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Emerging technologies for genetic control systems in cellular therapies

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Abstract

Progress in synthetic biology has enabled the construction of designer cells that sense biological inputs, and, in response, activate user-defined biomolecular programs. Such engineered cells provide unique opportunities for treating a wide variety of diseases. Current strategies mostly rely on cell-surface receptor systems engineered to convert binding interactions into activation of a transcriptional program. Genetic control systems are emerging as an appealing alternative to receptor-based sensors as they overcome the need for receptor engineering and result in cellular behaviors that operate over therapeutically relevant timescales. Genetic control systems include synthetic gene networks, RNA-based sensors, and post-translational tools. These technologies present fundamental challenges, including the requirement for precise integration with innate pathways, the need for parts orthogonal to existing circuitries, and the metabolic burden induced by such complex cell engineering endeavors. This review discusses the challenges in the design of genetic control systems for cellular therapies and their translational applications.

Introduction

Cellular engineering strategies for repurposing mammalian cells to respond to environmental cues in a user-controlled fashion have revolutionized the development of cellular therapeutics [1]. These sense-and-respond systems are designed to (1) sense a biological input, (2) transduce the signal, and (3) respond with a user-defined biomolecular program (Figure 1). This approach has enabled diverse applications ranging from diagnostics for real-time monitoring of disease biomarkers to therapeutics that translate biomarker detection

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into the execution of a therapeutic program for the treatment of a diverse range of diseases, including cancers and immune diseases [2–7]. The practicality of these designer cells that detect and correct human pathologies is rapidly growing as evidenced by the increasing number of clinical trials for cellular therapies, which have reached 1358 active trials as of 2021, with a sustained increase of 24% and 43% from 2019 to 2020 and 2020 to 2021 (Cancer Research Institute, URL: https://www.cancerresearch.org/en-us/blog/june-2021/io-cell-therapy-development-in-2020-pandemic). Cellular therapeutics provide alternative treatment options to address the global spread of infectious and metabolic diseases where more traditional methods have failed. Patient-derived T cells engineered to express chimeric antigen receptors (CAR-T cells) have been of particular interest for cancer immunotherapy and currently comprise 7 of the 17 FDA-approved cell and gene therapy products (FDA, URL: https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products). The sensing capabilities of designer cell therapies typically rely on natural or engineered receptors that detect a wide range of ligands and, in response, activate user-defined signal-transduction programs. However, the dynamic properties of these engineered cells ultimately depend on the signal-transduction mechanism, which may not accurately recapitulate the dynamics of the biomarker input [8,9•]. Gene activity determines cell functionality during physiological and pathological processes and is constantly and dynamically modulated to respond to environmental as well as intracellular stimuli. Recent advances in mammalian synthetic biology have provided an increasing number of cell engineering tools based on transcriptional and post-translational control elements 10,11. Such tools have opened the way to the design of cellular devices that sense and process intracellular as well as extracellular signals through genetic control elements. Genetic control systems overcome the need to engineer, rewire, or *de novo* design cellular receptors for biomarker detection and enable the design of diagnostic and therapeutic devices that operate over biologically relevant timescales [12,13]. Several technological considerations need to be addressed to equip designer cells with appropriately designed genetic control systems, ranging from the delivery of genetic materials to practical concerns associated with the source of cell type. In this review, we illustrate the potential of cellular therapies for the treatment of a wide range of diseases and discuss the current strategies for designing genetic control systems for sense-and-respond designer cells.

Control systems in cellular therapies

Current strategies to design cells that sense and respond to extracellular signals have capitalized on the recent progress in the field of receptor engineering, which provides innovative avenues to endow cells with seemingly endless sensing capabilities. A wide array of biomarkers can be processed by rewiring or evolving native ligand-receptor interactions or by designing cellular sensors *de novo*. A common approach to this end consists in linking single-chain variable fragments (scFvs) against antigens that are not recognized by known receptors to synthetic transduction systems [7,14–17]. Interesting features of cellular devices based on ligand-receptor systems are the signal-amplification mechanisms and design modularity, which enables the detection of a wide range of signals and the production of user-defined output responses [18]. The system's dynamic properties, however, are ultimately strictly determined by the signal-transduction mechanism embedded

in the receptor system. This may limit their clinical applications as efficient diagnostic and therapeutic devices that operate over therapeutically relevant timescales, requiring sensor–actuator mechanisms that recapitulate the dynamic properties of the disease biomarker.

