History

Animal models of diseases

Animal models, both vertebrates and invertebrates have been instrumental for dissecting the pathophysiology of human diseases. Despite divergent opinions over their use, animal models remain the most powerful tools to understand the mechanisms underlying physiological processes, and their pathological counterparts. They are also invaluable tools to search for disease modifiers and to develop and test novel treatment strategies. Although each model has intrinsic limitations, the use of animals as an entire systemic model is vital to biomedical research because they address metabolic and physiologic processes, which cannot be studied in isolated tissue culture.

The establishment of Mendelian genetics in the middle of the 19th century, and the later discovery of the structure of the DNA by Watson and Crick [1] were the basis of modern genetics that utilize the sequencing of the entire human genome to identify genes implicated in different diseases. A further application of modern genetics is recombinant DNA technology, resulting in the generation of the first transgenic animal (mouse) over three decades ago [2]. Since then, transgenesis, the artificial modification of an organism’s genome, has been extensively used to identify the role of genes in the occurrence of diseases. One of the key achievements in manipulating the genome was reached when Mario Capecchi successfully disrupted a single gene in the mouse, opening the era of gene targeting [3].

To date, the mouse remains the species most commonly used for genetic manipulation. Nonetheless, the recent advances of new technologies such as endonucleases designed to target and cleave specific DNA sequences have emerged as alternative methods to accelerate the process of genome editing, and apply it to virtually any mammalian species.

Engineered endonucleases

During the last decade, the development of ES-Cell free methods for genomic modifications simplified and accelerated drastically the process of gene manipulation. It also revived microinjection as the favorite method for producing precise (targeted) manipulations in the mammalian genome. The first generation of these engineered endonucleases consisted of three types of protein-based molecular scissors: Zinc Finger Nucleases (ZFN), Transcription activator-like effector nucleases (TALEN), and Meganucleases (MN). Although these types of nucleases can be discriminated by their recognition sequences or their modular assembly (Meganucleases having the longest recognition sequence), they all rely on the interaction of a defined sequence of the genomic DNA with protein recognition elements. All three classes of nucleases have been successfully applied to mouse transgenesis by direct oocyte microinjection [4-8]. However, these proteins remain quite complex to design and assemble [9,10], and the overall process can be cumbersome and time consuming.

Recently, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system associated to the Cas9 endonuclease (CRISPR/Cas9) superseded its predecessors [11]. Contrarily to the previous systems, the CRISPR system relies on the hybridization of the genomic DNA with a short complimentary RNA sequence. In particular, CRISPR being a RNA-guided endonuclease (RGEN) system, it guaranteed an unprecedented ease of design and seamless synthesis (discussed herein), and contributed to the recent advent of the CRISPR/Cas9 for genome editing [12].

Identification and description of a bacterial immune system

In 1987, an odd sequence repeat has been identified in the iap gene of the bacterium Escherichia coli [13]. Subsequently, it took researchers over a decade to realize that these bacterial sequence repeats were indeed part of the bacterial immunity mechanism [14]. Specifically, the type II CRISPR/Cas system constitutes a defense mechanism of the bacterium Streptococcus pyogenes to detect and destroy invading bacteriophages. This system relies on complexes made of three components: the Cas9 nuclease, guided by the CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA). In 2012, the mechanisms underlying the efficient targeting and cleavage of a specific DNA sequence were identified, and it has been shown that the crRNA and tracrRNA could be fused to a chimeric "single-guide" RNA (sgRNA), making up a two-component system sufficient for genome editing in vitro [15]. The short genomic
target sequence consists of twenty base pairs (bp) complimentary to
the sgRNA, and immediately upstream of a protospacer adjacent motif
(PAM). The PAM is a three-nucleotide sequence of the form NGG
(where N represents any nucleotide followed by two Guanines). The
PAM is the only limiting factor when choosing a targeted sequence in a
given genome. However, such target sites can be found on average every
8–12 bp in the human genome [16]. The second targeting limitations
of the CRISPR system are the intrinsic restrictions of the U6 or T7
promoters used in CRISPR tools that require a G or GG (respectively)
at the 5’ end of the sgRNA for efficient transcription [17]. Nonetheless,
these restrictions now tend to be completely ignored without many
problems when researchers perform CRISPR-based gene editing.
Consequently, the CRISPR system is far less restrictive than the repeat
variable diresidues (RVD) cipher of the TALENs [18].

