GENE INJECTION IN THE BOVINE: EFFECT OF TIME OF MICROINJECTION AND NUCLEAR TRANSFER TECHNOLOGIES

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ABSTRACT

Four experiments were conducted to investigate methods of producing transgenic bovine embryos entirely in vitro. Experiment 1 examined the effect of DNA microinjection at 11, 15 and 19 h after fertilization (haf) on survival rate and DNA detection frequency by polymerase chain reaction (PCR). There was no difference in transgene detection frequency between treatments (53% at 11; 50% at 15; 48% at 19 haf). Of all injected embryos developing to the morula or blastocyst stage after 7 d in culture, 89% tested positive for the presence of the transgene by PCR. Greater developmental efficiencies can be obtained when injection is performed early in pronuclear formation (7% (11/161) at 11; 4% (6/159) at 15; 1% (1/165) at 19 haf; $p<0.05$). Experiment 2 examined the effect of microinjection of DNA into the germinal vesicle (gv) of bovine oocytes on subsequent development and detection of the transgene. Injection of the transgene into the gv reduced developmental rates compared to controls (control=23% (89/384); non-injected=9% (23/250); GV injected=5% (12/259); $p<0.05$). Transgene detection frequency was 64% (37/58). Injection of bovine oocytes before

fertilization results in viable embryos containing the transgene, although at low frequencies. Experiment 3 was designed to examine whether the frequency of microinjected DNA detection by PCR in whole bovine embryos would decline over a 21 d culture period. At d 0, the transgene was detected in 100% (46/46) of embryos analyzed. At d 7, detection frequency was 84% (51/62) in viable embryos, at d 14 49% (18/37), and at d 21 38% (3/8). DNA detection frequency in microinjected bovine embryos by PCR analysis does not give a reliable indication of live transgenic birth rates until after 14 d in culture. Experiment 4 examined microinjected bovine embryos for their potential use as donor embryos in nuclear transfer, or cloning. There was no difference in development between embryos cloned from microinjected donor embryos and those from control donor embryos (injected=11% (37/377); control=9% (7/81); $p>0.05$). Of the embryos developing from microinjected donors, 32% (12/37) were PCR positive. Microinjected embryos can be successfully used in a nuclear transfer program to produce more viable embryos, and the resulting embryos may be more reliably screened by PCR. The efficiency of producing viable bovine embryos positive for the injected gene may be increased by performing microinjection early in pronuclear formation, and entering the resulting embryos into a nuclear transfer program.

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CHAPTER I.
INTRODUCTION

The production of transgenic livestock has the potential for creating a major impact on the scientific, medical, and agricultural industries. Genetic engineering of livestock will undoubtedly assist scientists in unraveling the mysteries of gene regulation, and the processes by which genes are able to precisely direct vital functions such as growth and reproduction, as well as disease. Transgenic animals may also be utilized as living bioreactors in the production of a large and inexpensive supply of pharmaceutical proteins important in treating human disease. Agricultural applications include the production of animals with improved feed efficiency, disease resistance, more desirable carcass characteristics, and milk with superior qualities for production of dairy products.

Before these goals can be realized, however, many limitations remain to be overcome. Microinjection, which continues to be the method of choice for producing transgenic animals, requires a large number of fertilized zygotes, pronuclear visualization, often embryo culture, and embryo transfer to a recipient animal. These procedures have proven to be both more difficult and more costly in domestic than in laboratory animals. A major problem, and source of expense, is the limited number and difficult recovery of recently fertilized one cell embryos, even from superovulated donor animals. Once recovered, centrifugation of zygotes from domestic animals is required before the pronucleus can be visualized because of the

opacity of the cytoplasm. Obtaining and maintaining donor and recipient animals is complicated by the cost and availability of facilities. Yet another difficulty is the low efficiency of current in vitro culture systems for early embryos of domestic animal species. Lastly, the long gestation period of most domestic species hampers progress in the production of any significant number of animals created from gene injection.

New biotechnologies are creating greater opportunities for increased production of transgenic animals. In vitro maturation and in vitro fertilization techniques yield many times more embryos for microinjection procedures. Ultrasound-guided follicular aspiration will allow the continuous collection of oocytes from genetically superior cows for use in transgenic programs. Intensive research on the metabolic requirements of domestic animal embryos and in vitro co-culture systems constitutes significant progress toward inclusion of in vitro culture systems in transgenic animal production strategies. In vitro culture to the morula or blastocyst stage creates the opportunity to test the embryo for both sex and the presence of the transgene with the polymerase chain reaction technique. Thus, only embryos that contain the inserted gene and are of the appropriate sex for the intended purpose of the program are transferred. This minimizes the need for purchasing, housing, and maintaining recipient animals. Continuing rapid progress in these areas will insure the realization of the many promises of transgenic animal biotechnology.

The objectives of these experiments were to 1) investigate the effect of DNA microinjection at various times after in vitro fertilization on DNA detection and survival rates of bovine embryos, 2) determine the effect of microinjection of DNA into the germinal vesicle of bovine oocytes on subsequent development and detection of the exogenous DNA, 3) test whether the frequency of DNA detection by polymerase chain reaction would decline over a 21 d culture period in whole bovine embryos, and 4) examine the potential of microinjected bovine embryos for use as donors in the nuclear transfer procedure.

CHAPTER II.

LITERATURE REVIEW

The following is a review of published research in the areas of bovine in vitro maturation, in vitro fertilization and in vitro culture, ultrasound guided transvaginal oocyte aspiration, gene transfer, polymerase chain reaction, bovine embryo transfer, and nuclear transfer, or cloning.

In Vitro Maturation of Bovine Oocytes

In vitro maturation (IVM) of bovine oocytes represents a method by which the number of one cell embryos for use in transgenic research may be greatly increased. For many years, early embryos could be recovered only by surgical laparotomy. This procedure results in a limited quantity of embryos of various cell stages, due to the unpredictable results of superovulation and the number of cows available to the researcher. Cost of the superovulatory drugs and surgical procedure, as well as housing facilities are also limiting factors. In vitro maturation allows the research scientist to collect many immature oocytes from a very inexpensive source-abattoir derived ovaries. These oocytes are then fertilized in vitro, and can be utilize at the desired cell stage. This section of the review will discuss the progress which has been made toward the successful maturation of bovine oocytes in vitro.

The oocytes of mammalian species are enclosed within a shell of granulosa cells specifically known as the cumulus oophorus. The entire structure is known as the cumulus oocyte complex, or COC. Cytoplasmic processes extend from the cumulus cells, through the zona pellucida, and contact the oolemma, the membrane surrounding the oocyte (Moor et al.,1980). At these contact points gap junctions exist between the follicle cell and the oocyte (Anderson and Albertini, 1976). Gap junctions provide the means for passage of small molecules from one cell to another. Molecules passed from cumulus cell to oocyte probably play a role in the nutritional support of the oocyte and regulation of maturation (Moor et al., 1980; Herlands and Schultz, 1984).

There are several methods by which the COC may be recovered from the post-slaughter bovine ovary. Follicular fluid may be aspirated from antral follicles using a needle and either a syringe or vacuum pump, or follicles may be dissected from the ovary and ruptured in a petri dish containing medium (Katska, 1984). Oocytes may also be recovered by slicing the ovary in a criss-cross pattern and rinsing with medium. More oocytes per ovary are recovered by slicing the ovary than aspirating the follicles (Hamano and Kuwayama, 1993). After the COC's are collected, they are commonly evaluated and classified before being placed into maturation medium.

One early classification scheme involved grouping oocytes according to the presence or absence of cumulus cells. Maturation

rates were better in oocytes with dense cumulus investments (97%) than in partially nude (90%) or totally nude (53%) oocytes (Shioya et al., 1988). Oocytes have also been classified by light microscope inspection. Oocytes were classified into four categories according to organelle distribution, penetration of the cortex of the oocyte by cumulus cell process endings, and degree of expansion of cumulus cells (de Loos et al., 1989). In this classification scheme, all oocytes displayed equal developmental capacity after in vitro maturation and fertilization excluding those which had clustered organelles, no cumulus process endings which penetrated the cortex, an expanded cumulus layer, and an overall irregular and dark appearance of the total COC (de Loos et al., 1989). The loss of metabolic coupling between cumulus cells and the oocyte before maturation in this group of oocytes probably accounts for their reduced developmental capacity. A more recent study has classified oocytes on a scale from one to nine based on number of layers and compactness of cumulus cell investment, homogeneity of the cytoplasm, and size of the oocyte (Hazeleger and Stubbings, 1992). Results of this study demonstrated that morphology of immature COC's has a direct effect on their ability to mature, fertilize, and develop in an in vitro system.

Once oocytes have been placed in culture, nuclear and cytoplasmic maturation occur spontaneously. During maturation, the oocytes undergo many changes which will later allow fertilization and cleavage to take place. Nuclear maturation encompasses the continuation of meiosis from the dictyate stage of prophase I until the oocyte re-arrests at metaphase II. Cumulus oocyte complexes

collected in prophase I contain a large germinal vesicle (GV). Once in culture the nuclear membrane dissolves after about six hours, a process known as germinal vesicle breakdown (GVBD; Motlik et al., 1978). After GVBD, the chromosomes progress through condensed chromatin (CC) at 8 hours post maturation (hpm), metaphase I (MI) at 10 hpm, anaphase I (AI) at 15 hpm, telophase I (TI) at 16 hpm, and reach metaphase II (MII) at 18 to 24 hpm (Suss et al., 1988;12). This sequence of nuclear maturation occurs more rapidly in vitro than in vivo (King et al., 1986; Suss et al., 1988).

Structural changes in the cytoplasm of maturing bovine oocytes have been well studied. Kruip et al. (1983) classified preovulatory oocytes into four successive groups. During the GV stage, mitochondria were located in a peripheral position, with rough endoplasmic reticulum (RER) and membrane bound vesicles just exterior to the mitochondria. During GVBD, the RER disappears and mitochondria form clustered associations with lipid droplets and smooth endoplasmic reticulum (SER). As the chromosomes move toward MII mitochondrial clustering continues, along with vesicle fusion and formation of ribosomes. At MII, the mitochondria disperse and most organelles move toward the center of the cell. Hytel et al. (1986) also observed movement of the mitochondria from peripheral positions to form aggregates with the SER. Transmission electron microscopy reveals that ER elements are actually formed from disrupted nuclear envelope remains. In contrast to these studies, Van Blerkholm (1990) suggests that there are five distinct cellular phenotypes in GV stage bovine oocytes, and that the cellular

organization of mitochondria, lipid droplets and vesicles remain unchanged during maturation. The cytoplasmic phenotype at the GV stage proved to be the most critical element in predicting the ability of an oocyte to successfully mature, be fertilized, and develop. These authors suggest that the cytoplasmic organization of the oocyte is actually pleiomorphic, and that different cytoplasmic organizations may reflect cellular changes due to atresia .

Resumption and completion of meiosis, along with cytoplasmic maturation, is critical for fertilization and subsequent development of the oocyte. Several factors during maturation in vitro may affect developmental capacity. These include hormonal environment, protein supplementation, follicle size from which the oocyte was recovered, temperature, and gas atmosphere.

Hormonal requirements for acquisition of developmental competence during oocyte maturation have been studied by several investigators, but no firm conclusions have been reached. Several studies have shown no positive effect of estradiol (E2), follicle stimulating hormone (FSH), and/or luteinizing hormone (LH) on oocyte maturation, fertilization, and development (Fukui et al., 1982; Leibfried-Rutledge et al., 1986; Sirard et al., 1988; Fukui and Ono, 1989; Sanbuissho and Threlfall, 1990; Olson et al., 1991). These studies, however, included either fetal calf serum (FCS) or estrus cow serum (ECS), which often contain varying amounts of gonadotropins and steroids. Fetal calf serum has been shown to be necessary for FSH induced cumulus expansion and completion of the first meiotic

division (Suss et al., 1990). Other reports indicate contrary effects of hormone addition on oocyte maturation and fertilization. Both FSH and LH increased cumulus expansion of cumulus oocyte complexes, and addition of either E2 alone, or human chorionic gonadotropin (hCG), FSH, LH, or FSH and LH in combination with estrogen, is reported to improve the proportion of oocytes completing maturation and developing successfully (Fukui et al., 1982; Hensleigh and Hunter, 1985; Stubbings et al., 1988; Younis et al., 1989; Stubbings et al., 1990; ,29a). Maturation conditions excluding E2, with FSH, LH, or FSH and LH are also reported to increase the percentage of oocytes that fertilize and develop to the blastocyst stage (Brackett et al., 1989; Saeki et al., 1990; Zuelke and Brackett, 1990; Rose and Bavister, 1992). The increased viability of oocytes matured with LH or FSH and LH has been confirmed using serum free, defined culture conditions (Brantmeier et al., 1987; Wise, 1987). Oocytes matured with LH exhibit increased glutamine metabolism, possibly enhancing oocyte quality (Zuelke and Brackett, 1993). These experiments show that bovine oocytes can develop successfully in serum free conditions, and provides the opportunity for further isolation of important factors during maturation which influence developmental capacity.

Because the majority of oocytes harvested from ovaries obtained at the abattoir are derived from follicles less than 6 mm in diameter (Personal communication, F. Barnes), it is important to know if large and small follicles react in the same manner when confronted with an in vitro maturation system and if different

factors are required to recruit a maximum number of competent oocytes from each population. Early studies reported that follicle size did not influence the ability of bovine oocytes to undergo meiotic maturation (Bae and Foote, 1975; Moor and Trounson, 1977; King et al., 1986), even though oocyte characteristics and follicular fluid components differ between follicle sizes (Edwards, 1965; Bae and Foote, 1975; Tsuji et al, 1985; Van Blerkom et al., 1990). In other species, it has been reported that oocytes recovered from large follicles completed meiosis and developed to the morula and blastocyst stage with greater efficiency than small follicle derived oocytes (Leibfried and First, 1979; Lenz et al., 1983; Katska and Smorag, 1985). Oocytes from small follicles may not yet have attained meiotic competence, and therefore are unable to respond to hormonal stimuli in an in vitro maturation system. As the follicle grows, oocytes attain the abilities to undergo maturation, fertilization, and development. Development of meiotic competence and completion of oocyte growth occur at various follicular stages among species. In the bovine, oocytes from 2-3 mm follicles are able to complete meiotic maturation even though the oocyte is not fully grown at this time. Recent studies have shown that bovine oocytes obtained from follicles greater than 6 mm in diameter produced a higher proportion of morula and blastocyst stage embryos than oocytes from 2 to 6 mm follicles (Lonergan, 1992). Embryos from medium and large follicles also exhibit similar nuclear function to in vivo derived embryos, while small follicle embryos do not (Pavlok, et al., 1993). This suggests that follicle size has a direct effect on oocyte viability.

Gas composition and temperature during in vitro maturation of bovine oocytes are two other critical factors. Although no study has been done strictly examining gas composition, medium for bovine oocyte maturation has been buffered with bicarbonate against 5% CO_2 in air from the inception of this technique (Edwards, 1965). Oocytes can mature in pH's ranging from 6.70 to 7.59, but a pH of 7.0 to 7.3 resulted in the highest maturation rates (Shea et al., 1976). The optimum temperature for bovine oocyte maturation was examined by Katska and Smorag (1985). They concluded that more oocytes reached metaphase II at 37°C to 39°C than at 33°C or 35°C. However, on the basis of a fluorescein diacetate viability test, oocytes matured at 35°C were more viable than those at 39°C. This test was brought into question for its value in determining the viability of bovine oocytes, as it had never been used for this purpose before. Another study demonstrated that oocytes matured at 39°C had the highest fertilization rates, suggesting that there is no problem with the quality of the oocyte cytoplasm at this temperature (Lenz et al., 1983a). At the present time, the majority of investigators mature bovine oocytes at 39°C, although 37°C is sometimes used as well.

It became clear in early attempts to mature bovine oocytes in vitro that a cumulus investment was critical for success (Leibfried and First, 1979), as discussed earlier in this review. When oocytes are recovered from ovaries, however, many are partially or totally nude. The challenge then becomes maturing these oocytes with a high degree of success rather than discarding them, thus increasing

the oocyte pool. Addition of granulosa cells during maturation of COC's improved development after fertilization (Critser et al., 1986; Fassi-Fihri et al, 1991). Cumulus cells around the oocyte during fertilization also play an important role in further development (Fukui, 1990).

Recent attempts to mature denuded oocytes with different concentrations of granulosa cells have met with limited success (Mochizuki et al., 1991). Fertilization rates were significantly lower, possibly due to hardening of the zona pellucida and incomplete cytoplasmic maturation caused by a lack of cumulus cell contacts. Susko-Parrish et al. (1992) alternatively suggest that reduced viability of denuded oocytes may be caused by the deletion of necessary metabolites which may normally be mediated by cumulus cells. Supporting this hypothesis, they found that pyruvate and glucose are necessary for meiotic maturation in denuded but not in cumulus intact oocytes.

In the bovine, RNA and protein synthesis by immature oocytes are thought to be required for the acquisition of meiotic competence. Oocytes from follicles less than 3 mm in diameter actively transcribe RNA, but as the follicle grows in size the nucleoli begin to compact and transcriptional activity decreases (Crozet et al., 1986). This RNA may be stored for use during maturation, fertilization, and early embryonic development. As the oocyte approaches GVBD, low levels of RNA continue to be synthesized. These new transcripts, produced as the oocyte resumes meiosis, are essential for further meiotic

progression (Hunter and Moor, 1987; Motlik, 1989; Kastrop et al., 1991). Protein synthesis is also required for bovine oocytes to resume meiosis (Hunter and Moor, 1987; Motlik, 1989; Kastrop et al., 1991). More specifically, protein synthesis is required at four distinct stages during oocyte maturation; to undergo GVBD, to progress into and out of MI, and to maintain the oocyte in MII until fertilization occurs (Sirard et al., 1989). It is unclear, however, whether synthesis of these regulator proteins is dependent upon transcription immediately prior to GVBD. After GVBD, protein synthesis patterns change in both in vivo and in vitro matured oocytes (Kastrop et al., 1988), and synthesis of new proteins declines gradually until the 8-cell stage (Frei et al., 1989).

In addition to protein synthesis, post-translational modification of pre-existing proteins also may play an important role in the regulation of meiosis. Extensive phosphorylation of some proteins has been observed in the bovine from 3 hr after the onset of maturation, before GVBD (Kastrop et al., 1990). These specific phosphoproteins may be essential for GVBD, specifically for the activation of the putative maturation promoting factor (MPF), or as MPF substrates (Kastrop et al., 1990; Kastrop et al., 1991a). Recent studies have shown that these phosphoproteins and/or the proteins involved in their phosphorylation are synthesized during the first 2 hr of in vitro maturation (Kastrop et al., 1991b). A puromycin analog that inhibits phosphorylation, 6-dimethylaminopurine (DMAP), inhibits GVBD and decreases the level of MPF activity (Fulka et al., 1991). These experiments give further credence to the hypothesis

that these proteins are associated with MPF. Cyclin, one of the subunits of MPF, must be newly synthesized and phosphorylated for MPF to become active (Murray and Kirschner,1989). Additional evidence presented for MPF involvement in bovine oocyte maturation is that two of the newly phosphoryated proteins have apparent molecular sizes similar to two known cyclins (Kastrop et al., 1991).

Another hypothesis for the induction of bovine oocyte maturation involves varying levels of cyclic adenosine monophosphate (cAMP). Cyclic adenosine monophosphate, produced by the granulosa cells, is maintained at constant levels inside the oocyte via gap junctions. A sudden change in cAMP content within the oocyte regulates resumption of meiosis (Sanbuissho et al., 1992; Sirard et al., 1992). This sudden shift in cAMP levels may involve both cumulus and granulosa cells, and is thought to occur as a consequence of the LH surge (Sirard et al., 1992). Experiments showing that bovine oocytes remain in an arrested state only when the COC's were not detached from the follicle wall provide supporting evidence for this scenario (de Loos et al., 1992). Co-culture of cumulus-enclosed oocytes with follicular hemi-sections including both theca and granulosa cells is able to maintain meiotic arrest (Sirard and Coenen, 1993). Also, when granulosa cells are added to oocyte cultures, maturation is inhibited (Sirard and Bilodeau, 1990b). The phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), which stimulates cAMP accumulation, also inhibits maturation but only in the presence of granulosa cells (Sirard and Bilodeau, 1990a).

Other agents which stimulate cAMP accumulation in cells, such as sodium fluoride and forskolin, also reduce the number of oocytes reaching MII (Sirard, 1990). Conflicting evidence indicates that removal of cumulus cells altogether does not prevent the inhibitory effect of dibutyryl-cAMP (db-cAMP; Sirard and First, 1988). In vitro, db-cAMP does not affect protein synthesis patterns in immature bovine oocytes, even though resumption of meiosis is inhibited (Bevers et al., 1992). Adenylate cyclase also inhibits maturation by elevating cAMP levels, although cAMP does not exert its effect through protein kinase (Sirard, 1989; Aktas et al., 1990; Aktas et al., 1992).

These two scenarios for the induction of maturation are not necessarily mutually exclusive. Cyclic adenosine monophosphate could target kinases or phosphatases which regulate key control proteins, such as MPF. The specifics of meiotic regulation in the bovine oocyte remain, for now, unclear.

Ultrasound Follicular Aspiration

A nonsurgical technique has recently been developed for the collection of oocytes from cows. Transvaginal ultrasound assisted follicle aspiration offers the opportunity for the repeated collection of large numbers of oocytes from genetically valuable cows. Because superovulation is not used with this technique, a higher quality oocyte and embryo may be produced resulting in a higher number of pregnancies per donor cow (Pieterse et al., 1988).

In this method, ovaries are palpated, and then brought in proximity to the vaginal wall. An ultrasound probe, inserted into the vagina, scans the surface of the ovary through the vaginal wall for the presence of antral follicles. A needle, attached to the probe, is inserted into the follicle and the follicular fluid aspirated by vacuum pressure. In the first reported study of this technique, 54 oocytes were recovered from 197 follicles, a recovery rate of 27% (Pieterse et al., 1988). This pioneering study also identified several factors that influence recovery rate, such as restraint of the animal, method of ovarian manipulation, follicular size, fixation of the oocyte in cumulus cells, vacuum pressure, tubing system, and needle size, sharpness and bevel. Subsequent studies showed an improvement in recovery rates to 50%, and demonstrated that repeated aspirations do not disturb normal cyclicity (Pieterse et al., 1991a; Pieterse et al., 1992). Recovery rates are decreased if superovulation is used, even though more large follicles are present (Pieterse et al., 1992).

Oocytes collected in this manner have been shown to be capable of successfully completing in vitro maturation and fertilization, and develop to the morula and blastocyst stages after culture in vitro (van der Schans et al., 1992). Aspirated oocytes may actually be more successful in an IVM/IVF system than slaughterhouse derived oocytes (Kruip , et al., 1990). Oocyte retrieval using ultrasound aspiration may become an alternative for the multiple superovulation and embryo transfer programs now in use (Kruip, et al., 1991; Pieterse, et al., 1991b). Currently, studies

suggest an average of 16 oocytes can be collected per cow, and each cow can be used for collection twice per week (van der Schans et al., 1992). At this rate, many embryos would be available for transfer to recipients from one donor cow.

In Vitro Fertilization

After oocytes are collected and matured, they must be fertilized before any further developmental processes can occur. In vitro fertilization of in vitro matured oocytes has many inherent advantages, some of which include overcoming some types of infertility, production of many genetically related offspring simultaneously, extension of semen reserves of valuable males, better assessment of male performance, and production of synchronously developing pronuclear stage ova (Brackett, 1983).

For successful fertilization in vitro, sperm must be acrosome reacted and capacitated to penetrate and activate the oocyte. This section of the review will discuss methods to acrosome react and capacitate sperm in vitro, and discuss the evolution of widely used protocols acceptable for bovine in vitro fertilization.

It is well known that mammalian sperm must undergo a period of incubation inside the female reproductive tract before they become capable of fertilizing an oocyte. This process is known as capacitation, and involves the removal of materials from the surface of the spermatozoa by an undefined enzymatic reaction (White et al.,

1992). Because this process remains relatively undefined, particularly in the bovine, a technique to capacitate bull sperm in vitro has taken years to develop.

Early attempts to capacitate bull sperm in vitro for use with in vitro fertilization involved incubating ejaculated semen in isolated female reproductive tracts. After incubation for 3 to 4 hr in isolated estrus cow tracts or 12 to 14 hr in vivo in ligated rabbit doe tracts, about 20% of the oocytes inseminated with incubated semen were successfully fertilized, showing that capacitation had been accomplished (Iritani and Niwa, 1977).

Modification of sperm completely in vitro became the next challenge. Byrd (1981) found that a minimum incubation time of 2 to 3 hr in chemically defined medium containing bovine serum albumin (BSA) could capacitate spermatozoa, as determined by a redistribution of surface lectin binding sites. In vitro penetration of oocytes is another assay frequently used to evaluate capacitation. Sirard et al. (1984) found that 14 to 46 % of inseminated oocytes were fertilized after hyperosmotic treatment of sperm and incubation with fatty acid free BSA. Differences between bulls, and between fresh and frozen semen in the ability to capacitate in vitro were also recognized (Sirard et al., 1984; Wheeler and Seidel, 1986).

The modern era of in vitro capacitation was ushered in when Parrish (1988) published a report on the ability of heparin to successfully capacitate bovine sperm in vitro. Heparin, a

glycosaminoglycan, could capacitate sperm after a 4 hr incubation at 39°C. Capacitation was verified by in vitro penetration of oocytes and by the ability of lysophosphatidylcholine to act on capacitated sperm and induce the acrosome reaction (Parrish et al., 1988). N-desulfation of heparin destroyed it's ability to bind to and capacitate sperm. Resulfation restored binding activity but did not result in capacitation, demonstrating that additional activities after heparin binding are essential (Miller and Ax, 1989). The percentage of sperm activated by heparin can be increased by exposure to 2mM calcium. This external calcium associates directly with the sperm in the presence of heparin (Handrow et al., 1989). Glucose inhibits heparin's effect on calcium association with and capacitation of sperm, but this inhibition can be overcome by addition of 8-bromo-cAMP (Handrow et al., 1989). These results imply a role for cAMP in sperm capacitation by heparin. However, 8-bromo-cAMP cannot capacitate sperm in the absence of heparin, although it can increase sperm associated calcium. Cyclic adenosine monophosphate may regulate associated calcium in sperm by affecting protein kinase activities, thus phosphorylation, and possibly calcium channels.

After capacitation has occurred sperm are able to undergo the acrosome reaction. The acrosome is an organelle that covers the anterior portion of the sperm nucleus which becomes modified during the acrosome reaction (Meizel, 1985). This reaction releases hydrolytic enzymes whose purpose is to digest the matrix of cumulus cells around the oocyte, permitting penetration of the zona pellucida (Lenz et al., 1983b). Substances isolated from the female

reproductive tract are thought to mediate the acrosome reaction, and are therefore candidates for substances to induce the acrosome reaction in vitro. Glycosaminoglycans (GAG's) chondroitin sulfate, heparin, and hyaluronic acid, all found in reproductive tract fluids, promote the acrosome reaction in bovine sperm, as evidenced by elevated rates of oocyte fertilization (Lenz et al., 1983b; Meizel, 1985; Parrish et al., 1985; Lee et al., 1986). The potency of these compounds for inducing the acrosome reaction is related to the degree of sulfation of the GAG (Handrow et al., 1982). Whether or not GAG's are the natural agents causing the acrosome reaction in vivo remains unclear. However, changes in the composition, concentrations, and potencies of GAG's, as well as in the ability of oviductal fluid to affect sperm fertilizing ability during the estrus cycle, suggest this is the case (Lee et al., 1986; Parrish et al., 1989; Killian and Grippo, 1992).

Because the acrosome reaction involves membrane fusion, compounds which either induce fusion or alter membrane fluidity also have been tested for their ability to induce the acrosome reaction. Liposomes made with phosphotidylcholine containing fatty acid chains of 10 to 12 carbons were able to induce the acrosome reaction in greater than 90 % of incubated semen (Graham et al., 1987).

Several other compounds have been tested for use in the induction of the acrosome reaction in vitro. Calcium is important in sperm binding to the zona pellucida and penetration of the oocyte

(Didion and Graves, 1989). In addition, calcium ions are required for capacitated sperm to undergo the acrosome reaction (Byrd, 1981). However, a conflicting report indicates that a low percentage of sperm exposed to calcium underwent the acrosome reaction (Didion and Graves, 1989). Platelet activating factor, a vasodilatory phospholipid identified in bull semen, also can be used to induce the acrosome reaction and promote in vitro fertilization of bovine ova (Parks and Hough, 1990). Lastly, bovine oviduct epithelial cells (BOEC) have been used in co-culture to acrosome react both fresh and frozen semen (Goldman et al., 1991). Recent studies have shown that unique polypeptides were synthesized by BOEC when co-cultured with sperm (Ellington et al., 1992). These polypeptides may be involved in sperm capacitation.

Research on capacitation and acrosome reaction of bovine sperm has contributed to the creation of successful in vitro fertilization (IVF) protocols for the bovine. The first report of successful IVF, as evidenced by birth of a normal live calf, treated sperm in high ionic strength medium to capacitate and acrosome react sperm (Brackett et al., 1982). Fertilization rates were low and quite variable. Later studies reported that high ionic strength medium was no more effective in preparing sperm for fertilization than standard medium (Bondioli and Wright, Jr., 1983). Sperm exposure to a calcium ionophore was found unnecessary for fertilization (Ball et al., 1983). Hypotaurine, epinephrine, and penicillamine improve fertilization rates up to 70 % (Leibfried and Bavister, 1982; Ball et al., 1983; Miller et al., 1992). Hypotaurine has

been found to be required for in vitro development of in vitro fertilized hamster ova, due possibly to its antioxidant nature (Barnett and Bavister, 1992). Bovine follicular fluid treated sperm were capable of fertilizing 46 % of inseminated oocytes, due to the presence of the GAG side chains of the proteoglycans present (Fukui et al., 1983; Lee and Ax, 1984). Fertilization rates were high and repeatable when the individual GAG, heparin, was used to treat sperm which had been separated by the swim up procedure (Parrish et al., 1986). Most current protocols are based on this study. Caffeine is reported to act synergistically with heparin to increase penetration of oocytes, presumably due to caffeine's ability to elevate intracellular cAMP, which may occur during capacitation in vivo (Niwa and Ohgoda, 1988). These studies have cumulated in a standardized protocol for in vitro fertilization which involves a sperm separation step, then treatment of sperm with heparin and usually a combination of penicillamine, hypotaurine, and epinephrine as well (Parrish, 1991).

