Analysis of Decorin Expression in the Uterine Endometrium during the Estrous Cycle and Pregnancy in Pigs

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ABSTRACT

Decorin (DCN) is a member of small leucine-rich proteoglycans which are ubiquitous components of the extracellular matrix. It regulates many physiological processes, such as matrix formation, collagen fibrillogenesis, angiogenesis, cancer growth, and cardiovascular diseases. It has been shown that DCN is expressed in the uterus during pregnancy and modulates implantation and decidualization for the establishment and maintenance of pregnancy in mice and humans. Expression of DCN in the uterine endometrium during pregnancy has not been investigated in pigs. Thus, this study investigated expression of DCN in the uterine endometrium during the estrous cycle and pregnancy in pigs. Uterine endometrial tissues were from day (D) 12 and 15 of the estrous cycle and D12, D15, D30, D60, D90, and D114 of pregnancy. Northern blot and real-time RT-PCR analyses showed that expression of *DCN* mRNA was detected throughout the estrous cycle and pregnancy with the highest levels during mid pregnancy. *In situ* hybridization analysis showed that *DCN* mRNA was localized to both luminal and glandular epithelia during the estrous cycle and pregnancy and also to chorionic membrane during mid pregnancy in pigs. To determine whether endometrial expression of DCN was affected by the somatic cell nuclear transfer (SCNT) procedure, *DCN* mRNA levels in the uterine endometrium from gilts with SCNT embryos on D30 of pregnancy were compared with those from gilts with normal embryos using real-time RT-PCR analysis. The result showed that *DCN* mRNA levels in the uterine endometrium were not significantly different between gilts with normal embryos and SCNT embryos. These results suggest that DCN may play an important role for endometrial tissue remodeling during mid pregnancy, and DCN expression is not affected by the SCNT procedure at the early stage of pregnancy in pigs.

(Key words : Pig, Uterus, DCN, Tissue remodeling)

INTRODUCTION

During pregnancy the uterine endometrium undergoes dramatic tissue remodeling for the establishment and maintenance of pregnancy. The species forming invasive type placentation such as humans and rodents show a unique tissue remodeling called as decidualization in which the elongated uterine stromal cells adjacent to the spiral arteries transform into enlarged and round shape of decidual cells and express numerous novel cellular products (Tang *et al.*, 1994). On the other hand, most domestic animals such as pigs and ruminants do not show any remarkable decidualization process, but the uterine endometrium undergoes a tissue remodeling during the estrous cycle and pregnancy (Johnson *et al.*, 2003). However, the molecular and cellular mechanisms of this endometrial tissue remodeling during pregnancy in domestic animals have not been much understood.

Small leucine-rich proteoglycans (SLRPs) are biologically active components of the extracellular matrix (ECM) and include biglycan (BGN), decorin (DCN), fibromodulin (FMOD), fibronectin (FN1) and lumican (LUM) (Merline *et al.*, 2009). Among the SLRP members, DCN is a ubiquitous component of paracellular matrices and a modulator of various physiological processes including collagen fibrillogenesis, angiogenesis, growth factor activity, cancer growth, and cardiovascular disease development (Merline *et al.*, 2009; Stander *et al.*, 1999; Kovanen and Pentikainen, 1999). DCN binds to various molecules such as ECM proteins, growth factors or growth factor receptors, and modulates their actions. For example, DCN binding with collagen can regulate fibril growth and assembly in extracellular matrix (Vogel *et al.*, 1984; Uldbjerg and Danielsen, 1988). DCN modulates the activity of TGFB1, 2, and 3 either directly or indirectly by regulating modulators of the TGFB signaling pathway or formation of DCN/TGFB complexes (Yamaguchi *et al.*, 1990; Li *et al.*, 2008; Schaefer *et al.*,

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2001). The interaction of DCN with EGFR induces intracellular signal transduction that up-regulates p21, a cyclin-dependent kinase inhibitor, and ultimately leads to arrest the cell cycle in the G1 phase (Santra *et al.*, 1995; Moscatello *et al.*, 1998; Wu *et al.*, 2008).

