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

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ABSTRACT

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

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

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

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

Another promising genetic engineering approach of CHO cells, which is based on post-transcriptional regulation by miRNAs, gained interest in the last years (Jadhav et al., 2013). The advantages of those short (21–24 nucleotides), non-coding RNAs (Finnegan and Pasquinelli, 2012) are that one miRNA can target hundreds of genes without burdening the translational machinery (Hackl et al., 2012a). MicroRNAs are encoded in intergenic and intronic regions,

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and are often located in close chromosomal proximity (Altuvia et al., 2005). According to miRBase version 21 (v21) (Kozomara and Griffiths-Jones, 2014), 31% of *Mus musculus*, 25% of *Homo sapiens*, and 54% of *Rattus norvegicus* annotated miRNAs are found in an inter-miRNA distance below 10 kilobases (kb), and are considered to be clustered. Individual or cooperative transcription, occurring for clustered miRNAs (Baskerville and Bartel, 2005), forms a long, primary transcript (pri-miRNA), which is processed in the nucleus by the microprocessor complex to release a stem-loop with a length of ~65 nucleotides, called precursor miRNA (pre-miRNA or mir) (Lee et al., 2003). After export into the cytoplasm by Exportin-5 (Yi et al., 2003), the stem-loop is recognized and processed by Dicer (Hutvágner et al., 2001) to produce a RNA duplex, consisting of the 5p and the 3p mature microRNA (miR) strands. While one is preferentially incorporated into the miRISC (microRNA-loaded RNA-induced silencing complex) (Bergauer et al., 2009), the other strand is usually, but not always, degraded (Krol et al., 2010). The miRISC recognizes target mRNAs, which bear mostly imperfect complementary regions in the three prime untranslated region (3′ UTR) to the miRNA, guiding translational repression (Zeng et al., 2002) or mRNA cleavage (Zeng et al., 2003).

