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PERSPECTIVE

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MicroRNA-mediated metabolic reprogramming of chimeric antigen receptor T cells

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Abstract

Advances made in chimeric antigen receptor (CAR) T cell therapy have revolutionized the treatment and management of certain cancers. Currently, B cell malignancies have been among the few cancers to which CAR T cells have shown persistent and resilient anti-tumor responses. A growing body of evidence suggests that the persistence of CAR T cells within patients following infusion is linked to the mitochondrial fitness of the CAR T cell, which could affect clinical outcomes. Analysis of CAR T cells from patients undergoing successful treatment has shown an increase in mitochondrial mass and fusion events, and a reduction in aerobic metabolism, highlighting the importance of mitochondria in CAR T cell function. Consequently, there has been recent interest and investment in approaches that focus on mitochondrial programming. In this regard, miRNAs are promising agents in mitochondrial reprogramming for several reasons: (1) natural and artificial miRNAs are non-immunogenic, (2) one miRNA can simultaneously modulate the expression of multiple genes within a pathway, (3) the small size of a sequence required for producing mature miRNA is ideal for use in viral vectors and (4) different precursor miRNAs (pre-miRNAs) hairpins can be incorporated into a polycistronic miRNA cluster to create a miRNA cocktail. In this perspective, we describe the latest genetic engineering strategies that can be used to achieve the optimal expression of candidate miRNAs alongside a CAR construct. In addition, we include an *in silico* analysis of rational candidate miRNAs that could promote the mitochondrial fitness of CAR T cells.

INTRODUCTION

Advances in cancer therapy have benefited from understanding the complexities of cellular and molecular biological processes, both in terms of the improvements to traditional treatments such as chemo- or radiotherapy, as well to more recent treatments such as immunotherapy. Immunotherapies seek to facilitate or engineer the patient's immune cells to identify and destroy cancerous cells within the body. In this regard, T cells are the primary engineering target of many immunotherapeutic targets owing to their native functions in targeting antigen-displaying cells for destruction. Chimeric antigen receptor (CAR) T cell therapy utilizes engineering of T cells to express a CAR that recognizes tumor-associated antigens to enable destruction of the target cell. Upon recognition, activation signals are propagated through ITAMs and costimulatory domains resulting in cytotoxic effects against the target cell, as well the initiation of activation

and cell survival mechanisms in CAR T cells. Generally, a CAR consists of an antigen specific single-chain variable fragment (scFv) from a monoclonal antibody, attached to intracellular signaling domains from the T cell receptor (TCR).[1, 2]

At the time of publication, only selected hematological malignancies, such as acute lymphoblastic leukemia, chronic lymphocytic leukemia, diffuse large B cell lymphoma and multiple myeloma, have been successfully treated with CAR T cell therapy. Failure of CAR T cell therapy may be due to poor long-term persistence and memory differentiation – factors which are critical in achieving a durable and effective response.[3] The treatment of solid tumors is further hindered by the development of a tumor microenvironment (TME) which imposes metabolic pressures and promotes the formation of dysfunctional CAR T cells as well as regulatory T cells (T_{reg}).[4–6] Recent studies have described a direct link between metabolism and the mitochondrial status of CAR T cells and the effect this has on their persistence.[4, 7] CAR T cells utilizing oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) as their main metabolic pathways as well as possessing a higher mitochondrial mass as result of mitochondrial fusion, have shown improved patient responses in clinical trials. Consequently, there has been growing interest in the metabolic reprogramming of CAR T cells to improve their efficacy. Approaches shift the metabolic pathway from glycolysis to OXPHOS/FAO or promote mitochondrial fusion by blocking fission factors have shown promising results.[7] Restricting glycolysis to favor OXPHOS and FAO can be achieved through limiting glucose uptake,[8] blocking glycolytic enzymes[9, 10] or inhibition of the positive regulators of glycolysis.[11, 12] Such approaches involve the use of loss-of-function strategies, small molecule inhibitors, or upregulation of negative regulators.[13–18]

MicroRNAs (also known as miRNAs and miRs) are small non-coding RNAs that regulate gene expression post-transcriptionally. miRNA biogenesis begins with the transcription of primary miRNA (pri-miRNA) via RNA polymerase II or RNA polymerase III in some cases. The pri-miRNA contains 5' cap and 3' polyadenylation and is processed into precursor miRNA (pre-miRNA) by a microprocessor complex that includes the RNase III enzyme, named Drosha, and the RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) within the nucleus. The pre-miRNA is a stem-loop structure, ~85 nt in length with a 5′-monophosphate and a 3′-2-nt overhang. Exportin5 and Ran-GTPase are responsible for the export of pre-miRNA from the nucleus to the cytoplasm, where the loop is cleaved by the RNase III Dicer. Next, the double-strand miRNA duplex (~20-22 nt) is loaded into the RNA-induced silencing complex (RISC). One of the strands from the miRNA duplex remains in the RISC complex (guide strand), while the complementary passenger strand one is ejected. Based on the direction of the guide strand in the pre-miRNA hairpin, the miRNA genes produce −5p or 3-p mature miRNAs.[19, 20]

MiRNAs predominantly bind mRNA through 3' untranslated regions (UTRs), resulting in mRNA degradation or translational interruption.[21] miRNAs are encoded from distinct miRNA genes or other genomic regions such as introns or exons.[19, 20] Although most miRNAs regulate gene expression in the cytoplasm, a fraction of miRNA known as mitomiRs are imported into the mitochondria.[22] The mitomiRs have been shown to regulate the expression of genes involved in mitochondrial function and metabolic regulation.[22]

T cell metabolism is dynamic and linked to function and differentiation state. Quiescent naïve T cells (T_N) have minimal metabolic requirements and use OXPHOS to generate ATP. Following T cell activation, rapidly proliferating effector T cells (T_{EFF}) undergo metabolic reprogramming, switching to glycolytic metabolism to generate both ATP and metabolic precursors needed to meet biosynthetic requirements of activation such as DNA and cell membrane synthesis. Following antigen clearance, T cells undergo contraction leaving only 5% of T cells to differentiate into long-lived memory cells (T_M) responsible for long-term protection. T_M cells utilize OXPHOS metabolism to maintain their cellular processes.[3,4]

Complete inhibition of metabolic gene expression or function through gene knock-out or small molecule inhibition can compromise the effector function of T cells. Deletion of glucose uptake receptor, Glut1[23] or AMP-activated protein kinase (AMPK),[24] have been shown to diminish the *in vivo* expansion of T cells or to increase the number of suppressive T_{reg} cells, respectively. In a similar fashion, blocking of pyruvate dehydrogenase kinase (PDHK), a positive regulator of glycolysis, with dichloroacetate reduces proinflammatory cytokine production and encourages T_{reg} differentiation.[25] Therefore, due to the relationship of metabolism, cell function and cell fate, immune homeostasis and functionality must be considered when metabolic genes are targeted.

The manipulation of metabolic pathways through miRNA expression is an ideal strategy for manipulation of CAR T cell therapy for several reasons. Firstly, miRNAs fine-tune rather than completely inhibit gene expression. A single miRNA can modulate the expression of multiple genes simultaneously within a pathway. The small sequence size required to produce mature miRNA is ideal for gene transfer as well as different precursor miRNA (pre-miRNAs) hairpins can be incorporated into

a polycistronic miRNA cluster to create a miRNA "cocktail".[26] Finally, artificial miRNA can be produced with a lower risk of immunogenicity and unwanted off-target effects.[26]

Few studies have investigated the use of miRNA in CAR T cell therapy; therefore, we will discuss potential miRNAs that can be used to enhance CAR T cell function through metabolic reprogramming. We have focused on genes whose downregulation resulted in a metabolic shift toward OXPHOS/FAO as well as mitochondrial fusion were selected. The functions of target genes are involved in one of four categories: glucose uptake receptors, glycolytic enzymes, positive regulators of glycolysis and mitochondrial fission. miRNAs that have at least one target gene in all categories were identified using bioinformatic analysis alongside published data. Lastly, we discuss genetic engineering approaches to express miRNAs within CAR T cells.

