CLONING AND EXPRESSION OF PLURIPOTENT FACTORS AROUND THE TIME OF GASTRULATION IN THE PORCINE CONCEPTUS

by

DOUGLAS ROBERT EBORN

B.S., Utah State University, 2000
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

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Abstract

Early in embryonic development a series of events occur whereby pluripotent cells undergo differentiation to give rise to the three germ layers and extraembryonic tissues of the developing conceptus. Nanog, Sox-2, and Oct-4 genes have been identified as having key roles in maintaining pluripotency in undifferentiated human and mouse cells but recent evidence suggests they may have different roles in farm animals. We cloned the coding sequence for porcine Nanog including 452 base pairs of the Nanog promoter, and partial coding sequences of Oct-4 and Sox-2. Embryos were flushed from sows 10, 12, 15, and 17 days post insemination. RNA was isolated from whole d-10 and -12 conceptuses, d-15 embryonic disk, distal and proximal extraembryonic tissue, and d-17 embryonic disk, distal and proximal extraembryonic tissue, and allantois for real-time PCR. RNA from d-40 maternal myometrium and endometrium, fetal placenta, and liver were also used in real-time PCR. The homeodomain and c-terminal tryptophan repeats are highly conserved in porcine Nanog compared to the mouse, human and bovine. In the promoter, the highly conserved Octamer and Sox binding sequences are also present. The Nanog expression pattern was different when compared to Oct-4 and Sox-2. Day-40 tissues demonstrated the highest expression including endometrium (7 fold) fetal liver (27 fold), placenta (40 fold) and myometrium (72 fold) when compared to day 15 distal extraembryonic tissue. Oct-4 and Sox-2 expression was lowest in d-40 tissues except for fetal liver which was 20 and 71 fold, respectively, higher than endometrium. Oct-4 levels were consistent in d-10, -12, and -15 conceptuses and disk but dropped 3 fold in d-17 disk. On the other hand, Sox-2 was upregulated a 1000 fold in the d-15 disk and 2000 fold in the d-17 disk when compared to the d-12 conceptus. Nanog may have other roles in than maintenance of pluripotency including a possible role in multipotent or progenitor stem cells. Expression of all 3 markers in fetal liver suggests a more primitive cell type is present such as hematopoietic stem cells.

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Early in embryonic development a series of events occur whereby pluripotent cells undergo differentiation to give rise to the three germ layers and extraembryonic tissues of the developing conceptus. Nanog, Sox-2, and Oct-4 genes have been identified as having key roles in maintaining pluripotency in undifferentiated human and mouse cells but recent evidence suggests they may have different roles in farm animals. We cloned the coding sequence for porcine Nanog including 452 base pairs of the Nanog promoter, and partial coding sequences of Oct-4 and Sox-2. Embryos were flushed from sows 10, 12, 15, and 17 days post insemination. RNA was isolated from whole d-10 and -12 conceptuses, d-15 embryonic disk, distal and proximal extraembryonic tissue, and d-17 embryonic disk, distal and proximal extraembryonic tissue, and allantois for real-time PCR. RNA from d-40 maternal myometrium and endometrium, fetal placenta, and liver were also used in real-time PCR. The homeodomain and c-terminal tryptophan repeats are highly conserved in porcine Nanog compared to the mouse, human and bovine. In the promoter, the highly conserved Octamer and Sox binding sequences are also present. The Nanog expression pattern was different when compared to Oct-4 and Sox-2. Day-40 tissues demonstrated the highest expression including endometrium (7 fold) fetal liver (27 fold), placenta (40 fold) and myometrium (72 fold) when compared to day 15 distal extraembryonic tissue. Oct-4 and Sox-2 expression was lowest in d-40 tissues except for fetal liver which was 20 and 71 fold, respectively, higher than endometrium. Oct-4 levels were consistent in d-10, -12, and -15 conceptuses and disk but dropped 3 fold in d-17 disk. On the other hand, Sox-2 was upregulated a 1000 fold in the d-15 disk and 2000 fold in the d-17 disk when compared to the d-12 conceptus. Nanog may have other roles in than maintenance of pluripotency including a possible role in multipotent or progenitor stem cells. Expression of all 3 markers in fetal liver suggests a more primitive cell type is present such as hematopoietic stem cells.

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Dedication

To my family; Mary Ann, Shaun, Megan, Camille, Elaine, and Julie Ann for their example of patience, long-suffering, faith, and love.

CHAPTER 1 - Literature Review

At fertilization, an oocyte and sperm merge into a single totipotent cell, or zygote, that has the ability to form every cell type of the conceptus. Eventually, during the process of cleavage, cells begin to differentiate. The first visual evidence of differentiation is in the formation of a blastocyst with the trophoblast surrounding the inner cell mass and blastocoele cavity. The inner cell mass is comprised of undifferentiated cells that will form the embryo proper and is the source of embryonic stem cells grown in culture. Embryonic stem cells have made an invaluable contribution to our early understanding of how gene regulation maintains pluripotency and events that lead to differentiation. Nanog, Sox-2 and Oct-4 are three transcription factors that are important for the development of the early mouse embryo and maintaining pluripotency in embryonic stem cells. This review will begin to characterize each factor in the pig, describe their expression patterns in embryonic development, and address their regulation and roles in regulation of other genes.

Gene Characterisitcs of Nanog, Oct-4 and Sox-2

Nanog

Named for the mythological Celtic land Tir nan Og (land of the ever young), Nanog is homeobox transcription factor expressed in pluripotent cells such as embryonic stem (ES), germ (EG) and carcinoma (EC) as well as the pluripotent cells of the developing embryo. It was first identified by Wang et al., (2003) who identified a transcription factor expressed in the early developing mouse embryo and ES cells. It was termed ENK (early embryo specific NK) due to the presence of a homeodomain that shared 50% identity with other NK-2 family proteins. Later two independent groups identified the same transcription factor which could maintain pluripotency in ES cells independent of leukemia inhibitory factor (LIF) stimulation and named it Nanog (Chambers et al., 2003; Mitsui et al., 2003).

Mouse

Located on chromosome 6, Nanog (GeneID: 71950) is comprised of 4 exons that encodes a 305 amino acid protein from a transcript of 1356 nucleotides (Accession NM_028016.2). This

RefSeq entry replaces an earlier version of Nanog that was 2188 nucleotides in length (Accession NM_028016.1). Found within the 3' untranslated region is a B2 repetitive element that may contribute to gene regulation (Chambers et al., 2003). The Nanog protein can be broken down into three domains, the N-terminal, homeodomain, and C-terminal. The homeodomain is contained within residues 98-155 and is unique as the family of NK-2 homeoproteins share the most identity with the homeodomain of Nanog but it's less than 50% (Mitsui et al., 2003). N-terminal to the homeodomain is a serine-rich motif and at the C-terminus is a tryptophan at every fifth position repeated ten times (Chambers et al., 2003; Mitsui et al., 2003). Both N- and C-terminal domains have transactivation abilities when fused to the binding domain of the yeast transcription factor, Gal4, but the C-terminal is 7 times more active than the N-terminus (Pan and Pei, 2003).

Human

Human NANOG (GeneID: 79923) is located on human chromosome 12 and encodes a 2098 nucleotide transcript (Accession NM_024865.2). The protein is also comprised of 305 amino acids with the homeodomain spanning from residues 98-154. Overall amino acid identity with the mouse is 57.7% but the homeobox is 87.5% similar. The trytophan repeats are conserved in the human except for a short deletion and a glutamine replacing a tryptophan at position 211 (Chambers et al., 2003). A high number of pseudogenes have been reported for human NANOG which include one tandem duplicate and ten processed pseudogenes and this is much higher than 2 pseudogenes found in the mouse genome (Booth and Holland, 2004). Unlike the mouse protein where both the N- and C-terminal domains show transactivation activity, only the C-terminal domain of human Nanog was shown to have activity (Oh et al., 2005).

Farm Animals

Bovine (GeneID: 538951) and porcine (GeneID: 595109) NANOG are located on chromosome 5. The bovine transcript (Accession NM_001025344.1) is generated from five exons and is 1644 nucleotides long resulting in a 300 amino acid protein. Amino acid identity between bovine and the mouse and human is 58.2 and 69.3% overall and 87.9 and 94.6% within the homeobox respectively. The full-length transcript has not been published for porcine Nanog.

Oct-4

Oct-4 belongs to a large family of transcription factors containing a DNA-binding domain called POU. The domain was named due to sequence similarity among 3 mammalian transcription factors Pit-1, Oct1 and Oct2, and UNC-86 (Herr et al., 1988). The POU domain is unique in that it is comprised of two subdomains, the POU-specific (POU_S) or POU domain and the POU homeodomain (POU_{HD}) connected by a linker. The two domains act independently of each other (Herr and Cleary, 1995). When binding to the consensus-binding motif, ATTAGCAT, the POU_S subunit binds to GCAT and POU_{HD} binds to ATTA without making contact between subunits. Oct-4 was first detected in F9 embryonal carcinoma cells and named NF-A3 (Lenardo et al., 1989). It was then independently cloned in P19 embryonal carcinoma (Okamoto et al., 1990) and in the early developing mouse (Rosner et al., 1990) and named Oct-3 or in the pre-implantation mouse embryo and named Oct-4 (Scholer et al., 1990).

Mouse

POU domain, class 5, transcription factor 1 (*Pou5f1*) (GeneID: 18999) is located on chromosome 17 in the mouse. Through five exons a 1346 nucleotide message (Genbank: NM_013633) is transcribed resulting in a 352 amino acid protein. The POU_S domain is found between amino acids 131-205 and the homeodomain is located at position 224-282.

Human

The human POU class 5 homeobox 1 gene generates a transcript of 1417 nucleotides (Genbank: NM_002701) from five exons. Within the 360 amino acids are the POU domain (138-212) and the homeodomain (231-289).

Farm Animals

In the bovine, POU5F1 (GeneID: 282316) is located on chromosome 23 and encodes a 1615 base pair transcript (Genbank: NM_174580) which translates to a 360 amino acid protein. It shares 90.6% and 81.7% overall identity with the human and mouse proteins respectively (van Eijk et al., 1999). Porcine POU5F1 (GeneID: 100127461) is located on chromosome 7 and has a coding sequence of 1083 base pairs (Genbank: NM_001113060) for a 360 amino acid. Identity between the pig and bovine protein is 96.4%. The POU_S region is between amino acids 138-212 and the POU_{HD} lies between 231-289 for both the bovine and the pig.

Sox-2

SOX-2 belongs to a superfamily of DNA-proteins (SRY-related HMG box) including SRY and its homologs. These proteins contain a single HMG box that binds the minor groove of DNA in a highly sequence-specific manner (A/TCAAAG/C).

Mouse

The mouse Sox-2 gene (GeneID: 20674) produces a 2457 base pair transcript (Genbank: NM_011443) that encodes a 319 amino acid protein. The HMG box is 81 amino acids (42-113).

Human

A 2518 base pair message (Genbank NM_003106) is transcribed by the Human SOX-2 gene (GeneID: 6657) resulting in a 317 amino acid protein.

Bovine

Bovine Sox-2 (GeneId: 784383) is 1477 nucleotides (Genbank NM_001105463) resulting in a protein of 320 amino acids.

Gene Regulation

Regulation of Nanog

A composite Octamer/Sox binding site is found in the mouse Nanog promoter approximately 180 nucleotides from the transcription start site (Kuroda et al., 2005; Rodda et al., 2005). Using constructs from both the mouse and human promoter, mutations to either or both elements dramatically reduced Nanog reporter expression in human and mouse ES cells (Kuroda et al., 2005). Sox-2 and Oct-4 protein were also shown to bind to the Nanog promoter by electrophoretic mobility shift assays and *in vivo* by chromatin immunoprecipitation assays (Rodda et al., 2005). However, in Oct-4 deficient embryos Nanog expression is still observed (Chambers et al., 2003). An unidentified factor, termed pluripotential cell-specific Sox element-binding protein (PSBP), was reported in R1 mouse embryonic stem cells but not embryonic germ or embryonal carcinoma cells (Kuroda et al., 2005). This finding could not be confirmed in a different embryonic stem cell line (Rodda et al., 2005).

Nanog expression levels dropped by 15% when promoter constructs were shortened from 2342 base pairs to 332 base pairs demonstrating other elements upstream of the Oct/Sox element

are present that can regulate Nanog expression (Kuroda et al., 2005). Originally termed Genesis because its expression was limited to pluripotent ES and EC cells (Sutton et al., 1996), FoxD3 belongs to the forkhead family of transcription factors and can up-regulate Nanog expression in mouse ES and EC cells by binding to an ES cell specific enhancer E2-like element in the Nanog promoter *in vivo* (Pan et al., 2006).

Pan et al., (2006) reported an Oct-4 dose-dependant effect on Nanog. At sub-steady concentrations Oct-4 up-regulates Nanog however at higher concentrations Oct-4 may repress Nanog. The tumor suppressor p53 has also been shown to down-regulate Nanog when embryonic stem cells have experienced DNA damage (Lin et al., 2005) and a member of the canonical Wnt signaling pathway, Tcf3, can also down-regulate Nanog expression (Pereira et al., 2006).

Overexpression of Nanog increased the stability of undifferentiated cells by becoming independent of LIF and are more resistant to differentiation procedures (Chambers et al., 2003). Evidence suggests that Nanog can be a repressor of genes, especially those of endoderm lineage. Differentiated Nanog null cells (-/-) expressed only endoderm markers such as gata4, gata6, tm, and bmp2 (Mitsui et al., 2003) and when Nanog was down-regulated by RNA interference in mouse embryonic stem cells, gata6, gata4, and laminin B1 were up-regulated (Hough et al., 2006). Results differed in heterozygous Nanog (+/-) ES cells. With a feeder layer they can remain undifferentiated but without out a feeder layer, the cells differentiated into endodermal, mesodermal, and ectodermal lineages (Hatano et al., 2005).

Initially Nanog was hypothesized to be only a repressor of genes leading to differentiation. However it has been shown the Nanog could be a potent activator of gene transcription. The 10 pentapeptide repeat that begins with tryptophan and a second subdomain located C-terminal to the repeat increased transcriptional activity of reporter constructs (Pan and Pei, 2005). When the tryptophans in the first subdomain were substituted with alanines, activity was abolished. More evidence suggests that Nanog, along with other transcription factors implicated in pluripotency are all involved in extensive autoregulatory feedforward loops that regulate their own expression as well as the expression of others. Nanog can activate expression of Oct-4 (Pan et al., 2006) and Sall4 (Wu et al., 2006), a transcription factor that is expressed in the inner cell mass of mouse embryos (Yoshikawa et al., 2006), embryonic carcinoma cells (Kohlhase et al., 2002), and in the trophectoderm (Sakaki-Yumoto et al., 2006; Yoshikawa et al.,

2006). Another marker of pluripotent cells, Rex-1, is also up-regulated by Nanog in cooperation with Sox-2 (Shi et al., 2006).

