Targeting miRNAs with CRISPR/Cas9 to improve recombinant protein production of CHO cells

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Abstract

MicroRNAs with their unique ability to target hundreds of genes have been highlighted as powerful tools to improve bioprocess behaviour of cells. The common approaches to stably deplete miRNAs are the use of sponge decoy transcripts or shRNA inhibitors, which requires the introduction and expression of extra genetic material. As an alternative, we implemented the CRISPR/Cas9 system in our laboratory to generate CHO cells which lack the expression of a specific miRNA for the purpose of functional studies. To implement the system, miR-27a/b was chosen as it has been shown to be upregulated during hypothermic conditions and therefore may be involved in controlling CHO cell growth and recombinant protein productivity. In this chapter, we present a protocol for the knockdown of miR-27 expression in CHO cells using CRISPR and the analysis of the resulting phenotype.

1 Introduction

Chinese Hamster Ovary (CHO) cells are the most prominent cell line used in biopharmaceutical production for therapeutic applications in treatment of various diseases such as haemophilia, cancer and inflammatory disorders. However, compared to other expression systems like bacteria or yeast, CHO cells generally yield considerably less amounts of protein product. Optimisation approaches like the improvement of media formulations and bioprocess regimes have achieved great benefits however, genetic engineering strategies to generate cell lines with improved bioprocess phenotypes are also being considered. Targeting of protein-coding genes for engineering of apoptosis (Baek, Noh, & Lee, 2017), secretion (Le Fourn, Girod, Buceta, Regamey, & Mermod, 2014), protein folding (Josse, Smales, & Tuite, 2012), metabolism (Toussaint, Henry, & Durocher, 2016) or glycosylation (Wang, Yin, Chung, &

Betenbaugh, 2017) have been shown to be very successful strategies to create tailored CHO producer cell lines.

MicroRNAs (miRNAs) have been demonstrated as useful tools for cell line engineering to improve the bioprocess characteristics such as growth, productivity, longevity, expression of difficult to express proteins and many more potentially critical processes (reviewed by (Barron et al; Jadhav et al., 2013). MiRNAs are small (~22 nts) non-coding RNAs and are involved in the regulation of hundreds of different mRNA. Two mechanisms of miRNA target interaction are well understood involving the block of translation due to a physical hindrance or the degradation of the target mRNA catalysed by the RNA-induced silencing complex (RISC). Besides being able to regulate entire pathways, miRNAs are processed from a non-coding precursor and therefore do not impose a translational burden if used as engineering tool. The non-coding primary transcript is processed into an immature pre-miR consisting of three regions: the mature sequence, star sequence and the stem loop (Fromm et al., 2015). The mature sequence is the more commonly selected strand of the duplex and is more likely to be incorporated into the RISC, whist the star sequence is degraded (reviewed by (Pasquinelli, 2012). Depending on the location in the pre-miR the miRNA is also referred as -5p and -3p e.g. miR-24-5p or miR-24-3p.

Differential expression of miR-27 was first identified as part of a temperature shift study by Gammel and colleagues (Gammell et al., 2007) where it was significantly reduced during hypothermic conditions, suggesting a role in proliferation as well as increased productivity and therefore represented an interesting target in cell line engineering. As part of a microRNA-cluster (miR-23-24-27 cluster), miR-27 consists of two genomic paralogues: miR-27a and miR-27b which only differ in one nucleotide. Several studies showed that miR-27a/b play a role in apoptosis by either targeting pro-apoptotic or anti-apoptotic proteins possibly dependent on the specific circumstances of the cell environment. Agrawal and colleagues showed that miR-27 targets APAF1 which inhibits apoptosis during hypoxia (Agrawal et al., 2014). Further studies also showed that miR-27 negatively regulates FADD which interacts with caspase-8 in the apoptotic signalling pathway and thereby prevents apoptosis in several human cell lines (Chhabra, Adlakha, Hariharan, Scaria, & Saini, 2009). In addition, miR-27 is involved in cell survival by targeting Myt-1 which has been shown to inhibit G_2-M phase (Mertens-Talcott, Chintharlapalli, Li, & Safe, 2007) presumably leading to a higher percentage of cells in S phase supporting our hypothesis in being involved in proliferation.

