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Mammalian synthetic biology by CRISPRs engineering and applications

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Technologies harnessing CRISPR systems have been rapidly evolving and expanding the capacity of researchers for understanding of mammalian cell behavior and its underlying mechanisms through genome and epigenome manipulations. In this review, we summarized the recent developments of CRISPR-based technologies for genetic and epigenetic modifications that include engineering of Cas9 for PAM simplification, non-cleaving base editing tools and alteration of gene expression. Applications such as genome-wide screening methods or CRISPR-based DNA barcoding for cellular lineage tracking are highlighted. Anticipated and upcoming development for mammalian synthetic biology that includes organelle engineering is also discussed.

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Introduction

To understand and control highly organized and complex mammalian biological systems, tools and technologies that allow spaciotemporal and precise manipulation are required. CRISPR (Clustered regularly interspaced short palindromic repeat) was first found in the 1980's as unknown repeat-containing sequences in *Escherichia coli* genome [1] and was later revealed to be an essential component of the microbial adaptive immune system [2]. It has recently been extensively studied to develop powerful genome and epigenome editing tools [3,4]. The CRISPR-based technologies have made mammalian cells genetically engineerable, greatly facilitating synthetic approaches towards understanding and controlling mammalian biological systems. Repurposing Cas9 as a programable DNA-binding module has been done by inactivating its nuclease activity to provide a Cas9 mutant (dead Cas9: dCas9) and recruiting diverse effector domains including transcriptional activators, repressors, epigenetic modifiers, and deaminases.

Here we describe recent developments of diverse CRISPR-based editing tools and applications useful for mammalian synthetic biology and discuss future perspectives of these revolutionary technologies (Figure 1).

'Basic' units of CRISPR toolbox and their updates

CRISPR and PAM varieties

CRISPR–Cas systems consist of Cas proteins and guide RNAs in general. An effector complex of Cas proteins with a guide RNA recognizes and cleaves a specific locus containing cognate oligonucleotide sequence which is complementary to the guide RNA targeting sequence. The CRISPR–Cas systems require a protospacer adjacent motif (PAM) sequences next to the target sequence. To date, numerous CRISPR systems have been identified from a wide variety of bacteria and archaea that exhibit diverse PAM sequences, expanding the scope of accessible genomic loci. A CRISPR–Cas system from *Streptococcus pyogenes*, namely SpCas9, has been the most widely used system as it shows robust activity and recognizes one of the simplest PAM 5′-NGG-3′ in nature. Through search for simpler PAM-requiring Cas9, *Streptococcus canis* Cas9, which has 89.2% homology to SpCas9, was reported to recognize a minimal 5′-NNG-3′ PAM [5]. Efforts for engineering of Cas9 have also been made extensively. An evolved SpCas9 (xCas9) created by phage-assisted continuous evolution (PACE) requires relaxed PAM that includes 5′-NG-3′, 5′-GAA-3′, and 5′-GAT-3′ [6], although its activity is somewhat compromised. The structural analysis of xCas9 suggested that one of the mutations E1219V leads to rotamer flexibility of a PAM interacting with residue R1335, resulting in its PAM compatibility [7]. Nishimasu *et al.* took a structure-based, rational engineering approach to generate an SpCas9 variant (SpCas9-NG) that recognizes a simplified PAM, 5′-NG-3′ with robust activity [8].

Base editing without DSB

DNA deamination for cytidine or adenosine can induce base transitions. Base editing is the technology that installs single base substitution by recruiting DNA deaminases using the nuclease-deficient CRISPR platform [9,10]. While cytidine deaminases had been known to exist in nature, no DNA adenosine deaminase had been identified. tRNA adenosine deaminase TadA was then

subjected to evolutionary engineering to direct its substrate specificity to DNA [11]. These deaminases target cytidine or adenosine only on single-stranded DNA (ssDNA) but not on double-stranded DNA (dsDNA). Binding of Cas9 to the target locus induces a single-stranded segment in an R-loop caused by base pairing between the guide RNA and the target DNA, which localizes the deamination activity to the specific sites in the genome, ensuring the high specificity of the base editors. Mutational windows are defined for each system that can be as wide as three nucleotides or more. Efforts to narrow the mutation window have been made, while often associated with compromised efficiency or target motif restriction [12–14].

