CRISPR/Cas9: a breakthrough in generating mouse models for endocrinologists

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Abstract

CRISPR/Cas9 is a recent development in genome editing which is becoming an indispensable element of the genetic toolbox in mice. It provides outstanding possibilities for targeted modification of the genome, and is often extremely efficient. There are currently two main limitations to in ovo genome editing in mice: the first is mosaicism, which is frequent in founder mice. The second is the difficulty to evaluate the advent of off-target mutations, which often imposes to wait for germline transmission to ensure genetic segregation between wanted and unwanted genetic mutations. However rapid progresses are made, suggesting that these difficulties can be overcome in the near future.

The CRISPR/Cas9 technology

CRISPR/Cas9 genome editing technology is a genuine technological breakthrough. Based on an immune defense mechanism discovered in bacteria (Terns & Terns 2011), the method was adapted and first used in mammalian cells in 2013 (Cong et al. 2013, Mali et al. 2013b). These initial studies opened a field of very intense investigation and technological development making genome editing very efficient in a number of animal species. The method also raises new hopes for human gene therapy (Xue et al. 2016).

As endocrinologists need animal models for in vivo integrated studies and make an intensive use of genetic manipulations in mice to study physiology and pathology, the advent of CRISPR/Cas9 genome editing is expected to deeply impact the discipline. This review focuses on the achievements and potential of this technology in the field of mouse genetics and the general benefit that it should bring to research in endocrinology.

Principle of CRISPR/Cas9 genome editing

The recent history of mouse genetics is marked by sudden accelerations due to technological revolutions. The first such revolution was the opportunity to generate transgenic mice by DNA microinjection into fertilized oocytes. This sometimes leads to random DNA integration, meaning that neither the integration site nor the number of integrated copies is controlled (Palmiter & Brinster 1985). The second revolution was the possibility to replace the process of random DNA integration by targeted integration, occurring if homologous recombination takes place between an exogenous DNA construct and the cognate genomic sequence. The occurrence of such homologous recombination events is not frequent. This strategy thus implies using embryonic stem (ES) cells, screening for homologous recombination event among a large number of cell clones, and then grafting the appropriate cells into recipient embryos. The chimeric mice born from the grafted embryos can then transmit the DNA modification through their germ line. This lengthy
process makes expression pattern more predictable and stable over generations. It also allows to generate knockout allele by disrupting endogenous gene (Capecchi 2005, Thomas & Capecchi 1987). The third major progress was the advent of the Cre/loxP technology, which permits to generate recombination events only in cells expressing the Cre recombinase and thus restrict the consequences of mutation and transgene expression to selected tissues (Gu et al. 1994, Kuhn et al. 1995, Wilson & Kola 2001). Generating somatic mutations in chosen tissues or cell types sometimes enable to avoid early lethality. Most importantly, for endocrinologists, Cre/loxP allows to uncouple the local consequences of a mutation and its systemic consequences, and provides a much deeper understanding of the gene functions (Davey & MacLean 2006). All these methods are used to integrate reporter constructs, to tag endogenous protein, and most of all to generate gain-of-function or loss-of-function mutations. The technology is difficult to master and poorly efficient, and the creation of a new mouse strain remains a very time-consuming process. Despite these difficulties, the International Mouse Phenotype Consortium (http://www.mousephenotype.org/) has been set to generate thousands of mouse models, mainly knockouts, which are now actively used in many laboratories. However, considering the degree of sophistication achieved in animal models that are cheaper and easier to breed, like Drosophila melanogaster, it is obvious that this systematic effort will never fulfill the growing need for new mouse models. So, even in mice, which have always been at the forefront of animal genetics, any new addition to the genetic toolbox is welcomed.

