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Production of Transgenic and Mutant Mouse Models

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Summary

Manipulation of the rodent genome by deliberately inserting (transgenic) or removing (knockout) a gene of interest or indeed by selectively breeding animals with a spontaneous or random mutation producing a trait of interest has been developed over several years. Mouse "fanciers" have been selectively breeding interesting mice since the turn of the last century to produce a plethora of different background strains of the common house mouse (*Mus musculus*). Rat (*Rattus norvegicus*) strain development has also proceeded with selective breeding, although the range of strains is more limited. The deliberate and targeted manipulation of the mouse genome has been with us for over two decades, with the rat genome a more recent addition, and yet this technology has been limited to a very narrow range of genes. With the complete mapping of the mouse genome (and the rat genome soon to follow), the powerful techniques of transgenic and knockout rodent production can be applied to the numerous genes whose expression is altered in existing stroke models.

Key Words

Transgenic; knockout; animal; mouse; rat; stroke; ischaemia; mutant; model; vascular; dementia.

1. Introduction

Transgenic models are those in which a protein from the gene of interest is overexpressed. This overexpression can be controlled in a temporal or spatial manner with appropriate use of DNA regulatory sequences. Knockout models are generated by removing the endogenous protein expression from a gene of interest. The first transgenic ***(1)*** and knockout ***(2)*** mouse lines followed earlier models of human disease using spontaneous mutants produced from selective breeding programs ***(3,4)*** or induced mutant models ***(5,6)***. Mutant models result from breeding that selects for specific traits, whereas transgenic and knockout

From: *Methods in Molecular Medicine, Vol. 104: Stroke Genomics: Methods and Reviews*
Edited by: S. J. Read and D. Virley © Humana Press Inc., Totowa, NJ

models result from the deliberate introduction of precise engineering into an identified gene.

The essential stages of overexpression transgenic model production start with cloning and construction of the relevant cDNA sequence (the construct). A supply of single-celled embryos for injection (harvesting embryos) is required before the DNA is introduced into the embryo (cDNA microinjection). Subsequently the surviving embryos are placed into a recipient mother (transfer of embryos), and finally offspring positive for the transgene are identified and bred on to produce experimental transgenic colonies. The essential stages of making a knockout model are very similar, with a few important differences. The process again starts with cloning and construction of a DNA but using relevant genomic DNA sequences in place of cDNA (the targeted construct). This genomic DNA is introduced into embryonic stem (ES) cells (transfection), and screening takes place to identify the correct recombination of the targeted construct (clone selection). Subsequently the correctly targeted ES cells are introduced into a blastocyst (ES cell microinjection), and surviving embryos are placed into a recipient mother (transfer of embryos) before identifying positive offspring and breeding the appropriate knockout lines.

Animal models of stroke have used nongenetic techniques ***(7,8)*** to reproduce the conditions seen in the ischemic human brain. These rodent models attempt to simulate the pathological sequelae that ensue following stroke in the human brain. The production of transgenic animals is routine in many laboratories around the world, and a gene of interest—GOI1—will be used to illustrate how a transgenic or knockout animal may be generated. These methods will help improve our understanding of the role specific genes play in ischemic stroke.

2. Materials

2.1. Microinjection Equipment

1. Microscope for microinjection (Leica, Milton Keynes, UK).
2. Antivibration table (cat. no.702, Leica).
3. Picoinjector (cat . no. P100, Wentworth Laboratories, Sandy, Bedfordshire, UK).
4. Microforge (cat. no. MF-900, Micro Instruments, Oxon, UK).
5. Compressed air supply for the antivibration table.
6. Incubator for 5% CO_2 and 37°C incubation.
7. Standard 4°C fridge with –20°C freezer compartment.
8. M2 media (Sigma-Aldrich, Poole, UK).
9. Micromanipulators (Zeiss, UK).
10. Needle puller/needles (Campden Instruments needle puller; Pre-pulled Needles from Eppendorf).

2.2. Surgical Equipment

1. Dissection equipment including fine scissors, fine forceps, Serafine clip (Fine Science Tools).
2. Surgical staples (9 mm Clay Adams Autoclips).
3. Dissection microscope with large swing-out arm (cat. no. MZ6, Leica).
4. Mouth transfer pipet and transfer capillaries (Sigma).

2.3. Animals

2.3.1. Donor Mice for Overexpression Transgenic Mouse Production

Single-cell embryo donor females are aged 3–6 wk. The precise age depends on the donor strain and health of the animals and will be determined by the quality of supply combined with trial and error. Generally younger or smaller mice give more embryos of poorer quality, and older mice give fewer, better quality embryos. Taking [C57Bl/6 × CBA]hybrid mice as an example, optimum embryo yield was obtained from mice with an average age of 4.5 wk and an average weight of 15.6 g (*see* **Note 1)** Strains of mice respond differently to superovulation (**Table 1**); however, an important consideration is the type of characterization to be carried out on the mice. In the study of vascular dementia, behavioral characterization is often required, and thus a mouse strain that is amenable to specific behavioral analyses is desirable. The inbred FVB/N strain is amenable to the production of transgenic lines, and, being inbred, it is isogenic. However, it has many problems with stereotypic behaviours that make behavioral characterization problematic. If hybrid mice are used, then consideration needs to be given to whether or not an isogenic colony is required. If this is the case, then the colony must be backcrossed to the strain of choice for subsequent generations of breeding. A strain typically used is the C57Bl6. This can be over three to six generations depending on whether speed congenics are used (*see* **Note 2**).

