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Types of genetically engineered animals

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1 Introduction

Generation and breeding of mutant animals is done by breeding of animals exhibiting spontaneous mutations or by inducing experimental alterations in the genome. This can be carried out by unspecific mutagenesis using conventional methods (e.g., chemical or physical mutagenesis) or much more precisely by using genetic engineering techniques. The induction of genetically engineered alterations which are stably transmitted to the offspring can result in mutations of defined endogenous DNA sequences in non-transgenic animals (see section 2) or - by integration of experimentally transferred gene constructs - in the generation of transgenic animals (see section 3). Genetically modified lines must be systematically analyzed on the occurrence for pain, suffering and harm of the animals.

Genetically engineered animals are used for the analysis of gene function and gene regulation (functional genome analysis), for the generation of disease models as well as for the production of biologically active proteins or modified animal products (gene farming). Generation of a high number of different genetically engineered lines is feasible for each of the ca. 20,000 protein coding mammalian genes and the additional regulatory genome loci.

Novel animal models are established by the generation of genetically engineered animals using two complementary approaches: the usually applied gene-based approach (reverse genetics) or the phenotype-based approach (forward genetics). The gene-based approach (reverse genetics) is carried out by modifying the function of a defined genome sequence chosen for a specific experimental purpose and by subsequently examining the consequences of the experimental alterations on the phenotype. Predictions on the specific phenotypic outcome are limited. For the complementary phenotype-based approach (forward genetics), transgenic animals are produced by random insertional mutagenesis via non-homologous DNA recombination and are subsequently analyzed to detect a defined phenotype chosen for a specific experimental purpose. Genetically engineered animals exhibiting the mutant phenotype of choice are further bred, and subsequently the host genome is analyzed to detect the causative mutation. Usually a loss of function is present in the host genome of these mutant lines.

Data about the numbers of animals used for experiments in Germany are published annually by the Ministry of Nutrition and Agriculture (BMEL) and show that far more than two out of three vertebrates used in animal experiments are mice; half of them are genetically modified animals. In addition, zebrafish is also used in higher numbers as genetically modified vertebrates in animal experiments (http://www.bmel.de).

The legal classification of non-transgenic animals harbouring genetically engineered mutations (see section 2) has not yet been implemented in the respective German regulations and is not within the scope of this manuscript. The induction of genetically engineered alterations in animals without germ-line transmission of the induced mutation to the offspring also is not within the scope of this manuscript.

1.1 Definitions

Using enzyme-mediated techniques like the application of sequence-specific nucleases (designer nucleases) can result in the induction of genome alterations (genome editing) without the integration of foreign DNA into the host genome. Animals derived from those experiments are genetically engineered, non-transgenic animals (see section 2).

Transgenic animals are experimentally generated mutants by transfer and integration of transgenes into the host genome. Animals exhibiting the stable genome alteration in the germ cells (gem-cell gene transfer) are further used to breed transgenic lines. Transgenesis of the animals is proven by identifying the transgene in the host genome. The function of the transgene is demonstrated by detection of transgene expression (transcribed mRNA or protein) and/or transgene-specific alterations of the host phenotype. Simply detectable marker genes (reporter genes) are used for various research purposes.

The choice of species and strain for the production of genetically engineered animals depends on the experimental purpose and comprises the classical laboratory mammals (mouse, rat, rabbit) as well as farm animals (pig, ruminants) and further vertebrates (birds, amphibians, fish). Published mutant mouse lines are collected in internet databases, where, however, only a

part of the published lines derived from the additive gene transfer as the type of induction of the mutation is covered (http://www.informatics.jax.org).

Production of valid data in such experimental projects generally requires the generation and analysis of several independent genetically engineered lines, as unintended genetic and/or epigenetic alterations due to the techniques used for the genetic modification may occur. If these unintended alterations are not linked to the mutant locus of choice, they can be removed by conventional breeding.

The use of inbred strains allows the analysis of the influence of the genetic background on the phenotype of the genetically engineered line by the generation of congenic strains. This is carried out by backcrossing the mutant or transgenic locus from the original strain (donor) into the genome of a novel strain (recipient) for ten subsequent generations which results in the exchange of the genetic background in the newly established strain except of a ca. 20 cM long genomic fragment (covering ca. 1% of the genome) harbouring the mutant or transgene locus.

The international nomenclature for genetically modified lines is described in the internet (http://www.informatics.jax.org).

1.2 Experimental purpose

The experimental purpose involving genetically engineered animals determines the strategy and the technique used for the genetic modification of the respective animal models. The experimentally induced mutation may result in an additional function by over- and/or ectopic expression of a transgene (gain of function) and/or in the partial or complete loss of function of host genes (loss of function). This includes the inactivation of specific genomic sequences (knockout), defined genomic modifications (knockin), suppression of the synthesis of specific gene products (knockdown, gene silencing) as well as random mutagenesis of the host genome (insertional mutagenesis).

The purpose of using genetically engineered, non-transgenic animals (see section 2) usually is the partial or complete loss of function of defined host genes chosen for the respective research projects.

The aim of the project may require transgene activity in specific cell types/tissues and/or at defined life stages (spatio-temporal control). Controllable transgenes may be reversibly or irreversibly functionally activated or inactivated.

1.3 General steps of the generation of mutant or transgenic animals

The generation of mutant or transgenic animals involves the use of assisted reproduction techniques (ART) as biotechnological methods which may cause unintended epigenetic alterations in the founder animals leading to phenotypic alterations. The process generally consists of the following steps:

- Production of a sequence-specific nuclease system or a functional transgene
- Transfer of the sequence-specific nuclease system or the transgene into host cells of donor animals. Depending on the technique used, gametes or progenitor cells thereof, early embryonic stages, embryonic stem cells or somatic cells are used.
- Generation of embryos derived from the treated cells and/or embryo transfer in pseudo-pregnant recipient animals (foster mothers)
- Analysis of the offspring of the recipients (founders = F0 animals) on the mutation or integration of the transgene in the host genome
- Breeding of genetically engineered lines with the genetically engineered founder animals
- Genotypic and phenotypic characterization of the genetically engineered lines

2 Non-transgenic animals harbouring genetically engineered, defined mutations

Enzyme-mediated genetic engineering techniques may induce alterations on defined genomic locations (site-specific recombination) of embryos without the integration of foreign DNA into the host genome. The purpose of using genetically engineered, non-transgenic animals

usually is the partial or complete loss of function of defined host genes chosen for the respective research projects. Therefore, artificial, programmable endonuclease systems harbouring components for binding specific DNA sequences like CRISPR-Cas9 (clustered regularly interspaced short palindromic repeat associated protein 9; or class 2 CRISPR endonuclease systems with analogous function), transcription activator-like effector nucleases (TALEN) or zinc-finger nucleases (ZFN) are constructed which bind to selected host genome sequences and cause double strand breaks at these sites. The CRISPR-Cas9 system consists of two parts, the enzyme (Cas9) and short project- and sequence-specific nucleic acids, that makes it superior to both other systems in view of the application of the technique. The subsequent DNA repair via the mechanism of non-homologous end joining (NHEJ) results in mutant animals harbouring alterations with a length of usually few base pairs (indels) at the site of the double strand break. Site and type of the mutations are not exactly controllable leading to different mutations in the affected animals. Control of the induction of mutations is increased using refined versions of the CRISPR-Cas9 system. Simultaneous mutation of multiple defined genomic sites in a given embryo has also been done.

