In vivo genome editing thrives with diversified CRISPR technologies

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ABSTRACT

Prokaryotic type II adaptive immune systems have been developed into the versatile CRISPR technology, which has been widely applied in site-specific genome editing and has revolutionized biomedical research due to its superior efficiency and flexibility. Recent studies have greatly diversified CRISPR technologies by coupling it with various DNA repair mechanisms and targeting strategies. These new advances have significantly expanded the generation of genetically modified animal models, either by including species in which targeted genetic modification could not be achieved previously, or through introducing complex genetic modifications that take multiple steps and cost years to achieve using traditional methods. Herein, we review the recent developments and applications of CRISPR-based technology in generating various animal models, and discuss the everlasting impact of this new progress on biomedical research.

Keywords: CRISPR/Cas9; Genome editing; Animal models

INTRODUCTION

Genome editing by manipulating functional DNA sequences in the host genome is a fundamental strategy for biomedical research. Starting from the discovery of the basic principles of DNA structure and genome organization, scientists have investigated various strategies for many decades to improve genome editing technology for different research and application purposes.

In the 1980s, gene targeting methods emerged together with a deepening understanding of DNA repair mechanisms. Back then, DNA conversion was found to occur between homology sequences, often termed homologous recombination (HR) (Zinn & Butow, 1985). Early studies took advantage of this finding to replace a selected endogenous genome DNA segment with a foreign DNA donor carrying homology sequences in living cells (Vasquez et al., 2001). Subsequently, by combining this with mouse embryonic stem cell (ESC) technology established at the same time, traditional gene targeting technology was developed to generate genetically modified mice (Koller et al., 1989). Since 1989, genetic modification by HR-based gene targeting in living mammals has become a fundamental approach to analyze gene functions and has revolutionized our understanding of mammalian development, metabolism, and genetic diseases (Capecci, 2005; Koller et al., 1989).

Traditional HR-based gene targeting is associated with low efficiency and requires laborious clonal expansions and sophisticated selections to identify target cells carrying the desired modifications (Koller et al., 1989). With pioneering studies finding that the introduction of double-strand breaks (DSBs) in target DNA by rare-cutting endonuclease I-Sce-1 could increase HR efficiency by several orders of magnitude in the subsequent DNA repair process (Rouet et al., 1994), extensive effort has been made to develop programmable endonucleases.

Zinc finger nuclease (ZFN), which was first reported in 1986 as an artificial nuclease to carry a zinc finger domain and a catalytic domain from restriction enzyme FokI, was suitable for introducing DNA cleavage and enhancing HR-dependent gene targeting (Bibikova et al., 2002; Kim et al., 1996). However, the laborious work involved in the design and identification of an efficient ZFN to a newly selected target sequence significantly limited its utility. Transcription activator-like effector protein (TALE), which

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originated in plant pathogen Xanthomonas sp., was found to recognize target DNA with highly conserved yet variable repetitive elements, each showing a preference to bind to specific nucleotides (Boch et al., 2009; Moscou & Bogdanove, 2009). Fusion of the programmable TALE domains and FokI catalytic domain thus yielded TALE-nuclease (TALEN), which is easier to construct and can introduce DNA cleavage and targeted genome modification equally efficiently as ZFN (Christian et al., 2010). More recently, an RNA-guided DNA-targeting approach was developed from the type II prokaryotic clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system (Bhaya et al., 2011; Wiedenheft et al., 2012). In this system, a programmable small guide RNA (sgRNA) complexes with Cas9 nuclease and anneals with a 20-nt target DNA sequence, at the presence of the adjacent NGG PAM (proto-spacer adjacent motif) sequence in a base-pairing manner. This process allows Cas9 to introduce DSB at the target region and enables genome modification in a site-specific manner (Jinek et al., 2012). The ease of constructing a sequence-specific sgRNA and the highly specific RNA-DNA recognition has made the CRISPR/Cas9 system superior to ZFN and TALEN, becoming the most popular tool for introducing programmed DNA cleavage as well as site-specific genome modifications in cells and animals (Barrangou & Doudna, 2016; Mali et al., 2013; Ran et al., 2013).

These recent advances in engineered nucleases, especially the CRISPR/Cas system, have opened new prospects for accomplishing robust gene targeting in previously non-permissive cell contexts. More importantly, it has widely revolutionized biomedical research by promoting quick generation of various animal models, which either carry complex genome modifications or are derived from species that could not be genetically modified previously (Dow et al., 2015; Swiech et al., 2015; Yin et al., 2016). Such progress has provided a wide range of methods as well as advanced animal models to study gene function and biological processes, significantly promoting research under in vivo conditions. Hence, in this review, we focus on summarizing the recent developments and applications of CRISPR-based technology in generating various animal models.

**OVERVIEW OF RECENT DEVELOPMENTS IN CRISPR-BASED ANIMAL MODELS**

Since early 2013, when the first successful CRISPR-based genome editing was demonstrated in mammalian cells (Mali et al., 2013), the number of studies using the CRISPR system has grown dramatically. Among the CRISPR-based in vivo studies, the majority (61.2%) have been conducted using mouse models (Figure 1, left panel). With the comprehensive knowledge and technologies established so far, research investigations using CRISPR technology in mouse models have covered various areas of biomedical research, including inherited metabolic disorders (Xue et al., 2014; Yang et al., 2016), cancer (Maddalo et al., 2014; Platt et al., 2014), neurology and neuroscience (Li et al., 2015c; Swiech et al., 2015), and virus-related studies (Jiang et al., 2017; Zhu et al., 2016).

In addition to mouse models, CRISPR-based genome editing has been demonstrated in large mammals such as pigs and monkeys to establish disease or genetic models for organ transplantation (Ni et al., 2014; Yu et al., 2016). At the same time, CRISPR/Cas9 technology has also been applied in various lower vertebrate and invertebrate models (Iriomote et al., 2014; Shi et al., 2015; Wen et al., 2016). The success of CRISPR technology is particularly valuable in lower vertebrate models, such as Xenopus and zebrafish (Iriomote et al., 2014; Shi et al., 2015), in which targeted genome editing could not be achieved previously.

**Molecular mechanisms for various genome editing strategies**

Sequence-specific DNA cleavage induced by any of the above engineered nucleases will elicit endogenous cellular responses to repair the damaged DNA in target cells. Utilizing various DNA repair mechanisms to induce mutations/deletions or to incorporate insertions of foreign DNA lays the foundation for genome editing. Cellular repair of DNA damage is mediated by two main pathways, namely, homology-directed repair (HDR) and non-homologous end joining (NHEJ). Despite their varied activities in different cell types and species, both pathways are highly conserved, from yeasts to mammals (Taylor & Lehmann, 1998).

The HDR pathway mediates a strand-exchange process to repair DNA damage based on existing homologous DNA sequences (Heyer et al., 2010), allowing precise insertion of foreign DNA at target regions by replacing endogenous genomic segments with donor DNA. CRISPR/Cas9-introduced site-specific DNA cleavage triggers DNA repair and greatly promotes HR at nearby regions, thus enhancing the efficiency of HDR-based genome editing (Yang et al., 2013). In contrast, the conventional NHEJ pathway initiates DNA repair with quick occupation by the Ku70/Ku80 complex at DNA broken ends, followed by recruitment of other components for end processing and subsequently DNA ligase IV for ligation. NHEJ-based DNA repair is a homology-independent and mechanistically flexible process, which often results in random insertions or deletions (indels) of a small number of nucleotides (Lieber, 2010). Hence, CRISPR/Cas9-induced NHEJ repair has been employed to generate loss-of-function alleles in protein-coding genes (Wang et al., 2013). In general, the NHEJ pathway mediates rapid DNA repair and plays an important role in various cellular contexts. Therefore, CRISPR/Cas9-induced NHEJ repair offers high efficiency and has been exploited to develop a variety of targeting strategies.

