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Developments in transgenic techniques in pigs

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Manipulation of the pig genome is currently restricted to the random insertion of new DNA using pronuclear microinjection. This method suffers from a number of inherent limitations, the majority of which result from the inability to control the site at which the transgene becomes integrated. These drawbacks, together with the need to be able to target existing genes, will result in the replacement of pronuclear injection by new methods that have the capability to direct insertion to a particular genomic site that does not influence expression. Currently, it is possible to control the site of insertion in mice using embryonic stem (ES) cell and homologous recombination technologies. However, pluripotent ES cells have yet to be isolated in pigs. The possibility of using nuclear transfer to reprogramme early differentiated embryonic cells as well as somatic cells from adult animals may provide an alternative method for generating precise genetic modifications. Methods that allow these changes to be carried out *in situ* are also likely to be developed in the future.

Introduction

The ability to manipulate the genome of the pig either by inserting new genetic material or deleting existing genetic information will greatly improve pig production in the coming decades and has a number of important biomedical applications such as xenotransplantation (reviewed by Pursel and Rexroad, 1993; Wall, 1996; Muller *et al.*, in press). In the first part of this review, the production of transgenic pigs using pronuclear injection is reviewed and areas where there is potential to improve existing methodology is highlighted. The second half of the review takes a more speculative approach and describes several approaches currently being developed, which if successful will greatly improve the efficiency of current transgenesis programmes.

Pronuclear Microinjection

Use of prepubertal gilts as embryo donors

Currently, transgenic pigs are produced by injecting DNA directly into the pronucleus of a recently fertilized egg or zygote. Several methods for obtaining pronuclear zygotes from prepubertal and sexually mature animals have been described (Brem *et al.*, 1990; Williams *et al.*, 1992a; Bolamba and Sirad, 1996). Embryos obtained from prepubertal gilts appear to be less competent in their developmental ability than those obtained from mature animals (Pinkert *et al.*, 1989). However, when more embryos are transferred back to recipients to take account for this, there would appear to be little difference in terms of pregnancy rates (Brem *et al.*, 1990). There are considerable cost advantages in using prepubertal gilts compared with mature animals as well as the potential for more embryos. We have compared the number of transgenic pigs that can be produced using both types of donors (M. Nottle, H. Nagashima, P. Verma, Z. Du, C. Grupen and S. MacIlfatrick, unpublished; Tables 1 and 2). The results from this study demonstrated that prepubertal gilts produce similar numbers of ova but produce fewer fertilized ova compared with adult animals. In

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Table 1. Number of corpora lutea, ova recovered, fertilized and injected from superovulated prepubertal or sexually mature donor pigs

Donor	Corpora lutea	Ova recovered	Ova fertilized	One-cell injected
Mature* (n = 63)	19.3±0.6[b]	15.1±0.7[b]	13.6±0.8[b]	10.7±1.0[b]
Prepubertal (n = 63)	20.5±1.5[b]	13.6±1.2[b]	9.3±1.3[c]	7.5±1.0[c]

*Sexually mature animals were treated as described by Nottle *et al.* (1995). Prepubertal gilts (23 week old) were treated as per sexually mature gilts except that 1500 iu of PMSG was used.
Values are means ± SEM and are expressed on a per donor basis. Within columns means with different superscripts are significantly different ($P < 0.05$).

this study pregnancy and integration rates were not different but litter size tended to be lower, although the difference was not significant ($P < 0.1$), when prepubertal donors were used. The efficiency with which transgenic pigs were produced was similar for prepubertal gilts compared with sexually mature donors (3.4% versus 4.0%).

