Embryo technology in laboratory animals.

by *Mia Buehr*

Centre for Genome Research, University of Edinburgh,

Kings Buildings, West Mains Road, Edinburgh EH9 3JQ Scotland.

The rapid progress of genetics in the past twenty years has been due largely to the development of new methods for analysing and manipulating the genome. These technical advances have had profound effects not only upon genetics itself, but on all areas of pure and applied biology. However, many of the exciting new approaches to mammalian genetics depend on the ability to manipulate early events of mammalian embryogenesis, and recent progress in such fields as gene targeting and transgenesis in mammals would not have been possible without advances in embryology that have parallelled progress in genetic techniques. In this paper I will briefly review some of the methods of embryology which have played an important role in the development of genetically manuipulated mammals, or which promise to be important in the future.

More is known about the embryology of the laboratory mouse than that of any other mammal, and almost all mammalian embryological techniques were originally devised for and practised on the mouse. Even now, a few methods (such as gene targeting in embryonic stem cells) are only possible in the mouse, and some other techniques may be conspicously less successful in other species. Although the embryos of eutherian mammals are similar in many ways, it is now clear that there are also many significant differences in the details of development between embryos of different groups. As a result, techniques developed for the mouse may not be appropriate for other species, and results from experiments with mouse embryos may not be valid for those of other species.

Post-implantation embryos

After the embryo implants in the uterus, opportunities for gaining access to it are limited, although there are techniques with which post-implantation embryos can be manipulated. For instance, the uterus of the pregnant female can be externalised, the embryo exposed, and simple surgery or injection performed in utero (Muneoka et al. 1990). Substances or cells can also be injected into the embryo or the placental circulation through the uterine wall (Papaioannou 1990). After treatment, the embryo can then continue its development in utero.

Alternatively, the embryo can be removed and cultured in vitro for a short time (see Cockroft 1990 and 1991 for a description of some techniques and media that have been used). With appropriate culture conditions rat embryos of various post-implantation stages can successfully be maintained in vitro for 48 hours or longer, and during this time their development appears to be normal. However, protein synthesis eventually slows, the circulation stops, and the embryos die after about 96 hours (New 1990)

So if it is desirable to obtain a living animal that has been manipulated as an embryo, it is for the most part necessary to confine experimental procedures to pre-implantation embryos which, after transfer to a foster mother, will be able to develop to term. Alternatively, gametes at various stages of development may be used as subjects.

Cryopreservation

One technique that has had a profound effect on the application of embryo technology is

that of cryopreservation of gametes and early embryos. Frozen embryos have many applications: for instance, strains and mutants of many animal species can be most conveniently and economically stored as frozen embryos, and germ cells from a particular individual (such as a unique transgenic animal) can be preserved. Frozen embryos are also more easily transported than live animals, especially across international boundaries.

The techniques of cryopreservation were developed in the early 1970s (e.g., Whittingham et al., 1972) and have since improved to the point where embryos of many species, at almost any stage of pre-implantation development, can be frozen and subsequently thawed with little effect on viability, although the degree of success depends upon the species of animal, the stage of development, and the protocol used. Embryonic stem cells can also be successfully frozen, as can spermatozoa, oocytes (Surrey & Quinn 1990), and primary follicles isolated from the ovary (Carroll et al. 1990). A discussion of the principles of cryopreservation, as well as several protocols, can be found in Wood et al., 1987.

When cells are frozen ice crystals form in the cytoplasm, and most freezing methods are designed to reduce the size of these intracellular ice crystals and the damage caused by them. It is thought that cells cooled slowly in a solution of cryoprotectant lose fluid to the medium and become dehydrated, and the number and size of ice crystals forming in the cells is therefore reduced. In early cryopreservation protocols cells were immersed in medium containing dimethylsulphoxide (DMSO) as a cryoprotectant, cooled slowly (of the order of 1 degree C per minute), stored in liquid nitrogen and then thawed by slow warming. Later studies (see Wood et al. 1987) tested the effect of cryoprotectants other than DMSO (such as glycerol, propylene glycol, ethylene glycol and others) on the number and size of intracellular ice crystals: the effect of different rates of cooling and warming were also analysed. As a result of these studies, the success of cryopreservation techniques has been significantly improved. However, freezing cells in this way usually requires complex apparatus and careful control of cooling and warming rates, which have in some cases limited its application. As a result, the techniques of conventional embryo freezing have in many labs been replaced by the simpler process of vitrification (also termed ultra-rapid freezing). In the process of vitrification, cells are cooled so rapidly that fluids are supercooled and transformed to a smooth glass rather than to ice crystals (Mazur 1990). In a typical vitrification protocol (e.g., Kasai et al. 1990, Valdez et al. 1992,) cells are placed in high concentrations of a cryoprotectant such as DMSO or ethylene glycol, and transferred to a suitable tube which is then dropped directly into liquid nitrogen. Thawing must be rapid enough so that crystallization does not occur as the cells are warmed, and is conveniently done in a 37° C water bath.

