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Developmental expression of the cellular prion protein (PrP^C) in bovine embryos

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Abstract

The mammalian cellular prion protein (PrP^C) is a highly conserved glycoprotein that may undergo conversion into a conformationally altered isoform (scrapie prion protein or PrP^{Sc}), widely believed to be the pathogenic agent of transmissible spongiform encephalopathies (TSEs). Although much is known about PrP^{Sc} conversion and its role in TSEs, the normal function of PrP^C has not been elucidated. In adult mammals, PrP^C is most abundant in the central nervous tissue, with intermediate levels in the intestine and heart, and lower levels in the pancreas and liver. PrP^C is expressed during neurogenesis throughout development, and it has recently been proposed that PrP^C participates in neural cell differentiation during embryogenesis. In order to establish the developmental timing and to address the cell-specific expression of PrP^C during mammalian development, we examined PrP^C expression in bovine gametes and embryos through gestation Day 39. Our data revealed differential levels of *Prnp* mRNA at Days 4 and 18 in pre-attachment embryos. PrP^C was detected in the developing central and peripheral nervous systems in Day 27, 32, and 39 embryos. PrP^C was particularly expressed in differentiated neural cells located in the marginal regions of the central nervous system, but was absent from mitotically active, periventricular areas. Moreover, a PrP^C cell-specific pattern of expression was detected in non-nervous tissues, including liver and mesonephros, during these stages. The potential participation of PrP^C in neural cell differentiation is supported by its specific expression in differentiated states of neurogenesis.

Keywords

Cellular prion protein (PrP^C); Protein expression; Gene expression; Bovine gametes; Bovine embryogenesis

Introduction

The mammalian cellular prion protein (PrP^C) is a host-encoded glycoprotein best known for its role in prion diseases or transmissible spongiform encephalopathies (TSEs). This group of neurodegenerative and infectious diseases affects several species, including sheep

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(scrapie, the prototypical prion disease), humans (Creutzfeldt-Jakob disease; CJD and Kuru), deer (chronic wasting disease; CWD) and cattle (bovine spongiform encephalopathy; BSE). Through a poorly understood process, PrP^C is post-transcriptionally converted into a protease-resistant and pathogenic isoform (scrapie prion protein, or PrP^{Sc}) that is widely believed to be the principal, if not the only, component of the TSE agent (Prusiner, 1998; Collinge, 2001; Abid and Soto, 2006).

Although much is known about the role of PrP^{Sc} in prion disease, the normal function of PrP^C is poorly understood. Mice and cattle null for PrP^C apparently develop and reproduce normally, although they are completely resistant to TSE infection (Bueler et al., 1992, 1993; Richt et al., 2007). Studies in vitro have provided evidence for the participation of PrP^C as an anti-apoptotic agent that blocks some of the internal or environmental factors that initiate apoptosis (Bounhar et al., 2001; Roucou et al., 2005). Alternatively, PrP^C may protect against oxidative stress through the binding of copper ions and modulation of a Cu/Zn superoxide dismutase (Vassallo and Herms, 2003). Others have suggested that PrP^C participates in the activation of signal transduction pathways involved in neuronal adhesion, proliferation, and differentiation (Zhang et al., 2006; Steele et al., 2006; Peralta et al., 2011).

Tissue-specific analyses in the adult mice and bovine have demonstrated a wide-spread expression of PrP^C, with an intense presence in neurons and lymphoreticular cells (Ford et al., 2002a; Peralta and Eyestone, 2009). Immune cells, including macrophages, lymphocytes, and dendritic cells, are believed to be responsible for the transport of PrP^{Sc} from the intestinal lumen through the enteric wall after oral inoculation (Aguzzi et al., 2003). Thereafter, PrP^{Sc} is transported by follicular dendritic cells to lymphatic tissues where it replicates and initiates colonization of the nervous system. After a long period of incubation that ranges in cattle from one to five years, PrP^{Sc} can infect the central nervous system (CNS) and induce a characteristic spongiform degeneration in the brain, leading to a variety of neuropathological symptoms (Harris and True, 2006).

During mouse embryogenesis, PrP^C mRNA has been detected in the CNS and developing peripheral organs, including the heart, lung, and intestine (Manson et al., 1992; Tremblay et al., 2007). In the developing mouse brain, undifferentiated neural progenitor cells in the mitotically active ventricular zone do not express PrP^C. In contrast, post-mitotic neurons express high levels of PrP^C after their last mitosis in the neuroepithelium as they migrate towards the marginal layers and differentiate, suggesting that PrP^C participates in neural cell differentiation (Tremblay et al., 2007; Steele et al., 2006). In order to establish the developmental timing and to address the cell-specific expression of PrP^C during bovine development, we examined the expression of the PrP^C in bovine gametes and during embryonic development. Our data showed a differential timing of PrP^C expression during early bovine development. The cell-specific expression of PrP^C in bovine embryos was revealed to included the developing brain and spinal cord, peripheral nervous system, liver, and mesonephros.

