Reproduction Supplement 58, 293–300

Cloning pigs: advances and applications

I. A. Polejaeva

PPL Therapeutics Inc., 1700 Kraft Drive, Suite 2400, Blacksburg, VA 24060, USA

Although mouse embryonic stem cells have been used widely for over a decade as an important tool for introducing precise genetic modification into the genome, demonstrating the great value of this technology in a range of biomedical applications, similar technology does not exist for domestic animals. However, the development of somatic cell nuclear transfer has bypassed the need for embryonic stem cells from livestock. The production of offspring from differentiated cell nuclei provides information and opportunities in a number of areas including cellular differentiation, early development and ageing. However, the primary significance of cloning is probably in the opportunities that this technology brings to genetic manipulation. Potential applications of gene targeting in livestock species are described with particular emphasis on the generation of pigs that can be used for xenotransplantation, and the production of improved models for human physiology and disease. The development of techniques for somatic cell nuclear transfer in pigs and the challenges associated with this technology are also reviewed.

Why clone pigs?

The continuously expanding gap between availability of organs and the number of patients awaiting an organ transplant is the major driving force behind most efforts to clone pigs. Between 1990 and 1999 the number of patients in the US waiting for organ transplants more than tripled from 21 914 in 1990 to 72 110 in 1999 (Transplant Patient Data Source, 2000). The organ donation programme initiated by the US Department of Health and Human Services was not able to reduce this gap. Annual cadaveric and living donor transplants over the same period increased at a far slower rate, from 15 009 in 1990 to 21 715 in 1999 (Fig. 1). This critical shortage of human organs for allotransplantation has forced researchers to look for alternative sources, one of which is xenotransplantation. Pig organs are the most compatible in terms of size and biology, and are ethically less controversial than alternative species (primates). However, pig organs must be genetically modified to overcome the natural destruction of pig organs by the human immune system. Gene targeting is likely to play a major role in preventing hyperacute rejection in organ xenotransplantation. Hyperacute rejection is the initial and most marked response to vascularized pig organs and is triggered by pre-formed antibodies binding to the endothelium lining of blood vessels in the pig organ. The bound human antibodies rapidly activate the complement cascade, as well as activating the endothelium and inducing a response causing it to become pro-coagulatory. The result of this process is total destruction of the graft within minutes to hours of transplantation.

Email: ipolejaeva@ppl-therapeutics.com

© 2001 Society for Reproduction and Fertility

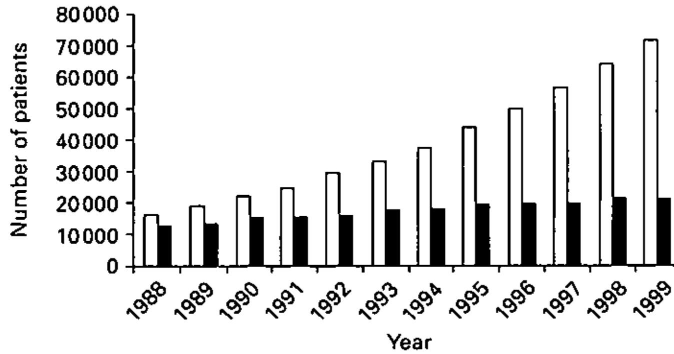

Fig. 1. Expanding gap between the number of patients waiting for organ transplantation (□) and the number of available donor transplants (■).Transplant Patient Data Source (2000).

