Identification of potential embryokines in the bovine reproductive tract

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ABSTRACT

Knowledge of the molecules used by the maternal reproductive tract to regulate development of the preimplantation embryo is largely incomplete. The goal of the present experiment was to identify candidates for this function. The approach was to assess expression patterns in the endometrium and oviduct of 93 genes encoding for hormones, growth factors, chemokines, cytokines, and WNT-related molecules. Results show that all of the genes were expressed in the reproductive tract. Expression in oviduct was affected by day of the estrous cycle for 21 genes with 11 genes having highest expression at estrus (*CCL21*, *CTGF*, *CXCL10*, *CXCL16*, *DKK3*, *FGF10*, *IL18*, *IL33*, *IL34*, *PGF*, and *SFRP2*), 1 gene at d 3 (*WNT4*), 8 at d 5 (*BMP7*, *HGF*, *IL6*, *SFRP1*, *TGFB1*, *WIF1*, *WNT2*, and *WNT5A*), and 1 at d 7 (*IK*). For endometrium, expression of 34 genes was affected by day of the estrous cycle with 11 having highest expression at d 0 (*BMP7*, *CCL14*, *CCL21*, *CCL26*, *CTGF*, *CXCL12*, *IGF2*, *IL16*, *IL33*, *SFRP2*, and *WIF1*), 2 at d 3 (*HDGF*, *IL15*), 14 at d 5 (*CSF2*, *CX3CL1*, *CXCL3*, *FGF1*, *FGF2*, *GRO1*, *HGF*, *IGF1*, *IL1B*, *IL8*, *SFRP1*, *SFRP4*, *WNT5A*, and *WNT16*), and 7 at d 7 (*CXCL16*, *FGF13*, *HDGFRP2*, *TDGF1*, *VEGFB*, *WNT7A*, and *WNT11*). Results are consistent with a set of genes regulated by estradiol early in the estrous cycle and another set regulated by progesterone later in the cycle. The cell-signaling genes identified here as being expressed in the oviduct and endometrium could serve to regulate early embryonic development in a stage-of-pregnancy-specific manner.

Key words: embryokine, histotroph, endometrium, oviduct, maternal-embryo crosstalk

INTRODUCTION

The environment established by the mother for the preimplantation embryo plays a key role in ensuring that development proceeds in a manner that optimizes pregnancy success and postnatal development. Disruption of maternal physiology during the preimplantation period can compromise embryonic survival. Examples include the effect of establishing an abnormal ratio of estradiol to progesterone in mice (Yoshinaga and Adams, 1966) and humans (Simón et al., 1995) and exposure of female embryo transfer recipients to bisphenol A in mice (Xiao et al., 2011). Other conditions can enhance maternal capacity for supporting development, as shown in cattle for treatment with somatotropin (Moreira et al., 2002). The maternal environment during the preimplantation period can also alter the developmental program of the embryo in a manner that alters postnatal phenotype [see review by Hansen et al. (2016)]. For example, feeding a diet low in protein during the preimplantation period modified postnatal growth and accumulation of body fat in rodents (Fleming et al., 2015).

The importance of the maternal environment for embryonic development is illustrated by the consequences of embryo production in vitro. In cattle, embryos produced in vitro experience altered gene expression (Corcoran et al., 2006; McHughes et al., 2009; Gad et al., 2012), metabolism (Khurana and Niemann, 2000), lipid content (Crosier et al., 2000; Sudano et al., 2012), ultrastructure (Boni et al., 1999; Rizos et al., 2002), DNA methylation (Niemann et al., 2010), and competence to establish pregnancy (Lonergan et al., 2007; Pontes et al., 2009). Moreover, properties of the resultant offspring can be disrupted in cattle (Siqueira et al., 2017) and other species (Fernandez-Gonzalez et al., 2004; Farin et al., 2006; Ceelen et al., 2008). Transfer of embryos produced in vitro to the oviduct can mitigate at least some of these abnormalities (Enright et al., 2000; Lazzari et al., 2010; Gad et al., 2012), indicating the importance of maternal signals for optimal development.

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An important mechanism by which the maternal oviduct and endometrium direct embryonic development is through secretion of regulatory molecules called embryokines. Several growth factors can affect embryonic development in various species. Among the most studied embryokines are colony-stimulating factor 2 (**CSF2**; Sjoblom et al., 1999; Loureiro et al., 2009, 2011; Denicol et al., 2014), IGF1 (Lin et al., 2003; Jousan and Hansen, 2007; Bonilla et al., 2011), and leukemia inhibitor factor (**LIF**; Kauma and Matt, 1995; Mohamed et al., 2004; Neira et al., 2010).

In many cases, it is unknown whether molecules that affect embryonic development in vitro are present in the reproductive tract at times coincident with development of the preimplantation embryo. In addition, other regulatory molecules are likely produced by the reproductive tract that can act on the preimplantation embryo. Indeed, the embryo is poised to respond to a plethora of maternal regulatory molecules because of the wide range of growth factor and hormone receptor genes that it expresses (Graf et al., 2014; Zuo et al., 2016).

The objective of the present study was to identify potential embryokines during the first 7 d after ovulation in the cow. It is during this period that the bovine embryo develops from the zygote to the blastocyst stage, where it spends the first 4 to 5 d in the oviduct, and then moves into the uterine lumen (Betteridge and Fléchon, 1988). The approach was to collect oviductal and endometrial tissue and determine the relative amounts of expression of genes for 93 hormones, growth factors, cytokines, chemokines, and WNT-related molecules that could potentially function as embryokines.

