Chapter 2

Practical Application of the 3Rs in Rodent Transgenesis

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Abstract

The principles of the 3Rs (replace, reduce, refine), as originally published by Russell and Burch, are internationally acclaimed guidelines for meeting ethical and welfare standards in animal experimentation. Genome manipulation is a standard technique in biomedical research and beyond. The goal of this chapter is to give practical advice on the implementation of the 3Rs in laboratories generating genetically modified rodents. We cover 3R aspects from the planning phase through operations of the transgenic unit to the final genome-manipulated animals. The focus of our chapter is on an easy-to-use, concise protocol that is close to a checklist. While we focus on mice, the proposed methodological concepts can be easily adapted for the manipulation of other sentient animals.

Key words Genome manipulation, Transgenesis, Genome editing, 3Rs (Replace, Refine, Reduce), Animal welfare, Harm-benefit analysis

1 Introduction

Genetic perturbation of protein networks is an important tool for understanding cellular physiology in steady state and disease. The mouse has been the first mammal to be routinely accessible for genetic manipulation, allowing the investigation of the roles of its genes, pathways, and genomic elements. Because of its abundant use, most genetic tools were initially developed for the mouse and later adapted for use in other species. Thus, the generation of genetically modified mice has become a routine technique that is widely used in basic and preclinical research. Because of it being a forerunner, animal welfare aspects surrounding genetic manipulation techniques have been most thoroughly assessed in the mouse. In this methodological guideline for improvement of animal welfare, notably based on the 3R concept [1], we will hence restrict ourselves largely to the mouse. Many state-of-the-art techniques that we will present for the amelioration of the experimental impact on animal well-being can be easily adapted to other species as well. A detailed discussion is given at the end. We will not introduce any

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of the methods for which we make recommendations on how to optimize compliance with the 3Rs. Rather we want to refer the reader to the other chapters of this book and other excellent literature [2, 3]. Our aim is to provide easy-to-use checklists for the practitioner. These checklists are rather extensive and based on our own interpretation of available information. Especially in the context of animal welfare assessment, room for interpretation exists depending on regional moral values and national legislation. Also, certain techniques are preferred over others by specific researchers, animal welfare officers, and government offices. We recommend that these decisions be based on available literature and hence should be considered evidence-based. Nevertheless, we found the literature in some aspects lacking evidence to truly allow an informed decision. More research on animal welfare aspects appears to be necessary.

The here-presented protocol has been structured according to the workflow of a typical facility for the genetic manipulation of rodents. We do not indicate which of the 3Rs a particular advice refers to, as this is generally clear, e.g., an increase in efficiency of a process usually results in the use of fewer animals.

2 Materials

The use of high-quality material and reagents, from known and tested sources, is pivotal for optimizing experimental success and therefore minimizes the number of animals used in the procedure until successful line establishment. Optimal general laboratory supplies will be found in the specialized methods section of this book. A non-exhaustive list of resources, including websites, is provided with a special focus on provision of 3R adherence. When writing a research protocol, the inclusion of such resources should be included but should be adapted to the individual protocol:

- Information on genomes, genes, and phenotypes: Mouse Genome Informatics (MGI), http://www.informatics.jax.org/; https://www.mousephenotype.org/.
- Information on existing mouse models: International Mouse Strain Resource (IMSR), http://www.findmice.org.
- Genome browsers: Ensembl (https://www.ensembl.org/index.html), UCSC (https://genome.ucsc.edu).
- Critical incident reporting: CIRS-LAS, https://www.cirs-las.de/home.
- >8 *g* of nesting material suitable for mice to build a full dome nest [4].

