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Developing genetically engineered mouse models using engineered nucleases: Current status, challenges, and the way forward

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The rapid development of engineered nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regulated interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonuclease 9 (Cas9) system has ushered in the era of 'renaissance in precision genome engineering' with profound potential to generate mouse models of human diseases. However, with accumulating experience, some drawbacks that we must seriously consider have appeared along with the recent advances in molecular genetics. Here, we highlight recent technical advances of engineered nucleases, discuss the challenges we have faced while using these 'state of the art' genome-editing technologies to generate genetically engineered mouse models (GEMs) and, and look toward the potential future uses of these technologies.

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Introduction

The GEMs have long been well-recognized as an essential platform for studying human diseases [1,2]. For the production of GEMs, engineered nucleases have been replacing the classical embryonic stem (ES) cell-based gene targeting technology that previously almost dominated the field. Pioneering works by our group [3] and others [4,5] have established standard protocols and technical know-how for modifying the genome of mouse embryos to generate GEMs using engineered nucleases. Even though there is abundant published literature describing how to generate GEMs, the scientific community still lacks clear insight into the best approaches for the efficient production, systematic screening, and validation of GEMs through the use of engineered nucleases. We review the current progress in this field and highlight concerns that should be considered regarding the screening and use of engineered nucleases for GEM production.

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Engineered nucleases: ZFNs, TALENs, and CRISPR/Cas9

The principles and characteristics of engineered nucleases have been extensively discussed in previous reviews [6–8]. Briefly, ZFNs and TALENs are generated by fusing a DNA-binding domain with the cleavage domain of the FokI endonuclease [6,7]. A pair of ZFNs or TALENs bind to opposite strands of adjacent sequences separated by a short spacer sequence where the target site is located [6,7]. However, the use of ZFNs and TALENs is still limited because their construction requires modular assembly technology for generating DNA-binding domains. The simplest and the most effective of the engineered nucleases is the CRISPR/Cas9 system, which consists of a Cas9 nuclease and a single guide RNA (sgRNA) [7,8]. The sgRNA contains a 20-nucleotide sequence that is complementary to a target site, which is immediately followed by a tri-nucleotide protospacer adjacent motif (PAM) in the genome, and recruits the Cas9 nuclease to the target sequence [6,8]. Because of its simplicity and precision, CRISPR/Cas9 has become the most prominent tool for genome engineering [9].

Mutations induced by engineered nucleases

To introduce a genomic mutation, a pre-designed nuclease is first used to create a unique double-strand break (DSB) at the desired genomic locus [6]. Such DSBs are generally repaired by DNA repair mechanisms such as non-homologous end-joining (NHEJ), homologous recombination (HR), and homology-directed repair (HDR) [10,11]. NHEJ-mediated repair frequently causes small indel (insertion/deletion) mutations, leading to frame-shift or nonsense mutations [10]. In contrast, HR or HDR allows a donor template to be introduced at a specific sequence between homologous arms flanking a DSB site [11]. Consequently, these DNA repair mechanisms can mediate gene editing.

Generation of knockout mice using engineered nucleases

Immediately after knockout rats using ZFNs was first created [12], the production of knockout mice using ZFNs followed [13]. Carbery et al. and other investigators targeted individual genes by injecting ZFN mRNAs into the pronuclei of mouse embryos, with targeting efficiencies of up to 68% [13–15]. However, ZFN mRNAs exhibited considerable toxicity to mouse embryos [14]. Nevertheless, ZFN-mediated gene-targeting experiments indicated that direct microinjection of ZFN mRNAs into one-cell embryos is still an efficient means for generating NHEJ-mediated knockout mice [13–15].

Our group first reported the generation of knockout mouse using TALENs. We obtained targeting efficiencies of 49–77% with increasing efficiency depending on the concentration of TALEN mRNA [16]. Later, the precise targeting of small genes, including the microRNAs mmu-mir-10a and −10b, was also reported to be feasible [17]. In the early days of this technology, researchers were primarily focused on increasing the efficiency of gene targeting. Using two pairs of TALENs for a gene (e.g., *Fats*) increased the targeting efficiency by more than threefold [18], and the cytoplasmic injection of TALEN mRNAs targeting *Ttc36* was more efficient than pronuclear injection [19]. Overall, TALENs yields higher mutation efficiencies and survival rates than ZFNs.

Recently, CRISPR/Cas9 has become available and represents a potent strategy for generating GEMs due to its simplicity, cost-effectiveness, efficiency, and versatility. The gene-targeting ability of CRISPR/Cas9 in mice was first confirmed by targeting the transgene in enhanced green fluorescent protein (EGFP) transgenic mice [20]. Wang et al. demonstrated that the co-injection of Cas9 mRNA and sgRNA into the cytoplasm of zygotes could efficiently and reliably generate knockout mice with bi-allelic mutations with a high targeting efficiency (67–100%) [21]. In practice, the targeting activity of CRISPR/Cas9 appears to depend on the concentration of sgRNA rather than that of Cas9 mRNA [3,21,22]. Similar to the experiments using TALEN, we and others achieved higher mutation rates by the cytoplasmic microinjection of Cas9/sgRNA compared to pronuclear microinjection [3,23]. Furthermore, we also obtained a high mutation rate by directly co-injecting recombinant Cas9 protein complexed with sgRNA (ribonucleoprotein [RNP] complex) into the cytoplasm (up to 71%) or pronuclei (up to 88%) [3]. Recently, the use of truncated sgRNAs (17/18 nucleotides) with CRISPR/Cas9 has increased the targeting efficiency (39.4–80.1%) compared with the use of standard gRNA controls (3.7–35.8%) [24]. Taken together, one-step CRISPR/Cas9-mediated gene targeting is an efficient method for generating mutant mice with high efficiency and low fetal toxicity even at relatively high doses of Cas9 mRNA and sgRNA [3,21–23].

Multiple gene targeting or chromosomal rearrangements by engineered nucleases

Several investigators have tried to simultaneously target multiple genes (up to 32) using engineered nucleases and have shown the generation of mutant mice with high mutation efficiencies (29% in TALEN and 80% in CRISPR/Cas9). [21,25–28]. This strategy is useful for generating knockouts of multiple neighboring genes that cannot be achieved by ES cell-based technology [26,29]. Furthermore, multiplexed sgRNAs can also be used to engineer chromosomal rearrangements including larger deletions, duplications, and inversions. The co-injection of two optimized long gRNAs together with Cas9 mRNA resulted in 33% of pups carrying large deletions across two target loci (~10 kb) [22]. The deletion efficiency depended on the concentration of gRNA rather than that of Cas9 mRNA [22]. Long deletions (up to 65 kb) were also introduced into genomic loci in mice [30,31]. Furthermore,