Gene expression control

The repressor domain Krüppel-associated box (KRAB) has been commonly adopted as one of the CRISPR interference (CRISPRi) gene repression tools, but the efficiency of gene silencing left room for improvement. An additional repression domain, MeCP2, fused to dCas9–KRAB induced more potent repression of the majority of endogenous genes tested in HEK293T cells compared to dCas9–KRAB [15].

The original CRISPR/Cas9-mediated transcriptional activation (CRISPRa) by fusing dCas9 with transactivating factor VP64 did not induce sufficient activation in mammalian cells. Improvement of dCas9-mediated activation systems by recruiting multiple transcriptional activation domains produced the second-generation CRISPRa systems including the tripartite activator system (dCas9-VPR) [16], synergistic activation mediator (SAM system) [17], and dCas9-Suntag [18]. Instead of dCas9, nuclease active Cas9 can be used with a short gRNA of 14-nucleotide or 15-nucleotide target sequence that serves as dead gRNA (dgRNA) as it abolishes the nuclease activity of its Cas9 complex [19,20]. Taking advantage of the smaller gRNA units that allowed packaging into the AAV delivery system, the SAM system employing MS2-P65-HSF1 fusion was combined and applied to postnatal mice to demonstrate efficient activation of endogenous genes *in vivo* [21].

Recent CRISPR applications in synthetic biology

Genetic circuits and CRISPR-CPU

Implementation of synthetic genetic circuits in mammalian cells may allow sophisticated control of cellular behavior and fate [22]. Since synthetic genetic circuits are generally based on transcriptional regulation of multiple genes and may require orthogonal operation devoid of cross talk, highly specific and multiplexable CRISPR-based tools should be ideal. AND-gates that combine inputs from cancer-specific and bladder cell-specific promoters via CRISPR-based repressor have achieved highly selective gene regulation and phenotype control by expressing transgenes in the targeted cells (i.e. bladder cancer cells) [23]. The improved CRISPRi technology, dCas9–KRAB–MeCP2 has enabled a three-layered synthetic gene circuit [24]. Nakamura *et al.* have demonstrated multiple layers regulation of synthetic gene circuits in mammalian cells by CRISPR-mediated transcriptional regulation along with an anti-CRISPR (Acr) protein AcrIIA4 [25]. Acr proteins interact with Cas proteins and inhibit their functions, which originate from bacteriophages that allow them to bypass the CRISPR immune systems of the host bacteria and archaea. Kim *et al.* have programmed cells to function as central processing units (CPUs), in which different sets of user-defined RNA inputs turned into the correct fluorescent result by arithmetic operations like a half adder [26].

Optogenetic devices

The optogenetics makes the CRISPR-based gene regulation more attractive as it enables precise spaciotemporal control. Shao *et al.* have developed a far-red light (FRL)-activated CRISPR–dCas9 effector (FACE) system and demonstrated the FRL-induced gene expression as well as epigenetic modulation at the intended targets in mice [27]. An optogenetic harnessing of AcrIIA4 with a photosensor LOV2 domain derived from *Avena sativa* phototropin-1 (Acr-LOV) has been performed to modulate the Cas9 activity in response to blue light: this technology was termed CASANOVA (for 'CRISPR–Cas9 activity switching via a novel optogenetic variant of AcrIIA4') [28]. With the CASANOVA system, dCas9–p300, which is composed of the core domain of histone acetyl transferase p300 domain tethered to dCas9, was successfully recruited to the promoter of interleukin 1 receptor antagonist (*IL1RN*) upon irradiation, resulting in upregulation of the *IL1RN* expression up to 10-fold compared to that without the light stimulation. Use of dCas9–3 × RFP along with CASANOVA visualized rapid localization of dCas9 after irradiation on a scale of minutes, which is reversible upon incubation in the dark state.

Cell barcoding

As timing and outcome of CRISPR-mediated genome editing are stochastic, cells may contain heterogenic combination of DNA sequences at the target sites especially in the case of multiplex editing. Such different pattern of the edited sites can mark each cells as barcoding elements especially for cell lineage tracing [29,30•] (also see the review by Schmidt and Platt [31]). Kalhor *et al.* utilized the homing CRISPR, in which a homing guide RNA (hgRNA) can target its own DNA locus by including PAM sequence in the scaffold sequence. By this self-editing cycle, hgRNA-expressing DNA sequence keeps diversifying over time [32]. A transgenic mouse (Mouse for Actively Recording Cells 1: MARC1) line that carries the distinct hgRNA-expressing units at 60 loci scattered over the whole genome was created and

