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Manipulating early pig embryos

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On the basis of established surgical procedures for embryo recovery and transfer, the early pig embryo can be subjected to various manipulations aimed at a long-term preservation of genetic material, the generation of identical multiplets, the early determination of sex or the alteration of the genetic make-up. Most of these procedures are still at an experimental stage and despite recent considerable progress are far from practical application. Normal piglets have been obtained after cryopreservation of pig blastocysts hatched *in vitro*, whereas all attempts to freeze embryos with intact zona pellucida have been unsuccessful. Pig embryos at the morula and blastocyst stage can be bisected microsurgically and the resulting demi-embryos possess a high developmental potential *in vitro*, whereas their development *in vivo* is impaired. Pregnancy rates are similar (80%) but litter size is reduced compared with intact embryos and twinning rate is approximately 2%. Pig blastomeres isolated from embryos up to the 16-cell stage can be grown in culture and result in normal blastocysts. Normal piglets have been born upon transfer of blastocysts derived from isolated eight-cell blastomeres, clearly underlining the totipotency of this developmental stage. Upon nuclear transfer the developmental capacity of reconstituted pig embryos is low and < 10% develop to morulae or blastocysts *in vitro*. Pig oocytes can be stimulated parthenogenetically and up to 10% grow to blastocysts in the *in vitro* culture. Sex determination can be achieved either by separation of X and Y chromosome bearing spermatozoa by flow cytometry or by analysing the expression of the HY antigen in pig embryos from the eight-cell to morula stage. Microinjection of foreign DNA has been successfully used to alter growth and development of transgenic pigs, and to produce foreign proteins in the mammary gland or in the bloodstream, indicating that pigs can be used as donors for valuable human pharmaceutical proteins. Another promising area of gene transfer is the increase of disease resistance in transgenic lines of pigs. Approximately 30% of pig spermatozoa bind considerable amounts of foreign DNA preferably at the post-acrosomal region, suggesting that transgenic animals can be obtained more efficiently than with the usual microinjection procedure. To increase gene transfer efficiency, considerable research efforts have been made to establish embryonic stem (ES) cells, but so far there is no definite proof of totipotency of the generated pig ES-like cells through viable chimaeras. In general, biotechnological procedures are much less advanced in pigs than in cows.

Introduction

Pig embryos with intact zona pellucida can be efficiently collected and transferred by established surgical procedures. The pregnancy rate upon transfer of embryos without an extended intermediate *in vitro* phase after collection reaches 82–85% and approximately 50–60% of the transferred embryos survive to term

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(see Polge, 1982), which is similar to the rate after artificial insemination or mating. Use of relatively simple regimens for the induction of superovulation allows the natural ovulation rate to be increased by an average of two to three times (Niemann and Elsaesser, 1987).

Thus, pig embryos from day 1–6 after insemination are readily and abundantly available, which makes the pig an attractive model for exploring basic aspects of early embryology and for the development of various biotechnological procedures involving manipulation of early embryos. These so-called embryo transfer related biotechnologies (Niemann, 1989) include cryopreservation of oocytes and embryos, bisection of embryos to produce identical twins, sex determination, the generation of chimaeras, nuclear transfer, gene transfer and *in vitro* maturation and fertilization. The purpose of this review is to summarize the current status in these areas with the exception of *in vitro* maturation and fertilization (IVM and IVF) which are covered by Niwa (this supplement).

Freezing of Pig Oocytes and Embryos

In contrast to embryos from cattle, sheep and goats (for review see Niemann, 1991), pig embryos with intact zona pellucida have not been frozen successfully so that the delivery of normal piglets has occurred. Earlier studies showed that pig embryos degenerate when cooled to temperatures of 5–10°C (Wilmut, 1972; Polge *et al.*, 1974) and 15°C has been identified as the critical temperature at which damage occurs (Polge, 1977). The rate of cooling to the critical temperature has no effect on survival (Wilmut, 1986). This sensitivity has been demonstrated in pig oocytes at the germinal vesicle stage as well as in pig morulae, which after chilling to temperatures below 15°C show lysis and no development to blastocysts (Didion *et al.*, 1990; Plante *et al.*, 1993). Damage to membrane lipids has been identified as the major reason for the lack of survival of pig embryos after exposure to decreased temperatures or freezing (Polge and Willadsen, 1978; Wilmut, 1986). In addition, it was suggested that the high lipid content of pig embryos may lead to uneven intracellular ice formation, which was considered the major cause of degeneration after freezing and thawing (Toner *et al.*, 1986). However, pig embryos with intact zona pellucida tolerate the osmotic alterations occurring upon addition and removal of cryoprotectants such as glycerol, dimethyl sulfoxide (DMSO) or sucrose. However, phosphatidylcholine, a potent membrane stabilizer, could not protect embryonic lipids (Niemann, 1985). The addition of antifreeze glycopeptides from antarctic fishes (AFGP) in a vitrification solution consisting of 7.5% propylene glycol, 2.5% glycerol, 20% fetal calf serum and 0.05 mol sucrose l^{-1} rescued 25% of immature pig oocytes and two-cell stage embryos from degeneration after exposure of the samples to −196°C. The AFGPs appeared to preserve the structural integrity of the cell membrane, but that was not always associated with the maintenance of viability (Rubinsky *et al.*, 1992). AFGPs are known to inhibit ice crystal growth below the freezing point and are found in abundance in antarctic fishes. When testing the toxicity of several vitrification solutions consisting of propylene glycol and glycerol, it was shown that embryos from compact morula to the hatched blastocyst had an impaired developmental potential and none survived the ultimate vitrification procedure (Weber *et al.*, 1992). However, it was shown that pig embryos from day 5 to day 7 tolerated the exposure to three different vitrification solutions (3.4 mol glycerol l^{-1} plus 3.4 mol propylene glycol l^{-1}, 8.0 mol ethylene glycol l^{-1}, 6.5 mol glycerol l^{-1} plus 6% BSA) and 70–90% continued development (Dobrinsky and Johnson, 1993). This finding suggests that pig embryos can withstand the conditions associated with high concentrations of cryoprotectants in vitrification media. In contrast to pig embryos with intact zona pellucida, a high percentage of advanced embryonic stages, preferably developed *in vitro*, can survive controlled freezing and thawing procedures. Expanded and hatched blastocysts appear to have a better tolerance to decreased temperatures and survive cooling to 6°C (Nagashima *et al.*, 1988a). Between 60 and 80% of hatched blastocysts even survived freezing to −20°C and hatched *in vitro* blastocysts have the highest survival rate (Nagashima *et al.*, 1989). Where controlled freezing and thawing procedure and termination of freezing at −35°C was used followed by an immediate thawing, five normal piglets were produced upon transfer of eleven expanded blastocysts (Hayashi *et al.*, 1989). Cryopreservation of blastocysts hatched *in vitro* in 1.5 mol glycerol l^{-1} and storage in liquid nitrogen resulted in 47% survival and four normal pigs upon transfer (Kashiwazaki *et al.*, 1991). Subsequent experiments (Nagashima *et al.*, 1992a) revealed that the freezability of pig blastocysts is greatest immediately after hatching at a size of 150–300 µm and then decreases rapidly when the blastocysts increase in

size. Moreover, it was shown that the type of culture medium supplementation is essential for achieving sufficient tolerance to freezing, and BSA was found to be superior to serum, suggesting a beneficial modification of membrane fluidity. However, significant differences in the freezability were observed among embryos derived from different breeds of pig (Nagashima *et al.*, 1992a). Despite this recent progress, there is still no procedure that can overcome the enormous sensitivity of pig embryos with intact zona pellucida to lower temperatures. From a hygienic point of view, protocols based on hatched blastocysts are not ideal, as the intact zona pellucida is an efficient barrier to various kinds of infective agent (see Singh, 1987). Thus, the use of frozen–thawed pig embryos and its benefit for the pig industry such as the international exchange of genetic material, the improvement of quarantine conditions, the maintenance of genetic resources through embryo banking and a facilitation of synchronization still await further exploitation.

