Biochimica et Biophysica Acta 1842 (2014) 1942–1950

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Endonucleases: new tools to edit the mouse genome ☆

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ARTICLE INFO

Article history:
Received 20 January 2014
Received in revised form 16 April 2014
Accepted 18 April 2014
Available online 30 April 2014

Keywords:
Genome editing
Mouse
Endonucleases
ZFN
TALEN
CRISPR/Cas

ABSTRACT

Mouse transgenesis has been instrumental in determining the function of genes in the pathophysiology of human diseases and modification of genes by homologous recombination in mouse embryonic stem cells remains a widely used technology. However, this approach harbors a number of disadvantages, as it is time-consuming and quite laborious. Over the last decade a number of new genome editing technologies have been developed, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats/CRISPR-associated (CRISPR/Cas). These systems are characterized by a designed DNA binding protein or RNA sequence fused or co-expressed with a non-specific endonuclease, respectively. The engineered DNA binding protein or RNA sequence guides the nuclease to a specific target sequence in the genome to induce a double strand break. The subsequent activation of the DNA repair machinery then enables the introduction of gene modifications at the target site, such as gene disruption, correction or insertion. Nuclease-mediated genome editing has numerous advantages over conventional gene targeting, including increased efficiency in gene editing, reduced generation time of mutant mice, and the ability to mutagenize multiple genes simultaneously. Although nuclease-driven modifications in the genome are a powerful tool to generate mutant mice, there are concerns about off-target cleavage, especially when using the CRISPR/Cas system. Here, we describe the basic principles of these new strategies in mouse genome manipulation, their inherent advantages, and their potential disadvantages compared to current technologies used to study gene function in mouse models. This article is part of a Special Issue entitled: From Genome to Function.

1. Introduction

Genome-wide association studies (GWAS) have been instrumental in the identification of single nucleotide polymorphisms (SNP)s associated with complex human diseases. The number of genetic associations has been steadily increasing each year since the introduction of this approach in 2005. Genomic regions marked by specific SNPs have attracted the attention of many researchers to potentially identifying the causal variant and understanding the pathophysiology of the disease [1,2]. These genomic regions can contain either protein-coding (direct protein variants) or non-coding regions that might regulate the expression of genes. However, discovering the causal variant and revealing the underlying biological mechanism of the associated disease is still a complicated process. For a number of reasons, the mouse is the most valuable and readily accessible animal model as a biological source to study genes within the candidate loci. The genome of the mouse has been fully sequenced, and most of the genes (~99%) in human are also present in mice. Mice are highly comparable to humans with respect to organs, tissues and physiological systems, enabling the study of gene-environment interactions in the whole organism. Furthermore, mice are easy to breed with a relatively short generation time, are small, and can be housed together, thereby keeping the costs relatively low. The discovery of gene editing via homologous recombination in mouse embryonic stem (ES) cells has further spurred the use of mice over other animal models [3–5]. Here, we will give an overview of the various tools for gene modification that have been developed during the last decades. Additionally, we will focus on new developments in mouse technology and the advantages these have over existing technologies to translate genetic findings into functional biological assessments.

2. Gene editing by homologous recombination

Most human diseases are studied from a candidate gene approach that has been identified by linkage or association studies, or deep

Abbreviations: CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR-associated; crRNA, CRISPR RNA; DSB, double-strand break; dsDNA, double-strand DNA; ES, embryonic stem; FLASH, fast ligation-based automatable solid-phase high-throughput; gRNA, guide RNA; GWAS, genome-wide association studies; HDR, homology-directed repair; iPS, induced pluripotent stem; NHEJ, non-homologous end joining; OPEN, oligomerized pool engineering; PAM, protospacer adjacent motif; RVD, repeat variable di-residue; SELEX, systematic evolution of ligands by exponential enrichment; SNP, single nucleotide polymorphism; SpCas9, *S. pyogenes* Cas9; TALEN, transcription activator-like effector nuclease; tracrRNA, trans-activating crRNA; ZFN, zinc finger nuclease; ZFP, zinc finger protein

☆ This article is part of a Special Issue entitled: From Genome to Function.

