EMBRYO CLONING: PRINCIPLES AND PROGRESS

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SUMMARY

Embryo cloning consists of transferring the nucleus of an embryonic cell to an enucleated ovum. The clone then develops into a genetic identical to the donor embryo. Although the principles of cloning are similar across species, differences exist. Depending on the source of recipient cytoplasm, the timing of activation may significantly effect subsequent clone development. Similarly, recent information regarding the cell cycle suggests that biphasic nuclear-cytoplasmic interactions effect development as well. The time of the maternal zygotic transition was once considered to be a point of irreversible cell commitment. Cloning studies in cattle and sheep indicate this is not the case for all mammals. Embryo cloning in cattle is now available commercially. The technology in its present form results in a 3 to 1 improvement in progeny production over standard embryo transfer procedures. The embryo cloning procedure promises to be a valuable tool for increasing the progeny of rare and superior cattle.

INTRODUCTION

In 1987, two publications described methods for cloning domestic animals (Willadsen, 1986; Prather et al., 1987). In the three years since its introduction, embryo cloning has rapidly moved from an experimental tool to a commercially attractive procedure for increasing the progeny production of genetically superior animals (Marx, 1988). Not withstanding its commercial merits, embryo cloning can be used to increase the number of progeny of rare transgenic cattle. This can improve the ability to analyze the effect of a transgene on growth and development. Similarly, the value of genetic identicals has long been recognized for research purposes (Reviewed by Biggers, 1986). Relatively large numbers of genetic identicals can improve the statistical validity of an experiment as well as reduce experiment cost, which would normally require a relatively large sampling of random-bred animals (Robl and Stice, 1989).

In principle, embryo cloning consists of dissociating a donor embryo into separate blastomeres and then fusing them to enucleated recipient ova. This effectively transfers the nucleus of the donor blastomere into the recipient cytoplasm where, if successful, it is reprogrammed and subsequently instructs development of an individual genetically identical to the donor embryo. Several factors may affect subsequent development of embryo clones. Among these are activation of the developmental program, nuclear cytoplasmic interactions, specifically as they relate to cell cycle hierarchy, and irreversible differentiation that may occur at specific developmental transitions.

This review will focus on some of the methods and principles of development that have been considered in the evolution of embryo cloning from frogs to cattle. Additionally, we present information reflecting the "state of the technology" of bovine embryo cloning and point out areas where future research is needed.

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METHODS OF EMBRYO CLONING

Embryo cloning was first described in the frog by Briggs and King (1952). They demonstrated that embryonic nuclei could replace the nuclei of fertilized ova and result in development. The cloning procedure as described by Briggs and King (1952) is as follows: 1) activation of the recipient oocyte, the process of initiating development in the absence of fertilization; 2) enucleation of the recipient ovum; and 3) transfer of a nucleus intact, lysed blastomere to the enucleated ovum. In principle the procedure is similar to that used in mammals today. Embryo cloning has been successfully performed in cattle, sheep, rabbits, pigs and mice (Prather et al., 1987; Willadsen, 1986; Stice and Robl, 1989; Prather et al., 1989; Tsunoda et al., 1987). The basic technique can be traced to the work of McGrath and Solter (1983a), who developed a non-invasive method for ovum enucleation and used a fusogenic agent for blastomere fusion. The procedure calls for pre-treatment of recipient ova with cytochalasin. This compound causes the recipient plasma membrane to become more elastic, making the recipient ovum more conducive to manipulation without lysis. To circumvent the zona pellucida, either a cut can be made with a glass knife to allow the insertion of a blunt enucleation pipette (Willadsen, 1986); or a sharpened beveled pipette can be used to puncture it and subsequently enucleate the ovum (McGrath and Solter, 1983a). Once inside the zona pellucida, a pipette is then placed against the oolemma (near the egg chromatin in the case of mice) and gentle suction is applied. The chromatin if visible, along with varying amounts of cytoplasm is "pinched" off in self contained karyoplast. In cattle, the karyoplast can then be placed into a recipient zona pellucida, and both halves stained with Hoechst 33342 to determine which demi-oocyte contains the metaphase chromosomes (Westhusin et al., 1990). The most significant finding, when investigating this procedure, was that Hoechst staining of the "enucleated" half does not result in reduced viability.