Standard technique

The generation of the mutant animals usually is carried out using the microinjection into zygotes and contains following steps:

- Production of the programmable, sequence-specific nuclease system (CRISPR-Cas9, TALEN, ZFN). The CRISPR-Cas9 system is superior to both other systems in view of the application of the technique.
- Hormonal super-ovulation of donor animals
- Mating to fertile males
- Breeding control
- Preparation of zygotes from the oviduct
- *In vitro* injection of the sequence-specific nuclease system into the pronuclei and/or in the cytoplasm of zygotes (RNA molecules or ribonucleoprotein (RNP) complexes but not DNA constructs are usually applied for to avoid unintended random integration of DNA fragments after unspecific degradation.). Alternatively, electroporation is used as transfer technique.
- Transfer of the embryos into the oviduct of the pseudo-pregnant recipients (foster mothers)
- Analysis of the offspring of the recipients for mutations in the selected genomic site. Animals exhibiting suitable mutations are subsequently chosen for further breeding.
- Breeding of mutant lines
- Genotypic and phenotypic characterization of the mutant lines

Genotype of animals

A considerable number of the offspring of the recipients (founders = F0 animals) may exhibit mutations in the selected gene sequence usually comprising few base pairs (indels). The mutant F0 animals can be classified in two groups: (1) Animals exhibiting the identical mutation in all cells of the organism, giving rise to heterozygous mutants, homozygous mutants, or alterations of both alleles by different mutations (bi-allelic mutation, compound heterozygosity). (2) Mosaic animals exhibit the mutation not in all, but only in a fraction of the cells. After mating the mutant founders to wild-type animals, heterozygous mutant and non-mutant offspring appear in the subsequent F1 generation if the experimentally induced mutation is present in the germ cells. Presence of more than one mutant allele for the selected gene sequence in the germ cells of the mated founder animals may result in mutant F1 offspring harbouring different mutations. In this context, the appearance of more than two different mutations in the germ cells of founder animals has already been described. After mating heterozygous mutant F1 offspring harbouring the identical mutation, homozygous mutant animals appear in the F2 generation according to the Mendelian rules of inheritance. The use of inbred strains as genetic background leads to genetically uniform homozygous mutant animals within a given line.

For obtaining valid data, generation and analysis of several independent lines is also recommended in projects with genetically engineered, non-transgenic animals. *Off-target* effects

of the enzyme system used like triggering unintended double strand breaks leading to mutations at non-selected sites of the host genome or unintended integration of foreign DNA are described.

Use of nucleases with binding sites within some distance to each other may result in the deletion of the genome fragments between the selected binding sites.

3 Genetically engineered, transgenic animals

For the generation of transgenic animals, a research project-specific DNA construct (transgene) is generated *in vitro* and transferred into the host genome by various techniques.

Defined enzyme-mediated transgene integration after co-injection of transgene and sequence-specific nuclease system (CRISPR-Cas9 or class 2 CRISPR endonuclease systems with analogous function, TALEN, ZFN) also allows the generation of mutations in the host genome where – when compared to spontaneous mutations - the use of transgenes is no longer detectable in the genetically engineered, mutant animals by DNA sequencing techniques (see section 3.2.2).

3.1 Transgenes

The transgene constructed *in vitro* with the use of cloning vectors and/or by applying artificial DNA synthesis is composed of genomic DNA or recombinant DNA. Bacterial cloning vector sequences are removed before the use of the transgene. The transgene may contain isogenic, allogenic and/or xenogenic DNA sequences including functional prokaryotic genome sequences referring to the chosen host organism. The size of the transgenes usually ranges between few kb and several 100 kb. The intact integration into the host genome has to be analyzed especially when using long constructs.

The performance of the transgene may be modified by neighbouring endogenous sequences and therefore strongly depends on the integration site of the host genome (position effect). Random or defined integration of the transgene into the host genome can be achieved by applying various transfer techniques. The defined integration via homologous recombination requires a specific type of transgene, the targeting vector (see section 3.1.2). For random transgene integration into the host genome, all established transfer techniques (see section 3.2) can be used. On the other hand, the defined integration of the transgene into the host genome usually is conducted by enzyme-mediated techniques using sequence-specific nuclease systems (see section 3.2.2) and/or by non-enzyme-mediated techniques using embryonic stem cells (see section 3.2.3.1) or somatic cell nuclear transfer (see section 3.2.3.2).

Transgenes may be tested in suitable *in vitro* systems before their use in animals. However, the results may not reliably represent their performance in *in vivo* systems.

3.1.1 Non-homologous recombination of transgene and host genome

Non-homologous recombination of the transgene and the host genome result in a random integration of the transgene into the host genome; only the enzyme-mediated transgene integration also enables a non-homologous recombination event at a defined site of the host genome (site-specific recombination; see section 3.2.2). Transgene integration into the host genome by non-homologous DNA recombination usually is carried out for the production of transgene-specific RNA and/or transgene-specific proteins (additive gene transfer). The transgene may contain one or multiple genes chosen for specific research purposes with the identical or different spatio-temporal expression. In addition, the CRISPR-Cas9 system may be used as a transgene by itself (see section 3.1.1.2). Trap constructs are applied for random mutagenesis of the host genome (see section 3.1.1.4). Transposons are used for the random, enzyme-mediated gene transfer (see section 3.1.1.5).

3.1.1.1 Additive gene transfer

Transgenes for the additive gene transfer consist of three basic domains: (1) regulatory domain, (2) coding region and (3) termination unit. (1) The regulatory domain determines the site

(ubiquitous or cell type-specific) and the time point/period (constitutive or at defined life stages; spatio-temporal expression; inducible or non-inducible; see section 3.1.3) as well as the strength of the transgene expression. Complete regulatory domains including *cis*-regulatory elements (specific promoter, binding sites of transcription factors) and *trans*-regulatory (long-range) elements (enhancer, insulator, locus control region [LCR], repressor/silencer) induce the optimal activity. Optimization of the transgene design covers the regulatory control of the transgene expression. (2) The coding region contains the chosen gene. Non-coding sequences including regulatory sequences and an exon/intron structure promote the effective expression. Alternatively, non-protein-coding RNA may be expressed (see section 3.1.1.3, gene silencing). (3) The termination unit provokes the proper expression of the transgene.

If an endogenous host gene is used for the additive gene transfer, discrimination of the expression of transgene and endogenous gene on the protein level may be done by applying different alleles or by linking additional short translated DNA sequences to the transgene which give rise to additional epitopes (peptide tags) to be used for detection by specific antibodies. However, they may also interfere with the protein activity.

Expression cassettes include tested regulatory and termination units as well as intermediate DNA sequences (multiple cloning site [MCS]) for the insertion of the coding sequences of choice. Using these tools, the expression of the transgene is predictable at some degree.