De novo design of transmembrane receptors has led to a dramatic improvement in the effector properties of designer cells [19,20]. A widely explored cell-receptor system applied to the development of mammalian therapeutics is the CAR. CARs consist of an extracellular scFv that can be programmed to recognize virtually any target antigen [21], fused to the intracellular signaling domain of the T-cell receptor, and costimulatory domains required for activation of the native T-cell therapeutic response. Patient T cells engineered to express appropriately designed CARs recognize and eliminate antigen-expressing tumor cells [22–24]. While CAR-T therapy has shown promise for the treatment of different types of cancers, this strategy presents limitations associated mainly with the lack of specificity and targeting of cancerous as well as healthy cells, leading to off-target toxicity [25,26••]. The specificity of CAR-T therapies may be improved by expressing the costimulatory domains onto separate receptors to create Boolean AND logic behaviors and prevent nonspecific activation of T-cell response from one input 3•. Another issue associated with current antiCD19 CAR-T therapies is the potential for antigen escape or the downregulation of the target antigen by cancer cells. A potential solution to this problem is based on the use of bispecific OR-gated CARs, such as the CD19-OR–CD20 CAR, which enables the CAR-T cell to target either one of the two antigens [27]. Finally, a limitation of the CAR is the innate lack of modularity of the sense-and-respond system, restricting applications requiring independent programming of the input signal and the output response, as the output behavior relies on native signaling pathways [21].

Recent efforts have focused on the development of universal platforms for facile customization of the relevant modules of designer cells. These platforms are typically based on the assembly of modular domains that mediate the detection of a wide array of biomarkers and activation of the desired, user-defined biomolecular outputs. The SynNotch platform consists of an extracellular sensing module, a nanobody, or an scFv targeting a cell-surface molecule of interest, a notch core domain with a cleavable sequence, and a programmable transcriptional effector in the intracellular domain 4••. Both the extracellular and the intracellular modules can be swapped to achieve customizable input–output cellular behaviors. When combined with other receptors such as the CAR, SynNotch enables programming Boolean logic behaviors. To overcome the lack of specificity of CAR T-cell therapy and the need to target antigens that are uniquely expressed by the cancer cells, the SynNotch system was further engineered to build an AND-gated circuit for conditional expression of a chimeric antigen receptor 3•. Additional attempts to generate a platform technology for the design of cells that sense and respond in a programmable fashion have led to the development of the Modular Extracellular Sensor Architecture (MESA), which is based on extracellular domains that bind to a wide variety of soluble molecules and trigger the activation of a programmable transcription factor 7. The sensor module of MESA is based on a target chain and a protease chain, each containing an extracellular domain for ligand recognition, a scaffold domain, and a transmembrane domain. The transmembrane domain of the target chain is fused to a cleavage sequence and a transcription factor, while the transmembrane domain of the protease chain is fused to tobacco etch virus (TEV)

protease. The binding of the target on the extracellular domains triggers dimerization of the receptor, which results in cleavage of the transcription factor domain on the target chain by the TEV protease on the protease chain. Release of the transcription factor from the sensor results in nuclear translocation and activation of user-defined targets. The MESA system was further engineered to rewire an endogenous pathway in human T cells and convert the immunosuppressive signal mediated by the vascular endothelial growth factor into activation of an immunostimulatory signal mediated by the interleukin 2 [26••].

The synthetic-receptor toolkit for mammalian cell engineering was further expanded with the development of the Generalized Extracellular Molecule Sensor (GEMS) platform [28]. This platform provides a novel technology to rewire innate cellular signaling pathways for the activation of desired biomolecular outputs and can be programmed to respond to both signaling molecules and molecules that do not activate endogenous signaling pathways, such as nicotine [28]. The modular architecture of GEMS accommodates a large range of homodimeric and heterodimeric molecules as extracellular-binding domains, including the widely used FK506-binding protein (FKBP)–rapamycin-binding protein (FRB)/FK506-binding protein (FRB–FKBP) system, heavy and light chains of antibodies, and scFvs. The extracellular domain is fused to an EpoR scaffold that spans the transmembrane domain, which, in turn, is fused to intracellular-dimerizing signal-transduction domains. Ligand-induced dimerization of the scaffold can be thus programmed to activate diverse endogenous pathways, including the Janus kinase (JAK)/signal transducer and activator of transcription (STAT), phosphoinositide 3-kinase (PI3K)/Akt serine/threonine kinase (Akt), and mitogen-activated protein kinase (MAPK) pathways by expressing the transgene under the control of the appropriate pathway-specific promoter. Collectively, these engineered receptor systems enable the design of sophisticated sensor–actuator devices capable of converting diverse extracellular cues into programmable cellular behaviors, opening the way to the design of a wide range of cellular therapies. A potential strategy for the construction of more sophisticated cell-based devices consists of integrating the cellular response to the activation of multiple receptors. Multiple CARs, SynNotch receptors, and MESA receptors can be used in parallel to implement cellular logic behaviors that respond to distinct ligands mediating surface-based or soluble interactions. Synthetic receptors can also be mixed and matched, as in the case of the AND-gated SynNotch with conditional expression of the CAR. To address the lack of quantitative performance metrics that challenge the design of synthetic receptors, the “metric-enabled approach for synthetic receptor engineering” consisting of six steps based on the ‘design–build–test–learn’ cycle was recently reported [29], providing a set of basic quantitative performance metrics with great potential for generating standardized design rules.

