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Advancing *in vivo* reprogramming with synthetic biology

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Abstract

Reprogramming cells will play a fundamental role in shaping the future of cell therapies by developing new strategies to engineer cells for improved performance and higher-order physiological functions. Approaches in synthetic biology harness cells' natural ability to sense diverse signals, integrate environmental inputs to make decisions, and execute complex behaviors based on the health of the organism or tissue. In this review, we highlight strategies in synthetic biology to reprogram cells, and discuss how recent approaches in the delivery of modified mRNA have created new opportunities to alter cell function *in vivo*. Finally, we discuss how combining concepts from synthetic biology and the delivery of mRNA *in vivo* could provide a platform for innovation to advance *in vivo* cellular reprogramming.

Introduction

There is an unmet need for medical technologies that can deliver or mediate therapeutic biomolecules and cellular treatments that are otherwise not possible with conventional pharmaceutical or surgical approaches. Cell therapy is a rapidly emerging field that involves the use of living cells to treat and potentially cure various diseases and medical conditions that were once considered untreatable [1–3].

The success of cell therapies lies in the ability of engineered cells to sense disease-associated biomarkers, perform complex computation to process this information, and produce a wide array of outputs to mitigate disease. A significant portion of initial efforts in synthetic biology was founded on assembling genetic parts to build synthetic gene circuits to respond to small molecules that diffuse through the cell membrane and

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activate synthetic circuits to implement new cellular functions [4–8]. The sophistication of sensing specific molecules within an environment has evolved with efforts to engineer receptors for improved target specificity that can be coupled to therapeutic responses, including activating cellular pathways, initiating a preprogrammed synthetic gene circuit, and participating in targeted payload delivery. Several cell types have been explored for the development of cell therapies, including bacteria, immune cells, and stem cells [9–11]. Engineering immune cells, specifically T cells, has become the focus of many studies due to the recent regulatory approval of chimeric antigen receptor (CAR) T cells, and the number of cancer patients who have been successfully treated with engineered CAR-T cells [12]. The premise of CAR-T-cell therapy is to couple the potency of a T cell with the specificity of an antibody to kill diseased cells [13]. However, the current high cost and complexity involved with manufacturing CAR-T cells *ex vivo* prohibits personalized T-cell therapy from being available to all cancer patients [14]. An exciting alternative is to target and reprogram circulating T cells for their direct reprogramming that would eliminate the need to perform costly manufacturing steps required to produce CAR-T cells *ex vivo*. The direct delivery of modified mRNA to T cells *in vivo* has been shown to enable T cells to translate new proteins, such as receptors, to target specific cell types for their destruction [15]. These early studies of delivering mRNA *in vivo* to alter the function of recipient cells strongly suggest that the delivery of mRNA *in vivo* may prove to be a new approach to advance cell therapies that can be used for treating many diseased and damaged tissues.

In recent years, the modification and optimization of mRNA for clinical applications has expanded the potential for *in vivo* reprogramming [16–18]. In 2020, the coronavirus disease 2019 (COVID-19) resulted in a global pandemic and catalyzed innovation in vaccine development with mRNA vaccines playing an essential role. Earlier studies by Kariko and Weissman demonstrated that base-modified mRNA prevents unintended immune responses [19] and increased protein production when delivered to immune cells compared with unmodified RNA [20,21]. With these discoveries, the concept of mRNA-based vaccines has been catapulted into the forefront of public awareness and has initiated additional research pursuits using mRNA in the clinic [22,23].

In this review, we highlight new developments in synthetic biology to reprogram cells using DNA- and RNA-based tools to emphasize the cellular reprogramming capabilities of synthetic biology, and we discuss recent studies that have successfully demonstrated the delivery of mRNA to cells *in vivo*. We propose that the success of delivering mRNA *in vivo* provides a roadmap for expanding synthetic biology devices to enhance the efficiency and precision of delivery, enable mRNA kinetics, and advance *in vivo* cellular reprogramming to address many challenges in medicine.

Reprogramming cells using synthetic biology

Boolean logic operations

A central goal of synthetic biologists is to reprogram cells with decision-making capabilities based on inputs from intrinsic and extrinsic cues for regulating target genes to produce a desired behavior. In this case, the processing of inputs has threshold states of ‘0’ or ‘1’ (‘OFF’ or ‘ON,’ respectively) [24] (Figure 1a). This Boolean logic can be used to

distinguish between healthy and diseased cells, and upon sensing a target cell, produces a response that destines specific cells for destruction. Additionally, engineered cells can be programmed to produce therapeutic molecules to be delivered at controlled levels in response to the degree of disease.