Normal live young of many mammalian species are now routinely obtained from embryos that have been frozen or vitrified at pre-implantation stages, and many protocols have been devised for the cryopreservation of laboratory species (for instance, Stein et al. 1993, Utsumi et al., 1992, Papis et al. 1993, al-Hasani et al. 1992, Kasai et al. 1992, Iida 1992, and others). However, the percentage of survival of cryopreserved embryos is usually lower (and sometimes much lower) than that of freshly collected embryos (e.g., al Hasani et al, 1992) . Factors affecting viability include the species or strain of embryo used, the stage at which the embryo was frozen, and the protocol used. In some studies, vitrified embryos are reported to be less viable than frozen (Wood et al. 1987). The reason for this reduced viability is not clear. There is evidence that both vitrification and freezing procedures can damage cytoplasm and DNA, and some studies show an increased

incidence of aneuploidy, polyploidy and sister chromatid exchange in cryopreserved embryos (e.g.,Sterzik et al 1992, Bouquet et al. 1993). However, much of the damage to cryopreserved embryos appears to be associated with the toxic effects of cryoprotectants rather than to the direct effect of low temperature itself.

Embryo transfer

If live young are required from pre-implantation embryos which have been manipulated in some way, the embryos must be transferred into a foster mother, where they can implant and develop normally to term. Embryo transfer procedures are routine in mouse, human and many other mammals, including the common laboratory species and many agricultural animals (see Betteridge and Rieger, 1993, for a detailed description of the procedure in several species). In a typical protocol, embryos are collected from donor females, or obtained from cryopreserved stocks or from oocytes fertilised in vitro, cultured for a short time and then transferred to the uterus or oviducts of a pseudopregnant foster mother. The details of this procedure will differ according to the species used and the age of embryos at transfer, and many factors can influence success. A suitable culture system is necessary: different culture media will be needed according to the species and in some cases the stage of embryo used. It is also essential that there be a reliable method for inducing pseudopregnancy in the recipient females, to ensure that they are in a suitable hormonal state to allow implantation. In mice and rats pseudopregnancy is normally induced by mating females with sterile males (Hogan et al., 1986), although in the rat, stimulation of the cervix may also be used. Embryos cultured in vitro are normally somewhat retarded in development compared to embryos of the same age which have been allowed to develop in vivo. To allow for this delay in development, pseudopregnant females intended as embryo recipients are normally mated later than embryo donors (a 24 hour delay is routinely used in rats and mice). Embryos are then transferred either to the oviduct or the uterus of the pseudiopregnant female, depending on the stage of their development. Embryo transfer is normally a surgical procedure: the oviduct and the anterior part of the uterus are externalised and the embryos injected into the appropriate site. Some methods for non-surgical, transcervical embryo transfer have been devised, but these are not widely used.

Techniques for manipulating gametes

At the time of writing, there has been no method described by which spermatocytes or spermatids can be cultured in vitro to yield functional spermatozoa. However, it is now possible to obtain embryos from oocytes that have been removed from the ovary, and matured and fertilised in vitro (Krimpenfort et al. 1991: also see Racowsky1991 for references). This procedure is now routinely used to provide cow embryos for transgenesis and other research, and may be extended to other animals, including humans. Developmentally competent mouse oocytes have also been obtained after in vitro culture of preantral follicles (Eppig & Schroeder 1989). These and other techniques of in vitro oocyte maturation will find many practical applications in medicine and in commercial animal production, and improved protocols, applicable to many species, will certainly be devised.

In vitro fertilisation

The fertilisation of mature oocytes with sperm in vitro, and the subsequent recovery of live young after short-term culture and embryo transfer is now routine in many mammalian species, including humans. The technique has many applications in medicine, agriculture, and in such fields as the preservation of endangered species. It is also of use in research studies in which it is necessary to synchronise the development of a large number of embryos. When a suitable system exists to capacitate the sperm, the

success rate of in vitro fertilization can be high (over 80% in animals such as the cow, the human, and laboratory rodents: see First 1991 for references. IVF protocols can be found in Hogan et al, 1986 and Wood et al., 1987).

