MSc in Regenerative Medicine and Technology
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Major Research Project

“Production of modRNA for *in vivo* prime editing and other therapeutic strategies”

Elena Jiménez Curiel

Supervisors

Indi Joore

Sabine Fuchs

Utrecht University

UMC Utrecht

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LAYMAN SUMMARY

Rare hereditary disorders are a group of diseases caused by mutations in the DNA. Although they are among the most common causes of child death in The Netherlands, treatments are difficult to come by because different mutations are responsible for different diseases and there can even be several mutations causing the same disorder.

Genetic editing techniques can solve this issue as they allow the correction of these genetic mutations. Several methods have been developed in the recent years, each with their own advantages and drawbacks. Prime editing is one of these newer techniques and the one chosen for this project. In brief, the prime editor complex can move to the site of the mutation, make a cut in the DNA, and create a small DNA sequence which contains the correct nucleotide. Then, this newer sequence is incorporated in the DNA strand, replacing the mutated version. The prime editing machinery can be delivered to the body using viruses, but they come with many disadvantages such as the risk of permanent incorporation into the host genome and the risk of causing cancer. To bypass this issue, we sought to use modified RNA.

Modified RNA (modRNA) refers to RNA molecules produced *in vitro*. Once they are introduced into the cell, they can be translated into protein by the cell's own machinery. ModRNA can be delivered using non-viral methods and it is degraded after ~72h. It has already been used for expression of different types of proteins, all of them with promising results. Therefore, the aim of this project was to produce modRNA of the prime editing tools to correct the mutations responsible for several metabolic disorders, with the final goal of achieving *in vivo* prime editing for treatment of patients. After several rounds of optimization, we were able to produce the needed modRNA molecules, which were capable of editing.

Taking advantage of the modRNA production platform, we sought to find proteins that could be interesting for the treatment of other disorders. The mitochondrial pyruvate carrier 1 (MPC1) is a channel protein that is in charge of transporting pyruvate, the resulting product of glycolysis, into the mitochondria. However, pyruvate can also be converted into lactate when deviated from this pathway and this is what happens in cancer cells. Moreover, MPC1 has been shown to have decreased expression in different cancer types and studies have shown that when expression is increased by using DNA delivery into the cells, the rate of proliferation, as well as the viability of cancer cells decreases. Therefore, we produced MPC1 modRNA, and after treating different cell lines, we saw that, indeed, cells slowed down their proliferation.

1. INTRODUCTION

Rare hereditary disorders are characterized by a wide heterogeneity in genetic causes and disease mechanisms, severely hindering the search for treatment. This heterogeneity impacts the different types of disorders and is also present among patients with the same disease. As such, developing treatments for rare hereditary disorders remains challenging, despite it being one of the most common causes of death in children in the Netherlands [1].

The main shared feature of these disorders, the pathogenic genetic mutations, can be used as a focal point for developing new therapies based on correcting these genetic aberrations. Several tools have already been developed to correct those mutations, such as base editing and Cas9-based homolog-directed repair (HDR). However, HDR-based methods generally achieve low editing efficiency and rely on double strand breaks, leading to unwanted indels [2]. Moreover, base editing is limited to the generation of four of the twelve transition mutations, which does not cover the wide variety of pathogenic mutations [2].

Prime editing is a genome editing technique that allows the introduction of any transition mutation, insertion and deletion [3]. Prime editing uses the prime editor, a Cas9 endonuclease fused to a reverse transcriptase (RT) [2]. The prime editing guide (pegRNA) contains the desired edit and guides the prime editor to the site of interest. The spacer sequence of the pegRNA enables the correct targeting of the genome, while the extension is homologous for the genome and contains the intended mutation [2]. The Cas9 nicks the target strand, creating a 3' flap that hybridizes with the PBS and is then used as a primer by the RT to synthesize the new strand which contains the desired mutation (Figure 1.1). Afterwards, the newly synthesized flap can be incorporated in the genome [2]. An optimized version of the pegRNAs was developed, known as engineered pegRNAs (epegRNAs), which contains a looped RNA structure in the 3' end of the pegRNA to decrease degradability [4]. Prime editing has already been shown to be capable of correcting pathogenic mutations in patient-derived organoids, iPSCs and mouse embryos [3].

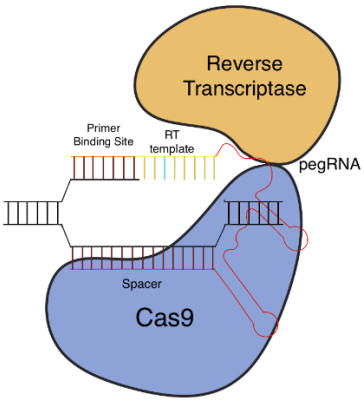

Figure 1.1 Prime editing machinery. Schematic figure showing the prime editor (Reverse transcriptase and Cas9 endonuclease) and pegRNA complex (red). The spacer sequence (purple) binds to the complementary strand while the primer binding sequence (orange) binds to the nicked strand. The RT synthesizes the new strand using the RT template (yellow), which contais the desired mutation (light blue). Figure adapted from *[2]*

Prime editing tools can be delivered to target cells using a DNA carrier, modified RNA (modRNA) or proteins (RNPs). Viral vectors, such as lentiviruses, are the most common method for delivering DNA to the cell, where they are transcribed and translated using the cell's endogenous machinery [5]. However, DNA delivery is inefficient since cytoplasmic DNA triggers cellular immune responses [6]. Moreover, DNA carries the risk of genome integration, leading to long-term expression of the foreign DNA [7], [8]. Furthermore, RNPs are unstable and effective delivery remains challenging [9]. ModRNA overcomes these problems and presents itself as a promising tool for delivering of the prime editing machinery.

modRNA is a messenger RNA molecule produced by *in vitro* transcription (IVT) using a T7 enzyme [10]. A DNA plasmid template is used for the IVT, which includes the desired gene, a T7 promoter sequence for transcription, a start codon, a Kozak consensus sequence for subsequent translation and a 5' and 3' untranslated regions. Moreover, modifications to decrease its immunogenicity and degradability are introduced in the modRNA molecule [7], [8], [10]. These include the use of 5'-methylcytidine and pseudouridine instead of cytosine and uridine, the addition of a 5' guanine cap and a phosphatase treatment after transcription (Figure 1.2) [10]. Furthermore, modRNA has a lower risk of genome integration, as it would first need to be reverse transcribed.