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http://dx.doi.org/10.1016/j.bbadis.2014.04.020
0925-4439/

sequencing approaches [6,7]. Although human diseases are usually very complex, typically involving gene-gene and/or gene-environment interactions, the most straightforward and commonly used method to study the function of candidate genes is by modifying these genes in mice. The development of gene targeting technology in ES cells was a major breakthrough that led to the generation of numerous mutant mouse models. The technique makes use of homologous recombination to mutagenize the genome in ES cells, which creates a deletion, insertion, or point mutation [8]. However, ~30% of all knockouts are embryonic or early postnatal lethal, which led to the development of other mutagenesis strategies, like Dre/Rox, Flp/Frt and the most widely used Cre/LoxP system. These systems provide the possibility of generating a tissue/cell-specific gene knockout (discussed below) [9–11]. Cre/LoxP is a site-specific recombination system that was discovered in bacteriophage P1 [11,12]. Cre recombinase drives recombination between two DNA recognition sites of 34 bp, also known as LoxP sites [8]. Genomic regions that are flanked by loxP sites in the same orientation, also termed a "floxed allele", will be excised in cells expressing Cre recombinase [13].

In general, this mutagenesis approach is commonly used if the gene of interest is vital for normal embryogenesis or if there is a necessity to investigate the function of the gene in a tissue/cell-specific context. Mice carrying the floxed alleles will be crossed with a mouse strain containing a transgene encoding the Cre recombinase under the control of a tissue-specific promoter, which results in conditional/tissue-specific knockout mice [14–16]. Over the last two decades, numerous, tissue-specific, Cre-driver lines have been developed. However, some drawbacks in the Cre/LoxP system have also started to emerge, which was recently extensively reviewed [16,17]. One major concern is the tissue-specificity of the chosen promoter that drives the Cre transgene. Expression of various genes assumed to be restricted to a specific tissue or cell type are actually expressed in multiple tissues/cells [16–18]. An additional problem is that Cre recombinase transgenic mice can have too high or low Cre activity, leading to toxicity or inefficient deletion of the gene, respectively [16]. Furthermore, Cre recombinase itself can also cause unwanted side-effects, such as random recombination, reduced proliferation and increased apoptosis, supporting the need to include the Cre recombinase transgenic mice as an additional control in the study design [16].

Another elegant method to examine gene function in a more physiological fashion is by engineering mice with reduced expression of the gene of interest. This can be accomplished by creating a hypomorphic allele that results in the expression of only a fraction of the normal protein levels. Combining a hypomorphic allele with either a wild-type, knockout, or hypomorphic allele enables generation of a series of mice with a gradual reduction in protein levels [19]. For example, this strategy has successfully been used to study the mitotic checkpoint proteins BubR1 and Bub1. Complete ablation of these genes results in embryonic lethality, but mice with reduced protein levels are born healthy and show an overt phenotype later in life [20,21]. The strategies to generate a hypomorphic allele have recently been described in detail [19,22].

Although gene editing by homologous recombination in ES cells is still the most widely used strategy to generate mutant mice the efficiency of homologous recombination is very low. Therefore this genetic editing method has to be performed in ES cells first instead of in the mouse directly. In addition, the availability of ES cells from different species is limited. All this combined has led to the development of new techniques, such as ZFNs, TALENs and CRISPR/Cas that harbor significantly improved efficiencies in gene editing [23–25]. The basic principles and advantages of these technologies will be discussed in the following sections.

