

MORPHOLOGIC AND HISTOLOGIC COMPARISONS BETWEEN *IN VIVO* AND
NUCLEAR TRANSFER DERIVED PORCINE EMBRYOS

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ABBREVIATIONS

BSA	Bovine Serum Albumin
CL	Corpus Luteum
DC	Direct Current
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic Acid
E ₂ S	Estrone Sulfate
FCS	Fetal Calf Serum
FGF-7	Fibroblast Growth Factor-7
IGF-I	Insulin-like Growth Factor-I
IGF-II	Insulin-like Growth Factor-II
IGFBP	Insulin-like Growth Factor Binding Protein
IGF-R	Insulin-like Growth Factor Receptor
KGF	Keratinocyte Growth Factor
MC	Manipulation Controls
MPF	Maturation Promoting Factor

MRP	Maternal Recognition of Pregnancy
NT	Nuclear Transfer
P ₄	Progesterone
PBS	Phosphate Buffered Saline
PGF _{2α}	Prostaglandin F _{2α}
RIA	Radio-Immunoassay

CHAPTER I

LITERATURE REVIEW

Introduction

During the course of a natural porcine pregnancy there are several time periods when embryonic losses occur. Of particular interest is the period of maternal recognition of pregnancy (MRP), thought to be regulated by conceptus-derived estrogens (Geisert et al., 1990), which occurs around days 11-14 of gestation as the embryo begins a morphological transformation from a spherical to a filamentous form. Due to the high failure rate of nuclear transfer (NT) pregnancies very early in gestation the possibility arises that NT derived embryos are developmentally compromised and have difficulty progressing through the critical points of elongation and successfully producing a signal for MRP. Because of the high incidence of embryonic loss during a nuclear transfer pregnancy, cloning remains a relatively inefficient process. However, as the only available method to produce livestock that are 'knockouts' (animals with a specific gene removed rendered nonfunctional in their genome), it is considered an invaluable tool for the biotechnical industry. The main objective of the study in Chapter II was to evaluate porcine nuclear transfer embryos for potential abnormalities that could contribute to the high mortality rate during early pregnancy. It was predicted that the period of elongation, a phase in which the porcine embryo undergoes extreme morphological changes as well as increased steroid secretion, would be a point of failure in the development of many

porcine embryos. The literature review in Chapter I details those events found in a normal porcine pregnancy as well as focuses on the technical and biological facets of nuclear transfer.

Embryonic Development

Embryogenesis: Day 0 to Day 14

Embryogenesis commences upon fertilization of a viable oocyte with a single viable spermatocyte, an event that occurs at the ampullary-isthmus junction of the oviduct and is denoted as day 1 of development (Senger, 2003). The appearance of pronuclei within the cytoplasm of the embryo occurs within 1.5 to 2 hours of fertilization and is soon followed by the first meiotic and cellular divisions at 12-13 hours and 14-16 hours, respectively. This two-cell stage of the embryo lasts only 6-8 hours before cleavage of the blastomeres occurs again to produce a 4-cell stage embryo (Hunter, 1974). This stage of development lasts anywhere from 20-24 hours and is an important milestone in porcine embryonic development as the embryo begins independent transcription of its own genome. Up until this time point, it depended solely on maternal mRNAs stored in the cytoplasm to produce the necessary proteins for early embryonic survival and development (Anderson et al., 1999).

The re-formation of nucleoli in the early embryo is integral to the initiation of embryonic protein production as the nucleoli are the sites of ribosomal RNA synthesis (Hyttel et al., 2000; Pederson, 1998). Under normal conditions, the nucleoli disassemble during the early M-phase of the oocyte cell cycle (which is then arrested at the meiosis II

stage awaiting fertilization (Senger, 2003)), and are reformed during the third cell cycle, or at the four-cell stage, after fertilization (Dundr et al., 2000; Hyttel et al., 2000). By the time the embryo reaches the blastocyst stage at day 6, its nuclei contain fully functional nucleoli that are comparable to somatic cell nucleoli (Hyttel et al., 2000).

The porcine embryo enters the uterus at the four-cell stage, approximately 46 hours after fertilization. Cleavage of the blastomeres at this stage represents the beginning of asynchronous cell divisions. Upon reaching the 16- to 32-cell stage late on day 3, they can be classified as morulae. Initial formation of a blastocele occurs reliably by day 5 and an inner cell mass, the primitive ectoderm that will eventually form the fetus, can be recognized. By day 7 the embryo has grown large enough to “hatch” from its outer membrane, also known as the zona pellucida (Hunter, 1974). Porcine embryos will continue to increase in diameter until day 10 of development (Patten, 1948).

A typical day 10 embryo can range in size from 3 to 10 mm in diameter. This time point is also when initial visualization of the embryonic disk, the putative fetus, can be made (Patten, 1948). On day 11, the fertilized embryo will begin to migrate through the uterine lumen (Waite and Day, 1967). By day 12, a rapid elongation of the trophoblast occurs, wherein the conceptus transitions from a tubular to a filamentous form and can reach 10 to 50 mm in length. By day 14, the filamentous conceptus can reach over 100 mm in length. The entire process of elongation can occur within 12-24 hours (Geisert et al., 1982).

Elongation

Elongation is a period of time in the rapidly developing embryo whereby “cellular

hyperplasia, plasma membrane re-arrangements, cellular re-modeling, or a combination of these processes” is used to drastically alter the physical form of the conceptus (Mattson et al., 1990). Initial growth of the embryo is thought to occur through cellular hyperplasia followed by a period of cellular migration and re-structuring to achieve the elongated morphological forms (Pusateri et al., 1990). Proliferation of the porcine embryonic trophectoderm is thought to occur through phosphorylation of the Keratinocyte Growth Factor (KGF) receptor which initiates the mitogen-activated protein kinase (ERK1/2) cascade (Ka et al., 2001). KGF, also known as Fibroblast Growth Factor-7 (FGF-7), mediates proliferation in epithelial cells via paracrine means (Finch et al., 1989; Rubin et al., 1989). The period of elongation has also been temporally correlated with the release of an embryonic signal for MRP (Geisert et al., 1982).

Embryonic Loss in the Pig

The majority of embryonic loss in the pig occurs within the first third of pregnancy. Anderson (1978) reported a 17% loss by day 18 of gestation which increased to a 30% loss by day 25 (Day et al., 1959). It is thought that the inability to smoothly transition through the extreme morphologic and physiologic changes that the early embryo undergoes probably contributes highly to this loss. Another hypothesis put forward is the possibility that less developed embryos within a litter are not as prepared to survive in a quickly changing uterine environment when compared to more advanced embryos in their ovulation cohort (Pope et al., 1982). For embryos that do survive past day 20-25, placental insufficiency is often considered a significant contributory factor in embryonic losses (Wilson and Ford, 2001).

Estrogen

At 7 mm, the spherical embryo develops the ability to synthesize estrogens (Gadsby et al., 1980). By the time the embryo initiates elongation; the rate of estrogen synthesis by the conceptus dramatically increases, elevating levels of estrone, estradiol, and an isomer of estriol in the uterine lumen. As development into the filamentous form progresses, estrogen production begins to decline slightly (Geisert et al., 1982).

The source of the estrogen produced by the conceptus is thought to originate from circulating maternal progesterone. Previous studies noted a positive difference in the plasma progesterone concentration between the uterine artery and the uterine vein indicating that the uterus was likely taking up progesterone (Bazer et al., 1979). *In vitro* studies where endometrial and conceptus tissues were co-incubated with [H^3]progesterone have demonstrated the production of estrogens by day 12 embryos (Fischer et al., 1985). It has been proposed that the endometrium is producing androgen pre-cursors, derived from maternal progesterone, which the conceptus then converts to estradiol-17 β (Knight and Kukoly, 1990). However, it appears much more likely that the conceptus itself is capable of converting maternal progesterone to estradiol-17 β due to the exclusive localization of cytochrome P450 17 α hydroxylase/17-20 lyase (P450_{c17}) to the embryonic trophectoderm (Conley et al., 1994).

Conjugated forms of estrogen, such as estrone sulfate, can be found in the maternal blood supply due to the activity of sulfatransferases and sulfatases in the endometrium. Estrone sulfate is easily titratable and has been reported to be an initial indicator of pregnancy in the pig (Pack and Brooks, 1974) and its levels in the maternal

blood serum and urine have also been used to predict litter size (Atkinson and Williamson, 1987).

Uterine responses to estrogen have included increases in arterial blood flow and elevation in levels of endometrial Ca^{2+} secretion (Geisert et al., 1982). The presence of estrogen also causes modification of secretion of progesterone-induced proteins such as uteroferrin (Simmen et al., 1991), plasmin/trypsin inhibitor (Fazleabas et al., 1982), antileukoproteinase (Simmen et al., 1991), and retinol-binding protein (Trout et al., 1992). Expression of growth factors such as Insulin-like Growth Factor-I (IGF-I) and FGF-7 are increased as estrogen acts on the uterine endometrium (Ka et al., 2001; Simmen et al., 1990).

