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Original Article

Analysis of cellular communication network factor (CCN) 4 and CCN6 expression in the endometrium during the estrous cycle and at the maternal-conceptus interface in pigs

Inkyu Yoo, Soohyung Lee, Yugyeong Cheon and Hakhyun Ka*

Department of Biological Science and Technology, Yonsei University, Wonju 26493, Korea

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***Correspondence**
Hakhyun Ka
E-mail: hka@yonsei.ac.kr

Author's Position and Orcid no.
Yoo I, Research professor,
https://orcid.org/0000-0003-2766-0634
Lee S, Graduate student,
https://orcid.org/0000-0002-1952-263X
Cheon Y, Graduate student,
https://orcid.org/0000-0002-9985-2013
Ka H, Professor,
https://orcid.org/0000-0003-0213-5884

ABSTRACT The cellular communication network factor (CCN) family proteins regulate many biological events such as angiogenesis, tumor growth, placentation, implantation, and embryogenesis. The expression and function of CCN1, CCN2, and CCN3 at the maternal-conceptus interface are established in humans and rodents, but little is known about the role of CCN4 to CCN6 in the reproductive organs in any other species. Several studies in transcriptome analysis in pigs have shown that the expression of *CCN4* and *CCN6* increases in the endometrium during early pregnancy. However, their expression, regulation, and function in the endometrium throughout the estrous cycle and pregnancy have not been fully understood in pigs. Thus, we determined the expression, localization, and regulation of *CCN4* and *CCN6* during the estrous cycle and at the maternal-conceptus interface in pigs. We found that the levels of *CCN4*, but not *CCN6*, changed during the estrous cycle. The levels of *CCN4* were greater during mid- to late pregnancy than in the early stage, and the levels of *CCN6* were greatest on Day 15 of pregnancy. *CCN4* and *CCN6* were detected in conceptus tissues during early pregnancy and in chorioallantoic tissues during the later stage of pregnancy. *CCN4* mRNA was mainly localized to epithelial cells, *CCN6* mRNAs to epithelial and stromal cells in the endometrium. In endometrial explant cultures, *CCN4* expression was increased by progesterone, and *CCN6* expression by interferon-γ. These results suggest that *CCN4* and *CCN6* may play roles in the establishment and maintenance of pregnancy by regulating the endometrial epithelial cell functions in pigs.

Keywords: endometrium, pig, pregnancy, CCNs

INTRODUCTION

The cellular communication network factors (CCNs) are a family of growth factors with six members, including cysteine-rich 61 (CYR61; CCN1), connective tissue growth factor (CTGF; CCN2), nephroblastoma overexpressed (NOV; CCN3), Wnt-induced secreted proteins-1 (WISP-1; CCN4), WISP-2 (CCN5), and WISP-3 (CCN6) (Brigstock, 2003). These CCN family members are 30-40 kDa proteins sharing four conserved modules, including insulin-like growth factor binding domain, von Willebrand type C domain, thrombospondin 1 domain, and C-terminal domain (Brigstock, 2003). CCN proteins form integrated constructions and scaffolds to place a variety of molecules in the

correct location for proper physiological events (Winterhager and Gellhaus, 2014). CCNs play important roles in mitosis, adhesion, apoptosis, and extracellular matrix production to regulate angiogenesis, tumor growth, placentation, implantation, and embryogenesis (Brigstock, 2003).

The endometrium is a tissue with a great transforming capacity and plays a pivotal role in the establishment and maintenance of pregnancy in mammalian species. Ovarian steroid hormones regulate the endometrial functions for the reproductive events such as luteolysis, implantation, maintenance of pregnancy, and parturition (Critchley et al., 2020). Endometrial expression of CCN members has been reported in humans and some animal species (Uzumcu et al., 2000; Rageh et al., 2001; Gashaw et al., 2006; Forde et al., 2010). CCN1 is expressed in epithelial and endothelial cells in the human endometrium and CCN1 expression is related to higher blood perfusion and increased expression of vascular endothelial growth factor (VEGF), suggesting that CCN1 plays a role in endometrial angiogenesis (Gashaw et al., 2006; Gashaw et al., 2008). CCN2 protein is localized to the epithelial and endothelial cells throughout the menstrual cycle and cells in the decidualized area during the secretory phase in the endometrium in humans (Uzumcu et al., 2000). In pigs and cows, CCN2 is expressed predominantly in the endometrial epithelial cells during the estrous cycle and stromal cells during early pregnancy (Moussad et al., 2002; Forde et al., 2010).

Studies using transcriptomic analysis in pigs have shown that *CCN2*, *CCN4*, and *CCN6* are expressed in the endometrium and the levels of *CCN2* are down-regulated during early pregnancy, while the levels of *CCN4* and *CCN6* are up-regulated (Zeng et al., 2019). Although the porcine endometrium does not undergo significant morphological alterations during the estrous cycle as in the human endometrium, the stage-specific expression of CCNs in the endometrium suggests that the CCN family may have important roles in the establishment and maintenance of pregnancy in pigs. However, the expression, regulation, and function of CCNs throughout the estrous cycle and pregnancy have not been fully understood in pigs. Therefore, this study was conducted to determine the expression, localization, and regulation of *CCN4* and *CCN6* in the endometrium during the estrous cycle and at the maternal-conceptus interface during pregnancy in pigs.

