



ALTERNATIVES FOR GENERATING GENETICALLY ENGINEERED ANIMALS

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PREFACE¹

The method used for the generation of a genetically engineered animal line will depend primarily on the type of genetic modification required. Nevertheless, when evaluating which method to use, potential welfare issues associated with the method should also be considered, as well as potential refinements to minimize any adverse welfare impact on the animals and to improve the efficiency of generation. Prior to generating any genetically engineered animal, confirmation should be sought that the line is not readily available elsewhere, as unnecessary repetition of generation is wasteful of animals.

Section 1 of this document lists currently used methods of generating genetically engineered animals, along with associated welfare concerns and possible ways in which each method could be refined to decrease the numbers of animals needed or improve the welfare of the animals involved. In terms of refinements, suggestions are made for consideration of other generation methods, as well as possible modifications to the current method to provide better control of gene expression (described in Section 2) or refine generation techniques (described in Section 3). For information on methods of identification, current best practices for obtaining genetic material and potential refinements for genotyping, see *Alternatives for Genotyping Genetically Engineered Animals* (in preparation).

1. Methods for Generating Genetically Engineered Animals

1.1 Pronuclear Injection

Pronuclear injection is used to generate animals that express gene sequences under the control of a heterologous promoter. The promoter and gene sequences may be derived from the same species or from different species. Pronuclear injection is often used in studies to examine the effects of overexpression or misexpression of specific gene sequences. However, transgenes that express so-called dominant negative (Levin et al., 1993, Inoue et al., 2010) or silencing RNA (siRNA, miRNA, RNAi) constructs (Casola, 2010) can be used to generate “knock-outs” in organisms not amenable to targeted mutagenesis, as an alternative to targeted mutagenesis for affecting function of multiprotein complexes, and/or as a method for conditional mutagenesis when combined with site-specific recombinases or other technologies.

Welfare Concerns

- Transgenes integrate randomly on the chromosome, thus transgene expression may differ depending on site of insertion (“position effect”) or cause insertional mutagenesis when inserted into native genes. This can affect the survival of the injected embryos or have unanticipated welfare impacts on the animals generated (Kirkwood and Hubrecht, 2010; Robinson et al., 2003).

¹ This best practices document was developed by a CCAC-facilitated committee involved in the development of guidelines for genetically engineered animals.

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- Multiple founder lines are required to properly control for position effect and insertional mutagenesis effect, which further increases the number of animals needed (especially when comparing multiple promoters and/or expressed sequences).
- Some integration sites may result in failure of transgene expression or subsequent transmission, which can mean that the animals used have been wasted (Robinson et al., 2003).
- Relatively large numbers of embryos are needed to produce relatively few animals with the desired transgene expression levels and patterns (Robinson et al., 2003).
- Large numbers of females may be needed as embryo donors or recipients (Robinson et al., 2003).

Experimental Considerations

- Success is variable from one type of animal to another; it is currently inefficient in medaka, xenopus and chickens, where foreign DNA usually does not integrate into the genome of the animals (Houdebine, 2002).
- Transgene expression may be extinguished after several generations of breeding due to epigenetic silencing of transgene promoters.

Potential Refinements

The following methods may help address problems associated with random integration of the transgene: targeted transgenics in ES cells (Section 1.2), virus-mediated gene transfer (Section 1.3), bacterial artificial chromosome (BAC) transgenics (Section 1.4) and targeted transgenics using zinc finger nucleases (ZFNs) and/or transcription activator-like effector nucleases (TALENs) (Section 1.5), as well as such refinements as conditional and inducible transgenic technologies (Sections 2.1 and 2.2), the use of insulators (Section 2.3) and careful selection of promoters (Section 2.4).

Other techniques that may address issues associated with the welfare of the animals involved in the generation of the genetically engineered animals include: non-surgical embryo transfer (NSET) (Section 3.1) and sperm-mediated gene transfer (Section 3.2).

In addition, there are a number of potential variables related to construct design and preparation, and the expression and transmission of the transgene that should be considered when undertaking pronuclear injection. These are described by Robinson et al. (2003).

To reduce the risk of transgene expression loss after several generations of breeding, early generation transgenics should be cryopreserved. In the event of loss of transgene expression, the line can then be recovered rather than re-created. Any additional founder lines that have not been selected for experimental use should also be cryopreserved, rather than breeding needlessly.

