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PROJECT NUMBER: W-171 (Rev.)

TITLE: Germ Cell and Embryo Development and Manipulation for the Improvement of Livestock.

DURATION: October 1, 1999 through September 30, 2004

STATEMENT OF THE PROBLEM:

Understanding of the underlying biological mechanisms and principles of methods used to produce genetically altered livestock is limited. Furthermore, most of these methods are very inefficient. These technologies will have to be substantially more efficient if we are to realize the advantages of transgenic farm animals for human food and fiber production. These advantages include the production of more desirable products, new products and increased efficiency of the utilization of natural resources.

JUSTIFICATION:

The development of transgenic animals used for food and fiber production has significant potential for consumers, animal producers, their communities and our environment. Potential examples of such transgenic animals are those producing a milk containing human proteins to make a more desirable human baby formula, those producing a leaner, more desirable meat, or those more efficient in growth, reproduction, wool production, or milk production including those with increased disease resistance (Wheeler and Choi, 1997). Increased efficiencies in production of animal products can be of economic benefit to both consumers and producers and have obvious advantages to the environment in terms of reduced use of natural resources.

Current procedures for the production of experimental transgenic animals involve the use of in vitro oocyte maturation, in vitro fertilization, and in vitro culture before and after gene injection (Hasler et al., 1995). This is more practical than recovery of in vivo fertilized embryos but still extremely time and labor-consuming. Currently, more than 10 hours of labor are required to produce a single gene-injected bovine embryo for recipient transfer. When this is coupled with a 20% pregnancy rate and a 12% incorporation rate of the injected gene into the cells contributing to the offspring, an estimated 5,000 hours are required to produce a single transgenic offspring. Before transgenic animals can contribute significantly to production systems, their production will have to be far more efficient. The inefficiencies occur during oocyte maturation and fertilization, during embryo culture and during the incorporation and expression of the microinjected DNA. Short and long term storage of the transgenic embryos is necessary for efficient production of transgenic animals and needs improvement as well (Hurtt et al., 1999).

The details of meiotic maturation of oocytes (particularly the details of nuclear maturation) are beginning to be understood. Much of this work has been done with mouse oocytes; yet differences are known to exist between murine oocytes and bovine, porcine, or ovine oocytes

(Cran and Moor, 1989). This is just one of the areas where the proposed regional research will contribute to animal production.

Although there have been recent advances in nuclear transfer technology in livestock and laboratory species (Wilmut et al., 1997; Schnieke et al., 1997; Wakayama et al., 1998), much is still to be learned regarding the biology and application of these methods to production of transgenic animals. This technology is very inefficient at present (Wilmut et al., 1997) and needs improvement before it can be widely used for livestock systems. Research to increase the practicality of making transgenic animals is directly in line with FAIR95 Goal 2 "Meet consumer needs in domestic and international markets for competitive and high-quality products from animals. Objective l. Increase efficiencies of production livestock. Objective 2. Enhance the quality of products from animals" (Anonymous, 1993). The economic significance of transgenic animals to U.S. animal agriculture in the future cannot be estimated with any confidence. However, the livestock and dairy industries generated over 68 billion dollars of on-farm receipts in 1992 (Anonymous, 1994). Even little effects on efficiency would repay research costs several times over.

A regional research approach is an extremely advantageous means to efficiently approach these problems. Alternative approaches can be tested in multiple laboratories and the effective procedures further tested in the remaining laboratories. Oocyte and embryo procedures appear particularly laboratory dependent; for example, the optimal exposure time for vitrification of mouse oocytes and mouse blastocysts varied significantly among laboratories (Wood et al., 1993; Valdez et al., 1993; Zhu et al., 1993; Shaw et al., 1992). Improvements in nuclear transfer methods and the development of embryonic/somatic cell lines to serve as nuclei donors are other areas that would benefit from this multiple laboratory approach.

The use of transgenic and nuclear transfer approaches are also very useful experimentally for obtaining a variety of information. Some examples are insight into the cell cycle, nuclear and cytoplasmic programing or re-programming, genomic imprinting, gene expression and developmental process to name a few. This information can be used in studies to examine basic biological questions, biomedical questions, genetic questions and evolutionary questions as well as applications for agriculture.

This proposal will evaluate two areas critically important to the future success of animal biotechnology: 1) the understanding of the developmental biology and underlying biological mechanisms of fertilization and embryonic development and 2) the refinement of methods for production of genetically modified animals to improve livestock production efficiency.

RELATED CURRENT AND PREVIOUS WORK:

Related regional projects:

Related regional research projects are NC-210 Mapping the Pig Genome, NCR-057 Reproductive Physiology, NCR-150 Application of Cellular and Molecular Biology to Animal

Science Research, NEC-80 Biotechnology-Animal Development, and S-248 Improving Reproductive Efficiency of Cattle. However, the work is complementary, rather than duplication.

The remaining description of current and previous work will be organized by objectives and references are typically to current reviews.

1). Understanding the developmental biology and underlying biological mechanisms of fertilization and embryonic development.

Oocyte Maturation

Use of in vitro matured oocytes in combination with in vitro fertilization for the production of livestock embryos in the laboratory is rapidly increasing (Hasler et al.,1995).These techniques have not only tremendous research value in studying the basic biological events that occur during oocyte maturation, fertilization and early embryo development, but also provide an inexpensive and readily procurable source of preimplantation livestock embryos. Successful maturation, fertilization and subsequent embryonic development depend heavily on initial oocyte quality. Procedures that could accurately determine oocyte viability and its likelihood to fertilize and develop as an embryo following in vitro maturation would dramatically improve the efficiency of in vitro embryo production. Alternatively, reliable methods for assessment of viability of in vivo- or in vitro- derived embryos would permit screening of embryos prior to manipulation or transfer procedures. Linking these indicators of embryo viability and successful development with the expression and function of specific genes could also afford valuable insights into mechanisms contributing to embryonic mortality.

An integral aspect of in vitro embryo production is successful oocyte maturation. Oocyte maturation is dependent on two separate events: nuclear maturation and cytoplasmic maturation (Fulka et al., 1998). Nuclear maturation refers to the progression of the oocyte from the dictyate stage in meiosis I to metaphase in a meiosis II and extrusion of the first polar body. The process of nuclear maturation occurs spontaneously upon removal of the oocyte-cumulus cell complex (OCC) from the follicle and does not represent a significant problem using established culture conditions. However, nuclear maturation alone is not necessarily indicative of the oocyte's ability to fertilize or sustain continued development (Izadyar et al., 1998). The process of cytoplasmic maturation appears to be the critical factor that determines the success of the subsequent steps to produce viable embryos. Determining and quantifying the indicators of cytoplasmic maturation in the oocyte would enable more efficient selection of oocytes for fertilization and embryo development in vitro.

Involvement of cyclin and $p34^{cdc2}$ kinase in nuclear maturation has been convincingly demonstrated in a number of species (Cran and Moor, 1989, Polanski et al., 1998). Other details of nuclear maturation are yet to be understood and exhibit some species variation. For example, protein synthesis of unknown molecules is required for appropriate nuclear maturation in ovine and porcine oocytes but is unnecessary in murine oocytes.

The biology of cytoplasmic maturation is even less well understood (Krisher and Bavister, 1998). The anatomy of the oocyte changes includes the development of microtubule organizing centers and the migration of intracellular organelles (Mattson and Albertini, 1990). The ability of the oocyte plasma membrane to fuse with sperm increases with increasing maturity at least in the hamster (Zuccotti et al., 1991).

There appears to be a lack of synchrony between nuclear and cytoplasmic maturation in vitro. Porcine oocytes may achieve nuclear maturation to metaphase II after as few as 24 hours of culture, but do not possess developmental competence until over 40 hours (Grupen et al., 1997). It is possible to prevent spontaneous nuclear maturation by addition of dibutyryl cAMP to the maturation medium (Nekola and Smith, 1975) and thus, allow more time for cytoplasmic maturation to occur while avoiding nuclear aging. Funahashi et al. (1997a) reported the addition of dibutyryl cAMP during the first 20 hours of maturation increased homogeneity of nuclear maturation among the maturing oocytes and resulted in higher pregnancy rates after fertilization and embryo transfer. Developmental competence might be improved further by delaying nuclear maturation for a longer period of time because oocyte maturation in vivo requires several days as compared with the 42 to 44 hour maturation period typically reported for porcine oocytes.

Studies have indicated that morphological characteristics such as appearance of cumulus cells, oocyte size, location in the ovary and the time of polar body extrusion are related to the ability of oocytes to fertilize and develop into viable embryos (Younis al., 1989; Arlotto et al., 1990, 1992; Dominko and First, 1992). There are distinct morphological differences between in vivo and in vitro matured porcine oocytes (Wang et al., 1998). Events following fertilization such as sperm decondensation, pronucleus formation and completion of meiosis appear delayed for in vitro matured porcine oocytes (Laurincik et al., 1994). The slow or incomplete cortical granule release following sperm penetration (Wang et al., 1997) and uneven distribution of cortical granules within the oocyte (Wang et al., 1998) might be related to the high incidence of polyspermy reported for porcine oocytes matured and fertilized in vitro. Overall, the limited developmental competence of porcine oocytes matured in vitro appears to be related to failure of adequate cytoplasmic rather than nuclear maturation (Nagashima et al., 1996).

Other reports suggested that activities of specific enzymes in OCC such as histone-1-kinase or quantities of biochemicals, such as prostaglandins, produced by OCC in culture are indicators of fertilization and potential embryo development (He et al., 1973). A multitude of studies have employed the addition of gonadotropins to maturation medium at the initiation of culture. However, there might be an advantage in either delaying or sequentially adding these hormones. Delaying the addition of gonadotropins by as little as 12 hours has been reported to improve developmental competence of porcine oocytes (Funahashi et al., 1997b). Studies investigating the long-term culture or preantral follicles have utilized FSH and estradiol in the culture medium (Hiro et al., 1994). Both estradiol (Eroglu, 1993) and FSH (Singh et al., 1993) have been shown to delay germinal vesicle breakdown (GVBD). Pre-culture of preantral follicles in FSH enhances GVBD and cumulus expansion in response to LH or hCG (Eppig, 1991). Therefore, sequential addition of FSH followed by LH might be effective in delaying nuclear maturation while promoting cytoplasmic maturation so that better maturation synchrony can be achieved.

Also, sequential addition of FSH and LH should prevent premature luteinization of cumulus cells and a shift from estradiol to progesterone production. In vivo, the shift from estradiol to progesterone production by follicles occurs late in maturation, as the oocyte approaches metaphase-II (Xie et al., 1990). There also might be an advantage in the addition of insulin to oocyte maturation medium during the initial stages of culture because insulin decreases follicular atresia in vivo (Matamoros et al., 1991). Follicular fluid composition has also been shown to be different between oocytes which develop to embryos and those which did not. These reports have demonstrated differences in concentrations of several proteins in follicular fluid, including plasminogen activator, that differ between fertilized and nonfertilized oocytes (Cannon and Menino, 1998).