MATERIALS AND METHODS

Animals and tissue preparation

All experimental procedures involving animals were conducted by the Guide for Care and Use of Research Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Yonsei University (No. YWC-P120) and the National Institute of Animal Science (No. 2015-137). Sexually mature cross-bred female gilts of similar age (6-8 months) and weight (100-120 kg) were assigned randomly to either cyclic or pregnant status. Gilts assigned to the pregnant uterus status group were artificially inseminated with fresh boar semen at the onset of estrus (Day 0) and 12 h later. The reproductive tracts of gilts were obtained immediately after slaughter on either Days 0 (onset of estrous behavior), 3, 6, 9, 12, 15, or 18 of the estrous cycle (21 Days of cycle; Days 0-3, estrus; Days 3-6, metestrus; Days 6-15, diestrus; Days 15-0, proestrus) and either Days 10, 12, 15, 30, 60, 90, or 114 of pregnancy (n = 3-6/day/status). Pregnancy was confirmed by the presence of apparently normal spherical to filamentous conceptuses in uterine flushings on Days 10, 12 and 15 and presence of embryos and placenta on the later Days of pregnancy (Oestrup et al., 2009). Uterine flushings were obtained by introducing and recovering 25 mL phosphate buffered saline (PBS; pH 7.4) into each uterine horn. Chorioallantoic tissues were obtained from Days 30, 60, 90, and 114 of pregnancy (n = 3-4/day). Endometrial tissues from prepubertal gilts (n = 8; approximately 6 months of age) that have not undergone the estrous cycle with no corpus luteum formed were obtained from local slaughterhouse. Endometrial tissue, dissected free of myometrium, was collected from the middle portion of each uterine horn, snap-frozen in liquid nitrogen, and stored at -80℃ prior to RNA extraction. For in situ hybridization and immunohistochemistry, cross-sections of endometrium were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 h and then embedded in paraffin as previously described (Yoo et al., 2022).

Explant cultures

To determine the effects of the steroid hormones estradiol and progesterone and interferon-γ (IFNG) on the expression of *CCN4* and *CCN6* mRNAs in the endometrium, endometrial explant tissues obtained from prepubertal gilts were cultured as previously described (Lee

et al., 2021; Yoo et al., 2020). Endometrium was dissected from the myometrium and placed into warm phenol red-free Dulbecco's modified Eagle's medium/F-12 culture medium (DMEM/F-12; Sigma) containing penicillin G (100 IU/mL) and streptomycin (0.1 mg/mL). The endometrium was minced into small pieces using scalpel blades (2-3 mm^3), and aliquots of 500 mg were placed into T25 flasks with serum-free modified DMEM/F-12 containing 10 μg/mL insulin (Sigma), 10 ng/mL transferrin (Sigma), and 10 ng/mL hydrocortisone (Sigma). Endometrial explants were cultured immediately after mincing in the presence of 0, 0.3, 3, 30 ng/mL progesterone (Sigma) or 0, 5, 50, 500 pg/mL estradiol-17β (Sigma) for 24 h with rocking in an atmosphere of 5% CO_2 in air at 37℃. To assess the effect of interferon-γ (IFNG), endometrial tissues obtained from gilts on Day 12 of the estrous cycle were cultured in the presence of progesterone (P4; 30 ng/mL), estradiol-17β (E2; 10 ng/mL), and interleukin-1β (IL1B; 10 ng/mL; Sigma) for 24 h with rocking in an atmosphere of 5% CO_2 in air at 37℃ and additional 24 h with 0, 1, 10, or 100 ng/mL of IFNG (R&D Systems) in the presence of 30 ng/mL P4, 10 ng/mL E2, and 10 ng/mL IL1B, as previously described (Yoo et al., 2020). Explant tissues were then harvested, and total RNA was extracted for real-time RT-PCR to determine the expression of *CCN4* and *CCN6* mRNAs.

Porcine uterine endometrial epithelial cell culture

To determine the effects of interleukin-10 (IL10) on the expression of *CCN4* and *CCN6* mRNAs in endometrial epithelial cells, a porcine uterine endometrial epithelial (pUE) cell line established from the endometrium on Day 15 of pregnancy (generously provided by Dr. R. Burghardt at Texas A&M University) (Yoo et al., 2020) for *in vitro* studies was cultured in DMEM/F-12 with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA) and 1X antibiotic-antimycotic mix (Invitrogen) in 5% CO_2 in air at 37℃. Monolayer cultures of pUE cells were grown to 80% confluence in culture medium in 100-mm tissue culture dishes. Cells were serum-starved for 24 h and then treated with increasing doses (0, 1, 10, 100 ng/mL) of IL10 (Sino Biological, Beijing, China) for 24 h. In order to obtain data for each individual experiment, whole process beginning from cell culture was independently replicated three times. Each treatment within individual trial was tested in triplicate (n = 3).

Total RNA extraction and RT-PCR for *CCN4* and *CCN6* cDNAs

Total RNA was extracted from endometrial, conceptus, and chorioallantoic tissues using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations as previously described (Yoo et al., 2022). The quantity of RNA was assessed spectrophotometrically, and the integrity of RNA was validated following electrophoresis in 1% agarose gels. Four micrograms of total RNA from endometrial, conceptus, and chorioallantoic tissues were treated with DNase I (Promega) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to obtain complementary DNAs (cDNAs). The cDNA templates were then diluted 1:4 with nuclease-free water and amplified by PCR using Taq polymerase (Takara Bio) and specific primers based on porcine *CCN4* and *CCN6* mRNA sequences. The PCR conditions, sequences of primer pairs for *CCN4* and *CCN6*, and expected product sizes are listed in Table 1. The PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The identity of each amplified PCR product was verified by sequence analysis after cloning into the pCRII vector (Invitrogen).