It has been shown that the expression and distribution of SLRPs are regulated during the estrous cycle and pregnancy for the preparation of decidualization and embryo implantation in the uterus (Martin and Zorn, 2003; Salgado *et al.*, 2009). Involvement of DCN in various reproductive processes has been shown in rodents (Hjelm *et al.*, 2002; Martin and Zorn, 2003), humans (Berto *et al.*, 2003), and sheep (Wu *et al.*, 2000). DCN is expressed in the endometrial stromal cells during the pre-implantation stage and involved in decidualization and invasion of trophoblasts in humans (Lysiak *et al.*, 1995). DCN and TGFB derived from decidua have inhibitory effect on proliferation, migration, and invasion of extravillous trophoblast cells, and the loss of DCN from endometrial stroma induces decidualization in mice (Martin and Zorn, 2003). Although DCN is important for the uterine tissue remodeling during pregnancy in mice and humans, expression and function of DCN during the estrous cycle and pregnancy are not known in pigs.

Somatic cell nuclear transfer (SCNT) technique is a useful tool to generate cloned animals for both basic and applied researches, but the efficiency of viable cloned animal production using the SCNT technique remains considerably low. Some reasons of the low efficiency include abnormal extra-embryonic tissue formation (Kim *et al.*, 2005; Chae *et al.*, 2006; Jouneau *et al.*, 2006), and the inappropriate uterine responsiveness to the developing conceptuses (Ka *et al*, 2008; Kim *et al.*, 2009). Our recent data also indicate that the uterine tissue remodeling is altered in the uterine endometrium with SCNT embryos compared to that with normal embryos on D30 of pregnancy in pigs (Ka *et al.*, 2008). However, expression of DCN in the uteri from gilts with SCNT embryos and normal embryos has not been studied.

Therefore, this study determined expression and distribution of *DCN* mRNA in the uterine endometrium during the estrous cycle and pregnancy and compared expression levels of *DCN* in the uterine endometrium with SCNT embryos to that with normal embryos on D30 of pregnancy in pigs.

MATERIALS AND METHODS

Animals, Embryo Transfer and Tissue Collection

All experimental procedures involving animals were conducted in accordance with the National Research Council publication *Guide for the Care and Use of Laboratory Animals* (National Academy of Science, 1996). Sexually mature naturally crossbred female pigs were assigned randomly to either cyclic or pregnant status. Oocyte collection, *in vitro* maturation, and SCNT procedures were done as previously described (Ka *et al.*, 2008). Crossbred prepubertal gilts weighing between 100 and 105 kg was used as recipients of the embryos. Estrus was induced in gilts for embryo transfer by intramuscular injection of 1,000 IU of eCG, followed by an injection of 1,500 IU hCG 72 h later. The embryos produced by SCNT were cultured for 1 or 2 days. A total of 150 embryos that were morphologically normal at the 1- or 2-stages were selected and transferred into the oviducts of the recipient gilts approximately 48 h after hCG injection. Twenty-four naturally crossbred gilts were hysterectomized on D12 and 15 of the estrous cycle and D12, 15, 30, 60, 90, or 114 of pregnancy (n=3 gilts/day/status) and three gilts with SCNT embryos on D30 of pregnancy were hysterectomized to compared with gilts with normal embryos on the same day of pregnancy. Pregnancy was confirmed by the presence of apparently normal conceptuses in uterine flushings or fetuses. Endometrium dissected from the myometrium was collected from the middle portion of the uterine horn. Endometrial tissues were snap-frozen in liquid nitrogen and stored at −80℃ for RNA and protein extraction. For in situ hybridization, cross-sections of endometrium and conceptuses were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 h and then embedded in paraffin.

Total RNA Extraction and Cloning of Porcine DCN Genes

Total RNA was extracted from endometrial tissues using TRIzol reagent (Invitrogen Life Technology, Carlsbad, CA) according to the manufacturer's recommendations. The quantity of RNA was assessed spectrophotometrically, and the integrity of the RNA was examined by gel electrophoresis using 1% agarose gels.

