



From Gene Targeting to Genome Editing: Transgenic animals applications and beyond

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ABSTRACT

Genome modification technologies are powerful tools for molecular biology and related areas. Advances in animal transgenesis and genome editing technologies during the past three decades allowed systematic interrogation of gene function that can help model how the genome influences cellular physiology. Genetic engineering via homologous recombination (HR) has been the standard method to modify genomic sequences. Nevertheless, nuclease-guided genome editing methods that were developed recently, such as ZFN, TALEN and CRISPR/Cas, opened new perspectives for biomedical research. Here, we present a brief historical perspective of genome modification methods, focusing on transgenic mice models. Moreover, we describe how new techniques were discovered and improved, present the paradigm shifts and discuss their limitations and applications for biomedical research as well as possible future directions.

Key words: Homologous recombination, DNA repair, Cre-LoxP, ZFN, TALEN, CRISPR/Cas9.

INTRODUCTION

A universal question in biology is how the genome translates into phenotypes giving rise to the endless forms of nature. It dates back to the first evidences that genes encode individual proteins and the seminal discovery of DNA's three-dimensional structure (Beadle and Tatum 1941, Watson and Crick 1953). Since then, molecular biology has

developed at a rapid pace guided by the Central Dogma (Crick 1958) and somehow impregnated by genetic determinism. Over the second half of the 20th century, advances in the generation of transgenic organisms made its way from prokaryotic to vertebrate model organisms, including mice.

Integration of a foreign DNA sequences into host genomes characterized the first attempts to study gene function *in vivo* (Brinster et al. 1981, 1982, Spradling and Rubin 1982). In mice, pioneering studies generated non-targeted

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genetic modifications in somatic cells through microinjection of exogenous DNA into fertilized eggs (Brinster et al. 1981, 1982, Palmiter et al. 1982). Shortly after, a series of groundbreaking studies described the disruption of endogenous gene expression through targeted modifications that were transmitted through germ line cells (reviewed in (Capecchi 2005)). The discoveries that allowed targeted genetic inactivation in the mouse genome, through the use of embryonic stem cells, granted the Nobel Prize award in Physiology and Medicine 2007 to Drs. Mario Capecchi, Martin Evans and Oliver Smithies.

The establishment of this technique led to a revolution in the ability to interrogate the relevance of specific genes revealing the molecular basis of numerous biological phenomena from animal diversity to human diseases. Advances in DNA sequencing technologies allowed the study of eukaryote's genomes at unprecedented resolution and taught us that the variations found in the coding regions were too small to account for the substantial organismal differences (King and Wilson 1975, Whitehead and Sackstein 1985, McGinnis and Krumlauf 1992, Adams et al. 2000, Venter et al. 2001). The lack of correlation between genome size or the number of protein-coding genes with organism complexity was puzzling. Additional findings supported the notion that organismal complexity arose from more elaborate regulatory networks rather than the appearance of new genes (Levine and Tjian 2003). This scenario exacerbated the need to go beyond sequence comparisons and made clear the need to study gene function in various contexts (e.g. cells, tissues, developmental stages and organisms) to elucidate the relationship between an organism's genome and its phenotype. Studies on gene targeting and genome edition developed over the last thirty years advanced transgenesis in an unimaginable scale. Nowadays, we have learned to manipulate the genome in more efficient (e.g. less time consuming) and refined ways (e.g. spatial and timing), to generate transgenics in non-model organisms and to edit the genome at a

systems level (e.g. target multiple genetic pathways simultaneously).

Here, we review and discuss the various methods to create targeted modifications in the genome and present a historical perspective of the generation of transgenic mice models that benefited from these advances. We describe recent technologies that greatly improved transgenesis, such as ZFNs, TALENs and CRISPR/Cas, its biological applications from basic research to biotechnology, while highlighting challenges as well as future perspectives.

GENE TARGETING BY HOMOLOGOUS RECOMBINATION

In the early 80's, a series of pioneer studies generated transgenic mice by microinjecting fertilized egg cells even before the establishment of homology based methods (Gordon et al. 1980, Brinster et al. 1981, 1982, 1984, Palmiter et al. 1982). These studies performed the implantation of eggs previously injected with plasmids encoding viral antigens into pseudo pregnant recipient females. The insertion of various copies of the transgene in the genome, its expression in different somatic cell types and the transmission through germ line cells was observed. However, integration occurred in a non-targeted manner and without copy number control (Brinster et al. 1981). Using similar methodologies of zygote microinjection, transgenic rabbits, pigs and sheep were also produced (Hammer et al. 1985). These studies represented a major advance in the areas of animal science and transgenic animal generation. Later, it became clear that these genetic modifications could disturb gene expression patterns, induce mutations and/or gene inactivation in the host genome.

The introduction of transgenes in the genome in a targeted manner was only possible with a better understanding of DNA repair by homologous recombination (HR) (Folger et al. 1982, 1984, Smithies et al. 1985). Using this knowledge the groups led by Mario Capecchi and Raju Kucherlapati generated targeted genetic modifications in

cultured mammalian cells (reviewed in Capecchi 1989, 2005). By electroporation or microinjection, exogenous DNA constructs could be introduced as a template of the target genomic sequence. These targeting vectors should have high homology with the targeted genomic sequence to be recognized by the HR machinery in order to introduce different types of modifications, including insertion or deletion. However, the targeting event occurred in a small percentage of the transfected mammalian cells making it unfeasible to efficiently target a fertilized egg and generate a whole transgenic animal (Capecchi 1989, Capecchi 2005).

Establishment of embryonic stem (ES) cells culture (Martin 1981) allowed the use of HR-dependent modifications described above to target specific *loci* of mouse pluripotent cells. Few successful strategies previously used in other mammalian cell types were employed to select the ES clones that underwent HR. Targeted ES cells carrying exogenous DNA sequences (antibiotic resistance genes - ARG), such as neomycin-resistance gene (*neo^r*), could be selected (positive selection). However, positive and negative strategies of selection became necessary, since the integration of a targeting vector and the *neo^r* at random sites through non-homologous recombination also occurred. The use of a vector combining the herpes virus thymidine kinase gene (HSV-tk), outside of the region to be recombined, with the *neo^r* allowed the selection of ES cells that contained the desired targeting. Integration through non-homologous recombination generated ES cells expressing the HSV-tk enzyme that were sensitive to FIAU (negative selection) (Mansour et al. 1988). In 1987, the groups of Drs. Capecchi and Smithies reported the targeting of the HPRT gene in mouse ES cells. As written by Dr. Capecchi, the protocol described in these papers “should be useful for targeting mutations into any gene” (Doetschman et al. 1987, Thomas and Capecchi 1987). Following the established methodology of ES cells targeted modification, several groups developed knockout mice for various genes.

In order to target desired genes *in vivo*, transgenic clones of ES cells were injected in the inner mass of blastocysts that were subsequently implanted into pseudo pregnant females which gave birth to chimeric animals. Mice with different coat patterns were used to screen the offspring chimeras containing cells derived from the transfected ES cells. The chimeric animals containing genetically modified germ line cells transmitted the transgenes to their offspring. Then, heterozygous mice crossing led to the generation of homozygous transgenic mice. Approaches to inactivate an endogenous gene included the replacement of the targeted region or the insertion of a *neo^r* within the coding region to disrupt the open reading frame (ORF) and gene expression. Altogether, the advances described above culminated in the generation of the first gene knockout mice (Joyner et al. 1989, Koller et al. 1989, 1990, Schwartzberg et al. 1989, Zijlstra et al. 1989, Thomas and Capecchi 1990). These pioneering methodologies also allowed the development of knockins with or without cell specific promoters, which could drive transgene expression in a restricted subset of cells or in the whole animal (Okabe et al. 1997, Tamamaki et al. 2003, Yang et al. 2004, Ma et al. 2014).

Transgenic mice generated through HR standard methods already enabled control of gene expression timing and reversibility by the use of drug-inducible transgenes. Two types of engineered tetracyclin receptors that work as drug-modulated transcription factors have been developed: the rTA is transcriptionally active after doxycycline treatment, while the rtTA receptor is prevented from binding DNA. To achieve drug-controlled transgene up- or down-regulation, two different lines are required: one with the expression of the receptor (rTA or rtTA) and the other with a transgene under the control of a promoter region responsive to the tetracyclin receptor. In mice containing both transgenes, gene expression is regulated by the tetracyclin treatment (Furth et al. 1994, Lewandoski 2001, Sun et al. 2007).