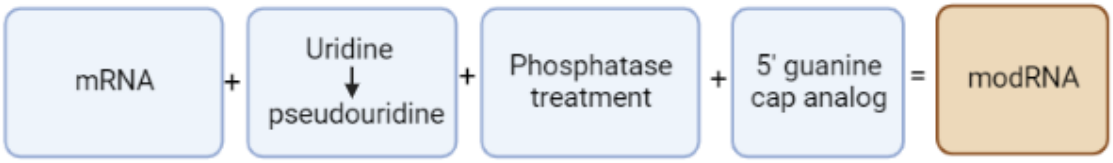

Figure 1.2. Modifications added to mRNA to produce modRNA

Therefore, we propose to set up a platform to produce the prime editing tools as modRNA for the correction of pathogenic variants. First, we will develop a plasmid (pcDNA4) enabling fast production of the prime editing tools as modRNA. Then, the modRNA is used to prime edit 2D cell cultures, patient-derived fibroblasts and patient derived organoids

ModRNA has already been shown to be an effective tool for expressing proteins for various applications, such as for reprogramming of iPSCs, the COVID-19 mRNA vaccines and expression of various therapeutic proteins [7], [8], [11]. Furthermore, p53 modRNA transcripts have been used in *in vivo* hepatocellular carcinoma models, showing decreased tumour viability and increased sensitivity to chemotherapy [12]. As cancer cells exhibit loss of p53 function, using it as a target for treatment is an effective way of selecting only for the tumour cells against the healthy cells [12]. Following this line of thought, we sought to target cancer-specific processes with modRNA production.

One such a cancer-specific process could be the Warburg effect. As opposed to healthy cells, which mostly rely on oxidative phosphorylation, tumour cells mostly rely on anaerobic glycolysis [13]. In order to keep glycolysis running, pyruvate is metabolized to lactate, thus sharply increasing cellular glycolysis [13]. This mechanism, also known as the Warburg effect, deprives the mitochondria of pyruvate, hindering the oxidative phosphorylation pathway (Figure 1.3) [14].

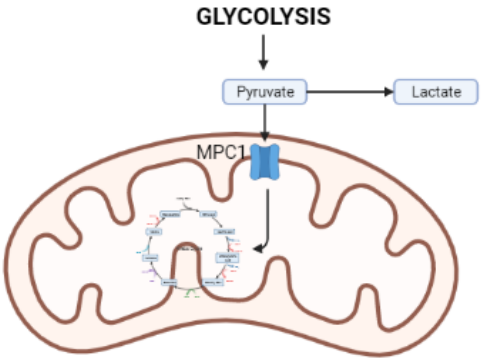

Figure 1.3. Schematic of the potential pathways followed by pyruvate

Even though oxidative phosphorylation yields more ATP molecules than anaerobic glycolysis per glucose molecule, cancer cells are thought to prefer glycolysis because of the higher availability of building blocks to sustain a high rate of proliferation [13], [14].

At the crossroads between both pathways the mitochondrial pyruvate carrier (MPC) can be found (Figure 1.3) [14]. It is a heterodimeric transporter channel, composed of MPC1 and MPC2, located in the inner mitochondrial membrane which transports pyruvate into the mitochondria [14], [15]. When cells shift towards carcinogenic phenotypes, their gene expression pattern is modified to match their new metabolic state [15]. Among these changes is the downregulation of MPC1, observed in most cancer types [15], [16]. With the decreased expression of MPC1, the transport of pyruvate for oxidative phosphorylation is inhibited, favouring the conversion of pyruvate into lactate for anaerobic glycolysis [17]. Possibly, inducing MPC1 expression in cancer cells could dysregulate the glycolysis-dependent metabolism in cancer cells, as pyruvate would be forced to enter the mitochondria, thus hindering the anaerobic glycolysis pathway [17], [18]. Indeed, previous studies have shown that overexpressing MPC1 by means of lentiviral infection leads to decreased proliferation and expression of stem cell markers in gastric cancer cells [18]. Similar results were observed for hepatocellular carcinoma (HCC), where cell viability and tumour size decreased when MPC1 was overexpressed [19]. As previously mentioned, the use of lentiviruses for gene therapy carries certain disadvantages that could be overcome by using modRNA and non-viral delivery methods. Thus, we sought to produce MPC1 modRNA to study its effect on the metabolism of cancer-derived immortalized cells.

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2. MATERIALS AND METHODS

1.1. Materials

1.1.1. Primers

TABLE 2.1. LIST OF PRIMERS USED

Name	Sequence (5’-3’)
Gene cloning	
pcDNA 4 PEmax Fw final	aatataagagccaccatgaaacggacagccgacggaagc
pcDNA 4 PEmax Rv final	cccgcagaaggcagcttagtccagcttcactctcttagcggcag
pcDNA4 GFP Fw	ccgcgaagacggcatgagcaagggcgaggagctgtt
pcDNA4 GFP Rv	gggggaagacggcagcttacttgtacagctcgtccatgccg
epegRNA-in-pcDNA Rv	tacgGAAGACggCAGCttctagttggtttaacgcgtaactagatag
modRNA MPC1 Fw	atggcgggcgcgttgg
modRNA MPC1 Rv	ttatgcagatgccgttttagtcatctcgt
PCR for IVT	
Xu-T120	tttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt tttttttttttttttttttttttttttttttttcttcctactcaggctttattcaaagacca
Xu-F1-pcDNA4	ctaccggtcgtacagaagctaatacg
Tevopreq1 IVT Rv	ttctagttggtttaacgcgtaactagatagaa
epegRNA IVT PCR Fw	atcttgtggaaaggacgaaacacc
Sequencing	
T7 promoter Fw	taatacgactcactatagg
Hu6-Prom-Seq-Fw2	ggctgttagagagataatta
PCR amplification	
HEK3-PCR-Fw	atgtgggctgcctagaaagg
HEK3-PCR-Rv	ggtgctgaaagccactgggc
MUT-ex12-Fw	cctagattgtcttccagggtttt
MUT-ex12-Rv	cactgtccacttttagaccttgt

2.2. Methods

2.2.1. pcDNA4 cloning

The template plasmid pcDNA3.3 was obtained in collaboration with the lab of Zhyong at the UMC. In order to optimize the insertion of target genes into the backbone, restriction sites for BbsI were created between the 5’ UTR and 3’ UTR.

