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Review

Synthetic biology in the clinic: engineering vaccines, diagnostics, and therapeutics

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SUMMARY

Synthetic biology is a design-driven discipline centered on engineering novel biological functions through the discovery, characterization, and repurposing of molecular parts. Several synthetic biological solutions to critical biomedical problems are on the verge of widespread adoption and demonstrate the burgeoning maturation of the field. Here, we highlight applications of synthetic biology in vaccine development, molecular diagnostics, and cell-based therapeutics, emphasizing technologies approved for clinical use or in active clinical trials. We conclude by drawing attention to recent innovations in synthetic biology that are likely to have a significant impact on future applications in biomedicine.

INTRODUCTION

As illustrated by current global pandemics, our approach to the diagnosis, treatment, and prevention of diseases requires the coordinated and efficient use of ever-increasing amounts of biological data and bioengineering techniques to maximize responsiveness and prepare us for future threats to human health. The speed or lack thereof of vaccine, diagnostic, and therapeutic development can have a tremendous impact on the human and economic cost of illnesses.

Synthetic biology emphasizes precise control over artificial biological systems. Although the definition of synthetic biology is relatively fluid, its central focus on iterative design and refinement to engineer modular and responsive biological systems differentiates this field from numerous related fields such as more foundational applications of protein or genetic engineering. This aspect of synthetic biology makes it highly and rapidly adaptable to respond to urgent needs. Since its inception as a distinct bioengineering discipline in the early 2000s with the creation of gene circuit designs such as toggle switches, oscillators, and logic gates (Gardner et al., 2000; Elowitz and Leibler, 2000), synthetic biology has played an increasingly important role in many sectors of our society, such as medicine, energy, agriculture, and environmental conservation.

Like other engineering disciplines, a critical goal of synthetic biology is the ability to predict and produce a desired level of output for any given input. This applies to simple outputs, such as maximizing protein production per nucleic acid during vaccination, and to complex outputs, such as controlled immune responses to specific cancer antigens in engineered T cells. The modularity of biological components allows synthetic biologists to create novel systems that provide genetically encoded computation and spatiotemporal control through the use of high-performance parts and the skillful assembly of these parts into a functional whole. The field of synthetic biology continues to benefit from biotechnological developments as newly discovered or created parts become integrated into its ever-growing toolkit. Concurrent developments in seamless nucleic acid assembly (Gibson et al., 2010) and large-scale genome editing (Annaluru et al., 2014; Richardson et al., 2017) have allowed scientists to rapidly iterate through numerous genetic designs to optimize system function. Widespread environmental metagenomic sequencing projects have also freed scientists from constraining themselves to parts harvested from culturable organisms and have demonstrably increased their utilization of molecular components from far more diverse sources than previously possible (Kunjapur et al., 2018). In addition to harnessing the fruits of natural evolution, synthetic biologists have used directed evolution, including phage-assisted continuous evolution (Esvelt et al., 2011; Badran and Liu, 2015), and design-driven engineering of proteins, nucleic acids, and gene circuits based on advanced modeling (Lillacci et al., 2018;

Nielsen et al., 2016) to select for and create biomolecules and synthetic systems with enhanced function. These techniques can be used alongside rational design to successfully implement complex biological systems (Mimee et al., 2018; Schmidts et al., 2019).

The availability of new biological parts, improved engineering of biological systems, and rapid design cycles made possible by facile nucleic acid synthesis have profoundly advanced the field of synthetic biology. In this review, we discuss recent examples of successful clinical translation of synthetic biology in vaccine development, molecular diagnostics, and living therapeutics. Many excellent reviews cited in each section, to which we refer interested readers, cover the astonishing breadth of inventiveness of our colleagues in the field. In this piece, we instead focus on describing technologies that have already entered clinical use or are in clinical trials. We conclude with a discussion of emerging trends that may influence future biomedical applications of synthetic biology.

SYNTHETIC BIOLOGY-BASED VACCINES

Vaccines are crucial components of public health and instrumental in reducing the morbidity and mortality of numerous diseases. The fundamental goal of training the human body to respond robustly to a pathogen without causing severe disease requires two main steps: (1) selecting an antigen and (2) delivering it into the body. Current vaccines use either whole (inactivated or live attenuated) microbes or viruses or selected components that are introduced into the body via diverse methods. Numerous innovations in genetics, biochemistry, structural biology, and bioinformatics have resulted in significant advancements in vaccine design and production (Kanekiyo et al., 2019). Below, we review some remaining challenges and discuss how synthetic biology has helped address these issues. We highlight synthetic biology approaches and techniques centered on large-scale nucleic acid manipulation that have been successfully applied in the creation of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines that have been approved or are currently in clinical trials, with a particular focus on genomic codon-deoptimized vaccines and DNA- and RNA-based vaccines. Additional methods such as viral vector-based vaccines (Humphreys and Sebastian, 2018) or virus-like particle vaccines (López-Sagaseta et al., 2015) have been extensively reviewed elsewhere.

Genomic codon-deoptimized vaccines

The balance between safety and efficacy of vaccines is often difficult to achieve and is compounded by multiple technical challenges. Attenuated live viruses yield highly effective vaccines that offer long-lasting protection. However, no suitable low-virulence species exist for most infectious diseases, and the commonly used method of attenuation through serial culture takes many years and may not produce safe strains (Schwarz, 1962; Alleman et al., 2020). Whole inactivated viruses are easier to generate but frequently lead to short-term protection that is primarily humoral and may even worsen disease outcomes (Rauh and Schmidt, 1965; Polack et al., 2003). Alternative methods for generating effective, attenuated live viruses that avoid prolonged culture and minimize reversion to virulent virus are urgently needed.

The advent of low-cost nucleic acid synthesis has allowed synthetic biologists to reengineer entire viral genomes using large-scale synonymous mutations. This method of viral attenuation (Le Nouën et al., 2019) uses the degeneracy of triplet codons and the non-random frequencies of specific codons, codon pairs, and dinucleotides that many species exhibit. The exact function of these biases remains an active area of research, but synthetic biologists have purposefully used under-represented codons and codon pairs to reduce viral protein production in human cells to rapidly and reliably create attenuated viruses without requiring detailed knowledge of viral function (Figure 1). The process was first used to create attenuated strains of poliovirus (Burns et al., 2006; Mueller et al., 2006) in which hundreds of synonymous mutations targeting the capsid-coding region suppressed viral replication more than 60-fold depending on mutation number and location. The mutated viruses remained infectious, but had severely attenuated virulence, and the vast majority of mutations remained stable over 25 passages. Subsequently, deoptimized pairs of codons using hundreds of synonymous mutations in poliovirus resulted in 1,000-fold attenuation, but maintained protection against paralysis and death in murine models (Coleman et al., 2008). These mutations were stable over 17 passages with no reversions to fully virulent viruses, which had occurred with the standard live attenuated oral polio vaccine through recombination with circulating Coxsackie A virus and led to local vaccine-derived polio outbreaks (Alleman et al., 2020). Similar techniques generated live attenuated influenza (Mueller et al., 2010; Fan et al., 2015), respiratory syncytial virus (Le Nouën et al., 2014), and dengue virus (Shen et al., 2015).

There are several benefits to using codon deoptimization as an attenuation technique. Speed is critical for a successful response to infectious outbreaks, and one benefit of this technique is that the method does not require detailed knowledge of viral function. Computational techniques allow the prediction of protein-coding regions from genomic data (Hyatt et al., 2012; Schlub et al., 2018) and the characterization of codon biases (Athey et al., 2017). A deoptimized genome takes 3–5 days to design, and genome synthesis, testing in cell lines, and handoff for clinical manufacturing can be achieved by day 48 (Tong, 2020). Live attenuated viruses often induce robust immune responses that are identical to exposure to wild-type virus (Centers for Disease Control and Prevention, 2015). A single dose may be sufficient to generate long-lasting protective immunity, which simplifies deployment, and the hundreds of mutations make virulent reversion highly unlikely.

Codon deoptimization vaccines have been used in several phase I clinical trials. These include CodaVax-H1N1, a live attenuated vaccine against influenza A H1N1, in both injectable (NCT03926416) and nasal spray (NCT04146623) formulations; CodaVax-RSV, a live attenuated vaccine against respiratory syncytial virus (RSV) (NCT04295070); and CDX-005, a live attenuated SARS-CoV-2 vaccine with phase I clinical trials planned for early 2021 (Codagenix, 2020).

