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Gap Junctional Connexin 37 Is Expressed in Sheep Ovaries

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The objective of current study was to evaluate the expression of Cx37 in ovarian follicles and in corpora lutea (CL) during the estrous cycle in sheep. Ovine Cx37 was cloned and characterized to design species-specific probe and primers. In Exp. 1, ovaries were collected on d 13, 14, 15, and 16 of the estrous cycle, or from FSH-induced ewes at 0, 2, 4, 8, 12, 24, and 48 h after hCG treatment on d 15 of the estrous cycle. In Exps. 2 and 3, CL were collected on d 5, 10, and 15 of the estrous cycle, or at 0, 4, 8, 12, and 24 h after prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)–induced luteal regression on d 10 of the estrous cycle, respectively. Ovarian tissues (e.g., granulosa cells, theca cells, ovarian follicles, and /or CL) were used for Cx37 immunostaining followed by image analysis or for determination of Cx37 mRNA expression by real-time RT-PCR. We demonstrated that (1) Cx37 protein was expressed in granulosa and cumulus oocyte complex compartments, ovarian blood vessels, and on the luteal cell borders, (2) expression of Cx37 mRNA was greater in granulosa than in theca cells of preovulatory follicles, (3) Cx37 mRNA expression in granulosa but not theca cells was affected by hCG treatment, (4) Cx37 protein and mRNA expression were dependent on the stage of luteal development, and (5) Cx37 expression changed during $PGF_{2\alpha}$-induced luteal regression. Thus, Cx37 may play a role in follicular development and ovulation as well as in luteal tissue growth, differentiation, and regression.

Key Words: Connexin 37; gap junctions; ovarian follicles; corpora lutea; sheep.

Introduction

Mammalian gap junctions permit direct exchange of ions, metabolites, and second messengers less than 1.5 kDa between neighboring cells *(1)*. Gap junctions are intercellular channels, composed of gap junctional proteins termed connexins *(2–5)*. Several studies demonstrated that connexin (Cx) expression is affected at different levels, from transcription to post-translational levels, and can be hormonally or developmentally regulated *(2,3,6,7)*. Gap junctions have been shown to be involved in regulation of cellular growth, metabolism, and differentiation *(2,3,8)*. In addition, gap junctions and connexins are expressed in majority of mammalian tissues including the ovaries *(2,3,7,9–5)*.

Ovarian folliculogenesis and the production of fertilizable oocytes depend on gap junctional intercellular communication (GJIC) *(14,15)*. During the growth of mammalian oocytes, there is a continuous oocyte coupling with the surrounding follicle cells via gap junctions *(16)*. Cx37 along with Cx43 seems to be involved in GJIC within granulosa cell and cumulus oocyte complex (COC) compartments in several species *(7,17–19)*.

During ovulation, structural and functional changes in the granulosa and theca layers of the preovulatory follicle cause transition of the preovulatory follicle into the corpora lutea (CL), which is a very dynamic structure exhibiting periodic growth, differentiation, and regression *(20–22)*. It has been demonstrated that granulosa, theca, and luteal cells interact through gap junctions to maintain normal ovarian function *(2,3,23)*.

Expression and function of Cx37 in the ovaries have clearly been demonstrated for mice *(9,10,14,19,24–28)*. In addition, expression of Cx37 protein or mRNA was detected in bovine ovaries *(9)* and human granulosa cells in vitro *(29)*. Cx37 is critical to ovarian function, because in mice with a targeted mutation of Cx37 gene oocytes do not achieve meiotic competence, follicles do not develop to the ovulatory stage and fail to ovulate, and numerous not fully functional CL are developing, all of which cause female infertility *(4,14,17,19,25–28)*. In addition, a recent functional study clearly demonstrated that Cx37 is specifically required in oogenesis in mice *(19)*. However, the role and expression of Cx37 in other species still remains to be elucidated.

Therefore, the aim of this study was to evaluate the protein and/or mRNA expression of Cx37 in ovarian follicles, during non-stimulated development or after treatment with FSH and hCG, and also in the CL during its growth, development, and non-induced and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)–induced regression in sheep.

