments were performed in duplicate flasks and in three independent experiments. Transfection efficiency reproducibly ranged between 80% and 90% for all constructs, as indicated by low standard deviations of 0.5–2.0%. This high reproducibility allowed good comparison of the effects of the respective miRNAs on the cellular phenotype. Starting viability was reliably >95%. Figure 3c–f shows the growth curves for miR-17, miR-221, miR-21, and miR-210 compared to the negative control transfection. After 24 h, viability was 80–90% for all constructs, probably due to transfection stress, but was restored to >90% after 48 h. The average of the growth rates calculated for each day from day 1 to 4 are shown in Figure 3g and show an increase of 15.4% for miR-17 and 7.2% for miR-221, respectively. For miR-21 and miR-210 no changes in growth rates were observed.

Screening for Production Characteristics

To address changes in the production characteristics upon miRNA overexpression in CHO-EpoFc cells, ELISA quantification of EpoFc was performed for all batches after 96 h (Fig. 4). The average titers achieved at 96 h as well as the cell specific productivity over the 4 days in culture reveal that despite the increase in growth rate, the specific productivity of miR-17 and miR-221 overexpressing cells was not affected, thus resulting in increased final titers. Overepression of miR-21 and miR210 on the other hand showed a decrease in specific productivity.

Analysis of Target Effects of miR-17 Overexpression

As miR-17 overexpression had the most significant effect on the phenotype, the effect on the intracellular concentration of mRNAs of known targets of this well characterized miRNA were analyzed by qPCR using the primers shown in Table II. The target messengers chosen were those of NCOA3, MAP3K12, JAK1, CCND1, and BCL2 (Cloonan et al., 2008; Grillari et al., 2010; Yu et al., 2008). Except for MAP3K12, all targets showed a reduction in mRNA content of 25–45% relative to the control, thus confirming that the achieved overexpression of miR-17 was sufficient to elicit a response on the target genes (Fig. 5).

Discussion

In order to address our previous hypothesis that miRNAs play a vital role in the regulation of biological processes in CHO cells (Muller et al., 2008) we have recently reported a comprehensive analysis of miRNA sequences as well as dynamics of miRNA expression during CHO-K1 batch cultivation (Bort et al., 2011; Hackl et al., 2011). In the current study we describe a reliable method for cloning CHO miRNAs and screening the functional effect of transient miRNA overexpression on growth and productivity of cells in small-scale batch cultivations. Several objectives were pursued in designing the experimental setup of the method and were shown to be met by the results obtained.

First, we considered it of prime importance to establish a protocol that enables the screening of multiple potential engineering targets (which may include miRNAs, miRNA-sponges, but also siRNAs or mRNAs) in a uniform protocol for ease of comparison. At the same time it was clear that modifications of the protocol must be easy to accomplish. Thus the protocol is on the one hand highly reproducible, on the other hand modifications can be easily introduced. For instance, if the expected effect of a miRNA was on apoptosis, then after transfection cells can be seeded at higher densities to simulate stationary culture phase. Due to the short observation window used, rapid screening of larger numbers of miRNAs is possible, but still standards of bioprocess setup are well mimicked. In addition to enabling the screening of multiple miRNAs, the protocol can also be used to evaluate the effect of a single miRNA on different production cell lines.

Second, we wanted to ensure that the protocol was easily up-scaled, in case larger amounts of cells or supernatant are required for analysis of the effect of miRNA engineering. For instance, for transcriptome or proteome analysis typically 10^7 cells are required, which can be easily achieved in the present protocol but which would not be possible in microtiter based screening systems.

Another consideration was the goal to achieve increases in miRNA levels that are within a physiologically acceptable range and therefore sustainable for long-term engineering of the cell line. Changes in miRNA expression are smaller than those observed for mRNAs, typically less than fourfold (Bort et al., 2011; Gammell et al., 2007; Lin et al., 2011).The fold-changes achieved in our evaluation study (1.3-2.3-fold) lie

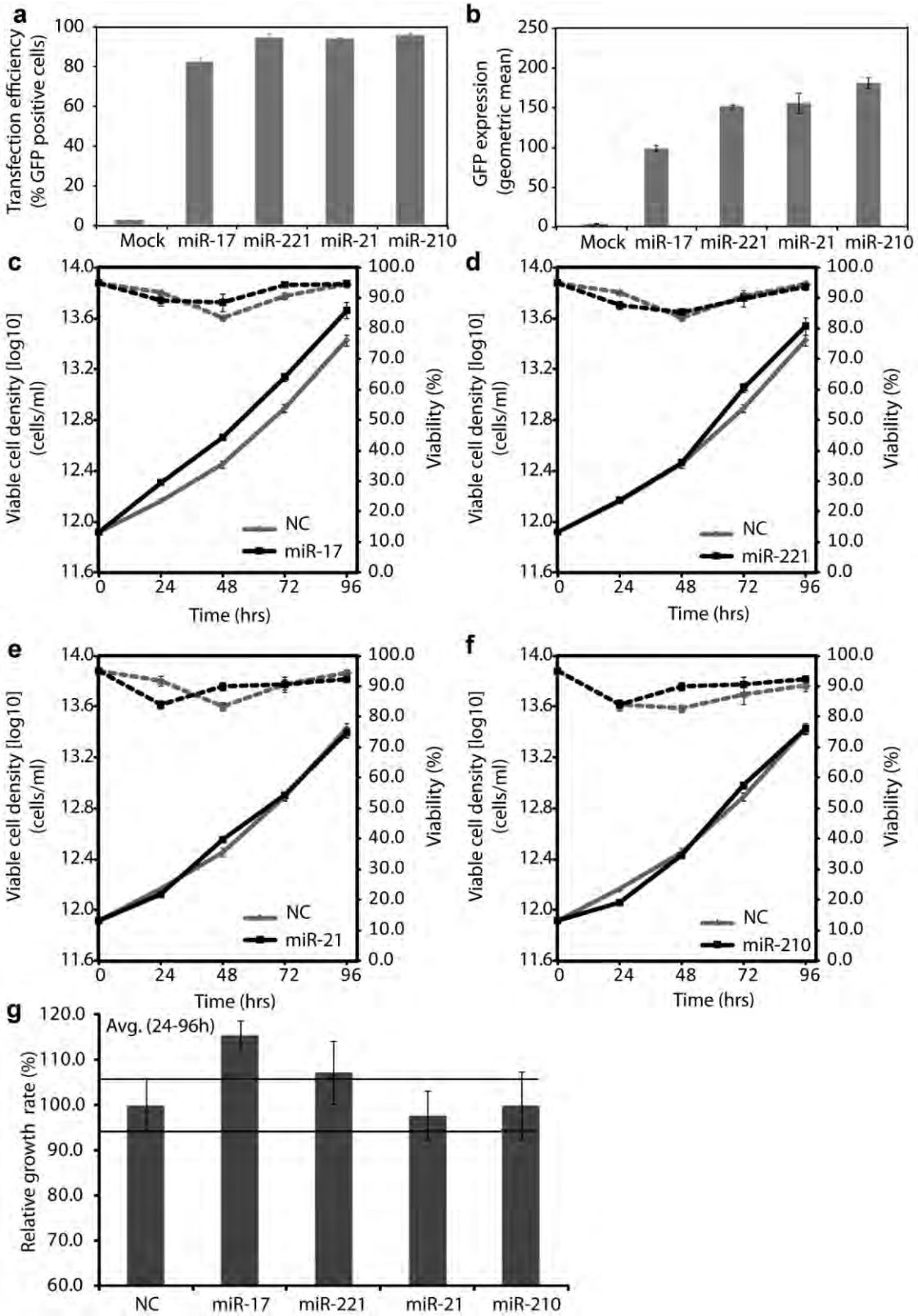

Figure 3. Transfection efficiency and effect of miRNA overexpression on growth. **a**: Transfection efficiency: percentage of GFP positive cells (10,000 cells per sample). **b**: GFP expression (geometric mean of fluorescence signal intensity of 10,000 cells) as indicator for co-cistronic expression of miRNA. **c**–**f**: Viable cell density (filled line) and viability (dotted line) up to 96 h post-transfection of miR-17, miR-221, miR-21, and miR-210 (black) and negative control (gray). Primary axis: mean cell numbers of three independent experiments with two technical replicates for each (in log scale). Secondary axis: viability (in percentage). **g**: Average daily growth rate (μ/day^{-1}) from 24 to 96 h relative to negative control (error bars represent: SEM).

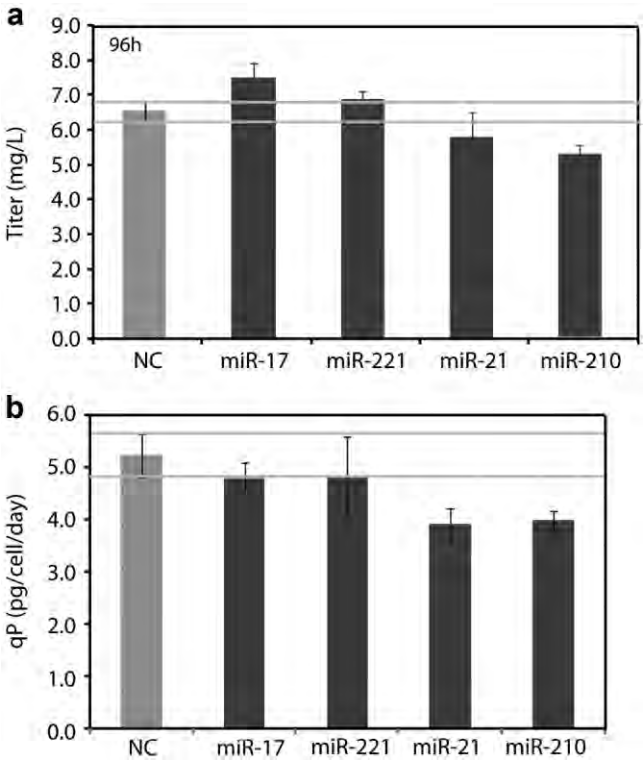

Figure 4. Effect of miRNA overexpression on titer and productivity. **a**: Final titres of EpoFc at 96 h in mg/L. **b**: Specific productivity (q_P) in pg/cell/day as calculated from titer and the integral of viable cells (error bars represent: SD).

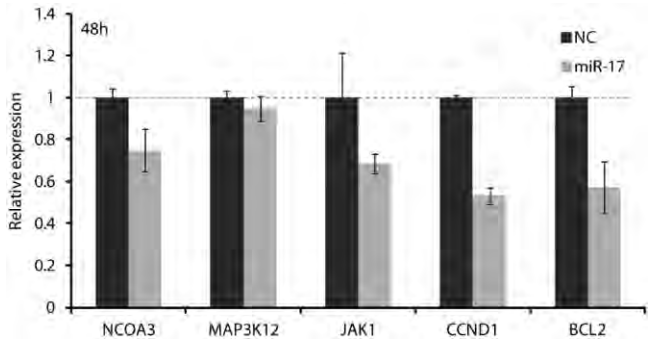

Figure 5. Cellular mRNA content of miR-17 targets. The cellular mRNA content of confirmed miR-17 targets during transient transfection of cgr-miR-17 were measured by RT-qPCR 48 h post-transfection of crg-miR-17 overexpressing cells and mock transfected cells. GAPDH expression levels were used as endogenous control; the data was analyzed by $\Delta\Delta C_t$ comparative method and further normalized to control transfection (NC). Bar graphs represent the results of two independent experiments (error bars = SD).

within this range. Despite the low fold-changes we were able to detect changes in the bioprocess relevant properties of these cells reliably and to corroborate them by demonstrating the downregulation of mRNA of confirmed target genes.

Finally, as the protocol was designed to study in the future a large number of miRNAs it was important that the protocol allows the identification of both beneficial and detrimental effects of the miRNA overexpression in a single experiment, as it is in many cases not possible to predict the direction of a phenotypic change a priori. Both positive and negative effects of miRNA overexpression were reproducibly observed in our experiments, so that based on these results a knowledge based decision on the next step in cell optimization can be reached: in the case of miR-17, where an increase in growth rate was observed, this is the generation of stable overexpressing cell lines, for which the same plasmid construct can immediately be used. In the case of miR-21 and miR-210, where a decrease in productivity was observed, the logical next step is the design of an appropriate knockdown approach, such as a sponge, to reduce the intracellular level of these miRs in the hope of increasing product yield. Interestingly, we observed in a previous study that expression of miR-21 was downregulated in recombinant K1 and DXB11 compared to their respective hosts (Hackl et al., 2011), suggesting indeed a possible negative role in protein production.

A surprising results of our study remains unexplained: as a strong viral promoter was used to drive miRNA overexpression, we would have expected larger differences in the fold-changes observed from miRNAs with low or high endogenous expression. Although we did observe such a dependence for three of the miRNAs overexpressed, the differences were relatively low and for miR-210 were not found at all. Currently we can only speculate that there are endogenous feedback-loops at work, which prevent overshooting of this important regulatory machinery by down-regulating the expression of endogenous miRNAs in the presence of artificial overexpression. In any case,

Table II. qPCR primer sequences for miR-17 targets.

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size	Intron-spanning[a]
JAK1	TTCGGGAAGCCTAAAGGAAT	CCGGTGAACATACTGTCGTG	127	Yes
CCND1	ACACCAACCTCCTGAACGAC	CTCCTCTTCGCACTTCTGCT	173	Yes
BCL2	CACTGAACAGAATGGGCAGA	CTTCAAAGCCCAGTTTGAGC	103	No
MAP3K12	AGCCATCATCTGGGGTGTAG	CTGAGGCGATATCCAGGTGT	158	Yes
NC0A3	CTCTACAGGACAGGGGGTCA	ACAATGTTGCCGTCTCGATT	12	Yes

[a]Based on blat aliment of amplicon sequence with mouse transcripts using UCSC genome browser.

overexpression of miRNAs from the here generated constructs using artificial pre-miRNAs resulted in active miRNAs as validated miRNA targets were indeed down-regulated upon overexpression.

The advances in the identification of functional targets of miRNAs have rapidly progressed by next-generation sequencing and interaction studies of miRNA-mRNA networks, so that the number of promising miRNA candidates as cell engineering targets has significantly increased during the last year. In the light of the availability of the CHO-K1 genome sequence (Xu et al., 2011), the here proposed method can be rapidly customized and further refined to fit the demands of this emerging field, for instance by comparison of the efficiency of endogenous pre-miR sequences to that of the here used artificial hairpin structures. In addition the method provides a general platform for further studies of miRNAs in CHO production cell lines, that also enables molecular analysis of their effects on the level of target mRNA and protein concentration.

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Appendix C

CHO microRNA Engineering Is Growing Up: Recent Successes and Future Challenges.

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Research review paper

CHO microRNA engineering is growing up: Recent successes and future challenges ☆

Vaibhav Jadhav [a], Matthias Hackl [a], Aliaksandr Druz [b,c], Smriti Shridhar [a], Cheng-Yu Chung [c], Kelley M. Heffner [c], David P. Kreil [a,d], Mike Betenbaugh [c], Joseph Shiloach [b], Niall Barron [e], Johannes Grillari [a,*], Nicole Borth [a,f,**]

[a] Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria
[b] Biotechnology Core Laboratory, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bldg 14A, Bethesda, MD 20892, USA
[c] Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD 21218, USA
[d] Life Sciences, University of Warwick, UK
[e] National Institute for Cellular Biotechnology, Dublin City University, Dublin 9, Ireland
[f] Austrian Center of Industrial Biotechnology, Austria

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ABSTRACT

microRNAs with their ability to regulate complex pathways that control cellular behavior and phenotype have been proposed as potential targets for cell engineering in the context of optimization of biopharmaceutical production cell lines, specifically of Chinese Hamster Ovary cells. However, until recently, research was limited by a lack of genomic sequence information on this industrially important cell line. With the publication of the genomic sequence and other relevant data sets for CHO cells since 2011, the doors have been opened for an improved understanding of CHO cell physiology and for the development of the necessary tools for novel engineering strategies. In the present review we discuss both knowledge on the regulatory mechanisms of microRNAs obtained from other biological models and proof of concepts already performed on CHO cells, thus providing an outlook of potential applications of microRNA engineering in production cell lines.

Contents

* Corresponding author.
** Correspondence to: N. Borth, Department of Biotechnology, 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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1. Introduction

1.1. MicroRNAs: from basics to applications

In the early 1990 small non-coding RNA molecules with the ability to regulate translation of target mRNAs by an antisense mechanism were discovered during developmental studies in the nematode worm (Lee et al., 1993; Wharton and Struhl, 1991). The much broader implications of these small RNAs were unacknowledged, until the identification of hundreds of similar small RNAs in a range of higher eukaryotes 7 years later. These newly discovered classes of small RNA molecules had striking similarities, such as lengths between 18 and 24 nucleotides and one or more, completely or partially complementary binding sites in 3′-UTR of mRNAs. These novel small RNAs were termed as microRNAs.

MicroRNAs were found to be expressed in a wide range of higher eukaryotes and to be highly conserved across species (Pasquinelli et al., 2000). With the availability of whole genome sequences, many more of these structurally and functionally distinct non-coding RNAs were discovered both experimentally and by bioinformatic prediction (Ambros and Lee, 2004; Ambros et al., 2003; Kozomara and Griffiths-Jones, 2011). The current version of miRBase (release 19.0) contains more than 20,000 entries from a wide variety of organisms, including viruses, plants and animals from nematodes to mammals (Griffiths-Jones, 2010). It is clear that such abundant molecules must have important functions and indeed, microRNAs have since been shown to be involved in all major cellular processes, such as cell division, death, embryonic development and timing, metabolism, host–virus interaction and tissue differentiation (Ambros, 2004; Scaria and Jadhav, 2007). Their function is based on antisense recognition of specific sequences in the target mRNA. They are able to regulate complex networks, due to the fact that a single microRNA can find targets in multiple mRNAs, while each mRNA may in turn contain binding sites for multiple microRNAs (Hobert, 2008).

The discovery and elucidation of the cellular machinery that controls this regulation enabled the development of siRNAs, a new toolbox whose discovery merited the Nobel Prize for Physiology in 2006 (Fire et al., 1998). In a recent review, the mechanism of action of microRNAs and their potential as biomarkers and novel drug targets was discussed (Bratkovic et al., 2012). In this review, we provide a detailed discussion of microRNAs with high relevance for optimizing cell lines used for biotechnological production of therapeutic proteins, with a focus on process-relevant properties such as growth, viability and apoptosis, productivity, stability and product quality. In addition to findings on microRNAs from other research areas that could be translated into cell engineering approaches, a comprehensive summary of CHO specific microRNA data will be given. The review concludes by discussing other non-coding RNAs with biological roles that might be of interest for cell culture technology.

2. CHO cell engineering — genome scale data is opening new doors

The most frequently used mammalian production cell line at industrial scales is the Chinese Hamster Ovary (CHO) cell line, isolated by Theodore Puck in the late 1950s (Puck et al., 1958). These lines were applied as recombinant hosts in 1987 with the commercial introduction of human tissue plasminogen activator (tPA) as the first recombinant therapeutic protein produced from mammalian cells (Finkle, 1988). Since then, the annual revenue of products from CHO cells has increased to more than 100 billion US dollars and continues to grow (Aggarwal, 2011). One of the major reasons for the success story of CHO cells is their adaptability and plasticity with respect to the phenotypic characteristics that are necessary for industrial production of therapeutic proteins (Jayapal et al., 2007): growth in suspension, adaptation to a number of steadily improving chemically defined media with ease, and production of proteins of high quality suitable for safe use in humans with low occurrence of immunologic reactions.

Silenced expression of specific surface proteins accounts for their low susceptibility to viral infections (Xu et al., 2011) and a high number of clones have been generated with distinct glycosylation patterns (Borisov et al., 2009; Imai-Nishiya et al., 2007; Yamane-Ohnuki et al., 2004) that enhance the therapeutic efficacy of the product (Jefferis, 2012). Despite the success story of CHO cells, the above mentioned plasticity also has drawbacks. First, a large number of clones need to be screened for each new production of cell line to identify the few that unite all the necessary phenotypic properties, such as high product quality, fast growth, high productivity and prolonged viable culture life in large scale bioprocesses. Second, once a suitable clone is identified, both phenotype and productivity can be subject to instability and phenotypic drift, resulting in a limited number of doublings over which a cell line can be used reliably.

These issues have been the focus of research over the last 25 years with only limited solutions having been found (Hacker et al., 2009). The most significant improvements to CHO culture so far have resulted from the optimization of media, feeding strategies and processes. This has resulted, at least for antibody products, in yields commonly ranging between 2 and 6 g/l (Wurm, 2004), with titers of 20 g/l being reported (Kim et al., 2012). Engineering of cell lines has resulted in improved product quality (Huang et al., 2012a; North et al., 2010; Pouilly et al., 2012; Raju et al., 2001) and some improvements in productivity (Figueroa et al., 2007); however, no successes comparable to the enhancements obtained by media optimization have been reported so far, and the most prominent problems of mammalian cell culture, such as the efficient energy utilization (Zeng et al., 1998) have not been completely resolved by metabolic engineering strategies (Kim and Lee, 2007a,b; Park et al., 2000). Most of these metabolic engineering strategies were limited to expressing single genes expected to shift metabolic pathways (Banmeyer et al., 2004; Hou and Li, 1987a,b; Jeon et al., 2011) or to making cells more resistant to apoptosis triggered by nutrient depletion or hyperosmolarity (Figueroa et al., 2007; Fussenegger et al., 2000; Lim et al., 2006; Park et al., 2000; Sauerwald et al., 2006; Sung et al., 2007; Wong et al., 2006). However, cells have redundant mechanisms to control cellular physiology, meaning that cells have different options to reach the same goal (Charaniya et al., 2009, 2010; Dinnis and James, 2005), which has been nicely demonstrated in recent years in several multiparameter -omics studies (Chong et al., 2010, 2011; Doolan et al., 2010; Meleady et al., 2012b; Wuest et al., 2012; Zhao et al., 2012). This has resulted in a paradigm shift from single gene engineering towards the control of signaling pathways, which can be achieved either by targeting regulatory hubs such as MAPK or mTOR (Dreesen and Fussenegger, 2011; Kim et al., 2011) or gene networks that control biological processes, which can be achieved by transcription factor or microRNA engineering (Barron et al., 2011b; Hackl et al., 2012a; Müller et al., 2008). microRNAs have the advantage that they do not add a translational burden onto production cells while being able to orchestrate complex gene networks in a coordinated fashion. However, as pointed out by Bratkovic et al. (2012), any research on CHO microRNA biology (or any other biology in CHO) has been severely restricted by the lack of genomic sequence information, at least until recently.

