NEW ADVANCES IN REPRODUCTIVE TECHNOLOGIES

Neal L. First
University of Wisconsin-Madison

Introduction:

Animal breeding is entering an exciting new era in which it will have the tools to rapidly multiply animals and to make new strains precisely tailored to meet product and environmental demands. The new reproductive tools which compliment the old tools of artificial insemination and embryo transfer include; the in vitro production of embryos, multiplication of embryos by splitting and cloning, as well as sexing of embryos and possibly sperm. The new genetic tools include the ability to transfer new genes into the genome of Domestic Animals and identification of genes or chromosome restriction fragment linked polymorphisms (RFLPs) which are associated with important production traits and which allow direct and rapid selection for that trait.

Commercial companies are already beginning to offer services such as in vitro production of embryos, embryo splitting, cloning and sexing while continuing research to increase efficiencies and to develop useful New Transgenic lines of cattle, sheep and swine.
The major effort to develop these technologies has been with cattle. This review will focus primarily on cattle and attempt to describe the state of the art and potential use of each of these new technologies.

In-Vitro Production of Embryos:

There are at least two reasons for producing embryos of cattle \textit{in vitro}. First, this technique provides large numbers of embryos for commercial transfer and calf production. In Europe and Japan, the value of dairy calves is sufficiently low relative to beef calves that there are economic incentives for transfer of \textit{in-vitro} produced beef embryos into dairy cow recipients, particularly with the goal of inducing twinning. In Britain and Japan, commercial ventures have been established for \textit{in-vitro} production of cattle embryos. In practice oocytes are recovered at slaughter from beef heifers coming from feedlots selected for high quality cattle of a desired breed. The oocytes are fertilized \textit{in-vitro} with sperm from a highly selected bull. Then the embryos are developed \textit{in-vitro} or in a sheep oviduct until the morula or blastocyst stage when they are transferred into recipient cows. These are usually dairy cows. By this system there is no need for a beef brood cow. Second, the economic feasibility of embryo cloning by nuclear transfer requires that
the enucleated oocytes be produced in-vitro from abattoir recovered ovaries and that the new zygote be developed in-vitro to a stage suitable for recloning. It is the use of oocytes from ovaries of thousands of slaughtered cattle that can make cloning economically feasible. This places great demand on development of systems for harvesting more oocytes from an ovary and on determining if the cytoplasm of the oocyte contributes to the offspring derived from nuclear transfer.

In-vitro production of embryos requires development of technology in three areas: oocyte maturation, in-vitro fertilization and in-vitro embryo development.

**Oocyte Maturation:**

In domestic species, oocytes recovered from follicles matured in vivo either with or without superovulation can be fertilized and proceed through embryo development with good success (cattle: Sirard et al., 1985, 1986; Leibfried-Rutledge et al., 1987, 1989; swine: Cheng et al., 1986; Nagai et al., 1988). Recovering oocytes after maturation in vivo is best accomplished by use of ultra sound guided vaginal laparoscopy. This is useful for recovery from valuable cows but it is an expensive technique and usually supplies only a few oocytes per cow (4 to 8).
co-culture with granulosa cells from the ovarian follicle and rigorous oocyte quality selection oocytes recovered at the abattoir from follicles greater than 3mm can yield a large supply and nearly normal rates of fertilization and embryo development. (Embryo development; to morula or blastocysts; in vivo matured and fertilized - 55%; in vivo matured - in vitro fertilized - 45%; in vitro matured and fertilized - 23-63%. Leibfried-Rutledge et al., 1989, Gordon and Lu 1990). The bovine ovary contains thousands of growing oocytes in follicles of < 1mm. A major challenge to scientists is how to mature and harvest from this early period of oocyte growth. Some success has been achieved with mouse oocytes (Eppig and Schroeder, 1989).