2.3.2. Donor Mice for Knockout Mouse Production

Blastocyst donor females are aged 6–8 wk. An inbred strain of mouse should be used that has proved to be suitable as a host for the ES cell line chosen. Often C57Bl/6 mice are used for this purpose. Superovulation of these donor mice does increase the embryo yield but not in the same numbers as for single-celled superovulation.

2.3.3. Sterile Males

Sterile males are required to mate with mature fertile females in order to generate pseudopregnant dams. Sterile males can be normal healthy hybrids

Table 1
Superovulation Response of Different Mouse Strains

Strain	No. of embryos
C57Bl/6	40–60
BalB/cByJ	40–60
129/SvJ	40–60
CBA/CaJ	40–60
SJL/J	40–60
C58/J	40–60
A/J	≤15
C3H/HeJ	≤15
129/J	≤15
129/ReJ	≤15
DBA2/J	≤15
C57/J	≤15
FVB/N	25
[C57Bl/6 × CBA]hybrid	40–60
C57Bl/6 normal ovulation	8–10

that have been vasectomized or can be a sterile breed of mice (e.g., T/Tw2 from the Jackson Laboratories). Vasectomized male mice maintain performance for up to 1 yr, whereas inbred sterile mice can last for up to 6 mo. In either case a test mating should be carried out once the mice are fit to mate to ensure sterility.

2.3.4. Pseudopregnant Recipient Females

Recipient females are healthy mature mice between 8 and 20 wk of age. Older or younger mice can be used, but as most strains of female mice become fertile at approx 6–8 wk and rapidly lose fertility after 26 wk of age, the percentage of successfully reared pups will decrease. The strain for recipient females should be the same as, or a hybrid of, that used to donate embryos for injection (e.g., C57Bl/6 used for microinjection and [C57Bl/6 × CBA]hybrid used for the recipient female). Recipient females at 0.5 d post-mating are required for single- or two-celled embryo transfer, and 2.5 d post-mating recipient females are required for blastocyst transfers.

2.4. Embryonic Stem Cells

ES cells are derived from the inner cell mass of an expanded blastocyst ***(9)***. Newly derived ES cells require testing for their ability to integrate into the germline when injected into a host blastocyst as well as their utility in transfec-

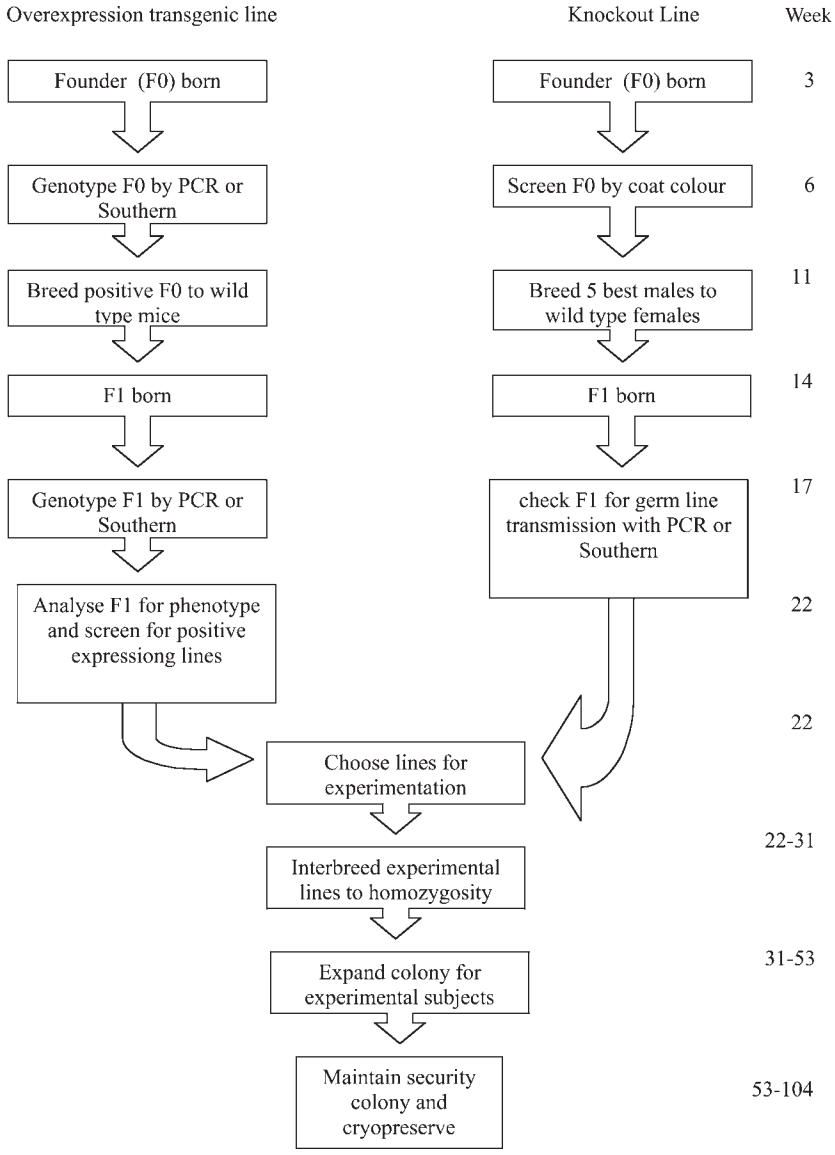

Fig. 1. Diagram of production and breeding timelines.