Evidence has emerged that hyperacute rejection is due primarily to a carbohydrate epitope, galactose, linked via an α (1→3) linkage to a second molecule of galactose (α-1,3 gal), to which about 1% of human immunoglobulins crossreact (Sandrin *et al.*, 1993). The high concentration of circulating antibodies to this epitope is thought to form a first line of defence against pathogens that express α-1,3 gal. The most direct method of preventing the adverse immunological response involves the production of α-1,3 galactosyl transferase gene knockout donor animals. Removal of this enzyme activity would lead to the total lack of expression of the α-1,3 gal epitope on the cell surface, which should reduce hyperacute rejection markedly. It should also prevent acute vascular rejection, which is anti-α-1,3 gal antibody-mediated, and which occurs approximately 5 days after transplantation. It has been reported that a homozygous knockout of α-1,3 galactosyl transferase is not lethal in mice (Thall *et al.*, 1995; Tearle *et al.*, 1996). Knockout mice have normal organ development and although tissues of α-1,3 galactosyl transferase knockout mice show a reduction in activation of human complement, they still retain binding capacity for human xenogenic antibodies (Tanemura *et al.*, 2000). Gene targeting in murine embryonic stem cells has been used widely for over a decade as a powerful tool for introducing modifications of the germ line (Moreadith and Radford, 1997). However, embryonic stem cells that contribute to the germ line are not available for any other species. Nuclear transfer using targeted somatic cells offers a method for producing precise genetic modification in a range of livestock species. Unfortunately, recombination frequencies in somatic immortal cells are on average much lower than in embryonic stem cells (Arbones *et al.*, 1994). Furthermore, homologous recombination events are even less frequent in primary cells than in immortalized cell lines (Finn *et al.*, 1989). Another problem is that primary cultures have a limited lifespan and many of the clonal populations reach proliferative senescence. Nevertheless, two reports indicate that gene targeting insertion (McCreath *et al.*, 2000) and deletions (Denning *et al.*, 2001) can occur in primary sheep fibroblasts and that animals can be generated from targeted primary cells using nuclear transfer. Production of α-1,3 galactosyl transferase knockout pigs has not yet been accomplished, but the recent achievement of cloning pigs (Betthauser *et al.*, 2000; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000) gives rise to increased expectation that it will be possible to produce knockout pigs.

In addition to their roles in xenotransplantation, pigs are often better models for human physiology and disease than rodents because of similarities in anatomy, physiology and size

(Petters, 1994). For example, pigs have a multipyramidal kidney with an undivided cortex; this occurs in only two other species, humans and dwarf water buffalo (Terris, 1986). Similarities in coronary anatomy make the pig an ideal model for ischaemic heart disease and atherosclerosis (Armstrong and Heistad, 1990). Pigs could also be a model for human eye diseases such as retinitis pigmentosa, because of the similarity in eye size and retinal anatomy (Adams, 1988). However, due to the previous low efficiency of transgenic livestock production and a lack of availability of homologous recombination techniques, these applications have been very limited. Somatic cell cloning could also be used as an alternative to microinjection for generating heteroplasmic animal models of mitochondrial DNA diseases. Genetic modifications in pigs also have a number of agricultural applications. A few examples of potential future benefits include: enhancement of resistance to disease and parasites, increased feed efficiency and modification of growth characteristics.

Techniques for nuclear transfer

The technique of nuclear transfer was proposed originally more than 60 years ago by Spemann (1938) as a method to study cellular differentiation. However, it was limited almost entirely to amphibians until McGrath and Solter (1983) demonstrated the possibilities of mammalian cloning. Robl and First (1985) were the first to describe nuclear transfer in pig embryos using a method for pronuclear exchange between zygotes as well as transfer of nuclei between two-cell stage embryos. Prather *et al.* (1988) demonstrated that metaphase II-arrested oocytes could be enucleated, activated and used as recipients for transferred nuclei. The nuclei of two- to eight-cell stage embryos were used as nuclear donors and after nuclear transfer they directed development to mid-gestation.

Techniques for nuclear transplantation involve a number of key factors, each of which potentially has a significant effect on cloning efficiency. These include: (i) removal of metaphase chromosomes from a metaphase II-arrested oocyte (enucleation); (ii) transfer of donor cell nuclei, in which a donor cell is either placed next to an 'enucleated' oocyte and fused using a precise electrical pulse, or the donor cell can be injected directly into the cytoplasm of the enucleated oocyte; (iii) activation of the reconstructed oocytes; (iv) embryo culture; and (v) transfer of the cloned embryos into a synchronized recipient.

Some of these factors have been addressed successfully and others require further investigation. Enucleation of recipient metaphase II oocytes using DNA-specific dyes to verify enucleation and transfer of donor cells are both highly efficient processes (near 100% efficiency).