Improving the response to superovulation

Other groups have reported that larger numbers of injectable embryos can be obtained from prepubertal gilts than we obtained in our original comparison (Pinkert *et al.*, 1989; Brem *et al.*, 1990; Williams *et al.*, 1992a). Individual prepubertal gilts appear to respond particularly well to superovulation (Fig. 1), suggesting that there is considerable potential for reducing the number of donors if the overall response could be improved. A number of methods have been examined for improving the response to superovulation in cattle and sheep (reviewed by Armstrong, 1993), some of which may be worthy of consideration in pigs. Several groups have shown that treatment with bovine growth hormone can improve the response to superovulation in cattle (Rieger *et al.*, 1991; Gong *et al.*, 1992; Kuehner *et al.*, 1993). Growth hormone (GH) has direct as well as indirect effects on follicular growth and oocyte maturation (reviewed by Bevers *et al.*, 1997). In a preliminary study, we found that treatment with recombinant pig GH tended to increase the number of small and medium follicles on the surface of the ovary of non-superovulated gilts treated before their second oestrus but the difference was not significant ($P > 0.05$; M. Nottle, C. Grupen and R. Campbell, unpublished; Table 3). Future work will determine whether GH can improve the response to superovulation in prepubertal gilts.

In pigs, PMSG is used exclusively for superovulation. In cattle and sheep, FSH has largely replaced PMSG as the gonadotrophin of choice because it overcomes a number of deficiencies associated with the relatively long half-life of PMSG (reviewed by Armstrong, 1993). The possibility of using FSH in pigs warrants further examination as work by us and others (M. Nottle and R. Ashman, unpublished; see also Bolamba *et al.*, 1996) suggests that larger numbers of injectable embryos may be obtained when FSH rather than PMSG is used to superovulate pigs. The use of GnRH as an alternative to hCG may also be worthy of investigation. Support for this suggestion comes from relatively large AI programmes which have demonstrated that a GnRH analogue increased pregnancy rates in gilts that were not superovulated compared with hCG (Brussow *et al.*, 1996).

The potential for IVM/IVF systems (reviewed by Nagai, 1996) to replace the need for embryo donors, together with further developments in the non-surgical collection and transfer of pig embryos (Li *et al.*, 1996) will result in further efficiencies in microinjection programmes in the future.

Increasing integration rate

Transgenes normally integrate in the form of multiple copies arranged in head to tail arrays at a single site in the genome (Hammer *et al.*, 1985; Palmiter and Brinster, 1986; Burdon and Wall, 1992).

Table 2. Pregnancy rate, litter size and integration rates using embryos obtained from sexually mature or prepubertal pigs

Donor	Transfers	Recipients farrowing (%)	Litter size	Transgenics/ piglets liveborn (%)
Mature	21[a]	17 (81)	8.1±0.5	26/137 (18.9)
Prepubertal	15	12 (80)	6.6±0.6	16/79 (20.3)

[a]Between 30 and 35 injected embryos were transferred per recipient for both groups.
Values are means ± SEM. Means are not significantly different ($P > 0.05$).

Between 0.3% and 4.0% of injected embryos result in the production of a transgenic pig (Pursel and Rexroad, 1993). Increasing the rate at which injected DNA becomes incorporated into the genome is one method whereby the efficiency with which transgenic pigs are generated could be improved. In mice, the concentration at which the DNA is microinjected (or more strictly the number of copies injected) does not appear to influence integration rate (Brinster *et al.*, 1985). However, in pigs, injecting DNA at concentrations greater than 5 ng μl^{-1} appears to result in relatively high integration rates (M. Nottle, H. Nagashima, P. Verma, R. Ashman, Z. Du, C. Grupen, S. MacIlfatrick, M. Harding, C. Cheah, D. Harrison, B. Luxford, R. Campbell, R. Crawford and A. Robins, unpublished; Table 4). Further work is needed to confirm these preliminary results.

