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Production of transgenic goats by somatic cell nuclear transfer

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PRODUCTION OF TRANSGENIC GOATS BY
SOMATIC CELL NUCLEAR TRANSFER

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Animal Science

by

Brett C. Reggio
B.S., Tulane University, 1992
M.S., Louisiana State University, 1997
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ABSTRACT

A series of experiments were conducted to produce transgenic goats by somatic cell nuclear transfer (NT). In all experiments, donor cells were electrically fused to enucleated metaphase II oocytes, then chemically activated. In a preliminary study to evaluate embryonic development following NT with proliferating or quiescent fibroblast or cumulus cells, significantly more embryos reconstructed with quiescent cumulus cells fused (77%) compared with proliferating cumulus cells (41%), proliferating fibroblasts (36%) or quiescent fibroblasts (37%). Improved development to the eight- to sixteen-cell stage was observed when fibroblast cells were serum starved (serum starved 39% vs. serum fed 15%). However, there was no benefit of serum starvation for cumulus cells nor was there a difference among the treatments for development to the blastocyst stage. Next, the in vivo developmental potential of NT embryos produced from the fusion of quiescent transgenic donor cells with cytoplasts prepared from either FSH-stimulated ovaries or nonstimulated abattoir-derived ovaries was compared. There was no difference in the number of transferable embryos produced, nor was there a difference in the number of pregnancies established per recipient between either treatment. All pregnancies from both groups culminated in the births of five healthy female kids. In the third and fourth experiments, proliferating and quiescent donor cells from two different transfected fibroblast cell lines were used to generate cloned goats capable of producing human recombinant antibodies in milk. There was no difference in the number of transferable embryos produced from proliferating donor cells compared with quiescent cells, nor was there a difference in the number of pregnancies established per recipient between either treatment. A twin pregnancy from the quiescent treatment resulted in the birth of two healthy transgenic kids. In the final study, oocytes were harvested either
from FSH-stimulated ovaries or from nonstimulated abattoir-derived ovaries to generate transgenic goats by NT using fetal fibroblast cells transfected with the MSP-142 gene. Following transfer of the reconstructed embryos to recipient females, one healthy transgenic kid was produced. There was no effect of oocyte source on the number of pregnancies established or on the number of offspring produced. In total, eight transgenic goats were produced.
CHAPTER I
INTRODUCTION

In 1997, the world was stunned and fascinated by the birth of a sheep named “Dolly” who was created not from the union of a sperm and egg, but by the transfer of a nucleus from a fully differentiated somatic cell into an oocyte devoid of its own nuclear DNA by the process of nuclear transfer (NT) (Wilmut et al., 1997). Since the birth of Dolly, six other mammalian species have been cloned from somatic cells, including the cow (Cibelli et al., 1998a; Kato et al., 1998), mouse (Wakayama et al., 1998), goat (Baguisi et al., 1999), pig (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000), gaur (Lanza et al., 2000c) and domestic cat (Shin et al., 2002). The significance of these accomplishments promises to impact livestock breeding practices (Wilmut et al., 2000), the biotechnology and pharmaceutical industry (Ziomek, 1998; Echelard et al., 2000), human medicine (Lanza et al., 1999a; b; Cibelli et al., 2001) and even the companion animal market (Westhusin et al., 2001; Shin et al., 2002).

Cloning by somatic cell NT involves removal of nuclear DNA from the host oocyte (enucleation) and replacing that DNA with a donor cell nucleus (reconstruction) derived from a somatic cell. The donor nucleus is then subjected to a complete “reprogramming” by undetermined factors located inside the ooplasm, which enable the complete set of instructions that were once turned off in the differentiated donor nucleus to become active and commence development, not as another somatic cell, but as a one-cell embryo (Campbell, 1999).

Oocytes from farm animal species can be obtained either by recovering in vivo ovulated mature oocytes or by aspirating oocytes from ovarian follicles and allowing
maturation to proceed in vitro. Immature oocytes can be harvested either from abattoir ovaries or by transvaginal or transabdominal oocyte retrieval from live animals.

Regardless of the source or method of retrieval, removal of the metaphase II (MII) chromosomes from a recently matured oocyte can be accomplished quickly by simply bisecting the oocyte (Willadsen, 1986) and relies on the assumption that one half will not contain the nucleus. However, this method removes a significant amount of cytoplasm, and may negatively influence subsequent embryonic development (Evsikov et al., 1990; Greising et al., 1994).

A more efficient enucleation method relies on the precise localization of nuclear DNA using the nuclear dye bis-benzimide (Hoechst 33342) to label the mature oocyte prior to enucleation. When exposed to ultraviolet light, the MII chromosomes become visible in the opaque cytoplasm (Westhusin et al., 1992), assuring complete removal of maternal nuclear DNA with minimal cytoplasmic loss.

The use of inverted microscopes equipped with micromanipulators has made the enucleation and reconstruction procedure a relatively straightforward procedure, although considerable skill is required to perfect the technique. To prevent damage during enucleation, mammalian oocytes are treated with cytoskeleton inhibitors (cytochalasins) to avoid rupturing the oolema while removing the MII chromosomes in a membrane-bound vesicle (McGrath and Solter, 1983b).

During reconstruction of enucleated mouse oocytes, the donor cell is first disrupted to isolate the nucleus, which is then injected directly into the ooplasm by piercing the oolema with a special piezo-driven pipette (Wakayama et al., 1998). For all other species, better success has been achieved when the entire intact donor cell (karyoplast) is inserted into the
perivitelline space of the enucleated oocyte (cytoplasm) to avoid penetration of the oolema during reconstruction, and then fused to the oolema (reviewed in Colman, 2000). An electrical pulse is used to induce a transient breakdown of the plasma membranes surrounding both the karyoplast and cytoplast, which causes fusion of the two cells (Kubiak and Tarkowski, 1985; Ozil and Modlinski, 1986). The donor nucleus is subsequently exposed to the reprogramming factors of the recipient ooplasm and becomes capable of giving rise to a complete organism. Following this fusion event, the newly reconstructed couplet is then activated by a two-step protocol to initiate embryonic development (Susko-Parrish et al., 1994).

A major obstacle in applying the NT technology for cloning valuable animals is the low efficiency (Dominko et al., 1999). The overall efficiency of the NT procedure, based on the number of live offspring produced per number of embryos transferred, ranges between 1% and 10% in the sheep (Schnieke et al., 1997; Wells et al., 1997; 1998a; Wilmut et al., 1997; McCreath et al., 2000), cow (Cibelli et al., 1998a; b; Wells et al., 1998b; 1999a; b; Shiga et al., 1999), mouse (Wakayama et al., 1998; 1999), goat (Baguisi et al., 1999; Keefer et al., 2001; 2002; Reggio et al., 2001) and pig (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000; Bondioli et al., 2001). In one remarkable study, eight calves were produced from the transfer of 10 NT embryos (80% efficiency) but four calves died at or soon after birth (Kato et al., 1998). Moreover, this study represents a small data set and is not indicative of the average efficiencies reported in this species.

Even with low efficiencies, the ability to propagate genetically identical animals with superior genotypes would be important for increasing the number of economically important animals from a food and fiber standpoint (Robl et al., 1987). Moreover, the production of
isogenic animals could lead to more efficient models for biomedical research by reducing the number of animals required for statistically valid experiments, especially in those species where animal or oocyte availability is limited (Wolf et al., 1999).

Cloning mammalian species by NT can also be applied to re-establish species on the brink of extinction. On the Enderby Island off the coast of New Zealand, the last remaining member of the Enderby breed of cattle was duplicated through somatic cell nuclear transfer (Wells et al., 1998b). This cloned female could help reintroduce the breed and create a diverse herd if a small number of clones were created and inseminated with the semen that was collected from the last 10 Enderby bulls before they died. In Spain, cloning technology will be used in a joint program with a U.S. biotechnology company to revive the now-extinct Bucardo mountain goat (*Capra pyrenaica pyrenaica*), which was eliminated when the last female of this species was killed by a falling tree (http://www.advancedcell.com/pr_10-18-2000-bucardo.html).

Perhaps the most exciting and promising use of the NT technology is in the creation of transgenic animals (Colman, 2000; Niemann and Kues, 2000; Polejaeva and Campbell, 2000; Wilmut et al., 2000). Until recently, microinjection of exogenous DNA into the pronuclei of zygote-stage embryos has been the only successful method for generating transgenic farm animals (Biery et al., 1988; Massey, 1990; Bowen et al., 1994; Hyttinen et al., 1994; Eyestone, 1998). Typical success rates for pronuclear injection are less than 5% (Simons et al., 1988; Murray et al., 1989; Pursel et al., 1989).

However, somatic cell nuclear transfer, combined with cell culture and molecular biology methodologies, has proven to be a more efficient method of producing transgenic animals than pronuclear DNA-microinjection (Schnieke et al., 1997; Cibelli et al., 1998a;
Transfected cells in which the gene of interest has been incorporated can be screened prior to NT to detect both the presence and location of the transgene in the donor cell DNA. The obvious advantage to this approach is that 100% of the resulting offspring will be transgenic (Niemann and Kues, 2000). Some of the proteins currently being produced in transgenic animals via somatic cell NT are human clotting factor IX (Schnieke et al., 1997) and antithrombin III (Baguisi et al., 1999).

The treatment of human disease will no doubt be impacted as NT becomes integrated into more and more research and development programs, not only for the production of therapeutic proteins, antibodies and vaccines, but for the creation of immune-compatible stem cells via autologous therapeutic cloning (Lanza et al., 1999b; 2000a). NT-derived stem cells may be coaxed into differentiated pathways leading to the production of specific cell types or even organ tissue masses, and may offer a promising solution to the immune rejection response normally associated with heterologous transfer (Cibelli et al., 2001).

Cardiomyocytes produced from stem cells generated through NT of autologous donor cells could be used to replace damaged heart tissue in heart-attack patients (Klug et al., 1996). Similarly, insulin-producing B-cells generated from NT-derived stem cells could be administered to diabetic patients to eliminate the reliance on daily injections of insulin (Soria et al., 2000).

Such applications are still years away from development, so a more immediate approach may be in the area of xenotransplantation from pigs to humans. The gene responsible for triggering an immune response in human transplant patients produces an enzyme (galactosyl transferase) that adds a sugar residue to the surface of pig cells that the human body recognizes as foreign. This leads to hyperacute rejection of the transplanted
organ or cell within minutes. Genetically engineered pigs could be produced via NT by modifying somatic porcine cells cultured in vitro to “knock-out” this gene. At least one study has reported the births of healthy piglets cloned from cells deficient in the galactosyl transferase gene (Lai et al., 2002).

Finally, NT technology is currently being considered as a method to produce vaccines to eradicate transmissible diseases such as malaria. Malaria afflicts an estimated 300 to 500 million people worldwide and accounts for more deaths than any other infectious disease except tuberculosis (http://www.who.int/inf-fs/en/fact094.html). Development of a malaria vaccine faces a major economic hurdle, since the populations that would benefit from the vaccine live in underdeveloped countries (particularly sub-Saharan Africa) that cannot afford immunization programs. Thus, the development of a vaccine production method capable of generating millions of doses at a low unit cost is essential. Preliminary results in a simian model prove that a recombinant vaccine (MSP-1 42) expressed in the milk of transgenic mice can elicit immunity against a lethal malaria challenge (Stowers et al., 2002). However, the limited milk yield from these transgenic mice limits the protein production to milligram quantities. Thus, efforts are now underway to generate cloned transgenic goats via NT with caprine donor cells transfected with the MSP-1 42 gene. If successful, cloned transgenic goats could have a significant impact on developing a high volume, low cost malaria vaccine.

Thus, NT technology offers an exciting approach to treating human disease. Xenotransplantation of genetically modified organ tissue harvested from cloned animals promises to provide a solution to the graft compared with host immune response in human transplant patients. Moreover, the ability to provide customized stem cells from NT embryos may offer better management of disease through isogenic cell replacement therapy. Finally,
NT holds particular promise for the commercialization of human therapeutic proteins at an affordable cost, with several such products in various stages of clinical trials.
CHAPTER II
LITERATURE REVIEW

Sea Urchins, Frogs and Salamanders

In the 1800’s August Weismann advanced a theory addressing the mechanism for cell specialization during development from the fertilized zygote to the adult. Weismann argued that differentiation results from the “differential and sequential partitioning of the genome” as the cells divide (reviewed by Wilson, 1928). In an effort to prove this theory, William Roux in 1888 used a hot needle to destroy one blastomere of a two-cell frog embryo and demonstrated that each daughter cell apparently contained “only half as much of the genetic information as the parent cell,” since the remaining blastomere failed to develop (Hamburger, 1988). However, he failed to realize that the destroyed blastomere prevented complete development due to the positional effect it had on the remaining intact blastomere.

When Hans Adolf Dreisch repeated the experiment in 1894 using a two-cell sea urchin embryo separated into individual blastomeres by shaking, each intact blastomere gave rise to a complete sea urchin, thus challenging Weismann’s hypothesis (Hamburger, 1988). Dreisch repeated the experiment with a four-cell embryo and each blastomere again produced an intact organism, suggesting that the entire genetic makeup was indeed present in each blastomere and was reproduced during cell division in contrast to Weismann’s theory.

In 1914, Hans Spemann further addressed the question of genetic constancy by partially constricting a newly fertilized salamander zygote with a strand of hair and forcing the nucleated portion of the embryo to one side. The nucleated side cleaved and developed to the 16-cell stage, at which time the ligature was loosened to allow one of the nuclei to pass through the constriction and into the nonnucleated portion. The ligature was then completely
tightened, cutting the embryo in half. Cell division occurred in the newly nucleated portion of
the cytoplasm, eventually resulting in twin larvae (one slightly older) (Spemann, 1938).
Spemann reported that nuclei from a 16-cell embryo were capable of directing the complete
growth and differentiation of adult amphibians, and proposed that future embryologists test
the genomic potential of older embryonic nuclei. At the time, the tools and technology were
not available. Nevertheless, both Spemann and Dreisch had effectively created the first
embryonic clones, one by rudimentary nuclear transfer and one by embryo splitting,
respectively.

In the early 1950s, Briggs and King reported the development of tadpoles from the
direct injection of embryonic blastula nuclei into enucleated frog eggs (Briggs and King,
1952), and solved some of the technical limitations inherent in Spemann’s earlier work.
Moreover, a later study using early gastrula cells showed that the injected nuclei could direct
complete development and generate adult frogs (Briggs and King, 1960). The totipotency of
embryonic cells was later demonstrated when injected nuclei developed into adult frogs that
subsequently produced normal progeny (Gurdon, 1961; McKinnell, 1962). Evidence from
these studies in amphibians suggested that differentiation is not caused by irreversible
modifications to the DNA. However, absolute proof would require that a truly differentiated
cell give rise to a normal reproducing adult.

Failure to generate adult frogs from differentiated cells led to the observation that the
 genotypic potential of a cell declined as it differentiated (King and Briggs, 1955), and therefore
cloning an organism from an adult, differentiated cell was not technologically feasible unless
the nucleus could be reverted to a more embryonic state. Other groups confirmed this
observation using several frog and amphibian species (McKinnell, 1972; 1978; Gurdon, 1974; Di Berardino, 1997), and it was generally accepted that adult cell nuclei were not totipotent.

A single report that differentiated cells were indeed capable of developing into a fully developed frog (Gurdon, 1962) was met with skepticism and never fully accepted (the adult intestinal cells that were used as nuclear donors may have been mixed with a small population of undifferentiated stem cells). Regardless, in a later study Gurdon summarized three lines of thinking that are still applicable today: 1) transferring a nucleus to an enucleated egg will change its gene activity, 2) these changes are immediate and are caused by the egg cytoplasm and 3) there is an exchange of proteins between the transferred nucleus and the egg cytoplasm which controls gene activity (Gurdon, 1970).

**Mammalian Nuclear Transfer**

The earliest reports of mammalian embryo cloning attempted to address the issue of embryonic cell differentiation, and to test the hypothesis that “prelocalized” cytoplasmic determinants were involved in this process. Experiments in the rat (Nicholas and Hall, 1942) and the rabbit (Moore et al., 1968) indicated that individual, isolated blastomeres from early embryos (two- to eight-cell stage) could develop into normal offspring when transferred to recipients, suggesting that cytoplasmic determinants were not in control during cell differentiation. Moreover, the concept of producing genetically identical offspring by embryo disaggregation set the stage for embryonic cloning in farm animals decades later.

The first successful cloning experiments in which fully differentiated adult cells developed into complete organisms were conducted in plants 44 years ago, when entire carrot plants were produced from single carrot cells (Steward et al., 1958). Steward demonstrated that by carefully defining and controlling the *in vitro* environment of the somatic cell, it could
indeed replace sexual reproduction (Steward, 1970). Researchers using vertebrate models overlooked this monumental achievement, and it took another 39 years for the same feat to be accomplished in mammals (Wilmut et al., 1997).

Nuclear transfer studies in mice were hampered because the techniques that worked well for enucleating and transferring nuclei in the large amphibian eggs were impractical for microscopic mammalian oocytes. Illmensee and Hoppe (1981) reported success in cloning mice by directly injecting inner cell mass (ICM) cell nuclei into enucleated pronuclear-stage oocytes using a procedure similar to the amphibian studies (Briggs and King, 1952). Controversy surrounding the techniques used in this study, and the inability to reproduce the same results in other laboratories, led to its rejection by the scientific community (Tsunoda and Kato, 2000).

However, a landmark report in 1983 described the use of the cytoskeletal inhibitor cytochalasin B (CB) and a virus-mediated cell fusion technique that allowed efficient pronuclear transplantation and full-term development in the mouse (McGrath and Solter, 1983b). Treating mouse pronuclear embryos with CB prevented damage to the oocyte plasma membrane and allowed the insertion of a glass pipette through the zona pellucida without lysing the oocyte. Both pronuclei could be removed as a membrane bound karyoplast and transferred to another enucleated pronuclear-stage oocyte (also a cytoplast). Instead of directly injecting the donor karyoplast, it was placed in the perivitelline space in close contact with the oolema. The karyoplast and cytoplast membranes were then induced to fuse using the inactivated Sendai virus (McGrath and Solter, 1983b).

Although pronuclear transfer in the mouse resulted in viable offspring (McGrath and Solter, 1983b), the transfer of nuclei from two-cell blastomeres into enucleated pronuclear-
stage oocytes produced very few blastocysts (13%) and no development to term (McGrath and Solter, 1984). Similar transfers using later stage nuclei (up to ICM) failed to produce any blastocysts, and led to the belief that donor nuclei reprogramming was impossible in the mammalian embryo. The authors concluded, “cloning of mammals by nuclear transfer is biologically impossible” (McGrath and Solter, 1984).

Pronuclear transplantation in bovine enucleated pronuclear-stage oocytes, using a modified version as described for the mouse (McGrath and Solter, 1983b), led to a 17% blastocyst rate and the birth of live calves following embryo transfer (Robl et al., 1987). The use of an electric current to induce the fusion of karyoplasts with cytoplasts was used in this study, as the Sendai virus (a rodent virus) proved ineffective as a fusogenic agent in cattle. However, similar to the mouse studies (McGrath and Solter, 1984), no in vitro development was observed when nuclei from later stage bovine embryos (two- to eight-cell stage) were transferred to enucleated pronuclear-stage bovine oocytes (Robl et al., 1987).

When enucleated two-cell stage mouse embryos, instead of enucleated pronuclear-stage oocytes, were used as the recipients for donor nuclei from eight-cell embryos, the rate of blastocyst formation increased (51% vs. 0%, respectively) (Robl et al., 1986). Also, 42% of the embryos that were transferred into recipients initiated pregnancies. However, no development beyond Day 12 was detected.

Shortly thereafter, fusing an enucleated two-cell stage embryo with the nucleus from a four-cell stage embryo resulted in the birth of live mice with normal fertility (Tsunoda et al., 1987; Kono and Tsunoda, 1989). In the same studies, nuclei from eight-cell embryos were also totipotent as evidenced by the birth of normal mice. However, the authors conceded that complete reprogramming of the transferred nucleus may not have occurred, because those
enucleated two-cell embryos that were reconstructed with eight-cell stage nuclei underwent compaction at the four- to eight-cell stage. In normal mouse embryos, compaction occurs at the eight- to 16-cell stage (Tsunoda and Kato, 2000).

Evidence regarding the inability of the enucleated two-cell embryo to fully reprogram the donor cell in the mouse was demonstrated by assessing the nuclear function of the transferred nucleus (Barnes et al., 1987). Several parameters were assessed following the transfer of eight-cell stage nuclei into enucleated two-cell stage embryos, including alterations in protein synthesis, changes in nuclear volume and timing of blastocyst formation to determine if reprogramming had occurred. Also, the types of proteins that were synthesized by the reconstructed embryo indicated that the nucleus was developing at least partly according to its own developmental program. Finally, the transplanted eight-cell nucleus did not enlarge upon transfer to the recipient two-cell cytoplasm. It should be remembered that nuclear volume diminishes with each cell division in cleavage-stage embryos. Thus, it is expected that the eight-cell nucleus should increase in volume following exposure to the enucleated two-cell cytoplasm if nuclear reprogramming occurs. The results indicated that complete nuclear reprogramming does not occur in the two-cell recipient. The authors suggested that the stage of the cell cycle of the recipient cytoplasm is more important than the donor cell’s degree of differentiation, and implied that metaphase oocytes would make better recipients for transferred nuclei compared with interphase zygotes and two-cell embryos (Barnes et al., 1987).