Culture of cleavage stage embryos and blastocysts

Because the first embryos successfully cultured in vitro were mouse embryos, many culture systems used today are modifications of protocols designed originally for the mouse. These systems may be sub-optimal for non-murine species, and may in part be responsible for the fact that some experimental procedures have proven difficult to repeat on embryos other than mouse. In particular, mouse embryo culture media have not always proven suitable for embryos of other groups. However, much work is now being devoted to the formulation of appropriate media for non-murine species, and as a result improvements in results with these species can be expected. One difficulty encountered in culturing embryos of many species is that of the two-cell block. In early studies of mouse embryos, the media used were unable to support development in some strains from the zygote to four cells: embryos blocked at the two-cell stage. But improvements to the formulation of media have meant that embryos of almost all mouse strains can now be successfully cultured through the two-cell stage (e.g., Chatot et al. 1990, Lawitts & Biggers 1991a, Erbach et al. 1994). However, the two-cell block or its equivalent can still be a problem when the embryos of other species (such as the rat and the golden hamster) are cultured. If it is necessary to maintain embryos of these species through the two-cell stage it has in the past been necessary to return them to the oviduct of a living animal, or to co-culture them with oviduct cells or conditioned medium. However, improvements to culture media are continually being made, and it is becoming increasingly possible to culture embryos of many species from the oocyte to the blastocyst stage completely in vitro. (McKiernan et al. 1991, Kishi et al. 1991, Barnett & Bavister 1992, Carney & Foote 1991). The formulation of a sucessful medium can be a difficult and complex process. Correct osmolarity, a correct balance of ions, and a suitable source of energy are all critical to the success of a medium, and these will differ between species. However, strategies such as simplex optimization (Lawitts & Biggers 1991b) now permit the development of increasingly successful media

Cloning embryos

In the process of cloning, two or more genetically identical embryos are derived from one. There are two ways in which this has been done (Willadsen 1989).

1. Embryo splitting

Splitting of a single embryo into two or more parts can be performed at most pre-implantation stages. Cleavage stage blastomeres can be separated from each other, placed in empty zonae pellucidae, and returned to the oviduct of a foster mother. In this way, each of the two blastomeres of the two cell stage of rats, rabbits and mice can give rise to a normal individual, while in the rabbit, an individual 8-cell blastomere can develop into a normal animal (Moore et al. 1968). Alternatively, the blastocyst may be split into two, three or four parts, and providing that cells of the inner cell mass are included in each, each part may then develop into a normal animal.

2. Nuclear cloning

In this method, blastomeres from a single early embryo are separated, and each nucleus is introduced into an enucleated oocyte (Fig. 1 and Willadsen, 1989: also see Prather & Robl, 1991 for a review, and Barton et al. 1987 for protocols). The recipient oocyte is an unfertilised secondary oocyte from which the metaphase chromosomes have been removed with a micropipette. A single donor blastomere (normally from an 8-cell embryo)

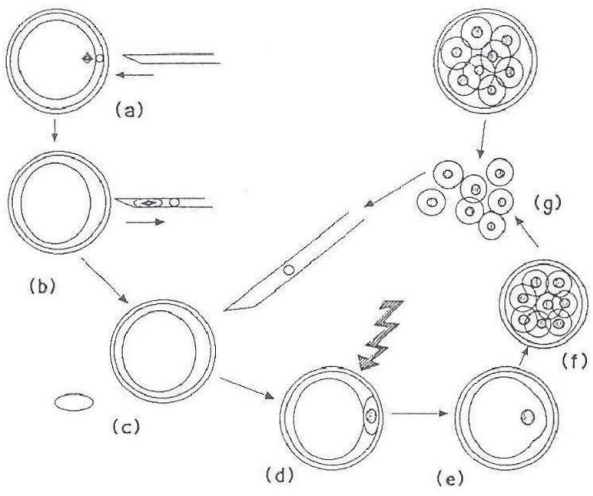

Fig. 1. Diagram of the strategy of nuclear cloning. (a) The recipient oocyte is an unfertilised secondary oocyte. (b) The metaphase chromosomes are removed with a micropipette. (c) A single 8-cell blastomere is injected under the zona pellucida (d) The blastomere and the enucleated oocyte are fused, with Sendai virus or electrofusion. (e) The nucleus from the blastomere is now in the oocyte cytoplasm. (f) Development may then proceed. (g) The resulting 8-cell embryo may then be disaggregated and its cells used for further nuclear transfers.

is then placed under the zona pellucida of the enucleated recipient and the two cells are fused, either with Sendai virus or by electrofusion, which also activates the oocyte. Activation is a critical step: failure of nuclear cloning in some species may be due to inadequate activation of the oocyte, and precise co-ordination of the activation stimulus and the developmental stage of the oocyte is necessary for a successful result. If activation is successful, normal development may then proceed. In this way, several individuals can be obtained from a single 8-cell embryo. Successful attempts have been made to clone nuclei from inner cell mass cells of rabbit blastocysts: the frequency of success was, however, lower than in experiments where 8-cell embryos were used, and it has not so far proved possible to clone embryos from stages older than the blastocyst. If necessary, cloned embryos can be allowed to develop to the 8-cell stage and the nuclei transferred again. As many as 6 successive serial transfers have been done with cow embryos, and by such serial cloning as many as 11 genetically identical live calves have been obtained from a single cloned embryo (reported in Prather & Robl 1991). Not all embryos produced in this way give rise to live young: in cattle 20 to 35% of embryos survive, and of these 20 to 30% develop to later embryonic stages after transfer to recipient females (data summarised in Prather & Robl 1991). Embryo cloning has been successful in several lab species, such as mouse and hamster, but the technique is particularly well advanced in cattle. It has many potential applications in animal production and breeding: it can, for instance, be used to to multiply an embryo of a particularly valuable genotype, or to produce genetically identical individuals in species where inbred lines are not available.