Strategies for building cellular sensors also rely on control systems that respond to small-molecule inducers. Two-way communication devices have been developed by generating a sender cell that secretes the signaling molecule L-tryptophan and a receiver cell that expresses an L-tryptophan-inducible sensor, which can be programmed to control a user-defined transcriptional response [30]. This system can be employed to study pattern formation, for instance, by programming a synthetic output mimicking the process of blood vessel formation. In another study exploring inducible synthetic switches, an isopropyl-β-thiogalactopyranoside (IPTG)-inducible system was designed to control the silencing of a

target gene through a small-hairpin RNA (shRNA) that mediates RNA interference and repressor protein elements [31]. Cell exposure to IPTG results in derepression of the transcriptional repressor TetR, and, in turn, TetR-mediated repression of a target-specific shRNA, ultimately activating the expression of the target gene. The two layers of negative regulation of the target gene result in a control system with improved tunability and reversibility and lowered leakiness. Finally, light-inducible control systems have also been employed to induce either protein production or silencing through photocaging of a T7 RNA polymerase that is responsive to 365 nm UV light [32]. Generally speaking, sense-and-respond systems that respond to small-molecule inducers enable precise control over the timing of the desired cellular response. However, they are inherently based on exogenous control and are not ideally suited to build feedback-responsive control systems that respond to cellular inputs dynamically.

Efficient diagnostics and therapeutics require sensitive adjustment of output generated in response to input fluctuations. Control mechanisms of current ligand-receptor devices rely mainly on the residence time of the biomarker/ligand, which is typically a downstream manifestation of the disease pathogenesis, and on the dynamics of the sensor–actuator device, which may not operate over timescales compatible with the therapeutic requirements. Implantable cellular devices require precise control systems to respond to rapidly changing environmental conditions. Cancer progression, for instance, is critically affected by the continuous remodeling of the tumor microenvironment (TME), a highly dynamic space consisting mainly of extracellular matrix, stromal cells, and immune cells [33] and characterized by hypoxia, oxidative stress, and acidosis [34,35]. Owing to its key role in tumor progression and multidrug resistance [36], the TME provides a target for the detection of prognostic biomarkers and the development of effective therapies [37–39]. Sense-and-respond devices that detect highly dynamic environments with high sensitivity and dynamic resolution provide a transformative technology for the development of diagnostic strategies for personalized medicine approaches and therapeutic systems for self-adjusted delivery. Smart delivery systems should also self-adjust drug dosage to avoid the induction of side effects. The main challenge associated with the systemic administration of immunomodulators for cancer treatment is the toxicity caused by therapeutically effective doses [40]. Controlling the pharmacokinetics and pharmacodynamics to achieve tailored efficacy and minimal side effects limits the use of many therapeutic proteins otherwise proven to be effective cancer drugs [41]. Continuous delivery improves patient survival with fewer unwanted side effects [42], but it is extremely difficult to achieve and represents a burdensome option not always accessible to patients, pointing to the need for cellular devices that sense unwanted effects and adjust drug release. This review will focus on emerging technologies for building genetic control systems for the detection of transcriptional signatures and other RNA-encoded inputs combined with post-translational control systems for superior resolution of protein dynamics [43]. We will illustrate the potential of these technologies as an alternative to the current receptor-based systems with respect to the unique opportunities they provide for building cellular sensors that control the production of therapeutic proteins with therapeutically relevant behaviors.

Genetic control systems for cellular therapies

DNA payload delivery methods

The construction of cellular therapies relies on the availability of tools for gene delivery and targeted gene integration designed specifically to minimize side effects, such as insertional oncogenesis, and ensure efficient transgene expression. The DNA-integration strategy must be chosen carefully in accordance with the size of the payload and the target cell type or tissue. The most used *ex vivo* gene-delivery vehicles in the context of cellular therapeutics are viral vectors. Cell therapies typically require large DNA payloads for creating genetic control systems with the appropriate complexity and efficient integration into the genome of target cells. Adeno-associated viruses (AAV) have a small packaging capacity of ~5 kb, but can be used to transduce multiple tissues and cell types [44], resulting in stable expression over time and a minimal immune response. The other class of viral vectors commonly employed for DNA payload delivery is retroviruses, and, particularly, lentiviral vectors. Retroviruses have a packaging capacity of ~9 kb, almost double the size of AAVs, enabling the delivery of multiple genes simultaneously. However, retroviruses infect dividing cells, limiting the types of cellular therapies that can be generated using retroviral delivery systems. Importantly, retroviral vectors have been the most commonly used DNA delivery method for creating cellular therapies based on SynNotch and CAR [45].

