Pharmaceutical BIOPROCESSING

Noncoding RNAs, post-transcriptional RNA operons and Chinese hamster ovary cells

Chinese hamster ovary cells are among the biotechnologically most relevant production systems for biopharmaceuticals. With the availability of the genomic sequences of Chinese hamster, rational systems biotechnology-driven approaches to optimize this cell factory have become available. Here, we review the current status of noncoding RNAs as members of the post-transcriptional operon concept in the context of Chinese hamster ovary cell line engineering and bioprocessing. In addition, we suggest that they already now keep their promises as tools for increasing specific productivity and thus time space yield of biopharmaceutical production, a feature that will allow for more cost efficient production processes in the future.

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Chinese hamster ovary cells

It can be considered remarkable that a non-human cell line is the most frequently used mammalian cell factory for the production of recombinant protein therapeutics. Concerns about merely 'human-like' post-translational protein modifications observed in Chinese hamster ovary (CHO) cells are outweighed by their ease of handling, the availability of metabolic mutants that enable antibiotic-free clone selection, resistance to human pathogens and consequently a long-standing regulatory track record as a safe biopharmaceutical production host. These advantages will likely warrant the future bioindustrial use of CHO cells in addition to the constant striving to overcome slow biomass and product accumulation compared with nonmammalian hosts [1], genome instability [2] and post-translational modifications negatively affecting product quality attributes [3]. Development of tools that allow to reach these aims strongly depends on the in-depth understanding of the molecular biology underlying CHO cell phenotype. Recent advances in massive parallel sequencing technologies have led to the rapid accumulation of sequenced CHO and Chinese hamster genomes as reference genomes [4–6] and transcriptomes, [7] which in their draft state revealed the urgent need for a universal reference genome for Chinese hamster [3]. The stage is now set to allow diving into the details of how the now familiar CHO genotypes are linked to specific phenotypes, and to use this know-how for rational design and product-quality-driven biopharmaceutical production processes.

Linking genotype to cell phenotype: the RNA operon concept

Essentially, the link between genotype and phenotype is gene expression, which is composed of two steps: transcription of DNA into RNA; and translation of RNA into proteins. This process of utilizing genomic information must, however, be performed in a highly controlled manner to ensure that cells adopt the 'right phenotype at the right time' by rapidly activating (or deactivating) certain functions in response to external or internal signals. Cells achieve this flexibility through the precise regulation of expression of whole sets of genes which together control specific cellular functions to maintain the biological balance [8].

In prokaryotic cells, RNA transcription and protein translation are tightly linked and often occur simultaneously. Therefore,

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Key terms

Noncoding RNA: The majority of transcribed RNAs in mammalian cells are not translated into protein and therefore termed as noncoding RNAs (ncRNAs). Such RNAs can further be subdivided into small noncoding RNAs (sncRNAs) predominantly ranging from 18–32 nt in length and long noncoding RNAs (lncRNAs) of more than 200 nucleotides.

Post-transcriptional RNA operon: A control mechanism by which higher eukaryotic cells can achieve coexpression of functionally related groups of monocistronic mRNAs. Noncoding RNAs (ncRNAs) play a key role in this process.

miRNA: SncRNAs that repress mRNA translation by binding and directing protein complexes to complementary regions in 3′UTRs.

PIWI-interacting RNA: The largest type of sncRNAs that function as epigenetic and post-transcriptional regulators of gene expression.

Long noncoding RNAs: LncRNAs are RNA transcripts, which are longer than 200 nt after the maturation and control gene expression transcriptional and post-transcriptional level.

prokaryotes have evolved into organizing functionally related genes in close genomic proximity, so-called gene clusters or operons, that are co-transcribed in a single, polycistronic mRNA (Figure 1A). Depending on the presence of activating factors, gene operons become expressed to give rise to functionally related proteins. One of the best studied, constantly revisited examples that is also used biotechnologically is the lac-operon, where all proteins required for the utilization of lactose are encoded in such a polycistronic RNA and therefore co-transcribed under the control of one promoter [9].

The higher complexity of eukaryotic and especially mammalian cells required the sequestration of chromosomal DNA from ribosomes by the nuclear envelope, resulting in the decoupling of mRNA transcription and translation [10]. Thereby, each mRNA 'is assumed to function as a free agent,' and can be selectively combined and coexpressed with other functionally related genes, which contributes to the higher complexity of eukaryotic systems (Figure 1B) [11]. However, the increase in complexity of organisms does not seem well reflected by the increase in the number of genes, if we consider 4500 genes to be present in *Escherichia coli*, and only around 4–5-times more (around 20,000–25,000) in humans [12].

In addition, it is unclear why such an efficient regulatory system like the operon should have been completely lost or neglected by evolution. Several attempts to explain the discrepancy of gene number and organismal complexity are currently available. One mechanism that clearly contributes to widely enhancing the amount of proteins to up to 100,000 is alternative splicing [13]. In addition, however, there is also increasing evidence that biological timing of translation of specific mRNAs that jointly function in a specific pathway is highly dependent on post-transcriptional regulation, which can be executed by RNA binding proteins (RBPs), noncoding RNAs (ncRNAs), and interactions between RBPs and ncRNAs [14]. These factors can influence mRNA sequences (via splicing) and cytoplasmic export, as well as activity and stability of transcribed RNA.

As a consequence, the theory of post-transcriptional RNA operons (PTROs) has been coined, which proposes that mammalian cells organize monocistronic mRNAs in functional groups based on specific sequence elements contained within untranslated regions (UTR) of mRNAs [15]. Sharing of these sequence elements allows mRNAs to become members of more than one RNA operon so that the encoded proteins can be produced coordinately in different combinations as functional groups via distinct UTR codes (Figure 1C).

Since single proteins can thus coordinately work in completely different functional units and pathways defined by sequence elements on their UTR of the mRNA, the translational context of a specific protein within one functional unit allows again to immensely increase the complexity of higher organisms.

Our understanding of such PTROs is at a very early stage. Still, it seems clear that specific sequence elements of mRNAs are recognized and post-transcriptionally regulated by mRNA binding proteins as well as by ncRNA species such as miRNAs. The knowledge that single miRNAs can target a large variety of mRNAs might suggest that they are a specifically valuable tool of the post-transcriptional operon concept, as they are able to regulate larger – potentially jointly functioning – sets of genes [16,17].

These miRNAs have been characterized in depth, so that applications of these molecules as diagnostic [18], therapeutic [19] or cell engineering tools have been accomplished [20,21]. Research with respect to other ncRNA species, such as PIWI-interacting RNAs (piRNAs) [22] that are likely contributing to post-transcriptional regulation of gene expression are, however, still in their infancy.

In the following, the aim of this review is to introduce the reader to the various types of ncRNAs that are part of the PTRO, their biosynthesis and mode of action, as well as methods of exploring their function and designing relevant tools for CHO cell culture technology.

