

RECONSTRUCTION OF NUCLEAR TRANSFER EMBRYOS
IN GOATS AND CATTLE

A Dissertation

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LIST OF ABBREVIATIONS

BO.....	Brackett-Oliphant
BLST	blastocyst
BRL	Buffalo Rat Liver
BSA	Bovine Serum Albumin
CH.....	Corpus Hemorrhagicum
CL.....	Corpus Luteum
eCG.....	equine Chorionic Gonadotropin
ES	Embryonic Stem
FISH	Flourescent In Situ Hybridization
FSH.....	Follicle Stimulating Hormone
GLM.....	General Linear Model
hCG.....	Human Chorionic Gonadotropin
i.m.	Intramuscular
IVF.....	In Vitro Fertilization
IVP.....	In Vitro Produced
LH.....	Luteinizing Hormone
LOS.....	Large Offspring Syndrome
MII.....	Metaphase II
NT.....	Nuclear Transfer
PBS.....	Phosphate-Buffered Saline
PCR.....	Polymerase Chain Reaction
RED	Ryan Embryo Development

SEM..... Standard Error of the Mean

2n Diploid

4n Tetraploid

6n Hexaploid

ABSTRACT

The low survival rates of nuclear transfer fetuses and neonates in goats and cattle have been linked to placental abnormalities. A series of studies was designed to investigate the possibility of supplementing nuclear transfer embryos with electrofused embryos to generate placental tissue in goats and cattle. The initial study was designed to determine if the breeding season of goats could be extended with hCG treatment. Progesterone concentrations in treated does increased but pregnancy rates were unaffected. In the second study, goat embryos were electrofused and combined with nuclear transfer embryos at the 8-cell stage to produce the first offspring as a result of electrofused embryo complementation in goats. The remainder of the studies focused on electrofused embryos in cattle. The method of electrofusion was studied and it was determined that fusion efficiency and developmental rates after two fusogenic pulses were not different from fusion efficiency and developmental rates after a single pulse. The latter study also showed that the time of cleavage following in vitro fertilization affected the cleavage and blastocyst rates of embryos after electrofusion. In the next study, electrofused embryos were aggregated with nuclear transfer embryos at the 8-cell stage. Aggregate embryos developed to the blastocyst stage at the same rate as electrofused and nuclear transfer control embryos. The final study was a series of experiments conducted to characterize the nuclear status of electrofused embryos. In the first and second experiments of the series, embryos were stained following electrofusion and it was found that more embryos were tetraploid and fewer were binucleate when embryos were electrofused later after cleavage. The third and fourth experiments in this series examined the stage of the cell cycle prior to electrofusion. These experiments indicated that the embryos electrofused at 30 hours post-insemination were in the G2 phase of the cell cycle. It was

concluded that the stage of the cell cycle would be an important factor in the production of tetraploid embryos via electrofusion and this should be the basis of future research in this area.

CHAPTER I

INTRODUCTION

AMPHIBIAN CLONING

Nuclear transfer, or cloning, of vertebrates began to develop with the experiments of Briggs and King in 1952 (Briggs and King, 1952; Di Berardino, 1997a; Di Berardino, 2001). These two researchers were studying the nuclei of differentiated cells to determine if the nucleus retained the potential to direct normal development or if the nucleus itself undergoes differentiation and loses totipotency (Briggs and King, 1952). They devised methods to do nuclear transfer by first activating *Rana pipiens* eggs with a glass needle prick and removing the nucleus with a glass needle. A blastula-stage or early gastrula-stage embryo was selected as the nucleus donor and a cell from the subsurface animal pole was drawn into a glass pipette, breaking the cell membrane. The cell contents were injected into the enucleated egg and development was allowed to proceed. Of the eggs injected with blastula-stage nuclei, 104 of 197 cleaved and 63 formed complete blastulae. From the 63 complete blastulae, 50 were allowed to continue development and 15 formed normal post-neurula embryos. From these results, Briggs and King (1952) showed that it was possible to transfer an embryonic nucleus to an enucleated egg and for that nucleus to direct the development of another organism.

Following this groundbreaking study and others from the same group of researchers, the generation of adult frogs (*Xenopus laevis*) was reported from the nuclei of vegetal hemisphere cells of blastulae and the gut of later stage tadpoles (Gurdon, 1962). These frogs were within normal ranges of controls produced in the same laboratory for size, morphology and reproduction. It was shown later that fully differentiated adult keratinized skin cells of *Xenopus laevis* were able to serve as nuclear donor cells that resulted in the production of

tadpoles with a heart beat but resulted in no adult frogs (Gurdon et al., 1975). This indicated that fully differentiated amphibian cell nuclei had the capacity to support the development of an organism to a certain extent, but that the nuclei were not truly totipotent.

MAMMALIAN CLONING

Successful nuclear transfer attempts in mammals were first reported in the early 1980s. The earliest report was of fertile adult mice produced via transfer of a nucleus from an inner cell mass cell into a fertilized zygote (Illmensee and Hoppe, 1981). Following nuclear transfer, both pronuclei were removed and the mouse embryos were cultured to the morula or blastocyst stage and transplanted into pseudopregnant recipients. The three offspring produced (two female and one male) had coat phenotypes and glucosephosphate isomerase (GPI) allele variants of the nuclear donors and when mated, had progeny resembling only that of the nuclear donor. Other groups were unable to reproduce these results but did show that enucleated single-cell mouse embryos were able to form blastocysts after transfer of pronuclei from another embryo (Di Berardino, 1997a; Di Berardino, 2001; McGrath and Solter, 1983; McGrath and Solter, 1984).

Early Embryonic Cell Nuclear Transfer

The first successful nuclear transfer in a large mammal was reported in 1986 (Willadsen, 1986). Cloned sheep were produced by microsurgically enucleating an oocyte at the metaphase II (MII) stage of development by aspirating the polar body and adjacent cytoplasm. A whole blastomere from an 8- to 16-cell embryo was fused to the enucleated oocyte by virus-mediated or electrically-induced cell fusion. Embryos were cultured in ligated sheep oviducts and examined on day 6 or 7 for development to the morula or blastocyst stage. Appropriately staged embryos were transferred to recipients and three of

four resulted in the birth of live lambs (Willadsen, 1986). The success in sheep was soon followed by reports from other species, including mice, rabbits, pigs, goats, cattle and nonhuman primates (Di Berardino, 1997a; Di Berardino, 2001). First reports of nuclear transfer offspring derived from early embryonic cells (on or before day 7 of development) are shown in Table 1.1.

Techniques for embryonic cell nuclear transfer were very similar between mammalian species with enucleation usually performed microsurgically after inhibition of microfilaments by cytochalasin B. The nucleus was visualized in darkly pigmented recipient cells by centrifugation of the recipient cell or after staining with Hoechst 33342 and viewing under epifluorescent illumination (Robl et al., 1987; Westhusin et al., 1992). The nucleus was then removed from the oocyte with a finely drawn glass pipette. The blastomere or embryonic cell to be fused to the enucleated oocyte was disaggregated from the embryo by culturing with calcium and magnesium-free medium, aspiration into a pipette or by trypsin treatment. The cell containing the desired nucleus, or karyoplast, was placed in close contact with the enucleated oocyte and exposed to a fusogenic treatment, either inactivated Sendai virus or electrofusion (Illmensee and Hoppe, 1981; Prather et al., 1987; Willadsen, 1986). Embryos were then returned to in vitro culture or transferred to recipient animals at the appropriate stage of the estrous cycle.

After these cloning successes with early embryonic blastomeres (from day 7 or earlier embryos), attempts were made to clone animals from cultured cells. The first report of achieving this goal was in cattle (Sims and First, 1994). The cells used as nuclear donors were derived from the inner cell mass cells of blastocysts that were maintained in culture for up to 28 days. This research was taken further by the birth of sheep from an epithelial cell

Table 1.1. Reports of first nuclear transfer offspring in different mammalian species produced from early embryonic cells (prior to day 7 of development)

Species	Recipient Cell Stage	Donor Cell Stage	Year	Author(s)
Mouse*	1-cell	inner cell mass	1981	Illmensee and Hoppe
Mouse	1-cell	1-cell	1983	McGrath and Solter
Mouse	2-cell	2- and 4-cell	1991	Kono et al.
Sheep	MII oocyte	8-cell	1986	Willadsen
Cow	MII oocyte	9-15-cell	1987	Prather et al.
Cow	1-cell	1-cell	1987	Robl et al.
Rabbit	MII oocyte	8-cell	1988	Stice and Robl
Rat	1-cell	1-cell	1988	Kono et al.
Pig	1-cell	4-cell	1989	Prather et al.
Mouse	MII oocyte	2-, 4- and 8-cell	1993	Cheong et al.
Rhesus monkey	MII oocyte	4-8-cell	1997	Meng et al.
Goat	MII oocyte	4- to 32-cell	1998	Yong et al.

*An unverified report

line that had been in culture up to 13 passages (Campbell et al., 1996). The cells used in this study were derived from the embryonic disc of day 9 sheep embryos and had been maintained in culture until used. Cells were synchronized in the G0 stage of the cell cycle by serum starvation prior to nuclear transfer and it was hypothesized that this method of inducing cell quiescence was important for nuclear reprogramming of the donor nucleus to direct development of a new reconstructed embryo (Campbell et al., 1996).

Somatic Cell Nuclear Transfer

The following year, researchers in the same laboratory utilized the serum starvation method of cell synchronization to clone a sheep from a cell line derived from a mature animal (Wilmut et al., 1997). The cells utilized as nuclear donors in this study were derived from mammary tissue of a 6-year old pregnant ewe. Oocytes at the MII stage were selected as nuclear recipients and were enucleated by inhibiting the cytoskeleton with cytochalasin B, staining the nucleus with Hoechst 33342 and removing the nucleus with a glass pipette under epifluorescent illumination. A single intact cell in G0 was inserted under the perivitelline space of the enucleated oocyte. Fusion of the cell to the oocyte and activation were both accomplished with electrical pulses. Embryos were cultured in either a defined medium or in ligated sheep oviducts. Cloned embryos (n=29) were then transferred surgically to 13 recipients on day 6. This study resulted in the birth of one lamb produced from adult somatic tissue (named “Dolly”), and showed that adult somatic cells could be totipotent (Wilmut et al., 1997).

After this initial report, researchers began trying to produce clones in other species, with success in many (Table 1.2). A variety of cell types including epithelial, fibroblast, cumulus and granulosa cells have been used as nuclear donors (Table 1.2). Sertoli cells and

Table 1.2. Reports of first nuclear transfer offspring in different mammalian species produced from cultured somatic cells

Species	Recipient Cell	Donor Cell Type	Year	Author(s)
Sheep	MII ovine oocyte	adult fibroblast	1997	Wilmut et al.
Cow	MII bovine oocyte	fetal fibroblast	1998	Cibelli et al.
Mouse	MII mouse oocyte	adult cumulus	1998	Wakayama et al.
Enderby Island cattle	MII domestic cow oocyte	adult mural granulosa	1998	Wells et al. (b)
Goat	MII caprine oocyte	fetal fibroblast	1999	Baguisi et al.
Pig	MII porcine oocyte; porcine zygote [†]	adult granulosa; pseudopronuclei [†]	2000	Polejava et al.
Gaur	MII domestic cow oocyte	adult fibroblast	2000	Lanza et al.
Muflon	MII domestic sheep oocyte	adult granulosa	2001	Loi et al.
Rabbit	MII rabbit oocyte	adult cumulus	2002	Chesne et al.
Cat	MII domestic cat oocyte	adult cumulus	2002	Shin et al.
Mule	MII equine oocyte	fetal fibroblast	2003	Woods et al.
Horse	MII equine oocyte	adult fibroblast	2003	Galli et al.
Rat	MII rat oocyte	fetal fibroblast	2003	Zhou et al.
White-tailed deer	deer oocyte	adult fibroblast	2003	Westhusin [‡]
Banteng	MII domestic cow oocyte	fibroblast	2004	Janssen et al.
African wild cat	MII domestic cat oocyte	adult fibroblast	2004	Gomez et al.

[†]Double nuclear transfer procedure was used. First nuclear transfer with adult granulosa cells as nuclear donors and enucleated MII porcine oocytes as nuclear recipients. Second nuclear transfer utilized pseudopronuclei from the first nuclear transfer procedure embryos with enucleated porcine zygotes as nuclear recipients.

[‡]This report has not yet been published in a scientific journal as of this writing.

embryonic stem cells have also been used as nuclear donor cells in mouse nuclear transfer (Ogura et al., 2000; Wakayama et al., 1999). The enucleation and reconstruction methods used in domestic ruminants are reported to be similar to those used by Wilmut and colleagues.

In the mouse and mule, a piezo-impact drive unit has been used as a means to enhance manipulation for nuclear transfer (Wakayama et al., 1998; Woods et al., 2003). Oocyte activation protocols used for nuclear transfer have varied with different species (Galli et al., 2003). Electrostimulation at cell fusion has been sufficient for activation in various species, although in others a calcium-releasing agent must be used, followed by an inhibitor of protein synthesis or kinase activity (Chesne et al., 2002; Cibelli et al., 1998; Galli et al., 2003; Kato et al., 1998; Shin et al., 2002; Wakayama et al., 1998; Wilmut et al., 1997).

CHAPTER II

REVIEW OF LITERATURE

NUCLEAR TRANSFER INEFFICIENCY

Although there have been many accomplishments and advancements in nuclear transfer technologies in various species, there have also been obstacles. Overall efficiency of cloning remains low, with less than 4% of cloned embryos surviving to term across species (Renard et al., 2002). Abnormalities in cloned pregnancies and offspring have been noted regardless of somatic cell type (Kato et al., 2000; Ogura et al., 2002), somatic cell age (Heyman et al., 2002), stage of the donor cell cycle (Campbell et al., 1996; Cibelli et al., 1998), recipient cytoplasm origin (Wells et al., 1997), nuclear transfer technique (Tecirlioglu et al., 2004; Wakayama et al., 1998; Wilmut et al., 1997) or embryo culture method (Wakayama et al., 1999; Wells et al., 1998a). Recloning, or utilizing blastomeres from embryos produced by somatic cell nuclear transfer for an additional nuclear transfer procedure, did not improve embryo survival rates in cattle although it did improve development rates in amphibians (Wells et al., 1999).

Nuclear transfer blastocysts have been found to have lower cell numbers than stage-matched control embryos (Boiani et al., 2003; Chesne et al., 2002; Koo et al., 2002). Abnormal cell allocation has also been noted in bovine nuclear transfer embryos, with a higher inner cell mass:trophectoderm ratio when compared with control embryos (Koo et al., 2002). Chromosomal abnormalities have been reported to be higher in cloned bovine embryos (Booth et al., 2003). However, karyotypic analysis of both normal and aberrant cloned sheep fetuses showed that there was no change in chromosomal number and

demonstrated that karyotypic abnormalities were not responsible for the abnormal fetal development noted in cloned lambs (De Sousa et al., 2001).

Large Offspring Syndrome

During the initial mammalian nuclear transfer experiments utilizing early embryonic cells, abnormal fetuses and placentas were noted, along with increased abortion and perinatal death rates, especially in cattle. Pregnancy rates after the transfer of cloned bovine embryos were lower than those reported from in vivo-derived embryos (Bondioli et al., 1990). Willadsen et al. (1991) reported that 10% of cloned bovine embryo pregnancies were lost between day 35 and 90 of gestation, with additional losses between 6 and 8 months, one of which was due to hydroallantois. In that report, gestation length of cloned pregnancies tended to be longer and only 16% of the births were unassisted with 32% requiring Caesarean sections. Many of the calves were larger than normal, weighing more than 58 kg, which was a contributing factor to calving difficulties. Eleven of the 100 calves delivered in that study died or were euthanized shortly after birth due to congenital abnormalities or calving difficulty.

Another study also reported that cloned calves were more variable and heavier at birth than in vivo-derived embryo transfer and artificial insemination/natural service calves, although this animal variation was not detectable at weaning or yearling stages (Wilson et al., 1995). As this variation was noted even between clones and their unmanipulated embryo transfer full siblings, it was concluded that the variation was probably due to the cloning procedure. Physiological characteristics of newborn cloned calves were monitored in another report by Garry et al. (1996) and the majority of the calves (85%) exhibited metabolic abnormalities with significantly elevated insulin and lower thyroid hormones as compared to

those of a reference population. Subsequent research corroborated these findings of abnormal gestational losses, elevated birth weights and metabolic abnormalities (Garry et al., 1998; Gartner et al., 1998; Kruip and den Daas, 1997).

The aberrations noted in embryonic cell nuclear transfer calves have been similar to pathologies noted in some in vitro produced (IVP) calves and lambs and have been termed Large Offspring Syndrome (LOS) (Young et al., 1998). LOS is characterized by elevated weight at birth, longer gestation, perinatal mortality and increased risk of pregnancy loss throughout gestation. One study reported placental abnormalities in large IVP calves, including 21% fewer cotyledons and changes in cotyledonary shape and size when compared to those of in vivo produced calves (Bertolini et al., 2002).

Some of the factors known to increase the possibility of LOS are in vitro culture of embryos and embryo manipulation, both of which are essential components of nuclear transfer (Young et al., 1998). LOS may be due to perturbations in gene expression after exposure of embryos to suboptimal culture conditions (Lazzari et al., 2002; Niemann and Wrenzycki, 2000; Wrenzycki et al., 2001; Young et al., 2001). Alterations in the maternally expressed *IGF2r* gene were found in sheep fetuses exhibiting LOS (Young et al., 2001). Over-expression of IGF2 in humans as a result of imprinting errors on chromosome 11 has been shown to lead to fetal overgrowth and has been termed Beckwith-Wideman Syndrome (Reik and Maher, 1997). Symptoms of Beckwith-Wideman Syndrome include macrosomia (somatic overgrowth), macroglossia (large tongue), liver and kidney abnormalities, abdominal wall defects and placental overgrowth (Eggenschwiler et al., 1997; Reik and Maher, 1997). Symptoms similar to those of Beckwith-Wideman Syndrome have been noted in mice and can result from loss of function of the *IGF2r* and *H19* genes (Eggenschwiler et al., 1997; Lau et

al., 1994; Leighton et al., 1995; Wang et al., 1994). These symptoms also appear to be very similar to the abnormalities seen in cattle and sheep with LOS.

Not surprising, LOS characteristics have also been noted in fetuses and offspring produced by somatic cell nuclear transfer. The classic LOS pathologies, as well as placental and internal organ abnormalities, have been noted in cattle and sheep pregnancies generated from cultured embryonic and fetal cell lines (Campbell et al., 1996; Schnieke et al., 1997; Stice et al., 1996; Wells et al., 1997). Abnormalities of cloned animals differ between species with some showing all of those previously detailed and others only describing decreased embryo survival from transfer to pregnancy determination. Somatic cell nuclear transfer offspring abnormalities have now been well documented in sheep, cattle and mice.

Abnormalities in Cloned Sheep

Sheep generated by somatic cell nuclear transfer have been reported to have high rates of pregnancy loss both early (prior to day 40) and late in gestation (after day 100) (Wells et al., 1998a). Prolonged gestation with little maternal evidence of impending parturition was noted in other studies (Schnieke et al., 1997; Wells et al., 1997). At birth, a high rate of perinatal mortality has been noted, generally from respiratory failure soon after birth (Campbell et al., 1996; Loi et al., 2002; Wells et al., 1997; Wilmut et al., 1997). Some cloned fetuses have exhibited organ abnormalities of the liver (De Sousa et al., 2001; Wilmut et al., 1997) and kidney (Loi et al., 2002; Wells et al., 1997; Wells et al., 1998a). A single report noted necrosing placental tissue present at the birth of a surviving cloned lamb (Wells et al., 1997). Abnormal placental development at day 35 of gestation, including reduced number of cotyledonary structures and reduced vascularization, was correlated with developmentally retarded fetuses and organ abnormalities in one study (De Sousa et al., 2001).

Abnormalities in Cloned Cattle

The abnormalities noted in cloned sheep have also been observed in bovine somatic cell nuclear transfer pregnancies and offspring. Early gestational losses, prior to day 90, have been reported to be as high as 82% (Hill et al., 2000). Late gestational losses (after day 90 and continuing until parturition) have been described in a multitude of studies (Cibelli et al., 1998; Heyman et al., 2002; Kato et al., 2000; Wells et al., 1999). Parturition signaling has been reported to be aberrant at term with cows showing little mammary gland development or signs of impending parturition (Kato et al., 1998). Longer than normal gestation has been noted, perhaps due to improper signaling of parturition, prompting many researchers to utilize Caesarean sections to recover the calves (Cibelli et al., 1998; Hill et al., 1999; Wells et al., 1999). Fetal oversize has commonly been reported in cloned bovine offspring surviving to term (Chavatte-Palmer et al., 2002; Heyman et al., 2002; Kato et al., 2000).

Placental anomalies have been described by many groups with some of the more common pathologies being placental edema, hydroallantois, decreased vascularization, decreased number of cotyledons and binucleate cells, larger umbilical cord diameter and increased cotyledonary size and cell numbers (Batchelder et al., 2005; Chavatte-Palmer et al., 2002; Cibelli et al., 1998; Hashizume et al., 2002; Heyman et al., 2002; Hill et al., 1999; Hill et al., 2000; Hill et al., 2001; Ravelich et al., 2004a; Stice et al., 1996; Wells et al., 1999; Zakhartchenko et al., 1999). Abortion throughout gestation and perinatal death of cloned calves has been attributed to placental abnormalities noted after nuclear transfer, although a live calf was delivered and survived despite placental malformation reported in one case study (Hill et al., 2001).

Cloned calves have a high rate of perinatal death (Heyman et al., 2002). Some of the deaths may have been due to fetal oversize and distress during calving, when it occurred naturally prior to Caesarean section (Kato et al., 1998; Kubota et al., 2000; Taneja et al., 2001). Cardiopulmonary malfunction has been reported in nearly all studies with somatic cell nuclear transfer calves (Cibelli et al., 1998; Hill et al., 1999; Hill et al., 2000; Li et al., 2005; Taneja et al., 2001). The cardiopulmonary pathologies have been reported as cardiac hypertrophy, immature lungs, elevated venous pressure, pulmonary hypertension and larger than normal pulmonary arteries (Cibelli et al., 1998; Hill et al., 1999; Li et al., 2005; Taneja et al., 2001). Liver and kidney abnormalities in cloned calves have been reported by various researchers (Chavatte-Palmer et al., 2002; Chavatte-Palmer et al., 2004; Kato et al., 2000; Li et al., 2005; Williamson et al., 2005).

The immune system of cloned cattle may be compromised, as one study noted lymphoid hypoplasia (Renard et al., 1999) and other studies have reported deaths in cloned offspring from systemic infections (Kato et al., 2000; Taneja et al., 2001). The endocrinology of newborn somatic cell nuclear transfer calves has also been described as abnormal with cloned calves having significantly lower plasma thyroxine concentrations and significantly higher plasma insulin-like growth factor II (IGF-II) and leptin concentrations (Chavatte-Palmer et al., 2002). It has been suggested that cloned calves cannot be considered healthy and normal until they are at least 60 days of age (Chavatte-Palmer et al., 2004).

Abnormalities in Cloned Mice

Mice cloned from somatic cells (tail-tip, cumulus and Sertoli cells) have been shown to have abnormalities. As in other species, it was found that the rate of embryonic loss in mice was very high after implantation but before term development with cloned fetuses

(Ogura et al., 2000; Ogura et al., 2002; Wakayama et al., 1998; Wakayama et al., 2000; Wakayama and Yanagimachi, 1999). Respiratory failure, retarded development and umbilical hernias in some cases were cited as the cause of death (Ogura et al., 2000; Ogura et al., 2002; Wakayama et al., 1998; Wakayama and Yanagimachi, 1999).

Enlarged placentas were also noted in these studies, with hyperplasia of the basal layer, including the spongiotrophoblast, as the primary cause (Ogura et al., 2002; Tanaka et al., 2001b). Cloned mice have presented other abnormalities in postnatal development. Cloned mice were found to have delayed eye opening, ear twitching and negative geotaxis after neurobehavior was assessed (Tamashiro et al., 2000). Cloned mice were also heavier than control animals after 8 to 10 weeks of age and were determined to be obese as these animals had increased amounts of adipose tissue, were hyperinsulinemic and hyperleptinemic. The tendency to obesity was noted primarily in animals cloned from specific strains with the agouti gene (Tamashiro et al., 2002; Tamashiro et al., 2000). It has been reported that somatic cell mouse clones die earlier than control animals with both liver malfunction and severe pneumonia cited as causes of death (Ogura et al., 2002). This has been attributed to immune system impairment that resulted in decreased antibody production in cloned somatic cell mice (Ogonuki et al., 2002).

NUCLEAR REPROGRAMMING

The chromatin structure and transcriptional activity of donor nuclei must revert to that of an early one-cell embryo after nuclear transfer in order to direct embryonic development. This process is termed nuclear reprogramming and involves both morphological and biochemical changes (Di Berardino, 1997b; Di Berardino, 2001; Kikyo and Wolffe, 2000; Sun and Moor, 1995). The process of nuclear reprogramming is thought to be similar to that

of paternal chromatin remodeling after fertilization (McLay and Clarke, 2003). In mammals, nuclear remodeling after nuclear transfer has been recorded as chromatin condensation, nuclear envelope breakdown and the formation of a pronuclear-like structure that undergoes swelling (Adenot et al., 1997; Collas and Robl, 1991; Czolowska et al., 1984; Prather et al., 1990; Stice and Robl, 1988; Szollosi et al., 1988).

The fine structure of the nucleolus, viewed with electron microscopy, has been reported to change after nuclear transfer, with structure of the transferred nucleus becoming more like that of early stage embryos (Baran et al., 2002; Hyttel et al., 2001; Kanka et al., 1991). TATA binding protein has been found to be released from chromatin after nuclear transfer in mice (Kim et al., 2002). Transcription from the donor nucleus ceased after nuclear transfer during nuclear remodeling and then reappeared later at genomic activation (Kanka et al., 1991; Kim et al., 2002). Protein and lamin expression have also been reported to change after nuclear transfer in mammalian studies (Kubiak et al., 1991; Prather et al., 1989; Prather et al., 1991; Prather and Rideout, 1992). In bovine nuclear transfer embryos, immunoreactive somatic histone H1 has been reported to disappear after nuclear transfer, only to reappear at the 8- to 16- cell stage, the time of genomic activation in bovine embryos (Bordignon et al., 1999). These studies all indicate that extensive chromatin remodeling takes place during nuclear reprogramming.

EPIGENETIC REPROGRAMMING

Nuclear reprogramming must occur for the nucleus to direct embryonic growth after nuclear transfer, and it is thought that inadequate reprogramming of the donor nucleus is the primary reason for the failure of cloned embryos to survive. Researchers have suggested that epigenetic changes in the donor cell genome are responsible for the abnormalities found in

cloned fetuses and offspring (Jaenisch et al., 2002). Epigenetics has been broadly described as the heritable differences in gene function and expression that result in phenotypic differences but cannot be explained by the DNA sequence itself (Smith and Murphy, 2004; Wolffe and Matzke, 1999; Wu and Morris, 2001).

Development may be affected by changes in epigenetic processes leading to differences in gene expression (Smith and Murphy, 2004). Histone modification and DNA methylation are two of the main routes for epigenetic alterations of the genome (Smith and Murphy, 2004). Epigenetic changes affect critical developmental processes, such as genomic imprinting, X-chromosome inactivation and gene expression. It is thought that cloned embryos produced by nuclear transfer must be able to epigenetically reprogram a somatic nucleus in an effort to restart embryonic growth. Evidence suggests that this is not a consistent event, with separate research groups describing different epigenetic changes within and among groups of embryos.

DNA Methylation in Cloned Animals

In cloned mice, individual clones showed differing aberrant methylation patterns of CpG islands in placental, skin and kidney tissues (Ohgane et al., 2001). Elevated methylation levels were found in cloned bovine embryos (Dean et al., 2001; Santos et al., 2003). One of these studies reported an initial demethylation of the donor nucleus immediately following transfer but no further demethylation during early embryogenesis (Dean et al., 2001). This was followed by precocious de novo methylation at the 8-cell stage (Dean et al., 2001). Different findings have been reported by other groups.

One study found demethylated euchromatin and hypermethylated heterochromatin in cloned bovine embryos (Bourc'his et al., 2001). Another reported global hypomethylation of

the genome after nuclear transfer (Cezar et al., 2003). Two reports from one group described hypermethylation of repeated satellite sequences in bovine cloned embryos when compared with in vitro produced, as well as demethylation of single copy sequences in both cloned embryos and in vitro produced embryos (Kang et al., 2001a; Kang et al., 2002). Sequences related to the control of the imprinted genes *H19* and *Snrpn* have been found to be demethylated in cloned mouse embryos (Mann et al., 2003a). Aberrant methylation patterns, generally hypermethylation, in the trophoctoderm of cloned bovine embryos have also been reported by two groups (Kang et al., 2002; Santos et al., 2003).

Several studies report that DNA methylation of cloned embryos is similar to that of the somatic cells from which they were derived (Bourc'his et al., 2001; Dean et al., 2001; Santos et al., 2003). An inhibitor of DNA methylation was used to treat donor cells prior to nuclear transfer in one study but the treatment did not improve developmental rates (Enright et al., 2003b). Two groups have reported abnormal DNA methyltransferase expression in cloned mice and cattle (Chung et al., 2003; Wrenzycki et al., 2001). These reports in mice and cattle all lead to the same conclusion that abnormal DNA methylation occurs in many cloned embryos. In contrast, when the methylation status of repeating elements of cloned porcine embryos were examined, no differences were detected from the patterns of in vitro fertilized embryos, demonstrating that different species could be more competent in genomic reprogramming than others (Kang et al., 2001b). Adult cloned animals were generally considered to be normal when analyzed for epigenetic changes, suggesting that perhaps only the most epigenetically normal embryos survive to adulthood (Cezar et al., 2003; Ohgane et al., 2001).

X-Chromosome Inactivation in Cloned Animals

X-linked inactivation in clones is one method to examine epigenetic reprogramming (Xue et al., 2002). X-linked inactivation has been reported to be functionally normal in cloned mice with random inactivation in embryonic tissues and the previously inactive X-chromosome in the nuclear donor chosen for inactivation in the extraembryonic tissues (Eggan et al., 2000). In cattle, X-chromosome inactivation was found to be abnormal (Xue et al., 2002). That report cited random X-inactivation in the placental tissues of nonviable cloned calves and abnormal methylation and expression patterns of X-linked genes. These results are in agreement with those of other studies that found altered expression of X-linked genes in bovine NT embryos (Li et al., 2005; Wrenzycki et al., 2002).

In contrast, surviving cloned calves were found to have normal X-linked gene expression patterns (Xue et al., 2002). A recent study of cloned mice reported a skewed X-inactivation ratio from the normal of 1:1 (Senda et al., 2004). The authors speculate that this was due to selection against cells in which the previously inactive X-chromosome from the somatic cell was not sufficiently activated, leading to impairment of gene function and cell death in some tissues (Senda et al., 2004).

Histone Modification in Cloned Animals

Just as X-chromosome dosage compensation can be impaired in cloned offspring, histone modification patterns may be abnormal in cloned embryos. The methylation of the ninth lysine residue on histone H3 has been shown to correlate with the pattern of DNA methylation in cloned bovine embryos and was been found to be abnormally elevated in many of the embryos (Santos et al., 2003). Histone acetylation levels have been altered in nuclear transfer donor cells after in vitro culture (Enright et al., 2003a). Trichostatin A, an inhibitor

of histone deacetylation (Yoshida et al., 1990), was utilized to treat bovine nuclear transfer donor cells and resulted in a higher developmental rate to the blastocyst stage (Enright et al., 2003b).

Gene Expression in Cloned Animals

As epigenetic mechanisms such as DNA methylation, X-chromosome inactivation and histone modification have been shown to be abnormal in cloned offspring, it is not surprising that gene expression patterns affected by epigenetic processes may not be correctly reestablished after nuclear transfer. Various studies have proposed aberrant gene expression in cloned embryos. Different NT protocols, including the method of nuclear delivery and activation times, have been indicated in changes in gene expression patterns in NT embryos (Daniels et al., 2001; Wrenzycki et al., 2001). Donor cell factors, including the specific cell line, the number of passages, cell cycle stage and cell culture media, have been linked to changes in gene expression (Baqir and Smith, 2003; Daniels et al., 2001; Humpherys et al., 2002; Wrenzycki et al., 2001). Gene expression in the early embryo (at or before the blastocyst stage) has been found to be altered as a result of in vitro culture (Blondin et al., 2000; Doherty et al., 2000; Khosla et al., 2001; Lazzari et al., 2002; Stojanov and O'Neill, 2001; Young et al., 2001).

Different groups of genes have been found to be abnormal in cloned embryos and fetuses from mice and cattle. The transcription factor *Oct4* was found to be incorrectly expressed in cloned mouse embryos, but not in cloned bovine embryos (Boiani et al., 2002; Daniels et al., 2000). Growth factors, including various fibroblast growth factors, members of the insulin and insulin-like growth factor family and their receptors, vascular endothelial growth factor and bone morphogenetic protein-4, have been reported to be abnormally

expressed after nuclear transfer in bovine embryos and tissues (Daniels et al., 2000; Daniels et al., 2001; Inoue et al., 2002; Li et al., 2005; Ravelich et al., 2004a). In one study of imprinted gene expression in cloned mice, 96% of the embryos did not correctly express one or more of the genes *H19*, *Meg3*, *IGF2r*, *Ascl2* and *Snrpn* at the blastocyst stage (Mann et al., 2003a). Another report of cloned mouse embryos found reduced expression of the imprinted genes *Meg1/Grb10* and *Peg1/Mest* (Inoue et al., 2002). In cloned bovine embryos, abnormal expression of the imprinted genes *Mash2* and *H19* were reported, although *IGF2r* expression was found to be normal (Wrenzycki et al., 2001; Zhang et al., 2004). Metabolic gene expression has been studied in cloned bovine embryos with the enzymes phosphoribosyl pyrophosphate synthetase 1, mitochondrial acetoacetyl-coenzyme A thiolase and α -glucosidase expressed at different levels than in vitro produced embryos (Pfister-Genskow et al., 2005). In contrast, another study of metabolic gene expression reported maintenance of normal expression of lactate dehydrogenase, citrate synthase and phosphofructokinase in cloned bovine embryos (Winger et al., 2000). Genes involved in the structural development of bovine embryos including cytokeratins, vimentin and nidogen 2 were found to be dysregulated in cloned bovine embryos (Pfister-Genskow et al., 2005). The stress-associated heat shock proteins have been reported by several groups to be underexpressed or not expressed at all in nuclear transfer derived embryos and tissues (Li et al., 2005; Pfister-Genskow et al., 2005; Wrenzycki et al., 2001). Interleukin-6 expression has also been found to be abnormal in cloned bovine embryos (Daniels et al., 2000).

Placental gene expression abnormalities have also been noted in cloned mouse and bovine embryos and fetuses. Abnormal expression of over 200 genes was noted in cloned mouse placentas (Humpherys et al., 2002). Elevated interferon tau expression was detected in

one study from bovine cloned embryos produced with less than optimal nuclear transfer techniques (Wrenzycki et al., 2001). Major histocompatibility complex I expression has been detected abnormally early in cloned bovine embryos and fetuses and has indicated immunologic rejection by the dam as a possible cause of early embryonic death (Hill et al., 2002; Pfister-Genskow et al., 2005). Insulin-like growth factor binding proteins 2 and 3 showed increased expression in the extraembryonic membranes of bovine nuclear transfer fetuses (Ravelich et al., 2004a).

Placental lactogen and pregnancy-associated glycoprotein levels were lower and heparanase levels were elevated in placental tissues derived from cloned bovine pregnancies (Hashizume et al., 2002). In contrast to the previous study, placental lactogen concentrations and leptin expression were found to be elevated in cloned bovine placental tissues (Ravelich et al., 2004b). These studies have indicated widespread abnormalities in gene expression from both fetal and placental tissues of cloned offspring.

EMBRYONIC STEM CELL CLONING

Various researchers have noted the similarities of nuclear transfer derived embryos to the donor cells they were derived from. This includes DNA methylation patterns, gene expression patterns and culture condition preferences (Chung et al., 2003; Gao et al., 2003; Mann et al., 2003a; Santos et al., 2003). These factors indicate incomplete reprogramming of the donor cell nucleus to function as an embryo. It has been suggested that greater cloning success would be possible from embryonic stem (ES) cells in species from which they were derived, such as in the mouse. It was found through the use of ES cells for cloning in mice that while fewer blastocysts were produced, more embryos survived to term and after birth (Eggan et al., 2001; Rideout et al., 2000; Wakayama et al., 1999).

Interestingly, ES cells have been described as epigenetically unstable, resulting in widely different DNA methylation patterns and gene expression levels (Humpherys et al., 2001; Humpherys et al., 2002). The expression of the imprinted genes *H19*, *IGF2*, *Peg1/Mest* and *Meg1/Grb10* were found to be abnormal in ES cell cloned mice produced by nuclear transfer (Humpherys et al., 2001). A subsequent study found over 200 abnormalities of gene expression in the placentas of cloned ES cell mice, although the number of genes with reportedly abnormal expression was lower than for cumulus cell clones (Humpherys et al., 2002). ES cell nuclear transfer-derived clones do show some characteristics of other nuclear transfer-derived clones from somatic cell lines in that fetal and placental overgrowth is common (Eggan et al., 2001; Humpherys et al., 2001; Rideout et al., 2000; Wakayama et al., 1999).

Although ES cells have been used for generating cloned animals via nuclear transfer, another method has also been used to produce ES cell clones. This method has been termed tetraploid embryo complementation and consists of combining ES cells with tetraploid embryos to produce a chimeric embryo (Eggan et al., 2001). These chimeric embryos have generated completely ES cell-derived mice, with tetraploid cells confined to the placental tissues (Eggan et al., 2001).

TETRAPLOID EMBRYO PRODUCTION METHODS

Tetraploid embryos were first experimentally produced to study the effects of polyploidy on embryonic development. Most of the initial work was conducted in mice, although influential studies have been conducted in rabbits. Tetraploid embryos have been produced in both rabbits and mice after heat shock treatment (Beatty and Fischberg, 1952; Pincus and Waddington, 1939). Rabbit embryos exposed to elevated temperatures (45 to

47.5°C) resulted in two tetraploid embryos of the five that could be analyzed (Pincus and Waddington, 1939). It was found that elevating the oviductal temperature to 45.5°C in mice with embryos nearing the time of the first cleavage resulted in 23% tetraploidy in the embryos recovered (Beatty and Fischberg, 1952).

Colchicine treatment to inhibit cell division was also utilized in early tetraploid experiments (Edwards, 1958; Pincus and Waddington, 1939). Rabbit embryos were exposed to various concentrations of colchicine for 19 to 21 hours following mating, which resulted in 56% tetraploid embryos. Female mice injected with colchicine either into the uterus or the ovarian capsule after mating yielded five tetraploid embryos from a total of 139 embryos examined (Edwards, 1958). This treatment also produced other types of polyploidy in this study, including triploids and mosaic embryos with a diploid ↔ tetraploid (2n ↔ 4n) makeup.

Micromanipulation has been utilized to produce tetraploid mouse embryos. Nuclei from blastomeres at the morula stage were transferred to 1-cell zygotes with low survival rates of less than 10% (Modlinski, 1978). Of the embryos that survived and cleaved following in vivo culture, four of the six that could be analyzed were determined to be tetraploid. A subsequent study found similar results when inner cell mass cell nuclei were used as nuclear donors, which resulted in six of 16 surviving embryos determined tetraploid (Modlinski, 1981).

Treatment of embryos with the cytoskeletal inhibitor cytochalasin B has been utilized to prevent cytokinesis but allow nuclear replication and therefore, generate tetraploid embryos. This method of tetraploid embryo production was first described using 2-cell mouse embryos (Snow, 1973). Embryos were placed in 10 µg/ml cytochalasin for ~12 hours and then observed for nuclear events. The nuclei of cytochalasin inhibited embryos were

reported to divide normally although cytokinesis did not occur and the resulting 2-cell embryo became binucleate. After removal from cytochalasin, the two nuclei migrated to the center of each cell and formed a single metaphase plate, and therefore a tetraploid embryo, with a single 4n nucleus enclosed in a single nuclear membrane per cell, in ~60% of the treated embryos (Snow, 1973). Some embryos were described as fragmenting quickly following removal from cytochalasin and those embryos were found to be diploid. Another study using cytochalasin to inhibit cleavage for 3 to 8.5 hours reported 52% of 2-cell embryos treated became tetraploid while 27% became 2n ↔ 4n mosaic embryos (Tarkowski et al., 1977). One report described treatment of pronuclear stage embryos with 5 µg/ml cytochalasin for 16 hours to produce tetraploid embryos although no chromosomal analysis of embryos treated with this method was conducted (Ueda et al., 1995)

Cell fusion has also been shown to be an effective method of tetraploid embryo production. Inactivated Sendai virus has been used to fuse cells to form tetraploid embryos. In mice, zona-free zygotes or separated blastomeres from 2-cell embryos were placed together in a Petri dish containing a Sendai virus preparation and were found to fuse within 6 hours of exposure to the virus (Graham, 1971). In a separate study, micromanipulation was utilized to inject Sendai virus between the blastomeres of a 2-cell mouse embryo resulting in a 21% fusion rate (O'Neill et al., 1990). Sendai virus-mediated cell fusion resulted in uniform tetraploidy in both of these studies.

Polyethylene glycol is another method of cell fusion that has been utilized to generate tetraploid embryos. In one study, 4-cell embryos were disaggregated after zona pellucida removal and were then attached together in pairs with phytohemagglutinin (Eglitis, 1980). Cell fusion was accomplished after the cells were incubated for 2 minutes in 45%

polyethylene glycol. This treatment resulted in fusion of 50% of the exposed blastomeres. Another study used a 1 minute exposure of 40% polyethylene glycol to fuse 82% of the zona pellucida-enclosed 2-cell embryos exposed to the treatment and demonstrated that the presence of the zona pellucida resulted in higher fusion rates (Spindle, 1981). Polyethylene glycol-mediated cell fusion resulted in uniform tetraploidy in both of these studies.

Treatments designed to induce tetraploidy by chemical methods have resulted in toxic effects on the embryos. Cytochalasin has been prepared in dimethyl sulphoxide for use in generating tetraploid embryos and control embryos exposed to a similar concentration of dimethyl sulphoxide had decreased cell numbers at the morula and blastocyst stage (Tarkowski et al., 1977). In cell fusion studies, toxicity of polyethylene glycol has been mentioned as a possible cause of decreased developmental rates from nonaggregated tetraploid blastomeres (Eglitis, 1980). Removal of the zona pellucida and exposure to inactivated Sendai virus has been found to be detrimental to mouse embryo development (Graham, 1971). Virus treatment of 2-cell mouse embryos has also been shown to decrease embryo survivability by the shortened crown-rump length and retarded development of diploid fetuses from treated embryos that did not fuse (O'Neill et al., 1990).

Tetraploid Embryo Production by Electrofusion

Electrofusion has been described as a less toxic method of cell fusion as it is characterized by a very short exposure time and can be performed without the removal of the zona pellucida (Bates et al., 1987; Kubiak and Tarkowski, 1985; McLaughlin, 1993). Electrofusion has also been recommended for cell fusion as it is less technically difficult, yields a high fusion rate, and is measurable and repeatable between replicates (Bates et al., 1987; Kubiak and Tarkowski, 1985; McLaughlin, 1993). Electrofusion utilizing a direct

current pulse has been shown to connect two cells by opening pores in the membranes between the two cells. The pores quickly close together and cytoplasmic bridges between the two cells widen until the individual cells fuse to form a single cell (Bates et al., 1987; McLaughlin, 1993).

Correct embryo orientation was essential for successful fusion between the two cells. It was found that cells must be aligned so that the plane of contact between the two cells was perpendicular to an imaginary line drawn between the electrodes (Kubiak and Tarkowski, 1985; McLaughlin, 1993). Embryos have been aligned for electrofusion either by precise placement with a pipette or by use of an alternating current field that polarizes the cell and rotates it to the proper orientation for electrofusion (McLaughlin, 1993). Electrofusion has been shown to occur in both electrolyte (phosphate buffered saline) and nonelectrolyte (0.3 M mannitol) solutions (Kubiak and Tarkowski, 1985). When an alternating current field has been used to align embryos, a nonelectrolyte solution such as mannitol has been used for electrofusion (Kubiak and Tarkowski, 1985; McLaughlin, 1993).

Electrofusion of mammalian embryonic blastomeres was first described in mouse oocytes and embryos (Berg, 1982). Fusion was accomplished by direct current pulses of 1 to 4 kV/cm for 1 to 5 minutes. That study reported the formation of embryos with half of the number of cells previously present after electrofusion (i.e., one cell from two, two cells from four, etc.) but no further embryonic development was reported. A separate group later examined parameters of electrofusion for 2-cell mouse embryos and found the highest rates of cell fusion occurred at 1 kV/cm with two pulses of 100 to 250 μ seconds (Kubiak and Tarkowski, 1985). The 11 fused embryos examined in that study were found to be tetraploid.

That report was followed in the next year by a description of electrofusion of 2-cell rabbit embryos (Ozil and Modlinski, 1986). Those researchers reported fusion rates of 85 to 90% after pulses of 1 to 3 kV/cm for 35 to 1000 μ seconds. Those researchers found that electrofusion of rabbit embryos resulted in diploid, $2n \leftrightarrow 4n$ mosaic and tetraploid embryos. After microscopic examination following electrofusion, they concluded that inappropriate cleavage occurred in up to 18% of the fused embryos. In those embryos, the two nuclei from each cell were unable to form a common metaphase plate and could not become tetraploid. Those researchers suggested selection of embryos that had cleaved after fusion into two equally-sized blastomeres to yield tetraploid embryos. However, even after the selection of evenly cleaved embryos post-fusion, a $2n \leftrightarrow 4n$ mosaic fetus was recovered (Ozil and Modlinski, 1986).

Research in tetraploid mouse development after electrofusion continued with a group located in Edinburgh (Henery and Kaufman, 1991; Henery and Kaufman, 1992; Kaufman and Webb, 1990). Researchers in this group exposed 2-cell embryos to a pulse of 3.3 kV/cm for 50 μ seconds to produce tetraploid embryos. Fusion efficiency was not reported in those studies but the authors stated that “a very high proportion of cases” fused after treatment. All studies from this group reported uniform tetraploidy in fused embryos after this electrofusion treatment (Henery and Kaufman, 1991; Henery et al., 1992; Henery and Kaufman, 1992; James et al., 1992; Kaufman and Webb, 1990).

Another group produced tetraploid embryos by fusing 2-cell embryos with a single, 100 μ second direct current pulse of 1 kV/cm (Nagy et al., 1990). They reported a 98% fusion rate from exposed embryos. A later report from the same group utilized a 3.6 kV/cm, 100 μ second direct current pulse to fuse 2-cell mouse embryos (Nagy et al., 1993). Other

researchers have utilized two 30 μ second direct current pulses of 4 kV/cm to fuse 2-cell mouse embryos (Sekirina et al., 1997). They found that the cell cycle stage of the embryo had an effect on fusion, where mid-stage embryos fused 98% of the time, which corresponded to only 24 and 31% fusion at the early and late 2-cell stages. From these results, a wide range of field strengths (1 to 4 kV/cm) and pulse durations (30 to 100 μ s) have been used successfully to electrofuse mouse embryos.

