Gene Expression, Vol. 13, pp. 283–297
Printed in the USA.

1052-2166/06 $90.00 + .00
E-ISSN 1555-3884
www.cognizantcommunication.com

Expression of the Prion Protein Gene (PRNP) and Cellular Prion Protein (PrPc) in Cattle and Sheep Fetuses and Maternal Tissues During Pregnancy

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We investigated the expression of prion protein gene both on mRNA and protein levels in bovine and ovine female reproductive organs during gestation and various tissues of their fetuses. The fetal tissues of both species included brain, cotyledon, heart, intestine, kidney, liver, lung, and muscle. In cattle, prion protein gene (PRNP) transcripts were detected by semiquantitative RT-PCR in reproductive tissues such as ovary, oviduct, endometrium, myometrium, follicles, and granulosa cells. In various tissues of 2-month-old fetuses, higher expression levels were found in brain and cotyledon compared to the other tissues. To detect the expression of the gene transcript in in vivo preimplantation embryos and 1-month-old fetuses, real-time PCR was performed showing that the level of gene expression in zygote stage was significantly higher ($p \leq 0.05$) than that of the other stages. Sheep were categorized as resistant (R1) or high susceptible (R5) to scrapie according to their PRNP genotype. In both genotype groups, the PRNP mRNA was detectable in all tissues studied including ovary, oviduct, endometrium, myometrium, and caruncle of ewes and all tissues of 2-month-old fetuses of both groups. Comparison between reproductive organs demonstrates the highest expression level in caruncle tissue of R1 ewes, whereas the level was high in brain and low in liver of both R1 and R5 fetuses. In addition, real-time RT-PCR was performed in immature oocytes, mature oocytes, in vivo embryos at morula stage, and 1-month-old fetuses. The results showed that the relative expression levels of the ovine PRNP mRNA in mature oocytes and morula stage embryos were significantly lower than those in immature oocytes and 1-month-old fetuses ($p \leq 0.05$). Western blot analyses revealed the immunoreactive bands corresponding to the cellular prion protein (PrPc) in all maternal and fetal tissues examined of both cattle and sheep. Moreover, immunohistochemical staining implicated localization of the PrPc in ovarian cortex and ovarian medulla of both species. However, PrPc was not detected in oocyte, granulosa cells, theca cells, and corpus luteum in this study.

Key words: Prion protein gene (PRNP); Cellular prion protein (PrPc); Prion; Preimplantation embryo; Reproductive organs; Pregnancy; Fetus

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INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorder of the central nervous system (CNS) caused by prions. Prion diseases include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, Creutzfeldt Jakob disease, Gerstmann Sträussler syndrome, fatal familial insomnia, kuru and alpers syndrome in humans. The primary cause of these diseases is a posttranslational conformation change in a host-encoded cellular prion protein (PrPc) to a proteinase-resistant form (PrPsc) (30).

Prion protein gene (PRNP) has been shown to be expressed at high levels in CNS but also in many nonneuronal tissues such as heart, skeletal muscle, lung, intestinal tract, spleen, testis, ovary, lymphocytes, and lymph nodes and some other organs in rodents and ruminants (3,20,22,28). PrPc is a normal protein located on the exterior cell surface and is attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (37). This protein is believed to be involved in several functions in the cell such as protection against antioxidant activity by regulating copper ion concentration (5,6,19), transduction of neuroprotective signals, and prevention of apoptosis in retinal cells (8), although its precise function(s) remains unclear.

Susceptibility to scrapie in sheep is influenced by polymorphisms at codons 136, 154, and 171 of the PRNP (40). The ovine PRNP genotypes could be classified into five risk groups: R1 genotype being the resistant and R5 genotypes being high susceptible to the disease (11). Tuo et al. (42) reported that accumulation of PrPsc in uterine placental epithelial cells in the placentome was determined by fetal PRNP genotype and the pregnancy status of scrapie-infected ewes. In cattle, so far no association of PRNP polymorphism with susceptibility to TSE has been be shown.

In natural TSE infections, the gastrointestinal tract is considered to be the major route of the infection (2,17,29). Oral and parenteral inoculation of PrPsc resulted in 100% infection of the prion disease in mice (21,26,27). Epidemiology shows that maternal vertical transmission of TSE is not of importance; however, embryo transfer experiments performed in sheep provide contradictory results about the possibility of this mode of infection, including those providing support for maternal vertical infection (14,15) or those showing prevention of vertical infection by embryo transfer (13,45,46).

Many studies showed that mice devoid of functional PRNP are resistant to scrapie and do not allow propagation of the infectious agent (4,7,32–34). Because the expression of PRNP is a prerequisite for the infection to start, studies on the temporospatial expression of PRNP in fetal and maternal tissue during gestation can provide further clues on the impact of maternal vertical transmission of TSE. Moreover, these expression profiles contribute to the elucidation of the physiological function of the protein. Therefore, the aims of this study were to determine the expression of the gene transcript and the normal protein during prenatal stages in cattle and sheep fetal and maternal tissues.

MATERIALS AND METHODS

Animals and Sampling

Bovine reproductive tissues such as ovary, oviduct, endometrium, myometrium, follicles, and granulosa cells of adult cows of the breeds Holstein-Frisian and Simmental at the first month of pregnancy were used in this experiment ($n = 2$). To study the expression of the PRNP in fetal tissues at the prenatal stage, the whole fetuses at 19 days and 1 month of age, and various tissues of brain, cotyledon, heart, intestine, kidney, liver, lung, and muscle from 2-month-old fetuses were collected ($n = 2$). To study the PRNP expression along preimplantation stages, in vivo embryos from different development stages (zygote, 8 cell, 16 cell, morula, and blastocyst) were obtained by oviduct flushing; oocytes were prepared from slaughterhouse-derived bovine ovaries by standard techniques. A total of 3–5 oocytes or single embryos were collected in 0.5 µl of PBS I (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 9.6 mM Na_2HPO_4 * 2 H_2O) and added to 1.5 µl lysis buffer [0.8% Igepal (Sigma, Taufkirchen, Germany), 1 U/µl RNasin (Promega, Mannheim, Germany), 5 mM DTT (Promega)], and snap frozen in liquid nitrogen before storage at −80°C.

Sheep of the breeds Black head and Bentheimer were classified as resistance (R1) or high susceptible (R5) to scrapie according to the polymorphisms at codons 136, 154, and 171 of the PRNP (11,40). R1 and R5 ewes were mated to rams of the same risk group. Reproductive organs such as ovary, oviduct, endometrium, myometrium, and caruncle as well as the fetuses were collected from ewes of both groups at the first month of pregnancy. Moreover, various tissues of brain, cotyledon, heart, intestine, kidney, liver, lung, and muscle were collected from 2-month-old ovine fetuses ($n = 2$). Immature oocytes, mature oocytes, and in vivo embryos at morula stage were collected from specifically genotype ewes using standard techniques as mentioned above.

PRNP Genotyping of Sheep

Genomic DNA from blood samples of sheep was isolated using standard method based on phenol/chloroform extraction. Primers were derived from the

published sequence using Primer Express® Software v2.0 (Applied Biosystems, Foster City, CA, USA) to PCR amplify coding sequences of ovine PRNP covering the polymorphic sites (external primers, Table 1). Direct DNA sequencing was employed to genotype the PRNP polymorphisms at codons 136 (^{136}A > ^{136}V), 154 (^{154}R > ^{154}H), and 171 (^{171}R > ^{171}Q or ^{171}H) (internal primers, Table 1) with the help of the SequiTherm EXCEL II DNA Sequencing Kit (BIOzym, Hamburg, Germany) and automatic sequencer (LI-COR DNA sequencer model 4200, Bad Homburg, Germany). Animals with the allelic combination ARR/ARR were assigned to risk group R1; animals of the risk group R5 showed the allelic combination VRQ/ARQ or VRQ/ARH or VRQ/VRQ (EU regulation).