Results

PrP^C is differentially expressed during early bovine embryogenesis

The relative Prnp mRNA levels were analyzed in bovine cumulus cells, spermatozoa, oocytes, and embryos from Day 4 through Day 39 of gestation using quantitative real-time PCR (Fig. 1). Prnp mRNA was detected in all cell types and developing embryos. Similar levels of Prnp mRNA were found in oocytes and sperm ($P>0.05$), whereas levels in cumulus cells were higher compared to oocytes (18.4-fold relative to oocytes; $P<0.05$). During in vitro development, Prnp mRNA levels rose sharply in Day 4 embryos (21.2-fold relative to oocytes; $P<0.05$), and then declined by Day 8. Similar Prnp mRNA levels were detected

Day-18 embryos (16.0-fold relative to oocytes; $P < 0.05$) but declined to levels similar to oocytes in Day-27, -32, and -39 embryos.

PrP^C protein was expressed at each developmental stage examined. No labeling was observed in the negative controls incubated with non-immune serum instead of SAF-32 antibody (Fig. 2D, H, L, P, T). PrP^C-associated immunofluorescence in oocytes and embryos until Day 14 generally reflected Prnp mRNA expression levels (Figs. 1 and 2), and thus appeared greater in embryos at Day 4 of gestation compared to other stages. Punctuate and disperse immunolabeling in the trophoblast of Day-18 embryos (Fig. 2Q–T) did not resemble levels of PrP^C mRNA at this stage, however. PrP^C immunoreactivity was observed in oocytes (Fig. 2A–D) and blastomeres in Day-4 embryos (Fig. 2E–H), and in both stages appeared to be located in the plasma membrane. PrP^C signal was detected both in the inner cell mass and trophoblasts of Day-8 blastocysts (Fig. 2I–L). A weak, specific PrP^C signal was observed in histological cross sections of Day-14 embryos (Fig. 2M–P).

PrP^C is expressed in the developing nervous system and non-nervous tissues during bovine organogenesis

The tissue-specific pattern of PrP^C distribution was studied using immunoperoxidase staining in sagittal sections of Day-27, -32 and -39 embryos. Low magnification images of Day-27 embryos demonstrated the presence of PrP^C staining in the developing nervous system and mesonephros (Fig. 3A–C). At higher magnification, PrP^C immunoreactivity was evident in the marginal region of the brain (Fig. 3D) and spinal cord (Fig. 3E). In visceral organs, PrP^C was detected in epithelial cells of the mesonephric duct and glomeruli (Fig. 3F) and in scattered multi-nucleated cells distributed in the liver parenchyma (Fig. 3G–I). A similar pattern was observed in Day-32 embryos, where PrP^C was restricted to the marginal layer of the developing brain (Fig. 4D) and spinal cord (Fig. 4E). In contrast, no immunoreactivity was observed in the periventricular layers of the CNS. PrP^C was also localized in the dorsal root ganglia (Fig. 3F), in the thoracic segment of the spinal cord. The relative size of the mesonephros decreased drastically compared to Day-27 embryos; however, epithelial cells in the mesonephric duct displayed intense PrP^C expression (Fig. 4G). Immunodetection in the liver showed PrP^C-positive cells in higher number compared the previous stage (Fig. 4H,I). Further development of the nervous system was observed in Day-39 embryos (Fig. 5A–C). As in earlier stages, PrP^C was observed in the brain and dorsal root ganglia as well as in the sympathetic trunks and peripheral nerves (Fig. 5B,C). Similar to previous stages, PrP^C immunoreactivity was restricted to the marginal region of the brain (Fig. 5D) and spinal cord (Fig. 5E). PrP^C staining was observed in the mantle layer (Fig. 5F), dorsal root ganglions (Fig. 5G), and peripheral nerves that originated from the cervical (Fig. 5H) and lumbar (Fig. 5I) segments of the spinal cord. Peripheral trunks stemming from the CNS were also positive for PrP^C (Fig. 5J). Neural tissue immunoreactive for PrP^C was also observed in near the intestine (Fig. 5K) and lungs (Fig. 5L). As the mesonephros continued to involute, epithelial and glomerular cells remained positive for PrP^C as in the previous stages (Fig. 5M). PrP^C-positive, multi-nucleated cells were observed in the liver in lower number compared with Day-32 embryos (Fig. 5N and O).