Quantitative real-time RT-PCR

To analyze levels of *CCN4* and *CCN6* mRNAs in endometrial and chorioallantoic tissues and pUE cells, real-time RT-PCR was performed using the Applied Biosystems StepOnePlus System (Applied Biosystems) using the SYBR Green method as previously described (Yoo et al., 2022). Complementary DNAs were synthesized from 4 μg total RNA isolated from different uterine endometrial tissues, and newly synthesized cDNAs (total volume of 21 μL) were diluted 1:4 with nuclease-free water and then used for PCR. The Power SYBR Green PCR Master Mix (Applied Biosystems) was used for PCR reactions. The final reaction volume of 20 μL included 2 μL of cDNA, 10 μL of 2X Master mix, 2 μL of each primer (100 nM), and 4 μL of dH2O. PCR conditions and sequences of primer pairs are listed in Table 1. The results are reported as expression relative to the level detected on Day 0 or Day 12 of the estrous cycle for endometrial tissues, Day 30 of pregnancy for chorioallantoic tissues, or the control group in endometrial explant tissues and pUE cells after normalization of the transcript amount to the endogenous porcine ribosomal protein L7 (*RPL7*), ubiquitin B (*UBB*), and TATA box binding

Table 1. Summary of primer sequences for RT-PCR, real-time RT-PCR, and in situ hybridization and expected product sizes

Primer	Sequence of forward (F) and reverse (R) primers (5' → 3')	Annealing temperature (℃)	Product size (bp)	GenBank accession no.
Real-time and RT-PCR				
CCN4	F: CCTCTGGAGGACACTTCTGC	60	246	XM_005662842.3
	R: GACCACCTGTGCACACACTC			
CCN6	F: TGTGACTACTCCGCAGATGG	60	203	XM_021091372.1
	R: CCAGAGCAGTGATTGTCAGC			
RPL7	F: AAG CCA AGC ACT ATC ACA AGG AAT ACA	60	172	NM_001113217
	R: TGC AAC ACC TTT CTG ACC TTT GG			
UBB	F: GCATTGTTGGCGGTTTCG	60	81	NM_001105309.1
	R: AGACGCTGTGAAGCCAATCA			
TBP	F: AACAGTTCAGTAGTTATGAGCCAGA	60	262	DQ845178.1
	R: AGATGTTCTCAAACGCTTCG			
In situ hybridization				
CCN4	F: CCTCTGGAGGACACTTCTGC	60	295	XM_005662842.3
	R: AGGACTGGCCGTTGTTGTAG			
CCN6	F: TTAAAAGGGATCCGGGAAAG	60	209	XM_021091372.1
	R: TCAGGTGCCGTGTCTAACAG			

protein (*TBP*) controls by the $2^{-\Delta\Delta Ct}$ method as previously described (Livak and Schmittgen, 2001).

In situ hybridization

The nonradioactive in situ hybridization procedure was performed as described previously (Braissant and Wahli, 1998; Yoo et al., 2022), with some modifications. Sections (5 μm thick) were rehydrated through successive baths of xylene, 100% ethanol, 95% ethanol, and diethylpyrocarbonate (DEPC)-treated water. Tissue sections were boiled in citrate buffer, pH 6.0, for 10 min. After washing in DEPC-treated PBS, they were digested using 5 μg/mL proteinase K (Sigma) in 100 mM Tris-HCl and 50 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, at 37℃. After postfixation in 4% paraformaldehyde, tissue sections were incubated twice for 15 min each in PBS containing 0.1% active DEPC and equilibrated for 15 min in 5X saline sodium citrate (SSC). The sections were prehybridized for 2 h at 68℃ in a hybridization mix (50% formamide, 5X SSC, 500 μg/mL herring sperm DNA, and 250 μg/mL yeast tRNA). Sense and antisense *CCN4* or *CCN6* riboprobes labeled with digoxigenin (DIG)-UTP were denatured for 5 min at 80℃ and added to the hybridization mix. The hybridization reaction was carried out overnight at 68℃. Prehybridization and hybridization reactions were performed in a box saturated with a 5X SSC and 50% formamide solution to avoid evaporation, and no coverslips were used. After hybridization, sections were washed for 30 min in 2X SSC at room temperature, 1 h in 2X SSC at 65℃, and 1 h in 0.1X SSC at 65℃. Probes bound to the section were detected immunologically using sheep anti-DIG Fab fragments covalently coupled to alkaline phosphatase and nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate (toluidine salt) as chromogenic substrate, according to the manufacturer's protocol (Roche).

Statistical analysis

Data from real-time RT-PCR for *CCN4* and *CCN6* expression during the estrous cycle and pregnancy were analyzed by ANOVA using the general linear models procedures of SAS (Cary, NC) as previously described (Yoo et al., 2022). As sources of variation, models included day, pregnancy status (cyclic or pregnant, Days 12 and 15 post-estrus), and interactions to evaluate steady-state levels of *CCN4* and *CCN6* mRNAs. Data from real-time RT-PCR to assess the effects of pregnancy day in the endometrium (Days 10, 12, 15, 30, 60, 90, and 114) and chorioallantoic tissue (Days 30, 60, 90, and 114), the effect of E2, P4, and IFNG doses in explant tissues, and the effect of IL10 dose in pUE cells on *CCN4* and *CCN6* expression were analyzed by least-squares regression analysis. Prior to the analysis, all data were tested for normality and homogeneity of variances, and log transformation

was performed when necessary. Data are presented as mean with SEM. A p-value < 0.05 was considered significant, and p-values 0.05-0.10 were considered to indicate a trend toward significance.