These technological developments have permitted the study of early morphological events of fertilization. Sequential changes in the oocyte after sperm penetration in vitro are important for utilization of IVM/IVF embryos in gene transfer technology. Although these time sequences vary slightly from bull to bull, a general time scheme can be presented (Leibfried-Rutledge et al., 1989). Sperm penetration is first observed at 3 hr post insemination (hpi), and continues until 6 hpi. Sperm decondensation follows 1 to 2 hr after penetration, and both pronuclei are formed in another 3 to 4 hr (Xu

and Greve, 1988; Saeki et al., 1991). Maturation-promoting factor, present in the cytoplasm of matured oocytes, may be responsible for the transformation of sperm nuclei to metaphase chromosomes (Abeydeera, et al., 1993).

One problem that has plagued scientists attempting to use IVF procedures is the large variability between bulls (Blottner et al., 1990; Hillery et al., 1990; Shi et al., 1990; Kroetsch and Stubbings, 1992). Pre-set heparin and sperm concentrations are not effective because each bull responds differently to IVF (Iritani et al., 1989). Characterization of individual bulls and even different ejaculates within a single bull is required. Adjusting heparin and sperm concentrations for individual ejaculates permits many bulls to be successful in an IVF program (Leibfried-Rutledge et al., 1989).

Gene Transfer

The ability to introduce foreign genes into domestic livestock species is a goal of many researchers in animal biotechnology. To date however, success rates for production of transgenic cattle are very low (Hansel and Godke, 1992). Success depends on many variables, including but not limited to the gene construct, technical experience, and the developmental competence of the zygote both before and after exposure to the foreign gene (Overstrom, 1992). The importance of optimizing IVM-IVF techniques to successful gene

transfer now becomes apparent. Several techniques have been used to produce transgenic animals. These are: 1) microinjection of pronuclei of one cell embryos, 2) retroviral vectors, 3) embryonic stem cells, 4) lipofection, and 5) sperm as DNA vectors. Only microinjection has resulted in the live birth of transgenic cattle. Because only microinjection will be used in these studies, this technique will be discussed in greatest detail in this review.

Microinjection of foreign DNA into one pronucleus of recently fertilized embryos is the most widely used method for producing transgenic animals (Simons and Land, 1987). A glass needle less than 1 micron in diameter is filled with buffer containing the foreign DNA. In mice and cows, DNA concentration between 1 and 2 mg/ml resulted in the best integration frequency (Brinster et al., 1985; Bondioli et al., 1988). Under high magnification, the needle is inserted into the pronucleus and DNA is allowed to flow in until pronuclear membrane swelling is discernible. The process usually places about 2 pl of solution containing tens to hundreds of copies of the gene into the pronucleus (Brinster et al., 1985; Simons and Land, 1987). In mice, 50 to 85% of the zygotes survive injection, 10 to 30% develop to term, and 10 to 30% of the live born mice carry the injected DNA in their genome (Wilmut et al., 1991). Efficiencies are lower in other species; only 4 to 10% in cows, sheep, and pigs (Simons and Land, 1987; Rexroad, Jr., 1992). The loss of embryos could be due to lethal physical damage which occurs at injection, inefficiency of embryo procedures, or lethal genetic damage during the insertion event (Simons and Land, 1987).

The process by which the injected DNA becomes part of the embryonic genome is not well understood. Injected genes nearly always integrate into random sites on a chromosome at a single site and often in multiple copies in a tandem array in the same orientation (Wilmut et al., 1991). Microinjection usually results in only random, or illegitimate recombination (Bishop and Smith, 1989). Homologous recombination after pronuclear microinjection has been reported only once (Brinster et al., 1989a). Insertion and formation of concatomers may both result from opportunistic repair ligation mechanisms within the cell. The frequency of insertional mutagenesis may indicate that DNA is integrated preferentially into transcriptionally active or replicative chromosomal locations. Another possibility is that the DNA is integrated at the site of chromosomal breaks which occur during pronuclear swelling (Brinster et al., 1985). This view is supported by the observation that the frequency of DNA integration is increased by irradiating transfected cells.

If DNA is integrated sometime after the first cleavage division of the embryo, a mosaic animal may result. A mosaic is made up of some cells that carry the transgene and some that do not (Wilmut et al., 1991). Studies in mice reveal that at least 30%, and probably greater than 60%, of G0 transgenic mice are mosaic (Wilkie, et al., 1986; Whitelaw, et al., 1993). This frequency could be accounted for by integration occuring after the first but before the second round of DNA replication (Whitelaw, et al., 1993). If the gene was

incorporated before DNA replication, one homologue of a pair of chromosomes in every cell of the animal all contain the new gene. This should result in transmission to 50% of progeny produced (Simons and Land, 1987; Wilmut et al., 1991).

Thus far, only about half of the transgenic livestock produced actually have functioning transgenes (Rexroad, Jr., 1992). Expression levels are extremely unpredictable, in part because they are subject to regulation by flanking DNA sequences. The current lack of knowledge concerning the mechanisms of multigene interactions and gene regulation, both between integrated genes and flanking sequences and transgenes and their promoters has been a limitation to progress in this field (Ebert and Schindler, 1993; Pursel and Rexroad, 1993b). Because insertion is a random event, site of insertion is a limitation which can not be controlled. The use of a genomic DNA construct instead of cDNA, because genomic constructs appear to improve expression, is one method by which expression may be enhanced (Pursel and Rexroad, 1993a).

When attempting to do microinjection in bovine, the first problem is visualization of pronuclei in living embryos. Two techniques have been utilized to accomplish this. The first is staining with 4'-6'-diamidino-2-phenylindole (DAPI) and then observing the embryo under fluorescence microscopy (Minhas et al., 1984; Kraemer et al., 1985). Live calves have shown that bovine zygotes can survive DAPI staining (Kraemer et al., 1985). The second and more widely used technique is centrifugation. After centrifugation for 3 to

4 min at 15,000 x g, pronuclei are more clearly visualized under differential interference contrast, or Nomarski optics (Luskutoff et al., 1986; Biery et al., 1988).

Many genes have been introduced into bovine embryos, including the herpes simplex virus thymidine kinase (HSV-TK) gene (Kraemer et al., 1985), the interferon alpha (IFN-a) gene (Loskutoff et al., 1986a), chloramphenicol acetyltransferase (CAT) gene (Biery et al., 1988; Bondioli et al., 1988), the bovine papilloma virus DNA (Roschlau et al., 1989), the alcohol dehydrogenase gene (Roschlau et al., 1989), human, rat, and bovine growth hormone (GH) gene (Roschlau et al., 1989; McEvoy and Sreenan, 1990; Jura et al., 1992), the ß galactosidase enzyme gene (McEvoy and Sreenan, 1990), the corticotropin releasing factor gene (Gagne et al., 1990), the human estrogen receptor gene (Hill et al., 1992), the insulin-like growth factor I (IGF I) gene (Hill et al., 1992), the human lactoferrin gene (Krimpenfort et al., 1991) and the human erythropoietin gene (Peura et al., 1992). These genes are fused to various promoter elements to direct expression in vivo. To date transgenic fetuses have been produced with the CAT gene (Biery et al., 1988; Bondioli et al, 1988). Only a small number of transgenic calves have been produced, containing the bovine GH, human lactoferrin, estrogen receptor and IGF-I genes (Roschlau et al., 1989; Krimpenfort et al., 1991; Hill et al., 1992). The calf transgenic for bovine GH was later found to contain only plasmid and promoter sequences, not the GH gene itself (Roschlau et al., 1989). Of the 6 calves transgenic for IGF-I, 3 were stillborn or died within 1 d of birth (Hill et al., 1992). In all, only 6

live transgenic calves have been reported in the literature (Krimpenfort et al., 1991; Hill et al., 1992). The efficiency of producing these animals, as determined by the number of transgenics per ova injected, is .02 to .2% (Krimpenfort et al., 1991; Hill et al., 1992). This is considerably lower than that typically obtained with mouse zygotes.

There are two distinct reasons for producing transgenic cattle. One is to produce an animal with better agriculturally important qualities, such as enhanced muscle development, less fat, increased feed efficiency, increased growth rates, or increased milk or milk protein production (Pursel et al., 1989a; Rexroad, Jr. et al., 1991; Pursel et al., 1992). In transgenic pigs producing bovine GH, growth was stimulated and conversion to protein was enhanced (Pursel et al., 1989b). However, detrimental effects on the health of the pig were observed due to the long term exposure to elevated GH levels. With more tissue specific expression and regulation, pigs have been produced that have high body weights, reduced backfat, and are healthy and fertile (Westphal, 1989; Brem, 1993). The second is called "transgenic pharming" (Bialy, 1991). The purpose is to have a cow produce human proteins in her milk. This process is superior to traditional purification from human derived tissues or fluids, or bacterial production of recombinant proteins. The reasons are high milk production of today's dairy cows, low operating costs, correct post-translational modification of complex proteins and the multiplication of the transgenic cow, which has recently been dubbed a "bioreactor" (Roschlau et al., 1989). It has already been

demonstrated that transgenic mice, sheep, goats, and pigs can produce high levels of a foreign protein in their milk (Ebert et al., 1991; Wall et al., 1991; Wright et al., 1991; Niemann et al., 1992; Velander, et al., 1992). These proteins would have an extremely high market value. For example, coagulation factors IX and VIII used in the treatment of hemophilia, have a value of $25,000 per gram and $150,000 per gram, respectively (Clark et al., 1987). The potential profit from sales of these proteins should offset the large financial investment which would be needed to produce the transgenic animals and harvest the proteins.

Retroviruses invade their hosts by converting their genes from RNA to DNA, incorporating the viral DNA into the chromosomes of the host cells, and relying on host expression mechanisms for their own gene expression (Varmus, 1988). Culture of pig embryos on canine cells producing an avian retrovirus and subsequent fetal development to 6 wk of gestation, demonstrate the feasibility of using retroviruses as a vector for gene transfer to early embryos (Jin et al., 1991). Retrovirus mediated gene transfer has also been reported in bovine embryos (Kim et al., 1993).

Embryonic stem cells are pluripotent cells isolated from early embryos (Anderson, 1992). These cells retain the ability to differentiate when cultured under appropriate in vitro conditions. When these cells are injected into host blastocysts, they can take part in the formation of all tissues of the resulting chimeric animal. A chimera is an animal consisting of two or more genetically distinct

cell types (Seidel, 1992). This has been accomplished in the mouse, but embryonic stem cells have not been available from domestic animal species (Baribault and Kemler, 1989; Anderson, 1992). Embryonic stem cells can be genetically transformed using such methods as calcium phosphate transfection or microinjection. These transformed cells are capable of colonizing the germ line of chimeric animals and thus stably transmitting the foreign gene to their offspring (Gossler et al., 1986; Robertson et al., 1986).

Lipofection is the process of complexing DNA with a cationic lipid, and fusion of the resulting liposomes with the cell membrane to deliver functional DNA into the cell. This technique is reproducible and efficient for the transformation of tissue culture cells (Felgner et al., 1987). Lipofected chicken blastodermal cells have been injected into chicken embryos and contributed to the fetal tissues (Brazlot et al., 1991). Liposomes alone or liposome DNA complexes have been injected directly into mice pronuclear stage embryos and mice blastocysts (Luskutoff et al., 1986b; Reed et al., 1988). Liposomes were not detrimental to embryo and fetal development, although no transgenic animals were produced in these studies.

The first report of producing transgenic mice using sperm as a vector to introduce DNA into an oocyte at fertilization was in 1989 (Lavitrano et al., 1989). The capacity of live sperm to bind DNA is very high although how or why sperm would bind and take up DNA is unknown (Birnstiel and Busslinger, 1989). This surprising study was immediately attempted to be repeated by other researchers. It

was confirmed that the spermatozoa of many species could indeed associate with DNA molecules (Castro et al., 1990; Atkinson et al., 1991; Horan et al., 1991). DNA-binding ability was confined to a specific region of the sperm head (equatorial segment and postacrosomal region; Camaioni et al., 1992). However, no other groups have been able to produce transgenic animals by this method (Brinster et al., 1989b; Maddox, 1989). Techniques to increase DNA uptake by sperm were then investigated. Electroporation of bovine sperm resulted in day 5 embryos that had incorporated the foreign gene (Gagne et al., 1991). Liposomes also were utilized, but no transgenic mice have been generated (Bachiller et al., 1991). The feasibility of using sperm to produce transgenic animals is still in question.

Polymerase Chain Reaction

After exposure of the embryo to a foreign gene, the embryo or the resulting animal must be tested for the presence of the transgene. Screening is an essential component of any transgenic animal production system. The state of the art screening method is the polymerase chain reaction, or PCR. This technique was first reported by Saiki et al. (1985, 1988). The technique involves primer mediated enzymatic amplification of specific target sequences in genomic DNA. The target sequence is amplified exponentially. The technique works in the following way. Oligonucleotide primers bind to opposite strands flanking the target region. Repeated heating and cooling cycles denature the DNA, anneal the primers, and extend

them across the target sequence (Van Brunt, 1990). The power of PCR lies in it's ability to amplify minute quantities of DNA. In transgenic programs, an injected embryo can be biopsied and tested for the presence of the transgene after culture and before transfer. This has been accomplished in the bovine (Behboodi, et al., 1993; Bowen, et al., 1993; Thomas, et al., 1993). Sex determination by PCR also has been accomplished in bovine embryos (Kirkpatrick and Monson, 1933; Machaty et al., 1993). Successful amplification of a single copy gene sequence in a single biopsied cell has been accomplished (Holding and Monk, 1989). Biopsy of 6.5 to 7.5 d in vivo produced embryos did not affect subsequent pregnancy rates (Lopes et al., 1992). However, PCR is not error free. Parameters are very strict and contamination occurs easily, so some risk accompanies using single cell PCR data (Arnheim, 1992). Another limitation of PCR is that it is only able to detect the presence of the transgene in the embryo, and cannot distinguish if the gene has been integrated or not. A method to identify integrated DNA based on methylation patterns (bacterial versus mammalian) has been developed (Jänne et al., 1992). This method has come into question, however, after studies showed that methylation patterns were changed independent of integration of the gene (Burdon and Wall, 1992).

Fluorescence in situ hybridization also has been used as a method to detect transgenes in microinjected embryos (Lewis-Williams et al., 1993). Integration can only be firmly established, however, if metaphase chromosome spreads are used. The

preparation of a metaphase spread from a biopsy of only a few cells of an embryo is difficult and time consuming. Another method of detecting integrated transgenes is to inject a DNA fragment containing dUTP, which is removed by the embryo if the gene does not integrate (Cousens et al., 1993). A problem with all of these methods is that mosaic embryos may be diagnosed as negative. In this case they would not be transferred and a potentially valuable embryo would be discarded. Despite problems, PCR is the most efficient and rapid method to analyze preimplantation embryos for the presence of the transgene prior to transfer to a recipient. By transferring only positive embryos, recipient animals and research funds are more efficiently managed. New PCR techniques to remove or destroy unintegrated DNA before analysis are currently being developed, and should make PCR a definitive test for integrated transgenes in microinjected embryos.

Embryo Culture

An integral part of any transgenic cattle production scheme is an embryo culture system. After microinjection, embryos must be cultured to an appropriate stage for PCR analysis and transfer. The development of cattle embryos in vitro has been a source of some difficulty, especially when in vitro matured, in vitro fertilized oocytes are used. It is unsure whether this difficulty results from sub-optimal culture conditions or reduced developmental capacity of IVM-IVF embryos (Trounson, 1992).

One reason that conditions necessary for in vitro development of early bovine embryos have not been perfected is the irreversible in vitro developmental block at the 8 to16 cell stage (Eyestone and First, 1986). The block results from exposure to in vitro conditions during the 4- to 8-cell stage, and although it is irreversible, is not the result of overt embryonic death (Eyestone and First, 1991). Because of this difficulty , the preferred method, until recently, has been in vivo culture in sheep or rabbit oviducts (Petters, 1992). In the last several years, advances in co-culture systems and even in defined culture medium for bovine embryo development have made in vitro culture more feasible. This review will discuss the evolution of bovine embryo culture through these various stages.

The earliest report of attempted bovine embryo culture came in 1952. One-to 8-cell embryos were cultured in follicular fluid or serum with very limited success (Brock and Rowson, 1952). Centrifuged follicular fluid and Eagles medium supplemented with serum supported cleavage of 8-cell embryos to the 16 and 24-cell stage (Sreenan et al., 1968). Using Ham's F10 or TCM199, maximal cleavage was from the 1-cell to the 12-cell stage (Seidel et al., 1971). These early frustrations in using different culture substratum led researchers to try other culture techniques for early cattle embryos.

One obvious choice was to culture bovine embryos in vivo in the oviducts of other species. Transfer of an 8-cell embryo to the ligated oviducts of pseudopregnant sheep resulted in a 64-cell embryo, although 2- and 4-cell embryos cleaved only to 10- or 20-

cells (Sreenan et al., 1968). In a later study, 1-cell embryos, when embedded in agar chips and transferred to the oviducts of synchronized ewes developed to morula and blastocyst stages at a rate up to 55% (Eyestone et al., 1985). Ovariectomized and/or anestrous ewes also were successful hosts for bovine embryo development (Eyestone et al., 1987a). Transfer of morula to immature mouse uteri in vivo for 24 hr resulted in viable embryos, although the low recovery rate reported hampered the feasibility of this method (Thuemmel et al., 1989). A more recent report comparing the sheep and rabbit oviduct to in vitro culture systems concluded that even though oviducts produce higher developmental rates, problems with recovery can be so great that they counteract this advantage (Fukui et al., 1989). The recovery rates from sheep in this study were lower (35%), compared to other studies (68%; Eyestone et al., 1987a; Fukui et al., 1989).

Another method which has been used for bovine embryo culture is the explanted mouse oviduct. This procedure requires considerably less preparation and animal handling, and eliminates the need for surgical procedures. This technique results in development from the 1-cell to morula and blastocyst at rates of about 24% (Sharif et al., 1991; Sharif et al., 1992a; Sharif et al., 1992b). However, one study reported that the isolated mouse oviduct did not support development of bovine embryos, although the organ culture technique was slightly different (Sparks et al., 1992).

The next logical step from in vivo culture was in vitro cell co-culture systems. These systems provide unknown nutrients to the embryo which cannot be directly added to medium alone, and alleviates the problems of in vivo culture. Trophoblastic vesicles were an early co-culture candidate, as they are thought to produce growth factors which allow exponential multiplication of embryonic cells (Heyman et al., 1987). Trophoblastic vesicles increased the development of 1- to 8-cell embryos from less than 20% to 50% when included in the culture system (Camous et al., 1984; Heyman et al., 1987; Aoyagi et al., 1990; Nakao and Nakatsuji, 1990). Cumulus and granulosa cell co-culture also has been tested with some success. Embryos from the 1- to 8-cell stage develop successfully at rates varying from 17% to 63% (Aoyagi et al., 1990; Nakao and Nakatsuji, 1990; Xiaoxia et al., 1991; Goto et al., 1992; Herrler et al., 1992; Xu et al., 1992; Zhang et al., 1992b; Thomas et al., 1993). Uterine cells, amniotic cells, skin cells, and testicular cells have been successful in supporting bovine embryo development (Voelkel et al., 1985; Aoyagi et al., 1990; Goto et al., 1992). These results demonstrate that the positive effect of co-culture does not seem to be specific to cells of reproductive tract origin.

Another cell type commonly used to support bovine embryo development is Buffalo Rat liver (BRL) cells. Buffalo rat liver cells are known to secrete polypeptides in culture that are similar to insulin-like growth factors (Marquardt et al., 1981). These cells also secrete differentiation inhibiting activity, which is homologous to leukemia inhibiting factor (LIF), both acting to inhibit differentiation

and stimulate growth (Smith and Hooper, 1987). Murine and ovine in vitro embryo development is improved by the addition of LIF to the culture medium, and LIF has been shown to be secreted by the murine uterus coincident with blastocyst implantation (Fry, 1992). Buffalo rat liver cells support the development of 1- and 2-cell bovine embryos to the morula and blastocyst stages (18% and 29% respectively: van Inzen et al., 1992; Voelkel and Hu, 1992). Co-culture with BRL cells has been shown to produce embryos with superior freeze survival when compared to co-culture with oviductal epithelial cells (Voelkel et al., 1992).

Probably the best studied co-culture system is that using oviductal epithelial cells (OEC). This method was first reported in 1987, and in the intervening years has burgeoned into a widely used and well characterized technique (Voelkel and Hu, 1992). There are two methods used with oviductal cells - epithelial clumps and monolayers. Free floating clusters of recently isolated oviduct epithelial cells produce impressive developmental rates depending on the medium used. Ham's F10 and OEC supported development of 5- to 8-cell embryos at a rate of 46% (Eyestone et al., 1987b; Eyestone and First, 1989). Synthetic oviductal fluid and OEC supported development of 1-cell, and 2- and 4-cell embryos to morula and blastocyst at rates of 32% and 64%, respectively (Fukui et al., 1991; Rorie et al., 1992). If TCM199 is used, developmental rates from 22% to 80% were reported (Fukui et al., 1991; McCaffrey et al., 1991; Rorie et al., 1992; Voelkel and Hu, 1992). The presence of oviductal epithelial cells has been shown to improve the quality of in

vitro cultured embryos as well (Shamsuddin et al., 1993). Menuzo's B2 medium, in conjunction with OEC, produces blastocysts with cell numbers and rates of development resembling in vivo embryos (Xu et al., 1992). Glycine and alanine, two amino acids that are secreted by oviductal cells in culture, improve embryo development when added to culture medium (Moore and Bondioli, 1993). This may be a mechanism by which oviductal cell co-culture enhances embryonic development.

Oviductal epithelial cell monolayers have been used with various cell culture medium. CZB culture medium and TCM199 were most successful, with developmental rates for 1- and 2-cell embryos reportedly well above 50% (Eyestone and First, 1989; Ellington et al., 1990a; Kim et al., 1990; Xiaoxia et al., 1991; Xu et al., 1992). Oviductal epithelial cells isolated from early and late luteal stages did not differ in their ability to support development, even though cells from early luteal stages had increased ciliary activity, viability and efficiency of attachment and growth (Thibodeuax et al., 1992). The use of millicell inserts treated with matrigel substratum maintained motile cilia while culture on plastic dishes did not, and promoted better development as well (75% vs 40%; Pollard et al., 1989). When compared to embryos recovered from donor cows, embryos cultured on OEC in vitro were not different in mean cell counts or the percentage of transferable embryos (Ellington et al., 1990b).

Embryos successfully develop after culture in oviduct tissue conditioned medium (Eyestone et al., 1991; Mermillod et al., 1992),

as well as BRL cell conditioned medium (Hernandez-Ledezma, et al., 1993). Stage of the estrous cycle when oviductal cells were collected did not affect development, but there was considerable variability between batches of oviductal cells (Nauta et al., 1992). Oviductal epithelial cells synthesize and secrete oviduct specific glycoproteins during estrus (Boice et al., 1990). These proteins associate with early bovine embryos, and may play a role in supporting embryonic development (Gandolfi et al., 1989; Boice et al., 1992). At least two different factors have been isolated from oviductal cell conditioned medium; one of low molecular weight important in development to the 8 cell stage, and one of high molecular weight allowing embryos to develop to the blastocyst stage (Mermillod et al., 1993). It is likely that these proteins are responsible for the increased development in OEC culture and conditioned medium.

The ultimate goal of researchers in this area is to create a completely defined culture medium for bovine embryos. A medium based on the biochemical analysis of oviductal fluid, called synthetic oviductal fluid (SOF), was developed for this purpose. Synthetic oviductal fluid contains lactate, pyruvate, and glucose as energy sources, and is efficient at supporting the development of bovine embryos (64%; Tervit et al., 1972; McLaughlin et al., 1990). When Brinster's medium, which contains only pyruvate, was used, 1-cell embryos cleaved to the 8-cell stage but no further (McKenzie and Kenney, 1973). This seems to point out that embryos need only pyruvate in early cell divisions, but that requirements for the continuation of cleavage change at the 8-cell stage. Another study

using CR1, which contains lactate, showed that pyruvate is not required at all if lactate is present (Rosenkrans et al., 1990).

A medium developed for hamster embryos (HECM), which is glucose and phosphate free, did not support bovine embryo development very well (4%) unless supplemented with heparin and estrogen (29%: Rose, 1990). Hamster embryo culture medium is a simple, chemically defined, protein free medium with 20 amino acids. Another study determined that HECM could support development to the morula stage, but that bovine calf serum was necessary for blastocyst formation (Pinyopummintr and Bavister, 1991). Serum has a biphasic effect on embryo development, both inhibiting the first cleavage division and stimulating morula and blastocyst formation (Bavister et al., 1992). The authors of this study suggested that embryo culture should be divided into more than one phase, each with specific requirements.

A study conducted by Takahashi and First (1992) examined the effect of glucose, lactate, pyruvate, amino acids and vitamins on IVM/IVF bovine 1-cell embryos. They found that glucose had a deleterious effect on development, while lactate and/or pyruvate was necessary. If lactate is present, the addition of pyruvate is not necessary (Rosenkrans et al., 1993). Other researchers have also observed that in vitro embryo development is improved when glucose is deleted from the medium during the first few days of culture (Kim et al., 1993a,b; Matsuyama et al., 1993). Supplementation with an amino acid mixture was beneficial in basal

medium Eagles (BME), minimal essential medium (MEM), or CR1, but not in SOF (Rosenkrans and First, 1991; Takahashi and First, 1992). Medium contained BSA in these experiments, and could not be substituted successfully. These semi-defined media supported development as well as co-culture, but the embryos had lower cell numbers. These nutritive requirements directly reflect the metabolism of the embryos. Unfortunately, little is known about the metabolic mechanisms of pre-implantation bovine embryos. Blastocysts were shown to metabolize glutamine and pyruvate, probably by the Kreb's cycle, but glycolysis is blocked and thus glucose is not greatly utilized (Rieger and Guay, 1988). The pentose phosphate pathway, which generates NADPH and ribose -5-phosphate, is most active at early cell stages and decreases with embryo development. The Embden-Meyerhof pathway, which is involved in the initial steps of glucose breakdown, shows the opposite trend (Javed and Wright, 1991). This may be the reason that glucose, while not necessary for early cleavages, is beneficial after the third day of development (Takahashi and First, 1992; Robl et al., 1991).

Studies directly comparing modified Eagles medium, TCM199, Ham's F10, SOF, Brinster's mouse ovum culture (BMOC) and Whitten's medium, in either 90% N, 5% CO_2 and 5% O_2 or 5% CO_2 in air atmospheres reported that maximal development occurred in Ham's F10 in 90%N/5%CO_2/5%O_2 atmosphere (Wright et al., 1976a; Thompson et al., 1990). Supplementation of Ham's F10 with 10% heat treated calf serum (HTFCS) was more effective than with 1.5%

bovine serum albumin (BSA) as a protein source (Wright et al., 1976b).

Although culture medium has evolved significantly over the past 20 yr, much remains unknown about the requirements of pre-implantation bovine embryos. In vitro produced embryos continue to exhibit significantly different morphology than in vivo produced embryos (Shamsuddin et al., 1992). More research needs to be conducted to make in vitro development more efficient and more like development in vivo.

Embryo Transfer of In Vitro Matured, Fertilized, and Cultured Embryos

To verify the viability of IVM/IVF/IVC techniques, pregnancies and live birth of calves must be produced. Pregnancies from embryos matured and fertilized in vitro, but cultured in vivo have been reported (Lu et al., 1987; Xu et al., 1987; Utsumi et al., 1991). The first pregnancies from embryos cultivated entirely in vitro were reported in 1988 (Goto, 1988). This report and another since then used a co-culture system with cumulus cells to produce viable embryos (Goto, 1988; Fukuda et al., 1990). Oviductal cell co-culture of IVM/IVF embryos also resulted in pregnancies (Xu et al., 1990; Monson et al., 1992). There have been pregnancies reported from IVM/IVF embryos cultured in medium CR1aa alone (Monson et al., 1992). Frozen thawed IVM/IVF/IVC embryos can produce pregnancies as well (Zhang et al., 1992a). Embryonic loss, possibly as

a result of lowered cell numbers with in vitro cultured embryos, is a limitation to the use of embryo transfer and needs to be further investigated (Betteridge and Loskutoff, 1993).

Nuclear Transfer

Nuclear transfer involves the transfer of a nucleus from a donor embryo to an unfertilized oocyte whose own nuclear material has been removed. The development of this technique, which has the capacity for producing large numbers of genetically identical animals, has enormous potential. This technology could be used to increase genetic gain in animal agriculture, reducing the number of animals needed for experiments, and investigating environmental and genetic interactions in animals (Robl and Stice, 1989). Genetically superior animals could be produced by cloning in 8 yr, while the same genetic advancement would take 17 yr in standard artificial insemination programs (Robl and Stice, 1989). Fewer clones, because they are more uniform than random bred animals, could be used in research to achieve greater statistical validity. The bottom line is that nuclear transfer, or cloning, could reduce and possibly eliminate genetic variation for scientific experimentation (Prather et al., 1992a). Commercial cloning of cattle has become a reality, and embryos of defined production potentials can now be produced. To date, there have been up to eight cloned calves produced from a single donor embryo (First, 1990). The advent of multiple generation cloning, using a nuclear transfer embryo as a nuclear donor, theoretically permits commercial companies to

produce large, and even unlimited, numbers of identical offspring (Stice, 1992).

Successful nuclear transfer of mouse isolated nuclei or whole blastomeres into enucleated donor cytoplasm was reported in the early 1980s (Illmensee and Hoppe, 1981; McGrath and Solter, 1983). The use of nuclear transfer technology to study the developmental biology of domestic animal species took several years longer to develop because of technical and practical difficulties (Willadsen, 1986). The first report of successful nuclear transfer in cattle came in 1987, as proven by normal live births (Prather et al., 1987; Robl et al., 1987). These early studies elucidated many important factors needed for the success of the technique. For isolated nuclei, Zimmerman's fusion medium at 100V for 20 to 40 ms produces the highest fusion rates (79%), and the optimal stage for donor embryos is greater than 8-cells (Zimmerman and Vienken, 1982; Robl et al., 1987). Whole blastomeres can be used successfully as nuclear donors in cattle, but the size of the blastomere affects cell fusion rates (Prather et al., 1987). Either fresh or frozen embryos can be used successfully as nuclear donors; IVF embryos can be used as well but with reduced success rates (Yang, et al., 1993). Most researchers have used in vivo culture techniques for micromanipulated embryos, because of their reduced viability and the inadequacies of current culture medium. The most common technique is to first embed the embryos in agar chips, and then to transfer the chips to the ligated oviducts of sheep or rabbits (Willadsen, 1979; Westhusin et al., 1989). This treatment results in high cell numbers and good embryo

quality (Northey et al., 1992). Recent studies have reported successful nuclear transfer using nuclear donors and oocytes derived entirely in vitro and culturing the resulting embryos in vitro as well (van Stekelenburg-Hamers, 1993).