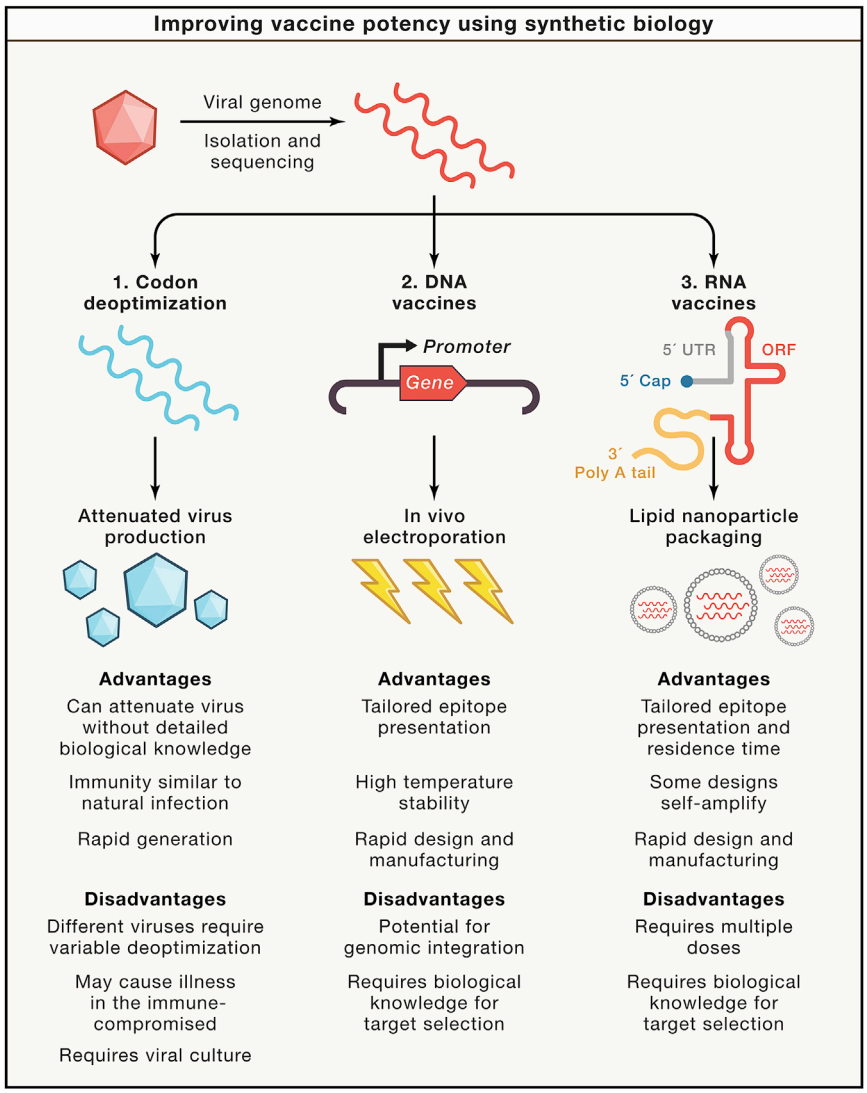

Figure 1. Synthetic biology and vaccine design

Several synthetic biology techniques have been utilized to create vaccines. (1) Genomic codon deoptimization uses genome-wide synonymous mutations to lowly represented codons and codon pairs to attenuate viruses. (2) DNA vaccines deliver plasmid-free dsDNA to cell nuclei to generate transcripts that are cytoplasmically translated. (3) RNA vaccines are typically delivered by lipid nanoparticles (NPs) and use several methods to avoid activation of the innate immune system to maximize antigen translation.

There are some drawbacks to using codon deoptimization to attenuate viruses. For example, there is a trade-off between the degree of attenuation and viral recovery for vaccine production, and the optimal mutational load may need to be empirically determined for each virus. This may increase the cost, effort, and time needed to produce vaccines that generate robust immunity, remain safe, and allow efficient industrial production. Additionally, the mechanism of attenuation is still under debate, with some researchers indicating that the increase in pro-inflammatory dinucleotides, such as CpG and UpA, which results from codon deoptimization, is responsible for cell- and organism-level immune-mediated viral attenuation (Tulloch et al., 2014). The attenuated virus must still be grown in culture, and resultant viruses have the same storage, handling, and refrigeration requirements of other live viruses. Finally, the administration of even highly attenuated live viruses may be dangerous for patients with compromised immune systems. Nucleic acid vaccines address several of these shortcomings and are discussed in the next section.

DNA- and RNA-based vaccines

The premise of nucleic acid vaccines centers on the introduction of DNA or RNA encoding viral components into human cells; these cells then produce viral antigenic peptides in a recapitulation of the natural infectious process to induce robust cellular and humoral immunity. Benefits of nucleic acid vaccines include their rapidity of design and streamlined manufacturing processes. Almost any protein epitope can be targeted, but increased size adds to the cost and complexity of production as well as reduced delivery efficiency, with most vaccines in the 5- to 12-kb range. By comparison, the SARS-CoV-2 genome is ~30 kb, so pre-existing biological knowledge is needed for epitope selection. Once the genomic sequence is obtained, a nucleic acid vaccine can be designed, manufactured, and started in trials on the order of weeks (Dowd et al., 2016).

DNA vaccines were initially favored due to their greater stability and reduced non-specific inflammation in comparison with early RNA formulations (Figure 1). One advantage of DNA vaccines is their relatively high thermostability. A DNA-based Ebola glycoprotein vaccine INO-4201 was stable for 1 month at 37°C, 1 year at 25°C, and 3 years at 4°C (Tebas et al., 2019). Another potential benefit is prolonged antigen expression of up to 1.5 years after rodent intramuscular injection (Wolff et al., 1992). However, DNA vaccine adoption has been limited by relatively weak immunogenicity in early human trials (Li and Petrovsky, 2016), the requirement for *in vivo* electroporation in order to facilitate intranuclear delivery, and the risk of undesirable genomic integration events (Wang et al., 2004). Modern DNA vaccines have increased immunogenicity via codon optimization, the co-administration of immune-stimulatory cytokines, streamlined plasmid and plasmid-free double-stranded DNA (dsDNA) designs, and needle-free intramuscular injections without electroporation (Gaudinski et al., 2018). A DNA-based vaccine expressing full-length SARS-CoV-2 S protein delivered via electroporation (Smith et al., 2020) is in a phase I clinical trial (NCT04336410).

RNA vaccines share many advantages of DNA vaccines, such as rapid design and ease of manufacturing, but they do not have the problem of potential genomic integration (Figure 1). RNA vaccines do not need electroporation since they must only cross one lipid bilayer for cytoplasmic translation to produce antigens. A major challenge of RNA vaccines is the delivery of intact transcripts into human cells, since RNA is inherently less stable than DNA and prone to rapid degradation by ubiquitous nucleases in the environment and inside cells. Most vaccine manufacturers use material chemistry and lipid nanoparticles (NPs) to condense, protect, and enhance the intracellular delivery of RNAs (Reichmuth et al., 2016). After the cytosolic delivery of intact vaccine RNAs, the second major challenge is consistent and robust expression of antigenic proteins from these RNAs, which is needed to maximally induce immune responses. Human cells have several defense mechanisms that recognize exogenous RNAs and induce RNA degradation and inflammatory responses that slow translation and lead to cellular cytotoxicity, all of which reduce antigen production and targeted immunity.

Synthetic biology and biochemical approaches have been used to increase the intracellular stability of vaccine RNAs, reduce cytotoxicity, and enhance protein production (Jackson et al., 2020b; Pardi et al., 2020; Kowalski et al., 2019). Biochemical removal of undesirable dsRNA contaminants generated during *in vitro* transcription of RNAs decreases innate immune activation and translational suppression (Karikó et al., 2011). Synthetic biology approaches to enhance RNA stability and translational efficiency broadly involve engineering RNA structure or base composition. One common structural alteration is the addition of a complete 5′ Cap1 (N7MeGpppN2′-OMe) to RNAs during *in vitro* transcription, which enhances translation and mRNA stability by avoiding innate immunological recognition of uncapped 5′-triphosphate RNAs (Devarkar et al., 2016), which can be further reduced using phosphatases (Warren et al., 2010). Other engineered alterations to RNA structure include the addition of modular 5′ untranslated regions (UTRs) and 3′ UTRs identified through high-throughput functional screening to stabilize mRNAs and increase protein translation (Orlandini von Niessen et al., 2019; Thess et al., 2015). Engineering low secondary structure in the 5′ UTR and first 30 nt of a coding region, but adding a high secondary structure region after these regions also improves mRNA translation (Mauger et al., 2019).

Modifications of mRNA base composition suppress innate immune recognition, reduce cytotoxicity, and enhance antigen production. For example, Moderna and BioNTech use modified nucleosides such as pseudouridine, *N*-1-methylpseudouridine, 5-methoxyuridine, or 5-methylcytidine to create mRNAs that evade innate immune effectors, such as protein kinase R, Toll-like receptors 3, 7, and 8, and retinoic acid-inducible gene I, which help detect exogenous RNAs (Karikó et al., 2008; Andries et al., 2015; Warren et al., 2010). By contrast, CureVac avoids modified nucleosides and instead uses whole-transcript engineering by replacing open reading frame (ORF) codons with synonymous codons that maximize GC content. This significantly enhances protein production through unclear mechanisms when matched to optimized 5′ and 3′ UTRs (Thess et al., 2015).