More recently, in addition to the conventional HDR and NHEJ pathways, studies have discovered the microhomology-mediated end joining (MMEJ) pathway, which is also termed as alternative NHEJ (Alt-NHEJ) pathway (Lieber, 2010; McVey & Lee, 2008). This MMEJ pathway repairs DNA damage by initiating single-strand resection similar to the HDR process, followed by microhomology-based alignment and ligation by DNA ligase III. In general, the MMEJ pathway mediates an error-prone repair process and plays a minor role to complement DNA repair by HDR and NHEJ (McVey & Lee, 2008). Collectively, the coupling of different DNA repair mechanisms with various strategies to design donor templates or select target sites in genomes, has resulted in a variety of targeting approaches, each having distinct advantages in different species (Table 1).
| Genome modifications and targeting strategies | Species | ESCs involvement | Efficiency*# | References |
|-----------------------------------------------|---------|-----------------|--------------|------------|
| NHEJ-based knockout by introducing indels     | Mouse:  | Multiple genes  | No           | 50%–80%    | Dow et al., 2015; Mandasari et al., 2016; Swiech et al., 2015; Wang et al., 2013; Xu et al., 2017 |
|                                               |         | Single gene     | 8%–90%       |            | Challa et al., 2016; Hay et al., 2017; Helsley et al., 2016; Hinze et al., 2017; Ishikawa-Fujwara et al., 2017; Jiang et al., 2017; Kasperek et al., 2016; Kim et al., 2017b; Li et al., 2015d; Mandasari et al., 2016; Meyer et al., 2016; Miannè et al., 2017; Miyata et al., 2016; Sweeney et al., 2017; Wang et al., 2015a; Zhong et al., 2015; Zhu et al., 2016 |
| Rats:                                        | Single gene | No               | 28.6%–45.5%  | Rannals et al., 2016; Wang et al., 2016c; Yoshimi et al., 2014 |
| Pig:                                         | Multiple genes | No               | N/A          | Wang et al., 2016a |
|                                               | Single gene | No               | 60%–83% for embryos | Park et al., 2017; Petersen et al., 2016; Wang et al., 2015e; Yu et al., 2016 |
|                                               | No         | 10.2% for offspring |              | Niu et al., 2014 |
| Monkey:                                      | Single gene | No               | 37.4%–87.6% for embryos | Crispo et al., 2015; Li et al., 2017b; Niu et al., 2017a; Zhang et al., 2017a |
| Sheep:                                       | Single gene | No               | 59.1%–83.3% for offspring |              |
|                                               | Goat:      | Single gene      | No           | 15%–28.6%   | Malpotra et al., 2017; Wang et al., 2015c; Zhou et al., 2017 |
|                                               | Zebrafish: | Single gene      | No           | ~32.9%      | Ablain et al., 2015; Anelli et al., 2017; Fujii et al., 2016; Gallardo et al., 2015; Gui et al., 2017; He et al., 2015; Homma et al., 2017; Hoodless et al., 2016; Lee et al., 2016; Narayanan et al., 2016; Perles et al., 2015; Shah et al., 2015; Varshney et al., 2015; Vejnar et al., 2016; Wang et al., 2014; Yuan et al., 2016a; Zhang et al., 2014 |
|                                               | Drosophila:| Multiple genes   | No           | N/A         | Port et al., 2014 |
|                                               |           | Single gene      | No           | 98.7% for embryos 100% for offspring | Bassett et al., 2014; Gao et al., 2015; Wakabayashi et al., 2016 |
|                                               | Rabbit:   | Single gene      | No           | 98.7% for embryos 100% for offspring | Lv et al., 2016; Yuan et al., 2016b |
|                                               | Mosquito: | No               | N/A          |            | Dong et al., 2015 |
| NHEJ-based knockout via deletion              | Mouse:    | Yes              | 10%–90%      | Han et al., 2014; Kraft et al., 2015; Seruggia et al., 2015; Wang et al., 2015a. 2017; Zhang et al., 2016 |
| NHEJ-based knock-in                          | Zebrafish:| No               | 4%–54%       | Auer et al., 2014; Hisano et al., 2015; Kimura et al., 2014; Li et al., 2015a |
|                                               | Frog      | No               | 8%–12%       |            | Shi et al., 2015 |
|                                               | Sheep     | No               | 34.7%        | Ma et al., 2017 |
| HDR-based knock-out                          | Drosophila:| No               | 47%          | Wen et al., 2016 |
| HDR-based knock-in                           | Mouse:    | dsDNA            | No           | 10%–88%     | Aida et al., 2015; Chu et al., 2016; Guo et al., 2016; Han et al., 2015; Ishizu et al., 2016; Lewis et al., 2016; Li et al., 2016; Mashiko et al., 2014; Wang et al., 2015b; Wu et al., 2013 |
|                                               | ssODN     | No               | 6%–66%       | Inui et al., 2014; Zhu et al., 2017 |
Enhanced genome editing via CRISPR-induced HDR

HDR is a major DNA repair mechanism broadly employed in CRISPR-based genome editing (Heyer et al., 2010). In the presence of Cas9 nuclease and specific sgRNA targeting a selected sequence in the genome, site-specific DNA cleavage is introduced at the target genomic locus, which then will trigger DNA repair. When the target cells are given a large quantity of donor templates carrying homology sequences, HDR-based repair will utilize the donors as templates to repair the damaged genome, thus introducing foreign DNA included in the donor construct into the recipient genome (Heyer et al., 2010).

The traditional gene targeting approach succeeded before the establishment of engineered nucleases. To accomplish sequence replacement in the genome, this approach relies on the HDR repair process triggered by spontaneous DNA damage that randomly occurs near target regions, (Koller et al., 1989). The desired targeting events occur at low frequency. Hence, successful genome targeting requires long homology arms in donor constructs, and needs sophisticated selection and clonal expansion in mouse ESCs before generating chimeric animals, and genetically modified offspring (Koller et al., 1989; Thomas & Capecchi, 1987). It often takes more than one year to establish a knock-in or knockout strain of mouse.

Site-specific DNA breaks trigger DNA repair around a target region. Hence, coupling this to the CRISPR system can greatly enhance the efficiency of HDR-based genome targeting and result in a high success rate of desired targeting. This improvement has bypassed the usage of ESC cells, allowing direct genome targeting in mouse zygotes (Yang et al., 2013). The direct genome targeting in zygotes via CRISPR-coupled HDR can produce a high percentage of chimeric animals and genetically modified mouse strains within 3–6 months, a much shortened period of time (Yang et al., 2013). Moreover, direct genome targeting in zygotes has also overcome the limitations of ESC unavailability, and made genome editing possible in many previously inaccessible organisms, such as pigs and monkeys (Peng et al., 2015). Furthermore, the introduction of site-specific DNA breaks allows the use of much shorter homology arms to achieve successful genetic modifications. Around 1000 bp homology fragments are usually sufficient, and around 100 bp single-stranded oligodeoxynucleotides (ssODN) carrying a 50–60 nt homology sequence at each side are effective in introducing small mutations/insertions to produce genetically modified animals (Inui et al., 2014; Zhou et al., 2016).

CRISPR-coupled HDR-mediated in vivo genome editing has been broadly used to introduce knock-in or knockout in the genome of various animal models for studying gene functions, modeling diseases, or developing novel treatment by correcting disease-associated mutations. Direct injection of Cas9 mRNA, sgRNA targeting only the mutant allele, and donor ssODN carrying a wild-type allele sequence into mouse zygotes carrying a heterozygous dominant-negative cataract-causing mutation in the Crygc gene resulted in cataract-free progeny (Wu et al., 2013). Besides rodents, large animals like pigs have also been used for disease modeling (Peng et al., 2015; Wang et al., 2015d; Zhou et al., 2016). In these studies, together with the use of the single blastocyst genotyping system and/or ssODN donors, researchers can assess sgRNA efficiency at the embryonic stage and achieve up to 80% targeting efficiency in producing animals carrying the desired genetic modification. Furthermore, successful targeting has also been reported in lower vertebrates and invertebrates (Irion et al., 2014; Li et al., 2015e; Lin & Potter, 2016; Liu et al., 2016; Ukken et al., 2016; Voutev & Mann, 2017; Yu et al., 2014). Targeted gene modification and tagging has been achieved in Drosophila based on the CRISPR/Cas9-
coupled HDR approach (Li et al., 2015e; Lin & Potter, 2016; Liu et al., 2016; Ukken et al., 2016; Voutev & Mann, 2017; Yu et al., 2014), with a similar method also applied in zebrafish, producing up to 50% targeted mutations in larvae (Irion et al., 2014). With modified ssODN templates and CRISPR components, gene editing efficiency has reached 85% in C. elegans (Paix et al., 2016). Targeted genes or long noncoding RNA (lncRNA) can be precisely replaced with fluorescence reporters to deplete target genes by inserting visible markers (Platt et al., 2014; Wen et al., 2016).