A promising development is genome editing, in which the genomic sequence is modified without necessarily inserting exogenous DNA. Genome editing was first performed using zinc finger nucleases (Pabo et al. 2001), and then transcriptional activator-like effector nucleases (TALENs) (Richter et al. 2016). Both endonucleases are modular proteins that can be engineered to fit a predefined sequence, by assembling the modules of coding sequences in an expression vector. They can thus recognize a unique genomic sequence and cut double-stranded DNA at a chosen locus. The presence of a double-strand break then activates the cellular machinery for DNA repair which can proceed in two distinct ways, each of them enabling genome editing. The first possibility is repair by nonhomologous end joining (NHEJ), an error-prone mechanism that often produces small insertions and deletions (indels) at the DNA break. The second is homology-directed repair (HDR), in which the cell uses a template to repair the cut DNA, either the remaining intact allele or the exogenous DNA introduced in the cell together with the nuclease. Genome editing can modify the existing genes in a more subtle manner than classical transgenesis, as only few nucleotides are modified.

The CRISPR/Cas9 genome editing method (for CRISPR)/Cas9) is the last addition to the genetic toolbox. The most common version (Ran et al. 2013b) was developed from a bacterial system of adaptive immune defense against bacteriophages present in Streptococcus pyogenes. For this purpose, complete synthesis of the nuclease reading frame was completed, optimizing translation in mammalian cells. Importantly, and unlike the other nucleases used for genome editing, Cas9 uses a short RNA as guide to target a genomic sequence (Fig. 1). This single-guide RNA (sgRNA) of 100 nucleotides (nt) long. Its 5′ end (17–20 nt) is chosen to be complementary to the target DNA strand, on which it anneals. If the 17–20 nt DNA target sequence is immediately followed by the so-called protospacer adjacent motif (PAM) (e.g., 5′-NGG-3′), Cas9 cleaves the target on both strands. The double-strand break, located 3 nt upstream to the PAM, will be repaired by either NHEJ or HDR. NHEJ errors generate random indels, whereas HDR can be used to copy precise modifications present in an exogenous DNA template. The key innovation is that the specificity of Cas9 for a genomic target stems from this sgRNA:DNA complementarity and not from protein structure, which does not have to be modified. Therefore, editing a chosen genomic sequence avoids laborious protein design, and simply involves the synthesis of a short nucleotide sequence to modify the 5′ end of the sgRNA (Table 1). The method is therefore straightforward, versatile, easily multiplexed, and, most of the time, extremely efficient. In our opinion, it already outperforms all the other methods of genetic modification. Although we only review its use to modify the mouse genome, it seems to be equally efficient in cultured cells and in all the plant and animal species where it has been tested (Yu et al. 2013, Xing et al. 2014, Zhu et al. 2014, Shen et al. 2014, Shi et al. 2015).

Possible use of CRISPR/Cas9

The potentiality of CRISPR/Cas9 seems unlimited, and a number of possibilities have already been explored (Fig. 2). Excellent reviews have been published, providing step-by-step procedures and detailed protocols (Ran et al. 2013b, Yang et al. 2014), and DNA constructs for Cas9 expression are freely available (http://addgene.org/). The simplest application of CRISPR/Cas9 is to generate
frameshift mutations in coding sequences, based on NHEJ, to generate null alleles. In ovo NHEJ generates mutations at high rate, often in more than 50% of the resulting pups. It is in many respects a rapid and efficient alternative to gene knockout. There is however a significant difference between a recessive frameshift mutation and a classical knockout, as the former usually leads to the insertion of selection cassettes.

Table 1  Some major landmarks in CRISPR/Cas9 development.

