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Full Length Article

Engineering transcriptional regulation for cell-based therapies

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ABSTRACT

A major aim in the field of synthetic biology is developing tools capable of responding to user-defined inputs by activating therapeutically relevant cellular functions. Gene transcription and regulation in response to external stimuli are some of the most powerful and versatile of these cellular functions being explored. Motivated by the success of chimeric antigen receptor (CAR) T-cell therapies, transmembrane receptor-based platforms have been embraced for their ability to sense extracellular ligands and to subsequently activate intracellular signal transduction. The integration of transmembrane receptors with transcriptional activation platforms has not yet achieved its full potential. Transient expression of plasmid DNA is often used to explore gene regulation platforms *in vitro*. However, applications capable of targeting therapeutically relevant endogenous or stably integrated genes are more clinically relevant. Gene regulation may allow for engineered cells to traffic into tissues of interest and secrete functional proteins into the extracellular space or to differentiate into functional cells. Transmembrane receptors that regulate transcription have the potential to revolutionize cell therapies in a myriad of applications, including cancer treatment and regenerative medicine. In this review, we will examine current engineering approaches to control transcription in mammalian cells with an emphasis on systems that can be selectively activated in response to extracellular signals. We will also speculate on the potential therapeutic applications of these technologies and examine promising approaches to expand their capabilities and tighten the control of gene regulation in cellular therapies.

1. Introduction

In the dynamic landscape of biotechnology and synthetic biology, the ability to harness the potential of living cells for therapeutic purposes has become a transformative force in recent years. Engineered cells hold immense promise in revolutionizing the field of medicine, offering innovative avenues for targeted interventions and precise treatment modalities [1,2]. One of the key paradigms driving the growth of this field is the regulation of gene expression through the activation of transmembrane receptors by extracellular signals [3].

Cells respond to external cues by initiating a cascade of intracellular events that often alter transcription. The tightly controlled orchestration of these signaling pathways enables cells to regulate their behavior in response to specific environmental stimuli. Strategically engineering cells to express constructs capable of recognizing and responding to defined ligands provides a powerful tool to precisely tune gene expression [4]. The ability to activate or repress specific genes of interest in a ligand-dependent manner not only offers insight into cell signaling, but also holds promise for the development of novel cell-based therapies. To date, transmembrane receptor engineering, inducible transcriptional activation, and immunotherapies have not reached their full potential and an in-depth understanding is required to develop the next generation of cellular signal actuators [5].

In this review article, we overview synthetic transcription factor technologies and strategies employed to control gene expression. We then explore the multifaceted benefits of using transmembrane receptors as molecular switches for the activation of transcriptional processes. We examine recent advances in cellular sensing capabilities that allow cells to sense and respond dynamically to their external environment, and this is followed by a discussion on the uses of ligand-responsive transcriptional regulation in the future of cell-based medicine. This review article illuminates the innovative endeavors in synthetic biology that are integrating biology, engineering, and medicine to discover new therapeutic strategies.

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1.1. Endogenous transcription

Transcription, serving as the central process in regulating gene expression, is crucial for cellular development, survival, and function. Transcription functions as the cornerstone of gene expression, playing a pivotal role in transcribing genetic instructions into functional molecules. This fundamental process, known as the central dogma of biology, involves the transcription of deoxyribonucleic acids (DNA) into ribonucleic acids (RNA), followed by RNA translation into proteins [6]. As DNA contains instructions for the structure of proteins vital for dictating cellular processes, transcription is widely considered to be the primary control point of cellular behavior. Understanding the intricacies of endogenous transcription is paramount for controlling gene expression and shaping fundamental life processes. Integrating inducible endogenous transcription into mammalian whole-cell-based systems provides a pathway to develop biologically-relevant, predictable, and adaptable platforms with diverse applications. Ultimately, comprehending gene expression marks the initial stride towards leveraging this knowledge for research endeavors in synthetic biology.

The intricate process of transcription hinges on the enzymatic action of RNA polymerase (RNAP) facilitating the transcription of genetic information from DNA into RNA [7]. Transcription yields various RNA molecules, each with distinct properties and functions. Messenger RNA (mRNA) conveys genetic information from the nucleus to the ribosomes in the cytoplasm, where it eventually undergoes translation into proteins (Fig. 1). Ribosomal RNA, transfer RNA, and microRNA molecules are not translated into proteins but play critical roles in the process and control of protein synthesis [8]. Intriguingly, eukaryotic cells harbor multiple RNA polymerase isoforms, with RNAPII being the predominant subunit responsible for transcribing DNA into protein-encoding mRNA. The initiation of transcription necessitates more than RNAPII recruitment; it mandates the formation of a pre-initiation complex (PIC). Typically, the minimal or 'basal' PIC comprises RNAPII and six generalized transcription factors (TFs; A, B, D, E, F, H), but can consist of fewer or considerably more than just these six subunits [9–12]. TFs, furnished with specific DNA-binding motifs, serve as pivotal protein regulators that can promote or repress genes of interest.