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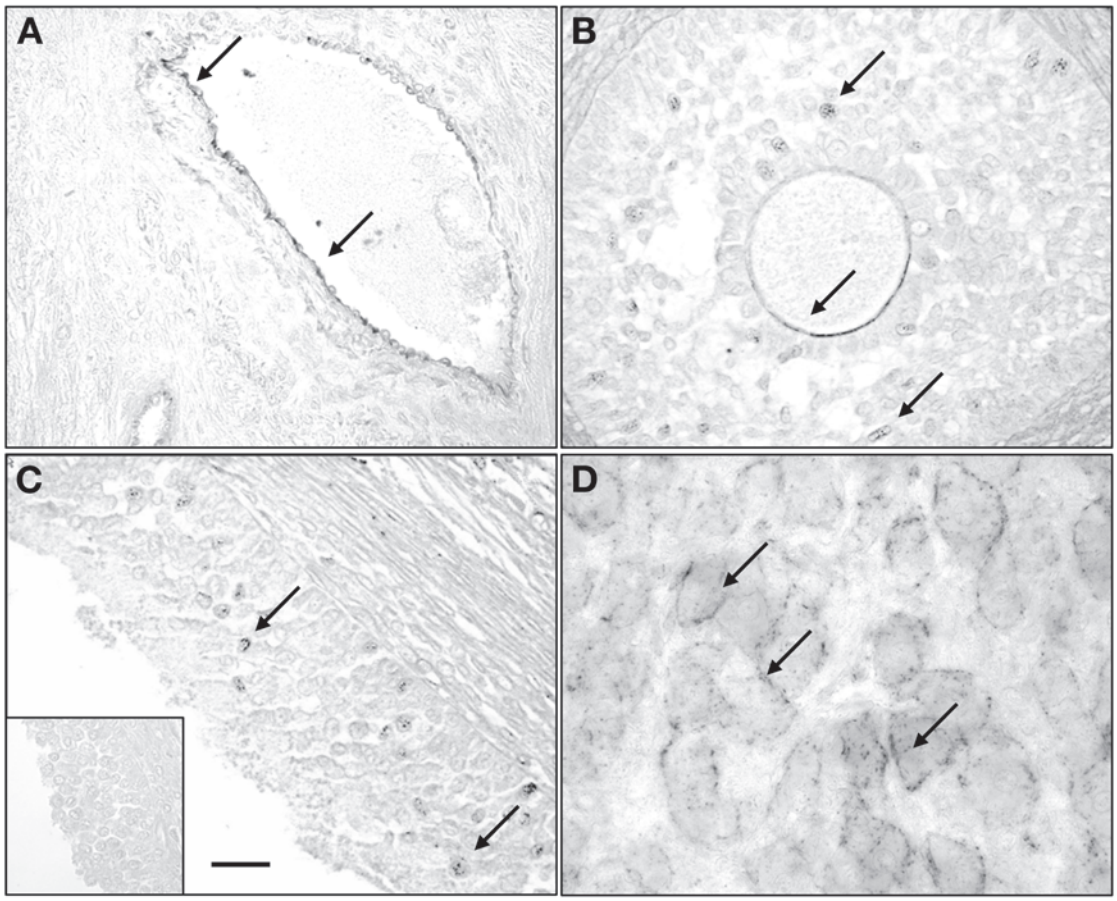

Fig. 1. Representative Cx37 protein expression (arrows) in the ovarian blood vessel (**A**), in early antral follicle (**B**), in preovulatory follicle (**C**), and in luteal tissue from d 10 of the estrous cycle (**D**). Note positive staining (arrows) in large blood vessels in ovarian stroma (**A**), in some granulosa cells (**B**, **C**), on the border between cumulus cells and oocyte (**B**), and on luteal cells borders (**D**). Control section (bottom inset in **C**) showed no positive staining for Cx37. Bar = 120 μm for **A**, and 40 μm for **B–D**.

Results

Experiment 1

Cx37 protein expression was detected in a few small and a majority of large blood vessels in the stromal tissue of ovarian cortex, medulla, and hilus (Fig. 1A). Cx37 was also detected on the border between oocyte and cumulus cells (Fig. 1B) and in some granulosa cells on cellular borders and in the cytoplasm during the follicular phase of the estrous cycle or after hCG treatment (Figs. 1B,C). The staining in theca cells was very weak and detected in only a few follicles (data not shown). However, Cx37 mRNA was detected in granulosa and theca cells of preovulatory follicles. At 0 h of hCG treatment, Cx37 mRNA expression was similar for granulosa and theca cells. In granulosa cells, Cx37 mRNA expression increased ($p < 0.01$) by fourfold at 12 h, and tended to increase ($p < 0.1$) at 8 and 48 h after hCG treatment compared with 0 h (Fig. 2A). In theca cells, Cx37 mRNA expression tended to increase ($p < 0.1$) at 12 h after hCG treatment (Fig. 2B).

