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MINI REVIEW

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Enhancing cell-based therapies with synthetic gene circuits responsive to molecular stimuli

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Abstract

Synthetic biology aims to contribute to the development of next-generation patient-specific cell-based therapies for chronic diseases especially through the construction of sophisticated synthetic gene switches to enhance the safety and spatiotemporal controllability of engineered cells. Indeed, switches that sense and process specific cues, which may be either externally administered triggers or endogenous disease-associated molecules, have emerged as powerful tools for programming and fine-tuning therapeutic outputs. Living engineered cells, often referred to as designer cells, incorporating such switches are delivered to patients either as encapsulated cell implants or by infusion, as in the case of the clinically approved CAR-T cell therapies. Here, we review recent developments in synthetic gene switches responsive to molecular stimuli, spanning regulatory mechanisms acting at the transcriptional, translational, and posttranslational levels. We also discuss current challenges facing clinical translation of cell-based therapies employing these devices.

KEYWORDS
cell therapies, designer cells, genetic switches, mammalian cell engineering, synthetic biology

1 | INTRODUCTION

Conventional therapies typically rely on chemical agents (small molecular drugs) or biological entities (monoclonal antibodies and other recombinant proteins) for disease treatment. The development of new therapeutic drugs usually involves multiple rounds of screening and optimization (Hughes et al., 2011), but once a drug has been approved for clinical use, treatment typically follows standardized procedures of administration, adjusting dosage and timing based on parameters such as gender, weight, and age. However, other factors such as the disease stage, quality of life, and route of administration may not be taken fully into account, and, importantly, the pleiotropic effects of chemical drugs may cause undesired side effects. To overcome such limitations, synthetic biologists have been exploring the revolutionary concept of living therapeutics, using engineered bacteria and eukaryotic cells to replace chemical drugs for treatment, diagnosis, or prevention of human diseases, and thereby laying the foundation for personalized, next-generation therapies (Figure 1a) (Charbonneau et al., 2020; Xie, Haellman, et al., 2016). The clinical application of engineered bacteria has so far primarily been limited to the gut environment, due mainly to safety and engineering constraints. Of note is the discovery and utilization of the strain *E. coli* Nissle 1917 (EcN) as a probiotic or as chassis organism due to its safety profile in humans (Fábrega et al., 2017; Isabella et al., 2018; Kurtz et al., 2019; Sonnenborn, 2016). In contrast, mammalian cell-based therapies offer greater safety and therapeutic potential. For example, mesenchymal stem cells (MSC) can be directly utilized without genetic

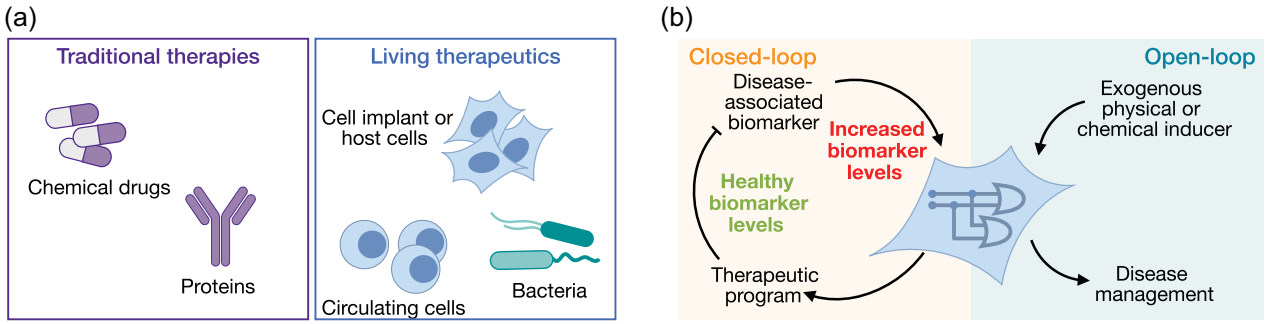

FIGURE 1 Traditional molecular therapies versus engineered living cell therapies. (a) Traditional therapies make use of chemical drugs or biological molecules and are frequently administered as standardized treatments. Living therapeutics involve the use of wild-type or engineered cells for tailored treatment of chronic diseases. (b) Designer cells can be engineered to activate their therapeutic program upon detecting abnormal levels of a disease-associated biomarker, creating a closed-loop system. Alternatively, they can respond to the administration of a user-provided signal, establishing an open-loop system.

manipulation. The procedure typically involves the isolation of MSCs from the patient, amplification ex vivo, and subsequent reinjection (Hmadcha et al., 2020; Margiana et al., 2022; Nebel et al., 2022). Nevertheless, for most diseases, natural cells alone do not suffice, as they are not naturally equipped with the desired therapeutic phenotype. This can be introduced through genetic engineering, resulting in what are often called "designer cells," that express a protein of interest in response to a defined stimulus. In this regard, the clinical approval of genetically engineered T cells equipped with cancer-targeting chimeric antigen receptors (CAR-T cells) for the treatment of certain types of leukemia or lymphoma was a groundbreaking advance (O'Leary et al., 2019; Sterner & Sterner, 2021).

In general, a designer cell must fulfill three main functions: (a) sensing specific inputs, such as disease-associated markers or externally provided signals; (b) integrating and processing the information received; (c) producing the desired therapeutic response. Successful clinical translation of cell-based therapies requires both safety and efficacy, with precisely controlled, time-dependent dosing intervention and minimal side effects. To meet these requirements, a major challenge of synthetic biology is the design of synthetic gene switches inducible by molecular signals or physical stimuli (light, temperature, electricity) (Xie & Fussenegger, 2018; Yamada et al., 2020). To equip cells with such regulatory networks, synthetic biologists draw inspiration from natural processes, which exhibit complex regulatory landscapes, with targets ranging from bacterial metabolism and communication to cell development and tissue specification.

In this review, we will first explore recent applications of gene switches inducible by molecular cues for the development of open- or closed-loop cell therapies, highlighting the advantages and disadvantages of each approach. Then, we will summarize recent advances in the design of chemically induced synthetic circuits, covering transcriptional, posttranscriptional, and posttranslational types of regulatory systems.