RNA Isolation and cDNA Synthesis

Three independent pools each containing 15–20 immature oocytes or 3 embryos of various stages of development were used for mRNA isolation using oligo(dT)25 attached magnetic beads (Dynal, Oslo, Norway) following the manufacturer's instruction. Briefly, samples in lysis buffer were mixed with 40 µl binding buffer [20 mM Tris-HCl, pH 7.5, 1 M LiCl, 2 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and incubated for 5 min at 65°C to obtain complete lysis of the sample. Ten microliters of oligo(dT) magnetic bead suspension was added to the samples and incubated for 30 min at room temperature. The hybridized mRNA and oligo(dT) magnetic beads were washed three times with washing buffer (10 mM Tris-HCl, pH 7.5, 0.15 mM LiCl, 1 mM EDTA, pH 8.0). Finally, mRNA samples were eluted in 12 µl DEPC-treated water and reverse transcribed in 20 µl reaction volume containing 2.5 µM oligo dT(12) primer, 4 µl of 5× first-stand buffer (375 mM KCl, 15 mM $MgCl_2$, 250 mM Tris-HCl, pH 8.3), 2.5 mM of each dNTP, 10 U RNase inhibitor (Promega), and 100 U of SuperScript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). In terms of the order of adding reaction components, mRNA and oligo dT(12) primer were mixed first and heated for 5 min at 70°C. The mixture was placed on ice until the addition of the remaining reaction components and then incubated for 90 min at 42°C and terminated by heat inactivation for 15 min at 70°C.

Total RNA was isolated from tissue samples using Tri-Reagent (Sigma) following the manufacturer's instruction. The RNA was treated with deoxyribonuclease I (DNase I, Promega) in the presence of RNase inhibitor (Promega) for 1 h at 37°C to remove the residual DNA and then purified by using RNeasy Mini kit (Qiagen, Hilden, Germany). The RNA integrity was documented on 1.5% formaldehyde containing agarose gel and the concentration was measured. First-strand cDNA was synthesized from 1 µg of total RNA using random primer, oligo dT(12), and superscript II (Gibco BRL, Karlsruhe, Germany). The thermal program was as mentioned above.

TABLE 1
INFORMATION OF PRIMERS USED FOR AMPLIFICATION AND SEQUENCING

Genes	Primer Sequence (5′–3′)	Position Within Ref. Sequence	Product Size (bp)	Annealing Temperature (°C)	Accession Number
Ovine PRNP (external)*	TCAGTGGAACAAGCCCAGTAAGC	22577	283	Touch down	U67922
	GGTGGTGACTGTGTGTTGC	22859	64–58		
Ovine PRNP (internal)†	AGCTGGAGCAGTGGTAGG	22637	198	60	U67922
	GTGATGTTGACACAGTCATGC	22814			
Ovine PRNP‡§	CCAAGCTGAAGCATCTGTCTTCC	89	131	58	NM_001009481
	TCACTCCACATGGCCACAAAG	219			
Ovine H2a§	CACCTCAAATCTAGGACGACTAGCC	210	117	60	AY074805
	CGATGCATTTCCTGCCAATTC	326			
18S rRNA‡§	GCGCGCAAATTACCCAC	89	159	54	AF176811
	GCTGGAATTACCGCGGCT	247			
Bovine PRNP‡§	CTTCCCAGAGACACAAATCCAAC	108	105	60	NM_181015
	CATGGCCACAAAGAGAACCAG	212			
Bovine H2a§	CTCGTCACTTGCAACTTGCTATTC	295	148	60	NM_174809
	CCAGGCATCCTTTAGACAGTCTTC	442			

*The primer used for DNA amplification in genotyping procedure.
†The primer used for PRNP sequencing.
‡The primer used for RT-PCR.
§The primer used for real time PCR.

Quantitative Real-Time PCR

Quantification of PRNP mRNA in preimplantation embryos was done by real-time quantitative PCR (polymerase chain reaction). PCR was conducted in an ABI Prism® 7000 SDS instrument (Applied Biosystems) using SYBR Green as a double-strand DNA-specific fluorescent dye. Quantitative analyses of embryo cDNA were performed in comparison to histone H2a (endogenous control) (21), and were run in separate wells. Standard curves were generated for both genes using serial dilutions of plasmid DNA (101–108 molecules). PCR was performed in 20 µl reaction volume containing 2 µl of cDNA, 10 µl of 2× SYBR®Green JumpStart™ Taq ReadyMix™ with internal reference dye for quantitative PCR (Sigma) and optimal levels of forward and reverse primers. During each PCR reaction, samples from the same cDNA source were run in duplicate to control the reproducibility of results. Thermal cycle parameter was programmed at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 s and 60°C for 60 s. After the end of the last cycle, dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements every 7-s interval until the temperature reached 95°C. Final quantitative analysis was done using the relative standard curve method as used in Tesfaye et al. (39), and results were reported as the relative expression level compared to the calibrator cDNA after normalization of the transcript amount to the endogenous control.

Polymerase Chain Reaction (PCR)

PCR was used to quantify the amount of gene transcript in sample tissues. In this study the level of 18S rRNA transcript was used as an internal standard. The appropriate cycle number of PCR was preoptimized. Each cDNA source was done in duplicate to control the reproducibility of results. Bovine PRNP PCR was performed using bovine PRNP primers, while ovine PRNP PCR was performed using ovine PRNP primers as described in Table 1. 18S rRNA PCR was preceded using the 18S rRNA primers for both types of animals (Table 1). The thermocycle condition of bovine PRNP PCR was programmed at 94°C for 5 min, 28 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s followed by a final extension step of 72°C for 5 min. Ovine PRNP PCR was programmed at 94°C for 5 min followed by 28 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension step of 72°C for 5 min. The 18S rRNA PCR was performed using cycles of 94°C for 5 min, 15 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s with a final step of 72°C for 5 min.

Western Blot Analyses

Twenty milligrams of tissue sample was homogenized in 200 µl of gel sample buffer (6.5% of 1 M Tris, pH 6.8, 3% of SDS, 5% of 2-mercaptoethanol, and 10% of glycerol) with a protease inhibitor cocktail (Sigma). Following boiling for 5 min at 95°C, 10 µl of protein extracts were electrophoretically separated on a 15% SDS-polyacrylamide gel and run at 10 mA for 2 h. Proteins were transferred to 0.45-µm pore diameter nitrocellulose membrane (Protran®, Schleicher & Schuell BioScience, Dassel, Germany) for 1 h at 100 mA using Trans-Blot® SD; Semi-Dry transfer Cell (Bio-Rad, Munich, Germany). After two times washing with PBS II (150 mM NaCl, 8 mM Na_2HPO_4, 17 mM NaH_2PO_4) containing 0.05% Tween® 20 (PBST), the membrane was stained with 0.2% Ponceau S to evaluate the transfer quality. The membrane was then blocked for 1 h in PBST containing 1% polyvinylpyrolidone (PVP) (Sigma) at room temperature and incubated overnight at 4°C with prion protein (PrP) monoclonal antibody 12F10 (dilution 1:250; Spibio, Montigny le Bretonneux, France). Membrane was washed six times in PBST and incubated for 1 h at room temperature with horseradish peroxidase goat anti-mouse (dilution 1:15,000; Jackson ImmunoResearch Laboratories, Dianova, Hamburg, Germany). After six times washing in PBST, immune complexes were detected using the ECL Plus Western Blotting Detection System (Amersham Biosciencs, Freiburg, Germany) following the manufacturer's instructions and visualized using Kodak Bio Max XAR film (Sigma).