tion experiments. It is advisable to use proven ES cell lines that are in general use by academic laboratories. Such information can be found in the literature and the relevant lab approached for a supply of ES cells. If possible, a low

passage (number of growth cycles since derivation) number should be used, as these provide better germline transmission. The vast majority of ES cell lines are of the 129 background strain of mice, as these have been found to be most amenable to efficient targeted ES cell line production. Other strains of ES cell are available and do offer significant advantages over the 129 strain. For example, if C57Bl/6 ES cells are used and a C57Bl/6 host is used, then the resulting offspring will be 100% C57Bl/6, removing the need for backcrossing to an isogenic population. However, C57Bl6 ES cells are not very amenable to current techniques and are an inefficient method of knockout mouse production.

2.5. Breeding Facility

A standard animal breeding facility with small animal operating suites is ample for the production of transgenic and knockout mice. One important consideration is the health status of such facilities. Animals with an altered genetic makeup may well be more susceptible to infection, and so the highest level of health is required. Full barrier containment procedures are required and should be adhered to in the areas where these animals are bred.

2.6. Gonadotropic Hormones

1. Follicle-stimulating hormone (FSH: Sigma, cat. no. F4021).
2. Luteinizing hormone (LH: Sigma, cat. no. C8554).

2.7 Molecular Biology Equipment

Standard equipment for genetic engineering is required. During the construction of transgenic or knockout animals DNA material for manipulation is obtained from cDNA and genomic libraries. Subsequent manipulations involve the use of mutagenesis, polymerase chain reaction (PCR), and cloning. Additionally, equipment for Southern blot analysis is required for screening the offspring that may carry the genetic manipulation of interest.

3. Methods

3.1. Summary of Process

The process for producing transgenic mice is very similar to that for producing knockout mice.

1. Initially DNA is manipulated to incorporate the key features required.
2. This transgene is introduced into a host embryo, either directly into the nucleus for a transgenic mouse or via an ES cell for a knockout mouse. Knockout mouse production requires a prescreening step to identify positive homologous recombination events within the ES cell.

3. Subsequently these embryos are replaced into a recipient dam, and the resulting offspring are screened for the presence of the transgene. Overexpression transgenic mouse lines with high copy numbers (and hence higher levels of protein) are often selected and bred on to produce experimental colonies.
4. Mice carrying a heterozygous targeted deletion of a gene are interbred to generate mice homozygous for the deletion, which are subsequently bred on for experimentation.
5. There are a number of differences in the breeding regime for overexpression and knockout mouse colonies. These are summarized in **Fig. 1**.

3.2. Expression Cassette Cloning Strategy

3.2.1. Elements Expressed

The manipulated DNA must have certain elements in order to be expressed successfully in the host genome. At the very basic level, a transgene requires a gene of interest and a polyadenylation signal driven by a suitable promoter (**Fig. 2A**). In order to follow the expression pattern of the transgene at a gross tissue level, a reporter gene can be included, separated from the transgene by an internal ribosomal entry sequence element (IRES) ***(10)***. This allows two gene products to be produced from one promoter, thus ensuring that the reporter gene will accurately report where the transgene has been expressed (**Fig. 2B**). The expression of a transgene can be temporally regulated by the inclusion of repressor sequences linked to the promoter. This will have the effect of activating gene transcription only when the repressor has been silenced (**Fig. 2C**) by an activator and a trigger. Examples of this activation/repression system include the use of doxycyclin or tetracyclin as the trigger ***(11)***. This can be administered in the diet of the mouse to activate the transcription of a gene or, conversely, to stop the repression of a gene knockout, thereby allowing temporal control of the gene expression.

3.2.2. Knockout Targeting Strategy

When engineering the target gene, an important consideration is ensuring that the expression of the endogenous gene is completely removed. If even part of a gene is transcribed, the resulting partial gene product may be translated into a functional protein and have unknown effects possibly entirely unrelated to its original function. To avoid this situation, the endogenous locus of the gene of interest must be genetically engineered to prevent any transcription. This is achieved by manipulating the endogenous locus as close to the transcription initiation site (ATG) as possible. This manipulation should remove at least the first exon of the locus and replace it with a reporter gene, *LacZ* for example. More of the locus can be removed so as to ensure that no endogenous functional gene product is transcribed (**Fig. 3**).