Another method to increase protein production from mRNA vaccines is the use of synthetic self-amplifying mRNAs (saRNAs) (Brito et al., 2015), which are made using parts of alphaviruses such as Semliki Forest virus (Zhou et al., 1995) and Sindbis virus (Herweijer et al., 1995). In saRNAs, an ~7-kb ORF encoding alphavirus RNA-dependent RNA polymerase (RDRP) is placed upstream of the vaccine antigen ORF together with a subgenomic promoter and replication recognition sequences. Once the positive-strand saRNA enters cells, host machinery translates RDRP, which replicates full-length negative-strand mRNA that serves as a template for replication of more positive-strand full-length mRNA and high levels of the 3′ antigen-coding subgenomic portion, leading to highly amplified antigen expression. The replicated mRNAs do not have modified nucleosides, but any immune-associated translational suppression seems to be overcome by increased mRNA copies. This approach increases the immunogenicity per unit vaccine by 64-fold in murine influenza (Vogel et al., 2018). Arcturus and Duke-National University of Singapore (Ramaswamy et al., 2017) and Imperial College (McKay et al., 2020) both have phase I clinical trials (NCT04480957, ISRCTN17072692) testing saRNAs expressing pre-fusion-stabilized SARS-CoV-2 spike protein delivered via lipid NPs.

One disadvantage of saRNAs is the tripling of transcript size, which is more challenging to produce. Recently, RDRP components were co-delivered as a second non-replicating mRNA in *trans* to a replication-competent mRNA encoding vaccine antigen (Beissert et al., 2020), which resulted in 10- to 100-fold higher antigen mRNA levels than standard unimolecular saRNAs. Murine vaccination with 50 ng of replicating vaccine mRNA plus 20 μg of RDRP mRNA was sufficient to induce protection against influenza.

There are several clinical trials of SARS-CoV-2 mRNA vaccine candidates created using combinations of the techniques described above. The first RNA-based SARS-Cov-2 vaccine candidate is Moderna mRNA-1273, which uses modified nucleosides to encode a transmembrane-anchored full-length spike protein stabilized in the pre-fusion state with two prolines and is delivered via lipid NPs. Moderna mRNA-1273 was well tolerated in a phase I trial (NCT04283461) and generated increases in neutralizing antibodies against SARS-CoV-2 spike protein and strong antigen-specific $CD4^+$, but low $CD8^+$, T cell responses. Two vaccine injections generated antibody levels similar to the upper half of levels detected in coronavirus disease 2019 (COVID-19) convalescent serum (Jackson et al., 2020a; Anderson et al., 2020). Recruitment of ~30,000 patients in a 1:1 ratio of controls to vaccine recipients has recently finished in their ongoing phase III trial (NCT04470427). Interim analysis by an independent data safety monitoring board indicated that Moderna mRNA-1273 had an efficacy of 94.1% in preventing COVID-19 at 42 days after initiation of the two 100 μg dose regimen (14 days after the last dose) (Baden et al., 2020).

CureVac CVnCoV has completed a phase I trial (NCT04449276) and started a phase II trial (NCT04515147) using a sequence-optimized mRNA with unmodified nucleosides encoding full-length SARS-CoV-2 spike protein delivered via lipid NPs. No results have been made available yet, and data collection is ongoing.

BioNTech created four separate SARS-CoV-2 mRNA vaccine candidates using nucleoside-modified mRNA, uridine-containing mRNA, or saRNAs. BNT162b1 encodes only the receptor binding domain of the SARS-CoV-2 spike protein trimerized

with a bacteriophage T4 fibritin foldon domain, and early studies (Mulligan et al., 2020) showed that two injections increased SARS-CoV-2 neutralizing immunoglobulin G (IgG) titers from 0.7- to 3.5-fold relative to COVID-19 convalescent plasma and expanded antigen-specific $CD8^+$ and $CD4^+$ T cells (Sahin et al., 2020). A combined phase I/II/III trial (NCT04368728/EU 2020-001038-36) tested two lipid NP-delivered versions of vaccine candidates, BNT162b1 and the related BNT162b2 encoding membrane-anchored full-length SARS-CoV-2 spike protein stabilized in the pre-fusion conformation with two proline mutations. BNT162b1 and BNT162b2 induced similar levels of neutralizing antibodies, but two injections were essential to generate strong antibody responses, and BNT162b2 produced significantly fewer systemic side effects, especially in older patients (65–85 years) (Walsh et al., 2020). The reason for reduced side effects with BNT162b2 is unclear, but may be related to differences in vaccine sequences or the 5-fold increased copies of BNT162b1 per 30 μg dose due to its shorter length. BNT162b2 was selected for the phase II/III stage of their ongoing trial with Pfizer. After enrolling ~43,000 participants in a 1:1 ratio of control-to-vaccine recipients, BNT162b2 was 95% effective at preventing COVID-19 at 28 days after the initiation of the two 30 μg dose regimen (7 days after the last dose) (Polack et al., 2020).

These preliminary vaccine trial results provide very welcomed hope as the COVID-19 pandemic continues, and both Moderna mRNA-1273 and BioNTech BNT162b2 were granted emergency use authorization (EUA) by the US Food and Drug Administration (FDA) in December 2020. However, there are some important caveats. The efficacy rate of both Moderna mRNA-1273 and BioNTech BNT162b2 is characterized as prevention of COVID-19, which consists of symptoms and a positive nucleic acid test for SARS-CoV-2 (Moderna, 2020a; Pfizer, 2020). Estimates of asymptomatic SARS-CoV-2 carriage vary, but may account for ~40% of all infections (Oran and Topol, 2020; Feaster and Goh, 2020), and it is likely that asymptomatic carriers spread the virus (Furukawa et al., 2020). Moderna mRNA-1273 and BioNTech BNT162b2 are highly effective at preventing symptomatic COVID-19, but there is currently no information about their effect on the spread of SARS-CoV-2, although this a secondary objective in the Moderna study protocol. The durability of immunity is unclear since titers of SARS-CoV-2-specific IgG vary widely between individuals after natural infection, with some reports of waning response within 3 months (Seow et al., 2020) and others reporting unchanged titers to at least 4 months after infection (Gudbjartsson et al., 2020). Additionally, the distribution of mRNA vaccines may be challenging since refrigeration at −80°C is required for storage of BioNTech BNT162b2, and equipment for this degree of cooling is not widely available. However, recent stability studies by Moderna (Moderna, 2020b) indicate its mRNA-1273 vaccine is stable between 2°C and 8°C for 30 days, at −20°C for up to 6 months, and at room temperature for up to 12 h, which is similar to most commonly administered vaccines. CureVac announced that its CVnCoV mRNA vaccine candidate is stable for at least 3 months when stored at 5°C and up to 24 h at room temperature (CureVac, 2020). Finally, most formulations required the administration of two doses separated by 14–28 days, which presents added logistical challenges. Additional engineering of subsequent vaccine versions may address some of the concerns with single-dose potency and long-term immunity and may be necessary in the event of antigenic drift.

SYNTHETIC BIOLOGY-BASED DIAGNOSTICS

One key step in addressing any illness is knowing whether or not it is present. Diagnostics is therefore an essential component of public health. Common goals of diagnostics development are focused on enhancements to clinical performance, such as increased sensitivity, specificity, and accuracy of quantification, and on improvements in assay characteristics, such as reduced time to results, lower cost, greater portability, simplified workflow, and resilience to contaminants. Synthetic biology techniques based on gene circuit construction and rapid, iterative prototyping have enabled the development of several innovative approaches to improve diagnostics. Synthetic biology devices, ranging from whole-cell living assays to engineered cell-free nucleic acid sensors and combinations in between that utilize aspects of native disease biology and reconstructed enzymatic functions (Slomovic et al., 2015; Wei and Cheng, 2016; Sedlmayer et al., 2018; Soleimany and Bhatia, 2020) have been successfully applied to non-communicable diseases such as cancer and coronary artery disease; communicable diseases such as Ebola, Zika, tuberculosis, malaria, HIV, and SARS-CoV-2; and other aspects of public health such as routine blood analyte quantification (McNerney et al., 2019) and water quality monitoring (Thavarajah et al., 2020). Although many of these approaches show great potential, the vast majority remain in the preclinical stage of development. We focus on two applications of synthetic biology in diagnostics that are in active clinical trials or are authorized for clinical use by the FDA: paper-based toehold switch RNA sensors and clustered regularly interspaced short palindromic repeat (CRISPR)-based diagnostics (Figure 2).

Toehold RNA switches and paper-based diagnostics

There is a critical shortage of diagnostic infrastructure in much of the world. In a study by the World Health Organization of 10 countries across three continents, only 1% of health centers and clinics were deemed to have full-service readiness for basic diagnostic tests (Leslie et al., 2017). Synthetic biology has led to the creation of novel diagnostics based on synthetic gene networks, but the vast majority have been limited to laboratory use and have not been able to address this important need due to the costly equipment and materials required to maintain the experimental conditions that are essential for their operation. Recent advances in cell-free expression systems have allowed for the dissemination and use of engineered RNA elements as multifunctional diagnostics at the point of need.

