Mammalian Synthetic Biology: Engineering Biological Systems

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Abstract

The programming of new functions into mammalian cells has tremendous application in research and medicine. Continued improvements in the capacity to sequence and synthesize DNA have rapidly increased our understanding of mechanisms of gene function and regulation on a genome-wide scale and have expanded the set of genetic components available for programming cell biology. The invention of new research tools, including targetable DNA-binding systems such as CRISPR/Cas9 and sensor-actuator devices that can recognize and respond to diverse chemical, mechanical, and optical inputs, has enabled precise control of complex cellular behaviors at unprecedented spatial and temporal resolution. These tools have been critical for the expansion of synthetic biology techniques from prokaryotic and lower eukaryotic hosts to mammalian systems. Recent progress in the development of genome and epigenome editing tools and in the engineering of designer cells with programmable genetic circuits is expanding approaches to prevent, diagnose, and treat disease and to establish personalized theranostic strategies for next-generation medicines. This review summarizes the development of these enabling technologies and their application to transforming mammalian synthetic biology into a distinct field in research and medicine.

Contents

1. INTRODUCTION

Synthetic biology aims to create new biological functions through the design and controlled assembly of genetic circuits. A genetic circuit is a combination of biological parts that together execute a defined function within a host organism. By deconstructing natural genetic circuits that have been refined by evolution and reconstructing them from modular components, synthetic biologists can gain insight into the structure–function relationship of natural biological systems and use this information to build systems with novel activity (1). Due to their robustness and low complexity, prokaryotic and lower eukaryotic host organisms served as the first platforms for building synthetic gene networks. Initial research demonstrated that gene circuits could be built to execute precise functions and recapitulate patterns of natural biological systems, including oscillating gene expression networks, multistate toggle switches, logic computation, and intercellular signaling networks (1–4). Inspired by these early successes, synthetic biologists have made significant progress in developing a wide range of modular genetic parts with standardized design and connectivity principles to streamline the construction of novel circuits with greater complexity. To date, this research has been successful in programming diverse cellular behaviors for applications in basic research, industry, and medicine (5, 6).

Gene circuit: a combination of biological parts (DNA, RNA, or protein) programmed to execute functions within a cell

As applications in synthetic biology advance into mammalian host organisms, the suite of genetic modules that form the basis of circuit architecture must evolve to adapt to the complex hierarchical regulation that governs cell phenotype. Mammalian cell gene networks are highly complex and involve regulation on the transcriptional, translational, and posttranslational levels. Consequently, synthetic biology efforts in mammalian cells require a set of precise and scalable tools to characterize and control gene expression and function. With the development of genome engineering tools, such as CRISPR/Cas9 (clustered regularly interspaced short

palindromic repeats/CRISPR-associated protein 9), researchers have begun to interrogate and control gene function and network dynamics (7). The CRISPR/Cas9 system in particular has served as the basis for the development of tools enabling programmable and site-specific control of chromatin and transcriptional states (8–10). This precise control has enabled the construction of multilayered gene circuits with higher-order functions in mammalian cells (11, 12). Moving beyond programming autonomous circuits in single cells, several research groups have recently made advances in the programming of synthetic intercellular communications to generate multicellular structures and organoids (13, 14). This recent research highlights the utility of synthetic biology approaches in tissue engineering and regenerative medicine applications.

Clustered regularly interspaced short palindromic repeats (CRISPR): bacterial and archaeal adaptive immune systems repurposed for gene editing and transcriptional regulation

Synthetic biology is a highly interdisciplinary field of research that integrates information and tools gained from basic research, technology development, and computational modeling. Thus, continued advances in synthetic biology will depend on coordinated and paralleled advances within a diverse realm of disciplines. In this review, we provide an overview of the techniques and tools that form the foundation of mammalian synthetic biology, describe applications in basic research and next-generation therapeutics, discuss the future outlook, and note important challenges ahead in the field.

2. METHODS AND TOOLS TO CHARACTERIZE AND MODULATE TRANSCRIPTIONAL ELEMENTS

The regulation of mammalian genomes involves a complex interaction among genetic sequence, chromatin structure, and tissue-specific transcription factors that coordinate to define the epigenome and impart diverse cellular phenotypes (15). Thus, methods and technologies to characterize and modulate gene expression in biological contexts are necessary to build synthetic circuits with predictable activity. Large-scale research projects such as the Encyclopedia of DNA Elements (ENCODE) (16) and the Roadmap Epigenomics Project (17, 18) have utilized next-generation sequencing (NGS) technologies in combination with advanced molecular analyses to map genome-wide epigenomic and transcriptomic states. Collectively, these projects are enabling the construction of integrated annotations of genome structure and function in mammalian cell types (19). Importantly, coordinated advances in bioinformatics have established data analysis pipelines, such as the widely adopted software ChromHMM, to compile and integrate these large data sets to categorize and predict epigenetic signatures of functional DNA elements (20).

The advent of NGS technologies, coupled with advances in de novo array-based oligonucleotide synthesis (21), has enabled researchers to test hypotheses on a high-throughput scale, facilitating the rapid acquisition of new functional genetic information (22). The exponential increase in NGS capabilities and DNA synthesis technologies are complemented by improvements in the assembly of large DNA constructs, including methods such as isothermal and Golden Gate assembly (23, 24). In parallel, new frameworks and protocols to construct multicomponent circuits with predictable behaviors from a hierarchical library of genetic parts provide standardization of circuit construction and establish synthetic biology as an engineering discipline (25).

2.1. High-Throughput Profiling of DNA Regulatory Elements

The complex cellular mechanisms that govern the regulation of any particular gene expression cassette in mammalian cells are still poorly understood and are therefore unpredictable in many cases. Thus, developments in the design and assembly of biological circuits must be accompanied by continued characterization of genetic modules imparting diverse regulatory functions. The systematic dissection and quantitation of the function of diverse DNA regulatory modules, such

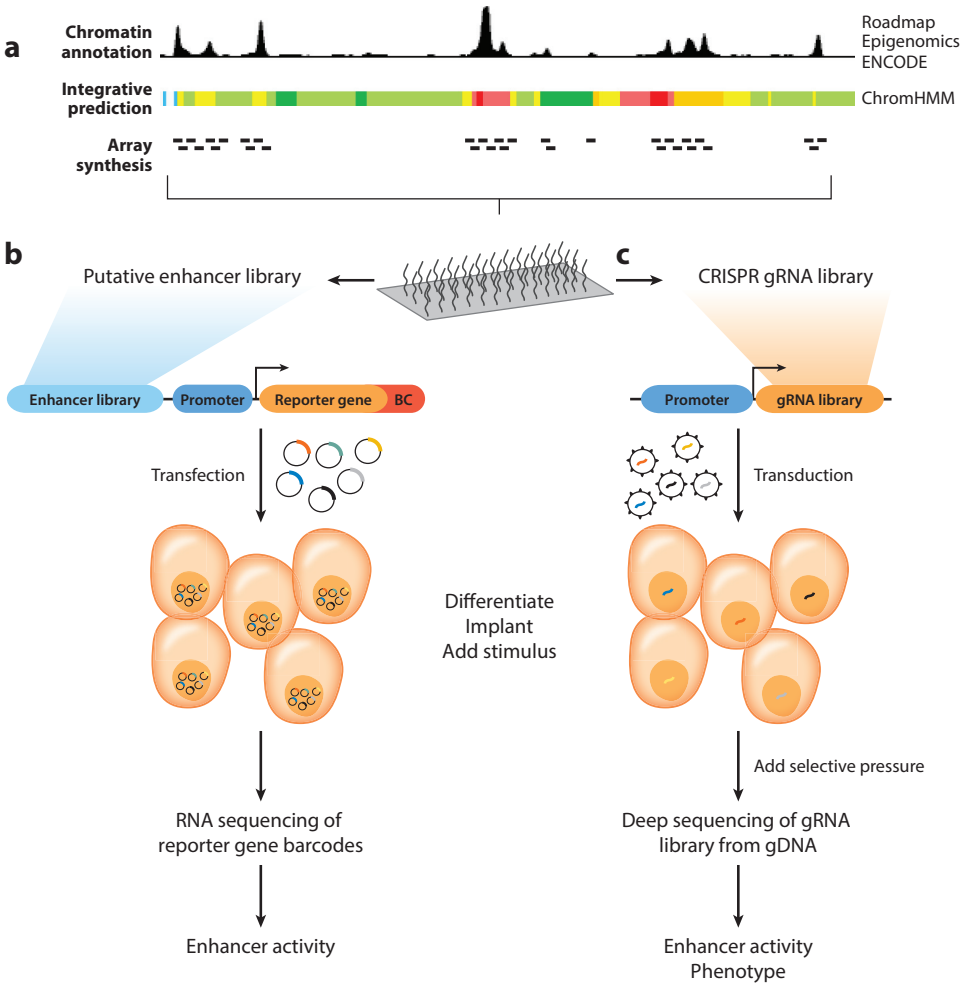

Figure 1

High-throughput characterization of genetic elements in mammalian cells. (*a*) International research initiatives such as the ENCODE Project and the Roadmap Epigenomics Project have provided annotated chromatin profiles of many human cell types. Bioinformatic pipelines, such as the software ChromHMM (20), have integrated these data sets to predict the functional potential of DNA elements on the basis of their chromatin signatures. Tools in synthetic biology, such as array-based synthesis and the CRISPR/Cas9 gene-editing platform, have enabled the functional dissection of diverse DNA elements on a high-throughput scale. (*b*) Massively parallel reporter assays assemble thousands of unique DNA sequences into a reporter vector and use next-generation sequencing to quantify their transcriptional activity. (*c*) The CRISPR system permits high-throughput interrogation of DNA elements in their genomic context via targeted mutagenesis using pooled gRNA libraries. Both of these approaches can be applied in vitro and in vivo to characterize functional genetic elements in diverse cellular contexts. Abbreviations: BC, bar code; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; gDNA, genomic DNA; gRNA, guide RNA.