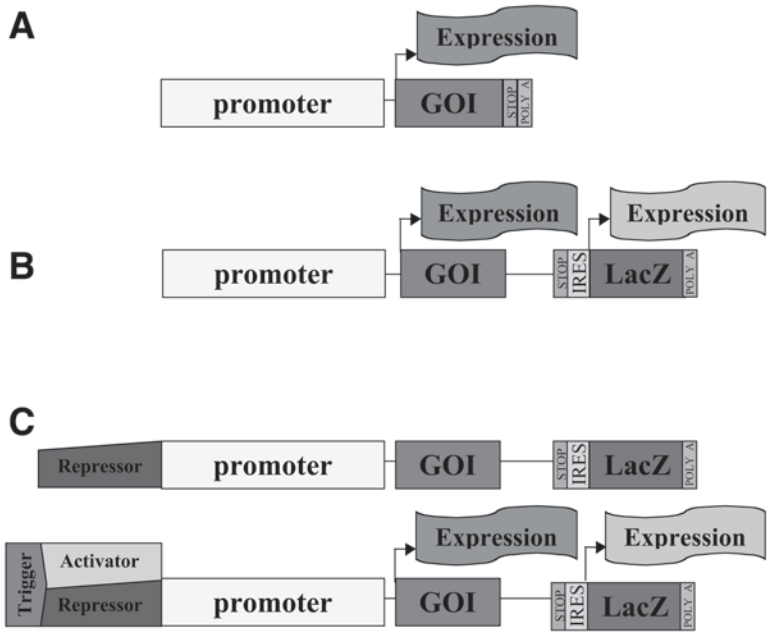

Fig. 2. (**A–C**) Expression vectors.

The example targeting vector in **Fig. 3** includes a stop sequence to prevent further transcription of the endogenous locus, an IRES element to reinitiate transcription of the downstream DNA, a *LacZ* reporter gene and polyadenylation sequence (to signal the end of the primary RNA transcript), and a selectable marker, neomycin. This is one of a number of selectable markers that can be used during targeting vector transfection into ES cells and confers resistance to antibiotic treatment, in this example neomycin. Thus cells that have incorporated the targeting vector correctly will survive when treated with neomycin in tissue culture.

The targeting vector must include regions of homology to the endogenous locus in order to integrate into the host genome. This is achieved by homologous recombination, as shown in **Fig. 4**. The lengths of the homologous arms have been found to be critical to successful integration, with a minimum of 1 kb and a maximum of 4 kb required. Specific sequences of DNA, LoxP (locus of recombination), flank the selectable marker as it is incorporated into the host genome along with the targeting vector. The marker may be expressed in the host animal and so is removed by the addition of cre protein into the transgenic or knockout mouse line (cre causes recombination of the LoxP sites), and the marker is removed ***(12)***.

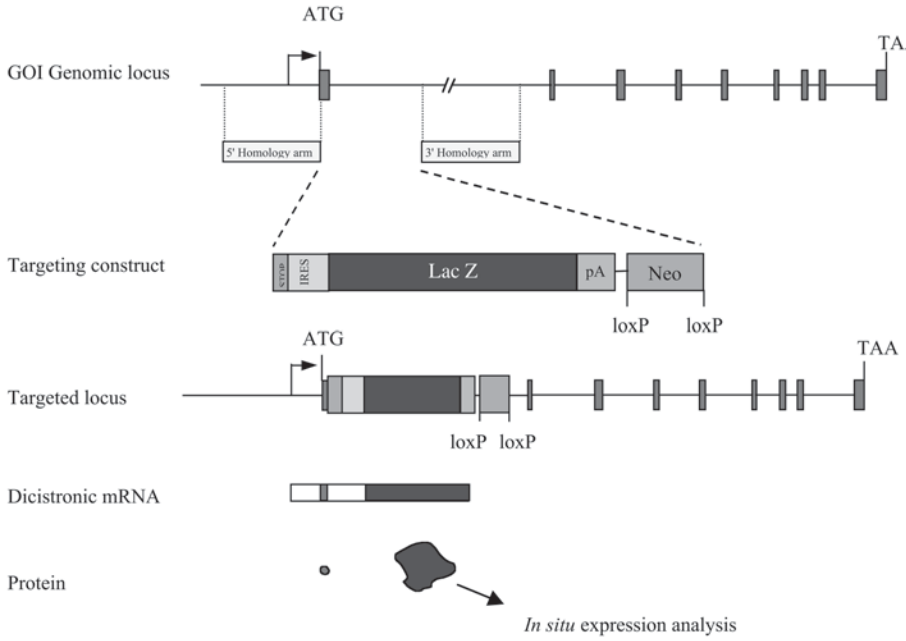

Fig. 3. Knockout targeting strategy. GOI, gene of interest.

3.3. Harvesting Embryos

1. Young females are used to provide the single-celled embryos for microinjection.
2. Females are dosed via the intraperitoneal route with 5 IU of FSH followed 48 h later by 5 IU of LH and are then paired with fertile mature males. The timing of the FSH injection is not critical, but the LH must be dosed before the middle of the second day after FSH administration.
3. Females are checked for vaginal plugs the following morning. In healthy hybrid donor mice, all the paired females will be plugged with an obvious vaginal plug of semen, indicating a successful mating. (*See* **Note 3**)
4. Embryos for injection are recovered from the euthanized donor female by dissecting the coiled oviduct away from the uterus and placing it into a 35-mm Petri dish filled with M2 medium supplemented with 10 mg/mL of hyaluronidase (cat. no. H4272, Sigma). This enzyme degrades the protein holding nutritive cumulus cells next to the embryo.
5. The embryos are gathered in a swelling called the ampulla. The wall of the oviduct is torn at the ampulla, and the embryos are released into the medium.
6. After approx 5 min of incubation, the cumulus cells fall away from the embryo. They are collected with a mouth micropipet and are washed twice in fresh M2. The embryos are then ready for microinjection.