Experiment 2

Cx37 protein was immunolocalized to the luteal cell borders in the CL during the estrous cycle (Fig. 1D). Expression of Cx37 protein and mRNA was the greatest ($p < 0.05$) on d 5, less on d 10, and least on d 15 of the estrous cycle (Figs. 3A,B).

Experiment 3

Compared to 0 h, Cx37 protein, but not mRNA expression in luteal tissue decreased ($p < 0.001$) at 4–24 h after $PGF_{2\alpha}$-treatment (Fig. 4). However, when the data for mRNA expression were evaluated using regression analysis, Cx37 mRNA expression was described by quadratic model ($y = 0.0015x^2 – 0.0447x + 0.996$), and tended to decrease ($p < 0.1$; $R^2 = 0.1455$) in luteal tissues from 0 to 24 h after $PGF_{2\alpha}$ treatment.

Discussion

The present study demonstrated that, in sheep, Cx37 protein is located primarily in the larger ovarian blood vessels,

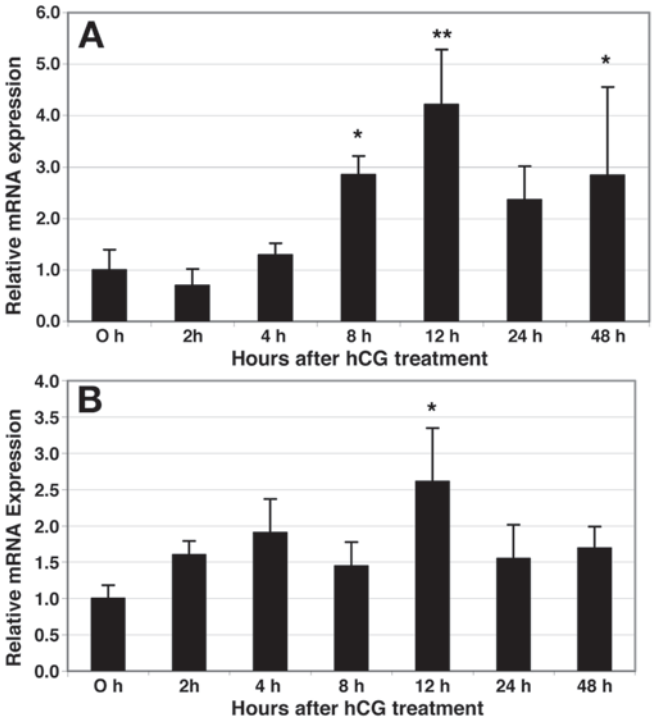

Fig. 2. Cx37 mRNA expression in granulosa cells (**A**) and in theca cells (**B**) of the preovulatory follicle after hCG treatment. Data are expressed as a fold of 0 h (arbitrary set at 1) after hCG treatment; $*p < 0.1$ and $**p < 0.01$; means ± SEM differ from 0 h.

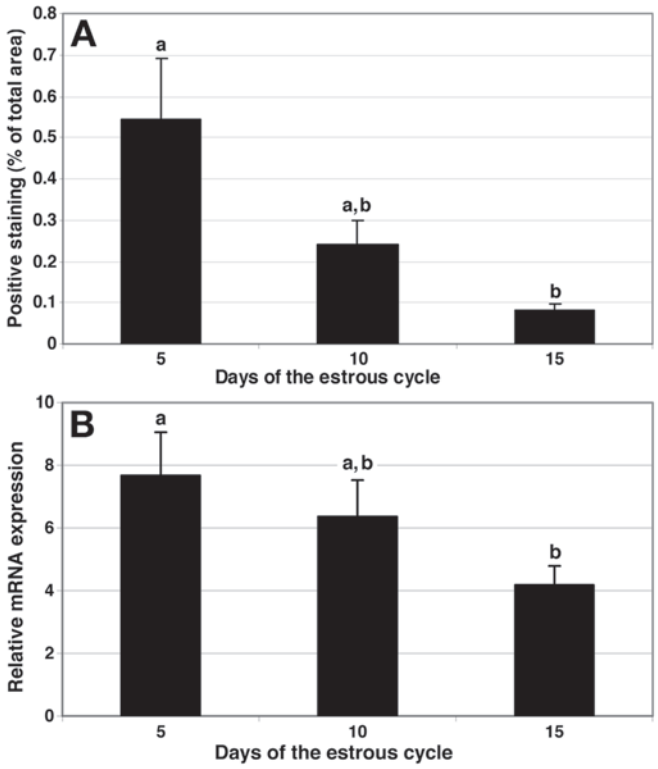

Fig. 3. Cx37 protein (**A**) and mRNA (**B**) expression on d 5, 10, and 15 of the estrous cycle; $^{a,b}p < 0.05$; means ± SEM with different superscripts differ.

