Chapter 1

Generating Genetically Modified Mice: A Decision Guide

Ivo J. Huijbers

Abstract

The generation of a new genetically modified mouse strain is a big hurdle to take for many researchers. It is often unclear which steps and decisions have to be made prior to obtaining the desired mouse model. This review aims to help researchers by providing a decision guide that answers the essential questions that need to be asked before generating the most suitable genetically modified mouse line in the most optimal timeframe. The review includes the latest technologies in both the stem cell culture and gene editing tools, particularly CRISPR/Cas9, and provides compatibility guidelines for selecting among the different types of genetic modifications that can be introduced in the mouse genome and the various routes for introducing these modifications into the mouse germline.

Key words Transgenesis, Genetic engineering, Mouse models, CRISPR/Cas9, Embryonic stem cell, Zygote, Gene targeting, Recombinase, Transposon, GEMM-ESC

1 Introduction

Genetically modified mice are used extensively in biomedical research to study gene function, to model a disease, or to examine a biological process in vivo. The mouse is considered the most suitable mammalian model system, because it is a small domesticated animal with the same organ and tissue systems as man. The generation time is relatively short, 3 months, and the litter size large, 7–10 pups, and importantly the mouse germline can be modified via genetic engineering. Though these technologies exist since the 1980s, generating a new genetically modified mouse line is still a big hurdle to take for many researchers. It is often unclear which steps and decisions have to be made prior to obtaining the desired mouse model. The experimental design process is further complicated by the fact that new tools for genetic engineering are constantly being developed, and it is often unclear how these tools can be applied most effectively to create a new mouse line. Another uncertainty is the time investment required to

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generate a new mouse line, since this differs with the type of modification to be introduced in the mouse germline.

This review aims to help researchers by providing a decision guide with answers to the essential questions that need to be asked before generating the most suitable genetically modified mouse line in the most optimal timeframe. The questions are divided in three sections: (1) prior considerations, (2) inventory of the requirements, and (3) design and production. The review includes the latest technologies in both the stem cell culture and gene editing tools, including CRISPR/Cas9. The review is not exhaustive but based on the most often used techniques and applications by transgenic facilities worldwide and on the specific technologies developed and applied at the transgenic facility of the Netherlands Cancer Institute in Amsterdam. Besides reading this review, researchers are advised to seek information from their local transgenic facility or commercial transgenic company to inquire about their capabilities and capacity for generating mice. Not all options mentioned in this review are routinely applied. Furthermore, researchers are encouraged to discuss their plans with experienced principal investigators that have a main focus on performing mouse experiments. Many decisions in the development of a new mouse model are based on practical experiences that are difficult to retrieve from literature. Online forums can also provide a wealth of information and an interactive exchange of experiences. A good example is the transgenic list provided by the International Society for Transgenic Technologies.

2 Prior Considerations

Any researcher considering developing a new mouse model should first take a close look at the scientific question that needs to be answered and identify whether a new strain is absolutely required. Ethical considerations involved in generating and performing the animal experiments should also be taken into account.

2.1 Is a New Genetically Modified Mouse Required?

For many scientific questions, generating a new mouse model might not be the most optimal way forward. If alternative approaches can provide similar answers, it is best to avoid making new mouse strains given the ethical considerations and the high financial and time costs associated with the design, creation, validation, and maintenance of transgenic mice. It is good to keep eyes open for in vitro or ex vivo alternatives as these are almost always faster and cheaper. Alternatively, identify a suitable non-germline mouse model that can be adapted to your needs [1]. For instance, a recombinant viral vector that infects a specific tissue can overexpress a target gene, induce gene repression using an shRNA, or delete or modify genes using CRISPR/Cas9 [2–4]. The flexibility of these

non-germline mouse models is high and the models are fast to develop. They are, however, limited in certain aspects, including their penetrance and their ability to infect specific organs or cell types.

2.2 Is a Suitable Mouse Line Already Available?

When a germline genetically modified mouse model is clearly required, it is important to research whether a mouse line that meets the requirements already exists. These mice can be identified through a literature search or via websites such as www.mousephenotype.org or www.findmice.org [5, 6]. For strains generated by consortia like KOMP or EUCOMM, it is important to identify whether the mouse line has already been generated and the mice or sperm are available for shipment. If only embryonic stem cells (ESCs) exist, it might be preferable to develop a new mouse strain from scratch. For instance, simple knockouts are easily generated with new gene editing tools, this in contrast to more complex gene modifications such as the knockout first, gene trap allele that can be converted into a conditional allele [7].

2.3 Is There Ethical Approval for Generating and Testing of the Mice?

All mouse experiments should comply with local and governmental regulations on performing mouse experiments. Ethical approval should be obtained before the actual generation of the mouse line starts. The approval should cover the generation of the genetically modified mice, breeding of the mice, and the actual experiments to be performed with the mice.

3 Inventory of the Requirements

Next, the researcher should identify all the characteristics to which the new mouse model should adhere and have a clear view on the final genotype and strain background in which the key mouse experiments will be performed.

