

## Bioprocess engineering: micromanaging Chinese hamster ovary cell phenotypes

Fundamental to the efficient production of quality biopharmaceuticals is the selection, optimization and tailored manipulation of the mammalian cellular production host. Engineering of these cell factories, predominantly the Chinese hamster ovary cell and advancements in bioprocess regimens have led to greatly increased product titres. The ability of miRNAs to regulate gene expression on a global level has generated considerable interest in these molecules as potential cell engineering targets. In this review, we briefly describe their organization and biogenesis and discuss their attributes as engineering tools in Chinese hamster ovary cells. The development of particular engineering strategies based upon further dissection of miRNA behavior will be considered, with particular emphasis on encouraging examples in Chinese hamster ovary cells and their potential for further development.

Chinese hamster ovary (CHO) cells continue to be the pre-eminent cell line for the production of recombinant therapeutic proteins. Their safety record, ability to reach reasonable densities in suspension culture, genetic tractability, human-like post-translational modification patterns and regulatory acceptance all contrive to ensure CHO cells will likely retain this favored position for some time to come. Optimization of their performance has been an area of intense research over the past two decades in order to continue to meet global market demands as well as internal demands to shorten cell line development timelines. Since the 1990s, product titres have improved at least 20-fold resulting primarily from expression vector improvement and an ability to isolate high-producing clones coupled with tailor-designed media formulations and the implementation of modified bioprocess regimes, to achieve industry standards ranging from 1 to 5 g/l for monoclonal antibodies (mAbs) [1,2]. However, the increasing demand for biologics and decreasing appetite for building large production facilities has maintained the pressure to further boost process efficiencies. Furthermore, these improvements cannot be

achieved at the expense of product quality. The recent proliferation of more 'exotic' therapeutic molecules, such as fusion proteins and various antibody fragments and chimeras, has also presented new manufacturing challenges for the CHO cell platform.

Enhancing CHO cell performance via genetic engineering strategies has been an area of considerable focus over the last decade as a means to compliment the previous breakthroughs in media and bioprocess design. Single and multigene genetic engineering strategies have been exploited to target CHO cell phenotypes critical to the bioprocess including cell cycle [3], apoptosis [4], metabolism [5] and secretion [6]. Manipulating these processes has been demonstrated to improve volumetric or specific productivity ( $Q_p$ ) by sustaining a prolonged production phase, manipulating bioenergetic pathways to reduce the accumulation of toxic byproducts and improving post-transcriptional processing. The most important advance in recent years has undoubtedly been the publication of the Chinese hamster [7] and CHO [8] genomes as well as the numerous 'omics' datasets that have been made available by various groups [9–12]. These datasets combined with

Paul S Kelly<sup>1</sup>, Colin Clarke<sup>1</sup>, Martin Clynes<sup>1</sup> & Niall Barron<sup>\*1</sup>

<sup>1</sup>National Institute for Cellular Biotechnology, Dublin City University, Glasnevin, Dublin, Ireland

\*Author for correspondence: niall.barron@dcu.ie



## Key Terms

**RNAi:** A biological process in which RNA molecules inhibit gene expression, typically by causing the destruction of specific mRNA molecules or blocking their translation into proteins. Two types of small RNA molecules – miRNA and siRNA – are central to RNAi.

**Seed region:** Six to eight nucleotide sequences at the 5'-end of a mature miRNA responsible for binding interaction with target mRNAs.

advanced bioinformatics analyses will help progress the deconvolution of genetic and metabolic networks underlying CHO cell performance and, hopefully, facilitate more predictable and robust cell line development processes. In parallel, there has been the effort to develop an increasing arsenal of tools and techniques for the manipulation of these expression networks in order to bend the CHO cell to the specific needs of individual projects and product molecules. Most of these tools are not specific to CHO cells of course, but do require modification and refinement having been developed for application in humans or other model organisms. Techniques such as site-specific genome targeting/editing using recombinase-mediated cassette exchange [13], zinc finger nucleases [14], TALENs [15] and CRISPR-Cas [16] hold great promise in this regard as well as the more traditional cDNA-based and siRNA-based gene engineering approaches. The use of transposons has also shown its utility as a means of introducing transgenes in a stable manner [17]. When it comes to influencing the expression of more than one gene there are limits in terms of how many can be engineered without running out of either selection markers or the cell's capacity to transcribe and translate multiple transgenes. This increases metabolic burden and can negatively affect growth or product synthesis and secretion.

One set of regulatory molecules that has received considerable attention in an effort to address this challenge has been miRNAs. **RNAi**, be it through siRNA or miRNAs, does not add any further translational burden on the cell, while in the case of miRNAs it can potentially target numerous molecular pathways [18,19]. In this review, we aim to present an overview of the current state of miRNA in CHO cell engineering, the pit falls, the success stories and their overall potential as a tool in biopharmaceutical manufacturing.

### miRNAs: rules & regulations

miRNA are small, nonprotein coding RNAs of approximately 22 nucleotides (nt) in length that exert their function at the post-transcriptional level and have emerged as an attractive alternative to gene-based CHO engineering (reviewed extensively in [20]). Typically transcribed as long primary transcripts

(pri-miRNAs), they are cleaved and processed by the Microprocessor complex (Drosha/DGCR8) to generate a short (~70 nt) hairpin loop structure [21]. This hairpin structure (pre-miRNA) is transported to the cytoplasm in a RAN-GTPase-dependent manner by Exportin-5 (XPO5) where it is further processed by another RNase-type protein, Dicer, thus removing the loop to yield an approximately 22 nt duplex. The active mature strand guides the effector function of the RNA-induced silencing complex, with strand selection being determined by thermodynamic stability of the duplex end and a number of bound cofactors [22,23].