1.2. Gene structure

Spatial and temporal expression dynamics are influenced considerably by the structural elements within the gene itself. A typical protein-encoding gene consists of core promoter regions, a 5′ untranslated region (UTR) housing a transcription start site, an open reading frame (ORF), a 3′ UTR, and transcription termination sequences (Fig. 1). These elements house RNA polymerase binding sites and several motifs responsible for mRNA stability and translocation [13]. Beyond this core framework, upstream regulatory elements, including enhancers, insulators, silencers, and core promoters exert additional regulation over gene expression, safeguarding the cell against erroneous protein synthesis [14]. Co-transcriptionally, a 5′ mRNA cap is added and post-transcriptional modifications such as the addition of a 3′ polyadenylation tail and intron splicing occur leaving protein-encoding exons to be translated into protein [15]. Transcription factors (TFs) play a vital role in binding specific sequences within these regulatory elements. Importantly, these regulatory elements and TFs exhibiting specificity to cell types can maintain homeostasis, govern protein concentrations, and regulate transcription rate, among other roles [16]. Many of these regulatory elements, including open reading frames [17], exons [18], and synthetic promoters [19], are currently being utilized to regulate the expression of genes of interest (GOI) in mammalian synthetic biology.

The integration of endogenous gene expression into mammalian whole-cell-based platforms enables the development of biologically relevant, predictable, and adaptable systems. Nevertheless, successful integration necessitates the careful consideration of safety, specificity, control, and delivery. Exploring strategies such as mimicking native signal transduction mechanisms or harnessing endogenous pathways may offer alternative avenues to overcome these challenges.

2. Induction of transcription using natural transcription factors

Activation of endogenous TFs can be used to control gene expression, providing an approach to control cell response to a given signal. In nature, TFs can be induced by various cell signals and stimuli, such as relative changes in cytokine concentration or mechanosensing-mediated activation of signaling pathways [20,21]. Signal transduction ultimately results in the activation and/or nuclear translocation of a TF. For example, a signaling molecule such as a cytokine or hormone can bind to its receptor on the cell surface. This triggers a cascade of intracellular signaling events that lead to the activation of TFs and ultimately protein expression, such as in the highly conserved JAK-STAT (Janus kinases-signal transducer and activator of transcription proteins), MAP/ERK (mitogen-activated protein kinases/extracellular signal-regulated kinases), and PI3K/AKT (phosphoinositide-3-kinase--protein kinase B/AKT) pathways [22,23]. Another mechanism is through changes in the availability of nutrients or metabolic intermediates. For example, glucose deprivation can induce the expression

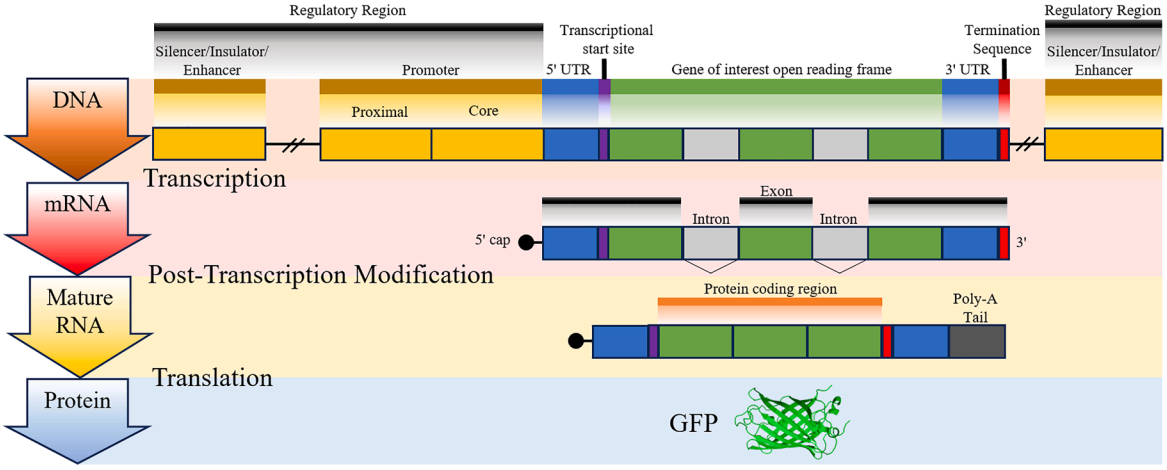

Fig. 1. Endogenous transcription and gene structure. Upstream and downstream gene regulatory elements (yellow boxes), 5′ and 3′ untranslated regions (UTR) (blue boxes), transcriptional start sites (purple boxes), termination sequences (red boxes), introns (gray boxes) and exons (green boxes) shown at the DNA level, through the process of transcription, post transcriptional modification, and translation into protein (e.g., green fluorescent protein, GFP).

of TF hypoxia-inducible factor-1 (HIF-1), which in turn activates genes involved in glucose metabolism [24]. However, these systems are subject to crosstalk with native regulatory pathways, which can make it challenging to achieve precise and predictable control over gene expression [25,26].