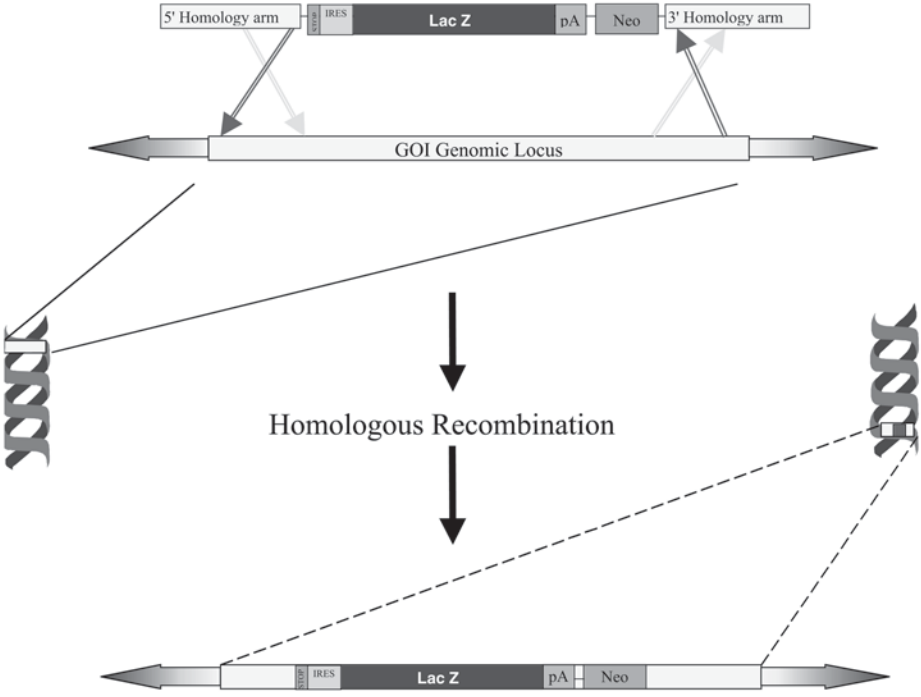

Fig. 4. Knockout targeting vector recombination. GOI, gene of interest.

7. Embryos not used immediately for injection should be stored in an incubator.
8. In order to relocate the embryos easily they are stored in a small volume of M2 medium in a 35-mm Petri dish.
9. Four such microdrops are placed on the Petri dish, one each at the 12, 3, 6, and 9 o'clock positions.
10. These drops of media are overlaid with low-density mineral oil (Sigma, cat. no. M8410) in order to prevent evaporation but allow the diffusion of gases.
11. This dish is stored in an incubator at 37°C and 5% CO_2. All embryos are stored in this Petri dish.
12. Uninjected embryos are stored at 12 o'clock and injected at 3 o'clock; poor or malformed embryos that cannot be injected are stored at 9 o'clock. The 6 o'clock position can be used to store embryos that have survived injection well and still look healthy after at least 1 h of incubation post injection.

3.4. Single-Cell Embryo Injection

1. A microinjection rig (**Fig. 5**) is required to introduce the DNA construct into single-celled mouse embryos.

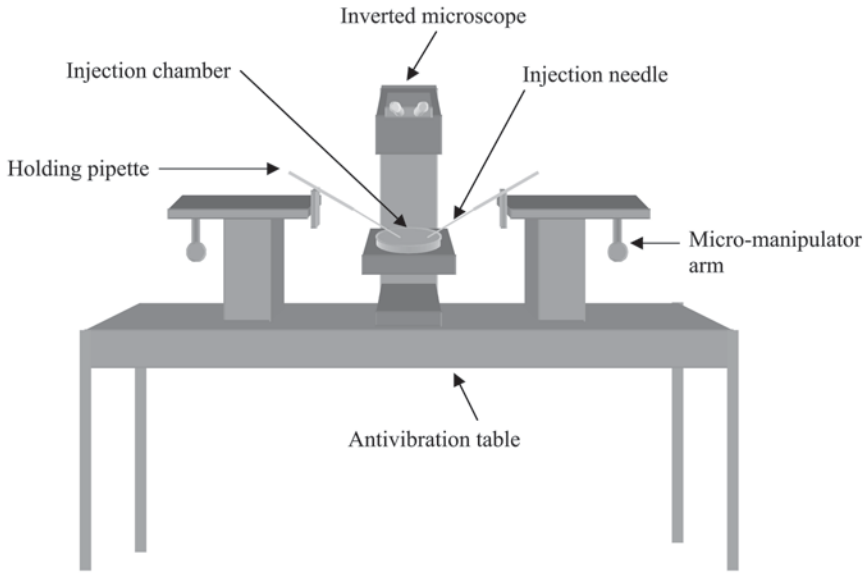

Fig. 5. Microinjection workstation.

