Chapter 11
Genome Editing in Mice Using TALENs

Tomomi Aida

Abstract Genetically modified animals such as knockout mice are essential for elucidating in vivo gene functions and identifying genetic contributions to the molecular pathophysiology of human diseases. For the past two decades, knockout mice have been created via embryonic stem (ES) cell-based gene targeting, a time-consuming, laborious, inefficient, and expensive process. The rapid emergence of targeted genome editing technologies is drastically revolutionizing this situation. Genome editing mediated by transcription activator-like effector (TALE) nucleases (TALENs), one of the popular genome editing tools, is a simple and powerful gene-targeting technology. With its extremely high efficiency, the mouse genome can be manipulated directly in fertilized eggs without any targeting vector or selection steps by a process called in vivo genome editing. TALEN-mediated in vivo genome editing provides an exciting opportunity for simple, convenient, and ultra-rapid production of precisely targeted knockout and knockin mice. Using this technology, researchers can freely and routinely manipulate mouse genomes and accelerate in vivo functional genomic research.

Keywords Glaucoma • Glutamate transporter • In vivo • Knockin • Knockout • Mouse • TALEN

11.1 Introduction

Historically, the development of genetic manipulation technologies has opened a new era for life sciences and drastically improved our understanding of, in particular, the in vivo gene functions of living organisms. Overexpression and knock-down of genes with transgenic and RNA interference (RNAi) technologies permits the control of specific gene dosage in vivo in many species from bacteria to animals to plants. However, these technologies are relatively coarse-grained with respect to

T. Aida (✉)
Laboratory of Molecular Neuroscience, Medical Research Institute,
Tokyo Medical and Dental University, Tokyo, Japan
e-mail: aida.aud@mri.tmd.ac.jp

T. Yamamoto (ed.), *Targeted Genome Editing Using Site-Specific Nucleases*,
DOI 10.1007/978-4-431-55227-7_11

control of gene expression. In transgenic animals, the expression state of a transgene differs from that under physiological conditions. The expression level of a transgene is much higher than that in the wild type, and it is constitutively active. The expression pattern is controlled by a tissue- or cell-specific or general promoter, but the expression pattern is strongly affected by the genetic locus where the transgene is inserted; this phenomenon is known as the positional effect. Thus, transgenic animals are useful tools, but often unrepresentative of physiological and pathophysiological conditions (Saito et al. 2014). RNAi-mediated knock-down technologies can be applied to living organisms by direct delivery of small interfering RNA (siRNA), a plasmid expressing short hairpin RNA (shRNA), or as a transgenic animal expressing shRNA. RNAi provides a method for specifically downregulating the expression of a target gene. The major problems of this technology are off-target effects and incompleteness of protein depletion (Echeverri et al. 2006).

In contrast to these transgenic and RNAi technologies, ES cell-based gene targeting allows specific and precise manipulation of the genome. The mouse is the most commonly used animal model in basic research because of the unexampled availability of homologous recombination in ES cells, an essential and core step in gene-targeting technology for precise replacement of a target gene with a selectable marker that can contribute to the organism and germ cells. Owing to the first success of gene targeting in the mouse, thousands of knockout and knockin mice have been created, revealing the in vivo functions of genes. Moving ahead, The International knockout Mouse Consortium (IKMC) has comprehensively constructed knockout mice for all protein-coding genes (Sung et al. 2012; Menke 2013). IKMC has produced targeted ES cell lines, including conventional knockout, conditional knockout, and gene-trapped alleles, for more than 18,000 mouse genes in addition to mice targeted for over 2,600 loci (Skarnes et al. 2011). These genome wide and large-scale knockout mouse resources are now publicly available and researchers can obtain chimeric mice by injection of targeted ES cells of interest derived from IKMC. This allows the researchers to focus their efforts on the functional analysis of target genes, rather than on the construction of targeted ES cells and mouse lines.

Recent advances in genome-wide association studies (GWAS) and high-throughput sequencing technologies have revealed the landscape of human genetic diversity, which is comprised of tens of millions of common and rare variants, known as single nucleotide variants (SNVs), associated with health and disease (1000 Genomes Project Consortium et al. 2012; Raychaudhuri 2011). Of these, loss-of-functional SNVs including that of nonsense, splice site, or frameshift, are easy to interpret those functional consequences because patients carrying loss-of-functional SNVs are comparable to knockout mice (Veltman and Brunner 2012). In contrast, a large proportion of SNVs are misssense, silent, intronic, or intergenic SNVs, thus, functional consequences of these SNVs are hard to estimate. Thus, further efforts are required to investigate the biological consequences of these SNVs. A powerful approach to address the biological function of these SNVs is the knockin mouse carrying precisely modified human SNVs (Veltman and Brunner 2012). The knockin mouse can be used to test whether these SNVs are causal for the human phenotype of interest, thus providing unique and direct opportunities for the

investigation of the functional consequence of SNVs in vivo to the complex human traits in health and disease. Südhof and colleagues showed a good example of this approach. They generated knockin mouse carrying a neuroligin-3 R451C SNV found in a subset of patients with autism, a major neurodevelopmental disorder. The neuroligin-3 R451C knockin mice showed abnormal behaviors that resembled those of human patients and abnormal synaptic transmission (Tabuchi et al. 2007). Importantly, these behavioral and electrophysiological abnormalities were not observed in neuroligin-3 knockout mouse, suggesting that the R451C SNV represents a gain-of-function mutation. Thus, knockin mouse provides a powerful approach for functional interpretation of SNVs. Although the demand for precisely modified knockin mouse is growing, Menke (2013) showed that just only 600 such mice could be found in the mutant mouse database. This situation is partially due to the several difficulties in generating such mice by conventional gene-targeting technology (Capecchi 2005).

