Developments in domestic animal embryo manipulation technology which support the application of molecular biology to animal production

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ABSTRACT

Currently most transgenic animals are produced by microinjecting DNA into a pronucleus of *in vivo* derived embryos. This is an inefficient procedure because of the costs of animals, superovulation and surgery, the low numbers of embryos produced, and their poor viability after injection. Thus, in most well run programmes, only about 10% of injected sheep, pig and cattle embryos result in offspring and only 0.5 to 1% in transgenic offspring. Methods for improving the efficiency are discussed and it is concluded that effective *in vitro* maturation (IVM), fertilisation (IVF) and culture techniques and alternative methods of gene insertion (particularly through stem cells or sperm - mediated insertion) would make significant contributions to improved results. Ruakura's successful sheep and cattle *in vitro* oocyte maturation, fertilisation and culture programme and its results are discussed. Recent developments in MOET techniques are discussed and it is concluded that, in the short term, it is possible that more effective multiplication of transgenics could arise from successful IVM/IVF and nuclear transplantation procedures than from significant improvements in current MOET technology. The *in vitro* produced egg therefore has considerable potential for increasing the efficiency of producing and multiplying transgenics.

Keywords Embryo, transgenic, DNA, clone, MOET, IVM, IVF, culture, pregnancy, sperm

INTRODUCTION

Gene transfer creates revolutionary new opportunities for the modification of animal performance. Its potential has been reviewed by many authors (e.g. Simons and Land, 1987) as has its problems (e.g. Pursel *et al.*, 1989). It is possible to produce transgenics by introducing genes into animal cells and then implanting the cells into intact animals where the gene continues to function for a short time (e.g. Selden *et al.*, 1987). However, since it is usually desirable that transgenics pass their altered characteristics to their offspring, the preferred methods for producing transgenics involve introduction of the genes into one-cell embryos. Ideally, all cells in the resulting animal are transgenic and this ensures that the gene is passed to succeeding generations. Thus, at present, the techniques of gene transfer and embryo manipulation are intimately linked.

This paper discusses developments in domestic animal embryo manipulation technology which support both the production of transgenics and the multiplication of the small numbers of animals expected to be produced.

PRODUCTION OF TRANSGENICS BY PRONUCLEUS INJECTION

Injection of *in vivo* produced embryos

Currently the favoured technique for producing transgenics is to inject the genes into one pronucleus of an embryo which has been recovered surgically from a superovulated donor animal. Whereas the technique is routine in mice, its application to domestic livestock is a formidable challenge. This is because of the expense of purchasing, housing and feeding the large numbers of donor and recipient animals involved, the cost of superovulation protocols and surgical procedures and the small numbers of embryos recovered, particularly from cattle and sheep. This is illustrated for sheep in Table 1 by the data of Nancarrow *et al.* (1987). These workers followed the usual procedures of synchronising oestrus with intravaginal sponges, superovulating with PMSG or FSH and controlling ovulation time with GnRH. Their harvest of pronuclear embryos is greater than reported by Walker *et al.* (1987) but similar to that reported by Clark (1988). In agreement with Walker *et*

al. (1987), they showed that animals superovulated with FSH produced more injectable embryos than those treated with PMSG. Their results also agree with others (e.g. Simons *et al.*, 1988) that careful microscopy, using differential interference optics, enables visualisation of most pronuclei and that a major problem with the technique is the poor survival of injected embryos. This results in low numbers of embryos transferred. This survival problem also occurs in microinjected pig embryos (Hammer *et al.*, 1986) and cattle embryos transferred to recipients immediately after injection (Roschlau *et al.*, 1988). The pig and cow embryo also have the added technical difficulty that they must be centrifuged to allow visualisation of the pronuclei. Pigs however, have the advantage that donors produce high yields of embryos (Hammer *et al.*, 1986) and that each recipient can receive a large number of embryos and give birth to litters of about 10 to 12 offspring. Cattle, on the other hand, have the disadvantages of large size, low embryo yields (Loskutoff *et al.*, 1986; Roschlau *et al.*, 1988) and low litter size.