Relevant findings in various areas of biomedical research (e.g. cancer, immunology, developmental biology) were achieved using knockout and knockin mice, including the use of transgenic mice to model human diseases (He et al. 1997). Importantly, as emphasized in the following section, transgenic mouse models generated by the use of HR standard methods have limitations, in particular embryo lethality, phenotypes caused by non-autonomous gene function and limited or complicated tools to generate gene knockout mice with temporal and cell type/tissue specificity. As described below, new biological tools were developed to bypass these issues.

RECOMBINASE-BASED CONDITIONAL TRANSGENICS: CRE-LOXP AND FLP-FRT SYSTEMS

In vivo germ line gene targeting can lead to premature death, making it impossible to evaluate phenotypes afterwards. In mice, about 30-40% of the germ line knockouts are embryonic or perinatal lethal (Adams et al. 2013). Moreover, some of the phenotypes of multiple organ malformation may be the consequence of non-autonomous effects, since the development of some tissues may depend on the correct development of others, complicating the task of fully understanding gene function (Clarke et al. 1992, Wu et al. 2003). Altogether, these negative features of transgenic organisms that carried genetic modification in all somatic cells increased the desire for alternative transgenic models with a tissue- or developmental stage- specific inactivation of the genes of interest.

Site-specific recombinases (SSR) have been used to modify the genome with temporal and cell type specificity. Different types of recombinases have been described, but here we will focus on the two SSR systems most widely used in transgenic models: the Cre-LoxP and Flp-FRT, members of the integrase family of recombinases (Turan and Bode 2011). The Cre-LoxP system was first characterized in the bacteriophage P1 and is responsible for genomic recombination during bacterial division. The Cre (cyclization

recombination) recombinase is a 38 kDa protein that can catalyze the recombination of two specific LoxP sequences (locus of crossing-over of P1) (Sauer and Henderson 1988). The Flp recombinase, characterized in *S. cerevisiae*, recognizes the FRT sites (Flp recombinase recognition target) (Gates and Cox 1988, Branda and Dymecki 2004). Although not identical, the sites recognized by Cre and Flp display high similarities. These sequences are formed by 34 bp consensus sequence with two 13 bp palindromic sequences separated by an 8 bp spacer region that is responsible for the sequences orientation (Sternberg and Hamilton 1981, McLeod et al. 1986). To enable recombination, two SSR enzymes monomers bind to each recognition site and mediate a Holliday junction between them before completing recombination. Both systems exhibit high specificity and do not need cofactors. The orientation of the recognition site determines the genetic modification catalyzed by SSR enzymes. When the sites have the same orientation the recombinase excise the sequence in between them irreversibly, while, in the case of opposite orientation, the enzyme drives the inversion of the flanked region (Grindley et al. 2006, Turan and Bode 2011).

Excision of genomic regions by SSR enzymes was a valuable tool to develop transgenic models that bypassed some of the limitations of the first generation of knockouts. To achieve genetic inactivation in cell type-specific manner, it is necessary that the recombinase is expressed in the cells containing specific exons or entire genes flanked by *FRT* or *LoxP* sequences. To do so, two independent transgenic mice are crossed: one carrying the recognition sites flanking the region to be excised and the other displaying the coding sequence of the SSR enzyme under the control of cell-type-specific promoters. Pups carrying both transgenes will have knockout (Cre- or Flp-expressing) and non-recombined (Cre- or Flp-negative) cells (Fig. 1A) (Sauer and Henderson 1988, Golic and Lindquist 1989). In addition to the excision of coding regions, SSR-mediated excision

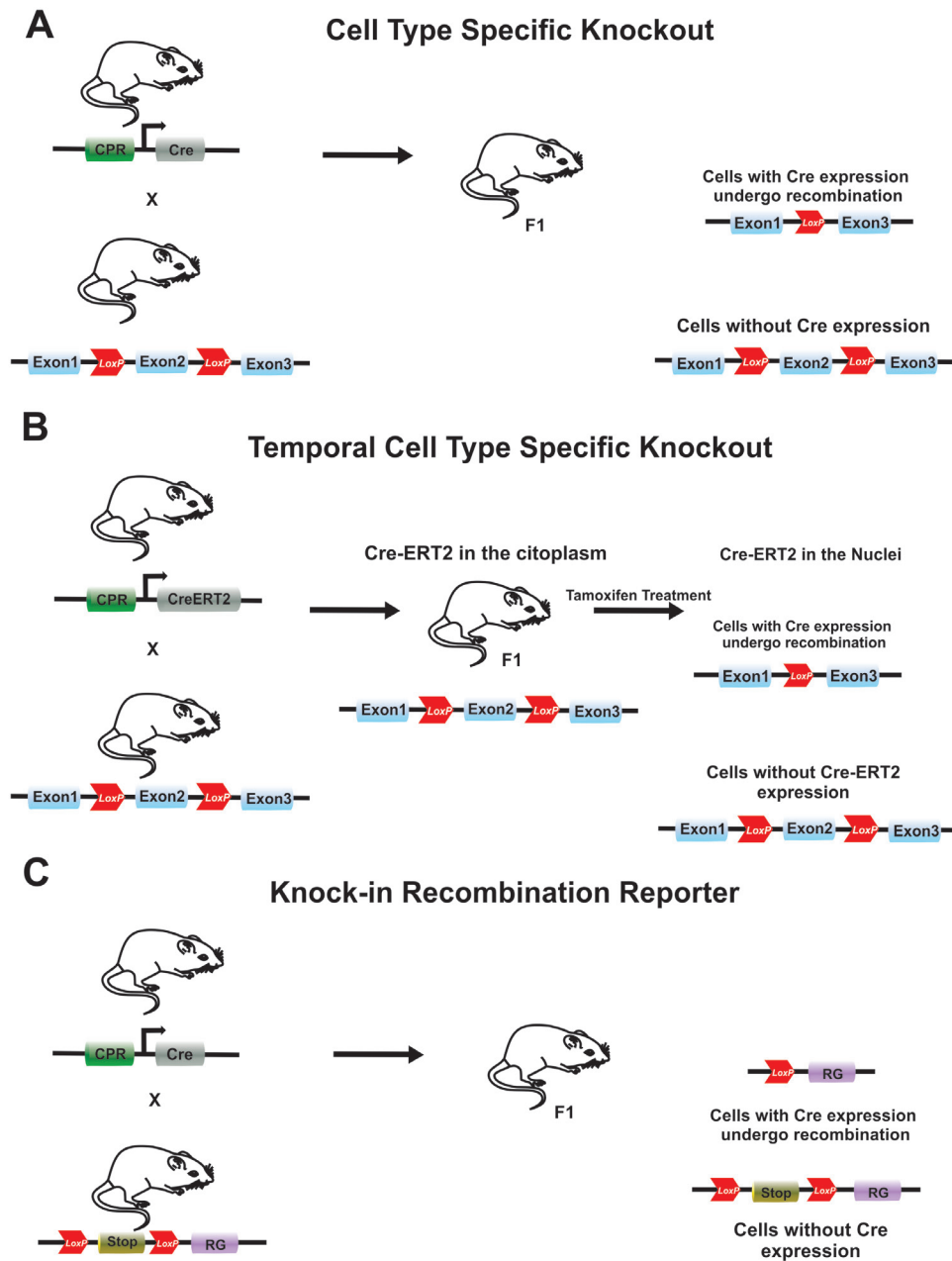


Figure 1 - *In vivo* transgenesis by SSR-systems: (A) Conditional knockout (cKO) mice may be generated by mating two different lines of transgenic mice: One carrying a SSR enzyme (e.g. Cre) under the control of a cell type-specific promoter (CPR) and the other containing coding regions of the gene of interest flanked by *LoxP* sequences. Offspring of this mating carrying both transgenes will undergo genetic inactivation only in cells expressing Cre that will recombine the *LoxP* flanked region. In cells without Cre expression no recombination or gene inactivation will occur. (B) Temporal control of Cre-mediate recombination may be achieved by the use of a chimeric Cre protein (Cre-ERT2). The Cre-ERT2 only translocates to the nucleus and catalyze the recombination after tamoxifen treatment. (C) Knockin recombination reporter mice usually carry a construct with a stop codon flanked by *LoxP* sequences followed by a reporter gene (RG). In mice carrying this transgene, RG is turned on only in cells expressing Cre, where the stop codon was excised. In the Cre negative cells, the RG is not expressed. These reporter lines allow researchers to track cells/tissues that underwent Cre-mediated recombination and to perform lineage tracing experiments.

was also used to generate chimeric proteins (e.g.: constitutively active beta-catenin) (Harada et al. 1999) and to remove entire chromosomes (Lewandoski and Martin 1997). The use of specific promoter regions to drive Cre or Flp expression allowed researchers to modify the genome in specific cells/tissues and/or specific developmental stages. Therefore, application of SSR enzymes represented a breakthrough for *in vivo* genetic modifications (Branda and Dymecki 2004).

