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Review

Regulation of CAR transgene expression to design semiautonomous CAR-T

Paweł Głowacki,[1] Cezary Treda,[1,2] and Piotr Rieske[1,2]

[1]Department of Tumor Biology, Chair of Medical Biology, Medical University of Lodz, Zeligowskiego 7/9 Street, 90-752 Lodz, Poland; [2]Department of Research and Development Personather Ltd, Inwestycyjna 7, 95-050 Konstantynow Lodzki, Poland

Effective transgene expression is critical for genetically engineered cell therapy. Therefore, one of CAR-T cell therapy's critical areas of interest, both in registered products and next-generation approaches is the expression of transgenes. It turns out that various constitutive promoters used in clinical products may influence CAR-T cell antitumor effectiveness and impact the manufacturing process. Furthermore, next-generation CAR-T starts to install remotely controlled inducible promoters or even autonomous expression systems, opening new ways of priming, boosting, and increasing the safety of CAR-T. In this article, a wide range of constitutive and inducible promoters has been grouped and structured, making it possible to compare their pros and cons as well as clinical usage. Finally, logic gates based on Synthetic Notch have been elaborated, demonstrating the coupling of desired external signals with genetically engineered cellular responses.

INTRODUCTION

The regulation of chimeric antigen receptor (CAR) transgene expression is crucial for eliciting the desired response in CAR-T cells. Typically, constitutive promoters are employed to drive CAR expression, leading to predictable levels of CAR and therefore antitumor activity.[1,2] However, due to the specific nature of CAR-T therapy, it can benefit from more precise transcriptional regulation in certain situations. To achieve this, inducible transcription systems are employed, utilizing transcription factors (TFs) and domains that bind to specific DNA sequences placed in proximity to the CAR sequence. This article provides an overview of the classification of transgene transcriptional regulation based on its mechanism of action, highlighting how variable gene expression can be harnessed to address current challenges associated with CAR-T cell therapy. The discussion begins with constitutive promoters and progresses to the utilization of the CRISPR system for inducing regulated expression.

As part of the description, several divisions can be made. The division proposed here is based on TF features. We distinguish the following TFs: endogenous (natural) TFs, exogenous (xenogenous) TFs, and artificial TFs (ATFs). Endogenous natural TFs are expressed in human cells naturally, for example, SP1 or HSF.[3,4] Exogenous TFs are not artificial proteins, but are expressed in other species and thus are not natural for human cells. Among that group, we can find bacterial Tet.[5] The last group of TFs is fully synthetic and therefore called artificial TFs (e.g., dCAS9sgRNA).[6] Examples of artificial TFs are dCas9, TALENs, or proteins containing zinc finger. Importantly, in the context of the immune system, both exogenous TFs and ATFs are recognized as not natural and potentially leading to triggering of the immune response.[7,8] Subsequently, all of these groups can be further subdivided into whether the expression is non-induced (constitutive Sp1 binding to the CMV promoter) or induced (variable Tet or dCAS9-CRY2-CIB activation). In the case of induced ones, on the other hand, a division can be made into chemically induced (by small molecules in the Tet system) and induced by physical factors (optogenetic LINTAD system, HSP promoter-based thermal/ultrasound control).[9,10]

From a clinical point of view, the goal of the aforementioned systems is to achieve a CAR-T that will be a more effective therapeutic agent. Control of CAR-T activity with induced transcription has been confirmed as an effective strategy and raises hopes in different aspects of four key immunological situations: (1) cytokine release syndrome (CRS), (2) CAR-T exhaustion, (3) escape of cancer cells from the CAR attack, and (4) CAR-T target change in an on-target off-tumor attack.[11–14] Since expression systems can drive various transgenes, not only the CAR but also other proteins such as cytokines can be controlled.[15]

In a cytokine storm, the triggering mechanisms of its key elements as well as eupotential markers (IL-1 or IL-6) and characteristic symptoms have already been, in some part, discovered.[16] For this reason, there are therapeutic strategies routinely used in cases of CRS occurrence based on the administration of steroids and/or receptor-binding antibodies for IL-6.[17] Despite this, the overall toxicity of CAR-T, for which CRS is mainly responsible, is significantly higher than in other types of immunotherapy.[18,19] Other strategies for circumventing this problem involve preparing the CAR-T cells themselves. An example is the modification of the manufacturing process

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Correspondence: Paweł Głowacki, Department of Tumor Biology, Chair of Medical Biology, Medical University of Lodz, Zeligowskiego 7/9 Street, 90-752 Lodz, Poland.
E-mail: pawel.glowacki@stud.umed.lodz.pl
Correspondence: Piotr Rieske, Department of Tumor Biology, Chair of Medical Biology, Medical University of Lodz, Zeligowskiego 7/9 Street, 90-752 Lodz, Poland.
E-mail: piotr.rieske@umed.lodz.pl

that enriches CAR-T with a stem-like population that is less likely to trigger macrophage activation and cytokine secretion, thus reducing the incidence of severe CRS in mice.[20] On the other hand, genetic modifications in CAR-T cells that limit or prevent CRS have been proposed. The first strategy tested in clinical trials is based on killing CAR-T cells by suicide switch.[21] Given that the cause of CRS is the large amount of damage-associated molecular patterns released from CAR-T-killed cells and the cytokines secreted by CARs, lowering the activity of CARs may reduce the intensity of the cytokine storm.[22] However, in order not to lose CAR-T cells, but only to temporarily limit their activity, a better strategy is to control the CAR protein expression itself. That can be achieved using the transcription-inducible systems described in Hotblack et al.[11] Another strategy is CAR-T autonomously secreting IL-6 or IL-1 binding factors so as to preemptively reduce pro-inflammatory interleukin concentrations and prevent triggering of the cytokine storm.[23] Despite the fact that proteins are driven by constant promoters, it would be possible to create a system that self reacts to CRS-triggering cytokines and secretes CRS binding factors in response. Such induced transcription, achieved with Synthetic Notch (SynNotch), for example, could reduce the cellular burden from the production of additional proteins.[24]

Another situation in which transcriptional regulation may be applicable is CAR-T depletion. The depletion phenomenon itself is complex and induced by many factors, both intrinsic (tonic signals from CARs) and extrinsic (influence of the tumor microenvironment).[13,25] In the case of tonic signals, the problem can be solved by limiting the expression of the CAR. Studies have shown that SynNotch systems autonomically regulate transcription limit depletion, contributing to increased survival and ultimately a better anticancer effect.[14] Pharmacologically regulated expression of CARs is also likely to have such an effect.[26] The second type of CAR-T depletion resulting from the tumor microenvironment can, in turn, be alleviated by secreting cytokines that boost CAR-T persistence.[27] However, such cytokines carry the risk of toxicity.[28] Transcriptional regulation of cytokine expression and secretion has shown promise in *in vivo* tests as a method of maintaining a balance between secreting them in the right amount and location and acting to reduce exhaustion.[29] Unlike in CRS, CAR-T depletion is more difficult to detect due to the lack of systemic markers. Instead, CAR-T cells present in the blood must be subjected to flow cytometry analysis to detect exhaustion markers like PD1 or TIM3.[30] For this reason, solutions for autonomous CAR-T seem more attractive.

The third situation negatively affecting the therapeutic value of CAR-T is antigen escape, i.e., circumstances in which a tumor cell loses its CAR-activating antigen or is undetectable.[31] This leads to the formation of a population of CAR-T-resistant tumor cells and, as a result, is a major cause of CAR therapy failure.[32] The basic strategy, in this case, is to increase the number of recognized antigens by using tandem-CAR (one chimeric receptor binds multiple antigens) or universal CARs (multiple adapters along the lines of bispecific T cell engagers).[33,34] In the case of adapters, a significant complication is their short half-life (which forces them to be continuously infused), while tandem chimeric antigenic receptors increase the pool of simultaneously recognized antigens, which can lead to increased killing of healthy cells.[34,35] Regulated transcription could make it possible to switch the transcription of the CAR recognition domain without the need for constant adapter infusion and without the simultaneous recognition of multiple antigens. The detection of an antigen escape could be done by cytometry (hematologic malignancies) or immunohistochemistry (solid tumors), which would allow medical personnel to react and change the specificity of the CAR antigen. This, however, may be more challenging due to some obstacles. First, primary TAA (tumor-associated antigen) can still be expressed on cancer cells, but in mutated form, so CAR-T cells lose their affinity or ability to activate CARs, but flow cytometry is still able to detect the antigen.[36] Likewise, it is difficult to determine the threshold at which CAR-T cells lose their ability to recognize such cells. At the same time, flow cytometry as a sensitive system still indicates some TAA expression.[37] Autonomous systems would have to recognize the antigen escape on their own; this is more challenging to do because, in the case of the loss of TAA, the T lymphocyte would not be able to determine whether it is in an area of cancer cells that lack TAA or in an area of normal healthy cells.