3.1 What Type of Genetically Modified Mouse Should Be Generated?

The majority of genetically modified mice are generated to study the effects of either a loss of expression, an increase in expression, or a change in the sequence of a target gene (Fig. 1). Genetically modified mice can also be designed to mark a specific cell population (reporter stain), monitor a biological process (reporter strain), allow Cre recombination in a specific cell type (Cre-driver strain), or deplete a specific cell population upon activation of caspase 8 (ATTAC-mice) [8–10]. Other types of genetically modified mice exist but are beyond the scope of this review.

3.2 How Should the Genetic Modification Be Controlled?

The required genetic modification can be stable, conditional, or inducible (Fig. 1a) [11]. A stable genetic modification cannot be altered and remains stable over time and generations. For instance,

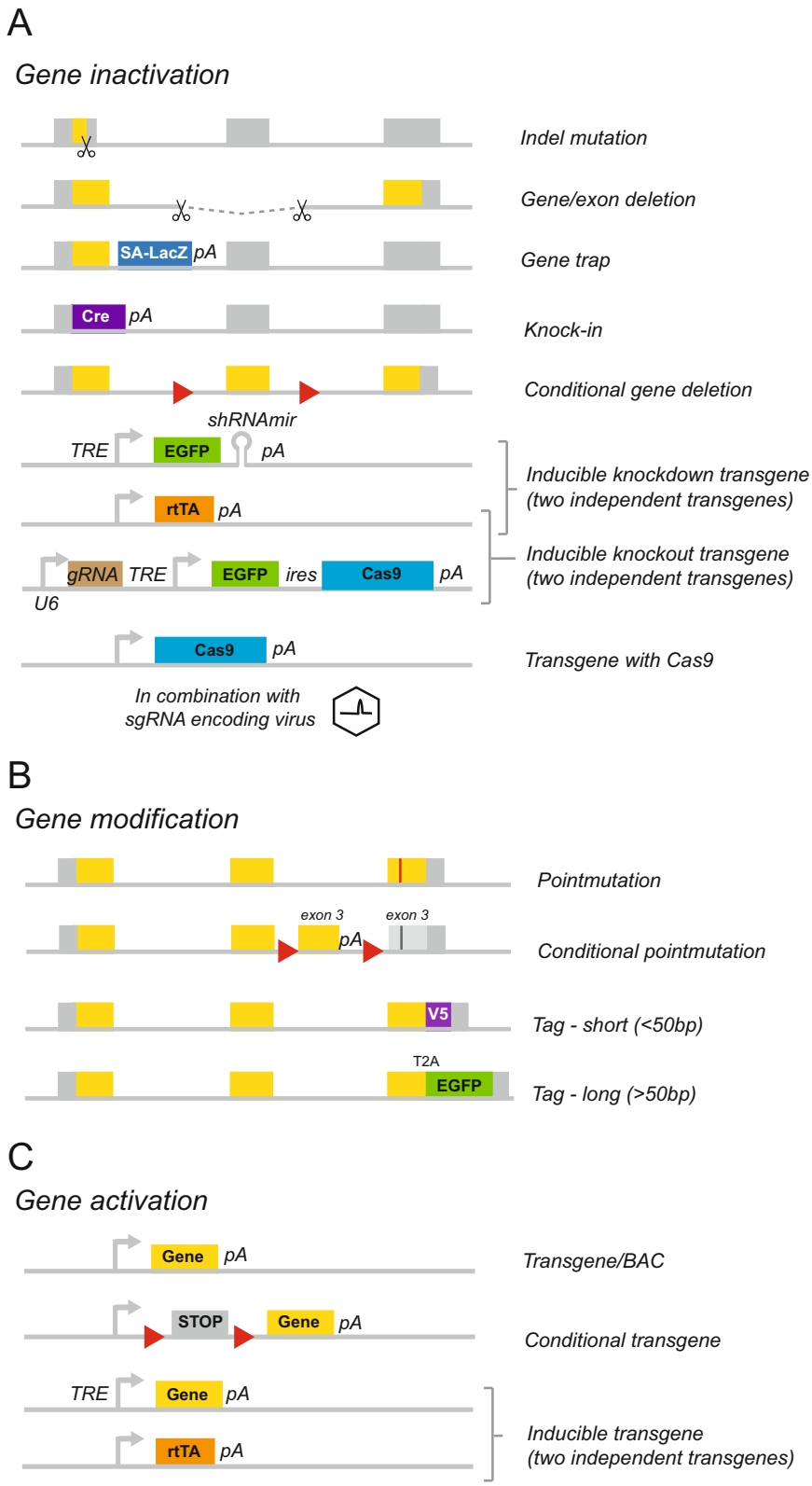

Fig. 1 Types of genetic modifications to introduce in the mouse germline. Schematic representation of the various genetic modifications that can be introduced in the mouse germline with the purpose of (**a**) gene

deleting a critical exon from a gene in the mouse germline will result in a stable gene knockout allele.

A conditional allele is controlled by a site-specific recombinase. For instance, in a conditional knockout allele, a critical exon of a gene is flanked by LoxP sites, and only upon expression of the Cre recombinase, the exon will be deleted resulting in gene loss [12]. In this situation, the pattern of Cre expression defines the cell type in which recombination and gene loss occur. The conditional allele itself is stable and transmitted over generations.

