Transferability of miRNA-technology to bioprocessing: Influence of cultivation mode and media

Ann-Cathrin Leroux¹,² | Elisabeth Bartels¹,³ | Luise Winter¹,⁴ | Melanie Mann¹ | Kerstin Otte⁵ | Christoph Zehe²

¹Product Development, Sartorius Stedim Cellca GmbH, Ulm, Germany
²Advanced Biotech Applications Corporate Research, Sartorius Stedim Cellca GmbH, Ulm, Germany
³Operations, Sartorius Stedim Cellca GmbH, Ulm, Germany
⁴Upstream Process Development, Rentschler Biopharma, Laupheim, Germany
⁵Biology, Cell- and Molecular Biology, Biberach University of Applied Sciences, Biberach an der Riß, Germany

Correspondence
Ann-Cathrin Leroux, Product Development, Sartorius Stedim Cellca GmbH, Marie-Goeppert-Mayer-Straße 9, Ulm 89081, Germany.
Email: ann-cathrin.leroux@sartorius.com

Abstract
The biopharmaceutical industry strives for improvement of their production processes. In recent years, miRNAs have been shown to positively impact the production capacity of recombinant CHO cells, especially with regard to difficult to express proteins. Effective and reliable gene regulation of process relevant target genes by miRNAs is a prerequisite for integrating them into the toolbox of industrial cell engineering strategies. However, most studies rely on transient transfection of miRNA mimics; there is low standardization in evaluation of miRNA function and little knowledge on transferability of effects found during transient expression to stable expression during industry relevant fed-batch cultivation. In order to provide more insight into this topic, we used the pcDNA6.2 vector for stable miRNA overexpression during batch and fed-batch cultivation in CHO DG44 cells, optimized the vector, and compared the miRNA levels and effects with those achieved by transfection of miRNA mimics. We found that miR-1 downregulated TWF1 mRNA in different recombinant CHO DG44 clones in a dose-dependent manner during transient batch cultivation. Cells stably overexpressing miR-1 also showed a TWF1 mRNA downregulation when cultivated in batch mode using in-house medium 1. However, when the cells stably overexpressing miR-1 were cultivated in fed-batch mode using in-house medium 2. Consequently, a change of cultivation mode and medium seems to have an impact on target gene regulation by miRNA. Taken together, our findings highlight the importance to standardize miRNA evaluations and test miRNAs in the final application environment.

KEYWORDS
bioprocess, CHO, miRNA

1 | INTRODUCTION

MiRNAs are small regulatory RNA molecules which are naturally encoded in the genome. They are under control of Polymerase II or Polymerase III promoters and form a hairpin structure after transcription.¹,² This pri-miRNA is processed by Drosha and DICER to form short mature miRNA duplexes. The guide strand of the mature miRNA duplex then associates with Argonaute 2 proteins forming the RNA-
induced silencing complex, RISC. RISC binds to various target mRNAs determined by the miRNA sequence and induces translational repression, for example, by mRNA decapping or deadenylation. This leads to a decrease in the respective protein levels, thus making miRNAs key regulators for various cell processes like cell differentiation, cell cycle progression or apoptosis. miRNAs as tool for engineering cell lines, coined as “engimiRs” by Hackl et al., 2012, have been also shown to be a useful tool for the regulation of productivity and cell growth in CHO cells. Most studies that have investigated miRNA function in CHO cells used transient mimic transfection, for example, a transient transfection of miR-30 family mimics as well as let-7e, miR-3072, and miR-330 increased cell-specific productivity in CHO cells expressing SEAP. Strotbek et al., 2013, transiently screened human miRNAs in an IgG1 producing cell line and identified miR-557 together with miR-1287 to increase productivity. Some studies also investigated miRNA function during stable batch expression, for example, it could be shown that stable overexpression of miR-557 together with miR-1287 and overexpression of miR-17 increased cell-specific productivity. Kelly et al., 2015, found that stable sponge based miRNA knockdown of miR-23b increased final product concentration in CHO-SEAP cells. Similarly, using CRISPR/Cas9 technology, a stable knockout of miR744-3p in IgG producing CHO DG44 cells was shown to result in significantly raised product titers in batch culture. Only few studies have tested miRNAs in a stable fed-batch environment, although this is the industry’s current standard for expression of biopharmaceuticals. For example, it could be shown that a stable plasmid based overexpression of miR-557 in CHO-GS cells cultivated in fed-batch mode increased productivity of difficult-to-express proteins. Similarly, overexpression of miR-106b in and CHO-IgG cell line increased final product concentration by 0.66-fold via extension of viability during a fed-batch process. Sanchez et al., 2014, discovered that reduced miR-7 levels increased final product concentration in CHO-SEAP cells during fed-batch cultivation. In addition to different expression (transient and stable) and cultivation modes (batch and fed-batch), also the used cell culture media as well as the amount of transfected miRNA mimics varies between the studies. Media used comprise but are not limited to ProCHO™5 culture medium (Lonza, Vervier, Belgium), chemically defined and serum-free HyCloneTM SFM4CHOTM (GE Healthcare, Chalfont St. Giles, England) or Bi proprietary, serum-free media (Boehringer-Ingeheim, Biberach, Germany). Transfected mimic concentrations range from 15 to 1,000 nM.