Types of ncRNAs

The majority of transcribed RNAs in mammalian cells are ncRNAs [23,24]. Such RNAs can be

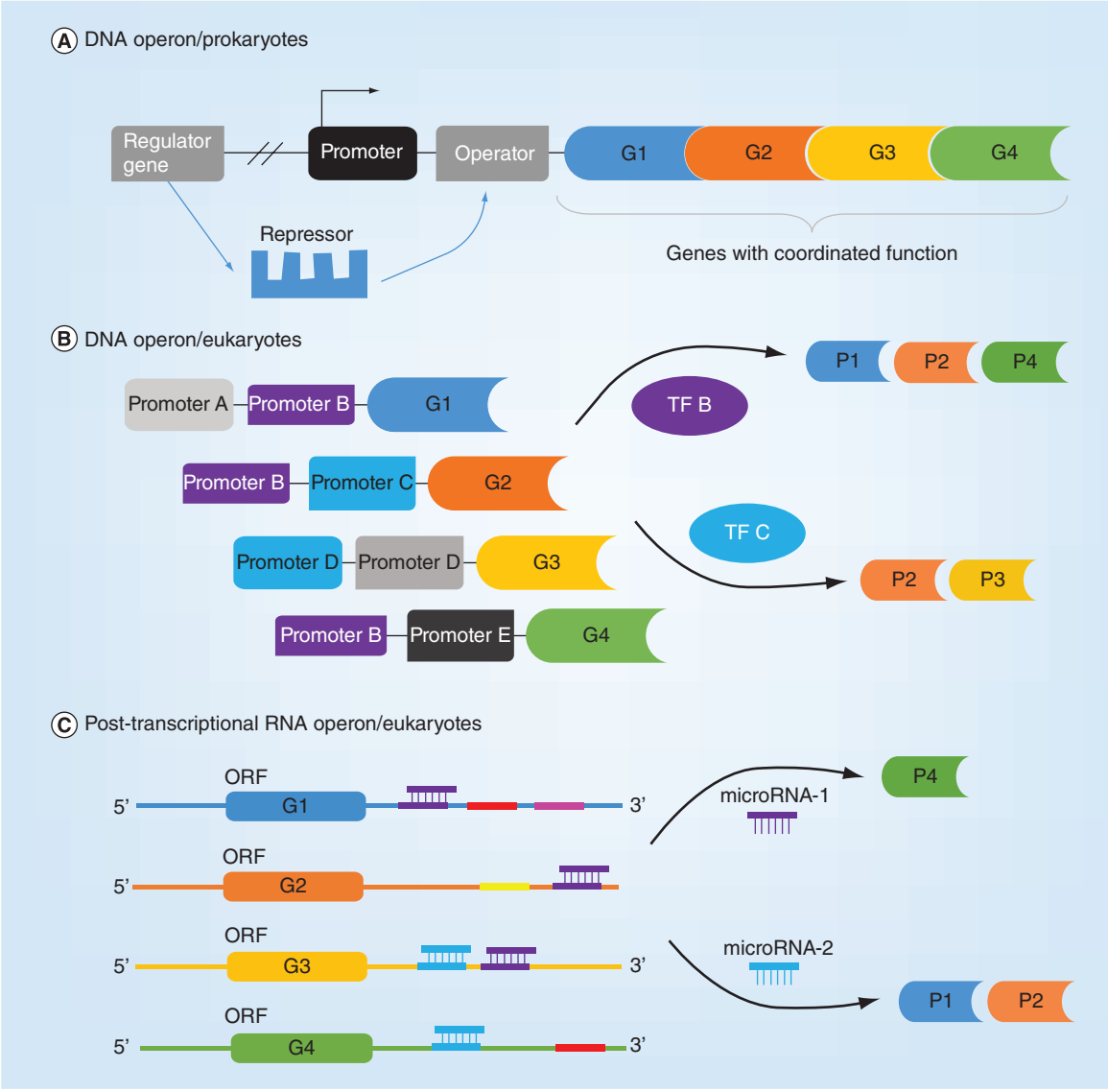

Figure 1. The operon concept for coordinated expression of functionally related genes. **(A)** DNA operon model in prokaryotes. A set of related genes labeled G1–G4 is located in physical proximity (polycistron). Co-transcription is regulated by promoter and operator elements in the DNA, which respond to the presence/absence of regulatory factors (e.g., 'repressors'). **(B)** DNA operon model in eukaryotes. Monocistronic genes that have a coordinated function are placed apart from each other in the genome. Coexpression of gene sets in response to environmental stimuli is achieved through shared promoter sites and transcription factor presence. **(C)** Post-transcriptional RNA operon model: transcribed mRNAs harbor regulatory sites in their 3′ untranslated regions. Genes with coordinated function share a specific RNA binding site; RNA stability and/or translation are regulated through hybridization of noncoding RNAs such as microRNAs.

further subdivided, based on the length of their mature construct, into small ncRNAs (sncRNAs) predominantly ranging from 18 to 32 nt in length and long noncoding RNAs (lncRNAs) of more than 200 nucleotides. A detailed overview on the biogenesis and function of regulatory ncRNAs in eukaryotic cells is illustrated in Figure 2. Within the sncRNAs, several classes have been defined such as small interfering RNAs (siRNAs), miRNAs and piRNAs, which are present in a wide range of higher eukaryotes [25]. A summary of ncRNAs exerting post-transcriptional gene regulation, which might be of interest for CHO cell engineering, is outlined in Table 1. In general, miRNAs act as regulators of endogenous genes, while siRNAs and piRNAs are considered predominantly to be defenders of genome integrity in response to invasive nucleic acids such as viruses, transgenes and transposons [26,27]. By now reports exist that piRNAs, which were originally identified in germ line cells, also occur in somatic cells and can silence gene expression [28,29].

While some sncRNAs already exert gene regulation on transcriptional level by silencing promoter DNA regions of coding genes and thereby blocking initiation of transcription [41], the majority of small regulatory RNAs act as post-transcriptional inhibitors of gene expression. These sncRNAs share several common mechanisms to provide highly specific inhibition of gene expression by binding to complementary mRNA sequences. The keystone of this silencing machinery is the RNA-induced silencing complex (RISC) comprising a core protein from the Argonaute (AGO) family and an sncRNA as guide for mRNA targets [42]. The sncRNA-loaded AGO complexes inhibit expression of their targets by either endonucleolytic cleavage or translational repression [27]. Despite their similar mode of action, the various classes of small RNAs arise from different biogenesis mechanisms. While miRNAs and siRNAs require cleavage of DICER prior to loading them onto AGO proteins, piRNAs are processed by DICER-independent mechanisms [43,44]. Even miRNAs and siRNAs diverge in their biogenesis mechanisms as siRNAs are excised from long fully complementary double-stranded RNAs (dsRNA), whereas miRNAs are processed from incomplete base-paired stem–loop structures [45]. In the following, we focus on the origin and biogenesis of the different small and lncRNAs.

Small-interfering RNAs

Since the first clear observation of RNA interference in *Caenorhabditis elegans* [46], post-transcriptional silencing of specific genes using siRNAs has become an established technology. Originating from exogenous sources like viruses [47], siRNAs can also be experimentally introduced as short hairpin RNA (shRNA) for silencing specific genes. Furthermore, siRNAs can derive from endogenous elements such as transposon transcripts, repetitive sequences, long stem–loop structures or sense–antisense transcripts [48–51]. siRNAs are 20–25 base pair long dsRNA molecules exhibiting complete sequence complementarity [52]. Exogenous dsRNAs are cleaved in the cytoplasm by DICER and loaded onto one of the four different AGO proteins [42,53], while precursor-derived and DICER-processed endogenous siRNA (endo-siRNA) is loaded onto an AGO2 protein (Figure 2C) [54]. Although single-stranded siRNAs can be directly loaded onto recombinant AGO2 proteins *in vitro* [55], dsRNAs processed by DICER into single-stranded RNAs require assembly of the entire siRNA-induced silencing complex [26]. The thermodynamic stability at the 5′-terminus of the dsRNA determines which strand will be favored as guide strand [45]. Despite the similarities with miRNAs regarding association with proteins from the AGO family, endo-siRNAs only depend on DICER activity but not on DROSHA [56,57]. Additionally, the selection of the different AGO proteins seems to be dependent on the precursor structure. Duplexes exhibiting mismatches, as in case of miRNAs, are preferably loaded onto AGO1 whereas perfectly base-paired duplexes are preferentially associated with AGO2 [54]. Notably, AGO2 represents the only protein of the AGO family having slicer activity [42]. This might be the reason why miRNAs predominantly induce translational inhibition in mammals, while artificial siRNA-mediated RNA interference leads to immediate cleavage and degradation of the target transcript [58]. In CHO cells, artificial siRNAs have been widely used for specific gene silencing and improving apoptosis resistance, glycosylation, metabolism and specific productivity [59–80].