CANDIDATE GENES TO TARGET FOR REPROGRAMMING CAR T CELL METABOLISM

Active metabolic pathways within T cells are linked to both their life cycle and subset differentiation. As such, the metabolic alteration can influence T cell differentiation and function. The promotion of OXPHOS/FAO metabolism or mitochondrial fusion has been shown to increase the number of T_M cells and to enhance anti-tumor activity.[7] Strategies used to induce metabolic reprogramming away from glycolysis and toward OXPHOS/FAO in T cells have included the targeting of glucose uptake receptors,[8] glycolytic enzymes,[9, 10] metabolic regulators[13] and mitochondrial fission factors to promote mitochondrial fusion.[27] Therefore, 36 genes described here as involved in these four functions could be used as targets to identify candidate miRNAs (Figure 1). It is worth noting that not all receptors or enzymes have a high expression level, or sometimes they are dispensable with their other family members in T cells, and therefore their inhibition may have little or no effect on T cell metabolism. Thus, out of these 36 genes, 20 of them are only high-value targets that have a predominant function in T cells or express at a higher level (discussed below).

Glucose uptake transporters

Glucose uptake in lymphocytes is carried out by five members of the GLUT family of non-concentrative glucose carriers (Glut 1, 3, 4, 6 and 8) and the Na^+-coupled glucose carrier SGLT1[28] (Figure 1). Glut1 and Glut3 are the main glucose uptake transporters in T cells among these transporters,[29] but others may be expressed following T cell activation or insulin stimulation.[30–33] Downregulation of Glut1 via overexpression miR143 in Her2-CAR T cells was shown to increase the number of central memory (T_{CM}) $CD8^+$ T cells, as well as to boost effector function as a result of metabolic reprogramming.[8]

Glycolysis enzymes

Glycolysis begins with the phosphorylation of glucose and ends with the conversion of phosphoenolpyruvate to pyruvate through a sequence of enzymatic reactions (Figure 1). The restriction of glycolysis through the targeting of glycolytic enzymes or positive regulators, favors the differentiation of T_M subsets and improves the anti-tumor activity of T cells.[4,7,9,10] Such an effect is seen via inhibition of the first glycolytic enzyme, hexokinase-1 and 2 (HK) using 2DG, which enhances $CD8^+$ T_M cell formation by shifting metabolism toward OXPHOS.[10] In contrast, upregulation of the glycolytic enzyme phosphoglycerate mutase-1 (PGAM1) diminishes the development of T_M cells.[10] It should be noted that only three reactions are rate-limiting and irreversible among the ten steps of glycolysis.[34] The three key rate-limiting enzymes include HK, phosphofructokinase 1 (PFKM) and pyruvate kinases (PKM).[34] Changing the level and activity of the reversible steps in glycolysis does not determine the direction of the pathway and is unlikely to have a significant effect on glycolysis.[35]

Metabolic regulators

Several metabolic regulators have been recognized in T cells. A metabolic shift toward OXPHOS/FAO metabolism, mitochondrial fusion and TM cell development occurs when these regulators are inhibited. These regulators include mammalian target of rapamycin (mTOR),[13,36,37] AMP-activated protein kinase (AMPK),[38] phosphoinositide 3-kinase (PI3K),[11,12,17,39–41] hypoxia-inducible factor 1-alpha (HIF1A),[42] basic leucine zipper ATF-like transcription factor (BTAF),[43,44] lactate dehydrogenase A (LDHA),[45,46] monocarboxylate transporters (MCT 1, 2 and 4),[9] AKT serine/threonine kinase (AKT1 and 2)[47] and Acyl-CoA cholesterol acyltransferase (ACAT1 and 2)[14,16] (Figure 1).

Some of the metabolic regulators have a controversial function in T cell development. For instance, deletion of the AMPK gene can cause defective $CD8^+$ T_M generation, or an increased level of AMPK promotes the T fitness, expansion and formation of T_M cells.[48,49] Recently, Mayer *et al.* showed that AMPK deficiency does not affect T cell fate, clonal diversity, the number of activated T cells and survival *in vivo,* rather it reduces the magnitude of T cell activation, expansion and protein translational capacity.[50] Conversely, others showed that reducing AMPK signaling

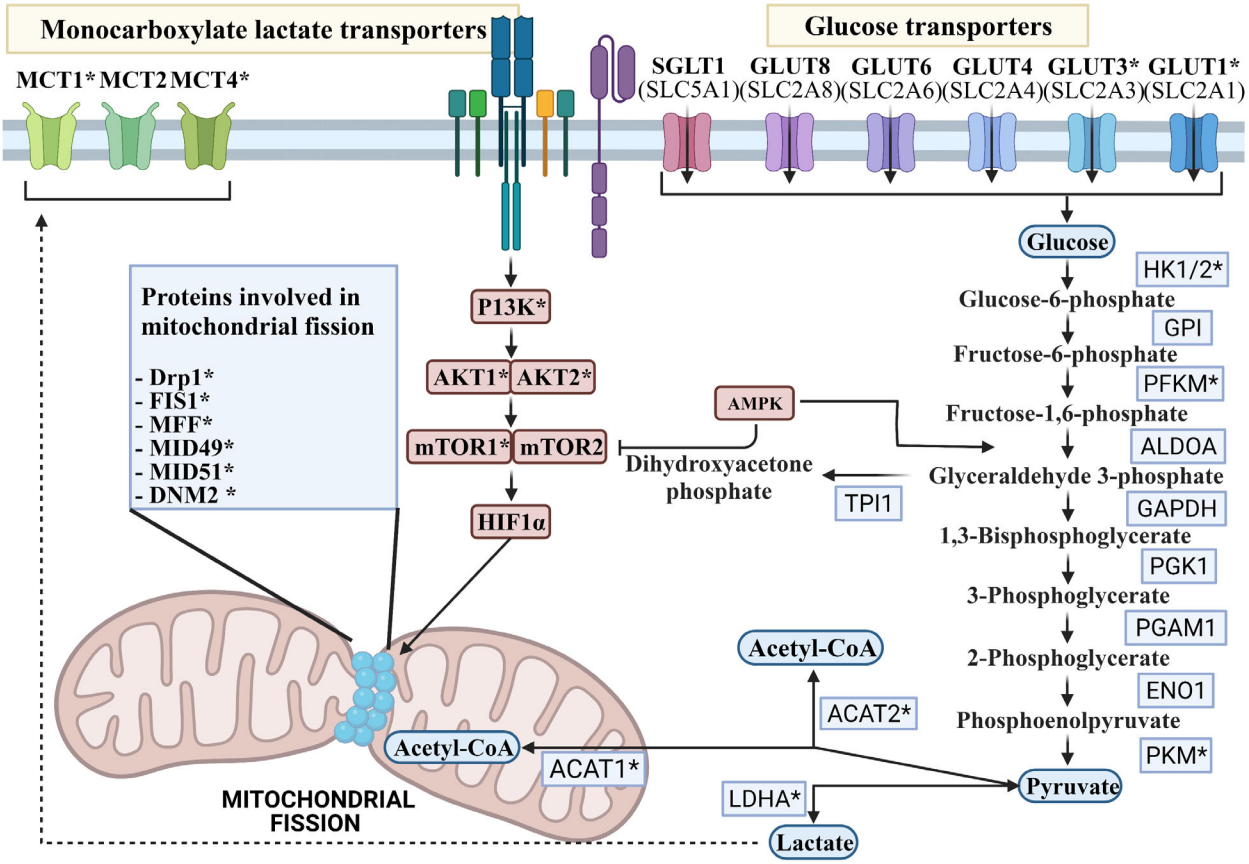

Figure 1. Schematic of T cell metabolic processes and the candidate genes for miRNA targeting. These include glucose transporters, glycolytic enzymes, metabolic regulators and mitochondrial fission factors. * High-value target genes in T cells. The figure was created with Biorender.com.