The use of TFs from other organisms, such as the GAL4 protein from yeast, provides researchers with isolation from regulatory signals and the ability to endow cells with new functions [27]. Inducible transcription using these noneukaryotic or synthetic TFs is achieved by placing the DNA recognition sequence upstream of the transgene of interest. Fluorescent proteins are often used as GOIs to easily quantify transcriptional activity using flow cytometry without requiring a cofactor [28,29]. Alternatively, quantitative polymerase chain reaction (qPCR) can be used as a tool to quantify changes in gene mRNA levels directly [30]. These noneukaryotic TF binding domains and GOIs can be expressed temporally in mammalian cells by transient transfection with plasmid DNA. Alternatively, the use of lentiviral systems allows for the stable integration of transgenes of interest into mammalian cells [31,32]. To provide control over specific cellular responses, synthetic systems using noneukaryotic TFs are typically constitutively expressed, activated by a ligand or light inducible switch, or are cleaved from a transmembrane receptor in response to a ligand as discussed in Section 5 (Inducible Split Systems) and Section 6 (Receptor Activation Mechanisms) [33,34]. These TFs taken from non-mammalian biological sources lack modularity and the capability to target endogenous genes, which are not limitations for synthetic DNA binding domains.

3. Synthetic DNA binding domains

Synthetic DNA binding domains provide control over target gene specificity. Directed DNA binding is the first step in synthetic TF design, as it enables the selective recruitment of effector proteins allowing for specific control over gene expression and downstream cell function. Synthetic DNA-binding domains have been designed and engineered for this application, as well as for genome editing and gene therapy. Three types of synthetic DNA-binding domains are: zinc fingers (ZFs) [35], TALE (transcription activator-like effector) domains [36], and clustered regularly interspaced short palindromic repeats (CRISPR) associated domains [37,38].

3.1. Zinc fingers

ZFs are small DNA-binding domains that recognize and interact with nucleotide triplets found in many natural eukaryotic TFs. In synthetic biology, ZF proteins can be used as tools to specifically target and modify DNA sequences. ZF nucleases have been widely used for the purpose of epigenetic silencing and gene knockout [39–41]. ZF triplets can be combined sequentially in a modular fashion to generate specificity to DNA sequences of 18–90 nucleotides producing larger, multi-finger domains with higher DNA-binding specificity and affinity (Fig. 2A) [42,43]. Computational approaches have also been explored to quantify the affinities to recognition sequences and to develop logical control into ZF-based technologies [44]. ZFs are potent tools in synthetic biology, but they suffer from poor nucleotide selectivity [45], which can result in off-target binding. To enhance safety and open the potential of genome editing, researchers are developing strategies to mitigate these off-target effects [39,46]. Despite these challenges, ZFs remain a promising tool for targeted gene regulation and genome editing in synthetic biology.

3.2. TALE domains

TALE domains, derived from bacterial plant pathogens, offer a unique approach in synthetic biology for precise DNA recognition through manipulation of amino acid sequence repeat domains [36,47]. Unlike ZFs, TALE repeat variable diresidue (RVD) domains recognize individual nucleotides with high specificity [36,48]. Although RVDs NN, NG, NI and HD that bind to guanine, thymine, adenine, and cytosine, respectively, are the most commonly used, over 400 RVDs have been identified with a variety of nucleotide specificities and affinities (Fig. 2A) [49]. This variety facilitates the design and engineering built around TALEs. TALE domains are commonly combined with nuclease units like FokI derived from *Flavobacterium okeanokoites* to create TALENs (transcription activator-like effector nucleases) for site-specific DNA cleavage [50–53]. TALENs have found success in target genetic engineering of human pluripotent stem cells and T-cell receptor modification

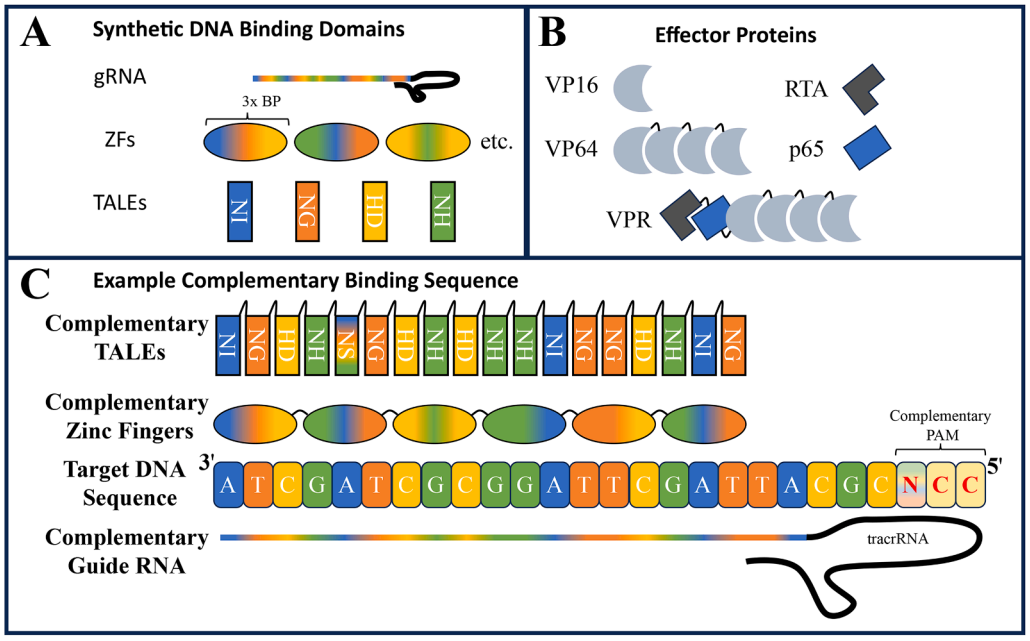

Fig. 2. Synthetic transcription factor components. (A) Examples of swappable DNA binding subunits include guide RNA (gRNA), zinc fingers (ZFs), and transcription-activator like effectors (TALEs). (B) Examples of complementary DNA recognition by DNA binding components. (C) Examples of transcriptional activator effector proteins including VP16, VP64, Rta, P65, and VPR.

for leukemia treatment [54–58]. However, TALENs face challenges due to their larger size (approximately 3 kb) compared to those of ZF nucleases (approximately 1 kb), limiting their delivery options and scalability [59]. The process of swapping out RVDs to select for a new DNA sequence is time consuming, but researchers have developed innovative methods for synthesizing TALEs to counteract this [50,51,60–62]. Ongoing efforts aim to enhance the specificity, efficiency, and delivery of TALE domains, but a great deal of recent focus has been on the alternative DNA binding tool, CRISPR.