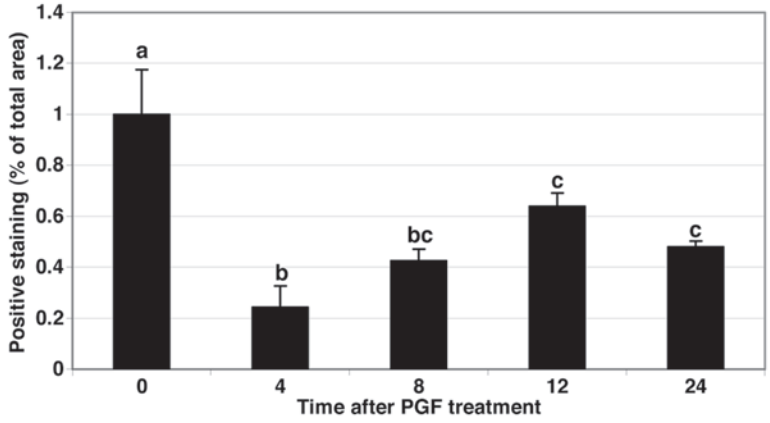

Fig. 4. Cx37 protein expression in luteal tissues after $PGF_{2\alpha}$-treatment. Data are expressed as a fold of 0 h (arbitrary set at 1) after $PGF_{2\alpha}$ treatment. Hour 0 of $PGF_{2\alpha}$ treatment was on d 10 of the estrous cycle. $^{a,b,c}p < 0.001$; means ± SEM with different superscripts differ. At 0 h, the area of Cx37 positive staining was 1.3 ± 0.2% of the total area (100%).

in some granulosa and theca cells of ovarian follicles, and on the borders between cumulus cells and the oocyte. Moreover, Cx37 mRNA is expressed in granulosa and theca cells of ovarian follicles and is affected by hCG treatment. Additionally, Cx37 protein and mRNA are expressed in luteal tissues and the expression pattern of Cx37 depends on the stage of luteal development and luteal regression.

In our study, localization of Cx37 protein in blood vessels in the ovary was consistent with detection of Cx37 protein predominantly in endothelium of large ovarian blood vessels in mice *(28,30)*. In addition, in several other tissues, Cx37 was predominantly expressed in endothelial cells and thus called a vascular connexin in numerous species *(8,31–36)*. Therefore, it is possible that Cx37 may play a role in coordinating ovarian vascular cell function.

In the current study, Cx37 protein was detected at the oocyte cumulus borders and in some granulosa and theca cells. In granulosa and theca cells, Cx37 protein was detected only in a portion of the cells. This may indicate that Cx37 forms a somewhat limited number of channels in ovar-

ian follicles in sheep, making it difficult to detect Cx37 by immunohistochemistry. In cows, Cx37 protein was detected in both oocyte and granulosa cell compartments, and was predominantly localized at the preantral stages of follicular development *(9)*. In mice, Cx37 protein was mostly restricted to cumulus cells and the oocyte, although some immunoreactivity was also found in granulosa cells more distant from the oocyte *(14,15,17,28)*. Therefore, it seems that Cx37 protein is predominantly located on oocyte–cumulus cell borders. In addition, Cx37 protein distribution in follicular tissues varies in a species-specific manner.

Previously, we have demonstrated intense staining and high expression of Cx43 protein and mRNA in granulosa cells in sheep *(11,18,37)*. In addition, based on Cx37 and Cx43 knockout models it has been demonstrated that both connexins are essential for follicular growth and development with different contributions of each of them in specific cell type within the ovarian follicle *(14,19,38)*. However, only Cx37 is obligatory for GJIC between oocyte and cumulus/granulosa cells, but Cx43 expression is not required for oocyte development in mice *(19)*. In cows, the expression pattern of Cx37 and Cx43 protein has been shown to be temporally and spatially regulated within follicular tissue indicative of their distinct physiological roles throughout folliculogenesis *(9)*. Because both Cx37 and Cx43 proteins were localized to granulosa and COC compartments, we hypothesized that both connexins contribute to building functional gap junctional channels in these compartments in sheep. However, this hypothesis remains to be tested.