In the conventional gene-targeting method in ES cells, generation of homozygous knockout mice generally takes more than a year and involves many processes: (1) construction of a targeting vector with long homology arms to replace a target gene with a drug selection marker, (2) electroporation of the targeting vector into ES cells and screening to isolate correctly targeted cells, (3) transplantation of correctly targeted ES cells into blastocysts, (4) obtaining and crossing F_0 chimeric mice with wild-type mice to identify germline-competent mutant lines, (5) crossing heterozygous mutants with each other, and (6) obtaining a homozygous mutant (Aida et al. 2014). Further, because ES cell lines derived from the 129/Sv mouse strain are generally used in conventional gene targeting, mutant mice must be crossed with the commonly used C57BL/6 strain mice many times to analyze the mutant in a C57BL/6 genetic background; these steps known as backcrossing generally take several years. Overall, even today, gene targeting in mouse is time consuming, laborious, inefficient, and expensive.

11.2 In Vivo Genome Editing in Mice

The recent emergence and drastic advances in targeted genome-editing technologies have opened a new era in biology, medicine, agriculture, and biotechnology (Carroll 2014; Hsu et al. 2014). Genome editing technologies enable the modification of almost any desired sequence from any living cell or organism. The technologies are based on molecular tools, including zinc-finger nucleases (ZFNs), transcription activator-like effector (TALE) nucleases (TALENs), and clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated endonuclease 9 (Cas9), known as the CRISPR/Cas9 system. The principle of genome editing is generation of a DNA double-strand break (DSB) at a specific target locus by introducing genome editing tools into cells (Urnov et al. 2010; Joung and Sander 2013). DSB is subsequently repaired by two major cellular endogenous DNA damage repair pathways. These pathways are the error-prone, nonhomologous end-joining

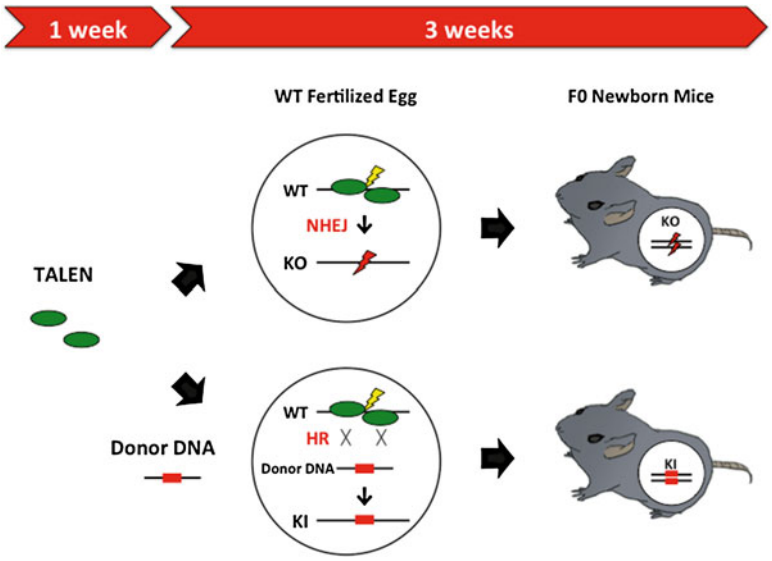

Fig. 11.1 Schematic diagram of TALEN-mediated in vivo genome editing in mouse. TALENs (*green*, 1 week for preparation) are microinjected into one-cell fertilized eggs (*circle in the middle*) derived from wildtype mice for knockout mouse production (*upper*). Donor DNA (ssOligo or targeting vector) is co-injected for knockin mouse production (*lower*). Then, DSB (*yellow knurl*) and subsequent NHEJ (*red knurl*) and/or HDR (*red box*) are induced within one-cell fertilized eggs, three weeks later, resulting in biallelically-targeted homozygous knockout (*upper*) and knockin (*lower*) mice at F_0

(NHEJ) route, which results in small deletions or sequence insertions into the DSB site, and the homology-directed repair (HDR) pathway, which relies on a donor DNA template with homology to the DSB site to achieve precise homologous recombination (Fig. 11.1). NHEJ occurs rapidly and preferentially, often leading to frameshift mutations and loss of function of the targeted genes, resulting in a gene knockout when a protein-coding sequence is targeted. HDR occurs infrequently and also results in precise and specific genome modifications such as SNV substitutions, insertions, deletions, or gene insertion, when a targeting vector or synthetic single-strand oligonucleotide (ssOligo) is co-delivered, which in turn results in a targeted knockin.

The selection steps for correctly targeted ES cells are required in conventional gene targeting in the mouse, because the efficiency of spontaneous homologous recombination is too low to perform the procedure directly in fertilized eggs (Brinster et al. 1989). The remarkable advantage of genome editing is its extremely high efficiency; NHEJ occurs in up to 50 % (or more) of cells in several primary and immortalized cells without drug selection, and HDR is stimulated by DSB and targeting efficiencies are enhanced at least 1,000-fold relative to the conventional method in several cells (Urnov et al. 2010; Hauschild-Quintern et al. 2013). Thus, one can easily imagine that by taking advantage of the highly efficient genome editing technology, researchers can directly manipulate the mouse genome in fertilized

eggs (perform in vivo genome editing) without any selection or use of ES cells. The principle of in vivo genome editing involves targeting the genome by direct microinjection of the mRNA that codes for genome-editing tools into the cytoplasm or pronuclei of one-cell mouse embryos for gene knockout, and co-injection of ssOligos or targeting vectors for gene knockin mice (Fig. 11.1). In vivo genome editing is a groundbreaking technology for mutant mouse production, enabling simple and daily experiments (Menke 2013; Aida et al. 2014). Here we review gene targeting in the mouse with a focus on TALEN-mediated in vivo genome editing.

11.3 Knockout Mice

The first success of gene knockout by in vivo genome editing in mammals was achieved in the rat with ZFNs (Geurts et al. 2009), and the technique was then rapidly applied to the mouse (Carbery et al. 2010). At the same time, the simple modular DNA recognition code of TALEN was cracked, and the technology was then rapidly expanded and emerged at center stage of genome editing (Anonymous 2012). The first knockout mice created by TALEN-mediated in vivo genome editing were reported in early 2013 (Sung et al. 2013). In this impressive work, two endogenous mouse genes were targeted with extremely high efficiencies (more than 50 %) in neonatal mice. When they used high doses of TALENs, a majority of neonatal mice were biallelically targeted homozygous knockout mice. They found no off-target effects and the targeted alleles were successfully transmitted to succeeding generations. This pioneering work showed the feasibility of highly efficient TALEN-mediated in vivo genome editing in mice. Because of the ease of target-specific TALEN construction, homozygous knockout mice can be obtained within one month.