MicroRNAs

First identified in 1993 as critical regulators of development in nematodes [81], miRNAs are known to play key roles in the coordination of almost every cellular process in eukaryotes, including proliferation, differentiation, apoptosis and development [82]. Furthermore, miRNAs are known to reorganize chromatin by elevating methylation of targeted mRNA promoters and inhibiting their expression [83]. Strikingly, most miRNAs are highly conserved among species [84,85], especially at the nucleotide positions 2–8, the so-called 'seed' sequence, which is supposed to be crucial for the recognition of the mRNA targets [42]. miRNAs with identical seed sequences are grouped into families [86]. However, miRNAs from the same seed family frequently can have different roles *in vivo*, increasing the challenge for a clear classification [87]. In mammals, roughly 50% of miRNA loci are in close proximity to other miRNAs [42] generating clusters which are transcribed from single polycistronic transcription units [88].

Prevailing transcription of miRNA genes is mediated from an RNA polymerase II into long primary transcripts (pri-miRNAs) containing a hairpin structure (Figure 2A) [89–91]. These transcripts are cleaved in the nucleus by the RNase III enzyme DROSHA and its cofactor DGCR8 (DiGeorge syndrome critical region 8) to produce a 60–80 nt long precursor miRNA (pre-miRNA) [92]. Exportin-5 (XPO5) transports the pre-miRNAs from the nucleus into the cytoplasm [93,94]. The RNase III enzyme DICER further processes the pre-miRNA into an 18–24 nt short RNA duplex intermediate consisting of a guide strand as well as a passenger strand, which in most cases is discarded [95–97]. The mature miRNA guide strand is then loaded onto an AGO protein of the miRNA-induced silencing

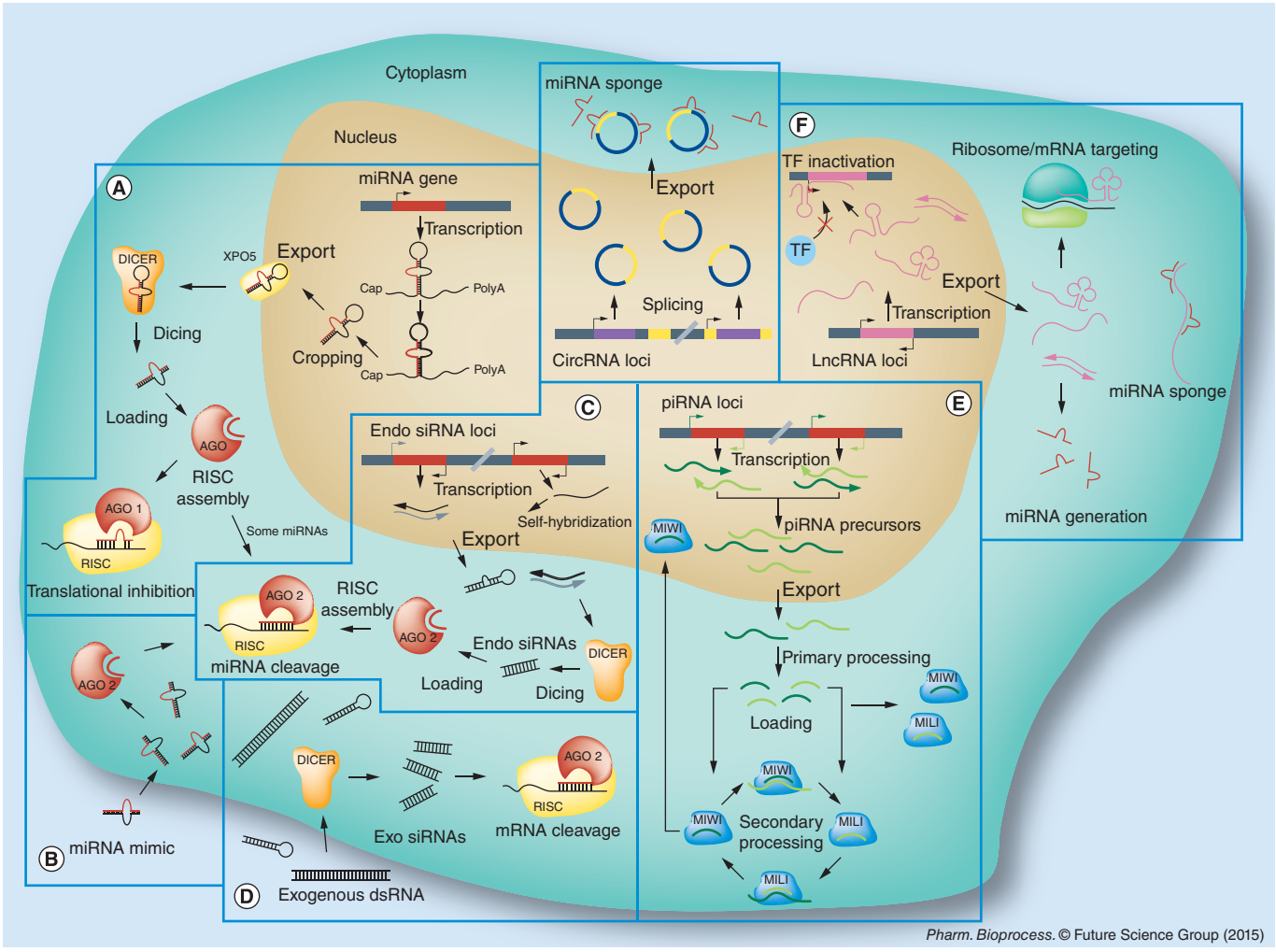

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Figure 2. Noncoding RNA biogenesis and function. (A) miRNAs are transcribed from RNA Pol II into long primary transcripts containing hairpin structures. The pri-miRNAs are processed in the nucleus by the DROSHA/DGCR8 complex to produce a 60–80 nt long pre-miRNAs. The pre-miRNAs are transported from the nucleus into the cytoplasm by XPO5. DICER removes the loop nucleotides from the pre-miRNA giving rise to a 18–24 nt short RNA duplex intermediate consisting of a guide strand and a passenger strand. The guide strand of the mature miRNA is loaded onto an AGO protein of the miRISC. The miRNA guides the miRISC complex to specific mRNA targets, where the miRNA binds the 3′UTR of the transcript leading to mRNA destabilization. **(B)** Exogenously introduced miRNA mimics are directly incorporated into the miRISC complex to bind to their target mRNAs. **(C)** Endo-siRNAs are transcribed from either transposon transcripts, repetitive sequences, long stem–loop structures or sense–antisense transcripts, and are exported into the cytoplasm. DICER is trimming the dsRNA and is involved in the loading of the single-stranded siRNA strand onto AGO2. The guide strand directs the siRISC to the mRNA target which is immediately cleaved by the endonucleolytic AGO2 domain. **(D)** Exogenously introduced siRNAs from viruses are also processed by DICER and loaded onto one of the four different AGO proteins of the siRISC complex. **(E)** piRNAs are processed from primary piRNA clusters or long precursor sequences. In contrast to siRNAs and miRNAs, piRNAs are DICER independent and after nuclear export, an amplification mechanism leads to an accumulation of the piRNAs in the cytoplasm. In mice, primary piRNAs associate with MILI which cleaves complementary transcripts. These so-called secondary transcripts then bind to MIWI which cleaves complementary transcripts, followed by binding to MILI again to accomplish the cycle. **(F)** lncRNAs are derived from promoter regions, intergenic regions, natural antisense transcripts, enhancer associated regions or pseudogenes. By preventing TFs to bind their genomic recognition sites lncRNAs can inhibit gene transcription. After export to the cytoplasm, lncRNAs are either binding to mRNAs and ribosomes or miRNAs. Furthermore, lncRNA can also be the source of miRNAs, which are excised from the lncRNA transcripts. **(G)** CircRNAs are generated by splicing of lariat introns or by back splicing circularization. CircRNAs usually exhibit multiple miRNA binding sites which serve as endogenous miRNA sponge molecules to regulate intracellular miRNA abundance.