Two micrograms of total RNA were treated with DNase I (Invitrogen) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to obtain cDNA. The cDNA templates were then diluted 1:5 with sterile water and amplified by PCR using Taq polymerase (Takara Bio, Shiga, Japan) and specific primers based on mRNA sequence of DCN (Forward: 5'-AGACTCCCTCAGCCATGAGA-3'; Reverse: 5'-GGGCAGGAAACATCAACAGT-3'). PCR conditions were 35 cycles at 94℃ for 45 sec, 54℃ for 45 sec, and 72℃ for 1 min. 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).

Northern Blot Analysis

Total RNA (20 μg) was loaded into each lane and electrophoresed on 1% MOPS-formaldehyde agarose gels. RNA was transferred overnight onto a nylon membrane in 20X SSC. The RNA probes for *DCN* mRNAs were labeled with DIG-UTP using the DIG RNA Labeling kit (Roche, Indianapolis, IN). After transferring, the RNA was fixed to the blot by UV-cross-linking (120 mJ). Prehybridization (30 min) and hybridization (7~8 h) was carried out at 68℃ using DIG Easy Hyb (Roche). The blot was washed in low stringency buffer (2× SSC and 0.1% SDS) twice for 5 min each at room temperature and in high stringency buffer (0.1× SSC and 0.1% SDS) twice for 15 min each at 68℃. After high stringency washing, signal was detected by an alkaline phosphatase reaction (Roche) and exposure to X-ray film (Agfa-Gevaert, Mortsel, Belgium).

Real-time Quantitative RT-PCR

To analyze level of *DCN* mRNAs in the uterine endometrium, real-time RT-PCR was performed using the Applied Biosystems StepOnePlus System (Applied Biosystems, Foster City, CA) using the SYBR Green method. Complementary DNAs were synthesized from 4 μg of total RNA isolated from different uterine endometrial tissues (total volume of 21 ul), diluted 1:4 with distilled water, and newly synthesized cDNAs were used for PCR. To maximize efficiency, specific primer based on *DCN* was designed to amplify cDNA of less than 200 bp. The Power SYBR Green PCR Master Mix (Applied Biosystems) was used for amplification of *DCN*. Final reaction volume was 20 μl including 2 μl of cDNA, 10 μl of 2X premix, 2 μl of each primer, and 4 μl of DEPC-treated ddH$_2$O. Specific primers based on mRNAs sequence for DCN (Forward: 5′-ATTCAGTAAGGGAAGGAGGAAGAC-3′; Reverse: 5′-GAGATCACCAAAGTGCGAAAG-3′) and RPL7 (Forward: 5′-AAGCCAAGCACTATCACAAGGAATACA-3′; Reverse: 5′-TGCAACACCTTTCTGACCTTTGG-3′) were used. PCR conditions were 50℃ for 2 min and 95℃ for 10 min followed by 40 or 45 cycles of 95℃ for 15 sec, 60℃ for 35 sec, and 72℃ for 35 sec. Data were analyzed using Applied Biosystems software. The results were reported as the expression relative to the level detected on D12 of the estrous cycle for stage or D30 of pregnancy for comparison with SCNT samples after normalization of the transcript amount to the endogenous *RPL7* control by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Non-Radioactive *In Situ* Hybridization

The non-radioactive in situ hybridization procedure was performed as described previously (Braissant and Wahli, 1998), with some modifications. Sections (5 μm thick) were rehydrated through successive baths of xylene, 100% ethanol, 95% ethanol, DEPC-treated water, and DEPC-treated PBS. Tissue sections were permeabilized with DEPC-treated PBS containing 0.3% Triton X-100. After washing in DEPC-treated PBS, tissue sections were digested with 5 μg/ml Proteinase K (Sigma) in TE (100 mM Tris-HCl, 50 mM EDTA, pH 7.5) at 37 ℃. After postfixation in 4% paraformaldehyde, sections were incubated twice for 15 min each in PBS containing 0.1% active DEPC, and equilibrated for 15 min in 5X SSC. The sections were prehybridized for 2 h at 68℃ in hybridization mix (50% formamide, 5X SSC, and 500 μg/ml herring sperm DNA; 200 μl on each section). Sense and antisense *DCN* riboprobes labeled with DIG-UTP were denatured for 5 min at 80℃ and added to the hybridization mix. The hybridization reaction was carried out at 68℃ overnight. Prehybridization and hybridization were performed in a box saturated with a 5X SSC - 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 immunologically detected using sheep anti-digoxigenin Fab fragments covalently coupled to alkaline phosphatase and NBT/BCIP as a chromogenic substrate, according to the manufacturer′s protocol (Roche).