Gene correction in somatic tissues has also been performed using the CRISPR system and donor DNA (Table 2). Targeting of deficient ornithine transcarbamylase in the mouse model showed more than 10% correction of the deficient gene in liver cells and significantly improved the survival rate in target groups (Yang et al., 2016). Similarly, somatic correction of Duchenne muscular dystrophy (DMD) caused by a mutation in the gene encoding dystrophin has been reported, showing a 70% increase in functional dystrophin and apparent improvement in the mouse model (Bengtsson et al., 2017).

| Genome modifications and targeting strategies | Species | Delivery system | Efficiency | References |
|----------------------------------------------|---------|----------------|------------|------------|
| NHEJ-based knockout via indel formation       | Mouse   | Virus          | 14.8%–86%  | Cheng et al., 2014; Chiou et al., 2015; de Solis et al., 2016; Ding et al., 2014; El Fatimy et al., 2017; Guo et al., 2017; Heckl et al., 2014; Hung et al., 2016; Kaminski et al., 2016; Kim et al., 2017a; Li et al., 2017a; Monetys et al., 2017; Ortinski et al., 2017; Takebordbar et al., 2016; Wang et al., 2015a, 2016b; Yin et al., 2017 |
| NHEJ-based knockout via deletion              | Mouse   | Virus          | N/A        | Long et al., 2016; Nelson et al., 2016 |
| HDR-based knockout                            | Mouse   | Virus          | 85%        | Platt et al., 2014 |
| HDR-based knock-in                            | Mouse   | Virus          | 2.3%–6%    | Bengtsson et al., 2017; Xie et al., 2016; Yang et al., 2016; Yin et al., 2016 |
| HDR-based knock-in                            | Mouse   | Virus          | 2.3%–6%    | Bengtsson et al., 2017; Xie et al., 2016; Yang et al., 2016; Yin et al., 2016 |
| HDR-based knock-in                            | Mouse   | Virus          | 2.3%–6%    | Bengtsson et al., 2017; Xie et al., 2016; Yang et al., 2016; Yin et al., 2016 |
| MMEJ-based knock-in                           | Mouse   | Virus          | 20%        | Yao et al., 2017 |
| NHEJ-based knock-in                           | Mouse   | Virus          | 3.4%–10%   | Suzuki et al., 2016 |
| Chromosomal rearrangement                     | Mouse   | Virus          | N/A        | Blasco et al., 2014; Maddalo et al., 2014 |

*: Data were converted into percentages, without normalization or additional statistical analysis.
#: Data presented were based on records at somatic tissue level, if stage not indicated.

Diverse targeting strategies through CRISPR-induced NHEJ-mediated DNA repair

Double-strand DNA breaks due to the disruption of phosphodiester bonds between adjacent nucleotides in double-helix DNA. While HDR repairs a broad range of DNA damage, NHEJ is the primary mechanism for repairing DSBs in mammalian cells. With site-specific DSBs able to be introduced at almost any target site in the genome with high efficiency and accuracy using the CRISPR system, the NHEJ repair mechanism has been broadly employed to introduce random mutations at selected target sites. This CRISPR-coupled NHEJ-based mutagenesis approach can disrupt protein coding potential of a target gene by causing frame shift or premature termination, and therefore deplete functional proteins and introduce loss-of-function effects (Figure 1). To date, most animal models established using CRISPR technology have employed this strategy to knockout a specific gene, especially model organisms that are incompatible with the traditional HDR-based strategy, such as zebrafish or Xenopus (broadly noticed via personal communications) (Table 1 and 2) (Auer & Del Bene, 2014; Irion et al., 2014; Won & Dawid, 2017).

Furthermore, due to its simple principles and procedures, CRISPR-NHEJ-based mutagenesis has been applied in high-throughput studies. Xu et al. reported successful loss-of-function screening to identify genes essential to tumorigenesis in mice using pre-constructed sgRNA libraries (Xu et al., 2017). Interestingly, in vivo application of a sgRNA library has also been reported in zebrafish (Shah et al., 2015). Combining CRISPR-based high-throughput screening with excellent...
accessibility to embryonic development, straight-forward phenotyping has allowed large scale analysis of gene function. Shawn M. Burgess and colleagues have verified more than 50 genes by this method (Varshney et al., 2015), and Stefania Nicolia’s team has succeeded in a similar screening using the sgRNA pool-targeting miRNA family (Narayanan et al., 2016).

In addition, NHEJ repair has been found to be highly efficient in re-ligating DNA ends from DSBs concurrently produced by the CRISPR system at two different genome loci, despite the long distance in genome. In support of these observations, the CRISPR-coupled NHEJ repair mechanism has also been employed to delete selected large DNA fragments by targeting two regions in the same chromosome (Dow et al., 2015; Han et al., 2014; Wang et al., 2015b) or catalyzing the desired genomic rearrangements by targeting two selected regions from different chromosomes (Blasco et al., 2014). These strategies have succeeded in generating mouse models carrying a 353-kb intragenic deletion of Laf4, which recapitulates a human malformation syndrome (Kraft et al., 2015), and engineering mouse models that harbor chromosomal rearrangements recurrently found in lung cancer to model carcinogenesis (Blasco et al., 2014; Maddalo et al., 2014). The functional study of lncRNA genes is another important application of NHEJ-mediated large fragment deletion. Knockout of the lncRNA gene Rian through a large deletion of up to 23 kb demonstrated efficiency as high as 33% (Han et al., 2014) can be achieved, with similar results reported for the tyrosinase (Tyr) associated lncRNA gene (Seruggia et al., 2015).

Rather strikingly, CRISPR-coupled NHEJ repair has also enabled high-efficiency knock-in of exogenous DNA at pre-selected locations. This is consistent with common observations that NHEJ is the predominant repair mechanism in mammalian cells. Since the early 1980s, transgenic technology has been established and applied broadly to render stable ectopic expression by introducing foreign DNA fragments carrying complete gene cassettes into host genomes (Palmiter et al., 1982). Later studies have found that the NHEJ repair mechanism is responsible for capturing foreign DNA fragments at spontaneously occurring DSBs in the genome, resulting in random integrations (Lin & Waldman, 2001). Consistently, traditional gene targeting studies have also shown that the frequency of random DNA integration via the NHEJ repair mechanism is significantly higher (over 1000-fold) than targeted insertion mediated by the HDR pathway (Vasquez et al., 2001). Due to the unavailability of programmable site-specific nucleases and their erroneous nature, the potential of the NHEJ mechanism in targeted DNA knock-in was largely neglected for a long time.

Until recently, after ZFN was successfully established, short oligonucleotides (<100 bp) were able to be inserted efficiently at ZFN-induced DSBs via NHEJ repair (Orlando et al., 2010). Subsequently, inclusion of a ZFN or TALEN target sequence in donor vectors showed that simultaneous cleavage of donor and genome DNA could enable targeted integration via NHEJ repair (Cristea et al., 2013; Maresca et al., 2013). Using promoterless fluorescence reporters followed by direct quantification using fluorescence-activated cell sorting (FACS), we compared the frequencies of NHEJ- and HDR-mediated knock-in after coupling with the CRISPR system (He et al., 2016). We found that knock-in via CRISPR/Cas9-induced NHEJ is superior to the commonly used HDR-based method in all human cell lines examined (He et al., 2016). This NHEJ-based knock-in approach has been applied in precise reporter knock-in in zebrafish (Auer et al., 2014; Hisano et al., 2015; Irión et al., 2014; Kimura et al., 2014; Li et al., 2015a) and Xenopus (Shi et al., 2015), with such gene targeting previously impeded by the deficiency of the HDR pathway. More recently, CRISPR/Cas9-induced NHEJ has been shown to mediate high efficiency knock-in in mouse somatic tissues (Suzuki et al., 2016), but success in targeting zygotes or blastocysts to generate genetically modified mice has not yet been reported.

Through CRISPR-coupled NHEJ repair, various genome targeting strategies have been established and utilized in generating genetically modified animal models. From studies published since early 2013, 75.9% (110/145) of in vivo genome editing studies have employed NHEJ-based targeting strategies. Extensive evidence has shown that NHEJ-based genome targeting is simpler, more flexible, and more efficient compared with HDR-based approaches. Without homology sequences involved, the design and system construction for NHEJ-based strategies are less laborious. On the other hand, however, the random nature of NHEJ repair incurs disadvantages including the unpredictability of indel-based mutagenesis as well as off-target cleavage and insertion.

Genome editing by CRISPR-induced MMEJ repair
Distinct from NHEJ and HDR, the two common forms of DNA repair, MMEJ requires microhomologous sequences of only 5–25 bp for the repair of DSBs in DNA. Sakuma et al. devised a detailed protocol for CRISPR-based gene knock-in using MMEJ, termed Precise Integration into Target Chromosomes (PITCh) (Sakuma et al., 2016).

In this system, DSBs are needed in both the genomic DNA and donor vector to insert a DNA fragment from the donor into the genome. As MMEJ repair requires the presence of microhomology both upstream and downstream of the DSB site, two microhomologous sequences need to be added to the donor vector at both sides of the purpose sequence (Sakuma et al., 2016). For the CRISPR system, two sgRNAs are required to generate DNA cleavages near the microhomology sequences on both sides, while one sgRNA is used to induce DSBs on the genome DNA (Figure 2). Longer microhomologies of around 20 bp are currently used to improve accuracy. After alignment between microhomologous sequences, the unmatched non-homologous sequences at the 3′-parts on both sides of the donor appear as single-strand tails and are removed. This results in the loss of a small part of the genome sequence at the target sites. Therefore, MMEJ-based genome editing is associated with deletion/insertions that are often larger than NHEJ-introduced indels (Villarreal et al., 2012).