Determination of Tetraploidy

Determination of the tetraploid status of embryos has been accomplished through various methods. Many groups have used karyotypic analysis to determine the chromosomal complement of tetraploid embryos (Eglitis, 1980; Graham, 1971; Kaufman and Webb, 1990; Koizumi and Fukuta, 1995; Kubiak and Tarkowski, 1985; Modlinski, 1978; O'Neill et al., 1990; Ozil and Modlinski, 1986; Snow, 1973; Spindle, 1981; Tarkowski et al., 1977). Karyotypic analysis has been criticized as underestimating the incidence of mosaic embryos due to the few spreads available for examination after slide preparation (Tarkowski et al., 1977). Fielgen staining and microdensitometry has been utilized to determine the amount of DNA present in the nucleus of embryos exposed to tetraploidy-inducing treatments (Graham, 1971). Light microscopy has also been used to monitor the fusion of nuclei after cytochalasin treatment or cell fusion treatments (Eglitis, 1980; Ozil and Modlinski, 1986; Sekirina et al., 1997; Snow, 1973). Presumptive tetraploid fetuses from transgenic mice generated through electrofusion at the 2-cell stage have been sectioned and examined with in situ hybridization to determine if the fetuses were in fact tetraploid (James et al., 1992). This study concluded that the fetuses were uniformly tetraploid as there was not a significant increase in the proportion of nuclei with only one transgenic signal in the tetraploid hemizygous tissues,

when compared with that of diploid homozygous tissues. However, the researchers acknowledged that the presence of at least 15% diploid nuclei would need to be found in the electrofused fetuses to detect mosaicism (James et al., 1992).

Tetraploid Embryonic Development

In mice, blastocyst rates of tetraploid embryos have been reported to range from 40 to 70% of the initial 2-cell embryos after cytochalasin treatment (Snow, 1973). Development rates to the blastocyst stage of 80% were noted with mouse embryos that did not cleave during cytochalasin treatment in a later study (Tarkowski et al., 1977). In the tetraploid embryos created by cell fusion with polyethylene glycol, cleaved tetraploid cells were either left single or aggregated with one additional fused cell or three additional fused cells to compare developmental rates of electrofused embryos with differing cell numbers with those of normal diploid control embryos (Eglitis, 1980). Blastocyst rates were lowest for single tetraploid cells at 32%, higher for two tetraploid cells aggregated together at 46% and highest for four tetraploid cells aggregated together at 73% (Eglitis, 1980). Another study described the formation of blastocysts in 80% of tetraploid embryos generated through polyethylene glycol-mediated cell fusion (Spindle, 1981). Blastocyst rates in tetraploid embryos from cell fusion utilizing inactivated Sendai virus were reported as 19% for fused 1-cell embryos, 68% for fused blastomeres of 2-cell embryos and 100% for fused blastomeres of 2-cell embryos aggregated in pairs (Graham, 1971). In a study using mouse embryo electrofusion, fused embryos were reported to reach the blastocyst stage although no rates of development were given (Kubiak and Tarkowski, 1985). Ozil and Modlinski (1986) reported that all rabbit embryos successfully electrofused at the 2-cell stage developed to the morula stage and that 67 to 84% reached the expanded blastocyst stage of development.

Tetraploid blastocysts produced from electrofused 2-cell mouse embryos have been reported to be of a similar size when compared to diploid blastocysts (Koizumi and Fukuta, 1995). The total number of cells in tetraploid mouse embryos has been shown to be reduced by more than half and the cell size was larger (Henery et al., 1992; Henery and Kaufman, 1992; Koizumi and Fukuta, 1995; Kubiak and Tarkowski, 1985; Snow, 1973; Spindle, 1981; Tarkowski et al., 1977). Tetraploid cell nuclear volume has also been reported as larger than that of diploid cells (Henery et al., 1992; Henery and Kaufman, 1992). Cell numbers and total area of the inner cell mass have been reported to be reduced in tetraploid embryos (Ozil and Modlinski, 1986; Spindle, 1981).

It was initially reported that there was no difference in the length of the cell cycle between diploid and tetraploid mouse embryos (Snow, 1973). Subsequent studies have found differences in cell cycle length between these two groups. Koizumi and Fukuta (1995) discovered a 2 hour increase in the cell cycle length of tetraploid embryos. This study also established that compaction and blastocoel formation occurred at the same time in diploid and tetraploid embryos, regardless of differences in cell number and cell cycle length (Koizumi and Fukuta, 1995). Mouse embryos produced by electrofusion also compacted at the same time as control embryos although they had fewer cells per embryo (Kubiak and Tarkowski, 1985).

In contrast, a delay in blastocyst formation of 7 hours was reported for tetraploid embryos produced through polyethylene glycol-mediated cell fusion but was attributed to the detrimental effects of zona pellucida removal (Eglitis and Wiley, 1981). The same study also noted the correct timing of expression of a stage-specific cell surface antigen in tetraploid embryos. A later study of tetraploid mouse embryos produced by electrofusion found no

difference in the cell doubling times of tetraploid embryos when compared with control embryos that had been collected, held in vitro and then transferred to recipient females (Henery and Kaufman, 1991). Both groups had significantly longer cell doubling times than did control embryos that remained in vivo during the experimental period. This study concluded that when experimental conditions were appropriately matched, tetraploid embryos did not develop at a slower rate than diploid embryos and that compaction and blastocoel formation occurred at the same time (Henery and Kaufman, 1991).

The sex chromosome ratio of tetraploid mouse embryos experimentally induced by cell fusion did not differ from a 1:1 ratio of XXYY to XXXX (O'Neill et al., 1990). A separate study examined X-chromosome inactivation in tetraploid mouse embryos (Webb et al., 1992). Two of the X chromosomes were found to be inactivated in XXXX embryos and no inactivation occurred in XXYY embryos. Inactivation was random in the embryonic and mesodermal tissue and paternal inactivation was preferred in endodermal tissue (Webb et al., 1992). The activity of malate dehydrogenase and RNA synthesis was examined in tetraploid mouse embryos generated through polyethylene glycol-mediated cell fusion and it was found that these parameters did not increase by a factor of two as the genetic material per cell increased, indicating that some dosage compensation was occurring in the tetraploid embryos (Eglitis and Wiley, 1981).

Tetraploid Fetal Development

The in vivo development of tetraploid embryos has been examined by various groups working with tetraploids generated through various methods. In cytochalasin-produced mouse tetraploids, most embryos have been found to not develop past day 8 of gestation with no organogenesis (Snow, 1973). One study reported three live offspring at term that were

eaten after birth (Snow, 1973), although no other researcher has been able to reproduce this work. Subsequent work by the same researcher reported that tetraploid embryos produced with cytochalasin lacked inner cell mass cells in 89% of the implantation sites between days 4.5 and 6.5 of gestation (Snow, 1976). A subsequent study reported a 32% implantation rate after transfer of tetraploid mouse embryos with evidence of placental tissue (trophoblast giant cells) but very little fetal tissue (Snow, 1975). Embryos that were present were described as normal up to day 13.5 of gestation but did weigh less than diploid controls although placental weights were not affected. The remaining fetuses present at day 14.5 and day 16.5 were described as abnormal with hemorrhagic areas in the lungs, presumably from difficulties in circulating larger than normal blood cells. Abnormal gonads and brain tissue were also found in the fetuses examined.

Another group reported implantation rates of over 50% from tetraploid embryos produced by cytochalasin treatment (Tarkowski et al., 1977). They concluded that embryonic development of tetraploid embryos was abnormal starting at day 8 of gestation. The primary abnormalities noted in that study were delayed embryonic development with very little mesoderm produced. No viable fetuses were obtained after day 11 of gestation in this study.

After cell fusion with inactivated Sendai virus, an implantation rate of 31% was reported in mice but only trophoblastic tissue was present in these implantation sites at 10 days of gestation (Graham, 1971). Another group recorded an implantation rate of 88% with one-half of the implantation sites containing mouse fetuses on day 10 of gestation (O'Neill et al., 1990). All of the recovered fetuses in that study were small for their age and delayed in their development. Placental membranes were not found to be abnormal with these fetuses (O'Neill et al., 1990).

Mouse tetraploid embryos produced by electrofusion were transferred to recipients and 12 of 52 embryos implanted, although none were viable at 16 and 20 days of gestation (Kubiak and Tarkowski, 1985). Rabbit tetraploid embryos were found to have an implantation rate of 54%, although only 8% remained viable when examined between days 11 and 20 of gestation. Some tetraploid rabbit fetuses recovered by Ozil and Modlinski (1986) were small for their gestational age and showed neural tissue abnormalities.

Subsequently, the group in Edinburgh reported post-implantation development of mouse tetraploid embryos up to day 15 of gestation (Kaufman and Webb, 1990). That study found that 69 to 96% of the electrofused embryos implanted after embryo transfer but that the number of viable fetuses present decreased with each increasing day of gestational age. The fetuses in this study were also found to be small for their gestational age and displayed craniofacial and neural tissue abnormalities (Kaufman and Webb, 1990). A subsequent study from the same group reported tetraploid fetal development as ~15% smaller than developmentally matched controls but morphologically normal fetuses except for the previously noted craniofacial and neural tissue abnormalities (Henery et al., 1992).

Mosaic $2n \leftrightarrow 4n$ embryo development has been described in mice after cytochalasin treatment of 2-cell embryos (Tarkowski et al., 1977). Those embryos were reported to have higher developmental capacity than their purely tetraploid counterparts. Normal development was observed in 11 of 12 mosaic fetuses at days 10 and 13 of gestation. Less than 4% tetraploid cells was found in the developing fetuses, although it was reported that a higher percentage of tetraploid cells were found in placental tissues of some of the fetuses. Development of a $2n \leftrightarrow 4n$ mosaic rabbit embryo (majority of $2n$ cells) was described as normal at day 18 of gestation (Ozil and Modlinski, 1986). It has been suggested by other

researchers that the development of live tetraploid offspring reported by Snow (1973) may in fact have been $2n \leftrightarrow 4n$ mosaic embryos (Eakin and Behringer, 2003).

TETRAPLOID EMBRYO COMPLEMENTATION

Attempts to rescue the tetraploid phenotype and produce adult mice with a tetraploid chromosomal makeup resulted in the production of chimeric embryos in several studies (Graham, 1971; Lu and Markert, 1980). A blastomere from a diploid embryo was aggregated with a tetraploid blastomere generated from virus-mediated cell fusion and three of the six chimeric blastocysts that were transferred resulted in the birth of live young but only of the diploid cell type (Graham, 1971). Whole tetraploid embryos produced through cytochalasin treatment have been successfully aggregated with either one or two diploid embryos in the presence of phytohemagglutinin to create chimeric embryos (Lu and Markert, 1980). Once embryos reached the morula or blastocyst stage they were transferred to recipient animals to complete gestation. In the latter study, 59 chimeric embryos were transferred, 13 of which survived to term. Of these 13, only two were chimeric at birth, with the remaining offspring derived only from the diploid embryo.

The outcome of these early studies resulted in the realization that tetraploid embryos aggregated with a diploid constituent could be used to produce offspring derived primarily from the diploid cells and extraembryonic tissues derived from the tetraploid cells. The first group to use this method of producing chimeric animals used a mass of diploid ES cells sandwiched between two tetraploid 8-cell embryos (Nagy et al., 1990). The ES cells had previously been shown not to contribute much to extraembryonic tissues after aggregation with diploid embryos but they did contribute to all tissues of the developing fetus (Beddington and Robertson, 1989).

The aggregation of ES cells and tetraploid embryos resulted in the birth of completely ES cell-derived mice with tetraploid cells confined to the placental tissues in most offspring (Nagy et al., 1990). Less than 10% tetraploid cells were detected in the blood and tail tissues of 21% of the ES cell-derived neonates. As a control in this study, tetraploid embryos were aggregated with diploid inner cell mass cells that resulted in the birth of six offspring, five of which were completely derived from the inner cell mass cells. The remaining chimera had less than 5% tetraploid cells present in blood and tail tissues. All ES cell mice in this study died soon after birth but inner cell mass ↔ tetraploid aggregate controls survived until adulthood, suggesting that the aggregation procedure was not responsible for the diminished viability of the ES cell offspring (Nagy et al., 1990). A subsequent study using the same method of tetraploid embryo aggregation with an outbred ES cell line that resulted in the birth of viable ES cell mice demonstrated that ES cell background played a crucial role in determining the survivability of offspring after tetraploid embryo complementation (Nagy et al., 1993).

Studies from other groups have reported similar results with $2n \leftrightarrow 4n$ aggregate embryos (Goto et al., 2002; Tarkowski et al., 2001; Ueda et al., 1995). Tetraploid embryos produced by cytochalasin treatment were aggregated either singly or in pairs with ES cells at the 4-cell stage and resulted in five live offspring derived completely from ES cells of which three survived to adulthood and were later proved to be fertile (Ueda et al., 1995). Another group aggregated either a single blastomere of a 4-cell or two blastomeres of an 8-cell diploid embryo with either one or two 2- to 4-cell tetraploid embryos (Tarkowski et al., 2001). This study resulted in the birth of eight live pups, three of which survived to adulthood and proved fertile. Only one of the six animals examined had any tetraploid cell contribution, and that

was confined to the heart and liver; however, 80% of the fetal membranes examined had tetraploid embryo-derived cells present. Another study reported that no tetraploid cells were present in fetal mouse tissues by day 12.5 after transfer if a single diploid 8-cell embryo was aggregated with a single tetraploid 4-cell embryo (Goto et al., 2002). That study did find that increasing the number of tetraploid cells at aggregation in proportion with diploid cells would increase the chances of fetal chimerism.

The mechanisms of tetraploid cell exclusion from the developing $2n \leftrightarrow 4n$ aggregate mouse fetus were extensively studied by researchers at the University of Edinburgh. The aggregates produced by these researchers were made by pushing zona-free 4-cell tetraploid and 8-cell diploid embryos together. In the first study, aggregate embryos were studied at days 7.5 and 12.5 of gestation (James et al., 1995). At these times, tetraploid cells were rarely found in derivatives of the primitive ectoderm (fetus proper, amnion and yolk sac mesoderm). Tetraploid cells were present in the derivatives of the primitive endoderm and trophoctoderm. Aggregate fetuses were smaller at 7.5 days of gestation than diploid controls but were of similar size at 12.5 days. Placental weights of aggregate fetuses were heavier than for the diploid controls.

Cell allocation studies of aggregate mouse blastocysts revealed that tetraploid-derived blastomeres were nonrandomly distributed to the mural trophoctoderm of the aggregate embryo (Everett and West, 1996). These researchers concluded that ploidy differences resulted in the differential cell allocation, as cell size and developmental stage of aggregated embryos did not seem to affect the distribution of blastomeres (Everett and West, 1996). A subsequent study reported that tetraploid cells were actively selected against as cell numbers from tetraploid embryos, but not diploid embryos, were lower in aggregate blastocysts

(Everett and West, 1998). Spatial analysis of aggregate embryos using three-dimensional reconstruction showed that mixing of the tetraploid and diploid cells in aggregates was very limited (Everett et al., 2000). This study also noted the tendency for blastocoel formation to occur where tetraploid cells were present, as did a previous report from the same group (Everett and West, 1996).

Research that followed these reports studied aggregate mouse blastocysts to determine the effects of cell size and ploidy on cell allocation (Tang et al., 2000). These researchers found that larger cells tended to localize to the trophectoderm when aggregated with smaller cells of the same ploidy. Tetraploid cells of the same size as diploid cells they were aggregated with were also found to localize to the mural trophectoderm. This finding suggested that both the ploidy of the cell as well as its size determined its location within the developing aggregate embryo.

Tetraploid Embryo Complementation for ES Cells

With the initial success of tetraploid embryo complementation, other researchers began utilizing this technology to exploit the ability of tetraploid cells to contribute to the placenta but not to the embryo proper. Traditional production of ES cell-derived mice involved either aggregation with diploid embryos or injection of ES cells into a diploid blastocyst. Chimeric animals were produced and mating tests were conducted to detect animals that produced germ cells derived from the ES cell constituent. These animals were then mated to produce ES cell-derived animals. This process has been characterized and may take up to 14 months to produce adult ES cell-derived animals (Seibler et al., 2003). With tetraploid embryo complementation, the process of producing an ES cell-derived mouse may be completed in as little as 6 months (Seibler et al., 2003).

The two main methods of tetraploid embryo complementation used for ES cells have been aggregation and blastocyst injection (Eakin and Behringer, 2003; Wood et al., 1993). Aggregation methods have been described as the simplest, quickest and least technically demanding, while blastocyst injection methods require more equipment and training for micromanipulation (Eakin and Behringer, 2003). Aggregation began with the previously described studies of Nagy and co-workers in the early 1990s (Nagy et al., 1990; Nagy et al., 1993). Aggregates were constructed with clumps of 10 to 15 ES cells sandwiched between two tetraploid 4-cell embryos (Nagy et al., 1993). This method has been utilized by other researchers for ES ↔ tetraploid aggregate embryo construction in mice (Peli et al., 1996).

Other researchers have aggregated clumps of ES cells with individual tetraploid embryos at the 4-cell to 8-cell stages or at the morula stage (Misra et al., 2001; Wang et al., 1997). Blastocyst injection was developed for tetraploid embryo complementation after the initial aggregation trials (Peli et al., 1996; Wang et al., 1997). Typically, 10 to 20 mouse ES cells were injected into the blastocoel near the inner cell mass of early blastocysts (Amano et al., 2001; Eggan et al., 2001; Eggan and Jaenisch, 2003; Humpherys et al., 2001; Schwenk et al., 2003).

At least two studies have compared the efficiency of tetraploid embryo aggregation with tetraploid blastocyst injection for the production of ES cell mice but with differing results. The first study reported that ES cell mouse fetuses were generated from 44% of embryos produced by aggregation, while only 22% of tetraploid blastocysts injected with 15 ES cells produced fetuses (Peli et al., 1996). In contrast, another group reported that 14% of tetraploid blastocysts injected with ES cells resulted in live offspring while only 6% of mouse embryos produced by aggregation resulted in live offspring (Wang et al., 1997). These

conflicting results may be due to differences among ES cell lines utilized by the different researchers, as other studies have noted that ES cell line differences may be responsible for successful production of live animals following tetraploid embryo complementation (Eggan et al., 2001; Schoonjans et al., 2003).

Tetraploid Embryo Complementation as a Research Tool

The first study to show that tetraploid embryo complementation could be utilized as a method of rescuing lethal placental phenotypes involved the aggregation of tetraploid 4-cell embryos with diploid morulae from ES cell chimeric mice that were homozygous null mutants for the *Mash2* gene (Guillemot et al., 1994). The diploid embryos lacking the product of the *Mash2* gene had been shown to die at day 10 of gestation due to placental failure. The utilization of tetraploid embryo complementation in this study resulted in the birth of live, viable animals from the diploid embryos that would normally not have survived to term (Guillemot et al., 1994). Tetraploid embryo complementation has become a powerful tool for studies of gene function in mice and has been used to rescue both embryos derived from gene-targeted ES cell chimeras, as well as from the targeted ES cells themselves (Rideout et al., 2002; Schreiber et al., 2000; Wu et al., 2003).

Tetraploid embryo complementation studies have been utilized by many research groups to examine the role of specific genes in placental function (Adelman et al., 2000; Fujiwara et al., 2001; Hirashima et al., 2003; Hobbs et al., 2002; Jaquemar et al., 2003; Schreiber et al., 2000; Sugimoto et al., 2003; Takahashi et al., 2003; Wu et al., 2003). This procedure has been used not only to study placental phenotypes, but also other tissue phenotypes, which would normally not be able to be studied since a placental defect would result in the loss of the fetus earlier than the target of research interest would appear (Eakin

and Behringer, 2003; Misra et al., 2001). The function of genes in tissues other than the placenta has also been studied through tetraploid embryo complementation (Chawengsaksophak et al., 2004; Fujiwara et al., 2002; Gallicano et al., 2001; Isermann et al., 2001; Rideout et al., 2002; Wang et al., 2002; Watt et al., 2004).

Tetraploid embryo complementation has also been used to rescue non-ES cell embryos with placental malfunctions. One study utilized tetraploid embryo complementation to rescue mouse embryos with an additional maternally inherited X-chromosome (Goto and Takagi, 1998). These embryos failed to form functional extra-embryonic endoderm and ectoplacental cone and died during gestation. When the embryos were aggregated at the 4-cell to 8-cell stage with 4-cell tetraploid embryos, two pups were born with an additional maternally inherited X-chromosome, showing that tetraploid cells were capable of rescuing these embryos (Goto and Takagi, 1998). Tetraploid mouse embryo aggregation has also been used in an attempt to rescue parthenogenetic embryos (Spindle et al., 1996). Fetuses produced in this study did not survive past day 13 of gestation due to defective chorioallantoic fusion but more developed as Class I embryos to that stage of development than did the nonaggregated parthenogenetic controls.

Another group of researchers utilized tetraploid embryo complementation to determine the developmental capabilities of the inner cell mass of ES cell nuclear transfer blastocysts (Amano et al., 2002). The inner cell mass of nuclear transfer blastocysts produced from ES cells was injected into tetraploid blastocysts. These embryos implanted at the same rate as control tetraploid blastocysts injected with an inner cell mass from normal diploid embryos (76% vs. 71%). However, significantly fewer fetuses survived to term from ES cell nuclear transfer inner cell mass aggregates than from control aggregate embryos (45% vs. 5%). Some

nuclear transfer embryos were exposed to electrofusion and the resulting blastocysts were injected with inner cell masses from normal diploid embryos to test the capacity of trophectoderm cells from ES cell nuclear transfer embryos. Since only 24% of these embryos implanted after transfer and no live fetuses were obtained, Amano et al. (2002) concluded that both inner cell masses and trophectoderm cells from ES cell nuclear transfer embryos had a diminished capacity to form fetuses and placental tissues.

Postnatal Characteristics of ES Cell ↔ Tetraploid Mice

Offspring and placentas from tetraploid embryo complementation were normally sized at birth, as compared with the overgrowth noted in ES cell clones produced by nuclear transfer (Eggan et al., 2001). The expression of *H19* was also found to be normal in ES cell mouse placentas after tetraploid embryo complementation (Humpherys et al., 2001). One study reported the production of both male and female mice after injection of male ES cells into tetraploid blastocysts (Eggan et al., 2002). It was found that the female embryos were derived from the ES cells but had an XO phenotype upon karyotypic analysis. The ES cells used in this experiment had been shown to be chromosomally unstable and the Y chromosome was lost from up to 2% of the cells used for blastocyst injection. The female mice were fertile and produced normal offspring after mating (Eggan et al., 2002).

The normality of ES cell ↔ tetraploid mice was examined through morphological, physiological and neurological parameters in one study (Schwenk et al., 2003). The ES cell ↔ tetraploid mice were found to be 21% heavier than in vivo controls but were not different from in vitro produced controls. Hematological analysis of 14 different factors found that ES cell ↔ tetraploid mice were normal but showed mildly elevated values for erythrocytes and hematocrit. Neurological behavior was evaluated through 17 different parameters and was

reported to be identical to that of control animals. These findings demonstrated that ES cell ↔ tetraploid mice are normal and healthy (Schwenk et al., 2003).

ELECTROFUSION IN OTHER SPECIES

Electrofusion has been used in several species, including cattle, pigs, rats and monkeys, to join blastomeres of 2-cell stage embryos an effort to create tetraploid embryos. Bovine embryo electrofusion has been studied by several different groups. The first report of electrofused cattle embryos found that the optimal fusion parameters for 2-cell embryos was 1 kV/cm with two 10 or 25 μsecond direct current pulses (Iwasaki et al., 1989). They reported fusion rates of 73 to 78% within 60 minutes and subsequent cleavage rates of 55%. However, most electrofused embryos in that study did not develop past the 8-cell stage and none beyond the morula stage. After electrofusion and subsequent cleavage, 14 fused embryos at the 2-cell stage were analyzed by karyotyping and 11 were found to be tetraploid (Iwasaki et al., 1989). A subsequent study from the same group found that with alterations in culture medium, 19% of the electrofused bovine embryos reached the blastocyst stage and that 82 and 86% of 2-cell to 4-cell and morula stage embryos were tetraploid, respectively, after karyotypic analysis (Iwasaki et al., 1999).

A later study reported optimal bovine embryo fusion parameters to be a single, 1.4 kV/cm, 100 μsecond direct current pulse (Curnow et al., 2000). These parameters resulted in a 76% fusion rate within 60 minutes of exposure and 73% of those fused embryos subsequently cleaving. This study also reported that 56% of the fused embryos developed to the blastocyst stage but that only three of 24 embryos examined by karyotyping were tetraploid. Of the remaining embryos, nine were found to be diploid and 10 were 2n ↔ 4n mosaic embryos.

Porcine embryo electrofusion has also been studied by two separate groups. In the first study, 2-cell embryos were electrofused with a single 1.2 kV/cm, 30 μ second direct current pulse (Prather et al., 1996). This treatment resulted in 55% fusion within 30 minutes and all but one of the electrofused embryos developed to the blastocyst stage. Relative DNA content as measured by 546 nm wavelength light absorption indicated that the electrofused embryos were uniformly tetraploid. A more recent study showed that embryos collected at the 2-cell stage rather than collected at the 1-cell stage and cultured in vitro had a higher blastocyst rate (90 and 29%, respectively) after electrofusion at the 2-cell stage with a single 0.75 kV/cm 50 μ second direct current pulse, indicating that in vitro culture could negatively affect porcine embryo development after electrofusion (Prochazka et al., 2004). Of the 28 electrofused embryos analyzed by fluorescent in situ hybridization in this study, 14 were determined to be tetraploid, eight mosaic and six diploid (Prochazka et al., 2004).

Rat embryos have been electrofused at the 2-cell stage by alignment with an alternating current and pulsing with two 20 μ second 60 V direct current pulses (Krivokharchenko et al., 2002). These embryos had a fusion rate of 96% and fused embryos cultured from the zygote stage reached the blastocyst stage of development at a rate of 14%, while fused embryos cultured from the 2-cell stage had a 60% blastocyst rate. This indicated an effect of in vitro culture on the developmental capacity of electrofused rat embryos, as the development rates from embryos cultured in vitro from the 2-cell stage were similar to those of control embryos and higher than those from embryos cultured in vitro from the zygote stage. Electrofused embryos at the blastocyst stage were smaller and had 43% fewer cells than the control diploid embryos but these electrofused rat embryos were uniformly tetraploid when examined via karyotypic analysis (Krivokharchenko et al., 2002).

Rhesus monkey embryos have also been electrofused to produce tetraploid embryos with two 1.5 kV/cm, 50 μ second direct current pulses (Schramm and Paprocki, 2004). Blastomere fusion rates of 82% were reported after 45 minutes and 51% of the electrofused embryos developed to the blastocyst stage. Karyotypic analysis of six electrofused 2-cell embryos revealed that all embryos were tetraploid (Schramm and Paprocki, 2004).

Some limited aggregation studies have been performed with electrofused embryos of cattle, pigs and rhesus monkeys. A study with bovine electrofused embryos reported blastocyst development from aggregations of two compacting morula-stage electrofused embryos into triangles with diploid cells, which were located between the two electrofused embryos (Iwasaki et al., 1999). After aggregation of electrofused embryos with inner cell mass cells, three of 18 aggregates formed blastocysts. Inner cell mass cells and mammary cells were also aggregated with electrofused embryos after labeling the diploid cells with Hoechst 33342 prior to aggregation. The aggregates developed to the blastocyst stage and fluorescence was noted in the inner cell mass of the resulting aggregate blastocyst. There was no mention of fluorescence in the trophectoderm of the aggregate blastocyst.

Another study by the same group reported aggregation of two electrofused zona-free 8-cell bovine embryos into triangles with a cluster of 10 to 15 ES-like cells (Iwasaki et al., 2000). Aggregate embryos developed to the blastocyst stage at a rate of 36% and 12 embryos were transferred to seven cows, resulting in the birth of six calves. Only two of the calves were chimeric for the ES-like cells. One calf had ES-like cell contribution to the hair roots while the other calf had ES-like cell contribution to the liver. Placental tissues were found to be chimeric in two other calves. It was determined that the calves produced were primarily derived from the electrofused embryos that were probably not completely tetraploid. Whether

the electrofused embryos were mosaic or had reverted to a diploid state was not known (Iwasaki et al., 2000).

In pigs, one third of a dye-labeled diploid inner cell mass was injected into the perivitelline space of a 4-cell electrofused embryos (Prather et al., 1996). Chimeric embryos constructed in this manner developed to the blastocyst stage 70% of the time. Only the inner cell mass of the aggregate blastocyst was labeled by the dye in 67% of the aggregates, indicating that the diploid cells formed the inner cell mass in most of the aggregate embryos.

In rhesus monkeys, aggregate embryos were constructed by combining a single blastomere from a 4-cell diploid embryo with a single blastomere from a 4-cell electrofused embryo, and also by combining two blastomeres from each of a 4-cell diploid and 4-cell electrofused embryo (Schramm and Paprocki, 2004). These aggregate embryos developed to the blastocyst stage at rates of 43 and 59%, respectively. Blastomeres from diploid and electrofused embryos were labeled with different Mitotracker[®] dyes (Molecular Probes, Oregon) prior to aggregation and showed that electrofused blastomeres contributed to both the inner cell mass and the trophectoderm of aggregate blastocysts (Schramm and Paprocki, 2004). These studies have shown that tetraploid embryo complementation is not as well defined in animal species other than the mouse.

CHAPTER III

THE EFFECT OF HUMAN CHORIONIC GONADOTROPIN ON CIRCULATING PROGESTERONE IN GOATS NEARING THE END OF THE BREEDING SEASON

INTRODUCTION

A major obstacle in the production of goats is the short time available for embryo transfer because of seasonality (Amoah et al., 1996; Gootwine et al., 1997). The natural breeding season for goats in North America has been reported to last from September to the end of January (Ott, 1982). Pregnancy rates decrease rapidly once does enter the transition period when coming into season or going out of season, and pregnancy rates remain low while the does are out of season (Amoah et al., 1996; Gootwine et al., 1997). Methods to increase the length of the breeding season would be beneficial to both meat and dairy goat producers by increasing the amount of time that meat and milk products are available to the consumer. Some techniques currently being used to extend the length of the breeding season are controlled lighting schemes, timing of exposure to male goats and administration of exogenous hormones during the transition and anestrus season (Haibel, 1990; Thimonier, 1981). It is hypothesized that near the end of the breeding season, serum progesterone concentrations in cyclic does decrease, which could account for the decrease in pregnancy rates at the end of the breeding season.

Mean reported peak progesterone levels during the breeding season occur between days 10 and 14 of the estrous cycle, ranging from 4 ng/ml to 8.9 ng/ml. Progesterone levels are reported to be nearly 0 ng/ml at estrus in the doe (de Castro et al., 1999; Greyling, 2000; Jones and Knifton, 1972; Ott et al., 1980; Sawada et al., 1994; Thibier et al., 1981; Thorburn and Schneider, 1972). In the goat, progesterone production by the corpus luteum (CL) is

essential for the maintenance of pregnancy throughout gestation and depends upon stimulation of the CL by luteinizing hormone (LH) (Gemmell et al., 1977; Kawate et al., 2000; Thorburn and Schneider, 1972). Human chorionic gonadotropin has LH activity in the goat and has also been shown to increase progesterone levels in superovulated goats (Saharrea et al., 1998) and in luteal cell cultures (Band et al., 1987).

Human chorionic gonadotropin (hCG) has successfully been used to increase plasma progesterone levels in beef and dairy cows and heifers (Diaz et al., 1998; Fricke et al., 1993; Schmitt et al., 1996a; Schmitt et al., 1996b). The cattle were treated either on day 4, 5 or 6 in the studies with doses of hCG that ranged from 1000 IU to 3000 IU. Differences in serum progesterone levels were detected by day 6, 8, and 9 in the four studies. An increased pregnancy rate was reported in Holstein heifers treated with hCG (Breuel et al., 1989).

The objective of this study was to determine if hCG treatment was effective in increasing serum progesterone levels and pregnancy rates in does approaching the anestrous season. If successful, this treatment could extend time available for embryo transfer in does. This would be especially beneficial for the generation of transgenic and nuclear transfer-derived goats as the short breeding season limits the time available for the production and transfer of valuable embryos to recipient animals.

EXPERIMENTAL DESIGN

Cycling does were selected at three times, once during the breeding season, and at two times during the spring transition period. Does in all groups were treated either with saline or hCG and plasma was collected for analysis of progesterone concentration during the estrous cycle. In addition, does in the second spring transition period were mated at estrus to determine pregnancy rates after the two treatments.

MATERIALS AND METHODS

Animals

This study utilized mature Spanish-type crossbred does. Does were housed at the Embryo Biotechnology Laboratory of the Reproductive Biology Center in St. Gabriel, Louisiana. Does were fed a commercially available pelleted ration once daily and had ad libitum access to hay and water. Body weight was recorded prior to treatment. All does used in this study were handled and maintained in accordance with the Louisiana State University Agricultural Center Institutional Animal Care and Use Committee Guidelines.

Group I. Does detected in estrus (standing to be mounted by a teaser buck) in November were assigned to one of two treatments, control (n=6) or hCG-treated (n=6). Control does received a sham injection of 0.5 ml saline i.m. on days 2 and 3 post-estrus. Does treated with hCG received an injection of 500 IU of hCG (0.5 ml Chorulon®, Intervet Inc., Millsboro, DE) IM on days 2 and 3 post-estrus.

Group II. Does detected in estrus in early to mid February were assigned to one of two treatments, control (n=11) or hCG-treated (n=12). Control does received a sham injection of 0.5 ml saline i.m. on days 2 and 3 post-estrus. Does treated with hCG received an injection of 500 IU of hCG (0.5 ml Chorulon®, Intervet Inc., Millsboro, DE) IM on days 2 and 3 post-estrus.

Group III. Does detected in estrus from mid to late February were assigned to one of two treatments, control (n=13) or hCG-treated (n=13). Does were exposed to an intact, fertile crossbred buck for 24 hours from the time of estrus detection for mating. No more than three does were placed with the buck within a single 24-hour period. Control does received a sham injection of 0.5 ml saline i.m. on days 2 and 3 post-estrus. Does treated with hCG received an

injection of 500 IU hCG (0.5 ml Chorulon®, Intervet Inc., Millsboro, DE) i.m. on days 2 and 3 post-estrus. Pregnancy was determined via ultrasonography at 90 days after mating. Gestation length, number of offspring born, sex ratio and birth weight were recorded at parturition.

Estrus Detection

Does were exposed to vasectomized teaser bucks once daily for the detection of estrus. Standing to be mounted and intromission were used as verification of estrous behavior.

Progesterone Samples

Blood samples were collected via jugular venipuncture from does at estrus (day 0) at ~6:00 pm and at 48 hour periods following estrus until the animal either returned to estrus or 26 days had elapsed for Groups I and II. Blood was collected every 7 days from does in Group III until day 28 post-estrus. Blood was collected into a 10-ml heparinized tube (Vacutainer; Becton and Dickinson, Franklin Lakes, NJ). Blood samples were placed on ice immediately after collection and centrifuged at 1000 x g at 4°C for 10 minutes. Plasma was collected and stored at -20°C until radioimmunoassay analysis. The plasma progesterone concentrations were determined by radioimmunoassay using a commercial kit (DSL-3400, Diagnostic Systems Laboratories, Inc., Webster, TX) and were compared between the two groups of does. Intra- and interassay coefficients of variation and assay sensitivity were 5%, 8% and 0.05 ng/ml, respectively.

Statistical Analysis

Data for doe body weight, doe body condition score, kid birth weight, estrous cycle length and progesterone concentration were analyzed using the mixed procedure of SAS (SAS

Institute, Inc., Cary, NC). Data for the rate of return to estrus, pregnancy rate and sex ratio of kids were analyzed using X² procedure of SAS (SAS Institute, Inc., Cary, NC).

RESULTS

Group I

Mean plasma progesterone data from days 0, 6, 14 and 20 of the estrous cycle are shown in Figure 3.1 and Table 3.1. Mean progesterone concentrations were near 0 ng/ml on day 0 and day 20. Elevated progesterone concentrations were detected during the luteal phase of the estrous cycle (day 6 and day 14). Plasma progesterone concentrations were higher in hCG-treated does than in control does during the luteal phase of the estrous cycle.

Only one of the control does failed to return to estrus following the treated cycle. There was no significant difference between treatment groups in the number of does returning to estrus following treatment (control does, 83%; hCG-treated does, 100%) or in the length of the estrous cycle following treatment (control does, 22.6 ± 0.8 ; hCG-treated does, 23.3 ± 0.6). There was also no significant difference in doe body weight (control does, 51.1 ± 1.8 kg; hCG-treated does, 58.0 ± 6.0 kg) or body condition score (control does, 3.5 ± 0.2 ; hCG-treated does, 3.8 ± 0.4) between the two treatments.

Group II

Mean plasma progesterone data from days 0, 6, 14 and 20 of the estrous cycle are shown in Figure 3.2 and Table 3.1. Mean progesterone values followed the general pattern found in Group I, with values near 0 ng/ml on day 0 and day 20 with elevated progesterone concentrations during the luteal phase of the estrous cycle. Does treated with hCG had elevated progesterone levels when compared to control does on day 7 and day 14.

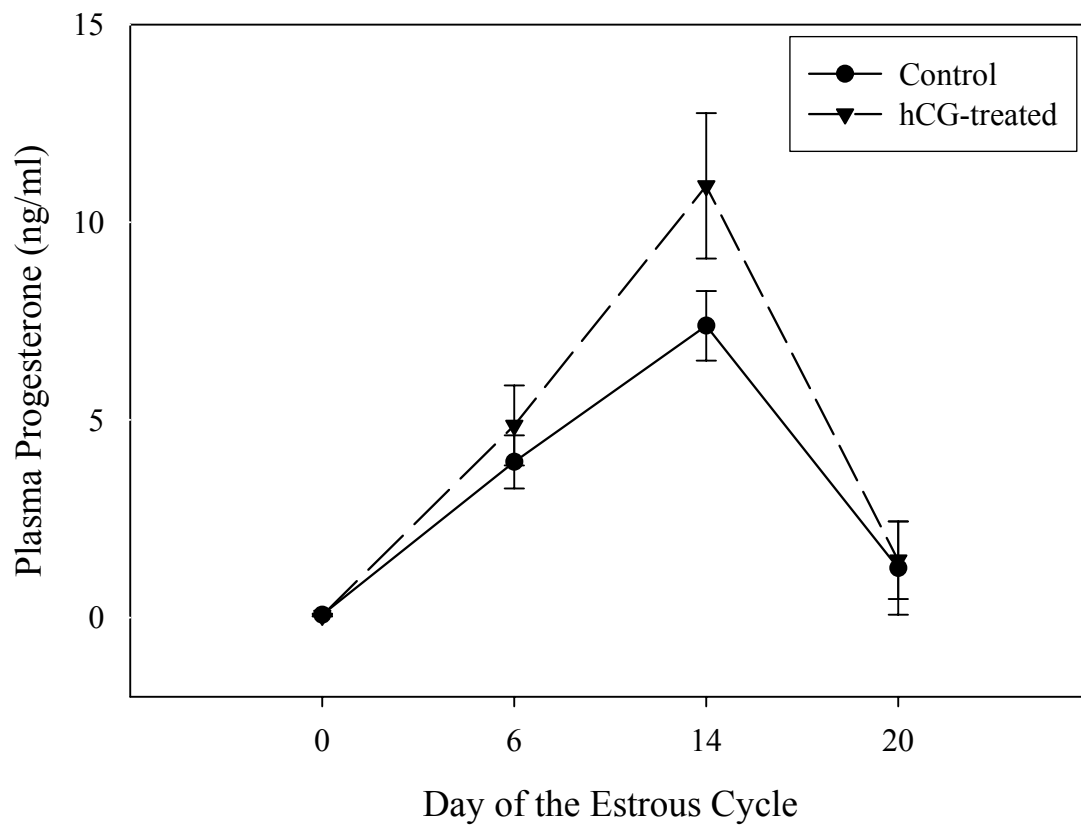


Figure 3.1. Mean plasma progesterone levels (\pm SEM) for control and hCG-treated does during the breeding season (Group I).

Table 3.1. Plasma progesterone concentrations (\pm SEM) from control or hCG-treated does in seasonal groups I, II and III following detection of estrus on day 0 and treatment with saline or hCG on days 2 and 3 post-estrus

Group	Treatment	Pregnant	n	Day 0	Day 6-7	Day 14	Day 20-21	Day 28
I	hCG	na	6	0.05 \pm 0.02	4.86 \pm 1.01	10.92 \pm 1.84	1.45 \pm 0.98	na
	control	na	6	0.07 \pm 0.03	3.94 \pm 0.67	7.38 \pm 0.88	1.25 \pm 1.18	na
II	hCG	na	11	0.06 \pm 0.01	6.25 \pm 1.13	8.96 \pm 1.31	0.14 \pm 0.05	na
	control	na	12	0.05 \pm 0.01	2.92 \pm 0.35	6.39 \pm 0.61	0.68 \pm 0.54	na
III	hCG	yes	7	0.03 \pm 0.01	6.64 \pm 1.58	10.51 \pm 2.53	6.46 \pm 1.43	4.98 \pm 1.36
	hCG	no	6	0.06 \pm 0.02	6.04 \pm 1.19	8.3 \pm 2.37	1.28 \pm 0.75	2.35 \pm 1.12
	control	yes	7	0.06 \pm 0.02	2.57 \pm 0.47	6.08 \pm 0.71	5.22 \pm 0.65	5.49 \pm 1.27
	control	no	5	0.06 \pm 0.03	2.53 \pm 0.77	5.11 \pm 0.54	1.74 \pm 1.02	1.30 \pm 0.54
	hCG	both	13	0.04 \pm 0.01	6.36 \pm 0.97	9.49 \pm 1.70	4.06 \pm 1.10	3.67 \pm 0.92
	control	both	13	0.06 \pm 0.01	2.52 \pm 0.37	5.86 \pm 0.48	3.84 \pm 0.69	3.74 \pm 0.97
	both	yes	14	0.04 \pm 0.01	4.76 \pm 1.03	8.30 \pm 1.41	5.84 \pm 0.78	5.25 \pm 0.89
	both	no	11	0.06 \pm 0.01	4.44 \pm 0.89	6.85 \pm 1.35	1.48 \pm 0.59	1.87 \pm 0.65

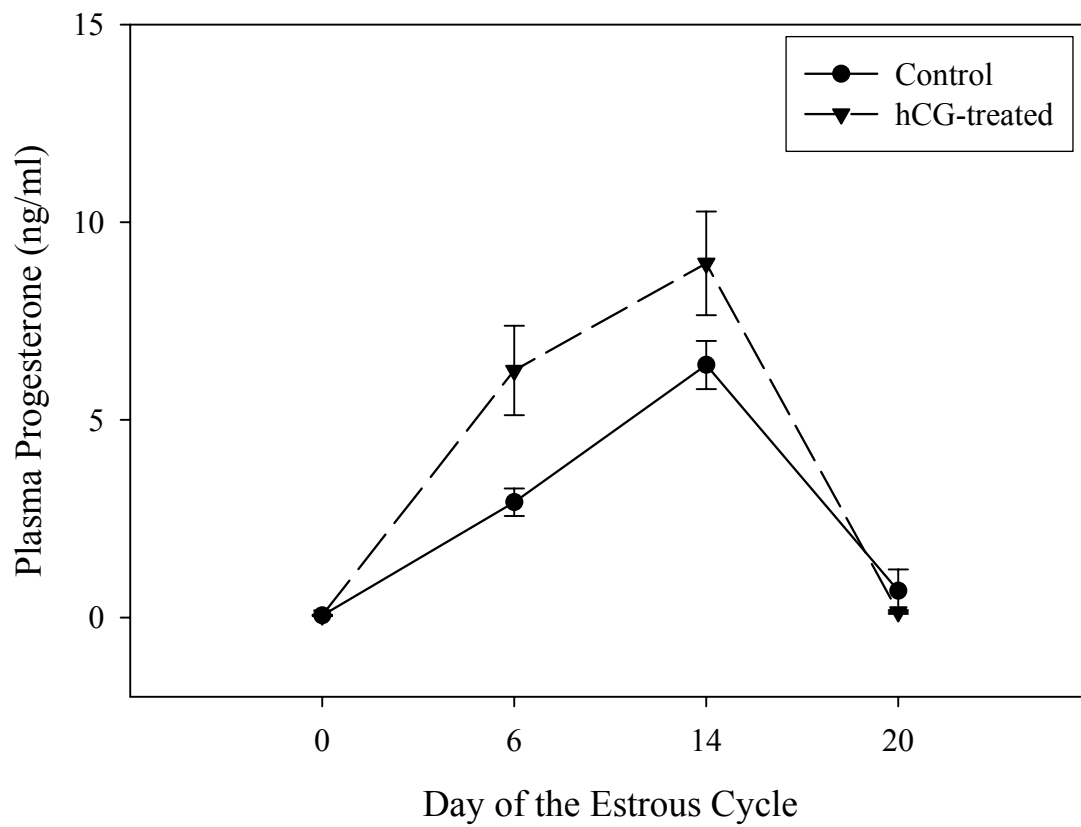


Figure 3.2. Mean plasma progesterone levels (\pm SEM) for control and hCG-treated does during the spring transition period (Group II).

Only five does returned to estrus following treatment (three hCG-treated and two control does). There was no significant difference in the number of does returning to estrus following treatment or in the length of the estrous cycle following treatment (control does, 21.5 ± 0.5 ; hCG-treated does, 25.7 ± 3.2). In addition, there was no significant difference in doe weight (control does, 49.1 ± 3.6 kg; hCG-treated does, 46.8 ± 2.3 kg) or body condition score (control does, 3.3 ± 0.2 ; hCG-treated does, 3.5 ± 0.2) between the two treatments.

Group III

Mean plasma progesterone levels are shown in Table 3.1 and Figures 3.3 through 3.6. Figures 3.3 and 3.4 present the mean concentrations of plasma progesterone from the pregnant and nonpregnant control and hCG-treated does, respectively. Plasma progesterone concentrations were significantly different in the pregnant does when compared to the nonpregnant does ($P < 0.01$). There was a significant pregnancy x time interaction ($P < 0.01$), which was expected as the progesterone concentrations from pregnant does will be elevated when compared with those of nonpregnant does depending upon the day of the estrous cycle. When plasma progesterone levels from pregnant does were analyzed, mean levels were higher on day 7 and day 14 in hCG-treated does when compared to control does (Figure 3.5). The mean progesterone levels between treatment groups were nearly identical by day 28 of pregnancy. The same pattern was noted in nonpregnant does with hCG-treated does having a higher mean progesterone level on days 7 and 14 post-estrus than did control does (Figure 3.6). The percentage of nonpregnant does continuing to cycle (plasma progesterone level ≥ 2 ng/ml after day 21) following treatment was not significantly different between groups but more does did have an increase in plasma progesterone levels on day 28 (three hCG-treated does compared with one control doe) (Table 3.2).