It has been shown that equine oocytes require a longer in vitro maturation period than bovine oocytes (Shabpareh et al., 1993). Considerable research was conducted on the effects of follicular fluid on maturation of bovine oocytes; we concluded that preovulatory follicular fluid before the LH surge inhibits maturation, while by 20 hours after the LH surge, follicular fluid stimulates oocyte maturation, even in the presence of inhibitory factors (Romero and Seidel, 1994, 1996). Furthermore, 20-hour follicular fluid during maturation produces embryos that develop to blastocysts more readily than controls. The primary stimulatory factor may be EGF (Kato and Seidel, 1996), whereas, it has been shown that follicular walls (theca interna cells) are the source of the inhibitory factors (Kato and Seidel, 1998).

Fertilization and Sperm Capacitation

Fertilization and initial cleavage stages occur in the mammalian oviduct. Although production and development of farm animal embryos have become routine, their development in vitro remains far below in vivo embryos. The inability to mimic in vivo conditions may contribute to sub-optimal development of in vitro-produced embryos. The secretion of several factor(s) from the mammalian oviduct may contribute to the superior development of in vivo produced embryos. A high molecular weight oviductal glycoprotein (OGP) is synthesized and secreted by the secretory cells of the oviduct. The presence of OGP is highest during the periovulatory period and declines thereafter, suggesting its potential role during in vivo fertilization and possibly during the initial cleavage stages (Malette et al., 1995; Verhage et al., 1998).

In most IVF systems sperm concentrations are adjusted to maximize fertilization rates without regard to exposure of oocytes to high concentrations of spermatozoa. However, under in vivo conditions, oocytes are only exposed to a few spermatozoa. The production of oxygen radicals from high concentrations of spermatozoa (Aitken et al., 1996) may adversely affect embryo development and implantation.

As part of the cytoplasmic maturation of oocytes, the protein composition of the oocyte plasma membrane changes including an increased ability to bind and/or fuse with sperm (Zuccotti et al, 1994; Cohen et al., 1996). Oocyte receptors for the sperm plasma membrane are either described/ hypothesized as the complementary receptors for putative ligands or in one case

as integrin heterodimers. Integrins are exposed on the surface of many mammalian oocytes and are complementary to one of the apparent sperm ligands in rodents. Hence, integrin molecules are likely to be one of the sperm receptors on rodent oocytes; integrin heterodimers do not meet the presumed criteria for tissue and species specificity. Continued synthesis/export of sperm receptors to the plasma membrane following ovulation was demonstrated previously in mice (Kellom et al., 1992). Wheeler et al. (1996) has verified that bovine oocytes increase their ability to bind sperm plasma membrane during the 22 hours of in vitro maturation and that this increase is dependent upon protein synthesis. There has previously been an evaluation of plasma membrane protein components in individual oocytes. Recently, a putative oocyte plasma membrane protein (POMP) receptor for sperm was identified based on western ligand blotting, affinity chromatography, localization on oocyte surface, and inhibition of sperm-oocyte binding by FAB to a MAP peptide of this protein (Berger et al., 1998; 1999; Yen and Berger, 1999).

Sperm gain the ability to bind and fuse with the oocyte plasma membrane in the equatorial segment during capacitation. Modification of one or more of the ligands or exposure of one or more of the ligands for the oocyte plasma membrane would appear to be an essential component of capacitation. A number of potential sperm ligands have been proposed to interact with the oocyte in different species; species specificity (at least among mammalian orders) would be expected. Ash et al. (1995) identified four predominant binders of the oocyte plasma membrane as potential porcine sperm ligands for the porcine oocyte plasma membrane.Using an antibody to the DRKD N-terminal peptide, antigenic cross-reactivity between this porcine sperm plasma membrane recognizing porcine oocyte plasma membrane and a bovine sperm plasma membrane recognizing bovine oocyte plasma membrane was demonstrated. Ligand/receptor similarity might be anticipated among species within a mammalian order since whole gametes demonstrate some cross species interaction.

Assisted Fertilization

It has been shown that capacitation status of bovine sperm for intracytoplasmic sperm injection (ICSI) is irrelevant, and that there is a benefit of activation of oocytes with the calcium ionophore A23187 (Chen and Seidel, 1997). Further, a beneficial effect has been shown by activating equine oocytes with ICSI (Kato et al., 1997). A novel method of increasing fertilization rates in vivo via a sperm-oocyte adhesive peptide (Amann et al., 1999) may also be promising in vitro. GnRH was also shown to increase the success rate of bovine IVF (Funston and Seidel, 1995).

Sex Determination

The ability to pre-select the sex of offspring would have tremendous economic benefit to beef and dairy producers alike. Numerous efforts have been directed toward development of procedures for pre-selecting the sex of offspring. One approach has been to attempt to physically separate X- from Y-bearing spermatozoa (Amann, 1989). To date, modified flow cytometry (Johnson et al., 1994) is the only separation procedure that has been successful in sorting X- from Y-bearing spermatozoa. However, this procedure does not have widespread application at the present time, due to the cost of the equipment and limitations on sorting rate.

It may be possible to significantly alter the sex ratio by screening and then selecting, ejaculates with a naturally altered ratio of X- to Y-bearing spermatozoa. Chandler et al. (1998) reported that within sires, the ratio of X- to Y-bearing spermatozoa from different ejaculates of semen can be skewed significantly toward one sex and this skewed ratio appears to translate into altered sex ratio of offspring. The ratio of X- to Y-bearing spermatozoa of ejaculates within bulls was found to be significantly skewed (~84%) in about 20% of the ejaculates evaluated. A number of physiological and environmental factors have been reported to be related to a male effect on sex ratio (Krackow, 1995). Studies are needed to confirm that the ratio of X- to Y bearing spermatozoa is skewed in some ejaculates of semen and to identify factors influencing or related to the skewed ratio.

Embryo Development

Although the methodology for maintaining embryos from livestock in culture has existed for many years, the ability of the present systems to support normal development is limited. This is especially true of early embryos that often exhibit developmental blocks in vitro (Beckmann and Day, 1993, Krisher and Bavister, 1998). These blocks are thought to be the result of inadequate culture systems since similar blocks are not found in vivo. In addition, numerous studies have shown that there is a decline in embryo viability corresponding to the length of time they are held in culture (Davis, 1985). More recently, co-culture systems have been developed to at least partially overcome the deficiencies associated with earlier systems (Trounson et al., 1994). Although a variety of cell types have been examined for use in co-culture systems, the greatest success has been achieved using oviduct cells. The benefits of co-culture systems in promoting in vitro development are well established, but the mechanisms by which these benefits are achieved have yet to be elucidated. Despite the increase of their use, co-culture systems are not without their limitations. First, by their very nature, co-culture systems are undefined. This makes comparisons of results difficult between laboratories as well as within laboratories when experiments are conducted over an extended period of time using different batches of oviduct cells. Second, co-culture systems require that researchers maintain cell lines or continually establish primary cell cultures to maintain the culture system. Third, it is difficult, if not impossible, to conduct basic studies in areas such as embryo metabolism, embryonic protein secretion and/or protein binding to embryos in co-culture systems due to the involvement by the oviduct cells.

Evidence is accumulating that growth factors have an autocrine and paracrine role in embryo development (Gandolfi, 1994; Heyner et al., 1994, Glover and Seidel, 1998).Tremendous effort will be required to define these effects but secretion of growth factors by the co-cultured cells may be at least one of the mechanisms for the beneficial effect of co-culture (Winger et al., 1997).

An extensive series of experiments confirmed the detrimental effects of high oxygen and beneficial effects of certain antioxidants on in vitro development of bovine embryos (Kato et al., 1998; Olson and Seidel, 1999). Optimal ways have been determined to condition media with buffalo rat liver cells for culturing bovine embryos (Funston et al., 1997). Studying gene

expression in elongating bovine embryos has shown the IGF system appears to have important regulatory roles (Keller et al., 1998). One of our stations (Colorado) has identified several genes with increased expression as embryos elongate (Glover and Seidel, 1998).

Extracellular matrix (ECM)-degrading proteinases are believed to play pivotal roles in early embryo development and embryo-uterine interactions (Cross et al., 1994). ECM-degrading proteinases include the plasminogen activator (PA)-plasmin system and the family of matrix metalloproteinases (MMP) (Vassalli and Pepper, 1994). To date, the laboratory of one of our contributing stations (Oregon) has demonstrated that endodermal cells express PA during migration in vitro in sheep and cattle. During the periods of endodermal cell migration and trophoblastic expansion and elongation, ovine embryos express PA, MMP-9 and three MMP inhibitors (TIMPs-1, -2 and -3).

Blastocyst production by in vitro methods has plateaued at around 40% despite various attempts to improve culture conditions. The 40% development rate falls short of the 85 to 95% that occurs in vivo. Various attempts have been made by using co-culture systems in conjunction with adding or varying media components or culture conditions (i.e. media, co-culture cell type, medium supplements, gas atmosphere, various environmental factors etc); however, in vitro production of blastocysts still hovers at or below 40% , with recent reports ranging from 20 - 30% (Eyestone and First 1989, Thibodeaux et al. 1992, Freeman et al. 1993, Choi et al. 1998, Krisher and Bavister 1998 and Lim et al. 1998). Furthermore, in vitro-produced blastocysts are usually developmentally retarded with fewer inner cell mass cells compared with embryos developed in vivo. The primary problem associated with current culture systems is that they do not mimic the changing oviductal/uterine environment. This appears to be supported by the fact that blastocyst development is influenced by the stage-specific composition of culture medium. The apparent lack of biologic culture conditions necessitates further innovation in tissue culture methodology and the pursuance of further research in this area.

2). Refinement of methods for production of genetically modified animals to improve livestock production efficiency.

Nuclear Transfer

Nuclear transfer, which involves the transfer of each nucleus from a multicellular embryo into an enucleated metaphase II oocyte, has been developed in several species (Willadsen, 1986; Prather et al., 1987; Stice and Robl, 1988.). Nuclear transfer has become an active field of research over the last decade, culminating in reports over the past two years of live offspring from differentiated cells of sheep, cattle, and mice (Schnieke et al., 1997; Wells et al., 1997; Cibelli et al., 1998; Kato et al., 1998; Wakayama et al., 1998). Dolly was not a fluke. Dozens of laboratories are producing calves and lambs whose genetic parents are somatic cells, often derived from adults. This technology has three broad applications: 1) applied animal breeding to copy outstanding animals (e.g., proven bulls), 2) a tool for basic research, (e.g., mechanisms of differentiation), and 3) a biotechnological tool (e.g., vastly simpler approaches to making transgenic farm animals).

Although offspring have been produced in livestock using nuclear transplantation, the efficiency rate has been low (Wilmut et al., 1997).In each of the species reported to date, the efficiency is 10% (1-6% in sheep, 1-4% in cattle, 1% in pigs, and 10% in rabbits). The factors affecting the efficiency of nuclear transplantation are: enucleation of the recipient oocyte, fusion of the transplanted nucleus to the enucleated oocyte cytosol, activation of the oocyte, and "re-programming" of the transferred nucleus. In each of the studies previously indicated, if we evaluate the efficiency of each of these steps, activation may be the factor responsible for the greatest loss in efficiency (Collas and Robl, 1990). However, the general consensus of those in the field is that nuclear transfer efficiency is much too low to be economically viable except in very limited applications. The low efficiency also makes it an expensive research tool. Any increase in efficiency will greatly enhance the value of this technology and contribute to our understanding of changes that must occur in chromosomes to allow embryonic gene expression patterns. It is also critical to begin to understand how potentially subtle modifications in the nuclear structure impact the ability of a cell to contribute to production of offspring and yet another to fail.