Epigenetic reprogramming

Epigenetics are stable alterations in gene expression that arise throughout the life of eukaryotic organisms without altering the DNA sequence. Epigenetic regulation can occur with the addition of methyl groups to cytosine or guanine nucleotides in the genomic DNA to silence genes [25], or when proteins bind to genomic DNA and alter the accessibility on chromatins for regulatory proteins to bind for activating or repressing gene expression [26]. Efforts to reprogram cells by targeting the genome directly are underway to create and optimize desired cell behavior. Specifically, synthetic transcription factors are designed to target specific sequences in the genome to either activate or repress gene expression. When transcription activator-like effectors and zinc finger proteins are fused to transcriptional activators or repressors, they can be custom-built to target specific locations in the genome to manipulate endogenous genes. Additionally, the clustered regulatory interspaced short palindromic repeat–Cas9 system enables programmable transcriptional regulation when the nuclease activity has been removed from the Cas9 protein (dCas9), and transcriptional activators or repressors are fused to dCas9 [27]. These synthetic transcription factors have been shown to be extremely versatile in a variety of applications in basic research and in biotechnology [28–31].

Transcriptional-based cellular reprogramming

Precise and sustainable control of gene expression is key to developing cell therapies. Enhancing cells' natural functions, redirecting their natural abilities, and engineering them with new functions has improved the therapeutic efficacy of using synthetic biology approaches to reprogram cells [32–34]. Controlling gene expression at the transcriptional level remains a corner-stone of developing new gene regulatory parts and devices. In these devices, gene expression is typically controlled by transcription factors and their associated DNA-binding sequences that either prevent or enable RNA polymerase to transcribe downstream genes [35] (Figure 1b). A challenge with this approach can be transgene silencing, the loss of gene expression over time, especially when engineering primary cells and stem cells to be used for therapeutic applications. This is an active area of research to better understand the mechanisms associated with transgene silencing to develop strategies to mitigate this challenge [36–40]. Never-theless, controlling gene expression at the transcription level has proven to be a powerful approach to reprogramming cells, especially when cells can implement Boolean logic operations to autonomously respond to intracellular or environmental molecules that trigger regulated therapeutic responses. Much of this work continues to provide the foundation for innovative reprogramming approaches and capabilities.

Extracellular molecules also strongly influence cell behavior. Cells are exposed to hundreds of different signals in their environment, and receptors enable them to distinguish which molecules to respond to for directing an appropriate cellular response. Efforts in synthetic

biology and receptor engineering have made it possible to repurpose natural cell receptors as custom environmental monitoring devices that can be linked to cell responses for defined cellular function. In general, synthetic receptors combine sensing capabilities to a desired extracellular molecule with the cell's natural function or activate synthetic circuits to implement new cellular function. This is a key step in engineering cell-based therapies for customized sensing of a specific extracellular signal, responding with an appropriate therapeutic output, and communicating with appropriate cells to mitigate disease. These synthetic receptors have been extensively reviewed elsewhere [41,42].

Synthetic receptors can also be designed to reprogram how cells communicate with each other and within their external environments [43]. Recently, a synthetic mammalian communication platform was engineered using synthetic receptors and coiled-coil peptides [44] (Figure 1c). In this work, the domains in the erythropoietin receptor (EpoR) were functionalized to bind to coiled-coil peptides that can be secreted by mammalian cells and specifically bind to the engineered EpoRs, activating them and initiating the expression of a reporter gene. In this study, sender cells were engineered to secrete coiled-coil ligands and upon binding to their cognate engineered EpoRs expressed by neighboring cells, activate a reporter gene in the recipient cell (Figure 1d). This engineered cellular communication system can also produce 2-input Boolean logic operations and advances the capabilities to program complex biological functions to better understand relationships between extracellular inputs and intracellular responses.

Post-transcriptional-based cellular reprogramming

RNA-based devices can be advantageous for cell therapies because they are delivered to the cytosol of cells where they control the translation of functional proteins, avoiding genomic integration and limiting epigenetic transgene silencing. Like transcription-based synthetic gene circuits, RNA-based genetic tools can sense inputs, process this information, and produce a controlled level of output in response to the degree of input. RNA is single-stranded and has the unique ability to base-pair with itself to form complex structures that enable the engineering of RNA-based tools to function by coupling the binding of a target molecule to alter its shape and facilitate translation [45,46]. This approach has been used to regulate the translation of mRNAs based on the presence or absence of RNA–protein interactions within the cytoplasm [47–52].