Targeted integration mediated by CRISPR-coupled MMEJ has been demonstrated in cultured cells and the generation of genetically modified zebrafish (He et al., 2015; Hisano et al., 2015; Nakade et al., 2014). Moreover, one-step knock-in of
Comparison between different targeting strategies

Conventional NHEJ repair does not require the presence of homology sequences and involves minimal processing of DNA broken ends. The activity of the NHEJ pathway is high and stable throughout the cell cycle. Distinctly, the HDR repair mechanism relies on long homology sequences (> 500 bp in general) to repair DNA lesions, and is only active from the late S phase to G2 phase during the cell cycle. The MMEJ pathway depends on microhomology sequences (5–25 bps) for DSB repair and is active during the M to early S phase (Taleei & Nikjoo, 2013). These differences explain why the activities of the different DNA repair pathways vary in different cell contexts.

The intrinsic activities of the two major pathways, HDR and NHEJ, also vary in different species, despite high conservation of these pathways across a broad range of organisms. Lower vertebrates, such as zebrafish and Xenopus, are deficient in HDR-based DNA repair. Hence, modification of genome sequences in these models has mainly succeeded with NHEJ-based strategies, such as transgenesis, indel-based targeted mutagenesis/deletion, or the recent knock-in approach based on coupling TALEN- or CRISPR-induced DNA cleavage to the NHEJ repair mechanism (Auer et al., 2014; Hisano et al., 2015; Irion et al., 2014; Kimura et al., 2014; Li et al., 2015a; Shi et al., 2015) (Table 1). In mammalian systems, although HDR was first employed to produce genetically modified mice, evidence shows that the NHEJ repair mechanism is predominant (Vasquez et al., 2001). Thus, the efficiency of NHEJ-based genome editing is generally superior to HDR-based approaches (He et al., 2016).

Scientists have attempted to manipulate the balance between the HDR and NHEJ pathways. Through inhibiting DNA ligase IV, a key component of the NHEJ pathway, studies have shown that the efficiency of HDR-based gene targeting can be increased substantially (Chu et al., 2015). Similarly, silencing KU70, KU80, or DNA ligase IV largely suppressed NHEJ-mediated introduction of indels at the junction and enhanced HDR-mediated genome editing (Pierce et al., 2001). To date, this type of approach has not been applied for in vivo gene targeting.

Besides efficiency, accuracy is another major concern. The HDR-based targeting strategy requires homology sequences as a template for DNA replication to repair induced DNA cleavage. It involves the cloning of homologous DNA and multi-step construction of donor plasmids. In return, the designed modifications can be introduced into the genome with high accuracy and off-target integrations can be largely reduced compared to other knock-in strategies. MMEJ-based targeting requires microhomologous sequences, which can be easily introduced into donor vectors through synthesized oligos, or during PCR amplification of the desired DNA for insertion.

Although the intrinsic MMEJ pathway often plays a minor role in overall DNA repair, the MMEJ-based targeting strategy has shown efficiency up to 10-fold higher than that of the HDR-based approach (Yao et al., 2017). Lastly, the NHEJ repair mechanism, which is completely independent of any homology sequences, offers the easiest path to modify an existing design for a new target site in the genome. In our recent study, a universal donor was established with the use of artificial sgRNA, which did not target any sequence in mice and humans (He et al., 2016). With the minimum work involved in constructing the new sgRNA to the genome, the whole system was easily orientated for targeting a new locus (He et al., 2016). However, the random errors potentially present at the integration/repair junctions with NHEJ-based targeting approaches should be considered during the design.

CONCLUSIONS

The recent advent of CRISPR technology has offered the simplest and possibly ultimate solution for introducing site-specific DSBs in genome DNA, which was once an insurmountable challenge in genome editing. Through coupling with different DNA repair mechanisms present in the endogenous cellular system, various targeting strategies have been developed to introduce a wide range of modifications in the genome through sequence-based editing. While further research is needed to evaluate the off-target issues and overcome the risks by developing improved CRISPR systems, the above technological advances have undoubtedly revolutionized biomedical research. The CRISPR-based genome editing approaches have significantly promoted studies on gene function via the rapid generation of animal models that carry genetic deficiencies of single or multiple genes. In addition, they have also enabled modeling of genetic diseases caused by chromosomal rearrangement or large deletions. Therefore, rapid progress could be foreseen in establishing various animal models for disease modeling or therapeutic intervention, which will significantly improve our understanding of human diseases and promote the development of new therapeutic strategies.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS

X.M. A.S.W., H.Y.T., S.Y.T, and D.L.C. wrote different parts of the manuscript; B.F. compiled and revised the manuscript. All authors read and approved the final manuscript.

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REFERENCES

Ablain J, Durand EM, Yang S, Zhou Y, Zon LI. 2015. A CRISPR/Cas9
vector system for tissue-specific gene disruption in zebrafish. Developmental Cell, 32(6): 756–764.

Aida T, Chiyoi K, Usami T, Ishikubo H, Imahashi R, Wada Y, Tanaka KF, Sakuma T, Yamamoto T, Tanaka K. 2015. Cloning-free CRISPR/Cas vector system facilitates functional cassette knock-in in mice. Genome Biology, 16: 87.

Aida T, Nakade S, Sakuma T, Izu Y, Oishi A, Mochida K, Ishikubo H, Usami T, Aizawa H, Yamamoto T, Tanaka K. 2016. Gene cassette knock-in in mammalian cells and zygotes by enhanced MMEJ. BMC Genomics, 17: 979.

Anelli V, Villefranc JA, Chhangawala S, Martinez-McFaline R, Riva E, Nguyen A, Verma A, Bareja R, Chen Z, Scognamiglio T, Elmento O, Houvras Y. 2017. Oncogenic BRAF disrupts thyroid morphogenesis and function via twist expression. Elife, doi: 10.7554/elife.20728.

Auer TO, Del Bene F. 2014. CRISPR/Cas9 and TALEN-mediated knock-in function via twist expression. Methods, 69(2): 142–150.

Barrangou R, Doudna JA. 2016. Applications of CRISPR/Cas systems in research and beyond. Nature Biotechnology, 34(9): 933–941.

Bassett AR, Azzam G, Wheatley L, Tibbit C, Rajakumar T, McGowan S, Auer TO, Del Bene F. 2014. CRISPR/Cas9 and TALEN-mediated knock-in approaches in zebrasfish. Developmental Cell, 33(5): 543–548.

Chiu VT, Weber T, Graf R, Sommermann T, Peths K, Sack U, Volchkov P, Rajewsky K, Kühn R. 2016. Efficient generation of Rosa26 knock-in mice using CRISPR/Cas9 in C57BL/6 zygotes. BMC Biotechnology, 16: 4.

Courtenay DG, Moore JE, Atkinson SD, Maurizi E, Allen EHA, Pedrioli DML, McLean WHI, Nesbit MA, Moore CBT. 2016. CRISPR/Cas9 DNA cleavage at SNP-derived PAM enables both in vitro and in vivo KRT12 mutation-specific targeting. Gene Therapy, 23(1): 108–112.

Crispo M, Mulet AP, Tesson L, Barrera N, Cuadro F, dos Santos-Neto PC, Nguyen TH, Créngay A, Brusselle L, Anégond I, Menchaca A. 2014. Efficient generation of myostatin knockout sheep using CRISPR/Cas9 technology and microinjection into zygotes. PLoS One, 10(9): e0136690.

Cristea S, Freyvert Y, Santiago Y, Holmes MC, Urmov FD, Gregory PD, Cost GJ. 2013. In vivo cleavage of transgene donors promotes nucleasemediated targeted integration. Biotechnology and Bioengineering, 110(3): 871–880.

de Sois CA, Ho A, Holehonnur R, Ploski JE. 2016. The development of a viral mediated CRISPR/Cas9 system with doxycycline dependent gRNA expression for inducible in vitro and in vivo genome editing. Frontiers in Molecular Neuroscience, 9: 70.

Ding QR, Strong A, Patel KM, Ng SL, Gosis BS, Regan SN, Cowan CA, Rader DJ, Musunuru K. 2014. Permanent alteration of PCSK9 with in vivo CRISPR-Cas9 genome editing. Circulation Research, 115(5): 488–492.

Dong SZ, Lin JY, Held NL, Clem RJ, Passarelli AL, Franz AWE. 2015. Heritable CRISPR/Cas9-mediated genome editing in the yellow fever mosquito, Aedes aegypti. PLoS One, 10(3): e0122353.