Target gene expression is regulated through imperfect miRNA binding to miRNA recognition elements located mainly within the 3' untranslated region (UTR) resulting in mRNA cleavage or translation inhibition depending on various criteria [24]. The primary binding criteria for a miRNA to elicit its effector function is the perfect complementarity between its 5'-'seed' region (nt 2–8) with its target mRNA, although alternative binding patterns have been observed to compensate for imperfect seed pairings [25,26]. The inconsistency in binding 'rules' makes the prediction of potential targets difficult thus presenting the researcher with a roadblock during the selection process of promising engineering candidates. Furthermore, the small nature of the seed site imparts the potential of a single miRNA to target multiple target transcripts with the addition of a single transcript being targeted by multiple miRNA, again contributing to the difficulty in identifying and predicting *bona fide* mRNA targets [27]. Although miRNA target prediction algorithms are a useful tool for generating lists of potential mRNA targets, these lists tend to contain a high degree of false positives and false negatives, thus highlighting the necessity of experimental identification and validation of true targets [28]. In relation to CHO specifically, the recent release of the genome sequence should help refine existing target prediction algorithms to encompass CHO sequence information, hopefully providing greater accuracy of target prediction. This will be explored further in a later section.

A particular feature of miRNAs is the apparent cell-type specific nature of their activity and this is the subject of considerable study and speculation. Until very recently, studying miRNA activity in CHO cells was confined to using cross-species tools owing to a lack of sequence information [29]. Since then next-generation sequencing (NGS) has provided data on 390 endogenous CHO miRNA that demonstrate a high level of conservation across human and mouse, as well as some CHO-specific sequences [11]. Furthermore, not only have miRNAs been shown to be highly conserved

across species but the elements in which they bind appear also to be under selective pressure suggesting functional conservation in target recognition across various species [30]. Notwithstanding this observed miRNA sequence or target site conservation, it has been postulated that miRNAs may act in a cell-type specific manner possibly due to the reciprocal relationship between expression of a particular miRNA and its target mRNAs [26]. In other words, the activity of a particular miRNA in a cell is intrinsically linked to the availability of cognate target mRNAs within that cell. Furthermore, dosage effects are evidenced by the observation that modest, exogenous overexpression of the miRNA-processing enzyme Dicer in CHO cells can boost cellular proliferation, but excessive expression or indeed downregulation, leads to suppression of cell growth [31]. These observations demonstrate some of the challenges to be overcome when considering miRNA-based strategies for CHO cell engineering.

miRNA organization within the genome (Figure 1) is diverse, ranging from intronic to intergenic, under the control of their own promoter or coexpressed with a protein encoding gene in addition to being present as single miRNA units (monocistronic) or in clusters (polycistronic) [32]. The existence of clusters enhances miRNAs' potential as pleiotropic engineering tools whereby several signaling networks might be manipulated simultaneously. Particular miRNA clusters have been the subject of extensive study, such as the miR-17~92 cluster. This cluster has been attributed to multiple cancerous phenotypes via several cellular pathways, including [33] apoptosis [33] and the cell cycle [34]. Profiling studies have identified this cluster to be highly expressed throughout the duration of fed-batch CHO culture [35] as well as being positively correlated with CHO cell growth rate [36]. The identification of previously validated targets of the miR-17~92 cluster (*E2F1*, *CCND1*, *PTEN* and *BCL211*) in CHO cells demonstrates target site conservation across species [11]. Furthermore, conservation of function, for this cluster at least, could be considered encouraging when assessing it as a potential engineering candidate, in light of the aforementioned cell-type specific activity of these molecules.

### miRNAs: a place in the bioprocess

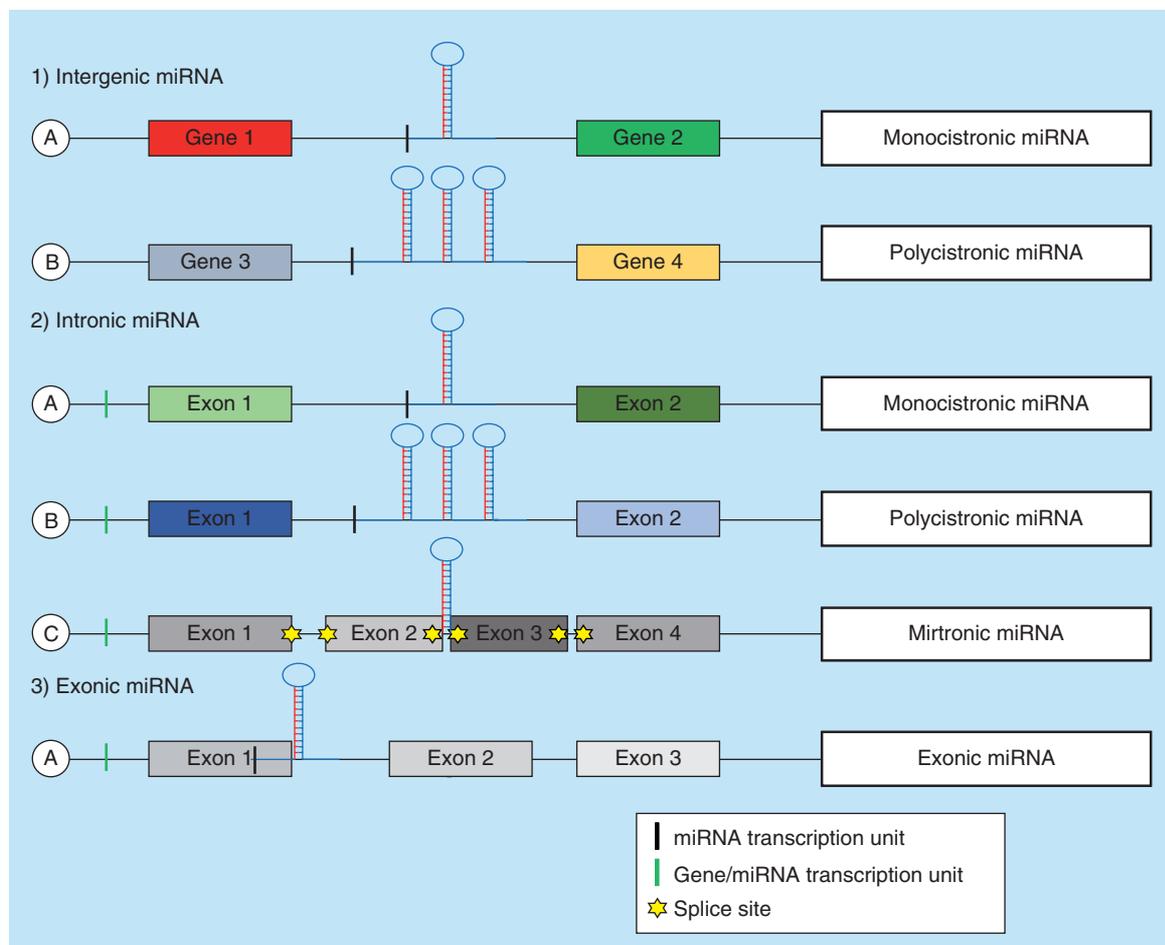
As mentioned above, the impact of individual miRNA expression on its targets tends to be modest. Indeed recent studies have suggested that in many cases the presence of a miRNA within a cell has no discernable effect at all [37]. This would suggest that miRNAs may be more subtle partners in global gene regulation, functioning in the background as a means of curbing excessive transcriptional responses in order to