Maximizing pregnancy rates

It is well established that microinjection reduces embryo survival (Williams *et al.*, 1992b; Hajdu *et al.*, 1994; Martin *et al.*, 1996). Several groups have examined the number of injected embryos that need to be transferred to maximize pregnancy rates in pigs (Wei *et al.*, 1993; Lancaster *et al.*, 1996; Pursel and Wall, 1996). On the basis of these results, transferring between 30 and 35 injected embryos would appear to be optimal. Similar numbers of embryos would appear to be appropriate for prepubertal donors (Brem *et al.*, 1990; M. Nottle, this supplement). Where sufficient numbers of embryos are not available, the co-transfer of two or three non-injected one-cell (i.e. those with attached spermatozoa but no visible pronuclei) or two-cell embryos may improve pregnancy rates (Hammer *et al.*, 1985; Pursel and Wall 1996; M. Nottle, H. Nagashima, P. Verma, Z. Du, C. Grupen and S. MacIlfatrick, unpublished).

Donor animals can also be used as recipients, reducing the number of animals required for microinjection programmes (Brem *et al.*, 1990; Pursel and Wall, 1996). Pursel and Wall (1996) reported that donors whose ovulation rate was 21 or more had fewer pregnancies than those whose ovulation rates were 20 or less. An increase in the production of ovarian steroids may have been responsible for this difference (reviewed by Armstrong, 1993). Acceptable pregnancy rates have also been reported when prepubertal gilts have been used as embryo recipients (Brem *et al.*, 1990). The method described by Bolamba and Sirad (1996) using prepubertal gilts induced to ovulate and used as donors at their subsequent oestrus may also be useful for producing suitably synchronized recipients.

Methods for Controlling Transgene Insertion

Pronuclear microinjection suffers from a number of inherent limitations, the majority of which arise from the inability to control the site at which the transgene integrates. This results in variable expression depending on the proximity of regulatory elements (so called 'position effects') as well as the potential to disrupt existing genes (reviewed by Bishop, 1997). Transgenes normally integrate as

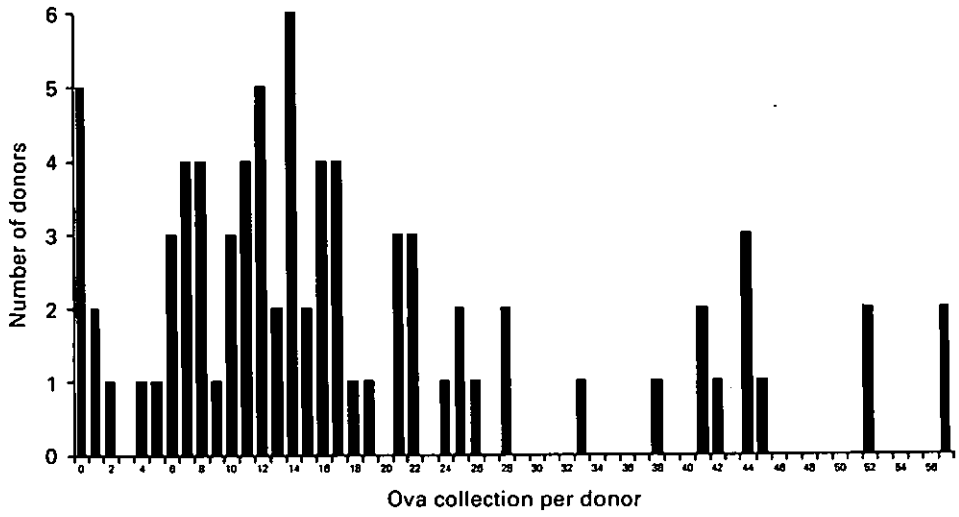

Fig. 1. The number of ova obtained from prepubertal gilts superovulated with 1500 iu PMSG and induced to ovulate 72 h later with 500 iu hCG. Animals were mated at 28–30 h, followed by AI at 30–32 h after hCG. Ova were recovered 50–56 h after hCG by mid-ventral laparotomy under general anaesthesia.

multiple copies, making them prone to methylation (Mehtali *et al.*, 1990) and heterochromatin formation which can also affect expression (Martin and Whitelaw, 1996). Furthermore, the injected DNA does not always integrate at the one-cell stage, resulting in the production of mosaics (Burdon and Wall, 1992; Whitelaw *et al.*, 1993). Mosaicism would appear to be a particular problem in pigs: more than half of transgenic founders transmit the transgene to less than half of their progeny (Brem *et al.*, 1990; Nottle *et al.*, 1996).

