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# RNA-based controllers for engineering gene and cell therapies

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Engineered RNA-based genetic controllers provide compact, tunable, post-transcriptional gene regulation. As RNA devices are generally small, these devices are portable to DNA and RNA viral vectors. RNA tools have recently expanded to allow reading and editing of endogenous RNAs for profiling and programming of transcriptional states. With their expanded capabilities and highly compact, modular, and programmable nature, RNA-based controllers will support greater safety, efficacy, and performance in gene and cell-based therapies. In this review, we highlight RNA-based controllers and their potential as user-guided and autonomous systems for control of gene and cell-based therapies.

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## Introduction

Over the last decade, advances in genome engineering, viral vectors, and stem cell biology have massively expanded the therapeutic potential of engineered cells. An expanding array of cell-based therapeutics is entering clinical trials for treatment of cancers [1–3], neurodegenerative disease [4], diabetes [5,6], and blood disorders [7,8]. Achieving the best safety and performance for gene and cell-based therapies requires tools for tuning, tailoring, and controlling expression of transgenes. Tapping into information-rich streams within the cell provides the potential to coordinate synthetic and native genetic programs and to build both autonomous and clinician-mediated control of gene and cell-based therapies. As a highly compact, modular, portable, and programmable substrate, RNA serves an ideal medium for interfacing native and synthetic programs to extract information and to program cellular responses for optimal control of gene and cell-based therapies (Figure 1).

## RNA as a highly compact, modular, portable, and programmable regulator

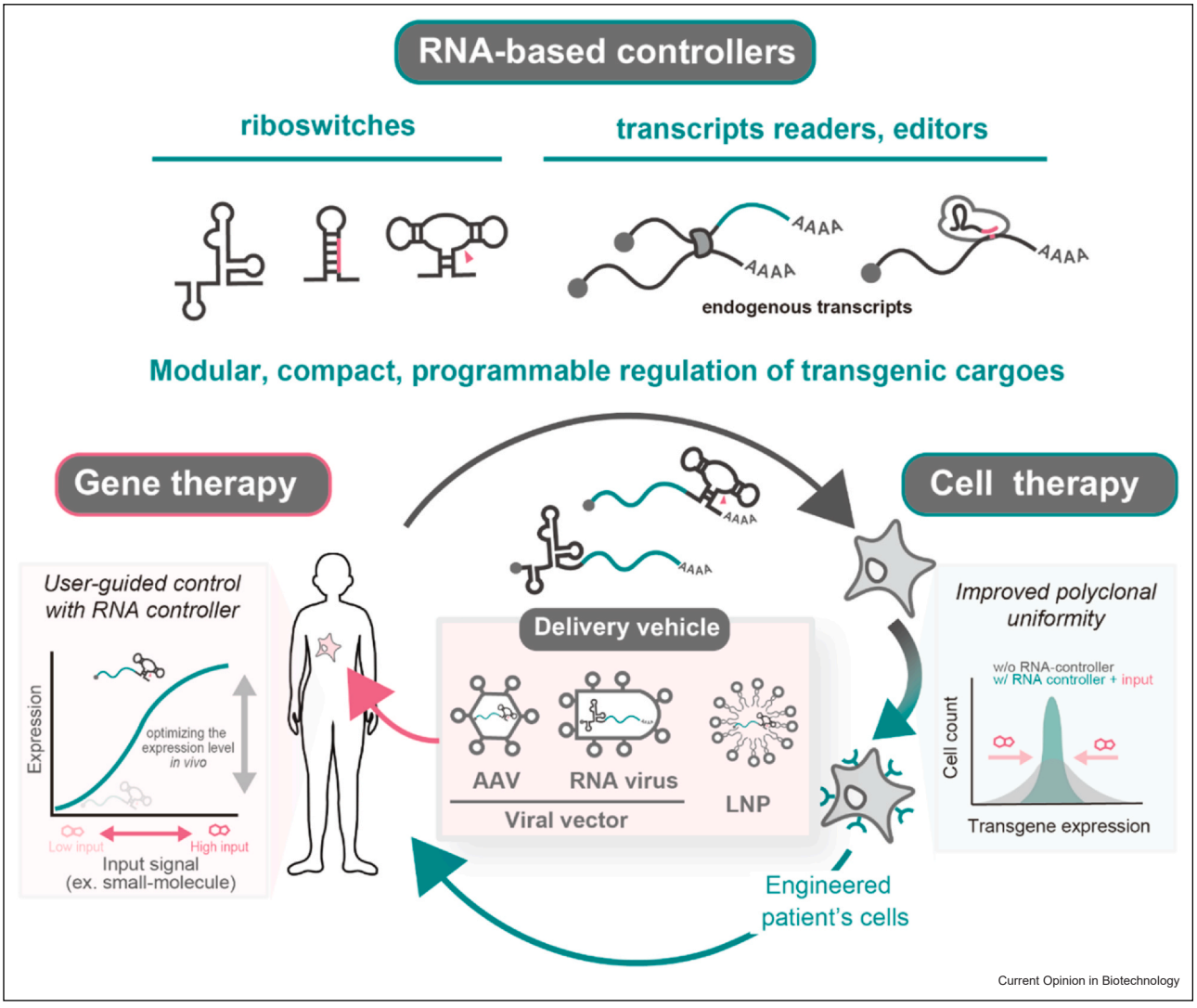
Over the last two decades, the expansion of synthetic biology has fueled the engineering of novel RNA-based devices and systems for regulating gene expression [9–24]. RNA-based genetic tools offer unique properties for building control into gene and cell therapies. RNA-based devices offer fast, compact, and modular gene regulation that is programmable. Importantly, RNA-based devices are typically small at a size of hundreds of nucleotides [25,26], which enables incorporation with transgenes and DNA-based regulators with negligible impact on delivery and integration efficiency in recipient cells. Additionally, the mechanism of regulation and small size makes RNA controllers compatible with a range of delivery methods, including nonintegrating viral vectors [25,27–30]. As many RNA control systems do not rely on auxiliary proteins, RNA-based systems can offer control without production of non-native proteins that may trigger an immune response through antigen presentation. Thus, RNA-based systems offer minimal immunogenicity compared with protein-based systems.