as promoters and enhancers, in high throughput and at high sensitivity provide a set of genetic components for customizable circuit construction. NGS technologies combined with recent advances in oligonucleotide synthesis allow high-throughput analysis of the regulatory potential of hundreds of thousands of unique genetic elements in parallel (**Figure 1**). Patwardhan et al. (26) first described a technique to quantify the transcriptional activity of a library of promoters by sequencing uniquely bar-coded transcripts that align to each promoter variant. These authors applied this strategy to single and combinatorial saturating mutagenesis of bacteriophage and mammalian promoter sequences and identified sequence variants that reduced, enhanced, or maintained transcriptional activity (26). Known as a massively parallel reporter assay (MPRA), this approach has now been applied to dissect the function of natural and synthetic DNA elements in vitro and in vivo (27, 28).

Massively parallel reporter assay (MPRA): high-throughput technology to evaluate activity of putative enhancer elements

DNA-binding domain (DBD): platform for sequence-specific DNA targeting

By quantifying the regulatory function of many individual DNA sequences, MPRAs provide a framework to construct a library of regulatory elements with defined transcriptional activity. They also serve as empirical screening platforms to optimize activity in a particular cellular context (29). More recent research has dissected the function of DNA elements in the context of specific signaling pathways or stimulus-responsive cellular phenotypes (30), which will provide additional options for context-specific function in the design of genetic circuits. Therefore, MRPAs are particularly useful in advancing the characterization of DNA modules to construct biological devices with predictable function for use in synthetic biology.

MPRA approaches are limited in that they quantify the activity of DNA elements outside of their natural genomic context and thus are unlikely to capture the influence of chromatin and local positioning within the genome. Murtha et al. (31) developed an assay to measure the activity of putative regulatory elements in a genomic context using integration of a lentiviral library. Although this method is useful for defining active regulatory elements in specific cell types, the random integration of lentiviral vectors into the genome is likely unable to recapitulate local chromosomal effects on gene expression (31, 32). In order to characterize the function of regulatory elements of mammalian cells in their natural chromosomal position, more recent efforts have focused on genome and epigenome engineering with CRISPR/Cas9 or other DNA-targeting tools.

2.2. Programmable DNA-Targeting Systems

The advent of technologies for engineering programmable DNA-binding domains (DBDs) permits targeting and interrogation of genomic elements in their native chromatin environment (**Figure 2**). DBDs based on the zinc-finger proteins (ZFPs), transcriptional activator–like effectors (TALEs), and the CRISPR/Cas system provide platforms for programmable DNA binding and have been adapted for diverse functionality in mammalian cells (33). The ZFP and TALE platforms were engineered as programmable endonucleases by fusion to the catalytic domain of the FokI nuclease (34–37), and the Cas9 protein harbors intrinsic endonuclease activity and is guided to a genomic site by an engineered guide RNA (gRNA) for targeted genome editing (38–40). Gene editing has been utilized for targeted mutagenesis of coding and noncoding genetic elements and for the insertion of transgenes at precise genomic locations with diverse applications in basic research and medicine (10, 41). Because target site specificity of the CRISPR system is programmed by an RNA molecule, libraries of oligonucleotides can be synthesized to encode hundreds of thousands of unique target sites for the high-throughput perturbation of DNA elements (**Figure 1**) (42), described in further detail in Section 4.1.

DBDs have also been adapted for targeted transcriptional regulation by fusion to transactivating and repressing scaffold domains (11, 43–50). These synthetic transcription factors are superior in many ways to nonprogrammable DBDs adapted for gene regulation, such as LacR and tTA

(51, 52), as they can target genomic sites and can be applied concurrently for multiplexed gene regulation. Although initial iterations required codelivery of multiple engineered factors to achieve robust transcriptional activation (48, 49, 53, 54), next-generation activators require only a single target site (55–57), which reduces the number of necessary components and therefore facilitates multiplexed targeting and the construction of multilayered circuits. Transcriptional activation has also been achieved by fusing self-association domains to DBDs to tether an enhancer to its corresponding promoter (58). Importantly, the genome-wide specificity of these tools has proven to be exceptionally precise, offering the ability to manipulate the expression of only a single target gene (49, 57–60). Together with targeted gene editing, transcriptional regulation via DBD-based fusions permits the control of mammalian genomes and epigenomes on multiple scales of gene regulation (**Figure 2**) (9, 11).

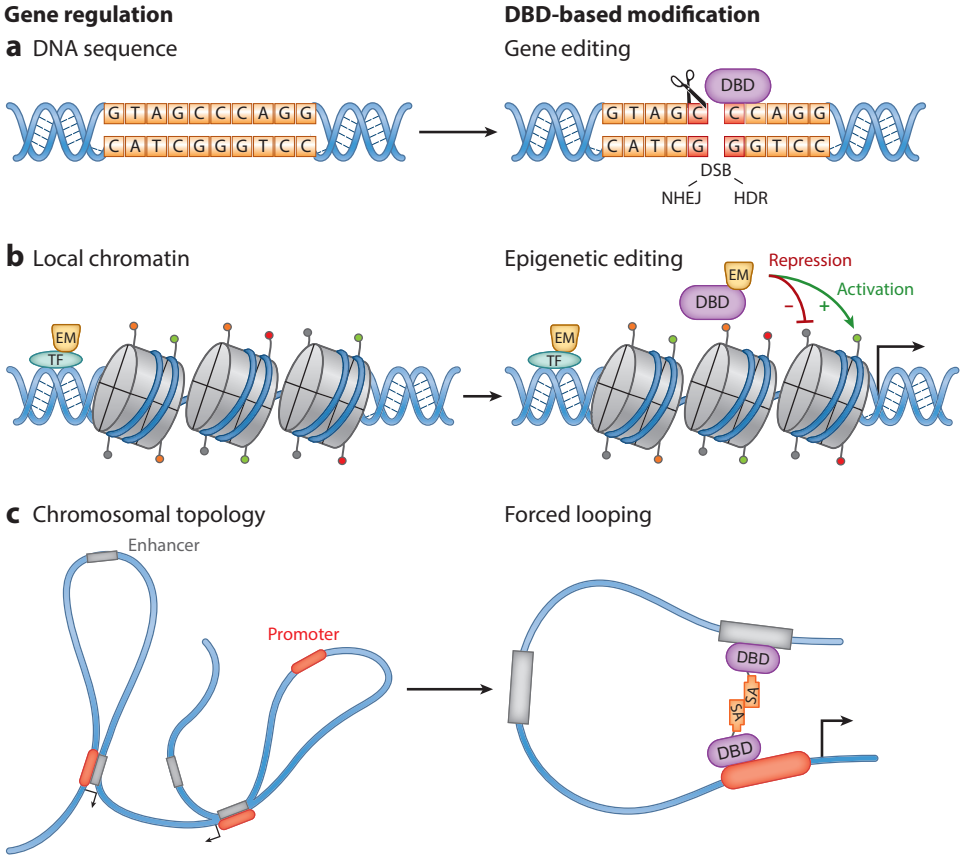

Figure 2

Hierarchical regulation of chromatin structure and gene expression with engineered factors. DBD technologies, such as zinc-finger proteins, TALEs, and the CRISPR/Cas9 system, enable synthetic biologists to edit the mammalian genome and epigenome on multiple scales, including modification of (*a*) the underlying DNA sequence, (*b*) the local chromatin environment, and (*c*) the three-dimensional chromosomal topology. Abbreviations: CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; DBD, DNA-binding domain; DSB, double-strand break; EM, epigenetic modifier; HDR, homology-directed repair; NHEJ, nonhomologous end joining; SA, self-association domain; TALE, transcriptional activator–like effector; TF, transcription factor.