- Ordering mice, ES cells, and/or targeting vectors [5]: The International Mouse Phenotyping Consortium (IMPC), https://www.mousephenotype.org/help/mouse-production/ordering-products/.
- goGermline sterile host embryos [6], https://www.ozgene.com/gogermline-knockout-and-knock-in-mice/.
- Kit Eazygote, zygote-stage frozen embryos for manipulation by injection or electroporation, https://janvier-labs.com/en/elevage/kit-frozen-embryos/.
- CARD HyperOva (Superovulation Reagent for mouse [7]), https://www.cosmobiousa.com/products/card-hyperova.
- Genetically sterile males (replacing vasectomies): CD1;B6D2-Tg(Prm1-EGFP)#Ltku/H; order: https://www.infrafrontier.eu/search?keyword=EM:12662; or Gapdhs^{tm1Dao} [8].
- Nonsurgical embryo transfer devices:
  - NSET: https://paratechs.com.
  - TCET: http://www.elimspringsbiotech.com/.

3 Methods

3.1 General

1. Ensure the facility generates an adequate number of lines per year to take advantage of the efficiency of scale. Consider centralizing efforts at your institution, between institutions and outsourcing. As a general rule, if stud males and sterile males are used on average less than once per week, centralization measures such as fusion with another facility should be evaluated.
2. Follow relevant literature and expert information to identify and develop efficient and effective state-of-the-art protocols and procedures. Attend conferences in the field and become a member of relevant mailing lists (e.g., ISTT mailing list).
3. All personnel must be properly trained and undergo continuous education.
4. Maintain detailed documentation for outcome evaluation:
   (a) Animal numbers must be recorded across all steps of a process.
   (b) Embryo number and quality must be recorded across all steps of a process.
   (c) If performance is low, measures must be taken to identify and solve apparent issues. Refer to the respective sections below for detailed minimal success standards.
   (d) Critical incidents and errors should be reported, preferably publicly, using reporting systems such as the German CIRS-LAS system (https://www.cirs-las.de/home).

5. Maintain optimal animal husbandry:
   (a) Husbandry conditions must adhere to internationally accepted standards (temperature, humidity, light cycle, cage size and occupancy, etc.), taking into account local applicable legislation.
   (b) High-energy food should be provided after the first trimester of pregnancy and during lactation (*see* **Note 1**).
   (c) At least 8 *g* of adequate, high-quality nesting material must be provided [9].
   (d) Hygiene status must be maintained in accordance with international standards such as those recommended by FELASA [10].
   (e) Animals should be acclimatized before use. However, acclimatization may not be possible in some cases (e.g., with prepubertal superovulation).
   (f) Non-aversive handling such as cup and tunnel handling should be performed.
   (g) Positive conditioning of animals should be considered to reduce stress as much as possible.
   (h) Disturbance of animals must be minimized and especially avoided for recipient females.
   (i) Animals must not be single-housed over longer periods without a specific reason. Consider companion animals when single-housing cannot be avoided.
   (j) Valid legislation and respective guidelines on humane euthanasia must be strictly followed.
6. Quality controlled materials must be employed for the manipulation procedure, including:
   (a) Media
   (b) Embryo culture equipment (incubators)
   (c) High-quality and highly purified macromolecules (DNA, RNA, proteins)
7. Use properly maintained, state-of-the-art inverted microscopes, including cooled injection tables and ancillary equipment for micromanipulation.
8. For endonuclease-based modifications, consider electroporation over microinjection for delivery of single-stranded DNA molecules (ssODN) smaller than 500 nucleotides, in order to reduce the number of embryos needed for manipulation and thus the number of donors and recipients. More complex genome alterations using larger DNAs, however, are mostly inefficient and increase the overall number of donors and recipients needed.