2 | DESIGNER CELLS WITH OPEN- OR CLOSED-LOOP PROGRAMS

Regulation of cellular functions (initiation, interruption, or termination), whether native or synthetic, can occur at the DNA, RNA, or protein level. Existing synthetic gene switches used in cell-based therapies have been designed to be responsive to either molecular cues or various physical stimuli, such as pressure (Zhao et al., 2022), temperature (Stefanov et al., 2021), or light (Mansouri et al., 2021) (Figure 1b). Molecular cues can either be externally provided, forming an open-loop system, or they can be endogenous disease-associated biomarkers, constituting a closed-loop system. The delivery of these genetic circuits can be achieved by direct engineering of host cells, often employing viral vectors, or by transplanting genetically engineered autologous or allogenic cells (Bulcha et al., 2021; Wang et al., 2019; Zhao et al., 2023). In the latter approach, engineered cells are typically encapsulated within a semipermeable polymer that allows nutrient transfer while shielding them from the host immune response (Ashimova et al., 2019; Farina et al., 2019).

In open-loop systems, the trigger molecule can be delivered orally, transdermally/topically, or via inhalation, entering the bloodstream to reach the cell implant. Since the introduction of the tetracycline gene switch in mammalian cells (Gossen et al., 1995), the repertoire of chemically inducible gene switches has expanded dramatically. Exogenous chemical inducers include organic and inorganic compounds, and peptides (Franko et al., 2021; Pistikou et al., 2023). From a therapeutic standpoint, food components or additives, such as caffeine (Bojar et al., 2018), menthol (Bai et al., 2019), xylose (Galvan et al., 2022), acetoin (Bertschi, Stefanov, et al., 2023), gluconate (Teixeira et al., 2023), and vanillic acid (Gitzinger et al., 2012), are preferable due to their safety and ease of incorporation into the patients' diet at concentrations well above the normal intake levels to activate the gene switches. Conversely, molecules such as vitamins (Weber et al., 2007), antibiotics (Fussenegger et al., 2000; Gossen et al., 1995), hormones (Rössger

et al., 2013), and other drugs are generally considered less promising candidates due to their broader toxicity spectrum and associated side effects. However, open-loop systems have limitations, depending on the therapeutic context. First, the inducer needs to be frequently and actively administered to the patient. Second, depending on the nature of the inducer, there may be associated toxicity or pleiotropic effects. Lastly, dosage adjustments may be required for each patient. Nevertheless, open-loop systems can be advantageous from the viewpoints of safety and dosage control, as delivery of the trigger molecule can be externally monitored and adjusted as needed.

In contrast, closed-loop systems employ cells rationally engineered to detect specific endogenous inputs and respond with dose-dependent activation of a therapeutic program (Mahameed & Fussenegger, 2022). Thus, a key advantage of closed-loop systems is that they continuously monitor the level of a disease-associated biomarker(s) and autonomously adjust the therapeutic output accordingly without the need of external intervention. Examples include the detection and autonomous correction of elevated glucose levels (Xie, Ye, et al., 2016), a hallmark of diabetes, the identification and normalization of high insulin levels, characteristic of patients with metabolic syndrome (Ye et al., 2017), and the detection and reduction of increased levels of uric acid, a characteristic marker of gout arthritis (Kemmer et al., 2010).

The design of both open- and closed-loop systems requires precise, specific, and safe regulation of the engineered cells, which can occur at the DNA, RNA, or protein level.

3 | TRANSCRIPTIONALLY REGULATED PROGRAMS

3.1 | Natural and engineered membrane receptors

The majority of inducible synthetic circuits developed so far act primarily at the level of gene transcription (Figure 2). For example, one class of gene switches is based on plasma membrane receptors with extracellular domains that recognize the input signal, and transmembrane and cytosolic domains that lead to the recruitment of effector proteins or to increased concentrations of second messengers, such as cyclic adenosine monophosphate (cAMP) or calcium. Native tissue-specific receptors can be ectopically expressed, thereby enabling transfer of the receptor's original function to a different cell type. For example, Bai et al. (2019) exploited the ability of the human transient receptor potential melastatin 8 (TRPM8) channel to trigger an intracellular calcium-dependent signaling cascade in response to menthol or a cooling environment. Receptor activation leads to a cytosolic calcium surge which ultimately turns on gene expression from an NFAT-responsive synthetic promoter (Figure 3a). Transplantation of encapsulated designer cells, genetically engineered with such a synthetic circuit controlling insulin production, mitigated hyperglycemia in experimental type-1 diabetes following daily transdermal application of menthol.

The G protein-coupled receptors (GPCR) are a class of membrane proteins well-suited for detecting therapeutically relevant molecules, as they have naturally evolved to sense and respond to changes in metabolites, hormones, and other ligands (Davenport et al., 2020). Since most mammalian cell lines share common intracellular signaling pathways, GPCR-triggered transgene expression can be achieved by using synthetic promoters harboring the DNA response elements recognized by the activated signaling pathway-specific transcription factors. Rössger et al. (2013) connected the human dopamine receptor D1 (DRD1) to the second messenger cAMP and rewired the signaling to a cAMP-responsive synthetic promoter for transgene expression (Figure 3b).

Alternatively, chimeric receptors can be designed by replacing the physiological signaling pathways with synthetic pathways, or by incorporating novel extracellular binding domains for desired ligands. For example, the GEMS (generalized extracellular molecule sensor) platform enables the flexible engineering of chimeric receptors by fusing new ligand-binding domains (e.g., single-chain antibodies or signal-induced dimerization domains) to the erythropoietin receptor (EpoR) (Figure 3c) (Scheller et al., 2018). The intracellular transduction domain can be exchanged to rewire the response to the desired signaling pathway. Strittmatter et al. (2022) have recently extended the scope of GEMS by introducing a high-throughput platform for generating customizable extracellular binding domains (AMBER, advanced modular bispecific extracellular receptor) based on DARPins (designed ankyrin repeat proteins). In another application, the GEMS platform was repurposed for recognition of ditopic coiled-coil (CC) ligands. This adaptation entailed the utilization of two receptor chains, each featuring a distinct CC peptide within its extracellular domain, which heterodimerize upon encountering their corresponding CC ligands (Figure 3d). Leveraging these new receptors, researchers engineered Boolean logic operations and established a synthetic communication network between sender and receiver cells.

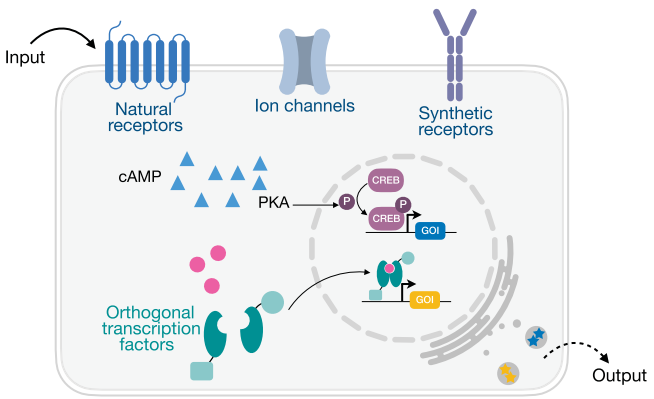

FIGURE 2 Gene switches regulating gene transcription in response to molecular stimuli. The input molecule can be recognized by natural or engineered receptors, activating native (such as the cAMP pathway illustrated here) or synthetic signaling pathways to promote the expression of the gene of interest (GOI). Prokaryotic transcription factors can be engineered to create orthogonal systems.

