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Genetic manipulations in the rat: progress and prospects

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Abstract

Purpose of review—Several advances have been made to manipulate the rat genome in the last 2 years. This review aims to describe these advances in rat genetic manipulations, with an emphasis on their current status and their prospects and applications in the postgenomic era.

Recent findings—Authentic rat embryonic stem cells were derived in 2008 using the 2i/3i culture system. This led to the generation of the first gene knockout rats via embryonic stem cell-based gene targeting. The development of zinc-finger nucleases (ZFNs) provided an alternative approach that avoids the necessity of germline competent embryonic stem cells. Meanwhile, improvements have been made to the well established random mutagenesis mediated by transposons or *N*-ethyl-*N*-nitrosourea (ENU). The in-vitro rat spermatogonial stem cell (SSC) system has greatly optimized these phenotype-driven approaches for future applications.

Summary—The rat has long been a prime model organism in physiological, pharmacological and neurobehavioral studies. The recent advances of rat reverse genetic approaches, together with the classical ENU and transposon mutagenesis system, will contribute tremendously to the deciphering of gene functions and the creation of rat disease models.

Keywords

gene targeting; knockout rat; mutagenesis; rat model

Introduction

The rat was the first animal species bred and kept for pure scientific purposes [1]. As early as 1828, albino rat mutants, which were selected from the offspring of rats trapped in rat-baiting sports in Europe, were being used in fasting studies by physiologists [2]. Rat genetic studies were also launched shortly after the rediscovery of Mendel's laws in 1900, with one of the outcomes being the identification of rat coat color as a Mendelian trait [2]. Since the development of the first inbred rat strain in 1909, more than 700 strains of laboratory rats have been bred for experimentation [2]. For example, the Zucker rat strain, which bears a mutation in the leptin receptor gene, is sugar metabolic deficient and insulin resistant and thus serves as a spontaneous genetic obesity model [3]. The spontaneously hypertensive rat (SHR) has been the most widely studied model of hypertension, as indicated by the number of publications on it [4*]. Historically, the rat has been a superior model organism for a variety of human health-related research fields, including physiology, pharmacology, neurobiology and drug discovery [5]. The rat shares a similar pathway with humans for

eradicating toxins, providing an ideal model for drug toxicology tests. Rats also own a natural advantage over mice in neurological and behavioral studies, because they are more sociable and skilled, and have complex cognitive abilities.

However, when it comes to reverse genetics and transgenic models, rats lag far behind mice. Thousands of gene knockout mouse strains have been generated through homologous recombination-mediated gene targeting in embryonic stem cells. The lack of authentic rat embryonic stem cells had, until recently, prevented this embryonic stem cell-based gene-targeting technology from being used in rats. Several alternative approaches aiming to circumvent this requirement have been developed for rat genetic manipulation. Classical transgenesis via pronuclear injection has been applied to rats since the early 1990s and contributed a large variety of transgenic rat disease models, such as hypertension [6], Alzheimer's disease [7] and Huntington's disease [8]. The use of *N*-ethyl-*N*-nitrosourea (ENU), as well as the mobile DNA technology of transposons and retrotransposons, has enabled generation of rat mutants in a random fashion. The first targeted knockout rats were generated in 2009 via zinc-finger nuclease (ZFN) technology [9], demonstrating an alternative approach to the classical embryonic stem cell-based gene-targeting technology. In this review, we summarize several strategies that have been used in rat genetic manipulations and describe the advantages as well as drawbacks of these advances in rat genetics for ongoing research in the postgenomic era.