1.2 Targeted Transgenics and Mutagenesis in ES Cells

1.2.1 Targeted Transgenics

Targeting refers to the site-specific integration or removal (knock-out) of genomic DNA sequences. Most commonly, targeting is done in mouse embryonic stem (ES) cells using homologous recombination. However, engineered site-specific nucleases have allowed targeting in mouse ES cells (Osiak et al., 2011) and in embryos of a range of animals including rats, rabbits, and zebrafish (Ben et al., 2011; de Souza, 2012; Duranthon et al., 2012; Jacob et al., 2010) (see Section 1.5). Site-specific recombinases (e.g., Cre and FLP) and integrases (e.g., PhiC31) are also being used in both ES cells and embryos (Lister, 2011; Monetti et al., 2011; Osterwalder et al., 2010; Tasic et al., 2011). These methods of targeted gene mutagenesis have been adapted for targeted transgenics to insert transgenes at defined genetic loci (Tchorz et al., 2012). Targeted transgenics enables better control over the genetic modification than pronuclear injection, and can therefore be used to minimize adverse welfare effects on the animals being generated (Robinson et al., 2003).

Welfare Concerns

- Mycoplasma contamination of ES cells affects various cells parameters, germline transmission and postnatal development of the resulting chimeras. It is important to screen ES cells for mycoplasma before their use (Markoullis et al., 2009).
- ES cell cultures are a potential source of pathogenic infection; they are susceptible to persistent infection with murine viruses such as murine hepatitis virus (MHV) (Nicklas and Weiss, 2000).
- Failure to achieve germline transmission can mean mice are wasted and that females used to provide host embryos and surrogate mothers have been used unnecessarily (Robinson et al., 2003).
- Off-target mutagenesis in the ES cell, either through genomic rearrangements, duplications or deletions (Liang et al., 2008) or by multiple insertions of the vector, have been widely reported. These can lead to unintended or unexpected welfare concerns.

Experimental Considerations

- The procedure is technically demanding and requires consistently high rates of germline transmission of the ES cell genome, excellent cell culture techniques to maintain ES cell pluripotency, and at least 2 generations of breeding to produce homozygotes (Robinson et al., 2003).
- A constitutive knock-out could be lethal or the gene could affect other pathways, making it difficult to study the physiology/gene of interest.

Potential Refinements

Targeting in the embryo (e.g., with site-specific recombinases, nucleases or integrases) can overcome pluripotency issues that arise with the use of ES cells, but the efficiency with which embryonic genomes are modified may not reduce the number of animals used. Parental ES cell

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lines with high germline transmission rates should be used for targeting to reduce animal numbers needed to generate targeted animal lines.

Control may be further advanced through the use of conditional knock-outs (Section 2.1) and inducible transgenes (Section 2.2) and may also help limit welfare impacts on derived animals.

ES cells may develop genomic and/or chromosomal abnormalities during culture (e.g., aneuploidy) that can reduce their pluripotency and germline transmission rates. Simple chromosome counts provide a method of identifying euploid versus aneuploid ES cell lines and can assist in the selection of clones that are more likely to provide germline transmission. Thus, chromosome counts should be considered before microinjection of ES cells. The general consensus in the field is that >50% euploid mitotic chromosome spreads (≥ 20 spreads assessed) is the minimum cut-off for use to produce chimeras; however, some facilities require >60–70% euploid spreads before use. When only single clones exist for a given targeted mutation and the ploidy does not meet minimum standards, subcloning to isolate euploid subclones is possible. However, care must be taken to maintain the pluripotency of ES cells during subcloning and a second simple chromosome count should be performed to verify the percentage of euploid cells.

Use of coisogenic ES cells (ES cells derived from the same strain as that in which animal experiments are to be done) eliminates the requirement of backcrossing to reach congenic status. Whenever possible, targeting in coisogenic ES cells should be performed.

In mice, techniques for producing chimeras using outbred host embryos (e.g., aggregation and/or 8-cell embryo microinjection) can reduce the number of embryo donors needed since outbred females produce more useable embryos than many commonly used inbred lines (Gertsenstein et al., 2010; Kiyonari et al., 2010; Poueymirou et al., 2007). The number of useable embryos can also be increased by modifying the number of ES cells injected.

Due to the potential of infectious contaminants confounding in vivo studies, pathogen testing is recommended as a best practice prior to ES cell microinjection (Peterson, 2008).

1.2.2 Targeted Mutagenesis

Targeted mutagenesis is used to mutate a specific gene in ES cells. The targeting vector is made up of DNA pieces identical to those of the gene of interest, a marker and promoter. The targeting vector is incorporated into ES cells by electroporation or within a virus. With the DNA pieces being an identical match, the targeting vector will become incorporated into the DNA of the ES cell gene. The marker then disrupts the gene, thereby ‘knocking’ it out. Live knock-outs are then retrieved from chimeras (Festing and Lutz, 2012). Two international consortia, KOMP and EUCOMM, are using gene targeting and trapping to knock out each of the mouse genes in order to find out their function.