Statistical Analyses

Data from real-time RT-PCR analysis for *DCN* mRNA levels were subjected to least squares ANOVA using the General Linear Models procedures of SAS (Cary, NC). As sources of variation, the model included day, pregnancy status (cyclic or pregnant), and the interactions of these two factors to evaluate the steady-state level of *DCN* mRNA. Data are presented as least squares means with standard error. Data from real-time RT-PCR analysis for comparison of DCN levels in the endometrium with SCNT and normal embryos were subjected to the *t*-test procedure of SAS, and are presented as means with standard error.

RESULTS

Expression of DCN mRNA in the Uterine Endometrium during the Estrous Cycle and Pregnancy in Pigs

To determine expression of *DCN* mRNA in the uterine endometrium during the estrous cycle and pregnancy in pigs, Northern blot and real-time RT-PCR analyses were performed using uterine endometrial tissue from day D12 and 15 of the estrous cycle and D12, D15, D30, D60, D90, and D114 of pregnancy. Northern blot and real-time RT-PCR analyses showed that *DCN* mRNAs in the uterine endometrium were detectable throughout the estrous cycle and during pregnancy (Fig.

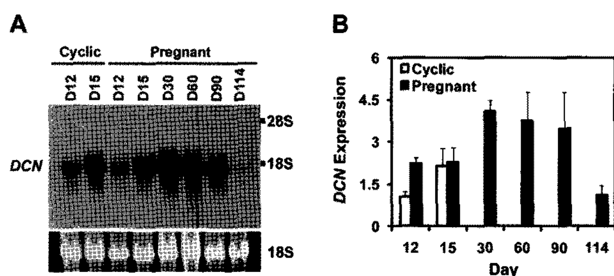

Fig. 1. **Analysis of *DCN* mRNA levels in the uterine endometrium by Northern blot (A) and real-time RT-PCR analyses (B) during the estrous cycle and pregnancy in pigs.** Endometrial tissue samples from cyclic and pregnant gilts (n=3 per day) were tested. Expression of DCN mRNA was highest during mid pregnancy, from D30 to D90 of pregnancy ($p<0.05$). Abundance of mRNA is presented as the expression relative to the level of *DCN* mRNA measured on D12 of the estrous cycle after normalization of the transcript amount to *RPL7* mRNA.