2.2.2. Gene cloning

Gene amplification was carried out by Phusion PCR (New England Biolabs) with 5X Phusion HF Buffer and 10mM dNTPs mix according to the protocol described by the manufacturer. Samples were then run on an agarose gel and isolated. Next, a PCR (Q5 HF Polymerase) was performed on them using primers containing overhangs compatible with the pcDNA4 plasmid (Table 2.1). Samples were again run on an agarose gel and isolated.

2.2.3. epegRNA cloning

epegRNAs were cloned following previously described protocols [20]. Briefly, the pU6-tevopreq1-GG-acceptor plasmid (Adgene) was digested with BsaI-HFv2 (NEB) for 16h and the 2.2kb fragment was isolated from gel. The corresponding oligonucleotides for the pegRNA spacer, pegRNA extension and pegRNA scaffold with the appropriate overhangs were ordered and annealed using T4 ligase buffer (Thermofisher). The annealed spacer, scaffold and extension were ligated into the pU6-tevopreq1-GG-acceptor plasmid following a Golden Gate assembly protocol with BsaI-HFv2 and T4 DNA ligase (Thermofisher), with 12 cycles of 5 min at 16ºC and 5 min at 37ºC and a final inactivation step of 5 min at 72ºC. The resulting epegRNA plasmids were transformed using competent Stellar cells (Takara), purified via miniprep using the PureLink Quick Plasmid Miniprep Kit (Invitrogen) and sent for Sanger sequencing to Macrogen Europe.

2.2.3. Cloning of genes into pcDNA4 plasmid

To clone the gene of interest into the pcDNA4 plasmid two different approaches were used. For GFP, a simple Golden Gate protocol was followed. The GFP with overhangs plasmid was digested with BbsI (NEB) for 1-2h at 37ºC and then ligated into the BbsI-digested pcDNA4 plasmid using a T4 ligase (NEB) reaction with vector:gene molar ratios of 1:3 and 1:5, according to manufacturer's protocol. For PEmax and MPC1, on the other hand, an In-Fusion protocol was used since the gene contained a BbsI restriction site in the coding sequence. For the PEmax, a 5µl reaction was carried out with 1µl In-Fusion 5X mix (Takara) and a vector:gene molar ratio of 1:1, since the PEmax gene was bigger than the pcDNA4 backbone (6396 bp vs 4300 bp). For the MPC1, a 5µl reaction was prepared as well, but with a vector:gene molar ratio of 1:3 and 1:5, since the MPC1 was significantly smaller than the pcDNA4 backbone (330 bp vs 4300 bp). The pcDNA4-gene plasmids were then transformed using competent stellar cells (Takara) following a heat-shock protocol where the cell-plasmid mix (1,5 µg plasmid + 15 µg cells) was incubated 10 minutes on ice, then heat-shock at 42ºC for 45s followed by 15 minutes incubation on ice and incubation with 100µl SOC medium for 45 min at 37ºC+shaking. Finally, cells were centrifuge at 3000g for 1:30 min and plated. Plates were incubated overnight at 37ºC and, if successful, colonies were picked and incubated in LB+Kana at 37ºC in a shaker overnight. Finally, a miniprep was performed using the PureLink Quick Plasmid Miniprep Kit for plasmid isolation and purification and the concentration was measured using a NanoDrop**.** Samples were prepared for sequencing with the appropriate sequencing primer (Table 2.1) and sent to Macrogen Europe for Sanger sequencing. The results were analyzed by the software Benchling.

2.2.3. Cell culture

HEK293T, HepG2 and HeLa cells were maintained in T75 flasks with DMEM + GlutaMax (1x, Gibco) supplemented with 10% FBS (Gibco) and 1x PenStrep (Gibco) and split every 3-4 days.

2.2.4 modRNA protocol

Modified RNA was produced according to the protocol described by Mandal et al. (2013) [10] with some slight modifications. In brief, the pcDNA4 plasmid containing the target gene was digested with the restriction enzyme SpeI (NEB) for 1h at 37ºC followed by a 5-minute inactivation step at 80ºC in a ThermalCycler T100 (Bio-Rad). The digested product was run on an agarose gel to check for by-products and then isolated using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) following manufacturer's instructions. The purified product was then added a poly-A tail via a PCR reaction using the following primers: XU-F1-pcDNA4 and XU-T120 (Table 2.1). For the epegRNAs modRNA no poly-A tail was added and the primer pair used instead was epegRNA IVT PCR Fw and pcDNA4 epegRNA IVT tevopreq Rv. The PCR reaction used a standard Q5 2x Hot Start (NEB) protocol with a final volume of 200µl. The master mix was aliquoted into eight 25µl reactions and cycled using the following protocol:

Table 2.2. PCR CYCLING PROTOCOL

Cycle	Denature	Anneal	Extend
1	95ºC (2-3min)		
2-31	98ºC (20s)	68ºC (15s) – pegRNAs 69ºC (15s) – Pemax, GFP, MPC1	72ºC (30s/kb)
32	72ºC (3min)		

The PCR product was then run on an agarose gel and purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Next, the in vitro transcription reaction was performed using the T7 MEGAScript kit (Thermo-Fisher), a 3'-O-Me-m7G cap analog (NEB) and pseudo-UTP (TriLink) and the tailed PCR product as indicated in Table 2.3 for the NTP mix and Table 2.4 for the IVT reaction.

Table 2.3. NTP MIX COMPOSITION

Component	Stock solution (mM)	Volume Per (IVT)
3′-O-Me-m7G cap analog	60	4
GTP	75	0,8
ATP	75	4

CTP	75	4
Pseudo-UTP	100	3

For the pegRNAs the 3'-O-Me-m7G cap analog is not included.

Table 2.4. IVT REACTION COMPOSITION

Component	Volume (for 40µl reaction)
DNAse/RNAse free water	Complete to 40 µl
NTP mix	15.8 µl (**11.8µl **)
Tailed PCR product (100ng/ul)	1 µg
T7 buffer mix 10x	10 µl
T7 enzyme mix 10x	10 µl

The reaction is then incubated in the thermocycler at 37ºC for 3h (MPC1, GFP), 4h (PEmax, PEMax SPRY and GFP) or 6h (pegRNAs) with 65ºC lid heat. The mixes for the IVT were prepared in an RNAse free hood and kept on ice during the process to avoid degradation of the components. After the incubation time is over, 4µl of TurboDNAse (Thermofisher) are added to the 40µl reaction and let to further incubate at 37ºC for 30 min. Next, the reaction is purified using the MEGAclear kit (Thermo-Fisher) following the heated elution buffer protocol. After purification, the reaction is treated with 2µl Antarctic Phosphatase (NEB) and 11µl of Antarctic Phosphate Buffer (NEB) and incubated for 1h at 37ºC. Finally, the reaction is purified using the MEGAclear kit and the concentration is measured with Qubit.

