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Article *in* Expert Review of Vaccines · January 2015

DOI: 10.1586/14760584.2015.997714 · Source: PubMed

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Synthetic biology devices and circuits for RNA-based "smart vaccines": a propositional review

Expert Review of Vaccines (SPECIAL FOCUS | RNA-Based Vaccines)

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Abstract/Summary

Nucleic acid vaccines have been gaining attention as an alternative to the standard attenuated pathogen or protein based vaccine. However, an unrealized advantage of using such DNA or RNA based vaccination modalities is the ability to program within these nucleic acids regulatory devices that would provide an immunologist the power to control the production of antigens and adjuvants in a desirable manner by administering small molecule drugs as chemical triggers. Advances in synthetic biology have resulted in the creation of highly predictable and modular genetic parts and devices that can be composed into synthetic gene circuits with complex behaviors. With the recent advent of modified RNA gene delivery methods and developments in the RNA replicon platform, we foresee a future in which mammalian synthetic biologists will create genetic circuits encoded exclusively on RNA. Here, we review the current repertoire of devices used in RNA synthetic biology and propose how programmable "smart vaccines" will revolutionize the field of RNA vaccination.

Synthetic biology is a radically new style of genetic engineering in which living organisms are "programmed" using genetic circuits to systematically engineer novel and useful biological properties. The earliest accomplishments in the field included the construction of simple genetic circuits such as oscillators [1] and toggle switches [2] in bacterial species using mathematical modeling and rational network design. Since then, increasingly more complex circuits have been engineered in prokaryotes as well as in mammalian systems using principles of synthetic biology [3-13]. This process typically involves the top-down decomposition of the high-level behavior (sensing-processing-actuation) of a genetic circuit followed by the physical implementation of the circuit via bottom-up assembly of categorized or novel biological devices with standardized functions [14,15]. The construction of synthetic gene circuits has been greatly facilitated by drastic improvements in our ability to assemble large DNA constructs as well as by the increase in the number of well characterized devices from which we can build such circuits.

By combining regulatory devices that function according to transcriptional, translational or post-translational logic, we and others have created various therapeutic circuits that operate in mammalian systems. These include circuits that selectively kill cancer cells [16], treat the symptoms of metabolic disorders [17-20], or profile allergies of people [21]. However, in contrast to the functional diversity of devices and circuits, the way in which these synthetic gene circuits have been physically encoded has lacked variation since, as of today, only DNA-based delivery platforms (plasmid DNA or DNA-based viral vectors) have been used. From the perspective of gene-based therapy, this presents a huge conundrum as DNA-based delivery has many limitations including possible mutation of the host genome due to vector integration, epigenetic silencing of the DNA construct, and the necessity of the DNA vehicle to overcome the nuclear barrier for gene expression in non- or slowly dividing cells, a particularly difficult challenge in an *in vivo* setting [22].

More recently, mRNA-based approaches have become increasingly popular as an alternative to DNA-based methods. We have previously shown that unlike DNA, which needs to enter the nucleus, mRNA offers immediate expression of a protein of interest even in non-dividing cells [23]. Furthermore, the transient nature of the vector and the extremely low risk of insertional mutagenesis make RNA a safer alternative to DNA. With the recent development of modified RNA-based expression strategies [24] and

rapid advances in replicating RNA technologies [25-27], we anticipate the arrival of a new era of mammalian synthetic biology in which synthetic gene circuits will be encoded on RNA rather than DNA.

An attractive area of application for such RNA circuits is the emerging field of RNA vaccination. While RNA-based vaccines are completely synthetic, provide compositional control, and cost five to ten times less to manufacture than protein-based therapeutics [24], the creation of effective and universal nucleic acid-based prophylactic solutions is still challenging. Additionally, researchers aim to create vaccines that would simplify the process of immunization and increase accessibility around the globe by offering effective one-shot injections, as booster injections can pose a challenge to communities with limited means of access to vaccination clinics. We propose here that "smart vaccines" with programmable adjuvant expression and prime-boost behavior could provide a solution to these problems.

With this in mind, the purpose of this review is to accomplish the following two objectives. First, we would like to introduce to the vaccine community the concept of synthetic gene circuits and how they could help create more effective vaccines with sophisticated programmable behavior. Second, we would like to challenge the mammalian synthetic biology community to engineer sophisticated gene circuits for vaccination by using the emerging modified or replicating RNA technologies. Towards this aim, we begin this review by providing a brief overview of the rapidly developing therapeutic mRNA platforms. Then, in the rest of the review, we examine the different categories of regulatory devices that can be used to create RNA encoded gene circuits. Finally, after a brief discussion of how regulatory devices can be composed into genetic circuits, we conclude by proposing specific ideas for "smart vaccines" with programmable RNA circuits inside. We hope this review will help establish a new paradigm for vaccine development in which immunologists will conceive ideas for "smart vaccination" strategies, and synthetic biologists will implement them in the form of gene circuits using the rational design principles of synthetic biology (as conceptualized in **FIGURE 1**). While a comprehensive discussion of the basic concepts of immunology or vaccine/adjuvant design is beyond the scope of this review, for those readers that are not specialists in these fields, we recommend the following articles that methodically cover these issues: [28-30].

mRNA platforms: modified and replicating

Modified mRNA and replicating mRNA are two of the most promising platforms on which therapeutic circuits may be encoded. One of the challenges that must be overcome when using such mRNAs for gene expression in mammalian cells is the antiviral innate immune response (i.e. activation of the interferon (IFN) and NF-κB pathways). mRNAs transfected into mammalian cells are subject to detection by pattern-recognition receptors (PRRs) such as the endosomal toll-like receptors (TLRs) TLR3, TLR7, and TLR8 and the cytosolic RIG-I-like receptors (RLRs) RIG-I, MDA-5, and LGP2 [31]. These sensors are involved in the recognition of RNA species that are "non-self" (e.g. viral RNA). Stimulation of these receptors leads to activation of the IFN and NF-κB signaling pathways and subsequent translation inhibition by protein kinase R (PKR), mRNA degradation by ribonuclease L (RNase L), inflammatory cytokine expression and programmed cell death. The innate immune response is particularly problematic when carriers such as cationic liposomes or polymers are used for the delivery of mRNAs into cells (for a recent review on nucleic acid delivery methods see [32]). Carrier-mRNA complexes, which have a net positive charge, bind the negatively charged cell membrane through electrostatic interactions and are subsequently taken up into endosomes via endocytosis, where the mRNAs are sensed by TLRs. Depending on the efficiency of the carrier, this may result in a very strong innate immune response. In contrast, when physical mRNA delivery methods such as electroporation or the gene gun approach are used, the mRNA does not encounter endosomal TLRs, and thus, the innate immune response may be less severe compared to when chemical carriers are used. However, the induction of an innate immune response is still a major concern in cells that are known to possess high levels of PRRs such as epithelial cells as shown by us and others [33,34]. This problem has now been largely solved by the pioneering research of Kariko and colleagues which demonstrated that the immunogenicity of mRNA molecules could be greatly reduced by the incorporation of base modifications such as pseudouridine (Ψ) into the mRNA [35]. Kariko and colleagues showed that mRNAs with Ψ can evade PRRs, activate PKR less, and are more resistant to RNase L [35-38]. Subsequently, others followed suit and identified other combinations of base modifications that provide similar types of effects [39,40] as described in **FIGURE 2A**. For the purpose of RNA vaccination, however, some level of innate immune activation may be beneficial to induce a potent adaptive immune response.

While cellular antiviral pathways have evolved into very complex innate immune signaling networks [31], viruses have also developed a myriad of sophisticated counterstrategies to dampen the IFN response or to avoid being recognized by the host cell [41]. Thus, RNAs derived from viruses provide another attractive option for a therapeutic platform. In particular, the RNA "replicon" approach in which non-essential structural proteins (but not RNA replicase proteins) are deleted from the genome of the virus and replaced with a gene of interest has gained popularity as a safe and robust mean of exogenous protein expression [42]. Major advantages of the RNA replicon approach include its strong expression level and long duration of expression due to its "self-replicating" properties. As an example, the mechanism of replication of an alphaviral RNA replicon has been depicted in **FIGURE 2B** (for review see [43]). Geall and colleagues recently showed that gene expression from alphaviral RNA replicons can last for at least seven weeks *in vivo* when replicon RNA was packaged in lipid nanoparticles and injected into the muscle of mice for vaccination [44]. Other groups have successfully used alphaviral replicons for the purpose of iPS cell reprogramming [45] or even *in vivo* artificial miRNA delivery [46] demonstrating their potential as a broad-purpose gene expression vector. More recently, to facilitate the use of alphaviral replicons as a platform for synthetic gene circuit engineering, our group created a mathematical model for *Alphavirus* gene expression kinetics using high-density time course data [47].

In **TABLE 1**, we summarize the differences in the properties of the non-replicating and replicating mRNA platforms discussed above. Strategies to improve protein expression from an mRNA molecule by optimizing the 5' cap, poly(A) tail, sequences of the 5' and 3' untranslated regions (UTRs), the sequence of the open reading frame (ORF), or by using advanced RNA purification methods have been recently described elsewhere [24] and will not be discussed here.