RESULTS

Expression of *CCN4* and *CCN6* mRNAs in the endometrium during the estrous cycle and pregnancy

To determine whether *CCN4* and *CCN6* mRNAs are expressed in the endometrium during the estrous cycle and pregnancy in pigs, we measured their relative abundance in the endometrium during the estrous cycle and pregnancy using real-time RT-PCR analysis (Fig. 1). During the estrous cycle, the expression of *CCN4* (linear effect of the day; $p < 0.05$; Fig. 1A), but not *CCN6* (Fig. 1B), was affected by day with the greatest levels on Day 18. During pregnancy, the expression of *CCN4* was affected by day (linear effect of the day; $p < 0.01$; Fig. 1A), and the expression of *CCN6* was greatest on Day 15 (linear effect of the day; $p = 0.055$; Fig. 1B). On Days 12 and 15 of postestrus, the expression of *CCN6* was affected by day ($p < 0.05$) and day x status ($p < 0.05$) interaction (Fig. 1B).

Expression of *CCN4* and *CCN6* mRNAs in conceptus tissues during early pregnancy and chorioallantoic tissues during later stage pregnancy

Next, we determined whether conceptuses during the peri-implantation period express *CCN4* and *CCN6* mRNAs by RT-PCR using cDNAs from conceptuses on Days 12 and 15. The expression of *CCN4* and *CCN6* mRNAs was detected in conceptus tissues on both days of pregnancy (Fig. 2A). We also performed real-time RT-PCR analysis to determine if the expression of *CCN4* and *CCN6* changed in chorioallantoic tissues during Day 30 to term pregnancy. The expression of *CCN4* and *CCN6* mRNAs did not change during pregnancy (Fig. 2B).

Localization of *CCN4* and *CCN6* mRNAs in the endometrium during the estrous cycle and pregnancy

After analyzing the expression patterns of *CCN4* and *CCN6* mRNAs in the endometrium, conceptus, and chorioallantoic tissues, we determined which cell type(s) express *CCN4* and *CCN6* mRNAs in the endometrium during pregnancy using nonradioactive in situ hybridization (Fig. 3). *CCN4* mRNA was predominantly localized to endometrial and chorionic epithelial cells and weakly to in stromal cells in the endometrium and chorioallantoic tissues during mid- to late pregnancy (Fig. 3A), while *CCN6* mRNA was localized to epithelial and stromal cells during early pregnancy (Fig. 3B).

Effects of E2, P4, and IFNG on the expression of *CCN4* and *CCN6* mRNAs in endometrial tissues

Because the endometrium is a major target of ovarian steroid hormones E2 and P4, and the expression levels

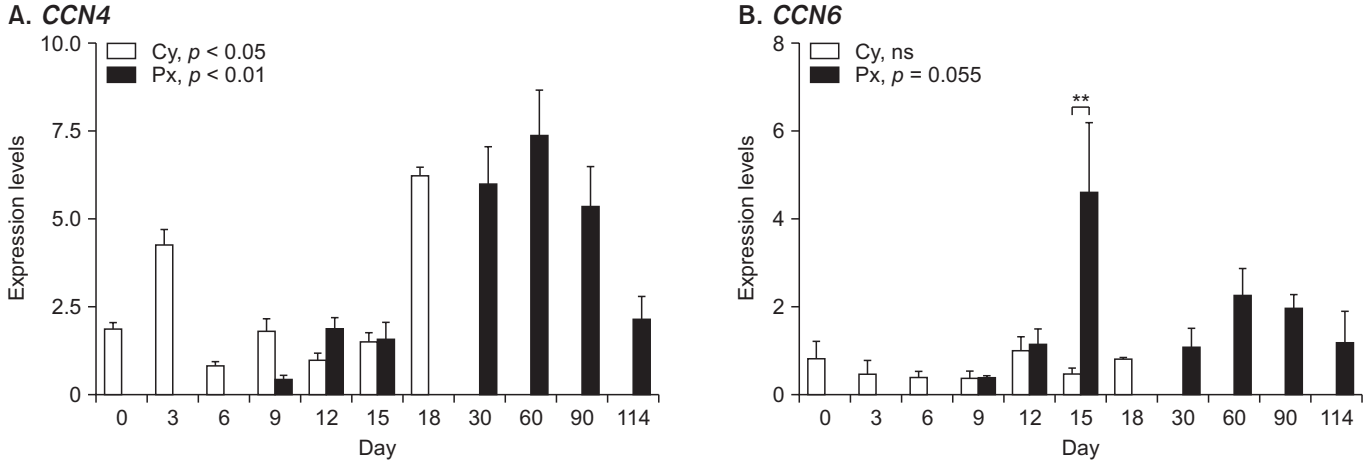

Fig. 1. Expression of *CCN4* (A) and *CCN6* (B) mRNAs in the endometrium during the estrous cycle and pregnancy in pigs. Endometrial tissue samples from cyclic (Cy) and pregnant (Px) gilts were analyzed by real-time RT-PCR, and data are reported as the expression relative to that detected on Day 12 of the estrous cycle after normalization to the transcript amount of the endogenous *RPL7*, *UBB*, and *TBP* mRNAs. Data are presented as the mean with standard error. RT-PCR, reverse transcription-polymerase chain reaction; *RPL7*, ribosomal protein L7; *UBB*, ubiquitin B; *TBP*, TATA binding protein; $^{**}p < 0.05$.