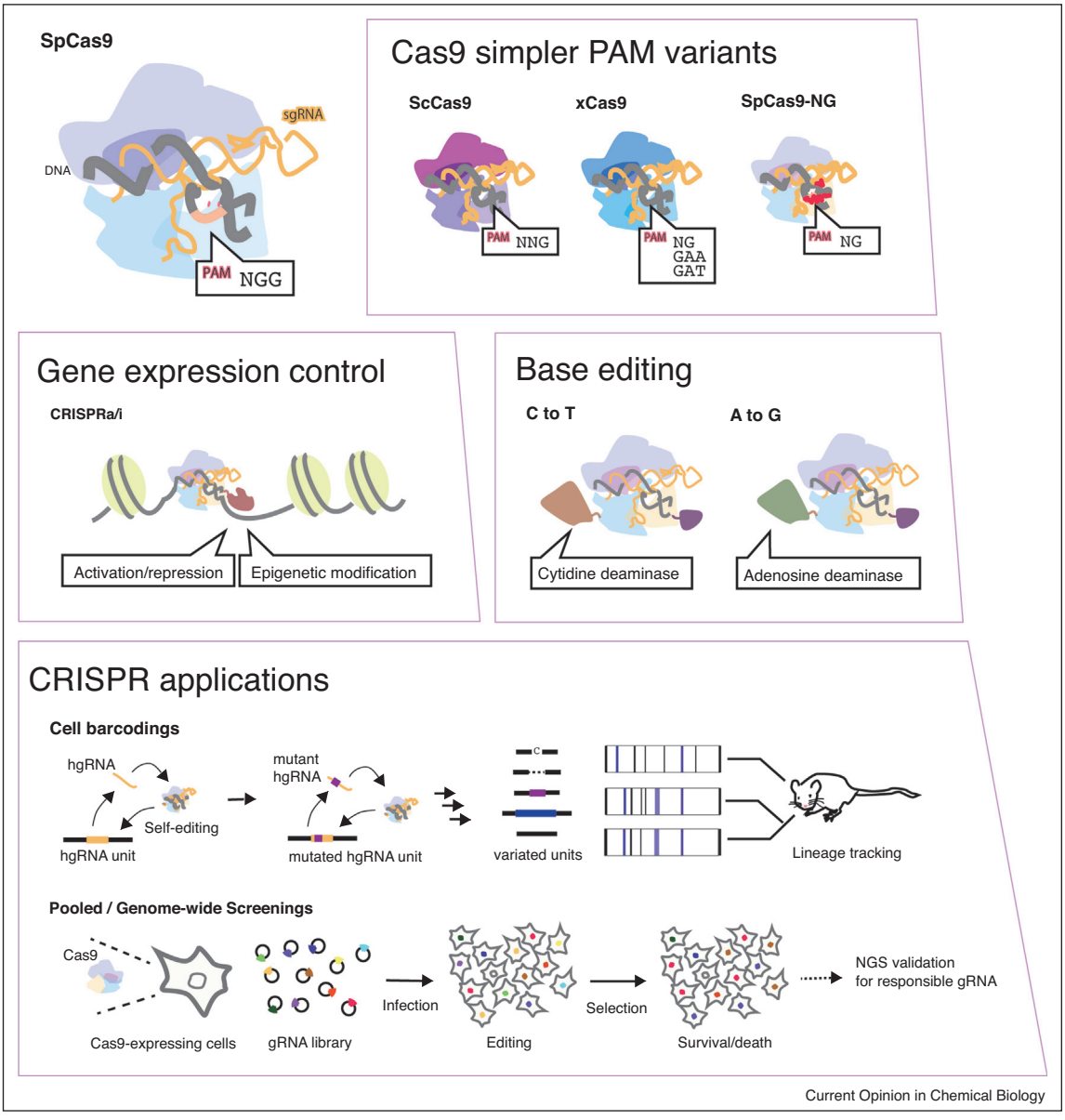
crossed with Cas9-expressing mice to initiate barcoding and recording of mouse cell lineages, elucidating the developmental order of mouse brain axis. Recently, cell barcoding with single cell RNA-seq information reveals higher-resolution recoding of cell lineage and transcriptional convergence [33•].