The latest CAR-T challenge, which is also the most pressing problem in the context of solid tumors, is on-target off-tumor attacks. This phenomenon is based on killing cells that possess CAR-activating antigens but are healthy cells.[38] The control of antigen receptor expression means that, if non-specific CARs begin to cause toxicity by attacking the patient's healthy tissue, they can be deactivated.[11] Manually controlled systems can be used in this case if there are visible symptoms, while autonomous systems can regulate expression if an antigen found only on healthy but not cancerous cells is recognized, providing an automatic safety button. What is more, in the case of SynNotch, it is even possible to use non-specific antigens specifically, as described in more detail in the section on autonomous CARs.[39]

Constitutive promoters used in the clinic

Endogenous and exogenous constitutive promoters are currently the most commonly used in CAR-T, both in registered therapies and in CAR-T undergoing clinical trials (Table 1). Among the clinically used CARs collected in our article, as many as half contain the human elongation factor 1α (EF1α) promoter classified as endogenous and non-inducible, presenting several advantages. EF1α is a potent promoter that ensures predictable transgene expression in lymphocytes.[40] Comparative studies have shown that the EF1α promoter enabled the most efficient transduction of T lymphocytes compared with transgenes containing the CMV, hPGK, and RPBSA promoters.[1] However, the second commonly clinically used promoter, the MND promoter, showed even greater lentiviral transduction capacity than EF1α.[2] The MND promoter is composed of two elements: the U3 region and the myeloproliferative sarcoma virus enhancer.[41] In addition to its greater transduction capacity, it allows

Table 1. Promoters used in CAR-T clinical trials

Clinical status	CAR cell name/NCT no.	Promoter
Registered	Axicabtagene ciloleucel (Yescarta)	MSCV[42]
Registered	Tisagenlecleucel (Kymriah)	EF1α[43]
Registered	Brexucabtagene Autoleucel (Tecartus)	MSCV[44]
Registered	Idecabtagene vicleucel (Abecma)	MND[45]
Registered	Ciltacabtagene autoleucel (Carvykti)	EF1α[46]
Phase III	CEA-CAR T (NCT04037241)	MSCV[47]
Phase III	JNJ-68284528 biepitopic CAR (NCT04181827)	EF1α[48]
Phase II	GPC3-CAR T (NCT05120271)	MND/MSCV[49]
Phase II	CARCIK-CD19 (NCT03389035)	MND[50]
Phase II	SLAMF7 CAR-T (NCT04499339)	EF1α[51]
Phase II	CD19-CAR T (NCT02535364)	MND[52]
Phase II	CD19-CAR-T with IL-6 shRNA (NCT03275493)	U6 (for shRNA)[53]
Phase I	GD2-CAR T (NCT04196413)	MSCV[54]
Phase I	Sleeping beauty generated CD19-CAR T (NCT00968760)	CMV[55]
Phase I	Off-the-shelf, IL13Rα2-CAR T (NCT02208362)	CMV + EF1α[56]
Phase I	CD19-CAR + PD-1/CD28 switch (NCT03258047)	EF1α + MSCV[57]
Phase I	CD19-CAR T (NCT02659943)	MSCV[58]
Phase I	CD19/CD22-CAR T (NCT03233854)	MSCV[59]
Phase I	GP3-CAR T (NCT03198546)	EF1α[60]

for a lower density of CARs on the membrane of lymphocytes relative to EF1α-controlled CARs. This can be perceived as an advantage, as it lowers susceptibility to exhaustion because of reduced tonic signaling while simultaneously not decreasing cytotoxic effect capacity.[2]

Another frequently used promoter is the mouse stem cell virus (MSCV) promoter, similar to EF1α, which provides a strong CAR expression.[61] Interestingly, although MSCV has demonstrated efficacy in clinical trials, its effectiveness may be unsatisfactory under some circumstances. When used in bispecific bicistronic CAR-CD19/CD22, it has shown significantly lower efficacy in contrast to EF1α. The reasons for this phenomenon are unknown, but it can be speculated that MSCV is less effective in the expression of long transcripts.[62] On the other hand, the MSCV promoter may find particular applications for CAR-T cells generated by CRISPR. A lentiviral vector delivering sgRNA and a transgene that achieved 3-fold higher efficiency than the traditional one has been designed, in which EF1α was replaced by MSCV. This change was due to the fact that MSCV is characterized by a much smaller sequence, which in turn is a great advantage in the case of limited CRISPR capacity.[63] Of course, this does not change the fact that EF1α can still be used in a CRISPR system with lower efficiency.[64]

The CMV promoter is another promoter used in gene therapies and *in vitro* studies that provides strong transgene expression comparable with EF1α; however, at the expense of lower transduction efficiency.[1] In clinical trials, it has been used, for example, in CAR-T generated with Sleeping Beauty and as a gene regulatory promoter for ZFN.[55,56]

Some preclinical CAR-T cells have been driven with a PGK promoter, which is usually considered a weaker promoter in T cells compared with those described previously.[31,65]

To induce the expression of shRNAs that are used to silence gene expression, for example, PD-1 or IL6, the U6 promoter is used.[53,66] Standard promoters described previously, despite being effective in expressing mRNA, are not optimal for small nuclear RNAs such as shRNA.[67]

A phenomenon that can limit promoter activity is transcription interference. It involves reciprocal silencing of the activity of closely spaced promoters (tandem structure) as a result of overlapping RNA polymerase complexes.[68] In the context of CAR-T, it is relevant both during lentivirus assembly and transgene expression in lymphocytes. Some studies indicate that the promoter directing the expression of a lentiviral vector (typically RSV) may interfere with the promoter directing the transgene (typically a stronger promoter than RSV).[69] For example, in the case of a study comparing the EF1α promoter with MND, greater efficiency in lentivirus production was achieved using the weaker MND promoter, which may be due to less interference with the RSV promoter driving the lentiviral genes.[2] In addition to the EF1α promoter, the CMV promoter is also characterized by interference-reducing transduction potential.[1] The second aspect is promoter interference within the transgene itself (internal promoters). It was shown that the combination of the EF1α and CMV promoters resulted in a significant reduction in the activity of both.[70] To avoid the use of more than one promoter, alternative methods to express two independent proteins can be applied. For example, IRES-mediated expression control (although in this case, the second gene achieves weaker expression) or self-cleaving A2 sequences (equivalent expression, but some proteins may have disrupted the structure).[71,72] However, if the genes turn out to be too large and it is necessary to use two independent promoters, in such a case a reduction in interferation can be achieved by installing an insulator sequence. This sequence helps prevent unwanted interactions between the promoters, thereby reducing interference.[73] If one wants to use only one promoter, there are also differences in terms of the efficiency of long transcripts, for example, EF1α performs much better than RPBSA promoter.[1]

Another problem-generating issue is transgene silencing as a result of epigenetic changes. In this case, some promoters are characterized by higher resistance (EF1α or MND) and some by lower resistance (CMV).[74–77] Promoter susceptibility to silencing also varies by cell type; for example, MSCV is resistant to silencing in mature T cells but given to silencing in stem cells.[78] Stem cells, especially iPSCs,

