Research Repository UCD

Title	Exploring the Potential Application of Short Non Coding RNA Based Genetic Circuits in Chinese Hamster Ovary Cells
Authors(s)	Valdés-Bango Curell, Ricardo, Barron, Niall
Publication date	2018-01-29
Publication information	Valdés-Bango Curell, Ricardo, and Niall Barron. "Exploring the Potential Application of Short Non Coding RNA Based Genetic Circuits in Chinese Hamster Ovary Cells." Wiley Online Library, January 29, 2018. https://doi.org/10.1002/biot.201700220.
Publisher	Wiley Online Library
Item record/more information	http://hdl.handle.net/10197/10564
Publisher's statement	This is the peer reviewed version of the following article: Valdés Bango Curell, R. and Barron, N. (2018), Exploring the Potential Application of Short Non Coding RNA Based Genetic Circuits in Chinese Hamster Ovary Cells. Biotechnol. J., 13: 1700220. doi:10.1002/biot.201700220. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.
Publisher's version (DOI)	10.1002/biot.201700220

Downloaded 2026-05-01 23:36:06

The UCD community has made this article openly available. Please share how this access benefits you. Your story matters! (@ucd_oa)

© Some rights reserved. For more information

Review

Exploring the potential application of short non-coding RNA-based genetic circuits in Chinese Hamster Ovary cells

Ricardo Valdés-Bango Curell[1]

Niall Barron [2, 3]

[1] The National Institute for Cellular Biotechnology, Dublin City University, Dublin, Ireland

[2] The National Institute for Bioprocessing Research and Training, Fosters Avenue, Blackrock, Dublin, Ireland

[3] University College Dublin, Dublin, Ireland

Correspondence: Dr. Niall Barron, The National Institute for Bioprocessing Research and Training, Fosters Avenue, Blackrock, Dublin 4, Ireland

E-mail: niall.barron@nibrt.ie

Keywords: Cellular engineering, CHO cells, Genetic circuits, miRNA, Protein expression control, RNA interference, Synthetic Biology

Abbreviations:

AGO2, Argonaute II protein;

ASO, Antisense Oligonucleotides;

Cas, CRISPR-associated;

CHO, Chinese Hamster Ovary;

CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats

1 **dsRNA**, double-stranded RNA;

2 **EPO**, Erythropoietin;

3 **GFP**, Green Fluorescent Protein;

4 **HEK,** human embryonic kidney;

5 **hPSCs**, human Pluripotent Stem Cells;

6 **HSL**, histone stem-loop;

7 **iPSCs**, induced pluripotent stem cells;

8 **IRE**, iron responsive element;

9 **KO**, knock-out;

10 **lncRNA**, long non-coding RNA;

11 **miRISC**, microRNA-induced silencing complex;

12 **miRNA**, microRNA;

13 **MMTV-LTR**, mouse mammary tumour virus . long terminal repeat;

14 **MRE**, microRNA responsive element;

15 **pORF**, primary Open Reading Frame;

16 **pre-miRNA**, precursor microRNA;

17 **pri-miRNA**, primary microRNA;

18 **RBP**, RNA binding protein;

19 **RepO,** repressor-dependent operon/promoter;

20 **RNAi**, RNA interference;

21 **rtTA**, reverse tetracycline-controlled transactivator;

22 **shRNA**, short-hairpin RNA;

23 **siRISC**, siRNA-induced silencing complex;

24 **siRNA**, short interfering RNA;

25 **SLBP**, Stem-Loop Binding Protein;

26 **sncRNA**, short non-coding RNA;

sxRNA, structural interacting RNA;

TINCR, Tissue Differentiation-Inducing Non-Protein Coding RNA;

TRE, transactivator-responsive element;

uORF, upstream Open Reading Frame;

UTR, untranslated region;

SEAP, Secreted embryonic alkaline phosphatase;

VEGF, Vascular endothelial growth factor;

Abstract

The majority of cell engineering for recombinant protein production to date has relied on traditional genetic engineering strategies, such as gene overexpression and gene knock-outs, to substantially improve the production capabilities of Chinese Hamster Ovary (CHO) cells. However, further improvements in cellular productivity or control over product quality are likely to require more sophisticated rational approaches to coordinate and balance cellular pathways. For these strategies to be implemented, novel molecular tools need to be developed to facilitate more refined control of gene expression. Multiple gene control strategies have been developed over the last decades in the field of synthetic biology, including DNA and RNA-based systems, which allow tight and timely control over gene expression. microRNAs have received a lot of attention over the last decade in the CHO field and have been used to engineer and improve CHO cells. In this review we focus on microRNA-based gene control systems and discuss their potential use as tools rather than targets in order to gain better control over gene expression.

1 Introduction

Chinese Hamster Ovary (CHO) cells are the preferred system for the production of biopharmaceuticals [1]. Improving their production capabilities has always been desirable in order to reduce the cost of producing therapeutic proteins [2]. Significant improvements in cell growth and productivity have been achieved in the last few decades, mainly via bioprocess and media optimization, increasing protein titres from the initial few milligrams per litre to more than 10 g/L. Although there have been large efforts to push the boundaries further by cell engineering, such strategies to date have been limited in terms of industrial application.

Since the first CHO genome was published in 2011, advances in next-generation sequencing and other 'omics technologies such as proteomics or metabolomics, have improved our ability to study CHO cells on a systematic and genome-wide scale [3–6]. In addition, the CHO field has recently entered the systems biology era with the development of the first consensus genome-scale model, integrating the available genomic, transcriptomic, proteomic and metabolomic data. This model should facilitate a better understanding of CHO cell biology, leading to novel rational genetic engineering approaches to optimize CHO cell lines for bioprocessing [7,8].

Several studies have acknowledged that improvements in cellular productivity will not likely be achieved by engineering a master gene or small subset of genes, but rather by coordinating and balancing a variety of complex cellular pathways in an optimal way [6,9–11]. In this context, it is likely that traditional cell engineering strategies, such as crude gene overexpression, gene knock-down or gene knock-out might not be nuanced enough to address some of these challenges [4]. More sophisticated approaches, such as metabolic pathway rewiring or gene dosage engineering will be required. For these to be implemented, gene control systems beyond the ON/OFF paradigm and allowing fine-tuning of gene expression need to be established. These systems would ideally utilize expression

vectors capable of linking transgene expression to endogenous molecules, for example microRNAs (miRNAs), which show specific expression profiles, in order to provide the right transgene dose at the right time, in response to a specific metabolic or environmental signal (Figure 1).