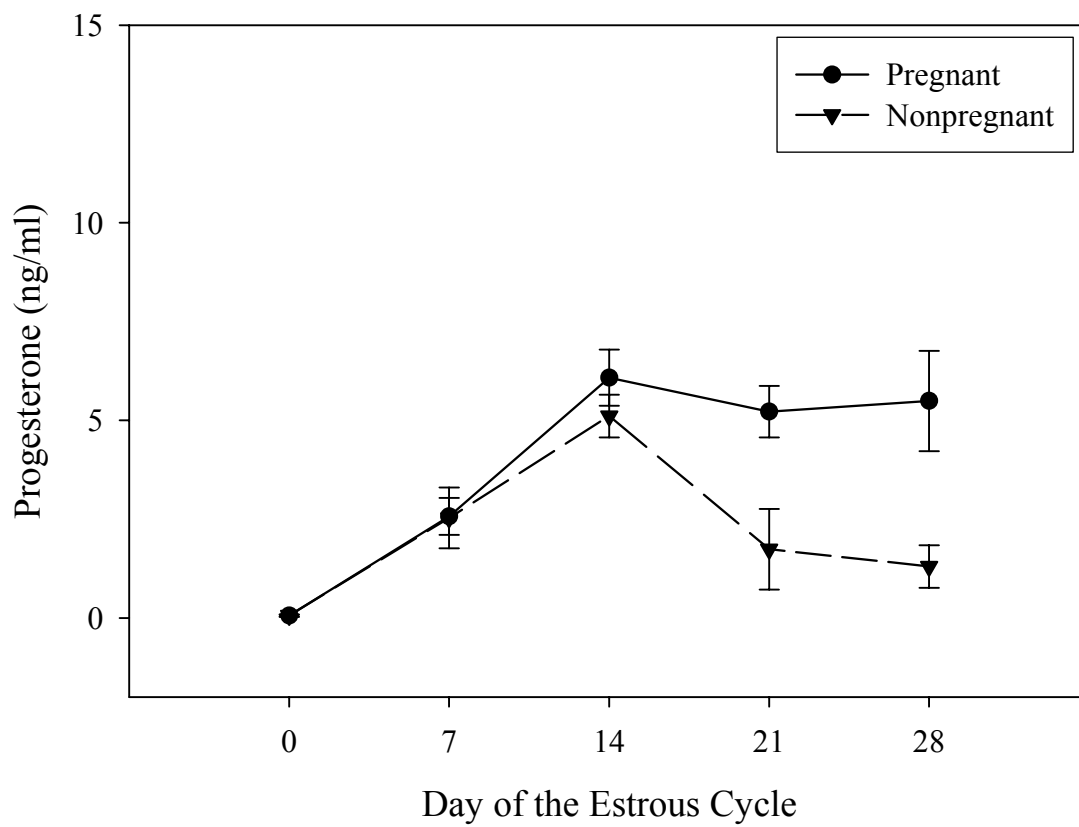


Figure 3.3. Mean plasma progesterone levels (\pm SEM) for control pregnant and nonpregnant does during the spring transition period (Group III).

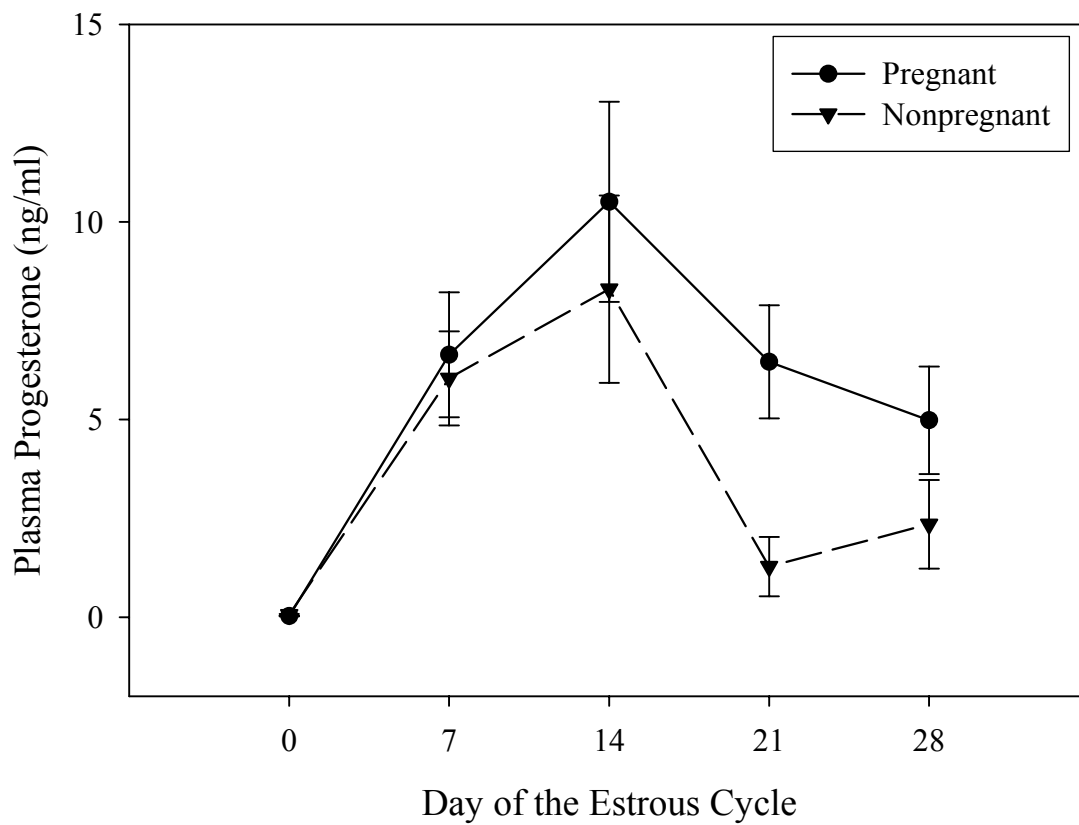


Figure 3.4. Mean plasma progesterone levels (\pm SEM) for hCG-treated pregnant and nonpregnant does during the spring transition period (Group III).

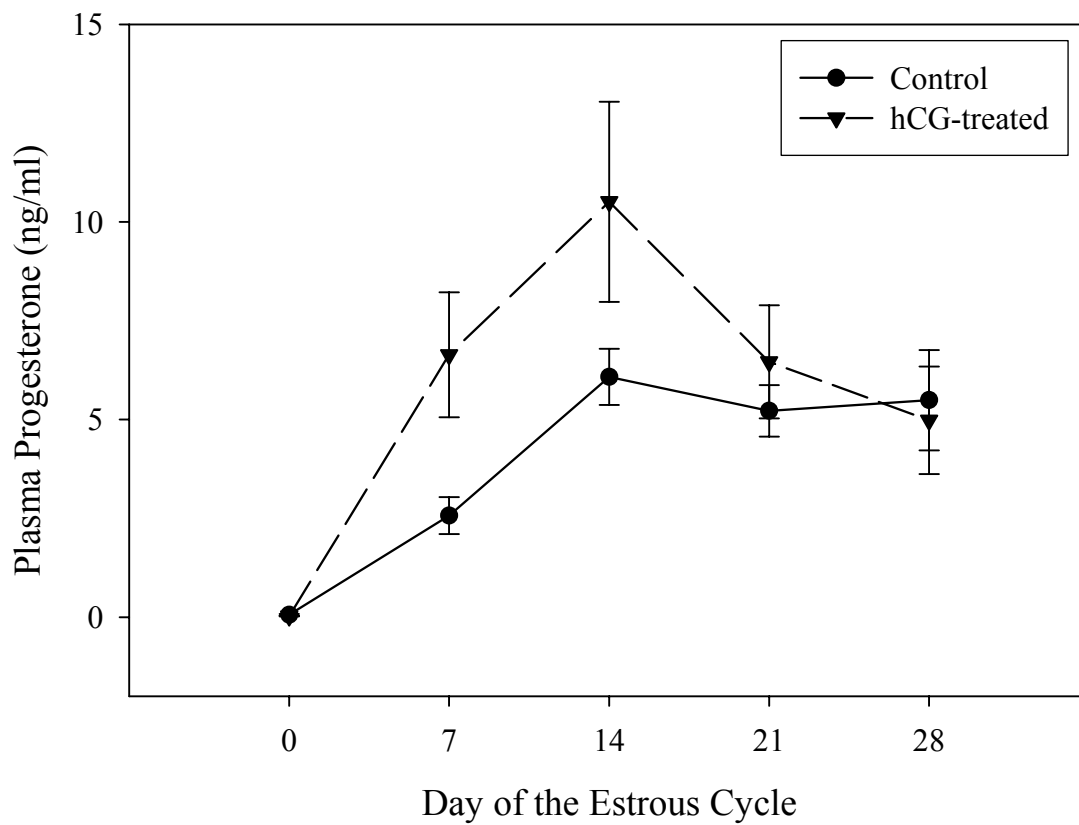


Figure 3.5. Mean plasma progesterone levels (\pm SEM) for control and hCG-treated pregnant does during the spring transition period (Group III).

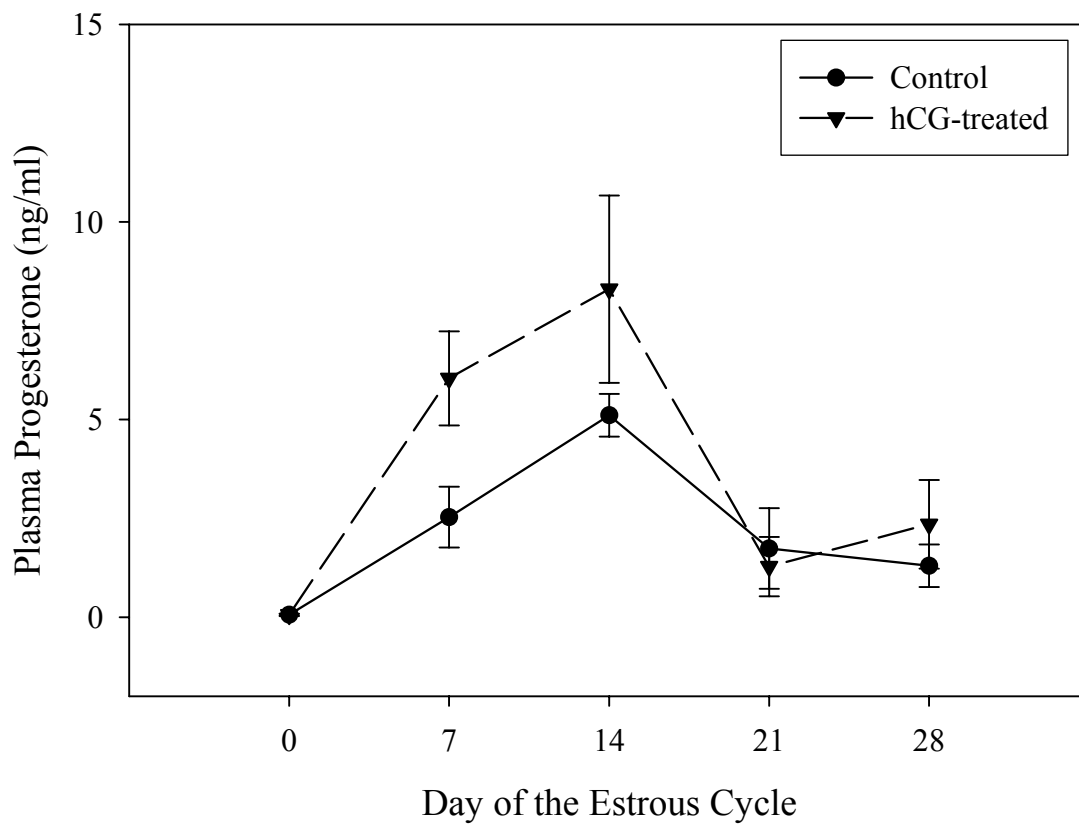


Figure 3.6. Mean plasma progesterone levels (\pm SEM) for control and hCG-treated nonpregnant does during the spring transition period (Group III).

Table 3.2. Pregnancy and continuing cyclicity status of does treated with hCG or saline during the spring transition period (Group III)

Treatment Group	n	Number Pregnant (%) [†]	Nonpregnant, Cycling (%) [‡]
Control	13 [*]	7 (58)	1 (20)
hCG-treated	13	7 (54)	3 (50)
Total	26	14 (56)	4 (36)

[†]Calculated from number of does remaining in study.

[‡]Cyclicity determined by plasma progesterone level of ≥ 2 ng/ml on day 28 post-breeding; percentage cycling calculated from number of does detected not pregnant.

^{*}One doe was removed from study prior to pregnancy detection due to illness.

One doe from the control group had to be removed from the study prior to pregnancy detection due to illness. Of the remaining does in the study, seven of 12 control does were detected pregnant (58%) on day 90 of gestation and delivered live kids. Of the 13 hCG-treated does, seven were detected pregnant (54%) on day 90 of gestation and delivered live kids (Table 3.2). There was no significant difference in pregnancy rate between the two groups. Kidding results are shown in Table 3.3. A total of 27 kids (16 male) were born to the 14 pregnant does (196% kidding rate). Kids were born as 11 pair of twins, two singletons and one set of triplets. The mean gestation length for both treatment groups was 150 days. Mean birth weight was 3.1 ± 0.1 kg. Kids born to hCG-treated does were significantly lighter at birth than kids born to control does ($P < 0.05$). There was no difference in the number of kids or in the number of male kids born per treatment group. There was no significant difference in doe weight (control does, 54.0 ± 3.0 kg; hCG-treated does, 50.7 ± 2.5 kg) or body condition score at mating (control does, 3.5 ± 0.2 ; hCG-treated does, 3.7 ± 0.2) between the two treatments.

Groups I, II and III

Progesterone levels across all experiments for control does are shown in Figure 3.7 (Group I – breeding season, Group II – spring transition during early to mid February, Group III – spring transition during mid to late February). Data from Group III are calculated only from nonpregnant does. There was no significant difference in circulating progesterone concentrations between control does across all seasonal groups. There was also no significant difference among groups of hCG-treated does for circulating progesterone concentrations across all seasonal groups (Figure 3.8). Mean circulating progesterone concentrations were higher in hCG-treated does than in control does in Groups I, II and III ($P < 0.01$). Progesterone

Table 3.3. Gestation length and kidding results of does bred during the spring transition period and treated with hCG or saline (Group III)

Treatment Group	n	Mean Gestation Length (day)	Mean Birth Weight (kg)	Number (%)	
				Kids	Male
Control	7	150	3.3 ± 0.1 ^a	13 (186)	9 (69)
hCG-treated	7	150	3.0 ± 0.1 ^b	14 (200)	7 (50)
Total	14	150	3.1 ± 0.1	27 (193)	16 (59)

^{a,b}Mean values with different superscripts in the same column are significantly different (P<0.05).

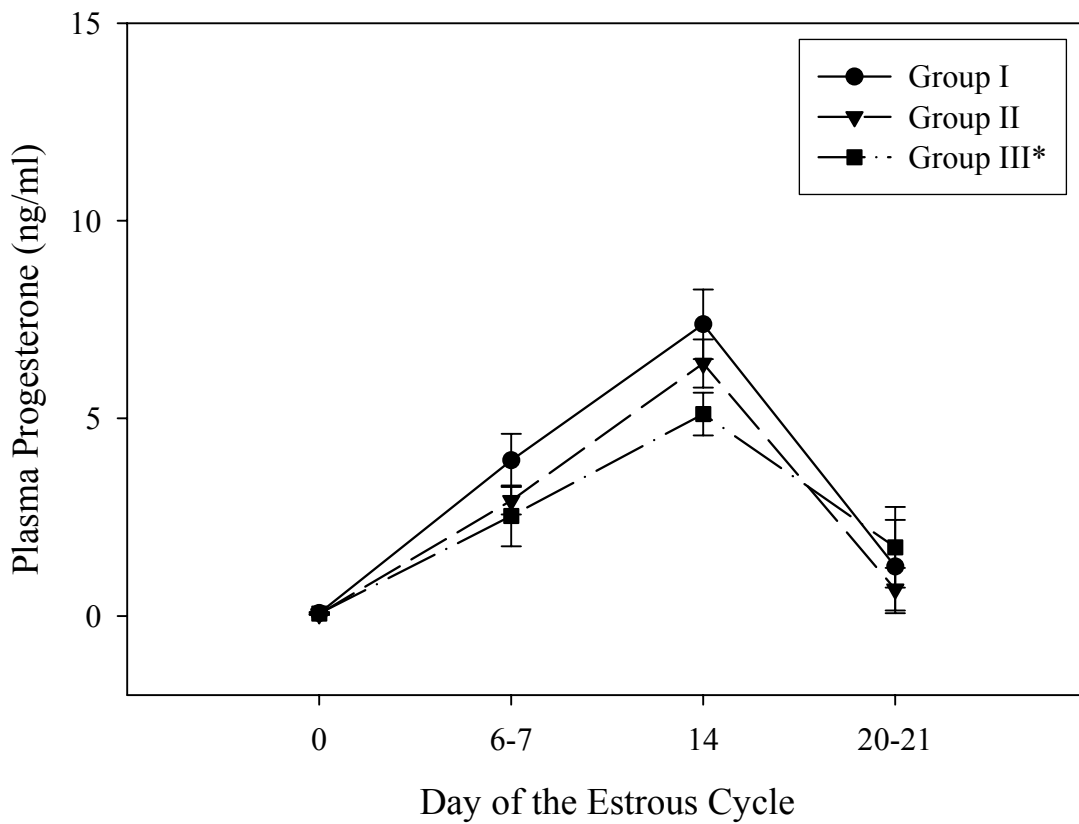


Figure 3.7. Mean plasma progesterone levels (\pm SEM) for control does during the breeding season (Group I) and spring transition period (Groups II and III).

*Mean progesterone of nonpregnant does from Group III, occurred 1 to 2 weeks later in spring transition season than Group II.

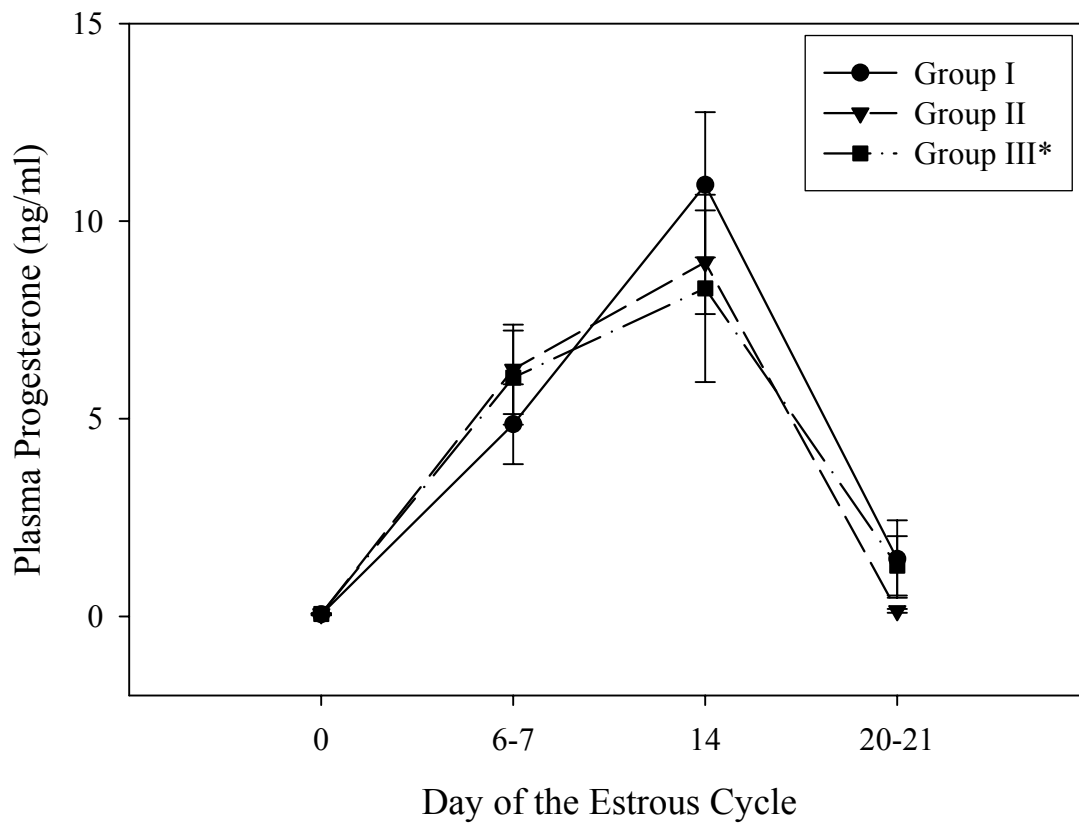


Figure 3.8. Mean plasma progesterone levels (\pm SEM) for hCG-treated does during the breeding season (Group I) and spring transition period (Groups II and III).
 *Mean progesterone of nonpregnant does from Group III, occurred 1 to 2 weeks later in spring transition season than Group II.

levels were significantly different with day of blood sample collection ($P < 0.01$). There was a significant treatment x time interaction ($P < 0.01$), which was expected as the data did follow the expected luteal phase curve for progesterone with low progesterone concentrations on day 0 and day 20-21 of the estrous cycle, and elevated progesterone on day 6-7 and day 14 of the estrous cycle (luteal phase).

DISCUSSION

In this study, the highest progesterone levels detected on day 14 for the control does were similar to those previously reported in the literature (Chemineau et al., 1984; de Castro et al., 1999; Greyling, 2000; Heap and Linzell, 1966; Jones and Knifton, 1972; Ott et al., 1980; Sawada et al., 1994; Thorburn and Schneider, 1972). Minor differences in plasma progesterone concentrations between the studies may be attributed to differences in assay procedures.

In one study, does were treated with three different doses of hCG (550, 650 and 750 IU) at 6 hours post-estrus (Dutta et al., 1993). In that study, there were no differences in peak progesterone values between the hCG-treated does and controls. This could be due to the relatively early treatment of the does after the end of estrus. In beef heifers, it was also noted that early administration of hCG (day 1 of the estrous cycle) did not affect serum progesterone concentrations in treated animals over that cycle (Breuel et al., 1989). In the same study, differences in circulating progesterone levels were noted when hCG was given on either day 4 or day 7 of the estrous cycle. Those findings are similar to the results in our study, with elevated progesterone in hCG-treated does across all seasonal groups (Groups I, II and III) when compared to control does.

In the present study, there are two possible explanations for the increase in progesterone noted in the hCG-treated does when compared to the control does. One explanation could be increased stimulation of the existing CL from the LH-like actions of hCG, which has previously been noted with in vitro luteal cell cultures in the goat (Band et al., 1987). Another possible explanation for the increase in plasma progesterone values between hCG-treated does when compared to the control does is the formation of accessory CL. Increased progesterone concentrations have been reported from does with two or more CL prior to day 30 of gestation when compared to plasma progesterone concentrations from does with a single CL (Jarrell and Dziuk, 1991). It has also been shown that the increase in plasma progesterone concentrations in hCG-treated Holstein heifers and beef cows compared with nontreated controls could be due the formation of an accessory CL (Diaz et al., 1998; Fricke et al., 1993; Schmitt et al., 1996a; Schmitt et al., 1996b).

Goats during the breeding season have been reported to have three or four follicular waves per estrous cycle (de Castro et al., 1999; Ginther and Kot, 1994; Menchaca and Rubianes, 2002). Wave 1 has been shown to emerge around day 0 to day 1 of the estrous cycle (de Castro et al., 1999; Ginther and Kot, 1994; Menchaca and Rubianes, 2002). These follicular dynamics would allow a follicle to be at the appropriate stage of development to be affected by hCG administered on days 2 and 3 and ovulate, forming an accessory CL. It is unknown if does in this study formed accessory CL as does were not laparotomized or monitored via ultrasonography for number of CL per female.

The elevation of plasma progesterone levels early in the estrous cycle could be important when considering the use of assisted reproductive techniques, such as embryo transfer, splitting embryos and the production of transgenic and nuclear transfer embryos.

One study reported no difference in peak progesterone values between does with different ovulation rates, but a significantly higher plasma progesterone concentration early in the estrous cycle among does with multiple ovulations compared with does with a single ovulation (days 0 to 6) (Pathiraja et al., 1991). A separate study demonstrated an increase in embryo survival rate when caprine embryos were transferred to does with increasing numbers of CL (52%, 63% and 75% embryo survival rates for does with 1, 2 and 3 CL, respectively) (Armstrong and Evans, 1983).

There was no significant difference in pregnancy rates between control and hCG-treated does during the spring transition period in this study. In sheep, it has been noted that exogenous progesterone given early (days 1 to 5) of gestation can enhance the growth of embryos in vivo (Kleemann et al., 1994; Nephew et al., 1991; Pope et al., 1995; Wallace et al., 2003). In studies on cattle, it has also been reported that elevated progesterone early in gestation (days 4 to 5) was associated with enhanced embryo growth (Garrett et al., 1988; Mann et al., 2003b; Mann and Lamming, 2001). A significant difference was noted in the birth weights of kids produced from control does and hCG-treated does in this study, with heavier kids born to control does ($P < 0.05$). This was likely due to the differences in the number of male kids born in the two treatment groups. In our study, although not significantly different, there was a higher number of male kids born to does in the control group. It was found that male kids were heavier at birth (3.3 kg) than females (3.0 kg) when averaged across both treatment groups in this study ($P < 0.05$). This would account for the lighter birth weights of kids born to hCG-treated does. This is in agreement with a prior study that showed male kids were heavier at birth than female kids (Amoah et al., 1996).

The results of the present study indicate that there is no significant difference in mean progesterone concentrations in control does during the luteal phase of the estrous cycle across seasonal groups (Figure 3.7). Previous studies in sheep and goats have shown that during the anestrus period, plasma progesterone concentrations are below normal luteal phase levels in season, and pregnancy rates are low as well (Gootwine et al., 1997; Haresign et al., 1975). This may be due to luteal insufficiency, or the lack of formation of functional CL during the anestrus season. In our study, transitional luteal insufficiency was not detected as expected (Gootwine et al., 1997). This was evidenced by no significant difference in plasma progesterone concentrations between control does across seasonal groups.

Premature luteal regression has been noted in many embryo transfer programs after eCG use in superovulation schemes (Armstrong et al., 1983; Battye et al., 1988; Saharrea et al., 1998; Stubbings et al., 1986). Treatment of superstimulated does with hCG 3.5 days after estrus elevated serum progesterone concentrations on days 5 and 6 of the estrous cycle and also prevented premature luteal regression in treated does (Saharrea et al., 1998). One group reported that norgestomet implants maintained demi-embryo pregnancies in five does with regressed CL (based on plasma progesterone of <1 ng/ml) at 25 days of gestation (Beckett et al., 1999). The results from the latter study indicate that exogenous progesterone in the pregnant doe with luteal insufficiency could be beneficial to pregnancy maintenance. It has also been reported that supplementation of progesterone through intravaginal sponges for days 7 through 50 of pregnancy after transfer of frozen-thawed embryos did improve pregnancy rates in treated does when compared with control animals (D'Alessandro et al., 2004). Both studies indicated that exogenous progesterone during pregnancy increased embryo survival rates (Beckett et al., 1999; D'Alessandro et al., 2004).

Treatment of does with hCG did increase circulating progesterone levels in the present study but was found not to affect pregnancy rates after natural mating during the spring transition period. Further research is warranted to determine if treatment of embryo transfer recipient does with hCG might be indicated for demi-embryo transfer, nuclear transfer or transgenic embryos. The value of transgenic nuclear transfer embryos for the production of therapeutic proteins gives good reason for additional research into methods of improving pregnancy rates, especially when approaching the spring transition period and there are concerns about embryo viability and adequate progesterone production in recipients.

CHAPTER IV

GENERATION OF CLONED TRANSGENIC GOATS DERIVED FROM NT EMBRYOS AGGREGATED WITH ELECTROFUSED IVF EMBRYOS

INTRODUCTION

Chimeric animals were first reported in the mouse after aggregation of two zona pellucida-free 8-cell embryos (different strains of mice (Tarkowski, 1961). Chimeras have since been produced in other species through various techniques, including the injection of cells (Gardner, 1968; Moustafa and Brinster, 1972) or inner cell masses (Butler et al., 1987; Polzin et al., 1987; Rorie et al., 1994; Rossant and Frels, 1980; Rossant et al., 1982; Roth et al., 1989) into blastocysts in mice, sheep and goats. This procedure results in a chimeric fetus but nonchimeric placental tissues derived from the host blastocyst (Markert, 1984; Rossant et al., 1983).

Another approach often used to produce chimeric embryos involves separation of the inner cell mass and trophoblast by microsurgery or immunosurgery. A separated inner cell mass of the desired fetal type is then injected into a trophoblast devoid of inner cell mass cells (Gardner et al., 1973; Gardner and Johnson, 1973; Papaioannou, 1982; Rorie et al., 1994). This technique, termed blastocyst reconstitution, is one method that has been used to produce fetuses and offspring derived from a genotype differing from that of their placental tissues (Rossant et al., 1983; Surani et al., 1987).

Aggregation of asynchronous embryos has also been shown to produce offspring derived from cells at one developmental stage at aggregation, while the placental tissues were derived from the other cells that were at a different developmental stage at the time of aggregation (Fehilly and Willadsen, 1986; Picard et al., 1990; Surani and Barton, 1984).

These studies demonstrated that the more advanced cells in bovine, sheep and mouse aggregate embryos were more likely to form the fetus, while the less advanced cells were more likely to form the placental tissues (Fehilly and Willadsen, 1986; Picard et al., 1990; Surani and Barton, 1984). In contrast, it was reported in asynchronous mouse aggregate embryos (2-cell stage with 8-cell stage) that the more advanced blastomeres formed the trophoblast, while the less advanced cells formed the inner cell mass (Prather and First, 1987).

Tetraploid embryo complementation is another method commonly used to produce fetal and placental tissues from differing cell lineages in mice (Eakin and Behringer, 2003; Nagy and Rossant, 2001; Tanaka et al., 2001a). Tetraploid mouse embryos, produced after cytochalasin treatment or electrofusion of 2-cell embryos, have not resulted in live offspring (Henery et al., 1992; Kaufman and Webb, 1990; Kubiak and Tarkowski, 1985; Snow, 1976; Tarkowski et al., 1977), with the exception of three live tetraploid mice reported by Snow (1975). However, when tetraploid and diploid mouse cells were combined, the tetraploid cells contributed to the placental tissue but not to the developing fetus (Everett and West, 1996; Everett et al., 2000; Lu and Markert, 1980; James et al., 1995; Tarkowski et al., 1977). This distribution of tetraploid cells in chimeric embryos has resulted in the use of tetraploid embryo complementation to successfully rescue mouse embryos with known lethal mutations that disrupt placental function (Guillemot et al., 1994; Wu et al., 2003).

Nuclear transfer (NT) has the potential to be a valuable tool in the production of transgenic animals that produce pharmaceutically valuable proteins (Baguisi et al., 1999; Cibelli et al., 1998; Chen et al., 2002; Keefer, 2004; McCreath et al., 2000; Meade, 1997; Reggio et al., 2001; Schnieke et al., 1997). Unfortunately, many NT pregnancies are lost throughout gestation and survival of neonates in some species is poor (Heyman et al., 2002;

Kato et al., 2000; Wells et al., 1999; Wilmut et al., 1997). The high abortion rate and perinatal death rates after NT are thought to be due to abnormal placental development (DeSousa et al., 2001; Hashizume et al., 2002, Hill et al., 2000; Wells et al., 1999).

The objective of this study was to generate caprine aggregate embryos constructed from NT and tetraploid embryos with the intention of replacing the NT placental tissue with a placenta derived from in vitro fertilized (IVF) embryos. IVF embryos were electrofused in an attempt to produce tetraploid embryos for aggregation with NT embryos. If the electrofusion and aggregation procedures were to be successful in goats, pregnancy rates and the number of offspring produced from NT embryos could be improved.

EXPERIMENTAL DESIGN

Caprine embryos were produced by both IVF and NT. The IVF embryos were generated using Spanish-type goat oocytes inseminated with Boer goat semen from a single buck. IVF embryos at the 2-cell stage were exposed to electrofusion to produce tetraploid embryos. NT embryos were made with Spanish-type goat oocytes and a transfected cell line from a female Saanen goat fetus. When NT and IVF-Fused embryos reached the 8-cell stage, a blastomere aggregation procedure was used to construct the aggregate embryos (Figure 4.1). A portion of the NT embryos were not micromanipulated and served as control embryos. Aggregate and control NT embryos were transferred immediately after the completion of aggregation to recipient does on day 3 of the estrous cycle.

MATERIALS AND METHODS

Oocyte Collection

Spanish-type crossbred does were superstimulated by injecting ovine FSH (Ovagen; Immuno-Chemical Products, Auckland, NZ) twice daily (i.m.) for 4 days, prior to the day of

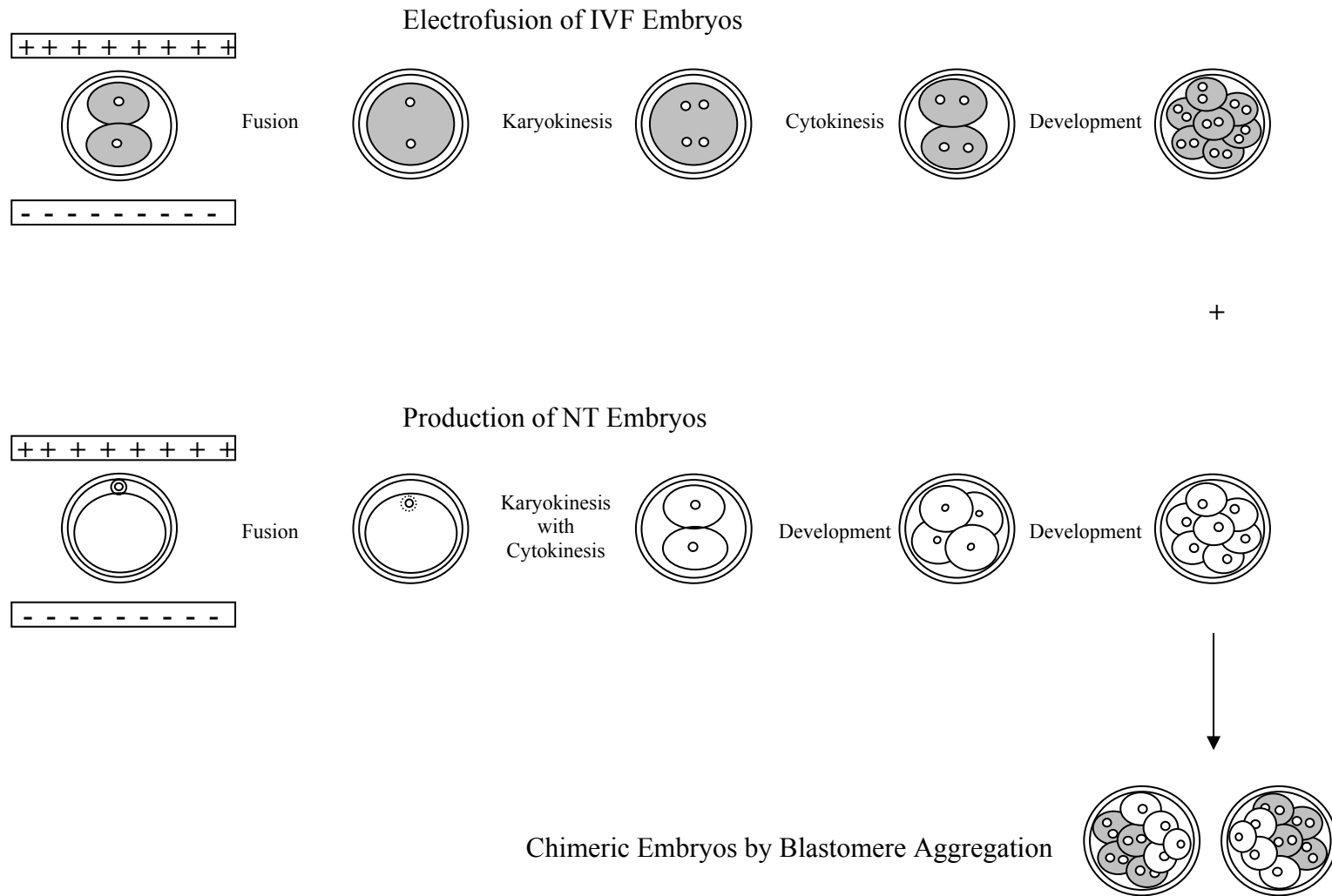


Figure 4.1. Production of caprine aggregate embryos from electrofused in vitro fertilized (IVF) embryos and nuclear transfer (NT) embryos.

follicle aspiration. Does were administered 1.32 mg of FSH at each injection, a total of 10.5 mg of FSH over 4 days for each doe. Estrus (day 0) was detected among the oocyte donor does and does were treated during the luteal phase of their estrous cycle, with the series of injections beginning between day 7 and day 16. All does (both oocyte donors and embryo transfer recipients) used in this study were handled and maintained in accordance with the Louisiana State University Agricultural Center Institutional Animal Care and Use Committee Guidelines.

Aspiration of oocytes occurred 24 hours after the last injection of FSH, as previously described (Reggio et al., 2001), adapted from the method previously reported by this laboratory (Graff et al., 2000). Does were administered general anesthesia (Halothane; Fort Dodge Animal Health, Fort Dodge, IA) and were placed on a hydraulic operating table for a mid-ventral laparotomy procedure while in dorsal recumbency. Ovaries were exteriorized and aspiration of all visible follicles was accomplished using an 18-gauge needle connected to a 20-gauge polyethylene tubing set (Cook Veterinary Products, Eight Mile Plains, Australia). An electric pump was used to aspirate medium at a rate of ~18 ml/minute. The medium for aspiration of oocytes consisted of phosphate-buffered saline (PBS) supplemented with 1% fetal bovine serum (FBS; Hyclone, Logan, UT), 0.4 of units/ml of sodium heparin (Elkins-Sinn, Cherry Hill, NJ) and 50 µg/ml of gentamicin (Gibco Laboratories, Grand Island, NY).

Centrifuge tubes containing warmed aspiration medium were used to collect oocytes, which were then washed eight times in maturation medium consisting of TCM-199 (Gibco) supplemented with 10% goat serum (Sigma Chemical Co., St. Louis, MO), 10 µg/ml of LH, 5 µg/ml of FSH and 1 µg/ml of estradiol-17β. Oocytes with two or more layers of compact cumulus cells were selected for maturation and allocated for either IVF or NT. Oocytes were

placed in groups of 10 to 20 into maturation medium in 35 μ l drops, and were overlaid with warmed, embryo-tested mineral oil (Sigma) for 18 to 20 hours at 38°C and 5% CO₂ in air.

Generation of NT Embryos

Isolation, Transfection and Preparation of Donor Cells. Fibroblast cells from a day 35 female goat fetus were isolated and cultured as previously described (Baguisi et al., 1999). Cells were transfected by lipofection with the MSP-1₄₂ gene (LipofectAMINE; Gibco) and selected by G418 challenge (Chen et al., 2002). The protein product of the MSP-1₄₂ gene, harvested from the milk of transgenic mice, has been shown to protect Aotus monkeys from a lethal challenge with *Plasmodium falciparum*, the organism that causes malaria (Stowers et al., 2002). Fibroblast colonies resistant to neomycin were genotyped prior to cell line establishment. Colonies were then expanded and cryopreserved (TCM supplemented with 20% serum and 5% DMSO) within four subpassages. This cell line has previously been used in NT studies to generate viable offspring (Chen et al., 2002; Reggio et al., 2002). Fibroblasts were thawed, washed in TCM-199 supplemented with 10% FBS (mTCM) and plated at a concentration of 12,000 cells per well in four-well culture plates 1 week prior to nuclear transfer. Actively growing cells were then cultured in TCM supplemented with 0.5% FBS for 4 days prior to nuclear transfer. Fibroblasts were removed from the culture well by treating with 0.5% trypsin (Sigma), were washed by centrifugation at 200 x g for 6 minutes in mTCM and were then transferred into cytoplasts (<1.5 hours).

Enucleation. NT embryos were produced as previously described (Reggio et al., 2001). Briefly, cumulus cells were removed from in vitro matured oocytes at 18 to 20 hours of maturation by mixing with a vortex (Vortex Genie 2, Fisher Scientific, Pittsburgh, PA) in 0.1% hyaluronidase (Sigma) in TL-HEPES (BioWhittaker, Walkersville, MD) for 2.25

minutes. Oocytes were then washed in modified TL-HEPES (mTLH; TL-HEPES supplemented with 10% FBS) and examined for the presence of a polar body. Oocytes with a polar body were stained with 1 $\mu\text{g}/\text{ml}$ of bis-benzimide (Sigma) in TL-HEPES for 2 minutes to label the DNA. Oocytes were then placed in the center of an elongated drop (200 μl) of manipulation medium (mTLH with 6.5 $\mu\text{g}/\text{ml}$ cytochalasin D) under warmed, embryo-tested mineral oil (Sigma). The polar body and metaphase spindle of each oocyte were removed by micromanipulation with a glass pipette (outer diameter of 25 μm , 35° bevel, spiked) under epifluorescent illumination. Cytoplasts were allowed to rest for at least 30 minutes at 38°C and 5% CO_2 in air prior to reconstruction.

Reconstruction, Fusion and Activation. Cytoplasts were transferred into elongated drops (200 μl) of manipulation medium overlaid with warmed, embryo-tested mineral oil containing prepared fibroblast cells. Using the same pipette, a single fibroblast was injected under the zona pellucida in contact with the oolemma, forming couplets. Two washes with increasing concentrations of fusion buffer (0.3 M mannitol, 0.1 mM MgSO_4 , 0.05 mM CaCl_2 , 0.5 mM HEPES and 4 mg/ml of BSA) were used to equilibrate couplets prior to electrofusion.

Couplets were manually aligned in groups of 10, so that the plane of contact between the cytoplast and karyoplast was in parallel with the electrode wires of a microslide fusion chamber filled with fusion buffer. A BTX Electrocell Manipulator 200 (Genetronics, San Diego, CA) was connected to the fusion chamber and used to generate a 1.3 kV/cm, 25 μsecond direct current pulse to fuse the couplets. Couplets were returned to mTCM for 30 minutes and then examined for fusion of the karyoplast and cytoplast. Couplets that fused were activated by exposure to ionomycin (5 μM) for 5 minutes followed by incubation in 10 $\mu\text{g}/\text{ml}$ of cyclohexamide (Sigma) and 6.5 $\mu\text{g}/\text{ml}$ of cytochalasin D (Sigma) prepared in

mTCM medium for 3 hours. Activated embryos were then washed extensively in mTCM and placed into culture. Embryos were co-cultured on buffalo rat liver (BRL) cells in mTCM and were allowed to develop to the 8-cell stage in 5% CO₂ in air at 38°C.

Generation of Electrofused IVF Embryos

In Vitro Fertilization. Sperm were prepared by thawing 0.5 ml straws of frozen, extended semen from a fertile, mature Boer buck in a 37°C water bath for 1 minute. Sperm were washed twice by centrifugation for 6 minutes at 200 x g in Brackett-Oliphant (BO) medium (Brackett and Oliphant, 1975) supplemented with 7.75 mM calcium lactate (Sigma) and 20% fetal bovine serum (Hyclone) (Han et al., 2001). Pelleted sperm were then resuspended in BO medium and incubated at 38°C and 5% CO₂ in air for 1 hour.

After 18 hours of in vitro maturation, cumulus-oocyte-complexes were washed twice in BO medium supplemented with 0.3 % bovine serum albumin (Sigma). Oocytes were then placed in groups of 15 into insemination drops of BO-sperm suspension (70 µl) containing ~12,000 sperm per cumulus-oocyte-complex. The time that sperm and oocytes were first placed in contact with each other was designated 0 hours post-insemination. Sperm and oocytes were co-incubated for 18 hours. Oocytes were then removed and washed to eliminate adherent sperm. Oocytes were then placed in groups of 25 to 50 into 500 µl wells mTCM and were cultured on a BRL cell monolayer at 5% CO₂ in air at 38°C.

Blastomere Fusion. Embryos were selected at the 2-cell stage between 24 and 30 hours post-insemination. Embryos were equilibrated through three washes of TCM-199 with increasing concentrations of fusion buffer and were placed in groups of five between the electrode wires of a microslide fusion chamber filled with fusion buffer that was connected to a BTX Electrocell Manipulator 200. Embryos were aligned with a 7.5 V, 5 second alternating

current pulse to orient the plane of contact between the blastomeres in parallel with the electrodes. A single 1.4 kV/cm direct current pulse of 100 μ seconds was used to fuse the blastomeres together. Embryos were then washed in mTCM and incubated for 1 hour. Embryos that had only a single cell upon evaluation after 1 hour were determined to have fused and were separated. Fused embryos were returned to co-culture on BRL cells in 5% CO₂ in air and 38°C until day 3 post-insemination. Electrofused IVF embryos at the 8-cell stage were selected for aggregation with NT embryos and were moved to mTLH.

Embryo Aggregation

Blastomere Removal. On day 3 of development (day 0 = enucleation and in vitro fertilization) electrofused 8-cell embryos were placed in an elongated drop (200 μ l) of mTLH under warmed, embryo-tested mineral oil. A pipette larger than that used for enucleation (outer diameter of 50 μ m, beveled at 40° with no spike) was used to remove four blastomeres (one at a time) from each embryo. The resulting “half-embryos” were moved to the opposite end of the drop from the separated blastomeres. The half-embryos were then moved to a new drop of mTLH while the separated blastomeres remained in the original drop. Blastomeres from NT 8-cell embryos were removed in the same manner, except from those NT 8-cell embryos designated to serve as controls, which were not micromanipulated.

Blastomere Insertion. For aggregation, NT half-embryos were transferred to the drop containing the separated blastomeres from the electrofused IVF embryos, while the electrofused IVF half-embryos were transferred to the drop containing the separated blastomeres from the NT embryos. The same pipette was used to inject four separated electrofused IVF blastomeres into each NT half-embryo and four separated NT blastomeres were injected into each electrofused IVF half-embryo. This procedure yielded aggregate

zona-enclosed 8-cell embryos with four blastomeres from the electrofused IVF embryo and four blastomeres from the NT embryo. Aggregate embryos were immediately pooled and washed in mTLH in preparation for embryo transfer to recipient does.

Embryo Transfer

Spanish-type crossbred does in natural estrus on day 0, the day of IVF and NT, were selected as embryo transfer recipients. NT control and aggregate 8-cell embryos were separately transferred into recipients on day 3 of their estrous cycle by a standard surgical procedure. Does were anesthetized and placed in dorsal recumbency for a mid-ventral laparotomy. The reproductive tract was exteriorized and ovaries were examined for indication of ovulation, the presence of one or more corpus hemorrhagicum (CH). Embryos (six to 17 per doe) were drawn into a small plastic Embryon Catheter (Rolon Medical, Watford, UK). The catheter was introduced through the ostium of the infundibulum of the oviduct ipsilateral to the ovary with a CH. The catheter was passed as far as possible without trauma into the oviduct and embryos were expelled into the oviduct. Recipients were allowed to recover from the surgical procedure and were returned to the herd.

Pregnancy Status

Recipients were first examined for pregnancy via transvaginal ultrasonography (Aloka 500-V; Aloka, Tokyo, Japan) 30 days post-estrus. Recipients were examined a second time 7 to 10 days later to verify pregnancy status. A positive diagnosis of pregnancy was dependant on the visualization of fetal heart beats. Recipients determined pregnant were examined throughout gestation at 2 week intervals. Transabdominal ultrasonography was used to monitor pregnancies after 90 days of gestation. Pregnant does were separated from the herd on day 120 of gestation and were then maintained in individual pens.

Parturition

Pregnant recipients underwent Caesarean sections on day 148 of gestation to ensure collection of placental tissues. Does received an injection (i.m.) of 10 mg of prostaglandin F_{2α} (Lutalyse; Upjohn, Kalamazoo, MI) and 12 mg of dexamethasone (Dexaject; Burns Veterinary Supply, Westbury, NY) 30 hours prior to surgery. Does were sedated immediately prior to surgery with 0.02 mg/kg body weight of xylazine (Butler Company, Columbus, OH) and anesthetized injection of 5 ml of 2% Lidocaine solution (Butler) into the epidural space at the lumbosacral junction.