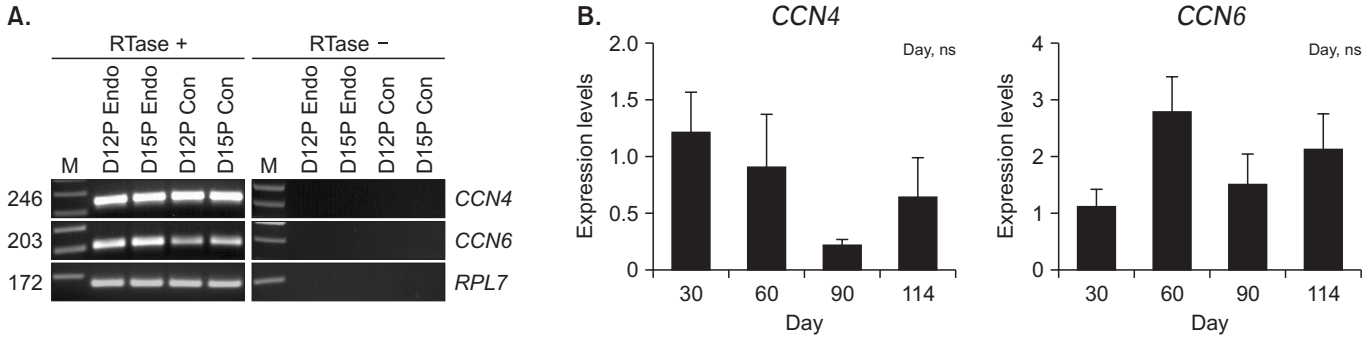

Fig. 2. Expression of *CCN4* and *CCN6* in conceptuses from Days 12 and 15 of pregnancy and chorioallantoic tissues during later pregnancy. (A) RT-PCR of *CCN4* and *CCN6* mRNA from pregnancy Days 12 and 15 conceptuses using total RNA. *RPL7* was used as a loading control. RTase +/-, with (+) or without (-) reverse transcriptase; M, molecular marker; D12P Endo, endometrium on Day 12 of pregnancy; D15P Endo, endometrium on Day 15 of pregnancy; D12 Con, Day 12 conceptus; D15 Con, Day 15 conceptus. (B) Real-time RT-PCR analysis of the expression of *CCN4* and *CCN6* mRNA in chorioallantoic tissues on Days 30, 60, 90, and 114 of pregnancy. Data are reported as expressions relative to that detected on Day 30 of pregnancy after normalization of the transcript amount to the endogenous *RPL7*, *UBB*, and *TBP* control, and are presented as mean with standard error. RT-PCR, reverse transcription-polymerase chain reaction; *RPL7*, ribosomal protein L7; *UBB*, ubiquitin B; *TBP*, TATA binding protein; ns, not significant.