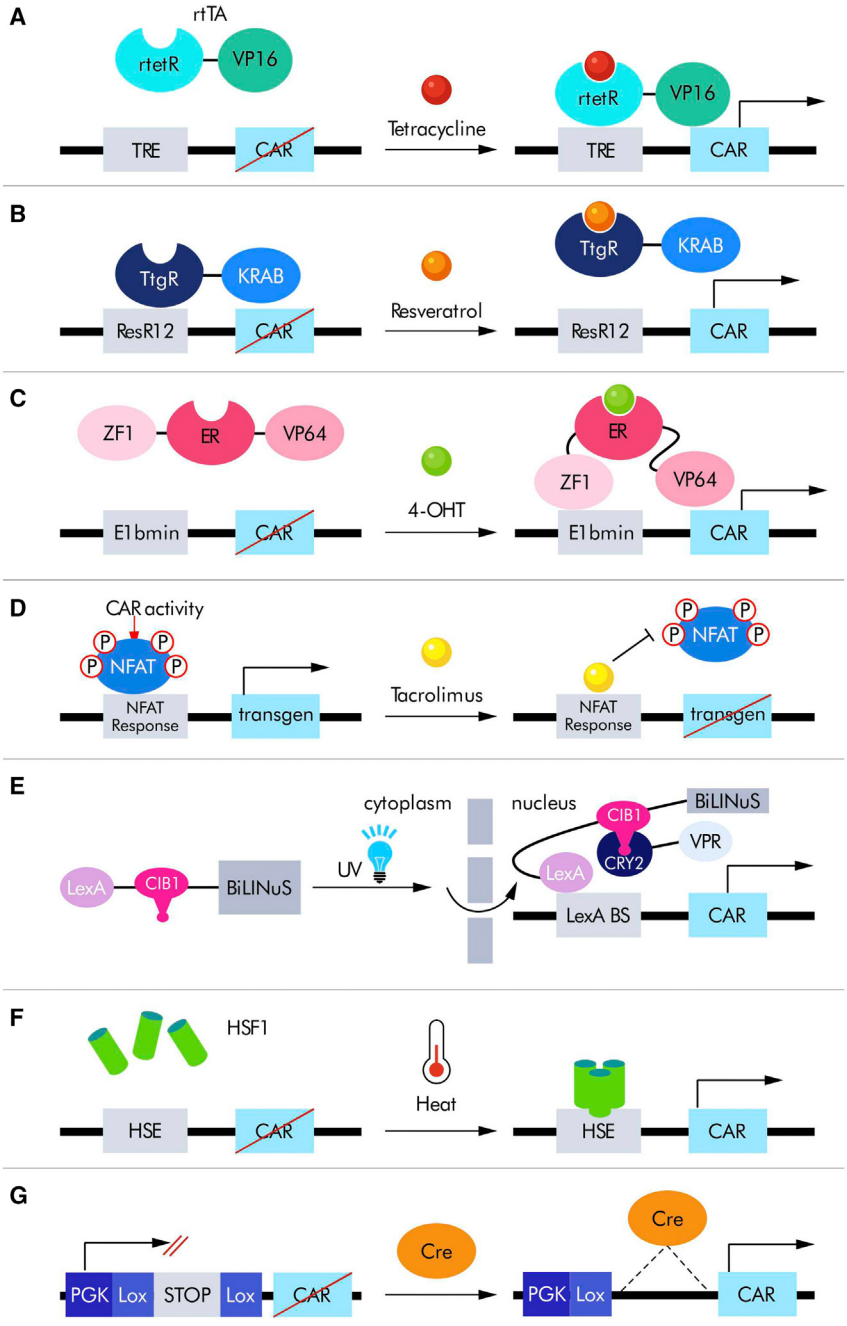

Figure 1. Inducible endogenous and exogenous transcription systems

(A) Tet-ON. An rtTA driven by the constitutive promoter is composed of the rtetR responsible for connecting to TRE upon tetracycline administration, and the VP16 transcription activator. Tetracycline administration results in rtetR bringing VP16 to the CAR sequence. (B) ResRep. TtgR and KRAB silencing factors are attached to the DNA binding sequence called ResR12. Upon resveratrol administration, TtgR dissociates from the DNA binding site resulting in the cessation of the KRAB influence on the CAR promoter and ultimately leading to CAR expression. (C) Zinc fingers. ER upon 4-OHT enables ZF1 to bind with E1bmin, leading to an approximation of the VP64 activating domain to the CAR sequence. (D) Tacrolimus system. CAR signaling (upon antigen recognition) phosphorylates NFAT leading to its binding to the NFAT response element and initiating transgene expression. Upon tacrolimus administration, NFAT dissociates, stopping transgene expression. (E) LINTAD. The LexA-CIB1-BiLINuS trimer is present outside the nucleus. Upon UV impulse, BiLINuS changes conformation, allowing the trimer to translocate inside the nucleus. Furthermore, LexA binds to its binding site, allowing dimerized CIB1-CRY2 to bring the VPR activation domain near the CAR cassette. (F) Heat shock system. Upon heating, HSF1 trimerizes and binds with HSE leading to the expression of CARs. (G) Cre system. The CAR gene is blocked by codon STOP following the constitutive promoter. Upon CRE recombinase, codon STOP is knocked out and the PGK promoter drives the expression of CARs.

may be relevant in the context of CAR-T when considering attempts to create off-the-shelf CARs.[79]

Before discussing inducible expression control systems, the key role of constitutive promoters should be noted. Even in systems where transgene induction is variable, the proteins in these systems must be controlled by constitutive promoters. This means that transgenes containing regulated TFs will still contain some constitutive promoter.

Inducible transcription systems

Chemically induced xenogenic TFs

The group of chemically induced xenogeneic TFs is the first to be described that offers induced gene expression depending on the presence of a given chemical molecule; in the case of the Tet-on/off system, tetracycline and its derivatives. The Tet system is based on the prokaryotic capability to eliminate tetracycline antibiotics from bacterial cells. When there are no tetracyclines, gene expression is silenced because it is unnecessary and even harmful to bacteria to produce redundant proteins, which is why this operon evolved.[15] In eukaryotic synthetic cells armed with this system, the production of the tet TF is constitutive; however, its activity or inactivation depends on the presence of doxycycline.[80] The functional linkage of the tet protein with human RNA polymerase is made possible by the engineering of a tet fusion protein (chimera) with the Vp16 protein. Vp16 together with tetR is called tTA (in the off-system) or rtetR, an rtTA protein (in the on-system) (Figure 1A). Vp16 has polymerase-regulating ability; thus, it is an element that is frequently used as a transcription activator, not only in the Tet system.[81] Vp16, commonly used in various described

systems, is naturally a protein of HSV and enables the transcription of the OFRs of this virus. Therefore, the tet system uses elements from the bacterial and viral proteome, making it prone to triggering an immune response, which is one of its biggest disadvantages in the context of clinical use in CAR therapy.[8] However, numerous *in vivo* studies have proved the Tet system to be an effective tool to activate and deactivate CAR expression and cytotoxic activity.[82,83]

Another system belonging to the same group is RESrep CARs. This system shares many similarities to Tet: the activation regulatory element is also derived from bacteria, acting as an efflux pump (TtgR), and a synthetic transcription activator VPR (composed of Vp16, p65, and Rta) is attached to induce expression. The system itself is operated by the CMV or SV40 promoter. TtgR protein, under the influence of resveratrol, a control molecule in this system, causes the attachment of an activation complex and TFs to initiate CAR expression (Figure 1B). An inverted version of this system has also been engineered, in which the absence of resveratrol initiates the expression and the addition stops the expression.[84]

There are other systems that fit the definition of exogenous chemical inducible, such as GAL4/UAS induced with auxin, an ecdysone-receptor-based system controlled with tebufenozide, or an RU486-inducible promoter induced upon RU486. However, this solution has not been tested yet in CAR-T, and they do not seem to resolve the problem of immunogenicity since they are exogenous as are the aforementioned systems.[85–87]

Chemically induced endogenous TF

Chemical induction of CAR expression can also be based on the use of sequences that bind synthetic variants of endogenous proteins such as estrogen receptors or nuclear factor of activated T cells (NFATs).[26,88] In the case of the 4-hydroxytamoxifen expression system (estrogen/zinc fingers), a synthetic transcription activator is driven by a constitutive promoter. Under the influence of a control molecule, it attaches to the E1bmin-binding region, and the TF initiates CAR transcription (Figure 1C). To minimize basal activity, the CAR sequence is reversed in the 3′ to 5′ direction. Another important modification to limit basal activity is to use a mutant variant of the estrogen receptor (G525R) so that the system is not activated by endogenous hormones.[89] The NFAT-based system works in a more complex mode and is half-autonomous. CAR expression is constant; however, under antigen detection, signaling pathways activate the expression of genes (interleukins) driven by the NFAT promoter. To mute these genes, a controlling substance (tacrolimus) is introduced (Figure 1D).[88] Because NFAT-based systems are activated by CAR signaling, they are also sensitive to TCR signaling, meaning basal activity may be higher than in estrogen/zinc fingers.[90] One way to overcome this is to knock out TCR and eliminate the source of background signaling.[91] What is interesting is that the gene delivery of the NFAT system as a single vector failed and had to be split into separate vectors. The authors indicate promoter interference as the reason for this phenomenon and justify that promoter competition may be an important and partially unknown occurrence.[88]

The evident advantage of systems induced with endogenous TFs is the low risk of immunization due to the use of proteins natural to human cells, in contrast to Tet-CARs or RESrep-CARs. On the other hand, they require additional modifications to eliminate the possibility of activation by endogenous signals. It is also difficult to determine how the activation of native genes using such systems will affect cell metabolism and function. Such a TF binding to genomic regulatory DNA (apart from the vector promoter) could start to affect the phenotype of the cell and possibly even reprogram it (reprogramming analogous to the preparation of iPSCs).[92] It seems theoretically possible to create a protein that binds only to the promoter of the vector, provided that the promoter has a DNA sequence that matches the receptor mutant (i.e., binds it) and is not present in the promoters of genes active in the cell.

Physically induced xenogeneic TFs

Chemicals used as controlling molecules have various disadvantages compared with physical control (heat or light). Apart from possible side effects and a more complex registration procedure, the pharmacokinetics of chemical substances are much more challenging to predict than the distribution of physical factors. For example, the metabolism rate of resveratrol (used in the RESrep system) may vary depending on different conditions affecting the patient's liver.[93] On the contrary, the transmission of heat, ultrasound, and even light through tissues is more constant.[94,95] Another advantage is precise local induction that can be compared with stereoradiotherapy rather than systemic therapy area-based control of CAR-T activity, which seems more promising in non-metastases cancer.

Various proteins contain blue light-absorbing chromophores, which cause the conformation of the protein to change upon light intensity fluctuation.[96] A widespread system is the CRY2 interaction system of the cryptochrome family 2 interacting with the CIB-1 protein (cryptochrome-interacting basic-helix-loop-helix protein).[97] Blue light induces dimerization of these proteins, and darkness reverses the process. Thus, the formation of their fusion forms (chimeras) of CRY2 and CIB with other proteins presents a wide range of possibilities in inducible gene transcription.[98] For example, with VP16 as an activating domain and biLINuS as a regulatory element in the system called LINTAD (light-inducible nuclear translocation and dimerization) (Figure 1E).[99] It has been shown that blue light can reach CAR-T cells *in vivo* and can activate LINTAD.[100]

Vivid (VVD) is another CRY2/CIB1 protein-based system, being a photoreceptor that can dissociate upon cessation of blue light exposure.[101,102] Blue light induces dimerization of CRY2/CIB1 reactivating split proteins such as CRE recombinase.[103,104] In practice, such systems resulted in the creation of light-controlled expression of a CAR protein.