TABLE 1 Efficacy of FSH and PMSG for sheep zygote production

Mean (per donor)	Treatment PMSG	FSH
Ovulation rate	10.8	12.7
Eggs recovered	5.5	8.5
Eggs fertilised	4.6	7.5
Pronuclear embryos	4.0	6.7
Embryos injected	4.0	6.7
Embryos transferred	2.7	4.2

The final parameter which makes production of transgenics from microinjected pronuclear domestic animal embryos such a daunting task is the poor survival to term of the transferred injected embryos. Results vary, depending on how vigorously embryos were screened for viability before transfer, but typical survival rates of sheep embryos are 7 to 25% (e.g. Simons and Land, 1987) and pigs 5 to 9% (Pursel *et al.*, 1989; Brem *et al.*, 1988). Overall, in well run programmes only about 10% of all injected sheep, pig and cattle (Briery *et al.*, 1988) embryos are expected to result in foetuses or offspring.

There are a number of ways the efficiency of this method of producing transgenics could be improved:

(1) Improved superovulation protocols (particularly in sheep and cattle)

Unfortunately, in spite of the considerable effort being put into developing new and pure sources of FSH and new hormone treatment protocols, superovulation responses and yields of embryos have improved only slightly and large between-animal variation in response is still present. Indeed, as stated by Mapletoft and Murphy (1989), the reponse of animals still appears to be more affected by non-hormonal factors such as breed, age, year, nutrition, stress and temperature than by hormonal factors. We believe that major advances in superovulation will only arise through improved understanding of the endocrinology and physiology of follicular and ovulation development and control processes.

(2) Alternative gene insertion procedures

The technique of microinjecting DNA into a pronucleus greatly reduces the viability of the embryo (e.g. Hammer *et al.*, 1986) resulting in lysis of 27 to 47% of mouse and sheep embryos injected (Walton *et al.*, 1987). The latter workers examined a number of aspects of the gene transfer procedure for effects on embryo viability and reported that, although embryo viability improves as the gene transfer operator becomes increasingly more efficient and consistent, there does not appear to be a simple conscious manipulation that the operator can make to significantly reduce embryo lysis. They did however recommend that the injection needles be as fine as possible and that the transfer not be conducted close to the time of first embryo cleavage. Unless membrane protecting compounds can be successfully used it is difficult to envisage how the incidence of embryo lysis can be reduced following pronucleus injection. For this reason other gene incorporating routes are being investigated.

Two of these, retroviral vectors and embryo stem cells, have been briefly discussed at this conference by Dr Clark and, as the Ruakura embryology programme is not investigating them, they will not be

discussed further in this paper.

Our group is however investigating the interaction of domestic animal sperm and DNA and are considering using this method to produce transgenic sheep. Our interest in the method was sparked by the report by Lavitrano *et al.* (1989) of the birth of transgenic mice following absorbtion of a transgene onto sperm prior to IVF procedures. The group have also reported the birth of transgenic pigs following surgical insemination of sperm which had been incubated with DNA (Gandolfi *et al.*, 1989). However, other groups have been unable to repeat these sperm - mediated DNA incorporation results (Barinaga, 1989) and so our investigations have so far been limited to studying aspects of DNA binding to sperm. The full results will be reported later in this conference (Peterson *et al.*, 1990) but in summary they show that: DNA binds to fresh ram, bull and buck sperm; it is totally removed with DNAase; and that for ram sperm, binding is inhibited by seminal plasma and heparin, is little affected by uterine secretions, and the motility of sperm with bound DNA is similar to unbound sperm. Our decision to proceed with this method for producing transgenics will largely depend on whether other groups can successfully repeat the technique.

(3) Screening embryos before transfer for viability and gene incorporation

Because of the expense of domestic animal transgenic production it is desirable that only viable embryos be transferred to recipients and preferably that the transferred embryos are proven transgenics.