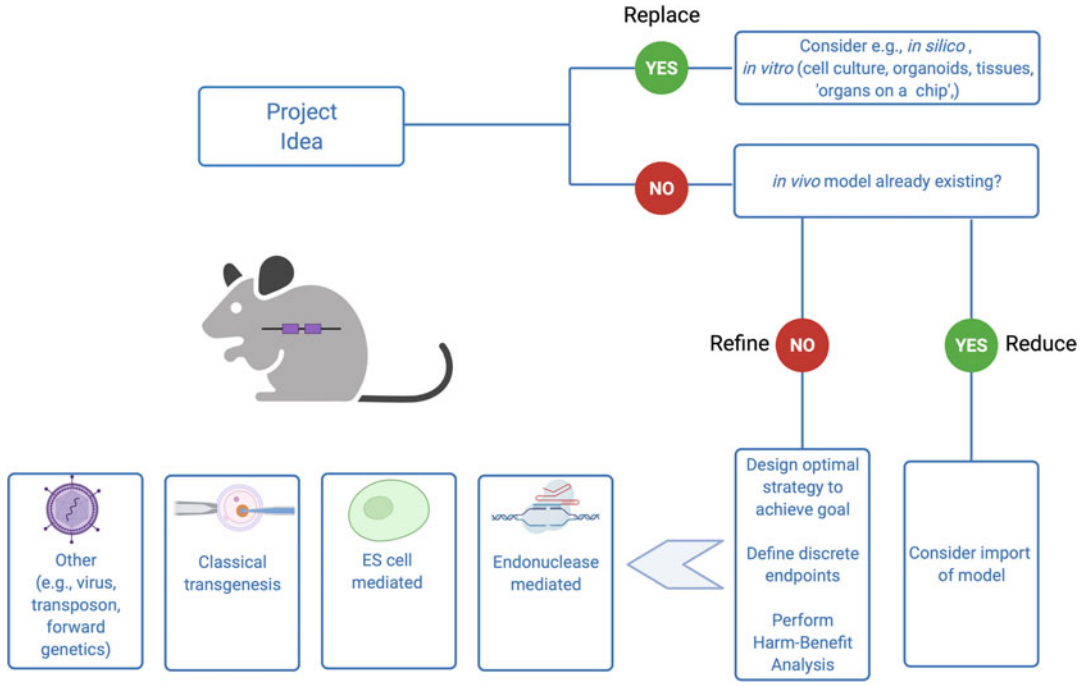

Fig. 1 3Rs decision process on a transgenic model

9. Consider in situ electroporation directly into embryos within the oviduct if applicable and properly trained. This sophisticated method eliminates the need to breed and kill embryo donors.

3.2 Choosing the Best Experimental Method

The decision on a specific transgenic model to be created by the transgenesis unit is the result of a scientific evaluation of the technical possibilities and the experimental objective. The decision process must involve all 3Rs. For a graphical illustration, *see* Fig. 1.

3.2.1 Non-sentient or Nonanimal Model

Assess if the use of nonanimal-based models or non-sentient animal models is possible before a project for model generation by transgenic methods is initiated. Evaluate alternative technologies alone or in combination:

(a) In silico modeling via bioinformatics and computing
(b) Simple cell culture models based on ex vivo material
(c) Simple cell culture models based on cell lines
(d) Embryonic or induced pluripotent stem cell-based assays
(e) Organoid and other 3D organ models
(f) Organ slices
(g) Organs-on-a-chip systems
(h) *Drosophila*, *Caenorhabditis*, and other invertebrate models

3.2.2 Existing Models

Assess reduction options if replacement is not an option. Evaluate the following:

(a) Screen database and literature for existing models.

(b) Check suitability of existing models for your purpose according to the following parameters:

(i) Does the type of mutation suit the experimental purpose?

(ii) Are the background strain genetics known and appropriate [11]?

(c) Evaluate importing versus de novo establishment according to the following parameters:

(i) Does the hygiene status allow direct import?

(ii) What harm or stress does a respective transport of live animals inflict (*see* **Note 2**)?

(iii) Are cryopreserved sperm or embryos available?

(iv) How many animals will be involved in harmful procedures (e.g., surgeries)?

(v) How many animals will be involved in total?

3.2.3 Type of Mutation

(a) Create inducible mutants by using recombination systems such as Cre-loxP or controllable expression systems such as CreER or Tet-On/Off when there is an indication that a harmful phenotype is likely with a conventional ubiquitous mutation. Limit harmful phenotypes to the tissue of interest or a defined time period whenever possible.

(b) Use an in vitro intermediate step in ES cells if there is a risk that inefficient manipulation of embryos could lead to high animal numbers:

(i) Evaluate the success probability of the in vivo (embryo) methods.

(ii) Assess the molecular design of the targeted gene alteration with respect to success probability (*see* **Note 3**).