MATERIALS AND METHODS

Synchronization of the Estrous Cycle

The reproductive status of nonlactating Holstein cows was assessed by transrectal ultrasonography and 20 cows with a detectable corpus luteum were subjected to a hormonal protocol to synchronize ovulation. On d -18 (day of expected ovulation = d 0), cows were injected i.m. with 25 mg of PGF_{2 α} (Lutalyse, Zoetis, Florham Park, NJ) followed by 100 μ g of gonadorelin (GnRH; Cystorelin, Merial Inc., Duluth, GA) on d -16 . A second, identical injection of GnRH was given on d -9 and a progesterone-containing controlled internal drug release device (CIDR, Zoetis) was inserted intravaginally. At d -4 , each cow was administered 25 mg of PGF_{2 α} i.m. and the intravaginal device was removed. Another 25 mg of PGF_{2 α} was injected at d -3 and 100 μ g of GnRH was injected i.m. at d -2 (i.e., 24 h after

PGF_{2 α}). Transrectal ultrasonography of ovaries was performed on d -4 , -1 , and 0 to confirm ovulation. A total of 15 cows were successfully synchronized and slaughtered at either d 0 ($n = 4$), 3 ($n = 4$), 5 ($n = 3$), or 7 ($n = 4$) relative to the day of ovulation. Cows within group were split into 2 groups that were slaughtered on 2 different days. Slaughter was by captive-bolt stunning and exsanguination at a commercial abattoir.

Collection of Oviductal and Endometrial Tissues and Flushings

Reproductive tracts were obtained immediately after slaughter and placed on ice. Processing of all organs was completed within a maximum of 4 h from slaughter of the first cow. Side of the reproductive tract was identified as being ipsilateral or contralateral to the side of ovulation. Ovulation of cows slaughtered at d 0 was confirmed by absence of a preovulatory follicle in 3 of 4 cows. For the remaining cow, ovaries were lost during processing and ovulation could not be confirmed.

After dissecting the oviduct free from the mesosalpinx, the caudal third of the oviduct, corresponding to the isthmus, was used to cut transversal 1-mm sections while the oviduct was gently stretched. Samples were snap frozen in liquid N₂ for evaluation of gene expression. Samples were stored in liquid N₂ until transport to the laboratory and storage at -80°C .

Uterine flushings and samples of endometrium were collected separately from both uterine horns. The mesometrium was removed and each uterine horn was clamped near the uterine body. The end near the uterotubal junction was opened with a 0.5-cm incision and 30 mL of Dulbeccos's PBS at room temperature were flushed into the uterine horn from the opposite end using an 18-gauge needle. The fluid was propelled by massage along the uterine horn through the incision. Recovered fluid was kept on ice; after centrifugation at $3,000 \times g$ for 15 min at 4°C , the supernatant fraction was obtained and stored at -20°C .

After flushing, each uterine horn was opened with a longitudinal incision along the major curvature. Intercaruncular regions of endometrial tissue were harvested from the middle section of uterine horns using a scalpel and tweezers. Three samples per cow were snap frozen, and 2 other samples were frozen in optimal cutting temperature medium (O.C.T., Sakura Finetek USA Inc., Torrance, CA) on dry ice covered with 2 methylbutyrate for immunofluorescence analyses. All samples were stored in liquid N₂ until transport to the laboratory where they were stored at -80°C .

Note that limited resources prevented collection of caruncular endometrium. Intercaruncular endometrium

was chosen for analysis because most of the surface of the endometrium is intercaruncular and the tissue is easy to identify and sample.

RNA Extraction and Gene Expression

Gene expression analysis was performed for endometrial and oviductal samples from all animals ($n = 4$ at d 0, $n = 4$ at d 3, $n = 3$ at d 5, and $n = 4$ at d 7). A total of 17 samples were subjected to analysis (from each of the 15 cows and with 2 cows being analyzed twice to assess technical variation). One snap-frozen sample of endometrium and one of oviduct of each animal were thawed and homogenized in lysis buffer from Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) using a tissue homogenizer (Tissue Master 125, OMNI International, Kennesaw, GA) for 10 s at speed 4. Homogenized tissue in lysis buffer was transferred to the silica columns of the Qiagen RNeasy Mini kit and RNA was extracted by following the manufacturer's instructions (<https://www.qiagen.com/us/shop/sample-technologies/rna/total-rna/rneasy-mini-kit/#resources>) including DNase treatment.

Abundance of specific mRNA molecules for 93 genes potentially involved in control of embryonic development was determined using the NanoString nCounter analysis system (NanoString Technologies, Seattle, WA; Geiss et al., 2008). This analytical procedure consists of gene-specific 100-mer probe pairs (i.e., one probe that captures the target gene and one probe that serves as reporter) that are hybridized to the sample in solution. The reporter probe carries a fluorescent signal, and only probe pairs that hybridize to transcripts in the sample are immobilized and captured for data collection. Probes were designed to quantify mRNA for 28 growth factors, 12 chemokines, 22 cytokines, 3 hormones, 19 WNT ligands, and 9 WNT regulatory molecules. Target genes were selected on the basis that they code for secretory molecules shown to affect preimplantation embryonic development, or that they participate in signaling pathways active during the preimplantation period such as WNT signaling. In addition to the 93 regulatory molecule genes, expression of an aminopeptidase (*ANPEP*) was also measured as an internal control because it has been shown to be highly expressed in bovine endometrium at d 5 and 7 after ovulation (Forde et al., 2009). Expression of 6 housekeeping genes (*ACTB*, *RPL19*, *ERK1*, *GAPDH*, *SLC30A6*, and *SUZ12*) was also assessed.

The 100-probe set was designed and synthesized by NanoString Technologies by identifying an optimal sequence within the target transcript that met the criteria of uniqueness in the genome and mutual independence

from the other probes in the set (Supplemental File 1; <https://doi.org/10.3168/jds.2017-13221>). In addition to the customized arrays of probes, internal controls were included in the hybridization reaction.

The level of expression of mRNA was assessed using the NanoString nCounter gene expression system as described by Geiss et al. (2008). The samples were analyzed in 6 multiplexed hybridization reactions. Purified reactions were subjected to the digital analyzer to automatically acquire images and collect data by counting the number of times the reporter probe for each gene was detected. Technical variation was assessed for both oviduct and endometrium by analyzing samples from 2 cows in duplicate. The coefficient of variation among technical replicates averaged 4.3%.