Since the first report, numerous knockout mice generated by TALEN-mediated in vivo genome editing have been reported (Aida et al. 2014). Davies and colleagues targeted the *Zic2* gene with TALENs in three different mouse strains: CD1, C3H, and C57BL/6J (Davies et al. 2013). The targeting efficiencies producing live newborns or blastocysts varied, with 10 %, 23 %, and 46 % for C57BL/6J, C3H, and CD1, respectively.

Li and colleagues generated a series of knockout mice for 10 genes, revealing the utility, convenience, and robustness of TALEN-mediated in vivo genome editing (Qiu et al. 2013). The targeting efficiencies varied from 13 to 67 %, with an average of 40 %, of live newborns. In contrast to the report of Davies et al. (2013), using one TALEN for the *Lepr* gene, which encodes the leptin receptor, they showed that the targeting efficiency did not differ between two different mouse strains (C57BL/6N and FVB/N). Among the F_0 founders, one had biallelic modifications with different frame-shift deletions and exhibited an obese phenotype resembling the Lepr mutant db/db mice phenotype. Moreover, there were no off-target effects, even at sites with only one mismatch to each TALEN. All tested F_0 founders transmitted the mutant alleles to F_1 mice with high efficiency. These results indicate that genome editing is highly accurate and efficient.

Han and colleagues generated knockout mice for the *Mlkl* gene (which encodes the mixed lineage kinase domain-like protein, essential for tumor necrosis factor-induced necrosis) by TALEN-mediated in vivo genome editing (Wu et al. 2013). They obtained 71 mutants from 390 newborns (18 % efficiency). Of these, four were homozygous mutants.

Zhou and colleagues generated knockout mice for the *Ttc36* gene (renal proximal tubule protein) by TALEN-mediated in vivo genome editing (Liu et al. 2014b). They compared the mutation efficiency between pronuclear and cytoplasmic injection and found that cytoplasmic injection works much (more than twofold) better than pronuclear injection; of these mice, a few were homozygous mutants.

Takada and colleagues applied in vivo genome editing to microRNAs (miRNAs) that are often present in intergenic regions where regulatory sequences are located (Takada et al. 2013). In this context, the advantage of in vivo genome editing is that it can delete only a target sequence, in contrast to traditional gene targeting, which leaves LoxP sequences in intergenic or intronic regions, potentially affecting transcriptional regulation. They targeted three different miRNAs (*mmu-mir-146a*, *mmu-mir-10a*, and *mmu-mir-10b*) and an intergenic region on chromosome 11, and successfully obtained each mutant mouse.

Genome-editing technologies enable gene targeting in mice, which was previously considered impossible. First, these methods permit gene targeting at loci where conventional homologous recombination cannot be applied, such as the Y chromosome. Because the Y chromosome has a unique structure containing many palindromes, conventional gene targeting in ES cells has failed. Jaenisch and colleagues targeted the *Sry* and *Uty* genes on the Y chromosome in mouse ES cells using TALENs and successfully obtained knockout mice lacking *Sry* or *Uty* (Wang et al. 2013). Takada and colleagues directly targeted the same *Sry* gene by TALEN-mediated in vivo genome editing and obtained an *Sry* knockout mouse (Kato et al. 2013). Thus, the high sequence specificity of TALENs provides a new approach for the genetic manipulation of the Y chromosome. Because *Sry* knockout male mice derived from either targeted ES cells or directly targeted fertilized eggs showed similar phenotypes such as anatomical femaleness, in vivo genome editing offers a great advantage in terms of time.

Second, in vivo genome editing allows multiple targeting of genes. Sherr and colleagues simultaneously targeted two and three genes including *Agouti*, *miR-205*, and *Arf* tumor suppressor locus by in vivo genome editing with a mixture of TALEN mRNAs against these targets. Both double and triple targeting worked well and newborns carrying mutations in two or three genes were obtained (Li et al. 2014). Previously, when researchers focused on two or more functionally redundant genes, they needed to cross each single-mutant mouse, a time-consuming and laborious process (Matsugami et al. 2006; Aida et al. 2012). Furthermore, when two genes are located next to each other on the same chromosome, double knockout mice can mostly not be obtained by crossing two single-knockout mice. Thus, researchers have generated double-targeted ES cells by sequential targeting, which is much more time consuming, laborious, and expensive than single-gene targeting (Kitajima et al. 2000). In this context, in vivo genome editing offers a great advantage for

simultaneous targeting of multiple genes and provides invaluable tools for studying cooperative roles of functionally redundant or clustered genes.

Third, these methods enable gene targeting in diverse genetic backgrounds of mouse strains. In conventional gene targeting, ES cells derived from 129 mouse strains are most often used owing to the high efficiency of gene targeting. However, subsequent analyses of targeted mice in a C57BL/6 genetic background are preferred. Thus, time-consuming backcrossing, which takes at least one year, is essential. As demonstrated previously (Davies et al. 2013; Qiu et al. 2013), in vivo genome editing can be applied to any mouse strain and provides the opportunity of analyzing the targeted mice immediately without backcrossing.

11.4 Knockin Mice

Although the success of in vivo genome editing has enabled the rapid generation of knockout mice, the development of this technique for producing knockin mouse models would fully exploit its potential. As with knockout mice, earlier successes in knockin mouse production by in vivo genome editing were achieved with ZFNs and conventional targeting vectors (Meyer et al. 2010; Cui et al. 2011). The construction of traditional targeting vector for the targeted integration of SNVs by HDR is disproportionately laborious and time consuming because only one or a few nucleotides substitutions are required. The use of ssOligos as donors for HDR allows bypassing this process, because ssOligos can be chemically synthesized and purchased from many companies within a few days. Davis and colleagues systematically investigated ssOligo-mediated targeted integration of point mutations by ZFN in several human cell lines. They found that ssOligo works very well as donor template for HDR, and surprisingly, its efficiencies were up to twice those achieved using conventional targeting vectors (Chen et al. 2011). Kühn and colleagues first applied an ssOligo donor to produce knockin mice carrying SNVs (Meyer et al. 2012). They generated a knockin mouse carrying several SNVs including a missense mutation in the *Rab38* gene by co-injecting ZFN mRNAs with a 144-mer ssOligo containing seven substitutions into one-cell mouse embryos. They obtained one partially targeted mutant from 60 newborns, an efficiency of 1.7 %. This work clearly reveals the enormous potential of ssOligos for the replacement of conventional gene-targeting vectors in in vivo genome editing, a technology that should greatly facilitate the rapid production of knockin mice.

