J. Reprod. Fert., Suppl. **43** (1991), 245–254
Printed in Great Britain
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Production of embryos by oocyte cytoplast–blastomere fusion in domestic animals

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Summary. Embryos of amphibians, sheep, cattle, pigs and rabbits have been multiplied by nuclear transfer. Successful nuclear transfer in these species has been accomplished by transfer of a blastomere from a late-stage embryo into an enucleated oocyte with large scale multiplication by repeating the procedure using blastomeres from the embryos produced from nuclear transfer. This allows the production of clonal lines which, when appropriately selected for performance in a given trait, can be reproduced to capture in the offspring expression of additive and non-additive inheritance.

The efficiency of these procedures is high only for amphibian embryos for which as many as 1000 offspring can be made from a blastula- to gastrula-stage embryo and the process repeated 60–100 times with descendant embryos. In domestic animals the largest number of offspring from one embryo has been 8 calves. Embryos as late as the 64-cell stage in cattle and 120-cell blastocyst in sheep have been used successfully as donors of blastomeres. Recloning has also been done in cattle. Nuclear transfer potentially provides a mechanism for multiplication and production testing of clonal lines, a method for rapid genetic improvement and rapid propagation of a selected genotype. Unfortunately the present efficiencies of subsequent embryo development, pregnancy and embryo survival are less than normal. This paper reviews variables contributing to reduced efficiency and research to improve nuclear transfer.

Keywords: nuclear transfer; embryo cloning

Introduction

Nuclear transfer is being developed in cattle, sheep and pigs as a method for clonal multiplication of genetically valuable embryos. It is usually accomplished by transfer of a blastomere from a late stage embryo into an enucleated oocyte, followed by large scale multiplication by repeating the procedure using blastomeres from the embryos produced from nuclear transfer (Fig. 1). This allows the production of clonal lines which, when appropriately selected for performance in a given trait, can be reproduced to capture in the offspring expression of both additive and non-additive inheritance.

Historically nuclear transfer was first used (Briggs & King, 1952) to show that nuclei of blastocyst-stage amphibian embryos, a differentiated state, when transferred into enucleated eggs could be reprogrammed to direct early embryo development again and could result in the production of live young (Fischberg *et al.*, 1958; McKinnell, 1962). Subsequently it was shown that success in amphibians resulted if the donor nuclei were obtained before gastrulation whereas nuclei from differentiated tissues were seldom reprogrammed for re-expression and term development (Gurdon, 1986).

In mammals, nuclear transfer by direct microinjection of mouse blastocyst inner cell mass or trophoblast into enucleated pronuclear mouse zygotes was performed by Illmensee & Hoppe (1981). Three mice were born from inner cell mass tissue whereas differentiated trophoblast cells produced neither blastocysts nor live offspring. A more efficient system for introducing nuclei by use of cell fusion was developed by McGrath & Solter (1983). Using cell fusion these authors and others (McGrath & Solter, 1984; Robl *et al.*, 1986) could not repeat the results of Illmensee & Hoppe (1981). So far, the only nuclear transfers in rodents resulting in pregnancies or live birth have been transfers between close embryonic stages such as 4- and 8-cell blastomeres into enucleated 2-cell embryos (Robl *et al.*, 1986; Tsunoda *et al.*, 1987). In domestic species offspring have resulted from fusion of late stage blastomeres into enucleated oocytes in sheep (Willadsen, 1986; Smith & Wilmut, 1989), cattle (Prather *et al.*, 1987; Bondioli *et al.*, 1990), pigs (Prather *et al.*, 1989a) and rabbits (Stice & Robl, 1989).

Failures in the mouse may relate to the early transition of the genome of the mouse embryo from maternal to embryonic control of development relative to the other species. This transition occurs at the 3000–4000 cell stage in amphibians (Nakakura *et al.*, 1987), but at the 1–2-cell stage in the mouse (Flach *et al.*, 1982), 4-cell stage in pigs (Tomanek *et al.*, 1989) and the 8–16-cell stage in sheep (Crosby *et al.*, 1988) and cattle (Barnes, 1988; Barnes & Eyestone, 1990).

For cattle, at least four companies are attempting to develop cloning procedures to an efficiency sufficient to allow mass production of cloned embryos and one company is already marketing embryos resulting from nuclear transfer. However, the frequency of developed blastocysts and maintained pregnancies after nuclear transfer has been less than normal with approximately one quarter of the nuclear transfers resulting in blastocysts and about one quarter of the blastocysts transferred into recipients resulting in maintained pregnancies (Prather *et al.*, 1987; Stice & Robl, 1989; Bondioli *et al.*, 1990; Smith & Wilmut, 1990).