Toehold switches are RNA devices that are engineered to repress translation through base pairing around the translation start site of the mRNA of interest, creating a hairpin structure and preventing translation. Toeholds were initially designed to function in prokaryotes and have become a significant component in cell-free synthetic biology for diagnostics [53,54]. A eukaryotic version of toehold switches has recently been shown to function in mammalian cells [55]. These eToeholds contain internal ribosome entry site (IRES) sequences that can recruit translational machinery and initiate the translation of downstream genes. Gene expression is in the off-state in the absence of the cognate trigger RNA due to the inhibitory loops within the IRES structure (Figure 2a). Like the prokaryotic toeholds, once the trigger RNA anneals to a complementary sequence in the eToehold, the inhibitory loops are disrupted, and translation of the downstream mRNA is initiated (Figure 2b). This

system is modular and can be designed to detect and respond to the presence of various intracellular RNA molecules, including viral RNA, that enables the translation of the mRNA linked to the toe-hold. eToehold devices can facilitate marking infected or diseased cells to be targeted by various therapies.

Both an advantage and a disadvantage, mRNA therapeutics only allow for short-term expression of proteins because mRNA is usually degraded within a few hours after entering the cytosol. Using mRNA as a long-term, high-level expression therapeutic would therefore require repeated treatments. To mitigate this challenge, efforts are underway to prolong protein expression from mRNA molecules by building synthetic self-amplifying mRNA (saRNA). saRNAs are derived from positive-sense stranded viruses that can produce RNA-dependent RNA polymerase complexes that amplify synthetic transcripts *in vivo* at high levels [56]. Recently, an inducible saRNA switch was engineered to control mRNA kinetics *in vivo* [57]. This was accomplished by fusing a destabilization domain to the RNA-binding protein, L7Ae, that undergoes rapid degradation in the absence of the small molecule, trimethoprim (TMP). Adding TMP stabilizes the L7Ae protein that binds to structural motifs and suppresses translation of the downstream mRNA. This switch showed a significant increase and prolonged expression of the therapeutic protein compared with other *in vivo* approaches. Moreover, a recent study identifies multiple modified nucleotides that can be incorporated into saRNA that result in immune evasion and enhanced protein expression [18]. Circular mRNA is another approach for prolonged protein expression because it is more stable than linear RNA, less prone to degradation, and is less likely to cause an immune response *in vivo* [58–60].

Delivery of modified mRNA *in vivo*

The ability to manipulate genes and pathways *in vivo* has largely been dependent on the generation of genetically engineered mouse models or the transfer of transgenes using a variety of vectors that include viruses, plasmids, and nanoparticles. Lipid nanoparticles (LNPs) have been an essential component in the COVID-19 vaccine for delivering mRNA *in vivo* and are the most clinically advanced delivery vehicle to date. LNPs are therefore promising vehicles to deliver a variety of therapeutics, including mRNA *in vivo*. LNPs are small fat droplets composed of cholesterol and lipids that stabilize the particle's structure, and the positively charged lipids bind to the negatively charged mRNA (Figure 3a). Once LNPs enter the cell, they are taken up by the endosome, which breaks open the LNP releasing the mRNA into the cytosol to be translated (Figure 3b). Tremendous progress has been made using LNPs for delivering mRNA *in vivo*.

Recent studies have demonstrated the potential for the targeted delivery of mRNA using LNPs to reprogram cells *in vivo* to direct cell behavior and improve clinical outcomes in animal models. In many chronic heart diseases and injuries, cardiac fibroblasts become activated and secrete excessive extracellular matrix, resulting in fibrosis that stiffens the myocardium and eventually leads to the loss in the ability to pump blood properly [61]. To address this, a recent study demonstrated that T cells could be reprogrammed *in vivo* to target and remove activated fibroblasts [62]. In this study, mRNA encoding a CAR designed against the fibroblast activation protein (FAP) was packaged into LNPs decorated with

antibodies for CD5, a marker for T cells (Figure 4a). Once the mRNA is delivered to the cytosol of T cells, it is translated into functional receptors against FAP, and these new CAR-T cells target and kill the activated fibroblasts. Using this *in vivo* platform demonstrated that cardiac function was improved in mice with cardiac injuries.