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<tr>
<th>Event</th>
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<tr>
<td>First description of short repeats located downstream of the iap gene in E. coli</td>
<td>1987</td>
<td>Ishino et al. (1987)</td>
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<td>CRISPR, together with associated cas genes, provides resistance against phages</td>
<td>2007</td>
<td>Barrangou et al. (2007)</td>
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<td>Cas9 is a RNA-guided endonuclease</td>
<td>2012</td>
<td>Garneau et al. (2010)</td>
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<td>CRISPR/Cas9 can be used for genome editing in mammalian cells, including human stem cells</td>
<td>2013</td>
<td>Cong et al. (2013), Mali et al. (2013b)</td>
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<td>CRISPR/Cas9 genome editing in mice</td>
<td>2013</td>
<td>Mashiko et al. (2013), Yang et al. (2013a)</td>
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<td>CRISPR/Cas9 genome editing in pigs and monkeys</td>
<td>2013</td>
<td>Niu et al. (2014), Whitworth et al. (2014)</td>
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<td>Cloning-free genome editing</td>
<td>2015</td>
<td>Aida et al. (2015), Hendel et al. (2015)</td>
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<td>RNA-guided nucleases with improved specificity</td>
<td>2015</td>
<td>Kleinstiver et al. (2016), Slaymaker et al. (2015)</td>
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<td>Controversial use of CRISPR/Cas9 genome editing in human embryos</td>
<td>2016</td>
<td>Kang et al. (2016)</td>
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These superfluous DNA sequences sometimes alter the modified locus in an unexpected manner (Rucker et al. 2000). To inactivate genes by NHEJ, one can also use two sgRNAs simultaneously to generate deletions (Vidigal & Ventura 2015), whose size can easily exceed 100 kb (Zhang et al. 2015, Song et al. 2016). Such mutations are easier to detect and provide a better guaranty that gene function is lost. NHEJ can also promote the insertion of exogenous DNA fragments, in a random orientation, if these are provided in large excess (Bachu et al. 2015). Although most experiments are designed to modify single genes, NHEJ has also been used to mutate nine genes simultaneously in fertilized oocytes (Sakurai et al. 2016) or 62 copies of an endogenous viral gene at once in a cell line (Yang et al. 2015). Multiplexing in mouse oocytes seems to be only limited by the necessity to dilute each guide RNA, and the risk of generating unwanted chromosomal rearrangements, if many double-strand breaks are generated simultaneously. In fact, CRISPR/Cas9 can also be applied to generate predefined chromosome rearrangements (Maddalo et al. 2014, Lupianez et al. 2015).

Another important application of CRISPR/Cas9 is to boost the efficiency of homologous recombination, which was previously only achieved in mouse ES cells. When a large DNA construct is introduced in ES cells, containing sequences that are homologous to a genomic locus, it can be spontaneously integrated in the targeted locus. The frequency of targeted integration increases with the size of the homologous sequence but remains usually low. A typical frequency is 1% of the DNA integration events when the homology spans 10 kb. Generating a double-strand break in the targeted locus with CRISPR/Cas9 results in an enormous increase in this frequency. The targeted insertion of large plasmid construct therefore becomes possible without using ES cells, by injecting mouse...
oocytes with Cas9 mRNA, sgRNA, and circular plasmid construct (Yang et al. 2013a). Although this remains to be confirmed independently, HDR frequency exceeding 30% has been reported for constructs with several kilobases of homology (Wang et al. 2015). In ovo, HDR can also be obtained with very short homologies and single-stranded oligonucleotides as templates. For the targeted integration of large constructs, an ingenious protocol suggests to combine several CRISPR-mediated cut and single-stranded oligonucleotides, making dispensable the introduction of any sequence homology between the construct and the targeted genomic locus (Yoshimi et al. 2016).

Currently, the main use of HDR is not to insert exogenous DNA, but to perform in ovo site-directed mutagenesis. A systematic analysis in cultured cells indicates that a 50-mer oligonucleotide with a 2nt central mismatch already gives an HDR frequency of 1% (compared with NHEJ) (Yang et al. 2013b), whereas a 100-mer oligonucleotide yields higher efficiencies. The PAM should be absent from the exogenous template, to prevent subsequent DNA cleavage and NHEJ after HDR. In ovo HDR, using synthetic oligonucleotide templates, has been used to introduce single-point mutation (Inui et al. 2014), to insert a 34bp loxP site, to extend protein-coding sequences to insert small tags, and even to perform the simultaneous insertion of two loxP sequences at two different sites, in a predefined orientation (Yang et al. 2013a). This single-step generation of ‘floxed’ alleles represents a spectacular shortcut for an otherwise tedious and time-consuming procedure. However, this astonishing success remains, 3 years after publication, unique in the literature, and it seems unlikely that this direct approach will become a routine.

