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Manipulation of gametes and embryos in the pig

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Summary. Several manipulation techniques including nuclear injections, nuclear transplantation, embryo splitting, chimaera production and sperm injection are discussed with special reference to their application in the pig. The nuclear injection technique is likely to be of greatest use for gene transfer. Gene transfer is feasible for pig embryos, but it is very inefficient. Efficiencies of various steps in the successful production of transgenic offspring as summarized from several published references are as follows: immediate survival of embryos following injection (60%), offspring produced from injected embryos (15%), offspring with an integrated foreign gene (24%) and offspring expressing the integrated foreign gene (60%). The overall efficiency is about 1%. Potential uses for nuclear transplantation are for the production of gynogenetic and androgenetic embryos and for cloning embryos. The feasibility of producing androgenetic and gynogenetic embryos will depend on whether in the pig, as in the mouse, the paternal and maternal genomes function differently. The feasibility of cloning by nuclear transplantation will depend upon the timing of developmental events in pig embryos and the development of methods for reprogramming nuclei. Pig embryos have been cloned by embryo splitting. Chimaera production and fertilization by sperm injection are likely to be feasible for pig embryos but have not yet found application.

Introduction

Originally, embryo manipulation techniques were developed as tools for the study of early embryogenesis. Injection, cell and nuclear transfer techniques have been the foundation for many studies investigating cell differentiation, regulation of development and cell lineage analysis in early embryos. Although they are still used primarily for research purposes in amphibian and laboratory mammalian species, manipulation techniques are now also being used commercially to clone cow embryos by splitting. In the future these techniques will probably be of importance to farm animal production through their use in cloning, gene transfer and fertilization. This paper deals with the application of manipulation techniques to the gametes and embryos of pigs. We will discuss methods for nuclear injection, nuclear transfer, sperm injection and blastomere manipulation. This will be followed by a discussion of information derived from using these techniques.

Methods

General

Micromanipulation of gametes and embryos is done using two glass micropipettes; one pipette for holding an embryo and the other for doing the microsurgery. Each is attached to a good quality micromanipulator and micromanipulation is done under a microscope at × 200 or, preferably,

× 400 magnification. The micropipettes are connected by a length of rigid-walled plastic tubing to syringes for control of fluid movement within the micropipettes (for review see Lin, 1971; Gardner, 1978).

Nuclear injection

A prerequisite for injecting a nucleus is a method of locating it. Although nuclei are easily visualized in mouse embryos, in pig embryos they are obscured by large lipid granules in the cytoplasm. Three methods have been used to locate nuclei: (1) labelling nuclei with a vital fluorescent dye, (2) using two-cell and later stage embryos and assuming nuclei are in the centre of the blastomeres and (3) centrifuging the embryos to clear the cytoplasm. Nuclei can be labelled with the DNA specific vital stains Hoechst 33342 (Critser, Ball, O'Brien & First, 1983) or DAPI (Minhas *et al.*, 1984). Nuclei thus stained must be visualized under ultraviolet light. However, our experience with this method indicates that exposure to ultraviolet light is very damaging to the embryo. In the mouse embryo, in which nuclei are easily visualized, the nuceli are nearly always located in the centre of the blastomere at the two-cell stage. We have used this information to locate nuclei in two-cell pig embryos without visualizing them. The injection tip is simply inserted into the centre of the blastomere. By the movement of the cytoplasm it is possible to discern when the tip is pushing against the nucleus. Although it is possible to locate nuclei in this way, it is very difficult to confirm proper injection. The best method of locating nuclei in one- and two-cell pig embryos is by centrifuging them to clear the cytoplasm (Wall, Pursel & Hammer, 1984; Fig. 1). Embryos are centrifuged at 15 000 ***g*** for 3 min. The large lipid granules which are lighter than the rest of the cytoplasm become displaced to the top edge of the embryo or form a mushroom-shaped pocket in the vitelline membrane. With this method, nuclei of most embryos are visible and nuclear injection is easily confirmed. Furthermore, if the embryos are centrifuged for a sufficient time before