Another demonstration of modifying cells *in vivo* is the recent study targeting hematopoietic stem cells (HSCs). HSCs reside in the bone marrow and have the potential to become all cells in the blood system throughout an individual's lifetime [9]. HSCs are a therapeutically relevant cell population to target because diseased HSCs can lead to leukemia, lymphoma, cardiac failure, immunodeficiencies, autoimmune diseases, bleeding disorders, and others [63]. Recently, it was shown that LNP–mRNA technology could be used to target HSCs *in vivo* for the stable gene editing of HSCs. In this study, the outside of LNPs was functionalized with antibodies against the mouse HSC marker, c-KIT, and filled with Cre recombinase mRNA to target HSCs in the Ai9 mouse line [64]. This mouse line has a loxP-flanked STOP cassette preventing the transcription of tdTomato in the absence of Cre recombinase (Figure 4b) [65]. After intravenous administration of c-KIT/LNP-Cre, Cre mRNA was delivered to HSCs and the expression of tdTomato was observed in peripheral blood cells for up to 4 months, demonstrating the delivery and translation of Cre mRNA in HSCs. Importantly, long-term HSCs are a critical population that replenishes the hematopoietic system and must persist for the lifespan of the organism [66,67]. Remarkably, in this study, the long-term HSCs were also reprogrammed and tdTomato expression was observed in all peripheral blood lineages, indicating that the edits made in progenitor cells are maintained throughout differentiation. This represents a promising modality to target HSCs *in vivo* for reprogramming stem cells with enhanced capabilities and open the possibilities for future cell therapies to treat hematologic diseases.

Conclusions and outlook

A powerful feature of using cells as therapeutic devices is their inherent compatibility, and their natural ability to sense, integrate, and respond to dynamic environments *in vivo*. Synthetic biology approaches offer platform technologies to augment these features to reprogram cells that can discriminate between cell states, produce a regulated dose of therapeutic biomolecules, and function to deliver payloads in a spatial and temporal fashion. Synthetic receptors can be built to sense specific molecules and cells within the environment and be coupled to synthetic gene circuits to facilitate tailored cell behavior. The recent success of mRNA-based COVID-19 vaccines shows promise for delivering mRNA to cells *in vivo*. In particular, the demonstration that mRNA for chimeric antigen receptors can be delivered to recipient T cells *in vivo* and translated into functional receptors to redirect immune cells for targeting specific cell types opens the door for a new wave of innovation in cell therapies and *in vivo* reprogramming.

Here, we highlight recent advances in synthetic biology to reprogram cells at the transcription and translation levels to emphasize the programmability of cells for sensing intracellular and extracellular molecules to activate native pathways, or synthetic circuits for implementing new cellular function. We envision the possibility of loading programmable synthetic gene circuits with multistep functions *in vivo* that can be used to enhance

information processing to produce appropriately graded outputs based on the level of therapeutic need to mitigate disease. For example, eToeholds were shown to detect the presence of intracellular viral RNA and, if infected, produced a reporter protein. Instead of producing a reporter protein, eToeholds could produce mRNA for a unique transmembrane peptide to mark the infected cell for CAR-T-cell destruction (Figure 5). This approach can be coupled to the recent multiplexing of functionalizing LNPs for diverse targeting and reprogramming of T cells [68]. Similar approaches could be used with other cell types for tissue regeneration to augment the healing process by recruiting specific cells to locations of injury and targeting progenitor cells to enhance their differentiation.

LNPs have shown tremendous success in vaccine development, however, challenges remain with low targeting specificity and toxicity, suggesting a need for improved delivery approaches [69,70]. Indeed, cells can be programmed for targeting molecules and cellular surface markers, however, nucleated cells come with their own set of safety risks. More recently, efforts have been made to utilize nonnucleated extracellular vesicles, red blood cells, and platelets as delivery vehicles that have shown promising early preliminary results for delivering payloads to potentially enhance targeted delivery and improve cell-based therapeutic approaches [71–74]. While *in vivo* reprogramming is still in its infancy, we envision that synthetic biology approaches will provide a platform for innovation that will offer next-generation cell therapy technologies.

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Data Availability

No data was used for the research described in the article.