Immunohistochemistry

Immunohistochemical staining was performed on 5–7-µm cryostat sections of snap frozen tissues. Sample sections were fixed in methanol and acetone for 10 and 1 min, respectively, at –20°C. The sections were air dried for 15 min and washed with three changes of PBST. Sections were incubated for 30 min in 0.3% H_2O_2 diluted in methanol to quench an endogenous peroxidase activity, washed twice with PBS II, and then incubated in 1.5% goat normal serum (Santa Cruz Biotechnology Inc., Heidelberg, Germany) for 1 h. Endogenous biotin activity was blocked by incubating the sections in egg white (diluted 1:1 with deionized H_2O) for 15 min followed by washing with three changes of PBS II. The PrP monoclonal antibody 12F10 (dilution 1:200) was then applied on the sections for 1 h followed by three times washing with PBST. To detect the PrP antibody, sections were incubated in biotinylated goat anti-mouse IgG (dilution 1:100; Santa Cruz Biotechnology Inc.) for 30 min and washed with three

changes of PBST. Avidine-biotinylated horseradish peroxidase (Santa Cruz Biotechnology Inc.) was added on the sections and allowed to stand for 30 min. To visualize the immune complexes, the samples were incubated with AEC (3-amino-9-ethylcarbazole, Sigma) substrate according to the manufacturer's instructions for at least 10–20 min until signals became visible. The samples were counterstained with hematoxylin (Merck, Darmstadt, Germany) for 5 s and immediately washed with several changes of deionized H_2O. Finally, the samples were mounted using glycerol gelatin (Merck) and covered with a cover glass slip and the staining was observed by light microscopy.

Statistical Analysis

PRNP mRNA expression in preimplantation embryos was analyzed based on the relative standard curve method. The relative expression data were analyzed using the Statistical Analysis System (SAS) version 8.0 (SAS Institute Inc., NC, USA) software package. Differences in mean values between developmental stages were tested using ANOVA followed by a multiple pairwise comparisons using *t*-test. Differences of $p \leq 0.05$ were considered to be significant.

RESULTS

Expression of PRNP mRNA

The relative expression levels of the PRNP transcript were investigated throughout the preimplantation developmental stages of in vivo embryos using quantitative real-time PCR. In order to detect the gene expression in separated organs of fetuses at the earliest stage of prenatal development, semiquantitative RT-PCR was done in various tissues of 2-month-old fetuses. The PRNP mRNA expression by RT-PCR of each sample was monitored as relative expression level compared to the level of 18S rRNA from the same cDNA source. The results showed that amplification of the 18S rRNA by RT-PCR produces similar quantities of product for all tissues investigated.

Cattle: Expression of PRNP Transcripts in Bovine Preimplantation Embryos. PRNP expression analysis was performed in immature oocytes ($n = 2$) and in vivo preimplantation embryos including zygote, 8-cell, 16-cell, morula, and blastocyst stage embryos ($n = 3$) as well as in the fetuses from day 19 and 30 of pregnancy ($n = 2$). The results showed that the gene transcripts were detected in the samples of all stages. The relative abundance of the transcript in embryos at zygote stage was found to be higher by 3.7-fold compared to the level at immature oocytes stage. The level at zygote stage was not significantly different compared to the level at 1-month-old stage, but was significantly higher than those in samples of other stages ($p \leq 0.05$). The lowest expression level was found in 19-day-old fetuses; however, no significant differences were observed between immature oocytes, 8-cell, 16-cell, morula, blastocyst, and day 19 stages ($p > 0.05$), as shown in Figure 1.

Cattle: Expression of PRNP Transcripts in Bovine Fetuses and Reproductive Organs of Pregnant Cows. PRNP transcripts were detected in all tissues studied of 2-month-old bovine fetuses. The high relative expression levels of PRNP mRNA were found in brain, cotyledon, heart, and kidney, while moderate expression levels were shown in the rest of the tissues of intestine, liver, lung, and muscle (Fig. 2).

The gene expression analysis in bovine female reproductive organs was performed in ovary, oviduct, endometrium, myometrium, follicles, and granulose cells. PRNP transcripts were detected by semiquantitative RT-PCR in all tissues investigated. The relative expression levels were similar between ovary, oviduct, endometrium, and myometrium. The level of transcript abundance in granulosa cells was very low when compared to other tissues (Fig. 3).

Sheep. In this study sheep were classified into two groups according to their PRNP genotype. The PRNP mRNA expression level in immature oocytes, mature oocytes, morula stage embryos, and 1-month-old fetuses was detected using quantitative real-time PCR, while the expression of this transcript in female reproductive organs of pregnant ewes and various tissues of 2-month-old fetuses was detected using semiquantitative RT-PCR.

Sheep: Expression of PRNP Transcripts in Ovine Preimplantation Embryos. Quantitative real-time RT-PCR was used to detect and compare the gene expression between two groups. This analysis was done in immature oocytes, mature oocytes ($n = 3$ per group), in vivo embryos at the morula stage ($n = 1$ for R1 group and $n = 3$ for R5 group), and in 1-month-old fetuses ($n = 2$ per group). PRNP mRNA was detected in samples of all stages. The relative expression patterns of R1 and R5 groups were in the same trend. The relative abundance levels of PRNP mRNA in mature oocytes and embryos at morula stage were significantly lower than the levels in immature oocytes and 1-month-old fetuses ($p \leq 0.05$). However, R5 group showed a significant difference in PRNP mRNA expression between immature oocytes

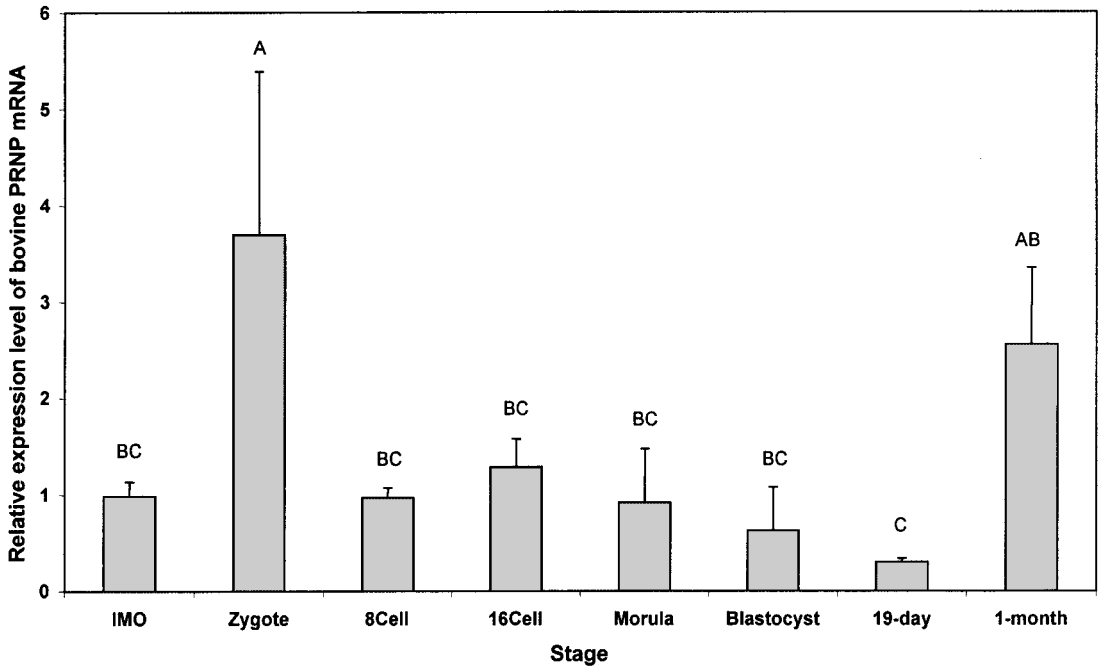

Figure 1. Relative expression levels of PRNP mRNA in in vivo bovine immature oocytes (IMO), preimplantation embryos, and 19-day and 1-month-old fetuses. The relative abundance of mRNA levels represents the amount of mRNA compared to the calibrator (immature oocyte), which is set to 1. Individual bars show the mean ± SE. Values with different letters (A, B, C) are significantly different ($p \leq 0.05$).