Even though nuclear transfer is widely used, efficiencies are still low. There are several factors affecting success, including the embryonic stage of the nucleus, source of recipient, and interactions between nucleus and cytoplasm (Smith and Wilmut, 1990).

When a blastomere is transferred into recipient cytoplasm, its nucleus becomes reprogrammed to direct the development of a new embryo. Researchers hypothesize that embryos which are more advanced in their development are unsuitable nuclear donors because of irreversible changes in the chromatin during differentiation (Westhusin et al., 1991). During differentiation, cells vary the combination of genes that are expressed, probably induced by the binding of transcription factors to the DNA (Wilmut et al., 1992). Another facet of the differentiation process is the transfer of transcription from maternal to embryonic messages (Smith and Wilmut, 1990). In cattle, the maternal zygotic transition occurs around the 8-cell stage, but there were no differences in development of nuclear transfer embryos derived from 2 to 8-cell versus 17- to 32-cell stage donors (Westhusin et al., 1991). Blastomeres from bovine embryos up to 6 d post estrous (approximately 64-cells) have, in fact, been used successfully as nuclear transfer donors (Bondioli et al., 1990; Westhusin et al., 1991).

These results suggest that transcriptional activation does not irreversibly change the genome.

After activation by an electric pulse, the recipient cytoplasm must be able to reprogram the donor nucleus back to the time of fertilization (Smith and Wilmut, 1990). The use of pronuclear stage cytoplasm is ineffective (Robl et al., 1987). The use of secondary oocytes, which have been enucleated, as cytoplasm recipients was successful (Prather et al., 1987). There are four methods used to enucleate oocytes (Smith, 1992). First, the embryo can be bisected and both halves used for cloning. This method produces 50% triploid embryos. Secondly, cytoplasm around the polar body is aspirated, hopefully removing the metaphase plate as well. This method still results in an unknown number of triploid embryos. The third technique involves certifying the removal of chromosomes after one of the first two methods with fluorescent stains and a brief exposure to ultraviolet light (Bondioli, 1993). However, even brief ultraviolet irradiation has been found to cause alterations to both membrane and intracellular components of the oocyte (Smith, 1993). Lastly, the chromosomes can be completely destroyed with chemical or physical agents. The reason that oocytes are superior to zygotes is unclear. Oocytes matured in vivo and recently ovulated were found optimal in one study, but another report found no differences in oocytes of different ages in their ability to serve as cytoplasm donors (Prather et al., 1987; Bondioli et al., 1990). The use of in vitro matured oocytes for cytoplasmic recipients has been an important advance in nuclear transfer, because of the ready availability of oocytes.

Although not statistically significant, 24 hr oocytes tended to be more efficient for activation, fusion, and development (First et al., 1992). Other studies comparing young to old oocytes found that 42 hr oocytes remodeled donor nuclei at higher frequencies than 24 hr oocytes (Heyman et al., 1992; Leibfried-Rutledge et al., 1992; Todorov et al., 1992).

After the blastomere has been fused to the oocyte, the nucleus begins to swell in size, synthesizes DNA, acquires cytoplasmic proteins, and releases nuclear proteins. The protein expression is thought to modify both gene expression and DNA replication (Prather et al., 1992b). Studies have shown that donor nuclei are arrested in morphology and function after transfer, and are re-activated at the time typical for normal embryos (Kanka et al., 1991). Synchronization of nuclei and cytoplasm at similar stages of the cell cycle is also important (Smith and Wilmut, 1990). For example, if a G2 nucleus is transferred in, after DNA synthesis occurs the resulting embryo will be polyploid. This is likely the reason that G1 donor cells result in higher rates of development (Prather et al., 1992). The time of activation relative to nuclear transfer has been found to affect the ploidy of the resulting embryo. Only G1 nuclei should be transferred at the time of activation, because MPF levels in the cytoplasm are high and stimulate replication of the DNA (Campbell, et al., 1993). At 10 hr post-activation, G2 nuclei may be used because MPF activity is absent and re-replication is prevented. The cell cycle stage of the recipient cytoplasm is also important. If donor nuclei are transferred into cytoplasm which is in the S phase of the

cell cycle, developmental potential is higher than if MII stage cytoplasm is used, possibly because DNA re-replication does not occur (Barnes et al., 1993).

While offspring have been produced from nuclear transfer in cattle, the frequency and survival of pregnancies have been low (Willadsen et al., 1991). This may result from inappropriate synchrony of nucleus and cytoplasm, or oocyte developmental stage, that causes incorrect DNA replication. One study showed that nuclear transfer embryos differ significantly in ultrastructural morphology from control embryos (King et al., 1992). Another observation is the occurrence of extremely large calves resulting from nuclear transfer embryos (Willadsen et al., 1991). The same clonal line can produce both normal and large calves. The cause of this problem is not yet known.

CHAPTER III.

INFLUENCE OF TIME OF GENE MICROINJECTION ON DEVELOPMENT AND DNA DETECTION FREQUENCY IN BOVINE EMBRYOS

ABSTRACT

The effect of DNA microinjection at various times after in vitro fertilization on DNA detection and survival rates of bovine embryos was investigated. Oocytes were fertilized 24 h after maturation with frozen/thawed semen prepared with a Percoll separation procedure. At 11, 15, and 19 h after fertilization (haf) embryos were centrifuged to visualize pronuclei and microinjected with a murine whey acidic protein-human protein C genomic DNA construct. After culture for 7 d on Buffalo Rat Liver cells, embryos were assessed for stage of development and assayed for the presence of the transgene by Polymerase Chain Reaction (PCR). Of zygotes in the 11 haf treatment, 16% (25/152) of non-injected and 7% (11/161) of injected embryos developed to the morula or blastocyst stage. Comparable development of non-injected and injected embryos at 15 haf was 15% (23/158) and 4% (6/159) and at 19 haf was 14 % (23/162) and 1% (1/165), respectively. Development of injected embryos was greater ($p<0.05$) when injection was performed at 11 haf compared to 19 haf. Development of non-injected embryos was greater ($p<0.01$) than that of injected embryos. There was no difference in transgene detection frequency between treatments (53% at 11 haf; 50% at 15 haf; 48% at 19 haf). Injected embryos testing positive for the presence of the transgene exhibited increased development over

negative embryos ($p<0.01$). Greater developmental efficiencies can be obtained in microinjected bovine embryos when injection is performed early in pronuclear formation.

INTRODUCTION

The production of transgenic livestock has immense potential in science, medicine, and agriculture. Transgenic mice and livestock have been produced that express high concentrations of a foreign protein in their milk (Gordon et al., 1987; Simons et al., 1987; Wall et al., 1991; Velander et al., 1992). However, microinjection, which continues to be the method of choice for producing transgenics, is considerably more difficult in domestic animals than in mice. In cattle, 10% of the animals born from injected embryos were transgenic (Krimpenfort et al., 1991). When expressed as a percentage of embryos injected, however, the efficiency of producing transgenic cattle was only 0.2 % (Bondioli et al., 1988; Krimpenfort et al., 1991). Engineering dairy animals to produce human pharmaceutical proteins has many benefits over conventional microbial production techniques, including high productivity, low operating costs, post-translational modification of proteins, and unlimited expansion potential of transgenic lines (Bialy et al., 1991; Janne et al., 1992).

The production of transgenic cattle is particularly difficult for several reasons. The supply of in vivo fertilized zygotes is limited, the embryos must be centrifuged to visualize pronuclei, and

developmental potential in vitro is low (Bondioli et al., 1988). In vitro embryo production procedures have been used to provide a large number of synchronous zygotes for pronuclear microinjection (Krimpenfort et al., 1991; Janne et al., 1992). Embryos that develop successfully in culture can be biopsied or bisected to determine transgene integration with Polymerase Chain Reaction (PCR) analysis (Janne et al., 1992) or with in situ hybridization (Lewis-Williams et al., 1993). Only the embryos containing the transgene are then transferred into recipient cows, thus increasing the probability of transgenic calves being born. Even with these recent advances, the process of producing transgenic cattle remains inefficient.

After a sperm penetrates an oocyte, the sperm and oocyte chromatin decondense and form pronuclei. As the sperm head decondenses, protamines are dissociated from the DNA and histones become associated during pronuclear formation (Bedford, 1982). During this time the sperm DNA is relatively free of proteins, and this may be when DNA replication occurs. Generally, DNA replication has been thought to occur after pronuclear formation. Integration of foreign DNA has been hypothesized to occur during replication, possibly because the replicating DNA is more susceptible to breakage. Pronuclei are first formed at 7 to 8 h after in vitro fertilization, and most zygotes contain two pronuclei at 9 to 14 h (Xu and Greve, 1988; Saeki et al., 1991). The first cleavage occurs approximately 28 h after fertilization (Xu and Greve, 1988). There is no G1 period in the cell cycle of early embryos, so DNA synthesis occurs immediately after the first cleavage division (McLaren, 1992). The DNA injected

at late pronuclear stages may be available for integration during this second replication event. The ability of the embryo to repair damage from micromanipulation at different pronuclear stages is unknown. Microinjection of in vitro fertilized bovine embryos has generally been carried out from 18-24 h after fertilization (Gagne et al., 1990; Hill et al., 1992). The present study was designed to investigate the effect of injection at various times after insemination on embryo development and DNA detection rates.

MATERIALS AND METHODS

Bovine oocytes were purchased (Golden Genes, Riverdale, CA) and shipped overnight in 1 ml of maturation medium held in a temperature-controlled (39°C) portable incubator (Minitube of America, Madison, WI). Maturation medium consisted of TCM199 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT), bFSH and bLH (0.01 U/ml each: Nobl Labs, Sioux Center, IA), and 1% penicillin-streptomycin. Oocytes were matured for 24 h and then washed in TL HEPES medium and placed into fertilization medium, 50 oocytes in 500 ml.

A Percoll-separation procedure was used to prepare frozen/thawed semen for in vitro fertilization (Mermillod et al., 1992). Frozen 0.5 ml straws were thawed at 35°C for 1 min. Sperm were layered on top of a percoll (Sigma, St. Louis, MO) density gradient in a 15 ml centrifuge tube and centrifuged for 30 min at 700 x g. After centrifugation, live sperm concentration was

determined. Sperm were added to the fertilization wells with the washed oocytes to give a final concentration of 0.5 x 10^6 sperm/ml. Heparin (0.4 mg/ml) and PHE (penicillamine, 20 mM; hypotaurine, 10 mM; epinephrine 1 mM) were included.

Embryos were removed from fertilization medium at 11, 15, and 19 h after fertilization (haf). Embryos were then vortexed to remove cumulus cells and centrifuged at 12,000 x g for 4.5 min to visualize pronuclei. The embryos were assessed for the presence of two pronuclei before microinjection. Embryos with two visible pronuclei were assigned randomly to either injected or non-injected groups. Embryos in which two pronuclei were not visible were assigned to a third treatment group, and were not microinjected. Microinjections were performed in TL HEPES medium on a heated stage.

The construct used for microinjection was a 12 kb fragment consisting of genomic human protein C (hPC) under the control of the murine whey acidic protein (WAP) promoter. The murine WAP-genomic human Protein C fusion gene was designed by Henryk Lubon and cloned in the Holland Laboratory, The American Red Cross (Rockville, MD). The genomic construct was purified by digestion with the restriction endonuclease Not I (Stratagene Cloning Systems, La Jolla, CA) followed by electrophoresis on a 1% LE agarose (FMC BioProducts, Rockland, ME) gel with 0.5 mg/ml ethidium bromide. Agarose containing the transgene was excised and the DNA purified using the Prep-a-Gene kit (BioRad Laboratories, Hercules, CA). The

construct was then resuspended in buffer (10 mM Tris-HCl, pH 7.4; 0.25 mM EDTA) and filtered through a 0.45 mm spin filter. Embryos were microinjected with 1 to 3 pl of the DNA solution (1.5 mg/ml DNA, at100 copies/pl).

After microinjection, embryos were co-cultured in wells prepared with Buffalo Rat Liver (BRL) cells as described by Voelkel and Hu (1992). Medium used for co-culture consisted of TCM199, 10% FCS, 1% BSA, and 1% penicillin-streptomycin. Embryos were cultured 25/well for 7 d in a 5% CO_2 in air atmosphere at 39°C. On the fourth day of culture, embryos were moved to fresh culture wells. At the end of the culture period, embryos were assessed for stage of development.

Polymerase chain reaction (PCR) was used to determine the presence of the transgene in 485 embryos of various developmental stages (Saiki et al., 1989). Two embryos were discarded from the data set because of an incomplete PCR reaction. Non-injected embryos were analyzed as negative controls. After examination for development, each embryo was transferred in 1 µl of medium into 4 µl of embryo-lysis buffer (20 mM Tris pH 8, 0.9% Tween 20, 0.9% Nonidet, Sigma, St. Louis, MO; and 0.4 mg/ml proteinase K, Amresco, Solon, OH) in a 0.5 ml microcentrifuge tube. Samples were then covered with 25 µl of paraffin oil and frozen at -80°C until they were analyzed. Immediately upon removal from the freezer, initial digestion of the embryos was performed at 55°C for 30 min followed by a 15 min denaturation period at 98°C, and held at 85°C until 20

ml of the PCR reaction mixture was added (water, 1X Taq buffer, 0.2 mM dNTP's, 1.5 mM $MgCl_2$, 0.5 mM of each primer, and 0.025 units/ml Taq polymerase) for a final volume of 25 µl. The primers used to amplify a 559-bp target sequence in the transgene were human ProC 5'2 (hPC-specific sense, 5'-TGG GAG AAG TGG GAG CTG GAC CTG) and ProC A9 (hPC-specific antisense, 5'-CAG CTC TTC TGG GGG GCT TCC TTG). This was followed by a 1 min denaturation at 96°C, and 45 cycles of annealing (55°C for 2 min), elongation (75°C for 30 sec), and denaturation (96°C for 15 sec). A dilution series of restriction enzyme digested plasmid was used as a positive control, and control bovine DNA was used as a negative control. Amplification products from embryos were evaluated concurrently with those from positive and negative controls on 1% agarose gels stained with 0.5 mg/ml ethidium bromide.

In a subsequent experiment, 275 embryos were injected at 11 haf and cultured for transfer to recipients. Embryos were cultured for 5 d either in vitro (n=150) on BRL cells or in vivo (n=125) embedded in double-layer agar chips in the ligated oviducts of sheep (Eyestone et al., 1987). After culture, 23 viable morulae and blastocysts (23/275; 8%) were transferred non-surgically, one embryo per recipient, to the uterine horn ipsilateral to a palpable corpus luteum of an estrous synchronized recipient. Embryos were transferred without biopsy and PCR analysis because of the high percentage of positive morulae and blastocysts detected in the previous experiment. Recipient heifers or cows were treated with 40 mg prostaglandin F_{2a} i.m. (Lutalyse; Upjohn, Kalamazoo, MI) on d 6

to 16 of the estrous cycle to synchronize estrus with the day of fertilization.

Statistical analysis was performed using GLM procedure in the Statistical Analysis System (SAS; 1985). Developmental data were coded as follows: 1 cell=1; 2 cell=2, 4 cell=3, 8 cell=4, 12-16 cell=5, compact morula=6, early blastocyst=7, blastocyst=8, expanded blastocyst=9. Polymerase Chain Reaction results were coded as follows: negative=0, positive=1. Developmental data and PCR status were analyzed using a model including treatment and repetition. Differences between treatment means were tested with linear contrasts using an improved Bonferroni F statistic.

RESULTS

Embryo Development

After microinjection at each time, three embryo populations were transferred to co-culture wells, including injected, non-injected, and those in which two pronuclei were not visible. Williams, et al. (1992) demonstrated that the decrease in developmental efficiencies of injected embryos is due to the DNA itself, not the injection procedure. At the end of the culture period, the developmental stage of all embryos was recorded (Table 1). Fewer embryos without two pronuclei at 15 or 19 haf reached the morula or blastocyst stage ($p<0.01$) than did those without two pronuclei at 11 haf. Development of non-injected embryos did not differ among treatment groups. More non-injected embryos developed ($p<0.01$)

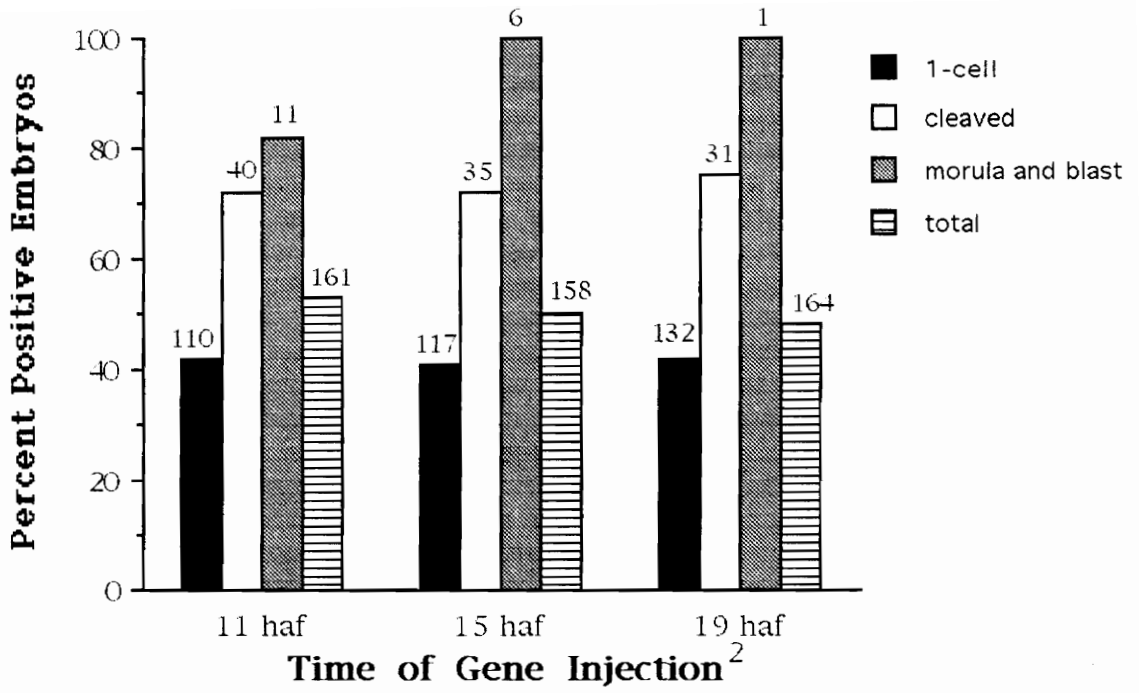
than did injected embryos. The number of injected embryos undergoing development was decreased when injection was performed later in pronuclear development. There was no difference in developmental competence between embryos injected 11 or 15 haf. Fewer embryos injected at 19 haf developed ($p<0.05$) than did those injected at 11 haf. Although development of injected embryos was greater at earlier than later stages, the percentage of viable, 11 haf injected embryos at the end of the culture period was low (11/161; 7%).

Table 1: Effect of Time of Gene Injection on Development of Bovine Embryos Matured and Fertilized in Vitro

HAF[a]	Group[b]	n	Number of 1 cell embryos (%)	Number of Morula and Blastocyst (%)	develop-mental score ± SEM
11	INJ	161	110 (68.3)	11 (6.8)	2.1 ± .2
	NON INJ	152	49 (32.2)	25 (16.4)	3.4 ± .2
	<2 PN	62	27 (43.5)	15 (24.2)	3.5 ± .3
15	INJ	159	118 (74.2)	6 (3.8)	1.8 ± .2
	NON INJ	158	63 (39.9)	23 (14.6)	3.0 ± .2
	<2 PN	41	27 (65.9)	4 (9.8)	2.2 ± .3
19	INJ	165	133 (80.6)	1 (0.6)	1.4 ± .2
	NON INJ	162	65 (40.1)	23 (14.2)	3.0 ± .2
	<2 PN	40	27 (67.5)	4 (10.0)	2.0 ± .3
TOTAL		1100	619 (56)	121 (11)	2.5 ± 2.2

[a] HAF=hours after fertilization

[b]INJ=injected, NON INJ=non injected, <2 PN=less than 2 pronuclei visible



[1] Numbers above bars represent total number of embryos in that category
[2] haf=hours after fertilization

Figure 1. Effect of time of DNA injection on detection of the transgene with PCR in d 7 bovine embryos

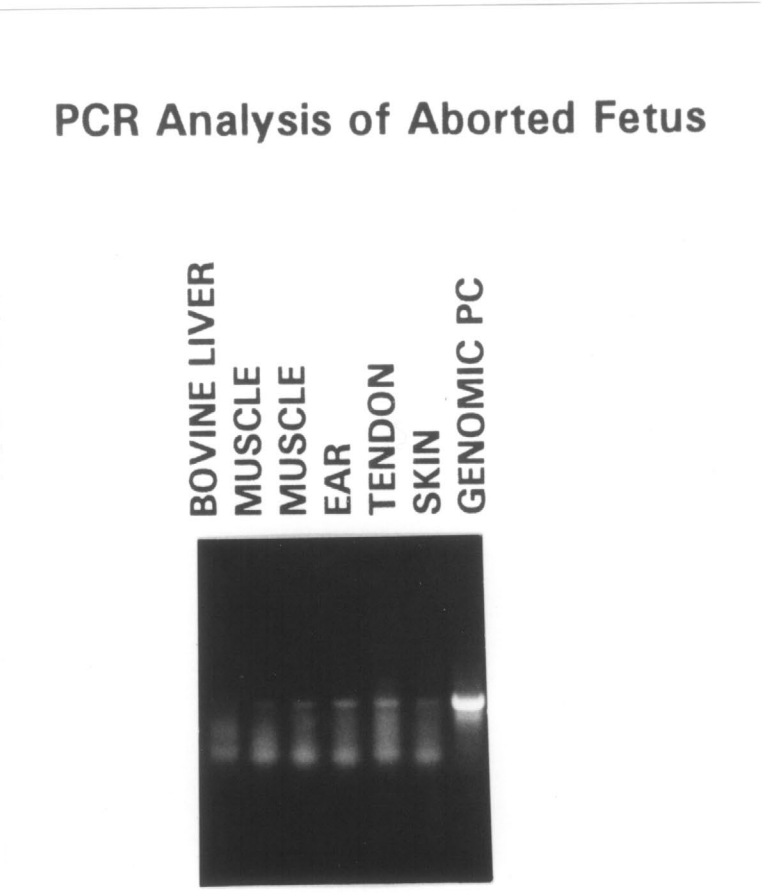


Figure 2. PCR analysis of a bovine fetus (6.5 months). Bovine liver is a control. Muscle, ear, tendon and skin were positive for the human genomic protein C (PC) DNA.

Transgene Detection

The percentage of positive embryos of all developmental stages did not differ among treatments (11 haf, 53%; 15 haf, 50%; 19 haf, 48%). A higher frequency of detection of the transgene occurred in injected embryos that developed to the morula or blastocyst stage ($p<0.01$) than in injected embryos which did not develop (Figure 1). Of the injected embryos developing to the morula or blastocyst stage after 7 d in culture, 89% tested positive for the transgene.

Embryo Transfer

After the completion of the culture experiment, embryos were injected at 11 haf and transferred to recipient cattle. Six recipients were confirmed pregnant by ultrasound analysis at 60 d of gestation, and another recipient had been pregnant but the fetus was not viable. This fetus was not recovered or analyzed. A pregnancy rate of 30% (3/10) was obtained with embryos first cultured in the ligated oviducts of sheep, although the non-viable fetus was from this group. Embryos cultured in vitro on BRL cells resulted in a pregnancy rate of 31% (4/13). Two pregnancies were lost, one at 6 and one at 6.5 mo. The cause of the spontaneous abortions was not determined. The fetus lost at 6.5 mo was recovered, and tested positive for the transgene by PCR analysis (Figure 2). Four calves were born from microinjected embryos, and all 4 were negative for the transgene by PCR analysis.

DISCUSSION

Results from microinjection of in vitro matured, in vitro fertilized oocytes at different times after fertilization indicate that the time of DNA microinjection does not affect the percentage of injected embryos containing the gene after 7 d in culture. However, injection of the gene at early pronuclear stages results in more embryos developing to the morula and blastocyst stages. The reason for this is not known. The embryo may be more resistant to damage at this time.

Embryos in which two pronuclei were not observed at the time of injection were less likely to develop if injection occurred later rather than earlier after fertilization. This reflects the increased probability of detection of pronuclei in fertilized oocytes with increased time after fertilization and, hence, the increased probability that ova without detectable pronuclei at later times are unfertilized. It is likely that many of the ova without visible pronuclei at 11 haf were fertilized, whereas more of those without visible pronuclei at 15 and 19 haf were unfertilized.

The mechanism of integration of injected DNA into the bovine genome is unknown. Injected DNA usually integrates randomly at a single site and often in multiple copy arrays (Wilmut et al., 1991). Possibly DNA is integrated at the site of chromosomal breaks that occur as a result of pronuclear swelling during the microinjection process (Brinster et al., 1985). Powell et al. (1992) found that

pronuclear stage bovine embryos generate significant amounts of large ligation products when DNA is injected into the cytoplasm. This extrachromosomal recombination probably occurs as a result of opportunistic repair-ligation mechanisms (Bishop and Smith, 1989). It is unclear how long these ligation products are able to endure inside the embryo. Because our PCR results showed a high number of viable embryos containing the transgene, and the observed integration rate based on live transgenic births is much lower, we believe that a positive PCR signal does not necessarily imply integration into the genome. Analysis by PCR only confirms the presence of the injected DNA in the cell, regardless of integration status. Although there was no difference in DNA detection among treatments, these results do not discount the possibility that differences in integration that were not detectable did occur. If the injected DNA was able to persist within the embryo for long periods of time in the unintegrated state, it could be available for integration at any time during development. A high percentage of the resulting transgenics would then be mosaic (Burdon and Wall, 1992), as is observed in mice, and there might be multiple insertion sites, as observed in mice.

Another possibility is that the PCR detects a high number of embryos with integrated transgenes. If so, the fate of the transgene is then questionable. The transgene could be subject to genomic rearrangements, possibly because there is no corresponding allele, to excise the foreign gene. If many of the positive embryos were indeed mosaic, the cells containing the gene may be preferentially

allocated to extra-embryonic tissues. Bondioli et al. (1988) reported expression of a transgene in placental but not in fetal tissue in the bovine. Canseco (1993), in our laboratory, described a higher proportion of positive placentas than fetuses in mice on day 18 of gestation after injection as one cell embryos . Such a mechanism could account for the disparity between positive embryos and transgenic cattle described by Bondioli et al. (1988).

A method to indisputably demonstrate transgene integration into the genome at the embryonic stage must be developed before these questions can be answered. A PCR method using methylation sensitive restriction enzymes has been developed (Janne et al., 1992). This system was based on the premise that when the DNA integrates into the genome, it's methylation pattern is transformed from bacterial to mammalian configurations during replication. Unintegrated DNA, retaining the bacterial pattern, was cut (within the target sequence) by the restriction enzyme before PCR. However, this method has been questioned by the recent observation that changes in DNA methylation patterns are independent of transgene integration in mouse embryos (Burdon and Wall, 1992). This study demonstrated that methylation patterns of injected DNA were transformed in all mouse embryos at the two cell stage. Injected DNA, converted to mammalian methylation patterns and present in multiple arrays, may be capable of remaining in the embryo and segregating independently until it incorporates into the genome or is degraded. The length of time before the DNA is finally degraded is unknown, and may vary among species. Cattle may not degrade DNA

as readily as mice, because the proportion of positive mouse blastocysts is closer to the proportion of transgenic mice (Burdon and Wall, 1992; Canseco et al., 1993), compared with cattle. There are differences in the ability of bovine and porcine oocytes to ligate and degrade DNA (Powell et al., 1992). Bovine embryos may create such large ligation products that they are unable to degrade them as quickly as do mouse embryos.

In conclusion, the results of this study indicate that although DNA detection frequencies were not different, injecting embryos at early pronuclear stages resulted in greater developmental efficiencies. Injecting embryos at 11 haf may assist in increasing the overall efficiency of producing transgenic dairy cattle.

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REFERENCES

Bedford, J.M. 1982. Fertilization. In: Austin CR, Short RV (ed.), Reproduction in mammals 1: Germ cells and fertilization. Cambridge: Cambridge University Press;156.

Bialy, H. 1991. Transgenic pharming comes of age. Biotechnology 9:786.

Bishop, J.O., and P. Smith. 1989. Mechanism of chromosomal integration of microinjected DNA. Mol. Biol. Med. 6:283-298.

Bondioli, K.R., K.A. Biery, K.G. Hill, K.B. Jones, and F.J. DeMayo. 1988. Production of transgenic cattle by pronuclear injection. In: First NL, Haseltine FP (ed.), Transgenic Animals. Boston: Butterworth-Heinemann; 265-273.

Brinster, R.L., H.Y. Chen, M.E. Trumbauer, M.K. Yagle, and R.D. Palmiter. 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Natl. Acad. Sci., USA 82:4438-4442.

Burdon, T.G., and R.J. Wall. 1992. Fate of microinjected genes in preimplantation mouse embryos. Mol. Reprod. Dev. 33:436-442.

Canseco, R.S., A.E.T. Sparks, R.L. Page, C.G. Russell, J.L. Johnson, W.H. Velander, R.E. Pearson, W.N. Drohan, and F.C. Gwazdauskas.

1993. Gene transfer efficiency during gestation and the influence of co-transfer of non-manipulated embryos on production of transgenic mice. Transgenic Res. (in press).

Eyestone, W.H., M.L. Leibfried-Rutledge, D.L. Northey, B.G. Gilligan, and N.L. First. 1987. Culture of one and two cell bovine embryos to the blastocyst stage in the ovine oviduct. Theriogenology 28:1-7.

Gagne, M., F. Pothier, and M.A. Sirard. 1990. Developmental potential of early bovine zygotes submitted to centrifugation and microinjection following in vitro maturation of oocytes. Theriogenology 34:417-425.

Gordon, K., E. Lee, S.A. Vitale, A.E. Smith, H. Westphal, and L. Henninghausen. 1987. Production of human tissue plasminogen activator in transgenic mouse milk. Biotechnology 5:1183-1187.