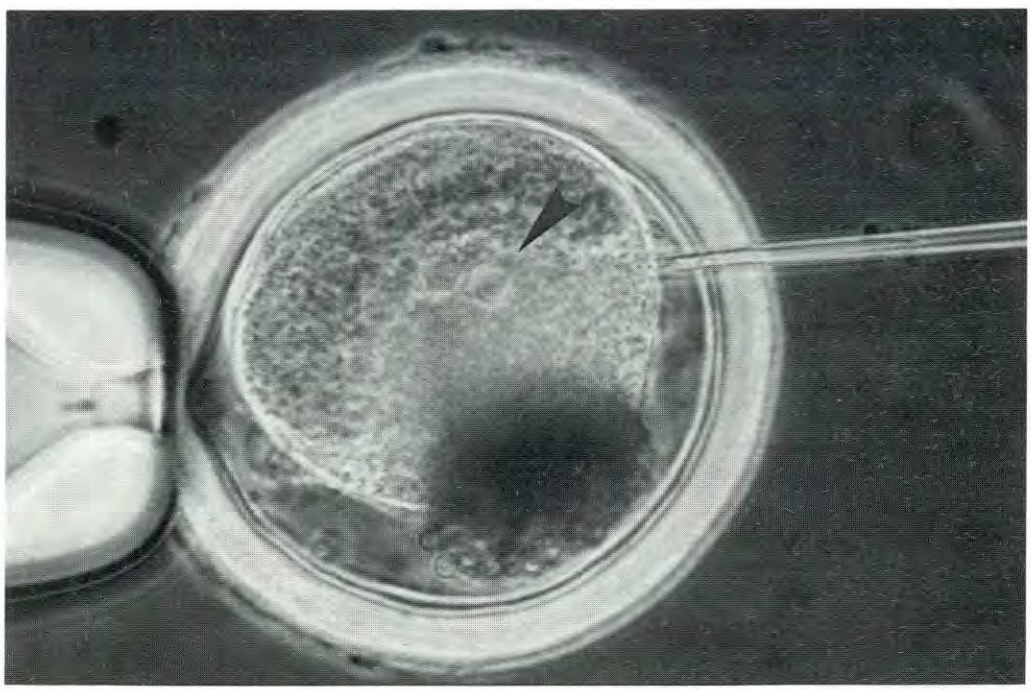

Fig. 1. Pronuclear injection of centrifuged pig embryo. Arrow indicates location of pronuclei.

cleavage the lipid granules become uniformly distributed throughout the embryo and development progresses normally.

Injection micropipettes are made by pulling capillaries on a pipette puller. The tips can either be bevelled to 1 to 2 μm on a grinding wheel or used unbevelled. Bevelled tips can be filled with injection solution through the back or by aspiration through the tip. Unbevelled tips, being very small ($< 0{\cdot}5$ μm), must be filled by capillary action through the back. Smaller tips cause less damage to the embryo, but they also plug more easily. Nuclei are injected using a pressure system with a syringe and nuclear injection is confirmed by swelling of the nucleus.

Nuclear transplantation

Two methods have been used for transplanting nuclei in mouse embryos. Illmensee & Hoppe (1981) used a totally surgical method. A nucleus, obtained by rupturing a cell and devoid of surrounding plasma membrane, is aspirated into a micropipette. The pipette is then inserted through the plasma membrane and into the cytoplasm of a pronuclear embryo. The donor nucleus is expelled and the pronuclei are then aspirated in a one-step procedure. McGrath & Solter (1983) have devised a nondisruptive method of transplanting nuclei. A large micropipette is inserted through the zona pellucida but not through the plasma membrane of an embryo. The pipette is then moved adjacent to the nucleus and with gentle aspiration the nucleus is pulled into the pipette without rupturing the plasma membrane. As the pipette is pulled away from the embryo the zona pellucida pinches the membrane off between the enucleated blastomere and the plasma membrane-bounded nucleus. Nuclei are thereby removed without disrupting the plasma membrane and the plasma membrane-bounded nucleus is inserted into an enucleated embryo by cell fusion. In the mouse, Sendai virus is used as the fusigenic agent. A small volume of Sendai virus is aspirated after nuclear removal and is injected into the perivitelline space of the recipient embryo just

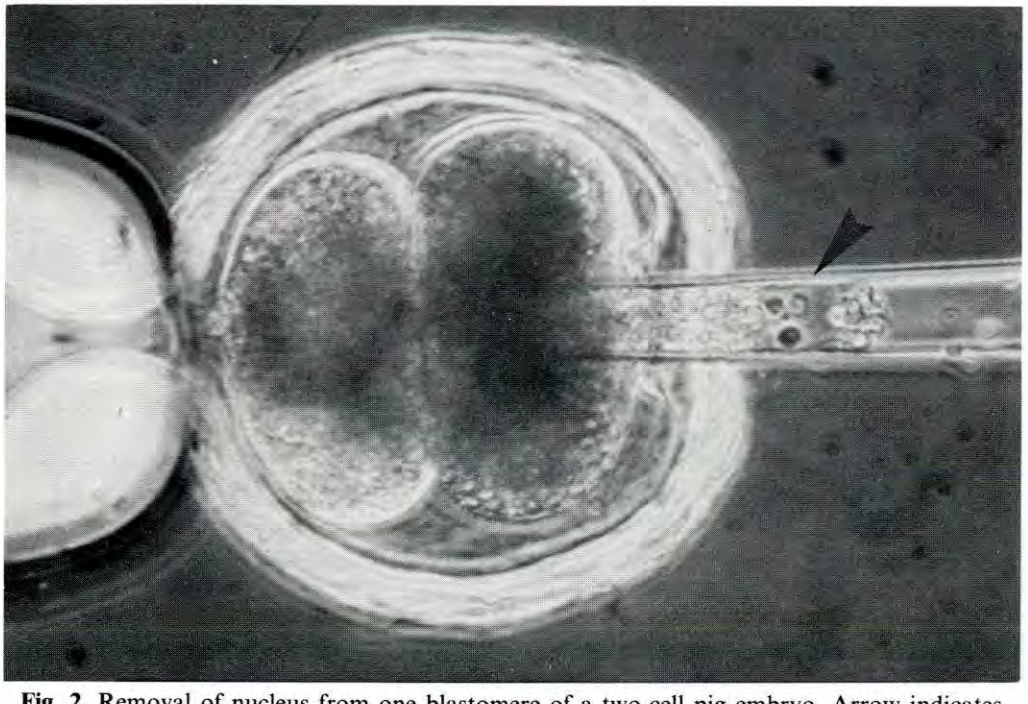

Fig. 2. Removal of nucleus from one blastomere of a two-cell pig embryo. Arrow indicates location of nucleus.