Chemical mutagenesis using *N*-ethyl-*N*-nitrosourea

ENU-driven target-selected mutagenesis is an effective approach for artificial introduction of point mutations throughout the genome. This alkylating agent induces mutagenesis by transferring its ethyl group to oxygen or nitrogen nucleophilic groups of nucleobases [10]. The modified DNA, in a vast majority of cases, undergoes nucleotide substitutions such as A–T base transversions [11]. In practice, ENU is intraperitoneally injected into adult male rats and primarily targets spermatogonial stem cells (SSCs) in rat gonads (Fig. 1). The expected outcome is the transmission of mutated alleles through the germ-line. To achieve this, female rats with a clean background are bred with ENU-treated male rats, thereafter giving birth to heterozygous G1 offspring. As ENU introduces mutations randomly at multiple sites, at intervals of approximately 1–2 Mb throughout the genome [12], a high throughput screening method is essential for identifying mutations in a predetermined gene of interest. Different strategies have been developed to identify ENU-induced mutations in the animal, including yeast-based screening [13], CEL I-based enzymatic cleavage [14] and Mu transposase-based heteroduplex identification [15]. The selected animals are intercrossed to generate homozygous offspring. In 2003, an important tumor suppressor gene, *BRCA2*, was knocked out in rats using ENU mutagenesis, with a point mutation on exon 11 resulting in a premature stop codon [13]. To date, several other gene knockout rat models have been generated using this method, and have become valuable research tools in tumorigenesis [13,16,17] and neurobehavioral studies [18,19].

The ENU approach does not require genetic manipulations in embryonic stem cells, oocytes or embryos: simple injection with ENU is sufficient to introduce mutations to the SSCs, and the mutations will be passed on to the next generation automatically. However, the efficacy of this strategy largely depends on mutagenesis efficiency, which in turn depends on the optimization of ENU dosage in each rat strain because of their differing genetic backgrounds [20]. Mutations generated by ENU are random, thus researchers who use it must maintain a large repository of G1 heterozygous animals and must undertake the costly and time-consuming process of high-throughput screening. Alternatively, an archive of cryopreserved rat sperms from G1 mutagenized animals was recently used for extensive screening of sperm-extracted DNA, as well as indefinite preservation of mutagenized specimens [15].

Mutants of interest can later be recovered by intracytoplasmic sperm injection (ICSI). Knockout mutants can only be generated by nonsense mutations, and therefore a precise manipulation of specific regions in a targeted gene would be extremely difficult. Consequently, ENU-driven mutagenesis is now mainly employed in phenotype-driven approaches (forward genetics), with no assumption of genes whose dysfunction causes diseases. One could map the genomic regions responsible for the disease after screening and identifying the phenotype of interest. Still, multiple background mutations need to be handled carefully, usually by serial outcrossing of G1 heterozygotes with rats of clean genetic background to wipe out confounding factors [10**].

Mobile DNA technology–transposon mutagenesis

Although several transposon/retro-transposon systems such as *Tol1*, *PiggyBac* and *Minos* have been developed and shown to be applicable in mammalian cells, the Sleeping Beauty transposon currently is the most widely used mutagenesis tool in rodents [21**]. Sleeping Beauty transposon, a DNA transposon in the Tc1/mariner superfamily, was originally derived from salmon fish genome yet later found to be transposably active after reconstruction in cells from a large variety of species [22]. The Sleeping Beauty sequence consists of a coding region of transposase and flanking inverted repeat/direct repeat elements as transposase recognition sites. The transposon sequence is excised from the donor vector upon transposase provision and re-integrates into a genomic locus whereby Sleeping Beauty mobilizes itself via a ‘cut-and-paste’ transposition process. Transpositions in gene open reading frame (ORF) will therefore lead to a disruption. In this system, two strains of transgenic rats have to be independently generated by injection of constructs into fertilized eggs (Fig. 2). One of the constructs bears the transposon vector and the other expresses the transposase enzyme. The resultant transgenic rats are crossed to generate double-transgenic ‘seed’ rats carrying transposed germ cells. The transposition events are fixed by mating ‘seed’ rats with wild-type females, and progeny mutants are identified by molecular biology techniques such as PCR and 3′-rapid amplification of cDNA ends [21**]. In most cases, a reporter or gene-trapping cassette is incorporated into the transposon vector to aid in the detection of transposition events. However, the induced transposition is accomplished by crossing two transgenic strains *in vivo*, making it costly and troublesome because it only allows scientists to identify mutants in animals. Recently, improvements have been made by inducing transposition events with high efficiency in rat SSC lines [23**, 24**]. This approach provides a shortcut by which researchers are able to conduct spermatogonial colony selection at the tissue culture stage, and generate animals that possess only the desired genotypes.