1.3 Virus-Mediated Gene Transfer (Lentivirus Transgenics)

Virus-mediated gene transfer allows for efficient delivery and sustained expression of transgenes (Clark and Whitelaw, 2003; Fässler, 2004; Hofmann et al., 2003; Park, 2007). This method is useful where a quick assessment of the phenotype associated with expression or deregulation of a specific gene is required (Le Provost et al., 2009), and allows for efficient transgenesis of

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livestock, extending the availability of appropriate species for particular studies (Le Provost et al., 2009). An alternative to making knock-outs can be done with lentivirus and RNAi constructs that are short enough to fit easily in lentiviral vectors.

Experimental Considerations

- Variation between laboratories may make it difficult to share protocols.
- Current viral vectors have size limitations so that virus-mediated gene transfer can only be used with very small transgenes.
- *In vitro* assessment of effectiveness of RNAi constructs (e.g., by measuring changes in mRNA abundance and/or protein level and/or activity) should be considered mandatory as different RNAi sequences have very different levels of effectiveness (reviewed in Podolska and Svoboda, 2011).
- It is often necessary to compare several different RNAi constructs to control for “off-target” effects (Campeau and Gobeil, 2011). If appropriate assays are available, this may be tested *in vitro* before selecting a smaller number of constructs for *in vivo* use.

1.4 Bacterial Artificial Chromosome (BAC) Transgenics

Creating transgenic animals with a BAC construct can help protect the transgene of interest from endogenous promoters and other regulatory elements; a BAC construct will most often include all the regulatory elements necessary for the transgene of interest to express properly (Giraldo and Montoliu, 2001; Heintz, 2001). This method may reduce position effects (Robinson et al., 2003).

Experimental Considerations

- Investigators using BAC transgenics must be aware of the potential effects of additional copies of passenger genes, especially if gene-rich regions of genome are source of BACs.
- Enhancers present in BACs may influence gene expression at or near site of BAC transgene insertion.
- Quality of BAC DNA is critical for success, otherwise animals used as embryo donors and recipients are wasted.

1.5 Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs)

ZFNs (Carbery et al., 2010) and TALENs (Miller et al., 2011) are efficient and convenient alternatives to conventional gene targeting (Carbery et al., 2010) and work in a wide variety of cell types, including one-cell stage embryos (Smits and Gould, 2009). These methods can be used to generate gene-targeted knock-outs in species where ES cells are lacking (drosophila and zebrafish; Smits and Gould, 2009) or where cloning techniques are not available (e.g., rats). When injecting zygotes/embryos, the method can be adapted to any genetic background, eliminating the need for generations of backcrossing to achieve congenic animals (Carbery et al.,

2010). Public databases are available for ZFNs and endonuclease design and construction of ZFNs and/or TALENs are available at reasonable prices.

Experimental Considerations

- Technically demanding.
- Efficiency of nucleases can differ between cell types and target loci.
- Can be done in both embryos and ES cells; therefore, deliberation about approach should be done with regard to desired experimental outcome and in consideration of the Three Rs.

2. Refinements to Control Transgene Expression

2.1 Conditional Knock-out and Transgenic Technologies (e.g. Cre/loxP and Flp/FRT recombination systems)

Cre/loxP and Flp/FRT can reduce harmful effects arising from undesirable spatial and temporal gene ablation or transgene expression, providing a mechanism for minimizing adverse effects on animal welfare and limiting pre- or post-natal deaths that may be associated with constitutive knock-outs or over-expression of the transgene (Robinson et al., 2003). Temporo-spatial control of gene expression can also be closely regulated, provided promoter sequences function properly in inserted locations (Lambert, 2006). Cre/lox technology requires production of a Cre-lox mouse, typically by breeding a Cre expressing mouse to a mouse containing the loxP-flanked (trans)gene of interest (Lambert, 2006) and FLP/FRT recombinase system is an alternative to Cre recombinase. Early reports showed that the Cre recombinase is more efficient than FLP recombinase (Bailey et al., 2009). However, a FLPo has recently been published that appears to be almost as efficient as Cre (Kranz et al., 2010).

Further temporo-spatial control can be conferred using a tissue-specific recombinase that is dependent on the presence of an exogenously supplied ligand for activation (e.g., tissue-specific Cre-ERT2 [estrogen receptor] and tamoxifen [ligand for estrogen receptor; tetracyclin-inducible promoter for Cre expression]).

Welfare Concerns

- Variable Cre expression efficiency, variable recombination, and potential genotoxicity (resulting from off-target recombination) using the Cre/loxP system (Bailey et al., 2009) can have detrimental effects on animal welfare and/or increase the number of animals needed for experiments.
- Ligand-dependent forms of Cre exhibit tighter spatial and temporal regulation of deletions and transgene expression (Bailey et al., 2009), but this requires optimization of ligand dose and delivery. Some ligands can result in welfare concerns, for example tamoxifen-induced dystocia in pregnant mice (Simpson, 2010 personal communication).