The present study demonstrated that Cx37 mRNA expression was greater in granulosa than in theca cells of ovarian follicles after hCG treatment. Because gap junctions allow for signal transduction and exchange of nutrients and regulatory molecules, they are especially important within avascular or poorly vascularized tissues, like the granulosa cell layer of ovarian follicles *(2,11)*. However, because theca cells are in direct contact with ovarian capillaries, the transport of nutrients, hormones, and other signaling molecules may not require gap junctional pathways *(39)*. Therefore, the increased Cx37 mRNA expression in granulosa cells seen in our study may indicate that Cx37 and likely other connexin(s) are needed for transfer of regulatory molecules and supplying nutrients within the avascular granulosa layer and COC during periovulatory period. In addition, it has been demonstrated that Cx37 is necessary to maintain oocyte health, and likely is involved in the regulation of final maturation of the oocyte in mice *(9,14,29)*. Detection of Cx37 protein in the ovine COC in our study supports observations by others that Cx37 may play a role in the regulation of COC function.

The present study demonstrated an increase in Cx37 mRNA expression after hCG treatment in granulosa cells of ovarian follicles. Interestingly, Simon et al. *(14)* demonstrated that Cx37-deficient mice lack mature (Graafian) follicles, and thus fail to ovulate, even in response to gonadotropin stimulation, indicating the importance of Cx37 in the ovulatory process. It has been demonstrated previously that gonadotropins including hCG, LH, and/or FSH modulate GJIC and expression of gap junctions in ovarian follicles in several species *(2,3,7,40)*. In contrast to Cx37 mRNA expression in ovine granulosa and theca cells (present study), which was stimulated or maintained by hCG treatment, Cx43 protein and/or mRNA expression in ovarian follicles was decreased by hCG or LH treatment in rats *(7, 41)*, sheep *(38)*, and mice *(17)*. Taken together, these observations suggest that an increase in Cx37 expression may be associated with the preovulatory LH surge and it is very likely that Cx37 is involved in preparation of follicles for ovulation. In addition, it seems that in sheep, regulation of expression of Cx37 mRNA and Cx43 mRNA during the preovulatory hours differs, therefore Cx37 and Cx43 may each have different roles before ovulation.

In the present study, expressions of Cx37 protein and mRNA in the CL were the greatest in the early luteal phase. A similar pattern of expression was observed for Cx43 protein and/or mRNA in our other studies with a similar experimental design *(11,38)*. In addition, in this and other studies, Cx37 and Cx43 were localized on the luteal cell borders *(2,3,11,42)*. Because these two connexins can form functional heterotypic channels *(15)*, it is very likely that Cx37 and Cx43 are present together within gap junctional channels in the same cells. However, owing to some technical difficulties, we were not able to co-localize Cx37 and Cx43 using a double-staining method.

The increased expression of Cx37 mRNA in the early CL observed in our study may be associated with the very rapid rate of CL growth and cell proliferation *(21,43)*. Cx37 is also expressed in other rapidly growing tissues, such as several types of carcinomas and in early human trophoblast *(34,44)*. Interestingly, on the basis of gene knockout studies in mice, it has been reported that Cx37 appears to be critical during the time of CL formation and lack of Cx37 causes development of inappropriate CL *(14)*. Expression of Cx37 mRNA was also relatively high in the mature CL in the present study, indicating that Cx37 may also be required for a fully developed and functional CL. However, in the fully regressed CL, Cx37 protein and mRNA expression were very low. Therefore, our data suggest that Cx37 may be involved in the regulation of luteal tissue growth and differentiation.

In the current study, expression of Cx37 protein decreased as early as 4 h after $PGF_{2\alpha}$-induced luteolysis. This decrease might be the consequence of functional luteolysis, which was manifested by a decrease in peripheral progesterone concentration *(45)*. Alternatively, the lower level of progesterone may be due to interrupted GJIC associated with the lower Cx37 protein and mRNA levels that were observed in our study. Therefore, based on our results showing reduc-

tion of Cx37 protein and mRNA expression during $PGF_{2\alpha}$-induced luteal regression, we postulate that Cx37 is involved in the functional luteolysis.