A potential pitfall of viral vectors for the delivery of genetic control systems is insertional oncogenesis, which is due to the lack of specificity of the transgene-integration sites. An alternative approach for DNA payload delivery is the use of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), a platform that leverages highly modular guide RNAs for targeting Cas9 to virtually any DNA locus and, in turn, enables the integration of exogenous DNA into the host genome with high specificity. Recently, PRIME editing, a modified form of CRISPR/Cas9 that does not produce double-stranded breaks, was combined with a site-specific recombinase to deliver DNA payloads over 5 kb [46]. CRISPR/Cas9 can also be fused to existing transposases such as the Piggybac system, for efficient and targeted transgene integration [47,48] with nearly absent off-target events. Significantly, in these systems, the transgene is integrated as a single copy, reducing the chances of insertional oncogenesis and addressing the safety concerns typically associated with viral delivery strategies for the development of cellular therapies. Furthermore, transposon-based technologies enable the targeting of a wide range of cell types, including primary cells [49].

RNA-based sensors and control systems

The efficacy of cellular therapies with genetic control systems depends on the sensitivity and dynamic resolution of the gene-activity sensor as well as of the transducer that converts the input signals into programmable outputs. RNA engineering provides avenues for the designs of both sensors and transducers with ideal properties and broad applicability for the design of cellular therapies. RNA sensors are highly versatile systems as they enable the detection of inputs at different hierarchical levels, including DNA, RNA, and proteins. Moreover, RNA sensors can be adapted for the detection of noncoding RNA, thus extending the sensing capabilities of these cellular devices to regulatory processes that are not ultimately

associated with a protein product. The folding and thermodynamic properties of RNA sensors rely on simple Watson–Crick base pairing, enabling the development of an array of modeling and experimental tools for functional prediction, structural validation, and design of RNA-based devices [50].

Unlike traditional, ligand-dependent receptors, RNA-based control elements allow detecting endogenous transcripts with unparalleled specificity and selectivity, opening the way to the development of cellular devices with potentially superior features compared with traditional ligand-receptor-based cellular sensors. Initial attempts to create RNA regulators such as small RNAs mainly focused on inhibition of translation through the use of appropriately designed sequences complementary to target mRNA [51]. Recent RNA-based regulators have leveraged the emerging regulatory role of a novel class of noncoding RNAs, namely microRNAs (miRNAs). miRNAs present cell- and tissue-specific patterns of expression that are also characteristic of developmental stages or cellular pathogenetic mechanisms [52•]. Genetic control systems for monitoring cell-state-specific miRNA expression profiles allow programming miRNA-controlled cellular behaviors. The MICR-ON system triggers the expression of the apoptotic protein hBax in response to HeLa cell-specific miRNAs. Specifically, a single-guide RNA with two miRNA-binding sites was used in this cell-classifier circuit to induce dCas9–viral protein R (VPR)-mediated expression of a fluorescent marker in response to miRNAs associated with cell differentiation [52•]. A synthetic toehold switch that can detect both endogenously and exogenously expressed miRNAs was also reported. In this system, the miRNA triggers the opening of the toehold structure that represses the target gene and results in the activation of the target gene's expression [53]. An important feature of miRNA sensors is their ability to implement Boolean logic for the construction of sophisticated, multi-input devices that can sense and respond to the complexity of endogenous miRNA expression profiles.

Recent advances in RNA-based control systems, mostly achieved in prokaryotic species, have led to the design of riboswitches, RNA molecules that comprise a sensing domain, or aptamer, and an actuator domain (Figure 2). Riboswitches convert a binding interaction in the sensor domain into a conformational change within the actuator domain embedded within an mRNA transcript, effectively resulting in ligand-controlled inhibition of translation [50]. Similar regulatory RNA motifs in eukaryotic cells are based on engineered ribozymes. Ribozymes are functional RNA molecules primarily known for catalyzing self-splicing or cleaving reactions that have been used for applications in mRNA degradation and editing. They have been engineered to regulate transgene expression post-transcriptionally, enabling precise, tunable control of gene expression. The *Tetrahymena* group-1 self-splicing intron provides an interesting ribozyme for building cellular sensors. Discovered in 1982, the first reported nonprotein catalytic molecule, this ribozyme catalyzes self-cleavage and ligation of an mRNA transcript. The *Tetrahymena* ribozyme has been engineered to integrate guide RNAs and sense and edit endogenous RNA transcripts. A guide RNA complementary to the desired RNA sequence directs the ribozyme to the target mRNA, triggering a cleavage reaction. This reaction can occur *in cis,* whereby the ribozyme cleaves itself out of the transcript, or *in trans,* which results in the cleavage and replacement of the 3' exon in the target mRNA [54]. A trans-splicing ribozyme was recently designed to target and replace the telomerase reverse transcriptase, a key pro-proliferative RNA transcript in hepatocellular

carcinoma (HCC), ultimately redirecting the cell toward apoptosis. The observed 70% reduction in telomerase expression in liver cells demonstrated the efficacy of the ribozyme for regulating gene expression. The antiHCC device hampered the cellular growth rate by up to 86% in a one-dose, 14-day observation period, demonstrating the potential clinical applicability of this technology [55].