#### Key Term

**miRNA sponge:** An exogenously expressed decoy mRNA containing binding sites to functionally deplete a mature miRNA or miRNA family.

maintain cellular homeostasis. However, that is not to say that their inappropriate expression cannot cause problems. The identification of miR-15 and miR-16 as frequently deleted in chronic lymphocytic leukemia was the first report that implicated these regulatory molecules in disease [38], which further led to the realization that a plethora of human miRNAs are frequently located in genomic fragile sites [39]. Since this discovery, miRNAs have been demonstrated to impact on almost every aspect of cellular activity including apoptosis, cell cycle, metabolism, differentiation and secretion to name a few (Table 1). It is for this reason that miRNAs have caught the interest of those engaged in efforts to refine CHO cell behavior via genetic engineering.

Efforts to enhance viable cell concentration, maintain a prolonged production phase, improve protein secretion and quality or secure resistance to late-stage culture conditions, such as hypoxia or toxin build-up, could potentially be achieved through miRNA intervention. Some of the cellular processes underlying these phenotypes have been shown to be impacted by miRNA regulation in other cell types and turn out to be conserved in CHO, such as the impact of miR-7 deregulation [70], whereas others have not been described elsewhere, as in the case of miR-466h and its role in inhibiting apoptosis in late-stage CHO culture [46]. Stable depletion of miR-7 using a **miRNA sponge** vector improved peak cell density, prolonged culture longevity and boosted secreted protein yield by almost twofold in a fed-batch process [71]. Similarly, Jadhav and colleagues enhanced the overall yield of an Epo-Fc protein through the stable overexpression of miR-17 [72]. Both studies demonstrate the utility of miRNA-mediated engineering to improve CHO cell growth without negatively impacting on specific productivity [73], a common trade-off for superior growth rates and vice versa [73]. From a product quality perspective, Strotbek *et al.* reported that the glycosylation profile of recombinant IgG1 was not compromised in CHO cells engineered to stably coexpress miR-557 and miR-1287, while inducing increased specific cellular productivity [74]. Besides the manipulation of individual miRNAs it has also been shown that tuning global, cellular miRNA levels via the RNAi processing machinery influences bioprocess-relevant phenotypes. Hackl *et al.* found that overall miRNA expression in CHO cells cultured in serum-containing medium was elevated [11] and upon further investigation found that Dicer mRNA and protein lev-



**Figure 1. Genomic organization of miRNA.** There are three ways in which miRNA can organize within the genome. **(1)** Intergenic miRNAs are located within the noncoding ‘junk’ DNA between coding genes, are under the transcriptional control of their own transcription unit (TU) and can be **(1A)** monocistronic or **(1B)** polycistronic. **(2)** Intronic miRNA are processed from the intron of protein coding genes subsequent to intron splicing and can be **(2A)** monocistronic, **(2B)** polycistronic and uncommonly **(2C)** miRtronic whereby the mature pre-miRNA constitutes the entire intron and bypasses cleavage by Drosha/*DSCR8* through intronic splicing. All intronic miRNA can be expressed under the control of their own TUs or be coexpressed with their host gene. **(3)** Exonic miRNA are located within the coding exon of a host gene and can be under either its own TU or the host genes TU.

els decreased in response to nutrient or serum removal. However, attempts to engineer Dicer expression demonstrated a very sensitive phenotypic response to the levels of Dicer present in the cells. Knockdown below endogenous levels or excessive overexpression both negatively affected growth but mild overexpression was found to be beneficial [31]. For this reason fine tuning the expression of single or small numbers of individual miRNAs, such as those mentioned previously, is probably a better approach for those interested in targeting a specific cellular function or behavior. A number of groups have reported on miRNA expression profiling in CHO cells representing different phenotypes, producing different products or grown under different conditions in order to identify potential engineering targets. These are discussed in more detail in Section 4 and, in particular, demonstrate the importance of gen-

erating CHO-specific miRNA expression data.