Many workers check embryo viability by transferring embryos to an intermediate host, usually a rabbit or sheep (e.g. Briery *et al.*, 1988). Others culture *in vitro* for a short time (e.g. 2 hours, Loskutoff *et al.*, 1986). Both of these procedures are suboptimal, the first because of the expense of maintaining animals and conducting surgery and the second because of the limited duration of culture. Efficient *in vitro* culture systems are now available and some results are presented in Table 2. These show that non-injected one-cell sheep embryos cultured for 1 or 3 days *in vitro* in either TCM 199 plus co-culture or SOF (Tervit *et al.*, 1972) without co-culture have viability similar to non-cultured embryos. Increasing the culture duration to 5 or 6 days is however accompanied by a substantial decrease in viability. Microinjected embryos are far less viable than non-injected embryos but can be cultured for at least one day (and possibly 3 days) without a decrease in viability. These results show that research programmes producing transgenic sheep should consider culturing the embryos for 1 to 3 days before transfer. This will ensure only cleaved embryos are transferred and will reduce the number of recipients used. It should be noted that the culture of embryos in SOF without co-culture cell support is an easy system to establish and that it also gives satisfactory results with cattle one-cell embryos (McLaughlin *et al.*, 1989).

TABLE 2 *In vitro* development of one-cell sheep embryos

Culture conditions	Percent embryos surviving* after various culture durations (d) 0	1	3	5/6	Ref
TCM 199[a]	-	-	80	30	1
SOF[b]	97	98	94	54	2
	88	-	92	56	3
SOF[c]	33	50	35	-	4
	29	25	14	-	4
	22	21	26	-	5

[a]non-injected embryos; co-culture with oviduct cells; gas, 5% CO_2/air
[b]non-injected embryos; no co-culture; gas 5% O_2/5% CO_2/90% N_2
[c]microinjected embryos; no co-culture; gas, 5% O_2/5% CO_2/90% N_2
*Surviving in recipients to Day 13, 14 or 50 of pregnancy

1, Gandolfi and Moor, (1987); 2, Walker *et al.* (1988); 3, Walker *et al.* (1989a); 4, Walker *et al.* (1989b); 5, Walker *et al.* (1990)

Unfortunately the culture systems described are not ideal for screening embryos for their transgenic status before transfer. This is because the embryos for analysis would need to comprise about 60-100 cells and would therefore have been cultured for about 6 days and have reduced viability. This, however, may not be a major problem as improvements in culture systems may match the development of the new techniques needed to distinguish between integrated and non-integrated DNA sequences in embryos.

(4) Techniques to improve embryo survival

There are a number of techniques for improving embryo survival through enhancing the antiluteolytic effect of the embryo. One of these is demonstrated in Table 3. It shows that the transfer of control pig embryos with injected embryos increases pregnancy and embryo survival rate and increases the efficiency of producing trangenics (Brem *et al.*, 1988). A similar effect has been shown in cattle when embryo trophoblastic vesicles are transferred together with embryos (Heyman *et al.*, 1987). Another technique is to treat recipients after transfer with supplementary progesterone, usually through an intra-vaginal progesterone releasing device. The response of sheep to this treatment depends on their level of nutrition (Parr *et al.*, 1986). Animals on a low or medium plane show no response but animals on a high plane show an increase in pregnancy rate. High nutritional intake stimulates metabolic activity which in turn accelerates the clearance of endogenous progesterone. Thus animals on a high plane of nutrition can require exogenous progesterone therapy to maintain pregnancy. Numerous other workers have treated sheep with progesterone and, not suprisingly, the results have been variable because of the varied levels of nutrition. However, we routinely treat recipients of "valuable" embryos (e.g. exotic sheep or experimental embryos) with supplementary progesterone as the treatment will not decrease embryo survival but may increase it. The value of progesterone supplementation for increasing embryo survival has also recently been demonstrated in dairy cattle (Macmillan *et al.*, 1990). Their results showed an increase in pregnancy rate of 13% when progesterone releasing devices were inserted on Days 6, 7 or 8. Finally, recent results with recombinant bovine interferon injected intramuscularly into sheep indicate that this treatment can increase both pregnancy rate and embryo survival (Nephew *et al.*, 1990). It remains to be seen whether interferon can be used to improve fertility in other species such as cattle. Also, a practical means for its delivery needs to be developed and some of its side effects such as hyperthermia, to which cattle seem particularly susceptible, need to be overcome.

A development which will have a major impact on the efficiency of transgenic production in sheep and cattle is the production of viable pronuclear embryos from *in vitro* matured and fertilised oocytes.