Does were placed in dorsal recumbency and a mid-ventral incision was made extending from ~5 cm anterior to the mammary gland to ~10 cm posterior to the umbilicus. Upon entering the abdomen, the uterus was located and an incision of ~10 cm was made at the greater curvature of the pregnant uterine horn. The kids were extracted from the uterus, the umbilicus was clamped and cut, and the kids were delivered to a technician for life support. Kids were placed on heating pads, dried and rubbed vigorously to stimulate bodily function. Pure oxygen (1 l/min) by an intranasal tube, injections (i.m.) of 0.25 ml each of lasix (Furosamide[®]) and epinephrine (1:1000), and topical application of doxapram (Dopram[®]) to the oral mucus membranes were given to those kids that failed to respond. All kids were fed 100 to 150 ml colostrum within 6 hours of birth.

Characterization of Offspring and Placental Tissues

Ovarian arteries and veins along with the uterine artery, vein and stump were ligated and the uterus of each recipient doe was removed. Cotyledons were carefully detached from the caruncles of the uterus to separate the placenta. The placenta was immediately weighed and cotyledonary size and number were recorded prior to sampling of placental tissues.

From each placenta, two or more biopsies (1 cm²) were taken from the chorioallantois and cotyledons. From each recipient and kid, two or more skin biopsies (6 mm²) were obtained from the caudal lateral aspect of the thigh over the semitendinosus muscle. Blood samples were collected from each recipient and kid. Blood and tissue samples were used for polymerase chain reaction (PCR) and fluorescent in situ hybridization (FISH) analysis.

For PCR analysis, the Genomic Prep Kit (Amersham Pharmacia Biotech, Piscataway, NJ) was used to isolate genomic DNA from whole biopsies (Laird et al., 1991). MSP-1₄₂ specific primers were used to analyze each sample and a control for goat genomic DNA (goat exon 7). The presence of the MSP-1₄₂ transgene was verified by analysis of two positive control samples, one from an adult female goat transgenic for MSP-1₄₂ and one from the transgenic fibroblast cell line, and one negative control blood sample from a nontransgenic female goat.

For FISH analysis, blood samples were treated to collect lymphocytes, as described previously (Klinger et al., 1992). Tissue biopsies were washed multiple times in mTCM and then minced using fine iris scissors in a 35-mm Petri dish to harvest fibroblast cells. Dispersed tissue samples were washed three times in Minimal Essential Medium alpha Medium (MEM- α ; Gibco) supplemented with 15% FBS (Hyclone) and 1% each of 200 mM glutamine, 50 mg/ml of gentamicin, 100 mM sodium pyruvate and 10 mM nonessential amino acids. Dispersed tissue samples were then aseptically moved to 35-mm Petri dishes and cultured in 1 ml of MEM- α at 38°C in 5% CO₂ in air. Tissue pieces were removed when outgrowth of cells was first noted (~4 days). Medium on fibroblast cells was changed to fresh MEM- α every 48 hours until cells were 70% confluent. Fibroblasts were enzymatically

dispersed with trypsin and were spotted (500 cells/15 μ l of PBS) onto polycarbonate filters on a vacuum flask apparatus adjusted to 375 mm of Hg of vacuum pressure.

Fibroblasts and lymphocytes were hybridized (Klinger et al., 1992) after pretreatment (van de Corput et al., 1998) and were probed, as previously described (Baguisi et al., 1999). Briefly, the 13.6 kb goat β -casein promoter sequence of the MSP-142 transgene was labeled with digoxigenin and used to identify the endogenous goat beta casein site, as well as the transgene integration site by fluorescence in situ hybridization. The TSA-Direct system was used according to the manufacturer's protocol to amplify the signal (NEN Life Science Products, Boston, MA). DAPI counterstain was used to visualize and identify R-bands (Di Bernardino et al., 1987) on a Zeiss Axioskop microscope equipped with a digital camera. Images were captured and processed using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Sex chromosome probes (ID Labs, Inc., London, ON, Canada) were also used with the FISH procedure. The X chromosome was labeled with biotin and the Y chromosome was labeled with Cy3 to identify the sex chromosomal composition of samples.

RESULTS

Oocyte Aspiration

In total, 864 oocytes were collected from 42 does stimulated with FSH in seven oocyte aspiration procedures (six donors per procedure). On average, 21 oocytes were recovered from each doe. Oocytes were matured and 406 were allocated to the IVF and electrofusion group, while 458 were allocated to the NT group (Table 4.1).

NT Embryo Production and Development

Upon examination of matured oocytes for the presence of the first polar body indicating the metaphase II stage of development, 286 (62%) of the oocytes allocated were

Table 4.1. Caprine oocyte aspiration, recovery and allocation for use in nuclear transfer (NT) or in vitro fertilization (IVF)

Replicate	No. Donor Animals	No. Follicles Aspirated	No. (%) Oocytes Recovered	No. Oocytes Allocated for IVF	No. Oocytes Allocated for NT
1	6	123	119 (97)	59	60
2	6	105	135 (100+)	67	68
3	6	105	126 (100+)	64	62
4	6	97	121 (100+)	76	45
5	6	118	132 (100+)	82	50
6	6	76	112 (100+)	58	54
7	6	133	119 (89)	0	119
Totals	42	757	864 (100+)	406	458

selected for use in NT (Table 4.2). Of those 286 oocytes, 261 (91%) were successfully enucleated and a donor cell was successfully injected under the zona pellucida. Of the 261 reconstructed couplets, the donor cell was successfully fused to 167 cytoplasts (64%). These 167 NT embryos were activated and cultured until day 3 of development, when it was determined that 128 (77%) had cleaved and 75 (45%) were at the 8-cell stage. For the NT controls, 29 8-cell embryos were selected and transferred to recipient does. For aggregation, 30 NT 8-cell embryos were selected and underwent additional micromanipulation.

IVF and Electrofusion

Of the 406 oocytes allocated for IVF, 251 (62%) had cleaved (2- to 4-cells) when evaluated between 24 and 30 hours post-insemination (Table 4.2). From those 251 cleaved embryos, 114 were good-quality 2-cell embryos and were subjected to the electrofusion procedure. The electrofusion procedure yielded a 75% fusion rate or 86 electrofused 1-cell embryos. On day 3 of development, 87% of the electrofused embryos had cleaved, and 27% had reached the 8-cell stage. Of the 23 electrofused 8-cell embryos, 18 morphologically normal embryos were selected for aggregation.

Embryo Aggregation and Transfer

After the aggregation procedure, 34 aggregate embryos were produced from the 30 NT embryos and the 18 electrofused IVF embryos (Table 4.3). The number of electrofused IVF 8-cell embryos was the limiting factor in the number of aggregate embryos that could be produced, as each electrofused IVF embryo could only contribute to two aggregate embryos, for a maximum of 36 aggregate embryos. From the 36 possible aggregates, 34 were successfully constructed, yielding a 94% embryo aggregation rate.

Table 4.2. Generation of caprine electrofused in vitro fertilized (IVF) embryos and nuclear transfer (NT) embryos

Treatment Group	No. Oocytes	No. (%) Matured	No. (%) Reconstructed	No. (%) 2- to 4-cell IVF Embryos	No. 2-cell IVF Embryos to Fuse	No. (%) Fused	No. (%) 2-cell	No. (%) 8-cell
NT	458	286 (62)	261 (91)	n.a.	n.a.	167 (64)	128 (77)	75 (45)
IVF/Electrofusion	406	n.d.	n.a.	251 (62)	114	86 (75)	75 (87)	23 (27)

Table 4.3. Results for embryo aggregation, embryo transfer and pregnancy results after aggregation of caprine electrofused in vitro fertilized and nuclear transfer (NT) embryos at the 8-cell stage

Treatment Group	Total No. 8-Cell Embryos	No. Embryos Used for Aggregation	No. Aggregate Embryos Produced	No. Embryos Transferred	No. Recips	No. (%) Pregnant	No. (%) Offspring [†]
NT Control	59	30	34 [*]	29	3	1 (33)	1 (3)
Aggregate	23 [‡]	18 [‡]		34	5	1 (20)	4 (12)

[†]Percentages based on the number of embryos transferred.

[‡]Electrofused in vitro fertilized embryos.

^{*}Aggregate embryos produced from nuclear transfer embryos and electrofused in vitro fertilized embryos used for aggregation.

A total of 63 embryos, 29 NT controls and 34 aggregates, were transferred to eight recipients. The number of embryos transferred to each doe ranged from six to 17, a mean of 7.9 embryos per doe (Table 4.3). Pregnancy was established in two does (25%) and five live kids were delivered. From the NT control group, one of the three recipient does became pregnant (33%) after transfer of eight embryos and one kid was born. From the aggregate group, one of five recipient does became pregnant (20%) after transfer of 13 embryos and four kids were born. The number of offspring produced per number of embryos transferred, or production efficiency, was (3%) for the NT control group and (12%) for the aggregate group.

Offspring Characterization

The characteristics of the offspring resulting from transfer of NT control and aggregate embryos at birth are described in Table 4.4. The kid resulting from transfer of NT control embryos (Offspring ID #2014) resembled other kids born following NT with the same cell line (Figure 4.2). Kid #2014 weighed 3.9 kg at birth, was female and had a solid white hair coat. After delivery, kid #2014 was alert and vigorous.

The kids resulting from the transfer of aggregate embryos (Offspring ID #2010, #2011, #2012 and #2013) were born as quadruplets to a single doe (Figure 4.3). These kids were small (average 1.6 kg, range of 1.4 to 1.8 kg) and lethargic at delivery. These kids had to be revived and required extensive care the first 4 hours after birth. All four kids survived, are healthy and are currently > 2 years of age. These kids averaged 10.7 kg (ranged from 9.5 to 11.4 kg) when weaned at 70 days of age.

The two female kids (Offspring ID numbers 2010 and 2012) are similar in appearance to the NT control kid, with white hair coats and features characteristic of the Saanen dairy

Table 4.4. Offspring resulting from the aggregation of electrofused in vitro fertilized (IVF) blastomeres with nuclear transfer (NT) blastomeres

Offspring ID #	Treatment	Recipient ID #	Date of Birth	Gestation Length (days)	Birth Weight (kg)	Sex	Coat Color	Health Status
2014	NT Control	452 ^a	5/15/2003	149	3.9	Female	All white	Excellent
2010	Aggregate	519 ^b	4/2/2003	148	1.8	Female	All white	Excellent
2012	Aggregate	519 ^b	4/2/2003	148	1.5	Female	All white	Excellent
2011	Aggregate	519 ^b	4/2/2003	148	1.7	Male	White/brown	Excellent
2013	Aggregate	519 ^b	4/2/2003	148	1.4	Male	White/brown	Excellent

^aTransferred 8 embryos into recipient 452, resulting in 1 NT control offspring.

^bTransferred 13 embryos into recipient 519, resulting in 4 aggregate quadruplets.



Figure 4.2. Transgenic female goat produced after nuclear transfer (NT control).



Figure 4.3. Kids produced by aggregation of electrofused in vitro fertilized embryos and nuclear transfer aggregate embryos.

goat breed. The phenotype of the female kids implies that they are derived from the NT cell line. The two male kids (Offspring ID #2011 and #2013) are smaller framed and heavier muscled, with brown and white hair coats and features characteristic of Boer goats. The phenotype of the male kids demonstrates that these kids were derived from the IVF embryos.

The placental characteristics of the offspring produced in this study are shown in Table 4.5. The placental weight of the NT control kid was 1.35 kg, while the average placental weight of the aggregate kids was 0.36 kg (ranged from 0.25 to 0.50 kg). This is not surprising as a singleton and its placenta will weigh more than quadruplets and their placentas. The placental weight of the NT control kid was 35% of its birth weight, while the mean placental weight of the aggregate kids was 23% of their average birth weight. There were 106 cotyledons on the placenta of the NT control kid, while the average cotyledonary number of the placentas from the aggregate kids was 25.2. Mean cotyledonary diameter for the NT control placenta was 3.15 cm (SEM = 0.42 cm) and was 3.63 cm (SEM = 0.16) for the aggregate placentas.

The presence of the MSP-1₄₂ gene was detected in lymphocytes and placental tissues of the NT control kid (#2014), as well as in the female aggregate kids (#2010 and #2012), by PCR analysis (Table 4.6). The MSP-1₄₂ gene was not present in the blood of the male aggregate kids and was not detected in placental tissues. As anticipated, the blood and skin tissues of recipient does were found to lack the MSP-1₄₂ gene. Blood cells of the aggregate kids were also analyzed using the FISH procedure and it was found that none of the aggregate kids had tetraploid lymphocytes (Table 4.6). The male aggregate kids had only nontransgenic lymphocytes. The female aggregate kids had transgenic lymphocytes, however these cells were not found to have Y chromosomes.

Table 4.5. Placental weights and cotyledon number and size from offspring produced from aggregation of caprine electrofused in vitro fertilized embryos and nuclear transfer embryos

Offspring ID #	Sex	Treatment	Offspring Birth Weight (kg)	Placental Weight (kg)	Ratio (%) of Placenta to Birth Weight	No. of Cotyledons	Mean Diameter \pm SEM of Cotyledons (cm)
2014	F	NT Control	3.9	1.35	35	106	3.15 \pm 0.42
2010	F	Aggregate	1.8	0.5	28	30	4.03 \pm 0.23
2012	F	Aggregate	1.5	0.35	23	28	3.55 \pm 0.25
2011	M	Aggregate	1.7	0.25	15	23	3.15 \pm 0.19
2013	M	Aggregate	1.4	0.35	25	20	3.78 \pm 0.29

Table 4.6. FISH analysis of cells harvested from blood and placental tissue from offspring produced from aggregation of caprine electrofused in vitro fertilized embryos and nuclear transfer embryos

Goat ID #	Sex	Lymphocytes	Chorioallantois cells	Cotyledon cells
2010	F	All cells are tg and diploid	Most cells are tg and diploid	Mixed (tg and nontg) diploid cells
		No cells are tetraploid	Some cells are tg and tetraploid	Mixed (tg and nontg) tetraploid cells
		No cells have Y chrom.	Some nontg diploid cells have 1 Y Some nontg tetraploid cells have 2 Y	
2012	F	All cells are tg and diploid	All cells are tg (most are diploid)	Mixed (tg and nontg) diploid cells
		No cells are tetraploid	Some cells are tetraploid	Mixed (tg and nontg) tetraploid cells
		No cells have Y chrom.	No cells have Y chrom.	No cells have Y chrom.
2011	M	All cells are nontg and diploid	All cells are nontg (most are diploid)	All cells are nontg
		No cells are tetraploid	All tetraploid cells have Y chrom.	Some cells are tetraploid
		All/most cells have Y chrom.		All/most cells have Y chrom.
2013	M	All cells are nontg and diploid	All cells are nontg (most are diploid)	All cells are nontg
		No cells are tetraploid	All/most cells have Y chrom.	Some cells are tetraploid
		All/most cells have Y chrom.	All tetraploid cells have Y chrom.	Some cells have Y chrom.

tg = transgenic.

nontg = nontransgenic.

When placental cell cultures from the female aggregate kids were analyzed using FISH, the transgene was detected in some cells and both diploid and tetraploid cells were present. Kid #2010 (female) had some chorioallantois cells which were nontransgenic and diploid with a Y chromosome, as well as nontransgenic tetraploid cells with two Y chromosomes present. This placental tissue data combined with information from blood analysis demonstrates that kid #2010 was derived from the NT blastomeres (transgenic and female) of the aggregated embryo while the electrofused IVF blastomeres (nontransgenic and male) formed placental tissues. All of the chorioallantois cells from kid #2012 were transgenic and no Y chromosome was present. The male aggregate kids (#2011 and #2013) were found to have diploid and tetraploid nontransgenic, Y chromosome-bearing chorioallantois cells.

DISCUSSION

The ability to rapidly and efficiently reproduce transgenic animals is very important to successfully supply pharmaceutically valuable proteins from these animals. Traditional microinjection procedures often yield transgenic animals from < 1% of the sperm-exposed ova that are injected (Meade, 1997). Cloning, or NT, has the potential to generate transgenic animals more quickly, by utilizing cells that have been proven to carry the transgene of choice at the desired location in the genome (Keefer, 2004; Meade, 1997). However, the efficiency of NT production is still low, partly due to low rates of pregnancy initiation and maintenance (Renard et al., 2002). In cattle and sheep, the percentage of pregnancy loss during the first trimester for NT-derived embryos has been reported to be > 50% (Cibelli et al., 1998; Kubota et al., 2000; Wells et al., 1997; Wells et al., 1998b; Wells et al., 1999; Wilmut et al., 1997). This is in sharp contrast to abortion rates of 7 to 11% for bovine IVF-derived embryos

throughout gestation (Hasler et al., 1995) and 5 to 10 % for in vivo fertilized embryos (Alexander et al., 1995; Forar et al., 1996). The high abortion rates noted after transfer of NT embryos has been related to poor placental formation and function by many researchers (De Sousa et al., 2001; Hashizume et al., 2002; Heyman et al., 2002; Hill et al., 2000; Hill et al., 2002; Ravelich et al., 2004b; Wells et al., 1999).

The high abortion rates noted after NT in cattle and sheep have not been reported after NT in goats (Baguisi et al., 1999; Keefer et al., 2001; Reggio et al., 2001). However, goats can be good model species for the production of transgenic dairy animals due to their shorter gestation time and generation interval (Meade, 1997). The objective of this study was to generate caprine aggregate embryos constructed from NT and tetraploid embryos with the intention of replacing the NT placenta with a placenta derived from in vitro fertilized (IVF) embryos. This work was intended serve as a model for subsequent aggregate embryo production in cattle.

Aggregation of NT embryos has been examined by other groups in mice (Eckardt et al., 2004) and in cattle (Wells and Powell, 2000) as a method to improve developmental rates. Those studies used up to three embryos (one NT embryo with one or two IVF embryos) to construct a single aggregate embryo, while the technique of blastomere aggregation used in this study generated two aggregate embryos from a single NT and electrofused IVF embryo.

In addition to reduced numbers of embryos necessary to produce aggregate embryos, the methods used in this study to produce aggregate embryos may be preferable to those used in the previous studies as aggregation of NT embryos with normal, diploid IVF embryos may lead to the production of chimeric embryos. In mice, aggregate embryos had NT cells in the inner cell mass only 34% of the time, contributing between 30% and 100% of the inner cell

mass cells (Eckardt et al., 2004). In cattle, aggregate embryos had NT cells in 100% of the inner cell masses, but contribution ranged from 19 to 100% of the inner cell mass cells (Wells and Powell, 2000). In mice, aggregation of electrofused (tetraploid) blastomeres to diploid blastomeres has been reported to produce offspring derived from the diploid cells only, with the tetraploid cells confined to the placental tissues (Everett and West, 1996; Everett et al., 2000; James et al., 1995).

Tetraploid embryo complementation has also been used with NT embryos in mice (Amano et al., 2002). In that study, ES cells were used as nuclear donors in a nuclear transfer procedure. The inner cell mass was then removed from the NT blastocyst and was injected into a tetraploid blastocyst. This procedure resulted in 5% live offspring, more than the 2% that were produced after transfer of NT embryos from the same cell line (Amano et al., 2002).

In this study, electrofused IVF and NT aggregation was successfully used to produce four live, healthy kids. Analysis of the four kids by PCR and FISH showed that one of the kids (Offspring ID #2010) was derived solely from the NT blastomeres in the aggregate embryo, while her placental tissues were derived from the electrofused IVF blastomeres. In contrast to a previous study in cattle that utilized electrofused IVF embryos for aggregation with embryonic stem-like cells (Iwasaki et al., 2000), no chimeric animals were produced in this study. The placental tissue of kid #2010 was chimeric, indicating that the electrofused blastomeres in the aggregate embryos were distributed to the extra-embryonic tissues but not to the fetus.

The birth of two male offspring in this study demonstrates that some electrofused embryos either revert back to a diploid state or were not completely tetraploid. It has been reported that 14% of putative tetraploid bovine embryos produced by electrofusion at the 2-

cell stage were in fact diploid (Iwasaki et al., 1999). Chromosomal analysis of the electrofused IVF embryos was not performed in this study since it was assumed that a majority of the fused embryos that developed to the 8-cell stage were tetraploid based previous reports in the literature.

Uniform tetraploidy has been reported after electrofusion of 2-cell embryos in mice (James et al., 1992), rats (Krivokharchenko et al., 2002) and pigs (Prather et al., 1996). In the cow, electrofusion of blastomeres at the 2-cell stage yielded 82% tetraploid embryos at the 2- to 4-cell stages and 86% tetraploid embryos at the morula stage (Iwasaki et al., 1999). However, another study has reported that only ~13% of electrofused bovine embryos were tetraploid, while ~42% were diploid ↔ tetraploid mosaic embryos (Curnow et al., 2000). In this study, all indications are that at least one of the electrofused IVF embryos continued development as a tetraploid embryo at least to the 8-cell stage, giving rise to placental tissues only and resulting in the production of a nonchimeric kid after aggregation with NT blastomeres.

CHAPTER V

THE EFFECT OF ELECTROFUSION METHOD AND TIME OF CLEAVAGE ON BOVINE EMBRYO DEVELOPMENT AFTER BLASTOMERE FUSION AT THE 2-CELL STAGE

INTRODUCTION

Tetraploidy in mouse embryos has been reported as a result of various treatments (Edwards, 1958; Eglitis, 1980; Kubiak and Tarkowski, 1985; O'Neill et al., 1990; Snow, 1973). Tetraploid embryos were produced after injecting female mice with colchicine prior to mating through the vaginal plug into both uterine horns, into the ovarian capsule when fertilized oocytes were at the pronuclear stage (~15 hours post-mating) or directly into the uterus prior to mating (Edwards, 1958). However, colchicine treatment tended to decrease embryonic development. Cytochalasin B has also been used to produce tetraploid embryos by preventing cytoplasmic division in mouse embryos (Snow, 1973). Mouse 2-cell embryos were cultured for 12 hours in the presence of 10 µg/ml of cytochalasin B in Whitten's medium and yielded tetraploid blastocysts at rates up to 75% (Snow, 1973). Reduced development noted after treatment of embryos with cytochalasin B may be due to toxic effects on the cell after long incubation periods (Tarkowski et al., 1977).

Fusion of isolated blastomeres is another method that has been used to generate tetraploid embryos. Polyethylene glycol (PEG) has been used to produce tetraploid mouse embryos by blastomere fusion (Eglitis, 1980). Inactivated Sendai virus may also be used as a method to fuse blastomeres for the production of tetraploid embryos (O'Neill et al., 1990). Electrofusion of blastomeres has been developed to produce mouse tetraploid embryos by the fusion of blastomeres within the zona pellucida without requiring exposure of the embryo to toxic chemicals or to Sendai viruses (Kubiak and Tarkowski, 1985).

Electrofusion has also been used in species other than the mouse to produce tetraploid embryos. Fusion of rabbit blastomeres at rates reaching 90% was achieved using variable field strengths and pulse durations (Ozil and Modlinski, 1986). Those fused rabbit embryos developed into hatched blastocysts, but subsequently did not develop past the second trimester of gestation. Upon examination, the rabbit fetuses developing from electrofused embryos were all tetraploid with the exception of a single diploid ↔ tetraploid mosaic fetus.

Porcine embryos have also been electrofused with a 5 V/mm alternating current for 10 seconds followed by a 120 V/mm direct current pulse of 30 μseconds (Prather et al., 1996). Fused embryos were cultured to the blastocyst stage and stained to assess ploidy. All fused porcine blastocysts were tetraploid when assessed for DNA content. Electrofusion has also been applied to rat and monkey embryos (Krivokharchenko et al., 2002; Schramm and Paprocki, 2004). Rat embryos were exposed to two 20 μsecond 60 V direct current pulses and fusion rates of 96% were reported (Krivokharchenko et al., 2002). Blastocysts from electrofused rat embryos in that study were found to be uniformly tetraploid upon analysis. Monkey embryos were exposed to two 1.5 kV/cm, 50 μsecond direct current pulses and fusion rates of 82% were reported (Schramm and Paprocki, 2004). As with porcine and rat embryos, blastocysts from electrofused monkey embryos were found to be tetraploid.

Electrofusion has been used by two groups in cattle to produce tetraploid embryos (Curnow et al., 2000; Iwasaki et al., 1989; Iwasaki et al., 2000). A field strength of 1 kV/cm with two direct current pulses of 10 or 25 μseconds yielded optimal fusion rates of 73 to 95% in bovine embryos (Iwasaki et al., 1989). A few of the fused embryos reached the morula stage, although most did not develop beyond the 8-cell stage. Chromosomal analysis of fused embryos showed that ~80% were tetraploid. Another study reported optimal fusion

parameters of 1.4 kV/cm with a single direct current pulse of 100 μ seconds (Curnow et al., 2000). The fused embryos resulting from treatment were returned to culture and developed to the blastocyst stage.

The objectives of this study were to evaluate electrofusion parameters for bovine embryos produced by in vitro fertilization (IVF) and determine their developmental potential through in vitro culture.

EXPERIMENTAL DESIGN

Bovine embryos were selected at cleavage to the 2-cell stage and exposed to two different methods of electrofusion (Method A, a single 1.4 kV/cm, 100 μ second direct current pulse; Method B, two 1.4 kV/cm, 50 μ second direct current pulses). Embryos were cultured to the blastocyst stage.

MATERIALS AND METHODS

Embryo Production

Bovine cumulus-oocyte-complexes were collected from ovaries at an abattoir (BoMed, Madison, WI) and shipped overnight in BoMed's TCM-199-based maturation medium to the Embryo Biotechnology Laboratory at Louisiana State University. After 22 to 24 hours maturation, oocytes were placed in a defined insemination medium (Brackett and Oliphant, 1975) with Holstein bull semen (bull #7H5188) at a concentration of 1.2×10^6 sperm/ml. The time that sperm and oocytes were first co-incubated was termed 0 hours post-insemination. After 6 to 9 hours of co-incubation, oocytes were washed in TCM-199 medium (Gibco Laboratories, Grand Island, NY) supplemented with 0.3% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and mixed with a vortex ((Vortex Genie 2, Fisher

Scientific, Pittsburgh, PA) with 0.1% hyaluronidase (Sigma) to remove cumulus cells.

Inseminated ova were then placed in CR1aa culture medium (Rosenkrans and First, 1994) with 0.3% BSA for the first 3 days of culture. Fetal bovine serum (Hyclone, Logan, UT) was added at 5% for days 4 through 7 of in vitro culture. Embryos were cultured in humidified 5% CO₂ in air at 39°C.

Electrofusion

Oocytes were observed for cleavage at 30, 32, 34 and 36 hours post-insemination and good quality 2-cell embryos were selected for fusion at those times. Fusion buffer consisted of 0.3 M mannitol, 0.1 M MgSO₄·7H₂O, 0.05 mM CaCl₂, 0.5 mM HEPES and 0.4% BSA (Sigma) and ranged in pH from 7.2 to 7.4. Embryos were equilibrated through a series of washes of TCM-199 with increasing concentrations of fusion buffer until embryos rested in fusion buffer in a microslide fusion chamber. A BTX Electrocell Manipulator 200 (Gentronics, San Diego, CA) was used for the electrofusion procedure. Embryos were aligned with a 5 second, 7.5 V alternating current pulse. Two protocols (treatments) were used to fuse embryos: Method A consisted of a single 1.4 kV/cm, 100 μsecond direct current pulse while Method B consisted of two 1.4 kV/cm, 50 μsecond direct current pulses. Treatments were performed at each timepoint on alternate weeks with four replicates for each treatment over 8 weeks.

Treated embryos were washed in TCM-199 medium supplemented with 0.3% BSA and were observed after 1 hour for fusion of the two blastomeres (Figure 5.1). Fused embryos were returned to CR1aa medium and cultured under the same conditions as in vitro produced embryos. Embryos that lysed after the fusion procedure were discarded. Cleavage was assessed at 60 hours post-fusion. Blastocyst rate was assessed on day 7 and day 8 post-

insemination (144 and 168 hours post-fusion). Selected electrofused embryos were stained with 2 µg/ml of Hoechst 33342 (Sigma) in TCM-199 for 10 minutes to label the nucleus and were viewed with epifluorescent illumination to visualize the nucleus.

Data Analysis

Data for fusion rates, cleavage rates and blastocyst rates were analyzed using analysis of variance by the general linear model procedure. Simple linear regression was used to describe the equation fitting blastocyst development of electrofused embryos (SAS, version 8.2; SAS Institute, Inc., Cary, NC).

RESULTS

In total, 1,981 oocytes were inseminated in eight replicates over a 9 week period. Of the oocytes subjected to in vitro fertilization, 1,545 (78%) cleaved following fertilization. Of the embryos that cleaved following in vitro fertilization, 709 good quality 2-cell embryos were subjected to electrofusion. Overall, 515 of 709 (73%) embryos exposed to electrofusion had fused and formed a single cell embryo at 1 hour post-fusion. In total, 63% of the 515 fused embryos cleaved following treatment. Bovine embryos prior to and following electrofusion are shown in Figure 5.1. Electrofused embryos stained with Hoechst 33342 (Sigma) are shown in Figure 5.2.

With electrofusion Method A, 345 embryos were exposed to a single, 1.4 kV/cm 100 µsecond pulse. With electrofusion Method B, 364 embryos were exposed to two, 1.4 kV/cm 50 µsecond pulses. Only 4% of the embryos lysed after the fusogenic pulse. The fusion method had no effect on the percentage of embryos that fused following treatment or on the percentage of embryos that cleaved following fusion (Table 5.1).

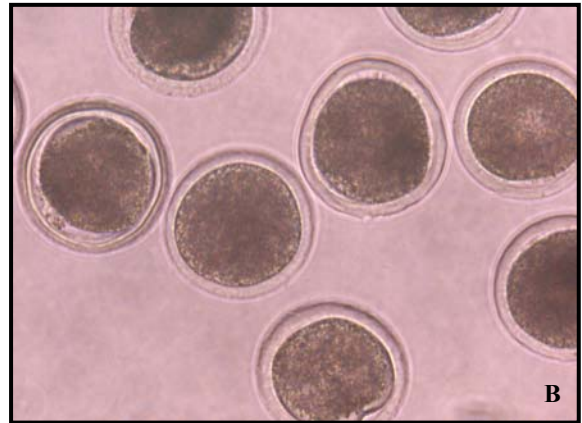


Figure 5.1. Bovine 2-cell embryos. A. Prior to electrofusion, 28 hours post-insemination. B. After electrofusion at 30 hours post-insemination, 1 hour post-fusion.

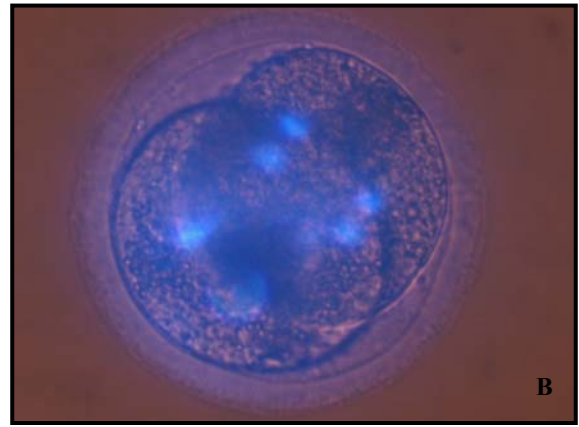


Figure 5.2: Bovine electrofused embryos stained with Hoechst 33342. A. Embryo electrofused at 28 hours post-insemination, photographed at 5 hours post-fusion. B. Embryo electrofused at 30 hours post-insemination, photographed at 20 hours post-fusion.

Table 5.1. Effect of fusion method on fusion and subsequent cleavage rate of electrofused bovine embryos

Treatment Method	Pulse	Duration	Replicates	No. Embryos Pulsed	No. Embryos Fused (%)	Mean % Fused \pm SEM	No. Embryos Cleaved (%) [*]	Mean % Cleaved \pm SEM
A	1 x 1.4 kV/cm	100 μ seconds	4	345	255 (73.9)	73.8 \pm 1.7 ^a	157 (61.6)	59.3 \pm 4.5 ^a
B	2 x 1.4 kv/cm	50 μ seconds	4	364	260 (71.4)	71.5 \pm 3.2 ^a	165 (63.5)	58.1 \pm 6.0 ^a
Total			8	709	515 (72.6)	72.6 \pm 1.8	322 (62.5)	58.7 \pm 3.7

^{*} Assessed at 60 hours post-fusion, based on number fused.

^aValues with different superscripts in the same columns are significantly different (P<0.05).

Embryos that cleaved and had fused at 30 hours post-insemination were more likely to cleave again following fusion than those that cleaved at 34 and 36 hours post-insemination (Table 5.2). Time of first cleavage post-insemination (30 to 36 hours after sperm and oocytes were co-incubated; also the time that electrofusion occurred) had no effect on the percentage of embryos that fused but did affect the percentage of embryos that cleaved again following fusion ($P < 0.05$). Replicate was also significant using this analysis.

These data may be described by the equation $y = 273.55 - 6.51x$, with y = the percentage of embryos cleaving following fusion and x = time of first cleavage (in hours) (Figure 5.3). This regression was highly significant ($P < 0.01$) and the coefficient of determination of these data was 0.50. Although replicate was significant when data were analyzed using general linear model of regression, the addition of replicate to the linear regression was not significant and only marginally improved the coefficient of determination (0.54).

Overall, 14% of the embryos that cleaved following electrofusion developed to the blastocyst stage by 168 hours post-fusion. There was no difference in the percentage of blastocysts that formed from embryos that cleaved following electrofusion by Method A or Method B at 144 and 168 hours post-fusion (Table 5.3). Time of cleavage post-insemination (30 to 36 hours after sperm and oocytes were co-incubated; also the time that electrofusion occurred) did have an effect on the percentage of cleaved, fused embryos that developed to the blastocyst stage ($P < 0.05$). Embryos that cleaved and were fused at 30 hours post-insemination were more likely to reach the blastocyst stage at 168 hours post-fusion than those embryos that initially cleaved at 34 and 36 hours post-insemination (Table 5.4). Blastocyst rates followed a similar pattern at 144 hours post-insemination but were not

Table 5.2. Effect of timing of first cleavage on fusion and subsequent cleavage rates of electrofused bovine embryos

Time (hours) [†]	n [‡]	Number (%) Fused	Mean % Fused ± SEM	Number (%) Cleaved*	Mean % Cleaved ± SEM
30	255	188 (73.7)	73.1 ± 2.8 ^a	140 (74.5)	77.3 ± 5.0 ^a
32	195	144 (73.8)	73.6 ± 3.6 ^a	93 (64.6)	65.3 ± 5.8 ^{ab}
34	149	99 (66.4)	68.2 ± 4.4 ^a	55 (55.6)	55.0 ± 4.4 ^{bc}
36	110	84 (76.4)	75.8 ± 3.4 ^a	34 (40.5)	37.3 ± 6.5 ^c
Total	709	515 (72.6)	72.6 ± 1.8	322 (62.5)	58.7 ± 3.7

[†]Post-insemination.

[‡]Number of 2-cell embryos exposed to electrical pulse.

*Assessed at 60 hours post-fusion, based on the number of fused oocytes.

^{abc}Values with different superscripts in the same columns are significantly different (P<0.05).

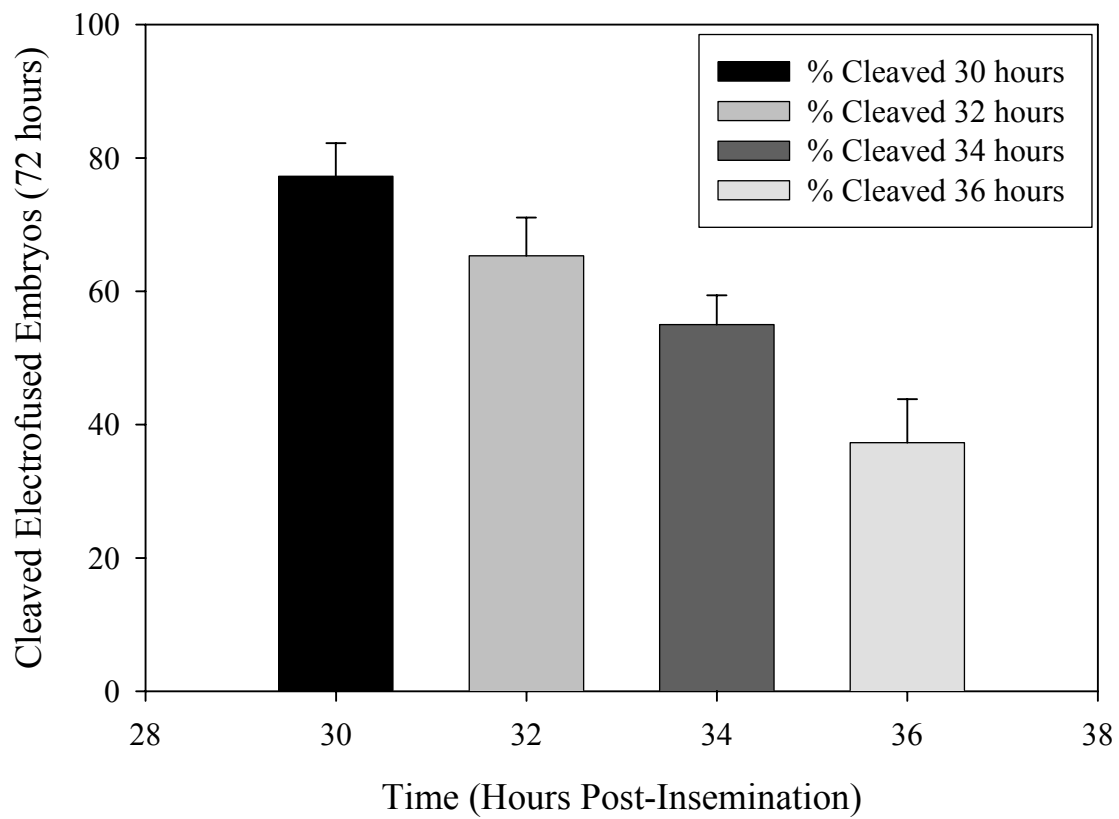


Figure 5.3. Percentage of bovine electrofused embryos (\pm SEM) that cleaved (determined at 72 hours post-insemination) following electrofusion at the 2-cell stage at 30, 32, 34 and 36 hours post-insemination.

Table 5.3. Effect of two fusion methods on blastocyst development of electrofused bovine embryos

Treatment Method	Replicates	Number of Embryos [†]	144 Hours [*]		168 Hours [*]	
			Number of Blastocysts (%) [‡]	Mean % Blastocysts ± SEM	Number of Blastocysts (%) [‡]	Mean % Blastocysts ± SEM
A ^{**}	4	120	15 (12.5)	8.4 ± 3.4 ^a	14 (11.7)	7.3 ± 2.7 ^a
B ^{***}	4	140	18 (12.9)	8.9 ± 3.2 ^a	23 (16.4)	10.9 ± 3.0 ^a
Total	8	260	33 (12.7)	8.6 ± 2.3	37 (14.2)	9.0 ± 2.0

[†]Cleaved following fusion (2- to 8-cells), assessed at 60 hours post-fusion.

[‡]Calculated from the number of embryos cleaved that remained in culture.

^{*}Post-fusion.

^{**} 1 X 1.4 kV/cm; 100 µseconds.

^{***} 2 X 1.4 kV/cm; 50 µseconds.

^aValues with different superscripts in the same columns are significantly different (P<0.05).

Table 5.4. Effect of time of cleavage (and fusion) on blastocyst development of electrofused bovine embryos

Time (hours) [*]	Replicates	Number of Embryos [†]	144 Hours ^{**}		168 Hours ^{**}	
			Number of Blastocysts (%) [‡]	Mean % Blastocysts ± SEM	Number of Blastocysts (%) [‡]	Mean % Blastocysts ± SEM
30	8	107	20 (18.7)	15.8 ± 4.0 ^a	24 (22.4)	19.4 ± 3.6 ^a
32	8	78	9 (11.5)	10.3 ± 6.1 ^a	10 (12.8)	10.8 ± 4.7 ^{ab}
34	8	45	2 (4.4)	4.2 ± 2.7 ^a	2 (4.4)	4.2 ± 2.7 ^b
36	8	30	2 (6.7)	4.2 ± 4.2 ^a	1 (3.3)	2.1 ± 2.1 ^b
Total	8	260	33 (12.7)	8.6 ± 2.3	37 (14.2)	9.0 ± 2.0

^{*} Post-insemination.

[†] Cleaved following fusion (2- to 8-cells), assessed at 60 hours post-fusion.

[‡] Calculated from the number of embryos cleaved.

^{**} Post-fusion.

^{ab} Values with different superscripts in the same columns are significantly different (P<0.05).

significantly different between groups when analyzed by general linear models procedures. Replicate was not significant when included in the analysis at either 144 or 168 hours post-fusion. The linear regression of the percentage of blastocysts formed at 144 hours post-fusion against time of first cleavage (and electrofusion) was significant ($P < 0.05$) with the equation that best fit the data being $y = 76.59 - 2.06x$, with y = the percentage of blastocysts at 144 HPF and x = time of first cleavage (in hours) (Figure 5.4). The resulting coefficient of determination was 0.14. The linear regression of the percentage of blastocysts formed at 168 hours post-fusion against the time of first cleavage was significant ($P < 0.01$). The best fitting equation was described as $y = 105.84 - 2.93x$, with y = percentage of blastocysts at 168 hours post-fusion and x = time of first cleavage (in hours) (Figure 5.5). The resulting coefficient of determination was 0.34.

Embryos that did not fuse following electrofusion were cultured as unfused embryo controls under the same conditions as the fused embryos. Of the 149 unfused cleaved embryos, 12 and 13% reached the blastocyst stage at 144 and 168 hours post-fusion, respectively. A total of 395 untreated embryos was also cultured as controls. At 168 and 192 hours post-insemination (corresponding to 144 and 168 hours post-fusion, respectively), 18 and 21%, respectively, had reached the blastocyst stage. Over eight replicates, approximately the same percentage of the electrofused, unfused and control embryos developed to the blastocyst stage at 144 and 168 hours post-fusion (Table 5.5).

DISCUSSION

Electrofusion at the 2-cell stage was first utilized specifically for the production of tetraploid embryos in mice by Kubiak and Tarkowski (1985). Those researchers cited the repeatability, exact measurement of parameters and short duration of embryonic exposure as

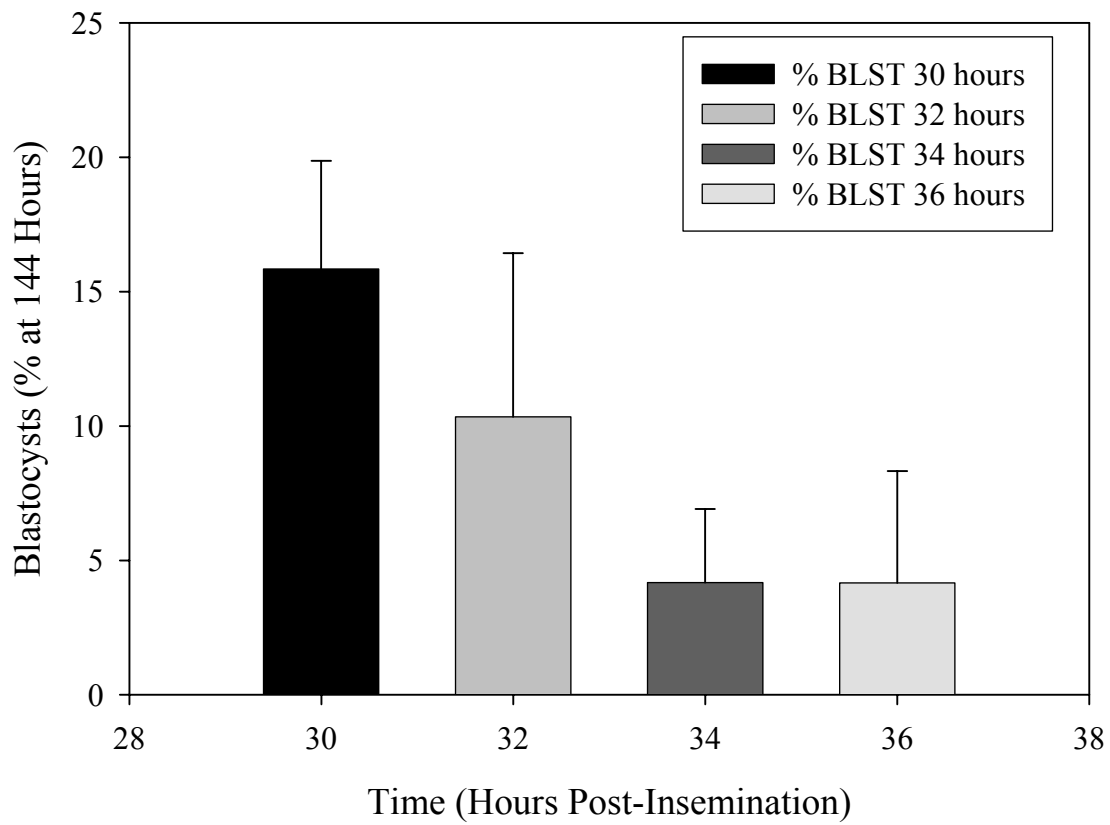


Figure 5.4. Percentage of bovine electrofused embryos (\pm SEM) that formed blastocysts (BLST) at 144 hours post-insemination following electrofusion at the 2-cell stage at 30, 32, 34 and 36 hours post-insemination.

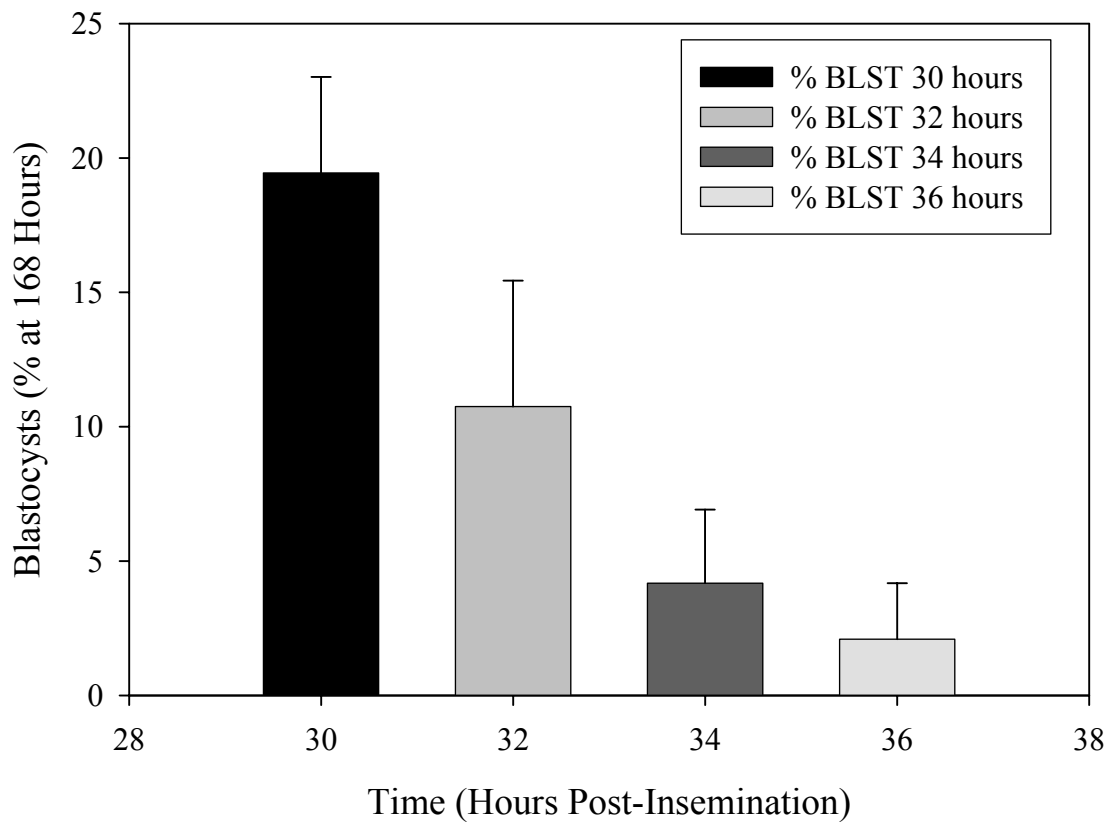


Figure 5.5. Percentage of bovine electrofused embryos (\pm SEM) that formed blastocysts (BLST) at 168 hours post-insemination following electrofusion at the 2-cell stage at 30, 32, 34 and 36 hours post-insemination.