This shows, that current investigations and the use of miRNAs in industrial settings does not follow standardized protocols or procedures, which raises the question, whether these non-standardized investigations are the reason for the identification of many different bioprocess relevant miRNAs with low overlap between the studies. In this work, we sought to study the transferability of miRNA-mediated effects found during transient expression to stable expression. In addition, we were interested in the transferability between different cultivation modes and media and in providing more insight into effective miRNA levels, as this is of interest for the application of miRNAs as engimiRs in a bioprocess environment.

2 | MATERIAL AND METHODS

2.1 | Vector construction

Two plasmids were tested for miRNA overexpression. First, the BLOCK-iT Pol II miR RNAi Expression Vector Kit with pcDNA 6.2-GW/EmGFP-miR vector and the negative control pcDNA 6.2-GW/EmGFP- miR-neg control (Thermo Fisher Scientific) was used following the standard kit protocol. In short, the miRNA sequence to be overexpressed (guide strand) and an artificially, fully complimentary passenger strand with two nucleotides deleted was synthesized (DNA oligosynthesis, biomers) and annealed to form a DNA duplex with overhangs. The duplex was ligated with the linearized pcDNA 6.2-GW/EmGFP-miR vector provided by the manufacturer. Additionally, the standard human Cytomegalovirus promoter (hCMV) was exchanged by mouse Cytomegalovirus (mCMV), Simian Virus 40 (SV40) and β-Actin promoter using SacI/SpeI restriction sites. Also, the miRNAs were chained following the standard kit protocol. Briefly, 2, 4, and 8 copies of miRNA were serially cloned using BamHI/BgIII restriction sites on the backbone and BamHI/XhoI restriction sites on the insert. Second, the mirVanaSelect™ pEGP-miR Cloning and Expression Vector and the negative control mirNASelect Null Control Vector both with hCMV promoter were used following the cloning procedures described in the product data sheet. The miRNA to be overexpressed and the 180–250 bp up- and downstream sequence was amplified from the CHO DG44 host cell genome using PCR. Then, the PCR fragment and the pEGP vector were ligated using BamHI/Nhel restriction sites. For more information on vector construction, see Figure 4.

2.2 | miRNA mimics

miRvana miRNA Mimic miR-1 Positive Control (Thermo Fisher Scientific), and self-annealed miRNA mimics were used. MiR-1 single strand RNAs were synthesized (oligosynthesis, biomers) and reconstituted in water at 100 μM. The 30-μl guide and 30-μl passenger strand were added to 15 μl annealing buffer (50 mM Tris, pH 8.0, 100 mM NaCl in DEPC-treated water) followed by denaturation at 95°C for 2 min. Reaction was left to cool down to room temperature (annealing), yielding a miRNA/siRNA mimic concentration of 40 μM.

2.3 | Cultivation of cells

Two different clonal CHO DG44 cell lines were used for transient experiments, clone A stably expressing Antibody A (IgG1) and clone B stably expressing Antibody B (IgG1). Cells were cultivated in an in-house medium (in-house medium 1) supplemented with 6 mM glutamine (200 mM stock solution) and 30 nM methotrexate (50 μM stock solution) at 36.8°C, 7.5% CO₂ on a linear shaker for 48 hr post transfection. For the generation of stable miRNA expressing cell lines, clone A was used. After transfection, resulting pools were seeded at...
4*10⁵ cells/ml, cultivated in in-house medium 1 supplemented with 30 nM methotrexate and 8 μg/ml Blasticidin (pcDNA6.2 vector) or 3.5 μg/ml Puromycin (miRNAsSelect vector) at 36.8°C, 7.5% CO₂ on a linear shaker. Cells were passaged every 3–4 days. After 14 days of cultivation in selective medium, cells were seeded at 3*10⁵ cells/ml in in-house medium 2 supplemented with 6 mM glutamine to start fed-batch cultivation in a volume of 25 ml using shake flasks. Cells were fed with two different in-house feed media and a glucose solution. Fed-batch cultivation was stopped when viability dropped below 70%, resulting in a cultivation time of 10–11 days.

2.4 | Transfection

Clonal CHO DG44 cell lines stably expressing IgG1 were seeded at 4*10⁶ cells/ml 24 hr prior to transfection. 1*10⁶ cells were transfected with miRNA/siRNA mimics at 50 nM or with 1.72*10¹¹ copies of plasmid (equal to 1 μg of control plasmid) in a culture volume of 4 ml using the Amaxa cell line Nucleofector kit (#VCA-1003, Lonza). As negative control, AllStars Negative Control siRNA (#1027280, Qiagen), pcDNA6.2 with a non-targeting miRNA (pcDNA 6.2-GW/EmGFP-mir-neg control plasmid, supplied with BLOCK IT kit) or empty miRNASelect vector (miRNASelect pEGBP-miR Null Control Vector) was transfected.