Insulin-Like Growth Factor-I (IGF-I)

IGF-I is a polypeptide growth factor that is responsible for cell differentiation, cell division, and tissue morphogenesis (Zapf and Froesch, 1986). IGF-I mRNA has been localized to the porcine endometrium during early pregnancy with the greatest intensity occurring at day 12. The peptide itself was isolated from uterine luminal flushings between day 8 and day 14, but again, the greatest levels were detected at day 12 and the lowest at day 8 (Letcher et al., 1989). The increase in IGF-1 is temporally associated with both elongation of the embryos as well as the onset of steroidogenic production of estrogens by the embryo (Geisert et al., 1982). It has been suggested, due to the presence of IGF-I receptors on the conceptus (Corps et al., 1990) that IGF-I plays a mitogenic role on the rapidly developing embryo (Simmen and Simmen, 1990).

Little can be said about IGF-I without mention of the Insulin-like Growth Factor

Binding Proteins (IGFBPs). There are six IGFBPs, molecules that have a high affinity for binding IGF. They can affect IGF bioavailability by preventing its interaction with the IGF receptor (IGF-R) (Rechler, 1993). IGFBPs lose their high affinity for IGF when they are proteolytically cleaved, leading to a concomitant rise in bioavailable IGF that is able to interact with the IGF-R (Blat et al., 1994; Lee and Rechler, 1996). Interestingly, Lee et al. (1998) demonstrated that IGFBP-3 protein found in uterine luminal flushings (ULF) became significantly decreased at day 12 although IGFBP mRNA in the uterine endometrium was unchanged indicating that the elongating porcine conceptus may be able to induce IGFBP proteolytic activity resulting in increased free intrauterine IGF.

Role of Progesterone in Pregnancy

Progesterone is a steroid hormone produced by the corpus luteum (CL) of the ovary during a normal estrous cycle as well as throughout gestation. Under normal conditions the production of progesterone begins to dramatically increase following the second day after estrus, reaching a peak of 28-48 ng/mL at days 11 and 12. In the cyclic pig, progesterone will then decrease to pre-estrus levels of <3 ng/mL by day 18 as the CLs are lysed under the influence of Prostaglandin F_{2α} (PGF_{2α}) (Henricks et al., 1972). Progesterone levels in the pregnant sow are maintained at a consistently high level throughout gestation until roughly three days before parturition. At this time they continue to decrease starting 3 days before parturition until day 1 post-partum where levels in the maternal serum reach <1 ng/mL (Killian et al., 1973). Progesterone is required for the maintenance of pregnancy in the pig, and removal of the CLs in pregnant pigs has been shown to terminate the pregnancy (Nara et al., 1981).

Synchrony between Uterus and Embryo

The synthesis and secretion of estrogen by the conceptus has previously been shown to cause the release of secretory vesicles from the epithelial cells of the uterine glands. These secretions, which include increases in protein, calcium, growth factors, and prostaglandins radically change the uterine environment (Davis and Blair, 1993; Geisert et al., 1982; Ka et al., 2001; Simmen et al., 1990).

In a two-part experimental series, Xie et al. (1990) were initially able to tag groups of aspirated pre-ovulatory and late ovulation oocytes with fluorescently labeled monobromobimane. These oocytes were then transferred back into the oviduct of the hCG-primed gilt from which they originated. The embryos were then surgically flushed 113-115 hours post-hCG injection and the embryos examined under incandescent light. It was found that later ovulating oocytes developed into lesser developed embryos while oocytes of earlier ovulating oocytes were more advanced in their development. A second experiment was also performed in which embryos were flushed from the tract of a gilt at day 4 and classified into groups as oldest, intermediate, or youngest based on cell number (4, 6, or 8). Equal numbers of oldest and youngest embryos were transferred into separate uterine horns that had been ligated at the external bifurcation. Embryos were recovered at day 12 and it was determined that embryos in the youngest experimental group eventually became lesser developed embryos. Also, uterine horns that contained the oldest group of embryos had greater levels of estradiol, protein, and acid phosphatase levels.

In conjunction with these findings, earlier studies by Pope et al. (1982) showed

that transferring embryos to a uterine environment greater than 24 hours more advanced than their embryonic stage resulted in embryonic death. As ovulations in the pig can be spread over a 6 hour time period, there exists the possibility that more advanced embryos (those ovulated and fertilized first and therefore producing estrogens first) are able to produce environmental changes in the uterus before their less developed embryo counterparts are physiologically capable of surviving these changes (Pope et al., 1982).

Maternal Recognition of Pregnancy

In 1966, Polge *et al.* determined that a minimum of four embryos must be present in the uterus to maintain pregnancy. It had also been shown that, after day 12, a gravid uterus with large areas unoccupied by conceptuses resulted in significant losses of pregnancy (Dhindsa and Dziuk, 1968). It was deduced that embryos must influence a significant portion of the uterus in order to successfully maintain a pregnancy (Polge and Dziuk, 1970).

Maternal recognition of pregnancy in the pig is thought to be signaled by conceptus-derived estrogens and initially occurs at day 12 (Geisert et al., 1990). It was hypothesized that the estrogens caused a change in secretion of $\text{PGF}_{2\alpha}$ from endocrine (where it entered the uterine stroma, made its way to the uterine vasculature and then to the ovary to exert a luteolytic effect) to exocrine (where $\text{PGF}_{2\alpha}$ was secreted harmlessly into the lumen of the uterus). This theory was initially proposed after uterine flushes revealed that prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$ (the luteolytic agent in the pig) levels were elevated in the histotroph of pregnant gilts as compared to cyclic gilts (Frank et al.,

1978). Further investigation using a bilateral perfusion device showed that in non-pregnant pigs, PGF_{2α} originated on the myometrial side of the endometrium. Yet endometrium taken from pregnant pigs exhibited PGF_{2α} secretion from the luminal side of the endometrium (Gross et al., 1988). The exact mechanism by which re-direction of PGF_{2α} is accomplished, either directly or indirectly, is still unknown although calcium cycling across the endometrium has been connected with the directional change (Spencer et al., 2004).

Experiments were performed by using a perfusion device on endometrial tissue (day 14 of an estrogen treated gilt) where the addition of a calcium channel blocker (verapamil) and an intracellular calcium inhibitor (3,4,5-trimethoxybenzoic acid 8-(diethylamino) octyl ester) showed no change in PGF_{2α} secretion. Yet when A23187 (a calcium ionophore) was administered, changes in PGF_{2α} secretion from myometrial endometrium to luminal endometrium were observed (Gross et al., 1988).

Studies have also shown a close relationship between prolactin and estrogen during porcine maternal recognition of pregnancy. *In vitro* studies found that while neither hormone had an effect on endometrial tissue when administered alone, when they were used in conjunction with each other a shift in PGF_{2α} secretion from luminal to myometrial was observed (Young et al., 1989).

Maternal recognition of pregnancy is established through complex interactions between embryo and dam that include multiple chemical mediators and possibly several different pathways none of which have been clearly established.

Uterine Endometrium

From the time of entry into the uterus (day 4) to the time of attachment (day 14), the porcine conceptus is a free floating entity that meets its nutritional requirements through the secretions of the uterine endometrium (Clawitter et al., 1990). These secretions, also known as histotroph, are in large part produced by the endometrial, or uterine, glands. Endometrial glands have been shown to be required in other species for pre-implantation embryonic survival through the use of a uterine gland knockout model (Gray et al., 2001). Maternal progesterone directs the secretory activity of the endometrial glands (Knight et al., 1973) while estrogens produced by the embryo modify those secretions, often upregulating proteins necessary for growth and development such as uteroferrin (Simmen et al., 1991) and retinol-binding protein (Trout et al., 1992). Uterine gland density within the endometrium of both pregnant and cycling pigs remains similar throughout the first three weeks but proliferation of the glands can be seen as early as day 30 in the pregnant porcine (Perry and Crombie, 1982).

Nuclear Transfer in the Pig

Introduction

The first report of the birth of a mammal cloned from an adult cell, was in 1996 with the introduction of Dolly the sheep (Campbell et al., 1996). Further success in somatic cell nuclear transfer has been reported in cattle (Cibelli et al., 1998), goats (Baguisi et al., 1999), mice (Wakayama and Yanagimachi, 1999), pigs (Onishi et al.,

2000; Polejaeva et al., 2000), monkeys (Meng et al., 1997), and cats (Shin et al., 2002) as well as a host of others. The ability to clone *Sus scrofa* has aroused much interest in the scientific community not just because of the pig's celebrated ability to transform feed into muscle mass, but because the pig itself is a good scientific animal model since it produces multiple offspring in one litter and can bear 2.5 litters per year, a much better ratio than other domestic livestock such as the sheep, which produces an average of only 2 offspring per year. The pig offers other advantages as well, such as organ size that is comparable to that of humans making it a possible reservoir for donor organs and its potential for human disease models such as cystic fibrosis and heart disease.