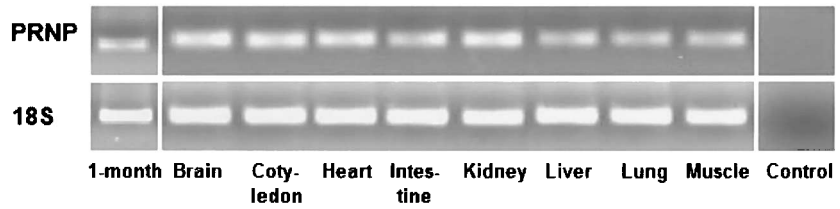

Figure 2. Representative semiquantitative RT-PCR detection of the PRNP and 18S mRNA in 1-month-old bovine fetuses and in various tissues of 2-month-old fetuses. The negative control reactions were done with no template DNA.

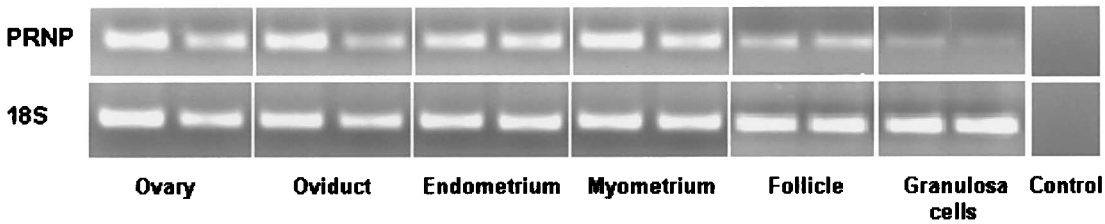

Figure 3. Semiquantitative RT-PCR detection of the PRNP and 18S mRNA in bovine reproductive tissues such as ovary, oviduct, endometrium, myometrium, follicles, and granulosa cells. The negative control reactions were done with no template DNA.

and 1-month-old stages ($p \leq 0.05$), while the levels at these stages were not different in the R1 group ($p > 0.05$). Compared within each stage between R1 and R5 groups, the results showed that the target gene expression in R1 group was higher than in the R5 group at immature oocytes and 1-month-old stages ($p \leq 0.05$), but no difference between these two groups was observed at mature oocytes and morula stages ($p > 0.05$) (Fig. 4).

Sheep: Expression of PRNP Transcripts in Ovine Fetuses and Reproductive Organs of Pregnant Ewes. The results of semiquantitative RT-PCR in 1- and 2-month-old ovine fetuses showed the presence of the gene transcript in all fetal tissues studied (Fig. 5). The PRNP mRNA level was high in the brain of both groups compared with the other tissues of heart, intestine, kidney, liver, lung, and muscle. The lowest expression level of the PRNP mRNA was found in the liver sample of R1 and R5 fetuses. However, the variations of the gene expression level compared between two independent samples of each group were observed in cotyledon, heart, and muscle.

PRNP mRNA expression was also detected in ovary, oviduct, endometrium, myometrium, and caruncle of both R1 and R5 ewes. In this experiment, their expression patterns were similar. Compared within each group, relative expression levels of PRNP mRNA in ovary, oviduct, endometrium and myometrium were not different. The highest expression level was found in caruncle tissue. However, when compared between R1 and R5 groups, the results showed that the expression levels of PRNP mRNA in caruncle tissue of two independent R1 ewes were higher than those of R5 ewes. Moreover, one of the sheep in R1 group showed higher expression level in endometrium when compared to the R5 group while the relative abundance of the transcript in other tissues were not different (Fig. 6).

Real time PCR was performed to quantify the amount of PRNP transcript in endometrium and caruncle of ewes with three replications using 18S rRNA as an internal standard. The results of real-time PCR supported the results obtained by RT-PCR. Relative PRNP expression levels were 25.5 and 5.4 for R1 endometrium, 10.2 and 13.3 for R5 endometrium, 28.5 and 67.7 for R1 caruncle, and 16.7 and 17.8 for R5 caruncle (Fig. 7). The means ± SE of the relative abundance of PRNP transcript in R1 and R5 endometrium were 15.5 ± 10.0 and 11.8 ± 1.5, respectively; the levels of R1 and R5 caruncle were 48.1 ± 19.6 and 17.2 ± 0.6, respectively. However, significant differences between R1 and R5 groups for both tissue types were not observed ($p > 0.05$).

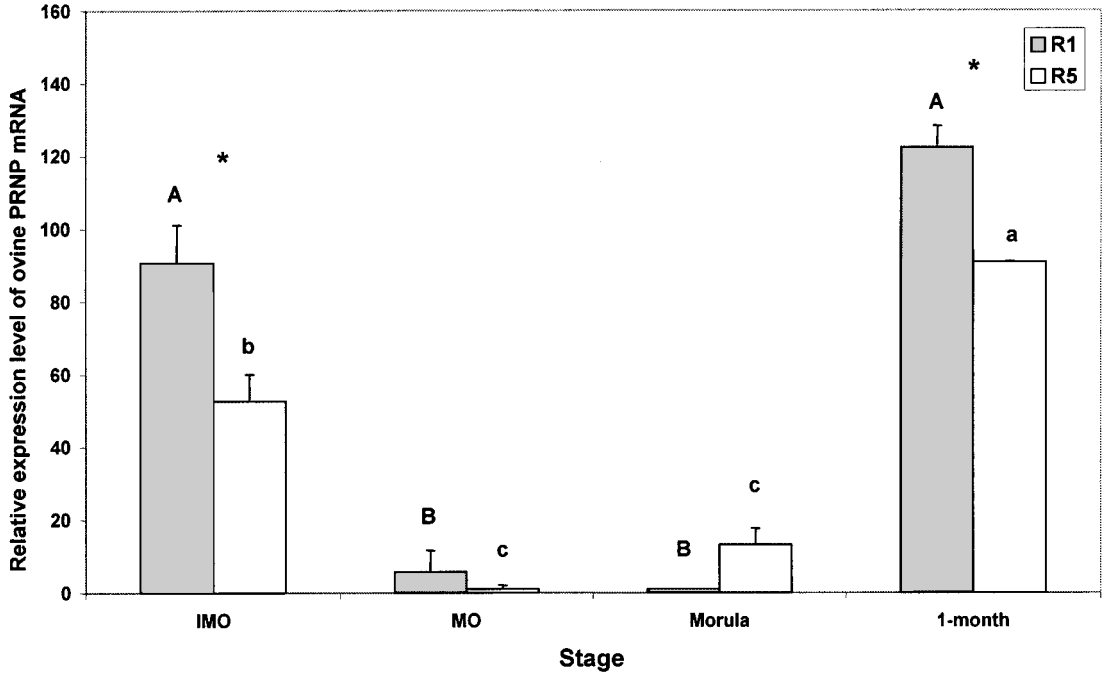

Figure 4. Relative expression levels of PRNP mRNA in in vivo ovine immature oocytes (IMO), mature oocytes (MO), morula stage embryos, and 1-month-old fetuses. The relative abundance of mRNA levels represents the amount of mRNA compared to the calibrator (R1 morula stage embryo), which is set to 1. Individual bars show the mean ± SE. Values with different letters (A and B for R1 group; and a, b, and c for R5 group) are significantly different within each group ($p \leq 0.05$), whereas the asterisk (*) shows a significant difference between two groups at the same stage ($p \leq 0.05$).