A common method for the stable depletion of miRNAs is the use of sponge decoys. The generation of miRNA sponges is straightforward and has been shown to be very reliant and effective (Kluiver et al., 2012). Several studies in CHO showed that stable inhibition of miRNAs can increase recombinant protein production and enhances longevity (Paul S. Kelly, 2014; Sanchez et al., 2014). Whilst miRNA sponges have a valuable tool for stable miRNA inhibition the necessity to introduce a reporter gene e.g. GFP or luciferase is not desired for the generation of industry cell lines and a complete knockout of a miRNA could potentially be more beneficial than a knockdown. To address this, we proposed to utilise the recently developed CRISPR/Cas9 system. This system consists of two main components: the single guide RNA (sgRNA) and a CRISPR-associated endonuclease (Cas9) which induces double strand breaks (DSBs). These DSBs can result in the insertion or deletion (indels) of base pairs which can disrupt gene function. Several studies have already proved the successful application of CRISPR/Cas9 in CHO cells with knockouts or insertion of genes aiming to influence e.g. product quality of monoclonal antibodies (Lee, Kallehauge, Pedersen, & Kildegaard, 2015; Ronda et al., 2014). Besides targeting protein-coding genes, it has been also shown that miRNAs can be targeted using CRISPR/Cas9 (Chang et al., 2016; Zhao et al., 2014). However, targeting non-coding RNA represents a special challenge as deletion of one or two base pair may not impair the function of the RNA. It was proposed that indels in the stem loop of pre-miRNAs could inhibit the recognition by Dicer or Drosha and therefore lead to lower levels of mature miRNA making this system a suitable tool for miRNA loss-of-function studies. In this protocol, we show that CRISPR/Cas9 can be successfully used to target miRNAs in CHO and show that this system is a valuable alternative to sponge decoy overexpression negating the requirement for introducing transgenes.

2 Materials

2.1 Expression plasmid construction

1. Guide RNA design tool e.g. GPP Web Portal (Broad Institute)
2. Genomic DNA sequence of miRNA target
3. Ampicillin (Sigma Aldrich)
4. PX459 sgRNA expression plasmid (Addgene #48139)
5. LB broth media and LB-Agar (Sigma Aldrich)
6. Petri dishes 90mm (Corning)
7. Subcloning Efficiency E. coli DH5-α (Thermo Fisher Scientific)
8. 250 mL Erlenmeyer flasks (glass)
9. DNA/RNA quantification device e.g. Nanodrop1000 (Thermo Fisher)
10. Sterile ultrahigh purity water
11. 1.7 mL reaction tubes (Starlab)
12. PCR thermo cycler (e.g. G-Storm)
13. BbsI FastDigest restriction enzyme (Thermo Fisher Scientific)
14. Agarose (Sigma Aldrich)
15. 50x Tris-Acetate-EDTA buffer (Sigma Aldrich)
16. Gel chamber and power supply (Bio-Rad)
17. Mini/midi prep kits for plasmid DNA extraction (Invitrogen)
18. Table top centrifuge (Sigma Aldrich)
19. Sterile pipette tips (Starlab)
20. Sterile spatula
21. T4 Polynucleotide Kinase (NEB)
22. Alkaline Phosphatase (Roche)

2.2 Transfection of CHO cell lines

1. TransIT-X2 Dynamic Delivery System (LLC Bio)
2. CHO-mAb cell line, IgG producing
3. Puromycin (Gibco)
4. Flat bottom 6-well plates (Corning)
5. DMEM/F12 media
6. Foetal Bovine Serum (Gibco)
7. BalanCD® CHO Growth A (Irvine Scientific)
8. T75 flasks vented lid (Corning) or 50 mL bioreactor tubes (TPP)
9. Kuhner, Incubator ISF1-X, Climo shaker
10. Static incubator 37°C, humidified

2.3 Indel analysis

1. Gel extraction kit (Invitrogen)
2. Surveyor® Mutation Detection Kit (IDT)
3. PCR thermo cycler (e.g. G-Storm)
4. Agarose (Sigma Aldrich)
5. Platinum® High fidelity Polymerase kit (Invitrogen)
6. Genomic DNA isolation kit (Qiagen)
7. Phosphate buffered saline (PBS)
8. TOPO®TA cloning kit (Invitrogen)
9. Plasmid DNA Mini prep kit (Invitrogen)
10. Primers (e.g. IDT)
11. SafeView™ Nucleic Acid Visualisation Kit (abm Inc.)

2.4 Analysis of mature miRNA expression

1) TaqMan™ assays (Applied Biosystems)
2) Real-time PCR cycler (Applied Biosystems 7500)
3) TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems)

4) MicroAmpTM Optical 96-well reaction plates (Applied Biosystems)
5) TRIzol reagent (Ambion)
6) Nuclease-free H_2O
7) Isopropanol (Sigma Aldrich)
8) Ethanol (Sigma Aldrich)

2.5 Analysis of phenotype in batch and fed-batch cultures

1. 50 mL Bioreactor tubes (TPP)
2. 250 mL Erlenmeyer flasks (Corning)
3. Orbital shaker, Kuhner ISF1-X, Climo shaker
4. BalanCD® CHO Growth A (Irvine Scientific)
5. BalanCD® CHO Feed 1
6. **Optional:** anti clumping agent e.g. Polyvinyl alcohol (PVA)
7. Guava easyCyte benchtop flow cytometer (Merck Millipore)
8. ViaCount reagent (Merck Millipore)
9. Human IgG ELISA Quantitation Kit (Bethyl Laboratories)

3 Methods

The following describes a general procedure for targeting miRNAs in CHO cells as established in our laboratory. Figure 1 describes the general workflow and approach of targeting a miRNA in CHO as well as screening for clones with complete knockouts. As there is no tool available for the design of gRNAs targeting non-coding RNAs in CHO we used the published and publicly available genomic information (https://www.ncbi.nlm.nih.gov/) and standard design tools as provided by the Broad Institute for spCas9 (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design).