Choice of Donor Cell

Previous work in cloning has explored the potential of different types of somatic cells as donors for nuclei. These included fetal fibroblast cells (Boquest et al., 1999), mammary epithelial cells (Campbell et al., 1996), granulosa cells (Collas and Barnes, 1994), pre-adipocytes (Tomii et al., 2005), and cumulus cells taken from the cumulus oophorus of the oocyte (Yang et al., 1993). Of these, the fetal fibroblast has shown the most success in producing cloned embryos (Park et al., 2001a; Tao et al., 1999; Uhm et al., 2000). The potential donor cells are initially harvested from a tissue sample. They can be cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, and antibiotics (Lai et al., 2001).

The lifecycle of a somatic donor cell, as for all cells, can be divided into 4 phases: G1, S, G2, and M. There is also a fifth stage known as G0, similar to G1, in which a cell

may lie dormant for periods of time. G1 is considered the pre-DNA synthesis stage, S the stage where DNA is replicated, G2 the post-DNA synthesis stage, and M the mitotic phase of the cell in which it physically divides into two daughter cells. G1 and G0 both contain only a single copy of genomic DNA while the G2 phase contains two in anticipation of dividing in the M phase (Cameron and Thrasher, 1971).

Several studies have been done to determine just what stage is the most appropriate for nuclear transfer: G0/G1 or G2/M. G0/G1 is most often used in order to maintain the correct ploidy without the need to eliminate extra chromosomes, as would be the case for cells at the G2/M stage. It should be noted however, that studies have been reported where, after activation, a second polar body has been extruded when G2/M stage donor nuclei are used for nuclear transfer. The G0/G1 developmental stage can be produced by culturing cells in low serum for 48 hours. The G2/M stage can be obtained by culturing in media containing 1.0 μ M colchicine for 24 hours (Lai et al., 2001).

Almost any stage of the cell cycle can be used but it is commonly observed that the donor cell must be in a pre-S phase of development to avoid the possibility of a lethal polyploidy condition.

Enucleation of Recipient Cell and Transfer of Donor Nuclei

Co-ordination of the cell cycle of the donor cell as well as the recipient cell is essential for successful nuclear transfer. The use of matured, unfertilized oocytes as recipient cells has proven beneficial since they are arrested at the metaphase II stage (MII), awaiting fertilization (Tao et al., 1999).

The most common method of nuclear transfer is to remove the metaphase chromosomes, known as enucleation, from a matured oocyte (i.e. one that has undergone cumulus expansion and has extruded a polar body) and replace this genetic material with the nucleus of a somatic cell. Porcine metaphase II oocytes are placed in culture medium (NCSU-23) containing 5 µg/mL cytochalasin B, a cytoskeletal inhibitor. Using the polar body as an indicator of the position of the metaphase plate, the polar body, and a small amount of cytoplasm containing the metaphase chromosomes, is then aspirated from the oocyte by using a microinjection pipette (Prather et al., 1999). Previous experiments have attempted to use a Hoechst stain to locate the DNA for better visualization during enucleation, but exposure to Hoechst has been shown to be detrimental to the development of the reconstructed embryo (Tao et al., 2000). Using the micropipette, a donor cell, in its entirety, is then placed within the perivitelline space of the recipient oocyte in close contact with the oolemma. Once nuclear transfer has been accomplished, the oocyte is then induced to begin cleavage by electrical and/or chemical activation (Park et al., 2001a).

Other methods of enucleation have been attempted such as chemical enucleation, where the MII oocyte is exposed to etoposide (a topoisomerase II inhibitor) (Elsheikh et al., 1998), etoposide and cyclohexamide (a protein biosynthesis inhibitor which blocks translational elongation) (Fulka and Moor, 1993), or ethanol and demecolcine (a mitotic inhibitor) (Ibanez et al., 2003). There has also been a “micromanipulator-free” enucleation process in which the area that contains the chromosomes is cut away from the rest of the oocyte (Vajta et al., 2004). These methods have met with varying degrees of

success and traditional use of the micromanipulators has produced the most consistent means of enucleation.

Activation

Activation is the use of physical and chemical means to artificially induce the reconstructed embryo to resume meiosis, behave as though it had been fertilized, and begin development. Normally, oocyte activation is triggered by fertilization, which causes an intracellular increase in free Ca^{2+} . Because no fertilization events are taking place, an alternative method of inducing activation must be used. Activation is most often a combination of two factors: appropriate medium and electrical stimulus although activation through chemical means alone has been studied (Tao et al., 2000).

A variety of reagents can be used in order to activate the oocyte. Ethanol and other agonists trigger the release of Ca^{2+} from the smooth endoplasmic reticulum by stimulating inositol 1,4,5,-triphosphate which gates specialized Ca^{2+} release channels. Ionophores like ionomycin and A23187 are also used to facilitate an increase in intracellular Ca^{2+} (Prather et al., 1999). Intracellular injection of CaCl_2 produces the desired result of inducing development in the quiescent oocyte as well (Machaty et al., 1996).

Once the enucleated oocyte has been combined with a nucleus, a common method of initiating meiosis is electrofusion. Using an electrical pulse, the plasma membrane is electroporated to allow entry of extracellular Ca^{2+} . Oocytes are placed between two platinum electrodes in a 0.3 mannitol solution supplemented with 0.5 mM Hepes, 0.01% BSA, 0.1 mM CaCl_2 , and 0.1 mM MgCl_2 (Park et al., 2001b). A direct current (DC)

pulse of 1.14 kV/cm for 30 μ s (Park et al., 2001a) is then used to “fuse” the donor nuclei to the oocyte cytoplasm and initiate the Ca^{2+} gradients necessary for further development by causing temporary openings in the plasma membrane of the reconstructed embryos allowing for exchange of intra- and extra- cellular molecules (Zimmermann and Vienken, 1982).

Methods of activating the oocyte without the use of Ca^{2+} have also been explored and include the use of puromycin and cyclohexamide to prevent cyclin B1 production (cyclin B1 is part of Maturation Promoting Factor (MPF) synthesis - eliminating MPF production has shown to induce oocytes to enter interphase) as well as activating or inhibiting protein kinases (for instance, MAPK activity is high in the oocyte in order to prevent the cell from leaving metaphase and entering interphase) (Prather et al., 1999).

There are many possible routes to take in activating the potential nuclear transfer embryo and there is a large body of work surrounding the variety and efficiency of such methods. Often, a combination of both chemical and electrofusion is used.

Embryo Culture and Transfer

After the nuclear transfer is performed, the reconstructed embryos are cultured for a period of time to ensure cleavage, an indicator of the beginning of mitosis. A common culture media for the re-constructed embryo is NCSU-23 supplemented with 4 mg/mL BSA (Hao et al., 2004), however, PZM-3 (Im et al., 2004) is also frequently used. The embryos are placed in the culture media, overlaid with mineral oil, and incubated for 24 hrs at 39 C in 5% CO_2 (Lai et al., 2001). The embryos are then surgically transferred to a recipient gilt. Recipients are heat checked twice a day with the first signs of estrus

denoting the recipient to be in day 0 of her estrous cycle. Embryos are transferred to recipients whose estrous cycle is synchronized with their day of development (Kolber-Simonds et al., 2004) or even before ovulation (Lai et al., 2002). It is worth noting that studies done on the development of normally fertilized embryos cultured *in vitro* have yielded evidence that artificial culture conditions may lead to abnormalities such as “large offspring syndrome” (Sinclair et al., 2000). As such, all-possible efforts are made to transplant the reconstructed embryos into a surrogate as soon as is feasible in order to limit exposure to adverse conditions in their environment.

Proper synchronization of the uterine environment is necessary for proper development of the conceptus. *In vitro* embryo transfer experiments have indicated a high mortality rate for embryos transferred to pigs whose day of estrous cycle differed by only one or two days from that of the day of embryonic development (Pope, 1988). Uterine secretion and hormone production are timed to optimize such gestational events as implantation, a gradual process that occurs between day 12 and day 30 when the layers of the placenta are essentially complete (King, 1993). Hormone signals from the conceptus are also necessary for maternal recognition and maintenance of pregnancy. Since the viability of nuclear transfer fetuses is very low, 70-100 embryos are often transferred at one time versus the 30 usually transferred when using *in vitro* produced embryos (Abeydeera, 2002).

Remodeling and Reprogramming

The physical changes that occur in the nucleus after nuclear transfer are collectively known as remodeling. These modifications include elongation of telomeres

to an embryonic length (Jiang et al., 2004), swelling of the nucleus (Prather et al., 1990), production of nuclear lamin-like proteins (normally depolymerized in the oocyte) (Prather et al., 1999), pronuclei becoming transcriptionally quiescent awaiting the 4-cell stage of development when the embryonic genome becomes active (Prather et al., 2004), and alterations in nucleolar structure such that they become more compact and lack vacuoles/reticulations (Ouhibi et al., 1996). All of these events are directed at pushing the more mature, differentiated, somatic cell nucleus into an earlier zygotic state.

For successful production of a cloned animal, several events in the genome of the nucleus of the reconstructed embryo must take place as the nucleus moves back to a totipotent state. In essence, the oocyte is “turning back the clock” in the donor nucleus and it is reprogrammed into a more primitive developmental stage that more closely resembles the pronucleus of a zygote wherein gene expression patterns are altered to match expression during an earlier period of development (Prather, 1998).