A requisite for encoding multilayered operations in synthetic circuits is the use of orthogonal modules that can concurrently execute diverse functions. Coexpression of multiple ZFPs or TALEs can impart distinct gene regulatory effects at different loci, as each DBD is engineered to target a unique DNA sequence (61). Cas9 lacks inherent orthogonality, as it will indiscriminately associate with all coexpressed gRNAs. Orthogonality can be obtained by using Cas9 variants from different species (62), gRNAs with orthogonal aptamers that recruit gene regulation domains (63), or Cas9s with altered PAM (protospacer-adjacent motif) specificities (64). Alternatively, gRNAs of different lengths can be used for orthogonal gene editing and gene regulation by use of the same Cas9 protein engineered to execute both functions (65, 66). In addition to recruiting orthogonal effector proteins, gRNAs have been engineered as a scaffold for the attachment of long noncoding RNAs (67), enabling the programming of diverse gene regulatory functions through recruitment of endogenous RNAs and RNA-containing complexes.

Orthogonal genetic devices: synthetic circuits whose activities do not influence the activity of other systems

Tunable and conditional control over the components of synthetic circuits permits the engineering of more extensible functionalities with improved spatial and temporal control of activity. All three programmable DBD platforms have been engineered for light-dependent control of gene editing and/or gene expression in mammalian cells and in vivo (68–73). ZFP and TALE activators have also been engineered to respond to chemical inputs (74–76), and several systems were recently demonstrated to regulate Cas9 activity in response to small molecules (77–80). Oakes et al. (81) assessed the effects of insertional mutagenesis on Cas9 activity, and identified sites within the protein amenable to sequence insertions. These authors inserted the estrogen receptor α ligand-binding domain within the Cas9 protein to impart allosteric regulation of activity in response to 4-hydroxytamoxifen (81).

The expression of the CRISPR gRNA from constitutive RNA polymerase III (Pol III) promoters, as done in most studies thus far, precludes inducible or tissue-specific regulation. Recent efforts have successfully expressed gRNAs from RNA polymerase II (Pol II) promoters by using flanking ribozymes or by positioning the gRNAs within introns of protein-coding genes in order to separate the gRNA from the capped and polyadenylated messenger RNA (mRNA) (11, 82). More recently, Liu et al. (83) built reprogrammed gRNAs modified with riboswitches to control gene regulatory activity in response to different signal inducers. These allosterically regulated gRNAs mediated activation and repression of endogenous genes in response to exogenous small molecules and endogenous signaling molecules. Importantly, the gRNA function is controlled posttranscriptionally, enabling a more rapid response to fluctuations in availability of the inducer.

The application of DBDs fused to catalytic epigenome-modifying domains that write or erase posttranslational changes to histone marks and DNA methylation states may enable the programming of more dynamic and diverse modes of gene regulation into synthetic gene circuits (8, 10). Two recent studies demonstrated that different epigenetic and transactivating effector domains could distinctly regulate gene expression from particular regulatory elements (57, 84). Hilton et al. (57) observed that fusion of the acetyltransferase core domain of the human EP300 protein to DBDs enabled targeted transcriptional activation of endogenous genes from proximal promoters and distal enhancers. Interestingly, fusion of the transcriptional activating scaffold domain VP64 to DBDs failed to activate gene expression from human distal enhancers in this study. Similarly, Kearns et al. (84) observed that fusion of the histone demethylase lysine-specific demethylase 1A (LSD1) to deactivated Cas9 (dCas9) could discriminately silence expression of *Oct4* from a distal enhancer but had no effect when targeted to a proximal regulatory element. The ability to discriminately modulate gene expression from diverse classes of genetic regulatory elements, as demonstrated in these studies, could enable the programming of more dynamic expression patterns that mimic the complex coordination of proximal and distal elements during differentiation

and development. These efforts will be accelerated by more comprehensive evaluations of many different epigenome-modifying effectors in different biological contexts (85).

The stability and heritability of the epigenetic and transcriptional effects induced by programmable transcription factors can vary depending on the effector domain used. For instance, targeting of the oncogene *SOX2* in breast tumor cells with a DNA methyltransferase 3A fusion to a ZFP led to stable repression that was maintained through cell divisions after the synthetic transcription factor was silenced (86, 87). Interestingly, fusion of the Krüppel-associated box (KRAB) heterochromatin-forming domain to the same ZFP resulted in transient silencing of the target gene that was lost upon depletion of the synthetic factor (86). In contrast, other studies have detected restoration of the chromatin state and gene expression level following transient delivery of targeting methyltransferases (88, 89). These conflicting observations might be explained by varying experimental conditions or cell type– and locus-specific regulatory mechanisms, which cannot be accurately predicted at this time. More recently, Amabile et al. (90) described an approach to achieve stable transcriptional silencing using transient delivery of three engineered repressors. Repression was heritable, highly specific, and reversed only via targeted demethylation (90). This type of heritable gene silencing could provide an approach to construct gene circuits that encode more complex behaviors, such as transcriptional memory or adaptation in response to transient stimuli.

3. DESIGN AND CONSTRUCTION OF SYNTHETIC CIRCUITS

3.1. Basic Transcriptional Circuits Constructed with DNA-Targeting Systems

The efficacy and versatility of DBD-based transcriptional regulation have led to its application in the wiring of transcriptional circuits in mammalian cells that impart precise functions and logic computation in order to control cellular phenotype. DBDs that lack sequence programmability have been incorporated into genetic circuits in mammalian cells that compute Boolean logic, produce time-delayed and oscillatory responses, and regulate bistable switches (91–95). There are also a diversity of ligand-responsive variants of these DBDs that enable inducible control and independent integration of multicomponent transcriptional units (96). However, these systems are limited to transcriptional control via synthetic promoters.

Because they can be designed to recognize any target DNA sequence of interest, programmable DBDs may enable the construction of more versatile genetic circuits that execute orthogonal functions and interface with regulation of the host genome. For example, ZFPs and TALEs have been used to construct multi-input logic gates in mammalian cells (97–99). The CRISPR/Cas9 system is particularly appealing for use in genetic circuits because new target sites can be encoded on a small RNA molecule, which minimizes the amount of genetic material that needs to be added to enhance circuit complexity. Kiani et al. (100) exploited the versatility of the CRISPR system to construct layered circuits using multiple gRNAs expressed from Pol II and Pol III promoters. Expression of gRNAs from Pol II promoters permits more dynamic control over gRNA expression and could interface with existing circuitries that use these promoters. Recently, Nissim et al. (11) provided a CRISPR/Cas9 tool kit for the construction of multilayered transcriptional circuits. In this study, the authors demonstrated multiplex expression of gRNAs from a single Pol II promoter and successfully constructed layered circuits with Pol II–only expression (11). Importantly, these authors were able to construct a circuit that integrated gRNA and microRNA (miRNA) activities to implement multioutput behaviors via distinct regulatory mechanisms.

3.2. RNA-Based Devices

RNA molecules found in nature coordinate diverse cellular processes through the transduction of extracellular and intracellular signals to modulate gene expression and function. RNA has

naturally evolved as sensor-actuator devices involved in diverse signaling and regulatory pathways. RNA can functionally interact with nucleic acids, proteins, and small molecules, enabling gene regulatory potential on the transcriptional, translational, and posttranslational levels (101). In addition, RNA structures are composed of combinations of only four fundamental nucleotides, reducing the parameter space and enabling more scalable and standardized approaches to the design and assembly of RNA-based synthetic devices (102).

The modular nature of RNA devices permits the assembly of RNA components that detect a wide array of signal inputs and execute diverse gene regulatory functions. A novel RNA device can be assembled from an existing set of defined sensing and regulatory modules or created using selection methods. Techniques such as systematic evolution of ligands by exponential enrichment (SELEX) screen libraries of RNA molecules against varied substrates to identify novel binding interactions (103). The linking of modular components to create multifunctional RNA devices can be achieved through rational design of linker sequences or through selection methods. Several groups have developed high-throughput methods and computational models for RNA device engineering (104, 105). Townshend et al. (105) utilized NGS technologies to develop a method to screen the activity of hundreds of thousands of unique RNA devices based on the hammerhead ribozyme. Notably, this platform can screen sequence variants that modulate RNA tertiary interactions, expanding the parameter space to identify aptamer pairs that sense and respond to stimuli with faster kinetics (105).