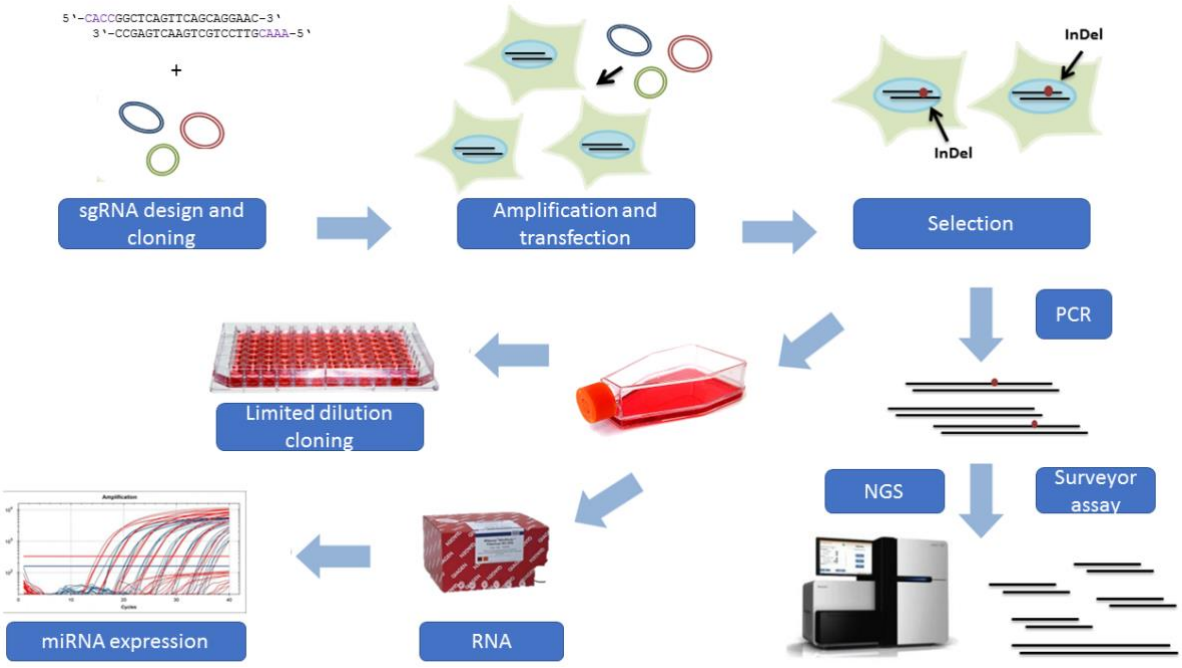


Figure 1: General overview of steps involved in the generation of cells with knockdown of miRNAs. The first steps involve the design and cloning of sgRNAs before transfection of cells. Depending on the approach, transfections can be performed transiently or mixed stable pools can be generated using selection markers e.g. puromycin resistance gene. High-fidelity PCR followed by TOPO-TA cloning as well as Surveyor® assay can be used to assess targeting or alternatively indels can be analysed using next-generation sequencing. Furthermore, miRNA expression after targeting can be analysed using qPCR assays for stable mixed populations and screening single cell clones.

3.1 Guide RNA design considerations for non-coding RNAs

1) Find the genomic sequence of the miRNA target by using publicly available sources e.g. (https://www.ncbi.nlm.nih.gov/).
2) Identify important regions of the miRNA hairpin i.e. seed sequence, mature miRNA and passenger strand.
3) Copy your sequence into a gRNA design tool e.g. provided by https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design
4) Select gRNAs ideally proximal to the seed region of the mature miRNA. Targeting the seed region however, can be limited by the presence of a PAM used by Cas9 for recognition. SpCas9 uses NGG which statistically occurs very frequently in the human genome. If no PAM site is located close to the seed region another Cas nuclease e.g. Staphylococcus aureus (NNGRRT with R=purine) or Cpf1 (YTN with Y=pyrimidine) can be selected.
5) Design your gRNA oligos with additional overhangs for BbsI recognition.
6) Oligos can be synthesised by various providers of primers as non-phosphorylated oligos e.g. IDT.
7) **Comment:** Once the gRNA is cloned into the expression plasmid it will be expressed as a single guide RNA (sgRNA) together with the scaffold which is located in the PX459 plasmid.

3.2 Generation and preparation of sgRNA expression plasmid

1) Purchase PX459 (Addgene, #48139) which is delivered as an agar stab. It encodes a human codon optimised spCas9 version as well as U6 promoter-driven scaffold for expression of sgRNAs.
2) Prepare glycerol stocks according to the supplier's protocol.
3) Inoculate 50 mL LB-broth cultures containing 100 µg/mL ampicillin using 100 µL sterile pipette tips. Incubate cultures over night at 37°C at 250 rpm in sterile 250 mL Erlenmeyer flasks.
4) Isolate plasmid DNA using a midi prep plasmid isolation kit according to the manufacturer's protocol and measure the plasmid concentration.