**Nuclear Reprogramming**

The first substantiated report of mammalian embryonic cloning occurred using enucleated MII sheep oocytes fused with eight- or 16-cell embryonic blastomeres (Willadsen,
1986). Since that report, it has been shown that transfer of an embryonic nucleus into an enucleated oocyte results in nuclear reprogramming as evidenced by the dispersion and growth of nucleoli in the mouse (Szollosi et al., 1988) and rabbit (Stice and Robl, 1988), and swelling of the donor nucleus as observed in the mouse (Czolowska et al., 1984), rabbit (Stice and Robl, 1988) and pig (Prather et al., 1990). Nuclear reprogramming is also indicated by precise regulation of DNA synthesis (Prather and First, 1990). Therefore, the combined effects of nuclear reprogramming induce the transferred nucleus to transform into a “developmentally equivalent pronucleus” (Collas and Robl, 1991).

Additional evidence of nuclear reprogramming is the development to term of healthy offspring. Numerous examples of successful nuclear transfer using embryonic donor cells have been reported for sheep (Willadsen, 1986; Smith and Wilmut, 1989), cows (Prather et al., 1987; Bondioli et al., 1990), rabbits (Stice and Robl, 1988; Collas and Robl, 1990), pigs (Prather et al., 1989a) mice (Kono et al., 1991; Kono et al., 1992), goats (Yong et al., 1991) and monkeys (Meng et al., 1997).

Moving toward more advanced cell types as nuclear donors, morula and blastocyst stage blastomeres have been shown to remain totipotent and support development to term when fused to enucleated MII oocytes in cows (Willadsen et al., 1991; Westhusin et al., 1992) and rabbits (Yang et al., 1992). Donor cells derived from the ICM of Day 7-9 blastocysts and used immediately are also totipotent, as shown by the birth of lambs (Smith and Wilmut, 1989) and calves (Collas and Barnes, 1994; Keefer et al., 1994) following nuclear transfer. Additionally, ICM cells that were derived from Day 7-9 blastocysts and cultured in vitro for an extended period (28 days as a primary culture) were proven capable of directing embryonic development to term in the cow (Sims and First, 1993).
The ability to produce live offspring from a cultured cell line was reported in 1996 (Campbell et al., 1996a). The cells used in this study were derived from the embryonic disk of an in vivo produced Day 9 sheep embryo and were cultured in vitro for 6 to 13 passages prior to nuclear transfer. There are two very important points to consider from this study. First, because the cells remained totipotent up to and including 13 passages, the potential exists to modify the cell line by gene targeting prior to nuclear transfer. This could provide a marked benefit in both research applications and biotechnology as a way to produce genetically modified offspring. Second, the discovery that donor cells had to be induced into a state of quiescence prior to transfer led to the next big breakthrough in mammalian cloning: producing a genetically identical clone from the cell of an adult animal.

**Quiescence**

Donor cells of sheep that are induced into quiescence by serum-deprivation exit the cell cycle and arrest at G₀ (Campbell et al., 1996a; Campbell, 1999). As mammalian cells progress through the cell cycle after cytokinesis, they require mitogens (growth factors) to progress through the G₁ phase (Zetterberg et al., 1995). Once past the checkpoint late in G₁, they can complete the rest of the cycle without further mitogenic stimuli (Reed, 1997; Connell-Crowley et al., 1998). Removing the mitotic signals by serum-deprivation will cause cells that are in G₁ to exit the cell cycle and arrest in a nondividing state of low metabolic activity, called G₀ (Krogstad, 1989; Tieqiao et al., 1999).

As a result, G₀ nuclei may be more easily modified by some “factor” in the oocyte cytoplasm, or become more amenable to respond to cytoplasmic signals, thus leading to complete nuclear reprogramming (Campbell, 1999). It has been suggested that the ability of a transferred nucleus to direct embryonic development to term depends on the “reprogramming
of gene expression” by these cytoplasmic factors (Szollosi et al., 1988), and G₀ nuclei may be more receptive to this action (Campbell et al., 1996a; Campbell, 1999).

First Mammal Cloned from an Adult-Derived Somatic Cell

After the success with cultured embryonic cells to create viable offspring from nuclear transfer in sheep (Campbell et al., 1996a), an adult-derived somatic cell was used to produce Dolly, the first animal cloned from an adult differentiated cell (Wilmut et al., 1997). Shortly after that report, reports of similar success with development to term in other species followed: cow (Vignon et al., 1998; Shiga et al., 1999; Wells et al., 1999a; b; Hill et al., 2000b), mouse (Wakayama et al., 1998; Wakayama and Yanagimachi, 1999; 2001), goat (Behboodi et al., 2001; 2002; Keefer et al., 2002), pig (Polejaeva et al., 2000; Bondioli et al., 2001) and cat (Shin et al., 2002).

In addition, many more successes using fetal cells were subsequently reported, most of them due to inducing quiescence in the donor cells prior to nuclear transfer: sheep (Schnieke et al., 1997; Wells et al., 1997; McCreath et al., 2000), cow (Cibelli et al., 1998a; Zakhartchenko et al., 1999b; 2001), mouse (Wakayama and Yanagimachi, 2001), goat (Baguisi et al., 1999; Keefer et al., 2001; Reggio et al., 2001; 2002; Chen et al., 2002) and pig (Betthauser et al., 2000; Li et al., 2000; Onishi et al., 2000).

All of the reports of successful mammalian nuclear transfer of donor nuclei either fused or injected into mature, MII oocytes required an additional activation step in the protocol for embryonic development to proceed, unlike the studies using embryos reconstructed with enucleated pronuclear stage or two-cell stage embryos.
**Activation**

Activation of mammalian oocytes arrested in metaphase of the second meiotic division (MII) involves exit from meiosis and re-entry into the mitotic cell cycle, resulting in pronuclear formation and subsequent cleavage and development (Wassarman and Albertini, 1994). In normal fertilization, the fusion of a sperm cell with the oocyte is sufficient for activation, causing a transient intracellular rise in calcium ions (Fissore et al., 1992; Sun et al., 1994; Lawrence et al., 1997), most often characterized as a series of spikes of short duration, or oscillations.

Calcium is released in a pulsatile manner from internal stores, including the endoplasmic reticulum and mitochondria (Yanagimachi, 1994), and can persist for several hours (Kline and Kline, 1992; Miyazaki et al., 1993; Carroll et al., 1996). These calcium oscillations are responsible for the cascade of events that follow, including the cortical reaction (Miyazaki, 1990), zona pellucida reaction (Yanagimachi, 1994) and escape from MII arrest (Whitaker and Patel, 1990). Sperm-induced activation has been observed in the mouse (Cuthbertson and Cobbold, 1985), hamster (Igusa and Miyazaki, 1986), pig (Sun et al., 1992), cow (Fissore et al., 1992) and rabbit (Fissore and Robl, 1992). Much of what we know regarding oocyte activation comes from the invertebrate world (Monroy, 1965; Jaffe, 1983; 1985; Metz and Monroy, 1985; Longo, 1987), and has been applied to mammalian species.

Protocols developed to artificially activate mammalian oocytes into parthenogenic development have attempted to mimic the biochemical and physiological events that normally occur during sperm-oocyte interaction. In the mouse, activation has been achieved with a variety of chemical and physical stimuli, including electrical stimulation (Tarkowski et al., 1970), hyaluronidase (Graham, 1970), Ca\(^{2+}\) ionophores (Steinhardt et al., 1974), exposure to
Ca²⁺- and Mg²⁺- free medium (Surani and Kaufman, 1977), inhibitors of protein synthesis (Siracusa et al., 1978; Clark and Masui, 1983) and ethanol (Cuthbertson, 1983). Sufficiently activated mouse oocytes will parthenogenetically develop to the blastocyst stage.

In cattle, these approaches are not effective unless the oocytes are allowed to age (>28 h post-maturation) prior to the activation stimulus (Ware et al., 1989). This implies that the sperm provides some “factor” needed for the activation of young oocytes (15–24 h post-maturation) that aged oocytes do not require (Whittaker and Irvine, 1984; Stice and Robl, 1990; Swann, 1990). In this scenario, aged bovine oocytes are easily activated by ethanol or electrostimulation (Nagai, 1987; Willadsen, 1989; Fukui and Sawai, 1992; Collas et al., 1993; Prochaszka et al., 1993) and can develop parthenogenetically to the blastocyst stage. Another possibility for this phenomenon is that the activation methods that cause an increase in intracellular calcium in young oocytes are not sufficient by themselves to overcome the cytostatic factor (CSF) and other factors that keep the oocyte arrested at MII (Masui, 1991).

Metaphase Promoting Factor

It has been shown that high levels of CSF and metaphase promoting factor (MPF) in young oocytes are responsible for maintaining meiotic arrest (Masui and Markert, 1971; Sagata et al., 1989; Masui, 1991). The inactivation of MPF is necessary for proper oocyte activation (Choi et al., 1991). In aged oocytes, levels of MPF are low, which allows activation to proceed (Campbell et al., 1996b).

MPF, first described by Masui and Markert (1971), is a complex of a catalytic subunit (p34^cdc2, a protein kinase) and a regulatory subunit (cyclin B). The activity of p34^cdc2 is regulated by changes in its state of phosphorylation and by its association with cyclin. The concentration of p34^cdc2 remains constant throughout the cell cycle, but cyclin concentrations
will vary (Campbell et al., 1996b). Activation of p34\textsuperscript{cdk2} by cyclin B will induce the onset of the M-phase and initiate nuclear envelope breakdown, chromosome condensation and spindle formation (Masui and Markert, 1971; Nurse, 1990; Maller, 1991; Masui, 1992).

In goat oocytes, the levels of p34\textsuperscript{cdk2} increase during maturation and are an indication of meiotic competence (Dedieu et al., 1998). MPF activity in oocytes is at its maximum during metaphase of meiosis I and meiosis II, and remains high at MII arrest. Upon sperm entry or artificial activation, MPF levels drop sharply due to the increase in intracellular Ca\textsuperscript{2+} (Kubiak et al., 1993), which causes a decline in cyclin B (Liu and Yang, 1999), allowing the oocyte to exit meiosis II and enter interphase of the subsequent cell cycle (Susko-Parrish et al., 1994). The sperm-induced repetitive spikes of intracellular Ca\textsuperscript{2+} maintain a low level of MPF to allow complete activation and exit from MII arrest. However, oocytes that are activated artificially must be treated to prevent MPF levels from rebounding, as there is no repetition of the calcium influx (Whitaker and Swann, 1993; Wu et al., 1998).

Recently matured bovine oocytes at MII do become activated by the methods described above to increase intracellular calcium, but then re-enter meiotic arrest if MPF levels are allowed to rebound (First et al., 1992; Susko-Parrish et al., 1994). Therefore, MII bovine oocytes require a combination of calcium influx to initiate the drop in MPF, followed by a further inhibition of MPF to become properly activated and develop parthenogenetically (Susko-Parrish et al., 1994). More specifically, 6-dimethylaminopurine (DMAP), a serine/threonine kinase inhibitor, prevents the phosphorylation of the serine and threonine kinases that are responsible for activating and deactivating MPF and CSF. By inhibiting phosphorylation of these kinases, MPF becomes inactive. This inactivation is necessary to allow the subsequent cortical reaction and zona pellucida reaction that are associated with
complete activation (Wassarman and Albertini, 1994), allowing the oocyte to escape from MII, enter the mitotic cycle and develop parthenogenetically. Additionally, DMAP interferes with the spindle apparatus and prevents expulsion of the second polar body, which allows the oocyte to enter interphase of the cell cycle and develop as a diploid parthenogenic embryo (Susko-Parrish et al., 1994).

In nuclear transfer (NT) procedures, enucleated oocytes fused with a diploid donor cell must be artificially activated to continue development, since somatic cell nuclei cannot initiate activation (Szollosi et al., 1986). The sequential treatment of NT couplets with ionomycin and DMAP is used in many NT programs to inactivate MPF (Cibelli et al., 1998a; Wells et al., 1999b; Rih et al., 2000; Daniels et al., 2001; Wrenzycki et al., 2001). However, cycloheximide (CHX), a nonspecific inhibitor of protein synthesis, has also been used in place of DMAP in other studies (Yang et al., 1994; Kato et al., 1998; Shiga et al., 1999; Zakhartchenko et al., 1999b; 2001). DMAP has the advantage of suppressing the second reduction division (preventing karyokinesis of the newly transferred diploid nucleus), thereby allowing diploid development to proceed (Susko-Parrish et al., 1994), whereas CHX does not interfere with the spindle apparatus and will result in haploid development if cytochalasin is not added to depolymerize the actin microfilaments of the spindle (Booth et al., 2001). Interestingly, in a direct comparison of parthenogenetic development of bovine oocytes activated with ionomycin followed by either DMAP or CHX, significantly more oocytes developed to blastocysts when DMAP was used compared with CHX (60% vs. 31%, P<0.001). However, when bovine oocytes were reconstructed with donor cells and then activated, again comparing DMAP with CHX, the use of CHX was superior to DMAP in blastocyst yield (Booth et al., 2001).
Finally, both DMAP and CHX are relatively nonspecific inhibitors of MPF activity (Susko-Parrish et al., 1994; Yang et al., 1994) that also affect several metabolic pathways in the oocyte (Alberio et al., 2000). Oocytes activated with the protein phosphorylation inhibitor DMAP may display an abnormal pattern of karyokinesis indicative of altered DNA content during the first cell cycle (De La Fuente and King, 1998), while the protein synthesis inhibitor CHX may prevent translation of cytoplasmic proteins responsible for controlling DNA replication (Soloy et al., 1997). Thus, several groups are now investigating more specific inhibitors of cyclin-dependent kinases, such as butyrolactone I (BLI), which is a highly selective inhibitor of the kinase subunit (cdc2) of MPF (Kitigawa et al., 1993; Motlik et al., 1998), and bohemine, a synthetic cyclin-dependent kinase (Alberio et al., 2000). In the cow, BLI was shown to be an effective compound when used in combination with ionomycin to activate cloned embryos (Hill et al., 1999b; 2000b), as evidenced by the birth of a healthy calf.

**Cell Cycles**

The cell cycle stage of the donor nucleus at the time of fusion with an enucleated, non-activated MII oocyte (cytoplast) has a pronounced effect on subsequent embryonic development (Smith et al., 1988; Collas et al., 1992a; Campbell et al., 1996b; Campbell, 1999). Morphological changes in donor nuclei following transfer into cytoplasts have been studied in several species including the mouse (Czolowska et al., 1984; Smith et al., 1988), pig (Prather et al., 1989b), rabbit (Collas and Robl, 1991) and cow (Kanka et al., 1991). Immediately upon fusion with a non-activated cytoplast, the nuclear envelope of the donor cell breaks down (NEBD) and the chromosomes undergo premature chromatin condensation (PCC), regardless of the position of the donor nuclei in the cell cycle (Czolowska et al., 1984;
NEBD, essential for exposing the donor chromatin to the “licensing factors” in the ooplasm and allowing DNA replication to commence (Blow and Laskey, 1988), is catalyzed by the high levels of maturation promoting factor (MPF) present in the ooplasm of non-activated cytoplasts, and causes PCC (Campbell et al., 1996b; Campbell, 1999).

Upon NEBD and PCC, diploid nuclei (G₀ and G₁) will condense to form single chromatids and maintain correct ploidy in the resulting embryo following the round of DNA replication that occurs after the donor cell chromatin decondenses and the nuclear envelope reassembles (Collas and Robl, 1991). Chromosome decondensation and nuclear envelope reformation is triggered by the fall in ooplasmic MPF following activation (Campbell et al., 1996b). However, tetraploid nuclei (those in S and G₂) will form double chromatids and lead to incorrect ploidy and defective embryos (Collas et al., 1992b). Additionally, the process of PCC will cause extensive DNA damage to S-phase nuclei (those that are actively replicating their DNA) (Schwartz et al., 1971) while not affecting the structure of G₀, G₁ or G₂ nuclei (Collas et al., 1992b; Cheong et al., 1993).

**Transgenic Clones**

In the relatively short time since sheep were cloned from a fully differentiated somatic cell fused with an enucleated MII oocyte (Wilmut et al., 1997), there has been an explosion of studies exploiting the techniques of somatic cell nuclear transfer. Donor cells from both fetal and adult cells have been reprogrammed in the MII cytoplasm and directed embryonic development to term in sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998a; Kato et al., 1998; Wells et al., 1999b), mice (Wakayama et al., 1998), goats (Baguisi et al., 1999; Keefer et al., 2001) and pigs (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000).
Taking advantage of somatic cell nuclear transfer techniques in dairy animals to generate valuable human recombinant proteins and monoclonal antibodies in the milk of transgenic offspring could potentially generate billions of dollars for the pharmaceutical industry (Echelard, 1996; Meade et al., 1998; Baguisi et al., 1999; Pollock et al., 1999). Prior to somatic cell NT, the most reliable method for generating transgenic animals was through pronuclear (PN) injection (Biery et al., 1988; Massey, 1990; Bowen et al., 1994; Hyttinen et al., 1994), a technique that relies on the microinjection of the gene of interest into the pronucleus of a pronuclear stage oocyte (Eyestone, 1998).

Mice were the first mammals to have foreign DNA incorporated into their genome via PN injection to generate transgenic offspring (Gordon et al., 1980). Transgenic sheep and pigs were produced by microinjection 5 years later (Hammer et al., 1985). However, only a low percentage (<5%) of the resulting offspring produced through PN injection are transgenic (Simons et al., 1988; Pursel et al., 1989; Eyestone, 1994). Those animals that have incorporated the transgene do so in a mosaic fashion, such that the gene is not present in every cell (Pursel et al., 1989; Rusconi, 1990). Even more problematic with PN injection is the unpredictable and random integration of the transgene into the genome, leading to variable and unpredictable expression levels among transgenic founders due to chromosome position effects (Pursel and Rexroad, 1993). In addition to PN injection, other methods of producing transgenic livestock have been reported, including sperm-mediated gene transfer (Gandolfi, 1998; Squires, 1999; Wall, 2002), intracytoplasmic injection of transgenic sperm heads (Perry et al., 1999), and the use of retrovirus vectors (Haskell and Bowen, 1995; Chan et al., 1998).
However, transfecting somatic cell lines with genes of interest, coupled with nuclear transfer, has become the method of choice for introducing foreign DNA into the genome of livestock species (Schnieke et al., 1997; Cibelli et al., 1998a; McCreath et al., 2000). Supplementing the foreign DNA with promoters that direct gene expression to the mammary gland (Ebert et al., 1994) enables a straightforward recovery of the protein of interest (Young et al., 1997). Moreover, combining cell culture and molecular biology methodologies with nuclear transfer techniques to produce transgenic offspring has eliminated the inherent problems associated with PN injection and increased the overall efficiency. Transfected cells in which the gene of interest has been incorporated can be screened prior to NT to detect both the presence and location of the transgene in the donor cell DNA. The advantage to this approach is that 100% of the resulting offspring will be transgenic (Niemann and Kues, 2000), and fewer animals are required (Schnieke et al., 1997).

The obvious benefits of utilizing transgenic animals to generate proteins and antibodies include 1) high product yield, 2) low capital investment compared with cell culture techniques, 3) the ability to perform reliable complex post-translational modifications (e.g., glycosylation and gamma-carboxylation) and 4) eliminating blood-derived pharmaceuticals, which may contain pathogens (e.g., human immunodeficiency virus and hepatitis viruses).

Dairy goats are excellent for the transgenic production of therapeutic recombinant proteins because of their high yield of purified product, relatively short generation interval and low incidence of the disease, scrapie (Meade et al., 1998). Up to 700 liters of milk per year can be obtained from a single transgenic goat, capable of expressing between 1 and 10 g of protein per liter (Echelard et al., 2000). Although milk yields are higher in dairy cows, dairy goats offer a shorter generation interval and thus are more attractive for use in
competitive transgenic programs (Ziomek, 1998). Moreover, transgenic female goats can be hormonally induced to lactate at 2 months of age (Cammuso et al., 2000) to provide an initial sample for product testing (e.g., expression levels and biological activity). Larger quantities of milk can then be produced following a normal gestation initiated through either natural mating or artificial insemination of the transgenic female. Purification of the protein from this milk can yield sufficient quantities of the protein for further testing, or for use in human clinical trials (Echelard et al., 2000).