Although observations from other cellular systems can provide useful hints as to which miRNA might be worth considering as a tool in CHO cells, the cell-specific nature of their action can lead to disappointing results. A good example of this is miR-34a. This miRNA is known to downregulate the glycotransferase  $\alpha$ -1,6-fucosyltransferase (*FUT8*), which adds an *N*-linked fucosyl group via an  $\alpha$ -1,6 linkage in the Fc region of IgG molecules [75,76]. This is known to reduce antibody-dependent cell cytotoxicity and therefore the efficacy of therapeutic mAbs. For example, two blockbuster mAbs produced in CHO cells – trastuzumab (Herceptin™), used for the treatment of breast cancer; and rituximab (Rituxan™) used for the treatment of non-Hodgkin’s lymphoma – exhibit low antibody-dependent cell cytotoxicity due

**Table 1. miRNAs for potential industrial application.**

Biological process	miRNA	Cluster	Phenotype	Target	Cell type	Ref.
Cell cycle	miR-7	No	Tumor suppressor	<i>Skp2</i> <i>Psmc3</i>	CHO	[40]
	miR-34a	No	Tumor suppressor	<i>CCND1</i> , <i>CCNE2</i> , <i>CDK4/6</i>	A549 NIH-3T3, HCT-116 TOV21G	[41,42]
	miR-24	miR-23a~24~22 miR-23b~24~21	Tumor suppressor	<i>E2F2</i> , <i>MYC</i>	K562.HL60	[43]
	miR-17	miR-17~92	Oncogenic	<i>CDKN1a/p21</i>	CHO	[44]
	miR-31	No	Oncogenic	<i>LATS2</i> <i>PPP2R2A</i>	Lung	[45]
	Apoptosis	Mmu-miR-466h	mmu-mir-297~669	Proapoptotic	<i>Bcl2/12</i> <i>Birc6</i>	CHO
Culture stress	miR-34a	No	Proapoptotic	<i>Bcl2</i> , <i>survivin</i>	Laryngeal carcinoma	[47,48]
	miR-15a/-16	miR-15a~16~11	Proapoptotic	<i>PDCD4</i>	CLL	[49]
	miR-21	No	Proapoptotic	<i>HSP60</i> , <i>HSP70</i>	MPNST	[50]
	miR-133	miR-1/-133	Proapoptotic	<i>Caspase 9</i>	Cardiomyocytes	[51]
	miR-126	No	Shear stress	<i>Bcl2</i> , <i>FOXO3</i> , <i>Irs1</i>	HUVEC	[52]
	miR-31	No	Hypoxia	<i>HIF</i>	Head and neck carcinoma	[53]
	miR-429	miR-200b~429	Osmolarity responsive	<i>OSTF1</i>	Tilapia	[54]
	miR-375	No	Lactate production	<i>LDHB</i>	Sinus carcinoma	[55]
	miR-144	miR-4732~451a	Oxidative stress	<i>NRF2</i>	Erythrocytes	[56]
Energy metabolism	miR-23a	miR-23a~24~21	Glutamine metabolism	<i>GLS</i>	B cells Prostate	[57]
	miR-124	No	Glycolysis	<i>PKM1/2</i>	Colorectal cancer	[58]
	Let-7	Let-7a/d/f	Glucose metabolism	<i>Irs2</i> , <i>INSR</i>	Multiple organs	[59]
	miR-23	miR-23a~24~21	Mitochondrial biogenesis	<i>TFAM</i>	Glioma	[60]
Protein quality	miR-122	miR-122~3591	Lipid metabolism	Cholesterol related genes	Liver	[61]
	miR-34a	No	Core fucosylation	<i>FUT8</i>	Hepatocarcinoma	[62]
	miR-148b	No	N-glycosylation	<i>C1GALT1</i>	Kidney	[63]
	miR-30b/d	miR-30b/d	O-glycosylation	<i>GALNT7</i>	Melanoma	[64]

<sup>†</sup>miRNA passenger strand.  
*Bcl2*: B-cell lymphoma 2; *Birc6*: Baculoviral AIP containing repeat 6; *C1GALT1*: Core 1 synthase glycoprotein-N-acetylgalactosamine 3- $\beta$ -galactosyltransferase 1; *CCND1/E2*: Cyclin D1/E2; *CDK4/6*: Cyclin-dependent kinase 4/6; *CDKN1a*: Cyclin-dependent kinase inhibitor 1a; CHO: Chinese hamster ovary; CLL: Chronic lymphocytic leukemia; *FOXO3*: Forkhead box O3; *GALNT7*: N-acetylgalactosaminyltransferase 7; *GLS*: Glutaminase; *GSIS*: Glucose stimulated insulin secretion; *HIF*: Hypoxia inducible factor; *Irs2*: Insulin receptor substrate 2; *INSR*: Insulin receptor; *LDHB*: Lactate dehydrogenase B; *LATS2*: Large tumor suppressor 2; *NRF2*: Nuclear factor erythroid 2-related factor 2; *OSTF1*: Osteoclast stimulating factor 1; *PPP2R2A*: PP2A regulatory subunit B  $\alpha$  isoform; *PDCD4*: Programmed cell death 4; *PKM1/2*: Pyruvate kinase 1/2; *Psmc3*: Proteasome activator subunit 3; *Skp2*: S-phase kinase associated protein 2; *SNAP25*: Synaptosomal-associated protein 25; *TFAM*: Mitochondrial transcription factor A; *UPR*: Unfolded protein response; *VAMP2*: Vesicular associated membrane protein 2; *XBP-1*: X-box binding protein-1.

**Table 1. miRNAs for potential industrial application (cont.).**

Biological process	miRNA	Cluster	Phenotype	Target	Cell type	Ref.
Protein productivity/ secretion	miR-33a/33b	No	GSIS	<i>Irs2</i>	Hepatic cell lines	[65]
	miR-30c <sup>†</sup>	No	UPR	<i>XBP-1</i>	NIH-3T3, MEFs, HeLa	[66]
	miR-410	mir-323b~656	Protein secretion	?	Min6	[67]
	miR-34a	No	Insulin secretion	<i>VAMP2</i>	β cells	[68]
	miR-124	No	Insulin exocytosis	<i>SNAP25 synapsin-1A</i>	β cells	[69]