In vitro Fertilization:

The second part of production of embryos in vitro is the sperm capacitation and fertilization system. Numerous capacitation systems have been used including high ionic strength media and glycosaminoglycans such as heparin and fucose sulfate, aging, pH shift, calcium ionophores and caffeine and oviduct fluid (First & Parrish, 1987, 1988; Parrish et al., 1989). In general, any agent which causes Ca++ entry into the sperm acrosome and causes a pH increase within the sperm
causes capacitation (First & Parrish, 1988). With appropriate sperm capacitation preincubation and incubation in serum-free medium at body temperature, in vitro fertilization has been successful with fertilization rates as high as 70-80% in cattle, sheep, swine and goats.

Development of Embryos In Vitro:

Embryos can be developed in surrogate oviducts of sheep or rabbits (Boland 1984, Sirard et al. 1985, 1986) or sheep (Willadsen, 1982; Eyestone et al. 1987) However, when culture is attempted in vitro embryos of cattle, sheep and swine are blocked in development at the stage where they transition from maternal to embryonic control of development, i.e. cattle and sheep 8-16 cells, swine 4-8 cells stage. (First and Barnes, 1989). Embryos can now be cultured through these periods of blocked development by adding oviduct cells or media conditioned by oviduct cells to the culture. Development is further enhanced if glucose is removed from the media (Eyestone and First, 1989, Ellington et al., 1990 Wang et al. 1990).

The identity of the embryotrophic material from oviduct cells is unknown except for sheep where it is believed to be a fucose rich glycoprotein (Gandolfi et al., 1989).

These systems usually result in normal pregnancy rates and fetal
development when fresh embryos are transferred. However, there are occasional reports that pregnancy is reduced after embryo transfer if in vitro produced embryos are also frozen.

The ability to culture embryos in vitro is essential for successful application of embryo cloning sexing and gene transfer.

**Multiplication of Embryos:**

The ability to produce multiple copies of an embryo provides a powerful selection and propagation tool especially useful for traits of low heritability.

A large number of genetically identical embryos provides a means for embryo selection wherein clonal lines descenddent from one embryo could be selected by progeny test for clonal multiplication to large numbers. This system approaches phenotypic selection and could permit rapid change in selected characteristics such as meat or milk production and, when combined with in vitro production of embryos, could provide a way to produce large numbers of high quality embryos for frozen storage and commercial transfer. Two methods of embryo multiplication will be discussed here. They are embryo bisection and nuclear transplantation.
Bisection of Embryos:

Embryos of cattle, sheep, swine, horses, rabbits and mice have been bisected at the morula or blastocyst stage to produce twin offspring and in a few cases trisected or quartered. This technique is now commonly used by the cattle embryo transfer industry to nearly double the number of embryos available for transfer. In one large study the pregnancy rate was 56.5% from transfer of 515 intact blastocyst stage embryos and 52.4% from transfer of 842 half embryos (Leibo, 1988). The emphasis in cattle has been on doubling the number of embryos rather than on producing identical twins. The technique was developed originally by Willadsen (1979) with sheep embryos. The embryo is bisected with a microknife (Williams et al., 1982) or pulled apart with two glass needles (Ozil et al., 1982; Willadsen and Godke, 1984). Highly efficient modifications of these techniques have now been developed (Rorie et al., 1985; Williams and Moore, 1988). When the blastocyst is split, care must be taken to bisect the inner cell mass into two equal halves. The bisection reduces the number of cells by half. There are still sufficient cells for normal embryo development unless another half or more are killed later by freezing, or quartering or any other procedure further reducing cell number. Embryos can be bisected
after freezing with reasonable subsequent pregnancy rates (Heyman, 1985; Suzuki and Shimohira, 1986). In a few cases pregnancies have resulted from bisected embryos frozen by techniques causing little damage (Lehn-Jensen and Willadsen, 1983) and from quartered embryos (Voelkel et al., 1985, 1986).

