Engineering of the Cellular Translational Machinery and Non-Coding RNAs to Enhance CHO Cell Growth, Recombinant Product Yields and Quality

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ABSTRACT

Chinese hamster ovary cells are the main mammalian cell expression system currently used for the production of recombinant protein biopharmaceuticals. One of the key processes determining the achievable biomass of cells in the bioreactor and the yield and quality of recombinant protein from such systems is mRNA translation. Translation is the process by which ribosomes and associated cellular machinery decode an mRNA to produce a polypeptide. In recent years the roles of different classes of non-coding RNAs in controlling global and transcript specific mRNA translation has also come to light. Here we review approaches to engineer the translational machinery and non-coding RNAs, particularly long non-coding RNAs and tRNAs in CHO cells and then outline the challenges and potential of such approaches to revolutionize the yields and quality of recombinant protein from CHO and other mammalian cell expression systems.

INTRODUCTION

For the production of biopharmaceuticals, Chinese hamster ovary (CHO) cells are the most widely used mammalian cell expression system, able to produce secretory yields of monoclonal antibody in fed-batch culture in excess of 5 g/L [1]. The secretory yield from such an expression system is governed by the number of cells in the bioreactor across the culture (the integral of viable cell concentration or IVC) and the average amount of material expressed by each cell, usually referred to as the cell specific productivity (qP) and expressed as pg of protein/per cell/per day [2]. mRNA translation is a key cellular process that is involve in determining global and protein specific synthesis, and hence control of the abundance of proteins that constitute the cellular machinery, cell growth, division and the IVC of culture. Likewise, mRNA translation plays a key role in determining the qP of a given cell line and hence is a key regulatory process impacting on the yields and quality of recombinant protein from CHO cells [3].

mRNA translation is the process by which the ribosome and associated cellular machinery decodes a target mRNA to yield a polypeptide. Translation is a key step in the gene expression pathway and is the predominant process by which protein cellular abundance is controlled [4]. Over the last few decades it has been established that the control in mammalian cells of mRNA translation, and hence protein synthesis, is not only determined by the translational machinery, modulation of the activity of various translation factors by phosphorylation, and the abundance, availability and makeup of a given mRNA, but also by availability, abundance and activity of non-coding RNAs [5]. Non-coding RNAs are generally described as either long non-coding RNAs of >200 nucleotides in length or small non-coding RNAs <200 nucleotides and includes microRNAs (also referred to as miRs) and tRNAs. The discovery of the mechanism(s) by which non-coding RNAs exert an influence on gene expression has opened up new opportunities for the engineering of cells to manipulate cell processes that underpin cell growth and recombinant protein production and quality. Further, manipulation of such non-coding RNAs offers the advantage of not placing an additional translational burden on the cell that over-expression of coding mRNAs does. Here we briefly review our understanding of the control of mRNA translation in CHO cells, describe approaches and outcomes to engineer the translational machinery and non-coding RNAs in CHO cells, and discuss current and future cell engineering opportunities and challenges such approaches present (Summarized in Table 1).

THE TRANSLATIONAL MACHINERY, mRNA ANALYSIS AND MANIPULATION

As mRNA translation is a key process in defining cell growth, biomass accumulation and recombinant protein yields and quality from cultured CHO cells [3,6], the translational machinery and the abundance and availability of global and recombinant mRNAs between cell lines and process conditions has been investigated using a variety of approaches. For example, the phosphorylation of the translation initiation factor eIF2α and attenuation of global protein synthesis during recombinant protein production in CHO cells is known to occur [7]. Culture temperature has been shown to impact mRNA translation and the quality of recombinant product produced [8] and the PERK-eIF2α pathway was reported to impact upon the aggregation of a recombinant TNFR-Fc fusion protein [9]. Indeed the activity and availability of translation factors has been shown to change during culture and under different culture conditions, where for example under reduced temperature translation elongation factor 2 (eEF2) becomes phosphorylated and a reprogramming of translation

occurs that means transcripts with particular codon usage can escape the general global attenuation of translation under such conditions and the translation of these transcripts is actually enhanced [10].