Sequences exhibiting shielding properties (insulator, matrix attachment region [MAR], scaffold attachment region [SAR]) against adjacent host genome sequences may increase specificity and copy number-dependence of the transgene expression, however, contrasting results are published after the application of these tools. Autonomously replicating transgenes without integration into the host genome (like mammalian artificial chromosomes [MAC]) result to date in an instable inheritance of the foreign DNA.

Inactivation or ablation of defined cells or cell compartments may be induced by spatio-temporal expression of toxic products or receptors of toxic substances as transgene (e.g., the diphtheria toxin or the receptor of the diphtheria toxin).

3.1.1.2 Use of CRISPR-Cas9 as a transgene

The CRISPR-Cas9 system (or class 2 CRISPR endonuclease systems with analogous function) may be used as a DNA construct by itself for the additive gene transfer to evoke spatio-temporal alterations in the host genome of the transgenic animals. The CRISPR-Cas9 system consists of two parts, the enzyme (Cas9) and short research project- and sequence-specific nucleic acids. Thus, both parts or the enzyme Cas9 alone may be applied as a transgene. The latter case requires the exogenous application of sequence-specific nucleic acids. Application of DNA constructs coding for an active Cas9 enzyme usually aims for the generation of short indels in selected genomic sequences. Alternatively, use of DNA constructs coding for enzymatically non-active Cas9 proteins in combination with additional effectors linked to the enzymatically non-active Cas9 proteins may lead to sequence-specific activation or deactivation of the expression of selected genomic sequences. Spatio-temporal control of the transgene system is feasible by using appropriate promoter sequences and/or controllable systems (see section 3.1.3). *Off-target* effects of these systems may occur.

Transgenes harbouring the coding sequences of sequence-specific nucleases (and potentially further genes, so-called cargo genes) flanked by parts of its own target sequence may induce sequence-specific double strand breaks by the expressed nuclease at the site of the complete target sequence in the host genome of the produced transgenic organisms. This increases the chance of homologous recombination events at this site leading to the subsequent insertion of the transgene at the site of the double strand break. Transfer of the transgene via self-duplication in diploid cells may directly induce homozygous transgenic genotypes of the cells. Thus, introduction of this system into a population may yield sequence-specific genetic modifications in the organisms of the population („mutagenic chain reaction“, gene drive).

3.1.1.3 Suppression of specific host gene products

RNA interference (RNAi) by additive transfer of DNA sequences expressing non-protein-coding regulatory RNA sequences (ncRNA) is applied for the targeted post-transcriptional suppression of the synthesis of defined host genome products (gene knockdown, gene silencing). Therefore, the transgene codes for the expression of naturally occurring or artificially designed RNA molecules that specifically inactivate complementary mRNA molecules of the host cell by producing RNA double strand molecules together with the mRNAs. This leads to the subsequent degradation of these structures thereby decreasing or completely inhibiting the production of the respective host protein. Specificity and level of inactivation vary in the publications of such projects, and the complete inactivation turned out to be difficult.

3.1.1.4 Trap constructs

Trap constructs contain regulatory elements in various versions (enhancer-, promoter-, gene-, poly (A)-trap) and marker genes (reporter gene). They are used for the induction of random insertional mutations via non-homologous DNA recombination in the host genome, typically in embryonic stem cells. In addition, retroviral vectors are used as trap constructs. The vectors may induce extensive alterations at the insertion site of the host genome. The mutant cells are clonally cultivated, analyzed for the successful expression of the marker gene as well as for the induced mutation caused by the integration of the trap construct into the host genome. After generating transgenic animals with the mutant cells of choice, *in vivo* analysis of the mutated host genome sequences (loss of function) is carried out. The mutant cells generated by this technique in various approaches worldwide are listed in several online databases (http://www.genetrap.org; http://www.mousephenotype.org; http://www.informatics.jax.org).

3.1.1.5 Transposons

Use of two-piece DNA transposon systems derived from various species like e.g., *sleeping beauty* (SB) or *piggyBac* (PB) gives rise to a random, enzyme-mediated gene transfer. The transgene is flanked on both sides by the target sequence (inverted terminal repeats, ITR) of the enzyme transposase. Transgene integration at random sites induces only small alterations of the adjacent host genome.

Usually DNA microinjection of transgene and transposase with temporally limited activity is carried out for the additive gene transfer that may result in increased efficiency of transgene integration, insertion of single copies per integration site and multiple segregating integration sites in the host genome when compared to the conventional technique (see section 3.2.1.1). The length of the transgenes used is limited for the successful *in vivo* application.

In addition, DNA transposons may be applied for the induction of random insertional mutations in the host genome of mammals which includes the generation of two transgenic lines. The first transgenic line harbours the transgene flanked by the target sequence of the transposase, and the second line is transgenic for the enzyme transposase. Breeding both lines may result in transgenic offspring exhibiting multiple random insertional mutations for the subsequent use in phenotype-based forward genetics approaches.

Published work described that complete reconstitution of the wild-type allele is possible by the enzyme-mediated removal of integrated transgenes of the *piggyBac* (PB) system through a subsequent transposition.

3.1.2 Homologous recombination of transgene and host genome

Targeted integration of the transgene into the host genome allows the induction of complete inactivation (knockout) or defined modifications (knockin) of specific host genes. It is conducted by enzyme-mediated techniques with the use of sequence-specific nuclease systems (see section 3.2.2), and is carried out directly in embryos or indirectly in embryonic stem cells (see section 3.2.3.1) or in cells that are subsequently used for the somatic cell nuclear transfer (see section 3.2.3.2). Usually double stranded or single stranded DNA targeting vectors are applied as transgenes. Due to the high efficiency of the sequence-specific nuclease systems,

targeting vectors without marker genes for the selection process may be used thereby allowing the generation of mutations in the host genome where – when compared to spontaneous mutations - the use of transgenes is no longer detectable in the genetically engineered, mutant animals by DNA sequencing techniques (see section 3.2.2).

Alternatively, the conventional, non-enzyme-mediated targeted integration of the transgene into the host genome is feasible *in vitro* by homologous recombination in embryonic stem cells (see section 3.2.3.1) or with the somatic cell nuclear transfer (see section 3.2.3.2). This method obligatorily requires a targeting vector as specific transgene and results in a low efficiency. In the targeting process, known genome sequences are replaced with the transgene leading to the defined mutation of endogenous DNA sequences. Thereby, homologous recombination of DNA sequences identical to the host genome on both sides of the transgene with the endogenous host sequences occurs (gene targeting). The length of the introduced mutation is highly variable covering point mutations as well as long DNA fragments. Subsequently, transgenic animals are generated using these *in vitro* specifically mutated cells.

Prerequisite for the construction of targeting vectors is the knowledge of the DNA sequence and the structure of the chosen host gene (http://www.ensembl.org). Integration site-specific expression cassettes (see section 3.1.1.1) are available as targeting vectors allowing the controlled production of transgene-specific proteins (additive gene transfer) by the integration of the transgene at a defined position of the host genome like e.g., the ROSA26 locus.