Cell-free systems contain all of the machinery and cellular components needed for gene expression. They have been used for decades to study fundamental biochemical processes, whose manipulation in living cells is challenging due to excessive toxicity and other deleterious issues (Silverman et al., 2020). An important development for the practical deployment of synthetic gene circuits outside of laboratories was the demonstration that freeze-drying and embedding cell-free expression systems in porous substrates such as paper could largely preserve their

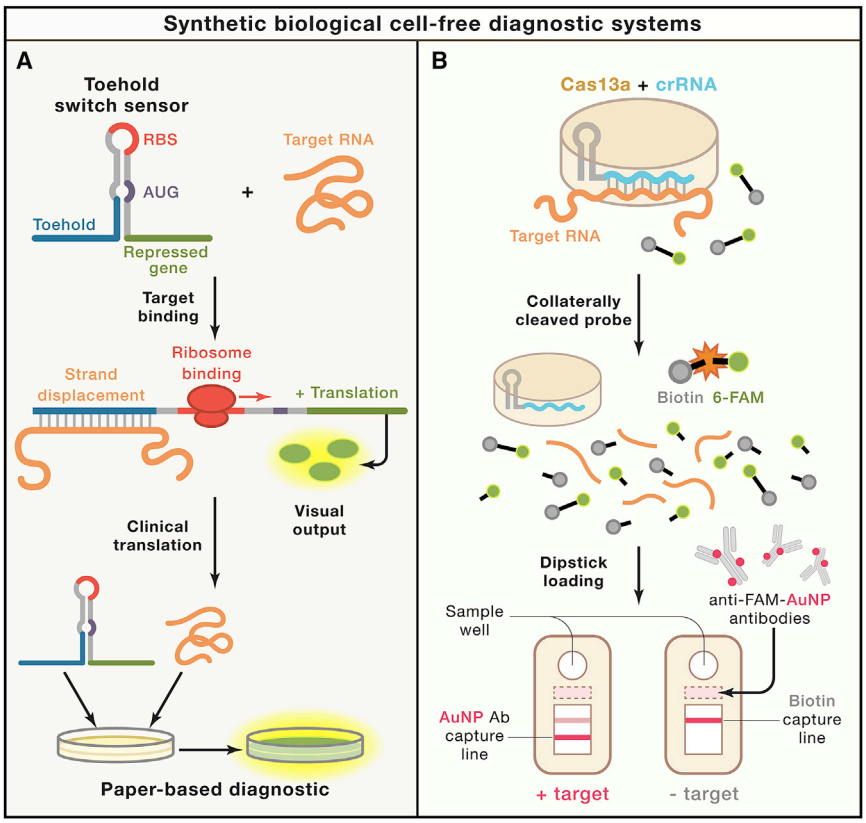

Figure 2. Synthetic biology-based cell-free diagnostics

(A) In the off state, a stem-loop sequesters the ribosome binding sequence (RBS) and AUG of a toehold switch to prevent reporter gene translation. Target RNA binding to the toehold frees the RBS and AUG and allows downstream translation. Switch-based detectors may be freeze-dried onto paper substrates and maintain activity even after prolonged storage at room temperature. Detection reactions proceed after rehydration with amplified sample.

(B) After activation by binding to crRNA and target RNA, some Cas12 and Cas13 effectors also cleave nearby nucleic acids. Here, activated Cas13a degrades dual biotin and 6-FAM-labeled RNA probes monitored via fluorescence or via lateral flow dipsticks that contain a pad with gold (Au)-NP conjugated rabbit anti-FAM antibodies, a streptavidin line to capture biotin, and a second capture line with immobilized anti-rabbit antibody. Clustering of Au-NP causes a visible line.

function for extended periods of more than 1 year despite storage at room temperature (Pardee et al., 2014). Synthetic gene circuits could be embedded within a piece of paper, stored or transported at room temperature, and reactivated with rehydration upon application of a sample. Almost any design could be used, including cell-free tests for antibiotics (Duyen et al., 2017), clinical concentrations of essential micronutrients (McNerney et al., 2019), heavy metals (Didovyk et al., 2017), sedative medications (Gräwe et al., 2019), and bioactive small molecules (Jung et al., 2020; Salehi et al., 2017). A class of engineered RNAs called toehold switches (Figure 2A) is particularly suited for use as biomedical diagnostics for specific nucleic acids. These toehold switches can be combined with cell-free expression systems to create highly portable, paper-based nucleic acid diagnostics.

The toehold switch is a prokaryotic riboregulator designed to detect the presence of arbitrary trigger nucleic acids and respond by driving proteinaceous output (Green et al., 2014). These high-performance RNA switches consist of a 5′ toehold region complementary to the trigger nucleic acid sequence, followed by a long stem-loop structure that incorporates a ribosomal binding sequence (RBS) within the loop and hides an AUG translation start site in-frame with a 3′ output gene. Without trigger nucleic acid, the stem structure keeps the switch off by limiting translation initiation. When trigger nucleic acid is present, the switch toehold region allows binding and opening of the stem by strand invasion, thus making the RBS and AUG accessible and initiating reporter gene translation. This design has several advantages over traditional RNA switches, including greater dynamic range, fewer target sequence restrictions, better orthogonality, and simplified rule-based construction (Green et al., 2014).

One of the first demonstrations of paper-stabilized toehold switches was the highly specific detection of Ebolavirus transcripts (Pardee et al., 2014), although practical application was limited by insufficient sensitivity. This was addressed by incorporating an isothermal nucleic acid amplification step involving nucleic acid sequence-based amplification (NASBA), which improved sensitivity ~10^6-fold to 3 fM of Zika virus mRNA and was tested successfully against serum samples from infected macaques (Pardee et al., 2016a). A colorimetric assay was developed based on β-galactosidase expression, which changes the color of an added reagent from yellow to purple. This color change is visually discernible, but a low-cost, multi-sample reader was also developed to enable higher throughput and finer quantitation. This platform has also been adapted to quantitatively detect 10 bacterial members of the human gut microbiome from clinical stool samples, fecal host mRNAs, and *Clostridioides difficile* toxin mRNA (Takahashi et al., 2018). Paper-based quantitation showed good agreement with quantitative real-time PCR with significantly lower cost and could be used to achieve basic microbiome profiling, assessment of host inflammation, potential responsiveness to medications, and differentiation of active *C. difficile* infection (CDI) from chronic colonization.

The combination of cell-free expression systems stabilized on paper, isothermal nucleic acid amplification, and modular toehold switches provides an adaptable, sensitive, and stable diagnostic platform that is capable of detecting specific nucleic acids at the point of care with a total cost of $0.10–$1.00 (USD) per test (Pardee et al., 2016a). Because toehold switch output can be any protein, there is nearly unlimited multiplexing capability. The system is easily adapted to fluorescent, bioluminescent, colorimetric, and bioelectric outputs, and complex gene

circuits have been constructed for multi-input logic (Green et al., 2014; Pardee et al., 2014). It is even possible to create combination theragnostic devices that not only detect but also treat infections, due to the capacity for cell-free expression systems to express anti-infective agents such as nanobodies or bacterial lysins (Pardee et al., 2016b). Paper-based toehold switches for Zika virus diagnosis are currently being tested in a coordinated study at sites in five countries across North and South America, with nearly 300 clinical samples processed thus far (K. Pardee, personal communication).

Despite the advantages of the toehold switch diagnostic platform, there are also some disadvantages. Toehold switches are easy to design using templates for RNA structure prediction packages, but only ~20% show high performance, which must currently be determined empirically. Recent efforts using deep learning have reported the ability to predict higher performance toeholds using sequence alone (Angenent-Mari et al., 2020; Valeri et al., 2020). The use of cell-free expression systems provides great flexibility in output, but also adds cost and potential susceptibility to chemical or physical inhibitors, including nucleic acid preservation, extraction, or amplification reagents. One-pot reactions combining amplification with detection have been challenging to develop due to mixed reagent effects on amplification efficiency and cell-extract output.

Additionally, toehold switches tolerate several mismatches in their binding region, which makes the detection of single-nucleotide polymorphisms (SNPs) highly challenging. This limitation was addressed with an elegant new switch design called a single-nucleotide-specific programmable riboregulator (SNIPR), which uses exquisitely balanced thermodynamic stability of two competing toehold stem structures that are driven to an on- or off-predominant state by specific SNPs on target nucleic acids (Hong et al., 2020). SNIPRs were used for SNP genotyping for human-disease genes, drug-resistance mutations, and Zika strain differentiation using a paper-based colorimetric platform with isothermal amplification via recombinase polymerase amplification (RPA). The sensitivity of ~250 aM for Zika SNIPRs with RPA is within the range achieved using standard toehold switches and NASBA (from ~2.5 fM to 250 aM). Additionally, the more complex SNIPRs also required double the screening of potential designs to find high-performance switches. The limit of detection of 250 aM (~150,000 copies/mL) is insufficient for many infectious diseases. For situations requiring simple detection of nucleic acids with maximal sensitivity, recently described CRISPR diagnostics based on collateral cleavage provide greater sensitivity and simplicity of reactions, while maintaining SNP specificity and low cost, as we discuss in the following section.