Cloned transgenic sheep (Wright et al., 1991; Schnieke et al., 1997; McCreath et al., 2000) and goats (Baguisi et al., 1999; Reggio et al., 2001) are capable of expressing in their milk many of these proteins that cannot be chemically synthesized nor manufactured by bacterial or cell culture systems, due to the extensive post-translational modifications required to maintain the biological activity of these compounds (Wright et al., 1991). Transgenic animals designed with lactation-specific transgenes have the potential to provide therapeutic proteins in large scale at an affordable cost, and may save millions of lives when used for the production of antibodies, proteins or vaccines (Stowers et al., 2002).
CHAPTER III

BOVINE NUCLEAR TRANSFER USING PROLIFERATING OR QUIESCENT ADULT DONOR CELLS: FIBROBLAST CELLS VS. CUMULUS CELLS

Introduction

The desire to produce genetically identical animals for experimentation and for propagating the genetics of superior animals led to the development of embryo micromanipulation techniques that could generate multiple offspring from one embryo (Robl et al., 1987). Before the advent of nuclear transfer (NT), embryos were either dissociated before the 8-cell stage and allowed to develop individually (Moore et al., 1968; Willadsen, 1981; Willadsen and Fehilly, 1980), or surgically bisected at later stages to generate multiple identical animals (Willadsen, 1979; 1980; Lambeth et al., 1983; Voelkel et al., 1985; Udy, 1987; Godke and Rorie, 1988). However, there is a limit to the number of identical embryos and offspring that can be produced from one bisected or disaggregated embryo. In commercial cattle embryo programs, embryo splitting at the morula and blastocyst stages was routinely used to produce identical twins at best (reviewed in Robl, 1999).

The first report of successful cloning by NT in mammals involved fusing embryonic blastomeres from eight-cell sheep embryos to enucleated oocytes to generate live lambs (Willadsen, 1986; Smith and Wilmut, 1989). Prior to this report, early experiments in mice led to the belief that cloning mammals by blastomere nuclear transfer was biologically impossible (McGrath and Solter, 1984). Moreover, studies in amphibians showed that differentiated nuclei from adult cells failed to generate fully developed adult frogs when transferred to enucleated oocytes (Briggs and King, 1952; Gurdon, 1962; Gurdon and
Uehlinger, 1966). Thus, subsequent cloning studies in mammals focused on transferring undifferentiated blastomeres of the early-stage embryo. Embryonic cloning (blastomere NT) was then applied to produce offspring in cattle (Prather et al., 1987; Willadsen, 1989; Bondioli et al., 1990; Barnes et al., 1993; Stice et al., 1994), mice (Tsunoda et al., 1987), pigs (Prather et al., 1989a), rabbits (Stice and Robl, 1988), goats (Yong and Yuqiang, 1998) and monkeys (Meng et al., 1997).

Combining embryo disaggregation with blastomere NT can increase the number of identical offspring produced from one embryo. In one report, eight identical calves were produced from a single embryo by transferring individual blastomeres from an eight- to 16-cell embryo into enucleated MII oocytes (Bondioli et al., 1990). Attempts to further increase the number of cloned calves by performing serial cloning from previously cloned embryos (i.e., the cloning of clones) met with limited success (Stice and Robl, 1988). In this scenario, blastomeres from the parent embryo are transferred singly into enucleated oocytes to generate multiple cloned embryos. These embryos are then used as blastomere donors in subsequent rounds of NT and the process can be repeated indefinitely, although efficiency declines dramatically with subsequent generations (Stice and Keefer, 1993). For large-scale cloning in the cow, serial NT is not considered a practical option. Nevertheless, serial cloning has produced over 30 isogenic progeny in mice (Park et al., 1993), 10 identical calves (Stice and Keefer, 1993) and 45 identical goats (Yong and Yuqiang, 1998).

Mammalian cloning was originally driven by questions of nuclear vs. cytoplasmic inheritance (McGrath and Solter, 1983a; Mann, 1986; Robl et al., 1988), events of imprinting during gametogenesis (Surani et al., 1986; Barra and Renard, 1988) as well as questions pertaining to nuclear differentiation in early development (Willadsen, 1986; Prather et al.,
1987; Stice and Robl, 1988). The availability of cloned offspring could also address the “nature vs. nurture” issue by separating genetic factors from environmental biases that may influence growth performance, milk production or even phenotype (Stice and Robl, 1988). In addition, varying the experimental conditions imposed upon genetically identical clones could reduce the number of experimental animals required to run statistically valid studies, especially in species (horse and primates) where animal or oocyte availability is limited (Wolf et al., 1999). This could lead to more efficient animal models for biomedical research.

Another limitation of embryonic cloning (in addition to the limited number of donor blastomeres) to generate large numbers of economically valuable animals is the unknown genetic potential of the parent embryo. These obstacles were overcome, however, with the recent breakthrough in somatic cell nuclear transfer (SCNT) using differentiated cells (Wilmut et al., 1997), and proved that millions of donor cells from the same adult could be harvested and used for NT into enucleated oocytes to produce an unlimited number of cloned offspring. Following the birth of “Dolly” the sheep, other species quickly joined the list of animals cloned from somatic cells: cattle (Cibelli et al., 1998a; Kato et al., 1998), mice (Wakayama et al., 1998; Wakayama and Yanagimachi, 1999), goats (Baguisi et al., 1999), pigs (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000) and gaur (Lanza et al., 2000c).

To date, the optimum cell type for success in NT has yet to be determined. Fibroblast cells harvested from adult animals have been used as nuclei donors in the sheep (Wilmut et al., 1997), cow (Kato et al., 1998; Shiga et al., 1999; Wells et al., 1999b), mouse (Wakayama et al., 1998; Wakayama and Yanagimachi, 1999) and pig (Polejaeva et al., 2000). Cumulus
cells collected from aspirated oocytes have also been used to generate cloned offspring in the cow (Kato et al., 1998) and mouse (Wakayama et al., 1998).

Using the approach of Campbell and Wilmut (1997), many groups have used quiescent donor cells (G0) to generate cloned offspring (Schlieke et al., 1997; Wells et al., 1997; 1998b; 1999b; Kato et al., 1998; Baguisi et al., 1999; Zakhartchenko et al., 1999a; Reggio et al., 2001). However, the requirement for donor cell quiescence for successful NT may not be absolute, as shown by the birth of calves from proliferating G1 donor cells (Cibelli et al., 1998a; b). Recent studies in the cow using fetal fibroblast cells showed that quiescent donor cells were better than proliferating cells in directing development to the blastocyst stage (Hill et al., 1999b; Zakhartchenko et al., 1999b), but no data was collected on fetal development. Moreover, none of these studies directly compared the effects of donor cell type from adult animals (fibroblast vs. cumulus) or cell cycle stage (proliferating vs. quiescent) on the developmental capacity of NT embryos to produce cloned offspring.

The objective of this study was to compare the developmental capacity of bovine embryos reconstructed with cumulus cells or adult fibroblast cells that were either induced into quiescence or allowed to proliferate prior to NT.

**Materials and Methods**

**Media Preparation**

Maturation medium was prepared and equilibrated at 39°C and 5% CO2 on site at the oocyte collection facility (BOMED, Inc., Madison, WI). All other media were prepared at the LSU Embryo Biotechnology Laboratory and equilibrated at 39°C and 5% CO2 for 3 h prior to use.
Oocytes were matured in tissue culture medium-199 with Earle’s salts buffered with 25 mM Hepes (TCM; Gibco Laboratories, Grand Island, NY) and supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), 6 µg/ml bovine luteinizing hormone (LH; NOBL Laboratories, Sioux Center, IA), 3 µg/ml bovine follicle stimulating hormone (FSH; NOBL Laboratories), 1 µg/ml estradiol-17β (E₂; Sigma Chemical Co., St. Louis, MO), and 25 µg/ml gentamicin (Sigma Chemical Co.).

Embryos were cultured on buffalo rat liver (BRL) cell monolayers in a semi-defined bovine embryo culture medium (BECM) consisting of 89 mM NaCl, 3.2 mM KCl, 2.0 mM CaCl₂, 0.5 mM MgCl₂, 0.35 mM NaH₂PO₄, 25 mM NaHCO₃, 0.5 mM sodium pyruvate, 10 mM sodium lactate, 1% (v/v) basal medium Eagle amino acid solution (100X solution), 1% (v/v) MEM nonessential amino acid solution (100X solution), 3 mg/ml fatty-acid-free bovine serum albumin (BSA) and 25 µg/ml gentamicin (Lim et al., 1996). All reagents were purchased from Sigma, except the gentamicin (Gibco).

Fibroblast cells and cumulus cells were initially cultured in modified TCM (mTCM; TCM supplemented with 10% FBS and 50 µg/ml gentamicin), then switched to a reduced-serum formulation (0.5% FBS) to induce quiescence. All other media were modifications of TL-Hepes (TLH; BioWhittaker, Walkersville, MD) or TCM and are described in detail later.

**Oocyte Collection and Maturation**

Bovine oocytes aspirated from abattoir ovaries were obtained from a commercial source (BOMED) and shipped via overnight service to the laboratory in 2 ml of maturation medium maintained at 39°C in a battery-powered incubator. Oocytes arrived within 19 h of exposure to maturation medium and were then transferred into 35-µl droplets of maturation medium under warmed mineral oil (M-8410, Sigma) to await enucleation.
**Donor Cell Collection**

Nuclear donor cells were obtained from two different sources: 1) skin biopsies from an adult cow and 2) in vitro matured bovine oocytes aspirated from abattoir ovaries. After shaving, scrubbing and disinfecting the biopsy site, a 6-mm diameter biopsy punch tool (Miltex Instrument Co, Bethphage, NY) was used to collect two skin samples from the dorsal aspect of the shoulder directly over the supraspinatus muscle of a 5-year-old crossbred beef cow maintained at the LSU Embryo Biotechnology Laboratory. The tissue was placed in sterile phosphate-buffered saline (PBS) at room temperature supplemented with 50 µg/ml gentamicin, then processed immediately. The epidermal layer was removed with a sterile scalpel and discarded, and the dermal tissue was finely minced with sterile iris scissors.

The processed tissue was washed three times in mTCM, then transferred to 35 x 10 mm Falcon™ plastic petri dishes (Becton and Dickinson, Lincoln Park, NJ) and cultured at 39°C and 5% CO₂. Fibroblast cells began to outgrow from the tissue by the fifth day in culture, and were maintained in petri dishes until 50% confluent, then trypsinized (T-3924, Sigma) and transferred to 25 cm² tissue culture flasks (Costar, Cambridge, MA). Cells were subpassaged (at 80% confluency) four times prior to cryopreservation in TCM supplemented with 20% FBS and 5% dimethylsulfoxide and then frozen in aliquots of 600,000 cells per vial.

Cumulus cells were recovered from a pool of in vitro matured oocytes harvested from abattoir ovaries, washed and then cultured in 25 cm² tissue culture flasks filled with 2.5 ml mTCM. Cells were maintained in culture at 39°C and 5% CO₂ until 80% confluent, then enzymatically dispersed and subpassaged four times before freezing in aliquots of 600,000 cells per vial.
Experimental Design

Adult fibroblast cells or cumulus cells were used as nuclear donors in a series of 11 NT replicates. The effect of donor cell type on the rate of fusion and subsequent development of reconstructed embryos was studied. In addition, donor cells were either serum-starved or used in the proliferative state to investigate the effect of cell cycle stage on subsequent embryo development (Table 3.1). Following reconstruction, fusion and activation, NT embryos were transferred to a BRL monolayer and cultured in BECM to continue development up to the blastocyst stage. Embryos were evaluated at 36 h, 60 h, 144 h and 192 h post-activation to assess development at the two- to four-cell stage, eight- to sixteen-cell stage, morula stage and blastocyst stage, respectively. The majority of blastocysts were nonsurgically transferred to crossbred beef cows on Day 8 of the estrous cycle (Day 0 = standing estrus). In addition, random blastocysts from each treatment were stained with Hoechst 33342 and observed under ultraviolet (UV) light to assess total cell number per blastocyst.

Statistical Analysis

The number of embryos reconstructed with fibroblast or cumulus donor cells in the proliferative or quiescent state that fused and developed to each stage was subjected to an analysis of variance (ANOVA) using the general linear model of SAS (SAS, 1992). When significance of the main effects of treatment on fusion rate or embryo development was detected, differences were compared by the least squares method. The differences in means at P<0.05 were regarded as statistically significant in this experiment.
Table 3.1. Experimental design

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Treatment identification&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast</td>
<td>Proliferating</td>
<td>FP</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>Quiescent</td>
<td>FQ</td>
</tr>
<tr>
<td>Cumulus</td>
<td>Proliferating</td>
<td>CP</td>
</tr>
<tr>
<td>Cumulus</td>
<td>Quiescent</td>
<td>CQ</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cells were used either in the proliferative state or induced into quiescent by treatment with a reduced-serum culture interval.

<sup>b</sup>FP = proliferating fibroblast cells; FQ = quiescent fibroblast cells; CP = proliferating cumulus cells; CQ = quiescent cumulus cells.
Microtool Preparation

Holding pipettes and enucleation pipettes were hand-forged from borosilicate glass tubing (1.0 mm outside diameter) using a Sutter Micropipette Puller (Sutter Instrument Company, Novato, CA) in conjunction with a Narishige Microforge and Narishige Microgrinder (Narishige Scientific Instrument, Tokyo, Japan). Holding pipettes were pulled to an outside diameter of 75 µm, then fire polished to create a 35-µm inner diameter. Enucleation pipettes were pulled to an outside diameter of 20 to 25 µm, then beveled to 35° before creating a sharp spike (<2 µm long) at the tip. In addition, both the holding and enucleation pipettes were bent near the distal end to an angle of 35°. Each microtool was attached to mineral-oil-filled polyethylene tubing connected to an oil filled syringe. Prior to micromanipulations, the microtools were allowed to equilibrate in a droplet of TLH overlaid with mineral oil.

All manipulations were performed on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan) equipped with Narishige micromanipulators, epifluorescent illumination and Hoffman Modulation Contrast objectives. Enucleation and reconstruction were conducted in a 200-µl elongated droplet of warmed micromanipulation medium (TLH supplemented with 7.5 µg/ml cytochalasin B).

Oocyte Enucleation

At 22 to 24 h of maturation, cumulus-enclosed oocytes were mechanically agitated for 2.25 min in TLH supplemented with hyaluronidase (Sigma; 1 mg/ml) to remove cumulus cells. Denuded oocytes were washed in modified TLH (mTLH; TLH supplemented with 10% FBS) and then selected for the presence of the first polar body. Prior to enucleation, mature oocytes were labeled with 1 to 5 µg/ml of the nuclear stain bis-benzimide (Sigma; Hoechst
33342) for 1 to 5 min to visualize the metaphase plate. Groups of 25 oocytes were then transferred to a micromanipulation droplet overlaid with warmed mineral oil.

Using the holding pipette to stabilize the oocyte, the metaphase plate was rotated into focus with the enucleation pipette while under a brief exposure (<10 sec) to UV-light. A slight negative pressure was applied to the holding pipette, and the enucleation pipette was pushed through the zona pellucida until it was adjacent to the metaphase chromosomes. While exposed to UV-light (<5 sec), the chromosomes and the first polar body were withdrawn from the oocyte by gentle suction applied to the enucleation pipette. Enucleated oocytes were washed and transferred into 35-µl droplets of mTCM and held for at least 30 min (39°C and 5% CO₂) to await reconstruction.

**Donor Cell Preparation**

Donor cells were thawed, washed and plated in 35 mm petri dishes 7 days prior to oocyte reconstruction and cultured in mTCM for the first 3 days. Cells were either allowed to proliferate or induced into quiescence by reducing the serum concentration to 0.5% for 4 days. Proliferating cells were maintained in 10% serum for the entire 7 days. Donor cells were trypsinized 1 to 2 h before transfer to enucleated oocytes. Briefly, cells were rinsed with two 5 ml portions of unsupplemented TCM-199 to remove traces of serum. The monolayers were trypsinized with 5 ml of warmed trypsin-EDTA solution (T-3924, Sigma) and incubated at 39°C for 3 to 4 min until cell rounding and loosening were observed. The cell-trypsin suspensions were transferred to 15 ml centrifuge tubes (Corning, New York, NY) and washed twice in mTCM to inactivate the trypsin. The cell pellets were resuspended and held in mTCM at 39°C and 5% CO₂.
Reconstruction and Fusion

Working in groups of 25, enucleated oocytes (cytoplasts) were transferred to an enucleation droplet overlaid with embryo-tested mineral oil. Cytoplasts were stabilized by the holding pipette and reconstructed by injecting a single donor cell (karyoplast) through the zona pellucida into the perivitelline space, using the tip of the injection pipette to push the cell tightly against the oolema.

Following reconstruction, cytoplast-karyoplast couplets were equilibrated (in groups of 10) in fusion buffer (0.3 M mannitol, 0.1 mM MgSO$_4$·7H$_2$O, 0.05 mM CaCl$_2$, 0.5 mM Hepes and 4 mg/ml BSA) for 5 min and then transferred to a buffer-filled fusion chamber with a 1-mm gap. The couplets were manually aligned between the two stainless steel electrodes so that the plane of contact between each cytoplast and karyoplast would be perpendicular to the direction of electric current (Figure 3.1). A single DC electrical impulse (1.5 kV/cm for 35 µsec) delivered by a BTX Electrocell Manipulator 200 (Genetronics, San Diego, CA) was applied across the electrodes to induce couplet fusion. Couplets were washed and held in microdroplets of mTCM for 1 h (39°C and 5% CO$_2$) before assessing fusion. Those couplets that did not fuse were exposed to a second treatment of electrical stimulation using the same parameters.

Couplet Activation and Culture

The method of Susko-Parrish was used to activate the fused couplets (Susko-Parrish et al., 1994). Following a 5 min incubation in 5 µM ionomycin, couplets were washed extensively in TLH supplemented with 30 mg/ml BSA to inactivate the ionomycin and then incubated in 2 mM 6-dimethylaminopurine (6-DMAP) for 4 h. Activated oocytes were then washed in BECM and co-cultured on a monolayer of BRL cells maintained in 35 µl droplets
Figure 3.1. Karyoplast-cytoplast couplets aligned in the fusion chamber. Note the position of the plane of contact between the cytoplast and karyoplast relative to the direction of the electrical flow.
of BECM overlaid with mineral oil. Embryo development was assessed from the cleavage stage up to the blastocyst stage. One blastocyst from each treatment was stained with 10 µg/ml bis-benzimide for 15 min and then mounted under glass and exposed to UV-light to determine total cell number.

**Embryo Transfer**

Crossbred beef cows exhibiting a natural estrus were selected from a herd of animals maintained at the LSU Embryo Biotechnology Laboratory and used as recipients on Days 7 and 8 of the estrous cycle (Day 0 = standing estrus). NT blastocysts were nonsurgically transferred into the uterine horn ipsilateral to the ovary with a corpus luteum. Recipients were returned to the herd to await subsequent ultrasonographic evaluations for pregnancy determination beginning on Day 30 post transfer.

**Results**

**Fusion**

A total of 797 cytoplast-karyoplast couplets were produced from the reconstruction of enucleated oocytes with proliferating fibroblast cells (FP), quiescent fibroblast cells (FQ), proliferating cumulus cells (CP) and quiescent cumulus cells (CQ) during the 11 replicates in this study. The overall fusion rate was 48% (380/797), and ranged from 15% to 90%.

No difference was detected in the fusion rate among couplets reconstructed with FP (36%; range, 15%–70%), FQ (37%; range, 20%–60%) or CP (41%; range, 25%–58%) donor cells (Table 3.2). However, significantly more couplets reconstructed with CQ cells fused (77%; range, 65%–90%) compared with couplets reconstructed with FP, FQ or CP cells (P<0.001).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. NT couplets</th>
<th>No. couplets fused (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP (fibroblast, proliferating)</td>
<td>199</td>
<td>72 (36)\textsuperscript{a}</td>
</tr>
<tr>
<td>FQ (fibroblast, quiescent)</td>
<td>202</td>
<td>75 (37)\textsuperscript{a}</td>
</tr>
<tr>
<td>CP (cumulus, proliferating)</td>
<td>200</td>
<td>82 (41)\textsuperscript{a}</td>
</tr>
<tr>
<td>CQ (cumulus, quiescent)</td>
<td>196</td>
<td>151 (77)\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{ab} Different superscripts within a column are significantly different (P<0.05).
When analyzed only by cell type, significantly more (P<0.0001) couplets reconstructed from cumulus cells fused compared with those couplets reconstructed from fibroblast cells (59% vs. 37%, respectively), regardless of serum treatment. When analyzed based on the serum treatment alone, significantly more (P<0.0001) couplets derived from quiescent donor cells fused (57%) compared with those couplets reconstructed with proliferating donor cells (38%).

**Embryo Development**

From a total of 380 fused couplets, 253 (66%) developed beyond the two-cell stage. Cleavage rates ranged from 33% to 100% over the 11 replicates. There was a significant effect of donor cell treatment on the cleavage rate (P<0.0001) of reconstructed embryos, as well as on the development of NT embryos to the 8- to 16-cell stage (P=0.003). No difference was detected among treatments for morula or blastocyst formation (P=0.21 and 0.06, respectively).

When reconstructed with FQ donor cells, significantly more fused couplets cleaved (79%; range, 67%–100%) compared with FP- (60%; range, 33%–75%) or CP- (58%; range, 50%–67%) derived embryos (Table 3.3). However, there was no difference in cleavage rates between FQ-derived embryos (79%) and CQ-derived embryos (68%; range, 50%–77%).