Variables influencing the efficiency of nuclear transfer include the efficiency of oocyte enucleation, the developmental competence of the recipient oocyte and oocyte age at nuclear transfer, the efficiency of fusion of the blastomere into an enucleated oocyte and the efficiency of oocyte activation by the fusion process. Also important may be cell cycle synchrony of blastomere and oocyte at fusion. These variables are being studied and high efficiency in each of the required steps is being rapidly achieved (Bondioli *et al.*, 1990; Prather & First, 1990b; Smith & Wilmut, 1990).

The number of clones produced depends on the number of identical donor cells or blastomeres from one donor embryo and on the number of generations of recloning performed. The latest nuclear donor cell stages used successfully have been the 3000–4000-cell blastula and gastrula stages in amphibians (Gurdon, 1986), the 8-cell stage in pigs (Robl & Stice, 1989), the 48- and 64-cell stage in cattle (Bondioli *et al.*, 1990) and the inner cell mass of the 120-cell blastocyst stage in sheep (Smith & Wilmut, 1989).

The late blastocyst is a stage in mice at which embryonic stem cells can be removed, multiplied in culture, injected into another blastocyst to form chimaeras and used to produce germ cells and mice derived from the cultured stem cells (Evans & Kaufman, 1981; Rossant & Joyner, 1989). Several laboratories are attempting to isolate and culture embryonic stem cells from cattle, sheep and pigs (Evans *et al.*, 1990).

This is being done in order to use stem cells as a means for gene transfer in which desired genes are introduced by transfection or by viral vector into the cultured stem cells which are then injected into blastocysts and used to make transgenic mice. These are stem cells and progenitors of all other cells. If they are identified in domestic animals, cultured to large numbers and totipotent as in mice, they will be useful as nuclear donor cells in nuclear transfer; this will allow clonal multiplication of embryos in the culture dish to numbers limited only by the multiplication rate of the cultured stem cells, perhaps resulting in as many as 10^3–10^6 clones from one embryo. This has not yet been accomplished and the largest number of cloned domestic animals born so far has been 8 calves (Bondioli *et al.*, 1990).

Use of nuclear transfer in livestock improvement

Several important questions must be answered for a given species concerning variables contributing to success of nuclear transplantation. These variables will be addressed in the following review with primary focus on cattle and sheep. The procedural steps for nuclear transfer in cattle are shown in Fig. 1.

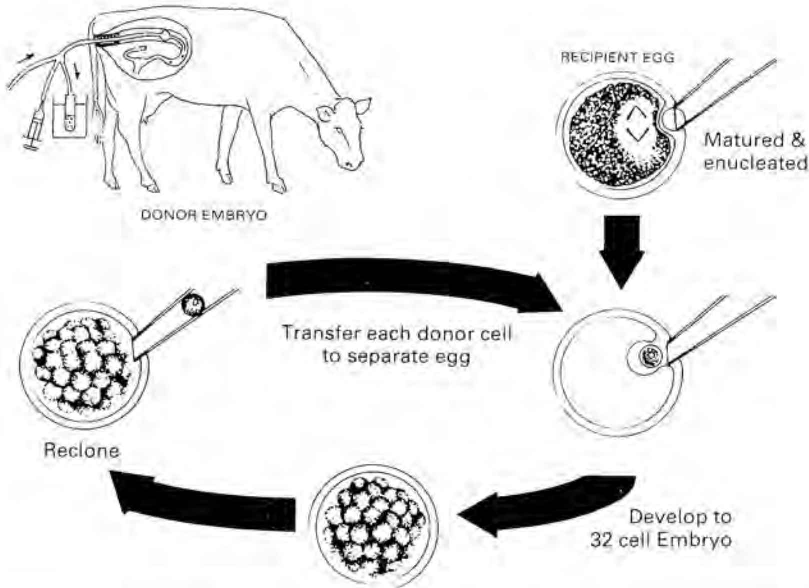

Fig. 1. Cloning bovine embryos by nuclear transfer. Morula- or blastocyst-stage embryos are flushed non-surgically from the uterus of a donor cow. The embryo cells (blastomeres without zona) are separated by aspiration and introduced by electrofusion into an enucleated secondary oocyte (with zona). The resulting embryo is cultured through preimplantation development to a multicellular stage of ≥ 30 cells and the process repeated.