Sex Determination

Recently, a major breakthrough has been reported for the separation of X and Y chromosome bearing spermatozoa. The procedure is based on the identification of the minimal difference in DNA contents (about 3%) between X and Y spermatozoa using a flow cytometer (Johnson *et al.*, 1989). When sorting boar semen for the Y chromosome, 68% of males and 32% of females could be obtained, whereas sorting for the X chromosome led to 26% male and 74% female piglets, which was significantly different from the normal 50:50 ratio (Johnson, 1991). The current technology, however, has three major obstacles that prevent a broader application. First, the number of spermatozoa that can be separated per hour is too small (3.5×10^5); second, labelling with the fluorochrome decreases sperm viability and leads to a higher embryonic mortality; and third, the expenses of an appropriate flow cytometer are enormously high. Use of X and Y sorted semen is therefore only practical for *in vitro* fertilization programmes. Recently it was shown that flow sorted pig spermatozoa can lead to normal, but reduced, fertilization and blastocyst formation (Rath *et al.*, 1993) and normal calves were obtained following *in vitro* fertilization with flow sorted bovine semen (Cran *et al.*, 1993).

From all of the methods used to determine sex in early embryos, only the immunological approach has been shown to some extent to be feasible in pigs. Use of an immunofluorescence test allowed the detection of the HY antigen on male pig embryos from the eight-cell to the early blastocyst stage (White *et al.*, 1987). The accuracy of sex determination was 81%, which is similar to the results obtained for other domestic species with this method. However, the zona pellucida had to be removed before incubation with the antibody owing to the number of spermatozoa usually attached to the pig zona pellucida which interfere with a proper sex diagnosis. The fluorescence of the HY antigen was verified by chromosome analysis, although pigs have three pairs of metacentric chromosomes, and the Y chromosome is a little smaller than the two other chromosomes (Anderson, 1987; White *et al.*, 1987). Up to now, the developmental potential *in vivo* of pig embryos that have undergone sex determination by an HY antigen test has not been reported. Other methods of sex determination that have been used successfully in mouse and cattle, such as the cytogenetic approach from demi-embryos, or from a biopsy of an early embryo, or from the trophoblastic tissue of a later stage, through measurement of X chromosome linked enzyme activities, the different developmental rate of male and female embryos (Avery *et al.*, 1992) or the use of Y chromosome specific DNA probes, have not been used for pig embryos (see Van Vliet *et al.*, 1989). The potential application of sex determination in pigs can be seen in a reduction of costs due to the elimination of supernumerary male embryos in particular in export programmes and the use of fewer recipients. However, the methods would have to be extremely efficient, otherwise field application is not feasible.

Formation of Chimaeras

A chimaera is defined as a composite animal whose cells are derived from two or more different sources. Mammalian chimaeras are usually produced by aggregation of two or more cleavage stage embryos (aggregation chimaeras) or through injection of embryonic cells into the blastocoel of a blastocyst (injection chimaeras). Chimaeras have been produced in mice, rats, rabbits, sheep, cows, goats (see Yang and

Anderson, 1992) and recently also in pigs (Kashiwazaki *et al.*, 1992). In the latter study day 6 blastocysts were collected from Landrace donors; the zona pellucida was removed, the inner cell mass (ICM) was isolated via immunosurgery, subsequently dissociated and 10–20 ICM cells were injected into the blastocoel of a day 6 blastocyst derived from a Duroc animal. In approximately 60% of the manipulated blastocysts, the injected cells could be identified and these blastocysts were transferred to synchronous recipients and resulted in two pregnancies. Among the 11 piglets, one male with an overt chimaerism was detected (Kashiwasaki *et al.*, 1992). Pig chimaeras could be useful tools in biomedical research, particularly when immunocompetent animals are needed in various kinds of transplantation or infection studies (Guzzetta *et al.*, 1991). In our experiments we attempted to generate chimaeras between Landrace pigs and Sinclair Miniature pigs (H. Niemann, N. M. Loskutoff and D. C. Kraemer, unpublished observation). This miniature pig strain is highly inbred, has a very low reproductive efficiency and is characterized by the formation of melanomas. Approximately 25% of the offspring are born with melanomas and these are used as a model for human skin cancer. The generation of chimaeras would allow the study of the growth of melanomas in a different tissue environment and could therefore increase basic knowledge of the aetiology of this tumour. The Sinclair Miniature and the Landrace pigs yielded a similar superovulatory response with approximately 30 corpora lutea and 22–24 viable four-cell stage embryos per donor. Embryos at the four-cell stage were collected from Sinclair and Landrace pigs; the zona pellucida was removed in a consecutive treatment of 0.5% pronase and acidified PBS (pH 3) and the embryonic cells were microsurgically separated and halves from Sinclair and Landrace embryos were aggregated within one zona pellucida. In total, 74 chimaeric embryos were produced and transferred to five synchronous recipients. Two of the five recipients were considered as pregnant by ultrasound but did not carry to term. At slaughter the uterine lumen contained only degenerated embryonic and extra-embryonic tissue.

Another attempt to create chimaeras revealed that microsurgical transplantation of a single blastomere into a recipient four-cell embryo did not impair development of micromanipulated embryos *in vitro*, but micromanipulated embryos contained fewer nuclei after 3 days culture *in vivo* in the porcine oviduct compared with controls. It was suggested that the slit of the zona pellucida in micromanipulated embryos made them more susceptible to a neutrophilic invasion thereby contributing to the reduced development *in vivo* (Martin *et al.*, 1991). The great potential of pig chimaeras, in particular for biomedical research, warrants further investigations to gain a better understanding on the fate of individual cells after aggregation or injection into host embryos.

Production of Identical Multiplets

Among the different methods to produce genetically identical animals, embryo bisection and nuclear transfer have been of greater interest in pigs, whereas the proliferation of single blastomeres and parthenogenesis have not been explored very much.