High producing antibody cell lines have been shown to maintain translation initiation factors at levels that allow such cells to maintain enhanced recombinant protein synthesis above that of lower producing cells [3]. With regard to monoclonal antibody synthesis in CHO and other cells, investigations have shown that recombinant antibody production is limited by translational efficiency [3,11-13]. Manipulation of the cellular translational machinery is however, not straightforward. One global regulator of ribosome biogenesis and translation is mTORC1, which coordinates cellular responses to signaling pathways involved in sensing growth factors, nutrient availability, intracellular energy status and other perceived cell stresses and modulates translation and ribosome biogenesis in response [14]. In particular, mTORC1 can influence translation initiation via phosphorylation of eukaryotic initiation factor 4E binding protein (4E-BP1), which when phosphorylated at multiple sites promotes dissociation of 4E-BP1 from the initiation factor eIF4E. Increased phosphorylation of 4E-BP1 has been correlated with increased interferon-γ production [15] whilst the stoichiometry of 4E-BP1 to eIF4E is reported to relate to recombinant antibody productivity [16]. Exogenous mTOR expression has also been shown to enhance recombinant protein expression in CHO cells by improving cell viability, growth, proliferation and cell specific productivity [17].

One approach applied to investigate mRNA amounts, and hence determine gene expression profiles of high producing or fast growing recombinant cell lines is transcriptomics. A general assumption of most such studies is that the amount of mRNA present at a given timepoint reflects the 'state' or 'need' of a cell with regard to the proteins that these mRNAs encode for. As such, transcriptomic profiling has been applied to identify mRNAs whose abundance correlates with cell growth and recombinant protein productivity and quality with a view to using the identification of such targets to engineer the cell for improved performance. Many of these initial studies were hampered by the lack of the CHO genome and appropriate arrays, however the elucidation of the genome [18,19] and advent of RNA-Seq has made it possible to undertake such studies with a higher degree of precision. Despite this, there has been little consensus across transcriptomic studies to date with regard to those mRNAs that correlate with cell growth and recombinant protein productivity [20]. Further, previous correlation analysis has shown that transcript amounts and translation efficiency are uncoupled for around 95% of investigated genes [21], providing strong evidence that global and mRNA specific translational control needs to be understood and determined to evaluate the impact of mRNAs on phenotype rather than simple mRNA abundance itself.

In order to address this issue, investigators have begun to apply ribosome footprint profiling or RiboSeq analysis to unravel the fine detail of translational control in CHO cells [22]. This powerful approach allows genome wide, but also transcript specific, detail on initiation and elongation stages of mRNA translation to be studied and identification of those mRNAs that are being translated at any given time (as opposed to just their abundance), the efficiency of mRNA translation and how this changes during a process or between cell lines to identify targets for cell line engineering [22]. Indeed, any given mRNA in the cell may be translated by one or multiple ribosomes (so call polysomes) at any one

time [23]. In some cases the number of ribosomes per transcript has been used to estimate translational efficiency of a transcript assuming that more ribosomes on a transcript indicates greater translational efficiency [24], but this does not account for elongation speed that RiboSeq analysis can. RNA-Seq approaches can also potentially be used to investigate translational activity at the single cell level. The application of such approaches is certain to provide a more detailed understanding of mRNA translation and its control in recombinant protein producing CHO cells, at the population and single cell level and at a global and transcript specific level, revealing new engineering approaches by which translation can be modulated to enhance protein production.

microRNAs and siRNAs

mRNA translation can also be tuned by non-coding RNAs. One such class of non-coding RNA that has been applied to reprogramming translation in CHO cells is that of microRNAs (also known as miRs or miRNAs). The potential application of microRNAs to CHO cell engineering has recently been reviewed elsewhere [25]. These RNAs are transcribed as long primary transcripts but then processed to yield small (20-23 nucleotide) non-coding RNAs and were first described in *C. elegans*. MicroRNAs tend to act as repressors of translation of target mRNAs by interacting with the 3'untranslated regions (3'UTRs) of such mRNAs. A given microRNA can in theory target multiple mRNAs via base pairing and hence modulate multiple mRNAs and pathways without placing an additional translational burden on the cell [25].