Data were first normalized to external RNA spike-in controls (to account for hybridization efficiency) and then normalized to housekeeping genes (*ACTB*, *ERK1*, *GAPDH*, *RPL19*, *SLC30A6*, and *SUZ12*) using 2 normalization factors (i.e., one to adjust for spike-in controls and one for housekeeping genes). Each normalization factor was calculated for each sample by dividing average geometric mean of spike-in controls or housekeeping genes for all samples by the geometric mean for an individual sample.

Genes were considered expressed if the number of reads was greater than 2 standard deviations above the mean of negative controls (i.e., 10.4). For cases in which transcript was detectable for some samples but not others, the value used for statistical analysis for nondetectable samples was the minimum detection level (10.4).

Data were analyzed by least-squares ANOVA using the GLM procedure of SAS for Windows, version 9.4 (SAS Institute Inc., Cary, NC). The statistical model for expression in endometrium included the fixed effects of day, side, the day by side interaction, and the random effect of cow nested within day. Orthogonal contrasts were performed to identify the pattern of variation over days, including linear, quadratic, and cubic effects of day. Two statistical models were used for ANOVA of gene expression in oviduct. Gene expression data for tissue ipsilateral to the side of ovulation were analyzed with day as a fixed effect and the random effect of cow nested within day. Day effects were separated into individual degree-of-freedom comparisons using the same contrasts described for endometrial tissue. In a separate analysis, side was included in the statistical model as a fixed effect along with day and day by side interaction. In this case, only changes between d 0 and 3 of the estrous cycle were evaluated because this is the period that coincides with embryonic development in the oviduct. Data are presented as least squares means $\pm$ standard error of the mean.

Immunofluorescence

Frozen tissue was processed on a Microm HM550 cryostatic microtome (ThermoFisher Scientific Inc., Waltham, MA) to obtain 4- μ m sections that were mounted on Superfrost plus slides (Fisher Scientific, Suwanee, GA) and kept at -80°C . Slides were fixed in ice-cold acetone for 10 min and allowed to dry for 1 h at room temperature. Rehydration was performed using Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5 containing 136.9 mM NaCl) for 20 min, followed by 1 h blocking with the same buffer containing either 10% (vol/vol) goat serum (Millipore, Billerica, MA) for WNT5A, WNT7A, and CSF2 or 10% (vol/vol) horse serum (Atlanta Biologicals, Flowery Branch, GA) for dickkopf-related protein 1 (DKK1). One tissue block and 3 to 6 tissue sections were processed for each cow. Primary antibodies included rabbit polyclonal antibodies against human WNT5A and human WNT7A (Abcam, Cambridge, MA; 98.4 and 98.6% predicted AA sequence identity with bovine WNT5A and WNT7A, respectively), a mouse IgG1 monoclonal antibody against bovine CSF2 (GM-CSF 17.2 IgG1, Washington State University Monoclonal Antibody Center, Pullman, WA); and goat polyclonal anti-human DKK1 antibody (R&D Systems Minneapolis, MN; 90.5% predicted AA sequence identity with bovine DKK1).

All antibodies were diluted with TBS containing 1% (vol/vol) goat serum, except for anti-human DKK1, which was diluted in TBS containing 1% (vol/vol) horse serum. Concentrations were 5, 10, 10, and 0.2 $\mu\text{g/mL}$ for anti-WNT5A, WNT7A, CSF2, and DKK1, respectively. As a negative control, primary antibody was substituted with IgG of the species corresponding to the primary antibody. Incubation with primary antibodies proceeded overnight at 4°C . Sections were then washed 3 times for 5 min using TBS containing 1% (vol/vol) goat serum, except for anti-human DKK1, which was washed with TBS containing 1% (vol/vol) horse serum. Secondary antibodies were diluted to a concentration of 6.66 $\mu\text{g/mL}$ and incubated with tissue at room temperature for 1 h. A goat anti-rabbit antibody conjugated to Alexa 555 was used for WNT5A, a goat anti-rabbit antibody conjugated to Alexa 488 was used for WNT7A, a goat anti-mouse antibody conjugated to Alexa 488 was used for CSF2, and a rabbit anti-goat antibody conjugated to Alexa 488 was used for DKK1. All secondary antibodies were from Life Technologies (Carlsbad, CA). Following labeling with second antibody and washing of sections with TBS, sections were incubated with 1 $\mu\text{g/mL}$ of Hoechst 33342 for 10 min to label nuclei.

Slides were mounted using ProLong antifade reagent (Life Technologies), covered with a coverslip, and

observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany) and Zeiss filter sets 02 (4,6-diamidino-2-phenylindole), 03 (fluorescein isothiocyanate filter), and 04 (rhodamine). Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera.

Western Blotting for CSF2

All reagents were purchased from Fisher Scientific unless otherwise stated. Uterine flushings were thawed and 1 mL aliquots were precipitated with ice-cold acetone (1:4 dilution, vol:vol) for 60 min at -20°C . After centrifugation at 13,000 $\times$ g for 10 min, pellets were allowed to dry and then reconstituted with double distilled water. Samples were mixed with Laemmli buffer, heated for 5 min at 95°C , and stored at -80°C until analysis. Equal volumes were loaded into precast polyacrylamide gels (Mini-PROTEAN TGX 4–15% polyacrylamide; Bio-Rad, Hercules, CA) and separated by SDS-PAGE at 110 V for 60 min. Proteins were then transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare Life Sciences, Pittsburgh, PA) in a wet system using Tris-glycine transfer buffer [0.06% (wt/vol) Tris base, 2.88% (wt/vol) glycine, 0.01% (wt/vol) SDS, 20% (vol/vol) methanol]. Protein transfer proceeded for 60 min at 65 V at 4°C . After 1 h incubation with blocking buffer (StartingBlock, Thermo Scientific), blots were probed with 1 $\mu\text{g/mL}$ of mouse IgG1 monoclonal antibody against bovine CSF2 (GM-CSF 17.2 IgG1, VMRD, Pullman, WA) diluted in blocking buffer containing 0.2% (vol/vol) Tween 20 and rocked overnight at 4°C . As a negative control, other blots were probed with an equivalent concentration of mouse IgG1. After washing, membranes were incubated with 0.1 $\mu\text{g/mL}$ goat anti-mouse IRDye 800CW conjugated anti-IgG antibody (LI-COR Biosciences, Lincoln, NE) for 1 h in the dark at 4°C . Bands were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences).