before inserting the nucleus. At best the microsurgical method of Illmensee & Hoppe (1981) is about 30–40% efficient whereas the nondisruptive method of McGrath & Solter (1983) is greater than 90% efficient. Furthermore, work of McGrath & Solter (1983) and our work (J. M. Robl, B. Gilligan, E. S. Critser & N. L. First, unpublished data) indicates that transplantation of nuclei between similar stages of embryos has little effect on their development to term.

We have adapted the nondisruptive method of nuclear transplantation for pig embryos. The procedure for nuclear removal is similar to the procedure used for the mouse. Embryos are treated with cytochalasin B (7·5 μg/ml; Sigma, St Louis, MO) and demecolcine (0·1 μg/ml; Sigma) for 15–20 min before and during micromanipulation. A bevelled, sharpened tip of ~ 25–33 μm in diameter is used for nuclear removal and transfer (Figs 3 & 4). We have not been successful in using either Sendai virus or polyethylene glycol in fusing nuclei to enucleated blastomeres of pig embryos. However, we have had success with the dielectrophoretic method of fusion. Embryos are placed in a fusion chamber and a voltage pulse is applied to the embryo. The plasma membrane-bounded nuclei fuse in 0·5–1·5 h.

As with nuclear injection, a method of locating nuclei is necessary for nuclear transplantation in pig embryos. For pronuclear eggs the centrifugation method is preferable. Nuclei may be removed efficiently from two-cell and later stage embryos by aspirating in the centre of the blastomere (Fig. 2).

Embryo splitting

Embryos can be split successfully at early cleavage stages or later as morulae or blastocysts. Willadsen (1982) has described the procedure for splitting embryos at early cleavage stages in detail. Splitting embryos at the morula or blastocyst stage is more difficult than at earlier stages

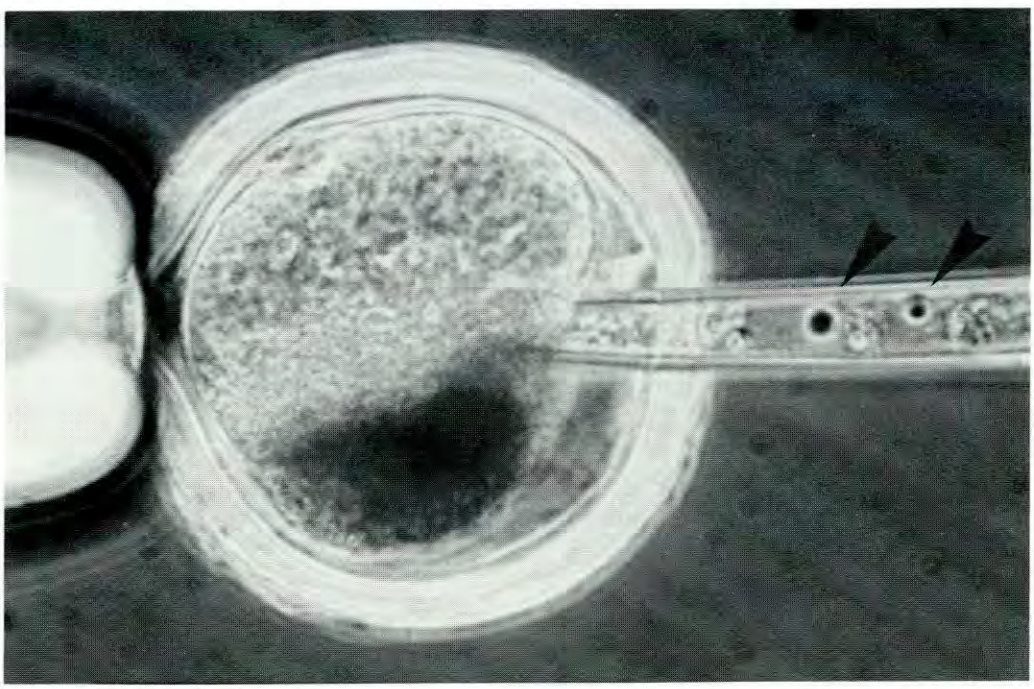

Fig. 3. Removal of pronuclei from centrifuged pig embryo. Arrows indicate location of pronuclei.