A much smaller catalyst than the *Tetrahymena* ribozyme, the hammerhead ribozyme, is of viral origin and performs a one-step, self-scission, reaction. The hammerhead ribozyme is the most common catalytic RNA motif used for generating RNA control systems in mammalian cells and has been widely used to alter mRNA stability by mediating the removal of the poly-A tail, which results in transcript degradation before translation. A guanine-responsive hammerhead aptazyme that when embedded within the 3' untranslated region (UTR) of viral transgenes, destabilizes the mRNA transcript by active-state cleavage of the poly-A tail, was recently reported [56]. This strategy was subsequently applied to the design of RNA-based devices for controlling p53 signaling using a p53-specific aptamer, generating a method for targeted growth inhibition and apoptosis induction of p53-deficient cancer cells that was demonstrated to inhibit tumor proliferation *in vivo* [57].

The design of RNA sensors combining RNA-based recognition of target sequences and protein-mediated target modification has opened new avenues for profiling gene expression and modulating endogenous mRNAs. Adenosine deaminases are a class of enzymes isolated from bacterial species that catalyze the conversion of the nucleotide adenosine (A) to inosine (I), providing ideal tools for programmable RNA modifications as inosine is recognized as guanosine by the cellular replication machinery, ultimately producing an A–G substitution. The reprogrammable adenosine deaminase acting on RNA (RADARS) system enables introducing targeted RNA modifications through RNA recognition by a complementary guide RNA and adenosine deaminase binding and removal of a premature amber stop codon (UAG) within the target transcript. This reprogrammable RNA-sensing technology has been validated for a variety of applications, including detection of siRNA-mediated transcript knockdown and cell-type classification with single or multiple marker transcripts. Importantly, RADARS enables the sensing of endogenous RNA with high sensitivity and negligible off-target activity, which is highly relevant for the development of diagnostics and synthetic mRNA therapeutics [58].

Bacterial CRISPR/Cas systems have also become widely employed for the design of cellular sensors. A CRISPR/Cas-inspired RNA targeting system (CIRTS) was recently reported that enables localized, small-molecule-inducible gene regulation at the transcript level. The modularity of the CIRTS system allows adapting the effector domain to mediate RNA degradation, activate RNA expression, or perform direct base editing of target RNAs. Remarkably, the CIRTS system was found to retain significant levels of activity *in vivo*. The CIRTS editor was employed to revert the driving mutation in human Methyl CpG Binding Protein 2 transcript known to cause Rett syndrome, a rare but threatening neurological disorder [59].

Protein sensors and post-translational control systems

The integration of tools for post-translational regulation of protein function (in addition to tools for transcriptional and translation regulation) into genetic control systems for designer cells can provide an additional source of control that holds promise to improve the dynamic properties of cellular therapies. Post-translational processes typically occur over faster timescales than transcriptional and translational processes, thus providing more efficient tools for controlling protein dynamics. Rapid control of protein levels in cells was achieved *via* targeted depletion through proteasomal degradation. Specifically, this technology relies on a bifunctional molecule (NanoDeg) consisting of the antigen-binding fragment from the Camelidae species heavy-chain antibody (nanobody) fused to a degron signal that mediates degradation of the nanobody-target complex through the proteasome 9. The features of the NanoDeg can be easily customized in terms of rate of synthesis, rate of degradation, and mode of degradation, enabling quantitative and predictable control of the target's levels. Importantly, integrating the NanoDeg within genetic control systems to achieve input-induced depletion of the NanoDeg's target results in enhancement of the dynamic range of the target and dynamic resolution of the input. Such a strategy could be implemented to improve the dynamic properties of designer cells and generate efficient cellular therapies.

Advancements in the design of genetic control systems rely on the development of tools to sense inputs from a wide range of biomolecular species. The rapid progress in the development of RNA sensors has not paralleled similar advances in the design of intracellular protein sensors for protein-controlled activation of programmable transcriptional responses. Remarkably, a modular intracellular protein sense–actuator device was developed by creating an intrabody, an antibody specifically designed to express intracellularly for recognition of intracellular proteins, engineered to release a transcriptional activator upon interaction with the ligand. Specifically, the intrabodies were fused to a TEV protease and to a TEV-recognition site linked to a transcriptional activator [60]. Ligand binding results in dimerization of the intrabody, which, in turn, triggers cleavage of a TEV- recognition site and release of the transcriptional activator. Because both the protein input and the transcriptional output are highly programmable due to the modularity of the construct, this system provides a plug-and-play technology for the design of cellular control systems based on intracellular protein sensors.