<sup>†</sup>miRNA passenger strand.  
*Bcl2*: B-cell lymphoma 2; *Birc6*: Baculoviral AIP containing repeat 6; *C1GALT1*: Core 1 synthase glycoprotein-N-acetylgalactosamine 3-β-galactosyltransferase 1; *CCND1/E2*: Cyclin D1/E2; *CDK4/6*: Cyclin-dependent kinase 4/6; *CDKN1a*: Cyclin-dependent kinase inhibitor 1a; CHO: Chinese hamster ovary; CLL: Chronic lymphocytic leukemia; *FOXO3*: Forkhead box O3; *GALNT7*: N-acetylgalactosaminyltransferase 7; *GLS*: Glutaminase; GSIS: Glucose stimulated insulin secretion; *HIF*: Hypoxia inducible factor; *Irs2*: Insulin receptor substrate 1/2; *INSR*: Insulin receptor; *LDHB*: Lactate dehydrogenase B; *LATS2*: Large tumor suppressor 2; *MRF2*: Nuclear factor erythroid 2-related factor 2; *OSTF1*: Osteoclast stimulating factor 1; *PPP2R2A*: PP2A regulatory subunit B α isoform; *PDCD4*: Programmed cell death 4; *PKM1/2*: Pyruvate kinase 1/2; *Psmc3*: Proteasome activator subunit 3; *Skp2*: S-phase kinase associated protein 2; *SNAP25*: Synaptosomal-associated protein 2; *SNAP25 synapsin-1A*: Synaptosomal-associated protein 2; *XBP-1*: X-box binding protein-1.

to high fucosylation and thus necessitate high patient doses. On the other hand, a 100-fold increase in antibody-dependent cell cytotoxicity was achieved via siRNA-mediated suppression of *FUT8* [77], which has the benefit of reducing the quantities of a therapeutic required to supply the same sized market. This suggests miR-34a overexpression to repress *FUT8* activity might represent a potential engineering strategy in CHO cells. However, miR-34a is also a potent tumor suppressor found to be deregulated in a multitude of cancerous phenotypes [78]. It causes cell cycle inhibition through its interaction with pro-cell cycle genes, such as *CCND1*, *CCNE2*, *CDK4* and *CDK6* [41,79], as well as direct repression of anti-apoptotic genes, such as *BCL2* and *survivin* [47,80]. Indeed this phenotypic effect was found to be conserved upon its overexpression in CHO cells, with a 90% reduction in cellular growth and induction of apoptosis, yet with no detectable change in fucosylation of secreted IgG [UNPUBLISHED DATA FROM THE AUTHORS' GROUP].

Yet a broad appreciation of the function of these molecules in other systems should still be considered worthwhile when considering the bioprocess. For example, the 'Warburg effect' is a process by which cancer cells avidly take up glucose and almost exclusively convert it to lactate in an energetically unfavorable process called aerobic glycolysis [81]. This phenomenon is typical of cultured mammalian cell lines and can be problematic during recombinant protein production where lactate accumulation impacts cell viability. Glucose metabolism has been linked to *c-Myc* expression, which is known to regulate the expression of numerous miRNAs [82]. Cellular reprogramming of the Warburg effect has been observed through the stimulation of glutamine metabolism mediated through the suppression of miR-23a/b, thus releasing their inhibitory function on glutaminase [57,83]. Glucose metabolism has also been demonstrated to be regulated by the miRNA let-7 family through interference with *Irs2* and *INSR* [59]. Other miRNAs known to be involved in various stages of metabolism are miR-122 (liver metabolism), miR-124/-137/-340 (glycolysis) and miR-33a/33b (fatty acid and insulin metabolism) [58,61,65]. These examples highlight the possibility of fine-tuning cellular metabolism via miRNA manipulation in a manner that could benefit cells in the bioreactor.

### The genomic revolution: implications for CHO cell engineering using miRNAs

The publication of multiple CHO cell line and parental hamster genome sequences has signaled the beginning of the systems biology era in these mammalian cell factories [7,84–85]. Genome-scale analysis promises to increase the efficiency of biopharmaceutical manu-

facture in areas such as optimisation of culture conditions (e.g., media formulation), identification of biomarkers for advanced process monitoring and indeed through manipulation of the cellular machinery. In this section, we will review how the availability of genomic sequence data is facilitating a deeper understanding of CHO miRNA biology and the influence these noncoding RNAs exert on industrially desirable phenotypes.

A typical CHO miRNA research program begins with a 'discovery phase', where a well-controlled experiment is designed to study expression patterns and prioritize miRNAs correlated with a particular characteristic, for example, rapid growth rate. The utilization of optimized miRNA profiling platforms is essential to ensure that false-positive rates are minimized and the most promising candidates progress to future functional studies. Pre-genome CHO miRNA expression profiling experiments exploited the high levels of mature miRNA sequence conservation and allowed the field to progress steadily without access to species-specific 'omics' analysis platforms. To date, multispecies (human, mouse and rat) microarrays [29,35,86] and human qPCR-based Taq-man low density arrays [36] have been utilized to successfully highlight deregulated CHO miRNAs in a range of cell culture conditions. Perhaps it is surprising that, despite the availability of genomic sequence and the potential of miRNA-based engineering, there is currently no miRNA hybridization array or qPCR assays available commercially for expression profiling in CHO cells. In recent years, researchers have begun to increasingly utilize NGS technology for miRNA expression profiling analysis. Several deep sequencing studies of miRNA expression, similar to the platforms mentioned above, have also relied on the conservation to identify miRNA. For instance, Johnson and coworkers identified 350 highly conserved miRNAs from NGS-based analysis following comparison to human, mouse and rat [87].

While mature miRNA sequences tend to be highly conserved the obvious drawback is that species-specific miRNAs are not detected by homology-based approaches. The potential of miRNAs that are unique to CHO cells is of particular interest to the community given the possibility that these molecules may underlie the advantages of this cell line for industrial applications. Hackl and coworkers demonstrated an approach to identify novel miRNAs without the genome sequence [11]. In that study, several CHO cell lines were cultured under a variety of conditions and miRNA analysis was carried out using NGS. To investigate miRNA expression patterns the authors initially employed a homology-based approach by align-

#### Key Term

**miRBase:** Database of published miRNA sequences including hairpin and mature forms as well as genomic location and any references. The current version (Release 20) contains 24,521 entries representing hairpin precursor miRNAs, expressing 30,424 mature miRNAs in 206 species – [www.mirbase.org](http://www.mirbase.org).

ing reads to a synthetic reference genome comprised of all known hairpin sequences within the **miRBase** repository [88]. A multistage bioinformatics pipeline was used to identify novel miRNAs from reads that did not map to known miRNAs (or indeed other small RNAs) by analyzing miRNA-like RNA secondary structure using RNA-fold, determining genomic location (i.e., intergenic) through comparison to mouse and utilizing a supervised algorithm to recognize miRNA sequence features (e.g., Dicer cleavage site). In total 235 conserved miRNAs were identified along with 11 putative novel miRNAs in CHO cells utilizing this approach.