Dow LE, Fisher J, O’Rourke KP, Muley A, Kastenhuber ER, Livshits G, Tschaharganeh DF, Socci ND, Lowe SW. 2015. Inducible in vivo genome editing with CRISPR-Cas9. Nature Biotechnology, 33(4): 390–394.

El Fatimy R, Subramanian S, Uhlmann EJ, Krichovsky AM. 2017. Genome editing reveals glioblastoma addiction to MicroRNA-10b. Molecular Therapy, 25(2): 368–376.

Fujii T, Tsumesumi S, Sagara H, Munakata M, Hisaki Y, Sekiya T, Furukawa Y, Sakamoto K, Watanabe S. 2016. Smyd5 plays pivotal roles in both primitive and definitive hematopoiesis during zebrafish embryogenesis. Scientific Reports, 6: 29157.

Gallardo VE, Varshney GK, Lee M, Bupp S, Xu L, Shinn P, Crawford NP, Inglesie J, Burgess SM. 2015. Phenotype-driven chemical screening in zebrafish for compounds that inhibit collective cell migration identifies multiple pathways potentially involved in metastatic invasion. Disease Models & Mechanisms, 8(6): 565–576.

Gao JL, Fan YJ, Wang XY, Zhang Y, Pu J, Li L, Shao W, Zhan S, Hao J, Xu YZ. 2015. A conserved intronic U1 snRNP-binding sequence promotes
trans-splicing in Drosophila. Genes & Development, 29(7): 760–771.

Gui HS, Schriemer D, Cheng WW, Chauhan RK, Antifloho G, Berrios C, Bleda M, Brooks AS, Brouwer RWW, Burns AJ, Cherry SS, Dopazo J, Egggen BUL, Grisner P, Jaloh B, Le TL, Lui VGH, Luzon -Toaro B, Matera I, Ngan ESW, Pelet A, Ruiz-Ferrer M, Sham PC, Shepherd IT, So MT, Sribudiani Y, Tang CSM, van den Hout MCGN, van der Linde HC, van Ham TJ, van Ljeken WJF, Verheij JBGM, Amiel J, Borrego S, Ceccherini I, Chakravarti A, Lyonnet S, Tam PKH, Garcia-Barceló MM, Hofstra RMW. 2017. Whole exome sequencing coupled with unbiased functional analysis reveals new Hirschsprung disease genes. Genome Biology, 18(1): 48.

Guo H, Cooper S, Friedman AD. 2016. In vivo deletion of the cebpa +37 kb enhancer markedly reduces cebpa mRNA in myeloid progenitors but not in non-hematopoietic tissues to impair granulopoiesis. PLoS One, 11(3): e0150809.

Guo YX, VanDusen NJ, Zhang LN, Gu WL, Sethi I, Guatimosim S, Ma Q, Jardin BD, Ai YL, Zhang DH, Chen BY, Guo A, Yuan GC, Song LS, Pu WT. 2017. Analysis of cardiac myocyte maturation using CASAaAV, a platform for rapid dissection of cardiac myocyte gene function in vivo. Circulation Research, 120(12): 1874–1888.

Han JX, Zhang J, Chen L, Shen B, Zhou Jk, Hu B, Du YN, Tate PH, Huang XG, Zhang WS. 2014. Efficient in vivo deletion of a large imprinted IncRNA by CRISPR/Cas9. RNA Biology, 11(7): 829–835.

Han Y, Silvano OJ, Christie CK, Cheng AW, Miano JM. 2015. CRISPR-Cas9 genome editing of a single regulatory element nearly abolishes target gene expression in mice—brief report. Arteriosclerosis, Thrombosis, and Vascular Biology, 35(2): 312–315.

Hay EA, Khalaf AR, Marini P, Brown A, Heath K, Sheddar P, MacKenzie A. 2016. Analysis of possible off target effects following Cas9/CRISPR targeted deletions of neuropeptide gene enhancers from the mouse genome. Neuropeptides, 64: 101–107.

He MD, Zhang FH, Wang HL, Wang HP, Zhu SY, Sun YH. 2015. Efficient ligase 3-dependent microRNA-mediated end joining repair of DNA double-strand breaks in zebrafish embryos. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 780: 86–96.

He XJ, Tan CL, Wang F, Wang YF, Zhou R, Cui DX, You WX, Zhao H, Ren JW, Feng B. 2016. Knock-in of large reporter genes in human cells via CRISPR/Cas9-induced homology-dependent and independent DNA repair. Nucleic Acids Research, 44(9): e85.

Heckl D, Kowalczyk MS, Yudovich D, Belizaire R, Puram RV, McConkey ME, Thielke A, Aster JC, Regev A, Ebert BL. 2014. Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. Nature Biotechnology, 32(9): 941–946.

Helsley RN, Sui YP, Park SH, Liu Z, Lee RG, Zhu BB, Kern PA, Zhou CC. 2016. Targeting lkb1 kinase β in adipocyte lineage cells for treatment of obesity and metabolic dysfunctions. Stem Cells, 34(7): 1883–1895.

Heyer WD, Ehmsen KT, Liu J. 2010. Regulation of homologous recombination in eukaryotes. Annual Review of Genetics, 44(1): 113–139.

Hinze SJ, Jackson MR, Lie S, Jolly L, Field M, Barry SC, Harvey RJ, Shoobridge C. 2017. Incorrect dosage of IQSEC2, a known intellectual disability and epilepsy gene, disrupts dendritic spine morphogenesis. Translational Psychiatry, 7: e1110.

Hisano Y, Sakuma T, Nakade S, Ohga R, Ota S, Okamoto H, Yamamoto T, Kawahara A. 2015. Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. Scientific Reports, 5: 8841.

Homma N, Harada Y, Uchikawa T, Kamei Y, Fukamachi S. 2017. Protanopia (red color-blindness) in medaka: a simple system for producing color-blind fish and testing their spectral sensitivity. BMC Genetics, 18: 10.

Hoodless LJ, Lucas CD, Duffin R, Dervin MA, Haslett C, Tucker CS, Rossi AG. 2016. Genetic and pharmaceutical inhibition of CDK9 drives neutrophil apoptosis to resolve inflammation in zebrafish in vivo. Scientific Reports, 5: 36980.

Hung SSC, Chrysostomou V, Li F, Lim JK, Wang JH, Powell JE, Tu L, Daniszewski M, Lo C, Wong RC, Crowston JG, Pébay A, King AE, Bui BV, Liu GS, Hewitt AW. 2016. AV-mediated CRISPR/Cas gene editing of retinal cells in vivo. Investigative Ophthalmology & Visual Science, 57(7): 3470–3476.

Inui M, Miyado M, Igarashi M, Tamano M, Kubo A, Yamashita S, Asahara H, Fukami M, Takada S. 2014. Rapid generation of mouse models with defined point mutations by the CRISPR/Cas9 system. Scientific Reports, 4: 5396.

Irion U, Krauss J, Nusslein-Volhard C. 2014. Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. Development, 141(24): 4827–4830.

Ishikawa-Fujitawa T, Shiraishi E, Fujikawa Y, Mori T, Tsujimura T, Todo T. 2017. Targeted inactivation of DNA polymerase genes in medaka fish (Oryzias latipes). Photochemistry and Photobiology, 93(1): 315–322.

Ishizu N, Uy DS, Hebisawa A, Aizawa H, Cui WP, Fujita Y, Hashimoto K, Ajikoa I, Mizusawa H, Yokota T, Watabe K. 2016. Impaired striatal dopamine release in homozygous Vps35 D620N knock-in mice. Human Molecular Genetics, 25(20): 4507–4517.

Jiang C, Mei M, Li B, Zhu XR, Zu WH, Tian YJ, Wang QN, Guo Y, Dong YZ, Tan X. 2017. A non-viral CRISPR/Cas9 delivery system for therapeutically targeting HBV DNA and pck9 in vivo. Cell Research, 27(3): 440–443.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. RNA-induced site-specific DNA cleavage by CRISPR-Cas nucleases. Science, 337(6096): 816–821.

Kalebic N, Taverna E, Tavano S, Wong FK, Suchold D, Winkler S, Hutner WB, Sarov M. 2016. CRISPR/Cas9-induced disruption of gene expression in mouse embryonic brain and single neural stem cells in vivo. EMBO Reports, 17(3): 338–348.

Kaminski R, Bella R, Yin C, Otte J, Ferrante P, Gendelman HE, Li H, Booze J, Gordon H, Wu WH, Khalili K. 2016. Excision of HIV-1 DNA by gene editing: a proof-of-concept in vivo study. Gene Therapy, 23(8–9): 690–695.