Nevertheless, some weaknesses of this solution have been identified precisely because of limitations in UV light penetration, and attempts are being made to improve the system's performance by increasing light penetration depth, using upconverting nanoparticles, or creating

proteins that would be activated by infrared light.[95,105] What is more, even if that problem were resolved, light systems are still based on xenogenous proteins and potentially immunogenic.[106]

Endogenous physically induced TFs

The weakness of shallow inducer penetration in light-controlled systems may be overcome with heat or ultrasound-based systems. In addition, these systems are based on heat shock proteins (HSPs), which are naturally expressed in human cells. Hsp proteins were found to regulate gene transcription not only as a result of temperature changes but also due to various environmental factors. Thus, their application in precise gene regulation requires additional modifications to decrease basal activity.[10,107,108]

The activation of thermal systems is based on heat shock factor 1 (HSF1). In native cells, exposure to moderate hyperthermia (39°C–42°C) induces a heat shock response, which is intended to lead to cytoprotection by HSPs. Under the influence of heat, HSF1 migrates to the cell nucleus and binds as a trimer within DNA motifs called heat shock elements (HSEs), activating transcription (Figure 1F). In gene-modified cells, HSEs drive the expression of transgenes, for example, CARs or interleukins. From the clinical side, increased temperature capable of activating the HSF1 system can be triggered by nanorods or ultrasound.[10,108,109] To prevent activation by other factors that naturally trigger the heat shock response, for example, hypoxia, additional optimization in structure has been made.[108] Ultimately, heat pulses can stimulate cells in specific areas of the body to millimeter-level accuracy, which now surpasses the resolution of most imaging methods.

The heat-activating system has also been engineered to work in a one-way, irreversible mode using CRE recombinase. CRE recombinase is expressed upon HSE heat activation, while the second sequence under the constantly active PKG promoter has a codon STOP sequence flanked by lox and followed by CD19CAR. Without CRE recombinase, the STOP sequence stops transcription before CD19CAR is reached. However, upon heat activation, CRE recombinase is expressed and deactivates the STOP sequence located between lox. Because the codon STOP is no longer present, transcription covers CD19CAR leading to its transcription (Figure 1G). Because the system has been lined with the ZsGreen protein, it is possible to accurately monitor the impact of ultrasound on the transcription. It turned out that a 15-min exposure to ultrasound can turn on a HSP-regulated promoter activated at 43°C, controlling the CAR transgene. The system was effective under *in vitro* conditions for eliminating tumor cells as well as *in vivo* conditions.[10] In another study, an HSP-based system was created that inactivated CAR expression under the influence of temperature.[110]

Shapiro and co-workers analyzed many heat shock-regulated promoters (HSPs), including natural and GE-modified promoters. Thus, HSPs with different transcriptional activity and sensitivity to temperature changes were detected.[111] This team admits that the term HSPs can be confusing. On the one hand, this gives a chance to turn them on using various factors such as temperature, hypoxia, cytokines, and cell division. On the other hand, it may lead to non-orthogonal activation, defined by them as a significant obstacle for this system to be effective in clinical usage. This is especially important in the context of CRS syndrome because this is usually accompanied by a fever, even in mild grade.[112] Another concern could be that major HSP-induced changes could occur in genes other than the transgenes in the cell. However, the team of Kwong and co-workers claims that CAR-T retained its function in the context of transgene stimulation, i.e., that there was no phenotype change in the effects of HSF on genomic HSE.[10,113]

Uninduced (constitutive) ATFs

Proposals are made to modify the CRISPR or TALEN systems to create artificial TFs as well as artificial inducible TFs.[6,114] It is worth starting the discussion of the problem with the constitutive ones. Generally, constitutive ATFs are a theoretical construct that is not the object of research because they lack specific advantages over traditional promoters. To use them, the presence of a synthetic dCas9 protein and gRNA are required, which need to be produced in the cell using a constitutive promoter. Nevertheless, on their basis, it is possible to describe the action of dCas9 itself and the activators and inhibitors of transcription attached to it. This is a prelude to more complex systems that enable the regulation of dCas9 activity through further modifications described in the section on chemical/physical ATFs.

CRISPR, TALEN, or ZFN technology was originally developed to edit the genome.[115] However, the basis of their operation is binding to the DNA of a specific sequence.[116] This is, therefore, a completely different situation than in the case of, for example, the estrogen receptor discussed in previous sections. It is the constructor that decides which DNA sequence the TF will be attached to, and it is much easier to avoid its presence in DNA at regulatory sites in the genome. To adapt CRISPR for use as a transcription activator, modifications have been performed. Firstly, unlike Cas9, capable of cutting DNA in expression systems, dCas9 lacking nuclease activity is used.[117] The cessation of nuclease activity is achieved by mutations in the RuvC and HNH Cas9 domains. The second difference is that dCas9 is linked with the transcriptional activator, for example, VP16, as discussed previously. dCas9, together with the sgRNA-like molecule, recognizes a specific sequence, and VP16 ensures the activation of the appropriate transcription machinery. Usually, several VP16 subunits are used. Other dCAS9 activation domains are p65 and Rta. Combining them in a trio with VP16 is termed VPR - V (VP16), P (p65) R (Rta).[117–119]

In addition to transcription activators, inhibitors are also being developed. Of course, the lack of an activator in the environment means slow silencing. However, it is possible to force a faster shutdown of the expression. In this case, known repression domains, such as KRAB, SID4X, MXl1, to dCas9/sgRNA are used[120,121]. One of the first such solutions was proposed by Gilbert and co-workers, examining the GFP reporter system in HEK293T-GFP cells.[113,122]

KRAB-ZFPs (Kruppel-associated box domain zinc-finger proteins) is a large family of transcriptional repressors. KRAB-ZFPs are characterized by the presence of two domains: the N-terminal KRAB domain and a tandem array of C2H2 zinc finger elements at the C terminus.[123] The KRAB proteins enable the recruitment of TRIM28 (tripartite motif-containing protein 28, also referred to as KAP1, Tif1b, and KRIP-1), which acts as a platform for transcription-limiting factors.[124] This is due to the binding of histone methyltransferases, which cause the formation of H3K9me3 (histone H3 trimethylated at lysine 9), SETDB1 binding (SET domain bifurcated 1), the histone deacetylase complex containing NuRD, and heterochromatin protein 1 (HP1).[125–127] All of these proteins catalyze changes that lead to the formation of heterochromatin (a transcriptionally inaccessible form of chromatin) and the consequent repression of transcription.

The fact of using dCAS9 makes it possible to combine sgRNA, recognizing a specific DNA sequence, with a hairpin sequence that can bind to a protein. Typical examples are RNA motifs such as MS2, Pp7, or Com. Such sequences allow joining chimeric sgRNAs recognizing DNA with hairpin RNA, which binds to proteins having MCP, PCP, or Com domains. MCP, PCP, and Com domains are bridging proteins that connect RNA on the one hand and transcription modulators on the other.[113,128]

Chemically induced ATF

To realize the true potential of the ATF, it is necessary to transition to a system using solutions such as dCAS9 VP16 but induced. Such systems are now under development. In this section, the focus is on selected examples of low-molecular-weight regulation systems. The physical-induced ATFs are discussed in the next section.

Most of the mechanisms designed to regulate Cas9 so far serve more to turn nuclease activity on and off (nuclease function as a cutting enzyme, not TF). However, few attempts to control transcription have been made. The first branch of systems used split Cas9, which can be coupled and uncoupled by chemical-induced dimerization. Cas9 is divided into two separate parts that are fused with FRB and FKBP proteins, respectively. Upon rapamycin administration, FRB and FKBP heterodimerize, linking split Cas9 into an operative protein (Figure 2A).[129] This system was originally made to control Cas9 nuclease activity; however, modifications adding VP64 or VPR to control transcription upon rapamycin have been made. Unfortunately, high background activity in that system was noticed, shrinking its usage in CAR regulation.[130] Thus, further modifications have been made. Previously described, 4-OHT was used to additionally control the presence of dCas9 outside of the nucleus. Here, the split dCas9 is fused with the ligand-binding estrogen receptor and dimerizing domain. Without 4-OHT, the Hsp90 protein binds to estrogen receptor binding sites preventing translocation to the nucleus. Upon 4-OHT, Hsp90 is displaced and thus no longer blocks migration of the dCAS9 complex into the nucleus. dCas9 can be armed in both Hsp90 and FRB/FKBP systems to minimize uncontrolled activation (Figure 2B).[130] 4-OHT has also been used to control Cas9 without splitting it by regulating only its presence outside/inside the nucleus.[131] FRB/FKBP chemical-induced dimerization has already been used in clinical trials as an iCasp9 system to control CAR-T activity, however, irreversibly killing it.[132] Thus, reversible transcription control would present a better perspective.