Accompanying the physical changes in the reconstructed embryo are changes in gene expression that more closely match those of the early embryo. The “re-programming” events occur at the epigenetic level without directly affecting the original DNA sequence and include changes in the patterns of ubiquitination, methylation, acetylation, phosphorylation, and poly ADP ribosylation of histones (Campbell et al., 2005). Errors in epigenetic re-programming have led to aberrant gene expression in the re-constructed embryo possibly leading to impaired development and high mortality. Those genes include mitogenic factors such as IGF-1, IGF-2, and IGF-1R (Schultz et al., 1996), nuclear lamins A/C and B (Hall et al., 2005), lactate dehydrogenase, phosphofructokinase, and citrate synthase (Winger et al., 2000) to name a few. Global

gene expression profiles of cloned embryos have shown extensive re-programming, however small errors in epigenetic regulation of the genome can be amplified into much greater problems farther down the developmental path (Smith et al., 2005).

Abnormalities Associated with Nuclear Transfer

Nuclear transfer is considered an inefficient process at best with success rates thought to be around 5% (Campbell et al., 2005). Losses of cloned fetuses are often attributed to abnormalities within the conceptus and conceptus-derived tissues such as the placenta.

Aberrant gene expression within the conceptus has often been cited as a primary reason for gestational losses of nuclear transfer produced animals (Humpherys et al., 2002; Niemann et al., 2002; Prather et al., 2004; Winger et al., 2000). Interestingly, these abnormalities are not considered to be caused by damage to the donor genomic DNA but were created due to incorrect epigenetic modifications during the re-modeling process (Campbell et al., 2005).

Maternal-fetal communications are also often considered compromised in nuclear transfer pregnancies as evidenced by poor placental morphology (Hill et al., 2000), lack of placental vasculature development (De Sousa et al., 2001), and development of hydroallantois (Heyman et al., 2002). Often, artificial means of maintaining the pregnancy are utilized. For the porcine, injections of estrogen-valerate can be given in early pregnancy to promote maintenance of the corpus luteum (Morgan et al., 1987). Recipients of nuclear transfer embryos often show no signs of impending parturition at the end of gestation, lacking obvious mammary development and little milk production.

As a result, many clones are often delivered C-section (Hill et al., 1999; Lai et al., 2002; Wells et al., 1999). Animals produced by nuclear transfer often require intense post-natal management to decrease their mortality rate.

Conclusions

As porcine embryos develop, they reach a period of profound morphologic and biochemical changes by day 14 that not only affects themselves but their environment as well. At this time there also exists considerable maternal-fetal communication, which when altered, ultimately results in the termination of that particular pregnancy. Nuclear transfer embryos not only must undergo many physical changes and establish maternal recognition of pregnancy, but they are also required to overcome the challenges of re-modeling and re-programming to survive.

CHAPTER II

MORPHOLOGIC AND HISTOLOGIC COMPARISONS BETWEEN *IN VIVO* AND NUCLEAR TRANSFER DERIVED PORCINE EMBRYOS

Abstract

Nuclear transfer (NT) is an inefficient but invaluable tool of the biotechnology industry. This study looks at abnormalities associated with NT porcine embryos at days 10, 12, and 14. *Methods:* 4 experimental groups were examined: non-pregnant animals, *in vivo* pregnant animals, NT recipients, and manipulation control recipients (MC). Maternal blood samples were collected until euthanasia (d10, 12, or 14) at which time embryos were collected and preserved. Serum samples were assayed for progesterone and insulin-like growth factor-1. All embryos were evaluated for embryonic disc diameter and gross morphology; they were then histologically evaluated for nucleoli density and mitotic figure index. *Results:* NT day 12 ($P \leq 0.03$) and day 14 ($P \leq 0.01$) embryos had increased nucleoli when compared to *in vivo* produced and MC embryos. NT day 14 embryos had an increased ($P \leq 0.03$) mitotic index when compared to *in vivo* produced day 14 embryos. *In vivo* produced day 12 embryos had greater ($P \leq 0.03$) embryonic disk diameters compared to NT and MC embryos; however *in vivo* produced day 14 embryos had increased ($P \leq 0.01$) embryonic disk diameters only when compared to NT day 14 embryos. *In vivo* produced day 14 embryos were morphologically more advanced ($P \leq 0.01$) than NT and MC day 14 embryos. There were no significant

differences between IGF-1 levels. *Conclusions:* Nuclear transfer embryos develop at a slower rate than their *in vivo* produced counterparts. The increase in nucleoli and mitotic index of NT embryos suggest the cell cycle may be affected or the NT embryos are employing other means to compensate for slow development. The techniques used during nuclear transfer also appear to compromise embryo development to a limited extent.

Introduction

During the course of a natural porcine pregnancy there are several time periods when embryonic losses occur. Of particular interest is the period of maternal recognition of pregnancy (MRP), which is thought to be due to conceptus-derived estrogens (Geisert et al., 1990) produced around days 11-14 of gestation as the embryo begins a morphological transformation from a spherical to filamentous form. Estrogen production by the conceptus, as measured by maternal serum estrone sulfate, peaks at day 12 before decreasing on day 14 when compared to estrone sulfate levels during a normal estrous cycle (Geisert et al., 1990). The synthesis and secretion of estrogen by the conceptus has previously been shown to cause the release of secretory vesicles from the epithelial cells of the uterine glands. These secretions, which include increases in protein, calcium, growth factors, and prostaglandins radically change the uterine environment (Davis and Blair, 1993; Geisert et al., 1982; Ka et al., 2001; Simmen et al., 1990) theoretically to provide the rapidly developing embryos with the environment needed for normal growth.

A typical day 10 embryo can range in size from 3 to 10 mm across with initial

visualization of an embryonic disk occurring at this time (Patten, 1948). On day 11, the spherical embryo will begin to migrate through the uterine lumen (Waite and Day, 1967). By day 12, a rapid trophoblastic elongation occurs in the developing embryo, wherein the conceptus becomes tubular in form, being anywhere from 10 to 50 mm in length. Once the embryo reaches day 14, it is filamentous in form and can reach over 1000 mm in length. The entire process of elongation can occur within 12-24 hours (Geisert et al., 1982).

Due to the high failure rate of nuclear transfer (NT) pregnancies very early in gestation it has been hypothesized that NT derived embryos have difficulty transitioning through the critical points of elongation and successfully producing a signal for MRP. Because of the high incidence of embryonic loss during a nuclear transfer pregnancy, cloning is considered a relatively inefficient process. However, as the only method currently available for the production of knockout livestock, it remains an invaluable tool for the biotechnical industry.

The goal of this study was to assess the pre-implantation nuclear transfer embryo at days 10, 12, and 14 for developmental competence by evaluating physical characteristics such as morphology and embryonic disk diameter to map proper development, and to use histological indicators of abnormality such as nucleoli number and mitotic figure density to appraise biologic potential.

Materials and Methods

Animals

Forty-eight gilts were randomly assigned to receive one of 4 embryo treatment groups (n=12): 1) *In Vivo* 2) Nuclear Transfer (NT) 3) Manipulation Controls (MC) and 4) Non-Pregnant (NP) used as a negative control for those experiments not involving embryos. Animals were heat checked every 12 hours for signs of estrus. When heat was first detected the animal was considered to be at day 0 of her estrous cycle. Embryo transfers were performed upon treatment groups receiving NT embryo and MC embryos as described (Betthauser et al., 2000; Lai et al., 2002). Gilts within the *in vivo* treatment group were bred by artificial insemination 12 hours after initial detection of heat.

Tissue Collection and Histology

Gilts were catheterized via the jugular vein on day 1 of the estrous cycle according to their assigned experimental group as previously described (Fudge et al., 2002). Blood samples were collected daily from day 5 to day of sacrifice. The blood was allowed to clot, and then centrifuged for 30 minutes at 4°C, 1500 g. The serum was then frozen and stored at -20°C. Gilts were euthanized at day 10, 12, or 14 at which time any embryos were recovered from the uterine horns by flushing them with phosphate buffered saline (PBS). Uterine horn sections and/or embryos were collected and preserved in 10% neutral buffered formaldehyde. Collected tissues were subsequently dehydrated through a series of ethanol washes and stored in 70% ethanol.

Hormone Assays

Serum samples were measured for progesterone (P₄) using Coat-A-Count® progesterone kits (Diagnostics Products Corporation, Los Angeles, CA). The intraassay and interassay coefficients of variation were $\leq 10\%$, respectively. Serum samples were measured for IGF-1 as previously described (Lamberson et al., 1995). The intraassay and interassay coefficients of variation were $\leq 10\%$, respectively.