Delivery of Cas9 and sgRNA to mouse oocytes and the problem of mosaicism

Different protocols have been used to create targeted germline mutations in mice with CRISPR/Cas9. As the method is relatively novel, a consensus on the best way to operate has not been reached yet. Although we focus here on in ovo protocols, using ES cells still offers some advantages and may not be abandoned: if CRISPR/Cas9 is used to generate complex genetic modifications, occurring at low frequency, it may still be easier to identify the expected genetic events in stem cell clones than directly in newborn mice. In ovo CRISPR/Cas9 remains otherwise a time-saving choice.

The simplest in ovo protocol is to inject a single-plasmid DNA construct, allowing the synthesis of both Cas9 and sgRNA into mouse fertilized oocytes (Mashiko et al. 2013). In most cases, the microinjected DNA is not integrated in chromosomes and Cas9 expression is only transient. Mutations often appear in the embryo after the first cell divisions, such that most animals are mosaics. This is immediately observable for the tyrosinase gene as homozygous mutations in this gene cause albinism (Mizuno et al. 2014). Mosaicism might result from the time taken for the construct to be transcribed and for Cas9 mRNA to be translated after pronucleus injection in the embryo. However, the first cell division of the mouse embryo takes place within 12-15h after pronucleus formation, therefore only few hours after DNA microinjection, whereas the time needed to resolve Cas9 lesions has been estimated to 15h (Kim et al. 2014). If applicable to oocytes, this timing is sufficient to explain the late appearance of mutations and the recurrent occurrence of mosaicism. Mosaicism is usually a source of difficulties, but could be sometimes beneficial, when one mosaic founder mouse can give rise to several mutant strains, with different nucleotide sequence changes, generating so-called allelic series. Limiting mosaicism necessitates to accelerate the editing process and to keep it transient. It is thus often preferred to inject premade Cas9 mRNA and sgRNA, both prepared by in vitro transcription with phage polymerases. Although this approach is more efficient than plasmid microinjection (Horii et al. 2014), it does not eliminate mosaicism (Yen et al. 2014). Cas9 protein produced by bacteria can also be purchased from several sources and is efficient in cultured cells (Kim et al. 2014), brain stem cells (Kalebic et al. 2016), and mouse oocytes (Aida et al. 2015). This recombinant protein is mixed with sgRNA in vitro before microinjection. Preparing sgRNAs by direct chemical synthesis (either as sgRNA or as two RNA molecules that will assemble into a guide RNA) goes one step further toward the simplification of the genome editing process, avoiding the need of molecular cloning (Aida et al. 2015, Hendel et al. 2015). Finally, the Cas9 protein can be encoded from a transgene and already present in the fertilized oocytes, in which case a single sgRNA microinjection is sufficient (Sakurai et al. 2016).

The off-target problem

Like other transgenesis and editing methods, CRISPR/Cas9 is not 100% specific and can generate ‘off-target’ mutations. Although off-target mutations are a major issue for any therapeutic applications, they are not as problematic when genome editing is used to generate mutant mouse lines. As long as their occurrence is not
too high and does not compromise genome integrity and cell viability (Kim et al. 2009), off-target mutations are acceptable, because they will segregate from the intended targeted mutation after germline transmission. The risk of confounding effects is significant only if an off-target mutation is located within 10Mb of the targeted locus (about 10cM, representing around 1% of the genome) or if the phenotype of ‘F0’ mice has to be analyzed.