After enucleation the recipient ovum is then ready to receive a donor blastomere. Donor blastomeres can be derived from a variety of embryonic cell stages ranging from the 2-cell stage to the blastocyst stage (McGrath and Solter, 1983b; Robl et al., 1986; Robl et al., 1987; Prather et al., 1987; Willadsen, 1986; Tsunoda et al., 1988; Smith and Wilmut, 1989; Stice and Robl, 1989; Prather, et al., 1989; Bondioli et al., 1990). In cases where compaction of the embryo makes manipulation difficult, a short time in Ca^{++} free medium improves blastomere separation (Westhusin and Looney, unpublished, 1989). Once separated, a single blastomere is placed inside the zona pellucida and positioned tightly against the ovum plasma membrane. The blastomere and enucleated ovum are now ready for fusion. Fusion, may be performed in one of two ways. Sendai virus ranging from 1000 to 9000 hemagglutinating units successfully fuses blastomeres to enucleated ova in sheep and mice (Grahm, 1969; McGrath and Solter, 1983a; Robl et al., 1986; Willadsen, 1986). In cattle, pigs and rabbits, as well as sheep and mice, fusion may be induced by electroporation. This is performed in a non-ionic solution such as 0.3 M mannitol in distilled water and a fusion chamber consisting of two electrodes, ranging from 200 μm to 1 mm apart (Kubiak et al., 1985; Robl et al., 1987, Willadsen, 1986; Smith and Wilmut, 1989). To achieve fusion it is critical that the plasma membranes of the ovum and blastomere be in close contact while the electrical current passes perpendicularly through the ovum/blastomere interface (Robl et al., 1987).

Oocyte Maturation and Activation Competence

It has long been recognized that oocyte maturation is composed of nuclear maturation and cytoplasmic maturation (Thibault et al., 1975). Nuclear maturation consists of nuclear envelope breakdown of the germinal vesicle, condensation of chromatin into chromosomes and progression through meiosis I resulting in the 2nd meiotic arrest at meiosis II. In bovine oocytes the process of nuclear maturation requires 19 hours in vivo and is similar to that observed in vitro, 18-21 h (Hyttel et al., 1986a; Hyttel et al., 1986b; Sirard et al., 1989). Cytoplasmic maturation involves changes in protein synthesis, post-translational modification of existing proteins and the repositioning of cytoplasmic organelles (Murray and Kirschner, 1989; Moor and Gandolfi, 1987; Hyttel et al., 1989). Concomitant with nuclear maturation, cytoplasmic organelles move centrally leaving a distinct cortical area (Cran, 1989; Hyttel et al., 1986b; Hyttel, 1989; Kruip et al., 1983). One noted exception to this centralized redistribution of cytoplasmic components is the migration of cortical granules to solitary positions beneath the oolemma (Hyttel et al., 1986a, 1986b, 1989; Cran 1989). The occurance of this migration in in vivo matured bovine oocytes closely resembles the time of completion of nuclear maturation, occurring at 21-22 h post LH surge (Hyttel et al., 1986a). However, in in vitro matured oocytes this migration occurs between 24 and 40 h after the onset of maturation, suggesting an asynchrony in nuclear and cytoplasmic events (Hyttel, 1986b). The significance of this finding becomes apparent when attempting to use in vitro matured oocytes for embryo cloning. One approach used to determine when oocyte maturation is complete is to apply an external stimulus and then detect whether activation has occurred; a procedure which has been used successfully in amphibian cloning systems (Hoffner and Di Berardino, 1980). Studies using bovine oocytes suggest that in vitro matured oocytes complete maturation at 26 to 30 h post-onset of maturation as determined by their ability to activate (Nagai, 1987; Ware et al., 1989). This observation is in general agreement with the ultrastructural observations of organelle redistribution of in vitro matured oocytes. Similarly, in vivo oocytes recovered approximately 24 h post onset of estrus readily activate, indicating a temporal correlation with the final ultrastructural changes of cytoplasmic maturation (Slapak and Westhusin, 1989).