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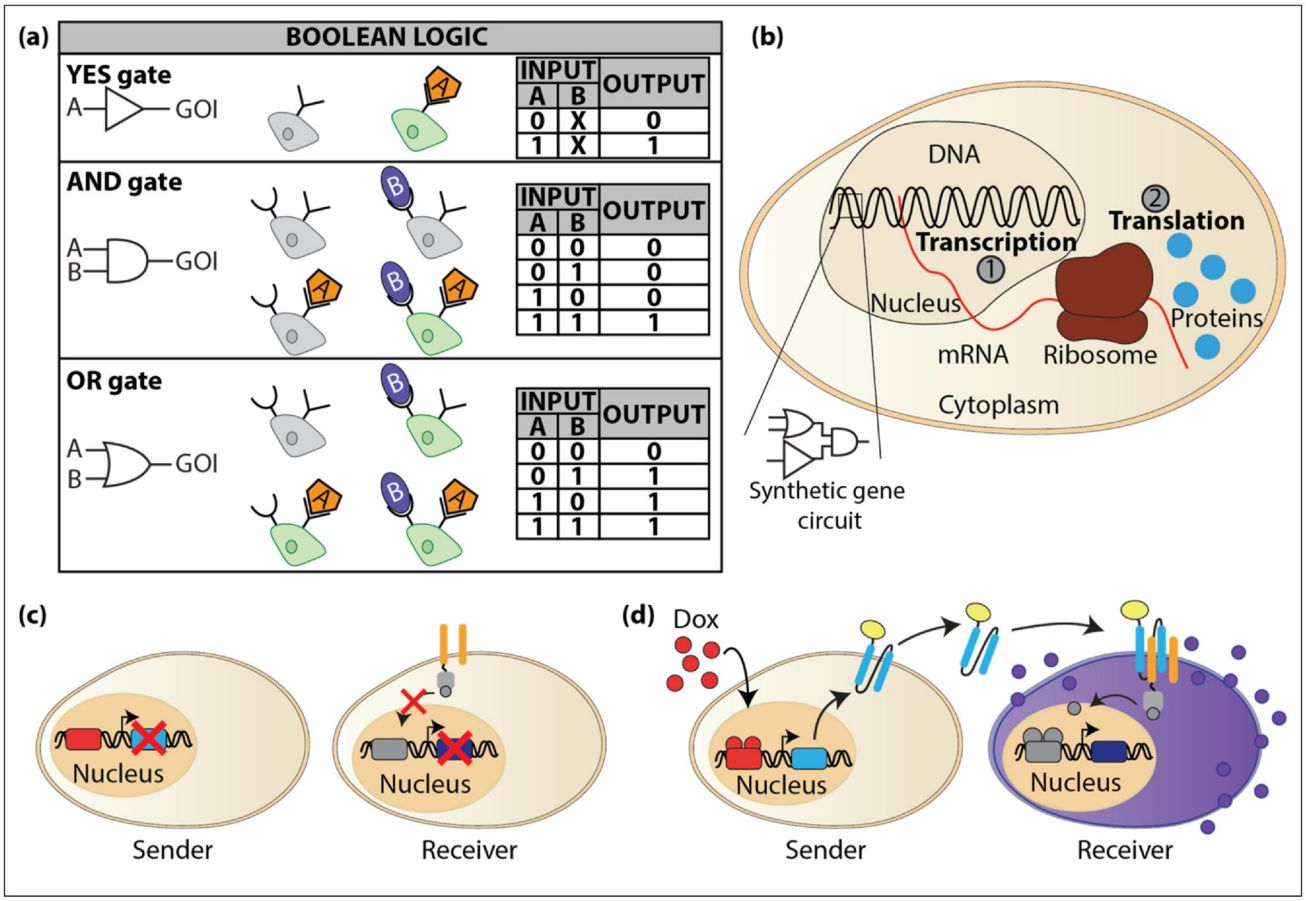

Figure 1.

Engineering cells. **(a)** Boolean logic can be programmed in cells using synthetic gene circuits to enable decision-making. Cells process inputs and when the logic input is true, the gene of interest (GOI) turns on (green). **(b)** DNA is transcribed into mRNA (red line) in the nucleus (1) and transported into the cytoplasm where it binds to a ribosome to be translated into protein (blue circles) (2). Transcriptional-based cellular reprogramming targets DNA located in the nucleus, whereas post-transcriptional-based reprogramming targets RNA in the cytoplasm. **(c)** An engineered communication system was built with engineered sender and receiver cells using coiled coils. In the absence of doxycycline (Dox), the coiled coils are not produced, and the receiver cell is in the off-state. **(d)** In the presence of Dox, the coiled coils are produced and bind to their cognate Epo receptors on the receiver cells. Upon binding, the STAT3 protein (gray ball) is released and translocates to the nucleus to activate the transcription of secreted alkaline phosphatase, which is secreted by the receiver cell (purple balls).