3.2.1 Annealing, ligation and amplification of sgRNA expression vector

1) Before the gRNA can be ligated into the vector, it is necessary to digest and purify the linear plasmid first.

2) Digest 1 µg of plasmid DNA using 1U of BbsI in a total volume of 20 µL for 1 hour at 37°C.
3) Treat digested plasmid additionally with alkaline phosphatase (AP) at 37°C for 1 hour. To remove enzymes and buffer which could inhibit the ligation reaction purify plasmid using a PCR purification kit with nuclease-free H_2O for elution.
4) **Optional:** Oligos are usually provided dephosphorylated and must be phosphorylated for a successful ligation using PNK. Annealing and PNK treatment can be performed in one step as following:

Component	Amount (µL)
Sense oligo (100µM)	1
Antisense oligo (100µM)	1
PNK buffer (10x)	1
PNK	1
H_2O	6
Total	10

5) Incubate at 37°C for 1 hour and inactivate PNK at 95°C for 20 minutes.
6) Anneal phosphorylated oligos into a duplex using a stepwise reduction of the temperature (-5°C per minute) until the reaction mix reaches 20°C.
7) Dilute duplex 1:200 for ligation and store at -20°C.
8) Set up ligation mix as follow:

Component	Amount (µL)
BbsI digested and AP treated PX459	X
Diluted annealed duplex gRNA	2

10x Ligation buffer	2
T4 DNA Ligase (1U/µL)	1
H_2O	Top up to 20
Total	20

9) Ligate over night at 16°C and store at -20°C until ready for transformation.

3.2.2 Transformation

1) Transform ligated plasmid using competent DH5-α E. coli.
2) Add 2 µL of the ligation mix into 20 µL chemically competent E. coli and incubate for 30 minutes on ice.
3) Heat shock at 42°C for 40 seconds.
4) Incubate on ice for 2 minutes and 700 µL of prewarmed SOC medium was added.
5) Recover at 37°C for 1 hour at 250 rpm in a shaker.
6) Plate on LB-Agar plates containing 100 µg/mL ampicillin and incubate over night at 37°C.
7) Pick colonies next day and cultivate over night at 37°C at 250 rpm in 5 mL LB-Broth containing 100 µg/mL ampicillin. Isolate plasmid DNA using a miniprep kit according to the manufacturer's protocol. Analyse positive ligation of gRNA using Sanger sequencing with specific primers targeting the regions upstream and downstream the scaffold sequence.
8) Expand positive clones into 50 mL LB-Broth cultures containing 100 µg/mL ampicillin. Isolate plasmid DNA using a midi prep kit according to the manufacturer's protocol and measure DNA concentration.

3.3 Transfection and generation of stable cell lines

For the transfection, a CHO-mAb cell line expressing an IgG was used. Transfection was performed in healthy cell cultures with viability above 95 %. Transfections can be performed in suspension as well as using adherent conditions when media is supplemented with FBS (5-

10%). For our transfections, we used serum free conditions. Cell culture conditions for all steps were at 37°C, 170 rpm and 5% CO2 with a humidity of 80%.

1) Refresh media 24 hours prior transfection.
2) Exchange media on day of transfection using 5 mL prewarmed DMEM/F12. **Note:** BalanCD® CHO Growth A can limit transfection efficacy and it is recommended to exchange the media for transfection.
3) **Optional:** Trypsinise cells if attached conditions are used.
4) Count cells and transfer $1x10^6$ cells into a final volume of 1 mL into 50 mL bioreactor spinner tubes. **Optional:** For attached cultures transfer $1x10^6$ cells in 6 well plates.
5) Perform complex formation according to the manufacturer's protocol. For plasmid DNA a ratio of 1:1 plasmid DNA/TransIT-X2 is common. Allow 30 minutes for complex formation.
6) Add complex to cell suspension and incubate for 3 hours.
7) Spin down and exchange media to BalanCD® CHO Growth A or preferred culture media.
8) Exchange media after 24 hours and apply selection pressure using 10 µg/mL puromycin. Maintain selection pressure for 2-3 weeks with routine passaging of cells using seeding densities of $2x10^5$ cells/mL.
9) Sample for RNA and DNA extraction after selection for further analysis of indels and for mature miRNA expression

Optional: After selection limited dilution cloning or FACS for generation of single cell clones can be performed if desired. Especially Cas9 versions fused to GFP can be effectively sorted using FACS.

Recommended: Usual concentration of puromycin varies between 1-10 µg/mL for mammalian cell lines. For each cell line used a kill carve is recommended.

Note: If the phenotype will be assessed transfer cells into the appropriate media and adapt cells for 3-4 passages prior to assessment.

