Transgenesis in Nonmurine Species

John J. Mullins, Linda J. Mullins

Although the mouse remains the species of choice for most transgenic experimentation, it may be preferable or even necessary to use alternative species for certain applications. We review the strategies by which transgenic technology has been applied to other animals, specifically, the rat, rabbit, pig, sheep, goat, and cow. Additionally, we outline the potential applications of alternative transgenic species with reference to the field of hypertension and cardiovascular research. (Hypertension. 1993;22:630-633.)

KEY WORDS • animals, transgenic • ruminants • animals, domestic

Classically, animal models of hypertension and cardiovascular research have arisen spontaneously or through selective inbreeding. Resultant disease models may mimic symptoms of the human condition but are not always helpful in discerning the etiology. The underlying genetic cause of the disease may remain unknown despite extensive pharmacologic and physiological characterization. A good example of this is the spontaneously hypertensive rat.

Genetic mapping strategies, using minisatellite and microsatellite polymorphisms, have come some way toward predicting loci associated with specific disease phenotypes. It is probable that a combination of these techniques, together with gene-transfer technology, will shed light on the underlying etiology of hypertension and cardiovascular disease.

Although the vast majority of transgenic experimentation has been and continues to be carried out in the mouse, the technology has been extended to include a large number of species, such as the rat, rabbit, pig, sheep, goat, and cow. Because of size constraints in the mouse, it may be preferable to design some animal modeling experiments in these alternative species, even those in which the generation time and litter size present a severe disadvantage (Table).

Here, we describe the various strategies by which transgenesis has been applied to other species and highlight the relevance to present and future cardiovascular research.

Transgenic Rats

Although for cardiovascular research the rat is the experimental species of choice, few reports in the literature describe the generation of transgenic rats. As with the other species described, the adaptation of the transgenic techniques to the rat was a direct result of a particular experimental and practical need. Although larger animals such as the sheep are important for certain research areas (e.g., reproduction and endocrinology), the rat is more widely used as an experimental species. The ability to introduce genes into the rat germ line has opened experimental possibilities for several disciplines, including neurobiology, pharmacology, physiology, and cardiovascular biology. However, the ability to perform technically difficult surgical procedures on the mouse is improving, and researchers will soon have a genuine choice of the most appropriate experimental species to use for a given study.

Most of the procedures used to introduce transgenes into the rat germ line are similar to those used for the mouse. One-cell embryos from naturally mated female rats may be successfully microinjected, but significantly larger numbers of healthy embryos can be obtained by hormonal superovulation.1 In this procedure, immature female rats are implanted with an osmotic minipump containing Folltropin (Vetrepharm, London, Ontario, Canada), a purified preparation of follicle stimulating hormone obtained from pig pituitaries. The minipump is implanted approximately 60 hours before the midpoint of the dark cycle during which the females will mate. To ensure ovulation, 20 IU of human chorionic gonadotropin is administered intraperitoneally approximately 12 hours before mating is expected to occur. With the use of such a protocol, 50 to 100 embryos can be obtained per female depending on the strain used. The embryos are microinjected by standard procedures and the surviving embryos implanted into pseudopregnant foster mothers. Routine testing of the resulting progeny by polymerase chain reaction or Southern blotting is normally used to ascertain which of the offspring harbor the transgene. At this stage, the primary difference between the mouse and rat is the logistic considerations of animal husbandry. The generation time of the rat and the space requirements for housing are significantly greater than for the mouse, and it is important to keep a tight control on the breeding stock. This simple, practical aspect is worth emphasizing because in a typical transgenic experiment, one may well initially be breeding five to six independent lines. The maintenance of a similar number of distinct rat strains would be a major undertaking.

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Comparison of Breeding Data for Murine and Nonmurine Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Gestation Time</th>
<th>Litter Size</th>
<th>Onset of Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>19-21 days</td>
<td>5-12</td>
<td>6-8 weeks</td>
</tr>
<tr>
<td>Rat</td>
<td>20-23 days</td>
<td>6-15</td>
<td>9-14 weeks</td>
</tr>
<tr>
<td>Rabbit</td>
<td>28-34 days</td>
<td>5-10</td>
<td>24-35 weeks</td>
</tr>
<tr>
<td>Goat</td>
<td>5 months</td>
<td>1-2</td>
<td>8 months</td>
</tr>
<tr>
<td>Sheep</td>
<td>5 months</td>
<td>1-2</td>
<td>7-8 months</td>
</tr>
<tr>
<td>Pig</td>
<td>4 months</td>
<td>9-10</td>
<td>7-8 months</td>
</tr>
<tr>
<td>Cow</td>
<td>9 months</td>
<td>1-2</td>
<td>12 months</td>
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Although a number of laboratories have established gene-transfer techniques in the rat, there are relatively few publications to date. The initial reports describing the use of the rat for in vivo gene manipulation were studies on renin gene expression and inflammatory disease. The former study demonstrated that a defined genetic change to the rat (integration of the mouse Ren-2 renin gene) resulted in severe hypertension in which the renin-angiotensin system played a critical role despite low circulating levels of active renin. The hypertensive transgenic rat line TGRmRen2-27 is presently being studied by a number of laboratories, and additional transgenic studies more meaningful.