The field of synthetic biology offers the most promising approach to gain better control over gene expression, and ultimately be able to rationally engineer organisms and control their behaviour [12]. A variety of DNA- or RNA-based expression control systems allowing fine control of gene expression has been developed. DNA-based systems are mainly transcriptional control elements or inducible promoters. Although the number of inducible promoters available in mammalian cells is small when compared with *E. coli* or yeast, there have been several examples of inducible promoters applied to the production of biopharmaceuticals in mammalian cells [13–15]. Some examples include the doxycycline-inducible Tet-ON / Tet-OFF system, the metallothionein expression system, responsive to heavy metals, the glucocorticoid-inducible mouse mammary tumour virus long terminal repeat (MMTV-LTR) or a biotin inducible promoter [16–20]. RNA-based systems are mainly riboswitches. Originally discovered in bacteria, these RNA-domains can undergo changes in their secondary structure after binding of a small-molecule ligand to modulate transgene expression at both transcriptional and post-transcriptional levels [21]. In mammalian cells, riboswitches responsive to small molecules, but also to proteins, have been developed [22–24]. One of the main drawbacks of these types of inducible elements is the need to add a controller molecule to the culture in order to modulate transgene expression. A more interesting group of RNA-based inducible systems is the one in which the regulating molecules are endogenously expressed miRNAs or synthetic siRNAs. This review focuses on the state-of-the-art miRNA/siRNA-based genetic switches and on their potential use to improve control over transgene expression in CHO cells. In addition, we discuss the challenges in adapting the currently developed miRNA-based gene control strategies, which

mainly focus on cell therapy or diagnostic applications, to the context of biopharmaceutical bioprocesses and CHO cells.

2 miRNAs and siRNA biogenesis and function

miRNAs are a type of short non-coding RNA (sncRNA) (21-25 nt) that play an important role in the regulation of gene expression at the post-transcriptional level [25]. The canonical biogenesis pathway of miRNAs involves several steps. First, a miRNA gene is transcribed in the nucleus by RNA polymerase II to produce a primary miRNA transcript (pri-miRNA), which comprises a hairpin domain flanked by two single stranded RNA domains [26]. The pri-miRNA is processed by the RNase-III enzyme DROSHA to form a 60-80 nt long precursor miRNA (pre-miRNA), that conserves the hairpin-like secondary structure and can be exported to the cytoplasm by Exportin5 [27,28]. There, the pre-miRNA is cleaved by a second RNase-III, Dicer, to form a dsRNA intermediate, from which the mature miRNA strand will be loaded into Argonaute II proteins (AGO2), as part of the miRNA-induced silencing complex (miRISC) [29,30]. Based on an imperfect sequence complementary between the loaded mature miRNA and its mRNA target, the miRISC complex can bind to the untranslated regions (UTR) of the target mRNA transcript and inhibit its translation, or in some cases of high level of complementarity between the miRNA and its target lead to endonucleolytic cleavage of the transcript [31].

Short interfering RNAs (siRNAs) can be derived from different biogenesis pathways, such as cleavage of long double stranded RNA (dsRNA) by Dicer, or be introduced artificially in the cell [29,32]. siRNAs are also loaded into AGO2 proteins and guide the binding of the siRNA silencing complex (siRISC) to their target mRNA molecules. Whereas typically miRNAs can have multiple targets due to their imperfect complementarity, siRNAs are specific to their target as they are fully complementary to their binding site. Perfect binding between siRNA and their target leads to its endonucleolytic cleavage by the RNAse Dicer, a

mechanism known as RNA interference (RNAi) [29]. To date, the role of endogenous siRNA in mammalian cells is still poorly understood. However, exogenously added siRNA have been broadly used as tools to investigate individual gene function [33,34].

For clarity, in this review, the term 'miRNA' will refer to any molecule originated from a miRNA precursor either transcribed from cellular genes and being endogenously expressed in the cell, or to a miRNA that comes from an exogenously introduced plasmid containing the miRNA precursor hairpin sequence and is therefore expressed and processed through the miRNA expression pathway. siRNA will only refer to exogenously short double stranded RNA introduced by transfection or electroporation.

3 Exploiting endogenous miRNA signatures to modulate transgene expression

Several studies have reported on using miRNAs to modulate the expression of transgenes in specific cell types or tissues. After identifying specific miRNA signatures in the desired cell type, miRNAs have been used as inputs for genetic switches containing a transgene, typically a reporter gene, under the control of microRNA-responsive elements (MREs). These MREs are simply miRNA-binding sites located in the UTR of the transgene.

One of the first examples of miRNA-controlled cell-specific transgene expression relied on a miRNA-sensitive OFF-type sensor and was used to study miRNA expression patterns during the development of mouse embryo [35]. An OFF-type switch comprises a reporter gene, a Green Fluorescent Protein (GFP) or ß-Galactosidase, which contains specific fully complementary miRNA-binding sites in its 3'-UTR. In the presence of the corresponding miRNA, the reporter gene mRNA transcript is sliced by RNAi, hence marking the areas where the miRNA is being expressed by the absence of fluorescence (GFP reporter) or lack of coloration in the presence of X-Gal (ß-Galactosidase reporter) (Figure 2 – A). Two later consecutive studies by Brown et al. used a similar approach in order to obtain selective transgene expression in different human cell types based on their specific miRNA

expression signatures. Again, the vector comprised a GFP reporter transgene, and specific 3'-UTR miRNA-binding sites, which lead to a down-regulation of the transgene in the presence of the complementary miRNA. By using a double reporter vector, with one reporter gene expressed constitutively and the second one under the control of miRNA-binding sites, specific populations of closely related cell types (including dendritic cells, hematopoietic and embryonic stem cells, and their progeny, which exhibit different miRNA signatures) or specific tissues, like retina, could be segregated based on the differential miRNA-controlled expression of the reporter transgene [36–38].

More recent studies used this type of miRNA-based transgene expression vector to classify neuron-type and brain-region-specific mice cells based on their cell-type specific miRNA signature [39]. Another example of OFF-type miRNA-based sensors is a study that identified and sorted different cell populations from complex mixtures of differentiated human pluripotent stem cells (hPSCs), using two types of OFF-switches: one based on miRNA-binding sites in the 3'-UTR of the reporter gene and one with a single miRNA-binding site in the 5'-UTR. The authors demonstrated the possibility of specifically selecting a given cell type, namely cardiomyocytes, using a miR-based switch driving the expression of the pro-apoptotic gene Bim in cells that do not show the proper miRNA signature (high miR-1 or high miR-208a). In addition, the authors proved that the obtained cells were still fully functional and could be used for medical applications such as in vivo transplantation therapy. The same technology was used to purify other cell types. In addition, the authors demonstrate the possibility of combining miRNA switches in order to simultaneously purify two different types of cells [40]. In a more complex version of a miRNA-based OFF-type switch, developed by Xie *et al.*, the expression of the output gene is controlled by a transactivator placed under the control of a particular miRNA by the presence of miRNA-binding sites at the 3'-UTR. The binding of the specific miRNA inhibits the expression of the transactivator, hence blocking the expression of the output gene (Figure 2 – B) [41]. A last