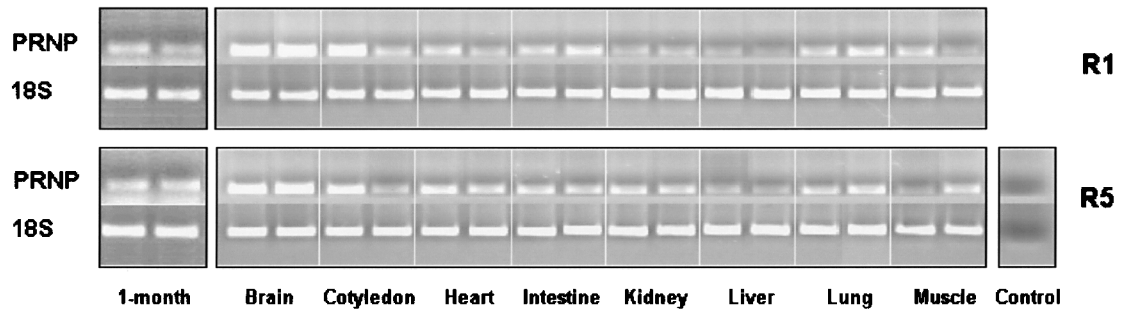

Figure 5. Comparison of the PRNP mRNA expression levels in 1-month-old fetuses and various tissues of 2-month-old fetuses between scrapie-resistant (R1) and -susceptible (R5) groups using semiquantitative RT-PCR technique. The negative control reactions were done with no template DNA.

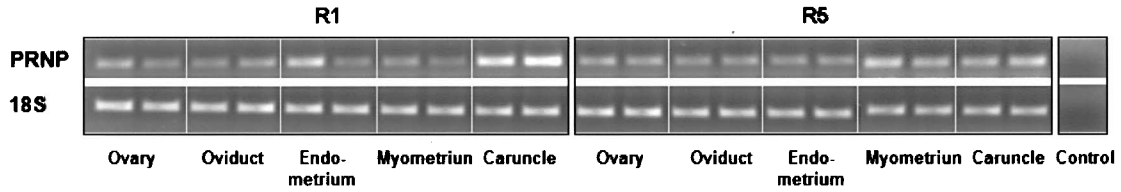

Figure 6. Comparison of the PRNP mRNA expression levels in reproductive organs such as ovary, oviduct, endometrium, myometrium, and caruncle of scrapie-resistant (R1) and -susceptible (R5) pregnant ewes using semiquantitative RT-PCR technique. The negative control reactions were done with no template DNA.

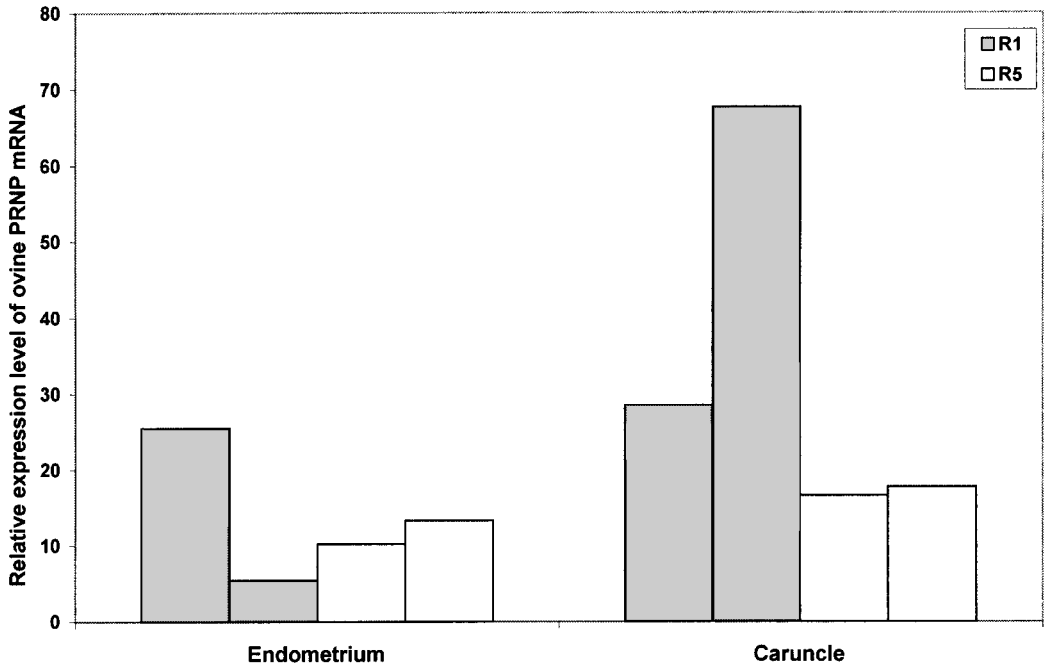

Figure 7. Relative expression levels of PRNP mRNA ($\times 10^{-6}$) in endometrium and caruncle of scrapie-resistant (R1) and -susceptible (R5) ewes ($n = 2$ per group). Individual bars show the mean ± SE.

Expression of PrPc

In order to study the PrPc expression in reproductive tissues of pregnant animals and various tissues of their fetuses, Western blot analysis and immunohistochemistry were performed. Western blot analysis was used to detect PrPc in the samples and to compare the expression level of the protein between samples, while immunohistochemistry was used to localize the PrPc in samples.

Cattle: Expression of PrPc in Bovine Fetuses. This study used fetuses at the first and second month of development to study the expression of PrPc in different organs. The immunoreactive bands corresponding to the PrPc at the expected molecular weight of ~28 and 35 kDa were detected in 1-month-old bovine fetuses and also in all separated single organs of 2-month-old bovine fetuses. The expression levels of the prion protein were high in brain and liver. Moderate levels were detected in cotyledon, intestine, and kidney, while low levels were detected in heart, lung, and muscle (Fig. 8a).

Cattle: Expression of PrPc in Reproductive Organs of Pregnant Cows. PrPc was detected in all reproductive tissues of pregnant cows including ovary, oviduct, endometrium, and myometrium. The levels of PrPc expression were not different between ovary, endometrium, and myometrium. Moreover, the protein level was low in oviduct compared to other tissues (Fig. 8b).

Ovary comprises many types of cells, which are involved in folliculogenesis such as follicular cells, granulosa cells, theca interna, theca externa, cortical stroma, or corpus luteum. Therefore, in this study, immunohistochemistry was used as a tool for localization of the PrPc in ovary tissue. The results demonstrated that PrPc diffused in all areas of ovarian cortex (Fig. 9A) while intense staining was found in ovarian medulla (Fig. 9B). However, in this present study, prion protein was not found in oocyte, cumulus oophorus, granulosa cells, theca cells (Fig. 9C), and corpus luteum (Fig. 9D).