Regulation of Oct-4

Typically regulation of transcription is thought of as being under an off-on type of control. To sustain self-renewal of mouse embryonic stem cells, the expression of Oct-4 must fall within a critical level. Using an Oct-4 transgene under tetracycline control, Niwa and coworkers (2000) were able to alter levels of Oct-4 expression. Relative to endogenous levels of expression, a 1.5 fold decrease resulted in dedifferentiation into trophectoderm whereas a 1.5 fold increase resulted in differentiation into primitive endoderm and mesoderm. One way that this steady-state regulation occurs is by negative feedback of Oct-4 on itself (Pan et al., 2006).

In comparing the bovine, mouse, and human Oct-4 promoters, van Eijk et al., (1999) reported the presence of a highly conserved Sp 1 binding site and an overlapping hormone responsive element. They also noted a Short Interspersed Nuclear Element (SINE) within the bovine reporter was not found in the mouse.

Regulation of Sox-2

Wiebe and coworkers (2000) described a CCAAT box -60 base pairs from the transcription start site that has a role in Sox-2 expression in undifferentiated F9 embryonal carcinoma cells. However, it was still active after cell differentiation. An enhancer in the 3'-flanking region of the Sox-2 gene has been described (Tomioka et al., 2002). Called Sox regulatory region 2 (SRR2), it contains an Octamer/Sox-2 like recognition sequence that both Oct-4/Sox-2 and Oct 6/ Sox-2 complexes can bind to and up-regulate expression. Zappone and coworkers (2000) describes a regionally restricted enhancer upstream of the Sox-2 gene that is active in mouse blastocysts but later becomes restricted to the developing telencephalon.

Gene Expression

Embryonic Development

Mouse

In the mouse, Nanog expression is first detected by northern blots in the compacted morula and is confined solely to the inner cell mass and epiblast where it is down-regulated at

the time of implantation (Chambers et al., 2003; Mitsui et al., 2003). Nanog null embryos at 3.5 dpc appeares normal but by 5.5 dpc they are disorganized with no discernible epiblast or extraembryonic ectoderm suggesting that Nanog is needed for maintenance of the epiblast (Mitsui et al., 2003). Nanog expression is absent in mouse primordial germ cells that express PGC7/Stella up to 7.5 dpc (Hatano et al., 2005; Yamaguchi et al., 2005) but by 7.75 dpc it is expressed by primordial germ cells throughout migration and population of the early gonad until female germ cells entered meiosis or the onset of mitotic arrest in male germ cells (Yamaguchi et al., 2005).

Oct-4 is present within the mouse oocyte (Rosner et al., 1990) but the maternal message is cleared by the 2-cell stage (Palmieri et al., 1994). Expression of Oct-4 occurs in the cells of the morula, the inner cell mass of the blastocyst, and the primitive ectoderm of the egg cylinder until the time of gastrulation (7.0 dpc) (Rosner et al., 1990). Rosner et al., (1990) also noted Oct-4 message in the trophectoderm at 3.5 dpc while it was gone by 4.5 dpc, but this pattern has not been observed by others (Palmieri et al., 1994). After gastrulation, Oct-4 was observed in primordial germs migrating at 10.5 dpc and populating the genital ridges (Rosner et al., 1990). Up-regulation of Oct-4 was observed in the early cells of primitive endoderm (Palmieri et al., 1994). Thus a model has been proposed that Oct-4 can direct cell differentiation to trophectoderm by down-regulation or to endoderm by up-regulation (Pesce and Scholer, 2001). Other evidence for this model is provided by loss of the inner cell mass with only trophoblast present in Oct-4 null embryos (Nichols et al., 1998), the up-regulation of trophectoderm markers Cdx2, Hand1, and PL-1 in Oct4 silenced embryonic stem cells (Hough et al., 2006), and the up-regulation of the endoderm marker gata4 in cells where Oct-4 was over expressed (Niwa et al., 2000).

Using a reporter construct, Sox-2 expression is first detected in the morula and the inner cell mass of the blastocyst and in later stages becomes confined to the neuroectoderm, the extraembryonic ectoderm or chorion, and gut endoderm (Avilion et al., 2003). Sox-2 is required for the maintenance of the epiblast and development of the extraembryonic ectoderm (Avilion et al., 2003).

Farm Animal

The expression pattern of Oct-4, Sox-2, and Nanog is incompletely described in farm animal species and appears to differ from that of the mouse where these markers are expressed in

only the undifferentiated cells of the mouse embryo. Oct-4 message was present in the inner cell mass but not in the trophoblast of d-7 in vitro-derived bovine blastocysts (Kurosaka et al., 2004) but was detected in the trophoblast of half of the bovine blastocysts derived by somatic cell nuclear transfer (Wuensch et al., 2007). In pre-implanting bovine embryos, Oct-4 message was present in both the embryonic and extraembryonic tissues but was detectable only in the embryonic tissues of filamentous embryos (Degrelle et al., 2005). They also showed by *in situ* hybridization, Oct-4 in the extraembryonic mesoderm of the elongating embryo. The Oct4 protein however, is present in both the inner cell mass and trophectoderm of bovine and porcine blastocysts (Kirchhof et al., 2000; van Eijk et al., 1999) but becomes confined to the epiblast of porcine and bovine embryos after hatching (Vejlsted et al., 2005; Vejlsted et al., 2006). By day 17 post-insemination, Oct-4 protein is generally cleared from the embryo except for the presumptive primordial germ cells in the yolk sac endoderm and allantois (Vejlsted et al., 2006).

Nanog and Sox-2 expression in farm animals has only been reported in pre-implanting bovine embryos, characterized as spherical, ovoid, and filamentous (Degrelle et al., 2005). Using semi-quantitative PCR, these investigators found both Nanog and Sox-2 are expressed in embryonic tissue at all stages and are up-regulated in the filamentous embryo. Sox-2 expression is low in the extraembryonic tissues at spherical and ovoid stages and Nanog expression increases in the extraembryonic tissues as development progresses.

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CHAPTER 2 - Cloning and Expression of Pluripotent Factors Around the Time of Gastrulation in the Porcine Conceptus

Introduction

After fertilization, the embryo will undergo a series of cleavage divisions resulting in the morula. The first appearance of cell differentiation occurs at blastocyst formation as the outer cells of the morula differentiate into trophoblast. The inner cell mass is comprised of pluripotent cells that will give rise to all three germ layers of developing embryo in the process of gastrulation. The transcription factors important in maintaining pluripotency in the inner cell mass and the ensuing epiblast include Nanog, Sox-2, and Oct-4. Nanog is a homeobox transcription factor that is first observed in the morula and becomes confined to the inner cell mass and epiblast in the mouse (Chambers et al., 2003; Mitsui et al., 2003). In the mouse, the loss of Nanog results in disorganized tissue with no discernable epiblast at day 5.5 post-coitum (Mitsui et al., 2003). Oct-4 is also expressed early in mouse development (Palmieri et al., 1994; Rosner et al., 1990) and is required for a pluripotent inner cell mass (Nichols et al., 1998). Consistent with Nanog and Oct-4, Sox-2 is also expressed in the inner cell mass and is required to maintain the epiblast (Avilion et al., 2003) but unlike the other factors it continues to be expressed in a differentiated tissue, the developing nervous system (Uchikawa et al., 1999; Uwanogho et al., 1995; Wood and Episkopou, 1999). In embryonic stem cells, these factors suppress differentiation and promote self-renewal by forming an autoregulatory and feedforward network (Boyer et al., 2005; Loh et al., 2006; Wang et al., 2006).

The expression pattern of these markers in farm animal species is not well characterized and may differ from that of the mouse. Oct-4 is observed in the trophoblast of porcine and bovine blastocysts (Kirchhof et al., 2000; van Eijk et al., 1999) and expression of these markers is observed in the extraembryonic tissues of the bovine prior to implantation (Degrelle et al., 2005). Therefore, we have partially cloned the porcine Oct-4, Nanog, and Sox-2 transcripts and characterize their expression in day 10, 12, 15, and 17 embryonic and extraembryonic tissues as well as endometrium, myometrium, placenta, and fetal liver at day 40 of pregnancy.

Material and Methods

Tissue Collection

Embryos were flushed from sows under general anesthesia 10 (n=3), 12 (n=4), 15 (n=5), and 17 (n=3) days post-insemination by exposing the reproductive tract through a mid-ventral incision. The uterine horn was clamped near the uterine body and 50 ml of 37^{o}C DMEM was injected at the utero-tubal junction. Medium was flushed through a small incision above the clamp into 100 mM Petri dishes. Day-10 and -12 embryos were processed as whole conceptuses (embryonic and extraembryonic tissues). Day-15 and -17 embryonic tissue (embryonic disk) was separated by closely trimming the adjacent extraembryonic tissues (proximal extraembryonic) with a scalpel under a stereo-microscope (5 to 50X). Additional extraembryonic tissue was collected after removal of the embryonic disks (distal extraembryonic). In addition, d-17 allantois and whole embryos (embryonic plus extraembryonic tissue still attached) were isolated. Day-40 endometrium, myometrium, placenta, and fetal liver were collected as previously described (Brown et al., 2007).

RNA and Protein Isolation

Total RNA was isolated from porcine embryonic and extraembryonic tissue at day 10, 12, 15, and 17 using the RNeasy Mini or RNeasy Micro Kits (Qiagen; Valencia, CA) according to manufacturer's instructions. Tissue was disrupted by a buffer containing guanidine thiocyanate and β-mercaptoethonal and homogenized by passing the lysate through a QIAshredder spin column (Qiagen). RNA was DNase treated by the on-column DNA digestion procedure in the kit instructions. Total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies; Wilmington, DE) and run on a 1% agarose-formaldehyde gel to assess quality. Protein from porcine subdermal skin cells, D3 mouse embryonic stem cells, d-15 extraembryonic tissue, and d-15 and -17 conceptus (embryonic and extraembryonic) were isolated by incubating cell cultures or tissues with M-PER Mammalian Protein Extraction Reagent (Pierce; Rockford, IL) for ~10 minutes and then samples were centrifuged at 13,200 *g* for 10 minutes. Supernatant was transferred to clean tube and stored at -70^{o}C.

Gene Cloning

Full-length, RNA ligase-mediated (RLM) rapid amplification of the 5' end of Nanog was performed with the FirstChoice RLM-RACE Kit (Ambion; Austin, TX) following manufacture's instructions. Briefly, total RNA was incubated at 37°C for 1 h with calf alkaline phosphatase to remove 5' phosphates from truncated mRNA and non-mRNA, and then treated with tobacco acid pyrophosphatase at 37°C for 1 h to remove the cap structure from full-length mRNA. An RNA oligonucleotide was then ligated to decapped RNA using T4 RNA ligase. The RT reaction for the 5' end was performed using the M-MLV reverse transcriptase and random decamer primers. The 3' ends of all transcripts were obtained using the GeneRacer Kit (Invitrogen, Carlsbad CA). First-strand cDNA was synthesized using SuperScript III reverse transcriptase and an Oligo dT primer creating a known priming site at the 3' end. The promoter sequence was cloned by PCR from DNA isolated from porcine whole blood.

Reaction conditions for the 5' outer (25 ul total) and inner (50 ul total) reaction were as follows; 0.5 ul first-strand cDNA (outer) or 2.0 ul outer PCR reaction (inner), 1X Amplitaq buffer with $MgCl_2$ [1.5mM], 5' RACE gene-specific outer primer and Nanog outer primer (outer) or 5' RACE gene-specific inner primer and Nanog inner primer (inner) [0.4 uM each], dNTPs [200 uM each], and 1.5 U of Amplitaq polymerase. Cycling conditions for both the initial and nested PCR of the 5' end were as follows: 3 minutes at 94°C followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec concluding with 72°C for 7 min.

5' RACE

Primer	Sequence (5' – 3')	Predicted Annealing Temp (ºC)
Nanog outer	GTCTGGTTGCTCCAGGTTG	55
Nanog inner	AGAAGCGTTCACCAGGCAT	57

3' RACE

β-actin outer	ACCACTGGCATTGTCATGGACTCT	63
Nanog outer	ATCCAGCTTGTCCCCAAAG	56
Nanog inner	AGCCTCCAGCAGATGCAAG	58

Oct-4 outer	AGGTGTTCAGCCAAACGACC	58
Oct-4 inner	GCTGCAGAAGTGGGTGGAGGAAG	65
Sox-2 outer	ACAACTCGGAGATCAGCAAGCG	62
Sox-2 inner	GCCTGGGCGCCGAGTGGA	69

Nanog Promoter

PromoterFwd	TGTGACCTTAGAGTGAACCAAAGA	57
PromoterRev	TGACATCTGCAAGGAGGCATA	58

Reaction conditions for the 3' end were similar to the 5' end conditions except primer concentrations in the outer reaction were 0.6 uM for the GeneRacer 3' primer and 0.2 uM for the gene-specific outer primer. Cycling conditions for the outer reaction were 94^{o}C for 2 minutes, 5 cycles at 94^{o}C for 30 sec and 70^{o}C for 2 minutes, 5 cycles at 94^{o}C for 30 sec and 68^{o}C for 2 minutes and then 25 cycles at 94^{o}C for 30 sec, 58^{o}C for 30 sec, and 70^{o}C for 2 minutes concluding with 70^{o}C for 10 min. The 3' nested reaction consisted of 2 minutes at 94^{o}C followed by 25 cycles at 94^{o}C for 30 sec, 64^{o}C for 30 sec, and 68^{o}C for 2 min concluding with 68^{o}C for 10 min. Cloning of the PCR products was performed by using the TOPO TA Cloning kit (Invitrogen; Carlsbad, CA). Plasmid DNA was isolated using Miniprep kit (Qiagen) and sequenced using M13 forward and reverse priming sites by DNA Sequencing Core Facility (University of Arkansas for Medical Sciences; Little Rock, AR).

Real-time PCR

One microgram of total RNA was reverse transcribed in a 50 ul reaction using TaqMan Reverse Transcription Reagents (Applied Biosystems; Foster City, CA). The reaction components included 5.0 ul RT buffer [1X], 11.0 ul $MgCl_2$ [5.5mM], 10.0 ul dNTPs [500 μM each], 2.5 ul random hexamers [2.5 μM], 1.0 ul inhibitor [20 U], and 3.2 ul Multiscribe reverse transciptase [160 U]. Cycling conditions were 25.0ºC for 10 minutes, 37.0ºC for 60 minutes and 95ºC for 5 minutes. Reactions using less the one microgram were adjusted proportionally.

Primer and probe sequences were obtained using Primer Express (Applied Biosystems) using the porcine Nanog and Sox-2 sequences and bovine Oct-4 sequence. Primers were tested before using the TaqMan probe in real-time PCR assays. cDNA was serially diluted 10 fold from 1:1 to 1:10000 and real-time PCR was performed at each dilution using 1X SYBR Green PCR Master Mix (Applied Biosystems) and 300 uM forward and reverse primers in a 20ul reaction. Melting curves were used to confirm the synthesis of a single PCR product. Threshold values for each dilution point were plotted to calculate the slope. Primer efficiency was estimated by efficiency = $10^{(-1/m)}$-1where m = slope. Primer efficiencies ranged from ~90-110%.