Synthetic RNA devices have been developed to modulate gene expression in mammalian cells through varied regulatory mechanisms on the levels of transcription, RNA splicing, mRNA stability, translation, and posttranslational processes for diverse applications in basic research and biomedicine (106). On the basis of this framework, synthetic RNA devices have been developed to control cell cycle dynamics (107, 108), regulate viral gene expression (109, 110), and modulate RNA interference (RNAi) activity (111), among other applications. Importantly, the use of circuits encoded by RNA can avoid immunogenicity of protein components and mitigate risks of genomic integration by DNA vectors. In addition to improving safety by preventing genomic integration, RNA-encoded circuits are transiently expressed, which may facilitate applications requiring rapid dynamics or the translation to therapeutic applications by reducing the likelihood of off-target activity associated with prolonged expression.

3.3. Engineering Synthetic Signaling Pathways

Natural biological processes often depend on the coordination of multiple different inputs in precise organization in order to execute complex cellular behaviors. To faithfully recapitulate these processes, synthetic biologists need tools to regulate cell signaling and phenotype at high spatial and temporal resolution. To this end, several groups have successfully built synthetic tools to link precisely controlled extracellular stimuli to intracellular signaling networks to regulate gene expression patterns and cell phenotypes (**Figure 3**).

Cells use mechanically sensitive receptors to survey their extracellular microenvironment in order to regulate diverse cellular processes (112). Thus, synthetic control of mechanical signaling could provide a means of programming cell behaviors. To this end, Seo et al. (113) developed a mechanically controlled signal transducer in mammalian cells by using micromagnetic tweezers to control positioning and force transduction of magnetoplasmonic nanoparticles. By functionalizing these particles with chemical ligands, these authors were able to determine the spatial and mechanical influences of Notch- and E-cadherin-mediated signal transduction in single cells (113). Similarly, mechanical inputs to cells have been controlled via light and ultrasonic pulses (114, 115). An important commonality between these methods of controlling mechanical signaling

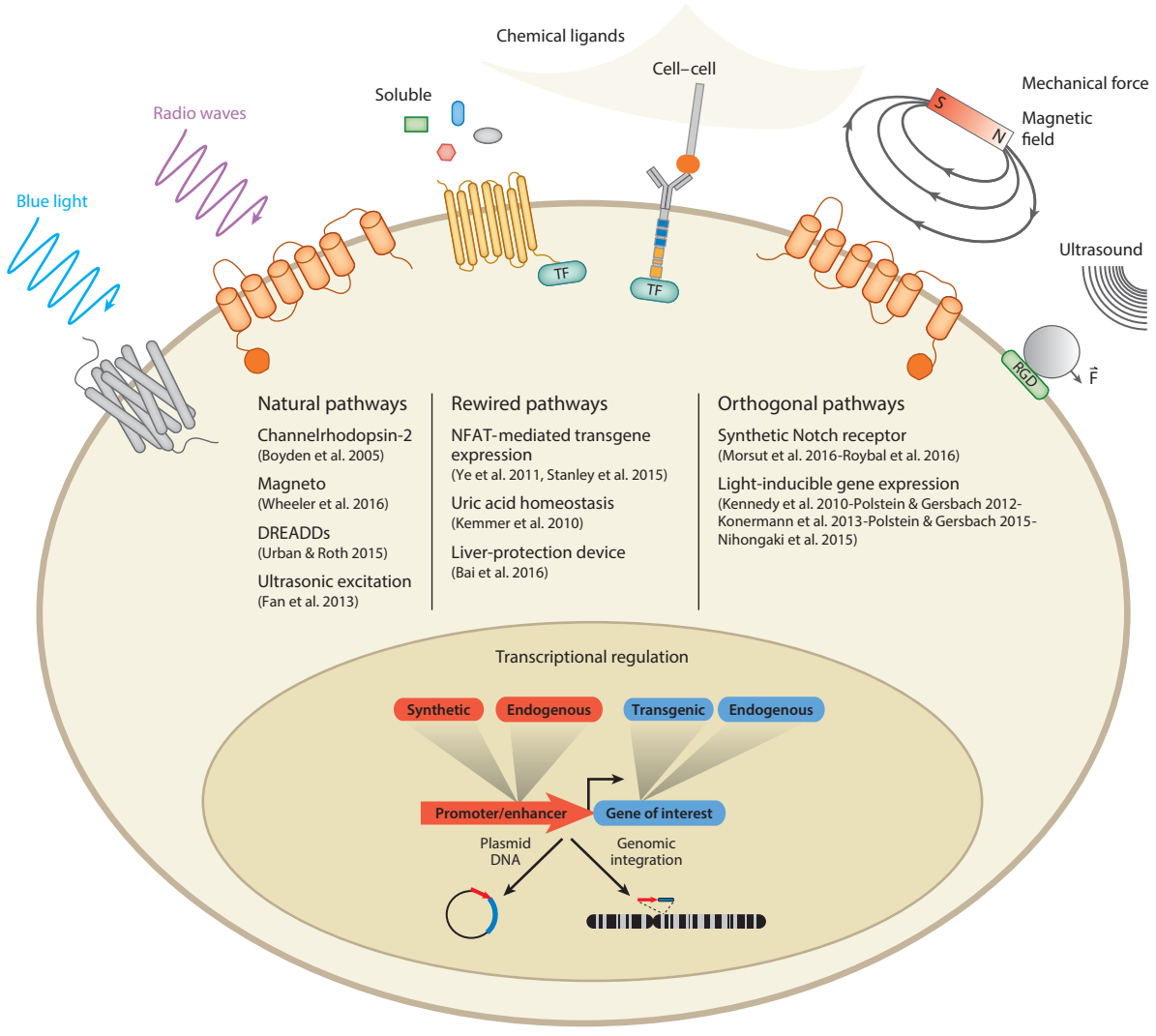

Figure 3

Synthetic signaling pathways. Several tools have been developed to control cellular signaling, gene expression, and phenotype at high spatial and temporal resolution in response to chemical, mechanical, and optical inputs. Many of these technologies are genetically encoded, and thus can be used for cell type–specific control. Synthetic signaling pathways can be assembled by (1) inducing natural endogenous signaling pathways, (2) rewiring endogenous signaling, or (3) providing entirely orthogonal pathways. These engineered signaling pathways commonly regulate cell activity via synthetic transcriptional circuits. Abbreviations: DREADD, designer receptor exclusively activated by designer drugs; NFAT, nuclear factor of activated T cells; TF, transcription factor.

is that they do not require genetic manipulation of the host cells. Although delivery of genetic components can be a challenge that limits the extensibility of experimental applications, genetically encoded systems have the advantage of imparting cell type–specific responses with dynamic, multicomponent regulatory potential. Consequently, several groups have developed strategies for precise spatiotemporal control of cell signaling and gene expression using genetically encoded actuator technologies.

In 2005, Boyden et al. (116) demonstrated light-inducible control of cell membrane potential depolarization using ectopic expression of Channelrhodopsin-2 in neurons, establishing the field of optogenetics. The high-resolution control of neural activity along with a genetically encoded single-component system enabled researchers to map the contribution of neuronal subtypes in establishing the behavior of neural networks. Subsequently, many research groups have refined and expanded this approach to the study of neural activity in diverse applications in cell and animal models (117). Other technologies have harnessed light-inducible modules from plant species to induce protein–protein interactions to control cell signaling, DNA recombination, and transcriptional regulation with light (68, 69, 118–120). Exposure to engineered cells by applying light across the skin, into the eye, or via implanted light-emitting devices has even enabled the optogenetic control of gene expression systems in vivo for biomedical applications (120, 121).

Recently, Stanley et al. (122) described a genetically encoded system for the control of calcium signaling through exposure to radio frequencies or a magnetic field. These authors tethered the magnetically sensitive ferritin protein to the transient receptor potential cation channel subfamily V member 1 (TRPV1) cation channel to program calcium-mediated regulation of a therapeutic transgene (122). Because radio waves are noninvasive and can penetrate biological tissue, they permitted control of an insulin transgene in vivo in a mouse model of hyperglycemia (122). A subsequent study used a similar approach to engineer a magnetically sensitive TRPV4 cation channel to control calcium signaling in neurons (123). Importantly, the authors of this study observed that the engineered TRPV4 channel remained responsive to endogenous stimuli, underscoring the advantage of using orthogonal components when designing synthetic systems to limit cross talk or unpredictable activity (123).

Genetically encoded actuators that respond to chemical, magnetic, or optical inputs provide a diverse set of synthetic tools for control of cellular signaling and gene expression at high spatial and temporal resolution (**Figure 3**). The exclusive use of genetically encoded components enables researchers to incorporate prolonged and dynamic expression patterns in a cell type–specific manner. An important consideration when designing and applying a genetically encoded actuator to mammalian cells is how the system interfaces with endogenous signaling pathways. Several actuator technologies use an orthogonal receptor to transduce a precisely controlled stimulus as a means of regulating naturally occurring endogenous pathways. Orthogonal receptors are advantageous because they lack sensitivity to endogenous stimuli, and thus can respond with high specificity to the ectopically programmed input. Dong et al. (124) developed a pipeline to engineer novel receptor–ligand pairs by using directed molecular evolution of G protein–coupled receptors in yeast, and then validated their activity in mammalian cells. These designer receptors exclusively activated by designer drugs (DREADDs) permit control of a particular signaling pathway in a cell type–specific manner without undesired off-target effects or high basal activity (124, 125).