2. After the microscope is turned on, the anti-vibration table is filled with compressed air and the picoinjector is turned on, a cavity slide filled with M2 is overlaid with light mineral oil, and the microinjection needle is pulled.
3. After a correctly pulled needle is obtained, it is backfilled with the DNA construct solution (*see* **Note 4**) and loaded onto the needle holder.
4. A holding pipet is mounted onto the opposite holder, and both are lowered into the M2 media under oil.
5. At this point the embryos for injection are introduced into the top part of the chamber. Batches of approx 20 embryos are a manageable number until the operator is proficient.
6. The holding pipet vacuum is turned on, most convieniently by use of a foot pedal, and maneuverd to pick up an embryo from the group. The embryo must be held at its center with the holding pipet resting very gently on the floor of the injection chamber.
7. Then the injection needle is brought into focus opposite the holding pipet (**Fig. 6**).
8. The needle must be in the same focal plane as the male pronucleus of the embryo prior to injection. To achieve this, the needle needs to be moved to a position either north or south of the embryo (in the microscope field of view).
9. The focus is adjusted to the male pronucleus, and then the needle is moved vertically up or down to be in the same focal plane as the pronucleus.
10. The needle is then moved away from, and brought into line with the embryo (the tip will move out of focus but that is acceptable) and then brought forward to inject.

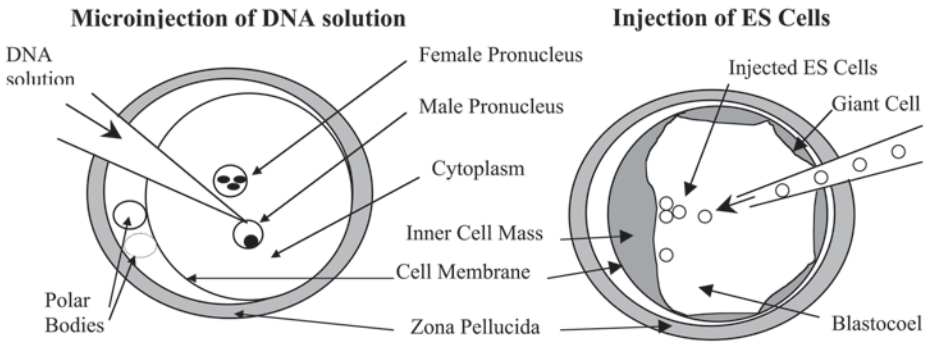

Fig. 6. Injection of embryos.

11. When the needle pierces the pronucleus, the DNA solution is delivered to the pronucleus by depressing the foot pedal of the picoinjector.
12. Immediately after injection the needle is removed from the embryo, care being taken not to touch the nucleolus.
13. The embryo is moved to the south of the injection chamber, and all the remaining embryos are injected in the same way.
14. If the DNA solution does not flow out of the needle tip it may be because the tip, of the needle has sealed closed when it was pulled. To solve this problem the tip of the needle must be broken on the clean, flat surface of the holding pipet ("tipping the needle"; *see* **Note 5**).
15. After all the embryos are injected in this way, they are removed to recover in the incubator, and another batch is introduced into the injection chamber for injection.
16. This continues until all the embryos are injected. If a needle becomes completely blocked or the tip too big, then the needle is discarded and another backfilled to replace it.

3.5. Blastocyst Injection

Knockout mice are generated by introducing the DNA construct into an embryonic stem cell, which is then injected into the vacuole of a mature blastocyst. Knockout mouse generation is more time-consuming and involves an additional in vitro culture step compared with making transgenic mice, although the injection part is not so difficult. This technology is the only way in which gene expression can be removed, thereby enabling the effect of removing or knocking out a particular gene to be observed.

1. Embryonic cells carrying the targeted locus are prepared in bulk so as to be single celled, with all contaminating debris removed. The ES cells are prepared in tissue culture conditions and stored in culture media in the incubator.
2. Just prior to injection, an aliquot of cells (several hundred) is introduced into a drop of M2 medium in the right-hand cavity of the main injection chamber.

3. Blastocysts are recovered from the donor females at 2.5 d post superovulation. The donor mice must be of the same strain or very closely compatible to the genetic strain of the DNA targeting construct.
4. Blastocyst recovery is achieved by flushing M2 medium through each uterine horn in turn.
5. The entire uterus is dissected out, severing just above the cervix.
6. The ovary and most of the oviduct coils are cut away, and a 25-gage needle is inserted into the top (ovary end) of each uterine horn.
7. With the bottom end of the uterus in a 35-mm Petri dish, a rapid flush of M2 medium is forced into the horn, expelling the embryos into the dish.
8. After flushing from the uterine horns, the blastocysts are washed with M2 and stored in the 12 o'clock position of a 35-mm Petri dish.
9. Just prior to injection, up to 20 blastocysts are placed in the injection holding chamber.
10. An ES cell injection needle is lowered into the drop of M2 to the right of the main injection chamber and is backfilled with M2 media.
11. Up to 20 clean, single ES cells are taken up into the needle.
12. The needle is then raised, moved to the injection chamber (by moving the stage, not the needle) and then lowered.
13. A single blastocyst is held by vacuum on the holding pipet and oriented so that the giant cells are nearest the needle tip.
14. Injection is by rapid movement of the needle tip into the blastocyst and subsequent expulsion of the ES cells. This will cause the blastocyst to collapse. Care should be taken to ensure that the ES cells adhere to the inside wall of the blastocoel and do not follow the tip of the needle as it is withdrawn from the injection site.
15. The needle is moved back to the ES cell chamber and refilled with cells, and the injection procedure is repeated until all the blastocysts are injected.
16. The blastocysts will recover quickly from injection and should have expanded back to their original size within 2 h.