via miR17-92 (indirect effect) or shRNA targeting of AMPK promotes metabolic reprogramming to aerobic glycolysis and restores T cell proliferation in senescent cells.[38,51] We omitted AMPK in the high-value target group due to the lack of research using knockdown approaches, but we cannot rule out the possible positive effects of miRNA targeting of the AMPK based on the scant data available.

Free cholesterols are enriched in microdomains known as lipid rafts in the plasma membrane. In T cells, TCRs and associated signaling molecules cluster at lipid rafts and disruption of lipid rafts impair the TCR signaling and T cell effector functions.[52] ACAT1 and ACAT2 esterify free cholesterol to be stored in the cytoplasmic lipid droplets. Within 6 hours after activation of $CD8^+$ T cell, the *ACAT1* mRNA level shows overexpression, whereas the *ACAT2* takes 24 hours for an increased level in mRNA.[14] The mRNA level of *ACAT1* is nearly 20 times higher in $CD8^+$ T cells and the protein level of ACAT2 is very low.[14] In addition, virus-specific T cells primarily expressed ACAT1 rather than ACAT2.[53] Deletion of ACAT2 does not affect $CD8^+$ T cell function, suggesting that ACAT1 is the main enzyme in cholesterol esterification in T cells.[14] Inhibition of ACAT1 by small molecules or gene deletion studies showed an increase in effector functions of $CD8^+$ T cells with an increase in T_{EM} cells.[14,52,53] CAR T cells treated with siRNAs against ACAT1 showed higher cytotoxicity, secretion of proinflammatory cytokines and enhanced tumor regression *in vivo*.[16] Although we excluded ACAT2 in our high-value target group, it should be noted that the *ACAT2* mRNA level in $CD4^+$ T cells is ≥ 2 fold higher that that in $CD8^+$ T cells.[14] Moreover, the positive impact of ACAT1 deletion is restricted to $CD8^+$ T cells suggesting that ACAT2 may compensate for the ACAT1 lost in $CD4^+$ T cells.[14,54] More studies are needed to uncover the function of ACAT2 in $CD4^+$ T cells.

Activation of T cells through TCR engagement, costimulatory molecules or IL-2 stimulation leads to activation of PI3K.[55] PI3K activates AKT and promotes mTOR signaling (Figure 1).[55] PI3K orchestrates with mTOR and AKT to promote T-cell glycolytic metabolism

and differentiation toward short-lived T_{EFF} cells, making PI3K an attractive target to enhance the quality of CAR T cell production. So far, several studies have shown that inhibiting PI3K in CAR T cells improves T cell expansion, anti-tumor activities *in vitro* and *in vivo*, reduces the expression of exhaustion markers, T_M phenotypes.[11,12,17,39–41]

Accumulating evidence suggests the positive effects of the AKT inhibition pathway on the CAR-T cell performance.[15,47,56,57] Treating the epithelial cell adhesion molecule (EpCAM)-CAR T cells with an AKT inhibitor, MK2206, promotes CAR T antitumor activity *in vivo* and increases CAR T cell expansion and the number of T_M cells.[15] The treatment was carried out 2 days post-transduction concurrently with CD3/CD28 stimulation and continued only 3 days after transduction. Interestingly, the authors showed that pre-treating with AKT inhibitor increases the transduction efficacy of T cells due to upregulation of low-density lipoprotein receptor that serves as a cellular receptor for the lentivirus with a VSVg envelope.[15] In addition, continuous culture of CAR T cells with AKT inhibitors also showed positive effects in CAR T cells, including lowering the level of glycolysis enzymes and MCT4, while FOXO1-dependent target genes such as *IL7R*, *KLF4*, *CD28*, *ICOS* and *CD95* showed upregulation.[47] Similar results were obtained where 40% of CAR T cells treated with an AKT inhibitor co-expressed CD28 and CD62L compared with 10% in untreated CAR T cells.[56]

mTOR regulates the T cell function and differentiation, and targeting mTOR has negative and positive impacts on T cells. The immunosuppressive effect of mTOR blockage by small molecule or gene deletion reduces T cell proliferation and increases the generation of non-functional T_M population and $CD4^+$ T_{reg} cells.[58,59] Conversely, lowering the level of mTOR by aptamer-targeted siRNA, IL-15 treatment or a low level of rapamycin treatment (20 nM vs. 100 nM) enhanced T_M phenotypes and antitumor activity of CAR T cells.[13,36,37] This highlights the potential of knockdown approaches, such as miRNA, for reducing the mTOR level rather than completely abolishing its activity.

Targeting lactate transporters (MCTs) to restrict glycolysis has also been investigated in the context of immunotherapy. T cells express three monocarboxylate transporters, MCT1, 2 and 4.[60] However, studies showed that MCT1 and MCT4 are the primary lactate transporter in T cells.[61] Upon T cell activation, MCT1 expression peaks at 12 hours while the MCT4 level induces with a delay, sometimes between 48 and 72 hours post-stimulation.[61] Blocking MCT1 and MCT4 seems a safe approach for reducing glycolysis and improving immunotherapy without compromising the anti-tumor activities of T cells.[9,62–64]

HIF-1, a member of the HIF transcription factor family, binds hypoxia response element (HRE) in the genome. Activation of HIF1 triggers a transcriptional program resulting in the adaptation of cells to the low oxygen level in hypoxia condition by minimizing oxygen consumption via promoting glycolytic program.[65] HIF-1 directly upregulates the expression of several glycolytic enzymes, LDHA, pyruvate dehydrogenase kinase 1 (PDK1), shifting the metabolic program away from TCA to glycolysis to generate ATP.[65] In combination with the PI3K-AKT-mTOR pathway following T cell activation, HIF-1 is a key modulator in the transition to glycolysis in T cells.[42] HIF-1 is critical for T cell's effector functions and promotes differentiation of T cells toward terminally differentiated T_{EFF} cells.[42] Genetic deletion of HIF-1 in T cells impaired acquisition of effector function.[66,67] Meanwhile, persistently elevated levels of HIF-1 cause lethal immunopathology due to the augmented effector capacity of CTLs. Plus, constitutive overexpression of HIF-1 results in the upregulation of the exhaustion markers (e.g. PD-1, CTLA-4, LAG-3 and TIM3) while T-bet, Emoes and TCF-1 are downregulated. Therefore, due to the variety of HIF-1 target genes, it seems wise to use knockdown approaches to reduce the HIF-1 in T cells for the purpose of metabolic reprogramming.