Production of Nuclear Transfer Embryos

Recipient oocytes were matured in TCM 199 (supplemented with 0.1% polyvinyl alcohol, cysteine (0.1 mg/ml), epidermal growth factor (10 ng/ml), 0.91 mM Na-pyruvate, 3.05 mM D-glucose, follicle-stimulating hormone (0.5 µg/ml), luteinizing hormone (0.5 µg/ml), penicillin (75 µg/ml), and streptomycin (50 µg/ml) (Lai et al., 2002)) for 48 hours after collection from the ovary. The matured oocytes were then vortexed in hyaluronidase for 4 minutes and rinsed twice in TL-HEPES to strip away cumulus cells. The oocytes were placed in culture medium (NCSU-23) containing 5 µg/mL cytochalasin B, a cytoskeletal inhibitor. Using the polar body as an indicator of the position of the metaphase plate, the polar body and a small amount of cytoplasm containing the metaphase chromosomes, were then aspirated from the oocyte by using a microinjection pipette (Prather et al., 1999). Using the same micropipette, a fibroblast cell was placed within the perivitelline space of the recipient oocyte in close contact with the plasma membrane. Reconstructed oocyte/fibroblasts were then placed in 0.3 M mannitol, 1 mM CaCl₂, 0.1 mM MgCl₂, 0.5 mM Hepes (Kuhholzer et al., 2001) and

fusion/activation was performed by using a BTX Electro-Cell Manipulator 200 (BTX, San Diego, CA) at 2 pulses of 1.2 kV/cm for 30 msec (direct current) (Lai et al., 2002). Presumptive embryos were then cultured overnight in NCSU-23 supplemented with 0.4% BSA (Abeydeera et al., 1998).

In Vitro Production of Manipulation Control Embryos

Matured oocytes were stripped of cumulus as above. Oocytes were then washed three times and co-incubated with boar sperm for 6 hrs in 50 μ L drops of modified Tris-buffered medium with 1mM caffeine and 0.1% BSA. Post-fertilization, the embryos' zona pellucida was pierced with a microinjection pipette and a small amount of cytoplasm removed being careful to avoid the polar body and cytoplasm directly surrounding it. Presumptive embryos were then cultured overnight in NCSU 23 medium supplemented with 0.4% BSA before being transferred to a recipient (Abeydeera et al., 1998).

Microscopy

Embryos recovered from the gilts were initially evaluated for morphology and were ranked as one of 4 stages: spherical, ovoid, tubular, and filamentous. After being imbedded in paraffin, uterine and embryonic tissues were sectioned and stained with a hematoxylin and eosin stain after placement on glass microscope slides. Uterine gland density was measured by visually counting uterine glands present in 3 random locations of a uterine section under 10x power. Embryonic disk measurements were made by using a dissection scope and a micrometer. Nucleoli density was evaluated by visually

counting the nucleoli in 10 nuclei in 5 random views within a single embryo at 100x power. The percentage of mitotic figures was evaluated by counting the number of figures within a view divided by the number of cells per view. This was done for 5 random views within a single embryo at 100x power.

Statistics

Data collected from uterine gland density, embryo nucleoli density, embryo mitotic figure percentage, and embryonic disk diameter was analyzed by ANOVA using the mixed model in the Statistical Analysis System program to determine treatment differences. Results are expressed as mean \pm SEM. A probability of $P \leq 0.05$ was considered to be statistically significant.

Data obtained from embryo morphology was analyzed by the chi-square test using the general linear model in the Statistical Analysis System program. A probability of $P \leq 0.05$ was considered to be statistically significant.

Data collected from progesterone, estrone sulfate, and IGF-I found in the maternal blood serum was analyzed by ANOVA using the mixed model in the Statistical Analysis System program to verify treatment differences. Results are expressed as mean \pm SEM. A probability of $P \leq 0.05$ was considered to be statistically significant.

Results

Embryo Nucleoli Density

When embryos within the same treatment groups were compared with each other, the *in vivo*-produced and MC embryos maintained the same number of nucleoli per cell through days 10-14 of gestation. The NT embryos also had similar numbers of nucleoli per cell on day 10, but had significant increases in nucleoli per cell by day 12 ($P \leq 0.003$) and day 14 ($P \leq 0.002$) of development (Figure 1).

When compared to embryos of the same day but different treatment, the embryos produced by NT had significantly increased numbers of nucleoli per trophoblast cell by day 12 ($P \leq 0.03$) and day 14 ($P \leq 0.01$) when compared to embryos produced by any other method (Figure 2).

Nuclear transfer day 12 and NT day 14 embryos were not found to be significantly different from each other either within day or treatment.

Embryo Mitotic Figure Index

In vivo-produced embryos exhibited no significant change in mitotic figure density over time, however, day 14 MC and day 14 NT embryos showed significant increases ($P \leq 0.009$ and $P \leq 0.008$, respectively) when compared to earlier embryos within the same treatment group (Figure 3).

The day 14 embryos produced by NT had a significantly increased ($P \leq 0.03$) number of mitotic figures per trophoblast cell population when compared to day 14 *in*

vivo-produced embryos. Day 14 MC embryos were found to have no significant differences when compared to either day 14 *in vivo*-produced or NT embryos (Figure 4).

Embryonic Disk Diameter

As *in vivo*-produced embryos developed from day 10 to day 14 there were significant increases in disk diameter between days 10 to 12 ($P \leq 0.003$) and days 12 to 14 ($P \leq 0.013$). In comparison, NT embryos showed no significant changes in embryonic disk growth as gestation progressed. MC embryos only showed significant embryonic disk growth between days 12 to 14 ($P \leq 0.008$) (Figure 5).

When compared to embryos of different treatment groups but at the same developmental stage, day 12 *in vivo*-produced embryos had significantly increased ($P \leq 0.02$) embryonic disk compared to MC and NT embryos, but by day 14, only NT embryos had a significant decrease ($P \leq 0.0002$) in embryonic disk size when compared to *in vivo*-produced embryos (Figure 6).

Embryo Morphology

Embryo morphology was determined on 146 embryos. Data analysis showed a difference in morphology between treatments ($P \leq 0.01$) on day 14, but not at other days. *In vivo* produced day 14 embryos were 6.41 times more likely to be in a more advanced stage of development than their NT and MC counterparts (Figure 7). There were no significant differences between any of the treatments at day 12. All embryos recovered at day 10 were morphologically spherical and were not analyzed.

Maternal Serum IGF-I

There were no significant differences in IGF-I levels between day, treatment, or treatment by day in any of the four groups.

Maternal Serum Progesterone and Uterine Gland Density

Maternal serum progesterone levels and uterine gland density were used as controls for determining gilt suitability as recipients and to assist in limiting the contribution of environmental variation. There were no significant differences for either parameter in the recipients of all four treatment groups.

Incidental Findings

Histologically, other abnormalities were observed between *in vivo* and nuclear transfer embryos but were not submitted to statistical analysis as they did not fit within the experimental model. Within certain of the NT embryos there were cytoplasmic and peri-nuclear vacuoles present as well as abnormally enlarged cells and “layering” of trophoblast cells where only a single cell layer should have been present (Figure 8). Also of note, several MC and NT recipients were found to have all embryos located within a single horn leading to the possibility that they failed to migrate normally.

Discussion

The main objective of the study was to evaluate porcine nuclear transfer embryos for potential abnormalities that could contribute to the high mortality rate during early

pregnancy. It was predicted that the period of elongation, a phase in which the porcine embryo undergoes extreme morphological changes as well as increased steroid secretion, would be a point of failure in the development of many porcine embryos.

As a result of this study, it was determined that as nuclear transfer embryos approached day 14, at which time elongation should be considered to be complete, many were unable to attain a filamentous trophoblast form. These embryos also had increasingly smaller embryonic disks when compared to normal embryos, another sign of their developmental retardation. An inability to develop morphologically could also signal abnormalities in other areas such as steroid production, contributing to a lack of maternal recognition of pregnancy and resulting in a subsequent termination of the pregnancy. It is also possible that the presence of a small number of advanced embryos could be signaling, via the secretion of estrogen, for the extreme changes in the uterine environment required for more advanced embryos to develop. As Pope (1988) demonstrated, this could create an environment incompatible for less developed embryos resulting in their mortality. Unable to maintain the minimum number of embryos required for pregnancy, the recipient once again experiences a return to estrus.

Specific histological abnormalities were also noted, with nuclear transfer embryos having increased numbers of nucleoli within their trophoblast as they approached day 14 of gestation. The re-formation of nucleoli in the early embryo is integral to the initiation of embryonic protein production as the nucleoli are the sites of ribosomal RNA synthesis (Hyttel et al., 2000; Pederson, 1998). Under normal conditions, the nucleoli disassemble during the early M-phase of the oocyte cell cycle (which is then arrested at the meiosis II stage awaiting fertilization (Senger, 2003)), and are reformed during the third cell cycle,

or at the four-cell stage, after fertilization (Dundr et al., 2000; Hyttel et al., 2000). By the time the embryo reaches the blastocyst stage at day 6, its nuclei contain fully functional nucleoli that are comparable to somatic cell nucleoli (Hyttel et al., 2000). During the course of the cell cycle in a somatic cell, the nucleolus is disassembled during mitosis and re-assembled during telophase (Olson et al., 2000). The increased presence of nucleoli does not necessarily indicate increased levels of gene transcription, but more likely indicates erroneous re-programming of the embryonic genome and possible production of inaccurate gene transcripts directing the cell down an abnormal developmental pathway.