Following the release of the genome sequence, NGS-based approaches, while still relying to some extent on conservation, researchers are increasingly utilizing the CHO genome sequence. For instance, Hammond *et al.* aligned reads to the CHO-K1 genome to examine miRNA organization and precursor miRNA sequences [88,89]. Analysis of the six miR-17–92 cluster members revealed a 95% sequence similarity between CHO cells and the mouse genome. Further analysis of miRNA sequences and organization across the CHO-K1 genome was described by Hackl *et al.* in 2012 [90]. In this study, 212 previously identified mature miRNAs were mapped to genomic loci for two independent CHO-K1 genomes and the pre-miRNA sequences were then submitted to miRBase. At the time of writing miRBase (Release 20) contains sequence data for 200 mature and 307 precursor *Cricetulus griseus* miRNAs and provides an essential resource that will enable miRNA expression profiling for future studies.

Access to genomic sequence is not only enhancing our ability to measure miRNAs directly but also enabling us to elucidate their impact on other levels of the biological system and complementing outputs from target prediction algorithms [91]. Recent studies in the CHO area have demonstrated the effectiveness of parallel global protein and gene expression profiling to understand the regulation of the biological pathways impacted by miRNAs at different phases of the culture [35] and the role miRNAs play in growth rate variation [36,92]. As with miRNA expression profiling, genome sequence availability is enhancing our ability to conduct high quality mRNA transcriptome and proteomic studies in CHO cells. The release of the

CHO-K1 genome sequence has led to the development of the first commercially available Affymetrix microarray comprised of probe sets targeting 19,670 genes [93]. In addition, the CHO-K1 and hamster genomes also permit improved analysis of CHO mRNA expression using NGS-based platforms. In particular, mass spectrometry-based proteomic analysis has improved greatly following the release of genome sequences. For instance, Meleady *et al.* reported a 40–50% improvement of protein identifications based on CHO sequence in comparison to using cross-species protein identification [94].

The availability of species specific expression profiling platforms in combination with genomic sequence will undoubtedly reduce false-positive rates when selecting candidates not just for miRNA engineering also confirming direct miRNA targets. A recent publication by Clarke *et al.* demonstrated the utility of multiple expression profiling technologies and the ability to place those data in the context of the genome to study the role of miRNAs in CHO cells [36]. In that study, miRNA, mRNA and proteomic expression data along with genomic sequence were integrated to investigate the variability in growth rate arising during cell line development. The first stage in the analysis identified 51 high-priority miRNAs that either increased or decreased in expression as cellular growth rate increased. Following individual analysis of protein and gene expression these data were combined to identify proteins that were translationally repressed (i.e., protein expression was altered while mRNA levels remained constant). Potential targets for those miRNAs associated with growth rate were then predicted using the TargetScan algorithm [95]. CHO cell genomic sequence not only increased the coverage of proteomic analysis but also allowed the identification of miRNA binding sites within the 3' UTR of translationally repressed proteins that were observed to be conserved in human, mouse, rat and, for the first time, in CHO cells

### miRNA sponge technology: a lesson from nature

A recent review by Park and colleagues on genetic knockout studies in mice indicated that “genetic ablation of miRNAs may not result in obvious phenotypes” [96]. This may be attributed in part to the functional redundancy inherent in the presence of related miRNA seed families as well as paralogs that share endogenous mRNA targets [25]. As such, multiple rounds of genetic knockout may be necessary to observe any phenotypic impact. A means to navigate around this inherent genetic fortitude has come from observations in nature of the apparent reciprocal relationship between

miRNAs and their target genes (reviewed in [26]). This lead to the hypothesis that miRNA targets could act as competitive inhibitors of miRNA function [97].

The first observation of an endogenous mRNA impacting on miRNA function was discovered in *Arabidopsis thaliana* and their response to phosphate starvation [98]. Upon deprivation of inorganic phosphate, upregulation of miR-399 resulted in the accumulation, rather than depletion, of its predicted target *PHO2* encoding a ubiquitin-conjugating E2 enzyme. It was discovered that the expected suppression of *PHO2* was circumvented due to increased expression of the noncoding (nc) gene, *IPSI*. Thus *IPSI* acted as a miRNA decoy, sequestering or titrating miR-399 function away from its endogenous targets. Furthermore, additional mismatches in the duplex formed between miR-399 and *IPSI* prevented Ago2-mediated slicing of the *IPSI* transcript, allowing persistence of this ‘target mimic’ in the cell. The presence of common miRNA recognition elements allows these competitive endogenous RNAs to mutually regulate each other in a titration-dependent manner, as observed recently for the *ZEB2* transcription factor and the tumor suppressor *PTEN* [99,100]. Not only did the observation demonstrate that target mRNA abundance dilutes miRNA activity, but it identified its presence in a mammalian system and in contributing to a disease state [101]. Indeed this phenomenon was found to go beyond pairs of protein-encoding genes to include long noncoding RNAs [102]. To support the role of *PTEN* as a tumor suppressor, the pseudogene *PTENP1* contains miRNA recognition elements within its 3' UTR similar to that of its coding counterpart *PTEN*. Expression of this pseudogene transcript retains biological activity as a decoy to sequester and fine tune the translation of the coding *PTEN* through competitive binding. Indeed, it has been shown that the *PTENP1* locus is frequently lost in human cancers [103].