These approaches were used in mice to switch on transgenes in a tissue-specific manner (Lakso et al. 1992, Orban et al. 1992). By the time the first SSR-dependent knockin mice was generated, it was already known that SV40 T overexpression triggered tumorigenesis in the lens (Mahon et al. 1987). Researchers aimed to establish a proof-of-principle that Cre expression could be restricted to a specific tissue and drive recombination of *LoxP* sites *in vivo*. A mouse line carrying a transgene containing the SV40 T antigen coding sequence downstream of a *LoxP*-flanked stop codon was mated with another harboring Cre under the control of the lens-specific α A-crystallin promoter. The offspring of these mice expressed SV40 T only in the lens and developed lens tumors, showing that SSR systems could function *in vivo* (Lakso et al. 1992).

SSR enzymes were also used to improve the procedures of transgenic mice generation. As explained, antibiotic resistance genes (ARG) are required for selection of ES-containing engineered transgenes. However, it was demonstrated that, in some cases, ARG constructs could disrupt gene expression nearby the transgene locus (Scacheri et al. 2001). To solve this problem, recognition sites of Cre or Flp surrounding the ARG region were inserted in the transgene. When mice containing this transgene were crossed with others expressing the correspondent SSR enzyme ubiquitously, the ARG region was excised from the genome of the offspring (Ren et al. 2002, Tamamaki et al. 2003, Favaro et al. 2009, Ma et al. 2014).

Mouse lines containing modified versions of the Cre allowed a more refined control of the timing of recombinase-mediated excision (Tronche et al. 2002, Branda and Dymecki 2004, Lao et al. 2012). The Cre-ERT2 is a chimeric protein that only translocates to the nucleus in the presence of tamoxifen. Therefore, regardless of ubiquitous or cell type-specific expression of Cre-ERT2, the Cre-mediated recombination will only occur after tamoxifen treatment, providing sophisticated timing control capabilities (Figure 1B) (Ahn and Joyner 2004, Lagace et al. 2007). Recently, a Cre-ERT2 under the control of the Axin promoter (*Axin-Cre-ERT2*) was used in an elegant lineage tracing experiment to label Wnt/ β -catenin responsive cells at different developmental time points. In addition to previously described recombinase-based lineage tracing experiments (Jensen and Dymecki 2014), these studies showed that Cre recombinase is suitable to leave a permanent genetic mark in the recombined cells that responded to a given ligand/morphogen/hormone. Interestingly, the daughters of the recombined cell will also express the reporter gene (van Amerongen et al. 2012, Bowman et al. 2013).

An extremely important requirement when using the transgenic lines that express SSR enzymes is to characterize the pattern of expression and activity of the recombinases in order to define where and when genetic inactivation will occur. Multiple approaches have been used, such as immunostaining for SSR enzymes and mouse lines in which the expression of a reporter gene is dependent on the SSR activity (Buchholz et al. 1996, Schwenk et al. 1997, Lobe et al. 1999, Yamamoto et al. 2009). In the latter, the transgene must contain a ubiquitous promoter and *LoxP* (or *FRT*) recognition sites flanking a stop codon (*LoxP*-Stop-*LoxP* - LSL) upstream of a reporter gene (e.g. GFP, RFP, β -galactosidase or alkaline phosphatase). In mice carrying this transgene, only cells with Cre activity will recombine the stop codon and express the reporter gene (Fig. 1C). That way it became possible to characterize where and/or

when the recombination and, most likely, the Cre-mediated genetic inactivation event occurred. As mentioned, these LSL cassettes were also used for Cre-dependent overexpression. In this case, usually the LSL is located in between the coding sequence of the gene and its transcription initiation site so the removal of the stop codon allows overexpression of the gene.

Even though the generation of transgenic animals using SSR based transgenesis represented a major advance in the field, there are several limitations. First, at least two different transgenic lines (Cre and *LoxP* lines) are required to achieve tissue and/or developmental stage-specific knockout or knockin mice. When double or triple knockout are intended or when a reporter of Cre recombination is needed in the same animal, the number of transgenes and the amount of crossings necessary can be extremely laborious and time consuming. Second, these SSR-mediated genetic modifications are not feasible to modify single or few nucleotides (e.g. point mutations). Importantly, the use of recombinase-based methods requires careful analysis of the biological effects of the SSR enzymes. In some Cre lines, the expression of Cre alone, regardless of genetic inactivation, was sufficient to induce some phenotype, such as cell death, chromosomal aberrations, DNA damage and sterility (Schmidt et al. 2000, Loonstra et al. 2001, Forni et al. 2006, Schmidt-Supprian et al. 2007). Another important issue in some Cre mouse lines is the allele parental inheritance. There are reports of different Cre expression and/or activity patterns depending on parental inheritance (Hayashi et al. 2003, MacPherson et al. 2004). Cre-mediated recombination of *LoxP* sequences may also vary depending on the position of the *LoxP* sequence in the genome (Vooijs et al. 2001). Some of these limitations were solved by the methods of genome editing to be described next.

GENOME EDITION BY NUCLEASES

In the last decade, new methodologies of genome manipulation have emerged. A series of custom target nucleases with the ability to specifically target the genomic region of interest and cleave the phosphodiester bonds have been developed (Urnov et al. 2005, Miller et al. 2007, Christian et al. 2010, Miller et al. 2010, Kim and Kim 2014). The broad concept underlying the new genome editing tools is that by stimulating DNA double-strand breaks (DSBs) at a target region, the endogenous repair machinery of the cell could be hijacked to introduce targeted mutations. In the absence of a homologous repair template, InDels (insertions/deletions) may occur via error-prone non-homologous end joining (NHEJ). These may alter the open reading frame (ORF) of the target gene leading to a premature stop codon or translation of a scrambled amino acid sequence. Notably, the InDels induced by NHEJ are random, therefore the outcome cannot be predicted (Bibikova et al. 2002, Sander and Joung 2014). The generation of InDels within a mutated genomic region may also restore gene function through the correction of disease causing mutations (Long, McAnally et al. 2014). Therefore, the engineered nucleases to be described in the next sessions, Zinc-finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and CRISPR-Cas systems, allowed the generation of transgenic organisms in a more efficient way and have introduced a new era in gene function analysis.

PROTEIN-GUIDED ENGINEERED NUCLEASES

The use of ZFNs (Zinc-Finger Nucleases) and TALENs (Transcription Activator-Like Effector Nucleases) facilitated the generation of transgenic animals. In comparison to the endogenous DNA break events, the activity of the engineered nucleases may increase the frequency of DSBs in a specific region in about 10,000-100,000 (Moehle et al. 2007). In addition to ZFNs and TALENs, other valuable tools for genome editing have been