Luteolytic effects of $PGF_{2\alpha}$ are mediated by nitric oxide (NO), primarily derived from endothelial cells *(46)*. Interestingly, Kameritsch et al. *(47)* demonstrated that NO has inhibitory effects on intracellular communication by suppressing the function of gap junctional channels formed by Cx37 in a human endothelial cell line. Therefore, it seems possible that enhanced NO levels during early luteolysis *(45)* could decrease Cx37 expression in our study. However, establishment of an interrelationship between Cx37 and the NO system in luteolysis would require further investigation.

In conclusion, we have demonstrated that (1) Cx37 protein was present in ovine ovarian blood vessels, in granulosa cells and oocyte compartments, and in the CL; (2) hCG treatment increased Cx37 mRNA expression in granulosa cells; (3) Cx37 protein and mRNA expression in the CL was the highest at the early luteal stage and decreased as the estrous cycle progressed; and (4) $PGF_{2\alpha}$ treatment decreased Cx37 protein and tended to decrease Cx37 mRNA expression. Because limited data are available about Cx37 among species the present study increases our knowledge about expression of Cx37 within ovarian tissues indicating that Cx37 may be involved in regulation of follicular development and ovulation, and luteal tissue growth, differentiation, and regression in sheep. In addition, these data may help us understand some of the hormonal and molecular mechanisms governing cellular communication during development and the periovulatory period in follicles and during growth, development, and regression of the CL. This information may ultimately be useful for enhancement of reproductive efficiency through our increased understanding of normal ovarian function.

Materials and Methods

Animals and Tissue Collection

The protocols and animal care for this study were approved by the Institutional Animal Care and Use Committee at NDSU. Crossbred ewes that exhibited at least one estrous cycle of normal duration (15–17 d) were used for this study. Day 0 of the estrous cycle (standing estrus) was determined by using vasectomized rams.

Experiment 1

Ewes were divided into two groups. In group 1, ovaries were collected on d 13, 14, 15, and 16 (n = 3/d) of the estrous cycle. Days 13–16 of the estrous cycle represent the follicular phase, which corresponds with dynamic follicular growth and a rise of peripheral estradiol-17β concentration *(48,49)*. The ovaries were perfused via the main artery with PBS (0.01 *M* phosphate and 0.14 *M* NaCl, pH 7.3) containing 0.1% (v:v) lidocaine to remove blood cells. Then, ovaries were fixed in Carnoy's solution for immunohistochemistry. In group 2, ewes were treated with twice daily (morning and evening) intramuscular injections of FSH with 10% LH (Sioux Biochemical, Sioux Center, IA, USA) on d 13 (5 units/injection) and 14 (4 units/injection), and both FSH (3 units/injection) and 600 IU hCG (Chorulon®, Intervet, Millsboro, DE, USA) on the morning of d 15 of the estrous cycle. Ewes were assigned randomly for collection of ovaries at 0, 2, 4, 8, 12, 24, and 48 h after hCG treatment (n = 5–7/group). Treatment with hCG was applied to mimic pre- and periovulatory period. A portion of each ovary containing two to four follicles was cut out and fixed in Carnoy's solution for immunohistochemistry. Another portion of each ovary was used to separate granulosa and theca tissues from all preovulatory follicles (>4 mm diameter) using a procedure described previously by us and others *(23,37, 50)*. Each ovary was placed into a 60-mm Petri dish containing serum-free Dulbecco's modified Eagle's medium (DMEM with 100 μg/mL heparin; Sigma, St. Louis, MO, USA). Follicular fluid was aspirated, and follicles were cut open. Granulosa cells were separated from the follicles by trituration with a siliconized Pasteur pipette. Then, theca tissue was separated from each follicle using a forceps. Granulosa and theca tissues were pooled for each ewe and snap-frozen for mRNA extraction and evaluation of Cx37 expression using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).

In addition, in order to validate the cloning and design of primers and Taqman® probe for ovine Cx37, adrenal, endometrial, kidney, skeletal muscle, small intestine, and spleen tissues were collected from four randomly chosen ewes and snap-frozen for further analysis.

Experiment 2

Ewes were treated with twice-daily injections of FSH on d 13, 14, and 15 of the estrous cycle to induce superovulation as described previously *(42)*. In the subsequent estrous cycle, ovaries were collected at the early luteal (d 5; n = 7), mid luteal (d 10; n = 6), and late luteal phase (d 15; n = 8) of the estrous cycle. Day 5 of the estrous cycle represents rapidly growing and developing CL, d 10 represents fully differentiated mature CL, and d 15 represents regressing CL *(43)*. Corpora lutea were dissected from the ovaries, one to three CL were fixed in Carnoy's solution for immunohistochemical detection of Cx37, and the remaining CL were snap-frozen for RNA extraction and qRT-PCR.