More recently the phenomenon of head-to-tail splicing of exons has been shown to generate an exciting new class of noncoding RNAs with regulatory potential, known as circular RNAs [104–106]. For example, an antisense transcript from the cerebellar degeneration-related protein 1 (CDRIAs) [107] was found to generate a circular decoy that harbored 63 conserved binding sites for the well-characterized tumor suppressor miRNA, miR-7 [108].

The discovery of these endogenous miRNA decoys led to the development of ‘miRNA sponge technology’ as an experimental tool to evaluate miRNA function [109]. Named for their ability to soak up endogenous miRNAs, miRNA sponges contain multiple sites of complementarity to the miRNA of interest, usually in an artificial 3' UTR placed downstream of a reporter

gene such as GFP (Figure 2). Drawing from lessons in nature, mismatches were introduced as a means to inhibit miRNA-mediated mRNA cleavage, thus prolonging the life-time of the sponge decoy [110]. As seed pairing has been observed to be sufficient to drive miRNA-target interactions, miR-sponges have the potential to sequester entire miRNA seed families. Although this can complicate efforts to understand the network of downstream molecular events that accompany multi-miRNA inhibition, research has proven the miR-sponge approach to be a viable tool for industrial rCHO cell line engineering [71]. In this study prolonged sequestration of miR-7 by a stably expressed sponge resulted in increased peak cell density, prolonged viability and ultimately enhanced yield in a CHO fed-batch culture.

Another potential application of this approach is the use of sponge sequences as a means of controlling the expression of transgenes in a miRNA-dependent manner. Consider a situation whereby overexpression of a particular gene was desirable only at a particular point in the culture but otherwise it should be silent. By identifying an endogenous miRNA whose expression is anticorrelated with the desired transgene expression profile, a sponge for that miRNA placed downstream of the transgene sequence would suppress its expression during the chosen culture phase. Conversely it would become derepressed later when the miRNA was naturally downregulated (Figure 2). This application is in some ways analogous to the use of inducible promoters.

### Overexpression: performance enhancing miRNAs

There are a limited number of reports on the impact of miRNA overexpression on CHO cell behavior. As mentioned earlier, CHO Dicer mRNA and protein levels are reduced in response to serum starvation and nutrient depletion [31]. Conversely, Dicer expression was increased threefold in fast growing CHO cells cultured in protein-free media when compared with slow-growing CHO cells. Furthermore, modest Dicer overexpression in these slow-growing cells enhanced cellular growth rate by 20%. This observation complimented a previous observation by this group that overall miRNA abundance correlated with cellular growth comparing CHO cells cultured in SFM and serum-containing medium [11].

In terms of perturbing individual miRNAs, transient transfection of a miR-7 mimic was shown to increase specific productivity in CHO cells while arresting cell growth [70] potentially via pathways associated with protein translation and RNA/DNA processing including inhibition of genes involved in cell growth such as stathmin and catalase [111].

Stable overexpression is typically achieved using

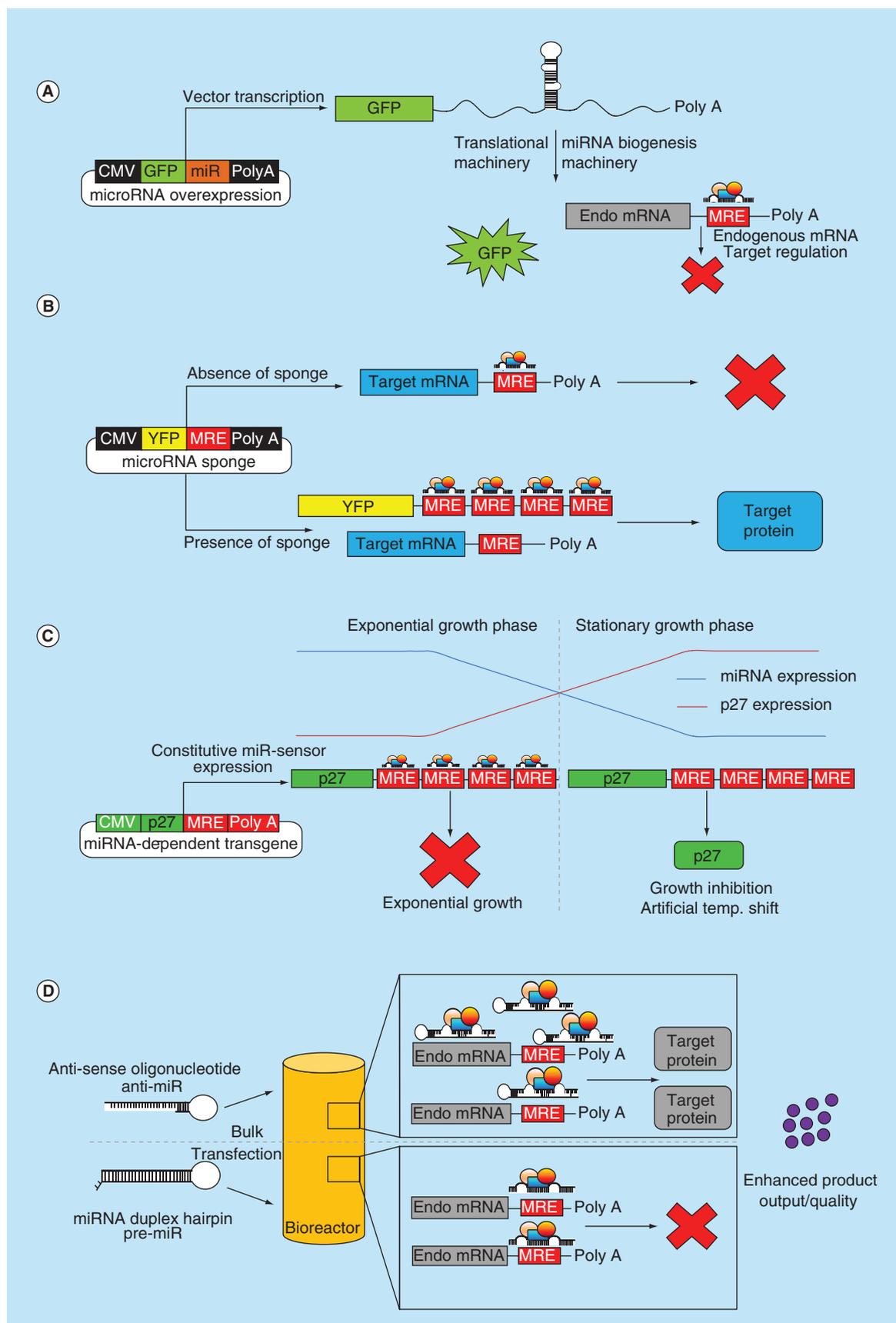
either a RNA PolIII promoter to express a short pre-mir that is processed by Drosha and Dicer into the mature form or by co-expression with a protein-encoding reporter gene in a PolII-dependent manner (Figure 2). Both transient and stable overexpression studies have been explored in CHO cells for miR-17 and miR-557/1287 [72,74,112]. Overexpression of miR-17 resulted in enhanced cell growth and specific productivity of an EpoFc protein resulting in a threefold improvement in titre. An extensive functional screen by Strotbek *et al.* identified several miRNAs associated with improved Qp and subsequent stable co-expression of miR-557/1287 enhanced both cell density and productivity without compromising IgG product quality [20].

