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The prenatal expression of mRNA and protein of the prion protein gene, PRNP, in sheep

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Dedicated to my beloved parents and all members of my family

Die pränatale Expression der mRNA und des Protein vom Prion-Proteingen PRNP beim Schaf

Die Expression des Prion-Protein (PRNP) Gens wurde auf den Ebenen mRNA und Protein in den Geschlechtsorganen des weiblichen Schafs sowie verschiedenen Geweben von Schafsföten unterschiedlicher Trächtigkeitsstadien untersucht. Reproduktive Organe wie Ovar, Eileiter, Endometrium, Myometrium und Karunkel wurden während dem ersten, dritten und fünften Trächtigkeitsmonat beprobt. Im ersten Monat der Entwicklung wurde der gesamte Fötus beprobt, beim zweimonatigen Fötus wurden Proben von Gehirn, Kotyledonen, Herz, Darm, Niere, Leber, Lunge und Muskel entnommen. Bei den drei und fünf Monate alten Föten wurden zusätzlich Proben von Rückenmark und Gehirn entnommen. Die Schafe wurden anhand ihrer PRNP Genotypen in die Kategorien resistent gegen (R1) oder anfällig für (R5) Scrapie eingestuft. In beiden Genotypgruppen konnten die Gentranskripte in allen Stadien und allen untersuchten Geweben mit einer RT-PCR nachgewiesen werden. Das Profil der Genexpression der R1 und R5 Gruppen war ähnlich. Der Vergleich der reproduktiven Organe zeigte das höchste Expressionslevel im Karunkelgewebe der Mutterschafe, während das Level im Gehirn hoch und in der Leber der Feten niedrig war. Zusätzlich wurde eine Real-Time RT-PCR in unreifen Oozyten, reifen Oozyten, *in vivo* Embryonen zum Morulastadium und in einmonatigen Föten durchgeführt. Die Ergebnisse zeigten, dass das relative Expressionslevel der PRNP mRNA in reifen Oozyten und Embryonen im Morulastadium signifikant niedriger war als in unreifen Oozyten und einmonatigen Föten ($p \leq 0{,}05$). Fluoreszenz in situ Hybridisierung von adulten Ovarien und einmonatigen Föten wies die Gentranskripte in Oozyten, Granulosazellen, Thekazellen, Ovarrinde, Ovarmark und Gelbkörpern der Ovarien und im Gehirn, Wirbelsäule, Dermatom, Herz, Leber und Niere der Feten beider Gruppen nach. Eine Westernblot-Analyse zeigte die zu PrP^C korrespondierenden immunreaktiven Banden in allen weiblichen Reproduktionsgeweben ebenso wie in den einmonatigen Föten. Das PrP^C wurde ebenfalls in allen Geweben des zweimonatigen Fötus gefunden. Zusätzlich implizierte die immunohistochemische Färbung die Lokalisation des PrP^C in Gehirn, Herz und Niere des einmonatigen Fötus. Das PrP^C wurde in dieser Untersuchung ebenfalls in beiden Gruppen in der Rinde und im Mark des Ovars jedoch nicht in Oozyten, Granulosazellen, Thekazellen und Gelbkörpern nachgewiesen.

The prenatal expression of mRNA and protein of the prion protein gene, PRNP, in sheep

The expression of the prion protein gene both on mRNA and protein levels were investigated in ovine female reproductive organs and in various tissues of their foetuses during the prenatal stage. Reproductive organs such as ovary, oviduct, endometrium, myometrium and caruncle were collected at the 1st, 3rd and 5th month of pregnancy. Foetal tissues were the whole foetuses at 1 month of age, brain, cotyledon, heart, intestine, kidney, liver, lung and muscle of 2-month-old foetuses. At 3 and 5 months of age the spinal cord and spleen were added. Sheep were categorized as resistant (R1) or high susceptible (R5) to scrapie according to their PRNP genotype. In both genotype groups, the gene transcript was detectable in all stages and all tissues examined by RT-PCR. The gene expression profiles of R1 and R5 groups were similar. Comparisons between reproductive organs demonstrated the highest expression level in caruncle tissue of ewes, whereas the level was high in brain and low in liver of their foetuses. In addition, real-time RT-PCR was performed in immature oocytes, mature oocytes, *in vivo* embryos at morula stage and in 1-month-old foetuses. The results showed that the relative expression levels of PRNP mRNA in mature oocytes and morula-stage embryos were significantly lower than those in immature oocytes and 1-month-old foetuses ($p \leq 0.05$). Fluorescent in situ hybridisation in adult ovaries and 1-month-old foetuses demonstrated the presence of the gene transcript in oocytes, granulosa cells, theca cells, ovarian cortex, ovarian medulla and corpus lutuem of the ovaries, and in brain, vertebral column, dermatome, heart, liver and kidney of the foetuses of both groups. Western blot analyses revealed the immunoreactive bands corresponding to PrP^C in all female reproductive tissues as well as their foetuses collected at the 1st month gestation. The PrP^C was also detected in all tissues of 2-month-old foetuses. In addition, immunohistochemical staining implicated localisation of PrP^C in brain, heart and kidney of 1-month-old foetuses. The PrP^C was also found in ovarian cortex and ovarian medulla of the two groups however, it was undetectable in oocytes, granulosa cells, theca cells and corpus luteum, in this study.

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List of abbreviations

AEC	: 3-amino-9-ethylcarbazole
BLAST	: Basic local alignment search tool
bp	: Base pair
°C	: Degree Celsius
cDNA	: Complementary deoxy ribonucleic acid
DEPC	: Dimethyl pyrocarbonate
DIG	: Digoxigenin
DMF	: Dimethylformamide
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DNase	: Deoxyribonuclease
dNTP	: Deoxynucleotide triphosphate
DTT	: 1, 4, Dithio theritol
E. coli	: *Escherichia coli*
EDTA	: Ethylenediaminetetra acetic acid
Fab	: Fragment antigen binding
g	: Earth's gravity (9.8 m/sec^2)
HCl	: Hydrochloric acid
IPTG	: Isopropyl ß-D-thiogalactopyranoside
ISH	: In situ hybridisation
kDa	: Kilodaton
M	: Molar
mg	: Miligram
min	: Minute
ml	: Mililitre
mM	: Milimolar
MAb	: Monoclonal antibody
MOPS	: 3-Morpholinopropanesulfonic acid
mRNA	: Messenger RNA
NaCl	: Sodium chloride
NaOH	: Sodium hydroxide

ng : Nanogram
nM : Nanomolar
OD : Optical density
PBS : Phosphate bufferred saline
PCR : Polymerase chain reaction
PFA : Paraformaldehyde
POD : Peroxidase, commonly horseradish peroxidase
PRNP : Prion protein gene
PrP : Prion protein
PrP^{C} : Cellular prion protein
PrP^{Sc} : Infectious isoform of prion protein
PVP : Polyvinyl pyrolidone
RNA : Ribonucleic acid
RNase : Ribonuclease
rpm : Rotations per minute
RT-PCR : Reverse transcriptase-polymerase chain reaction
SE : Standard error
sec : Second
SDS : Sodium dodecyl sulphate
SSC : Sodium chloride – sodium citrate buffer
TAE : Tris-acetate-EDTA
TBE : Tris-Boric acid-EDTA
TE : Tris-EDTA
TEA : Triethanolamine
TEMED : N, N, N', N'-Tetramethylendiamine
tRNA : Transfer RNA
TSA : Tyramide signal amplification
U : Unit
w/v : Weight by volume
X-gal : 5-Bromo 4-chloro-3-indolyl-ß-D-galactoside
µg : Microgram
µl : Microlitre
µM : Micromolar

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1 Introduction

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders 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 post-translational conformation change in a host-encoded cellular prion protein (PrP^{C}) to a proteinase-resistant form (PrP^{Sc}) (Prusiner 1991). Various studies showed that mice devoid of functional PRNP gene are resistant to scrapie and do not allow propagation of the infectious agent (Brandner et al. 1996, Bueler et al. 1993, Sailer et al. 1994, Sakaguchi et al. 1996, 1995). Moreover, it has been suggested that the absence of PrP^{C} expression is not the cause of neuronal dysfunction, animals devoid of PrP^{C} are not damaged by exogenous PrP^{Sc}, and the amount of PrP^{C} is a rate-limiting step in the development of the prion disease (DeArmond and Prusiner 1996).

PRNP has been shown to be expressed at high levels in CNS but also in many non-neuronal tissues such as heart, skeletal muscle, lung, intestinal tract, spleen, testis, ovary, lymphocytes, lymph nodes and some other organs in rodents and ruminants (Bendheim et al. 1992, Mabbott et al. 1997, McBride et al. 1992, Pammer et al. 1998). PrP^{C} is a normal protein located on the exterior cell surface and is attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (Stahl et al. 1990). This protein is believed to be involved in several functions in the cell such as protection against antioxidant activity by regulating copper ion concentration (Brown et al. 2001, 2002, Klamt et al. 2001) transduction of neuroprotective signals or even prevention of apoptosis in retinal cells (Chiarini et al. 2002). Although, it’s precise function(s) remains unclear.

Susceptibility to scrapie in sheep is influenced by polymorphisms at codons 136, 154 and 171 of the PRNP gene (Tranulis 2002). The ovine PRNP genotypes were classified into 5 risk groups according to the susceptibility to the disease; R1 genotype being the resistant and R5 genotypes being high susceptible to the disease (Erhardt et al. 2002). Tuo et al. (2002) reported that accumulation of PrP^{Sc} in uterine-placental epithelial cells in the placentome was influenced by the foetal PRNP genotype and the pregnancy status of scrapie-infected ewes.

In natural TSE infections the gastrointestinal tract is considered to be the major route of the infection (Andreoletti et al. 2000, Hadlow et al. 1982, Pattison et al. 1972), and the scrapie infectious prions diffused to sheep and goat are hypothesized to originate from placenta of infected ewes (Andreoletti et al. 2000, Pattison et al. 1972, Tuo et al. 2001). Oral and parental inoculation of PrP^{Sc} resulted in 100% infection of the prion disease in mice (Manolakou et al. 2001, Narang 1996, Narang 2001). 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 (Foster et al. 1996) or those showing prevention of vertical infection by embryo transfer (Foote et al. 1993, Wang et al. 2002, 2001).

Since the expression of PRNP is a prerequisite for the infection, studies on the temporo-spatial expression of PRNP in foetal 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:

1) Quantitative expression profiling of the PRNP gene in R1 and R5 ovine immature oocytes, mature oocytes and *in vivo* preimplantation embryos using real-time PCR.

2) Semi-quantitative expression profiling of the PRNP gene during prenatal stages in R1 and R5 ovine foetal and maternal tissues using RT-PCR technology.

3) Detection of PrP^{C} in R1 and R5 female reproductive tissues of ewes and whole foetuses at the 1^{st} month of pregnancy as well as various tissues of 2-month-old foetuses by western blot.

4) Detection and localisation of PRNP mRNA and PrP^{C} in adult ovaries and 1-month-old foetuses of both groups.

2 Literature review

2.1 Prion diseases

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders of the central nervous system that occur in a number of species. The clinical signs of these diseases are variable but include progressive dementia, cerebellar ataxia and behavioural disturbances. Gross neuropathology can include reduced brain weight, cortical atrophy and enlargement of the ventricles (reviewed in Moore and Melton 1997). Histophatological changes of the brain comprise a fine vacuolation termed spongiosis, reactive changes of astrocytes called gliosis, and variable loss of neurons (reviewed in Brandner et al. 2000). Widespread apoptosis has been described in the brain of infected sheep, mice and human and may be a major mode of cell death (Fraser 1976).

Prion diseases are found in both animals and human including bovine spongiform encephalopathy (BSE) or mad cow disease in cattle, chronic wasting disease (CWD) in elk and deer, transmissible mink encephalopathy (TME) in mink, feline spongiform encephalopathy (FSE) in cat, puma, cheetah, ocelot and tiger, and ovine spongiform encephalopathy (OSE) or scrapie in sheep and goat. The prion diseases in human are Alpers syndrome, Kuru, fatal familial insomnia (FFI), Gerstmann–Sträussler–Scheinker syndrome (GSS), Creutzfeldt–Jakob disease (CJD) and a new variety of Creutzfeldt–Jakob disease (nv CJD) which is caused by the transmission of BSE via dietary exposure (Moore and Melton 1997, Will et al. 1996) (Table 1). The possibility that the infectious agent of prion disease consists of viral particles can be ruled out, since no nucleic acid has been detected. The protein-only hypothesis has been formulated firstly by Griffith (1967), and supported by additional data of Prusiner (1982) who coined the term prions, composed from “proteinaceous infectious particle” to identify the agents that cause a novel type of fatal brain diseases. The hypothesis states that the infectious prion protein is devoid of nucleic acid, and the primary cause of these diseases is a post-translational conformation change in a host-encoded cellular prion protein (PrP^{C}) to the disease-***c***ausing isoform (PrP^{Sc}) (Prusiner 1991, 2004).