From the preceding discussion it is reasonable to suggest that the activation competence of an oocyte may be used to determine when oocyte maturation is complete, the timing of which may vary depending on the maturation system used. With regard to embryo cloning, the procedure used for the fusion of embryonic blastomeres (electrofusion) to in vivo or in vitro matured oocytes also results in activation. The necessity of activation cannot be overstated, as the nucleus of an embryo clone that fails to activate undergoes premature chromatin condensation (PCC) and remains developmentally quiescent unless activated at some later time (Johnson and Rao, 1970; Czolowska et al., 1984; Barnes unpublished, 1989). Although nuclear and cytoplasmic asynchrony exists in in vitro matured bovine oocytes, this does not appear to significantly inhibit development, as demonstrated by the ability of in vitro matured and fertilized oocytes to progress to the blastocyst stage and result in offspring (Sirard et al., 1988; Gordon and Lu, 1990). Future experiments designed to examine the cause of this asynchrony may improve the developmental competence of in vitro matured oocytes.

The Cell Cycle

The cell cycle is composed of two distinct phases, M phase and interphase. M phase refers to the period of mitosis and entails karyokinesis and cytokinesis of a parent cell resulting in two daughter cells. Interphase is comprised of three periods: S, which is the period when DNA replication is conducted, and G1 and G2, which comprise the gap periods between M phase and S; G1 = pre-replication and G2 = post-replication. An exception to this general scheme is the cell cycle of early cleaving embryos, which is composed primarily of M phase and S up until the time when the embryonic genome begins to be expressed (Newport and Kirschner, 1982; First and Barnes, 1989; Barnes and Eyestone, 1990).

When cells of different periods of the cell cycle are fused, a hierarchy is established based on the two prominent periods of the cell cycle, M phase and S. This hierarchy controls the ensuing cycle of the hybrid cell (Johnson and Rao, 1970; Ord, 1979). Generally speaking, when G1 and G2 cells are fused to S cells, S phase is induced in G1 nuclei and completed in S nuclei but G2 nuclei remain unaltered. G2 cells require nuclear envelope breakdown before they can replicate or re-replicate their DNA (Blow and Laskey, 1988). In contrast, when interphase cells of all periods (G_1, S and G_2) are fused to M-phase cells, PCC is induced due to the pleiotropic factor MPF (maturation promoting factor; Masui and Markert, 1971; Johnson and Rao, 1970). MPF activity is high during meiosis II and rapidly declines (within 8 mins. in some frog species and 2 hr. in the mouse) upon activation or fertilization (Czolowska et al., 1984; Gerhart et al., 1984; Szöllösi et al., 1988; Hashimoto and Kishimoto, 1988). Furthermore, subsequent cell cycles are dominated by cyclic MPF activity, which reaches a plateau during M-phase. MPF is responsible for nuclear envelope breakdown, chromatin condensation and spindle assembly (Murray and Kirschner, 1989; Lohka and Maller, 1985; Gerhart et al., 1984).