For real-time PCR reactions, 1 ul of cDNA was added to the following; TaqMan Universal PCR Master Mix [1X], fwd primer [900 nM], reverse primer [900 nM], TaqMan TAMRA probe [250 nM] in a 20 ul reaction. 18s ribosomal RNA was used as the normalization control using TaqMan Ribosomal RNA Control Reagents (Applied Biosystems). Cycling conditions were 50.0ºC for 2 min, 95.0ºC for 10 minutes, and 40 cycles of 95.0ºC for 15 sec and 60ºC for 1 min. The GLM procedure of SAS (SAS Institute Inc.; Cary, NC) was used to analyze threshold values adjusted for 18s with tissue as the fixed effect.

Primer	Sequence (5' – 3')	Position[a]	Predicted Annealing Temp (ºC)	Predicted Size (bp)
Nanog fwd	CCCGGGCTTCTATTCCTACCA	715	61	68
Nanog rev	TACCCCACACGGGCAGGTT	782	62	
Nanog Probe	CAAGGATGCCTGGTGAACGCTTCTG	737	68	
Oct-4 fwd	GCAAGGCAGAGACCCTTGTG	31	59	68
Oct-4 rev	GCCTCTCACTCGGTTCTCGAT	98	59	
Oct-4 probe	AGGCCCGAAAGAGAAAGCGGACG	52	69	
Sox-2 fwd	TTCCATGGGCTCAGTGGTCAA	493	62	
Sox-2 rev	TGGAGTGGGAAGAAGAGGTAAC	563	56	
Sox-2 probe	TCCGAGGCGAGCTCCAGCCC	515	70	71

[a]Position based on clone sequences. See Figures 1, 5, and 6

Northern Blotting

10-20 ug of total RNA was separated in a 1% denaturing agarose/formaldehyde gel for 4 hours at 50 volts. Two micrograms of RNA Millennium Markers (Ambion) was used as RNA standards. The gel was rinsed 4X in distilled H_20 before alkaline transfer to Nytran SuPer Charge nylon membrane using the Turboblotter Rapid Downward Transfer System and blotter pack following manufacture's instructions (Schleicher & Schuell, Keene, N.H.). Transfer buffer consisted of 3 M NaCl and 8 mM NaOH. Transfer was allowed to occur for 4 hours and then membrane was neutralized for 5 min. in neutralization buffer (1 M phosphate, pH 6.8). Membranes were wrapped in clear plastic wrap and exposed to UV light for 30 seconds to covalently bind RNA to membrane.

Probe labeling and detection of RNA was done through the Amersham Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare, Piscataway, NJ) according to manufacturers instructions. Probes were synthesized by PCR using plasmid DNA generated from the cloning experiments as the template. Bands were cut from the gel and isolated from the agarose gel by the MinElute Gel Extraction Kit (Qiagen). Primer sequences were as follows:

Primer	Sequence (5' – 3')	Predicted Annealing Temp (ºC)	Predicted Size (bp)
Nanog fwd	TCCAGCTTGTCCCCAAAGC	59	556
Nanog rev	AGAAGCGTTCACCAGGCAT	57	
Oct-4 fwd	GCTGCAGAAGTGGGTGGAGGAAG	65	414
Oct-4 rev	TCAGGGAAAGGCACCGAGGAGTA	64	
Sox-2 fwd	GCCTGGGCGCCGAGTGGA	69	563
Sox-2 rev	TGGAGTGGGAGGAAGAGGTAAC	69	
β-actin fwd	ACCACTGGCATTGTCATGGACTCT	63	545
β-actin rev	ATCTTGATCTTCATGGTGCTGGGC	64	

To label probes, DNA (10 ng/uL) was first denatured by placing in a vigorously boiling water bath for 5 minutes and then cooled 5 minutes on ice. Reaction buffer, labeling reagent, and cross-linker working solution were mixed with the DNA and incubated at 37ºC for 30 minutes. Blots were placed in pre-warmed hybridization buffer containing NaCl [0.5 M] and blocking reagent [4% w/v] for 15 minutes at 55ºC. Labeled probe (200 ng total) was added at 20 ml of buffer and left to hybridize overnight. Blots were washed 2X in a primary washing buffer (2M Urea, SDS (0.1% w/v), 50 mM NaH_2PO_4 (pH 7.0) 150 mM NaCl, 1 mM $MgCl_2$, and blocking reagent (0.2% w/v)) at 55ºC for 10 minutes. Blots were then washed 2X in a secondary wash buffer (in mM; 50 Tris, 100 NaCl, and 2 $MgCl_2$) at room temperature for 5 minutes. Two mL of CDP-Star detection reagent was placed on the blot for 5 minutes and excess was drained before blot was wrapped in clear plastic wrap. Blots were exposed to Classic Blue Autoradiography Film BX (MIDSCI, St. Louis, MO) initially for 15 min-1 hour and then a second film was exposed overnight. Blots were stripped in 0.5% (w/v) SDS at 60ºC for 60 minutes and rinsed for 5 minutes in 100 mM Tris (pH 8.0) at room temperature.

Western Blotting

Approximately 20 ug of protein in Laemmli Sample buffer was loaded onto a 12% Tris-HCl pre-cast gel (Bio-Rad) and run at 200V for 45 minutes. Prestained kaleidoscope and biotinylated SDS-PAGE broad range were included as standards. Protein was transferred to Immun-Blot PVDF membrane in a 1x Tris/Glycine buffer (25 mM Tris, 192 mM Glycine, 20% Methanol (v/v), pH 8.3) for 1 hour at 100 volts. The Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) was used for detection of protein following manufacturer's instructions. Briefly, membranes were blocked for 30 minutes in a 1x casein solution which was used in all remaining steps and washes. Membranes were incubated with primary antibody for 30-60 minutes. Primary antibodies and dilutions were as follows: rabbit anti-Nanog (Chemicon International; Temecula, CA), goat anti-Oct3/4 (Santa Cruz Biotechnology; Santa Cruz, CA), and goat anti-Sox-2 (R&D Systems, Inc; Minneapolis, MN) at 1:2500, 1:200, and 1:1000 respectively. After 3-4 washes, membranes were incubated with biotinylated secondary antibody (1:200) for 30 minutes with gentle agitation, followed by incubation in Vectastain ABC reagent for 30 minutes. The TMB Substrate Kit for Peroxidase (Vector Laboratories, Inc) was used for staining.

Results

Cloning

A putative full length cDNA transcript of Nanog was synthesized comprised of 1181 bp which encodes a protein of 304 amino acids (see Figure 1). The homeodomain is comprised of 58 amino acids (position 97-154) and c-terminal to the homeodomain is a tryptophan at every fifth position repeated nine times. Comparison of the amino acid sequence with sequences of other species (see Figure 2) revealed 75% identity with the human (Genbank NP_079141), 81% with the bovine (Genbank NP_001020515), 83% with the caprine (Genbank AY786437), and < 60% in the mouse (Genbank NP_082292). Similarity within the homeodomain is high in all species including 89% within the mouse, 98% in the bovine and caprine, and 94% with the human protein

A 769 bp product was cloned through PCR which included 453 bp upstream of the transcription start site and 316 bp of coding sequence (see Figure 3). The Octamer/Sox element was identified at position -149 relative to the start site. The Octamer sequence (5'-CTTTGCAT-3') differs slightly from the consensus sequence by either a T deletion or C addition at the first position. The Sox sequence is conserved. A putative binding site for FoxD3 is also found at position -259. Comparison of the non-coding region with the homologous regions of the bovine and human resulted in 70 and 71% identity in 419 and 328 bp respectively. Comparing those sequences with the mouse resulted in ~75% identity but only in a 94 bp region (see Figure 4).

The Oct-4 sequence included 452 bp of coding sequence which resulted in 149 amino acids of the protein and included the 57 amino acid POU homeodomain (see Figure 5). Overall sequence similarity with the bovine, human, and mouse Oct-4 proteins were 96, 94, and 86% respectively and identity within the homeodomain was 100, 96, and 88% respectively. Porcine Oct-4 is similar to mouse Oct-4 in that 13 of the 71 amino acids C-terminal to the homeodomain are prolines (Okamoto et al., 1990). Results when blasted against the porcine genome resulted in 2 matches on chromosome 7. The first was 164 base pairs with 100% identity to the genomic sequence. The second match was separated by 98 base pairs and resulted in a 99.6% match. We hypothesize that this corresponds to exon 4 and 5. A thymidine at base pair 87 was changed in our cloned sequence to a cytosine based on comparison with the genomic sequence. This resulted in a stop codon being translated to an arginine.

Two products differing by 57 bp in length at the 3' end were cloned for Sox-2. Combining the longer sequence with an earlier cloned sequence (Genbank: DQ159208) an 860 bp product was generated. The coding sequence is 755 bp in length and results in 250 amino acids (see Figure 6). This includes a partial sequence of the HMG binding domain. This protein is highly conserved; identity between mouse, human, and bovine protein is greater than 98%.

Real-time PCR

Expression of Nanog, Oct-4, and Sox-2 were measured in d-10 and -12 whole conceptus, embryonic and extraembryonic tissues at d-15 and -17, d-40 fetal liver, and placental, endometrial, and myometrial tissues recovered from d-40 of pregnancy. Adjusted threshold means and standard errors are reported in Table 1.

Expression of Nanog was lowest for all extraembryonic tissues at day 15 and 17 (see Figure 7). Nanog expression levels were similar in the allantois, d-10 and -12 conceptus, and d-40 endometrium. Expression increased 1.5 fold in the d-15 and -17 disk, however this was not significant. The highest expression of Nanog was surprisingly observed in the d-40 tissues fetal liver, placenta, and myometrium which were 27, 39, and 72 fold higher than extraembryonic tissue expression respectively.

Oct-4 expression (see Figure 8) was low in all d-40 tissues except fetal liver where expression was approximately 26 fold higher than the other d-40 tissues. Expression was highest in d-10 and -12 conceptuses, and d-15 disk but decreased 3.5 fold by d-17. Higher expression (4-5 fold) was also observed in the proximal extraembryonic tissue compared to the distal extraembryonic tissue but may also be declining between day 15 and day 17 (1.5 fold). Expression in the allantois was 10 fold higher than compared to myometrium

Sox-2 expression (Figure 9) was significantly up-regulated by 45 fold in the d-15 disk and 80 fold in the d-17 disk when compared to the d-12 conceptus. Expression in the fetal liver was also high; 70 fold higher when compared to myometrium and endometrium expression which showed the lowest expression. Allantois also had higher levels of Oct-4 expression (14 fold) when compared to the endometrium.

Northern Blots

Total RNA (10 ug) from d-12 conceptus, and d-15 disk, distal and proximal extraembryonic tissues were blotted and probed for Sox-2. A band of approximately 2300 base

pairs in length was detected for embryonic disk (see Figure 10). The membrane was stripped and reprobed for Oct-4 but no bands were detected. Tissues having the highest Nanog expression and 20 ug of total RNA, from d-17 disk, allantois, and d-12 conceptus were used to generate a new blot for Nanog. Even after ~18 hours exposure no bands could be visualized. Both blots were then probed for β-actin and bands of ~1750 and 1550 bp were observed (see Figure 11). The smaller bands were observed in the d-15 extraembryonic tissues and are likely a transcript variant. According to the description of Human β-actin (GeneID 60), the gene encodes 6 different highly conserved proteins. Based on these results we conclude that our current detection protocol is not sensitive enough to detect Oct-4 and Nanog.

Western Blots

Nanog protein was detected in porcine fibroblasts, mouse D3 cells, and d-15 conceptus and extraembryonic tissue as a 50 kDa band (see Figure 12). A second band of 23 kDa was observed in the porcine samples and a third band was observed at 63 kDa in the d-15 samples.

Discussion

Sequencing of the porcine genes resulted in the complete coding sequence for Nanog and partial sequences for Sox-2 and Oct-4. Nanog appears to be the least conserved when compared to other species including the bovine, human, and mouse as overall identity with the porcine protein ranges from 83% to < 60%. The low identity with the mouse is similar to the less than 60% identity between human and mouse (Chambers et al., 2003; Hart et al., 2004). However the identity within the homeobox is high (> 89%) and the tryptophan repeat motif of five amino acids (WXXXX) is conserved. The tryptophan repeats nine times as it does in the human but maintains the tryptophans in each repeat whereas in the human a tryptophan is substituted with a glutamine in the forth repeat. The repeat forms a subdomain that has transcriptional activation properties that become lost when alanines are substituted for the tryptophans (Pan and Pei, 2005). The Nanog nucleotide sequence was blasted in the porcine genome and resulted in one match on chromosome 1. We hypothesize that this may be a pseudogene because porcine Nanog has been mapped to chromosome 5 (Yang et al., 2004) and that the matched sequence was intronless, similar to the mouse pseudogene described by (Hatano et al., 2005). The porcine chromosome 5 sequence has not yet been made available. The 50 kDa band for Nanog was larger than the expected (37 kDa) but is consistent with other results for porcine Nanog including the

23 kDa band (Kei Miyamoto; Kyoto University, Japan; personal communication). Smaller bands are seen in mouse Nanog (Hatano et al., 2005; Wu et al., 2006) and it is hypothesized that Nanog is easily degraded.

Conserved within the porcine Nanog promoter are the Octamer/Sox element and a putative FoxD3 binding site. The element is invariant among 5 species (Rodda et al., 2005) and can up-regulate Nanog expression by Oct-4 and Sox-2 binding (Kuroda et al., 2005; Rodda et al., 2005). The potential effect of a single base change observed in the porcine octamer sequence is unknown but could be addressed by targeted mutagenesis.

Of the three transcription factors, Sox-2 expression in the pig is the most consistent with the expression pattern seen in mouse development. Sox-2 is necessary for the maintenance of the epiblast (Avilion et al., 2003) and is expressed during neural development in the embryonic disk (Uchikawa et al., 1999; Uwanogho et al., 1995; Wood and Episkopou, 1999). The up-regulation in the d-15 disk is concurrent with the formation of the neural tube in the pig (Vejlsted et al., 2006) and similar up-regulation is observed in the elongated bovine embryo (Degrelle et al., 2005). The high expression of Oct-4 in d-10 and -12 conceptuses and d-15 embryonic disk is consistent with its role in maintaining pluripotency of the inner cell mass (Nichols et al., 1998) but down-regulation at d-17 suggests that those cells are undergoing differentiation and that gastrulation is occurring. The presence of Oct-4 message and protein in the extraembryonic tissues of farm animals (Degrelle et al., 2005; Kirchhof et al., 2000; van Eijk et al., 1999) remains to be clarified. The greater expression seen in the proximal extraembryonic tissue as opposed to the distal extraembryonic tissue may be due to a population of trophoblast stem cells that delays commitment to differentiate until elongation (Degrelle et al., 2005) and promotes trophoblast proliferation through Oct-4-directed secretion of FGF4 (Nichols et al., 1998; Tanaka et al., 1998).