3.3. CRISPR-Associated domains

Derived from the immune system of *Streptococcus pyogenes* and designed in 2012, the CRISPR-Cas9 system immediately gained prominence as the guided nuclease of choice, overcoming many of the challenges associated with ZFs and TALE domains [37,38]. Specifically, CRISPR-Cas9 increases target specificity and lowers production costs and complexity, and therefore decreases barriers of entry for researchers [37,38]. This reduction in complexity exists because the Cas protein and the DNA recognition sequence are decoupled from each other, eliminating the arduous cloning process to reorganize DNA recognition domains inherent in other systems. The engineered design of guide RNA (gRNA) which combines target specific features of CRISPR RNA (crRNA) and Cas recruitment from trans-activating CRISPR RNA (tracrRNA) into one construct further reduces design complexity [37,63]. gRNA acts as a complement to 20 base pairs on the target DNA sequence, upstream of a short protospacer adjacent motif (PAM) sequence and recruits the CRISPR-Cas9 protein using short hairpins, forming a complex with nuclease activity (Fig. 2A). Defined libraries of gRNA incorporated with CRISPR technology lead to controlled, highly customizable genetic circuit platforms. For gRNA sequences with high specificity, computational tools such as Basic Local Alignment Search Tool (BLAST), CRISPOR, E-CRISP, CHOPCHOP, and Benchling are often used [64–68].

To facilitate transcriptional regulation, CRISPR-Cas9 has been mutated to remove the endonuclease domains, termed dead Cas9 (dCas9) [69,70]. Like wild type Cas9, dCas is recruited to DNA by bound gRNA; however, it does not cleave it [71]. This makes dCas9 a useful tool for binding to endogenous DNA and driving gene expression. At over 4000 base pairs, dCas9 is relatively large, making it difficult to package into lentiviral or adeno-associated viral vectors for gene delivery. To aid in delivery, other Cas proteins are also being explored and characterized, such as those from other species like *Staphylococcus aureus* or the smaller CRISPR-Cas12 [72–74].

All three synthetic DNA binding domains, ZFs, TALEs, and gRNA can be reengineered to bind to essentially any DNA sequence of interest (Fig. 2B). When paired with effector proteins, these DNA binding molecules have extensive applications in the field of mammalian cell engineering.

4. Effector proteins

Endogenous transcriptional activators contain a DNA binding domain and an acidic, proline, serine and/or glutamine-rich activating region responsible for recruiting transcriptional machinery [75]. The earliest example of transcriptional activation using a synthetic protein was accomplished by conjugating the acidic activating region of the herpes simplex TF, virus protein 16 (VP16), to the DNA binding fragment of the yeast TF, GAL4 [76]. This hybrid protein supported the potent activation of a reporter gene harbored on a plasmid containing multiple GAL4 binding sites. The potency of VP16 can be amplified further by placing four activation domains in series, referred to as VP64 [35,71]. Using a similar approach, the p65 subunit of the NF-κB (nuclear factor kappa light chain enhancer of activated B cells) transcription factor [27], and the transactivator replication and transcription activator (Rta), encoded by the Epstein-Barr virus, were also shown to activate transcription when bound to Gal4 [77]. Furthermore, the tripartite activator VP64-p65-Rta, termed VPR, has been shown to be more effective at transcriptional programming than each individual component (Fig. 2C) [78,79]. While these platforms are constitutively expressed after initial transfection, this strategy of attaching a DNA binding domain to a potent transcriptional effector remains the general approach to induce targeted transcription.

Protein multimerization using RNA binding molecules or single chain variable fragments (scFv's) can be utilized, such as in the SunTag platform to recruit transcriptional effectors to the Cas9 DNA binding domain [80,81]. This platform has the potential to amplify the transcriptional activation signal dependent on how many tags are present and can also minimize the total amount of DNA required to activate two separate reporters. The synergistic activation mediator (SAM) system also utilizes protein multimerization to achieve effector protein and DNA binding domain dimerization with RNA adapter placing on an exposed section of a Cas9 gRNA molecule [82,83]. The combination of a DNA binding region and an effector protein has the potential to activate transcription of genes of interest. Being able to accomplish this gene activation in an inducible manner has substantial applicability for novel cell therapies and is an ongoing avenue of research.