Early microRNA studies were limited by the lack of available Chinese hamster sequence annotation of microRNA primary transcripts, and hence chimeric microRNAs that contained the mature miR sequence but flanking sequences in the primary transcript from other species were used. Subsequent studies showed that endogenous CHO microRNA flanking sequences gave rise to higher expression when over-expressing microRNAs [26]. Recent engineering approaches harnessing microRNAs include studies that look to enhance the ability of CHO cells to produce so called 'difficult to express proteins'. For example, one such study showed that a CHO cell line constitutively over-expressing miR-557 and a difficult to express antibody produced twice the antibody yield of cells engineered to express a negative control microRNA [27]. A further study reported that both transient and stable miR-143 over-expression resulted in enhanced difficult to express protein production and targeted MAPK7 in CHO [28]. The natural repertoire of microRNAs has also been harnessed to repress expression of the DHFR selection marker during cell line construction and allow the generation of higher producing cell pools [29]. Others have shown that microRNA fingerprints or signatures can be correlated with growth rate across a number of different CHO cell lines [30].

However, although microRNA engineering appears an attractive approach by which to tune translation of multiple mRNAs and translation of specific targets, the potential large number of predicted targets of any given microRNA means that the outcome of such engineering approaches can be difficult to predict as is identifying which targets a given microRNA interacts with. Barron and colleagues have described a system termed 'miR-CATCH' that allows the investigator to identify those microRNAs that interact with a given target and thus validate these for potential cell engineering approaches [31•]. The authors had identified that the overexpression of the X-linked inhibitor of apoptosis (XIAP) enhanced CHO cell productivity, growth and culture longevity. To avoid overexpressing this

gene and placing an additional translational burden on the cell, microRNA regulators of XIAP were identified using a biotin-labelled antisense DNA for XIAP resulting in the capture of interacting microRNAs. Inhibition of two of these microRNAs resulted in increased XIAP protein expression, validating the microRNA catch approach and the utility of this for identifying cell engineering targets.

The use of small interfering RNAs (siRNAs) has also proved to be an effective strategy for CHO cell line engineering to selectively knockdown expression of target genes detrimental to cell growth or productivity. The advantage of knockdown, as opposed to knockout strategies, is that essential genes can be reduced in their expression and the impact on cell phenotype assessed when knockout proves fatal. One successful application of siRNA engineering has been the inhibition of lactate dehydrogenase-A (LDH-A) on its own [32], or in combination with pyruvate dehydrogenase kinases (PDHKs) [33] to significantly reduce lactate accumulation in cultured CHO cells without negatively impacting cell growth and enhancing cell specific productivity. A further group undertook ribosomal profiling in CHO cells and identified the resistance marker NeoR as being highly transcribed and translated, and as expression of this exogenous gene in CHO cells is not required, used siRNA knockdown to reduce its expression with a resultant improvement in production and growth of the host observed [34]. Finally, an siRNA approach was used to knockdown the expression of the endoplasmic reticulum localized proteins ceramide synthase 2 (CerS2) and Rab1 GAP Tbc domain family member 20 (Tbc1D20) in CHO IgG producing cells with a subsequent observed increase in recombinant protein specific productivity and enhanced cell growth [35].

LONG NON-CODING RNAs (lncRNAs) AND THEIR MANIPULATION

Recent genome wide analysis in mammalian cells estimates that 75% of the transcriptome is composed of non-coding sequences [36] and led to the identification of a heterogeneous class of transcripts known as Long non-coding RNAs (lncRNAs) [37]. LncRNAs are defined as transcripts longer than 200 nucleotides that lack a significant open reading frame (ORF) and are usually transcribed by RNA polymerase II and spliced, with or without, 3' polyadenylation [38]. These molecules are emerging as key regulators in various biological processes both in the nucleus and the cytoplasm [39], including epigenetic regulation, transcriptional control, splicing events, and mRNA translation. While most of our current understanding into lncRNAs and the underlying mechanism(s) by which they elicit their responses has come from studies relating to disease and developmental studies, their potential as targets for cell engineering in mammalian cell factories remains largely unexplored.

The first analysis of the non-coding transcriptome in CHO cells under batch and fed-batch conditions has recently been published, unveiling a number of differentially regulated lncRNAs depending on feed and culture time which could be targets for cell engineering [40•]. One of the main challenges in identifying lncRNAs is the low sequence conservation between species. This, coupled with incomplete genome sequences and partial annotations of coding and non-coding genes of most vertebrates including Chinese hamster, have impaired an effective lncRNAs annotation outside from model organisms (Figure 1).