The first knockin mouse by TALEN-mediated in vivo genome editing was also achieved by Kühn and colleagues in early 2013 (Wefers et al. 2013). They generated knockin mice carrying *Rab38* missense SNV G19V by TALENs using an ssOligo. They first prepared TALENs targeting the same region of the *Rab38* gene previously targeted by themselves using ZFNs; they found that the activity of the TALENs was approximately twice that of ZFNs. They then co-injected TALENs and an ssOligo into one-cell mouse embryos and obtained one founder mouse carrying a partially targeted G19V allele from 117 newborns (an efficiency of 0.9 %).

Because TALENs are easier to construct as compared with ZFNs, knockin mice can be rapidly generated using a combination of TALENs and ssOligos. However, the relatively low knockin efficiency of TALENs is a bottleneck that limits the broad application of the method. Recently, they reported improved knockin efficiencies of up to 8 %, using TALEN mRNAs transcribed from plasmids containing a poly A tail, which may increase mRNA stability and translation efficiency (Panda et al. 2013).

Jones and Meisler (2014) reported the production of knockin mice carrying a missense mutation in *Scn8a,* which encodes the neuronal sodium channel Nav1.6 (the mutation causes human epilepsy), by TALEN-mediated in vivo genome editing. They used a conventional targeting vector as a knockin donor and obtained correctly targeted newborns with 7 % efficiency. *Scn8a* is a member of a sodium channel family with nine paralogs sharing high DNA sequence identity. To investigate the specificity of their TALENs, the authors analyzed candidate regions of *Scn5a* and Scn4a, with 3 and 4 mismatches with *Scn8a* TALEN binding sequences, respectively. Although they found no off-target modifications in *Scn5a* or Scn4a in their 5 correctly targeted knockin mice, surprisingly, they found several off-target modifications in both genes in NHEJ-mediated knockout mice. This work suggests the power of TALEN-mediated in vivo genome editing and the considerable problem of off-target effects, a point to be discussed later.

Wiles and colleagues systematically investigated the optimal conditions for TALEN-mediated in vivo genome editing with ssOligos (Low et al. 2014). They chose as a model the mouse *Crb1rd8* gene mutation, which is present in many inbred laboratory strains derived from C57BL/6N and leads to retinal external limiting membrane fragmentation and outer retinal dysplasia. They extensively compared TALEN mRNA concentrations (10, 25, and 50 ng/μL), and ssODN length (200- or 52-mer), strand orientation (sense or antisense), and concentration (0.3, 1, 2, 6 ng/μL). As results, they showed that higher concentrations of TALEN mRNA and longer sense ssODNs improved knockin efficiency. Under the best conditions (50 ng/μL TALEN mRNA, 200-mer sense ssODN), the knockin efficiency was 27 % of newborns. This work contributes useful fundamental information to the research community and accelerates knockin mouse production by in vivo genome editing.

Although TALEN-mediated in vivo genome editing is improving rapidly, the efficiency of knockin is still low compared to that of knockout mouse production. A key step to improve knockin efficiency is TALEN activity. Recently, Yamamoto and colleagues developed a novel TALEN platform, named Platinum TALEN, which shows extremely high efficiency (Sakuma et al. 2013). We focused on *Slc1a3*, a glial glutamate transporter, in knockout mice has been reported as the first model for normal tension glaucoma (Watase et al. 1998; Harada et al. 1998, 2007; Bai et al. 2013a, b; Namekata et al. 2013). We recently discovered deleterious rare missense SNVs in *SLC1A3*, a human ortholog of *Slc1a3*, in patients with glaucoma (Yanagisawa and Aida et al. unpublished observation). To test whether the causality of these rare SNVs for glaucoma pathophysiology in mice, we generated knockin mice with these SNVs in the *Slc1a3* gene. We co-injected highly active Platinum TALENs targeting *Slc1a3* into one-cell mouse embryos with ssOligos carrying each SNV. We obtained several germline-competent knockin founders with a targeting

efficiency of approximately 20 % and there were no off-target effects (Aida et al., unpublished observation). Because a single microinjection is sufficient to produce several knockin founders, Platinum TALEN technology provides a fast and efficient approach for producing genetic mouse models that reproduce the disease-associated SNVs of complex diseases. When the Platinum TALEN is used under the optimal conditions reported by Low et al. (2014), it is expected that knockin efficiency will be further improved.

Mice carrying a large functional gene cassette such as fluorescent reporter proteins, Cre recombinase and Lox-flanked sequences are the fundamental tools for basic and pathophysiological biology. In contrast to the situation with SNV, there are few reports of the production of knockin mouse carrying a large cassette by in vivo genome editing. Beyer and colleagues reported the first knockin mice carrying a cassette for conditional targeting consisting of EGFP, two pairs of Lox sequences known as FLEX, and genomic fragments including two exons of the *Satb1* gene (Sommer et al. 2014). They identified a precisely targeted knockin mouse from 11 newborns under optimal conditions and confirmed that the conditional targeting of the cassette derived from the knockin mouse worked as expected. This work showed that knockin mouse production by TALEN-mediated in vivo genome editing is not limited to the insertion of SNVs with ssOligos to generate conditionally targeted alleles.

Thus, knockin mice carrying not only an SNV but also a large functional gene cassette can now be created within a month using TALEN-mediated in vivo genome editing with high efficiency. In summary, almost everything achieved by conventional ES cell-based gene targeting can now be performed by TALEN-mediated in vivo genome editing technologies. Further, this revolutionary technology allows previously impossible achievements, such as ultra-rapid production, biallelic targeting in F_0 mice, and multiplexing, making TALEN-mediated in vivo genome editing the first choice for gene targeting (Fig. 11.1).