Sheep: Expression of PrPc in Ovine Fetuses. To study the expression of PrPc in ovine prenatal fetuses, Western blot analysis was performed. The proteins extracted from the whole prenatal fetuses at the first month of pregnancy and from separated organs of 2-month-old fetuses were used to detect and compare the expression level of the protein between R1 and R5 groups. The results showed that the immunoreactive bands of the PrPc were detected in all samples examined. The expression patterns were not different between the two groups. The intensity of the PrPc band at the expected molecular weight of ~33 kDa was strongest in liver compared to the other tissues. The level of the protein in brain was also high when compared to those in heart, intestine, kidney, lung, and muscle. In this study, PrPc levels detected in cotyledon samples of both groups were very low (Fig. 10a).

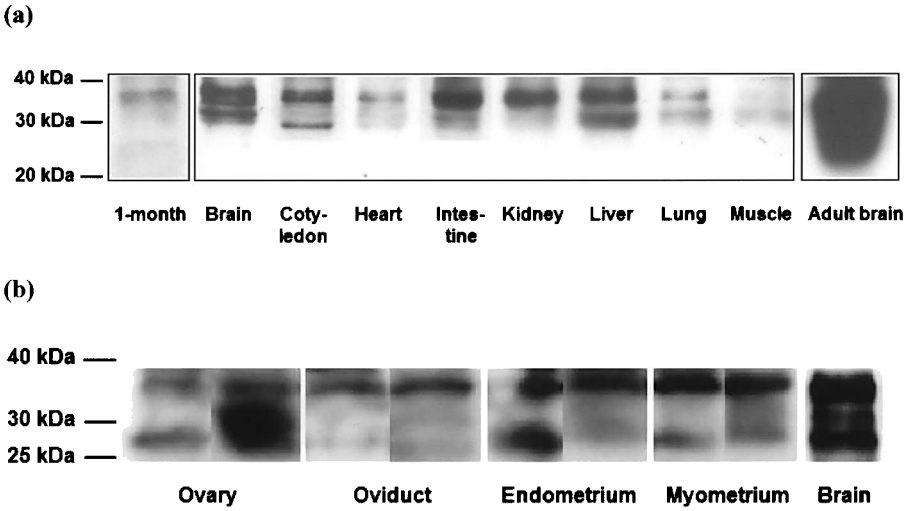

Figure 8. Representative Western blot analysis of PrPc in bovine tissues showed the immunoreactive band corresponding to the PrPc at the expected molecular weight of ~35 kDa. (a) One-month-old fetuses and various tissues of 2-month-old fetuses. (b) Reproductive tissues of pregnant cows. In this study, the adult brains were used as positive control. Protein extracts from 1 mg of each sample, but 0.5 mg from adult brain, were loaded per lane. The PrPc band was not observed in negative control membranes, which were devoid of the PrP MAb 12F10 incubation (data not shown).

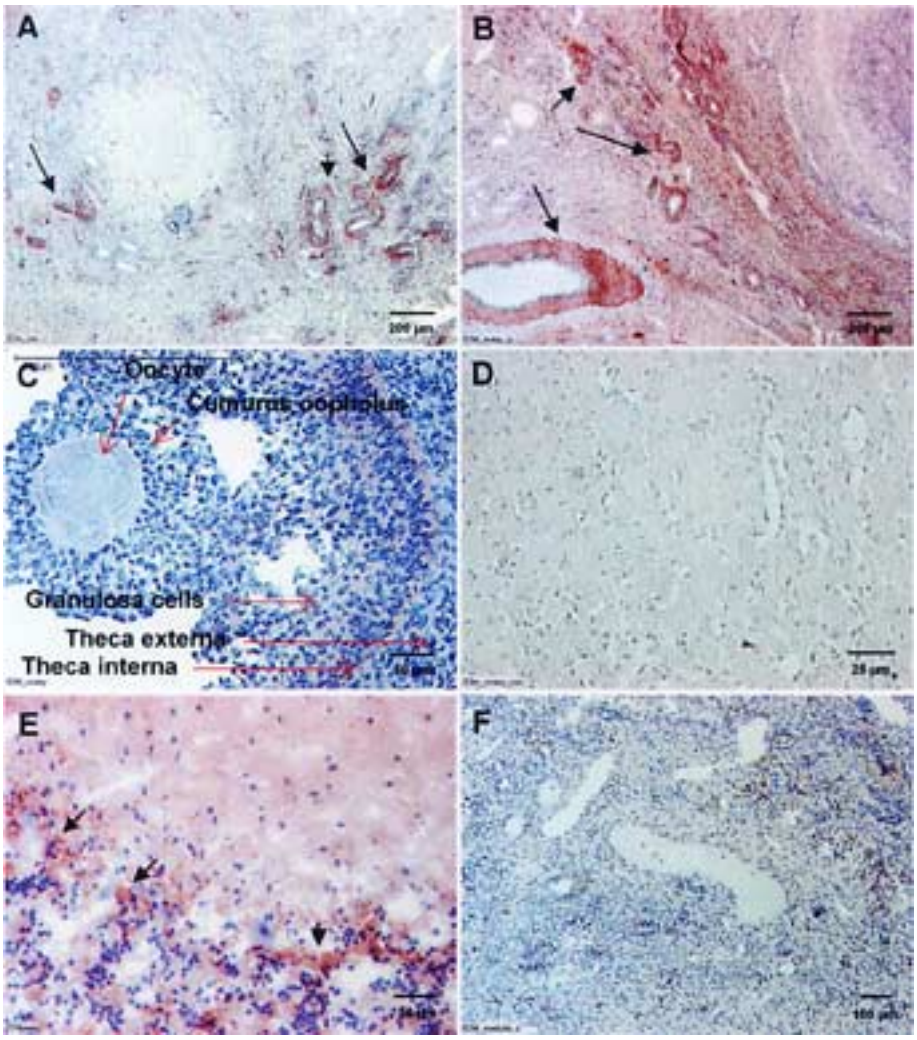

Figure 9. Immunohistochemistry of the prion protein with PrP MAb 12F10 in bovine ovaries using HRP-AEC substrate, which produces the red-rose color during the emzymatic reaction as a chromogen. PrPc (black arrow) was detected in ovarian cortex (A) and ovarian medulla (B). Immunostainning of the PrPc was not found in oocyte, granulosa, and theca cells (C), and corpus luteum (D). Strong staining was also found in brain that served as positive control tissue (E). Control sections were devoid of specific staining with the PrP MAb (F).

Sheep: Expression of PrPc in Ovine Female Reproductive Organs. The PrPc could be detected in all female reproductive organs investigated including ovary, oviduct, endometrium, myometrium, and caruncle. The prion protein expression patterns of the two risk groups were similar. The intensity of the PrPc bands in endometrium, myometrium, and caruncle tissue was higher compared to those of ovary and oviduct tissue. Moreover, the protein levels in ovary, endometrium, myometrium, and caruncle were not different when compared within each tissue between R1 and R5 groups, but it was higher in R5 oviduct than that of R1 oviduct (Fig. 10b).

Immunohistochemical localization was performed in ovine ovaries of both R1 and R5 groups due to the same reason as in bovine. The results in R1 and R5 ovaries were not different and similar to the results in cattle. PrPc was detectable in ovarian cortex (Fig. 11A, D) and ovarian medulla (Fig. 11B, E) but not in corpus luteum (Fig. 11C, F) of R1 and R5 ovaries, respectively. Moreover, ovine PrPc was not found in oocyte, granulosa, and theca cells (Fig. 11G), whereas the expression of the protein was found intense in ovarian medulla.