Genetic control systems

Current approaches to design cells that sense extracellular inputs and, in response, activate a user-defined biomolecular program, are mostly based on input-dependent transcriptional control regulated by a small-molecule-responsive repressor or transactivator. The construction of robust genetic programs for cellular therapeutics largely depends on the availability of well-characterized, reliable components. Though large libraries of genetic parts have been developed for prokaryotic species, such diversity of genetic parts is not available to the mammalian synthetic biology community, limiting the design of complex genetic networks. To address these limitations and expand the mammalian synthetic biology toolbox, a collection of orthogonal transcription factors and promoters was recently engineered from zinc-finger DNA-binding domains. This system, Composable

Mammalian Elements of Transcription, comprises an array of 56 transcription factors and associated promoters that can modulate gene expression from genomically integrated or transiently transfected transgenes over three orders of magnitude. Increasing the number of zinc-finger –transcription factor (ZF–TF) target sites enhanced target gene expression in a cooperative manner but demonstrated a propensity to sequester cellular resources at high ZF–TF expression levels, decreasing transgene expression [61].

The expression of complex genetic networks often results in resource loading, which occurs when exogenous genetic parts compete with native systems for transcriptional or translational resources, often leading to gene coupling and loss of orthogonal behavior. Key challenges in implementing synthetic gene circuits are thus the variability of cellular outcome due to context-dependent resource loading and the resulting genetic burden. For example, transcriptional activators recruit a sizable number of guanosine 5' triphosphates (GTPs) and acetyl-CoAs, resulting in the coupling of otherwise independent genes in a process known as squelching. Resource loading leads to the unpredictable behavior of synthetic gene circuits. To address this issue, the expression of genes associated with resource loading was decoupled using synthetic feedforward loop circuits. Specifically, burden-mitigation circuits were developed using an incoherent feedforward loop (iFFL), a core regulatory motif in biology. Endogenous and synthetic miRNA-mediated repression of target genes was found to reduce the coupling between coexpressed genes through an iFFL network topology [62]. This strategy has provided insights into the cellular mechanisms that result in the coupling of coexpressed genes, including genes that are part of synthetic gene networks and endogenous genes, through a common resource pool, and suggested that the decoupling observed with miRNA-based iFFL circuits results from increased availability of translational resources.

In a separate study, an endoribonuclease-based feedforward controller was engineered for robust adaptation of gene-expression levels between genes whose expression would otherwise be coupled due to the loading of cellular resources. In this system, a Cas6-family endoribonuclease CasE was utilized to knock down a target mRNA. The expression of CasE and a target gene is coupled to generate an iFFL, whereby depletion of resources leads to derepression of gene expression. This system demonstrated stable protein expression, despite changes in resource levels, including high performance across cell lines independent of DNA copy number, promoter, or transcriptional activators affecting resource loading [63].

Gene expression in mammalian cells is the product of multiple, coordinated processes guided by layers of control at different levels of biological organization, ranging from protein–protein interactions to chromatin rearrangement. As a result, it is important to build sensors of endogenous transcriptional processes that recapitulate the complexity of these processes from the native chromosomal context. To this end, a gene signal amplifier platform was developed to monitor gene expression through a signal-amplification system that recapitulates the endogenous regulatory mechanisms underlying the control of gene expression while maintaining a high dynamic resolution of the target gene activity (Figure 3) 12. An orthogonal gene network was built that links the activity of a chromosomal gene to the transcriptional and post-translational regulation of a fluorescent reporter. Transcriptional control was used to modulate reporter output sensitivity, and post-translational control

(through the NanoDeg technology) to ensure temporal resolution of the gene-expression dynamics. This gene amplifier platform holds great potential for engineering genetic control systems for a variety of applications ranging from fundamental synthetic biology studies of common design challenges such as silencing and other epigenetic modifications that affect the expression of large genetic circuits integrated into the genome, to the design of cell therapies that sense transcriptional signatures associated with disease markers.

Conclusions

Mammalian cellular therapies designed to sense and respond to biological inputs with programmable genetic outputs have continued to grow worldwide. While current products mostly rely on receptor-based control mechanisms, emerging technologies for the design of genetic and post-translational control systems provide unique opportunities to expand the synthetic biology toolbox for creating cellular therapies that would overcome the need for cell-surface receptor and their potential lack of specificity and enable the design of cellular devices that operate over therapeutically relevant timescales.

Genetic control systems are based on strategies for the detection and modulation of transcriptional as well as translational mechanisms, which can be combined to improve the system dynamics and achieve more stringent control. Implementing such control systems can improve the safety profile of therapeutic approaches requiring narrow therapeutic windows. Advancements in synthetic biology have generated efficient methods for introducing large DNA payloads into human as well as laboratory designer cell lines through transposon-based integration technologies and engineered CRISPR/Cas9 systems. This growing mammalian synthetic biology toolkit of genetic controllers holds great promise to improve the design of strategies for optimizing the performance of cellular therapies for applications requiring precise temporal and spatial control of drug delivery for the treatment of a diverse range of diseases.