Temporal control of a genetic lesion can be achieved by applying ligand-dependent inducible systems. CreERT2, a fusion protein with the Cre recombinase and the mutated ligand-binding domain of the estrogen receptor (ER), is sensitive to tamoxifen. Upon treatment of mice with either tamoxifen or an analog such as 4-hydroxytamoxifen, the CreERT2 protein translocates to the nucleus and recombines conditional alleles [13]. Other major inducible expression systems used in transgenic mice are based on tetracycline-controlled transcriptional activation and named Tet-Off and Tet-On [14–16]. The Tet-On system makes use of the reverse tetracycline transactivator (rtTA), a fusion protein of the tetracycline repressor (TetR) with the activation domain of VP16. The rtTA is able to bind TetO operator sequences only in combination with treatment of tetracycline or one of its derivates, such as doxycycline. When the TetO sequences are placed upstream of a minimal promoter, the resulting promoter element is called a tetracycline responsive element (TRE), and upon binding of the rtTA element, the transcription of the downstream gene or shRNAmir is initiated. The Tet-Off system works similarly but uses the tTA protein, which constitutively binds the TRE elements, leading to expression of the gene unless doxycycline is added and binding is abolished.

3.3 Should the Gene Modification Be Restricted to Certain Cell Types?

In genetically modified mice, all cells will contain the same genetic modification. However, addressing certain scientific questions requires tissue-specific gene modifications. In these cases the target gene can be placed under the control of a tissue-specific promoter allowing for the expression of a gene in a particular cell type. Most often, tissue specificity is achieved by placing Cre recombinase under control of a tissue-specific promoter, allowing Cre to delete a conditional gene (Fig. 1a) or activate a conditional transgene (Fig. 1c) in a cell type of interest. A large collection of

Fig. 1 (continued) inactivation, (**b**) gene modification, and (**c**) gene activation. *Yellow box*—open reading frame. *Scissor*—cleavage site. *Red triangle*—LoxP site. *Arrow*—promoter. *SA* splice acceptor, *pA* polyadenylation sequence, *U6* a PolIII promoter, *TRE* tetracycline responsive element, and *IRES* internal ribosomal entry site

tissue-specific Cre transgenic mice have already been generated and characterized (for an overview, visit www.jax.org). These Cre-driver lines are also indispensible for studying the effect of loss of genes that confer embryonic lethality when deleted from the germline.

3.4 Are Additional Features Required That Are Linked to the Genetic Modification?

To answer this question, it is essential to specify what the readout of the final experiment will be. In many cases the phenotype might be subtle and not immediately visible, and therefore utilizing genetic tools to help to get the right output might be useful. Such tools include fluorescent markers for identifying and isolating target cells, genetic tags for staining the gene of interest when good antibodies are not available, and thiouracil tags for in vivo labeling, purification, and analysis of cell-type-specific RNAs [17].

3.5 What Is the Genotype of the Mouse Model to Be Used in the Final Experiment?

It is important to realize in advance all the modified alleles that need to be present in the final mouse and whether these need to be heterozygous or homozygous, since the fastest route to obtain these mice might be different. In some cases, crossbreeding might be faster, while in other cases methods for modifying the germline of complex genotypes might be preferred. Answering this question also provides a realistic timeframe estimate of how long it will take until the final experiment can start. This can be much longer that initially thought, since crossbreeding is time consuming.

In many cases the final genotype is simple. For instance, when creating a new knockout allele, only a single cross is required to obtain homozygosity of the allele, at which point the mouse can be studied for the observed phenotype. In other cases the newly modified allele needs to be combined with other genetically modified alleles to perform the study, resulting in a complex genotype. For instance, a newly generated conditional gene knockout allele has to be crossed to homozygosity. A suitable Cre-driver allele (heterozygous) has to be crossed in, and often it is also preferably to cross in a conditional reporter allele (heterozygous) in order to identify the cells in which the conditional gene has been deleted. Complex genotypes are also common in genetically engineered mouse models (GEMMs) of diseases. For instance, to mimic a human tumor, a specific combination of tumor suppressor alleles and oncogenes needs to be deleted or activated, respectively, in a specific cell type [18, 19]. These GEMM models typically contain multiple conditional alleles (homozygous) and a Cre-driver allele (heterozygous).

3.6 What Is the Required Strain Background?

Since the strain background can influence the observed phenotype in a genetically modified mouse, the decision of which background to use for the final mouse experiment is important. The choice of the most suitable background is often based on practical and historical data. When a new mouse strain needs to be compared to other genetically modified mouse lines, it is best to use matching strain backgrounds. Also, when the newly generated mouse line

needs to be crossed to another mouse line with a modified allele to obtain a complex genotype, it is highly recommended to match the strain background. Mixed strain backgrounds are a source of variability and should be prevented.