The expression of Nanog early in development is also consistent with maintaining pluripotent stem cells in the mouse epiblast (Chambers et al., 2003; Mitsui et al., 2003) but the dramatic up-regulation in d-40 tissues was unexpected. It may be relevant that Nanog expression in porcine umbilical cord matrix cells was similar to d-15 embryonic disk while Oct-4 and Sox-2 expression were reduced (Carlin et al., 2006). Nanog is thought to be a repressor of endoderm (Hough et al., 2006; Mitsui et al., 2003) and mesoderm differentiation (Suzuki et al., 2006) and an activator of pluripotent markers such as Sall4 (Wu et al., 2006), Rex1 (Shi et al., 2006), and

Oct-4 (Pan et al., 2006). However, in a pattern like Sox-2, Nanog may continue to be expressed in a differentiated tissues and this warrants further study to determine it's role and how it is being regulated.

Relatively high expression of all three transcription factors in the d-40 liver suggests a population of more primitive type cells. Progenitors of red blood cells from the yolk sac and hematopoietic stem cells from the embryo populate the fetal liver where blood cell formation occurs until near the end of gestation when it will moves to it's final location in the bone marrow (McGrath and Palis, 2008). Fetal tissues may have some expression of these factors based on the number of reports of multipotent progenitor cells in adult tissues. From human adult liver, Oct-4 and Nanog expressing multipotent adult stem cells have been isolated (Beltrami et al., 2007). Oct-4 has been reported in many adult tissues (Cervello et al., 2007; Matthai et al., 2006; Tai et al., 2005) as well a Nanog (Hart et al., 2004). Stem cells in adult tissues serve to replace dying cells or regenerate damaged tissue and these cells may have a role in tissue generation in the developing embryo.

Nanog regulation has been characterized in mouse pluripotent stem cells (Kuroda et al., 2005; Pan et al., 2006; Rodda et al., 2005; Wu et al., 2006) but to better understand Nanog expression in tissues such as d-40 myometrium or placenta, we plan to further characterize regulatory elements and transcription factors involved in Nanog expression using porcine umbilical cord matrix cells. This would be the first report describing Nanog regulation in cells other than the mouse from a non-embryonic source. The long-term goal is to create reporter constructs that can be tested *in vivo* using embryos generated through somatic cell nuclear transfer.

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Figures and Tables

Figure 1 Porcine Nanog Nucleotide and Amino Acid Sequence.

Overall transcript length is 1181 base pairs encoding a 304 amino acid protein. Start and stop codons at nucleotide positions 194 and 1106 respectively. Homeodomain and tryptophan repeats begin at amino acid positions 97 and 195 respectively and are shaded.

```
   1 ATTTTGCTAG ATTGGGGTGG TTAGCTCCTG TTCTCGTAAG GGTGACTCAC TTCATCCCAT TTTTTGATAC TTTTAACACC
  81 TGGAGGAGAT CTTCATGATT CTAAGCTCTT CTATCTAGAC ACTTAAGCCT GGACTTTTCC TACAATCCAG CTCTTTGGTG
   1                                    M  S   V  D  P  A   C  P  Q   S  L  L   C  P  E  A
 161 GTTTTTTTTT TTTTCCCCCT TCTTCAACTC AACATGAGTG TGGATCCAGC TTGTCCCCAA AGCCTGCTTT GCCCCGAAGC
  17   S  I  S   S  E  S   S  P  M  P   E  V  Y   G  P  E   E  N  Y  A   S  L  Q   M  S  S
 241 ATCCATTTCC AGCGAATCTT CACCAATGCC TGAGGTTTAT GGGCCTGAAG AAAATTATGC CTCCTTGCAG ATGTCATCTG
  43 A  E  T  L   D  T  E   T  V  S   P  L  P  S   S  M  D   L  L  I   Q  D  S  P   D  S  S
 321 CTGAGACCCT CGACACCGAG ACTGTCTCTC CTCTTCCTTC CTCCATGGAT CTGCTTATTC AGGACAGCCC TGATTCTTCC
  70  T  S  P   R  V  K  P   L  P  T   S  A  E   K  S  T  E   K  E  E   K  V  P   V  K  K  Q
 400 ACAAGCCCCA GAGTAAAACC ACTGCCCACA TCTGCAGAGA AGAGCACAGA GAAGGAGGAA AAGGTCCCAG TCAAGAAGCA
  97   K  I  R   T  V  F   S  Q  T  Q   L  C  V   L  N  D   R  F  Q  R   Q  K  Y   L  S  L
 481 GAAGATCAGA ACTGTGTTCT CGCAGACCCA GCTCTGTGTC CTCAACGACA GATTTCAGAG GCAGAAGTAC CTCAGCCTCC
 123 Q  Q  M  Q   E  L  S   N  I  L   N  L  S  Y   K  Q  V   K  T  W   F  Q  N  Q   R  M  K
 561 AGCAGATGCA AGAACTTTCC AACATCCTGA ACCTTAGCTA CAAACAGGTT AAAACCTGGT TCCAGAACCA GCGAATGAAA
 150  C  K  R   W  Q  K  N   H  W  P   R  N  S   N  S  V  I   Q  G  S   A  S  T   E  Y  P  G
 641 TGTAAGAGGT GGCAGAAAAA CCACTGGCCA AGGAATAGCA ACAGTGTGAT TCAGGGCTCA GCCAGTACAG AATACCCGGG
 177   F  Y  S   Y  H  Q   G  C  L  V   N  A  S   G  N  L   P  V  W  G   N  Q  S   W  S  N
 721 CTTCTATTCC TACCACCAAG GATGCCTGGT GAACGCTTCT GGAAACCTGC CCGTGTGGGG TAATCAGAGC TGGAGTAACC
 203 P  T  W  S   N  Q  T   W  N  S   Q  S  W  S   N  Q  T   W  N  S   Q  T  W  C   P  Q  A
 801 CAACCTGGAG CAACCAGACC TGGAACAGCC AGTCTTGGAG CAACCAAACC TGGAACAGCC AGACCTGGTG CCCCCAAGCC
 230  W  N  N   Q  T  W  N   S  Q  L   N  N  Y   V  E  E  F   L  Q  P   Q  L  Q   F  Q  Q  N
 881 TGGAATAACC AGACTTGGAA TAGCCAGCTC AACAACTATG TTGAGGAATT CCTGCAGCCC CAGCTCCAGT TTCAGCAAAA
 257   S  I  S   D  L  E   A  V  L  E   T  A  G   E  N  H   N  V  I  Q   Q  T  S   K  Y  C
 961 TTCTATCAGT GATTTGGAGG CCGTCTTGGA AACTGCTGGG GAAAATCATA ATGTAATACA GCAGACTTCA AAGTACTGCG
 283 G  T  Q  Q   Q  I  M   D  L  F   P  N  Y  S   M  N  I   Q  P  E   D  M  &
1041 GTACCCAGCA GCAAATCATG GATTTATTCC CAAATTACTC CATGAACATA CAGCCTGAAG ATATGTGACG ATCATTTTAT
1121 TTTTTTAAAA AATTTTATTG GAATATAGTT GATTTACAAA AAAAAAAAAA AAAAAAAAAA A
```

Figure 2 Porcine Nanog amino acid alignment with the Human, Bovine and Mouse proteins.

Upper case letters represent complete consensus (4/4), lower case represent partial (3/4). Homedomain and tryptophan repeats are highlighted in the consensus sequence.

```
Porcine    1 MSVDPACPQS LLCPE-ASIS SESSPMPEVY GPEENYASLQ MSSAETLDTE TVSPLPSSMD LLIQDSPDSS TSPRVKPLPT
Human      1 MSVDPACPQS LPCFE-ASDC KESSPMPVIC GPEENYPSLQ MSSAEMPHTE TVSPLPSSMD LLIQDSPDSS TSPKGKQPTS
Bovine     1 MSVGPACPQS LLGPE-ASNS RESSPMPE-- ---ESYVSLQ TSSADTLDTD TVSPLPSSMD LLIQDSPDSS TSPRVKPLSP
Mouse      1 MSVGLPGPHS LPSSEEASNS GNASSMPAVF HP-ENYSCLQ GSATEMLCTE AASPRPSSED LPLQGSPDSS TSPKQKLSSP
Consensus    MSV pacPqS L   E AS s  esSpMP       EnY sLQ  Ssae l Te tvSPlPSSmD LliQdSPDSS TSP  K

Porcine   80 SAEKSTEKEE –KVPVKKQKI RTVFSQTQLC VLNDRFQRQK YLSLQQMQEL SNILNLSYKQ VKTWFQNQRM KCKRWQKNHW
Human     80 AEKSVAKKED –KVPVKKQKT RTVFSSTQLC VLNDRFQRQK YLSLQQMQEL SNILNLSYKQ VKTWFQNQRM KSKRWQKNNW
Bovine    75 SVEESTEKEE –TVPVKKQKI RTVFSQTQLC VLNDRFQRQK YLSLQQMQEL SNILNLSYKQ VKTWFQNQRM KCKKWQKNNW
Mouse     80 EADKGPEEEE NKVLARKQKM RTVFSQAQLC ALKDRFQKQK YLSLQQMQEL SSILNLSYKQ VKTWFQNQRM KCKRWQKNQW
Consensus          ekEe  kVpvkKQK  RTVFSqtQLC vLnDRFQrQK YLSLQQMQEL SnILNLSYKQ VKTWFQNQRM KcKrWQKN W

Porcine  159 PRNSNSVIQ- GSASTEYPGF YS–YHQGCLV NASGNLPVWG NQSWSNPTWS NQTW-----N SQSWSNQTWN SQTWCPQAWN
Human    159 PKNSNGVTQ- KASAPTYPSL YSSYHQGCLV NPTGNLPMWS NQTWNNSTWS NQTQ-----N IQSWSNHSWN TQTWCTQSWN
Bovine   154 PRNSNGMPQ- GPAMAEYPGF YS-YHQGCLV NSPGNLPMWG NQTWNNPTWS NQSW-----N SQSWSNHSWN SQAWCPQAWN
Mouse    160 LKTSNGLIQK GSAPVEYPSI HCSYPQGYLV NASGSLSMWG SQTWTNPTWS SQTWTNPTWN NQTWTNPTWS SQAWTAQSWN
Consensus    p nSNg  Q  g a  eYP   ys YhQGcLV N  GnLpmWg nQtW NpTWS nQtw     N  QsWsN  Wn Sq Wc Q WN

Porcine  238 NQTWNS-QLN NYVEEFLQPQ LQFQQNS-IS DLEAVLETAG ENHNVIQQTS KYCGTQQQIM DLFPNYSMNI QPEDM
Human    238 NQAWNS-PFY NCGEESLQSC MQFQPNSPAS DLEAALEAAG EGLNVIQQTT RYFSTPQT-M DLFLNYSMNM QPEDV
Bovine   233 NQPWNN-QFN NYMEEFLQPG IQLQQNSPVC DLEATLGTAG ENYNVIQQTV KYFNSQQQIT DLFPNYPLNI QPEDL
Mouse    240 GQPWNAAPLH NFGEDFLQPY VQLQQNFSAS DLEVNLEATR ESH------- AHFSTPQA-L ELFLNYSVTP PGEI
Consensus    nQ WN      N  EefLQp   Q QqNs  s DLEa Le ag E  nviqqt   yf t Q    dLF NYs n  qpEd
```

Figure 3 Porcine Nanog Promoter

Numbers at left are position relative to transcription start site and at right are overall. A putative ES specific enhancer and Oct/Sox element are highligted at position -259 and at -148. Transcription start site (+1) and start codon (+194) are also highlighted.

```
-453 GAAAAATGGA GCTAACATGT TTCTGCAGAA TAAGCCTGAA CTGGAGACCC AAAGGAGTCT 60
-393 CAGGTCAAGA AATTCATTGT CCCAGCGGGA GTTTCAGTCA CCGGAAATAG CCTCAGGAAC 120
-333 TGGAGGTGCA TCTTCCATTT GATCTGATTT TTTTTTTTTT TAATTTTTAA AAAAATTTTT 180
-273 TGCATCTTTG ATTTTAAAAA GTGGAAACAC GGTGGACCTG CAAGTAGTTC ACTGCGGGGT 240
-213 TTATTTTGTT TCCAGGTTCC ATGGTCCCAG TTCCCCACCC AGTCTGGGTT ACTCAGCAGC 300
-153 CCTCTCTTTG CATTACAATG GCCTTGGTGA GGCTGGCAGA CGGGATTAAC TGGGAATTCG 360
 -93 CAAGGGTGTG TGTGGGCGTG GGGCTGCCAG GAGGGGCGGG CTTAAGTATG GTCGATCCTT 420
 -33 CCTTATAAAT CTAGAGCCTC CAAAATTTTT CTCATTTTGC TAGATTGGGG TGGTTAGCTC 480
  28 CTGTTCTCGT AAGGGTGACT CACTTCATCC CATTTTTTGA TACTTTTAAC ACCTGGAGGA 540
  88 GATCTTCATG ATTCTAAGCT CTTCTATCTA GACACTTAAG CCTGGACTTT TCCTACAATC 600
 148 CAGCTCTTTG GTGGTTTTTT TTTTTTTCCC CCTTCTTCAA CTCAACATGA GTGTGGATCC 660
 208 AGCTTGTCCC CAAAGCCTGC TTTGCCCCGA AGCATCCATT TCCAGCGAAT CTTCACCAAT 720
 268 GCCTGAGGTT TATGGGCCTG AAGAAAATTA TGCCTCCTTG CAGATGTCA             769
```

Figure 4 Porcine Nanog Sequence Alignments with Bovine, Human and Mouse

A region of high sequence similarity as described by Rodda et al., (2005). The Oct/Sox element is conserved except for the first base pair in the porcine. M1 is also involved in Nanog regulation but is uncharacterized.