We would like to emphasize that one mRNA platform is not generally better than the other, and the specific application of interest will ultimately determine which platform to choose to bring out the maximum potential of RNA-based synthetic gene circuits. In the following section, we describe in detail the types of regulatory devices that can be encoded on these RNA platforms to create RNA-based gene circuits for "smart vaccines."

Devices for post-transcriptional gene regulation

Devices that can be used in RNA-based genetic circuits include: RNA binding proteins (RBPs), synthetic riboswitches, devices that modulate the RNAi machinery, devices that modulate protein stability and devices that sense the environment (see **TABLE 2**). Some of these devices, including a few widely used RBPs and their cognate binding motifs, were transferred from other species (e.g. phage, archaea and bacteria) in their original form into mammalian systems, whereas others such as aptamers were engineered from scratch. The majority of these devices function by inhibiting translation initiation or inducing RNA degradation. However, other devices may regulate splicing, modulate innate immune activation, control protein stability or act as an interface module between the environment and other regulatory devices. **FIGURE 3** provides a summary of the representative mechanisms by which these devices function.

Apart from the advantages discussed above, post-transcriptional devices have additional benefits such as their fast response time (they directly modulate the expression of proteins) and their resource-friendliness (they bypass the use of cellular metabolites and energy involved in transcription). Furthermore, RNA-based devices can be versatile compared to DNA as they can carry the information of a protein output as well as form three-dimensional structures with enzymatic activities [48] or even rearrange into higher order assemblies [49]. However, general disadvantages of RNA include its inherent instability and immunogenicity, although moderate levels of innate immune stimulation by the RNA may be beneficial for certain applications such as cancer vaccination. In the following sections, we discuss in more detail the properties of these RNA encoded devices and how they have been used to regulate RNA related processes.

RNA binding proteins

Many RNA binding protein (RBP)-based devices discussed in this section function by inhibiting translation initiation. Eukaryotic translation initiation is an elaborate process in which various proteins/riboprotein complexes including the eukaryotic initiation factors (eIFs), poly(A)-binding protein (PABP) and the small and large ribosome subunits act in concert to facilitate the assembly of the 80S (ribosome) translation initiation complex over the initiation codon of an open reading frame (ORF) of a gene (reviewed in [50,51]). Briefly, this process begins by the recruitment of the eIF4 complex (eIF4E/eIF4G/eIF4A) to the

m7G 5' cap of an mRNA (a process facilitated by PABP), followed by binding of the 43S pre-initiation complex (40S small ribosomal subunit + tRNAi^{Met}) to eIF4. The eIF4 complex then unwinds the secondary structure of the 5'UTR of the mRNA as the 43S pre-initiation complex scans the mRNA sequence in the 5' to 3' direction until it reaches the initiation codon of the gene. Not surprisingly, thermodynamically stable secondary structures within the 5'UTR of an mRNA have been shown to be inhibitory for translation [52]. Thus, this provides an opportunity for RBPs to regulate translation by binding to the 5'UTR of mRNAs to prevent scanning of ribosomes through steric hindrance, secondary structure formation or both.

L7Ae

The archaeal ribosomal protein L7Ae binds with high affinity to RNA motifs known as kink-turns (K-turns) and K-loops [53,54]. L7Ae was first used to regulate translation by Saito and colleagues who inserted a K-turn motif into the 5'UTR region of a reporter gene in HeLa cells [55]. Similarly, insertion of the K-loop motif, which binds L7Ae with slightly lower affinity, can also be inserted into the 5'UTR of a gene for repression [56]. The level of repression by L7Ae can be increased by positioning the K-turn or K-loop motifs closer to the 5'-end of the mRNA or by increasing the number of motifs inserted into the 5'UTR [57]. The L7Ae/K-turn system can also be inverted to an ON switch by coupling it with the nonsense-mediated decay (NMD) pathway in which mRNAs with premature termination codons (PTCs) are rapidly degraded [58]. This ON switch was created by inserting an NMD "bait ORF" with PTCs upstream of an IRES and a reporter gene. While this mRNA is normally rapidly degraded by NMD, if the bait ORF is translationally repressed by L7Ae, then the PTCs are no longer recognized by the NMD pathway. Thus, the mRNA remains intact, and the reporter gene can be translated.

L7Ae can also be used to create interesting ribonucleoprotein (RNP) nanostructures with therapeutic potential [59,60]. Binding of L7Ae to a K-turn motif is known to bend the RNA at an angle of ~60º [61]. Saito and colleagues used this property to design an equilateral triangular RNP nanostructure containing a dsRNA circle with three K-turn motifs bound by three L7Ae proteins. Formation of the triangular structure was confirmed by atomic force microscopy (AFM) [59]. Formation of this RNP nanostructure provides enhanced stability to the RNA when incubated in serum [60]. By incorporating a fusion protein between L7Ae and a HER2 receptor affibody (a 6 kDa engineerable three-helix peptide

affinity motif) into the triangular nanostructure labeled with Alexa-647, the RNP was able to function as a detector of HER2-positive cancer cells. Finally, when the RNA strands in the nanostructure were redesigned so that three siRNA modules would protrude perpendicularly from the sides of the triangular RNP, the siRNA modules were able to undergo processing by Dicer and reporter gene expression was knocked-down in HeLa cells [60].

MS2 coat protein

The coat protein of the MS2 RNA bacteriophage (MS2-CP), in its native context, is a bifunctional protein which may exist in one of two distinct higher-order structures. When MS2-CP aggregates, it becomes the bacteriophage capsid, which functions to encapsulate and protect the bacteriophage genome. However, when MS2-CP forms an anti-parallel homodimer, it binds a stem loop region within its genomic RNA that contains the start codon of the MS2 replicase gene, thereby inhibiting translation of the gene. As expression of MS2-CP is tolerated well in eukaryotic cells, the MS2-CP/stem loop system has been used extensively in the field of RNA biology to tether and study the effect of a protein of interest on reporter RNAs (reviewed in [62]). MS2-CP is also capable of directly affecting various eukaryotic RNA processes via steric hindrance. Hentze and colleagues targeted MS2-CP to the 5'UTR of a reporter gene in HeLa cells and achieved ~16-fold repression of gene expression [63]. Repression was strictly translational as the abundance of the reporter mRNA was not affected by MS2-CP binding as shown by Northern blotting and a primer extension assay. Smolke and colleagues recruited MS2-CP to various locations within the introns of a three exon-two intron mini gene RNA and showed that the inclusion/exclusion rate of the middle exon can be increased or decreased depending on the where MS2-CP was recruited to [64]. Modulation of the splicing pattern was speculated to be due to decreased binding of spliceosome components or trans-acting splicing factors through steric hindrance or by recruitment of such factors by MS2-CP.

TetR

The *E. coli* Tet repressor (TetR) protein and the various TetR fusion proteins (e.g. tetracycline-controlled transactivator: tTA [65] and reverse tetracycline-controlled transactivator: rtTA [66]) are arguably the most

commonly used regulatory devices for creating synthetic gene circuits on DNA. Recently, the Suess group and Niles group performed SELEX (Systematic evolution of ligands by exponential enrichment [67,68]) and independently identified RNA aptamers that tightly bound TetR (K_d in the low nM range in the absence of tetracycline derivatives) [69-71]. The aptamers shared a similar stem loop structure with two stems and an inner loop. The inner loop portion of the identified aptamers contained conserved sequence motifs that were shown to directly interact with the TetR protein using in-line probing [69]. It was shown using site-directed mutagenesis that, not surprisingly, the aptamer binding domain of the TetR protein was located within the DNA binding domain of TetR (the N-terminal helix-turn-helix motif). Using rational design and functional testing, the Niles group engineered a minimal TetR aptamer that could repress translation when placed in the 5'UTR of several genes in *S. cerevisiae* in the presence of TetR [72]. Translational repression was relieved when a tetracycline derivative such as doxycycline was added to the culture media. Thus this system provides a general mechanism for small molecule regulated control of gene expression using an RNA binding protein.