Ideally, the genetic modification is introduced in the strain background that is subsequently used to perform the experiments. Historically, the introduction of a genetic modification was only possible on a limited number of strain backgrounds, mainly the 129 and C57Bl6, as these were the only strains for which ESC clones were available that were amenable for genetic engineering [20]. New technologies have paved the way for genetic engineering in the strain background of choice. New culture conditions for mouse ESCs allow for the derivation, culture, and genetic engineering of mouse ESCs from all strain backgrounds [21–25]. Moreover, the efficient use of gene editing tools in fertilized mouse eggs (zygotes) allows for more flexibility in choice of strain [26–29].

When a genetically engineered mouse line has been created on an undesirable strain background, it is advisable to backcross the mouse line to the desired background, typically for ten generations. Backcrossing will result in a genome that originates for the vast majority from the backcrossed strain. Still, sequences flanking the modified locus will by definition be from the original, undesired, strain. These can be of considerable length and encode for passenger mutations that possibly confound the interpretation of the phenotype [30].

4 Design and Production

With all the requirements of the new genetically modified mouse line in clear sight, the design of the specific genetic modification can be made. At this point a decision needs to be made about the precise genetic modification to be introduced in the genome (Fig. 1). Also, the most suitable process or method for introducing that specific modification in the mouse germline needs to be chosen in light of the feasibility of the various available routes (Table 1 and Fig. 2). The design also requires some forward thinking in order to develop a proper screening method to both identify the correctly modified mouse or ESC clone and to minimize the risks of undesired off-target phenotypes. Finally, with all parameters in clear view, a realistic time estimate can be made.

4.1 What Is the Precise Genetic Modification to Introduce in the Mouse Genome?

As discussed before, the purpose of a genetic modification in mice is typically gene inactivation, gene modification, or gene activation. Various options are available to inactivate a gene (Fig. 1a). The endogenous gene can be inactivation via one of four methods. First, an insertion/deletion (indel) mutation can be used to cause a frameshift, which in turn leads to either a truncated protein or absence of protein due to instability of the mRNA. Second, the

Table 1
Suitable process or method to introduce a specific genetic modification

	NHEJ	HDR with ssOligo	Transposon-mediated integration	HDR with dsDNA	Recombinase-/integrase-mediated integration
Indel mutation	Yes[a]	No	No	Yes[a]	No
Gene/exon deletion	Yes[a]	No	No	Yes[a]	No
Gene trap	No	No	Yes[b]	Yes[a]	No
Gene knock-in	No	No	No	Yes[a]	No
Conditional gene deletion	No	Yes[a]	No	Yes[a]	No
Point mutation	Yes[b]	Yes[a]	No	Yes[a]	No
Conditional point mutation	No	No	No	Yes[a]	No
Tag—short (<50 bp)	No	Yes[a]	No	Yes[a]	No
Tag—long (>50 bp)	No	No	No	Yes[a]	No
Constitutive transgene	Yes[b]	No	Yes[b]	Yes[c]	Yes[c,d]
Tissue-specific transgene/BAC	Yes[b]	No	Yes[b]	No	No
TRE transgene	Yes[b]	No	Yes[b]	Yes[c]	Yes[c,d]
Conditional transgene	No	No	No	Yes[c]	Yes[c,d]

[a]Modification in gene of interest
[b]Integration into a random genomic location
[c]Integration into a defined permissive locus, e.g., Rosa26 or Col1a1 locus
[d]Provided locus is equipped with suitable docking site

inactivation can be achieved via a deletion of a critical exon or an entire gene. The third method involves a gene trap, where a promoterless reporter gene flanked by an upstream splice acceptor (SA) and a downstream transcriptional termination sequence (polyadenylation sequence, pA) is inserted in an intron. The gene trap results in a fusion transcript under control of the endogenous promoter. The gene is effectively inactivated, but the reporter gene allows for monitoring of promoter activity of the endogenous gene. Lastly, gene loss can be achieved with a knock-in, where the sequence after the transcription start site is replaced by another sequence, for instance, coding for a Cre(ERT2) recombinase. This strategy is primarily used to get the Cre recombinase expressed in the same cells that express the endogenous gene.

Conditional gene inactivation and inducible gene inactivation have been introduced earlier. It is important to realize that Tet-Off/Tet-On inducible gene inactivation typically requires two independent transgenes: one coding for the tTA/rtTA element under control of a promoter and the other a shRNAmir element or gRNA/Cas9 element coupled to a reporter and under control of