5. Inducible split systems

Split protein systems provide researchers with the ability to control the temporal activation of platforms and to track biomolecular interactions. Split proteins allow for the conditional assembly of a functional protein in response to specific signals. As early as 1996, this was accomplished by attaching the FKBP12 ligand binding protein (FKBP) to both the VP16 effector and GAL4 DNA binding proteins [84]. Upon its administration, the lipid-soluble FK1012 dimeric ligand effectively assembled these proteins into a synthetic transcription factor to express secreted embryonic alkaline phosphatase (SEAP), a reporter protein [84]. In later iterations of protein multimerization, one of the FKBP binding proteins is swapped out for the rapamycin binding protein (FRB), which allows rapamycin to heterodimerize two split constructs with higher affinity [85]. Beyond this, other systems have been developed in a comparable manner that dimerize in response to diverse inputs including caffeine [86], light [87,88], asunaprevir [89], and telaprevir [89], which do not have overlapping crosstalk, allowing for logical control over platform induction. Relevant to the clinical applicability of these platforms, split inducible systems have been developed which rely on FDA-approved small molecules such as gibberellin [90], and grazoprevir [91]. Often, in split systems, a nuclear export sequence (NES) is attached to the effector protein to minimize off-target gene expression and a nuclear localization sequence (NLS) is attached to the DNA binding domain to initiate transport of the construct while bound (Fig. 3) [92,93].

While protein multimerization of a DNA binding domain and an effector protein together is the most straightforward way to elicit a transcriptional response, other strategies have been investigated. For

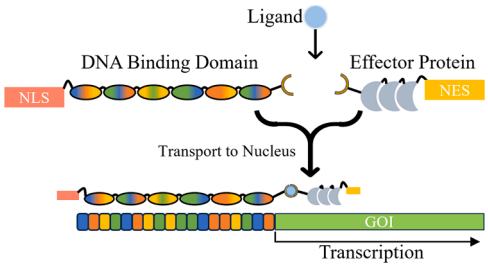

Fig. 3. Ligand dependent split synthetic TF induction. Upon ligand binding, a DNA binding domain (which contains the nuclear localization sequence, NLS) and an effector protein (which contains the nuclear export sequence, NES) will assemble into a synthetic TF and translocate to the nucleus where it can initiate transcription of a gene of interest (GOI).

example, researchers have split Cas proteins into two halves which regain function once reconstituted [94]. This allows for inducible nuclease activity for the unmutated split Cas proteins and added specificity to the dead Cas transcriptional activation designs with the added benefit of reducing the size of each component [87,94–96]. Moreover, in doxycycline induced tetracycline (Tet) on/off systems, chemical control is mediated at the transcription level, allowing for constitutive protein expression to either commence or cease in response to the tetracycline ligand [97–99]. Additionally, the split tobacco etch virus protease (TEV) allows for specific and controlled cleavage at recognition sequences [100]. Split TEV serves as a valuable tool for detecting intracellular protein-protein interactions within mammalian cells and can be used to release TFs from membrane bound or cytoplasmic proteins, allowing for their transport into the nucleus to initiate transcription [101–103].

While these platforms are useful for temporally activating synthetic platforms for the purpose of transcriptional activation, they all require user-defined inputs which can be difficult to administer accurately in clinical practice. Enabling cells with the ability to sense their dynamic external environments and respond by initiating a transcriptional response would enable for more autonomous control and therapeutic relevance.

6. Receptor activation mechanisms

In mammalian biology, the phospholipid bilayer is responsible for isolating intracellular cytosolic protein-protein interactions from the extracellular space. The complexity of multicellular organisms demands that cells have the capacity to recognize, decipher, and respond to external cues. This need gives rise to transmembrane receptors, responsible for initiating intracellular responses to extracellular stimuli. Transmembrane receptors consist of three major components: the extracellular ligand binding domain, the transmembrane domain, and the intracellular domain. To empower cell-based therapies with the ability to sense and react dynamically, an ongoing and concerted effort has been dedicated to developing synthetic transmembrane receptors with a modular array of inputs, actuators, and outputs, which in many cases, include transcription (Table 1).

6.1. Transmembrane receptor architecture

On synthetic transmembrane receptors, the extracellular ligand binding domain is responsible for sensing a target ligand of interest, resulting in the actuation of a cellular response. Numerous synthetic protein binding domains have been developed with the purpose of targeting soluble [23,104,105] or membrane-bound [34,106] ligands. ScFv's are a class of molecules derived from the heavy and light chains of antibodies and possess the ability to selectively bind to target protein epitopes. ScFv's have been used for a myriad of treatments including blocking malignant protein domains, diagnostic imaging, tumor therapy, and the treatment of neurodegenerative and infectious diseases [107–110]. As early as 1993, scFv's were adapted for the use in cell therapies within a transmembrane receptor by linking them through the transmembrane domain to the cd3ζ T-cell receptor activator [111]. Considerable work has been done towards expanding the number of targets available to scFv's, including directed evolution and computational modeling [112]. Additionally, a new class of non-antibody-based receptors known as monobodies have been developed, adapting the type-III binding domain of the fibronectin molecule [113,114]. These monobodies expand the number of available targets and physiological environments that can be explored and allow for more rapid development of novel targets using site-directed mutagenesis and computational approaches [115]. Monobody binders have favorable characteristics, such as small size, lack of disulfide bond formation, and simplicity of fusion protein synthesis [116,117].