The donor nuclei

The latest stage from which development will occur after embryonic cells are used as nuclear donors in nuclear transfer is as yet unknown for domestic animals. Donor cells from the inner cell mass of the sheep blastocyst resulted in 56% development to the blastocyst stage (Smith & Wilmut, 1989) and donor cells from cattle embryos recovered as late as Day 6 after hCG, a late morula stage averaging 48 cells and maximally 64, resulted in 35% morulae or blastocysts (Bondioli *et al.*, 1990; Marek *et al.*, 1990). As shown in Table 1 developmental potential from Day 6 donor embryos of cattle was higher than from Day 5. The latest usable stage is still unknown and as yet the developmental fate of trophoblast cells *versus* inner cell mass has not been studied in ruminants. Evidence from mice and knowledge of the differentiation states of trophoblast cells indicate they should not result in development when used as nuclear donors (Illmensee & Hoppe, 1981). The 64-cell and blastocyst stages used as nuclear donors in cattle and sheep are not far from the late blastocyst stage in mice and hamsters from which totipotent stem cells can be recovered from the epiblast and cultured to large numbers *in vitro* (Evans & Kaufman, 1981; Doetschman *et al.*, 1988). If such stem

cells can be identified and cultured in sheep and cattle (Evans *et al.*, 1990; Notarianni *et al.*, 1991) and if they are truly totipotent or become so after exposure to oocyte cytoplasm, then large numbers of clones (10^3 or 10^6 or more) can be produced from one valuable embryo.

Experiments in amphibians provide a basis for predicting the outcome of transferring nuclei from advanced-stage embryos. These experiments show that limited development occurs when nuclei beyond the blastula stage are used and that an irreversible differentiation occurs for an increasing population of cells as development progresses further and further past the blastula (Gurdon, 1964, 1986). Failure of development from differentiated nuclei can be assumed to result from the irreversible modification of embryonic chromatin (King, 1966; DiBerardino & Hoffner, 1970).

It is also possible that failed development results from harvesting donor cells at an inappropriate stage of the cell cycle. The decreased rate of development observed from nuclei more advanced in development may not be entirely explained by differentiation alone. A major confounding factor is the change in the length of the cell cycle that occurs at the midblastula transition. Before the midblastula stage the length of the *Xenopus* cell cycle is about 35 min with no pause between M and S phases. This omission of G_1 and G_2 may explain the absence of transcription as RNA synthesis does not occur in M or S phases. As the embryo develops beyond the midblastula stage the cell cycle begins to lengthen progressively with the addition of both G_1 and G_2 phases while length of M and S phases remains unchanged, effectively lengthening the cell cycle as development proceeds. Nuclei that are transferred from cells that have long cell cycles would be required to divide earlier in their respective cell cycle after nuclear transfer as compared to nuclei from the blastula stage. Thus nuclei from more slowly dividing cells promote development at a higher rate if in G_2 when transferred (Von Beroldington, 1981), whereas the stage of the cell cycle may be less important for nuclei from rapidly dividing cells (McAvoy *et al.*, 1975; Ellinger, 1978).

Although nuclei in G_1 undergo DNA synthesis after nuclear transfer to activated, enucleated metaphase II oocytes, in many instances the replication is not complete. This incomplete DNA replication results in chromosomal breakage and unequal inheritance among the daughter cells (Gurdon, 1964; DiBerardino & Hoffner, 1970). Later in development the chromosomal abnormalities are manifest as developmental restriction points or stages that the nuclear transfer embryo is unable to progress beyond. That these restriction points are stably inherited is shown by retransfer, i.e. serial nuclear transfer (Briggs *et al.*, 1964; DiBerardino & King, 1965).

There are no studies in domestic species identifying the importance of or defining the stage of the cell cycle of blastomere donors most conducive to maximal development. In mice synchrony of stage of the cell cycle of donor and recipient cell was shown to be important for transfers between embryonic stages (Smith *et al.*, 1988).

Source of recipient cytoplasm

The recipient cell stage appears to be critical for development after nuclear transfer. When donor cells are fused into enucleated 1-cell zygotes, development does not occur in mice (McGrath & Solter, 1984; Robl *et al.*, 1986; Tsunoda *et al.*, 1987; Smith *et al.*, 1988), rats (Kono *et al.*, 1988) or cattle (Robl *et al.*, 1987). In the mouse, when the recipient stage is the enucleated 2-cell embryo and the donor cell is within one or two stages of the 2-cell (i.e. 4–8 cell) embryo, development and in one case birth have occurred (Robl *et al.*, 1986; Tsunoda *et al.*, 1987). With neither the zygote nor 2-cell recipient stage is there evidence of nuclear remodelling or swelling.