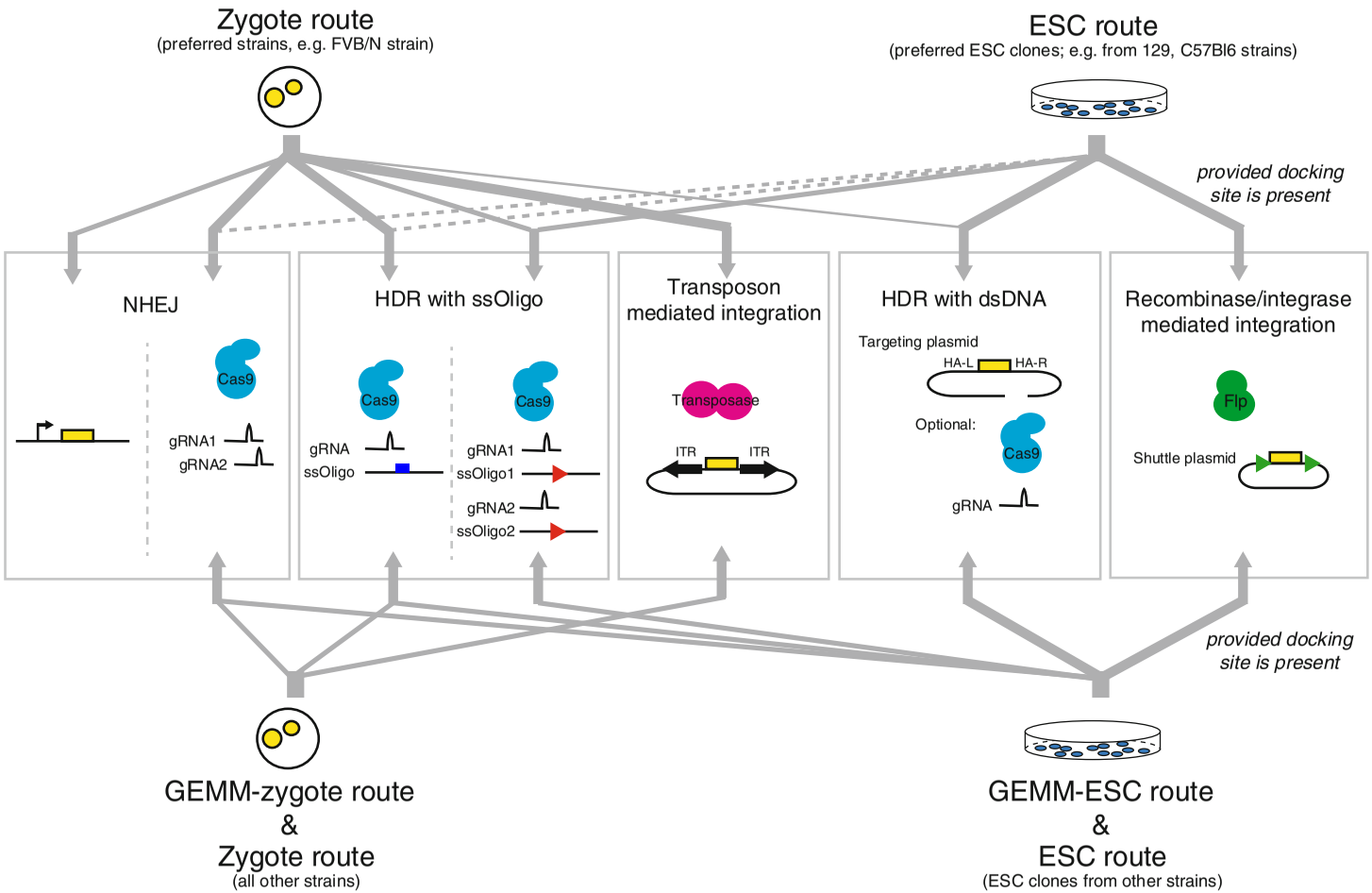

Fig. 2 The feasibility of routes to introduce genetic modifications in the mouse germline. Five strategies are available to modify the mouse genome: (1) via NHEJ, (2) via HDR with ssOligo, (3) via transposon-mediated integration, (4) via HDR with dsDNA, and (5) via recombinase-/integrase-mediated integration. The main routes to introduce these modifications in the mouse germline are (1) the zygote route, (2) the ESC route, (3) the GEMM-zygote route, and (4) the GEMM-ESC route. *Arrows* indicate the compatibility of each route with a specific strategy to modify the genome. *Thick arrow*—very compatible. *Thin arrow*—poorly compatible. *Dotted line*—compatible, but only interesting under certain conditions. For instance, the zygote route is ineffective when a gene is embryonic lethal upon deletion. In that case, the ESC route is preferential

a TRE promoter [14, 31, 32]. An alternative to the complex inducible gene knockdown strategy is the use of transgenic mice expressing Cas9 ubiquitously or tissue specifically [4]. These mice can be infected with a recombinant virus that expresses a specific gRNA. The target gene will be mutated in the infected tissue, and the majority of these cells will lose target gene expression due to indel mutations. This approach is not restricted to one gene and is very flexible, since the gRNA in the virus dictates which gene is targeted.

More subtle gene modifications can also be created in endogenous genes (Fig. 1b). Defined point mutations can be introduced in the open reading frame of a gene. The precise point mutations to be introduced are identified by basic protein biochemistry or from patient data and typically affect essential domains such as a catalytic site or a site for protein–protein interactions. Conditional point mutations are also possible but are more complicated. For example, a LoxP-flanked cDNA can be introduced into an intron that codes for the remainder of the gene followed by a downstream pA sequence. The defined point mutation is introduced in the exon behind this insert. This results in a normal expression of the endogenous gene from the cDNA. Upon Cre recombination, the cDNA is lost, and the gene now includes the defined point mutation. This approach has been successfully applied to oncogenes such as Kras and Braf [33, 34]. Expressed sequence tags are another gene modification strategy which is used either to facilitate gene expression analysis via immunohistochemistry, e.g., with V5-tag, or to allow for fluorescent imaging, e.g., with EGFP tag, without affecting endogenous gene expression.