Other uses of 4-OHT can be based on the intein protein. Here, unlike in the split version, Cas9 is not divided but enriched with an intein sequence that hinders its activity. That synthetic intein is derived from *M. tuberculosis* bacteria and is fused with the human estrogen ligand-binding domain. The presence of intein in the Cas9 structure disrupts its activation; however, upon 4-hydroxytamoxifen administration, estrogen-binding sites trigger conformational changes and intein self-splicing activates Cas9[133] (Figure 2C). Despite this, the system has not yet been used to trigger transcription modifications similar to the aforementioned systems, but may be effective in triggering transcription. Intein can also be used for split Cas9 reconstruction working similarly to FRB/FKBP, but without chemical triggering.[134]

The alternative solution used a plant-based ABA (abscisic acid-inducible) and GA (gibberellin-inducible) dimerization Cas9 system. ABA and GA are compounds that induce heterodimerization, similar to rimiducid.[135] For transcriptional control, dCas has been fused with ABI, and the VPR activation domain is connected to PYL1. Upon ABA administration, ABI dimerizes with PYL1, increasing the proximity of VPR to DNA and thus activating transcription (Figure 2D). In the GA-activated system, dimerizing proteins are GAI and GID1. For reverse control, the KRAB repressor can be fused instead of VPR. Further modifications allowed to engineer the OR gate (both ABA and GA dimerizing domains attached to Cas9), AND gate (GA and ABA are fused to Cas9 in tandem mode), and even switch (ABA dimerization triggers VPR proximity while GA dimerization triggers KRAB repression) (Figures 2E and 2F).[136]

Finally, control of Cas9 activity can be achieved by controlling its degradation. A system made of DHFR, PP7, and VP64 was proposed. The PP7 protein recruits VP64 to dCas9, activating the transcription. However, DHFR is vulnerable to proteasomal degradation, resulting in no transcriptional activity. To induce transcription, a controlling molecule (trimetophrin) is administered stop DHFR degradation[137] (Figure 2G). Degradation control has already been used to directly control the CAR presence.[138]

Physically induced ATFs

The last group of division indicated in this article is ATFs induced by a physical factor. Light control with the use of CAS9 mutants can also be distinguished (in this study, as a rule, the regulation of genome editing is not considered, but the regulation of transcription is).[139] These systems are derived from the already discussed CRY2/CIB1 solutions. An example of an optogenic hybrid system consisting of two elements, a DNA-binding part, a dCas9 element, and a fused system with a light-sensitive basic-helix-loop-helix (CIB1) cryptochrome was proposed. In this system, the dCas9-CIB1 and cryptochrome CRY2 complexes are formed under the influence of blue light. These proteins are fused to the effector domain. During the blue light

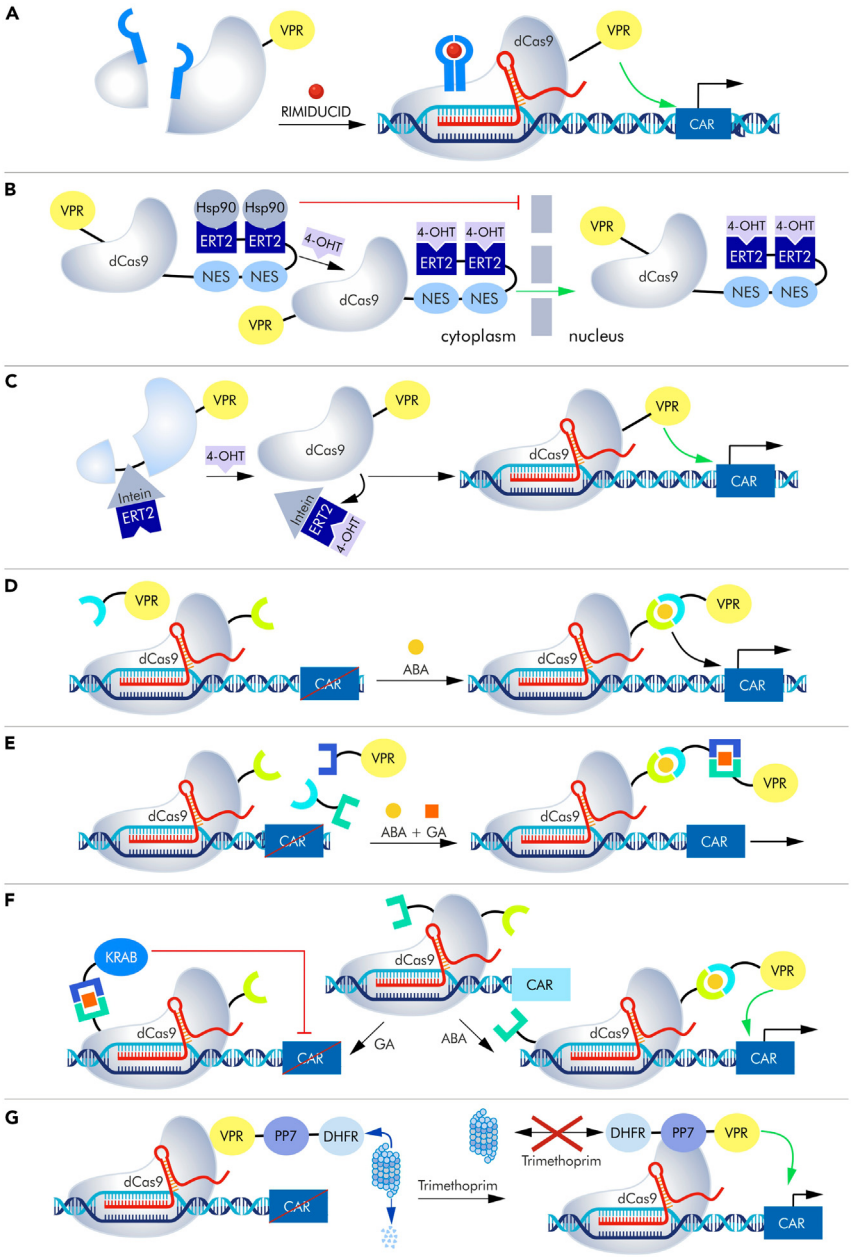

Figure 2. Inducible artificial transcription factors

(A) Split dCas9. Split dCas9 connected to VPR is activated upon rimiducid administration. Rimiducid triggers chemical-induced dimerization of FKBP/FRB proteins (drawn as semicircles), leading to the assembly of split dCas9. The complex of dCas9, VPR, and gRNA activates the transcription of CARs. (B) Hsp90 dCas9. ERT2 is the domain responsible for the inducible binding of Hsp90. Hsp90, if connected to ERT2, prevents the translocation of the whole dCas9 complex from the cytoplasm to the nucleus. Upon 4-OHT administration, Hsp90 is released, resulting in the translocation of ATF into the nucleus and the initiation of transcription. (C) Intein dCas9. The intein structure deactivates dCas9's ability to bind to DNA by changes in spatial conformation. Upon 4-OHT administration, intein is excised, allowing dCas9 to return to the normal conformation. (D) ABA dCas9. dCas9 and its activating domain are split. However, upon ABA administration dimerization occurs, resulting in the creation of an active complex. (E) ABA + GA dCas9. Similar to the ABA system, an additional intermediate activated by GA is added. Only upon ABA and GA administration, is the active complex assembled. (F) ABA-ON GA-OFF dCas9. dCas9 has the ability to bind both VPR and KRAB, depending on the presence of ABA or/and GA. (G) dSpCas9. VPR-PP7-DHFR is constantly eliminated in the proteasome. However, trimethoprim binds to DHFR, abolishing its susceptibility to proteasomal degradation. Next, PP7 is able to bind to dCas9, leading to the activation of transcription by VPR.

stimulation (peak ~450 nm), the CIB1-effector fusion protein can form the dCas9-CIB1-CYR2-effector complex, which enhances the transgene activity in this system. In addition, similar to previous systems, cessation of light exposure abolishes activation.[140,141]

As has been explained, different proteins are found in different systems, as can be seen in the example of VP16. Various items are transferred from system to system and are regulated by physical-chemical factors in various contexts by their natural or ATFs. Moreover, there is nothing to prevent various activities from being combined to create more compiled circuits characteristic of synthetic biology.

Engineered signaling receptors

SynNotch biology

In addition to the constitutive and controlled expression of CAR-T genes, there is also the concept of self-controlling (autonomous) CARs, i.e., CARs that can decide to change their expression depending on the surrounding environment. The basis for this strategy is SynNotch.[24] SynNotch is a modified version of the naturally expressed Notch receptor in humans, responsible for cell-cell interactions.[142] From the original Notch, only the central (core) part is found in the SynNotch. The ligand-binding and transcription-activating domains have been modified. The mechanism of action of SynNotch contains several steps: a T cell encounters an antigen called a "priming" antigen, i.e., one that is recognized by the ligand-binding region of the Notch receptor (scFv). The spatial conformation within SynNotch is then altered, which results in enzyme-sensitive S2 site exposition. As a result, the proximal end of SynNotch is cut off and can penetrate the nucleus (Figure 3). Depending on the desired activity, this fragment can be either a transcription enhancer or a transcription inhibitor.[143] In the case of CAR-T, this usually leads to activation of transcription and further production of CAR protein.