example of a practical application of these miRNA-based genetic switches is a cell-type-specific genome editing platform developed based on a miRNA-responsive element developed using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas9) system, which was able to drive targeted genome editing of human cell lines (HeLa and induced pluripotent stem cells) in a modulated fashion in response to endogenous miRNA signatures [42]. The system comprises a Cas9 gene, modified by adding a miRNA-binding site to the coding sequence, complementary to the miRNA of interest. These miR-Cas9 switches are able to control genome editing based on the presence/absence of specific miRNAs. In the presence of the miRNA, the Cas9 transcript is degraded and no genome editing activity can occur. In the absence of the particular miRNA, the transcript is not degraded and can be translated, thus the Cas9 protein can drive the desired targeted genome edition based on the provided single guide RNA. The authors show how a miR-21-Cas9 switch responded to the cell-specific miR-21, which is highly expressed in HeLa cells, switching OFF the CRISPR-Cas9 KO of a stably transfected GFP reporter gene. On the other hand, a miR-302-Cas9 switch was not functional, as miR-302 is not expressed in these cells, therefore resulting in the GFP gene being knocked out. The authors also provide evidence that the presence of specific miR-21 inhibitors switched ON Cas9 protein expression, re-instating genome editing activity . The authors used the same miR-Cas9 switch to target other endogenous regions in the genome, including the Alu1 element, leading to selective cell death based on the chosen miRNA-binding site present in the switch.

All the above examples rely on OFF-type miRNA-based switches, in which specific miRNA signatures act as the input, switching OFF the expression of the transgene. There are other examples of miRNA-based circuits whereby the presence of specific miRNA input turns ON the expression of the transgene. An example of this type of ON-circuit has been developed and tested in human embryonic kidney cells (HEK) [43]. It comprises an endogenous miRNA

which controls the expression of a synthetic miRNA using an OFF-switch configuration as explained above. The output gene also contains binding sites for the synthetic miRNA/siRNA in its UTR. Hence, in the presence of the endogenous miRNA, the expression of the synthetic miRNA is repressed and the output protein can be expressed. Another example of miR-based ON-switches comprises a repressible promoter driving the expression of an output protein and a repressor protein containing miRNA-binding sites in its UTR. In the presence of the input miRNA, the expression of the repressor protein is blocked, allowing the output protein to be expressed (Figure 2 – C).

Combining these different types of miRNA-based switches, it is possible to build more complex multi-input circuits, which can react to specific miRNA signatures and allow the final expression of an output protein. Xie *et al.* describe a cell classifier able to detect specific cancer cells in a mixed pool of different human cancer cell lines, based on more complex miRNA signatures, for example the one found in HeLa cells, which show high expression of miR-17, miR-21 and miR-30a and low expression of miR-141, miR-142(3p) and miR-146a. By using a double inversion module circuit design, a reverse tetracycline-controlled transactivator (rtTA) controls the expression of an inducible LacI repressor, which represses the transcription of the output gene. In addition, both trans-activator and repressor have miRNA binding sites specific to the highly expressed miRNAs (17, 21, and 30a). In the presence of these miRNAs, both transactivator and repressor proteins are repressed due to specific binding sites in their UTRs and the output protein can be expressed (Figure 2 – D). To incorporate a sensing capacity for low expressed miRNAs, the output gene also contains binding sites for the low expressed miRNAs (miR-hsa-141, miR-hsa-142(3p) and miR-hsa-146a). When low expressed, these miRNAs are not sufficient to efficiently inhibit the output gene; therefore, it is expressed. Any change in expression of any of the controller miRNAs (high expression changing to low expression or vice versa) will result in repression of the output gene [41]. In a similar way, other cell classifiers able

to detect complex miRNA signatures in different human cell lines (HeLa cells and HEK293 cells) have been developed using different circuit designs. For example, Wroblewska *et al.* developed a cascade circuit, in which a synthetic siRNA controlled the expression of a RNA-binding protein (RBP), which in turn controlled the expression of the output gene, and a more complex two-way signal transmission and feedback circuit, in which two different siRNA inputs are able to modulate the expression of two different reporters, by controlling different RBPs [44]. Other examples can be found as well, in which miRNA-based circuits are combined with other genetic control elements such as transcription repressors or transactivators and which are able to react to specific miRNA signatures [45,46].

4 Synthetic miRNAs to modulate transgene expression

4.1 Inducible expression of synthetic miRNA/siRNAs

Synthetic miRNAs have also been widely used in genetic circuits to modulate transgene expression. Some studies have combined them with inducible promoters to be able to control expression in a dose and time-dependent manner. Leisner *et al.* have built and characterized different synthetic genetic circuits which comprise miRNAs controlled by transcription factor-dependent promoters, which down-regulate the expression of an output reporter gene by RNAi. They show how multiple combinations of different modular circuits can lead to different programmable protein expression patterns, based on the different inputs present in the circuit [47].

In another study, Malphetes *et al.* described the first adjustable RNA polymerase II promoter-based siRNA expression system, which allows the expression of siRNA hairpins (or miRNAs) based on the absence/presence of an antibiotic (erythromycin or tetracycline), in a dose-dependent manner, for the precise control of transgene expression in CHO cells. In a later study, the previously described siRNA expression system is used to fine tune the expression of different transgenes in CHO cells, including secreted embryonic alkaline

phosphatase (SEAP), vascular endothelial growth factor (VEGF), or erythropoietin (EPO) [48]. This study is the only one in CHO cells where a systematic characterization of different circuit relevant parameters, such as the number of binding sites for the specific siRNA is performed. Combining the above controllable siRNA expression module with other transcriptional regulatory elements such as a tetracycline-dependent inducible promoter allowed direct control of transgene expression.

In a more recent study, an OFF genetic control device based on RNAi that combines ligand-responsive ribozyme switches with synthetic miRNA regulators has been built and characterized [49]. The genetic device comprises a ribozyme, where cleavage is dependent on the presence/absence of a ligand and which controls the expression levels of a synthetic miRNA. Finally, synthetic miRNAs have been used as add-ons to improve other transgene expression control systems. miRNAs expressed from an artificial intron in the trans-regulator gene and targeting the output reporter gene have been used to reduce leaky expression, leading to tighter control of transgene expression in the OFF state [41,50,51].

4.2 Controlling miRNA expression by affecting their processing

Another interesting strategy to control the expression of synthetic or ectopic miRNAs or siRNAs is to fuse them with aptamers. An aptamer is an RNA domain which can bind to a specific ligand and change its secondary structure. One of the best-characterized aptamers is based on a previously described riboswitch, which is able to drive the termination of the transcription of a gene of interest in the absence of its binding ligand theophylline [52]. By introducing an aptamer sequence in between functional regions of the miRNA gene, such as the hairpin loop region or the basal stem region, it is possible to alter its secondary structure, hence affecting miRNA processing and consequently, its expression [53–55].