Mitotic figures, condensed DNA, are present in a cell population and are indicators of mitosis that appear during the metaphase period. The increase in the mitotic index may indicate a potential problem with control of the cell cycle resulting from improper re-programming of the re-constructed nucleus. There are reports of a mutant mouse line with the inability to transition from metaphase to anaphase as a result of a mutation in the anaphase-promoting complex component APC10/DOC1 (Pravtcheva and Wise, 2001) leading to a higher than normal mitotic index (Magnuson and Epstein, 1984). Yet another mouse mutation affecting *Omcg-1*, a gene coding for a nuclear zinc finger protein, exhibits a high mitotic index as well as the inability to develop past the pre-implantation stage (Artus et al., 2005). There are numerous possibilities as to the reasons why this abnormality is present. However, the inability of a cell to exit mitosis, as the increase in the mitotic index might suggest, may be contributing to the notably slower development seen in NT embryos.

Another possibility is that the nuclear transfer embryos are attempting to augment their development by altering cell division, in this instance increasing it. This

phenomenon has been seen before in the production of mouse aggregation chimeras. The addition of blastomeres to an already developing mouse embryo does not produce a larger than normal fetus, but through a decreased rate of cell division, a normal sized embryo is obtained (Buehr and McLaren, 1974; Lewis and Rossant, 1982). A reversal of this event can be seen in the production of multiple embryos by splitting a single embryo. This time an increase in cell division results in the development of a normal sized embryo (Rands, 1985). Comparisons to embryos further in development will be necessary to understand the mechanisms nuclear transfer embryos utilize to adjust their cell numbers.

Conclusions

Nuclear transfer embryos appear to develop at a slower rate than their *in vivo* counterparts as demonstrated by their less advanced morphology and decreased embryonic disk size as gestation progresses. Due to the apparent increase in nucleoli and mitotic figures within the trophoblast cell population of NT embryos, it would seem that the cell cycle is either adversely affected, possibly by incomplete re-programming of that portion of the embryonic genome, or the embryo itself is attempting to compensate for earlier, inadequate growth. The mechanical perturbations caused by the nuclear transfer technique itself also appear to retard development but not to the extent that the actual cloning process does as MC embryos often appear to re-cover some of the developmental ground they lost as they approach day 14. It would appear from these results that increasing the accuracy of the re-programming events would contribute to increasing the

survival rate of NT embryos; however the exact means by which to accomplish this are still unknown and require further study.

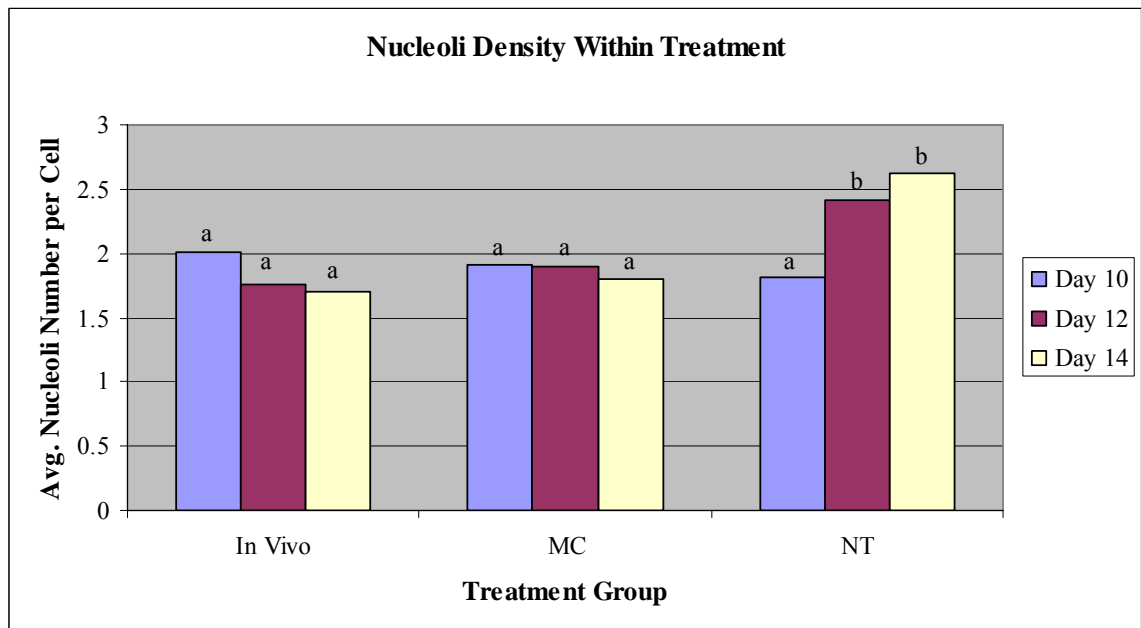


Figure 1. Nucleoli density by treatment group. $P \leq 0.05$ was considered to be statistically significant.

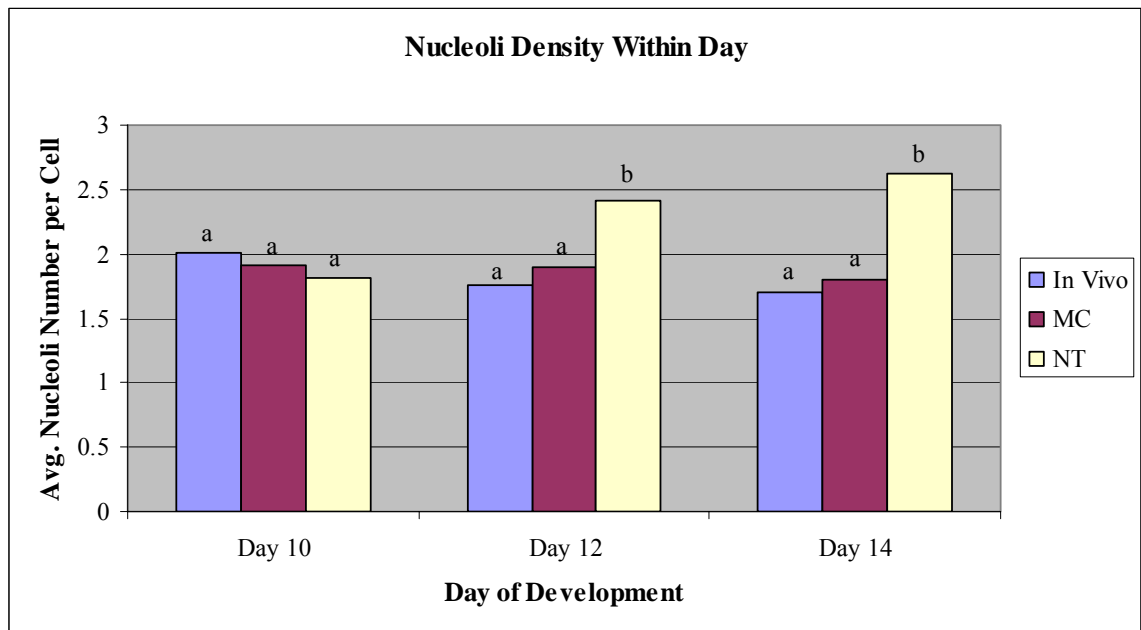


Figure 2. Nucleoli density by day of development. $P \leq 0.05$ was considered to be statistically significant.

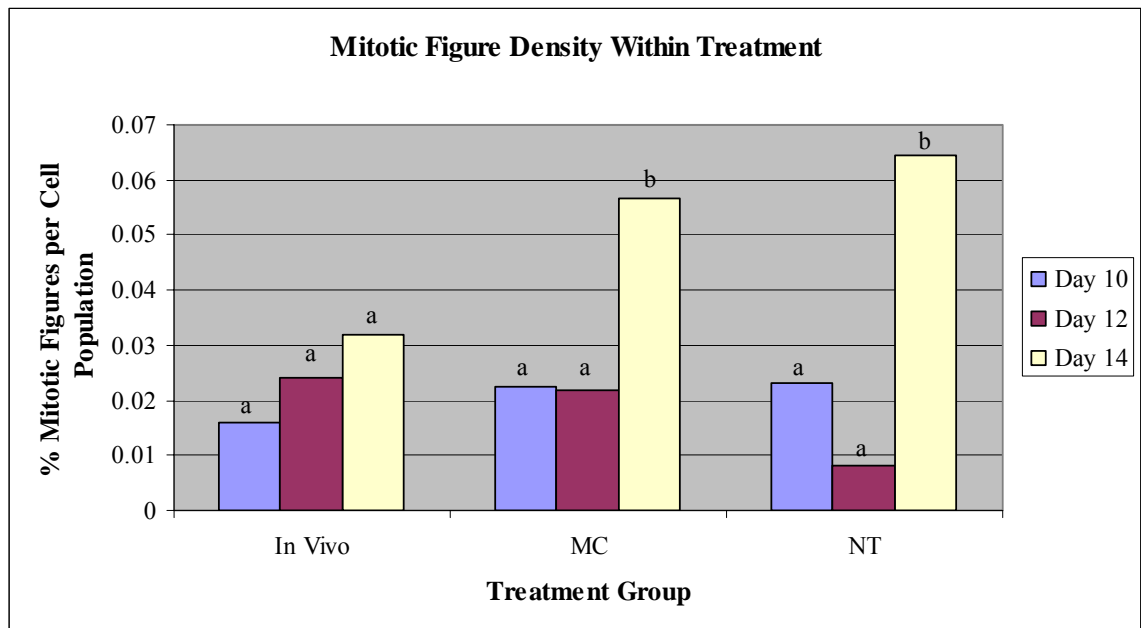


Figure 3. Mitotic index by treatment group. $P \leq 0.05$ was considered to be statistically significant.