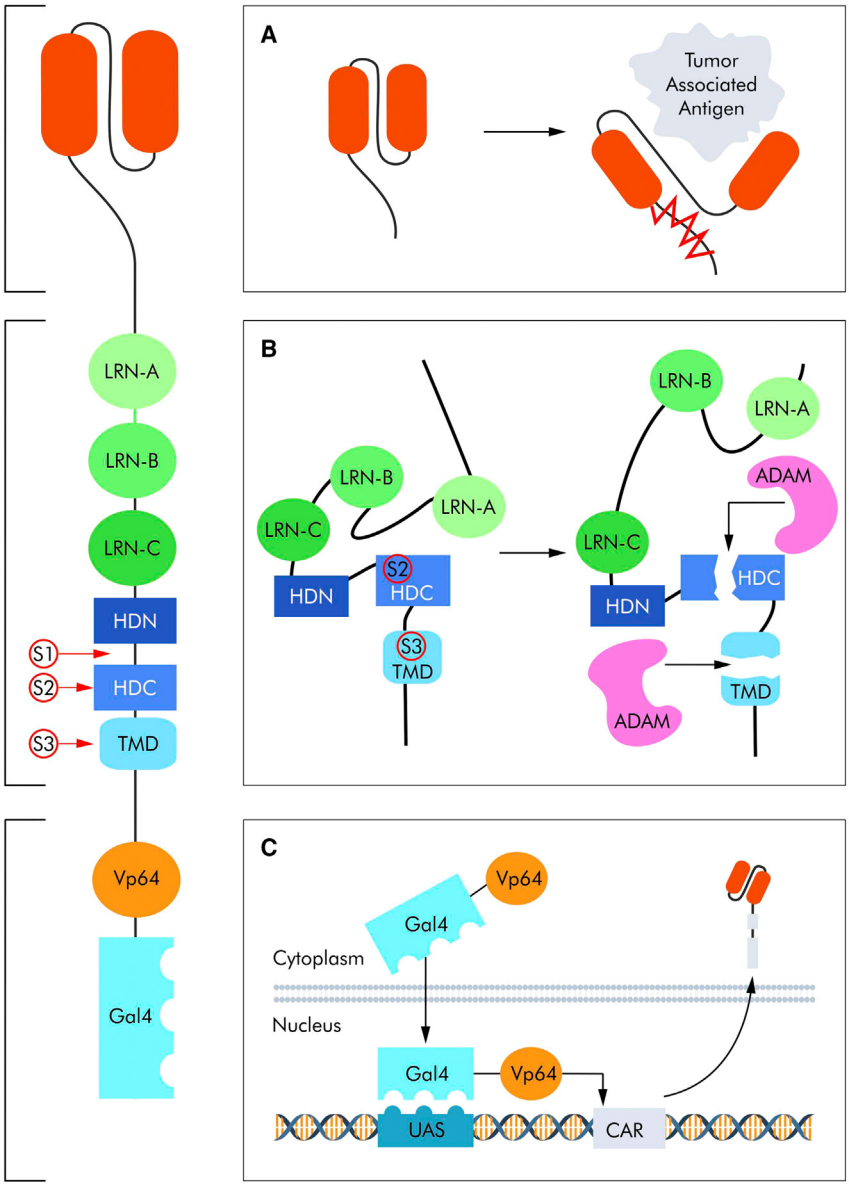

Figure 3. Synthetic notch

SyntNotch. Structure: SynNotch consists of three main parts. The first is an extracellular region that binds the ligand, made of scFv. The middle one is the "Notch Core," which controls ligand-binding-dependent enzymatic cleavage, and can be subdivided into the negative regulatory region (NRR), consisting of three cysteine-rich Lin12-Notch repeats (LNR A, B, and C), and two heterodimerization domains (HD-N and HD-C). Situated proximal to the NRR is the transmembrane domain (TMD). The last part is the intracellular region equipped with a transcription factor. Within the NRR and TMD, there are three cleavage sites (S) susceptible to enzymatic cleavage. S1 is located between HD-N and HD-C, S2 is located in the HD-C domain, while S3 is located in the TMD. Steps of SynNotch activation: (A) to initiate activation, the extracellular ligand-binding domain has to encounter the corresponding antigen. (B) As a result, conformational changes in the NRR region lead to exposure of S2, previously flanked by LNR-A, linker LNR-A/B, and HD-C. The exposed S2 domain is now susceptible to being cut by ADAM (a disintegrin and metalloprotease) proteases. The S3 domain is then cleaved by γ-secretase. (C) As a result, the intracellular part is detached, which allows the transcription factor, previously attached to the Notch Core, to fuse with the DNA near the CAR region. The last step is the triggering of CAR protein expression which makes it possible for lymphocytes to be activated by antigen.

Each SynNotch must have a suitable TF that will interact with the nucleus. Due to its high modularity, many different TFs can be used in SynNotch; however, Gal4-vp64 has been used most often. In this solution, Galt4 binds specifically to the UAS domain located near the SynNotch-controlled gene (e.g., the CAR gene), while VP64 is responsible for the transcription-activating signal. The SynNotch system itself must be produced under its own (constantly active) promoter, e.g., PGK. What is more, other activating TFs have been used, such as tTA binding to TRE and CymR binding to pCuO. It is also possible to make a reverse-acting system, i.e., one that is constitutively active but deactivated after SynNotch activation (NOT gate). For this purpose, an independently acting SV40 promoter is inserted after the UAS sequence. The control element attached to SynNotch is Gal4 linked to the promoter quenching factor: KRAB. When Notch is activated, the TF penetrates into the nucleus, and Gal4, as in other accidents, binds to the UAS. For that reason KRAB silences the SV40 promoter, leading to the interruption of transcription.[24,142,144]

SynNotch was created based on the original Notch Core, which had detectable basal activity, which reduces the effectiveness of SynNotch by ligand-independent activation of transcription. To reduce ligand-independent activation, the hN1RAM7 domain, which is a hydrophobic amino acid sequence (QHGQLWF), was incorporated at the C-terminal end of the Notch Core, thus proximal to the nucleus behind the negative regulatory region (NRR) and transmembrane domain (TMD). This provided an almost 15-fold reduction in ligand-unbound activity.[145]

Several studies have confirmed that SynNotch's undeniable advantage is to counteract CAR-T depletion. Lymphocyte depletion is a multifactorial phenomenon caused by external factors (tumor microenvironment) as well as internal (tonic signaling from CARs).[146,147] In the case of tonic signaling, as previously mentioned, lower CAR expression reduces tonic signaling, leading to increased resistance of CARs to exhaustion.[2] Similar to the

weaker promoters, downregulating CAR expression in the SynNotch system also made it possible to reduce tonic signals and, consequently, exhaustion. A biochemical analysis revealed that CAR-T cells regulated by SynNotch exhibited, to a greater extent, the oxidative metabolism characteristic of naive T cells with an increased capacity for proliferation. The effectiveness of the Notch receptor is also supported by a reduction in markers of exhaustion PD1 and LAG3 measurements.[148] In another study, the increased resistance to exhaustion was confirmed by decreased expression of CD39 and an elevated level of T cell factor 1 (TCF1).[14] Other studies have demonstrated a lower contribution of the NF-κB pathway in SynNotch CARs relative to cells with constant expression, which is further evidence against depletion since this pathway is strongly associated with depletion.[149] Thus, SynNotch, like other transcriptional regulators or Dasatinib (a pharmacological inhibitor of CARs), reduces tonic signals to control CAR activity without causing depletion.[150] Although, unlike them, it does so autonomously.[13] Reducing CAR expression until the lymphocytes reach the tumor is preferable from both a safety and efficacy standpoint.

Logic gates and biological circuits

The human immune system is a complex network that relies on information exchange to function effectively. CAR-T therapy, which uses genetically modified immune cells to recognize cancer, was initially developed with a simple 0/1 decision CAR. However, as CAR-T technology advances, it is moving toward more intricate decision-making models that increasingly mimic the complexity of the nature of the human immune system. The fundamental role is carried out by inducible transcription and synthetic receptors, whose usage leads to the creation of a basic information processing unit, the Logic Gate. Multiple Logic Gates can be further connected in circuits creating an intracellular grid.

The underlying assumption of how the SynNotch works in CAR-T is the creation of an AND logic gate. This means that a T cell will only start a cytotoxic effect if it encounters two signals at the same time. The first signal is recognized by the SynNotch receptor and is the so-called Priming (antigen). Upon detecting it, the T lymphocyte initiates the start of CAR protein expression. The second signal is (as in classical CARs) an antigen recognized by a CAR. Such construction allows for the elimination of the problem of non-specific tumor antigens and avoids on-target off-tumor attacks. At this point, it should be mentioned that not every cancer cell needs to have a priming antigen to be destroyed. Studies have shown that it is sufficient for only 10% of the cell population to contain the priming antigen, which already enables CAR activation AND regionalized killing.[148] SynNotch-activating tumor cells (which contain the priming antigen) are killed in the CIS mechanism; that is, they simultaneously arm lymphocytes with CARs and are killed by them. In contrast, cells that do not have the priming antigen but have the antigen for CARs are killed in the TRANS mechanism; that is, they fall prey to lymphocytes armed with CARs by neighboring cancer cells.