Both ends of the classical targeting vector harbour DNA sequences which are usually a few kb in length, homologous to the chosen integration site of the host genome and derived from isogenic DNA of cells planned to be used for the generation of the specific mutation. Double stranded or single stranded targeting vectors with only short homologous DNA sequences work well when combined with the use of sequence-specific nuclease systems. The intended mutation is inserted between both homologous ends. The targeting vector also includes marker genes which are essential for the selection of the cell clones with the desired alteration. The positive marker gene usually remains in the genome of the established transgenic animals, but may be also removed from the host genome *in vitro* or *in vivo* in the case that appropriate transgene constructs are used.

3.1.3 Control of the transgene function

Transgene activity is primarily controlled transcriptionally by using appropriate regulatory elements. This does not modify the integrated transgene by itself. Furthermore, the defined modification of the transgene locus in the transgenic animals is feasible by using conditional mutagenesis. Both systems can be combined.

3.1.3.1 Induction of transgenes

On the transcription level, the spatio-temporal transgene expression can be reversibly regulated. Inducible transgenes with appropriate promoter sequences allow the functional activation or inactivation of the transgene. The passive state of the transgene shows no or only small activity, whereas reversible induction increases the activity. Inactivation of the transgene function is conducted in the reverse direction. In both cases, for practical reasons, the inactivation of the inducible promoter should be almost complete.

Promoter control is conducted (1) by application of exogenous substances. Endogenous substances combined with their target promoters do usually not give rise to a stringent regulation of the induction of transgene expression. Triggering the transgene induction by synthetic substances or extrinsic proteins may avoid additional unintended effects of the organism or unintended endogenous cross reactions. Recently, controlled application of essential amino acids via food combined with the use of optimized promotor elements (amino acid response elements) has been described to result in a low basal expression and a high inducible and reversible expression of transgenes. Experiments for the spatio-temporal, reversible induction of transgene expression via exposure to blue light combined with synthetic transactivator and promoter elements (*LightOn* system) have principally been successful, but depend on the penetration of the light into the body tissues.

On the other hand, promoter control is conducted (2) by two-piece transgene systems. This requires the generation of two transgenic lines each of them harbouring one part of the system as a transgene. After breeding double transgenic animals by mating both transgenic lines, the spatio-temporal control of the expression of the first transgene is conducted by tissue-specific and/or time point-specific promoter sequences of the second transgene which drive the expression of a product that subsequently interferes with the promoter of the first transgene thereby resulting in its activity or inactivity. (3) The two-piece transgene system can be combined with exogenously supplied substances.

Often systems using the application of tetracycline or derivates thereof (e.g., doxycycline) are administered. Here, the expression of the chosen transgene is based on the transgenic expression of the tetracycline-dependent, active transactivator tTA (tet-off system) or the tetracycline-dependent, inactive reverse transactivator rtTA (tet-on system) under tissue-specific promoter control. The tet-off system relies on the expression of the chosen transgene via binding of the usually tissue-specifically expressed active transactivator tTA to the promoter element TRE (tetracycline responsive element) of the chosen transgene. Application (via food, drinking water or injection) of tetracycline or doxycycline leads to the binding of this exogenous factor to the transactivator tTA which subsequently prevents the binding of the now inactive transactivator tTA to the promoter element TRE and thereby prevents the expression of the chosen transgene.

The reverse tet-on system relies on the absence of the binding of the usually tissue-specifically expressed, reverse transactivator rtTA to the promoter element TRE. Thus, no expression of the chosen transgene takes place. Application of tetracycline or doxycycline leads to the binding of the newly forming rtTA-doxycycline complex to the promoter element TRE, and subsequently to the induction of the expression of the chosen transgene.

3.1.3.2 Conditional mutagenesis

The spatio-temporal function of genes can be analyzed with inducible systems allowing the activation or inactivation of transgenes (conditional mutagenesis). The induction gives rise to a gene knockout/-in and is irreversible.

For this purpose, two-piece recombination systems like the bacterial *cre/loxP* system (*cre* = causes recombination, *loxP* = locus of crossover (X) of bacteriophage P1) are applied. The *cre* recombinase catalyzes the recombination of the DNA fragment between the two short identical *loxP* recognition sites. Therefore, two transgenic lines are generated: the first line exhibits the usually cell type-specific expression of the *cre* recombinase as a transgene, and the second line exhibits the chosen gene that is flanked on each side with the *loxP* sequences (floxed target gene). The transgenic lines are generated by using enzyme-mediated, sequence-specific nuclease systems and/or by non-enzyme-mediated techniques via targeting vectors. Breeding both lines yields double transgenic animals where the recombination is triggered by the *cre* recombinase. Tissue- and/or time point-specific promoter sequences of the transgene harbouring the *cre* recombinase control the spatio-temporal regulation of the conditional mutagenesis. The spatio-temporally controlled and/or incomplete conditional mutagenesis results in the generation of mosaic animals. Prolonged and/or high expression of recombinases may induce unintended interactions with endogenous sites of the host genome as erroneous target sites (*off-target* effects) and the phenotypic consequences resulting thereof. This may be overcome by using heterozygous *cre* recombinase-transgenic animals. Moreover, use of the *cre*-transgenic animals as an additional negative control group is recommended in such experiments. Published *cre* recombinase-transgenic mouse lines are collected in online databases (e.g., http://www.informatics.jax.org).

Position and orientation of the *loxP* recognition sites determine the arising recombination event. Position of the *loxP* recognition sites on one DNA molecule and identical orientation give rise to the deletion of the intermediate DNA fragment. Position of the *loxP* recognition sites on two DNA molecules (e.g., on an integrated transgene and an exogenously supplied transgene; or on two integrated transgenes at different genome loci) and identical orientation give rise to the translocation of both DNA molecules at these sites. Position on one DNA molecule and inverse orientation result in the *cre*-mediated inversion of the intermediate DNA fragment which may induce a change in the function of the corresponding DNA fragment. Possible applications of the recombination systems are the generation of genome recombinations with a length of up to

several 100 kb (deletion, inversion, translocation), the removal of marker genes, gene knockin applications, or the generation of defined point mutations in the host genome. For instance, stop sequences flanked by *loxP* sequences can be inserted in front of specific gene sequences, thereby transcriptionally or translationally preventing the expression of the respective gene. The *cre* recombinase removes the stop sequence and irreversibly gives rise to the expression of the gene.

The application of the induction approach (see section 3.1.3.1) on the *cre/loxP* system can be done by using the respective inducible promoter sequences for the expression of the *cre* recombinase. Thus, transcription of the transgenic *cre* recombinase can be reversibly controlled by combining the *cre/loxP* system with the tet-on system or the tet-off system and the exogenous application of tetracycline or doxycycline in a three-piece transgene system: Transgene 1 harbouring the tissue-specific promoter for the expression of the transactivator, transgene 2 with the promoter element TRE (tetracycline responsive element) for the expression of the *cre* recombinase and transgene 3 with the *loxP* recognition sequences of the *cre* recombinase. This requires the breeding of triple transgenic animals (see section 3.1.3.1). The genome recombination caused by the transgenic *cre* recombinase is irreversible.