Barcoded information can also contain cellular events. Tang and Liu utilized base editing under control of a stimuli-inducible promoter (named after 'CRISPR-mediated analog multi-event recording apparatus', CAMERA), and the relative editing rate record a history of events in bacteria and mammalian cells [34]. An RNA recoding system, Record-seq, has been developed in *E. coli*, in which exploits a spacer-acquisition system of type I-E CRISPR from *Fusicatenibacter saccharivorans* [35••]. This system stores transcriptome-scale gene expression as memories of transcriptional events over time within the CRISPR array. In the future, such system could be an attractive cell linage tracking device for multicellular organisms as each cell should contain distinct transcriptomes through development.

Pooled or genome-wide CRISPR screen

Genome-wide screening via CRISPR-mediated technologies is one of the most powerful applications. In the case

Figure 1



Recent updates on diverse CRISPR techniques.

of mammalian cells, cells were infected with virus containing a pooled gRNA library, which targets all or selected genes in the genome and can be utilized as a barcode corresponding to the mutating gene. After the given selection condition, changes in gRNA distribution easily analyzed by next generation sequencing reflect genes involved in the fitness, while the responsible gene of each mutant is difficult to be determined in ethyl methanesulfonate (EMS) mutagenesis. Since two proof-of-concept studies were reported in 2014 [36,37], several studies have revealed novel genetic mechanisms in the mammalian research. The CRISPRi and CRISPRa systems have also been used for such screenings [38]. To perform effective screenings practically, well-designed gRNA libraries are vital. Accumulated knowledge of effectiveness of the previous gRNA libraries now allows better optimization for CRISPR knock-out, CRISPRi, and CRISPRa [39]. Hart *et al.* reanalyzed and evaluated sets of genome-wide CRISPR screening conducted by three research groups, and designed a highly effective genome-scale CRISPR library for human cell lines [40]. Recently pooled or genome-wide CRISPR screens have been combined with multiple single cell omics technologies including genome, epigenome, transcriptome, proteome, and immunological profiles [41]. These studies will enlighten the center of key elements of cell function, further providing more effective synthetic units.

Super-Mendelian inheritance by gene drive

A gene drive unit consists of a guide RNA that induces a cleavage at a native genome locus, and the unit is expected to be inserted into the locus via the endogenous homology-directed repair (HDR). The inserted gRNA unit then facilitates the cleavage of homologous chromosome and insertion of its own copy, converting to homozygous genotype. The system was first demonstrated in insects and then applied to mammalian cells [42•]. Through mating, biased inheritance of desired alleles over Mendelian inheritance is expected.

Conclusions and perspectives

The engineered Cas proteins and various orthologs from diverse CRISPR systems have been overcoming the PAM limitation to enable targeting of virtually any region of the genome. Engineering for PAM alteration can also be applied to Cas orthologs that have beneficial features such as smaller size to overcome delivery issue. The recently developed circularly permutated Cas9 variants (Cas9-CPs) further expanded the CRISPR toolkit for safer and more efficient genome editing [43••]. In addition, Cas9-CPs can be adopted as an optimized scaffold for fusing other proteins including activators, repressors, and deaminases. Enormous efforts have been invested to improve the capacity, fidelity, and efficiency of CRISPR-based editing technologies. Accumulating knowledge and development further accelerates evolution of the technologies. One of the remaining challenges is precise editing with high efficiency and rate while reducing the toxicity of DSB. HDR is not always efficient while base editors are also limited by their changeable type of nucleotides and window range. Employing alternative mechanisms for sophisticated editing may be needed. Another challenge for CRISPR technology is editing of mitochondria genome. As gRNA cannot enter into the organelle through mitochondrial transport machineries, MitoTALEN, mitochondrial localized TAL effector-nuclease complex, is preferred to edit mitochondrial DNA [44,45]. However, even MitoTALEN has not yet achieved editing of mitochondrial DNA (mtDNA) because it rather induces elimination of the whole of target-containing mtDNA molecules than editing the target presumably due to poor activity of non-homologous end joining repair pathway in the organelle. Therefore, manipulation of mitochondrial DNA may be the last frontier of genome editing, because it encodes many disease causative genes.

The efficiency, versatility, and orthogonality of CRISPR systems enable researchers to manipulate genomic and epigenomic profiles in mammalian cells. The diverse CRISPR-based technologies reviewed here and to be developed in the future will be essential tools to dissect and understand the complex and highly coordinated mammalian cellular mechanisms and processes.

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Conflict of interest statement

Nothing declared.

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