2.2.5. Cell transfections

HEK293T cells were transfected with modRNA using lipofectamine 2000 (Invitrogen) following manufacturer's instructions. HepG2 and HeLa cells were transfected using lipofectamine 3000 (Invitrogen) following manufacturer's instructions. Cells were plated to be at 2/3 confluency for the day of the transfection. For the MPC1 exCELLigence and cell counting experiments, cells were first plated in 6-well plates and transfected with 1 µg of MPC1 modRNA and 1 µg of GFP modRNA. For the ATP and Annexin/PI assays, cells were plated in 96-well plates to have 40.000 cells (AnnexinV/PI) or 20.000 cells (ATP) the day of the assay. Approximately 24h after plating, cells were transfected with 120ng of MPC1 modRNA. To prepare the mixes for the PEmax experiments, the amount of the different modRNAs per well in a 48-well plate were: 400ng PE and 50ng of the pegRNA. To check for editing, the corresponding fluoPEER plasmid was added to the mix (100ng). For each pegRNA four mixes were prepared: PE DNA + pegRNA DNA, PE DNA + pegRNA modRNA, PE modRNA +

pegRNA DNA and PE modRNA + pegRNA modRNA. The amount of plasmid DNA added was double that of the modRNA.

2.2.6. Fluorescent Activated Cell Sorting (FACS)

For the PE experiments, cells were harvested using Trypsin (Gibco) approximately 36h after transfection and resuspended in FACS buffer (phosphate-buffered saline with 2mM ethylenediaminetetraacetic acid and 0.5% bovine serum albumin) with 1x DAPI. Cells were then filtered through a 5 ml Falcon polystyrene test tube (Corning). Flow cytometry and sorting were performed on the FACS FUSION (BD) using FACS Diva software (BD). GFP+ sorted cells were recovered in culture medium and spun down. For the MPC1 exCELLigence and cell counting experiments, cells were instead harvested ~16h after transfection. GFP+ sorted cells were recovered and plated either in a 96-well exCELLigence plate, normal 96-well plate/48-well plate or 6-well plate as shown in Table 2.5.

TABLE 2.5. NUMBER OF CELLS SORTED WITH FACS FOR EACH EXPERIMENT

	HEK293T	HepG2	HeLa
exCELLigence plate	3500	2500	2500
6-well plate	6500	4000	4000
96-well plate	3500	2500	2500
48-well plate	12000	8000	8000

2.2.7. Genotyping

Genomic DNA was isolated from the prime-edited FACS-sorted cells using the Quick-DNA MicroPrep kit (Zymo Research), following manufacturer's protocol. The genomic region of interest was amplified by PCR using the Q5-HF polymerase (NEB). The PCR product was purified by the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and sent for Sanger sequencing to EZSeq Macrogen Europe. The PCR and sequencing primers used are listed in Table 2.1.

2.2.8. exCELLigence

Cells transfected with MPC1 (1µg MPC1 modRNA + 1µg GFP modRNA) or only GFP (2µg GFP modRNA) were FACS sorted, recovered and plated as shown in Table 2.5. The exCELLigence plate was then placed in the exCELLigence machine. The parameters set for the test were 999 sweeps with a 15min interval and it run for 7 days. At day 7, the data was recovered and analyzed using Excel and GraphPad.

2.2.9. FACS cell counting

Cells transfected with MPC1 (1µg MPC1 modRNA + 1µg GFP modRNA) or only GFP (2µg GFP modRNA) were FACS sorted, recovered, plated in a 48-well or 6-well plate and placed back in a 37ºC incubator. At day 4 after transfection, cells in the 48-well

plate were harvested for FACS, as detailed in the sections above. Flow cytometry was performed using a FACS Fortessa (BD) and FACS Diva software (BD). The number of cells was measured for the first 20s. At day 7 after transfection, the process was repeated with the 6-well plate. Data was analysed using FlowJo software.

2.2.10. ATP quantification assay (ATPlite)

HepG2 cells were plated in a 96-well clear culture plate at ~5000 cells/well. 24h after plating, cells were transfected with 120ng of MPC1 or control modRNA or 100ng of GFP modRNA using lipofectamine 3000 and following manufacturer's instructions. 48h after transfection, the ATPlite assay was performed according to the manufacturer's protocol. Briefly, the cells were equilibrated at room temperature and lysis using the Mammalian lysis buffer. After incubating at 700rpm for 5min, the cells were transferred to a white opaque plate compatible with luminescent reading. Next, the Substrate solution was added and again incubated for 5 min at 700rpm. Then, the plate was further incubated protected from light for 10 min without shaking. A microplate reader (Clariostar) was used to measure luminescence.

3. RESULTS

3.1. Plasmid and gene cloning

To produce modRNA, a DNA template is required. This plasmid serves as a backbone for cloning the desired genes and contains important sequences for transcription and translation such as the T7 promoter and the 5' and 3' UTRs. The plasmid pcDNA3.3 was developed with this aim. However, the cloning protocol for adapting it for each gene was cumbersome and time consuming. Therefore, a version of this plasmid, pcDNA4, was cloned with BbsI restriction sites that allowed the quick introduction of the target gene into the backbone.

3.2. GFP modRNA production

A batch of green fluorescent protein (GFP) was produced using the pcDNA4-GFP and pcDNA3.3-GFP plasmids as the IVT template to assess the efficiency of modRNA production for both plasmids. When cells successfully express GFP, the signal can be easily visualized using a fluorescence-compatible microscope, since the cells will appear green. ModRNA was produced following the protocol described in the Methods section, which is briefly outlined in Figure 3.1. After the protocol was completed, modRNA concentration was measured using Qubit.

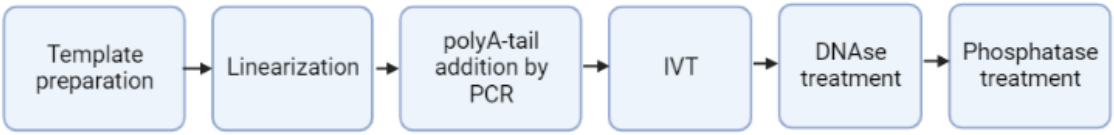

Figure 3.1 Outline of modRNA production protocol

After the protocol was completed, the concentration of the modRNAs was measured using a Qubit, due to its higher accuracy compared to the NanoDrop. The obtained concentrations are shown in Table 3.1.