(iii) Follow accepted standards, such as the recommendations for the development of knockout alleles in protein-coding genes [12].

3.2.4 Harm-Benefit Analysis (HBA)/Weighing of Interests

(a) Perform a HBA (also referred to as "weighing of interests") before engaging in a project to generate a genetically modified animal model:

(i) Generally, HBA needs to be performed with regard to national laws, local ethical standards, and animal procedure guidelines. The two parts of the report of the AALAS-FELASA Working Group on Harm-Benefit Analysis may help with this endeavor [13, 14] (*see* **Note 4**).

Table 1
Factors in a HBA for a genome modification project

Pros	**Increase gain**
Gain of knowledge	Provide model to the scientific community
Elucidation of physiological processes (involved in disease)	Provide detailed information in publication (ARRIVE guidelines)
Modeling disease, humanized animal models	Optimize translatability
Drug development	Publish open access
Preclinical drug testing	Provide data according to FAIR principles
(Preclinical) compound safety, pharmacokinetics	
Cons	**Decrease harm**
Pain: injections, surgery	Minimize number of injections
Death: animal use per se, animal numbers to be considered	Use best practice methods, e.g., adequate anesthesia and analgesia
Stressful holding and handling conditions	Use humane method for euthanasia
The inherent worth of the animal (sometimes called dignity)	Limit animal numbers as much as possible; avoid necessity for backcrossing
Harm: temporal or permanent negative effect on physical or mental condition, such as obesity, amputation, infertility, disturbance of behavior, etc., caused by the intended genetic modification	Provide adequate nesting and enrichment; avoid single-housing whenever possible; consider non-aversive handling methods; ensure adequate hygiene
	Consider use of inducible modifications to minimize number of affected animals; balance harm against increase in animal numbers
	Define line-specific mitigation strategies as soon as a harmful phenotype occurs

(ii) Assess the anticipated severity of the model in the planning phase of the project (Table 1) (*see* **Note 5**):

1. Include veterinarians, animal welfare experts, and scientists who are experts in the research field in the evaluation.
2. Consult publications on similar models as well as databases on GM animals and human conditions for information on potential phenotypes.
3. Consider genetic background effects.
4. Consider additional genome manipulations that may increase severity.

(iii) Choose the method with the least potential for unintended mutations, depending on the requirements of the scientific question.

(b) Make a Go/No-Go decision before the start of the project. Document how you weighed the potential benefit of the model against the potential harm inflicted.

(c) Choose optimal protocols and methods to generate the GM model in question for further reduction of the 3Rs footprint.

(d) In case of outsourcing, assess welfare standards of external partners before commencing the project.

3.3 Transgenic Techniques

3.3.1 Classical Transgenesis

In classical transgenesis, after microinjection into zygotes, a transgene inserts into the host genome in an uncontrollable fashion [15]. Evaluate whether the given research question cannot be better answered by precise GM employing ES cell technology or genome editing via CRISPR/Cas (*see* **Note 6**):

1. Consider a transposon system for integration if size requirements can be met [16].
2. Generate new transgenic lines on the background of interest, if possible. This obviates the later need for backcrossing.
3. Meet the minimal standards for efficiency [17]:
   (a) >70% of injected zygotes should survive microinjection.
   (b) >80% of injected zygotes should develop to the two-cell stage.
   (c) > 8% of transferred zygotes should result in live-born pups.
   (d) Of founder animals, 8–16% should be transgenic, in case of average size transgenes (*see* **Note 7**).
4. Examine several transgenic founder lines resulting from a given experiment for expression (*see* **Note 8**).
5. Choose for further analysis founding lines that show the desired expression profile without unwanted side effects causing unnecessary harm.
6. Identify the precise transgene location and integration within the genome by, e.g., targeted locus amplification (TLA), Samplix Xdrop®, or sequencing.
7. Backcross the founder animals to establish lines with one stable integration each (*see* **Note 9**).
8. Cryopreserve independent lines. This allows further control experiments [18].
9. Consider alternatives to classical DNA microinjection for increasing efficiency. Among others, consider the following:
   (a) Transduction with lentiviral vectors.
   (b) Using transposons can make integration of BACs at least five times more efficient. Note, however, that size limitations apply.