An important aspect of this field in the past and the future concerns understanding the abnormalities of calves cloned by nuclear transplantation (Seidel et al., 1997; Garry et al., 1998). Such calves and lambs have high rates of fetal and neonatal death. At birth, they are hypoxic, hypoglycemic, hypothermic, and readily die (Garry et al., 1998). It has been hypothesized that these animals have a genetically or epigenetically abnormal placenta, since the calves themselves appear normal genetically (Garry et al., 1996).

Transgenic Methodologies

The ability to produce novel proteins in the mammary gland of domestic animals will have an important impact on agriculture. With recent success in the ability to use somatic cells as nuclear donors which result in the production of offspring, this objective appears finally to be attainable at rates which will allow use of domestic animals. The ability to routinely introduce novel genes into tissue culture cells will greatly increase the types of genes utilized for production of transgenic animals, other than mice. Several years ago one of our contributing stations (Colorado) combined transgenics with in vitro-produced bovine embryos (Thomas et al., 1993), and produced the first transgenic calf demonstrated to, in fact, express a transgene (Bowen et al., 1994). Additional previous work has included the production and study of transgenic mice incorporating a variety of transgene constructs to determine optimum promoter elements (Gutierrez-Adan et al., in press) or fusion gene constructs for use in dairy goats and cattle. Targeted milk properties include processing functionality (Maga et al., 1995; Gutierrez-Adan et al. 1996), anti-microbial properties (Maga et al., 1997), and the application of antisense constructs to obtain efficient down-regulation of protein secretion into the milk (Sokol et al., 1998).

Transgenic animals have great potential to livestock production but the potential is still unrealized. The low efficiency in their production is a primary reason (Ebert and Schindler, 1993; Halter et al, 1994). These inefficiencies occur at all stages. An aspect of great importance in this regard is the isolation and manipulation of undifferentiated, pluripotent embryonic cells. There

are two types of pluripotent/totipotent embryonic cells, embryonic stem (ES) cells and embryonic germ (EG) cells. ES cells are isolated in culture from blastocyst-stage embryos, and EG cells are isolated from cultured primordial germ cell (PGC). Development of a stem cell line for a species that could be used as a recipient of the desired DNA and subsequent nuclear transfer of the stem cell nuclei or the incorporation of the stem cells into an embryo with the resulting birth of a chimeric animal are potential solutions to the inefficient incorporation of the injected DNA. ES cells first were isolated from mouse embryos (Evans and Kaufman, 1981; Martin, 1981) and since have been used as a model for mammalian embryogenesis and more recently for genetic manipulation. Due to their ability to integrate foreign DNA and thereafter differentiate into any and all tissues of a normal individual, ES cells are effective vehicles for genetic engineering and for creating laboratory animal models of human diseases. Despite considerable effort, progress toward isolation of ES cells from agricultural species has been slow. Some of the more promising results have been achieved with pigs (Wheeler, 1994). To date, production of embryonic stem cell lines has been much more difficult in livestock species than in mice (Sims and First, 1994; Wheeler, 1994, Wheeler et al., 1995).

In the early 1990s, several laboratories reported isolation of ES-like cells from cultures of primordial germ cells (PGC's)(Matsui et al., 1992; Resnick et al., 1992). These cells were called EG cells to distinguish them from blastocyst-derived ES cells, but EG and ES cells appear to share many characteristics, including the ability to differentiate into numerous cell types. One of our contributing stations (California) successfully isolated EG cells from porcine PGC (Shim et al., 1997, Shim and Anderson, 1998;), the first example in a species other than the mouse. Recent successes with isolation of ES and EG cells from agricultural species have been exciting, but generally these ES and EG cells have a low efficiency of incorporation into normal, developing embryos. Moreover, in no instance have livestock ES or EG cells been shown to develop into gametes, a prerequisite to their use in genetic engineering. Several laboratories involved in the W-171 project have a history of productive research with ES and EG cells. By combining the resources, ingenuity and collaborative efforts from these laboratories, this Regional Research Project could yield undifferentiated cell lines of practical value to animal agriculture, a phenomenal contribution to animal agriculture.

Cryopreservation

Cryopreservation of in vivo produced bovine embryos is quite successful and very limited success has recently been achieved with other species such as late-stage porcine embryos (Pollard and Leibo, 1994; Nagashima et al., 1994; Dobrinsky, 1997). Initially, in vitro matured and fertilized embryos appeared more susceptible to damage during freezing and thawing (Leibo and Loskutoff, 1993, Arau et al., 1996). Alterations in in vitro maturation and culture can affect the susceptibility of the in vitro-produced oocytes to cryodamage. Recently, some of our group have had an emphasis on equine oocytes and embryos. The large equine embryo routinely collected nonsurgically is particularly challenging to cryopreserve. Recently, a new concept was developed, step-down equilibration, to cryopreserve such embryos successfully (Young et al., 1997). This evolved from basic research of permeability of equine embryos to cryoprotectants (Pfaff et al., 1994). Recently, equine and bovine oocytes have successfully been vitrified (Hurtt et al., 1999). They showed that matured oocytes survived vitrification better than immature ones.

OBJECTIVES:

Objective 1: Understanding the developmental biology and underlying biological mechanisms of fertilization and embryonic development.

Objective 2: Refine methods for production of genetically modified animals to improve livestock production efficiency.

PROCEDURES:

Objective 1: Understanding the developmental biology and underlying biological mechanisms of fertilization and embryonic development.

The overall aim of the research to be conducted under this objective is to gain a more precise understanding of the biological requirements for successful oocyte maturation, fertilization, and subsequent embryonic development. The production of live offspring is dependent upon all of these events occurring in a well-orchestrated fashion.

Collaboration in the area of porcine oocyte maturation will occur. California will examine the presence of a putative oocyte plasma membrane protein (POMP) receptor before and after partial in vitro maturation and during incubation under maturation conditions with the presence of cycloheximide to inhibit protein synthesis. Oocytes will be examined using a confocal microscope, and the intensity of fluorescence in ellipses spaced around the oocyte plasma membrane will be measured and analyzed. In a related study, porcine ovaries will be fixed in Bouin's solution, embedded in paraffin, and sections subjected to immunohistochemistry. Alternate sections will be incubated with pre-immune IgG or stained with hematoxylin and eosin. Presence of POMP at the oocyte surface during oocyte maturation in vivo will be examined.

Arkansas, Iowa and Illinois will investigate the effects of maturation time and media supplementation on the developmental competence of porcine oocytes matured and fertilized in vitro. Porcine oocyte-cumulus complexes (OCC) will be recovered from the ovaries of gilts at slaughter and will be matured for 24, 36 or 48 hours in M-199 supplemented with various combinations of serum, FSH, estradiol, insulin and dibutyryl cAMP. After the initial culture, a portion of the OCC will be removed for evaluation of germinal vesicle breakdown. The remaining OCC will be matured for an additional 24 to 48 hours in M-199 supplemented with serum, LH and cysteine. Following final maturation, oocytes will be evaluated for nuclear maturation and electrically activated to assess parthenogenic development. After the maturation treatments have been assessed, the best treatment/time combination will be used in conjunction with IVF to evaluate oocyte developmental competence. The rate of polyspermy will be compared after pre-exposure of oocytes and/or spermatozoa to oviductal cell secretions.

Colorado will use bovine and equine oocytes from slaughterhouse ovaries for studies on in vitro maturation. Connecticut will use bovine oocytes from pre-pubertal ovaries for studies on in vitro maturation in collaboration with Louisiana. Oocytes will be matured in vitro using standard procedures (Romero and Seidel, 1996), except that chemically defined media will be used for most studies (Olson and Seidel, 1999). The dose-response of gonadotropins; dose-responses of sulfhydryl-donating agents such as glutathione, cysteamine, and ß-mercaptoethanol; addition of amino acids present in oviductal and follicular fluid; benefits of the growth factors EGF and IGF-1; and dose responses of decreasing sodium ion will be the main topics studied. Colorado also will evaluate success via metabolic rates, cortical granule distribution, and zona hardening, as well as the more conventional fertilization and embryonic developmental rates.

Louisiana, Colorado and Illinois will collaborate on methods to assist fertilization of equine oocytes. Intracytoplasmic sperm injection (ICSI) seems to be the most probable method to produce live foals via in vitro embryo production techniques (Squires et al., 1996; Cochran et al., 1998). Joint efforts are planned to continue development of ICSI technology for in-field use in the cow (Colorado, Oklahoma, Louisiana), small ruminants (Louisiana, Iowa) and swine (Illinois). Colorado will use the new piezo-injection technology for ICSI in cattle and horses. An important key to the success of ICSI is proper oocyte activation. Colorado will research injection of the sperm activating protein that causes oscillations of free intracellular Ca^{++} in oocytes (Fissore et al., 1998). This protein will be isolated from bull and stallion semen, and dose responses will be generated by injection at different times relative to ICSI (Chen and Seidel, 1997).

Colorado also will study other methods of attaining physiological oocyte activation including injecting IP3 and other agents affecting this pathway. This work will coincide with studies conducted by Utah where mechanisms associated with activation of bovine oocytes will be evaluated. The potential role of integrins in the interactions between the sperm and oocyte; any association between integrin associated intracellular calcium transients and either IP_3 or ryanodine receptors and their possible link with specific integrin subunits found on the surface of bovine oocytes will be the focus of these investigations.

Studies will be conducted on in vitro sperm capacitation because capacitation is needed for optimal in vitro production of embryos. California will determine, using flow cytometry, if bovine sperm with varying abilities to interact with oocytes vary in the binding of the DRKD antibody to the sperm surface, either initially or after normal in vitro capacitation. Initial comparisons will be made among samples used for in vitro fertilization within a single laboratory, but subsequent studies will focus on semen samples obtained from the collaborating stations (Colorado, Louisiana, Utah) that use slightly different in vitro embryo production conditions.

Fresh and frozen epididymal sperm, and techniques for capacitation, will be studied by several stations (California, Colorado, Iowa, Illinois, Louisiana) for use in IVF procedures in various farm animal species. California and Illinois will investigate porcine sperm, California and Colorado will study bovine sperm, and Colorado, Iowa, and Louisiana will examine equine sperm. Prior research (Louisiana) has identified differences among species, and it has been noted

that epididymal stallion sperm, for example, does not require capacitation in order to produce equine embryos in vitro.

A major collaborative effort among stations will focus on fertilization and subsequent development of in vitro matured bovine oocytes. All oocytes will originate from a single source (Wisconsin) and will be shipped to participating stations (Colorado, Connecticut, Iowa, Louisiana, Oklahoma, and Utah) for subsequent investigations. To the best of our knowledge, this will be the first time that oocytes from a common source will be utilized in such a manner. This uniformity in pre-fertilization conditions will enable a more clear-cut interpretation of experimental results obtained across stations. Likewise, this approach affords the opportunity to investigate various methods of oocyte maturation on subsequent embryonic development using standardized protocols for fertilization and culture.