Embryo bisection

In contrast to the well-developed bisection technology in ruminants, reports for pigs are scarce and only a few identical twin piglets have been generated after transfer of bisected pig embryos (Polge, 1985; Nagashima *et al.*, 1988b; Ash *et al.*, 1989; Reichelt and Niemann, in press). The main obstacles to generating more identical twin piglets relate to the fact that in pigs pregnancy is maintained only when a minimum of two to four embryos implant and at least one quarter of the uterine surface is covered by embryonic tissue (Polge *et al.*, 1966; Webel *et al.*, 1970). Furthermore, the transuterine migration during the preimplantation phase (Dziuk *et al.*, 1964) does not allow a separate transfer of demi-embryos into each uterine horn and thereby prevents an easy identification of monozygosity. Thus, in pigs, identification of monozygosity requires analysis of polymorphic traits at specific gene loci such as those for blood groups, isoenzymes and plasma proteins or restriction mapping of specific simple repetitive DNA sequences (i.e. DNA fingerprinting) (Jeffreys *et al.*, 1985; Nakamura *et al.*, 1987). Recently, we studied systematically the developmental potential of bisected pig morulae and blastocysts *in vitro* and *in vivo* and found that 66% of 1162 bisected embryos were evaluated as transferable after 24 h of culture *in vitro* (Reichelt and Niemann, in press). Upon transfer, 22 of 27 recipients (81.5%) remained pregnant and 21

recipients delivered 126 piglets of which six were born dead. The mean litter size was reduced ($P < 0.01$) compared with controls (6.0 ± 2.5 versus 10.5 ± 2.1 piglets). Among the piglets we could identify seven sets of identical twins via analysis of blood genetic markers at 25 different gene loci and by DNA fingerprinting corresponding to a twinning rate of 2.3% (seven sets of 311 pairs of demi-embryos transferred to pregnant recipients, Reichelt and Niemann, in press). Within this study we analysed a number of potential factors affecting the percentage of transferable demi-embryos after 24 h of culture *in vitro* such as the experimental day, donor animal, boar, embryo quality, the duration of the culture *in vitro* before microsurgical bisection, and the size of the halves after bisection (Table 1).

Table 1. Various effects of factors (experimental day, donor, boar) that influenced percentage of transferable pig demi-embryos after 24 h of culture *in vitro*

	Percentage embryo survival within		
	Experimental day (n = 26) %	Donor (n = 24) %	Boar (n = 22) %
	66	63	68
	57	46	58
	66	55	35*
	50	37	72
	55	80	76
	39	79	79
	83	90	65
	35	52	69
	75	87	69
	73	78	68
	77	75	75
	77	60	60
	76	88	72
	81	81	86
	75	79	64
	43	83	89**
	78	39	79
	85**	66	74
	65	96**	70
	78	76	38
	69	78	41
	60	67	46
	73	65	
	39	35*	
	64		
	34*		
Means ± within factor SD	64.3 ± 15.7	69.0 ± 17.4	66.0 ± 14.6

*Minimum; **maximum.

A total of 26 different experimental days were analysed in which more than 20 embryos were bisected and we found that the percentage of transferable pairs of demi-embryos was different ($P < 0.01$) among experimental days and ranged from 34% to 85%. The study of the effect of donor animal involved 583 embryos from 24 different donors with 20–35 bisected embryos per donor. The percentage of

transferable demi-embryo pairs was different ($P < 0.01$) among donors and ranged from 35% to 96%. The influence of a boar factor included analysis of 906 embryos derived from matings with 22 different boars and 20–130 bisected embryos per boar. A considerable difference among boars ranging from 35% to 89% was observed. However, it did not reach a statistically significant level mainly because of the small number of mated donors per boar. With regard to embryo quality, embryos classified as excellent (perfect morphology) before bisection resulted in a higher ($P < 0.01$) percentage of transferable pairs (661 out of 969; 68%) than did embryos classified as good (minor morphological deviations) before bisection (103 out of 193; 53%). Embryos cultured for less than 6 h before bisection yielded 69% transferable embryos (601 embryos), whereas after culture for more than 6 h, the percentage of transferable pairs was 62% (561 embryos) ($P < 0.05$). Finally, the percentage of transferable pairs was higher (73% versus 38%) ($P < 0.01$) when microsurgical bisection yielded halves of an identical (50:50) size ratio (922 embryos) compared with bisection resulting in halves of a size of 60:40% (217 embryos).

This analysis showed that several factors have a significant effect on the success rate of pig embryo bisection. The factor 'experimental day' represents a very complex mixture of influences which are difficult to specify. The significant effects of both the female and the male on the percentage of transferable bisected pig embryos suggests that certain genetic combinations yield embryos that are better suited for microsurgical intervention, whereas others are less appropriate. This would, however, be difficult to investigate under our experimental conditions because the donors are killed. In mice, embryos from certain strains survived cryopreservation better than those from other strains and an individual influence from a particular maternal–paternal combination was shown (Schmidt *et al.*, 1985; Pomp and Eisen, 1990). This finding suggests that the suitability for other manipulations can also be affected by the genetic origin of the embryo. However, interactions with other factors, such as developmental stage and embryo quality, cannot be completely ruled out.

Similar to our findings, it has been shown that bovine embryos of a better quality after bisection yielded higher pregnancy rates than did embryos with minor morphological deviations (Voelkel *et al.*, 1984). Moreover, the time elapsed from embryo collection until bisection significantly influenced the percentage of transferable pairs after 24 h of culture *in vitro*. The small but significant difference of 7% suggests that bisection should be done as soon as possible after recovery of embryos. Finally, our results show that the smaller half of a pair of demi-embryos possessed much less developmental potential than did the larger half. The optimal size ratio was easier to achieve in blastocysts than it was in morulae in which there was early compaction and frequently a nonsymmetrical shape, which meant that it was more difficult to achieve even bisection. Collectively, the results of this analysis indicate that the highest success rates can be expected when pig embryos at the blastocyst stage with an excellent morphological quality, immediately after recovery, are bisected into two even halves.

The further exploitation of the technology to produce identical twin piglets could benefit the pig industry because of a facilitated implementation of specific breeding strategies, but could also benefit basic biomedical research. Our identical twin piglets have been used in reciprocal organ transplantation studies serving as a model for human pancreas transplantation. They proved to be extremely valuable because no rejection of the respective graft occurred.

Proliferation of isolated blastomeres

Use of isolated blastomeres derived from mammalian embryos has gained particular significance for purposes such as multiplication of genotypes from economically superior animals (Bondioli *et al.*, 1990; Prather and First, 1990a; First and Prather, 1991a), DNA probes for sex determination (Bondioli *et al.*, 1989; Herr and Reed, 1991) or screening for the transmission of genetic disorders (Handyside *et al.*, 1990). Previous investigations have demonstrated that single blastomeres from two-cell mouse embryos (Tarkowski, 1959; Rossant, 1976), four- and eight-cell rabbit embryos (Moore *et al.*, 1968) and two-, four- and eight-cell sheep embryos (Willadsen, 1979, 1981) can still give rise to normal progeny. In pigs, approximately one third of blastomeres derived from four- to six-cell embryos can develop to blastocysts after transfer to recipient gilts (Moore *et al.*, 1969). When single blastomeres derived from four- and eight-cell pig embryos were cultured *in vitro* low blastocyst formation rates (4–13%) were obtained (Menino and Wright, 1983). *In vitro* culture of blastomeres from pig embryos up to the eight-cell stage in Whitten's

medium supplemented with BSA and 10% fetal calf serum or in a coculture system using porcine oviductal cells, either alone or after reaggregation of two or more embryonic cells, yielded insufficient blastocyst formation rates, particularly for single blastomeres, whereas aggregated blastomeres showed a modestly higher rate of development (Smith *et al.*, 1992). In another study, approximately 40–50% of normal blastocysts were obtained after reaggregation of single blastomeres from four- and eight-cell embryos (Saito *et al.*, 1991). Lee *et al.* (1993) showed that 55–77% of single blastomeres derived from four-cell embryos could develop to normal morulae and blastocysts after being subjected to an electrical pulse and electrofusion medium, which indicates that the development of isolated pig blastomeres could be stimulated by this method.