(c) The use of recombinases such as Flp and Cre or integrases such as Φ31 together with sequence-matched constructs and one-cell embryos carrying the respective sites for recombination allows very efficient targeted integration. However, this approach requires additional lines in the colony [19–21].

3.3.2 ES Cell Mutagenesis

1. Perform extensive characterization of the mutation already in ES cells by, e.g., Southern blotting, PCR, TLA, Samplix Xdrop®, and sequencing technologies. Characterization should also be performed when ES cells are obtained from repositories [22].
2. Ensure pluripotency of ES cells by adhering to stringent quality control measures:
   (a) Maintain a master cell bank with germline-tested wild-type ES cells.
   (b) Tightly adhere to optimized cell culture conditions.
   (c) Use only karyotypically normal ES cell batches.
3. Use ES cells derived from the background strain to be used for planned research.
4. Analyze gene-targeted ES clones for aneuploidies before generating chimeras by chromosome count or PCR-based methods. Use only those ES clones for injection with more than two thirds displaying a correct chromosome count of 40 X/Y.
5. Choose appropriate combinations of ES cell line and host embryos for optimal germline transmission rate (*see* **Note 10**):
   (a) ES cells derived from inbred strains (e.g., C57BL/6) can be combined with host embryos also derived from inbred strains (e.g., B6(Cg)-*Tyr*$^{c-2J}$ or BALB/c) but not with those derived from outbred strains.
   (b) ES cell lines from F1 hybrids (e.g., B6D2F1 or B6129F1) can be successfully combined with outbred embryos (e.g., CD1).
   (c) Consider using host embryos lacking the ability to develop sperm (*see* **Note 11**).
6. Inject up to 50 embryos/ES clone.
7. Meet the minimal standards for efficiency:
   (a) >90% of manipulated embryos should survive.
   (b) Use approximately three recipients per ES clone.
   (c) >50% of recipients should establish a pregnancy.
   (d) Birth rates should reach 25–50% of embryos transferred.
   (e) Roughly 50% of pups born should be chimeric with an ES cell coat color contribution of 70% or more.

8. Breed max. three chimeras per ES clone for germline transmission (*see* **Note 12**):
   (a) Germline transmission should be obtained from about 40–100% of chimeras mated and 50–70% of ES cell clones injected.
   (b) Terminate parallel experiments with chimeras of different ES cell clones harboring the same mutations immediately upon obtaining germline transmission (sperm from these males can be frozen as a backup if it should be needed later).

3.3.3 Endonucleases

1. For efficiency reasons, consider the use of the CRISPR/Cas system over Zn-fingers or TALEN systems.
2. Use only gRNAs with high cutting efficiency and low off-target probability. Evaluate gRNAs by:
   (a) Use of dedicated algorithms such as those reviewed in [23]
   (b) Testing cutting efficiency in vitro
   (c) Multiplexing of gRNAs against the target
   (d) Manipulation of a small number (20–30) of zygotes and analysis of mutations, e.g., by sequencing, either directly or after in vitro development into blastocysts [24] (*see* **Note 13**)
3. Reduce the chances of undesired mosaic founder animals [25]:
   (a) Use Cas9 ribonucleoprotein instead of Cas9 mRNA.
   (b) Lower the concentration of Cas9.
4. Consider increasing the efficiency of the CRISPR/Cas9 homology-directed repair system (*see* **Note 14**) by methods such as:
   (a) Chemical stabilization of donor DNA and gRNAs [26, 27]
   (b) Silent mutations in template DNA [28]
   (c) Optimization of the distance between cutting site and mutation target [28]
   (d) Design of asymmetric donor single-stranded oligodeoxynucleotides relative to the PAM site [28]
   (e) Cas9 variants [29]
5. Meet the minimal standards for efficiency:
   (a) >70% of embryos should survive manipulation with endonucleases.
   (b) >80% of manipulated embryos should reach the two-cell stage after overnight culture.