Experimental Considerations

- The generation of Cre-expressing mouse lines should be well planned, including location of transgene insertion, promoter used to drive expression, and ligand choice and delivery method.
- Requires generation of two congenic lines, one with the loxP target sequences and one with Cre expressed under proper temporal and/or spatial control.
- Sequences similar to loxP (Cre targets) may be present in vertebrate genomes which could result in “off-target” recombination.
- Insertional mutagenesis used to produce Cre-expressing or loxP-flanked transgenes can effectively yield one mutant allele, which may confound experimental outcomes (Bailey et al., 2009).
- A sequence similar to a loxP may be present, in this case the CRE will “misread” the sequence and may cut the DNA at the wrong place changing the expected mutation. Scientists have molecular tools to identify “off-target” mutation.
- While Cre/ER does not respond to endogenous estrogen, endogenous estrogen receptors will be activated by the most commonly used ligand, tamoxifen. Controls must be used with Cre-dependent mutagenesis, including each transgene (Cre and floxed alleles) alone, and in the case of ligand-dependent Cre, with and without ligand treatment (e.g., of tamoxifen effects in mice; Chen et al., 2002; Esmaili et al., 2009; Hardman et al., 2008; Ishii et al., 2008; Mehassab et al., 2010; Perry et al., 2005).

2.2 *Insulators*

Insulators may reduce position effects (Robinson et al., 2003) by blocking interference between an integrating vector and target cell (Emery, 2011).

2.3 *Promoter Selection for Transgenics*

Identification of appropriate promoter sequences can allow greater control over the temporal and spatial pattern of gene deletion or expression (Bailey et al., 2009). Tissue-specific promoters are often used to create Cre transgenic mice to be used with mice containing loxP-flanked DNA to control gene expression or ablation. Tissue-specific promoters offer finer control of the transgene expression pattern (Robinson et al., 2003).

Welfare Concerns

- An inducible promoter should be well characterized to avoid deleterious effects arising from unexpected expression of the transgene (Robinson et al., 2003).
- Not all promoters will work “out of context” so several different constructs may have to be tested prior to identifying a good promoter sequence.

Experimental Considerations

- Promoters inserted into other locations in the genome may not recapitulate expected patterns of gene expression due to influence of enhancers or other genomics sequences at the site of insertion.

2.4 Gene Traps

Gene traps are used for genome-wide high-throughput random mutagenesis in ES cells (using plasmid or viral vectors) or in embryos (using viral vectors or transposable elements). Before undertaking generation of new gene-trap screens in animals, publicly available gene trap resources that may be useful for genomic structure/function studies should be checked (e.g., www.knockoutmouse.org for mice, http://zfin.org/zf_info/catch/catch.html for zebrafish, and www4.utsouthwestern.edu/hamralab/hamra_rats.htm for rats).

Welfare Concerns

- Gene trapping is a random insertional process most suited to production of genome-wide publicly available resources and not for acquisition of mutations in specific genes, due to the large number of animals used in an embryo- or germline-based gene trap screen.
- When mutations in a specific gene or set of genes is needed, ES cell-based approaches or embryo-based targeting or other methods are more likely to be suitable in the context of the Three Rs.

3. Refinements for Embryo Transfer

Embryo transfer into the oviduct or uterus through the body wall is an invasive procedure.

3.1 Non-Surgical Embryo Transfer (NSET) in Mice

NSET uses methods similar to artificial insemination, and eliminates the pain and distress of invasive surgery. No anesthesia or analgesics are required and this method eliminates complications such as post-surgical infection and is equally as efficient as standard oviduct transfer for the production of transgenic mice via microinjection. NSET has been successfully used to transfer blastocysts generated by the aggregation of morulas with gene-targeted R1 ES cells, and to transfer cryopreserved embryos (Green et al., 2009).

Experimental Considerations

- Transillumination is necessary for accurate visualisation of cervix.
- Still some debate as to whether this method is as efficient as surgical embryo transfer for generating live born mice.
- Cannot be used to transfer embryos earlier than blastocyst stage.

3.2 Sperm-Mediated Gene Transfer

Sperm-mediated gene transfer involves the introduction of DNA into the sperm cells of a live male. The male is then mated with females, allowing germline transmission of the required genetic modification to some of the offspring.

Experimental Considerations

- Results are inconsistent; the yield of transgenic animals is usually low and largely unpredictable, and usually the integrated DNA is profoundly rearranged and not functional (Houdebine, 2002).

Potential Refinements

In vivo electroporation of undifferentiated spermatogonia (Deathless Transgenesis) (Dhup and Majumdar, 2008) reduces the number of animals required to generate transgenic founders, and the amount of time required for generation (Dhup and Majumdar, 2008). This method may also facilitate transgenesis in large animals whose gestation period is protracted (Dhup and Majumdar, 2008).

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