Harnessing CRISPR for genome editing

A milestone has been achieved when simultaneous reports showed
that the CRISPR system could be harnessed in vivo [19,20]. In mice, the
CRISPR technology has effectively targeted several genes at the same
time (multiplex) and can induce either gene Knock-Out (KO) via the
Non Homologous End Joining (NHEJ) pathway, or induce gene Knock-
In (KI) upon Homologous Recombination (HR) of a “donor” template
(plasmid or single strand oligonucleotides). Since then, the CRISPR
system has pushed in all types of genetic manipulations, and genome
editing has been achieved in an unprecedented number of species
(animals and plants) including rats, rabbits, pigs, zebrafish, and even
axolotls [21] or Rhesus monkeys [22]. To the best of our knowledge,
there is no report to date of any species resistant to CRISPR editing.
Ultimately, research on human cell lines [23], human stem cells [24],
or human iP cells [25] using CRISPR will tremendously speed up
direct applications such as regenerative medicine. Nonetheless, such
ubiquitous and unreviled efficiency recently raised alarm over ethical
consequences underlying genetic engineering in humans [26].

Genome editing in mice using CRISPR

For more than twenty years, ES-cell injection into blastocysts was
the predominant way of editing the mouse genome. The main drawbacks of
this method are the variable availability and potency of ES-cell lines,
the time to obtain chimeras, and the inefficiency of transmission upon
breeding of the chimeras. Despite sensible improvements over the
years [27,28] this lengthy process may become obsolete. As previously
mentioned, the direct injection of nucleases into the one-cell embryos
has repositioned microinjection at the forefront of genome editing in
mice.

Speed, precision, efficiency

Although the production of CRISPR edited mice via direct injection
of CRISPR components is a very recent technique (the first report was
published less than two years from the date of the present review), the
plethora of publications [29-36] argue in favor of an incredibly fast
pace. In average, from the design of the sgRNAs to the screening of
genetically modified mice, the process takes about eight weeks, and
most facilities and transgenic cores now produce modified mice within
two to four months.

In the early days of CRISPR, potential off-target effects of the
CRISPR system (Cas9 tolerates mismatches, especially in the 5’
upstream region of the target site) have been documented [16,37].
The system has subsequently been fine tuned for increased specificity.
The first modification consisted in engineering a “nickase” form of the
Cas9 (referred to as Cas9-D10A). This mutated version of
the nuclease cuts only one strand of DNA, and the double nicking
approach greatly improved specificity both in human and mouse cells
[38,39]. Furthermore, it has been shown that truncated guides could
also increase specificity [40]. Finally, an attractive approach consisted in
coupling sgRNAs to the FokI endonuclease, which induces DNA
cleavage only upon dimerization, thus doubling the length of the
recognition sequence [41,42]. It is important to note here that off-target
effects in mice are not as critical as in human since they can easily be
outcrossed by way of breeding scheme.

The efficacy of the nucleases is generally dependent on chromatin
accessibility and epigenetic mechanisms such as DNA methylation
or histone modifications [43]. Nonetheless, several reports tend to
prove that CRISPR is a much more efficient system than any other
programmable endonuclease [44]. Several genes that failed to be edited
using other nucleases were successfully targeted using CRISPR, and
successful targeting of both alleles is much more efficient using
CRISPR [34].

Seamless synthesis

There are many protocols to generate the two necessary components (Cas9 and sgRNA) for genome editing in mice [45]. Some of
these protocols detail the complete procedure, from design of the
target sequence to the identification of founders [46]. In short, there
are few different ways of producing the readily available reagents
for microinjection. One such fast method consists in cloning the desired
20bp sequence into a dual expression plasmid (e.g px330, Addgene
#42230) expressing both sgRNA and Cas9. The direct injection of this
circular plasmid into the pronucleus of fertilized eggs is a fast method
to obtain KO mice [47]. However, this method is limited by the time
required for cloning (sequencing is necessary for quality control) and
the relative inefficiency of the expression vector to edit both alleles.

Traditionally, the injection mixture contains both sgRNA and
Cas9 mRNA, rather than DNA. The in vitro transcription (IVT) of
these two RNAs is then necessary, and they can both be generated
using the same expression plasmid (e.g. px330). However, the Cas9
mRNA is slightly more difficult to synthesize because of its size and
the polyA capping. An easy way to get fully validated Cas9 mRNA is
to buy it from a commercial provider, as microinjection requires very
low concentration of Cas9 reagent. Consequently, transgenic cores may
obtain few micrograms of Cas9 mRNA, a very cheap investment that
can last several months or up to a year.

This leaves the transgenic facilities with only one reagent to produce:
the guide RNA. This is classically achieved by cloning the 20bp sequence
into an expression vector, and then using this plasmid as template for
IVT. Yet, the simplest and fastest way of producing several sgRNAs
is the PCR-based “non-cloning” method. This method becomes very
popular to create transgenic mice [48], as it takes a technician only one
day to synthesize multiple sgRNAs (Figure 1).

The entire procedure can be divided into three steps.

It starts with the identification of guide sequences. This step is
computerized and it takes only few minutes to get multiple guides
(assessed against off-target likelihood) using one of several freely
accessible computational tools (e.g. http://crispr.mit.edu). Some of
these tools have been optimized and take genomic context (such as
CpG islands) into account [49].