Although a consortium has been working on sequencing a CHO EST library since 2004 (Wlaschin et al., 2005), it was only during the last two years that a flood of sequencing data on CHO cells has become publically available, thus setting the stage for a new era of scientific exploration and innovation in CHO biology and engineering. The milestones of published genomic and transcriptomic sequence data are summarized in Fig. 1 (Gammell, 2007; Birzele et al., 2010; Hammond et al., 2011; Johnson et al., 2011; Hackl et al., 2011; Xu et al., 2011; Becker et al., 2011; Hackl et al., 2012b; Hammond et al., 2012b; Baycin-Hizal et al., 2012; Gerstl et al., 2013; Lewis et al., in press; Brinkrolf et al., 2013) and the respective datasets including updates are now assembled and

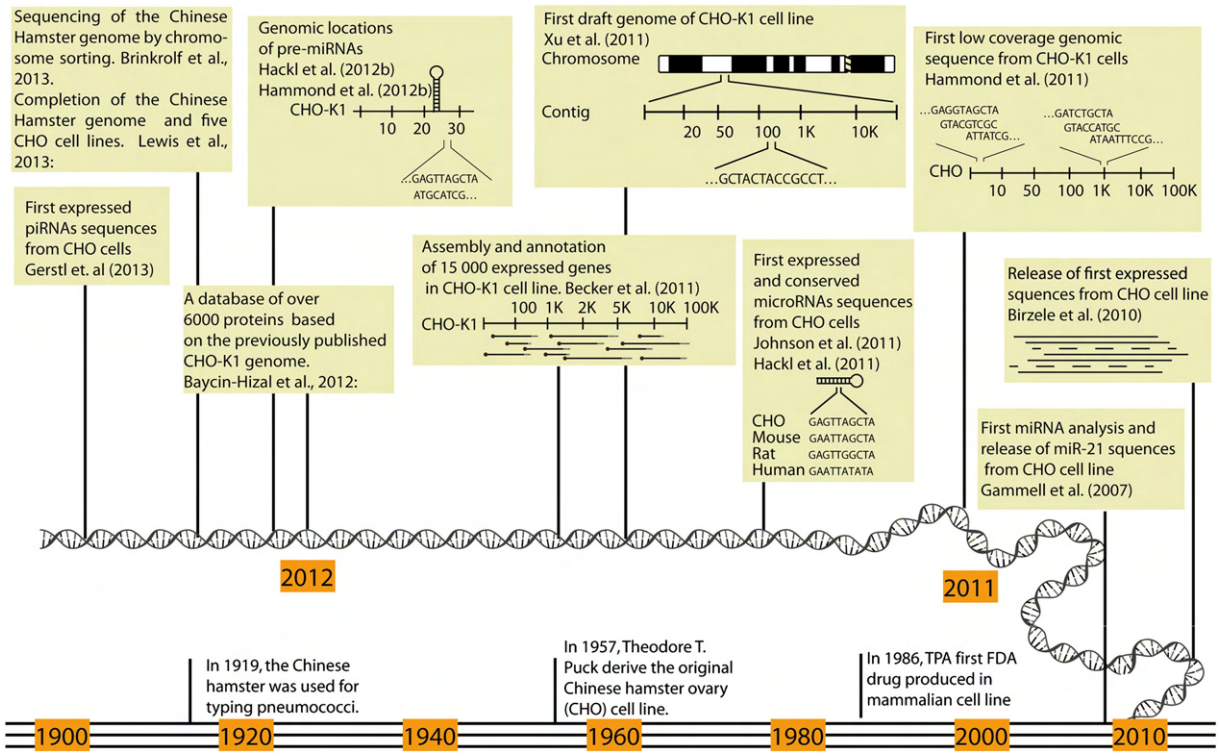

Fig. 1. Timeline showing recent boom in Chinese Hamster Ovary (CHO) cell genome science with increase in publications on sequence information and annotation. These developments have significantly advanced the establishment of new and improved tools for cell engineering and bioprocess development.

available online at www.CHOgenome.org for easy access and reference (Hammond et al., 2012b). With this information, several essential tools can be developed that facilitate an improved understanding of CHO cell physiology and provide the impetus for groundbreaking novel engineering strategies: i) CHO-specific mRNA, cDNA or whole-genome microarrays can be designed based on CHO sequence data, and analysis of next-generation sequencing data and proteomics data will be simplified by well-annotated references; ii) sequence alignment and primer design tools will facilitate gene cloning as well as site-specific gene knock-in and knock-out approaches during cell line development; and iii) mature and stemloop microRNA sequences will enable the design of anti-sense inhibitors (termed antagomirs or anti-miRs) or mimics for overexpression (Krützfeldt et al., 2005). The importance of a sequenced host genome can be deduced from the fact that overexpression of mature microRNAs in CHO cells was higher when the autologous CHO stemloops were used rather than an artificial stemloop routinely used for expression of siRNAs (personal communication).

Even without such tools and CHO-specific sequence data, the potential of using microRNAs to engineer the most important process-relevant properties was recognized very early, and multiple groups began to explore their use (Barron et al., 2011b; Müller et al., 2008). In the following chapter we discuss those cellular characteristics that are relevant for recombinant protein production (Fig. 2), summarizing both knowledge obtained from other biological models (Table 1) and experiments and proof of concepts already performed on CHO cells as production cell lines (Table 2), thus setting the stage for future developments of microRNA engineered cell lines.

3. Process relevant properties as targets for microRNA based cell engineering

3.1. Cell growth

Since their discovery, microRNAs were thought to play critical roles in modulating cell cycle arrest, cell proliferation and cell death. A clear link came from studies of microRNA expression profiling in human cancer that lead to an understanding of the relationship between microRNA function and cancer phenotype (Blenkiron et al., 2007; Jiang et al., 2005; Porkka et al., 2007; Solomides et al., 2012; Yang et al., 2008; Yao et al., 2009). Differential expression studies revealed that the majority of microRNAs are expressed at significantly lower levels in a variety of tumors compared to normal tissues. Deregulation of microRNA expression can be both tumor suppressive or oncogenic (oncomirs), with differentially expressed microRNAs associated with pathways such as cell cycle, cell growth and cell death (Lee and Dutta, 2006).

Tumor suppressor microRNAs function by down-regulating oncogenes. The first identified tumor suppressor microRNAs shown to regulate the expression of an oncogene were let-7 family members. They regulate Ras genes, which are membrane-associated GTPases involved in signaling of cellular growth and differentiation (Johnson et al., 2005). In addition, miR-143 and miR-145 negatively regulate mitogen-activated protein kinase 7 (MAPK7) at a posttranscriptional level, thus reducing growth rate in human cell lines (Lin et al., 2009; Noguchi et al., 2011).

Similarly, oncogenic microRNAs interact with tumor-suppressor genes and have either pro-proliferative or anti-apoptotic function. Recent studies revealed that miR-17–92 cluster overexpression drives tumorigenesis under the intricate network of c-Myc and E2F, and was also shown to have bi-functional effects, acting either as oncogene or as tumor suppressor in a cell type dependent manner (Cho, 2007; Grillari et al., 2010; Mendell, 2008). For example, the miR-17-5p and miR-20a target E2F1 are transcription factors that promote cell proliferation in normal human cells but induce apoptosis in cancer cell lines (Cloonan et al., 2008; Hackl et al., 2010; Li et al., 2011c; Olive et al., 2010).

Another oncogenic microRNA – miR-21 – was described as playing a critical role in the development and progression of lung cancer by regulating multiple genes controlling several pathways including JAK/STAT, MAPK, PPAR signaling and cell cycle related pathways, based on a systematic analysis of literature and gene network studies

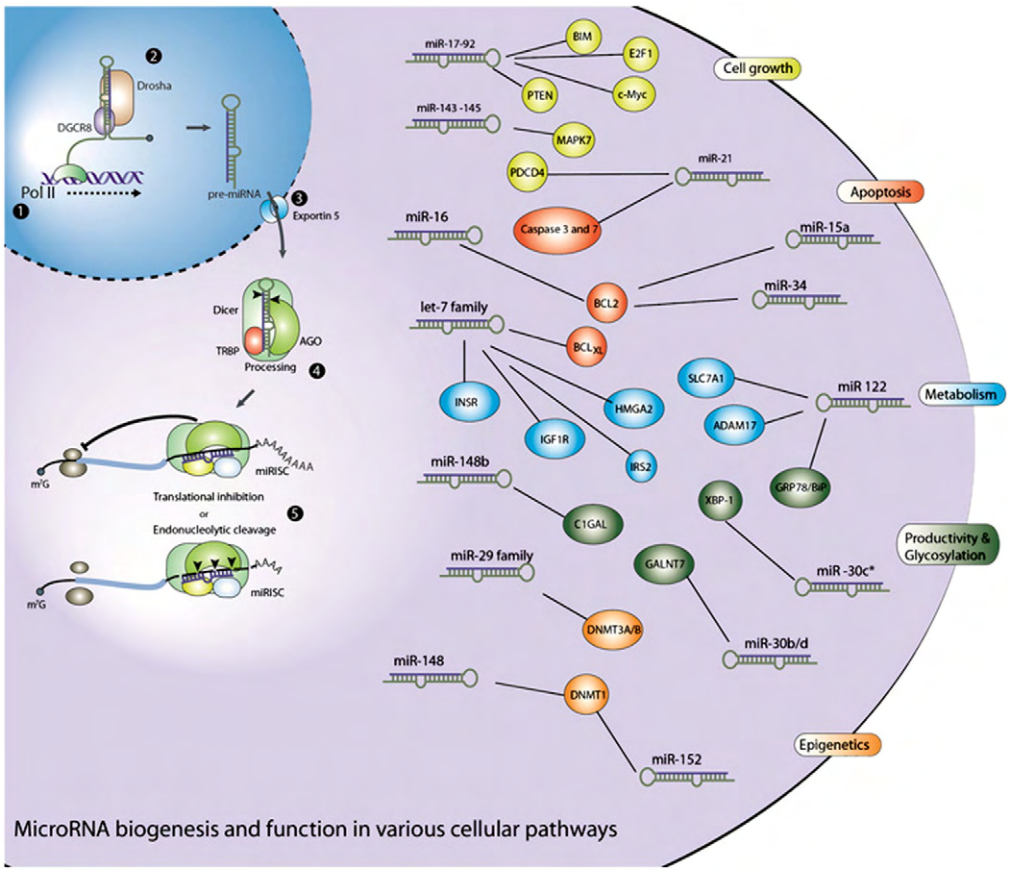

Fig. 2. Biogenesis and function of microRNA in controlling process relevant cellular properties. Biogenesis: (1) canonically, microRNAs are transcribed by RNA polymerase II to generate primary transcript (pri-microRNAs), long capped and polyadenylated RNAs with hairpin structure. (2) First processing steps are mediated by the microprocessor complex, consisting of Drosha and DiGeorge syndrome critical region 8 (DGCR8) which produces a ~70 nt hairpin structured RNA known as a precursor-microRNA (pre-microRNA). (3) Pre-microRNA are exported from the nucleus by the Exportin-5–Ran–GTP complex. (4) In the cytosol further processing occurs by Dicer together with TRBP and Argonaut proteins 1–4 (AGO), resulting in the active microRNA-induced silencing complex (miRISC). (5) miRISC binds to its target mRNA, mediating translational inhibition or cleavage. Function of microRNA: selected examples of microRNA discussed in the text that influence process relevant cellular processing by post-transcriptionally controlling expression of genes, highlighting the complexity of the regulatory network and interactions between the different pathways.

(Frezzetti et al., 2011; Guan et al., 2012; Hatley et al., 2010). It can also promote migration and invasion of human hepatocellular carcinoma by targeting PDCD4 in a negative feedback loop (Lu et al., 2008; Si et al., 2007). Furthermore, several microRNAs were described to act similarly in support of cell proliferation in different cell types (Esquela-Kerscher and Slack, 2006).

Identification and application of oncomirs or tumor suppressor microRNAs in CHO cell engineering are interesting challenges (Müller et al., 2008). Here two different aims are of relevance: the ideal bioprocess consists of an initial extremely fast growth phase to reach full biomass quickly, followed by a non-growing, high-productivity state in which cells accumulate high yields of product. Thus microRNAs could potentially be used both for their growth-enhancing and -repressing function. In a detailed transcriptomic analysis in CHO cells aimed at understanding the microRNA–mRNA network dynamics during the course of a batch culture a set of 10 microRNAs were identified as down-regulated during stationary phase, with their target mRNA levels up-regulated (Bort et al., 2012). The functions of these mRNAs were enriched for cell cycle and programmed cell death, suggesting them as good engineering targets to control cell death and proliferation during the late stages of bioprocess. In a similar approach, Barron et al. (2011a) identified miR-7 as significantly down-regulated during a temperature shift from 37 °C to 31 °C. Interestingly, contrary to expectations, the transient overexpression of miR-7 led to growth arrest, resulting in increased recombinant protein production, generally observed during temperature shift culture conditions. In a follow-up study, the effect of miR-7 overexpression on the proteome was analyzed, revealing proteins involved in protein folding and secretion to be up-regulated, while targets that control protein translation and nucleic acid processing were down-regulated (Meleady et al., 2012a). The productivity enhancement was thus affected by an improvement in protein processing, while two mRNAs, Stathmin and catalase, were identified as potential direct targets of miR-7, which caused the growth arrest. More recent work, in which miR-7 levels were stably depleted in CHO cells using a 'sponge', showed a marked increase in cell proliferation and improved longevity later in batch-fed culture (personal communication).

In the microRNA sequencing experiments, Hackl et al. (2011) found a significant difference in the overall expression of microRNAs in CHO cells when comparing cells grown in serum-containing medium or adapted to protein free media. In a follow-up study on this global microRNA regulation, the relevance of Dicer, one of the key enzymes during microRNA biogenesis, for maintenance of growth in CHO cells was studied (Hackl et al., under review). Dicer mRNA and protein levels quickly decrease in response to nutrient depletion or serum removal. Conversely, Dicer expression during the exponential growth phase is 3 fold higher in fast growing, protein-free and suspension-adapted CHO cells ($\mu \sim 1.0\ d^{-1}$) compared to slow growing cells ($\mu \sim 0.5\ d^{-1}$), and siRNA mediated down-regulation of Dicer expression reduces the proliferation rate of CHO cell lines. Growth of such slow-growing cells could be increased by 20% following recombinant expression of human Dicer, suggesting a link between the overall microRNA load in CHO cells and growth behavior.

Table 1
miRNAs controlling cellular processes and their identified targets.

Biological process	Manuscript section	MicroRNA identifier	Effect	Selection of confirmed targets	Annotated in CHO (miRBase v20)
Cell growth	3.1.	let-7	Tumor-suppressive	Ras, BCL-XL	Yes
		miR-143–145	Tumor-suppressive	MAPK7	Yes
		miR-17–92	Oncogenic	c-Myc, E2F1, PTEN, Bim, HIF-1α	Yes
		miR-21	Oncogenic	PDCD4, Caspases 3 and 7	Yes
		miR-7a	Growth arrest	Stathmin	Yes
Apoptosis & cell death	3.2.	miR-1	Pro-apoptotic	HSP60, HSP70	Yes
		miR-133	Pro-apoptotic	Caspase 9	No
		miR-144/155	Pro-apoptotic	Caspase 3	Yes
		miR-15a-16	Pro-apoptotic	BCL-2, BCL-XL	Yes
		miR-218	Pro-apoptotic	ECOP	Yes
		miR-297–669	Pro-apoptotic	BCL2L2, DAD1, BIRC6, STAT5a, SMO	No
		miR-34	Pro-apoptotic	BCL-2, SIRT1 Deacetylase	Yes
Hypoxia & oxidative stress	3.2.	miR-107/210/26	Hypoxia inducible, prevent apoptosis	Multiple pro-apoptotic genes	Yes
		miR-31	Supports HIF-1α induction	FIH	Yes
		miR-144/451	Oxidative stress protective	NRF2, 14-3-3ξ	Yes/no
Shear & osmotic stress	3.2.	miR-200b/717	Osmolarity responsive	OREBP	Yes/no
		miR-7b	Osmolarity responsive	FOS	Yes
Energy metabolism	3.3.	let-7	Glucose metabolism	INSR, IGF1R, IRS2, HMGA2	Yes
		miR-122	Liver metabolism	Multiple cholesterol related genes	Yes
		miR-124/137/340	Glycolysis rate	Pyruvate Kinase Isozymes (PKM1/2)	Yes
		miR-23a	Glutamine metabolism	Suppressed by C-Myc, targets GLS	Yes
		miR-33a/33b	Fatty acid and insulin metabolism	Multiple enzymes in cholesterol synthesis	Yes
Productivity protein expression	3.4.	miR-122/30/181d/199a-5p	UPR	GRP78/BiP	Yes
		miR-204	ER-stress	SERP1/RAMP4, M6PR	Yes
		miR-221/222	Induce ER-stress	p27Kip1, MEK/ERK	Yes
		miR-30c*	UPR	XBP-1	Yes
		miR-7	Shift from growth to translation	Multiple ribosomal genes	Yes
		miR-708	ER-stress inducible	Rhodopsin	Yes
Protein quality	3.4.	miR-148b	N-glycosylation	C1GALT1	Yes
		miR-30b/d	O-glycosylation	GALNT7	Yes
Epigenetic	3.5.	miR-29	DNA methylation	DNMT3A/3B	Yes
		miR-148/152	DNA methylation	DNMT1	Yes

However, since the underlying limit is likely to be found in microRNA transcription as opposed to post-transcriptional processing by Dicer, the preferential approach is to titrate the expression of specific microRNAs with high impact on specific growth to an optimal expression level that will facilitate fast proliferation. Such an approach was recently taken by Clarke et al., who performed integrated analysis of microRNA, mRNA and protein expression in a set of clones with variable growth rate (Clarke et al., 2012). In total, 35 miRNAs were identified to be up-regulated with increased growth, and 16 miRNAs that were down-regulated. By combining this information with mRNA and protein expression data, certain biological processes such as mRNA processing and protein synthesis were found to be relevant for enhanced proliferation. In silico analysis of microRNA-mediated regulation of these pathways resulted in a high-priority list of microRNAs for use as cell engineering targets or biomarkers, such as microRNA-17-92.

3.2. Apoptosis, cell viability and culture stress

Apoptosis, or programmed cell death, is a necessary physiological function which helps eliminate unhealthy cells, however, the cascade presents difficulties in maintaining high viable cell densities in mammalian bioprocess applications (Müller et al., 2008). Stress conditions in bioreactors, including nutrient limitation, byproduct accumulation, shear and oxidative stress, osmolality and hypoxia, can trigger apoptosis

Table 2
Summary of miRNA analysis and engineering in CHO cells.

Experimental setting	Type of analysis	Outcome	Reference
Temperature shift	Microarray & qPCR	26 regulated miRNAs	Gammell (2007)
Transcription in recombinant cell lines	qPCR	16 miRNAs with de-regulation in recombinant DG44 cell lines	Lin et al. (2010)
Temperature shift	qPCR	10 regulated miRNAs (miR-7) miR-7 reduces growth and enhances qP	Barron et al. (2011a)
MicroRNA repertoire in various cell lines	NGS	380 conserved 22 novel miRNAs	Hackl et al. (2011)
MicroRNA repertoire in various cell lines	NGS	350 conserved miRNAs	Johnson et al. (2011)
Batch cultivation	Microarray & qPCR	118 miRNAs regulated during batch cultivation between lag, exponential and stationary growth phase	Bort et al. (2012)
Nutrient depletion & apoptosis	Microarray	70 miRNAs with regulation upon nutrient limitation	Druz et al. (2012)
MicroRNA overexpression screen	Engineering	miR-17 improves growth miR-21 reduces qP	Jadhav et al. (2012)
Transcription in recombinant cell lines	NGS	190 conserved microRNAs 93 microRNA regulated in two distinct recombinant cell lines	Hammond et al. (2012a)
Genomic context of microRNAs	In silico	Genomic annotation of 350 miRNAs	Hackl et al. (2012b)
Correlation to growth rate	Microarray	35 miRNAs with positive correlation to growth rate 16 miRNAs with negative correlation to growth rate	Clarke et al. (2012)
Specific microRNA knockdown	Engineering	miR-466h-5p knockdown improves batch performance of CHO cells	Druz et al. (2013)

during CHO cell cultures. Apoptosis onset in bioreactors lowers the Integral Viable Cell Density (IVCD) and affects product yield and quality (Druz et al., 2011; Gammell, 2007; Müller et al., 2008). As a result, apoptosis prevention is one of the most widely implemented approaches in CHO cell engineering (Hacker et al., 2009; Majors et al., 2007; Zanghi et al., 1999).