Preimplantation embryo development in vivo depends on the interaction between the embryo and the oviductal/uterine environment. Interactions occur primarily between proteins (e.g. changes in type, quantity and location of specific proteins such as receptors, enzymes etc.) and the embryo and/or oviductal and uterine epithelial cells. Colorado, Illinois, Louisiana, Oklahoma, and Utah will investigate the role of various glycoproteins on embryonic development. Oklahoma will focus on bovine oviduct-specific glycoprotein (Thibodeaux et al., 1995), while Colorado will study the secreted oviduct glycoprotein that binds to oocytes (Verhage et al., 1998) and a seminal plasma peptide, Fert+, that recently was shown to improve fertility of subfertile bulls with artificial insemination (Amann et al., 1999). Utah will study changes in protein profiles (i.e., type, amount and distribution) of OVEP and UEC co-cultures, which will provide a window through which external processes (epigenetic) of embryonic development can be studied. They will use electrophoresis (i.e. 2-D and capillary IEF and standard electrophoresis procedures) to investigate kinetic changes of protein patterns, and ICC (immunocytochemistry) and FISH techniques will be used to detect changes of specific proteins (receptors). Colorado and Iowa will investigate the role sulfhydryl-donating agents such as glutathione, cysteamine, and ß-mercaptoethanol in embryonic development using a chemically defined medium (Romero and Seidel, 1996; Olson and Seidel, 1999; Caamano et al., 1996; Caamano et al., 1998). Colorado will continue their dose- response studies with various antioxidants, particularly Vitamin E and ß-mercaptoethanol (Olson and Seidel, 1999) and carbohydrate sources (e.g., fructose; Chung et al., 1996. Oregon plans to evaluate expression of components of the PA-plasmin system and the MMP during the periods of endodermal cell migration from the inner cell mass, formation of the bilayered trophoblast, and trophoblastic expansion and elongation in sheep and cattle embryos. They intend to determine the complete phenotypic expression pattern and the cellular localizations of the ECM-degrading proteinases and their associated inhibitors during these periods of development. Understanding these systems during specific developmental events may afford valuable insights into embryonic mortality as well as provide targets for investigation of abnormal placenta formation in pregnancies produced by nuclear-transferred embryos.

Novel approaches to in vitro culture will be investigated by Colorado, Iowa, Illinois, and Louisiana. Colorado has confirmed data of others (Vajta et al., 1997) that submarine culture (using a water bath) appears to improve embryonic development greatly compared to standard

incubators, and they will attempt to determine the reasons for this enhanced development. Illinois has devised a new micro-fluidic embryo culture device, and this will be tested at other stations (Iowa, Louisiana,)

Oregon, Utah, Illinois and Colorado will investigate gene expression in developing preimplantation embryos. Colorado will characterize the genomic organization and functions of genes that are expressed in greater amounts in day-17 than in day-15 bovine embryos, and gene expression between in vitro and in vivo embryos will be compared using differential display (Glover and Seidel, 1998). Illinois will characterize the gene expression of porcine embryos from the 1-cell to blastocyst stage using, RT-PCR, differential display PCR and capillary electrophoresis. Oregon will collect embryos from superovulated donor cows and ewes, and RT-PCR will be used for determining the phenotypic expression pattern of the genes of interest. Localization of the cells expressing the specific genes will be conducted using in situ hybridization. For experiments involving analysis of secreted proteins by cultured embryos, isolated inner cell masses or trophoblast, conditioned medium will be recovered and analyzed by immunoblotting and casein and gelatin zymography. Utah will also use RT-PCR to trace sequential gene expression during embryo development.

Recently, great interest has been expressed in the ability to control the sex of offspring. Arkansas, Colorado, and Iowa will be involved with studies on offspring sex ratios. Arkansas will investigate if a naturally occurring skews occurs in the ratio of X- to Y-bearing spermatozoa among ejaculates, and, if so, will study factors that potentially influence the altered ratio of or X- to Y-bearing spermatozoa. Semen will be collected from bulls at varying intervals, and the ratio of X- to Y- bearing spermatozoa of each ejaculate will be determined using X- and Y-specific DNA probes in conjunction with fluorescence in situ hybridization (Hassanane et al., 1998). Seminal plasma will be evaluated for components that might be related to altered sex ratio on semen. Ejaculates identified as having the sex ratio skewed (70% or greater X- or Y-bearing spermatozoa) will be used in conjunction with in vitro fertilization to confirm that altered sex ratio of semen translates into altered gender ratio in vivo. Embryos will be sexed using commercially available male-specific DNA probes. If possible, arrangements will be made to confirm through artificial insemination and fetal sexing, that altered sex ratio of semen translates into altered sex ratio of offspring. Colorado will continue its studies on non-surgical deep intrauterine insemination using sperm sorted with the aid of a modified flow cytometer, while Iowa will continue its studies on the use of a vaginal conductivity probe to pinpoint an insemination time that may lead to an altered calf sex ratio.

Understanding ovarian follicular dynamics is important for optimizing the production of oocytes for in vitro embryo production and for in vivo embryo production via conventional superovulation. In an effort to produce excellent quality oocytes for IVF, collaborative efforts are planned to evaluate protocols to maximize follicular growth and oocyte collection by transvaginal ultrasound-guided oocyte recovery (Louisiana, Iowa, Connecticut). These studies will focus primarily on prepuberal, pregnant, and postpartum females. Preliminary studies have indicated that transvaginal ultrasound oocyte recovery is possible in goats (Han et al., 1996; Graff et al., 1999) and horses (Bruck et al., 1992; Cook et al., 1992; Meintjes et al., 1994), as well as cows (Pieterse et al., 1988, 1991a,b; Looney et al.,1994. One station (Louisiana) has

offered to train personnel from other stations to enhance collaborative efforts among the contributing stations.

An electronic heat detection system (Heat Watch) has recently become available to livestock producers in the United States, and a joint effort is planned to evaluate this system in different regions (Louisiana, Illinois, Iowa, Utah, Connecticut) using dairy and beef females. The initial focus of this research will be to evaluate differences in the onset and duration of behavioral estrus in similar genotypes during different climates/seasons of the year. Ultimately, these data will be used to predict the best time for transvaginal oocyte collection that will optimize the number of oocytes collected from each donor female. Harvesting the best quality oocytes is important not only for IVF, but also for nuclear transfer or transgenic research programs.

Objective 2: Refine methods for production of genetically modified animals to improve livestock production efficiency.

The overall aim of the research to be conducted under this objective is to enhance the success of each step in the embryo manipulation process that is required for successful production of genetically modified embryos and offspring. Continual advances in gene transfer techniques and nuclear transfer methodology have led to a crucial need for more research that will lead to increased incorporation of these methods into livestock production systems.

Colorado, Louisiana, Connecticut, Illinois and Utah will conduct studies on nuclear transfer. Utah will investigate methods for increasing activation rates of bovine nuclear transfer embryos. They also will conduct experiments designed to evaluate the ability of the early process of fetal and embryonic development to "reset" the developmental clock of adult bovine fibroblasts. These studies will evaluate subtle modifications in nuclear components that result in some cells having the developmental competence to produce offspring and others to result in failure (Blackburn, 1991; Harley et al., 1990; Allsopp et al., 1992; Vaziri et al., 1993; Bodnar et al., 1998). Utah also will continue to study the developmental potential of hybrid (sheep-cow) nuclear transfer embryos to produce viable offspring.

Utah, Louisiana, Illinois, Connecticut and Colorado will investigate the use of somatic cells for nuclear transfer. Louisiana and Illinois will develop methodology to establish caprine, ovine, equine, and bovine somatic cell lines that are useful for nuclear transfer, while Colorado will optimize methods of transplanting nuclei from fetal fibroblasts and cumulus cells into activated enucleated oocytes. Connecticut will test the nuclear transfer efficiency of different donor cell types for nuclear transfer and explore the possibility of transfecting cultured cells for transgenesis. Utah will investigate the use of somatic cell lines (embryonic, fetal and adult) from domestic animal species as vectors for the production of transgenic animals using nuclear transfer.

Louisiana and Colorado also will study the normalcy of embryonic and fetal development in vitro and in vivo because it has been well documented that a portion of the nuclear transfer offspring have been abnormally large at birth (Willadsen et al., 1991). Efforts are needed to

identify the cause of this developmental problem for the success of this new technology to be accepted by the livestock community. Colorado will continue to search for genetic or epigenetic changes in cloned embryos vs. control embryos. They will particularly examine the placenta, by using differential display (Glover and Seidel, 1998) to compare differences in mRNA content of various genes in the cotyledons of cloned vs IVF vs control day-45 pregnancies (collected by a novel, nonsurgical procedure). Differential expression of candidate genes will be confirmed by Northern blots; genes will be cloned, sequenced, and identified and/or characterized.

California, Colorado, Connecticut, Illinois, Iowa, Louisiana, and Utah will investigate techniques for the efficient production of transgenic livestock. California and Illinois will test in vivo differentiation of porcine ES and EG cells after being combined with embryos of various developmental stages. In addition, Illinois will test in vivo differentiation of porcine ES cells in the SCIDs mouse tumor system. Porcine cell lines that have been screened for markers of undifferentiated cells and that have been shown using tests for in vitro differentiation to be pluripotent will be tested for in vivo differentiation. California will share cell lines among participating laboratories in order to test these cell lines under the varying conditions found in the various laboratories. Iowa will investigate methods for more efficient isolation of porcine primordial germ cells. Colorado, Connecticut, Utah, Illinois and Louisiana will conduct studies with transfected cell lines. Colorado will conduct experiments with cattle and sheep and will use GFP (green fluorescent protein) as a reporter gene to study placental expression driven by the regulatory regions of molecules such as placental lactogens, interferon tau, and molecules from other species such as hCG and eCG subunits. Illinois will conduct transfection experiments with the bovine alpha-lactalbumin gene and promoter to drive mammary expression of IGF-1 and beta 1,4 glactosyltransferase. Somatic cells will be transfected in vitro, characterized, and frozen in aliquots for later nuclear transplantation. Similarly, Utah will transfect somatic cell lines with genes designed for expression of foreign proteins in the mammary gland and for enhancement of the resistance of animals to disease of economic importance to agriculture. Louisiana will conduct transfection studies using embryo-derived stem cell lines from goats and cattle.

Colorado, Iowa, and Louisiana will investigate methods for cryopreservation of oocytes and embryos. Colorado, Louisiana, and Iowa will investigate the potential use of vitrification for the freezing of mammalian oocytes and embryos. Louisiana will compare conventional and vitrification methodologies for the cryopreservation of oocytes from the mouse, goat and cow, while Colorado will study vitrified oocytes (cattle and horses) by evaluating fixed and stained specimens (Hurtt et al., 1999). Louisiana and Iowa will evaluate various non-conventional apparatuses (e.g., electron microscope grids) for their ability to enhance the cooling rate during the cryopreservation process. Having a supply of excellent quality oocytes and embryos available at any time would certainly enhance research on nuclear transfer, ICSI, and/or transgenesis.

Much remains to be learned concerning concentration, timing of addition, and timing of removal of cryoprotectants for both vitrification and conventional cryopreservation. Colorado and Iowa will investigate cryopreservation of equine and porcine blastocyst stage embryos. Colorado will expand step-down equilibration studies with large equine blastocysts (Young et al., 1997), compare concentrations of glycerol and ethylene glycol (4 vs 6 M), investigate whether

the step-down procedure (decreasing cryoprotectant) before freezing is, in fact, beneficial. Iowa will examine the biophysical properties of porcine blastocysts in an attempt to develop a suitable method for cryopreservation of intact, nonmanipulated porcine blastocysts.