Oocyte activation has been the most difficult technical component of the nuclear transfer procedure to refine. In all species, when metaphase II oocytes are used as recipients, the method of activation is crucial for subsequent development. Under normal conditions the fertilizing spermatozoon induces oocyte activation by generating a transient increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). Activation of oocytes can be induced artificially by a variety of physical and chemical agents (for reviews see Whittingham, 1980; Prather *et al.*, 1999). Activation can be achieved either by a calcium-dependent mechanism or by a pathway downstream of the calcium signal through inhibition of protein synthesis or kinase inhibition. An increase in $[Ca^{2+}]_i$ can be generated by the entry of external Ca^{2+} through the oocyte plasma membrane, by exposing the oocytes to electric field pulses resulting in the formation of plasma membrane pores (Zimmermann and Vienken, 1982). This method of oocyte activation resulted in the production of viable offspring after transfer of a nucleus from a four-cell stage embryo (Prather *et al.*, 1989). Another method to increase $[Ca^{2+}]_i$ is by stimulating the release of Ca^{2+} from the smooth endoplasmic reticulum stores through Ca^{2+}

release channels using inisitol 1,4,5-triphosphate (IP_3) agonists. Ca^{2+}, Mg^{2+} ionophore is able to increase $[Ca^{2+}]_i$. Presicce and Yang (1994) reported that a combination of an increase in $[Ca^{2+}]_i$ and inhibition of protein synthesis or protein kinase resulted in higher rates of pronuclear formation.

Methods of embryo culture, which are not as advanced in pigs as in cows, may also play a crucial role in cloning. The results presented by Machaty *et al.* (1998) indicate that cultured embryos are developmentally competent (formed conceptuses), even though *in vitro* culture is not able to provide an environment comparable to *in vivo* conditions (lower cell number). A detailed study conducted by Wang *et al.* (1999) showed that abnormal embryonic division begins with the first cell cycle under *in vitro* culture conditions. Morphological abnormalities include fragmentation and binucleation. These morphological abnormalities were not observed in *in vivo*-derived embryos. Day 6 pig blastocysts produced *in vitro* have more than four times fewer cells than do *in vivo*-derived embryos (37.3 ± 11.7 versus 164.5 ± 51.9 nuclei per blastocyst, respectively). Wang *et al.* (1999) also observed an abnormal distribution of actin filaments in the *in vitro*-cultured embryos, which is a possible explanation for abnormal embryo cleavage. A combination of low efficiency of activation with suboptimal culture conditions can be detrimental to the success rates of nuclear transfer procedures.

The relative stage of the cell cycle of the donor and recipient cell is also crucial to the success of nuclear transfer and the production of live offspring. In mammalian species, enucleated metaphase II oocytes are the preferred recipient, owing to the lack of development obtained using enucleated zygotes (Robl *et al.*, 1987; Prather *et al.*, 1989). The use of a diploid donor cell allows the cycles of the donor and recipient to be co-ordinated, while the use of metaphase II oocytes as recipients maximizes the number of mitotic events that the donor chromatin undergoes before initiation of zygotic transcription. The importance of co-ordination of the cell cycle between the recipient cytoplasm and the incoming nuclear component has been discussed in great detail elsewhere (Collas *et al.*, 1992; Campbell *et al.*, 1993; Cheong *et al.*, 1993).

Somatic cell nuclear transfer

Successful somatic cell nuclear transfer using an embryo-derived differentiated cell population was first demonstrated in sheep by Campbell *et al.* (1996). The technique was repeated and extended subsequently using cell populations derived from fetal and adult donors in sheep (Wilmut *et al.*, 1997). The technique has been developed successfully for cattle (Cibelli *et al.*, 1998), goats (Baguisi *et al.*, 1999), mice (Wakayama *et al.*, 1998) and pigs (Polejaeva *et al.*, 2000). The somatic cell nuclear transfer system has an advantage compared with embryonic stem cell technology for producing transgenic animals, because the entire animal is derived from a single transgenic donor nucleus, thereby eliminating the need for generation of an intermediate chimaera before the effect of genetic modification can be assessed (Polejaeva and Campbell, 2000).

At the time of writing, three groups have reported the birth of cloned pigs (Table 1). In the first published report of cloned pigs, Polejaeva *et al.* (2000) used *in vivo*-matured oocytes and a double nuclear transfer procedure. Granulosa cell nuclei were transferred into enucleated recipient oocytes by electrofusion. Oocytes were activated at the time of fusion and an additional electrical activation pulse was applied 30–60 min later to induce a second wave of calcium. The fused embryos were placed into culture. The following day, a second round of nuclear transfer was performed by removing karyoplasts from 1-day-old nuclear transfer embryos, and transferring them into *in vivo*-derived zygotes from which the two pronuclei had been removed. Couplets were fused using an electrical pulse and transferred into