The reversible control of the *cre* function on the posttranslational level is done by linking the transgenic *cre* recombinase to a mutant ligand binding domain of the estrogen receptor. Endogenous estrogen does not bind to the mutant ligand binding domain. Without the exogenous application of the estrogen derivate tamoxifen, the transgenic fusion protein of *cre* recombinase and estrogen receptor cannot enter through the cell membrane to the nucleus as the site of its function. This occurs only after exogenous application of tamoxifen which subsequently binds to the fusion protein. The genome recombination caused by the transgenic *cre* recombinase is irreversible. Soybeans contain phytoestrogens which may interfere with the estrogen receptor and cause unintended reactions. Therefore, food free of or containing only low levels of phytoestrogens should be used. The same is true for bisphenol A that is found especially in the plastic of water bottles.

Conditional mutagenesis is also induced by similar tools like the yeast-derived *flp/FRT* system and further yeast-derived systems, the bacterial *dre/rox* system, mutant systems of the *cre/loxP*- and *flp/FRT* system, and the bacteriophage-derived *ΦC31/attP/attB* system.

These recombinases can be also combined for the efficient integration of transgenes at target sites. Simultaneous use of two different systems with non-interacting recognition sequences and with the recognition sequence located both in the host genome and on an exogenously applied transgene construct may cause *in vitro* or *in vivo* the exchange of the DNA fragment between the different recognition sequences via the activity of the two respective recombinases (recombinase-mediated cassette exchange, RMCE).

Simultaneous use of two different systems with non-interacting recognition sequences and with inverse orientation of the respective recognition sites on a transgene may cause a first recombination in form of an inversion (and usually the loss of function of the intermediate DNA fragment), and a second recombination in form of the repeated inversion (and the rescue of the function of the intermediate DNA fragment).

3.2 Techniques for the generation of transgenic animals

The function of the transgene potentially depends on the integration site in the host genome (position effect). Non-enzyme-mediated direct transfer methods include DNA microinjection, viral vectors and sperm-mediated gene transfer that result in the random insertion of the transgene into the host genome (non-homologous DNA recombination, see section 3.2.1). Enzyme-mediated transfer methods by using sequence-specific nuclease systems are much more efficient and allow the targeted insertion of the transgene into the host genome (see section 3.2.2). Alternatively, targeted insertion of the transgene into the host genome is possible indirectly by non-enzyme-mediated, *in vitro* operating tools via homologous DNA recombination in embryonic stem cells (ES cells) or in cells subsequently used for the somatic cell nuclear transfer (cloning), however this is much less efficient (see section 3.2.3).

Transgenic animals harbouring the genetic modification in one allele after non-homologous DNA recombination are named as hemizygous transgenic animals. Transgenic animals harbouring the genetic modification in one allele after homologous DNA recombination

are named as heterozygous transgenic animals. Transgenic animals harbouring the genetic modification in both alleles are named as homozygous transgenic animals irrespective of the kind of DNA recombination.

Compared to the low efficiency of the basal DNA microinjection technique, the establishment of novel techniques for the generation of transgenic animals may have the potential to lower the number of animals needed in the experiment.

3.2.1 Random transgene integration by non-homologous recombination

Non-homologous recombination of transgene and host genome without the use of sequence-specific nuclease systems results in the random insertion of the transgene into the host genome. Transgenes for the additive gene transfer are suitable tools for this techniques (see section 3.1.1).

3.2.1.1 DNA microinjection

Microinjection of DNA constructs is the basal standard method of the additive gene transfer for all vertebrate species. The efficiency of the method, i.e. the number of transgenic offspring related to the number of microinjected embryos, without the use of sequence-specific nuclease systems is described to be 1-5 % for laboratory animals.

Standard technique

The generation of transgenic animals via DNA microinjection contains following steps:

- Production and *in vitro* testing of the transgene construct
- Hormonal super-ovulation of donor animals
- Mating to fertile males
- Breeding control
- Preparation of zygotes from the oviduct
- *In vitro* injection of the transgene into the pronuclei of zygotes
- Transfer of the embryos into the oviduct of the pseudo-pregnant recipients (foster mothers) (Alternative: Prolonged *in vitro* incubation of the embryos (blastocysts) and trans-cervical transfer into pseudo-pregnant recipients)
- Analysis of the offspring of the recipients for transgenesis
- Parallel breeding of several transgenic founder lines
- Genotypic and phenotypic characterization of the transgenic lines

Genotype of animals

Up to 25% of the offspring of the recipients exhibit the random insertion of few or up to several hundred transgene copies usually in one non-segregating site of the host genome. The insertion site may contain complete and fragmented transgene copies in both orientations mixed with endogenous host sequences. The host genome may be altered at the boundaries to the transgene by recombinations with a length of several kb.

The transgenic animals (founders = F0 animals) can be classified in two groups, while both kinds of mutations may also appear combined in a given founder animal: (1) Hemizygous transgenic animals exhibit the transgene at the identical genomic site in all cells of the organism, (2) while mosaic animals exhibit the transgene not in all, but only in a fraction of the cells. After mating the founders to non-transgenic wild-type animals, hemizygous transgenic and non-transgenic offspring occur in the subsequent F1 generation if the experimentally induced mutation is present in the germ cells. Founder animals harbouring segregating transgene insertion sites may give rise to the production of independent transgenic lines. After mating hemizygous transgenic F1 littermates, homozygous transgenic animals appear in the F2 generation according to the Mendelian rules of inheritance. The use of inbred strains as genetic background leads to genetically uniform homozygous transgenic animals within a given line. Usually transgene locus and transgene expression remain stable after the establishment of the first few generations of a transgenic line.

Insertion site and transgene locus of the transgenic lines are random that may give rise to potential differences in time point, site and strength of the transgene expression in the established lines of a given research project. This makes it necessary to generate and analyze several independent lines for the project.

Using methods leading to the random insertion of the transgene into the host genome results in the unintended alteration of host genes (insertional mutation) in a low number of the established transgenic animals. This may give rise to unforeseen, not transgene-specific alterations of the physiological phenotype of the usually affected homozygous transgenic animals (in the case of a recessive mutation). In the event that the insertional mutation is lethal, no hemizygous transgenic and/or homozygous transgenic animals appear further on from the affected life stage.

3.2.1.2 Viral vectors

Various viral vectors are used for the insertion of transgenes into the host genome. Generation of transgenic vertebrates is carried out with genetically modified viral particles. Using their own enzymes, retroviruses complementarily transcribe their RNA genome into double stranded DNA that is subsequently inserted into the host genome. Transgenic lines showing stable integration of the gene construct into the host genome and function of the transgene are usually produced with recombinant lentiviral vectors. Translocation of the lentiviral genome into the host cell nuclei takes place via nucleopores which allows the direct transgene insertion. Translocation of other retroviral genomes into the host cell nucleus requires the division of the respective cell. Lentiviral vectors offer the possibility of the virus-independent, spatio-temporal control of the transgene by using suitable promoter sequences. The injection technique used causes a less strong physical manipulation of the embryos when compared to the conventional DNA microinjection method.