Early investigations in frogs, on the effect of cell cycle on embryo cloning, resulted in mixed conclusions. Some reports demonstrated increased developmental potential with some cell cycle combinations while others found no difference (McAvoy et al., 1975; Ellinger, 1978; von Beroldingen, 1981). However, in those experiments, transfer of the donor nucleus occurred between 0 to 75 minutes post-activation (McAvoy et al., 1975; Ellinger, 1978; von Beroldingen, 1981), and therefore, donor nuclei may have been subjected to different types of oocyte cytoplasm relative to MPF activity. The consensus of opinion concerning cell cycle effects in frogs is that DNA replication must occur normally in the donor nucleus within its new environment and that most lethal chromosomal abnormalities occur within the first cell cycle (Di Berardino and Hoffner, 1970; Di Berardino, 1979; Gurdon et al., 1979). The significance of MPF activity in the success of embryo cloning is further implicated in a recent experiment in mammals. When mouse thymocytes are fused to activated oocytes, complete nuclear remodeling of the thymocyte is only possible when fusion occurs prior to extrusion of the 2nd polar body, a period of suspected high MPF activity. The thymocyte nucleus then progresses through the cell cycle synchronously with the host nucleus. The apparent key to this synchronous cell cycle is the exposure of the thymocyte chromatin to the oocyte cytoplasm, an event which results from the nuclear envelope breakdown properties of MPF. When fusions are performed during interphase of the activated oocyte, a period with low MPF activity, thymocyte nuclei show only limited signs of oocyte induced changes and maintain their inherent interphase characteristics (Czolowska et al., 1984; Szöllösi et al., 1988).

From our present perspective, we postulate that nuclei transferred into oocytes at the time of activation undergo nuclear envelope breakdown, nuclear envelope reformation and progression through the cell cycle in harmony with what can be described as a normal first cell cycle. However, it is not known how the oocyte and transferred nucleus interact when asynchrony exists. The impact of varying amounts of DNA present in a random population of donor cells on the ultimate survival of embryo clones would most likely be detected by the appearance and number of chromatids found during the subsequent metaphase. Any abnormalities would be suspected to have occurred during the first cell cycle. Alternatively, nuclear transfers occurring substantially post-activation would also be subject to imprecise synchrony, as the egg cytoplasm would retain the developmental timing (Aimar, 1988; Gerhart et al., 1984; Newport and Kirschner, 1984) while some transferred nuclei may not complete normal DNA replication. In both cases we can hypothesize that only embryo clones that are in precise synchrony result in significant development beyond the period of maternal control. Data presented in Ellinger (1978) and von Beroldingen (1981) appear to support this hypothesis. In both reports the highest frequency of development beyond the period of maternal control was obtained with donor nuclei in S. If nuclear transfer occurred after MPF activity had declined (Ellinger, 30-35 min; von Beroldingen, 35-75 min), then the oocyte cytoplasm would be expected to be in S and, thus, more synchronous with donor nuclei in S.

More recently, the effect of cell cycle on embryo cloning has been tested in mice. There it has been demonstrated that cell cycle stage of recipient cytoplasm and donor nuclei are critical for optimal development (Smith et al., 1988). Presently, there are no reports describing cell cycle affects on embryo cloning of domestic animals. Clearly such experiments should be preceded by a careful characterization of the cyclic changes occurring within the recipient cytoplasm and the donor nucleus.

The Maternal/Zygotic Transition

Early development is controlled by products of the maternal genome. These products, in the form of mRNA and proteins, are synthesized and stored within the oocyte during growth. The point during early development when genes unique to the embryo are first expressed is the maternal/zygotic transition (MZT; Reviewed by: Davidson, 1986; First and Barnes, 1989; Barnes and Eyestone, 1990).

Maternal control of development was suggested in amphibians when it was found that anucleate egg fragments could undergo cleavage (Fankhouser, 1934; Briggs et al., 1951). This led to the conclusion that stores of maternal products in the cytoplasm of eggs were responsible for the subsequent anucleate or nuclear dysfunctional development. The MZT in amphibians occurs during the mid-blastula stage and is often termed the mid-blastula transition (MBT, Newport and Kirschner, 1982). Embryo cloning experiments in frogs suggested that embryonic nuclei taken near the time of the MBT resulted in the highest frequency of development, whereas nuclei from post MZT embryonic tissue resulted in limited development. The apparent difference between those two cell stages being transcription of embryonic genes, which results in a lengthening of the cell cycle (Kimmelman et al., 1987). This led to the hypothesis that differentiation results in a change within the chromatin that increases the time required for normal DNA replication. When such nuclei are used for cloning they fail to complete DNA replication in the abbreviated cell cycle of the egg (Di Berardino and Hoffner, 1970; Di Berardino, 1979; Gurdon, 1986).