Kasperek P, Ileninova Z, Haneckova R, Kanchev I, Jerickova I, Sledacek R. 2016. A viable mouse model for Netherton syndrome based on mosaical inactivation of the Spink5 gene. Biological Chemistry, 397(12): 1287–1292.

Katigbak A, Cencic R, Robert F, Senecha P, Scuoppo C, Pelletier J. 2016. A viable mouse model for Netherton syndrome based on mosaic inactivation of the Spink5 gene. Biological Chemistry, 397(12): 1287–1292.

Katigbak A, Cencic R, Robert F, Senecha P, Scuoppo C, Pelletier J. 2016. A viable mouse model for Netherton syndrome based on mosaic inactivation of the Spink5 gene. Biological Chemistry, 397(12): 1287–1292.

Kim E, Koo T, Park SW, Kim D, Kim K, Cho HY, Song D, Lee KJ, Jung MH, Kim S, Kim JH, Kim JM. 2017a. In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. Nature Communications, 8: 14500.

Kim K, Park SW, Kim JM, Lee SH, Kim D, Koo T, Kim KE, Kim JM. 2017b. Genome surgery using Cas9 ribonucleoproteins for the treatment of age-related macular degeneration. Genome Research, 27(3): 419–426.

Kim YG, Cha J, Chandrasegaran S. 1996. Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. Proceedings of the National

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Kraft K, Geuer S, Will AJ, Lee Chan W, Paliou C, Borschower M, Harabula I, Wittler T, Franke M, Ibrahim DM, Krage Steven BK, Spielmann M, Mundlos S, Lupliádez DG, Andrey G. 2015. Deletions, inversions, duplications: engineering of structural variants using CRISPR/Cas in mice. Cell Reports, 10(5): 833–839.

Latella MC, Di Salvo MT, Cocchiarella F, Benati D, Grisendi G, Comitato A, Marigo V, Recchia A. 2016. In vivo editing of the human mutant rhodopsin gene by electroporation of plasmid-based CRISPR/Cas9 in the Mouse Retina. Molecular Therapy Nucleic Acids, 5: e389.

Lee RT, Ng AS, Ingham PW. 2016. Ribozyme mediated gRNA generation for in vitro and in vivo CRISPR/Cas9 mutagenesis. PLoS One, 11(11): e0166020.

Lewis WR, Malarkey EB, Treschler D, Bower R, Pasek RC, Porath JD, Birdet SE, Sannier S, Antignac C, Knowles MR, Leigh MW, Zarivala MA, Challia AK, Kesterson RA, Rowe SM, Drummond IA, Parent JM, Hildebrandt F, Porter MY, Yoder BK, Berbari NF, Dutcher SK. 2016. Mutation of growth arrest specific 8 reveals a role in motile cilia function and human disease. PLoS Genetics, 12(7): e1006220.

Li J, Zhang BB, Ren YG, Gu SY, Xiang YH, Huang C, Du JL. 2015a. Intron targeting-mediated and endogenous gene integrity-maintaining knockin in zebrafish using the CRISPR/Cas9 system. Cell Research, 25(5): 634–637.

Li L, Song LJ, Liu XW, Yang X, Li X, He T, Wang N, Yang S, Yu C, Yin T, Wen YZ, He ZY, Wei XW, Su WJ, Wu QJ, Yao SH, Gong CY, Wei YQ. 2017a. Artificial virus delivers CRISPR-Cas9 system for genome editing of cells in mice. ACS Nano, 11(1): 95–111.

Li MC, Feng B, Wang L, Guo S, Zhang P, Gong J, Zhang Y, Zheng AK, Li HL. 2015c. Tollip is a critical mediator of cerebral ischaemia-reperfusion injury. The Journal of Pathology, 237(2): 249–262.

Li MY, Huang R, Jiang X, Chen YX, Zhang Z, Zhang XY, Liang PP, Zhan SQ, Cao SB, Zhou SY, Huang JJ. 2015d. CRISPR/Cas9 promotes functional study of tests specific X-linked gene in vivo. PLoS One, 10(11): e0143148.

Li QY, Barish S, Okuwa S, Volkman PC. 2015. Examination of endogenous rotund expression and function in developing Drosophila olfactory system using CRISPR-Cas9-mediated protein tagging. G3 (Bethesda), 5(12): 2809–2816.

Li WR, Liu CX, Zhang XM, Chen L, Peng XR, He SG, Lin JP, Han B, Wang LQ, Huang JC, Liu MJ. 2017b. CRISPR/Cas9-mediated loss of FGFR5 function increases wool staple length in sheep. FEBS Journal, 284(17): 2764–2773.

Li XJ, Li YQ, Han GY, Li XR, Ji YS, Fan ZR, Zhong YL, Cao J, Zhao J, Mariuza G, Zhang MZ, Wen JG, Nesland JM, Suo Zh. 2016. Establishment of mitochondrial pyruvate carrier 1 (MPC1) gene knockout mice with preliminary gene function analyses. Oncotarget, 7(48): 79981–79994.

Li MY, Huang R, Jiang X, Chen YX, Zhang Z, Zhang XY, Liang PP, Zhan SQ, Cao SB, Zhou SY, Huang JJ. 2015d. CRISPR/Cas9 promotes functional study of tests specific X-linked gene in vivo. PLoS One, 10(11): e0143148.

Liang WC, Liang PP, Wong CW, Ng TB, Huang JJ, Zhang JF, Waye MMY, Fu WM. 2017. CRISPR/Cas9 technology targeting fas gene protects mice from concanavalin-a induced fulminant hepatic failure. Journal of Cellular Biochemistry,118(3): 530–536.

Lieber MR. 2010. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annual Review of Biochemistry, 79: 181–211.

Lin CC, Potter CJ. 2016. Non-mendelian dominant maternal effects caused by CRISPR/Cas9 transgenic components in Drosophila melanogaster. G3 (Bethesda), 6(11): 3586–3691.

Lin Y, Waldman AS. 2001. Capture of DNA sequences at double-strand breaks in mammalian chromosomes. Genetics, 158(4): 1665–1674.

Liu Y, Lin JJ, Zhang MJ, Chen K, Yang SX, Wang Q, Yang HQ, Xie SS, Zhou YJ, Zhang X, Chen F, Yang YF. 2016. PINK1 is required for timely cell-type specific mitochondrial clearance during Drosophila midgut metamorphosis. Developmental Biology, 419(2): 357–372.

Long CZ, Amosii L, Mireault AA, McNally JR, Li H, Sanchez-Ortiz E, Bhattachararya S, Shelton JM, Bassel-Duby R, Olson EN. 2016. Postnatal genome editing partially restores dystrophin expression in a mouse model of muscular dystrophy. Science, 351(6271): 400–403.

Lv QY, Yuan L, Deng JC, Chen M, Wang Y, Zeng J, Li ZJ, Lai LX. 2016. Efficient generation of Myostatin gene mutated rabbit by CRISPR/Cas9. Scientific Reports, 6: 25092.

Ma T, Tao JL, Yang MH, He CJ, Tian XZ, Zhang XS, Zhang JL, Deng SL, Feng JZ, Zhang ZZ, Wang J, Ji PY, Song YK, He PL, Han HB, Fu JC, Lian ZX, Liu GS. 2017. An AANAT/ASMT transgenic animal model constructed with CRISPR/Cas9 system serving as the mammary gland bioreactor to produce melatonin-enriched milk in sheep. Journal of Pineal Research, 63(1): e12406.

Maddalo D, Manchado E, Concepcion CP, Bonetti C, Vidigal JA, Han YC, Ogrodowski P, Crippa A, Rektmann N, de Stanchina E, Lowe SW, Ventura A. 2014. In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. Nature, 516(7531): 423–427.

Maili P, Yang LH, Eszeit KM, Aach J, Guell M, Dicarlo JE, Norville JE, Church GM. 2013. RNA-guided human genome engineering via Cas9. Science, 339(6121): 823–828.

Malpota S, Vats A, Kumar S, Gautam D, De S. 2017. Generation of genomic deletions of (Rig-I GENE) in goat primary cell culture using CRISPR/CAS9 method. Animal Biotechnology, doi: 10.1080/10495398.2017.1331915.

Mandasari M, Sawangarun W, Katsube K, Kayamori K, Yamaguchi A, Maresch R, Mueller S, Veltkamp C, Öllinger R, Friedrich M, Heid I, Unger K, Yang FT, Schmid RM, Vassiliou GS, Braren R, Schneider G, Steiger AJ, Heiwenlander M, Bradley A, Saur D, Rad R. 2016. Multiplexed pancreatic genome engineering and cancer induction by transfection-based...
CRISPR/Cas9 delivery in mice. Nature Communications, 7: 10770.