TABLE 3.1. CONCENTRATIONS OF modRNA GFP PRODUCED

modRNA	Concentration (ng/µl)
pcDNA4-GFP	506
pcDNA3.3-GFP	688

The concentration of the modRNA produced from the original plasmid was 1.3-fold times higher than that of the pcDNA4-GFP modRNA, which, in turn, was 1.08-times higher than the previously prepped plasmid modRNA.

To check if the modRNA was functional, HEK293T cells plated in a 24-well plate were transfected with GFP modRNA using lipofectamine 2000. Three days after transfection, cells were examined for GFP expression using an EVOS, as seen in Figure 3.2. As can be observed in the images, GFP expression is relatively high, as most of the cells appear green. Subsequent experiments using GFP as a transfection control for FACS sorting showed that transfection efficiency was around 80%, higher than when using a GFP plasmid.

Therefore, we showed that the protocol for modRNA production allows for production of functional GFP, which in turn present relatively high transfection efficiency.

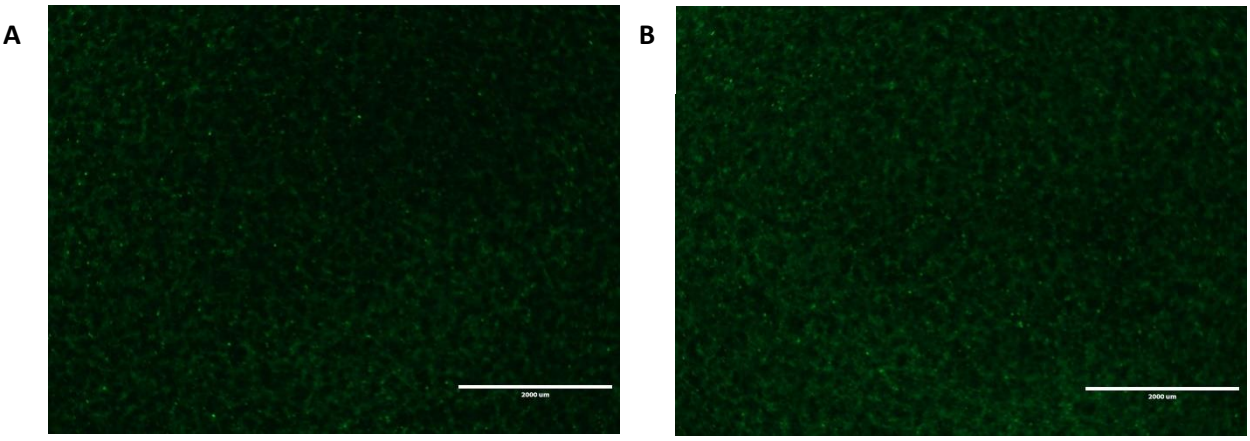

Figure 3.2. GFP expression in HEK293T cells. (A) Cells transfected with pcDNA4-GFP modRNA (B) Cells transfected with pcDNA3.3-GFP modRNA.

3.3. Prime editing modRNA production

The next step after establishing the functionality of the modRNA protocol was producing modRNA of the prime editing tools. For this purpose, the NGG prime editor and two epegRNAs, HEK3 X>R (referred as H1e) and Mut700 (referred as M1e) were cloned into the pcDNA4 plasmid.

The same modRNA IVT protocol was performed for both transcripts, resulting in modRNAs yield of 700ng/µl for the PEmax and ~300ng/µl for the epegRNAs. Next, the prime editing machinery was introduced in cells through lipofectamine transfection, in which the modRNA molecules are packaged in lipid droplets which fuse with cell membranes when added to a cell culture. HEK293T cells were co-transfected with 400ng of PEmax modRNA, 50ng of H1e modRNA and 150ng of GFP modRNA in a 48-wells plate. GFP is used as a transfection control because transfection of cells with the transcripts can be quantifed through expression of a green fluorescent signal. Approximately three days after transfection, the cells were FACS sorted, and the GFP+ positive cells were recovered. Genomic DNA was isolated, and a PCR was performed to amplify the sequence of interest then sent for sequencing. After analysing the results, no editing was observed in the target sequence. This experiment was repeated another time, yielding the same negative result.

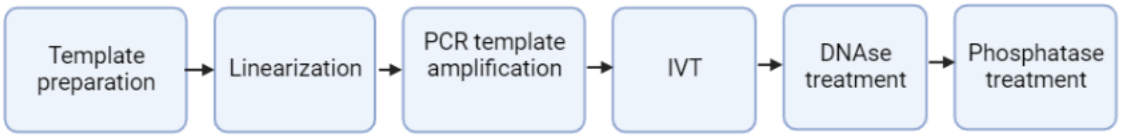

Figure 3.3 Updated modRNA protocol without the polyA-tail addition.

Consequently, the modRNA protocol was repeated for the epegRNAs, but this time the polyA-tail was not added, as it was thought it could be interfering with the functioning

of the epegRNAs (Figure 3.3). The newly produced modRNA had a concentration of ~400ng/µl for M1e but <100 ng/µl for H1e. The constructs were again tested by transfection in HEK293T cells as described above. However, no editing was observed.

To rule out degradation of modRNA during the production process, cDNA was made from the modRNA transcripts using an iScript cDNA synthesis Kit (Bio-Rad) and then run on an agarose gel. Single bands with minimal smear were observed for the transcripts, meaning the sample had not degraded and the issue was something relating to the transcript itself.

Next, we hypothesized different transcript sizes would benefit from different IVT incubation conditions, as PEmax (± 7kb), GFP (± 1.5kb) and the epegRNAs (± 0.15kb) differ significantly in size. As such, different IVT incubation times were used, as well as different T7 concentrations to uncover the optimal protocol. The IVT was tested at 4 hours, 6 hours and overnight (~16 hours) with 10x or 5x T7 enzyme mix. After the DNAse treatment, the modRNA concentration was measured and cDNA was made out of it to run on an agarose gel. PEmax yield was highest with the 4h 5x T7 enzyme conditions and epegRNA yield was highest with 6h 10x T7 enzyme. Therefore, we adapted the IVT protocol accordingly and attempted production of the prime editing machinery again.