Bovine embryos display a distinct pattern of PrP^C isoforms

Relative expression of the PrP^C was analyzed by quantitative Western blot in Day-27, -32 and -39 embryos. PrP^C immunoreactive bands were observed at all stages analyzed (Fig. 6A). A predominant band was observed at 31 kDa, with a less intense band at 29 kDa. Comparison with adult bovine brainstem (obex, positive control) suggested the presence of presumptive mono-glycosylated and non-glycosylated PrP^C isoforms of 31 and 29 kDa, respectively (Stimson et al., 1999). The immunoreactive band at 35 kDa (di-glycosylated isoform), normally present in several adult bovine tissues (Peralta and Eyestone, 2009), was

scarcely detected in embryonic lysates. Computerized quantification of bands showed a progressive increase in the level of the 29-kDa isoform from Day-27 to -39 fetuses (1.32 and 3.48 expression relative to β -Actin; $P < 0.05$) (Fig. 6B). In contrast, no significant differences were detected for the 31- and 35-kDa isoforms or for total PrP^C expression between stages.

Discussion

In the present study, we evaluated the timing and tissue-specific distribution of PrP^C expression in bovine oocytes and embryos from Day 4 until Day 39 of gestation as little data is available on the expression of PrP^C during bovine development (Thumdee et al., 2007). Studies in early stages of mouse embryogenesis reported intense expression in the CNS and wide distribution in peripheral organs, including heart, lung and intestine (Manson et al., 1992; Miele et al., 2003; Tremblay et al., 2007). PrP^C transcript expression peaked on Day 4 in in vitro-produced embryos and on Day-18 in pre-attachment in vivo derived embryos. Levels of mRNA expression corresponded with the intensity of the protein immunolabeling until Day 14 of gestation. Disparities between PrP^C transcript level and protein immunolabeling in Day-18 embryos are difficult to explain, although similar variations in the expression of PrP^C in the CNS have been associated with differences in translation of message and/or of trafficking and protein degradation (Ford et al., 2002b). Besides embryonic tissues, our analysis by quantitative PCR revealed the presence of the PrP^C transcript in bovine cumulus cells and mature sperm. The function of PrP^C in these cells has not been elucidated; however, a cytoprotective role has been suggested after sperm collected from PrP^C-null mice showed higher susceptibility to copper toxicity compared to wild-type counterparts (Shaked et al., 1999).

We have previously reported the expression of PrP^C in the nervous system of Day-27 and -39 bovine embryos (Peralta et al., 2011). Here, we observed a similar tissue-specific pattern of staining for PrP^C in the same stages and in Day-32 embryos. Expression of PrP^C was strong in the brain, spinal cord, dorsal root ganglia, cervical ganglia, sympathetic trunks, and peripheral nerves. This pattern of expression in neural tissues was consistent throughout stages of development, although the staining was more intense in Day-39 embryos, suggesting higher levels of expression. In the brain, PrP^C staining was observed primarily in the marginal and outer intermediate layers. Expression of PrP^C followed a similar pattern in the spinal cord. The marginal layer is rich in dendrites and axons, and later in development will be part of the gray matter where most PrP^C is localized at the adult stage (Sales et al., 2002). Throughout these stages, PrP^C was particularly expressed in differentiated neural cells located in the marginal regions of the CNS, but was absent from mitotically active periventricular areas. Similar results have been reported previously, where PrP^C was found exclusively in differentiated, post-mitotic neurons that have ceased proliferation and migrated to marginal layers of the neuroepithelium (Manson et al., 1992; Tremblay et al., 2007; Peralta et al., 2011). This potential association of PrP^C with neural differentiation has been supported by in vitro experiments where PrP^C expression was positively correlated with differentiation of multipotent neural precursors and embryonic stem cells into neurons (Steele et al., 2006; Peralta et al., 2011). Moreover, embryonic hippocampal neurons have shown intense neurite outgrowth after treatment with recombinant PrP^C (Chen et al., 2003; Kanaani et al., 2005). These studies and others have cumulated considerable evidence that implicate PrP^C in cell signaling regarding the potency, self-perpetuation, and differentiation of embryonic stem cells (reviewed by Lopes and Santos, 2012).

In Day-27, -32 and -39 embryos, expression of PrP^C in developing peripheral organs was evident in the mesonephros and the liver. The mesonephric duct displayed intense labeling for PrP^C in glomerular and tubule epithelial cells. In the adult stage, the mesonephric duct

differentiates into the epididymis, where strong PrP^C expression has been previously reported (Ford et al., 2002a). In the liver, PrP^C expression was confined to multi-nucleated cells, presumably macrophages scattered throughout the hepatic parenchyma.