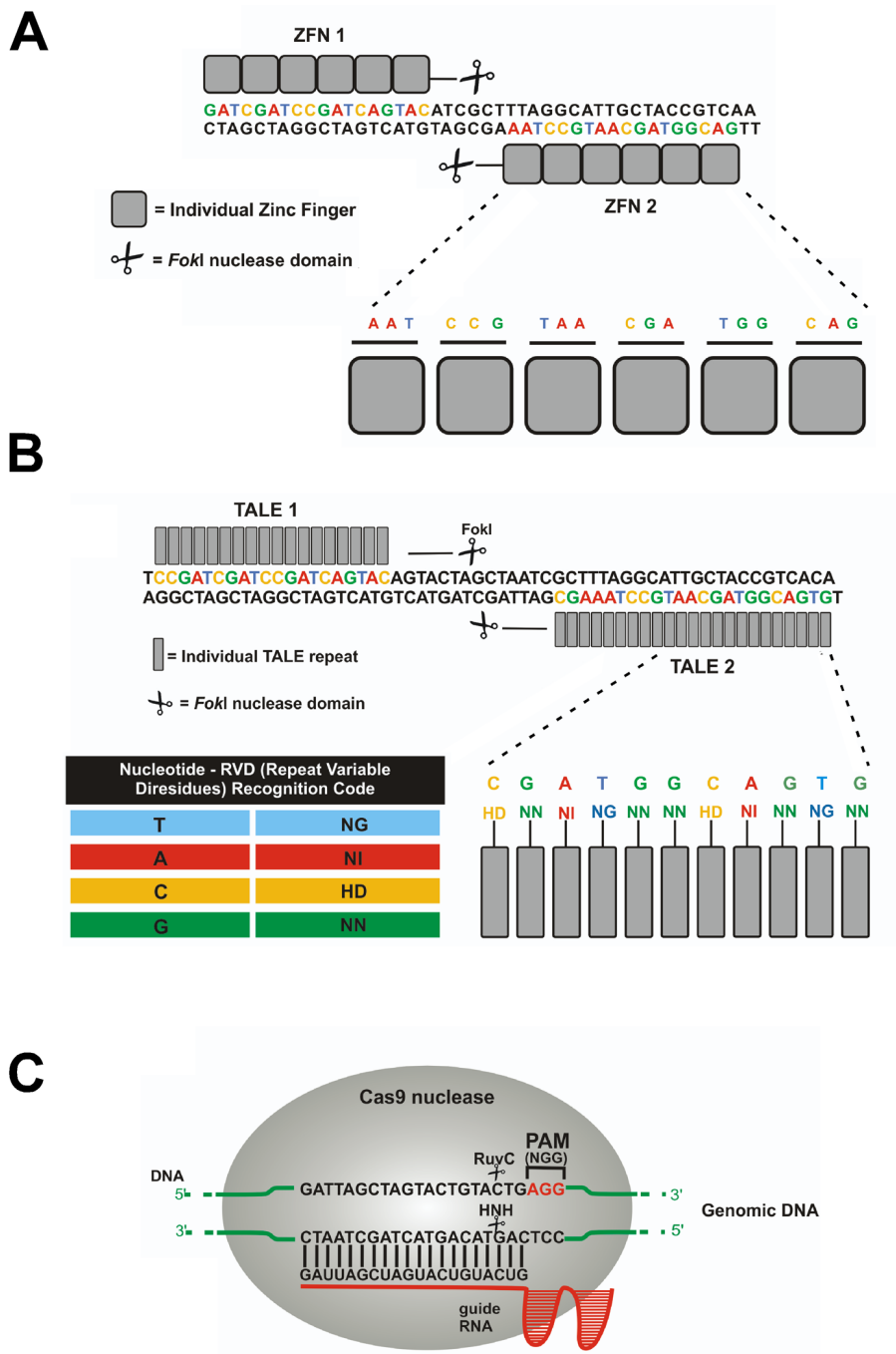


Figure 2 - Molecular mechanisms of DNA recognition: ZFNs, TALENs and CRISPR/Cas9: (A) Illustration of a ZFN pair bound to DNA sequence on opposite strands. In this case, each individual zinc finger domain (gray square) binds to a nucleotide triplet. (B) Illustration of a TALEN pair bound to DNA sequence on opposite strands. Note that each individual TALEN module (gray rectangle) is bound to a specific nucleotide, through interactions of two amino acid residues (RVD – repeat variable diresidues) and the nucleotide (inset in TALEN 2). The RVD-nucleotide recognition code is shown on the left. The FokI DNA cleavage domains of each ZFN (A) or TALEN (B) are juxtaposed and present a spacer region in between them. (C) The CRISPR/Cas9 recognition system consists of a guide RNA (gRNA, red) transcript and the Cas9 nuclease. The gRNA requires an adjoining NGG sequence (PAM) for Cas9 recognition and directs DNA cleavage through base pair complementarity. RuvC and HNH nuclease domains of Cas9 create DSBs at the genomic target site (green).

developed (e.g. meganucleases and transposons), but these are not in the scope of this review.

One of the biggest limitations imposed by restriction endonucleases was the small size of the DNA site to be recognized, what hampered their use when working with larger DNA sequences due to cleavage activity in multiple sites. Also, most restriction enzymes present DNA binding and cleavage activity within the same domain, limiting their targeting capability (Lanio et al. 2000). However, a few of them have separate binding and cleavage domains, such as *FokI*. In order to increase the possible binding sites for restriction enzymes, Dr. Srinivasan Chandrasegaran and colleagues envisioned an approach of linking DNA binding region of homeodomain transcription factors to the cleavage domain of *FokI*. The resultant artificial nuclease contained a DNA binding homeodomain fused to *FokI* nuclease, being able to cleave DNA sequences nearby homeodomain-binding sites (Kim and Chandrasegaran 1994). Later on, this same group assembled the first ZFNs (Kim et al. 1996), paving the way to genome tailoring at custom sites.

ZFNs are chimeric proteins composed of a modular array of Cys2-His2 DNA-binding zinc fingers fused to a *FokI* nuclease domain (Kim et al. 1996, Bibikova et al. 2001, Urnov et al. 2010). These modular arrays are usually composed of 3-6 zinc fingers that recognize three nucleotides each, providing DNA binding specificity to the nuclease (Greisman and Pabo 1997, Wolfe et al. 2000, Maeder et al. 2008, Ramirez, Foley et al. 2008) (Figure 2A). A pair of ZFNs must be designed to target a given genomic region, since the *FokI* nuclease domain requires dimerization to work (Bitinaite et al. 1998). Each zinc finger array binds to close DNA regions, on opposite strands, so that a DSB is induced in a specific genomic site (Fig. 2A). The repair of the DSB may lead to DNA insertion, deletion, inversion, point mutations or translocations, depending on the targeting strategy used and the DNA repair pathway chosen by the cell (Fig. 3).

ZFNs can be assembled by merging previously described individual zinc fingers characterized for specific nucleotide triplets. However, the resulting ZFNs may not necessarily bind to the expected sequence (Ramirez et al. 2008). Efficient DNA recognition by ZFNs is highly dependent on the position of the zinc fingers in the array and on the 3-dimensional conformation of the entire engineered protein (Ramirez et al. 2008). This aspect led to the extensive research on the development of methods for the selection of functional ZFNs. Recently, new platforms for ZFN assembly arose (Maeder et al. 2008, Sander et al. 2010, Bhakta et al. 2012), however commercial custom-made ZFNs are still the quickest and safest design option.

TALE proteins were described as key players during plant infection by bacteria of the genus *Xanthomonas*. These secreted bacterial proteins can bind specific DNA promoter regions of the plant cells regulating host gene expression (Romer et al. 2007). TALEs are modular proteins composed of TALE repeats (34 amino acids each) that contain two hypervariable amino acid residues called RVDs (repeat variable diresidues) at positions 12 and 13. These variable residues determine a surprisingly simple code of DNA binding specificity: the combination of these two amino acid residues dictates the specific binding to one nucleotide (Boch et al. 2009, Moscou and Bogdanove 2009) (Fig. 2B).

Similar to ZFNs, TALENs were developed by fusing the *FokI* endonuclease domain with an array of TALE repeats. TALEN assembly is much less problematic in comparison to ZFN, although some difficulties are present. Different from ZFNs, modular assembly of TALE repeats results in the predicted DNA binding pattern. On the other hand, cloning steps to generate the desired TALEs can be challenging due to the high DNA sequence homology between the different repeats. However, advances in cloning systems are helping to overcome these difficulties (Engler et al. 2008). As an example, libraries of TALENs targeting most