Alternatively, other technologies for genetically encoded actuators rewire or repurpose endogenous signaling pathways or create entirely orthogonal pathways. For instance, synthetic transcription factors can be tethered to the intracellular domain of engineered transmembrane receptors (126, 127). Receptor activation initiates cleavage and subsequent release of the transcription factor, which shuttles to the nucleus to regulate gene expression. These synthetic receptors can be enginered to respond to different soluble cues, providing an extensible method to rewire diverse input–output relationships. More recently, Morsut et al. (13) engineered the Notch receptor to respond to mechanical forces applied by novel cell-surface ligands and initiate varied downstream responses, discussed in more detail in Section 4.2.

The utility of genetically encoded actuator technologies for applications in basic research and biomedicine depends on the modular nature of the components and the degree of spatial and temporal resolution attained. It is important to define genetic control modules to interface

these technologies with precise cell type–specific expression patterns. Thus, continued advances in functional genomics to characterize cell type– and stimulus-specific regulatory element activity will help provide gene regulatory cassettes for applications in synthetic biology (10).

3.4. Resources for the Modular Design of Synthetic Gene Circuits

A goal of synthetic biology is to develop platforms that facilitate prototyping and validation of synthetic circuits in a controlled manner. Duportet et al. (128) developed a framework for the standardized construction and validation of synthetic gene circuits. These authors engineered cell lines with landing pads for the site-directed insertion of large genetic circuits via Bxb1 site-specific recombination. This method enabled efficient insertion of gene constructs that resulted in homogeneous transgene expression across a population of edited cells (128). In addition, circuit design and construction can be expedited through the use of in silico models to assemble multicomponent systems and accurately predict their behavior. Davidsohn et al. (129) developed an algorithm, termed empirical quantitative incremental prediction (EQuIP), to predict gene expression patterns within a synthetic circuit based on the genetic components used and the empirically derived models of activity. Finally, several groups have made advances in the assembly of large circuits (25) and the production of new sensor components optimized for mammalian systems (130). Some synthetic tools, such as programmable transcription factors, follow a set of engineering principles in their design and construction, but the design of an entire regulatory circuit with defined behavior remains an ongoing challenge in synthetic biology (131).

4. APPLICATIONS IN BASIC RESEARCH AND ENGINEERING

4.1. Dissecting Mechanisms of Genomic and Epigenomic Regulation

In addition to providing a tool kit to construct transcriptional circuits with programmable behaviors, DBD-based synthetic transcription factors and nucleases provide tools to uncover mechanisms of epigenetic and transcriptional regulation and map interaction nodes within signaling networks. The knowledge thus gained regarding the mechanisms of gene regulation can then feed back on the circuit design to incorporate novel regulatory functions. The highly scalable and versatile targeting of the CRISPR system permits the synthesis of gRNAs for genome-wide gain-of-function and loss-of-function screening (55, 56, 132, 133). The extensive protein engineering required to construct a ZFP or TALE has limited its application in large-scale genomic perturbation, although TALE–LSD1 fusions have been used to screen putative enhancers in low throughput (134).

CRISPR screens have been employed to identify genes involved in viability and growth (55, 132, 133), drug resistance (56, 132), in vivo tumor maturation and metastasis (135), and immune cell inflammatory signaling networks (136), among other applications. More recently, several groups have performed screens targeting annotated regulatory elements or transcription factor–binding sites (137–141). Interestingly, Rajagopal et al. (138) discovered that targeted mutations to a noncoding region lacking common annotations of regulatory elements, such as deposition of H3K27ac or DNase I hypersensitivity, was sufficient to modulate gene expression. Thus, this regulatory region would not have been discovered by conventional epigenetic annotation approaches. All of these studies analyzed the effect of a single gRNA on cell phenotype, but combinatorial gRNA delivery permits the study and determination of how synergistic and interconnected gene relationships influence cell phenotype (142, 143). In addition, most of the screens performed so far have evaluated expression of a single gene or simple phenotype, such as growth or survival. More

recently, several studies have demonstrated the utility of combining single-cell sequencing technologies with pooled CRISPR screens (144, 145). Because these screens analyze whole-transcriptome effects of single gRNAs, they can determine the influence of genomic and epigenomic pertubations on more complex phenotypes, such as cell fate specification and reprogramming.

Another strategy utilizing synthetic biology approaches to dissect mechanisms of epigenetic regulation is in the construction of synthetic histones by use of genetic code expansion. The expansion of the genetic code by the incorporation of novel amino acids into proteins has enabled many applications in basic research and biotechnology. Genetic code expansion is achieved through the use of an orthogonal aminoacyl–tRNA (transfer RNA) synthetase that has been engineered to recognize and load a novel amino acid onto an orthogonal tRNA. This tRNA then must recognize a rare codon, often the amber stop codon, to catalyze the site-specific incorporation of the unnatural amino acid into a gene of interest (146).

Elsässer et al. (147) utilized this approach to incorporate *N*-ε-acetyl-lysine at various lysine positions within the histone H3.3 protein in mouse embryonic stem cells. These authors verified the incorporation of the synthetic histones into chromatin, and they identified a set of differentially expressed genes in response to the expression of certain site-specific acetylated H3.3 transgenes (147). David et al. (148) described a method to generate novel histone modifications with split intein technologies to expand the scope of modifications and enable the programming of novel gene regulatory behaviors. Although these approaches could be used to determine whether specific chromatin modifications are causative or correlative with transcriptional activity, they are limited by the lack of positional specificity within chromatin and the inefficient incorporation of certain synthetic histone modifications relative to natural histone variants. Future research may involve combining genetic code expansion with targeted epigenetic modification by programmable DBD platforms. An advantage of using genetic code expansion strategies is that the researcher has control over which residues are modified, whereas the extent of modifications imparted by DBD-based epigenetic modifiers to other substrates in the cell is uncertain. A possible strategy could be to use genetic code expansion to prevent, maintain, or impart modifications to certain residues concurrent with targeting of a programmable epigenetic modifier to a specified genomic locus to better resolve the role of epigenetic marks in coordinating gene expression.

4.2. Engineering Cell Fate and Multicellular Structures

Cellular differentiation and organogenesis during development, maintenance of tissue homeostasis, and regeneration involve the complex coordination of spatial and temporal cues that govern cell behaviors and interactions. Recent research has utilized tools and principles from synthetic biology to recreate natural developmental processes or create artificial processes with utility in basic research and medicine (**Figure 4**). For instance, significant effort has been focused on engineering cell fate in precise ways. Transcription factors are considered the master regulators of cell type specification and can be used to program cell fate decisions (149), as exemplified by the demonstration that ectopic overexpression of transcription factors is sufficient to reprogram one cell type into another (150, 151). However, this method of artificial reprogramming relies on stochastic processes that can limit the efficiency and kinetics of generating the desired cell type (152). Consequently, several groups have utilized tools and approaches from synthetic biology to enhance natural transcription factors and improve reprogramming efficiency and fidelity.

A successful way to enhance natural transcription factors is to fuse additional potent transactivation domains to the termini of the protein (153). This approach has been most extensively demonstrated with the pluripotency factors OCT4, SOX2, KLF4, and NANOG (OSKN) used