The extent to which off-target mutations occur has been quite controversial (O’Geen et al. 2015b). It is apparently less common in mouse zygotes than in cell lines (Iyer et al. 2015). Cas9 tolerates mismatches throughout the guide sequence in a manner that is sensitive to the number, position, and distribution of the mismatches (Hsu et al. 2013). Although computational prediction reliability remains limited, it is advisable to carefully search the genome sequence with appropriate software solutions (see below) and to ascertain that the predicted off-target mutations are not located in exonic fraction of the genome. Several methods based on deep sequencing, including GUIDE-Seq (Tsai et al. 2015), digenome-seq (Kim et al. 2015, 2016), BLESS (Crosetto et al. 2013), and HTGTS (Frock et al. 2015), have been developed to detect de novo mutations on a genome-wide scale. When CRISPR/Cas9 is used to integrate exogenous DNA constructs, fluorescent in situ hybridization on metaphase chromosomes can also be performed (Paulis et al. 2015). They all confirm the existence of off-target mutations, whose locations were not always predicted, but also indicate that the frequency of these events is low in most experimental applications.

It is important to stress that CRISPR/Cas9 is a two-step process (Sternberg et al. 2014, Jiang et al. 2015): Cas9 only needs the annealing of 10nt of the guide RNA to bind DNA, whereas its endonuclease activity requires the annealing of more than 16nt to the genomic target (Kiani et al. 2015). Direct microscopy study indicates that off-target binding events are, on average, short-lived (Knight et al. 2015). ChipSeq analysis confirms that, although sgRNA allows Cas9 to bind to thousands of genomic sites, mutations are detected almost exclusively at the targeted locus (Kuscu et al. 2014, Wu et al. 2014, O’Geen et al. 2015a). Although there is little correlation between the chromatin occupancy by Cas9 and the occurrence of double-strand breaks leading to mutations (Tsai et al. 2015), chromatin accessibility has a significant influence on editing efficiency (Chari et al. 2015).

A number of possibilities have been explored to reduce the frequency of off-target mutation. Shorter sgRNAs tend to have fewer off-target sites, and an homology stretch of 17nt was proposed to be a better option than the most common 20nt choice (Fu et al. 2014). Adding two extra G at the 5′ end of the sgRNA, which do not match the genomic sequence, can also increase cleavage specificity, but sometimes reduces cleavage efficiency (Cho et al. 2014). A way to drastically reduce the frequency of off-target effects is by using Cas9D10A, a Cas9 with an amino acid change that alters one of the two active sites of the nuclease. This turns Cas9D10A into a ‘nickase’, which only cuts one DNA strand (Mali et al. 2013a, Ran et al. 2013a, Shen et al. 2014a). Cas9D10A nickase is not mutagenic, unless two sgRNAs are combined to target two opposite strands simultaneously, introducing two independent single-strand breaks at nearby sites (Shen et al. 2014a). In that case, the specificity of double-stranded DNA cleavage relies on the formation of two neighboring 17–20nt RNA:DNA heteroduplexes instead of one. Another interesting attempt to limit off-target cleavages is the use of the Cas9–Fok1 fusion protein, in which the nuclease activity of Cas9 is eliminated and replaced by the nuclease activity of Fok1, which can only cleave DNA as a homodimer. Therefore, Cas9–Fok1 cuts only if two sgRNAs target neighboring genomic sequences (Hara et al. 2015). Both strategies require that two PAM sequences are found at the appropriate distance on opposite strands in the targeted locus. A similar approach is to combine CRISPR and zinc finger proteins (Bolukbasi et al. 2015). Although the reduction in the rate of off-target mutations was experimentally demonstrated (Cho et al. 2014), the available data do not permit to fully evaluate whether these alternate strategies maintain the efficacy of on-target genome editing. The most promising developments have come very recently (Kleinsteiver et al. 2015, Slaymaker et al. 2015). It consists in a rational engineering of modified Cas9, with amino acid substitutions introduced to increase the sensitivity to single- and double-base mismatches between the guide RNA and the target DNA. The enhanced nucleases, called eSpCas9 and SpCas9-HF1, display a significant decrease in their capacity to generate off-target mutations, without loss of on-target efficiency. If these enhanced nucleases fulfill the expectations, they should rapidly replace the original version of the nuclease.