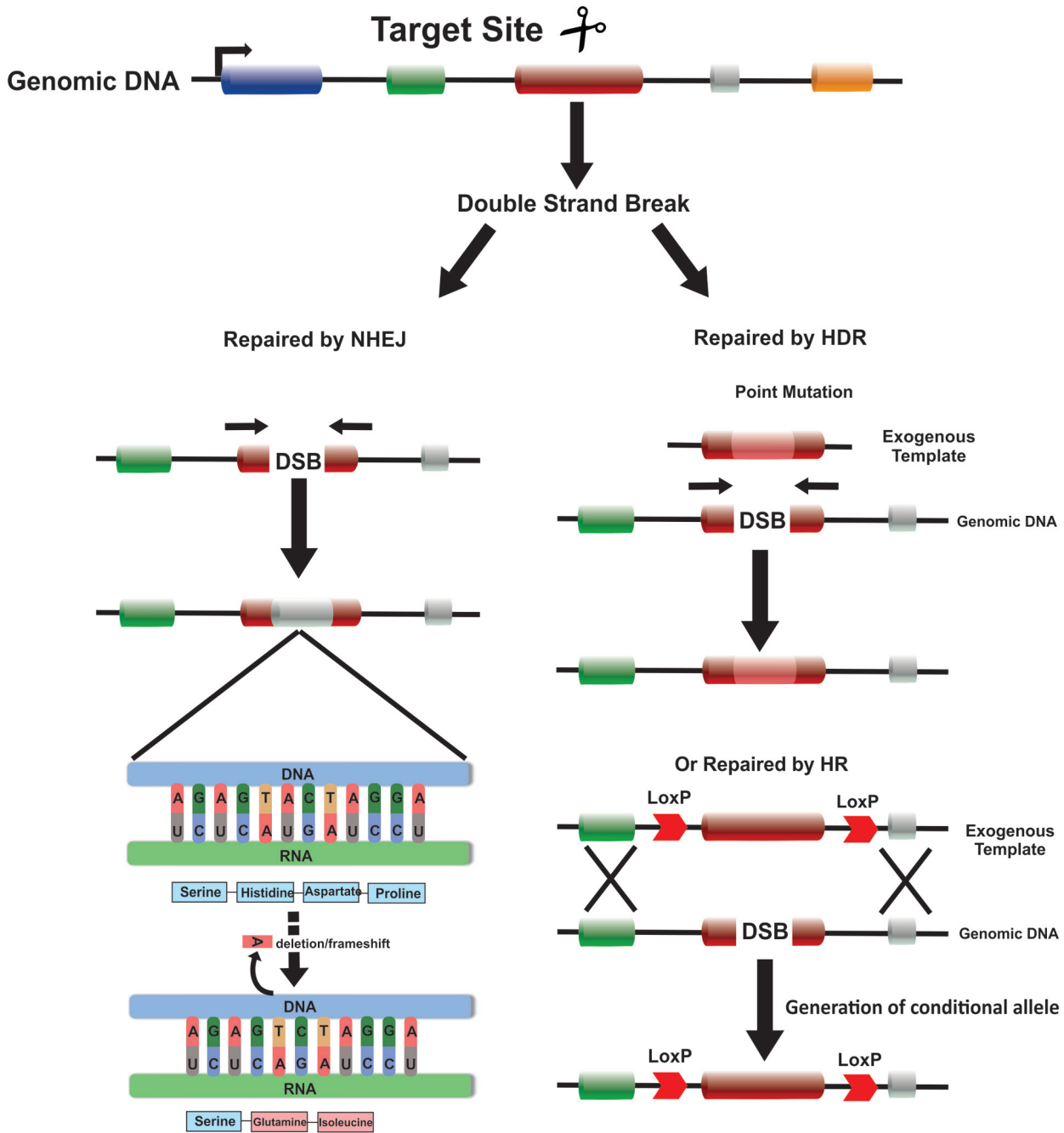


Figure 3: Nuclease-induced genome editing: modes and applications: Custom target nucleases (ZFNs, TALENs and CRISPR/Cas9) stimulate DSBs at the genomic region of interest. The cell can choose between different DNA repair pathways: non-homologous end joining (NHEJ), homology-directed repair (HDR) or homologous recombination. In the absence of a homology repair template, the endogenous repair machinery may introduce InDels (insertions/deletions) via error-prone NHEJ, which can result in a frameshift downstream of the DSB. To produce animals with defined editing, an exogenous template carrying a modified sequence (e.g. point mutation or *LoxP* sites) flanked by homology arms is used to trigger either HDR or HR.

of human coding-genes and microRNAs were recently developed (Kim et al. 2013a, b).

The extent of off-target effects of ZFNs and TALENs was shown to vary between experimental designs (Gabriel et al. 2011). Generally, when using ZFNs and TALENs, it is desirable to give these nucleases a narrow time-window to work within the cell. A common approach is to directly deliver the mRNAs encoding these proteins into the cell, in order to quickly and transiently generate the nucleases that will induce sequence-specific DSBs. This avoids unspecific cleavage events that may happen due to prolonged nuclease presence (e.g. integrated transgenes coding ZFNs or TALENs). Another option for transient expression is the use of non-integrating viral vectors. In addition, a recent report indicated that ZFNs may enter the cells by itself. Although this requires previous purification of each nuclease, it has the advantage of reducing off-target effects due to the rapid degradation of the nuclease inside the cell (Gaj et al. 2012).

By using direct injection of ZFN mRNAs into one-cell stage embryos, the first ZFN-based transgenic mice were generated (Carbery et al. 2010). In this study, either *Mdr1a*, *Jag1* or *Notch3* genes were targeted. Around 20-75% of pups presented insertions or deletions in the coding sequence as a consequence of the activation of the NHEJ pathway. Notably, most of the founder mice generated were mosaic for the genomic alterations, indicating that ZFN activity primarily occurred after first cell division. The authors reported successful transmission of the modifications to the F1 generation. Later, another study co-injected a one-cell stage embryo with ZFN mRNA targeting the *ROSA26* locus and a vector carrying the β -galactosidase gene flanked by *ROSA26* locus homologous sequences (Meyer et al. 2010). This resulted in the integration of the β -galactosidase in the endogenous *ROSA26* locus, providing the first indication that, following fertilization, ZFN-induced DSB may activate distinct DNA repair pathways: either the mutation-inducing NHEJ or the homology-directed repair (HDR) (Fig. 3).

Additionally, the authors reported efficient gene targeting in 1.7 – 4.5% of co-injected mouse zygotes.

Despite the fact that ZFNs emerged earlier than TALENs as a tool for genome editing, the first transgenic mice created using each technology appeared relatively at the same time. This may be the consequence of the technical challenges imposed by ZFN design and suggests that, for future applications, TALENs will be preferred given its easier assembly. The first transgenic mouse created by TALEN-mediated genome editing method was reported in 2013 (Sung et al. 2013). Cytoplasmic injection of TALEN mRNAs targeting either *Pibf1* or *Sepw1* genes in mouse pronuclear-stage embryos resulted in TALEN activity already at the one-cell stage. It was suggested that the presence of mosaicism in a minority of the founders was a consequence of TALEN activity at the late one-cell stage after DNA duplication. Another work reported that the majority of founder mice present mosaicism, indicating that TALENs' activity after first round of cell division may vary (Qiu et al. 2013). These studies provided evidence that following DNA damage induced by TALENs, the fertilized egg may induce the NHEJ repair pathway and that these mutations were transmitted through the germ line. HDR-mediated incorporation of exogenous templates following TALENs-induced DSBs was also reported (Sommer et al. 2014).

Of great relevance for the mouse transgenesis field is the fact that only few strains of mouse ES cells have been successfully cultured, limiting the number of strains used to generate transgenic mice (Kawase et al. 1994). ZFNs and TALENs methods are poised to overcome this limitation allowing the generation of transgenic mice of diverse backgrounds without time-consuming backcrossing. Notably, the generation of mouse knockouts for Y-linked genes using traditional gene targeting methods has been quite unsuccessful, mainly due to Y chromosome structural particularities. In the mouse, most of the knowledge about the *in vivo* function of Y-linked genes has come from spontaneous mutations or