Embryos have been bisected and transferred both with and without zona pellucida with little difference in pregnancy rate (Warfield et al., 1987). In litter bearing species the bisected embryos must be placed in a zona pellucida before transfer because the zona free embryos will aggregate and fuse forming large chimeric embryos. Thus far there has been no evidence for increased incidence of birth defects or abnormal offspring from this procedure. The frequency of an additional splitting and spontaneous twinning has however been increased by bisection (Seidel, personal communication). In sheep and cattle this technique is being combined with sexing of the embryo wherein a few cells (2-8) are removed from the trophoblast at the time of bisection and sexed before transfer 3-7 hr later. (Herr et al., 1990a).

**Nuclear Transfer:**

The second method for producing multiple copies of an embryos
is by nuclear transplantation. A nuclear transplantation procedure has recently been shown successful in producing viable embryos and offspring in cattle (Prather et al., 1987), sheep (Willadsen, 1986; Smith & Wilmut, 1989), rabbits (Stice & Robl, 1988) and swine (Prather et al., 1989). This procedure is a modification of a procedure originally developed and shown successful for the frog (Briggs & King, 1952).

The procedure (Figure 1) involves transfer of a blastomere or nucleus from a valuable embryo of a multicellular stage into an enucleated metaphase II oocyte with subsequent development to a multiple cell stage and use as a donor in a serial recloning. This procedure is being developed in private industry as well as by University research. Collectively in the USA and Canada, several hundred pregnancies have been produced in cattle by this procedure and recloning has been performed. Thus far the largest number of calves cloned from one embryo has been eight and groups of 11 or more clones are known to be gestating. The 8 calves were born at Granada Genetics in 1987.

A system for cloning of embryos useful to the livestock industry depends on the ability to produce offspring from donor embryos of large cell number and the ability to reclone as the clones develop to advanced
cell number or to multiply donor cells in culture. Studies with sheep at Edinburgh, Scotland, suggest this should be possible. The frequency of development to blastocyst after use of donor cells from blastocyst inner cell mass was 56% and pregnancies resulted (Smith & Wilmut, 1989). In cattle, embryos of morula and early blastocyst stage recovered as late as day-6 have produced good results as donors in cloning (Bondioli et al., 1990). This is very close to a stage where embryonic stem cells can be recovered and multiplied in culture in the mouse (Evans, 1990). If similar stem cell multiplication can be done in domestic animals and if stem cells should prove useful in cloning by nuclear transfer then the number of clones possible is unlimited.

**Sexing of Embryos:**

Sexing of embryos before transfer is especially sought by the dairy cattle industry where females are the desired milk producing unit. To be useful, sexing techniques must be accurate, efficient, rapid and without detrimental effects on the embryos. Embryos can be sexed by use of antibodies to male specific antigens such as the H-Y antigen. When a fluorescent second antibody approach is used, this method is non-damaging to embryos and approximately 85% accurate for cattle.
embryos (White et al., 1987; Wachtel et al., 1988; White, 1988).

Recently highly accurate methods for sexing embryos which use only a few cells and provide a quick answer have been developed. These methods involve the use of Y chromosome-specific bands on a gel from a labeled homologous recognition probe.

Y-specific fragments are used as probes to locate homologous sequences present in DNA from blastomeres or trophoblast cells of embryos. As few as two to five blastomeres can be biopsied from embryos and, using an oligonucleotide polymerase chain reaction for signal amplification, embryonic sex can be determined in 6 hrs or less. Several Y specific probes are currently available for sex selection of cattle embryos (Leonard et al., 1987; Ellis et al., 1988; Popescu et al., 1988; Reed et al., 1988; Bondioli et al., 1989; Herr et al., 1989a,b, 1990) and one has been developed for swine. Recently the Y chromosome deletion assay has been simplified to a cow side field assay kit of excellent accuracy (Herr et al., 1990b,c).