AGO: Argonaute; CircRNA: Circular RNA; DGCR8: DiGeorge syndrome critical region 8; dsRNA: Double-stranded RNA; Endo-siRNA: Endogenous siRNA; lncRNA: Long noncoding RNA; miRISC: miRNA-induced silencing complex; piRNA: PIWI-interacting RNA; pre-miRNA: Precursor miRNA; RNA Pol II: RNA polymerase II; siRISC: siRNA-induced silencing complex; TF: Transcription factor; UTR: Untranslated region; XPO5: Exportin-5.

Table 1. Successful applications of engimiRs in Chinese hamster ovary cells.

miRNA name	miRNA function	Type of stable miRNA engineering	Putative target genes in CHO	Ref.
cgr-miR-7a	Antiproliferative and pro-productive	Inhibition	*STMN1*; *CAT*; *PSME3*; *RAD54L*; *SKP2*	[30–32]
cgr-miR-17	Pro-productive	Overexpression	*NCOA3*; *JAK1*; *BCL2*; *CCND1*; *CFL2*; *DDX5*	[33–35,36]
cgr-miR-466h	Pro-apoptotic	Inhibition	*BCL2L2*; *BIRC6*; *DAD1*; *STAT5a*; *SMO*	[37,38]
hsa-miR-557	Pro-proliferative	Overexpression	Unknown	[39]
hsa-miR-1278	Pro-productive	Overexpression	Unknown	[39]
cgr-miR-19b	Pro-productive	Overexpression	*HNRNPF*	[34,36]
cgr-miR-20a	Pro-productive	Overexpression	*CFL2*; *DDX5*	[34,36]
cgr-miR-17–92a cluster	Pro-productive	Overexpression	–	[34,35]
cgr-miR-30a	Pro-proliferative	Overexpression	Unknown	[40]
cgr-miR-30c	Pro-productive	Overexpression	Unknown	[40]
cgr-miR-30e	Pro-productive	Overexpression	Unknown	[40]

CHO: Chinese hamster ovary.

complex. The miRNA guides the complex to specific mRNA targets, where the miRNA imperfectly binds the 3′-untranslated region (3′UTR) of the transcript leading to its silencing [98–100]. This imperfect target recognition lowers target specificity of the miRNA and allows single miRNAs to fine-tune the expression of several dozens of mRNA targets and genes [30,58,101]. Targeting over 60% of mRNA transcripts [102] and regulating complex networks by evading an increase in translational burden makes them interesting targets for phenotype modification [103]. For *Cricetulus griseus* (*C. griseus*), 307 mature miRNAs and 200 precursor sequences are currently annotated in the latest miRBase version (release 21). However, when compared with human (2588 mature miRNAs) and mouse (1982 mature miRNA) this indicates that the number of mature miRNAs in *C. griseus* is likely to be underestimated, mainly due to the experimental limitation to CHO cell lines during the identification process [104,105]. This implies considerable potential for the discovery of novel miRNA sequences in this biopharmaceutical relevant expression host in the future. Still, already now, different strategies for exploiting miRNAs for CHO cell line engineering were applied such as transient transfection of a human miRNA mimics library [39] or the utilization of chimeric hairpin vectors for miRNA overexpression [33]. However, until the recent publication of the hamster genome [4–6], the lack of genomic sequence information substantially hindered miRNA research in CHO cells [106] as chimeric vectors were shown to be outperformed by vectors encoding endogenous miRNA sequences from *C. griseus* [40,107]. Despite these hurdles, the application of different miRNAs successfully improved process relevant parameters such as growth, specific productivity, apoptosis and stress resistance.

Key terms

miRNA mimic: Synthetic small RNA molecules that are delivered to biological systems to mimic the activity of specific mature miRNA species.

miRNA sponge: RNA transcripts that harbor several preferential binding sites for mature miRNAs, thereby preventing the binding of miRNAs to endogenous mRNA targets and reducing their biological activity.

PIWI-interacting RNAs

piRNAs are 24–32 nt long single-stranded sncRNAs. First discovered in small RNA profiling studies in *Drosophila melanogaster* (*D. melanogaster*) development [108], piRNAs were later also found in mammals through immunoprecipitation of the PIWI protein from testis and subsequent sequencing of the attached RNAs [109,110]. The best known function of piRNAs is the silencing of mobile transposon elements in the germ line [27]. These elements threaten the genomic integrity by moving through insertion or transposition to new sites and thereby disrupting the host genome [111]. Enriched in the germ line, there are also indications that piRNA function reaches beyond silencing of transposable elements. In *D. melanogaster*, Fasciclin 4 (FAS3) is a target of PIWI-associated piRNAs and is

important for the intermingling of the germ line and somatic cells in ovaries [28]. Several studies also reveal their involvement in epigenetic regulation [29] where MILI (also known as PIWIL2) and MIWI (also known as PIWIl4) from knockout mice indicate that PIWI homologs have similar function in heterochromatin control in both mice and flies [112]. For their biogenesis, two different pathways are proposed: the primary transcription pathway and an amplification mechanism (Figure 2E). In contrast to miRNAs and endo-siRNAs, piRNAs do not require DICER for their processing [43]. piRNAs possess a high degree of diversity with hundreds of thousands of individual sequences mapping to relatively small numbers of genomic loci called piRNA clusters. piRNA clusters have neither phasing nor overlapping sequences [109–110,113]. From these locations they are either transcribed directionally [113–115], directly from piRNA clusters [113–114,116] or from long precursor sequences as single strands without significant secondary structures [27]. Transcriptional tendency might be species specific [117]. The different processing steps generating mature piRNAs still remain unclear. However, in mice, the mature piRNA has been shown to be loaded onto MIWI proteins [110,114]. There are also reports that piRNAs can form piRNA-induced silencing complexes with PIWI proteins, which recognize and cleave complementary RNA targets [27,118]. After piRNA export from the nucleus, a ping-pong- amplification mechanism leads to an accumulation of piRNAs in the cytoplasm [113]. Originally proposed in *D. melanogaster,* this ping-pong mechanism also applies to mouse prepachytene piRNAs [115]. In mice, the primary piRNA is associated with MILI which cleaves complementary transcripts. These so-called secondary transcripts then bind to MIWI, which again cleaves complementary transcripts that can bind to MILI, thus completing the cycle [113].

Recently, piRNA sequences and expression were characterized in six different CHO cell lines [22]. Computational analysis using pro-TRAC and the CHO genome resulted in the identification of more than 25,000 individual piRNAs in 540 piRNA clusters. The functions of piRNAs and their high abundance in CHO cell lines indicate their potential application in cell line engineering, even though experimental evidence for their effects in CHO cells is still missing.