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

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

2. Material and methods

2.1. Sample ID generation

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

2.2. Cell culture

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

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

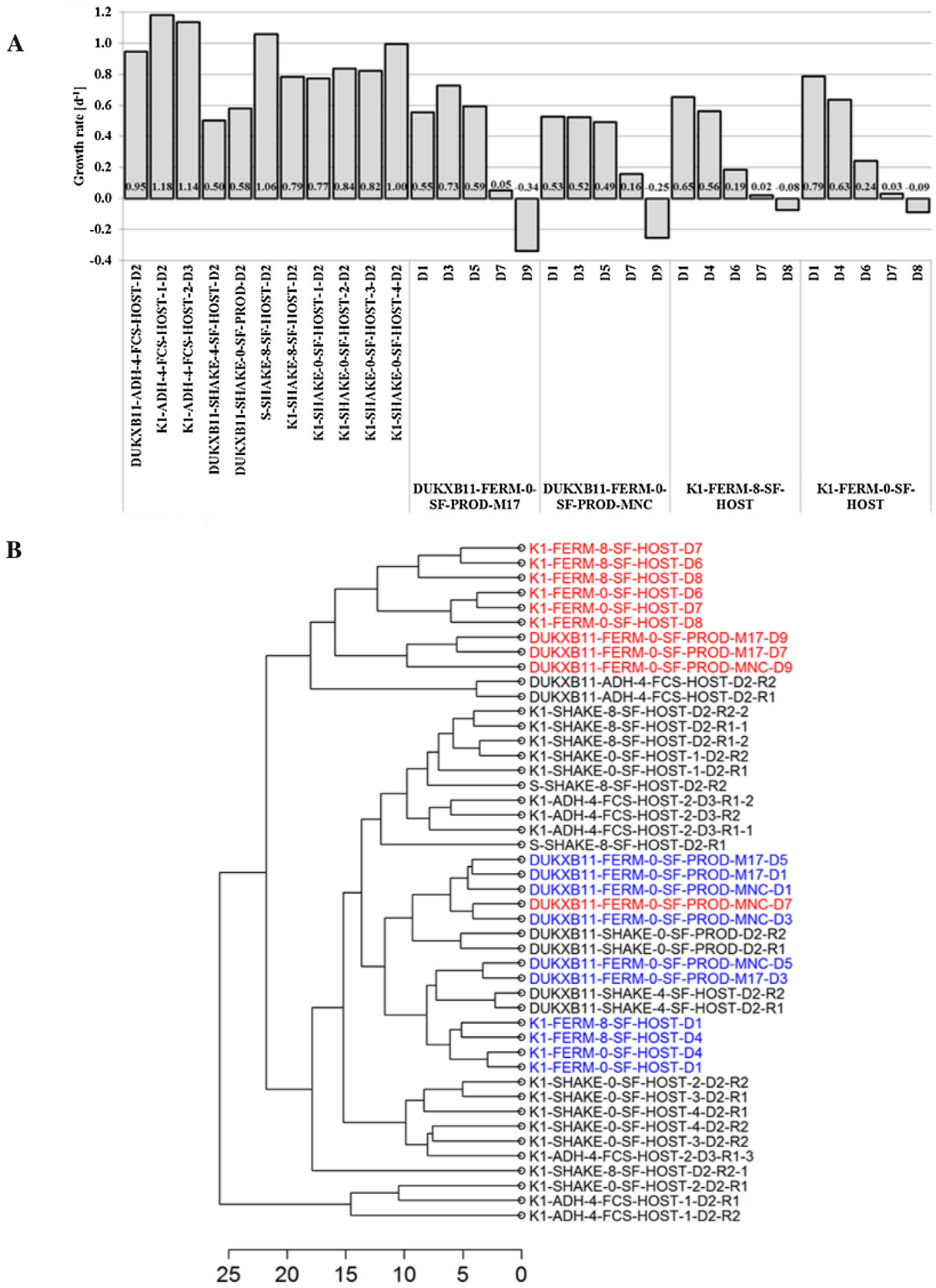

Fig. 1. Sample characteristics. (A) Growth rates of all CHO cell lines analyzed in this study at the time of RNA sampling. (B) Unsupervised sample clustering based on miRNA expression patterns. Red: Samples drawn from the fermenter in stationary/decline phase. Blue: Samples drawn from the fermenter in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

DUKXB11-SHAKE-0-SF-PROD were cultivated in shaker flasks at 140 revolutions per minute (rpm) and a 25 mm shaking diameter.

K1-FERM-8-SF-HOST, K1-FERM-0-SF-HOST, DUKXB11-FERM-0-SF-PROD-M17 and DUKXB11-FERM-0-SF-PROD-MNC were cultivated in DASGIP bioreactor system (DASGIP AG, Jülich, Germany) controlled by DASGIP Control 4.0 software with similar settings as described before (Taschwer et al., 2012). Briefly, advanced spinner vessels with 800 ml working volume, magnetic drive and pitch blade impellers for constant stirring (80 rpm) were used for the cultivation. Temperature was maintained at 37 °C, constant 30% oxygen concentration was held via probe (Broadley James Oxyprobe) by air saturation and neutral pH (Mettler Toledo) was regulated with 0.5 M NaOH and CO_2.

2.3. RNA isolation

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

2.4. RNA quality and quantity assessment

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

2.5. MicroRNA microarray hybridization

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

2.6. Data processing

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

2.7. Growth rate determination

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

2.8. Probe mapping

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

2.9. Clustering

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

2.10. Growth rate correlation

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

3. Results

3.1. Growth and miRNA profiling of diverse CHO cells

Growth profiles of different CHO cell cultures were collected, including adherent and suspension-adapted host and producer cell lines, cultivated under different conditions. A total of 46 miRNA profiles were generated from 31 biological samples by microarrays, and growth rates were calculated, ranging from −0.34 to 1.18 d^{-1} (see Fig. 1A and Supplementary Table 1). MicroRNA microarrays based on miRBase v16 were hybridized using a common reference design, and the resulting raw data were normalized. Log_2 fold changes against the common reference pool (LogFC) and average expression values (AvExp) were retrieved for all miRNA probes. Out of 2367 probes, 295 showed a signal intensity (AvExp) above background criteria (see Supplementary Table 2). The annotated miRNA IDs for these probes were updated from miRBase v16 to miRBase v21. While the majority of the probes (n = 277) were still valid in