The implementation of this mobile DNA technology in rats has led to 92 transposon-mediated knockouts, which are available from the KnockOut Rat Consortium (www.knockoutrat.org) or Transposogen (www.transpo-sagenbio.com). Transposons introduce only a few insertions per genome, which serve as DNA ‘tags’ for rapid identification of mutated alleles by PCR. Identification of mutations using transposon insertion as a tag has been remarkably efficient when adapted to the rat SSC system. On the contrary, ENU-mediated random mutagenesis usually causes thousands of point mutations throughout the genome and the less sophisticated detection methods [13–15] often leave unknown background mutations outside the gene of interest that may contribute to phenotypes. Some progress has been made to increase the number of gene hits of the Sleeping Beauty system, making it more effective for function annotation of genes [25]. However, one factor researchers need to consider is the nonrandom integration produced by the transposon system, as indicated by preferential recognition sequence of transposons that delineate ‘hotspots’ and ‘cold regions’ on a genome-wide scale. For example, Sleeping

Beauty transposon has exhibited a preference for palindromic AT repeats but recognition is primarily based on bending DNA structure [26].

Zinc-finger nuclease-mediated knockout rats

ZFNs have been invaluable tools for genomic manipulation in many model organisms, including *Caenorhabditis elegans*, *Drosophila*, zebrafish and finally the rat [27^{**},28]. ZFNs are a class of artificial chimeric proteins fused by two functional domains: a DNA-binding domain consisting of zinc-finger motif repeats and a nuclease domain from type II restriction endonuclease *FokI*. Each ZFN recognizes a triplet of DNA, and typically three to six individual ZFN ‘modules’ are aligned together to ensure the targeting of 9–18 specific nucleotides. This specificity is doubled by ZFN dimerization because *FokI* nuclease must work in pairs to cleave DNA. ZFN takes advantage of the DNA repair mechanism in eukaryotic cells to induce gene disruption (Fig. 3). When ZFN-expressing mRNAs predesigned to target a particular locus are injected into fertilized oocytes, a DNA double-strand break (DSB) is introduced at that locus, which activates the innate cellular DNA repair system to fix it. The majority of DSBs in cells are rescued by a mechanism called nonhomologous end joining (NHEJ), which functions when no homologous DNA template is available in proximity to the DSB [29]. NHEJ uses a ligation reaction to eliminate DSBs and restore the physical integrity of the chromosome, but the imprecise rejoining of the ends results in a loss of information content, usually a deletion of several base pairs at the DSB site. If this deletion happens to occur in a coding exon of a gene, a frameshift mutation and knockout genotype will be generated [30^{**}]. Targeted integration of new sequences can also be achieved by stimulating the homology directed repair (HDR) mechanism (Fig. 3). A donor DNA template containing the gene to be inserted, with flanking sequences homologous to genomic DNA at the ZFN action site, is co-administered with ZFNs. After a DSB is introduced, cells will preferentially activate the innate HDR to repair the break, leading to precise integration of the exogenous sequence at the ZFN cleavage site.

ZFN-mediated gene disruption is favorable because of its high targeting efficiency and the reduced time needed to generate knockouts [31^{*}]. However, a major concern surrounding ZFN technology is its high expense. The commercialized custom-designed ZFN reagents from Sigma–Aldrich currently cost US\$ 25 000 [28], and this must be joined with the costs of microinjecting multiple rat embryos as well as breeding and maintaining the rats. Despite some standardized ZFN modules now being available from public sources, these ZFN modules either have reduced targeting specificity or require research to design, assemble, clone and validate the ZFNs on their own, bringing in new technological burdens. Moreover, the possibility of off-target cleavage remains a critical issue to be addressed, as it can induce embryonic cyto-toxicity and confound the phenotype of gene disruption [27^{**}].

Embryonic stem cell-based gene targeting

Over the years, embryonic stem cells have been routinely used to create loss-of-function mutations (knockouts) or gene replacement (knockin) by homologous recombination in mice [32–34]. But numerous attempts to apply this gene-targeting technique to the rat, until recently, have all failed because of the imperfect methodology for isolating and manipulating authentic rat embryonic stem cells. In 2008, we developed a new strategy to maintain embryonic stem cell self-renewal using three small molecule inhibitors (3i: CHIR99021, PD184352 and SU5402) [35]. CHIR99021 specifically inhibits glycogen synthase kinase 3 (GSK3), whereas PD184352 and SU5402 inhibit mitogen-activated protein kinase (MAPK) and fibroblast growth factor receptor (FGFR) tyrosine kinase, respectively. Later we found that PD184352 and SU5402 can be substituted with a more potent MAPK inhibitor, PD0325901 (2i: CHIR99021 and PD0325901) [35]. Use of this 3i/