When analyzed with respect to cell type only, there was no difference (P=0.42) in cleavage rates between embryos reconstructed from fibroblast cells or cumulus cells (69% and 65%, respectively). However, significantly (P<0.01) more embryos produced from quiescent cells cleaved (72%) compared with proliferating donor cells (59%) in this study.
Table 3.3. Effect of donor cell treatment on NT embryo development after fusion

<table>
<thead>
<tr>
<th>Donor cell treatment</th>
<th>2- to 4-cell [36]†</th>
<th>8- to 16-cell [60]†</th>
<th>Morula [144]†</th>
<th>Blastocyst [192]†</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP</td>
<td>43 (60)(^{a})</td>
<td>11 (15)(^{a})</td>
<td>1 (1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>FQ</td>
<td>59 (79)(^{b})</td>
<td>29 (39)(^{b})</td>
<td>4 (5)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>CP</td>
<td>48 (58)(^{a})</td>
<td>12 (15)(^{a})</td>
<td>3 (4)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>CQ</td>
<td>103 (68)(^{ab})</td>
<td>30 (20)(^{a})</td>
<td>8 (5)</td>
<td>6 (4)</td>
</tr>
</tbody>
</table>

†Time (h) post-fusion.
\(^{ab}\)Different superscripts within a column are significantly different (P<0.05).
Embryonic development to the 8- to 16-cell stage was significantly influenced by the
 donor cell treatment (P=0.003). Significantly more embryos reconstructed with the FQ cells
developed to this stage (39%; range, 12%–50%) compared with FP-, CP- and CQ-derived
embryos (15%–20%; ranges, 0%–33%). With respect to donor cell type, significantly more
(P=0.04) embryos developed to the 8- to 16-cell stage when fibroblast cells were used
compared with cumulus cells (27% vs. 18%, respectively). In addition, when analyzed with
respect to serum treatment only, significantly more (P<0.01) embryos developed to the 8- to
16-cell stage when quiescent donor cells were used compared with proliferating donor cells
(26% vs. 15%, respectively).

**Blastocyst Cell Number and Embryo Transfer**

Blastocysts derived from oocytes reconstructed with FQ, CP and CQ donor cells were
either stained or transferred to recipients (Table 3.4). Although very few embryos were
analyzed (n=3), the total cell number for NT blastocysts was similar among treatments and
varied from 103 nuclei to 122 nuclei. As these embryos were flattened under glass to more
accurately determine cell number, they could not be used for embryo transfer.

The remaining blastocysts were transferred to three recipient cows (Table 3.4). One
recipient received four blastocysts from the CQ treatment; the remaining two recipients
received one embryo each from the CP and CQ treatment group. All six blastocysts failed to
initiate pregnancies.

**Discussion**

In this comparative study, both fibroblast and cumulus cells were reprogrammed
following nuclear transfer and directed embryonic development up to the blastocyst
Table 3.4. Effect of donor cell treatment on total cell number of NT blastocysts

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. blastocysts</th>
<th>No. stained</th>
<th>Cell number</th>
<th>No. transferred&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. recipients</th>
<th>No. cows pregnant&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FQ</td>
<td>1</td>
<td>1</td>
<td>122</td>
<td>0</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>CP</td>
<td>2</td>
<td>1</td>
<td>103</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CQ</td>
<td>6</td>
<td>1</td>
<td>119</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup>No blastocysts developed from the FP treatment.

<sup>b</sup>Stained blastocysts were not transferred.

<sup>c</sup>Recipients were evaluated on Days 30, 40 and 47 post-transfer.
stage, although with disappointingly low frequency. The cytoplasm of mature enucleated oocytes is capable of reprogramming a variety of cell types, including embryonic inner cell mass cells (Keefer et al., 1994), embryonic blastomeres (Yong and Yuqiang, 1998), embryonic stem cell-like cells (Campbell et al., 1996a; Wells et al., 1997), mammary gland cells (Wilmut et al., 1997), fibroblasts obtained from fetal and adult tissues (Schnieke et al., 1997; Cibelli et al., 1998a; Baguisi et al., 1999; White et al., 1999; Hill et al., 2000b), muscle cells (Vignon et al., 1998; Shiga et al., 1999), cumulus granulosa cells (Kato et al., 1998; Wakayama et al., 1998; Keefer et al., 2002), mural granulosa cells (Wells et al., 1999b; Polejaeva et al., 2000), Sertoli cells (Ogura et al., 2000) and leukocytes (Galli et al., 1999).

The application of adult cell nuclear transfer lies in the ability to replicate a particular male (Campbell et al., 1996a; Wells et al., 1999b) or female genotype (Shiga et al., 1999; Wakayama and Yanagimachi, 1999). Finding the best donor cell type to accomplish this is key to increasing the efficiency of NT, but as of yet there have been no direct comparisons made between different adult cell types in a single experiment. In this study, improved fusion rates were observed when cumulus cells were used in the quiescent state, but serum deprivation had no beneficial effect on fusion rates for fibroblast cells. Embryonic development up to the 16 cell stage was enhanced by inducing fibroblast cells into quiescence prior to NT, but development to this stage was not improved by serum starvation of cumulus cells.

Conflicting reports on the positive effects of serum starving fetal fibroblasts on subsequent embryo development further complicates the issue. Improved development of NT embryos to the blastocyst stage is observed when fetal fibroblast cells are serum starved prior to NT in some reports (Zakhartchenko et al., 1999b; Hill et al., 2000b). Conversely, serum
deprivation has no effect on cleavage or blastocyst formation in others (Kuhholzer et al., 2001).

Although the high cell numbers observed in the stained blastocysts in this study were indicative of viable embryos, poor embryonic development may have been due to improperly synchronized cell cycles between the donor cell and recipient ooplasm (Campbell, 1999), and may account for the lack of pregnancies.

Although no pregnancies were established in this study, several important modifications to the NT technique were developed which may prove beneficial in future experiments. The amount of time that MII oocytes were exposed to Hoechst dye was significantly reduced from the 20 min commonly reported in the literature (Campbell et al., 1996a; Wells et al., 1997; 1999b; Vignon et al., 1998; Baguisi et al., 1999; Polejaeva et al., 2000) to 5 min in this study. In addition, exposure of the labeled oocytes to UV-light was kept to a minimum during enucleation. It has been reported that the combination of Hoechst staining with a 20-sec exposure to UV-light is detrimental to early stage embryos (Sharma et al., 1999). After locating the metaphase plate with a brief exposure to UV-light, the chromosomes were aspirated while exposed to a second brief UV pulse. This method insured complete removal of nuclear DNA while minimizing the damaging effects of UV-light on oocyte organelles. Finally, after injecting the donor cell into the perivitelline space (PVS), it was pushed tightly against the oolema and then re-aspirated into the enucleation pipette with a small amount of ooplasm; the ooplasm and donor cell were then expelled back into the PVS. This technique insured very close contact of the donor cell membrane with the oolema of the oocyte, and may improve fusion rates.
CHAPTER IV

CLONED TRANSGENIC OFFSPRING RESULTING FROM SOMATIC CELL NUCLEAR TRANSFER IN THE GOAT*

Introduction

A relatively short time has elapsed since cloned sheep were produced by transferring nuclei from an established cell line isolated from the embryonic discs of 9-day-old embryos into enucleated metaphase II oocytes (Campbell et al., 1996a). Since that time, the use of donor nuclei from fetal and adult cells has resulted in the births of sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998a; Kato et al., 1998; Wells et al., 1999b), mice (Wakayama et al., 1998), goats (Baguisi et al., 1999; Keefer et al., 2001) and pigs (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000). The use of these nuclear transfer (NT) techniques in farm animals to efficiently generate cloned transgenic offspring capable of producing valuable proteins could have a marked impact on the pharmaceutical industry (Echelard, 1996).

The mammary gland is well suited for the production and expression of human recombinant proteins (Maga and Murray, 1995) (e.g., alpha-1-antitrypsin (Wright et al., 1991), fibrinogen (Prunkard et al., 1996), human clotting factor IX (Schnieke et al., 1997), and antithrombin III (Baguisi et al., 1999) and antibodies (Pollock et al., 1999). Obvious benefits of using transgenic animals to provide such human pharmaceuticals include 1) high product yield, 2) low capital investment compared with cell culture techniques, 3) the ability to perform complex post-translational modifications (e.g., glycosylation and gamma-carboxylation) and 4) elimination of reliance on products derived from human blood, which may contain pathogens (e.g., human immunodeficiency virus and hepatitis viruses). Dairy

goats are ideal for the transgenic production of therapeutic recombinant proteins because of their high yield of purified product and relatively short generation interval (Meade et al., 1998).

One of the limitations of somatic cell nuclear transfer in sheep and cattle is the very low success rate, with a high proportion of fetal loss (Campbell et al., 1996a; Schnieke et al., 1997; Wells et al., 1997; Wilmut et al., 1997; White et al., 1999; Zakhartchenko et al., 1999b), and an increase in perinatal morbidity/mortality (Cibelli et al., 1998a; Kato et al., 1998; Vignon et al., 1998; Hill et al., 1999a; Shiga et al., 1999; McCreath et al., 2000). Reported causes of fetal wastage include abnormal liver development (Wilmut et al., 1997), insufficient placentation leading to spontaneous abortions or prolonged gestations (Cibelli et al., 1998a; Hill et al., 1999a; Wells et al., 1999b), and oversized fetuses (Vignon et al., 1998). Reported causes of perinatal death include metabolic and cardiopulmonary abnormalities (Cibelli et al., 1998a; Hill et al., 1999a; 2000b; Wells et al., 1999b; Zakhartchenko et al., 1999b) and lymphoid hypoplasia (Renard et al., 1999).

Calves and lambs resulting from embryos cultured in vitro may be heavier at birth (Willadsen et al., 1991; Keefer et al., 1994; Behboodi et al., 1995; Sinclair et al., 1995; Walker et al., 1996; Bertolini and Anderson, 2002), present with an increased incidence of neonatal mortality (Schmidt et al., 1996; Kruij and den Daas, 1997), and are associated with longer gestation lengths (Walker et al., 1996) than offspring resulting from in vivo-produced embryos. Moreover, placental development is also affected, resulting in fewer placentomes (Farin and Farin, 1995) and increased incidences of hydrallantois (Hasler et al., 1995). In addition, blastomere NT in the sheep and the cow results in increased fetal wastage and perinatal mortality (Behboodi et al., 1995; Wilson et al., 1995; Garry et al., 1996; Schmidt et
al., 1996; Kruip and den Daas, 1997), which may be attributable to the culture conditions (serum in the medium), co-culture systems, or the manipulation procedure itself (Garry et al., 1996; Kruip and den Daas, 1997).

Conversely, the incidence of perinatal loss associated with somatic cell NT has not been reported in the goat (Baguisi et al., 1999; Keefer et al., 2000), although this lack of information may be due to 1) the relatively low number of goat clones produced to date or 2) a minimal in vitro culture period in which reconstructed embryos were transferred at the two- to four-cell stage in both previous studies. However, Yong and Yuqiang produced 45 cloned goats from the transfer of 141 serially reconstructed embryos into 29 recipients in a cloning study using blastomere donor nuclei, in which embryos were cultured up to the morula stage prior to transfer (Yong and Yuqiang, 1998). Thus, manipulated goat embryos may not be as sensitive as cow and sheep embryos to micromanipulation procedures and in vitro culture conditions.

Although cloned transgenic goats have been produced using oocytes collected from stimulated donor animals (Baguisi et al., 1999; Keefer et al., 2001), abattoir-derived ovaries could also be a source of oocytes for the production of transgenic goats and may improve the cost effectiveness of generating pharmaceutically important proteins. Large numbers of goat oocytes can be harvested from abattoir-derived ovaries and matured in vitro (Han, 1995) to provide an inexpensive supply of cytoplasts suitable for NT procedures, apparently without the risk of generating oversized or moribund offspring, as reported in the sheep (Thompson et al., 1995; Maxfield et al., 1997) and cow (Behboodi et al., 1995; Sinclair et al., 1995; Schmidt et al., 1996; Kruip and den Daas, 1997). The expense and difficulty of obtaining caprine oocytes from a donor herd can thus be eliminated.
Although the use of abattoir-derived oocytes for the production of human pharmaceuticals is restricted by federal Food and Drug Administration regulations, the economic benefit of their use in a research setting becomes obvious as it is more cost-effective to utilize oocytes harvested from an open herd of animals rather than from a closed herd requiring intense management practices.

Our objective in this study was to compare the fusion rates of couplets and then the \textit{in vivo} development of NT embryos produced from the fusion of transgenic fetal fibroblast cells with that of cytoplasts prepared from oocytes aspirated from either FSH-stimulated or abattoir-derived caprine ovaries.

**Materials and Methods**

**Isolation of Caprine Transgenic Fetal Fibroblast Cell Line**

The primary caprine fetal fibroblast cells used as karyoplast donors were derived as previously described (Baguisi \textit{et al.}, 1999). Fetal tissues from a Day-40 transgenic female fetus (Toggenberg) were minced, washed, and transferred into 25-cm$^2$ tissue culture flasks. Cells were cultured in fetal cell medium (FCM) consisting of Tissue Culture Medium-199 (TCM-199; Gibco Laboratories, Grand Island, NY) supplemented with 10\% fetal bovine serum (FBS; Hyclone, Logan, Utah), nucleosides, 2 mM L-glutamine, 0.1 mM $\beta$-mercaptoethanol as an anti-oxidant (Sigma Chemical Co., St. Louis, MO) and 50 $\mu$g/ml gentamicin sulfate (Gibco). After three subpassages, cell stocks were frozen and stored in liquid nitrogen. This transgenic female line was designated F-6 and used for all NT procedures within four to eight subpassages. Each passage was maintained in culture and required 6.2 $\pm$ 0.43 days to reach 80\% confluency.
Oocyte Collection

FSH-stimulated ovaries. Prior to oocyte retrieval, mixed-breed Spanish-type goats were implanted (Day 1) on the dorsal side of the ear (s.c.) with a 3-mg norgestomet implant (Synchro-Mate-B, Rhone Merieux, Athens, GA) and then subjected (Day 4) to ovarian superstimulation by twice daily administration (i.m.) of FSH (Sioux Biochemical, Sioux Center, IA) in a decreasing dose schedule (10 and 10, 7.5 and 7.5, 5 and 5, and 2.5 and 2.5 units) totaling 50 units/doe (Graff et al., 1999). All goats (both donor and recipient females) used in this study were handled and maintained in accordance with the Institutional Animal Care and Use Committee Guidelines at Louisiana State University.

Implants were removed on treatment Day 8 and oocytes were then aspirated 24 h after the final FSH injection. Following general anesthesia (Halothane; Fort Dodge Animal Health, Fort Dodge, IA), does were placed in dorsal recumbency on a hydraulic operating table for a mid-ventral laparotomy procedure. Oocytes were harvested using techniques previously described (Graff et al., 2000) with slight modifications. Ovaries were exteriorized, and all visible follicles were aspirated with a 20-gauge needle attached via polyethylene tubing (20-gauge) to an electric vacuum pump (Cook Veterinary Products, Eight Mile Plains, Australia). The pump was set to aspirate ~18 ml of fluid/min. Oocytes were collected in 50-ml centrifuge tubes containing warmed PBS supplemented with 1% FBS, 25 µg/ml gentamicin and 0.4 units/ml sodium heparin (Elkins-Sinn, Cherry Hill, NJ). All oocytes with a compact investment of cumulus cells were washed eight times in maturation medium (TCM-199 supplemented with 10% normal goat serum (Sigma), 10 µg/ml LH, 5 µg/ml FSH, and 1 µg/ml estradiol-17β) and allowed to mature for 18 to 22 h (38°C and 5% CO₂) in
groups of 10 to 20 in 35-µl microdrops of maturation medium overlaid with warmed embryo-tested mineral oil (Sigma).

**Abattoir ovaries.** Ovaries harvested from nonstimulated does at an abattoir located in San Angelo, TX, were placed in insulated thermos bottles containing PBS at ambient temperature and transported by air courier within 12 h of collection. Upon arrival at the laboratory, oocytes from these ovaries were similarly aspirated, selected, washed, and matured as described for oocytes from stimulated ovaries.

**Nuclear Transfer**

**Enucleation.** Cumulus-oocyte complexes were vortexed at 18 to 22 h postmaturation for 2.25 min in TL-Hepes (BioWhittaker, Walkersville, MD) containing 0.6 mg/ml hyaluronidase (Sigma) to aid in the final removal of the cumulus cells. Oocytes were washed in modified TL-Hepes (mTLH; TL-Hepes supplemented with 10% FBS), selected based on the presence of a polar body (M II), and labeled with 3 µg/ml Hoechst 33342 stain (Sigma) for 1 min. Mature oocytes were enucleated in a 200-µl elongated droplet of warmed micromanipulation medium (mTLH with 7.5 µg/ml cytochalasin B) overlaid with warmed mineral oil on the stage of an inverted microscope (Nikon Diaphot) equipped with Hoffman Modulation Contrast objectives, Narishige micromanipulators, and epifluorescent illumination.

Oocytes were held at their 9 o’clock position by negative pressure applied to a holding pipette (75-µm outside diameter). After a brief exposure (<10 sec) to ultraviolet light to visualize nuclear DNA, the oocytes were rotated against the holding pipette to bring the metaphase plate into focus at the 3 o’clock position. Using a borosilicate glass enucleation pipette (20- to 25-µm outside diameter) with a 35° bevel and a spike, the metaphase plate and
first polar body were removed under short-term exposure to UV-light (<5 sec). This procedure was generally effective in removing both the polar body and the metaphase plate. The resulting cytoplasts were allowed to recover at 38°C in modified TCM (mTCM; TCM supplemented with 10% FBS) for at least 30 min prior to reconstruction with donor cells.

**Donor cell preparation.** Actively dividing fibroblast cells from the transgenic female line (F-6) were maintained in FCM with 10% FBS for 3 to 4 days and then synchronized by an additional 4 days of culture in reduced-serum (0.5 %) FCM. Donor cells were trypsinized, washed, and held (<1.5 h) in mTLH immediately before transfer into the recently prepared cytoplasts.

**Reconstruction and fusion.** A single fibroblast cell (15- to 20-µm diameter) was injected into the perivitelline space of each enucleated oocyte in a droplet (200 µl) of micromanipulation medium using the same enucleation pipette. Close contact of the donor cell membrane with the vitelline membrane of the cytoplast was visually confirmed prior to fusion. Karyoplast-cytoplast couplets were manually aligned between two stainless steel electrodes (1-mm gap) in a microslide fusion chamber filled with fusion buffer (0.3 M mannitol, 0.1 mM MgSO₄·7H₂O, 0.05 mM CaCl₂, 0.5 mM Hepes, and 4 mg/ml BSA) and fused by a single DC pulse (1.30 kV/cm for 25 µsec) delivered by a BTX Electrocell Manipulator 200 (Gentronics, San Diego, CA). Couplets were evaluated for fusion after a 1-h incubation period in mTCM and then activated.

Fused couplets were activated by a 5-min exposure to 5 µM ionomycin (Sigma), washed extensively in mTLH, and then incubated for 3 h in 2 mM 6-dimethylaminopurine prepared in G1.2 medium (Zander IVF, Vero Beach, FL). Following activation, the
reconstructed embryos were washed and cultured under oil in 35-µl droplets of G1.2 medium for 33 to 36 h (two- to four-cell stages) prior to transfer to recipient females.

**Embryo Transfer**

Spanish-type crossbred recipient does were selected from those exhibiting a natural estrus 2 days prior to the scheduled embryo transfer. NT embryos, produced from either FSH-stimulated ovaries or nonstimulated abattoir-derived ovaries, were transferred into recipients 54 to 60 h after donor oocytes were aspirated.

The same general anesthesia protocol used for the oocyte aspiration was followed for the embryo transfer procedure. Does were placed in dorsal recumbency, and a mid-ventral laparotomy procedure was performed. After the ovaries were examined for evidence of ovulation, the uterus was exteriorized and embryos were transferred to the oviduct ipsilateral to the ovary with the most ovulation points. Embryos (1–15/female) were transferred via a small plastic catheter (Embryon Catheter; Rolon Medical, Watford, UK) through the ostium of the infundibulum, gently advancing the catheter as far as possible distally into the oviduct. Recipients were returned to the herd to await subsequent examinations of pregnancy status.

**Pregnancy Status and Parturition**

Beginning on Day 30 of gestation, recipients were subjected to a transvaginal ultrasonographic evaluation (Model 500-V; Aloka, Tokyo, Japan), followed 7 and 10 days later by additional ultrasonographic assessments. The presence of a fetal heartbeat was used to diagnose pregnancy. Pregnant recipients were subsequently monitored at 14-day intervals and then separated from the herd on Day 90 of gestation and placed in groups of two and three females per pen. Monitoring of pregnant recipients past Day 90 was performed via abdominal ultrasonography.
Parturition was induced in recipient females if they had not given birth by Day 152 of gestation. Induced females received one single 15 mg injection (i.m.) of prostaglandin F$_{2\alpha}$ (Lutalyse; Upjohn, Kalamazoo, MI) and a single 12 mg injection (i.m.) of dexamethasone and were monitored closely until parturition. The kids were allowed to remain with the dams and nurse freely until weaning.

**Characterization of Cloned Animals**

Skin biopsies (6-mm diameter) were obtained from each offspring (1.5–3 months-of-age) from the caudal lateral aspect of the thigh directly over the semitendinosis muscle and then subjected to genomic DNA isolation (Laird *et al.*, 1991) using the Genomic Prep Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Each sample and an internal control for goat genomic DNA (goat exon 7) were analyzed by polymerase chain reaction using human antithrombin (hAT)-specific primers. The hAT sequence was detected by amplification of a 367-base pair (bp) sequence with oligonucleotides GTC11 (CTCCATCAGTTGCTGGAGGGTGTCATTA) and GTC12 (GAAGGTTTATCTTTTGTCCTGCTGCTCA).