In mammals the affect of donor nuclei obtained beyond the time of the MZT on embryo cloning has been mixed. Initial experiments in mice, where enucleated pronuclear eggs were used as recipients, demonstrated that only donor nuclei obtained around the time of the MZT (2 cell stage) resulted in significant development (McGrath and Solter, 1984; Robl et al., 1986). Additionally, it has been postulated that development resulting from embryo cloning in mice is due to the overlap of developmental periods, (i.e. from the MZT to compaction). The transitions of which may mark irreversible points of commitment (Johnson, 1981; Robl et al., 1986; Barnes et al., 1987; Howlett et al., 1987). In those experiments, it was demonstrated that advanced stage nuclei (8-cell stage) when transferred to pronuclear cytoplasm could be reprogrammed to undergo embryonic gene activation a second time. However, development beyond the 2-cell stage was limited. When 8-cell nuclei were transferred to 2-cell cytoplasm, development to the blastocyst stage was obtained at a high frequency. Protein profiles of those embryo clones suggested that development of the 8-cell nucleus was continuing in part on its own accord. Additionally compaction occurred earlier than controls, although, the timing of blastulation did not appear to differ (Barnes et al., 1987; Howlett et al., 1987). The data support the above stated hypothesis. Moreover, it has been demonstrated that 8-cell nuclei transferred to 2-cell cytoplasm can result in the production of offspring (Tsunoda et al., 1987).

Recent studies on embryo cloning of cattle and sheep suggest that the MZT does not represent an irreversible barrier of commitment. In sheep and cattle the MZT occurs at the 8- to 16-cell stage and 4- to 8-cell stage, respectively (Crosby et al., 1988; Camous et al., 1986; King et al., 1989; Kopečný et al., 1989; Barnes and Eyestone, 1990). Nuclei from inner cell mass cells of sheep, when transferred to meiosis II cytoplasm, result in substantial development to the blastocyst stage (Smith and Wilmut, 1989). In cattle, nuclei from 16- to 64-cell stage donor embryos, when transferred by similar methods, also result in blastocyst formation and are capable of resulting in offspring (Prather et al., 1987; Bondioli et al., 1990). The main difference between these studies and those discussed in the mouse is the use of enucleated meiosis II oocytes as recipients. In mice, this has recently been tested using inner cell mass cells as nuclear donors. In a few cases development progressed to the blastocyst stage (5%) (Tsunoda et al., 1988). Embryo cloning experiments in rabbits and pigs have yet to test the affect of post-MZT nuclei on clone viability. The outcome of future investigations in these animals should be interesting, as both procedures currently in use utilize enucleated meiosis II oocytes as recipients (Stice and Robl, 1989; Prather et al., 1989).

It appears that development to the blastocyst stage is not totally dependent on reprogramming. Developmental overlap of donor nuclei and recipient cytoplasm may in some cases be adequate for insuring clone viability (Barnes et al., 1987; Howlett, 1987; Tsunoda et al., 1987) whether or not the MZT represents an irreversible barrier of commitment in some animals remains to be proven. That some development in mice occurs from post-MZT nuclei under different cloning conditions suggests that the recipient cytoplasm and cell cycle timing may be the most important factors to consider (Tsunoda et al., 1988; Smith et al., 1988).