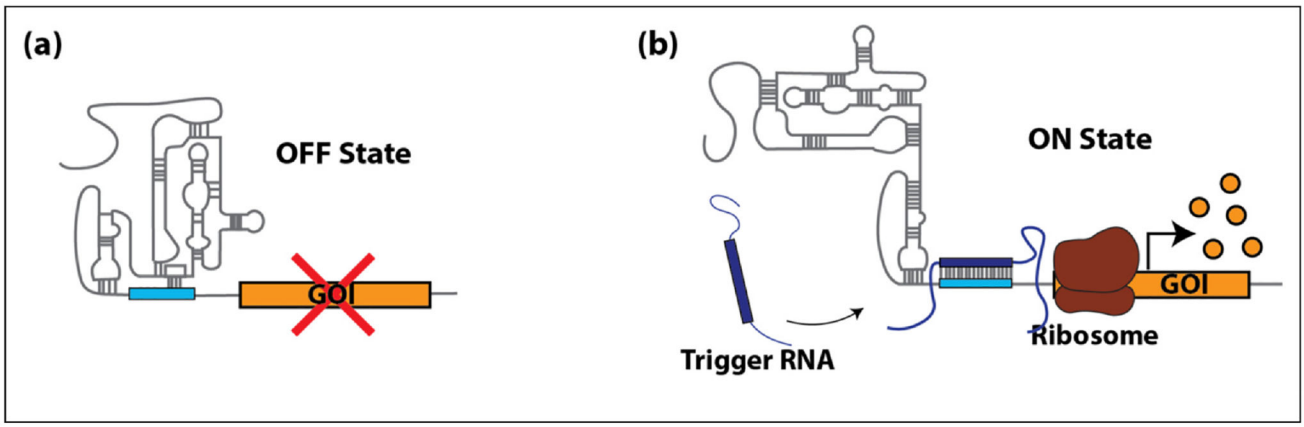

Figure 2.

eToeholds. **(a)** Gene expression of the GOI is in the off-state in the absence of the trigger RNA due to inhibitory loops within the RNA structure. **(b)** In the presence of the trigger RNA (dark blue), it binds to the complementary RNA (teal) within the eToehold, causing disruption of the toehold structure to enable the assembly of ribosomes and translation of the downstream GOI.

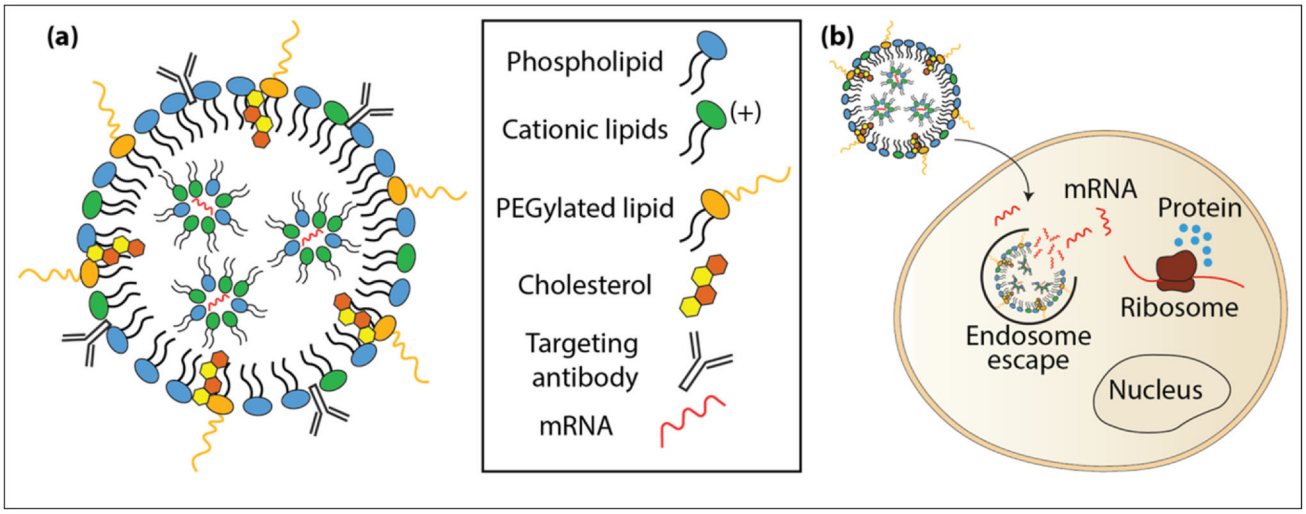

Figure 3.