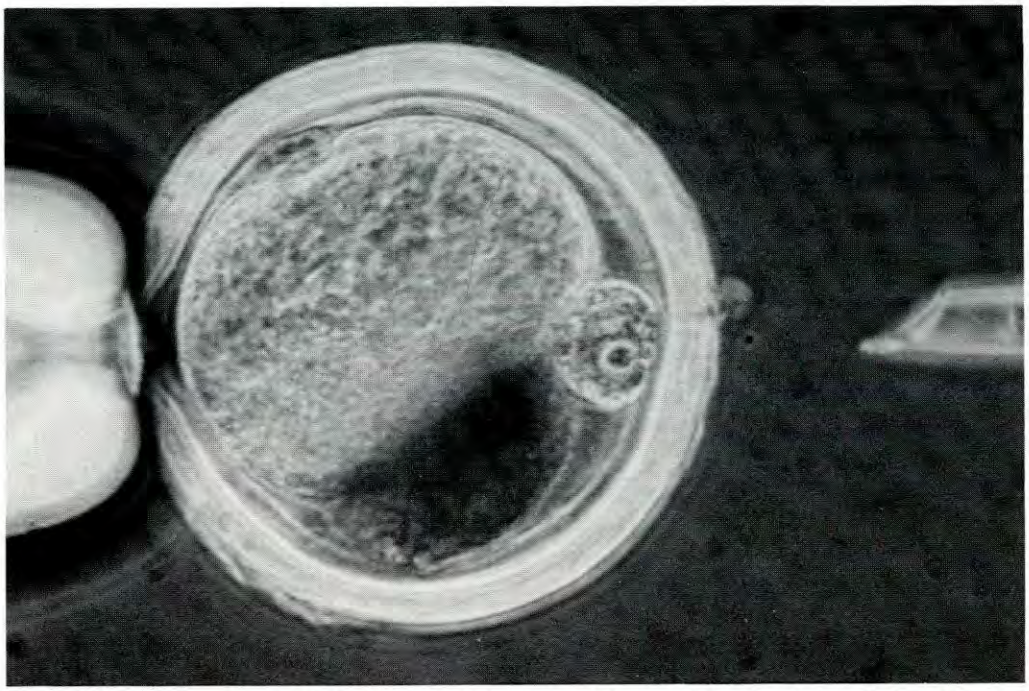

Fig. 4. Plasma membrane-bounded pronuclei reinserted into perivitelline space of pig embryo. The plasma membrane-bounded pronuclei can then be fused to the enucleated blastomere by electrofusion.

because the cells of the embryo are tightly adhered to one another by cell junctions. The embryos must be cut into halves using a fragment from a fractured razor blade (Baker & Shea, 1985) or finely drawn glass needle (Voelkel, Viker, Humes & Godke, 1984). The optimal stages for splitting pig embryos may be the late 4-cell to early 16-cell embryos because these embryos can be easily cultured, they have not yet developed cell junctions and they can be transferred to the uterus.

Chimaera

Chimaeric embryos can be made using the procedures of Willadsen (1982) for splitting early stage embryos. Alternatively, Gardner (1978) described a procedure for making chimaeric embryos by injecting cells into the blastocoele cavity of blastocysts.

Sperm injection

Spermatozoa can be injected into oocytes by using a bevelled tip that is larger than the diameter of the spermatozoon but as small as possible to minimize damage to the oocyte. Uehara & Yanagimachi (1976) described a procedure for injecting spermatozoa into hamster oocytes. Markert (1983) described a procedure in which the spermatozoon is attached by suction to the end of the injection tip and is pushed through the zona pellucida and into the egg.

Discussion

Nuclear injection

The nuclear injection technique is likely to be of greatest importance to farm animal production

through its use for gene transfer. Within the past few years it has been demonstrated that cloned genes can be injected into nuclei of mammalian embryos and may integrate into the host DNA. The foreign genes are usually expressed and regulated in the resulting transgenic offspring. Furthermore, in many instances the genes are transmitted as single heterozygous traits and can be bred to homozygosity. Although each of these steps has been accomplished in the mouse their overall efficiency is very low. In the pig there are no published reports of the production of transgenic offspring, but workers in several laboratories are currently studying towards this goal. In the next few paragraphs we will discuss problems associated with the injection, integration, expression, regulation and inheritance of exogenous genes in the mouse.

Gene injection. After injection, some embryos lyse within a few minutes, others survive injection but fail to cleave or cleave abnormally *in vitro* and others are lost *in vivo* after transfer to recipients Immediate survival following injection is usually greater than 60% and, with care, can be as high as 95%. The immediate survival rate depends partly on the type of tip used and the quantity of fluid injected. The detrimental effect of injection can also be assessed by short-term development *in vitro*. Table 1 shows the development *in vitro* of embryos injected with various solutions or not injected. It appears that most of the embryo loss was caused by the mechanical insertion of an injection tip into the nucleus. The effect of injection was even more pronounced *in vivo* after transfer to recipient females. Non-manipulated embryos developed at a rate of 58% (62/106) to offspring whereas only 14% (13/94) of injected embryos resulted in offspring. This loss may be due to chromatin damage caused by the injection pipette. Due at least partly to the effects of injection, only about 5–15% of the total embryos injected survive to offspring.