Table 1: Transmissible spongiform encephalopathies (Adapted from Moore and Melton 1997, Prusiner 1994, 2004)

Species	Disease	Description	Distribution
Sheep	Scrapie	Field scrapie; infection in genetically susceptible sheep. Experimentally transmitted to sheep, goats, mice, hamsters and cattle (Gibbs et al. 1990, Okamoto et al. 2003).	Worldwide (1936)
Goat	Scrapie	Natural cases rare	
Mink	Transmissible mink encephalopathy	Infection in farmed mink; source of infection probably scrapie infected offal. Experimentally transmitted to hamsters.	North America; Europe
Mule Deer	Chronic wasting disease	Infection of farmed deer probably from scrapie contaminated feed. Experimentally transmitted to other deer.	North America
Elk	Chronic wasting disease	Infection in farmed elk, probably derived from scrapie infected feed.	
Cattle	Bovine spongiform encephalopathy	UK BSE epidemic mostly in Holstein Friesian dairy cattle; scrapie contamination of feed. Experimentally transmitted to mice (Fraser et al. 1992), pigs (Ryder et al. 2000), monkeys (Lasmezas et al. 2005, 1996) and mink (Robinson et al. 1994).	UK; Republic of Ireland; Europe
Domestic cat		Infection with prion-contaminated bovine tissues or MBM in domestic cats. Transmitted to mice (Fraser et al. 1994)	UK; Norway
Cheetah		Infection in a zoo specimen	UK
Puma		Infection in a zoo specimen	UK

MBM: meat and bone meal

Table 1: Transmissible spongiform encephalopathies (Continued, adapted from Moore and Melton 1997, and Prusiner 1994, 2004)

Species	Disease	Description	Distribution
Human	Sporadic CJD	Sporadic: $1:10^6$ incidence; not associated with prion protein (PrP) mutations. Experimentally transmitted to chimpanzees, monkeys, cats and mice	Worldwide
	Iatrogenic CJD	Iatrogenic: transmission following neurosurgery, corneal transplant and dura mater graft	Worldwide
	Familial CJD	Familial: linked to mutations in prion protein; autosomal dominant, some have variable penetrance	Worldwide
	New variant CJD	Unusual CJD variant in UK (10 cases) and France (1 case) with early onset (<42 years), unusual clinical presentation and neuropathology. Infection from bovine prions. Transmissibility to rodents not yet demonstrated.	UK; France
	Gerstmann-Scheinker-Sträussler Syndrom	Familial disorder tightly linked to mutations in the prion protein	Worldwide
	Fatal familial insomia	Familial disorder linked to germline mutation in prion protein in association with a common PrP polymorphism	Worldwide
	Kuru	Infection: Sporadic CJD spread and maintained by cannibalism; endemic to a remote highland region of Papua New Guinea. Experimentally transmitted to goats, primates and rodents	Papua New Guinea (1950)

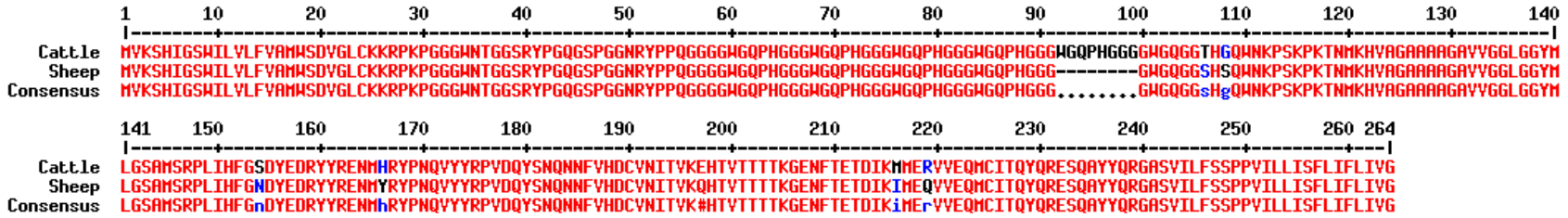

Figure 1: Amino acid sequence alignment of bovine and ovine prion protein

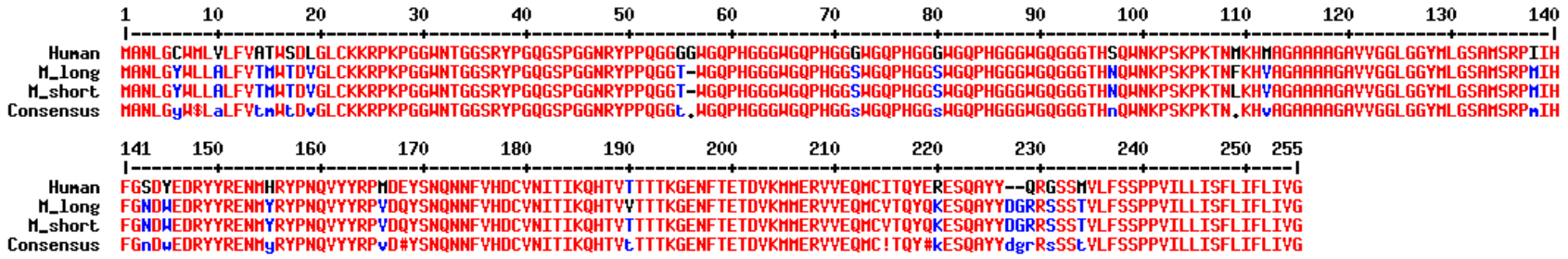

Figure 2: Amino acid sequence alignment of human and mouse prion protein

2.2 Incubation time and transmission of prion diseases

When the titre of infectious prion reaches a critical threshold, animals develop signs of neurologic dysfunction. The time interval from inoculation to clear signs of the disease is referred to as the incubation period. The length of incubation period can be modified by the dose of the infectious agent, the route of inoculation, the level of PrP^{C} and polymorphism in the sequence of PrP^{C} (Prusiner et al. 2004). Since the mean incubation time for BSE is ~4-5 years, most cattle do not manifest the disease because they are slaughtered between 2-3 years of age (Spongiform Encephalopathy Advisory Committee 1995). For the scrapie in sheep, the incubation period range from 3.5 – 23 months after intracerebral injection with a source of scrapie known as SSBP/1 (Dickinson 1976).

2.2.1 Transmission of human prion diseases

The infection route of Kuru is composed of a combination of consumption, ocular exposure and skin exposure to the infected, uncooked and cooked tissues of dead patients at mortuary rituals of the Fore people in Papua New Guinea (Spongiform Encephalopathy Advisory Committee 1995). For the CJD, it seems unlikely that the disease is spread among humans by infection except in the cases of accidental inoculations. Most of CJD cases are sporadic and are the result of a somatic mutation, the spontaneous conversion of PrP^{C} to PrP^{Sc}, or reduced clearance of low levels of PrP^{Sc} that are normally present (Prusiner et al. 2004).

2.2.2 Transmission of BSE and scrapie

BSE in cattle appears to be transmitted through concentrate feeds containing ruminant-derived meat and bone meal (MBM). The MBM is prepared from the offal of sheep, cattle, pigs and chicken as a high-protein nutritional supplement. The hydrocarbon-solvent extraction method is used in the rendering of offal results in MBM with much higher fat content. This process allowed scrapie prions from sheep to survive rendering and subsequently to be passed into cattle (Prusiner et al. 2004, Spongiform Encephalopathy Advisory Committee 1995). However, an ability of the bovine

gastrointestinal microbiota to degrade the infectious prions during digestion was observed. Scherbel et al. (2006) demonstrated that after incubation of the microbial consortia collected from rumen and colon with brain homogenates of scrapie infected hamsters for 20 hours, PrP^{Sc} was digested up to immunochemically undetectable levels. Especially polymyxin resistant (mainly gram-positive) bacteria expressed PrP^{Sc} degrading activity.

In sheep and goat, the natural route by which infectious agents are transmitted among these species is unknown. However, in natural scrapie, the gastrointestinal tract is considered to be the major route of infection (Hadlow et al. 1982). Some investigations have suggested that horizontal transmission occurs orally, and the scrapie infectious prions diffused to sheep and goat are hypothesized to originate from placenta of infected ewes (Andreoletti et al. 2000, Pattison et al. 1972, Tuo et al. 2001). Nevertheless, there is no evidence for vertical transmission or for transmission through semen and embryos (Wrathall 1997).

For experimentally transmission, scrapie and BSE can be transmitted through multiple routes (Wrathall 1997). The disease was first transmitted by intraocular inoculation (Cuille and Chelle 1939, reviewed in Prusiner 2004) and later by intracerebral (Dickinson 1976, Renwick and Zlotnik 1965), oral (Prusiner et al. 1985), subcutaneous (Buyukmihci et al. 1985), intravenous (Kimberlin and Walker 1989) and intragastrical, intracardiac and intramuscular (Zhang et al. 2004) injections. However, many of the inoculated animals often failed to develop the disease (Dickinson and Stamp 1969, Hadlow et al. 1980, 1982). Moreover, many studies showed that different PRNP genotypes of sheep influenced the incubation time and susceptibility to scrapie prions inoculated, suggesting that the genetic background influence host permissiveness (Bulgin et al. 2006, Goldmann et al. 1991a, Maciulis et al. 1992, O'Rourke et al. 1997).

2.2.3 Maternal –vertical transmission

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 (Foster et al. 1996) or those showing prevention of vertical infection by embryo transfer (Foote et al. 1993, Wang et al. 2002, 2001).

Foster et al. (1996) examined the effect of washing the embryos and the viability of highly susceptible offspring derived from scrapie-affected and uninfected donors. The results showed that scrapie occurred in both washed and unwashed embryos from both groups of donor ewes. This is in contrast to the study of Foote et al. (1993), who measured scrapie transmission via the embryo by using offspring from embryos of scrapie-inoculated donors and scrapie-free recipients, and via the uterus by using offspring from embryos of scrapie-free donors and scrapie-inoculated recipients taken by cesarean section. The results showed that none of the scrapie-free donor/recipients, including those gestating embryos from scrapie-inoculated donors, and the offspring from reciprocal cross, via embryo, or via the uterus developed scrapie. These investigations were supported by the studies in cattle (Wrathall et al. 2002) and sheep (Wang et al. 2002, 2001). The results reported that embryos collected from donors with a high incidence of clinical or natural prion disease did not develop the diseases even many embryos had the high risk genotype. In a study of experimental BSE of Foster et al. (2004), Cheviot ewes challenged orally with BSE cattle brain produced lambs of various PRNP genotypes. Of 72 surviving to >30 months of age, 29 are of the most susceptible PRNP genotype ($A_{136}Q_{171}/A_{136}Q_{171,}$ the underscript numbers present the order of amino acid) and born to mothers that were challenged with BSE. However, none of the progeny have shown any signs of BSE disease. From these studies it could be concluded that prion diseases were not neither transmitted to offspring via the embryo nor was the infective agent transmitted to recipient animals during embryo transfer procedures even when the offspring have the high risk genotype. In addition, since PrP^{Sc} was detected in placenta from scrapie-infected ewes (Race et al. 1998) and oral inoculation is a major route of scrapie infection, by which lambs may be exposed to scrapie by ingesting the transmissible agent during contact with contaminated wool and mammary gland of the dam (Tuo et al. 2001).

2.3 Prion protein

2.3.1 Structure of prion protein

The mammalian PRNP encodes a protein of ~250 amino acids (aa) that contains several distinct domains, including an amino-terminal signal peptide, a series of five peptide repeats, a central hydrophobic segment and a carboxy-terminal hydrophobic region that is a signal for addition of a glycosyl phosphatidyl inositol (GPI) anchor (Harris et al. 2004). The amino acid sequences of prion protein (PrP) in human, cattle, sheep and mouse with long and short incubation period for prion disease are shown in Figure 1 and Figure 2. Codon and amino acid abbreviations are shown in appendix 1.

The human prion protein contains 253 aa (AAG21693) while cattle and sheep prion proteins contain 264 (CAA39368) and 256 (CAA04236) aa, respectively. Mouse PrP with long and short incubation time have the same number of 254 aa (AAD41440 and AAC02804, respectively). The amino acid sequences of prion protein in these four species are quite similar especially when comparing between human and mouse (Figure 2), and cattle and sheep (Figure 1). Differences in the sequence of prion protein in mouse with long and short incubation time are a substitution of leucine (L) to phenylalanine (F) at codon 109, and threonine (T) to valine (V) at codon 190. Between human and mouse, the differentiation in prion protein sequences consist of 26 aa was observed, in which a deletion at codon 56, and an insertion of two amino acids between codons 226 and 227 in mouse PrP sequence (residual number refer to the number of human PrP). Comparison between bovine and ovine prion protein sequences shows an insertion of eight amino acids in bovine PrP at codons 92-99, and substitution of serine (S) -> threonine (T), serine (S) -> glycine (G), asparagine (N) -> serine (S), tyrosine (Y) -> histidine (H), and isoleucine (I) -> methionine (M) at codons 106, 108, 154, 166 and 216, respectively (residual number refer to the number of bovine PrP).

The characteristic secondary structure elements of prion protein are three alpha helices, with two small beta-sheets. From the human PrP structure shown in Figure 3A, three helices are shown as HA, HB and HC while two beta-sheets are shown as S1 and S2, and two N-linked polysaccharides are shown at top left and center. HA is a short helix, spanning residues 144-154, HB is a regular helix spanning 173-194, and HC is longer. The two glycosylation sites are asparagine 181 on the HB, and asparagine 197 between

HB and HC (human sequence). One disulfide bond is present in the protein connecting the second and third helices via cysteine179-cysteine 214 (Govaerts et al. 2004).

(A) **(B)**

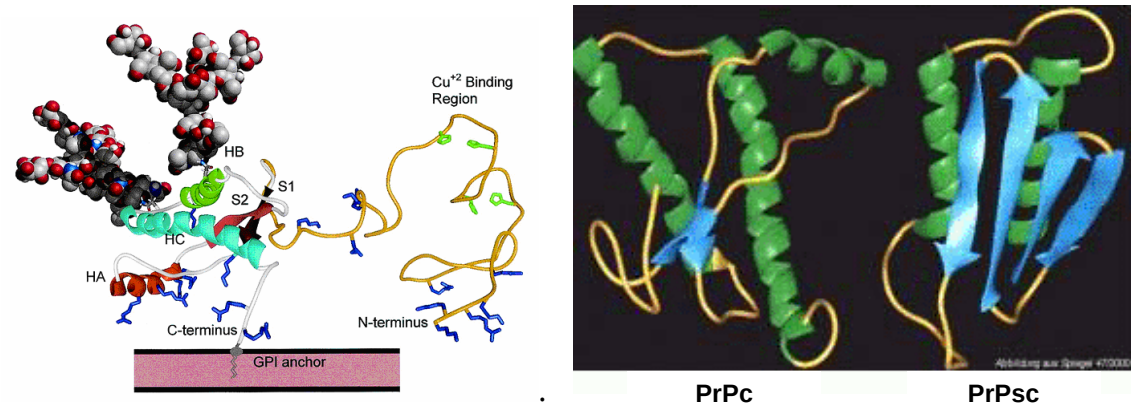

Figure 3: Model of the C-terminus of human prion protein (A). Structure model of PrP^{C} and PrP^{Sc} (B)

2.3.2 Biochemical and biophysical differences between PrP^{C} and PrP^{Sc}

PrP^{C} is a normal cellular host-encoded glycoprotein found in neuron system of mammals. It is located on the exterior cell surface and is attached to the plasma membrane via a GPI anchor (Stahl et al. 1990). PrP^{Sc} is a modified isoform of PrP^{C} originated by a conformation change of PrP^{C} during a posttranslational process. This modification is non genomic protein and causes TSEs in humans and animals (Prusiner 1982).

Since both of PrP^{C} and PrP^{Sc} have the same primary amino acid sequence and similar N-linked carbohydrate side chain and GPI anchors, chemical differences between PrP^{C} and PrP^{Sc} are not found (Stahl et al. 1993). However, biochemical and biophysical differences between PrP^{C} and PrP^{Sc} have been identified. PrP^{C} is a non-infectious agent, soluble in soft detergent and sensitive to proteinase K digestion. In contrast, PrP^{Sc} is an infectious agent associated with prion diseases infectivity, insoluble in soft detergents and resistant against digestion of proteinase K. Moreover, infrared spectroscopy

indicated that the secondary structure of PrP^{C} contains approximately 42% alpha-helix and 3% beta-sheet, whereas PrP^{Sc} contains 30% alpha-helix and 43% beta-sheet (Figure 3B, Pan et al. 1993). In addition, while a tertiary structure of PrP^{C} included a long flexible N-terminal tail, three alpha-helix and two small anti-parallel ß-strands that flank the first alpha-helix, a tertiary structure of PrP^{Sc} has not yet been obtained (Harris 1999).