The second step is the synthesis of a linearized DNA template
generated by PCR using a High Fidelity enzyme (e.g. Plusion
polymerase or equivalent). The Forward primer is of the form 5’TTA
Figure 1: Flowchart of the “non-cloning” strategy to synthesize small guide RNAs for CRISPR genome editing in mice. Design and selection of suitable guides are performed using freely accessible computer tools (step 1). The use of a Forward primer containing the T7 minimal sequence (orange), the guide sequence selected in step 1 (green), and a sequence complimentary to the sgRNA scaffold (red) allows the synthesis of a suitable DNA template without any cloning step (step 2). Finally, in vitro transcription (IVT) is performed using a commercially available kit following manufacturer’s recommendations (step 3).
Outstanding Challenges

The mechanisms underlying the targeting and cleavage activities of the CRISPR system are not completely understood. Several studies of the mode of action provided valuable information that might help improving the system [59-62].

Finally, the physical form of the CRISPR reagents (DNA, RNA, or protein) used for microinjection in mice might also be critical. Since mouse genome editing is more powerful using the mRNA form than the DNA one, it is possible that direct injection of the Cas9 ribonucleoprotein might also improve the efficiency, as it has been successfully applied to human cell lines [63,64].

Mode of delivery

CRISPR is considered a “disruptive” technology, which is a technological innovation that creates a paradigm shift. In the mouse community, it indeed created several shifts on the way scientists create genetically modified mice.

Interestingly, early reports of factors influencing the outcome of microinjection in mice showed that cytoplasmic transgenesis, although quite inefficient, could still be successful [65].

Because the CRISPR components are made of mRNA, it makes sense to target the cytoplasm when microinjecting the mouse oocytes (even when containing a DNA donor, successful cytoplasmic injections have been reported [20]). The first reports of CRISPR edited mice used a piezo-assisted method for injecting into the cytoplasm of the oocytes [66]. However, non-assisted injection is also possible and the efficiency seems overall higher using cytoplasmic injection over pronuclear injection [67]. There are an increasing number of publications using cytoplasmic injection in mice, where the oocytes can accommodate very high concentration of reagents with no obvious toxicity. Another advantage of cytoplasmic injections is that the fertilized eggs do not need to have apparent pronuclei to be injected, thus allowing injection in a wider range of oocytes, eventually reducing the number of mice superovulated, and offering more flexibility in the timing of hormonal stimulation. In our hands, switching from pronuclear to cytoplasmic did not result in any pregnancy. However, this problem (probably due to the physical characteristics of the micropipettes once pulled) has easily been overcome in our laboratory by a short pre-incubation (five minutes) of the eggs with cytoskeletal inhibitors (e.g. Cytochalasin B - Sigma C6762 – 5 ug/ml), known for increasing the survival rate [68].

Another interesting change in the way investigators perform CRISPR genome editing in mice is the fact that the efficiency of the sgRNA tends not to be pre-assessed anymore. In the first publications, the investigators used to test their guides in vitro, using mouse cell lines (e.g. N2a or NIH3T3). There might be several reasons explaining why this step became less popular. First, this procedure is time-consuming and requires a lot of manpower. Secondly, high throughput experiments showed that only a very small proportion of the guides are inactive [69,70]. Additionally, the degree of activity of a given guide in vitro does not always correlate completely with the degree of activity in vivo. For that reason, the activity of the sgRNA is sometimes assessed directly by in vivo assay [71].

Since the mode of delivery of the CRISPR reagents to the oocytes is critical, we anticipate that alternative methods to microinjection [72,73] might ultimately change completely the way genetically modified mice are produced.

Concluding remarks

The flexibility and adaptability of the CRISPR–Cas9 system offer vast potential for genome manipulations. Despite controversy over the discovery of CRISPR genome engineering and patent disputes
transgenic facilities around the world now offer this service. In mice, work is ongoing to elucidate discrete mechanisms inherent to the editing capacities of CRISPR elements. For instance, mosaicism following CRISPR injection has not been explored thoroughly [75].

New strategies for controlling the outcome of DNA cleavage are on their way. For example, when creating KO mice, the repair mechanisms of the oocyte following NHEJ generally create small deletions (indels) of random sizes. Conversely, the coinduction of two guides targeting two close sequences in opposite orientations allows efficient excision of a piece of DNA of a predefined size [76], which is convenient for genotyping of the progeny. Likewise, the efficiency of homologous recombination and the characteristics of the donor DNA for successful gene repair are being assessed [76,77]. Besides, the two main assays used to detect induced genomic modifications (e.g. Surveyor and T7E1 assays) lack sensitivity, and other methods are currently developed [78-81].

Collectively, nuclease-based technologies are revolutionizing contemporary molecular genetics, and are particularly applicable to the mouse genome. We anticipate that each of these systems will be thoroughly studied and enhanced [82], allowing researchers to take advantage of each type of nucleases according to their specificity and mechanisms of action. Other technologies based on nucleases [83,84] or not [85] may also appear as fast as the CRISPR/Cas9 system did.

Competing interests statement
The authors declare no competing financial interest.

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