**Sexing Sperm:**

While the sexing of embryos provides a way to predetermine sex, the commercial production of offspring of a chosen sex would be greatly
facilitated if sperm could be sexed and the sexed semen distributed through artificial insemination. A dairyman may wish all females while specific superior cows might be inseminated with male sperm to produce beef or dairy bulls. Until recently the prospects for sexing semen have been dim. It has been known for some time that sperm containing either an X or a Y chromosome could be separated by fluorescence of stained DNA of X and Y sperm using a fluorescence activated cell sorter. This became a standard confirmatory assay but the sperm were dead after the separation. Recently Johnson et al., (1989) have reported a modification of this method wherein sperm remain alive. When female rabbits were inseminated with sorted sperm from the X chromosome population 94% of the offspring born were females. After insemination of the Y bearing sperm 81% of the offspring were male. Because of the large sample required for insemination there was overlap between the base of the X and Y peaks. If in vitro fertilization and small sperm numbers were used it should be possible to use only the completely separated peaks of X and Y sorted populations thereby increasing the accuracy to nearly 100%. Commercial use through artificial insemination will be limited until more efficient sorting systems are developed and damaging effects of the fluorescence staining on
Gene Transfer:

Methods for transferring genes into embryos by micro injection of DNA into the pronucleus of an egg have been developed for cattle, sheep, swine, rabbits, mice and rats. While large numbers of new strains of mice have been made (Table 1), the efficiency is relatively low for all species (Table 1). More efficient and effective methods are being developed; these include the infection of genes into embryos by replication defective viral vectors, a method well developed for mice and chickens but not yet for cattle, sheep or swine. Secondly, the introduction of genes into cultured embryonic stem cells which are then used to form the germ cells of an embryo. The later is well developed for mice and hamsters but not for cattle, sheep or swine. Use of the stem cell gene transfer method and selection of cultured cells for homologous recombination between an introduced and native gene sequence allows gene insertion or deletion at a specific chromosome site. This could be used for correction of genetic defects or for adding a new gene.

The time and tissue in which a gene is expressed are dependent
on gene promoter-enhancer sequences associated with a given gene. A catalogue of promoter-enhancer sequences able to provide gene expression only in a specific tissue and at a specific time is developing.

Already muscle specific promoters such as α-actin allow direction of gene expression specifically to skeletal muscle (Shani, 1986). If the appropriate genes were known this should allow changing the marbling of meat, the growth of the muscle and perhaps it’s flavor. Promoter sequences specific for mammary gland epithelial cells such as the acidic whey promoter, β Lactoglobulin α-lactalbumin or casein, all proteins produced exclusively by the mammary gland should allow production of new proteins, perhaps pharmaceutical proteins in milk or allow changing the composition of milk, for example - it’s casein, fat or sugar content might be changed. Already the pharmaceuticals, "clotting factor 9" (Simons et al., 1987) and "tissue plasminogen activator" (Andres et al., 1987) have been produced in milk.

The exciting aspect of gene transfer is that it provides a way to create new strains of animals which are transgenic for useful genes never found in their species as well as facilitating the introduction of allelic genes existing in a breed or strain but at low frequency. The usefulness of gene transfer will depend on identification and
understanding of potentially useful genes for transfer.

Knowing the genes associated with desirable traits such as milk production, disease resistance, reproduction, growth and carcass quality not only provides candidate genes for transfer, it also provides probes or chromosome RFLPs which can be used as powerful and accurate animal selection tools.

Major efforts are needed in mapping the genome of Domestic Animals to identify gene sequences or RFLPs linked to production traits which can be used as animal selection tools and to identify promising gene candidates for transfer into embryos.

A likely future scenario of animal reproduction will be breeding companies selling sperm and embryos of precisely tailored genetic ability to resist disease and efficiently produce a high quality meat or milk product. Additionally, the sex of the offspring will be preselected and the embryos sold will be from tested and highly selected clonal lines which are mass produced to be cost affordable. With herds of identical cows, breeders will test several bulls on a sample of the herd to identify favorable specific combining ability of the sire and damline then breed large numbers of cows commercially to specific bulls to take advantage of the specific combining ability and further increase the level of
performance.

References


characterization of pluripotent cell lines from porcine and bovine blastocysts. Theriogenology 33:1.


Shani, M. 1986. Tissue specific and developmentally regulated expression of a chimeric