2.3.3 Conversion of PrP^{C} into PrP^{Sc}

The mechanism of the conversion of PrP^{C} to PrP^{Sc} is not well understood. However, cell-free conversion studies indicate that PrP^{Sc} aggregates are able to convert PrP^{C} into a protease-resistant PrP isoform (Bessen et al. 1995, Kocisko et al. 1996). In order to explain the mechanism by which an infectious form of PrP could induce the refolding of native PrP molecules into the abnormal conformation, three distinct models have been postulated: (i) the template-directed model (ii) the template-assisted model and (iii) the nucleation-polymerisation model (Figure 4). The first and second models postulate an interaction between exogenously introduced PrP^{Sc} and endogenous PrP^{C}, and a prevention of spontaneous conversion of PrP^{C} and PrP^{Sc} by high energy barrier. The first model (Figure 4A) suggests that PrP^{Sc} forms a heterodimer with PrP^{C} and causes PrP^{C} to undergo an induced conformational change to yield PrP^{Sc} (Prusiner 1991). The template-assisted model (Figure 4B) states that in an uninfected cell, PrP^{C} with wild type sequence is likely to exist in equilibrium with its monomeric alpha-helical, protease-sensitive state or bound to some other proteins, such as protein X. The conformation of PrP^{C} that is bound to protein X is denoted PrP*. This PrP*-protein X complex will bind PrP^{Sc}, thereby creating a replication-competent assembly. When PrP* is transformed into a nascent molecule of PrP^{Sc}, protein X is released and a dimer of PrP^{Sc} remains. In this model, a fraction of infectious PrP^{Sc} dimers dissociates into uninfectious monomers as the replication cycle proceed, while a majority of the dimers accumulate in accord with the increase in prion titer that occurs during the incubation period (Prusiner 2004). The nucleation model (Figure 4C) proposes that PrP^{C} and PrP^{Sc} are in equilibrium, strongly favouring PrP^{C}. The PrP^{Sc} is only stabilized when it adds onto a crystal-like seed or aggregate of PrP^{Sc}. If a stable aggregate needs to consist minimally of a substantial number of PrP^{Sc} molecules, then its spontaneous formation

would be a very rare event. However, once a seed is present, monomer addition could ensue at a rapid rate. Fragmentation of PrP^{Sc} increases the number of nuclei which can recruit further PrP^{Sc} and results in apparent replication of the agent (reviewed in Weissmann 1999).

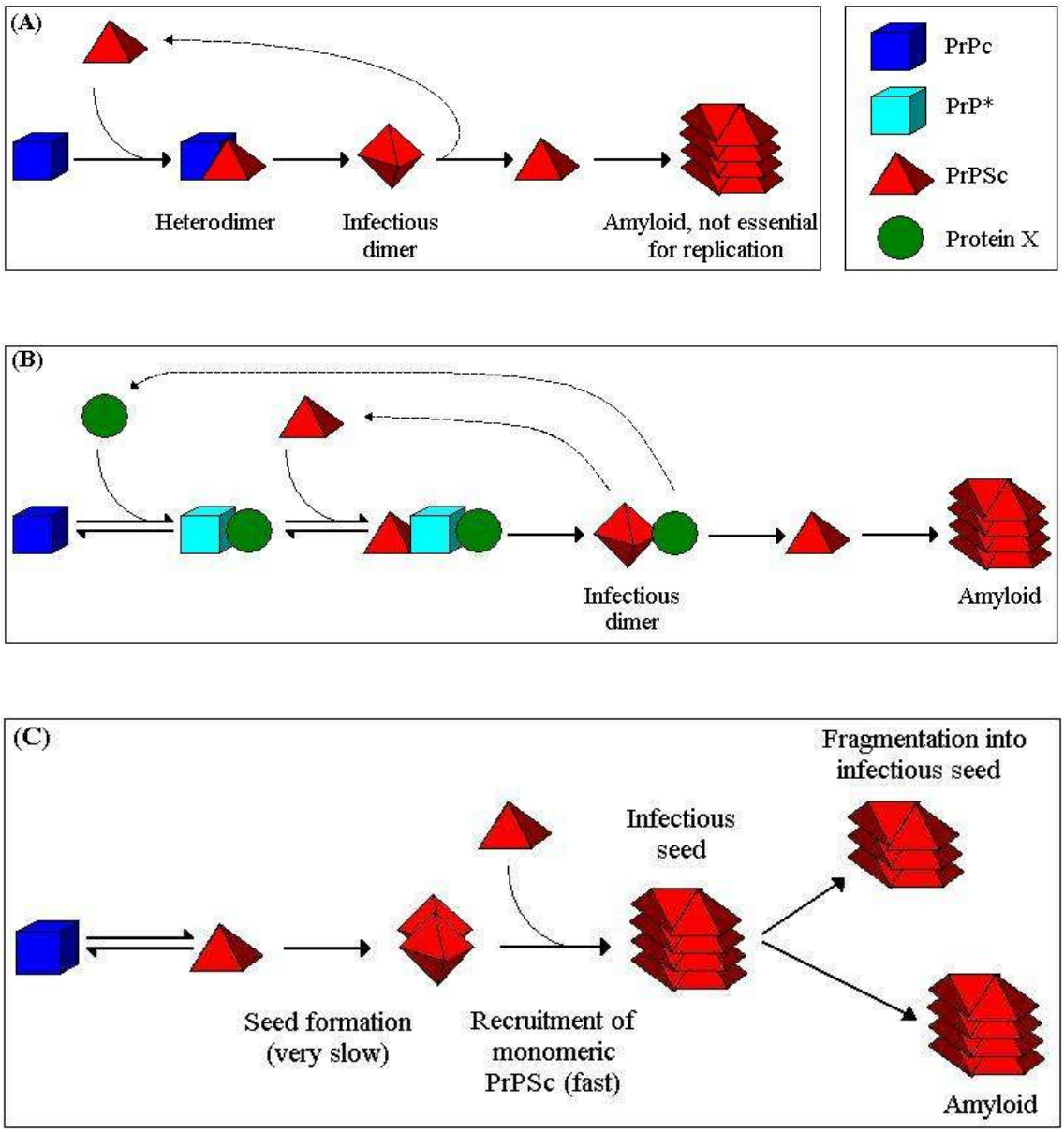

Figure 4: Hypothetical scheme for the conformational conversion of PrP^{C} into PrP^{Sc}. The template-directed model (A), the template-assistance model (B) and the nucleation-polymerisation model (C)

2.3.4 Function of PrP^C

The precise function(s) of PrP^C remains unclear, but the localisation of PrP^C on the cell surface would be consistent with roles in cell adhesion and recognition, ligand uptake, or transmembrane signalling. Defining the physiological role of PrP^C may be relevant to understanding the disease state, since the protein may fail to perform its normal function when it is converted to the PrP^{Sc} isoform (Harris 1999). It has been shown that mice homozygous for disrupted PRNP ($PRNP^{-/-}$) developed and behaved normally for at least 7 months, and no immunological defects were apparent (Bueler et al. 1992). On the other hand, some studies presented that $PRNP^{-/-}$ mice revealed an alteration in circadian activity rhythms and sleep patterns (Tobler et al. 1997, 1996), abnormalities of synaptic neurophysiology in the hippocampus (Colling et al. 1996, Whittington et al. 1995), loss of Purkinje cells in the vast majority of cerebellar folia (Sakaguchi et al. 1996) and a disturbance in latent learning and long-term memory retention (Nishida et al. 1997). However, other investigators did not observe certain of these features (Herms et al. 1995, Lledo et al. 1996)

PrP^C has been suggested to be involved in several functions in the cell, such as copper metabolism, since PRNP-null mice display a reduction in the copper content of membrane-enriched brain extracts and synaptosomal fractions, and a reduced activity of copper/zinc superoxide dismutase (Brown et al. 1997). Additional possible functions of the PrP^C are protection against oxidative stress by regulating copper ion concentration (Brown et al. 2001, 2002, Klamt et al. 2001), transduction of neuroprotective signals and prevention of apoptosis function in retinal cells (Chiarini et al. 2002). An additional study on antiapoptotic function of PrP^C was done by Liang et al. (2006), who introduced either the PrP^C or two pairs of RNAi into the gastric cancer cell line. The results showed that PrP^C slowed down apoptosis in transfected cells. In addition, the apoptosis-related protein Bcl-2 was upregulated, whereas Bax was downregulated in the PrP^C-transfected cells. These contrast the results in RNAi-transfected cells that showed the downregulated Bcl-2 and upregulated Bax. These results suggested that PrP^C might play a role as an effective antiapoptotic protein through Bcl-2-dependent apoptotic pathways in gastric cancer cells.

2.3.5 Role of PrP^C in prion diseases

According to the protein only hypothesis, PrP^C is a substrate for the infection of prion diseases to start by the conversion of PrP^C into PrP^{Sc}. An important corollary to this hypothesis is that an organism lacking PrP^C should be resistant to prion disease and unable to propagate the infectious agent. To this end, many groups generated mice with a targeted disruption of the PRNP gene. The results showed that after challenged with infectious prions, PRNP knockout mice were completely protected against scrapie disease and did not allow propagation of the infectious agent (Bueler et al. 1993, Sailer et al. 1994, Sakaguchi et al. 1996, 1995). Moreover, after grafting neural tissue with PRNP over expression into the brain of PRNP-deficient mice followed by intracerebral inoculation with scrapie prions, the grafts accumulated high levels of PrP^{Sc} and infectivity, and developed the severe histopathological changes characteristic of scrapie, whereas no pathological changes were seen in PRNP-deficient tissue, not even in the immediate vicinity of the grafts (Brander et al. 1996). In addition, heterozygous mice with only one disrupted allele ($PRNP^{+/0}$) showed partial resistance to scrapie infection as manifested by prolonged incubation times of ~290 days compared to ~160 days in $Prnp^{+/+}$ mice (Bueler et al. 1994). It has been showed that when a Syrian hamster (SHa) PrP transgene was introduced into $PRNP^{0/0}$ mice by crossing them with transgenic (SHaPrP) mice, the transgenic (SHaPrP) mice became susceptible to SHa prions but not to mouse prions (DeArmond and Prusiner 1996). These suggest that the absence of PrP^C expression is not the cause of neuronal dysfunction, animals devoid of PrP^C are not damaged by exogenous PrP^{Sc}, and the amount of PrP^C is a rate-limiting step in the development of the prion disease.

2.3.6 Expression of PrP^C

PrP^C has been observed at high level in central nervous system (CNS) and could be detected in several non-neuronal tissues such as squamous epithelia of the skin and upper gastrointestinal tract of human (Pammer et al. 1998), leukocytes, heart, skeletal muscle, lung, intestinal tract, spleen, testis, ovary, lymph nodes, Peyer's patches, thymus, and pancreas of mice and cows (Bendheim et al. 1992, McBride et al. 1992), as well as on the surface of lymphocytes in humans and mice (Mabbott et al. 1997).

A prion protein isoform truncated at the C terminus has been detected in ram (Ecroyd et al. 2004), human and cattle mature sperm cells (Shaked et al. 1999), which retain only the minimal components required for their unique function. Horiuchi et al. (1995) demonstrated the distribution of PrP^{C} in sheep organs and showed that PrP^{C} can be detected in several tissues besides the brain such as spleen, lymph node, lung, heart, kidney, skeletal muscle, uterus, adrenal gland, parotid gland, intestine, proventriculus, abomasum and mammary gland.

In brain, immunohistochemical studies have described a somatic expression of PrP^{C} in neurons with no or only a minor signal in the neuropil, which is a network of unmyelinated neuronal processes (axonal and dendritic) within the gray matter of the CNS. By electron microscopy, PrP^{C} labelling in the neuropil was found on the plasma membrane of dendrites, including spines, as well as dendritic transport vesicles, endosomes, axolemma, axonal transport vesicles and myelin sheaths. In both neuronal and glial cells, PrP^{C} was detected in the endoplasmic reticulum, Golgi complex, endosomes, uncoated transport vesicles and the plasma membrane, which are important parts of the biosynthetic and endocytic pathway (reviewed in Harris et al. 2004).

2.3.7 Accumulation of PrP^{Sc}

In natural scrapie, the gastrointestinal tract is believed to be the major route of infection. The earliest accumulations of PrP^{Sc} are found in ileal Peyer's patch, tonsil, retropharyngeal lymph nodes, mesenteric-portal lymph nodes, and intestine. Accumulation of PrP^{Sc} in the enteric nervous tissue has been reported to appear later but still in an early phase of the disease (Andreoletti et al. 2000, Hadlow et al. 1982, Maignien et al. 1999). PrP^{Sc} also aggregates in other tissues such as tingible body macrophages (TBMs), follicular dendritic cells (FDCs), distal jejunal lymph node, medial retropharyngeal lymph node (RPLN), spleen and placenta in sheep with both clinical and nonclinical disease (Ersdal et al. 2003, Jeffrey et al. 2000, Race et al. 1998, Schreuder et al. 1998, van Keulen et al. 1996). The earliest traces of PrP^{Sc} in the brain have been found in the autonomic myenteric nervous plexus and in the nucleus parasympathicus nervi X of the brain stem when naturally infected lambs are 9 months old (Andreoletti et al. 2000). The scrapie prions seem to reach the spinal cord at the same time since the PrP^{Sc} was detected in the dorsal motor nucleus of the vagus and the

intermediolateral column of the thoracic segments T8-T10 in naturally infected lambs at the age of 10 months (van Keulen et al. 2000).

The presence of PrP^{Sc} in placentas from clinically scrapie-affected goats and ewes was demonstrated by mouse bioassays. PrP^{Sc} accumulation was detected in foetal chorion from pregnant scrapie-incubating ewes. Interestingly, the placenta from a scrapie-incubating ewe can be found as PrP^{Sc}-positive in its first gestation and negative in the following gestation (Andreoletti et al. 2002). Moreover, Tuo et al. (2002) indicated that accumulation of PrP^{Sc} is eliminated or reduced to undetectable levels in reproductive and placental tissues if infected ewes are not pregnant or conceive conceptuses with a resistant PRNP genotype.

2.4 The prion protein gene

2.4.1 Gene structure

The prion protein gene is located on the short arm of chromosome 20 in human, the syntenic region chromosome 2 in mouse (Prusiner 2004), and chromosome 13 in cattle and sheep (Castiglioni et al. 1998, Schlapfer et al. 1998). The gene structures of all species are similar. All of them contain three exons, which are separated by two large introns, and the coding regions (CDS) of all are located on exon 3. The length of the exons, introns and CDS of human, cattle, sheep and mouse PRNP are shown in Table 2

Table 2: Size (base pairs) of exons, introns and CDS of human, cattle, sheep and mouse PRNP

Species	Exon 1	Exon 2	Exon 3	CDS	Intron 1	Intron 2
Human[1]	134	99	2,354	762	2,622	9,975
Cattle[2]	53	98	4,091	795	2,436	13,552
Sheep[3]	52	98	4,028	771	2,421	14,031
Mouse						
- long incubation time[4]	47	98	2,009	765	2,190	11,127
- short incubation time[5]	47	98	2,153	765	2,190	17,733

Reference number [1/] U29185, [2/] AJ298878, [3/]U67922, [4/] U29187, [5/] U29186

2.4.2 Polymorphisms of human, cattle and mouse PRNP

In the human prion protein gene, a polymorphism at codon 129 encoding either methionine (M) or valine appears to influence human prion disease expression in the Caucasian population, not only in the inherited forms but also in the iatrogenic and sporadic forms. Genotype frequencies for the codon 129 polymorphisms in Caucasians are 12% V/V, 37% M/M and 51% M/V. In sixteen patients (fifteen white and one black) from United Kingdom, United States and France with iatrogenic CJD, eight (50%) were V/V, five (31%) were M/M, and three (19%) were M/V. Thus, a disproportionate number of patients with iatrogenic CJD were homozygous for valine at human PrP codon 129. This contrasts to the frequency of the V allele in the Japanese population which is much lower and heterozygosity at codon 129 is more frequent in Japanease CJD patients (18%) than in the general population where the polymorphisms are 0% V/V, 92% M/M and 8% M/V (reviewed in Prusiner 1996). Wadsworth et al. (2004) also reported that the generation of variant CJD in transgenic mice required expression of human prion protein with methionine 129; expression of human PrP with valine 129 resulted in a distinct phenotype and persistence of a barrier to transmission of BSE-derived prions on subpassage. In addition, the homozygosity for methionine at codon 129 of the human PrP appears to influence the onset of neurological and hepatic symptoms in patients with the copper storage disorder Wilson disease as well (Merle et al. 2006).