11.5 Off-Target Effects

Off-target effects, which involve nonspecific recognition and digestion in non-targeted regions by ZFNs, TALENs, and the CRISPR/Cas9 system, is the foremost problem of genome editing and has been extensively discussed in the field. Compared to ZFNs, TALENs produce only minimal off-target effects (in less than a tenth), even at highly similar nonspecific target sites with only two mismatches in the TALEN recognition sequence in human cells (Mussolino et al. 2011).

Researchers at Cellectis, a leading supplier of custom-made TALENs, comprehensively analyzed the specificity of TALENs (Juillerat et al. 2014). By computational off-target site analysis of more than 15,500 putative TALENs against the human genome, they found that nearly two-thirds of these TALENs had potential off-target sites with more than 4 mismatches that strongly reduced the binding capacity of TALENs. For the experimental confirmation of TALEN specificity, they chose six TALENs that had at least one potential off-target site containing one to

four mismatches and investigated their DNA modification activities in human cell lines. They analyzed potential off-target sites by deep sequencing and found that off-target modifications were detected at only a few loci, with extremely low modification efficiency.

Liu and colleagues performed unbiased prediction of potential off-target sequences of two TALENs using in vitro selection and high-throughput sequencing (Guilinger et al. 2014). They found 76 potential off-target sites in the human genome, 16 of which, surprisingly, were modified by TALENs in human cells. However, when they used engineered TALENs by substitution of several amino acids, off-target effects were tenfold lower than those of standard TALENs in human cells.

In contrast to the in vitro situation using human cell lines, off-target effects appear to be much rarer in TALEN-mediated in vivo genome editing. I previously reviewed off-target effects in mutant mice generated by TALEN-mediated in vivo genome editing described in three reports, and found no off-target effects in 15 potential off-target sites containing only one mismatch for four TALEN pairs (Aida et al. 2014; Sung et al. 2013; Panda et al. 2013; Qiu et al. 2013). Lower off-target effects in mutant mice produced by in vivo genome editing compared to those in human cell lines have also been reported for CRISPR/Cas9 (Yang et al. 2013). One possible explanation is differences in properties of fertilized eggs and transformed human cell lines, which may consist of heterogeneous populations with different and increased mutagenic possibility. Another is the means of delivery: short-lived RNA for fertilized embryos, and stable plasmid DNA, which shows longer and higher expression, for human cell lines. Thus, the specificity of in vivo genome editing in fertilized mouse eggs is higher than that in human cell lines, and useful for mutant mouse production. However, further extensive characterization of off-target effects in TALEN-mediated in vivo genome editing should be performed.

11.6 Applications

The mouse is already in an impregnable position in the field of gene targeting in mammals, and thousands of various knockout and knockin mice have been produced. However, the ease and speed of TALEN-mediated in vivo genome editing further accelerates and expands the utility and application of genetically targeted mice. In the conventional ES cell-based gene targeting method, it takes at least a year to obtain a homozygous mutant at best (Aida et al. 2014). Also, it is common to spend a year or more obtaining germline-competent chimeric founders. However, TALEN-mediated in vivo genome editing is revolutionizing these complex and long processes and enables the ultra-rapid production of precisely targeted homozygous mice within a month (Fig. 11.1; Sung et al. 2013; Qiu et al. 2013; Wu et al. 2013; Liu et al. 2014; Li et al. 2014). Thus, now researchers can freely and rapidly generate gene-targeted mice as they handle cell lines.

One promising application of this groundbreaking technology is the functional characterization of millions of common and rare human SNVs discovered in GWAS,

and exome- and whole genome sequencing in vivo by generating "humanized" knockin mice carrying equivalent variants. Because the vast majority of rare SNVs are missense, synonymous, intronic, or intergenic, instead of gene-disrupting nonsense, splice site, or frameshift variants, they are equivalent to "knockout" alleles (Veltman and Brunner 2012), and the production of knockin mouse models carrying such SNVs is a much better approach than the production of conventional knockout mouse models. Jones and Meisler (2014) described a good example of this application. By TALEN-mediated in vivo genome editing, they generated knockin mice carrying a de novo missense mutation N1768D in *Scn8a* discovered by exome sequencing of patients with epileptic encephalopathy or intellectual disability. Interestingly, the authors noted that their N1768D/+heterozygous mice exhibited the key clinical features of patients with epileptic encephalopathy: behavioral abnormalities, seizures, and sudden, unexpected death. Thus, the ultra-rapid production of precisely modified mouse models by TALEN-mediated in vivo genome editing drastically accelerates the functional investigation of rare SNVs in human genetic diseases.

TALEN-mediated genome editing technology also accelerates functional research on common SNVs in regulatory noncoding regions catalogued by the ENCODE (Encyclopedia of DNA Elements) project and GWAS studies (ENCODE Project Consortium et al. 2012; Maurano et al. 2012). Orkin and colleagues found common SNVs associated with fetal hemoglobin level identified by GWAS located in the noncoding erythroid enhancer region of *BCL11A* (Bauer et al. 2013). They showed that the SNV disrupts a motif sequence for the binding of a transcription factor, resulting in reduced *BCL11A* expression and elevated fetal hemoglobin level. Further, they showed by generating a transgenic mouse carrying the human enhancer that the noncoding sequence functions as a developmental stage–specific, lineage-restricted enhancer in vivo. To investigate directly whether the enhancer regulates *BCL11A* expression, they identified a 10-kb orthologous enhancer signature in the noncoding region of the mouse *Bcl11a* and deleted the region using two pairs of TALENs in a mouse erythroleukemia cell line. Surprisingly, biallelic excision of the region by TALENs profoundly reduced *Bcl11a* transcript and completely removed BLC11A protein. This excellent work suggests that TALEN-mediated genome editing is an invaluable tool for functional genomics and potentially for cell-specific gene therapy. Although they deleted enhancer regions from a mouse cell line in this study, it is very interesting to generate, by using TALEN-mediated in vivo genome editing, knockin mice carrying GWAS-associated human SNVs in the orthologous enhancer, and investigate those phenotypes in vivo (Sur et al. 2012; Kamberov et al. 2013).