The involvement of microRNAs in apoptosis regulation was initially described in peripheral blood cells of people diagnosed with chronic lymphocytic leukemia (CLL) where a deletion of miR-15a/16 cluster was reported in the majority of patients (Calin et al., 2002). Later studies revealed that the members of this cluster, miR-15a-5p and miR-16, promote apoptosis in malignant B cells by targeting Bcl-2 expression at the post-transcriptional level (Cimmino et al., 2005). Another member of miR-15a/16 cluster, miR-15a-3p, was shown to induce apoptosis by targeting Bcl-xL, thus activating Caspase-3/7 and reducing viability in several cancers (Druz et al., 2013). Another microRNA, miR-21 was found to be up-regulated in several human cancers and characterized as an oncogenic microRNA. Its silencing in glioblastoma cells led to increased apoptosis by activation of Caspases 3 and 7 (Chan et al., 2005; Meng et al., 2006; Si et al., 2007). Cheng and colleagues identified several microRNAs involved in apoptosis regulation using large-scale antisense microRNA inhibition in HeLa cells. The inhibition of miR-1d, 7, 148, 204, 210, 216 and 296 increased apoptosis by activation of Caspase 3, while the inhibition of miR-214 had the opposite effect (Cheng et al., 2005).

Other examples include miR-218 which was found to be involved in NF-kappaB response and apoptosis induction by targeting expression of ECOP gene (Gao et al., 2010). miR-34 family members function as potent mediators of the p53-induced apoptotic pathway by targeting anti-apoptotic genes including Bcl-2, and also participate in a positive feedback loop of p53 activation via increased acetylation by targeting SIRT1 deacetylase (Hermeking, 2010). miR-30 affects the levels of the Ubc9 and ITGB3 genes in breast tumor-initiating cells, thus restricting their self-renewing capacity and targeting them for apoptosis (Yu et al., 2010). miR-10a was shown to participate in the TRAIL-induced apoptosis pathway leading to Caspase 3 activation in human lung carcinomas (Ovcharenko et al., 2007). The members of let-7 microRNA family, let-7c and let-7g, target Bcl-xL directly and Mcl-1 indirectly which leads to Caspase-3/7 activation and apoptosis induction in hepatocytes (Shimizu et al., 2010).

Two of the stresses that cells are exposed to under bioreactor conditions are hypoxia and oxidative stress. Here, involvement of microRNAs has been demonstrated in several instances, however, not yet in CHO cells. miR-15a-5p, miR-16, and miR-20a are down-regulated during hypoxic conditions in human carcinomas (Hua et al., 2006), while miR-26, miR-107, and miR-210 are up-regulated in neoplastic cells. These microRNAs are likely to decrease the pro-apoptotic signaling in a hypoxic environment (Kulshreshtha et al., 2007). miR-210 is progressively up-regulated in endothelial cells in hypoxic conditions and inhibits the receptor tyrosine-kinase ligand Ephrin-A3 which is critical for vascular development (Fasanaro et al., 2008). The up-regulation of the miR-34 family, as part of the p53 network, can be implicated in stress responses to DNA damage, hyperactive cytokine signaling, and hypoxia (He et al., 2007). The miR-17–92 cluster was shown to target hypoxia-inducible factor alpha (Hif-1α), a transcriptional factor known to regulate cellular response to hypoxia and to play an important role in various biological processes such as glucose metabolism, pH regulation and angiogenesis (Taguchi et al., 2008). miR-31 was also shown to activate Hif-1α via the inhibition of factor-inhibiting hypoxia-inducible factor (FIH) (Liu et al., 2010).

With respect to oxidative stress, the bicistronic transcript miR-144/451 was shown to modulate oxidative stress in erythroid cells. miR-144 directly affects NRF2 gene expression in K562 and primary erythroid progenitor cells, which induces the expression of several antioxidant enzymes (Sangokoya et al., 2010), while miR-451 protects erythrocytes against oxidative stress and rescues erythroid cells' differentiation defect by inhibiting the intracellular regulator of cytokine signaling, 14-3-3ξ gene (Patrick et al., 2010). miR-34a and miR-93 are involved in the loss of oxidative stress defense and repress expression of genes associated with oxidative stress regulation and defense mechanism such as Sp1, Sirt1, Mgst1, and Nrf2 (Li et al., 2011b). miR-1 and miR-133 produce opposing effects on apoptosis induced by oxidative stress in rat cells (Xu et al., 2007). miR-1 has a pro-apoptotic function in response to oxidative stress by targeting heat shock proteins HSP60 and HSP70 and miR-133 seems to have an anti-apoptotic role by repressing Caspase 9 gene expression.

Some microRNAs have been associated with other stress types that might be encountered within a bioreactor such as osmotic pressure, shear stresses, and nutrient depletion/gradients. miR-200b and miR-717 are down-regulated by isotonic and hypertonic treatments in renal medullary epithelial cells. However, when up-regulated, these microRNAs inhibit the activity of a transcriptional factor called osmotic response binding protein, OREBP, a major cellular osmoregulator in kidney cells and T-lymphocytes (Huang et al., 2010). miR-7b is over-expressed in hyperosmolar conditions to down-regulate the protein levels of Fos, which reduces the activity of transcription factor activator protein 1 (AP1), a regulator of cellular processes, which is formed by dimerization of Fos and Jun proteins (Lee et al., 2006). miR-21 and miR-19a are induced by shear stress in endothelial cells (Qin et al., 2010; Weber et al., 2010).

Druz and colleagues recently showed the up-regulation of the large miR-297–669 cluster during apoptotic conditions induced by nutrient depletion in CHO cells. One member of this cluster, miR-466h-5p was shown to alter the expression of five anti-apoptotic genes from different apoptosis-initiating pathways (bcl2l2, dad1, birc6, stat5a, and smo). This microRNA was shown to be activated in response to glucose depletion (Druz et al., 2012). Antisense knockdown of miR-466h-5p delayed apoptosis onset in nutrient-depleted conditions by decreasing Caspase-3/7 activation and increasing cell viability (Druz et al., 2011). Stable inhibition of miR-466h-5p in CHO cells enhanced apoptosis resistance and increased protein production (Druz et al., 2013). One other member of miR-297–669 cluster, miR-669c-5p, has been previously associated with impairments in glutathione metabolism which activates the apoptosis cascade (Lanceta et al., 2010; Maes et al., 2008).

The utilization of microRNAs with roles in apoptosis regulation and stress response should provide researchers with an additional tool to minimize or eliminate apoptotic effects that result from different stress conditions that reduce recombinant protein production. The recent sequencing of the CHO cell genome and microRNA transcriptome revealed conserved sequences of the apoptosis and stress-regulating microRNAs such as miR-1, let-7 family, miR-7b, miR-10a, miR-15/16 cluster, miR-93, miR-107, miR-144, miR-200b, miR-210, miR-214, miR-218, and mi-R708. These microRNAs and the miR-297–669 cluster are promising targets for apoptosis pathway engineering. Due to the complexity of apoptosis and the diversity of apoptotic stimuli, it may be useful to investigate the combined effects of several microRNAs affecting genes from different stages of the apoptosis cascade as well as those that seem to be involved in global regulation of the pathway. It might be also worthwhile to consider the engineering of whole clusters of apoptosis-relevant microRNAs since clustered microRNAs are known to be transcribed together as polycistronic transcripts to regulate mRNA of genes with similar functions (Druz et al., 2011), thus enabling a more global approach to modification of cellular phenotypes. An important aspect here is the need to test CHO cell-specific microRNAs, their biological role and their effects on CHO cell-specific gene targets not only in small scale, but also in an industrial-scale bioprocess environment to finally identify the most suitable candidates and combinations.

3.3. Energy metabolism

Metabolic homeostasis is crucial for maintenance of cellular physiology for optimal growth and adaptation to culture conditions. Culture

adaptations are controlled by complex regulatory networks and have to continuously evolve to monitor and respond to such changes during a bioprocess to keep it efficient. In a recent review the role of microRNAs and their integration into multilayered cellular networks functioning to maintain physiological conditions was discussed (Rottiers and Naar, 2012). The regulation of microRNA expression in response to genetic, epigenetic or environmental cues, to metabolic wastes or stress may contribute to our understanding of cellular physiology and metabolism (Lynn, 2009; Tomankova et al., 2010). The first link connecting microRNAs to metabolic control is miR-122, which is involved in lipid metabolism and regulates genes involved in cholesterol biosynthesis, such as 7-dehydrocholesterol reductase, 3-hydroxy-3-methylglutaryl-CoA synthase-1 and 3-hydroxy-3-methylglutaryl-CoA reductase (Jopling, 2012; Sacco and Adeli, 2012). Application of an LNA antagomir of miR-122 leads to 25–30% reduction of plasma cholesterol levels (Elmen et al., 2008; Esau et al., 2006). Similarly, miR-33a and miR-33b were shown to co-express from an intron of the transcription factor of sterol-regulatory element-binding proteins, a family of proteins that controls the expression of genes central to fatty acid homeostasis. They thus coordinate the regulation of fatty acid, triglyceride and cholesterol biosynthesis and uptake (Davalos et al., 2011; Rayner et al., 2010). Recent interesting findings suggested functions for let-7 in regulation of mammalian glucose metabolism by targeting IGF1R, INSR, and IRS2 components of the insulin–PI3K–mTOR pathway (Zhu et al., 2011). The ratio of Pyruvate Kinase Isozymes M1/M2 (PKM) is involved in control of glycolysis rate and it was suggested that miR-124, miR-137 and miR-340 are involved in the regulation of PKM1/2 ratios in colorectal cancer cells (Sun et al., 2012). In human leukemic Jurkat cells, it was shown that the expression of miR-23a was controlled by NF-κB and could play a critical role in glutamine metabolism (Rathore et al., 2012). Mitochondria are central to all energy, related functions of the cell, and recently it was proposed that several microRNAs are associated with them and could control their function (Carrer et al., 2012; Sripada et al., 2012a,b). Over several decades, studies have suggested that cellular and physiological responses to nutrients such as glucose, lipids, growth factors and metabolic wastes and their respective levels, induce drastic changes in gene expression patterns and altering metabolic homeostasis. The link between nutrient levels and microRNAs could be exploited by using them as metabolic sensors and/or modulators, both to fine tune and to monitor biologically controlled bioprocesses (Carrer et al., 2012; Druz et al., 2012; Kochanowski et al., 2012; Zanghi et al., 1999).

3.4. Productivity and product quality

The ability of a CHO clone to synthesize and secrete correctly modified recombinant proteins in abundance is a key attribute. Clearly, there are numerous steps, enzymes, co-factors, quality control points and internal structures involved in ensuring this process operates efficiently. All of them are subject to regulation within the cell and depend on both extracellular and intracellular stimuli, with microRNAs playing an important role in several of these processes. As mentioned above, increasing the levels of miR-7 in CHO cells has been reported to increase cellular productivity – at the expense of cell growth – which is reflected in a shift in the abundance of particular ribosomal proteins in the cell and other proteins involved in translation elongation (Meleady et al., 2012a). Lin and colleagues screened microRNAs in recombinant human IgG producing CHO cells and found miR-221 and miR-222 to be significantly down-regulated in all cell lines when compared with the parental DG44 cell line, indicating good targets for engineering high producer cell lines (Lin et al., 2010). The miR-221/222 cluster was also found down-regulated during ER stress in human hepatocellular carcinoma cells. The ectopic introduction of miR-221/222 mimics increased ER-stress and induced apoptosis which was associated with p27Kip1 and MEK/ERK-directed cell cycle regulation (Dai et al., 2010).

In other model systems, microRNAs have been implicated in regulating proteins involved in the unfolded protein response — a key cellular stress response that impacts on recombinant protein production. miR-30c-2* can down-regulate the expression of XBP-1, a critical mediator of cellular adaptation to increased protein processing load (Byrd et al., 2012). This gene has been successfully engineered in CHO cells to improve productivity in the past (Tigges and Fussenegger, 2006).

Several microRNAs including miR-122 (Yang et al., 2011), miR-30, 181d and 199a-5p have been shown to suppress GRP78/BiP, another cellular chaperone involved in UPR and one that has been given some attention in the context of recombinant protein production in CHO cells (Morris et al., 1997; Van Dyk et al., 2003). miR-708 was shown to be induced during ER stress by the transcription factor CCAAT enhancer-binding homologous protein (CHOP) and may facilitate the enhancement of ER protein-folding capacity under the stress of accelerated protein synthesis (Behrman et al., 2011). miR-204 supported ER and oxidative stress induction in human trabecular meshwork cells by inhibition of two genes involved in the elimination of damaged and misfolded proteins (SERP1/RAMP4 and M6PR), thus enhancing the expression of carbonylated proteins (Li et al., 2011a). microRNAs are also known to be key regulators of pancreatic beta cell function. In particular, miR-375 can influence glucose-induced insulin secretion by modulating the expression of myotrophin, a protein potentially involved in cytoskeleton dynamics (Poy et al., 2004). In addition, a number of microRNAs are involved in various aspects of exocytosis that have implications in other human diseases (Lovis et al., 2008; Sullivan et al., 2012; Zhang et al., 2011). Finally, mTOR overexpression has recently been shown to confer benefit to both CHO cell productivity and growth (Dreesen and Fussenegger, 2011) and mTOR has also been identified as a protein whose expression can be regulated by microRNA binding (Liu and Wilson, 2012). These observations demonstrate the potential that manipulation of particular microRNAs may have in engineering aspects of the secretory and high productivity function of CHO cells. This is also supported by consistent patterns of microRNA expression observed between different host cell lines and their recombinant, high producing subclones (Hackl et al., 2011; Lin et al., 2010).

A major aspect of recombinant protein production is product quality, specifically the pattern of glycosylation on the therapeutic product. Several instances indicate that microRNAs may also play a role in the control of this important property. It was recently found that a specific microRNA, miR-148b, modulates the expression of β1,3-galactosyltransferase-1 (C1GALT1), an important enzyme in the synthesis of O-glycosylation (Coppo and Amore, 2004; Novak et al., 2001).

In another study focused on the regulation of glycosylation and its impact on cancer metastasis, it was shown that the up-regulation of microRNA clusters suppresses N-acetylgalactosamine transferases (GALNTs) which initiate O-linked glycosylation (Gaziel-Sovran and Hernando, 2012). Specifically, miR-30b/30d expression was shown to silence GALNT7, resulting in defective glycosylation and changes in protein exocytosis. To this end, no study has looked in detail on microRNA target sites in enzymes mediating the formation of N- and O-glycosylation in animal cells. However, the above examples provide evidence that microRNAs are involved in these processes. Therefore, this justifies a systematic analysis of microRNA target site enrichment in CHO glycosylation genes, which will unveil the potential value of microRNAs as diagnostic and engineering tools to control the precise pattern of glycosylation required for production of biosimilars.

3.5. Clonal stability and epigenetics

At an early stage of the biopharmaceutical product development pipeline, a major R&D challenge is to accelerate progress from the point of having cloned an appropriate gene for a biopharmaceutical product into a CHO cell, to having established the best CHO cell clone – in terms of growth rate, productivity, product quality and stability –

to place in the bioreactor for manufacture of clinical trial batches (and with a reasonable level of confidence that the same clone can be used for subsequent large-scale production, in order to avoid expensive and time-consuming new cell line development and re-validation of the process for regulatory approval).

Clone to clone variation is hugely important with respect to many bioprocess-relevant cellular phenotypes, including productivity (O'Callaghan et al., 2010; Pichler et al., 2011; Pilbrough et al., 2009; Porter et al., 2010; Prieto et al., 2011; Sigal et al., 2006; Sunley et al., 2008). Currently, clone selection is done on a trial-and-error basis, and many initially promising clones prove, at a later stage in the process, to be unstable, thus making the process unpredictable. Methylation of the viral promoters commonly used in mammalian expression vectors is believed to play a role (especially in slow loss of productivity over time) (Osterlehner et al., 2011; Yang et al., 2010). Loss of transgene amplification when certain selection systems (e.g., DHFR) have been used and DNA rearrangements in and around the transgene can occur; these mechanisms are probably most important in rapid loss of productivity (Kim et al., 2011).

A considerable literature exists in relation to stability/instability of cells in culture (e.g., Bailey et al., 2012). Substantial recent research has been done on the effects of including features in the expression vector that discourage epigenetic silencing. These include insulators such as the chicken β-globin HS4 element; (S) MARs — ((Scaffold) Matrix Attachment Factors); STARs (stabilizing anti-repression elements); and UCOEs (ubiquitous chromosome opening elements) (Allen and Antoniou, 2007; Galbete et al., 2009; Harraghy et al., 2012). While the proximate biochemical mechanisms of silencing (e.g. DNA methylation, histone deacetylation, reduction of copy number, and DNA rearrangement) and the identity of corresponding enzymes are reasonably well understood, there is no in-depth understanding of the molecular regulatory events that lead to these modifications in some clones, but not in their sister clones, which are being cultured at the same time under the same conditions. Of course, the impact of epigenetic changes in CHO cells goes beyond transgene stability; it may also influence the stability of other characteristics including growth and product quality.

Since microRNAs are believed to regulate expression of over 50% of proteins, it is likely that they have a role in regulation of expression of the enzymes involved in DNA and chromatin modification (Lorio et al., 2010). However, limited information exists in this regard, and no information at all is available for CHO. One of the few microRNAs with a recognized role in epigenetic modifications in cancer is the miR-29 family, which was shown to target DNA Methyltransferases (DNMT) 3A and 3B (Fabbri et al., 2007). In doing so, these microRNAs prevent inappropriate methylation at the promoters of tumor-suppressor genes and their expression has been shown to be down-regulated in tumor cells. On the other hand there are several reports of epigenetic changes impacting on microRNA expression.

A recent publication by Druz et al. (2012) demonstrated how the biogenesis of microRNAs can be influenced by epigenetic events. In this case, glucose depletion in the culture medium led to histone deacetylase inhibition, increased promoter acetylation and subsequently increased transcription of miR-466h-5p. Although this work was performed in mouse cells, this microRNA had previously been shown by the same group to increase resistance to apoptosis in CHO cells (Druz et al., 2011). As with most genetic regulatory networks, there is evidence of feedback loops between microRNAs and their target genes or proteins. A good example of this feedback is the link between miR-148 and miR-152 and DNMT1. The promoters of these microRNAs are silenced by DNMT1-dependent methylation, and DNMT1 itself is, in turn, a target for repression by these miRs (Xu et al., 2012). It is becoming more apparent, as an increasing number of reports appear, that there is a strong inter-relationship between microRNA activity and the epigenetic status of cells, and it will be interesting to see how this relationship might be exploited in the bioprocessing area.

3.6. Tools for probing and engineering microRNA function in CHO cells

Sections 3.1 to 3.5. give clear evidence of microRNA-mediated regulation of gene expression in a range of biological processes that are of high relevance for a robust and excellent performing CHO cell line (Table 1). But should one directly translate these valuable insights into experimental engineering approaches in CHO? The answer unfortunately has to be no, since microRNA function strongly depends on the cellular mRNA transcriptome, and can therefore be extremely diverse between different cell types or even different states of the same cell type (Shu et al., 2012) (which likely includes different CHO host cell lines as well). Hence, two roads can be taken in order to prioritize microRNAs for stable engineering: i) promising microRNA candidates from expanded literature searches can be probed for their applicability as engineering targets in CHO cells using classical reverse genetic approaches (Jadhav et al., 2012; Müller et al., 2008) and ii) transcriptomic experiments can be helpful in reducing the list of engineering candidates to a few microRNAs that can be directly taken to transient and stable functional analyses, as in the case of miR-7 (Barron et al., 2011a) and miR-466h-5p (Druz et al., 2013). In the following an overview of currently available methods for transient and stable overexpression and knockdown of microRNA is given.

3.6.1. miRNA loss of function by antagomirs

Antagomirs are antisense miRNA oligonucleotides, which are currently the most widely used molecules for targeted miRNA inhibition (Kaur et al., 2007; Krützfeldt et al., 2005). They have been applied successfully to test miRNA function in cell culture systems and as well as animal models (Pasquinelli, 2012). Some antagomirs contain chemical modifications to increase their binding to a target miRNA and/or serve as protection from nucleases. One of the most common chemical modifications are 2-O-methyl or 2-O-methoxyethyl and locked nucleic acid (LNA) (Fabani and Gait, 2008). A prominent example for using antagomirs is the blocking of miR-122 in the liver in order to reduce replication of hepatitis-C virus, which is dependent on high miR-122 levels in the liver (Gottwein, 2013). Besides microRNA sequestration through antagomirs, targeted microRNA cleavage was established by introducing a catalytic domain from DNAzymes to the antagomir. These molecules are called “antagomirzymes” and function by binding and cleaving complementary microRNA sequences (Jadhav et al., 2009).

Antagomirs are well suited as transient tools for testing microRNA function in animal cell models if they can be efficiently delivered into the cytoplasm of a cell. For long-term effect (i.e. during a fed-batch or continuous bioprocess), antagomirs would need to be repeatedly delivered to the cell via a media feed, thus, requiring highly effective and cheap delivery methods (Stein et al., 2010) for suspension cells. While there have been reports of transfection reagent free uptake of small RNAs into cells (“naked” or “gymniotic” uptake) (Lingor et al., 2004; Moschos et al., 2011), these are challenging protocols and still would require large amounts of synthetic RNA to be delivered to the culture media. Therefore, methods have been developed that employ synthetic antagomir molecules produced by transcription from simple expression vectors and are termed “decoys” or “sponges” (Yang et al., 2012).