EXPECTED OUTCOMES/TECHNOLOGY TRANSFER

Results from the proposed research are expected to have tremendous impacts on scientists, on persons engaged in animal production, and on consumers. Gaining insights into the biological mechanisms underlying early embryonic development will enable researchers to devise better systems for the production of embryos genetically capable of more efficient production of food and fiber. Some of the immediate tangible benefits from this research will include the generation of scientific publications which reflect the advancement of science, development of new methodologies that can be utilized in the private sector, and education of society through extension reports and popular press publications. Once the new techniques have been incorporated into biomedical research and production agriculture, consumers will see new products emerging onto the marketplace as well as traditional products being sold at a reduced price because of their more efficient production. Of course, it will also offer the opportunity to decrease the number of animals needed to produce products that reach market, lessening animal waste production and reducing potential environmental concerns.

Studies on oocyte maturation, fertilization, and embryonic development are crucial to enhance livestock reproductive efficiency. Once the underlying biological mechanisms are better understood, problems with infertility that arise in the field may be more easily overcome. It will also enable a more prolific generation of embryos for research purposes, which ultimately will lead to further advances in many fields. Information generated from this research may be used to increase fertility of livestock species, decrease embryonic death, and ameliorate the declining fertility of older animals. There also will be more direct benefits for farmers who use assisted reproductive technologies such as superovulation, in vitro fertilization, and embryo transfer. These technologies increase reproductive rates of valuable females, even enabling generation of in vitro produced embryos from cows that are pregnant. These techniques also enable older, infertile females to continue to reproduce and provide products to market in the form of pregnant recipients and frozen embryos. The latter can be marketed easily internationally without a concern for transmitting diseases.

Studies on nuclear transfer, transgenesis, and cryopreservation are important to the future of society. It is likely that the research on cell cycle control, conducted as a part of research on somatic cell nuclear transfer, may lead to cures for cancer, treatments to reduce the aging process, and methods to generate organs for transplant. When nuclear transfer is combined with transgenesis, even greater possibilities exist for both animal agriculture and biomedicine.

Significant research effort concerning selective genetic modification of animals is occurring at public institutions and in private companies, and it is imperative that research of this type be publicly funded to ensure availability of results to animal agriculture and to private industry. Combining technologies such as in vitro fertilization, nuclear transfer, and cryopreservation provides phenomenal opportunities for increased efficiency of livestock

production. Efficacious cryopreservation of oocytes enables storage of female gametes. Although embryos from ruminant species can be frozen reasonably well, the ability to cryopreserve oocytes will increase flexibility in animal production and in research. In vitro embryo production could be timed to use naturally cycling recipients. Oocytes from genetically elite females could be better utilized, leading to a faster rate of genetic improvement due to an increased female selection intensity. Cryopreservation of routinely collected embryos would provide many benefits to the livestock industry, such as not needing recipients in "real time" and being able to export genetic material.

ORGANIZATION:

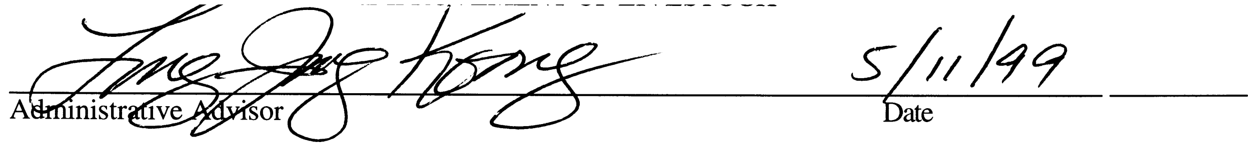
The organization and conduct of the revised project will be in accordance with the Manual for Cooperative Regional Research (revised January, 1986) and according to guidelines established for the original regional project. Briefly, elected officers of the Technical Committee will be a chair, vice chair, and secretary. Election of secretary will be held annually. Secretary will rotate to vice chair and vice chair to Chair. The agenda for the annual meeting of the Technical Committee will be set by the chair and she/he will preside over the meeting. The secretary will prepare minutes of the annual meeting of the Technical Committee and prepare the annual report.

ASSURANCES:

The project leaders and their institutions recognize the importance of complying with the intent and procedures of the National Institutes of Health (NIH) "Guidelines for Research Involving Recombinant DNA Molecules" and other applicable federal/state guidelines and regulations. The project leaders and their institutions also recognize the importance of complying with the Animal Welfare Act of 1966 and 9CFR Subchapter A.

SIGNATURES:

REGIONAL PROJECT TITLE: GERM CELL AND EMBRYO DEVELOPMENT AND MANIPULATION FOR THE IMPROVEMENT OF LIVESTOCK



Administrative Advisor Date

CHAIR, REGIONAL ASSOCIATION OF DIRECTORS 7/14/1999 Date

Administrator, Cooperative State Research Service Date

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PROJECT LEADERS

REGIONAL PROJECT TITLE: GERM CELL AND EMBRYO DEVELOPMENT AND MANIPULATION FOR THE IMPROVEMENT OF LIVESTOCK

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A.	EXPERIMENT STATIONS		
	Arkansas	R. W. Rorie	Gamete Biology
		C.F. Rosenkrans, Jr	Gamete Biology
	California	G. B. Anderson	Developmental Physiology
		P. J. Berger	Gamete Biology
	Colorado	G. E. Seidel, Jr.	Embryo Biology
		R. A. Bowen	Bovine Embryo Biology
	Connecticut	X. Yang	Embryo Biology
	Illinois	M. B. Wheeler	Embryo/Developmental Biology
		C. N. Graves	Embryo Biology
		D. J. Miller	Gamete Biology
	Iowa	C. R. Youngs	Embryo Biology
		S. P. Ford	Embryo Survival
	Louisiana	R. A. Godke	Embryo Biology
		S.P. Leibo	Cryobiology
	Oregon	A. R. Menino, Jr.	Embryo Biology
	Oklahoma	J.K. Thibodeaux	Embryo Biology
	Utah	T. D. Bunch	Embryo Biology and Cytogenetics
		K. L. White	Embryo Biology and Immunology
	Washington	R. W. Wright, Jr.	Gamete Biology
	Wisconsin	J. Rutledge	Genetics/ Gamete Biology

RESOURCES PAGE

PARTICIPANT	OBJECTIVES 1		2	RESOURCES SY		APPT SPLIT PY	TY	PI R	T	E
Arkansas SAES										
R.W. Rorie		X			.45	.25	.25	80	20	0
C.F. Rosenkrans, Jr.		X			.2			80	20	0
California SAES										
Davis										
P.J. Berger		X			.1	.2	.5	60	40	0
G.B. Anderson			X		.2	.5	.5	70	30	0
J.D. Murray			X		.2	.5	.5	70	30	0
Colorado SAES										
G.E. Seidel Jr		X	X		.15		.2	40	35	25
R.A. Bowen			X		.1		.1	40	35	25
Connecticut										
X. Yang		X	X		.1	2.5	1.0	60	20	20
J. Riesen		X	X		.1	1.5		60	20	20
Illinois SAES										
M.B. Wheeler		X	X		.2	.2		70	30	0
C.N. Graves		X	X		.2	.2		70	30	0
D.J. Miller	X	X		.2	.2					
Iowa SAES										
C.R. Youngs		X	X		.25			70	30	0
Louisiana SAES										
R.A. Godke		X	X		.6	.1	.8	65	35	0
S.P. Leibo			X		.2	.1	.2	85	15	0
Oklahoma										
J.K. Thibodeaux		X			.2	.1	.2	100	0	0
Oregon SAES										
A.R. Menino Jr.		X			.4	.5		60	40	0

Utah SAES								
T.D. Bunch	X	X	.5			80	20	
0								
K.L. White,	X	X	.1		.5	80	20	
0								
Washington SAES								
R.W. Wright Jr	X	X	.2		.2	43	23	34
Wisconsin SAES								
J. Rutledge	X	X	.3	.4	.4	50	50	0
TOTALS			**4.75**	**6.75**	**4.85**			

W-171 Federal Regional Project

Critical Review

Final W-171 Progress Summary Report
W-171 Regional Research Project
Germ Cell and Embryo Development and Manipulation
for the Improvement of Livestock
July 1, 1994-December 31, 1998

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Progress of Work and Principal Accomplishments:

Objective 1. Identification of Marker Systems for Oocyte Quality and Embryo Development.

a. Harvesting Viable Oocytes for In Vitro Fertilization.

Harvesting oocytes from the slaughterhouse has been used for years by researchers to develop *in vitro* maturation and *in vitro* fertilization (IVF) procedures for farm animals. Now that progress has been made in the scientific community and IVF offspring have been produced in all the farm animals, the technology was not being utilized by the seedstock producers. Unfortunately this approach of obtaining oocytes for embryo production, although acceptable for researchers, clearly is not acceptable for those livestock producers wanting to improve the genetic base of their breeding stock. An effort was made to make this new technology applicable to the livestock producer by developing transvaginal ultrasound oocyte collection procedures.

Several groups in the USA and Europe established this methodology for the cylic dairy cow. Subsequently, several contributing stations have continued in efforts during this last 4 years to develop and refine this technology for use in young calves (Louisiana), early postpartum cows (Iowa), sheep and goats (Louisiana) and horses (Colorado, Louisiana). In recent studies, transvaginal ultrasound oocyte collection procedures have been shown to be highly successful for harvesting oocytes from first trimester cows and pregnant mares without terminating gestation (Louisiana). In fact, more good quality oocytes (20 to 45%) can be harvested from pregnant cows and mares than from their cyclic counterparts. These findings stimulated a great deal of interest among livestock producers; and we are pleased to report that this new technology is presently being used commercially by producers in the North America and in other countries.

Since new life starts with the oocyte, contributing stations initiated experiments to identify viable oocytes and characteristics of follicles containing viable oocytes (Louisiana, Utah) under *Objective 1*. In a joint study with Texas A&M University, oocyte quality was evaluated in English breed and Brahman donor cattle both in the cool winter months and during the hot humid summer months using transvaginal oocyte collection procedures (Louisiana). Findings from this study showed that English breed donor cattle produced good quality oocytes for IVF only during the winter months and consistently poor quality oocytes during the summer months. In contrast, the Brahman donor cattle produced good quality oocytes during both the winter months and during the hot summer months. This finding is of importance to the cattle producers using AI and IVF technologies in the hot humid summer months, especial those in the southern and western states. In addition, the nutritional status of an oocyte donor cow has now been established to be strongly implicated in affecting bovine embryo development *in vitro* as well as *in vivo* (Utah, Louisiana).

b. Developing Methods to Identify and Enhance Oocyte Quality.

Contributing stations executed a number of experiments to identify and enhance oocyte quality (Colorado, Louisiana, Utah, Oklahoma, Washington, Oregon). In a well planned study, it was found that small and atretic bovine follicles contain IGF-binding proteins -2, -3, -4 and -5, but as a normal follicle becomes pre-ovulatory, the only IGF-1 binding protein remaining is IGFBP-3, the one found in high concentration in blood (Colorado). This disappearance of IGFBPs likely increases the availability of free IGF-1. Changing characteristics of bovine follicular fluid between the LH surge and ovulation was also evaluated (Colorado); follicular fluid at the time of the LH surge inhibits oocyte maturation, but 20 hours later this fluid stimulates maturation, even in the presence of inhibitory type of follicular fluid. In addition, oocyte maturation in 20% follicular fluid followed by IVF results in a higher percentage of blastocysts than the control group. The same hypothesis relating IVF success rates is presently being tested with follicular fluid after hCG treatment of oocyte donor cattle and horses at another contributing station (Louisiana). Preliminary results, relating to results hCG treatment, support those reported by the former station (Colorado). To further our understanding of follicular fluid's effect on oocyte maturation and IVF, experiments have been underway to evaluate the effects of cystic follicular fluid on the maturation rate of oocytes recovered from normal bovine follicles (Oregon). Although experiments are still in progress, preliminary results do not demonstrate an

inhibitory effect of cystic follicular fluid on *in vitro* maturation when compared with maturation and development in medium supplemented with estrous cow serum.