TABLE 3 Increase in efficiency of pig gene transfer programmes by simultaneous transfer of control embryos

	No controls	Controls
No. transfers	20	11
No. injected embryos	755	304
No. control embryos	0	94
Pregnancy rate (%)	40	64
Survival rate (%)	5	9
Efficiency *(%)	0.5	1.0

* No transgenic piglets/no. injected embryos

Production of embryos by *in vitro* technology

In this technology, oocytes are collected from the ovaries of animals either at slaughter, laparoscopy or surgery. They are then matured during culture *in vitro* (IVM) for about 24 h, fertilised *in vitro* (IVF; during about 18 h culture) and then finally either cultured *in vivo* (in the sheep or rabbit oviduct) or *in vitro* (IVC) to a stage where they can be transferred or deep-frozen. Successful techniques have been reported for sheep (e.g. Crozet *et al.*, 1987) and considerable research effort worldwide is being put into the development of techniques for cattle (e.g. Fukui and Ono, 1989; Leibfried-Rutledge *et al.*, 1989; Gordon and Lu, 1990). Over the last year at Ruakura we have developed successful techniques for both species and results are presented in Figure 1 (sheep) and 2 (cattle).

It can be seen from Figure 1 that high levels of maturation and fertilisation are achieved in sheep - the latter achieved using either frozen or fresh sperm (Pugh, P.A., Fukui, Y., Tervit, H.R. and Thompson, J.G., unpublished results). Seventy-six percent of the embryos cleave during the first day of culture in SOF and, when these cleaved embryos are transferred to the oviducts of recipient ewes, about forty percent develop into lambs. This very satisfactory result is different to that achieved when the embryos are cultured for a total of 7 days *in vitro* before transfer. Under these circumstances, 27% of the fertilised eggs develop to morulae/blastocysts but only about 3% develop to lambs after transfer. This highlights the problem of poor

viability after extended culture periods and, comparison of the result with that of *in vivo* produced embryos cultured for similar durations (Table 2), also highlights the relatively poor viability of *in vitro* produced embryos.

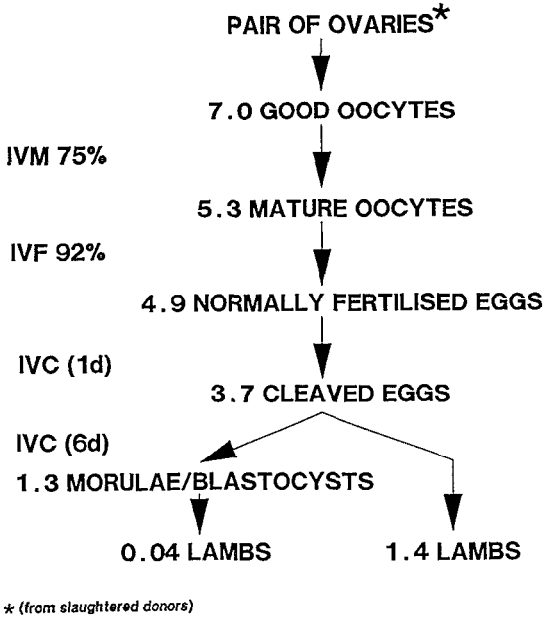

FIG 1 Efficiency of sheep oocyte IVM/IVF

We also achieve high levels of maturation and fertilisation (with frozen sperm) in cattle oocytes (Fukui, Y., McGowan, L.T., James, R.W., Pugh, P.A. and Tervit, H.R., unpublished results, Fig. 2). Degenerate oocytes are discarded at the end of fertilisation so that, on average, about 5 embryos from each pair of ovaries are cultured for 7 days. The culture system used is unique in that it does not involve the co-culture of embryos with oviduct cells and during culture about 42% of embryos develop to blastocysts. This is as good as or better than most reports on the development of cattle IVM/IVF embryos during culture with various somatic cells. We have not yet transferred blastocysts to recipients and, although there is increasing evidence from overseas literature that satisfactory survival rates can be achieved from selected transferred embryos (e.g. Lu *et al.*, 1990; Xu *et al.*, 1990), we are anticipating a maximum survival of about 40%.