CRISPR-based diagnostics

The discovery and characterization of the prokaryotic adaptive immunity systems mediated by CRISPR and CRISPR-associated (Cas) proteins have revolutionized numerous aspects of biological research and clinical medicine (Ishino et al., 2018). There are two major classes of CRISPR-Cas systems based on whether the Cas effectors are multicomponent complexes (class 1) or single proteins (class 2). All known CRISPR systems constitute RNA-guided nucleases that protect the host from invading phage and plasmids by recognizing target RNA or DNA and cutting a variety of nucleic acids both specifically and non-specifically (Makarova et al., 2020). Class 2 systems include CRISPR-associated protein (Cas9) (type II) as well as newer members Cas12 (type V) and Cas13 (type VI) with novel functions that have been adapted to create ultrasensitive nucleic acid diagnostics.

Cas13a (C2c2), the prototypical type VI effector, is guided by a CRISPR RNA (crRNA) to bind and cut a target RNA in *cis*, which additionally triggers the activation of two surface nuclease domains that then non-specifically degrade nearby RNAs in *trans* in a highly processive manner (Abudayyeh et al., 2016). Cas12a (Cpf1), a prototypical type V effector, is guided by crRNA to bind and cut dsDNA in *cis*, which then activates non-specific nuclease activity to degrade nearby single-stranded DNAs (ssDNAs) (Chen et al., 2018; Li et al., 2018). This process of non-specific in *trans* "collateral cleavage" after in *cis* target cleavage is also present in many other type V effectors, including Cas12b (C2c1), Cas12d, Cas12f (Cas14), and Cas12g.

As first described using Cas13a (East-Seletsky et al., 2016), Cas effectors with collateral cleavage can function as highly sensitive and easily programmable nucleic acid detectors by providing a crRNA complementary to a target nucleic acid together with labeled nucleic acid probes that produce a signal when degraded in *trans* (Figure 2B). Cas effectors with collateral cleavage combined with nucleic acid amplification techniques such as RPA or loop-mediated isothermal amplification (LAMP) have been used to create highly sensitive and specific nucleic acid diagnostics (Gootenberg et al., 2017, 2018; Li et al., 2018, 2019). Two of these CRISPR-based techniques, SHERLOCK (specific high sensitivity enzymatic reporter unlocking) and DETECTR (DNA endonuclease-targeted CRISPR trans reporter), have been granted EUA by the FDA for testing SARS-CoV-2 in human clinical samples and are discussed below.

SHERLOCK combines RPA amplification, Cas13a detection and collateral cleavage, and signal output from RNA probes (Gootenberg et al., 2017). Since Cas13a detects RNA targets, a T7 promoter is incorporated into one RPA primer, and T7 polymerase generates RNAs from amplicons that are subsequently detected by Cas13a, which cleaves reporter RNA probes consisting of a fluorophore linked by a short RNA oligomer to a quencher. This initial version of SHERLOCK demonstrated low attomolar sensitivity for the detection of several RNA and DNA targets, including infectious agents such as Zika virus, dengue virus, and bacterial pathogens. Through the use of engineered mismatches between crRNAs and target RNAs, SHERLOCK could detect and discriminate SNPs of viral strains and cancer-associated mutations with mock abundance as low as 0.1%. Lyophilization and incorporation into paper created portable diagnostics that cost $0.61 per reaction. Further refinements for SHERLOCKv2 (Gootenberg et al., 2018) included the introduction of same-reaction multiplexing through the concurrent use of Cas13 and Cas12 enzymes from different species with orthogonal cleavage preferences for RNA or DNA probes labeled with different fluorophores. Reliable quantitation was possible to 2 aM (~1,200 copies/mL) and optimization of RPA, including primer concentration adjustment, increased input sample volume, increased amplification reaction volume, and extended

reaction time, led to detectable signal down to input of 8 zM (~5 copies/mL).

The use of 6-carboxyfluorescein (6-FAM) and biotin dual-labeled RNA oligomer probes with commercially available lateral flow dipsticks allowed for the creation of instrument-free diagnostics with visual outputs (Figure 2B). The dipsticks contain a pad embedded with mobile rabbit anti-FAM antibodies conjugated with gold (Au)-NPs, a capture line with immobilized streptavidin, and a second capture line with immobilized anti-rabbit antibody. Detection reactions using Cas13, crRNA, amplified sample, and 6-FAM biotin RNA probes are incubated and then applied to the dipstick. Intact probes cause Au-NPs to cluster at the streptavidin line by binding biotin, while cleaved probes cause Au-NP to cluster at the anti-rabbit antibody line. By incorporating a simplified nuclease inactivation step called HUDSON (heating unextracted diagnostic samples to obliterate nucleases), lateral flow SHERLOCK reactions successfully detected specific strains of Zika virus and dengue virus from patient saliva, urine, and serum, although with reduced analytical sensitivity of 9–90 aM depending on the sample matrix (Myhrvold et al., 2018). This protocol was optimized and combined with a smartphone-based application to detect viral infections and graft rejection in urine samples from kidney transplant recipients, which largely matched assessment with clinical qPCR and biopsy with analytical sensitivity in the low attomolar range (Kaminski et al., 2020). The first FDA approval for a CRISPR-based diagnostic was awarded as an EUA to Sherlock Biosciences for their SARS-CoV-2 test based on a modified SHERLOCK workflow with RT-LAMP amplification, Cas13a detection and collateral cleavage, and fluorescent output assayed on a plate reader (FDA, 2020c).

As mentioned above, Cas12a uses a crRNA to guide binding and in *cis* cutting of target dsDNA (Chen et al., 2018). Binding of target dsDNA is sufficient to generate in *trans* collateral cleavage of nearby ssDNAs. In a process similar to that described for SHERLOCK, RPA amplification, Cas12a detection and collateral cleavage, and quenched fluorescent ssDNA probes were combined to create a diagnostic system called DETECTR (Chen et al., 2018). Because Cas12a detects dsDNA, RPA amplicons can be detected directly, which simplifies the reaction. However, a 5′ TTTN protospacer flanking site (PFS) on the target is essential for function and limits the ability to detect some nucleic acids. This system detected exogenous plasmids at low attomolar concentrations, and its clinical utility was demonstrated with swabs containing different strains of human papilloma virus (HPV). An adaptation of one-pot concurrent Cas12a detection with RPA amplification and optimized nucleic acid extraction from parasites allowed high-performance point-of-care detection of malaria (Lee et al., 2020). There are two active phase I clinical trials using DETECTR-based methods: one for the rapid identification of *Mycobacterium tuberculosis* complex (NCT04074369) and one for early diagnosis and treatment selection in patients with pneumonia (NCT04178382).

Recently, a modified DETECTR assay was used to detect SARS-CoV-2 in clinical nasopharyngeal swabs with RT-LAMP for amplification, Cas12a for detection and collateral cleavage, and either quenched fluorescent ssDNA probes or 6-FAM biotin ssDNA probes for output (Broughton et al., 2020). This diagnostic has an analytical sensitivity of 10,000 copies/mL (~16.6 aM), which is ~17-fold less sensitive than the CDC quantitative real-time PCR SARS-CoV-2 assay (~1 aM) (FDA, 2020a). Both methods showed 95% concordance for positive samples (n = 40) and 100% concordance for negative samples (n = 42) using either fluorescent or lateral flow outputs. The University of California, San Francisco (UCSF) and Mammoth Biosciences were granted an EUA by the FDA for their jointly developed modified DETECTR assay for SARS-CoV-2 based on this method (FDA, 2020b).

Collateral cleavage-based CRISPR diagnostics are easily adaptable, highly sensitive, and suitable for use at the point of care. However, some limitations remain. Both SHERLOCK and DETECTR rely heavily on isothermal amplification steps to improve sensitivity, which in general is from ~2-fold (SHERLOCK) to ~17-fold (DETECTR) lower than equivalent qPCR tests (FDA, 2020d). Although greater sensitivity has been reported for SHERLOCK, this was only achieved for certain targets and required the use of larger sample input and amplification reaction volumes that increase cost and is impractical in most settings. The currently approved diagnostics maximize sensitivity by using separate amplification and detection steps due to cross inhibitory effects. This increases the risk of cross-contamination and handling errors. A new one-pot SHERLOCK diagnostic combining RT-LAMP with a thermostable Cas12b from *Alicyclobacillus acidiphilus* and single guide RNAs based on *trans*-activating crRNA from *A. acidoterrestris* called STOP (SHERLOCK testing in one pot) showed excellent performance and streamlined sample handling in clinical nasopharyngeal swabs with SARS-CoV-2 through the use of magnetic beads to concentrate virus prior to amplification (Joung et al., 2020) with a reported limit of detection that was similar to quantitative real-time PCR. It is interesting to note that both SHERLOCK and DETECTR were developed using RPA, but both of their FDA-approved SARS-CoV-2 commercial diagnostics use RT-LAMP for amplification. To our knowledge, there currently is no clinically approved commercial diagnostic based on RPA, perhaps due to issues with non-specific amplicons or excessive sensitivity to mixing steps (Zou et al., 2020). For field applications, the stability of the reagents, including single-stranded nucleic acid probes on which these methods rely, is unclear. However, SHERLOCK-based diagnostics for Ebola were used successfully at a government hospital in Sierra Leone (Barnes et al., 2020).