2.5 | qPCR

Cell samples for qPCR were taken 48 hr post transfection (transient experiments), or on day 17 post transfection (stable batch experiments) on day 5 of fed-batch cultivation (stable fed-batch experiments). Total RNA was isolated from 2.5*10⁶ cells with the NucleoSpin miRNA extraction kit (#740971.50, Macherey-Nagel) following the standard protocol. For miRNA analysis, universal transcription was conducted following the miRCURY LNA RT Kit manual (#339340, Qiagen). The cDNA was diluted 1:10, quantified using the miRCURY LNA SYBR Green PCR Kit (#339346, Qiagen) and taqman gene expression assays (TWF1, #YP00204344; mmu-miR-34b-3p, YP00205086; Qiagen) on a QuantStudio 3 qPCR cycler. For GFP and YP00204344; mmu-miR-34b-3p, YP00205086; Qiagen) on a QuantStudio 3 qPCR cycler. For evaluation, the ΔΔCt method was used.

The graphs either depict ΔCt or FC values. ΔCt values are Ct values normalized to the reference gene or reference miRNA. They can be interpreted like Ct values, meaning high ΔCt stand for low expression levels. The reference gene for mRNA qPCR is β-Actin and the reference miRNA for miRNA qPCR is an endogenous expressed miRNA, cgr-miR34b-3p (Figure S1).

2.6 | Statistical evaluation

Statistical evaluation was performed using GraphPad Prism version 8.0.1 for Windows, GraphPad Software, La Jolla, CA. For comparison of two values, two-tailed unpaired t-test, α = .05 was performed. For comparison of more than two values, ordinary one-way ANOVA and Dunnett’s multi-comparison test, α = .05 were performed. For multi-factor analysis, a two-way ANOVA was performed with α = .05. Significance in graphs is illustrated as follows: * p < .05; ** p < .01; *** p < .001; **** p < .0001.

2.7 | MiRNA target analysis

MiRNA targets were predicted using mirWalk 3.0 selecting mouse as reference species. The resulting target gene lists were analyzed using PANTHER gene ontology version 15.21

3 | RESULTS

3.1 | Transient miRNA mimic transfection induces reliable gene regulation in industrial production cell lines independent of chemical miRNA modification

MiRNAs have been described as effective modulators of cellular production systems (#111618). Initial studies to identify or validate endogmir# with regard to effects on process relevant cellular parameters are usually undertaken by transient transfection of miRNA mimics into production cell lines. It is then supposed that the same effects will be triggered by stable miRNA overexpression during cell line development. In order to elucidate the reliability and transferability of results observed during transient miRNA mimics experiments to stable overexpression applications in CHO production cell lines, we established a system to evaluate effective miRNA levels and miRNA target gene regulation during transient and stable expression in CHO DG44 production cell lines. For that purpose, two different miRNAs, miR-1 and miR-30a, were evaluated. MiR-1 was previously shown to downregulate Twinfilin (TWF1), a protein involved in β-Actin polymerization,23,24 whereas miR-30a has been experimentally proven to downregulate S-phase kinase associated protein 2 (SKP2) mRNA in CHO cells.25 We utilized two industrial CHO DG44 production clones producing an anti-TNFα antibody (Antibody A, clone A) or an anti-VEGF antibody (Antibody B, clone B) and transiently transfected them with 50 nM miR-1 or miR-30a mimics (Table 1). Controls were transfected with 50 nM AllStars Negative Control. Subsequently, qPCR experiments were performed 48 hr post transfection to quantify transfected miRNAs and their target mRNA levels (Figure 1). The basal miRNA expression levels are shown in Figure 1a,b as expression after mock transfection and are depicted as negative controls (white bars). The basal expression of miR-1 is lower compared to miR-30a. For both miR-1 and miR-30a, elevated levels were observed compared to basal expression (control) (Figure 1a,b). Because cellular overexpression of functional miRNAs should induce an efficient downregulation in the
expression of target genes, target mRNA levels were quantified using qPCR analysis of the same experimental samples. Results are shown as fold change values in Figure 1c,d, where miR-1 downregulated TWF1 mRNA to 0.30- and to 0.37-fold change, respectively. In contrast, miR-30a induced a less pronounced effect on SKP2 mRNA.

In subsequent experiments we aimed to test whether observed cellular miRNA levels after transfection and target gene regulation induced by miRNA mimics were clone independent. Here, we focused on miR-1, which had shown a reliable regulation in the previous experiments. After transfection of additional six stable CHO DG44 clones producing Antibody A or Antibody B with 50 nM miR-1 mimics, miRNA and mRNA levels were determined by qPCR. The basal miRNA expression levels are depicted as controls (white bars) and varied between clones (Figure 2a). Focusing on normalized miR-1 levels ($\Delta Ct$) after mimic transfection, the miR-1 levels were similar across all clones, independent of the basal miR-1 expression (Figure 2b). The observed rather large range of fold change in miRNA levels (Figure 2b) might be due to varying basal miR-1 expression between individual clones (Figure 2a). When analyzing target gene regulation, a significant regulation by miR-1 was visible in all CHO production clones independent of the produced antibody (Figure 2c).