Experiment 3

Luteal regression was induced by one injection of Estrumate (cloprostenol sodium, analog of $PGF_{2\alpha}$; 2 mL/injection; 250 μg/mL; Beyer, Shawnee Mission, KS) on d 10 of the estrous cycle. Changes in the CL function and morphology in this model have been characterized by Vonnahme et al. *(45)*. Ovaries were collected at 0, 4, 8, 12, and 24 h after induction of luteal regression (n = 5 ewes/group). Then, CL were dissected and one to three CL were fixed in Carnoy's

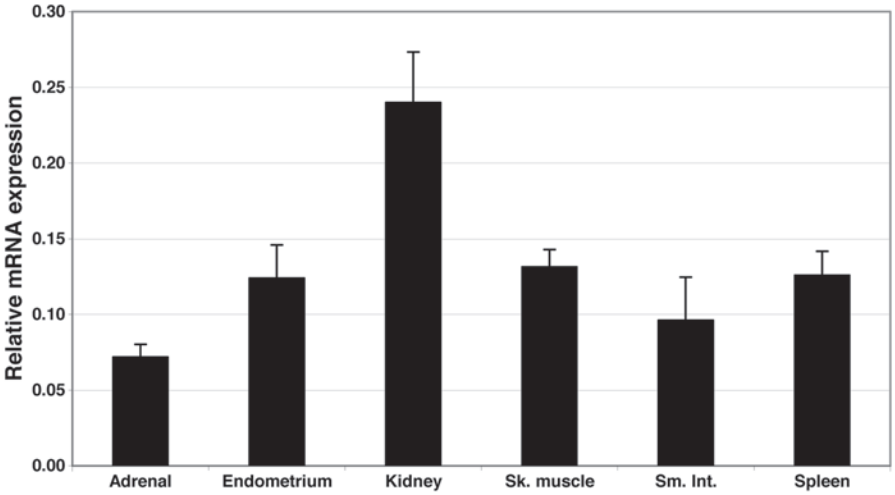

Fig. 5. Cx37 mRNA expression (means ± SEM) in ovine adrenal, endometrium, kidney, skeletal muscle (Sk. muscle), small intestine (Sm. Int.) and spleen tissues (n = 4 ewes), which was used to validate the cloning of the sheep Cx37 gene.

solution for immunohistochemical detection of Cx37. The remaining CL were snap-frozen for RNA extraction and qRT-PCR.

RNA Isolation and Reverse Transcription

RNA was isolated from the granulosa, theca, and luteal tissues by using Trizol® (Molecular Research Center, Cincinnati, OH) according to the manufacturer's recommendations. For granulosa cells, polyacrylamide carrier solution was added to facilitate better RNA yields. The Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used to determine the quantity and quality of total cellular RNA samples (Agilent, 2000). All RNA samples were reverse transcribed in triplicate 20-μL reactions using Taqman® Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Then, all cDNAs from the reverse transcription reaction were stored at –20°C prior to PCR analysis. In order to remove the inhibitory effects of heparin on RT and PCR reactions, isolated RNA from granulosa cells was processed as described before *(37)*.

Cloning of Cx37

Ovine Cx37 was cloned (accession no. AY745977) and characterized so that species-specific Taqman® probe and primers could be designed for qRT-PCR. Thus, sheep-specific cDNA clones of a portion of Cx37 were prepared by RT-PCR of tcRNA isolated from sheep adrenal tissue. For cloning, the amplicon was selected from a unique region of the Cx37 gene and the primers for Cx37 were taken from consensus regions of the gene with human sequences as the template for Cx37. Cloning primers were designed using the internet Primer3 software *(51)*. The cDNA was amplified in two rounds of PCR (5 cycles 94°C, 50°C, 72°C, and 30 cycles 94°C, 54°C, 72°C, respectively) using nested primers. The forward outside primer for Cx37 was 5'-ACTGG GGCTTCCTGGAGAA-3', starting at base 8 of the CDS, and the reverse outside primer was 5'- CCACTGGCCATA GAGGAAGC-3', starting at base 522. The forward inside primer was 5'-ACGGTGCTCTTCATCTTCC-3' starting at base 79 of the CDS and the reverse inside primer was 5'-CACCGAGATCTTGGCCATC-3' starting at base 417 of the CDS. Then, the PCR products were extracted from the gel, purified, ligated into the pGEM-T Easy vector system (Promega Corporation, Madison, WI), cloned into *Escherichia coli* JM109, and sent for sequencing to MWG Biotech (High Point, NC). The sequences were compared with connexin sequences from other species and tissues by using Entrez (National Center for Biotechnology Information; NIH), DNASIS, and CLUSTALW multiple alignments *(52)*. In order to determine if the developed probe could be used to detect Cx37 mRNA in tissues known to express Cx37, Cx37 mRNA expression was evaluated in ovine adrenal tissues, endometrium, kidney, skeletal muscle, small intestine, and spleen, as described below (Fig. 5).