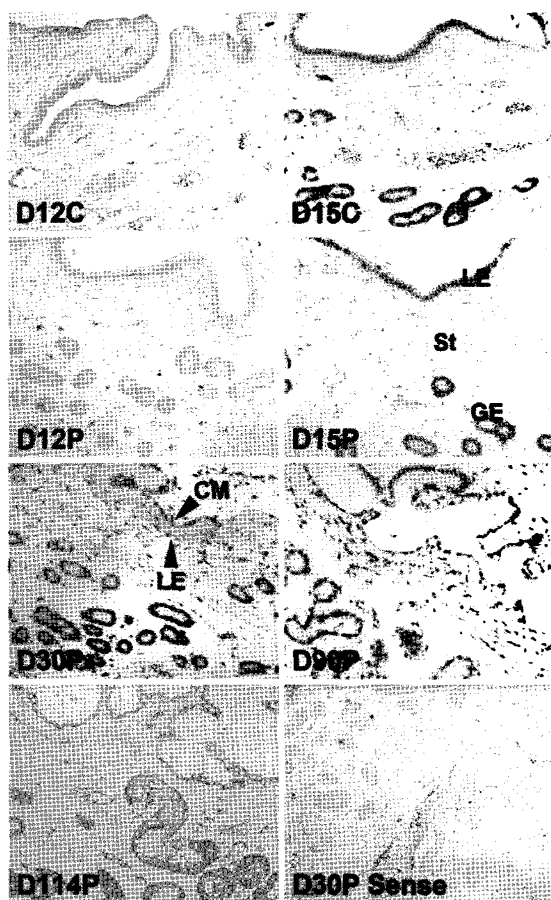

Fig. 2. **In situ hybridization analysis of *DCN* mRNA in the uterine endometrium during the estrous cycle and pregnancy in pigs.** *DCN* mRNA was localized to luminal (LE) and glandular epithelial cells (GE) during the estrous cycle and early pregnancy, increased in chorionic membrane (CM) during mid pregnancy, and decreased in LE, GE, and CM at term pregnancy. A representative uterine section from D30 of pregnancy is shown hybridized with a DIG-labeled sense *DCN* cRNA probe (Sense) as a negative control. D, day; C, estrous cycle; P, pregnancy; ST, stroma. Original magnification x100.

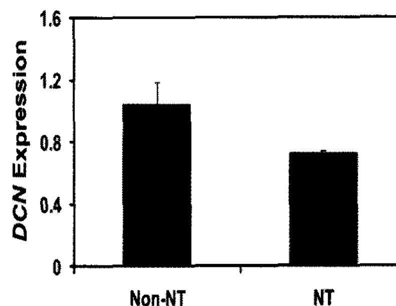

Fig. 3. **Analysis of *DCN* mRNA levels in the uterine endometrium from gilts with normal embryos and those from gilts with SCNT embryos on D30 of pregnancy using real-time RT-PCR.** Levels of *DCN* mRNA in uterine endometrium from gilts with SCNT embryos was not significantly different compared with those from gilts with normal embryos. Abundance of m-RNA is presented as the expression relative to the level of *DCN* mRNA measured in uterine endometrium from gilts with normal embryos after normalization of the transcript amount to *RPL7* mRNA.

1). Expression levels of *DCN* mRNA changed during pregnancy with the highest levels between D30 and D-90 of pregnancy ($p<0.05$) (Fig. 1).

Localization of DCN mRNA in the Uterine Endometrium during the Estrous Cycle and Pregnancy in Pigs

In situ hybridization analysis was performed to investigate localization of *DCN* mRNA in the uterine endometrium during the estrous cycle and pregnancy in pigs. Results showed that *DCN* mRNA was localized to luminal (LE) and glandular (GE) epithelia during the estrous cycle and early pregnancy and to LE, GE, stromal cells (ST) and chorionic membrane (CM) during mid pregnancy. At term pregnancy expression of *DCN* mRNA decreased in the uterine LE, GE, ST, and CM (Fig. 2).

Comparison of DCN mRNA Levels in the Uterine Endometrium with SCNT Embryos to that with Normal Embryos on D30 of Pregnancy in Pigs

To determine whether SCNT procedure affects DCN expression in the uterine endometrium on D30 of pregnancy in pigs, we performed real-time RT-PCR anaylsis to compare *DCN* mRNA levels in the uterine endometrium from surrogates with normal embryos to those with SCNT embryos on D30 of pregnancy. Abundance of *DCN* mRNA in the uterine endometrium from gilts carrying SCNT embryos was not significantly different compared to those from gilts carrying normal embryos ($p>0.05$) (Fig. 3).

DISCUSSION

This study investigated the expression and localiza-

tion of *DCN* mRNA in the uterine endometrium during the estrous cycle and pregnancy in pigs, a species forming true epitheliochorial type placentation, and found that 1) *DCN* mRNA was expressed in the uterine endometrium during the estrous cycle and pregnancy with the highest level during mid pregnancy; and 2) *DCN* mRNA was localized to LE and GE during the estrous cycle and early pregnancy, and to LE, GE, ST, and CM during mid pregnancy.