Success in cloning mammalian embryos came from adaptation of methods used in amphibians, namely use of the metaphase II-arrested oocyte as a recipient stage, with the hope that the oocyte would treat the introduced nucleus as it does a fertilizing spermatozoon. This method was first reported in mammals by Willadsen (1986) who showed that nuclei from sheep 8-cell blastomeres were able to support blastocyst development (42–48%) and the birth of lambs. This is also the recipient stage that has resulted in transferable morulae and blastocysts as well as offspring in sheep

(Willadsen, 1986; Smith & Wilmut, 1989), cattle (Prather *et al.*, 1987; Bondioli *et al.*, 1990), rabbits (Stice & Robl, 1989) and pigs (Prather *et al.*, 1989a). Activated cytoplasm of secondary oocytes has the unique ability to promote nuclear envelope breakdown (Szollosi *et al.*, 1988), chromatin condensation and decondensation (Newport & Kirschner, 1984; Fisher, 1987), nuclear swelling (Gurdon, 1964; Prather *et al.*, 1990) and translocation of cytoplasmic proteins into the nucleus and to some extent reprogramming of the genome (Merriam, 1969; DiBerardino & Hoffner, 1975; Korn & Gurdon, 1981; Prather *et al.*, 1989b).

The stage of maturation of the oocyte at enucleation and nuclear transfer may be important. In amphibians fewer chromatin abnormalities and greater embryo development occur with introduction of the nucleus before metaphase II (Orr *et al.*, 1986).

Most mammalian studies have attempted to use oocytes at metaphase II. In sheep (Smith & Wilmut, 1989) and cattle, the optimum age for in-vivo matured oocytes has been reported to be 36 h after hCG or after oestrus (Prather *et al.*, 1987; Bondioli *et al.*, 1990). For cattle little difference was seen in one study for metaphase II oocytes collected 25–48 h after hCG (Bondioli *et al.*, 1990), while 36 h after oestrus was superior to 48 h in another study (Prather *et al.*, 1987). Studies pertaining to effects of time of insemination in relation to ovulation on pregnancy and embryo survival (Barrett, 1948; Casida, 1950) suggest that eggs 36 and 48 h after hCG or the beginning of oestrus should result in lower pregnancy and embryo survival. With the great lack of precision in estimating the physiological state of each cow at 24, 36 and 48 h, great overlap exists at all time points and time differences may not be distinguishable. Additionally, if oocyte activation and cell fusion are more efficient at later times as shown by Ware *et al.* (1989), this may bias the outcome in favour of the more aged egg. Oocytes matured *in vitro* require 18–24 h to reach metaphase II (Edwards, 1965; King *et al.*, 1986; Suss *et al.*, 1988; Sirard *et al.*, 1989) and could be activated and fertilized by spermatozoa at that time (J. J. Parrish, personal communication). However, maximal artificial activation and hence suitability for nuclear transfer is not achieved until more than 30 h after removal from the ovarian follicle (Ware *et al.*, 1989). This enigma needs resolution.

Enucleation of the oocyte

Removal of the oocyte metaphase chromatin and polar body appears essential for a high frequency of embryo development. This is best illustrated by the original experiment of Willadsen (1986) with sheep oocytes in which nuclei were transferred to enucleated and nucleated oocytes (Table 2). Enucleation has been performed by bisection of the oocyte, and rejection of the half with evidence of polar body or chromatin (Willadsen, 1986) or by aspiration of polar body and adjacent cytoplasm (Prather *et al.*, 1987, 1989a; Stice & Robl, 1989; Smith & Wilmut, 1989). The efficiency of both methods can be greatly improved by staining the chromatin with a fluorescent chromatin dye such as DAPI or Hoechst 33258 (Critser & First, 1986; Tsunoda *et al.*, 1988; Prather & First, 1990a).

Cell fusion

Puncture of the oocyte plasma membrane by a needle of sufficient size to introduce a blastomere or nucleus is damaging to the oocyte (Illmensee & Hoppe, 1981). Alternatively, a non-damaging introduction of blastomere or nuclei was accomplished originally in mice by use of Sendai virus-induced membrane fusion (McGrath & Solter, 1983). In cattle this Sendai virus method or use of herpes virus gave little success (Robl *et al.*, 1987). Electrofusion has been successful with ruminant eggs (sheep: Willadsen, 1986; cattle: Prather *et al.*, 1987; Robl *et al.*, 1987) and has become the method of choice when nuclear transfer has been successful in cattle, sheep, pigs and rabbits.