Table 1. Live births resulting from somatic cell nuclear transfer in pigs

Cell type	Cell donor	Donor cell culture conditions	Oocyte maturation	Oocyte activation	Embryo stage at the time of transfer	Live births/ number transferred (%)*	Reference
Granulosa	Adult	Confluent 0–2 days	*In vivo*	Electrical	One-cell	5/72 (6.9)	Polejaeva *et al.* (2000)
Fibroblast	Fetal	Confluent 16 days	*In vivo*	Electrical	Two- to eight-cell	1/36 (2.8)	Onishi *et al.* (2000)
Fibroblast	Fetal	Confluent 0–4 days	*In vitro*	Ionomycin and DMAP	One-cell	2/143 (1.4)	Betthauser *et al.* (2000)
Genital ridge	Fetal	Confluent 0–4 days	*In vitro*	Ionomycin and DMAP	Four-cell stage or later	2/164 (1.2)	Betthauser *et al.* (2000)

*This figure does not include the trials that have resulted in no offspring.

synchronized recipient gilts within 2 h after fusion. Five cloned piglets were produced. This system, which uses fertilized oocytes as cytoplast recipients, bypasses the inefficiencies of artificial activation procedures and may promote more successful development. However, it is very labour intensive and time-consuming. With the recent success in cloning pigs using a standard (single round) nuclear transfer (Betthauser *et al.*, 2000; Onishi *et al.*, 2000), coupled with the further optimization of activation and embryo culture conditions, the double nuclear transfer approach may be replaced by single nuclear transfer techniques.

Onishi *et al.* (2000) produced one cloned piglet by microinjection of somatic cell nuclei into enucleated oocytes, similar to a technique used previously to produce cloned mice (Wakayama *et al.*, 1998). Onishi *et al.* used *in vivo*-matured oocytes as recipients, as did Polejaeva *et al.* (2000), and fetal fibroblast cells were used as nuclear donors. Oocytes were activated by an electrical pulse applied 3–4 h after nuclear microinjection. After immersion in a short-term culture, 110 cloned embryos (two- to eight-cell stage) were transferred to four recipients, resulting in one pregnancy, which yielded one live offspring. It has been suggested that mitochondrial DNA heteroplasmy, resulting from the fusion of donor and recipient cells, could result in high rates of death and abnormal development in fused nuclear transfer embryos. Factors contained within the cytoplasm of a donor cell such as protein and mRNA transcripts could theoretically interfere with reprogramming and development of cloned embryos. This would favour the technique of nuclear transfer by microinjection, which removes much of the donor cell cytoplasm selectively. However, Steinborn *et al.* (2000) demonstrated that mitochondrial DNA heteroplasmy in cloned animals does not necessarily impede normal development.

Betthauser *et al.* (2000) applied techniques similar to those used in bovine cloning. *In vitro*-matured oocytes were used for nuclear transfer and electrofusion was applied to deliver the cell nucleus into an enucleated oocyte. The activation procedure involved increasing calcium concentrations using calcium ionophore (ionomycin) and inhibition of the activity of maturation-promoting factors using the kinase inhibitor, 6-dimethylaminopurine (DMAP). Two types of cell were used in this study: cells from the genital ridge and a population of cells derived from 47- to 51-day-old pig fetuses. Cloned embryos were cultured for up to 3 days and transferred into recipients, resulting in four cloned male pigs.

Many factors contribute to the development of reconstructed embryos. These factors include the quality of the recipient oocyte, method and timing of activation, and culture

methodology. Similarly, induction and maintenance of pregnancy are dependent upon a range of factors, influenced by the quality of the transferred embryos, in combination with the age and hormonal status of the recipient. At the present time it is difficult to determine to what extent each factor or combination of factors has contributed to making pig cloning successful.

We have observed, as have a number of other scientists, significant pre- and post-natal mortality in both ovine and bovine nuclear transfer programmes. Placental (hydroallantois, reduced number of placentomes) and fetal (kidney defects, liver and brain pathology, metabolic and cardiovascular problems) developmental abnormalities associated with somatic cell nuclear transfer have been reported by several research groups (Cibelli *et al.*, 1998; Kato *et al.*, 1998; Wells *et al.*, 1999; Hill *et al.*, 2000). However, problems with developmental abnormalities and death at birth or soon after birth have not been observed in the cloned pigs (Polejaeva *et al.*, 2000; Betthauser *et al.*, 2001). In addition, no fetal losses have been observed after day 40 of gestation. Two factors that may contribute to the low rate of fetal loss are the very different type of placentation in pigs and the limited duration of embryo culture *in vitro*.