Analysis by Western blot indicated a distinct pattern of PrP^C isoforms in Day-27, -32 and -39 embryos. In adult tissues, variable glycosylation of the two PrP^C asparagine-linked oligosaccharide sites by N-glycans results in three PrP^C bands, representing diglycosylated, monoglycosylated, and unglycosylated PrP glycoforms (Stimson et al., 1999). Due to the interaction with the environment, N-glycans may contribute to the function of membrane proteins, for instance, modulation of cell-to-cell or cell-to-matrix adhesion or binding to extracellular ligands (Varki, 1993). Moreover, it has been suggested that different PrP^C glycoforms may influence the timing of TSE infectivity by directly influencing the interaction with the infectious agent (Glatzel and Aguzzi, 2000). The absence of diglycosylated isoform in bovine fetuses, normally predominant in several adult tissues, suggests that this isoform arises at later stages of development. Regulation of enzymes involved in N-glycans addition to PrP^C, including N-acetylglucosamine transferase-III, has been described during aging of the CNS (Goh et al., 2007).

In the present study we documented the relative levels of PrP^C mRNA and the cell-specific expression of the PrP^C protein in gametes and during bovine embryonic development. We found differential PrP^C mRNA levels during pre-attachment stages of embryogenesis. The cell-specific expression of PrP^C in early bovine embryos was revealed to include the developing brain and spinal cord, peripheral nervous system, liver, and mesonephros. The potential participation of PrP^C in neural cell differentiation is supported by its specific expression in differentiated neurons.

Material and methods

All procedures have been approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech.

Oocytes, spermatozoa and cumulus cells

Ovaries were obtained from cattle at a local abattoir within 30 min of slaughter, and transported to the laboratory at room temperature. Cumulus-oocyte complexes (COC) were obtained by aspirating 3- to 5-mm follicles with an 18-g needle and syringe. Frozen-thawed semen from bulls of proven fertility (ABS, American Breeders Service, DeForest, WI) was used for in vitro fertilization (IVF) and for PrP^C expression analyses. Cumulus cells were obtained from COC by mechanical stripping for 5 min, washed three times in HEPES-buffered synthetic oviduct fluid (SOFH), and expanded in SOF media for 2 weeks to obtain sufficient cells for mRNA extraction. Pools of oocytes, spermatozoa, and cumulus cells were collected and fixed in 10% formalin for immunofluorescence or resuspended in RLT buffer (Qiagen Incorporated, Valencia, CA) for quantitative PCR.

Embryos

Day 4–8 embryos—Bovine embryos were generated by in vitro maturation (IVM), IVF, and in vitro culture (IVC) using our laboratory's protocol (Eyestone, 1999). Briefly, COCs were obtained from abattoir-derived ovaries as described above, matured in SOF media supplemented with 5 µg/ml of follicle stimulating hormone (FSH) and 10% of fetal calf serum (FCS) for 22 h at 38.5°C under a humidified atmosphere of 5% CO₂ in air. For IVF, COC were washed three times in SOFH and placed in 48-µl fertilization droplets of IVF medium under mineral oil. The IVF medium consisted of SOF without glucose and supplemented with 10 µg/ml of heparin (Sigma, St. Louis, MO). Frozen-thawed semen was

enriched for motile sperm using the swim-up method (Parrish et al., 1986), and added to fertilization droplets at a concentration of 25×10^6 per ml. Sperm and COC were co-incubated as described for IVM. After 18 h, COC were stripped of cumulus cells by vortex agitation for 5 min and washed three times in SOFH. Aliquots of 30 presumptive zygotes were transferred to 10 μ l droplets of SOF media under mineral oil for IVC under a humidified atmosphere containing 5% CO₂, 5% O₂ and 90% N₂ in a modular incubator chamber (Billups-Rothenberger, Del Mar, CA). Pools of Day-4 and -8 embryos were collected and immediately fixed in 10% formalin or in RLT buffer, as described above.