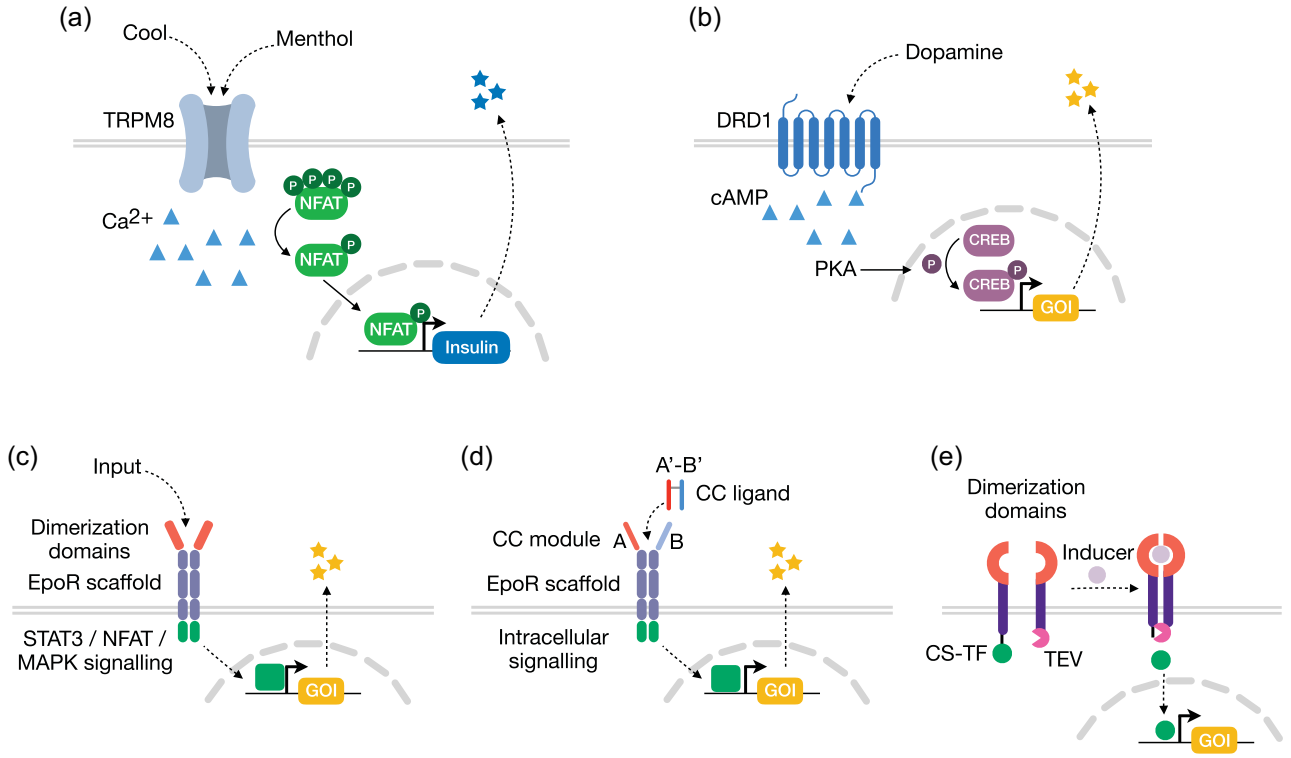

FIGURE 3 Gene switches relying on natural or engineered membrane receptors/channels. (a) Overexpression of the TRPM8 ion channel leads to a transient increase of intracellular calcium (Ca^{2+}), thereby promoting insulin expression from an NFAT promoter. (b) The overexpression of DRD1 GPCR induces cAMP signaling (via PKA and CREB), resulting in transgene expression in response to dopamine. (c) Different ligands can activate GEMS receptors by employing corresponding inducible dimerization domains. The signal is rerouted intracellularly to different endogenous pathways (STAT3/NFAT/MAPK). (d) CC ligand (A'-B') induces the heterodimerization of the CC module (A, B) exposed by the CC-GEMS, inducing transgene expression. (e) Inducer-dependent dimerization of MESA receptor chains triggers TEV protease-mediated release of a transcription factor (TF) linked to a transmembrane domain, and the TF then promotes transgene expression.

In this system, sender cells encoded communication signals in the form of CC ligands, which were regulated by chemical gene switches (Pistikou et al., 2023).

The activation of either native or synthetic receptors linked to increased intracellular concentration of a second messenger can potentially disrupt endogenous signaling pathways, resulting in undesirable crosstalk with the natural functions and metabolism of the cells. To overcome this issue, orthogonal systems offer a promising alternative. For example, the MESA (modular extracellular sensor architecture) platform relies on the dimerization of two receptor chains, consisting of extracellular sensor domains tailored for the desired ligand, each combined with intracellular domains that house either a tobacco etch virus (TEV) protease or an attached transcription factor connected via a TEV cleavage site (Figure 3e) (Daringer et al., 2014). Ligand-induced dimerization triggers proximity between these two fragments, enabling TEV-mediated release of the transcription factor.

The Notch receptor, a pivotal single-pass transmembrane protein, operates as a crucial mediator of short-range cell-cell communication through ligand interaction across adjacent cell surfaces. Upon activation, it undergoes cleavage of its extracellular segment by the ADAM10 protease, followed by intracellular cleavage facilitated by γ-secretase. Subsequently, the liberated cytosolic tail translocates to the nucleus, where it orchestrates the expression of downstream genes (Zhou et al., 2022). The modular nature of the Notch receptor has been demonstrated in various studies, showcasing the feasibility of replacing its extracellular or intracellular domains (Gordon et al., 2015; Lecourtois & Schweisguth, 1998; Struhl & Adachi, 1998). Morsut et al. (2016) simultaneously exchanged both domains while retaining the core transmembrane domain, creating a platform for customizable, synthetic, and orthogonal receptors known as synNotch (Figure 4a). The synNotch receptor is activated upon direct contact with sender cells that display the corresponding ligand on their membrane. Additionally, synNotch receptors that respond to soluble small molecule ligands were recently developed (Mahameed, Wang, et al., 2022). In this case, the Notch1 receptor transmembrane domain was fused N-terminally to inducible dimerizing domains. The presence of the dimerizer molecule promotes a conformational change in the receptor, activating the cleavage of the attached intracellular transcription factor. Rational mutagenesis targeting cysteine residues within the dimerization domains resulted in functional engineered synNotch receptors for soluble ligands.