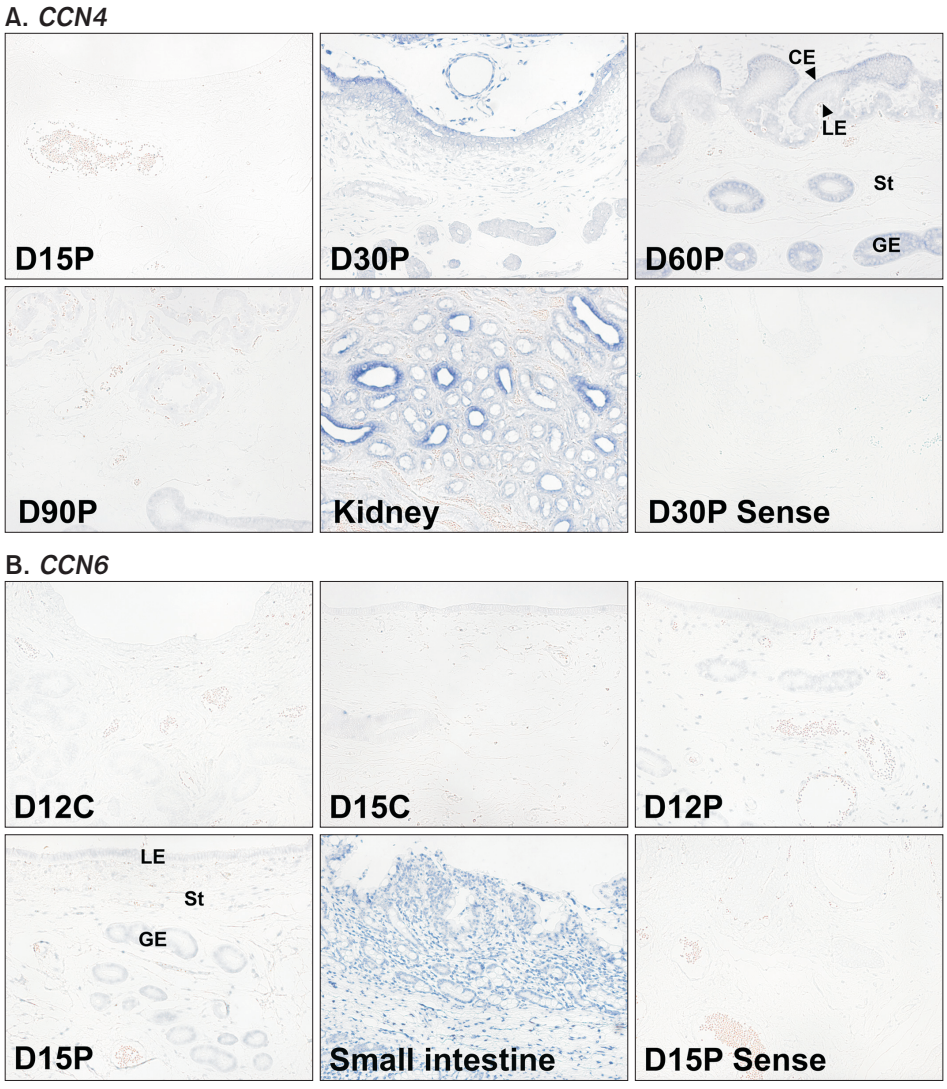

Fig. 3. Localization of *CCN4* and *CCN6* mRNA by in situ hybridization in the endometrium during the estrous cycle and pregnancy in pigs. Representative uterine sections from Day 30 and 15 of pregnancy stained with sense RNA probes are shown as negative controls for *CCN4* and *CCN6*, respectively. Tissue sections from the kidney and small intestine are shown as positive controls for *CCN4* and *CCN6* mRNA, respectively. D, Day; C, estrous cycle; P, pregnancy; LE, luminal epithelium; GE, glandular epithelium; CE, chorionic epithelium; St, stroma. Bars = 100 μm.

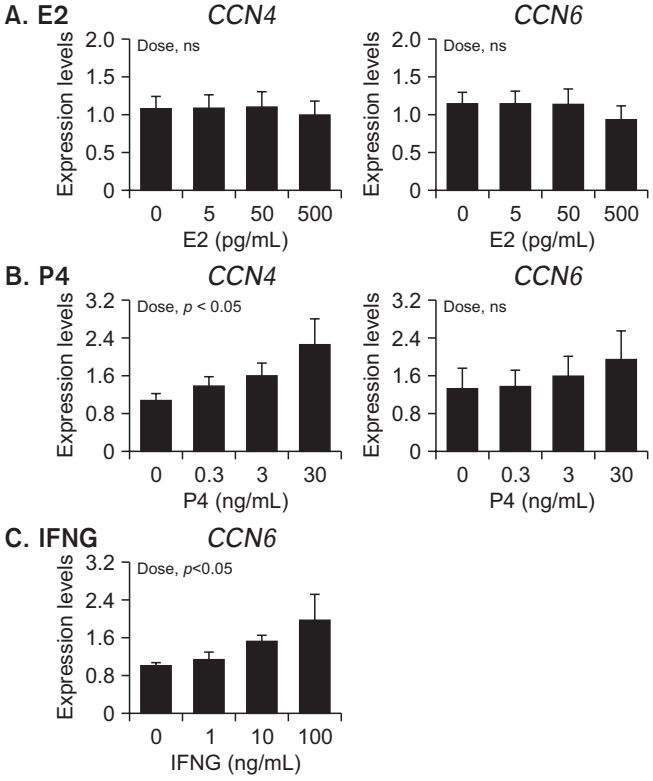

Fig. 4. Effects of E2 (A), P4 (B), and IFNG (C) on the expression of *CCN4* and *CCN6* mRNAs in endometrial explant cultures. Endometrial explants from prepubertal gilts were cultured with 0, 5, 50, 500 pg/mL E2 (estradiol-17β) or 0, 0.3, 3, 30 ng/mL P4 (progesterone) and endometrial explants from gilts on Day 12 of the estrous cycle were cultured with 0, 1, 10, 100 ng/mL IFNG. The abundance of mRNA expression determined by real-time RT-PCR analyses was relative to that for *CCN4* and *CCN6* mRNAs in the control group (0 ng/mL) of endometrial explants after normalization of transcript amounts to *RPL7*, *UBB*, and *TBP* mRNA. Data are presented as mean with standard error. These treatments were performed in triplicate using tissues obtained from each of the three gilts. RT-PCR, reverse transcription-polymerase chain reaction; *RPL7*, ribosomal protein L7; *UBB*, ubiquitin B; *TBP*, TATA binding protein; ns, not significant.

of *CCN4* mRNAs in the endometrium changed during the estrous cycle, we hypothesized that ovarian steroids may affect the expression of *CCN4* and *CCN6* in the endometrium. We treated endometrial explant tissues from prepubertal gilts with increasing doses of E2 or P4. E2 did not affect the endometrial expression of *CCN4* and *CCN6* mRNAs (Fig. 4A), whereas the abundance of *CCN4* mRNA, but not *CCN6* mRNA, was increased by increasing doses of P4 in a dose-dependent manner (linear effect of dose, $p < 0.05$) (Fig. 4B).