3.6. Transfer of Injected Embryos Into Recipient Female

Protocols for small animal surgery must be adhered to when one is transferring the surviving embryos back into the recipient mothers. The basic surgery for both operations is the same.

1. Initially embryos are loaded into a transfer capillary. Up to 20 single- or two-celled embryos or up to 10 blastocysts are loaded into the capillary preceded by a small bubble and some M2 medium. This is to allow the operator to see when the embryos have been transferred, as they are too small to see with the naked eye.
2. The appropriate post-mating recipient females (*see* **Subheading 2.3.4.**) are anesthetized using a long-acting anesthetic (halothane is ideal or combination Hypnorm/Hypnorvel) and are laid on their belly.
3. A 10-mm incision is made longitudinally in the skin over the spine approximately halfway down the back (**Fig. 7A**).

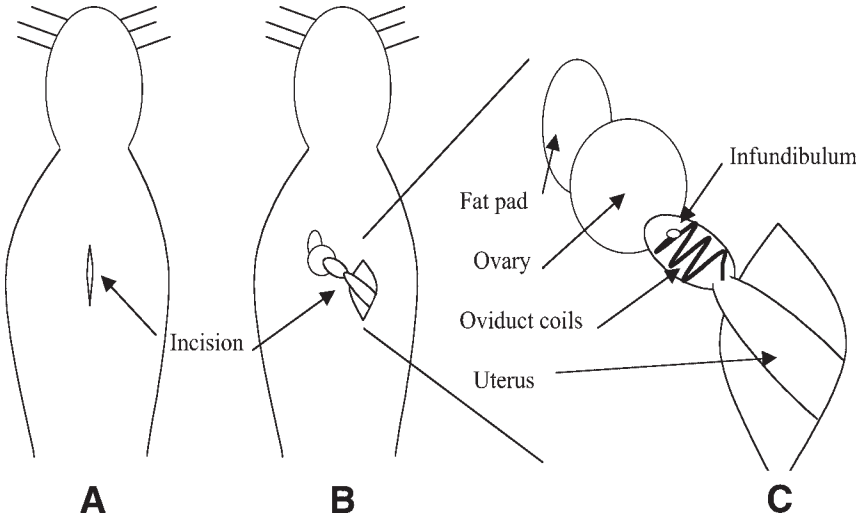

Fig. 7. Surgery.

4. This incision can then be used as a "window" to move around the body wall and search for a cream-colored fat deposit that is attached to the ovary.
5. Once this is found, the body wall is cut with a small 5-mm incision and the fat pad is exteriorized along with the ovary and top of the uterus (**Fig. 7B**).
6. The Serafine clip is used to hold the fat pad out of the body cavity while the binocular microscope if moved into place.
7. This is where the surgery for replacing the different embryos differs. To transfer single- or two-celled embryos, the mouse is examined under low magnification to find the infundibulum (**Fig. 7C**). This opening receives the eggs during natural ovulation and is located at the end of the oviduct coils at the top of the uterus.
8. The injected embryos must be inserted into the infundibulum by hand under low-power magnification of the binocular microscope. This technique requires a steady hand and it is advisable to practise on dead animals with a colored dye prior to live transfer.
9. Transfer is achieved by tearing the bursa near the infundibulum, clasping the bursa with fine forceps to stop the coils from moving, and then gently inserting the tip of a transfer capillary into the infundibulum. This opening is approx 150 μm in diameter, and a good capillary should be approx 100 μm in diameter.
10. The tip of the transfer capillary should be maneuverd around the first oviduct coil bend and the embryos expelled into the ampulla by gently blowing into the mouthpiece of the capillary tubing. The bubble preceding the embryos is used to assess when the capillary has been emptied.
11. The transfer of blastocysts into the recipient mouse is an easier technique. A 27-gage needle is used to make a hole in the uterus wall just before the point where the uterus begins to narrow to the oviduct (**Fig. 7C**).

12. The transfer capillary loaded with blastocysts is inserted into the hole and the embryos expelled again using the bubble before the embryos to assess when the capillary has been emptied.
13. After transfer, the uterine horn, ovary, and fat pad are replaced into the body cavity, and the incision is closed with a suture.
14. Once the same procedure has been repeated on the other uterine horn, the skin incision is closed with a Michel clip, and the animal is allowed to recover from surgery on a heated pad.
15. Saline may be administered to avoid dehydration.

3.7. Colony Expansion

3.7.1. Initial Breeding Strategy for Overexpression Transgenic Mice

1. After identification of the desired mouse line by screening the first founder generation, the colony will need to be expanded in order to generate animals for further characterization and breeding.
2. Initial breeding regimes will be determined by the properties of the gene of interest, for example, sex specificity or copy number (in the case of transgenic animals).
3. The rapid expansion of the colony is best achieved by breeding founder male mice to two wild-type females.
4. This "trio" of mice can be left in the cage to produce litter after litter until enough F_1 generation mice are produced.
5. Gestation in mice is approx 19–21 d, and, when left in the same cage as the male, the female will mate within 12–24 h of parturition (a postpartum mating) and give birth another 19–21 d later.
6. This cycle can be repeated successfully up to six times before breeding performance is affected.