Mitochondria have several functions within T cells which are vital for the elimination of cancer. These functions include energy generation, T cell activation, biosynthesis, cell fate, cellular survival and cellular migration.[4] Mitochondrial morphology refers to the fused or fragmented state of the mitochondria within a cell. The morphological state of the mitochondria is coupled with its function in T cells and as such different T cell subsets possess different mitochondrial morphologies which best suit their function. Mitochondrial fission factors include dynamin-related protein 1 (Drp1), fission mitochondrial 1 (FIS1), mitochondrial fission factor (MFF), mitochondrial dynamics protein of 49 Kda (MID49), mitochondrial dynamics protein of 51 Kda (MID51) and dynamin 2 (DNM2).[68]

Potential miRNA candidates

We will discuss miRNAs identified using DIANA-microTCDS and TarBase v.8, as well as published miRNAs experimentally verified through qRT-PCR, western blot and reporter assay (Supporting Information 1). There are 455 miRNAs with a 7 - 8-mer match in their seed sequence to the 36 target genes involved within the categories mentioned above. Of the 36 target genes, only five genes, *Glut6*, *SGLT1*, *BTAF*, *FIS1* and *MID49*, had

fewer than ten potential miRNAs. As mentioned before, 20 target genes are either highly expressed in T cells, or have a crucial function in T cell metabolism, making them more suitable as miRNA targets (Supporting Information 1; Figure 1).

In general miRNAs often target more than one gene. The implication of this is critical when determining which miRNA to overexpress within CAR T cells to prevent unintended gene regulation. In our analysis, approximately two-thirds of identified miRNAs modulate more than two target genes (Figure 2). Potentially, 22 miRNAs have ≥ 10 target genes and 59 of them had at least one target within all gene categories (Supporting Information 1; Figure 2).

It is not within the purview of this paper to elucidate the functions and implications of all miRNAs identified, but previous research has investigated the function of miR146a and miR29 both of which have target genes within all gene groups.[69] The expression of miR146a is upregulated following TCR stimulation to support the T_M establishment. miR146a has minimal expression in T_N cells but increased expression in effector memory (T_{EM}) and T_{CM} cells.[69] The role of miR146a in T_M development is unknown, though targeting IL-2 production has been proposed as a mechanism.[69] In addition, ectopic expression of miR146 protects T cells from activation-induced cell death (AICD) by directly targeting the Fas-associated death domain (FADD).[69] In our analysis, miR146a has seven target genes (*Glut3*, *PGK1*, *AMPK*, *AKT2*, *MCT4*, *HIF1A* and *DNM2*) which make it among the few miRNAs that have at least one target in all gene groups (Supporting Information 2; Figure 2b).

The miR29 family is amongst the most highly expressed miRNAs in T_N and T_M populations.[70] The miR29 family includes three members: miR29a and miR29b located at chromosome 7 and expressed as a polycistronic primary transcript, whereas miR29c is located in chromosome 1. The expression of all miR29 members is upregulated in response to IL-21, a cytokine used to support CAR T cell expansion and to increase the T_{CM} and T_{SCM} development.[71] Overexpression of miR29 in $CD8^+$ T cells reduces the T_{EFF} cell number while boosting the frequency of T_M cells.[72] Three members of the miR29 family were among the miRNAs with the highest target number in our analysis (≥ 10 targets, Supporting Information 1). In addition, miR29 has at

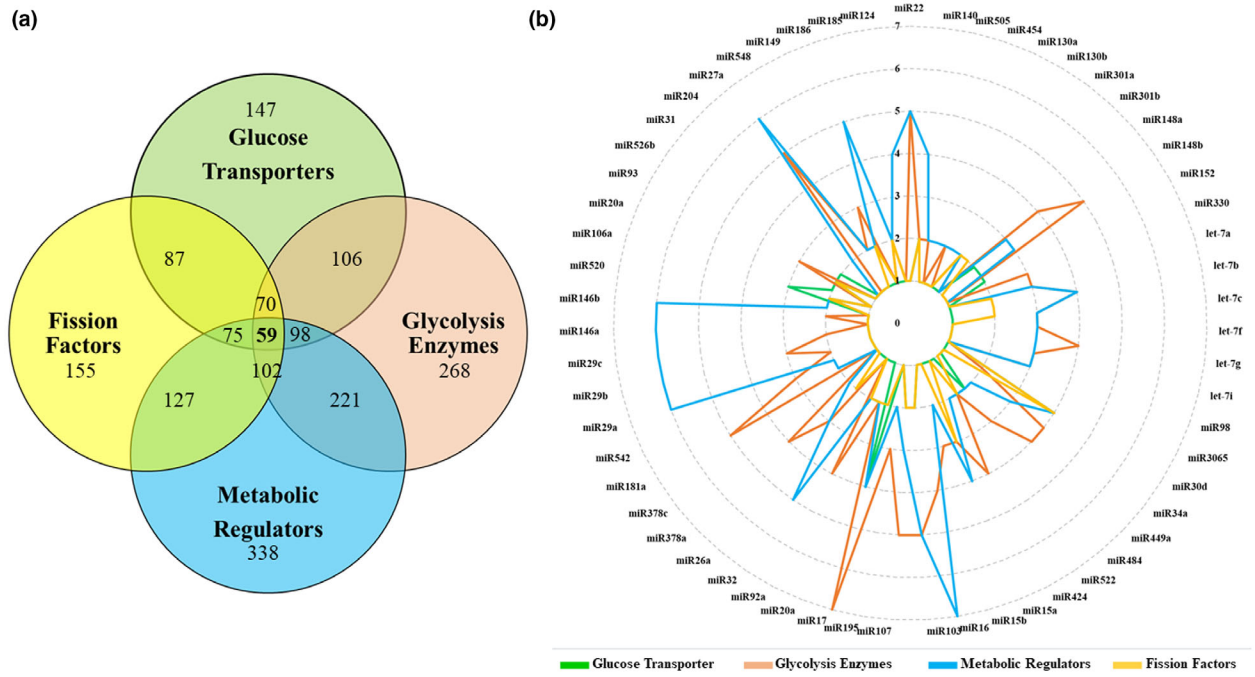

Figure 2. Most of the identified miRNAs have shared targets. **(a)** The Venn diagram shows the number of miRNAs identified for each category of genes and the number of shared miRNAs between groups. **(b)** The radar chart illustrates the 59 miRNAs with at least one target gene in each group of genes. The circular lines represent the number of target genes that a miRNA is predicted to target in each category. T_{EFF} cells have fragmented mitochondria that utilize glycolytic metabolism, while T_N and T_M cells have fused mitochondria which use OXPHOS to generate ATP. Therefore, miRNAs that target glycolytic enzymes might be under-expressed in T_{EFF} cells. To determine this, we looked at the miRNA profile expressed during $T_N \rightarrow T_{EFF} \rightarrow T_M$ stages of development based on published data.[75,79,115] A comparison of our identified miRNAs with the miRNA expression profile of each T cell subset found 12 miRNAs that are expressed at a low level in T_{EFF} cells, whilst being upregulated in T_M cells. These miRNAs have several targets among genes involved in glucose uptake, glycolysis and mitochondrial fission (Table 1).