6. Do not rely solely on analysis of founder animals for verification of the expected mutation; definitive genotype confirmation should be performed in subsequent generations (*see* **Note 15**):
   (a) Confirm the presence of the correct mutation through a thorough quality control screening in G1 animals.
   (b) Exclude most likely aberrant and off-target mutations in G1 animals (*see* **Note 16**).
7. Consider adding additional mutations by genome editing on preexisting animal models instead of intercrossing separately generated mutations.

3.4 Donor Females

1. Choose donor strain such that mutations are introduced into the background, on which subsequent research will be carried out. For the reduction of animal numbers, backcrossing should be avoided.
2. Consider superovulation. It will increase embryo yield per donor and therefore reduce the number of animals involved.
   (a) Consult literature before titrating hormones to optimize superovulation yields for individual strains.
   (b) Consider hyperovulation [7, 30] followed by IVF to generate embryos for manipulation (this can be especially useful when using donors which are in short supply).
   (c) Meet the minimal standards for efficiency:
      (i) >80% superovulated donor females should be plug positive after mating (>50% without superovulation making use of the Whitten effect) [31].
      (ii) A superovulated female should produce at least 30 zygotes, with more than 70% of them intact.
      (iii) The number of injectable morulae or blastocysts may vary between five (for strains like BALB/c) and ten (for strains like C57BL/6) (*see* **Note 17**).
   (d) Consider in vitro fertilization instead of natural mating. However, male mice have to be killed to collect sperm. If possible, use archived sperm to reduce the number of donor males to be killed.
   (e) Try to cryopreserve excess embryos for future use or to use them for tests and optimizations.
   (f) Consider purchasing cryopreserved embryos when your own colony, including stud males, is underused.
   (g) Do not superovulate for in utero manipulation.

3.5 Surgical Procedures

1. Use aseptic conditions.
2. Provide preemptive analgesia. We recommend subcutaneous administration of 0.1 mg/kg buprenorphine 30 minutes before the start of the procedure.
3. Provide anesthesia with isoflurane/O_2 or ketamine/xylazine (*see* **Note 18**).
4. After initiation of the anesthesia, 50 μL 0.5% bupivacaine in NaCl can be injected subcutaneously at the cutting site as infiltration anesthesia.
5. Support body temperature maintenance by the use of a heat mat (or similar).
6. Apply eye ointment to protect eyes from dehydration.
7. Shave and disinfect the surgical area.
8. For bilateral embryo transfer, perform only a single dorsal skin cut.
9. After embryo transfer, stitch the peritoneum and close the skin with a clamp or tissue adhesive. Clamps should be removed 7 days after the surgery.
10. Apply analgesia immediately after surgery (while still under narcosis), the same evening, and the next morning. This may be a subcutaneous injection of 10 mg/kg bodyweight carprofen. Analgesia using slow-release buprenorphine may be considered, if available.
11. Monitor animal health and absence of pain for at least the first 3 days after surgery by, e.g.:
    (a) The Mouse Grimace Scale [32, 33]
    (b) Posture, e.g., signs of abdominal pressings (belly pressing), twitching, and writhing
12. If the animals continue to show signs of pain, analgesia must continue (*see* **Note 19**). Predefine humane endpoints for termination of the experiment.

3.6 Embryo Transfer

3.6.1 Sterile Males

1. Favor the use of genetically sterile males over surgical sterilization.
2. In case of surgical sterilization (vasectomy or epididymectomy):
    (a) Use males at an early age (typically 6 weeks) so that the animals can be used for as long as possible.
    (b) Access the vas deferens or the epididymis via the scrotum instead of the abdomen.
    (c) Avoid test matings. Instead, start using sterile males as early as possible for mating to embryo-transfer dams and record the outcomes. Exclude fertile males, in the rare event of failed surgery.

(d) In the case of using genetically sterile males:

(i) Consider the use of dominant genetic male sterility (***for source of animals***, *see* Subheading 2) or ensure alternative use of surplus animals when recessive male sterility is used [8].