Figure 5 Partial Porcine Oct-4 Sequence

725 bp of the porcine Oct-4 coding sequence including 425 bp of coding sequence. Highlighted are the 59 amino acid POU homeodomain and the stop codon.

```
  1    D  N  N   E  N  L   Q  E  I   C  K  A  E   T  L  V   Q  A  R
  1 CTGACAACAA CGAGAATCTG CAGGAGATAT GCAAGGCAGA GACCCTCGTG CAGGCCCGGA
 20 K  R  K  R   T  S  I   E  N  R   V  R  G  N   L  E  S   M  F  L
 61 AGAGAAAGCG GACAAGTATC GAGAACCGAG TGAGAGGCAA CCTGGAGAGC ATGTTCCTGC
 40 Q  C  P  K   P  T  L   Q  Q  I   S  H  I  A   Q  Q  L   G  L  E
121 AGTGCCCAAA GCCCACTCTG CAGCAGATCA GCCACATCGC CCAGCAGCTC GGGCTAGAGA
 60 K  D  V  V   R  V  W   F  C  N   R  R  Q  K   G  K  R   S  S  S
181 AGGATGTGGT CCGCGTGTGG TTCTGCAACC GTCGCCAGAA GGGCAAACGA TCAAGCAGTG
 80 D  Y  S  Q   R  E  D   F  E  A   A  G  S  P   F  P  G   G  P  V
241 ACTATTCGCA ACGAGAGGAT TTTGAGGCTG CTGGGTCTCC TTTCCCAGGG GGACCAGTAT
100 S  F  P  L   A  P  G   P  H  F   G  T  P  G   Y  G  G   P  H  F
301 CCTTTCCTCT GGCGCCAGGG CCCCATTTTG GTACCCCAGG CTATGGGGGC CCTCACTTCA
120 T  T  L  Y   S  S  V   P  F  P   E  G  E  A   F  P  S   V  S  V
361 CCACCCTGTA CTCCTCGGTC CCATTCCCTG AGGGTGAGGC CTTTCCCTCG GTGTCTGTCA
140 T  P  L  G   S  P  M   H  S  N   &
421 CCCCTCTGGG CTCCCCCATG CATTCAAACT GAGGTGCCTG CCCTTCCCAG GAGTGGGGGG
481 GGTGAGGAAG GGGTGAGCTA GGGAGAGAAG CCTGGGGTTT GTACCAGGGC TTTGGGATTA
541 AGTTCTTCAT TCACTAAGAA AGGAATTGGG AACACAAAGG GTGTGGGGGC AGGGAGTCTA
600 GGGGAACTGG TTGGAGGGAA GGTGAAGTTC AATGATGCTC TTGATTTTAA TCCCCACATC
661 ACTCATCACT TTGTTCTTAA ATAAAGAAGC CTGGGACCCA GAAAAAAAAA AAAAAAAAAA
721 AAAAA
```

Figure 6 Partial Porcine Sox-2 Sequence

861 bp of Sox-2 sequence including 733 bp of coding sequence. Highlighted are 37 residues of the HMG binding domain and stop codon.

```
  1   L  G  A   E  W  K   L  L  S  E   T  E  K   R  P  F   I  D  E  A
  1 GCTGGGCGCC GAGTGGAAAC TTTTGTCGGA GACGGAGAAG CGGCCGTTCA TCGACGAGGC
 21   K  R  L   R  A  L   H  M  K  E   H  P  D   Y  K  Y   R  P  R  R
 61 CAAGCGGCTG CGAGCGCTGC ACATGAAGGA GCACCCGGAT TATAAATACC GGCCCCGGCG
 41   K  T  K   T  L  M   K  K  D  K   Y  T  L   P  G  G   L  L  A  P
121 GAAAACCAAG ACGCTCATGA AGAAGGATAA GTACACACTG CCCGGAGGGC TGCTGGCCCC
 61   G  G  N   S  M  A   S  G  V  G   V  G  A   G  L  G   A  G  V  N
181 GGGAGGCAAC AGCATGGCGA GCGGGGTCGG GGTGGGCGCT GGCCTCGGCG CGGGCGTGAA
 81   Q  R  M   D  S  Y   A  H  M  N   G  W  S   N  G  S   Y  S  M  M
241 CCAGCGCATG GACAGCTACG CGCACATGAA TGGCTGGAGC AACGGCAGCT ACAGCATGAT
101   Q  D  Q   L  G  Y   P  Q  H  P   G  L  N   A  H  S   A  A  Q  M
301 GCAGGACCAG CTGGGCTATC CGCAGCACCC GGGCCTCAAT GCGCACAGCG CGGCTCAGAT
121   Q  P  M   H  R  Y   D  V  S  A   L  Q  Y   N  S  M   T  S  S  Q
361 GCAGCCCATG CACCGCTACG ACGTGAGCGC CCTGCAGTAC AACTCCATGA CCAGCTCGCA
141   T  Y  M   N  G  S   P  T  Y  S   M  S  Y   S  Q  Q   G  T  P  G
421 GACCTACATG AACGGCTCGC CCACCTACAG CATGTCCTAC TCGCAGCAGG GCACCCCTGG
161   M  A  L   G  S  M   G  S  V  V   K  S  E   A  S  S   S  P  P  V
481 CATGGCGCTC GGTTCCATGG GCTCAGTGGT CAAGTCCGAG GCGAGCTCCA GCCCCCCCGT
181   V  T  S   S  S  H   S  R  A  P   C  Q  A   G  D  L   R  D  M  I
541 GGTTACCTCT TCTTCCCACT CCAGGGCGCC CTGCCAGGCC GGGGACCTAC GGGACATGAT
201   S  M  Y   L  P  G   A  E  V  P   E  P  A   A  P  S   R  L  H  M
601 CAGCATGTAC CTCCCCGGCG CTGAGGTGCC AGAGCCCGCC GCCCCCAGCA GACTTCACAT
221   S  Q  H   Y  Q  S   G  P  V  P   G  T  A   I  N  G   T  L  P  L
661 GTCCCAGCAC TACCAGAGCG GCCCGGTGCC CGGCACGGCC ATCAACGGTA CACTGCCTCT
241   S  H  M   &
721 CTCTCACATG TGAGGGCCGG ACAGTGAACT GGAGGGGGCG GGGGGAGAAA ATTTTCAAAG
781 AAAAAGAGGG AAATGGGAGG AGAGTAAGAA ACAGTATGGA GAAAAACCCG GTACGCTTAA
841 AAAAAAAAAA AAAAAAAAAA A
```

Table 1 Adjusted Threshold Means for Nanog, Sox-2, and Oct-4 by Tissue.

Tissue	Sample #	Gene	Mean[a]	Std. Error
D10 Conceptus	3	Nanog	21.59	0.74
		Oct-4	21.05	0.74
		Sox-2	21.20	0.87
D12 Conceputs	4	Nanog	21.76	0.64
		Oct-4	21.27	0.64
		Sox-2	21.61	0.75
D15 Disk	5	Nanog	20.94	0.57
		Oct-4	20.95	0.57
		Sox-2	15.92	0.67
D15 Distal	5	Nanog	24.56	0.57
		Oct-4	23.90	0.57
		Sox-2	25.57	0.67
D15 Proximal	3	Nanog	22.58	0.74
		Oct-4	21.31	0.74
		Sox-2	23.17	0.87
D17 Allantois	3	Nanog	21.75	0.74
		Oct-4	23.54	0.74
		Sox-2	22.27	0.87
D17 Disk	3	Nanog	21.08	0.74
		Oct-4	22.84	0.74
		Sox-2	15.09	0.87
D17 Distal	3	Nanog	24.21	0.74
		Oct-4	23.83	0.74
		Sox-2	24.08	0.87
D17 Proximal	2	Nanog	22.86	0.90
		Oct-4	21.87	0.91
		Sox-2	25.58	1.06

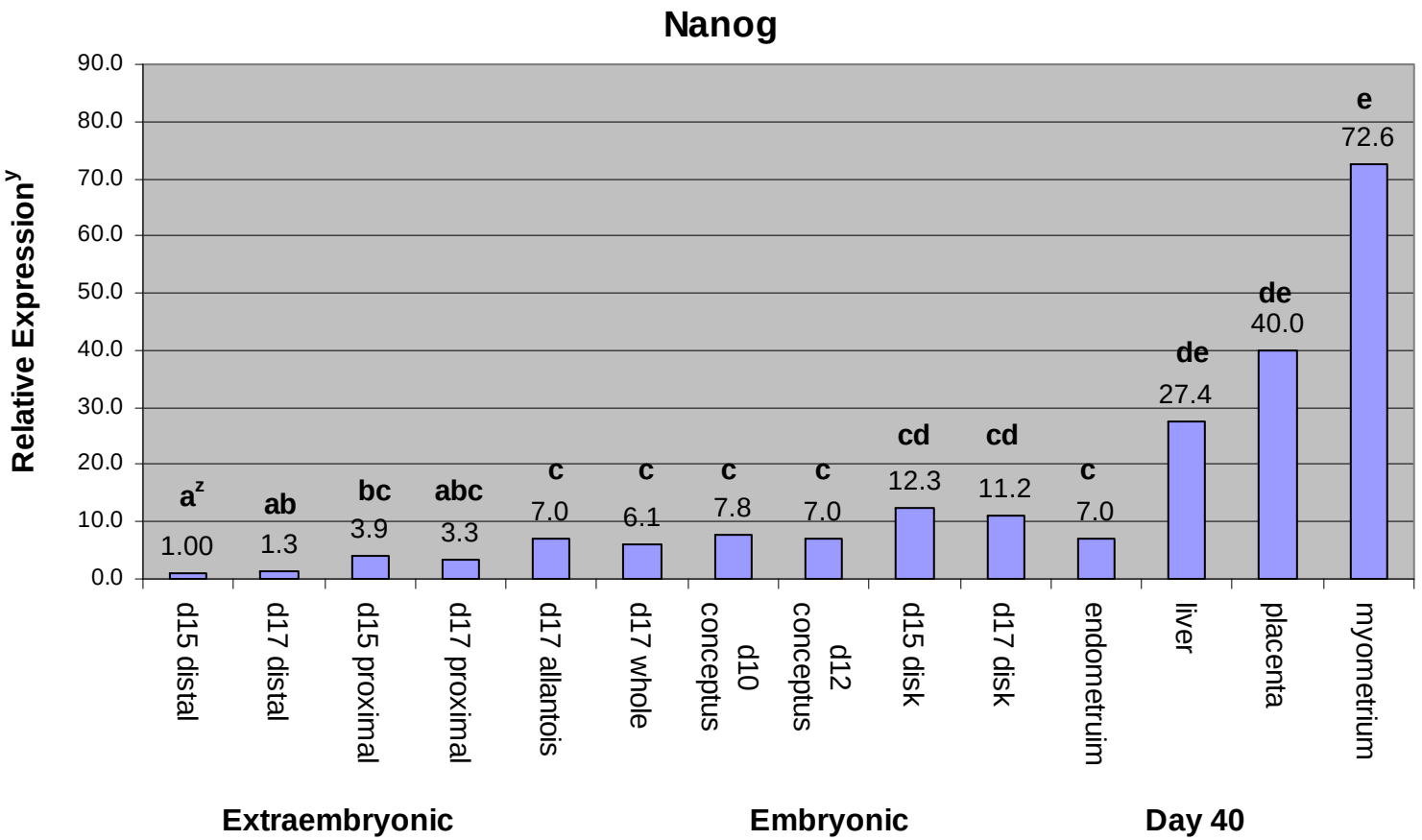
[a]Normalized to 18s ribosomal RNA

Table 1 continued Adjusted Threshold Means Nanog, Sox-2, and Oct-4 by Tissue

Tissue	Sample #	Gene	Mean[a]	Std. Error
D17 Whole	3	Nanog	21.96	0.74
		Oct-4	21.63	0.74
		Sox-2	16.85	0.87
D40 Endometrium	3	Nanog	21.75	0.74
		Oct-4	26.60	0.74
		Sox-2	26.07	0.87
D40 Liver	3	Nanog	19.78	0.74
		Oct-4	22.25	0.74
		Sox-2	19.91	0.87
D40 Myometrium	3	Nanog	18.38	0.74
		Oct-4	26.99	0.74
		Sox-2	25.21	0.87
D40 Placenta	3	Nanog	19.24	0.74
		Oct-4	26.39	0.74
		Sox-2	23.67	0.87

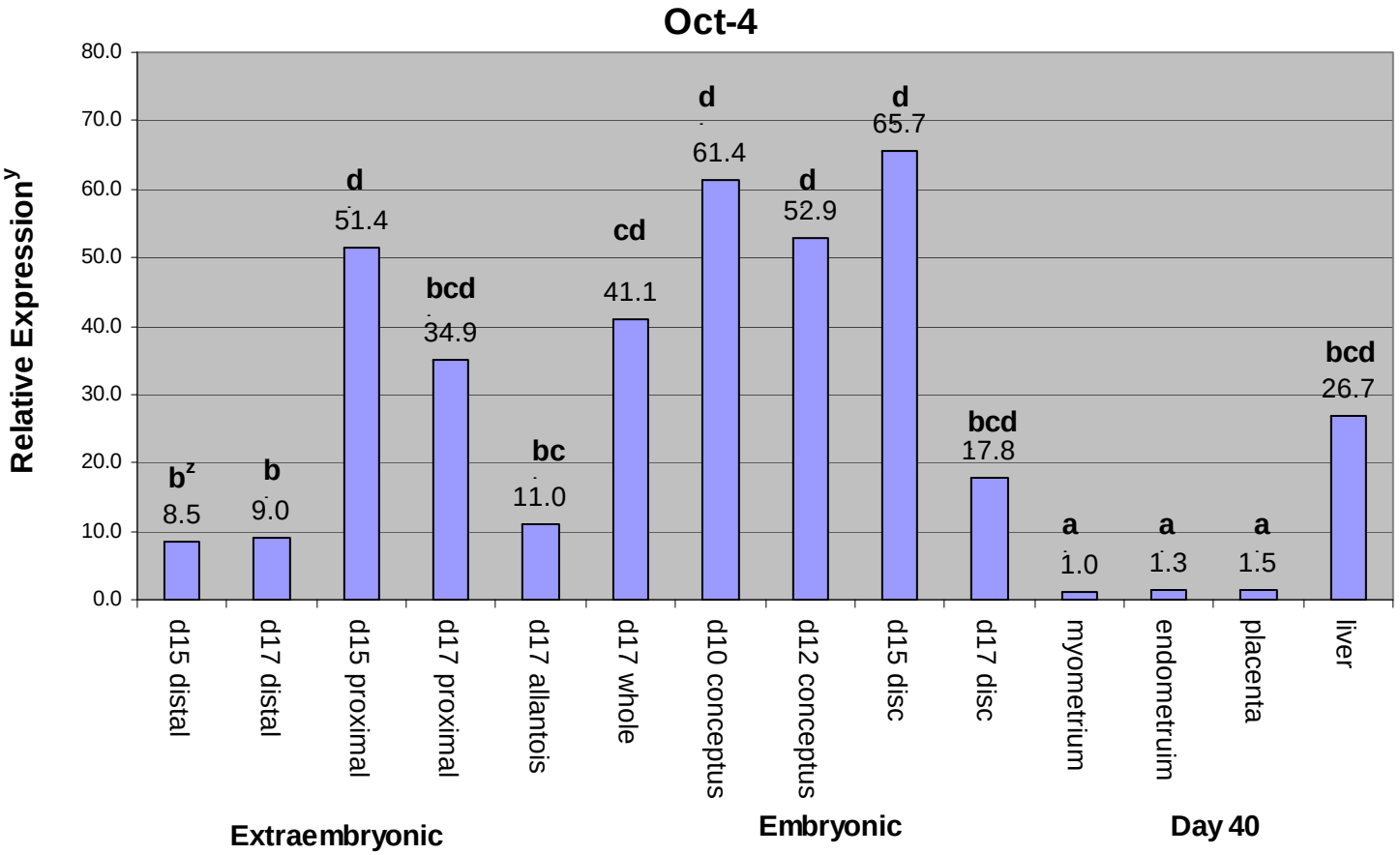
[a]Normalized to 18s ribosomal RNA

Figure 7 Relative Nanog Expression in the Early Porcine Conceptus, Fetal Liver, Placenta, and Maternal Endometrium and Myometrium