Due to their versatility and modularity, synNotch receptors have attracted significant clinical interest for immunotherapies (Choe et al., 2021; Roybal et al., 2016; Srivastava et al., 2019). Roybal et al. programmed T cells to be activated by the

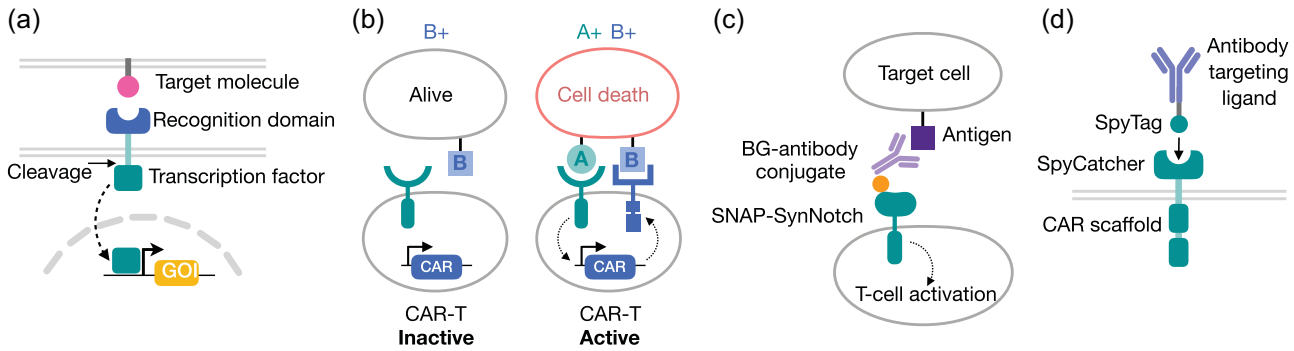

FIGURE 4 Gene switches activated by membrane-bound ligands. (a) A synNotch receptor comprises an extracellular recognition domain, the Notch transmembrane domain and an intracellular transcription factor. This transcription factor is released upon cell-cell interaction between the recognition domain and target antigen. (b) Combinatorial antigen-sensing genetic switch to target tumor cells. T cells are engineered to constitutively express a synNotch receptor (green) for antigen A recognition. This promotes CAR (blue) expression for interaction with antigen B. Activation of the CAR-T cell occurs only when both antigens (A and B) are present on the target cell, leading to its death. (c) Universal synNotch receptor for targeting different antigens. An antigen-specific antibody (light purple) conjugated to a benzylguanine (BG)-motif (orange) is covalently bound to a universal SNAP-tag synNotch receptor expressed by engineered T cells. The antibody specifically binds to its cognate antigen (purple), activating the SNAP-tag synNotch receptor and ultimately triggering T-cell signaling and effector functions. (d) The SpyCatcher immune receptor consists of a versatile CAR scaffold paired with an extracellular SpyCatcher domain. When a ligand-targeting antibody is fused with the SpyTag domain, it forms a covalent bond with the exposed SpyCatcher, facilitating precise and robust antigen recognition.

combinatorial presence of two tumor-specific antigens, employing a synNotch receptor, which, upon binding to its cognate tumor-specific surface antigen, promotes the expression of a CAR designed to recognize a second antigen. This approach ensures T-cell activation only when both antigens are present, thereby enhancing safety and reducing off-tumor toxicity (Figure 4b). To further expand the flexibility of CARs and synNotch receptors, recent studies have focused on developing adaptable systems, in which the engineered receptor binds to a general-purpose molecule conjugated to an antigen-specific antibody. Patients with infused CAR-T cells equipped with such systems would receive a bifunctional molecule consisting of an antigen-specific antibody for tumor cell recognition and a domain that interacts with a universal CAR expressed by the infused T cells (Cho et al., 2018; Ruffo et al., 2023). For example, Ruffo et al. have developed universal CAR and synNotch receptor systems that are genetically fused to a self-labeling enzyme capable of forming a covalent bond with molecules containing a benzylguanine (BG) motif. Therefore, by co-administering a BG-conjugated antibody against the target antigens, CAR-T cells can be activated upon engagement of all three components. Infused CAR-T cells can be retargeted to new tumor antigens simply by providing new BG-conjugated antibodies (Figure 4c). The SpyCatcher immune receptor represents a versatile CAR system. In this design, the CAR's extracellular domain comprises a SpyCatcher protein, while the soluble antibody targeting tumor antigens is engineered with the SpyTag domain. This strategic fusion enables a covalent interaction between SpyCatcher and SpyTag, facilitating T cell activation upon encountering cells bearing the target antigen (Figure 4d) (Minutolo et al., 2020).

3.2 | Engineered orthogonal transcription factors

Alternative orthogonal systems can be constructed by harnessing transcription factors derived from other kingdoms of life. In general, synthetic transcription factors (TFs) share basic features: a DNA-binding domain (DBD) that recognizes a specific DNA sequence (operator sequence) in the promoter region upstream of a gene of interest (GOI); an actuator domain, which inhibits or promotes transgene expression by recruiting the necessary components; and a ligand-binding domain that specifically recognizes the input molecule. Many bacterial transcription factors have been adapted for the regulation of transgene expression in mammalian cells by fusing them with mammalian cell-compatible transactivation domains (VPR, VP16, VP64) and by optimizing the TF-binding region upstream of the GOI. Utilizing such systems offers several advantages, including orthogonality, the abundance of naturally occurring ligands, modularity, and the potential for combining multiple switches to achieve a multi-input response (Bacchus et al., 2012; Folcher et al., 2013; Galvan et al., 2022; Mazé & Benenson, 2020). However, the design of mammalian gene switches based on heterologous TFs requires a series of trial-and-error optimization steps to achieve acceptable signal-to-noise ratios, even when their native DNA-binding sequences have been characterized or the structure of the transcription factor has been elucidated. Usually, multiple tandem repeats of the DNA binding sites and different spacings between them are screened to obtain the best-performing gene switch design. To simplify and rationalize the process, Bertschi, Wang, et al. (2023) implemented LOGIC (large orthogonal gates based on inducer-controlled cascades of protein fusions), a system that exploits bacterial helix-turn-helix transcription factors to engineer ON and