A recent study used this strategy to modulate the expression of the Tissue Differentiation-Inducing Non-Protein Coding RNA (TINCR), a well known long non-coding RNA (lncRNA) that acts as a master regulator in somatic differentiation and which is up-regulated in

bladder cancer tissues and cells, contributing to oncogenesis and cancer progression [56,57]. Here, the expression of the transgene could be controlled by the theophylline aptamer using two newly developed genetic switches, both based on the conditional expression of specific siRNAs [58]. The OFF-switch relies on the expression of a mature miRNA domain, in the presence of theophylline, which silences the TINCR lncRNA, switching it OFF and causing a reduction in cell growth. The ON-switch is based on the conditional expression of a mature miRNA, which is able to inhibit the processing of a second short-hairpin RNA (shRNA), which acts a repressor of TINCR. By conditionally silencing TINCR, cell growth can be affected. This is another good example of how miRNA-based genetic switches can be used to modulate the expression of relevant target genes and lead to programmed cellular behaviour.

5 Novel miRNA responsive elements based on new modes of action

So far, most examples of miRNAs being incorporated in genetic circuits relied on the best known mechanism of action of miRNAs or siRNA in the cell: translation inhibition or RNAi-mediated transcript degradation. However, some studies have exploited different mechanisms of action of miRNAs, providing new miRNA responsive elements for use in synthetic genetic circuits.

5.1 miRNA regulating RNA-binding protein motifs

Doyle *et al.* proposed alternative mechanisms through which miRNA can bind to mRNA molecules and affect how certain RBPs interact with them [59]. The model proposes that miRNA can act as structural interacting RNAs (sxRNA) and participate in forming 3-way RNA junctions to regulate gene expression. Post-transcriptional regulation of histone genes, by means of a histone stem-loop (HSL) structure located at the 3' end of the mRNA transcripts is used as an example. The Stem-Loop Binding Protein (SLBP) is able to bind to this structure and greatly increase the translation of the associated mRNA. By using

secondary structure and nucleic acid interaction prediction software, the authors explored how miRNA could be responsible for generating or stabilizing the HSL structure, and identify several target miRNAs which could potentially do it. In a similar way, the authors identified miRNAs that could potentially help form sxRNA structures in the iron- responsive element (IRE), which is targeted by IRE-binding proteins to regulate post-transcriptional expression of iron metabolism-related genes. The authors predict how specific miRNAs can influence alternative splicing of the ALA synthase gene by forming two different sxRNAs structures. In a follow-up study from the same group, a sxRNA-based reporter consisting of a luciferase gene upstream of the sxRNA switch, was designed, built and tested in vitro, using *in silico* folding studies and gel-based and cell-based binding studies [60]. The sxRNA switch comprises the sequence required to form the functional stem-loop structure, in order to bind the RBP, flanked by two paired regions complementary to the desired trigger miRNA (Figure 3 – A).

5.2 miRNAs to improve translation efficiency

A recent study has shown the possibility of using competing upstream open reading frames (uORF) and antisense oligonucleotides (ASO) to modulate expression of a transgene at the post-transcriptional level (Figure 3 – B) [61]. uORFs are caused by the presence of initiation sequences and/or start codons upstream of the correct initiation sequence and start of the gene of interest, which initiates the primary ORF (pORF). The presence of these alternative uORFs down-regulates protein synthesis from the pORF due to competing translation events. To counteract this, ASOs can be designed to specifically target uORFs, hence blocking translation events starting in those without leading to any transcript degradation. This leads to the up-regulation of the translation occurring from the main pORF. If we consider that the proposed ASOs are synthetic 16-18bp oligonucleotides (DNA/RNA), it is possible to imagine scenarios in which uORFs could be designed to be targeted by endogenous miRNAs and incorporated upstream of a transgene to modulate its expression.

6 miRNA-based CHO cell engineering – from targets to tools

Since the identification of the first CHO-specific process relevant miRNA, there has been a significant effort to identify miRNAs involved in the regulation of bioprocess-relevant pathways as potential engineering targets to improve CHO cellular phenotypes [62,63]. Several miRNAs have been identified and manipulated to improve process relevant cell characteristics such as cell growth, apoptosis, and recombinant protein production [64–69]. In addition, several studies have already highlighted miRNAs that exhibit expression profiles which vary depending on specific conditions, such as different culture stages [63,70], different culture conditions like a temperature shift [64,71] or different cellular phenotypes such as high/low Qp, producers/non-producers and fast/slow growth rate [6,72]. However, little attention has been dedicated to utilizing these molecules as control elements, rather than direct targets, in order to implement more refined cell engineering strategies. In order to do so, re-evaluating these datasets from this new perspective could help to identify specific miRNA signatures that would allow implementation of some of the reviewed miRNA-based circuits in CHO cells for particular bioprocess relevant applications, such as conditional expression after a temperature shift or selective killing of low producers to improve selection methods. There are some challenges that can be anticipated in terms of introducing miRNA-based gene control in CHO cells. For instance, the use of miRNA-based genetic switches could result on the knock down of the 'controller' miRNAs, i.e. the sponge effect, potentially leading to unwanted cellular effects. In addition, full ON/OFF control is likely to be difficult to achieve using the currently available miRNA-based gene control technologies, so combinations with other gene control elements such as promoters or other genetic elements should be further investigated.

7 Conclusion

In the context of the more complex challenges that will arise from the new systems biology approaches in studying CHO cells, it is necessary to start thinking about developing more refined solutions to improve the CHO cell platform. Further developments and refinement of the current CHO genome-scale models will surely be crucial not only to identify which targets/pathways require these interventions but also to assess the potential benefits that would arise from them. Hypothetical examples of these more sophisticated approaches include conditional expression of specific product genes or gene-dosage balancing to fine-tune the expression of specific genes or pathways and achieve a more energetically efficient CHO cells free of unnecessary metabolic burden. In order to implement these new engineering approaches, the proper molecular tools have to be available.

Synthetic biology advances have provided us with a large variety of miRNA-based genetic switches or circuits, which allow us to gain tighter control over transgene expression. Most of the available miRNA-based gene expression control elements are based on tissue-specific miRNA signatures or diseased versus control cell profiles. Although miRNAs have been typically studied in CHO cells as targets for cellular engineering, we anticipate that meaningful miRNA signatures or expression patterns in the context of CHO cells and bioprocessing can be identified from the CHO miRNA profiling datasets that have been published to date. This should present new opportunities in terms of using miRNA-based genetic-switches in the next generation of rational genetic engineering strategies and to facilitate more refined control over the CHO cell as a therapeutic protein production platform.