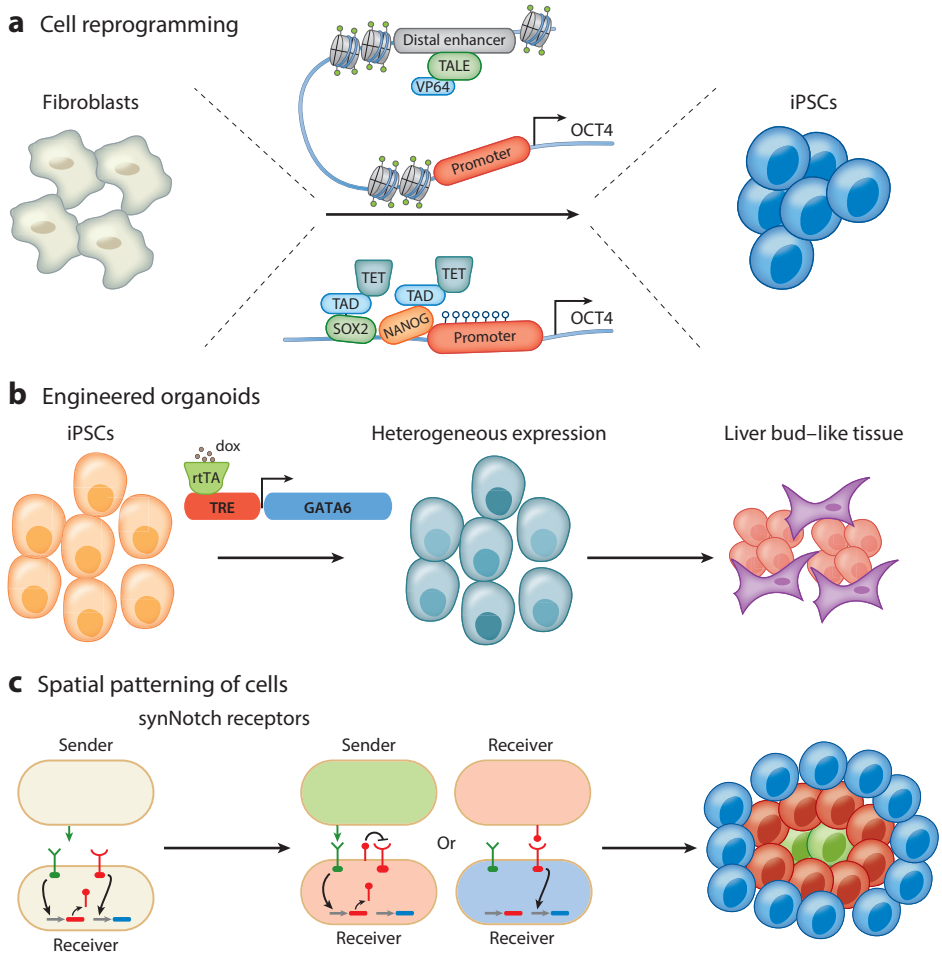

Figure 4

Engineering cell fate and organization using tools in synthetic biology. (*a*) Direct reprogramming of fibroblasts to iPSCs by use of synthetic transcription factors. (*Top*) TALE activators targeting a distal enhancer of *Oct4* remodeled local chromatin and activated endogenous *Oct4* expression more rapidly than achieved with overexpression of *OCT4* cDNA (157). (*Bottom*) Fusion of OCT4, SOX2, and NANOG to the transactivation domain of the Yes-associated protein improved iPSC generation 100-fold and coordinated with endogenous Tet proteins to demethylate targeted promoters (155). (*b*) A simple gene circuit that controlled expression of the transcription factor GATA6 produced heterogeneous GATA6 expression in iPSCs, enabling the differentiation of diverse cellular subtypes to form a liver bud–like organoid (14). (*c*) A synthetic Notch receptor coordinated the self-assembly of multilayered spatial patterns of sender and receiver cells using synthetic cell interaction pathways (13). Abbreviations: cDNA, complementary DNA; iPSC, induced pluripotent stem cell; rtTA, reverse tetracycline-controlled transactivator; TAD, transactivation domain; TALE, transcriptional activator–like effector.

in the reprogramming of fibroblasts to induced pluripotent stem cells (iPSCs) (151). Wang et al. (154) demonstrated that fusion of the herpes simplex virus transactivator domain VP16 to OCT4, SOX2, and NANOG improved reprogramming efficiency 100-fold over that obtained with the natural factors. These authors were also able to reduce the pool of transcription factors required

for successful reprogramming by using OCT4–VP16 alone, and they successfully generated iPSCs through episomal expression of the engineered factors, mitigating the undesired effects of genetic modifications imparted by random vector integration of lentiviral vectors (154). A more recent study fused the transactivation domain of the Yes-associated protein to OSKN to enhance the generation of iPSCs (155). Notably, this study demonstrated that the synthetic transcription factors more effectively interacted with endogenous epigenome-modifying enzymes compared with the natural transcription factors, leading to enhanced binding to and reactivation of the endogenous pluripotency factors (155). A similar approach has been applied to promote the direct conversion of differentiated cell types, such as the conversion of fibroblasts to skeletal myocytes by MyoD (156). These studies have established methods for efficient manipulation of cell identity through transcription factor engineering. They also shed light on mechanisms of transcriptional regulation and cell fate specification.

A more recent strategy to engineer cell fate has been to use synthetic transcription factors based on programmable DBDs (157–162). These studies prove that targeting programmable transactivators to particular regulatory elements controlling expression of endogenous genes involved in cell fate specification is sufficient to reprogram cell identity (**Figure 4*a***). In a recent study, multiplexed activation of three endogenous proneural genes using CRISPR/Cas9-based activators was sufficient to convert mouse fibroblasts to induced neuronal cells (162). In this case, targeting the endogenous loci more rapidly remodeled the epigenome and induced transcriptional activation of the target loci compared with expression of reprogramming factors from ectopic transgenes. The more deterministic chromatin remodeling and transcriptional activation of the endogenous master regulatory factors by programmable transactivators as demonstrated in this study may facilitate the use of transient delivery of the synthetic factors by more rapidly activating autonomous endogenous gene networks (162). In addition, because engineered factors based on DBDs can be programmed to target any locus in the genome, they can serve as a platform to identify novel genes and regulatory elements involved in cell fate conversions (163).

The process of induced cell differentiation and reprogramming is heterogeneous and often stochastic (152). The chromatin state of the starting cell type can influence transcription factor binding and thus limit its ability to regulate gene expression (164). Interfacing reprogramming or differentiation studies with synthetic circuits that can monitor or manipulate aspects of the cell state could enable the identification of cell subpopulations that are more amenable or resistant to differentiation or reprogramming. Transcriptional memory circuits in human cells can track cell subpopulations that have been exposed to transient stimuli to study long-term phenotypic responses (165). A similar approach could be employed to study or manipulate how cell subpopulations respond to differentiation or reprogramming stimuli. For example, a synthetic lineage-control circuit in human cells was used to coordinate the kinetics of activation and repression of lineage-specific transcription factors by use of looped circuitry (166). The circuit directed the differentiation of iPSC-derived pancreatic progenitor cells into glucose-sensitive insulin-secreting β-like cells (166).

The development of multicellular structures and tissues depends on cell–cell and cell–environment interactions and signaling. Morsut et al. (13) described a synthetic Notch receptor that is capable of mediating contact-dependent cellular signaling (**Figure 4*c***). These authors combined this synthetic receptor with downstream signaling via a synthetic transcription factor to enable contact-dependent, and thus spatially defined, regulation of gene expression (13). These receptors could be engineered for the spatial patterning of cell contact and differentiation, and could also be used to construct self-organized patterns of cellular structures (13). This research establishes an approach to rewire natural signaling topologies to control cellular behavior in precise ways. Importantly, the synthetic Notch receptor is modular and orthogonal, and thus supports an

integrated coordination of multiple engineered receptors for more complex logic computation to connect extracellular cues to intracellular signaling and gene expression patterns.

Several other groups have created synthetic circuits and rewired endogenous signaling pathways to detect and regulate cell fate and organization. For example, Deans et al. (167) described an approach to incorporate genetic inducers within the three-dimensional microenvironment of a biomaterial to provide a link to intracellular signaling pathways and initiate activity of gene circuits in a spatially controlled manner. In another instance, an inducible transgene system was utilized to direct the differentiation of iPSCs to a heterogeneous liver bud–like two-dimensional organoid (**Figure 4*b***) (14). Interestingly, lentiviral delivery of a dox-inducible *GATA6* vector generated wide variation in GATA6 expression levels, which governed the differentiation into distinct cell types (14). These approaches enable the control of more complex cellular structures and attempt to recapitulate key steps in developmental processes, which has tremendous potential to advance applications in tissue engineering and regenerative medicine. Furthermore, improvements in our understanding of how a cell naturally computes cell fate decisions will enable better engineering of novel cellular states and reprogramming of lineage specification.

In addition to developing methods to control the identity of single cells or small, organized cellular structures, it is important to continue establishing methods that aim to construct entire functional tissues ex vivo. Synthetic biology approaches could enable more elegant designs of engineered tissue constructs by programming logic circuits that assess cell fate and local environmental conditions and compute desired functional outputs or generate measurable signals. Substantial efforts are under way in tissue engineering to functionalize extracellular substrates with ligands and small molecules to influence cell behavior. Examples such as the synthetic Notch receptor (13) may permit a connection between those extracellular inputs to intracellular signaling pathways that govern specific cellular behaviors and phenotypes to improve the function and adaptability of tissue-engineered constructs.