During pregnancy, the abundance of *CCN6* mRNA changed in the endometrium with the greatest levels on

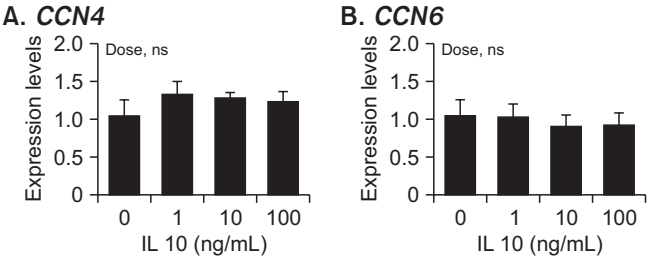

Fig. 5. Effects of IL10 on the expression of *CCN6* mRNAs in pUE cell cultures. pUE cells were cultured with 0, 1, 10, or 100 ng/mL IL10. The abundance of mRNA expression determined by real-time RT-PCR analyses was relative to that for *CCN4* and *CCN6* mRNAs in the control group (0 ng/mL) of pUE cells after normalization of transcript amounts to *RPL7*, *UBB*, and *TBP* mRNA. Data are presented as mean with standard error. These treatments were performed in triplicate using tissues obtained from each of the three gilts. pUE, porcine uterine epithelial; RT-PCR, reverse transcription-polymerase chain reaction; *RPL7*, ribosomal protein L7; *UBB*, ubiquitin B; *TBP*, TATA binding protein; ns, not significant.

Day 15 of pregnancy, when the implanting porcine conceptus secretes a significant amount of IFNs (Bazer and Johnson, 2014; Ka et al., 2018). Thus, we hypothesized that IFNG may affect the expression of *CCN6* in the endometrium during early pregnancy. When we treated endometrial tissues from Day 12 of the estrous cycle with different doses of IFNG, the abundance of *CCN6* mRNAs increased with increasing doses of IFNG (linear effect of dose, $p < 0.05$; Fig. 4C).

Effects of IL10 on *CCN4* and *CCN6* mRNA expression in pUE cells

Because the expression of *CCN4* is induced by IL10 in intestinal epithelial cells (Quiros et al., 2017) and the expression of *IL10* mRNA is the greatest on Day 15 of pregnancy in the porcine endometrium (Han et al., 2022), we hypothesized that IL10 may affect the expression of *CCN4* and *CCN6* mRNAs in pUE cells. We treated pUE cells with an increasing dose of IL10 and found that IL10 did not affect the expression of both *CCN4* and *CCN6* mRNAs in pUE cells (Fig. 5).

DISCUSSION

The novel findings of this study in pigs were 1) *CCN4* and *CCN6* were expressed in the endometrium during the estrous cycle and pregnancy in a stage- and pregnancy status-specific manner; 2) conceptuses on Days 12 and

15 of pregnancy and chorioallantoic tissues during mid- to late pregnancy expressed *CCN4* and *CCN6*; 3) *CCN4* mRNAs were predominantly localized to epithelial cells at the maternal-conceptus interface during mid- to late pregnancy and *CCN6* mRNAs were localized to endometrial epithelial and stromal cells during the peri-implantation period; and 4) P4 induced the expression of *CCN4* and IFNG induced the expression of *CCN6* in endometrial explant tissues.

Endometrial and placental CCN expression has been confirmed in both humans and rodents. In humans, CCN1 and CCN2 are expressed in the endometrium and placenta during the menstrual cycle and pregnancy and involved in stromal remodeling and neovascularization (Uzumcu et al., 2000; Gashaw et al., 2008). CCN3 is also expressed in the human placenta and plays important role in proliferation and migration of extravillous trophoblast cells (Yang et al., 2011). Increased expression of CCN1 is associated with endometriosis and polycystic ovary syndrome (Absenger et al., 2004; MacLaughlan et al., 2007), and decreased levels of CCN1 and CCN3 in the placenta and sera are associated with preeclampsia (Gellhaus et al., 2007). However, the expression and function of other CCN members, CCN4 to CCN6, in the endometrium or placenta have not been fully understood in any species. The present study demonstrated the expression of *CCN4* and *CCN6* in the endometrium throughout the estrous cycle and pregnancy and conceptus and chorioallantoic tissues during pregnancy in pigs.

Results of this study showed that the expression of *CCN4* varied during the estrous cycle with the peak level at the proestrus phase and during pregnancy with greatest levels at mid- to late pregnancy. Although morphology of the porcine endometrium during the estrous cycle does not change dramatically as does in humans and rodents, some minor morphological changes undergo in the endometrium and the distribution of immune cells in the endometrium changes during the estrous cycle depending on plasma levels of E2 and P4 in pigs (Kaeoket et al., 2001). During pregnancy, the endometrium undergoes dramatic changes in morphology and function to support the formation of epitheliochorial type placenta and fetal and placental development. In humans, CCN4 induces the expression of vascular cell adhesion molecule-1 (VCAM1) and promotes monocyte adhesion (Liu et al., 2013B). CCN4 also regulates migration and proliferation of vascular smooth muscle cells of rats (Liu et al., 2013A). Our study in pigs shows that the expression of cell adhesion molecules, including VCAM1, intercellular adhesion molecule-1, and platelet and endothelial cell adhesion molecule-1 increases in the endometrium during mid- to late pregnancy, which corresponds to the time of dramatic fetal and placental growth (Yoo and Ka, unpublished data), and the expression of macrophage markers is greatest in the endometrium during mid-pregnancy (Han et al., 2022). Thus, based on the findings of this study that *CCN4* expression was high at the proestrus phase of the estrous cycle and at mid- to late stages of pregnancy, one can speculate that CCN4 may be involved in induction of cell adhesion molecules, including VCAM1, in the endometrium and at the maternal-conceptus interface, migration and recruitment of immune cells into the endometrium, and development of placenta. However, the detailed actions of CCN4 in the endometrium during the estrous cycle and pregnancy still need to be studied.