During cell line development, relevant engimiRs are stably overexpressed using a plasmid based overexpression system. In contrast to commercially produced miRNA mimics (miRVana, thermofisher.com), the resulting endogenously produced mature miRNAs are not chemically modified. To elucidate whether the chemical modifications of routinely used miRNA mimics have any effect on transfected miRNA levels and target gene regulation, we established unmodified mature miRNAs by self-annealing unmodified HPLC purified RNA oligos to form miR-1 mimics without chemical modification (Table 1). These unmodified miR-1 mimics were transfected into clone A and six additional stable CHO DG44 clones producing Antibody A or Antibody B (clone 1 to 6) at a concentration of 50 nM. MiR-1 levels were determined 48 hr after transfection by qPCR. When using unmodified miR-1 mimics, miR-1 levels were significantly elevated compared to control (Figure 2d,e). These elevated miR-1 levels led to

### TABLE 1 MiRNA mimic sequences used for transient transfection

| miRNA name       | miRNA sequence                                                                 | Description                                    |
|------------------|-------------------------------------------------------------------------------|-----------------------------------------------|
| mirVana™ miRNA mimic | Not available, requires a confidential disclosure agreement                    | Mimics mature miR-1, chemically modified      |
| miR-1 (modified miR-1) | 5’-caauacuucuuauaggcuaaagc3’ 3’-uauguaugaagaaugua-aggu 5’ | Mimics mature miR-1                          |
| Modified miR-1 (mmu-miR-1a-1) | 5’-gcauacuucguacacgagc3’ 3’-acauacuucguacacgagc 5’ | Mimics mature miR-30a                        |

### FIGURE 1 Two CHO DG44 clones expressing two different products (clone A/antibody A; clone B/antibody B) were transfected with 50 nM miRNA mimics. MiRNA and mRNA levels were measured 48 hr post transfection by qPCR. For this measurement, samples from two biological replicates were pooled. (a, b) MiR-1 levels of clone A and B which were transfected with chemically modified miR-1 (miRVana) or miR-30a mimics; values are normalized to reference miRNA cgr-miR-34b. (c, d) TWF1 mRNA levels of miRNA transfected cells depicted in (a) and (b). For transgene expression data, see Figure S2.
a significant downregulation of TWF1 mRNA (Figure 2f). Comparing unmodified and modified miR-1 mimics, the transfection of unmodified miR-1 mimics resulted in higher miR-1 levels than modified miR-1 mimics (Figure 2a vs. d). On the other hand, modified miR-1 reduced TWF1 mRNA levels to a greater extent than unmodified miR-1 (Figure 2c vs. f). This indicates that modified miR-1 mimics are possibly more effective in TWF1 downregulation.

Generally, these data suggest that independent of the clone used for transfection and the chemical modification of the miRNA mimic, miRNA levels are reliably increased and the target mRNA is significantly reduced upon miRNA mimic transfection, underlining the reliability and usefulness of this in-vitro system.

### 3.2 Gene regulation by miR-1 mimics is dose-dependent

For a comparison of transient and stable miRNA expression and the resulting target gene downregulation, it is necessary to understand which levels of miRNA can mediate effective downregulation and whether this regulation might be dose-dependent. Therefore, a dilution series of miR-1 was tested in transient transfection experiments (1:2 dilutions, starting at an amount of 100 nM). In order to mirror the molecular mechanisms during stable miR-1 expression, where the resulting mature miRNAs are not chemically modified, unmodified miR-1 mimics were used for the following experiments. Following
transfection into clone A, miR-1 and TWF1 mRNA levels were measured 48 hr after transfection using qPCR. Results showed that transfection of increasing amounts of miR-1 resulted in increasing concentrations detectable within the cells (Figure 3a) downregulating TWF1 mRNA levels accordingly (Figure 3b). These results also show that there is a clear dose–response relationship visible between miRNA levels and TWF1 downregulation. This dose–response curve is a valuable tool to later correspond miR-1 levels to their effects on TWF1 mRNA during stable overexpression.

3.2.1 Stable miRNA overexpression results in lower miRNA levels than transient miRNA mimic transfection

The overexpression of process relevant engimiRs to optimize bioproduction is implemented in the pharmaceutical industry for example by integrating relevant miRNAs into expression vectors.18 In order to analyze whether the obtained results from transient overexpression of miR-1 are transferrable to stable overexpression, we used two commercially available miRNA expression vectors pcDNA™6.2-GW/EmGFP-miR (pcDNA 6.2) and miRNASelect™pEGP-miR (miRNASelect) (Figure 4). In both vectors, miRNA expression is under control of an hCMV promoter and is integrated into the transcription unit of GFP. However, there are differences in the organization of the transcription unit as well as miRNA sequence and structure. For pcDNA6.2, the miRNA flanking regions are derived from mmu-miR-155 and therefore the resulting miRNA precursor is a chimeric molecule. Additionally, the miRNA is processed directly from the 3’UTR of the GFP transcript without prior splicing. On the other hand, the miRNA select vector allows for cloning of the miRNA’s natural genomic sequence and the miRNA is located in the human β-globin intron. Thus, the miRNA is spliced from the 5’UTR of the GFP transcript prior to miRNA processing (Figure 4).