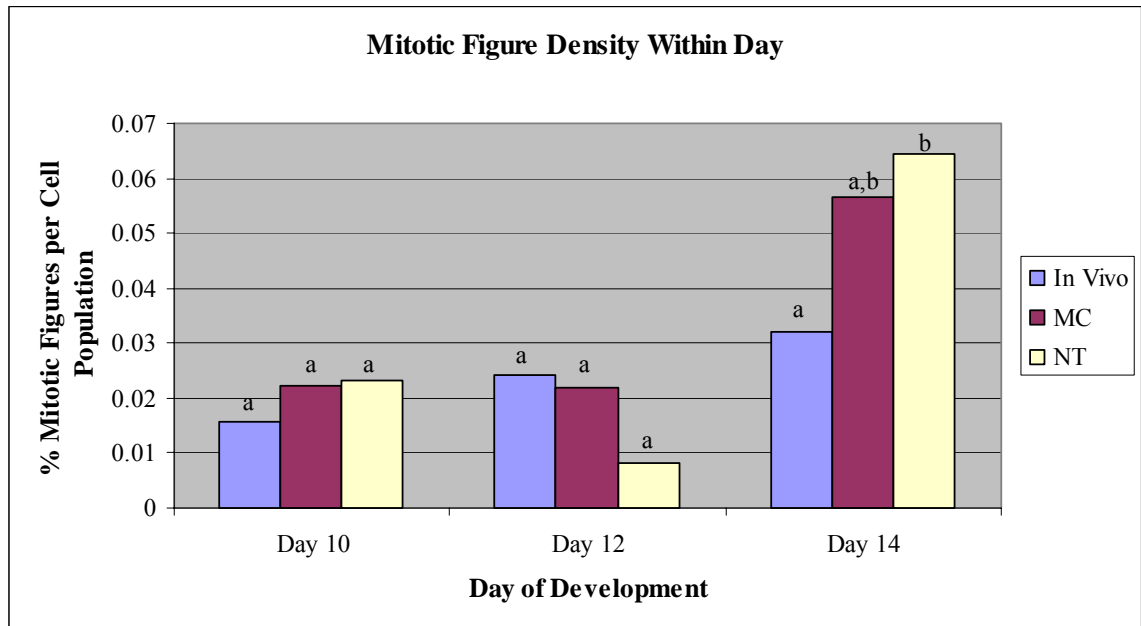


Figure 4. Mitotic index by day of development. $P \leq 0.05$ was considered to be statistically significant.

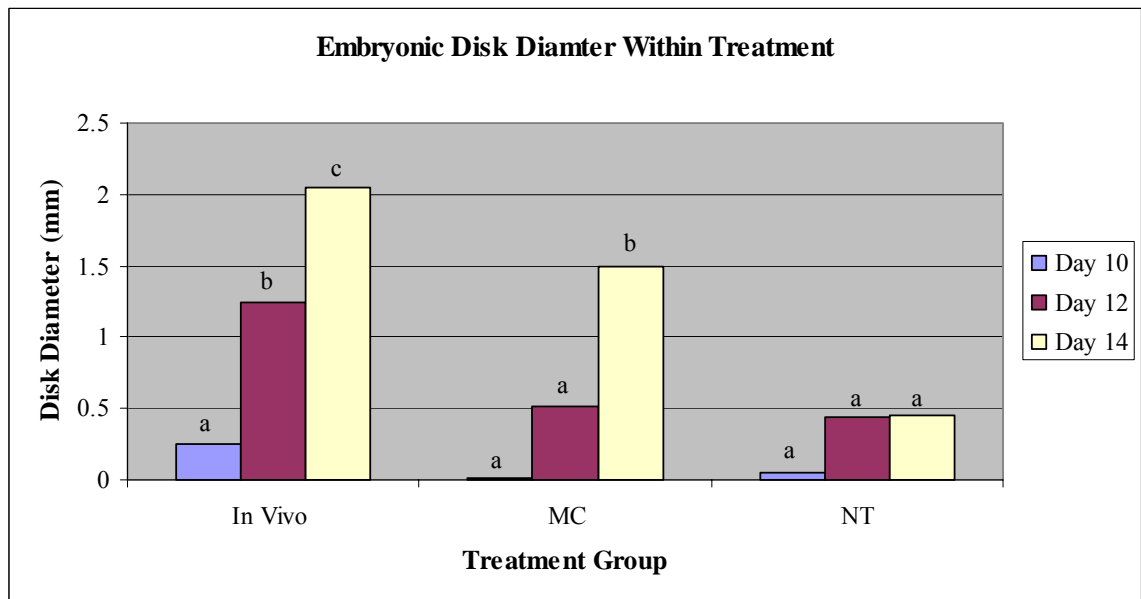


Figure 5. Embryonic disk diameter by treatment group. $P \leq 0.05$ was considered to be statistically significant.

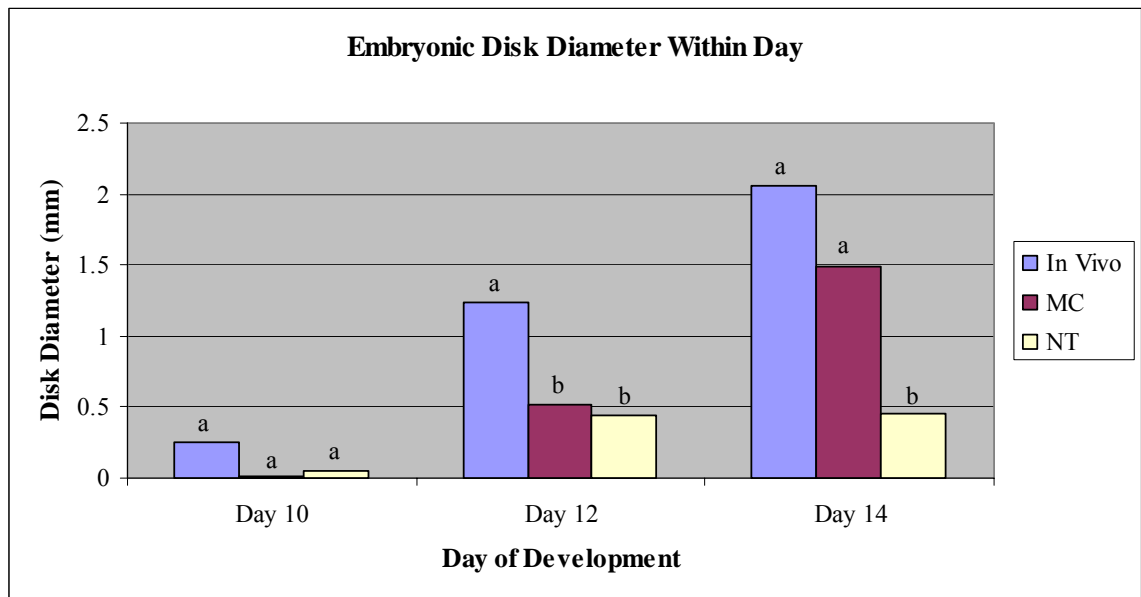


Figure 6. Embryonic disk diameter by day of development. $P \leq 0.05$ was considered to be statistically significant.