Hill, K.G., J. Curry, F.J. DeMayo, K. Jones-Diller, J.R. Slapak, and K.R. Bondioli. 1992. Production of transgenic cattle by pronuclear injection. Theriogenology 37:222.

Jänne, J., J.M. Hyttinen, T. Peura, M. Tolvanen, L. Alhonen, and M. Halmekytö. 1992. Transgenic animals as bioproducers of therapeutic proteins. Ann. Med. 24:273-280.

Krimpenfort, P., A. Rademakers, W. Eyestone, A.S. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. Platenburg, F. Pieper, R. Strijker, and H. de Boer. 1991. Generation of transgenic dairy cattle using in vitro embryo production. Biotechnology 9:844-847.

Lewis-Williams J., T. Houseal, M. Harvey, P. DiTullio, and C. Ziomek. 1993. Selection of preimplantation mouse embryos using flourescence in situ hybridization. Theriogenology 39:258.

McLaren, A. 1982. The embryo. In: Austin CR, Short RV (ed.), Reproduction in mammals 2: Embryonic and fetal development. Cambridge: Cambridge University Press; 3.

Mermillod, P., C. Wils, A. Massip, and F. Dessy. 1992. Collection of oocytes and production of blastocysts in vitro from individual, slaughtered cows. J. Reprod. Fert. 96:717-723.

Powell, D.J., C. Galli, and R.M. Moor. 1992. The fate of DNA injected into mammalian oocytes and zygotes at different stages of the cell cycle. J. Reprod. Fert. 95:211-220.

Saeki, K., H. Kato, Y. Hosoi, M. Miyake, K. Utsumi, and I. Iritani. 1991. Early morphological events of in vitro fertilized bovine oocytes with frozen thawed spermatozoa. Theriogenology 35:1051-1058.

Saiki, R.K., P.S. Walsh, C.H. Levenson, and H.A. Ehrlich. 1989. Genetic analysis of amplified DNA with immobilized sequence-specific oligonucleotide probes. Proc. Natl. Acad. Sci., USA 86:6230-6234.

SAS Institute, Inc. 1985. SAS User's guide: Statistics. Cary, NC.

Simons, J., M. McClenaghen, and A. Clark. 1987. Alteration of the quality of milk expression of ß-lactoglobulin in transgenic mice. Nature 328:530-532.

Velander, W.H., J.L. Johnson, R.L. Page, C.G. Russell, A. Subramanian, T.D. Wilkins, F.C. Gwazdauskas, C. Pittius, and W.N. Drohan. 1992. High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. Proc. Natl. Acad. Sci., USA 89:12003-12007.

Voelkel, S.A., and Y.X. Hu. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. Theriogenology 37:1117-1131.

Wall, R.J., V.G. Pursel, A. Shamay, R.A. McKnight, C.W. Pittius, and L. Hennighausen. 1991. High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. Proc Natl. Acad. Sci., USA 88:1696-1700.

Williams, B.L., A.E.T. Sparks, R.S. Canseco, J.W. Knight, J.L. Johnson, W.H. Velander, R.L. Page, W.N. Drohan, J.M. Young, R.E. Pearson, T.D. Wilkins, and F.C. Gwazdauskas. 1992. In vitro development of zygotes from prepubertal gilts after microinjection of DNA. J. Anim. Sci. 70:2207-2211.

Wilmut, I., M.L. Hooper, and J.P. Simons. 1991. Genetic manipulation of mammals and its application in reproductive biology. J. Reprod. Fert. 92:245-279.

Xu, K.P., and T. Greve. 1988. A detailed analysis of early events during in-vitro fertilization of bovine follicular oocytes. J. Reprod. Fert. 82:127-134.

CHAPTER IV.

GENE MICROINJECTION INTO THE GERMINAL VESICLE OF BOVINE OOCYTES

ABSTRACT

Microinjection of DNA into the germinal vesicle of bovine oocytes was conducted to determine the effect of the procedure on subsequent development to the morula and blastocyst stages and detection of the exogenous DNA. Oocytes were held in meiotic arrest with dimethylaminopurine (DMAP) for 18 h after they were aspirated from abbatoir ovaries. Upon removal from DMAP, a portion of the oocytes were vortexed to remove cumulus cells (denuded oocytes) and centrifuged to visualize pronuclei. One half of these oocytes were then microinjected with a whey acidic protein-human protein C genomic DNA construct (denuded injected oocytes). Injected and non-injected denuded oocytes were placed into maturation medium with cumulus-intact oocytes (controls). Oocytes were fertilized 22-24 h after maturation with frozen/thawed semen prepared with a Percoll separation procedure. After culture for 7 d on buffalo rat liver cells, embryos were assessed for stage of development and assayed for the presence of the transgene with Polymerase Chain Reaction (PCR). A higher percentage of control embryos developed to the morula or blastocyst stage than did denuded, non-injected embryos (90/385; 23.4% versus 23/250; 9.2%; $p<0.01$). Both of these control groups had a higher developmental percentage than denuded, DNA-injected embryos (12/259; 4.6%;

p<0.01). Overall detection frequency of the gene construct with PCR was 37/58 (63.8%). There was no difference in development between embryos testing positive for the transgene and those testing negative. The procedure described for the injection of bovine oocytes before fertilization results in viable embryos containing the transgene, although at low frequencies.

INTRODUCTION

Pronuclear microinjection, the current method used for producing transgenic cattle, is difficult, costly, and inefficient. Only 0.2% of the oocytes that are used in microinjection programs become transgenic calves (Bondioli et al., 1988; Krimpenfort et al., 1991). Because transgenic cattle that produce pharmaceutical proteins in their milk have enormous commercial potential, a method is needed to produce these animals more efficiently.

Injection of DNA into pronuclei may not be the optimum method for introducing transgenes. In vitro-fertilized bovine zygotes are usually microinjected from 18-24 h after fertilization (Gagne et al., 1990; Hill et al., 1992). Replication, which is thought to occur approximately 12 hours after fertilization, has been hypothesized as an opportunity for foreign DNA to integrate. If this is the case, pronuclear injection would not place the DNA in contact with replicating chromosomes until after the first cleavage division, which ocurs approximately 28 h after fertilization (Xu and Greve, 1988). If DNA integrates after the first cleavage division, mosaic animals may

result (Burdon and Wall, 1992). In the mouse model, DNA degradation is slight over the initial 24 h (Chen et al., 1986). If the same is true in the bovine, injection of DNA into the germinal vesicle may provide an opportunity to ensure its presence at the first replication. The present study was designed to determine the effect of DNA microinjection into the germinal vesicle of bovine oocytes on subsequent embryo development in vitro and detection of the exogenous DNA.

MATERIALS AND METHODS

Bovine oocytes were purchased (Golden Genes, Riverdale, CA); they were shipped overnight in 1.5 ml of meiotic arrest medium that was held at 39°C in a portable incubator (Minitube of America, Madison, WI). Meiotic arrest medium consisted of TCM199 (Gibco, Grand Island, NY) supplemented with 10% vol/vol fetal calf serum (FCS; HyClone, Logan, UT), 333 μg/ml of dimethylaminopurine (DMAP; Sigma Chemical, St. Louis, MO; Fulka et al., 1991) and 1% vol/vol penicillin-streptomycin (Gibco).

Approximately 18 h after aspiration from the ovary, oocytes were removed from meiotic arrest medium and washed four times in TL HEPES medium. Nearly 50% of the oocytes were placed directly into maturation medium. The remaining oocytes were vortexed to remove cumulus cells and centrifuged at 12,000 x g for 6 min to visualize the germinal vesicle. One half of the vortexed oocytes were microinjected, and one half used as non-injected controls.

Manipulations were performed in TL HEPES medium on a heated microscope stage. Injected and non-injected oocytes were matured for 24 h with cumulus intact oocytes at a ratio of 1:1 to assist in maturation. Maturation medium consisted of TCM199 supplemented with 10% fetal calf serum, bFSH, bLH (0.01 U/ml of gonadotropin each: Nobl Labs, Souix Center, IA), and 1% vol/vol penicillin-streptomycin.

The DNA construct used for microinjection was a 12 kb fragment consisting of genomic human protein C (hPC) under the control of the murine whey acidic protein (WAP) promoter. The murine WAP-genomic human Protein C fusion gene was designed by Henryk Lubon and cloned in the Holland Laboratory, The American Red Cross (Rockville, MD).

After maturation, oocytes were washed three times in TL HEPES medium and placed into fertilization medium (Bavister and Yanagimachi, 1977), approximately 65 oocytes in 500 µl. A Percoll-separation procedure was used to prepare frozen/thawed bull semen for in vitro fertilization (Mermillod et al., 1992). Frozen 0.5 ml straws of semen were thawed at 35°C for 1 min. Spermatozoa were layered on top of a percoll (Sigma Chemical) density gradient in a 15 ml centrifuge tube and centrifuged for 30 min at 700 x g. After centrifugation, live spermatozoa concentration was determined. Spermatozoa were added to fertilization wells with the washed oocytes to give a final concentration of 0.5 x 10^6 sperm/ml. Heparin

(4.0 mg/ml) and PHE (penicillamine, 20 mM; hypotaurine, 10 mM; epinephrine, 1 mM) were included.

Embryos were co-cultured in wells prepared with buffalo rat liver (BRL) cells (Voelkel and Hu, 1992). Medium used for co-culture consisted of TCM199, 10% FCS, 1% bovine serum albumin, 0.1 mM non-essential amino acids (Gibco), 2 mM glycine, 1 mM alanine (Sigma Chemical), and 1% vol/vol penicillin-streptomycin. Embryos were cultured 25 per well in Nunclon (Roskilde, Denmark) four well plates for 7 d in a 5% CO_2:95% air atmosphere at 39°C. On the fourth day of culture, embryos were moved to fresh culture wells. At the end of the culture period, embryos were assessed for stage of development.

Polymerase Chain Reaction (PCR) was used to determine the presence of the transgene in 58 embryos of various developmental stages (Saiki et al., 1989). Non-injected embryos were analyzed as negative controls. After examination for development, each embryo was transferred in 1 µl of medium into 4 µl of embryo-lysis buffer (20 mM Tris, pH 8, 0.9% vol/vol Tween 20, 0.9% Nonidet P-40, Sigma Chemical; 0.4 mg/ml of proteinase K, Amresco, Solon, OH) in a 0.5 ml microcentrifuge tube. Samples were covered with 25 µl of paraffin oil and frozen at -80°C until analysis. Embryos were digested at 55°C for 30 min, denatured for 15 min at 98°C, and held at 85°C until 20 µl of the PCR reaction mixture was added (1X Taq buffer {500 mM KCl, 100 mM Tris-HCl pH 8, 1% Triton X-100}, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 mM of each primer, and 25 units/ml of Taq polymerase)

for a final volume of 25 µl. The primers used to amplify a 559-bp target sequence in the transgene were human ProC 5'2 (hPC-specific sense, 5'-TGG GAG AAG TGG GAG CTG GAC CTG-3') and ProC A9 (hPC-specific antisense, 5'-CAG CTC TTC TGG GGG GCT TCC TTG-3'). This was followed by 45 cycles of annealing (55°C for 2 min), elongation (75°C for 30 sec), and denaturation (96°C for 15 sec).

Statistical analysis was performed using GLM procedures in the Statistical Analysis System (SAS Institute, Inc., 1985). Developmental data were coded: 1 cell=1; 2 cells=2, 4 cells=3, 8 cells=4, 12-16 cells=5, compact morula=6, early blastocyst=7, blastocyst=8, expanded blastocyst=9, hatched blastocyst=10. Polymerase Chain Reaction results were also coded: negative=0, positive=1. Developmental data and PCR status were analyzed using a model including treatment and repetition. Differences between treatment means were evaluated using linear contrasts and tested with an improved Bonferroni F statistic.

RESULTS

Three embryo populations were cultured, including cumulus intact, and denuded non-injected and injected oocytes. At the end of the culture period, the developmental stage of all embryos was recorded (Table 1). Embryos derived from oocytes that were matured with intact cumulus cells and not injected (control) exhibited improved development over embryos derived from oocytes which were denuded and not injected, and both groups had

Table 1. Development of embryos microinjected at the germinal vesicle (gv) stage and matured and fertilized in vitro.

	Number of Embryos		
cell stage	control[a]	non injected	gv injected
1	144	96	177
2	55	51	23
4	41	39	20
8	40	32	20
12-16	15	9	7
compact morula	6	3	2
early blastocyst	7	1	2
blastocyst	18	5	3
exp. blastocyst[b]	30	11	4
htc. blastocyst[c]	28	3	1
total	385	250	259
dev[d]/total (%)	89/385 (23.1)	23/250 (9.2)	12/259 (4.6)
developmental score ± SEM	3.6 ± 3.1[e]	2.7 ± 2.2[f]	1.9 ± 1.7[g]

[a] cumulus intact

[b] exp. = expanded

[c] htc. = hatching

[d] Number of embryos developing to the compact morula stage or further.

[e,f,g] Treatments with different superscripts differ at $p<0.01$.

greater development than embryos which had been denuded and injected before maturation ($p<0.01$). There was no difference in development between injected embryos testing positive for the transgene and those testing negative.

The percentage of embryos detected as positive for the exogenous DNA by PCR from all developmental stages at d 7 was 64% (37/58). After the completion of the culture experiment, additional oocytes were microinjected into the germinal vesicle for transfer to recipient cattle. After culture, 14 of 229 (6%) viable morulae were transferred non-surgically, one embryo per recipient, to the uterine horn ipsilateral to a palpable corpus luteum of a synchronous recipient. Embryos were transferred without biopsy and PCR analysis because of the high percentage of positive morulae and blastocysts detected in this experiment. Recipient heifers or cows were treated with 40 mg of prostaglandin $F_{2\alpha}$ i.m. (Lutalyse; Upjohn, Kalamazoo, MI) on d 6 to 16 of the estrous cycle to synchronize estrus with the day of fertilization. None of the recipients were confirmed pregnant by ultrasound on d 45.

DISCUSSION

Results from microinjection of the germinal vesicle of in vitro matured, in vitro fertilized bovine oocytes indicate that these embryos can survive to d 7 and contain the transgene. The developmental rate of oocytes that had DNA microinjected into the germinal vesicle (4.6%) was lower than the development of

pronuclear-injected embryos processed at the same time in our laboratory (61/477, 12.8%). The lack of pregnancies produced from these germinal vesicle injected embryos, though only a small number of transfers were performed, raises questions as to the long-term viability of these embryos. Based on previous results, our laboratory averages a pregnancy rate of 26% at 60 d of gestation with DNA microinjected embryos (Krisher et al., 1993). Another possibility is that the lack of cumulus cells during maturation severely compromises the ability of the resulting embryo to produce a pregnancy.

The frequency of transgene detection in this experiment was similar to other results obtained in this laboratory (Krisher et al., 1993). The percentage of d 7 embryos detected as positive for the exogenous DNA by PCR was greater than reported integration rates, based on live transgenic births. For this reason, it is not clear whether PCR results represent integrated DNA. Detected DNA could be the result of large ligation products, which are not degraded by the embryo. Powell et al. (1992) found that approximately 60% of the DNA injected into bovine germinal vesicles remained in a monomeric form, while the remaining DNA formed ligation products. Another possibility is that PCR represents integrated DNA, and the cells containing the transgene die or become allocated to extra-embryonic tissues later in development. This hypothesis seems plausible, as mosaicism in transgenic animals has been reported to be as high as 60% (Whitelaw et al., 1993). Mosaicism could occur if the integration event takes place after the first round of DNA replication.

If DNA is able to persist in bovine embryos for long periods it may be available for integration at many times during embryo development, leading to a mosaic animal. Unequal segregation of the positive cells to the placenta could occur, or the positive cells could die, without compromising the fetus (Wilkie et al., 1986; Whitelaw et al., 1993). There have been two reports describing positive fetuses but negative placentas in mice and cattle (Bondioli et al., 1988; Canseco et al., 1993).

In conclusion, the results of this study indicate that the germinal vesicle of bovine oocytes can be microinjected, and the resulting embryo can successfully develop to d 7 in culture and contain the transgene. However, in our laboratory, this technique appears to be inferior to pronuclear microinjection in the production of transgenic embryos, due to reduced developmental efficiencies.

REFERENCES

Bavister, B.D., and R. Yanagimachi. 1977. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa in vitro. Biol. Reprod. 16: 228-237.

Bondioli K.R., K.J. Hill, K.B. Jones, and F.J. DeMayo. 1988. Production of transgenic cattle by pronuclear microinjection. In: Transgenic Animals. F. P. H. N.L. First, Eds. Butterworth-Heineman, Stoneham, MA, 265-273.

Burdon, T.G., and R.J. Wall. 1992. Fate of microinjected genes in preimplantation mouse embryos. Mol. Reprod. Dev. 33: 436-442.

Canseco, R.S., R.L. Page, C.G. Russell, J.L. Johnson, W.H. Velander, R.E. Pearson, W.N. Drohan, F.C. Gwazdauskas. 1993. Gene transfer efficiency during gestation and the influence of co-transfer of non-manipulated embryos on production of transgenic mice. Transgenic Res., in press.

Chen, H.Y., M.E. Trumbauer, K.M. Ebert, R.D. Palmiter, and R.L. Brinster. 1986. Developmental changes in the response of mouse eggs to injected genes. In: Molecular Developmental Biology. L. Bogorad, Eds. Alan R. Liss, Inc., New York, 149-159.

Fulka, J., M.L. Leibfreid-Rutledge, and N.L. First. 1991. Effect of 6-dimethylaminopurine on germinal vesicle breakdown of bovine oocytes. Mol. Reprod. Dev. 29: 379-384.

Gagne, M., F. Pothier, and M.A. Sirard. 1990. Developmental potential of early bovine zygotes submitted to centrifugation and microinjection following in vitro maturation of oocytes. Theriogenology 34: 417-425.

Hill, K.G., J. Curry, F.J. DeMayo, K. Jones-Diller, J.R. Slapak, and K.R. Bondioli. 1992. Production of transgenic cattle by pronuclear microinjection. Theriogenology 37: 222.

Krimpenfort, P., W. Eyestone, A. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. Platenburg, F. Pieper, R. Strijker, and H. de Boer. 1991. Generation of ransgenic dairy cattle using in vitro embryo production. Bio/Technology 9: 844-847.

Krisher, R.L., J.G. Gibbons, F.C. Gwazdauskas, C.G. Russell, T.D. Wilkins, and W.H. Velander. 1993. Influence of time of gene microinjection on development and DNA integration in bovine embryos. Biol. Reprod. 48 (suppl. 1): 172.

Mermillod, P., C. Wils, A. Massip, and F. Dessy. 1992. Collection of oocytes and prouction of blastocysts in vitro from individual, slaughtered cows. J. Reprod. Fert. 96: 717-723.

Powell, D.J., C. Galli, and R.M. Moor. 1992. The fate of DNA injected into mammalian oocytes and zygotes at different stages of the cell cycle. J. Reprod. Fert. 95: 211-220.

Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of b-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

SAS Institute, Inc. 1985. SAS Users Guide: Statistics. Cary, NC.

Voelkel, S.A., and Y.X. Hu. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. Theriogenology 37:1117-1131.

Whitelaw, C.B.A., A.J. Springbett, J. Webster, and J. Clark. 1993. The majority of Go transgenic mice are derived from mosaic embryos. Transgenic Res. 2:29-32.

Wilkie, T.M., R.L. Brinster, and R.D. Palmiter. 1986. Germline and somatic mosaicism in transgenic mice. Dev. Biol. 118:9-18.

Xu, K.P., and T. Greve. 1988. A detailed analysis of early events during in vitro fertilization of bovine follicular oocytes. J. Reprod. Fert. 82:127-134.

CHAPTER V.

DNA DETECTION FREQUENCY IN MICROINJECTED BOVINE EMBRYOS FOLLOWING EXTENDED CULTURE IN VITRO

ABSTRACT

Attempts to accurately detect integrated transgenes by polymerase chain reaction (PCR) in bovine embryos after pronuclear DNA injection have been frustrated by an apparent high frequency of positive results. The objective of this experiment was to test whether the frequency of detection of PCR positive results in whole bovine embryos would decline over a 21 d culture period. After in vitro maturation (22-24 h) and in vitro fertilization (14-16 h), embryos were microinjected with either a murine whey acidic protein-human protein C (WAP-hPC) genomic DNA construct or an ovine ß-lactoglobulin-human alpha-lactalbumin (bLG-HA) genomic DNA construct. Noninjected embryos were used as controls. Approximately 5% of the injected embryos were prepared for PCR analysis immediately after injection (d 0). A 559-bp (hPC) or a 407-bp (HA) sequence was targeted for PCR analysis. After culture for 7 d on Buffalo Rat liver (BRL) cells, embryos were assessed for stage of development attained. Embryos developing to the morula or blastocyst stage were assigned randomly to 2 groups; PCR analysis (d 7) or extended culture. Extended culture embryos were cultured for an additional 7 d. Embryos maintaining a blastocoel at d 14 were considered viable, and were again assigned randomly to either further culture or PCR analysis. All remaining embryos were

analyzed on d 21. On d 21, viable embryos were those maintaining a blastocoel. Statistical analysis was performed using Chi-square. Of zygotes examined after d 7 of culture, 25.8% of control and 11.7% of injected embryos had developed to the morula or blastocyst stage (control vs. injected; $p<0.01$). After 14 d of culture, 33.3% of control and 50.8% of injected embryos were viable ($p>0.05$). After 21 d of culture, 28.6% of control and 13.6% of injected embryos were viable ($p>0.05$). Analysis of d 0 injected embryos resulted in a transgene detection frequency of 100%. At d 7, detection frequency was 84% of morulae and blastocysts, and was 83% in non-viable embryos. Detection frequencies decreased to 49% and 50% on d 14, and 38% and 22% on d 21 in viable and non-viable embryos, respectively. Transgene detection frequency between viable embryos on d 0, 7, and 14 was different ($p<0.01$). The PCR-based transgene detection frequency declined during a 21-d culture period following pronuclear DNA injection. DNA detection frequency in microinjected bovine embryos by PCR analysis does not give a reliable indication of live transgenic births, based on reported rates, after 7 d in culture.

Key Words: Transgenic cattle, Polymerase Chain Reaction, Co-culture

INTRODUCTION

Production of heterologous proteins in the milk of transgenic livestock has enormous potential as a relatively inexpensive source of pharmaceutical proteins. To date, there have been successful reports of transgenic sheep (Niemann, et al., 1992; Wright, et al.,

1991), goats (Ebert, et al., 1991), swine (Velander, et al., 1992), and cattle (Krimpenfort, et al., 1991) that produce human proteins in their milk. Because of the large volume of milk produced by dairy cows, transgenic cattle would be the logical best choice as a commercially viable bioreactor. However, the production of transgenic cattle has proven to be particularly difficult. With other livestock species, which are multiple ovulators, in vivo systems to produce embryos for microinjection can be used. These manipulated embryos can immediately be transferred into a recipient animal. Because cattle produce only one offspring during their 9 month gestation period, such a system is cumbersome. In vitro maturation/in vitro fertilization procedures were used to provide a large number of bovine zygotes from abattoir-derived ovaries for pronuclear microinjection (Thomas et al., 1993; Hill et al., 1992; Krimpenfort et al., 1991). After in vitro culture, viable preimplantation stage embryos can be transferred nonsurgically to recipients for the duration of pregnancy.

The efficiency of producing live transgenic offspring from microinjected bovine zygotes is low (Krimpenfort et al., 1991). One method to increase the number of transgenic animals born while decreasing production costs would be to screen embryos for the transgene before transfer to a recipient animal. The polymerase chain reaction (PCR; Saiki et al., 1988) can be used to analyze biopsies of injected embryos for the presence of the transgene. Bovine embryos have been bisected, with one half used for PCR analysis and the other transferred to establish pregnancies (Sparks et al., 1993;

Jänne et al., 1992). With mice, PCR analysis of morulae and blastocysts overestimates the number of positive offspring born by approximately 10% (Burdon and Wall, 1992). In cattle, there are conflicting reports concerning the usefulness of PCR analysis to correctly identify embryos with integrated transgenes. Thomas et al. (1993) reported that PCR-positive blastocysts represent only 4.2% of microinjected blastocysts tested. Behboodi et al. (1993) report that 54% of bovine blastocysts that developed from microinjected ova were PCR positive. However, in our laboratory, 89% of morulae and blastocysts are PCR-positive (Krisher et al., 1993). The high percentage of apparent false positive PCR results makes it difficult to accurately detect integrated transgenes. The objective of this experiment was to determine if the detection frequency of PCR positive embryos would decline over an extended culture period in vitro.

MATERIALS AND METHODS

Bovine oocytes were purchased (EmTran, Inc., Elizabethtown, PA) and shipped overnight in 1 ml of maturation medium held in a temperature-controlled (39°C) portable incubator (Minitube of America, Madison, WI). Maturation medium consisted of TCM199 (Gibco, Grand Island, NY) supplemented with 10% vol/vol fetal calf serum (FCS; HyClone, Logan, UT), bFSH and bLH (0.01 U/ml each: Nobl Labs, Sioux Center, IA), and 1% vol/vol penicillin-streptomycin. Oocytes were matured for 24 h and then washed three times in TL

HEPES medium and placed into fertilization medium (Bavister and Yanagimachi, 1977), 50 oocytes in 500 μ l.

A Percoll-separation procedure was used to prepare frozen/thawed Holstein semen for in vitro fertilization. Frozen 0.5 ml straws were thawed at 35°C for 1 min. Semen was layered on top of a Percoll (Sigma Chemical, St. Louis, MO) density gradient (90%:45%) in a 15-ml centrifuge tube and centrifuged for 30 min at 700 x g. After centrifugation, live sperm concentration was determined. Sperm were added to fertilization wells with the washed oocytes to give a final concentration of 1.0 x 10^6 sperm/ml. Heparin (5 mg/ml) and PHE (penicillamine, 20 mM; hypotaurine, 10 mM; epinephrine, 1 mM; Bavister, 1989) were included.

Embryos were removed from fertilization medium at 14-16 h after fertilization, vortexed to remove cumulus cells and centrifuged at 12,000 x g for 6 min to permit visualization of pronuclei. All embryos were examined for the presence of two pronuclei before microinjection. Embryos without two pronuclei were not injected, classified as unfertilized, and cultured separately. Microinjections were performed in TL HEPES medium on a heated stage.

Two DNA constructs were used for microinjection. The first was a 12 kb fragment consisting of genomic human protein C (hPC) under the control of the murine whey acidic protein (WAP) promoter. The murine WAP-genomic human Protein C fusion gene was designed by Henryk Lubon and cloned in the Holland Laboratory, The American

Red Cross (Rockville, MD). The second construct was a 20.4 kb fragment consisting of genomic human alpha-lactalbumin (HA) under the control of the ovine ß-lactoglobulin (bLG) promoter. The bLG-genomic HA fusion gene was designed by Angelika Schnieke at Pharmaceutical Proteins Limited (Edinburgh, United Kingdom). Embryos were microinjected with 1 to 3 pl of the DNA solution (1.5 mg hPC/ml; 7.5 mg HA/ml).

After microinjection, embryos were cocultured in wells prepared with Buffalo Rat liver (BRL) cells as described by Voelkel and Hu (1992). Medium used for coculture consisted of TCM199, 10% FCS, 1% bovine serum albumin, 0.1 mM nonessential amino acids (Gibco), 2 mM glycine, 1 mM alanine (Sigma Chemical), and 1% vol/vol penicillin-streptomycin (Gibco). Embryos were cultured 25/well for the first 7 d in a 5% CO_2 in air atmosphere at 39°C. On the fourth day of culture embryos were moved to fresh culture wells. Embryos were assessed for stage of development after 7 d of culture. Nonviable embryos were prepared for PCR analysis. On d 7, morulae and blastocysts were considered viable embryos. Of embryos developing to the morula or blastocyst stage, approximately 30% were prepared for PCR analysis. The majority of viable control embryos were prepared as negative controls for PCR analysis on d 7. The remaining embryos were cultured for an additional 7 d on BRL cells, 1 per well. Embryos were examined for viability on d 14. There were three groups of embryos present at this time; alive, dead, and those that were no longer intact. Approximately 35% of both live and dead embryos were prepared for PCR analysis. The

remaining live embryos were cultured for an additional 7 d on BRL cells, 1 per well. On day 21, embryos were classified as live, dead, or not intact, and all embryos were prepared for PCR analysis. On d 14 and 21, embryos with a blastocoel were considered alive.

The PCR was used to determine the presence of the transgene in embryos of various developmental stages. Noninjected embryos were analyzed as negative controls. After determination of developmental classification, each embryo was transferred in 1 µl of medium to 4 µl of embryo-lysis buffer (20 mM Tris pH 8, 0.9% Tween 20, 0.9% Nonidet [Sigma] and 0.4 mg/ml of proteinase K [Amresco, Solon, OH]) in a 0.5 ml microcentrifuge tube. Samples were covered with 25 µl of paraffin oil and frozen at -80°C until they were analyzed. Initial digestion of the embryos was performed at 55°C for 30 min, followed by a 15 min denaturation period at 98°C, and held at 85°C until 20 µl of the PCR reaction mixture was added (1X Taq buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 mM of each primer, and 0.025 units/ml Taq polymerase) for a final volume of 25 µl. The primers used to amplify a 559-bp target sequence in the hPC transgene were human ProC 5'2 (5'-TGG GAG AAG TGG GAG CTG GAC CTG) and ProC A9 (5'-CAG CTC TTC TGG GGG GCT TCC TTG). For hPC, this was followed by 45 cycles of annealing (55°C for 1 min), elongation (75°C for 1 min 15 sec), and denaturation (96°C for 15 sec). The primers used to amplify a 407-bp target sequence in the HA gene construct were α11 (5'-GTC ACC AAG TGG TTA TTG AGG ATA TGC TG) and α12 (5'-GTG AGC ACT CCA GCC TGG CAA CAG AG). These two primers flank the Xho 1 site in the 3' non-coding region of

the gene construct, 183-bp upstream and 168-bp downstream. For HA, this was followed by 35 cycles of annealing (60°C for 1 min), elongation (72°C for 1 min), and denaturation (94°C for 45 sec), followed by a final cycle at 72°C for 10 min. A dilution series of restriction enzyme-digested plasmid for both constructs was used as a positive control, and control bovine DNA was used as a negative control. Day 21 embryos, both injected and control, were evaluated with PCR using primers to the bovine trophoblastic protein-1 (TP-1) to verify its presence. Amplification products from embryos were evaluated concurrently with those from positive and negative controls on 1% agarose gels stained with 0.5 mg/ml ethidium bromide.

Statistical analyses was performed on developmental percentages and PCR detection frequencies using Chi-square.