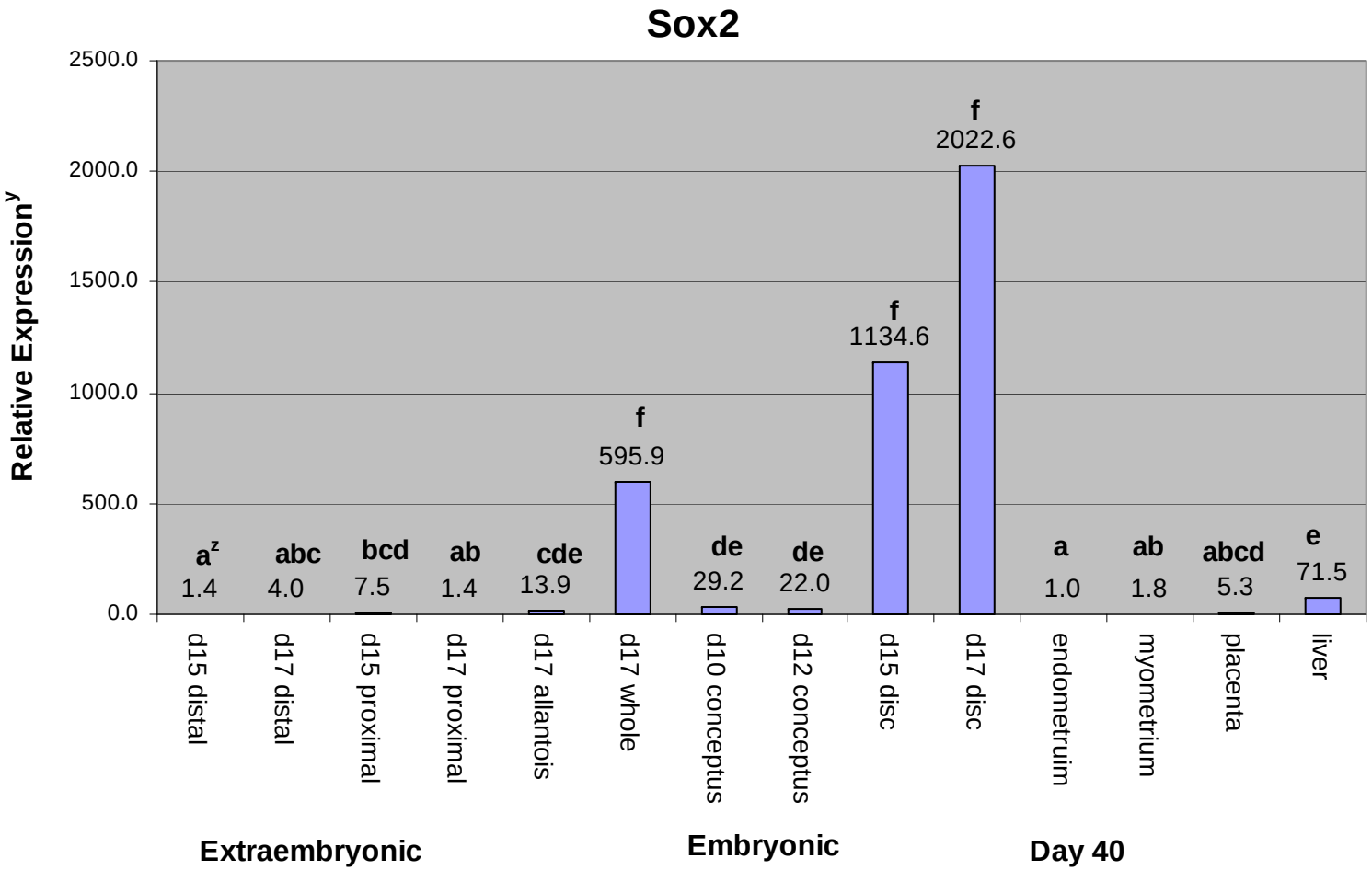
[y]Fold difference relative to the tissue with lowest expression (d15 distal). See Table 1.
[z]Tissues with different letters differ $P < 0.05$.

Figure 8 Relative Oct-4 Expression in the Early Porcine Conceptus, Fetal Liver, Placenta, and Maternal Endo- and Myometrium



[y]Fold difference relative to the tissue with lowest expression (myometrium). See Table 1.
[z]Tissues with different letters differ $P < 0.05$.

Figure 9 Relative Sox-2 Expression in the Early Porcine Conceptus, Fetal Liver, Placenta, and Maternal Endo- and Myometrium



[y]Fold difference relative to the tissue with lowest expression (endometrium). See Table 1.
[z]Tissues with different letters differ $P < 0.05$.

Figure 10 Northern Blot of Porcine Sox-2

Ten ug of total RNA from d-12 conceptus (A), d-15 disk (B), d-15 distal extraembryonic (C) and d-15 proximal extraembryonic (D) were probed for Sox-2 mRNA. Film was exposed for 1hr 30min. A band of ~2300 bp was observed in the d-15 disk (B).

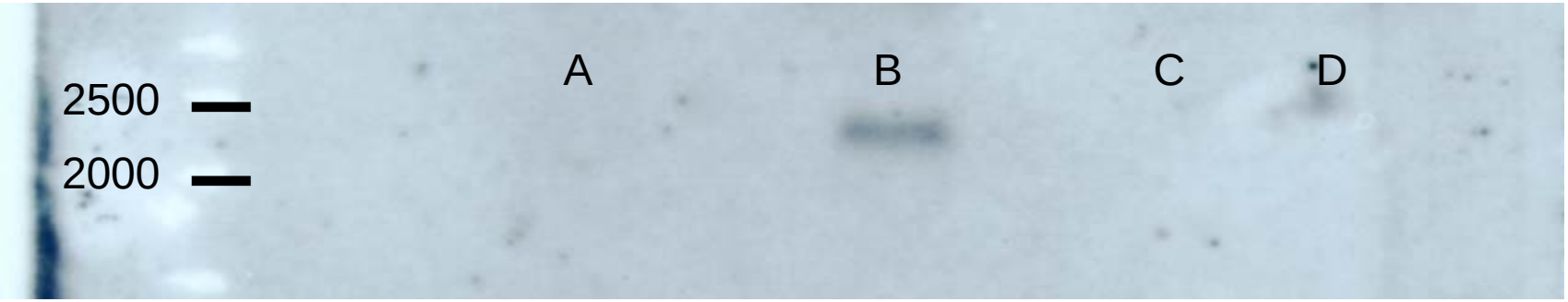


Figure 11 Northern Blots of Porcine β-Actin

Ten ug (A-D) and 20 ug (E-G) of total RNA from d-12 conceptus (A,G), d-15 disk (B), d-15 distal extraembryonic (C) d-15 proximal extraembryonic (D), d-17 disk (E), and allanotis (F) were probed for β-Actin. Filmed was exposed overnight. Two bands for β-Actin were observed (~ 1550 and 1750).

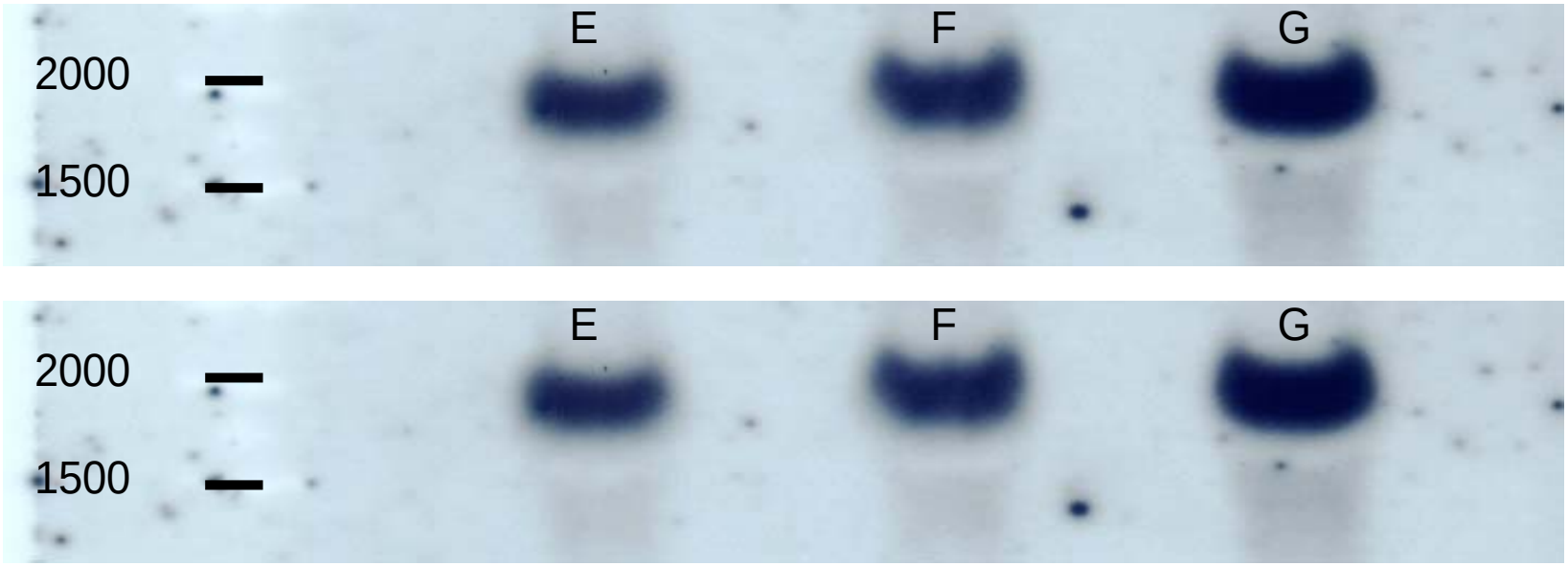
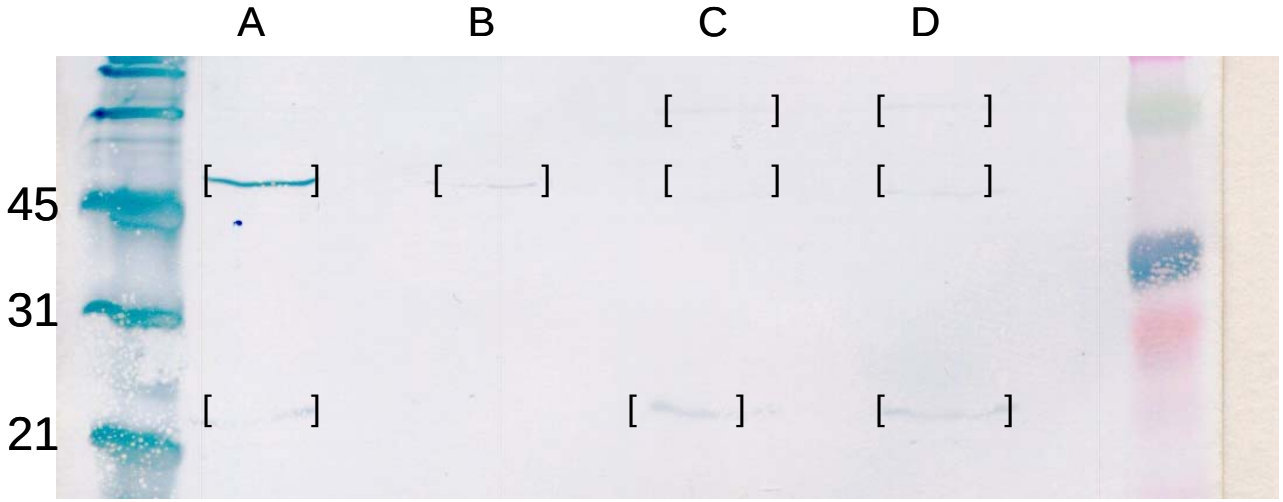


Figure 12 Western Blot of Porcine Nanog

Approximately 20 ug of protein, from porcine fibroblasts (A), mouse ES D3 cells (B), d-15 extraembryonic (C), and d-15 conceptus (D), were probed for Nanog. It is believed the 50 kDa band is Nanog.



CHAPTER 3 - Timed Insemination of Beef Heifers using the 7-11 Synch Protocol

Abstract

In Exp. 1, 179 yearling heifers were either fed melengestrol acetate (MGA; 7-11 Synch) or given an intravaginal progesterone (P4)-releasing insert (CIDR; 7-11 CIDR) for 7 d. Prostaglandin $F_{2\alpha}$ (PG) was administered on the last day of MGA feeding or at CIDR removal followed by the Cosynch protocol (GnRH – PG – GnRH) beginning 4 d after MGA withdrawal or 2 d after CIDR removal. Heifers were fixed-time AI (TAI) 48 h after the second PG. Blood samples were collected at d -10, d 1 (start of MGA feeding) and d 18 (second PG injection). In Exp. 2, 298 yearling heifers were treated with the 7-11 Synch protocol or with the 7-11 Synch protocol without the first GnRH injection (7 Synch) and TAI beginning 54 hr after PG. Blood samples were collected at d -10 and d 1 in yr 1 and d -10, 1, 18 and at TAI in yr 2. In Exp. 1, there was no difference between treatments in inducing ovulation in prepubertal heifers (94 vs 78; $P = 0.21$), the proportion of heifers that had luteal tissue on d 18 (87 vs 83%; $P = 0.39$) or pregnancy rates to 48 hr TAI (47 vs 46%; $P = 0.84$) between 7-11 Synch and 7-11 CIDR treatments respectively. In Exp. 2, the administration of the GnRH after MGA removal tended ($P = 0.07$) to induce more prepuberal heifers to cycle (88 vs 61%) and increased ($P < 0.01$) the proportion of heifers with luteal tissue on d 18 (88 vs 72%). Pregnancy rates for a 54 hr TAI were higher ($P < 0.01$) for the 7-11 Synch treatment (55%) compared to 7 Synch (38%). We conclude that there is no difference in pregnancy rates between MGA and CIDR when included before the Cosynch protocol. However, the use of GnRH induces more prepubertal heifers to ovulate and improves the proportion of heifers with luteal tissue at the PG injection which increased pregnancy rates to a timed artificial insemination..

Introduction

Melengestrol acetate (MGA) has been included in artificial insemination protocols because it is a cost effective and easy to administer progestogen that can induce prepubertal heifers or anestrus cows to become cyclic. Long-term feeding (14 d) of MGA ($0.5 mg \cdot animal^{-1} \cdot d^{-}$

[1]) has been effective in estrus synchronization of heifers (Brown et al., 1988; Jaeger et al., 1992) and cows (Bader et al., 2005; Patterson et al., 1995; Stegner et al., 2004c; Stegner et al., 2004a). Recent synchronization protocols using MGA have focused on reducing the length of MGA feeding and use of GnRH to synchronize the follicular wave for a fixed-timed AI (TAI).