Guide RNA design

Genome editing by CRISPR/Cas9 can be performed at virtually any genomic site with a PAM: 5′-NGG-3′. When such a PAM cannot be used, alternatives can be used. First, Cas9 can cleave sites with 5′-NAG-3′ or 5′-NGA-3′ PAM albeit less efficiently (Hsu et al. 2013).
Cas9 has also been engineered to alter PAM specificity (Kleinstiver et al. 2015). Furthermore, microorganisms host a variety of other RNA-guided nucleases, the use of which as genome editing tools is just starting to be explored (Shmakov et al. 2015). Streptococcus thermophilus Cas9 uses the 5′-NNAGAAW-3′ sequence as PAM, but has lower cleavage efficiencies than S. pyogenes Cas9. Structure-guided engineering of Francisella novicida Cas9 produced a variant specific for the relaxed 5′-YG-3′ PAM (Hirano et al. 2016). The recently studied Cpf1 nuclease, which belongs to a different class of CRISPR–Cas system (Zetsche et al. 2015a), has distinct properties that make it promising for future development: it uses a T-rich PAM, a shorter sgRNA (42 nt), and cuts the two DNA strands at different positions, leaving 5′ overhangs that should facilitate foreign DNA insertion.

A growing number of free bioinformatics tools have been developed to help in sgRNA design. The aim is to maximize the cutting efficiency while minimizing off-target cleavages (Ren et al. 2014, Koo et al. 2015, MacPherson & Scherf 2015, Park et al. 2015, Oliveros et al. 2016). The sequence of predesigned sgRNA is also available for the whole mouse exome (http://www.sanger.ac.uk/htgt/wge/) (Hodgkins et al. 2015). Most software tools evaluate the probability of off-target mutations, based on sequence similarities. However, experimental data indicate a poor predictive capacity of these algorithms (Tsai et al. 2015). Some programs also try to predict the on-target cutting efficiency, based on large experimental datasets (Doench et al. 2014, Liu et al. 2016). The most recent one, sgRNA Scorer, takes into account the observation that a G located immediately upstream to the PAM greatly increases genome editing efficiency (Chari et al. 2015). The size of the sgRNA 5′ end that is complementary to the genomic sequence is between 17 and 20 nt. The most common choice is 20 nt as most investigators assume that a longer sequence will stabilize RNA:DNA hybrids and increase Cas9 cutting efficiency. However, 17 nt has been claimed to be an optimal compromise between efficiency and specificity (Fu et al. 2014). In any case, it is usual to test at least two sgRNAs for a given targeted genomic site, as cleavage efficiency remains difficult to predict and to avoid superfluous nucleotides in sgRNA. However, in vitro transcription by phage polymerases imposes a 5′ GG dinucleotide (Stump & Hall 1993), whereas in vivo transcription initiation from the U6 promoter by RNA polymerase III starts with a G. It is thus necessary to append at least one extra G at the 5′ of the sgRNA if the 17–20 nt guide sequence does not begin with G.