Acknowledgement

The authors would like to acknowledge funding from the European Union's Horizon 2020 Research and Innovation programme under the Marie Sklodowska-Curie grant agreement

No 642663 (eCHO systems). The authors would like to thank Krishna Motheramgari for his contribution to correcting this manuscript.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

8 References

[1] Walsh, G., Biopharmaceutical benchmarks 2014, Nat. Biotechnol. 2014, 32, pp. 992–1000.

[2] Butler, M., Meneses-Acosta, A., Recent advances in technology supporting biopharmaceutical production from mammalian cells, Appl. Microbiol. Biotechnol. 2012, 96, pp. 885–894.

[3] Xu, X., Nagarajan, H., Lewis, N. E., Pan, S., et al., The genomic sequence of the Chinese hamster ovary (CHO)-K1 cell line., Nat. Biotechnol. Nature Publishing Group 2011, 29, pp. 735–741.

[4] Fischer, S., Handrick, R., Otte, K., The art of CHO cell engineering: A comprehensive retrospect and future perspectives, Biotechnol. Adv. Elsevier B.V. 2015, 33, pp. 1878–1896.

[5] Datta, P., Linhardt, R. J., Sharfstein, S. T., An 'omics approach towards CHO cell engineering, Biotechnol. Bioeng. 2013, 110, pp. 1255–1271.

[6] Clarke, C., Henry, M., Doolan, P., Kelly, S., et al., Integrated miRNA, mRNA and protein expression analysis reveals the role of post-transcriptional regulation in controlling CHO cell growth rate, BMC Genomics. BMC Genomics 2012, 13, p. 656.

[7] Kaas, C. S., Fan, Y., Weilguny, D., Kristensen, C., et al., Toward genome-scale models of the Chinese hamster ovary cells: incentives, status and perspectives, Pharm. Bioprocess. 2014, 2, pp. 437–448.

[8] Harcum, S. W., Lee, K. H., CHO Cells Can Make More Protein, Cell Syst. Elsevier Inc. 2016, 3, pp. 412–413.

[9] Harreither, E., Hackl, M., Pichler, J., Shridhar, S., et al., Microarray profiling of preselected CHO host cell subclones identifies gene expression patterns associated with in-creased production capacity, Biotechnol. J. 2015, 10, pp. 1625–1638.

[10] Wilkens, C. a., Gerdtzen, Z. P., Comparative Metabolic Analysis of CHO Cell Clones Obtained through Cell Engineering, for IgG Productivity, Growth and Cell Longevity, PLoS One. 2015, 10, p. e0119053.

[11] Ley, D., Seresht, A. K., Engmark, M., Magdenoska, O., et al., Multi-omic profiling -of EPO-producing Chinese hamster ovary cell panel reveals metabolic adaptation to heterologous protein production, Biotechnol. Bioeng. 2015, 112, pp. 2373–2387.

[12] Purnick, P. E. M., Weiss, R., The second wave of synthetic biology: from modules to systems, Nat. Rev. Mol. Cell Biol. Nature Publishing Group 2009, 10, pp. 410–422.

[13] Weinhandl, K., Winkler, M., Glieder, A., Camattari, A., Carbon source dependent promoters in yeasts, Microb. Cell Fact. 2014, 13, p. 5.

[14] Vogl, T., Glieder, A., Regulation of Pichia pastoris promoters and its consequences for protein production, N. Biotechnol. Elsevier B.V. 2013, 30, pp. 385–404.

[15] Wang, T., Ma, X., Du, G., Chen, J., Overview of Regulatory Strategies and Molecular Elements in Metabolic Engineering of Bacteria, Mol. Biotechnol. 2012, 52, pp. 300–308.

[16] Santos, A. K., Parreira, R. C., Resende, R. R., Expression System Based on an MTIIa Promoter to Produce hPSA in Mammalian Cell Cultures, Front. Microbiol. 2016, 7, pp. 1–10.

[17] Weber, W., Lienhart, C., Daoud-El Baba, M., Fussenegger, M., A biotin-triggered genetic switch in mammalian cells and mice, Metab. Eng. 2009, 11, pp. 117–124.

[18] Huang, E. P., Marquis, C. P., Gray, P. P., Process development for a recombinant

Chinese Hamster Ovary (CHO) cell line utilizing a metal induced and amplified metallothionein expression system, Biotechnol. Bioeng. 2004, 88, pp. 437–450.

[19] Figueroa, B., Ailor, E., Osborne, D., Hardwick, J. M., et al., Enhanced cell culture performance using inducible anti-apoptotic genes E1B-19K and Aven in the production of a monoclonal antibody with Chinese hamster ovary cells, Biotechnol. Bioeng. 2007, 97, pp. 877–892.

[20] Lipscomb, M. L., Mowry, M. C., Kompala, D. S., Production of a Secreted Glycoprotein from an Inducible Promoter System in a Perfusion Bioreactor, Biotechnol. Prog. 2004, 20, pp. 1402–1407.

[21] Serganov, A., Nudler, E., A Decade of Riboswitches, Cell. 2013, 152, pp. 17–24.

[22] Beilstein, K., Wittmann, A., Grez, M., Suess, B., Conditional Control of Mammalian Gene Expression by Tetracycline-Dependent Hammerhead Ribozymes, ACS Synth. Biol. 2015, 4, pp. 526–534.

[23] Kennedy, A. B., Vowles, J. V, D'Espaux, L., Smolke, C. D., Protein-responsive ribozyme switches in eukaryotic cells., Nucleic Acids Res. 2014, pp. 1–16.

[24] Ausländer, S., Stücheli, P., Rehm, C., Ausländer, D., et al., A general design strategy for protein-responsive riboswitches in mammalian cells, Nat. Methods. 2014, 11, pp. 1154–1160.

[25] Bartel, D. P., MicroRNAs: Target Recognition and Regulatory Functions, Cell. 2009, 136, pp. 215–233.

[26] Lee, Y., Kim, M., Han, J., Yeom, K.-H., et al., MicroRNA genes are transcribed by RNA polymerase II, EMBO J. 2004, 23, pp. 4051–4060.

[27] Zeng, Y., Cullen, B. R., Efficient Processing of Primary microRNA Hairpins by Drosha Requires Flanking Nonstructured RNA Sequences, J. Biol. Chem. 2005, 280, pp. 27595–27603.

[28] Yi, R., Qin, Y., Macara, I. G., Cullen, B. R., Exportin-5 mediates the nuclear export of

pre-microRNAs and short hairpin RNAs, Genes Dev. 2003, 17, pp. 3011–3016.

[29] Kim, V. N., Han, J., Siomi, M. C., Biogenesis of small RNAs in animals, Nat. Rev. Mol. Cell Biol. 2009, 10, pp. 126–139.