PUF proteins

The Pumilio and FBF homology (PUF) proteins are a family of highly conserved eukaryotic translational regulators that play a role in a wide array of processes including differentiation, mitochondrial biogenesis, cell cycle regulation and memory formation (reviewed in [73]). In the native context, PUF proteins are recruited to the 3'UTRs of target mRNAs through their RNA binding domains (Pumilio homology domain: PUM-HD). By doing so, PUF proteins exert their effects as repressors or activators by interacting with or influencing the binding of other proteins such as decapping enzymes, deadenylases and possibly poly(A)-polymerases [73]. The RNA binding PUM-HD consists of eight α-helical PUM repeat motifs which assemble into a "half-doughnut" shaped structure [74]. PUF proteins are attractive targets for engineering due to their highly modular nature: each of the eight PUM repeats within a PUM-HD recognizes a single nucleotide base of an RNA sequence according to a simple RNA recognition "code" [75]. Thus, using this code, it is possible in theory to engineer PUF proteins that target any arbitrary eight-nucleotide RNA sequence. Wang and colleagues demonstrated the potential for using PUF proteins as targeting domains for regulation of RNA related processes by fusing them to glycine-rich splicing repressors and

arginine/serine-rich splicing activators [76]. When targeted to specific exons, these PUF-splicing activator/repressor fusion proteins were capable of promoting/suppressing exon skipping or influencing alternative splicing of reporter mRNAs in 293T cells. Strikingly, by engineering PUF-splicing repressor fusion proteins that bind to an exon within the cancer related *Bcl-X* pre-mRNA, the authors were able to facilitate splicing of the pro-apoptotic Bcl-xS isoform of the mRNA. This induced apoptosis of the HeLa, MDA-MB-231 (breast cancer) and A549 (lung cancer) cell lines. Subsequently, Wang and colleagues also fused a RNA endonuclease to a PUF protein to create synthetic RNA "restriction enzymes" [77]. Wickens and colleagues demonstrated the use of PUF-deadenylase or poly(A) polymerase fusion proteins for downregulation or upregulation of reporter/endogenous gene expression in *Xenopus* oocytes [78] and human cells [79]. Similarly, Schaffer, Kane and colleagues repressed translation of reporter genes by using PUF to cause steric hindrance or activated translation by recruitment of a PUF-eIF4E (i.e. an eukaryotic translation initiation factor) fusion protein [80]. Furthermore, by connecting eIF4E and PUF to CRY2 and CIB1 (components of a light inducible heterodimerization system) the authors were able to activate translation of a reporter gene using light. Other efforts to facilitate the use of PUF proteins as RNA devices include work from Zhao and colleagues who created a PUM repeat library for high-throughput cloning of synthetic PUF proteins [81] using Golden Gate cloning [82] and work from Rackham and colleagues who engineered synthetic PUF proteins with 16 PUM repeats to increase targeting specificity [83].

Another family of RNA binding proteins with great engineering potential is the pentatricopeptide repeat (PPR) protein family. PPR proteins are highly modular RNA binding proteins made up of an array of 2-30 modular PPR repeats. Like the PUM repeats of PUF proteins, each PPR motif can recognize a base of one nucleotide within a target RNA sequence. While the underlying RNA recognition code for PPR proteins was only recently elucidated [84,85], the potential for using PPR proteins as versatile tools for manipulating RNA has been recognized and reviewed elsewhere [86].

Synthetic riboswitches

Natural riboswitches, frequently found in bacteria, are RNA based molecular switches with a defined three-dimensional structure that undergo conformational changes upon intracellular metabolite binding

and affect the outcome of specific biological processes including transcription, translation, and RNA processing (reviewed in [87]). Unlike most other RNA based regulators, riboswitches do not require additional protein factors to sense metabolites of interest or influence downstream biological processes. Synthetic riboswitches work in a similar manner except that they have been artificially engineered by combining synthetic small molecule binding aptamers with various RNA devices such as ribozymes. Thus by creating synthetic riboswitches that respond to non-toxic exogenous small molecules, orthogonal control of RNA based processes can be achieved.

Engineering small molecule binding aptamers

RNA aptamers are short highly structured RNA motifs that can bind with high affinity and selectivity to specific ligands. Using SELEX, hundreds of aptamers that can bind to a wide variety of molecules such as metal ions, nucleotides, carbohydrates, amino acids, peptides, proteins, and antibiotics have been engineered (reviewed in [88]). However, while SELEX has been successful in discovering aptamers that bind to molecules of interest *in vitro*, very few of these aptamers can be engineered into riboswitches that function *in vivo*. Recently, Suess and colleagues compared the thermal stability and conformation of various neomycin-binding aptamers (some that are functional *in vivo* and others that are non-functional) in the presence or absence of ligand using UV melting analysis and NMR [89]. Indeed, they found that high ligand-binding affinity and thermal stability upon ligand binding is required but not sufficient for the aptamer to serve as a functional switch. Instead they showed that the functional aptamers are those that have a destabilized basal unbound state and undergo extensive conformational changes upon ligand binding. Another issue related to the use of aptamers is the often cytotoxic high ligand concentration required for regulatory activity. It has been speculated that this may be due to discrepancies between the intracellular environment and the experimental conditions of SELEX [90]. For instance, folding or accessibility of an aptamer may be disrupted by RBPs inside a cell or the ionic concentrations *in vivo* may be different from SELEX conditions. Thus, ultimately, to engineer an aptamer that functions *in vivo*, functional screening must be performed in cells [91].

Non-catalytic synthetic riboswitches

Despite the challenges described above, aptamers have been used successfully to modulate cellular processes. Green and colleagues inserted an aptamer for Hoechst 33258 upstream of a beta-galactosidase reporter gene and showed that small molecule dependent repression can be achieved in eukaryotic cells using aptamers [92]. Subsequently, Pelletier and colleagues definitively demonstrated this concept in wheat germ extracts and *Xenopus* oocytes by inserting aptamers for biotin or theophylline in the 5'UTR of reporter genes [93]. Translational inhibition was due to reduced 40S ribosome complex binding as well as 80S ribosome complex assembly. Similarly, Suess and colleagues developed a synthetic riboswitch that responded to the cell permeable and non-toxic small molecule tetracycline [94]. The tetracycline riboswitch functioned in a dose and position dependent manner by blocking 43S initiation complex formation when inserted in the proximity of the cap or by blocking ribosome scanning when positioned close to the AUG initiation codon [94,95]. The strength of repression increased as more aptamers were inserted in the 5'UTR [96]. Smolke and colleagues rationally designed trans-acting RNA sequences termed "antiswitches" that hybridized to regions encompassing the initiation codon of a reporter mRNA in yeast [97]. These antiswitches contained aptamer domains and were designed so that the portion of the antiswitch that hybridizes to the reporter mRNA would only be exposed upon small molecule binding to the aptamer. They were able to engineer antiswitches that repressed reporter gene translation in the presence of theophylline or tetracycline. Furthermore, they were also able to design an "on" antiswitch that responded to theophylline in the reverse manner (repressed translation in the absence of theophylline). More recently, using a rational design approach, Ogawa showed that internal ribosome entry site (IRES) mediated translation can also be regulated with small molecules using a theophylline aptamer [98]. Ogawa accomplished this using a Plautia stali intestine virus (PSIV) IRES by first inserting an anti-IRES (aIRES) sequence within the IRES that forms an aberrant hybrid and disrupts its function. He then inserted an anti-anti-IRES (aaIRES) sequence into the IRES so that the aaIRES hybridizes with the aIRES and restores the function of the IRES. Finally, he inserted an aptamer between the aIRES and aaIRES so that in the presence of theophylline, the aIRES-aaIRES hybrid will preferentially form, thereby facilitating theophylline dependent translation from an IRES. Ogawa has also modulated a phenomenon known as "ribosome shunting" observed in certain viruses such as the cauliflower mosaic virus (CaMV). Ribosome shunting is a process by which a ribosome translates an

upstream short ORF (sORF) and is then shunted to a downstream ORF (dORF) after encountering a properly positioned rigid stem structure. By modifying the CaMV 35S RNA and replacing the rigid stem structure with a theophylline aptamer, Ogawa achieved ~14 fold induction of a reporter dORF in a theophylline dependent manner [99].

Aptamers have also been used to regulate RNA related processes other than translation in a small molecule dependent manner. Gaur and colleagues showed *in vitro* that a theophylline aptamer inserted near a 3' splice site of a pre-mRNA can inhibit splicing [100] and that one inserted near the branch point can inhibit splicing *in vitro* or *in vivo* [101] in a theophylline dependent manner. Similarly, Suess and colleagues demonstrated that a tetracycline aptamer positioned near the 5' splice site of a pre-mRNA in yeast inhibited splicing in a tetracycline dependent manner [102]. The theophylline aptamer has also been used to control the replication of the positive strand RNA virus, tombusvirus. By replacing a stem loop structure whose stability is required for replication with a theophylline aptamer, White and colleagues were able to induce replication of the viral RNA by ~10-fold using theophylline [103].

Finally, Fussenegger and colleagues created an aptamer that was a fusion between the TetR aptamer (that binds the TetR protein) described above and a theophylline aptamer [104]. This TetR-theophylline fusion aptamer enabled proper folding of the TetR aptamer portion only when the theophylline aptamer portion was stabilized by theophylline. This fusion aptamer enabled disruption of tTA mediated transcriptional activation in a theophylline or doxycycline dependent manner, by inhibiting tTA binding to the promoter of a reporter gene by blocking the DNA binding domain with the TetR aptamer (by theophylline administration) or by inducing a conformational change in the structure of TetR (by doxycycline administration), respectively. In theory, this fusion aptamer could also be used to regulate TetR mediated translational regulation of a reporter RNA using two small molecule inputs.