(ii) Consider using extra females (produced from the breeding to generate sterile males) as surrogate dams for training purposes or sentinel animals for your health monitoring program.

(iii) Alternatively, consider using natural hybrid sterility by crossing *Mus musculus domesticus* females with *Mus musculus musculus* males. All male offspring are sterile. However, you will need to maintain two parental strains to breed interspecies hybrids (***for source of animals***, *see* Subheading 2).

3. Record copulation success per male.
4. Replace males according to declining mating performance and not at fixed intervals.

3.6.2 Surrogate Dams and Embryo Implantation

1. Choose dams from a mouse strain with a good record of reproduction and rearing of newborns such as CD1(ICR), Swiss Webster, NMRI, or F1 hybrid strains (e.g., B6CBAF1 or B6D2F1).
2. Ensure that sexual maturity is reached (6–8 weeks old, depending on strain).
3. Ensure that animals are in the optimal weight and age range.
4. Synchronize females by exposing them to male pheromones (Whitten effect):
   (a) Place dams on bedding of a male before their planned use.
   (b) Mate females 48 hours after exposure.
5. Mate sufficient dams to match the expected number of embryos to be transferred.
6. Identify dams in proestrus or estrus for mating with sterile males [34].
7. Non-plugged females should be used repeatedly in their next estrous cycles.
8. Consider transcervical instead of surgical embryo transfer to avoid surgery (***for source of materials***, *see* Subheading 2).
9. For transcervical implantation:
   (a) Implant embryos transcervically from the morula stage onward (*see* **Note 20**).
   (b) This is done by implantation in a non-anesthetized female using a speculum and a pipette.

10. Avoid individual housing:
    (a) Place surrogate dams in groups.
    (b) After confirming the gravidity of the dams, reduce to two animals per cage to avoid crowding after birth (*see* **Note 21**).
11. The reuse of females upon weaning of their litter for a second embryo transfer should be considered [35].

3.7 Identification of Harmful Phenotypes

1. Assess whether the new mutation leads to a harmful phenotype by analyzing animals according to Table 2.

Table 2
Template for welfare assessment for harmful phenotypes as a result of genome modification

Mortality	Consider necropsy to investigate the cause of death
Reproduction data	Litter size Infertile pairs Care by dam/cannibalism Death between birth and weaning Frequency of gravidity
Preweaning animals	Size Coloration Size differences Food intake (milk spot)
General condition	Weight/body condition score Food/water intake Skin/fur condition Senses (sight, hearing, balance) Body orifices Externally visible deformities Respiration/breathing Abnormal posture
Behavior and motor functions	Apathy Jumpiness Stereotypic behavior/barbering Aggression and bite wounds Self-mutilation Nest construction and nest condition Reaction to handling
Clinical symptoms	Tremors Seizures/convulsions Lameness Writhing Discharge (ocular, nasal) Prolapse (rectal, penis, vaginal) Tumors Malformations
Project-specific indicators	Depending on the model generated

2. The number of animals assessed should be sufficient to provide statistically significant results, but animals should not be bred for the sole purpose of a welfare assessment.
3. Assessments should be carried out after birth, at weaning and at a mature age over their entire lifetime.
4. Define specific strategies to reduce the number of affected individuals or to mitigate the harmful phenotype, e.g., by intensified care and husbandry.
5. Welfare assessments should be repeated on a regular basis until harmful phenotypes are clearly identified or ruled out.
6. Assessments should be documented and the information, including mitigation strategies or termination criteria, reported to other potential users, together with a general description of the GM line.

3.8 Beyond Rodents

Obviously, genome modification is not limited to rodents. Wherever researchers work with sentient animals, the measures outlined here must be considered to optimize animal welfare during a transgenic project. The sentient animals to which these stringent welfare requirements apply generally include vertebrates, cephalopods, and certain crustaceans (e.g., decapods). When working with non-rodents, we recommend taking our list of recommendations and adapting them to species-specific needs.