3.4 Analysis of indels generated by CRISPR/Cas9 using TOPO®TA cloning and Surveyor® assay

For the analysis of Indels generated, DNA can be isolated and amplified using PCR. Primers targeted the region ± 400bp of selected miRNA. PCR products can be ligated into TOPO®TA cloning vectors and analysed by Sanger sequencing. It is recommended to optimise primers as single specific bands for the performance of the Surveyor® assay are necessary. Furthermore, high fidelity enzymes for PCR amplification are required to exclude amplification errors.

1) Isolate genomic DNA using $1x10^6$ cells following the manufacturer's protocol and measurement of DNA concentration.
2) Perform high-fidelity PCR according to the manufacturer's protocol using optimised annealing temperatures, 100ng of genomic DNA and 10nM final concentration of forward and reverse primer.
3) Purify the PCR product using a PCR purification kit according to the manufacturers protocol.
4) Run an analytical gel to ensure specificity of PCR. If non-specific bands are detectable perform gel extraction of the correct amplicon.

Note: Same purified PCR amplicon can be used for TOPO®TA and Surveyor® assay.

3.4.1 TOPO®TA cloning

Important: many high-fidelity PCR kits will give a mix of blunt- and sticky- end amplicons which can be used for TOPO®TA cloning. If high-fidelity polymerases are used e.g. Pfu-polymerase which will result in a blunt end PCR amplicon a TOPO® kit with corresponding vector can be purchased or the addition of an A-overhang using Taq-polymerase is recommended.

1) PCR product can be directly ligated according to the manufacturer's protocol into TOPO®TA plasmids without purifying the PCR reaction.
2) Transformation according to 3.2.2.
3) Spread 40µL of a 20mg/mL X-Gal solution in DMF on a kanamycin (50µg/mL) containing LB-agar plate and incubate for 30 minutes as 37°C.

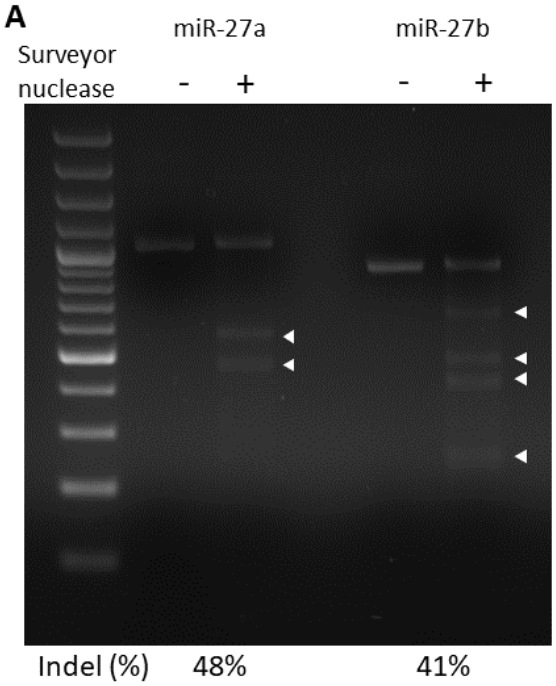
4) Select positive clones and expand into 5mL LB-broth cultures containing 50µg/mL kanamycin as well as incubation over night at 37°C.
5) Plasmid isolation using miniprep kits and analysis using primers provided by the manufacturer.
6) Sequence inserts for comparison to wildtype genomic sequence.

3.4.2 Surveyor® assay for detection of indels

5) Purify PCR product and anneal wildtype DNA with mutated DNA. Denature double stranded DNA at 95°C for 10 minutes. A stepwise reduction of the temperature is recommended and described in the manufacturer's protocol.
6) Treat wildtype and reannealed PCR amplicon with Surveyor® nuclease.
7) Analysis of treated PCR product on 2% agarose gel.

3.4.3 Next generation sequencing for analysis of indels

For higher resolution information of the frequency of indels as well as the efficacy of targeting we recommend the use of next-generation sequencing. It will give more information whether both alleles are targeted efficiently. First, the targeted region is amplified using high-fidelity PCR (see 3.4). Primers are designed to amplify approximately 200-300 base pairs around the targeted region. For library generation linkers are added to the primers. Linker sequence depends on the NGS core facility used.



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miR-27a	Sequence	Indel
WT	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	--
1	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	0
2	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	0
3	CCGCAGCAAGTCGTGTTCAACAGTGGCTAAGTT	+1
4	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	0
5	CCGCAGCAAGTCGTGTTC---CAGTGGCTAAGTT	-1
6	CCGCAGCAAGTCGTGTTCCCCAGTGGCTAAGTT	+2
7	CCGCAGCAAGTCGTGTTCA----GT-AGCTAAGTT	-2
8	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	0
9	----------CAGTGGCTAAGTT	-62
10	CCGCAGCAAGTCGTGTTCAACAGTGGCTAAGTT	+1
11	CCGCAGCAAGTCGTGTTCAACAGTGGCTAAGTT	+1
12	CCGCAGCAAGTCGTGTTCACCAGTGGCTAAGTT	+1