[30] Ha, M., Kim, V. N., Regulation of microRNA biogenesis, Nat. Rev. Mol. Cell Biol. Nature Publishing Group 2014, 15, pp. 509–524.

[31] Lytle, J. R., Yario, T. A., Steitz, J. A., Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR, Proc. Natl. Acad. Sci. 2007, 104, pp. 9667–9672.

[32] Tam, O. H., Aravin, A. A., Stein, P., Girard, A., et al., Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes, Nature. 2008, 453, pp. 534–538.

[33] Cullen, L. M., Arndt, G. M., Genome-wide screening for gene function using RNAi in mammalian cells, Immunol. Cell Biol. 2005, 83, pp. 217–223.

[34] Papageorgiou, A., Rapley, J., Mesirov, J. P., Tamayo, P., Avruch, J., A Genome-Wide siRNA Screen in Mammalian Cells for Regulators of S6 Phosphorylation, in: Cota, D. (Ed.), PLoS One. 2015, 10, p. e0116096.

[35] Mansfield, J. H., Harfe, B. D., Nissen, R., Obenauer, J., et al., MicroRNA-responsive "sensor" transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression, Nat. Genet. 2004, 36, pp. 1079–1083.

[36] Brown, B. D., Gentner, B., Cantore, A., Colleoni, S., et al., Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state, Nat. Biotechnol. 2007, 25, pp. 1457–1467.

[37] Brown, B. D., Venneri, M. A., Zingale, A., Sergi, L. S., Naldini, L., Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer, Nat. Med. 2006, 12, pp. 585–591.

[38] Karali, M., Manfredi, A., Puppo, A., Marrocco, E., et al., MicroRNA-Restricted transgene

expression in the retina, PLoS One. 2011, 6.

[39] Sayeg, M. K., Weinberg, B. H., Cha, S. S., Goodloe, M., et al., Rationally Designed MicroRNA-Based Genetic Classifiers Target Specific Neurons in the Brain, ACS Synth. Biol. 2015, 4, pp. 788–795.

[40] Miki, K., Endo, K., Takahashi, S., Funakoshi, S., et al., Efficient Detection and Purification of Cell Populations Using Synthetic MicroRNA Switches, Cell Stem Cell. Elsevier Inc. 2015, 16, pp. 699–711.

[41] Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R., Benenson, Y., Multi-Input RNAi-Based Logic Circuit for Identification of Specific Cancer Cells, Science (80-.). 2011, 333, pp. 1307–1311.

[42] Hirosawa, M., Fujita, Y., Parr, C. J. C., Hayashi, K., et al., Cell-type-specific genome editing with a microRNA-responsive CRISPR–Cas9 switch, Nucleic Acids Res. 2017, pp. 1–11.

[43] Rinaudo, K., Bleris, L., Maddamsetti, R., Subramanian, S., et al., A universal RNAi-based logic evaluator that operates in mammalian cells, Nat. Biotechnol. 2007, 25, pp. 795–801.

[44] Wroblewska, L., Kitada, T., Endo, K., Siciliano, V., et al., Mammalian synthetic circuits with RNA binding proteins for RNA-only delivery, Nat. Biotechnol. Nature Publishing Group 2015, 33, pp. 839–841.

[45] Li, Y., Jiang, Y., Chen, H., Liao, W., et al., Modular construction of mammalian gene circuits using TALE transcriptional repressors, Nat. Chem. Biol. Nature Publishing Group 2015, 11, pp. 207–213.

[46] Prochazka, L., Angelici, B., Haefliger, B., Benenson, Y., Highly modular bow-tie gene circuits with programmable dynamic behaviour., Nat. Commun. 2014, 5, p. 4729.

[47] Leisner, M., Bleris, L., Lohmueller, J., Xie, Z., Benenson, Y., Rationally designed logic integration of regulatory signals in mammalian cells, Nat. Nanotechnol. 2010, 5, pp.

666–670.

[48] Malphettes, L., Fussenegger, M., Improved transgene expression fine-tuning in mammalian cells using a novel transcription?translation network, J. Biotechnol. 2006, 124, pp. 732–746.

[49] Bloom, R. J., Winkler, S. M., Smolke, C. D., Synthetic feedback control using an RNAi-based gene-regulatory device, J. Biol. Eng. 2015, 9, p. 5.

[50] Greber, D., El-Baba, M. D., Fussenegger, M., Intronically encoded siRNAs improve dynamic range of mammalian gene regulation systems and toggle switch, Nucleic Acids Res. 2008, 36.

[51] Lapique, N., Benenson, Y., Digital switching in a biosensor circuit via programmable timing of gene availability, Nat. Chem. Biol. Nature Publishing Group 2014, 10, pp. 1020–1027.

[52] Wachsmuth, M., Findei??, S., Weissheimer, N., Stadler, P. F., M??rl, M., De novo design of a synthetic riboswitch that regulates transcription termination, Nucleic Acids Res. 2013, 41, pp. 2541–2551.

[53] An, C., Trinh, V. B., Yokobayashi, Y., Artificial control of gene expression in mammalian cells by modulating RNA interference through aptamer – small molecule interaction Artificial control of gene expression in mammalian cells by modulating RNA interference through aptamer – small molecule i, Rna. 2006, 12, pp. 710–716.

[54] Beisel, C. L., Bayer, T. S., Hoff, K. G., Smolke, C. D., Model-guided design of ligand-regulated RNAi for programmable control of gene expression, Mol. Syst. Biol. 2008, 4.

[55] Beisel, C. L., Chen, Y. Y., Culler, S. J., Hoff, K. G., Smolke, C. D., Design of small molecule-responsive microRNAs based on structural requirements for Drosha processing, Nucleic Acids Res. 2011, 39, pp. 2981–2994.

[56] Kretz, M., Siprashvili, Z., Chu, C., Webster, D. E., et al., Control of somatic tissue

differentiation by the long non-coding RNA TINCR, Nature. Nature Publishing Group 2013, 493, pp. 231–235.

[57] Xu, T., Liu, X., Xia, R., Yin, L., et al., SP1-induced upregulation of the long noncoding RNA TINCR regulates cell proliferation and apoptosis by affecting KLF2 mRNA stability in gastric cancer, Oncogene. 2015, 34, pp. 5648–5661.

[58] Chen, Z., Liu, Y., He, A., Li, J., et al., Theophylline controllable RNAi-based genetic switches regulate expression of lncRNA TINCR and malignant phenotypes in bladder cancer cells, Sci. Rep. Nature Publishing Group 2016, 6, p. 30798.

[59] Doyle, F., Tenenbaum, S. A., Trans-regulation of RNA-binding protein motifs by microRNA, Front. Genet. 2014, 5, pp. 1–10.