A transfection experiment in HEK293T cells was performed using the newly synthesized PEmax and H1e epegRNA. This time, however, the DNA version of the constructs was also included in the conditions to elucidate if the lack of editing was due to prime editor, the epegRNA or both. Furthermore, we included a reporter (fluoPEER) allowing fast visualization of prime editing activity by FACS. Therefore, the transfection set up was as follows:

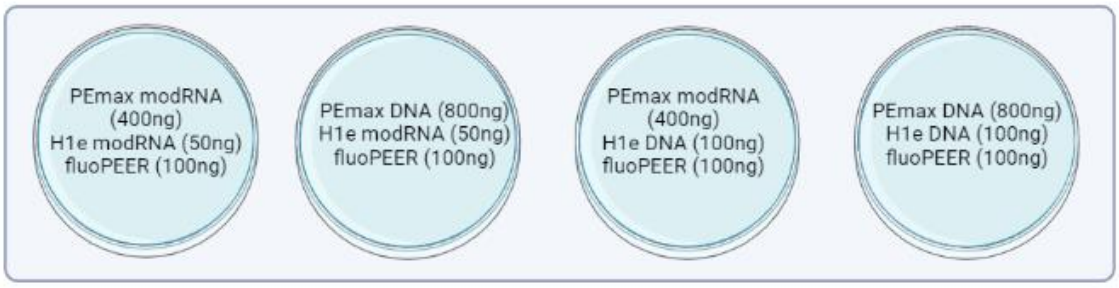

Figure 3.4. Transfection set up for testing the prime editing modRNA

FluoPEER is a prime editing reporter plasmid that contains constitutively expressed GFP and red fluorescent protein (RFP) sequences (Figure 3.5) [20]. In between both cassettes, the target genomic sequence is inserted; such that it can be edited by the appropriate epegRNA [20]. Therefore, if editing is successful, it will result in the expression of the mCherry sequence and cells will be both GFP and RFP positive, which can be quantified using FACS.

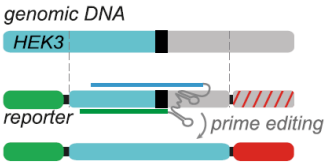

Figure 3.5. Schematic of fluoPEER working mechanism. Figure extracted from *[20]*

After recovering the cells that show editing by the fluoPEER (GFP+ and RFP+) and amplifying the target sequence by PCR, editing (T>A mutation) is observed with a 20% efficiency in the GFP+ population of the PEmax modRNA+H1e DNA condition (Figure 3.6A). From the RFP+ cell population (those cells that exhibit editing in the fluoPEER plasmid), the observed editing efficiency was 50% (Figure 3.6B). Therefore, the modRNA PEmax was capable of introducing the desired mutation in HEK293T cells. However, the PEmax DNA + H1e DNA condition exhibited ~60% editing efficiency, 3x higher than the codition using PEmax modRNA (Figure 3.6C).

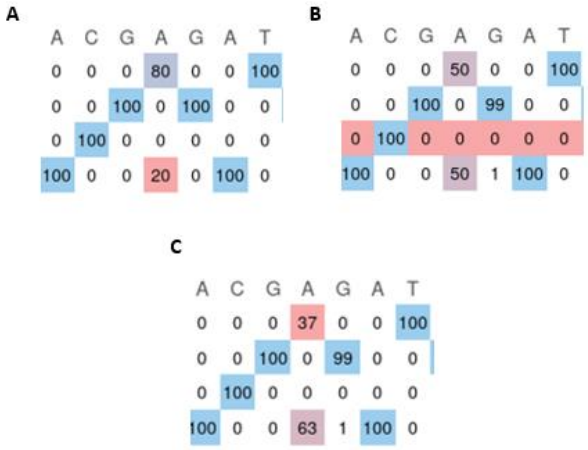

Figure 3.6. Predicted T>A editing from Sanger sequencing for the (A) GFP+ sorted Pemax modRNA+H1e DNA condition, (B) RFP+ sorted Pemax modRNA+H1e DNA condition and (C) GFP+ sorted Pemax DNA+H1e DNA condition.

Therefore, the focus was now on the epegRNA modRNA, since no editing was measured in the PEmax DNA + H1e modRNA transfection condition. Since the H1e epegRNA plasmid had successfully edited when used in previous experiments [3], we hypothesized something intrinsic to the modRNA production protocol hindered with its functionality. As the polyA-tail had already been removed after the first try, the only additional element still present was the 5' guanine cap analog, which adds an extra G to the beginning of the spacer sequence. Thus, it was decided to remove the cap analog for the upcoming tries.

Finally, another batch of H1e modRNA was produced without adding neither the polyA-tail nor the cap analog. This new epegRNA modRNA was tested by repeating the same transfection protocol as before (Figure 3.7).

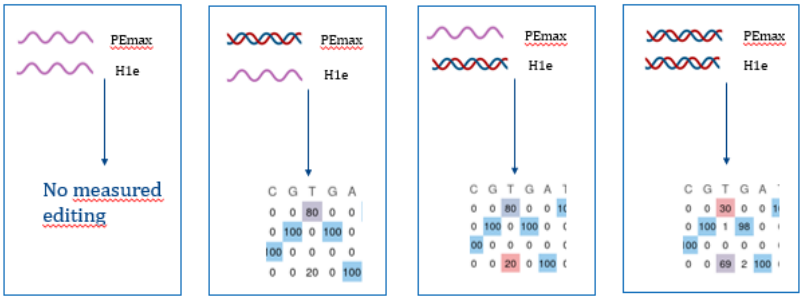

Figure 3.7. Transfection protocol with the editing efficiencies for each condition.

When combining H1e modRNA with PEmax DNA, an editing efficiency of 20% was measured. 20% was also obtain for the PEmax modRNA + H1e DNa condition, matching the results of the previous experiment. However, still the DNA+DNA condition results in more than 3x higher editing efficiency.

Therefore, we were able to successfully produce modRNA of the prime editing tools, which were capable of introducing the desired mutation in HEK293T cells. Moreover, modRNA of other transcripts have also been produced using our platform and are currently being used for different projects with promising results. One of those projects is the production of modRNA for mitochondrial base editing (mBE). mBE is another gene editing technology focused on induction of mutations within mitochondrial DNA [21]. This is of interest for those metabolic hereditary disorders that originate due to mitocondrial mutations. The tools required for mBE were produced by and tested for mutation correction or creation in patient-derived fibroblasts and organoids. Experiments on organoids showed that mBE modRNA was capable of editing the cells with a bulk efficiency of ~60%, with some organoids clones being 100% edited (Figure 3.8). Moreover, when transfecting the patient-derived fibroblasts with the modRNA via electroporation, higher cell viability was observed compared to using DNA, although further experiments are needed to confirm this.