An insert of 144 bp containing six octarepeats at codon 53 (5 octarepeats are normally present) was described in patients with CJD from four families residing in southern England. It has been demonstrated that inserts of 2, 4, 5, 6, 7, 8 or 9 octarepeats in addition to the normal five are found in individual with inherited CJD. Moreover, a polymorphism resulting in substitution of glutamate (E) -> lysine (K) at codon 219 has been reported in the Japanese (Shibuya et al. 1998) and Korean populations (Jeong et al. 2005). The lysine at codon 219 seems likely act dominant and protects against CJD over the glutamine in both populations. In addition, it seems that PrP^{C} is not converted to PrP^{Sc} in the people carrying K/K genotype (reviewed in Prusiner 2004).

Many polymorphisms of bovine PRNP have been reported as shown in Figure 5 (Sander et al. 2004). The positions of 60 polymorphisms detected in German cattle are indicated with respect to the PRNP reference sequence AJ298878. The nucleotide sequence

variants detected in the coding region of the bovine PRNP are a variation in the number of octapeptide repeats and the substitution of base G -> A, C -> T and C->T at position numbers 234, 339 and 576 of the coding region, respectively. For the octapeptide repeats polymorphism, most cattle have five repeats, but some have six repeats (Goldmann et al. 1991b, Hunter et al. 1994) and even seven repeats in Swiss brown cattle (Schlapfer et al. 1999). The amino acid sequences of the octapeptide repeats are Pro-His-Gly-Gly-Gly-Try-Gly-Gln or its longer variants Pro-Gln/His-Gly-Gly-Gly-Gly-Try-Gly-Gln (see amino acid abbreviations in Appendix 1). In contrast to human and sheep, no bovine PRNP polymorphism associated to the prion disease has been reported. An analysis of 370 cattle in Scotland revealed no difference between the frequencies of the PRNP octapeptide repeats genotypes (6/6, 6/5 and 5/5 repeats) in healthy cattle and cattle with BSE (Hunter et al. 1994). Eventhough significant differences in allele frequencies for the 23 bp insertion/deletion (indel) and 12 bp indel polymorphisms (Figure 5) were observed in comparison between healthy and BSE infected German cattle (Sander et al. 2004), these differences were not found in Holstein cattle in Japan (Nakamitsu et al.2006).

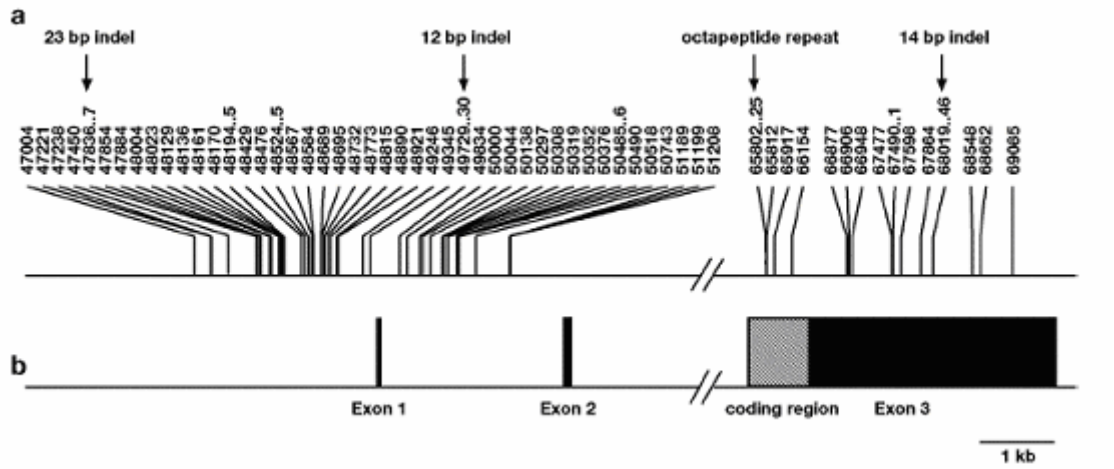

Figure 5: Diagram illustrating the genomic architecture of the bovine PRNP. (a) The positions of 60 polymorphisms detected in German cattle. (b) The three PRNP exons are presented by black boxes; the protein coding region is shown as a hatched box

In mouse, classical genetic analysis of the control of ME7 scrapie agent incubation period identified the presence of a single gene encoding two alleles; s7 for the one which shortens the incubation time (180-200 days) and p7 for the one which prolongs the incubation time (300 days or more) (DeArmond and Prusiner 1996, Dickinson et al.

1968). It has been shown that these two alleles have an association to the polymorphisms at codons 108 [leucine (L) -> phenylalanine (F)] and 189 [threonine (T) -> valine] of the murine PRNP. Threonine at codon 189 is retained in mouse strains carrying allele s7 but is absent in mice carrying p7. At codon 108, mice with short incubation time encode leucine while mice with long incubation time encode phenylalanine. The association between these two polymorphisms and incubation period of prion disease in mice was supported by Barron et al. (2005) which presented that both codons 108 and 189 control TSE incubation time and that each polymorphism plays a distinct role in the disease process.

2.4.3 Polymorphisms of ovine PRNP and association to scrapie susceptibility

In sheep, within the PRNP coding sequence, several polymorphisms have been identified up to date as described in Table 3 (DeSilva et al. 2003, Lan et al. 2006, Seabury and Derr 2003, Tranulis 2002). The polymorphisms at codons 136 [alanine (A) or valine(V)], 154 [histidine (H) or arginine (R)] and 171 [glutamine (Q), arginine or histidine] of the ovine PRNP have been found to be linked with phenotypic expression differences of scrapie including incubation period, clinical signs and pathology. On the other hand, further polymorphisms are rare, and no association between these polymorphisms and any disease phenotype has been reported (Tranulis 2002). The presence of valine at codon 136 was detected mostly in natural scrapie-infected sheep and is believed to be associated with high susceptibility to scrapie and short survival times. Whereas alanine at codon 136, which was found mostly in healthy sheep of the same flocks, is believed to be linked to resistance or incubation times that span beyond the lifetime of sheep (Hunter et al. 1996, 1993, 1994, Laplanche et al. 1993, Tranulis et al. 1999). The polymorphism at this position appears, in addition, to be associated to control the disease incidence and to modulate the incubation period even in experimentally induced scrapie sheep (Goldmann et al. 1991a, Maciulis et al. 1992).
Clouscard et al. (1995) and Tranulis et al. (1999) demonstrated that the major genetic factor controlling the susceptibility to natural scrapie in sheep is also represented by glutamine at codon 171 of the PRNP. Even the PRNP allele encoding valine at codon 136 confers a high risk of scrapie in many sheep breeds, in some breeds i.e. Suffolk breed, this allele is vanishingly rare. Hunter et al. (1997) showed that in a single closed

flock of Suffolk sheep in Scotland, scrapie occurred primarily in animals which were homozygous for glutamine at codon 171, a genotype which is significantly less frequent in healthy flocks. Moreover, O'Rourke et al. (1997) presented that after being inoculated orally with a scrapie agent into a Suffolk flock, all sheep developing scrapie during the observation period were homozygous for the glutamine at codon 171, whereas all homozygous for arginine and heterozygous remained free of scrapie. There was only one homozygous for arginine at codon 171 diagnosed with scrapie in Japan reported by Ikeda et al. (1995).

The association between susceptibility to scrapie and the polymorphism at codon 154 is unclear; however, there was evidence suggesting that a histidine substitution at codon 154 enhances the resistance to scrapie (Elsen et al. 1999, Thorgeirsdottir et al. 1999, Tranulis et al. 1999).

Table 3: Polymorphisms in the coding region of ovine PRNP

Codon number	Amino acid variant	Codon number	Amino acid variant
85[1]	G <-> R	146[1]	N <-> S
101[1]	Q <-> R	151[2]	R <-> C
112[2]	M <-> T	154[2]	R <-> H
116[3]	A <-> P	167[4]	R <-> S
127[2]	G <-> S	171[2]	Q <-> K
136[2]	A <-> T	171[2]	Q <-> R or H
136[2]	A <-> V	176[2]	N <-> K
137[2]	S <-> A	180[4]	H <-> Y
138[2]	M <-> T	189[1]	Q <->L
138[4]	S <-> R	195[4]	T <-> S
141[2]	L <-> F	196[4]	T <-> S
143[2]	H <-> R	211[2]	R <-> Q

[1] Lan et al. (2006), [2] Tranulis (2002), [3] Seabury and Derr (2003), [4] DeSilva et al. (2003)

2.4.4 Ovine PRNP genotypes and risk groups

From the three polymorphisms of ovine PRNP described above, twelve alleles are possible derivable, but only five alleles are commonly seen. They are $A_{136}R_{154}R_{171}$, $A_{136}R_{154}Q_{171}$, $A_{136}R_{154}H_{171}$, $A_{136}H_{154}Q_{171}$ and $V_{136}R_{154}Q_{171}$. The underscript numbers present the order of amino acid, here after ARR, ARQ, ARH, AHQ and VRQ, respectively (Belt et al. 1995, Tranulis 2002). In natural scrapie flocks, the VRQ allele was detected with high frequency in scrapie affected sheep, whereas the ARR variant was almost exclusively present in the healthy sheep of the flock-mates and believed to be associated with resistance to developing the clinical signs of scrapie (Belt et al. 1995, Goldmann et al. 1994, O'Doherty et al. 2002, Thorgeirsdottir et al. 1999). A study of Bossers et al. (1996) demonstrated that sheep with at least one AR allele (polymorphic amino acids at codons 136 and 171 are indicated) did not develop scrapie.

For the rest of the alleles, ARH allele is likely to be neutral, while the AHQ allele decreased the risk of developing scrapie. The AHQ allele was not found in scrapie-affected animals in Icelandic sheep (Thorgeirsdottir et al. 1999, 2002, Tranulis 2002). However, VRQ and ARR alleles act dominant over the ARQ and ARH alleles. The mechanisms by which the different PRNP alleles contribute to differences in scrapie susceptibility and incubation time are not clear.

Table 4: Ovine PRNP genotypes and risk groups according to the polymorphisms at codons 136, 154 and 171

Risk group	Genotype
R1	ARR/ARR
R2	ARR/AHQ, AHQ/AHQ
R3	ARR/ARQ, ARR/ARH, ARQ/AHQ, ARH/AHQ
R4	ARH/ARH, ARQ/ARH, ARQ/ARQ, VRQ/ARR, VRQ/AHQ
R5	VRQ/ARQ, VRQ/ARH, VRQ/VRQ

Based upon the common five alleles, fifteen genotypes have been investigated (Table 4). These genotypes have been divided into five risk groups according to the susceptibility to scrapie. The ARR/ARR genotype (risk group 1, R1) is classified as resistant to scrapie group, while the VRQ/ARQ, VRQ/ARH and VRQ/VRQ genotypes (risk group 5, R5) are classified into the high susceptible group (Erhardt et al. 2002).

2.4.5 Expression of PRNP mRNA

PRNP mRNA is predominantly expressed in many regions of the central nervous system (CNS) of humans and animals (Han et al. 2006, Harris et al. 1993, Kubosaki et al. 2000, Manson et al. 1992, McLennan et al. 2001, Tichopad et al. 2003). In the brain, the mRNA was predominantly localised within pyramidal cells of the hippocampus, large neurons of the thalamus and neocortex, and Purkinje cells of the cerebellum (Tanji et al. 1995). In the septum, the levels of PRNP transcripts and choline acetyltransferase were found to increase in parallel during development (reviewed in Prusiner 2004).

In non-neuron tissues, PRNP mRNA was observed in spleen, lymph nodes, thymus, muscle, liver, kidney and lung of sheep and cattle (Han et al. 2006, Tichopad et al. 2003), but unable to be detected in kidney and liver of mice (Miele et al. 2003). The gene transcript was also found in reproductive tissues such as caruncle, myometrium, stratum longitudinale tunicae muscles and circular layers of muscular tunics of ewes (Kubosaki et al. 2000), and in spermatogenic cells of mice (Fujisawa et al. 2004).

During embryogenesis, PRNP transcripts were found in developing brain and spinal cord of embryonic day 13.5 as well as in ganglia and nerves of the central and peripheral nervous system of embryonic day 16.5 in mice. The gene transcript was also detected in extra-embryonic tissues from day 6.5 and in specific non-neuronal cell populations such as intestine, dental lamina, tooth bud and kidney of the 13.5 and 16.5 day embryos. However, no PRNP transcript could be detected by days 6.5 and 9.5 of mouse embryogenesis (Manson et al. 1992). This is in contrast to the later study of Miele et al. (2003), who represented the expression of PRNP mRNA in developing neurons of mice since days 8.5 to 9.5. In chicken, mRNA for a chicken PrP was detected in the brain and spinal cord as early as day 6 (the earliest stage studied) and was also found in dorsal root ganglia, retina, intestine and heart of day 11 embryos (Harris et al. 1993).

3 Material and methods

This study was started with the PRNP genotyping in sheep of various breeds according to the reported polymorphisms at codons 136, 154 and 171 (Tranulis 2002). The sheep were then classified into 5 risk groups as described in Erhardt et al. (2002). A number of resistant (R1) and high susceptible to scrapie (R5) ewes were selected and mated to ram of the same risk group. PRNP mRNA expression analysis was performed in preimplantation embryos by real time RT-PCR, in various reproductive tissues of pregnant ewes and various tissues of their conceptuses during prenatal stage using semiquantitative RT-PCR. Moreover, normal cellular prion protein (PrP^C) was detected in reproductive tissues of pregnant ewes, 1-month-old foetuses and various tissues of 2-month-old foetuses using western blotting. Localisation of the gene transcript and the protein was performed in adult ovaries and 1-month-old foetuses by in situ hybridisation and immunohistochemistry, respectively.