MIR-1 was cloned into pcDNA 6.2 and miRNASelect vectors and transfected into clone A followed by 2 weeks of selection to generate stable expression pools. Controls were transfected with pcDNA6.2 or miRNASelect negative control vector. Subsequent fed-batch cultivation, which is a standard cultivation technique for production of biopharmaceuticals, included a medium exchange from in-house medium 1 to in-house medium 2. Samples were taken on day 5 of the fed-batch process and qPCR was performed to quantify overexpression of miR-1. Stable integration of both vectors caused high miR-1 overexpression of 1914-fold change (for miRNASelect and of 13,318-fold change for pcDNA6.2 compared to negative control (Figure 5a), indicating that pcDNA6.2 outperforms miRNASelect by inducing a higher level of miR-1 expression. However, a parallel transient transfection of 100 nM unmodified miR-1 mimic showed that the resulting cellular miR-1 levels are higher than levels achieved during stable overexpression (Figure 5a). A downregulation of TWF1 mRNA was only induced with transient 100 nM miR-1 mimic transfection but not for stable miR-1 expression (Figure 5b). Because downregulation of miRNA target genes may depend on cellular levels of miRNAs,27,28 we aimed at elevating stable miR-1 levels. As pcDNA6.2 clearly outperformed miRNASelect for stable miR-1 overexpression, pcDNA6.2 was chosen for optimization of stable miR-1 expression.

3.3 Exchanging promoters in pcDNA6.2 vector does not reliably increase miRNA levels during fed-batch cultivation

In order to increase stable miR-1 transcription levels to an extent that it is comparable to transient miR-1 mimic transfection at 100 nM

![Figure 3](https://example.com/figure3.png)

**FIGURE 3** TWF1 regulation by miR-1 mimics is dose-dependent. Unmodified miR-1 was diluted 1:2 starting at 100 nM and transfected into clone A. (a) MiR-1 levels were measured 48 hr after transfection via qPCR. MiR-1 expression is significantly higher for all concentrations compared to control (0 pmol), one-way ANOVA, \( p = .0001 \). (b) TWF1 mRNA levels 48 hr after miR-1 mimic transfection. TWF1 mRNA expression is significantly lower compared to control (0 pmol), except for 1.563 nM miR-1 transfection, one-way ANOVA \( n = 3 \), bars indicate mean, error bars indicate SD. For transgene expression data, see Figure S2.
FIGURE 4  (a) PcDNA™ 6.2-GW/EmGFP-miR (pcDNA 6.2) vector and (b) its GFP-precursor-miR transcript. (c) MirNASelect™ pEGP-miR vector (mirNASelect) and (d) its precursor-miR-GFP-Puromycin transcript.

FIGURE 5  Comparison of transient and stable overexpression of miR-1 with pcDNA6.2 and mirNA select vector. Linearized vector was transfected into clone A followed by 2 weeks of selection in Blasticidin supplemented in-house medium 1 and 2 weeks of fed-batch cultivation in in-house medium 2. Samples were taken on day 5 of fed-batch cultivation and miR-1 levels were measured by qPCR. For transient experiments 100 nM unmodified miR-1 mimics were transfected and miR-1 levels were measured 48 hr after transfection by qPCR (a) miR-1 levels and (b) TWF1 mRNA levels. T-test was performed for statistical analysis. * p < .05, *** p < .001, n = 3. For transgene expression data, see Figure S2.
(Figure 5), a selection of different promoter sequences including the mCMV, SV40 and β-Actin promoter were inserted into the pcDNA6.2 vector. All expression plasmids, including the pcDNA 6.2 negative control vector were transfected into clone A and stable miRNA expressing cell pools were generated. MiR-1 and TWF1 mRNA levels were measured on day 5 of fed-batch cultivation by qPCR. The standard pcDNA6.2 as well as the modified vectors with exchanged promoter sequences all significantly increased miR-1 levels when compared to negative control pools (Figure 6a,b). When comparing these data to the standard pcDNA6.2 vector harboring the hCMV promoter, the highest increase in miR-1 levels at 1.8-fold change was observed with mCMV promoter as shown in Figure 6c. However, this increase was not significant, due to high variability within the biological replicate group. In contrast, SV40 and β-Actin promoter did not show any increase of miRNA levels compared to standard pcDNA6.2 during fed-batch cultivation (Figure 6c).