OFF responses and multi-input logic gates. For example, a bacterial TF that dimerizes in the presence of its effector molecule can be used to build a new mammalian gene switch in which the cumbersome promoter optimization process is avoided by relying on a well-characterized DBD such as the tetracycline-dependent TetR or Gal4, whose DNA-binding regions have been optimized for high performance in mammalian cells. Briefly, the dimerizing TF is fused to either a transactivation domain (TA) or to the chosen DBD, and upon exposure to the dimerizing molecule, both fusion proteins come together, co-localizing the TA and DBD in the promoter region and thereby activating transgene expression (Figure 5a). This approach was utilized to build gene switches responsive to vanillic acid (VA), virstatin, xylose, or gluconate (Teixeira et al., 2023), as well as more complex multi-input/output logic gates. For example, multioutput control was obtained by simultaneously using VA-inducible ON and OFF switches to co-regulate the two reporter proteins SEAP and Nanoluc in different ways, that is, to activate one protein and repress the other at low or high VA concentrations, and to express both at intermediate concentrations of VA (Figure 5b). Moreover, a band-pass filter was obtained by replacing SEAP and Nanoluc with two dimerizing partners allowing the expression of the reporter gene only at intermediate concentrations of VA.

3.3 | Inducible tools for endogenous gene editing and regulation

The CRISPR-Cas9 technology has revolutionized in vivo genome editing for disease correction. Several studies have underscored the need for gene switches to precisely control Cas9 expression to ensure spatiotemporally controlled delivery, reduce off-target effects, and enhance overall safety (Schmidt et al., 2023). Zetsche et al. (2015) developed a rapamycin-inducible split-Cas9 system characterized by low background activity in the absence of the inducer, achieved by spatially sequestering one split component in the cytosol. Cas9 devoid of nucleolytic activity, referred to as dead Cas9 (dCas9), has found use in directing other proteins of interest to target DNA sites, significantly expanding applications of the CRISPR system for creating new genetic switches (Du et al., 2021; Zhuo et al., 2021). For example, Gao et al. (2016) developed a platform based on dCas9 utilizing multiple orthogonal dCas9 systems combined with multiplexed sgRNAs to activate or repress different target genes within the same cell. They created AND and OR Boolean logic gates in which the dCas9 is connected to a transactivation domain to promote transgene expression only in the presence of both or either one of the inducers, respectively (Figure 5c). Krawczyk et al. (2020) harnessed endogenous signaling pathways and dCas9 to activate native or synthetic promoters in a system termed GEAR (generalized engineered activation regulators). The system comprised three main components: (i) a fusion of the MS2 bacteriophage coat protein (MCP) with a regulatory domain from a chosen signaling pathway (the GEAR); (ii) the expression of dCas9; (iii) a synthetic guide RNA (sgRNA) with two binding loops (MS2) recognizable by the MCP protein. The dCas9 binds the target DNA sequence complementary to the sgRNA-MS2. Upon endogenous signaling activation, the GEAR is translocated to the nucleus and associates with dCas9 via MS2-MCP interaction, ultimately driving transgene expression. The versatility of the GEAR system was demonstrated with various signaling pathways including the calcium, TGFβ/SMAD, NFKB, and MAPK/ERK pathways. Alongside the regulation of (d)Cas9

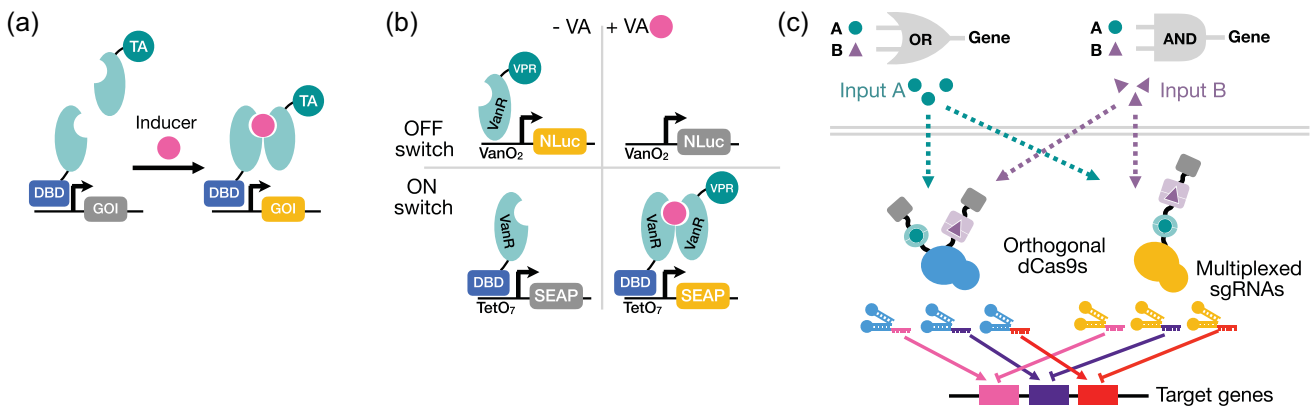

FIGURE 5 Intracellular orthogonal gene switches activated by soluble ligands (a) Design of an orthogonal system in which dimerizing partners are fused to a DNA-binding domain (DBD) or to a transactivation domain (TA). Transgene expression is activated only in the presence of the inducer. (b) Design of a multi-output control system responsive to different concentrations of vanillic acid (VA). In the absence of VA, VanR-VPR binds to the $VanO_2$ promoter, inducing Nanoluc expression (OFF switch). With increasing VA concentration, VanR-VPR is released from the $VanO_2$ promoter, and VanR-TetR, bound to the $TetO_7$ promoter, dimerizes with VanR-VPR promoting SEAP expression (ON switch). (c) Boolean logic gates for inducible expression of orthogonal dCas9 regulators. Orthogonal dCas9s (blue and yellow) are activated by OR and AND gate circuits. On the left, either input A (green) or B (purple) is required to promote dimerization of dCas9 with a dimerizing partner fused to a transactivation domain (gray), creating an OR gate. On the right, inputs A and B are both necessary to connect the dCas9 with dimerizing partners and, subsequently, to the transactivation domain. Additional layers of control are introduced by expressing various dCas9-specific activating or repressing sgRNAs.

expression with inducible gene switches, substantial engineering effort has been directed toward improving the performance of the (d)Cas9 protein by enhancing fidelity and specificity, and decreasing off-target effects (Bratovič et al., 2020; Casini et al., 2018; Chen et al., 2017; Kim et al., 2023; Kleinstiver et al., 2016; Slaymaker et al., 2016).