3. Zinc finger nucleases

Zinc finger nucleases (ZFNs) facilitate genetic modification through the introduction of a double strand break (DSB) in a DNA sequence of interest. Subsequent DNA break repair then enables the introduction of the desired modification, which is discussed in detail below [26,27]. The DSB is produced by a ZFN, which is a sequence-specific endonuclease that can be designed to cleave at a precise DNA sequence [27]. A ZFN consists of a varying number of zinc finger proteins (ZFPs) or Cys_2His_2 fingers which are usually fused to the nuclease domain of *Fok*I, a restriction enzyme that cleaves non-specific DNA sequences [27–31] (Fig. 1A). Each ZFP is able to recognize a distinct three-base-pair DNA sequence and a typical ZFN consists of 3–6 fused zinc finger proteins. Optimal *Fok*I cleavage by ZFNs requires two independent ZFNs to bind on opposite DNA strands in the appropriate orientation and at the correct distance from each other [27,32] (Fig. 1A).

The introduction of a DSB by ZFNs at a predefined DNA locus provokes activation of a conserved DNA repair pathway, namely non-homologous end joining (NHEJ) or homology-directed repair (HDR) [33–35] (Fig. 1B). In most cases the DSB is repaired by the NHEJ pathway, which efficiently ligates the two broken ends. However, the NHEJ pathway is error-prone and the repair can result in small deletions and/or insertions (indels), which can lead to gene disruption [27,33] (Fig. 1B). Gene inactivation was initially applied by expression of two ZFNs directed against the *yellow* gene in the larvae of *Drosophila melanogaster*, which resulted in germline mutations [36,37]. Subsequently, ZFN technology has successfully been applied to mutagenize genes in various organisms, including zebrafish, rats and mice with varying frequency [23, 38–42]. For example, microinjection of engineered ZFNs in embryos was used to generate *Mrd1a* and *Tnfrsf9* knockout mice, respectively [23,42]. In addition to single gene disruption, ZFN technology has also been used to target two or three genes simultaneously in mammalian cells [43,44]. Furthermore, larger deletions, translocations, duplications and inversions can be introduced with ZFN [44–48].

HDR enables the introduction of single nucleotide changes (gene correction) after DSB induction by ZNF upon simultaneous delivery of a donor DNA repair template, which contains homology arms flanking the site of alteration [37,49,50] (Fig. 1B). This opens the possibility to study the functional consequences of human disease-associated point mutations in the preferred cells and/or model organisms [23,51–54]. In addition, this approach can be used to engineer larger modifications, including insertions of loxP sites, fluorescent proteins, antibiotic resistance markers, or other tags [52,55–59]. There are limitations to gene correction and gene addition via HDR: the need for co-delivery of a designed DNA donor template together with a specific-ZFN, and the strong preference of a cell for NHEJ over HDR-mediated repair of the DSB. Possible solutions are either to use ZFN nickases or a vector carrying multiple copies of linear donor fragments, which both increase HDR-driven genome editing while reducing unwanted mutations caused by NHEJ [60–63].

Importantly, ZFN-mediated gene modification has great therapeutic potential. ZFN has the advantage over known knockdown or blocking strategies because it is efficient and persistent, which could avoid the need for life-long treatment. For example, independent studies have shown that disruption of the *CCR5* and *CXCR4* gene, which encode HIV co-receptors, protects against HIV-1 infection *in vitro* and *in vivo*. Based on the *CCR5* studies, ZFN-mediated therapies for HIV have been designed and are currently being used in Phase 2 clinical trials [27, 64–68]. ZFN-induced HDR can also be exploited to correct genetic disease-causing mutations, as demonstrated in human induced pluripotent stem (iPS) cells carrying mutations underlying Parkinson's disease, α1-antitrypsin deficiency, or sickle-cell anemia [69–71]. Furthermore, ZFN-driven gene correction has been demonstrated to be effective in a mouse model of hemophilia, raising the possibility of *in vivo* genome editing by ZFN as a strategy for the treatment of genetic diseases [72]. The risk for potential off-target DNA cleavage when using ZFN technology raises some concerns. Increased ZFN specificity and simultaneous reduction of off-target cleavage can be achieved by linking more ZFPs in a ZFN, optimizing the orientation of protein-DNA interaction and using a heterodimer ZFN pair [51,73]. Although some reports have