Table 1. Changes in miRNA expression of T cells during $T_N \rightarrow T_{EFF} \rightarrow T_M$ development. These miRNAs potentially target several genes involved in glycolytic pathway and mitochondrial fission.

miRNA	Targets
miR15a	Glut3, HK1, ALDOA, PKM, PI3K, AKT1, AKT2, Drp1, FIS1 & DNM2
miR15b	Glut3, HK1, ALDOA, PKM, PI3K, MCT1 & Drp1
miR26a	Glut3, TIP1, GAPDH, PGK1, ACAT2, AMPK, MCT1, MFF & MID51
miR26b	Glut3, TIP1, PGK1, LDHA & AMPK
miR146a, b	Glut3, ALDOA, PGK1, AMPK, AKT1, MCT4, HIF1A & DNM2
miR101	TIP1, mTOR, MCT, MFF & DNM2
Let-7f	Glut3, ALDOA, HK2, PGK1, AMPK, AKT2, MCT4 & MID51
miR142	Glut3, GPI, PFKM, ALDOA, PI3K, AKT2, MCT2 & HIF1A
miR150	Glut3, GAPDH, PKM, LDHA & MCT1
miR16	Glut4, HK1, ALDOA, PGK1, PKM, LDHA, AMPK, PI3K, MCT1, MCT2, MCT4, HIF1A & Drp1
miR29a	Glut3, GPI, ALDOA, PI3K, AKT2, MCT1, 2, 4, HIF1A & MID51

least one target in all gene groups (Supporting Information 2). However, the role of miR29 in T cell subsets is controversial due to the potential targeting of TBET, Eomes and IFN-γ.[72]

It is always possible that selected miRNA may have undesirable off-targets, particularly in regulating the expression of genes involved in effector functions and master regulators of T_M development. Due to the vast number of potential genes, transcription factors and regulators involved in such processes, it is time-consuming to screen via bioinformatic tools. However, if researchers choose to perform screening prior to the functional assays, software such as DIANA-microT-CDS and TargetScan are helpful in providing extensive lists of potential target genes. A single complementary sequence with a weak binding (< 7-mer) in a distal 3'UTR site may not be considered off-target. In contrast, multiple complementary sequences throughout the 3'UTR, or a strong binding site in the proximal region of 3'UTR (> 7-mer, with full seed complementary) will likely be considered off-target.

We instead propose that it is more relevant to screen for deleterious off-targets by monitoring CAR T cell function following miRNA overexpression. It is arguably less relevant to be concerned about off-target effects of miRNA that still yield the desired outcomes. Endpoints must be carefully selected to predict maximal clinical effects. Such desirable endpoints should include enhanced anti-tumor effects against a range of blood and solid cancers, as well as optimal T cell longevity and memory cell formation. To this end, an approach of using cluster pooled miRNA is particularly helpful. If the high number of potential miRNAs will be tested (such as all the proposed 59 miRNAs), we suggest using them in the backbone of miRNA clusters, for example based on a miR17-92 backbone (see Figure 5). Hence, only < 10 final constructs will be tested rather than ~60 constructs. Observing any negative effects within individual clusters makes it easier to narrow an analysis down to find the culpable miRNA.

miRNAs that translocate to the mitochondria are referred to as mitochondrial miRNAs or mitomiRs. The mitomiRs have been shown to influence various mitochondrial functions such as OXPHOS, TCA, lipid and amino acid metabolism and Ca^{2+} homeostasis by targeting mitochondrial transcripts or nuclear-encoded genes inside the mitochondria.[22,73] The mitomiRs can cause metabolic reprogramming by regulating gene expression at the pre-translational level.[74] So far, several human mitomiRs and their targets have been recognized.[73] There are around 60 potential mitomiRs that are predicted to alter gene expression and mitochondrial activity (Supporting Information 1). Most of these mitomiRs have several potential target genes, with at least one target in each of our target categories (Supporting Information 2). Such miRNAs may have a more direct role in metabolic and mitochondrial regulations.

CONSIDERATIONS WHEN SELECTING miRNAS FOR EXPRESSION IN CAR T CELLS

In T cells, miRNAs are precisely regulated during the lifespan and during subset differentiation, therefore selection of miRNA for overexpression within T cells should take into account not only the potential of multiple target genes, but also the timing and magnitude of miRNA expression. A large number of target genes are targeted by miRNAs belonging to families such as miR17-92 and miR15-16 (Supporting Information 1). The use of these families is appealing as they not only target genes of interest but they also express polycistronic clusters that make it easier to express multiple miRNAs with a single DNA cassette. However, previous studies have shown that continuous expression of these families might impair T_M development.[75,76] For example, exogenous upregulation of miR15-16 family members restricts the T_M development by downregulation Eomes and CD127.[77] Moreover, only a transient expression of miR19-72 during expansion time is required for normal memory formation.[75]

Another example of the temporal regulation of miRNAs during T_M development is the let-7 family. It has been shown that the let-7 family is expressed in T_N cells, downregulated in T_{EFF} cells and re-expressed in T_M cells.[78,79] Downregulation of the let-7 family during the expansion phase is necessary for T cell proliferation and expansion.[80] Loss of let-7 increases T cell proliferation and effector function while it also promotes differentiation of terminal effector cells, mitochondria fission and AICD in T cells.[78,80]

Many studies investigating the role of miRNAs in T cell function utilize murine models for infection settings. Whilst conserved miRNAs tend to have comparable targets and functions both in humans and mice, some miRNAs are divergent in both their function and targets. For example, in the murine T cell model, the downregulation of miR17-92 after the initial expansion phase is necessary for T_M development.[75] In contrast, data from human T cells showed that there is continued expression of several members of the miR17-92 in human T_M phenotypes, including, miR20a, miR19b and miR92 which are preferentially expressed in human $CD8^+$ T_{CM} cells.[81] In addition, miR17-92 clusters were experimentally verified to increase T cell survival and persistence by downregulating the proapoptotic protein Bim.[82]

The expansion of CAR T cells is not an equivalent process to the expansion seen in physiological T cells.[3] Under physiological conditions, following antigen triggered TCR activation, T cells differentiate to T_{EFF} cells which then expand to a higher number. After antigen clearance, T_{EFF} cells undergo a contraction phase as a result of AICD leaving only ~5% of cells as a potential pool to differentiate to T_M cells. The environmental conditions contributing to each physiological T cell expansion event is influenced by surrounding immune cells that produce a unique combination and concentration of cytokines and other activating molecules to influence T cell response and cell fate. In contrast, CAR T cells undergo expansion both *in vitro* and *in vivo*. The initial expansion involves isolated patient T cells which are activated in the absence of antigen via CD3 and CD28 antibodies for a period of 2 to 3 days. During this time, T cells are also cultured with one or more gamma-chain cytokines such as IL-2, IL-7, IL-15 and IL-21 to support the homeostatic proliferation of T cells. Currently, only IL-2 has the FDA approval for the CAR T cell therapy, although IL-7/IL-15 and IL-21 have been studied in some clinical trials and pre-clinical studies.[83,84] After transduction, commonly using retro- or lentiviral vectors, T cells are expanded with gamma-chain cytokines for another ~10 days to provide sufficient numbers for infusion.[1,3] In other words, in the absence of antigen, the expansion phase is continued *in vitro* by controlling the media, cell number and cytokine treatment (Figure 3).