## Ligand-responsive riboswitches for user-guided control of transgenes

Riboswitches are biochemically responsive gene switches that are composed of RNA. Formally identified two decades ago, native riboswitches enable bacteria to sense and respond to a diversity of metabolites by modulating transcript stability or translation rate to induce changes in gene expression [31–34]. Riboswitches consist of three modules: (1) an aptamer domain that binds the small molecule, (2) an actuator for RNA processing, and (3) a communication module that transmits information between the aptamer and the ribozyme (Figure 2) [14,35,36]. Often located in the 5' untranslated region in bacterial genes, native riboswitches can sequester the ribosome binding site in response to metabolite binding to reduce expression. Alternatively, metabolite binding to RNA can induce catalytic cleavage and degradation of the transcript [37,38].

The modular nature of riboswitches supports engineering of novel riboswitches via integration of aptamers that

**Figure 1**



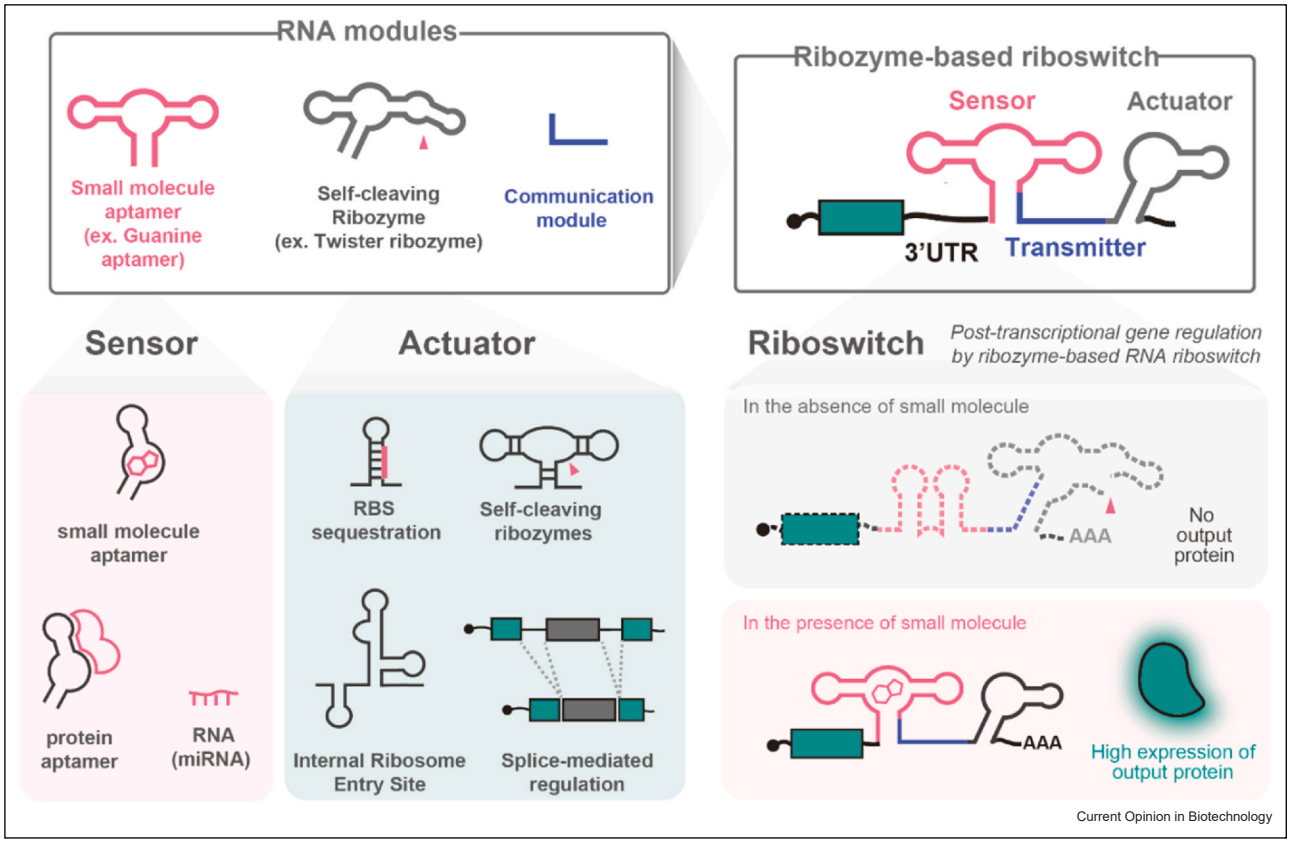

RNA-based tools and their application to gene and cell therapy. Since RNA-based tools are compact in size compared with DNA- and protein-based tools, RNA-based tools can be easily integrated into various types of genetic delivery methods. Thus, RNA-based tools are highly compatible with gene- and cell therapy application.

serve as 'sensors' with 'actuators' (Figure 2). Actuators transmit molecular binding events at the sensor into changes in RNA activity that manifest as changes in gene expression. Engineered riboswitches use a diverse array of mechanisms of RNA regulation (Figure 2).

Binding of metabolites to trigger riboswitches is mediated by aptamers, RNA sequences that offer precise molecular discrimination through structured binding [39]. These structure-dependent RNA tools can be easily programmed through varying the nucleotide sequence. For example, RNA aptamers, which bind with their corresponding target molecules, have been used as molecular sensors and can be engineered to bind with a specific target molecule (e.g. theophylline, tetracycline, guanine, and folinic acid) by changing the sequence of binding domain. Systematic Evolution of Ligands by Exponential Enrichment is a method used to engineer novel RNA aptamers responsive to various types of chemical or biological inputs. An increasing number of molecules, including small molecules, RNAs, and proteins, can be detected by aptamers [39,40].

One such RNA controller, the ribozyme-based riboswitch, regulates gene expression in response to small molecules, RNAs, and proteins [17,26,28,41]. Engineered RNA-based genetic controllers such as ribozyme switches control gene expression post-transcriptionally. Binding of a cognate ligand increases or decreases transcript stability, resulting in increased or decreased gene expression [28].