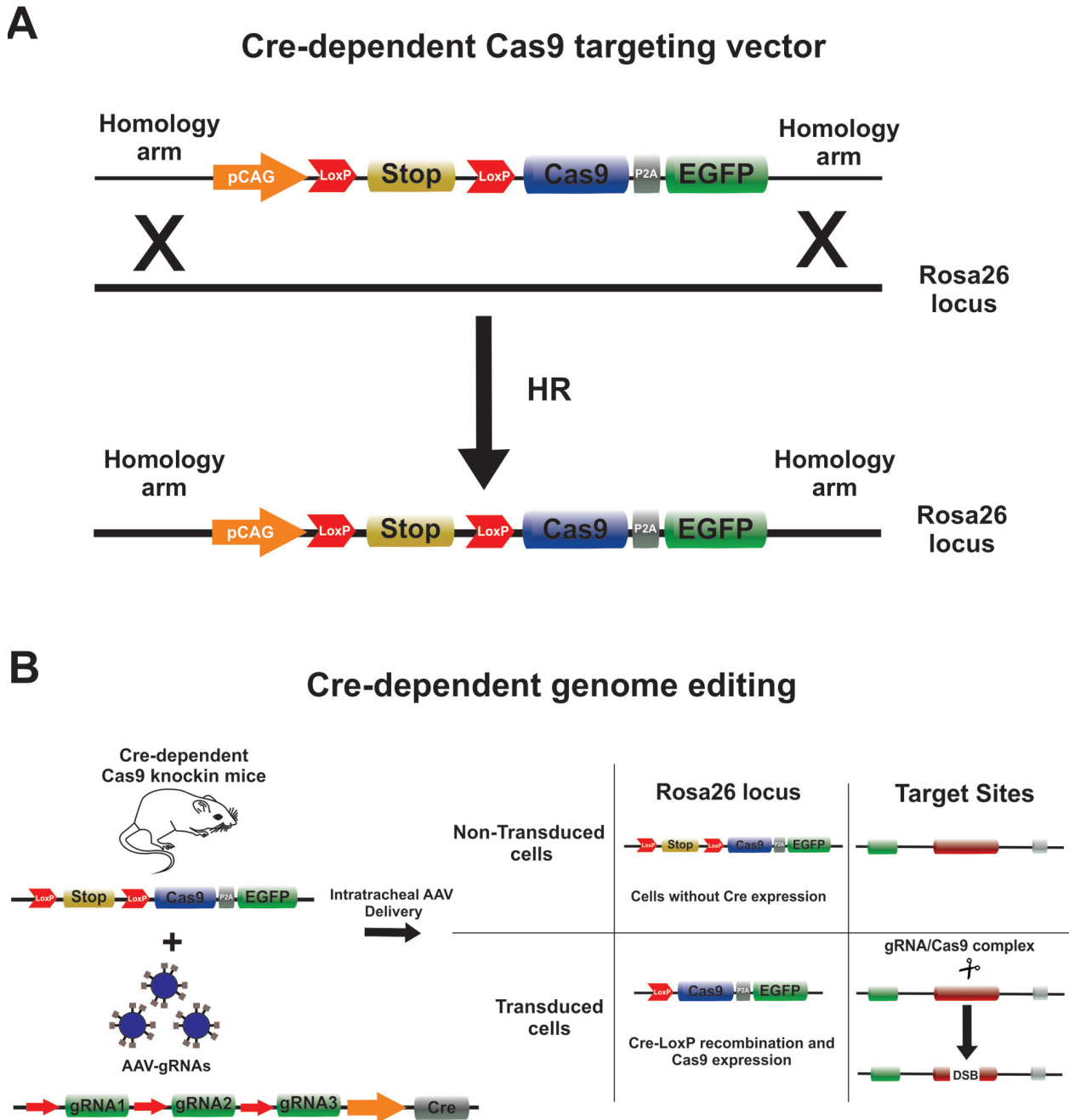


Figure 4: Generation and applications of the Cre-Dependent Cas9 mouse knockin: (A) Illustration of the Cre-dependent Cas9 Rosa26 targeting vector. Exogenous template contained ubiquitous CAG promoter (pCAG), *LoxP*-*Stop*-*LoxP* cassette (LSL), Cas9 nuclease gene, self-cleaving P2A peptide and the reporter gene (EGFP) flanked by two homology arms. The transgene expression cassette was introduced through homologous recombination into the Rosa26 locus. (B) Spatial and temporal control of multiplex CRISPR/Cas9-mediated genome editing. A single AAV vector integrating guide RNAs (gRNA) for three different genes as well as a Cre recombinase expression cassette was delivered into Cre-dependent Cas9 mice. In this experiment, Cre recombinase activity on transduced cells lead to excision of LSL and Cas9 expression. Cas9 nuclease activity directed by gRNAs introduce DSB at the target sites of interest. In contrast, genomic DNA of non-transduced cells remain intact, since Cas9 expression and activity relies on Cre recombinase and gRNAs, respectively.

non-targeted induced mutations in Y-linked *loci* (Lovell-Badge and Robertson 1990, Mazeyrat et al. 2001). TALEN technologies allowed researchers to induce targeted mutations in Y-linked genes (e.g. Sry and Uty) (Wang et al. 2013) and multiplexed genome editing (Li et al. 2014).

Although both ZFNs and TALENs brought advances for transgenic mice generation, drawbacks presented by whole-embryo knockin or knockout mice are still possible. As explained, some cases of embryonic lethality or organ malformation due to non-autonomous effects can be prevented by cell type/tissue-specific gene inactivation. Therefore, applications that combine engineered nucleases with other transgenesis tools, such as the Cre-*LoxP* system, can be used to reach tissue- and spatial-specificity in gene inactivation (Bedell et al. 2012, Brown et al. 2013) (Fig. 4). Another way to restrict the action of the nucleases to specific tissues is through delivery of ZFN and TALEN DNA or mRNA by electroporation as performed in the ascidian *Ciona intestinalis* (Treen et al. 2013).

The potential of ZFNs- and TALENs-based genome editing approaches to generate mouse models of human diseases is only starting to be explored (Perez et al. 2008, Li et al. 2011, Anguela et al. 2013). For instance, in a mouse model of haemophilia B, which carries a coagulation factor IX (F9) mini-gene that phenocopies the human mutation, delivery of ZFNs to the liver of adult mice along with a donor DNA template containing a region of wild-type F9 gene, stimulated gene replacement (Li et al. 2011). This procedure resulted in haemophilia recovery, as indicated by the reestablishment of the normal clotting times. Interestingly, treated animals maintained the functional recovery even following partial hepatectomy, indicating that the recipient quiescent cells replicated normally. Another example of experimental models of liver diseases was the generation of a murine model of hepatocellular carcinoma. In this study, TALENs delivery to the liver of healthy mice induced mutations in genes associated with liver tumorigenesis (Zhang et al.

2014). Delivery of TALEN mRNA to fertilized mouse oocytes carrying a retinal degeneration phenotype linked to the *Crb1* (rd8) allele led to gene correction and generation of phenotypically normal mice (Low et al. 2013). Advances in the mitigation of off-targets effects of both ZFNs and TALENs point to promising advances towards the translation of these technologies to the clinic for a number of diseases (Miller et al. 2007, Szczepek et al. 2007, Doyon et al. 2010, Anguela et al. 2013).

Another envisioned approach to treat human disease using these nucleases was the ZFN-mediated mutation of the *CCR5* gene, which codifies a HIV co-receptor. This resulted in CD4+T cells survival after HIV infection *in vitro* (Perez et al. 2008). When transplanted into the mouse, CD4+T edited cells were able to resist viral infection and populate murine tissues (Holt et al. 2010). Currently, a ZFN-based clinical trial to control AIDS is recruiting patients for a phase 2 study. ZFN-based therapies for diabetic neuropathy and glioblastoma are also in clinical trials. *In vitro* correction of mutations linked to disease, such as sickle-cell anemia (Sebastiano et al. 2011), amyotrophic lateral sclerosis (Kiskinis et al. 2014) and α 1-antitrypsin deficiency (Yusa et al. 2011) have been achieved through ZFN-based methods or as in the case of epidermolysis bullosa (Osborn et al. 2013) through the use of TALEN technology.

RNA-GUIDED NUCLEASES: CRISPR/CAS9

As mentioned, a challenging aspect of the ZFN and TALE protein-guided approaches is the requirement to design new modular DNA-binding proteins for each gene target. It became clear that engineering nucleases guided by alternative modes of DNA recognition would simplify genome editing. At this point, the RNA-guided endonuclease technology CRISPR/Cas9 is established as the less time-consuming method of reduced cost that allows targeting of multiple DNA sites. These features brought the possibility of transgenesis at a system

level one step closer (Cong et al. 2013, Mali et al. 2013).

The development of the Cas9 endonuclease for genome editing became possible due to more than a decade of research on the biological function of the repetitive elements now known as CRISPR (clustered regularly interspaced short palindromic repeats). A study on the chromosomal DNA segment that contains *iap* gene in *E. coli*, an enzyme responsible for isozyme conversion of alkaline phosphatase, reported an unusual series of repeat sequences (direct repeats) interspaced by variable sequences (spacers) (Ishino et al. 1987). Sequencing of other microbial genomes revealed that these interspaced repeat sequences were present across many species (~40% of sequenced bacteria and ~90% of archaea) and these were classified as a unique family of clustered repeat elements (Mojica et al. 2000).

Despite the detailed mapping and annotation of CRISPR loci, their role remained elusive. Some suggested that the spacers descend from foreign genetic elements, such as bacteriophages (Bolotin et al. 2005, Mojica et al. 2005, Pourcel et al. 2005). In 2007, Horvath and colleagues demonstrated that CRISPR spacers dictated target specificity through

base-pair complementarities between nucleic acids, while Cas enzymes were required for the acquisition of new spacer sequences and defense from phage infection (Barrangou et al. 2007). This was the first experimental evidence for the biological significance of a type II CRISPR system as an adaptive immune mechanism. Subsequent CRISPR discoveries unraveled details of each CRISPR system and elucidated the components required for engineering a simple RNA-programmable endonuclease for genome editing.

The type II CRISPR/Cas9 system is composed of an array of small CRISPR RNAs (crRNAs), auxiliary trans-activating crRNA (tracrRNA) and a significantly reduced number of nuclease associated with the CRISPR locus (CRISPR associated - Cas). Cas9 is the only enzyme within the *cas* gene cluster that mediates DNA cleavage (Garneau et al. 2010). TracrRNA hybridizes with crRNA, this dual RNA hybrid, together with Cas9 and endogenous RNase III, is required for processing the CRISPR array transcript into mature crRNAs (Deltcheva et al. 2011). The small crRNAs containing individual spacers require an adjoining NGG sequence known as the protospacer adjacent motif (PAM)

TABLE I
Comparison of ZFN, TALEN and CRISPR/Cas9 engineered nucleases.

	ZFN	TALEN	CRISPR/Cas9
Recognition mode	Protein-DNA	Protein-DNA	RNA-DNA
Recognition site	9 – 18 bp x 2	10 – 20 bp x 2	20 bp
Nuclease type	FokI	FokI	Cas9
Size	~ 1 kb x 2	~ 3 kb x 2	~ 4.2 kb (Cas9) + 0.1 kb (gRNA)
Permissive mismatches	Not known	1 *	3
Multiplex targeting	Very Limited	Limited	Possible (e. g. 8 alleles)
Sensitivity to DNA methylation	Sensitive	Sensitive **	Non-Sensitive

* reported to greatly reduce cleavage activity of the TALEN (Meckler et al. 2013).