Further variations between CAR T cell expansion and physiological T cell expansion include the composition of the CAR T cell being implemented. There are several CAR T cell generations, predominantly categorized based on the number of costimulatory domains. Second-generation CAR T cells have CD3ζ with only one costimulatory domain, while third-generation CAR T cells are composed of CD3ζ with two costimulatory domains.[1] Each costimulatory domain confers different functions to the CAR T cell and promotes different metabolic programs.[1] CD28, ICOS and OX40 costimulation results in a more pronounced glycolytic phenotype.[1] whilst CD137 (4-1BB) promotes less efficient glycolysis while more efficiently enhancing mitochondrial respiration and fusion.[85] Furthermore, cytokines used during *ex vivo* expansion impact metabolism and mitochondrial functions. For example, IL-2 drives T cells toward effector-like phenotypes and a metabolic program characterized by enhanced glycolysis.[86] Whilst CAR T cells expanded under IL-15 have a higher mitochondria mass, spare respiratory capacity (SRC) and FAO metabolism.[13] The implications of the expansion conditions on the efficacy of miRNA action in CAR T cells has not been thoroughly investigated; however, the effect of these conditions on the metabolic state of the CAR T cell should be considered when choosing miRNA for overexpression.

It should also be noted that T cell stimulation is sustained in CAR T cells due to CAR tonic signaling in an antigen-independent manner.[87] Continuous tonic signaling from different costimulatory domains will impact CAR T cell metabolism and the anti-tumor function of CAR T cells.[88] Therefore, the overexpression of a miRNA may exhibit differing effects based on the CAR design and have a different effect on murine or human T cell models.

The abundance of target gene transcripts, as well as the number of miRNA binding sites influence the effectiveness of miRNA. It has been suggested that miRNAs set a threshold in which, at a low level of target transcript, miRNAs act as a "switch" to repress the gene expression (Figure 4a). When the target gene transcript levels are high, miRNAs act instead as a fine-tuner.[89,90] Interestingly previous research has shown that when the abundance of target gene transcripts is low, the presence of two and seven miRNA binding sites in the 3'UTR resulted in 2-fold and 10-fold reporter inhibition, respectively.[89] However, when target gene transcripts levels are high, regardless of the number of complementary binding sites, the gene repression stayed still at 2-fold.[89] Therefore, it would be expected that

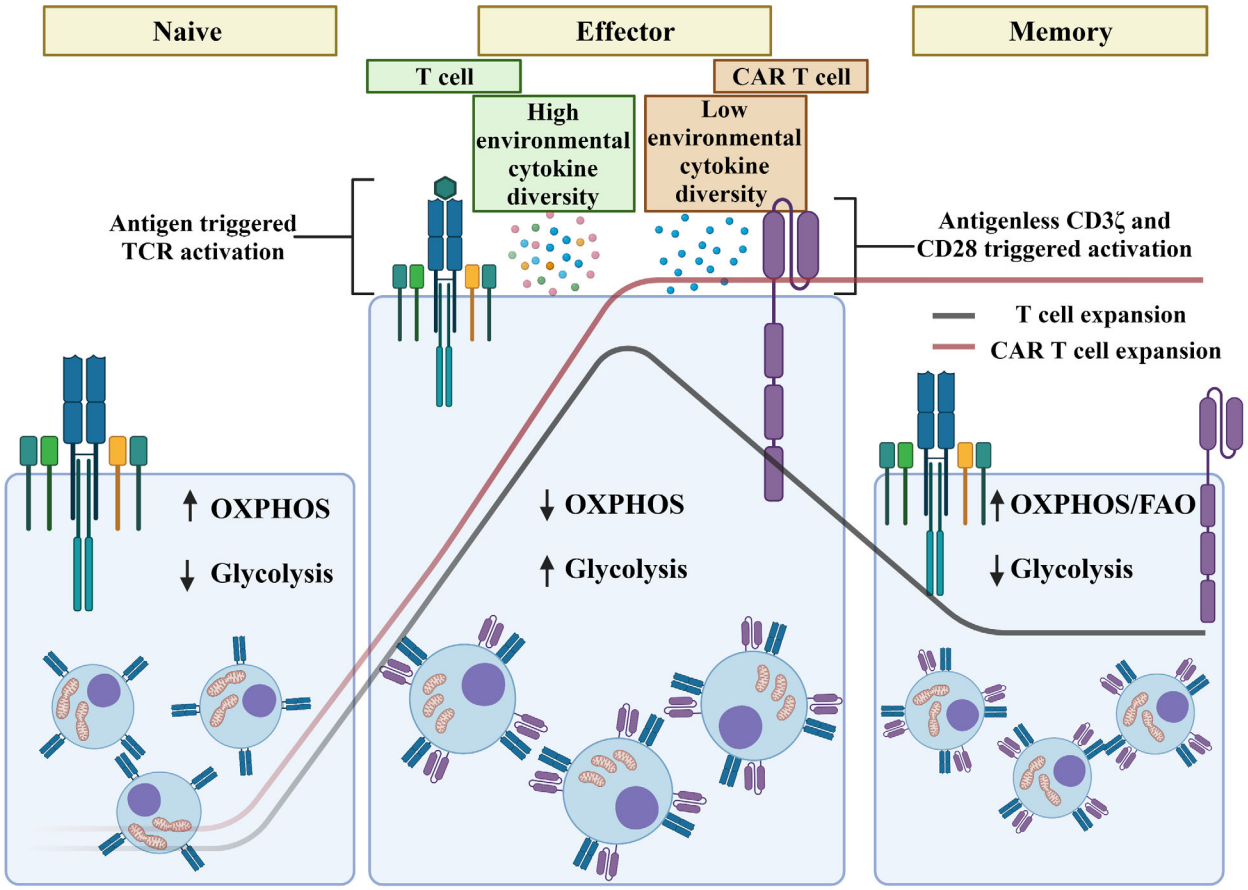

Figure 3. Physiological T cell activation and expansion is distinct from CAR T cell culture. The mitochondria within naïve T cells utilizes a OXPHOS and FAO which is reprogrammed when T cells are activated through antigen presentation and a diverse range of cytokines. Following activation effector T cells possess fragmented mitochondria with a glycolytic metabolism to facilitate effector function. Contraction (the green line) of these effector T cells into a memory population once again reprograms the mitochondria toward OXPHOS and FAO. In contrast CAR T cells are activated through CD3 and CD28 antibody stimulation in the presence of one or more cytokines. Furthermore, CAR T cell expansion is maintained, without allowing the natural contraction of the population (the red line). The figure was created with Biorender.com.

glycolytic inhibiting miRNAs would act as a fine-tuner in T_{EFF} cells where the glycolysis is at its highest. This might be an advantage of using miRNAs since abolishing glycolysis negatively affects the effector function and tumor killing ability of T cells.[66, 67]

Lastly, the location of the miRNA complementary binding sequence within the 3'UTR also influences the inhibition of the target. It is well known that many genes have different mRNA isoforms that vary in their 3'UTR length. In addition, genes in cells with higher proliferation (such as T cells during expansion) tend to have a shorter 3'UTR due to proximal polyA site usage caused by alternative polyadenylation (APA).[91] The 3'UTR harbors regulatory sequences including miRNA binding sites and AU-rich elements that negatively regulate gene expression.[92] Hence, a longer 3'UTR is more likely to possess an abundance of miRNA interacting elements (Figure 4b). The length of 3'UTRs vary among tissues, genes in tissue such as the brain tend to have longer 3'UTR, while genes in blood cells prefer isoforms with shorter 3'UTR.[93] It is therefore possible that miRNA used to inhibit a gene within hepatocytes may not be able to inhibit the same gene within T cells.