Earlier experiments indicated that the efficiency of cell fusion decreased rapidly as smaller and later stage donor blastomeres were used (cattle: Prather *et al.*, 1987; sheep: Smith & Wilmut, 1989). However, more recent reports show the opposite (Kinis *et al.*, 1989), or only a small reduction in

fusion efficiency with donor cells as late as the 40–64-cell stage (mean = 48; Bondioli *et al.*, 1990; Table 1). Efficient fusion depends on healthy cell membranes, physical contact of both oocyte and blastomere membranes to be fused, and direction of the fusion current primarily through the point of membrane contact (Zimmerman & Vienken, 1982). Present methods allow fusion efficiencies of approximately 70% (Bondioli *et al.*, 1990; Table 1).

Table 1. The effect of donor embryo age on the development of embryos produced by nuclear transfer (from Bondioli *et al.* (1990) and Marek *et al.* (1990))

Donor embryo age (days)	Average cell no.	No. of attempted fusions	No. (%) of successful fusions	No. of transferred sheep	No. (%) of recovered sheep	No. (%) of morulae and blastocysts*
5·0	28·2	882	636 (72)[a]	882	842 (95)	196 (23)[a]
5·5	30·8	212	149 (70)[a]	212	197 (93)	55 (28)[a,b]
6·0	48·3	87	56 (64)[a]	87	84 (97)	29 (35)[b]

*Compact morula or blastocyst after 6 days of culture in the ligated sheep oviduct.
[a,b]Different superscripts within the same column denote a significant difference (χ^2 analysis, $P < 0{\cdot}05$).

Oocyte activation

For completion of meiosis II and progress through subsequent stages of development, an oocyte must be activated. Oocytes are normally activated at fertilization by the fertilizing spermatozoon. For cattle this occurs optimally *in vivo* almost as soon as the oocytes reach the oviduct since the spermatozoa have been previously deposited in the reproductive tract and are already at the site of fertilization. However, *in vitro* only a few of the oocytes are capable of activation by electrofusion at 24 h after the start of culture and do not reach maximal activation until ≥ 30 h (Ware *et al.*, 1989) and with a narrow window for successful later development.

Normally the electrostimulation used for cell fusion is sufficient to activate oocytes during nuclear transfer (Prather *et al.*, 1987, 1989a; Robl & Stice, 1989; Ware *et al.*, 1989; Bondioli *et al.*, 1990; Smith & Wilmut, 1990). These observations suggest that, because of late activation, oocytes used for nuclear transfer, whether recovered *in vivo* or *in vitro*, are relatively aged at the time of cell fusion. The window of opportunity for subsequent development may be very narrow. In mice, when thymocyte nuclei are fused into enucleated metaphase II oocytes, proper nuclear membrane breakdown and further reassembly occur only when cell fusion occurs immediately before or up to 30 min after oocyte activation (Czolowska *et al.*, 1984; Szollosi *et al.*, 1988). A similar narrow window has been reported for sheep oocytes receiving nuclei from nuclear transfer of 32- to 64-cell stage blastomeres (Sun *et al.*, 1989). Much more needs to be learned about the time of oocyte activation, nuclear transfer, the dedifferentiation of nuclei after transfer and subsequent embryonic development.

Culture and development of the embryo resulting from nuclear transfer

In nearly all cases the production of late stage embryos (morula or blastocyst) or pregnancies and calves after transfer have resulted from culture of the nuclear transfer embryos in agar blocks placed in the ligated oviducts of sheep (sheep: Willadsen, 1986; Smith & Wilmut, 1989; cattle: Prather *et al.*, 1987; Bondioli *et al.*, 1990). In one reported study the frequency of embryo development to morula or blastocyst for nuclear transfer embryos cultured *in vitro* appeared to be approximately half (13%) that obtained from culture of nuclear transfer embryos in sheep oviducts (23–35%; Bondioli *et al.*, 1990). This may reflect a sensitivity of embryos derived from nuclear transfer to in-vitro culture or an inadequacy of the in-vitro culture system. Embryos cultured *in vitro* by co-culture with oviduct

epithelial cells or media conditioned by oviduct epithelial cells develop to morulae or blastocysts at nearly the same frequency as those cultured in sheep oviducts (Eyestone & First, 1989) but the number of cells in the embryo is reduced by in-vitro culture after the 8-cell stage (Vergos *et al.*, 1989; Barnes & Eyestone, 1990) and development equivalent to the sheep oviduct occurs only for early cleaving in-vitro cultured embryos (Gordon & Lu, 1990).