Two positive control samples, from an antithrombin III (ATIII) transgenic adult female (B21) and from the transgenic fetal fibroblast F-6 cell line, and one negative control blood sample from a nontransgenic female goat were analyzed to verify the presence of the human ATIII (hATIII) transgene.

At 150 days of age, the cloned offspring were subjected to a hormonal lactation induction protocol developed for prepubertal goats (Cammuso *et al.*, 2000) and were then hand-milked once daily to collect samples to assay for hATIII expression. Western blots were performed as previously described (Edmunds *et al.*, 1998).
Statistical Analysis

Data for maturation rate, number of couplets produced, rate of fusion, *in vitro* embryo development and subsequent production of offspring were all analyzed by a general linear model of regression using a SAS analysis of variance procedure (SAS, 1992).

Results

Oocyte Recovery and Embryo Reconstruction

The rate of oocyte recovery and maturation from stimulated and nonstimulated abattoir-derived ovaries is shown in Table 4.1. A total of 833 oocytes were recovered from a series of aspirations (12 replicates) from 34 FSH-stimulated donor animals (68 ovaries). An average of 12.2 oocytes were recovered per stimulated ovary (from 7 to 33 oocytes/ovary). Eight replicates of follicular aspiration from 944 nonstimulated abattoir-derived ovaries produced a total of 769 oocytes (0.81 oocytes/ovary).

Following a maturation interval of 18 to 22 h, 50% of the oocytes from the FSH-stimulated ovaries and 68% of the oocytes from the abattoir-derived ovaries reached the MII stage, as evidenced by extrusion of the first polar body. Of the matured oocytes used for NT, 91% and 85% resulted in successful couplets derived from oocytes from FSH-stimulated and abattoir-derived ovaries, respectively. The rate of fusion of NT embryos reconstructed from oocytes from either FSH-stimulated or abattoir-derived ovaries was 63% (range, 22%–79%) and 57% (range, 45%–81%), respectively. There was no significant difference between the two treatment groups for maturation rate, couplet production rate and fusion rate.
Table 4.1. Oocyte maturation and embryo reconstruction rates from oocytes derived from FSH-stimulated and nonstimulated abattoir-derived goat ovaries

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of ovaries processed</th>
<th>Oocytes recovered</th>
<th>Oocytes per ovary</th>
<th>No. (%) oocytes matured</th>
<th>No. (%) of NT attempts&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number (%) of couples produced</th>
<th>Number (%) of couples fused</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH-stimulated</td>
<td>68</td>
<td>833</td>
<td>12.25</td>
<td>415 (50%)</td>
<td>367 (88%)</td>
<td>336 (91%)</td>
<td>213 (63%)</td>
</tr>
<tr>
<td>Abattoir-derived</td>
<td>944</td>
<td>769</td>
<td>0.81</td>
<td>524 (68%)</td>
<td>378 (72%)</td>
<td>320 (85%)</td>
<td>183 (57%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Only grade-1 mature oocytes were enucleated; oocytes exhibiting a distinct polar body but poor cytoplasm were marked as mature, but not used in this study.
Embryo Development and Cloned Transgenic Offspring

There was no effect of oocyte source on the number of fused couplets that developed to the two- to four-cell stages (57% vs. 56%; Table 4.2). Only high-quality reconstructed embryos (those with equal size blastomeres and no fragmentation) at the two- to four-cell stages were transferred into recipient females. Moreover, 92% of the two- to four-cell embryos resulting from oocytes derived from FSH-stimulated ovaries were classified as high quality, but the rate was not different when compared with those reconstructed embryos produced from oocytes from abattoir-derived ovaries (70%).

A total of 23 similar recipient does received an average of 8 embryos/female (range, 1–15 embryos/female). Five of the recipients maintained conceptuses throughout gestation, and each gave birth to a single offspring. The three recipients that maintained pregnancies from the FSH-stimulated group each received an average of 7.6 embryos (range, 7–8 embryos). The two recipients that maintained pregnancies in the abattoir ovary group each received nine reconstructed embryos. The pregnancy rate (defined as the number of pregnant recipients per total number of recipients) at 30 days of gestation was 21% (3/14) and 22% (2/9) for the FSH-stimulated group and the abattoir-derived group, respectively. The overall pregnancy rate was 21.7% (5 of 23 recipients).

Based on the number of offspring produced per number of embryos transferred, the cloning efficiency was 2.7% for both the FSH-stimulated and abattoir-derived groups (3/112 and 2/72, respectively). All pregnancies were produced from donor cells that originated from the fifth or sixth passage of the transgenic fetal fibroblast cell line.
Table 4.2. Recipient cytoplasts derived from FSH-stimulated and nonstimulated abattoir-derived goat ovaries

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. fused couplets</th>
<th>2- to 4-cell embryos$^a$</th>
<th>No. transferred$^b$</th>
<th>No. recipients</th>
<th>Pregnant day 30$^c$</th>
<th>No. offspring$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH-stimulated</td>
<td>213</td>
<td>122 (57%)</td>
<td>112 (92%)</td>
<td>14</td>
<td>3 (21%)</td>
<td>3</td>
</tr>
<tr>
<td>Abattoir-derived</td>
<td>183</td>
<td>103 (56%)</td>
<td>72 (70%)</td>
<td>9</td>
<td>2 (22%)</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$Includes both good and poor quality embryos.

$^b$Only good quality embryos were transferred.

$^c$Determined via ultrasonography starting on d 30 post-transfer; percentage based on the number of recipients.

$^d$All offspring in excellent health and beyond 90 days.
In this study, 100% of the Day 30 pregnancies culminated in the births of live, healthy offspring, with no fetal loss and no postpartum morbidity. Two of the recipients gave birth at 149 and 151 days of gestation. Parturition was induced in the other three recipient females at Day 152 of gestation, and they gave birth between 24 and 36 h postinduction. All kids were vigorous and healthy at birth and weighed an average of 3.8 kg (±SE = 0.18 kg; range, 3.4–4.3 kg). Phenotypically, all five offspring had a similar coat color pattern and physical stature (Figure 4.1). At this writing, all cloned animals have been weaned (Day 90) and are past 12 months of age. Mean weaning weight was 20.9 kg (±SE = 0.98 kg), with a range of 19.1 to 24.5 kg (Table 4.3). Each cloned animal has exhibited estrus and has been bred by an intact buck.

To verify that the cloned offspring were derived from the F-6 cell line, skin biopsies were screened for the presence of the hATIII transgene (Figure 4.2). Induced milk samples also were positive for the presence of recombinant hATIII by Western blotting (data not included).

**Discussion**

Five cloned transgenic goats were produced through NT using a cell line derived from a transgenic fetus and oocytes obtained from either FSH-stimulated or abattoir-derived ovaries. At birth, all 5 kids were healthy and weighed between 3.4 and 4.3 kg, which is within the normal range for the breed. At weaning (90 days), body weights ranged from 19.1 to 24.5 kg, and all kids are continuing to grow as expected.

There was no significant difference in the percentage of matured oocytes successfully enucleated and reconstructed regardless of oocyte source (FSH-stimulated ovaries vs. abattoir-derived ovaries). There was also no difference in the fusion rate for oocytes derived
Figure 4.1. Female goats produced by NT using transgenic fetal fibroblast cells
<table>
<thead>
<tr>
<th>Offspring identification</th>
<th>Cytoplast source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Karyoplast passage no.</th>
<th>Date of birth</th>
<th>Gestation length (d)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Induced birth</th>
<th>Birth weight (kg)</th>
<th>90-d weaning weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2001 (Louise)</td>
<td>FSH-stimulated</td>
<td>5</td>
<td>3/29/00</td>
<td>154</td>
<td>yes</td>
<td>4.3 kg</td>
<td>19.5 kg</td>
</tr>
<tr>
<td>2002 (Anna)</td>
<td>FSH-stimulated</td>
<td>5</td>
<td>3/29/00</td>
<td>154</td>
<td>yes</td>
<td>3.4 kg</td>
<td>19.5 kg</td>
</tr>
<tr>
<td>2003 (Ellie)</td>
<td>FSH-stimulated</td>
<td>5</td>
<td>5/07/00</td>
<td>151</td>
<td>no</td>
<td>3.4 kg</td>
<td>19.1 kg</td>
</tr>
<tr>
<td>2004 (Sue)</td>
<td>Abattoir</td>
<td>6</td>
<td>5/13/00</td>
<td>149</td>
<td>no</td>
<td>3.9 kg</td>
<td>24.5 kg</td>
</tr>
<tr>
<td>2005 (Gabrielle)</td>
<td>Abattoir</td>
<td>6</td>
<td>5/17/00</td>
<td>153</td>
<td>yes</td>
<td>4.3 kg</td>
<td>21.8 kg</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cytoplasts were derived from oocytes aspirated from either FSH-stimulated live animals or from abattoir ovaries (nonstimulated).

<sup>b</sup>Gestation calculated from day of recipient estrus (Day 0).
Figure 4.2. PCR analysis of cloned offspring: lanes A and K are the 100 bp ladders; lane B is a blank (negative control); lanes C through G are from clones 2001 through 2005; lane H is from a nontransgenic female goat (ATIII negative control); lane I is an ATIII clone (B521) positive control; lane J is the fibroblast cell line (CFF6) used for donor cells. The upper 440 bp band is a Goat Exon 7 (GEX 7) internal control. The lower 370 bp band is an ATIII specific sequence.
from FSH-stimulated ovaries (63%) and those derived from abattoir ovaries (57%). Similar fusion rates (59%) of fetal fibroblasts with cytoplasts from abattoir ovaries in the cow have also been reported (Vignon et al., 1998; Zakhartchenko et al., 1999b; Hill et al., 2000b), and fusion rates (85%) of fetal fibroblasts with cytoplasts from FSH-stimulated sheep tend to be higher (Wilmut et al., 1997). Oocyte source also had no effect on embryo development and overall pregnancy rate in the two treatment groups.

Recipient synchronization with NT embryo age likely affects pregnancy rates. In our study, all two- to four-cell stage NT embryos were transferred into recipients that were in estrus 36 to 48 h previously. Because mixed-breed goats usually ovulate 24 to 36 h after the onset of estrus, we calculated that at the time of embryo transfer the recipients would be 12 to 24 h postovulation. This would then be the time of first embryonic division, and the asynchrony between recipient and NT embryo stage was used in an effort to compensate for slowly developing NT embryos. Following in vitro maturation, the time for NT embryos to reach the two- to four-cell stage (36–40 h) appears to be slightly longer than required for embryos produced by in vitro fertilization (30–36 h) (Han, 1995).

An interesting aspect of this study was the fact that all detected pregnancies resulted in the births of healthy kids, with no fetal loss occurring from Day 30 to parturition and no postnatal morbidity. However, we cannot speculate on the number of pregnancies that were initiated and lost before the first ultrasound evaluations on Day 30 nor can we rule out the possibility of undetected twins before Day 30 that resorbed. Although it is possible to detect fetal heartbeats in the sheep (and possibly the goat) as early as Day 25 (Romano and Christians, 2000), this method places considerable stress on the animal because it must be restrained and placed in dorsal recumbency. To minimize this stress to our potentially
pregnant recipients, this method was not used. Other groups developing NT procedures in the goat have recently reported similar perinatal viability in NT offspring (Keefer et al., 2000; 2001).

In contrast, nearly 50% of the pregnancies resulting from cattle NT embryos are lost during the first trimester of gestation; most of these are thought to be caused by poor placentae and placentome development (Hill et al., 1999a). In addition, lethal postnatal complications (pneumonia, cardiac abnormalities) arising in cloned newborn calves have been a major problem (Hill et al., 1999a). These variations between the cow and the goat could be due to inherent differences between the two species or differences in the NT procedure. In our study, reconstructed caprine embryos were transferred into the oviduct at the two- to four-cell stages. Therefore, the reconstructed embryos underwent a relatively short in vitro culture period.

In this study, a total of 184 embryos were transferred into 23 recipient females. Five of the 23 recipients (21%) maintained pregnancies and subsequently gave birth to healthy offspring. This pregnancy rate, based on the number of pregnant recipients per total number of recipients, is greater than the 5.3% pregnancy rate previously reported by Baguisi et al. (1999) in the goat but lower than the 38% to 44% pregnancy rate reported by Keefer et al. (Keefer et al., 2000; 2001). Although 100% of the recipients that were determined pregnant by ultrasonography on Day 30 in this study delivered normal kids, the efficiency of the cloning procedure (based on the number of offspring per number of embryos transferred) was 2.7%, similar to that reported by Baguisi et al. (1999). The cloning efficiency reported by Keefer et al. (2000; 2001) ranged from 6.2% to 7.3%. In the sheep, overall cloning efficiency
ranges from 4.8% to 20%, depending on the cell type used (Schnieke et al., 1997; Wilmut et al., 1997).

Another important outcome of this study was the production of cloned offspring using abattoir-derived oocytes as a source of recipient cytoplasts. Until now, cloned goats have only been produced using cytoplasts obtained from oocytes aspirated from FSH-stimulated live donor animals (Baguisi et al., 1999; Keefer et al., 2000; 2001). Abattoir oocytes would likely be easier to obtain and more cost effective than maintaining and stimulating a herd of donor goats.

There was no difference in the number of cloned goat offspring produced from oocytes harvested from FSH-stimulated donor animals and from oocytes aspirated from abattoir ovaries. All of the conceptuses were maintained throughout gestation, with no fetal wastage or abortions, and all transgenic kids have remained healthy after parturition.
CHAPTER V
PRODUCTION OF RECOMBINANT HUMAN ANTIBODIES IN GOATS CLONED FROM A TRANSFECTED FETAL FIBROBLAST CELL LINE

Introduction

Somatic cell nuclear transfer (SCNT) has proven to be a more efficient method of producing transgenic animals than pronuclear DNA-microinjection (Schnieke et al., 1997; Cibelli et al., 1998a; Ziomek, 1998; McCreath et al., 2000). The ability to generate valuable human recombinant proteins and monoclonal antibodies in the milk of transgenic animals by SCNT has caught the interest of major biotechnology firms wishing to capitalize on this new technology (Echelard, 1996; Meade et al., 1998; Baguisi et al., 1999; Pollock et al., 1999). Cloned sheep (Wright et al., 1991; Schnieke et al., 1997; McCreath et al., 2000) and goats (Baguisi et al., 1999; Reggio et al., 2001) have proven reliable as sources of several of these proteins that cannot be synthesized in vitro nor manufactured by traditional bacterial or cell culture systems due to the complex post-translational modifications required to confer biological activity and/or stability to these compounds (Wright et al., 1991).

Transfecting cell lines with genes of interest, coupled with SCNT, has become a viable method for introducing foreign DNA into the genome of animals (Schnieke et al., 1997; Cibelli et al., 1998a; McCreath et al., 2000). Supplementing the foreign DNA with promoters that direct gene expression to the mammary gland (Ebert et al., 1994) enables a straightforward recovery of the protein of interest (Young et al., 1997). Although milk yields are higher in dairy cows, dairy goats have a shorter generation interval and thus are more attractive for use in transgenic programs (Ziomek, 1998). Also, transgenic female goats can
be induced to lactate at 2 months of age (Cammuso et al., 2000) to provide an initial sample for product testing (e.g., expression levels and biological activity). Larger quantities of milk can then be produced following a normal pregnancy. Purification of the protein of interest from this milk can yield sufficient quantities for further testing, or for use in human clinical trials (Echelard et al., 2000).

Fibroblast cells harvested from skin biopsies taken from adult animals have been used as nuclear donors in many NT programs (Wilmut et al., 1997; Kato et al., 1998; Wakayama et al., 1998; Shiga et al., 1999; Wakayama and Yanagimachi, 1999; Wells et al., 1999b; Hill et al., 2000b; Polejaeva et al., 2000; Keefer et al., 2001) because of the relative ease in procuring the tissue, as well as in culturing, harvesting and cryopreserving this particular cell type. While SCNT using nontransfected adult fibroblast donor cells has proven useful in generating clones of valuable individual animals, the main advantage of cloning lies in the generation of nondescript animals capable of producing pharmaceutical proteins in their milk. Incorporating genes of interest into cell lines (transfection) can be accomplished via standard lipofection (Watanabe et al., 1994; Dass et al., 1997; Bell et al., 1998) or electroporation protocols (Wong and Neumann, 1982; Takahashi et al., 1991; Oshima et al., 1998).

A potential limitation of using adult transfected fibroblast cells as nuclear donors is the prolonged culture period required to propagate, transfect, select and expand the cell line. Each of these steps requires repeated subpassaging and an extended culture period that may induce perturbations in the donor cells (such as chromosomal damage due to physical manipulation during transfection, or senescence brought on by lengthy in vitro culture), thereby decreasing the efficiency of NT (Forsberg et al., 2001; Zakhartchenko et al., 2001).
Also, there is a limit to the number of passages that a primary cell line can be subjected to before a state of senescence is reached (Hayflick and Moorhead, 1961; Pignalo et al., 1992). Moreover, adult human and bovine cells are capable of fewer population doublings before reaching senescence than are fetal cells (Cristofalo et al., 1998; Lanza et al., 2000b). Despite the inherent limitations of using donor cells from aged animals or from cell lines allowed to reach senescence by extended *in vitro* culture periods, recent data have shown that NT using aged or senescent donor fibroblast cells does not affect the viability of NT embryos (Kubota et al., 2000; Lanza et al., 2000b; Kasinathan et al., 2001), as evidenced by the birth of healthy offspring. However, fetal fibroblast cells have become the donor cells of choice in transgenic NT programs wishing to introduce foreign genes into the donor cell line (Schnieke et al., 1997; Cibelli et al., 1998a; McCreath et al., 2000).

Serum starvation of donor cells prior to NT induces the cells to exit the growth cycle and arrest in a state of quiescence (G0 of the cell cycle), and has been proposed to enhance embryonic development by allowing undefined “licensing” factors in the ooplasm to reprogram the donor nucleus (Blow and Laskey, 1988; Campbell et al., 1996a; Wilmut et al., 1997). Cells induced into the G0 phase possess an altered chromatin configuration that presumably allows increased access of these ooplasmic licensing factors that may enhance nuclear reprogramming (Campbell, 1999).

Following the lead of Campbell and Wilmut, many groups have successfully used serum starvation of donor cells prior to NT to generate offspring (Schnieke et al., 1997; Wells et al., 1997; 1998b; 1999b; Kato et al., 1998; Baguisi et al., 1999; Zakhartchenko et al., 1999a; Reggio et al., 2001), but only one group has recommended the use of proliferating (non serum-starved) cells (Cibelli et al., 1998a; b). However, none of these studies directly
compared proliferating or quiescent donor cell treatments to produce cloned animals. Recent studies using bovine fetal fibroblasts showed that quiescent donor cells significantly improved development to the blastocyst stage compared with proliferative cells (Hill et al., 1999b; Zakhartchenko et al., 1999b), but did not follow up with embryo transfer into recipient cows. In the pig, serum starvation of transfected fetal fibroblast donor cells did not have a positive effect (compared with nonstarved cells) on the development of NT embryos that cleaved or developed to the morula or blastocyst stage (Kuhholzer et al., 2001). Moreover, several groups have found no difference in blastocyst development or development to term of transferred NT embryos between quiescent or proliferative fibroblast cells (Shiga et al., 1999; Vignon et al., 1999; Wakayama and Yanagimachi, 1999; Hill et al., 2000b; Jones et al., 2001).

The ability to generate live offspring using proliferating donor cells may be advantageous in transgenic NT programs that must rely on extended in vitro culture periods of the cell line to allow incorporation of the gene of interest. Bondioli et al. (2001) produced transgenic live piglets after NT using proliferating transfected fibroblasts, a noteworthy accomplishment as it has been reported that porcine fibroblast cells undergo extensive DNA fragmentation during just three days of serum deprivation (Kues et al., 2000). It should also be noted that the donor cells used to produce the cloned pigs in this study were subjected to transfection, selection, expansion from a single cell, freezing and thawing—in short, an extensive in vitro culture period.

Reducing the overall time in culture by eliminating the extra step of inducing quiescence in the donor cells by serum starvation might prove important. Moreover, serum deprivation may have other detrimental effects on the donor cell, causing perturbations in
gene and protein expression (Hayashida et al., 1997) as well as unintentional changes of epigenetic DNA modifications, including DNA methylation (Dean et al., 1998; Jones et al., 2001).

Drawing on our recent success with SCNT using a nontransfected, quiescent fetal fibroblast cell line to produce cloned transgenic goats expressing rhATIII in milk (Reggio et al., 2001), we used the cloning technology to generate transgenic goats from two separate transfected cell lines (designated T9 and D2). Also, based on the previous studies suggesting that serum-starvation may be detrimental to donor nuclei, a comparison was made to determine if non-starved (proliferating) cells could be used to improve the efficiency of the NT procedure in goats.