Additional control has been leveraged by systems such as bi-specific scFv's. This design consists of two scFv domains linked together in series to target different antigens [118]. Additionally, the supra-CAR and SNAP-CAR/SNAP-synNotch are designed to respond to an array of scFv's and/or antibodies that can be administered at a later stage of treatment [119,120]. This design enables user-defined adaptability to different targets and mitigates the risk of antigen escape in cancer, which occurs when tumor cells evolve and no longer display the original target antigen [124,125].

The methods for autonomously inducing the signaling pathways, split systems, and synthetic TFs we have previously discussed in response to extracellular ligands involve synthetic receptor activation mechanisms. Currently there are two main approaches for this process, including the induction of the endogenous native signaling pathways previously discussed, and the proteolytic release of a synthetic TF from the membrane. Novel means of actuating cellular pathways to induce transcription using these approaches has been a focus in the field of synthetic biology [3,121].

6.2. Activation of native signaling pathways

Relying on some of the endogenous signaling pathways previously discussed herein, researchers can replace native response elements with new outputs that respond to user-defined target molecules. The most well-characterized receptor system which utilizes this approach is the CAR. The intracellular region in the first generation of CARs relies on the

Table 1
Classical Engineered Transmembrane Receptors: A focused overview of impactful classical transmembrane receptor constructs. It is important to note that while this list is not exhaustive, these transmembrane receptors represent pivotal frameworks, serving as cores for many future iterations and advancements in engineered receptor technology. The columns detail essential information, including the intracellular mechanism of action, extracellular target, and functional output.

Receptor Name	Intracellular Mechanism	Intracellular Action	Extracellular Target	Output
synNotch [34,106] & Synthetic Intramembrane Proteolysis Receptor (SNIPR) [126]	Force transduced proteolytic cleavage	Release of synthetic TF	Cell surface antigen	Transcription of GOI
Chimeric Antigen Receptor (CAR) [111]	Endogenous signal transduction	Endogenous T-cell/B-cell activation pathway	Cell surface antigen/ cytokines	T-cell/B-cell differentiation and activation
Receptor Activated Solely by Synthetic Ligand (RASSL) [125]	Endogenous signal transduction	G-protein recruitment	Orthogonal synthetic small molecule ligands	Endogenous transcriptional targets associated with GPCR signaling pathways (PLCB, AC, Gby RhoEGF)
Transcriptional Activation Following arrestin translocation (TANGO) [127]	Intracellular recruitment induced proteolytic cleavage	Release of synthetic TF	Endogenous ligand	Transcription of GOI
Modular Extracellular Sensor Architecture (MESA) [103]	Proteolytic cleavage	Release of synthetic TF, dCas9	Dual binding domain soluble ligand	Transcription of GOI
Generalized Extracellular Molecular Sensor (GEMS) [23]	Dimerization induced endogenous signal transduction	Endogenous JAK/STAT, MAPK/ERK, PLCG, PI3K/AKT pathways	Dual binding domain soluble ligand	Multiple endogenous transcriptional targets

T-cell receptor derived CD3ζ domain to elicit the activation of a Zap70 protein, leading to downstream T-cell proliferation and differentiation (Fig. 4A) [122,123]. In the second generation of CARs, either the CD28 or 4–1BB costimulatory domains were added directly to the intracellular component of these receptors to provide co-stimulation, similar to natural T-cell activation, thereby resulting in a potent response. Chimeric B cell receptors have been developed in a similar fashion, replacing the T-cell costimulatory domain with the CD79 domain used in natural B-cell activation [124]. The receptor activated solely by synthetic ligand (RASSL) [125] relies on a seven-transmembrane receptor system to activate the endogenous G-protein-coupled receptor (GPCR) pathway [131]. The generalized extracellular molecular sensor (GEMS) platform uses extracellular ligand-dependent dimerization to dimerize an array of intracellular endogenous pathways [23]. These platforms are beneficial due to their simplicity, but the dependance on natural pathways makes it difficult to control the potency of response or enable the transcription of genes outside of the scope of the selected activation pathway.

6.3. *Proteolytic release of a synthetic TF*

The other methodology for gene activation relies on the proteolytic release of a TF or other components relevant to transcription, enabling it to travel to the nucleus and initiate transcription of a GOI. Three seminal studies introduce this methodology: the modular extracellular sensor architecture (MESA) [103], the synthetic notch (synNotch) [34] pathway, and PRESTO-Tango [127] (parallel receptorome expression and screening via transcriptional output, with transcriptional activation following arrestin translocation) [106,128,129].

The MESA receptor is a two transmembrane domain system which uses extracellular dimerization to recruit a TEV on one receptor towards the TEV recognition amino acid sequence and release an intracellular transcription factor (Fig. 4B) [103]. The synNotch platform uses the regulatory core found in native notch receptors to undergo intramembrane proteolysis [34]. This process involves disintegrin-mediated shedding of the extracellular domain followed by ɣ-secretase mediated cleavage of the transmembrane domain and TF release [126]. This platform was optimized to expand the available ɣ-secretase transmembrane core domains and quantify the effect of linker lengths on net activation [126]. In addition, a tension-tuned synNotch platform has been developed to investigate mechanotransduction-related phenomena, and may eventually find utility in cancer therapeutics for differentiating the stiff tumor microenvironment from healthy tissue [130]. The Tango synthetic seven transmembrane receptor platform was developed so that the β-arrestin, protein recruited to turn off and therefore regulate GPCR activation, contains a TEV and the receptor contains its recognition sequence [127]. When a ligand is bound to this receptor, the intracellular GPCR machinery recruits this TEV to the receptor to cleave off a TF [125,127]. In MESA, synNotch, Tango and its derivatives, upon ligand activation, the protease cleaves the domain to which the TF is bound. This results in TF release, nuclear translocation, and subsequent downstream transcription. A limitation of these platforms is that once the TF is released, the receptor is rendered useless and remains on the surface until it is naturally degraded by ubiquitination, as there are no systems capable of recycling the receptor to replace its synthetic TF [131]. This latency limits the reversibility of these receptors and their ability to respond multiple times to signals at the same potency, reducing their long-term predictability. Bridging these strategies with CARs modified to induce TF release may open additional control strategies for the next generation of cell therapies [132].