Nevertheless, the CRISPR-Cas9 technology has some disadvantages for in vivo cell engineering applications. These include the relatively large size of the system, which makes delivery to the host cells challenging, and the enhanced immunogenic potential arising from its bacterial origin (Charlesworth et al., 2019; Chew, 2018; Wagner et al., 2019). In contrast, zinc finger (ZF) transcription regulators are widespread in the human genome and are significantly smaller in size (Cassandri et al., 2017). Consequently, there has been great interest in the engineering of ZFs for remote control of CAR-T cells. For example, Kotter et al. (2021) constructed anti-CD20 CAR primary T cells regulated by 4-hydroxytamoxifen (4OHT), the active metabolite of the clinically approved drug tamoxifen. In this design, a tamoxifen-responsive ZF transcription factor regulates the expression of the CAR. These engineered T cells completely eradicated lymphoma in vivo, with a slight delay compared with T cells constitutively expressing the same anti-CD20 CAR. Similarly, Li et al. devised novel synthetic ZF-based genetic switches active in primary T cells and responsive to various small molecules, including the FDA-approved compounds grazoprevir (GZV) and 4OHT, and the plant-derived hormone abscisic acid. These synthetic ZF systems exhibited dose-dependent responses to their respective inducers and were validated across multiple in vivo applications, including CAR expression in T cells and stimulation of immune cell proliferation. Remarkably, a dual and synergistic effect was achieved in vivo by co-delivering engineered CAR-T cells with GZV-inducible and 4OHT-inducible ZFs. This approach allowed for simultaneous modulation of CAR expression to target the tumor and control of IL-2 expression for cellular proliferation (Li et al., 2022).

An exciting recent study by Saito et al. (2023) unveiled a new RNA-guided endonuclease termed Fanzor, distinguished by its smaller size relative to Cas9 and by its eukaryotic origin. They demonstrated that Fanzor can be programmed for precise genome editing in human cells, showcasing significant potential for future applications in human genome engineering.

4 | POSTTRANSCRIPTIONALLY REGULATED PROGRAMS

4.1 | RNA-based synthetic regulation

An additional layer of control of synthetic gene switches is provided by posttranscriptional regulation targeting mRNA stability, splicing, or translation (Ausländer & Fussenegger, 2017). Specific RNA structures capable of binding a small molecule are called RNA aptamers. These can occur naturally as a part of riboswitches or can be produced in vitro using techniques such as SELEX (systematic evolution of ligands by exponential enrichment) (Famulok & Mayer, 2014). Upon binding of their respective ligands, RNA aptamers typically undergo conformational changes that impact the secondary structure of RNA, thereby influencing transcript stability and, consequently, protein expression. Two commonly employed aptamers in mammalian synthetic biology studies are the theophylline and tetracycline aptamers. These aptamers are often incorporated into self-cleaving ribozymes, known as aptazymes. Yen et al. (2004) showed for the first time the functionality of ribozymes in a variety of mammalian cell lines and in vivo. Following this work, other studies focused on the development of small molecule-inducible aptazymes, active both in mammalian cell cultures and in vivo (Ausländer et al., 2014; Mustafina et al., 2020; Nomura et al., 2012; Zhong et al., 2016). Ausländer et al. (2010) developed a theophylline aptazyme in mammalian cells, by means of in vivo screening starting from an artificial aptazyme.

RNA interference (RNAi) is a naturally occurring genetic switch in the cytoplasm of eukaryotic cells. Small RNA molecules, such as short hairpin RNA (shRNA), siRNA, or microRNA (miRNA), can be designed to recognize complementary mRNA sequences, triggering their degradation and thereby blocking or decreasing protein production (Frei et al., 2022; Matsuura et al., 2018; Nordick et al., 2022; Schmiedel et al., 2015). RNA-based therapeutics can impact protein expression by upregulating the expression of a GOI or by altering the splicing, resulting in an aberrant protein (Anthony, 2022; Brentari et al., 2023). Matsuura et al. (2018) designed multiple logic circuits using miRNAs to target the expression of RNA-binding proteins, which, in turn, regulate the expression of the protein of interest. Notably, they created an AND gate based on two miRNAs, capable of inducing apoptosis in target cells (Figure 6a).

Advantages of using RNA-based synthetic regulation, especially for in vivo delivery of genetic circuits, include the short half-life in cells and the fact that RNA does not randomly integrate into the genome, avoiding disruption of endogenous genes or regulatory sequences. New vaccine technologies based on the delivery of nucleic acids, in particular RNA, have generated enormous interest, especially since the SARS-CoV-2 pandemic, thanks to their potential for different applications (Ho et al., 2021; Liu, 2019; Rojas et al., 2023). New vaccines can easily be developed once a pathogen's genome has been sequenced, offering tremendous advantages compared with the classic whole-organism vaccine platforms, which rely on the cultivation of the pathogen and long manufacturing procedures (Pollard & Bijker, 2021). mRNA-based tools are also showing promise as therapies for cancer (Liu et al., 2023; Xie et al., 2023). Rojas et al. (2023) reported successful results from a phase I clinical trial in patients with resected PDAC (pancreatic ductal adenocarcinoma), which is lethal in almost 90% of cases even after surgery. After surgical removal of PDAC and in combination with chemotherapy and monoclonal antibody therapy, patients received a personalized mRNA vaccine based on sequencing-predicted tumor-specific