Aptazymes

Another type of synthetic riboswitch, which combines small molecule sensing and cleavage of RNA, is the aptazyme. Aptazymes are allosteric ribozymes that undergo self-cleavage based on whether or not a small molecule is bound to the aptamer domain. The first aptazyme was created by Breaker and colleagues who modified a minimal hammerhead ribozyme (a ribozyme which consists of an 11

nucleotide conserved core sequence flanked by three stem regions) by replacing stem II of the ribozyme with an aptamer that binds ATP [105]. Depending on the "connector" sequence between the aptamer and core region of the aptazyme, ATP binding to the aptamer either inhibited or induced self-cleavage activity presumably by causing steric hindrance or stabilizing folding of the aptazyme, respectively. However, activity of a minimal hammerhead ribozyme requires a Mg^{2+} concentration much higher than that inside a cell. Thus for intracellular operation, the full-length hammerhead ribozyme which contains additional sequence elements that stabilize folding of the structure through tertiary interactions must be used [106]. Smolke and colleagues modified a hammerhead ribozyme from tobacco ringspot virus (TRSV) satellite RNA to create such an aptazyme that could function in yeast. Theophylline or tetracycline aptamers were embedded within loop II of the ribozyme so that binding of a small molecule to the aptamer would either disrupt or facilitate the proper formation of loop II and influence folding of the entire aptazyme. The aptamer sequences were rationally designed so that the aptazyme would be turned ON or OFF upon ligand binding via "strand-displacement" or "helix-slipping" based mechanisms. Insertion of these aptazymes into the 3'UTR of an mRNA enabled small molecule induction of gene expression in yeast [107]. Subsequently, by inserting two ON or OFF aptazymes that respond to different or identical small molecule inputs in the 3'UTR of a reporter mRNA, the authors were able to regulate reporter gene expression according to AND or NOR logic using theophylline and tetracycline [108]. They were also able to induce reporter gene expression when theophylline was within a certain concentration range but not higher or lower that that range (bandpass filter). Furthermore, by simultaneously inserting two different aptamers in loop I and loop II of the same hammerhead ribozyme or by connecting two aptamers in tandem in loop II, NAND or OR logic gates were, respectively, created. More recently, Hartig and colleagues created a theophylline responsive aptazyme based on the *Schistosoma mansoni* hammerhead ribozyme that functions as an OFF switch in mammalian cells [109], and Smolke and colleagues adapted their TRSV hammerhead aptazymes to engineer T cells by expressing IL-2 or IL-15 in a small molecule dependent manner in mice [110]. Finally, most recently, Yokobayashi and colleagues created a genomic hepatitis delta virus (HDV) aptazyme OFF switch which can repress reporter gene expression ~30-fold in mammalian cells upon guanine administration [111].

RNAi modulation

Since its original discovery over two decades ago, RNA interference as a technology has transformed into one of the most predictable and effective tools to silence gene expression (reviewed in [112]). Most commonly, RNAi based silencing is induced by either delivery of small interfering RNA (siRNA) duplexes which consist of ~20-30 nucleotide long RNAs characterized by perfect base-pairing or in the form of primary miRNAs (pri-miRNAs; long single RNA molecules which contain characteristic stem loop structures) or short hairpin RNAs (shRNAs; engineered single RNA molecules which consist of minimal stem loop structures that resemble either pri-miRNAs or precursor miRNAs [pre-miRNAs] with perfectly base-paired stems) expressed from a vector. The unique stem loop structures of pri-miRNAs can be divided into four modular domains: the terminal loop, the upper stem, the lower stem, and the basal segments (5' and 3' single stranded RNA regions) [113]. Whereas siRNA duplexes are loaded directly onto the RNA induced silencing complex (RISC) with the "guide strand" retained by RISC as siRNA, pri-miRNAs and shRNAs must first undergo processing by the endogenous miRNA biogenesis machinery. Pri-miRNA stem loop structures are first recognized by the Microprocessor complex (Drosha/DGCR8) and then cleaved between the upper and lower stems to produce pre-miRNAs. Subsequently, the Dicer endonuclease recognizes the pre-miRNA structure and clips off the terminal loop region from the pre-miRNA. Finally, the miRNA duplex (typically containing a 1 bp mismatch or "bulge") originating from the upper stem region of the miRNA is loaded onto RISC, and the guide strand is selected as the mature miRNA to silence its target mRNA (reviewed in [114]).

Relatively recently, several groups have engineered regulatory devices based on aptamers, aptazymes and RBPs, or have just used small molecules to regulate gene expression by modulating shRNA or miRNA processing. Yokobayashi and colleagues replaced the loop region of an shRNA with a theophylline aptamer and showed that processing of shRNA by Dicer was inhibited upon administration of theophylline, preventing Dicer mediated generation of siRNAs in HEK293 cells using theophylline inhibited reporter gene silencing (ON switch) [115]. Subsequently, Yokobayashi's group attached a theophylline aptazyme (a hammerhead ribozyme derived from *Schistosoma mansoni* with an aptamer inserted into stem III) to the 5' end of an shRNA so that Drosha processing of the shRNA would be inhibited due to base paring in the 5' portion of the basal segment. Upon theophylline administration to

HEK293 cells expressing this aptazyme-shRNA fusion, the aptazyme cleaved itself away from the shRNA thus enabling production of an siRNA duplex and knockdown of reporter gene expression (OFF switch) [116]. Similarly, the same group attached a stem loop structure to an shRNA to prevent Drosha processing but this time dissolved the base pairing of the stem and enabled processing by transfecting a modified oligonucleotide that competes with the stem. Using this oligonucleotide induced OFF switch, they demonstrated reporter and endogenous gene knockdown in HEK293 cells [117]. Smolke and colleagues also modulated Drosha processing by inserting aptamers into the basal region of an shRNA. They showed using three aptamers (theophyline, tetracycline, and hypoxanthine) that small molecule binding to the aptamers inhibited shRNA processing by Drosha and prevented knockdown of reporter genes (ON switch) [118]. Saito and colleagues replaced the terminal loop of an shRNA with a K-turn motif and demonstrated that steric hindrance caused by L7Ae binding to the terminal loop can prevent siRNA processing by Dicer [119]. They used this ON switch to control reporter genes as well as expression of the pro-apoptotic *Bim* and anti-apoptotic *Bcl-xL* genes to regulate cell fate. Disney and colleagues used a computational approach termed Inforna to predict that a heterocyclic aromatic compound benzimidazole may bind the Drosha cleavage site of miR-96, a miRNA upregulated in cancer, and prevent processing [120]. Briefly, the Inforna pipeline uses a combination of experimentation and computation to identify RNA sequence motifs that may bind small molecule compounds of interest. The experimental part consists of a small molecule-RNA motif interaction screen, termed two-dimensional combinatorial screening (2DCS) [121]. In 2DCS, RNA hairpin structures with short randomized internal loops (e.g. six random nucleotide loops) are hybridized to small molecule ligands immobilized on an agarose microarray. Following gel extraction and sequencing of the RNA hairpins bound to a ligand of interest, the random nucleotide loop sequences are analyzed using the RNA Privileged Space Predictor (RNA-PSP) program for statistically enriched motifs by calculating Z-statistics for each motif [122]. Strikingly, when the experimentally determined binding affinities of RNA hairpin loops to a small molecule were plotted against the sum of the Z-statistics for the statistically enriched motifs identified by RNA-PSP included within that specific internal RNA loop sequence, the data points could be fit well to a simple inverse first-order equation ($R^2 = 0.85$) [123]. This method termed Structure-activity relationships through sequencing (StARTS) was then used to successfully predict the binding affinities of various RNA hairpin loops (that were not captured by 2DCS)

to the small molecule of interest. Finally, *in silico* folding of all human pri-miRNA sequences in miRBase [124] was performed using the RNAstructure program [125], and all secondary structural elements within the pri-miRNAs were extracted and queried against the 2DCS data for various small molecule compounds using StARTS or RNA-PSP v. 2.0 [120]. This Inforna platform predicted that benzimidazole would inhibit processing of miR-96. Indeed, when tested in primary cells, benzimidazole inhibited miR-96 processing by 90%. Importantly, benzimidazole inhibition of miR-96 in the MCF7 cancer cell line caused upregulation in the protein levels of *FOXO1* (Forkhead box protein O1), a target of miR-96, and induced apoptosis. This demonstrates the potential of this method for identifying drugs that could treat diseases by intervening with RNA related processes. Finally, an alternative way to modulate miRNA activity has been described previously by Sharp and colleagues who showed that miRNA target sites themselves (with perfect or mismatch complementarity to the miRNA), when overexpressed, could act as "sponges" that titrate away endogenous mature miRNAs and prevent them from degrading their exogenous or native RNA targets [126].