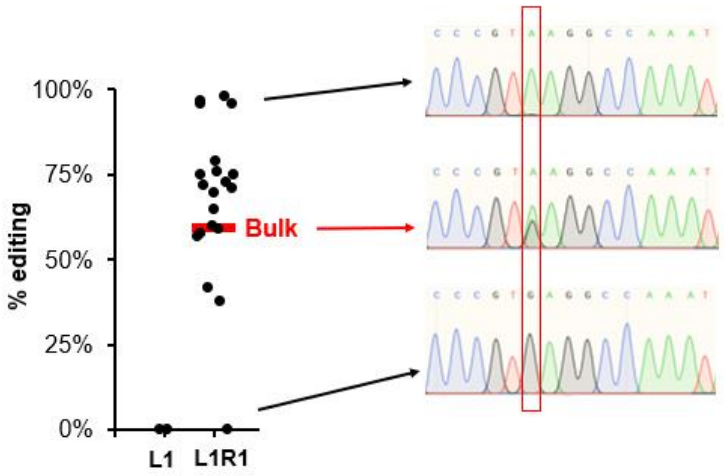

Figure 3.8. Editing efficiency of mitochondrial base editing modRNA in organoids. Figure provided by Martijn Koppens

3.4. MPC1 modRNA production

To produce the MPC1 modRNA transcript, the protocol described above was followed without introducing any extra alterations.

To study if MPC1 modRNA could alter cell proliferation, an exCELLigence experiment was set up. For this, HEK293T, HepG2 and HeLa cells were transfected with MPC1 + GFP modRNA or only GFP modRNA. Around 16h after transfection, the cells were FACS sorted and the GFP+ cells were plated in an exCELLigence culture plate and incubated at 37ºC for 7 days. The proliferation curves can be seen in Figure 3.9.

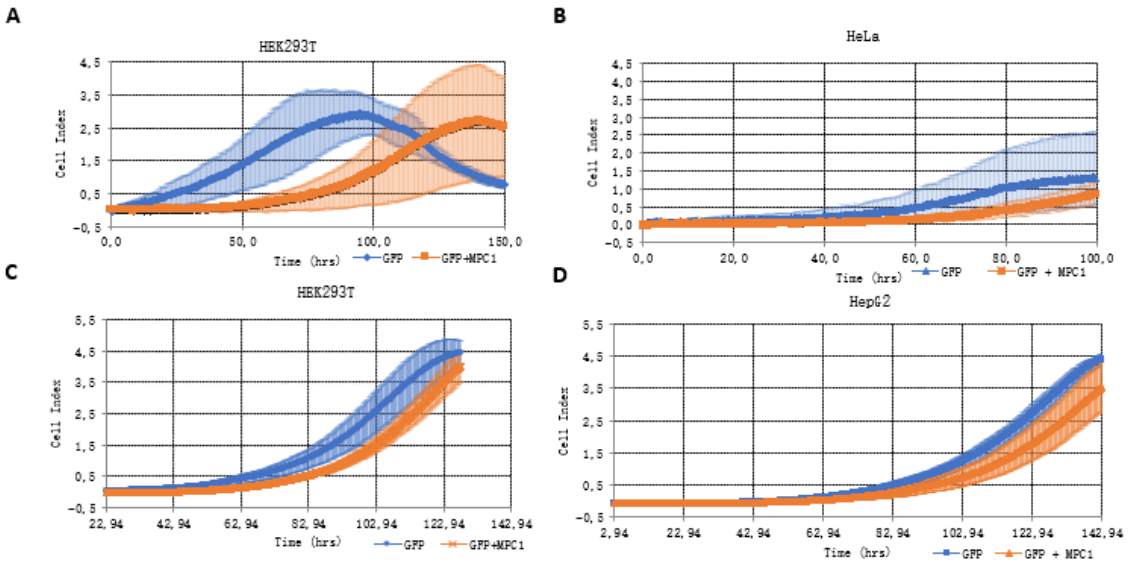

Figure 3.9. Cell proliferation curves for MPC1-treated (orange) and GFP control groups (blue) in HEK293T (A, C), HeLa (B) and HepG2 (D) cell lines.

Cells treated with MPC1 showed a slower proliferation rate than the GFP only controls for the three different cell types. Moreover, the difference was maximum at around 80h and afterwards the MPC1 cells slowly picked up their normal growth rate.

A second batch of MPC1 modRNA was later produced. To determine batch-to-batch variability and functionality, another exCELLigence experiment was performed using both transcripts. After 7 days of measurement, the following results were obtained (Figure 3.10). Cell proliferation when treated with each MPC1 transcript was similar and both showed differences with the GFP control, replicating what was seen in the previous experiment.

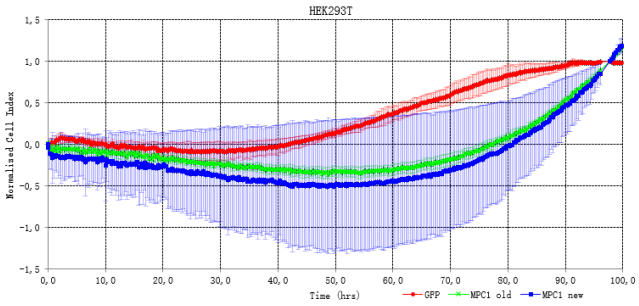

Figure 3.10. Functional comparison for different batches of MPC1 modRNA.

A cell counting experiment was also performed to see if overexpressing MPC1 decreased cell viability, as previously reported by others [19]. This was achieved by transfecting cells with MPC1 + GFP or GFP only, FACS sorting 16h after and plating back the GFP+ cells into either a 48 well-plate or a 6-well plate. The cells in the 48-well plate were then harvested 4 days after transfection and counted using FACS while the 6-well plate was harvested at day 7. As seen in Figure 3.11, at day 4, the number of cells treated with MPC1 were half of the number of the control cells. While the difference between them slightly decreased at day 7, the MPC1 cell population was still lower than the control.