Chimaeras

A chimaera can be defined as an animal derived from 2 separate zygotes and consisting of two genetically distinct populations of cells (see Ng & Iannaccone 1992 for a review). In the past, chimaeras have been useful in the analysis of many developmental mechanisms, and have also been used to rescue cells that by themselves cannot make a viable embyo (such as parthenogenetic cells, or those expressing some mutant genes). Because chimaeras are immunologically tolerant to tissues of the strains from which they are derived, they have also been of use in immunological studies. Chimaeras can be generated by combining cells from two (or more) pre-implantation embryos, transferring the chimaeric embryos to recipient females and allowing them to develop to term (although most chimaeras are generated from two embryos, three or more donor embryos have occasionally been used). There are two ways in which chimaeras are commonly made (Fig. 2): the first (aggregating embryos) has been used for many years, while the second (blastocyst injection) is a more recently developed technique.

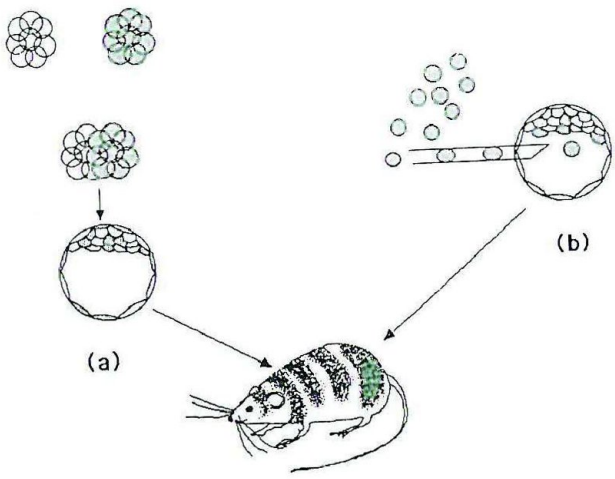

Fig. 2. Chimaeras are commonly made by one of two methods. Aggregation chimaeras originate from two pre-implantation embryos which are aggregated together to form a single embryo (a). Injection chimaeras are derived by injecting blastomeres or embryonic stem cells into the blastocyst cavity of a host embryo (b).

1. Aggregation chimaeras

If two 4 or 8-cell embryos are removed from their zonae pellucidae and pressed together, they will adhere and, their cells will mingle to form a single embryo. (Protocols are given in Hogan et al., 1986, Bradley 1987 and Pratt, 1987.) The animal developing from this embryo will be normal, and will contain cells of both contributing genotypes. Chimaeric mice have been made for many years by this method, and if a suitable culture system is available, aggregation chimaeras of other species (Mayer & Fritz, 1974), or even interspecific chimaeras (Fehilly et al. 1984) can be made.

2. Injection chimaeras

In this method, cells of embryonic origin are injected into the cavity of a recipient blastocyst (see Hogan et al., 1986, and Bradley 1987 for protocols). The injected cells may be from an inner cell mass, or embryonic stem cells, and they can become incorporated in the host inner cell mass. Descendants of those cells can contribute to all the tissues of the animal developing from the blastocys, which will then be a chimaera. If the injected cells contribute to the germ cells, appropriate breeding of the chimaera may then yield progeny that are completely of the genotype of the injected cells. Although blastocyst injection has become routine procedure in many laboratories, it is laborious and technically demanding, and a modification has recently been developed (Wood et al. 1993) in which embryonic stem cells are co-cultured with morulae stage embryos. Embryonic stem cells will aggregate with the embryos and contribute to the animal that develops from them.

Embryo biopsy

In the process of embryo biopsy, one or several cells may be removed from a pre-implantation stage with little effect on embryo viability (Kola & Wilton 1991, Cui et al., 1993a). Single cells can be detached from 4 or 8 cell stages, or patches of trophoblast removed from a blastocyst by means of a micropipette, and the cells then be analysed in one of several ways to yield information about the embryo. For instance, they may be subjected to PCR analysis, treated with a fluorescent probe to a specific sequence, or examined by conventional cytogenetic techniques. The technique of embryo biopsy has found many practical applications in biomedical science. It can, for instance, be used to sex embryos (Cui et al 1993b), to determine if an embryo carries a specific genetic defect, or to check whether DNA injected into the embryo at the pronuclear stage has been incorporated into the genome.