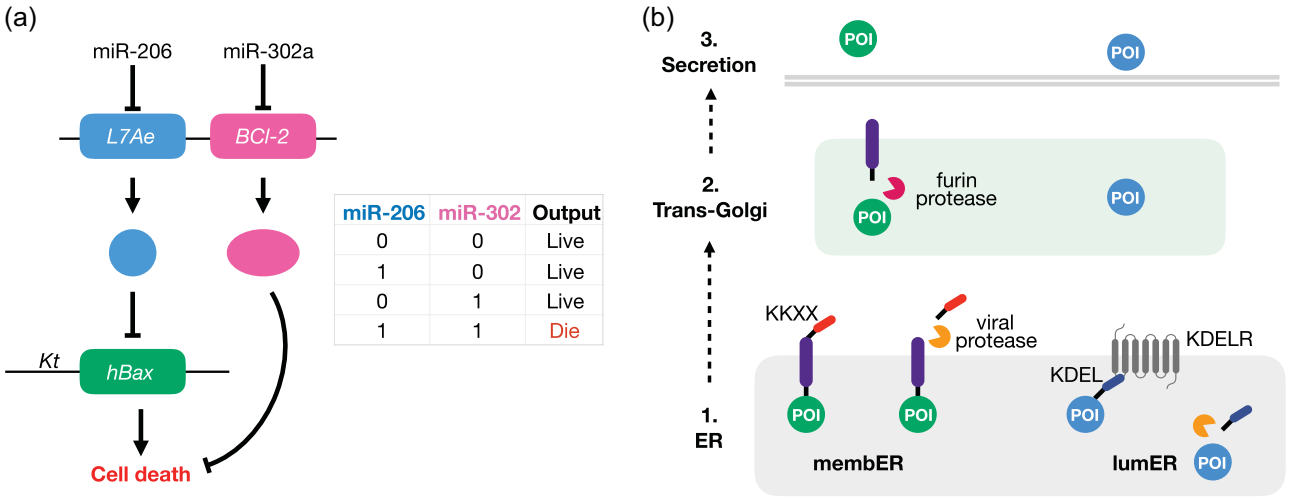

FIGURE 6 Post-transcriptionally regulated systems. (a) miRNA-dependent 2-input AND circuit regulating apoptosis. Two miRNAs (miR-206 and miR-302a) control the expression of the human *Bax* (*hBax*) proapoptotic gene by regulating the transcript of the antiapoptotic BCl-2 fused to L7Ae, a kink-turn (*Kt*) RNA-binding protein. Interaction between L7Ae and the Kt motif at the 5′-UTR inhibits the translation of the hBax transcript. The survival table on the right indicates that when no miRNA or either one of them alone is present, the cell survives. However, when both miRNAs are present, miR-302a suppresses the expression of the antiapoptotic BCl-2 protein, and miR-206 inhibits the translation of L7Ae. This prevents L7Ae from binding to the Kt motif, leading to hBax expression and cell apoptosis. (b) Regulation of protein secretion. In the membER system, the protein of interest (POI), fused with a furin cleavage site, a transmembrane domain, a protease cleavage site and a KKXX tag, is sequestered into the ER membrane (1). When the viral protease is present in the cytosol, it cleaves the KKXX tag enabling the protein complex to transit to the trans-Golgi (2). There, the furin protease releases the POI from the membrane, facilitating its secretion (3). In the lumER system, the POI is fused to a protease cleavage site and a KDEL tag for interaction with the KDEL receptor (KDELR) (1). When the viral protease is present in the ER, the POI is released from the KDELR interaction and proceeds to the trans-Golgi (2) before being secreted (3).

neoantigens and intended to further stimulate T-cell cytotoxicity. Half of the patients that received the mRNA therapy remained cancer-free during the 18-month follow-up. mRNA vaccines directed toward a tumor's neoantigens, particularly when combined with existing immunotherapies, offer several advantages. They are personalized for each patient, are simple and rapid to manufacture, and have reduced off-target effects (Blass & Ott, 2021).

4.2 | Engineered protein switches

Designer cells relying on transcriptional or posttranscriptional control might not be suitable for time-sensitive therapeutic applications, as they exhibit a delayed response to the input signal due to the time required for transcription and translation of the protein of interest. Recent research has sought to address this limitation by developing fast-release systems, wherein the protein of interest is released within minutes after the detection of the input molecule. In nature, processes that require a rapid response often rely on the release of presynthesized proteins that are stored within intracellular vesicles and released in response to specific environmental stimuli. Examples of such natural processes can be seen in neurons, which release small molecule neurotransmitters and neuropeptides upon detection of action potentials, and in β-cells, which release insulin in response to postprandial elevated glucose levels. However, the majority of proteins are constitutively secreted via a well-established pathway involving the endoplasmic reticulum (ER), the Golgi apparatus, trans-Golgi, and plasma membrane (Benham, 2012). To ensure proper trafficking, secreted proteins are tagged with an N-terminal signal peptide or an internal signaling sequence, directing the newly synthesized protein to the ER. Proteins that are retained in the ER are marked with specific C-terminal motifs such as KDEL (Lys-Asp-Glu-Leu), for interaction of soluble proteins with the KDEL receptor (KDELR), or KKXX (Lys-Lys-X-X, where X can be any amino acid), commonly found in transmembrane proteins. Recently, engineered systems have been developed in which the protein of interest is trapped in the ER and released by protease cleavage (Mahameed, Xue, et al., 2022; Mansouri et al., 2023; Praznik et al., 2022). For example, Mahameed et al. fused an engineered ER-localized split-TEV protease to the FKBP or FRB domains for rapamycin-inducible heterodimerization. The protein of interest is localized in the ER and a TEV cleavage site is placed between the protein and the KDEL tag. Upon rapamycin supplementation, the two protease fragments combine to generate active TEV that acts on the cleavage site to release the protein of interest, which is then secreted within minutes (Mahameed, Xue, et al., 2022). In another study, Praznik et al. (2022) utilized a split protease to develop two systems for the rapid release of presynthesized proteins accumulated in the ER (Figure 6b). In the membER system, the protein of interest was anchored to the ER membrane by fusion