3.1 Material

3.1.1 Animals and sampling

Sheep of various breeds were used for PRNP genotyping. However, after grouping and selection, Black head and Bentheimer breed were used for gene and protein expression analyses. All of the Bentheimer sheep were R5 genotypes, whereas most of the Black head sheep were R1 genotype. Immature oocytes, mature oocytes and in vivo embryos at morula stage were collected from specifically selected ewes using standard techniques and subjected to RNA isolation as mentioned in 3.2.4.1. In addition, foetuses and female reproductive organs such as ovary, oviduct, endometrium, myometrium and caruncle were collected from R1 and R5 ewes at the 1^{st}, 2^{nd}, 3^{rd} and 5^{th} month of pregnancy (n = 2 from each group). The foetuses at the 2^{nd}, 3^{rd} and 5^{th} month were separated into single organs of brain, cotyledon, spinal cord, heart, intestine, liver, lung, muscle and spleen. The samples were snap frozen in liquid nitrogen and kept at -80°C until used for RNA isolation (in 3.2.4.2), fluorescence in situ hybridisation (in 3.2.9), western blotting (in 3.2.10) and immunohistochemistry (in 3.2.11).

3.1.2 Laboratory equipment

Amersham Biosciences (Buckinghamshire, UK):

Ultraspec 2100 pro spectrophotometer

Applied Biosystems (Foster City, CA, USA):

ABI Prism® Sequence Detection System Instrument

BioRad (Munich, Germany):

Electrophoresis, Power Supply PAC 3000, Power Supply Mini-Protran®,

Trans-Blot SD Semi-Dry Transfer Cell

Carl-Roth (Karlsruhe, Germany):

Hellendahl jars, Rotilabo®-Probenröhrchen

Heraeus (Hanau, Germany):

Incubator (BB16)

Hermle (Wehingen, Germany):

Centrifuge

Leica (Nussloch, Germany):

Cryostat CM 3050 S

Li-cor Biotechnology (Lincoln, USA)

Li-cor autonated DNA sequencer (model 4200)

MJ Research (USA):

PCR thermal cycler (PTC 100)

Menzel-Gläser (Braunschweig, Germany):

SuperFrost® Plus slide

Schleicher & Schuell BioScience (Dassel, Germany):

Nitrocellulose transfer membrane (Protran®, pore size 0.45 µm)

3.1.3 Biological materials, reagents, kits and buffers

3.1.3.1 Biological materials

Applichem (Darmstadt, Germany):

50x Denhardt's solution

Biomol (Hamburg, Germany):

Lambda DNA *Eco*91I (BstE II), Lambda DNA *Hind*III, Phenol

Biozym Diagnostik (Hessisch-Oldendorf, Germany):

Sequagel XR sequencing gel, Sequagel complete buffer reagent

Fermentas (St. Leon-Rot, Germany):

PageRuler™ Unstained Protein Ladder, Ribonuclease A, Ribonuclease T1

Invitrogen (Karlsruhe, Germany):

Dynal beads® Oligo (dT)25

Kodak (Japan):

Autoradiography film (Kodak® Biomax XAR film)

Merck Eurolab GmbH (Darmstadt, Germany)

Kaiser's glycerol gelatine, Hematoxylin

MWG Biotech (Eberberg, Germany):

Oligonucleotide primers

Promega (Mannheim, Germany):

Random primer, RQ1 RNase-free Dnase and 10x RNase-free buffer, RNasin® Ribonuclease inhibitor inhibitor, DTT 0.5 M

Roche (Mannheim, Germany)

Fish sperm DNA, Horseradish peroxidase anti-digoxigenin antibody

Roth (Karlsruhe, Germany):

Acetic acid, Agar-Agar, Ampicillin, Ammonium peroxydisulfate, Boric acid, 5-bromo-4-chloro-3-indolyl-β-D-galactopyra-noside (X-gal), Bromophenol blue, Chloroform, DEPC, Dimethyl sulfoxide (DMSO), Di-sodium hydrogen phosphate dihydrate (Na_2HPO_4*$2H_2O$), dNTPs set, Ethanol, Ethylenediamine tetra acetic acid di sodium salt, Ethidium bromide, 37% Formaldehyde, Formamide, Glycerine, Hydrochloric acid,

Hydrogen peroxide (30%), Isopropyl β-D-thiogalactoside (IPTG), Korsolin® FF, Methanol, Mop, N,N´-dimethylformamide, Pepton, Proteinase K, Sodium acetate, Sodium chloride, Sodium di-hydrogen phosphate, Sodium dodecyl sulfate (SDS), Sodium hydroxide, TEMED, Tris, Tri-sodium-citrate*$2H_2O$, Xylene cyanol, Yeast extract

SERVA Electrophoresis GmbH (Heidelberg, Germany):

Acrylamide, Bisacrylamide

Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany):

Acetic anhydrid, Acetone, Agarose, 3-amino-9-ethylcarbazole (AEC), 6-aminohexanoic acid, Blue dextran, Dextran sulphate, Dimethylformamide, Glycine, Guanidine hydrochloride, Igepal, Isopropanol, Lithium chloride, 2-Mercaptoethanol, Paraformaldehyde, Polyvinyl pyrolidone (PVP), Ponceau S, Protease inhibitor cocktail, *Tag* DNA polymerase and 10x Buffer, Triethanolamine, Tri Reagent, Tween® 20, Yeast tRNA

Stratagene (Amsterdam, NL):

5α DH *Escherichia coli* competent cells

3.1.3.2 Kits

Amersham Biosciences (Buckinghamshire, UK):

ECL Plus Western Blotting Detection

Biozym Diagnostik (Hessisch-Oldendorf):

SequiTherm Excel™II DNA sequencing kit

Invitrogen (Karlsruhe, Germany):

Superscript™ II RNase H^- Reverse Transcriptase

PerKin Elmer (Rodgau Juegesheim, Germany)

TSA™-Plus Fluorescein System

Promega (Mannheim, Germany):

pGEM®-T vector

Qiagen (Hilden, Germany):

RNeasy® Mini Kit

Roche (Mannheim, Germany):

DIG RNA labelling kit

Santa Cruz Biotechnology (Heidelberg, Germany)

Mouse ABC Staining System

Sigma (Steinheim, Germany):

GenElute™ Plasmid Miniprep Kit

SYBR® Green JumpStart™ *Tag* ReadyMix™

3.1.3.3 Buffers and reagents

All solutions were prepared with deionised and demineralised millipore water and pH was adjusted with sodium hydroxide (NaOH) or hydrochloric acid (HCl).

General reagents

DEPC-treated water:	DEPC	1.00	ml
	Water added to	1,000.00	ml
	Incubate overnight at 37°C and heat inactivate by autoclaving		
dd H_2O:	Deionised millipore water		
	Autoclave (for in situ hybridisation)		
10x PBS:	NaCl	87.67	g
	$Na_2HPO_4.2H_2O$	15.00	g
	NaH_2PO_4	2.04	g
	Water added to	1,000.00	ml
	For in situ hybridisation, treat with 1 ml DEPC, incubate overnight at 37°C and heat inactivate by autoclaving		

1x PBS:	10x PBS	100.00	ml
	Water added to	1,000.00	ml
	For in situ hybridisation, treat with 1 ml DEPC, incubate overnight at 37°C and heat inactivate by autoclaving		
1x PBS-Tween (PBST):	1x PBS	999.50	ml
	Tween®20	0.50	ml
Tris-HCl (1 M):	Tris	121.14	g
	Water added to	1,000.00	ml
	Adjust pH to the required pH		
EDTA (0.5 M, pH 8.0):	Ethylendiamin tetra acetic acid disodium salt	186.12	g
	Water added to	1,000.00	ml
EDTA (5 mM, pH 8.0)	EDTA (0.5 M, pH 8.0)	10.00	ml
	Water added to	1,000.00	ml

DNA isolation

Digestion buffer:	9% NaCl	6.50	ml
	Tris (1 M, pH 7.5)	5.00	ml
	EDTA (0.5 M, pH 8.0)	0.20	ml
	SDS (10%)	1.00	ml
	Mercaptoethanol	2.00	ml
Proteinase K	10 mg/ml		
Phenol-Chloroform	1/1 (v/v)		

1x TE buffer:	Tris-HCl (1 M, pH 8.0)	0.50	ml
	EDTA (0.5 M, pH 8.0)	0.10	ml
	Water added to	50.00	ml

RNA isolation

Binding buffer:	Tris-HCl (1 M, pH 7.5)	1.00	ml
	Lithium chloride (5 M)	10.00	ml
	EDTA (5 mM, pH 8.0)	20.00	ml
	Water added to	50.00	ml
Washing buffer:	Tris-HCl (1 M, pH 7.5)	0.50	ml
	Lithium chloride (5 M)	1.50	ml
	EDTA (5 mM, pH 8.0)	1.00	ml
	Water added to	50.00	ml

Protein isolation

Guanidine hydrochloride (0.3 M):	Guanidine hydrochloride	1.43	g
	95% Ethanol added to	50.00	ml
4x Sample loading buffer:	Tris (1 M, pH 6.8)	13.00	ml
	SDS	6.00	g
	2-Mercaptoethanol	10.00	ml
	Glycerine	20.00	ml
	Bromophenol blue	10.00	mg
	Water added to	50.00	ml

DNA electrophoresis

50x TAE:	Tris	242.00	g
	Acetic acid	57.10	ml
	EDTA (0.5 M, pH 8.0)	100.00	ml

	Water added to	1.000.00	ml
10x TBE:	Tris	108.00	g
	Boric acid	55.00	g
	EDTA (0.5 M, pH 8.0)	40.00	ml
	Water added to	1,000.00	ml
Blue dextran loading buffer:	Blue Dextran	50.00	mg
	EDTA (0.5 M, pH 8.0)	50.00	µl
	Water	0.95	ml
	Formamide	5.00	ml
PAA loading buffer:	Formamide	47.50	ml
	NaOH	0.40	g
	Bromophenol blue	125.00	mg
	Xylene cyanol	125.00	mg
	Water added to	50.00	ml

RNA electrophoresis

10x FA buffer (pH 7.0):	MOPS	41.85	g
	Sodium acetate	4.10	g
	EDTA	20.00	ml
	Water added to	1000.00	ml
	1 litre of 1x FA buffer containing 20 ml of 37% formaldehyde		
1.5% FA gel:	Agarose	1.50	g
	10x FA buffer	10.00	ml
	DEPC-treated water added to	100.00	ml
	Boil for 3 to 5 min until agarose is completely dissolved, add 5µl of Ethidium bromide and 1.8 ml of 37% formaldehyde		

Protein electrophoresis

Anode buffer I:	Tris-HCl (1 M, pH 10.4)	150.00	ml
	Methanol	50.00	ml
	Water added to	500.00	ml
Anode buffer II:	Tris-HCl (1 M, pH 10.4)	12.50	ml
	Methanol	50.00	ml
	Water added to	500.00	ml
Cathode buffer:	Tris-HCl (1 M, pH 9.4)	12.50	ml
	Methanol	50.00	ml
	6-aminohexanoic acid	3.94	g
	Water added to	500.00	ml
Seperation gel (15%):	Acrylamide/Bis-acrylamide [30%/0.8% (w/w)]	7.50	ml
	Tris-HCl (1 M, pH 8.8)	5.60	ml
	SDS (10%)	0.15	ml
	Water	1.75	ml
	Temed	10.00	µl
	APS (20%)	30.00	µl
Stacking gel:	Acrylamide/Bis-acrylamide [30%/0.8% (w/w)]	1.50	ml
	Tris-HCl (1 M, pH 6.8)	1.30	ml
	SDS (10%)	0.15	µl
	Water	7.05	ml
	Temed	10.00	µl
	APS (20%)	30.00	µl

10x Running buffer:	Tris	30.30	g
	Glycine	144.00	g
	SDS	10.00	g
	Water added to	1,000.00	ml
0.2% Porceau S:	Ponceau S	1.00	g
	Acetic acid	50.00	ml
	Water added to	500.00	ml

Cloning

Amplicillin:	Amplicillin	10.00	mg
	Sterilized water	1.00	ml
IPTG solution (0.5 M):	IPTG	1.20	g
	Water added to	10.00	ml
	Filter-sterilize and store at -20°C		
LB-agar plate:	NaCl	8.00	g
	Peptone	8.00	g
	Yeast extract	4.00	g
	Agar-agar	12.00	g
	NaOH (1 N)	0.48	ml
	Water added to	800.00	ml
	Autoclave-sterilize		
LB-broth:	NaCl	8.00	g
	Peptone	8.00	g
	Yeast extract	4.00	g
	NaOH (1 N)	0.48	ml
	Water added to	800.00	ml
	Autoclave-sterilize		

X-Gal working solution:	X-Gal	50.00	mg
	N, N´-dimethyl-formamide	1.00	ml
	Store at -20°C.		

In situ hybridisation

50% Dextran sulphate:	Dextran sulphate	2.00	g
	DEPC-treated H_2O	2.50	ml
	Leave to dissolve overnight. Adjust to 4.0 ml with DEPC-treated H_2O. Prepare always fresh in 25.0 ml measuring cylinder one day prior to preparation of hybridisation buffer		
70% DMF:	Dimethylformamide (DMF)	3.50	ml
	DEPC-treated H_2O	1.50	ml
	Prepare fresh		
Hybridisation buffer:	50% Dextran sulphate	4.00	ml
	NaCl (2.5 M)	1.20	ml
	Formamide, deionised	10.00	ml
	20x SSC	2.00	ml
	Yeast tRNA (10 mg/ml)	0.40	ml
	50x Denhardt's solution	0.40	ml
	Fish sperm DNA (10 mg/ml)	2.00	ml
	Mix thoroughly, wait until air bubbles have disappeared, and store 1 ml aliquots at -20°C		
NaCl (2.5 M):	NaCl	14.61	g
	Water added to	100.00	ml
	Treat with 0.1% (100 µl) DEPC, incubate overnight at 37°C and heat inactivate by autoclaving		

4% PFA (pH 7.3):	Paraformaldehyde	10.00	g
	1 x PBS added to	250.00	ml
	Bring to 65°C under ventilation hood, add 5 µl of 5 M NaOH for solution to become clear, store protected from light and use within 2 weeks		
20x SSC (pH 7.0):	NaCl	87.66	g
	Tri-Na-citrate*2 H_2O	44.12	g
	Water added to	500.0	ml
	Treat with 1% (500 µl) DEPC. Incubate overnight at 37°C and heat inactivate by autoclaving		
2x SSC:	20x SSC	100.00	ml
	DEPC-treated water	900.00	ml
0.1 M TEA buffer (pH 8.0):	Triethanolamine	13.20	ml
	DEPC-treated water added to	1,000.00	ml
	Autoclave. Store at RT protected from light		
TNB blocking buffer:	Blocking reagent	0.50	g
	TN buffer	100.00	ml
	Add blocking reagent slowly in small increments to TN buffer while stirring, if necessary heat to 60°C to dissolve completely. Store aliquots at -20°C		
TN buffer (pH 7.5):	Tris	12.11	g
	NaCl	8.77	g
	Water added to	1,000.00	ml
TNT wash buffer:	TN buffer	995.00	ml
	10% Tween 20	5.00	ml

10% Tween 20:	DEPC-treated H_2O	45.00	ml
	Tween®20	5.00	ml
Yeast tRNA (10 mg/ml):	Yeast tRNA	25.00	mg
	DEPC-treated H_2O	2.50	ml
	Dissolve by incubating on ice for at least 1 h.		
	Store aliquots at -20°C.		