It has recently been demonstrated that Epidermal Growth Factor (EGF) mimics this oocyte maturation effect in cattle but not in horses (Colorado). Also, it has been shown that HEPES buffer was not toxic during bovine oocyte maturation as once thought (Colorado). It has recently been shown by the same group that co-culture with follicle walls containing theca cells markedly inhibits bovine oocyte maturation. Also, it has now been established that follicular fluid can be a good medium for maturing equine oocytes when compared with other more common culture media (Colorado, Louisiana). Recently it has been shown that pre-ovulatory equine follicles contain fluid with high IGF1 concentrations (Louisiana).

Since the mammalian oviduct is the site of fertilization and the initial stages of embryonic development, bovine oviductal-specific glycoprotein (bOGP) may play important roles in enhancing early embryo development during *in vitro* culture. An effort has been made to investigate high molecular weight proteins (Oklahoma) and their effects on fertilization of bovine oocytes (Washington) and early embryo development (Oklahoma). Several studies were conducted to determine if a partially purified bovine oviductal glycoprotein (bOGP) would influence fertilization rates of bovine oocytes during *in vitro* fertilization. In an initial study, one station (Oklahoma) co-incubated sperm and oocytes with or without bOGP during the 16- to 18-hour insemination period. In subsequent studies, sperm concentrations were lowered to provide a more physiological experimental system to assess the effects of bOGP on fertilization rates. These studies demonstrated an enhanced *in vitro* fertilization rate when bOGP was included in the IVF system for cattle oocytes.

Furthermore, the pre-incubation of oocytes with bOGP resulted in a significant enhancement of fertilization rates, similar to that observed when bOGP was included in the 16- to 18-hour fertilization period (Oklahoma). However, when sperm were pre-incubated with bOGP no enhancement of fertilization was noted, nor was there a synergistic effect when both gametes were preincubated with bOGP. These results suggest that the faciliatory effect of bOGP on early reproductive events is primarily mediated through its interaction with the oocyte, perhaps because of its association with the zona pellucida and perivitelline space. Studies were also conducted to determine the effects of bOGP on *in vitro* development of IVF-derived bovine embryos. These results indicated that bOGP may have an important role in regulating bovine embryonic development. The effects of bOGP appear to be mediated by a rapid progression of embryos to the morula or blastocyst stages earlier compared with control embryos. Collectively, the studies have demonstrated that bovine proteins, a constituent of the oviduct environment, directly influences *in vitro* fertilization and development of bovine oocytes and subsequently embryos (Oklahoma, Washington). These studies also provide new information to establish the biochemical requirements of bovine embryos *in vivo* that may be used in *in vitro* culture systems to enhance the production of embryos for research and commercial purposes.

c. Sperm Quality and Its Potential for Use in IVF.

Sperm plasma membrane proteins in different breeds of boars were evaluated in a collaborative effort by two stations (California, Illinois). Sperm plasma membrane proteins with an affinity for the zona pellucida were similar between the two genotypes (Meishan and European), except the P23 kD protein represented a much higher proportion of the bound zona pellucida (15.5% *vs.* 5.4%) for Meishan and European genotypes, respectively ($P<0.05$). The profile of oocyte plasma membrane binding proteins between the two genotypes was similar although the E1 kD protein was more prominent in the European genotype and the E28 kD protein was less prominent ($P<0.05$). The E11 kD protein appeared more prominent in the Meishan than the European (7.8 *vs.* 3%) but this was not statistically different.

Complimentary experiments have identified four sperm ligands believed to function in sperm-oocyte interactions and provided further evidence for a role for each in the fetilization process (California). Each of these proteins provides a potentially relevant marker molecule to evaluate oocyte maturation and quality. Also, the relationship between differences in plasma membrane protein composition and potential differences in efficiency of gamete function is under further study by two stations (California, Illinois) during the final year of this Federal Regional Project. These cooperative efforts will help establish the semen characteristics for comparison between Chinese and European pigs, so breeding management programs can be established for the livestock producer.

Several stations have conducted a series of experiments to identify the optimal concentration of percoll and heparin to aid in capacitating bull sperm for bovine IVF procedures (Utah, Washington, Colorado). A chemically-defined bull sperm extender has been found to improve post-thaw viability, velocity and survival in culture (Washington). Plant polysaccharide gradients have been used to wash bull sperm, which subsequently improved *in vitro* fertilization and embryo development rates (Washington). Researchers at the same station have developed a new sperm oviduct co-culture test to evaluate the fertility of stallions.

This new approach using oviduct co-culture is presently being evaluated to test the fertility of different bulls for IVF at another station (Louisiana). A new test using resazurin to evaluate the quality of ram semen for AI and IVF has also been recently reported by one of the contributing stations (Utah). It has now become evident that metabolic rate of ram sperm is a good indicator of its fertilizing capacity. A quantitative assay has now been developed to access sperm cell DNA quality in relationship to fertilization rate and embryo quality (Washington). The results of these new methodologies, thus far, are very encouraging for potential use with IVF procedures. Another station made a new discovery with fresh and frozen bovine sperm that is considered to be directly applicable to the AI industry (Colorado). After a series of trials, it was concluded that the AI technician could markedly reduce the number of sperm cell used per insemination and maintain acceptable pregnancy rates. This finding will likely lead the way for artificial insemination of cattle with X- and Y-separated sperm in the future. Trials are presently underway to re-establish the optimal number of sperm needed per insemination for cattle.

During this Federal Regional Project interval both the first IVF-derived and the first frozen-thawed IVF-derived goat offspring were produced in the world from uterine- stage embryos (Louisiana). Several stations have been working to develop improved sperm injection

procedures, now commonly known as ICSI (intracytoplasmic sperm injection), for bovine and equine oocytes (Colorado, Louisiana). It has become evident that this technology has potential when limited frozen semen is available from sick, injured or deceased genetically-valuable animals. Although live offspring have not resulted from ICSI thus far in cattle, we are pleased to report that the first live ICSI foals in the world have been produced during this Federal Regional Project by two contributing stations (Colorado, Louisiana).

Objective 2: Refinement of Methods for Short- and Long-Term Embryo Maintenance.

a. Identification of Optimal Systems for Producing Quality Embryos.

Several stations conduct either joint or compatible experiments to refine the present oocyte maturation, IVF and embryo culture systems (Arkansas, Louisiana, Oklahoma, Colorado, Iowa, Washington, Utah). Contributing stations investigated the effects of various culture components [e.g., free radicals, UV light, antibiotics, protein supplements (bovine serum albumin, fetal bovine serum), granulosa cells, oviduct cells (follicular and luteal phases of the estrous cycle), culture media (Whitten's medium, CR2 medium, modified oviduct fluid, chemically defined medium, synthetic culture medium) in caprine, ovine, porcine and bovine culture systems] (Arkansas, Oklahoma, Utah, Iowa, Illinois, Louisiana).

Fetal bovine serum has been verified to be generally superior to bovine serum albumin (BSA) in promoting embryo development, blastulation and hatching (Utah). The effects of BSA lot and breed type on *in vitro* development of early porcine embryos in modified Whitten's medium was evaluated (Illinois). No difference was detected among the multiple lots of BSA for development to the 8-cell, compact morula, blastocyst and expanded blastocyst stages following 96 hours of *in vitro* culture. Results from these studies indicate that fatty acid-free BSA is not necessary for *in vitro* culture of early stage porcine embryos, although this has not been shown to be the case when culturing bovine embryos (Louisiana). In a recent study, the addition of hemoglobin to the granulosa co-culture system significantly improved the developmental rate of IVF-derived bovine embryos to the blastocyt stage (Louisiana).

Oviduct and uterine cells, whether collected during the follicular or luteal phases of the estrous cycle, have similar beneficial effects on *in vitro* culture of bovine embryos (Utah, Louisiana, Oklahoma). In a comparitive study, modified oviduct fluid produced a higher developmental rate for embryos when compared with CR2 medium (Utah). It was found that fructose was superior to equimolar glucose in the embryo culture medium as measured by cleavage and development to blastocysts (Colorado). In an interesting study, evidence was presented that GnRH enhances IVF as measured by the percentage of oocytes cleaving and developing to blastocysts (Colorado).

A series of studies have investigated the effect of oxygen concentration on the *in vitro* maturation and fertilization of bovine oocytes, and development of cleaved embryos (Arkansas, Utah, Colorado). Results indicated that the subsequent development rate of cleaved embryos is improved by maturing bovine oocytes in a reduced oxygen atmosphere (Arkansas). Cleavage was

reduced when oocytes were fertilized in the presence of reduced oxygen, but development to the blastocyst stage benefited from reduced oxygen. In another study, it was found that there was no advantage of adding antioxidants to media where 5% CO_2, 5% O_2, 90% N_2 is used in the culture procedure (Utah). It was also confirmed that 5% O_2 is vastly superior to 20% O_2 when culturing bovine embryos (Colorado). In addition, resazurin dye has been shown to be a good metabolic indicator for sperm (see *Objective 1*) but has not yet been shown to be effective in evaluation the status of individual embryos (Utah).

It is now evident that free radicals are a problem when oocytes/embryos are cultured under gas atmospheres of >5% O_2 and antioxidants (SOD or catalase) are then useful in medium (Utah). A number of experiments focused on antioxidants for *in vitro* culture of embryos were conducted by contributing stations. One of the important finding was that Vitamin E in culture medium for 5 days resulted in significantly larger embryos after transfer to the bovine uterus for one week of additional *in vivo* development (Colorado, Washington). In a cooperative effort, contributing stations completed a series of experiments on the addition of *beta*-mercaptothanol to the culture medium to culture IVF-derived embryos (Colorado, Iowa, Utah, Louisiana). Overall, these studies concluded that *beta*-mercaptoethanol improved the *in vitro* development of early stage IVF-derived embryos to the blastocyst stage. In fact, one station reported that *beta*-mercaptoethonal markedly improved the development of IVM/IVF-derived bovine embryos to the blastocyst stage and beyond (Utah). These findings will likely aid in developing culture systems for the incubation of IVF-derived and reconstructed farm animal embryos in the years to come.

UV light has been found to be detrimental to all phases of oocyte/embryo development (Utah, Louisiana), however, this negative effect can be eliminated with a yellow filter placed over the light (Utah). In an applied study, embryos cultured in the presence of ceftiofur sodium (Naxel) at 50 mcg/ml significantly retarded embryo development; whereas, amphotericin B, penicillin G and streptomycin used at concentrations commonly used in tissue culture systems have no detrimental effects on development (Utah). Swainsonine (locoweed active component) was evaluated on *in vitro* oocyte maturation, fertilization and culture in a bovine and an ovine embryo culture system (Utah). It appears that swainsonine is embryotrophic without having adverse side effects on embryo development. Another contributing station uncovered that consumption of endoophyte-infected tall fescue by beef cattle can affect embryo viability *in vitro* (Arkansas). It was concluded that this could be one of the causes of unexplained infertility in cattle herds in some of the southern states. The important IGF molecules, their receptors, their binding proteins and mRNA for these molecules in elongating bovine conceptuses and the uterus have recently been quantified (Colorado). These components of the IGF system are almost ubiquitous in reproductive tissues, except that the bovine conceptus appears not to make any of the IGF-binding proteins.