A recent study compared lncRNAs among 16 vertebrates and the echinoid sea urchin finding thousands of human lincRNAs homologs with conserved genomic position sharing 5'-biased patches of sequence nested in rewired exonic architectures [41]. The FANTOM consortium applied a cap analysis of gene expression (CAGE) to data obtaining more than 27,919 human

lncRNA genes with high-confidence 5′ ends and expression profiles across 1,829 samples from the major human primary cell types and tissues [42]. Through the incorporation of conservation and expression data, the consortium was able to identify 19,175 potentially functional lncRNAs in the human genome. Due to the tissue-specificity of lncRNAs, comparing the expression among several cell types has led to a more robust identification of functional targets. By modelling their effects on the activity of transcription factors, RNA-binding proteins, and microRNAs in 5,185 TCGA tumors and 1,019 ENCODE assays, it was possible to identify potential lncRNAs involved in dysregulated cancer pathways. This approach indicated OIP5-AS1, TUG1, NEAT1, MEG3, and TSIX, as synergic lncRNAs leading to dysregulated cancer pathways in multiple tumor contexts [43]. A similar effort using nascent RNA capture sequencing identified 1145 temporally expressed S-phase-enriched lncRNAs across TCGA data sets in several cancer models showing effects on pathways including FGF/FGFR and its downstream PI3K/AKT and MAPK pathways [44].

The NEAT1 lncRNA is a central component of paraspeckles, nuclear bodies that regulate multiple aspects of gene expression, promoting their formation through ATR signaling in response to replication stress and p53 activation [45]. The RNA-binding NONO–PSF heterodimer binds a large number of expressed pri-miRNAs in the paraspeckles to promote processing by the Drosha–DGCR8 Microprocessor. NEAT1 thus regulates efficient processing of potentially an entire class of small non-coding RNAs in the nucleus by interaction with the NONO–PSF heterodimer as well as other ribosome binding proteins (RBPs) [46].

The relationship between lncRNAs and the translational machinery was further elucidated with the discovery of a long nucleolus-specific lncRNA (LoNA) [47•]. LoNA is expressed at high levels at resting state suppressing rRNAs transcription in the nucleoli through the combined effects of its 5′ portion, which binds and sequesters nucleolin, and its snoRNA like 3′ end, which recruits and diminishes fibrillarin activity to reduce rRNA methylation. When the cell needs to sustain an elevated translational load, LoNA expression decreases leading to elevated rRNA and ribosome levels, an increased proportion of polysomes, mRNA polysome loading, and eventually protein synthesis.

The first successful engineering of lncRNAs for enhanced recombinant protein production involved manipulation of SINEUPs, natural and synthetic antisense lncRNAs that can activate translation in a gene-specific manner using an inverted SINEB2 sequence [48]. A Binding Domain (BD) located towards the 5′ region of the SINEUP overlaps a target mRNA of choice conferring specificity, while an inverted SINEB2 element defined as the Effector Domain (ED) provides the translation activation function [49]. Synthetic SINEUPs have been used to increase translation and secretion of recombinant proteins in a range of mammalian cell lines, including CHO [50••] and HEK293 [51•]. As further studies define those lncRNAs present in CHO cells and how these influence cell growth, fate and recombinant protein production, engineering of these non-coding RNAs is sure to offer potential to further tune and enhance mRNA specific and global mRNA translational efficiency.

tRNAs AND TRANSLATION

The use of specific codons with high gene copy number and high codon bias coupled with the modulation of intracellular tRNA concentration has been shown to improve protein production in CHO cells [52]**.** However, despite translational efficiency often being considered the mere result of codon optimization based on the correlation between codon bias and tRNA gene copy numbers (Figure 2), recent evidence suggests a considerably more

intricate picture where ribosome collisions, co-translational folding, mRNA stability, composition, charge status and post-transcriptional modifications of the tRNA pool all contribute to finely tune protein production in response to the environment [53]. Controlling the translational capacity of an expression system through the use of alternative codon combinations modulates ribosome decoding speed, impacting protein quality as well as final yield [54]. The use of suboptimal codons has been reported to slow translation at key structural motifs in order to facilitate correct co-translational polypeptide folding and signal recognition particle (SRP) recognition, which assists in protein translocation across membranes [55]. Thus, although recombinant genes are often 'codon optimized', we do not currently have all the information required around codon usage, context, tRNA abundance, modifications and charging to fully harness codon usage in recombinant sequences or to engineer tRNA abundance.