Genome editing technology provides the potential for application of precise gene targeting not only in mouse but also in higher species such as in nonhuman primates, to model human development and disease and investigate higher brain function. Recently, two teams applied TALEN-mediated in vivo genome editing to rhesus and cynomolgus monkeys to target *MeCP2,* a causal gene for Rett syndrome (Liu et al. 2014a, c). Male monkeys carrying *MeCP2* mutations died at midgestation (Liu et al. 2014a) or soon after birth (Liu et al. 2014c), consistent with male embryonic

lethality in humans carrying *MeCP2* mutations. In contrast, female newborn monkeys carrying *MeCP2* mutations were obtained, consistent with the observation that patients with Rett syndrome are female, with no detectable off-target effects. These works provide proof of concept for gene targeting in non-human primates and opportunities to investigate higher brain function and develop novel therapeutics for human diseases that are difficult to study in mice.

TALEN consists of a TALE domain for specific DNA binding and an effector domain, which is a FokI nuclease domain for introducing a double-strand break into target DNA. Thus, in addition to targeted genome editing, TALEs can be applied for targeted genome regulation, and for visualization by exchanging the effector domain with other functional domains such as transcriptional and epigenetic effector domains or fluorescent proteins (Hsu and Zhang 2012). When a TALE is engineered to fuse with a transcriptional activator domain, the specific target gene can be specifically activated. Zhang and colleagues showed TALE-mediated specific transcriptional and epigenetic control in the adult brain of freely moving mice in vivo by a combination of genome editing with optogenetics, a high-precision spatiotemporal control of many cellular functions using light-responsive proteins (Konermann et al. 2013). They engineered a TALE to fuse with CIB1 protein, which physically binds, under blue light, with its interacting partner light-sensitive CRY2 protein fused with a VP64 transcriptional activator domain, leading to transcription of a target gene. They engineered TALEs to target the *Grm2* gene, packaged these components into viral vectors (adeno-associated viruses, AAVs), and delivered AAVs into adult brains. By blue light stimulation through a fiber optic cannula system, the expression level of endogenous *Grm2* was increased by more than twice. Further, they engineered the CRY2 to fuse with histone deacetylases (HDACs), histone methyltransferases (HMTs), histone deacetylases, histone acetyltransferase inhibitors, HDACs, and HMT recruiting proteins. When they tested these epigenetic TALEs with *Grm2* in mouse neural cells, the expression levels of *Grm2* were suppressed via histone modifications.

TALE can also be engineered with fluorescent proteins and be used as fluorescent probes to visualize specific chromosomal configurations in mouse in vivo. Torres-Padilla and colleagues reported TALE-mediated fluorescent genomic visualization of endogenous repetitive mouse genome sequences and showed nuclear positioning and chromatin dynamics in living mouse embryos (Miyanari et al. 2013). They engineered TALE to target mouse major satellite repeats, highly abundant tandem repeats in pericentromeric regions, and fused it with monomeric GFP mClover. Using this fluorescent TALE probe, they successfully visualized the replication and proliferation of major satellite repeats in living mouse embryos throughout development.

These advanced approaches suggest that TALE is a highly generalizable, robust and versatile platform for the manipulation of endogenous mammalian genomes and has unlimited potential for broad application. Overall, TALE and TALEN-mediated in vivo genome editing are revolutionary technologies and dramatically facilitate our understanding of in vivo gene functions in health and disease.

Acknowledgements This work was supported in part by Strategic Research Program for Brain Sciences and Grant-in-Aid for Science Research from Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from Medical Research Institute, Tokyo Medical and Dental University. I thank K Tanaka, R Imahashi, H Ishikubo, and T Usami (Tokyo Medical and Dental University), and T Yamamoto, and T Sakuma (Hiroshima University) for technical support and useful discussions.

References

Aida T, Ito Y, Takahashi YK, Tanaka K (2012) Overstimulation of NMDA receptors impairs early brain development in vivo. PLoS One 7:e36853

Aida T, Imahashi R, Tanaka K (2014) Translating human genetics into mouse: the impact of ultra-rapid in vivo genome editing. Dev Growth Differ 56:34–45

Anonymous (2012) Method of the Year 2011. Nat Methods 9:1

1000 Genomes Project Consortium, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, Handsaker RE, Kang HM, Marth GT, McVean GA (2012) An integrated map of genetic variation from 1,092 human genomes. Nature 491:56–65

Bai N, Aida T, Yanagisawa M, Katou S, Sakimura K, Mishina M, Tanaka K (2013a) NMDA receptor subunits have different roles in NMDA-induced neurotoxicity in the retina. Mol Brain 6:34

Bai N, Hayashi H, Aida T, Namekata K, Harada T, Mishina M, Tanaka K (2013b) Dock3 interaction with a glutamate-receptor NR2D subunit protects neurons from excitotoxicity. Mol Brain 6:22

Bauer DE, Kamran SC, Lessard S, Xu J, Fujiwara Y, Lin C, Shao Z, Canver MC, Smith EC, Pinello L, Sabo PJ, Vierstra J, Voit RA, Yuan GC, Porteus MH, Stamatoyannopoulos JA, Lettre G, Orkin SH (2013) An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. Science 342:253–257

Brinster RL, Braun RE, Lo D, Avarbock MR, Oram F, Palmiter RD (1989) Targeted correction of a major histocompatibility class II E alpha gene by DNA microinjected into mouse eggs. Proc Natl Acad Sci U S A 86:7087–7091

Capecchi MR (2005) Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. Nat Rev Genet 6:507–512

Carbery ID, Ji D, Harrington A, Brown V, Weinstein EJ, Liaw L, Cui X (2010) Targeted genome modification in mice using zinc-finger nucleases. Genetics 186:451–459

Carroll D (2014) Genome engineering with targetable nucleases. Annu Rev Biochem 83:409–439

Chen F, Pruett-Miller SM, Huang Y, Gjoka M, Duda K, Taunton J, Collingwood TN, Frodin M, Davis GD (2011) High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases. Nat Methods 8:753–755

Cui X, Ji D, Fisher DA, Wu Y, Briner DM, Weinstein EJ (2011) Targeted integration in rat and mouse embryos with zinc-finger nucleases. Nat Biotechnol 29:64–67