RESULTS

Embryo Development

Fertilization rates were monitored by examining the development of embryos that did not have two pronuclei at the time of microinjection (Table 1).

Development of control and injected embryos after 7, 14, and 21 d in culture are presented in Table 2. There was no statistically significant difference in development between embryos injected with

hPC and HA at any time point, so these data were combined. On d 7, control embryos had greater development than injected embryos (25.8% versus 11.7%; $p<0.01$). Of viable embryos that were placed into extended culture, 33% of control and 50.8% of injected embryos remained viable at d 14, whereas 28.6% of controls and 13.6% of injected embryos survived to d 21. The variation in development of control and injected embryos over 12 replicates is shown in Appendix Figure 1.

Transgene Detection

Detection of the transgene by PCR analysis in viable and nonviable embryos at 0, 7, 14, and 21 d of culture is presented in Figure 1. There was no difference in transgene detection frequency between the two constructs; therefore, PCR results were combined. Embryos that were classified as not intact did not display a positive signal after PCR analysis with TP-1 primers, and thus were discarded from the data set. At d 0, 100% (46/46) of the embryos contained the transgene. After 7 d of culture, 84% (51/62) of viable embryos and 83% (513/610) of nonviable embryos contained the transgene. After 14 d of culture, the transgene was detectable in 49% (18/37) of viable and 50% (41/82) of nonviable embryos. At the termination of culture on d 21, 38% (3/8) viable and 22% (5/23) nonviable embryos were PCR positive. When data for both viable and nonviable embryos were combined, there was a statistically significant decrease in PCR detection frequencies at each progressive time point ($p<0.05$). However, when only viable embryos were examined, there

was a significant decrease in detection of the transgene up to d 14 ($p<0.05$), but no difference ($p>0.05$) between detection rates on d 14 and d 21.

Table 1. Classification of zygotes examined for microinjection and resulting development of zygotes classified as unfertilized.

	Classification		Unfertilized
Total	Injected (%)	Unfertilized (%)	Viable/total (%)
2877	2509 (87.2)	368 (12.8)	25/305 (8.2)

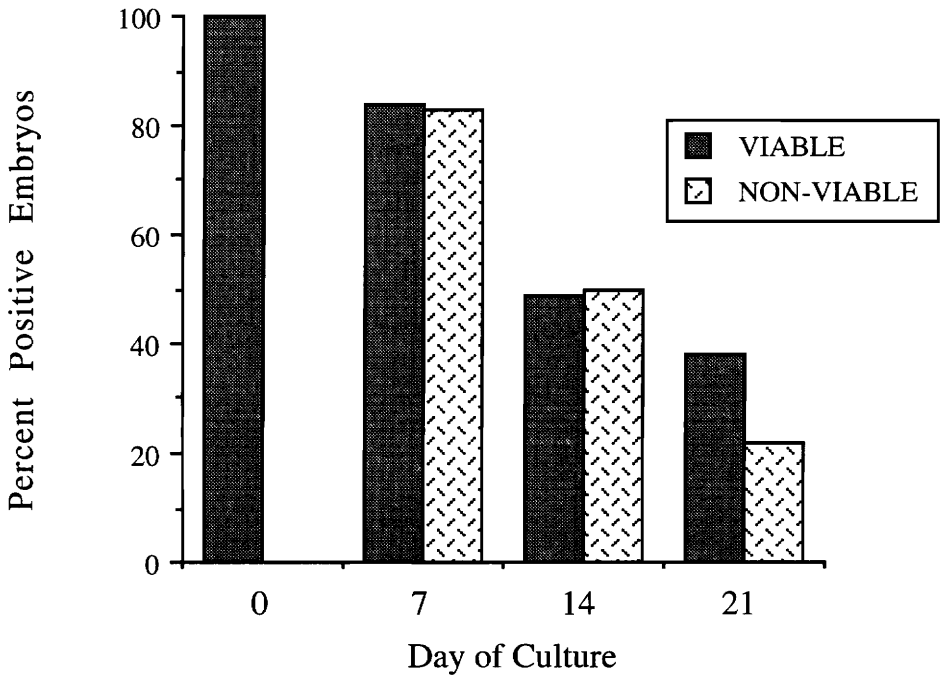
Table 2. Development of control and injected bovine embryos after extended culture in vitro.

	Day of culture viable/total (%)		
Treatment	day 7[a]	day 14[b]	day 21[b]
Control	189/733[c] (25.8)	10/30[c] (33.3)	2/7[c] (28.6)
Injected	294/2509[d] (11.7)	100/196[c] (50.8)	9/66[c] (13.6)

[a] Viable embryos on d 7 are morulae and blastocysts.

[b] Viable embryos on d 14 and 21 are those maintaining a blastocoel.

[c,d] Values with different superscripts within the same day of culture are different, $p<0.05$).



[a] Numbers above bars represent total number of embryos in that category.

[b,c,d] Values with different superscripts are different, $p<0.05$.

Figure 1. Transgene Detection Frequency in Microinjected Bovine Embryos After Extended Culture In Vitro[a].

DISCUSSION

Culturing microinjected bovine embryos for extended periods provides an opportunity to examine the fate of injected genes past the point when the embryos are usually transferred to a recipient. Although this is an artificial system, and the embryos do not develop as they normally would in utero, we believe that the transgenes behave similarly in both systems. Because the rate of transgene detection by PCR on d 7 was greater than reported transgenic rates in live calves, the fate of the transgene between the time of transfer and birth of the animal is unclear. The present experiment clarifies this question for the first three weeks following fertilization.

By injecting genes into pronuclear stage embryos, the intent is to allow integration of the foreign gene before the first round of replication. In cattle, DNA replication is thought to occur approximately 12 hours after fertilization. Unfortunately, injection at or before this time is difficult because of the absence or small size of the pronuclei and often results in the misidentification of fertilized ova as unfertilized. In our laboratory, PCR analysis could not distinguish any differences in transgene detection in embryos that had been microinjected at 11, 15, or 19 h after fertilization, although these results could not rule out the possibility that differences in integration rates did occur (Krisher et al., 1993). Studies in mice have shown that pronuclear microinjection most often results in integration after the first and before the second round of DNA

replication, resulting in a high number of mosaic mice (Whitelaw et al., 1993; Wilkie et al., 1986).

The fate of microinjected DNA immediately after injection has been investigated. Little degradation of the foreign DNA takes place in mouse embryos during the initial 24 h (Chen et al., 1986). It was thought that the injected DNA forms head to tail concatamers by an opportunistic repair-ligation mechanism before integration takes place (Bishop and Smith, 1989). These ligation products are detected almost immediately after injection in mice (Burdon and Wall, 1992). In cattle, the cytoplasm of pronuclear stage zygotes is efficient in ligating DNA into large concatamers (Powell, et al., 1992). During microinjection, DNA leaks out of the pronucleus and into the cytoplasm (Chen et al., 1986). It seems that DNA in the cytoplasm can exist in a stable form. The possibility that this cytoplasmic DNA may be capable of integrating into the genome has been raised, but there are no data at present to support this hypothesis (Powell et al., 1992). Microinjected genes that remain unintegrated segregate independently as the blastomeres of the embryo divide until they are either integrated or degraded (Burdon and Wall, 1992; Wilkie et al., 1986).

In mice, PCR is able to detect transgene products in all one-, two-, and four-cell injected embryos, but detection frequency drops to 44% of morulae and 26% of blastocysts tested (Burdon and Wall, 1992). The results in mice are similar to the results of this experiment with bovine embryos, although in a condensed time

interval. This may be a reflection of the increased time period of maternal control in bovine embryos relative to mice. In mice, expression of the embryonic genome begins at the two-cell stage (Bolton et al., 1984), while in cattle this does not occur until the 8-16 cell stage (Barnes and Eyestone, 1990). If cytoplasmic activities are required to support synthesis of DNA until activation of the embryonic genome (Powell et al., 1992), bovine embryos would have a longer period to form and stabilize cytoplasmic concatomers of the injected transgene than would mouse embryos. These large ligation products may then be able to persist for long periods in the cytoplasm. This could be one explanation for the high percentage of PCR positive results in this experiment, and the extended time period for these positive results to decline.

If this hypothesis accurately represents the fate of injected genes in bovine embryos, PCR as it is currently used is not an effective method for screening embryos because of the inability of this technique to distinguish between integrated and unintegrated transgenes. Another method must be developed to screen preimplantation stage embryos before transfer. A PCR-based method is desirable, because of the sensitivity and speed of the technique. Methods using PCR and based on the methylation patterns of the foreign gene have been considered, because the transgene has bacterial methylation patterns at the time of injection. It was originally thought that these patterns would only be replaced by mammalian methylation patterns upon integration and replication, but it has been shown that a change in methylation

patterns occurs regardless of integration status (Burdon and Wall, 1992). Fluorescent in situ hybridization also has been considered (Lewis-Williams et al., 1993), but only metaphase chromosome spreads would positively identify a transgene as integrated. It is difficult to successfully prepare a metaphase spread from an embryo biopsy of only a few cells. In addition, this method requires more time. Another method currently being researched is the microinjection of DNA containing dUTP (Cousens et al., 1993). Unintegrated uracil-DNA should be removed by uracil DNA glycosylase before PCR. A procedure that would allow the biopsied cells of an injected embryo to be processed so that unintegrated transgenes would be removed or destroyed before PCR analysis would be optimal.

In conclusion, these results demonstrate the slow decline of PCR-detectable transgenes in microinjected bovine embryos over a 21 d culture period. The results of this experiment indicate that PCR analysis on d 7 is not useful as a screening method for microinjected bovine embryos before transfer to a recipient.

REFERENCES

Barnes, F.L., and W.H. Eyestone. 1990. Early cleavage and the maternal zygotic transition in bovine embryos. Theriogenology 33:141-152.

Bavister, B. D. 1989. A consistently successful procedure for in vitro fertilization of golden hamster eggs. Gamete Res. 23:139-158.

Bavister, B. D., and R. Yanagimachi. 1977. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatazoa in vitro. Biol. Reprod. 16:228-237.

Behboodi, E., G.B. Anderson, S. Horvat, J.F. Medrano, J.D. Murray, and J.D. Rowe. 1993. Microinjection of bovine embryos with a foreign gene and its detection at the blastocyst stage. J. Dairy Sci. 76:3393-3399.

Bishop, J.O., and P. Smith. 1989. Mechanism of chromosomal integration of microinjected DNA. Mol. Biol. Med. 6:283-298.

Bolton, V.N., P.J. Oades, and M.H. Johnson. 1984. The relationship between cleavage, DNA replication and activation of transcription in the 2-cell mouse embryo. J. Embryol. Exp. Morph. 79:139-163.

Burdon, T.G., and R.J. Wall. 1992. Fate of microinjected genes in preimplantation mouse embryos. Mol. Reprod. Dev. 33:436-442.

Chen, H.Y., M.E. Trumbauer, K.M. Ebert, R.D. Palmiter, and R.L. Brinster. 1986. Developmental changes in the response of mouse

eggs to injected genes. In L. Bogorad (ed):"Molecular Developmental Biology". New York: Alan R. Liss, 149-159.

Cousens, C., G.T. O'Neill, I. Wilmut, A.S. Carver, and I. Garner. 1993. Selection of pre-implantation G0 transgenic embryos. J. Reprod. Fert. (Suppl. 11): 29.

Ebert, K.M., J.P. Selgrath, P. DiTullio, J. Denman, T.E. Smith, M.A. Memon, J.E. Schindler, G.M. Monastersky, J.A. Vitale, and K. Gordon. 1991. Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: Generation of transgenic goats and analysis of expression. Bio/Technology 9:835-838.

Hill, K.G., J. Curry, F.J. DeMayo, K. Jones-Diller, J.R. Slapk, and K.R. Bondioli. 1992. Production of transgenic cattle by pronuclear injection. Theriogenology 37:222.

Jänne J, Hyttinen JM, Peura T, Tolvanen M, Alhonen L, Halmekytö 1992: Transgenic animals as bioproducers of therapeutic proteins. Ann Med 24:273-280.

Krimpenfort, P., A. Rademakers, W. Eyestone, A. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. Platenburg, F. Pieper, R. Strijker, and H. de Boer. 1991. Generation of transgenic dairy cattle using in vitro embryo production. Bio/Technology 9:844-847.

Krisher, R.L., J.R. Gibbons, F.C. Gwazdauskas, C.G. Russell, T.D. Wilkins, and W.H. Velander. 1993. Influence of the time of gene microinjection on development and DNA integration in bovine embryos. Biol. Reprod. 48(suppl 1):172.

Lewis-Williams, J., T. Houseal, M. Harvey, P. DiTullio, and C. Ziomek. 1993. Selection of transgenic preimplantation mouse embryos using fluorescence in situ hybridization. Theriogenology 39:258.

Niemann, H., R. Halter, K. Huter, E. Lemme, A. Guzik, and D. Paul. 1992. Generation of transgenic sheep harbouring the human blood clotting factor VIII gene. Theriogenology 37:265.

Powell, D.J., C. Galli, and R.M. Moor. 1992. The fate of DNA injected into mammalian oocytes and zygotes at different stages of the cell cycle. J. Reprod. Fert. 95:211-220.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mulis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Sparks, A.E.T., R.S. Canseco, C.G. Russell, J.L. Johnson, R.L. Page, W.H. Velander, M.L. McGilliard, and F.C. Gwazdauskas. 1993. Development of bovine morulae after bisection and biopsy and

assessment of DNA amplification by the polymerase chain reaction. Anim. Reprod. Sci. (In press).

Thomas, W.K., A. Schnieke, and G.E. Seidel, Jr.. 1993. Methods for producing transgenic bovine embryos from in vitro matured and fertilized oocytes. Theriogenology 40:679-688.

Velander, W.H., J.L. Johnson, R.L. Page, C.G. Russell, A. Subramanian, T.D. Wilkins, F.C. Gwazdauskas, C. Pittius, and W.N. Drohan. 1992. High level expression of a heterologous protein in the milk of transgenic swine using cDNA encoding human protein C. Proc. Natl. Acad. Sci., USA 89:12003-12007.

Voelkel, S.A., and Y.X. Hu. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. Theriogenology 37:1117-1131.

Whitelaw, C.B.A., A.J. Springbett, J. Webster, and J. Clark. 1993. The majority of G_0 transgenic mice are derived from mosaic embryos. Transgenic Res. 2:29-32.

Wilkie, T.M., R.L. Brinster, and R.D. Palmiter. 1986. Germline and somatic mosaicism in transgenic mice. Dev. Biol. 118:9-18.

Wright, G., A. Carver, D. Cottom, D. Reeves, A. Scott, P. Simons, I. Wilmut, I. Garner, and A. Coleman. 1991. High level expression of

active human alpha-1-antitrypsin in the milk of transgenic sheep. Bio/Technology 9:830-834.

CHAPTER VI.

NUCLEAR TRANSFER IN THE BOVINE USING MICROINJECTED DONOR EMBRYOS: ASSESSMENT OF DEVELOPMENT AND DNA DETECTION FREQUENCY

ABSTRACT

Bovine embryos that had been microinjected with DNA were examined for their potential use as donor embryos in nuclear transfer. Donor embryos were obtained from oocytes collected by transvaginal oocyte aspiration, matured and fertilized in vitro, microinjected with a murine whey acidic protein-human protein C (hPC) genomic DNA construct, and cultured in vitro on Buffalo Rat liver (BRL) cells. Blastomeres from these embryos were transferred into enucleated bovine abbattoir-derived oocytes 40 h after maturation with electrofusion. Developmental stage following 7 d of culture on BRL cells was recorded and resulting embryos prepared for PCR analysis. Embryos derived from microinjected donor embryos did not differ in development to the morula and blastocyst stage than those from control donor embryos (37/337; 11.0% versus 7/81; 8.6%: $p>0.05$). Of biopsies from 20 microinjected donor embryos, 19 were PCR positive for the injected DNA (95%). Of 37 embryos developing normally, only 12 were positive for the injected DNA (32.4%). These results indicate that microinjected embryos can be successfully used in a nuclear transfer program to produce

additional viable embryos, and that these embryos may be reliably screened by PCR for transfer to recipients.

Key words: transgenic, cloning, polymerase chain reaction

INTRODUCTION

Transgenic cattle that produce heterologous proteins in their milk have great value as a source of human pharmaceuticals. The low efficiency of integration into the embryonic genome, and the high cost of maintaining recipients carrying nontransgenic offspring are major are major limitations to this technology. The polymerase chain reaction (PCR; Saiki et al., 1988) is one technique that has been used to screen preimplantation embryos (Krisher et al., 1993). Only embryos containing the injected DNA are then transferred to recipients. However, DNA detection frequency by PCR is higher than expected based on live transgenic birth rates (Behboodi et al., 1993; Krisher et al., 1993). It is believed that unintegrated DNA may be responsible for these high detection frequencies. To date, no other reliable method has been developed to screen embryos before transfer.

For transgenic technology to be feasible in cattle a method is needed to produce a greater number of viable preimplantation embryos that can be reliably screened before transfer. Nuclear transfer, or cloning, of microinjected embryos offers this possibility. A single embryo developing successfully to the early morula stage

can be greatly multiplied by transferring each blastomere into an enucleated oocyte, fusing the two with an electrical pulse, and allowing the resulting embryo clones to develop (Prather et al., 1987; Bondioli et al., 1990). The additional culture period may provide time for unintegrated DNA to be degraded by the embryo. At this point, PCR could be successfully used to screen embryos before transfer. This experiment was designed to investigate the effect of using microinjected embryos as donor embryos for nuclear transfer and to test the effectiveness of PCR as a screening method on the resulting embryo clones.

MATERIALS AND METHODS

Donor Embryo Production

Donor embryos were produced entirely in vitro. Oocytes were obtained by ultrasound-guided transvaginal follicular aspiration. Aspiration was performed twice per week on 1 Jersey and 8 Holstein cows for a period of 12 weeks. Aspirations began on d 3 of the cycle (estrus=d 0). The ovaries of each cow were visualized using an Aloka 500V ultrasound machine (Corimetrics, Wallingford, CT). The follicles were visualized and aspirated with the aid of a 5 MHz sector scanner (Corimetrics) attached to a vaginal probe. The oocytes were collected through 17 ga, 50 cm needles affixed to the dorsal surface of the vaginal probe by a 16 ga needle guide. Fluid was aspirated directly into an embryo filter (PETS, Canton, TX). The oocytes were rinsed away from the filter with phosphate buffered saline (PBS; Gibco,

Long Island, NY) supplemented with 10% vol/vol newborn calf serum (NCS; Gibco), 1% vol/vol penicillin-streptomycin (pen-strep, Gibco) and 25 U/ml heparin (Sigma Chemical, St. Louis, MO). After the oocytes had been recovered, they were washed in TL HEPES medium and held at 39°C. Oocytes were then placed into 0.5 ml gassed maturation medium and transported to the laboratory in a temperature-controlled (39°C) portable incubator (Minitube of America, Madison, WI). Maturation medium consisted of TCM199 (Gibco) supplemented with 10% vol/vol fetal calf serum (FCS; HyClone, Logan, UT), bFSH and bLH (0.01 U/ml each: Nobl Labs, Sioux Center, IA), and 1% vol/vol penicillin-streptomycin (Gibco). Oocytes were matured for 24 h, washed three times in TL HEPES medium, and placed into fertilization medium (Bavister and Yanagimachi, 1977), 50 oocytes in 500 μ l.

A Percoll-separation procedure was used to prepare frozen/thawed Holstein semen for in vitro fertilization. Frozen 0.5 ml straws were thawed at 35°C for 1 min. Semen was layered on top of a Percoll (Sigma Chemicals) density gradient (90%:45%) in a 15 ml centrifuge tube and centrifuged for 30 min at 700 x g. After centrifugation, live sperm concentration was determined. Sperm were added to fertilization wells with the washed oocytes to give a final concentration of 1.0×10^6 sperm/ml. Heparin (5 mg/ml) and PHE (penicillamine, 20 mM; hypotaurine, 10 mM; epinephrine, 1 mM; Bavister, 1989) were included.

Embryos were removed from fertilization medium at 14 to 16 h after fertilization. Approximately 15% of the zygotes were placed directly into culture as controls at this time. The remaining zygotes were vortexed to remove cumulus cells and centrifuged at 12,000 x g for 6 min to permit visualization of pronuclei. Microinjections were performed in TL HEPES medium on a heated stage.

The construct used for microinjection was a 12 kb fragment consisting of genomic human protein C (hPC) under the control of the murine whey acidic protein (WAP) promoter. The murine WAP-hPC fusion gene was designed by Henryk Lubon and cloned in the Holland Laboratory, The American Red Cross (Rockville, MD). The genomic construct was purified by digestion with the restriction endonuclease Not I (Stratagene Cloning Systems, La Jolla, CA) followed by electrophoresis on a 1% LE agarose (FMC BioProducts, Rockland, ME) gel with 0.5 mg/ml ethidium bromide. Agarose containing the transgene was excised and the DNA purified using the Prep-a-Gene kit (BioRad Laboratories, Hercules, CA). The construct was then resuspended in buffer (10 mM Tris-HCl, pH 7.4; 0.25 mM EDTA; Brinster et al., 1985) and filtered through a 0.45 µm filter. Embryos were microinjected with 1 to 3 pl of the DNA solution (1.5 mg/ml DNA, at 100 copies/pl).

After microinjection, embryos were cocultured in wells prepared with Buffalo Rat Liver (BRL) cells as described by Voelkel and Hu (1992). Medium used for coculture consisted of TCM199, 10% FCS, 1% BSA, and 1% penicillin-streptomycin. Embryos were cultured

25/well for 5 d in a 5% CO_2 in air atmosphere at 39°C. On the fifth day of culture, embryos at the early morula stage were removed for nuclear transfer. The remaining embryos were moved to fresh BRL wells and cultured for an additional two days.

Recipient Oocytes

Oocytes used as cytoplasm donors were collected from abattoir ovaries. Bovine oocytes were purchased (Utah State University, Logan, UT) and shipped overnight in 1 ml gassed maturation medium as described above, held in a temperature-controlled (39°C) portable incubator (Minitube of America). Oocytes were matured for 22 to 24 h, and selected for the presence of the first polar body. Selected oocytes were placed in aging medium consisting of TCM199 (Gibco) supplemented with 10% vol/vol FCS (HyClone) and 1% vol/vol pen-strep (Gibco) for an additional 16 to 18 h (Barnes et al., 1993). Aged oocytes were enucleated in PBS supplemented with 5 µg/ml cytochalasin B (Sigma Chemical) using a bevelled micropipette to puncture the zona pellucida and aspirate a small amount of cytoplasm adjacent to the first polar body (Collas and Robl, 1990).

Nuclear Transfer

Embryos that had been selected as donors for nuclear transfer were placed into calcium/magnesium free PBS (Gibco) for 30 min before manipulations. Donor embryos along with enucleated oocytes were then placed into a 100 µl drop of cytochalasin B PBS (described

above) for micromanipulation. A bevelled micropipette was used to puncture the zona pellucida of the donor embryo. Cells were aspirated from the embryo and placed into an enucleated oocyte, one blastomere per oocyte (Wolfe and Kraemer, 1992). A biopsy of two cells from each donor embryo was left in the zona pellucida and used for PCR analysis.

Blastomere-oocyte (fusion) units were allowed to equilibrate in cell fusion medium (Wolfe and Kraemer, 1992) for 15 min before membrane fusion. Fusion units were then placed into fusion medium in a chamber consisting of two wire electrodes mounted on a plexiglass slide. The fusion units were manually aligned so that the two membranes to be fused were parallel to the two electrodes. Electrofusion was accomplished with a BTX (San Diego, CA) 200 cell fusion machine (110 V, 15 μs single pulse). Immediately after fusion embryos were transferred to BRL culture wells and cultured for 7 d as described above. Embryos were moved to fresh culture wells on d 4 of culture.

Polymerase Chain Reaction

Polymerase chain reaction (PCR; Saiki et al., 1989) was used to determine the presence of the transgene in 20 donor embryo biopsies and 288 cloned embryos of various developmental stages. Noninjected embryos were analyzed as negative controls. After examination for development, each embryo was transferred in 1 μl of medium to 4 μl of embryo-lysis buffer (20 mM Tris pH 8, 0.9%

Tween 20, 0.9% Nonidet [Sigma, St. Louis, MO], and 0.4 mg/ml proteinase [K, Amresco, Solon, OH] in a 0.5 ml microcentrifuge tube. Embryo biopsies were handled in the same manner. Samples were then covered with 25 µl of paraffin oil and frozen at -80°C until they were analyzed. Initial digestion of the embryos was performed at 55°C for 30 min followed by a 15 min denaturation period at 98°C, and held at 85°C until 20 µl of the PCR reaction mixture was added (water, 1X Taq buffer, 0.2 mM dNTP's, 1.5 mM $MgCl_2$, 0.5 mM of each primer, and 0.025 units/ml Taq polymerase) for a final volume of 25 µl. The primers used to amplify a 559-bp target sequence in the transgene were human ProC 5'2 (hPC-specific sense, 5'-TGG GAG AAG TGG GAG CTG GAC CTG) and ProC A9 (hPC-specific antisense, 5'-CAG CTC TTC TGG GGG GCT TCC TTG). This was followed by 45 cycles of annealing (55°C for 1 min), elongation (75°C for 1 min 15 sec), and denaturation (96°C for 15 sec). A dilution series of restriction enzyme digested plasmid was used as a positive control, and control bovine DNA was used as a negative control. Biopsies from control embryos were evaluated with PCR using primers to the endogenous bovine trophoblastic protein-1 (BTP-1) gene to verify the sensitivity of the PCR procedure with only two cells. Amplification products from embryos were evaluated concurrently with those from positive and negative controls on 1% agarose gels stained with 0.5 mg/ml ethidium bromide.

Statistical Analysis

Developmental and PCR data were analyzed by Chi-Square.

RESULTS

Development of cloned embryos from control and microinjected donor embryos is presented in Table 1. Five control donor embryos yielded 81 clones (16.2 per donor). Twenty microinjected donor embryos yielded 337 clones (16.9 per donor). Microinjected donor embryos (37/337, 11.0%) did not differ in development of resulting cloned embryos from control donor embryos (7/81, 8.6%; $p<0.05$). For each control embryo cloned, 1.4 viable embryos were produced. For each microinjected embryo cloned, 1.9 viable embryos were produced.

Developmental data also were examined based upon the number of fusions per donor embryo. Nuclear transfer embryos derived from donors producing at least 15 cloned embryos (n=12) had greater development to the morula and blastocyst stage (36/274, 13.1%) than embryos from donors producing 14 or fewer clones (n=8; 1/63, 1.6%; $p<0.05$).

Data from PCR analysis are presented in Figure 1. Of 20 embryo biopsies analyzed by PCR, 19 (95%) were positive for the presence of the transgene. All 5 control embryo biopsies were positive for the endogenous TP-1 gene. Of 288 embryos at all developmental stages analyzed by PCR, 176 (61.1%) were positive for the transgene. Of the 37 viable embryos produced by nuclear transfer, 12 (32.4%) were positive for the transgene. The number of PCR positive embryos decreased as cell number increased, while the

number of negative embryos increased as cell number decreased. In other words, embryos testing positive for the presence of the transgene had a greater number of one cell embryos and fewer viable embryos than those embryos testing negative ($p<0.05$).

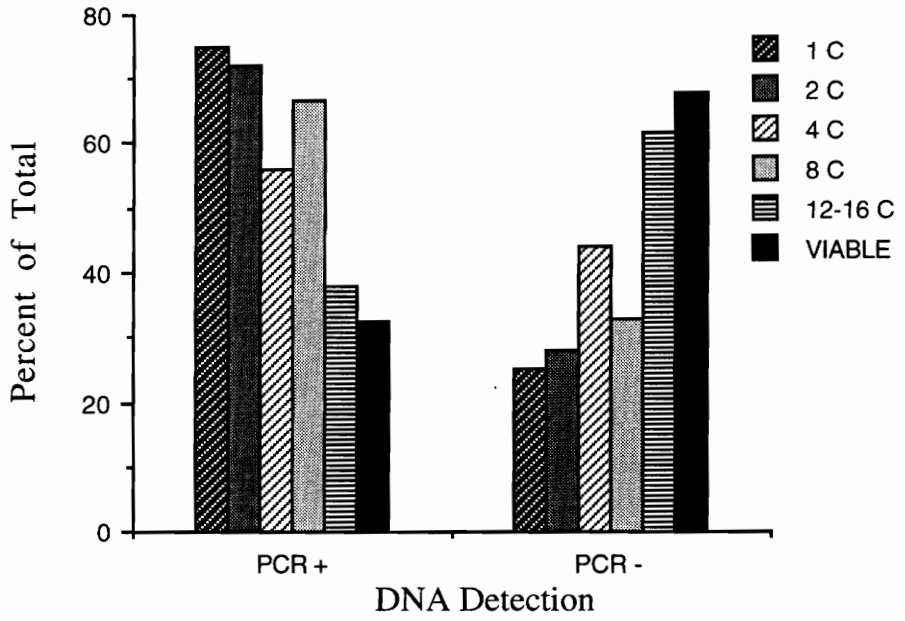
The 12 positive, viable, cloned embryos produced were derived from 7 different positive donor embryos. The distribution of viable, nonviable, and PCR positive and negative nuclear transfer embryos for each positive microinjected donor embryo is shown in Figure 2. Five of these seven donor embryos also produced viable embryos that tested negative for the presence of the transgene. Only two positive donor embryos produced exclusively positive viable clones, although both of these embryos produced nonviable embryos that tested negative. Three positive donor embryos produced no positive viable embryos of 13 viable embryos in total. Nine positive donor embryos produced no viable clones. Six of these nine donors produced 14 or fewer clones. All positive donor embryos produced cloned embryos (viable and nonviable) testing both positive and negative for the transgene. Figure 3 depicts the PCR analysis of embryos resulting from nuclear transfer of two microinjected, cloned embryos which tested positive for the transgene. Embryo 01 produced 5 viable embryos, 2 of which tested positive for the transgene. Embryo 02 produced 3 viable embryos, 1 of which was positive for the transgene.

Table 1. Development of Nuclear Transfer Embryos from Control and Microinjected Donor Embryos After 7 d of Culture In Vitro.

Stage of Development	CONTROL	INJECTED
1 CELL	17	107
2 Cell	11	49
4 Cell	23	77
8 Cell	13	46
12-16 Cell	10	21
Morula	1	14
Early blastocyst	2	8
Blastocyst	3	11
Hatched blastocyst	1	4
TOTAL	81	337
Viable[a]/Total (%)	7/81 (8.6)[b]	37/337 (11.0)[b]

[a] Viable embryos are morulae and blastocysts on d 7 of culture

[b] Columns with different superscripts are different at $p<0.05$



[a] C = cell.