Table 1. Effect of injection and injection media on development of one-cell mouse embryos *in vitro*

	72 h in-vitro development	
Treatment	Morulae or blastocysts/total	%
Immediate culture	20/22	91
Culture at end of manipulation	15/15	100
Tip inserted into nucleus (no injection)	9/14	64
Nuclear injection of PBS (phosphate buffered saline)	8/12	67
Nuclear injection of Whitten's + Hepes*	8/11	73
Nuclear injection of PBS + pMK†	7/12	58
Nuclear injection of Whitten's + Hepes + pMK	9/14	64

* Whitten's medium with part of the bicarbonate replaced with Hepes buffer.

† pMK is the plasmid pBR322 which contains the promoter region of the mouse metallothionein I gene fused to the structural region of the herpes simplex virus thymidine kinase gene.

Gene integration. Of those embryos surviving to offspring a low and variable percentage contain the exogenous gene integrated into host DNA (Table 2). Little is known of the factors that determine the rate of integration, but this is not dependent on the number of gene copies injected (Table 2). Furthermore, attempts to increase the rate of integration have not proved entirely successful. Palmiter, Chen & Brinster (1982a) injected DNA from the plasmid pMK in several forms (supercoiled circle, linearized or ligated to mouse DNA sequences) and found no differences in integration rates. Expecting that integration might be mediated by recombination of homologous sequences, Gordon, Scangos, Plotkin, Barbosa & Ruddle (1980) also injected a recombinant plasmid containing sequences isolated from mouse DNA. None of the 30 offspring analysed retained the sequence, indicating that sequence homology between donor and recipient DNA does not increase the frequency of integration. Integration of foreign DNA can take place at almost any

Table 2. Efficiencies of various steps in gene transfer

Number (%)							
Embryos injected	Embryos transferred to recipients	Offspring	Offspring with gene integrated	Offspring expressing integrated gene	Plasmid	Copies of plasmid injected	Reference
—	240	19	4 (21)	1 (25)	pMK	200	Brinster *et al.* (1981)
300	192 (64)	11 (6)	6 (55)	—	pB_1-14	440	Brinster *et al.* (1983)
—	170	21 (12)	7 (33)	6 (86)	pMGH	600	Palmiter *et al.* (1982b)
169	91 (54)	18 (20)	—	—	2-pCRI/RchrβG-1	2500	Wagner, T. E., *et al.* (1981)
229	143 (62)	20 (14)	6 (30)	—	pHGH	6000	Wagner *et al.* (1983)
—	33	10 (30)	1 (10)	—	pIf	10 000	Gordon & Ruddle (1981)
—	—	69	10 (14)	7 (70)	pMK	—	Palmiter *et al.* (1982a)
—	—	44	11 (25)	—	P17	500 140	McKnight *et al.* (1983)
—	—	78	2 (3)	—	pST6	1000	Gordon *et al.* (1980)

Mean percentages 60 × 15 × 24 × 60 = 1% overall efficiency.

time during embryo development. Early integration has been demonstrated in several mice by the presence of the foreign gene in consistent levels in a variety of tissues (Gordon *et al.*, 1980; Gordon & Ruddle, 1981; Stewart, Wagner & Mintz, 1982). Later integration has been shown by mosaicism in transgenic animals. In at least two instances differences have been observed between placental and fetal integration sites (Wagner, Stewart & Mintz, 1981; Burki & Ullrich, 1982). It is also possible for foreign genes to be transiently retained in animals without integration (Gordon *et al.*, 1980) and genes that integrate may be modified before integration, particularly for single copy insertions (Gordon *et al.*, 1980; Stewart *et al.*, 1982). Multiple copy insertions are also frequently observed. These generally take the form of large tandem repeats with up to 150 copies in a single insertion site (Wagner, E. F., *et al.*, 1981; Palmiter *et al.*, 1982a). Only 25–30% of transgenic animals have 1–2 copies and most have about 7–30 copies (McKnight, Hammer, Kuenzel & Brinster, 1983; Brinster *et al.*, 1984). These studies illustrate the types of integration patterns that are observed in transgenic offspring. The optimal form, an early integration of single or few unmodified copies, probably occurs in fewer than 15% of offspring obtained from injected embryos.

Gene expression. Not all animals with an integrated foreign gene express the protein product of the gene. The percentages of animals expressing an integrated gene range from 20 to 90% (Brinster *et al.*, 1981; Palmiter *et al.*, 1982a, b) and it is not known precisely what determines whether or not a gene will be expressed. It may be related to its site of integration and how the DNA at this site is packaged. The level of expression in some cases is closely related to the number of gene copies integrated (Palmiter *et al.*, 1982b) and in other instances is not (Palmiter *et al.*, 1982a). Jahner *et al.* (1982) reported that gene expression was inversely related to methylation, but Brinster *et al.* (1984) found this relationship to be inconsistent.