Davies B, Davies G, Preece C, Puliyadi R, Szumska D, Bhattacharya S (2013) Site specific mutation of the Zic2 locus by microinjection of TALEN mRNA in mouse CD1, C3H and C57BL/6 J oocytes. PLoS One 8:e60216

Echeverri CJ, Beachy PA, Baum B, Boutros M, Buchholz F, Chanda SK, Downward J, Ellenberg J, Fraser AG, Hacohen N, Hahn WC, Jackson AL, Kiger A, Linsley PS, Lum L, Ma Y, Mathey-Prévôt B, Root DE, Sabatini DM, Taipale J, Perrimon N, Bernards R (2006) Minimizing the risk of reporting false positives in large-scale RNAi screens. Nat Methods 3:777–779

ENCODE Project Consortium, Bernstein BE, Birney E, Dunham I, Green ED, Gunter C, Snyder M (2012) An integrated encyclopedia of DNA elements in the human genome. Nature 489:57–74

Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Ménoret

S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R (2009) Knockout rats via embryo microinjection of zinc-finger nucleases. Science 325:433

Guilinger JP, Pattanayak V, Reyon D, Tsai SQ, Sander JD, Joung JK, Liu DR (2014) Broad specificity profiling of TALENs results in engineered nucleases with improved DNA-cleavage specificity. Nat Methods 11:429–435

Harada T, Harada C, Watanabe M, Inoue Y, Sakagawa T, Nakayama N, Sasaki S, Okuyama S, Watase K, Wada K, Tanaka K (1998) Functions of the two glutamate transporters GLAST and GLT-1 in the retina. Proc Natl Acad Sci U S A 95:4663–4666

Harada T, Harada C, Nakamura K, Quah HM, Okumura A, Namekata K, Saeki T, Aihara M, Yoshida H, Mitani A, Tanaka K (2007) The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma. J Clin Invest 117:1763–1770

Hauschild-Quintern J, Petersen B, Cost GJ, Niemann H (2013) Gene knockout and knockin by zinc-finger nucleases: current status and perspectives. Cell Mol Life Sci 70:2969–2983

Hsu PD, Zhang F (2012) Dissecting neural function using targeted genome engineering technologies. ACS Chem Neurosci 3:603–610

Hsu PD, Lander ES, Zhang F (2014) Development and applications of CRISPR-Cas9 for genome engineering. Cell 157:1262–1278

Jones JM, Meisler MH (2014) Modeling human epilepsy by TALEN targeting of mouse sodium channel Scn8a. Genesis 52:141–148

Joung JK, Sander JD (2013) TALENs: a widely applicable technology for targeted genome editing. Nat Rev Mol Cell Biol 14:49–55

Juillerat A, Dubois G, Valton J, Thomas S, Stella S, Maréchal A, Langevin S, Benomari N, Bertonati C, Silva GH, Daboussi F, Epinat JC, Montoya G, Duclert A, Duchateau P (2014) Comprehensive analysis of the specificity of transcription activator-like effector nucleases. Nucleic Acids Res 42:5390–5402

Kamberov YG, Wang S, Tan J, Gerbault P, Wark A, Tan L, Yang Y, Li S, Tang K, Chen H, Powell A, Itan Y, Fuller D, Lohmueller J, Mao J, Schachar A, Paymer M, Hostetter E, Byrne E, Burnett M, McMahon AP, Thomas MG, Lieberman DE, Jin L, Tabin CJ, Morgan BA, Sabeti PC (2013) Modeling recent human evolution in mice by expression of a selected EDAR variant. Cell 152:691–702

Kato T, Miyata K, Sonobe M, Yamashita S, Tamano M, Miura K, Kanai Y, Miyamoto S, Sakuma T, Yamamoto T, Inui M, Kikusui T, Asahara H, Takada S (2013) Production of Sry knockout mouse using TALEN via oocyte injection. Sci Rep 3:3136

Kitajima S, Takagi A, Inoue T, Saga Y (2000) MesP1 and MesP2 are essential for the development of cardiac mesoderm. Development 127:3215–3226

Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM, Zhang F (2013) Optical control of mammalian endogenous transcription and epigenetic states. Nature 500:472–476

Li C, Qi R, Singleterry R, Hyle J, Balch A, Li X, Sublett J, Berns H, Valentine M, Valentine V, Sherr CJ (2014) Simultaneous gene editing by injection of mRNAs encoding transcription activator-like effector nucleases into mouse zygotes. Mol Cell Biol 34:1649–1658

Liu H, Chen Y, Niu Y, Zhang K, Kang Y, Ge W, Liu X, Zhao E, Wang C, Lin S, Jing B, Si C, Lin Q, Chen X, Lin H, Pu X, Wang Y, Qin B, Wang F, Wang H, Si W, Zhou J, Tan T, Li T, Ji S, Xue Z, Luo Y, Cheng L, Zhou Q, Li S, Sun YE, Ji W (2014a) TALEN-mediated gene mutagenesis in rhesus and cynomolgus monkeys. Cell Stem Cell 14:323–328

Liu Y, Lv X, Tan R, Liu T, Chen T, Li M, Liu Y, Nie F, Wang X, Zhou P, Chen M, Zhou Q (2014b) A modified TALEN-based strategy for rapidly and efficiently generating knockout mice for kidney development studies. PLoS One 9:e84893

Liu Z, Zhou X, Zhu Y, Chen ZF, Yu B, Wang Y, Zhang CC, Nie YH, Sang X, Cai YJ, Zhang YF, Zhang C, Zhou WH, Sun Q, Qiu Z (2014c) Generation of a monkey with MECP2 mutations by TALEN-based gene targeting. Neurosci Bull 30:381–386

Low BE, Krebs MP, Joung JK, Tsai SQ, Nishina PM, Wiles MV (2014) Correction of the Crb1rd8 allele and retinal phenotype in C57BL/6N mice via TALEN-mediated homology-directed repair. Invest Ophthalmol Vis Sci 55:387–395

Matsugami TR, Tanemura K, Mieda M, Nakatomi R, Yamada K, Kondo T, Ogawa M, Obata K, Watanabe M, Hashikawa T, Tanaka K (2006) Indispensability of the glutamate transporters GLAST and GLT1 to brain development. Proc Natl Acad Sci U S A 103:12161–12166