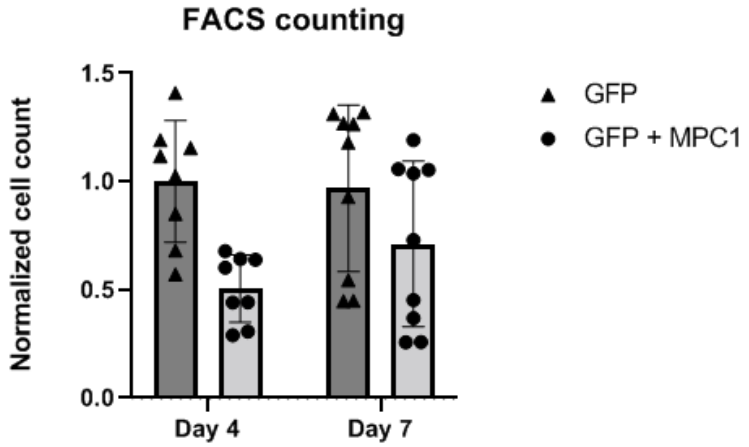

Figure 3.11. Cell numbers present at days 4 and 7 after treatment with MPC1

To obtain a more accurate measurement of cell viability after MPC1 treatment, a cytotoxicity assay was carried out using the ATPlite kit. This assay links cell death to ATP presence and measures it using luminescence. When comparing cells treated with a modRNA control (a mitochondrial base editor transcript which should not be functional on its own) to MPC1-treated cells, we observed that modRNA by itself does not have any effect on ATP production. However, overexpressing MPC1 decreases ATP production by half compared to the controls.

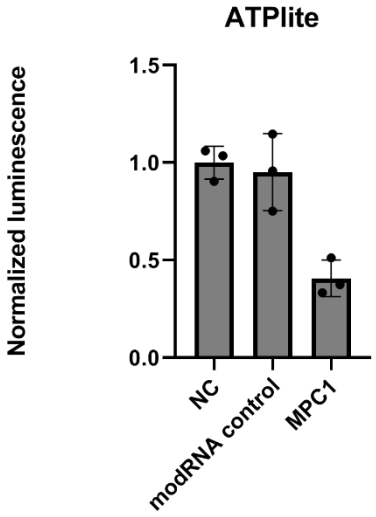

Figure 3.12. Cell viability results for modRNA and MPC1 modRNA

4. DISCUSSION

Patients suffering from rare hereditary diseases are at a disadvantage when developing therapies due to the high heterogeneity among each disease and even within the same patient group. However, all such disorders arise due to pathogenic mutations. Therefore, exploiting this common ground could be the key to developing therapies for such a large group of patients. Prime editing has a lot of therapeutic potential, since it is capable of safely correcting and creating pathogenic mutations with high efficiency. By using a modRNA version of the prime editing tools, we can bypass the issues presented by DNA delivery.

We presented an in-lab system to support the production of modRNA for any desired transcript. Several optimization steps were introduced to the original protocol to adapt it to produce the prime editing tools. First, the polyA-tail was removed from the epegRNA modRNA molecules since it most likely interfered with their correct functioning. Next, the IVT incubation times were optimized according to transcript size since the number of transcription initiation events and/or available T7 enzyme are correlated with the size of the gene of interest. Once the highest yield conditions for each of the transcripts were obtained, a PEmax batch capable of editing in HEK293T with 20% efficiency was produced. Regarding the epegRNA, the 5' cap analog was removed from the IVT protocol to obtain a functional transcript. We hypothesized that this additional G decreases the efficiency of the epegRNA as spacer sequences are susceptible to size variations [22]. However, editing efficiency with the epegRNA modRNA was again 20%, compared to the 60% obtained using the DNA-encoded prime editing tools. The reason behind the large difference between conditions could be the extra DNA amplification step that takes place when transfecting plasmid DNA, which could be solved by increasing the epegRNA modRNA. Moreover, further optimization of the epegRNA transcripts is probably needed in order to achieve their full potential.

The next steps in developing the production platform would be testing the prime editing modRNA in patient-derived organoids and fibroblast, since it has only been tested in HEK293T cells as of yet. Furthermore, a delivery strategy needs to be developed for *in vivo* applications. A non-viral method would be ideal, such as lipid nanoparticles or engineered virus-like particles, as they carry less disadvantages than viral vectors [5].

Establishing this production platform shows great promise, as modRNA can be used in several applications. Just the prime editing machinery alone can be used to target most rare hereditary disorders, since it will only require cloning the epegRNA with the corresponding mutation. Thus, prime editing can bring this group of patients who currently lack standard treatments and therapies closer to possible clinical trials. Moreover, as stated earlier, other types of gene editing are also being developed in modRNA form within our group with successful results. In addition, many proteins with possible therapeutic potential could be produced as a modRNA treatment.

Among these potential therapeutic proteins is MPC1. MPC1 is a key player in the cell metabolism due to its location at the bifurcation between the anaerobic glycolysis and oxidative phosphorylation pathways. Particularly, cancer cells, which mostly rely on

anaerobic glycolysis, have been shown to downregulate MPC1. Thus, it was hypothesized that overexpressing MPC1 via virus delivery in tumour cells could force them to switch to a non-ideal oxidative phosphorylation-based metabolism. Indeed, previous studies showed that when MPC1 expression is recovered, cancer cells decrease their proliferation rate and viability [18], [19]. By using modRNA to express MPC1 in cancer-derived immortalized cells, we observed similar findings. Those cells that received MPC1 modRNA transcripts presented a slower proliferation rate than the control groups. This was corroborated by counting the number of cells present at 4 and 7 days after transfection. Moreover, modRNA by itself was shown to be non-toxic for cells and a decrease in ATP was only observed when using MPC1 modRNA. However, since MPC1 is directly related to the ATP production pathways, we cannot conclude that this change in ATP levels correlates with a decrease in cell viability, only that indeed overexpressing MPC1 has an effect in the cell metabolism. Thus, other non-ATP-based cell viability assays should be performed in order to make a final conclusion. Furthermore, due to the transient nature of modRNA, its therapeutic effect wears off around a few days after transfection, as seen in the proliferation assays. Therefore, using it in combination with other treatments could improve its long-term efficacy. As cancer cells rely on a different pathway for fatty acid production than healthy cells, this difference can be exploited to target cancer cells [23]. By blocking the conversion of the resulting by-products, accumulation of toxic molecules is achieved, leading to cancer cell death [23]. Thus, combining MPC1 overexpression with treatment with these fatty acid pathway inhibitors could be a promising strategy to target cancer cells.

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