2i culture system enabled the first successful isolation and propagation of germline competent rat embryonic stem cells [36,37]. Recently, we reported the generation of the first gene knockout rats via homologous recombination in embryonic stem cells [38**]. As one would expect, the strategy for embryonic stem cell-based gene targeting in the rat is similar to the strategy used for the mouse (Fig. 4). A gene-targeting vector, usually harboring a positive selection cassette and two flanking arms that are homologous to the targeting site on the genomic locus, is electroporated into rat embryonic stem cells. After selection and expansion of embryonic stem cell colonies, targeting events are screened by PCR followed by Southern blot and/or sequencing. Targeted embryonic stem cells are then microinjected into rat blastocysts, which are implanted in the uterus of foster female rats. The resulting male chimeric offspring are crossed with wild-type females to produce heterozygotes carrying the targeted mutation. Confirmed heterozygotes are intercrossed to produce homozygous offspring.

Conditional knockouts offer a highly flexible control of gene disruption in either a specific type of cells/tissue or a defined time window during development, allowing a more accurate dissection of gene functions *in vivo*. A large variety of such models have been established in mice, using embryonic stem cell-based gene targeting in combination with the Cre (Flp) site-specific recombinase system [39]. Given that it is relatively easy to generate Cre-bearing transgenic rats via pronuclear microinjection [40,41], we anticipate that many conditional knockout rats will be generated in the near future using the same strategy developed for the mouse. There has been some progress concerning targeted integration via ZFN technology [42**]. However, the preparation of sophisticated genetic modifications, such as conditional knockout and insertion of large DNA fragments (>1 kb) into targeting site, still requires the use of embryonic stem cell-based gene-targeting technologies.

Notably, strain combination can be critical to germline transmission capacity in rat embryonic stem cell-based gene targeting. Using the 3i/2i systems, we have derived embryonic stem cells from various strains of rats, such as Dark Agouti, Fischer 344 (F344), Sprague–Dawley, Brown Norway, Wistar, Long–Evans and SHR ([36,37] and unpublished data). It is essential for researchers to test whether the rat embryonic stem cell line of the chosen strain is germline competent before conducting gene-targeting experiments.

Conclusion

In 2004, the completion of genomic sequencing of the Brown Norway rat has enabled the rat to join the mouse and human as the third species to enter the postgenomic era [43]. An increasing number of commonly used rat strains are also being added to the database [4*,44]. So far, genome-wide association study (GWAS) in rats has mapped thousands of quantitative trait loci (QTLs) correlated with human diseases (see <http://rgd.mcw.edu/>), indicating the huge potential of rats as disease models. ENU-mediated chemical mutagenesis and transposon-mediated mutagenesis are commonly used approaches in forward genetic screening applications. These approaches depend on the establishment of large-scale rat mutant pools and aim to analyze mutant phenotypes without assumption of genetic basis of the mutations. The use of rat SSCs has enabled insertional mutagenesis to be performed *in vitro*, thereby greatly facilitating the set-up of a mutant library. ZFN technology and rat embryonic stem cell-based gene targeting are two highly complementary methods for generating rat knockouts, allowing a genotype-driven strategy for interrogation of gene functions. Each of these two methods has its unique advantages that assist investigators in achieving their specific objectives: ZFNs can generate knockout rats in a short time with high targeting efficiency, whereas embryonic stem cell-based gene targeting is the superior choice for sophisticated genetic modifications. In this review, we listed some rat knockout models (established or under development) for investigators in the cardiac and/or renal field

to utilize in their research (Table 1). For those who intend to create their own models, the comparison of different technologies discussed above is available in Table 2 and investigators should choose the most appropriate method based on their individual purposes. The rapid development of all these technologies, together with advances in rat genomic resources, will usher the rat research community into a great future of prosperity.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 439–440).

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Key points

- The adaptation of embryonic stem cell-based gene-targeting technology to the rat may result in many sophisticated rat genetic manipulations such as conditional knockouts.
- The development of zinc-finger nuclease (ZFN) technology has provided an alternative for targeted gene disruption without the need of embryonic stem cells.
- Random mutagenesis using *N*-ethyl-*N*-nitrosourea or transposons remains an important forward genetic approach for systematically generating rat mutant alleles in large-scale studies.