Immunohistochemistry

AEC stock solution:	AEC	100.00	mg
	DMF	10.00	ml
	Store at 4°C and protect from light		
AEC substrate (10 ml):	AEC stock solution	0.50	ml
	Sodium acetate (50 mM, pH 5.0)	9.50	ml
	Sodium hydroxide (30%)	5.00	µl
Sodium acetate	Sodium acetate	4.10	g
(50 mM, pH 5.0):	Water added to	1000.00	ml

3.1.4 Sources of software and Internet facilities

ABI Prism® Sequence Detection System Software	Applied Biosystems (Foster City, USA)
BLAST program	http://www.ncbi.nlm.nih.gov/blast/
Image analysis program (version 4.10)	Li-cor Biotechnology (Lincoln, USA)
Multiple sequence alignment	http://prodes.toulouse.inra.fr/multalin/multalin.html
Primer Express® Software version 2.0	Applied Biosystems (Foster City, USA)
SAS version 8.0	SAS Institute Inc. (Cary, NC)
Weight to Molar Quantity (for nucleotide)	http://www.molbiol.ru/eng/scripts/01_07.html

Table 5: Details 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) [a]	TCAGTGGAACAAGCCCAGTAAGC GGTGGTGACTGTGTGTTGC	22577 22859	283	Touch down 64-58	U67922
Ovine PRNP (internal) [b]	AGCTGGAGCAGTGGTAGG GTGATGTTGACACAGTCATGC	22637 22814	198	60	U67922
Ovine PRNP [c, d]	CCAAGCTGAAGCATCTGTCTTCC TCACTCCACATGGCCACAAAG	89 219	131	58	NM_001009481
Ovine H2a [d]	CACCTCAAATCTAGGACGACTAGCC CGATGCATTTCCTGCCAATTC	210 326	117	60	AY074805
18S-rRNA [c, d]	GCGCGCAAATTACCCAC GCTGGAATTACCGCGGCT	89 247	159	54	AF176811

[a] The primer used for DNA amplification in genotyping procedure, [b] The primer used for PRNP sequencing,

[c] The primer used for RT-PCR, [d] The primer used for real time PCR

3.2 Methods

3.2.1 Primers design

All of the primers used in this study were designed for PCR amplification and *in vitro* transcription using Primer Express® Software v2.0 (Applied Biosystems, Foster City, CA, USA). Primer sequences, size of amplified products, annealing temperature and the GenBank accession number are shown in Table 5.

3.2.2 PRNP genotyping

3.2.2.1 Blood sampling

To identify the PRNP genotype of ewes and rams, 10 ml of blood was taken from the jugular vein of the animal, kept in a tube containing 500 µl of 0.5 M EDTA as an anticoagulant and chilled immediately on ice. After centrifugation of blood samples at 1,100 rpm for 30 min, the blood was separated into: 1) a lower red blood cell phase, 2) a middle phase of white blood cells, and 3) a colourless upper aqueous phase of plasma. The middle layer was transferred to a new sterile 15 ml tube. Nine ml of deionised and demineralised water was added to the sample and the mixture was then mixed by inversion for 20 sec followed immediately by adding 1 ml of 9% NaOH. The mixture was centrifuged at 1,100 rpm for another 15 min. Supernatant was discarded and the pellet was used for DNA isolation.

3.2.2.2 DNA extraction from blood samples

Genomic DNA from blood samples of sheep was isolated using standard phenol/chloroform extraction method. All centrifugation steps were conducted at 12,000 x g. To the white blood cell pellet from previous step 0.7 ml of digestion buffer and 20 µl of proteinase K were added. The mixture was incubated at 50°C overnight at 100 rpm in a shaking incubator. Complete pellet digestion resulted in a viscous homogeneous solution. An equal volume of phenol-chloroform was added and mixed by several inversions. The sample was then centrifuged for 10 min at room temperature.

After centrifugation, the sample was separated into: 1) a lower phenol-chloroform phase, 2) an interphase of protein and 3) an upper aqueous phase of DNA. The aqueous phase was transferred into a 2.0 ml microcentrifuge tube and washed by mixing with an equal volume of chloroform to remove residual phenol. After centrifugation for another 10 min at room temperature, the DNA containing the aqueous phase was transferred to a new 2.0 ml tube and precipitated by mixing with 1/10 volume of sodium acetate (3 M, pH 8.5) and a double volume of isopropanol. The mixture was incubated overnight at -20°C or 2 hours at -80°C followed by centrifugation for 20 min at 4°C. Then, the supernatant was drained and the pellet was washed with 0.5 ml of 75% ethanol to remove excess salt. After centrifugation for another 10 min at 4°C, the DNA pellet was briefly air dried and dissolved in 0.3 to 0.5 ml of 1x TE buffer. The DNA concentration was measured by the formula: 50 x absorbance at 260 nm x dilution factor. A value of more than 1.5 of the absorbance 260/280 ratio and integrity of high molecular weight indicates a good quality of DNA. A working DNA solution was prepared by diluting stock DNA with 1x TE buffer to the concentration of 50 ng/µl. The stock DNA solution was kept at -20°C and the working solution was kept at 4°C.

3.2.2.3 PCR amplification

The ovine PRNP was amplified using Ovine PRNP (external) primers (Table 5). PCR was performed by combining 2 µl of working DNA solution and 18 µl of master mix containing 2 µl of 10x PCR buffer, 0.5 µl of dNTPs (10 pmol/µl), 0.5 µl of each primer (10 pmol/µl) and 0.2 µl of Taq polymerase (5 units/µl). The thermal cycler PTC (MJ Research Inc.) was used to run the PCR at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, a final extension step was run at 72°C for 5 min. Five microlitre of the PCR product was electrophoresed on a 1.5% agarose gel and UV-Transilluminator's documented. The rest of the PCR product was kept at 4°C for the next step of sequencing.

3.2.2.4 Sequencing

Direct DNA sequencing was employed to genotype the PRNP polymorphisms as previously described by using the SequiTherm EXCEL™ II DNA Sequencing Kit (Biozym Diagnostik, Germany) and LI-COR automated DNA sequencer (LI-COR Biotechnology, USA). The sequencing was performed by dye the termination method. The primers used were fluorescent labelled primers [Ovine PRNP (internal) primers, Table 5] which can be read by laser of the sequencer. For each sample, four reactions were performed; each reaction had one dideoxy nucleotide termination mix (ddATP, ddTTP, ddCTP and ddGTP) which would terminate the elongation reaction once the nucleotide binds to the elongating strand. Each reaction consists of 1 µl of termination mix and 2 µl of premix solution [3.6 µl of 3.5x Excel II sequencing buffer, 0.25 µl of forward primer (10 pmol/µl), 0.25 µl of reverse primer (10 pmol/µl), 0.5 µl of Taq polymerase (5 units/µl) and 3.9 µl of PCR product from step 3.2.2.3]. The reaction was run on a thermocycler at 94°C for 5 min followed by 20 cycles of 94°C for 20 sec, 60°C for 20 sec and 68°C for 20 sec. Immediately after PCR, 1.5 µl of stop buffer was added and the product was stored at –20°C till loading. The sequencing reactions were denatured at 90°C for 5 min and immediately loaded onto the 41 cm 6% Sequagel-XR (Biozyme Diagnostik, Germany). Electrophoresis was performed on a LI-COR model 4200 automated DNA sequencer in 1x TBE buffer at 50°C, 50 W and 1500 V. Sequence data was analysed by using the Image Analysis program, version 4.10 (LI-COR Biotechnology).

3.2.3 Oocytes and *in vivo* embryo collection

Ovaries and uteri were taken from specifically mated ewes at a specific time range. The ovaries were removed and kept in 35°C warm 0.9% physiological salt solution supplemented with 0.5 ml Streptocombine® per litre. Before aspiration of cumulus oocyte complexes (COCs), the ovaries were washed once in 70% ethanol followed by two times of washing in 0.9% salt solution. The COCs were aspirated from follicles using 5 ml syringe attached with 18G needle. The follicle fluid was allowed to precipitate for 15 min and the competent COCs were picked out using a glass pipet. For the morula stage embryo collection, ewes were slaughtered and the uteri were dissected

at the uterotubal junction and the cervix. Both uterine horns were divided at the bifurcation and flushed with 40 to 50 ml of Phosphate buffer saline (PBS, 39°C) each. After precipitation, the embryos were picked using a glass pipet. A total of 3 to 5 oocytes and single embryos were washed two times with PBS and treated with acidic Tyrode pH 2.5 to 3 to dissolve the zona pellucida. The zona free oocytes and embryos were snap frozen in cryotubes containing 1.5 µl of lysis buffer [0.8% Igepal, 1 U/µl RNasin, 5 mM DTT], before storage at –80°C for the further work of RNA isolation.

3.2.4 RNA isolation and cDNA synthesis

3.2.4.1 Oocytes and preimplantation embryos

RNA isolation

Three independent pools of each containing 15 to 20 immature oocytes or 3 embryos were used for mRNA isolation using oligo (dT)25 attached magnetic beads following the manufacturer's instruction. Briefly, samples in lysis buffer were mixed with the total volume of 40 to 60 µl of binding buffer and incubated for 5 min at 70°C to obtain complete lysis of the sample. Twenty microlitres of oligo(dT) magnetic bead suspension was washed twice with double volume of binding buffer and resuspensed in 4 µl of binding buffer before added to the samples. The mixture was then incubated for 30 min at room temperature and chilled on ice after this step until finish. The hybridised mRNA and oligo(dT) magnetic beads were washed three times with 100 µl of washing buffer. Finally, mRNA samples were eluted in 11 µl of DEPC-treated water and heated at 90°C for 4 min.

cDNA synthesis

The mRNA samples were reverse transcribed in 20 µl reaction volume containing 1 µl of oligo dT(12)N primer (where N = G, A or C), 1 µl of random primer (500 ng/µl), 4 µl of 5x first strand buffer, 2.5 µl of 0.1 M DTT, 1 µl of each dNTPs (40 pmol/µl) and RNase inhibitor, and 0.5 µl of SuperScript II reverse transcriptase (200 unit/µl). In terms of the order of adding the reaction components, mRNA, random primer and

oligo(dT) primer were mixed and heated for 5 min at 70°C. The mix was placed on ice until the addition of the remaining reaction components and then incubated for 90 min at 42°C. The reaction was terminated by heat inactivation for 15 min at 70°C. The cDNA solution was kept at -20°C until used for gene expression analyses using real time PCR technique.

3.2.4.2 Tissue samples

RNA isolation

Total RNA was isolated from tissue samples using TRIZOLE Reagent following the manufacturer's instruction. The tissue samples stored at –80°C were taken out just before isolation. About 30 to 40 mg of tissue was ground by using mortal and pestle, and incubated with 1 ml of TRIZOL for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. The clear homogenate was then mixed with 0.2 ml of chloroform and vigorously shaken by hand for 15 sec. After centrifugation at 12,000 x g for 15 min at 4^{o}C, the mixture was separated into: 1) a lower red phenol-chloroform phase, 2) a white interphase and 3) a colorless upper aqueous phase containing RNA. The aqueous phase was transferred to a fresh 2.0 ml tube and the RNA was precipitated by adding 0.5 ml of isopropanol. The sample was then incubated at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 4^{o}C. After centrifugation, the RNA precipitate becomes visible as white pellet at the bottom of the tube. The supernatant was drained off and the pellet was washed once with 1 ml of 75% ethanol before being centrifuged at 12,000 x g for 5 min at 4°C. The ethanol supernatant was removed and the RNA pellet was briefly air dried for 5 to 10 min and dissolved with 22 µl of DEPC-treated H_2O. The quality of RNA was checked by running 2 µl of total RNA in 1.5% FA gel.

DNase digestion and RNA purification

All good quality RNA samples were subjected to DNase digestion to avoid the residual DNA by mixing 20 µl of RNA extract with the premix of 7.5 µl of deoxyribonuclease I (RQ1 DNase), 4 µl of 10x RQ1 buffer, 1 µl of RNase inhibitor and 7.5 µl of DEPC

treated water. The mixture was incubated for 1 hour at 37°C. The digested RNA was purified using RNeasy mini kit (Qiagen, Germany) following the manufacturer's instruction. The quality of the RNA was checked by running 2 µl of total RNA in 1.5% FA gel. The OD spectrometer value was taken to estimate the concentration of total RNA by the formula: 40 x absorbance at 260 nm x dilution factor. A working RNA solution was prepared by diluting stock RNA with DEPC-treated H_2O to the concentration of 200 ng/µl. Both of stock and working RNA solution was kept at -80°C for further use.

cDNA synthesis

One microgram of total RNA was reverse transcribed using reverse transcriptase to synthesize cDNA. Five microlitre of total RNA working solution and 1 µl of each oligo dT(12)N (100 pmol/µl) and random primers (500 ng/µl) were added in a 0.2 ml PCR tube. The mixture was incubated for 5 minutes at 70°C and then immediately chilled on ice. Thirteen microlitre of the premix containing 4 µl of 5x first strand buffer, 2.5 µl of 0.1 M DTT, 5 µl of dNTPs (10 pmol/µl) and 1 µl of each RNase inhibitor and SuperScriptII^{TM} reverse transcriptase (200 unit/µl) was mixed and incubated for 90 minutes at 42^{o}C followed by heat inactivation for 15 min at 70°C. The synthesized cDNA was diluted with 150 µl of DEPC-treated H_2O and stored at –20^{o}C for further use.

3.2.5 Semiquantitative PCR

Polymerase chain reaction was used to quantify the amount of gene transcript in sample tissues. In this study the levels of 18S-rRNA transcript were used as an internal standard. The appropriate cycle number of PCR was pre-optimised. Each cDNA source was done in duplicate to control the reproducibility of results. Ovine PRNP PCR was performed using Ovine PRNP primers, and 18S-rRNA PCR was done using the 18S-rRNA primers (Table 5). The thermo cycle condition of ovine PRNP PCR was programmed at 94°C for 5 min followed by 28 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec 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 sec, 54°C for 30 sec and 72°C for 30 sec followed by a final extension step of 72°C for 5 min.

3.2.6 Plasmid DNA preparation

3.2.6.1 PCR amplification

The ovine PRNP, histone H2a and 18S-rRNA PCR for plasmid DNA preparation were amplified by using Ovine PRNP, Ovine H2a and 18S-rRNA primers, respectively, as shown in Table 5. cDNA from adult brain was used as template for every reaction. The PCR reaction was combined as described in step 3.2.2.3 and the thermo cycler was programmed at 95°C for 5 min followed by 35 cycles of denaturing at 94°C for 30 sec, annealing at temperatures as indicated in Table 5 for 30 sec and extension at 72°C for 30 sec. Following the last cycle, a 5 min elongation step at 72°C was performed.