Long noncoding RNAs

lncRNAs are RNA transcripts, which are longer than 200 nt after the maturation step (Figure 2F) [119,120] and are involved in many levels of gene regulation such as transcription by preventing binding of transcription factors, translation by binding to mRNA and ribosomes, chromatin remodeling, splicing and protein stability [121–124]. They have a cell-specific expression pattern [125] and subcellular distribution [126]. Promoter regions, intergenic regions, natural antisense transcripts, enhancer-associated regions or pseudogenes are considered as sources for transcription [122,127–128]. Similar to mRNAs, post-transcriptional processing steps such as 5′-capping, RNA editing, polyadenylation and alternative splicing are present during lncRNA maturation [23,129]. lncRNAs have also been associated with a variety of diseases such as cancer [130–132]. Expression levels of the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), for instance, are elevated in several cancer types and increased cell proliferation was observed when MALAT1 is overexpressed [133]. Moreover, MALAT1 is known as a prognostic biomarker for lung cancer and has critical regulatory functions in lung cancer metastasis [134]. Recent experiments including MALAT1 showed that not all lncRNAs possess a poly(A) tail to protect for degradation [135]. Instead, a triple-helical secondary structure at the 3′-end of nonpolyadenylated MALAT1 prevents degradation of this lncRNA.

Recently, regulation of miRNA activity has been demonstrated for a particular lncRNA entity in eukaryotic cells termed competing endogenous RNAs (ceRNAs) [136]. ceRNAs harbor miRNA response elements which are complementary to specific miRNAs thus competing with miRNA binding sites in the 3′-UTR of target mRNAs (Figure 2F). This feature enables to precisely modulate the abundance of certain miRNAs in the cell [136]. However, these sponges seem to be expressed only at basal levels, often contain low numbers of miRNA targets sites and are prone to miRNA-mediated destabilization [137–139]. Moreover, the question if ceRNAs are also expressed by CHO cells still remains to be elucidated. Further subdivision of lncRNA classes is difficult as many lncRNAs are associated with different regulatory pathways and many functions still seem to be unknown [140].

Circular RNAs

Very recent studies discovered another interesting and novel class of ncRNAs capable of regulating miRNA activity in the cell. First shown to encode subviral agents in plants [141], circular RNA (circRNA) was generally dismissed for years as an experimental artifact or genetic accident [142]. However, computational evidence from mammals indicates that circRNAs are more abundant than previously anticipated with thousands of circRNAs present in human and mouse tissues [143,144]. circRNAs are considered as a large class of endogenous RNAs acting as post-transcriptional regulators due to their involvement in the regulation of miRNA abundance by functioning as miRNA sponges (Figure 2G) [144,145]. In comparison to linear

Key terms

engimiRs: Prefix for microRNAs that can be used for cell line engineering to improve traits such as growth rate or cell-specific protein productivity ('engineering miRNAs').

Anti-miRs: Synthetic small RNA molecules with or without chemical modifications, which are designed to hybridize to endogenous miRNA sequences, thereby reducing their biological activity.

ceRNA sponges, circRNAs possess ten-times higher miRNA binding capacity than any reported ceRNA transcript [144]. Due to their circular nature they possess no accessible termini, rendering them resistant to miRNA-mediated RNA destabilization [146]. Owing to these properties circRNAs are interesting tools for the control of endogenous miRNA expression levels *in vivo*.

Cell engineering using ncRNAs

Driven by an increasing demand for more efficient animal cell-based expression systems for recombinant proteins, novel genetic tools have entered the field of cell engineering. Among these new technologies, non-coding regulatory RNAs such as siRNAs and miRNAs have emerged as smart instruments to control gene expression [147,148]. MicroRNAs do not add to the translational burden of producer cell lines, and as they might act as controller elements of post-transcriptional operons, they might help to shift the overall cell behavior by targeting multiple, synergistic pathways. Furthermore, a huge variety of other ncRNAs like piRNAs, lncRNAs or circRNAs that certainly play a substantial role in the cell's fate exist [22,29,145,149], and might, therefore, be of special interest for biotechnological applications in the future.

miRNA expression profiling in CHO cells

One bioprocessing strategy that seems ideal involves a short growth phase characterized by high proliferation rates in the beginning followed by a nongrowing highly efficient production phase, which is maintained for as long as possible in order to achieve high total product yield [150,151]. One frequently applied approach to achieve these goals is to reduce temperature in a biphasic bioprocess. Shifting the temperature at the end of the exponential growth phase expands the longevity of the cells in bioreactors and ultimately increases final product yields [152,153]. Evaluation of the positive effect of a temperature by establishing miRNA profiles on a cross-species microarray revealed miR-21 and miR-24 to be upregulated during a biphasic process as well as in the stationary phase of a standard batch cultivation [154]. In another study, differentially expressed miRNAs were investigated 24 h after a temperature shift [31]. Here, six miRNAs (miR-219, miR-518d, miR-126, miR-30e, miR-489 and miR-345) were identified to be significantly up and four to be down regulated (miR-7, miR-320, miR-101 and miR-199). In a combined approach to monitor both mRNA and miRNA expression pattern of CHO-K1 suspension batch cultures, more than 1400 mRNAs and 100 miRNA were found to be differentially expressed comparing lag, exponential and stationary growth phase [155]. These results underscore the highly dynamic nature of a cell's mRNA and miRNA transcriptome and have identified several targets that might be able to mimic the temperature shift effects on growth versus productivity [31].

To prolong cultivation time and to adapt cells to the stress environment of a bioreactor, inhibition of apoptosis is a common approach for cell line engineering and an interesting topic for miRNA research [156]. Cross-species microarray studies between CHO cells cultivated in fresh or nutrient-depleted media showed an upregulation of the miR-297–669 cluster during apoptotic cell death [37]. Of the 28 miRNA members of the miR-297–669 cluster, 18 were found to be upregulated in depleted culture media [37]. In mice, the miR-297–669 cluster is located in intron 10 of the *Sfmbt2* gene on chromosome 2. In CHO cells, differential miRNA expression of members of the miR-297–669 cluster was confirmed by qRT-PCR for miR-466h and miR-669c. However, localizing of the miR-297–669 cluster onto the chromosomal locus within the CHO genome is not yet possible as genomic sequence annotations are not yet fully accomplished, but will certainly be available in the near future.

By comparing low and high-producing CHO cell lines, several miRNAs have been identified to be potentially involved in production and secretion of different recombinant proteins [157,158]. In microarray screenings comparing parental CHO-DG44 with IgG-producing cells, miR-221 and miR-222 were detected to be significantly downregulated in IgG-producing cells [158]. As the transcriptome and the miRNA profiles of a cell are changing in response to progressive nutrient consumption and the accumulation of metabolites [155], miRNA profiles of non, low and high-producing CHO cells were investigated during a steady-state cultivation [157]. In addition to 83 differentially expressed miRNAs, the authors observed that producer cells generally exhibit higher miRNA expression levels than nonproducing cells indicative of a critical role of miRNAs in recombinant protein production.

miRNAs as tools for CHO cell engineering

As described above, miRNAs play a critical role in CHO cell behavior and thus are obvious candidates for state-of-the-art genetic engineering to improve

of CHO production cell lines [156,159]. The term 'engimiR' describes a miRNA that is manipulated to improve bioprocess relevant cellular characteristics of mammalian manufacturing cell lines, turning it into a valuable instrument for cell engineering [21,160]. Indeed, there are multitudes of conceivable cell functions, which can be exploited by engimiRs such as the protein production machinery, secretory pathway, cell cycle, metabolism or cell death represented by apoptosis, necrosis or autophagy [156,161–165]. A comprehensive list of successful engimiR applications for CHO cell engineering is summarized in Table 2.