[60] Doyle, F., Lapsia, S., Spadaro, S., Wurz, Z. E., et al., Engineering Structurally Interacting RNA (sxRNA), Sci. Rep. Nature Publishing Group 2017, 7, p. 45393.

[61] Liang, X., Shen, W., Sun, H., Migawa, M. T., et al., Translation efficiency of mRNAs is increased by antisense oligonucleotides targeting upstream open reading frames, Nat. Biotechnol. Nature Publishing Group 2016, 34, pp. 875–880.

[62] Barron, N., Sanchez, N., Kelly, P., Clynes, M., MicroRNAs: Tiny targets for engineering CHO cell phenotypes?, Biotechnol. Lett. 2011, 33, pp. 11–21.

[63] Gammell, P., Barron, N., Kumar, N., Clynes, M., Initial identification of low temperature and culture stage induction of miRNA expression in suspension CHO-K1 cells, J. Biotechnol. 2007, 130, pp. 213–218.

[64] Barron, N., Kumar, N., Sanchez, N., Doolan, P., et al., Engineering CHO cell growth and recombinant protein productivity by overexpression of miR-7, J. Biotechnol. Elsevier B.V. 2011, 151, pp. 204–211.

[65] Druz, A., Son, Y. J., Betenbaugh, M., Shiloach, J., Stable inhibition of mmu-miR-466h-5p improves apoptosis resistance and protein production in CHO cells, Metab. Eng. Elsevier 2013, 16, pp. 87–94.

[66] Strotbek, M., Florin, L., Koenitzer, J., Tolstrup, A., et al., Stable microRNA expression enhances therapeutic antibody productivity of Chinese hamster ovary cells, Metab. Eng. Elsevier 2013, 20, pp. 157–166.

[67] Kelly, P. S., Breen, L., Gallagher, C., Kelly, S., et al., Re-programming CHO cell metabolism using miR-23 tips the balance towards a highly productive phenotype, Biotechnol. J. 2015, 10, pp. 1029–1040.

[68] Fischer, S., Mathias, S., Schaz, S., Emmerling, V. V., et al., Enhanced protein production by microRNA-30 family in CHO cells is mediated by the modulation of the ubiquitin pathway, J. Biotechnol. Elsevier B.V. 2015.

[69] Jadhav, V., Hackl, M., Klanert, G., Hernandez Bort, J. a., et al., Stable overexpression of miR-17 enhances recombinant protein production of CHO cells, J. Biotechnol. Elsevier B.V. 2014, 175, pp. 38–44.

[70] Bort, J. a H., Hackl, M., Höflmayer, H., Jadhav, V., et al., Dynamic mRNA and miRNA profiling of CHO-K1 suspension cell cultures, Biotechnol. J. 2012, 7, pp. 500–515.

[71] Emmerling, V. V., Fischer, S., Stiefel, F., Holzmann, K., et al., Temperature-sensitive miR-483 is a conserved regulator of recombinant protein and viral vector production in mammalian cells, Biotechnol. Bioeng. 2016, 113, pp. 830–841.

[72] Maccani, A., Hackl, M., Leitner, C., Steinfellner, W., et al., Identification of microRNAs specific for high producer CHO cell lines using steady-state cultivation, Appl. Microbiol. Biotechnol. 2014, 98, pp. 7535–7548.

Figure legends

Figure 1. A theoretical example of transgene expression control based on an endogenously expressed microRNA. Transgene expression is repressed during growth phase, when microRNA levels are high, and it is turned on to the right dose when the culture enters in stationary phase and the "controller" microRNA levels drop.

Figure 2. Schematic representation of different miRNA-based genetic switches or circuits able to react to specific miRNAs signatures. OFF-type switches or circuits are able to repress the expression of the output gene in the presence of specific miRNA signatures. (A) – The output gene is controlled by miRNA-binding sites at its UTR, which lead to translation inhibition in the presence of the specific miRNA(s) signature. (B) – The output gene is controlled by a transactivator-responsive element (TRE). The transactivator protein is controlled by miRNA-binding sites at its UTR, which inhibit its translation in the presence of the specific miRNA signature. ON-type switches or circuits are able to allow the expression of the output gene in the presence of specific miRNA signature. (C) - The output gene is controlled by a repressor-dependent promoter (RepO). The repressor protein is controlled by miRNA-binding sites at its UTR, which inhibit its translation in the presence of the specific miRNA(s) signature. (D) – Double inversion module. The output gene is controlled by a repressor-dependent promoter. The repressor protein is controlled by a transactivator protein. In addition, both the repressor protein and the transactivator protein have miRNA-binding sites in their 3'-UTR, specific to high abundance miRNAs. Only in the presence of the specific high miRNA the output protein can be expressed.

Figure 3. Schematic representation of potential novel miRNA-based responsive elements. (A) – The binding of a Structural Interacting RNA (sxRNA) leads to the formation of a

particular stabilized secondary structure, which can be recognized by a RNA-binding protein (RBP). (B) – The presence of upstream open reading frames (uORF) reduces the translation efficiency of the primary ORF (pORF) leading to lower levels of full length protein. The binding of an antisense oligonucleotides (ASO) complementary to the start codon region of the uORF blocks translation initiation events on this ORF, hence increasing the translation of the pORF.