The in vitro evidence for species specificity of the cleavage of angiotensinogen by renin suggested that it may be possible to obtain similar specificity in vivo. This was demonstrated by the introduction of the human angiotensinogen gene into the rat germ line. The infusion of recombinant renin into these transgenic rats resulted in a blood pressure elevation that could be inhibited by a human renin-specific inhibitor. A similar pressor response produced by the infusion of rat renin was not affected by this treatment, confirming the in vivo specificity of angiotensinogen cleavage. This demonstration provides the basis for future transgenic experiments aimed at targeted manipulation of the renin-angiotensin system.

Although most of the current lines of transgenic rats are linked to cardiovascular studies, the rat is now being used successfully for other areas of research. Hochi et al have initiated studies on the secretion of a-plactalbumin into the milk of transgenic mice. Although high-level expression of recombinant proteins has been obtained in transgenic mice (see below), the increased volume of milk produced by the rat may be advantageous for preliminary pharmaceutical development studies.

Thus, transgenic rats are being increasingly used in a variety of research areas, and it is likely that within the next few years the rat will become an established experimental species for in vivo gene manipulation.

Transgenic Rabbits

Transgenic rabbits were first reported by Hammer et al, who obtained germ line integration of a fusion gene consisting of the mouse metallothionein promoter and the human growth hormone structural gene MT-hGH. This construct had previously been shown to be highly expressed in transgenic mice. Fertilized one-cell ova were flushed from the oviduct of superovulated New Zealand White females 19 hours after mating. Nuclear structures in the ova were readily discernible by interference-contrast microscopy, allowing standard techniques of microinjection to be used. The authors reported the integration frequency (defined as the number of animals [fetuses, stillborns, and neonates] that retained the injected DNA/total number of animals resulting from injected ova) of the transgene to be 12.8% in the rabbit. This is lower than that of the mouse (27%) and could be due to factors such as DNA concentration, buffer composition, and age of ovum.

An alternative strategy to germ line integration of a transgene is ex vivo gene therapy, in which the transgene is targeted to specific somatic tissues. This involves the isolation of cells from an animal, the transfer of functional genetic material into the cells in vitro, and subsequent transplantation of the suitably modified cells back into the animal. (A classic example of this would be bone marrow–directed gene therapy.)

Attempts at liver-directed ex vivo gene therapy have been hampered by the limited proliferative potential and maintenance of hepatocytes in culture. However, Wilson and Grossman demonstrated the feasibility of the technique using the Watanabe heritable hyperlipidemic (WHHL) rabbit, which has an inherited deficiency of the receptor for low-density lipoprotein. Hepatocytes from the rabbit were isolated and exposed to recombinant retroviruses carrying the functional human low-density lipoprotein receptor gene. Infusion of the genetically modified cells into the portal vein of recipient WHHL rabbits led to a transient decrease in serum cholesterol. The ultimate goal will be permanent correction of the equivalent human genetic disease of familial hypercholesterolemia.

Transgenic Sheep

Transgenic sheep were first reported by Hammer et al using the MT-hGH construct. Ewes were superovulated with a progesterone-gonadotropin regimen, and fertilized ova were recovered from the reproductive tract. Pronuclei and nuclei of the sheep ova are difficult to locate and were visualized using interference-contrast microscopy. After microinjection, the ova were introduced into the fimbriated end of the oviduct of synchronized recipient ewes. Because of the concern of multiple births, only five or six embryos were transferred per recipient. The transgene integration efficiency reported in this set of experiments was very low (1.3%). Early attempts at generating transgenic farm animals were marred by low integration frequencies, low numbers of animals expressing the recombinant
Transgenic Pigs

Major constraints in large animal transgenic projects include the number of fertilized ova obtainable and the availability of recipient animals for microinjected embryos. On average, two to three times more injectable ova can be recovered from a donor gilt than from a cow, doe, or ewe. Secondly, a recipient sow can carry fivefold more fetuses, and the generation interval of swine is approximately 11 months. This makes the pig an attractive large animal for transgenic manipulation. Ovulation can be controlled hormonally, and the eggs are recovered under general anesthesia. Pig ova are optically opaque, so their nuclear structures are not visible. Centrifugation at 15,000g for 3 to 8 minutes has been found to displace the opaque material in the cytoplasm, allowing pronuclei to be visualized while having no detectable effect on development.27

Recently, high-level synthesis (approximately 1 g/L) of mouse whey acidic protein (WAP) was achieved in the mammary glands of transgenic pigs.28 The WAP gene product is a major constituent of mouse, rat, and rabbit milk but has not been identified in the milk of livestock (including swine). In this experiment, mammary gland regulatory elements from the murine WAP gene were demonstrated to function specifically across the species boundary. Approximately 1% of the injected ova resulted in transgenic founders. Although transgenic progeny were successfully recovered from three of the founders, pigs from two of these lines were unable to sustain lactation. This suggests that high expression of some transgenes could have adverse effects on the physiology of the mammary gland. (There are indications that the WAP construct also causes abrogated lactation in transgenic mice.) Low-level WAP expression was additionally found in the salivary gland of one transgenic pig. Such “leakiness” of tissue-specific expression has been reported with other mammary gland-specific constructs.29

Swanson et al30 generated transgenic pigs that express functional human hemoglobin. The construct contained the locus control region from the human α-globin locus, together with two copies of the human α-gene and a single copy of the human β-gene. All three transgenic founders expressed human hemoglobin, consistent with position-independent expression of the transgenes. The relative amounts of the human and endogenous globins varied among the pigs, however, and this could be due to mosaicism.30 Because functional human hemoglobin could be purified from pig hemoglobin, such transgenic animals could become a potential source for an oxygen-carrying red blood cell substitute.