Figure 1. Percentage of Embryo Stages with Positive or Negative PCR analyses of Nuclear Transfer Embryos from Microinjected Donors Positive for the Transgene.

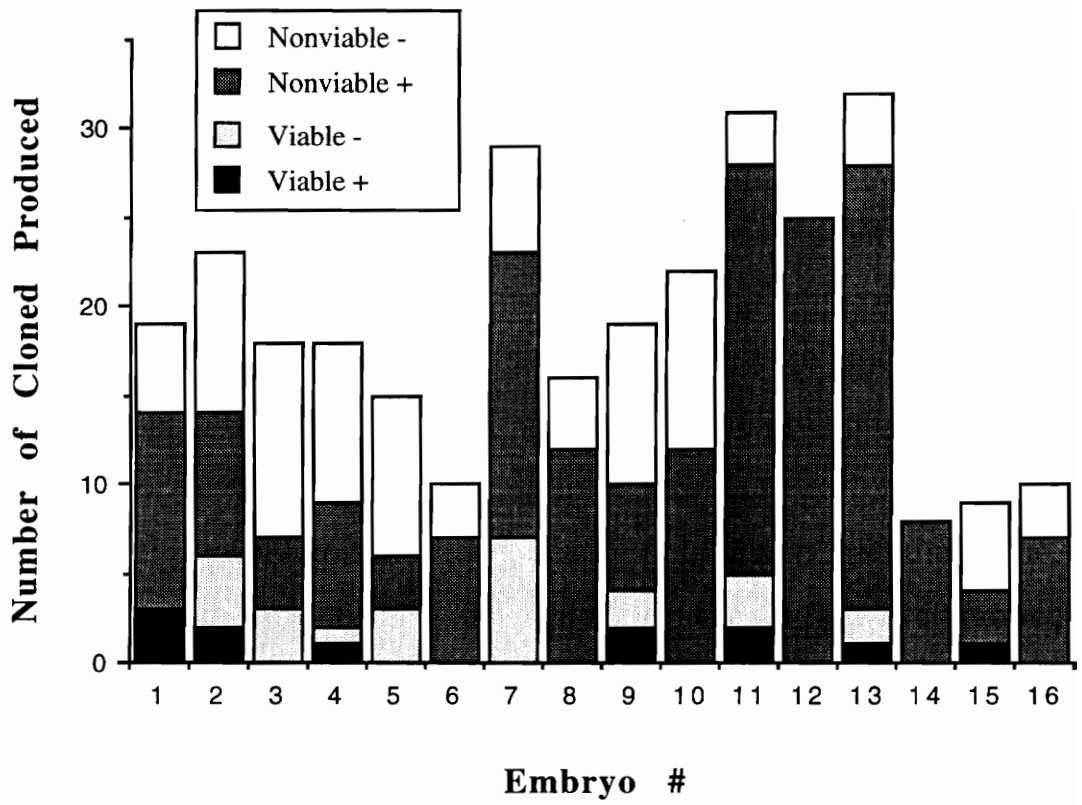


Figure 2. Distribution of Viable, Nonviable, and PCR Positive and Negative Clones from Each Positive, Microinjected Donor Embryo.

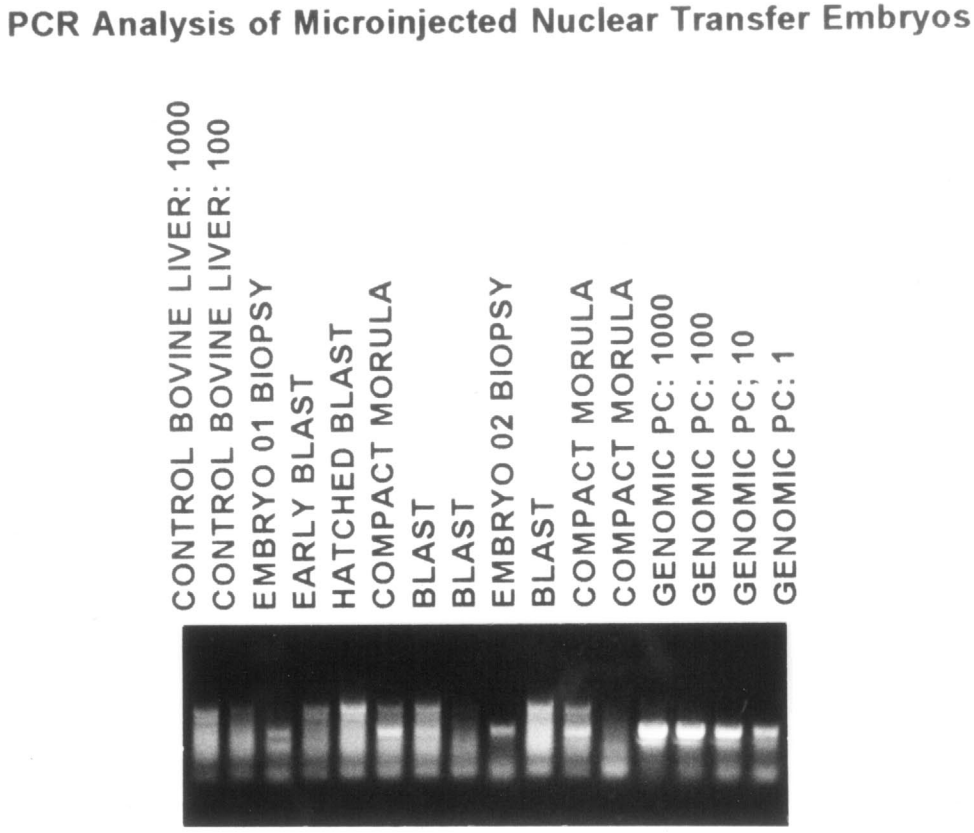


Figure 3. PCR Analysis of Viable Nuclear Transfer Embryos from Two Positive, Microinjected Donor Embryos.

DISCUSSION

The production of transgenic cattle is hampered by problems that make the procedures both labor intensive and extremely expensive to perform. In vitro maturation and in vitro fertilization procedures has been used successfully to produce transfer quality embryos and a transgenic calf (Krimpenfort et al., 1991; Behboodi et al., 1993; Thomas et al., 1993). However, efficiencies are still low (Krimpenfort et al., 1993). Many zygotes must be injected to produce a few transferrable embryos. Finally, there is currently no good method for screening embryos for the transgene before transfer to a recipient. Polymerase chain reaction analysis produces a high number of positive results, possibly because it detects unintegrated DNA as well as integrated transgenes (Behboodi et al., 1993; Krisher et al., 1993). Injected DNA may form large concatomers (Burdon and Wall, 1992), which may be difficult for the embryo to degrade. Positive PCR results decline over a 21-d culture period, possibly reflecting degredation of the DNA (Krisher et al., 1994). On d 7, the PCR may detect this unintegrated DNA as well as integrated transgenes. Nuclear transfer may help eliminate these problems.

This experiment demonstrates that microinjected embryos can be used succesfully in a nuclear transfer program. Injected embryos are no less viable than control embryos when used as donors for the cloning procedure. For each microinjected embryo cloned, 1.85 viable embryos were produced. Donor embryos having at least 17 blastomeres (2 for biopsy) produced 3 viable embryos per each

donor embryo cloned. Thus, the number of viable embryos available for transfer is tripled.

Another advantage of cloning microinjected embryos for the production of transgenic cattle is that PCR becomes a more reliable test. The additional 5 d of culture may allow the embryo additional time to degrade unintegrated DNA. Analysis of biopsied d 5 microinjected embryos revealed that 95% (19/20) of these embryos was positive for the transgene by PCR. After nuclear transfer, only 32.4% (12/37) of the clones produced from positive donor embryos was still positive by PCR analysis. By transferring only clones testing positive for the transgene, the number of recipients needed could be reduced and the efficiency of producing a transgenic calf may be increased. Even though fewer embryos are ultimately transferred, the possibility of producing a transgenic calf is increased. The PCR assay used in this study does not permit discrimination of integrated from unintegrated DNA. Only transfer of positive cloned embryos to produce transgenic calves will determine the reliability of PCR analysis of cloned embryos.

An observation made in this study is that the number of positive embryos decreases as cell number increases in cloned embryos. This suggests embryos that are positive for the transgene are less viable than negative embryos. In pronuclear injected embryos that have not been cloned, this effect is difficult to distinguish from the mechanical damage of the injection process. The loss of embryo viability due to integrated DNA may be an alternative

hypothesis for the disparity between DNA detection frequency at d 7 and reported transgenic birth rates. The PCR may detect integrated transgenes on d 7, but cells containing the gene may die later in fetal development, or may be allocated to extra-embryonic tissues.

Another facet of transgenic animal production is brought to light by this experiment. Because each nuclear transfer embryo is derived from a single blastomere, the transgenic composition of the donor embryo can be examined cell by cell. This study indicates that microinjected bovine embryos may be highly mosaic. From 30 to 60% of microinjected murine embryos have been found to be mosaic (Wilkie et al., 1986; Whitelaw et al., 1993) All positive donor embryos produced cloned embryos that were both positive and negative for the presence of the transgene. Therefore, simply because a cloned embryo is derived from a donor embryo that is PCR positive does not mean that it will be positive as well. In fact, there were twice as many negative (n=25) transferrable embryos produced by cloning PCR positive embryos than positive embryos clones (n=12). Nuclear transfer embryos reconstructed with a positive blastomere may not be mosaic, because all cells of the embryo are derived from the same cell. This is another advantage of using nuclear transfer technology in a bovine transgenic system.

In conclusion, microinjected embryos may be used successfully to produce cloned embryos in a nuclear transfer program. Embryos produced by nuclear transfer may be more accurately screened by PCR, a simple and expedient technique. Animals produced from

cloned embryos also may have the additional advantage of reduced mosaicism.

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REFERENCES

Barnes, F., M. Endebrock, C. Looney, R. Powell, M. Westhusin, and K. Bondioli. 1993. Embryo cloning in cattle; the use of in vitro matured oocytes. J. Reprod. Fert. 97:3317-320.

Bavister, B. D. 1989. A consistently successful procedure for in vitro fertilization of golden hamster eggs. Gamete Res. 23:139-158.

Bavister, B. D., and R. Yanagimachi. 1977. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatazoa in vitro. Biol. Reprod. 16:228-237.

Behboodi, E., G.B. Anderson, S. Horvat, J.F. Medrano, J.D. Murray, and J.D. Rowe. 1993. Microinjection of bovine embryos with a foreign gene and its detection at the blastocyst stage. J. Dairy Sci. 76:3393-3399.

Bondioli, K.R., M.E. Westhusin, and C.R. Looney. 1990. Production of identical bovine offspring by nuclear transfer. Theriogenology 33:165-174.

Collas, P. and J.M. Robl. 1990. Factors affecting the efficiency of nuclear transplantation in the rabbit embryo. Biol. Reprod. 43:877-884.

Krimpenfort, P., A. Rademakers, W. Eyestone, A. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. Platenburg, F. Pieper, R. Strijker, and H. de Boer. 1991. Generation of transgenic dairy cattle using in vitro embryo production. Bio/Technology 9:844-847.

Krisher, R.L., J.R. Gibbons, F.C. Gwazdauskas, and W.H. Eyestone. 1994. DNA detection frequency in microinjected bovine embryos following extended culture in vitro. Theriogenology 41:229.

Krisher, R.L., J.R. Gibbons, F.C. Gwazdauskas, C.G. Russell, T.D. Wilkins, and W.H. Velander. 1993 Influence of the time of gene microinjection on development and DNA integration in bovine embryos. Biol. Reprod. 48(suppl 1):172.

Prather, R.S., F.L. Barnes, M.M. Sims, J.M. Robl, W.H. Eyestone, and N.L. First. 1987 Nuclear transplantation in the bovine embryo: Assessment of donor nuclei and recipient oocyte. Biol. Reprod. 37:859-866.

Saiki, R.K., P.S. Walsh, C.H. Levenson, and H.A. Erlich. 1989. Genetic analysis of amplified DNA with immobilized, sequence specific oligonucleotide probes. Proc. Natl. Acad. Sci., USA 86:6230-6234.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mulis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Thomas, W.K., A. Schnieke, and G.E. Seidel, Jr. 1993. Methods for producing transgenic bovine embryos from in vitro matured and fertilized oocytes. Theriogenology 40:679-688.

Voelkel, S.A., and Y.X. Hu. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. Theriogenology 37:1117-1131.

Whitelaw, C.B.A., A.J. Springbett, J. Webster, and J. Clark. 1993 The majority of G_0 transgenic mice are derived from mosaic embryos. Transgenic Res. 2:29-32.

Wilkie, T.M., R.L. Brinster, and R.D. Palmiter. 1986. Germline and somatic mosaicism in transgenic mice. Dev. Biol. 118:9-18.

Wolfe, B.A., and D.C. Kraemer. 1992. Methods in bovine nuclear transfer. Theriogenology 37:5-15.

GENERAL CONCLUSIONS

The first experiment demonstrated that although transgene detection frequencies by PCR were not different, injecting embryos at early pronuclear stages resulted in greater developmental efficiencies. Injecting embryos at 11 h after fertilization may improve the overall efficiency of producing transgenic dairy cattle. This experiment also brought to light the high frequency of transgene detection by PCR in d 7 in vitro fertilized bovine embryos. Transgene detection by PCR does not indicate the integration of the transgene, only that it is present in the embryo. Analysis by PCR may detect unintegrated DNA, or the DNA may be integrated and positive cells later die or are subject to exclusion from the fetus proper.

Results of the second experiment indicateded that DNA injection into the germinal vesicle, followed by in vitro fertilization and culture, can result in successful development of embryos containing the transgene. However, this technique does not seem to be as efficient in production of embryos with the injected DNA as pronuclear microinjection.

The third experiment demonstrated the slow decline of PCR-detectable transgenes in microinjected bovine embryos over a 21-d culture period. This experiment confirmed that PCR analysis on d 7 is not useful as a screen for bovine embryos containing the integrated transgene before transfer to a recipient. These results are

consistent with reports which suggest that genes injected into embryos probably form large ligation products (Burdon and Wall, 1992) which are difficult for the embryo to destroy, and these ligation products may be what is being detected by PCR analysis on d 7. It seems to take the embryo at least three weeks to completely digest this exogenous DNA. This experiment points out the necessity of developing a screening method that can unequivocally identify integrated transgenes. These results also indicate that, if these injected genes are able to remain inside the embryo for extended periods of time, the opportunity to integrate into the genome is available over many cell cycles, thus increasing the possibility of producing a highly mosaic animal.

The last experiment in this series demonstrated that microinjected embryos can be used successfully as donor embryos in a nuclear transfer protocol. Embryos produced by nuclear transfer from injected donors may be more accurately screened by PCR, a currently available method that is both simple and expedient. Embryos produced by this technique may also have the additional advantage of reduced mosaicism, because all of the cells of the resulting embryo are derived from only one cell of the donor embryo.

In conclusion, the efficiency of producing transgenic cattle may be increased by microinjecting in vitro matured, in vitro fertilized embryos early in pronuclear formation, and entering the resulting embryos into a nuclear transfer protocol. The resulting embryos can

then be screened by existing PCR methods before transfer to recipient animals. Until a technique is developed to screen preimplantation embryos for integrated transgenes, these procedures represent the best method to decrease costs and increase efficiencies of transgenic cattle production.

REFERENCES

Abeydeera, L.R., K. Niwa, and K. Okuda. 1993. Maturation-promoting factor (MPF) is responsible for the transformation of sperm nuclei to metaphase chromosomes in maturing bovine oocytes in vitro. J. Reprod. Fert. 98:409-414.

Aktas, H., M. L. Leibfried-Rutledge, and N. L. First. 1992. A phorbol ester accelerates bovine oocyte maturation. In: 12'th International Congress on Animal Reproduction. p 297.

Aktas, H., M. L. Leibfried-Rutledge, M. B. Wheeler, C. F. Rosenkrans, Jr., and N. L. First. 1990. Maintenance of meiotic arrest in bovine oocytes. Biol. Reprod., Suppl. 42:91.

Anderson, E., and D. F. Albertini. 1976. Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. J. Cell Biol. 71:680-686.

Anderson, G. B. 1992. Isolation and use of embryonic stem cells from livestock species. Anim. Biotechnol. 3:165-175.

Aoyagi, Y., Y. Fukui, Y. Iwazumi, M. Urakawa, and H. Ono. 1990. Effects of culture systems on development of in vitro fertilized bovine ova into blastocysts. Theriogenology. 34:749-759.

Arnheim, N. 1992. Genetic mapping and preimplantation diagnosis in agriculturally important species using single cell PCR. Anim. Biotechnol. 3:55-65.

Atkinson, P. W., E. R. Hines, S. Beaton, K. I. Matthaei, K. C. Reed, and M. P. Bradley. 1991. Association of exogenous DNA with cattle and insect spermatozoa in vitro. Molec. Reprod. Dev. 29:1-5.

Bachiller, D., K. Schellander, J. Peli, and U. Ruther. 1991. Liposome mediated DNA uptake by sperm cells. Molec. Reprod. Dev. 30:194-200.

Bae, I-H., and R. H. Foote. 1975. Effects of hormones on the maturation of rabbit oocytes recovered from follicles of different sizes. J. Reprod. Fert. 42:357-360.

Ball, G. D., M. L. Leibfried, R. W. Lenz, R. L. Ax, B. D. Bavister, and N. L. First. 1983. Factors affecting succesful in vitro fertilization of bovine follicular oocytes. Biol. Reprod. 28:717-725.

Baribault, H., and R. Kemler. 1989. Embryonic stem cell culture and gene targetting in transgenic mice. Mol. Biol. Med. 6:481-492.

Barnes, F.L., P. Collas, R. Powell, W.A. King, M. Westhusin, and D. Shepherd. 1993. Influence of recipient oocyte cell cycle stage on DNA synthesis, nuclear envelope breakdown, chromosome constitution, and development in nuclear transplant bovine embryos. Molec. Reprod. Dev. 36:33-41.

Barnett, D.K., and B.D. Bavister. 1992. Hypotaurine requirement for in vitro development of golden hamster one-cell embryos into morulae and blastocysts, and production of term offspring from in vitro-fertilized ova. Biol. Reprod. 47:297-304.

Bavister, B. D., T. A. Rose-Hellekant, and T. Pinyopummintr. 1992. Development of in vitro matured/in vitro fertilized bovine embryos into morulae and blastocysts in difined culture media. Theriogenology. 37:127-147.

Bedford, J. M. 1982. Fertilization. In: C. R. Austin and R. V. Short (Ed.) Reproduction in mammals 1: Germ cells and fertilization. p 156. Cambridge University Press, Cambridge.

Behboodi, E., G.B. Anderson, S. Horvat, J.F. Medrano, J.D. Murray, and J.D. Rowe. 1993. Microinjection of bovine embryos with a foreign gene and its detection at the blastocyst stage. J. Dairy Sci. 76:3392-3399.

Betteridge, K.J., and Loskutoff, N.M. 1993. Prospects for improving the survival rate of transferred embryos. Molec. Reprod. Dev. 36:262-265.

Bevers, M. M., H. T. M. van Tol, and F. A. M. de Loos. 1992. Protein patterns of immature bovine oocytes incubated with ^{35}S-methionine in either medium or medium supplemented with dibutyryl-cyclic AMP or follicular fluid. In: 12'th International Congress on Animal Reproduction. p 312-314.

Bialy, H. 1991. Transgenic pharming comes of age. Biotechnology. 9:786-788.

Biery, K. A., K. R. Bondioli, and F. J. DeMayo. 1988. Gene transfer by pronuclear injection in the bovine. Theriogenology. 29:224.

Birnstiel, M. L., and M. Busslinger. 1989. Dangerous liasons: Spermatozoa as natural vectors for foreign DNA? Cell. 57:701-702.

Bishop, J. O., and P. Smith. 1989. Mechanism of chromosomal integration of microinjected DNA. Mol. Biol. Med. 6:283-298.

Blottner, S., H. Nehring, and H. Torner. 1990. Individual differences in capacitation of bull spermatozoa by heparin in vitro: relationship to fertility. Theriogenology. 34:619-628.

Boice, M. L., R. D. Geisert, R. M. Blair, and H. G. Verhage. 1990. Identification and characterization of bovine oviductal glycoproteins synthesized at estrus. Biol. Reprod. 43:457-465.

Boice, M. L., P. A. Mavrogianis, C. N. Murphy, H. G. Verhage, R. S. Prather, and B. N. Day. 1992. Immunocytochemical analysis of the association of bovine oviduct-specific secretory glycoproteins with early embryos. Theriogenology. 37:194.

Bondioli, K.R. 1993. Nuclear transfer in cattle. Molec. Reprod. Dev. 36:274-275.

Bondioli, K. R., K. A. Biery, K. G. Hill, K. B. Jones, F. J. DeMayo. 1988. Production of transgenic cattle by pronuclear injection. In: N. L. First, and F. P. Haseltine, (Ed.) Transgenic Animals. p 265. Butterworth-Heinemann, Boston.

Bondioli, K. R., M. E. Westhusin, and C. R. Looney. 1990. Production of identical bovine offspring by nuclear transfer. Theriogenology. 33:165-174.

Bondioli, K. R., and Wright, R. W. 1983. In vitro fertilization of bovine oocytes by spermatozoa capacitated in vitro. J. Anim. Sci. 57:1001-1005.

Bowen, R.A., M. Reed, A. Schnieke, G.E. Seidel, Jr., Z. Brink, and A. Stacey. 1993. Production of transgenic cattle from PCR-screened embryos. Theriogenology 39:194.

Brackett, B. G. 1983. A review of bovine fertilization in vitro. Theriogenology. 19:1-15.

Brackett, B. G., D. Bousquet, M. L. Boice, W. J. Donawick, J. F. Evans, and M. A. Dressel. 1982. Normal development following in vitro fertilization in the cow. Biol. Reprod. 27:147-158.

Brackett, B. G., A. I. Younis, and R. A. Fayrer-Hosken. 1989. Enhanced viability after in vitro fertilization of bovine oocytes matured in vitro with high concentrations of luteinizing hormone. Fert. Steril. 52:319-324.

Brantmeier, S. A., M. E. Bellin, S. K. Boehm, S. M. Bushmeyer, C. L. Kubajak, M. R. Dentine, R. R. Grummer, and R. L. Ax. 1987. Influence of stage of cycle, corpus luteum location, follicle size, and number of large follicles on estradiol-17b concentrations in bovine follicles. J. Dairy Sci. 70:2138-2144.

Brazolot, C. L., J. N. Petitte, R. J. Etches, and A. M. Verrinder Gibbins. 1991. Éfficient transfection of chicken cells by lipofection, and introduction of transfected blastoderm cells into the embryo. Mol. Reprod. Dev. 30:304-312.

Brem, G. 1993. Inheritance and tissue-specific expression of transgenes in rabbits and pigs. Molec. Reprod. Dev. 36:242-244.

Brinster, R. L., R. E. Braun, D. Lo, M. R. Avarbock, F. Oram, and R. D. Palmiter. 1989a. Targetted correction of a major histocompatability class II Ea gene by DNA microinjected into mouse eggs. Proc. Natl. Acad. Sci., USA. 86:7087-7091.

Brinster, R. L., H. Y. Chen, M. E. Trumbauer, M. K. Yagle, and R. D. Palmiter. 1985. Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Natl. Acad. Sci., USA. 82:4438-4442.

Brinster, R. L., E. P. Sandgren, R. R. Behringer, and R. D. Palmiter. 1989b. No simple solution for making transgenic mice. Cell. 59:239-241.

Brock, H., and L. E. Rowson. 1952. The production of viable bovine ova. J. Agric. Sci., Camb. 42:479-482.

Burdon, T.G., and R.J. Wall. 1992. Fate of microinjected genes in preimplantation mouse embryos. Molec. Reprod. Dev. 33:436-442.

Byrd, W. 1981. In vitro capacitation and the chemically induced acrosome reaction in bovine spermatozoa. J. Exp. Zool. 215:35-46.

Camaioni, A., M. A. Russo, T. Odorisio, F. Gandolfi, V. M. Fazio, and G. Siracusa. 1992. Uptake of exogenous DNA by mammalian spermatozoa: specific localization of DNA on sperm heads. J. Reprod. Fert. 96:203-212.

Camous, S., Y. Heyman, W. Meziou, and Y. Menezo. 1984. Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. J. Reprod. Fert. 72:479-485.

Campbell, K.H.S., W.A. Ritchie, and I. Wilmut. 1993. Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: Implications for deoxyribonucleic acid replication and development. Biol. Reprod. 49:933-942.

Castro, F. O., O. Hernandez, C. Uliver, R. Solano, C. Milanes, A. Aguilar, A. Perez, R. de Armas, L. Herrera, and J. de la Fuente. 1990. Introduction of foreign DNA into the spermatozoa of farm animals. Theriogenology. 34:1099-1110.

Chen, H. Y., M. E. Trumbauer, K. M. Ebert, R. D. Palmiter, and R. L. Brinster. 1986. Developmental changes in the response of mouse eggs to injected genes. In: L. Bogorad (Ed.) Molecular Developmental Biology. p 149. Alan R. Liss, Inc., New York.

Clark, A. J., Simons, P., I. Wilmut, and R. Lathe. 1987. Pharmaceuticals from transgenic livestock. Tibtech. 5:20-24.

Cousens, C., G.T. O'Neill, I. Wilmut, A.S. Carver, and I. Garner. 1993. Selection of preimplantation G0 transgenic embryos. J. Reprod. Fert. (Suppl. 11):29.

Critser, E. S., M. L. Leibfried-Rutledge, W. H. Eyestone, D. L. Northey, and N. L. First. 1986. Aquisition of developmental competence during maturation in vitro. Theriogenology. 25:150.

Crozet, N., J. Kanka, J. Motlik, and J. Fulka. 1986. Nucleolar fine structure and RNA synthesis in bovine oocytes from antral follicles. Gamete Res. 14:65-73.

Didion, B. A., and C. N. Graves. 1989. Influence of calcium on the true acrosome reaction and capacity of bull spermatozoa to penetrate oocytes in vitro. J. Dairy Sci. 72:1540-1546.

Ebert, K.M., and J.E.S. Schindler. 1993. Transgenic farm animals: Progress report. Theriogenology 39:121-135.

Ebert, K. M., J. P. Selgrath, P. DiTullio, J. Denman, T. E. Smith, M. E. Memon, J. E. Schindler, G. M. Monastersky, J. A. Vitale, and K. Gordon. Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: Generation of transgenic goats and analysis of expression. Biotechnology. 9:835-838.

Edwards, R. G. 1965. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey, and human ovarian oocytes. Nature. 208:349-351.

Ellington, J. E., E. W. Carney, P. B. Farrell, M. E. Simkin, and R. H. Foote. 1990a. Bovine 1-2-cell embryo development using a simple medium in three oviduct epithelial cell coculture systems. Biol. Reprod. 43:97-104.

Ellington, J. E., P. B. Farrell, and R. H. Foote. 1990b. Comparison of six day bovine embryo development in uterine tube (oviduct) epithelial cell culture versus in vivo development in the cow. Theriogenology. 34:837-844.

Ellington, J. E., G. G. Ignotz, W. B. Currie, and B. A. Ball. 1992. Spermatozoa induce the secretion of new proteins by bovine

oviductal epithelial cells during co-culture. In: 12'th International Congress on Animal Reproduction. p 635-637.

Epel, D. 1990. The initiation of development at fertilization. Cell Diff. Dev. 29:1-12.

Eyestone, W. H., and N. L. First. 1986. A study of the 8- to 16-cell developmental block in bovine embryos cultured in vitro. Theriogenology. 25:152.

Eyestone, W. H., and N. L. First. 1989. Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. J. Reprod. Fert. 85:715-720.

Eyestone, W. H., and N. L. First. 1991. Characterization of developmental arrest in early bovine embryos cultured in vitro. Theriogenology. 35:613-624.

Eyestone, W. H., J. M. Jones, and N. L. First. 1991. Some factors affecting the efficacy of oviductal tissue-conditioned medium for the culture of early bovine embryos. J. Reprod. Fert. 92:59-64.

Eyestone, W. H., M. L. Leibfried-Rutledge, D. L. Northey, B. G. Gilligan, and N. L. First. 1987a. Culture of one- and two-cell bovine embryos to the blastocyst stage in the ovine oviduct. Theriogenology. 28:1-7.

Eyestone, W. H., D. L. Northey, and M. L. Leibfried-Rutledge. 1985. Culture of one-cell bovine embryos in the sheep oviduct. Biol. Reprod., Suppl. 32(1):100a.

Eyestone, W. H., J. Vignieri, and N. L. First. 1987b. Co-culture of early bovine embryos with oviductal epithelium. Theriogenology. 27:228.

Fassi-Fihri, N., D. Chupin, B. Marquant-Le Guienne, and M. Thibier. 1991. Critical effect of granulosa cells from preovulatory follicles on IVM, IVF, and embryonic development in cattle. Theriogenology. 35:198.

Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, and M. Danielsen. 1987. Lipofection:

A highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci., USA. 84:7413-7417.

First, N. L. 1990. New animal bredding techniques and their applications. J. Reprod. Fert., Suppl. 41:3.

First, N. L., M. L. Leibfried-Rutledge, D. L. Northey, and P. R. Nuttleman. 1992. Use of in vitro matured oocytes 24 hours of age in bovine nuclear transfer. Theriogenology. 37:211.

Frei, R. E., G. A. Schultz, and R. B. Church. 1989. Qualitative and quantitative changes in protein synthesis occur at the 8-16 cell stage of embryogenesis in the cow. J. Reprod. Fert. 86:637-641.

Fry, R.C. 1992. The effect of leukaemia inhibitory factor (LIF) on embryogenesis. Reprod. Fertil. Dev. 4:449-458.

Fukuda, Y., M. Ichikawa, K. Naio, and Y. Toyoda. 1990. Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells in vitro up to the blastocyst stage. Biol. Reprod. 42:114-119.

Fukui, Y. 1990. Effect of follicle cells on the acrosome reaction, fertilization, and developmental competence of bovine oocytes matured in vitro. Mol. Reprod. Dev. 26:40-46.

Fukui, Y., M. Fukushima, and H. Ono. 1983. Fertilization in vitro of bovine oocytes after various sperm procedures. Theriogenology. 20:651-660.

Fukui, Y., M. Fukushima, Y. Terawaki, and H, Ono. 1982. Effect of gonadotropins, steroids and culture media on bovine oocyte maturation in vitro. Theriogenology. 18:161-175.