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Data Availability

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

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Papers of particular interest, published within the period of review, have been highlighted as:

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•• of outstanding interest

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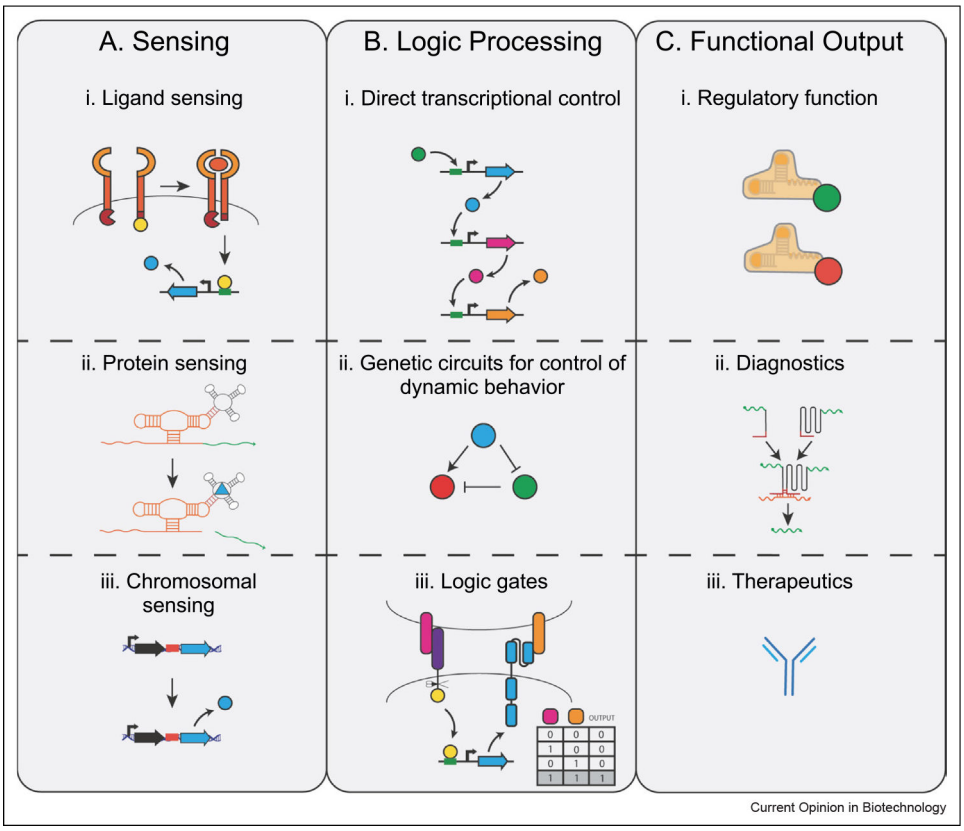

Figure 1.

Engineering cells to sense and respond requires the integration of cellular modules that mediate input sensing, input processing, and input translation into a functional output. **(a)** Cellular sensors typically depend on small-molecule or ligand-based interactions, intracellular protein sensors, and gene-activity systems. Most cell-surface sensors are based on (i) synthetic cell-surface receptors that can be programmed to sense either surface ligands (e.g. SynNotch) or soluble molecules (e.g. MESA and GEMS) and produce a transcriptional response through the generation of cleavable transcription factors. Such receptors are highly modular, as the input–output relationships can be rewired by programming the input sensing domain and the output transcription factor. (ii) Protein sensors are highly modular systems that allow programming transcriptional outputs in response to a diverse range of protein ligands. Hammerhead ribozymes (shown as an example) can be fused to a protein-sensing aptamer domain that upon ligand binding activates the ribozyme and enables translation of a target mRNA. (iii) Gene signal amplifier circuits enable monitoring gene activity from the native chromosomal context and are typically developed by editing the chromosome to link the expression of a gene of interest to a user-defined output such as a transcription factor or a fluorescent reporter. **(b)** Cellular units for processing inputs from cellular sensors rely on the design of (i) transcriptional control systems that regulate the target expression through direct transcriptional control or cascades, (ii) genetic circuit topologies for achieving the desired output dynamics (an oscillatory circuit is shown as an example), and (iii) logic gates for integrating multiple inputs. **(c)** User-defined outputs include (i) regulatory proteins, such as synthetic transcription factors (e.g. dCas9 effectors), (ii) diagnostic signals (e.g. activation of

a trans-cleaving ribozyme), and (iii) therapeutic programs (e.g. expression of a monoclonal antibody).