3.2.6.2 PCR product extraction

The PCR product was extracted from agrose gel using basic method base on phenol-chloroform extraction. All the centrifugation steps were carried out at 12,000 x g at 4°C. Briefly, the PCR fragment was electrophoresed and sliced out from the 1.0% agarose gel. The gel slice was frozen in a 1.5 ml microcentrifuge tube for 30 min at -20°C and chopped into small pieces. The gel was then homogenized with 0.5 ml of 1x TE buffer by repeatedly forcing through a syringe and needle. The extraction was carried out by vigorously vortexing the gel solution with 0.6 ml of phenol-chloroform. By centrifugation for 15 min, the mixture was separated into a lower phenol-chloroform phase, an interphase of precipitated protein, and an upper aqueous phase containing the PCR product. The aqueous phase was taken to a fresh tube, shaken with an equal volume of chloroform to remove possibly carried over phenol, and recentrifuged for another 10 min. The clear aqueous phase was precipitated by gentle mixing with 50 µl (or 1/10 volume) of sodium acetate solution (3 M, pH 5.3) and 1.5 ml of 100% ethanol (or 2.5 volume). Precipitation was maximized by placing at -20°C for 2 hour. The precipitated PCR product was pelleted at 12,000 x g for 30 min at 4°C. The supernatant was then removed and the pellet was washed with 1.0 ml of 70% ethanol to remove

excess salt. The mixture was recentrifuged to remove the supernatant and then the pellet was air dried briefly. Finally, the pellet was gently dissolved in 7 µl of ddH_2O.

3.2.6.3 Ligation

The pGEM®-T Vector System I ligation kit was used. The ligation was performed by mixing 2.5 µl of 2x ligation buffer, 1.5 µl of gel purified PCR product, 0.5 µl of pGEM vector (50 ng) and 0.5 µl of T4 DNA ligase (3 units/µl), and by an incubation for 2 hours at 20°C or overnight incubation at 4°C.

3.2.6.4 Competent cell preparation

Competent cells were prepared from stock bacteria *E. coli* strain DH5α (Clontech). Stock solution of bacteria was streaked on LB plate without ampicillin and incubated overnight at 37°C. A single colony was picked and inoculated in 50 ml of LB broth (without ampicillin). Inoculums were shaken gently at 37°C approximately 6 hours, during this OD was periodically measured to keep the OD no more than 0.3. Then, the cell culture was centrifuged at 5,000 x g for 10 min at 4°C. The pellet was resuspended in 25 ml of cold sterile 0.1 M CaCl and incubated on ice for 20 min. The cells were recentrifuged at 5,000 x g for 5 min at 4°C and then resuspended in 5 ml of 0.1 M CaCl containing 20% glycerol. The cell suspension was aliquoted into 1.5 ml microcentrifuge tubes and stored at -80°C.

3.2.6.5 Transformation

Transformation was performed by combining 60 µl of competent *E. coli* cells DH5α and 5 µl of ligation reaction in a 15 ml falcon tube and incubating on ice for 30 min. After incubation, heat-shock was performed by placing the tube for 90 sec in water bath at exactly 42°C and immediately returning to ice bath for 2 min. Then, 0.7 ml of LB broth was added to the reaction and the mixture was shaken 110 rpm for 90 min at 37 °C.

3.2.6.6 Clonning

Two LB-ampicillin plates were used for each ligation solution. The plates were prepared 30 min prior to use by spreading 20 µl of 0.5 M IPTG and 20 µl of 50 mg/ml X-Gal over the surface of LB-ampicillin plates with a glass spreader. The bacteria culture was then plated onto duplicate LB-ampicillin plates and incubated overnight (~16 hours) at 37°C until colonies are visible. Successful cloning of an insert in the pGEM-T vectors interrupts the coding sequence of β-galactosidase, hence the vectors with insert produce white colour colonies in plate having X-gal medium against blue colony producing vectors which doesn't have inserts. Two white colonies and a blue colony (negative control) were picked from each plate in 30 µl of 1x PCR buffer. During that time, 5 ml of LB-broth prepared with amplcillin (5 µl/ml) in a 15 ml tube was inoculated with each clone separately, which was later used as a resource for plasmid DNA isolation. The bacteria in 1x PCR buffer were lysed by heating for 15 min at 95^{o}C. The colonies were screened for the insert by performing a PCR with primers designed in the M13 promoter region of the vector. Twenty microlitre of reaction volume containing 10 µl of lysate, 1 µl of 10x buffer, 0.8 µl of dNTPs (10 pmol/µl), 0.4 µl of each primers (forward: 5´-ttgtaaaacgacggccagt-3´, reverse: 5´-caggaaacagctatg acc-3´, concentration 10 pmol/µl) and 0.1 µl of Taq polymerase (5 units/µl) were amplified in the PTC-100 thermal cycler for 35 cycles of 95°C for 30 sec, 65°C for 30 sec and 70°C for 30sec followed by another 5 minutes of final extension at 70°C. The products were electrophoresed in a 2% agarose gel. Clones were size fractionated on an agarose gel. Clones with an insert have higher molecular weight fragments than the blue clones.

3.2.6.7 Sequencing

The identity of inserts was confirmed by sequencing. Only colonies with right insert sequences were used in the next step. The protocol of this step was similar as described in 3.2.2.4. The sequencing was done using fluorescent labelled primers with the help of the SquiTherm EXCEL ^{TM} II DNA Sequencing Kit and LI-COR automated DNA sequencer. However, the primers used in this step were designed from SP6 (5'-taaatccatgtgtatcttatg-3') and T7 (5'-attatgctgctgagtgatatatcccgct-3') promoter regions of

the pGEM-T vector and the templates were M13 PCR products from the previous step of cloning.

3.2.6.8 Plasmid DNA isolation

Plasmid DNA was isolated from the cultured E. coli cells using GenElute^{TM} Plasmid mini kit (Sigma, Germany). Five mililitre of bacterial culture was centrifuged at 12,000 x g for 1 min and the supernatant was discarded. Cells were resuspended in 200 µl of resuspension solution. Two hundred mililitre of lysis solution was added to lyse the cells. The sample was then gently inverted for 8 to 10 times to mix them, until the mixture became clear and viscous. The mixture was incubated at room temperature for exactly 2 min. Precipitation of cell debris was done by adding 350 µl of neutralisation binding buffer into lysed cells, and mixed by inversion. The solution became cloudy and developed a flocculent white precipitant. The tubes were centrifuged for 10 min at ≥12,000 x g. The clear supernatant was transferred to GenElute miniprep binding column and centrifuged at ≥12,000 x g for 1 min. The flow-through was carefully poured off from the tube and 750 µl of wash solution was added to the column, and then centrifuged at ≥12,000 x g for another 1 min. The flow-through was discarded and recentrifuged for 2 min without any additional washing buffer to remove excess ethanol. The column was transferred to a fresh collection tube, 50 µl of elution solution was added to the column followed by centrifugation at ≥12,000 x g for 1 min. Size and quality of plasmid DNA were checked by agarose gel electrophoresis. Quantity and quality were determined by spectrophotometer. The concentration of plasmid DNA was measured by the formula: 50 x absorbance at 260 nm x dilution factor.

3.2.6.9 Plasmid serial dilutions

The copy number per microlitre of plasmid DNA was calculated based on the size and concentration. Serial dilutions were then prepared from the concentration of 10^1 to 10^9 copies/µl. The isolated plasmid DNA and serial dilutions were stored at -20°C. These DNA serial dilutions were used as template to generate the standard curve during real time PCR analysis.

3.2.7 Real time PCR

The quantification of PRNP mRNA in oocytes, preimplantation embryos, 1-month-old foetuses as well as tissue samples with varying expression levels during semi-quantitative PCR was done by real time quantitative PCR. PCR was conducted in an ABI Prism 7000 SDS instrument using SYBR® Green as a double-strand DNA-specific fluorescent dye. Quantitative analyses of cDNA from oocytes, embryos and 1-month-old foetuses were performed in comparison to histone H2a (endogenous control) (Robert et al. 2002), and were run in separate wells whereas that of sample tissues were compared to 18S-rRNA. Standard curves were generated using serial dilutions of plasmid DNA (10^1 to 10^9 copies/µl). The PCR was performed in 20 µl reaction volume containing 2 µl of cDNA, 10 µl of 2x 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 were programmed at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 sec and 60°C for 60 sec. After the end of the last cycle, a dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements every 7 sec interval until the temperature reached 95°C. Final quantitative analysis was done using the relative standard curve method as used in Tesfaye et al. (2004) and results were reported as the relative expression level compared to the calibrator cDNA after normalization of the transcript amount to the endogenous control.

3.2.8 Statistical analysis

PRNP mRNA expression by real time PCR was analysed based on the relative standard curve method. The relative expression data were analysed using the Statistical Analysis System (SAS) version 8.0 software package. Differences in mean values between developmental stages were tested using ANOVA followed by a multiple pair wise comparisons using t-test. Differences of $p \leq 0.05$ were considered to be significant.

3.2.9 PRNP mRNA localisation by fluorescent in situ hybridisation

To localise the PRNP mRNA in ovaries and 1-month-old foetuses fluorescent in situ hybridisation was performed on 7 to 10 µm cryostat sections of snap frozen tissues. A non-radioactive probe with a reporter molecule digoxigenin (DIG) detected by the indirect method with the help of tyramide signal amplification (TSA) was applied in this experiment including six main steps of (I) generation of DIG-RNA probe, (II) tissue sectioning and fixation, (III) pre-treatment, (IV) hybridisation of probe to the target RNA, (V) posthybridisation washes and (VI) immunological detection of hybrids and visualisation.

3.2.9.1 Generation of RNA probes

The RNA probes used for in situ hybridisation (ISH) were generated and simultaneously labelled with DIG by in vitro transcription from cDNA template. The cDNA template required for the probe synthesis consisted of purified RT-PCR products from ovine brain RNA. Two different templates were required allowing the separate synthesis of antisense (as) and sense (s) probes. This was achieved by extending one of the two PRNP primers at its 5'-end by the sequence of the T7- or SP6-RNA-Polymerase promoter as shown in Table 6. The PCR with the oPRNP_for and SP6+oPRNP_rev primers led to the template for the antisense probe, and with T7+oPRNP_for and oPRNP_rev primers led to the template for the sense probe. Both antisense and sense PCRs were programmed at 94°C for 5 min, 40 cycles of 94°c for 30 sec, 60°C for 30 sec, 72°C for 1 min followed by a final extension step at 72°C for 5 min. PCR products were assessed on a 2% agarose gel for quality measurement. The subsequent purification of the obtained PCR products provided optimal conditions for the transcription reaction by removing contaminations of primers, buffers and enzymes, using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. The concentration was measured by the formula: 50 x absorbance at 260 nm x dilution factor. Finally, the PCR product was diluted to 100 ng/µl.

The in vitro transcription of the DNA template to a single-stranded RNA probe in the presence of Dig labelled UTPs by the T7 and SP6 RNA polymerase was carried out according to the manufacturer's instructions of the DIG RNA labelling kit (Roche,

Germany). Ten microlitre of DNA template and 3 µl of DEPC-treated H_2O were added in a 0.2 ml PCR tube and left at room temperature until the addition of the Master Mix. This Master mix was prepared at room temperature with 2 µl of 10x Dig NTP labelling mixture, 2 µl of 10x transcription buffer, 1 µl of RNase inhibitor and 2 µl of T7 or SP6 RNA polymerase according to the promoter presented on the template. After gentle mixing and brief centrifugation, the mixture was incubated for 2 hours at 37°C. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0). The probe was purified to remove undesirable components of the transcription reaction by precipitation in 2 µl (0.1 volume) of 4 M LiCl and 50 µl (2.5 volume) of 100% ice cold ethanol for a minimum of 1 hour at -20°C. After centrifugation at ≥ 12,000 x g for 15 min at 4°C, the pellet was washed in 200 µl of 70% ice cold ethanol and centrifuged for another 10 min. The pellet was finally air dried and dissolved in 20 µl of DEPC-treated H_2O. The quality of RNA probes was checked by running 2 µl of RNA plus 2 µl of PAA loading buffer in a 1.5% FA gel. OD spectrometer values were taken to estimate the concentration of the probe by the formula: 40 x absorbance at 260 nm x dilution factor. A stock RNA probe was diluted in DEPC-treated H_2O to a working concentration of 50 ng/µl. Stock and working RNA probes were stored at -20°C.

Table 6: Ovine PRNP primers for generating templates required for the probe synthesis. Forward primer (for) extended by the T7 promoter sequence and the standard reverse primer (rev) was used to generate a DNA template for ISH sense probe synthesis. While normal forward primer and reverse primer extended by SP6 promoter sequence were used to amplify DNA template for antisense probe synthesis.

	Primer sequence	Position within ref. sequence	Accession number
oPRNP_for	5’-accaagctgaagcatctgtcttc-3’	88	
SP6+oPRNP_rev	5’-gatttaggtgacactatagaatggc ttactgggcttgttcc-3’	-	NM_001009481
T7+oPRNP_for	5’-gtaatacgactcactatagggacca agctgaagcatctgtcttc-3’	-	NM_001009481
oPRNP_rev	5’-tggcttactgggcttgttcc-3’	484	

3.2.9.2 Tissue sectioning and fixation

The tissue samples stored at -80°C were transferred into the cryomicrotome for a minimum of 3 hours to allow the sample to warm to the chamber temperature of approximately -20°C. The specimen temperature (-20°C to -25°C) and speed of sectioning were pre-optimised for each kind of tissue. Sections of a thickness of 7 to 10 µm were prepared at the optimal specimen temperature and a cutting angle of 2 to 3°, and were placed on room temperature warm SuperFrost® Plus slides (Menzel-Glaeser, Germany). The slides were then immediately placed on the quick freeze shelf (-45°C) for the section to refreeze and then kept in a precooled Hellendahl glass jar (Roth, Germany) within the cryomicrotome until fixation. All steps of in situ hybridisation were carried out in Hellendahl glass jars at room temperature unless noted otherwise. The sections were firstly fixed in 4% paraformaldehyde (PFA) for 15 min to inhibit cellular metabolism, deactivate lytic enzymes and endogenous RNases, and to conserve cellular morphology and nucleic acid integrity (Morel and Cavalier 2000). The sections were then washed twice with 1x PBS for 5 min each, and left in PBS until the next step of pretreatment. Once the sections were immersed in the fixation solution it was important to ensure that the sections never dried out during any subsequent steps until the staining was completed.

3.2.9.3 Pretreatment of sections

The main aim of the pretreatments is to make the target nucleic acid accessible to a probe. This may include modifying or destroying membrane and histone proteins, improving signal/background ratio as well as denaturing the target nucleic acid and the probe (Morel and Cavalier 2000). In this study, after tissue fixation, the sections were incubated in an ascending alcohol series of 50%, 70%, 90% and 100% ethanol for 5 min each (prepared fresh in DEPC-treated H_2O), respectively. These steps were followed by a descending alcohol series of 90%, 70%, 50% ethanol for 5 min each, respectively (reused the solutions from the ascending series). The sections were washed in 1x PBS for 5 min, blocked in 0.6% H_2O_2 diluted in 1x PBS for 1 hour to quench an endogenous peroxidase activity and then washed twice with 1x PBS for 5 min each.