Figure 1

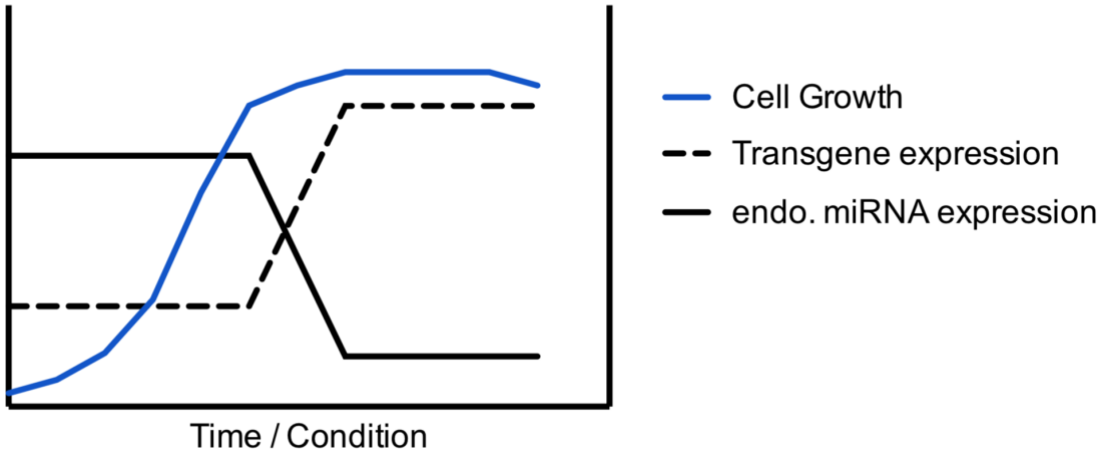
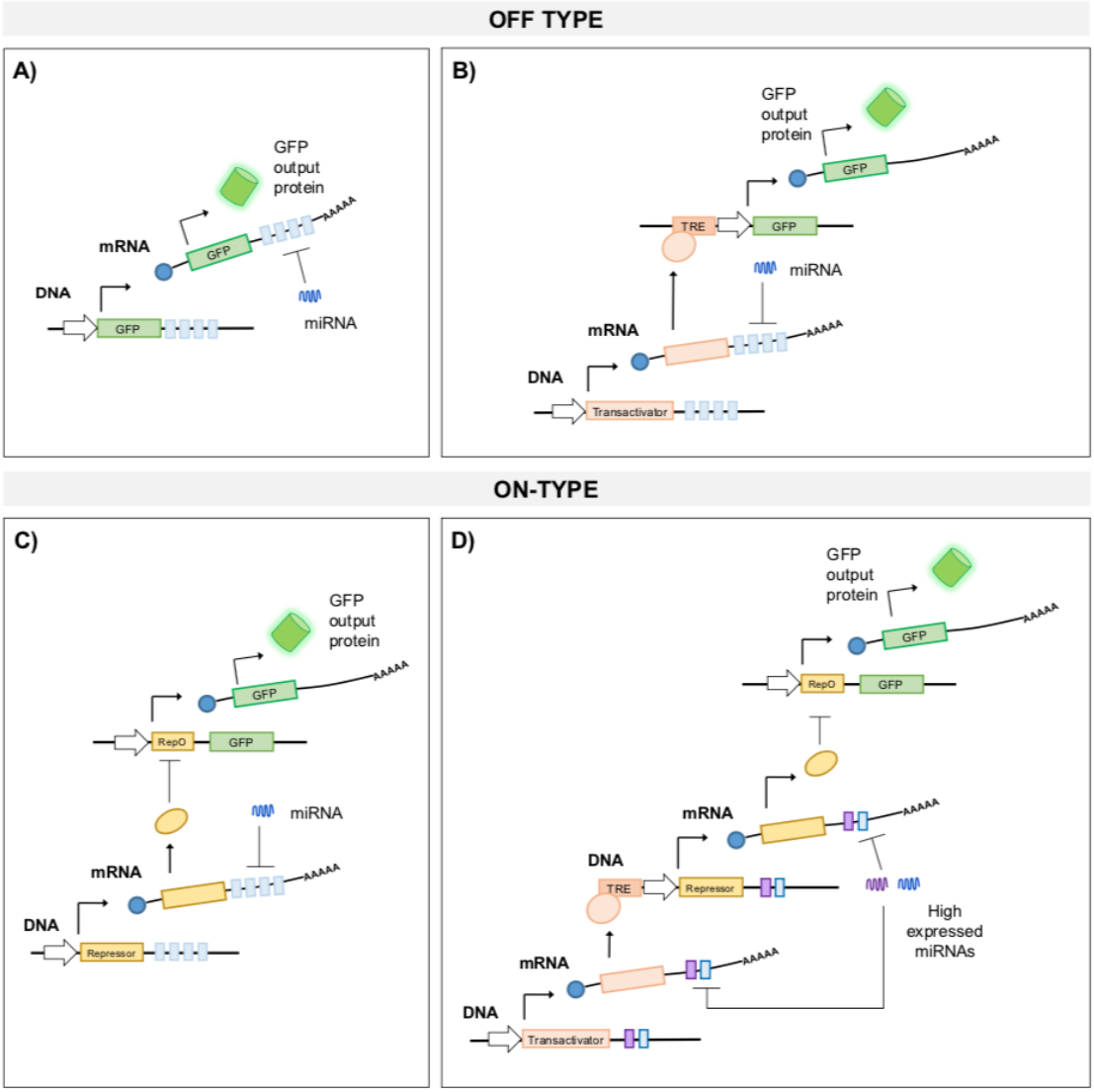
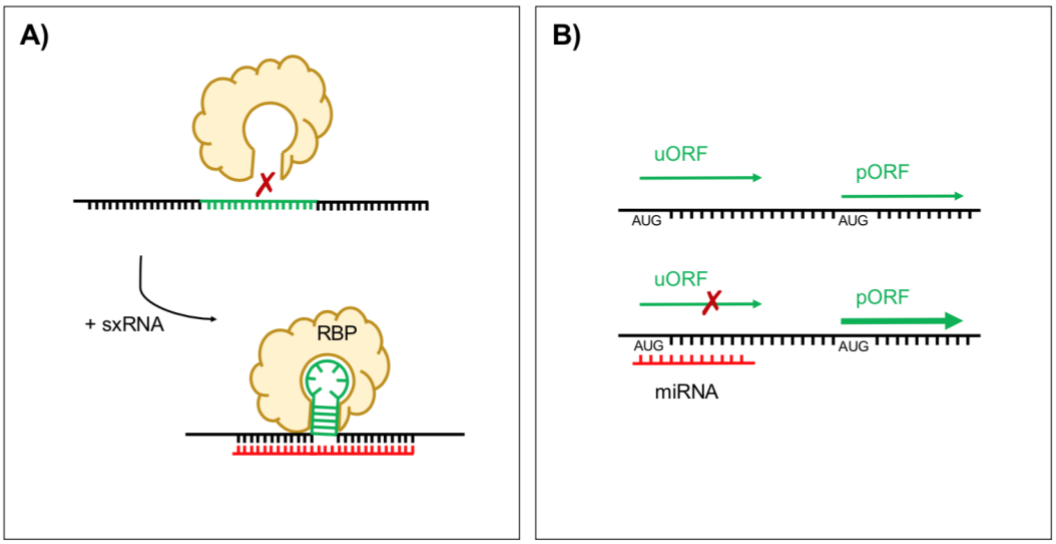

Cell Growth
Transgene expression
endo. miRNA expression
Time / Condition

Figure 2


OFF TYPE
A)
GFP
output
protein
mRNA
GFP
AAAAA
DNA
GFP
miRNA
B)
GFP
output
protein
GFP
AAAAA
TRE
GFP
miRNA
mRNA
AAAAA
DNA
Transactivator
ON-TYPE
C)
GFP
output
protein
GFP
AAAAA
RepO
GFP
miRNA
mRNA
AAAAA
DNA
Repressor
D)
GFP
output
protein
GFP
AAAAA
RepO
GFP
mRNA
AAAAA
DNA
TRE
Repressor
High
expressed
miRNAs
mRNA
AAAAA
DNA
Transactivator

1

Figure 3



1

2