** can be overcome since TALEs recognize methylated cytosines as thymines in the major groove.

site for Cas9 recognition (Deveau et al. 2008) (Fig. 2C).

By 2013, various groups began to harness the CRISPR system for genome edition (Cong et al. 2013, Mali et al. 2013). The endogenous bacterial crRNA and tracrRNA were engineered into a single chimeric guide RNA (gRNA) transcript that combines the targeting specificity of the crRNA with the scaffolding properties of the tracrRNA. When the gRNA and the Cas9 were expressed in a cell, the Cas9 HNH and RuvC-like nuclease domains cleave the complementary and non-complementary strands (Fig. 2C). The DSB elicit an error-prone DNA repair pathway (Jinek et al. 2012). The guide sequence within these CRISPR RNAs, that typically derives from phage sequences (Barrangou et al. 2007), can be easily replaced by a sequence of interest to retarget the Cas9 nuclease. The use of a small gRNA to target DNA dramatically facilitated site-specific genomic modifications.

As expected, one major concern when using CRISPR/Cas9 system is the occurrence of high off-target mutagenesis, since gRNA-DNA target binding tolerate up to three mismatches (Cradick et al. 2013, Pattanayak et al. 2013). In order to improve specificity without compromising on-target cleavage efficiency, Cas9 was converted to a nicking enzyme. Individual nicks in the genome are repaired with high fidelity, and simultaneous nicking by using a pair of gRNAs is necessary to effectively introduce targeted DSBs. In this case, the increase in the number of specifically recognized bases in the target site minimized the off-target activity (Ren et al. 2013). Specificity can be further tuned by titration of CRISPR/Cas9 components and reduction of the regions responsible for target complementarity (spacer region) (Hsu et al. 2013, Fu et al. 2014). Truncated gRNAs composed by shorter spacer regions (<20 nucleotides) display less non-specific cleavage activity mutagenesis without compromising on-target activity (Fu et al. 2014). Even without undesired DSBs, a screening process is required, since the InDels induced by NHEJ repair pathway are still random.

The CRISPR/Cas9 technology was rapidly applied for gene modification in a variety of experimental model systems (*Caenorhabditis elegans* (Dickinson et al. 2013), *Drosophila melanogaster* (Gratz et al. 2013), *Arabidopsis thaliana* (Jiang et al. 2013), *Rattus norvegicus* (Ma et al. 2013), *Danio rerio* (Hwang et al. 2013), and *Macaca fascicularis* (Niu et al. 2014) due to advantages over other genome editing methods (Table I) (Meckler et al. 2013). Cas9 and gRNA can be microinjected into embryos through the use of plasmid expression vectors or *in vitro* transcribed RNA (Hsu et al. 2013, Fei et al. 2014, Fujihara and Ikawa 2014, Niu et al. 2014). However, generation of knockin lines expressing Cas9 nuclease, as done for mice and *Drosophila*, provides a straightforward method to optimize efficiency of mutagenesis and recovery of injected animals (Ren et al. 2013, Platt et al. 2014).

Multiplexed gene editing at unprecedented scale through a battery of short gRNAs rather than a library of large proteins is a major advantage of this approach (Cong et al. 2013, Jao et al. 2013, Mali et al. 2013, Wang et al. 2013, Ma et al. 2014, Ma et al. 2014, Xing et al. 2014). For example, simultaneous disruption of functionally redundant genes from the same gene family (Tet1, 2, 3, Sry, Uty - 8 alleles) was accomplished in mouse ES cells. These results showed that out of 96 clones screened, 10% carried mutations in all eight alleles of the five genes (Wang et al. 2013). Thus, CRISPR/Cas9 has the potential to circumvent the need of sequential recombination and intercrossing of lines to generate animal models carrying mutations in multiple genes.

As described for ZFNs and TALEN (Bedell et al. 2012, Brown et al. 2013), conditional gene modification that allows temporal and spatial control of gene inactivation (Cre-Lox system) was also achieved using Cas9-triggered HR (Yang et al. 2013, Lee and Lloyd 2014, Ma et al. 2014). For example, to generate rats with LoxP-containing alleles, Cas9 and a single gRNA designed to target (*cytosine-5*)-methyltransferase 1 (*Dnmt1*) were injected with an

exogenous template containing the *LoxP* sites and homologous sequences flanking the DSB (Fig. 4). A circular donor vector containing exon 1 flanked by 2 *LoxP* sites and 2 homology arms of ~800 bp each was used as a template to repair the DSB by HR and produce animals with targeted integration (Ma et al. 2014). Two out of 12 founder rats contained floxed exon 1 on the same allele and one of them presented a biallelic modification. Other four rats only carried NHEJ-mediated mutations (Ma et al. 2014). Moreover, because the characterization of gene expression relies on the availability of high-quality antibodies, Cas9-triggered HR can also be explored to introduce tags or reporter transgenes to endogenous locus (Yang et al. 2013, Krentz et al. 2014). This technique has proven efficient to create both reporter and conditional lines in a single step.

Apart from the fact that the generation of transgenic strains can take several months, delivery of Cas9 nuclease and gRNAs into developing and adult organisms allows phenotyping of controlled perturbation in the 'F0' (Liu et al. 2014, Platt et al. 2014, Stolfi et al. 2014, Straub et al. 2014, Wang et al. 2014). CRISPR/Cas9-mediated knockout is more efficient than controlled silencing of targeted mRNA via RNA interference (RNAi) or morpholino (Harrison et al. 2014, Schulte-Merker and Stainier 2014). Moreover, it enables targeting of non-coding regions, such as promoters, enhancers, splice site sequences and mRNA 5' and 3' untranslated regions. For example, Wang and collaborators interrogated whether a putative enhancer (B108) is required for *Blimp1* expression during retinogenesis through *in vivo* electroporation of a vector plasmid encoding both Cas9 and a gRNA. The transcription factor *Blimp1* is a key component of the gene regulatory network that determines whether a retinal progenitor cell becomes a rod photoreceptor or a bipolar interneuron. Deletion of the genomic B108 enhancer by CRISPR/Cas9 recapitulated *Blimp1* conditional knockout phenotype with a relative increase in the proportion of bipolar cells, demonstrating that B108 is a cis-regulatory module necessary for proper *Blimp1* expression

in the developing retina. More importantly, they determined that CRISPR/Cas9 effectively allows genomic edition in heterogeneous somatic tissues *in vivo* (Wang et al. 2014).

Development of plasmids or mouse lines with Cas9 expression under the control of tissue-specific promoters/enhancers will further increase the spatial and temporal control over genome editing (Liu et al. 2014, Xue et al. 2014). In mice, the establishment of a Cre-dependent Cas9 knockin strain facilitates *in vivo* applications to somatic tissues (Platt et al. 2014). In brief, ES cells were transfected with a targeting vector containing a constitutive promoter, a *LoxP*-stop-*LoxP* (LSL) cassette and the Cas9 coding sequence linked via P2A peptide to an EGFP. Therefore, Cas9 expression depended on the excision of the LSL cassette by Cre-mediated recombination (Fig. 4A). ES cells that incorporated the transgene cassette by homologous recombination were subsequently implanted in blastocysts using the standard procedures to generate the Cre-dependent Cas9 knockin mice (Platt et al. 2014). This strategy combines the tissue-specificity driven by Cre recombinase with Cas9 genome editing capabilities, what allows modeling of multigenic human diseases. Platt and colleagues used a single AAV-vector composed of three distinct gRNAs and Cre recombinase coding sequence to target three frequently mutated genes in lung cancer (p53, *Lkb1*, and K-Ras) (Fig. 4B). The viruses were introduced directly into the trachea of Cre-dependent Cas9 mice. Cre recombinase activity on transduced cells lead to excision of the LSL and induction of Cas9 expression. Then, Cas9 nuclease activity directed by gRNAs introduced DSB at the three target sites of interest (Fig. 4B). In contrast, genomic DNA of non-transduced cells remained intact, since Cas9 expression was not induced due to the absence of Cre-mediated recombination of the LSL (Fig. 4B). In this study, Cas9-induced mutations in p53, *Lkb1*, and K-Ras were sufficient to induce multiple tumor nodules in the lung. This was an excellent example that the traditional methods of transgenesis (HR), the

Cre-LoxP system and genome edition by nucleases are not mutually exclusive. In fact, they can be combined to achieve state-of-the-art functional analysis of multiple genomic regions.