The limitations of dependance on native signaling pathways and receptor depletion should be addressed, as well as the need for a system that is reversible at the protein level prior to activation of transcription. The numbers of potential targets and mechanisms of stimulating transcription via transmembrane receptors continue to rise, and so do the number of cell-based therapies that rely on these receptors for the treatment of disease.

7. Cell therapies

7.1. *CAR T-Cell therapy*

Cell-based therapies harness the potential of living cells to address a diverse spectrum of diseases and conditions, holding significant promise in modern science and medicine [2,133,134]. One of the most prominent examples of cell-based therapies, and a prime illustration of applied synthetic biology, is CAR T-cell therapy. This therapy uses synthetically engineered CAR receptors to target specific cell surface antigens and activate T-cell activation pathways [5,135]. These engineered T-cells bypass regulatory major histocompatibility complex (MHC)-dependent reactions present in native T-cells in the interest of heightening T-cell activation and the attack of target cells. CAR T-cell therapies have found extensive application in cancer treatment, with a primary focus on blood-borne cancers such as acute and chronic lymphoblastic leukemia (ALL and CLL, respectively), lymphoma, and multiple myeloma [134]. CD19, along with B-cells maturation antigen (BCMA), serve as optimal targets for CAR-T cell therapy due to their substantial bioavailability in hematological cancers [136]. Notably, CD19 CAR T-cells show high rates of remission and favorable long-term outcomes in the management

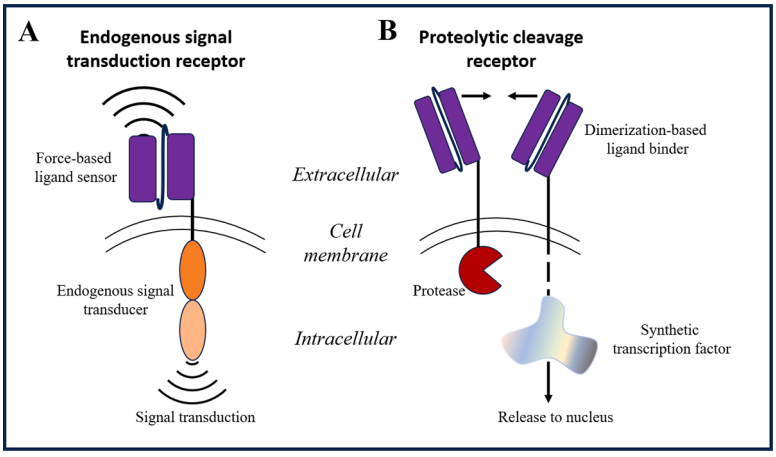

Fig. 4. Transmembrane receptor activation mechanisms. Endogenous signal transduction receptors (i.e., CAR T-cell therapy) induce signal transduction through a transducer such as CD3ζ, upon stimulation by a synthetic extracellular receptor, often composed of an scFv linked to a coiled transmembrane domain. Proteolytic cleavage receptors (i.e., MESA) depend on cleavage of a recognition sequence. This releases a synthetic TF, such as dCas9 or an effector protein attached to a DNA binding component, to control cell function in response to a given ligand.

of ALL and CLL [136]. Beginning in 2017, the U.S. Food and Drug Administration (FDA) has authorized six CAR T-cell therapies (Abecma, Breyanzi, Carvykti, Kymriah, Tecartus and Yescarta) at the time of this publication, highlighting the transformative potential of these therapeutic approaches [137]. There are, however, some limitations to CAR T-cell therapy. Antigen escape, solid tumor infiltration, targeting, and control over post-infusion cell behavior and phenotype remain among the considerations which synthetic biology aims to address [138–141].

In the context of blood cancers, cell surface antigens have little spatial restrictions when targeting. However, when addressing solid tumors, cell membrane-bound antigens are concealed and less accessible [142]. Consequently, there is a pressing need for therapeutic strategies to broaden targeting to include the extracellular matrix of solid tumors that surrounds cancerous cells. Of note there is a dysregulation of many extracellular matrix proteins found in the tumor microenvironment which may serve as potential targets [143]. While some extracellular matrix binding receptors exist, like the extra domain B splice variant of fibronectin (EDB-FN) binding CAR, targeting the extracellular matrix remains a relatively untapped avenue for the treatment of solid tumors [144]. Several scFv's have been developed for imaging and tagging various extracellular matrix components found in cancers, but few have been adapted for cell therapy [145].