Because the analyzed miRNAs are processed from a primary transcript, their final transcript level within the cell might depend on the level of produced primary transcript. In addition to the encoded miRNA, the primary transcript contains a coding sequence for GFP. Therefore, we next evaluated GFP transcription levels to assess promoter strength independently of the miRNA processing machinery. QPCR analysis showed that GFP mRNA levels resulting from the mCMV promoter were drastically increased displaying a 3.01-fold change when compared to hCMV (Figure 6d). In addition, GFP transcript levels were also significantly elevated with 2.99-fold for SV40, while GFP levels resulting from the β-Actin promoter were again similar compared to standard pcDNA6.2 vector (hCMV) (Figure 6d). Comparing the increase in miRNA with the increase in GFP mRNA (Figure 6c,d), mCMV promoter increased GFP mRNA levels to 3.01-fold change compared to standard pcDNA6.2 but only increased miR-1 levels to 1.8-fold change with high variability. SV40 promoter increased GFP mRNA levels to 2.99-fold increase but decreased miR-1 levels to 0.77-fold change and β-Actin promoter did only slightly increase GFP mRNA levels to 1.22-fold change which had no effect on miR-1 levels (1.00-fold change). Consequently, clear correlation of GFP mRNA levels with mature miR-1 levels was not observed for any promoter tested (Figure 6e). Thus, an increase in precursor miRNA did not result in a reliable increase of mature miRNA levels.

These data showed that an increase in miRNA precursor transcript levels by using stronger promoters (mCMV and SV40) did not lead to a reliable increase in mature miR-1 levels during stable overexpression in fed-batch mode. In order to reliably elevate miR-1 levels in fed-batch mode, alternative vector optimization strategies had to be tested.

### 3.4 | MiRNA-chaining significantly increased miR-1 levels during fed-batch cultivation but does not induce TWF1 downregulation

An alternative attempt to increase miRNA levels is to clone repetitive miRNA copies into the expression vector, an approach called chaining. For that purpose, two, four and eight copies of miR-1 were inserted into the standard pcDNA6.2 vector containing the hCMV promoter. Stable pools were generated by transfecting the vectors with chained miRNAs and the negative control vector into clone A. After selection, the resulting stable cell pools where cultivated in fed-batch mode. qPCR analysis of miR-1 levels on day 5 of fed-batch cultivation revealed that chaining four and eight copies of miR-1 significantly outperformed the standard pcDNA6.2 vector (Figure 7a,b). Finally, we analyzed the functionality of the produced miRNA by measuring the target TWF1 mRNA levels in the same samples by qPCR. During fed-batch cultivation, there was no downregulation of TWF1 mRNA observable compared to negative control, but in contrast a significant upregulation to a maximum of 1.43-fold was detected (Figure 7c). Although the miR-1 levels during stable expression in fed-batch mode (Figure 7a) were even higher compared to a transient miR-1 mimic transfection at 100 μM (Figure 3a), a functional regulation was only observed using transient transfection of miRNA mimics. In summary, despite higher levels of miR-1 were detected upon chaining no further downregulation of TWF1 was observed.

### 3.5 | Change in cultivation mode-induced functional downregulation of TWF1

A further difference between transient and stable miRNA overexpression experiments is the mode of cultivation (batch vs. fed-batch), including the utilization of different culture media. While transient experiments are routinely performed in in-house medium 1 in batch mode, experiments with stable expression cell lines are carried out using in-house medium 2 in fed-batch mode. To account for these differences, the same stable cell pools tested in standard fed-batch mode were also analyzed for miR-1 and TWF1 mRNA levels during cultivation in in-house medium 1 in batch mode. Here, as well, all chained vectors significantly outperformed the standard pcDNA6.2 vector (Figure 7d). These results were similar to those generated in fed-batch mode (Figure 7a). The miR-1 levels were also significantly elevated compared to the standard pcDNA6.2 (Figure 7e). Statistical testing of these data showed even higher levels of significance in batch mode (p < .0001) compared to fed-batch mode (p < .05). Finally, when analyzing the induced target regulation by miR-1 overexpression, there was a significant downregulation of TWF1 mRNA observable in batch mode (Figure 7f). These data are in strong contrast to the target regulation during fed-batch cultivation, where despite high miR-1 expression an upregulation of TWF1 mRNA was induced.