DISCUSSION

At the present, the precise molecular function of prion protein gene is unclear. This study was carried on the basis that the timing and distribution of prion protein mRNA and the protein expression knowledge may lead our understanding of its function. Semi-

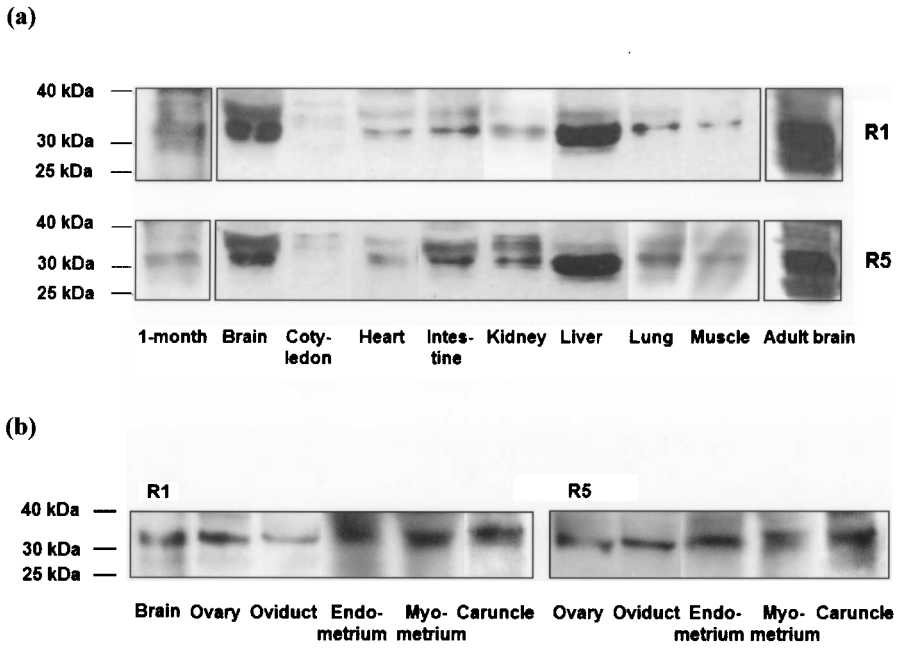

Figure 10. Representative Western blot analysis of PrPc in ovine tissues showed the immunoreactive band corresponding to the PrPc in various tissues of scrapie-resistant (R1) and -susceptible (R5) animals. (a) One-month-old fetuses and various tissues of 2-month-old fetuses. (b) Reproductive tissues of pregnant ewes. The adult brains were used as positive control. Protein extracts from 0.5 mg of adult brain in (a), 0.25 mg of R1 brain in (b), and 1 mg of the rest of the samples were loaded. The PrPc band was not observed in negative control membranes, which were devoid of the PrP MAb incubation (data not shown).

quantitative RT-PCR and quantitative real-time PCR were used to characterize PRNP expression, while Western blot analysis and immunohistochemistry were used to investigate the expression of protein in both amount of expression and location in organs. In this study, we used cattle and sheep as animal models to study the expression of prion protein gene at mRNA and protein levels.

PRNP mRNA Expression in Preimplantation Embryos

This study is the first report of PRNP mRNA expression in preimplantation embryos. The results showed that the bovine PRNP transcript abundance increased at zygote, was decreased along embryonic development, and was elevated at early fetal stage again. We hypothesized the increasing of this transcript at zygote stage due to sperm activity after fertilization. This study did not quantify the expression of PRNP transcript in bovine sperm; however, PRNP mRNA was found in spermatogenic cells of mice (16). Moreover, a C-terminally truncated isoform of PrPc was shown in bovine (36) and ovine (10) mature sperm by Western immunoblotting. Thus, sperm could be the source of PRNP after fertilization.

The quantitative gene expression levels in R1 and R5 ovine preimplantation embryos were in the same trend, showing that relative expression levels were significantly lower at mature oocytes and morula stages compared to the levels at immature oocytes and 1-month-old stages. However, comparison between R1 and R5 groups demonstrates that PRNP mRNA expression levels of R1 immature oocytes and 1-month-old fetuses were more abundant than those of R5 group.

Maternal–zygotic transition (MZT) is a complex phenomenon characterized by the initiation of transcription in the embryo and the replacement of maternal mRNA with embryonic mRNA (44). MZT comprises a period of minor gene activation in one-cell embryos, followed by a period of major gene activation in two-cell embryos in mice (35), occurs at the 4000- to 8000-cell stage in *Xenopus* (12), and at the 4- to 8-cell stage in humans [reviewed in (38)]. The bovine MZT occurs at the 8- to 16-cell stage, and is characterized by a major onset of transcription, while minor transcription is observed as early as the one-cell embryo (23). A switch from maternal to embryonic genome control appears to occur at the 8- to 16-cell stage in sheep embryos. As in the cow embryo, a relatively constant pattern of protein synthesis is observed during the first three cell cycles (1-, 2-, or 4-cell embryos), but a distinctly different pattern is observed in 16-cell embryos and at later stages [reviewed in (38)]. Similar to those studies, it seems

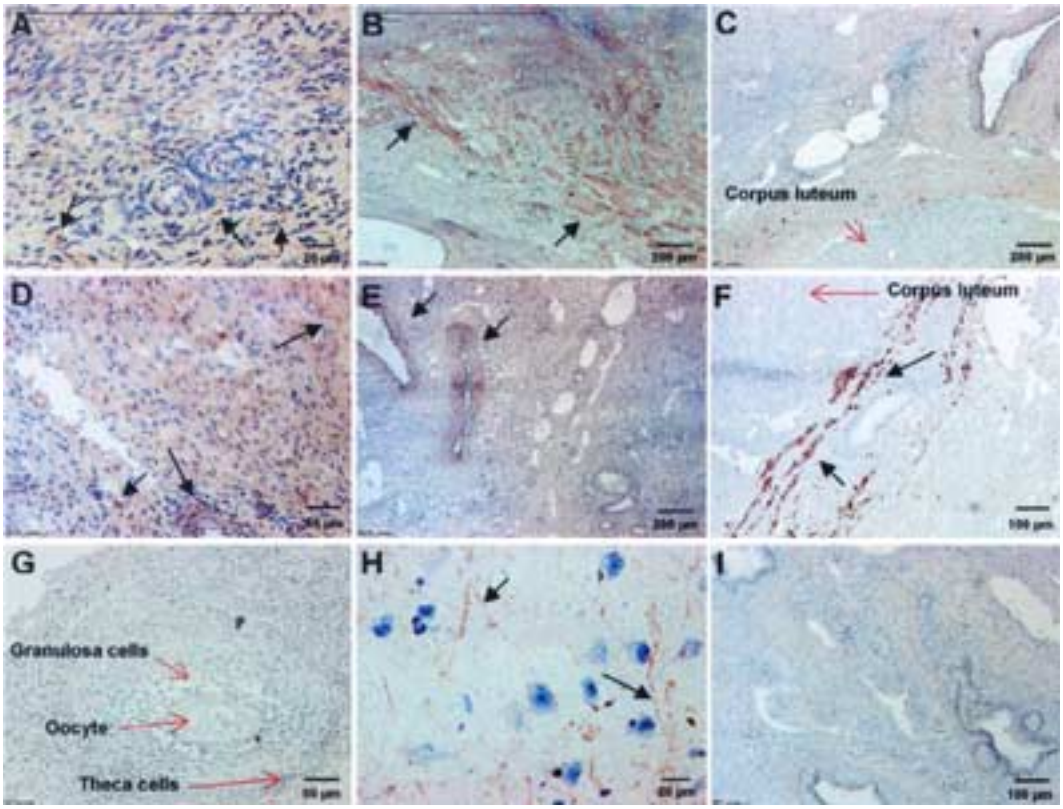

Figure 11. Immunohistochemical localization of PrPc in ovine ovaries (black arrow). Strong staining was found in ovarian cortex (A, D) and ovarian medulla (B, E) of scrapie-resistant (R1) and high susceptible (R5) ewes, respectively. PrPc was not detected in oocyte, granulosa cells, and theca cells of R5 animal (G) and in corpus luteum (C, F) of R1 and R5 ewes, respectively. Ovine brain was stained in parallel as positive controls for PrPc immunostaining (H). Control sections were devoid of specific staining with the PrP MAb (I).

likely that the minor embryonic transcription of the PRNP occurs at zygote stage followed by the major transcription after blastocyst stage in cattle and sheep. This implies that PRNP might have role in normal embryo development.