Figure 1. *N*-Ethyl-*N*-nitrosourea-mediated chemical mutagenesis in the rat

Random germline mutations are introduced by *N*-ethyl-*N*-nitrosourea (ENU) injection. Mutagenized male rats are crossed with wild-type females to generate G1 mutant progeny. Screening is performed in the G1 mutant pool to identify a nonsense mutation inherited from germline. In this case, a ‘knockout’ genotype is selected, in which a mutation of the ‘TTG’ codon in the coding region of a gene to stop codon ‘TAG’ results in premature termination of gene product. The G1 heterozygote mutant is then bred to homozygosity.

Figure 2. Sleeping Beauty transposon-mediated mutagenesis in the rat

Transgenic rats carrying transposon sequence throughout their genome are crossed with transposase-bearing transgenic rats. A reporter gene with flanking inverted terminal repeat (ITR) elements is cloned in the transposon vector to facilitate screening. The produced double-transgenic progeny serve as 'seed' rats to pass the mutagenized allele to the heterozygous offspring. If an insertion occurs in the open reading frame (ORF) of a gene, a frameshift mutation will be induced to disrupt the gene.

Figure 3. Zinc-finger nuclease-mediated genome modification in the rat

Zinc-finger nuclease (ZFN)-expressing mRNA is microinjected into one-cell stage rat embryos. ZFNs recognize the targeting site and generate a double-strand break (DSB). The DSB is repaired either by nonhomologous end joining (NHEJ), which usually results in out-of-frame deletions, or by homology-directed repair (HDR), if a donor template is co-administered with ZFN mRNA. The HDR mechanism achieves a targeted integration of the template DNA. The ZFN-treated embryos are transferred to a pseudo-pregnant female. The resulting progeny are genotyped for targeted mutations (founder animal, red circle) before breeding to homozygosity.

Figure 4. Embryonic stem cell-based gene targeting in the rat

Rat embryonic stem (ES) cells are derived from rat blastocysts and maintained under the 2i/3i culture system. After transfection with a gene-targeting vector incorporating a selection marker, single colonies are selected, expanded and screened for targeting events. Correctly targeted cells are injected into a host blastocyst and transferred to foster females. Resulting chimeras are easily identified by the appearance of coat color (a strain recombination with distinct coat color is required) and crossed with wild-type females to generate G1 progeny of heterozygotes.

Table 1

Gene knockout rat models in the cardiac and/or renal field of research

Gene	Method	Mutation	Gene description	Function and disease	Current status (as of 18 March 2011)
Abat	Transposon	Insertion, 1st intron	4-Aminobutyrate aminotransferase	Hypertension	Available
Adra2a	ZFN	Disruption	Adrenergic, alpha-2A-, receptor	Hypertension	Phenotyping
Agr1a	ZFN	Disruption	Angiotensin II receptor, type 1a	Blood vessel Kidney diseases	Intercrossing
Agrap	ZFN	Disruption	Angiotensin II receptor-associated protein	Hypertension Cardiomegaly	Phenotyping
Aldh2	ZFN	Disruption	Aldehyde dehydrogenase 2 family (mitochondrial)	Hypertension	Phenotyping
Apoe	ZFN	Disruption	Apolipoprotein E	Cardiovascular diseases	Phenotyping
Atp2b1	ZFN	Disruption	ATPase, Ca ²⁺ transporting, plasma membrane 1	Hypertension	Backcrossing
Brinp3 (Fam5c)	Transposon	Insertion, 7th intron	Family with sequence similarity 5, member C	Hypertension	Available
Comt	ZFN	Disruption	Catechol-O-methyltransferase	Hypertension	Backcrossing
Cypa4a2	ZFN	Disruption	Cytochrome P450, family 4, subfamily A, polypeptide 2	Polycystic kidney, autosomal recessive	Phenotyping
Cypa4a3	ZFN	Disruption	Cytochrome P450, family 4, subfamily A, polypeptide 3	Polycystic kidney, autosomal recessive	Phenotyping
Erbp4	Transposon	Insertion, 2nd intron	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	Congestive heart failure	Available
Grk1	Transposon	Insertion, 3rd intron	G-protein coupled receptor kinase 1	Renal cell carcinoma Hypotension	Available
Kcnab1	Transposon	Insertion, 1st intron	Potassium voltage-gated channel, shaker-related subfamily, beta member 1	Ventricular tachycardia	Available
Kcnq1	ZFN	Disruption	Potassium voltage-gated channel, KQT-like subfamily, member 1	Hypertension	Intercrossing
Ldlr	ZFN	Disruption	Low-density lipoprotein receptor	Myocardial infarction Atherosclerosis	Intercrossing
Lep	ZFN	Disruption	Leptin	Hypertension	Backcrossing
Lrrc7	Transposon	Insertion, 22nd intron	Leucine-rich repeat containing 7	Diabetic nephropathy	Available
Mas1	ZFN	Disruption	MAS1 oncogene	Hypertension	Phenotyping
Nox4	ZFN	Disruption	NADPH oxidase 4	Hypertension	Phenotyping
Nppa	ZFN	Disruption	Natriuretic peptide precursor A	Hypertension	Phenotyping
Nppb	ZFN	Disruption	Natriuretic peptide precursor B	Hypertension Heart failure	Phenotyping
Nrg1	Transposon	Insertion, 1st intron	Neuregulin 1	Heart development	Available