Days 14–18 embryos—Day-14 and Day-18 embryos were collected from 32 single-ovulated and 4 super-ovulated Angus cows. Estrus was synchronized by injecting 2 mg estradiol cyprionate (Pfizer Animal Health Incorporated, New York, NY), 50 mg progesterone (P₄; Sigma), and intravaginal insertion of a controlled internal drug releasing (CIDR) device containing 1.9 g of P₄ (Vetrepharm, Canada Inc., London, Canada) (treatment Day 0). On Day 8, cows were injected with 500 μ g cloprostenol (Estrumate, Schering Plough, Union, NJ) and the CIDR were removed. Estruses were detected with a radiotelemetric-pressure sensing system (HeatWatch, Denver, CO). For superovulation, cows were given 50 mg of FSH (Bioniche, Belleville, Ontario, Canada) spread over eight injections given every 12 h, beginning on treatment Day 4. On Days 7 and 8, 500 μ g of cloprostenol was given in the afternoon and morning, respectively. Cows were artificially inseminated with one unit of frozen-thawed semen (20×10^6 sperm) at 12 h, and then again at 24 h after detection of estrus. Embryos were collected on Days 14 or 18 of gestation, by standard non-surgical uterine flushing techniques using an 18-g embryo collection catheter (Bioniche) and approximately 250 ml lactated Ringer's solution per horn. Embryos were washed in phosphate-buffered saline (PBS) under a stereomicroscope and fixed in 10% formalin or resuspended in RLT buffer, as described above.

Days 27–39 embryos—Embryos at Days 27, 32, and 39 of gestation were collected surgically from 10 Holstein Friesian cows. For this purpose, estrus was synchronized on Day 0 by administration of a 100 μ g injection of GnRH (Cystorelin, Merial, Duluth, GA) followed the same day by insertion of a CIDR. At Day 7, the CIDR was removed and 500 μ g of cloprostenol was injected, followed by a second injection of GnRH on Day 9. Cows were observed daily for standing estrus behavior and inseminated immediately upon detection with approximately 20×10^6 sperm. At Day 26 of gestation, cows were evaluated for pregnancy by rectal palpation and confirmed using transvaginal ultrasound (Aloka, Tokyo, Japan). On Days 27, 32, and 39 of gestation, cows were sedated with 0.2 mg/kg xylazine hydrochloride (Rompun, Bayer AG, Germany) and restrained in dorsal recumbency. A dose of 0.06 mg/100 kg of clenbuterol hydrochloride (Planipart, Boehringer Ingelheim, Germany) was administered to induce uterine relaxation. After the surgical site was disinfected, 80 ml of 2% lidocaine hydrochloride (Vedco Incorporated, Saint Joseph, MO) was administered by local infiltration on the ventral midline between the sternum and mammary gland. A 20-cm incision was made through the skin and linea alba, and the gravid uterine horn exteriorized and incised for fetus extraction. After fetal removal, the uterine wall, peritoneum, abdominal muscles, and skin were sutured. Additional intact Day-27, -32 and -39 embryos were obtained from 6 clinically healthy cows at a local abattoir. Reproductive tracts were obtained at slaughter and gently palpated for the presence of a four-to six-week old fetus (bovine gestation interval=283 days, or about 40 weeks). Though detailed descriptions of early bovine nervous system development have not been made, neural tube closure occurs on Day 22 and the formation of sulci and gyri commences around Day 60 (Winters et al., 1942). Thus, fetuses of four-to-six week gestational age clearly represent early stages of neural development. After extraction from the uterus and placental membranes, crown-rump length of each embryo was measured in order to estimate fetal age,

as described in Winters et al., 1942. After removal, all embryos were immediately frozen in dry ice for Western blot or immersed in 10% formalin or in RNA later buffer for immunohistochemistry or PCR analyses.

RNA Extraction and cDNA synthesis

Total RNA was extracted using RNeasy extraction mini kit (Qiagen) according to the manufacturing's instructions. Pools of oocytes (~150), Day-4 (~100), and Day-8 (~100) embryos were homogenized by pipetting in RLT buffer. Pools of spermatozoa ($\sim 75 \times 10^5$), cumulus cells ($\sim 9 \times 10^4$), Day-14 (n=5), and Day-18 (n=4) embryos were homogenized by centrifugation in Qias shredder columns (Qiagen). Day-27 (n=3), -32 (n=3), and -39 (n=3) embryos were homogenized in RLT buffer using a tissue homogenizer rotor (Tissuemiser, Fisher Scientific, Pittsburgh, PA). The concentration and purity of the RNA in each sample was determined using Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). Total RNA was eluted in 30–50 μ l of RNase-free water. Samples were subjected to RT-PCR using an iScript cDNA synthesis kit (Bio-Rad Laboratories., Hercules, CA). The reaction protocol consisted of incubation for 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, and hold at 4°C using a DNA engine PCR thermocycler (Bio-Rad).