Post-translational regulatory mechanisms

Synthetic biology devices for RNA vaccination need not directly act on RNA but may function at the post-translational level. Post-translational devices are capable of actuating even more rapidly than devices that regulate RNA. An example of such a device is the destabilizing domain (DD) developed by Wandless and colleagues [127]. A DD is comprised of a small-molecule ligand binding domain and a degron domain, which targets proteins for degradation in an ubiquitin and proteasome-dependent manner. When a DD is fused to a protein of interest, the half-life of the protein is dramatically decreased. However, binding of a ligand to the DD induces a conformational change that masks the degron thus preventing subsequent ubiquitination and degradation. (De)stabilization occurs in a reversible manner, and the stability of the protein can be tuned by adjusting the concentration of the cognate ligand. The first DD developed (referred to here as DDf) was based on the human FKBP12 protein and was stabilized by a synthetic small molecule Shield-1 [127]. When a vaccinia virus harboring a fusion protein between DDf and the cytokine IL-2 was systemically delivered to tumor bearing mice, administration of Shield-1 to the mouse led to stabilization of IL-2 and a reduction in the size of the tumor [128]. Later, DDs that respond to the

FDA-approved small molecule drug trimethoprim (TMP) and 4-hydroxytamoxifen (4-OHT; the active metabolite of another FDA-approved drug, tamoxifen citrate) were engineered using *E. coli* dihydrofolate reductase (DDd) and human estrogen receptor (DDe) [129,130]. Since TMP can traverse the blood-brain barrier, DDd-fluorescent reporter proteins delivered to the brain of a rat using lentiviruses were capable of being stabilized by TMP administration. Thus, there is potential for using this system for clinical applications related to the brain. More recently, using the same FKBP protein, Wandless and colleagues developed a ligand-induced degradation (LID) domain, which operates in the opposite manner as a DD [131]. LIDs induce degradation of a protein by exposing a cryptic degron upon binding of a ligand. DDs and LIDs are useful devices for simple protein (de)stabilization. However, another way to use these domains would be to fuse them to RBPs such as L7Ae, TetR and MS2 to regulate translation in a small molecule dependent manner as proposed later.

Sensor modules

Biological sensor modules sense endogenous or environmental signals such as small molecules, proteins, miRNAs, mRNAs, or enzymatic activity and relay information to other devices within a circuit. Thus, sensor modules are the interfaces between input signals and insulated processing modules of a circuit. For instance, Fussenegger and colleagues have developed sensor modules which use G protein-coupled receptors (GPCRs) to detect small molecules such as dopamine [20] and histamine [21] or changes in the pH [132] and communicate this information to downstream actuation devices through the cAMP signaling pathway. Smolke and colleagues adapted their MS2-CP based splicing modulation device described above to sense the p50 or p65 subunits of NF-κB or the β-catenin protein of the Wnt signaling pathway [64]. However, since these sensors are connected to processing modules which actuate through transcription of a transgene (for GPCR/cAMP signaling based sensors) or splicing, they cannot be directly integrated into RNA encoded circuits (at least in their current form).

In contrast, one type of device that can be easily embedded into an RNA encoded circuit is a miRNA sensor. The basic unit of a miRNA sensor consists of a miRNA target site inserted into the 3'UTR of an mRNA. Using this simple setup, Naldini and colleagues demonstrated the proof of concept that tissue specific miRNAs such as miR-142-3p could be exploited to suppress gene expression in

undesirable cell types using a lentiviral gene therapy vector in mice [133]. More recently, tenOever and colleagues applied the same concept to modulate the host tropism of an influenza A virus [134]. They incorporated into the viral genome a target site for a miRNA (miR-192) that is differentially expressed in different host species so that transmission of a virus would occur in ferrets but be attenuated in mice (or humans, in theory). The concept of using RNAi for complex Boolean logic evaluation was demonstrated by Benenson and colleagues in collaboration with our group [135]. In the study, logic gates were created by incorporating up to five different siRNA target sites into 3'UTRs of two reporter mRNAs or alternatively, by incorporating siRNA target sites into lacI or lacI-KRAB fusion repressor-encoding mRNA(s) which in turn repressed a reporter mRNA. Subsequently, Benenson and colleagues demonstrated that such Boolean logic gates can similarly be implemented in mammalian cells using artificial miRNAs embedded within the introns of genes regulated by transcriptional activators or repressors [136]. Finally, Benenson and colleagues and our group created a miRNA-classifier circuit which "senses" the distinct miRNA expression pattern of certain types of cells and identifies them based on evaluation of the following Boolean logic function: miR-21 AND miR-17/miR-30a AND NOT(miR-141) AND NOT(miR-142-3p) AND NOT(miR-146a) [16]. This was implemented using a combination of six miRNA target sites regulating three distinct ORFs encoding repressors, activators, and reporter or actuator proteins. This circuit was used to distinguish a HeLa cancer cell from a HEK cell and selectively kill the HeLa cell by expression of the pro-apoptotic *BAX* (Bcl2-associated X protein) gene.

Other types of sensing devices that are compatible with RNA encoded circuits include an mRNA sensor developed by Benenson and colleagues which was based on an "RNA strand displacement" mechanism [137]. In this device, input mRNA molecules release cryptic antisense strands of siRNAs from "protecting strand" RNAs through strand exchange. This results in the generation of siRNA duplexes which are loaded onto the RISC complex to knock-down downstream target RNAs. This mRNA sensing device was used to create simple Boolean logic evaluators in Drosophila extracts. Saito and colleagues developed an shRNA based protein sensing device which could potentially be integrated into RNA encoded circuits [138]. In the study, structural modeling was used to observe the amount of steric hindrance that would be generated between Dicer and a protein of interest when the terminal loop of the shRNA was replaced by an aptamer which binds the protein of interest. Based on this information, it was

possible to predict aptamer configurations that would maximize inhibition of Dicer mediated processing of the shRNA. This design process was used to create a device that senses the levels of the NF-κB p50 subunit in 293FT cells. Finally, Covert and colleagues created synthetic sensors for kinase activity dubbed "kinase translocation reporters" (KTRs) [139]. KTRs have a modular structure which consists of a kinase docking site, a nuclear export signal (NES), and a nuclear localization signal (NLS). Phosphorylation of the NES and NLS moieties of the KTR enhances nuclear export and decreases nuclear localization activities, respectively. Thereby, KTRs sense kinase activity and communicate that information in the form of a nucleocytoplasmic shuttling event. KTRs were successfully engineered for the JNK, p38, ERK, and PKA kinases demonstrating the universality of this approach.

RNA circuits

The RNA devices discussed thus far with single inputs and outputs can be connected with one another to create modules with more complex behavior. A key aspect to consider when connecting devices is their "composability." For instance, in order to directly connect device 1 (which operates in the form of: input 1 -> device 1-> output 1) with device 2 (input 2 -> device 2-> output 2), output 1 of device 1 must be able to become input 2 of device 2. Thus, only devices with compatible inputs/outputs are considered composable. In over a decade, researchers in the field of synthetic biology have used composable devices to create numerous circuit modules including oscillators, toggle switches, and cascades. These modules can be assembled further into integrated systems with more sophisticated functions.

There are two complementary approaches by which devices can be assembled into modules and modules into systems: the first approach involves the rational matching of parts based on mathematical modeling and the other involves experimental testing of many circuit configurations by screening variations of individual parts. In actuality, gene circuit optimization cannot be accomplished solely by model-based methods and still involves a significant amount of experimental trial and error. While many sophisticated circuit modules and systems have been engineered to date, to our knowledge, none have been encoded entirely on RNA for use in mammalian systems. Although Fussenegger and colleagues used the L7Ae and MS2 devices to create "mammalian biocomputers" which perform programmable calculations based on NOT, AND, N-IMPLY, and XOR logic gates, this was done by combining

transcriptional regulation and L7Ae/MS2 based translational repression [140]. In the following section, we propose examples in which RNA devices could be composed into circuits and encoded exclusively on RNA for the purpose of vaccination.

Synthetic gene circuit ideas for "smart vaccination"

Over the years, mRNA and replicating RNA have become well established as platforms for vaccination and immunotherapy (reviewed in [25-27,141,142]). RNA based devices such as aptamers or aptazymes have also been used for immunomodulation ([110] and reviewed in [143]), cell specific targeting of antigens [144] and presentation of *de novo* antigens [145]. However, such efforts to improve vaccines/immunotherapies using RNA-based tools have thus far been limited to the use of standalone devices. Here, we propose how RNA-based "smart vaccines" with complex regulatory gene circuits inside may be used to solve unmet needs in this area, highlighting their potential as an enabling technology.

"One-shot" vaccination

The development of one-shot vaccines that do not require booster shots would be particularly beneficial in communities with limited means of transportation. Chadambuka *et al.* reported that a significant number of children (~35%) drop-out from vaccination programs in rural Zimbabwe due to transportation barriers [146]. Here we propose a "smart vaccine" solution to this problem in which prime-boost expression of an antigen can be achieved using a small molecule drug rather than a follow up injection of the antigen. This can be done as shown in **FIGURE 4**, using a replicon with two subgenomic promoters (SGPs) where one SGP expresses an RNA binding protein (RBP) fused to a DD domain and the other has a motif which binds the RBP upstream of an antigen of interest. In this circuit, administration of a small molecule drug stabilizes DD-RBP and represses translation of the antigen. Thus, a small molecule can be used to suppress antigen expression, in effect, creating the prime and boost phases of vaccination.