Real-Time Quantitative RT-PCR

Expression of Cx37 mRNA was determined using the ABI PRISM® 7000 Sequence Detection System and software. The primers and Taqman® probe were designed using the Primer Express software version 2.0 (Applied Biosystems) (Table 1). For standard curves, which were included in each assay, cDNA was prepared from ovine placentome RNA and used for Cx37 and for 18S determinations. Then, Cx37 mRNA was normalized to 18S rRNA by dividing each of the mRNA values by their corresponding 18S rRNA value *(37)*.

Immunohistochemistry

After fixation, ovarian and luteal tissues were dehydrated, embedded in paraffin (Paraplast X-TRA, Tyco Healthcare Group, Mansfield, MA), then sectioned (4 μm) and mounted onto glass slides. Sections of tissues were rinsed several times

Table 1
Sequence of TaqMan Primers and Probes for Cx37

Oligonucleotide[a]	Nucleotide sequence	Accession number[b]
Sheep Cx37 FP	5'-CTG CAC CAA CGT CTG CTA TGA-3'	AY745977
Sheep Cx37 RP	5'-GCA CCC AGT AGC GGA TGT G-3'	
Sheep Cx37 Probe	5'-(6FAM) CAG GCC TTC CCC ATC-(MGBNFQ)-3'	

[a]FP, forward primer; RP, reverse primer.
[b]Nucleotide sequences for ovine-specific genes were obtained from the National Center for Biotechnology Information (NCBI, 2003) database.

in PBS containing Triton-X100 (0.3%, v/v) and then were treated for 20 min with blocking buffer [PBS containing Triton-X100 (0.3%) and normal goat serum (1–2%, v/v)] followed by incubation with a rabbit polyclonal antibody against Cx37 (Alpha Diagnostics, San Antonio, TX). After overnight incubation at 4°C, primary antibody was detected by using a biotinylated secondary antibody (goat anti-rabbit IgG; Boehringer Mannheim, Indianapolis, IN) and the ABC Vectastain Elite kit with Vector SG (Vectors Labs, Burlingame, CA). Control sections were incubated with normal rabbit serum in place of primary antibody *(11,42,45)*.

Image Analysis

Digital images of ovarian tissues were obtained with a Nikon DXM 1200F digital camera and Nikon Eclipse E800 microscope. Image acquisition and microscope shutters were controlled by ACT-1 (Nikon ACT-1 2.63, 2000) software and *z*-axis focus was controlled by MetaMorph (Universal Imaging Corp., Downingtown, PA) using Remote Focus Accessory (Nikon Inc., Melville, NY, USA). Z-series optical sections through each analyzed tissue area were obtained in 0.5 μm steps and the stack of eight images was created. The stack arithmetic operation of MetaMorph (Version 6.2r6) was performed in order to reconstruct the anatomical details of the tissue within the 4 μm tissue depth of the analyzed area. Images of luteal tissues were used to determine Cx37 protein expression pattern in the CL. The percentage of the total area that exhibited positive staining was evaluated quantitatively with image analysis (Image Pro-Plus, Media Cybernetics, Silver Spring, MD) as described previously *(42)*. For each CL, 10 randomly chosen fields were evaluated in each tissue section (n = 10 measurements/CL).

Statistical Analysis

Data were analyzed using the general linear model (GLM) procedure of SAS *(53)* and presented as means ± SEM. When the F test was significant ($p < 0.05$), differences between specific means were evaluated by using least significant differences test *(54)*. In addition, the pattern of change of Cx37 expression over time was evaluated by regression analysis using PROC REG of SAS *(53)* and the best-fit curves (greatest R^2) were selected.

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