One of the most successful applications of the AND gate represents the EGFRvIII and IL-13R α2/EphA SynNotch antigen pair used in glioma. This follows from the unusual location of TAA antigens: interleukin-13 receptor α2 (IL-13R α2) or ephrin type A receptor 2 (EphA), which are low specific but expressed on all cancer cells (low chance for antigen escape but big risk of on-target off-tumor), and EGFRvIII, which is a highly specific TAA for glioblastoma but usually not expressed on all cancers cells (high risk of antigen escape and low chance for on-target off-tumor). The second crucial factor is that IL-13R α2/EphA are expressed in healthy tissue only outside the CNS. Obtaining specific and effective CAR-T comes from combining the features of both of these antigen groups and is based on the physical separation of activated CAR-T from healthy tissues potentially exposed to their attack.[151] SynNotch activates CAR expression only in the close presence of tumor cells while keeping them far from healthy cells.[14] Promising results have also been obtained in mouse studies on the pair of GD2-B7H3 antigens, where complete eradication of glioblastoma cells was achieved as well as significantly prolonged survival using as targets antigens normally causing lethal on-target off-tumor attacks.[148]

SynNotch has also achieved success in other types of cancer; however, a certain disadvantage has been detected. In *in vivo* studies in the 4T1 breast tumor model, priming Ep-CAM and CAR anti-ROR1 did not induce toxicity and specifically killed only cancer cells. However, in a murine model with metastasized or circulating cancer cells, SynNotch showed similar (high) toxicity against contiguous CAR-T and healthy cells. This demonstrates the importance of spatially separating the cancer cells from the reservoir of healthy cells expressing antigens detected by CARs.[152]

The description so far has involved a SynNotch that works by recognizing two or more different antigens, although it is also possible to create a SynNotch that responds to the same antigen as a CAR. An experiment was performed in which scFv domains with different affinities to HER2 were generated. Both priming receptors and CARs (SynNotch anti-HER2 AND concomitantly CAR anti-HER2) were constructed against the same antigen, but they varied with different affinities. The purpose was to produce an "ultra-sensitive" CAR, i.e., one that will not linearly but rapidly activate under the influence of a certain antigen density threshold. Concerning CAR SynNotch, a priming receptor against HER2 with low affinity was made, and a CAR also against HER2 but with high affinity. This action ensures that healthy cells with HER2 (which physiologically have less of it than cancerous ones) will not cross the threshold for activation and will not trigger CAR transcription. In contrast, tumor cells with a higher density of HER2 will cross the threshold and spike the cytotoxic effect of T lymphocytes against each other in all or nothing in the mechanics. The discrimination of HER2 antigen density that has been achieved is about 10^5 HER2 molecules per cell for cells that do not activate CARs and 10^7 for cells that do.[39]

The AND gate alone has achieved tangible research results, which has encouraged researchers to construct more complex variants of logic gates and their circuits based on SynNotch. Figure 4 defines

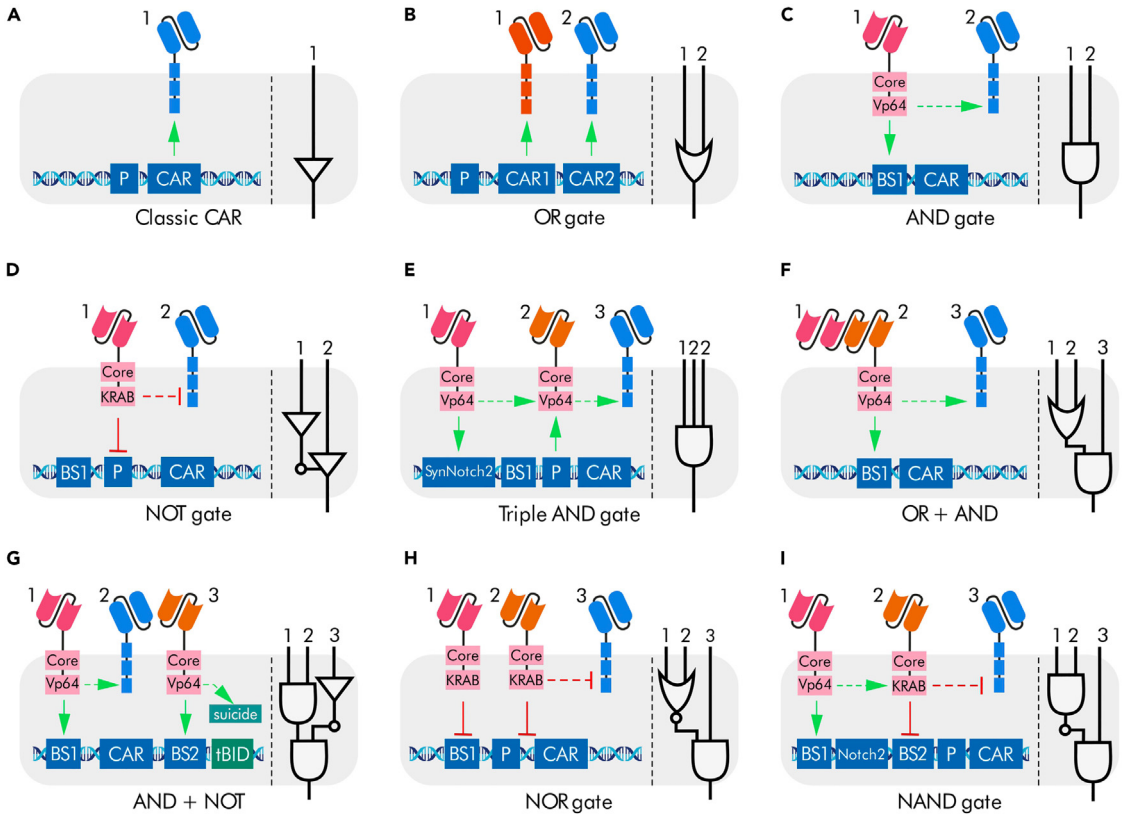

Figure 4. Logic gates and circuits

The left side shows the logic gate's biological construction, written schematically as logic gates on the right. BS, binding site for TF; P, promoter constitutive. (A) Classic CAR detection works in simple 0–1 mode. (B) OR gate. OR gate induces a cytotoxic effect with at least one signal detected by one of two CARs with different specificity. Both CARs are under constitutive promoters. (C) AND gate. SynNotch is under a constitutive promoter. The first SynNotch signal activates transcription by releasing activating TF, leading to the expression of CARs detecting the second signal. Only the presence of both signals induces cytotoxic effects. (D) NOT gate. Both SynNotch and CARs are under constitutive promoters. Upon SynNotch signal detection, inhibiting TF, which stops the expression of CARs, is released leading to the cessation of CAR presence. (E) Triple AND gate. The first SynNotch is under constitutive promoter; the second SynNotch and CAR are silent by default. Upon the first signal, SynNotch 1 activates the expression of the second SynNotch. Upon a signal from the second SynNotch, expression of the CAR is triggered. (F) OR + AND gate circuit. OR + AND are designed similarly to a single AND gate. However, SynNotch is bispecific, leading to a situation where only one of the paired signals is sufficient to activate the expression of the CAR. (G) AND + NOT gates. This circuit is engineered similarly to a basic AND gate. Additional constitutively expressed SynNotch recognizing signal 3 may trigger the expression of basally muted tBID, a protein acting as a suicide switch. (H) NOR gate + AND. Both SynNotch receptors and CARs are under constitutive promoters. Upon detection of the SynNotch 1 or 2 signal, inhibiting TF (KRAB) is released, leading to the cessation of CAR expression. (I) NAND gate + AND. SynNotch 1 is under constitutive promoter as well as CARs, while SynNotch 2 is silent by default. Cessation of CAR expression will only be possible if two inhibiting signals are present. The first signal is detected by SynNotch 1, and it induces the expression of the second Notch. The second SynNotch, unlike the first, inhibits TF (KRAB) leading to the cessation of CAR expression.

SynNotch variants as logic gates carved out in CAR-T. Besides the AND gate, other types are OR, NOT (basic used in CAR-T), NOR, and NAND, as well as "triple-AND." The OR gate is activated if at least one signal is detected (tandem CAR), while the NOT gate induces a response until it detects a given signal that deactivates the NOT logic gate (Not-SynNotch).

NOR and NAND gates are more complex; however, they could be created using other basic gates. NOR is a series connection of OR & NOT while NAND is AND & NOT. They have already been created in bacterial cells using tetR/lacI and in mammalian cells by utilizing miRNA.[153,154] Autonomous CAR-T cells could be engineered using SynNotch as indicated in Figure 4.

It is worth mentioning that, in CAR-T, in addition to the use of induced transcription, there is another family of solutions that produce logic gates. Alternatively, protein adapters are also used. However, their significant drawback is that their short half-life time enforces the need for constant infusion. What is more, they can only regulate the activity of CARs or kill CAR-T cells, while transcriptional regulators can control the expression of any given protein.[155]

Logic gates can also be formed through systems induced by small-molecule compounds. For example, in the ATF-based switch described earlier, the combination of FRB/FKBP and split dCas9 (AND gate) has provided a significant reduction in the background. In the future, there is potential for the development of hybrid systems