Studies in our laboratory showed that by a modification of the *in vitro* culture conditions isolated blastomeres derived from four- and eight-cell embryos (1/4, 1/8) can give rise to normal blastocysts, and normal piglets have been obtained after transfer of blastocysts derived from cultivated blastomeres originating from eight-cell embryos (Saito and Niemann, 1991). The most beneficial effects were observed when the culture dishes were coated with the extracellular matrix component fibronectin and modified Krebs ringer bicarbonate solution (mKRB) supplemented with 10% heat inactivated lamb serum were used as the culture medium. Under these conditions, a 40–50% blastocyst formation rate was achieved and cell number (about 50 nuclei) and diameter (200–230 μm) measured in a representative number of blastocysts showed that these parameters were similar to those of conventional blastocysts at a comparable day of development. This study shows that 1/8 blastomeres had a greater potential *in vitro* than 1/4 and 1/16 blastomeres (Saito and Niemann, 1991). This finding was in apparent contrast to previous studies in which a progressive loss of developmental capacity in isolated blastomeres with increasing cell stage of the parent embryo was found in various species. In autoradiographic studies eight-cell pig embryos displayed a significantly higher rate of nuclear DNA synthesis than did earlier stages, which coincided with a functional restructuring of the nuclei in the eight-cell embryos (Tomanek *et al.*, 1989). It was therefore concluded that the protein synthesis and mitotic activity in eight-cell pig embryos is greater than at earlier stages, and this notion is supported by our finding of a higher development in 1/8 blastomeres. Our results suggest that it should be feasible to use isolated blastomeres from embryos at least up to the eight-cell stage to produce groups of identical piglets.

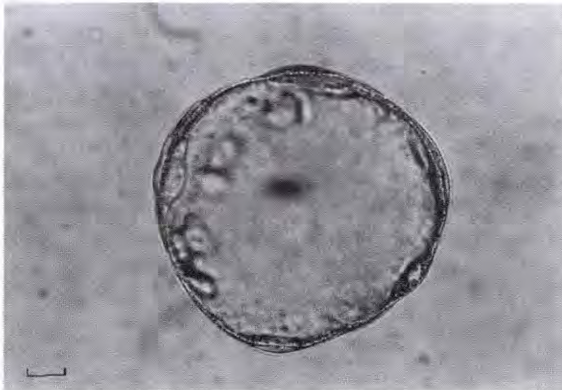

Fig. 1. Expanded blastocyst developed from a 1/8 porcine blastomere after 96 h of *in vitro* culture. Scale bar = 25 μm.

In an extension of the study from Saito and Niemann (1991), we investigated the effects of several growth factors such as epidermal growth factor (EGF) and insulin-like growth factors I and II (IGF-I and -II) on the development of isolated pig eight-cell blastomeres *in vitro*. The blastomeres were isolated as described by Saito and Niemann (1991) and were cultured in mKRB medium that was either supplemented with 10% lamb serum or contained 0.01% polyvinyl alcohol to avoid adhesion of the blastomeres to the bottom of the dish. EGF was used at concentrations of 1, 10 and 100 ng ml^{-1} and IGF-I and -II at concentrations of 1, 10, 100 and 200 ng ml^{-1}. In some groups, the growth factors were renewed after

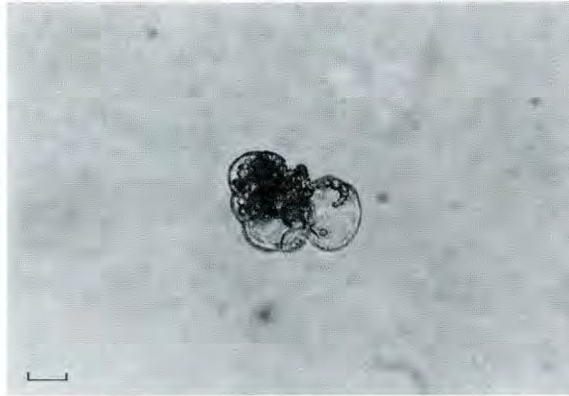

Fig. 2. Non-integrated form of a 1/8 porcine blastomere after 96 h of *in vitro* culture. Scale bar = 25 μm.

Table 2. Development of pig 1/8 blastomeres *in vitro* after 96 h in serum-enriched mKRB supplemented with various concentrations of epidermal growth factor (EGF)

EGF ($ng\ ml^{-1}$)	Total*	No development *n*	(%)	Non-integrated forms *n*	(%)	Blastocysts *n*	(%)
0	248	64	(25.8)	64	(25.8)	120	(48.4)
1	248	83	(33.5)	60	(24.2)	105	(42.3)
10	248	65	(26.2)	73	(29.4)	110	(44.4)
100	249	75	(30.1)	63	(25.3)	111	(44.6)

*15 replicates.
mKRB: modified Krebs ringer bicarbonate.

culture for 48 h. As the rates of development were similar in groups with and without renewal of growth factors, the data were pooled. All blastomeres were cultured for 96 h and the development to regular blastocysts was recorded (Figs 1 and 2). At the end of the culture, the diameter and the number of cells were determined. A general feature in this experiment was that the development in serum-free culture medium was insufficient and the percentage of blastocysts obtained ranged from 0 to 5.6% for EGF, 0 to 5% for IGF-I and 0 to 11% for IGF-II. No stimulating effects of the three growth factors were detected. In contrast, the development of isolated blastomeres in serum enriched culture medium was significantly improved compared with development in serum-free culture and the maximum percentage of blastocysts reached 51% for EGF, 54% for IGF-I and 53% for IGF-II (Tables 2, 3 and 4). Although significant differences in the rates of blastocyst development were observed among the different concentrations of IGF-I and -II, the presence of these growth factors could not consistently support the rate of development of 1/8 blastomeres to normal blastocysts compared with controls. Some of the blastocysts reached the diameter and the number of cells of conventionally grown blastocysts. However, on average, blastocysts derived from 1/8 blastomeres had diameters of 150–170 μm and 15–20 cells; number of cells and cell diameter were closely correlated ($r = +0.73$).