3.6.2. miRNA loss-of-function by miRNA sponges

miRNA sponges are synthetic RNA molecules that act as pseudo target by presenting a dominant amount of miRNA binding sites to a cell, thus acting as scavenger for miRNA function. Fittingly, these synthetic RNA molecules are termed “sponges” or “decoys”. Ebert and colleagues described for the first time the application of miRNA sponges for knockdown of miRNA function. They designed and engineered tandem repeats of specific miRNA binding sites into the 3′-UTR of green fluorescent protein reporter genes and demonstrated more effective inhibition of miRNA function compared to other methods such as antagomirs. Furthermore sponges can be designed in such a

way, that an entire family of miRNAs can be inhibited. Thereafter, several studies have successfully applied miRNA sponges in both in vitro cell culture systems and in vivo for inhibiting miRNA function (Brown and Naldini, 2009). Druz and colleagues have already applied this tool to generate engineered CHO cells (Druz et al., 2013), which overexpress miR-466h-5p sponge and thereby exhibit a modulated growth behavior. The future directions in CHO cell engineering include the design and development of microRNA-sponge expression systems that are based on an inducible system for culture-stage specific fine tuning of microRNA activity.

3.6.3. miRNA gain-of-function by miRNA mimics and vector based expression

Modulating miRNA function by overexpression is a prominent alternative for cell engineering and commonly termed "miRNA-targeting" or "miRNA-gain-of-function" strategy. Two main approaches have been developed for ectopic expression, which depend on delivery of a synthetic microRNA (termed "microRNA mimic") (Wang, 2011) or vector-based transcription of microRNA precursors, which are further processed to yield the mature microRNA of interest.

The miRNA mimic technology results in silenced target gene translation by introducing synthetic double-stranded RNA molecules with sequences equivalent to an endogenous miRNA, thus reinforcing the biological effect. Compared to vector-based screening of microRNA function, mimics have the advantage of being readily available without the need for cloning. Hence, high-throughput screenings of microRNA function usually employ large synthetic libraries that cover the entire miRNome available for a specific organism. The shortcomings of this strategy are i) the limitation to transient overexpression with short-term effects, especially in cell lines exhibiting rapid proliferation and therefore dilution of synthetic RNA sequences, ii) the synthetic nature of these molecules and their chemical modifications, which could have cytotoxic effects, and iii) the usually high initial increase in microRNA copies per cell (usually several hundred-folds) might result in off-target effects. Therefore, the alternative strategy uses the endogenous miRNA maturation pathway of a cell to ectopically produce mature miRNAs from plasmids containing a primary microRNA transcript. Such constructs can be used for both transient and stable expression of miRNAs to study long term effect in gain-of-function. Furthermore, the choice of specific promoters allows to time microRNA expression with the entry of specific culture stages and provides a further opportunity to control miRNA function. Jadhav and colleagues have developed vector based expression system for screening microRNA function in CHO cells. The reported protocol uses in silico designed stem-loop sequences that are synthesized and readily cloned into a commercial expression vector. Thus, albeit the need for cloning, a medium-throughput protocol for gain-of-function screen of selected microRNAs could be established, which identified positive effects for miR-17 on CHO growth performance (Jadhav et al., 2012).

3.7. The need for better computational tools to predict microRNA function

Theoretically, the labor- and time-intensive phase of functional screening could be replaced by computational tools that reliably predict the biological function of a microRNA in a certain transcriptomic environment. However, the improvement of tools for the identification of interactions between microRNAs and their respective targets remains a key challenge of microRNA research. When microRNAs were first identified, sequence analysis tools were developed for the prediction of such interactions, exploiting conserved seeds and sequence complementarity (Lewis et al., 2005). Limitations of early seed-motif matching approaches led to the integration of thermodynamic models for binding strength. The challenges of reliable de novo prediction are, however, reflected in the lack of agreement between different tools (Rajewsky, 2006; Sethupathy et al., 2006). While focusing on predictions common to several tools has been a popular strategy to try and reduce false positives, this comes at a considerable cost in terms of false negatives. High false positive/negative rates therefore motivate more elaborate attempts at integration of multiple tools (Zhang and Verbeek, 2010).

In a complementary trend, individual tools now consider and combine additional data sources. Algorithms may (1) incorporate other relevant computational predictions, like target site accessibility (Kertesz et al., 2007), (2) take advantage of multi-track measurements, such as matched microRNA and mRNA expression profiles (Bonnet et al., 2010), or (3) use expression profiles to refine sequence based predictions (Stingo et al., 2010; Wang and Li, 2009). Increased enrichment of targets in known pathways suggests that the incorporation of additional information as filters for sequence based predictions may be a promising strategy for reducing false positive rates (Muniategui et al., 2012).

Lack of agreement across methods has nevertheless remained an issue and concordance with experimentally validated or refuted interactions was found to be poor in an evaluation of a large independent data set (Shridhar and Kreil, in press). Prediction performance as well as true interactions may be specific to certain experimental settings. Indeed, some approaches have been tailored for particular experimental designs like time-course data (Jayaswal et al., 2009) or try to exploit data from different tissues or heterogeneous cell mixtures to identify additive interactions between microRNAs and multiple targets (Wang and Li, 2009). The unavailability of an experimentally validated comprehensive 'gold standard' list of interactions and absence of interactions, on the other hand, has been a major limitation for the development of improved analysis tools. Recent collection of data from high-throughput techniques like HITS-CLIP and TAP-tar for probing physical interactions (Yang et al., 2011) may over time, fill this need. Collections of evidence covering a variety of experimental scenarios will provide more powerful data to train and validate new analysis methods.

4. Conclusions and future perspectives

With respect to microRNA engineering of CHO cells for enhanced phenotypes, an important aspect is that many of the phenotypes discussed in the previous sections overlap (Fig. 2). Therefore, it is likely that a cell line with optimal properties needs to express a variety of microRNAs in a coordinated and balanced way. Apart from new engineering strategies, a better understanding of the microRNAs' roles in determining phenotypes could also lead to the development of novel screening tools that allow better prediction of cell behavior at industrial scale, by analyzing the precise expression pattern of those microRNAs that were previously identified to control these properties. With improvements in analysis methods, these could be assessed during an early stage in cell line development, from small scale cultures, as a novel screening tool for the identification of suitable clones and as tools for monitoring cell behavior and state in production processes. Emerging technologies, such as flow cytometry assisted RNA quantification (Chapin et al., 2011) that could be directly applied to a cell homogenate without prior RNA isolation, might become valuable tools for integration of biomarkers in the process of cell line development.

Finally, even with the research on microRNAs and their application for CHO cell engineering in full swing, we would like to point out that microRNAs are by no means the only interesting non-coding RNAs that could be used for cell line optimization, specifically in the field of genomic and phenotypic stability. A recent manuscript identifies piRNAs expressed in CHO cells (Fig. 1) (Gerstl et al., 2013), another group of small non-coding RNAs that have been linked to epigenetic and post-transcriptional gene silencing, specifically of retrotransposons, by their interaction with PIWI proteins (Sienski et al., 2012). In addition, long non-coding RNAs are considered to be involved in the regulation of gene transcription, both by controlling the basal transcriptional machinery and by gene-specific regulation via recruitment of epigenetic modifying factors to genomic loci, and modulation of mRNA splicing, as well as translation (Huang et al., 2012b). Both of these classes of RNA

promise to enable exciting new approaches to cell line optimization for industrial purposes in the near future.

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Appendix D

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

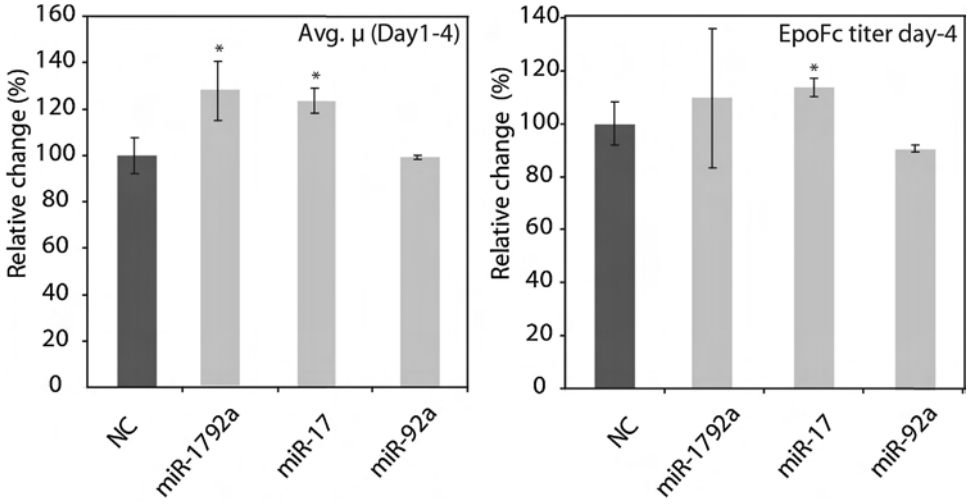

Fig. 1. Effect of transient overexpression of miRNAs on growth rate and recombinant protein titers: (A) effect of miRNA overexpression on the growth rate is represented by the average growth rate (μ) calculated until day 4 of transient miRNA over-expression relative to the negative control transfection (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

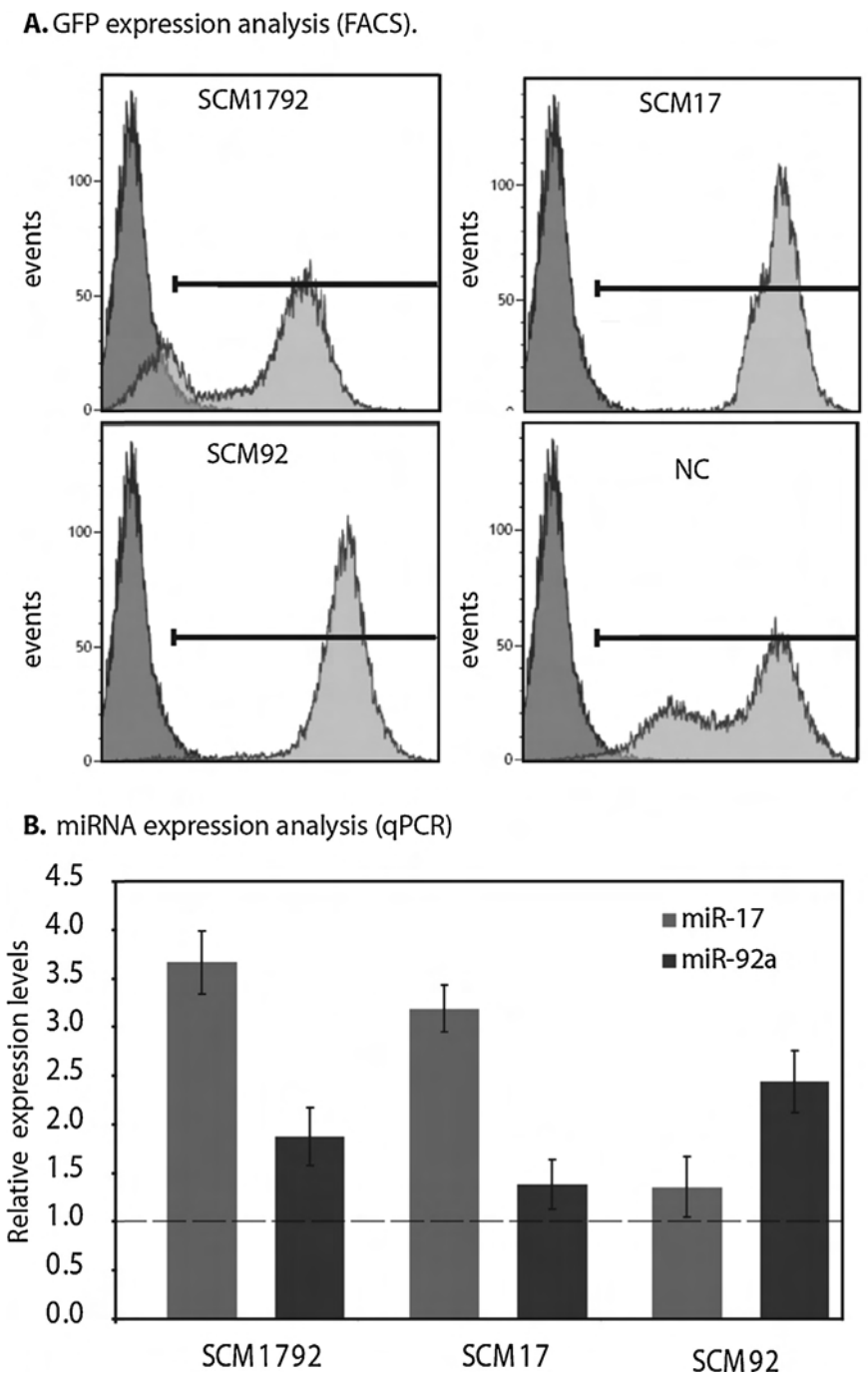

Fig. 2. Selection and characterization of stable miRNA expression clones: miRNA overexpression was driven by a constitutive CMV promoter with co-expression of GFP. (A) The panel shows GFP-expression (Fl-1H) of 4 stable-pools overexpressing the miR-17–92 cluster (SCM-1792), miR-17 (SCM-17), miR-92a (SCM-92a) 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.

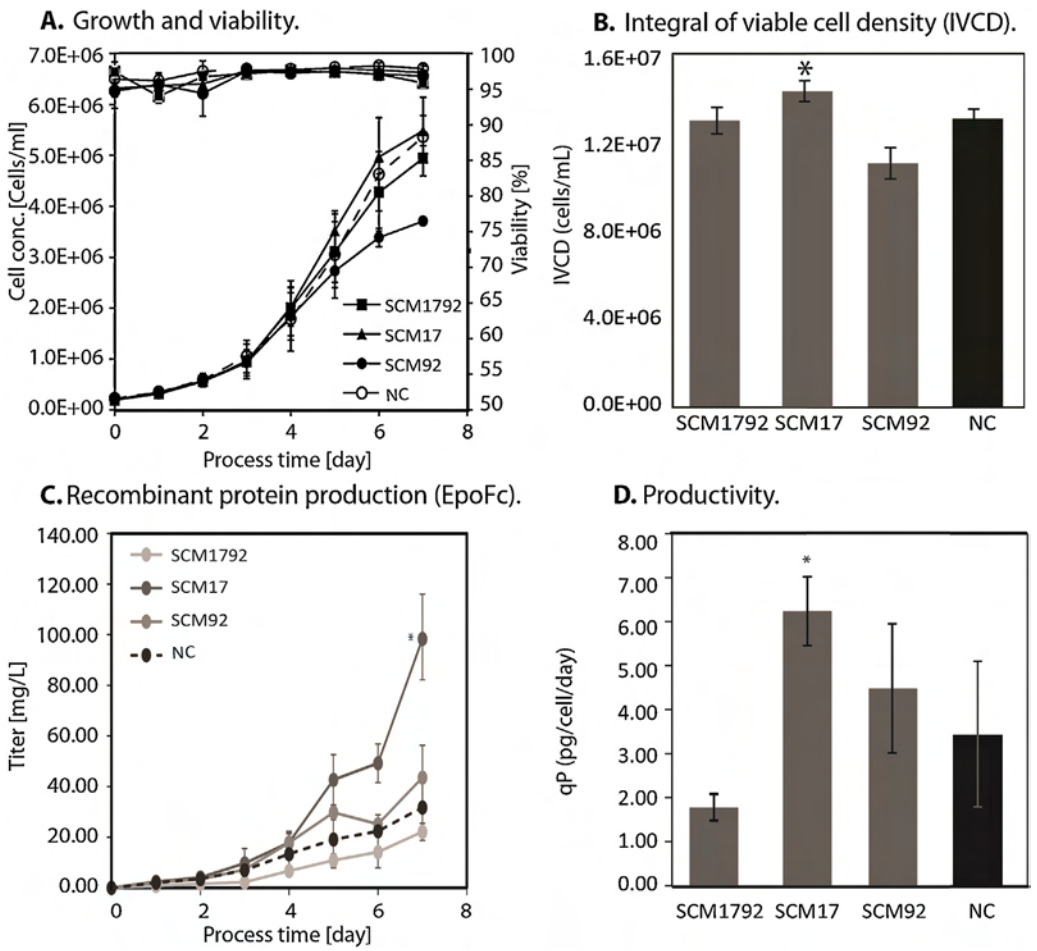

Fig. 3. Analysis of culture performance of stable miRNA overexpressing EpoFc producers in shaken batch cultures: (A) mean viable cell density (primary *y*-axis) and viability (secondary *y*-axis) of three independent batches of SCM-1792(■), SCM-17(▲), SCM-92 (●), and NC (○). (B) Integral of viable cell 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

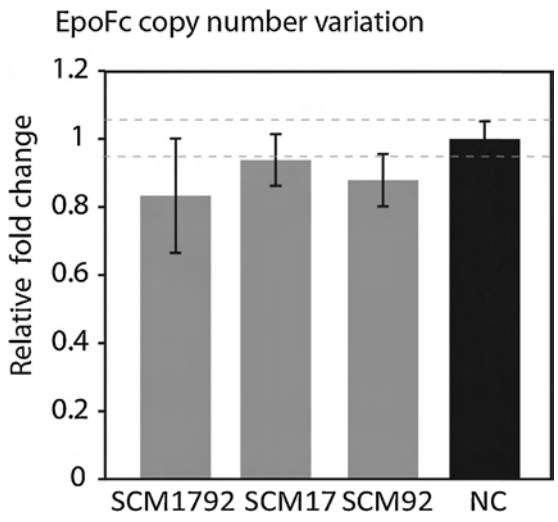

Fig. 4. EpoFc gene copy number analysis: EpoFc gene copy numbers in the miRNA overexpressing pools were analyzed using real-time Q-PCR. Shown is the relative copy number of EpoFc relative to the reference gene, normalized to the negative control. The data are represented as mean (±standard deviation;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

Dynamic mRNA and miRNA Profiling of CHO-K1 Suspension Cell Cultures.

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Research Article

Dynamic mRNA and miRNA profiling of CHO-K1 suspension cell cultures

Juan A. Hernández Bort[1,], Matthias Hackl[1,*], Helga Höflmayer[1], Vaibhav Jadhav[1], Eva Harreither[1], Niraj Kumar[1], Wolfgang Ernst[1,2], Johannes Grillari[1] and Nicole Borth[1,2]*

[1] Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna, Austria
[2] ACIB GmbH, Austrian Centre of Industrial Biotechnology, Graz, Austria

In spite of the importance of Chinese hamster ovary (CHO) cells for recombinant protein production, very little is known about the molecular and gene regulatory mechanisms that control cellular phenotypes such as enhanced growth under serum-free conditions or high productivity. Most microarray analyses to this purpose are performed with samples taken during the exponential growth phase. However, the cellular transcriptome is dynamic, changing in response to external and internal stimuli and thus reflecting the current functional capacity of cells as well as their ability to adapt to a changing environment. Therefore, during batch or fed-batch cultivations it can be expected that the transcription pattern of genes will change and that such changes may give indications on the cellular state in terms of viability, growth, and productivity. In the current study we monitored the change in expression patterns of mRNAs and microRNAs (miRNA) during lag, exponential, and stationary phases in CHO-K1 suspension cell cultures. In total, over 1400 mRNAs and more than 100 miRNAs were differentially regulated ($p<0.05$) relative to the batch culture at the starting point. Functional clustering revealed groups of genes with similar expression patterns, which were subjected to functional pathway analysis. In addition, as miRNAs generally act as negative post-transcriptional regulators of mRNAs, we looked for changes in their expression that were inverse to those of their predicted target mRNAs.

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1 Introduction

Chinese hamster ovary (CHO) cells are currently the most important mammalian host cell line for the expression of recombinant proteins, as CHO cells are used for the production of approximately 60% of all marketed recombinant therapeutics. Thus, an excellent molecular understanding of the cellular processes that guide growth and recombinant protein productivity in CHO cells, including the important aspect of protein quality, is a prerequisite for both cell line development and process optimization, and will enable new approaches for targeted cell engineering and selection procedures [1–3].

Correspondence: Dr. Juan A. Hernández Bort, Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria
E-mail: juan.hernandez-bort@boku.ac.at

Abbreviations: 3'UTR, 3'-untranslated region; **BRCA1**, breast cancer 1; **Eme1**, essential meiotic endonuclease 1; **FDR**, false-discovery rate; **Gapdh**, glyceldehyde-3-phosphate-dehydrogenase; **H2-Q7**, histocompatibility 2, Q region locus 7; **Hspa7**, Heat shock 70 kDa protein 7; **LNA**, locked-nucleid acid; **MCM7**, mini-chromosome maintenance 7; **miRNA**, micro RNA; **Tsen34**, tRNA-splicing endonuclease subunit Sen34

*These authors contributed equally to this study and share first authorship.
Additional corresponding author: Matthias Hackl, Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria
E-mail: matthias.hackl@boku.ac.at

For the characterization of different cell phenotypes and to achieve a profound molecular understanding, different "Omic" technologies, including transcriptomics and proteomics [4–11] as well as metabolomics [12–14], are used to identify individual genes that correlate with a certain phenotypic behavior [15]. In a recent publication a large number of industrially used clones were analyzed to reveal a "short" list of ~200 genes involved in and predictive of cellular productivity, within the range of conditions tested [16]. However, most work in this context [5–7, 17–20] typically is carried out using samples harvested during the exponential growth phase to compare different cell lines or culture conditions. This approach disregards the fact that biological systems are dynamic and subject to change, continuously altering the transcription of mRNA and miRNA as well as protein translation and activity in response to external and internal stimuli. Thus analysis of these parameters at a given time point will only reflect the present state of the cells and their environment, underlining the ability to adapt to changing conditions [21]. Batch culture, as the most commonly used culture system in publications, in fact is the perfect example for this, as exponential growth spans a time phase of continuously altered media composition. Slight shifts in the time of sampling, though all executed during "exponential growth", will imply significant changes in the environment. Few papers have so far addressed how cells adapt to these changes at the level of transcription [22]. In view of the increasing number of studies that use transcriptomics to screen for phenotype relevant changes in gene expression, it appears important to obtain an overview over these dynamic changes and the number of genes affected as a background to identify changes that are relevant to phenotypic differences between cells or conditions.