**Figure 2**




Modular composition of riboswitches using sensor and actuator modules. Riboswitches are composed of sensors and actuators that interact through the communication module (transmitter). At the right, a ribozyme-based riboswitch in the 3' untranslated region degrades the eukaryotic transcript by removal of the poly-A (AAA) tail. Addition of the small molecule reduces ribozyme cleavage, stabilizing the transcript and increasing output.

As ribozyme switches are generally small and do not require protein cofactors, these elements have been used to control gene expression from DNA and RNA viral vectors [29,42]. Theophylline-responsive ribozyme switches were able to control overexpression of a cytokine, enabling ligand-inducible expansion of T cells engineered with chimeric antigen receptors [43]. In a different approach, ligand-responsive microRNA-based controllers targeted native cytokines to mediate expansion of T cells [44]. Riboswitches such as ligand-responsive ribozymes rely on relatively small differences in binding energetics to bias RNA folding toward inactive or active states. Owing to the need to switch between folded states, the dynamic range of these devices remains small and thermodynamically limited.

Alternative mechanisms of RNA regulation that allow greater signal amplification may improve switch range. For example, splice-based riboswitches offer large dynamic ranges [45]. In splice-based switches, the riboswitch is located within an intron in the pre-mRNA. The switch adopts different conformations in the presence or absence of guanine. These conformational states influence the pre-mRNA splicing process to dictate which combination of exons is included in the mature mRNA. The splicing switch may achieve dynamic ranges that are 5–10-fold higher than similar ribozyme-based switches [46–48].

## RNA-responsive RNA controllers for detection of transcriptional state

RNA-responsive controllers allow for detection of endogenous transcripts. Recently, the RNA-responsive controllers have expanded with the advent of Cas13-mediated systems [49,50], adenosine deaminases acting on RNA (ADAR) systems [9–11,51], eToeholds [12], and split ribozymes [13]. Detection of endogenous transcripts enables RNA controllers to actuate changes in response to specific transcriptional profiles. In sensing transcriptional profiles, cells can respond to diverse

states, cues, disease profiles, and events. Cas13 uses a guide RNA to selectively identify RNA species. Active Cas13 targets RNA for degradation. Alternatively, an inactive Cas13 can be used to recruit the ADAR enzyme for editing of the target transcript [49]. More recently, ADAR-based systems have used specific synthetic and endogenous transcripts that trigger activation of a transgene via ADAR-mediated editing [9–11,51]. In the presence of the target RNA (e.g. the desired molecular trigger), ADAR induces editing of a stop codon, allowing translation of the regulated transgene. While native and synthetic RNAs can serve as trigger species, robust activation requires expression of the trigger transcript to be relatively abundant as the editing frequency remains low. To solve the challenge of limited dynamic range from low editing efficiency, a feedback loop can be introduced to amplify editing and improve the dynamic range of an ADAR-based system [11]. As dynamic range continues to be a challenge for some forms of RNA-based regulation, network approaches such as the introduction of positive feedback loops may make RNA-based regulation tractable for cellular processes that require large fold changes in transgene expression.

Relying on only endogenous protein machinery, Zhao et al. engineered *cis*-acting a synthetic internal ribosomal entry site (IRES), eToeholds, that senses and responds to the presence of target RNA by altering the rate of mRNA translation [12]. The eToehold system controls accessibility of ribosomes to the translation initiation site of the mRNA encoding an output gene by integrating a short complementary RNA sequence into the IRES to form inhibitory loops. Binding between the target RNA and inhibitory loops allows activation of translation from the IRES, triggering expression of the transgene [12].

In terms of RNA sensing technology, guide RNAs provide programmable control of CRISPR-mediated gene regulation and genome editing. Hochrein et al. controlled guide RNA activity by integrating target RNA-binding motif into the gRNA and demonstrated target RNA-responsive transcriptional activation [52]. In microbial systems, Siu et al. demonstrated programmable and multiplexed gene expression regulation by using engineered gRNA equipped with toehold riboswitches integrated into gRNA scaffolds [53].