Maurano MT, Humbert R, Rynes E, Thurman RE, Haugen E, Wang H, Reynolds AP, Sandstrom R, Qu H, Brody J, Shafer A, Neri F, Lee K, Kutyavin T, Stehling-Sun S, Johnson AK, Canfield TK, Giste E, Diegel M, Bates D, Hansen RS, Neph S, Sabo PJ, Heimfeld S, Raubitschek A, Ziegler S, Cotsapas C, Sotoodehnia N, Glass I, Sunyaev SR, Kaul R, Stamatoyannopoulos JA (2012) Systematic localization of common disease-associated variation in regulatory DNA. Science 337:1190–1195

Menke DB (2013) Engineering subtle targeted mutations into the mouse genome. Genesis 51:605–618

Meyer M, de Angelis MH, Wurst W, Kühn R (2010) Gene targeting by homologous recombination in mouse zygotes mediated by zinc-finger nucleases. Proc Natl Acad Sci U S A 107:15022–15026

Meyer M, Ortiz O, Hrabé de Angelis M, Wurst W, Kühn R (2012) Modeling disease mutations by gene targeting in one-cell mouse embryos. Proc Natl Acad Sci U S A 109:9354–9359

Miyanari Y, Ziegler-Birling C, Torres-Padilla ME (2013) Live visualization of chromatin dynamics with fluorescent TALEs. Nat Struct Mol Biol 20:1321–1324

Mussolino C, Morbitzer R, Lütge F, Dannemann N, Lahaye T, Cathomen T (2011) A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity. Nucleic Acids Res 39:9283–9293

Namekata K, Kimura A, Kawamura K, Guo X, Harada C, Tanaka K, Harada T (2013) Dock3 attenuates neural cell death due to NMDA neurotoxicity and oxidative stress in a mouse model of normal tension glaucoma. Cell Death Differ 20:1250–1256

Panda SK, Wefers B, Ortiz O, Floss T, Schmid B, Haass C, Wurst W, Kühn R (2013) Highly efficient targeted mutagenesis in mice using TALENs. Genetics 195:703–713

Qiu Z, Liu M, Chen Z, Shao Y, Pan H, Wei G, Yu C, Zhang L, Li X, Wang P, Fan HY, Du B, Liu B, Liu M, Li D (2013) High-efficiency and heritable gene targeting in mouse by transcription activator-like effector nucleases. Nucleic Acids Res 41:e120

Raychaudhuri S (2011) Mapping rare and common causal alleles for complex human diseases. Cell 147:57–69

Saito T, Matsuba Y, Mihira N, Takano J, Nilsson P, Itohara S, Iwata N, Saido TC (2014) Single App knock-in mouse models of Alzheimer's disease. Nat Neurosci 17:661–663

Sakuma T, Ochiai H, Kaneko T, Mashimo T, Tokumasu D, Sakane Y, Suzuki K, Miyamoto T, Sakamoto N, Matsuura S, Yamamoto T (2013) Repeating pattern of non-RVD variations in DNA-binding modules enhances TALEN activity. Sci Rep 3:3379

Skarnes WC, Rosen B, West AP, Koutsourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T, Jackson D, Severin J, Biggs P, Fu J, Nefedov M, de Jong PJ, Stewart AF, Bradley A (2011) A conditional knockout resource for the genome-wide study of mouse gene function. Nature 474:337–342

Sommer D, Peters A, Wirtz T, Mai M, Ackermann J, Thabet Y, Schmidt J, Weighardt H, Wunderlich FT, Degen J, Schultze JL, Beyer M (2014) Efficient genome engineering by targeted homologous recombination in mouse embryos using transcription activator-like effector nucleases. Nat Commun 5:3045

Sung YH, Baek IJ, Seong JK, Kim JS, Lee HW (2012) Mouse genetics: catalogue and scissors. BMB Rep 45:686–692

Sung YH, Baek IJ, Kim DH, Jeon J, Lee J, Lee K, Jeong D, Kim JS, Lee HW (2013) Knockout mice created by TALEN-mediated gene targeting. Nat Biotechnol 31:23–24

Sur IK, Hallikas O, Vähärautio A, Yan J, Turunen M, Enge M, Taipale M, Karhu A, Aaltonen LA, Taipale J (2012) Mice lacking a Myc enhancer that includes human SNP rs6983267 are resistant to intestinal tumors. Science 338:1360–1363

Tabuchi K, Blundell J, Etherton MR, Hammer RE, Liu X, Powell CM, Südhof TC (2007) A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. Science 318:71–76

Takada S, Sato T, Ito Y, Yamashita S, Kato T, Kawasumi M, Kanai-Azuma M, Igarashi A, Kato T, Tamano M, Asahara H (2013) Targeted gene deletion of miRNAs in mice by TALEN system. PLoS One 8:e76004

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD (2010) Genome editing with engineered zinc finger nucleases. Nat Rev Genet 11:636–646

Veltman JA, Brunner HG (2012) De novo mutations in human genetic disease. Nat Rev Genet 13:565–575

Wang H, Hu YC, Markoulaki S, Welstead GG, Cheng AW, Shivalila CS, Pyntikova T, Dadon DB, Voytas DF, Bogdanove AJ, Page DC, Jaenisch R (2013) TALEN-mediated editing of the mouse Y chromosome. Nat Biotechnol 31:530–532

Watase K, Hashimoto K, Kano M, Yamada K, Watanabe M, Inoue Y, Okuyama S, Sakagawa T, Ogawa S, Kawashima N, Hori S, Takimoto M, Wada K, Tanaka K (1998) Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. Eur J Neurosci 10:976–988

Wefers B, Panda SK, Ortiz O, Brandl C, Hensler S, Hansen J, Wurst W, Kühn R (2013) Generation of targeted mouse mutants by embryo microinjection of TALEN mRNA. Nat Protoc 8:2355–2379

Wu J, Huang Z, Ren J, Zhang Z, He P, Li Y, Ma J, Chen W, Zhang Y, Zhou X, Yang Z, Wu SQ, Chen L, Han J (2013) Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. Cell Res 23:994–1006

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