The addressed adaption to culture conditions spans all levels of molecular regulation in cells, from transcription to translation. However, most immediately connected to mRNAs are probably microRNAs (miRNA), a class of short non-coding RNAs that control the fate of gene expression via post-transcriptional repression of mRNA translation or mRNA destabilization [23]. The ~22 nt mature forms of miRNAs are derived from RNA Pol II-transcribed primary transcripts that are processed in the nucleus by the RNaseIII Drosha into ~70 nt precursor transcripts [24] and subsequently exported into the cytoplasm, where RNase Dicer completes biogenesis by cleaving the precursors into complementary RNA duplexes [25]. It is assumed that both sequence features as well as the thermodynamic stability at the duplex ends are crucial for the selection of the guide miRNA for incorporation into the RNA-induced silencing complex (RISC), while the opposite strand (miRNA-star) is rapidly degraded [8, 26]. By directing this protein complex to mRNAs with complementary sequence motifs that are predominately found in the 3′-untranslated regions (3′-UTR), the mature miRNA enables RISC to either destabilize or repress the translation of the target mRNA [27]. In silico predictions of miRNA–target interactions suggest up to hundred distinct targets for a single miRNA species and several different miRNA binding sites in individual mRNAs, thus creating a complex and interwoven network of regulation [28]. This, together with the high degree of conservation of many miRNA sequences as well as miRNA binding sites throughout mammalian as well as other eukaryotic species [29], underlines the importance of this post-transcriptional layer of regulation.

Recently, the importance of miRNA regulation has also been recognized in CHO cells, where so far 365 highly conserved miRNAs have been identified and annotated [30, 31]. Speculations about the ability of miRNAs to alter cell proliferation, increase robustness, or enhance productivity of CHO cells [18] have been confirmed, by showing that miR-7 is able to drastically diminish cell growth [32]. In addition, comparisons of miRNA transcription between serum-free adapted and serum-dependent CHO cell lines as well as between recombinant and host cell lines [30, 33] have contributed to the understanding of miRNA function in CHO cells. However, as with mRNAs, comparisons are mostly done using samples from a single time point.

Interestingly, despite the obvious connection between miRNAs and mRNA [34, 35], many transcriptome profiling studies have been published either about mRNA [8, 20, 36, 37] or miRNA [38, 39], but very few studies have combined both [40, 41]. To assess the role of miRNAs on mRNA profiles it may be even more important to analyze a time course, as it is likely that the effect of a higher expression of certain miRNAs may take some time to be observable in the mRNA profiles.

The aim of this study therefore was the dynamic characterization and correlation of mRNA and miRNA expression patterns during lag, exponential, and stationary phase of CHO-K1 suspension cells in batch culture using a validated cross-species mRNA microrray platform [17, 18] and a mouse LNA miRNA microarray platform [42]. The samples were taken during a previously published experiment on adaptation of cell lines to growth in glutamine-free media [43]. Initially the goal was to study differences in gene expression between cells grown with and without glutamine, however, the

high dynamics of gene expression observed made it clear that a background of normal changes needs to be defined and that this is of general importance even outside the comparison of the cell lines described in [43]. In the present paper we therefore focus on the dynamics changes in gene and miRNA expression during batch culture. Differentially regulated genes were subjected to functional clustering analysis and pathway enrichment. In total, more than 1400 mRNAs and more than 100 miRNAs were differentially regulated (adj. $p<0.05$) over the different batch phases (lag, exponential and stationary) relative to the batch starting point. Significant effects of changes in miRNA expression on the mRNA transcript profile were shown. Our data underline the dynamic nature of gene expression and stress the importance of time course experiments for the comparison of phenotypes or culture conditions.

2 Materials and methods

2.1 Cell lines and media

The CHO-K1 suspension cell lines adapted to growth in 8 and 4 mM L-glutamine used in this study have been described previously [43]. Cells were cultivated in CD CHO medium (Gibco, Invitrogen, Carlsbad, CA, USA) and supplemented with L-glutamine. Batch cultures were cultivated in spinner flasks with a working volume of 100 mL at 37°C in a humidified atmosphere containing 7% carbon dioxide and with constant stirring at 50 rpm.

2.2 RNA isolation

For each cell line (4 and 8 mM L-glutamine-adapted CHO cells) two biological replicates were monitored for 12 d. Samples for RNA isolation were harvested from each batch culture replicate at day 0, 2, 4, 8, and 12 covering the different growth phases as shown in Fig. 1. Samples harvested at day 12 (decline phase) were only considered for miRNA profiling since it has been shown that miRNAs are more stable than mRNAs and can therefore be reliably profiled, even from cell samples exhibiting low viability and hence potentially degraded RNA [44]. In brief, harvested cells ($1–5 \times 10^6$ cells) were washed with phosphate buffered saline (PBS) at 170 g for 10 min and homogenized in 1 mL Trizol reagent (Invitrogen) by vigorous mixing followed by a 5-min incubation step at room temperature and storage at –80°C. Total RNA was extracted using chloroform and pellets were resuspended in 30 μL of RNase free H_2O. Absorbance at 230, 260, and 280 nm were measured using a ND-1000 spectrophotometer (NanoDrop technologies, DE, USA) and total RNA quality was assessed using the RNA Nano 6000 Kit (Agilent, Germany). Only total RNA extracts with RNA integrity numbers (RIN) >7 were used for microarray hybridization.

2.3 mRNA analysis

2.3.1 Hybridization and labeling

Two-color microarray platforms were used to detect changes in the mRNA expression pattern of CHO-K1 cell lines adapted to growth in 8 and 4 mM L-glutamine during the batch process. As specific

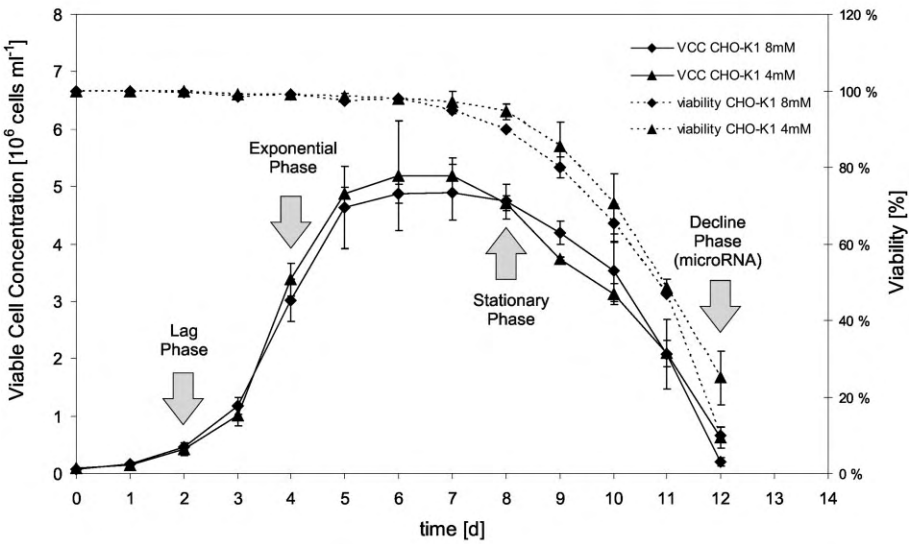

Figure 1. CHO-K1 growth curves and sampling points for microarray analysis. Viable cell concentration as well as viability during batch cultivation is shown for two CHO cell lines adapted to growth at 4 and 8 mM L-glutamine, respectively. Samples for microarray analysis were harvested at the starting time point (day 0), lag phase (day 2), exponential phase (day 4), stationary phase (day 8) for mRNA and miRNA analysis. At the end of batch cultivation (day 12) only samples for miRNA analysis were considered.

CHO chips for microarray analysis are not publicly available, cross-species hybridization between hamster and mouse cRNA was performed [45] using a mouse Agilent 22 k microarray (G4121B) platform (Table 1), based on 60-mer oligonucleotide probes [17, 18]. Total RNA isolated from two biological replicates at day 0, 2, 4, and 8 was pooled and subsequently labeled using the Agilent Quick Amp Labeling protocol for two-color microarray-based gene expression analysis (Agilent). In brief, 500 ng of total RNA per sample were reverse transcribed into cDNA at 40°C for 2 h. Cy3 or Cy5 dye incorporation was performed as suggested by the manufacturer using T7 RNA polymerase and labeled cRNA was subsequently purified using RNeasy mini spin columns (Qiagen, Germany). Uniform dye corporation was checked using a ND-1000 spectrophotometer (NanoDrop Technologies) and equal amounts of cRNA were hybridized at 60°C for 17 h against the same standard (batch day 0 of CHO-K1 8 mM cells) including dye swaps as technical replicates. Prior to scanning, the slides were washed for 10 min at room temperature in 6% SSC and 0.005% Triton X-102, followed by a second washing step with 0.1% SSC and 0.005% Triton X-102 at 4°C for 5 min before they were dried with nitrogen gas. Slides were scanned with the two laser Agilent microarray scanner (G2565A) at 10-µm-pixel resolution and 100% photo multiplier tube (PMT) power setting.

2.3.2 Data processing and statistical analysis

Agilent Feature Extraction software (v10.5) together with GeneSpring GX software (Agilent) were used to extract median spot intensities and to normalize intensities using local weighted linear regression (Loess) [46]. *t*-Test against day 0 with asymptotic *p*-value computation was performed for all samples and the obtained *p*-values were adjusted for multiple testing using Benjamini–Hochberg false discovery rate (FDR) correction [47]. Genes with an absolute fold change of ≥1.5 and an adjusted *p*-value of ≤0.05 were considered differentially expressed.

Table 1. Overview of microarray platforms.

	mRNA	microRNA
Profiling Platform	Agilent Inc., mouse 22k oligo	Exiqon Inc., miRBase v 9.2
Hybridization Design	Two color, common reference	Two color, common reference
Number of Probes	20 868	806
Number of Probe Replicates	2	8
Normalization	Loess	Loess
Present call cut-off	>∅ (BG) + 2x SD	>∅ (BG) + 2x SD
Expressed Genes	13 082	199
Statistical cut-off	adj. p<0.05 dynamic range > 1.5	adj. p<0.05 dynamic range > 1.5
Number of Regulated Genes	1455	118

2.3.3 Quantitative real-time PCR

For confirmation of microarray data selected genes were examined by qRT-PCR. cDNA was synthesized from extracted RNAs according to DyNAmo™ cDNA Synthesis kit for qRT-PCR's protocol (Finnzymes). The Primers were designed using the online software tool Primer 3 [48] available online on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). In-house sequenced hamster cDNAs (unpublished data) were BLAST aligned (BLASTN 2.2.24 software) against murine mRNA sequences from selected genes in order to optimize primer binding location. Primers were selected with a length of 20 bases and melting temperatures (T_m) ranging between 57 and 60°C (Table 2). Amplicons between 90 and 350 bp were preferred, as PCR efficiency decreases with very long (>1000 bp) amplicons. To distinguish between amplification of mature mRNA from that of genomic DNA, amplicons which span over one or more exons were selected. Assays were performed using SensiMix™ SYBR kit (Bioline, Taunton, MA, USA). Each 10 µL reaction consisted of the following: 5 µL SensiMix, 3.5 µL nuclease free H_2O and 0.25 µL sense and anti-sense primers. The following qRT-PCR program was used: step 1, 94°C for 2 min, step 2, 94°C for 15 s; step 3, 57°C for 30 s; step 4, 72°C for 20 s; step 4, 72°C for 5 min and step 5, 4°C in hold phase. Each reaction was performed in quadruplicates.

2.4 miRNA analysis

2.4.1 Hybridization and labeling

Cross-species microRNA microarrays were spotted on epoxy-coated Nexterion glass slides (Schott AG, Germany) using the MicroGrid II (Zinsser Analytic, Germany) Microarrayer and miRBase version 9.2 locked nucleic acid probe set (Exiqon, Denmark). The spotted probe set consisted of 415 mouse and 314 complementing human miRNAs, which were spotted in 8 replicates on each array. Spotting was performed according to guidelines provided by the manufacturer. In order to assure the quality of spotted miRNA microarrays, scanning of all slides was performed. Weak buffer aut-

Table 2. Primer sequences used for qRT-PCR.

Gene	Primer Sequence (5'–3')	Length	T_m (°C)[a)]	GC content (%)	GenBank/ miRBase Accession #
Hspa5 sense	CGGGCCGAGGAGGAGGACAA	20	59.97	70	NM_022310.3
Hspa5 antisense	GTGCGTCCGATGAGGCGCTT	20	60.11	65	
H2-Q7 sense	ACCAGAGCGAGGGCGGCTCT	20	62.08	70	NM_010394.4
H2-Q7 antisense	ACTCCACGCACGTGCCCTCC	20	61.42	70	
Mcm7 sense	TGCAGCTGAGGCTGGAGAGC	20	58.78	65	NM_008568.2
Mcm7 antisense	GGCCACACCAGGGTCTCCCA	20	60.11	70	
Eme1 sense	CCACAGCTCCTGGTACAGGC	20	56.68	65	NM_177752.4
Eme1 antisense	GCGGGAGGTGGATGTCACAC	20	57.35	65	
Gapdh sense	AACTTTGGCATTGTGGAAGG	20	64.20	45	NM_008084.2
Gapdh antisense	ACACGTTGGGGGTAGGAACA	20	66.80	55	
miR-20a	CAAAGTGCTTACAGTGCAGGTAG	23	63.07	48	MI0000568
miR-210	CTGTGCGTGTGACAGCGGCT	20	72.68	65	MI0000695
miR-221	AGCTACATTGTCTGCTGGGTTTC	23	65.70	48	MI0000709
miR-222	AGCTACATCTGGCTACTGGGTCT	23	64.51	52	MI0000710
miR-23a	ATCACATTGCCAGGGATTTCC	21	67.20	48	MI0000571

a) Calculated using modified Breslauer's parameters for dH and dS [76]

ofluorescence confirmed the presence of more than 98% of spots.

For hybridization total RNA extracts from two biological replicates per time point were pooled as described above. Samples were then labeled with Cy-3 and hybridized against a Cy-5 labeled common reference pool, which was prepared by pooling equal amounts from all samples in the experiment. Cy-3 and Cy-5 labeling of 1-μg aliquots was performed using the Exiqon miRCURY LNA miRNA Array labeling kit including a set of synthetic oligonucleotides (Exiqon). Cy-3 and Cy-5 labeled samples were then pooled, heat denatured at 95°C for 2 min and hybridized onto LNA microarrays at 56°C for 16 h using the TECAN HS 400 hybridization station (Tecan, Austria) followed by washing and drying with nitrogen. Immediately after hybridization, all slides were scanned at 532- and 635-nm wavelengths at 10-μM resolution using a GenePix 4000B scanner and GenePixPro 4.1 software (Molecular Devices, Sunnyvale, CA, USA).

2.4.2 Data processing and statistical analysis

Feature extraction was performed in GenePix 4.1, and the resulting GPR-files were processed using a previously described LIMMA pipeline in R/Bioconductor [42, 49] that allowed quality control, background correction, and intensity normalization. In brief, the $\log_2$ ratios (M) and $\log_2$ average intensities (A) were calculated for each spot and the resulting M–A datasets for each array were background corrected using the normexp function and normalized using loess regression. The influence of background correction and normalization was diagnosed using M–A plots as well as PrintTip boxplots. Subsequently, M–A values from the eight replicates per miRNA present on each array were correlated and linear models and moderated t-statistics in LIMMA [50] were used to calculate $\log_2$ fold changes in miRNA expression for the contrasts of interest. The resulting p-values were adjusted for multiple testing according to a method by Benjamini and Hochberg [47]. miRNA that showed expression intensities exceeding the average background intensity plus twice standard deviation were considered as expressed. Significant regulation over batch cultivation time was assumed for miRNAs with adjusted p-values <0.05 and fold changes >1.5 at either exponential, stationary or decline phase versus lag phase.

2.4.3 Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed using 200 ng of total RNA extracts that had been poly-adenylated and reverse-transcribed using the NCode™ system (Invitrogen). PCRs were run using the Platinum SYBR Green kit system, a universal poly(A) primer and gene specific primers that were designed to yield a melting temperature of 60°C (Table 2) using the available Chinese hamster miRNA sequence information [30]. qRT-PCRs were run on the Corbett Rotorgene rotorcycler (Qiagen) including four technical replicates per sample. Data was analyzed using the delta–delta-Ct method [51] where miR-23a served as internal controls. The resulting $\log_2$ fold changes were used for

correlation of qPCR and microarray expression data.

2.5 Functional data analysis

2.5.1 Clustering

Prior to *k*-Means clustering, expression values of 4 and 8 mM glutamine cultivations were averaged. Genes or miRNAs with an absolute fold change of ≥1.5 (relative to lag phase) and a multiple testing adjusted *p*-value of ≤0.05 were considered for supervised *k*-Means clustering using Genesis software [52] and a maximum of 50 iterations.

2.5.2 Functional annotation analysis

Up- or down-regulated genes classified in different *k*-Means clusters were classified in four groups as either down-regulated or up-regulated in stationary phase or in exponential phase. For pathway analysis of gene groups (Table 3) DAVID functional analysis online tool was used [53, 54].

2.5.3 Correlation of miRNA and mRNA expression

The web-based tool GeneSet2miRNA [55] was used to evaluate whether specific miRNA binding sites were enriched in sets of similarly regulated genes ("regulatory model"), which for this study are genes allocated to a *k*-Means cluster based on similar expression profiles. Therefore, miRNA binding sites for each gene were predicted by GenSet2miRNA using 11 established miRNA target prediction algorithms available in the miRecords database [56]. In order to decrease the number of false-positive predictions, GeneSet2miRNA considers only interactions that are predicted by at least four or more algorithms. For testing the hypothesis whether a specific miRNA binding site is enriched within a set of genes, GeneSet2miRNA uses hypergeometric distribution tests. The resulting *p*-values are further corrected for multiple testing using the Monte-Carlo simulation approach. In brief, the Monte-Carlo simulation approach compares *p*-values of a submitted gene list to a randomly sampled gene list of the same size *N*. The distribution of random *p*-values p_d (derived for the random set of the size *N*) is compared to the hypergeometric *p*-value for each regulatory model r (p_r) inferred from the input gene list. If *k* is the number of times p_r is equal to or inferior ($p_r \geq p_d$) than the *p*-values from the random distribution, the corrected *p*-value is given by formula $p_{corrected} = (k + 1)/N$ [55].

Transcriptional levels of miRNAs with enriched binding sites ($p<0.05$) and expression levels above the background were retrieved from the available microarray data and checked for inverse correlation with the respective mRNA targets at specific time points.

Table 3. Functional pathway enrichment of genes differentially expressed during the batch.

Genes	KEGG Pathway	Count	Percent	*p*-value	p_{adj}-value[a)]
Up in stationary phase	Antigen processing and presentation	13	3.1	4.60E-06	6.20E-04
	Lysosome	14	3.3	1.50E-05	1.00E-03
	Pathways in cancer	18	4.3	5.60E-03	2.20E-01
	Type I diabetes mellitus	7	1.7	6.50E-03	2.00E-01
	Glycolysis/Gluconeogenesis	7	1.7	9.40E-03	2.30E-01
Down in stationary phase	Homologous recombination	6	1.4	3.30E-04	3.90E-02
	DNA replication	6	1.4	1.20E-03	6.70E-02
	Small cell lung cancer	8	1.9	3.40E-03	7.80E-02
	Cell cycle	10	2.4	2.70E-03	7.90E-02
	Base excision repair	6	1.4	2.10E-03	8.20E-02
Up in exponential phase	Insulin signaling pathway	12	4.3	6.90E-06	7.60E-04
	Pathways in cancer	17	6.1	2.50E-05	1.40E-03
	Jak-STAT[b)] signaling pathway	10	3.6	4.90E-04	1.80E-02
	Endometrial cancer	6	2.2	1.10E-03	3.00E-02
	Progesterone-mediated oocyte maturation	7	2.5	1.80E-03	3.90E-02
Down in exponential phase	Spliceosome	9	5.7	5.40E-05	4.30E-03
	Cell cycle	6	3.8	1.30E-02	4.00E-01
	Glutathione metabolism	4	2.5	1.90E-02	4.00E-01

a) Benjamini and Hochberg [47].
b) Jak-STAT, Janus kinase signal transducer and activator of transcription.

3 Results

3.1 Analysis of global gene expression

To identify mRNAs and miRNAs that are regulated during lag, exponential, and stationary phase in CHO-K1 suspension cell lines, cellular pellets were harvested for transcriptome analysis at day 0, 2, 4, and 8 (Fig. 1) from batch culture replicates.

For microarray analysis, the batch starting point (day 0) was used as the common reference for all other samples harvested during the batch culture at day 2, 4, and 8 (Fig. 1). The differential gene expression calculated with a fold change (FC) >1.5 and an adjusted p-value <0.05 (Benjamini–Hochberg) showed a constant increment in the number of regulated genes during batch culture. The analysis revealed that a total of 1455 genes were differentially regulated at least at one time point compared to the starting point (Table 1). From these genes, 660 (45.4%) were down-regulated and 795 (55.6%) up-regulated.