STRATEGIES TO OVEREXPRESS miRNA IN CAR T CELLS

The primary miRNA (pri-miRNA) sequence along with flanking can be expressed under RNA polymerase II promoters to produce mature miRNAs.[19,20] Following transcription, pri-miRNA is cleaved by Drosha to produce a ~85 bp stem-loop structure known as the pre-miRNA. A subset of intronic miRNAs produces pre-miRNA during RNA splicing which is independent from

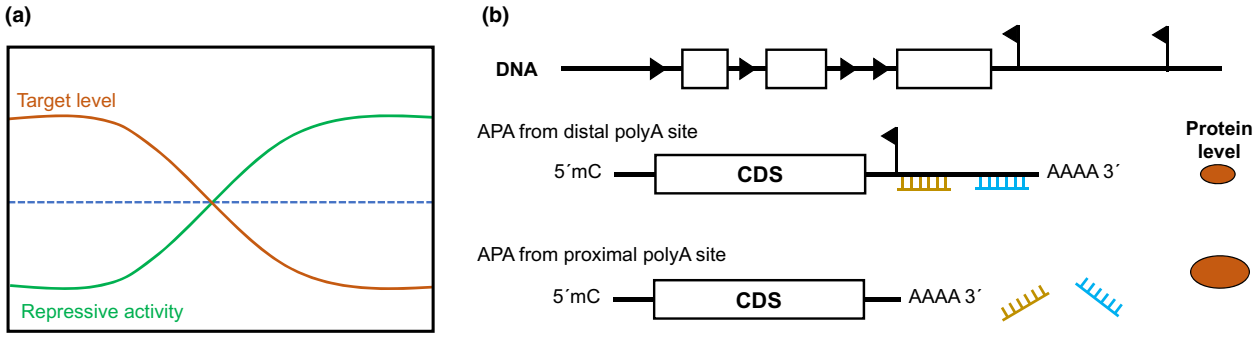

Figure 4. (a) miRNAs are more potent in repressing genes when the target gene is expressed below the miRNA threshold. **(b)** miRNA binding sites may be lost due to alternative polyadenylation (APA) mechanisms. This attenuation of miRNA sites can affect the protein expression of the target gene. Potential alternative poly-A sites of 36 candidate genes using 3′ end sequencing data[114] may be predicted by APAatlas[93] (Supporting Information 3). Therefore, the selection of miRNAs with several binding sites for expression within CAR T cells, is crucial to circumvent the possible remove of target regions by alternative poly adenylation sites.

Drosha.[94] The DNA sequence necessary for optimal Drosha cleavage contains pri-miRNA and a flanking region, which vary in size depending on the miRNA.[95]

The overexpression of miRNA within CAR T cells can be achieved through a variety of strategies. The use of dual transduction to express a miRNA and a CAR within a T cell involves the use of two separate viral vectors, one containing the CAR and a reporter gene such as green fluorescent protein (GFP), whilst the other contains the miRNA and a second reporter gene such as red fluorescent protein.[96] This approach is costly, time consuming and, due to the use of multiple reporters to identify successful dual-transduced T cells, limits the available fluorescent channels for phenotypic analysis by flow cytometry.[97] Due to these disadvantages, we will focus on alternative approaches which involve the selected miRNA and CAR being encoded within a single DNA cassette.

As detailed in Figure 5, these strategies involve the use of either a single or dual promoter construct within a lenti/retroviral or transposon gene transfer system. The main advantage of single promoter constructs is the reduced size of the overall cassette, which increases the efficiency of gene transfer.[98] Additional promoters within viral vectors decreases the viral titration and therefore efficacy.[97]

miRNA can be positioned upstream or downstream of the CAR coding sequence. However, placing a miRNA at 5' of the mRNA diminishes the expression of coding gene, as pri-miRNA cleavages by Drosha in the nucleus removes the 5'mC from mRNA necessary from mRNA exportation to cytoplasm.[99] It has been shown that placing the internal ribosome entry site (IRES) sequence upstream of the coding region (Figure 5a) leads to optimal production of miRNA and translation of the coding gene.[100] However, some reports still showed sporadic reporter gene expression in this design.[99] Alternatively, intronic miRNA can be used to preserve the 5'UTR after miRNA excision (Figure 5b). Several known single or cluster intronic miRNAs are included in our identified miRNAs. Using intronic miRNAs yields a high level of mature miRNA and coding protein.[101] However, miR26b and miR208a may induce lower exon ligation leading to a low protein level of coding genes.[101] Lastly, positioning miRNA at the 3' end of mRNA (Figure 5c) also produces a sufficient level of both coding gene and miRNA simultaneously.[99]

Bidirectional promoters may be used to express miRNAs and a CAR with a single, compact promoter (Figure 5d). Recently, we and others have shown that several commonly used human and viral promoters have bidirectional activities in human cell lines.[102,103] Synthetic bidirectional promoters also can be used by fusing two minimal promoters back-to-back, or by duplicating TATA and other core elements in the reverse direction.[104] This might be useful when one direction is more robust and therefore allows a control over the expression level of miRNA or CAR.

Dual-promoter constructs have been used widely to express two GOI where both promoters are constitutive (Figure 5e, f) or inducible (Figure 5g, h). In a constitutive manner, we have compared the function of EF1 (driving CAR and GFP) and hPGK (driving miR429) in both uni- or reverse directions in the lentiviral system. Although both orientation CAR and GFP expression were similar, the level of mature miR429 was slightly higher in the reverse orientation (Unpublished data Rad SMAH and McLellan AD 2020).

For a controlled expression, inducible promoters can be utilized either through drug or auto-inducible promoters.