Gene regulation. The level of expression and in what tissues a gene is expressed appears to depend mainly on regulatory sequences within the gene. Foreign immunoglobulin (Brinster *et al.*, 1983; Grosschedi, Weaver, Baltimore & Constantini, 1984) and transferrin (McKnight *et al.*, 1983) genes are preferentially expressed in tissues similar to those in which the endogenous genes are expressed, presumably because of an internal tissue-specific regulator sequence. The most informative work on foreign gene regulation has been with the metallothionein-I gene promoter sequence linked to the thymidine kinase gene (Palmiter *et al.*, 1982a) or the human growth hormone gene (Palmiter *et al.*, 1982b). For both gene constructs, expression closely paralleled the level and site of expression of the endogenous metallothionein-I gene. Furthermore, expression could be induced by heavy metals which are the natural inducers of metallothionein-I expression. Regulation by the foreign metallothionein-I promoter was not completely equivalent to endogenous regulation since glucocorticoids, also natural inducers of metallothionein-I, failed to induce the foreign genes. Another example of faulty gene regulation has been shown for the rabbit β-globin gene introduced into mice (Lacy, Roberts, Evans, Burtenshaw & Constantini, 1983). Expression of β-globin, which normally occurs in erythroid cells, was found in skeletal muscle and testis. The authors suggested that the inappropriate expression was due to integration at an abnormal chromosomal position. These studies indicate that, although the expression of a foreign gene may be influenced by the number of genes integrated and their site of integration, expression is predominately controlled by regulatory sequences within the gene construct.

Inheritance of foreign genes. Foreign genes are generally inherited as single heterozygous traits, but expression of the inherited gene can be quite variable. Palmiter *et al.* (1982a) analysed offspring from two transgenic mice containing multiple copies of the fusion plasmid pMK and expressing thymidine kinase at high levels. Many of the offspring did not express the gene, some expressed it at diminished levels and others at enhanced levels. In most of the offspring the genes were present in an unmodified form, although several offspring with enhanced expression had lost several copies of the gene. In some animals there was a correlation between changes in expression and DNA methylation. The variability in expression in the progeny of transgenic animals may be peculiar to the pMK fusion plasmid since the expression of inherited chicken transferrin (McKnight *et al.*,

1983) and immunoglobulin genes (Brinster *et al.*, 1983) is fairly stable. In general, the physical inheritance of foreign genes is not a problem. However, further investigation will be required to determine why some transmitted gene constructs give variable expression.

Upon breeding foreign genes to homozygosity their mutagenic effects are revealed. Wagner, Covarrubias, Stewart & Mintz (1983) have found two cases of homozygous recessive prenatal lethals which they attribute to disruption of native DNA sequences. Palmiter, Wilkie, Chen & Brinster (1984) discovered a transgenic line in which the foreign gene is not transmitted through males although the males are fertile. The foreign gene evidently disrupted an endogenous gene that must be expressed during the haploid stages of spermatogenesis. Although the mutagenic effects of foreign genes are interesting, they may pose serious problems for producing transgenic farm animals. Testing of foreign genes in the homozygous state will be required before transgenic animals can be used extensively for breeding.

Table 2 summarizes the efficiencies of each of the steps in the production of transgenic offspring. The overall efficiency for producing mice that express a foreign gene is about 1%. Most of the loss occurs in the low survival rates of transferred injected embryos and in the low rates of gene incorporation. It is expected that similar efficiencies will be observed in the production of transgenic pigs by nuclear injection.

Nuclear transplantation

Production of parthenogenetic, gynogenetic and androgenetic embryos. One application of the nuclear removal and transfer technique is in the production of parthenogenetic, gynogenetic and androgenetic embryos. Gynogenetic embryos contain only maternally derived genes whereas androgenetic embryos contain only paternally derived genes. Gynogenetic and androgenetic embryos can be homozygous, if the diploid genome is derived from one female or male pronucleus, or heterozygous, if the diploid genome is derived from two different pronuclei. Gynogenetic embryos that contain genes derived from only one egg are parthenogenetic. Early attempts at producing gynogenetic embryos were made by activating a mature oocyte with an artificial stimulus and diploidizing the oocyte by suppressing cytokinesis with cytochalasin or by some other means. No offspring were produced using these methods (Graham 1974; Whittingham, 1980). The inability to produce gynogenetic offspring was attributed to improper egg activation, homozygosity or a lack of some important extragenetic contribution by the spermatozoon. Subsequently, it became possible to remove pronuclei microsurgically (Modlinski, 1975). Using microsurgery the spermatozoon could be allowed to fertilize and activate the egg and a pronucleus could then be removed, leaving behind any extragenetic sperm component. Of several attempts to produce gynogenetic and androgenetic offspring by this technique (Markert & Petters, 1977; Modlinski, 1980; Surani & Barton, 1983) only one was apparently successful (Hoppe & Illmensee, 1977). With the development of a technique to remove and reinsert pronuclei with high efficiency (McGrath & Solter, 1983) it became possible to evaluate thoroughly the requirements for development in androgenetic and gynogenetic embryos. These studies (Surani, Barton & Norris, 1984; McGrath & Solter, 1984a; Mann & Lovell-Badge, 1984) have not confirmed the work of Hoppe & Illmensee (1977) but more importantly have shown that complete development of offspring in the mouse requires both the maternal and paternal genomes. Gynogenetic and androgenetic embryos develop to midgestation then die. The results of Barton, Surani & Norris (1984) suggest that the paternal genome is essential for the normal development of extraembryonic tissues while the maternal genome may be essential for embryogenesis. Parthenogenetic embryos can develop to term as chimaeras with normal embryos (Surani, Barton & Kaufman, 1977; Stevens, 1978), indicating that parthenogenesis is not cell lethal.