miRBase v21, some of the probe IDs (n = 18) had been disproved as mature miRNAs in miRBase v21 and were excluded from further analyses. Around 46% (n = 128) of the probe sequences perfectly matched to already annotated stem-loop sequences.

3.2. Sample clustering indicates factors influencing global miRNA expression

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

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

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

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

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

4. Discussion

4.1. Global miRNA expression and evaluation

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

4.2. Factors influencing global miRNA expression

As indicated by hierarchical clustering of sample IDs, the growth phase is the factor with the most impact on global miRNA expression, as all but one sample in stationary or death phase cluster isolated from all exponential samples (see Fig. 1B). The miRNA expression of CHO cells is known to be dynamic during batch cultures (Bort et al., 2012). Also, serum-free and suspension-adaptation seems to have an influence on the miRNA expression, as part of the FCS-supplied adherent cells cluster apart from suspension and serum-free adapted cell lines. The change in miRNA expression in CHO cells upon serum-free and suspension adaptation has also been shown before (Hackl et al., 2011). The cell line itself, the L-Gln-free adaptation, the cultivation method (shaken

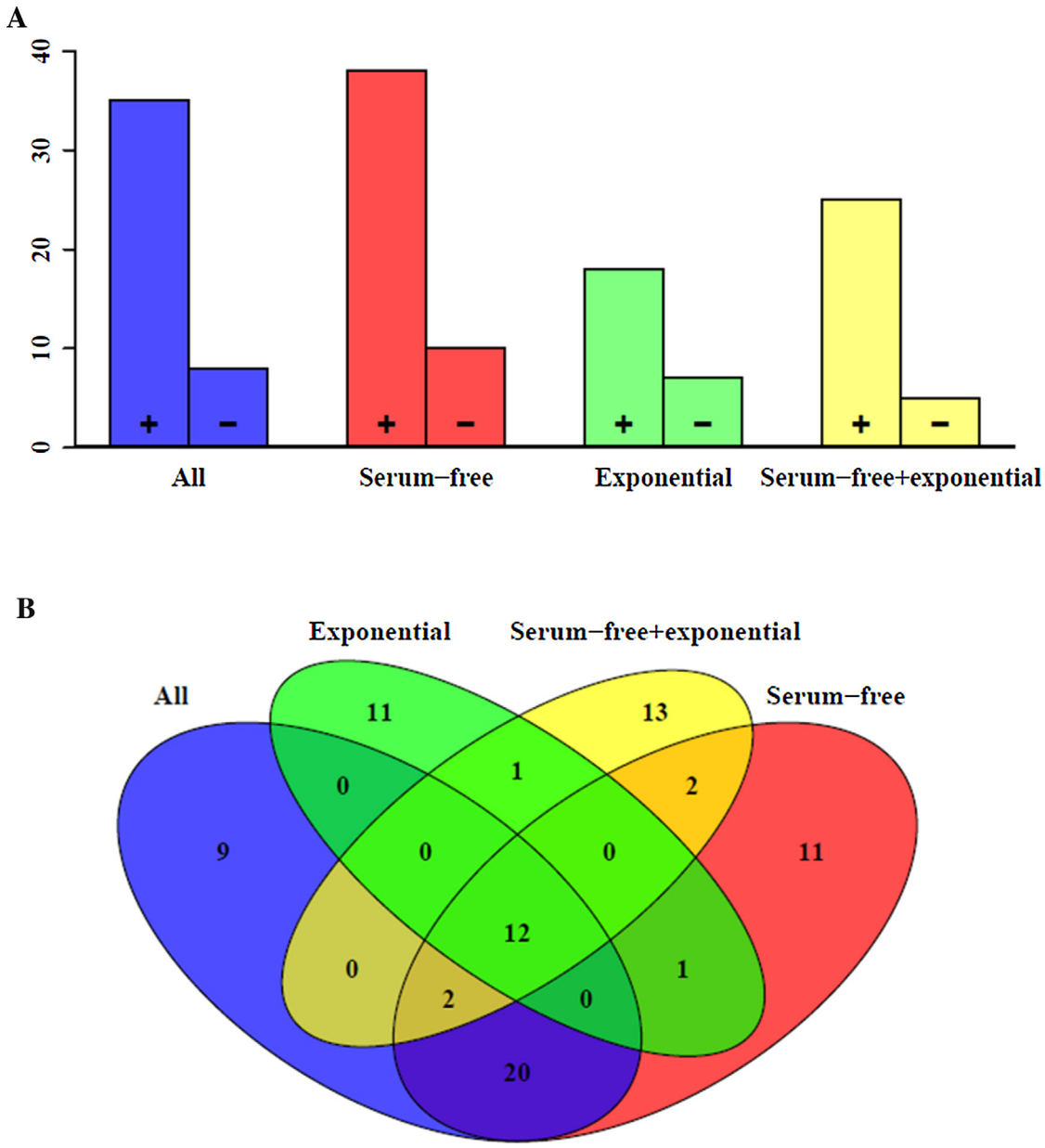

Fig. 2. Growth-correlating miRNAs. (A) Number of positively (+) and negatively (−) growth-correlating miRNAs including either all samples (All), samples of the exponential growth phase (Exponential), serum-free samples (Serum-free) or serum-free samples in exponential growth phase (Serum-free + exponential).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

Table 1
Growth-correlating miRNAs.