PRNP mRNA and PrPc Expression in Prenatal Fetuses

In order to investigate the expression of PRNP transcript and PrPc in prenatal fetuses, the whole fetuses at the first month of pregnancy and various tissues of 2-month-old fetuses were used as the sample. Amselgruber et al. (1) presented that PrPc was detected in pancreas of bovine prenatal fetuses ranging from 7 to 86 cm in crown–rump length. The present study demonstrated, for the first time, that PRNP and PrPc were expressed in some additional tissues of brain, cotyledon, heart, intestine, kidney, liver, lung, and muscle of bovine and ovine prenatal embryos since the second month of the embryogenesis. These results were in the same trend as previous studies that showed the presence of PrPc since day 8.5 and day 9 of embryogenesis in mouse (24) and in brain, kidney, liver, and heart of zebrafish from 24 h postfertilization (9).

The study of PrPc expression in prenatal fetuses established that the levels of PrPc in some tissues were not related to the level of the gene transcript. PRNP mRNA levels were high in brain and cotyledon in both bovine and ovine fetuses, while the PrPc was highly expressed in brain and liver. The liver is an organ that plays a major role in several metabolism functions, detoxification, and blood protein formation. In the fetus, blood leaving the placenta via the umbilical vein passes through the ductus venosus and other veins of the fetal liver (43). Because PrPc was found on peripheral blood mononuclear cells (18), the accumulation of PrPc in embryonic liver might result from the circulation of PrPc from its maternal placenta. In addition, our finding suggested that organs, for which infectivity has been demonstrated (brain and intestine), contain more PrPc than tissues shown not to support infection (cotyledon, heart, lung, and muscle) (17).

Prion protein is a cell surface glycosylated protein with two *N*-glycosylation sites; therefore, forms of mature PrPc or glycoforms are found with different

degrees of glycosylation. The glycoform signature of PrPc in the brain, as obtained with most anti-PrP antibodies described in the literature, is in general characterized by the presence of three bands with decreasing intensity, representing the bi-, mono-, and unglycosylated isoform of PrP of ~33, ~30, and ~28 kDa, respectively (25). In this study, PrPc could be detected by loading protein extracts from only 1 mg of tissue per lane of almost all tissues except cotyledon of the ovine fetuses. The PrPc reactive bands detected in cotyledon tissue of both risk groups were very weak and unclear. Due to these results, Western blot analysis was done in addition in double concentration of protein extracted from cotyledon of 2-, 3-, and 5-month-old ovine fetuses (R1 and R5 groups). The results clearly revealed the existence of PrPc with the molecular weight of ~30 kDa in cotyledon of 3- and 5-month-old R1 and R5 fetuses, and assured the presence of PrPc in the 2-month-old fetuses (Fig. 12). Interestingly, most of ovine fetal tissues examined produced mainly the biglycosylated isoform, and minimally the monoglycosylated isoform of PrPc. Nevertheless, the cotyledon of both R1 and R5 fetuses produced only the monoglycosylated isoform. These suggest that the PrPc in ovine fetal cotyledon is differentially glycosylated from other tissues, and could be involved in different physiological functions of the protein. Moreover, because PrPc expression levels in various tissues of R1 and R5 ovine fetuses were in the same trend, and comparison within each tissue showed similar level between the two groups, it could be concluded that there is no relationship between PRNP genotype and the tissue expression of the PrPc in ovine prenatal fetuses at this developing period.

PRNP mRNA and PrPc Expression in Reproductive Tissues of Ewes

In this study expression pattern of PRNP transcript and PrPc in female reproductive organs of cattle and sheep were not different. Comparison between tissues within each type of animal displays the similar expression level of PRNP transcript in ovary, oviduct, endometrium, and myometrium. However, the PrPc expression level in oviduct was low compared to the level in ovary, endometrium, and myometrium of both species. Compared between R1 and R5 ewes, the expressions of PRNP mRNA in ovary, oviduct, endometrium, and myometrium were not different, whereas the gene expression in caruncle of R1 sheep was higher than that in R5 sheep. However, the expressions of PrPc in caruncle between R1 and R5 groups were not different.

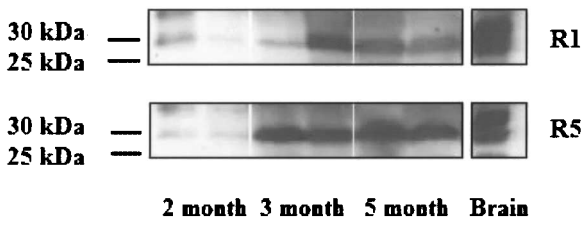

Figure 12. Western blot analysis, revealing the immunoreactive bands of PrPc in ovine fetal cotyledon of scrapie-resistant (R1) and high susceptibility (R5) groups at the second, third, and fifth month of pregnancy. The adult brains were used as positive control. Protein extracts from 2 mg of each sample, but 0.5 mg of adult brain, were loaded per lane. The PrPc band was not observed in negative control membranes, which were devoid of the PrP MAb incubation (data not shown).

The ruminant placenta (bovine and ovine) is classified as synepitheliochorial (47) and has a cotyledonary organization in which both fetal and maternal villi are discernible as discrete structures (placentome) on the uterine epithelium. Intimate contact between maternal and fetal tissue occurs only in the placentome (comprising the fetal cotyledon and maternal caruncle), which is the most highly vascularized portion of the placenta. This study also found that the expressions of both mRNA and protein level in caruncle of sheep were high compared to the other tissues. These results were in the same trend as the results of Tuo et al. (41), who found higher levels of PrPc in caruncular endometrium than that in intercaruncular endometrium, myometrium, oviduct, and ovary. Moreover, PRNP mRNA expressions was also found at high level in cotyledon of both cattle and sheep fetuses compared to other tissues investigated as well.

Western blot has been showed as an excellence tool to study the expression of protein. Localization of the protein in tissue is also important to understanding the function of target genes at cellular level as well. We used immunohistochemistry as a tool for localization of the PrPc in tissues, and the ovary was chosen. In this study the presence of PrPc was detected in cortex and medulla of both bovine and ovine (R1 and R5) ovaries but not in the oocyte and corpus luteum.

In summary, this is the first report of the expression of PRNP mRNA and PrPc during the prenatal stage of cattle and sheep. We have documented the relative levels of PRNP mRNA and protein in various tissues of prenatal fetuses and various female reproductive tissues. We have shown that transcriptional activity was found in most of the tissues, but the levels of those activities were different between tissues. Moreover, our study showed that the translational activity was not related to the amount of the gene transcript. These might be involved in the function of the PRNP. So far, the switch-on period of the prion protein gene both on mRNA and protein levels during prenatal stage of mammalian fetuses is not available.

In order to understand the mechanism and function of PrPc in embryogenesis, this information is very important. Our present study showed the expression of the gene transcript and the cellular prion protein already at the first month of embryogenesis and in all tissues of 2-month-old fetuses.

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