Standard technique

The generation of transgenic animals with lentiviral vectors contains following steps:

- Production of the DNA transgene construct
- Production of infectious lentiviral particles which contain the transgene in the virus genome in form of RNA sequences, by using packaging constructs and specific cell lines
- Hormonal super-ovulation of donor animals
- Mating to fertile males
- Breeding control
- Preparation of zygotes from the oviduct
- Infection of the zygotes (or alternatively of non-fertilized oocytes and subsequent *in vitro* fertilization [IVF]) with the recombinant retroviral particles by injection of the lentiviral particles into the perivitelline space or by co-incubation of the embryos after removal of the z*ona pellucida* with the lentiviral particles
- Transfer of the infected embryos into the oviduct of the pseudo-pregnant recipients (foster mothers)
- Analysis of the offspring of the recipients for transgenesis
- Parallel breeding of several transgenic lines
- Genotypic and phenotypic characterization of the transgenic lines

Genotype of animals

Up to a high percentage of the offspring of the recipients may be transgenic, and transgene copies may be inserted in several segregating sites of the host genome of a founder animal. Thus, production of independent transgenic lines may be feasible from a given founder animal. Each of the random insertion sites harbours one transgene copy and exhibits alterations of the host genome on the boundaries to the transgene with a length of only few nucleotides.

The transgenic animals (founders = F0 animals) can be classified in two groups: (1) Hemizygous transgenic animals exhibit the transgene at the identical genomic site in all cells of the organism, (2) while mosaic animals exhibit the transgene not in all, but only in a fraction of

the cells. After mating the founders to non-transgenic wild-type animals, hemizygous transgenic and non-transgenic offspring appear in the subsequent F1 generation if the experimentally induced mutation is present in the germ cells. After mating hemizygous transgenic F1 littermates, homozygous transgenic animals appear in the F2 generation according to the Mendelian rules of inheritance. The use of inbred strains as genetic background leads to genetically uniform homozygous transgenic animals within a given line.

The insertion site of the transgenic lines is random that may give rise to potential differences in time point, site and strength of the transgene expression in the established lines of a given research project. This makes it necessary to generate and analyze several independent lines for the project. Using methods leading to the random insertion of the transgene into the host genome results in the unintended alteration of host genes in a low number of the established transgenic animals (insertional mutation, see section 3.2.1.1).

Disadvantages of the system are increased demands on the biosecurity level for the work with infectious lentiviral particles, the limited length of the transgene and a potential loss of function of integrated transgenes in the following generations via endogenous methylation of the transgene locus. Afterwards, the specific methylation status of the transgene locus and the transgene expression level of the produced lines seem to be stable during the breeding of further generations.

3.2.1.3 Sperm-mediated gene transfer

The sperm-mediated gene transfer is a simple technique to generate transgenic animals. However, up to now published results demonstrate the principal successful use in mammals but considerable difficulties in establishing the technique and achieving reproducible results. In total, up to now especially the successful incubation of sperm and transgene is not implemented as a routine method.

Several methods and combinations of methods are described for the sperm-mediated gene transfer, and various methods are published for the incubation of sperm or its progenitor cells and the transgene.

(1) *In vitro* incubation of sperm and transgene; subsequently applying *in vitro* fertilization (IVF):
- Preparation of sperm from fertile donor animals
- *In vitro* incubation of sperm and transgene
- Preparation of oocytes after super-ovulation of donor animals
- *In vitro* fertilization (IVF) of non-fertilized oocytes with the treated sperm (in special cases by intracytoplasmic injection of the treated sperm [ICSI] into the non-fertilized oocytes)
- Transfer of the zygotes or higher developmental stages after *in vitro* culture into pseudo-pregnant recipients (foster mothers)

(2) *In vitro* incubation of sperm and transgene; subsequently applying direct artificial insemination (AI):
- Preparation of sperm from fertile donor animals
- *In vitro* incubation of sperm and transgene
- Artificial insemination of recipients with the treated sperm

(3) Injection of the transgene into the testes:
- Direct injection of the transgene into the testes of fertile animals with or without subsequent electroporation (alternative: use of viral vectors)
- Mating of the treated recipients to untreated females

(4) *In vitro* incubation of sperm progenitor cells and transgene:
- Preparation of sperm progenitor cells from the testes of fertile donor animals
- *In vitro* incubation of sperm progenitor cells and transgene
- Injection of the transgenic sperm progenitor cells into the testes of unfertile recipients
- Mating of the recipients to untreated females

Using these methods may give rise to offspring that may be transgenic up to a high percentage. The transgenic animals exhibit the random insertion of transgene copies in one or more segregating site(s) of the host genome. Little is known about these transgene loci.

The transgenic animals (founders = F0 animals) can be classified in two groups: (1) Hemizygous transgenic animals exhibit the transgene at the identical genomic site in all cells of the organism, (2) while mosaic animals exhibit the transgene not in all, but only in a fraction of the cells. No mosaic animals appear when using transgenic sperm progenitor cells for the gene transfer. After mating the founders to non-transgenic wild-type animals, hemizygous transgenic and non-transgenic offspring appear in the subsequent F1 generation if the experimentally induced mutation is present in the germ cells. After mating hemizygous transgenic F1 littermates, homozygous transgenic animals appear in the F2 generation according to the Mendelian rules of inheritance. Little is known about the stability of transgene loci and transgene expression levels after gene transfer via incubation of sperm and transgene.

The insertion site of the transgenic lines is random that may give rise to potential differences in time point, site and strength of the transgene expression in the established lines of a given research project. This makes it necessary to generate and analyze several independent lines for the project. Using methods leading to the random insertion of the transgene into the host genome results in the intended alteration of host genes in a low number of the established transgenic animals (insertional mutation, see section 3.2.1.1).

3.2.2 Defined, enzyme-mediated transgene integration by non-homologous or homologous recombination

The defined, enzyme-mediated transgene integration by non-homologous or homologous recombination can be directly applied *in vivo* in the embryo at the site of the double strand break that is sequence-specifically triggered in the host genome. Co-injection of a transgene and artificially programmable endonuclease systems harbouring components for binding specific DNA sequences like CRISPR-Cas9 (clustered regularly interspaced short palindromic repeat associated protein 9; or class 2 CRISPR endonuclease systems with analogous function), transcription activator-like effector nucleases (TALEN) or zinc-finger nucleases (ZFN) (see section 2) may result in the defined transgene integration by non-homologous recombination of transgene and host genome via the repair of the double strand break by the mechanism of non-homologous end joining (NHEJ). On the other hand, co-injection of an appropriate targeting vector (see section 3.1.2) in the form of double stranded or single stranded DNA molecules and a nuclease system may give rise to the enzyme-mediated transgene insertion by homologous recombination of targeting vector and host genome via the mechanism of homology-directed repair (HDR). Refined versions of the CRISPR-Cas9 system demonstrate a higher level in the occurrence of the HDR mechanism. *Off-target* effects of the enzyme system may appear.

The relatively high efficiency of the systems allows the use of targeting vectors without exogenous selection markers (see section 3.1.2) that may give rise to mutations in the host genome where – when compared to spontaneous mutations - the use of transgenes is no longer detectable in the genetically engineered, mutant animals by DNA sequencing techniques.