Transgenic Goats

The dairy goat has a gestation period of 5 months, is ready to breed at 8 months old, and produces an average of 4 L of milk per day, making it potentially important as a producer of recombinant proteins. Ebert et al31 have reported the generation of transgenic goats expressing bovine growth hormone.16 To overcome these difficulties, attempts were made to target protein synthesis to an exocrine organ, namely, the mammary gland, where expressed proteins are sequestered away from the general circulation.

The commercial value of transgenic livestock arises from the prospect of producing large quantities of therapeutic proteins in milk. (Milk yield in sheep, for example, ranges from 250 to 800 L per lactation, depending on the breed.) The main advantages of this procedure over cell culture are the accessibility of the expressed protein, the high production capability of the mammary gland, and the potential to increase the number of expressing animals through animal husbandry. Typically, the gene of interest is fused to the regulatory sequences of a milk protein gene, such that it will be specifically expressed in the mammary gland of the transgenic animals. The mammary gland should be capable of performing most posttranscriptional modifications necessary for activity or stability of the protein.

Several model systems have demonstrated the feasibility of targeting gene expression to the lactating mammary gland, for example, in mice17-18 and rabbits.19 Efficient generation of transgenic livestock and production of foreign proteins in their milk have proved more difficult to achieve, however (for review, see Hennighausen et al20). Transgenic sheep expressing human factor IX in their milk exhibited no physiological or reproductive problems,21 but expression levels were low, despite the use of the ovine β-lactoglobulin gene promoter.

More recently, Wright et al22 have achieved high-level expression of active human α1-antitrypsin in transgenic sheep. In one animal, expression levels stabilized at approximately 35 g/L—approximately 50% of total protein production and substantially higher than that previously reported for any nonmilk protein in a transgenic system. Interestingly, the same minigene construct gave lower yields (ranging from 0.5 to 7 g/L) in transgenic mouse lines.23 The variation in expression levels between different lines and species may be attributed to host genetic background, the site or sites of chromosomal insertion, and absence of specific transcription factors. Improvements in expression were obtained when native, foreign, or hybrid introns were included in the transgene construct.24 Despite improvements in expression levels, the integration frequency of transgenes in sheep remains approximately 1.2%.25

Transgenic Cows

Dairy cows can produce 10,000 L of milk per year and would seem the optimal species for the production of large quantities of heterologous protein. However, the generation interval of cattle is approximately 2 years, with only one offspring produced per gestation. Additionally, the standard microinjection protocol—collection of fertilized eggs from superovulated and artificially inseminated cows, microinjection of the zygotes, and their surgical transfer into the oviduct of recipient cows—is extremely labor intensive. This is particularly apparent when one considers the logistics of regularly supplying the large numbers of bovine zygotes necessary to produce transgenic animals.

Several of these problems appear to have been obviated by combining gene transfer with in vitro embryo
production technology. Bovine ovaries obtained from slaughterhouses were used as a source of oocytes. The oocytes were aspirated from the follicles and were assessed for maturation, and in vitro fertilization was achieved with frozen semen from proven bulls under optimized conditions. Pronuclei were visualized by centrifugation at 14,500g for 8 minutes. After microinjection, the embryos were allowed to develop to the compact morula/blastocyst stage (approximately 9 days; development of the microinjected embryos was somewhat retarded). Healthy embryos were transferred singly to recipients that had been synchronized to start estrus on the day that oocytes were collected. Out of 21 pregnancies, two resulted in transgenic calves carrying the human lactoferrin gene under the bovine αS-casein regulatory elements. It should be noted that the in vitro culture of embryos would allow additional manipulations such as biopsy for sexing, transgenesis detection, and cloning.

Conclusion

Since transgenic technology has been extended to include a wide range of rodents and ruminants, the benefits for cardiovascular and hypertensive research are clear. Animal models can be designed in species that are far better suited to the physiological analyses under consideration. For example, the rat is the animal of choice for kidney transplantation, telemetry, and vascular studies; the rabbit has classically been used for balloon angioopathy studies and provides a good animal model for familial hypercholesterolemia; transgenic manipulation of the pig may lead to advances in transfusion and transplantation technology. Additionally, pharmacologically important proteins can potentially be synthesized in large amounts in the mammary glands of livestock. Transgenesis is a tool for basic, clinical, and pharmaceutical research and with ongoing developments will be refined still further over the next decade.

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