miR-27b	Sequence	Indel
WT	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	--
1	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
2	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0
3	TTGGTTTCCGCTTT---------CAGTGGCTAAGTT	-5
4	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
5	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0
6	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0
7	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
8	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
9	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0
10	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
11	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
12	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0

miRNA seed

C

sgRNA	% (wt)	% (indel)
miR-27a	16.29%	83.71%
miR-27b	11.16%	88.84%

Figure 2: Indel analysis using TOPO-TA cloning combined with Sanger sequencing, Surveyor® assay and next-generation sequencing. A) Analysis of Surveyor® Mutation Detection Assay shows digestion of the amplified genomic region due to mismatch formation for both targeted miRNAs. B) PCR products cloned into TOPO®TA and analysed clones shows a variety of indels proximal to the targeted region. C) Next-generation sequencing shows high efficacy of targeting of both miRNAs up to 88%.

3.5 Analysis of mature miR-27 expression after targeting using CRISPR/Cas9

For the analysis of mature miRNA expression, RNA was isolated and reverse transcribed using specific stem loop primers for miR-27b. Expression was analysed using TaqMan™ assays for miR-27b and miR-27b* on a qPCR cycler and normalised to U6 snRNA expression.

1) Total RNA isolation using $1x10^6$ cells. Spin down at 800g for 5 minutes and discard supernatant.
2) Wash pellet 3 times with PBS.

3) Resuspend cell pellet in 1 mL TriZOL and isolate RNA following the manufacturer's protocol. Store at -80°C
4) Measure quality and concentration.
5) Use a total of 100ng RNA for reverse transcription according to the manufacturer's protocol. Specific stem loop primers for U6 snRNA, miR-27b and miR-27b* are used.
6) **Optional:** To avoid the risk of contaminating genomic DNA the RNA can be treated with DNaseI. However, the final concentration must be adjusted for RT-qPCR.
7) Analyse mature miRNA expression on a qPCR cycler using the corresponding assays and normalise to U6 snRNA expression. Use ddCt method for analysis of differential expression.
8) Same principle can be applied for a larger clone screen to find clones which show an appropriate knockdown of miR-27b or any other miRNA.

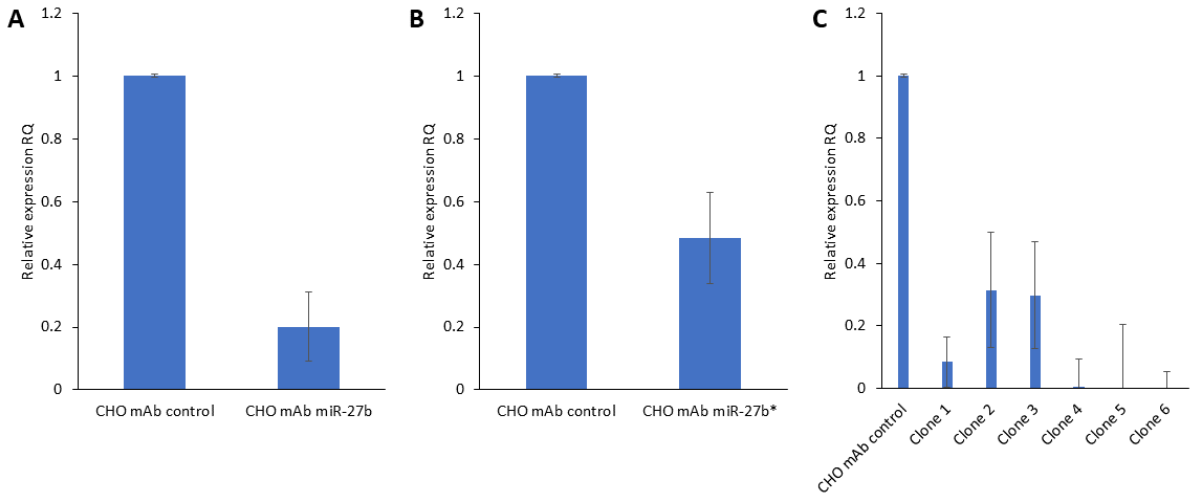

Figure 3: Expression of miR-27b and miR-27b* as well as selected clones. A and B) Targeting miR-27b using CRISPR/Cas9 led to reduced levels of mature miRNAs in mixed population with a knockdown of up to 80%. Furthermore, mature levels of the passenger strand (miR-27b*) were reduced as well. C) Analysis of mature miR-27b expression after limited dilution cloning and isolation of single cell clones showed clones which show a significant reduced level or no miR-27b expression.

3.6 Analysis of phenotype in batch and fed-batch cultures after knockdown of miR-27b using CRISPR/Cas9

For the analysis of the phenotype, the mixed miR-27b depleted population was compared to a control population expressing Cas9 as well as a non-targeting scaffold, in batch cultures of 5mL as well as fed-batch cultures of 30 mL.