5. BIOMEDICAL APPLICATIONS

5.1. Modeling Genetic Disease

Thousands of genome-wide association studies have identified genetic variation in the human genome that is associated with normal and disease phenotypes (168). Notably, the vast majority of this genetic variation falls within putative gene regulatory elements of the non-protein-coding genome (169, 170). Thus, the precise manipulation of the genome and epigenome enabled by DBD-based tools constitutes a strategy to assign causal roles of genetic and epigenetic variation to disease states. These manipulations may inform potential therapeutic strategies or serve as the therapy. Spisak et al. (171) described a pipeline to characterize the causality of disease-associated genetic variation in the noncoding genome determined from genome-wide association studies. These authors used annotated chromatin marks and programmable synthetic transcription factors to validate the regulatory potential of candidate genetic variants within a prostate cancer risk locus (171). They then applied genome engineering tools to introduce precise genetic modifications via homology-directed repair into an isogenic background and assessed the effects on epigenetic marks at the target loci, global gene expression profiles, and cell phenotypes (171). Although this research modeled genetic variation using programmable nucleases, recent technology for site-specific editing of single base pairs using a CRISPR/Cas9 cytidine deaminase fusion potentially provides a means to directly induce point mutations relevant to disease (**Figure 5*c***) (172, 173). Finally, disease states can manifest from aberrant epigenetic remodeling in the absence of any genetic alteration (174). Consequently, recent studies

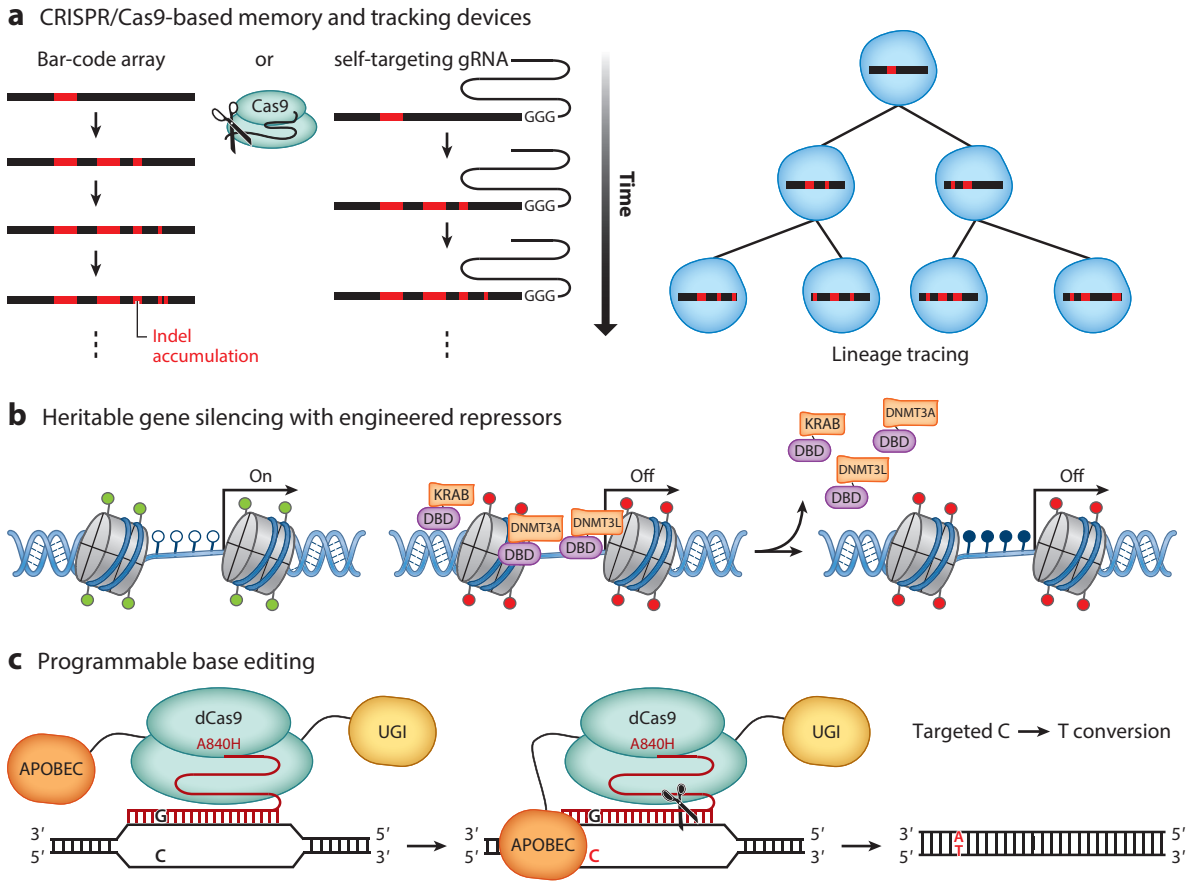

Figure 5

Next-generation technologies for applications in synthetic biology. (*a*) CRISPR/Cas9-based gene editing can be applied to record cell lineage and memory via DNA mutagenesis. In one case, a self-targeting gRNA continuously mutagenizes the DNA that encodes the gRNA, thereby permitting an analog recording of gRNA activity. Expression of Cas9/gRNA can be linked to the magnitude and duration of an external stimulus; thus, the evolution of gRNA mutations can serve as a metric for stimulus exposure. A similar approach using bar-code arrays of gRNA target sites was used to track cell lineage connections via comparison of the bar-code mutations shared between cells. (*b*) Transient coexpression of a combination of engineered repressors can mediate stable and heritable epigenetic silencing of endogenous genes via DNA and histone methylation. Importantly, this strategy mitigates the need for genetic modification or constitutive expression of synthetic factors to achieve permanent silencing. (*c*) The CRISPR/Cas9 system has been adapted for programmable single-base editing through fusion of the cytidine deaminase APOBEC1 enzyme to the N terminus of a Cas9 nickase. The addition of a uracil glycosylase inhibitor fused to the C terminus of Cas9 improved efficiency of the desired base edit. Abbreviations: APOBEC1, apolipoprotein B messenger RNA editing enzyme, catalytic polypeptide–like 1; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9; DBD, DNA-binding domain; DNMT3A, DNA methyltransferase 3α; DNMT3L, DNA methyltransferase 3–like; gRNA, guide RNA; KRAB, Krüppel-associated box; UGI, uracil DNA glycosylase inhibitor.

Epigenome editing: site-specific and programmable control of chromatin and transcriptional states

have established the causal role of epigenetic states in models of addiction using programmable epigenome-modifying enzymes (175, 176). The modular nature of genome and epigenome-editing tools enable the application of these tools to study the effects of diverse genetic and epigenetic variation.

5.2. High-Throughput Discovery of Disease Drivers

Gene and cell therapy: the ectopic delivery of engineered genes or cells to treat disease

In addition to providing a means to validate genetic correlates of normal and disease states, genome- and epigenome-editing tools constitute a strategy for the unbiased identification of novel loci causing disease states. For instance, the versatility and scalability of the CRISPR technology enable genome-wide perturbation of the genome and epigenome. Chen et al. (135) used a CRISPR screen to identify genes involved in cancer maturation and metastasis in a mouse tumor model. These authors used a library of more than 60,000 gRNAs targeting all protein-coding genes and miRNAs to generate a population of mutagenized cells. After implantation of this cell population into a mouse, the authors identified a narrowing of diversity of gRNA representation at later stages of tumor maturation in vivo, and they were able to identify gRNAs targeting known and novel genes that drove this maturation and could be further explored as therapeutic targets (135). Wallace et al. (177) studied the role of miRNAs in coordinating the phenotypes of cancer cell lines using a CRISPR-based unbiased loss-of-function screen. They identified novel miRNAs that control cellular fitness as assessed by survival and proliferation in a leukemia cell line (177). Future research may entail the direct in vivo delivery of CRISPR libraries to enable screening in the host tissue in models of spontaneous tumor development.

5.3. Genome and Epigenome Editing for Gene and Cell Therapy

Genomic and epigenomic editing tools can also be employed as potential therapeutics to treat genetic and epigenetic disease (41). Targeted activation and repression of endogenous genes for gene therapy purposes can have distinct advantages over ectopic expression of transgenes (178). For instance, targeting the promoter of vascular endothelial growth factor (VEGF) with ZFP transactivators to promote angiogenesis produced all VEGF isoforms, which facilitated improved vasculature maturation relative to the delivery of a transgene encoding a single isoform (179). Programmable transcription factors can also be engineered for allele-specific targeting, which has shown efficacy in targeting the mutant form of *Htt* in Huntington's disease (180). Engineering these tools to be sensitive to single-nucleotide mismatches could enable the targeting of disease-associated SNPs, which are commonly found in noncoding regulatory elements (169, 170). Lastly, the versatility of chromatin marks that can be deposited by a suite of epigenome-editing tools could eventually enable therapies that modulate a particular chromatin state in order to generate the desired expression dynamics. For instance, stable and heritable silencing has been demonstrated in a few cases (86, 87, 90) and could enable transient delivery of synthetic transcription factors for the stable repression of a therapeutically relevant gene target (**Figure 5*b***). Programmable nucleases are also being applied to diverse biomedical applications to knock out disease-causing genes (181), restore expression of mutated genes (182–184), or construct logic gates to identify and selectively eliminate cancer cells (185).