The outcome of the above-mentioned studies has already provided a preliminary list of potential engimiRs that might have beneficial influence on CHO cell behavior. However, reported data sets from profiling studies first had to be functionally validated to confirm that these miRNAs will actually confer the expected phenotype. The fact that the expression of a miRNA changes under certain culture conditions could be causative for the changes behavior or it could be a consequence. In the latter case, manipulating the expression of the miRNA would probably not have the desired effect. In addition, given the dynamics of gene expression in cells, the impact of miRNAs is always depending on the presence and the pattern of target mRNAs available under a given condition. Thus, overexpressing a miRNA that has a certain effect on cell phenotype when present in the stationary growth phase might have a different or no effect during exponential growth. The precise effect of each stably manipulated engimiR therefore needs to be individually verified.

Depending on the expected mode of action, miRNA expression can be either enforced or diminished resulting in an increased or decreased target gene repression, respectively. Transiently elevated miRNA expression levels can be facilitated by introducing small RNA duplexes, called miRNA mimics (Figure 3A), into cultured mammalian cells, thereby imitating the endogenous miRNA function [169,170]. In this context, miR-7a was discovered to be downregulated upon temperature downshift in CHO cells, and unexpectedly, ectopic overexpression of miR-7a-5p led to a decrease in cell growth, but increased specific recombinant protein productivity [31]. Similar controversial observations were made by Loh and colleagues for the miR-17–92a cluster which was found to be downregulated in high-producing CHO cells compared with low-producing clones [34]. Nevertheless, stable overexpression of selected members of the miR-17–92a cluster, but not their inhibition as expected, enhanced cell-specific IgG productivity, whereas combined enforced expression of all members of the miR-17–92a cluster did

Table 2. Successful applications of small interfering RNAs in Chinese hamster ovary cells.

Targeted pathway	siRNA target gene	Engineered phenotype	Ref.
Cytoskeleton	*CFL1*	65% (SEAP) and 47% (tPA) increase in specific productivity	[63]
Cell cycle	*ATR*	Fourfold increase in specific IgG productivity and threefold improved IgG titer	[166]
Metabolism	*LDHA*	45–79% reduced lactate concentrations and diminished glucose consumption	[68]
	PDHK	68–90% increase in IgG titer	[80]
Apoptosis	*Caspase 3* and *7*	Enhanced cell viability and 55% increase in hTPO titer	[60]
	ALG2, REQ, FAIM	Elevated cell density and culture longevity; 1.2–2.5-fold increase in IFN-γ titer	[76]
	Bax and *Bak*	Enhanced cell viability and 35% increase in IFN-γ titer	[59]
Protein expression	*DHFR*	>100% increase in specific IgG productivity and 30% improved stability of transgene expression	[64]
Glycosylation	*NEU3*	60% decrease in sialidase activity led to increased sialic acid content in IFN-γ	[72]
	NEU1 and *NEU3*	98% decrease in sialidase activity led to 26–33% increase in sialic acid content of IFN-γ	[79]
	FUT8	Reduction in core fucose by 60–88% resulted in 100-fold improved ADCC of the produced IgG	[61,70]
	GMD	Production of 100% defucosylated recombinant antibodies if culture medium lacks L-fucose	[66]
	FUT8 and GMD	Production of fully nonfucosylated antibodies with improved ADCC	[168]

not improve culture performance [34]. These data were also in line with findings by Jadhav *et al.* who discovered that miR-17–5p represents an engimiR as this miRNA increased recombinant protein expression in CHO cells by about threefold [35].

In contrast to miRNA overexpression, short-term inhibition of cellular miRNAs can be achieved using specific antisense oligonucleotides, so-called antagomiRs or miRNA inhibitors (Figure 3B), which are often chemically modified to increase half-life [171–173]. AntagomiRs directly bind to their target miRNA leading to the formation of RNA duplexes which are finally degraded in the cell [174]. While Barron and coworkers could not observe any reversed effects after transient