Fukui, Y., L. T. McGowan, R. W. James, P. A. Pugh, and H. R. Tervit. 1991. Factors affecting the in-vitro development to blastocysts of bovine oocytes matured and fertilized in vitro. J. Reprod. Fert. 92:125-131.

Fukui, Y., and H. Ono. 1989. Effects of sera, hormones, and granulosa cells added to culture medium for in vitro maturation, fertilization, cleavage and development ofbovine oocytes. J. Reprod. Fert. 86:501-506.

Fukui, Y., M. Urakawa, C. Sasaki, N. Chikamatsu, and H. Ono. 1989. Development to the late morula or blastocyst stage following in vitro maturation and fertilization of bovine oocytes. Anim. Reprod. Sci. 18:139-142.

Fulka, Jr., J., M. L. Leibfried-Rutledge, and N. L. First. 1991. Effect of 6-dimethylaminopurine on germinal vesicle breakdown of bovine oocytes. Mol. Reprod. Dev. 29:379-384.

Gagne, M., F. Pothier, and M. A. Sirard. 1990. Developmental potential of early bovine zygotes submitted to centrifugation and microinjection following in vitro maturation of oocytes. Theriogenology. 34:417-425.

Gagne, M. B., F. Pothier, and M. A. Sirard. 1991. Electroporation of bovine spermatozoa to carry foreign DNA in oocytes. Molec. Reprod. Dev. 29:6-15.

Gandolfi, F., T. A. L. Brevini, L. Richardson, C. R. Brown, and R. M. Moor. 1989. Characterization of proteins secreted by sheep oviduct epithelial cells and their function in embryonic development. Development. 106:303-312.

Goldman, E. E., J. E. Ellington, P. B. Farrell, and R. H. Foote. 1991. Use of fresh and frozen-thawed bovine oviduct cells for acrosome reacting fresh and frozen-thawed bull sperm in vitro. Theriogenology. 35:204.

Gossler, A., T. Doetschman, R. Korn, E. Serfling, and R. Kemler. 1986. Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc. Natl. Acad. Sci., USA. 83:9065-9069.

Goto, K., N. Iwai, Y. Takuma, and Y. Nakanishi. 1992. Co-culture of in vitro fertilized bovine embryos with different cell monolayers. J. Anim. Sci. 70:1449-1453.

Goto, K., Y. Kajihara, S. Kosaka, M. Koba, Y. Nakanishi, and K. Ogawa. 1988. Pregnancies after co-culture of cumulus cells with bovine embryos derived from in-vitro fertilization of in vitro matured follicular oocytes. J. Reprod. Fert. 83:753-758.

Graham, J. K., R. H. Foote, and S. R. Hough. 1987. Penetration of zona free hamster eggs by liposome treated sperm from the bull, ram, stallion, and boar. Biol. Reprod. 37:181-188.

Hamano, S., and Kuwayama, M. 1993. In vitro fertilization and development of bovine oocytes recovered from the ovaries of individual donors: A comparison between the cutting and aspiration method. Theriogenology. 39:703-712.

Handrow, R. R., N. L. First, and J. J. Parrish. 1989. Calcium requirement and increased association with bovine sperm during capacitation with heparin. J. Exp. Zool. 252:174-182.

Handrow, R. R., R. W. Lenz, and R. L. Ax. 1982. Structural comparisons among glycosaminoglycans to promote an acrosome reaction in bovine spermatozoa. Biochem. Biophys. Res. Comm. 107:1326-1332.

Hansel, W., and R. A. Godke. 1992. Future prospectives on animal biotechnology. Anim. Biotechnol. 3:111-137.

Hazeleger, N. L., and R. B. Stubbings. 1992. Developmental potential of selected bovine oocyte cumulus complexes. Theriogenology. 37:219.

Hensleigh, H. C., and A. G. Hunter. 1985. In vitro maturation of bovine cumulus enclosed primary oocytes and their subsequent in vitro fertilization and cleavage. J. Dairy Sci. 68:14561562.

Herlands, R. L., and R. M. Schultz. 1984. Regulation of mouse oocyte growth: Probable nutritional role for intracellular communication between follicle cells and oocytes in oocyte growth. J. Exp. Zool. 229:317-325.

Hernandez-Ledezma,J.J., C. Villanueva, J.D. Sikes, and R.M. Roberts. 1993. Effects of CZB versus medium 199 and of conditioning culture media with either bovine oviductal epithelial cells or buffalo rat liver cells on the development of bovine zygotes derived by in vitro maturation-in vitro fertilization procedures. Theriogenology 39:1267-1277.

Herrier, A., A. Lucas-Hahn, and H. Niemann. 1992. Effects of insulin-like growth factor-I on in vitro production of bovine embryos. Theriogenology. 37:1213-1224.

Heyman, Y., Y. Menezo, P. Chesne, S. Camous, and V. Garnier. 1987. In vitro cleavage of bovine and ovine early embryos: Improved development using coculture with trophoblastic vesicles. Theriogenology. 27:59-68.

Heyman, Y., V. H. Rao, P. Chesne, and J. P. Renard. 1992. Bovine nuclear transfer: Embryo development is influenced by age of the recipient oocyte. In: 12'th International Congress on Animal Reproduction. p 688-690.

Hill, K. G., J. Curry, F. J. DeMayo, K. Jones-Diller, J. R. Slapak, and K. R. Bondioli. 1992. Production of transgenic cattle by pronuclear injection. Theriogenology. 37:222.

Hillery, F. L., J. J. Parrish, and N. L. First. 1990. Bull specific effect on fertilization and embryo development in vitro. Theriogenology. 33:249.

Holding, C., and M. Monk. 1989. Diagnosis of beta-thalassaemia by DNA amplification in single blastomeres from mouse preimplantation embryos. Lancet. 8662:532-535.

Horan, R., R. Powell, S. McQuaid, F. Gannon, and J. A. Houghton. 1991. Association of foreign DNA with porcine spermatozoa. Arch. Androl. 26:83-92.

Hunter, A. G., and R. M. Moor. 1987. Stage dependent effects og inhibiting ribonucleic acids and protein synthesis on meiotic maturation of bovine oocytes in vitro. J. Dairy Sci. 70:1646-1651.

Hyttel, P., K. P. Xu,S. Smith, and T. Greve. 1986. Ultrastructure of in-vitro oocyte maturation in cattle. J. Reprod. Fert. 78:615-625.

Illmensee, K., and P. C. Hoppe. 1981. Nuclear transplantation in Mus musculis: Developmental potential of nuclei from preimplantation embryos. Cell. 23:9-18.

van Inzen, W. G., Y. A. Wurth, C. Verstrate-Voolstra, Th. A. M. Kruip, and S. M. Weima. 1992. Culture of bovine embryos to the blastocyst stage in the presence of embryotrophic factors secreted by buffalo rat liver (BRL) cells. In: 12'th International Congress on Animal Reproduction. p 1360-1362.

Iritani, A., and K. Niwa. 1977. Capacitation of bull spermatozoa and fertilization in vitro of cattle follicular oocytes matured in culture. J. Reprod. Fert. 50:119-121.

Iritani, A., K. Utsumi, M. Miyake, and Y. Yamaguchi. 1989. Individual variation in the in vitro fertilizing ability of bull spermatozoa. Dev. Growth Diff., Suppl. 28:45.

Jänne, J., J.M. Hyttinen, T. Peura, M. Tolvanen, L. Alhonen, and M. Halmekytö. 1992. Transgenic animals as bioproducers of therapeutic proteins. Ann. Med. 24:273-280.

Javed, M. H., and R. W. Wright, Jr. 1991. Determination of pentose phosphate and Embden-Meyerhof pathway activities in bovine embryos. Theriogenology. 35:1029-1037.

Jin, D. I., R. M. Petters, B. H. Johnson, and R. M. Schuman. 1991. Survival of early preimplantation porcine embryos after co-culture with cells producing an avian retrovirus. Theriogenology. 35:521-526.

Jura, J., J. J. Kopchick, W. Y. Chen, J. A. Modlinski, M. A. Reed, and Z. Smorag. 1992. Production of transferable bovine embryos from exogenous DNA microinjected zygotes and 2-cell embryos. In: 12'th Internation Congress on Animal Reproduction. p 697-699.

Kanka, J., J. Fulka, Jr., J. Fulka, and J. Petr. 1991. Nuclear transplantation in bovine embryo: Fine ultrastructural and autoradiographic studies. Molec. Reprod. Dev. 29:110-116.

Kastrop, P. M. M., M. M. Bevers, O. H. J. Destree, and Th. A. M. Kruip. 1988. Protein synthesis of cultured bovine oocytes. J. Reprod. Fert., Suppl. 2:37.

Kastrop, P. M. M., M. M. Bevers, O. H. J. Destree, and Th. A. M. Kruip. 1990. Changes in protein synthesis and phosphorylation

patterns during bovine oocyte maturation in vitro. J. Reprod. Fert. 90:305-310.

Kastrop, P. M. M., M. M. Bevers, O. H. J. Destree, and Th. A. M. Kruip. 1991a. Protein synthesis and phosphorylation patterns of bovine oocytes maturing in vivo. Mol. Reprod. Dev. 29:271-275.

Kastrop, P. M. M., S. C. J. Hulshof, M. M. Bevers, O. H. J. Destree, and Th. A. M. Kruip. 1991b. The effects of a-amanitin and cyclohexamide on nuclear progression, protein synthesis, and phosphorylation during bovine oocyte maturation in vitro. Mol. Reprod. Dev. 28:249-254.

Katska, L. 1984. Comparison of two methods for recovery of ovarian oocytes from slaughter cattle. Anim. Reprod. Sci. 7:461-463.

Katska, L., and Z. Smorag. 1985. The influence of culture temperature on in vitro maturation of bovine oocytes. Anim. Reprod. Sci. 9:205-212.

Killian, G. J., and A. A. Grippo. 1992. Incubation in ampullary oviductal fluid improves the ability of sperm to fertilize bovine oocytes in vitro. In: 12'th International Congress on Animal Reproduction. p 650-652.

Kim, C. I., J. E. Ellington, and R. H. Foote. 1990. Maturation, fertilization, and development of bovine oocytes in vitro using TCM199 and a simple defined medium with co-culture. Theriogenology. 33:433-440.

Kim, J.H., H. Funahashi, K. Niwa, and K. Okuda. 1993a. Glucose requirement at different developmental stages of in vitro fertilized bovine embryos cultured in semi-defined medium. Theriogenology 39:875-886.

Kim, J.H., K. Niwa, J.M. Lim, and K. Okuda. 1993b. Effects of phosphate, energy substrates, and amino acids on development of in vitro matured, in vitro fertilized bovine oocytes in a chemically defined, protein-free culture medium. Biol. Reprod. 48:1320-1325.

Kim, T., M.L. Leibfried-Rutledge, and N.L. First. 1993. Gene transfer in bovine blastocysts using replication-defective retroviral

vectors packaged with gibbon ape leukemia virus envelopes. Molec. Reprod. Dev. 35:105-113.

King, W. A., D. Bousquet, T. Greve, and A. K. Goff. 1986. Meiosis in bovine oocytes matured in vitro and in vivo. Acta. Vet. Scand. 27:267-279.

King, W. A., D. L. Shepherd, L. Plante, R. Powell, C. R. Looney, and F. L. Barnes. 1992. An ultrastructural study of bovine embryos produced by nuclear transfer. Theriogenology. 37:238.

Kirkpatrick, B.W., and R.L. Monson. 1993. Sensitive sex determination assay applicable to bovine embryos derived from IVM and IVF. J. Reprod. Fert. 98:335-340.

Kraemer, D., B. Minhas, and J. Capehart. 1985. Gene transfer into pronuclei of cattle and sheep zygotes. In: F. Costantini and R. Jaenisch (Ed.) Genetic manipulation of the early mammalian embryo. p 221. Cold Spring Harbor, N.Y..

Krimpenfort, P., A. Rademakers, W. Eyestone, A. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. Platenburg, F. Pieper, R. Strijker, and H. de Boer. 1991. Generation of transgenic dairy cattle using in vitro embryo production. Biotechnology. 9:844-847.

Kroetsch, T. G., and R. B. Stubbings. 1992. Sire and insemination dose does effect in vitro fertilization of bovine oocytes. Theriogenology. 37:240.

Kruip, T. A. M., D. G. Cran, T. H. van Beneden, and S. J. Dieleman. 1983. Structural changes in bovine oocytes during final maturation in vivo. Gamete Res. 8:29-47.

Kruip, T.A.M, M.C. Pieterse, T.H. van Beneden, P.L.A.M. Vos, Y.A. Wurth, and M.A.M. Taverne. 1991. A new method for bovine embryo production: a potential alternative to superovulation. Vet. Rec. 128:208-210.

Kruip, T.A.M., M.C. Pieterse, T.H. van Benenden, P. Vos, Y.A. Wurth, and M.A.M. Taverne. 1990. Increased success rate of IVM and IVF in the bovine after sonographic guided transvaginal collections of the oocytes. Theriogenology 33:269.

Lavitrano, M., A. Camaioni, V. M. Fazio, S. Dolci, M. G. Farace, and C. Spadafora. 1989. Sperm cells as vectors for introducing foreign DNA into eggs: Genetic transformation of mice. Cell. 57:717-723.

Lee, C. N., and R. L. Ax. 1984. Concentrations and composition of glycosaminoglycans in the female reproductive tract. J. Dairy Sci. 67:2006-2009.

Lee, C. N., M. K. Clayton, S. M. Bushmeyer, N. L. First, and R. L. Ax. 1986. Glycosaminoglycans in ewe reproductive tracts and their influence on acrosome reactions in bovine spermatozoa in vitro. J. Anim. Sci. 63:861-867.

Leibfried, M. L., and B. D. Bavister. 1982. Effects of epinephrine and hypotaurine on in vitro fertilization in the golden hamster. J. Reprod. Fert. 66:87-93.

Leibfried-Rutledge, M. L., E. S. Critser, and N. L. First. 1986. Effects of fetal calf serum and bovine serum albumin on in vitro maturation and fertilization of bovine and hamster cumulus-oocyte complexes. Biol. Reprod. 35:850-857.

Leibfried-Rutledge, M. L., E. S. Critser, J. J. Parrish, and N. L. First. 1989. In vitro maturation and fertilization of bovine oocytes. Theriogenology. 31:61-74.

Leibfried-Rutledge, M. L., D. L. Northey, P. R. Nuttleman, and N.L. First. 1992. Processing of donated nucleus and timing of post-activation events differ between recipient oocytes 24 or 48 hours of age. Theriogenology. 37:244.

Lenz, R. W., G. D. Ball, M. L. Leibfried, R. L. Ax, and N. L. First. 1983a. In vitro maturation and fertilization of bovine oocytes are temperature-dependent processes. Biol. Reprod. 29:173-179.

Lenz, R. W., G. D. Ball, J. K. Lohse, N. L. First, and R. L. Ax. 1983b. Chondroitin sulfate facilitates an acrosome reaction in bovine spermatozoa as evidenced by light microscopy, electron microscopy and in vitro fertilization. Biol. Reprod. 28:683-690.

Lewis-Williams, J., T. Houseal, M. Harvey, P. DiTullio, and C. Ziomek. 1993. Selection of transgenic preimplantation mouse embryos using fluorescent in situ hybridization. Theriogenology 39:258.

Lonergan, P., H. Sharif, P. Monaghan, H. Wahid, M. Gallagher, and I. Gordon. 1992. Effect of follicle size on bovine oocyte morphology and embryo yield following maturation, fertilization and culture in vitro. Theriogenology. 37:248.

de Loos, F. A. M., M. Jeuken, E. Zeinstra, and M. M. Bevers. 1992. Maintenance of meiotic arrest in bovine oocytes cultured in vitro. In: 12'th International Congress on Animal Reproduction. p 357-359.

de Loos, F., P. Kastrop, P. Van Maurik, Th. H. Van Beneden, and Th. A. M. Kruip. 1991. Heterologous cell contacts and metabolic coupling in bovine cumulus oocyte complexes. Mol. Reprod. Dev. 28:255-259.

de Loos, F., C. van Vliet, P. van Maurik, and Th. A. M. Kruip. 1989. Morphology of immature bovine oocytes. Gamete Res. 24:197-204.

Lopes, R. F. F., J. L. Rodrigues, R. J. Chebel, H. M. M. Maia, J. A. S. Perseu, C. Termignoni, and G. M. Stoll Rial. 1992. Survival of bovine embryos after micromanipulation for sex determination using the polymerase chain reaction (PCR). In: 12'th International Congress on Animal Reproduction. p 712-714.

Loskutoff, N. M., B. R. Coren, D. R. Barrios, E. Bessoudo, M. J. Bowen, G. Stone, and D. C. Kraemer. 1986a. Gene microinjection in bovine embryos facilitated by centrifugation. Theriogenology. 25:168.

Loskutoff, N. M., C. A. Roessner, and D. C. Kraemer. 1986b. Preliminary studies on liposome mediated gene transfer: Effects on survivability of murine zygotes. Theriogenology. 25:169.

Lu, K. H., I. Gordon, M. Gallagher, and H. McGovern. 1987. Pregnancy established in cattle by transfer of embryos derived from in vitro fertilization of oocytes matured in vitro. Vet. Rec. 121:259-260.

Machaty, Z., A. Paldi, T. Csaki, Z. Varga, I. Kiss, Z. Barandi, and G. Vajta. 1993. Biopsy and sex determination by PCR of IVF bovine embryos. J. Reprod. Fert. 98:467-470.

Maddox, J. 1989. Transgenic route runs into sand. Nature. 341:686.

Marquardt, H., G.J. Todaro, L.E. Henderson, and S. Oroszlan. 1981. Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures. J. Biol. Chem. 256:6859-6865.

Matsuyama, K., H. Miyakoshi, and Y. Fukui. 1993. Effect of glucose levels during the in vitro culture in synthetic oviduct fluid medium on in vitro development of bovine oocytes matured and fertilized in vitro. Theriogenology 40:595-605.

Marx, J. L. 1988. Cloning sheep and cattle embryos. Science. 239:463-464.

McCaffrey, C., T. G. McEvoy, M. G. Diskin, F. C. Gwazdauskas, M. T. Kane, and J. M. Sreenan. 1991. Successfulco-culture of 1-4-cell cattle ova to the morula or blastocyst stage. J. Reprod. Fert. 91:119-124.

McEvoy, T. G., and J. M. Sreenan. 1990. The efficiency of production, centrifugation, microinjection and transfer of one- and two-cell bovine ova in a gene transfer program. Theriogenology. 33:819-828.

McGrath, J., and Solter, D. 1983. Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science. 220:1300-1302.

McKenzie, B. E., and R. M. Kenney. 1973. In vitro culture of bovine embryos. Am. J. Vet. Res. 34:1271-1275.

McLaren, A. 1982. The embryo. In: C. R. Austin and R. V. Short (Ed.) Reproduction in mammals 2: Embryonic and fetal development. p 3. Cambridge University Press, Cambridge.

McLaughlin, K. J., D. M. McLean, G. Stevens, R. A. Ashman, P. A. Lewis, B. D. Bartsch, and R. F. Seamark. 1990. Viability of bovine

embryos cultured in synthetic oviductal fluid medium. Theriogenology. 33:1191-1199.

Meizel, S. 1985. Molecules that initiate or help stimulate the acrosome reaction by their interaction with the mammalian sperm surface. Amer. J. Anat. 174:285-302.

Mermillod, P., C. Boccart, C. Wils, A. Massip, and F. Dessy. 1992. Effect of oviduct-conditioned medium and of cumulus cells on bovine embryo development in vitro. Theriogenology. 37:256.

Mermillod, P., A. Vansteenbrugge, C. Wils, J-L. Mourmeaux, A. Massip, and F. Dessy. 1993. Characterization of the embryotropic activity of exogenous protein-free oviduct-conditioned medium used in culture of cattle embryos. Biol. Reprod. 49:582-587.

Miller, D. J., and R. L. Ax. 1989. Chemical N-desulfation of heparin negates its ability to capacitate bovine spermatozoa. Gamete Res. 23:451-465.

Miller, G. F., D. L. Gliedt, T. D. Lester, J. N. Pierson, J. M. Rakes, and R. W. Rorie. 1992. Addition of bovine oviductal epithelial cells (BOEC) and/or penicillamine, hypotaurine and epinephrine (PHE) to bovine in vitro fertilization (IVF) medium increases the subsequent embryo cleavage rate. Theriogenology. 37:259.

Minhas, B. S., J. S. Capehart, M. J. Bowen, J. E. Womack, J. D. McCrady, P. G. Harms, T. E. Wagner, and D. C. Kraemer. 1984. Visualization of pronuclei in living bovine zygotes. Biol. Reprod. 30:687-691.

Mochizuki, H., Y. Fukui, and H. Ono. 1991. Effect of the number of granulosa cells added to culture medium for in vitro maturation, fertilization and development of bovine oocytes. Theriogenology. 36:973-986.

Monson, R., D. L. Northey, R. Gottfredson, D. R. Peschel, J. J. Rutledge, and D. M. Schaefer. 1992. Pregnancy rates of in vitro produced bovine embryos following nonsurgical transfer. Theriogenology. 37:261.

Moor, R. M., M. W. Smith, and R. M. C. Dawson. 1980. Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. Exp. Cell Res. 126:15-29.

Moor, R. M., and A. O. Trounson. 1977. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. J. Reprod. Fert. 49:101-109.

Moore, K., and K.R. Bondioli. 1993. Glycine and alanine supplementation of culture medium enhances development of in vitro matured and fertilized cattle embryos. Biol. Reprod. 48:833-840.

Motlik, J. 1989. Cytoplasmic aspects of oocyte growth and maturation in mammals. J. Reprod. Fert., Suppl. 38:17.

Motlik, J., H. H. Koefoed-Johnsen, and J. Fulka. 1978. Breakdown of the germinal vesicle in bovine oocytes cultivated in vitro. J. Exp. Zool. 205:377-384.

Murray, A. W., and M. W. Kirschner. 1989. Cyclin synthesis drives the early embryonic cell cycle. Nature. 339:275-280.

Nakao, H., and N. Nakatsuji. 1990. Effects of co-culture, medium components and gas phase on in vitro culture of in vitro matured and in vitro fertilized bovine embryos. Theriogenology. 33:591-600.

Nauta, W., G. E. Seidel, Jr., and S. E. Olsen. 1992. Development of bovine embryos in conditioned media made with oviduct epithelial cells obtained at four stages of the estrous cycle. Theriogenology. 37:264.

Nieman, H., R. Halter, K. Huter, E. Lemme, A. Guzik, and D. Paul. 1992. Generation of transgenic sheep harbouring the human blood clotting factor VIII gene. Theriogenology. 37:265.

Niwa, K., and O. Ohgoda. 1988. Synergistic effect of caffeine and heparin on in vitro fertilization of cattle oocytes matured in culture. Theriogenology. 30:733-741.

Northey, D. L., M. L. Leibfried-Rutledge, P. R. Nuttleman, and N. L. First. 1992. Development of bovine nuclear transfer embryos in in vivo versus in vitro culture systems. Theriogenology. 37:266.

Olson, S. E., W. K. Thomas, and G. E. Seidel, Jr. 1991. Effects of gonadotropins during in vitro maturation of bovine oocytes on subsequent embryonic development. Theriogenology. 35:250.

Overstrom, E. W. 1992. Manipulation of early embryonic development. Anim. Reprod. Sci. 28:277-285.

Parks, J. E., and S. R. Hough. 1990. Effects of platelet activating factor on the motility and acrosome reaction of bovine spermatozoa. Theriogenology. 34:903-912.

Parrish, J. J. 1991. Application of in vitro fertilization to domestic animals. In: P. M. Wasserman (Ed.) Elements of Mammalian Fertilization, vol. II. p 111. CRC Press, Boca Raton, FLA.

Parrish, J. J., J. L. Susko-Parrish, and N. L. First. 1985. Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm in vitro. Theriogenology. 24:537-549.

Parrish, J. J., J. L. Susko-Parrish, R. R. Handrow, M. M. Sims, and N. L. First. 1989. Capacitation of bovine spermatozoa by oviduct fluid. Biol. Reprod. 40:1020-1025.

Parrish, J. J., J. L. Susko-Parrish, M. L. Leibfried-Rutledge, E. S. Critser, W. H. Eyestone, and N. L. First. 1986. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology. 25:591-600.

Parrish, J. J., J. Susko-Parrish, M. A. Winer, and N. L. First. 1988. Capacitation of bovine sperm by heparin. Biol. Reprod. 38:1171-1180.

Pavlok, A., V. Kopecny, A. Lucas-Hahn, and H. Niemann. 1993. Transcriptional activity and nuclear ultrastructure of 8-cell bovine embryos developed by in vitro maturation and fertilization of oocytes from different growth categories of antral follicles. Molec. Reprod. Dev. 35:233-243.

Petters, R. M. 1992. Embryo development in vitro to the blastocyst stage in cattle, pigs, and sheep. Anim. Reprod. Sci. 28:415-421.

Peura, T. T., J-M. Hyttinen, M. Tolvanen, J. Aalto, V. Rainio, and J. Janne. 1992. Pregnancies resulting from in vitro produced,

pronucleus injected and bisected bovine embryos. In: 12'th International Congress on Animal Reproduction. p 733-735.

Pieterse, M. C., K. A. Kappen, Th. A. M. Kruip, and M. A. M. Taverne. 1988. Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. Theriogenology. 30:751-762.

Pieterse, M. C., P. L. A. M. Vos, Th. A. M. Kruip, A. H. Willemse, and M. A. M. Taverne. 1991a. Characteristics of bovine estrous cycles during repeated transvaginal, ultrasound-guided puncturing of follicles for ovum pick-up. Theriogenology. 35:401-413.

Pieterse, M.C., P.L.A.M. Vos, T.A.M. Kruip, Y.A. Wurth, T.H. van Benenden, A.H. Willemse, and M.A.M. Taverne. 1991b. Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology 35:857-862.

Pieterse, M. C., P. L. A. M. Vos, Th. A. M. Kruip, Y. A. Wurth, Th. H. van Beneden, A. H. Willemse, and M. A. M. Taverne. 1992. Repeated transvaginal ultrasound-guided ovum pick-up in ECG-treated cows. Theriogenology. 37:273.

Pinyopummintr, T., and B. D. Bavister. 1991. In vitro matured/in vitro fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined, protein free culture media. Biol. Reprod. 45:736-742.

Pollard, J. W., K. P. Xu, R. Rorie, W. A. King, and K. J. Betteridge. 1989. Influence of various oviductal epithelial cell culture systems on the development of early cleavage stage bovine embryos in vitro. Theriogenology. 31:239.

Powell, D. J., C. Galli, and R. M. Moor. 1992. The fate of DNA injected into mammalian oocytes and zygotes at different stages of the cell cycle. J. Reprod. Fert. 95:211-220.

Prather, R. S., F. L. Barnes, M. M. Sims, J. M. Robl, W. H. Eyestone, and N. L. First. 1987. Nuclear transplantation in the bovine embryo: Assessment of donor nuclei and recipient oocyte. Biol. Reprod. 37:859-866.

Prather, R. S., T. T. Stumpf, and L. F. Rickords. 1992a. Nuclear transplantation as a method of producing genetically identical livestock. Anim. Biotechnol. 3:67-69.

Prather, R. S., T. T. Stumpf, and L. F. Rickords. 1992b. Reprogramming the nucleus and synchronizing it with the cytoplasm. In: Proc. Symp. Nuclear Transfer, January 1992, Colorado State University, Fort Collins. p 26-27.

Pursel, V. G., K. F. Miller, D. J. Bolt, C. A. Pinkert, R. E. Hammer, R. D. Palmiter, and R. L. Brinster. 1989a. Insertion of growth hormone genes into pig embryos. In: R. B. Heap, C. G. Prosser, and G. E. Lamming (Ed.) Biotechnology of Growth Regulation. Butterworths, London.

Pursel, V. G., C. A. Pinkert, K. F. Miller, D. J. Bolt, R. G. Campbell, R. D. Palmiter, R. L. Brinster, and R. E. Hammer. 1989b. Genetic engineering of livestock. Science. 244:1281-1287.

Pursel, V.G., and C.E. Rexroad, Jr. 1993a. Recent progress in the transgenic modification of swine and sheep. Molec. Reprod. Dev. 36:251-254.

Pursel, V.G., and C.E. Rexroad, Jr. 1993b. Status of research with transgenic farm animals. J. Anim. Sci. 71(suppl. 3):10-19.

Pursel, V. G., P. Sutrave, R. J. Wall, A. M. Kelly, and S. H. Hughes. 1992. Transfer of c-SKI gene into swine to enhance muscle development. Theriogenology. 37:278.

Reed, M. L., C. A. Roessner, J. E. Womack, C. G. Dorn, and D. C. Kraemer. 1988. Microinjection of liposome-encapsulated DNA into murine and bovine blastocysts. Theriogenology. 29:293.

Rexroad, C. E. Jr. 1992. Transgenic technology in animal agriculture. Anim. Biotechnol. 3:1-13.

Rexroad, C. E., Jr., K. Mayo, D. J. Bolt, T. H. Elsasser, K. F. Miller, R. R. Behringer, R. D. Palmiter, and R. L. Brinster. 1991. Transferrin- and albumin-directed expression of growth-related peptides in transgenic sheep. J. Anim. Sci. 69:2995-3004.

Rieger, D., and P. Guay. 1988. Measurement of the metabolism of energy substrates in individual bovine blastocysts. J. Reprod. Fert. 83:585-591.

Robertson, E., A. Bradley, M. Kuehn, and M. Evans. 1986. Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature. 323:445-448.

Robl, J. M., R. Prather, F. Barnes, W. Eyestone, D. Northey, B. Gilligan, and N. L. First. 1987. Nuclear transfer in bovine embryos. J. Anim. Sci. 64:642-647.

Robl, J. M., and S. L. Stice. 1989. Prospects for the commercial cloning of animals by nuclear transplantation. Theriogenology. 31:75-84.

Rorie, R. W., T. D. Lester, G. F. Miller, and D. L. Gliedt. 1992. In vitro development of bovine embryos co-cultured with bovine oviductal epithelial cells (BOEC) in either synthetic oviductal fluid (SOF) or M-199 medium. Theriogenology. 37:287.

Roschlau, K., P. Rommel, L. Andreewa, M. Zackel, D. Roschlau, B. Zackel, M. Schwerin, R. Huhn, and K. G. Gazarjan. 1989. Gene transfer experiments in cattle. J. Reprod. Fert., Suppl. 38:153-160.