Conclusion

The use of nuclear transplantation for livestock species promises to provide enormous benefits. The impact of nuclear transfer on the fields of biotechnology, biomedicine and agriculture looks increasingly promising as new technology and scientific research continue to refine the process of nuclear transfer. However, the efficiency of this procedure is still low in relation to pregnancy and development-to-term rates. Significantly more research is needed to determine how cloning by somatic cell nuclear transfer is achieved. The mechanism of somatic cell nuclear reprogramming, the effect of karyoplast source and its differentiation on reprogramming, the effect of mismatches between nuclear and mitochondrial genes on development, as well as potential species-specific differences, are still unknown. For applied research, somatic cell nuclear transfer offers a new method for transgenesis and allows the production of disease models in species that are physiologically more similar to humans, thereby allowing the progression of disease and the benefits of any potential new therapies to be assessed more effectively. The successful development of nuclear transfer in pigs provides opportunities for multiple applications of gene targeting technology, allowing very precise genetic modifications, including gene knockouts, to be made.

The author would like to acknowledge the staff at PPL Therapeutic Inc. who have contributed to the nuclear transfer experiments: S-H. Chen, T. Vaught, R. Page, P. Jobst, S. Walker, B. Gragg and S. Ball. In addition, the support and helpful advice of D. Ayares, R. Greene and J. Robl in preparation of this manuscript are greatly appreciated.

References

Adams RJ (1988) Ophthalmic system. In *Experimental Surgery and Physiology: Induced Animal Models of Human Disease* pp 125–153 Eds MM Swindle and RJ Adams. Williams and Wilkins, Baltimore

Arbones ML, Austin HA, Capon DJ and Greenburg G (1994) Gene targeting in normal somatic cells: inactivation of the interferon-gamma receptor in myoblasts *Nature Genetics* **6** 90–97

Armstrong ML and Heistad DD (1990) Animal models of atherosclerosis *Atherosclerosis* **85** 15–23

Baguisi A, Behboodi E, Melican D *et al.* (1999) Production of goats by somatic cell nuclear transfer *Nature Biotechnology* **17** 456–461

Betthauser J, Forsberg E, Augenstein M *et al.* (2000) Production of cloned pigs from *in vitro* systems *Nature Biotechnology* **18** 1055–1059

Betthauser J, Forsberg E, Jurgella G *et al.* (2001) Cloning pigs using *in vitro* systems *Theriogenology* **55** 255 (Abstract)

Campbell KH, Ritchie WA and Wilmut I (1993) Nuclear–cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos:

implications for deoxyribonucleic acid replication and development *Biology of Reproduction* **49** 933–942

Campbell KH, McWhir J, Ritchie WA and Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line *Nature* **380** 64–66

Cheong NT, Takahashi Y and Kanagawa H (1993) Birth of mice after transplantation of early cell-cycle-stage embryonic nuclei into enucleated oocytes *Biology of Reproduction* **48** 958–963

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon A and Robl JM (1998) Cloned transgenic calves produced from non-quiescent fetal fibroblasts *Science* **280** 1256–1258

Collas P, Pinto-Correia C, Ponce de Leon A and Robl JM (1992) Effect of donor cell cycle stage on chromatin and spindle morphology in nuclear transplant rabbit embryos *Biology of Reproduction* **46** 501–511

Denning C, Burl S, Ainslie A *et al.* (2001) Deletion of the α(1,3) galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep *Nature Biotechnology* **19** 559–562

Finn GK, Kurz BW, Cheng RZ and Shmookler Reis RJ (1989) Homologous plasmid recombination is elevated in immortally transformed cells *Molecular Cell Biology* **9** 4009–4017

Hill JR, Winger QA, Long CR, Looney CR, Thompson JA and Westhusin ME (2000) Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells *Biology of Reproduction* **62** 1135–1140

Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H and Tsunoda Y (1998) Eight calves cloned from somatic cells of a single adult *Science* **282** 2095–2098

McCreath KH, Howcroft J, Campbell KH, Colman A, Schnieke AE and Kind AJ (2000) Production of gene-targeted sheep by nuclear transfer from somatic cells *Nature* **405** 1066–1069

McGrath J and Solter D (1983) Nuclear transplantation in the mouse embryo by microsurgery and cell fusion *Science* **220** 1300–1302

Machaty Z, Day BN and Prather RS (1998) Development of early porcine embryos *in vitro* and *in vivo*. *Biology of Reproduction* **59** 451–455