Gene activation in genetically modified mice is typically achieved by overexpression from a transgene (Fig. 1c). In its simplest form the transgene contains a promoter, the cDNA of the gene of interest, and a poly-A sequence. The promoter can be ubiquitous or tissue specific. The most used ubiquitous promoter is the CAG promoter, a strong synthetic promoter that includes an intron [35]. Of note, for all transgenes it is advisable to include an intron in the construct as it increases gene expression in transgenic mice [36]. Conditional gene activation is achieved by including a LoxP-STOP-LoxP (LSL) element in the transgene upstream of the gene of interest. This LSL element can stop transcription by virtue of a splice acceptor (SA) site and one or more poly-A sequences (for an example, see ref. 37). Alternatively, the LSL element can also encode a cDNA followed by a poly-A sequence as, for instance, in the mTmG reporter line. Here the transcription of the tdTomato gene prevents expression of the downstream EGFP gene. Upon Cre recombinase expression, the cells delete the LoxP-flanked tdTomato cDNA and express EGFP [38].

For inducible gene activation, the Tet-On or Tet-Off system can be used, again requiring two transgenes to achieve suitable gene

regulation. Many transgenes include additional features linked to the gene of interest, such as fluorescent proteins or luminescent proteins. A bicistronic expression vector can be made by using an internal ribosomal entry site (IRES) or by including a self-cleaving 2A peptide [39, 40].

4.2 Where in the Genome Is the Genetic Modification Introduced?

A defined genetic modification can be introduced at several positions in the genome depending on the purpose. Genetic modifications that lead to gene inactivation or modifications are often introduced in the endogenous locus. In contrast, transgenes can be introduced into the genome either randomly or at defined "safe-harbor" locations. Several of these safe-harbor loci, including the *Rosa26* and *Col1a1* loci [41, 42], have been identified as both are permissive for genetic engineering and capable of facilitating reliable transgene expression. In the *Rosa26* locus, the endogenous promoter is typically used in constructs and provides ubiquitous expression. In the *Col1a1* locus, the transgene is integrated downstream of the 3'untranslated region of the *Col1a1* gene, and a promoter is required in the transgene. Typically, ubiquitous promoters are used. Avoid the use of tissue-specific promoters in targeted transgenes for the *Rosa26* and the *Col1a1* locus, since the expression pattern is often not as desired and few successful examples have been published. Tissue-specific expression is best achieved by a gene trap or knock-in allele or alternatively by a transgene with a tissue-specific promoter that is integrated randomly in the genome and selected for on basis of the correct expression pattern.

4.3 What Process Is Used to Introduce the Genetic Modification?

The introduction of specific genetic modifications in the mouse genome can be achieved either by exploiting the cellular processes involved in DNA repair or by the use of an external enzyme, such as an integrase, a transposase, or a site-specific recombinase.

4.3.1 Genetic Modifications by Exploiting DNA Repair Processes

Double-strand DNA breaks (DSB) in the genome can occur either spontaneously or when induced by site-specific nucleases such as Cas9. Two DNA repair mechanisms resolve these DSBs: (1) non-homologous end joining (NHEJ) and (2) homology-directed repair (HDR). The principles and proteins involved in the two types of repair differ (for review [43, 44]). An essential difference between the two is the fact that HDR uses a DNA template to repair a DSB, whereas NHEJ does not. Consequently, HDR is less error prone than NHEJ. The use of a template for DSB repair is exploited when creating defined genetic modifications. Typically, a DNA template containing two homology arms matching the sequences flanking the location of the desired genetic alteration is introduced into the cell of interest. When a DSB occurs, this template can be used in HDR instead of the sister chromatid, thereby introducing the defined genetic alteration at a specific

location in the genome. The DNA template can be a double-stranded DNA (dsDNA) vector, also known as a targeting vector, or a single-stranded oligonucleotide (ssOligo). The dsDNA vectors tend to be large, since they include a short homology arm (1.5–3 kb), a long homology arm (3–5 kb), and the desired genetic modification that can span multiple kilobases, often including an antibiotic selection cassette (for review, *see* [45]). The ssOligos are small, with homology arms of 60–100 bp flanking both sides of the oligo. This ssOligo template is typically used to introduce small genetic alterations, such as a point mutation or a small expressed sequence tag (<50 bp).

The types of genetic alterations introduced by NHEJ are different and are based on faulty repair of DSBs, which in turn result in deletions and insertions. These indel mutations can be directed to a defined location in the genome when combined with gene editing tools, such as CRISPR/Cas9, and allow the efficient generation of gene inactivating mutants. Though the DNA repair via NHEJ is not very error prone in itself, Cas9 in combination with a specific gRNA will constantly create a DSB at a target site and will only stop cutting when NHEJ has introduced a mistake and the gRNA does not recognize that target site anymore. The use of a single gRNA in a gene can create a frameshift mutation, while two nearby gRNAs flanking an exon or gene can create a null allele (Fig. 1a). Errors in NHEJ are also responsible for the incorporation of a transgene or a concatemer of transgenes into the genome when a spontaneous DSB occurs. Integration can occur when the transgene is present in excess in the nucleus of a cell. Where this integration will take place is uncertain, since the DSB occurs randomly in the genome.