Overall, current IVM/IVF technology results in fewer offspring than where donors are subjected to established MOET procedures [i.e. MOET cattle produce, on average, about 3 calves (Dixon, 1990) and sheep 3 to 5 lambs (Tervit, 1989) from each flush]. There is however, little doubt that the efficiency of the IVM/IVF technology will improve and that it has enormous potential to improve the efficiency of gene transfer. This is because: large numbers of embryos can be produced cheaply from slaughterhouse material; it is easy to obtain the correct stages for injection; culture systems exist to screen embryos for viability before transfer and to grow cattle embryos to a stage where they can be transferred non-surgically or deep-frozen; and no surgery is involved. Disadvantages are that the embryos are currently of reduced viability compared to *in vivo* embryos and, since transgenic livestock need to be genetically superior for other characters of interest, it may not always be possible or desirable to recover oocytes from slaughtered animals. In these cases the technique will be more expensive as the donors will need to be subjected to surgery or repeated oocyte recovery by laparoscopy (Sirard *et al.*, 1985).

Interestingly, there are as yet no reports of workers injecting IVM/IVF pronuclear embryos with DNA. However, the group at DSIR, Palmerston North, New Zealand have recently begun injecting IVM/IVF produced cattle embryos (Amos, pers. comm.) and we intend shortly to inject cattle and sheep embryos.

DEVELOPMENTS IN EMBRYO TECHNOLOGY FOR RAPIDLY MULTIPLYING UNIQUE GENOTYPES

When small numbers of transgenics are produced it will be desirable to multiply these using techniques such as artificial insemination and embryo manipulation.

Unfortunately, as stated previously, there have not been any recent major improvements in yields of embryos or reduction in between-animal variability following superovulation. There have however been a number of advances in sheep and goat embryo production which improve the practicality of the technique. These include the widespread adoption in Australia of single injection FSH/PMSG sheep superovulation regimes (e.g. Maxwell and Wilson, 1989); increasing use of intra-uterine insemination of donors; development of

improved surgical techniques enabling sheep donors to undergo up to 14 embryo recoveries (Tervit, H.R., Thompson, J.G. and McGowan, L.T., unpublished); increasing use of laparoscopic embryo recovery techniques which enable the donor uterus to remain within the abdominal cavity during flushing (McKelvey and Robinson, 1986); laparoscopic transfer of embryos to recipients; development of effective sheep regimes for use in the non-breeding season (Tervit *et al.*, 1989); simpler goat superovulation regimes (Batt *et al.*, 1989) and the use of anti-prostaglandin to prevent premature luteal regression in goats (Battye *et al.*, 1988). In addition, satisfactory embryo transfer procedures are now available for deer. Overall though, we still expect donor cattle, sheep and goats to produce, on average 5, 5 to 8, 11 to 20 transferable embryos, respectively, from each flush and 3, 3 to 5, 7 to 13 offspring born to recipients. These figures have changed little over the past 5 years.

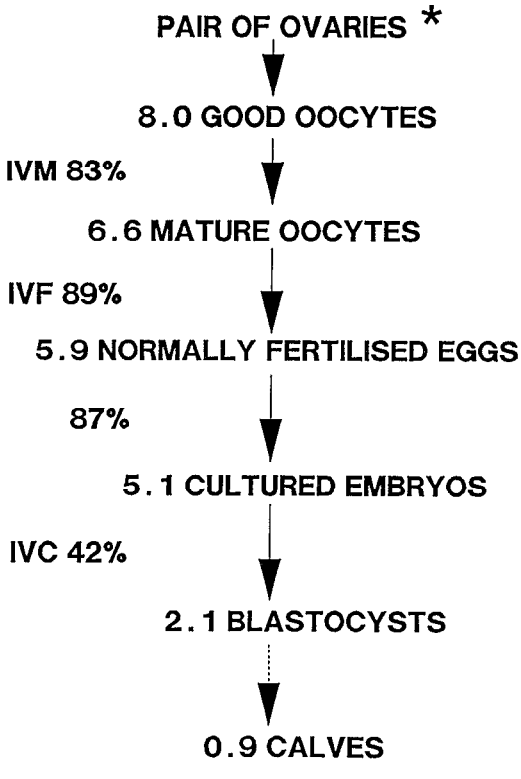

FIG 2 Efficiency of cow oocyte IVM/IVF

There have however been recent remarkable advances in embryo manipulation technology which will assist the multiplication of transgenics. These advances are discussed below.