Moreadith RW and Radford NB (1997) Gene targeting in embryonic stem cells: the new physiology and metabolism *Journal of Molecular Medicine* **75** 208–216

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H and Perry ACF (2000) Pig cloning by microinjection of fetal fibroblast nuclei *Science* **289** 1188–1190

Petters RM (1994) Transgenic livestock as genetic models of human disease *Reproduction, Fertility and Development* **6** 643–645

Polejaeva IA and Campbell KHS (2000) New advances in somatic cell nuclear transfer: application in transgenesis *Theriogenology* **53** 117–126

Polejaeva IA, Chen S, Vaught TD *et al.* (2000) Cloned pigs produced by nuclear transfer from adult somatic cells *Nature* **407** 86–90

Prather RS, Sims MM and First NL (1988) Nuclear transplantation in early porcine embryos *Theriogenology* **29** 290 Abstract

Prather RS, Sims MM and First NL (1989) Nuclear transplantation in early pig embryos *Biology of Reproduction* **41** 414–418

Prather RS, Tao T and Machaty Z (1999) Development of the techniques for nuclear transfer in pigs *Theriogenology* **51** 487–498

Presicce GA and Yang X (1994) Nuclear dynamics of parthenogenesis of bovine oocytes matured *in vitro* for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment *Molecular Reproduction and Development* **37** 61–68

Robl JM and First NL (1985) Manipulation of gametes and embryos *Journal of Reproduction and Fertility Supplement* **33** 101–114

Robl JM, Prather R, Barnes F, Eyestone W, Northey D, Gilligan B and First NL (1987) Nuclear transplantation in bovine embryos *Journal of Animal Science* **64** 642–647

Sandrin MS, Vaughan HA, Dabkowski PL and McKenzie IF (1993) Anti-pig IgM antibodies in human serum react predominantly with Gal(alpha 1-3)Gal epitopes *Proceedings National Academy of Sciences USA* **90** 11 391–11 394

Spemann H (1938) *Embryonic Development and Induction* pp 210–211. Yale University Press, New Haven

Steinborn R, Schinogl P, Zakhartchenko V, Achmann R, Schernthaner W, Stojkovic M, Wolf E, Muller M and Brem G (2000) Mitochondrial DNA heteroplasmy in cloned cattle produced by fetal and adult cell cloning *Nature Genetics* **25** 255–257

Tanemura M, Maruyama S and Galili U (2000) Differential expression of alpha-Gal epitopes (gal alpha1-3Gal beta1-4GlcNAc-R) on pig and mouse organs *Transplantation* **69** 187–190

Tearle RG, Tange MJ, Zannettino ZL *et al.* (1996) The alpha-1,3-galactosyltransferase knockout mouse: implications for xenotransplantation *Transplantation* **61** 13–19

Terris JM (1986) Swine as a model in renal physiology and nephrology: an overview. In *Swine in Biomedical Research* pp 1673–1689 Ed. ME Tumbleson. Plenum Press, New York

Thall AD, Malý P and Lowe JB (1995) Oocyte Gal alpha 1,3Gal epitopes implicated in sperm adhesion to the zona pellucida glycoprotein ZP3 are not required for fertilization in the mouse *Journal of Biochemistry* **270** 21 437–21 440

Transplant Patient Data Source (2000) February 16. Richmond, VA: *United Network for Organ Sharing*: http://www.patients.unos.org/data.html

Wakayama T, Perry ACF, Zucotti M, Johnson KR and Yanagimachi R (1998) Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei *Nature* **394** 369–374

Wang W, Abeydeera LR, Han Y, Prather R and Day B (1999) Morphologic evaluation and actin filament distribution in porcine embryos produced *in vitro* and *in vivo*. *Biology of Reproduction* **60** 1020–1028

Wells DN, Misica PM and Tervit HR (1999) Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells *Biology of Reproduction* **60** 996–1005

Whittingham DG (1980) Parthenogenesis in mammals. In *Oxford Reviews of Reproductive Biology* pp 205–231 Ed. CA Finn. Clarendon Press, Oxford

Wilmut I, Schnieke AE, McWhir J, Kind AJ and Campbell KHS (1997) Viable offspring derived from fetal and adult mammalian cells *Nature* **385** 810–813

Zimmermann U and Vienken J (1982) Electric field-induced cell-to-cell fusion: topical review *Journal of Membrane Biology* **67** 158–174