Transgenic animals

In the early 1980s, it was found that fragments of DNA injected into the pronucleus of a zygote could become incorporated into the host genome (Gordon et al., 1980, Hammer et al., 1985, Palmiter & Brinster 1985). The animal that developed from the injected zygote carried the exogenous DNA sequence in its cells, and was termed a transgenic. Since then, transgenic animals of many species have been made, and transgenic tech-

nology has found many applications in the biological sciences. But though the genetic manipulations of transgenesis have only recently been developed, they are based on the well-established techniques of embryo culture and transfer described above. A typical protocol for the production of transgenic mice (such as can be found in Hogan et al., 1986) will include the superovulation of young mice to yield large number of eggs for microinjection and the collection of fertilised eggs in a suitable medium. Cloned DNA is then injected into one of the pronuclei (the male pronucleus is generally chosen as it is larger and closer to the surface than the female). The injected embryos are usually cultured in vitro overnight (so that dead or damaged embryos can be identified and removed) and then transferred to foster mothers. Animals derived from injected zygotes may carry the exogenous construct in some or all of their cells, but it is normally not possible to predict exactly where in the genome the transgene is incorporated, as DNA injected into a pronucleus will normally integrate at random into host chromosomes.

Gene targeting

When a transgenic animal is made, chance alone normally determines whether the exogenous DNA will integrate into a host gene. With the technique of gene targeting, however, it is possible to make alterations to specific host genes. These changes can be, for instance, small base alterations which may result in an altered gene product, but targeting has usually been used to disable the function of a gene (knocking out the gene). There are two general types of targeting vectors, or sequences of DNA designed to integrate at a specific place in the host genome. When a replacement vector is used, a sequence of host DNA is excised and replaced with the exogenous sequence. An insertion vector, on the other hand, inserts into the genome without loss of host DNA. Both types of vector contain two elements. First, there will be exogenous DNA to be inserted into the site of interest. In many cases, this element will contain a bacterial gene whose expression confers resistance to an antibiotic (often neomycin), which is of use in the later step of selecting cells containing the correctly integrated construct. Second, there must be a region of homology between the vector and the site to be targeted. These homologous sequences (up to about 14 kb in all) increase the likelihood that the vector will integrate at the desired site, but they do not guarantee it. Indeed, when a targeting vector is introduced into a cell (by such techniques as elecroporation, microinjection, calcium phosphate precipitation, or others: Lovell-Badge, 1987), the rate of homologous recombination with the host DNA will be low. In most cells, the vector will not be incorporated at all. In others, it will have incorporated at the wrong place. Only rarely will the construct be located correctly. Because correct integrations are so rare, attempting gene targeting by injecting constructs into zygotes is usually extremely inefficient. Rather, targeting constructs are normally introduced into embryonic stem cells, or ES cells. Large numbers of cells can be transfected with targeting constructs, and various strategies used to select those few cells in which targeting has been sucessful. These cells can them be cloned to yield large numbers of cells with which chimaeras can be made, either by blastocyst injection or aggregation techniques. More detailed descriptions of gene targeting strategies and techniques can be found in Capecchi, 1989a and b.

Embryonic stem cells

The ES cells which are a vital part of most gene targeting protocols are embryo-derived cell lines, which differ from most cell lines in that they retain their totipotency through many generations in vitro, and can, when injected into a host blastocyst or aggregated with a morula, contribute to the tissues of the resulting chimaera. If the ES cells contribute to the germ line of the chimaera, a suitable breeding programme can then be followed

which will yield progeny wholly derived from the genetically modified ES cells that contributed to the chimaera. ES cells are in many ways similar to embryonal carcinoma cells (EC cells). Lines of EC cells can be derived by explanting genital ridges, pre-implantation embryos, or ectoderm of 6 day embryos into sites such as the kidney capsule of recipient mice (Damjanov et al. 1987). When introduced into the blastocyst cavity of a host embryo EC cells will participate in the formation of chimaeras, but they rarely contribute to the germ line. For this reason, ES cells are more widely used in gene targeting work. The first lines of ES cells were derived by culturing blastocysts or isolated inner cell masses on feeder layers (Evans & Kaufman, 1981) and this approach is still widely practiced (Robertson, 1987). However, ES cell lines have also been obtained from primordial germ cells of early embryos (Matsui et al. 1992). The great value of ES cells is their ability to remain undifferentiated until they are incorporated into an embryo. If culture conditions are not suitable, they can lose their totipotency in vitro and differentiate into trophoblast and other cell types. However, lines of ES cells are now routinely maintained in many labs, and their culture has been facilitated by improvements in media formulation, and by the discovery that feeder layers are not necessary if the medium is supplemented with the growth factor DIA/LIF (Smith 1991) or some other cytokines (e.g., Nichols et al. 1995).

Although the culturing of established ES cell lines is relatively straightforward, the establishment of ES cell lines can present problems. ES cells were first derived from blastocysts of the inbred mouse strain 129, and it has proven difficult to produce ES cells from other mouse strains. And at the time of writing, no cell line with the same properties as mouse ES cells has been established from any other species. Several cell lines have been established in the rat, the pig, and some other animals that resemble ES cells in culture, and in the case of the rat, chimaeric animals have been produced from these cells (Iannaccone et al., 1994). However, only in the mouse have ES cells successfully populated the germ line, and therefore to date it is only in the mouse that genetic modifications made to ES cells can be passed to subsequent generations. But with improvements in culture techniques, and improved understanding of the events of early embryology in animals other than mouse, embryonic stem cells of many species will surely become available, and with them, the ability to modify the genome directly by the techniques of gene targeting.