Codon bias has been referred to as a secondary genetic code that impacts on the fidelity of translation, efficiency of translation, polypeptide/protein folding and mRNA stability/half-life [56]. The cell utilizes such codon effects to tailor the proteome and allow reprogramming, such as under cold stress whereby reprogramming and synthesis of specific proteins is enhanced through codon bias [10]. Codon bias or optimization is also linked to tuning mRNA stability and stable mRNAs are found to be enriched in codons that are considered optimal whilst also impacting on ribosome translocation [57]. Specific combinations of adjacent codons in yeast and mammalian cells can have an effect on translation efficiency resulting in reduced expression, proving how the focus must be on global translation efficiency and codon context as opposed to single codons optimality [58•-60]**.** On top of this, mRNA secondary structure combined with tRNA abundance modulate translational elongation speed among different regions of the same transcript to avoid excessively slow or fast ribosome movement [61]. As such, there remains enormous potential to enhance recombinant protein yields from further manipulation of codon usage.

In order to further enhance recombinant protein yields by manipulation of codon usage it is necessary to further understand the abundance and modifications of tRNAs and the role these play in their activity. Determination of tRNA copy numbers can now be undertaken using RNA-Seq approaches. tRNA secondary structure and nucleotide modifications, mainly methylations, impair the efficiency of standard sequencing. Dedicated protocols based on an initial de-methylation step were recently developed to overcome this limitation, allowing for direct measurement of each tRNA abundance and detailed mapping of modifications [62,63]. While some methods focus exclusively on mature tRNAs [64], partial alkaline RNA hydrolysis complemented with tRNA precursors enrichment identified tRNA leaders, trailers, and introns and showed that around half of all predicted tRNA genes are transcribed in human cells [65]. While tRNA abundance is a major modulator of translational elongation, the aminoacylation state has to also be considered. The addition of chemical steps that specifically remove the 3'A residue in uncharged tRNA coupled with the aforementioned de-methylation RNA-Seq protocols showed most cytosolic tRNAs in HEK293T cells are charged at >80% levels, whereas tRNASer and tRNAThr are charged at lower levels [66].

An additional layer of regulation during elongation is chemical modification of nucleotides among tRNAs [67]. One of the key enzymes to regulate the methylation state of tRNAs is the demethylase ALKBH1, which acts dynamically in response to specific conditions such as variations in glucose availability to impact translation at both the initiation and the elongation

phases [68]**.** These modifications can have different effects depending on the target tRNA and the position in the transcript, as it was shown ALKBH1 is required for the formation of essential methylations at position 34 of anticodon in cytoplasmic tRNALeu and mitochondrial tRNAMet [69]. Advances in high-throughput sequencing and data analysis have also allowed the identification of new classes of small non-coding RNAs derived from tRNAs: stress-induced tRNA halves (tiRs) and tRNA-related fragments (tRFs). These RNAs act on cell proliferation, priming of viral reverse transcriptase, regulation of gene expression, RNA processing, modulation of the DNA damage response, tumor suppression, and stress response [70]. The application of such approaches to study tRNAs in CHO cells will further elucidate the mechanism(s) by which tRNAs and their modifications modulate translation.