Mashiko D, Young SAM, Muto M, Kato H, Nozawa K, Ogawa M, Noda T, Kim YJ, Satoh Y, Fujihara Y, Ikawa M. 2014. Feasibility for a large scale mouse mutagenesis by injecting CRISPR/Cas plasmid into zygotes. Development, Growth & Differentiation, 56(1): 122–129.

McVey M, Lee SE. 2008. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. Trends in Genetics, 24(11): 529–538.

Meyer MB, Benkusky NA, Onal M, Pike JW. 2016. Selective regulation of zinc-finger nuclease-driven targeted integration into mammalian genomes using donors with limited chromosomal homology. Nucleic Acids Research, 38(15): e152.

Ortinski PI, O'Donovan B, Dong XY, Kantor B. 2017. Integrase-deficient lentiviral vector as an all-in-one platform for highly efficient CRISPR/Cas9-mediated gene editing. Molecular Therapy. Methods & Clinical Development, 5: 153–164.

Ou ZH, Niu XH, He WY, Chen YC, Song B, Tian YX, Fan D, Tang DL, Sun XF. 2016. The combination of CRISPR/Cas9 and iPSC technologies in the gene therapy of human β-thalassemia in mice. Scientific Reports, 6: 32463.

Palminteri RD, Brinster RL, Hammer RE, Trumbauer ME, Rosenfeld MG, Birmberg NC, Evans RM. 1982. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature, 300(5893): 611–615.

Park KE, Kaucher AV, Powell A, Waqaas MS, Sandmaier SES, Oatley MJ, Park CH, Tibary A, Donovan DM, Blomberg LA, Lillico SG, Whitelaw CB, Mileham A, Telugu BP, Oatley JM. 2017. Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. Scientific Reports, 7: 40176.

Perles Z, Moon S, Ta-Shma A, Yaacov B, Francescatto L, Edvardson S, Rein AJ, Elpeleg O, Katsanis N. 2015. A human laterality disorder caused by a homozygous deleterious mutation in MMP21. Journal of Medical Genetics, 52(12): 840–847.

Platt RJ, Chen SD, Zhou Y, Miy M, Swiech L, Kempston HR, Dahlman JE, Parnas E, Eisenhaure TM, Jovanovic M, Graham DB, SuJ3hunwala S, Heidenreich M, Xavier RJ, Langer R, Anderson DG, Hacozen N, Regev A, Feng GP, Sharp PA, Zhang F. 2014. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell, 159(2): 440–455.

Port F, Chen HM, Lee T, Bullock SL. 2014. Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. Proceedings of the National Academy of Sciences of the United States of America, 111(29): E2967–E2976.

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. 2013. Genome engineering using the CRISPR-Cas9 system. Nature Protocols, 8(11):
Rannals MD, Page SC, Campbell MN, Gallo RA, Mayfield B, Maher BJ. 2016. Neurodevelopmental models of transcription factor 4 deficiency converge on a common ion channel as a potential therapeutic target for Pitt Hopkins syndrome. *Rare Diseases*, 4(1): e1220468.

Rouet P, Smith F, Jasin M. 1994. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, 91(13): 6064–6068.

Sakuma T, Nakade S, Sakane Y, Suzuki KT, Yamamoto T. 2016. MMEJ-assisted gene knock-in using TALENs and CRISPR-Cas9 with the PITCh system. *Nucleic Acids Research*, 43(10): 4855–4867.

Shah AN, Davey CF, Whitebirch AC, Miller AC, Moens CB. 2015. Rapid reverse genetic screening using CRISPR in zebrafish. *Biotechnology*, 12(6): 535–540.

Shi ZY, Wang FQ, Cui Y, Liu ZZ, Guo XG, Zhang YQ, Deng Y, Zhao H, Chen YL. 2015. Heritable CRISPR/Cas9-mediated targeted integration in *Xenopus tropicalis*. *FASEB Journal*, 29(12): 4914–4923.

Shimmyo Y, Tanaka S, Tsunoda S, Hosomichi K, Tajima A, Kawasaki H. 2016. CRISPR/Cas9-mediated gene knockout in the mouse brain using in utero electroporation. *Scientific Reports*, 6: 20611.

Staub C, Granger AJ, Saulinier JL, Sabatini BL. 2014. CRISPR/Cas9-mediated gene knock-down in post-mitotic neurons. *PLoS One*, 9: e105584.

Suzuki K, Tsunekawa Y, Hernandez-Benitez R, Wu J, Zhu J, Kim EJ, Hatanaka F, Yamamoto M, Azaroka T, Li Z, Kurita M, Hishida T, Li M, Aizawa E, Guo SC, Chen S, Goebel A, Soligalla RD, Qu J, Jiang TS, Fu X, Jafari M, Esteban CR, Berggren WT, Lajara J, Nufiez-Delicedo E, Guillen P, Campistol JM, Matsuzaki F, Liu GH, Magistretti P, Zhang K, Callaway EM, Zhang K, Belmonte JC. 2016. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature*, 540(7631): 144–149.

Sweeney CL, Choi U, Liu C, Koontz S, Ha SK, Malech HL. 2017. CRISPR-mediated knockout of cyyb in NSC mice establishes a model of chronic granulomatous disease for human stem-cell gene therapy transplants. *Human gene therapy*, 28(7): 565–575.

Swiech L, Heidenreich M, Banerjee A, Habib N, Li Y, Trombetta J, Sur M, Zhang F. 2015. *In vivo* interrogation of gene function in the mammalian brain using CRISPR-Cas9. *Nature Biotechnology*, 33(1): 102–106.

Tabeobdorbar M, Zhu KK, Cheng JK, Cheng WL, Widrick JJ, Yan WX, Maesner C, Wu EY, Xiao R, Ran FA, Cong L, Zhang K, Vandenbergh LH, Church GM, Wagers AJ. 2016. In vivo gene editing in dystrophic mouse muscle and stem cell targets. *Science*, 351(6271): 407–411.

Taleei R, Nikjoo H. 2013. Biochemical DSB-repair model for mammalian cells in G1 and early S phases of the cell cycle. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 756(1–2): 206–212.

Taylor EM, Lehmann AR. 1998. Conservation of eukaryotic DNA repair mechanisms. *International Journal of Radiation Biology*, 74(3): 277–286.

Thomas KR, Capecchi MR. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem-cells. *Cell*, 51(3): 503–512.
2016c. Characterization of novel cytochrome P450 2E1 knockout rat model generated by CRISPR/Cas9. Biochemical Pharmacology, 105: 80–90.

Wang XL, Zhou JW, Cao CW, Huang JJ, Hai T, Wang YF, Zheng QT, Zhang HY, Qin GS, Miao XN, Wang HM, Cao SZ, Zhou Q, Zhao JG. 2015d. Efficient CRISPR/Cas9-mediated biallelic gene disruption and site-specific knockin after rapid selection of highly active sgRNAs in pigs. Scientific Reports, 5: 13348.

Wang XL, Yu HH, Lei AM, Zhou JK, Zeng WX, Zhu HJ, Dong ZM, Niu YY, Shi BB, Cai B, Liu JW, Huang S, Yan HL, Zhao XE, Zhou GX, He XL, Chen XX, Yang XZ, Li XT, Shi L, Tian XE, Wang YJ, Ma BH, Huang XX, Gao L, Chen YL. 2015c. Generation of gene-modified goats targeting FGF5 and FGR5 via zygote injection of Cas9/sgRNA system. Scientific Reports, 5: 13878.

Wang XL, Cao CW, Huang JJ, Yao J, Hai T, Zheng QT, Wang X, Zhang HY, Qin GS, Cheng JB, Wang YF, Yuan ZQ, Zhou Q, Wang HM, Zhao JG. 2016a. One-step generation of triple gene-targeted pigs using CRISPR/Cas9 system. Scientific Reports, 6: 20620.

Wang Y, Du YA, Shen B, Zhou XY, Li J, Liu Y, Wang JY, Zhou JK, Hu B, Kang NN, Gao JM, Yang XQ, Huang XX, Wei H. 2015e. Efficient generation of gene-modified pigs via injection of zygote with Cas9/sgRNA. Scientific Reports, 5: 8256.

Weber J, Öllinger R, Friedrich M, Ehrler U, Barenboim M, Steiger K, Heid I, Mueller S, Maresch R, Englertner T, Gross N, Geumann U, Fu BY, Segler A, Yuan DT, Lange S, Strong A, de la Rosa J, Liszt P, Cadiñanos Jan, Vassiliou GS, Schmid RM, Schneider G, Unger K, Yang FT, Braren R, Heikenwälder M, Varela I, Saur D, Bradley A, Rad R. 2015. CRISPR/Cas9 somatic multiplex-mutagenesis for high-throughput functional cancer genomics in mice. Proceedings of the National Academy of Sciences of the United States of America, 112(45): 13982–13987.