Quantitative PCR

Real-time PCR primers and TaqMan probes were designed using PrimerExpress software (Applied Biosystems Incorporated, Foster City, CA) to amplify a segment of cDNA that spans the exon 2-exon 3 junction in the bovine Prnp sequence. Equivalence of amplification efficiencies among all primer-probe sets was confirmed using serial 5-fold dilutions of mouse or bovine brain cDNA (Huckle and Eyestone, unpublished). TaqMan probe sequence (CACAGCAGATATAAGTCATCATGGTGAAAAGCC) specific for the target was designed to contain a fluorescent 5' reporter dye (FAM) and 3' quencher dye (TAMRA). Sequence of forward and reverse bovine Prnp primers were as follows: 5' - CCAGAGACACAAATCCAACCTTGAG and 5' - AACCAGGATCCAACCTGCCTATG. Each RT-PCR reaction (25 μ l) contained the following: 2X Master Mix without uracil-N-glycosidase (12.5 μ l), 40X Multiscribe and RNase Inhibitor Mix (0.63 μ l), target forward primer (60 nM), target reverse primer (60 nM), fluorescent-labeled target probe (4 nM) designed for the RNA sequence isolated from bovine Prnp gene and a total RNA (40 ng). The PCR amplification was carried out in the 7300 Real Time PCR System (Applied Biosystems Incorporated). Thermal cycling conditions were 48°C for 30 min and 95°C for 10 min, followed by 40 repetitive cycles at 95°C for 15 sec and 60°C for 1 min. As a normalization control for RNA loading, parallel reactions in the same multiwell plate were performed using TaqMan Ribosomal RNA as a target (18s control kit, Applied Biosystems). Quantification of gene amplification was made following RT-PCR by determining the threshold cycle (C_T) number for FAM fluorescence within the geometric region of the semilog plot generated during PCR. Within this region of the amplification curve, each difference of one cycle is equivalent to a doubling of the amplified product of the PCR. The relative quantification of the target gene expression across treatment was evaluated using the comparative $\Delta\Delta C_T$ method. The C_T value was determined by subtracting the ribosomal C_T value from the target C_T value of the sample. Calculation of $\Delta\Delta C_T$ involved using oocyte PrP^C mRNA expression (sample with the highest C_T value or lowest target expression) as an arbitrary constant to subtract from all other C_T sample. Relative PrP^C mRNA expression levels in various cell types and stages of development were calculated as fold change in relation to unfertilized oocyte sample and expressed as $2^{-\Delta\Delta C_T}$ value. (Livak and Schmittgen 2001).

Immunofluorescence

Bovine oocytes, and embryos sampled on Days 4 and 8 of gestation were processed for whole-mount immunofluorescence, as described previously (Favetta et al., 2007). Briefly, oocytes and embryos were fixed in 4% paraformaldehyde in PBS for 10 min, washed in PBS, and then stored at 4° C for up to 1 week. Fixed cells were permeabilized in 0.01% Triton X-100 in PBS for 1 h followed by incubation in 5% normal horse serum for 1 h at 37°C. Cells were then incubated overnight at 4°C with SAF-32 mouse monoclonal anti-PrP (1:100; Cayman Chemical, Ann Arbor, MI) in 1% normal horse serum diluted in PBS. Cells were washed three times in PBS for 30 min each, and then incubated overnight at 4°C with Alexa Fluor 594 goat anti-mouse secondary antibody (1:200; Invitrogen Corporation, Camarillo, CA). Processed oocytes and embryos were mounted under coverslips in a solution containing 4', 6-diamino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Immunofluorescence analysis of PrP^C in oocytes and embryos included the following controls: 1) replacement of the primary antibody with non-immune serum and 2) omission of both primary and secondary antibodies. Samples were examined under epifluorescence and the results captured by digital photomicroscopy (Olympus, Tokyo, Japan).

Immunohistochemistry

Formalin-fixed embryos from gestation Days 27, 32, and 39 were embedded in paraffin and sectioned to 5–7 µm using a microtome (Historange, LKB, Bromma, Sweden). Tissue sections were mounted on adhesive slides (Newcomer Supply, Middleton, WI) and incubated overnight at 37°C. Mounted tissues were deparaffinized in xylene and dehydrated in serial alcohol solutions. Slides were subjected to an antigen unmasking protocol by autoclaving at 120°C for 5 min in an unmasking solution (Vector, Laboratories). Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide diluted in 0.1 M PBS (pH 7.4) for 30 min. Tissues were then rinsed twice in PBS, and blocked in 2.5% horse serum for 15 min. PrP^C was detected by overnight incubation at room temperature with primary antibody SAF-32 (1:400) diluted in 1.5% equine serum solution (Vector Laboratories). After two washes in PBS, bound primary antibody was detected using a horseradish peroxidase-tagged horse anti-mouse secondary antibody (Vector Laboratories) for 10 min at room temperature. Immune complexes were visualized using 3, 3'-diaminobenzidine (DAB) substrate for 5 min, or until the signal became visible. Probed sections were then counterstained with hematoxylin and rehydrated in serial alcohol solutions. Sections were mounted under coverslips with Permount (Fisher Scientific). Neighboring sections were used for the following experimental controls: 1) substitution of primary antibody with non-immune serum; 2) substitution of the secondary antibody with non-immune serum; and 3) omission of both primary and secondary antibodies, followed by incubation in DAB alone. Digital photos of tissue sections were obtained using bright microscopy (Olympus Vanox-T, Tokyo, Japan).