3.7.2. Independently Segregating Transgenes

When a transgene is directly injected into an embryo, multiple copies of the transgene can randomly integrate into the host genome in a concatenation at one integration site or at multiple integration sites. When the mice breed, homologous recombination may separate the transgenes at multiple integration sites, leading to segregation of the transgene. This can result in F_1 offspring having different copies of the transgene and hence different protein expression levels. A Southern blot analysis is required to assess the transgene copy number of different F_1 litters and check for this segregation event. After the F_1 generation, transgene segregation, although not impossible, is far less likely.

3.7.3. Initial Breeding Strategy for Knockout Mice

Founder knockout mice are selected on the basis of the percent contribution of the injected ES cell to the coat color. If the host strain of mouse used is

C57Bl/6 then the coat color of the host will be black. If 129 ES cells are used, then the donor coat color is a light brown. Thus one can assess the percent contribution of the donor ES cells, and hence the proportion carrying the targeted locus, by looking at coat color. A 100% black mouse will not have incorporated many ES cells but will breed vigorously whereas a 100% light brown mouse will have incorporated many ES Cells but will not breed well. Five founder mice with more than 50% of their coat color as light brown should be chosen for breeding onto the F_1 generation. The resulting F_1 generation mice need to be genotyped to assess whether the targeted locus has transmitted through the germline successfully. F_1 mice carrying the targeted mutation can be interbred at this point to generate mice homozygous for the targeted locus. Assuming they are viable, these mice can be analysed to discover if the locus has been targeted correctly and the protein expression is indeed knocked out.

3.7.4. Background Strain

The background strain can have an enormous influence on the phenotype of transgenic and knockout mice. If isogenic donor single-celled embryos are used, then overexpression transgenic mouse lines will be isogenic from birth. However, knockout mouse lines will usually be a mix of C57Bl/6 and 129 strains. When the F_1 generation mouse is a hybrid mix of strains, then more reliable phenotypic data will be obtained from the mouse line being backcrossed to produce an isogenic background. This decision to backcross is made by considering the reasons for making the mouse line. If just one protein is to be investigated in the mouse line, then other proteins from the different strains are less likely to have an impact on the data. However, if many interacting proteins are to be investigated or behavioral observations made, then an isogenic background will produce more reproducible data.

3.8. Summary

Historically mice have been used in transgenic and mutant rodent models, and now rats are becoming more widely employed for this technique. Novel rat transgenic techniques will contribute to the knowledge already acquired from existing nontransgenic rat models of stroke. As an example, the SOD1 rat has been produced ***(13)***, following on from the SOD1 mouse ***(14)***. Rodent-directed transgenic technologies have allowed researchers to follow the consequences of increasing or decreasing expression of genes selected from the human genome in a live mammalian host. This technique has huge advantages over ex vivo or cell-based technology, as there are many more biochemical interactions among proteins, DNA, and RNA possible in vivo than in other expression systems. As specific genes involved in stroke are identified by existing technologies such as subtractive cDNA cloning ***(15,16)*** and by newer technologies

such as microarray chips ***(17)***, it is possible to manipulate these genes and create new models of stroke in which the consequence of very specific gene manipulations can be examined (*see* **Note 6**). With the sequencing of the human genome, it is possible to identify genes as risk factors for stroke ***(18)***. Using this information to generate models in which these risk factor genes have been manipulated will allow researchers to follow the consequence of inherited genetic susceptibility to stroke.

4. Notes

1. Different strains of embryos respond differently to culture and microinjection. For example, C57Bl/6 inbred mouse embryos lyse very easily on injection, whereas [C57Bl6 × CBA]hybrid embryos survive very well. Similarly, the inbred embryos will not all proceed to the two-cell stage of development if cultured overnight, whereas the hybrid embryos will
2. Speed congenics is a technique whereby specific allelic variants of the required mouse strain are identified by PCR and appropriate animals selected for breeding. This can reduce the number of generations needed to breed to the selected background from six to three generations (18–9 mo).
3. If very large sterile males are paired with small superovulated females, some females may die because of too much stress and bullying during mating.
4. The needle may clog when it is being back-filled. To clear the capillary, approx 3 mm of capillary can be cut from the needle base in order to provide a clean surface to draw up the DNA solution. The cut is made by scoring with a diamond-tipped pencil and sharply tapping the blunt end of the capillary.
5. If the needle is not pulled accurately, it may be sealed at the tip. In this case the tip of the needle needs to be broken to provide a hole through which the DNA solution can travel. The needle tip is broken by gently dragging the tip across the holding pipet until it breaks. If the hole is visible at high (400×) magnification it is too big, and the needle must be replaced.
6. As with all animal work, the protocols used should be reviewed by a local ethical review board. When one is making novel transgenic or knockout mouse lines, the potential for suffering of these animals is unknown. A wide literature search for related lines should be carried out as a guide to the potential harm that the planned gene manipulation will have. This information can then serve as a guide for the likely level of harm that may be inflicted on the animals as they are produced and hence the desirability to progress with the work.

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