These results suggest that the three growth factors tested under our experimental conditions do not have a consistent stimulating effect on the development of isolated 1/8 porcine blastomeres. In contrast, supplementation of the culture medium with insulin increased the proportion of 1/8 blastomeres that developed to normal blastocysts significantly compared with controls (Saito and Niemann, 1991). The presence of EGF and IGF-I and -II in culture medium stimulated murine embryos as shown by an increased

Table 3. Development of pig 1/8 blastomeres *in vitro* after 96 h in serum-enriched mKRB supplemented with various concentrations of insulin-like growth factor I (IGF-I)

IGF-I (ng ml^{-1})	Total*	No development *n*	(%)	Non-integrated forms *n*	(%)	Blastocysts *n*	(%)
0	121	27	(22.3)	45	(37.2)	49	(40.5)^{a,b}
1	122	37	(30.3)	43	(35.2)	42	(34.4)^{a,b}
10	122	35	(28.7)	32	(26.2)	55	(45.1)^{a}
100	122	24	(19.7)	57	(46.7)	41	(33.6)^{b}
200	121	16	(13.2)	47	(38.8)	58	(47.9)^{a}

Numbers in the same column with different superscripts are significantly different ($P < 0.05$).
*15 replicates.
mKRB: modified Krebs ringer bicarbonate.

Table 4. Development of pig 1/8 blastomeres *in vitro* after 96 h in serum-enriched mKRB supplemented with various concentrations of insulin-like growth factor II (IGF-II)

IGF-II (ng ml^{-1})	Total*	No development *n*	(%)	Non-integrated forms *n*	(%)	Blastocysts *n*	(%)
0	130	46	(35.4)	36	(27.7)	48	(36.9)^{a}
1	130	30	(23.1)	31	(23.9)	69	(53.1)^{b}
10	130	43	(33.1)	34	(26.2)	53	(40.8)^{a}
100	130	48	(36.9)	34	(26.2)	48	(36.9)^{a}
200	130	49	(37.7)	19	(14.6)	62	(47.7)^{b}

Numbers in the same column with different superscripts are significantly different ($P < 0.05$).
*15 replicates.
mKRB: modified Krebs ringer bicarbonate.

rate of blastocyst formation, an increase in the proliferation of cells and protein synthesis (Wood and Kaye, 1989; Paria and Dey, 1990; Werb, 1990; Harvey and Kaye, 1991, 1992). Isolated blastomeres from *in vitro* derived four-cell bovine embryos have been successfully grown to blastocysts and upon transfer at least in one case four identical multiplets were obtained from a single embryo (Loskutoff *et al.*, 1993). This observation clearly shows the great potential of obtaining small groups of identical multiplets through proliferation of single blastomeres from embryos of domesticated animals.

Parthenogenesis

The development of an oocyte without the genetic contribution of a spermatozoon is called parthenogenesis and potentially provides a good method for obtaining large numbers of identical multiplets. However, parthenogenetic progeny could exhibit some unknown variation because chromosomal distribution occurs at random during meiosis. Parthenogenetic development can be induced by various chemical and physical treatments, but complete parthenogenetic development has not been reported in mammals. In mice, gestation was interrupted at the 25 somite stage (day 11) at the latest and it was shown that the paternal contribution is necessary for complete fetal development (Kaufman *et al.*, 1977; Barton *et al.*, 1984). After a simulation of the 22 physiological Ca^{2+} releases at fertilization, it was possible to obtain high rates of regular blastocysts *in vitro* from rabbit oocytes and high implantation rates upon transfer to foster mothers (Ozil, 1990). In pigs, electric pulses have been shown to be more

effective at induction of parthenogenetic development than is ethanol. The cleavage rate reached 67% after exposure to electrical pulses, whereas only 45% cleavage was obtained after ethanol stimulation. A maximum of 9–12% blastocysts (with reduced cell number) was achieved after 8 days of culture (Saito *et al.*, 1993). It was also shown that okadaic acid, a specific inhibitor of the protein phosphatase type 1a and 2a, increased parthenogenetic activation of *in vitro* matured pig oocytes to 89% when present in the electrofusion medium (Rickords *et al.*, 1993). As with pig oocytes, approximately 10% of bovine oocytes stimulated by cytochalasin B developed to blastocysts and one pregnancy was detected by ultrasound, but lost at day 48 (Fukui *et al.*, 1992). These recent findings should stimulate further basic studies to explore the developmental potential of parthenogenetically activated oocytes from domestic species.

Nuclear transfer

After the birth of the first offspring resulting from nuclear transfer in sheep (Willadsen, 1986), this method has been of interest for both basic research and applied aspects. The whole technology has been reviewed extensively over the last few years (Prather and First, 1990a,b; First and Prather, 1991a,b; Seidel 1992). The main emphasis of all nuclear transfer experiments has been in cows, and up to now only one piglet has been generated as a result of nuclear transfer (Prather *et al.*, 1989). This piglet was derived from the fusion of single four-cell blastomeres with metaphase II oocytes and was one of 88 reconstituted embryos that had been transferred together with control embryos to increase the likelihood of maintenance of pregnancy. Recently, some important parameters concerning nuclear transfer in pigs have been elaborated. As in cattle, pig oocytes after maturation *in vitro* are appropriate as recipients for blastomeres (Prochazka *et al.*, 1990; Prather *et al.*, 1991; Saito *et al.*, 1992a). The transportation temperature from the abattoir to the laboratory should be 36°C; the optimum pH of the electroporation medium is 7.0; and the most efficient pulse strength is 120 V. Under the best conditions, 75% of the stimulated ova showed pronuclear formation (Prather *et al.*, 1991). In addition, follicular oocytes from hormonally stimulated gilts can be used as recipient cytoplasts. Gilts stimulated with 1500 iu PMSG delivered better quality oocytes than did those stimulated with 2000 iu PMSG. The whole micromanipulation procedure could be simplified by the presence of 0.25 mol sucrose l^{-1} in the holding medium (Nagashima *et al.*, 1992b). For pig oocyte enucleation, it seems generally accepted that about one third of the cytoplasm has to be aspirated including the polar body (Prochazka *et al.*, 1990; Nagashima *et al.*, 1992b). Enucleation rates between 60 and 90%, activation rates of 70–85%, fusion rates of 70–90% and initial cleavage rates of 40–65% can be obtained. Further development is significantly reduced and only a few reconstituted embryos (< 5%) reach the blastocyst stage (Prochazka *et al.*, 1990; Nagashima *et al.*, 1992b; Terlouw *et al.*, 1992; Saito *et al.*, 1992a). It has been clearly demonstrated that pig embryos undergo nuclear swelling which is considered as the main sign for nuclear reprogramming. Nuclear swelling depends on the maturational stage of the recipient oocytes and the interval between fusion and activation (Stumpf *et al.*, 1993). Nuclei from eight-, 16- and 32-cell embryos display a 100% increase in size (from approximately 11–14 µm to 21–27 µm), whereas nuclei from four-cell embryos increase from 18 to 27 µm (approximately 55%) in size (Prather *et al.*, 1990). This result shows that nuclear swelling is not limited by the amount of cytoplasm represented by the cyto- or karyoplast but does not indicate the subsequent developmental potential.