**Materials and Methods**

**Transgene Construction**

To create donor cells carrying two transgenes on the same chromosome, an IgG fusion protein light chain transgene was constructed by inserting the light chain fusion gene into the mammary specific expression vector B80. This vector contained the caprine beta casein promoter, a 3’untranslated region (3’UTR) and flanking sequences, and a PGK-neo gene (to confer neomycin resistance) flanked by the chicken beta globin insulator sequences. Similarly, to construct the IgG fusion protein heavy chain transgene, the fusion gene was inserted into the mammary specific expression vector B35 containing the same caprine beta casein promoter and the 3’UTR and flanking sequences.

**Isolation and Transfection of Caprine Fetal Fibroblast Cells**

**Isolation of primary cell line.** An initial population of female fetal fibroblast cells was generated as previously described (Baguisi et al., 1999) with slight modifications. Briefly, a
Day-40 female fetus (Saanen) produced by artificial insemination was surgically removed from the dam. The conceptus was then finely minced after removing the head and viscera. The tissue was washed and transferred into 25-cm² tissue culture flasks containing fibroblast culture medium (FCM) consisting of Tissue Culture Medium-199 (TCM-199; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah), nucleosides, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO) and 50 µg/ml of gentamicin sulfate (Gibco). Cell cultures were maintained in FCM at 38°C in a humidified atmosphere of 5% CO₂ in air until 60% confluent, then transfected.

**Transfection.** Transgenes were introduced into the fetal fibroblast cells by lipofection using the protocol supplied with LipofectAMINE® (Gibco) and allowed to proliferate for 72 h in FCM. Neomycin resistant cells were selected by subsequent culture in modified FCM (mFCM; FCM supplemented with 0.6 mg/ml neomycin), and individual colonies were isolated and expanded (3–4 subpassages) in mFCM. Individual cell lines were assayed by the polymerase chain reaction and Southern blotting to detect the presence of both transgenes. Further characterization of candidate cell lines by fluorescence in situ hybridization (FISH) confirmed co-localization of the heavy and light chain transgenes on a single chromosome.

Only those cell lines having both transgenes located on the same chromosome were cryopreserved for use as nuclear donors. Cells were frozen in TCM-199 supplemented with 20% FBS, 5% DMSO and 0.6 mg/ml neomycin. Prior to the NT procedure, two transgenic female cell lines (designated as T9 and D2) were thawed and subpassaged once more to establish stocks of donor cells frozen at a concentration of 200,000 cells/ml and used within six subpassages.
Oocyte Collection and Maturation

All oocytes used in this study were recovered either from a herd of mixed-breed donor goats maintained at the LSU Embryo Biotechnology Laboratory (St. Gabriel, LA) or from an abattoir located in San Angelo, TX during the latter part of the breeding season (mid-February to mid-March).

Ovaries from the abattoir were collected from mixed-breed goats, placed in insulated thermos bottles containing PBS at ambient temperature and transported to the laboratory (Ovagenix, San Angelo, TX) within 2 h of collection. All follicles ≥2 mm in diameter were aspirated using a 20-gauge aspiration assembly (Cook Veterinary Products, Eight Mile Plains, Australia) connected to an electric vacuum pump and collected in 50 ml centrifuge tubes containing warmed PBS supplemented with 1% FBS, 0.4 units/ml sodium heparin (Elkins-Sinn, Cherry Hill, NJ) and 50 µg/ml gentamicin (aspiration medium). Only those oocytes with a compact layer of cumulus cells were selected, washed extensively and placed in groups of 50/ml in 1 ml cryovials (Corning, New York, NY) filled with warmed, equilibrated (38°C, 5% CO₂) maturation medium (TCM-199 supplemented with 10% normal goat serum, 10 µg/ml LH, 5 µg/ml FSH and 1 µg/ml estradiol-17β [Sigma]). Cryovials were loaded into portable shipping incubators maintained at 38°C and shipped overnight via air courier to the LSU Embryo Biotechnology Laboratory. Upon arrival, oocytes were transferred to a standard incubator (38°C and 5 % CO₂) and allowed to complete maturation (18–22 h post aspiration).

Oocytes were harvested from the donor herd following an ovarian superstimulation protocol developed at this laboratory (Graff et al., 1999). All goats were handled and maintained in accordance with the Louisiana State University Institutional Animal Care and Use Committee Guidelines. Briefly, a 3-mg norgestomet implant (Synchro-Mate-B, Rhone
Merieux, Athens, GA) was inserted (Day 1) on the dorsal side of the ear (s.c.), followed by a twice daily injection (i.m.) of FSH (Sioux Biochemical, Sioux Center, IA) beginning on Day 4 in a decreasing dose regimen (10 and 10, 7.5 and 7.5, 5 and 5, and 2.5 and 2.5 units) totaling 50 units/doe.

On treatment Day 8 (24 h after the final FSH injection), implants were removed and oocytes were aspirated from the donors using a slight modification of the techniques previously described in this laboratory (Graff et al., 2000). Does were placed in dorsal recumbency and general anesthesia was induced (Halothane; Fort Dodge Animal Health, Fort Dodge, IA) prior to a midventral laparotomy procedure to exteriorize the ovaries. Oocytes from all visible follicles were similarly aspirated, selected and washed as described for oocytes from the abattoir ovaries. Cumulus-oocyte-complexes were placed in groups of 10 to 20 in 35-µl droplets of maturation medium overlaid with equilibrated mineral oil (Sigma) and allowed to mature for 18 to 22 h at 38°C and 5% CO₂.

**Experimental Design and Statistical Analysis**

In Experiment 5.1, transfected T9 cells were used as nuclear donors either in the proliferative (n=157) or quiescent (n=150) state and transferred into enucleated oocytes. In Experiment 5.2, transfected D2 donor cells were similarly used in either the proliferative (n=87) or quiescent (n=106) state. Both experiments were replicated 5 times.

In both Experiments 5.1 and 5.2, the rate of fusion, *in vitro* embryo development, pregnancy at Day 30 and subsequent production of offspring in each treatment were recorded. The effect of donor cell treatment on fusion rates and embryo development was analyzed using a logistic regression procedure of the SAS statistical package (SAS, 1992). Differences where P<0.05 were regarded as statistically significant. Pregnancy and birth rates were
compared by an analysis of variance procedure (ANOVA) using the general linear model with Tukey’s Studentized Range (HSD) Test post ANOVA analysis.

**Nuclear Transfer**

**Enucleation.** At 18 to 22 h postmaturation, cumulus cells were stripped from the oocytes by mechanical agitation for 2.25 min in 1 ml of TL-Hepes (TLH; BioWhittaker, Walkersville, MD) containing 0.6 mg/ml hyaluronidase (Sigma). Mature oocytes were washed in modified TLH (mTLH; TLH supplemented with 10% FBS) and selected based on the presence of a polar body (MII), an intact oolema and evenly granulated cytoplasm. Morphologically normal oocytes were incubated for 1 to 3 min with 3 µg/ml bis-benzimide (Hoechst 33342, Sigma) diluted in mTLH to stain nuclear DNA. Enucleation and reconstruction were carried out in 200-µl elongated droplets of micromanipulation medium (TLH supplemented with 6.5 µg/ml cytochalasin B).

To facilitate removal of the nucleus, the polar body was rotated to the 3 o’clock position while the oocyte was held at the 9 o’clock position with negative pressure applied to the holding pipette. A brief exposure (<10 sec) to UV-light confirmed the location of the underlying metaphase plate. To assure complete removal of nuclear DNA, both the polar body and the metaphase plate were aspirated with the enucleation pipette while subjecting the oocyte to short-term exposure to UV-light (<5 sec). Enucleated oocytes (cytoplasts) were allowed to recover at 38°C in modified TCM (mTCM; TCM supplemented with 10% FBS) for at least 30 min prior to reconstruction with donor cells.

**Donor cell preparation.** Frozen transfected fibroblast cells were thawed, washed and maintained in mFCM for 3 to 4 days. Actively dividing cells were either allowed to proliferate or induced into quiescence (presumptive G0/G1) by an additional 4 days of culture.
in mFCM formulated with 0.5% serum. Approximately one hour prior to transfer into the recently prepared cytoplasts, donor cells (karyoplasts) were enzymatically dispersed, washed and held in mTLH.

**Reconstruction and fusion.** Each cytoplast was reconstructed by injecting a single fibroblast donor cell (15–20 µm diameter) through the zona pellucida and into the perivitelline space, pushing the donor cell tightly against the oolema with the tip of the enucleation pipette. Groups of 5 to 10 karyoplast-cytoplast couplets were manually aligned between two stainless steel electrodes (1 mm gap) in a microslide fusion chamber filled with fusion buffer (0.3 M mannitol, 0.1 mM MgSO₄·7H₂O, 0.05 mM CaCl₂, 0.5 mM Hepes and 4 mg/ml BSA). Orientation of the couplets was such that the plane of contact between karyoplast and cytoplast was perpendicular to the direction of the electric current. A single DC pulse (1.30 kV/cm for 25 µsec) delivered by a BTX Electrocell Manipulator 200 (Gentronics, San Diego, CA) was applied across the electrodes to induce membrane fusion. Couplets were evaluated for fusion after a 1-h incubation period in mTCM and then activated. Couplets that had not fused after the initial pulse were subjected to another round of electrofusion using the same parameters.

**Activation.** Fused couplets were exposed for 5 min to 5 µM ionomycin (Sigma) prepared in TLH, then washed extensively in mTLH. Next, couplets were incubated for 3 h in 2 mM 6-dimethylaminopurine prepared in G1.2 medium (Zander IVF, Vero Beach, FL). Following activation, embryos were washed in G1.2 medium and cultured overnight in groups of 10 to 20 in 35-µl droplets of G1.2 medium under oil prior to transfer to recipient females the next day.
**Embryo Transfer**

Recipient does exhibiting a natural estrus 2 days prior to the scheduled embryo transfer were selected from a herd of crossbred Spanish-type goats in good body condition. NT embryos were surgically transferred into the oviduct ipsilateral to the ovary with the ovulation point(s), using the same general anesthesia and laparotomy protocols used for oocyte aspiration.

A soft plastic Embryon catheter (Rolon Medical, Watford, UK) loaded with NT embryos was inserted into the ostium of the infundibulum and gently pushed as far as possible toward the distal end of the oviduct. In Experiment 5.1, an average of 20 embryos (range 5–43) was transferred into each of 11 recipients, while 8 to 23 embryos/recipient (average = 13.8) were transferred into 9 females in Experiment 5.2. Following a brief recovery period, recipients were returned to the herd to await subsequent pregnancy determination.

**Pregnancy Status and Parturition**

Recipient does were subjected to transvaginal ultrasonographic evaluations (Model 500-V; Aloka, Tokyo, Japan) to detect fetal heartbeats beginning on Day 30 of gestation (Ayres *et al.*, 1999). Ultrasound assessments were repeated on all females 7 and 10 days later. One pregnant recipient carrying twins was subsequently monitored at 14-day intervals, then separated from the herd on Day 90 of gestation. Abdominal ultrasonography was used to monitor this pregnancy past Day 90. Kids were allowed to remain with the dam and nurse freely until weaning.

**Characterization of Cloned Animals**

At 2 months of age, a 6-mm diameter punch biopsy was obtained from each kid from the caudal lateral aspect of the thigh directly over the semitendinosus muscle. Genomic DNA
was isolated (Laird et al., 1991) using the Genomic Prep Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Skin samples and an internal control for goat genomic DNA (goat exon 7) were analyzed by the polymerase chain reaction (PCR) using transgene-specific primers and detected by amplification of a sequence specific for the transgene. A positive control from the transfected T9 cell line and a negative control from a nontransgenic female goat were also analyzed to verify the presence of the transgene.

Clones were subjected to a lactation induction protocol at 150 days of age (Cammuso et al., 2000) and then hand-milked once daily to collect samples for Western blot analysis to assay for protein expression.

**Results**

**Oocyte Maturation and Embryo Reconstruction**

**Experiment 5.1.** Following a maturation interval of 18 to 22 h, 41% of the 854 oocytes recovered in Experiment 5.1 reached the MII stage (Table 5.1). A total of 307 couplets were produced over 5 replicates (a mean of 61 couplets per replicate; range, 40–93 couplets per replicate), resulting in an overall reconstruction rate of 88% (307 couplets/347 mature oocytes) for this experiment. The overall fusion and cleavage rates were 78% and 91%, respectively. The mean fusion rate and cleavage rate per replicate was 76% (range, 58%–90%) and 90% (range, 81%–100%), respectively.

**Experiment 5.2.** In Experiment 5.2, 47% of the 515 recovered oocytes were matured in vitro (Table 5.1). The overall reconstruction rate was 79% (193 couplets/243 mature oocytes), with a mean of 39 couplets (range, 17 couplets–76 couplets) produced per replicate over 5 replicates. The overall fusion and cleavage rates were 88% and 79%, respectively.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Donor cell line</th>
<th>No. oocytes recovered</th>
<th>No. (%) oocytes matured</th>
<th>No. (%) couplets produced</th>
<th>No. (%) couplets fused</th>
<th>No. (%) embryos&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>T9</td>
<td>854</td>
<td>347 (41)</td>
<td>307 (88)</td>
<td>241 (78)</td>
<td>220 (91)</td>
</tr>
<tr>
<td>5.2</td>
<td>D2</td>
<td>515</td>
<td>243 (47)</td>
<td>193 (79)</td>
<td>170 (88)</td>
<td>135 (79)</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>1369</td>
<td>590 (43)</td>
<td>500 (85)</td>
<td>411 (82)</td>
<td>355 (86)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Two- to four-cell embryos.
The mean fusion rate per replicate was 88% (range, 82%–96%) and the mean cleavage rate per replicate was 76% (range, 57%–100%).

Embryo Development

Experiment 5.1: Proliferating vs. quiescent T9 donor cells. The development of 307 NT couplets reconstructed from either proliferating or quiescent T9 donor cells was assessed (Table 5.2). The rate of fusion of NT embryos reconstructed with donor cells in either the proliferative or quiescent state was 80% (range, 64%–91%) and 77% (range, 54%–89%), respectively. Of the 125 successfully fused embryos produced from 157 proliferating karyoplast NT couplets, 112 (89%) cleaved and were subsequently transferred to recipients. The cleavage and transfer rate for the 116 fused embryos reconstructed from 150 quiescent karyoplast NT couplets was 93%. There was no significant difference between the two treatment groups for cytoplast/karyoplast fusion, cleavage and subsequent embryo transfer.

Experiment 5.2: Proliferating vs. quiescent D2 donor cells. From a total of 193 NT couplets reconstructed using D2 donor cells, 87 originated from a proliferating cell population and 106 were generated with quiescent cells (Table 5.3). Of the 78 NT fused embryos generated from proliferating cells, 60 cleaved and were subsequently transferred into recipient does. Also, 75 of the 92 fused embryos reconstructed with quiescent cells cleaved and were similarly transferred. The rate of fusion of NT embryos reconstructed with either proliferating or quiescent cells varied from 82% to 94% and 79% to 96%, respectively. There was no effect of donor cell source on the number of NT couplets fused (90% vs. 87%), nor on the number of embryos that cleaved and were subsequently transferred (77% vs. 82%).
Table 5.2. Embryo development from nuclear transfer couplets reconstructed with either proliferating or quiescent transfected fetal fibroblast donor cells (T9)

<table>
<thead>
<tr>
<th>Donor cell treatment</th>
<th>No. couplets produced</th>
<th>No. (%) couplets fused</th>
<th>No. (%) embryos cleaved and transferred</th>
<th>No. recipients</th>
<th>No. (%) does pregnant, Day 30</th>
<th>No. healthy offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferating</td>
<td>157</td>
<td>125 (80)</td>
<td>112 (89)</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Quiescent</td>
<td>150</td>
<td>116 (77)</td>
<td>108 (93)</td>
<td>5</td>
<td>1 (20)</td>
<td>2</td>
</tr>
</tbody>
</table>

*Twin pregnancy; 43 embryos originating from donor cells at passage 5 were transferred.*
Table 5.3. Embryo development from nuclear transfer couplets reconstructed with either proliferating or quiescent transfected fetal fibroblast donor cells (D2)

<table>
<thead>
<tr>
<th>Donor cell treatment</th>
<th>No. couplets produced</th>
<th>No. (%) couplets fused</th>
<th>No. (%) embryos cleaved and transferred</th>
<th>No. recipients</th>
<th>No. does pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferating</td>
<td>87</td>
<td>78 (90)</td>
<td>60 (77)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Quiescent</td>
<td>106</td>
<td>92 (87)</td>
<td>75 (82)</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>
Embryo Transfer and Cloned Transgenic Offspring

Experiment 5.1 An average of 20 embryos/recipient were transferred into 11 similar females (range, 5–43 embryos/recipient). The six recipients receiving embryos reconstructed with proliferating donor cells each received an average of 18.6 embryos/female (range, 5–27 embryos); no pregnancies were detected in this group (Table 5.2). A total of 108 NT embryos reconstructed from quiescent cells were transferred into five recipients; each female received an average of 21.6 embryos (range, 19–43). The recipient receiving 43 embryos maintained a twin pregnancy throughout gestation. This pregnancy was produced from donor cells originating from the fifth passage of the transfected T9 cell line.

One set of twin transgenic kids (ID No. 2006 and No. 2007) was born at 147 days of gestation. Both offspring were healthy and vigorous at birth, weighed 2.95 kg and 3.06 kg, and were similar in physical stature and coat color (Figure 5.1). At weaning (90 days), both clones weighed 26.3 kg and are now past 12 months of age (Table 5.4). Skin biopsies from each clone analyzed by PCR verified that the clones were positive for the transgene and were derived from the T9 cell line. Milk samples from an induced lactation were positive for the presence of the recombinant protein by Western blotting.

The pregnancy rate, based on the number of pregnant recipients per total number of recipients, was 0% (0/6) and 20% (1/5) for the proliferating group and the quiescent group, respectively. The overall pregnancy rate for this study was 9.1% (1 of 11 recipients). The cloning efficiency, defined as the number of offspring produced per number of NT embryos transferred, was 0% (0/112) for the proliferating treatment and 1.8% (2/108) for the quiescent treatment, respectively. The overall cloning efficiency for this experiment was less than 1%
Figure 5.1 Cloned transgenic female goats (No. 2006 and No. 2007) produced from a transfected fetal fibroblast cell line (T9).
Table 5.4. Origin, development and growth performance of cloned transgenic goats produced from cell line T9

<table>
<thead>
<tr>
<th>Offspring identification</th>
<th>Karyoplast treatment</th>
<th>Karyoplast passage no.</th>
<th>Date of birth</th>
<th>Gestation length (days)a</th>
<th>Birth weight (kg)</th>
<th>90-day weaning weight (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td>quiescent</td>
<td>5</td>
<td>07/13/00</td>
<td>147</td>
<td>2.95</td>
<td>26.3</td>
</tr>
<tr>
<td>2007</td>
<td>quiescent</td>
<td>5</td>
<td>07/13/00</td>
<td>147</td>
<td>3.06</td>
<td>26.3</td>
</tr>
</tbody>
</table>

aGestation calculated from day of recipient estrus (Day 0).
(2 offspring/220 embryos transferred). Because of the low number of recipients, there were no significant differences either in pregnancy rate or cloning efficiency between embryos reconstructed with proliferating or quiescent donor cells.

Experiment 5.2 All NT embryos were reconstructed with donor cells originating from the second passage of the D2 transfected cell line. From a total of 135 cleaved embryos, an average of 15 embryos was transferred into each of 9 similar recipients (range, 10–23 embryos/recipient) (Table 5.3). The four females receiving embryos derived from proliferating cells also received an average of 15 embryos/female (range, 11–22 embryos/recipient). A total of 75 NT embryos reconstructed from quiescent cells was transferred into five recipients, and each female received an average of 15 embryos (range, 10–23 embryos/recipient). No pregnancies were detected in either group up to 55 days posttransfer.

Discussion

Two cloned transgenic goats, both expressing a pharmaceutically important protein, were produced by NT using a transfected fetal fibroblast cell line (T9). As in our previous study (Reggio et al., 2001), and consistent with reports from other goat NT programs (Baguisi et al., 1999; Keefer et al., 2001, 2002), there were no incidents of prenatal loss in this study. Although the number of cloned offspring in this study was small, we observed no neonatal loss compared with previous reports in which up to 50% of clones derived from fetal fibroblasts died shortly after birth in the goat (Keefer et al., 2001, 2002), sheep (Schnieke et al., 1997) and cow (Zakhartchenko et al., 1999b).

Both kids were healthy at birth and weighed within the normal range for the breed (Morand-Fehr, 1981). Body weights at weaning were similar (26.3 kg) and the goats are
growing as expected. At 1 year of age, clone No. 2007 was mated to a crossbred Boer goat and delivered two healthy kids (1 male, 1 female) following an uneventful gestation and a normal parturition at 150 days of gestation (Figure 5.2). The male weighed 3.6 kg and the female weighed 2.7 kg.

The overall fusion rates using transfected fetal fibroblast cells for both experiments in this study were higher (77%–90%) than those reported in previous goat NT studies using nontransfected fetal fibroblasts (54%–63%) (Keefer et al., 2001, 2002; Reggio et al., 2001), but similar to those reported in a recent study using nontransfected caprine granulosa donor cells (87%) (Keefer et al., 2002). This ability to maintain high fusion rates using transfected cells is advantageous to programs wishing to generate transgenic animals from cell lines that have been subjected to prolonged in vitro transfection and selection processes.