Multivalent cancer vaccine

Intratumoral heterogeneity (the presence of many subclones of cancer cells within a tumor that are genetically different from one another) is one of the greatest hurdles in treating cancer. For cancer vaccination, heterogeneity of the tumor and the diverse gene expression pattern of individual cancer cells are a problem since not all cancer cells within a population may be targetable by immunization with a single tumor antigen. Thus, in order to attack and clear a heterogeneous population of cancer cells, it may be necessary to perform vaccination with multiple tumor antigens. However, induction of immune responses against multiple antigens by simultaneous injection/expression of antigens may be difficult for certain combinations of proteins due to "immunodominance". Immunodominance causes CD4^{+} and/or CD8^{+} T cells to preferentially respond to the most immunogenic epitopes and leave other epitopes unattended [147,148]. Here, we propose a method to overcome this problem by creating a small molecule inducible sequential antigen expression cascade with additional adjuvant pulsing capabilities (**FIGURE 5**). This circuit is encoded on a replicon with three SGPs: the first SGP expresses DD-RBP1, the second SGP contains a binding motif for RBP1 and expresses RBP2 connected to Antigen 1 via a 2A "ribosome skipping" peptide [149] which enables co-translational separation of the antigen from RBP2, and the last SGP contains a binding motif for RBP2 followed by Antigen 2 fused to an adjuvant by a 2A peptide. In the absence of a DD-stabilizing small molecule drug, DD-RBP1 is degraded and allows expression of RBP2 and Antigen 1 (Antigen 2 is repressed by RBP2). Upon administration of the drug, DD-RBP1 is stabilized and represses RBP2-2A-Antigen 1 thereby allowing expression of Antigen 2 and the adjuvant. Here, an additional benefit of the cascade is that the potent adjuvant, which may be highly toxic when delivered systemically, is only expressed when TMP is administered to the body.

Conclusion

Roughly a decade and a half has passed since the first synthetic gene circuits created in *E. coli* launched a field of research that has now come to be known as synthetic biology. By creating and cataloging standardized genetic parts and devices that can be assembled into modules and systems for reprogramming living organisms, synthetic biologists have transformed the field of biotechnology into a rigorous engineering discipline. In particular, mammalian synthetic biology has been experiencing rapid expansion over the past few years with successful implementations of genetic circuits in cell culture as

well as in model organisms. However, we believe that mammalian synthetic biology is in fact at a crossroads. Ultimately, therapeutic gene circuit applications must graduate from the academic proof-of-concept phase and find a place in the real-world. Will mammalian synthetic biologists be able to identify society's pressing needs and deliver gene circuit solutions that can withstand the public field test? Efforts aimed in the right direction could indeed make this happen. One absolute requirement for this would be that synthetic gene circuits for therapeutic purposes be safe. Encoding genetic circuits on RNA using the emerging modified or replicating RNA-based platforms rather than DNA-based platforms will greatly facilitate this transition. Building circuits that do not trigger unnecessary innate or adaptive immune responses against regulatory components of the circuit will also be necessary. An area of particular interest for synthetic biology applications using RNA is vaccination. The proven success of antigen-encoding RNA in eliciting protective immunity combined with the desire to control the dynamics of antigen/adjuvant expression to maximize an immune response makes vaccination an optimal target for RNA circuit applications. With the ever-expanding list of parts and devices for RNA regulation and our rapidly-developing ability to rationally compose devices into regulatory circuits, it is only a matter of time before RNA "smart vaccines" with programmable antigen/adjuvant circuits inside will deliver a solution to a real-world problem: the development of potent vaccines to protect humanity from the threats of infectious diseases.

Expert commentary

While there has been a long history of creating and optimizing regulatory devices that function at the post-transcriptional level, regulatory gene circuits using such devices have only been encoded on DNA-based platforms. Towards the creation of RNA-based therapeutic circuits with improved safety profiles, synthetic biologists must start transferring post-transcriptional devices onto the emerging modified and replicating RNA platforms. Whether such devices would still work in a robust, modular and predictable manner when moved to RNA is yet to be determined. One aspect that must be kept in mind when using RNA devices from an RNA-based platform is that these devices will often end up operating on or modifying the RNA vector itself. Thus, it is highly conceivable that certain devices that function well on DNA will not be optimal for RNA and *vice versa*. With respect to device and circuit operation, despite the improvements in

computational modeling approaches, synthetic biology, to a large extent, is still largely dependent on trial and error.

Five-year view

The future will certainly bring us plenty of novel RNA-based devices and methodologies. Devices such as PUF or PPR proteins that can directly target any endogenous sequence of interest will be further developed and optimized. RNA-encoded devices that act transiently but cause a heritable change in the phenotype of a cell/organism in a precisely controlled manner at the genetic (e.g. CRISPR/Cas9 based genome engineering) or epigenetic (e.g. chromatin regulation) level will become popular. Devices that regulate or use long non-coding RNA (lncRNA)-based methods will be developed. Elaborate RNA nanostructure-based devices will find more areas of application. Circuits that interact with endogenous signaling pathways will be implemented. RNA strand displacement as a method to perform molecular computations will be further developed and optimized.

RNA platforms will also be further developed. Existing replicating RNA platforms such as alphaviral replicons will be engineered to be stealthier and even less cytopathic. Novel superior replicating RNA technologies will be developed and replace current replicon platforms. New RNA base modifications that can "program" expression and/or other properties of mRNA (for more/less expression, longer/shorter duration of expression, more/less stimulation of innate immune pathways) will be discovered/developed further. Organisms or circuits that use synthetic XNAs as novel and orthogonal carriers of genetic information will be developed.

Computational approaches will improve all aspects of RNA circuit construction. Tools to predict RNA structure, RBP binding, ribosome recruitment, translation efficiency and the extent of innate immune activation will improve and be used to guide strategies for RNA sequence optimization. Methods to more accurately predict the behavior of RNA devices will improve our ability to rationally design devices. More accurate mathematical models will allow us to assemble RNA-based circuits in a top-down manner.

Synthetic biology and genetic circuits will revolutionize the way vaccines are designed. DNA assembly technologies will greatly shorten the time that it takes to manufacture vaccines that can prevent pandemic outbreaks. Programmable personalized vaccines that modulate antigen/adjuvant expression

levels based on the individual (e.g. children, adolescents and the elderly) will be created using systems biology approaches. Antibody expressing circuits that enhance passive immunoprophylaxis will be developed. Delivery vehicles using novel materials that can better protect and deliver nucleic acid vaccines as well as synergize with the circuits encoded on them will be invented.

Finally, proven success in the field of RNA vaccination will position RNA as a superior alternative to DNA for the creation of therapeutic circuits for mammalian synthetic biology.

Financial & competing interests disclosure

T Kitada, K Bodner and R Weiss are named as inventors on a U.S. provisional patent application (62/047137 "RNA-based Logic Circuits with RNA Binding Proteins, Aptamers and Small Molecules") related to the subject of this review. Ownership of the patent, if granted by the USPTO, will be transferred to the Massachusetts Institute of Technology. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Key issues

- Synthetic biology aims to engineer living organisms with unprecedented precision, predictability and sophistication.

- Synthetic biologists have been compiling a catalog of highly modular and predictable genetic parts and devices.

- RNA binding proteins, synthetic riboswitches, RNAi modulators and protein destabilization tags are among the parts and devices that can be used for post-transcriptional gene regulation.

- Composable genetic devices can be assembled into synthetic gene circuits with complex behavior.

- Modified and replicating RNA are emerging platforms for gene delivery.

- Mammalian synthetic biologists must encode therapeutic gene circuits on RNA for increased safety.

- RNA vaccines have been gaining attention as an alternative to standard attenuated pathogen or protein based vaccines.

- RNA-based gene circuits may enable antigen or adjuvant expression to be controlled in a sophisticated manner.

- One shot prime-boost circuits or multivalent sequential antigen/adjuvant expression circuits are just a few of the many possible synthetic gene circuit applications for vaccination.

- RNA-based "smart vaccines" will revolutionize the field of vaccination by providing solutions to unmet societal needs.

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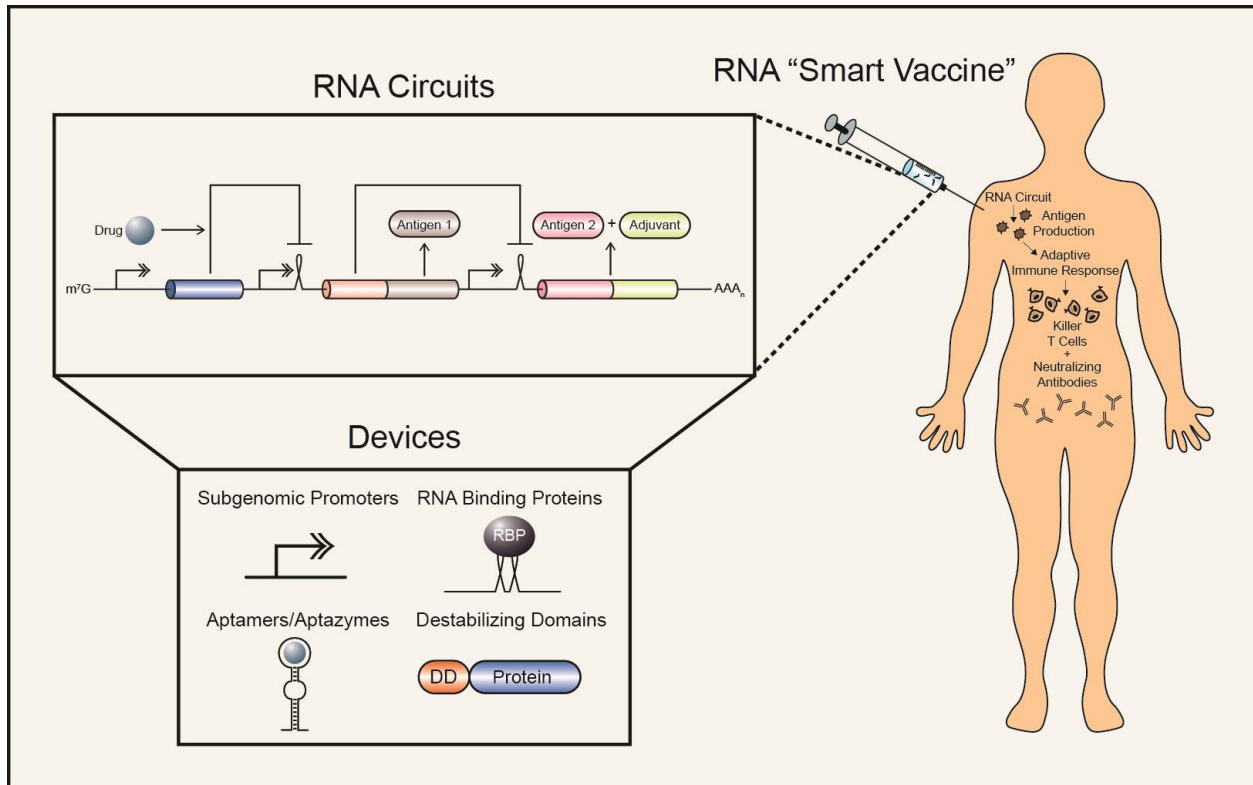
Figure 1. The RNA "smart vaccine" paradigm. Composable devices for post-transcriptional gene regulation can be assembled into synthetic gene circuits in the form of RNA. Such RNA circuits may be used to control the expression kinetics of antigens and adjuvants using small molecule drugs to create potent RNA "smart vaccines."