The reasons for the poor development of reconstituted pig embryos are unknown. It has been shown that transcription after transfer of porcine or bovine blastomeres decreased or even ceased after fusion for at least 24 h or up to three consecutive cell cycles and some fused embryos displayed abnormal nuclear development (Hyttel *et al.*, 1990; Kanka *et al.*, 1991). Further studies aimed at a better basic understanding of the events following nuclear transfer in pigs have revealed that small ribonucleoproteins (snRNP) are not expressed after transfer of a 16-cell stage nucleus (Prather and Rickords, 1992), leading to a lack of RNA synthesis which is normally detectable in pig embryos from the four-cell and later stages (Freitag *et al.*, 1991). Further studies should focus on the cell cycle and the understanding of basic molecular and ultrastructural events, to improve the success rates of nuclear transfer in pigs. In mice, the cell cycle has been shown to have a significant effect on the success of nuclear transfer (Smith *et al.*, 1988, 1990). Furthermore, nuclear transfer can significantly interfere with the normal molecular and ultrastructural events of early embryogenesis (Kopecny and Niemann, 1993).

Gene Transfer in Pigs

The alteration of the genetic make-up of domestic animals holds great promise for distinct improvements in animal production. However, progress has been rather slow mainly because of a lack of basic understanding of gene regulation and gene expression. The research has focused primarily on the alteration of growth and development, but recently has also included the generation of foreign proteins in milk and blood and the increase of disease resistance.

Progress in methodology and understanding of gene transfer in pigs

Microinjection of foreign DNA into the pronuclei of zygotes is still the only available method for transferring foreign DNA in domestic animals, although the efficiency of this process with $< 1\%$ transgenic animals is very low. Moreover, visualization of pronuclei in pig zygotes requires centrifugation (Wall *et al.*, 1985). The decreased viability of pig zygotes is to some extent attributed to the presence of DNA in the injected solution, as the number of blastocysts developed *in vitro* is significantly lower than in noninjected controls or buffer-injected zygotes (Williams *et al.*, 1992a). The yield of appropriate pig zygotes could be optimized by treating sexually mature animals with a 5–9 day altrenogest regimen or prepubertal gilts should be used (Williams *et al.*, 1992b). Twenty to 29 microinjected zygotes were found to be the optimal number to transfer for the highest possible pregnancy rate (Wei *et al.*, 1993). Integration rates can be increased, in particular of large transgenes, by the use of overlapping fragments (Pieper *et al.*, 1992) and the co-injection of promotor and structural genes (Clark *et al.*, 1992) have been successfully used in mice and await verification in large domestic species.

In mice, after pronuclear microinjection DNA could be found in all of the resultant one- to four-cell stage embryos, but not all of the blastomeres contained the foreign DNA. The injected DNA was detected in 44% of resultant morulae and 26% of the blastocysts. These observations suggest that for a screening at the embryonic level blastocysts should be used and that already in the very early embryonic phase foreign DNA is eliminated to a large extent (Burdon and Wall, 1992). Upon injection into the cytoplasm of porcine oocytes at the germinal vesicle stage, approximately 30–40% of the detected DNA was found to be ligated to higher molecular weight forms. In contrast to the situation in cows and sheep, pig metaphase II and pronuclear stage embryos display a reduced ability to ligate the exogenous DNA (Powell *et al.*, 1992). Obviously there are species-specific differences in DNA metabolism and it was suggested that cytoplasmic activity in zygotes was required to support DNA replication during the early post-fertilization period before activation of the embryonic genome. Collectively, the findings discussed above point towards some areas in which improvements in gene transfer in pigs can be achieved and provide some explanations for the low success rates.

Sperm-mediated DNA transfer

As an alternative to microinjection, sperm-mediated DNA transfer has been reported in mice (Lavitrano *et al.*, 1989); however, the results were not reproducible (Brinster *et al.*, 1989). The ability of mouse and pig spermatozoa to carry foreign DNA has been studied to develop this potentially attractive procedure further. Epididymal sperm cells of mice obviously bind exogenous DNA specifically at the nuclear area of the sperm head. Seminal plasma contains a factor that controls the interaction of DNA with the spermatozoa mainly by abolishing permeability of spermatozoa and thereby is a powerful inhibitor of uptake of DNA (Lavitrano *et al.*, 1992). In addition, it was found that 15–22% of DNA bound by the spermatozoa is directly associated with the nucleus as determined examination by microscope of isolated specimens. The proportion of spermatozoa with foreign DNA increased with time from 45% after incubation for 10 min to 65% after incubation for 2 h. Ultrastructural autoradiography further supported the suggestion that foreign DNA was internalized by the sperm head (Francolini *et al.*, 1993). Foreign DNA (λ Hind III DNA fragment) was found to bind strongly to pig spermatozoa and preferably to motile spermatozoa (Horan *et al.*, 1991). Approximately 30% of pig sperm cells carried the foreign DNA with 25 ng DNA bound to approximately 10^7 spermatozoa even after five washes. It was calculated

that 4 × 10^2 molecules were taken up by each pig spermatozoon which is similar to levels found for mouse spermatozoa. The DNA was exclusively attached to the sperm head and electroporation was found to increase DNA uptake by only 5–10%. In contrast to mouse spermatozoa (Lavitrano *et al.*, 1992) mainly smaller fragments were retained in pig spermatozoa (Horan *et al.*, 1991, 1992). Transgenic pigs have been reported upon sperm-mediated DNA transfer, whereas in cattle, rabbit and sea-urchin, DNA was detected only in embryonic cells (Lauria, 1991). These results, together with the data published for a number of different species, ranging from sea-urchin and honeybee to cattle (see Francolini *et al.*, 1993), show that DNA transfer through spermatozoa is feasible and might have played an important role in the evolutionary process. This recent progress should stimulate further research to develop repeatable procedures in the laboratory for sperm-mediated DNA transfer.

Pig embryonic stem cells

Embryonic stem (ES) cells are pluripotent or totipotent cell lines derived from the ICM of blastocysts. They hold great promise for significant improvements in gene transfer as indicated by studies reported in mice (see Stewart, 1991). Murine ES cell lines possess pluripotent properties and chimaeras were obtained after their injection into blastocysts and subsequent transfer of the chimaeric blastocysts into appropriate recipients (Evans and Kaufman, 1981; Martin, 1981). Until recently, the establishment of pluripotent or totipotent ES cell lines has been restricted to mice but ES-like cell lines have now been produced for cattle (Saito *et al.*, 1992b), sheep (Notarianni *et al.*, 1991), hamsters (Doetschman *et al.*, 1988), mink (Sukoyan *et al.*, 1992), rabbits (N. Strelchenko and H. Niemann, unpublished) and pigs (Notarianni *et al.*, 1990; Piedrahita *et al.*, 1990a). By definition, the morphology of ES-like cells does not alter over repeated passages and they can form embryoid bodies under appropriate culture conditions, but have not yet shown their potential to contribute to chimaeric offspring, whereas true ES cells have demonstrated ability to differentiate to every cell type and are thus totipotent. In mice, stem cells have been generated from sublines of mouse strain 129/SV. ES cell lines have also been generated from C57BL/6 inbred mice (Ledermann and Bürki, 1991).