RESULTS

Expression of Putative Embryokines Expressed in Oviduct

Levels of expression of the 93 evaluated genes at each day of the estrous cycle are shown in Supplemental File 2 (<https://doi.org/10.3168/jds.2017-13221>) and the 50-highest expressed on each day are presented in Figure 1. Overall, wide variation was present in the magnitude of expression between genes. Average reads varied from 13,625 to 16 for the 50 most expressed genes. Of the 93 genes evaluated, all were expressed

in the oviduct ipsilateral to the side of ovulation on d 0 and 3 of the estrous cycle. By d 5, however, only 71 genes were expressed. Those genes whose transcripts were not detectable were *AMH*, *BMP15*, *CCL4*, *DKK4*, *IFNB1*, *IL2*, *IL3*, *IL4*, *IL5*, *IL13*, *IL17A*, *IL21*, *SFRP5*, *TSHB*, *WNT1*, *WNT3*, *WNT3A*, *WNT7B*, *WNT8A*, *WNT8B*, *WNT9B*, and *WNT10B*. Transcripts for these genes were also nondetectable at d 7. Moreover, 10 other genes were not expressed at d 7 (*FGF11*, *FGF1*, *GDF9*, *IL10*, *IL1B*, *INHBA*, *PGF*, *WNT7A*, *WNT10A*, and *WNT11*). The 10 most highly expressed genes in descending magnitude were *CXCL12*, *CTGF*, *IGF2*, *SFRP2*, *WNT5A*, *IK*, *CXCL10*, *CXCL3*, *HDGFRP2*, and *HDGF* at d 0; *CXCL12*, *CTGF*, *IGF2*, *IK*, *IGF1*, *HDGF*, *WNT5A*, *CXCL3*, *CXCL10*, and *CCL14* at d 3; *CTGF*, *GRO1*, *CXCL3*, *WNT5A*, *SFRP1*, *IL8*, *DKK3*, *CXCL12*, *IK*, and *HDGF* at d 5; and *IK*, *CTGF*, *HDGF*, *CXCL12*, *HDGFRP2*, *CXCL3*, *GRO1*, *VEGFA*, *IGF2*, and *VEGFB* at d 7 after ovulation. Of these, *CTGF*, *CXCL3*, and *IK* were among the 10 most abundant transcripts at each day.

A total of 21 genes were significantly affected by day of the estrous cycle (Table 1). Of these genes, 11 genes were most highly expressed at estrus (*CCL21*, *CTGF*, *CXCL10*, *CXCL16*, *DKK3*, *FGF10*, *IL18*, *IL33*, *IL34*, *PGF*, and *SFRP2*), 1 at d 3 (*WNT4*), 8 at d 5 (*BMP7*, *HGF*, *IL6*, *SFRP1*, *TGFB1*, *WIF1*, *WNT2*, and *WNT5A*), and 1 at d 7 (*IK*).

Further analysis was conducted to determine if gene expression was affected by side of the oviduct relative to ovulation during the first 3 d of the estrous cycle when the embryo is resident within the oviduct. There was an effect of side of the oviduct for only 5 genes (*DKK2*, *IL10*, *IL13*, *TGFB3*, and *WNT4*). In each case, expression was higher for the oviduct ipsilateral to the side of ovulation than for the oviduct contralateral to the side of ovulation (Table 2). For 9 genes (*BMP4*, *CXCL12*, *CXCL16*, *FGF10*, *GRO1*, *HDGFRP2*, *IL34*, *SFRP2*, and *VEGFB*), the effect of side of ovulation depended on the day of the estrous cycle (Table 3). All of these genes were more abundant in the oviduct ipsilateral to the side of ovulation at d 0, whereas side had little effect at d 3. Expression of *BMP4*, *CXCL12*, *GRO1*, *HDGFRP2*, *SFRP2*, and *VEGFB* was higher for the ipsilateral side at d 0 but lower for the ipsilateral side at d 3.

Expression of Putative Embryokines Expressed in Endometrium

Data for all 93 evaluated genes are summarized in Supplemental File 3 (<https://doi.org/10.3168/jds.2017-13221>) and the transcript abundance for the top 50 expressed genes at each day of the estrous cycle are

displayed in Figure 2. Overall, there was wide variation in the magnitude of gene expression. For example, average reads varied from 26,609 to 21 for the top 50 most expressed genes across days.

All genes were expressed at d 0, 3, and 7 of the estrous cycle, but only 69 of the 93 genes were expressed on d 5. Genes whose transcripts were not detected at d 5 were *AMH*, *BMP15*, *CCL21*, *CCL26*, *DKK4*, *FGF14*, *GDF9*, *IL2*, *IL3*, *IL4*, *IL5*, *IL10*, *IL13*, *IL21*, *SFRP5*, *TSHB*, *WNT1*, *WNT2B*, *WNT3*, *WNT7B*, *WNT8A*, *WNT8B*, *WNT9B*, and *WNT10B*.