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

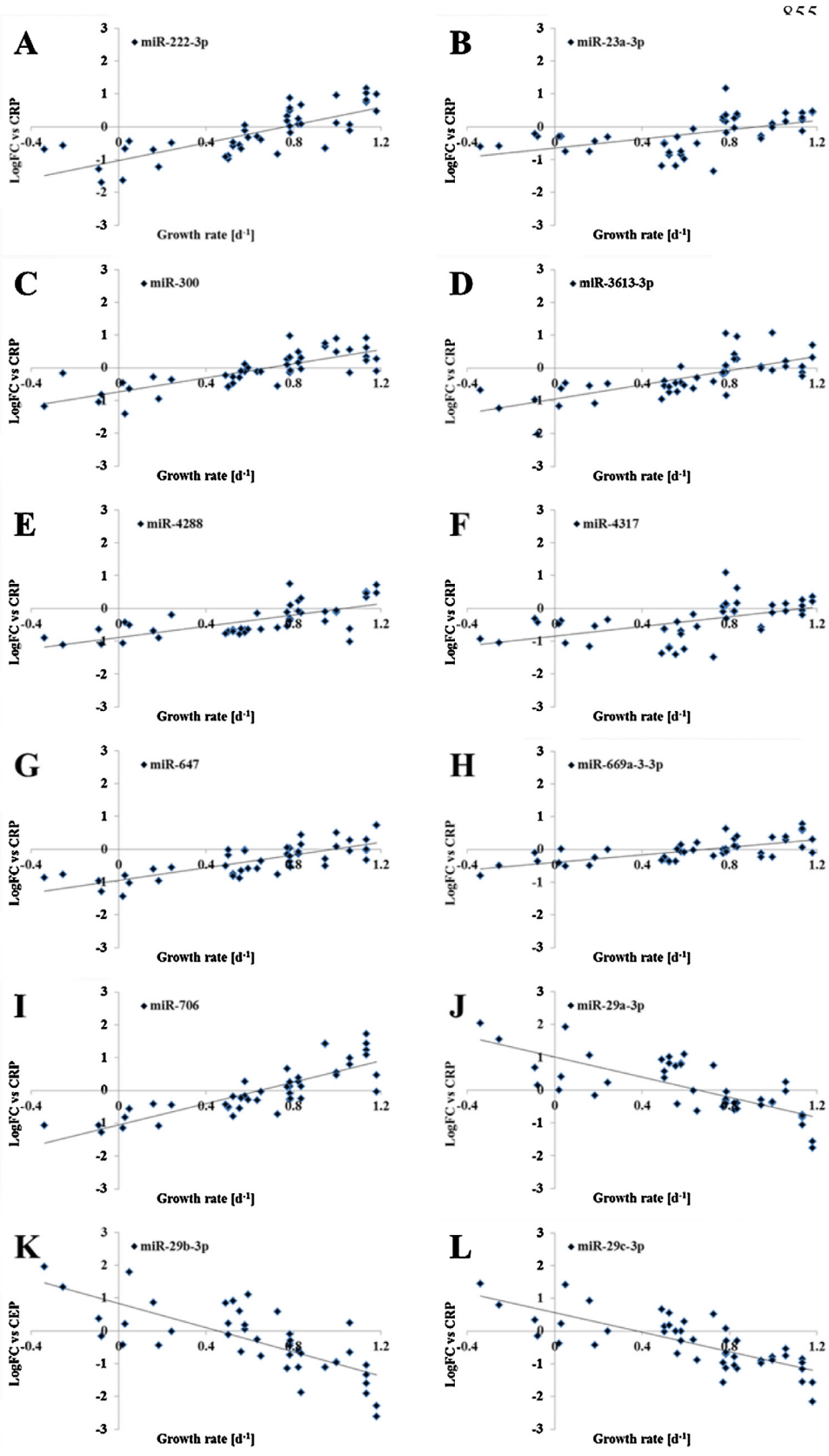

Fig. 3. MiRNA expression versus growth rate. LogFC of each sample was aligned to the growth rate for all constantly growth-correlating miRNAs. The line depicts the linear correlation. (A–I) Positively growth-correlating miRNAs. (J–L) Negatively growth-correlating miRNAs.

or in a fermenter) and miRNA engineering exhibit only a marginal effect on global miRNA expression in comparison to the above mentioned factors, as no distinct clustering pattern was observed.

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

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

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

4.4. Function of positively growth-correlating miRNAs

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

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

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

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

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

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

4.5. Function of negatively growth-correlating miRNAs

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

addition, it is able to upregulate and activate p53, which is capable of inducing apoptosis (Park et al., 2009). The miR-29 family is found upregulated in quiescent cells (Bandyopadhyay et al., 2011), promotes differentiation (Wang et al., 2008) and is capable of inducing senescence in aged cells (Ugalde et al., 2011). But it is also found upregulated in certain tumor cells (Hong et al., 2014), and can have a positive effect on the migrational potential (Franceschetti et al., 2013). It controls global gene expression by targeting several genes involved in DNA (de) methylation (Fabbri et al., 2007). The family members are associated with extracellular matrix (ECM) remodeling, as they inhibit ECM gene expression (Maurer et al., 2010), target genes involved in the extracellular matrix breakdown (Ramdas et al., 2013), and negatively regulate EMT (Rostas et al., 2014). They are also involved in nutrient metabolism control, as the expression is upregulated by nutrient limitation (Liang et al., 2013), and downregulated by high glucose levels (Du et al., 2010). The miR-29 family itself is downregulated by oncogenes (Zhang et al., 2012).

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

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

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

5. Conclusion

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2016.03.022.

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