Our results showed that the expression of *CCN6* did not change during the estrous cycle, but increased significantly on Day 15 of pregnancy, which is the time when the implanting conceptus secretes maximum levels of IFNG (Bazer and Johnson, 2014). It has been shown that CCN6 inhibits cell death by suppressing caspases and poly (ADP-ribose) polymerase activity in humans and rodents (Wei et al., 2018). Previously, we have shown in pigs that endometrial epithelial cells express caspase (CASP) 3, cleaved-CASP3, and CASP7 proteins as well as inhibitors of apoptosis proteins (IAPs) during the implantation period (Jung et al., 2021; Yoo et al., 2022), suggesting that IAPs may protect endometrial epithelial cells from undergoing apoptosis by caspases during the implantation period in pigs. Results of this study suggest that CCN6, in addition to IAPs, may also be involved in regulation of epithelial cell survival from apoptosis in the endometrium during early pregnancy in pigs.

Porcine conceptus tissues during early pregnancy and chorioallantoic tissues during mid- to late pregnancy expressed *CCN4* and *CCN6* mRNAs in this study. The expression and function of CCNs have been demonstrated in the placenta of both humans and rodents (Uzumcu et al., 2000; Rageh et al., 2001; Gashaw et al., 2006; Forde et al., 2010), but most of these studies have examined the roles of CCN1, CCN2, and CCN3, and no studies have been performed on the role of CCN4, CCN5, and CCN6 in

placental tissues in any species. Our results showed that *CCN4* mRNA was localized primarily to luminal, glandular, and chorionic epithelial cells and weakly to stromal cells during mid- to late pregnancy, while *CCN6* mRNA was localized to endometrial epithelial and stromal cells during the early pregnancy. Approximately on Day 30 of pregnancy in pigs, the attachment between the trophectoderm and the endometrial luminal epithelium is complete and rapid placental development occurs until Days 60 to 70 with an increased proliferation of endometrial and trophoblast cells (Hong et al., 2017; Almeida and Dias, 2022). The uterine-placental associations form numerous placental folds, which become increasingly complex as pregnancy progresses (Almeida and Dias, 2022). Because CCN4 promotes cell proliferation, migration, and adhesion (Liu et al., 2013A; Liu et al., 2013B; Quiros et al., 2017) and CCN6 inhibits apoptosis of epithelial cells in humans and rodents (Wei et al., 2018), it is possible to speculate that CCN4 is involved in proliferation, differentiation, migration, and adhesion of endometrial epithelial cells and cells in chorioallantoic membrane during mid- to late pregnancy, and *CCN6* is involved in survival of epithelial cells at the maternal-conceptus interface in pigs.

Because the endometrial expression of *CCN4* changed during the estrous cycle with the greatest level at the proestrus phase in this study, we hypothesized that endometrial *CCN4* and *CCN6* expression was regulated by ovarian steroids E2 and P4. Our explant culture study showed that the expression of *CCN4* was induced by P4, but not by E2, in endometrial tissues in a dose-dependent manner. The expression of *CCN6* was not affected by E2 or P4. In addition, the greatest level of *CCN6* expression in the endometrium on Day 15 of pregnancy led us to postulate that *CCN6* may be regulated by conceptus-derived IFNs. Indeed, our result showed that *CCN6* expression was upregulated by IFNG in a dose-dependent manner in endometrial explants. Because CCN4 is increased by IL10 in the intestinal epithelial cells in mice (Quiros et al., 2017), and IL10 and its receptor proteins are detected at the maternal-conceptus interface in pigs (Han et al., 2022), we postulated that epithelial expression of *CCN4* and *CCN6* was induced by IL10. However, our result showed that the expression of *CCN4* and *CCN6* was not induced by IL10 in pUE cells *in vitro*. It has been shown that CCN4 and CCN6 are induced by the WNT signaling pathway (Winterhager and Gellhaus, 2014), and WNT family members, including WNT4, WNT5A, and WNT7B, are differentially expressed in the endometrium during the estrous cycle and early pregnancy in pigs (Kiewisz et al., 2011; Zeng et al., 2019). Thus, in addition to P4 on *CCN4* expression and IFNG on *CCN6* expression, other factors, including WNT family members, may be related to the expression of *CCN4* and *CCN6* at the maternal-conceptus interface in pigs.

CONCLUSION

In conclusion, our study showed that *CCN4* and *CCN6* were expressed in the endometrium during the estrous cycle and pregnancy and in conceptus and chorioallantoic tissues during pregnancy; *CCN4* and *CCN6* mRNAs were expressed in a cell-type specifically at the maternal-conceptus interface; and P4 and IFNG increased the expression of *CCN4* and *CCN6* in endometrial tissues, respectively. Although the roles of CCN4 and CCN6 still need to be further determined, our findings suggest that CCN4 and CCN6 expressed stage-specifically at the maternal-conceptus interface may play important roles in proliferation, migration, adhesion, and survival of cells to regulate the estrous cycle and to establish and maintain pregnancy in pigs.

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