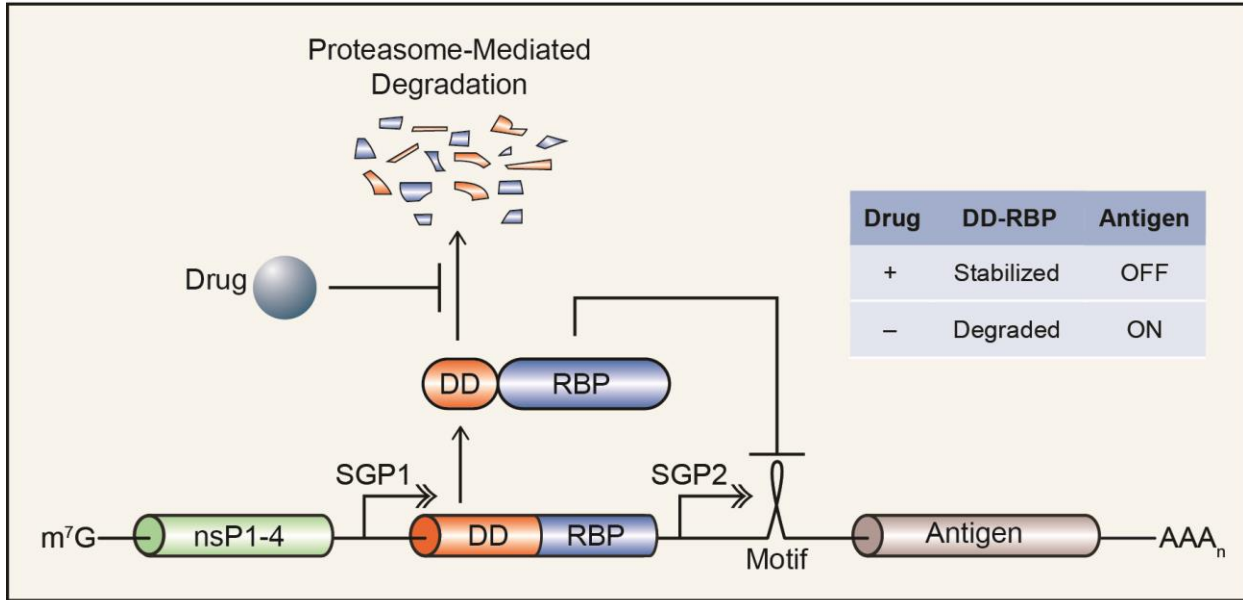m^7G: 7-methyl-guanosine; AAA_n: poly(A) tail; RBP: RNA binding protein; DD: destabilizing domain.
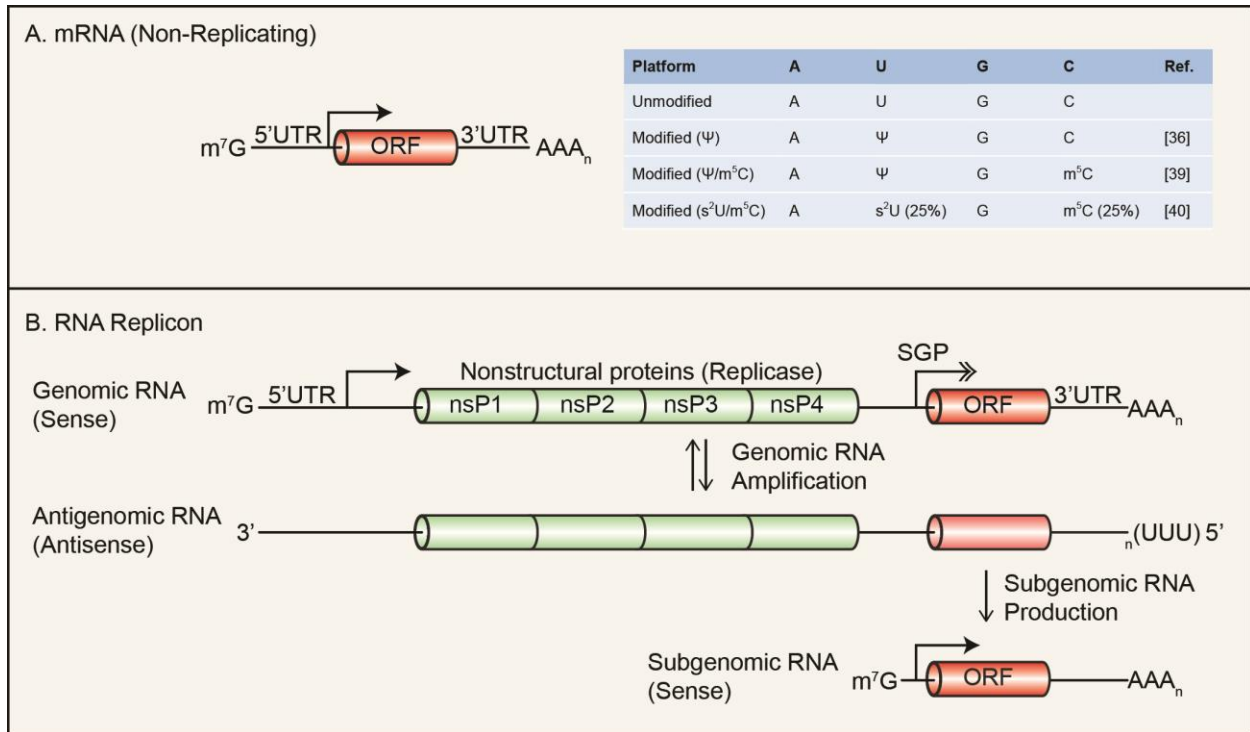
Figure 2. Structures of RNA platforms for vaccination. (A) Unmodified and modified (non-replicating) RNA structures. **(B)** Alphaviral RNA replicon structure.