The 10 most highly expressed genes in descending magnitude were *CTGF*, *CXCL12*, *WNT5A*, *WNT6*, *IGF2*, *CXCL3*, *WIF1*, *HDGF*, *IK*, and *VEGFA* at d 0; *WNT5A*, *CTGF*, *CXCL10*, *HDGF*, *CXCL3*, *IK*, *IGF2*, *CXCL12*, *VEGFA*, and *SFRP1* at d 3; *WNT5A*, *TDGF1*, *CXCL3*, *CTGF*, *SFRP1*, *GRO1*, *IK*, *HDGF*, *IGF1*, and *VEGFA* at d 5; and *TDGF*, *WNT5A*, *CTGF*, *VEGFB*, *IK*, *HDGF*, *SFRP1*, *VEGFA*, *HDGFRP2*, and *WNT7A* at d 7 after ovulation. A total of 5 genes (*CTGF*, *HDGF*, *IK*, *VEGFA*, and *WNT5A*) were among the 10 most highly expressed genes at each day of the estrous cycle.

Day of the estrous cycle affected expression of 34 genes (Table 4). Of these, 11 were most highly expressed at d 0 (*BMP7*, *CCL14*, *CCL21*, *CCL26*, *CTGF*, *CXCL12*, *IGF2*, *IL16*, *IL33*, *SFRP2*, and *WIF1*), 2 at d 3 (*HDGF*, *IL15*), 14 at d 5 (*CSF2*, *CX3CL1*, *CXCL3*, *FGF1*, *FGF2*, *GRO1*, *HGF*, *IGF1*, *IL1B*, *IL8*, *SFRP1*, *SFRP4*, *WNT5A*, and *WNT16*), and 7 at d 7 (*CXCL16*, *FGF13*, *HDGFRP2*, *TDGF1*, *VEGFB*, *WNT7A*, and *WNT11*). Only one gene (*IK*) was differentially expressed between uterine horns, being higher for endometrium ipsilateral to the side of ovulation than for endometrium contralateral to the side of ovulation (900 ± 42 vs. 788 ± 43). There were also significant interactions between day and side for 6 genes (Table 5).

Immunolocalization of Selected Embryokines Within Endometrium

Protein localization in the endometrium was evaluated for 2 known embryokines (*CSF2* and *DKK1*) and 2 WNT signaling molecules that were highly expressed in endometrium (*WNT5A* and *WNT7A*). Labeling for immunofluorescence was conducted using tissue from the day of the estrous cycle at which gene expression was highest (i.e., d 5 for *CSF2* and *WNT5A* and d 7 for *DKK1* and *WNT7A*).

The *CSF2* was localized to the luminal epithelium, glandular epithelium, and stroma (Figure 3A). The same was true for *WNT5A* (Figure 3C) except labeling in the luminal epithelium was much more intense than labeling in other cell types. The *DKK1* was lo-

Figure 1. Expression of the top 50 expressed genes in the oviduct at d 0, 3, 5, and 7 after ovulation. Data are LSM.

Table 1. Genes whose expression in the oviduct ipsilateral to the side of ovulation was affected by day of the estrous cycle within the first 7 d after ovulation¹

Gene	Normalized number of transcripts				P-value		
	d 0	d 3	d 5	d 7	Linear	Quadratic	Cubic
<i>BMP7</i>	148 ± 61	82 ± 43	151 ± 61	57 ± 61	0.011	—	—
<i>CCL21</i>	192 ± 57	100 ± 41	24 ± 57	37 ± 57	0.076	—	—
<i>CTGF</i>	3,123 ± 1,760	1,973 ± 1,244	13,625 ± 1,760	1,020 ± 1,760	—	0.013	0.002
<i>CXCL10</i>	799 ± 193	435 ± 136	249 ± 193	198 ± 193	0.058	—	—
<i>CXCL16</i>	184 ± 33	76 ± 23	126 ± 33	66 ± 33	0.080	—	0.082
<i>DKK3</i>	244 ± 355	158 ± 251	1,015 ± 355	62 ± 355	—	—	0.096
<i>FGF10</i>	173 ± 33	70 ± 23	84 ± 33	41 ± 33	0.040	—	—
<i>HGF</i>	160 ± 52	70 ± 37	166 ± 52	38 ± 52	—	—	0.093
<i>IK</i>	845 ± 150	888 ± 106	976 ± 150	1,314 ± 150	0.065	—	—
<i>IL18</i>	227 ± 59	206 ± 42	114 ± 59	78 ± 59	0.084	—	—
<i>IL33</i>	545 ± 69	360 ± 49	114 ± 69	77 ± 69	0.002	—	—
<i>IL34</i>	121 ± 33	38 ± 23	46 ± 33	17 ± 33	0.082	—	—
<i>IL6</i>	32 ± 29	60 ± 21	98 ± 29	14 ± 29	—	0.086	—
<i>PGF</i>	61 ± 18	36 ± 13	29 ± 18	10 ± 18	0.092	—	—
<i>SFRP1</i>	528 ± 350	364 ± 248	1,151 ± 350	129 ± 350	—	0.092	—
<i>SFRP2</i>	991 ± 216	284 ± 153	131 ± 216	85 ± 216	0.024	—	—
<i>TGFB1</i>	132 ± 30	70 ± 21	145 ± 30	60 ± 30	—	—	0.046
<i>WIF1</i>	370 ± 77	256 ± 55	605 ± 77	48 ± 77	—	0.022	0.004
<i>WNT2</i>	28 ± 108	39 ± 77	338 ± 108	16 ± 108	—	—	0.076
<i>WNT4</i>	61 ± 16	76 ± 11	30 ± 16	55 ± 16	—	—	0.077
<i>WNT5A</i>	916 ± 355	622 ± 251	1,341 ± 355	274 ± 355	—	—	0.092

¹Data are LSM ± SEM. — indicates $P > 0.10$.

calized primarily to endometrial stromal cells. It was not detectable in glandular epithelium but there was some labeling of luminal epithelial cells (Figure 3B). Immunoreactive WNT7A was localized to the apical surface of luminal and glandular epithelial cells and was present in stromal cells (Figure 3D).