Rose, T. A. 1990. Bovine morula/blastocyst development in vitro in simple medium. Biol. Reprod. 42(suppl. 1):58.

Rose, T. A., and B. D. Bavister. 1992. Effect of oocyte maturation medium on in vitro development of in vitro fertilized bovine embryos. Mol. Reprod. Dev. 31:72-77.

Rosenkrans, C. F., Jr., and First, N. L. 1991. Culture of bovine zygotes to the blastocyst stage: Effects of amino acids and vitamins. Theriogenology. 35:266.

Rosenkrans, C. F., Jr., G. Z. Zeng, P. K. Schoff, and N. L. First. 1990. A simple medium for in vitro development of bovine embryos. J. Anim. Sci., Suppl. 68:430.

Rosenkrans, C.F., Jr., G.Q. Zeng, G.T. McNamara, P.K. Schoff, and N.L. First. 1993. Development of bovine embryos in vitro as affected by energy substrates. Biol. Reprod. 49:459-462.

Saeki, K., M. Hoshi, M. L. Leibfried-Rutledge, and N. L. First. 1990. In vitro fertilization and development of bovine oocytes matured with commercially available follicle stimulating hormone. Theriogenology. 34:1035-1039.

Saiki, K., H. Kato, Y. Hosoi, M. Miyake, K. Utsumi, and I. Iritani. 1991. Early morphological events of in vitro fertilized bovine oocytes with frozen thawed spermatozoa. Theriogenology. 35:1051-1058.

Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of ß-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 230:1350-1354.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Sanbuissho, A., S. Coskun, and Y. C. Lin. 1992. Role of cyclic adenosine monophosphate (cAMP) in vitro on bovine oocyte maturation. Theriogenology. 38:153-163.

Sanbuissho, A., and W. R. Threlfall. 1990. The influence of serum and gonadotropins on in vitro maturation and fertilization of bovine oocytes. Theriogenology. 34:341-348.

van der Schans, A., B. T. T. M. van Rens, L. A. J. van der Westerlaken, and A. A. C. de Wit. 1992. Bovine embryo production by repeated transvaginal oocyte collection and in vitro fertilization. In: 12'th International Congress on Animal Reproduction. p 1366-168.

Seidel, G. E. 1992. Transgenic animals. Embryo Transfer. 7:1-5.

Seidel, G. E., Jr., L. L. Larson, C. H. Spilman, J. Hahn, and R. H. Foote. 1971. Culture and transfer of calf ova. J. Dairy Sci. 54:923-926.

Shamsuddin, M., B. Larsson, H. Gustafsson, S. Gustari, J. Bartolome, and H. Rodriguez-Martinez. 1992. Comparative morphological evaluation of in vivo and in vitro produced bovine embryos. In: 12'th International Congress of Animal Reproduction. p 1333-1335.

Shamsuddin, M., B. Larsson, H. Gustafsson, and H. Rodriguez-Martinez. 1993. In vitro development up to hatching of bovine in vitro-matured and fertilized oocytes with or without support from somatic cells. Theriogenology 39:1067-1079.

Sharif, H., P. Lonergan, P. Monaghan, M. Gallagher, and I. Gordon. 1992a. Comparison of bovine embryonic development in an isolated mouse oviduct culture system with bovine oviductal epithelial cell co-culture. In: 12'th International Congress on Animal Reproduction. p 1336-1338.

Sharif, H., P. Lonergan, P. Monaghan, H. Wahid, M. Gallagher, and I. Gordon. 1992b. Development of early bovine embryos in isolated mouse oviducts maintained in organ culture under two gas atmospheres. Theriogenology. 37:297.

Sharif, H., E. Vergos, P. Lonergan, M. Gallagher, A. Kinis, and I. Gordon. 1991. Development of early bovine embryos in the isolated mouse oviduct maintained in organ culture. Theriogenology. 35:270.

Shea, B. F., J. P. A. Latour, K. N. Bedirian, and R. D. Baker. 1976. Maturation in vitro and subsequent penetrability of bovine follicular oocytes. J. Anim. Sci. 43:809-815.

Shi, D. S., K. H. Lu, and I. Gordon. 1990. Effects of bulls on fertilization of bovine oocytes and their subsequent development in vitro. Theriogenology. 33:324.

Shioya, Y., M. Kuwayama, M. Fukushima, S. Iwasaki, and A. Hanada. 1988. In vitro fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cells and matured in vitro. Theriogenology. 30:489-496.

Simons, J. P., and R. B. Land. 1987. Transgenic livestock. J. Reprod. Fert., Suppl. 34:237-250.

Sirard, M. A. 1989. Temporary inhibition of in vitro meiotic resumption by adenylate cyclase stimulation in immature bovine oocytes. Theriogenology. 31:257.

Sirard, M. A. 1990. Temporary inhibition of meiosis resumption in vitro by adenylate cyclase stimulation in immature bovine oocytes. Theriogenology. 33:757-767.

Sirard, M. A., and S. Bilodeau. 1990a. Effects of granulosa cell co-culture on in-vitro meiotic resumption of bovine oocytes. J. Reprod. Fert. 89:459-465.

Sirard, M. A., and S. Bilodeau. 1990b. Granulosa cells inhibit the resumption of meiosis in bovine oocytes in vitro. Biol. Reprod. 43:777-783.

Sirard, M.A., and Coenen, K. 1993. The co-culture of cumulus-enclosed bovine oocytes and hemi-sections of follicles: Effects on meiotic resumption. Theriogenology 40:933-942.

Sirard, M. A., K. Coenen, and S. Bilodeau. 1992. Effect of fresh or cultured follicular fractions on meiotic resumption in bovine oocytes. Theriogenology. 37:39-57.

Sirard, M. A., and N. L. First. 1988. In vitro inhibition of oocyte nuclear maturation in the bovine. Biol. Reprod. 39:229-234.

Sirard, M. A., H. M. Florman, M. L. Leibfried-Rutledge, F. L. Barnes, M. L. Sims, and N. L. First. 1989. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol. Reprod. 40:1257-1263.

Sirard, M. A., R. Lambert, and R. Beland. 1984. Evaluation of bovine capacitation by in vitro fertilization. Theriogenology. 21:261.

Sirard, M. A., J. J. Parrish, C. B. Ware, M. L. Leibfried-Rutledge, and N. L. First. 1988. The culture of bovine oocytes to obtain developmentally competent embryos. Biol. Reprod. 39:546-552.

Smith, A.G., and M.L. Hooper. 1987. Buffalo rat liver cells produce a diffusable activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Developmental Biol. 121:1-9.

Smith, L. C. 1990. Factors affecting the viability of nuclear transplanted embryos. Theriogenology. 33:153-164.

Smith, L. C. 1992. Removal or destruction of meiotic chromosomes of oocytes. In: Proc. Symp. Nuclear Transfer, January, 1992, Colorado State University, Fort Collins. p 21-23.

Sparks, A. E. T., F. C. Gwazdauskas, and M. L. McGilliard. 1992. Culture of one cell bovine embryos in explanted mouse oviduct and bovine oviductal epithelial cells. Theriogenology. 37:587-594.

Sreenan, J., P. Scanlon, and I. Gordon. 1968. Culture of fertilized cattle eggs. J. Agric. Sci., Camb. 70:183-185.

van Stekelenburg-Hamers, A.E.P., W.G. van Inzen, T.A.E. van Achterberg, T.A.M. Kruip, S.W. De Laat, and S.M. Weima. 1993. Nuclear transfer and electrofusion in bovine in vitro-matured/in vitro-fertilized embryos: Effect of media and electrical fusion parameters. Molec. Reprod. Dev. 36:307-312.

Stice, S. L. 1992. Multiple generation bovine embryo cloning. In: Proc. Symp. Nuclear Transfer, January, 1992, Colorado State University, Fort Collins. p 28.

Stubbings, R. B., K. J. Betteridge, and P. K. Basrur. 1988. Investigations of culture requirements for bovine oocyte maturation in vitro. Theriogenology. 29:313.

Stubbings, R. B., R. M. Liptrap, K. J. Betteridge, J. S. Walton, D. T. Armstrong, and P. K. Basrur. 1990. Requirements for bovine oocyte maturation in vitro. Reprod. Dom. Anim. 25:158-166.

Susko-Parrish, J., H. Atkas, and M. L. Leibfried-Rutledge. 1992. The effect of energy substrates during maturation on the fertilization and development of bovine oocytes. Theriogenology. 37:305.

Suss, U., J. Kasner, K. Wuthrich, and G. Stranzinger. 1990. Cumulus expansion, in vitro fertilization, and embryonic development after in vitro maturation of bovine oocytes in the presence of follicle stimulating or luteinizing hormone. Reprod. Dom. Anim. 25:3-13.

Suss, U., K. Wuthrich, and G. Stranzinger. 1988. Chromosome configurations and time sequence of the first meiotic division in bovine oocytes matured in vitro. Biol. Reprod. 38:871-880.

Takahashi, Y., and N. L. First. 1992. In vitro development of bovine one cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology. 37:963-978.

Tervit, H. R., D. G. Whittingham, and L. E. A. Rowson. 1972. Successful culture in vitro of sheep and cattle ova. J. Reprod. Fert. 30:493-497.

Thibodeaux, J. K., Y. Menezo, J. D. Roussel, W. Hansel, L. L. Goodeaux, D. L. Thompson, Jr., and R. A. Godke. 1992. Coculture of in vitro fertilized bovine embryos with oviductal epithelial cells originating from different stages of the estrus cycle. J. Dairy Sci. 75:1448-1455.

Thomas, W.K., A. Schnieke, and G.E. Seidel, Jr. 1993. Methods for producing transgenic bovine embryos from in vitro matured and fertilized oocytes. Theriogenology 40:679-688.

Thompson, J. G. E., A. C. Simpson, P. A. Pugh, P. E. Donnelly, and H. R. Tervit. 1990. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. J. Reprod. Fert. 89:573-578.

Thuemmel, A. E., F. C. Gwazdauskas, R. S. Canseco, and M. L. McGilliard. 1989. Culture of bovine embryos in tissue repair factor-supplemented media and immature mouse uteri. J. Dairy Sci. 72(suppl. 1):401.

Todorov, J., T. Todorova, U. Zimmerman, W. M. Arnold, C. Leiding, R. Hahn, and J. Hahn. 1992. The influence of a prolonged in vitro maturation time and of a repeated field pulse sequence upon the development rates of the oocytes in bovine nuclear transfer. In: 12'th International Congress of Animal Reproduction. p 754-756.

Trounson, A. 1992. The production of ruminant embryos in vitro. Anim. Reprod. Sci. 28:125-137.

Tsuji, K., M. Sowa, and R. Nakano. 1985. Relationship between human oocyte maturation and different follicular sizes. Biol. Reprod. 32:413-417.

Utsumi, K., H. Kato, and A. Iritani. 1991. Full term development of bovine follicular oocytes matured in culture and fertilized in vitro. Theriogenology. 35:695-703.

Van Blerkholm, J., H. Bell, and D. Weipz. 1990. Cellular and developmental biological aspects of bovine meiotic maturation, fertilization, and preimplantation embryogenesis in vitro. J. Electron Microsc. Technique. 16:298-323.

Van Brunt, J. 1990. Amplifying genes: PCR and its alternatives. Biotechnology. 8:291-293.

Varmus, H. 1988. Retroviruses. Science. 240:1427-1435.

Velander, W.H., J.L. Johnson, R.L. Page, C.G. Russell, A. Subramanian, T.D. Wilkins, F.C. Gwazdauskas, C. Pittius, and W.N. Drohan. 1992. High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. Proc. Natl. Acad. Sci., USA. 89:12003-12007.

Voelkel, S. A., G. F. Amborski, K. G. Hill, and R. A. Godke. 1985. Use of a uterine cell monolayer culture system for micromanipulated bovine embryos. Theriogenology. 24:271-281.

Voelkel, S. A., and Y. X. Hu. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. Theriogenology. 37:1117-1131.

Voelkel, S.A., Y.X. Hu, K. Moore, and K.R. Bondioli. 1992. Freeze survival of bovine embryos produced by in vitro maturation, fertilization and culture of oocytes. Theriogenology 37:317.

Wall, R. J., V. G. Pursel, A. Shamay, R. A. McKnight, C. W. Pittius, and L. Hennighausen. 1991. High level synthesis of a heterologous milk protein in the mammary gland of transgenic swine. Proc. Natl. Acad. Sci., USA. 88:1696-1700.

Westhusin, M. E., J. H. Pryor, and K. R. Bondioli. 1991. Nuclear transfer in the bovine embryo: A comparison of 5-day, 6-day, frozen-

thawed, and nuclear transfer donor embryos. Molec. Reprod. Dev. 28:119-123.

Westhusin, M. E., J. R. Slapak, D. T. Fuller, and D. C. Kraemer. 1989. Culture of agar embedded one and two cell bovine embryos and embryos produced by nuclear transfer in the sheep and rabbit oviduct. Theriogenology. 31:271.

Westphal, H. 1989. Transgenic mammals and biotechnology. FASEB. 3:117-120.

Wheeler, M. B., and G. E. Seidel, Jr. 1986. Time course of in vitro capacitation of frozen and unfrozen bovine spermatozoa. Theriogenology. 25:216.

White, I. G., L. Belanger, S. Hough, J. Ellington, and R. H. Foote. 1992. Biochemical changes in bull spermatozoa during capacitation in vitro. Theriogenology. 37:571-578.

Whitelaw, C.B.A., A.J. Springbett, J. Webster, J. Clark. 1993. The majority of G0 transgenic mice are derived from mosaic embryos. Transgenic Res. 2:29-32.

Wilkie, T.M., R.L. Brinster, and R.D. Palmiter. 1986. Germline and somatic mosaicism in transgenic mice. Dev. Biol. 118:9-18.

Willadsen, S. M. 1979. A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. Nature. 227:298-300.

Willadsen, S. M. 1986. Nuclear transplantation in sheep embryos. Nature. 320:63-65.

Willadsen, S. M., R. E. Jansen, R. J. McAlister, B. F. Shea, G. Hamilton, and D. McDermand. 1991. The viability of late morula and blastocysts produced by nuclear transplantation in cattle. Theriogenology. 35:161-170.

Wilmut, I., K. H. S. Campbell, and G. T. O'Neill. 1992. Sources of totipotent nuclei including embryonic stem cells. In: Proc. Symp. Nuclear Transfer, January, 1992, Colorado State University, Fort Collins. p 8.

Wilmut, I., M. L. Hooper, and J. P. Simons. 1991. Genetic manipulation of mammals and its application in reproductive biology. J. Reprod. Fert. 92:245-279.

Wise, T. 1987. Biochemical analysis of bovine follicular fluid: Albumin, total protein, lysosomal enzymes, ions, steroids, and ascorbic acid content in relation to follicle size, rank, atresia classification and day of estrous cycle. J. Anim. Sci. 64:11531169.

Wright, G., A. Carver, D. Cottom, D. Reeves, A. Scott, P. Simons, I. Wilmut, I. Garner, and A. Coleman. 1991. High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. Biotechnology. 9:830-834.

Wright, R. W., Jr., G. B. Anderson, P. T. Cupps, and M. Drost. 1976a. Blastocyst expansion and hatching of bovine ova cultured in vitro. j. Anim. Sci. 43:170-174.

Wright, R. W., Jr., G. B. Anderson, P. T. Cupps, and M. Drost. 1976b. Successful culture in vitro of bovine embryos to the blastocyst stage. Biol. Reprod. 14:157-162.

Xiaoxia, Z., J. Zhanqun, J. Shie, L. Jianmin, and Z. Yuding. 1991. In vitro co-culture of in vitro fertilized (IVF) bovine embryos with different cell monolayers. Theriogenology. 35:295.

Xu, K. P., and T. Greve. 1988. A detailed analysis of early events during in-vitro fertilization of bovine follicular oocytes. J. Reprod. Fert. 82:127-134.

Xu, K. P., T. Greve, H. Callesen, P. Hyttel. 1987. Pregnancy resulting from cattle oocytes matured and fertilized in vitro. J. Reprod. Fert. 81:501-504.

Xu, K. P., J. W. Pollard, R. W. Rorie, L. Plante, W. A. King, and K. J. Betteridge. 1990. Pregnancy rates following transfer of bovine embryos produced by in vitro maturation, fertilization, and co-culture. Theriogenology. 33:351.

Xu, K.P., B.R. Yadav, R.W. Rorie, L. Plante, K.J. Betteridge, and W.A. King. 1992. Development and viability of bovine embryos derived from oocytes matured and fertilized in vitro and co-

cultured with bovine oviductal epithelial cells. J. Reprod. Fert. 94:33-43.

Xu, X. J., Q. Z. Yang, R. C. Qian, Z. Nakahara, K. Niwa, and P. C. Qin. 1992. Development on in vitro fertilized bovine ova in two co-culture systems. In: 12'th International Congress on Animal Reproduction. p1372-1374.

Yang, X., S. Jiang, P. Farrell, R.H. Foote, and A.B. McGrath. 1993. Nuclear transfer in cattle: Effect of nuclear donor cells, cytoplast age, co-culture, and embryo transfer. Molec. Reprod. Dev. 35:29-36.

Younis, A. I., B. G. Brackett, R. A. Fayrer-Hosken. 1989. Influence of serum and hormones on bovine oocyte maturation and fertilization in vitro. Gamete Res. 23:189-201.

Zhang, L., D. M. Barry, Denniston, R. S., T. D. Bunch, and R. A. Godke. 1992a. Successful transfer of frozen thawed IVF-derived bovine embryos. Theriogenology. 37:331.

Zhang, L., M. R. Flood, T. D. Bunch, W. Hansel, and R. A. Godke. 1992b. Evaluating bovine oviductal cells used in combination with bovine cumulus cells to co-culture IVF-derived bovine embryos in vitro. In: 12'th International Congress on Animal Reproduction. p1375-1377.

Zimmerman, U., and Vienken, J. 1982. Electric field-induced cell-to-cell fusion. J. Memb. Biol. 67:165-182.

Zuelke, K.A., and B.G. Brackett. 1993. Increased glutamine metabolism in bovine cumulus cell-enclosed and denuded oocytes after in vitro maturation with luteinizing hormone. Biol. Reprod. 48:815-820.

Zuelke, K. A., and B. G. Brackett. 1990. Luteinizing-hormone enhanced in vitro maturation of bovine oocytes with and without protein supplementation. Biol. Reprod. 43:784-787.

APPENDIX

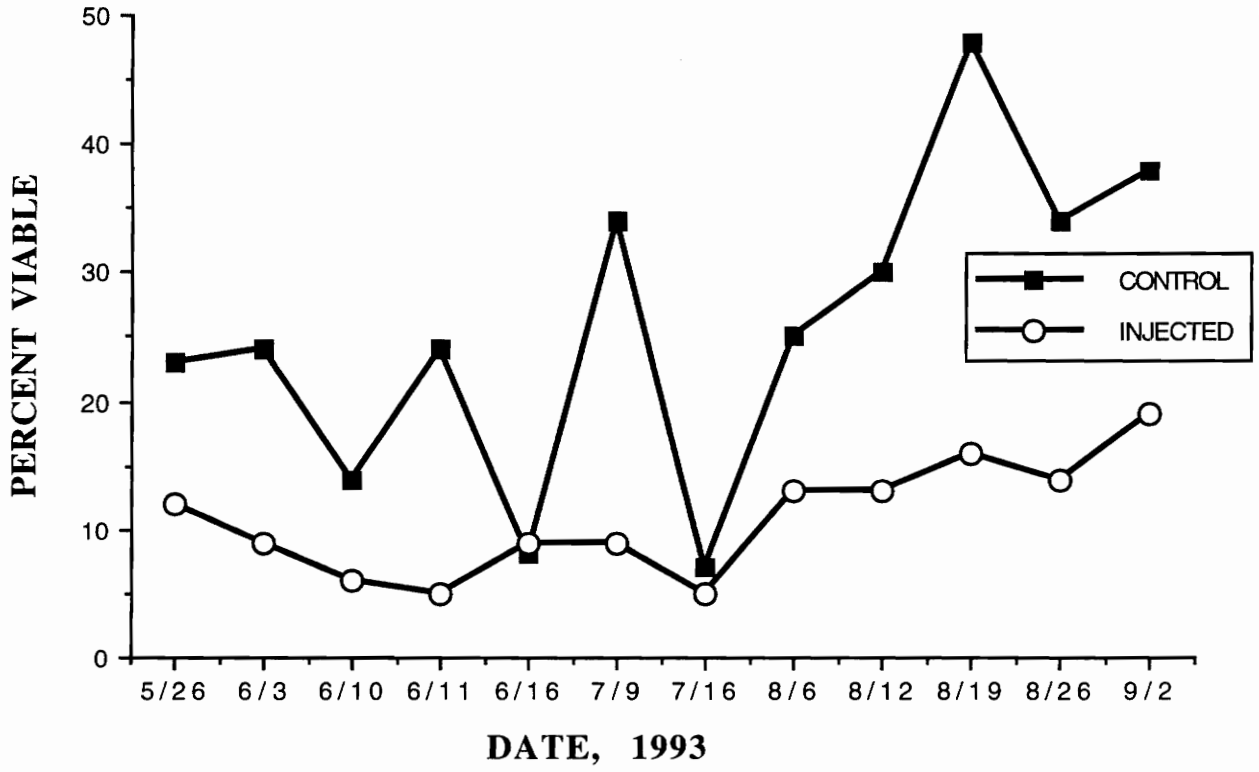

Figure 1. Replicate Variation in Development to Morula and Blastocyst of Injected and Control Bovine Embryos.

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EDUCATION

Doctor of Philosophy, Virginia Polytechnic Institute and State University
Dr. Frank Gwazdauskas, advisor
Major: Animal Science/Dairy
Title of Dissertation: Gene Injection in the Bovine: Effect of Time of Microinjection and Nuclear Transfer Technologies
Expected Graduation Date: January, 1994

Texas A&M University, August, 1990 through December, 1991
Dr. Frank L. Barnes, co-advisor
Dr. Tom Welsh, co-advisor
Major: Animal Science

Master of Science, North Carolina State University, August, 1989
Dr. Robert Petters, advisor
Major: Animal Science Minor: Interdisciplinary
Title of Thesis: Development of Porcine Embryos in Mouse Oviducts in Organ Culture

Bachelor of Arts, Hanover College, May, 1987
Major: Biology Minor: Chemistry and History
Independent Study: Investigation of the Role of the *Agrobacterium tumefaciens* Ti Plasmid in Crown Gall Tumor Formation

Study Abroad: Goethe Institute, Staufen, West Germany, Spring 1985

EMPLOYMENT EXPERIENCE

Marshfield Medical Research Foundation; Marshfield, WI, January, 1994
Research Associate
- perform in vitro fertilization and assisted reproduction techniques with human embryos
- develop methods to screen embryos for genetic diseases
- develop in vitro maturation techniques for human embryos

Virginia Polytechnic Institute and State University; Blacksburg, VA, January 1992 to January, 1994
Graduate Research Assistant
- perform pronuclear microinjections of cattle, pig, goat, and mouse embryos
- prepare and analyze embryos and tissue samples by polymerase chain reaction for the presence of the transgene
- assist in ultrasound-guided follicular aspiration of cattle
- accomplish daily in vitro maturation and fertilization of cattle oocytes
- responsible for estrus synchronization of cattle and embryo transfer of microinjected embryos
- conduct research in bovine embryo culture
- assist in teaching graduate courses

EMPLOYMENT EXPERIENCE (CONT'D)

TransPharm, Inc.; Blacksburg, VA, March 1993 to January, 1994
Consultant
- responsible for production of transgenic cattle from slaughterhouse oocytes
- assist in development of novel techniques for transgene detection in pre-implantation embryos

Granada BioSciences, Inc.; College Station, TX, July 1989 through December, 1991
Research Associate
- conduct research in bovine oocyte in vitro maturation, in vitro fertilization, and embryo culture
- compose project proposals and completion reports
- assist in designing experimental protocols
- utilize cell culture techniques with several types of cells to improve current maturation and co-culture systems
- collaborate in the study of nuclear maturation of non-human primate oocytes
- investigate methods to maintain meiotic arrest in bovine oocytes
- aid in grant authorship

North Carolina State University; Raleigh, NC, September 1987 through June 1989
Graduate research assistant
- conduct research in porcine, murine, and bovine embryo culture
- assist in teaching undergraduate and graduate courses
- perform and assist surgical recovery of porcine embryos
- responsible for estrus synchronization and detection in mice, rats, cows, and pigs

PUBLICATIONS

Krisher, R.L., Gibbons, J.R., Gwazdauskas, F.C., Russell, C.G., Wilkins, T.D., Velander, W.H. (1993) Influence of the time of gene microinjection on development and DNA integration in bovine embryos. Biol. Reprod. 48 (suppl 1):172.

Krisher, R.L. (1992) Expanding the genetic impact of the super cow in the 90's. Virginia Dairyman 56(6):60.

Krisher, R.L., Petters, R.M., Johnson, B.H., Bavister, B.D., Archibong, A.E. (1989) Development of porcine embryos from the one-cell stage to blastocyst in mouse oviducts maintained in organ culture. J. Exp. Zool. 249:235-239.

Krisher, R.L., Petters, R.M., Johnson, B.H. (1989) Effect of oviductal condition on the development of one-cell porcine embryos in mouse or rat oviducts maintained in organ culture. Theriogenology 32(6):885-892.

Krisher, R.L., Gibbons, J.R., Gwazdauskas, F.C., Eyestone, W.H. DNA detection frequency in microinjected bovine embryos following extended culture in vitro. Theriogenology 41:229.

Gibbons, J.R., Beal, W.E., **Krisher, R.L.,** Faber, E.G., Pearson, R.E., Gwazdauskas, F.C. Efects of once versus twice weekly transvaginal follicular aspiration on bovine oocyte recovery and embryo development. Theriogenology 41:206.

PUBLICATIONS (CONT'D)

Hajdu, M.A., Knight, J.W., Canseco, R.S., **Krisher, R.L.,** Velander, W.H., Pearson, R.E., Gwazdauskas, F.C. Effect of culture conditions, donor age, and injection site on in vitro development of DNA microinjected porcine zygotes. Theriogenology 41:211.

Accepted for publication:

Krisher, R.L., Gwazdauskas, F.C., Page, R.L., Russell, C.G., Canseco, R.S., Sparks, A.E.T., Velander, W.H., Johnson, J.L., Pearson, R.E. Ovulation rate, zygote recovery and follicular populations in FSH-superovulated goats augmented with $PFG_{2\alpha}$ and/or GnRH. Theriogenology.

Krisher, R.L., Gibbons, J.R., Canseco, R.S., Johnson, C.G., Notter, D.R., Velander, W.H., Gwazdauskas, F.C. Influence of time of gene microinjection on development and DNA detection frequency in bovine embryos. Transgenic Res.

Hajdu, M.A., Knight, J.W., Canseco, R.S., **Krisher, R.L.,** Velander, W.H., Pearson, R.E., Gwazdauskas, F.C. Effect of culture conditions, donor age, and injection site on in vitro development of DNA microinjected porcine zygotes. J. Anim. Sci.

PRESENTATIONS

1994	DNA detection frequency in microinjected bovine embryos following extended culture in vitro. Student Competition, International Embryo Transfer Society, Melbourne, Australia
1993	Influence of the time of gene microinjected on development and DNA integration in bovine embryos. Society for the Study of Reproduction, Fort Collins, CO
1992	The effect of follicle size and hormone supplementation on meiotic and developmental competence of in vitro matured, in vitro fertilized bovine oocytes. Virginia Tech Graduate Student Association Poster Presentation, Blacksburg, VA
1989	Development of porcine embryos in mouse oviducts maintained in organ culture. American Society of Animal Science, Southern Section, Nashville, TN
1989	Development of porcine embryos in mouse oviducts maintained in organ culture. North Caroline State University Biotechnology, Molecular Biology Interest Group Retreat, Duke University Marine Laboratory, Beaufort, NC
1989	Development of porcine embryos in mouse oviducts maintained in organ culture. Southeast Regional Developmental Biology Conference, Raleigh, NC

MEETINGS ATTENDED

1994	Annual Conference of the International Embryo Transfer Society, Melbourne, Australia
1993	Annual Conference of the Society for the Study of Reproduction, Fort Collins, CO
1993	Annual Conference of the International Embryo Transfer Society, Baton Rouge, LA
1992	Annual Conference of the Society for the Study of Reproduction, Raleigh, NC
1992	Annual Meeting of the American Society of Andrology, Bethesda, MD

MEETINGS ATTENDED (CONT'D)

1992	Ninth International BioTechnology Symposium, Crystal City, VA
1990	Annual Conference of the International Embryo Transfer Society, Denver, CO
1990	Advances in Reproductive Medicine and Surgery: A Comprehensive Review, Houston, TX
1990	VIII Ovarian Workshop: Regulatory Processes and Gene Expression in the Ovary, Maryville, TN

HONORS AND ACHIEVEMENTS

Sigma Xi, The Scientific Research Honor Society, 1993
Gamma Sigma Delta, The Honor Society of Agriculture, 1993
Graduate Honor Society, Virginia Tech, 1992 to present
- Investigator

Phi Mu Sorority, 1984
- Discipline/Ethics Chairman, 1986
- Executive Council, 1986
- Chaplain, 1985

Hanover College Varsity Field Hockey Team, 1983-1986
- Captain, 1986

Deans List, Hanover College
- Winter 1985, Fall 1986, Winter 1987

Hanover College Pan Hellenic Council
- Vice President, 1986
- Sorority Selection Committee, 1986
- Rush Counselor, 1986

Alpha Phi Omega Service Fraternity, 1985

COURSE WORK AND LABORATORY SKILLS

Animal Biotechnology: Embryo Manipulation
- embryo recovery and culture, in vitro fertilization, parthenogenetic activation, embryo transfer, embryo aggregation, cryopreservation, and DNA microinjection techniques

Biotechnology
- isolation and characterization of nucleic acids and proteins, molecular cloning of eukaryotic genes, and expression, detection and purification of recombinant proteins

Experimental Biochemistry
- radioimmunoassay, enzyme purification, protein, DNA and RNA isolation and electrophoresis, northern dot hybridization, autoradiography, and DNA sequencing

Experimental Statistics for Biological Sciences, I and II
- student's t distribution, chi square analysis, analysis of variance, co-variance, multiple regression, curvilinear regression, latin square design, factorial analysis, split plot design, and use of Statistical Analysis System (SAS)

Biochemical Genetics
- tools of molecular biology, eukaryotic gene regulation, transcriptional control in yeast systems, tissue specific gene expression, and advanced lambda phage gene regulation

REFERENCES

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