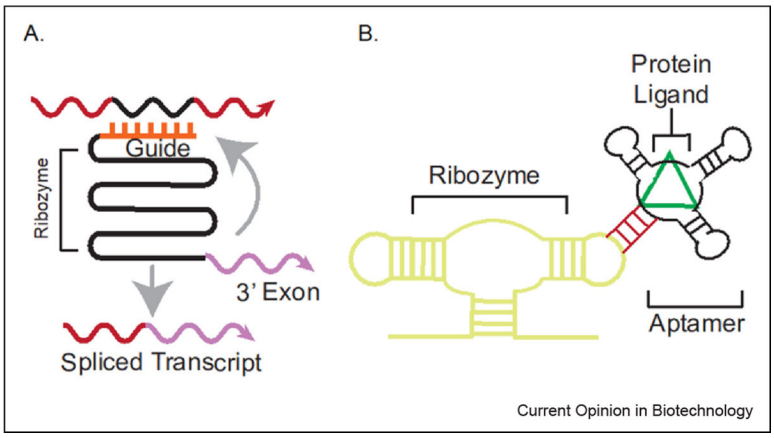

Figure 2.

RNA-based devices for genetic control systems. **(a)** RNA-sensing *Tetrahymena* ribozyme: complementation of the guide RNA (orange) with the target mRNA transcript (red/black) directs the ribozyme to the target mRNA. The guide RNA forms a scaffold with target mRNA, triggering a splicing reaction *in trans*, resulting in the replacement of the 3' end of the target transcript (red) with a user-defined 3' exon (purple). **(b)** Protein-sensing hammerhead ribozyme: a hammerhead ribozyme (yellow) fused to an aptamer (black) is embedded within the 3'UTR of a target mRNA. The binding of a protein ligand induces a conformational change in the aptamer that, in turn, is transmitted through a communication module (red) to the ribozyme, switching the ribozyme to the active state and enabling translation of the target mRNA.

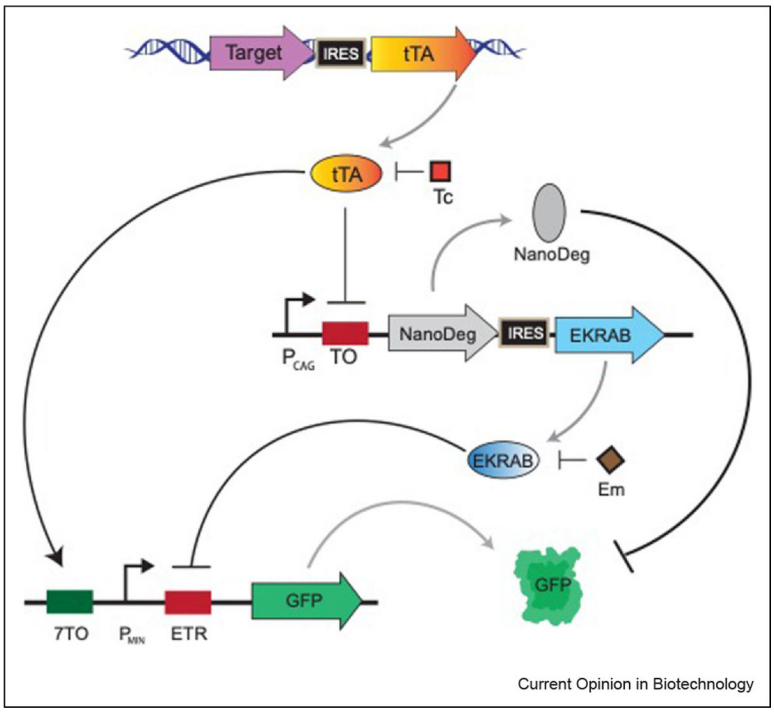

Figure 3.

A gene signal amplifier for sensing the activity of chromosomal genes. The gene signal amplifier circuit allows monitoring the activity of a target gene by linking the expression of the target gene from its native chromosomal context to that of a master transcriptional regulator (i.e. tetracycline-controlled transactivator [tTA]) through an internal ribosome-entry site (IRES), such that the transcriptional regulator is under the same transcriptional and post-translational control as the target gene. Specifically, the chromosome is edited to insert an IRES and the gene encoding the transcriptional regulator downstream of the target gene. The transcriptional regulator activates the expression of a fluorescent reporter and represses the expression of negative transcriptional (i.e. erythromycin-dependent transrepressor [EKRAB]) and post-translational regulators (i.e. NanoDeg) of the reporter. The two layers of negative regulation endow the circuit with superior signal amplification and dynamic resolution of the input compared with circuit topologies based on direct activation of reporter outputs or reporter output with reduced half-life. P_{CAG}, CMV early enhancer/chicken b-actin promoter; TO tetracycline operator; P_{MIN} minimal promoter; ETR erythromycin operator.