The described revolution in genomic editing possibilities offered new ways to expand the range of studied organisms, including species where gene targeting was not yet available (Daimon et al. 2013, Flowers et al. 2014, Guo et al. 2014, Niu et al. 2014). For instance, CRISPR/Cas9 technology allowed functional characterization of the regenerative capacity of salamanders, the only tetrapod that regenerates the spinal cord and all cell types of the limb at an adult stage. Researchers addressed for the first time the regeneration phenotype in the axolotl by deleting a key neural stem gene (Sox2) (Fei et al. 2014). Although Sox2-CRISPR RNAs injection into single-cell fertilized eggs had no effect on spinal cord organization, upon tail amputation, cell proliferation did not increase in the spinal cord, preventing the regrowth of this tissue in the regenerating tail (Fei et al. 2014). These studies in salamanders may shed light on the limited regenerative abilities of other organisms.

CRISPR-Cas9 methods also have a potential utility to either treat or prevent human genetic disorders (Schwank et al. 2013, Li et al. 2014, Long et al. 2014, Wu et al. 2014, Yin et al. 2014). As proof of concept, this system was deployed *in vivo* to correct a homozygous point mutation of fumarylacetoacetate hydrolase (Fah) in a mouse model of hereditary tyrosinemia type I (HTI) (Yin et al. 2014). FAH deficiency causes accumulation of toxic metabolites, such as fumarylacetoacetate in hepatocytes, resulting in severe liver damage. In adult mice, hydrodynamic tail vein injection of CRISPR-Cas9 system components and a short DNA repair template for gene correction resulted in initial expression of the wild-type Fah protein in ~1/250 liver cells. Due to survival advantage, Fah-positive hepatocytes expanded and rescued the body weight phenotype of the adult mice (Yin et al. 2014). Therapeutic strategies through genome editing look promising; however, safety issues

cannot be underappreciated. One must consider possible immune responses against the bacterial nuclease as well as off-target nuclease activities.

Cas9 targeting has become a versatile tool for engineering biology due to its high efficiency as a site-specific nuclease and the possibility for multiplexed modifications. So far, CRISPR/Cas9 system has broadened the repertoire of target sites and biological applications from basic research to biotechnology.

CONCLUSIONS

ADVANCES IN THE GENERATION OF TRANSGENIC MICE

In the past, most methods employed for genetic alterations were restricted to model organisms, such as mice, whose ES cells could be manipulated at endogenous genomic loci via homologous recombination (HR). Historically, the low frequency of desired recombination events (1 in 10^6 – 10^9 cells), characteristic of traditional transgenesis methods (Capecchi 1989, Capecchi 2005), presented considerable pitfalls for systems level applications (e.g. targeting multiple genetic pathways). For instance, more than 15,000 *Drosophila* genes and 30,000 mouse genes have been annotated (Adams et al. 2000, Waterston et al. 2002) and several loss-of-function mutants have been generated, however many genes still lack functional characterization (Bradley et al. 2012, Koscielny et al. 2014). Traditional methods allowed great advances in understanding gene function *in vivo*, but the generation of transgenic animals is a laborious and time-consuming process. Engineered nucleases are turning this task much more precise and efficient, by allowing direct genetic modification of target loci, increasing the rate of site specific DSBs and thus improving transgenesis by homologous recombination. Curiously, somehow the modern methods resemble the first attempts to generate transgenic mice, since genetic manipulation occurs directly in the zygote, eliminating the need of ES cell culture and selection steps.

COMPARING THE NUCLEASES

A comparison of the features of ZFNs, TALENs and CRISPR/Cas9 is summarized in Table I. In general, the RNA-guided nuclease CRISPR/Cas9 is advantageous over protein-guided nucleases. The design process is easier, as CRISPR/Cas9 does not require different proteins for each target and eliminates laborious cloning steps. Moreover, CRISPR/Cas9 is much more amenable for multiplex applications and does not present sensitivity to DNA methylation, being the method of choice if one plans to target a GC-rich region. Although considerable efforts into increasing the specificity of these nucleases have been made (Miller et al. 2007, Szczepek et al. 2007, Doyon et al. 2010), additional work is required to precisely detect the off-target events (Gabriel et al. 2011). New methodologies to map off-target activity are arising, such as spontaneous integration of Integrase-Defective Lentiviral Vectors (IDLV) in DSBs (Kim et al. 2013, Wang et al. 2015) and targeted sequencing of translocated DSB regions (Frock et al. 2015). Regarding specificity, TALENs might be considered a favorite, since reports indicate that one mismatch in the binding site may impair TALE binding. In addition, to avoid DNA cleavage in undesired locations, TALENs have been engineered to prevent homodimerization and Cas9 to work as a nicking enzyme.

ADVANCES IN NON-MODEL ORGANISMS

The study of genome function in non-model animal species has been widely hampered by the lack of an efficient and stable system to promote genetic loss-of-function. Although, in principle, homologous recombination could be used to modify the genome of any animal species, ES cell targeting would be required to generate whole-animal genetic modifications. Challenges in establishing ES cell culture for a wide range of models hindered the production of transgenic non-model animals. RNAi technology brought substantial advances to understanding and interfering with regulatory

networks in non-model species. However, RNAi often produces multiple off-target effects and provides transient loss-of-function, making it difficult to analyze long-term effects of gene inactivation. Also, RNAi is a gene-oriented technology, not allowing targeting of genomic regulatory regions. Engineered nucleases offer a remarkable opportunity to study the function of genomic loci in non-model organisms. The current vision is to turn any organism into a genetic trackable model, the same way the scientific community has been able to easily engineer *S. cerevisiae* and *E. coli*.

ADVANCES IN SPATIAL AND TEMPORAL CONTROL

Although a big number of SSR enzyme lines have been developed, one of the limitations for their use is the relatively restricted number of cell type specific expression transgenes available. Recent developments on GFP binding proteins may improve *in vivo* manipulation of SSR enzymes. Cepko and colleagues established a system in which the activation of a transgene expression depends on the presence of GFP. In brief, two different proteins recognize distinct portions of GFP: one fused to a DNA binding domain and the other to the transcriptional activator p65. When GFP brings these proteins together, gene expression is triggered. Using this technique, authors demonstrated that Cre expression can be restricted to GFP expressing cells (Tang et al. 2013). This methodology can bypass the need to generate new cell/tissue-specific Cre lines, given the huge number of cell type-specific GFP lines already available. Other possible application is the cell type specific control of ZFN, TALEN or Cas9 expression.

EPIGENOME EDITION

Some of the exciting possibilities offered by the advances in nuclease engineering might go beyond genome editing applications. The fusion of DNA binding modules (zinc-finger proteins or TALEs) to different effector domains points

to a new era of synthetic biology in complex systems. One of the most astounding possibilities is to “edit the epigenome” by, for example, introducing or removing specific postranslational histone modifications and DNA methylation at target loci (Maeder et al. 2013, Mendenhall et al. 2013). TALEs fused to a light-responsive domain were also used to control gene expression using optogenetics in both primary neurons *in vitro* and in the mouse brain *in vivo* (Koneremann et al. 2013). Another novel application derived from TALENs is the visualization of chromatin dynamics through live imaging, using a TALE domain fused to a monomeric form of GFP (Miyanari et al. 2013). Furthermore, the CRISPR/Cas9 system has also been engineered to allow flexible recruitment of desired perturbations, such as transcriptional activation, to a locus of interest (Xu and Bestor 1997, Beerli et al. 2000, Koneremann et al. 2013, Maeder et al. 2013, Mendenhall et al. 2013).

RESUMO

Tecnologias de modificação do genoma são ferramentas poderosas para a biologia molecular e áreas afins. Avanços na transgênese animal e em tecnologias de edição do genoma durante as últimas três décadas permitiram interrogar de modo sistemático a função de genes, o que pode ajudar a compreender como o genoma influencia a fisiologia celular. A engenharia genética através de recombinação homóloga (HR) tem sido o método padrão para modificar sequências genômicas. No entanto, métodos de edição do genoma por nucleases que foram desenvolvidos recentemente, como ZFN, TALEN e CRISPR/Cas, trouxeram novas perspectivas para pesquisa biomédica. Aqui, apresentamos uma breve perspectiva histórica de métodos de modificação do genoma com foco em modelos de camundongos transgênicos. Além disso, descrevemos como novas técnicas foram descobertas e aperfeiçoadas, apresentamos as mudanças de paradigma e discutimos suas limitações e aplicações para a investigação biomédica, bem como possíveis direções futuras.

Palavras-chave: Recombinação homóloga, Reparo de DNA, Cre-LoxP, ZFN, TALEN, CRISPR/Cas9.

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