Wen KJ, Yang LJ, Xiong TL, Di C, Ma DH, Wu MH, Xue ZY, Zhang XD, Long L, Zhang WM, Zhang JY, Bi XL, Dai JB, Zhang QF, Lu ZJ, Gao GJ. 2016. Critical roles of long noncoding RNAs in Drosophila spermatogenesis. Genome Research, 26(9): 1233–1244.

Wiedenheft B, Sternberg SH, Doudna JA. 2012. RNA-guided gene silencing systems in bacteria and archaea. Nature, 482(7385): 331–338.

Won M, Dawid IB. 2017. PCR artifact in testing for homologous recombination in genomic editing in zebrafish. Won M, Dawid IB. 2017. PCR artifact in testing for homologous recombination in genomic editing in zebrafish. Won M, Dawid IB. 2017. PCR artifact in testing for homologous recombination in genomic editing in zebrafish. Won M, Dawid IB. 2017. PCR artifact in testing for homologous recombination in genomic editing in zebrafish. Won M, Dawid IB. 2017. PCR artifact in testing for homologous recombination in genomic editing in zebrafish. Won M, Dawid IB. 2017. PCR artifact in testing for homologous recombination in genomic editing in zebrafish. Won M, Dawid IB. 2017. PCR artifact in testing for homologous recombination in genomic editing in zebrafish.

Wu Y, Cai H, Wang YH, Bai MZ, Tang W, Bao SM, Yan ZQ, Li DS, Li JS. 2013. Correction of a genetic disease in mouse via CRISPR Cas9. Cell Stem Cell, 13(6): 659–662.

Xie C, Zhang YP, Song L, Luo J, Qi W, Hu JL, Lu DB, Yang Z, Zhang J, Xiao J, Zhou B, Du JL, Jing NH, Liu Y, Wang Y, Li BL, Song BL. 2016. Genome editing with CRISPR/Cas9 in postnatal mice corrects PRKAG2 cardiac syndrome. Cell Research, 26(10): 1099–1111.

Xu CL, Qi XL, Du XG, Zou HY, Gao F, Peng T, Lu HX, Li SL, An XM, Zhang LJ, Wu Y, Liu Y, Li N, Capocchi MR, Wu S. 2017. piggyBac mediates efficient in vivo CRISPR library screening for tumorigenesis in mice. Proceedings of the National Academy of Sciences of the United States of America, 114(4): 722–727.

Xue W, Chen SD, Yin H, Tammela T, Papagiannakopoulos T, Joshi NS, Cai WX, Yang G, Bronson R, Crowley DG, Zhang F, Anderson DG, Sharp PA, Jacks T. 2014. CRISPR-Cas9-mediated direct mutation of cancer genes in the mouse liver. Nature, 514(7522): 380–384.

Yao H, Wang HY, Shivalila CS, Cheng AW, Shi LY, Jaenisch R. 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell, 154(6): 1370–1379.

Yang Y, Wang LL, Bell P, McMenamin D, He ZN, White J, Yu HW, Xu CY, Moritzono H, Musunuru K, Batshaw ML, Wilson JM. 2016. A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. Nature Biotechnology, 34(3): 334–338.

Yao X, Wang X, Liu JL, Hu XD, Shi LY, Shen XW, Ying WQ, Sun XW, Wang X, Huang PY, Yang H. 2017. CRISPR/Cas9 mediated precise targeted integration in vivo using a double cut donor with short homology arms. EBioMedicine, 20: 19–26.

Yin CR, Tang Z, Xu QY, Zhang YG, Putatunda R, Xiao X, Li F, Xiao WD, Zhao HQ, Dai S, Qin XB, Mo XM, Young WB, Khalili K, Hu WH. 2017. In vivo excision of HIV-1 provirus by saCas9 and multiplex single-guide RNAs in animal models. Molecular Therapy, 25(5): 1168–1168.

Yin H, Song CQ, Dorkin JH, Zhu LJ, Li YX, Wu QQ, Park A, Yang J, Suresh S, Bizhanova A, Gupta A, Bolukbasi MF, Walsh S, Bogorad RL, Gao GP, Wang ZP, Dong YZ, Kolteniansky V, Wolfe SA, Langer R, Xue W, Anderson DG. 2016. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. Nature Biotechnology, 34(3): 326–333.

Yoshimura K, Kaneko T, Voigt B, Mashimo T. 2014. Allele-specific genome editing and correction of disease-associated phenotypes in rats using the CRISPR-Cas platform. Nature Communications, 5: 4240.

Yu HH, Zhao H, Qing YB, Pan WR, Jia BY, Zhao HY, Huang XX, Wei HJ. 2016. Porcine zygote injection with Cas9/sgRNA results in DMD-modified pig with muscle dystrophy. International Journal of Molecular Sciences, 17(10): 1668.

Yu ZS, Chen HQ, Liu JY, Zhang HT, Yan Y, Zhu NN, Guo YW, Yang B, Chang Y, Dai F, Liang XH, Chen XY, Shen Y, Deng WM, Chen JM, Zhang B, Li CQ, Xiao J. 2014. Various applications of TALEN- and CRISPR/Cas9-mediated homologous recombination to modify the Drosophila genome. BioOpen, 4(4): 271–280.

Yuan BL, Wang XS, Fan CY, You J, Liu YC, Weber JD, Zhong HB, Zhang YD. 2016a. DHX33 transcriptionally controls genes involved in the cell cycle. Molecular and Cellular Biology, 36(23): 2903–2917.

Yuan L, Sui TT, Chen M, Deng JC, Huang YY, Zeng J, Lv QY, Song YN, Li ZJ, Lai LX. 2016b. CRISPR/Cas9-mediated GJA8 knockout in rabbits recapitulates human congenital cataracts. Scientific Reports, 6: 22024.

Zhang DH, Golubkov VS, Han WL, Correa RG, Zhou Y, Lee S, Strongin AY, Dong PD. 2014. Identification of Annexin A4 as a hepatopancreatic factor involved in liver cell survival. Developmental Biology, 395(1): 96–110.

Zhang T, Yin YJ, Liu H, Du WL, Ren CH, Wang L, Lu HZ, Zhang ZY. 2016b. Generation of VDR knockout mice via zygote injection of CRISPR/Cas9 system. PLoS One, 11(9): e0165351.

Zhang XM, Li WR, Liu CX, Peng XR, Lin JP, He SG, Li XJ, Han B, Zhang N, Wu YS, Chen L, Wang LQ, MaYila, Huang JG, Lu MJ. 2017a. Alteration of sheep coat color pattern by disruption of ASIP gene via CRISPR Cas9. Scientific Reports, 7: 8149.

Zhang YH, Wu LZ, Liang HL, Yang Y, Qiu J, Kan Q, Zhu W, Ma CL, Zhou X. 2017b. Pulmonary surfactant synthesis in miRNA-26a-1/miRNA-26a-2
double knockout mice generated using the CRISPR/Cas9 system. *American Journal of Translational Research*, 9(2): 355–365.

Zhong H, Chen YY, Li YM, Chen R, Mardon G. 2015. CRISPR-engineered mosaicism rapidly reveals that loss of *Kcnj13* function in mice mimics human disease phenotypes. *Scientific Reports*, 5: 8366.

Zhou WJ, Wan YJ, Guo RH, Deng MT, Deng KP, Wang Z, Zhang YL, Wang F. 2017. Generation of beta-lactoglobulin knockout goats using CRISPR/Cas9. *PLoS One*, 12(10): e0186056.

Zhou XY, Wang LL, Du YN, Xie F, Li L, Liu Y, Liu CH, Wang SQ, Zhang SB, Huang XX, Wang Y, Wei H. 2016. Efficient generation of gene-modified pigs harboring precise orthologous human mutation via CRISPR/Cas9-induced homology-directed repair in zygotes. *Human Mutation*, 37(1): 110–118.

Zhu QM, Ko KA, Ture S, Mastrangelo MA, Chen MH, Johnson AD, O'Donnell CJ, Morrell CN, Miano JM, Lowenstein CJ. 2017. Novel thrombotic function of a human SNP in STXBP5 revealed by CRISPR/Cas9 gene editing in mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 37(2): 264–270.

Zhu W, Xie K, Xu YJ, Wang L, Chen KM, Zhang LZ, Fang JM. 2016. CRISPR/Cas9 produces anti-hepatitis B virus effect in hepatoma cells and transgenic mouse. *Virus Research*, 217: 125–132.

Zinn AR, Butow RA. 1985. Nonreciprocal exchange between alleles of the yeast mitochondrial 21S rRNA gene: kinetics and the involvement of a double-strand break. *Cell*, 40(4): 887–895.