When bovine embryos derived from nuclear transfer are cultured in oviducts of sheep, the highest frequency of embryos reported to reach the morula to blastocyst stage is 23–35% of the oocyte–blastomere complexes which were subjected to fusion (Table 1; Bondioli *et al.*, 1990). If 70% complete fusion, this represents 33–50% of the fusion products. In sheep maximal development has been 48% (Table 2; Willadsen, 1986) and 56% (Smith & Wilmut, 1989) of morulae or blastocysts.

Table 2. Cloning sheep embryos by blastomere transfer (from Willadsen, 1986)

		Recipient-metaphase oocyte			
		Enucleated		Nucleated	
		8-cell donor	16-cell donor	8-cell donor	16-cell donor
Embryos cultured		76	29	35	19
Blastocysts developed	No.	32	14	4	1
	%	42*	48	11	5

*Of 4 transferred blastocysts, 3 resulted in blastomere donor type lambs (Willadsen, 1986).

Pregnancies and pregnancy maintenance

While offspring have been born from nuclear transfer in amphibians (Gurdon, 1986), mice (Illmensee & Hoppe, 1981; Tsunoda *et al.*, 1987), sheep (Willadsen, 1986; Smith & Wilmut, 1989), cattle (Prather *et al.*, 1987; Robl *et al.*, 1987; Bondioli *et al.*, 1990), pigs (Prather *et al.*, 1989a) and rabbits (Stice & Robl, 1989), the frequency of pregnancies and their survival have been lower than normal in all the above species. In cattle 42-day pregnancy rates after transfer of embryos formed by nuclear transfer to recipient cows were reported to average 22% and to be 33% for Grade I, 15% for Grade II and 11% for Grade III embryos (Bondioli *et al.*, 1990). This is less than half the expected 50–60% pregnancy rate from normal embryo transfer. Of 104 pregnancies at Day 42, 92 resulted in live calves (Bondioli *et al.*, 1990).

Why pregnancy rate and/or early pregnancy maintenance are reduced after nuclear transfer is unknown. In amphibians after nuclear transfer and especially with inappropriate timing, incomplete DNA replication occurs and results in chromosomal breakage and unequal inheritance among daughter cells (Gurdon, 1964; DiBerardino & Hoffner, 1970). Later in development these chromosomal abnormalities are known to be manifest as developmental restriction points beyond which nuclear transfer embryos are unable to proceed. These restriction points are stably inherited as shown by serial retransfer of nuclei (Briggs *et al.*, 1964; DiBerardino & King, 1965). Further research is needed to improve pregnancy survival if nuclear transfer embryos are to be used for commercial embryo transfer.

Offspring resulting from nuclear transfer are not expected to be identical clones. Studies with bisected cattle embryos show differences in colour pigmentation probably due to differences in melanoblast migration patterns while embryos reside in different uterine environments (Seidel, 1985). The mitochondrial DNA and cytoplasmic environment will differ for each oocyte into which identical nuclei are transferred. We do not know the cytoplasmic or mitochondrial contribution to cattle traits but there is evidence for maternal influences on milk production in cattle (reviewed by

Kirkpatrick & Dentine, 1988). Additionally, any translocation (King & Linares, 1983), diminution (Beerman, 1977), gene rearrangements (Alt *et al.*, 1987), gene amplification (Tobler, 1975) or mutation in one or more cells of a donor embryo will be propagated and multiplied in descendant embryos by serial recloning.

To test identity of clones, numerous clones will need to be produced. At present the usual is 2–4 and the maximum is one set of 8 (Bondioli *et al.*, 1990). At the least it is expected that embryos resulting from nuclear transfer will be useful in genetic improvement because they will transmit through generations the benefits of non-additive as well as additive inheritance.

Conclusions

At present nuclear transfer has been accomplished in ruminants to produce multiple offspring from one donor embryo. Further research is needed to improve the efficiency of the procedure, its embryo multiplication potential, pregnancy rate and survival. Potentially, the procedure provides a mechanism for rapid genetic improvement and rapid propagation of a given genotype. It also provides the possibility for exciting research concerning the interaction of the nucleus, cytoplasm and environment in inheritance.

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison and a grant from W. R. Grace and Co.-Conn. We thank Dr Lorraine Leibfried-Rutledge for assistance and advice on this manuscript and Julie Busby for typing and editing.

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