SYNTHETIC BIOLOGY-BASED THERAPEUTICS

Cellular immunotherapy

Engineered cell therapies are a powerful modality to complement pharmaceutical and surgical intervention for treating human diseases. Specific advantages of cell-based therapies include (1) providing persistence in patients for long-term disease management, (2) engaging endogenous cells to create a coordinated response, and (3) engineering precise control over their functionality using synthetic biology. Designed cell systems have demonstrated an improved ability to detect and attack cancer cells (Roybal et al., 2016), control the spatiotemporal activity of therapies in a drug- or antigen-dependent fashion (Schukur

et al., 2015; Wu et al., 2015), and differentiate stem cells to form complex tissues (Toda et al., 2018; Saxena et al., 2016). Here, we focus on clinical translation of engineered control systems and discuss how synthetic biology has improved the safety and efficacy of cell therapies currently in clinical use or in clinical trials.

Logic computation improves specificity

Chimeric antigen receptor (CAR) T cell therapies have emerged as groundbreaking, clinically approved treatments for several blood cancers, and they are effective even in patients who have failed radiation and chemotherapy. CARs consist of an external single-chain variable fragment (scFv), a CD8α transmembrane domain, an intracellular CD3ζ domain from the T cell receptor, and a co-stimulatory domain (CD28 or 4-1BB) (Figure 3A). When a target antigen is bound by the scFv, activation of both stimulatory and co-stimulatory domains is required to promote T cell proliferation and target cell killing. However, patients treated with CAR T cells can relapse due to antigenic escape if killing is inefficient (Majzner and Mackall, 2018). Conversely, CAR T cell therapies that induce potent killing often target healthy tissues or cause systemic immune activation that can lead to life-threatening cytokine release syndrome (CRS) and neurotoxicity (Schirrmacher, 2019; Majzner and Mackall, 2018). To balance safety and efficacy, improved control systems for CAR T cell therapies are necessary.

One approach to creating more precise treatments is to design cell therapies with greater specificity by sensing and responding to antigen combinations. Genetic logic circuits, such as AND, OR, and NOT gates, are foundational cellular decision systems in synthetic biology that can integrate several input signals. Multi-input CARs using AND (Roybal et al., 2016; Lanitis et al., 2013; Sukumaran et al., 2018), OR (Grada et al., 2013; Ruella et al., 2016; Bielamowicz et al., 2018), and NOT (Fedorov et al., 2013) logic have recently been developed to improve specificity and killing efficacy. To decrease patient relapse due to tumor antigenic escape, bispecific OR-gate CARs display two unique scFv fragments on the extracellular surface of engineered immune cells (Figure 3A) and can be activated by multiple antigens. Two orthogonal, full-length CARs may also be used (Ruella et al., 2016; Bielamowicz et al., 2018), but bispecific CARs (Grada et al., 2013; Zah et al., 2016) offer similar efficacy while reducing genetic payload size, which may increase CAR T production efficiency.

A bispecific tandem CAR (TanCAR) targeting human epidermal growth factor 2 (HER2) and CD19 demonstrated that two scFvs joined via a short, flexible linker could target both antigens (Grada et al., 2013). Subsequent studies using bispecific CD19/CD20 CARs showed the relative order of scFvs and a more rigid peptide linker improve CAR activation, promote targeting of $CD19^+/CD20^+$ cells *in vitro*, and eliminate CD19 antigenic escape in murine $CD19^+$ tumor models (Zah et al., 2016). Phase I clinical trials (NCT03019055, NCT04007029) showed maximal response rates of 82% for relapsed, refractory, $CD19^+/CD20^+$ non-Hodgkin's lymphoma. Importantly, relapsed patients retained expression of either CD19 or CD20 (Shah et al., 2019). These promising results demonstrate the power of logic processing in cell-based therapies.

While OR-gate CARs reduce antigenic escape, their drawbacks still include potentially fatal CRS, neurotoxicity, and B cell aplasia (Bonifant et al., 2016; Boyiadzis et al., 2018). Neurotoxicity is a dangerous side effect of current CAR therapies that leads to confusion, delirium, language disturbance, and seizures in ~22% of patients (Cao et al., 2020) and may be due to on-target off-tumor effects of CD19-specific therapies. Mural cells lining the blood-brain barrier (BBB) express CD19, and killing them can compromise the BBB and cause CAR T-related encephalopathy syndrome and death (Gust et al., 2019; Parker et al., 2020). This highlights the need for precise tumor targeting using multi-input cellular logic, which may be used in combination with other solutions to curb their toxicity.

Greater control using kill switches and activity switches

Several synthetic biology strategies have been developed to improve the safety of cell therapies, including drug-inducible safety switches (Stanton et al., 2018). Small-molecule-mediated dimerization of a split functional protein is widely used in synthetic biology to impart user-defined activation of a cell circuit (Voß et al., 2015) including drug-inducible cell-death circuits as safety switches. One of the more successful designs is a drug-inducible caspase 9 (iCasp9) kill switch licensed by Bellicum Pharmaceuticals and used in >20 clinical trials for CAR T cell and histocompatibility leukocyte antigen (HLA)-mismatched hematopoietic stem cell transplants (HSCTs).

The iCasp9 switch consists of two parts: (1) a genetically encoded split caspase-9 protein fused to a chemically inducible dimerization system based on mutated FKBP12 (F36V) homodimerization domains (Clackson et al., 1998; Straathof et al., 2005) and (2) the dimerization-inducing small-molecule AP1903. Native caspase-9 is dimerized by cytochrome *c* to activate apoptosis (Kuida, 2000). Fusing caspase-9 to FKBP12 (F36V) in engineered cells allows induction of apoptosis after AP1903 administration (Straathof et al., 2005). A phase I clinical trial (NCT00710892) using T cells carrying iCasp9 found that 4 of 4 patients given AP1903 after the onset of acute graft-versus-host disease (GvHD) experienced a 90% reduction in iCasp9+ cells within 30 min of administration and had complete reversal of GvHD (Di Stasi et al., 2011). Pediatric patients receiving HLA-mismatched HSCT and donor T cells expressing iCasp9 (BPX-501) showed successful engraftment in a phase I clinical trial (NCT03301168, NCT02065869) (Shaw et al., 2018), and 2 of 4 patients who developed GvHD symptoms improved after two administrations of AP1903. The iCasp9 kill switch has also been incorporated into several clinical trials for novel CAR designs as a safety mechanism.

A major limitation to using kill switches is their finality—once activated, therapeutic cells are removed from the patient's body. An alternative to killing constitutively active CAR T cells when they become overactive is instead to use small molecules to control their activation and prevent overactivity. To this end, activity switches based on the FKBP12/AP1903 dimerization system using non-caspase effectors have been developed (Figure 3B). One strategy utilizing an inducible MyD88/CD40 (iMC) costimulatory domain with a first generation CAR targeting prostate stem cell antigen (PSCA) has shown promising results in a combined phase I/II clinical trial (NCT02744287). Native homodimerization of MyD88, a Toll-like receptor adaptor molecule, and CD40, a tumor necrosis factor family member, act to stimulate nuclear factor κB (NF-κB), Activator protein 1 (AP-1), and