Beyond genetic and epigenetic editing, DBDs can be employed as sensor and actuator devices for applications in diagnostics and therapeutics. Slomovic & Collins (186) recently described a novel technique to initiate a transactivating signal in response to the recognition of a particular DNA sequence. The authors used pairs of ZFPs to recognize adjacent DNA sequences and initiate intein-induced splicing to release a synthetic transcription factor for activation of a downstream promoter (186). To demonstrate the utility of this system, the authors used it to program cell death and detect viral infection. In another study, a self-targeting gRNA was designed by incorporating the targeting motif directly downstream of the target site recognition sequence within the gRNA expression cassette (187). The gRNA target site recognition sequence evolves as it accumulates mutations via the self-targeting Cas9 nuclease, and therefore serves as a bar code to track

cell lineage (**Figure 5*a***). When inserted into cells under the control of a tumor necrosis factor α–inducible promoter, this system served as an analog memory storage device by recording mutations in response to the degree of lipopolysaccharide-induced inflammation following implantation in vivo (187). Future applications of this device could entail the use of distinct stimuli-responsive promoters with multiplexed gRNAs to track how cellular subtypes and signaling pathways respond to environmental inputs, such as exposure to pharmacologic therapies (187).

Chimeric antigen receptor (CAR): engineered receptor expressed on T cells to target cancer-specific antigens

5.4. Building Designer Cells

The elucidation of signaling networks involved in normal and disease states facilitates the construction of synthetic theranostic circuits that rewire natural pathways to sense and respond to pathological states for therapeutic benefit. For instance, excess bile acid accumulation in the peripheral bloodstream can serve as a marker of liver injury or liver disease (188). A recent study showed that rewiring bile acid–triggered activation of G protein–coupled bile acid receptor 1 (TGR5)- and cAMP-response element–binding protein 1 (CREB1)-mediated transcriptional activation to induce the expression of hepatocyte growth factor can attenuate acute drug-induced liver damage in an animal model (189). Importantly, this synthetic circuit could sense and respond in a closed-loop, autonomous fashion, enabling tunable and reversible response dynamics. Similar circuits based on the same downstream signaling cascade were built to sense and respond to environmental pH level (190) and blood dopamine level (191) for the treatment of diabetes and hypertension, respectively. Rossger et al. (191) exploited the relationship between blood and brain dopamine levels and rerouted a dopamine receptor to stimulate release of atrial natriuretic peptide in hypertensive mice in response to increases in blood dopamine levels. Another group recently identified an injury-responsive enhancer in regenerating tissues of the zebrafish, and used it to program expression of a regenerative factor in response to injury (192). Surprisingly, this regulatory element displayed injury-specific expression in mouse tissues as well, even though the enhancer lacked sequence homology within the mouse genome. Lastly, several synthetic circuits have been described to monitor and maintain aspects of metabolic homeostasis in vivo (193–195).

The use of engineered chimeric antigen receptor (CAR) T cells for the targeted killing of antigen-positive cells has been a transformative advance in cancer therapy (196, 197). Some recent developments in this field include the use of a synthetic Notch receptor to construct AND gate T cells that respond only to the combination of two antigens (198). In this study, the authors separated the T cell activation cascade from the recognition of the first antigen, thereby limiting false positives and reducing likelihood of activation in response to a single antigen (198). The modularity of the Notch receptor makes this approach amenable to many targeted antigens by altering the ligand-binding domain. Wu et al. (199) developed an ON-switch CAR T cell that responds to small molecules in vivo for activation, thus providing tunable and reversible control over CAR T cell activity. Furthermore, like the AND gate CAR T cells, this system is modular and amenable to diverse small molecule–responsive modules (199).

The ability to detect disease signatures at multiple levels of gene regulation from transcription to translation could enable more effective combinatorial therapies. Synthetic circuits are primed for this capability, as they can execute precise logic computation in response to activity at diverse levels within the cellular regulome. For instance, Nissim & Bar-Ziv (200) developed an AND gate circuit with a dual promoter system to detect transcriptional signatures of cancer cells. The use of a dual-promoter architecture minimized basal activity and false-positive detection (200). In another instance, Xie et al. (201) constructed a multi-input logic circuit that can detect miRNA signatures within a cell and, if all conditions are met, execute downstream expression of a toxic gene product.

These authors used this circuit to selectively identify and destroy HeLa cells in a heterogeneous population of HeLa and HEK293 cells (201). Lastly, Culler et al. (202) described an RNA-based sensor-actuator device that monitors nuclear protein levels by inserting protein-binding RNA aptamers within introns of therapeutic transgenes. Binding of the target proteins to the intronic aptamers increases the frequency of exclusion of a neighboring exon encoding a premature stop codon (202). Notably, these authors demonstrated the modular nature of this system to detect diverse protein inputs. An exciting future strategy will be to integrate sensor-actuator devices with multilevel regulatory control to provide combinatorial targeting of disease pathways.

6. CONCLUSION AND FUTURE OUTLOOK

Synthetic biology is a broad, multidisciplinary field at the intersection of engineering and the biological sciences. The overall objective of this field is to build biological systems with novel behavior from a toolbox of modular parts and predictable connectivity. Consequently, advances in synthetic biology are contingent on progress in many other fields of research, including basic science. For instance, as our understanding of how genetic and epigenetic mechanisms govern gene expression and cellular phenotype improves, we can begin to exploit and repurpose these systems to encode new functions. Furthermore, the development of next-generation tools in synthetic biology described herein, such as CRISPR/Cas9 memory and tracking devices (Section 5.3), programmable transcriptional repressors for heritable gene silencing (Section 2.2), and programmable base-editing devices (Section 5.1), will enable researchers to address more complex biological questions in the future (**Figure 5**). Notably, basic research is also critical to enabling these technological developments, as tools like CRISPR, RNAi, and green fluorescent protein are inevitably discovered in unexpected areas of biology.

Tools and circuits in synthetic biology have tremendous potential to revolutionize how disease is diagnosed, prevented, and treated. Similar to how the "genomic revolution" has facilitated approaches to personalized medicine through understanding how individual genetic backgrounds can guide and influence therapeutic strategies, applications in synthetic biology enable the intelligent dissection of signaling pathways and phenotypes in real time to best respond to pathological states. However, there are several key challenges to overcome in order to adapt tools and strategies in synthetic biology to applications in biomedicine. For instance, it will be important to standardize how synthetic genetic circuits are characterized ex vivo. This could entail using a cell line with safe harbor loci to insert the genetic payload and limit the parameter space that could influence circuit behavior (203). Alternatively, some applications in biomedicine may necessitate autologous cell sources for cell-based therapies; thus, approaches for characterization of gene circuits in primary cells with diverse genetic backgrounds will need to be established.

In addition, the implementation of synthetic biological circuits in biomedicine will be contingent on the development of circuits with minimal basal activity and cross talk with neighboring endogenous pathways. It is likely that methods in directed evolution and expansion of the genetic code will continue to become more prevalent in the development of synthetic tools with orthogonal function within the host organism. Lastly, the implantation of ex vivo engineered cells or the direct delivery of genetic circuits in vivo will require optimization of efficient delivery strategies (204). For the implantation of designer cells, several studies have demonstrated the utility of using immunoprotective microcontainers to encapsulate the engineered cells during subcutaneous implantation (205). The direct delivery of genetically encoded circuits in vivo will be limited by the payload size permitted by various viral and nonviral delivery platforms (204). We expect that engineering viruses to carry larger payloads and target specific tissues and cellular subtypes will continue to be an active area of research (206). Ultimately, mammalian synthetic biology has the

potential to revolutionize how we interface with biology in diverse disciplines in basic research, pharmaceutical development, and biomedicine.

FUTURE ISSUES

1. For the field of synthetic biology to progress effectively, it will be critical to develop approaches to standardize the construction and characterization of gene circuits. Commonly, new tools or circuits are characterized in immortalized cell lines, and their activity in primary cells or in vivo is often uncertain. In addition, differences in the description of genetic parts and metrics used to assay circuit function limit the accessibility of these new technologies.
2. In vivo applications of synthetic biology are often hindered by limitations in the effective delivery of genetic payloads. Gene circuits and synthetic tools can be large relative to the packing limit of delivery vehicles. A critical focus will need to be placed on the design of smaller circuit components and the improvement of engineered delivery platforms to permit efficient tissue-specific targeting and expression.
3. Off-target activities of engineered factors, such as programmable nucleases, transcription factors, and sensor-actuator devices, pose a concern for translating these technologies into the clinic. Significant research has focused on characterizing and limiting off-target effects, and there are likely to be continued advances in this area. In addition, it will be important to establish benchmarks for specificity and the long-term consequences of off-target effects.

DISCLOSURE STATEMENT

The authors are named inventors on patent applications related to mammalian synthetic biology.

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