A high proportion of heifers exhibit estrus in response to short-term MGA feeding but with lower fertility (Beal et al., 1988) (Chenault et al., 1990). Therefore, a program that synchronized first-wave follicles after MGA feeding with GnRH was developed called 7-11 Synch (Kojima et al., 2000). This protocol has been used successfully in postpartum beef cows by inseminating after observed estrus (Kojima et al., 2000; Stegner et al., 2004c) and at a fixed time (Bader et al., 2005; Kojima et al., 2002; Kojima et al., 2003) but has not been reported in beef heifers. The objectives of this study were to compare MGA and an intravaginal progesterone (P4)-releasing insert (CIDR) using the 7-11 Synch protocol and the effect of GnRH on prepubertal heifers to induce cyclicity and improve pregnancy rates to a fixed-time AI after short-term MGA feeding.

Material and Methods

Experiment 1

Experimental Design.

Crossbred Angus heifers from two locations (CCR; n=51and CCU; n=79) and purebred Angus, Hereford, and Simmental heifers (PBU; n=50) from a third location were randomly assigned to two treatments (Figure13). The 7-11 Synch treated heifers were fed MGA (0.5 $mg \cdot animal^{-1} \cdot d^{-1}$ MGA 200 Premix, Pharmacia & Upjohn Company, Kalamazoo, MI) in a sorghum grain carrier for 7 days beginning on d 1 followed by an injection of $PGF_{2\alpha}$ (**PG**; 25 mg i.m. of Lutalyse, Pharmacia & Upjohn Company) on d 7. An injection of GnRH (100 µg i.m. of OvaCyst, IVX Animal Health, Inc., St. Joseph, MO) was given on d 11, followed by PG on d 18, and GnRH at the time of insemination. Heifers assigned to 7-11 CIDR received an Eazi-Breed CIDR (Pharmacia & Upjohn Company) beginning on d 3. On d 9, CIDRs were removed and given PG, followed by GnRH on d 11, PG on d 18 and GnRH at the time of insemination.

AI and Pregnancy Determination

Heifers were TAI beginning 48 h after PG on d 18. The same two inseminators were used for all locations. Two different sires were used at the CCR and CCU locations and 11 sires were used at the PBU location. Purebred heifers were checked twice daily for 45 d after TAI and were bred 12 h after the onset of estrus. Crossbred heifers were exposed to bulls for 60 to 80 days beginning 7 days after insemination. Conception rate to AI was determined by transrectal ultrasonography (Aloka 500V with a 5.0-MHz linear array probe; Aloka, Wallingford, CT) 30 to 35 days after insemination. Final pregnancy status was determined 50-60 days after the natural service breeding season by rectal palpation.

Blood Collection and Progesterone Concentrations

Blood samples were collected via coccygeal venipuncture on d -10, d 1, and d 18 to determine heifer cyclicity and treatment response. Blood was allowed to clot overnight at 4^{o}C and serum was separated by centrifugation the following day. Serum was frozen at -20^{o}C until assays for P4 concentration by RIA were performed. Heifers with P4 concentrations > 1 ng/mL either on d -10 or d 1 were considered to have obtained puberty before treatments. Heifers not cycling before treatment but had P4 concentrations > 1 ng/mL at d 18 or conceived to TAI was considered to have been induced to ovulate during treatments.

Experiment 2

Experimental Design

Experiments took place at two locations and over two years using crossbred Angus heifers (CCU05; n=73 and CCU06; n=91) and purebred Angus, Hereford, and Simmental heifers (PBU05; n=58 and PBU06; n=66). A third location (SF06) consisting of 21 purebred Angus heifers was included in year 2. Beginning on d 1, all heifers in both treatments (Figure 14) were fed MGA for 7 days and injected with PG on d 7. MGA was fed in a sorghum grain carrier at locations 1 and 2 and in a grain pellet at location 3. Heifers randomly assigned to the 7-11 Synch treatment received a GnRH injection on d 11, PG on d18, and GnRH at the time of breeding. The 7 Synch treated heifers were given PG on d 18 and GnRH at the time of breeding.

AI and Pregnancy Determination

Timed insemination began 54 h after PGF injection. The same two AI technicians were used both years and in all herds. In year one, eight sires and four sires were used at PBU05 and

CCU 05, respectively. In year two, five sires were used at PBU06, two sires at CCU06, and two sires were used SF06. In herd one, heifers were heat checked twice daily for approximately 30 d and inseminated 12 h from the onset of estrus and then exposed to bulls for another 30 d. In herd two and three, heifers were exposed to bulls 7 d after the timed insemination for a 60 d breeding season.

Blood Collection and Progesterone Concentrations

In year one, blood samples were collected via the coccygeal vein d -10 and d 1 to determine heifer cyclicity before treatment. In year two, blood samples were collected on d -10, d 1, d 18, and at the time of insemination. Blood was allowed to clot overnight at 4^{o}C and serum was separated by centrifugation the following day. Serum was frozen at -20^{o}C until assays for progesterone concentration by RIA were performed. Serum progesterone concentrations on the day of breeding were determined for only those heifers who did not conceive to the timed insemination. Heifers with progesterone concentrations > 1 ng/mL on d -10, d 1, or d 18 were considered to have obtained puberty.

Semen Analysis

Semen analysis was done by the Andrology Laboratory at the Kansas State University Veterinary Medical Teaching Hospital (Manhattan, KS) on 2 sires used in experiment 1. Semen from eight sires used in experiment 2 was evaluated at the Bovine Andrology Lab (Penn Veterinary Medicine, Kennett Square, PA) for sperm motility, sperm morphology, sperm concentration, volume, and total sperm/dose. Results are presented in Table 10.

Statistical Analysis

Each experiment was analyzed by the Glimmix procedure of SAS (SAS Inst., Inc., Cary, NC). Pretreatment cyclicity and overall cyclicity at d18 were analyzed using the model of location, treatment, and location x treatment. Pretreatment cyclicity was included in the model when proportion of heifers with P4 > 1ng/ml and actual P4 concentrations at d18 was analyzed. The full model to predict pregnancy rate included treatment, cyclicity, and presence of CL at d18 as fixed effects and location, AI technician, and sire nested within location as random effects. Significance of random effects was tested by likelihood ratio tests. Terms that were not significant were dropped from the models.

Results

Experiment 1

Results from Exp. 1 are summarized in Table 2. There was no difference in the proportion of cycling heifers at the beginning of treatment between treatments (P = 0.97) or between locations (P = 0.18). Non-cycling heifers that were induced to ovulate were not different (P = 0.21) between MGA (94%) and CIDR (78%) treated heifers. Proportion of heifers with high P4 (≥ 1ng/mL) at d 18 was not different (P = 0.39) between 7-11 Synch and 7-11 CIDR treatments (87 vs 83%) but was different between locations (*P* = 0.04) (see Table 4). At one location only 78% of the heifers had high P4 at d 18 compared to > 90% at the other 2 locations and the cycling heifers tended to be higher compared to the non-cycling heifers (87 vs 77%). Day 18 progesterone concentrations were significantly higher in the 7-11 Synch compared to 7-11 CIDR (*P* < 0.01), was lower in the CCU herd (*P* < 0.01) when compared to the other herds, and was higher in heifers that were cycling (*P* = 0.03) before treatment compared with those that were not. For pregnancy rates to a timed AI, sire was a significant source of variation (*P* = 0.04) but location (variance estimate = 0) and inseminator (*P* = 0.09) were not therefore they were dropped in the final model. Pregnancy rates for 7-11 Synch (47%) and 7-11 CIDR (46%) were similar (P = 0.82). Pregnancy rates by herd and pubertal status are described in Table 5.

Experiment 2

Results from Exp. 2 are summarized in Table 3. There was no difference in the proportion of cycling heifers at the beginning of treatment between treatments or locations. The percentage of prepubertal heifers induced to ovulate by d 18 were higher (*P* = 0.07) in the 7-11 Synch treatment (88%) compared to the 7 Synch treatment (61%). More 7-11 Synch treated heifers (*P* < 0.01) had a CL (88%) on d 18 then did the 7 Synch treated heifers (72%). P4 concentrations at d 18 differed by treatment (*P* = 0.03) and herd (*P* < 0.02) and cycling status before treatment (p=0.02) (see Table 6). Pregnancy rates to a TAI were higher (*P* < 0.01) in the 7-11 Synch treated heifers (55%) compared to 7 Synch (38%). Pregnancy rates by herd and pubertal status are described in Table 7.

Discussion

Artificial insemination gives producers access to bulls that have high accuracy, are superior in a particular trait(s), and/or they couldn't afford otherwise. A successful synchronization protocol should get more females to conceive earlier in the breeding season, achieved in part by inducing pre-pubertal or anestrous females to cycle, and be economical and efficient.

The 7-11 Synch is a synchronization protocol that uses short-term MGA feeding of 7 days followed by GnRH-PG to synchronize follicular growth and luteal regression (Kojima et al., 2000). For a timed AI, GnRH is given at breeding so that 7-11 Synch is simply a Cosynch protocol preceded by a presynchronization with a progestogen. Use of a progestogen in synchronization induces ovulation in heifers (Jaeger et al., 1992) (Plugge et al., 1990) and cows (Fike et al., 1997; Patterson et al., 1995), prevents a short luteal cycle from GnRH-induced ovulations in postpartum cows (Thompson et al., 1999) and prevents early heats before or around PG in a GnRH-PG-based protocol (Kojima et al., 2000).

Cyclicity

More than 95% of heifers had ovulated by d 18 after MGA or CIDR treatment when followed by GnRH. Administration of GnRH after MGA withdrawal increased the number prepubertal heifers to ovulate by 17%. Relative to other studies in cow, a high proportion of the heifers were already cycling (> 75%) before treatment. An estrous response of 91% has been observed in cows that were 90% anestrous at the beginning of the 7-11 synch treatment (Stegner et al., 2004c). Breed, season, and nutritional status are other factors that can influence initiation of puberty in heifers (Kinder et al., 1995).

In Exp. 1 and Exp. 2, prepubertal heifers in the 7-11 Synch treatment had pregnancy rates 4% and 10% higher than pubertal heifers but was not significant (p=0.72). Thus conception rates are expected not to differ if a large proportion of heifers are not cycling at the beginning of treatment.

Synchronization and Pregnancy Rates

Synchronization rates, the proportion heifers with high P4 at PG (d 18), were similar between experiments (87 and 88%) for the 7-11 Synch treatment and for the 7-11 CIDR treated

heifers (83%). Our synchronization rates are similar to those reported for cows which have ranged from 66 to 91% (Bader et al., 2005; Stegner et al., 2004c). In Exp. 2, 16% more heifers were synchronized when given GnRH following MGA withdrawal. In two locations the synchronization rate for 7-11 Synch was 96.4 and 90% and differed from the 7 Synch treatment by 41 and 35 %. Kojima (Kojima et al., 2000) reported that only half of the females that did not receive GnRH responded to the PG injection due to delayed ovulation after MGA withdrawal and unresponsive early-developing CL.

Pregnancy rates to a timed AI using MGA or CIDR in the 7-11 Synch protocol were 47% and 46% respectively which is lower than the pregnancy rates (> 60%) reported in postpartum cows (Bader et al., 2005; Kojima et al., 2003). Average interval to estrus using 7-11 Synch has been reported to be about 54 hr in heifers (Kojima et al., 2000) and from 52-64 hr in cows (Kojima et al., 2000; Stegner et al., 2004c; Stegner et al., 2004b). Timed inseminations 60 hr after PG have been suggested for cows (Bader et al., 2005; Kojima et al., 2003). Our lower pregnancy rates maybe due to an earlier insemination time of 48 hr. In Exp. 2, a 54 hr TAI was used and pregnancy rates ranged from 51 to 68% (55% overall) for 7-11 Synch. The 7 Synch treatment had an average of 38%. Of the 7-11 Synch heifers that did not conceive to the TAI, 21 had high P4 at breeding; 15 had high P4 at d18 and 6 had low P4 at d18.

Differences in P4 concentrations can occur due to the hormonal environment under which the dominant follicle develops. In 7-11 Synch, the first-wave dominant follicle is developing under higher estradiol-17β concentrations and lower progesterone concentrations compared to higher progesterone and lower estradiol-17β in a second-wave or mid-luteal follicle (Stegner et al., 2004b). Since the dominant follicle in both treatments is a first wave follicle, higher P4 concentrations may be due to a more mature CL induced by the d11 GnRH after MGA withdrawal.

Since many of the response variables in our study had a binomial distribution such as presence of luteal function on d 18 (yes/no) and pregnancy rate to TAI (pregnant/open), we used the Glimmix Procedure in SAS which is a relatively new method for analysis of generalized linear mixed models (GLMM). GLMM can be used when the response variable in not necessarily normally distributed and can have any distribution in the exponential family which includes binary, binomial, and Poisson distributions and when random effects are included in the model assuming they are normal. The ability to include random effects offers an advantage over

other commonly used SAS procedures including Logistic and Genmod. In the absence of random effects, the Glimmix procedure fits generalized linear models in the same manner as the Genmod procedure. In the model for pregnancy rate, the variables location, inseminator, and sire were treated as random effects likelihood ratio tests were used to test their significance in the model.

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Figures and Tables

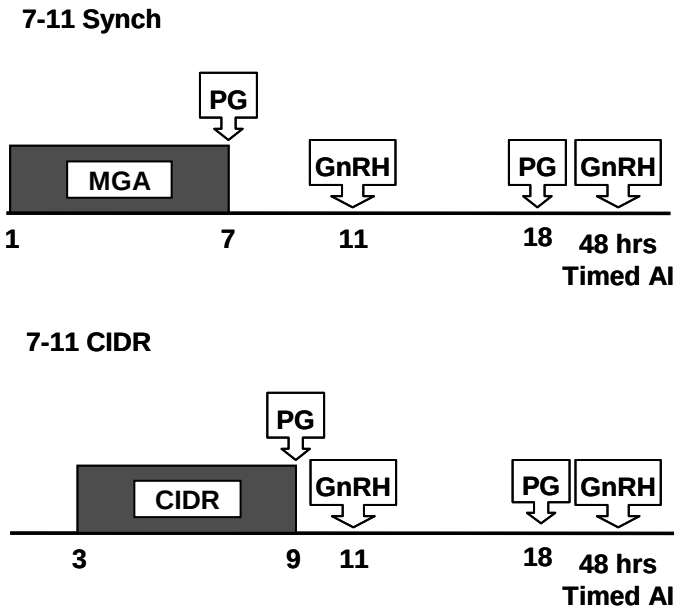

Figure 13. Experiment 1 treatment schedule for heifers assigned to the 7-11 Synch and 7-11 CIDR protocols.

Heifers assigned to the 7-11 Synch treatment were fed melengestrol acetate (MGA) for 7 d beginning on d 1 and injected with $PGF_{2\alpha}$ (PG) on day of MGA withdrawal (d 7). The Cosynch protocol followed 4 d later; a GnRH injection (d 11), PG on d 18, and GnRH at breeding 48 h after PG. The 7-11 CIDR treatment began on d 3 with insertion of a CIDR for 7 d and a PG injection at CIDR removal. The Cosynch protocol began 2 d after CIDR removal (d 11).