Standard technique

The generation of the transgenic animals is carried out using the microinjection technique (see section 3.2.1.1) into zygotes and contains following steps:

- Production of the programmable, sequence-specific nuclease system (CRISPR-Cas9, TALEN, ZFN) and the transgene construct. The CRISPR-Cas9 system consists of two parts, the enzyme (Cas9) and short research project- and sequence-specific nucleic acids, that makes it superior to both other systems in view of the application of the technique.
- Hormonal super-ovulation of donor animals
- Mating to fertile males
- Breeding control
- Preparation of zygotes from the oviduct
- *In vitro* co-injection of the sequence-specific nuclease system and the transgene into the pronuclei and the cytoplasm of zygotes
- Transfer of the embryos into the oviduct of pseudo-pregnant recipients (foster mothers)
- Analysis of the offspring of the recipients for transgene insertion in the selected genomic site
- Breeding of transgenic lines

- Genotypic and phenotypic characterization of the transgenic lines

Sequence-specific nuclease systems are also used for the targeted transgene insertion *in vitro* in embryonic stem cells (see 3.2.3.1) or in cells which are subsequently used for the somatic cell nuclear transfer (see 3.2.3.2).

Genotype of animals

Compared to methods for the defined, non-enzyme-mediated transgene insertion by homologous recombination (see section 3.2.3), a considerable number of the offspring of the recipients may be transgenic. The transgenic animals harbouring the desired mutation (founders = F0 animals) can be classified in two groups: (1) Hemizygous transgenic (derived from non-homologous recombination events) or heterozygous transgenic (derived from homologous recombination events) animals exhibit the transgene in all cells of the organism, (2) while mosaic animals exhibit the transgene not in all, but only in a fraction of the cells. In transgenic F0 animals, also both alleles may be affected and/or may show different mutations. After mating the founders to non-transgenic wild-type animals, hemizygous transgenic or heterozygous transgenic animals and non-transgenic offspring appear in the subsequent F1 generation if the experimentally induced mutation is present in the germ cells. Founder animals harbouring different mutant alleles of the selected genome sequence in the germ cells may give rise to the production of independent transgenic lines. After mating hemizygous transgenic or heterozygous transgenic F1 littermates of the same line, homozygous transgenic animals appear in the F2 generation according to the Mendelian rules of inheritance. The use of inbred strains as genetic background leads to genetically uniform homozygous transgenic animals within a given line.

Off-target effects of the enzyme systems used like triggering mutations at non-selected sites of the host genome or unintended integration of foreign DNA are described. Thus, generation and analysis of several independent lines is recommended for obtaining valid data.

Co-injection of a transgene with the respective recognition sites and specific recombinases/integrases combined with endogenous target sequences in the host genome or in genetically modified host strains (e.g., by two-piece recombination systems like the *cre/loxP* system) may result in the enzyme-mediated exchange of the DNA fragment between the recognition sites (recombinase-mediated cassette exchange, RMCE) (see section 3.1.3.2). This principally allows the insertion of an intact transgene copy into defined chromosomal loci with high efficiency. Possible *off-target* effects of the enzymes used are to be considered.

3.2.3 Defined, non-enzyme-mediated transgene integration by homologous recombination

The defined mutation of the genome by homologous recombination of extended DNA sequences (gene targeting) without the use of sequence-specific nuclease systems requires the use of targeting vectors as transgenes (see section 3.1.2). In this case, *in vitro* targeted mutant cells are produced and applied to the blastocyst injection of embryonic stem cells or to the somatic cell nuclear transfer. The aim of the method usually is the functional inactivation (knockout) or defined modification (knockin) of (a) specific gene(s).

The defined *in vitro* mutagenesis of cells is much more efficiently feasible by using sequence-specific nuclease systems without or with transgene(s) (see sections 2 and 3.2.2).

Technical developments

Research projects for the production of germ cells, germ cell-specific stem cells or induced pluripotent stem cells (iPSC) have been carried out in different species with the purpose of the untargeted or targeted insertion of transgenes into the host genome by non-homologous or homologous DNA recombination *in vitro* in these cells. These methods are technically demanding and not established as routine procedures. The principal occurrence of genetic and/or epigenetic lesions as consequences of *in vitro* cell culture or genomic reprogramming processes and the phenotypic outcome thereof has to be considered.

3.2.3.1 Embryonic stem cells (ES cells)

The generation of transgenic vertebrate lines by using ES cells with targeted mutations up to now is established only for mice, and in addition, for rats. A functional analogous cell system exists for poultry in the form of primordial germ cells (PGC). ES cell lines are derived from the inner cell mass (ICM) of blastocysts and are available for only few inbred strains. Murine ES cell lines were originally developed from various sublines of the inbred strain 129; thereafter functional ES cells were established from additional inbred strains like C57BL/6. The homologously recombined murine ES cells have the potential to colonize the germ line and subsequently provide cells for the development of the mutant organism.

Standard technique

The generation of transgenic lines by using ES cells contains following steps:

- Production of the targeting vector (knockout/-in construct) for the homologous recombination
- *In vitro* enzyme-mediated or non-enzyme-mediated gene targeting in ES cells (originally mainly in ES cells of the inbred strain 129) that results in the heterozygous or homozygous homologous recombination in the selected mutant cell clones
- Hormonal super-ovulation of donor animals (e.g., BALB/c or C57BL/6 inbred mice, for to use the coat colour as a marker for transgenesis in the offspring)
- Mating to fertile males
- Breeding control
- Preparation of blastocysts from the uterus
- *In vitro* injection of the genetically modified ES cell clones into blastocysts (alternative: co-cultivation of homologously recombined ES cells and embryos after removal of the *zona pellucida* [aggregation])
- Transfer of the injected (or aggregated) embryos into pseudo-pregnant recipients (foster mothers)
- Analysis of the offspring of the recipients for transgenesis
- Breeding of transgenic lines
- Genotypic and phenotypic characterization of the transgenic lines

Genotype of animals

A low percentage of the offspring of the recipients is transgenic. The transgenic animals (founders = F0 animals) are chimeras; they consist of non-transgenic cells derived from the cells of the blastocyst and of transgenic cells derived from the injected (or aggregated), homologously recombined ES cells. Certain combinations of the genetic backgrounds of ES cells and blastocysts are described to yield an increased chance that the homologously recombined ES cells participate in the germ cell development (germ line chimerism). ES cells and blastocysts are used from different mouse strains or mouse lines harbouring different coat colours (originally mainly ES cells of the inbred strain 129 and blastocysts e.g. of the inbred strain C57BL/6) that allows the use of the coat colour for identifying transgenic offspring. Chimeras show both coat colours in the case that they are derived from non-transgenic as well as transgenic cells (and both cell populations contribute to the development of the coat). In addition, the offspring of the recipients are molecular genetically analyzed for transgenesis. The transgene locus reflects the desired mutation that was verified in the ES cell clones chosen for the injection or aggregation.

The sex of the chimeras and its functionality depend on the mostly male sex of the established ES cell lines and the sex of the blastocysts used for the injection or aggregation.

Usually male chimeras are mated to non-transgenic, wild-type animals of the inbred strain of the blastocyst donor for the production of transgenic lines. In the event that the transgenic cells participate in the germ cell development (germ line chimerism) heterozygous transgenic animals appear in a varying percentage in the F1 generation which are F1 hybrids in their genetic background (in the example described: F1 hybrids of the inbred strains 129 and C57BL/6). After mating heterozygous transgenic F1 littermates, homozygous transgenic animals appear in the F2 generation according to the Mendelian rules of inheritance. For the described example, the

absent genetic uniformity of the genetic background of the mutant line often requires the backcross of transgenic F2 hybrids to the genetic background of an inbred strain. This results in homozygously mutated transgenic animals with high genetic uniformity (see section 1.1) after ten backcross generations (= congenic strain). In addition, this also removes potential genetic lesions of the ES cell line used which are not linked to the targeted locus from the transgenic lines.