It has been shown that DCN is expressed in uterine stromal cells and myometrial cells, but not in LE and GE, except implantation site in human (Hjelm *et al.*, 2000; Berto *et al.*, 2003) and mice (Martin and Zorn, 2003; Salgado *et al.*, 2009). In sheep, DCN expression has been shown in the endometrium, myometrium and fetal membrane during labor (Wu *et al.*, 2000). Our results showed that *DCN* mRNA was mainly localized to both LE and GE during the estrous cycle and early pregnancy and to LE, GE, ST, and CM during mid pregnancy. However, it was not detected in uterine myometrial cells throughout the estrous cycle and pregnancy (data not shown). These results suggest that the difference in localization of uterine *DCN* expression among species may result from the difference in implantation and placentation type. Localization of *DCN* in ST during mid pregnancy in pigs suggests that DCN may be synthesized by endometrial ST and act as a component of ECM. In addition, *DCN* mRNA expression in endometrial epithelial cells during the estrous cycle and pregnancy suggests that DCN synthesized by the epithelial cells may be secreted into the uterine lumen and play a role in regulating histotroph function in pigs. However, secretion of DCN into the uterine lumen has not been determined so that it needs to be elucidated in the further study.

Uterine expression of *DCN* mRNA has shown to be regulated by steroid hormones in mice (Salgado *et al.*, 2009) and sheep (Wu *et al.*, 2000). DCN expression in the endometrium and myometrium is enhanced by estrogen during the estrous cycle in mice (Salgado *et al.*, 2009). In sheep, myometrial DCN expression at parturition is increased by estrogen (Wu *et al.*, 2000). In the present study, we have not determined whether DCN expression in the uterine endometrium is regulated by steroid hormones during the estrous cycle and pregnancy in pigs, but it is likely that endometrial *DCN* mRNA expression is regulated by progesterone and estrogen of ovary/placenta origin.

Molecular mechanism of DCN function in various physiological processes is not much known, but some studies suggest that DCN controls cell proliferation by interacting with TGFB. In human first trimester placenta, interaction of DCN and TGFB inhibits proliferation, migration, and invasion of extravillous trophoblast cells into the uterine endometrium (Lysiak *et al.*, 1995; Xu *et al.*, 2002). In pigs, TGFB expression in the uterine endometrium during the implantation period has been reported (Gupta *et al.*, 1998). Thus, it may be possible that TGFB and DCN interaction inhibits invasion of trophoblastic cells into the uterine endometrium because DCN is expressed in the endometrial epithelial cells at the time of conceptus implantation. Further study to test this hypothesis is needed for understanding the DCN function during implantation in pigs.

The SCNT technique is a useful tool to generate cloned animals, but the very low efficiency to produce viable offspring is a challenge to use the technique for animal cloning (Campbell *et al.*, 2005). It has been suggested that abnormal extra-embryonic tissue formation (Kim *et al.*, 2005; Chae *et al.*, 2006; Jouneau *et al.*, 2006) and inappropriate uterine responsiveness to the developing conceptuses (Ka *et al.*, 2008; Kim *et al.*, 2009) result in the low efficiency of the SCNT technique. This study investigated DCN expression to see whether uterine endometriual tissue remodeling was affected by the SCNT procedure. Results showed that expression of *DCN* mRNA was not altered in the uterine endometrium from gilts with SCNT embryos compared to that with normal embryos. This suggests that the SCNT procedure does not influence expression of DCN in the uterine endometrium and the uterine tissue remodeling mediated by DCN action.

In conclusion, this study determined that *DCN* mRNA in the uterine endometrium was expressed during the estrous cycle and pregnancy with highest levels during mid pregnancy, and localized to LE and GE during the estrous cycle and pregnancy and to LE, GE, ST, and CM after mid pregnancy. These results suggest that DCN may play an important role in the uterine endometrial tissue remodeling for the establishment and maintenance of pregnancy in pigs. Further study of the detailed cellular and molecular mechanisms of DCN action will help understanding the role of DCN in the uterine endometrium during pregnancy in pigs.

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