Table 5.5. Blastocyst development of control embryos and embryos that did not fuse following treatment compared to the development of electrofused bovine embryos

Treatment Group	Replicates	Number of Embryos [†]	144 Hours [*]		168 Hours [*]	
			Number of Blastocysts (%) [‡]	Mean % Blastocysts ± SEM	Number of Blastocysts (%) [‡]	Mean % Blastocysts ± SEM
Electrofused Embryos	8	260	33 (12.7)	12.1 ± 3.3 ^a	37 (14.2)	13.6 ± 2.4 ^a
Unfused Embryos	8	149	18 (12.1)	13.3 ± 3.3 ^a	19 (12.8)	11.4 ± 3.8 ^a
Control Embryos	8	395	70 (17.7)	20.0 ± 6.4 ^a	82 (20.7)	21.4 ± 6.5 ^a

[†]That cleaved (2- to 8-cells), assessed at 60 hours post-fusion.

[‡]Calculated from the number of embryos cleaved.

^{*}Post-fusion.

^aValues with different superscripts in the same columns are significantly different (P<0.05).

advantages of the use of electrofusion. That study utilized two pulses of 1 kV/cm for varying durations to produce tetraploid mouse embryos.

Increased fusion rates between the two blastomeres of rabbit embryos was described after four pulses when compared to a single pulse (Ozil and Modlinski, 1986). More bovine embryos were reported to fuse after application of two pulses during electrofusion except when the pulse duration was 100 μ seconds as this led to lysis of the cell membrane (Iwasaki et al., 1989). Another study reported the optimal fusion parameters for bovine 2-cell embryos to be a single 1.4 kV/cm 100 μ second pulse (Curnow et al., 2000). It was hypothesized based on the previous reports that multiple pulses could enhance electrofusion by increasing the number of pores between the cell membranes. However, in this study, the electrofusion method (Method A: single 100 μ second, 1.4 kV/cm pulse; Method B: two 50 μ second, 1.4 kV/cm pulse) had no effect on the number of embryos that fused, cleavage rate following fusion or blastocyst formation following fusion in the present study.

The observation that the time of first cleavage after insemination has an effect on cleavage and blastocyst rates post-fusion was not surprising. Several studies have reported increased blastocyst rates from in vitro produced embryos that cleaved earlier in the post-insemination period (Lonergan et al., 1999; Van Soom et al., 1997; Ward et al., 2001). Those authors reported that the embryos that cleaved first were the most likely to form blastocysts in vitro. It has also been reported that viable in vitro produced embryos (those that reached the blastocyst stage) completed their first few cell cycles faster than did nonviable embryos (Holm et al., 1998). Holm et al. (1998) reported a mean first cleavage time of viable embryos to be 32 hours post-insemination compared with 35 hours post-insemination for nonviable embryos. Other factors have also been implicated in the different developmental kinetics of

dividing bovine embryos including culture conditions (Bredbacka and Bredbacka, 1996; Peippo et al., 2001; Van Soom et al., 1996;), embryo sex (Bredbacka and Bredbacka, 1996; Dominko and First, 1993; Peippo et al., 2001; Yadav et al., 1993), chromosomal abnormalities (Viuff et al., 2001; Yadav et al., 1993), gene product modifications (Brevini et al., 2002) and genetic influences (Boediono et al., 2003; Comizzoli et al., 2000; Hillery et al., 1990; Rubeš et al., 1999; Shi et al., 1990; Ward et al., 2001). Most recently, the stress-related protein P66^{SHC} has been shown to play a role in early developmental kinetics of bovine embryos (Favetta et al., 2004). This demonstrates that the time of cleavage post-insemination, while an indicator of developmental capacity, is not the only factor influencing embryonic growth and developmental rates.

When data for cleavage rates after electrofusion of 2-cell embryos were analyzed in the present study, replicates were significantly different ($P < 0.05$). The variance among weeks did not show any obvious trends, indicating that the variance was not due to technique problems at the beginning of the study but perhaps due to oocyte variation on a weekly basis. In contrast, replicates were not significantly different in blastocyst rates following electrofusion. The coefficients of determination (R^2 values) were not high in this study ($R^2 < 0.50$), which implies some outside source(s) of variation within the model. The addition of replicate to the statistical analysis was not significant, which implies that some source of variation not controlled by experimental conditions was affecting the results.

This study demonstrates that electrofusion can produce good rates (73%) of fused bovine embryos. Those fused embryos can also go on to divide and form blastocysts. However, the percentage of the oocytes that were fused and became blastocysts after electrofusion was low. The percentage of electrofused embryos that formed blastocysts was

comparable to the number of unfused and control embryos that formed blastocysts, although the blastocyst rates among these groups were low when compared with rates using the same culture system in our laboratory. The low rates may be due to the increased handling and disturbance of the embryos at cleavage to select for those embryos that were to be electrofused. Also, the good quality 2-cell embryos were selected for electrofusion leaving the poorer quality embryos to serve as culture controls, which could have affected the results, yielding lower blastocyst rates.

The percentage of the electrofused embryos that were tetraploid after electrofusion needs to be determined through further study. Previous studies of bovine electrofused embryos report differing percentages of embryos actually determined to be tetraploid by karyotypic analysis. Tetraploid rates of 78.6% were reported following electrofusion in one study (Iwasaki et al., 1989), while another reported only 12.5% tetraploidy and 41.7% diploid ↔ tetraploid mosaic embryos (Curnow et al., 2000). Mouse embryos have been found to be uniformly tetraploid following electrofusion at the 2-cell stage (James et al., 1992). Electrofusion of 2-cell rat embryos resulted in uniform tetraploidy after treatment (Krivokharchenko et al., 2002), as did electrofusion of pig and rhesus monkey embryos (Prather et al., 1996; Schramm and Paprocki, 2004). Possible methods for verifying the ploidy of these electrofused embryos are karyotyping and fluorescent in situ hybridization (FISH). Microscopy may be another important tool in analyzing these embryos as fluorescent visualization of some of the electrofused embryos in this study indicated that the embryos might be binucleate rather than tetraploid (Figure 5.2).

If a high percentage of these electrofused bovine embryos are tetraploid, this could be a useful method to produce tetraploid embryos for aggregation with other embryos that might

be developmentally compromised. Studies in the mouse have shown that when tetraploid embryos are aggregated with diploid embryos, the tetraploid embryos show a preferential allocation to the trophectoderm (Everett and West, 1996; James et al., 1995; Tang et al., 2000). The result of preferential allocation of tetraploid cells to the extraembryonic tissues in diploid/tetraploid chimeras is that the developing fetus is made up almost completely of diploid cells (Tarkowski et al., 2001). Mouse “knockout” embryos that lack the product of the *Mash-2* gene, which is necessary for placental formation, have been rescued by aggregation with tetraploid embryos (Guillemot et al., 1994). Thus, the aggregation of tetraploid bovine embryos with diploid, developmentally compromised embryos may enable researchers to produce offspring from embryos that would not normally produce a live calf due to placental insufficiency.

CHAPTER VI

DEVELOPMENT OF BOVINE AGGREGATE EMBRYOS CONSTRUCTED FROM NUCLEAR TRANSFER EMBRYOS AND ELECTROFUSED IVF EMBRYOS

INTRODUCTION

The production of animals by nuclear transfer has been hindered by placental and developmental abnormalities in the fetus. Placental abnormalities have been noted in cloned mice (Ogura et al., 2002; Tanaka et al., 2001b)), sheep (De Sousa et al., 2001) and cattle (Heyman et al., 2002; Hill et al., 2000). The abnormalities reported include malformations of placental structure as well as disrupted gene expression. Overly large placentas are commonly found in cloned mice (Ogura et al., 2002; Tanaka et al., 2001b). In cattle and sheep, fewer cotyledonary structures and reduced vascularization have been found in placentas from cloned animals (De Sousa et al., 2001; Hashizume et al., 2002; Hill et al., 2000; Hill et al., 2001). Excessive amounts of allantoic fluid have also been noted in cloned bovine pregnancies (Heyman et al., 2002; Wells et al., 1999).

Gene expression has been shown to be disrupted in cloned placentas in multiple studies (Hashizume et al., 2002; Hill et al., 2002; Ravelich et al., 2004a; Ravelich et al., 2004b). Specific genes that have been found to be aberrantly expressed are placental lactogen, pregnancy-associated glycoprotein, heparanase, insulin-like growth factor binding proteins -2 and -3, leptin and trophoblast major histocompatibility complex 1 (Hashizume et al., 2002; Hill et al., 2002; Ravelich et al., 2004a; Ravelich et al., 2004b). These disruptions in gene expression could be the underlying mechanism initiating placental malformation in cattle (Hashizume et al., 2002; Ravelich et al., 2004a). Placental abnormalities may be a major factor in the decreased survivability of cloned bovine offspring (Wells et al., 1999).

Studies in mice have shown that tetraploid embryo complementation can be used to rescue embryos with lethal placental deficiencies and produce live offspring. Mouse embryos that are homozygous null mutants for the genes *Mash2* and *Rb* will not survive until birth due to abnormal placental function (Guillemot et al., 1994; Wu et al., 2003). Aggregation with tetraploid embryos rescued embryos with these mutations and allowed development of offspring derived only from the diploid portion of the aggregate, with placental tissues were constructed from the tetraploid component (Guillemot et al., 1994; Wu et al., 2003).

Tetraploid embryos for aggregation experiments in mice are routinely generated through the use of electrofusion at the 2-cell stage (Eakin and Behringer, 2003; Tanaka et al., 2001a). Electrofusion of 2-cell embryos has been reported to result in uniform tetraploidy in mice (James et al., 1992). The objectives of this experiment were to produce bovine electrofused embryos by blastomere fusion and to utilize those embryos for aggregation with nuclear transfer (NT) embryos.

EXPERIMENTAL DESIGN

Bovine embryos were produced by in vitro fertilization and nuclear transfer. In vitro fertilized embryos were electrofused at the 2-cell stage and fused embryos were cultured to the 8-cell stage. Nuclear transfer embryos were also cultured to the 8-cell stage. At the 8-cell stage, a portion of the nuclear transfer and fused embryos were allocated to serve as controls, while the remaining 8-cell embryos were used to form aggregate 8-cell embryos consisting of four nuclear transfer blastomeres and four electrofused blastomeres. Aggregate embryos, along with fused and nuclear transfer control embryos, were cultured to the blastocyst stage and were evaluated through fluorescent in situ hybridization, karyotyping and scoring for embryo quality and development at the blastocyst stage.

MATERIALS AND METHODS

In Vitro Embryo Production

Oocytes were obtained from a commercial source (BoMed, Madison, WI) and were shipped overnight in BoMed's TCM-199-based maturation medium to the LSU Embryo Biotechnology Laboratory. After 22 to 24 hours of maturation, oocytes were washed two times in Brackett-Oliphant (BO) medium (Brackett and Oliphant, 1975). Frozen semen from a single bull was thawed for 1 minute in a 38°C water bath and washed twice for 5 minutes each in 10 ml of warmed BO medium by centrifugation at 200 x g. The resulting pellet was resuspended at a concentration of 1.25×10^6 cells/ml in BO medium. Oocytes (~50) were co-incubated with 500 µl of sperm suspension in four-well plates (Nunc, Denmark) and overlaid with warmed, embryo-tested mineral oil (Sigma Chemical Company, St. Louis, MO).

The time that sperm and oocytes were first co-incubated was designated as 0 hours post-insemination. Sperm and oocytes were co-incubated for 6 hours at 39°C in humidified 5% CO₂ in air. Oocytes were then removed from insemination and mixed with a vortex (Vortex Genie 2, Fisher Scientific, Pittsburgh, PA) in TL-HEPES (BioWhittaker, Walkersville, MD) containing 1 µg/ml of hyaluronidase (Sigma) to remove cumulus cells and spermatozoa from the zona pellucida. Oocytes were then washed twice in CR1aa (Rosenkrans and First, 1994) supplemented with 0.3% bovine serum albumin (BSA; Sigma) and cultured in vitro under warmed, embryo-tested mineral oil at 39°C in humidified 5% CO₂, 5% O₂ and 90% N₂ until 28 hours post-insemination.

Electrofused Embryo Production

Zygotes from in vitro embryo production were observed for cleavage at 28, 30, 32 and 34 hours post-insemination. Good quality 2-cell embryos were removed at each time and

equilibrated through three washes with increasing concentrations of fusion buffer consisting of 0.3 M mannitol, 0.1 mM MgSO₄, 0.05 mM CaCl₂, 0.5 mM HEPES and 4 mg/ml of BSA (Sigma). Embryos were placed in fusion buffer between the electrode wires of a microslide fusion chamber. Embryos were aligned using a 5 second, 7.5 V alternating current pulse to orient the plane of contact between the two blastomeres parallel to the electrode wires. Embryos were then fused using a single 100 µsecond direct current pulse of 1.4 kV/cm delivered from a BTX Electrocell Manipulator 200 (Gentronics, San Diego, CA). Treated embryos were washed in TCM-199 medium and were observed after 1 hour for fusion of cell membranes between the two blastomeres (IVF-Fused) and were returned to culture in CR1aa medium under warmed, embryo-tested mineral oil at 39°C in humidified 5% CO₂, 5% O₂ and 90% N₂ until ~72 hours post-insemination.

Nuclear Transfer Embryo Production

Oocytes were obtained as described for in vitro embryo production. After 18 to 20 hours of maturation, oocytes used for nuclear transfer (NT) were stripped of cumulus cells by mixing with a vortex (Vortex Genie 2, Fisher Scientific) in TL-HEPES supplemented with 1 µg/ml of hyaluronidase. Oocytes with a single polar body were selected and stained with 2 µg/ml of bis-benzimide (Sigma). The metaphase spindle and polar body of the oocyte were removed with a finely drawn glass pipette (15 to 20 µm outer diameter) under epifluorescent illumination in micromanipulation media consisting of TL-HEPES supplemented with 5% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 6.5 µg/ml of cytochalasin D (Sigma).

Bovine fibroblast cells obtained from GTC Biotherapeutics (Framingham, MA) carrying a proprietary transgene were maintained in active culture for less than 10 sub-

passages until used as nuclear donors for the nuclear transfer procedure. Cells were trypsinized and washed by centrifugation for 5 minutes at 200 x g in 10 ml of TCM-199 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone). Cells were placed into micromanipulation medium with the enucleated oocytes and a single cell was inserted between the oocyte plasma membrane and the zona pellucida using the same glass pipette. All micromanipulation was performed on an inverted microscope at 400X magnification (Diaphot, Nikon Inc., Garden City, NY).

Couplets were then equilibrated through a series of washes from TL-HEPES into fusion buffer (composed as described previously). Couplets were placed between the wires of a microslide fusion chamber and aligned so that the point of contact between the cell and the cytoplasm was parallel with the electrode wires. Couplets were exposed to a single 2 kV/cm, 30 μ second direct current pulse and examined for fusion of the cell membranes after 30 minutes. Couplets that did not fuse were exposed to the fusogenic pulse for a second time. Successfully fused couplets were activated by exposure to 5 μ M of ionomycin for 5 minutes and were then cultured in 10 μ g/ml of cyclohexamide and 6.5 μ g/ml of cytochalasin D in CR1aa medium for 3 hours. Activated embryos were then washed thoroughly in CR1aa medium and cultured under warmed, embryo-tested mineral oil at 39°C in humidified 5% CO₂, 5% O₂ and 90% N₂ until the 8-cell stage.

Embryo Aggregation

Good quality 8-cell embryos produced from the NT and IVF-Fused groups were selected for aggregation at 72 hours post-insemination. Selected NT embryos were equilibrated in a 0.25% sucrose (Sigma) solution in calcium-free and magnesium-free phosphate-buffered saline (PBS) (Gibco) supplemented with 10% fetal bovine serum.

Removal of four blastomeres from the 8-cell NT embryo was accomplished by aspiration of single blastomeres into a glass pipette with an outer diameter of 50 μm , a 45° bevel and no spike. Blastomeres that were removed were discarded and the remaining NT half-embryos were allowed to rest in PBS with 10% fetal bovine serum (with calcium and magnesium but without sucrose) (Gibco) before additional manipulation.

The zona pellucida of an 8-cell IVF-Fused embryo was then removed by placing the embryo in a 0.25% pronase solution for ~1 minute. After the zona pellucida was removed, blastomeres were separated from the IVF-Fused embryo by placing them in calcium-free and magnesium-free PBS. To form the NT/IVF-Fused aggregate embryos, the same glass pipette was then used to inject four IVF-Fused blastomeres into the NT half-embryo in PBS with calcium and magnesium and supplemented with 10% fetal bovine serum. Aggregate embryos and control NT and IVF-Fused embryos were then returned to culture in CR1aa supplemented with 5% fetal bovine serum under oil at 39°C in humidified 5% CO₂, 5% O₂ and 90% N₂ until days 7 and 8 of in vitro culture (168 and 192 hours post-insemination).

Ryan Embryo Development Score

Embryos were examined at 168 and 192 hours post-insemination for blastocyst development. At 192 hours post-insemination, some embryos were evaluated for morphological quality and developmental stage and were given a score using the Ryan Embryo Development (RED) system (Ryan et al., 1992).

Fluorescent In Situ Hybridization (FISH)

At 192 hours post-insemination, 30 IVF-Fused embryos were selected as negative controls for the transgene, 33 NT embryos were selected as positive controls and 38 aggregate embryos were selected for analysis. Blastocysts selected for FISH were either those that had

hatched from the zona pellucida or the zona was removed by manipulation of the embryo with a holding pipette and a small glass pipette (10 µm outer diameter) to open the zona pellucida and free the embryo. Zona-free blastocysts were placed onto polycarbonate filters on a vacuum flask apparatus adjusted to 375 mm Hg of vacuum pressure. Filters were then stored at -80°C and shipped on dry ice to GTC Biotherapeutics for the completion of analysis.

Karyotypic Analysis

Some blastocysts in the NT and IVF-Fused groups not selected as controls for FISH analysis at 192 hours post-insemination that were still enclosed in the zona pellucida were subjected to karyotyping. This included data from 16 additional IVF-Fused blastocysts that were not selected for aggregation at the 8-cell stage but did reach the blastocyst stage at ~192 hours post-insemination. Blastocysts were cultured in 0.28 µg/ml of colcemid (Sigma) for 12 hours. Embryos were washed in a hypotonic solution of 0.9% sodium citrate (Sigma) for 2 minutes. Embryos were fixed by dropping them onto a cleaned glass slide in a 1:3 dilution of acetic acid and methanol (Sigma). Slides were then immersed in 0.4% Giemsa stain (Sigma) for 5 minutes for chromosome staining. Slides were examined with an upright microscope (Optiphot; Nikon) at 400x magnification and readable metaphase spreads were counted.

Data Analysis

Data for blastocyst rates were analyzed using a Chi square test by the frequency procedure of SAS. Data for RED scores were examined by analysis of variance with the general linear model procedure (SAS, version 8.2; SAS Institute, Inc., Cary, NC).

RESULTS

At 28, 30, 32 and 34 hours post-insemination, 1,334 2-cell embryos were selected from in vitro embryo production across 14 replicates. Electrofusion yielded 980 fused

embryos (IVF-Fused), a 73% fusion rate. Following fusion, 66% of the electrofused embryos cleaved and 129 good quality 8-cell embryos were used for aggregation. From nuclear transfer, 242 good quality 8-cell embryos were utilized for aggregation. The production of IVF-Fused embryos was the limiting factor in the number of aggregates that could be constructed and only 58 embryos (half of the number of aggregate embryos constructed) were allocated to serve as the IVF-Fused culture control group for this study.

From the 242 8-cell NT embryos selected, 115 were allocated to serve as the NT control group. For aggregation, 127 NT embryos were combined with blastomeres from 71 IVF-Fused embryos to produce 115 aggregate embryos in 14 replicates. Blastocyst development rates calculated from the number of 8-cell IVF-Fused, NT and aggregate embryos placed into culture are shown in Table 6.1. At 168 hours post-insemination, IVF-Fused, NT and aggregate embryos had developed to the blastocyst stage at rates of 52%, 56% and 49%, respectively. At 192 hours post-insemination, IVF-Fused, NT and aggregate embryos had developed to the blastocyst stage at rates of 60%, 57% and 53%, respectively. There was no significant difference in the percentage of embryos that reached the blastocyst stage among the three groups at either 168 or 192 hours post-insemination as analyzed by Chi square. Aggregate embryos at 192 hours post-insemination are shown in Figure 6.1.

Results from the embryos given a score with the Ryan Embryo Development (RED) system at 192 hours post-insemination are presented in Table 6.2. IVF-Fused, NT and aggregate blastocysts resulted in mean RED scores of 3.14 ± 0.23 , 2.56 ± 0.31 and 1.89 ± 0.37 , respectively. The RED score of IVF-Fused blastocysts was significantly greater than that of the aggregate blastocysts, although the NT blastocyst RED score was not different from that of the NT or aggregate blastocysts, as determined by analysis of variance.

Table 6.1. Blastocyst rates of NT/IVF-Fused aggregate bovine embryos constructed at the 8-cell stage

Treatment Group	n [†]	No. (%) Blastocysts	
		168 Hours [‡]	192 Hours [‡]
IVF-Fused Control Embryos	58	30 (52) ^a	35 (60) ^a
NT Control Embryos	115	64 (56) ^a	66 (57) ^a
NT/IVF-Fused Aggregate Embryos	115	56 (49) ^a	61 (53) ^a

[†]Number of 8-cell embryos.

[‡]Post insemination, calculated from number of 8-cell embryos.

^aValues with different superscripts in the same column are significantly different (P>0.05).

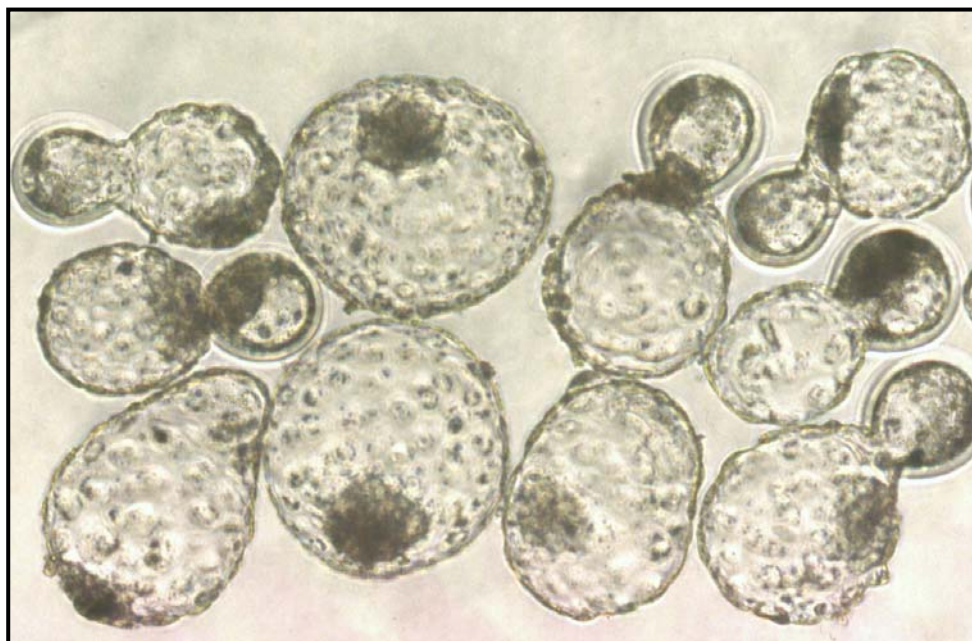


Figure 6.1. Bovine NT/IVF-Fused aggregate blastocysts at 192 hours post-insemination.

Table 6.2. Ryan Embryo Development (RED) scores for bovine blastocysts evaluated at 192 hours post-insemination

Treatment Group	n	Embryo Score \pm SEM*
IVF-Fused Control Embryos	29	3.14 \pm 0.23 ^a
NT Control Embryos	7	2.56 \pm 0.31 ^{ab}
NT/IVF-Fused Aggregate Embryos	6	1.89 \pm 0.37 ^b

* Scored at 192 hours post-insemination.

^{ab} Values with different superscripts in the same columns are significantly different (P<0.05).

For FISH analysis, 38 aggregate blastocysts, 33 NT blastocysts and 30 IVF-Fused blastocysts were placed on filters. Due to technical difficulties with the FISH procedure, only six aggregate embryos yielded data after the FISH procedure. Data were recovered from only four NT embryos serving as positive controls and only from three IVF-Fused embryos that served as negative controls. The FISH results are shown in Table 6.3. IVF-Fused negative control embryos were not positive for the transgene in any nucleus at analysis. These embryos had a mean of 180 ± 58 nuclei per embryo.

Transgenic nuclei were present in all NT positive control embryos. The NT embryos had a mean of 70 ± 10 nuclei per embryo. All of the NT embryos displayed the presence of two transgenic signals in a portion of their nuclei (mean, 20% of nuclei), and were scored as transgenic tetraploid. Also, some nuclei of the NT embryos did not display a signal from the transgene, and were classified as nontransgenic. On average, 10 nontransgenic cells of 70 total cells (14%) were found in NT embryos. In the aggregate embryos, the mean number of nuclei was low, with 27 ± 8 nuclei per blastocyst. Only one of the six aggregate embryos that data were available from had more than three nontransgenic cells (presumably derived from IVF-Fused cells). This embryo also had the highest cell number of the aggregate embryos analyzed. Unfortunately, there were too few embryos with data from the FISH procedure to perform statistical analysis.

Results from the karyotypic analysis of NT and IVF-Fused blastocysts not used for FISH analysis are presented in Table 6.4. Data were limited as only 10 embryos from the IVF-Fused group and two embryos from the NT group had readable metaphase spreads after mounting on slides. None of the NT embryos had more than one readable spread per embryo and only seven of the IVF-Fused group had more than one readable spread per embryo. Both

Table 6.3. Fluorescent in situ hybridization analysis of bovine blastocysts from NT, IVF-Fused and NT/IVF-Fused aggregate embryos at 192 hours post-insemination

Treatment Group	Embryo	Number of				
		Nuclei [†]	Transgenic	Transgenic Tetraploid [‡]	Nontransgenic	Fragmented [*]
IVF-Fused Negative Control Embryos	1	104	0	0	104	0
	2	142	0	0	142	11
	3	294	0	0	294	16
	Mean =	180	0	0	180	9
NT Positive Control Embryos	1	89	71	11	7	2
	2	81	67	6	8	4
	3	68	22	38	8	5
	4	43	22	5	16	3
	Mean =	70	41	15	10	4
NT/IVF-Fused Aggregate Embryos	1	20	14	6	0	1
	2	33	29	1	3	0
	3	10	7	2	1	2
	4	66	23	1	42	1
	5	16	10	5	1	2
	6	16	14	0	2	1
	Mean =	27	16	3	8	1

[†]Number of nuclei present as determined with DAPI staining.

[‡]Number of nuclei with two transgenic signals in a single nuclei.

^{*}Number of nuclei that appeared to be fragmented at analysis.

Table 6.4. Karyotyping results from bovine NT and IVF-Fused blastocysts after examination at 192 hours post-insemination

Treatment Group	Number			Classification			
	Mounted	Spread [†]	>1 Spread [‡]	2n	4n	2n ↔ 4n	Other
IVF-Fused Control Embryos	21	10	7	4	2	3	1
NT Control Embryos	8	2	0	2	0	0	0

[†]Number of embryos with a metaphase spread that could be counted.

[‡]Number of embryos with more than one metaphase spread that could be counted.

NT embryos had a diploid chromosomal composition, although it must be taken into consideration that only one spread was readable from each embryo and could have biased the data. In the IVF-Fused group, four were diploid (2n), two were tetraploid (4n), two were diploid ↔ tetraploid mosaic and one was hexaploid (6n). Again, it must be considered that with only one readable metaphase spread in three of the embryos, thus the data may be biased. It is possible that the embryo with the 4n and 6n karyotype were more than one nuclei that were overlapping. This technique did not allow for the differentiation of multinucleate cells. Due to the small number of embryos with readable metaphase spreads, statistical analysis was not performed on karyotyping results. Representative photographs of a tetraploid and diploid metaphase spread are shown in Figure 6.2.

DISCUSSION

Bovine nuclear transfer embryos have been found to have an abnormally high ratio of inner cell mass cells to total cell numbers at the blastocyst stage with fewer numbers of trophoctoderm cells than in vivo produced or in vitro produced control embryos (Koo et al., 2002). Those researchers hypothesized that lower numbers of trophoctoderm cells could be related to poor placental development and fetal loss in nuclear transfer pregnancies. A decrease in total cell number was detected in mouse blastocysts produced by nuclear transfer, and was proposed to be related to abnormal function of the *Oct4* gene (Boiani et al., 2003). When mouse nuclear transfer embryos were aggregated at the 4-cell stage with other cloned mouse embryos, more of the resulting blastocysts had a normal pattern of *Oct4* gene expression and thus, full-term fetal development rates were increased. Those results strongly suggest that supplying more epigenetically normal cells to cloned mouse embryos resulted in better fetal development (Boiani et al., 2003).

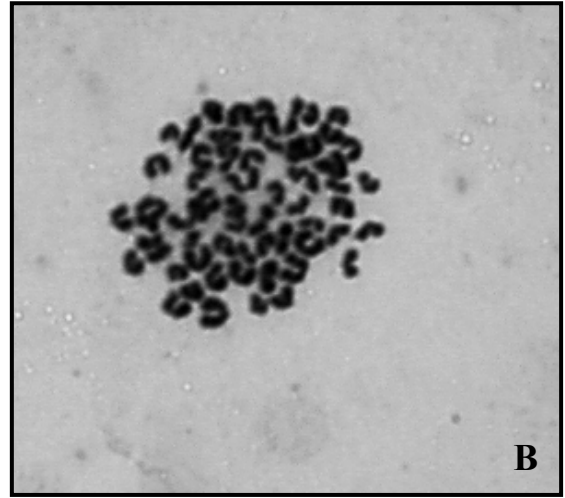
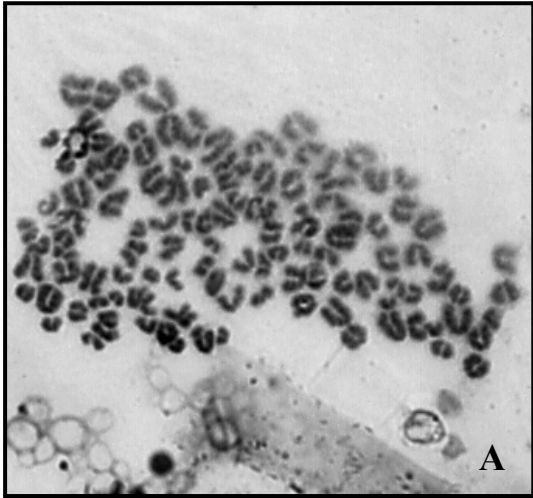


Figure 6.2. Metaphase spreads of tetraploid bovine embryos (A) and diploid bovine embryos (B) after karyotyping.

In a study using bovine embryos, Wells and Powell (2000) reported increased pregnancy rates at 90 days of gestation from in vitro fertilized ↔ nuclear transfer aggregate embryos than from single nuclear transfer embryos. No further results have been published to date on those pregnancies, and it is not known if live offspring were born after this aggregation procedure or if chimerism was detected in the fetuses or offspring. Wells and Powell (2000) also aggregated cloned bovine embryos with parthenogenetic and other nuclear transfer embryos, as well as in vitro produced embryos, and noted that nuclear transfer embryos tended to be biased against contributing to the trophectoderm and were more likely to be located in the inner cell mass in aggregate embryos.

The bias of nuclear transfer blastomeres against contributing to the trophectoderm of aggregate embryos is reminiscent of the bias noted with embryonic stem cells (Wells and Powell, 2000). Embryonic stem cells have been found to rarely contribute to the trophectoderm after aggregation with normal diploid embryos in mice, but did contribute to the inner cell mass (Beddington and Robertson, 1989). The diploid embryo ↔ embryonic stem cell aggregation process has resulted in the production of chimeric embryonic stem (ES) cell mice; however, purely ES cell mice have been produced after aggregation of ES cells with tetraploid embryos (Eggan et al., 2001; Nagy et al., 1990; Nagy et al., 1993). It has been reported that the ES cells in the aggregate embryo form the inner cell mass and later, the fetal tissues (Nagy and Rossant, 2001; Tanaka et al., 2001a). The tetraploid cells in the aggregate embryo form the trophectoderm and later, the placental tissues (Nagy and Rossant, 2001; Tanaka et al., 2001a).

The objective of this study was to produce electrofused ↔ nuclear transfer aggregate embryos to evaluate their subsequent development and pattern of blastomere distribution after

aggregation. This research was intended to lay the groundwork for future studies designed to determine if it is possible to produce placental tissue derived from the electrofused embryo component and the fetus from the nuclear transfer component. If the electrofused embryos were tetraploid at the 8-cell stage, this should bias the electrofused cells toward the trophoctodermal lineage of the aggregate blastocyst and reduce the possibilities of producing chimeric offspring in addition to providing placental support from a nonnuclear transfer-derived source (Everett et al., 2000; Everett and West, 1996; Everett and West, 1998; James et al., 1995; Tang et al., 2000).

Across 14 replicates, the developmental rates to the blastocyst stage of the aggregate embryos constructed in this study were the same as those of nonmanipulated IVF-Fused control embryos and NT control embryos. This indicates that the aggregation procedure used in this study did permit the production of aggregate embryos that would develop to the blastocyst stage. Bovine electrofused embryos have been previously utilized for aggregation with cultured bovine mammary cells and ES-like cells (Iwasaki et al., 1999; Iwasaki et al., 2000). In their first experiment, two electrofused embryos were combined at the morula stage with either a clump of bovine mammary cells or inner cell mass cells (Iwasaki et al., 1999). Approximately 17% of the aggregates produced in this manner formed blastocysts. Their second study reported ~36% blastocyst formation after aggregation of two 8-cell electrofused embryos with embryonic stem cell-like cells (Iwasaki et al., 2000). Some of these embryos were transferred to recipients and six calves were born. Only two of the calves had any contribution from the embryonic-stem like cells. The calves were primarily derived from the electrofused (presumptive tetraploid) embryos, which had either reverted to a diploid state or would have been $2n \leftrightarrow 4n$ mosaic to generate normal calves (Iwasaki et al., 2000). In another

study, 42% of bovine blastocysts were found to be mosaic after electrofusion at the 2-cell stage (Curnow et al., 2000). In the latter study, three of the 10 electrofused blastocysts that had readable metaphase chromosome spreads after karyotyping were mosaic and two were diploid, indicating that electrofusion does not result in uniform tetraploidy in bovine embryos. This is in contrast to earlier reports of uniform tetraploidy after electrofusion at the 2-cell stage in mice (James et al., 1992).

In the present study, aggregate embryos did score significantly lower with the Ryan Embryo Development (RED) System than the IVF-Fused control embryos, but did not have a significantly lower RED score than the NT control embryos. The total cell number of the aggregate embryos was also low, but was low when compared with the NT and IVF-Fused control embryos. Unfortunately, fewer than five embryos were able to be analyzed by FISH in the NT and IVF-Fused control groups. These results indicate that the aggregation procedure might be detrimental to embryo quality, although the number of embryos was too low to verify this conclusion in the present study. It is also possible that the developmental potential of the nuclear transfer embryo is compromised from the nuclear transfer procedure, reducing the viability of the aggregate embryos.

It was reported by Amano et al. (2002) that the injection of mouse inner cell mass cells from nuclear transfer embryos into tetraploid blastocysts in mice only resulted in 5% live offspring after Caesarean-section compared with 45% live offspring from inner cell mass cells from blastocysts collected as 2-cell embryos and cultured in vitro, and then injected into tetraploid blastocysts. The latter study indicated that the nuclear transfer inner cell mass cells were not as developmentally competent as those from nonnuclear transfer embryos.

When NT blastocysts were analyzed with FISH in our study, all nuclear transfer embryos were found to have on average 20% of nuclei with two transgenic signals. It has been shown by Booth et al. (2003) that ~10% of the nuclei from bovine nuclear transfer embryos are tetraploid at the blastocyst stage. In our study, nuclear transfer embryos did not have a transgenic signal from each nucleus (mean, 10% of nuclei), and other researchers have reported that not all transgenic cells will have a signal with in-situ hybridization (James et al., 1992).

The present study demonstrates that NT/IVF-Fused aggregate embryos can be constructed at the 8-cell stage and develop into blastocysts at the same rate as NT and IVF-Fused control embryos. It was attempted to determine the nuclear status of the electrofused embryos but technical limitations in the karyotyping procedure did not permit a straightforward and unequivocal differentiation between diploid, tetraploid and multinucleate cells. Also, due to technical complications, the distribution of NT cells in aggregate embryos was not determined. Further research is needed to resolve the nuclear status of IVF-Fused embryos and the allocation of NT and IVF-Fused cell lineages within the developing embryo.

CHAPTER VII

COMPARISON OF NUCLEAR CHANGES AFTER ELECTROFUSION OF BOVINE 2-CELL EMBRYOS

INTRODUCTION

Successful electrofusion of two cells was first reported between two plant protoplasts (Senda et al., 1979). Electrofusion was reported to open up pores in the cell membranes between the cells exposed to the fusogenic pulse that would then allow the cell membranes to fuse together, forming a hybrid cell (Tsong, 1983). This was soon followed by reports of electrofusion of animal cells (Bates et al., 1987; Zimmermann and Vienken, 1984), including the electrofusion of two sea urchin eggs (Richter et al., 1981). Following fusion, the sea urchin eggs were found to have cytoplasmic mixing and were capable of being fertilized but the fertilized eggs did not cleave. Electrofusion was first reported between mouse blastomeres at the 2-cell, 4-cell, 8-cell and blastocyst stages (Berg, 1982). That study showed that electrofusion might be useful in the formation of polyploid embryos, but the author did not report subsequent development of embryos after the electrofusion procedure.

Electrofusion is currently the most commonly used method to produce tetraploid mouse embryos (Eggan and Jaenisch, 2003; Kubiak and Tarkowski, 1985). Electrofused mouse embryos have been found to be uniformly tetraploid when analyzed by in situ hybridization (James et al., 1992). Electrofusion of blastomeres at the 2-cell stage has also been used to produce tetraploid embryos in other species, including rabbits (Ozil and Modlinski, 1986), rats (Krivokharchenko et al., 2002), pigs (Prather et al., 1996) and monkeys (Schramm and Paprocki, 2004). After cytogenetic examination of embryos and fetal tissues produced from diploid embryos electrofused at the 2-cell stage, it was reported that the

electrofusion procedure resulted in uniform tetraploidy in all of the species listed previously, with the exception a single embryo in the rabbit.

Bovine embryos have also been exposed to an electrical pulse (electrofusion) at the 2-cell stage to produce tetraploid embryos (Curnow et al., 2000; Iwasaki et al., 1989; Iwasaki et al., 1999; Iwasaki et al., 2000). Iwasaki et al. (1989) reported that a field strength of 1 kV/cm with two direct current pulses of 10 or 25 μ seconds yielded fusion rates of 73 to 95%. In the latter study, the embryos that fused after treatment were placed into culture medium and development was monitored in vitro. Only 10% of the embryos that fused following treatment developed past the 4-cell stage and only two of 136 fused embryos reached the morula stage in that study. Following chromosomal analysis of 14 electrofused bovine embryos, 78.6% were found to be tetraploid. Another group reported optimal fusion parameters of 1.4 kV/cm with a single direct current pulse of 100 μ seconds for bovine 2-cell embryos (Curnow et al., 2000). The embryos that fused following exposure to an electrical pulse at the 2-cell stage were returned to culture and did develop to the blastocyst stage. In the latter study, only 12.5% of the blastocysts from embryos that were electrofused were determined to be tetraploid after karyotyping, while 41.7% of blastocysts were determined to be diploid \leftrightarrow tetraploid mosaic. This rate of diploid \leftrightarrow tetraploid mosaicism was not noted in mouse embryos after electrofusion at the 2-cell stage (James et al., 1992).

The reports of nuclear status of bovine embryos after electrofusion at the 2-cell stage have been conflicting, with tetraploidy rates reported to range from 13 to 86% (Curnow et al., 2000; Iwasaki et al., 1989; Iwasaki et al., 1999). In our laboratory, preliminary staining of bovine embryos electrofused at the 2-cell stage with bis-benzimide (Hoechst 33342) has indicated that electrofused bovine embryos could be binucleate, tetraploid, mosaic or that the

embryo has perhaps cleaved without nuclear division and has reverted back to a normal diploid embryo.

The objectives of this study were to examine the nuclear status of bovine IVF embryos electrofused at the 2-cell stage compared with those of embryos that were not exposed to electrofusion and to determine if the stage of the cell cycle has an effect on the number of embryos that become tetraploid after electrofusion at the 2-cell stage.

EXPERIMENTAL DESIGN

Experiments 7.1 and 7.2 utilized the nuclear stains Hoechst 33342 and orcein to examine the nuclear status of embryos electrofused at the 2-cell stage and compared the results to those of embryos that were not exposed to electrofusion. Experiment 7.3 utilized the cell cycle inhibitors genistein, daidzein and nocodazole prior to electrofusion to determine if the stage of the cell cycle was affecting the number of embryos that became tetraploid after electrofusion. In Experiment 7.4, embryos that cleaved at 28 hours post-insemination were selected and half were labeled with the S phase indicator 5-Bromo-2'-deoxyuridine (BrdU) immediately, while the remaining embryos were labeled with BrdU at 30 hours post-insemination, in an attempt to determine differences in the cell cycle stage for 2-cell embryos at those times.

MATERIALS AND METHODS

In Vitro Embryo Production

Bovine cumulus-oocyte-complexes (BoMed, Madison, WI) were collected and matured while in transit to the Embryo Biotechnology Laboratory at Louisiana State University. Oocytes were matured for 22 to 24 hours and were then co-incubated with ~6,000 sperm from a fertile Holstein bull (#7H5188) per cumulus-oocyte-complex for 6 to 9 hours in

defined insemination medium (Brackett and Oliphant, 1975). The time that oocytes were placed in contact with sperm was designated 0 hours post-insemination. Oocytes were then washed in TCM-199 medium (Gibco Laboratories, Grand Island, NY) supplemented with 0.3% bovine serum albumin (BSA) (Sigma-Aldrich Inc., St. Louis, MO) and mixed with a vortex (Vortex Genie 2, Fisher Scientific, Pittsburgh, PA) in 0.1% hyaluronidase to remove cumulus cells and adherent spermatozoa. CR1aa culture medium (Rosenkrans and First, 1994) supplemented with 0.3% BSA and overlaid with warmed, embryo-tested mineral oil (Sigma) was used to culture inseminated oocytes in vitro. Embryos were cultured in 5% CO₂ in air at 39° C in a humidified environment.

Electrofusion of Embryos

Bovine embryos were selected for electrofusion at the 2-cell stage. Embryos were equilibrated through three washes of TCM-199 medium supplemented with 0.3% BSA with increasing concentrations of fusion buffer until embryos rested in undiluted fusion buffer composed of 0.3 M mannitol, 0.1 M MgSO₄·7H₂O, 0.05 mM CaCl₂, 0.5 mM HEPES and 0.4% BSA (Sigma). Embryos were placed in a microslide fusion chamber filled with fusion buffer. An electrical pulse for fusion was delivered to the embryos through a BTX Electrocell Manipulator 200 (Gentronics, San Diego, CA). Embryos were aligned so that the plane of contact between the two blastomeres was parallel to the electrode wires using a 5 second, 7.5 V alternating current pulse. A single 1.4 kV/cm, 100 μsecond direct current pulse was applied to the embryos for electrofusion. Treated embryos were washed in TCM-199 supplemented with 0.3% BSA and were observed after 1 hour for fusion of the two blastomeres. Fused and nonfused embryos were separately cultured in CR1aa medium under warmed, embryo-tested mineral oil and were cultured in 5% CO₂ in air at 39° C in a

humidified environment. Embryos that lysed after the electrofusion procedure were discarded.

Experiment 7.1

Hoechst 33342 Staining of Embryos. Normal diploid 2-cell embryos (n=60) were selected at 28, 30 and 32 hours post-insemination for staining with Hoechst 33342 (Sigma-Aldrich Inc., St. Louis, MO). Fused embryos at the 2-cell stage were selected from 8 to 16 hours post-fusion for staining with Hoechst 33342 (bis-benzimide) (n=36). Embryos were incubated in 2 µg/ml of Hoechst 33342 in TL-HEPES (Bio Whittaker, Walkersville, MD) for 10 minutes prior to viewing.

Embryos were placed on an inverted microscope (Diaphot, Nikon Inc., Garden City, NY) in a 60 mm Petri dish (Becton Dickinson Labware, Franklin Lakes, NJ) and visualized with a 20X objective under epifluorescent illumination. Embryos were manipulated using microtools to facilitate optimal viewing of the nuclei within the embryo. Photographs were then taken of embryos using a 35 mm camera (Nikon Inc.). Embryos were classified as having one nucleus per cell, two nuclei per cell or unequal numbers of nuclei per cell (mixoploid). Embryos stained with Hoechst 33342 are shown in Figure 7.1.

A photograph was taken of a slide micrometer with 10 µm divisions on the same microscope used to visualize the embryos. The developed slide from this photograph was used to project an image onto a dry erase board, where it was measured to determine the scale. Photographs of both control and electrofused embryos were projected onto the dry erase board and nuclear diameter was measured at the widest point of the nucleus.