CURRENT STATE OF TECHNOLOGY

Parameters Influencing the Commercial Application of Embryo Cloning

In the advent of commercializing embryo cloning in cattle a number of parameters fundamental to its success have been evaluated. Among these are recipient oocyte age, cell

stage of donor embryo, effect of freezing donor embryos prior to cloning and serial recloning of embryo clones (Bondioli et al., 1990). Initial investigations suggested that oocyte age (36 or 48 h post estrus) could significantly affect embryo clone survival (Prather et al., 1987). More recently in a larger study, no effect was observed when oocytes from 25 to greater than 48 h post HCG were used for embryo cloning as determined by development to the morula or blastocyst stage (Bondioli et al., 1990a). A variety of donor embryo cell stages have been used for embryo cloning. As previously described, blastomeres from 16- to 64-cell stage embryos non-surgically recovered on days 5 through 6 of the estrous cycle appear to be totipotent when used for embryo cloning (Marek et al., 1990).

The ability to freeze donor embryos collected at various points throughout the world for use at embryo cloning centers is primary to the establishment of a commercial cloning enterprise. Table 1 provides a representative sampling of embryo cloning efficiencies at a commercial cloning laboratory using fresh, frozen and serially multiplied donor embryos. The field data indicates that pregnancy rates of cloned embryos derived from donor embryos (16- to 64-cell stage) recovered and frozen in California, Indiana, Wisconsin and Texas and subsequently transported to our cloning laboratory in Marquez, Texas are equivalent to those of fresh donor embryos. The viability of embryo clones, as determined by development to the morula or blastocyst stage, may differ, however, this is confounded by the acknowledged inability of early embryos to survive freezing. As in any commercial setting, all intact blastomeres of frozen donor embryos are used to provide the client the most pregnancies possible.

Table 1. Commercial Embryo Cloning Efficiencies.

Donor Embryo	Viable/Attempted Fusions	Viable/Successful Fusions	Viable/ Recovered	Pregnant/ Transferred
Fresh Embryo	337/1629 (21)	337/1428 (23)	337/1539 (22)	88/280 (31)
Frozen Embryo	182/1375 (13)	182/1164 (16)	182/1293 (14)	35/111 (31)
Serial Cloned Embryo	61/424 (14)	61/362 (17)	61/371 (16)	15/48 (31)

Methods for serial multiplication of donor embryos by cloning can increase the number of progeny produced from a given donor embryo. The process calls for cloning of a fresh or frozen donor embryo, allowing development to proceed to approximately the 16- to 30-cell stage, followed by recloning. As demonstrated in Table 1, pregnancy rates of morula and blastocyst stage embryos produced in this fashion do not differ from fresh embryos. Whether or not the frequency of viable embryos by attempted fusions reflects a true difference between fresh and serially multiplied donor embryos or our inability to select viable embryos from cloned populations during the early precompacting and compacting cell stages is presently unknown.

The preceding discussion indicates that a variety of fresh and frozen oocyte and embryo material can be used for bovine embryo cloning. The ability to improve these procedures will depend on better methods for freezing early embryo stages as well as developing methods, other than morphology, for assessing viability of pre- and early-compaction stage embryo clones.

The Use of In Vitro Matured Oocytes for Cloning

One of the more recent advancements in embryo cloning technology is the use of in vitro matured oocytes (Table 2). Progress in this area is due to improved methods for oocyte maturation, selection based on follicle size and determination of activation competence. In our laboratory, we have modified the procedure described by Critser et al (1986), by substituting an equivalent amount of recombinant bovine FSH (rbFSH, Chappel et al., 1988) for NIADDK-oFSH and completely omitting NIADDK-oLH. Additionally, we have observed, as have others (Tan et al., 1990), that the size follicle from which an oocyte is derived can greatly affect its developmental competence. Finally, as discussed previously, the oocyte must be activated to insure subsequent development. In properly matured oocytes, this occurs at the time of fusion of the donor nucleus to the enucleated oocyte. These three parameters, when adhered to, produce IVM oocytes that are equivalent to in vivo recovered oocytes when used for bovine embryo cloning.