A number of methods have been developed in mice for improving transgene expression, including the use of locus control regions (Grosveld *et al.*, 1987) or sequences that insulate the transgene from surrounding elements (Stief *et al.*, 1989). Simpler methods for inserting DNA have also been developed, such as the retroviral infection of early embryos and the use of adenoviruses (Rubenstein *et al.*, 1986; Tsukui *et al.*, 1996). However, none of these has the ability to control where the injected DNA becomes inserted in the genome. The use of yeast artificial chromosomes (YACS) in providing a method for delivering transgenes with the necessary regulatory elements to guarantee expression may prove useful in this regard (reviewed by Montoliu *et al.*, 1994; Brem *et al.*, in press).

The costs associated with producing large numbers of founder animals in order to screen for animals in which the transgene is appropriately expressed is particularly prohibitive for domestic species. The ability to insert a single copy of a transgene at a site in the genome that does not interfere with its expression will be essential for the pig as well as other livestock species.

Embryonic stem cells

The use of embryonic stem (ES) cells (Evans and Kaufman 1981; Martin, 1981) and primordial germ (PGC) cells (Matsui *et al.*, 1992; Resnick *et al.*, 1992) can overcome many of the limitations associated with pronuclear injection and is the subject of a separate review in these proceedings (Piedrahita *et al.*, this supplement). Together with homologous recombination (Capecchi, 1989), it is possible to transform and select for the appropriate insertion *in vitro* before injecting these cells into the developing embryo to produce a chimaera. This is then bred to produce progeny that are heterozygous for the genomic modification. This approach can be used to disrupt the function of existing genes (gene knockout), allowing the effect of a particular gene to be studied (reviewed by

Table 3. Effect of porcine growth hormone on ovulation and follicular growth in pigs

	Ovulation rate	Small follicles (1.0–2.9 mm)	Medium follicles (3.0–5.9 mm)	Large follicles (> 6.0 mm)
Control (n = 11)	13.1±0.9	30.7±6.5	25.1±4.8	1.1±.04
pST[a] (n = 9)	15.2±0.7	43.4±6.0	36.3±6.0	3.0±1.3

[a]50 μg kg^{-1} porcine growth hormone (Reporcin, Southern Cross Biotech, Melbourne) was administered for 14 days from 7 days after the start of the first oestrus.
Values are means ± SEM. Means are not significantly different ($P > 0.05$).

Joyner, 1993). However, pluripotent ES cells are yet to be isolated in pigs (Piedrahita *et al.*, 1990; Notariana *et al.*, 1991; Wheeler, 1994). The ability of nuclear transfer to reprogramme early differentiated embryonic cells (Campbell *et al.*, 1996) as well as somatic cells from adult animals (Wilmut *et al.*, 1997) may circumvent the need for pluripotent cells. The use of nuclear transfer would also overcome the need to generate chimaeras and screen for germline transmission. Nuclear transfer is still in its infancy in pigs (Prather *et al.*, 1989; Nagashima *et al.*, 1992, 1997). However, we have recently demonstrated that it is possible to generate blastocysts from blastomeres obtained as late as the morula stage (Nagashima *et al.*, 1997; Table 5).

Alternative methods for gene insertion

In mice, the time required to transform and select cells that have undergone homologous recombination and reintroduce these into the developing embryo to produce a chimaera can be 6–12 months by the time germline transmission is demonstrated. In pigs the same process would take longer and cost considerably more because of the longer generation interval involved. Furthermore, a number of generations may be required to breed the mutation on to the desired genetic background. Given these limitations, it is likely that alternative methods that allow transgenes to be inserted *in situ* without the need to transform cells *in vitro* may be developed in the future.