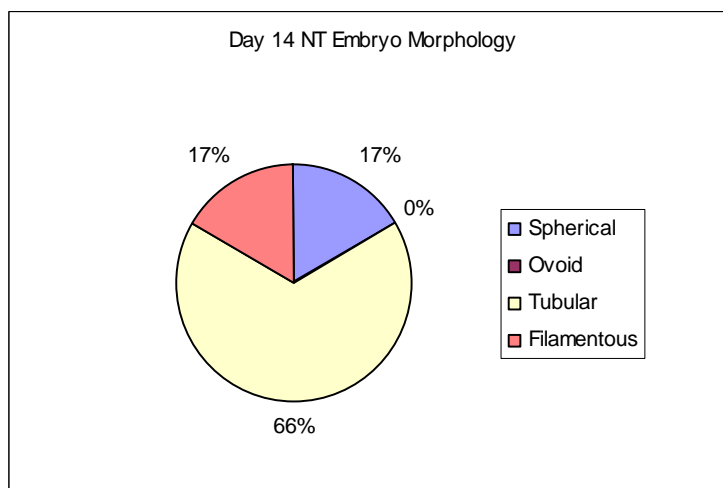
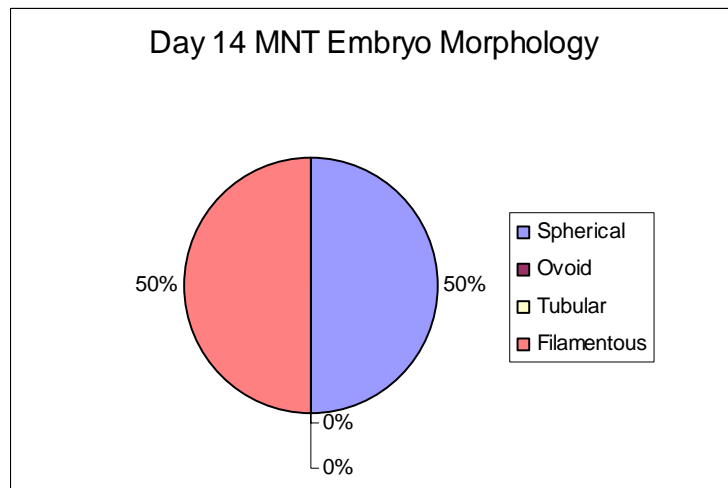
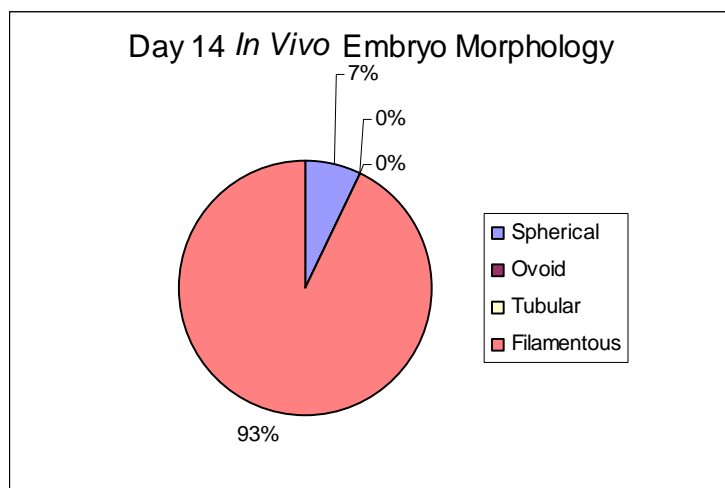


Figure 7. Percentage of embryo morphology by treatment group. Difference in developmental stage found at day 14 ($P \leq 0.01$). $P \leq 0.05$ was considered to be statistically significant.

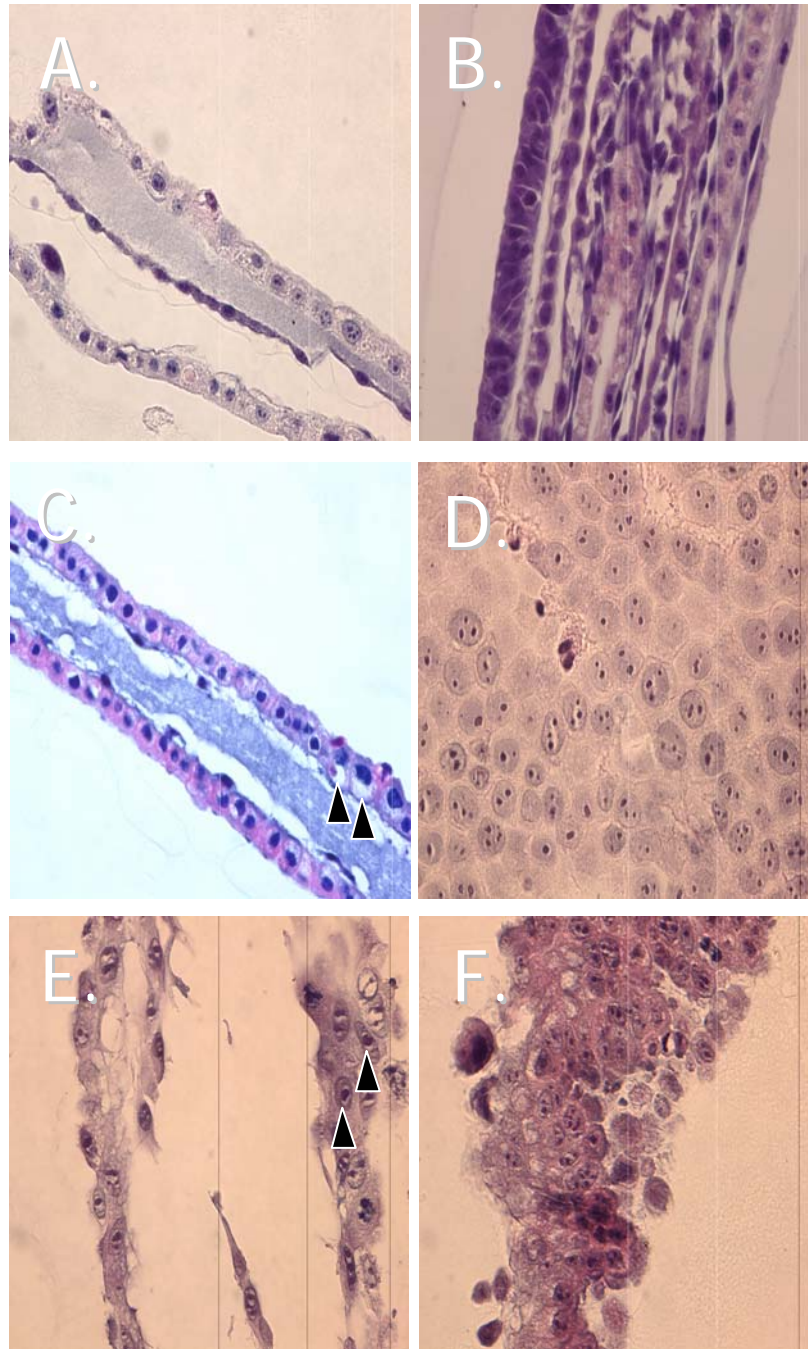


Figure 8. Histologic sections of embryos A.) day 10 *in vivo* produced embryo (40x) B.) day 10 *in vivo* produced embryo (40x) C.) day 10 nuclear transfer embryo (40x) with cytoplasmic vacuoles (black arrows) D.) day 14 nuclear transfer embryo (40x) with enlarged cells E.) day 14 nuclear transfer embryo (40x) with perinucleolar vacuoles (black arrows) F.) day 14 nuclear transfer embryo (40x) with abnormal “layering” of cells

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APPENDIX A: 2005 IETS ABSTRACT

Histological Comparisons between Nuclear Transfer and *In Vivo* Porcine Embryos

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Due to the high incidence of embryonic loss during a nuclear transfer pregnancy, cloning is considered a relatively inefficient process. However, as the only method of producing knockout domestic animals it is considered an invaluable tool for the biotechnical industry. By taking a comparative histological look at embryos at significant stages in the porcine pregnancy (d 10, 12, and 14), factors contributing to early embryonic loss may be revealed. *Methods:* This study included 3 gilts per stage of pregnancy and 4 different experimental groups for each stage studied: non-pregnant animals (negative controls), *in vivo* pregnant animals, nuclear transfer (NT) recipients, and *in vitro* manipulated recipients (IVM). IVM embryos were *in vitro* produced embryos upon which a mock nuclear transfer has been performed in an effort to account for the variability introduced by the actual technique. Animals were catheterized and either bred or underwent a surgical embryo transfer on d1 of the estrous cycle according to their assigned experimental group. Fifty embryos were transferred per embryo transfer. Blood samples were collected from d5 to day of euthanasia (d10, 12, or 14) at which time embryos were collected and preserved in 10% neutral buffered formalin. All embryonic

disc diameters and gross morphology were evaluated as parameters for normal development. Embryos were then dehydrated in ethanol, paraffin-embedded, sectioned, stained with hemotoxylin and eosin, and day 14 embryos were evaluated for abnormalities such as higher than normal nucleoli numbers, increased cytoplasmic vacuoles, and higher than normal numbers of mitotic figures. *Results:* There were significant differences ($p < .0001$) between diameter of the embryonic disks with the diameter of the NT embryonic disks being smaller than the *in vivo* controls at all stages studied. Morphologically, the *in vivo* controls were more developmentally competent than their NT counterparts by the time they reached d 14 ($p = .0002$) in that most had achieved the more advanced elongated form of growth as opposed to remaining spherical in shape. Significant histological differences in the number of nucleoli per nuclei were also found between *in vivo* and NT embryos ($p = .05$) as well as between MC and NT embryos ($p = .05$). *Conclusions:* Nuclear transfer embryos develop at a much slower rate than their *in vivo* counterparts and often exhibit histological abnormalities that could contribute to this slow growth. Due to the apparent increase in nucleoli it is possible that NT embryos are being arrested at a specific stage in the cell cycle. Other abnormalities noted but not quantitatively evaluated were enlarged nuclei, apparent layering of the trophoblast within the embryo, and nuclear vacuoles.