Summary:
Recent progress in genetics has been in part due to progress in embryo technology, as new developments in embryology have made increasingly sophisticated genetic manipulations possible. Although post-implantation embryos may in some cases be used as experimental subjects, most procedures make use of pre-implantation stages. Techniques such as in vitro culture and fertilisation of embryos, embryo transfer and the cryopreservation of gametes and embryos are now routine in many laboratories, and play an important part in the creation of genetically manipulated animals. The production of chimaeras has also become routine. Aggregation chimaeras have been made for some years, while injection chimaeras (created by the injection of embryonic stem cells or blastomeres into the blastocyst cavity) play an important part in the production of mice with alterations to specific genes (the process of »gene targeting«). The techniques of embryo cloning and embryo biopsy have also found practical applications in animal production and medicine. But one of the most significant advances in embryo technology has been the development of embryonic stem cells (ES cells), lines of cultured cells that can, when introduced into a host blastocyst, participate in the formation of all tissues. Animals can thus be produced carrying genetic alterations induced in ES cells. Although at present ES cells are available for only a few strains of mice, it is expected that in the future ES cell lines from other species will be developed.

Resume:
De seneste års fremskridt inden for genetik har været delvist betinget af fremskridt indenfor embryoteknologi, og en nu udvikling inden for embryologi har gjort de stadig mere forfinede genetiske manipulationer mulige. Skønt nogle procedurer kan gennemføres med postimplantationsembryoer, er de fleste eksperimentelle fremgangsmåder begrænset til præimplantationsstadier. Teknikker såsom in

vitro dyrkning og befrugtning af embryoer, embryo overflytning og nedfrysning af kønsceller og embryoer er nu rutine i mange laboratorier og er af væsentlig betydning for fremstillingen af genetisk modificerede dyr. Også produktionen af kimærer er blevet rutine. Aggregationskimærer er blevet fremstillet i nogle år, mens injektionskimærer (fremstillet ved injektion af celler fra embryoner eller blastomerer i blastocyst hulen) har en vigtig plads i fremstillingen af mus med specifict ændrede gener. Teknikken til embryokloning og embryo biopsi har også fundet praktisk anvendelse indenfor husdyravl og medicin. En af de vigtigste fremskridt indenfor embryoteknologi har været udviklingen af embryonale stamceller (ES-celler), linier af dyrkede celler der kan deltage i dannelsen af alle væv, hvis ES-cellerne indføres i blastocyster. Dyr kan således produceres med genetiske ændringer, som er introduceret i ES-celler. Skønt ES-celler indtil nu kun er tilgængelige fra nogle få stammer af mus, må det forventes at ES-celler fra andre dyr vil blive udviklet i fremtiden.

References:

al Hasani, S., Hepnar, C., Diedrich, K., van der Ven, H., Krebs, D. 1992. Cryopreservation of rabbit zygotes. Hum Reprod 7, Suppl. 1:81-83.

Barnett, D.K., Bavister, B.D., 1992. Hypotaurine requirement for in vitro development of golden hamster one-cell embryos into morulae and blastocysts, and production of term offspring from in vitro-fertilized ova. Biol. Reprod 47:297-304.

Barton, S.C., Norris, M.L., Surani, M.A.H., 1991. Nuclear transplantation in fertilized and parthenogenetically activated eggs. In: Mammalian Development: A Practical Approach, ed. M. Monk, IRL Press, pp. 235-253.

Betteridge, K.J., Rieger, D., 1993. Embryo transfer and related techniques in domestic animals, and their implications for human medicine. Human Reprod 8:147-167.

Bouquet, M., Selva, J., Auroux, M., 1993. Cryopreservation of mouse oocytes: mutagenic effects in the embryo? Biol Reprod 49:764-769.

Bradley, A., 1987. Production and analysis of chimaeric mice. In:Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. E. Robertson, ed. IRL Press, Oxford. pp 113-151.

Capecchi, M.R., 1989a. Altering the genome by homologous recombination. Science 244:1288-1292.

Capecchi, M.R., 1989b. The new mouse genetics: altering the genome by gene targeting. Trends in Genetics 5:70-76.

Carney, E.W., Foote, R.H., 1991. Improved development of rabbit one-cell embryos to the hatching blastocyst stage by culture in a defined, protein-free culture medium. J Reprod Fert 91:113-123.

Carroll, J., Whittingham, D.G., Wood, M.J., Telfer, E., Gosden, R.G., 1990. Extra-ovarian production of mature viable mouse oocytes from frozen primary follicles. J Reprod Fert 90:321-327.