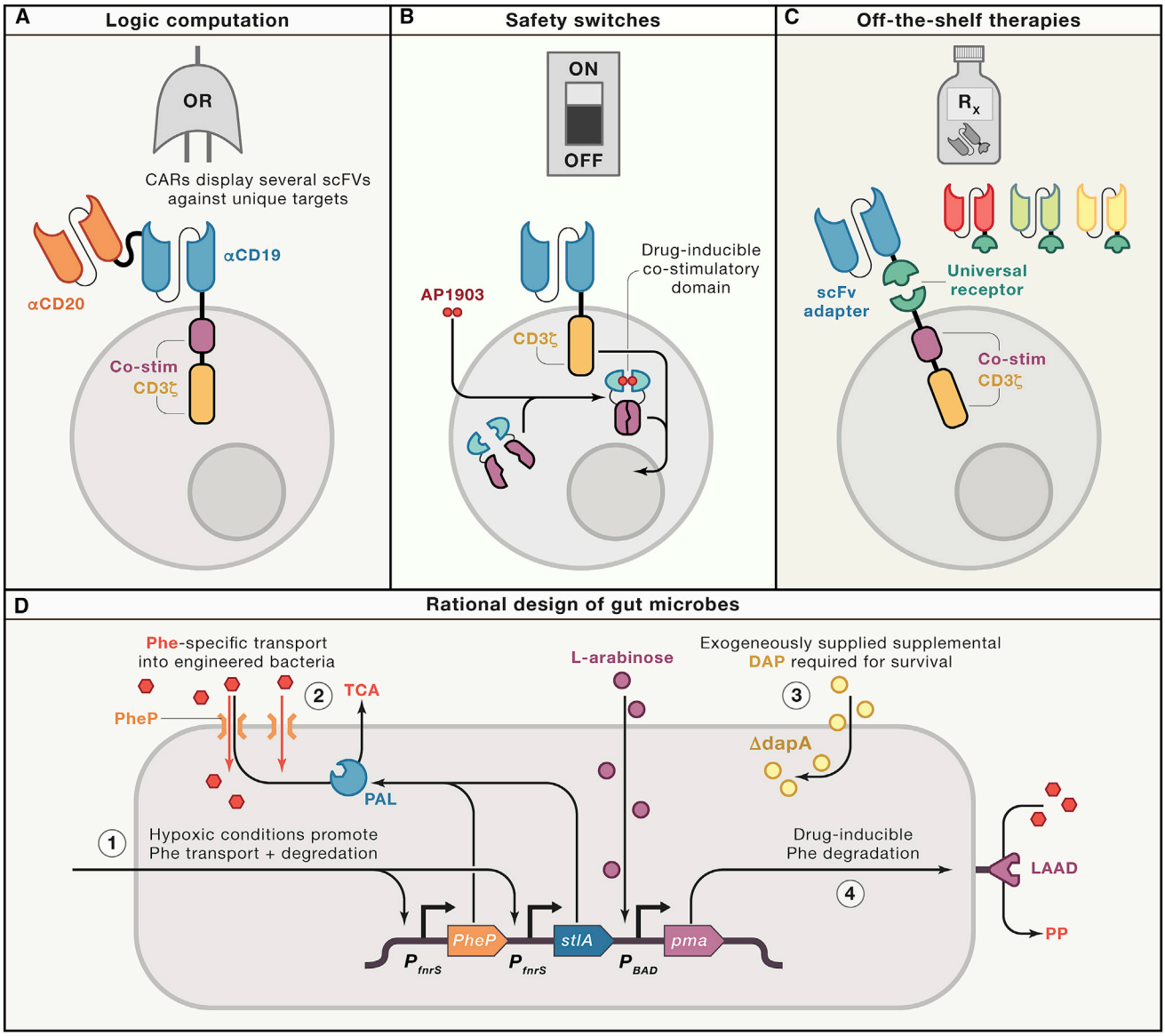

Figure 3. Expanding the repertoire of cellular therapeutics using synthetic biology

(A) Logic gates such as AND, NOT, and OR gates (shown here) allow greater specificity and range of antigens for targeting by engineered cells.
(B) Safety switches, such as drug-inducible kill switches and on/off switches (shown here), allow control over the duration or level of therapeutic activity.
(C) Universal receptors facilitate the switching of antigenic targets or alteration of activity levels by varying the types or amount of scFv adaptor molecules.
(D) Engineered synthetic microbes can help reestablish healthy microbial communities after antibiotic use, reduce levels of toxic or unmetabolized waste, or secrete therapeutic molecules for disease treatment. Shown here, SYNB1618 metabolizes excess Phe in PKU patients. Phe transport channels (PheP) and degrading enzymes (PAL) are placed under the control of hypoxia-sensing promoters (1) to limit the import and degradation of Phe (2) to the anaerobic gut environment and increase biomass yield during aerobic industrial production. The *E. coli* Nissle 1917 chassis is rendered auxotrophic as a biocontainment strategy by knocking out the *dapA* gene (3), necessitating supplemental diaminopimelate (DAP) for survival. Finally, supplemental Phe processing enzymes are placed under the P_{BAD} promoter to further tailor Phe degradation upon L-arabinose drug addition (4).

other immune activating and anti-apoptotic proteins (Deguine and Barton, 2014; Elgueta et al., 2009). Fusion of MyD88 and CD40 to two copies of FKBP12 (F36V) creates a costimulatory domain inducible by AP1903 binding and enhances T and natural killer (NK) cell residence time by promoting cell proliferation and CAR activation in murine models (Foster et al., 2017; Wang et al., 2020b). Cells co-transduced with iMC and a first generation CAR lacking co-stimulatory domains display little activation without dual CAR/iMC activation. T cells expressing a first-generation PSCA-specific CAR and the iMC go-switch demonstrated prolonged T cell residence time in patients, and 8 of 11 (66%) showed stable disease 9.8 weeks after infusion (Becerra et al., 2019).

Universal platforms for off-the-shelf cell therapies

Another strategy for controlling receptor-mediated cell therapy is to split the receptor into two separate pieces: a universal CAR

consisting of an intracellular signaling component plus an extracellular docking domain, and modular docking adapters used to select different targets and titrated to yield specific degrees of activation. Immune cells engineered to express universal CARs are unable to bind target antigens directly. Instead, they bind adaptor molecules comprised of an antigen-specific scFv attached to a docking ligand recognized by the CAR, such as leucine zippers (Cho et al., 2018), unique epitopes (Rodgers et al., 2016; Raj et al., 2019; Kudo et al., 2014), or chemical tags (Lohmueller et al., 2017; Tamada et al., 2012) (Figure 3C). Universal CARs allow continuous changes in the adaptor molecules given to patients in response to evolving or heterogeneous disease states and to control the level of immune-cell activation through different adaptor concentrations or binding strengths.

The first split CAR design to enter clinical testing is made by Cogent Bio (previously Unum Therapeutics) and fuses an antibody-binding CD16 domain to CD3ζ stimulatory and either 4-1BB (ACTR087) or CD28 (ACTR707) costimulatory domains. A significant benefit of this platform is the ability to use any antibody to control antigen targeting, and an infusion of CAR T cells can be redirected as needed by administering different antibodies. A similar platform was developed at the California Institute for Biomedical Research (Calibr). Their switchable CAR (sCAR) contains an extracellular scFv specific for a peptide neo-epitope (PNE) that can be conjugated to a second scFv or antibody that binds to a cancer cell target (Viaud et al., 2018). The PNE is linked to the scFv adaptor and is injected after sCAR T cell infusion to direct a tumor antigen-specific response. Mice bearing $CD19^{+}$ tumors survived up to 5 months after cotreatment with sCAR T cells and PNE adapters. Additionally, temporarily removing PNE adapters prior to repeat activation provided sCAR T cells with rest periods that were essential for their expansion and the induction of memory sCAR T cells.

In clinical trials of ACTR087 (NCT02776813) and ACTR707 (NCT03189836), up to 50% of patients showed a complete response after cotreatment with CAR Ts and the CD20 monoclonal antibody (mAb) rituximab, and engineered cells were detectable up to 1 year post infusion. These studies were halted by the FDA due to safety concerns including severe neurotoxicity (grade 3) and respiratory distress (grade 4) (Cogent Biosciences, 2019), but a third phase I clinical trial using ACTR707 with the HER2 mAb trastuzimab (NCT03680560) has successfully concluded with results pending (Cogent Biosciences, 2020). The PNE system from Calibr (Rodgers et al., 2016) was granted FDA clearance to begin testing in partnership with Abbvie (The Scripps Research Institute, 2020). Improved synthetic receptors promise to greatly increase the ability of clinicians to precisely tailor the timing, strength, and specificity of engineered CAR T cells. The highly flexible designs described above may lead to off-the-shelf cell therapies for cancer that significantly reduce development cost and time.

Engineered bacteria and microbial communities for disease treatment

As the intimate relationship between our resident bacteria and the myriad of human health states they influence continues to be explored, rationally designed microbes and synthetic microbial communities offer an advantageous platform for treating human inherited and communicable disease. Gut dysbiosis, characterized by significant perturbations in the normal microbial composition, has been linked to a number of disease states including CDI (Carding et al., 2015). Modeling the interactions and dynamics of bacteria and metabolites in the human gut has shed light on how dysbiosis promotes disease (Kumar et al., 2019; Magnúsdóttir and Thiele, 2018). In CDI, which affects ~10% of hospitalized intensive care unit patients worldwide (Balsells et al., 2019), lower biodiversity among Firmicutes and Bacteroidetes species following antibiotic use is a key indicator of increased susceptibility to infection (Jurburg et al., 2019; Buffie et al., 2015; Fletcher et al., 2018). Supplementation with Firmicutes that convert primary to secondary bile acids inhibits the growth of *C. difficile* up to 1,000-fold (Reed et al., 2020), and secondary bile acids, such as deoxycholate and lithocholate, can bind the *C. difficile* TcdB toxin and reduce its cytotoxicity (Tam et al., 2020). Using this information, synthetic microbial communities can be designed to colonize the human gut, outcompete *C. difficile* for its preferred carbon sources, and produce secondary bile acids to mitigate *C. difficile* growth and toxicity. Such an engineered solution could hold a significant advantage over antibiotic use for the treatment of recurrent CDI by addressing the underlying problem of dysbiosis and preventing *C. difficile* overgrowth, as well as offering a standardized manufactured treatment unlike donor-sourced fecal microbiota transfer.