At one station (Oregon), experiments have been undertaken to investigate changes in the electrophoretic profile and solubility of bovine zonae pellucidae (ZP) induced by bovine plasmin or embryonic plasminogen activator (PA). ZP exposed to plasmin exhibited significant reductions in the high molecular weight polypeptides and greater solubilities in a ZP dissolution assay. When ZP were treated with embryonic PA for durations similar to that used for plasmin, no differences were

observed compared with controls. However, if plasminogen was added to treatments containing bovine embryonic PA, changes in ZP solubility and polypeptide distribution were similar to plasmin treatment. These results suggest that embryonic PA does not exert a direct proteolytic effect on the ZP, and that any proteolytic modification by embryonic PA is mediated through plasminogen conversion to plasmin.

The endodermal cell outgrowth from bovine and porcine inner cell masses (ICM) were evaluated on matrices of collagen IV, fibronectin and laminin (Oregon). Percentages of porcine ICM generating cellular outgrowth on fibronectin and laminin were similar; whereas, for bovine ICM, fibronectin was the superior matrix compared with laminin. Collagen IV failed to support outgrowth for either of these two species. A comparative study was conducted to evaluate the involvement of tissue inhibitors of matrix metalloproteinases (TIMP) and integrins in bovine and porcine endodermal cell migration *in vitro*. The results of these studies suggested that porcine endodermal cells migrate over fibronectin with an integrin that does not utilize the common tripeptide fibronectin recognition sequence (R-G-D). Cell migration was enhanced from bovine ICM by TIMP suggesting a stimulatory effect on endodermal cell proliferation in this species.

In an effort to improve *in vitro* embryo co-culture systems, experiments were conducted to determine if *in vitro* protein secretion by bovine oviduct cells and uterine epithelial cells could be altered by the supplementation of culture medium with serum collected from cattle at different stages (0 to 8 days) of the estrous cycle (Arkansas). Secretion of proteins by oviduct cells was found to change in response to different serum, but this was not the case for uterine cell secretions. A subsequent study showed that the percentage of *in vitro*-produced bovine embryos cleaving to the blastocyst stage increased by changing serum supplementation to match the stage of embryo development.

In a continuing effort to enhance the quality of mammalian embryos prior to transfer novel co-culture experiments were conducted (Louisiana). In the first experiment, bovine oocytes (n=240) harvested from abattoir ovaries were randomly assigned either to a TCM-199 (A), to TCM-199 plus a bovine follicular granulosa cell monolayer (B), to TCM-199 and co-cultured with ten 4- to 8-cell stage mouse embryos (C) or to TCM-199 with ten 4- to 8-cell ICR mouse embryos along with a granulosa cell monolayer (D). The embryos were *in vitro* cultured in TCM-199 with 10% fetal bovine serum in an atmosphere of 5% CO_2 in air for <9 days. In summary, the developmental rate of the bovine was greater than when co-cultured in medium alone. A second experiment using the same experiment design with 200 bovine embryos, only in this case, a condition medium from the granulosa cells and the developing mouse embryos were used in (B) through (D). Continued replications of this experiment have given similar results. These findings indicate that mouse embryos could be used in place of somatic cells in co-cultures to enhance *in vitro* development of cattle embryos.

A cooperative effort has been underway to evaluate the potential usefulness of the prolific Chinese Meishan pig in breeding management schemes at two contributing stations. A classic study showed that breed type was involved in early embryo developmental rates of Meishan and Yorkshire embryos when placed (cross transferred) into either Meishan or Yorkshire recipient females (Iowa). In addition, Meishan x Yorkshire crossbred embryos were shown to develop into

more morulae, and Meishan embryos developed at a higher rate to more advanced pre-implantation stages than did Yorkshire or crossbred embryos (Illinois).

b. Efforts to Enhance Cryopreservation of Embryos.

When freezing day-7 or day-8 equine embryos it was found that success rates are related to morphological stage of embryo development (Colorado), however, this was not the case when similar stage bovine embryos were subjected to a standard freezing protocol (Louisiana). Preliminary results showed that vitrification procedures work well for equine and bovine oocytes (Colorado), and is presently being evaluated in a freezing protocol for IVF-derived bovine embryos (Utah). It was also found that permeability of equine embryos to cryoprotectants decreased markedly between day 6 and day 7 post-ovulation (Colorado). This enabled the latter station to develop an entirely new procedure for cryopreservation of larger equine embryos, termed *"Step-Down Equilibration"*. Embryos are exposed to 2 M, 4 M and then 2 M glycerol + galactose prior to cooling them. Pregnancies were achieved with this new freezing procedure (Colorado). Other stations (Illinois, Louisiana) have requested working on a cooperative project with Colorado to evaluate and refine new embryo freezing procedure in the final year of this Federal Regional Project. Freezing porcine embryos is one of the most difficult to achieve among farm animals. The problem has been evaluated at one of the contributing stations having pigs available for embryo production (Iowa). The most used embryo cryoprotectants were included in this study, however, most were toxic to the swine embryos.

Occasionally mammalian embryos frozen in liquid nitrogen are mistakenly thawed in the laboratory. If these embryos would survive refreezing, valuable genetic material would not be lost. In an initial experiment (Louisiana), mouse embryos were obtained from female mice 78 to 82 hours following hCG. Excellent quality 8- to 16-cell embryos (n=264) were washed and placed in modified PBS containing 0.4% BSA. After equilibration, 5 to 10 embryos were loaded into 0.25 ml straws and placed in a freezing unit at 6°C. Straws were seeded at -6°C before cooling to -33 C at a rate of -0.3°C per min. Thawed embryos were then randomly allotted to one of three treatment groups. Embryos in (A) were placed in Whitten's medium and cultured to expanded blastocysts *in vitro*. Embryos in (B) were co-cultured for 18 to 30 hours then refrozen, thawed and again cultured in Whitten's medium, those in (C) were placed back into the freezing machine and refrozen within 2 min thawed and cultured, and those in (D), serving as a nonfrozen control group, were cultured in Whitten's medium to the expanded blastocyst stage. In summary, 76% of the once-frozen embryos in (A) developed to expanded blastocysts after thawing. Unexpectedly, 50% of the twice-frozen embryos in (B) and 42% of the twice-frozen embryos in (C) developed to expanded blastocysts following culture; whereas, 87% of the nonfrozen embryos developed to expanded blastocysts in (D). Based on the embryos frozen and thawed the first time, 55 to 65% of the murine embryos refrozen and thawed should develop to expanded blastocysts in culture.

In a second experiment (Louisiana), embryos were recovered nonsurgically on day 7 to 7.5 from Holstein and crossbred beef cows treated with FSH-P. Excellent quality embryos (compact morulae and early blastocysts) (n=135) were randomly allotted to treatment groups similar to those outlined above. Unexpectedly, the bovine embryos that were frozen a total of

three times in this experiment, 37% of these developed to expanded blastocysts and 30% of the total continued on to hatched blastocysts during *in vitro* culture. These findings indicate that day 7 to 7.5 bovine embryos can tolerate two freeze-thaw cycles and still maintain development through the hatching blastocyst stage *in vitro*. Furthermore, one could expect comparable *in vitro* development rates among embryo groups frozen-thawed three times. It was proposed that the next logical step would be to establish if refrozen bovine embryos are able to produce live ET offspring. This study is presently underway at two stations.

Two contributing stations worked on a joint project to establish a method to freeze bovine granulosa cells for use in embryo co-culture systems (Oklahoma, Louisiana). A successful protocol was developed, and the resulting frozen-thawed bovine granulosa cells were capable of co-culturing bovine IVF-derived embryos to morula and blastocyt stages similar to that of the control granulosa cells. Another contributing station focused on developing effective procedures for freezing bovine trophoblastic vesicles (Arkansas). This station reported that the tissue thawing process was uneventful, successful reactivation of the bovine trophoblastic vesicles occurred *in vitro*, and recommended a simple procedure that could be used by all those using trophoblastic cells/tissues in an embryo co-culture system.

Objective 3. Development of genetically modified animals to enhance desirable Traits.

a. Developing Transgenic Embryo Technologies.

Progress has recently been made by the contributing station members of W-171 under *Objective No. 3*. One station (Colorado) was successful at producing a transgenic beef calf that was subsequently found to express the *c-ski* gene. A second station (Louisiana), working with a commercial company, was able to produced transgenic goat offspring that expressed the anti-thrombin III gene in their milk.

Success has been reported in the production of transgenic cattle and swine over these last few years using similar technologies (Illinois). This group has been successful incorporating the bovine *alpha*-lactalbumin and the *beta*-casein gene into the embryos of cattle and pigs. Presently, this station is working on germline gene incorporation of the same genes. This station has also become successful in producing transgenic mice. Transgenic mouse results suggest that *alpha*-lactalbumin (LA) maybe limiting for lactose synthesis, and lactose may be limiting for milk production (Illinois). This may provide an avenue to increase milk production in beef and dairy cattle as well as sheep, goats and pigs. Transgenic pig results suggest that (LA) maybe limiting for lactose synthesis, and lactose may be limiting for milk production. Results suggest that over-expressing (LA) will provide an avenue to increase milk production. Another station as recently developed a line of transgenic mice carrying the *HPRT* gene to study early-stage embryo development (Louisiana).

The first transgenic calf reported (Colorado) in the scientific literature that in fact expressed a transgene; the gene was *c-ski*, and it induced hypertrophy of skeletal muscle. The same station also identified three genes expressed more in day-17 than day-15 bovine embryos; none of these genes have yet to be studied in mammalian embryos. Two of the genes were identified from studies in nonembryonic tissues of other species: Allograft Inflammatory Factor 1 and *Lerk-5*; their functions in embryos are unknown. The third gene found had no significant homology with any sequence in the Genbank, thus it was considered a new gene not yet reported in the literature for any species. Another study involved methods of transfecting bovine trophoblast cells (Colorado). This is considered to be exceedingly difficult using conventional procedures, but this station was successful transfecting bovine trophoblast cells using the new *gene gun.*

The expression and cellular localization of components of the plasminogen activator and matrix metallo-proteinase systems in porcine and ovine embryos during pre- and peri-implantation development were evaluated by zymography, RT-PCR and *in situ* hybrid- ization (Oregon). Day 15.75 porcine embryos expressed transcripts for uPA, MMP-2 and -9, and TIMP-1, -2 and -3. PA was detected by zymography in ovine embryo conditioned medium throughout days 9 to 15 of gestation. MMP-2 was not detected in ovine embryo-conditioned medium and MMP-9 was observed only in medium recovered from day-15 embryos. Transcripts for MMP-2 were not found in any of the ovine embryonic stages investigated; whereas, transcripts for MMP-9 were observed

only in day-15 embryos. Ovine embryos expressed transcripts for TIMP-1 and -3 during days 9 to 15 and for TIMP-2 during days 11 to 15.

b. Developing DNA and Embryonic Stem Cell Technologies.