Although there was no significant difference in the percentage of embryos produced from either proliferating or quiescent cells, only the latter supported development to term in this study. In sheep, it has been suggested that donor cell quiescence (G0) is the key in allowing reprogramming of the donor nucleus and subsequent development to term (Campbell et al., 1996a). However, a different approach followed by Cibelli et al. implies that proliferating donor cells in the G1 phase may be a better alternative for SCNT (Cibelli et al., 1998a).

The rationale in choosing actively dividing cells rather than quiescent cells was two-fold: 1) proliferating cells are an indication of a relatively undifferentiated state and 2) actively dividing cells may be more compatible with the rapid rate of cell divisions that occur in early embryonic development (Cibelli et al., 1998a). Fetal fibroblast cells may be the optimal choice as nuclear donors because they proliferate rapidly in culture and have an
Figure 5.2. Transgenic clone No. 2007 and her two kids (48 h old) from natural mating.
inherently long G1 phase (Gadbois et al., 1992), thus abrogating the need to artificially arrest the cell cycle at G1 (chemically or by serum starvation). In addition, inducing a state of quiescence by serum deprivation in donor cells has been associated with altered patterns of DNA methylation (Dean et al., 1998; Jones et al., 2001) and gene and protein expression (Hayashida et al., 1997), which may help explain the low efficiencies reported in all NT programs. Moreover, findings in the rabbit indicate that chemically synchronizing donor cells to arrest in the G1 phase can result in fewer pregnancies following transfer of NT embryos (Collas et al., 1992a).

Although donor cells from an actively proliferating population of cells are capable of supporting development to term following NT in cattle (Cibelli et al., 1998a; b), the exact cell cycle stage (G1, S, G2 or M) of those donor nuclei was not determined. Similarly, we cannot conclude that the proliferating donor nuclei used in this NT study were all in the G1 phase, which may be a possible explanation for the lack of pregnancies from the proliferating cells in both Experiment 5.1 and Experiment 5.2.

It has been shown that the cell cycle stage of the donor nucleus at the time of fusion with an enucleated, nonactivated MII oocyte (cytoplast) has a pronounced effect on subsequent embryonic development (Smith et al., 1988; Collas et al., 1992a; Campbell et al., 1996b; Campbell, 1999). Diploid karyoplast nuclei (G0 and G1 cells) will condense to form single chromatids and maintain correct ploidy in the resulting embryo following karyoplast/cytoplast fusion (Collas and Robl, 1991). However, tetraploid nuclei (those in S and G2) will form double chromatids and lead to polyploidy and defective embryos (Collas et al., 1992b). Additionally, the process of chromosome condensation will cause extensive DNA damage to S-phase nuclei (those that are actively replicating their DNA) (Schwartz et
al., 1971) while not affecting the structure of $G_0$, $G_1$ or $G_2$ nuclei (Collas et al., 1992b; Cheong et al., 1993). Thus, NT using enucleated MII oocytes reconstructed with $G_0$ and $G_1$ nuclei results in normal diploid embryonic development, while transferring $S$ and $G_2$ nuclei results in damaged and/or tetraploid embryos.

In Experiment 5.1, the pregnancy rate (20%) for embryos reconstructed with quiescent transfected fibroblasts was similar to the pregnancy rates (21% and 17%) reported for quiescent nontransfected fibroblasts (Reggio et al., 2001; Keefer et al., 2002) but considerably lower than that reported for either transfected (50%) or nontransfected (50%) quiescent donor cells in another study (Keefer et al., 2001). NT embryos derived from proliferating donor cells in this study did not initiate a pregnancy, which may have been a result of choosing cells in the population that were in $S$ or $G_2$, leading to cell cycle asynchrony between donor cells and MII cytoplasts.

The failure to generate pregnancies in Experiment 5.2 from either quiescent or proliferating donor cells may have been a result of several factors, including low numbers of NT embryos transferred. Compared with Experiment 5.1, 30% fewer embryos reconstructed with quiescent donor cells and more than 50% fewer proliferating cell-derived embryos were transferred into recipients. Additionally, the cell line used in Experiment 5.2 may have been compromised, either from the transfection process or from the gene itself, which could have prevented successful reprogramming of the donor cell DNA (Zakhartchenko et al., 2001). It is worthwhile to note that no cloned offspring have been produced either at the LSU laboratory or at the Genzyme facility in Massachusetts when using donor cells transfected with this particular gene. Another plausible explanation is that these embryos were produced and transferred after the end of the breeding season (mid-March) for goats in the southern
United States, which may have led to luteal insufficiency (Gordon, 1997). However, more studies are required to test the validity of these assumptions.

Although the cloning efficiency (kids per embryos transferred) in this study with respect to the transfected T9 fibroblast cell line was similar (1.8%) to a previous report using nontransfected cells in this species (Reggio et al., 2001), it was lower than more recent studies using both transfected and nontransfected cells (Keefer et al., 2001; 2002). Nevertheless, it is within the range of average NT efficiencies (1–10%) reported for sheep, cattle and mice (Dominko et al., 1999).

In summary, this study has shown that G1 donor cells are just as effective as G0 cells in generating pre-implantation stage NT embryos in the goat. Further experiments are needed to determine whether G1 cells are superior to G0 cells for supporting development to term. Low efficiencies notwithstanding, NT will continue to be a viable method for producing transgenic animals capable of generating pharmaceutically important human recombinant proteins and antibodies.
CHAPTER VI

PRODUCTION OF TRANSGENIC GOATS AS A SOURCE FOR A MALARIA VACCINE

Introduction

Malaria, a tropical disease caused by single-celled parasites, afflicts an estimated 300 to 500 million people worldwide, causing 1 to 2 million deaths per year. According to recent World Health Organization (WHO) estimates, the majority of these casualties occurs among children under the age of 5 and pregnant women, and accounts for more deaths than any other infectious disease except tuberculosis (http://www.who.int/inf-fs/en/fact094.html).

Malaria is endemic in more than 90 countries and is transmitted by the anopheline mosquito, acting as a vector for the four species of Plasmodium protozoa that cause malaria in humans (P. falciparum, P. vivax, P. ovale and P. malariae). The most lethal species, P. falciparum, also accounts for the majority of the reported cases of this disease and is responsible for the drug resistant strains now appearing throughout Asia (http://www-micro.msb.le.ac.uk/).

The parasite enters the bloodstream from the bite of an infected mosquito as motile sporozoites, which then quickly arrive in the liver and invade hepatocytes. After a short incubation period in the liver cells, the sporozoites multiply and are transformed into merozoites. Merozoites burst from the infected liver cells and enter back into the bloodstream and infiltrate erythrocytes, initiating the blood stage of infection. The blood cells eventually lyse, releasing more merozoites that propagate the infection by invading other red blood cells (Krogstad, 1989). If not treated, infected individuals become severely anemic and die.
Mosquitoes that transmit the disease have become pesticide-resistant, and only a limited number of agents (chloroquine, mefloquine, quinine) are available to treat malaria. In many cases, drug toxicity and decreased efficacy precludes the use of these anti-malarials. Moreover, the most lethal strain of malaria (*P. falciparum*) has become resistant to the agent of choice (chloroquine) in many parts of Africa (WHO Fact Sheet No. 94; [http://www.who.int](http://www.who.int)).

Combating malaria has been a priority for the WHO since its founding in 1948. At the Amsterdam Summit of 1992, the Global Malaria Control Study was adopted which promised a deeper commitment to win the fight against malaria. In October of 1998, the WHO launched the “Roll Back Malaria” initiative, which has since spawned significant progress in developing a malaria vaccine (WHO Fact Sheet No. 203; [http://www.who.int](http://www.who.int)).

Vaccines to combat malaria fall into three categories: 1) anti-transmission vaccines designed to eliminate transmission of *Plasmodium* by arresting parasitic development in the mosquito, 2) anti-sporozoite vaccines designed to prevent infection following an encounter with an infected mosquito and 3) anti-blood stage vaccines designed to interfere with the merozoite stage of the malaria life-cycle.

The WHO has given priority to the anti-blood stage vaccines. Merozoite surface proteins (MSPs), associated with the merozoite stage of the malaria life-cycle, have shown considerable promise for a vaccine against the blood stage of *P. falciparum* (Tieqiao *et al.*, 1999). These surface proteins include a group of integral membrane proteins (Smythe *et al.*, 1988; Anders and Saul, 1993) and peripheral membrane proteins (Miller *et al.*, 1993; Marshall *et al.*, 1996; Tieqiao *et al.*, 1999) that have been shown to inhibit parasite growth or
illicit protection against malaria in exposed experimental animals (Hoffman et al., 1991; Anders and Saul, 1993).

Both the public and private sector, supported in part by the pharmaceutical industry, are currently involved in the development of over a dozen potential MSP vaccines. Genzyme Transgenics Corp., in collaboration with the National Institute of Allergy and Infectious Diseases (NIAID), is developing a recombinant MSP-based vaccine in transgenic animals (http://www.transgenics.com/). By modifying portions of the nucleotide sequence of the merozoite surface protein-1 gene (MSP-1) without disturbing the amino acid sequence, high-level expression (2–4 mg/ml) of the antigenic 42 kD fragment of MSP-1 (MSP-1$_{42}$) was achieved in the milk of transgenic mice. In a preclinical vaccine study conducted by the NIAID, the mouse-derived MSP-1$_{42}$ antigen successfully protected _Aotus nancymai_ monkeys against malaria (_P. falciparum_) (Stowers et al., 2002).

Generating transgenic animals capable of producing recombinant anti-malarial proteins in an active form and in sufficient quantities to manufacture vaccines is a major advantage over conventional _in vitro_ protein production systems (Clark, 1998; Meade et al., 1998). Bacterial systems cannot perform the proper post-translational modifications necessary to confer biological activity, and are unable to produce complex proteins. Cell culture systems require expensive bioreactors, huge quantities of expensive media and generate very low yields. However, transgenic dairy goats are ideal for producing recombinant proteins in high yield (Echelard et al., 2000) and may be an excellent choice for producing a potential malarial vaccine using the MSP-1$_{42}$ gene in a somatic cell nuclear transfer (NT) program.
Fibroblast cells have been used successfully as nuclear donors to generate cloned offspring in every mammalian species cloned to date, which includes sheep (Schnieke et al., 1997; Wilmut et al., 1997), cow (Cibelli et al., 1998a; Wells et al., 1998b; Zakhartchenko et al., 1999b), mouse (Wakayama and Yanagimachi, 1999), goat (Baguisi et al., 1999; Reggio et al., 2001; Keefer et al., 2002) and pig (Onishi et al., 2000). Moreover, cell-mediated transgenesis using fibroblast cells has been demonstrated in the sheep (Schnieke et al., 1997; McCreath et al., 2000), cow (Cibelli et al., 1998a) and goat (Keefer et al., 2001).

Using oocytes harvested from either FSH-stimulated or abattoir-derived caprine ovaries, the objective of this study was to generate transgenic founder goats by NT using fetal fibroblast cells transfected with the MSP-1<sub>42</sub> malaria gene. The development of a vaccine based on this gene product, as shown in the mouse model, would be expected to interrupt the blood stage of infection and afford protection against malaria. A much greater quantity of the purified protein can be recovered from the milk of transgenic goats compared with mice, and may therefore speed the development of a vaccine against <em>P. falciparum</em>. Such a vaccine would represent a significant advance in global public health, as this disease has become an epidemic in many regions of the world and efforts to control its spread are failing.

**Materials and Methods**

**Isolation of Donor Cells**

Fibroblast donor cells were established from a Day-35 female fetus by mechanical and enzymatic dispersion following removal of the head and internal organs (Baguisi et al., 1999; Reggio et al., 2001). Fetal tissues were finely minced, then exposed to 0.025 % trypsin/0.5 mM EDTA (porcine-derived; Sigma Chemical Co., St. Louis, MO) for 10 min at 38°C and 5% CO<sub>2</sub>. The minced preparation was washed in fetal cell medium (FCM) consisting of
tissue culture medium-199 (TCM-199; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah), nucleosides, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol (Sigma) and 50 µg/ml gentamicin sulfate (Gibco), then transferred into 25-cm² tissue culture flasks. Cells were maintained in FCM at 38°C in 5% CO₂ until 70% confluent, then trypsinized and frozen in liquid nitrogen prior to transfection with the DNA construct.

**Transgene Construction and Donor Cell Transfection**

The MSP-142 gene was inserted into the mammary specific B80 expression vector containing the caprine beta casein promoter, the 3’ untranslated region and flanking sequences. The vector also contained the neomycin selection PGKneo gene, flanked by the chicken beta globin insulator sequences. The transgene was introduced into the donor cells through lipid-mediated transgenesis using LipofectAMINE® (Gibco) according to protocols supplied by the manufacturer.

Transfected cells remained in culture for 72 h prior to challenge with 0.6 mg/ml geneticin (G-418 sulfate; Gibco). Several geneticin-resistant colonies were isolated, expanded (3–4 subpassages) in the presence of G-418 and cryopreserved in TCM-199 supplemented with 20% FBS, 5% DMSO and 0.6 mg/ml geneticin prior to analysis for expression of the MSP-142 gene.

Individual cell lines were assayed by the polymerase chain reaction to detect the presence of the transgene, then subjected to a Southern blotting procedure to determine the transgene copy number. Further characterization of the candidate cell lines by fluorescence in situ hybridization confirmed the integration sites. Two potential lines (T6 and T4) were subsequently used as NT donors within six subpassages.
**Oocyte Aspiration**

Oocytes were harvested and NT embryos were transferred from October to February during the 2000-2001 breeding season. All goats (oocyte donors and embryo recipients) used in this study were handled and maintained in accordance with the Institutional Animal Care and Use Committee Guidelines at Louisiana State University.

**FSH-stimulated ovaries.** An 8-day ovarian superstimulation protocol, beginning on Day 1 with a 3-mg norgestomet ear implant (s.c.; Synchro-Mate-B, Rhone Merieux, Athens, GA) was initiated on a group of mixed-breed Spanish-type goats. On Day 4 following the implant, oocyte donors were administered twice daily injections (i.m.) of FSH (Sioux Biochemical, Sioux Center, IA) in a decreasing dose schedule (10 and 10, 7.5 and 7.5, 5 and 5, and 2.5 and 2.5 units) totaling 50 units/doe (Graff et al., 1999). Implants were removed and oocytes were aspirated 24 h after the final FSH injection (Day 8). All donors were feed restricted for 36 h prior to a mid-ventral laparotomy procedure, induced into general anesthesia with an administration of atropine/ketamine mix (i.v.; 5 mg/kg body weight) and maintained via endotrachial intubation (Halothane; Fort Dodge Animal Health, Fort Dodge, IA).

Does were placed in dorsal recumbency to exteriorize the ovaries (Graff et al., 2000), and oocytes were aspirated into 50-ml centrifuge tubes containing warmed PBS supplemented with 1% FBS, 50 µg/ml gentamicin, and 0.4 units/ml sodium heparin (Elkins-Sinn, Cherry Hill, NJ). Only those oocytes with a compact investment of cumulus cells were selected and placed in groups of 10 to 20 in 35-µl microdrops of maturation medium (TCM-199 supplemented with 10% normal goat serum [Sigma], 10 µg/ml LH, 5 µg/ml FSH, and 1 µg/ml estradiol-17β) and allowed to mature for 18 to 22 h at 38°C and 5% CO₂.
Abattoir ovaries. Ovaries from mixed-breed goats were collected from an abattoir in San Angelo, TX. Oocytes were aspirated into 50-ml centrifuge tubes containing warmed aspiration medium (PBS supplemented with 1% FBS, 0.4 units/ml sodium heparin and 50 µg/ml gentamicin), washed extensively and placed in maturation medium (TCM-199 supplemented with 10% normal goat serum, 10 µg/ml LH, 5 µg/ml FSH and 1 µg/ml estradiol-17β). Oocytes with a compact investment of cumulus cells were transferred in groups of 50 into 1 ml cryovials (Corning, New York, NY) filled with warmed, equilibrated (38°C, 5% CO₂) maturation medium. Cryovials were shipped overnight via express air courier to the LSU Embryo Biotechnology Laboratory in portable shipping incubators maintained at 38°C.

Donor Cell Preparation

Prior to NT, frozen-thawed donor cells were maintained in modified TCM-199 (mTCM; TCM supplemented with 10% FBS) for 3 days followed by an additional 3 days of culture in reduced-serum (0.5%) TCM to induce quiescence. Donor cells (karyoplasts) were trypsinized, washed and suspended in mTCM immediately before transfer into enucleated MII oocytes.

Enucleation

Following an 18 to 22 h oocyte maturation interval, cumulus cells were removed by vortexing for 2.25 min in TL-Hepes (BioWhittaker, Walkersville, MD) containing 1 mg/ml hyaluronidase (Sigma). Denuded oocytes were washed in modified TL-Hepes (mTLH; TL-Hepes supplemented with 10% FBS) and selected based on the presence of a polar body (MII). Mature oocytes were labeled with 3 µg/ml Hoechst 33342 (Sigma) for 1 to 2 min, then enucleated in an elongated droplet (200 µl) of micromanipulation medium (mTLH with 6.5
µg/ml cytochalasin B) overlaid with warmed mineral oil. All manipulations were conducted on the unheated stage of an inverted microscope (Nikon Diaphot) equipped with Hoffman Modulation Contrast objectives, hydraulic and electric Narishige micromanipulators, and epifluorescent illumination.

After a brief exposure (<10 sec) to ultraviolet light to determine the location of chromosomes, the metaphase plate and first polar body were removed under short-term exposure to UV-light (<5 sec). Cytoplasts were allowed to recover in mTCM for at least 30 min but less than 1.5 h prior to reconstruction with donor cells.

Reconstruction, Fusion and Activation

A suspension of fibroblast cells was dispersed into a 200 µl elongated droplet of micromanipulation medium containing 20 to 25 enucleated oocytes (cytoplasts). A single, small (15- to 20-µm diameter) donor cell was inserted into the perivitelline space of each cytoplast and pushed firmly against the oolema. Couplets were equilibrated in room temperature fusion buffer (0.3 M mannitol, 0.1 mM MgSO₄·7H₂O, 0.05 mM CaCl₂, 0.5 mM Hepes, and 4 mg/ml BSA) for 3 to 5 min prior to fusion.

Couplets were then manually aligned between the electrodes of a 1-mm gap fusion chamber pre-filled with room temperature fusion buffer. A single DC pulse (1.30 kV/cm for 25 µsec) delivered by a BTX Electrocell Manipulator 200 (Gentronics, San Diego, CA) was applied to induce membrane fusion. Couplets were removed from the fusion chamber then washed and held in mTCM for 1 h prior to evaluation. Couplets that had not fused within 1 h were subjected to a second electrofusion pulse using the same parameters.

Fused couplets were activated by the method of Susko-Parrish et al. (Susko-Parrish et al., 1994) using a 4-min exposure to 5 µM ionomycin (Sigma), followed by a 3-h incubation
in 2 mM 6-dimethylaminopurine (Sigma) prepared in G1.2 medium (Zander IVF, Vero Beach, FL). Activated embryos were washed extensively and cultured in groups of 10 to 20 in 35-µl droplets of G1.2 medium for 33 to 36 h prior to transfer to recipient females.

**Embryo Transfer**

Using the same general anesthesia protocol used for oocyte donors, does that had exhibited a natural estrus on the same day of the scheduled NT procedure were used as embryo recipients. Thus, Day 1.5 embryos (Day 0 = day of fusion) were transferred into Day 1.5 recipients (Day 0 = estrus). Following a midventral laparatomy, the uterus was exteriorized and embryos were transferred into the oviduct ipsilateral to the most active ovary using a soft plastic catheter (Embryon Catheter; Rolon Medical, Watford, UK). Following a supervised recovery period, all recipients were returned to the herd.

**Pregnancy Status and Parturition**

On Day 30 of gestation, recipient does were examined by ultrasonography (Aloka 500-V; Aloka, Tokyo, Japan) to detect fetal heartbeats (Ayres *et al.*, 1999), followed 7 and 10 days later by additional assessments. Pregnant recipients were monitored at 14-day intervals up to Day 90, then separated from the herd and placed in groups of two females per pen. Offspring were delivered by scheduled cesarean section on Day 147 and provided with colostrum.

**Genotyping of Cloned Animals**

Tissue and blood samples from the fetuses and kids were subjected to genomic DNA isolation (Laird *et al.*, 1991). Samples were first assayed by polymerase chain reaction (PCR) using MSP-142 specific primers, followed by Southern blot analysis. Genomic DNA was digested with EcoRI (New England Biolabs, Beverly, MA) and electrophoresed on 0.7%
agarose gels. DNA fragments were immobilized on nylon membranes (MagnaGraph, MSI, Westboro, MA) by capillary action using standard procedures (Maniatis et al., 1982), then probed with a $^{32}$P dCTP label according to protocols supplied with the Prime-It® kit (Stratagene, La Jolla, CA). Following an overnight hybridization at 65°C (Church and Gilbert, 1984), the membranes were washed and exposed to X-OMAT™ AR film for 48 h.

Amplification of the second exon of the caprine major histocompatibility complex Class II DRB gene was performed exactly as described (Amills et al., 1996). Following digestion of the PCR product, restriction fragments were separated on a nondenaturing polyacrylamide precast gel (Stratagene) at room temperature in the presence of ethidium bromide.