1) Count cells using Guava and ViaCount reagent. **Note:** Use only cultures with viability >95%.
2) For batch cultures seed cells with a density of $2x10^5$ cells/mL in either 50mL bioreactor tubes with a total volume of 5mL. For fed-batch cultures use 250mL Erlenmeyer flasks with a total volume of 25mL at day 0. Cultures are seeded in triplicate and assayed for viability every day or every second day using ViaCount reagent on a Guava benchtop flow cytometer. Take conditioned media samples every second day for IgG quantification using ELISA. Addition of feed corresponds to the media used and can be optimised according to the media provider. For growth curves using BalanCD CHO Growth A we added 10% BalanCD CHO Feed 1 on days 1, 5 and 7. **Note:** Growth curves can take up to 10-12 days depending on medium used for batch cultures and are usually stopped once viability falls below 80%.
3) Spin down conditioned media samples for 5 minutes at 800g and store at -80°C until usage.
4) Use ELISA IgG quantification kit to assess titre and productivity calculation

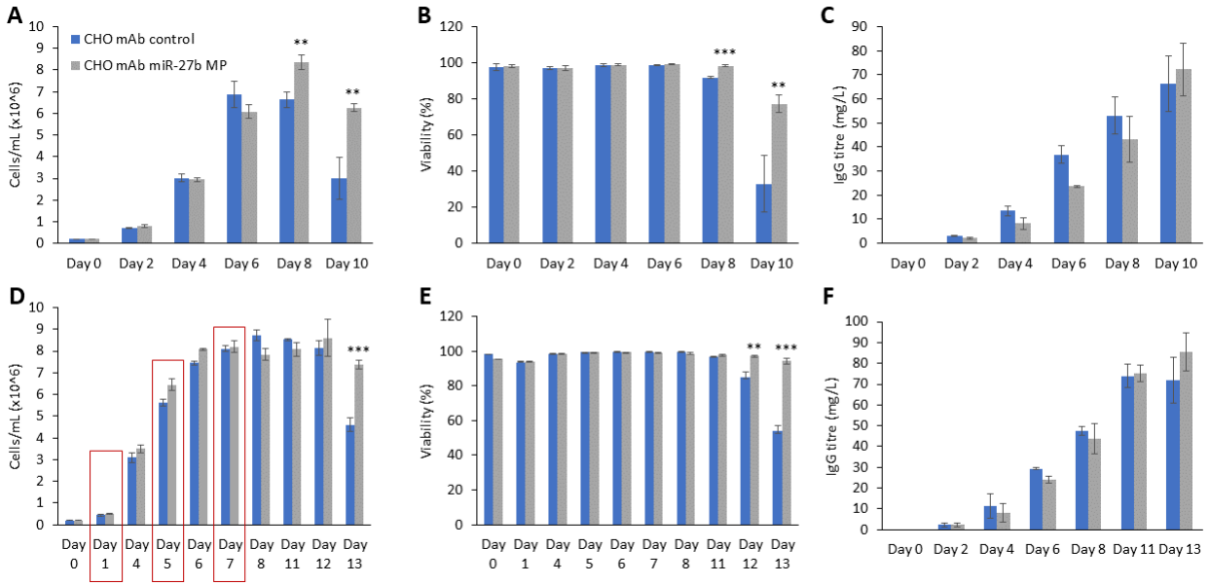

Figure 4: Phenotype after depletion of miR-27b in batch and fed-batch cultures. A and B) Batch cultures showed increased viable cell densities and increased viability on day 8 and 10 of the cultures. C) IgG titre was increased but not significantly due to increased longevity. D and E) Fed-batch cultures showed increased viable cell density on day 13 and increased viability on day 12 and day 13 compared to the control. F) IgG titre was not significantly in fed-batch.

4 Conclusions

In conclusion, we showed that it is possible to target miRNAs in CHO cells using the recently developed CRISPR/Cas9 system. We implemented the system on miR-27a/b and achieved ≥ 80% targeting efficiency. Indel analysis and TOPO-TA cloning combined with Sanger Sequencing showed a range of different indels. Furthermore, it was possible to identify clones with no detectable expression of mature miR-27. Depletion of miR-27 led to improved viability in late stages of batch and fed-batch cultures making it a potentially interesting target to improve bioprocess performance of CHO cells.

5 Literature

Agrawal, R., Pandey, P., Jha, P., Dwivedi, V., Sarkar, C., & Kulshreshtha, R. (2014). Hypoxic signature of microRNAs in glioblastoma: Insights from small RNA deep sequencing. *BMC Genomics, 15*, 686-2164-15-686. 10.1186/1471-2164-15-686 [doi]

Baek, E., Noh, S. M., & Lee, G. M. (2017). Anti-apoptosis engineering for improved protein production from CHO cells. *Methods in Molecular Biology (Clifton, N.J.), 1603*, 71-85. 10.1007/978-1-4939-6972-2_5 [doi]

Barron, N., Sanchez, N., Kelly, P., & Clynes, M. (2011). MicroRNAs: Tiny targets for engineering CHO cell phenotypes? *Biotechnology Letters, 33*(1), 11-21. 10.1007/s10529-010-0415-5 [doi]