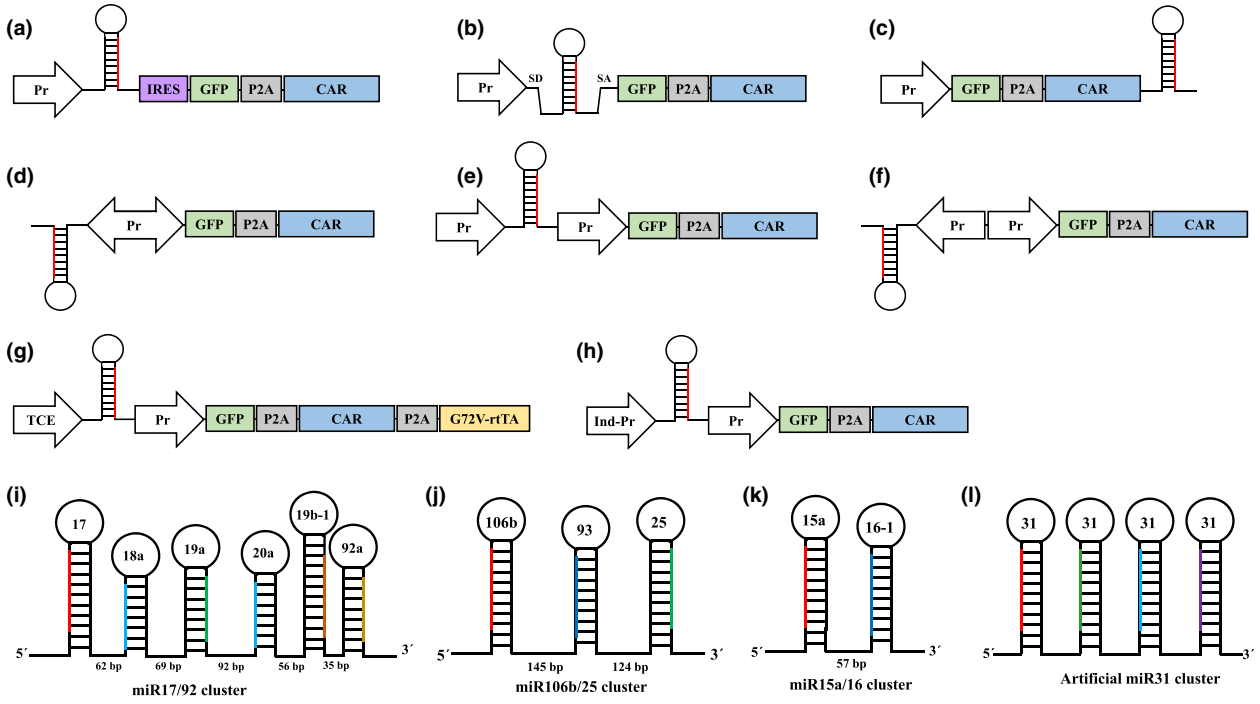

Figure 5. Strategies to express miRNA in CAR T cells. One single promoter drives both miRNA and CAR genes in a, b, c & d strategies. **(a)** A promoter derives miRNA and green fluorescent protein (GFP)-P2A-CAR coding sequence and internal ribosome entry site (IRES) sequence enhance the translation, **(b)** Intronic miRNAs use alternative splicing for maturation result in 5′UTR of mRNA vital for preserved translation, **(c)** placing miRNA downstream of CAR sequence, **(d)** bidirectional promoters to express miRNA and GFP-P2A-CAR with a single promoter. Alternatively, miRNAs and GFP-P2A-CAR can be expressed separately using two promoters in **(e)** uni-directional or **(f)** reverse-orientation fashion. Controlled miRNA expression using **(g)** Tet-On system with G72V-rtTA or **(h)** an auto-inducible promoter. Clusters of natural miRNAs to express **(i)** six miRNAs, **(j)** three miRNAs, or **(k)** two miRNAs. **(l)** Structure of artificial polycistronic miR31 by joining several repeats of pri-miR31 sequences.

The tetracycline inducible system is one of the tightest rheostats for controlling gene expression in mammals (Figure 5g). This system is composed of two elements, the TCE (tet-responsive) promoter and the rtTA (reverse tetracycline-controlled trans-activator). In the presence of tetracycline or doxycycline, conformational changes in rtTA make it able to bind and drive the transcription from the TCE promoter. Recently, our group developed a Tet-On system for CAR T cell applications. We showed that introducing a G72V mutation in rtTA (G72V-rtTA) described previously for yeast,[105] significantly enhanced the Tet-On system function in large gene cassettes containing a CAR.[106] Such a system might be beneficial when the expression of a miRNA needs to be regulated during T cell differentiation. For instance, let-7 has an increased expression within T_N and T_M cells whilst is downregulated in T_{EFF} cells. The downregulation of let-7 during expansion is necessary for T_M development.[75,76,80]

There have been several auto-inducible promoters investigated in T cells with potential use in CAR T cell therapy. Nuclear factor of activated T-cells (NFAT), nuclear receptor subfamily 4 group A member 1 (NR4A1) and CD69 promoters are the leading examples of auto-inducible promoters.[107] The activity of these promoters depends on the activation status of T cells; ON when T cells are engaged with antigen and activated, OFF when T cells are in resting condition. These promoters are ideal when the expression of GOI or miRNA is needed only during activated T cells (Figure 5h).

Finally, a cocktail of miRNAs can be used to inhibit the expression of several genes by using natural or artificial polycistronic miRNAs (Figure 5i–l). Because miRNA families tend to have shared seed sequences and common targets, mature miRNA sequences could replace the natural miRNAs. It should be noted that these replacements should not change the nucleotide compositions critical for the miRNA maturation process. Further sequence optimization may be applied to achieve a desirable level of mature miRNAs.[101]

Interestingly, the degree of processing individual miRNAs within the cluster might be distinct, which gives the advantage of a less inhibitory effect on sensitive targets. For instance, in the miR19-72 cluster (Figure 5i), miR17, miR19a and miR20a have a higher level of

mature miRNA than miR18a, miR19b and miR92a.[108] Examples of natural miRNA clusters with potential application in CAR T cells are shown in Figure 5i–k. Multimeric miRNA systems have been used to inhibit HIV-1 and HCV replication by replacing the miRNA sequence with small interfering RNAs (siRNA) sequences in the miR17-92 backbone.[109,110] A more significant intrinsic inhibitory activity with multimeric miRNA was achieved compared with conventional short hairpin (shRNA) design.[109] Artificial polycistronic miRNAs by joining several repeats of a single pri-miRNA sequence and is another way to get a ubiquitous inhibition effect on all targets (Figure 5l).[110]

CONCLUSION

Metabolism impacts on T cell function and differentiation. Promoting T cell metabolism toward OXPHOS/FAO and mitochondrial fusion has been shown to improve the T_M differentiation and anti-cancer effects of CAR T cell therapy. However, complete inhibition of gene expression or activity can compromise the effector function of T cells. In this regard, miRNA-mediated gene downregulation offers an alternative strategy to boost metabolically reprogramming of CAR T cells toward a fitter mitochondria and metabolism. However, our understanding of the function of miRNAs in T cell metabolism remains minimal and therefore, there is need for identification and characterization of miRNA functions in both T cells and CAR T cells. In this study, we identified potential miRNAs that target the genes involved in glycolytic metabolism and mitochondrial fission. We focused our analysis on miRNAs that are conserved and experimentally validated. However, miRNA acts in a cell and tissue specific manner, therefore the tissue or cells used in their validation must be considered. In addition, the differences between CAR T cells and native T cells adds to the complexity of the miRNA functions. The location of the miRNA complementary sequence in 3'UTR is another consideration and target sequences in proximal regions are less likely to be affected by APA. Moreover, multiple miRNAs expressed in a clustered manner against either a common target or differing targets may be beneficial to enhance the effect of miRNA-mediated gene modulation. Costimulatory molecules and culture condition also impact the CAR T cell metabolism; hence it is necessary to validate candidate miRNA function in the conditions relevant to their final application. Therefore, in the search for candidate miRNA or combination of miRNA candidates, we suggest that several miRNA candidates should be investigated in the context of CAR T cells.

METHODS

Potential miRNAs were identified using three bioinformatics software and verified published data. TargetScan7.2[111] was used to predict miRNAs with a high binding probability to target genes, filtering conserved miRNA with a seed match 7mer-m8 and context++ score percentile 80%. Verified miRNAs were identified using high-throughput techniques by DIANA-microT-CDS at threshold 0.7[112] and TarBase v.8 filtered for *Homo sapiens*, negative regulation, validated as positive and, direct validation.[113] Experimentally published miRNAs with references are provided in Supporting Information 1.

The polyA sites for each gene were collected from 3' end sequencing data available in PolyASite2.0[114] containing the location of the polyA site (chromosome, position and strand). Sites supported by more than one 3' end sequencing protocol are shown in bold.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Seyed Mohammad Rad: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Software; Supervision; Writing – original draft; Writing – review & editing. **Joshua Colin Hosseini Halpin:** Data curation; Formal analysis; Methodology; Writing – original draft; Writing – review & editing. **Supannikar Tawinwung:** Conceptualization; Formal analysis; Project administration; Supervision; Writing – review & editing. **Koramit Suppipat:** Conceptualization; Investigation; Resources; Supervision; Writing – review & editing. **Nattiya Hirankarn:** Conceptualization; Funding acquisition; Methodology; Project administration; Resources; Supervision; Writing – review & editing. **Alexander Donald McLellan:** Conceptualization; Formal analysis; Funding acquisition; Investigation; Project administration; Resources; Supervision; Writing – original draft; Writing – review & editing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.