Immunofluorescence on sections of embryos at Day 14 and Day 18 of gestation was performed following a similar protocol described for immunohistochemistry, with the following modifications: To achieve the correct orientation, embryos were pre-embedded in 4% agar (Bacto-agar, Difco Lab, Detroit, MI) under a stereomicroscope. Specimens were trimmed, embedded in paraffin, and sectioned transversely at 5–7 µm using a microtome. The peroxidase inhibition step was omitted. After detection with SAF-32 (1:400), slides were incubated in Alexa Fluor 594 goat anti-mouse antibody (Invitrogen Corporation). Slides were coverslipped using mounting solution with DAPI (Vector Laboratories) for visualizing nuclei and examined under epifluorescence. Immunofluorescence analysis of PrP^C in sectioned embryos included the following controls: 1) replacement of the primary antibody with non-immune serum and 2) omission of both primary and secondary antibodies.

Western blot

Frozen Day-27 (n=3), -32 (n=3), and -39 (n=3) embryos were thawed and homogenized (10 w/v) in cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton-X-100, 1% deoxycholate, 0.1% SDS) using a pestle homogenizer (Fisher Scientific, Hampton, NH). Homogenates were centrifuged at 13,500 rpm for 5 min, and the supernatants transferred into a new tube. Total protein concentrations were determined using a bicinchoninic acid (BCA) kit (Pierce; Rockford, IL) according to the manufacturer's instructions. Proteins were denatured by mixing the samples 1:1 (v/v) with Laemmli buffer (BioRad Laboratories) and heating to 98°C for 5 min. Aliquots containing 20 µg of total protein were loaded into each lane of a 12% gels SDS-PAGE Ready-Gel (BioRad Laboratories) and separated at 125 V. Proteins were then transferred onto PDVF membranes by electroblotting at 100 V for 1h. After blocking for 1 h in blocking buffer (LI-COR Corporation, Lincoln, NE), membranes were probed for PrP^C with a monoclonal antibody SAF-32 diluted 1:400 for 1 h at room temperature. All membranes were co-probed for β-actin using a monoclonal antibody (1:1000; Santa Cruz Biotechnology). Primary antibodies were diluted in 0.1% Tween-20 in blocking buffer (LI-COR Corporation). After four 5 min washes in 0.1% Tween-20 in PBS, membranes were incubated in the appropriate anti-mouse or anti-rabbit IRDye-conjugated secondary antibody (LI-COR Corporation) diluted 1:5000 in 0.1% Tween-20 in blocking buffer for 30 min. Immunoreactive band intensities of PrP^C and β-actin were detected and quantified as integrated intensity values using an Odyssey infrared imaging system (LI-COR Corporation). Relative expression of PrP^C was normalized to β-actin expression.

Data Analysis

Analyses for PrP^C expression were repeated three times, and each analysis was quantified separately. Embryo lysates for each of the replicates were run in three independent gels. Values of expression of PrP^C mRNA and protein were transferred to a spreadsheet and then analyzed using SAS software (version 9.3.1, SAS Institute Inc., Cary, NC). Data was normalized to a logarithmic scale in base-10 for normality. Mean values for each replicate were compared by one-way ANOVA. PrP^C expression values for each day were compared to the lowest value using Dunnett's t-test. Significant differences (P<0.05) between days were analyzed using Duncan's multiple comparison test.

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Abbreviations

CNS	central nervous system
<i>Prnp</i>	prion gene
PrP^C	cellular prion protein
PrP^{Sc}	scrapie prion protein
TSE	transmissible spongiform encephalopathy

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Figure 1.

Relative *Prnp* mRNA levels in bovine cumulus cells, gametes, and embryos during early development. *Prnp* mRNA levels were detected using quantitative PCR, normalized to 18s rRNA, and compared to oocyte expression. Cumulus cells (CC) showed significantly higher *Prnp* mRNA levels compared to oocytes. During development, the highest ($P < 0.05$) *Prnp* mRNA levels were detected in Day-4 and Day-18 embryos. (*) Indicates significant difference ($P < 0.05$). Abbreviations: Sp, spermatozoa; Oc, oocytes.