Embryonic stem (ES) cells are generally considered to be pluripotent, undifferentiated cells isolated from blastocyst-stage embryos. Contributing stations have been working together under this Federal Regional Project to develop embryonic stem cell technology for farm animals (California, Illinois, Colorado). These stations have now developed pluripotent cell lines that heretofore were unavailable. Recently, the identification of differentially expressed transcripts during ES cell differentiation has been reported by one station (Illinois). After years of experiments, it is now well known that the Vitamin A analog, retinoic acid (RA), is an inducer of ES cell differentiation. In one study, a total of 27 differentially expressed transcripts were identified (Illinois). Of the transcripts detected, 21 were found in cells treated with RA and not in undifferentiated cells, and six were found in undifferentiated cells but not in RA-treated cells. The *RA-1* expression was not observed in undifferentiated cells or in adult mouse liver.

It has recently been discovered that 5 mg of prolactin will release mink embryos from embryonic diapause (delayed implantation) and is necessary for the establishment of mink ES-like cells (Utah). Inositol 1,4,5-triphosphate (IP3) and ryanodine receptors in mature bovine embryos have been identified and when stimulated, results in calcium release. The ryanodine receptor appears to be synthesized during oocyte maturation. Parthenogenetic development in the bovine and rabbit occurs after oocytes are activated by IP3 and ryanodine. Methylase activity in the embryo has been shown to decrease to the 16-cell stage in bovine embryos, and then increases shortly thereafter (Utah).

It has been recently found, however, that lactogenic hormones can induce expression of bovine *beta*-casein gene promoter activity in a mouse epithelial cell line (Utah). At another station, stage-specific embryonic antigen-1 (SSEA-a), a cellular marker commonly used to identify undifferentiated murine embryonic cells, was used as a marker for bovine pluripotent cells (California). Although SSEA-1 was expressed on murine pre-implantation embryos, it was found not to be a useful cell marker for pluripotent bovine embryonic cells and bovine primordial germ cells.

The effects of phytohemaglutinin (PHA) on adherence of ES cells was evaluated by co-culturing embryonic stem (ES) cells with mouse embryos (Illinois). Co-culture of zona-free, 8-cell mouse embryos with embryonic stem cells has produced chimeric mice. This study was conducted to test the effect of PHA on adherence of ES cells to embryos in co-culture. The number of embryos with adherent cells and the percentage of embryos that developed to blastocysts were decreased by treatment with Hoescht stain. It was noted that 50 mg PHA/ml partially compensated for this effect, 400 mg PHA/ml increased the percentage of blastocysts formed, the number of embryos with cells attached and the number of cells attached per embryo above control levels.

Contributing stations have focused their cell line culture efforts under *Objective 3* on either primordial germ cells (California) or first-trimester fetal somatic cells (Louisiana). The former station now has been successful in establishing porcine cell lines that retain their ability to differentiate normally *in vivo* (California). In an effort to production of chimeric swine from embryonic stem (ES) cells, *in vivo* differentiation of pluripotent ES cells was tested by their ability to participate in the formation of chimeric offspring (Illinois). Twenty-one piglets exhibited coat-color chimerism at birth. Chimerism was analyzed and confirmed by polymerase chain reaction amplification of a Meishan-specific 120 bp SW16 microsatellite allele and/or of a 192 bp porcine Y-chromosome specific sequence.

Live offspring with predicted genotypes using PCR-RFLP analysis of polar bodies from oocytes (Illinois). Accurate identification of genotypes in gametes and early embryos could facilitate the efficient production of offspring with desirable traits. The Polymerase Chain Reaction (PCR) was used to amplify genes in the IA subregion of the major histocompatibility complex of the mouse. The genotype of the gamete was predicted by subtraction of haplotypes observed in the polar bodies. Micromanipulated, fertilized oocytes were transferred to recipient females and live offspring were born. The predicted maternal contribution to the embryonic genome was confirmed in 10 of 12 offspring from which polar body analysis was comprehensive. Recently, integrins or integrin-like molecules have been identified on the surface of bovine oocytes, which appear to serve as sperm receptors (Utah).

c. Activation of In Vivo-Matured Oocytes for Nuclear Transfer.

Experiments were designed to develop an efficient method for activation of *in vivo*-matured porcine oocytes that could be integrated into porcine nuclear transfer technology (Illinois). Ethanol (7% for 5 min) had little effect on oocyte activation as only 8% activation was achieved for both treated and control oocytes. Cold shock treated oocytes activated at higher rates than control oocytes (74% *vs.* 50%; $P < 0.05$). Results indicated that a higher percentage of oocytes ($n = 36$) activated following sham enucleation (62%) than control oocytes (27%; $P < 0.05$). The next objective was to investigate the effects of (1) electroactivation and (2) electroactivation followed by culture with cycloheximide on oocyte activation ($n = 156$). The treatments were: (1) *in vitro* culture (CNTRL), (2) electroactivation and culture (ELECTRO), and (3) electroactivation and culture with cycloheximide. Activation rates were highest for cycloheximide (CYCLO) oocytes (76%), intermediate for electroactivation (ELECTRO) oocytes (49%) and lowest for control oocytes (13%). The CYCLO treatment was the most efficient activation method to produce pronuclear-stage cytoplasm for use in porcine nuclear transfer procedures. Another station used a similar ethanol activation for bovine oocytes and then made reconstructed parthenogenetic embryos with IVF-derived marker embryos (4- to 16-cells) (Louisiana). Three ET pregnancies resulted producing a live phenotypic chimeric IVF-derived reconstructed calf.

d. Nuclear Transfer Embryo Technologies.

An effort has been made characterize bovine pregnancies resulting from embryonic cell nuclear transplantation (Colorado, California, Louisiana). Pregnancy rates per frozen, nuclear transfer embryo declined from 21% on day 15 to 6% by day 65; however, all day 65 pregnancies continued to term (Colorado). Similar findings were recorded by another station from similar recipient females carrying the same breed of NT calves (Louisiana). In a more basic study, nuclear transfer pregnancies and controls were evaluated by catheterizing fetal arteries at 255 to 265 days of gestation (Colorado). It was noted that IGF_1, IGF_2, and PO_2 were lower in blood of the nuclear transfer calves than the control calves. One possible explanation is that these fetuses had experienced earlier excessive growth followed by decreased growth due to homeostatic mechanisms.

One station has produced Argali Wild sheep nuclear reconstructed embryos using bovine and domestic ovine enucleated cytoplasts (Utah). From a total of 166 constructed nuclear donor cell - bovine cytoplast embryos, 77% fused, 78% developed to the 8- to 16- cell stage and 2% developed to the blastocyst stage. Thirty-five enucleated oocytes reconstructed with domestic sheep fibroblasts resulted in a 69% fusion rate, with 83% of the fused embryos developing to the 16- to 32-cell stage. Forty-two enucleated oocytes reconstructed with Argali fibroblasts resulted in a 71% fusion rate, with 73% of the fused embryos developing to the 16- to 32-cell stage. Twenty-four enucleated oocytes reconstructed with domestic sheep reconstructed embryo blastomeres resulted in a 96% fusion rate, with 71% of the fused embryos developing to the 16- to 32-cell stage. Twenty-five enucleated oocytes reconstructed with Argali reconstructed embryo blastomeres resulted in a 96% fusion rate, with 88% of the fused embryos developing to the 16- to 32-cell stage. No live offspring were produced from this study.

Another station (Louisiana), working with a commercial transgenic animal company, has recently produced the first nuclear transfer goat offspring in the world. These offspring have now been verified as being able to express the appropriate transgene. From initial studies, it is becoming more evident that the use of cell lines, DNA transfection and nuclear transfer methodologies will likely be the most efficient approach to producing transgenic offspring.

Usefulness of the Findings:

A major problem embryo biologists have faced for years was how to maintain *in vitro* embryo development and viability of early stage embryos until they are at an optimal stage for transfer to recipient females. Our *W-171 Technical Planning Committee* feels that the scientists of the contributing stations of the W-171 Federal Regional Project have played a major role in the progress made in this area of reproductive physiology in recent years. Maintaining embryos of farm animals *in vitro* for research, evaluation and study now is common place; and we are proud to be a member of the group that contributed to this success. Success in research is achieved when the planning group identifies the problem and focuses the objectives outlined to solve the problem. With the advancements in cell and molecular biology, there are clearly new challenges in *embryo world*, and the *W-171 Technical Planning Committee* is anxious to begin.

One of the problems is to maintain viable development of embryos produced from *in vitro* fertilization and embryo reconstructed procedures. Developing alternative systems to culture for cell lines, oocytes and embryos will benefit by aiding the advancements in *in vitro* fertilization, gene incorporation procedures, nuclear transfer methodologies for farm animals. Major advances have been made in gamete and embryo biology in recent years, and new discoveries are occurring daily. These new findings will add to the body of knowledge and add to the long-term chances of success for improving the efficiency of livestock production.

Research findings, *no matter whether they are large or small*, may be the part of the puzzle that makes the differences in success. To use examples extracted from this summary report……"These findings enabled this station (Colorado) to develop an entirely new procedure for cryopreservation of embryos, termed *"Step-Down Equilibration"* or "These findings indicate that mouse embryos could be used in place of somatic cells in co-cultures to enhance *in vitro* development of cattle embryos (Louisiana)" or "Recently, integrins or integrin-like molecules have been identified on the surface of bovine oocytes, which appear to serve as key sperm receptors (Utah)", or what about……"After a series of trials, it was concluded that the AI technician could markedly reduce the number of sperm cells used per artificial insemination and maintain good acceptable pregnancy rates (Colorado). This finding leads the way for artificial insemination with X- and Y-separated sperm in the future". Evidence of the research productivity from contributing stations is presented in the *W-171 Publication Summary Report for the Calendar Years 1994 Through 1998* and a listing of publications in the subsequent pages of this report.

Work Planned for the Future:

We are planning to develop modified methods for the culture of animal embryos with various improved media types and synthetic alternatives and compare these systems with our standard culture systems. Optimal culture systems are needed to continue our research efforts in oocyte maturation, *in vitro* fertilization, understanding embryo development, cryopreservation, evaluating embryo viability, developing nuclear transfer methodologies and refining procedures for the production of transgenic embryos. Our plans call for conducting new experiments under new objectives in a revised W-171 Federal Regional Project, and to become more productive than ever before. We have invited several new members to join in our contributing scientist group, which we feel will be beneficial to our joint research efforts under the new objectives of the revised project.

From initial studies during the last four years of this W-171 Federal Regional Project, it is becoming more evident that the use of cell lines, DNA transfection and nuclear transfer methodologies will likely be the most efficient approach to producing transgenic offspring in the future. Freezing oocytes and refreezing embryos would augment assisted reproductive technology procedures in animal laboratories. Furthermore, if the advancements continue as they have recent years, it is likely that we will use nuclear transfer methodologies for genotypes that rare or endangered.

W-171 Publication Summary By Participating Stations (1994 -1998)

Refereed Pubilcations/Manuscripts Approved

Colorado State University

Romero, A.R. and Seidel, G.E., Jr. 1994. Effects of bovine follicular fluid on maturation of bovine oocytes. Theriogenology 41:383-394.

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Iowa State University

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