Pronuclear injection. The most direct way of inserting genes at a predetermined site would be to use existing pronuclear injection technology and simply inject targeting constructs that contained sufficient homology to guarantee the insertion of a transgene at a predetermined site. However, homologous recombination appears to be a rare event in pronuclear embryos (Brinster *et al.*, 1989a). Methods are being designed to improve this frequency, such as the possibility of using exonuclease-resistant hairpin bends to allow injected DNA to persist to embryonic stages capable of supporting homologous recombination (Horie and Shimada, 1994).

Gene insertion using the gametes. Spermatogonia and oogonia undergo homologous recombination during meiosis. In oogonia this occurs before birth and as such is not readily accessible. Several workers have suggested that spermatozoa can bind DNA *in vitro* and can be used to carry transgenes into the oocyte, which then become incorporated in the genome (Brackett *et al.*, 1971; Lavitrano *et al.*, 1989; Hochi *et al.*, 1990). Considerable controversy surrounds this approach and the consensus appears to be emerging that, although foreign DNA can be detected in the developing embryo or fetus, it is rearranged (Brinster *et al.*, 1989b; Wall, 1996; Kim *et al.*, 1997).

Several groups have demonstrated that injection of DNA–liposome complexes into the testes can transform sperm cells *in situ* (Sato *et al.*, 1994; Ogawa *et al.*, 1995; Kim *et al.*, 1997). Of particular interest is the suggestion (Kim *et al.*, 1997) that this method may transform the male stem cell population. In this study, treatment with busulfan destroyed the majority of developing

Table 4. Summary of BresaGen Pig Transgenesis Programs

DNA concentration (ng μl^{-1})[a]	Transgenics liveborn/ total liveborn (%)	Transgenics liveborn/ ova injected (%)
10	43/148 (29)	43/1327 (3.2)
10	45/141 (32)	45/1835 (2.4)
7.5	76/302 (25)	76/1810 (4.1)
5.0	18/192 (9)	18/1012 (1.8)
1.0	8/185 (4)	8/1137 (0.7)

[a]Constructs ranged in size from 1.8 to 7.1 kb.

Table 5. Development of pig nucleus transfer embryos reconstructed with blastomeres from cryopreserved delipated four–eight-cell or morula stage embryos

Donor blastomeres	Fusion rates (%)	Number of embryos cultured	Number developed to (%)			
			Two-cell	Four-cell	Morula	Blastocyst
4–8 cell	104/113 (92)	100	53 (53)	34 (34)	17 (17)	6 (6)[a]
Morula	56/58 (97)	53	29 (55)	20 (38)	16 (30)	9 (17)[b]

[a,b]Significantly different ($P < 0.05$).
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spermatocytes, causing the stem cells to undergo meiosis and proliferate. Whether homologous recombination would occur at frequencies high enough simply to allow transgenes containing sufficient homology to be injected, animals to be mated and progeny to be screened for the appropriate insertion (or deletion) remains to be determined. Alternatively, it may be possible to screen spermatozoa or embryos for homologous recombination before their transfer. It may also prove possible in the future to isolate spermatogonia and use them in the same way as ES cells are used presently in mice to insert transgenes (Brinster and Zimmermann, 1994).

In situ insertion. A number of possible approaches involving the use of DNA or DNA–RNA oligonucleotides to generate genetic modifications in mammalian cells have been reported. Perhaps the most promising of these is the recent report that DNA–RNA duplexes can be used to correct single base mutations at relatively high frequencies in tissue culture (Cole-Strauss *et al.*, 1996). It may be possible in the future to use a similar approach in fertilized eggs using pronuclear injection as an alternative method for gene knockout applications. Whether similar technology can be used to direct transgene insertion remains to be determined.

Conclusion

Technologies aimed at improving the efficiency with which transgenic pigs can be produced using pronuclear injection continue to be developed. In the future, methods will be developed for pigs as well as other domestic species and these methods will allow transgenes to be inserted at genomic sites that do not influence their expression. It is possible to direct insertion in the mouse using ES cells and homologous recombination technologies. The use of nuclear transfer to reprogramme differentiated cells as well as the development of other methods that allow genetic modifications to be carried out *in situ* may circumvent the need for pluripotent cells in the future.

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