Table 2. Bovine Embryo Cloning Using Oocytes Derived by In Vitro or In Vivo Methods (%)

Oocyte Source	Viable/Attempted Fusions	Viable/Successful Fusions	Viable/ Recovered	Pregnant/ Transferred
In Vitro Matured	161/961 (17)	161/840 (19)	161/906 (18)	32/125 (26)
In Vivo Matured	161/1074 (15)	161/884 (18)	161/1008 (16)	40/135 (30)

A comparison of embryo clone development when using oocytes derived by in vitro or in vivo methods. In vitro matured bovine oocytes, aspirated from follicles 3 mm or greater, were compared to in vivo matured oocytes recovered from superovulated cows as described by Bondioli et al. (1990). Blastomeres from a donor embryo on any given day were randomly distributed between oocyte source. Embryo cloning was subsequently carried out by the methods of Willadsen (1986) as modified by Westhusin et al (1990). Viability was morphologically assessed by the frequency of embryo clones progressing to the morula or blastocyst stage of development after 5 to 6 days of culture in the sheep oviduct. There was no difference in the developmental competence of in vitro matured and in vivo matured oocytes for any of the parameters measured. Analysis by Chi-squared, $P>.05$ for all parameters.

State of the Technology

Embryo cloning is still in the development phase, however, it can be used to obtain more pregnancies per embryo than conventional embryo transfer (Table 3). Highly efficient methods are now commercially available for sexing bovine embryos. The cattle producer can now select pre-sexed embryos for use in the embryo cloning procedure. This will result in groups of identical offspring of the desired sex. The ability to freeze some cloned embryos and transfer others, allows performance testing of the genotype. Recloning and transfer of performance selected genotypes should greatly improve performance predictability.

Table 3. Comparison of Conventional Embryo Transfer to Embryo Cloning for Pregnancy Production

	Conventional ET	Nuclear Transfer	Advantage
Viable Embryos per Recovery	6	6	0
No. Non-Surgical Transfers	6	38	+ 32
No. Pregnancies	3-4	10-11	+ 7

A number offspring have been produced using the embryo cloning process. All offspring produced from a given donor embryo have been confirmed to be genetically identical, either by blood typing or DNA fingerprinting. As predicted, coat color patterns are not identical and large variability in birth weights has been observed. To date, 7 bulls represent the largest group of identicals born at Granada Biosciences. A total of 14 cloned female pregnancies have been confirmed, some of which were the result of embryos produced by serial recloning.

CONCLUSION

The application of embryo cloning to animal agriculture, as well as research is exciting. The techniques can be used to increase progeny production of valuable animals, as well as quicken our understanding of the complex interaction of nucleus and cytoplasm.

Several areas of future research have been implied. Improving in vitro maturation systems for bovine oocytes may improve the rate of cytoplasmic maturation and result in improved rates of development. Investigations into the cyclic nature of MPF in the maturing oocytes, as well as the 1st cell cycle, should provide insight into the requirements for nuclear reprogramming. Similar studies defining the cell cycle of donor embryos will aid in assessing the influence of cell cycle asynchrony on subsequent development. Additional studies investigating improved methods for freezing early stage embryos, as well as methods for assessing viability, by other than morphological means, should improve selection and production procedures required for commercial application.

Several factors influencing the successful commercial application of embryo cloning have also been discussed. Recent improvements have been made in oocyte selection and in vitro maturation based on developmental competence by follicle size and cytoplasmic maturation. This results in the use of a more economically feasible recipient oocyte which can be obtained as a abattoir by-product. The observation that non-surgically collected bovine morula can be sexed, frozen and subsequently used for embryo cloning provides great flexibility regarding the establishment of embryo cloning centers.

It is clear that livestock producers will enter the 21st century with yet another reproductive technique to improve the quality of their product.

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