The inability of androgenetic and gynogenetic embryos to develop to term suggests a differential function of maternal and paternal genome. Other examples of differential functioning are in X-chromosome activity and development of embryos with the T^{hp} mutation. In normal mouse

embryos the paternal X-chromosone is preferentially inactivated in the extraembryonic tissues (Takagi & Sasaki, 1975; Harper, Fosten & Monk, 1982). The T^{hp} mutation is a deletion on chromosome 17. Heterozygous embryos that inherit the mutation from their male parent are viable whereas those that inherit it from their female parent are not (Johnson, 1974). Furthermore, the defect is not cytoplasmically inherited (McGrath & Solter, 1984b). The production of androgenetic and gynogenetic pig embryos will depend on whether the maternal and paternal genomes in the pig function differently, as they do in the mouse.

Cloning. A second application of the nuclear transplantation technique is in the cloning of embryos. The feasibility of cloning was first demonstrated in amphibians. When nuclei are transplanted from blastula- or gastrula-stage embryos, which contain thousands of nuclei, to enucleated ooctyes, 30–40% develop normally to young frogs (Gurdon, 1964). Transplantation of later stage nuclei to enucleated oocytes yields a much lower percentage of normally developing embryos (Gurdon, 1964). No one has yet obtained complete development of nuclear transplant embryos with nuclei from any adult tissue (DiBerardino, 1980). The progressive decrease in developmental potential of a donor nucleus is probably due to differentiation of the nucleus and the inability of the oocyte cytoplasm to induce complete dedifferentiation.

Very little has been done in applying the nuclear transplantation procedure towards developing a method of cloning mammalian embryos. Illmensee & Hoppe (1981) were the first to report the successful transplantation of a donor nucleus to an enucleated recipient mouse embryo. In their study nuclei from inner cell mass or trophectodermal cells were transplanted to enucleated zygotes. Embryos with donor nuclei from the inner cell mass developed at a higher rate to blastocysts than embryos with trophectodermal nuclei and when transferred to recipient females gave rise to 3 offspring. However, McGrath & Solter (1984c) failed in a very extensive and thorough attempt to repeat this study. They found the developmental potential of donor nuclei to be much more restricted than reported by Illmensee & Hoppe (1981). Transplantation of nuclei from 2-cell embryos to enucleated zygotes resulted in only 13% of the embryos forming blastocysts and only one or two divisions were observed with donor nuclei from more advanced stages.

In comparing the mouse nuclear transplantation experiments to those in the frog several possible explanations arise as to the discrepancy between the results obtained for the two species. First, nuclear differentiation begins at a later stage of development in the frog than in the mouse. The nucleus of the frog embryo becomes active only at the blastula stage or after about 12 cleavage divisions (Newport & Kirschner, 1982). In contrast, the nucleus of the mouse embryo begins synthesizing RNA after the first cleavage division (Johnson, 1981). It is, therefore, possible to transplant nuclei between stages of frog embryos that are functionally equivalent whereas in the mouse each stage is slightly different from the previous one. Secondly, transplanted nuclei in frog embryos have the initial 12 cleavage divisions to adjust to the recipient cytoplasm before they must begin functioning normally. Transplanted nuclei in mouse embryos must function normally after one division. Thirdly, in most frog experiments the mature oocyte is used as a recipient rather than the pronuclear embryo that is used as the recipient for transplanted mouse nuclei.

We have tried to overcome some of the obstacles in the mouse by using recipient stages of embryos that might be more compatible with the donor nucleus. As indicated by the types of polypeptides synthesized, a major transition occurs at the 2-cell stage (Johnson, 1981). The one-cell embryo is very different from the two-cell embryo, but little change occurs from the late 2-cell stage to the 8-cell stage in the mouse (Johnson, 1981). This observation prompted us to consider the 2-cell embryo as a recipient for transplanted 8-cell nuclei. We have found that these nuclear transplant embryos develop for 8–12 days *in vivo* in comparison to only one or two cleavage divisions when the one-cell embryo is used as a recipient (unpublished data), but we have not observed development beyond Day 12 of gestation. These results indicate that the differentiated state of donor and recipient embryos does influence the developmental potential of nuclear transplant embryos. Although there is considerable overlap in function between the 2- and 8-cell stages the differences that do exist are sufficient to cause death of the embryos in midgestation. In amphibians

the mature oocyte causes limited dedifferentiation of a transplanted nucleus (DiBerardino, 1980). We have tested the mature oocyte as a recipient for donor 8-cell nuclei and have found that the oocyte causes complete morphological remodelling of the transplanted nucleus. We have not yet tested the developmental potential of this combination. To date, cloning of mammals by nuclear transplantation has not been successful; however, testing various combinations of donor and recipient embryos or testing other species, such as the pig, which have patterns of development different from the mouse may show that cloning is possible.