LNPs used to deliver modified mRNA. **(a)** LNPs are fat droplets composed of a mixture of lipids and cholesterol. They can be designed to target specific cells by functionalizing them with targeting antibodies. **(b)** Once inside the cell, the LNPs enter the endosome where they are broken apart and the mRNA escapes into the cytosol where it is translated into functional protein.

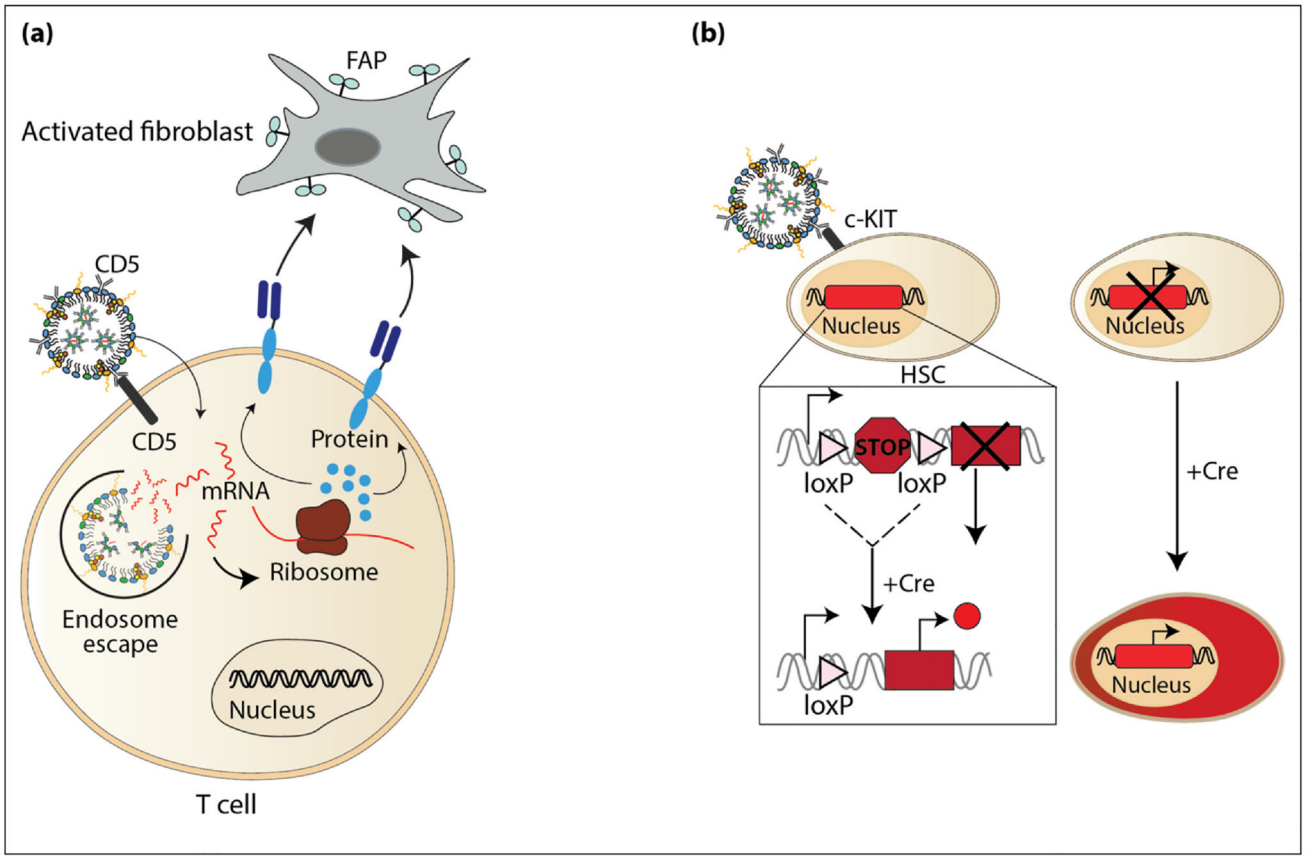

Figure 4.