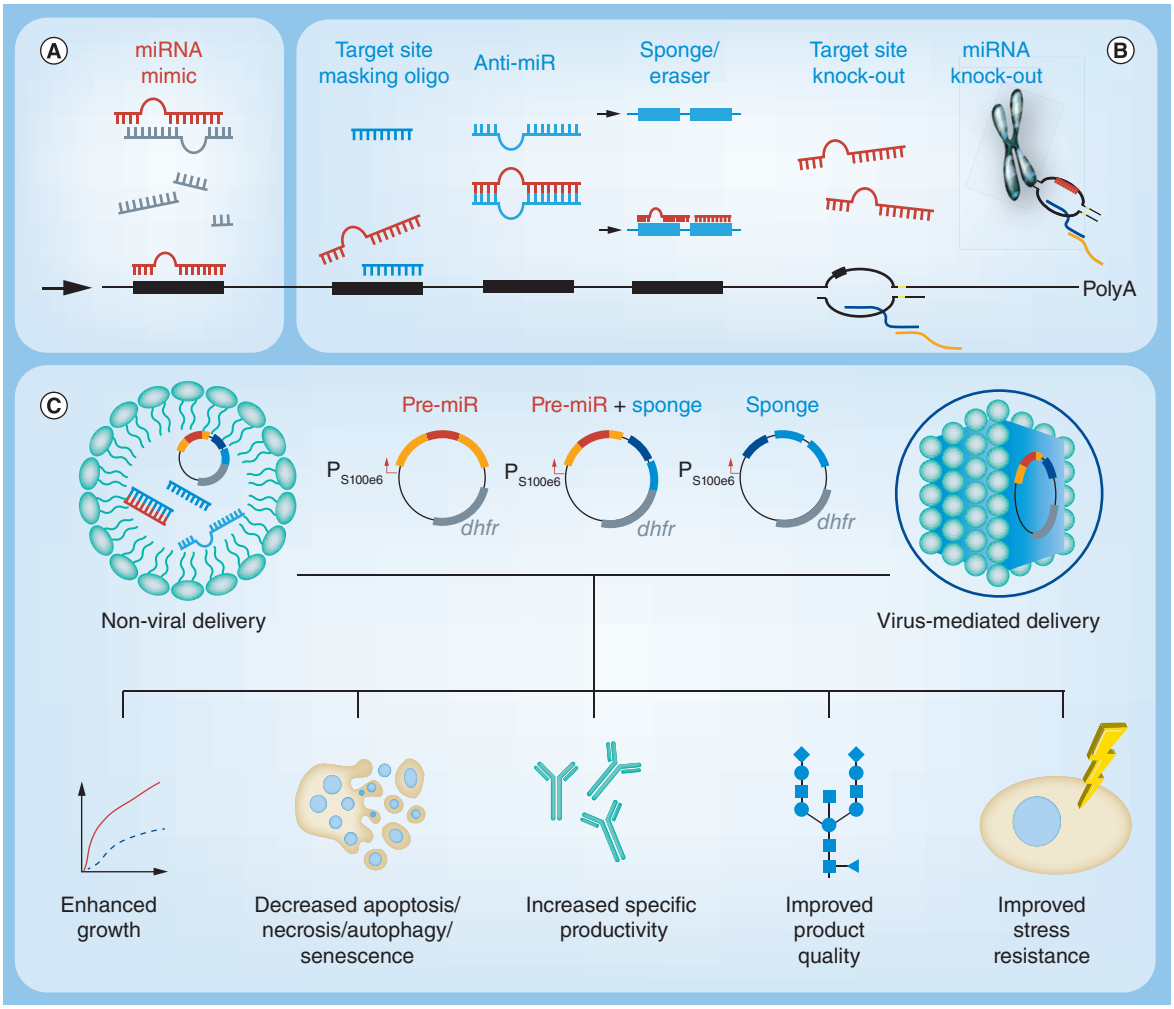

Figure 3. Modulation of miRNA expression or function in cell engineering. (A) miRNA mimics are small double-stranded RNA molecules that can be directly introduced into the cytoplasm of mammalian cells to transiently increase the cellular abundance of a miRNA. miRNA mimics are chemically modified such that only one of the two RNA strands is specifically loaded into the miRISC complex to post-transcriptionally regulate the endogenous target mRNAs. **(B)** miRNA function can be experimentally modulated by different modes of action: to transiently prevent a specific gene from being regulated by miRNAs, short single-stranded RNA oligonucleotides which are complementary to the target mRNA and thereby blocking the binding site (target site masking oligo), can be delivered to inhibit miRNA target recognition. For transient sequestration of a given miRNA, single-stranded antisense oligonucleotides (antagomiRs or anti-miRs) or miRNA sponge/eraser molecules can be introduced into the cell to inhibit the targeted miRNA. Genomic deletion of the miRNA binding site in the 3′UTR of a target mRNA can be used to stably prevent the regulation of a single gene by a specific miRNA. In contrast, genomic removal of a pre-miRNA sequence leads to stable loss of miRNA function and to dysegulation of several target genes. **(C)** Long-term ectopic overexpression or loss of miRNA function can be facilitated by stable genomic integration or knockdown/out of pre-miRNA genes. In this conjunction, intracellular transfer of miRNA expression or sponge/decoy vectors is usually enabled by either nonviral or virus-mediated delivery systems followed by a selection process to obtain stable transfectants.
miRISC: miRNA-induced silencing complex; pre-miRNA: Precursor miRNA; UTR: Untranslated region.

inhibition of miR-7a-5p, stable inhibition resulted in an increase in both culture longevity and recombinant secreted alkaline phosphatase (SEAP) yields in CHO cells [32]. Stable repression of endogenous miRNAs with negative effects on CHO cell behavior thus represents another way to take advantage of engimiRs in cell engineering. Besides miRNA sponge or decoy molecules, which are reporter genes such as the green fluorescent protein harboring multiple miRNA binding sites within their 3′UTR [175], there are also other possibilities to suppress cellular abundance of a given miRNA (Figure 3B) [176]. Druz *et al.* stably suppressed miR-466h, a proapoptotic miRNA in CHO cells by shRNA-mediated knockdown of pre-miR-466h, resulting in prolonged cultivation periods and a 53.8% increased IVC in recombinant CHO–SEAP cells due to delayed onset of caspase 3/7 activation [37,38]. The authors illustrated that the knockdown of pre-miR-466h led to an upregulation of antiapoptotic genes *bcl2l2*, *dad1*, *birc6*, *stat5a*, *smo* which are supposed to be targets of miR-466h-5p according to bioinformatics target prediction tools [37,38].

An alternative strategy for stable miRNA knockout represents genome editing using the CRISPR/Cas9 system [177–179]. Toward this end, pre-miRNA sequences of disadvantageous engimiRs can be specifically excised from genomic DNA of CHO production cells, presumably leading to improved phenotypes as miRNA activity will be completely lost (Figure 3B). The recently published 'CRISPy' tool, which represents a novel bioinformatics database that can be used to identify small guide RNA target sequences in CHO cells necessary for CRISPR/Cas9-mediated genome editing [180], has the potential to substantially drive miRNA knockout studies in biotechnological context.

A broader functional investigation on miRNAs capable of increasing the yield of an antibody-producing CHO cell line was reported by Strotbek *et al.* [181]. Upon screening of an entire human miRNA mimics library, they discovered that two human miRNAs (hsa-miR-557 and hsa-miR-1287) lacking a homolog in CHO positively impact cell growth and specific IgG production in CHO cells. Although the application of high-throughput miRNA screenings is promising techniques for elucidating miRNA function [182], to gain deeper insights into the complex nature of miRNA-mediated gene regulation, screening protocols should be geared to a multifaceted cellular readout [40]. This is supported by the fact that common concepts of miRNA function generally comprise: the cumulative reduction in expression of several components of a cellular pathway mediated by a single miRNA; the cooperative function of different miRNAs targeting various essential effector genes of the same regulatory network; and the fact that a single miRNA can maintain a cellular balance by fine-tuning the expression of both activators and inhibitors of a functional regulator simultaneously [183–186]. It is therefore fundamental to comprehensively analyze miRNA function in CHO cells and not only the impact of single miRNAs on a particular phenotype. The widespread regulation of crucial cellular pathways by miRNAs in mammalian cells was recently unveiled by a functional, genome-wide, multiparametric miRNA screen in recombinant CHO–SEAP cells [40]. The authors discovered an unexpectedly large number of engimiRs substantially improving bioprocess relevant cellular characteristics such as cell proliferation, recombinant protein production or cell death (represented by apoptosis and necrosis). Interestingly, this comprehensive screen revealed a whole 'engimiR family', the miR-30 miRNA family, to substantially increase recombinant protein yields by enhancing either growth or cell-specific productivity [40]. Taken together all these data indicate that engimiRs are indeed attractive tools for next-generation cell engineering strategies of CHO production cells in the post genomic era.

Stable overexpression of miRNAs represents a valuable tool to improve CHO cell phenotypes and there are several technical methods to facilitate stable miRNA expression in CHO cells (Figure 3C). Since miRNAs are transcribed as precursor (pre-)miRNAs, consisting of a stem–loop structure which is subsequently processed by the endogenous RNase III enzyme Dicer, these sequences are used for the construction of expression vectors, where the miRNA coding sequence is cloned into the 5′ or 3′-UTR of a reporter gene (e.g., green fluorescent protein or antibiotic resistance). Importantly, the generated pre-miR must exhibit several key features allowing for correct binding and processing by Dicer [187,188], and exact loop sequences which are supposed to critically influence miRNA functionality [189]. Before the publication of the CHO genome, plasmid-based overexpression was accomplished by piecing together the sequences of mature CHO miRNAs and ectopic flanking and loop sequences from mouse ('artificial chimeric miRNA constructs') [33,181]. However, these artificial constructs were shown to perform inferior to native constructs, which use amplified and subcloned endogenous pri-miRNAs based on the CHO genome sequence [190], and were successfully applied for overexpression studies [34,40].

Additional currently available methods for miRNA overexpression are viral vector approaches such as adeno-associated viral (AAV) (Figure 3C), retroviral or adenoviral vectors [191]. AAV vectors emerged as one of the most studied vectors for gene therapy [192], and

have been successfully used to transduce a variety of genes into a number of cell types *in vitro* as well as *in vivo* [193–195]. However, there are no studies available which have evaluated the compatibility of AAV vectors and CHO cells regarding transduction efficiency and the ability to confer stable transgene expression. Although lentivirus-mediated miRNA overexpression has been widely applied in conjunction with human or murine cell lines [196–198], no miRNA overexpression has yet been reported for CHO cells employing viral vectors. This might be due to the inherent resistance of CHO cells to viral infection [199], or the requirement for more restrictive laboratory safety levels when working with recombinant viruses and the unsuitability of using viral vectors in cell lines to be used for production of safe human therapeutics. Nonetheless, the ability of viral vectors to mediate persistent miRNA (over) expression in CHO cells as well as the rapid generation of stable cell lines may promote their application in the future as a valuable alternative to traditional plasmid-based miRNA introduction, at least for the purpose of high-throughput screening experiments.

Besides the cellular production of therapeutic proteins, there will be alternative biopharmaceutical formats, which might be enhanced by the use of engimiRs. Preclinical as well as clinical grade manufacturing of viral vectors for gene therapy, oncolytic viral therapy or vaccination, using, for eample, AAV, adeno or lentiviral vectors, is preferably accomplished by stable cell lines, but also includes transient production processes [200–203]. Among many others, the predominantly applied cell types comprise HeLa, BHK, Vero or HEK293 cells as well as the baculovirus/SF9 expression system [204–209]. These production platforms might profit from the stable expression of engimiRs, which would raise virus titers by increased vector replication, improved growth characteristics of the host cell or enhanced protein production capacity. Furthermore, host cell defense mechanisms, which can hinder efficient viral vector production, might be attenuated by engimiRs [210–213]. However, this will require the identification and verification of appropriate engimiRs for each of the specific production host cell systems.