3.2 Functional classification of significantly regulated genes

Due to good correlation of the differentially expressed genes between CHO cells grown at 4 and 8 mM glutamine (Supporting information, Fig. S1) expression values were averaged and used for k-Means clustering (k = 13). Clustering resulted in four main groups representing genes with up- (317 genes = Group 1) or down-regulation (192 genes = Group 2) during exponential phase and up- (478 = Group 3) or down-regulation (468 = Group 4) during stationary phase (Fig. 2). Within each group the clusters differed by degree of regulation. The assignment of differentially regulated genes to Keggs pathways was carried out using the "functional annotation clustering" tool of DAVID [53, 54]. As shown in Table 3, enriched pathways of genes up-regulated in stationary phase were associated with secretion-related pathways, e.g., Antigen processing and presentation (adj. $p = 6.20 \times 10^{-4}$) and Lysosome (adj. $p = 1.00 \times 10^{-3}$), whereas genes down-regulated in stationary phase were assigned to Homologous recombination (adj. $p = 3.90 \times 10^{-2}$), a DNA-related pathway. On the other hand, genes up-regulated in exponential growth phase were associated with growth enhancing signaling pathways like insulin signaling pathway (adj. $p = 7.60 \times 10^{-4}$), cancer-related pathways (adj. $p = 1.40 \times 10^{-3}$) or the Jak-STAT signaling pathway (adj. $p = 1.80 \times 10^{-2}$). Interestingly, genes involved in the spliceosome pathway (adj. $p = 4.30 \times 10^{-3}$) were down-regulated in the exponential growth.

3.3 qRT-PCR confirmation of mRNA data

For confirmation of microarray data, we used in-house sequenced CHO-K1 cDNA sequences for primer design of selected genes from enriched Keggs pathways for qRT-PCR. All designed primers showed high specificity to cDNA CHO-K1 samples. As *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) transcription levels fluctuated significantly over batch cultivation (Supporting information, Fig. S2), alternative candidates showing minimal variation in their transcript levels over batch-time were investigated. *Tsen34* fulfilled these criteria and was used as reference for normalization (Supporting information, Fig. S2). Four genes were selected for confirmation based on their significant association with "Homologous recombination" (*Eme1*), "DNA replication" (*Mcm7*), and "Antigen processing and presentation" (*H2-Q7*, *Hspa5*) Keggs pathways. Transcript levels of all four genes as obtained from microarray data and qRT-PCR, are depicted in Fig. 3A as $\log_2$ fold changes relative to day 2.

3.4 miRNA transcription in CHO batch cultivation

As it has recently been shown that mature miRNA sequences are highly conserved between human, mouse, and the Chinese hamster [30], an established two-color miRNA microarray platform comprising LNA probes against 729 mature miRNAs from mouse and human [42] was used to profile miRNA expression of CHO-K1 cells during different stages of batch cultivation, i.e., lag, exponential, stationary phase, and decline phase. Decline phase was included as it has been shown that miRNAs are more stable than mRNAs and can therefore be reliably profiled from cell samples with low viability and therefore potentially degraded RNA [44]. In total, 199 miRNAs showed signal intensities that exceeded the background intensity level plus twice its standard deviation, and were hence considered as expressed in CHO-K1 cells (Table 1). Using the R/Bioconductor package "linear models for microarray analysis" [49] $\log_2$ fold changes for all expressed miRNAs in exponential, stationary, and death phase relative to lag phase at day 2 were calculated together with Benjamini and Hochberg adjusted p-values [47]. After imposing statistical cut-offs (adj. $p<0.05$, dynamic fold change >1.5), 118 miRNAs were identified as significantly regulated at least at one stage during batch cultivation (Table 1). Similar to mRNA expression, also miRNAs expression values exhibited good correlation between CHO-K1 cells grown at 4 and 8 mM glutamine (Supporting information, Fig. S1), which

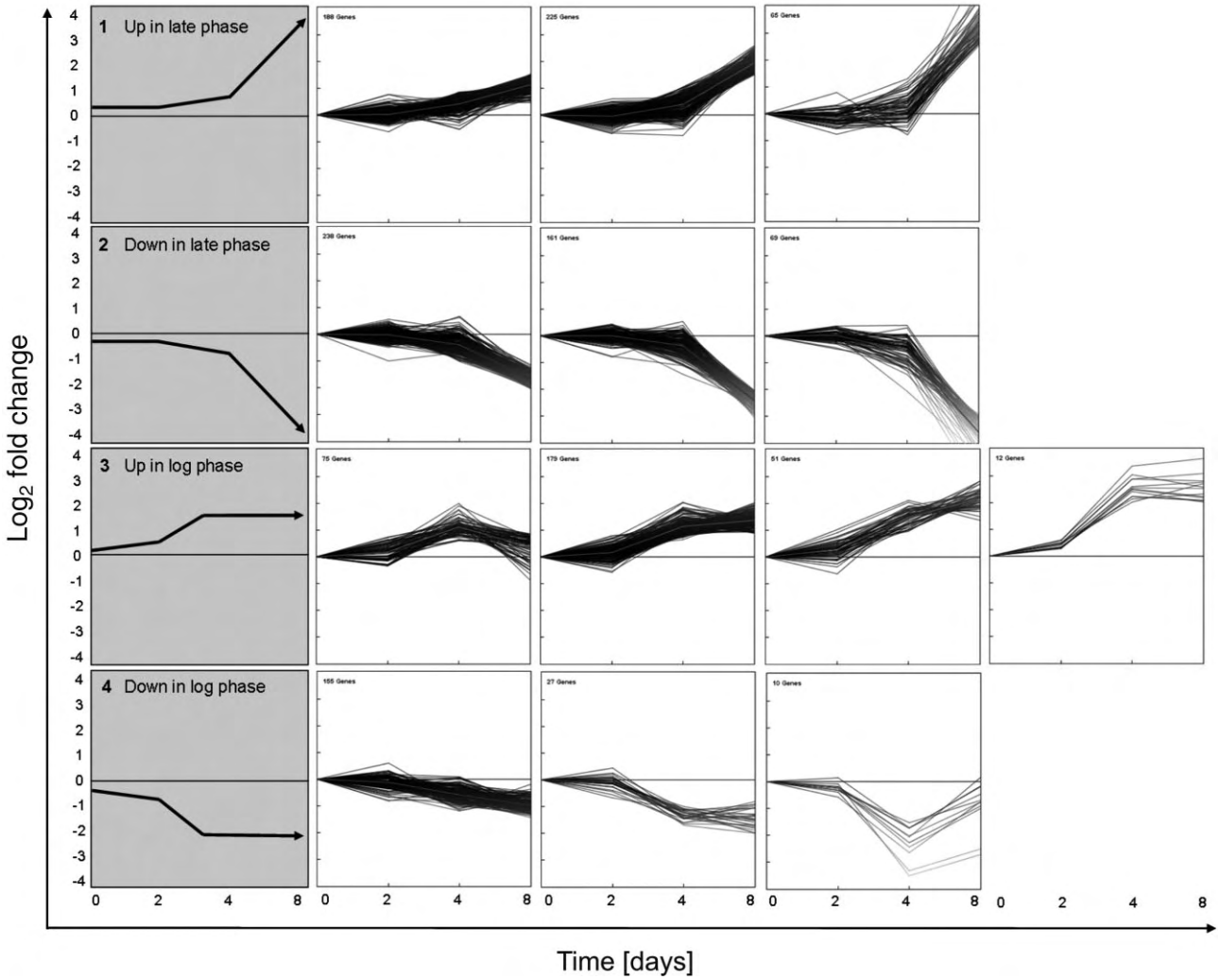

Figure 2. k-Means clustering of differentially expressed mRNAs during CHO-K1 batch growth. Supervised clustering (n = 13) of 1455 regulated mRNAs (adj. $p<0.05$, FC > 1.5) was performed and results are shown in expression plots. Clusters were sorted into four groups representing genes with up- or down-regulation during stationary growth phase, or exponential growth phase (denoted as log phase).

is why *k*-Means ($n = 4$) supervised clustering was applied using average expression values. Clustering identified two principal groups of miRNAs sharing similar transcription patterns (Fig. 4): 43 miRNAs (36%) were up-regulated at either stationary (A) or death phase (B), while the majority of 75 miRNAs (64%, clusters C and D) exhibit moderate or no up-regulation during exponential growth phase followed by down-regulation during stationary and death phase.

3.5 qRT-PCR confirmation of miRNA expression

For confirmation of microarray results qRT-PCR was performed on four miRNAs that were found differentially regulated (Fig. 3B). For the choice of internal standards, initially the commonly accepted reference gene *gapdh* was used. But since gapdh transcription levels fluctuated significantly over batch cultivation (Supporting information, Fig. S2), the miRNA, and mRNA array data at hand was used to look for alternative candidates that display average transcription levels and minimal fluctuation over batch-time as previously described [57]. Among others, miR-23a fulfilled these criteria and was consequently used as internal control for qRT-PCR analysis.

3.6 Functional classification of miRNAs

Since the biological role of a miRNA is mainly defined by its mRNA targets, many algorithms have been developed for the in silico prediction of miRNA–mRNA interactions, often resulting in sev-

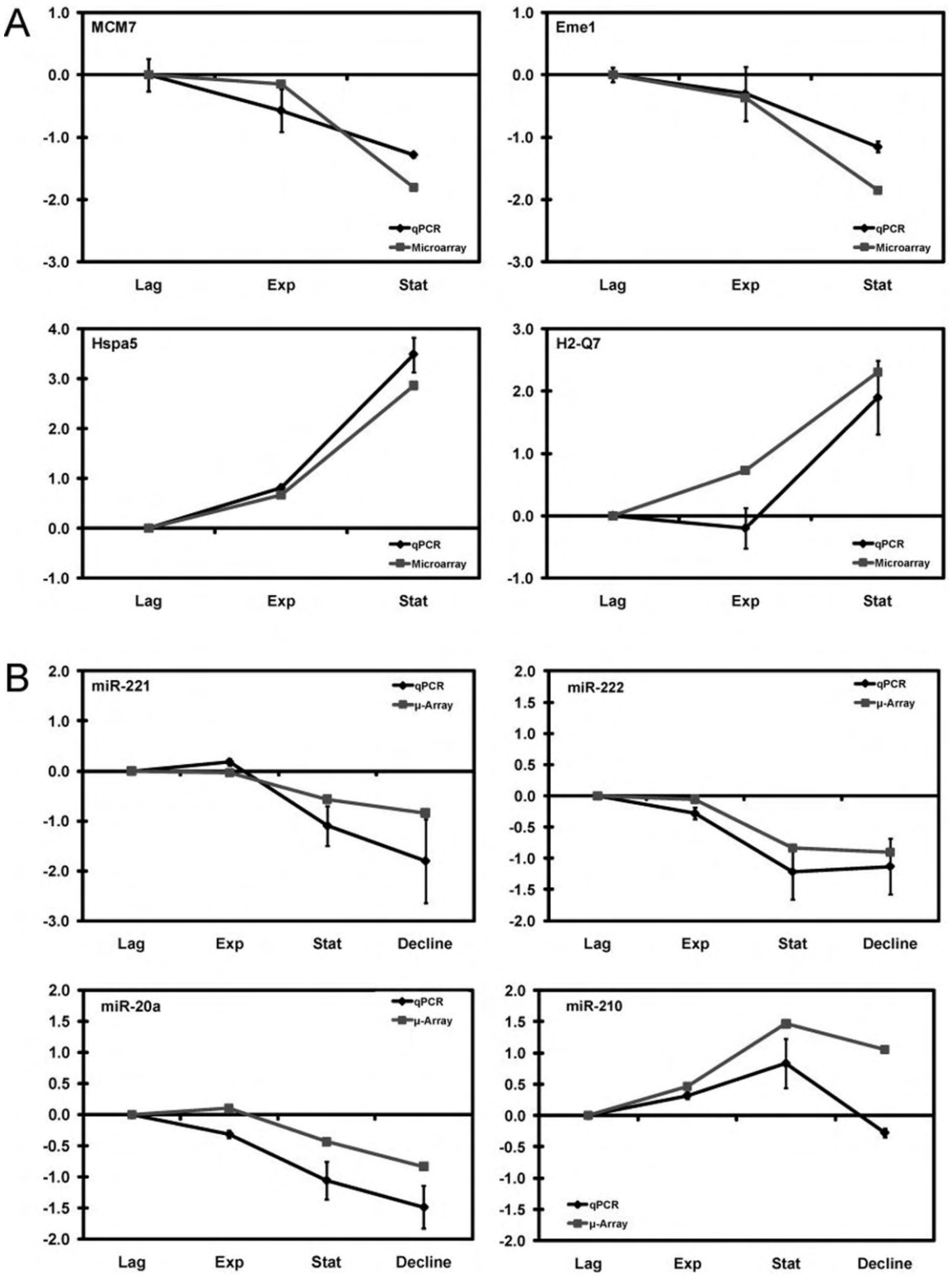

Figure 3. Confirmation of microarray data by qRT-PCR. Log_2 fold changes in transcription relative to day 2 (lag phase) as determined by microarray and qRT-PCR (mean ± SD, n = 4) are given for selected mRNAs and miRNAs. (**A**) mRNAs: Four genes were selected for qPCR confirmation based on their significant association with "Homologous recombination" (*Eme1*), "DNA replication" (*Mcm7*), and "Antigen processing and presentation" (*H2-Q7*, *Hspa5*) pathways. The tRNA splicing factor *Tsen34* was used as internal control for normalization. (**B**) miRNA: Two miRNAs which represent the mir-221 family (miR-221 and miR-222), one miRNA representing the miR-17 family (miR-20a), and miR-210 were chosen for qPCR, using miR-23a was as internal control for normalization.

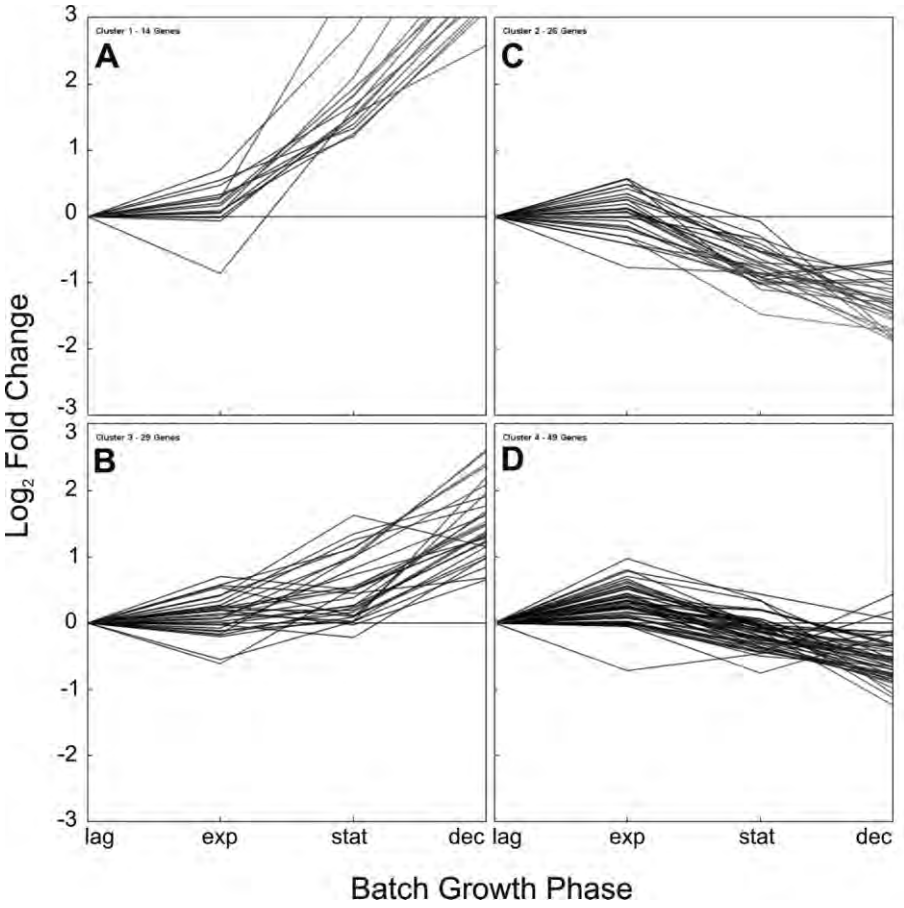

Figure 4. k-Means clustering of differentially expressed miRNAs during CHO-K1 batch growth. Supervised clustering (n = 4) of 118 regulated miRNAs (adj. $p<0.05$, FC>1.5) was performed and results are shown in expression plots.

eral hundred possible interactions. Applying these predictions to long lists of miRNAs derived from deep-sequencing or microarray experiments may, however, result in a large number of false-positive predicted targets. Alternatively, meaningful information from miRNA profiling results can be gained by grouping miRNAs into categories such as families (characterized by identical seed sequences), clusters (characterized by close genomic proximity), and published functions. The novel web-based "Tool for annotations of microRNAs (TAM)" [58] allows for such an analysis and was used to identify clusters and families within miRNAs showing initial up-regulation during exponential growth followed by down-regulation in stationary and decline phase (Fig. 4, clusters C and D). While in total six different miRNA clusters and five different families were present in our list (Table 4), only members of the growth related mir-106a cluster (miR-106a, miR-18b, miR-20b, and miR-92b, FDR = 0.04) and miR-17 family (miR-17, miR-20a, miR-93, miR-106a, miR-18b, and miR-20b, FDR = 0.003) were found overrepresented [59]. Additionally, both miR-221 and miR-222, which are located together on chromosome X in human and mouse and share some of the targets of the miR-17 family [60] are present among the miRs in clusters C and D. In general, enrichment analysis of the biological functions associated with miRNAs in cluster C and D mainly resulted in growth-related processes such as cell proliferation (FDR = 0.02) and cell-cycle (FDR = 0.03) as well as apoptosis (FDR = 0.02). In case of the 43 miRNAs up-regulated during stationary and decline phase (Fig. 4, cluster A and B), TAM analysis identified only two miRNA clusters, mir-1283 and mir-182 but no families or significantly enriched functions (Table 4). Yet, it is worth noting that miR-182 was recently found to impair DNA-repair in breast tumors by inhibiting BRCA1 [61], a tumor suppressor gene with DNA repair activity that is commonly deleted in cancer.

3.7 Correlation of mRNA and miRNA expression

To answer the question whether genes that are similarly regulated over batch time-course (groups

Table 4. Functional annotation of differentially transcribed miRNAs

k-Means Cluster	Category	Term	Count	Percent	Fold	P-value	FDR
A/B	Cluster	mir-1283 cluster	6	0,15	2,41	2,93E-02	1,00E+00
A/B	Cluster	mir-182 cluster	2	0,67	10,69	1,08E-02	1,00E+00
C/D	Cluster	mir-106a cluster	4	0,67	5,87	1,88E-03	4,44E-02
C/D	Cluster	mir-17 cluster	3	0,50	4,40	2,16E-02	2,00E-01
C/D	Cluster	mir-221 cluster	2	1,00	8,80	1,27E-02	1,54E-01
C/D	Cluster	mir-29b cluster	2	1,00	8,80	1,27E-02	1,51E-01
C/D	Cluster	mir-34b cluster	2	1,00	8,80	1,27E-02	1,50E-01
C/D	Cluster	mir-424 cluster	3	0,60	5,28	1,17E-02	1,42E-01
C/D	Family	mir-17 family	6	0,75	6,60	3,82E-05	3,43E-03
C/D	Family	mir-221 family	2	1,00	8,80	1,27E-02	1,67E-01
C/D	Family	mir-29 family	2	0,67	5,87	3,53E-02	2,93E-01
C/D	Family	mir-30 family	3	0,60	5,28	1,17E-02	1,46E-01
C/D	Family	mir-34 family	2	0,67	5,87	3,53E-02	2,99E-01
C/D	Function	onco-miRNAs	14	0,45	3,98	6,50E-07	1,46E-04
C/D	Function	Hormones regulation	17	0,27	2,41	1,14E-04	6,39E-03
C/D	Function	immune system	8	0,44	3,91	2,92E-04	1,31E-02
C/D	Function	Cell proliferation	10	0,36	3,14	4,02E-04	1,56E-02
C/D	Function	Apoptosis	13	0,30	2,60	4,16E-04	1,64E-02
C/D	Function	Immune response	13	0,28	2,44	8,62E-04	2,56E-02
C/D	Function	Cell cycle related	16	0,24	2,13	9,71E-04	2,57E-02

from Fig. 2) might be controlled by cooperative miRNA activity, an enrichment of miRNA binding sites within the 3′-UTRs of sets of co-regulated genes was calculated using the software GeneSet2miR [55]. Indeed, a subset of genes up-regulated during stationary growth phase of CHO-K1 cells (group 1), were found to contain binding sites for 17 miRNAs at a frequency significantly higher than expected by chance ($p<0.05$), of which let-7, miR-17, and miR-30 family members were the most prominent miRNAs. In addition, eight miRNA binding sites were significantly enriched in a subset of genes in cluster group 2 (genes down-regulated during stationary phase), four in cluster group 3 (up-regulated during exponential phase), and three in cluster group 4 (down-regulated during exponential phase). The expression of significantly ($p<0.05$) enriched miRNAs for each gene cluster is visualized as heatmap in Fig. 5, where miRNAs with expression values inversely correlating to that of their predicted target genes at the relevant time points are marked with an asterisk. The majority of miRNA–mRNA target pairs with inverse correlation in their transcription levels was found for genes up-regulated and miRNA down-regulated upon entry into stationary growth phase, indicating a role for miRNAs in regulating the transition from exponential growth to growth arrest.