### 3.6 | MiR-1-mediated TWF1 mRNA downregulation is dependent on the mode of cultivation and media

Figure 8 shows a correlation of miR-1 levels with TWF1 mRNA levels during transient mimic transfection, stable expression in batch mode
FIGURE 6  The hCMV promoter of pcDNA6.2-miR-1 vector was exchanged for mCMV, SV40, and β Actin promoter. Linearized vector was transfected into clone A followed by 2 weeks of selection in Blasticidin supplemented in-house medium 1 and 2 weeks of fed-batch cultivation in in-house medium 2. Samples were taken on day 5 of fed-batch cultivation. (a) Mirna expression levels as ΔCt during fed-batch cultivation. (b) Mirna expression levels as fold-change compared negative control during fed-batch cultivation. (c) Mirna expression levels as fold-change compared to standard pcDNA6.2 vector (hCMV promoter) during fed-batch cultivation. (d) GFP mRNA levels compared to standard pcDNA6.2 in cells cultivated in fed-batch mode. (e) Correlation of GFP mRNA levels with mature miR-1 levels during fed-batch mode. Statistical testing: ANOVA and Dunnett’s multiple comparison testing with α = .05 was performed comparing standard pcDNA6.2 with mCMV, SV40, and β Actin. p-Values: * p < .05; ** p < .01; *** p < .001; **** p < .0001; if nothing is indicated, the statistical test result was not significant. For transgene expression data, see Figure S2
(in-house medium 1) and stable expression in fed-batch mode (in-
house medium 2). TWF1 mRNA downregulation is dependent on
miR-1 levels, however the potency to downregulate decreases when
comparing stable batch to transient mimic cultivation and vanishes
during stable fed-batch cultivation. These results show that in the
applied CHO expression system transient miR-1 mimic results can be
transferred to stable expression with cultivation in batch mode. How-
ever, the miR-1-mediated effect is less prominent: although the

FIGURE 7  A series of 2, 4, and 8 miR-1 copies were cloned into standard pcDNA6.2 vector. Linearized vector was transfected into clone A
followed by 2 weeks of selection in Blasticidin supplemented in house medium 1 and 2 weeks of fed-batch cultivation in in-house medium
2. Samples were taken on day 14 of batch cultivation and on day 5 of fed-batch cultivation. (a) miRNA expression levels as ΔCt during fed-batch
and (d) batch cultivation. (b) miRNA expression levels as fold-change during fed-batch and (e) batch cultivation. (c) TWF1 mRNA levels upon
stable miR-1 overexpression during fed-batch cultivation and (f) batch cultivation. Statistical testing using ANOVA and Dunnett’s multiple
comparison testing was performed with an α = .05. p-values: * p < .05; ** p < .01; *** p < .001; **** p < .001. For transgene expression data during
fed batch cultivation, see Figure S2
highest miR-1 levels during transient expression are lower than those during stable expression in batch mode, the downregulation of TWF1 mRNA is stronger during transient than during stable expression in batch mode.

4 | DISCUSSION

Various studies have been conducted to identify miRNAs that regulate productivity or cell growth in CHO cells and the term “engimiR” (Hackl et al., 2012) has been coined for miRNAs used as tools to improve recombinant protein production processes. However, most studies rely on transient miRNA mimic transfection, which has been shown to lead to unspecific gene regulation at high mimics concentrations. Only few studies have investigated effects under stable conditions during fed-batch cultivation, the current standard for biopharmaceutical production. Additionally, there is a low degree of standardization concerning amount of transfected mimics, media used for transfection and production, as well as the cultivation modes used. In order to generate a better understanding of miRNA-mediated effects in the context of transient testing and final application in bioprocesses, we investigated transient and stable miRNA overexpression in CHO DG44 production cells during batch and fed-batch mode using different media.

In a first step, two miRNAs, miR-30a and miR-1, were tested for specific target gene mRNA downregulation. MiR-30a has been experimentally validated to downregulate SKP2 mRNA in CHO and is annotated in miRTarBase. MiR-1 was shown to downregulate mouse TWF-1 in a luciferase reporter assay. MiR-1 as well as the miR-1 target mRNA sequence Twinfilin-1 are well conserved between hamster and mouse. Although experimentally validated, miR-30a did not downregulate SKP2 mRNA in the CHO cell lines and experimental setup used in this study. This discrepancy could be due to different expression levels of the target mRNA during cultivation, too low miR-30a levels achieved by transient transfection or more general due to different experimental conditions such as media or cultivation mode used in other studies.

When looking at the ability of mature miR-1 to functionally regulate its target TWF1, we could observe that miR-1 downregulated TWF1 mRNA in batch cultivation mode after transient transfection and during stable expression, although the downregulation was less prominent during stable expression. During stable expression in fed-batch mode, miR-1 not only failed to downregulate but significantly upregulated TWF1 mRNA. The main differences between these experimental setups include transient versus stable miR-1 expression and batch versus fed-batch cultivation in two different media.

When comparing transient versus stable miRNA expression, one must consider that during stable expression, miR-1 levels are constantly elevated over 14 days compared to 48 hr upon miRNA mimic transfection. During stable miR-1 overexpression TWF1 might be upregulated in order to compensate for elevated miR-1 levels. TWF1 is a protein that belongs to the actin-depolymerizing factor/cofilin superfamily and inhibits actin polymerization. No difference was
identified in TWF2 knockout mice compared to wildtype mice and thus, redundancy in the function of TWF1 and was suggested. This also indicates, that TWF1 is not crucial for cell survival or development. Consequently, the upregulation of TWF1 upon prolonged (stable) miR-1 overexpression seems not to be related to TWF1 function being required for cell survival.