m^{7}G: 7-methyl-guanosine; UTR: untranslated region; ORF: open reading frame; AAA$_n$: poly(A) tail; Ψ: pseudouridine; m^{5}C: 5-methylcytidine; s^{2}U: 2-thiouridine; nsP: nonstructural protein; SGP: subgenomic promoter.
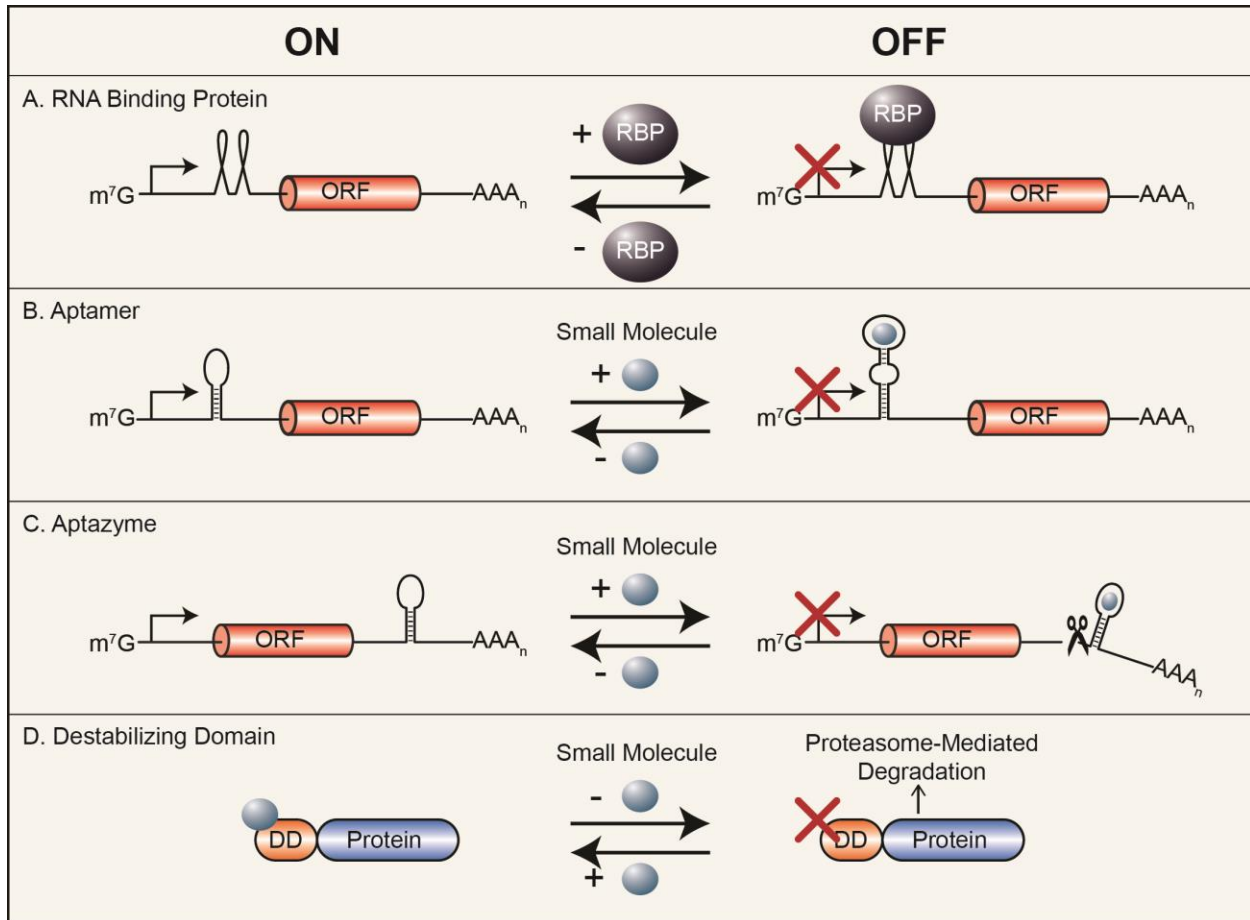
Figure 3. Devices for post-transcriptional gene regulation and their modes of action. Operation of (A) RNA binding proteins, (B) aptamers (C) aptazymes and (D) destabilizing domains.

m^{7}G: 7-methyl-guanosine; ORF: open reading frame; AAA$_n$: poly(A) tail; RBP: RNA binding protein; DD: destabilizing domain.

Figure 4. One-shot "smart vaccine" with small molecule enabled prime-boost.

m^{7}G: 7-methyl-guanosine; nsP: nonstructural protein; SGP: subgenomic promoter; DD: destabilizing domain; RBP: RNA binding protein; AAA$_n$: poly(A) tail.
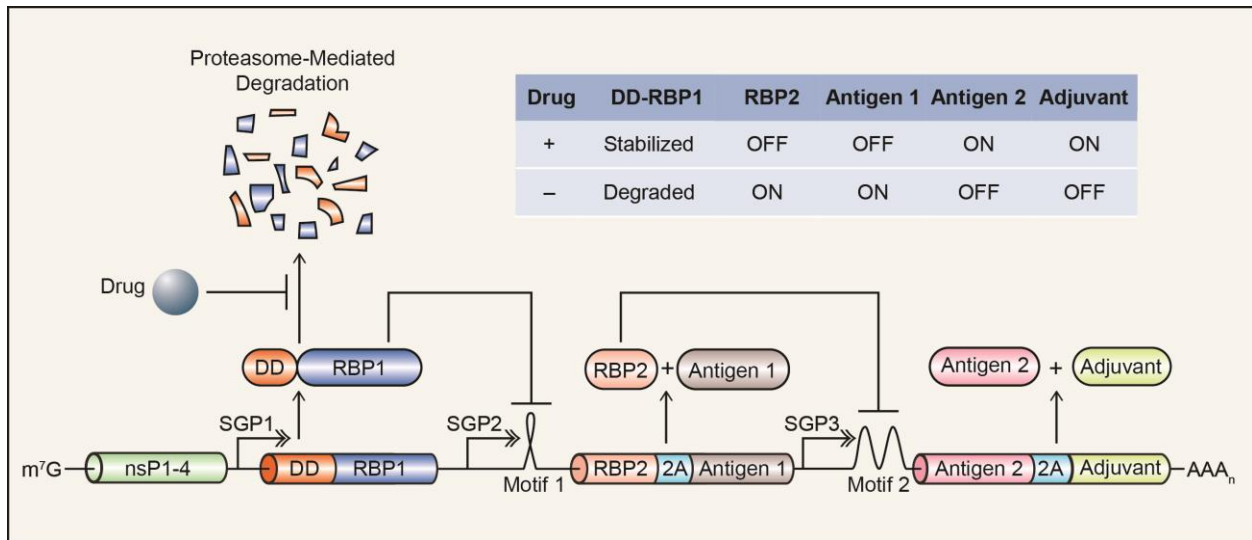
Figure 5. Multivalent cancer "smart vaccine" with small molecule induced antigen cascading and adjuvant pulsing.

m^{7}G: 7-methyl-guanosine; nsP: nonstructural protein; SGP: subgenomic promoter; DD: destabilizing domain; RBP: RNA binding protein; AAA$_n$: poly(A) tail.

Table 1. Comparison of RNA platforms for vaccination.

Platform	Size	Expression level	Duration of expression *in vivo* (i.m. injection)	Innate immune stimulation	Amplification in cells	Ref.
Unmodified mRNA	Typically > ~500 nt	Low	~1 week*	High	No	Reviewed in [25,141,142]
Modified mRNA	Typically > ~500 nt	Medium	~4 weeks*	Low	No	[36,39,40]
RNA replicon	> ~8000 nt	High	~7 weeks	High	Yes	Reviewed in [25-27]

*Authors' unpublished results.
i.m.: intramuscular.

Table 2. Devices for post-transcriptional gene regulation.

Device	Origin	Function(s)	(Potential) vaccine application	Ref.
RNA binding proteins				
L7Ae	*Archaeoglobus fulgidus*	• Translational regulation	• ON/OFF switch for expression of antigens and adjuvants	[55-58]
		• RNP nanostructure	• Immunomodulation	[59,60]
		• shRNA processing regulation	• Immunomodulation	[119]
MS2-CP	Bacteriophage MS2	• Translational regulation	• ON/OFF switch for expression of antigens and adjuvants	[63,140]
TetR	*E. coli*	• Translational regulation	• ON/OFF switch for expression of antigens and adjuvants	[69-72]
		• RNA-localization regulation	• Immunomodulation	[150]
PUF	Eukaryotes	• Splicing regulation	• Cell fate regulation of immune cells	[76]
		• RNA cleavage	• ON/OFF switch for expression of antigens and adjuvants	[77]
		• Translational regulation	• ON/OFF switch for expression of antigens and adjuvants	[78-81,83]
Synthetic riboswitches				
Aptamer	Synthetic	• Translational regulation	• ON/OFF switch for expression of antigens and adjuvants	[89,91-99]
		• Splicing regulation	• Cell fate regulation of immune cells	[100-102]
		• Viral RNA replication regulation	• ON/OFF switch of vaccine circuit	[103]
		• shRNA processing regulation	• Immunomodulation	[115,118]
		• Receptor targeting	• Immunomodulation, antigen delivery to APCs and *de novo* antigen presentation	[144,145]* and reviewed in [143]*
Aptazyme	Synthetic	• Translational regulation	• ON/OFF switch of vaccine circuit	[107-111]
		• shRNA processing regulation	• Immunomodulation	[116]
RNAi modulators (other than those listed above)				
Oligonucleotide	Synthetic	• Drosha inhibition	• Immunomodulation	[117]
Small molecule	Synthetic	• Dicer or Drosha inhibition	• Immunomodulation	[120]
miRNA sponges	Synthetic	• Endogenous miRNA sequestration	• Immunomodulation	[126]
Protein (de)stabilization domains				
DD	Synthetic	• Protein stability regulation	• Immunomodulation	[127-130]
LID	Synthetic	• Protein stability regulation	• Immunomodulation	[131]
Sensors				
miRNA target site	Synthetic	• miRNA sensing	• Cell type specific vaccine circuit activation	[16,133-136]
mRNA strand displacement	Synthetic	• mRNA sensing	• Cell type specific vaccine circuit activation	[137]
Protein aptamer	Synthetic	• Protein sensing	• Detection of immune cell activity	[138]
Kinase translocation reporter	Synthetic	• Kinase activity sensing	• Detection of immune cell activity	[139]

*References in which devices were used for vaccination.
RNP: ribonucleoprotein; shRNA: short hairpin RNA; TetR: Tet repressor; PUF: Pumilio and FBF homology; APC: Antigen presenting cell; RNAi: RNA interference; miRNA: microRNA; DD: destabilizing domain; LID: ligand-induced degradation.