Embryo splitting

The discovery that individual blastomeres of embryos each have the potential to give rise to normal young has resulted in a limited method of producing identical offspring. Identical twins have been produced from isolated blastomeres from embryos of many different species and one set of identical quadruplet lambs has been produced (Willadsen, 1982). Pig embryos split at the early cleavage stages (Willadsen, 1982) and at the blastocyst stage (Rorie, Voelkel, McFarland, Southern & Godke, 1985) also give rise to normal offspring. The number of identical offspring that can be produced by this method is limited because the greater the number of times an embryo is split the lower the rate of survival of the resulting embryos. As an example, the rate of survival to young of individual blastomeres from 2-, 4- and 8-cell rabbit embryos is 30%, 19% and 11%, respectively (Moore, Adams & Rowson, 1968). The reduced survival rate appears to be due mainly to an inadequate number of cells for proper differentiation into trophectoderm and inner cell mass at the time of blastulation (Willadsen, 1982). Blastulation in individual blastomeres occurs on a schedule similar to that for an intact embryo but with fewer cells (Tarkowski, 1965: O'Brien, Critser & First, 1984). At the time of blastulation the inner cell mass is derived from cells of the embryo that are completely surrounded by other cells which form trophectoderm. A minimum of 8–16 cells is required for any of the cells to reside in an inside position. If blastulation occurs with fewer than this minimum, as sometimes occurs with split embryos, all the cells of the embryo will form only trophectoderm (Tarkowski & Wroblewska, 1967). As with other species individual blastomeres from 8-cell pig embryos form a higher proportion of trophoblastic vesicles than do blastomeres from earlier stage embryos (Menino & Wright, 1983). The limit to the number of identical offspring that can be produced by splitting embryos, therefore, is about 4.

Chimaeras

Aggregation of cells from different embryos to form chimaeras has been used extensively to study the regulation of early embryo development. The developmental ability of early embryos has been shown by studies in which cells are aggregated as late as the morula or blastocyst stage or even of different stages and gave rise to one complete embryo (reviewed by McLaren, 1976). Experiments in which labelled blastomeres were aggregated in various ways have shown that the position of a blastomere relative to the other blastomeres of the embryo determines whether it will form inner cell mass or trophectoderm (Hillman, Sherman & Graham, 1972). Knowledge of the regulation of early development may be useful in formulating methods of cloning or gene transfer.

Aggregation of blastomeres in appropriate positions may be useful to extend cloning by embryo splitting. As discussed previously the major factor limiting cloning by embryo splitting is an inadequate number of cells at the time of blastocyst formation. Additional cells could be added in such a way as to form mostly placenta leaving the individual blastomere of interest to form the embryo (Willadsen & Fehilly, 1983).

The transfer of genes into the germ line of embryos would be greatly facilitated if embryos were like tissue culture cells. A possible alternative is to form a chimaera between an embryo and a tissue culture cell such as embryonal carcinoma cells. Embryonal carcinoma cells can be injected into the

blastocoele cavity and aggregate to the inner cell mass and can form part of the developing embryo. Recent advances in methods of producing embryonal carcinoma cells have resulted in cells which participate, at a rate of 35–45%, in normal development of the embryo and contribute to many different organs (Evans, Robertson, Bradley & Kaufman, 1983) including the germ line (Stewart & Mintz, 1981).

Sperm injection

With the use of micromanipulation techniques, oocytes can be fertilized with spermatozoa from a variety of sources. Spermatozoa from species foreign to the oocyte have been injected and form normal pronuclei (Uehara & Yanagimachi, 1976; Thadani, 1980). The chromosomes align at the first metaphase plate and undergo at least one mitotic division (Thadani, 1980). Sperm nuclei from the testis and cauda epididymidis also form normal pronuclei (Uehara & Yanagimachi, 1977). Storage of spermatozoa, frozen or freeze dried (Uehara & Yanagimachi, 1976), and the capacitated state of the spermatozoa (Westhusin, Anderson, Harms & Kraemer, 1984) do not affect the ability of injected spermatozoa to form pronuclei. It appears that the most limiting factor in pronuclear formation is the state of the oocyte rather than the spermatozoon (Komar, 1982; Westhusin *et al.*, 1984). Little information is available on the fate of oocytes fertilized by injected spermatozoa beyond the first cleavage. Markert (1983) has observed blastocyst formation from injected oocytes, but he presented no data on the efficiency of development. No-one has reported the birth of offspring from oocytes fertilized by sperm injection.

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