SYTO 24 Staining of Embryos. The cell-permeant nucleic acid stain SYTO 24 (Molecular Probes Inc., Eugene, OR) was used to label the nucleus of 8-cell electrofused

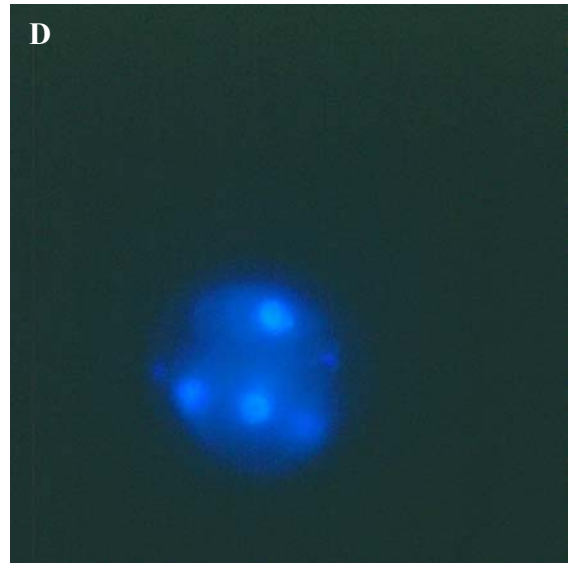
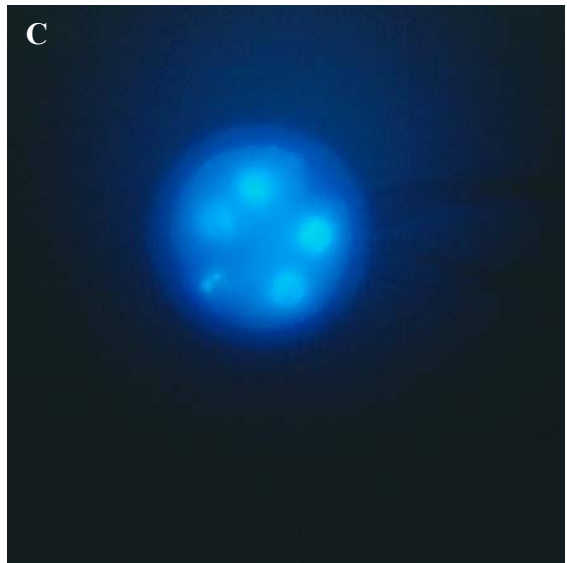
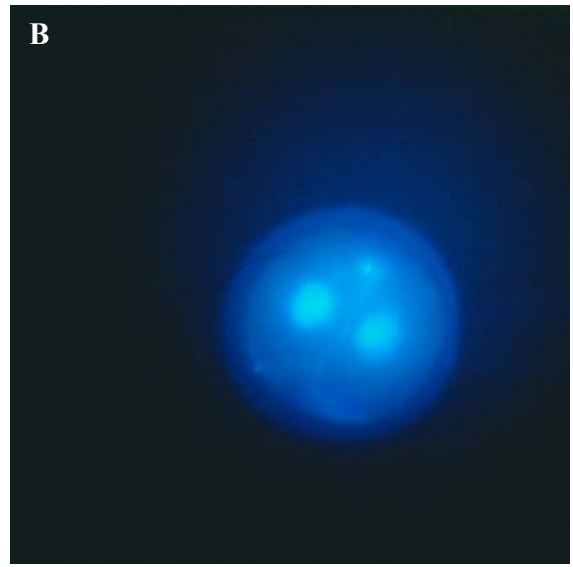
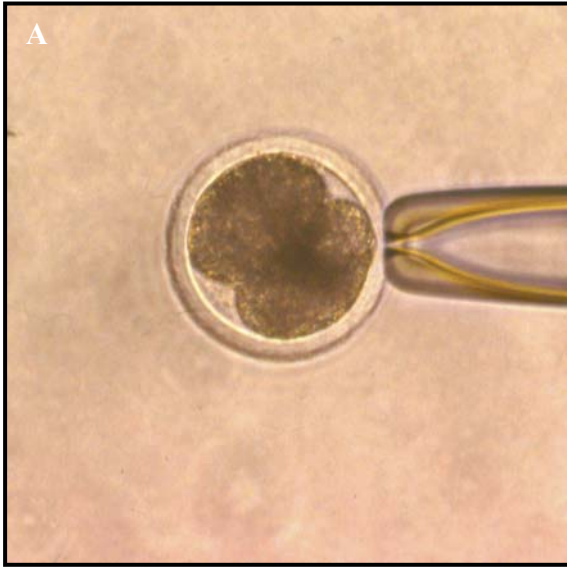


Figure 7.1. Embryo photographs at ~12 hours post-fusion: (A) Electrofused 2-cell embryo, (B) Hoechst stained 2-cell electrofused embryo, single nucleus per cell, (C) Hoechst stained 2-cell electrofused embryo, two nuclei per cell, (D) Hoechst stained 2-cell electrofused embryo, mixoploid nuclear status.

embryos. Embryos at 8-cell stage from control and electrofused embryo groups were selected at ~72 hours post-insemination for staining and analysis. Embryos were stained with 5 μM of SYTO 24 in TL-HEPES for 30 minutes prior to viewing. Embryos were mounted on glass slides in 0.5 μM SYTO 24 in TL-HEPES to prevent leaching of the stain. Embryos were examined using confocal laser scanning microscopy with a 20X objective (Leica DMIRE2 Confocal Microscope, Leica Inc., Bannockburn, IL; located at the Socolofsky Microscopy Center at Louisiana State University). Embryos stained with SYTO 24 are shown in panels A, B and C of Figure 7.2,

SYTO 83 Staining of Embryos. Embryos were also stained with SYTO 83 (Molecular Probes Inc., Eugene, OR) under the same conditions as SYTO 24. Embryos were cultured in staining media of TL-HEPES with 5 μM SYTO 83 for 30 minutes. Embryo washing and mounting media contained 0.5 μM SYTO 83 in TL-HEPES. Both electrofused and control embryos at the 8-cell stage were selected and stained at ~72 hours post-insemination. Embryos were examined using confocal laser scanning microscopy with a 20X objective (Leica DMIRE2 Confocal Microscope, Leica Inc., Bannockburn, IL). One focal plane of an 8-cell embryo stained with SYTO 83 is shown in panel D of Figure 7.2.

Data Analysis. Nuclear diameter data are reported as mean \pm the standard error of the mean (SEM) and were analyzed by analysis of variance using the general linear model (GLM) procedure of SAS (SAS Institute). Nuclear diameter data from control embryos at the 2-cell to 4-cell stage were considered and any embryo with different numbers of interphase nuclei in cells was excluded from analysis. Data for numbers of nuclei per cell after Hoechst 33342 staining were analyzed by the logistic procedure of SAS and by Chi square analysis with the frequency procedure of SAS.

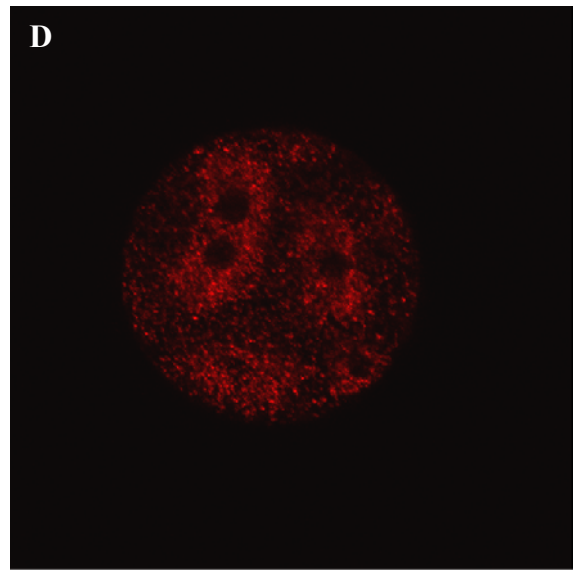
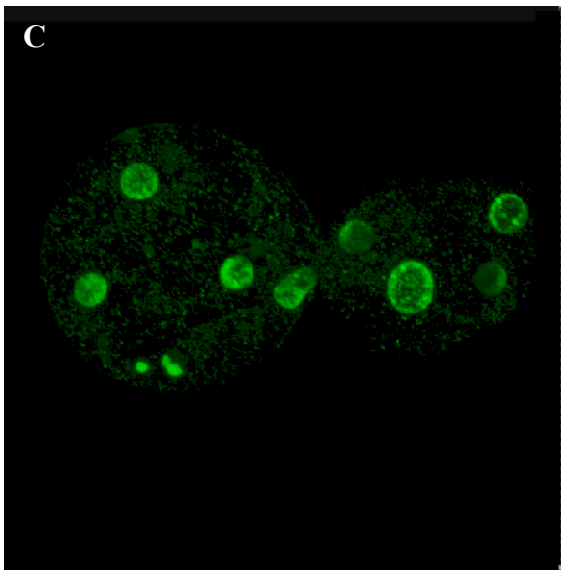
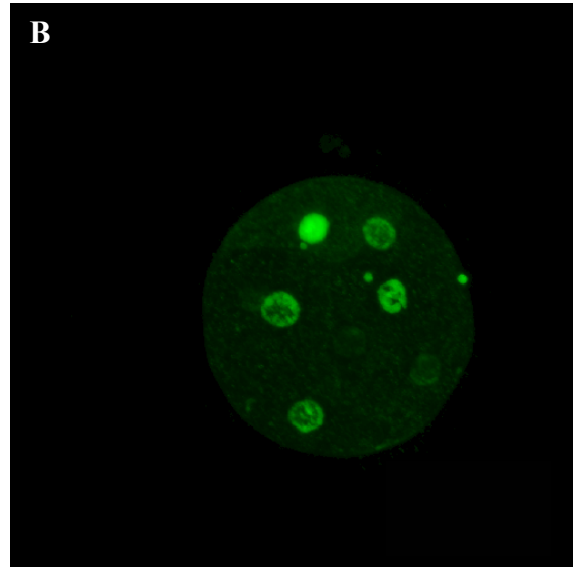
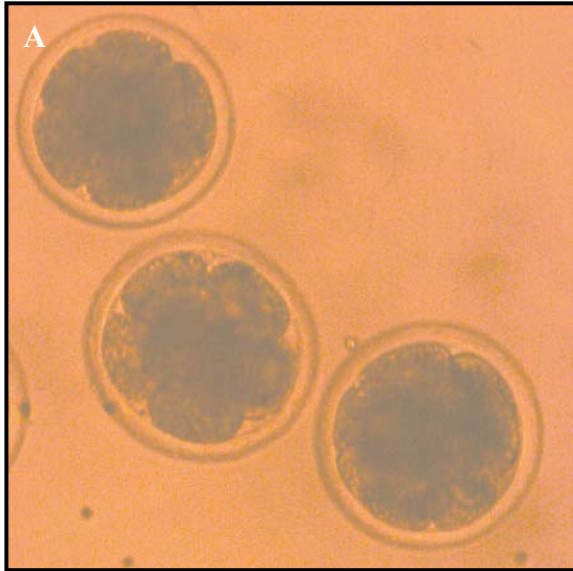


Figure 7.2. Embryo photographs at ~72 hours post-insemination: (A) 8-cell embryos, (B) and (C) 8-cell embryos stained with 5 μ M SYTO 24, (D) 8-cell embryo stained with 5 μ M SYTO 83.

Experiment 7.2

Orcein Staining of Embryos. At 30 hours post-insemination, good quality 2-cell embryos were selected and were either allocated to controls or were exposed to electrofusion. Embryos were examined for fusion of cell membranes at 1 hour post-fusion and embryos that successfully fused following treatment were separated and further analyzed (electrofused embryos). Embryos were stained every 2 hours beginning at 1 hour post-fusion until the last embryos were stained at 13 hours post-fusion. For the control embryos, 1 and 13 hours post-fusion were equivalent to 32 and 45 hours post-insemination, respectively. In the control group, 70 embryos were divided randomly for staining at seven periods (10 embryos per period). In the electrofused embryo group, 140 embryos were divided randomly for staining at seven periods (20 embryos per period). Embryos for each period were then mounted on slides at the predetermined time. Data were collected in three replicates.

Embryos were pipetted onto clean slides in as little medium as possible (~1 μ l) for mounting and fixing prior to staining. Coverslips were placed over the embryos and embryos were slightly flattened. Coverslips were attached to slides with rubber cement at two corners and slides were carefully immersed into a Coplin jar containing a fixative composed of 1 part glacial acetic acid (Sigma) to 3 parts methanol (Fisher). Slides were maintained in fixative for 3 to 7 days. To stain embryos, a drop of 1% orcein stain (100 mg orcein powder (Sigma) dissolved in 10 ml of 60:40 water:glacial acetic acid) was placed at the edge of the slide. The stain was drawn across the slide by the wicking action of a Kimwipe tissue (Kimberley-Clark Corporation, Roswell, GA) placed on the opposite side of the slide.

The number of nuclei in each blastomere of the stained embryos was counted with both an upright microscope (Labophot, Nikon) and inverted microscope (Diaphot, Nikon).

Embryos were photographed using digital imaging software (Dazzle, Inc., Fremont, CA). Nuclear diameter was calculated by measuring the nuclear diameter at the widest point with the scale of a slide micrometer photographed at the same magnification on the same microscope with the same digital imaging software. Data were collected in three replicates. Orcein stained embryos are shown in Figure 7.3.

Data Analysis. Data for numbers of nuclei per cell after orcein staining were only analyzed from embryos with cell numbers that were multiples of two by the logistic procedure of SAS. Nuclear diameter results are reported as mean \pm SEM, and were analyzed by analysis of variance using the general linear model (GLM) procedure of SAS (SAS Institute). Nuclear diameter results from control embryos with less than or equal to four cells were analyzed and any embryo with different numbers of interphase nuclei in individual blastomeres were excluded from analysis.

Experiment 7.3

Cell Cycle Inhibition of Embryos. Good quality 2-cell embryos were selected at 30 hours post-insemination and were allocated to one of three treatment groups: genistein (n=100), daidzein (n=100) or nocodazole (n=100). Embryos were cultured in CR1aa medium containing either 100 μ M genistein, 100 μ M daidzein or 0.33 μ M nocodazole for 8 hours at 37°C. Genistein has been shown to block cells at the G2/M border of the cell cycle (Casagrande and Darbon, 2001). Daidzein has been shown to block cells in the G1 phase of the cell cycle (Casagrande and Darbon, 2001). Nocodazole has been shown to block cells in the M phase of the cell cycle (Samake and Smith, 1996a). Embryos were removed from cell cycle inhibition solution and were washed two times in CR1aa medium for 5 minutes each. Embryos were then checked for successful cell cycle inhibition and scored as successfully

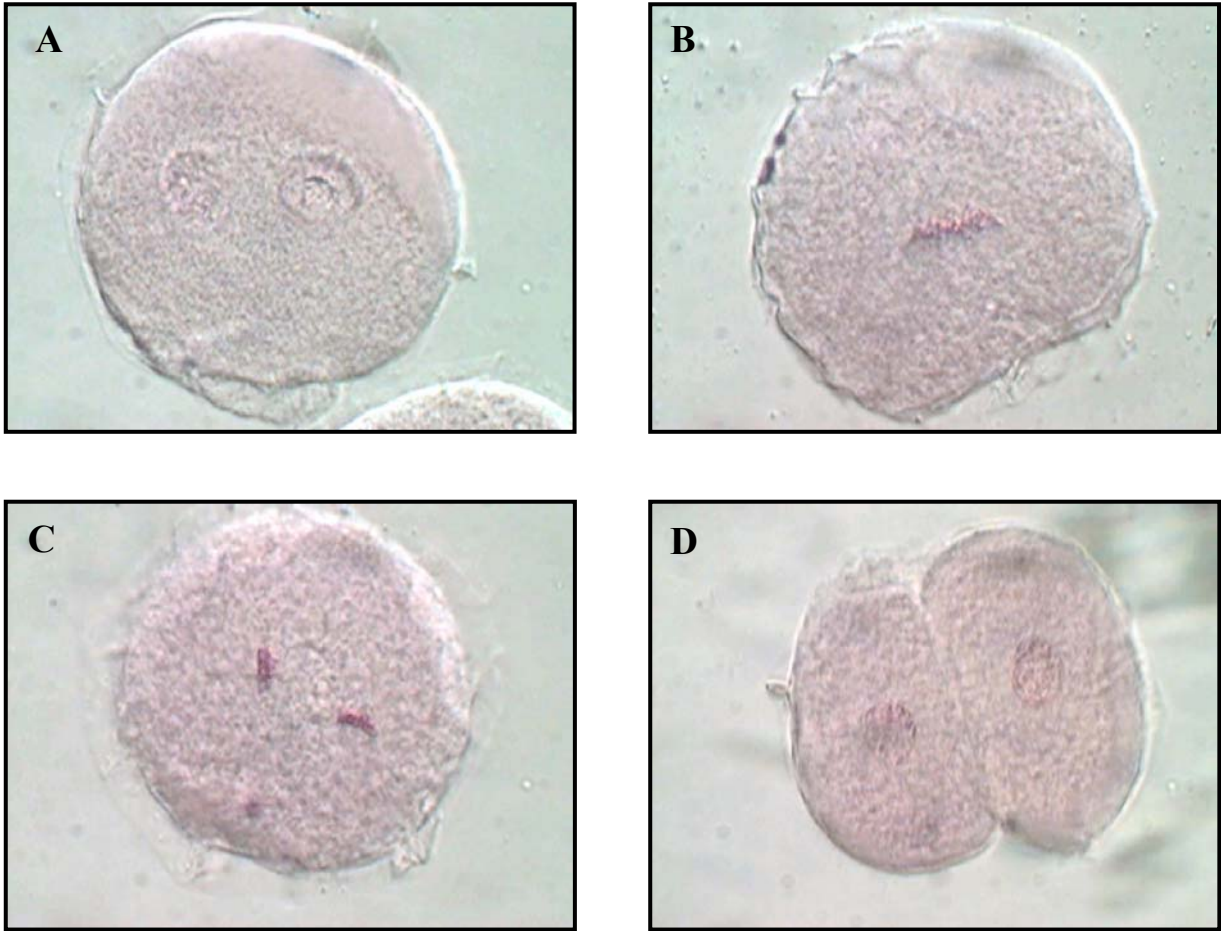


Figure 7.3. Embryos stained with orcein: (A) Electrofused embryo with two nuclei in one cell at 5 hours post-fusion, (B) Electrofused embryo with a single metaphase nucleus at 9 hours post-insemination, (C) Electrofused embryo with two metaphase nuclei in one cell at 9 hours post-fusion, (D) Electrofused 2-cell embryo with one nucleus per cell at 5 hours post-fusion.

inhibited if only two cells were present, and as unsuccessfully inhibited if more than two cells were present.

Successfully inhibited embryos were exposed to electrofusion as previously described. Embryos that successfully fused were selected at 1 hour post-fusion and were divided evenly into two groups for staining at 5 and 11 hours post-fusion. These embryos were mounted on slides and fixed as described previously at their respective time. Embryos were then stained with orcein and examined for the number of nuclei.

Data Analysis. Data for the effectiveness of cell cycle inhibition by genistein, daidzein or nocodazole were analyzed by the logistic procedure of SAS. Data for the fate of embryos that were electrofused after cell cycle inhibition were analyzed by the logistic procedure of SAS. The number of nuclei present in 1-cell embryos that fused after cell cycle inhibition were analyzed by Chi square, with the frequency procedure of SAS.

Experiment 7.4

BrdU Staining of Embryos. Good quality 2-cell embryos (n=105) were selected at 28 hours post-insemination and were separated from the remaining oocytes. Half of the embryos selected at 28 hours post-insemination were allocated for staining at that time (28-hour group, n=52) and the other half were held in CR1aa culture medium for an additional 2 hours and then stained (30-hour group, n=53). Embryos were incubated in 20 μ M BrdU (Sigma) dissolved into CR1aa medium at 37°C for 1 hour. Embryos were then washed three times in phosphate-buffered saline (PBS) (Gibco) supplemented with 5% calf serum (Gibco) for 3 minutes each. The zona pellucida of the embryo was removed by placing embryos into 0.25% protease (Sigma) dissolved in TL-HEPES (Bio Whittaker) for ~1 minute. Zona-free embryos

were washed an additional three times in serum-supplemented PBS (5%) and were placed in 70% ethanol for 1 to 3 hours at -20°C to fix the cells.

After removal from ethanol, embryos were washed once in serum-supplemented PBS and were placed in 2 N HCl (Sigma) for 30 minutes. Embryos were then washed one time in serum-supplemented PBS and were placed in permeabilization buffer for 30 minutes (Reagent B from Fix and Perm Kit, Caltag Laboratories, Burlingame, CA). Embryos were then washed once in serum-supplemented PBS and were incubated at room temperature in a 1:20 dilution of Anti-BrdU antibody conjugated with Alexa-Fluor (Caltag) for 45 minutes. Hoechst 33342 (Sigma) was added for the final 5 minutes of antibody incubation at 2 µg/ml. Embryos were washed three times for 3 minutes each in serum-supplemented PBS and were placed in serum-supplemented PBS for 15 minutes prior to mounting.

Embryos were mounted on clean slides by pipetting embryos with as little medium as possible (~1 µl) into microdrops with 2 µl of ProLong Gold Antifade (Molecular Probes) and were then covered with a coverslip. Slides were sealed with nail polish and were viewed with an inverted microscope (Eclipse TE200, Nikon) and deconvolution microscope (DMRXA2, Leica). Embryos were evaluated for the presence or absence of BrdU staining in the nucleus. Embryos were also scored for the intensity of BrdU staining and given a score (BrdU score) ranging from 0 to 3, with 0 indicating the lack of fluorescence, 1 = dim fluorescence, 2 = average fluorescence and 3 = very bright fluorescence. Examples of BrdU stained bovine embryos are shown in Figure 7.4.

Positive and negative control embryos were also stained with the three replicates of this experiment. Cleaved embryos were selected at 28 hours post-insemination to serve as either positive or negative controls. Positive control embryos (n=8) were incubated in BrdU

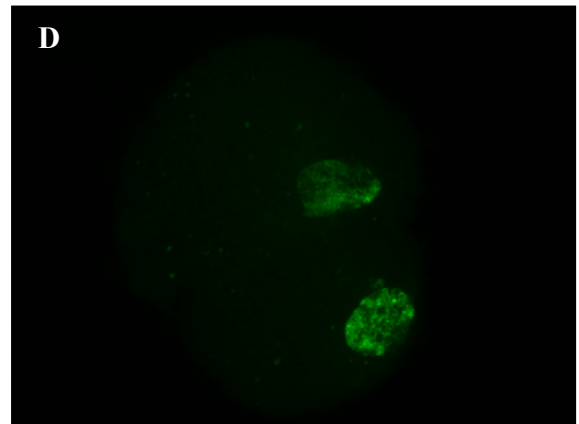
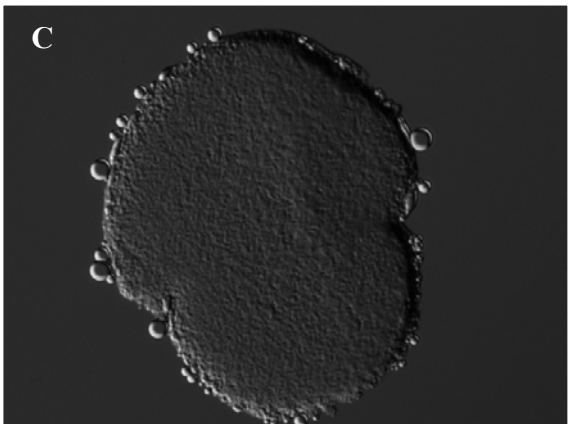
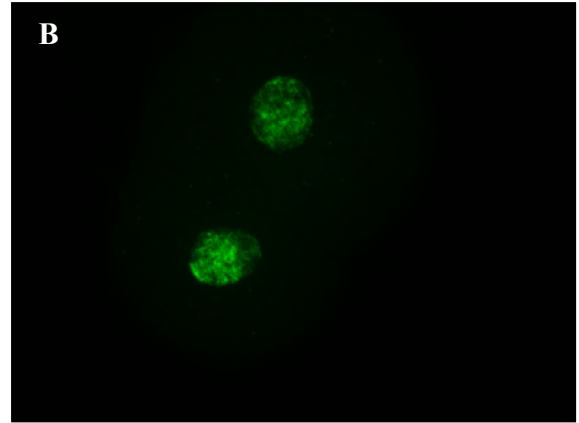
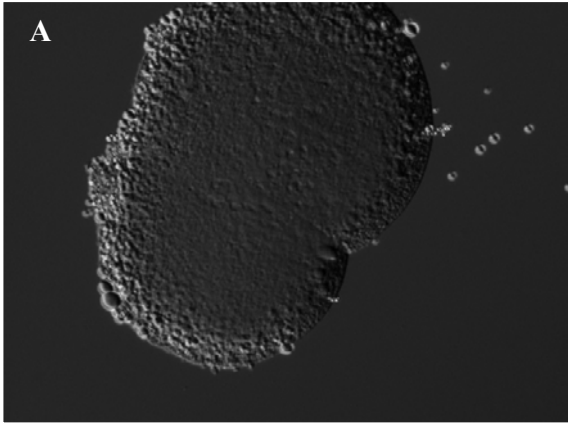


Figure 7.4. Embryos stained with BrdU: (A) and (B) 2-cell embryo stained at 28 hours post-insemination, (C) and (D) 2-cell embryo stained at 30 hours post-insemination.

for 6 hours and then treated identically to the tested embryos. Negative control embryos (n=8) were not incubated in BrdU but were exposed to the remainder of the staining protocol. Data were collected in three replicates.

Data Analysis. Data for the number of blastomeres in S phase at 28 and 30 hours post-insemination were analyzed by Chi square with the frequency procedure of SAS. Data for the intensity of BrdU staining (BrdU scores) were analyzed with analysis of variance through the general linear models procedure of SAS.

RESULTS

Experiment 7.1

Staining of 2-Cell Embryos. In total, 60 control embryos were stained over four replicates, and 36 electrofused embryos were stained over seven replicates. Embryos were classified as having a single nucleus per cell, two nuclei per cell (binucleate), or an unequal number of nuclei in each cell (mixoploid) (Figure 7.1). Control embryos were found as: 53 with a single nucleus, one binucleate and six mixoploid. Electrofused embryos were found as: 21 with a single nucleus, 11 binucleate and four mixoploid. Results for nuclear status are shown in Table 7.1.

Nuclear diameter was analyzed for the embryos that did not appear to be mixoploid. Not all embryos were measured due to a camera malfunction. For control embryos with a single nucleus per cell, 43 embryos were measured and had a mean nuclear diameter of $20.55 \pm 0.27 \mu\text{m}$. Electrofused embryos with a single nucleus per cell had a mean nuclear diameter of $23.00 \pm 0.65 \mu\text{m}$ while electrofused binucleate embryos had a mean nuclear diameter of $21.16 \pm 0.31 \mu\text{m}$. Data for nuclear diameter are reported in Table 7.2. Mean nuclear diameter

Table 7.1. Nuclear status of control and electrofused 2-cell bovine embryos as determined by staining with Hoechst 33342

Treatment Group	n	Single Nucleus	Binucleate	Mixoploid [*]
Control	60	53 (88) ^a	1 (2) ^a	6 (10) ^a
Electrofused	36	21 (58) ^b	11 (31) ^b	4 (11) ^a

^{*}Differing number of nuclei present in the cells of the embryo.

^{a,b}Percentages with different superscripts in the same column are significantly different (P<0.01).

Table 7.2. Mean nuclear diameter of control and electrofused 2-cell bovine embryos stained with Hoechst 33342

Treatment Group	n	Mean Nuclear Diameter (μm) \pm SEM
Control, Single Nucleus per Cell	43	20.55 \pm 0.28 ^a
Electrofused, Single Nucleus per Cell	19	23.00 \pm 0.65 ^b
Electrofused, Binucleate*	8	21.16 \pm 0.33 ^a

*Two nuclei present in each cell.

^{a,b}Values with different superscripts in the same column are significantly different (P<0.01).

of electrofused embryos with a single nucleus per cell was significantly greater ($P < 0.01$) than the nuclear diameter of control embryos with a single nucleus or electrofused binucleate embryos.

Staining of 8-Cell Embryos. After selection at the 8-cell stage, 12 control and 11 electrofused embryos were stained with SYTO 24, mounted on slides and viewed with confocal laser scanning microscopy. Another six electrofused embryos were stained with SYTO 24, mounted on slides and viewed with the Noran confocal laser scanning microscopy system (Thermo NORAN, Middleton, WI) on a single occasion when the Leica confocal microscope was unavailable. SYTO 24 did stain the nuclei of embryos. After mounting, the embryos were flattened to a thickness of $\sim 30 \mu\text{m}$ for viewing with confocal microscopy. This made distinguishing between cells and determining the number of nuclei in each cell impossible (Figure 7.2, Panels B and C).

Another four control embryos and two electrofused embryos were stained with SYTO 83 and examined with confocal microscopy. The SYTO 83 did not stain nuclei of embryos but did appear to stain perinuclear areas of the cytoplasm, which could have been mitochondria. Staining with SYTO 83 did not allow individual cells to be distinguished within the embryo (Figure 7.2, Panel D).

Experiment 7.2

A total of 70 control and 140 electrofused embryos were mounted on slides although only 69 and 116 could be read, respectively, due to losses during the staining procedure. The results of the numbers of nuclei present in the control and electrofused embryos are presented in Table 7.3. In the control group, 62 of the 69 readable embryos (90%) had only one nucleus per cell while the remaining 7 (10%) were in what appeared to be normal mitotic division. In

Table 7.3. The numbers of nuclei present in control and electrofused bovine embryos after orcein staining between 1 and 13 hours post-fusion (32 to 45 hours post-insemination)

	Number of Embryos		Number of Nuclei per Cell (%)			
	Mounted	Read	1	2	4	other*
Control	70	69	69 (100)	-	-	-
Electofused	140	116	30 (25)	63 (54)	13 (11)	10 (8)

*Differing numbers of nuclei in individual blastomeres or fragmented nuclei in the embryo.

the electrofused group, 30 of the 116 readable embryos (25%) had one nucleus per cell, 63 (54%) had two nuclei per cell, 13 (11%) had four nuclei per cell and 10 (8%) were classified as “other” due to differing numbers of nuclei in individual blastomeres or what appeared to be fragmented nuclei.

Results for the number of nuclei per cell expressed by the number of cells present in each electrofused embryo are shown in Table 7.4. Of the 83 1-cell electrofused embryos analyzed, six had only one nucleus in that cell, 62 had two nuclei in that cell and 13 had four nuclei in that cell. Of the 18 2-cell embryos analyzed, 16 had one nucleus per cell and one had two nuclei per cell. None of the 3-cell embryos analyzed had a consistent number of nuclei present in each cell and were discarded from statistical analysis. Of the eight 4-cell embryos, seven had one nucleus per cell. Only one 6-cell embryo was present and had only one nucleus in each cell and was excluded from statistical analysis.

Statistical analysis showed that the probability of electrofused embryos having a single nucleus per cell was greater for embryos at the 2-cell and 4-cell stages than at the 1-cell stage. The probability of electrofused embryos having a single nucleus per cell and their respective 95% confidence intervals are shown in Figure 7.5.

The mean nuclear diameter of embryos stained with orcein is shown in Table 7.5. The mean nuclear diameter of control embryos stained with orcein was $19.5 \pm 0.44 \mu\text{m}$. The mean nuclear diameter of electrofused embryos stained with orcein with a single nucleus per cell was $20.1 \pm 1.88 \mu\text{m}$. The mean nuclear diameter of electrofused embryos stained with orcein with two nuclei per cell was $16.0 \pm 1.25 \mu\text{m}$. The mean nuclear diameter of electrofused embryos with a single nucleus per cell was not different from that of control embryos but was significantly different from that of electrofused embryos with two nuclei per cell ($P < 0.02$).

Table 7.4. Number of nuclei present in electrofused bovine embryos at different embryo stages after orcein staining between 1 and 13 hours post-fusion

Embryo Stage	n [†]	Number of Nuclei per Cell (%)			
		1	2	4	Other [*]
1-Cell	83	6 (7)	62 (75)	13 (16)	2 (2)
2-Cell	18	16 (89)	1 (6)	-	1 (6)
3-Cell	6	-	-	-	6 (100)
4-Cell	8	7 (88)	-	-	1 (13)
6-Cell	1	1 (100)	-	-	-
Total	116	30	63	13	10

*Differing numbers of nuclei in individual blastomeres or fragmented nuclei in the embryo.

†The total number of embryos stained at each stage across all time points between 1 and 13 hours post-fusion.

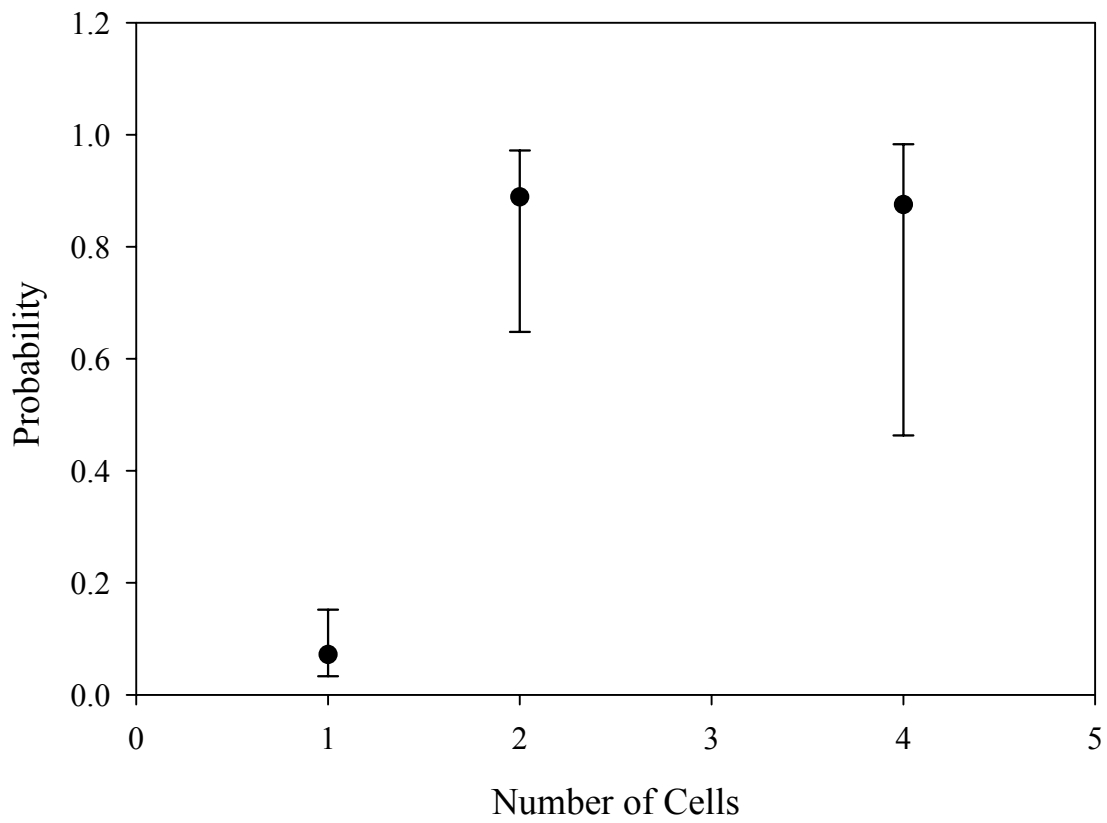


Figure 7.5. Probability (\pm 95% confidence interval) of electrofused bovine embryos at the 1-cell, 2-cell and 4-cell stages having only one nucleus per cell.

Table 7.5. The nuclear diameter of electrofused bovine embryos stained with orcein between 1 and 13 hours post-fusion (32 and 45 hours post-insemination)

Group	Number of		Embryo Stage	Mean Nuclear Diameter (μm) \pm SEM
	Embryos	Nuclei		
Control, Single Nucleus per Cell	14	52	2- to 4-cell	19.54 \pm 0.44 ^{ab}
Electrofused, Single Nucleus per Cell	9	19	1- to 2-cell	20.05 \pm 1.88 ^a
Electrofused, Binucleate*	14	36	1- to 2-cell	16.03 \pm 1.25 ^b

^{a,b}Mean values with different superscripts in the same column are significantly different ($P < 0.02$).

Experiment 7.3

A total of 300 electrofused embryos (100 per group) were exposed to the cell cycle inhibitors genistein, daidzein and nocodazole. Data for the successful inhibition of embryos exposed to the different cell cycle inhibitors are shown in Table 7.6. Successfully inhibited embryos (remained at the 2-cell stage) for genistein, daidzein and nocodazole were recovered at rates of 52%, 22% and 94%, respectively. The rate of inhibition was significantly higher for nocodazole-treated embryos than for daidzein-treated embryos, although the rate of inhibition of genistein-treated embryos was not significantly different from either of the two groups.

Results from the electrofusion of successfully inhibited embryos are presented in Table 7.6. Genistein-treated embryos were classified as fused, unfused or lysed at rates of 50%, 17% and 33%, respectively. Daidzein-treated embryos were classified as fused, unfused or lysed at rates of 73%, 0% and 27%, respectively. Nocodazole-treated embryos were classified as fused, unfused or lysed at rates of 36%, 6% and 56%, respectively. The fusion rate of daidzein-treated embryos was significantly greater than that of nocodazole-treated embryos ($P < 0.05$). The genistein-treated embryos did not fuse at rates different from either of the other two treatment groups. Unfused and lysed embryo rates were not significantly different between the treatments, although there was a significant treatment x result (fused, unfused or lysed classification) interaction. These data are shown graphically in Figure 7.6.

Results for nuclear status of embryos electrofused after cell cycle inhibition are shown in Table 7.7. Of the 76 fused embryos, 70 (92%) were successfully mounted on slides and examined for nuclear status. Genistein-treated embryos were classified as having one nucleus per cell, two nuclei per cell or four nuclei per cell at rates of 10%, 70% and 5%, respectively.

Table 7.6. Cell cycle inhibition rates and electrofusion results from bovine embryos treated at the 2-cell stage

Treatment Group	n	No. Inhibited	Number of Embryos (%) [*]		
			Fused	Unfused	Lysed
Genistein	100	52 ^{ab}	26 (50) ^{ab}	9 (17) ^a	17 (33) ^a
Daidzein	100	22 ^a	16 (73) ^a	0 (0) ^a	6 (27) ^a
Nocodazole	100	94 ^b	34 (36) ^b	6 (6) ^a	53 (56) ^a

^{*}Percentage of fused, unfused and lysed embryos calculated from the number of embryos successfully inhibited and subsequently exposed to electrofusion.

^{a,b}Values with different superscripts in the same column are significantly different (P<0.05).

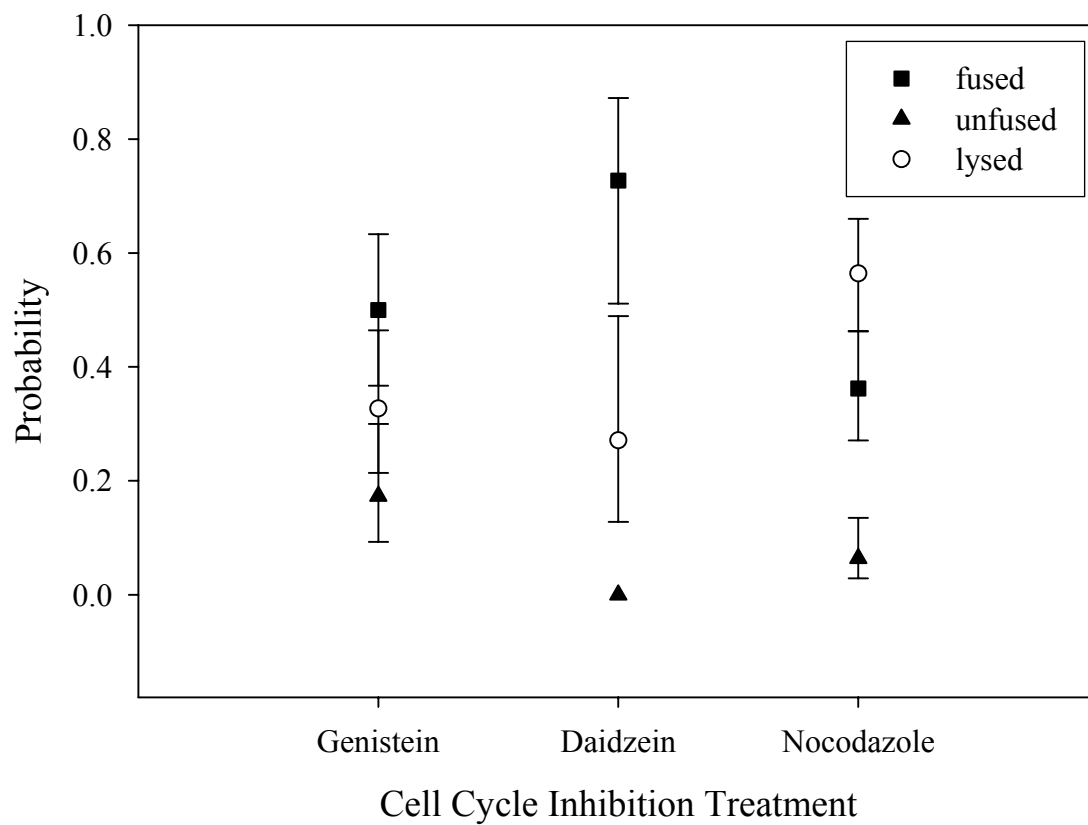


Figure 7.6. The probability (\pm 95% confidence interval) of bovine embryos classified as fused, unfused or lysed following electrofusion after inhibition of the cell cycle with either genistein, daidzein or nocodazole.

Table 7.7. Nuclear status of bovine embryos electrofused after cell cycle inhibition

Treatment Group	n	Number of Nuclei per Cell (%)			
		1	2	4	Other [*]
Genistein	20	2 (10) ^a	14 (70) ^a	1 (5) ^a	3 (15) ^a
Daidzein	14	1 (7) ^a	10 (71) ^a	1 (7) ^a	2 (14) ^a
Nocodazole	29	0 (0) ^a	7 (24) ^b	21 (72) ^b	1 (3) ^a

^{a,b}Values with different superscripts in the same column are significantly different (P<0.01).

Daidzein-treated embryos were classified as having one nucleus per cell, two nuclei per cell or four nuclei per cell at rates of 7%, 71% and 7%, respectively. Nocodazole-treated embryos were classified as having one nucleus per cell, two nuclei per cell or four nuclei per cell at rates of 0%, 24% and 72%, respectively.

The remaining 1-cell embryos in each group were classified as abnormal, most often with fragmented nuclei present. Only six embryos from each group were not at the 1-cell stage when stained and these six embryos were removed from statistical analysis.

Nocodazole-treated embryos had a significantly lower percentage of embryos with two nuclei per cell and a significantly higher percentage of embryos with four nuclei per cell after electrofusion when compared with genistein- and daidzein-treated embryos ($P < 0.01$).

Experiment 7.4

Results from embryos stained with BrdU are shown in Table 7.8. Positive and negative control embryo staining data are shown in Table 7.8 as well, but the results were not statistically compared to those from embryos stained at 28 and 30 hours post-insemination. Positive and negative control embryos were found to have BrdU staining in the nucleus at rates of 83% and 0%, respectively. Embryos from the treatment groups at 28 hours and 30 hours were found to have BrdU staining in the nucleus at rates of 61% and 41%, respectively. The percentage of embryos that did stain for BrdU was significantly higher for the treatment group at 28 hours ($P < 0.02$). The average BrdU score (\pm SEM) of all embryos in the treatment groups at 28 hours and 30 hours was 1.34 (\pm 0.15) and 0.73 (\pm 0.11), respectively. The average BrdU score from only those embryos that did stain for BrdU in the treatment groups at 28 hours and 30 hours was 2.21 (\pm 0.11) and 1.79 (\pm 0.10), respectively. The average BrdU

Table 7.8. Results of BrdU staining in bovine embryos at 28 and 30 hours post-insemination

Treatment Group	Number of			Average BrdU Score \pm SEM	
	Embryos	Blastomeres	BrdU Positive Cells (%)	All Embryos	BrdU Positive Embryos
28 Hours	52	64	39 (61) ^a	1.34 \pm 0.15 ^a	2.21 \pm 0.11 ^a
30 Hours	53	71	29 (41) ^b	0.73 \pm 0.11 ^b	1.79 \pm 0.10 ^b
Positive Controls	8	30	25 (83)	2.27 \pm 0.21	2.72 \pm 0.11
Negative Controls	8	20	0 (0)	0	0

^{a,b}Values with different superscripts in the same column are significantly different (P<0.02).

score was higher for embryos stained at 28 hours post-insemination than for embryos stained at 30 hours post-insemination ($P < 0.02$).

DISCUSSION

Uniform tetraploidy has not been reported in cattle embryos after electrofusion. Three studies reported differing tetraploidy rates of electrofused embryos after karyotypic analysis with diploid and mosaic embryos also present (Curnow et al., 2000; Iwasaki et al., 1989; Iwasaki et al., 1999). Many early studies of tetraploid embryo production in mice utilized karyotyping to determine the chromosomal composition of the resulting embryos (Eglitis, 1980; Graham, 1971; Kaufman and Webb, 1990; Koizumi and Fukuta, 1995; Kubiak and Tarkowski, 1985; Modlinski, 1978; O'Neill et al., 1990; Snow, 1973; Spindle, 1981; Tarkowski et al., 1977). Karyotypic analysis may underestimate the incidence of mosaic mouse embryos as not all nuclei were found to be in metaphase and had readable chromosome spreads after slide preparation (Tarkowski et al., 1977).

Microdensitometry has also been used in mice and pig embryos to determine relative DNA content and tetraploidy in embryos after fusion of blastomeres (Graham, 1971; Prather et al., 1996). Fetuses produced after transfer of transgenic mouse embryos electrofused at the 2-cell stage were sectioned and exposed to in situ hybridization to determine tetraploidy (James et al., 1992). The fetuses were determined to be uniformly tetraploid, as the proportion of nuclei with only one transgenic signal in the tetraploid hemizygous tissues was not significantly different from the proportion of nuclei with only one transgenic signal in the diploid homozygous tissues. It was conceded that at least 15% of the nuclei from the electrofused embryo fetuses in the latter study must have been diploid to be detected as mosaic.

Microscopy has also been used to monitor nuclear fusion after tetraploidy-inducing treatments (Eglitis, 1980; Ozil and Modlinski, 1986; Sekirina et al., 1997; Snow, 1973). The formation of a tetraploid nucleus appears to be similar for both cytochalasin and fusogenic treatments of embryos at the 2-cell stage (Eglitis, 1980; Ozil and Modlinski, 1986; Snow, 1973; Tarkowski et al., 1977). Fusogenic treatments and cytochalasin were both found to generate binucleate cells after treatment of the embryos. The two nuclei present in each blastomere must travel to the center of the cell and become positioned closely together to form a single metaphase spindle (Eglitis, 1980; Ozil and Modlinski, 1986; Snow, 1973; Tarkowski et al., 1977). The embryo then proceeds through the remainder of mitosis and results in a tetraploid state. The process of the nuclei coming together to form a tetraploid nucleus is visible without nuclear staining techniques in mouse and rabbit embryos (Eglitis, 1980; Ozil and Modlinski, 1976).

This study utilized microscopy with the nuclear stains Hoechst 33342 and orcein, as the nuclei of bovine embryos are not visible without staining. Hoechst 33342 is a stain that binds specifically to double stranded DNA within cells (Sigma Product Information) and has been used in many mammalian species for the visualization of nuclei in embryos (Critser and First, 1986).

Of the 60 control embryos examined in Experiment 7.1, 88% had a single nucleus per cell. A single control embryo (2%) appeared to be binucleate and 10% of the control embryos appeared to be mixoploid. Of the 36 electrofused embryos examined, 58% had a single nucleus. Of the remaining electrofused embryos, 31% appeared to be binucleate and 11% were mixoploid (Table 7.1). There were significantly more embryos with a single nucleus per cell in the control group and significantly more embryos with two nuclei per cell in the treated

group ($P < 0.01$). The percentage of mixoploid embryos was similar for both groups of embryos. This may be due to polyspermic insemination of oocytes during in vitro fertilization or uneven cleavages due to asynchronous cell cycles between blastomeres.

Orcein has been used previously to stain the nucleus of cattle oocytes (Lonergan et al., 1997). Of the 69 control embryos viewed after staining with orcein in Experiment 7.2, all had a single nucleus present in each cell, except for those cells visibly in mitosis at the time of staining. Of the 116 electrofused embryos viewed after staining with orcein, only 2 % of the electrofused embryos had a single nucleus present in each cell, 54 % were binucleate and 11% had four nuclei present (Table 7.3). More than four 1-cell embryos viewed after orcein staining looked as if they were in the process of becoming tetraploid as they had two nuclei in close contact. After electrofusion, no 1-cell embryos were found to have a single interphase nucleus because all electrofused embryos with a single nucleus at the 1-cell stage were in mitosis. The process of forming tetraploid nuclei is similar to that of forming a single nucleus from the two pronuclei after fertilization (Hyttel et al., 1988; Hyttel et al., 1989; Longo, 1973; Ozil and Modlinski, 1986). As the electrofused embryos cleaved after fusion, the majority of cells had only one nucleus present in each cell, implying that these embryos could have a tetraploid nucleus (Table 7.4).