## RNA control for DNA- and RNA-based vectors

Nonintegrating viruses, including adeno-associated viruses and RNA-based viruses, represent the next generation of translational vectors for gene and cell-based therapies [29,42,54,55]. While AAVs significantly improve safety over other DNA-based viruses such as retro- and lentiviruses, concerns remain around rare, unpredictable patterns of genomic integration. Alternatively, cytoplasmically localized RNA-based viruses, which do not transit through DNA intermediates, avoid the risk of unintended genomic integration. In addition to an improved safety profile, RNA-based viruses display unique tropism, offering the potential to selectively target *in vivo* cell populations from neurons to cardiomyocytes [56]. However, a limited set of tools to regulate gene expression from RNA-based viruses has slowed the broad adoption of these vectors.

To date, a number of major viral vectors such as adenoviral and adeno-associated viral (AAV) vectors have been engineered to deliver a genetic payload, including a therapeutic transgene to mammalian cells. Ketzer et al. first used theophylline riboswitches in adenoviral vectors to control transgene expression and viral replication [57]. Similarly, riboswitch-mediated transgene expression control from adenoviral and AAV vectors has been demonstrated by Strobel et al. and Reid et al. [42,58]. Strobel et al. increased the yields of the AAV vectors. By tuning transgene expression, they were able to increase production by 23-fold using a guanine-responsive riboswitch. Reid et al. controlled the intraocular concentration of antivascular endothelial growth factor synthetic protein (Eylea), which is used as a therapeutic for wet age-related macular degeneration. This AAV vector expressed Eylea under the control of a tetracycline-responsive ribozyme and inhibited choroidal neovascularization in a mouse model of wet AMD. As demonstrated, the compactness of RNA devices allows for user-defined control of cell and gene therapies with minimal expansion of the encoded genetic cargoes.

## Future opportunities for RNA-based controllers for cell and gene therapies

RNA-based tools can improve the efficiency and safety of gene and cell therapies. Some challenges and opportunities remain for predictably engineering functions with RNA-based controllers. Computational models and chemical probing can improve our understanding of structures and dynamics [59]. Predicting the function of *de novo* RNA sequences remains challenging, limiting forward design. Machine learning techniques have already shown remarkable progress in the design of novel and desired proteins. Similarly, leveraging the power of machine learning can substantially enhance our ability to predict RNA structure and function within the intricate cellular environment [60]. Integrating known endogenous mechanisms and pathways that affect the RNA's structure and function will improve the predictive potential of machine learning models. By developing machine learning models that can account for the multifaceted factors affecting RNA behavior *in vivo*, we can improve

the accuracy of predictions and increase the reliability of RNA-based tools in gene and cell therapies.

By limiting transgene expression, RNA controllers offer a mechanism to achieve performance in polyclonal cell populations by constraining transgene expression across cells bearing different vector copy numbers. Additionally, as cell state can substantially influence transcriptional activity, RNA controllers can limit the variance introduced through cell-specific differences in transcription rate. While gene therapy necessarily relies on a control system that performs within polyclonal populations, cell therapy can allow for isolation of clones that perform the desired therapeutic functions before delivery. However, RNA controllers may accelerate cell engineering procedure by controlling for variance in gene expression and thus eliminating the need for time-intensive screening, isolation, and expansion of clones. By rejecting natural sources of noise, RNA controllers will improve the predictability and performance of designs. By ensuring robust performance, RNA controllers will support more rapid development of cell therapies and expand the sophistication and precision of gene therapies.

Harnessing the incredible potential of RNA-based controllers will open new opportunities in the future of cell and gene therapies. With the expansion of tools for the detection of native transcripts, we anticipate the development of systems that finely tune therapeutics to unique cell states, disease-specific contexts, and patient-specific genotypic variations. Moreover, RNA-based controllers offer the possibility of clinician-guided control and intervention through tunable switches and safety mechanisms such as kill switches [20,43]. As we continue to develop these tools, there is an exciting opportunity to expand the dynamic range and test their efficacy in a myriad of different contexts, further enhancing their clinical utility. While the field is advancing rapidly in detection of endogenous RNAs, protein-responsive RNA-based controllers remain somewhat limited. This limitation presents an ongoing opportunity for improvement. Harnessing the modular and adaptable nature of RNA, RNA-based controllers will expand the range and potential of cell and gene therapies.

## Data Availability

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

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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