Chatot, C.L., Lewis, J.L., Torres, I., Ziomek, C.A. (1990). Development of 1-cell embryos from different strains of mice in CZB medium. Biol Reprod 42:432-440.

Cockroft, D.L., 1990. Dissection and culture of post-implantation embryos. In: Postimplantation Mammalian Embryos: A Practical Approach. Eds A. J. Copp & D. L. Cockroft, IRL Press, pp. 15-40.

Cockroft, D.L., 1991. Culture media for postimplantation embryos. Reprod Toxicol 5:223-238.

Cui, K.H., Verma, P.J., Matthews, C.D., 1993a. Hatching rate: an optimal discriminator for the assessment of single-blastomere biopsy. J Assist Reprod Genet 10:157-162.

Cui, K.H., Putland, R.A., Seamark, R.F., Matthews, C.D., 1993b. Precise sex selected births of mice following single cell embryo biopsy and Y-linked testis-specific gene analysis. Hum Reprod 8:621-626.

Damjanov, I., Damjanov, A., Solter, D., 1987. Production of teratocarcinomas from embryos transplanted to extra-uterine sites. In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. E. Robertson, ed. IRL Press, Oxford. pp1-18.

Eppig, J.J., Schroeder, A.C., 1989. Capacity of mouse oocytes from pre-antral follicles to undergo embryogenesis and development to live young after growth, maturation and fertilization in vitro. Biol. Reprod. 41:268-275.

Erbach, G.T., Lawitts, J.A., Papaioannou, V.E., Biggers, J.D. (1994). Differential growth of the mouse preimplantation embryo in chemcally defined medium. Biol Reprod 50:1027-1033.

Evans, M.J., Kaufman, M.H., 1981. Establishment and culture of pluripotential cells from mouse embryos. Nature 292:154-156.

Fehilly, C.B., Willadsen, S.M., Tucker, E.M., 1984. Interspecific chimaerism between sheep and goat. Nature 307:634-636.

Gordon, J.N., Scangos, G.A., Plotkin, D.J., Barbosa, J.A., Ruddle, F.H., 1980. Genetic transformation of mouse embryos by microinjection of purified DNA. PNAS 77:7380-7384.

Hammer, R.E., Pursel, V.G., Rexroad, C.E., Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D., Brinster, R.L., 1985. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315:680-683.

Hogan, B., Costantini, F., Lacy, E., 1986. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbour Laboratory.

Iannaccone, P.M., Taborn, G.U., Garton, R.L., Caplice, M.D., Brenin, D.R., 1994. Pluripotent stem cells from the rat are capable of producing chimaeras. Dev. Biol. 163:288-292.

Iida, T., 1992. The effects of cryopreservation on early development and chromosome constitution in Chinese hamster embryos. Asia Oceania J. Obstet Gynaecol 18:407-412.

Kasai, M., Komi, J.H., Takakamo, A., Tsudera, H., Sakurai, T., Machida, T., 1990. A simple method

for mouse embryo cryopreservation in a low toxicity vitrification medium, without appreciable loss of viability. J Reprod Fert 89:91-97.

Kasai, M., Hamaguchi, Y., Zhu, S.E., Miyake, T., Sakurai, T., Machida, T., 1992. High survival of rabbit morulae after vitrification in an ethylene glycol-based solution by a simple method. Biol Reprod 46:1042-1046.

Kishi, J., Noda, Y., Narimoto, K., Umaoka, Y., Mori, T., 1991. Block to development in cultured rat 1-cell embryos is overcome using medium HECM-1. Hum. Reprod. 6:1445-1448.

Krimpenfort, P., Rademakers, A., Eyestone, W., van der Schans, A., van den Broek, S., Kooiman, P., Kootwijk, E., Platenburg, G., Pieper, F., Strijker, R., de Boer, H., 1991. Generation of transgenic dairy cattle using 'in vitro' embryo production. Bio/Technology 9:844-847.

Kola, I., Wilton, L., 1991. Preimplantation embryo biopsy: detection of trisomy in a single cell biopsied from a four-cell mouse embryo. Mol Reprod Dev 29:16-21.

Lawitts, J.A., Biggers, J.D. 1991a. Overcoming the 2-cell block by modifying standard components in a mouse embryo culture medium. Biol Reprod 45:245-251.

Lawitts, J. A., Biggers, J. D., 1991b. Optimization of mouse embryo culture media using simplex methods. J Reprod Fert 91:543-556

Lovell-Badge, R.H., 1987. Introduction of DNA into embryonic stem cells. In:Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. E. Robertson, ed. IRL Press, Oxford. pp 153-182.

Matsui, Y., Zsebo, K., Hogan, B.L.M., 1992. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70:841-847.

Mayer, J.F., Fritz, H.I., 1974. The culture of preimplantation rat embryos and the production of allophenic rats. J Reprod Fert 39:1-9.