In pigs, several attempts have been made to elucidate significant methodological and embryological details required for establishment of ES cells. Embryos from day 9 and 10 were cultivated for 6–7 days on a pig uterine fibroblast layer and a total of seven cell lines was obtained after three to five passages that resembled morphologically undifferentiated embryonic cells (Strojek *et al.*, 1990). Other investigators used hatched blastocysts from day 7–9 after fertilization and ES-like cells were obtained upon mechanical isolation of the ICM. Conditions that allow the isolation of ES cells from murine embryos could also lead to the isolation of porcine ES-like cells that exhibit some but not all of the characteristics of murine ES cells (Piedrahita *et al.*, 1990a). These ES-like cells showed a slower proliferation rate than did murine ES cells and formed embryoid bodies and possessed large nuclei and sparse cytoplasm (Notarianni *et al.*, 1990, 1991). Intact pig embryos or isolated ICM gave rise to cell lines with an ES-like morphology when plated onto mouse STO feederlayers or STO with BRL (Buffalo rat liver) conditioned medium (Evans *et al.*, 1990; Piedrahita *et al.*, 1990a,b). These pig cell lines could be maintained almost indefinitely in an undifferentiated stage and formed embryoid bodies in suspension culture (Evans *et al.*, 1990). Apart from pig ES-like cells, cells with an epithelial-like morphology were also isolated from pig blastocysts. A test for pluripotency of the ES-like cells in pigs by the production of chimaeras was, however, unsuccessful (Piedrahita *et al.*, 1990a). Conditioned medium derived from the embryonic carcinoma (EC) cell line PCC4 containing high concentrations of transforming growth factor β (TGF-β) activity, is known to enhance the establishment of murine ES cells. The use of TGF-β-enriched medium for the establishment of bovine and porcine ES-cell lines failed. In the presence of TGF-β, pig embryos did not undergo passage number two (Strelchenko and Niemann, 1993). Although several groups have apparently generated cell lines that resemble characteristics of murine ES cells, the final proof of true pig ES cells via chimaeras has not yet been published. There is one report of a true chimaeric pig that was generated upon injection of ES cells into blastocysts and their subsequent transfer to appropriate recipients (M. Wheeler, personal communications). Collectively, the data suggest that it will soon become clear whether ES cells can be established in domestic species or whether this is a special characteristic of murine species. Embryonic germ (EG) cells recently generated from the mouse might help in clarifying

this question and in extending the whole technology to farm animals, and thereby open a new route for transgenesis (McLaren, 1992).

Growth and development

Most gene transfer studies in pigs have used growth regulating gene constructs. The results have been reviewed extensively (Pursel *et al.*, 1990a,b; McEvoy *et al.*, 1992; Ebert and Schindler, 1993). Some transgenic pigs expressing a metallothionein promotor–bovine growth hormone (MTI-bGH)-fusion gene gained weight 23% faster and had an 18% better feed efficiency on an 18% crude protein diet. In addition, expression of the bGH genes in pigs resulted in a dramatic reduction in backfat thickness from approximately 21 mm to 7.5 mm. However, most of the transgenic pigs showed an overexpression of GH from early development, associated with serious side effects such as depressed appetite, lethargy, arthritis, poor fertility and a high incidence of peptic ulcers (Pursel *et al.*, 1990a,b). Some of these problems were overcome by testing bGH gene constructs with the rat phosphoenolpyruvate carboxy kinase (PEPCK) promotor, which is activated at birth and not during gestation, as is the MT-I promotor usually used (Wieghart *et al.*, 1990). The incidence of side effects was postponed to a later phase in the development of piglets, but not eliminated. PEPCK is primarily expressed in the liver and its transcription is regulated by cAMP administration or alteration in the dietary carbohydrate and protein concentration (Wieghart *et al.*, 1990). In another approach to avoid the side effects mentioned above, the cDNA of the chicken ski gene (c-*ski*) was introduced into pig zygotes and five transgenic pigs exhibited various degrees of muscular hypertrophy that was first detected at between 3 and 7 months of age (Pursel *et al.*, 1992). In two pigs, this hypertrophy was evident only in the shoulders, whereas in the three others, both hams and shoulders were enlarged. However, histological examinations revealed pathological vacuoles in muscular tissue. The c-*ski* gene was under the control of the mouse sarcoma virus (MSV) LTR (long terminal repeats) promotor. It was expressed specifically in pig skeletal muscle and obviously had profound effects on muscle development depending on the level and the time of onset of expression (Pursel *et al.*, 1992). Although this new generation of growth-promoting gene constructs still needs further investigation, it has recently been shown that a modification of the pig growth hormone (pGH) construct had beneficial effects on important parameters of development without deleterious side effects (Seamark, 1993). In these experiments the cDNA of the pGH was used and the hMT-I promotor was modified at one end to reduce the expression of growth hormone to 30–40 ng ml^{-1}, thus avoiding reported negative side effects. Lines of these pGH transgenic pigs will possibly be available soon for human consumption in Australia (Seamark, personal communication).

Table 5. Expression of non-growth-related foreign proteins in transgenic pigs

Promotor	Gene/cDNA	kb	Transgenic pigs/ injected zygotes (%)	Site of expression	Highest expression	References
mWAP	mWAP	7.2	5/850 (0.6)	Milk	1 mg ml^{-1}	Wall *et al.*, 1991
mWAP	mWAP	7.2	9/732 (1.2)	Milk	1.5 mg ml^{-1}	Shamay *et al.*, 1991
mWAP	hPrC	8.8	7/320* —	Milk	1 mg ml^{-1}	Velander *et al.*, 1992, Williams *et al.*, 1992b
LCR	$\alpha_1\alpha_1\beta^A$	16.9	3/709 (0.4)	Blood	10% of red blood cells	Swanson *et al.*, 1992
α, κ	α, κ	14.8	2/542 (0.4)	Serum	1.4 mg ml^{-1}	Lo *et al.*, 1991
κ, γ	κ, γ	14.8	—	Serum	1 mg ml^{-1}	Weidle *et al.*, 1991

*Approximately 40 microinjected zygotes transferred to 8 recipients.
mWAP: murine whey acidic protein; hPrC: human protein C; LCR: locus control region; α_1: human α_1 gene; β^A: human β^A gene.