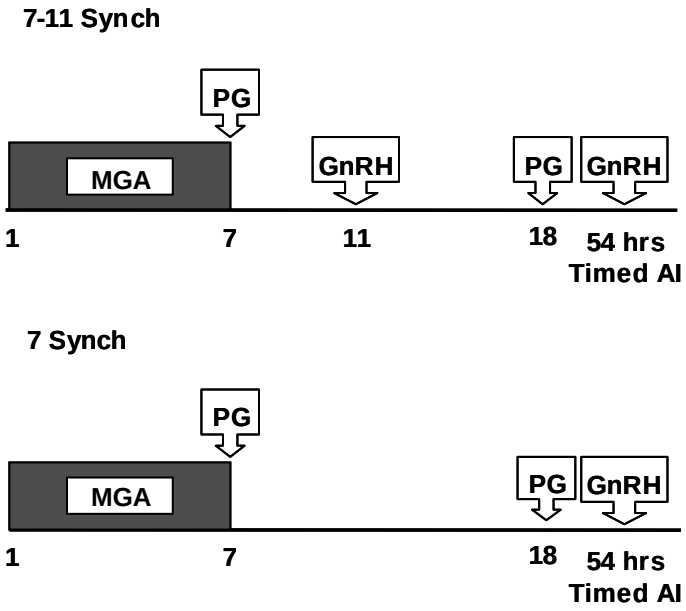

Figure 14. Experiment 1 treatment schedule for heifers assigned to the 7-11 Synch and 7 Synch protocols.

Heifers assigned to the 7-11 Synch treatment were fed melengestrol acetate (MGA) for 7 d beginning on d 1 and injected with $PGF_{2\alpha}$ (PG) on day of MGA withdrawal (d 7). The Cosynch protocol followed 4 d later; a GnRH injection (d 11), PG on d 18, and GnRH at breeding 48 h after PG. In the 7 Synch treatment, the d 11 GnRH injection is omitted.

Table 2 Comparison of 7-11 Synch using MGA (7-11 Synch) or CIDR (7-11 CIDR) on different reproductive traits of heifers (Exp. 1)

Item	Treatment[a]	
	7-11 Synch	7-11 CIDR
	% (total No. of heifers)	
Cycling before initiation of treatment[w]	79.8 (89)	78.9 (90)
Prepubertal heifers induced to cycle by d 18[x]	94.4 (18)	78.9 (19)
High progesterone on d 18 before PG[y]	87.5 (88)	83.2 (89)
Pregnancy rates to TAI		
Overall[z]	47.1 (89)	46.6 (90)
Cycling Status		
Pubertal	46.5 (71)	52.1 (71)
Prepubertal	50.0 (18)	26.3 (19)
Pregnancy rate at end of breeding season	92.1 (89)	91.1 (90)

[a]7-11 Synch: beginning on d 1, melengestrol acetate (MGA) was fed for 7 d followed by injections of $PGF_{2\alpha}$ (PG) on day of MGA withdrawal, GnRH on d 11, PG on d 18 and GnRH at breeding. 7-11 CIDR: insertion of CIDR for 7 d beginning on d 3. PG was injected at CIDR removal and GnRH was injected 2 d later (d11), PG at d 18, and GnRH at breeding. Timed inseminations began 48 h after PG.

[w]Treatment difference P = 0.97

[x]Treatment difference P = 0.21

[y]Treatment difference P = 0.39

[z]Treatment difference P = 0.82

Table 3 Comparison of 7-11 Synch with (7-11 Synch) or without (7 Synch) GnRH on d 11 of treatment on different reproductive traits of heifers (Exp. 2)

Item	Treatment[a]	
	7-11 Synch	7 Synch
	% (total No. of heifers)	
Cycling before initiation of treatment	75.1 (149)	73.8 (149)
Prepubertal heifers induced to cycle by d 18	88.2 (17)	61.9[x] (21)
High progesterone on d 18 before PG	88.1 (84)	72.9[y] (85)
Pregnancy rates to TAI		
Overall	55.3 (150)	38.0[z] (150)
Cycling Status		
Pubertal	52.6 (112)	39.0 (110)
Prepubertal	62.1 (37)	35.9 (39)
Pregnancy rate at end of breeding season	87.5 (144)	84.2 (146)

[a]7-11 Synch: beginning on d 1, melengestrol acetate (MGA) was fed for 7 d followed by injections of $PGF_{2\alpha}$ (PG) on day of MGA withdrawal, GnRH on d 11, PG on d 18 and GnRH at breeding. 7-11 Synch: the GnRH injection at d 11 was omitted. Timed inseminations began 54 h after PG.

[x]Different ($P = 0.07$) from 7-11 Synch

[y]Different ($P < 0.01$) from 7-11 Synch

[z]Different ($P < 0.01$) from 7-11 Synch

Table 4 Puberty status before treatment, overall puberty status after treatment, heifers with serum progesterone (P4) concentrations ≥1 ng/ml, and d 18 serum progesterone concentration of heifers by location

	Heifer cyclicity[1]	Cyclicity at d18[2]	CL present at d18	P4 concentrations at d18
	% (total # of heifers)	% (total # of heifers)	% (total # of heifers)	(ng/mL ± SE)
Overall				
7-11 Synch	79.8 (89)	98.8 (89)	87.5 (88)	2.75 ± 0.16[x]
7-11 CIDR	78.9 (90)	95.5 (90)	83.2 (89)	2.20 ± 0.15[w]
Combined	79.3 (179)	96.0 (179)	85.3 (177)	
CCR				
7-11 Synch	76.0 (25)	100 (25)	87.5 (24)	2.92 ± 0.27
7-11 CIDR	84.6 (26)	100 (26)	96.0 (25)	2.75 ± 0.28
Combined	80.4 (51)	100 (51)	91.8 (49)	2.82 ± 0.20[y]
CCU				
7-11 Synch	82.1 (39)	97.4 (39)	84.6 (39)	1.84 ± 0.22
7-11 CIDR	87.2 (39)	94.8 (39)	71.8 (39)	1.33 ± 0.23
Combined	84.6 (78)	96.1 (78)	78.2 (78)	1.57 ± 0.17[z]
PBU				
7-11 Synch	80.0 (25)	100 (25)	92.0 (25)	3.53 ± 0.27
7-11 CIDR	60.0 (25)	92.0 (25)	88.0 (25)	2.55 ± 0.26
Combined	70.0 (50)	96.0 (50)	90.0 (50)	3.03 ± 0.19[y]

[a]Percentage of heifers that had progesterone serum concentrations ≥1 ng/mL in one of the two blood samples collected before treatment.
[b]Percentage of heifers that was cyclic before treatement, had progesterone serum concentrations ≥1 ng/mL at d 18 or conceived to timed insemination.
[w,x]Treatment means differ, $P < 0.01$
[y,z]Location means differ, $P < 0.01$

Table 5 Pregnancy rates to 48 hr timed AI

	Overall	Pubertal	Prepubertal	Pregnancy Rate at the end of breeding season
	% (total # of heifers)	% (total # of heifers)	% (total # of heifers)	% (total # of heifers)
Overall				
7-11 Synch	47.1 (89)	46.5 (71)	50.0 (18)	
7-11 CIDR	46.6 (90)	52.1 (71)	26.3 (19)	
Combined	46.9 (179)	49.3 (142)	37.8 (37)	
CCR				
7-11 Synch	48.0 (25)	42.1 (19)	60.0 (6)	100 (25)
7-11 CIDR	42.3 (26)	50.0 (22)	0.0 (4)	92.3 (26)
Combined	45.0 (51)	46.3 (41)	40.0 (10)	96.0 (51)
CCU				
7-11 Synch	56.4 (39)	59.4 (32)	42.9 (7)	92.3 (39)
7-11 CIDR	48.7 (39)	50.0 (34)	40.0 (5)	92.3 (39)
Combined	52.5 (78)	54.5 (66)	41.7 (12)	92.3 (78)
PBU				
7-11 Synch	32.0 (25)	30.0 (20)	40.0 (5)	83.0 (25)
7-11 CIDR	48.0 (25)	60.0 (15)	30.0 (10)	88.0 (25)
Combined	40.0 (50)	42.9 (35)	33.3 (15)	

Table 6 Puberty status before treatment, overall puberty status after treatment, heifers with serum progesterone (P4) concentrations ≥1 ng/ml, and d 18 serum progesterone concentration of heifers by location

	Pretreatment cyclicity[1]	Cyclicity at d18[b]	P4 ≥1 ng/ml at d18	P4 concentrations at d18
	% (total # of heifers)			(ng/mL ± SE)
Overall				
7-11 Synch	75.1 (149)	97.6 (84)	88.1 (84)	2.91 ± 0.30[w]
7 Synch	73.8 (149)	90.5 (85)	72.9 (85)	2.09 ± 0.29[x]
Combined	74.5 (298)	94.0 (169)	80.4 (169)	
CCU06				
7-11 Synch	72.9 (37)	-	-	
7 Synch	66.7 (36)	-	-	
Combined	69.9 (73)	-	-	
CCU07				
7-11 Synch	82.6 (46)	95.6 (46)	82.6[s] (46)	3.05 ± 0.33
7 Synch	75.5 (45)	97.7 (45)	88.8[s] (45)	3.21 ± 0.33
Combined	79.1 (91)	96.7 (91)	85.7 (91)	3.13 ± 0.25[u]
PBU06				
7-11 Synch	64.2 (28)	-	-	
7 Synch	78.5 (28)	-	-	
Combined	71.4 (56)	-	-	
PBU07				
7-11 Synch	71.4 (28)	100 (28)	96.4[s] (28)	3.24 ± 0.40
7 Synch	65.5 (29)	75.8 (29)	55.1[t] (29)	1.95 ± 0.39
Combined	68.4 (57)	87.7 (57)	75.4 (57)	2.59 ± 0.29[uv]
SF07				
7-11 Synch	90.0 (10)	100 (10)	90.0[st] (10)	2.44 ±0.68
7 Synch	100 (11)	100 (11)	54.5[t] (11)	1.10 ± 0.66
Combined	95.2 (21)	100 (21)	71.4 (21)	1.77 ± 0.49[v]
Cycling				
Yes				2.96 ± 0.21[y]
No				2.03 ± 0.37[z]

[a]Percentage of heifers that had progesterone serum concentrations ≥1 ng/mL in one of the two blood samples collected before treatment.
[b]Percentage of heifers that had progesterone serum concentrations ≥1 ng/mL before treatment, at d 18, or conceived to timed insemination.
[s,t]Treatment means within location differ, P<0.01
[u,v]Location means differ, P<0.05
[w,x]Treatment means differ, P<0.05
[y,z]Cycling means differ, P<0.05

Table 7 Pregnancy rates to 54 hr timed AI

	Overall	Pubertal	Prepubertal	Pregnancy Rate at the end of breeding season
	% (total # of heifers)	% (total # of heifers)	% (total # of heifers)	% (total # of heifers)
Overall				
7-11 Synch	55.3[y] (150)	52.6 (112)	62.1 (37)	87.5 (144)
7 Synch	38.0[z] (150)	39.0 (110)	35.9 (39)	84.2 (146)
Combined	46.6 (300)	45.9 (222)	48.6 (76)	85.8 (290)
CCU06				
7-11 Synch	51.3 (37)	40.7 (27)	80.0 (10)	86.4 (37)
7 Synch	52.7 (36)	66.6 (24)	25.0 (12)	80.5 (36)
Combined	52.0 (73)	52.9 (51)	50.0 (22)	83.5 (73)
CCU07				
7-11 Synch	56.5 (46)	60.5 (38)	37.5 (8)	91.3 (46)
7 Synch	33.3 (45)	26.4 (34)	54.5 (11)	77.7 (45)
Combined	45.0 (91)	44.4 (72)	47.3 (19)	84.6 (91)
PBU06				
7-11 Synch	68.9 (29)	66.6 (18)	70.0 (10)	82.7 (29)
7 Synch	44.8 (29)	40.9 (22)	66.6 (6)	89.2 (28)
Combined	56.9 (58)	52.5 (40)	68.7 (16)	85.9 (57)
PBU07				
7-11 Synch	53.5 (28)	55.0 (20)	50.0 (8)	91.3 (23)
7 Synch	24.1 (29)	31.5 (19)	10.0 (10)	96.1 (26)
Combined	38.6 (57)	43.5 (39)	27.7 (18)	93.8 (49)
SF07				
7-11 Synch	30.0 (10)	22.2 (9)	100.0 (1)	77.7 (9)
7 Synch	27.2 (11)	27.2 (11)	-	81.8 (11)
Combined	28.5 (21)	25.0 (20)	100 (1)	80.0 (20)

Table 8 Pregnancy rate of heifers with low P4 on d 18

2005	% (total #)	2007	% (total #)
Overall		Overall	
7-11 Synch	63.6 (11)	7-11 Synch	40.0 (10)
7-11 CIDR	26.7 (15)	7 Synch	8.6 (23)
Combined	42.3 (26)	Combined	18.1 (33)
CCR		CCU07	
7-11 Synch	66.6 (3)	7-11 Synch	25.0 (8)
7-11 CIDR	0.0 (1)	7 Synch	20.0 (5)
Combined	50.0 (4)	Combined	23.0 (13)
CCU		PBU07	
7-11 Synch	66.6 (6)	7-11 Synch	100 (1)
7-11 CIDR	36.7 (11)	7 Synch	7.6 (13)
Combined	47.1 (17)	Combined	14.2 (14)
PBU		SF07	
7-11 Synch	50.0 (2)	7-11 Synch	100 (1)
7-11 CIDR	0.0 (3)	7 Synch	0.0 (5)
Combined	20.0 (5)	Combined	16.6 (6)

Table 9 P4 status of heifers that did not conceive

	Day 18 P4	Breeding P4	
		High (Total #)	Low (Total #)
7-11 Synch	High	6	28
	Low	2	3
7 Synch	High	15	24
	Low	6	15

Table 10 Semen Analysis

Sire	0 hr Motility (%)	2 hr Motility (%)	Normal Morphology (%)	Intact Acrosomes (%)	Conception Rate	
					%	No.
Bingo[1,3]	48	10	75	77	35.9	(14/39)
Sleep Easy[1,3]	38	48	68	86	69.2	(27/39)
Bingo[2,4]	9	-	55	69	37.5	(9/24)
Paramount[2,4]	32	-	57	68	76.9	(10/13)
Sleep Easy[2,4]	58	-	80	90	50.0	(12/24)
Traveler[2,4]	44	-	72	93	58.3	(7/12)
Conservative[2,6]	74	-	69	87	60.0	(6/10)
Domino[2,6]	40	-	47	66	14.3	(1/7)
Mo Better[2,6]	25	-	38	64	57.1	(4/7)
New Level[2,5]	48	-	61	87	50.0	(23/46)

1. Analysis by Andrology Laboratory, Veterinary Medical Teaching Hospital, Kansas State University, Manhattan Kansas.
2. Analysis by Reference Andrology Laboratory, Penn Veterinary Medicine, Kennett Square Pennslyvania.
3. 2005 Cow-calf Unit
4. 2006 Cow-calf Unit
5. 2007 Cow-calf Unit
6. 2007 Purebred Unit