The mean nuclear diameter of Hoechst-stained electrofused embryos in Experiment 7.1 with a single nucleus per cell was significantly larger ($P < 0.01$) than the diameter of both the binucleate electrofused embryos and the control embryos with a single nucleus per cell after Hoechst staining (Table 7.2). The ratio of electrofused nuclear diameter (one nucleus per cell) to control nuclear diameter is 1.12:1. The ratio of electrofused nuclear diameter (one nucleus per cell) to electrofused embryos with two nuclei per cell is 1.08:1. These ratios are

less than the calculated ratio of 1.26:1 if the nuclear volume of tetraploid cells is two times greater than that of diploid cells as previously described (Epstein, 1986; James et al., 1992).

Results differing from those of bovine embryos stained with Hoechst 33342 in Experiment 7.1 were obtained after measuring the nuclei of orcein-stained bovine embryos in Experiment 7.2 (Table 7.5). Embryo diameters were smaller for the orcein-stained embryos than for the Hoechst-stained embryos in the present study. This may have been due to differences in the cell cycle stage at the time of staining or possibly due to fluorescence from the nucleus of Hoechst-stained embryos causing the nuclei to be measured as slightly larger than the actual diameter. Electrofused embryos with a single nucleus per cell did have a significantly larger nucleus than electrofused embryos with two nuclei per cell ($P < 0.02$), but the nuclear diameter from both electrofused embryo groups was not different from the nuclear diameter of unfused embryos. The ratio of electrofused nuclear diameter (single cell per nucleus) to the nuclear diameter of electrofused embryos with two nuclei per cell was 1.26:1, which was the same as the calculated ratio for tetraploid cells if they have a nuclear volume twice that of diploid cells (Epstein, 1986; James et al., 1992).

Although interesting, these data are not conclusive that the electrofused embryos with a single nucleus per cell are truly tetraploid, as the position of the nucleus after mounting and cell cycle stage may alter the nuclear shape. In addition, cells that appear binucleate may have undergone karyokinesis in preparation for cytokinesis that had not yet occurred at the time of staining. Further research with karyotypic or in situ hybridization analysis of the electrofused cells will likely be necessary to determine the ploidy of electrofused bovine cells.

The nucleic acid stain SYTO 24 may stain DNA, RNA and mitochondria in live or dead cells (Molecular Probes Handbook). In Experiment 7.1, SYTO 24 was used to stain the

nuclei of control and electrofused embryos at the 8-cell stage. There was some cytoplasmic staining that may have been mitochondria. The dye did stain the nuclei of bovine embryos at 5 μ M; however, the nuclear status of individual blastomeres was unable to be determined due to the flattening of the embryo during mounting.

Another of the SYTO family of dyes was used to stain 8-cell control and electrofused embryos. The SYTO 83 dye was used in an attempt to stain the nucleus of the cell with a dye that would fluoresce at a different wavelength than SYTO 24, which has a peak emission wavelength that is very close to that of fluorescein isothiocyanate (FITC). This was done in anticipation of being able to label cell membranes with a lectin bound to FITC to identify the nuclear status of the blastomeres within the embryo. The SYTO 83 dye did not label the nucleus but did label areas of the cytoplasm, possibly mitochondria, that were clustered around the nuclei in the embryos. The staining of 8-cell electrofused embryos was not pursued further after these results.

There tended to be a greater number of embryos at the 2-cell stage following electrofusion with two nuclei per cell in Experiment 7.1 than in Experiment 7.2 (31% compared with 6%, respectively). After examining the experimental procedures, it was noted that embryos for Experiment 7.1 were electrofused at three different timepoints, 28, 30 and 32 hours post-insemination. All 2-cell embryos present were removed at each timepoint and immediately electrofused. In Experiment 7.2, embryos were only selected at a single timepoint, 30 hours post-insemination, and were electrofused at that time. This led to the hypothesis that embryos selected in Experiment 7.2 were further along in the cell cycle as they had possibly been cleaved for 2 or more hours by the time of selection and electrofusion; whereas embryos in Experiment 7.1 were selected and fused shortly after cleavage.

Bovine zygotes have been reported to cleave to the 2-cell stage between 28 and 36 hours post-insemination, completing the first cell cycle (Holm et al., 1998). The second cell cycle has been reported to be 9 to 12 hours long, resulting in cleavage to the 4-cell stage (Barnes and Eystone, 1990; Holm et al., 1998). The second cell cycle of the bovine embryo has been described as having no G1 phase, a S phase of 8 hours and a G2 phase of 0 to 2 hours (Barnes and Eystone, 1990).

In the mouse, the second cell cycle of zygotes derived from in vitro fertilization have characterized by a short G1 phase of ~2 hours, a S phase of 6 to 7 hours and a long G2 of 14 hours, due to the maternal-zygotic transition at that stage (Moore et al., 1996). This corresponds with results from in vivo fertilized mouse embryos that have been reported to enter the second cell cycle at 19 hours post-insemination with a G1 phase of 1 to 2 hours, a S phase of 6 hours and a G2 phase of 15 hours (Sawicki et al., 1978).

Mouse 2-cell embryos are typically collected from in vivo fertilized animals and electrofused between 28 and 38 hours post-insemination (Eggan et al., 2001; Kubiak and Tarkowski, 1985; Nagy et al., 1993). Embryos that are exposed to electrofusion should therefore be in G2 of the second cell cycle. One study utilizing polyethylene glycol for electrofusion in mice reported that embryos collected and treated during the G2 phase of the cell cycle yielded uniform tetraploidy after fusion of blastomeres at the 2-cell stage (Spindle, 1981). Another study reported highest fusion rates from mouse embryos that were electrofused at 28 to 30 hours post-insemination (Sekirina et al., 1997). In that study, the time of embryo electrofusion should correspond to embryos in the G2 stage of the cell cycle.

In this study, embryos in Experiment 7.3 were exposed to the cell cycle inhibitors genistein, daidzein or nocodazole. Genistein and daidzein are soy isoflavones that have been

found to affect the cell cycle (Casagrande and Darbon, 2001). Genistein has been found to inhibit cells at the G2/M border of the cell cycle through its action as a specific inhibitor of tyrosine kinase and directly inhibits the cell cycle-related cdc25 phosphatase and cdk1 kinase (Casagrande and Darbon, 2001; Cui et al., 2005; Kim et al., 1998; Mansour et al., 2004; Matsukawa et al., 1993; Wang et al., 2003). Genistein has been used to successfully inhibit cleavage in mouse embryos (Besterman and Schultz, 1990; Goyal and Alexandre, 2000). Daidzein has been found to inhibit cells in G1 by acting as an inhibitor of the cell cycle-related kinases cdk2 and casein kinase II (Casagrande and Darbon, 2001; Higashi and Ogawara, 1994; Jing et al., 1993; Matsukawa et al., 1993). Nocodazole is an inhibitor of microtubule polymerization and has been shown to effectively arrest cleavage-stage mouse embryos (Samake and Smith, 1996a; Samake and Smith, 1996b).

In Experiment 7.3, genistein and nocodazole were used to arrest bovine embryos at the G2 to M phase of the cell cycle, while daidzein was employed to arrest embryos at the G1 phase. These cell cycle inhibition treatments did not arrest embryos at the same rate, as nocodazole-treated embryos were significantly more likely to remain at the 2-cell stage than were daidzein-treated embryos ($P < 0.01$). Genistein-treated embryos were not significantly different from either of the other two treatment groups, however, our data imply that the majority of embryos selected at 30 hours for cell cycle inhibition and subsequent electrofusion were likely past the G1 stage of the cell cycle (Table 7.6).

After electrofusion following cell cycle inhibition, daidzein-treated embryos had significantly higher rates of fusion than nocodazole-treated embryos ($P < 0.05$), while fusion rates of genistein-treated embryos were not different from either group. Nocodazole-treated embryos had the highest rates of cell lysis following electrofusion although there was no

significant difference between the groups (Table 7.6). This may be due to the destabilization of the cytoskeleton after treatment with a microtubule inhibitor.

Embryos that fused following cell cycle inhibition were stained with orcein and examined for nuclear status (Table 7.7). Embryos at the 1-cell stage following electrofusion in the genistein-treated and daidzein-treated groups were found to be binucleate at rates of ~70%, similar to the rate of two nuclei per cell noted in 1-cell electrofused embryos in Experiment 7.2 (75%), even though only 24% of embryos treated with nocodazole were binucleate ($P < 0.01$). In contrast, ~70% of 1-cell embryos treated with nocodazole and electrofused had four nuclei per cell when stained with orcein, which was significantly different from the rates of 5% and 7% found in genistein-treated and daidzein-treated embryos ($P < 0.01$). This was likely due to differences in the mechanism of cell cycle inhibition between the treatments.

In Experiment 7.4, 2-cell stage embryos were selected at 28 hours post-insemination and were allocated either to immediate staining with the S phase indicator, BrdU (28 hour group), or were held for an additional 2 hours and then stained with BrdU (30 hour group). More embryos were found to be in S phase at 28 hours ($P < 0.02$) than when embryos were held for an additional 2 hours prior to BrdU staining. Embryos in the group labeled at 28 hours also fluoresced more brightly than did embryos in the group labeled at 30 hours ($P < 0.02$). These data indicate that an additional 2 hours of incubation resulted in embryos that progressed further through the second cell cycle (Table 7.8).

In summary, although no definite conclusions on the ploidy of electrofused embryos may be drawn from the results of these studies, the results from Experiments 7.1 through 7.4 indicate that bovine embryos allowed to progress further through the second cell cycle prior to

electrofusion are more likely to become tetraploid following electrofusion. It is proposed that bovine embryos electrofused at 30 hours post-insemination would be more likely to form tetraploid embryos than embryos electrofused at 28 hours post-insemination. The nuclear status of 2-cell and 8-cell electrofused embryos can not be further defined without karyotyping or in situ hybridization analysis of the electrofused embryo. Additional important information on the ploidy of the electrofused bovine embryos at the 8-cell stage could be generated by analysis of individual cells separated from the embryo.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

Pregnancies and offspring of mice, sheep and cattle produced from nuclear transfer have been found to have a multitude of abnormalities. Placental malformation and malfunction are thought to be causative factors for excessive pregnancy loss and various postnatal pathologies noted with these cloned offspring. A technique involving the aggregation of tetraploid cells with normal diploid cells has been successfully utilized to generate live offspring in mice from embryos that would not survive to birth if left unaided due to placental abnormalities. The overall objective of the studies in this dissertation was to examine the possibilities of applying tetraploid embryo complementation to nuclear transfer embryos derived from goats and cattle. Electrofusion of 2-cell embryos was the method utilized in an effort to produce tetraploid cells in these studies, since it is the most commonly used method for tetraploid embryo production in mice.

The first experiment dealt with the possibility of increasing the time available for research in the goat by attempting to extend the reproductive season (Chapter 3). Does were treated with hCG to determine if elevating serum progesterone levels would enhance pregnancy rates in does approaching the spring transition to anestrus. If successful, this treatment could extend the amount of time available for developing assisted reproductive technologies for goats, including the production of cloned, transgenic offspring. After treatment with hCG, circulating progesterone concentrations increased, but pregnancy rates were unaffected after natural mating. These results may be useful for embryo transfer in does approaching the end of the breeding season, when recipient luteal function or embryo viability is questionable, such as with nuclear transfer or transgenic embryos.

The second experiment examined the possibility of improving nuclear transfer pregnancy rates by aggregating nuclear transfer embryos with electrofused in vitro produced embryos in the goat. If the electrofused embryos in goats were to be tetraploid and the mouse model for tetraploid embryo aggregation were applicable to the goat, this would supply placental tissue from a nonnuclear transfer source after embryo reconstruction. Four blastomeres from an 8-cell nuclear transfer and tetraploid embryo were aggregated together and four kids were born from the aggregate embryos produced in this experiment (Chapter 4). After tissue analysis, at least one kid was derived from the nuclear transfer component with the electrofused embryo contributing to the placental tissues. The birth of two male kids of the breed type used for in vitro fertilization indicated that not all electrofused embryos are completely tetraploid and that diploid cells after electrofusion can generate live offspring.

The third experiment examined electrofusion parameters for bovine 2-cell embryos and the subsequent development of the electrofused embryos (Chapter 5). Two fusion methods were examined but no difference was detected between the two methods for the rate of fusion, cleavage following fusion or blastocyst development after fusion. The time that the embryos cleaved after in vitro fertilization and were selected for electrofusion did affect cleavage and blastocyst rates following electrofusion. The embryos that cleaved earlier following in vitro fertilization had higher rates of cleavage and blastocyst development following electrofusion. Fusion rates were not affected by the time of cleavage following in vitro fertilization. These results indicated that the most developmentally competent embryos cleaved earlier following in vitro fertilization and this information was utilized to reduce the time of 2-cell embryo selection for electrofusion in later studies.

The objectives of the fourth experiment were to produce bovine electrofused embryos and to utilize them for aggregation with nuclear transfer embryos in a similar manner to the caprine aggregate embryos produced in the second experiment. This study (Chapter 6) attempted to determine the composition of the resulting aggregate embryos as well. Aggregate embryos were successfully constructed and developed to the blastocyst stage at the same rate as 8-cell non-manipulated electrofused and nuclear transfer control embryos. However, the aggregate embryos tended to be of poorer quality when evaluated by the Ryan Embryo Development Score. Due to difficulties with the procedure for fluorescent in situ hybridization, the pattern of distribution of the nuclear transfer cells could not be definitively determined. Also, karyotypic analysis did not provide a clear distinction of the ploidy of electrofused embryos and was not able to distinguish differences between ploidy and the number of nuclei in each cell. This study showed that aggregate embryos would develop to the blastocyst stage from the aggregation procedure used but that modifications to the aggregation procedure to enhance embryo quality and an improved fluorescent in situ hybridization procedure may be necessary.

The final study consisted of a series of experiments designed to evaluate the nuclear status of bovine electrofused embryos. In Experiments 7.1 and 7.2, embryos were stained with Hoechst 33342 and orcein after electrofusion. Fewer binucleate embryos were present at the 2-cell stage in Experiment 7.2 than in Experiment 7.1. This led to the realization that embryo selection for electrofusion differed between the studies, with embryos in Experiment 7.1 selected as soon as they reached the 2-cell stage and embryos in Experiment 7.2 selected at a time when most were at the 2-cell stage for a few hours. The possibility that the stage of the cell cycle could influence the likelihood of producing tetraploid embryos was then

examined in Experiment 7.3. In this experiment, embryos were exposed to differing cell cycle inhibition treatments to manipulate the stage of the cell cycle at the time of electrofusion. More embryos were successfully inhibited with chemicals that blocked cells at the G2 to M phases of the cell cycle than with a chemical that blocked cells in G1 phase. The results indicated that most embryos at the time of selection used had passed through G1.

The final experiment, 7.4, utilized an indicator of S phase to examine 2-cell embryos at the times used for electrofusion in Experiments 7.1 and 7.2 to determine if embryos were in different stages of the cell cycle. A higher percentage of embryos in S phase were noted at the first time of embryo electrofusion than at the second of electrofusion, leading to the conclusion that the embryos selected at the second timepoint for electrofusion had passed into the G2 phase of the cell cycle. It was concluded that this stage of the cell cycle may be more permissive in allowing the formation of tetraploid embryos after electrofusion. Some 8-cell electrofused embryos were also stained in this study, but due to the method of preparation, the number of nuclei present in each cell could not be determined.

In conclusion, electrofusion was applied to 2-cell bovine and caprine embryos and cell membranes did fuse and form single cells. These electrofused embryos did develop to the blastocyst stage and did contribute to the formation of an embryo after aggregation with nuclear transfer blastomeres at the 8-cell stage. In the goat, at least one kid was produced from this procedure that was derived from the nuclear transfer cells, while placental tissue had a contribution from the electrofused cells. The male kids produced after aggregation in this experiment demonstrated that not all electrofused embryos are tetraploid. In the final study, it was found that the stage of the cell cycle at electrofusion would be an important factor to consider in the production of tetraploid embryos.

Unfortunately, no definitive conclusions on the ploidy of electrofused caprine and bovine embryos can be drawn from the results of these studies and further research is necessary to determine if embryos fused later in the second cell cycle are tetraploid. It has become clear from this series of experiments that the pattern of distribution of nuclear transfer and electrofused cells in aggregate embryos warrants further study.

LITERATURE CITED

- Adelman, D. M., M. Gertsenstein, A. Nagy, M. C. Simon, and E. Maltepe. 2000. Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. *Genes Dev.* 14:3191-3203.
- Adenot, P. G., M. S. Szollosi, P. Chesne, S. Chastant, and J. P. Renard. 1997. In vivo aging of oocytes influences the behavior of nuclei transferred to enucleated rabbit oocytes. *Mol. Reprod. Dev.* 46:325-336.
- Alexander, B. M., M. S. Johnson, R. O. Guardia, W. L. Van de Graaf, P. L. Senger, and R. G. Sasser. 1995. Embryonic loss from 30 to 60 days post breeding and the effect of palpation per rectum on pregnancy. *Theriogenology* 43:551-556.
- Amano, T., Y. Kato, and Y. Tsunoda. 2001. Comparison of heat-treated and tetraploid blastocysts for the production of completely ES-cell-derived mice. *Zygote* 9:153-157.
- Amano, T., Y. Kato, and Y. Tsunoda. 2002. The developmental potential of the inner cell mass of blastocysts that were derived from mouse ES cells using nuclear transfer technology. *Cell Tissue Res.* 307:367-370.
- Amoah, E. A., S. Gelaye, P. Guthrie, and C. E. Rexroad, Jr. 1996. Breeding season and aspects of reproduction of female goats. *J. Anim Sci.* 74:723-728.
- Armstrong, D. T. and G. Evans. 1983. Factors influencing success of embryo transfer in sheep and goats. *Theriogenology* 19:31-42.
- Armstrong, D. T., A. P. Pfitzner, G. M. Warnes, and R. F. Seamark. 1983. Superovulation treatments and embryo transfer in Angora goats. *J. Reprod. Fertil.* 67:403-410.
- Baguisi, A., E. Behboodi, D. T. Melican, J. S. Pollock, M. M. Destrempe, C. Cammuso, J. L. Williams, S. D. Nims, C. A. Porter, P. Midura, M. J. Palacios, S. L. Ayres, R. S. Denniston, M. L. Hayes, C. A. Ziomek, H. M. Meade, R. A. Godke, W. G. Gavin, E. W. Overstrom, and Y. Echelard. 1999. Production of goats by somatic cell nuclear transfer. *Nat. Biotechnol.* 17:456-461.
- Band, V., S. M. Kharbanda, K. Murugesan, and A. Farooq. 1987. Steroid production in vitro by granulosa, theca, and luteal cells from goat ovaries. *Biol. Reprod.* 36:799-806.
- Baqir, S. and L. C. Smith. 2003. Growth restricted in vitro culture conditions alter the imprinted gene expression patterns of mouse embryonic stem cells. *Cloning Stem Cells* 5:199-212.
- Baran, V., X. Vignon, D. LeBourhis, J. P. Renard, and J. E. Flechon. 2002. Nucleolar changes in bovine nucleotransferred embryos. *Biol. Reprod.* 66:534-543.

- Barnes, F. L. and W. H. Eyestone. 1990. Early cleavage and the maternal zygotic transition in bovine embryos. *Theriogenology* 33:141-152.
- Batchelder, C. A., M. Bertolini, K. A. Hoffert, J. B. Mason, A. L. Moyer, S. G. Petkov, and G. B. Anderson. 2005. Comparison of term placentas in cloned and control pregnancies in cattle. *Reprod. Fertil. Dev.* 17:164.
- Bates, G. W., J. A. Saunders, and A. E. Sowers. 1987. Electrofusion. Principles and applications. In: A. E. Sowers (Ed.). *Cell Fusion*. Plenum Press, New York. pp. 367-395.
- Battye, K. M., R. J. Fairclough, A. W. N. Cameron, and A. O. Trounson. 1988. Evidence for prostaglandin involvement in early luteal regression of the superovulated nanny goat (*Capra Hircus*). *J. Reprod. Fertil.* 84:425-430.
- Beatty, R. A. and M. Fischberg. 1952. Heteroploidy in mammals. III. Induction of tetraploidy in pre-implantation mouse eggs. *J. Genet.* 50:471-479.
- Beckett, D. M., S. M. Oppenheim, A. L. Moyer, R. H. BonDurant, J. D. Rowe, and G. B. Anderson. 1999. Progestin implants can rescue demi-embryo pregnancies in goats: A case study. *Theriogenology* 51:1505-1511.
- Beddington, R. S. and E. J. Robertson. 1989. An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. *Development* 105:733-737.
- Berg, H. 1982. Biological implications of electric field effects. Part V. Fusion of blastomeres and blastocysts of mouse embryos. *Bioelectrochem. Bioenerg.* 9:223-228.
- Bertolini, M., J. B. Mason, S. W. Beam, G. F. Carneiro, M. L. Sween, D. J. Kominek, A. L. Moyer, T. R. Famula, R. D. Sainz, and G. B. Anderson. 2002. Morphology and morphometry of in vivo- and in vitro-produced bovine concepti from early pregnancy to term and association with high birth weights. *Theriogenology* 58:973-994.
- Besterman, B. and R. M. Schultz. 1990. Regulation of mouse preimplantation development: inhibitory effect of genistein, an inhibitor of tyrosine protein phosphorylation, on cleavage of one-cell embryos. *J. Exp. Zool.* 256:44-53.
- Blondin, P., P. W. Farin, A. E. Crosier, J. E. Alexander, and C. E. Farin. 2000. In vitro production of embryos alters levels of insulin-like growth factor-II messenger ribonucleic acid in bovine fetuses 63 days after transfer. *Biol. Reprod.* 62:384-389.
- Boediono, A., T. Suzuki, and R. A. Godke. 2003. Comparison of hybrid and purebred in vitro-derived cattle embryos during in vitro culture. *Anim. Reprod. Sci.* 78:1-11.
- Boiani, M., S. Eckardt, N. A. Leu, H. R. Scholer, and K. J. McLaughlin. 2003. Pluripotency deficit in clones overcome by clone-clone aggregation: epigenetic complementation? *EMBO J.* 22:5304-5312.

- Boiani, M., S. Eckardt, H. R. Scholer, and K. J. McLaughlin. 2002. Oct4 distribution and level in mouse clones: consequences for pluripotency. *Genes Dev.* 16:1209-1219.
- Bondioli, K. R., M. E. Westhusin, and C. R. Looney. 1990. Production of identical bovine offspring by nuclear transfer. *Theriogenology* 33:165-174.
- Booth, P. J., D. Viuff, S. Tan, P. Holm, T. Greve, and H. Callesen. 2003. Numerical chromosome errors in day 7 somatic nuclear transfer bovine blastocysts. *Biol. Reprod.* 68:922-928.
- Bordignon, V., H. J. Clarke, and L. C. Smith. 1999. Developmentally regulated loss and reappearance of immunoreactive somatic histone H1 on chromatin of bovine morula-stage nuclei following transplantation into oocytes. *Biol. Reprod.* 61:22-30.
- Bourc'his, D., D. Le Bourhis, D. Patin, A. Niveleau, P. Comizzoli, J. P. Renard, and E. Viegas-Pequignot. 2001. Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr. Biol.* 11:1542-1546.
- Brackett, B. G. and G. Oliphant. 1975. Capacitation of rabbit spermatozoa in vitro. *Biol. Reprod.* 13:260-274.
- Bredbacka, K. and P. Bredbacka. 1996. Glucose controls sex-related growth rate differences of bovine embryos produced in vitro. *J. Reprod. Fertil.* 106:169-172.
- Breuel, K. F., J. C. Spitzer, and D. M. Henricks. 1989. Systemic progesterone concentration following Human Chorionic Gonadotropin administration at various times during the estrous cycle in beef heifers. *J. Anim. Sci.* 67:1564-1572.
- Brevini, T. A., P. Lonergan, F. Cillo, C. Francisci, L. A. Favetta, T. Fair, and F. Gandolfi. 2002. Evolution of mRNA polyadenylation between oocyte maturation and first embryonic cleavage in cattle and its relation with developmental competence. *Mol. Reprod. Dev.* 63:510-517.
- Briggs, R. and T. J. King. 1952. Transplantation of living nuclei from blastula cells into enucleated frogs eggs. *Proc. Natl. Acad. Sci. U. S. A.* 38:455-463.
- Butler, J. E., G. B. Anderson, R. H. BonDurant, R. L. Pashen, and M. C. T. Penedo. 1987. Production of ovine chimeras by inner cell mass transplantation. *J. Anim. Sci.* 65:317-324.
- Campbell, K. H. S., J. McWhir, W. A. Ritchie, and I. Wilmut. 1996. Sheep cloned by nuclear transfer from a cultured cell line. *Nature* 380:64-66.
- Casagrande, F. and J. M. Darbon. 2001. Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1. *Biochem. Pharmacol.* 61:1205-1215.

- Cezar, G. G., M. S. Bartolomei, E. J. Forsberg, N. L. First, M. D. Bishop, and K. J. Eilertsen. 2003. Genome-wide epigenetic alterations in cloned bovine fetuses. *Biol. Reprod.* 68:1009-1014.
- Chavatte-Palmer, P., Y. Heyman, C. Richard, P. Monget, D. LeBourhis, G. Kann, Y. Chilliard, X. Vignon, and J. P. Renard. 2002. Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. *Biol. Reprod.* 66:1596-1603.
- Chavatte-Palmer, P., D. Remy, N. Cordonnier, C. Richard, H. Issenman, P. Laigre, Y. Heyman, and J. P. Mialot. 2004. Health status of cloned cattle at different ages. *Cloning Stem Cells* 6:94-100.
- Chawengsaksophak, K., W. de Graaff, J. Rossant, J. Deschamps, and F. Beck. 2004. *Cdx2* is essential for axial elongation in mouse development. *Proc. Natl. Acad. Sci. U. S. A.* 101:7641-7645.
- Chemineau, P., N. Poulin, and Y. Cognie. 1984. Progesterone secretion in the Creole goat during male-induced ovarian cycles : seasonal effects. *Reprod. Nutr. Dev.* 24:557-561.
- Chen, L. H., E. Behboodi, B. C. Reggio, M. F. Shen, M. O'Coin, M. M. Destrempe, M. Sansinena, S. D. Nims, R. S. Denniston, and H. M. Meade. 2002. Generation of transgenic goats from transfected fibroblast cell lines for the production of a malaria vaccine antigen. *Theriogenology* 57:777.
- Cheong, H. T., Y. Takahashi, and H. Kanagawa. 1993. Birth of mice after transplantation of early cell-cycle-stage embryonic nuclei into enucleated oocytes. *Biol. Reprod.* 48:414-8.
- Chesne, P., P. G. Adenot, C. Viglietta, M. Baratte, L. Boulanger, and J. P. Renard. 2002. Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat. Biotechnol.* 20:366-369.
- Chung, Y. G., S. Ratnam, J. R. Chaillet, and K. E. Latham. 2003. Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. *Biol. Reprod.* 69:146-153.
- Cibelli, J. B., S. L. Stice, P. J. Golueke, J. J. Kane, J. Jerry, C. Blackwell, F. A. Ponce de Leon, and J. M. Robl. 1998. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* 280:1256-1258.
- Collas, P. and J. M. Robl. 1991. Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos. *Biol. Reprod.* 45:455-465.

- Comizzoli, P., B. Marquant-Le Guienne, Y. Heyman, and J. P. Renard. 2000. Onset of the first S-phase is determined by a paternal effect during the G1-phase in bovine zygotes. *Biol. Reprod.* 62:1677-1684.
- Critser, E. S. and N. L. First. 1986. Use of a fluorescent stain for visualization of nuclear material in living oocytes and early embryos. *Stain Technol.* 61:1-5.
- Cui, H. B., X. L. Na, D. F. Song, and Y. Liu. 2005. Blocking effects of genistein on cell proliferation and possible mechanism in human gastric carcinoma. *World J. Gastroenterol.* 11:69-72.
- Curnow, E. C., I. M. Gunn, and A. O. Trounson. 2000. Electrofusion of two-cell bovine embryos for the production of tetraploid blastocysts in vitro. *Mol. Reprod. Dev.* 56:372-377.
- Czolowska, R., J. A. Modlinski, and A. J. Tarkowski. 1984. Behaviour of thymocyte nuclei in non-activated and activated mouse oocytes. *J. Cell Sci.* 69:19-34.
- D'Alessandro, A. G., G. M. Terzano, A. Borghese, G. Ficco, and G. Martemucci. 2004. Progestagen supplementation after transfer of embryos improves pregnancy rate in goats. *Anim Reprod. Sci.* 82-83:404.
- Daniels, R., V. Hall, and A. O. Trounson. 2000. Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. *Biol. Reprod.* 63:1034-1040.
- Daniels, R., V. J. Hall, A. J. French, N. A. Korfiatis, and A. O. Trounson. 2001. Comparison of gene transcription in cloned bovine embryos produced by different nuclear transfer techniques. *Mol. Reprod. Dev.* 60:281-288.
- de Castro, T., E. Rubianes, A. Manchaca, and A. Rivero. 1999. Ovarian dynamics, serum estradiol and progesterone concentrations during the interovulatory interval in goats. *Theriogenology* 52:399-411.
- De Sousa, P. A., T. King, L. Harkness, L. E. Young, S. K. Walker, and I. Wilmut. 2001. Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. *Biol. Reprod.* 65:23-30.
- Dean, W., F. Santos, M. Stojkovic, V. Zakhartchenko, J. Walter, E. Wolf, and W. Reik. 2001. Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc. Natl. Acad. Sci. U. S. A.* 98:13734-13738.
- Di Berardino, D., M. Ronne, I. Burguete, M. B. Lioi, L. Taibi, and D. Matassino. 1987. R-banding pattern of the prometaphase chromosomes of the goat. *J. Hered.* 78:225-230.

- Di Berardino, M. A. 1997a. Jean Brachet Memorial Lecture. Ninth International Conference of the International Society of Differentiation: Genomic potential-Acetabularia to mammals. *J. Cell Physiol* 173:119-123.
- Di Berardino, M. A. 1997b. Nuclear potential of mammalian cells. In: *Genomic Potential of Differentiated Cells*. Columbia University Press, New York, New York. pp. 180-213.
- Di Berardino, M. A. 2001. Animal cloning-the route to new genomics in agriculture and medicine. *Differentiation* 68:67-83.
- Diaz, T., E. J. P. Schmitt, R. L. de la Sota, M. J. Thatcher, and W. W. Thatcher. 1998. Human Chorionic Gonadotropin-induced alterations in ovarian follicular dynamics during the estrous cycle of heifers. *J. Anim. Sci.* 76:1929-1936.
- Doherty, A. S., M. R. Mann, K. D. Tremblay, M. S. Bartolomei, and R. M. Schultz. 2000. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol. Reprod.* 62:1526-1535.
- Dominko, T. and N. L. First. 1993. Male predominance of bovine embryos can be observed at the 2-cell stage. *Biol. Reprod.* 48 :168.
- Dutta, A., R. N. Baruah, J. C. Dutta, S. C. Talukdar, B. C. Sarmah, and P. Chakravarty. 1993. Serum progesterone profile during oestrous cycle in goat treated with different doses of HCG. *Indian J. Anim. Reprod.* 14:14-15.
- Eakin, G. S. and R. R. Behringer. 2003. Tetraploid development in the mouse. *Dev. Dyn.* 228:751-766.
- Eckardt, S., N. A. Leu, and K. J. McLaughlin. 2004. Developmental potential of clone cells in murine clone-fertilized aggregation chimeras. *Reprod. Fertil. Dev.* 16:141.
- Edwards, R. G. 1958. Colchicine-induced heteroploidy in the mouse. *J. Exp. Zool.* 137:349-362.
- Eggan, K., H. Akutsu, K. Hochedlinger, W. Rideout, R. Yanagimachi, and R. Jaenisch. 2000. X-chromosome inactivation in cloned mouse embryos. *Science* 290:78-81.
- Eggan, K., H. Akutsu, J. Loring, L. Jackson-Grusby, M. Klemm, W. M. Rideout, R. Yanagimachi, and R. Jaenisch. 2001. Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proc. Natl. Acad. Sci. U. S. A.* 98:6209-6214.
- Eggan, K. and R. Jaenisch. 2003. Differentiation of F1 embryonic stem cells into viable male and female mice by tetraploid embryo complementation. *Methods Enzymol.* 365:25-39.

- Eggan, K., A. Rode, I. Jentsch, C. Samuel, T. Hennek, H. Tintrup, B. Zevnik, J. Erwin, J. Loring, L. Jackson-Grusby, M. R. Speicher, R. Kuehn, and R. Jaenisch. 2002. Male and female mice derived from the same embryonic stem cell clone by tetraploid embryo complementation. *Nat. Biotechnol.* 20:455-459.
- Eggenschwiler, J., T. Ludwig, P. Fisher, P. A. Leighton, S. M. Tilghman, and A. Efstratiadis. 1997. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev.* 11:3128-3142.
- Eglitis, M. A. 1980. Formation of tetraploid mouse blastocysts following blastomere fusion with polyethylene glycol. *J. Exp. Zool.* 213:309-313.
- Eglitis, M. A. and L. M. Wiley. 1981. Tetraploidy and early development: effects on developmental timing and embryonic metabolism. *J. Embryol. Exp. Morphol.* 66:91-108.
- Enright, B. P., B. S. Jeong, X. Yang, and X. C. Tian. 2003a. Epigenetic characteristics of bovine donor cells for nuclear transfer: levels of histone acetylation. *Biol. Reprod.* 69:1525-1530.
- Enright, B. P., C. Kubota, X. Yang, and X. C. Tian. 2003b. Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin A or 5-aza-2'-deoxycytidine. *Biol. Reprod.* 69:896-901.
- Epstein, C. J. 1986. The consequences of chromosome imbalance. Cambridge University Press, New York, NY. pp. 195-200.
- Everett, C. A., M. H. Stark, J. D. West, D. Davidson, and R. A. Baldock. 2000. Three-dimensional reconstruction of tetraploid diploid chimaeric mouse blastocysts. *J. Anat.* 196:341-346.
- Everett, C. A. and J. D. West. 1996. The influence of ploidy on the distribution of cells in chimaeric mouse blastocysts. *Zygote* 4:59-66.
- Everett, C. A. and J. D. West. 1998. Evidence for selection against tetraploid cells in tetraploid diploid mouse chimaeras before the late blastocyst stage. *Genetic Res.* 72:225-228.
- Favetta, L. A., C. Robert, W. A. King, and D. H. Betts. 2004. P66^{SHC}, but not P53 is involved in early arrest of in vitro produced bovine embryos. *Reprod. Fertil. Dev.* 16:124.
- Fehilly, C. B. and S. M. Willadsen. 1986. Embryo manipulation in farm animals. In: J. R. Clarke (Ed.) *Oxford reviews of reproductive biology*. Clarendon Press, Oxford. pp. 389-413.

- Forar, A. L., J. M. Gay, D. D. Hancock, and C. C. Gay. 1996. Fetal loss frequency in ten Holstein dairy herds. *Theriogenology* 45:1505-1513.
- Fricke, P. M., L. P. Reynolds, and D. A. Redmer. 1993. Effect of Human Chorionic Gonadotropin administered early in the estrous cycle on ovulation and subsequent luteal function in cows. *J. Anim. Sci.* 71:1242-1246.
- Fujiwara, T., D. B. Dehart, K. K. Sulik, and B. L. Hogan. 2002. Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse. *Development* 129:4685-4696.
- Fujiwara, T., N. R. Dunn, and B. L. Hogan. 2001. Bone morphogenetic protein 4 in the extraembryonic mesoderm is required for allantois development and the localization and survival of primordial germ cells in the mouse. *Proc. Natl. Acad. Sci. U. S. A.* 98:13739-13744.
- Galli, C., I. Lagutina, G. Crotti, S. Colleoni, P. Turini, N. Ponderato, R. Duchi, and G. Lazzari. 2003. Pregnancy: A cloned horse born to its dam twin. *Nature* 424:635.
- Galli, C., I. Lagutina, and G. Lazzari. 2003. Introduction to cloning by nuclear transplantation. *Cloning Stem Cells* 5:223-232.
- Gallicano, G. I., C. Bauer, and E. Fuchs. 2001. Rescuing desmoplakin function in extra-embryonic ectoderm reveals the importance of this protein in embryonic heart, neuroepithelium, skin and vasculature. *Development* 128:929-941.
- Gao, S., Y. G. Chung, J. W. Williams, J. Riley, K. Moley, and K. E. Latham. 2003. Somatic cell-like features of cloned mouse embryos prepared with cultured myoblast nuclei. *Biol. Reprod.* 69:48-56.
- Gardner, R. L. 1968. Mouse chimaeras obtained by the injection of cells into the blastocyst. *Nature* 220:596-597.
- Gardner, R. L. and M. H. Johnson. 1973. Investigation of early mammalian development using interspecific chimaeras between rat and mouse. *Nat. New Biol.* 246:86-89.
- Gardner, R. L., V. E. Papaioannou, and S. C. Barton. 1973. Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from isolated trophoblast and inner cell mass. *J. Embryol. Exp. Morphol.* 30:561-572.
- Garrett, J. E., R. D. Geisert, M. T. Zavy, and G. L. Morgan. 1988. Evidence for maternal regulation of early conceptus growth and development in beef cattle. *J. Reprod. Fertil.* 84:437-446.

- Garry, F., R. Adams, M. D. Holland, W. W. Hay, J. P. McCann, A. Wagner, and G. E. J. Seidel. 1998. Arterial oxygen, metabolite and energy regulatory hormone concentrations in cloned bovine fetuses. *Theriogenology* 49:321.
- Garry, F. B., R. Adams, J. P. McCann, and K. G. Odde. 1996. Postnatal characteristics of calves produced by nuclear transfer cloning. *Theriogenology* 45:141-152.
- Gartner, K., K. Bondioli, K. Hill, and K. Rapp. 1998. High variability of body sized within nucleus-transfer-clones of calves: Artifacts or a biological feature? *Reprod. Dom. Anim.* 33:67-75.
- Gemmell, R. T., B. D. Stacy, and C. D. Nancarrow. 1977. Secretion of granules by the luteal cells of the sheep and the goat during the estrous cycle and pregnancy. *Anat. Rec.* 189:161-167.
- Ginther, O. J. and K. Kot. 1994. Follicular dynamics during the ovulatory season in goats. *Theriogenology* 42:987-1001.
- Gomez, M.C., C. E. Pope, A. M. Giraldo, L. Lyons, R. F. Harris, A. King, A. Cole, R. A. Godke, and B. L. Dresser. 2004. Birth of African wild cat cloned kittens. *Reprod. Fertil. Dev.* 16:141.
- Gootwine, E., I. Barash, A. Bor, I. Dekel, A. Friedler, M. Heller, U. Zaharoni, A. Zenue, and M. Shani. 1997. Factors affecting success of embryo collection and transfer in a transgenic goat program. *Theriogenology* 48:485-499.
- Goto, Y., J. Matsui, and N. Takagi. 2002. Developmental potential of mouse tetraploid cells in diploid <--> tetraploid chimeric embryos. *Int. J. Dev. Biol.* 46:741-745.
- Goto, Y. and N. Takagi. 1998. Tetraploid embryos rescue embryonic lethality caused by an additional maternally inherited X chromosome in the mouse. *Development* 125:3353-3363.
- Goval, J. J. and H. Alexandre. 2000. Effect of genistein on the temporal coordination of cleavage and compaction in mouse preimplantation embryos. *Eur. J. Morphol.* 38:88-96.
- Graff, K. J., M. Meintjes, Y. Han, B. C. Reggio, R. S. Denniston, W. G. Gavin, C. Ziomek, and R. A. Godke. 2000. Comparing follicle-stimulating hormone from two commercial sources for oocyte production from out-of-season dairy goats. *J. Dairy Sci.* 83:484-487.
- Graham, C. F. 1971. Virus assisted fusion of embryonic cells. *Acta Endocrinol. Suppl.* (Copenhagen) 153:154-167.
- Greyling, J. P. C. 2000. Reproduction traits in the Boer goat doe. *Small Ruminant Res.* 36:171-177.

- Guillemot, F., A. Nagy, A. Auerbach, J. Rossant, and A. L. Joyner. 1994. Essential role of Mash-2 in extraembryonic development. *Nature* 371:333-336.
- Gurdon, J. B. 1962. Adult frogs derived from the nuclei of single somatic cells. *Dev. Biol.* 4:256-273.
- Gurdon, J. B., R. A. Laskey, and O. R. Reeves. 1975. The developmental capacity of nuclei transplanted from keratinized skin cells of adult frogs. *J. Embryol. Exp. Morphol.* 34:93-112.
- Haibel, G. K. 1990. Out-of-season breeding in goats. *Vet. Clin. N. Am.: Food Anim. Pract.* 6:577-583.
- Han, Y., M. Meintjes, K. Graff, R. Denniston, L. Zhang, C. Ziomek, and R. Godke. 2001. Caprine offspring born from fresh and frozen-thawed in vitro-produced embryos. *Vet. Rec.* 149:714-716.
- Haresign, W., J. P. Foster, N. B. Haynes, D. B. Crighton, and G. E. Lamming. 1975. Progesterone levels following treatment of seasonally anoestrous ewes with synthetic LH-releasing hormone. *J. Reprod. Fertil.* 43:269-279.
- Hashizume, K., H. Ishiwata, K. Kizaki, O. Yamada, T. Takahashi, K. Imai, O. V. Patel, S. Akagi, M. Shimizu, S. Takahashi, S. Katsuma, S. Shiojima, A. Hirasawa, G. Tsujimoto, J. Todoroki, and Y. Izaike. 2002. Implantation and placental development in somatic cell clone recipient cows. *Cloning Stem Cells* 4:197-209.
- Hasler, J. F., W. B. Henderson, P. J. Hurtgen, Z. Q. Jin, A. D. McCauley, S. A. Mower, B. Neely, L. S. Shuey, J. E. Stokes, and S. A. Trimmer. 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* 43:141-152.
- Heap, R. B. and J. L. Linzell. 1966. Arterial concentration, ovarian secretion and mammary uptake of progesterone in goats during the reproductive cycle. *J. Endocrin.* 36:389-399.
- Henery, C. and M. H. Kaufman. 1991. Cleavage rates of diploid and tetraploid mouse embryos during the preimplantation period. *J. Exp. Zool.* 259:371-378.
- Henery, C. C., J. B. L. Bard, and M. H. Kaufman. 1992. Tetraploidy in mice, embryonic cell number, and the grain of the developmental map. *Dev. Biol.* 152:233-241.
- Henery, C. C. and M. H. Kaufman. 1992. Relationship between cell size and nuclear volume in nucleated red blood cells of developmentally matched diploid and tetraploid mouse embryos. *J. Exp. Zool.* 261:472-478.

- Heyman, Y., P. Chavatte-Palmer, D. LeBourhis, S. Camous, X. Vignon, and J. P. Renard. 2002. Frequency and occurrence of late-gestation losses from cattle cloned embryos. *Biol. Reprod.* 66:6-13.
- Higashi, K. and H. Ogawara. 1994. Daidzein inhibits insulin- or insulin-like growth factor-1-mediated signaling in cell cycle progression of Swiss 3T3 cells. *Biochim. Biophys. Acta* 1221:29-35.
- Hill, J. R., R. C. Burghardt, K. Jones, C. R. Long, C. R. Looney, T. Shin, T. E. Spencer, J. A. Thompson, Q. A. Winger, and M. E. Westhusin. 2000. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. *Biol. Reprod.* 63:1787-1794.
- Hill, J. R., J. F. Edwards, N. Sawyer, C. Blackwell, and J. B. Cibelli. 2001. Placental anomalies in a viable cloned calf. *Cloning* 3:83-88.
- Hill, J. R., A. J. Roussel, J. B. Cibelli, J. F. Edwards, N. L. Hooper, M. W. Miller, J. A. Thompson, C. R. Looney, M. E. Westhusin, J. M. Robl, and S. L. Stice. 1999. Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). *Theriogenology* 51:1451-1465.
- Hill, J. R., D. H. Schlafer, P. J. Fisher, and C. J. Davies. 2002. Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is associated with a pronounced endometrial lymphocytic response. *Biol. Reprod.* 67:55-63.
- Hillery, F. L., J. J. Parrish, and N. L. First. 1990. Bull specific effect on fertilization and embryo development in vitro. *Theriogenology* 33:249.
- Hirashima, M., Y. Lu, L. Byers, and J. Rossant. 2003. Trophoblast expression of fms-like tyrosine kinase 1 is not required for the establishment of the maternal-fetal interface in the mouse placenta. *Proc. Natl. Acad. Sci. U. S. A.* 100:15637-15642.
- Hobbs, N. K., A. A. Bondareva, S. Barnett, M. R. Capecchi, and E. E. Schmidt. 2002. Removing the vertebrate-specific TBP N terminus disrupts placental beta2m-dependent interactions with the maternal immune system. *Cell* 110:43-54.
- Holm, P., N. N. Shukri, G. Vajta, P. Booth, C. Bendixen, and H. Callesen. 1998. Developmental kinetics of the first cell cycles of bovine in vitro produced embryos in relation to their in vitro viability and sex. *Theriogenology* 50:1285-1299.
- Humpherys, D., K. Eggan, H. Akutsu, A. Friedman, K. Hochedlinger, R. Yanagimachi, E. S. Lander, T. R. Golub, and R. Jaenisch. 2002. Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. *Proc. Natl. Acad. Sci. U. S. A.* 99:12889-12894.

- Humpherys, D., K. Eggan, H. Akutsu, K. Hochedlinger, W. M. Rideout, D. Biniszkiewicz, R. Yanagimachi, and R. Jaenisch. 2001. Epigenetic instability in ES cells and cloned mice. *Science* 293:95-97.
- Hyttel, P., T. Greve, and H. Callesen. 1988. Ultrastructure of in-vivo fertilization in superovulated cattle. *J. Reprod. Fertil.* 82:1-13.
- Hyttel, P., T. Greve, and H. Callesen. 1989. Ultrastructural aspects of oocyte maturation and fertilization in cattle. *J. Reprod. Fertil. Suppl.* 38:35-47.
- Hyttel, P., J. Laurincik, V. Zakhartchenko, M. Stojkovic, E. Wolf, M. Muller, R. L. Ochs, and G. Brem. 2001. Nucleolar protein allocation and ultrastructure in bovine embryos produced by nuclear transfer from embryonic cells. *Cloning* 3:69-82.
- Illmensee, K. and P. C. Hoppe. 1981. Nuclear transplantation in *Mus musculus*: Developmental potential of nuclei from preimplantation embryos. *Cell* 23:9-18.
- Inoue, K., T. Kohda, J. Lee, N. Ogonuki, K. Mochida, Y. Noguchi, K. Tanemura, T. Kaneko-Ishino, F. Ishino, and A. Ogura. 2002. Faithful expression of imprinted genes in cloned mice. *Science* 295:297.
- Isermann, B., S. B. Hendrickson, K. Hutley, M. Wing, and H. Weiler. 2001. Tissue-restricted expression of thrombomodulin in the placenta rescues thrombomodulin-deficient mice from early lethality and reveals a secondary developmental block. *Development* 128:827-838.
- Iwasaki, S., K. H. S. Campbell, C. Galli, K. Akiyama, and S. Iwasaki. 2000. Production of live calves derived from embryonic stem-like cells aggregated with tetraploid embryos. *Biol. Reprod.* 62:470-475.
- Iwasaki, S., Y. Ito, and S. Iwasaki. 1999. In vitro development of aggregates of bovine inner cell mass cells or bovine mammary cells and putative tetraploid embryos produced by electrofusion. *J. Reprod. Dev.* 45:65-71.
- Iwasaki, S., T. Kono, H. Fukatsu, and T. Nakahara. 1989. Production of bovine tetraploid embryos by electrofusion and their developmental capability in vitro. *Gamete Res.* 24:261-267.
- Jaenisch, R., K. Eggan, D. Humpherys, W. Rideout, and K. Hochedlinger. 2002. Nuclear cloning, stem cells and genomic reprogramming. *Cloning Stem Cells* 4:389-396.
- James, R. M., M. H. Kaufman, S. Webb, and J. D. West. 1992. Electrofusion of mouse embryos results in uniform tetraploidy and not tetraploid/diploid mosaicism. *Genetic Res.* 60:185-194.