### Favoring HDR vs NHEJ

When short DNA templates are used, NHEJ is largely predominant over HDR after double-strand break in fertilized mouse embryos. It is thus easier to generate random indels in the targeted locus than to perform a predefined modification of the sequence. Having full control over the genetic modification is however much more desirable and requires favoring HDR over NHEJ. Impressive HDR rates have been obtained with long-synthetic single-stranded oligonucleotide templates in a cell line, by following two simple rules (Richardson et al. 2016): (1) the sequence of the oligonucleotide should be identical to that of the ‘target strand’ (i.e., complementary to the sgRNA), and (2) the position of the sequence should be asymmetrical, being longer on the PAM-proximal side of the double-stranded DNA break. A chemical modification of the single-stranded template oligonucleotide, introduced to increase its stability, also favors in ovo HDR (Renaud et al. 2016). In addition, a recent study showed that HDR accuracy can be dramatically increased by incorporating silent mutations, along with the desired mutation, to prevent subsequent NHEJ events (Paquet et al. 2016). The knowledge of the molecular mechanisms that underlie each DNA repair pathway offers some other interesting possibilities for manipulating the outcome of the repair. Scr7 is an anticancer agent that inhibits DNA ligase IV, and thus hampers NHEJ, favoring the occurrence of HDR in both cultured cells and mouse embryos without apparent toxicity (Maruyama et al. 2015). However, Scr7 did not significantly increase the frequency of in ovo HDR for large DNA templates (Wang et al. 2015). The same inhibition of NHEJ can be obtained by knocking down the mRNA of ligase IV or KU70 with shRNA. Adenovirus 4 (Ad4) E1B55K and E4orf6 proteins, which mediate the ubiquitination and proteasomal degradation of DNA ligase IV, also inhibit NHEJ and increase HDR/NHEJ ratio (Chu et al. 2015). An ingenious alternative strategy is to destabilize Cas9 during the G1 and S phases of the cell cycle, by fusing it to a domain of Geminin, as HDR mainly takes place during the G2 phase (Gutschner et al. 2016). Finally, pharmacological screening identified several small molecules that also favor HDR compared with NHEJ (Yu et al. 2015). It is currently too early to clearly evaluate the benefit of all these approaches.

### Using nuclease-defective Cas9 to engineer new RNA-guided proteins

The general concept that a protein can be targeted to a specific genomic locus by a guide RNA has far-reaching
consequences. Nuclease-deficient versions of Cas9 have already been engineered to direct a variety of effectors to target loci. This strategy has been used to activate (Maeder et al. 2013, Tanenbaum et al. 2014, Zetsche et al. 2015b) and repress transcription either by tethering a transcriptional repressor (Gilbert et al. 2013, Qi et al. 2013) or by inducing DNA methylation in regulatory sequences (McDonald et al. 2016). Inhibition and activation of different genes can even be achieved within the same cell (Zalatan et al. 2015). This is a field of very active investigation, as genome editing could perhaps advantageously be replaced by targeted deregulation of gene expression. However, the fact that the native form of Cas9 binds DNA at many genomic sites, but introduces double-strand breaks in only few of them, where sequence homology is maximal (Wu et al. 2014), raises a major concern about the possible specificity of these approaches. A clever recent novelty was the fusion of nuclease-defective Cas9 and a cytidine deaminase enzyme (Komor et al. 2016). This hybrid RNA-guided enzyme mediates the direct conversion of cytidine to uridine, thereby effecting a C→T (or G→A) substitution. This makes the outcome of genome editing more predictable than the one mediated by NHEJ. More work is required to fully evaluate all these strategies, as most of the new developments are very recent, and often still preliminary.

**CRISPR/Cas9 and the future of endocrinology**

Only the novelty of the CRISPR/Cas9 genome editing technology explains why only few publications already used it for endocrine research. One can however foresee deep changes in the way genetic investigations will be performed. One immediate advantage is that it will renew interest for cellular models, primary cultures, or cell lines, in which the capacity to perform genome editing will bring a lot of interesting possibilities. Genome-wide genetic screen will permit to bring system biology, currently limited to few model cell lines, to more relevant cellular models. For example, it may help to define the functions of the thousands of binding sites that have been identified for several nuclear hormone receptors by ChipSeq analysis.