FUTURE DIRECTIONS AND CHALLENGES

Our understanding and ability to manipulate the translational machinery and harness non-coding RNAs to enhance global and recombinant protein synthesis in CHO cells has advanced rapidly in the last decade. Further, the advent of the Chinese hamster and CHO cell line genomes has helped in the identification of non-coding RNAs such that these can be studied and manipulated. The ability of non-coding RNAs, in particular microRNAs, siRNAs, lncRNAs and tRNAs to tune both global and transcript specific translation, and hence protein synthesis, offers enormous opportunities to use these to enhance cell growth and proliferation, extend culture lifetimes, and increase recombinant protein yields and quality. However, our ability to harness these non-coding RNAs by engineering of CHO cells is currently limited by our knowledge of the mechanisms and targets by which many of these non-coding RNAs elicit their responses. The manipulation of microRNAs that can, in theory, tune multiple target transcripts appears an appealing approach, however in our view this approach alone is unlikely to deliver new commercially viable host cells with dramatically enhanced phenotypes due to the fact these are 'tuning' molecules and tend to be negative regulators and off target approaches can be difficult to control. Where these might be more applicable is for the tuning of transcript targets with a specific role, such as enzymes involved in glycosylation or to harness modulation of the cells own endogenous microRNA pool as inducible controllers of exogenous gene circuits. The potential of lncRNA engineering is very much in its infancy and would appear to offer the potential to act as negative and positive regulators of gene expression. The limitation here is that many of these are, as the name suggests, long RNAs and thus the manipulation is more challenging and we do not yet understand what, if any, role many of these play in the cell. The control of gene expression via the elongation step of mRNA translation and tRNA availability, charging and modification, linked with improved predictive models for how such changes in abundance or modification change elongation rates of target mRNAs is likely to offer advances that can be directly applied industrially to engineering of the target recombinant gene(s) and of pathways in the cell to deliver new engineered host cell lines with improved growth, productivity and post-translational modification abilities. However, the major challenge will be to unravel the mechanisms by which the control on gene expression that these different non-coding RNAs provide are coordinated together, in order to reprogram the translational efficiency of current CHO cell chassis, under appropriate bioprocessing conditions (including continuous processes) to generate new chassis with enhance bioprocessing properties.

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Table 1. Summary of non-coding RNA cell engineering studies discussed in this review grouped by type of approach, with the reported experimental effect and the relative reference.

Method	Effect	Reference
siRNA	LDH-A activities were decreased by 75-89%, while the specific glucose consumption rates reduced by 54-87% and the specific lactate production rates reduced to 45-79% of the control cell line level.	**[32]**
	siRNA mediated inhibition of PDHKs and LDH-A in CHO cells expressing a therapeutic monoclonal antibody reduced lactate production, increased specific productivity and volumetric antibody production by 90%, 75% and 68%, respectively.	**[33]**
	Ribosome profiling identified NeoR as a highly transcribed and translated gene in an IgG-producing CHO cell line. Viable cell concnetration was increased by 35% upon siRNA knock-down of NeoR, which was accompanied by an 18% increase in product titer.	**[34]**
	Combined transient siRNA-mediated knockdown of the expression of the endoplasmic reticulum localized proteins CerS2 and Tbc1D20 resulted in a 50-66% increase in specific productivity of CHO-IgG cells.	**[35]**
miRNA	Co-expression of miR-557 and a difficult-to-express antibody resulted in a two-fold increase in product titer.	**[27]**
	miR-143 overexpression resulted in a 20% final increase in mAb productivity.	**[28]**
	Addition of a synthetic 3'UTR to destabilize DHFR expression allowed the generation of stable DG44-derived cell pools expressing a model monoclonal antibody (mAb) with low MTX concentrations.	**[29]**
	Inhibition of miR-124-3p and miR-19b-3p in CHO increased X-linked inhibitor of apoptosis protein levels, enhancing CHO cell growth and prolonged culture longevity while additionally boosting productivity.	**[31]**
lncRNA	Engineering of CHO cells with SINEUP long non-coding RNAs resulted in a 150% increase in periostin levels in cell supernatant at 72 h post-transfection.	**[50]**
	The development of a 'universal' protein expression enhancer tool based upon long non-coding RNAs gave expression enhancement in various mammalian cells of recombinant proteinsin the order of 50-1000%, with more than 200% enhancement in most cases.	**[51]**
Codon optimization	Modification of human interleukin-2 (IL-2) through codons with high gene copy number and high codon usage bias significantly increased protein productivity in CHO-K1 cells.	[52]
	Codon de-optimization of a bispecific antibody sequence through the introduction of less frequently occurring codons in CHO gave a 2-fold final yield increase.	[55]
	In vivo expression of various codon context (CC) optimized IFN-γ in CHO cells exhibited at least 13-fold increase in expression compared to the wild-type IFN-γ while a maximum of 10-fold increase was observed for the individual codon usage (ICU) optimized genes.	[60]