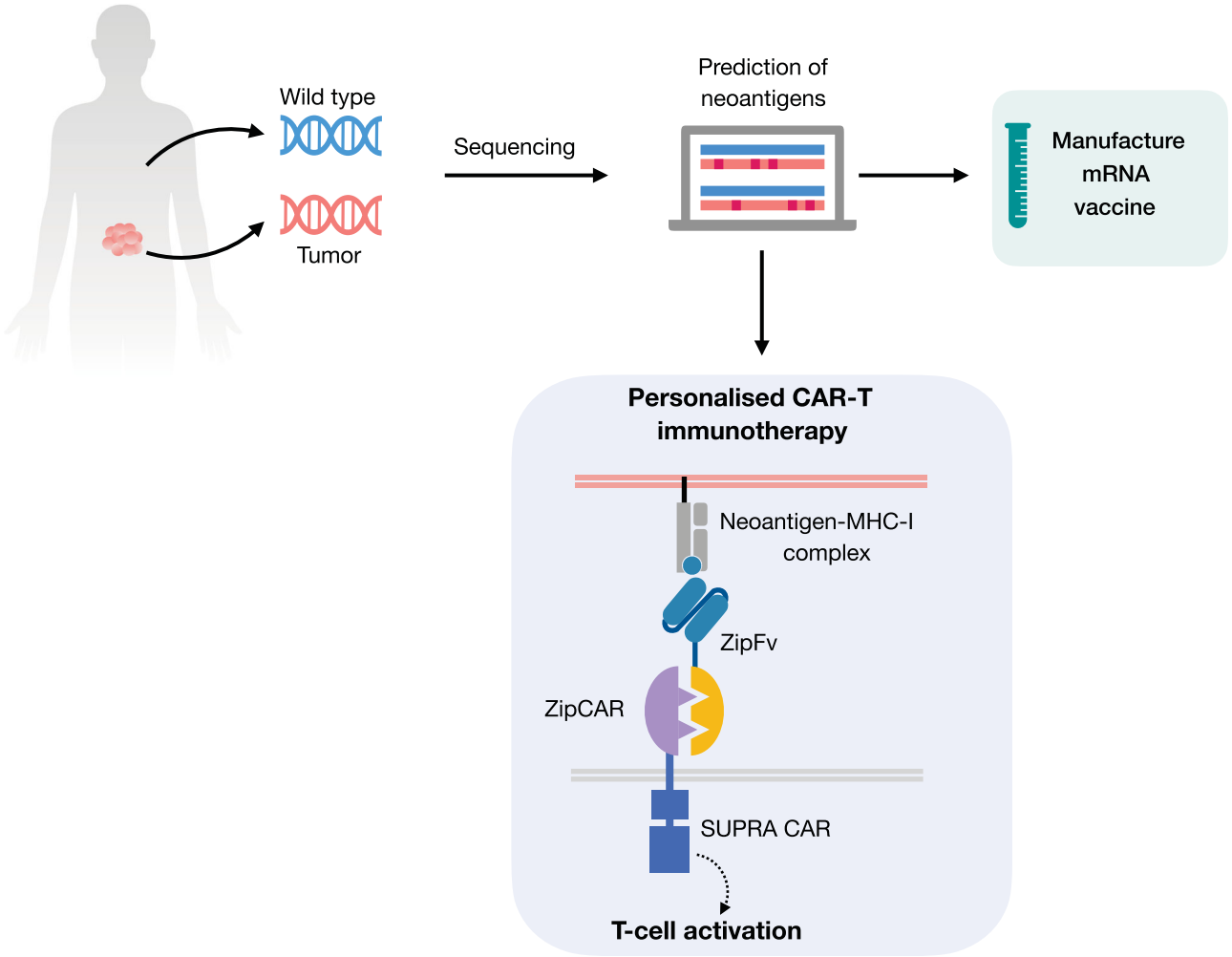

FIGURE 7 Neoantigens-based personalized next-generation therapies. Normal and tumor cells are isolated from the patient and next-generation sequencing is performed to predict tumor-specific neoantigens. Custom mRNA neoantigen vaccines can be manufactured (right panel), or universal CAR-T cells can be retargeted (bottom panel). Knowing the neoantigen sequence increases the specificity of the therapy and reduces the engineering effort by combining machine learning approaches with an adaptor CAR system. A standardized ZipCAR of a SUPRA CAR system (Cho et al., 2018) is combined with an ad-hoc predicted and engineered ZipFv to recognize the neoantigen-MHC-I-complex on the tumor cell, leading to the activation of the engineered T cell. The safety and specificity of the therapy are ensured through the incorporation of genetic switches that tune the expression of the genetic parts, encode a kill-switch in case of emergency and program the engineered T cell to be activated only when multiple (neo)antigens are present.

to the B-cell antigen receptor complex-associated protein beta chain (CD79B) and to a KKXX motif. A protease cleavage site was placed between the ER retention motif and the transmembrane domain, allowing for cleavage by a cytosolic protease. To ensure the release of the protein of interest from the membrane in the Golgi apparatus, a furin cleavage site was inserted between the protein and the transmembrane domain. In the lumER system, a soluble protein was trapped in the ER by fusion to the KDEL retention signal. A protease cleavage site placed between the protein of interest and the KDEL tag ensures that protein release via interaction with KDELR and further processing through the secretory pathway occur only when the protease is present.

Alternative strategies to tune protein levels/activity include the regulation of protein degradation, for example by introducing specific destabilizing tags (degrons), by engineering natural protein regulatory mechanisms or by developing very rapid and efficient protease-dependent protein switches (Chassin et al., 2019; Franko et al., 2024; Zhang et al., 2022).

5 | CONCLUSION AND PERSPECTIVES

Cell-based therapies represent a promising path forward in medicine, potentially offering solutions to critical therapeutic challenges that have evaded traditional drug-based approaches. While numerous gene- and cell-based therapies discussed are still in the preclinical stages, the market approval of various CAR-T therapies for cancer treatment underscores the remarkable potential of engineered cell-based treatments in clinical practice (Sadelain et al., 2017; Wang et al., 2023; Yip & Webster, 2018).

Moreover, the utilization of CRISPR-Cas9 technology is experiencing rapid expansion, with its applications extending to both ex vivo and in vivo genome editing. This trend is underscored by the proliferation of ongoing clinical trials (e.g., Stadtmauer et al., 2020) and the emergence of several ex vivo applications that have already entered the market (Li et al., 2020; Witkowsky et al., 2023). By programming cells to execute personalized therapeutic programs, we can achieve unprecedented controllability and minimize the toxicity often associated with traditional chemical drugs. The incorporation of synthetic genetic switches, as discussed in this review, is key to ensuring the safety, controllability, and therapeutic efficacy of these advanced therapies (Bashor et al., 2022). Addressing challenges facing clinical translation, such as establishing long-term effectiveness, mitigating uncontrolled immune system reactions, that is, the cytokine storms and off-target effects common in CAR-T therapy, and mitigating risks of tumorigenicity in the case of stem cell-based treatments, are active areas of research (Sterner & Sterner, 2021; Yamanaka, 2020). For example, the elucidation of TCR CD3 signaling chain mechanisms has sparked advancements in CAR receptor engineering, incorporating CD3ε. This innovation has led to increased antitumor efficacy while mitigating cytokine production (Wu et al., 2020).

More advanced cell-based therapies will directly benefit from the development of new tools and technologies in medicine, biology, and material science. For example, future developments in biocompatible materials, precise genetic control systems, and advanced techniques for nucleic acid manipulation and delivery will further enhance the capabilities of cell-based therapies (Maity et al., 2023; Milone and O'Doherty, 2018; Neves et al., 2020). In addition, the integration of next-generation sequencing technologies (NGS) and deep learning modeling approaches, exemplified by innovations such as AlphaFold for protein structure prediction, will contribute to the advance of engineered cell-based therapies (Hassoun et al., 2021; Jumper et al., 2021; Thornton et al., 2021). In the realm of cancer treatment, the rapid prediction of tumor neoantigens by NGS holds immense potential to design tailored cancer mRNA vaccines (Liu et al., 2023; Rohner et al., 2022; Rojas et al., 2023; Xie et al., 2023), or modularly updating adaptor molecules in universal CAR approaches, redirecting infused engineered T cells toward new tumor-specific antigens (Cho et al., 2018; Lajoie et al., 2020; Ruffo et al., 2023) (Figure 7). These developments offer the prospect of widespread introduction of personalized treatments, significantly enhancing patients' outcomes.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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