Furthermore, a medium exchange and a cultivation in fed-batch mode resulted in TWF1 mRNA upregulation despite miR-1 expression levels similar to those observed in batch culture. Consequently, in-house medium 2 might contain inhibitors generally inhibiting miRNA processing or function or the fed-batch cultivation process influences miRNA function. To the authors’ knowledge, no studies have been conducted so far on media related effects on miRNA function. A general inhibition of miRNA processing or function related to the medium might be driven by potentially inhibiting substances. As mature miR-1 levels were elevated during all cultivation modes compared to control, these potential inhibitors have to impact miRNA processing downstream of Dicer, leaving Argonaute proteins 1–4, GW182, the subunits of the poly(A)-nuclease deadenylation complex (PAN2 and PAN3) and CCR4-NOT as potential targets. Inhibiting the loading of mature miRNA onto AGO proteins could be shown for trypaflavine, aurintricarboxylic acid, suramin, and oxidopamine and inhibition of Caf1, one of the catalytic domains of CCR4-NOT has been shown for purine-2,6-dione derivatives. However, none of these substances is contained in any of the used in-house media. In-house medium 2 is generally richer in amino acids than to in-house medium 1 in order to promote recombinant protein production. Comparing both media, none of the substances present in in-house medium 2 but absent in in-house medium 1 are directly related to inhibition of RISC formation or inhibition of Argonaute protein, which would have explained a general loss-of-function of miRNAs when cultivated in in-house medium 2. Focusing on fed-batch cultivation, miRNA expression patterns are highly dependent on the cultivation phase. A general change of miRNA expression levels could for example decrease incorporation of miR-1 to RISC when miRNAs with higher abundance are preferably incorporated in RISC. This would explain a miRNA independent loss-of function during fed-batch cultivation. On the other hand, the loss of miR-1-mediated TWF1 downregulation could also be miRNA-specific, as TWF1 is responsible for high actin turnover which is important for cell division and cells grow to higher cell densities in fed-batch mode.

Considering a more general approach, the reason for the lack of miRNA regulation during stable expression and a cultivation in fed-batch mode may be found in the natural function of miRNAs. On the one hand, miRNAs function as direct or indirect viral defense and as such, are involved in rapid regulation of highly expressed viral genes or host genes involved in viral defense as a response to infection. Integrating miRNAs in the toolbox for cell line engineering and using them for long-term overexpression in contrast to temporary upregulation as a reaction to high gene expression might explain the differences in TWF1 miRNA regulation during transient overexpression for 48 hr and stable overexpression for over 3 weeks. On the other hand, miRNAs balance mammalian gene expression to reduce expression noise. Disturbing this balance by long-term overexpression of a single miRNA at very high unnatural levels might induce regulatory mechanisms to restore the balanced state of miRNAs.

Further investigation could include evaluation of other miRNAs with known targets in stable batch and fed-batch mode, in order to elucidate whether cultivation in fed-batch mode and different media generally inhibits miRNA function or if this effect is miRNA-specific.

5 CONCLUSION

Various miRNAs have been tested for their effects on growth and productivity in a transient and/or stable environment in different cultivation modes with low experimental standardization between studies. We compared transient miRNA mimic transfection with stable miRNA overexpression in batch and fed-batch mode and found, that effects visible during transient expression are not as prominent as during stable batch cultivation in in-house medium 1 and not existent during stable fed-batch cultivation in in-house medium 2. This highlights the importance of the testing procedure for miRNA function evaluation and comparing results from different miRNA testing setups. In order to evaluate engimiRs, we state that a transient screen might be the first choice, as it is easy to perform and mature miRNA is directly available in the cell. However, relevant miRNAs found on transient level are not necessarily having the same effects on stable level or another cultivation mode or media, although the respective miRNA levels are the same. Therefore, a subsequent stable evaluation in a specified cultivation mode and medium is of value, in order to clarify miRNA-mediated downregulation of target genes in a stable environment. This is necessary for the application of engimiRs in biopharmaceutical production processes.

AUTHOR CONTRIBUTIONS

Ann-Cathrin Leroux: Conceptualization; data curation; formal analysis; project administration; writing-original draft; writing-review and editing. Elisabeth Bartels: Data curation; formal analysis; writing-review and editing. Luise Winter: Data curation; formal analysis; writing-original draft. Melanie Mann: Conceptualization; project administration; resources; writing-original draft; writing-review and editing. Christoph Zehe: Conceptualization; project administration; resources; supervision; writing-original draft.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

PEER REVIEW

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ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors. The authors confirm that
principles of ethical and professional conduct have been followed in this research and in the preparation of this article.

NOTATIONS

in-house medium 1  selective medium DHFR
in-house medium 2  production medium
RISC  RNA-induced silencing complex
TNFα  tumor necrosis factor alpha
TWF1  Twinfilin-1
VEGF  vascular endothelial growth factor

ORCID

Ann-Cathrin Leroux  https://orcid.org/0000-0002-1893-2848

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