Accumulation of CSF2 in Uterine Flushings

Immunoreactive CSF2 was detected in uterine flushings at d 3, 5, and 7 of the estrous cycle and, faintly, at d 0 (Figure 4). In each sample where immunoreactive CSF2 was present (2 of 4 samples at d 0, 4 of 4 samples at d 3, 3 of 3 samples at d 5, and 3 of 4 samples at d 7), the most prominent immunoreactive protein was of molecular weight = 24,600. This band was of higher

molecular weight than either of the 2 immunoreactive bands identified in a preparation of recombinant CSF2 produced in yeast (22,700 and 19,600). Additional lower and higher molecular weight bands were identified in some samples, particularly at d 7.

DISCUSSION

These results reveal that a large number of genes encoding for cell signaling proteins are expressed by the oviduct and endometrium of the reproductive tract of the cow during the first 7 d after ovulation. It is during this time when the bovine embryo develops to the blastocyst stage, first in the oviduct and, after d 4 to 5, in the uterus (Betteridge and Fléchon, 1988). Many of the proteins encoded by these genes are likely to play important roles in development of the preimplantation embryo. Indeed, several proteins whose genes were expressed by the oviduct and endometrium in this study have been shown to modify embryonic development in the cow. These include activin A (Trigal et al., 2011; Kannampuzha-Francis et al., 2016), bone morphogenetic protein 4 (**BMP4**; La Rosa et al., 2011), CSF2 (Loureiro et al., 2009; Denicol et al., 2014), connective tissue growth factor (**CTGF**; Kannampuzha-Francis et al., 2016), DKK1 (Denicol et al., 2014), epidermal growth factor (**EGF**; Sakagami et al., 2012), fibroblast growth factor 2 (**FGF2**; Fields et al., 2011), hepatoma-derived growth factor (**HDGF**; Gómez et al., 2014), IGF1 (Jousan and Hansen, 2007; Bonilla et al., 2011),

Table 2. Genes whose expression in the oviduct was differentially expressed between sides ipsilateral and contralateral to the side of ovulation within the first 3 d after ovulation¹

Gene	Normalized number of transcripts		P-value
	Ipsilateral	Contralateral	
<i>DKK2</i>	96 ± 24	28 ± 19	0.052
<i>IL10</i>	46 ± 12	17 ± 10	0.093
<i>IL13</i>	46 ± 12	17 ± 10	0.082
<i>TGFB3</i>	137 ± 22	61 ± 18	0.025
<i>WNT4</i>	69 ± 08	41 ± 07	0.024

¹Data are LSM ± SEM.

Figure 2. Expression of the top 50 expressed genes in the endometrium at d 0, 3, 5, and 7 after ovulation. Data are LSM.

Table 3. Genes whose expression was affected by the interaction between location of the oviduct relative to the side of ovulation and day of the estrous cycle during the first 3 d after ovulation¹

Gene	Normalized number of transcripts				<i>P</i> -value
	d 0		d 3		
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	
<i>BMP4</i>	191 ± 30	93 ± 21	114 ± 21	196 ± 21	0.004
<i>CXCL12</i>	12,385 ± 3,463	1,988 ± 2,449	3,085 ± 2,449	4,124 ± 2,449	0.063
<i>CXCL16</i>	184 ± 22	74 ± 15	76 ± 15	75 ± 15	0.010
<i>FGF10</i>	173 ± 31	56 ± 22	70 ± 22	73 ± 22	0.037
<i>GRO1</i>	202 ± 61	74 ± 43	79 ± 43	254 ± 43	0.010
<i>HDGFRP2</i>	773 ± 140	327 ± 99	274 ± 99	511 ± 99	0.011
<i>IL34</i>	121 ± 26	25 ± 18	38 ± 18	16 ± 18	0.095
<i>SFRP2</i>	991 ± 257	280 ± 181	284 ± 181	469 ± 181	0.052
<i>VEGFB</i>	473 ± 60	253 ± 42	313 ± 42	390 ± 42	0.010

¹Data are LSM ± SEM for effect of day by side interaction.

interleukin 1 β (**IL1 β**) (Paula-Lopes et al., 1998), LIF (Mohamed et al., 2004; Neira et al., 2010; Mo et al., 2014), transforming growth factor beta (**TGF β** ; Neira et al., 2010), and WNT7A (Tribulo et al., 2017). Other

proteins encoded by genes expressed in the oviduct and endometrium have been implicated in regulation of preimplantation embryonic development in other species include nerve growth factor (**NGF**; Menino et

Table 4. Genes whose expression in the endometrium was affected by day of the estrous cycle within the first 7 d after ovulation¹