Embryo freezing, splitting and sexing

Appropriately treated embryos with an intact zona pellucida are generally regarded as disease free and are readily transported between countries. Cattle, sheep and goat embryos are now routinely deep-frozen and embryo splitting is increasingly being used in the cattle industry to increase the number of calves born from each embryo recovered. We have investigated a technique for splitting sheep embryos and, although the technique can be successful, it has a number of problems which preclude its routine use. The details of the technique will be discussed later in this conference (Wells *et al.*, 1990). Herr *et al.* (1988) appear to have overcome many of the problems with the technique and New Zealand veterinarians are now using their technique to split cattle, sheep and deer embryos. Herr *et al.* (1990) also have developed a rapid Y-chromosome-detection assay for sexing embryos and are offering a commercial service.

IVM/IVF

The efficiency of this technique in sheep and cattle and its use for producing pronuclear embryos has already been discussed. With improved success rates, the technique could be used to produce large numbers of embryos for transfer (or other manipulations such as cloning) from oocytes repeatedly recovered laparoscopically from living transgenic donors. Also, oocytes could be recovered from the ovaries when the donor is finally slaughtered. The IVF technique uses relatively low numbers of sperm and thus is an ideal technique for using the low numbers of sexed sperm produced using current flow cytometer sorting technology (Johnson *et al.*, 1989). In this way sexed transgenic embryos could be produced for further manipulations. The technique also may be useful for shortening the generation interval in domestic animals by recovering oocytes from immature animals.

Cloning

This is the production of identical offspring from embryos and can be achieved either by bisecting morulae or blastocysts, separation of the blastomeres of early embryos or nuclear transplantation. The first method has already been discussed. The second has produced identical offspring in cattle, sheep, pigs and horses (Willadsen, 1982). Also, up to 5 identical offspring have been produced in sheep after the blastomeres of an 8-cell embryo were separated and each combined with a blastomere of a 4-cell embryo (Fehilly and Willadsen, 1986). The blastomere separation method is not widely used because of the problems of culturing the clones *in vivo* or *in vitro* to the morula/blastocyst stage and the low numbers of clones produced. The potential of the above two methods for producing clones is surpassed by the technique of nuclear transplantation. This technique was first demonstrated in domestic animals by Willadsen (1986) and involves separation of the blastomeres of the embryo to be cloned and the transfer of each blastomere individually to an unfertilised oocyte which has had its DNA removed. The blastomere and oocyte are then fused by electro- or viral-fusion. The resulting one-cells are clones as their nuclear DNA has come from a common embryo. They are then cultured *in vivo* or *in vitro* to a stage where they can either be recloned or transferred to a recipient. Thus, in theory, a 64-cell transgenic embryo could give rise to 64 clones which would allow very rapid multiplication of the genotype. It is envisaged that ultimately the system used would involve production of embryos from the transgenic donor (by *in vivo* or *in vitro* techniques), determination that the embryo was transgenic and of the desired sex, fusion of the separated embryo cells with enucleated oocytes produced by *in vitro* technology, and culture of the embryos *in vitro* to a transferable or reclonable stage.

Considerable research is being conducted into the technique (e.g. Bondioli *et al.*, 1990; Smith and Wilmut, 1990) and it has been suggested that it should be commercial in cattle in about 3 years (Polge, 1989). In practice though, the overall efficiency is currently low with a cloned 32-cell embryo expected to produce only about 2 offspring. Third generation clones have been produced and one cow embryo has produced 8 clones (Bondioli *et al.*, 1990). *In vitro* matured material has been used in cattle (Prather *et al.*, 1987; Kinis *et al.*, 1989) and pigs (Prochazka *et al.*, 1990) and at this stage, there is insufficient data to indicate whether the material is substantially different to *in vivo* material.