Expansion of synthetic receptor systems provides an avenue for new cancer cell-based therapeutics. For example, synNotch receptors in murine T-cells have displayed multi-antigen specificity and increased efficacy and persistence in mouse models for mesothelioma and ovarian cancers compared to traditional CAR T-cell therapy [146]. Additionally, a synNotch receptor capable of activating the transcription of the pro-inflammatory cytokine IL-10 is specific to the tyrosine kinase receptor, Axl, which is upregulated in breast, pancreatic, lung, and colon cancers [147]. Encouraged by the successes of CAR T-cell therapies against blood cancers, and cognizant of the limitations of T-cells, researchers are exploring additional cell types to develop more advanced and robust immunotherapies.

7.2. Additional cell types

Developing cell therapies using immune cells outside of T-cells has been of broad interest to researchers in recent years and can open the door to their unique abilities and the potential for these therapies to work in concert. One constraint of T-cells is their limited infiltration into cold tumor tissue, a task which other immune cell types including macrophages may help address [148,149]. Depending on their post-polarization phenotypes, macrophages can either be potent anti-tumor cells (M1 phenotype) or tumor-promoting cells (M2 phenotype and tumor-associated macrophages) [150,151]. Thus, CAR-macrophages must be capable of sensing tumor-associated antigens and maintain their M1 phenotype to transcribe and release pro-inflammatory cytokines and to phagocytose cancerous cells. Engineered macrophages against human epidermal growth factor receptor 2 (HER2), are currently in phase I clinical trials for patients with recurrent breast or gastric cancers who have failed at least one treatment (NCT04660929) [150,152]. A CAR-macrophage was also developed that targets the mesothelin antigen, which is overexpressed in a number of solid cancers, potentially offering a much-needed solid tumor-infiltrating cell-based therapy [150,153]. These CARs utilize the CD3ζ intracellular region found in first generation CARs due to its homology to the phagocytosis-initiating signaling molecule FcεRI-γ (Fc common γ-chain), found in macrophages [150]. While phagocytosis is capable of clearing cancer cells, there is a window of opportunity to develop macrophage-based therapies that respond to cancer antigens by inducing the transcription of genes that maintain the M1 phenotype and release targeted pro-inflammatory cytokines.

Along with macrophages, natural killer (NK) cells are being investigated for cancer therapy for their ability to drive an inflammatory response and eliminate tumor cells. When activated, NK cells secrete elevated levels of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), perforin, and granzyme-containing granules, making them a potent cell type for fighting cancer [154]. However, the immunosuppressive tumor microenvironment can render native NK cells inactive [154]. Engineered NK cells aim to harness the targeted cell killing potential of endogenous NK cells to eliminate tumor cells. Such therapies are currently being investigated preclinically for B and T cell malignancies and for solid cancers including glioblastoma, breast, and ovarian cancers [155]. Furthermore, engineered natural killer (NK92) cells functionalized with synNotch receptors programmed to release interleukin-12 (IL-12) in response to glypican-3 (GPC3) aid CAR-T tumor elimination, suggesting an assortment of engineered cells may present a more effective cell-based therapy strategy in the future [156].

Apart from the targeting of solid tumors, the expansion of cell types in immune cell therapies has the potential to assist in autoimmune diseases and tissue regeneration [157]. Of particular interest, regulatory T-Cells (Tregs) suppress the immune system and prevent autoimmunity. Tregs have been engineered to recognize the human leukocyte antigen (HLA) A2 in order to prevent organ rejection, and are currently in clinical trials [158,159]. It is suggested that engineered regulatory T-cells are to be at the forefront of the next generation of autoimmune and inflammatory disease treatment and could serve as a potential safety mechanism in traditional CAR T-cell therapy [159]. The expansion of available cell types and control over cell sensing and response capabilities will improve the safety and efficacy of future cell-based therapies. Furthermore, the development of new synthetic receptor platforms will enhance these therapies and allow for better outcomes in oncology as well as autoimmune diseases and tissue regeneration.

8. Conclusions

Considerable progress has been achieved in the pursuit of a fully autonomous cell therapy system able to perceive its surroundings and initiate transcriptional responses. Currently, most immune cell therapies under clinical investigation harness endogenous pathway activation for circuit induction. However, as we look towards the future, iterations require improvements in terms of increasing specificity and expanding the number of available targets, diversified modes of activation, and the integration of orthogonal intracellular pathways focused on gene activation. The trajectory of this research field is set to evolve as platforms become more sophisticated and diverse. In tandem with this evolution, we must now face a host of additional considerations.

One paramount consideration is the inherent limitations associated with gene size and the concomitant challenges of efficient delivery and stable host cell integration. Researchers should be conscious of the ethical concerns related to genetic engineering and limit their work to somatic cells. Genes should be responsibly designed, and integration should be undertaken with extreme caution and care for containment. Crafting genetic constructs that can navigate these constraints will be a pivotal undertaking for researchers and potentially enable the translation of these concepts from bench to bedside. Moreover, fine-tuning the engineering of these autonomous systems necessitates meticulous attention to safety, specificity, and regulatory compliance, ensuring that the promises of this innovative field can be harnessed responsibly and ethically.

As we progress towards realizing fully autonomous cell therapies, creative strategies of innovation and interdisciplinary collaboration will be crucial. This emerging era promises to revolutionize the landscape of cellular therapeutics, offering new approaches to develop therapeutic strategies towards a myriad of diseases and medical conditions.

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Declaration of competing interest

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