Gene	Normalized number of transcripts				P-value		
	d 0	d 3	d 5	d 7	Linear	Quadratic	Cubic
<i>BMP7</i>	184 ± 27	130 ± 25	82 ± 27	28 ± 27	<0.001	—	—
<i>CCL14</i>	227 ± 49	170 ± 46	138 ± 49	90 ± 49	0.056	—	—
<i>CCL21</i>	78 ± 23	17 ± 22	10 ± 23	12 ± 23	0.064	—	—
<i>CCL26</i>	40 ± 11	17 ± 11	10 ± 11	12 ± 11	0.089	—	—
<i>CSF2</i>	43 ± 32	13 ± 30	85 ± 32	12 ± 32	—	—	0.091
<i>CTGF</i>	3,310 ± 705	2,297 ± 652	2,038 ± 705	1,462 ± 705	0.079	—	—
<i>CX3CL1</i>	98 ± 68	66 ± 63	215 ± 68	25 ± 68	—	—	0.090
<i>CXCL3</i>	951 ± 986	895 ± 913	3,560 ± 986	98 ± 986	—	0.093	0.051
<i>CXCL12</i>	2,079 ± 686	701 ± 635	604 ± 686	213 ± 686	0.077	—	—
<i>CXCL16</i>	107 ± 28	140 ± 26	167 ± 28	349 ± 28	<0.0001	0.013	—
<i>FGF1</i>	33 ± 15	51 ± 13	89 ± 15	62 ± 15	0.069	—	—
<i>FGF2</i>	186 ± 68	95 ± 63	273 ± 68	63 ± 68	—	—	0.036
<i>FGF13</i>	77 ± 8	41 ± 8	30 ± 8	106 ± 8	0.063	<0.0001	—
<i>GRO1</i>	474 ± 295	171 ± 273	986 ± 295	31 ± 295	—	—	0.035
<i>HDGF</i>	825 ± 62	1,104 ± 58	827 ± 62	743 ± 62	0.072	0.007	0.011
<i>HDGFRP2</i>	452 ± 38	374 ± 36	370 ± 38	472 ± 38	—	0.026	—
<i>HGF</i>	137 ± 37	65 ± 34	165 ± 37	55 ± 37	—	—	0.025
<i>IGF1</i>	391 ± 133	306 ± 123	759 ± 133	415 ± 133	—	—	0.030
<i>IGF2</i>	1,015 ± 212	710 ± 196	606 ± 212	424 ± 212	0.060	—	—
<i>IL1B</i>	33 ± 114	14 ± 105	333 ± 114	12 ± 114	—	—	0.060
<i>IL8</i>	47 ± 163	17 ± 151	487 ± 163	16 ± 163	—	—	0.054
<i>IL15</i>	53 ± 14	107 ± 14	53 ± 14	19 ± 14	—	—	0.054
<i>IL16</i>	60 ± 8	48 ± 8	57 ± 8	35 ± 8	0.019	0.004	0.044
<i>IL33</i>	178 ± 44	109 ± 41	99 ± 44	38 ± 44	0.040	—	—
<i>SFRP1</i>	513 ± 188	553 ± 174	1,516 ± 188	677 ± 188	0.098	0.027	0.003
<i>SFRP2</i>	195 ± 68	28 ± 63	22 ± 68	22 ± 68	0.099	—	—
<i>SFRP4</i>	47 ± 52	27 ± 48	169 ± 52	53 ± 52	—	—	0.074
<i>TDGF1</i>	159 ± 980	287 ± 907	3,654 ± 980	26,610 ± 980	<0.0001	<0.0001	0.001
<i>VEGFB</i>	442 ± 71	396 ± 66	380 ± 71	1,160 ± 71	<0.0001	<0.0001	0.021
<i>WIF1</i>	832 ± 111	202 ± 102	122 ± 111	129 ± 111	<0.001	0.008	—
<i>WNT5A</i>	1,625 ± 620	3,998 ± 574	4,185 ± 620	2,083 ± 620	—	0.001	—
<i>WNT7A</i>	36 ± 22	161 ± 20	192 ± 22	443 ± 22	<0.0001	0.007	0.003
<i>WNT11</i>	81 ± 39	15 ± 36	45 ± 39	214 ± 39	0.021	0.005	—
<i>WNT16</i>	129 ± 39	81 ± 36	178 ± 39	83 ± 39	—	—	0.058

¹Data are LSM ± SEM. — indicates $P > 0.10$.

Table 5. Genes whose expression was affected by the interaction between day of the estrous cycle and side of the reproductive tract relative to ovulation¹

Gene	Normalized number of transcripts								P-value
	d 0		d 3		d 5		d 7		
	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	
<i>DKK3</i>	222 ± 149	805 ± 129	291 ± 129	379 ± 129	814 ± 129	447 ± 149	202 ± 129	178 ± 149	0.01
<i>IL16</i>	33 ± 13	86 ± 11	42 ± 11	53 ± 11	64 ± 11	50 ± 13	40 ± 11	31 ± 13	0.04
<i>IL34</i>	18 ± 12	62 ± 10	18 ± 10	13 ± 10	25 ± 10	17 ± 12	17 ± 10	21 ± 12	0.09
<i>NGF</i>	16 ± 21	41 ± 18	16 ± 18	10 ± 18	19 ± 18	20 ± 21	184 ± 18	281 ± 21	0.06
<i>WNT2</i>	51 ± 52	330 ± 45	108 ± 45	110 ± 45	184 ± 45	148 ± 52	34 ± 45	21 ± 52	0.01
<i>WNT5B</i>	13 ± 12	61 ± 11	18 ± 11	13 ± 11	31 ± 11	26 ± 12	25 ± 11	19 ± 12	0.07

Data are LSM ± SEM.

¹Data are LSM ± SEM.

al., 1989), transforming growth factor alpha (**TGFα**) (Paria and Dey, 1990; Dardik et al., 1993), and WNT3A (Krivega et al., 2015). Further studies on the effects of the proteins encoded by each of the 93 genes expressed in the oviduct and endometrium can be useful to identify additional cell-signaling molecules that act as embryokines.

Many of the cell signaling molecules encoded by the transcripts under investigation may function to regulate physiology of the reproductive tract itself. The angiogenic factor *VEGFA* was among the 20 most highly expressed genes in the oviduct and among the 10 most highly expressed genes in the endometrium at each day of the estrous cycle. Differential expression of genes involved in angiogenesis have been related to capacity of heifers to become pregnant after embryo transfer (Ponsuksili et al., 2014). In addition, various chemokine genes were highly expressed in both oviduct (including *CXCL3*, *CXCL10*, *CXCL12*, *CCL14*, and *GRO1*) and endometrium (*CXCL3* and *CXCL10*). Chemokines participate in limiting bacterial infection in the reproductive tract (Sheldon, 2014). Another highly expressed gene in the endometrium, *WNT7A*, is involved in uterine gland morphogenesis (Dunlap et al., 2011).

Accumulation of transcripts does not necessarily mean that the protein is synthesized and placed in a location where it can act on the embryo. This is particularly a concern for genes expressed in the oviduct because tissue sections analyzed included all layers of the oviduct. Nonetheless, it is likely that accumulation of mRNA is indicative of protein synthesis in many cases. Indeed, all the proteins examined immunohistochemically (CSF2, DKK1, WNT5A, and WNT7A) were present in the endometrium. Moreover, CSF2 was secreted into the uterine lumen as indicated by the presence of immunoreactive protein in uterine flushings.