4.3.2 Genetic Modifications by Transposons, Recombinases, or Integrases

Besides naturally occurring DNA repair processes, genetic tools can also be used to introduce genetic modifications in the genome. Two are relevant for the generation of genetically modified mice: (1) transposon-mediated integration and (2) recombinase-/integrase-mediated integration. In transgenesis, DNA transposons are applied as a biocomponent vector system. The DNA sequence of interest (or transgene) can be cloned between the transposon's inverted terminal repeats (ITRs) and mobilized by expression of the transposase enzyme [46, 47]. In the cell, the transposase excises the genetic element from the donor plasmid and integrates it randomly into the mouse genome. Multiple integrations of single-copy transgenes are likely, depending on how long the two components are present in the cells. The other genetic tool that can incorporate a transgene is based on the expression of a recombinase or an integrase. This is a two-stage process first requiring the targeting of a docking site for future transgenes in a safe-harbor locus, such as the *Rosa26* or *Col1a1* locus. This docking site typically consists of a selection cassette and recombination sites for

either the FLP recombinase, i.e., Frt or mutant Frt sites, or ΦC31 integrase, i.e., *attB* or *attP* sites [42, 48, 49]. Cells equipped with the docking site are permissive for the integration of transgenes flanked by matching recombination sites upon expression of either the recombinase or the integrase. This integration process is controlled and very efficient.

4.4 Which Route Is Most Suitable to Introduce the Genetic Modification?

The main routes to modify the mouse germline were developed in the 1980s, with the first transgenic mouse dating from 1980 and the first knockout mouse from 1989 (for review, *see* [50]). These mice were generated by introducing a recombinant piece of DNA in either a zygote or an ESC line. The techniques have been refined but are still valid today and applied routinely in transgenic facilities. In brief, for the zygote route, the recombinant DNA is injected in the pronucleus of a zygote. The zygotes are cultured overnight in embryonic medium. The embryos that cleave into the two-cell stage are implanted in the oviduct of a pseudopregnant surrogate mother who brings the embryos to term. The offspring is screened for the integration of the transgene in the genome by PCR and/or Southern blot. For the ESC route, a targeting vector is introduced in ESCs. Cells are selected for on the basis of their antibiotic resistance, and ESC clones with the correct integration of the targeting vector in the genome are used for microinjection into blastocysts (3.5-day embryo). The injected embryos are implanted in the uterus of a pseudopregnant surrogate mother who brings the embryos to term. The extent of the contribution of the modified ESCs to the individual offspring is screened for via coat color or using quantitative PCR. For both routes, a newly genetically modified strain is established once a first-generation pup (F0) transmits the modified locus to the next generation, also called germline transmission (GLT).

The arrival of gene editing tools, in particular CRISPR/Cas9, has simplified genetic engineering both in zygotes and ESCs. Protocols for the "one-step generation of mice" have proven to be very effective in creating mouse strains with indel mutations, point mutations, and small gene modifications [26, 28, 29]. Here, Cas9 mRNA or protein is injected in a zygote together with one or more gRNAs and possibly a DNA template for HDR repair (Fig. 2). This application to create defined genetic lesions via the zygote route has completely shifted the decision matrix for the most efficient route to introduce a genetic lesion in a mouse strain. In Fig. 2, a comparison is made between the zygote and ESC route and their compatibility with the process for introducing a genetic modification in the mouse genome (Table 1). The thickness of the lines shows whether a process is efficient (thick line), possible (thin line), or only relevant in exceptional situations (dotted line). It should be noted that the targeting efficiency with a large targeting construct can be increased

by creating a DSB at the site of integration using a gRNA. This CRISPR-assisted targeting can be applied in ESCs, but is not necessary. In zygotes, CRISPR-assisted targeting is required when using large targeting construct, although only a few successful attempts have been published [28, 51].

Genetic modifications can also be introduced on already complex genetic backgrounds, for instance, from GEMMs. In the GEMM-ESC route, ESC clones derived from existing validated mouse strains can be modified to introduce additional genetic modifications [25, 52, 53]. As a consequence, no or minimal crossbreeding is required to obtain the final experimental mice, thereby gaining time and requiring less mice [54]. The GEMM-zygote route offers similar benefits, although the isolation of a sufficient quantity of high-quality zygotes from a GEMM background can be challenging for several reasons. First, sufficient breeding pairs are not easily available (10–15 female GEMM mice are typically required per injection session). Second, the superovulation protocol needs to be optimized to obtain sufficient zygotes per female donor. Third, the strain background can complicate the injectability of a zygote, for instance, the visibility and size of the pronucleus can vary dramatically between strains. In summary, the GEMM-zygote route is only suitable for relatively simple genetic modifications and only when the genotype is sufficiently complex that crossbreeding of mice is considered too time consuming. The overview for the compatibility of the GEMM-zygote and GEMM-ESC route with the process to introduce a genetic modification in the mouse genome is shown at the bottom of Fig. 2.