- James, R. M., A. H. E. M. Klerkx, M. Keighren, J. H. Flockart, and J. D. West. 1995. Restricted distribution of tetraploid cells in mouse tetraploid diploid chimaeras. *Dev. Biol.* 167:213-226.
- Janssen, D. L., M. L. Edwards, J. A. Koster, R. P. Lanza, and O. A. Ryder. 2004. Postnatal management of cryptorchid Banteng calves cloned by nuclear transfer utilizing frozen fibroblast cultures and enucleated cow ova. *Reprod Fertil. Dev.* 16:224.
- Jaquemar, D., S. Kupriyanov, M. Wankell, J. Avis, K. Benirschke, H. Baribault, and R. G. Oshima. 2003. Keratin 8 protection of placental barrier function. *J. Cell Biol.* 161:749-756.
- Jarrell, V. L. and P. J. Dziuk. 1991. Effect of number of corpora lutea and fetuses on concentrations of progesterone in blood of goats. *J. Anim. Sci.* 69:770-773.
- Jing, Y., K. Nakaya, and R. Han. 1993. Differentiation of promyelocytic leukemia cells HL-60 induced by daidzein in vitro and in vivo. *Anticancer Res.* 13:1049-1054.
- Jones, D. E. and A. Knifton. 1972. Progesterone concentration in the peripheral plasma of goats during the oestrus cycle. *Res. Vet. Sci.* 13:193-195.
- Kang, Y. K., D. B. Koo, J. S. Park, Y. H. Choi, A. S. Chung, K. K. Lee, and Y. M. Han. 2001a. Aberrant methylation of donor genome in cloned bovine embryos. *Nat. Genet.* 28:173-177.
- Kang, Y. K., D. B. Koo, J. S. Park, Y. H. Choi, H. N. Kim, W. K. Chang, K. K. Lee, and Y. M. Han. 2001b. Typical demethylation events in cloned pig embryos. Clues on species-specific differences in epigenetic reprogramming of a cloned donor genome. *J. Biol. Chem.* 276:39980-39984.
- Kang, Y. K., J. S. Park, D. B. Koo, Y. H. Choi, S. U. Kim, K. K. Lee, and Y. M. Han. 2002. Limited demethylation leaves mosaic-type methylation states in cloned bovine pre-implantation embryos. *EMBO J.* 21:1092-1100.
- Kanka, J., J. Fulka, Jr., J. Fulka, and J. Petr. 1991. Nuclear transplantation in bovine embryo: Fine structural and autoradiographic studies. *Mol. Reprod. Dev.* 29:110-116.
- Kato, Y., T. Tani, Y. Sotomaru, K. Kurokawa, J. Kato, H. Doguchi, H. Yasue, and Y. Tsunoda. 1998. Eight calves cloned from somatic cells of a single adult. *Science* 282:2095-2098.
- Kato, Y., T. Tani, and Y. Tsunoda. 2000. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J. Reprod. Fertil.* 120:231-237.
- Kaufman, M. H. and S. Webb. 1990. Postimplantation development of tetraploid mouse embryos produced by electrofusion. *Development* 110:1121-1132.

- Kawate, N., N. Morita, M. Tsuji, H. Tamada, T. Inaba, and T. Sawada. 2000. Roles of pulsatile release of LH in the development and maintenance of corpus luteum function in the goat. *Theriogenology* 54:1133-1143.
- Keefer, C. L. 2004. Production of bioproducts through use of transgenic animal models. *Anim. Reprod. Sci.* 82-83:5-12.
- Keefer, C. L., H. Baldassarre, R. Keyston, B. Wang, B. Bhatia, A. S. Bilodeau, J. F. Zhou, M. Leduc, B. R. Downey, A. Lazaris, and C. N. Karatzas. 2001. Generation of dwarf goat (*Capra hircus*) clones following nuclear transfer with transfected and nontransfected fetal fibroblasts and in vitro-matured oocytes. *Biol. Reprod.* 64:849-856.
- Khosla, S., W. Dean, D. Brown, W. Reik, and R. Feil. 2001. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol. Reprod.* 64:918-926.
- Kikyo, N. and A. P. Wolffe. 2000. Reprogramming nuclei: Insights from cloning, nuclear transfer and heterokaryons. *J. Cell Sci.* 113 (Part 1):11-20.
- Kim, H., T. G. Peterson, and S. Barnes. 1998. Mechanisms of action of the soy isoflavone genistein: emerging role for its effects via transforming growth factor beta signaling pathways. *Am. J. Clin. Nutr.* 68:1418S-1425S.
- Kim, J. M., A. Ogura, M. Nagata, and F. Aoki. 2002. Analysis of the mechanism for chromatin remodeling in embryos reconstructed by somatic nuclear transfer. *Biol. Reprod.* 67:760-766.
- Kleemann, D. O., S. K. Walker, and R. F. Seamark. 1994. Enhanced fetal growth in sheep administered progesterone during the first three days of pregnancy. *J. Reprod. Fertil.* 102:411-417.
- Klinger, K., G. Landes, D. Shook, R. Harvey, L. Lopez, P. Locke, T. Lerner, R. Osathanondh, B. Leverone, T. Houseal, and . 1992. Rapid detection of chromosome aneuploidies in uncultured amniocytes by using fluorescence in situ hybridization (FISH). *Am. J. Hum. Genet.* 51:55-65.
- Koizumi, N. and K. Fukuta. 1995. Preimplantation development of tetraploid mouse embryo produced by cytochalasin B. *Exp. Anim* 44:105-109.
- Kono, T., Y. Shioda, and Y. Tsunoda. 1988. Nuclear transplantation of rat embryos. *J. Exp. Zool.* 248:303-305.
- Kono, T., O. Y. Kwon, and T. Nakahara. 1991. Development of enucleated mouse oocytes reconstituted with embryonic nuclei. *J. Reprod. Fertil.* 93:165-172.

- Koo, D. B., Y. K. Kang, Y. H. Choi, J. S. Park, H. N. Kim, K. B. Oh, D. S. Son, H. Park, K. K. Lee, and Y. M. Han. 2002. Aberrant allocations of inner cell mass and trophoctoderm cells in bovine nuclear transfer blastocysts. *Biol. Reprod.* 67:487-492.
- Krivokharchenko, A., V. Galat, D. Ganten, and M. Bader. 2002. In vitro formation of tetraploid rat blastocysts after fusion of two-cell embryos. *Mol. Reprod. Dev.* 61:460-465.
- Kruip, T. and J. H. G. den Daas. 1997. In vitro produced and cloned embryos: Effects on pregnancy, parturition and offspring. *Theriogenology* 47:43-52.
- Kubiak, J. Z., R. S. Prather, G. G. Maul, and G. Schatten. 1991. Cytoplasmic modification of the nuclear lamina during pronuclear-like transformation of mouse blastomere nuclei. *Mech. Dev.* 35:103-111.
- Kubiak, J. Z. and A. K. Tarkowski. 1985. Electrofusion of mouse blastomeres. *Exp. Cell Res.* 157:561-566.
- Kubota, C., H. Yamakuchi, J. Todoroki, K. Mizoshita, N. Tabara, M. Barber, and X. Yang. 2000. Six cloned calves produced from adult fibroblast cells after long-term culture. *Proc. Natl. Acad. Sci. U. S. A.* 97:990-995.
- Laird, P. W., A. Zijderveld, K. Linders, M. A. Rudnicki, R. Jaenisch, and A. Berns. 1991. Simplified Mammalian DNA Isolation Procedure. *Nucleic Acids Res.* 19:4293.
- Lanza, R.P.; J. B. Cibelli, F. Diaz, C. T. Moraes, P. W. Farin, C. E. Farin, C. J. Hammer, M. D. West, P. Damiani. 2000. Cloning of an endangered species (*Bos gaurus*) using interspecies nuclear transfer. *Cloning* 2:79-90.
- Lau, M. M., C. E. Stewart, Z. Liu, H. Bhatt, P. Rotwein, and C. L. Stewart. 1994. Loss of the imprinted *IGF2/cation-independent mannose 6-phosphate receptor* results in fetal overgrowth and perinatal lethality. *Genes Dev.* 8:2953-2963.
- Lazzari, G., C. Wrenzycki, D. Herrmann, R. Duchi, T. Kruip, H. Niemann, and C. Galli. 2002. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. *Biol. Reprod.* 67:767-775.
- Leighton, P. A., R. S. Ingram, J. Eggenschwiler, A. Efstratiadis, and S. M. Tilghman. 1995. Disruption of imprinting caused by deletion of the H19 gene region in mice. *Nature* 375:34-39.
- Li, S., Y. Li, W. Du, L. Zhang, S. Yu, Y. Dai, C. Zhao, and N. Li. 2005. Aberrant gene expression in organs of bovine clones that die within two days after birth. *Biol. Reprod.* 72:258-265.

- Loi, P., G. Ptak, B. Barboni, J. Fulka, Jr., P. Cappai, and M. Clinton. 2001. Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nat. Biotechnol.* 19:962-964.
- Loi, P., M. Clinton, B. Barboni, J. Fulka, Jr., P. Cappai, R. Feil, R. M. Moor, and G. Ptak. 2002. Nuclei of nonviable ovine somatic cells develop into lambs after nuclear transplantation. *Biol. Reprod.* 67:126-132.
- Lonergan, P., H. Khatir, C. Carolan, and P. Mermillod. 1997. Bovine blastocyst production in vitro after inhibition of oocyte meiotic resumption for 24 h. *J. Reprod. Fertil.* 109:355-365.
- Lonergan, P., H. Khatir, F. Piumi, D. Rieger, P. Humblot, and M. P. Boland. 1999. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *J. Reprod. Fertil.* 117:159-167.
- Longo, F. J. 1973. Fertilization: A comparative ultrastructural review. *Biol. Reprod.* 9:149-215.
- Lu, T. Y. and C. L. Markert. 1980. Manufacture of diploid/tetraploid chimeric mice. *Proc. Natl. Acad. Sci. U. S. A.* 77:6012-6016.
- Mann, M. R., Y. G. Chung, L. D. Nolen, R. I. Verona, K. E. Latham, and M. S. Bartolomei. 2003a. Disruption of imprinted gene methylation and expression in cloned preimplantation stage mouse embryos. *Biol. Reprod.* 69:902-914.
- Mann, G. E., M. P. Green, K. D. Sinclair, K. J. Demmers, M. D. Fray, C. G. Gutierrez, P. C. Garnsworthy, and R. Webb. 2003b. Effects of circulating progesterone and insulin on early embryo development in beef heifers. *Anim. Reprod. Sci.* 79:71-79.
- Mann, G. E. and G. E. Lamming. 2001. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* 121:175-180.
- Mansour, A., B. McCarthy, S. K. Schwander, V. Chang, S. Kotenko, S. Donepudi, J. Lee, and E. Raveche. 2004. Genistein induces G2 arrest in malignant B cells by decreasing IL-10 secretion. *Cell Cycle* 3:1597-1605.
- Markert, C. L. 1984. Genetic control of cell interactions in chimeras. *Dev. Genet.* 4:267-279.
- Matsukawa, Y., N. Marui, T. Sakai, Y. Satomi, M. Yoshida, K. Matsumoto, H. Nishino, and A. Aoike. 1993. Genistein arrests cell cycle progression at G2-M. *Cancer Res.* 53:1328-1331.

- McCreath, K. J., J. Howcroft, K. H. Campbell, A. Colman, A. E. Schnieke, and A. J. Kind. 2000. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. *Nature* 405:1066-1069.
- McGrath, J. and D. Solter. 1983. Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. *Science* 220:1300-1302.
- McGrath, J. and D. Solter. 1984. Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. *Science* 226:1317-1319.
- McLaughlin, K. J. 1993. Production of tetraploid embryos by electrofusion. *Methods Enzymol.* 225:919-930.
- McLay, D. W. and H. J. Clarke. 2003. Remodelling the paternal chromatin at fertilization in mammals. *Reproduction* 125:625-633.
- Meade, H. M. 1997. Dairy gene. *The Sciences*. September/October:20-25.
- Menchaca, A. and E. Rubianes. 2002. Relation between progesterone concentrations during the early luteal phase and follicular dynamics in goats. *Theriogenology* 57:1411-1419.
- Meng, L., J. J. Ely, R. L. Stouffer, and D. P. Wolf. 1997. Rhesus monkeys produced by nuclear transfer. *Biol. Reprod.* 57:454-459.
- Misra, R. P., S. K. Bronson, Q. Xiao, W. Garrison, J. Li, R. Zhao, and S. A. Duncan. 2001. Generation of single-copy transgenic mouse embryos directly from ES cells by tetraploid embryo complementation. *BMC. Biotechnol.* 1:12.
- Modlinski, J. A. 1978. Transfer of embryonic nuclei to fertilised mouse eggs and development of tetraploid blastocysts. *Nature* 273:466-467.
- Modlinski, J. A. 1981. The fate of inner cell mass and trophectoderm nuclei transplanted to fertilized mouse eggs. *Nature* 292:342-343.
- Moore, G. D., T. Ayabe, G. S. Kopf, and R. M. Schultz. 1996. Temporal patterns of gene expression of G1-S cyclins and cdks during the first and second mitotic cell cycles in mouse embryos. *Mol. Reprod. Dev.* 45:264-275.
- Moustafa, L. A., and R. L. Brinster. 1972. Induced chimaerism by transplanting embryonic cells in mouse blastocysts.
- Nagy, A., E. Gocza, E. M. Diaz, V. R. Prideaux, E. Ivanyi, M. Markkula, and J. Rossant. 1990. Embryonic stem cells alone are able to support fetal development in the mouse. *Development* 110:815-821.
- Nagy, A. and J. Rossant. 2001. Chimaeras and mosaics for dissecting complex mutant phenotypes. *Int. J. Dev. Biol.* 45:577-582.

- Nagy, A., J. Rossant, R. Nagy, W. Abramow-Newerly, and J. C. Roder. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 90:8424-8428.
- Nephew, K. P., K. E. McClure, T. L. Ott, D. H. Dubois, F. W. Bazer, and W. F. Pope. 1991. Relationship between variation in conceptus development and differences in estrous cycle duration in ewes. *Biol. Reprod.* 44:536-539.
- Niemann, H. and C. Wrenzycki. 2000. Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. *Theriogenology* 53:21-34.
- O'Neill, G. T., S. Speirs, and M. H. Kaufman. 1990. Sex-chromosome constitution of postimplantation tetraploid mouse embryos. *Cytogenet. Cell Genet.* 53:191-195.
- Ogonuki, N., K. Inoue, Y. Yamamoto, Y. Noguchi, K. Tanemura, O. Suzuki, H. Nakayama, K. Doi, Y. Ohtomo, M. Satoh, A. Nishida, and A. Ogura. 2002. Early death of mice cloned from somatic cells. *Nat. Genet.* 30:253-254.
- Ogura, A., K. Inoue, N. Ogonuki, J. Lee, T. Kohda, and F. Ishino. 2002. Phenotypic effects of somatic cell cloning in the mouse. *Cloning Stem Cells* 4:397-405.
- Ogura, A., K. Inoue, N. Ogonuki, A. Noguchi, K. Takano, R. Nagano, O. Suzuki, J. Lee, F. Ishino, and J. Matsuda. 2000. Production of male cloned mice from fresh, cultured, and cryopreserved immature Sertoli cells. *Biol. Reprod.* 62:1579-1584.
- Ohgane, J., T. Wakayama, Y. Kogo, S. Senda, N. Hattori, S. Tanaka, R. Yanagimachi, and K. Shiota. 2001. DNA methylation variation in cloned mice. *Genesis* 30:45-50.
- Ott, R. S. 1982. Dairy goat reproduction. *Compend. Cont. Ed. Pract. Vet.* 4:164-172.
- Ott, R. S., D. R. Nelson, and J. E. Hixon. 1980. Peripheral serum progesterone and luteinizing hormone concentrations of goats during synchronization of estrus and ovulation with prostaglandin. *Am. J. Vet. Res.* 41:1432-1434.
- Ozil, J. P. and J. A. Modlinski. 1986. Effects of electric field on fusion rate and survival of 2-cell rabbit embryos. *J. Embryol. Exp. Morph.* 96:211-228.
- Papaioannou, V. E. 1982. Lineage analysis of inner cell mass and trophectoderm using microsurgically reconstituted mouse blastocysts. *J. Embryol. Exp. Morphol.* 68:199-209.
- Pathiraja, N., E. O. Oyedipe, E. O. Gyang, and A. Obasi. 1991. Plasma progesterone levels during oestrous cycle and their relationship with the ovulation rate in Red Sokoto (Maradi) goats. *British Vet. J.* 147:57-62.

- Peippo, J., M. Kurkilahti, and P. Bredbacka. 2001. Developmental kinetics of in vitro produced bovine embryos: the effect of sex, glucose and exposure to time-lapse environment. *Zygote* 9:105-113.
- Peli, J., F. Schmoll, J. Laurincik, G. Brem, and K. Schellander. 1996. Comparison of aggregation and injection techniques in producing chimeras with embryonic stem cells in mice. *Theriogenology* 45:833-842.
- Pfister-Genskow, M., C. Myers, L. Childs, J. C. Lacson, T. Patterson, J. M. Betthausen, P. J. Goueleke, R. W. Koppang, G. Lange, P. Fisher, S. R. Watt, E. J. Forsberg, Y. Zheng, G. H. Leno, R. M. Schultz, B. Liu, C. Chetia, X. Yang, I. Hoeschele, and K. J. Eilertsen. 2005. Identification of differentially expressed genes in individual bovine preimplantation embryos produced by nuclear transfer: Improper reprogramming of genes required for development. *Biol. Reprod.* 72:546-555.
- Picard, L., I. Chartrain, W. A. King, and K. J. Betteridge. 1990. Production of chimaeric bovine embryos and calves by aggregation of inner cell masses with morulae. *Mol. Reprod. Dev.* 27:295-304.
- Pincus, G. and C. H. Waddington. 1939. The effects of mitosis-inhibiting treatments on normally fertilized pre-cleavage rabbit eggs. *J. Hered.* 30:515-518.
- Polejaeva, I. A., S. H. Chen, T. D. Vaught, R. L. Page, J. Mullins, S. Ball, Y. Dai, J. Boone, S. Walker, D. L. Ayares, A. Colman, and K. H. Campbell. 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 407:86-90.
- Polzin, V. J., D. L. Anderson, G. B. Anderson, R. H. BonDurant, J. E. Butler, R. L. Pashen, M. C. Penedo, and J. D. Rowe. 1987. Production of sheep-goat chimeras by inner cell mass transplantation. *J. Anim Sci.* 65:325-330.
- Pope, W. F., H. Cardenas, T. M. Wiley, and K. E. McClure. 1995. Dose-response relationships of exogenous progesterone shortly after ovulation on estrous cycle length, blastocyst development and fertility in sheep. *Anim. Reprod. Sci.* 38:109-117.
- Prather, R. S., F. L. Barnes, M. M. Sims, J. M. Robl, W. H. Eyestone, and N. L. First. 1987. Nuclear transplantation in the bovine embryo: Assessment of donor nuclei and recipient oocyte. *Biol. Reprod.* 37:859-866.
- Prather, R. S., and N. L. First. 1987. Developmental fate in chimeras derived from highly asynchronous murine blastomeres. *J. Exp. Zool.* 242:27-33.
- Prather, R. S., K. E. Hoffman, R. A. Schoenbeck, T. T. Stumpf, and J. Li. 1996. Characterization of DNA synthesis during the 2-cell stage and the production of tetraploid chimeric pig embryos. *Mol. Reprod. Dev.* 45:38-42.

- Prather, R. S., J. Kubiak, G. G. Maul, N. L. First, and G. Schatten. 1991. The expression of nuclear lamin A and C epitopes is regulated by the developmental stage of the cytoplasm in mouse oocytes or embryos. *J. Exp. Zool.* 257:110-114.
- Prather, R. S. and W. Rideout. 1992. Developmental regulation of an snRNP core protein epitope during pig embryogenesis and after nuclear transfer for cloning. *Mol. Reprod. Dev.* 33:119-123.
- Prather, R.S., M. M. Sims, and N. L. First. 1989. Nuclear transplantation in early pig embryos. *Biol. Reprod.* 41:414-418.
- Prather, R. S., M. M. Sims, and N. L. First. 1990. Nuclear transplantation in the pig embryo: Nuclear swelling. *J. Exp. Zool.* 255:355-358.
- Prather, R. S., M. M. Sims, G. G. Maul, N. L. First, and G. Schatten. 1989. Nuclear lamin antigens are developmentally regulated during porcine and bovine embryogenesis. *Biol. Reprod.* 41:123-132.
- Prochazka, R., P. Vodicka, D. Zudova, R. Rybar, and J. Motlik. 2004. Development of in vivo derived diploid and tetraploid pig embryos in a modified medium NCSU 37. *Theriogenology* 62:155-164.
- Ravelich, S. R., B. H. Breier, S. Reddy, J. A. Keelan, D. N. Wells, A. J. Peterson, and R. S. Lee. 2004a. Insulin-like growth factor-I and binding proteins 1, 2, and 3 in bovine nuclear transfer pregnancies. *Biol. Reprod.* 70:430-438.
- Ravelich, S. R., A. N. Shelling, A. Ramachandran, S. Reddy, J. A. Keelan, D. N. Wells, A. J. Peterson, R. S. Lee, and B. H. Breier. 2004b. Altered placental lactogen and leptin expression in placentomes from bovine nuclear transfer pregnancies. *Biol. Reprod.* 71:1862-1869.
- Reggio, B. C., H. L. Green, M. Sansinena, L. H. Chen, E. Behboodi, R. S. Denniston, Y. Echelard, and R. Godke. 2002. Production of cloned transgenic goats as a potential source for human pharmaceuticals. *Theriogenology* 57:445.
- Reggio, B. C., A. N. James, H. L. Green, W. G. Gavin, E. Behboodi, Y. Echelard, and R. A. Godke. 2001. Cloned transgenic offspring resulting from somatic cell nuclear transfer in the goat: Oocytes derived from both follicle-stimulating hormone-stimulated and nonstimulated abattoir-derived ovaries. *Biol. Reprod.* 65:1528-1533.
- Reik, W. and E. R. Maher. 1997. Imprinting in clusters: Lessons from Beckwith-Wiedemann syndrome. *Trends Genet.* 13:330-334.
- Renard, J. P., S. Chastant, P. Chesne, C. Richard, J. Marchal, N. Cordonnier, P. Chavatte, and X. Vignon. 1999. Lymphoid hypoplasia and somatic cloning. *Lancet* 353:1489-1491.

- Renard, J. P., Q. Zhou, P. Chavatte-Palmer, I. Hue, Y. Heyman, and X. Vignon. 2002. Nuclear transfer technologies: Between successes and doubts. *Theriogenology* 57:203-222.
- Richter, H.-P., P. Scheurich, and U. Zimmermann. 1981. Electric field-induced fusion of sea urchin eggs. *Dev. Growth Diff.* 23:479-486.
- Rideout, III, W. M., K. Hochedlinger, M. Kyba, G. Q. Daley, and R. Jaenisch. 2002. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* 109:17-27.
- Rideout, W. M., T. Wakayama, A. Wutz, K. Eggen, L. Jackson-Grusby, J. Dausman, R. Yanagimachi, and R. Jaenisch. 2000. Generation of mice from wild-type and targeted ES cells by nuclear cloning. *Nat. Genet.* 24:109-110.
- Robl, J. M., R. Prather, F. Barnes, W. Eyestone, D. Northey, B. Gilligan, and N. L. First. 1987. Nuclear transplantation in bovine embryos. *J. Anim Sci.* 64:642-647.
- Rorie, R.W., S. H. Pool, J. F. Prichard, K. J. Betteridge, and R. A. Godke. 1994. A simplified procedure for making reconstituted blastocysts for interspecific and intergeneric transfer. *Vet. Rec.* 135:186-187.
- Rosenkrans, Jr., C. F., and N. L. First. 1994. Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. *J. Anim Sci.* 72:434-437.
- Rossant, J., B. A. Croy, D. A. Clark, and V. M. Chapman. 1983. Interspecific hybrids and chimeras in mice. *J. Exp. Zool.* 228:223-233.
- Rossant, J., and W. I. Frels. 1980. Interspecific chimeras in mammals: Successful production of live chimeras between *Mus musculus* and *Mus caroli*. *Science* 208:419-421.
- Rossant, J., V. M. Mauro and B. A. Croy. 1982. Importance of trophoblast genotype for survival of interspecific murine chimeras. *J. Embryol. Exp. Morph.* 69:141-149.
- Roth, T. L., G. B. Anderson, R. H. Bon Durant, and R. L. Pashen. 1989. Survival of sheep x goat hybrid inner cell masses after injection into ovine embryos. *Biol. Reprod.* 41:675-682.
- Rubeš, J., M. Machatková, E. Jokešová, and D. Zudová. 1999. A potential relationship between the 16;20 and the 14;20 Robertsonian translocations and low in vitro embryo development. *Theriogenology* 52:171-180.
- Ryan, D. P., E. G. Blakewood, J. W. Lynn, L. Munyakazi, and R. A. Godke. 1992. Effect of heat-stress on bovine embryo development in vitro. *J. Anim Sci.* 70:3490-3497.

- Saharrea, A., J. Valencia, A. Balcazar, O. Mejia, J. L. Cerbon, V. Caballero, and L. Zarco. 1998. Premature luteal regression in goats superovulated with PMSG: Effect of hCG or GnRH administration during the early luteal phase. *Theriogenology* 50:1039-1052.
- Samake, S. and L. C. Smith. 1996a. Effects of cell-cycle-arrest agents on cleavage and development of mouse embryos. *J. Exp. Zool.* 274:111-120.
- Samake, S. and L. C. Smith. 1996b. Synchronization of cell division in eight-cell bovine embryos produced in vitro: Effects of nocodazole. *Mol. Reprod. Dev.* 44:486-492.
- Santos, F., V. Zakhartchenko, M. Stojkovic, A. Peters, T. Jenuwein, E. Wolf, W. Reik, and W. Dean. 2003. Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. *Curr. Biol.* 13:1116-1121.
- Sawada, T., M. Hou, H. Tamada, and J. Mori. 1994. Secretion of progesterone and 20 alpha-dihydroprogesterone during the estrous cycle in goats. *Steroids* 59:672-675.
- Sawicki, W., J. Abramczuk, and O. Blaton. 1978. DNA synthesis in the second and third cell cycles of mouse preimplantation development. A cytophotometric study. *Exp. Cell Res.* 112:199-205.
- Schmitt, E. J. P., C. M. Barros, P. A. Fields, M. J. Fields, T. Diaz, J. M. Kluge, and W. W. Thatcher. 1996a. A cellular and endocrine characterization of the original and induced corpus luteum after administration of a Gonadotropin-Releasing Hormone agonist or Human Chorionic Gonadotropin on day five of the estrous cycle. *J. Anim. Sci.* 74:1915-1929.
- Schmitt, E. J. P., T. Diaz, C. M. Barros, R. L. de la Sota, M. Drost, E. W. Fredriksson, C. R. Staples, R. Thorner, and W. W. Thatcher. 1996b. Differential response of the luteal phase and fertility in cattle following ovulation of the first-wave follicle with Human Chorionic Gonadotropin or an agonist of Gonadotropin-Releasing Hormone. *J. Anim. Sci.* 74:1074-1083.
- Schnieke, A. E., A. J. Kind, W. A. Ritchie, K. Mycock, A. R. Scott, M. Ritchie, I. Wilmut, A. Colman, and K. H. Campbell. 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* 278:2130-2133.
- Schoonjans, L., V. Kreemers, S. Danloy, R. W. Moreadith, Y. Laroche, and D. Collen. 2003. Improved generation of germline-competent embryonic stem cell lines from inbred mouse strains. *Stem Cells* 21:90-97.
- Schramm, R. D. and A. M. Paprocki. 2004. In vitro development and cell allocation following aggregation of split embryos with tetraploid or developmentally asynchronous blastomeres in rhesus monkeys. *Cloning Stem Cells* 6:302-314.
- Schreiber, M., Z. Q. Wang, W. Jochum, I. Fetka, C. Elliott, and E. F. Wagner. 2000. Placental vascularisation requires the AP-1 component fra1. *Development* 127:4937-4948.

- Schwenk, F., B. Zevnik, J. Bruning, M. Rohl, A. Willuweit, A. Rode, T. Hennek, G. Kauselmann, R. Jaenisch, and R. Kuhn. 2003. Hybrid embryonic stem cell-derived tetraploid mice show apparently normal morphological, physiological, and neurological characteristics. *Mol. Cell Biol.* 23:3982-3989.
- Seibler, J., B. Zevnik, B. Kuter-Luks, S. Andreas, H. Kern, T. Hennek, A. Rode, C. Hiemann, N. Faust, G. Kauselmann, M. Schoor, R. Jaenisch, K. Rajewsky, R. Kuhn, and F. Schwenk. 2003. Rapid generation of inducible mouse mutants. *Nucleic Acids Res.* 31:e-12.
- Sekirina, G. G., N. A. Bogoliubova, N. V. Antonova, and A. P. Dyban. 1997. The behaviour of mitochondria and cell integration during somatic hybridisation of sister blastomeres of the 2-cell mouse embryo. *Zygote* 5:97-103.
- Senda, M., J. Takeda, S. Abe, and T. Nakamura. 1979. Induction of cell fusion of plant protoplasts by electrical stimulation. *Plant Cell Physiol.* 20:1441-1443.
- Senda, S., T. Wakayama, Y. Yamazaki, J. Ohgane, N. Hattori, S. Tanaka, R. Yanagimachi, and K. Shiota. 2004. Skewed X-inactivation in cloned mice. *Biochem. Biophys. Res. Commun.* 321:38-44.
- Shi, D. S., K. H. Lu, and I. Gordon. 1990. Effects of bulls on fertilization of bovine oocytes and their subsequent development in vitro. *Theriogenology* 33:324.
- Shin, T., D. Kraemer, J. Pryor, L. Liu, J. Rugila, L. Howe, S. Buck, K. Murphy, L. Lyons, and M. Westhusin. 2002. A cat cloned by nuclear transplantation. *Nature* 415:859.
- Sims, M. and N. L. First. 1994. Production of calves by transfer of nuclei from cultured inner cell mass cells. *Proc. Natl. Acad. Sci. U. S. A.* 91:6143-6147.
- Smith, L. C. and B. D. Murphy. 2004. Genetic and epigenetic aspects of cloning and potential effects on offspring of cloned mammals. *Cloning Stem Cells* 6:126-132.
- Snow, M. H. L. 1973. Tetraploid mouse embryos produced by cytochalasin B during cleavage. *Nature* 244:513-515.
- Snow, M. H. L. 1975. Embryonic development of tetraploid mice during the second half of gestation. *J. Embryol. Exp. Morph.* 34:707-721.
- Snow, M. H. L. 1976. The immediate postimplantation development of tetraploid mouse blastocysts. *J. Embryol. Exp. Morph.* 35:81-86.
- Spindle, A. 1981. Polyethylene glycol-induced fusion of two-cell mouse embryo blastomeres. *Exp. Cell Res.* 131:465-470.

- Spindle, A., K. S. Sturm, M. Flannery, J. J. Meneses, K. Wu, and R. A. Pederson. 1996. Defective chorioallantoic fusion in mid-gestation lethality of parthenogenone tetraploid chimeras. *Dev. Biol.* 173:447-458.
- Stice, S. L. and J. M. Robl. 1988. Nuclear reprogramming in nuclear transplant rabbit embryos. *Biol. Reprod.* 39:657-664.
- Stice, S. L., N. S. Strelchenko, C. L. Keefer, and L. Matthews. 1996. Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. *Biol. Reprod.* 54:100-110.
- Stojanov, T. and C. O'Neill. 2001. In vitro fertilization causes epigenetic modifications to the onset of gene expression from the zygotic genome in mice. *Biol. Reprod.* 64:696-705.
- Stowers, A. W., L. H. Chen Lh, Y. Zhang, M. C. Kennedy, L. Zou, L. Lambert, T. J. Rice, D. C. Kaslow, A. Saul, C. A. Long, H. Meade, and L. H. Miller. 2002. A recombinant vaccine expressed in the milk of transgenic mice protects Aotus monkeys from a lethal challenge with *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. U. S. A.* 99:339-344.
- Stubbings, R. B., W. T. Bosu, C. A. Barker, and G. J. King. 1986. Serum progesterone concentrations associated with superovulation and premature corpus luteum failure in dairy goats. *Can. J. Vet Res.* 50:369-373.
- Sugimoto, M., Y. Karashima, K. Abe, S. S. Tan, and N. Takagi. 2003. Tetraploid embryos rescue the early defects of tw5/tw5 mouse embryos. *Genesis* 37:162-171.
- Sun, F. Z. and R. M. Moor. 1995. Nuclear transplantation in mammalian eggs and embryos. *Curr. Top. Dev. Biol.* 30:147-176.
- Surani, M. A. H., and S. C. Barton. 1984. Spatial distribution of blastomeres is dependent on cell division order and interactions in mouse morulae. *Dev. Biol.* 102:335-343.
- Surani, M. A. H., S. C. Barton, and M. L. Norris. 1987. Experimental reconstruction of mouse eggs and embryos: an analysis of mammalian development. *Biol. Reprod.* 36:1-16.
- Szollosi, D., R. Czolowska, M. S. Szollosi, and A. J. Tarkowski. 1988. Remodeling of mouse thymocyte nuclei depends on the time of their transfer into activated, homologous oocytes. *J. Cell Sci.* 91:603-613.
- Takahashi, Y., N. Carpino, J. C. Cross, M. Torres, E. Parganas, and J. N. Ihle. 2003. SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. *EMBO J.* 22:372-384.
- Tamashiro, K. L., T. Wakayama, H. Akutsu, Y. Yamazaki, J. L. Lachey, M. D. Wortman, R. J. Seeley, D. A. D'Alessio, S. C. Woods, R. Yanagimachi, and R. R. Sakai. 2002. Cloned mice have an obese phenotype not transmitted to their offspring. *Nat. Med.* 8:262-267.

- Tamashiro, K. L. K., T. Wakayama, R. J. Blanchard, D. C. Blanchard, and R. Yanagimachi. 2000. Postnatal growth and behavior of mice cloned from adult cumulus cells. *Biol. Reprod.* 63:328-334.
- Tanaka, M., A. K. Hadjantonakis, and A. Nagy. 2001a. Aggregation chimeras. Combining ES cells, diploid and tetraploid embryos. *Meth. Mol. Biol.* 158:135-154.
- Tanaka, S., M. Oda, Y. Toyoshima, T. Wakayama, M. Tanaka, N. Yoshida, N. Hattori, J. Ohgane, R. Yanagimachi, and K. Shiota. 2001b. Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. *Biol. Reprod.* 65:1813-1821.
- Taneja, M., R. French, H. Levine, D. Tauro-Miller, and X. Yang. 2001. Clinical and pathological status of cloned calves born pre-term. *Theriogenology* 55:293.
- Tang, P. C., W. A. Ritchie, I. Wilmut, and J. D. West. 2000. The effects of cell size and ploidy on cell allocation in mouse chimaeric blastocysts. *Zygote* 8:33-43.
- Tarkowski, A. K. 1961. Mouse chimaeras developed from fused eggs. *Nature* 190:857-860.
- Tarkowski, A. K., W. Ozdzanski, and R. Czolowska. 2001. Mouse singletons and twins developed from isolated diploid blastomeres supported with tetraploid blastomeres. *Int. J. Dev. Biol.* 45:591-596.
- Tarkowski, A. K., A. Witkowska, and J. Opas. 1977. Development of cytochalasin B-induced tetraploid and diploid/tetraploid mosaic mouse embryos. *J. Embryol. Exp. Morph.* 41:47-64.
- Tecirlioglu, R. T., A. J. French, I. M. Lewis, G. Vajta, N. A. Korfiatis, V. J. Hall, N. T. Ruddock, M. A. Cooney, and A. Trounson. 2004. Birth of a cloned calf derived from a vitrified hand-made cloned embryo. *Reprod. Fertil. Dev.* 15:361-366.
- Thibier, M., D. Pothelet, N. Jeanguyot, and G. De Montigny. 1981. Estrous behavior, progesterone in peripheral plasma and milk in dairy goats at onset of breeding season. *J. Dairy Sci.* 64:513-519.
- Thimonier, J. 1981. Control of seasonal reproduction in sheep and goats by light and hormones. *J. Reprod. Fertil. Suppl.* 30:33-45.
- Thorburn, G. D. and W. Schneider. 1972. The progesterone concentration in the plasma of the goat during the oestrous cycle and pregnancy. *J. Endocrin.* 52:23-36.
- Tsong, T. Y. 1983. Voltage modulation of membrane permeability and energy utilization in cells. *Biosci. Rep.* 3:487-506.

- Ueda, O., K. Jishage, N. Kamada, S. Uchida, and H. Suzuki. 1995. Production of mice entirely derived from embryonic stem (ES) cell with many passages by coculture of ES cells with cytochalasin B induced tetraploid embryos. *Exp. Anim.* 44:205-210.
- van de Corput, M. P. C., R. W. Dirks, R. P. van Gijlswijk, F. M. van de Rijke, and A. K. Raap. 1998. Fluorescence in situ hybridization using horseradish peroxidase-labeled oligodeoxynucleotides and tyramide signal amplification for sensitive DNA and mRNA detection. *Histochem. Cell Biol.* 110:431-437.
- Van Soom, A., M. Boerjan, M. T. Ysebaert, and A. de Kruif. 1996. Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media. *Mol. Reprod. Dev.* 45:171-182.
- Van Soom, A., M. T. Ysebaert, and A. de Kruif. 1997. Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. *Mol. Reprod. Dev.* 47:47-56.
- Viuff, D., P. J. Hendriksen, P. L. Vos, S. J. Dieleman, B. M. Bibby, T. Greve, P. Hyttel, and P. D. Thomsen. 2001. Chromosomal abnormalities and developmental kinetics in in vivo-developed cattle embryos at days 2 to 5 after ovulation. *Biol. Reprod.* 65:204-208.
- Wakayama, T., A. C. F. Perry, M. Zuccotti, K. R. Johnson, and R. Yanagimachi. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 394:369-374.
- Wakayama, T., I. Rodriguez, A. C. F. Perry, R. Yanagimachi, and P. Mombaerts. 1999. Mice cloned from embryonic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 26:14984-14989.
- Wakayama, T., Y. Shinkai, K. L. K. Tamashiro, H. Niida, D. C. Blanchard, R. J. Blanchard, A. Ogura, K. Tanemura, M. Tachibana, A. C. F. Perry, D. F. Colgan, P. Mombaerts, and R. Yanagimachi. 2000. Cloning of mice to six generations. *Nature* 407:318-319.
- Wakayama, T. and R. Yanagimachi. 1999. Cloning of male mice from adult tail-tip cells. *Nat. Genet.* 22:127-128.
- Wallace, J. M., D. A. Bourke, P. Da Silva, and R. P. Aitken. 2003. Influence of progesterone supplementation during the first third of pregnancy on fetal and placental growth in overnourished adolescent ewes. *Reproduction* 126:481-487.
- Wang, J., J. Mager, E. Schnedier, and T. Magnuson. 2002. The mouse PcG gene *eed* is required for *Hox* gene repression and extraembryonic development. *Mamm. Genome* 13:493-503.
- Wang, S., V. L. DeGroff, and S. K. Clinton. 2003. Tomato and soy polyphenols reduce insulin-like growth factor-I-stimulated rat prostate cancer cell proliferation and

apoptotic resistance in vitro via inhibition of intracellular signaling pathways involving tyrosine kinase. *J. Nutr.* 133:2367-2376.

- Wang, Z. Q., M. R. Fung, D. P. Barlow, and E. F. Wagner. 1994. Regulation of embryonic growth and lysosomal targeting by the imprinted *Igf2/Mpr* gene. *Nature* 372:464-467.
- Wang, Z. Q., F. Kiefer, P. Urbanek, and E. F. Wagner. 1997. Generation of completely embryonic stem cell-derived mutant mice using tetraploid blastocyst injection. *Mech. Dev.* 62:137-145.
- Ward, F., D. Rizos, D. Corridan, K. Quinn, M. Boland, and P. Lonergan. 2001. Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development in vitro and fertility in vivo. *Mol. Reprod. Dev.* 60:47-55.
- Watt, A. J., M. A. Battle, J. Li, and S. A. Duncan. 2004. GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 101:12573-12578.
- Webb, S., T. J. de Vries, and M. H. Kaufman. 1992. The differential staining pattern of the X chromosome in the embryonic and extraembryonic tissues of postimplantation homozygous tetraploid mouse embryos. *Genet. Res.* 59:205-214.
- Wells, D. N., P. M. Misica, A. M. Day, A. J. Peterson, and H. R. Tervit. 1998a. Cloning sheep from cultured embryonic cells. *Reprod. Fertil. Dev.* 10:615-626.
- Wells, D. N., P. M. Misica, A. M. Day, and H. R. Tervit. 1997. Production of cloned lambs from an established embryonic cell line: a comparison between in vivo- and in vitro-matured cytoplasts. *Biol. Reprod.* 57:385-393.
- Wells, D. N., P. M. Misica, and H. R. Tervit. 1999. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.* 60:996-1005.
- Wells, D. N., P. M. Misica, H. R. Tervit, and W. H. Vivanco. 1998b. Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the Enderby Island cattle breed. *Reprod. Fertil. Dev.* 10:369-378.
- Wells, K. D. and A. M. Powell. 2000. Blastomeres from somatic cell nuclear transfer embryos are not allocated randomly in chimeric blastocysts. *Cloning* 2:9-22.
- Westhusin, M. E., M. J. Levanduski, R. Scarborough, C. R. Looney, and K. R. Bondioli. 1992. Viable embryos and normal calves after nuclear transfer into Hoechst stained enucleated demi-oocytes of cows. *J. Reprod. Fertil.* 95:475-480.
- Westhusin, M. 2003. Texas A&M scientists clone world's first deer. Texas A&M University www.cvm.tamu.edu/news/releases/deer_clone.shtml

- Willadsen, S. M. 1986. Nuclear transplantation in sheep embryos. *Nature* 320:63-65.
- Willadsen, S. M., R. E. Janzen, R. J. McAlister, B. F. Shea, G. Hamilton, and D. McDermand. 1991. The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. *Theriogenology* 35:161-170.
- Williamson, M., R. Tecirlioglu, A. French, and M. Holland. 2005. Anatomical abnormalities in calves produced by nuclear transfer. *Reprod. Fertil. Dev.* 17:1039.
- Wilmut, I., A. E. Schnieke, J. McWhir, A. J. Kind, and K. H. S. Campbell. 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature* 385:810-813.
- Wilson, J. M., J. D. Williams, K. R. Bondioli, C. R. Looney, M. E. Westhusin, and D. F. McCalla. 1995. Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating. *Anim. Reprod. Sci.* 38:73-83.
- Winger, Q. A., J. R. Hill, T. Shin, A. J. Watson, D. C. Kraemer, and M. E. Westhusin. 2000. Genetic reprogramming of lactate dehydrogenase, citrate synthase, and phosphofructokinase mRNA in bovine nuclear transfer embryos produced using bovine fibroblast cell nuclei. *Mol. Reprod. Dev.* 56:458-464.
- Wolffe, A. P. and M. A. Matzke. 1999. Epigenetics: Regulation through repression. *Science* 286:481-486.
- Wood, S. A., N. D. Allen, J. Rossant, A. Auerbach, and A. Nagy. 1993. Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* 365:87-89.
- Woods, G. L., K. L. White, D. K. Vanderwall, G. P. Li, K. I. Aston, T. D. Bunch, L. N. Meerdo, and B. J. Pate. 2003. A mule cloned from fetal cells by nuclear transfer. *Science* 301:1063.
- Wrenzycki, C., A. Lucas-Hahn, D. Herrmann, E. Lemme, K. Korsawe, and H. Niemann. 2002. In vitro production and nuclear transfer affect dosage compensation of the X-linked gene transcripts G6PD, PGK, and Xist in preimplantation bovine embryos. *Biol. Reprod.* 66:127-134.
- Wrenzycki, C., D. Wells, D. Herrmann, A. Miller, J. Oliver, R. Tervit, and H. Niemann. 2001. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. *Biol. Reprod.* 65:309-317.
- Wu, C. and J. R. Morris. 2001. Genes, genetics, and epigenetics: A correspondence. *Science* 293:1103-1105.
- Wu, L., A. de Bruin, H. I. Saavedra, M. Starovic, A. Trimboli, Y. Yang, J. Opavska, P. Wilson, J. C. Thompson, M. C. Ostrowski, T. J. Rosol, L. A. Woollett, M. Weinstein,

- J. C. Cross, M. L. Robinson, and G. Leone. 2003. Extra-embryonic function of Rb is essential for embryonic development and viability. *Nature* 421:942-947.
- Xue, F., X. C. Tian, F. Du, C. Kubota, M. Taneja, A. Dinnyes, Y. Dai, H. Levine, L. V. Pereira, and X. Yang. 2002. Aberrant patterns of X chromosome inactivation in bovine clones. *Nat. Genet.* 31:216-220.
- Yadav, B. R., W. A. King, and K. J. Betteridge. 1993. Relationships between the completion of first cleavage and the chromosomal complement, sex, and developmental rates of bovine embryos generated in vitro. *Mol. Reprod. Dev.* 36:434-439.
- Yong, Z. and L. Yuqiang. 1998. Nuclear-cytoplasmic interaction and development of goat embryos reconstructed by nuclear transplantation: Production of goats by serially cloning embryos. *Biol. Reprod.* 58:266-269.
- Yoshida, M., M. Kijima, M. Akita, and T. Beppu. 1990. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J. Biol. Chem.* 265:17174-17179.
- Young, L. E., K. Fernandes, T. G. McEvoy, S. C. Butterwith, C. G. Gutierrez, C. Carolan, P. J. Braodbent, J. J. Robinson, I. Wilmut, and K. D. Sinclair. 2001. Epigenetic change in IGFR2 is associated with fetal overgrowth after sheep embryo culture. *Nat. Genet.* 27:153-154.
- Young, L. E., K. D. Sinclair, and I. Wilmut. 1998. Large offspring syndrome in cattle and sheep. *Rev. Reprod.* 3:155-163.
- Zakhartchenko, V., G. Durcova-Hills, M. Stojkovic, W. Scherthaner, K. Prella, R. Steinborn, M. Muller, G. Brem, and E. Wolf. 1999. Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts. *J. Reprod. Fertil.* 115:325-331.
- Zhang, S., C. Kubota, L. Yang, Y. Zhang, R. Page, M. O'Neill, X. Yang, and X. C. Tian. 2004. Genomic imprinting of h19 in naturally reproduced and cloned cattle. *Biol. Reprod.* 71:1540-1544.
- Zhou, Q., J. P. Renard, G. L. Fricc, V. Brochard, N. Beaujean, Y. Cherifi, A. Fraichard, and J. Cozzi. 2003. Generation of fertile cloned rats by regulating oocyte activation. *Science* 302:1179.
- Zimmermann, U. and J. Vienken. 1984. Electric field-mediated cell-to-cell fusion. In: R. F. Beers Jr. and E. G. Bassett (Eds.) *Cell fusion: Gene Transfer and Transformation*. Raven Press, New York. pp. 171-187.

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