The main form of CSF2 detected in uterine flushes was of larger molecular weight (24,600) than the 2 immunoreactive bands in recombinant bovine CSF2 (22,700 and 19,600). Although the recombinant CSF2 used was produced in yeast and is subject to posttranslational modifications, it is likely that native CSF2 is more glycosylated than the recombinant protein. Multiple forms of CSF2 have previously been reported in bovine uterine fluid, with bands of 26,000, 28,000, and 40,000 (de Moraes et al., 1999). Similarly, 9 species of immunoreactive CSF2 were isolated from human lymphocytes with differences in size being due to differential glycosylation (Cebon et al., 1990). In that latter study, glycosylation was associated with reduced biological activity.

There was a great deal of variation in transcript abundance among genes. It would be overly simplistic,

Figure 3. Representative images for immunolocalization of CSF2, DKK1, WNT5A, and WNT7A in endometrium. The DNA was labeled with Hoescht 33342 (gray/blue). (A) CSF2 (gray/green) localized to luminal epithelium (LE), glandular epithelium (GE), and stroma at d 5. (B) DKK1 (gray/green) localized primarily to stroma and with some labeling in the LE. (C) WNT5A (gray/red) localized to LE, GE, and stroma at d 5. (D) WNT7A (gray/green) localized to LE, GE, and stroma at d 7. Note that the magnification varies between panels. Scale bars represent 100 μ m. Color version available online.

however, to assume that genes that are most highly expressed are more important for embryonic development. For cell-signaling molecules, active concentra-

tions required for receptor binding are usually low, in the nanomolar range (see, for example, Kannampuzha-Francis et al., 2016). Endometrial expression of 2 well-

Figure 4. Detection of CSF2 in uterine fluid by Western blotting. Each lane represents a sample of uterine flushings from individual cows at d 0 (n = 4), 3 (n = 4), 5 (n = 3), and 7 (n = 4) after ovulation. The location of molecular weight (Mol. wt) standards at 25 and 20 kDa is shown to the left of the blot, whereas the migration distances of immunoreactive bands of recombinant bovine (rBo) CSF2 are shown by the arrows to the right of the blot.

characterized embryokines, *CSF2* and *DKK1*, was low compared with many other genes. Nonetheless, transcript abundance was sufficient to allow production of detectable amounts of immunoreactive protein in the endometrium.

Examination of cyclic changes in gene expression in both oviduct and endometrium is consistent for a role of ovarian steroids in regulation of some of the genes examined. In oviduct, all 93 genes were expressed at d 0 and d 3 of the estrous cycle, but only 71 were expressed by d 5 and 61 by d 7. Of the genes significantly affected by day of the estrous cycle, 11 were most highly expressed at d 0, when concentrations of estradiol were high and those of progesterone low; only one was most highly expressed at d 7. For the endometrium, some genes were upregulated at d 0, whereas another set was upregulated at d 7, when estradiol concentrations would be low and progesterone concentrations elevated. While all genes were expressed at d 0, 3, and 7 of the estrous cycle, only 69 of the 93 genes were expressed on d 5. Of genes significantly affected by day of the estrous cycle, 12 were most highly expressed at d 0 and another 21 at either d 5 or 7. In other studies on changes in oviductal and endometrial gene expression during the estrous cycle of the cow, a fraction of genes were differentially expressed between estrus and diestrus (Bauersachs et al., 2004, 2005; Mitko et al., 2008). Steroids play a key role on the regulation of the reproductive tract. Exogenous supplementation of estradiol and progesterone during early stages of the estrous cycle alters the transcriptome of oviduct and endometrium (Groothuis et al., 2007; Forde et al., 2010). Additionally, there are differences in gene

expression in the oviduct (Gonella-Diaz et al., 2015, 2017) and endometrium (Mesquita et al., 2015) between cows having a large preovulatory follicle and large corpus luteum and cows having a small preovulatory follicle and small corpus luteum.

Earlier studies indicated that side of the reproductive tract relative to ovulation had an effect on secretion of proteins by oviduct and endometrium (Malayer et al., 1988; Williams et al., 1992) and on expression of specific genes in the endometrium including genes for the progesterone and oxytocin receptors (Araújo et al., 2014). Such a result is consistent with local regulation of the reproductive tract by the preovulatory follicle or incipient corpus luteum. In contrast, expression of few of the genes in the present experiment were affected by side of the reproductive tract. Only 14 genes were differentially expressed between oviducts ipsilateral and contralateral to ovulation, with the majority being upregulated in the ipsilateral oviduct. Moreover, expression of only 7 genes in the endometrium differed between uterine horns at 1 or more days of the estrous cycle. Consistent with our findings was the general lack of effect of side of the reproductive tract on the endometrial transcriptome (Bauersachs et al., 2005). Differences in systemic concentrations of progesterone and accumulation of hexoses and proteins in the uterus between left and right sides of the reproductive tract have been reported (Trigal et al., 2014), and it is possible that some differences between ipsilateral and contralateral sides of the reproductive tract were obscured by differences between the left and right sides of the reproductive tract as well as by the limited number of cows per group.

Endometrial tissue was collected from the middle of the uterus relative to the oviduct and vagina. This is below the upper third of the endometrium where the embryo is located at d 7 of pregnancy (Sponchiado et al., 2017). There is regional variation in endometrial gene expression, with transcript abundance for many genes varying between the anterior, middle, and posterior third of the uterus (Araújo et al., 2014; Sponchiado et al., 2017). It may be, therefore, that some of the differences in transcript abundance reported in the current paper may not be identical at other regions of the endometrium. The effects of follicle and corpus luteum size on endometrial gene expression were similar for all regions of endometrium examined, however (Araújo et al., 2014).

Taken together, results indicate that the oviduct and endometrium express a wide range of cell-signaling genes that have the potential to participate in regulation of development of the preimplantation embryo. Expression of many of these genes varies with stage of the estrous cycle, suggesting importance of both estradiol and progesterone in regulation of gene expression.

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