Ruakura is currently developing cloning technology using mouse embryos and sheep oocytes. We have optimised electrofusion parameters so that a high proportion of 2-cell mouse embryos fuse to produce developmentally competent tetraploids and electrically activated *in vitro* produced sheep oocytes are developing as far as the blastocyst stage (Pugh, P.A., Thompson, J.G. and Tervit, H.R., unpublished results). The latter observation is very exciting and it will be interesting to see whether such effective activation equates to successful cloning in our sheep blastomere/oocyte fusion programme due to begin shortly.

Interestingly, the cloning technique can also be used to produce transgenics as well as to multiply them. Production of transgenics would involve fusing either a transfected stem cell or a transgenic micro-injected embryo blastomere with enucleated oocytes.

It must be remembered that the clones produced by the three methods described are clones of an embryo, not of an adult. In the case of nuclear transplantation, possible cytoplasmic effects on the genetic identity of the clone will need to be evaluated.

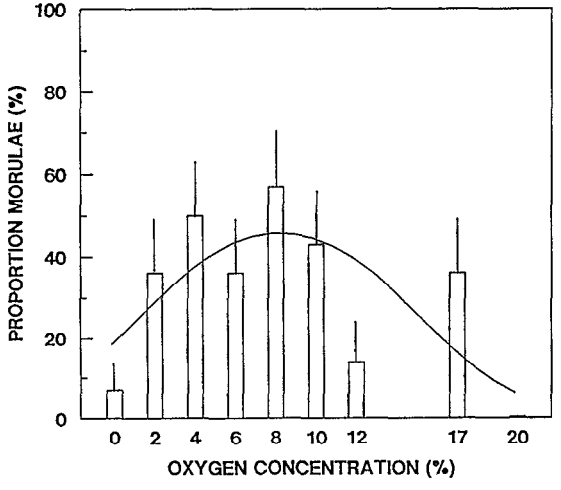

FIG 3 Proportion of sheep 2-cell embryos developing to morulae (≥24 nuclei) during culture for 5 days in SOF + 32 mg/ml BSA under different oxygen concentrations. Bars represent standard errors. The line represents the back-transformed estimates from the quadratic regression.

Embryo handling *in vitro*

The above very detailed techniques rely on efficient *in vitro* gamete and embryo manipulation procedures. Compared to the rodent, relatively little is known of the metabolic and other requirements of domestic animal embryos and this, as already discussed, is reflected in the inability to maintain viability in embryos cultured *in vitro* for greater than 3 days. This should change in the near future as our laboratory, as well as a number of laboratories overseas, conduct extensive research into factors affecting embryo development *in vitro*. Our new programme involves studying: energy substrates utilised by embryos; the effects of antioxidants on *in vitro* development; the nature of oviduct and uterine epithelial cell secretions; the effect of growth promotants and oxygen concentrations on development. Typical data on the effect of oxygen concentrations is shown in Figure 3 (Thompson *et al.*, 1990). Overall the data shows that oxygen concentration in the atmosphere is a factor involved in the development of sheep and cattle pre-implantation embryos and that, in support of the observations of Tervit *et al.* (1972), optimum levels appear to be in the range of 5 to 10%. Although the exact mechanism of the effect of oxygen has not yet been ellucidated, we suggest that auto-oxidative damage caused by free radical production occurs in embryos cultured in air.

CONCLUSION

It is clear that embryological techniques are vital to the production of transgenics and important for the multiplication of the small numbers produced. The production of transgenics by micro-injection of *in vivo* produced pronuclear embryos is currently the preferred method but is inefficient because of poor superovulation responses, the need for surgery, a relatively traumatic gene insertion procedure, inability to reliably culture embryos to viable blastocysts *in vitro*, and poor embryo viability. The solution to most of these problems will be through intensive basic research into ovarian physiology and embryology - our laboratory is ideally placed to make a major contribution to the latter. The first two problems could be largely circumvented by the development of improved IVM/IVF procedures and the third by further studies on alternative methods of gene insertion, particularly stem cells and sperm-mediated insertion as the latter technique has the potential to produce transgenic embryos *in vivo*. More effective multiplication of the transgenics produced could arise in the short term from successful nuclear transplantation and IVM/IVF procedures rather than through significant improvements in current MOET technology. Overall, it is clear that the *in vitro* produced egg has considerable potential for increasing the efficiency of the production and multiplication of transgenics.

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