The mutations produced may also turn out to be recessive mutations, thereby showing an altered phenotype only in homozygous transgenic animals. Moreover, in homozygous transgenic knockout animals also a grossly normal phenotype may appear depending on the chosen gene and/or mutation.

The ES cell clones with the verified mutation used in the procedure may contain random genetic lesions as a consequence of the *in vitro* cell culture which may affect the phenotype of the transgenic animals. Thus, generation and analysis of several independent lines established with independent, genetically modified ES cell clones is recommended for obtaining valid data.

Technical developments

Numerous modifications are published for the ES cell technology. In some cases, the general practicability as a routine procedure is still under analysis:

The absent uniformity of the genetic background in the following generations after breeding the chimeras is avoided by using ES cells and embryos from two co-isogenic inbred strains, e.g., ES cells of the normal pigmented inbred strain C57BL/6 and blastocysts of unpigmented, tyrosinase mutant sublines of C57BL/6.

After having used two different inbred strains as ES cell donor and blastocyst donor, the heterogeneity of the genetic background in der F2 generation can be avoided by basal breeding techniques and the subsequent analysis of the established animals. This includes the mating of F0 chimeras to the inbred strain of the ES cell donor after they have been previously identified as germ cell chimeras in the normal mating to the inbred strain of the blastocyst donor by the occurrence of heterozygous transgenic F1 hybrids. The offspring show identical coat colour, therefore molecular genetic analysis is used to identify the heterozygous transgenic F1 animals which are subsequently used for breeding homozygous transgenic F2 animals on the genetic background of the inbred strain of the ES cell donor (= co-isogenic strain). So the transgenic co-isogenic strain as well as transgenic congenic strains can be bred and analyzed.

Generation of ES cells harbouring the specific mutation on both alleles allows the production of germ line chimeras which can deliver twice the number of heterozygous transgenic animals in the F1 generation when compared to the standard method. Homozygous mutant ES cells can also be generated by simultaneously or consecutively using two targeting vectors harbouring different resistance genes.

The use of specific ES cells derived from F1 hybrids and pretreated embryos as recipients (tetraploid embryos) may also improve the procedure. The tetraploid cells do not participate in the development of the animals that leads to the exclusive appearance of transgenic animals in the F0 generation. Depending on the zygosity of the mutation in the ES cells, all animals are heterozygous transgenic or homozygous transgenic. The disadvantage of the procedure up to now is the absent uniformity of the genetic background of the animals in the following generations.

The laser-based penetration of the *zona pellucida* of early embryos (one- to eight-cell-stage) and subsequent injection of genetically modified ES cells derived from inbred strains led to the generation of founder animals that developed almost completely from the ES cells transferred. This makes it possible to breed transgenic lines with uniform genetic background.

Moreover, research is carried out in mice and other species to identify additional cell types which contribute to the development of germ cells in chimeras after the targeted alteration of their genome. For this purpose, particularly progenitor cells of germ cells are in the focus. In addition, induced pluripotent stem cells (iPSC) derived from differentiated somatic cells that show ES cell-specific potential may serve as an alternative to ES cells. Such cells are generated by using a number of various techniques that are not established as routine procedures. Genetic and/or epigenetic alterations due to *in vitro* cell culture or genomic reprogramming processes may occur that may lead to consequences on the phenotype of the established animals.

3.2.3.2 Somatic cell nuclear transfer (cloning)

The somatic cell nuclear transfer allows the generation of animals harbouring defined mutations in a high number of mammal species including the classical laboratory species mouse, rat and rabbit. Up to now the success rate of the technique in laboratory species is low. On the other side, the technique is well established especially for pigs and ruminants for the production of mutant or transgenic animals – including the additive gene transfer - and has a higher efficiency compared to conventional techniques, e.g. DNA microinjection.

Standard technique

The generation of transgenic animals by using the somatic cell nuclear transfer technique contains the following steps:

- Production of the targeting vector (knockout/-in construct) for homologous recombination
- *In vitro* enzyme-mediated or non-enzyme-mediated gene targeting in diploid somatic nucleus donor cells usually resulting in the heterozygous gene knockout/-in in the selected cell clones
- Preparation of mature oocytes as recipient cells
- Enucleation of the oocytes
- Transfer of the specifically mutated donor cells or their nuclei into the enucleated oocytes
- Oocyte activation and embryo culture
- Transfer of the cloned embryos into pseudo-pregnant recipients (foster mothers)
- Analysis of the offspring of the recipients for transgenesis
- Breeding of transgenic lines
- Genotypic and phenotypic characterization of the transgenic lines

Genotype of animals

After using transgenic donor cells, all animals of the resulting F0 generation are transgenic; they may be heterozygous transgenic or homozygous transgenic depending on the zygosity of the genetic alteration in the donor cells. The transgene locus exhibits the desired mutation that was verified in the cell clones selected for the nuclear transfer.

Long periods of *in vitro* cell culture and extended manipulation of the nucleus donor cells usually decrease the cloning efficiency. Therefore, transgenic nucleus donor cells still mixed with various portions of non-transgenic cells due to the incomplete execution of the purification procedure are also applied to the cloning technique. In this case, also non-transgenic animals occur in the F0 generation.

In the F0 generation, animals showing an abnormal pre- and postnatal development as a consequence of the incomplete or incorrect genomic reprogramming process of the transferred somatic cell nuclei may appear. Founder animals without obvious transgene-independent alterations of the normal phenotype may frequently show transgene-independent alterations of the expression of endogenous genes in the molecular genetic analysis. In the case of intact fertility of the founder animals showing apparent transgene-independent alterations, the animals of the subsequently bred F1 generation usually do not exhibit this phenotype any more. The use of inbred strains as genetic background leads to genetically uniform homozygous transgenic animals within a given line.

The cell clones exhibiting the desired mutation and being selected for the nuclear transfer may harbour random genetic lesions as a consequence of the *in vitro* cell culture that may affect the phenotype of the transgenic animals. Thus, generation and analysis of several independent lines established with independent, genetically modified cell clones is recommended for obtaining valid data.

Technical developments

The current research on the genomic reprogramming of somatic cell nuclei may contribute to increase reproducibility and efficiency of the somatic cell nuclear transfer for laboratory species. Moreover, application of different cell types as nucleus donors and their pretreatment

are under investigation. In addition, re-cloning of cells derived from cloned embryos or fetuses may increase the efficiency of the somatic cell nuclear transfer.

For the *in vitro* transfer of the transgene into the somatic nucleus donor cells, applied techniques include viral vectors like recombinant adeno-associated viruses (rAAV) that may cause increased rates of homologous recombination.

For the additive gene transfer, cumulative transfer of embryos that are cloned by using independent transgenic cells, is done in recipient animals. This may lead to the generation of transgenic F0 animals harbouring different transgene loci in one litter.

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