4 Notes

1. High caloric water gel is an alternative (www.clearh2o.com).
2. Take strain and line characteristics, such as harmful phenotypes, into consideration.
3. In the case of complex genome alterations, especially homologous recombination of large genome segments, the success rates of direct generation in embryos are sometimes low and can lead to the use of excessive numbers of animals.
4. While most people agree on what interventions and consequences need to be considered, there is inherent disagreement about the individual weight of each factor. While we must leave the weighing to the reader, Zintzsch et al. provide extensive guidance [36, 37].
5. The actual severity of the phenotype of the GM model must be assessed and documented and should be published after its generation.
6. Increase the probability of obtaining the desired expression pattern and strength by the use of optimized expression constructs for large vectors (BACs, YACs) [38].

7. To increase efficiency, the use of a transposon system can be considered if construct size does not exceed the maximum payload of those systems [16].
8. Integrated large constructs may be fragmented. In addition, transgenes may integrate into functional regions of the genome with deleterious effects [39, 40].
9. The transgene may have been integrated at multiple genomic locations that segregate upon breeding, resulting in variable phenotypes.
10. As a rule, ES cells must be at least as vital as the host embryo, to allow sufficient participation in embryonic development.
11. A mouse line with improved germline transmission has been developed under the name "goGermline" [6]. Male animals developing from goGermline embryos lack germ cells due to testicular atrophy and are therefore infertile. Therefore, chimeras generated with such host embryos are fertile only if ES cells take part in development and generate functional male sperm. This ensures that when the chimeras are mated, only offspring resulting from the injected ES cells are produced. Moreover, it is possible to distinguish between fertile and sterile males by palpating their testes before mating. However, the males must be anesthetized prior to palpation; otherwise, they will not tolerate the procedure, and there is a risk that the testes will be retracted into the abdominal cavity.
12. Combinations of ES cell lines and host embryos resulting in the expression of different coat colors (e.g., "black" C57BL/6 ES cells with "white" BALB/c embryos) are often used to identify chimeras with a substantial contribution of ES cells by coat color. The strain for subsequent mating should be identical to the ES cell strain to obtain a pure genetic background.
13. The improved predictability of successful genome editing comes at the cost of using more zygotes for pre-screening. However, in comparison, the increased number of donor animals needed to obtain the test embryos will likely be considerably less than the number of additional animals that would be needed to repeat an unsuccessful editing procedure. In addition, surplus colony animals that might otherwise be killed may primarily be used for such purposes, as can be surplus embryos from other experiments, provided that they are of the same strain background used to design the sgRNA.
14. Injection of single-stranded DNA (ss) or supercoiled plasmid vectors into embryos at the G2 cell cycle stage can significantly increase the efficiency of homology-mediated repair in CRISPR/Cas9 experiments [41–43]. Thus, it is possible to use CRISPR/Cas9 directly in embryos for precise integrations. Constructs can have a size of up to about 11 kilobases (kb).

15. In case of large phenotype screens, it is advisable to screen for different mutations in founders.
16. Aberrant mutations include off-target mutations, on-target insertions or deletions at the mutation site, and duplications or integration of templates away from the target site. Techniques used may include, e.g., Southern blotting, PCR, TLA, Samplix Xdrop®, or sequencing technologies.
17. Significantly lower numbers have to be expected for non-superovulated females.
18. Isoflurane anesthesia through a face mask requires a relatively rigid fixation of the narcotized mouse, which can complicate the microsurgical embryo transfer using a binocular microscope.
19. The analgesic carprofen may be given per os in the 3 days following surgery by voluntary intake, a particularly refined method [44, 45]. To facilitate this protocol, the animals must be accustomed to the oral intake. This can be done, for instance, by offering them sweetened condensed milk mixed with water (3:10) from a 200 μL micropipette over the 2 days before surgery. On the following days, the animals voluntarily drink a drug dissolved in condensed milk.
20. Multiday culture of manipulated embryos (at least up to the morula stage) before transcervical transfer may negatively affect viability and birth rate.
21. Consider that litter size after embryo transfer is usually smaller than after mating. Reduce chances of neglect and associated death of newborns by the joint rearing of the offspring of two surrogate dams.

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