Delivery of mRNA in vivo**. (a)** LNPs functionalized with antibodies for CD5, a marker for T cells, loaded with mRNA encoding a CAR designed against the FAP. Once the mRNA is released from the endosome, it is translated into a functional CAR and the newly engineered CAR-T cells target and kill activated fibroblasts. **(b)** LNPs functionalized with antibodies against c-KIT, a marker for mouse HSCs, were filled with mRNA encoding the enzyme Cre recombinase. HSCs in the transgenic mouse model harboring a loxP-flanked STOP cassette that prevents the expression of tdTomato were targeted. In the absence of Cre recombinase, the cells do not express tdTomato and are not red. However, when Cre is delivered to the cells, it causes a homologous recombination event at the loxP sites and removes the STOP cassette and turns on the expression of tdTomato turning the cells that turn red.

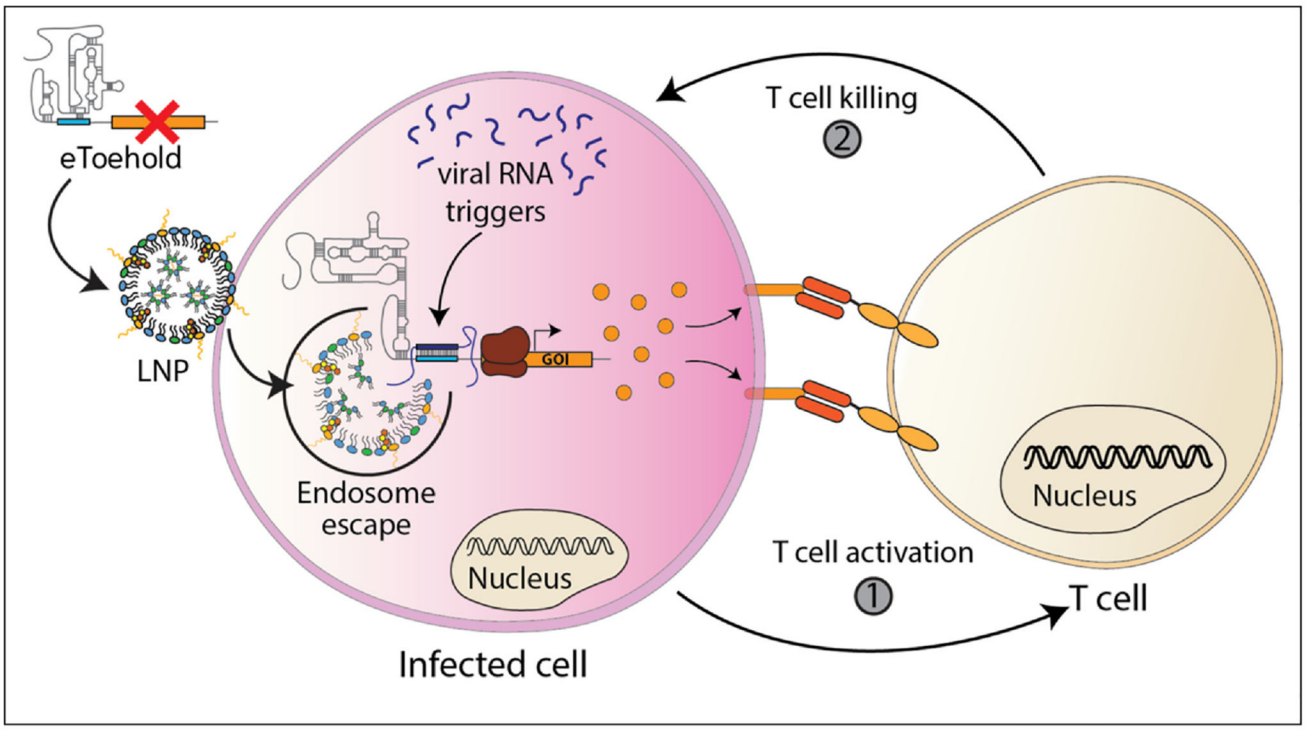

Figure 5.

eToeholds for detecting infected cells. eToeholds can be delivered by LNPs or other delivery mechanisms and upon detecting viral RNA in cells, it activates the translation of transmembrane proteins that function as T-cell beacons (orange) for T-cell destruction. (1) Upon T binding to the infected cell, it is activated and (2) the T cell is signaled to kill the infected cell.