Offspring were to be subjected to a hormone-induced lactation protocol specifically developed for prepubertal goats (Cammuso et al., 2000) and hand milked once daily for milk analysis to detect expression of the transgene product.

**Statistical Analysis**

The effect of ovary source on the rates of oocyte maturation, embryo reconstruction and embryo development was analyzed using a logistic regression procedure of SAS (SAS, 1992), where the odds-ratio estimates were compared. Differences where P<0.05 were regarded as statistically significant. Pregnancy and birth rates were compared using the Fisher’s exact two-tailed test (Metha and Patel, 1983), and differences where P<0.05 were considered significant.
Results

Oocyte Maturation and Embryo Reconstruction

The rates of oocyte maturation and embryo reconstruction are presented in Table 6.1. In ten replicates, a total of 1,055 oocytes were recovered from FSH-stimulated donor animals (range, 56 – 152 oocytes/replicate). A total of 10 replicates of follicular aspiration from the nonstimulated abattoir-derived ovaries produced a total of 1,784 oocytes (range, 112–230 oocytes/replicate). Significantly more (P<0.001) oocytes from abattoir-derived ovaries reached the MII stage (54%) compared with the maturation rate for oocytes aspirated from FSH-stimulated ovaries (47%). However, a higher proportion (P<0.001) of embryos were reconstructed from oocytes harvested from the FSH-stimulated ovaries compared with nonstimulated abattoir-derived ovaries (82% vs. 72%, respectively).

It should be noted that only grade-1 mature oocytes were enucleated and reconstructed. Those oocytes exhibiting a distinct polar body but uneven or granular cytoplasm, compromised membranes and/or abnormal chromatin configurations were marked as mature but not used in this study. Accordingly, 95% (450/500) of the mature oocytes aspirated from FSH-stimulated ovaries were suitable for enucleation, whereas significantly fewer (75%; 727/969) mature oocytes from abattoir-derived ovaries were acceptable as potential cytoplasts (P<0.001).

There was no difference between the two treatment groups for the fusion of karyoplasts with cytoplasts (P = 0.86). The rate of fusion of NT couplets reconstructed from oocytes harvested from either FSH-stimulated or abattoir-derived ovaries was 61% (range, 40%–72%) and 62% (range, 33%–70%), respectively.
Table 6.1. Oocyte maturation and embryo reconstruction using oocytes harvested from FSH-stimulated vs. nonstimulated abattoir-derived goat ovaries

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. oocytes</th>
<th>No. (% matured)</th>
<th>No. (%) reconstructed†</th>
<th>No. (%) fused‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH stimulated</td>
<td>1055</td>
<td>500 (47)a</td>
<td>411 (82)a</td>
<td>252 (61)</td>
</tr>
<tr>
<td>Abattoir derived</td>
<td>1784</td>
<td>969 (54)b</td>
<td>694 (72)b</td>
<td>428 (62)</td>
</tr>
</tbody>
</table>

†Based on the number matured. Only grade-1 mature oocytes were enucleated.
‡Based on the number reconstructed.

a,bNumbers within columns with different superscripts are different (P<0.05).
Embryo Development and Cloned Offspring

There was a significant effect of oocyte source on the number of fused couplets that developed into cleavage-stage embryos (P<0.0001, Table 6.2). Compared with embryonic development in the FSH-stimulated treatment, significantly more two- to four-cell stage embryos developed from fused couplets of abattoir-derived origin (46% vs. 82%, respectively). Of the 252 fused couplets reconstructed with oocytes harvested from FSH-stimulated ovaries, 116 developed into two- to four-cell stage embryos (range, 0%–70%). From a total of 428 fused couplets reconstructed with oocytes harvested from abattoir-derived ovaries, 351 developed into two- to four-cell stage embryos (range, 67%–100%).

Moreover, there was a significant effect of treatment on the number of cleaved embryos that were considered transferable (P<0.005). Only those embryos with equal size blastomeres and no fragmentation were transferred to recipient females. Although more embryos were produced in the abattoir-derived group compared with the FSH-stimulated treatment, significantly more NT embryos produced from FSH-stimulated ovaries were transferable compared with the number of transferable embryos produced from nonstimulated abattoir-derived ovaries (87% vs. 74%, respectively). More succinctly, only 13% of the embryos produced from FSH-stimulated oocytes exhibited fragmentation or uneven blastomeres, while 26% of the embryos produced from the abattoir-derived oocytes were considered poor quality and thus, not transferred.

There was no effect of oocyte source on pregnancy rate, NT efficiency or on the number of cloned offspring produced. Overall, an average of 11 embryos was transferred into each of 33 similar recipient does (range, 1–32 embryos/female) in this study. Four recipients were determined pregnant on Day 30 of gestation (Table 6.2). Three of those recipients each
Table 6.2. NT embryo development and offspring production from FSH-stimulated goat ovaries and nonstimulated abattoir-derived goat ovaries

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. (%) cleaved/fused</th>
<th>No. (%) transferred†</th>
<th>No. recipients</th>
<th>No. does pregnant, Day 30</th>
<th>No. offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH stimulated</td>
<td>116/252 (46)\textsuperscript{a}</td>
<td>101 (87)\textsuperscript{a}</td>
<td>12</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Abattoir derived</td>
<td>351/428 (82)\textsuperscript{b}</td>
<td>261 (74)\textsuperscript{b}</td>
<td>21</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{†}Only good-quality embryos transferred.
\textsuperscript{ab}Numbers within columns with different superscripts are different (P<0.05).
received five embryos produced from the fusion of FSH-stimulated oocytes with donor cells at passage 5. The remaining recipient received 19 embryos (produced from cell line T6 at passage 5) from the abattoir-derived group and carried a twin pregnancy until Day 127 of gestation, then spontaneously aborted both fetuses (Figure 6.1). It appeared that one of the twins was mummified and had died earlier in gestation (second trimester), eventually causing the death of the other morphologically normal twin.

Ultrasonographic evaluation revealed a resorbing fetus (produced from cell line T6) in one of the three recipients from the FSH-stimulated group, and was surgically removed on Day 58 of gestation (Figure 6.2). The remaining two recipients from this group maintained conceptuses throughout gestation and underwent elective cesarean section on Day 147 of gestation. Clone No. 2008 was delivered uneventfully, weighed 3.8 kg and is growing as expected (Figure 6.3). Clone No. 2009 (2.6 kg) was also delivered with no complications, and showed initial signs of vigor (bleating, rapid movement) upon extraction, but died within 30 min from cardiac failure (Figure 6.4). Clones No. 2008 and No. 2009 were both produced from cell line T4 at passage 4, and were phenotypically similar.

The pregnancy rate (defined as the number of pregnant recipients per total number of recipient animals) at 30 days of gestation was 25% (3/12) and 4.7% (1/21) for the FSH-stimulated group and the abattoir-derived group, respectively. NT efficiency (number of offspring per number of embryos transferred) in this study was less than 1%. One healthy kid was delivered from the FSH-stimulated group.

Skin biopsies from clones No. 2008, No. 2009 and from the three aborted fetuses screened positive for the presence of the MSP-142 transgene and verified that the cloned offspring were derived from the T4 cell line. In addition, milk samples from clone No. 2008
Figure 6.1. Aborted twin fetuses at 127 days of gestation, produced from oocytes harvested from nonstimulated abattoir-derived ovaries.
Figure 6.2. Resorbing fetus from the FSH-stimulated ovary group removed at 57 days of gestation.
Fig. 6.3. Cloned transgenic goat, No. 2008, derived from the FSH-stimulated ovary group, expressing the malaria antigen MSP-142.
Fig 6.4. Clone No. 2009, derived from oocytes aspirated from FSH-stimulated ovaries, 30 min post delivery. Cause of death was diagnosed as cardiac failure.
were positive for the presence of the MSP-1_{42} malarial antigens. Studies to determine the expression levels from the milk of this animal are ongoing.

**Discussion**

In this study, caprine donor cells transfected with the MSP-1_{42} gene were used to produce a healthy cloned goat expressing the anti-malarial protein in her milk. MSP-1_{42} produced in the milk of transgenic mice has afforded protection to primates against a lethal challenge with *P. falciparum*, and although expression levels in the milk of these transgenic mice exceeds 2 mg/ml, total milk volume is extremely low. The ability to express the malaria antigen in high yield in the milk of transgenic dairy goats constitutes a tremendous advantage in developing a malaria vaccine.

Fetal wastage during the first trimester of gestation accounts for up to 50% of the loss observed in sheep and cattle NT studies (Campbell *et al.*, 1996a; Schnieke *et al.*, 1997; Wells *et al.*, 1997; Wilmut *et al.*, 1997; Hill *et al.*, 1999a; 2000b; Zakhartchenko *et al.*, 1999b). However, such losses have not been reported for the goat (Baguisi *et al.*, 1999; Keefer *et al.*, 2001; 2002; Reggio *et al.*, 2001). Compared with sheep or cow NT programs in which blastocyst stage embryos are transferred, cloned goat embryos are transferred at the two- to four-cell stage and therefore spend very little time in *in vitro* culture. Large offspring syndrome in the sheep and cow has been associated with prolonged *in vitro* culture in the presence of serum (Walker *et al.*, 1996; Young *et al.*, 1998; Van Wagtendonk-De Leeuw *et al.*, 2000), and perhaps it is this difference that accounts for the high rate of prenatal and neonatal loss in these species compared with the goat.

In contrast, 60% of the pregnancies in this study were lost prior to parturition (one at Day 58 and a set of twins at Day 127 of gestation). This is the first report of fetal wastage
occurring in cloned goats, and may become important to future somatic cell nuclear transfer studies in this species. A possible explanation for the loss at Day 58 was a lack of complete reprogramming of the donor cell (Sun and Moor, 1995; Campbell, 1999). Donor cells used to create that embryo were used immediately post-thaw and had not been serum-deprived, and therefore may have been at an inappropriate stage of the cell cycle to insure compatibility with the MII oocyte.

The overall pregnancy rate in this study (12%) was similar to the 22% pregnancy rate previously reported from this laboratory (Reggio et al., 2001), but significantly lower than the 36% to 38% pregnancy rates recently reported (Keefer et al., 2001; 2002). Although the overall kidding rate (number of offspring per number of pregnant recipients) in this study was significantly lower (50%) than in any other goat NT study (100%), it was similar to a recent study involving the transfer of IVM/IVF/IVC caprine embryos in which 62% of the pregnant recipients maintained pregnancies to term (Han et al., 2001).

Additionally, one of the two offspring died from cardiac failure shortly after delivery, consistent with other goat NT programs reporting neonatal losses as high as 50% (Keefer et al., 2001). Similar neonatal and postnatal losses have also been reported in sheep (Schnieke et al., 1997; Wells et al., 1998a) and cattle (Kato et al., 1998; Renard et al., 1999) and have been associated with poor placentation, metabolic disorders and cardiopulmonary abnormalities (Cibelli et al., 1998a; Hill et al., 1999a; 2000a; b; Renard et al., 1999; Wells et al., 1999b; Zakhartchenko et al., 1999b).

Results from a study in pigs show that different clonal cell lines from the same fetus results in significantly different development of NT embryos (Kuhholzer et al., 2001). A larger study in cows also suggest that different cell lines from the same animal can influence
the rate of development, pregnancy and number of offspring produced when used for NT (Kato et al., 1998). Although there were too few numbers to determine what significance the cell line had in determining pregnancy, it is interesting to note that only T4 cells produced a viable kid in this study. The resorbed and aborted fetuses were derived from the T6 cell line. More studies are needed to elucidate the effect of donor cell source and treatment on the development of NT embryos.

In sheep, it has been reported that oocytes harvested from abattoir ovaries tended to produce better quality IVF-derived embryos compared with oocytes aspirated from FSH-stimulated live donor animals (Smith, 1994). While the source of oocytes used in this study did not affect the number of pregnancies or offspring produced, there was a difference in oocyte quality between FSH-stimulated ovaries and nonstimulated abattoir-derived ovaries. A higher percentage of oocytes matured from the pool of oocytes harvested from abattoir ovaries compared with FSH-stimulated ovaries (54% vs. 47%, respectively), but better quality mature oocytes (based on cytoplasmic morphology, membrane integrity and nuclear configuration) were observed in the FSH-stimulated treatment compared with the nonstimulated group (82% vs. 72%, respectively). Similarly, although a significantly greater percentage of NT embryos were produced in the nonstimulated group (82%) compared with the FSH-stimulated treatment (46%), better quality embryos were observed in the FSH-stimulated treatment. Less fragmentation and uneven blastomeres were noted in embryos reconstructed from FSH-stimulated oocytes, and was reflected in the percentage of NT embryos transferred into recipient females.

In summary, we have shown that healthy offspring can be generated from a cell line transfected with an antimalarial antigen. Although the efficiency of the NT procedure was
low, somatic cell nuclear transfer will continue to be a viable option for producing cloned transgenic animals having the potential to manufacture pharmaceutically important proteins in their milk.
CHAPTER VII
SUMMARY AND CONCLUSIONS

Cloning a sheep by somatic cell nuclear transfer has definitively answered the question of totipotency of a terminally differentiated cell. Since the reported birth of Dolly produced from the mammary epithelium of an adult ewe in 1997 (Wilmut et al., 1997), six other species have been similarly cloned from adult donor cells. The application of this new assisted reproductive technology to animal reproduction promises to impact livestock breeding practices by producing exact duplicates of genetically and economically important adult animals (Wilmut et al., 2000). Rare, exotic and endangered species can also benefit from this technology, as witnessed by the birth of Noah (an endangered gaur) (Lanza et al., 2000c) and the last remaining member of the Enderby Island cattle breed (Wells et al., 1998b).

In addition, cloning by NT may have a major impact on the treatment of human diseases by generating transgenic animals capable of producing in their milk valuable pharmaceuticals (Ziomek, 1998; Echelard et al., 2000). Several companies have produced herds of cloned transgenic dairy animals expressing a variety of proteins, including alpha-1-antitrypsin for the treatment of emphysema (Polejaeva and Campbell, 2000), human clotting factor IX for managing hemophiliac patients (Schnieke et al., 1997), and antithrombin III to supplement individuals that are deficient in this anticoagulant (Baguisi et al., 1999).

Dairy goats are an ideal bioreactor for the production of these complex therapeutic proteins. Increasing the overall efficiency of the cloning process in goats may lead to cost-effective strategies to generate these pharmaceuticals. Previous reports in the goat have relied on harvesting oocytes from FSH-stimulated animals maintained in a donor herd, and have
incurred the additional cost of the superstimulation protocol as well as the care and feeding of the donor animals (Baguisi et al., 1999; Keefer et al., 2001; 2002).

An important outcome of this study was the production of cloned goats by the transfer of donor cells into enucleated oocytes aspirated from abattoir ovaries. Although abattoir-derived oocytes have been used successfully for NT in other species, this is the first such report in goats. There was no difference in the number of cloned offspring produced from oocytes harvested either from FSH-stimulated donor animals or from abattoir-derived ovaries (3 and 2, respectively). It is expected that abattoir oocytes would be easier to obtain and more cost effective than maintaining and stimulating a herd of oocyte donor goats.

The ability to transfect a cell line prior to NT has made it possible to generate transgenic animals more efficiently than pronuclear injection. Prior to somatic cell NT, pronuclear injection was the only consistent method of introducing foreign genes into the genome of livestock (Hammer et al., 1985; Niemann and Kues, 2000). However, this method suffers from severe limitations including random integration and variable expression of the transgene (Wilmut and Clark, 1991; Pursel and Rexroad, 1993; Wall, 1996). In addition, less than 5% of the microinjected pronuclear-stage oocytes develop into transgenic offspring and a considerable proportion of those that are born are mosaic (Pursel et al., 1989; Rusconi, 1990). Therefore, the generation of several transgenic founder lines would be required to produce the desired phenotype linked to germ line transmission (Polejaeva and Campbell, 2000). Producing transgenic animals using transfected somatic cells coupled with NT would circumvent the limitations of PN injection and accelerate the establishment of a transgenic herd, since 100% of the resulting offspring would be transgenic (Niemann and Kues, 2000).
In this study, two transgenic goats were produced from a transfected donor cell line via somatic cell NT. As expected, both offspring tested positive for the presence of the transgene and have expressed the gene product in milk samples obtained from both an induced lactation and following the birth of healthy kids produced from a natural mating.

Because of conflicting reports in the literature concerning the optimum cell cycle stage of the nuclear donor prior to NT (Wilmut et al., 1997; Cibelli et al., 1998a), this study also compared the use of transfected cells in the proliferative (G1) or quiescent (G0) state. It has been proposed that to allow complete reprogramming of the donor cell by the recipient ooplasm, a state of nuclear quiescence must be induced in the donor cell prior to NT (Wilmut et al., 1997). However, actively dividing cells have also been used to generate transgenic calves (Cibelli et al., 1998a). Although the use of transfected donor cells in G1 of the cell cycle were just as effective as G0 cells in contributing to early embryonic development in this study, no development to term was observed in those embryos generated from G1 donor cells.

A better understanding of the relationship between cell cycle synchronization and subsequent reprogramming of the donor cell is required to further improve upon the NT technique, as it has been shown that inducing G0 prior to NT by serum starvation can lead to DNA damage (Kues et al., 2000), alterations in gene and protein expression (Hayashida et al., 1997), or unintentional modifications of DNA methylation patterns (Dean et al., 1998; Jones et al., 2001).

By directing the production of recombinant human proteins to the milk of transgenic animals, many of the problems inherent in bacterial or mammalian cell culture systems can be overcome (Clark, 1998; Meade et al., 1998). For example, cultured bacteria cannot properly fold complex proteins and lack adequate post-translational modifications required to confer
biological activity to these pharmaceutical proteins, whereas mammalian cell culture bioreactors require a huge initial investment, consume large quantities of expensive media, and generate low yields (Echelard et al., 2000).

Transgenic animals have the capacity to express complex biotherapeutics in the appropriate conformation and at the high yields required for human clinical applications. A recombinant vaccine against malaria has recently been developed in transgenic mice (Stowers et al., 2002). This vaccine is based on a protein (MSP-1) located on the surface of the malaria parasite that prompts the immune system to attack the merozoite stage of the life cycle. Although the expression level of the malaria antigen was adequate (0.9 g/liter) for a trial using monkeys lethally challenged with malaria (Stowers et al., 2002), the total milk volume was too low to consider using this model for manufacturing a malaria vaccine in mass quantities. However, developing a malaria vaccine in transgenic goats using the same MSP-1 transgene described in the mouse model could enable the large-scale manufacturing of vaccines against malaria. Dairy goats can produce 600 to 800 liters of milk per lactation per doe, and are capable of expressing between 1 to 10 grams of therapeutic protein per liter of milk (Rudolf, 1999; Echelard et al., 2000). Based on the expression levels obtained in the mouse, a herd of three transgenic goats could supply enough antigen to vaccinate 20 million people annually (Stowers et al., 2002).

Therefore, in the final experiment in this study, caprine donor cells were transfected with the MSP-1 gene and used as nuclear donors to create cloned transgenic goats expressing the MSP-1 protein. To determine the effect of oocyte source on subsequent development of reconstructed embryos, a comparison was made between oocytes obtained from either FSH-stimulated donor animals or from abattoir-derived ovaries. One healthy offspring was born
from the FSH-stimulated treatment, and has tested positive for the presence of the MSP-1 transgene. Although the efficiency of the NT procedure was low in this study, this system may be useful in generating potential malaria vaccines in the milk of cloned goats.

In summary, eight cloned transgenic goats were produced in this study from the transfer of both nontransfected and transfected donor cells into oocytes that were harvested either from a herd of donor animals or from an abattoir. This study demonstrated that abattoir-derived caprine oocytes were capable of supporting embryonic development to term, resulting in the birth of cloned goats. Moreover, the production of cloned transgenic goats from a transfected cell line has confirmed the ability to use this technology to introduce foreign genes into the caprine genome. Incorporating genes that direct the production of valuable pharmaceutical proteins in the milk of these cloned goats has become an attractive option for producing a high volume of therapeutic products at a relatively low cost.
LITERATURE CITED


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APPENDIX

LETTER OF PERMISSION

April 9, 2002

Dear Brett C. Reggio:

You have requested permission to use the following article in your dissertation:


"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,"

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Brett C. Reggio was born in Lakehurst, New Jersey, on June 24, 1964, and raised in Metairie, Louisiana, by his parents Ron and Mary Reggio. Upon graduation from Archbishop Rummel High School in Metairie, Louisiana, he attended Tulane University in New Orleans and received his Bachelor of Science degree in biology in 1992.

Following a two-year employment at Tulane University School of Medicine, he enrolled at Louisiana State University in Baton Rouge to pursue a graduate degree in animal science. Under the direction of Professor Robert Godke, Brett earned a Master of Science degree in reproductive physiology in the summer of 1997, then immediately enrolled in the doctoral program. During the course of his studies, he accepted an internship at Women and Infants’ Hospital of Rhode Island and Brown University to learn nuclear transfer procedures and applied them to his current research. He is now a candidate for the degree of Doctor of Philosophy in reproductive physiology in the Department of Animal Science at Louisiana State University, Baton Rouge, Louisiana, under the supervision of Professor Godke.