4 Discussion

4.1 Variation of mRNA expression in batch cultures

Our study revealed significant changes and dynamics in gene expression during CHO-K1 suspension batch culture. By the time the cells were in stationary phase more than 1400 genes had been regulated with respect to the culture starting point (day 0). The number of differentially regulated genes was continuously increasing with elapsed time: 157 genes were differentially regulated on day 2, 819 genes on day 4 and finally 1455 genes in stationary phase on day 8.

Statistical analysis of genes differentially regulated by *k*-Means clustering showed four defined profiles over time: up- or down-regulated in exponential phase and up- or down-regulated in stationary phase (Fig. 2). Interestingly, six genes down-regulated in stationary phase (*Blm*, *Rad51c*, *Rad54l*, *Eme1*, *Pold1*, and *Top3b*) were associated significantly ($p = 3.30 \times 10^{-4}$) with "Homologous recombination". Homologous recombination is a natural safety mechanism conserved among species and evolution for maintaining genome integrity, protecting chromosomes against DNA double-strands breaks and allowing the replacement of one defective DNA sequence by its allelic homolog

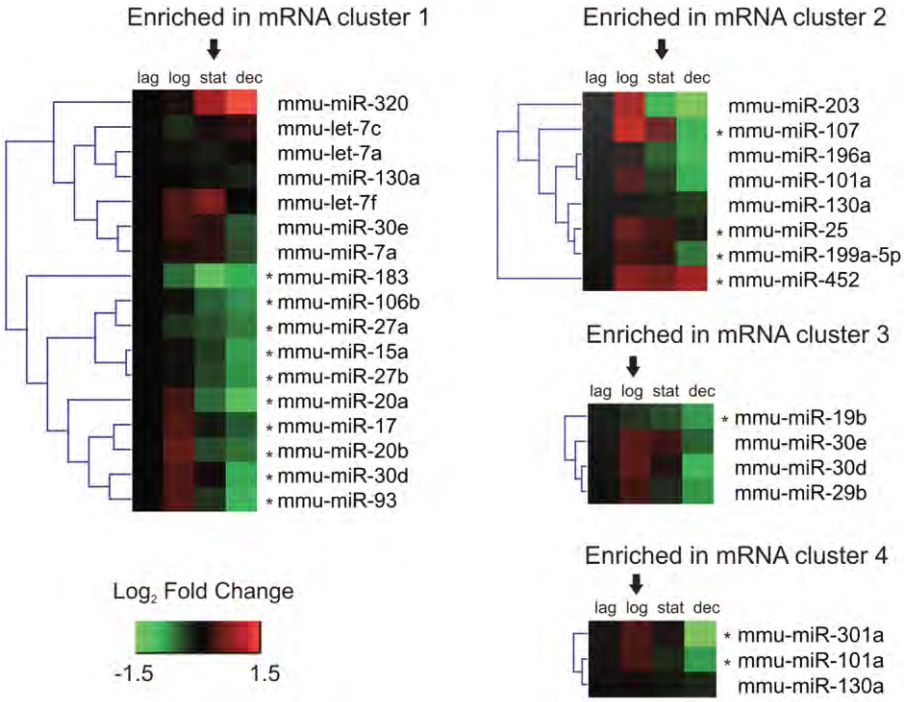

Figure 5. Identification of miRNA binding sites with significant enrichment in co-regulated mRNAs. Starting from 1488 mRNAs that had been clustered and subsequently assigned to four distinct groups with similar expression dynamics during batch cultivation, the software GenSet2miRNA was used to identify miRNA target sites present within the 3'-UTRs of a list of genes belonging to one group. Statistical analysis (hypergeometric *t*-test with Monte Carlo adjustment for multiple testing) identified miRNA target sites that were significantly enriched ($p<0.05$) among a group of similarly regulated genes. The transcriptional profiles (log_2 fold changes relative to lag phase) of the respective miRNAs were retrieved from the microarray data and are given as heatmap. Asterisks mark miRNAs with inverse regulation compared to their mRNA targets at the relevant time points (marked by arrows). mRNA cluster 1, up-regulated in stationary phase; mRNA cluster 2, down-regulated in stationary phase; mRNA cluster 3, up-regulated in log phase; mRNA cluster 4, down-regulated in log phase.

with high precision [62–64]. We found that expression of Eme1, involved in the stability of genome [65], decreased continuously, but most significantly when cells reach stationary phase (Fig. 3A). This loss in genomic stability is further enhanced by the upregulation of miR-182, an inhibitor of BRCA1, a DNA-repair enzyme. In a recent study Bort et al. [43] describe a method for rapidly adapting cell lines to new media conditions by sorting surviving cells at the end of a batch culture when viability dropped below 10%. After several sorting cycles, cells were able to survive in the absence of glutamine, a usual supplement in media to achieve high cell densities, without a significant loss in IVCD, or viability. One explanation for the successful isolation of phenotypic variants under these conditions could be that cells sorted during such a death phase bear a higher likelihood of achieving an altered phenotype, due to reduced activity of the genome mismatch repair machinery. In fact, late stationary phase appears like a mutagenic treatment without any added mutagenic reagent. Even though DNA strand breaks that occur during stationary, non-dividing phase per definition are not a mutation, once the cells are transferred into fresh medium, they will start dividing again, so that it is possible that such mutations are passed on and thus become permanent. Obviously, although setting the stage for the selection of phenotypic variants, this situation is undesirable for the maintenance of production cell lines, where passage of cells early in stationary phase or even already toward the end of the exponential growth phase should be recommended to maintain stability. The fact that genes involved in the DNA replication were down-regulated during stationary phase, including proliferating cell nuclear antigen (*Pcna*), a cofactor of DNA polymerases that encircles DNA during the replication fork and replicative helicases like *Mcm7* or clamp loaders *Rfc2* and *Rfc3* support this theory.

Genes found to be up-regulated during exponential growth were involved in growth stimulating signaling pathways like the "Jak-STAT signaling pathway", critical for growth regulation, survival, and differentiation [66], as well as the "Insulin signaling pathway", controlling glucose storage and

uptake, protein synthesis, regulation of lipid synthesis, and mitogenic responses [67–69]. The expression of these genes was low during lag phase and again declined as cells reached the stationary phase. The inverse effect was observed in genes down-regulated during the exponential phase which included growth and proliferation inhibitory genes like *Cdkn2c* [70], involved in the cell cycle as well as genes involved in "Spliceosome".

4.2 Dynamics of miRNA transcription during batch growth

By studying the regulation of miRNA transcription during batch growth of CHO-K1 cells, significant changes in the levels of more than 50% of the expressed miRNA genes were found, thus expanding the previously identified set of miRNAs with dynamic expression during batch growth of HEK293 cells [39]. In contrast to this study, however, no clear trend toward a consistent up- or down-regulation of all miRNAs was observed, but rather a fairly equal distribution into two principal profiles: (i) up-regulation upon growth arrest in stationary phase, and (ii) up-regulation during exponential growth phase and/or down-regulation in stationary phase. Among the latter group several miRNAs were identified that either shared important parts of their sequence (miRNA families) or are known to be located in genomic clusters (miRNA clusters). Even more intriguing is the fact that the functions allocated to these groups include onco-miRNAs, cell proliferation, and cell cycle as well as apoptosis, which suggests an important role for miRNAs in regulating and timing growth stages during batch cultivation. In this respect, the role of the miR-17-92 cluster deserves special attention, as it is known to be amplified in several types of lymphoma and solid tumors and it has been shown that most mRNA target sites of miR-17-92 are conserved in CHO cells [30]: according to previous reports in human and mouse, an up-regulation of miR-17-92, as observed here during exponential growth, could repress the translation of cyclin-dependent kinase (CDK) inhibitors CDKN1A (p21) and CDKN1C (p57), retinoblastoma (Rb1), phosphatase tensin homolog (PTEN), or the pro-apoptotic effector BCL2L11 [59, 71–74]. Therefore, miR-17-92 overexpression can either indirectly, by repression of the cell cycle inhibitor p21 and subsequent activation of CyclinD/CDK4 complex, or directly, by repressing Rb1, result in the activation of E2F, thus driving cell proliferation. On the other hand, by down-regulation of both Bim and PTEN, miR-17-92 increases the levels of anti-apoptotic BCL-2, and hence adds an anti-apoptotic component to its oncogenic activity upon overexpression [75]. In a different context, down-regulation of miR-17-92, as observed during stationary and decline phase in CHO batch cultivation, has been shown to be a common signature in senescent cells of both in vitro cultivations and ex vivo tissue samples [42], suggesting that its down-regulation during stationary growth phase could contribute to the halt in cellular growth.

It is therefore tempting to conclude that the natural rhythm of miR-17-92 expression over batch cultivation correlates with growth kinetics. Further experiments including both overexpression and knockdown of individual members of miR-17-92 as well as the whole cluster will be performed to assess its importance for cell growth and survival, and hence the overall performance of CHO cell lines.

4.3 miRNA–mRNA networks

Explanations for the dynamics of mRNA transcription during batch growth can be found both upstream of transcription, for example, in the form of transcription factors that are released upon cell signaling events and migrate to the nucleus to activate or silence transcription of their targets, as well as post-transcriptional in the form of RNA interference caused by miRNAs. Here we identified 10 miRNAs that are predicted to target a significant fraction of mRNAs up-regulated during stationary phase and whose transcription levels negatively correlate to their mRNA target levels. By applying gene-set analysis tools we found that 12 of these mRNAs (Table 5) have functions related to programmed cell death (GO:0012501, $p = 2.5 \times 10^{-2}$) while sharing target sites for inversely expressed miRNAs, thus suggesting that CHO cell death during the late stages of batch culture might in part be controlled by cooperative down-regulation of miRNAs.

5 Conclusion

Advances in transcriptome profiling by high-throughput techniques and bioinformatics allow a rapid identification of genes, miRNAs, and biological pathways which, by increasing our knowledge on the molecular cell biology of production cell lines, can lead to both optimized cell lines and processes. In this study we have used microarray expression profiling to identify mRNA and miRNA that are differentially expressed during CHO-K1 suspension cell batch cultures. Our data underline the dynamic nature of gene expression and high-

Table 5. Programmed cell death related genes with predicted miRNA binding sites.

Gene Symbol	GeneID	Cell death related function	Predicted miRNA target sites[a)]
Atp7a	11977	Regulation of cytochrome c release from mitochondria	miR-15a, miR-16
Ddit4	74747	Inhibits cell growth via mTOR upon DNA damage or hypoxia	miR-30d
Rhob	11852	Apoptosis related function in response to DNA damage	miR-30d
Ypel3	66090	Nuclear protein described to be involved in proliferation and apoptosis	miR-27a
Gsk3b	56637	Ser/Thr kinase downstream of PI3K/Akt involved in apoptosis	miR-15a, miR-16, miR-27a
Bdnf	12064	Anti-apoptotic effect during neuron development	miR-15a, miR-16, miR-30d
Rybp	56353	DNA binding protein with possible involvement in apoptosis	miR-15a, miR-17, miR-27a
Btg2	12227	Part of p53 dependent anti-proliferative action	miR-15a, miR-16, miR-27a
Bax	12028	Pro-apoptotic effect by binding and inhibiting Bcl-2	miR-27a[b)]
Mcl1	17210	Bcl-2 family protein that inhibits apoptosis	miR-17, miR-27a
Vegfa	22339	Multifaceted protein with roles in angiogenesis, proliferation and apoptosis of endothelial cells	miR-15a, miR-16, miR-17
Eya1	14048	Dephosphorylates H2AX by which it switches from apoptosis to DNA repair	miR-15a, miR-16, miR-27a

a) Only target sites of miRNAs with inverse correlation of expression considered (according to Fig. 5).
b) Only predicted for human.

light the importance of time course experiments for the comparison of phenotypes or culture conditions. The final proof, of course, would require analysis of changes of the actual amount of the relevant proteins in the cells.

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Appendix F

Endogenous microRNA Clusters Outperform Chimeric Sequence Clusters in Chinese Hamster Ovary Cells.

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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.

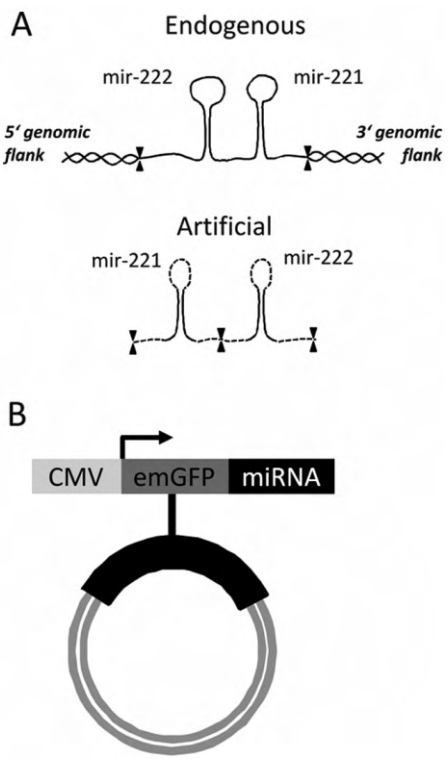


Figure 1. Schematic representation of endogenous and artificial constructs for over-expression of microRNA clusters. **(A)** Endogenous mir-221/222 was PCR amplified from CHO-K1 genome, using primers 70 nt up and downstream of the genomic location. Primers contained restriction sites, which were used 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.

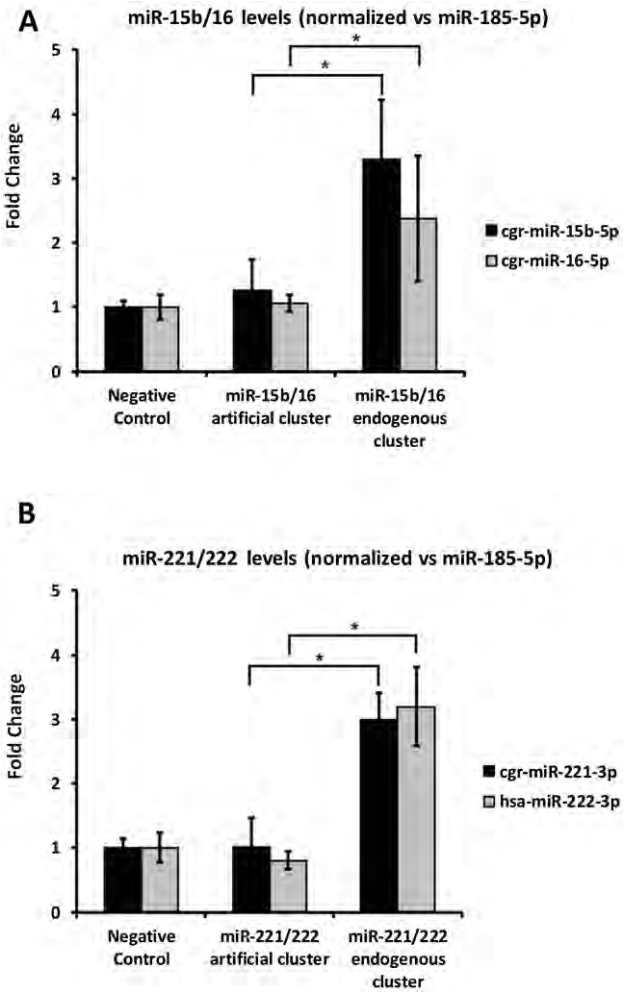
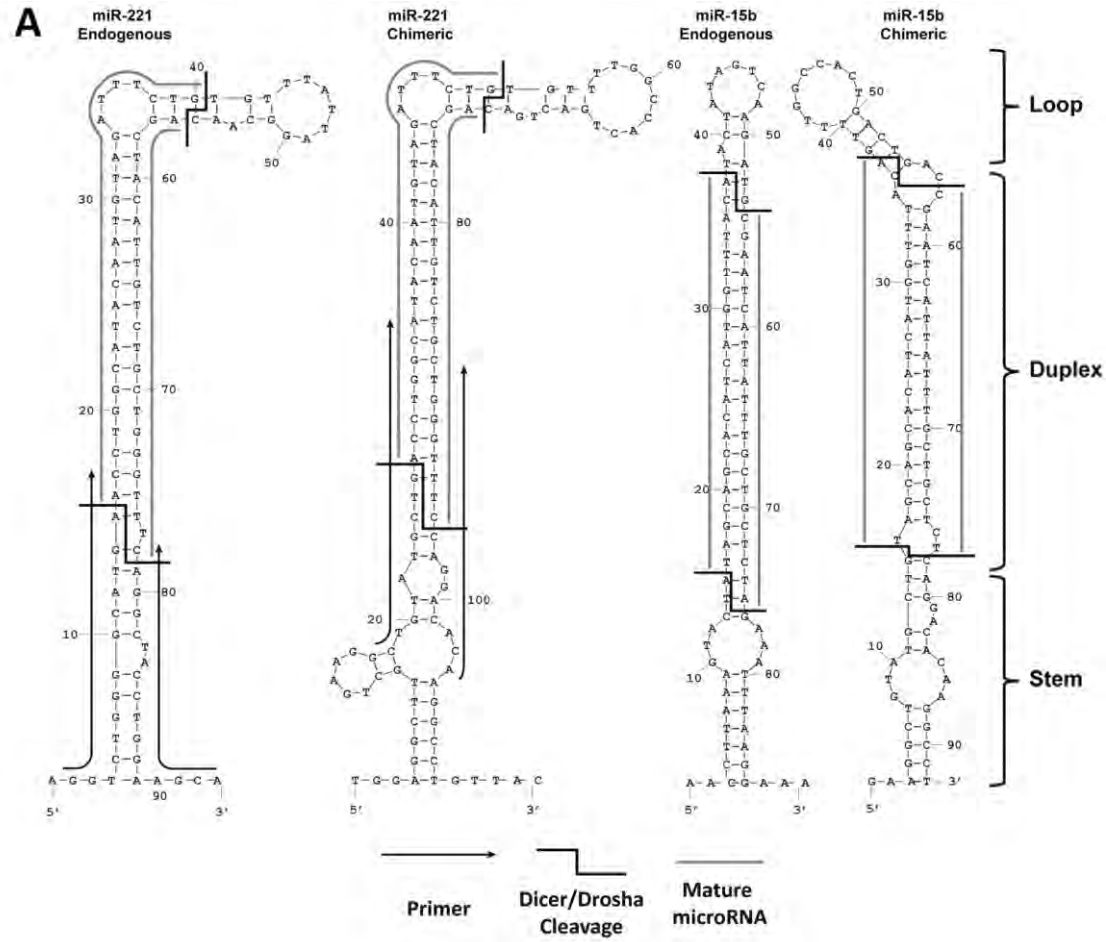


Figure 2. RT-qPCR analysis of mature miRNA levels. Fold changes in mature miRNA levels are shown relative to the negative control (mean ± standard deviation of three individual transfections). miR-185-5p was used as reference miRNA to assess miRNA over-expression after transfection of artificial and 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



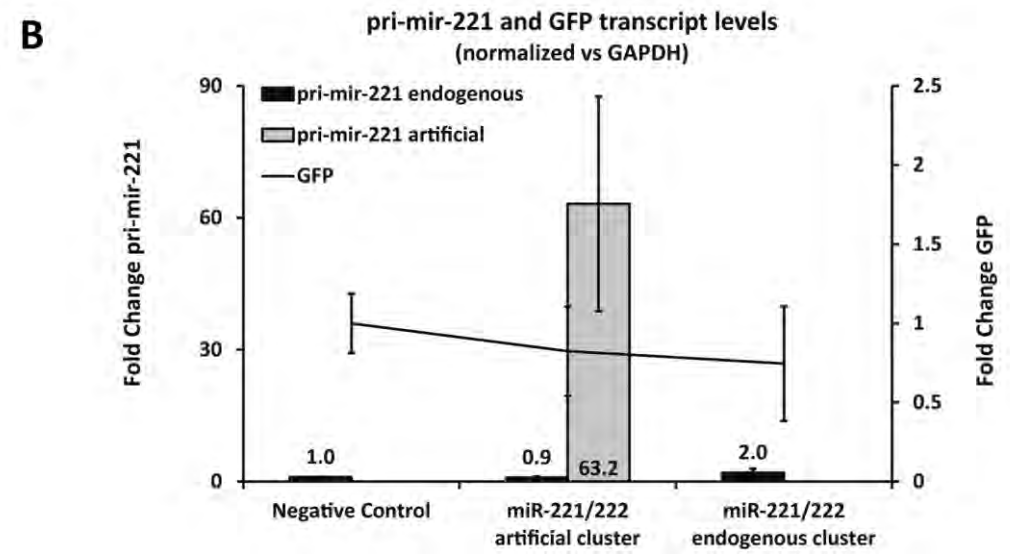


Figure 3. Analysis of pri-miRNA folding and transcription. **(A)** Illustration of putative secondary structures for both artificial and endogenous mir–221 and mir-15b using Quikfold [38, http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold] with energy rules for RNA(3.0) and default settings. The location 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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Appendix G

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

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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

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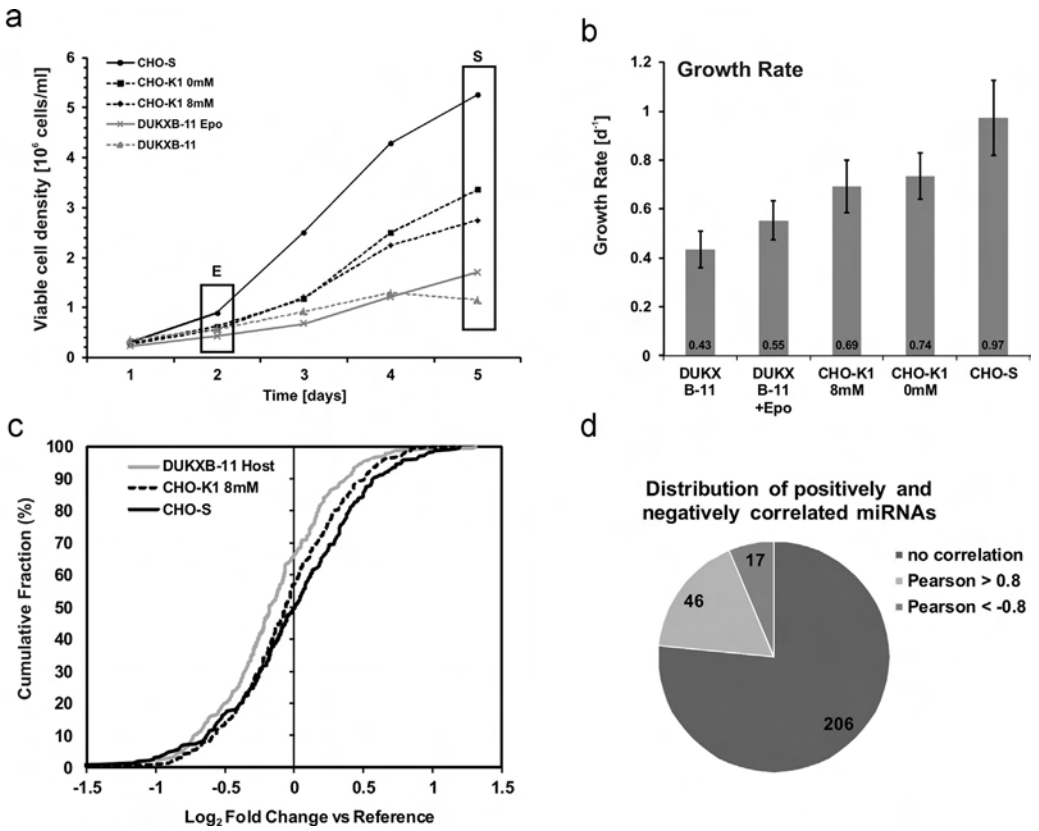

Fig. 1. Global microRNA transcription correlates with growth-rate in protein-free and suspension adapted CHO cell lines. (a) Five CHO cell lines were selected for cultivation in chemically defined media. Two individual batch cultivations were performed and samples were harvested during exponential and 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.