Mazur, P., 1990. Equilibrium, quasi-equilibrium and non-equilibrium freezing of mammalian embryos. Cell Biophys 17:53-92.

McKiernan, S.H., Bavister, B.D., Tasca, R.J., 1991. Energy substrate requirements for in vitro development of hamster 1- and 2-cell embryos to the blastocyst stage. Hum Reprod 6:64-75.

Moore, N.W., Adams, C.E., Rowson, L.E.A., 1968. Developmental potential of single blastomeres of the rabbit egg. J Reprod Fert 17:527-533.

Muneoka, K., Wanek, N., Trevino, C., Bryant, S.V., 1990. Exo utero surgery. In: Postimplantation Mammalian Embryos: A Practical Approach. Eds A. J. Copp & D. L. Cockroft, IRL Press, pp. 41-59.

New, D.A.T., 1990. Introduction. In: In: Postimplantation Mammalian Embryos A Practical Approach. Eds A. J. Copp & D. L. Cockroft, IRL Press, pp. 1-14.

Ng, Y.K., Iannaccone, P.M., 1992. Experimental chimeras: current concepts and controversies in normal development and pathogenesis. In: Current Topics in Developmental Biology, vol. 27, Academic Press. pp. 235-274.

Nichols, J., Chambers, I., Smith, A., 1994. Derivation of germline competent embryonic stem cells with a combination of interleukin-6 and soluble interleukin-6 receptor. Exp Cell Res 215:237-239.

Palmiter, R.D., Brinster, R.L., 1985. Transgenic mice. Cell 41:343-345.

Papaioannou, V.E., 1990. In utero manipulations. In: Postimplantation Mammalian Embryos: A Practical Approach. Eds A. J. Copp & D. L. Cockroft, IRL Press, pp. 61-80.

Racowsky, C., 1991. Gamete resources: origin and production of oocytes. In: Current Communications in Cell and Molecular Biology 4: Animal Applications of Research in Mammalian Development. Cold Spring Harbour Laboratory Press. pp. 23-82.

Robertson, E.J., 1987. Embryo-derived stem cell lines. In:Teratocarcinomas and Embryonic Stem Cells: A Practical Approach. E. Robertson, ed. IRL Press, Oxford. pp 71-112.

Papis, K., Fujikawa. S., Kojima, T., Oguri, N., 1993. Effect of the composition of vitrification media on the survival of rabbit embryos. Cryobiology 30:98-105.

Prather, R.S., Robl, J.M., 1991. Cloning by nuclear transfer and embryo splitting in laboratory and domestic animals. In: Current Communications in Cell and Molecular Biology 4: Animal Applications of Research in Mammalian Development. Cold Spring Harbour Laboratory Press. pp. 205-232.

Pratt, H.P.M., 1987. Isolation, culture and manipulation of pre-implantation mouse embryos. In: Mammalian Development: A Practical Approach, ed. M. Monk, IRL Press, pp.13-42.

Smith, A.G., 1991. Culture and differentiation of embryonic stem cells. J. Tiss. Cult. Meth. 13:89-94.

Stein, A., Fisch, B., Tadir, Y., Ovadia, J., Kraicer, P.F., 1993. Cryopreservation of rat blastocysts: a comparative study of different cryoprotectants and freezing/thawing methods. Cryobiology 30:128-134.

Sterzik, K., Rosenbusch, B., Grab, B., Wahl, A., Beier, H.M., Lauritzen, C., 1992. Numerical chromosome anomalies after fertilization of freeze-thawed mouse oocytes. Arch Gynecol Obstst 251:133-138.

Surrey, E.S., Quinn, P.J., 1990. Successful ultrarapid freezing of unfertilized oocytes. J In Vitro Fert Embryo Transf 7:262-266.

Utsumi, K., Hochi, S., Iritani, A., 1992. Cryoprotective effect of polyols on rat embryos during two-step freezing. Cryobiology 29:332-341.

Valdez, C.A., Abas-Mazni, O., Takahashi, Y., Fujikawa, S., Kanagawa, H., 1992. Successful vitrification of mouse blastocysts using a new vitrification solution. J Reprod Fert 96:793-802.

Whittingham, D.G., Leibo, S.P., Mazur, P., 1972. Survival of mouse embryos frozen to -196°C and -269°C. Science 178:411-414.

Willadsen, S.M., 1989. Cloning of sheep and cow embryos. Genome 31:956-962.

Wood, M.J., Whittingham, D., Rall, W.F., 1987. The low temperature preservation of mouse oocytes and embryos. In: Mammalian Development: A Practical Approach, ed. M. Monk, IRL Press, pp. 255-280.

Wood, S.A., Pascoe, W.S., Schmidt, C., Kemler, R., Evans, M.J., Allen, N., 1993. Simple and efficient production of embryonic stem cell-embryo chimeras by co-culture. Proc Nat Acad Sci 90:4582-4585