Production of foreign proteins in milk from pigs

Recent experiments clearly indicate that the mammary gland of pigs can be used to produce recombinant pharmaceutical proteins (see Clark *et al.*, 1987; Hennighausen *et al.*, 1990; Halter *et al.*, 1993) (Table 5). The mouse whey acidic protein gene (mWAP) is almost exclusively expressed in the mammary gland of pigs and is weakly expressed in the salivary gland (less than 1%) (Wall *et al.*, 1991). The 7.2 kb mWAP gene, consisting of four exons, three introns and a flanking region was expressed in milk of pigs at similar amounts as in mice. In total, 0.5–1.5 g l^{-1}, corresponding to approximately 2% of all milk proteins, could be isolated. The gene was transmitted to the offspring but in five of the eight transgenic founder pigs, each harbouring 10–15 copies of the gene, lactation stopped within a few days after parturition and the mammary gland showed signs of complete involution. This effect was WAP specific and only to some extent related to the amount of expression. The other three animals completed a whole lactation (Shamay *et al.*, 1991). Transgenic pigs were also generated that harboured the cDNA of the human protein C gene (hPrC) regulated by the mWAP promotor. The 8.8 kb protein C cDNA was expressed in amounts that varied from 100 to 1000 μg ml^{-1}, which is 1000 fold higher than in mice. Different post-translational modifications of the protein C were found and from 1000 μg ml^{-1} approximately 380 μg biologically active, X-carboxylated substance ml^{-1} could be isolated (Velander *et al.*, 1992). Protein C is a vitamin K dependent serine protease that inactivates clotting factors Va and VIIa. The gene shows strong homology to the factor X and IX genes. For physiological action, protein C requires interaction with the membrane surface. Protein C is mainly required as an anticoagulant in patients that are homozygous for protein C deficiency or suffer from a severe sepsis. However, protein C can also stimulate fibrinolysis (Esmon, 1987). It has been shown that 400–1000 ml of pig milk can be collected with each milking and milking six times per day can provide 1–6 l day^{-1} (Williams *et al.*, 1993). Calculations in the United States have revealed that approximately ten pigs would be necessary to cover the demand for blood clotting factor VIII based on the assumption that 500 l milk are produced per year with 50% loss at purification and an expression of 0.05 g l^{-1} (W. N. Drohan, personal communication). The data available clearly show the suitability of pigs for the production of certain important recombinant pharmaceutical proteins.

Generation of foreign proteins in pig blood

Recently, functional human haemoglobin has been produced in transgenic swine. The 16.9 kb construct consisted of the locus control region (LCR) from the human β-globin locus together with two copies of the human $\alpha 1$ gene and a single copy of the human β^A gene. In total, 709 zygotes underwent microinjection and were transferred to 19 recipients yielding 13 pregnancies and 112 piglets of which three were transgenic (Table 5). These were healthy, not anaemic and grew at a rate comparable to that of nontransgenic littermates. All these animals expressed the human haemoglobin gene and the product could be purified to more than 99% and exhibited a similar oxygen affinity to the human derived haemoglobin (Swanson *et al.*, 1992). The expression efficiency, however, needs to be improved since only 15% of the red cells of animals contained the human haemoglobin. The concept of producing human blood substitutes in domestic animals has several advantages compared with fresh human blood that is now used for transfusions. Clearly it would have a long shelf life and not require refrigeration. Furthermore, these substitutes would not be contaminated with various human pathogens such as HIV, hepatitis or other viruses. Human haemoglobin can also be successfully produced in yeast and which of the two systems is better will be determined (Moffat, 1992). This recent success raises the question of the extent to which domestic animals, in particular pigs, could be used as organ donors for humans. The pig offers major advantages, as large numbers are available and they are easy to breed and to maintain and have a similar anatomy and physiology to humans. Pigs have a great ability to breed under quarantine or specific pathogen free (SPF) conditions and would have a much better public acceptance than would the use of primates. Although the immunological hurdle is great, it is conceivable that genetically engineered pigs could be produced that express complement inhibiting proteins that lead to a blocking of autologous complement, but not of xenogeneic complement of distantly related species (Cooper, 1992). These exciting and also ethically challenging visions need to be further pursued.

Table 6. Status of the various embryo manipulation methods in pigs as compared with cattle, sheep and goats

Methods	Pig	Cattle	Sheep/goat
Embryo transfer	++	+++	++
IVM/IVF	+	++	+
Freezing	+	+++	+++
Chimaeras	+	+	+
Sexing	–	++	–
Embryo bisection	++	+++	++(+)
Nuclear transfer	+	+(+)	+
Gene transfer	+(+)	+	+(+)

–: Experimental stage, no offspring; +: experimental stage, first offspring; ++: practical application possible, but not yet realized on a wide scale; +++: broader practical application; ( ): on the edge to next category in some sections.

Increase in disease resistance

The following principal approaches must be explored to increase the resistance of animals to certain diseases: transfer of specific disease resistance genes, genes producing antisense RNA against viruses, genes for specific monoclonal antibodies or antigens or genes expressing lymphokines or T-cell receptors. In pigs, an attempt has been made to increase the resistance against influenza A infections by microinjection of the murine *Mx* gene. The *Mx* gene was injected in the form of three different constructs either under control of the human MT-I promotor, the SV40 early enhancer or the murine *Mx-I*. Expression of the first two gene constructs early in embryogenesis was deleterious to further development. The use of the interferon (IFN) and virus inducible *mMx-I/Mx* construct resulted in two transgenic pig lines that expressed the gene to produce mRNA but no protein could be detected (Müller *et al.*, 1992). The interferon inducible intracellular *Mx-I* protein was orginally discovered in certain inbred mouse strains that exhibited a high degree of resistance to influenza virus (Staeheli, 1991). Mouse strains that were extremely susceptible to experimental infection with the influenza virus had no functional *Mx-I* gene. Other mammals and nonmammalian vertebrates seem to have a functional *Mx* defence system that can be activated by virus infections. It is therefore unclear whether additional transgenic expression of *Mx* proteins would further increase the natural resistance to influenza virus. Indeed, *Mx* transgenic mice were still significantly less resistant to the influenza virus than were mice with a functional endogenous *Mx* system. Another problem with this approach would be the selection of the most appropriate promotor because the interferon response to an infection has to be very quick to protect the cells from the deleterious viral effects. Finally, *Mx* proteins are effective against viruses only if the proteins are abundantly available in the cells (Staeheli, 1991). Because of these problems, the whole approach to the generation of specific disease resistant transgenic domestic pigs needs further intensive investigation. IgA genes have been expressed in pigs, mice and sheep to increase the resistance against infections and led to high amounts of murine IgA in mice and pigs but not in sheep (Lo *et al.*, 1991) (Table 5). Two lines of transgenic pigs were generated that expressed the 14.8 kb fragment (Brinster *et al.*, 1983) of the mouse IgA gene from the age of four months. In contrast to mice, in pigs the endogenous production of IgA was not suppressed. However, the mouse IgA included pig light chains and showed only little binding to phosphorylcholine. The endogenous B-cell production was not affected (Lo *et al.*, 1991). Finally, up to 1000 μg monoclonal murine antibodies ml^{-1} have been expressed in transgenic pigs. These showed antigen-binding activity (Weidle *et al.*, 1991).

The results discussed above show that the transgenic technology that was originally used in pigs to alter growth and development can be successfully applied to modify other important characteristics of the

animals. However, although progress is evident, commercial applications are in most instances still far away.

Conclusion

It can be seen from the above that in pigs biotechnological procedures involving various manipulations of the early pig embryo have recently undergone progress but on the whole the development is far behind that in cattle and sheep (Table 6). This is to some extent inherent to the reproductive characteristics of pigs but is certainly also related to too little scientific effort being devoted to explore these technologies further. Embryo transfer and embryo bisection can now be used routinely in research and in the pig industry. All other biotechnologies need further intensive research to allow the generation of their inherent potential benefits. The high fertility of pigs, however, provides a solid foundation for further successful experimental approaches in this area.

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