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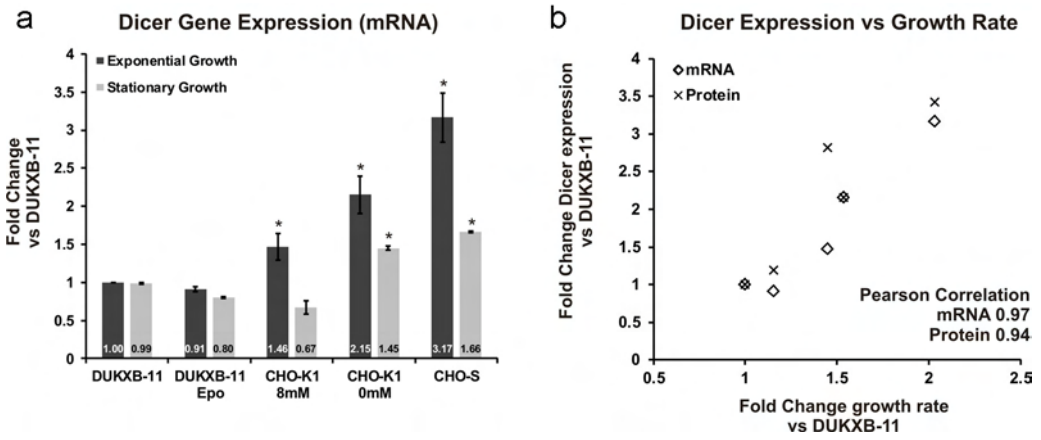

Fig. 2. Dicer expression correlates with growth-rate in protein-free and suspension adapted CHO cell lines. (a) qPCR analysis of Dicer transcript levels at two time-points during batch cultivation is shown (dark, exponential growth phase; bright, stationary growth phase). Fold changes are given relative 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

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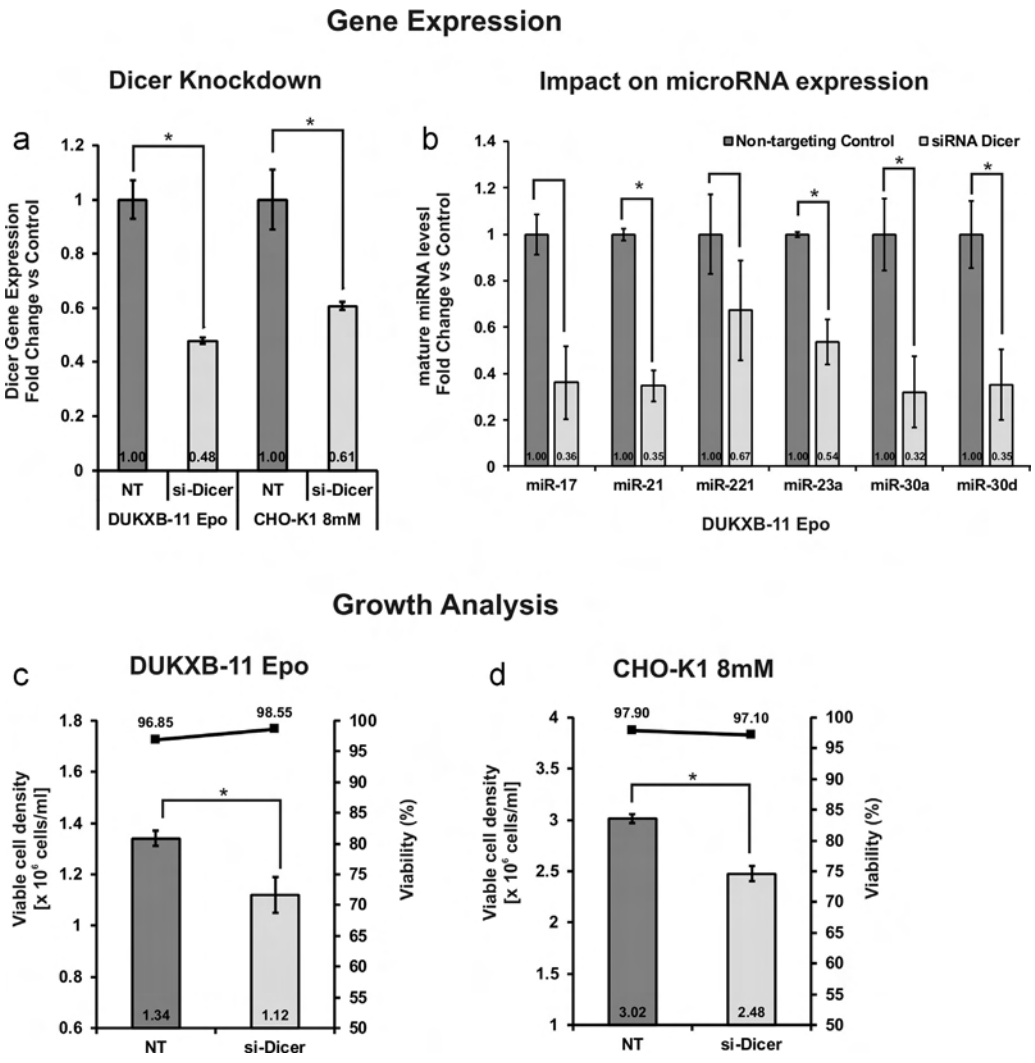

Fig. 3. Targeted transient knockdown of Dicer expression using siRNA. Transfection of 30 nM siRNAs targeting Dicer and non-targeting control (NT) was performed in CHO-K1 8 mM and DUKXB-11 Epo cells. Cells were analyzed 72 h post-transfection: Dicer transcript levels (a) were analyzed by qPCR ($n = 3$, 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 (*).

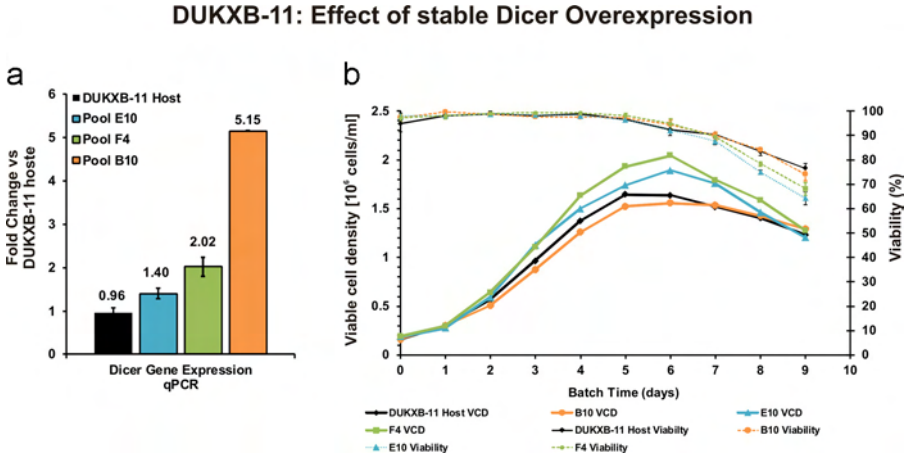

Fig. 4. Engineering Dicer expression in CHO DUKXB-11 cells affects growth behavior. (a) Overexpression of Dicer in three stable bulk cell lines compared to DUKXB-11 host cells was analyzed on day 2 during three individual batch cultivations by qPCR ($n = 3$, Actb used as reference gene, error bars represent 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.

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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).

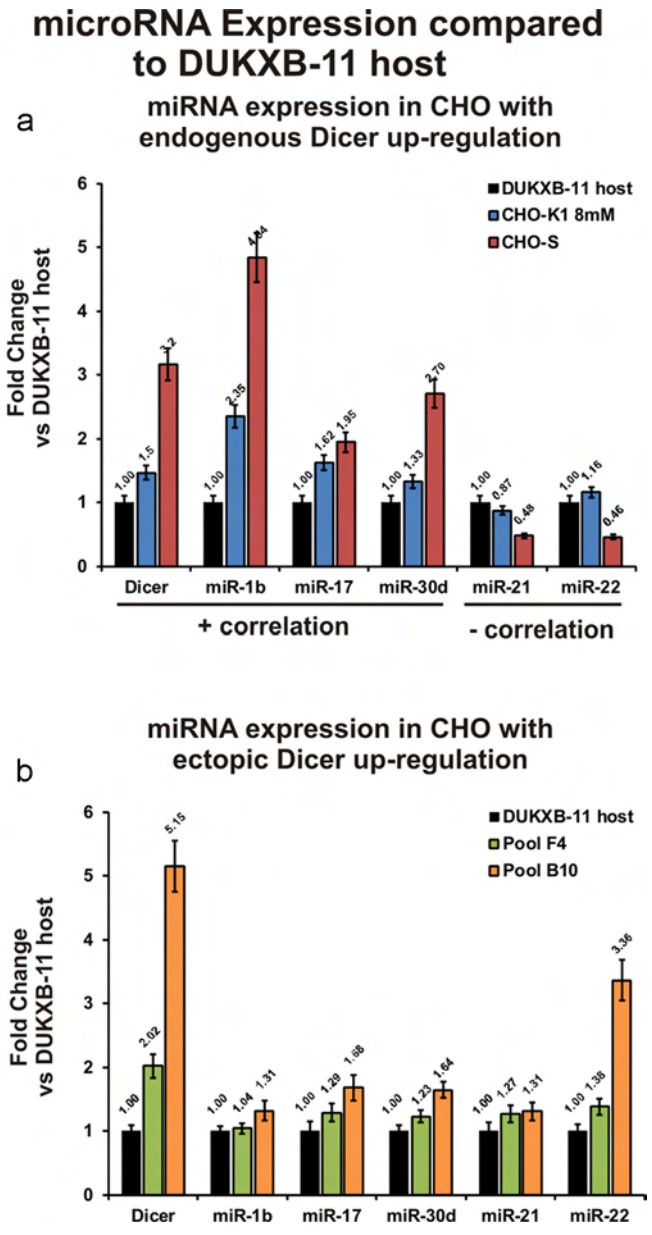

Fig. 5. Comparison of miRNA transcription in cell lines with endogenous and ectopic up-regulation of Dicer. DUKXB-11 host cells were chosen as reference for the comparison of miRNA expression in response to endogenous (a) and ectopic (b) Dicer up-regulation. (a) Microarray fold changes for 5 selected 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 H

Curriculum vitae

VAIBHAV JADHAV

vaibhav.jadhav@boku.ac.at

PERSONAL DETAILS:

Sex	Male.
Date of Birth	05-Feb-1981.
Place of Birth	Bijapur, Karnataka, India.
Family status	Married.
Nationality	India.

EDUCATION

2010-present **PhD**, Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria.
Thesis: *MicroRNA engineering in Chinese hamster ovary (CHO) cells: its biological effects on cell growth and productivity*
Supervisor: Dr. Johannes Grillari

2004-2006 **Master of Science,** Applied Genetics, Karnatak University, Dharwad, India. **1st class with distinction, 2nd Rank.**
Thesis: *Cloning and Characterization of anti-viral gene from Bougenvilla Spectabilis.*
Supervisor: Dr. Pramod B. Gai

2001-2004 **Bachelor of Science,** Chemistry, Genetic and Botany**.** Karnatak University, Dharwad, India. **1st class with distinction.**

HONOURS

2010 **BioTop Doctoral Fellowship**, University of Natural Resources and Life Sciences and Austrian Science Fund (FWF),Vienna, Austria.

2009 **SEMM International Student Fellowship,** European Institute Oncology, Milan, Italy.

2006 **Research assistantship**: Institute of Genomics and Integrative Biology, New Delhi, India.

RESEARCH EXPERIENCE

2013 (Mar–Sep)
Visiting Scholar, Doctoral level,
Johns Hopkins University, Baltimore, MD, USA.

Project: *Comparative proteomics analysis of miRNA engineered Chinese hamster ovary (CHO) cells.*

Supervisors: Dr. Michael Betenbaugh

2009-2010

Research Assistant,

European institute oncology, Milan, Italy.

Project: Purification and crystallization multimeric protien complex for x-ray crystallography.

Supervisors: Dr. Marina Mapelli

2006-2008

Research Assistant,

Institute of Genomics and Integrative Biology, Delhi, India.

Project 1: *Application of DNAzymes in microRNA knock down.*

Project 2: *Application of artificial microRNA in antiviral therapeutics.*

Project 3: *Cationic agents-DNA interactions.*

Supervisors: Dr. Souvik Maiti and Dr. Vinod Scaria.

2004 (Apr-Jun)

Summer Internship,

Indian Institute of Technology Bombay, Mumbai, India.

Project: *Isolation & Characterization of NADP-GDH enzyme from Aspergilus niger*

Supervisors: Dr. N. S. Punekar.

SKILLS & ACTIVITIES

SKILLS	Transcriptomics, Cell Culture, RNAi, Genomics, Proteomics, Bioprocess and Fermentation Technology, Cancer Biology.
LANGUAGES	English (fluent), Hind, Kannada and Marathi (Native)
SCIENTIFIC MEMBERSHIPS	Austrian Association of Molecular Life Sciences and Biotechnology, Vienna, Austria
ACADEMIC COMMUNITY INVOLVEMENT	Designed a comic book "Know your genome" for school children: Highlighting importance of modern biology in a fun way.
INTERESTS	Photography and designing graphics. Designed cover arts for Anural report IGIB 2007-08 and scientific journals “Zebrafish”, “Biotechnology and Bioengineering”. Designer and editor daily Newsletter “HUGO samachar” during HUGO-2008 conference.
ORGANIZATIONAL ACTIVITIES	Founding member of Morgan club, a discussion forum for student of Dept. of Applied Genetics at Karnataka University. Organized talks from eminent scientists across India under the Morgan club activities.

PUBLICATIONS

- **Vaibhav Jadhav**, Matthias Hackl, Gerald Klanert, Juan A Hernandez Bort, Renate Kunert, Nicole Borth, and Johannes Grillari: *Stable overexpression of miR-17 enhances recombinant protein production of CHO cells.* Journal of Biotechnology 02/2014;
- Matthias Hackl, **Vaibhav Jadhav**, Gerald Klanert, Michael Karbiener, Marcel Scheideler, Johannes Grillari, Nicole Borth: *Analysis of microRNA transcription and post-transcriptional processing by Dicer in the context of CHO cell proliferation.*. Journal of Biotechnology 01/2014;
- Gerald Klanert, **Vaibhav Jadhav**, Konstantina Chanoumidou, Johannes Grillari, Nicole Borth, and Matthias Hackl: *Endogenous MicroRNA Clusters Outperform Chimeric Sequence Clusters in Chinese Hamster Ovary Cells.*. Biotechnology Journal 12/2013;
- **Vaibhav Jadhav**, Matthias Hackl, Aliaksandr Druz, Smriti Shridhar, Cheng-Yu Chung, Kelley M Heffner, David P Kreil, Mike Betenbaugh, Joseph Shiloach, Niall Barron, Johannes Grillari, and Nicole Borth: *CHO microRNA engineering is growing up: Recent successes and future challenges*. Biotechnology Advances 01/2013;
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- Matthias Hackl, **Vaibhav Jadhav**, Tobias Jakobi, Oliver Rupp, Karina Brinkrolf, Alexander Goesmann, Alfred Pühler, Thomas Noll, Nicole Borth, and Johannes Grillari: *Computational identification of microRNA gene loci and precursor microRNA sequences in CHO cell lines.*. Journal of Biotechnology 01/2012; 158(3):151-5.
- Juan A Hernández Bort, Matthias Hackl, Helga Höflmayer, **Vaibhav Jadhav**, Eva Harreither, Niraj Kumar, Wolfgang Ernst, Johannes Grillari, and Nicole Borth: *Dynamic mRNA and miRNA profiling of CHO-K1 suspension cell cultures.*. Biotechnology Journal 06/2011; 7(4):500-15.
- **Vaibhav M Jadhav**, Vinod Scaria, and Souvik Maiti: *Antagomirzymes: oligonucleotide enzymes that specifically silence microRNA function.*. Angewandte Chemie International Edition 03/2009; 48(14):2557-60.
- Mahantappa Halimani, S Prathap Chandran, Sudhir Kashyap, **Vaibhav M Jadhav**, B L V Prasad, Srinivas Hotha, and Souvik Maiti: *Dendritic Effect of Ligand-Coated Nanoparticles: Enhanced Apoptotic Activity of Silica-Berberine Nanoconjugates*. Langmuir 01/2009; 25(10.1021/la802761b):2339-2347.
- **Vaibhav M Jadhav**, Rebecca Valaske, and Souvik Maiti: *Interaction between 14mer DNA oligonucleotide and cationic surfactants of various chain lengths.*. The Journal of Physical Chemistry B 08/2008; 112(29):8824-31.
- **Vaibhav Jadhav**, Souvik Maiti, Antara Dasgupta, Prasanta Kumar Das, Rita S Dias, Maria G Miguel, and Björn Lindman: *Effect of the head-group geometry of amino acid-based cationic surfactants on interaction with plasmid DNA.*. Biomacromolecules 08/2008; 9(7):1852-9.
- Nicholas A A Rossi, **Vaibhav Jadhav**, Benjamin F L Lai, Souvik Maiti, and Jayachandran N Kizhakkedathu: *Stimuli-Responsive Cationic Terpolymers by RAFT Polymerization: Synthesis,*

Characterization, and Protein Interaction Studies. Journal of Polymer Science Part A Polymer Chemistry 03/2008; 46(10.1002/pola.22743):4021-29.

- Rajeshwari Meli, Abhiranjan Prasad, Ashok Patowary, Mukesh K Lalwani, Jayant Maini, Meenakshi Sharma, Angom Ramcharan Singh, Gaurav Kumar, **Vaibhav Jadhav**, Vinod Scaria, and Sridhar Sivasubbu: *FishMap: a community resource for zebrafish genomics*. Zebrafish 02/2008; 5(2):125-30.
- Antara Dasgupta, Prasanta Kumar Das, Rita S Dias, Maria G Miguel, Björn Lindman, **Vaibhav M Jadhav**, Muthaiah Gnanamani, and Souvik Maiti: *Effect of headgroup on DNA-cationic surfactant interactions.* The Journal of Physical Chemistry B 08/2007; 111(29):8502-8.
- Vinod Scaria, **Vaibhav Jadhav**: *microRNAs in viral oncogenesis.* Retrovirology 02/2007;
- 4:82.

PRESENTATION AND TALKS

2013 IBCss 9th Cell line development and engineering, May 20-22, San Diego, USA.

Talk: Application of Genomic Technologies to Cell Line Development.

2012 Cell Culture Engineering (CCE) XIII, April 22 -27, Scottsdale, Arizona, USA.

Poster*: CHO-Engimirs: Growth enhancement by the miR-17-92 cluster in CHO cells.*

2011 22nd ESACT meeting, May 15-18 2011, Vienna, AUSTRIA

Poster*: Establishment of a large scale functional screening method for microRNAs in Chinese hamster ovary cells.*

2011 Keystone Symposia on "Non-coding RNAs in cancer and disease", Feb 11-16, Banff, CANADA

Poster*: Chinese Hamster Ovary (CHO) cell transcriptional profiling reveals the role of c-Myc and the mR-17-92 cluster in cellular physiology.*

2008 HUGO's 13th Human Genome Meeting, Sep 27-30, in Hyderabad, INDIA.

Poster: *Application of artificial microRNA in antiviral therapeutics*

2007 International workshop on Cationic amphiphiles-DNA Systems: Basics to Technology.

Organized jointly by Physical Chemistry 1, Lund University, Sweden and Institute of Genomics and Integrative Biology, Delhi

March-2014. Wien.