It is also worth noting that prior to the availability of CHO sequence information, miRNA overexpression vectors incorporated murine flanking and loop sequences from miR-155 to generate a chimeric miRNA expression vector. Although conservation in miRNA hairpin flanking elements has been documented [113], a recent study has shown that the use of endogenous, CHO-specific miRNA sequences over chimeric constructs produced significantly higher expression levels of mature miRNA in the case of miR-221/222 and miR-15b/16 [114]. This improved vector design provides another example of how CHO genome sequence information has enhanced and refined the tools available to engineer this host.

Finally, one must consider the practicalities of manipulating miRNA levels in a commercial process. The most obvious route for any genetic engineering approach would be to generate a stable CHO cell line – either subsequent to insertion of the product encoding sequence or more likely into a parental CHO line – that would be used in subsequent cell line development projects. These cell lines would obviously require a full regulatory filing. However, there is the potential to use miRNA mimics or inhibitors in an existing approved process via transient transfection – almost like a media supplement – which would have considerably less regulatory implications (Figure 2). This approach has been demonstrated in scale-down models recently [115].

### Summary & future perspective

As the number of biological drugs in development and entering the market continues to grow the necessity of improving and refining the CHO cells used to manufacture them also increases. Marked advances have been achieved in the area over the last 25 years and it has even been suggested that the limit of specific productivity (~100 pg/cell/day) has been reached [1]. However, reaching these productivities is still far from routine and we still lack understanding of the mechanisms



**Figure 2. A series of vector- and nonvector-based engineering strategies are available for the engineering of rCHO using miRNA in both a stable and transient manner (see facing page).**

**(A)** Stable miRNA overexpression can be achieved through the introduction of a reporter construct (GFP) with the desired miRNA sequence (miR) inserted into its 3' untranslated region (UTR). Upon transcription, the reporter gene is translated while the miRNA-duplex hairpin is processed by the cell's miRNA biogenesis machinery. The mature processed, RNA-induced silencing complex loaded, miR subsequently inhibits the translation of its Endo mRNA by association with MREs with the mRNAs 3' UTR. **(B)** Stable miRNA depletion/inhibition is achieved through the introduction of a decoy mRNA (YFP) containing multiple MREs specific for the miRNA of interest. In the presence of the sponge decoy, endogenous miRNA is sequestered from its 'normal' targets (Target mRNA) through competitive inhibition resulting in the translation of endogenous mRNA targets. **(C)** In the event of knowing the miRNA expression profile of a CHO cell over the course of the various culture phases, an miRNA-dependent transgene can be utilized as a rudimentary inducible system. The cell cycle inhibitor *p27*, engineered with MREs, can be expressed at low levels during exponential growth phase as a result of high target miRNA levels. Once miRNA levels decline *p27* is derepressed, thus mediating cell cycle arrest in the stationary phase, potentially negating the implementation of hypothermic growth conditions for example. **(D)** Large-scale bulk transfection can be performed transiently by introducing small working concentrations of either mimics (overexpression) or inhibitors (knockdown) at various culture time points in a bioreactor to achieve a multitude of phenotypes such as enhanced product quality or yields.

Endo mRNA: Endogenous mRNA; GFP: Green fluorescent protein; MRE: miRNA-recognition element; YFP: Yellow fluorescent protein.

by which some clones and not others, can achieve these specific productivities. Increasing maximal cell densities within the bioreactor could also push volumetric yields further. Yet many would suggest that product yields from most current processes, particularly in the case of mAbs, are adequate and perhaps the next most desirable outcomes would be better predictability and control over properties such as product quality, cell line stability, process robustness during scaling or tech transfer and perhaps yield in the case of some of the more difficult to express products. The expanding range of fragments and fusions in development may also require some case-by-case attention in order to manufacture them efficiently. Future efforts will utilize the expanding number and range of 'omics datasets to start building and testing genomic and metabolomic network models of CHO cell behavior with a view to gaining greater understanding of the platform and ultimately, to develop strategies to control some of

these traits. In parallel, the development and testing of various genetic tools to implement these strategies continues apace. Among this expanding toolkit miRNAs and their manipulation represent a valuable and useful option and will no doubt play a role in shaping the CHO cell hosts of the future.

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**Executive summary**

- miRNAs are small regulatory molecules capable of influencing the expression of multiple target genes simultaneously.
- Their activity is often tissue- or cell-type dependent, therefore it is important to study their expression and the effect of deregulation in the cell of interest.
- Several practical examples of miRNA manipulation in Chinese hamster ovary cells have demonstrated improvements in phenotypes such as growth, culture longevity and cell productivity.
- In the post-genome era of Chinese hamster ovary cell engineering miRNAs represent a promising additional tool for researchers and biopharmaceutical companies to improve recombinant protein production.

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