Chang, H., Yi, B., Ma, R., Zhang, X., Zhao, H., & Xi, Y. (2016). CRISPR/cas9, a novel genomic tool to knock down microRNA in vitro and in vivo. *Scientific Reports, 6*, 10.1038/srep22312. 10.1038/srep22312 [doi]

Chhabra, R., Adlakha, Y. K., Hariharan, M., Scaria, V., & Saini, N. (2009). Upregulation of miR-23a-27a-24-2 cluster induces caspase-dependent and -independent apoptosis in human embryonic kidney cells. *PloS One, 4*(6), e5848. 10.1371/journal.pone.0005848 [doi]

Fromm, B., Billipp, T., Peck, L. E., Johansen, M., Tarver, J. E., King, B. L., . . . Peterson, K. J. (2015). A uniform system for the annotation of vertebrate microRNA genes and the evolution of the human microRNAome. *Annual Review of Genetics, 49*, 213-242. 10.1146/annurev-genet-120213-092023 [doi]

Gammell, P., Barron, N., Kumar, N., & Clynes, M. (2007). Initial identification of low temperature and culture stage induction of miRNA expression in suspension CHO-K1 cells. *Journal of Biotechnology, 130*(3), 213-218. S0168-1656(07)00289-1 [pii]

Jadhav, V., Hackl, M., Druz, A., Shridhar, S., Chung, C. Y., Heffner, K. M., . . . Borth, N. (2013). CHO microRNA engineering is growing up: Recent successes and future challenges. *Biotechnology Advances, 31*(8), 1501-1513. 10.1016/j.biotechadv.2013.07.007 [doi]

Josse, L., Smales, C. M., & Tuite, M. F. (2012). Engineering the chaperone network of CHO cells for optimal recombinant protein production and authenticity. *Methods in Molecular Biology (Clifton, N.J.), 824*, 595-608. 10.1007/978-1-61779-433-9_32 [doi]

Kluiver, J., Gibcus, J. H., Hettinga, C., Adema, A., Richter, M. K., Halsema, N., . . . van den Berg, A. (2012). Rapid generation of microRNA sponges for microRNA inhibition. *PloS One, 7*(1), e29275. 10.1371/journal.pone.0029275 [doi]

Le Fourn, V., Girod, P. A., Buceta, M., Regamey, A., & Mermod, N. (2014). CHO cell engineering to prevent polypeptide aggregation and improve therapeutic protein secretion. *Metabolic Engineering, 21*, 91-102. 10.1016/j.ymben.2012.12.003 [doi]

Lee, J. S., Kallehauge, T. B., Pedersen, L. E., & Kildegaard, H. F. (2015). Site-specific integration in CHO cells mediated by CRISPR/Cas9 and homology-directed DNA repair pathway. *Scientific Reports, 5*, 8572. 10.1038/srep08572 [doi]

Mertens-Talcott, S. U., Chintharlapalli, S., Li, X., & Safe, S. (2007). The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. *Cancer Research, 67*(22), 11001-11011. 67/22/11001 [pii]

Pasquinelli, A. E. (2012). MicroRNAs and their targets: Recognition, regulation and an emerging reciprocal relationship. *Nature Reviews.Genetics, 13*(4), 271-282. 10.1038/nrg3162 [doi]

Paul S. Kelly. (2014). *Enhancing CHO cel productivity through the stable depletion of microRNA-23*

Ronda, C., Pedersen, L. E., Hansen, H. G., Kallehauge, T. B., Betenbaugh, M. J., Nielsen, A. T., & Kildegaard, H. F. (2014). Accelerating genome editing in CHO cells using CRISPR Cas9 and CRISPy, a web-based target finding tool. *Biotechnology and Bioengineering, 111*(8), 1604-1616. 10.1002/bit.25233 [doi]

Sanchez, N., Kelly, P., Gallagher, C., Lao, N. T., Clarke, C., Clynes, M., & Barron, N. (2014). CHO cell culture longevity and recombinant protein yield are enhanced by depletion of miR-7 activity via sponge decoy vectors. *Biotechnology Journal, 9*(3), 396-404. 10.1002/biot.201300325 [doi]

Toussaint, C., Henry, O., & Durocher, Y. (2016). Metabolic engineering of CHO cells to alter lactate metabolism during fed-batch cultures. *Journal of Biotechnology, 217*, 122-131. 10.1016/j.jbiotec.2015.11.010 [doi]

Wang, Q., Yin, B., Chung, C. Y., & Betenbaugh, M. J. (2017). Glycoengineering of CHO cells to improve product quality. *Methods in Molecular Biology (Clifton, N.J.), 1603*, 25-44. 10.1007/978-1-4939-6972-2_2 [doi]

Zhao, Y., Dai, Z., Liang, Y., Yin, M., Ma, K., He, M., . . . Teng, C. B. (2014). Sequence-specific inhibition of microRNA via CRISPR/CRISPRi system. *Scientific Reports, 4*, 3943. 10.1038/srep03943 [doi]