Table 2. Comparison of expression systems for CAR

Promoter/DNA recognition element	Regulation	Immunogenicity	Silencing susceptibility	Transduction efficiency	Exhaustion prevention	Expression level	Test stage
EF1α[1,2]	no, constitutive	no	low (T cells)	high	no	higher	clinically
CMV[1]	no, constitutive	no	high (T cells)	medium	not	higher	clinically
MND[1,2,156]	no, constitutive	no	low (T cells)	high	yes, reducing tonic signaling	lower	clinically
MSCV[1,61]	no, constitutive	no	low (T cells)	high	not known	lower	clinically
U6[66,157]	no, constitutive	no	medium (T cells)	not comparable	not comparable	high (RNA)	clinically
hPGK[1]	no, constitutive	no	medium (T cells)	medium	could reduce tonic signaling	low	CAR-T, *in vivo*
RPBSA[1]	no, constitutive	no	medium (T cells)	medium	could reduce tonic signaling	low	CAR-T, *in vitro*
Tet[8,80,158]	yes, doxycycline	yes	high (stem cells)	low (need selection)	possible, if deactivated	regulated	CAR-T, *in vivo*
RESrep[84]	yes, resveratrol	yes	unknown	neutral/not known	possible, if deactivated	regulated	CAR-T, *in vivo*
ZF1-4-OHT[159]	yes, 4-OHT	yes	unknown	neutral/not known	possible, if deactivated	regulated	CAR-T, *in vivo*
NFAT[160]	yes, tacrolimus	not known	unknown	low (need selection)	possible, if deactivated	regulated	CAR-T, *in vivo*
LexA[100]	yes, light	yes	unknown	neutral/not known	possible, if deactivated	regulated	CAR-T, *in vivo*
HSE[10]	yes, temperature	no	unknown	neutral/not known	possible, if deactivated	regulated	CAR-T, *in vivo*
RU486-inducible[87]	yes, RU486	yes	unknown	not known	not known	regulated	non-CAR
Auxin-inducible[85]	yes, auxine	yes	unknown	not known	not known	regulated	non-CAR
Ecdysone receptor[86]	yes, tebufenozide	yes	unknown	not known	not known	regulated	non-CAR
dCAS9-based systems[110,119,136,161]	yes, various controlling substances	yes	low (hematopoietic stem cells)	neutral/not known	not known	regulated	non-CAR

that integrate external control determined by medical professionals with SynNotch-regulated autonomous CAR-T. A good combination may be an externally controlled light/ultrasound system and SynNotch. The disadvantage of SynNotch is that, if the cancer cells are outside the area of the original focus (metastasis), the system may decompensate due to disruption of antigen compartments (as described above in SynNotch). In such a case, the use of activation control space would limit the problem (Hsp, UV).[152]

Summary

Transcription of transgenes in CAR cells is an important issue in designing modern CAR-T therapy. The selection of an appropriate constitutive promoter can contribute to both therapeutic and production efficacy. Based on current research, it seems that for both CAR-T persistence and the manufacturing process, weaker constitutive promoters might be more favorable.[1] With the evolution of expression systems, the inducible on/off mechanisms have opened the door to a potentially even greater role for transcriptional regulation in CAR-T. The TFs and the systems harvesting their advantages despite serving the same purpose of regulating transgene expression are rich in differences. Pros and cons are summarized in Table 2. Crucial factors that would determine if a given system is clinically significant are safety and orthogonality.

Immunogenicity, the biggest safety concern, is undoubtedly an unfavorable factor that is hard to diminish in the group of exogenous inducible systems. The anti-CAR T cell immune response is known to be reported against both extracellular (mouse-based scFv) and intracellular (HSV-TK safety switch) exogenous proteins. Rejection of CAR-T by the patient's body raises risks due to the ineffectiveness of the therapy. However, it is also possible that risks arise from directly dangerous side effects. HAMA-triggered mast cell degranulation is a dangerous (but rare) side effect based on the immune response against non-human-derived components of CARs.[162]

On the other hand, systems that are not immunogenic (inducible endogenous) are usually not orthogonal since their components can be activated by matching native signaling pathways.[136,163] Here, mutated or computational-biology-engineered variants may overcome that leakage while keeping the privilege of being non-immunogenic.

Further aspects to consider are how to control the inducible systems. Clinically approved or under clinical trial, regulatory molecules can be found in both endo and exogenic and even ATF groups.[15,88,130] However, without clinical trials, it is difficult to determine the dose of a substance that will ultimately be required to predictably control cells. Physical activator-based systems, in addition to the inherent limitations (or advantages) of a small activation area, may prove less amenable to differences in the pharmacokinetics than controlling molecules, meaning that the

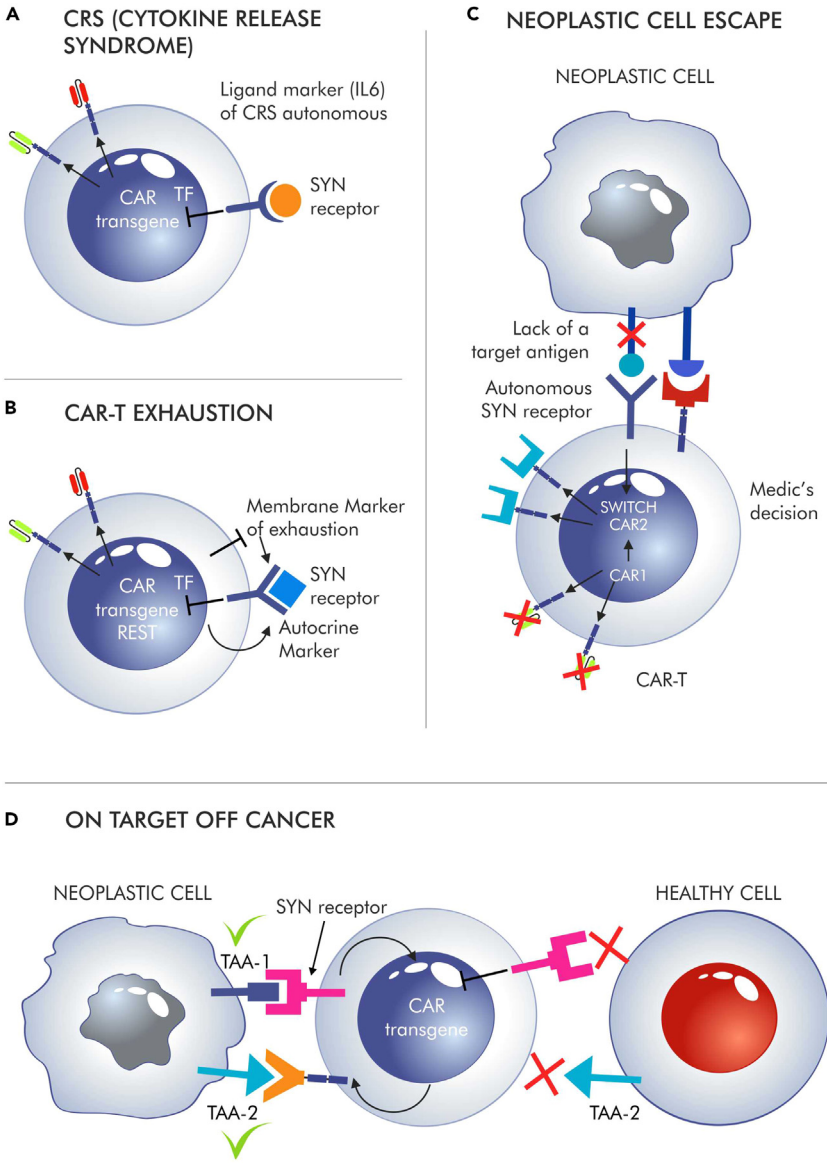

Figure 5. Inducible transcription overcoming CRS, depletion, antigen escape, and on-target off-tumor toxicity

(A) CAR expression can be turned off thanks to remote (chemical physical inducers of transcription factors regulating CAR expression) or autonomous (synthetic receptors) control since markers of a cytokine storm are well-defined. (B) CAR expression could be autonomously silenced if CAR-T exhaustion starts. The detection of exhaustion can be realized based on autocrine mechanisms. Although remote control is effective in reducing exhaustion, the problem might be the detection of exhaustion by hospital staff since CAR-T cells represent too small a percentage of blood or bone marrow cells. (C) Lack of CAR antigen on neoplastic cells can be autonomously detected (lack of synthetic receptor antigen). Immunocytochemistry or flow cytometry can be used to detect such a change in the phenotype of the neoplastic cells, and proper actions can be realized by medical doctors. (D) SynNotch will activate CAR expression only if it encounters a priming antigen. It makes it possible to effectively and safely target antigens present in both cancer and healthy cells (unspecific antigens). When cells with non-specific TAA—healthy and cancerous—are physically separated from each other, CAR-T can be activated only within the tumor, which means that healthy cells outside the tumor perimeter are not destroyed even though they have an antigen that could activate CARs.

distribution of chemical compounds may be more variable due to their metabolism and excretion rate, while physical factors can offer constant penetration within tissues.[4,102]

The last aspect is promoter silencing through epigenetic phenomena. One of the primary means of silencing artificial transgenes is methylation, which depends on CpG islands, among other factors.[164] However, it is hard to assess the impact of silencing on current CAR-T therapies because these changes do not occur immediately. For example, the PGK or MSCV promoter can function for at least 7 weeks before it is silenced in T cells, providing stable expression during this time.[61] A greater effect of silencing could be observed in off-the-shelf CAR-T concepts in which transgenes are maintained in cells for longer periods. Stem cells used to produce such CAR-T cells also have different methylation kinetics for their promoters.[165] Bypassing the problem of methylation of artificial transgenes could be solved by placing CAR under the TCR promoter so that expression of the artificial protein occurs under the influence of the natural promoter.[166]

Ultimately, control of transgene expression can be handed over to CAR-T itself. Autonomous CARs are being created to form biological circuits that have a chance to respond to four of the biggest CAR-T problems (Figure 5).

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AUTHOR CONTRIBUTIONS

All authors were involved in writing the article, as well as collecting and analyzing the literature cited. All authors have read and accepted the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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