A phase III clinical trial of Seres Therapeutics SER-109 (NCT03183128), a microbiome product containing bacterial spores purified from healthy human donors, showed that SER-109 reduces recurrent CDI after standard antibiotic treatment by ~30% compared with placebo (Seres Therapeutics, 2020). Seres SER-262 is a rationally designed synthetic microbial community designed to include highly prevalent bacterial species isolated from healthy donors from the Human Microbiome Project (Turnbaugh et al., 2007) and successful iterations of SER-109 (J.R. Wortman et al., 2016, American Society for Microbiology conference). SER-262 contains a proprietary blend of spores from 12 bacterial strains including several Firmicutes species from the Lachnospiraceae, Erysipelatrichaceae, Peptostreptococcaceae, and Clostridiaceae families (Ford et al., 2019). SER-262 is able to compete with *C. difficile* for up to 90% of its carbon sources, and 10 of 12 strains included in the synthetic community convert primary to secondary bile acids to inhibit the growth and reduce the toxicity of *C. difficile*. SER-262 was safe and well tolerated in patients in a phase I clinical trial (NCT02830542), but it did not meet its primary endpoint of reducing overall rates of CDI in patients following antibiotic treatment (Ford et al., 2019), although efficacy may differ depending on the specific antibiotic used.

Individual bacterial strains can also be engineered to treat human disease. Biocontainment is a necessary precaution to ensure the treatment does not become a pathogen (Pedrolli et al., 2019; Lee et al., 2018). One widely adopted approach is to render microbes auxotrophic, whereby a critical gene in the host bacteria is replaced with a therapeutic gene of interest, and the missing essential product is externally supplied or internally produced under the control of a drug- or environment-inducible rescue cassette. SYNB1618, a rationally designed clinical stage microbial therapeutic made by Synlogic, uses

auxotrophic biocontainment through deletion of the *dapA* gene encoding 4-hydroxytetrahydropicolinate synthase in *E. coli* Nissle 1917. This renders the bacteria dependent on supplemental diaminopimelate to maintain cell wall integrity (Isabella et al., 2018). SYNB1618 (Figure 3D) was engineered to treat phenylketonuria (PKU), a rare genetic disorder characterized by the inability to metabolize excess phenylalanine (Phe). Genes encoding PheP, a Phe transporter, and Phe ammonia lyase (PAL), a protein that converts Phe into easily metabolized *trans*-cinnamic acid (TCA), are placed under the control of the hypoxia-specific promoter P_{fnrS}, which limits their expression to the anaerobic gut environment. This allows for efficient uptake and processing of Phe in the gut while minimizing the metabolic burden on bacteria during manufacturing. Additionally, to allow Phe degradation in the aerobic portion of the stomach and small bowel, an L-amino acid deaminase (LAAD) gene under the control of the inducible P_{BAD} promoter metabolizes extracellular Phe upon addition of L-arabinose. SYNB1618 has demonstrated specific conversion of Phe to TCA and subsequently hippurate (HA) in mice (Isabella et al., 2018) and in a phase I/IIa trial (NCT03516487) of PKU patients and healthy controls (Synlogic, 2019).

Engineered microbes are also an attractive platform for the development of cancer treatments. Certain bacteria such as *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* naturally localize to the tumor microenvironment via unclear mechanisms, and several clinical trials utilize this innate ability to colonize tumors and induce local immune responses. An ongoing phase I trial (NCT03762291) investigates the utility of CVD908ssb-TXSVN, an attenuated *Salmonella* strain engineered to constitutively express the tumor-associated antiapoptotic antigen Survivin (BIRC5). Survivin has previously been used to elicit an immune response to Survivin+ tumors in both preclinical and clinical studies (Onodi et al., 2018). The engineered *Salmonella* displaying Survivin will naturally colonize tumor sites and are designed to promote cytotoxic T cell infiltration of tumors to enhance recognition and killing of Survivin+ tumor cells. Other approaches induce immune responses using secreted chimeric proteins. The biotechnology firm Advaxis engineered several *L. monocytogenes* strains to secrete a non-cytotoxic, highly immunogenic version of the Listeriolysin O protein fused with a tumor antigen, such as the HPV-16 E7 epitope found on HPV+ cervical carcinomas (ADXS-001) or prostate-specific antigen (PSA) (ADXS-PSA). In a phase II HPV+ cervical cancer trial (NCT01266460), administration of ADXS-001 led to an overall survival rate of 38.5% (10 of 26) at 12 months compared with 30% in the standard chemotherapy group (Basu et al., 2018). These technologies illustrate how synthetic biology can be harnessed to address unmet needs when traditional therapeutic options fail.

ENGINEERING THE FUTURE OF SYNTHETIC BIOLOGY: FROM BENCH TO BEDSIDE

From humble beginnings with the creation of bistable switches and oscillating circuits in *E. coli* to now enabling rapid development of vaccines, high-performance diagnostics, and the cure of previously untreatable cancers, tremendous progress has been made in synthetic biology and its applications over the past two decades. Because of its highly interdisciplinary nature, synthetic biology continues to develop together with advancements in both basic and applied research in biochemistry, microbiology, protein engineering, and systems biology, among others. For example, combined modeling techniques have led to the stabilization and expression of peptides not previously achievable and enabled the creation and clinical testing (NCT03814720) of a universal influenza vaccine candidate targeting the hemagglutinin stem (Corbett et al., 2019). Synthetic biologists have also incorporated non-biological inputs such as temperature, magnetic fields, light, electrical fields, and ultrasound (Weinberg et al., 2019; Guntas et al., 2015; Mannix et al., 2008; Weber et al., 2009; Pan et al., 2018) in addition to using increasingly complex genetic logic (Weinberg et al., 2017; Zúñiga et al., 2020; Hsiao et al., 2016) to create more robust biological computation and auto-regulating closed-loop circuits in functional cellular therapeutics (Ye et al., 2017). Ongoing efforts to concurrently engineer the mechanical (Ackerman et al., 2020), chemical (Fuchs et al., 2019), and biological (Wessels et al., 2020) aspects of molecular detection have led to the development of next generation diagnostics with greater accuracy, higher throughput, simpler operation, and lower cost.

It is clear that developments in computational biology will continue to have an outsized effect. In particular, machine learning has recently been applied to several aspects of synthetic biology (Volk et al., 2020) including finding (Padilha et al., 2020; Gussow et al., 2020; Eitzinger et al., 2020) or creating (Alley et al., 2019) novel biological components; characterizing, classifying, and improving their performance (Angenent-Mari et al., 2020; Valeri et al., 2020; Wang et al., 2020a; Yang et al., 2019), and guiding the selection of parts and design of complete gene circuits (Nielsen et al., 2016; Radivojević et al., 2020). These nascent techniques have only begun to be incorporated into the synthetic biology toolkit, and additional breakthroughs are sure to come. In the near term, this may take the form of concurrently evolved multifunctional proteins, metabolic pathways, or multicellular co-dependent synthetic consortia. Eventual applications may shift away from specific products and instead aim to create and stabilize specific physiological states, such as pan-resistance to all known infectious agents using a universal vaccine that regulates its own activity to account for variability in immune response due to genetic factors such as HLA type (Leitman et al., 2016) and acquired factors such as prior exposure to infectious agents (Linderman and Hensley, 2016; Benn et al., 2020). Another goal may be the creation of a tumor-free state via automated surveillance and destruction of all neoplastic cells by universal engineered immune cells.

Regardless of the specific application, the use of synthetic biology also illustrates the accelerating pace of technological innovation and adoption with highly productive partnerships between academia and industry. For example, CRISPR-based collateral cleavage was first described in 2017, led to the creation of two biotechnology startups within 1 year with a combined capitalization of $124 million, and two FDA-approved SARS-CoV-2 diagnostics by 2020. Additionally, multiple functional vaccines against the newly discovered SARS-CoV-2 virus were developed, tested in clinical trials, granted emergency approval,

and deployed within 10 months. Given the extraordinary progress thus far and numerous ongoing efforts in diverse applications, synthetic biology is sure to have a continued and lasting impact on the future of biomedical enterprise.

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DECLARATION OF INTERESTS

X.T. is a consultant for Sherlock Biosciences. J.J.C. is a co-founder of Senti Biosciences, Sherlock Biosciences, and Synlogic. W.W.W. is a co-founder of Senti Biosciences.

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