Cell engineering using miRNAs might not only be restricted to the utilization of the small RNA molecule itself. The application of engimiRs improving CHO production cells may be further promoted by modulating the endogenous miRNA biogenesis machinery in these cells such that an engineered cell is able to increase cellular engimiR abundance more efficiently. Expression of crucial processing or cargo proteins could be enforced to accelerate intracellular miRNA maturation. In this conjunction, most promising candidates are, for example, DROSHA, DGCR8, XPO5, DICER, TRBP, AGO1–4 or GW182, representing key mediators for the generation and function of the single-stranded mature miRNAs [86]. Hackl and coworkers reported that the expression level of DICER in CHO cells correlates to an increase in overall expression levels of mature miRNAs which finally resulted in an increased cell growth [214]. The authors suggested that increased levels of DICER proteins allowed for more rapid maturation of endogenous miRNAs which had beneficial influence on cell proliferation [214]. Of note, induction of NDP52-mediated selective autophagy has been demonstrated to be causative for targeting DICER and AGO2 for degradation, thus regulating miRNA activity in the cell [215]. This underscores the interconnected nature of cellular processes and miRNA biogenesis to ensure a homeostasis in the cell. Although overexpressing particular effector proteins of the canonical miRNA biogenesis pathway will further increase translational burden of the cell, this example nicely illustrates the potential of engineering the endogenous miRNA biogenesis in CHO production cells. Furthermore, the combined overexpression of such proteins and engimiRs might be an attractive approach for further achievements in the field of miRNA-mediated cell engineering in the near future.

Potential of other ncRNAs for cell engineering

Since their discovery as a novel class of sncRNAs influencing germline development and function [216], piRNAs have further been shown to be critically involved in epigenetic gene regulation by mediating histone modifications and DNA methylation [217]. PIWI proteins have been demonstrated to be functionally associated with HP1a at the piRNA target site and thereby mediating methylation of histone H3 lysine9 in *D. melanogaster* ovarian somatic cells [218–220]. Moreover, piRNAs are supposed to direct DNA methylation on both transposon and nontransposon loci in mammals, albeit the exact mechanisms are still to be elucidated [115,221]. In this conjunction, acetylation is another key epigenetic modification and alterations in histone acetylation, for example, by inhibition of histone deacetylases have already been connected to improved protein production in CHO cells [222,223]. Yet, the question remains whether certain piRNAs can actually affect histone acetylation legitimating their more detailed examination. However, these examples, together with the fact that piRNA function appears to be evolutionarily well conserved, illustrate that piRNAs might indeed be valuable alternatives to current strategies where other sncRNAs are applied for cell engineering. As mentioned above, the presence of piRNAs in CHO cells has already been confirmed by Gerstl and colleagues who successfully identified

>25,000 piRNA species, and additionally presented a number of differentially expressed piRNAs in different CHO cell lines [22]. Of note, considering that the number of piRNAs per cell can include up to 100,000 different species [29], it is needless to mention that the large number of piRNAs will significantly increase the difficulty to identify piRNAs suitable for cell engineering and to control their expression precisely to enable fine-tuned changes in phenotype.

For many years the prevailing opinion about lncRNAs had been that lncRNAs are rare and only few functional transcripts possess relevant activity in the cell [149]. However, rising numbers of recently published literature examining biological functions of lncRNAs enabled deeper insights into this exciting class of ncRNAs. The broad functionality of lncRNAs comprising gene transcription control, regulation of alternative splicing, nuclear organization, epigenetic gene silencing, chromatin modification and modulation of miRNA expression might attract researchers to explore the potential of lncRNAs for CHO cell engineering [126,138,224–226]. The first hint of a successful implication of lncRNAs for CHO cell line optimization was the stable overexpression of a lncRNA that transcriptionally silences the NF-κB inhibitor α (NFKBIA) [227]. NFKBIA inactivates NF-κB, which is a positive regulator of cell growth [228,229], thus stable inhibition of NFKBIA using the lncRNA improved culture performance of an antibody-producing CHO cell line finally resulting in enhanced product titers [227]. This example demonstrates that it might be worth to further investigate this exciting class of ncRNAs in CHO cells. Moreover, the phenomenon that lncRNAs can also bind to and therefore regulate cellular miRNA expression – so-called ceRNAs – adds another level of complexity onto ncRNA-mediated regulatory networks, but also provides new opportunities for tailored cell engineering strategies. In this context, endogenous miRNA 'sponge' molecules represented by circRNAs [145], which modulate cytosolic abundance of particular miRNAs, is another smart cellular tool to fine-tune post-transcriptional gene silencing. No circRNAs have been identified in CHO cells yet, but re-analysis of existing NGS data sets from CHO transcriptomics may presumably enable the discovery of these endogenous miRNA regulators in CHO cells and their exploitation for cell line engineering in the future. The recently identified circRNA ciRS-7, which targets miR-7 by more than 70 complementary binding sites [146], speculatively has high potential to be applicable in CHO cells for stable attenuation of miR-7 expression increasing culture longevity and thus recombinant protein yields [32]. The advantages of circRNAs over ceRNAs or classical artificial miRNA 'sponge' constructs would be that circRNAs lack accessible termini, rendering them resistant to miRNA-mediated RNA destabilization and exonucleolytic decay [146].

Conclusion & future perspective

Research on ncRNAs is currently boosted by the availability of next-generation sequencing methods and new and surprising classes of ncRNAs have been identified over the last few years. When looking at next-generation sequencing data sets for CHO, it also becomes clear that not *yet* all ncRNAs have been identified and annotated and most probably we will live through a couple of exciting surprises during the next few years. In addition, proof of principle has been

Executive summary

Background

- Chinese hamster ovary (CHO) cell factories represent one of the most important production systems for biopharmaceuticals.
- Novel approaches to CHO cell line development and optimization will benefit from the wealth of genomic and transcriptomic data by enabling precise control over cell metabolism by regulating gene expression.

Noncoding RNAs for the regulation of gene expression

- Noncoding RNAs (ncRNAs) are essential for precise regulation and fine-tuning of gene expression in mammalian cells, and consequently the control of cellular behavior.
- This is achieved by coordinated regulation of gene expression through ncRNAs, which resembles the well-known operon concept.

The use of ncRNAs in CHO cell line development

- Specific types of ncRNAs, namely microRNAs and piwiRNAs, have been sequenced and annotated as well as thoroughly characterized in CHO cells.
- Proof of concept has been given that by engineering the transcription of specific miRNAs protein productivity can be improved.

Future perspective

- Albeit the progress, numerous types of ncRNAs are yet to be discovered and characterized in CHO cells.
- It is very likely that ncRNAs will be one of the most valuable tools for future cell factory engineering.

provided that at least miRNAs can markedly enhance the biotechnologically relevant characteristics of CHO cells in terms of proliferation, avoiding apoptosis, stress resistance as well as specific productivity. Still, one missing link for industrial-scale application of miRNA engineered cell lines is their application to CHO producer cells with industry scale productivity of around 6 g/l. If such productivity can still be boosted, it is just a matter of time until such engineered CHO cell lines will produce biopharmaceuticals that will make it to the clinics. While miRNA engineering is the most advanced application of ncRNAs, the other variants discussed in this review also bear the potential of sophisticated, post-transcriptional operon-based regulatory engineering strategies over the next years.

Financial & competing interests disclosure

J Grillari and M Hackl are co-founders of TAmiRNA, J Grillari is co-founder of Evercyte. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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