Most of all, CRISPR/Cas9 makes genetic engineering in mice easier, faster, and cheaper. Considering that synthetic guide RNA can be synthetized quickly, transgenic pups homozygous for knockout mutation can be produced using the cloning-free procedure within 1 month. If the phenotype manifests at birth, the entire process could take not more than 1 month. Considering the problems of mosaicism and off-target mutations, immediate phenotyping of these F0 animals remains risky, but already provides important indications. By contrast, the equivalent experiment performed with the traditional ES-based protocol would take on average more than 1 year. It is even possible to target several genes simultaneously, as up to nine different sgRNAs can be microinjected in oocytes at the same time (Sakurai et al. 2016). One main outcome should be that genetic investigations of a gene function in mice will not be limited to the analysis of a single-mutated allele anymore, but will be expanded to allelic series, as this is common practice in D. melanogaster. This will produce a range of phenotypic severity and identify the respective functions of the different proteins that a single gene often produces. Introducing single amino acid substitutions to alter different protein–protein interactions separately will uncouple the different functions of a protein. Another possibility will be to produce ‘custom-made’ models for human genetic diseases in which mice will carry a point mutation equivalent to that found in a given patient. Finally, tackling the poorly explored noncoding part of the genome, that is, the noncoding RNAs and regulatory sequences, becomes feasible (Korkmaz et al. 2016). The production of new transgenic lines is not a rate-limiting step anymore. Only the difficulty of maintaining and phenotyping many mouse strains will constrain research projects.

The other expected change is that genome editing should become feasible for any laboratory animal from which fertilized oocytes can be explanted and reimplanted. CRISPR/Cas9 has already been used in rats (Menoret et al. 2014), hamster (Fan et al. 2014), and rabbit (Yuan et al. 2016), which all bring some specific benefit in endocrinology (Duranthon et al. 2012, Bouscsein et al. 2016). These alternative models will certainly provide a useful complement and might eventually challenge the predominance of mice as model species to study mammalian physiology.

Finally, the advent of CRISPR/Cas9 may rejuvenate the traditional genetic approach of biological questions. We considered here only the reverse genetic approach, in which the relation between genes and characters is studied from bottom to top: mutations are first produced for a known gene, and then the unknown phenotypic consequences of the gene mutation are analyzed.
Classical genetics goes in the opposite direction, and first chooses a character before searching for the genes that determine it. Chemical (ENU) mutagenesis has sometimes been used for this purpose in mice, but experience proves that identifying the causative mutations of a phenotypic trait remains in this case a daunting challenge (Weiss et al. 2012). Several CRISPR libraries are already available, offering the possibility to set phenotype-based screening strategies, by introducing NHEJ-based frameshift mutations in virtually all the genes. This approach has already been implemented in cell lines (Wang et al. 2014), zebrafish (Varshney et al. 2015), and Drosophila (Bassett et al. 2015). It is in essence similar to ENU mutagenesis but has a key advantage: here the DNA corresponding to sgRNA persists in a variant individual and can easily be sequenced a posteriori. Therefore, the gene that has been mutated can be easily inferred from the sgRNA sequence. A second round of confirmatory experiments, performed on a gene-by-gene approach, could confirm the gene identification process. The rate-limiting step for such a CRISPR-based genetic screening in mice is oocyte microinjection, which could be replaced by oocyte electroporation (Hashimoto & Takemoto 2015, Qin et al. 2015, Takahashi et al. 2015).

In that respect, somatic gene transfer is certainly an alternative to consider (Mikuni et al. 2016, Williams et al. 2016, Yang et al. 2016). If in vivo screening has to be done by oocyte microinjection, it would be facilitated if germline transmission of mutations was not required. This would imply that a large fraction of F0 animals are homozygous, as many of the mutations are recessive. A way to increase the rate of homozygosity is a genetic trick, called mutagenic chain reaction, which consists in integrating the Cas9 reading frame itself within the mutated locus (Gantz & Bier 2015).

The gold rush that follows a major technical innovation is usually followed by a phase of soberness, where the limitations of the new technology become visible. We are not at this point yet for genome editing and important progresses are still expected.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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