Figure 2.

Expression of PrP^C in bovine oocytes and pre-attachment embryos. PrP^C was immunodetected in whole-mounted oocytes and in Day-4 and -8 embryos. Sagittal histological sections (oriented as dashed line) were obtained from Day-14 and Day-18 embryos (M–Q). PrP^C was detected using SAF-32 antibody followed by Alexa Fluor 594 secondary antibody, and counterstained with DAPI. Specific PrP^C immunofluorescence was observed in the oocyte cytoplasm (B red and C merged), blastomeres in Day-4 embryos (F red and G merged), trophoblast (tf) and inner cell mass (icm) in Day-8 embryos (J red and K merged), and trophoblastic cells in Day-18 embryos (white arrow, R red and S merged). A weak PrP^C immunostaining was observed in Day-14 embryos (N red and O merged). No labeling was observed in the negative controls incubated with non-immune serum instead of SAF-32 antibody (D,H,L,P,T). Phase contrast (PC) (A, E, I, M and Q). Abbreviation: ed, embryonic disc. Scale bars: 25 μ m (B,C,D,F,G,H); 50 μ m (J,K,L).

Figure 3.

Expression of PrP^C protein in Day-27 bovine embryos. PrP^C was immunodetected using SAF-32 antibody followed by peroxidase-conjugated goat anti-mouse IgG, and counterstained with hematoxylin. Low magnification show PrP^C specific immunostaining associated to the developing brain (arrow), spinal cord (arrowhead), and mesonephros (*). Stereoscope image (A). Negative control incubated with non-immune horse serum instead of SAF-32 and counterstained with hematoxylin (B). Higher magnification showed PrP^C-associated peroxidase in the marginal regions (mr) of the brain (D), spinal cord (E) (arrow), and mesonephros (F) (arrowhead). Cell-specific staining was detected in the liver (G–I). Abbreviations: drg, dorsal root ganglion; he, heart; liv, liver; me, mesonephros, mes, mesencephalon; met, metencephalon; mye, myelencephalon; sc, spinal cord; sto, stomach; tel, telencephalon.

Figure 4.

Expression of PrP^C protein in Day-32 bovine embryos. PrP^C-specific immunostaining was detected in the dorsal root ganglion, spinal cord (C, arrow), and mesonephros (arrowhead). Stereoscope image (A). Negative control incubated with with non-immune horse serum instead of SAF-32 and counterstained with hematoxylin (B). PrP^C specific immunostaining was detected in the marginal region (mr) of the brain (D), spinal cord (E), and dorsal root ganglion (F). PrP^C cellular-specific immunostaining was observed in epithelial cells and glomeruli of the mesonephros (G) and scattered multi-nucleated cells in the liver (H–I). Abbreviations: drg, dorsal root ganglion; he, heart; liv, liver; me, mesonephros, mes, mesencephalon; mye, myelencephalon; tel, telencephalon.

Figure 5.

Expression of PrP^C protein in Day-39 bovine embryos. At low magnification, PrP^C-specific immunostaining was detected in the CNS (arrow) and mesonephros (arrow-head) (C). Stereoscope image (A). Negative control incubated with with non-immune horse serum instead of SAF-32 and counterstained with hematoxylin (B). At higher magnification, PrP^C labeling was observed in the marginal region (mr) of the brain (D) and the spinal cord (E), but not in the periventricular region (pv). Staining was also detected in the mantle layer (F), dorsal root ganglions (G), sympathetic trunks (H–J), and peripheral nerves associated to the intestine (K) and lungs (L). In peripheral organs, PrP^C expression was evident in the mesonephros (M) and in multi-nucleated cells in the liver (N–O). Abbreviations: cc, cord canal; drg, dorsal root ganglion; he, heart; li, liver; lu, lung; me, mesonephros; met, metencephalon; tel, telencephalon; sc, spinal cord; st, sympathetic trunks.

Figure 6.

Western blot analysis of PrP^C expression in bovine embryos. PrP^C was detected by Western blot using SAF-32 anti-PrP antibody in Day-27, -32 and -39 embryos. PrP^C displayed two predominant migratory bands at 31 and 29 kDa, representative of different isoforms of the protein (A). Obex tissue was used as a positive control. Relative levels of 29-kDa PrP^C isoform were higher ($P < 0.05$) in Day-39 compared to Day-27 embryos (B). Levels of 31- and 35-kDa PrP^C isoforms and total PrP^C were not different between days of development. (*) Indicates significant difference ($P < 0.05$).