Gene	Method	Mutation	Gene description	Function and disease	Current status (as of 18 March 2011)
Plcb3	Transposon	Insertion, 18th exon	Phospholipase C, beta 3	Myocardial infarction	Available
Plce1	Transposon	Insertion, 2nd Intron	Phospholipase C, epsilon 1	Nephrotic syndrome	Available
Pparg	ZFN	Disruption	Peroxisome proliferator-activated receptor gamma	Hypertension, cardiovascular diseases Kidney failures	Backcrossing
Ptpn11	ZFN	Disruption	Protein tyrosine phosphatase, nonreceptor type 11	Heart defects	Intercrossing
Rasgrp3	ZFN	Disruption	RAS guanyl releasing protein 3 (calcium and DAG-regulated)	Hypertension	Phenotyping
Ren	ZFN	Disruption	Renin	Kidney diseases Hypertension	Phenotyping
Slc7a11	Transposon	Insertion, 6th intron	Solute carrier family 7, member 11	Hypertension Kidney filtering disorders	Available
Slc16a12	Transposon	Insertion, 1st intron	Solute carrier family 16, member 12	Renal glucosuria	Available
Tmem22	Transposon	Insertion, 22nd intron	Transmembrane protein 22	Renal cell carcinoma	Available
Trdn	Transposon	Insertion, intron 9	Triadin	Cardiac hypertrophy	Available
Trpc4	Transposon	Insertion, 1st intron	Transient receptor potential cation channel, subfamily C, member 4	Hypertension	Available
Umod	ZFN	Disruption	Uromodulin	Kidney diseases	Backcrossing

The information concerning the rat knockout models was adapted from <http://rgd.mcw.edu/wg/physgenknockouts> and <http://www.knockoutat.org/>. ZFN, zinc-finger nuclease.

Table 2

Comparison of different methods for genetic manipulation in the rat

Method		Time	Cost	Efficiency (mutational/targeting)	Screening	Confounding factors	Advantages
Forward genetics (random)	ENU mutagenesis	<1 Year	Relatively low	Very high (one mutation in every 1–2 Mb in genome)	In adult animals	Substantial background mutations	Indefinite cryopreservation of rat sperms from G1 mutagenized animals
Reverse genetics (targeted)	Transposon mutagenesis	<1 Year (in SSC system)	Low	Low (1–10 hits per genome)	In rat SSC lines	Genomic ‘hot spots’ and ‘cold regions’ of insertional events	Rapid detection of insertion by reporter or gene-trapping cassettes
	ZFN-mediated gene-targeting	6–9 Months	High	Relatively high	In adult animals	Off-target effects	Embryonic stem cell-independent
	Embryonic stem cell-based gene targeting	1 Year	Low	Low (1–3%)	In rat embryonic stem cell lines	Germline competent embryonic stem cells of the chosen strain are necessary	Enables sophisticated genetic manipulations (knock-ins, conditional knockouts)

ENU, *N*-ethyl-*N*-nitrosourea; SSC, spermatogonial stem cell; ZFN, zinc-finger nuclease.