4.5 How Is the Correct Genetic Modification Identified?

The method to screen for the correct genetic modification in the mouse genome should be included in the initial design. Small genetic alterations in the endogenous gene introduced by CRISPR/Cas9 can be screening for by PCR using primers flanking the region of interest. The PCR product can be subcloned and Sanger sequenced to identify the precise genetic modifications. The screening of the integration of a targeting vector or transgene is often performed by Southern blot analysis. To confirm correct targeting, a minimum of two probes are used, including an external probe and an internal probe (for detailed protocol, *see* [52]). The external probe is located in the endogenous locus just outside the homology arm of the targeting vector and confirms whether the integrated vector elements are intact. The internal probe is unique to the targeting vector and identifies the number of copies that are integrated in the genome. In most cases correct successful targeting should lead to the observation of a single genomic copy of the probe. This is different for transgene integration, where a Southern blot using an internal probe can be used to determine the number of copies of the transgene that are integrated and whether there is more than one integration site. An alternative approach for

determining the number of transgene copies in the genome is quantitative real-time PCR on genomic DNA amplifying a unique sequence from the transgene [55].

4.6 How to Minimize Risk of Undesired Off-Target Phenotypes?

Genetic engineering of the mouse germline can lead to undesired genetic alterations that influence the phenotype observed in final mouse experiment. It is important to realize what these risks are and how they can be minimized. Three types of off-target events are most relevant: (1) integration of a transgene in the genome can alter nearby gene expression, either directly due to the site of integration of the transgene or indirectly due to the often strong promoter present on the transgene that affects expression of nearby genes [56]; (2) indel mutations at off-target cleavage sites of a gRNAs can affect gene expression [57, 58]; and (3) long-term tissue culture of mouse ESCs can result in small chromosomal gains and loses [25, 59]. Activity screens for undesired effects can be used to detect off-target effects. For instance, the integration site of the transgene can be mapped, expression levels of nearby genes can be monitored, all potential off-targets sites can be sequenced, and copy number variations can be identified by low-coverage sequencing. This is, however, a tremendous effort and is often unrealistic in practice. It can even be unnecessary in some cases, for instance, genetically modified mice produced by zygote injections have a low rate of off-target mutations introduced by CRISPR/Cas9 [57]. A more practical approach is to generate two independent lines with the same modification and determine whether the phenotype is consistent. Alternatively, backcrossing the new line for a few generations to the matching wild-type strain background can dilute out off-target modifications that are not coupled to desired genetic modification. Besides these solutions, further efforts can be made to optimize procedures. For instance, the use of Cas9 D10A nickase to introduce defined genetic alterations will theoretically reduce the number of off-target events [60]. Also, more high-fidelity Cas9 proteins are available [61, 62].

4.7 What Is the Projected Timeframe?

The time to obtain the final experimental mouse is dependent on the route and procedure to introduce the genetic modification and on the number of crossbreeding required to obtain the final genotype. For the zygote route, design and cloning of a CRISPR/Cas9 application is typically fast (~1 month). Only when a transgene or targeting vector is required, the cloning will be more time consuming, typically taking 1–4 months. After zygote injections, it takes little over a month before the F0 can be screened for the presence of the genetic modification. The potentially interesting mice are bred, and the offspring (F1) again needs to be carefully analyzed to confirm the presence of the correct genetic modification. This step is important because the F0 mouse is considered a mosaic mouse with potentially different genetic modifications in different

cells. The ESC route requires similar time in design and cloning, but the introduction of the genetic modification in cells is more time consuming; 2–3 months are minimally required for the isolation and validation of correctly modified ESC clones. Only at that point blastocyst injections can be performed. The best chimeras are used for breeding and GLT. Screening for the right genetic event is done by a simple genotyping PCR. It is important to realize that the zygote route takes a relatively short time before injection but requires detailed screening of the F0 and F1 offspring, whereas the ESC route requires more development time but little efforts to screen the offspring.

When extensive crossbreeding is required to obtain the desired genetic background for experiments, the GEMM-ESC route might be suitable. Deriving GEMM-ESC clones takes 2–3 months and the subsequent validation of the clones another 4–5 months. When a validated GEMM-ESC clone is already available, for instance, from a public resource (www.infrafrontier.eu, NKI GEMM-ESC archive), the timing is identical to the classic ESC route. Similarly, there is in principle no time difference between the zygote route and the GEMM-zygote route. It is important to realize that the risk of delay or failure is higher in both the GEMM-ESC and the GEMM-zygote routes, because the conditions for introducing the genetic modifications are not as standardized as for the broadly used ESC clones or the permissive strains for zygote injection. The same higher risk applies to introducing genetic modifications on strain backgrounds that are not generally used in transgenic facilities (Fig. 2). Still, the benefit of being able to immediately start work on the desired strain or GEMM background can outweigh the risks.

5 Concluding Remarks

Novel technologies have created new avenues for generating genetically modified mice. Mice can now be generated in a shorter timeframe and starting with nearly any genetic background. This has lowered the threshold for generating the most suitable genetically modified mouse strains and has improved the quality of the resulting mouse models. This has had a positive impact on biomedical research, making studies that provide biologically relevant in vivo data within everyone's reach.

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