The acetylation reaction was performed to reduce background by transforming the reactive amine group (NH^{3+}) of proteins into a substituted amine group (-NH-CO-CH_3) (Morel and Cavalier 2000). For this purpose the sections were incubated in 0.1 M TEA buffer plus 0.25% acetic anhydride for 10 min. This solution was prepared just prior to usage due to the instability of acetic anhydride. Finally, the samples were equilibrated in 2x SSC for 10 min.

3.2.9.4 Hybridisation of probe to the target RNA

The successful hybridisation is characterised by the formation of a stable and specific anti-sense probe complimentary to the target nucleic acid. Factors for a successful hybridisation are hybridisation buffer, hybridisation temperature, Na^+ concentration, type and length of probe. The composition of the hybridisation buffer was based on the protocol of Thielen (2006). In this experiment, the sense probe served as negative control, since its sequence is identical to the target mRNA and does not attach to the target mRNA. A section hybridised with the sense probe was always prepared in parallel to each section hybridised with the antisense probe. In addition, different levels of probe amounts were used to optimise the hybridisation procedure. The probe was applied to the section as follows: after thawing the probe on ice, the RNA probes (sense or antisense) were added to the hybridisation buffer to yield a total volume of 100 µl/section and the concentration of 500 ng/ml. This solution was mixed gently, exposed for 5 min at 80°C to denature the RNA probe and then immediately placed in ice to preserve the denatured structure. Following the final step of pretreatment, the section was placed horizontally in a humid chamber filled with DEPC-treated H_2O, carefully overlaid with 100 µl of the hybridisation mixture and covered with a glass cover slip (24 mm x 24 mm) before being incubated overnight at 52°C.

3.2.9.5 Posthybridisation washes

The aim of this step is to reduce background by removing unbounded and unspecifically bounded probe. This purpose can be achieved by controlling temperature, salinity of washing buffer, and RNase treatment. After the hybridisation step, the slips were rinsed off by flushing 2x SSC between slip and slide. The slides were washed twice in 2x SSC

at 45°C for 10 min each, twice in a mixture between formamide and 2x SSC (ratio 1:1) at 45°C for 10 and 20 min, respectively, and finally washed twice in 0.2x SSC on an orbital shaker at speed 100 rpm for 10 min at room temperature. The sections were then subjected to RNase digestion. Two different types of enzymes, RNase A and RNase T1 were used. The sections were incubated for 30 min at 37°C in the combination of 5 µg of RNase A and 50 U of RNase T1 per ml of 2x SSC (Thielen 2006) followed by three times washing in 2x SSC on an orbital shaker at speed 100 rpm for 10 min at room temperature.

3.2.9.6 Immunological detection

TSA or tyramide signal amplification (fluorophore system) was integrated into the protocol. Tyramide is a phenolic compound that precipitates after oxidation. This technology uses HRP to catalyze the deposition of a fluorophore-labelled tyramide amplification reagent onto tissue sections surfaces. The reaction results in the deposition of numerous fluorophore labels immediately adjacent to the immobilised HRP enzyme (Figure 6).

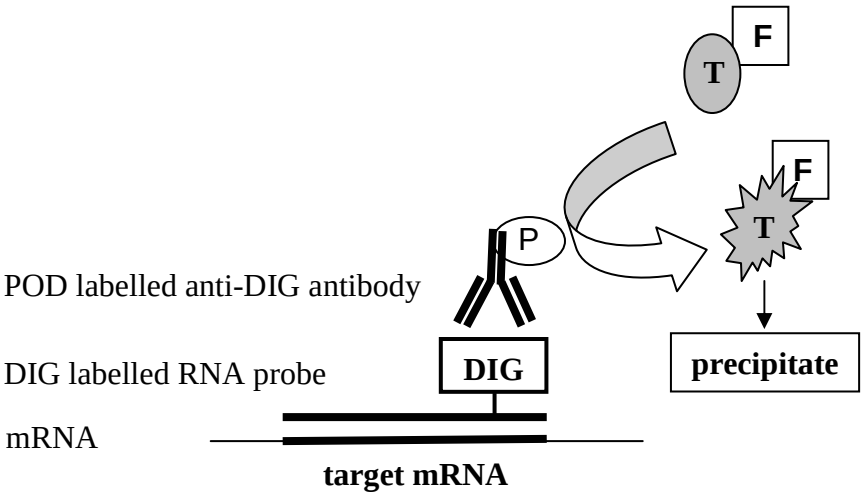

Figure 6: Hybrid detection system including tyramide signal amplification

The TSA fluorophore system was carried out according to the manufacturer's instruction. Briefly, after posthybridisation washes (see 3.2.9.5) the sections were incubated in Tris-sodium (TN) buffer in a Hellendahl glass jar for 5 min. The slides were placed horizontal in a humidity chamber and incubated with TN blocking buffer (TNB) for 30 min. Next, the sections were incubated in peroxidase conjugated anti-DIG diluted 1:100 in TNB buffer (200 µl/section) for 1 hour and washed with three changes of TN buffer plus 0.05% Tween 20 (TNT) on an orbital shaker at speed 100 rpm for 5 min each. Then, the fluorescent solution was added to the sections. The developing time was pre-optimised for each kind of tissue, 5 min for the brain, and 10 min for ovary and foetus. After another three times of washing in TNT buffer, the samples were finally counterstained in 0.5 µg/ml of propidiumiodide (diluted in TN buffer, 200 µl/section) for 15 min followed by three times washing in TNT buffer on an orbital shaker at speed 100 rpm for 5 min each. A drop of Vecta shield mounting media was applied on the section; a glass cover slip was then placed over and fixed with nail polish. The staining of the sections was assessed by light microscopy (Microscope DM LB, Leica).

3.2.10 Detection of PrP^C expression by western blotting

3.2.10.1 Protein extraction

Protein was extracted using TRIZOLE reagent following the manufacturer's instruction. The tissue samples were taken out from storage at -80^{o}C just before isolation. All centrifugation steps were performed at 4°C. Twenty miligramm of tissue was ground by using mortal and pestle and incubated with 0.5 ml of TRIZOL for 5 minutes at room temperature in a microcentrifuge tube. The clear homogenate was then mixed with 0.1 ml of chloroform, vigorously shaked by hand for 15 sec and centrifuged at 12,000 x g for 15 min. A colourless upper aqueous phase was moved out and the DNA from the interphase and organic phase was precipitated by adding 150 µl of 100% ethanol. The mixture was mixed by inversion, allowed to stand for 2 to 3 min at room temperature and centrifuged at 2,000 x g for 5 min. The supernatant was transferred to a new 2.0 ml tube, 750 µl of isopropanal was then added and allowed to stand at room temperature for 10 min followed by centrifugation at 12,000 x g for 10 min. The protein pellet was

washed three times in 1 ml of 0.3 M guanidine hydrochloride/95% ethanol solution. During each wash, the sample was stored in wash buffer for 20 min at room temperature and centrifuged at 7,500 x g for 5 min. After the three washes, 1 ml of 100% ethanol was added and the mixture was allowed to stand for 20 min and then centrifuged at 7,500 x g for another 5 min. Finally, the protein was dried under a vacuum for 5 min and dissolved in 200 µl of sample buffer containing 1% protease inhibitor cocktail followed by boiling for 5 min at 95°C and storing at -20°C.

3.2.10.2 SDS-PAGE gel preparation

SDS is an anionic detergent which denatures proteins by wrapping around the polypeptide backbone and confers a negative charge to the polypeptide in proportion to its length. An appropriate concentrated SDS-PAGE gel was used for the protein of interest. For the prion protein of ~ 30 kDa, a 15% acrylamide gel was prepared. Glass plates were cleaned thoroughly using normal detergent and distilled water. The sandwich glass plates were introduced into the support piece. It was confirmed that the lower border of the glass plates were well aligned and fixed on the rubber piece.
The separation gel and stacking gel were prepared. The separation gel is used to separate proteins into their respective sizes allowing sharp bands to be seen, whereas the stacking gel organizes proteins before they enter the separating gel. First, the separating gel was prepared, quickly mixed and poured in 3/4 volume of each sandwich plates and the remaining space (1/4 volume) was filled with isopropanol to ensure that no air bubbles would be formed at the surface of the gel during gel polymerization. The isopropanol was poured off after polymerization; the stacking gel was filled in the remaining space of the sandwich plates and, finally, a 10 well comb was inserted on the top of the staking gel.

3.2.10.3 SDS-PAGE gel electrophoresis

One lit of 1x running buffer was prepared. After the gel was already polymerized, the comb was removed; two support pieces of the gel sandwiches were mounted with the U-shape rubber piece and then put into a chamber. The upper reservoir was filled with

running buffer and then 10 µl of each protein extract was added in the prepared well. The gel electrophoresis was run at 10 mA per one gel for 1.5 hours.

3.2.10.4 Transferring proteins to the membrane

Proteins were transferred into nitrocellulose transfer membrane using the Trans-Blot Semi-Dry transfer Cell. For each gel, one piece of transfer membrane and nine pieces of filter paper with the same size as the gel were prepared. Each of three pieces of the filter paper were wetting in anode buffer I, anode buffer II and cathode buffer, while the transfer membrane was soaked in distilled water. After the electrophoresis, the whole setup was dismounted, the stacking gel was discarded and the separation gel was moved and placed on the transfer membrane. Filter papers soaked in anode buffer I were placed on the anode plate of the blotter first followed by those paper soaked in anode buffer II. The transfer membrane and gel were removed from water and placed on top of filter paper stack followed by the paper in cathode buffer (Figure 7). The cathode plate of the blotter was placed on top of transfer stack. The time of transfer was 1 hour using 100 mA per each gel (1.75mA/cm^2/h). After the transfer was completed, the blot membrane was washed with distilled water for 30 sec and subsequently stained with 0.2% ponceau S to evaluate the transfer quality. The membrane was then washed twice in PBST (10 min/time) on an orbital shaker.

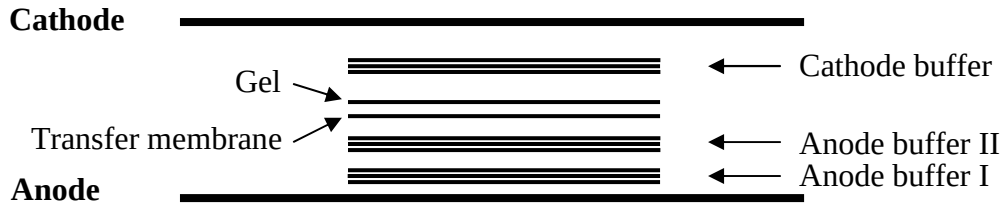

Figure 7: Diagram of transferring system

3.2.10.5 Antibody incubation

To block non-specific sites, the membrane was incubated in PBST containing 1% polyvinylpyrolidone (PVP, Sigma) for 1 hour at room temperature. The primary antibody was prepared by diluting PrP monoclonal antibody 12F10 in 0.1% PVP

(dilution 1:250, Spibio, Montigny le Bretonneux, France). The membrane was incubated with the primary antibody overnight at 4°C or for 1 hour at room temperature followed by washing with six changes of PBST (10 min/time) at room temperature. Horseradish peroxidase goat anti-mouse (Jackson ImmunoResearch Laboratories, USA) diluted 1:15,000 in 0.1% PVP in PBST was then added to the membrane and incubated at room temperature for 1 hour. After incubation, the membrane was washed with six changes of PBST (10 min/time). All incubations, blockings and washing steps were done on an orbital shaker.

3.2.10.6 Protein visualisation

Immune complexes were detected using the ECL plus Western Blotting Detection System and visualized using Kodak BioMax XAR film. Briefly, the detection reagents were removed from storage at 2 to 8°C and allowed to equilibrate to room temperature. Solution A and B were mixed in a ratio 40:1. The final volume of detection reagent required was 2 ml per membrane. The membrane was taken out from the wash buffer and placed protein side up on a clean surface. The mixed detection reagent was applied on the membrane and incubated for 5 min at room temperature. For chemiluminescent detection, the membrane was placed on a fresh piece of saran wrap placed in an x-ray film cassette, wrapped up to gently remove any air bubbles. In the dark room, a sheet of autoradiography film was placed on top of the membrane and exposed for the appropriate time period of 1 to 5 min.

3.2.11 Protein localisation by immunohistochemistry

The cellular prion protein was localised in ovaries and 1-month-old foetuses using the immunohistochemical technique with the help of avidine-biotin complex to increase the signal of the staining. Primary and secondary antibodies used in this experiment were the same as used in western blot analysis. Immunohistochemical staining was performed on 5 to 7 µm cryostat sections of snap frozen tissues. Sample sections were placed on SuperFrost® plus slides and 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. To quench an endogenous peroxidase activity, sections were

incubated in 0.3% H_2O_2 in methanol for 30 min, washed twice with PBS and then incubated in 1.5% goat normal serum in PBS for 1 hour. Endogenous biotin activity was blocked by incubating the sections in egg white (diluted 1:1 with ddH_2O) for 15 min followed by washing with three changes of PBS. The PrP antibody 12F10 diluted 1:200 in PBS was then applied on the sections and incubated for 1 hour at room temperature in an humidity chamber followed by three times washing with PBST. To detect the PrP antibody, sections were incubated in biotinylated goat anti-mouse IgG (dilution 1:100) for 30 min and washed with three changes of PBST. Avidine-biotinylated horseradish peroxidase was prepared 30 min prior to use following the manufacturer's instruction. This solution was added on the sections and allowed to stand for 30 min in the humidity chamber. The immune complexes were then visualised by incubating the samples with AEC (3-amino-9-ethylcarbazole) substrate for 30 min or until a desirable signal was obtained. The samples were counterstained with hematoxylin for 5 sec and immediately washed with several changes of deionised H_2O. Finally, the samples were mounted using glycerol gelatine and covered with a cover glass slip and the staining was observed by light microscopy.

4 Results

4.1 PRNP genotyping

So far, many polymorphisms have been identified in the ovine PRNP gene. Correlations between those polymorphisms and the susceptibility to scrapie have been analysed resulting that the polymorphisms at codon 136, 154 and 171 within exon 3 of the PRNP gene are strongly linked to the variation in the phenotypic expression of scrapie. Based on the three polymorphisms twelve alleles can be derived; however, only five alleles and fifteen genotypes, which can be divided into five risk groups according to scrapie susceptibility, are commonly seen (Table 7 and 8).

Table 7: The PRNP genotype distribution in sheep of the present study

Risk group	Genotype	Number	Genotype frequency (%)	Risk group frequency (%)
R1	ARR/ARR	74	22.84	22.84
R2	ARR/AHQ	4	1.23	1.54
	AHQ/AHQ	1	0.31	
R3	ARR/ARQ	75	23.15	29.63
	ARR/ARH	1	0.31	
	ARQ/AHQ	20	6.17	
	ARH/AHQ	0	0.00	
R4	ARH/ARH	0	0.00	33.33
	ARQ/ARH	8	2.47	
	ARQ/ARQ	74	22.84	
	VRQ/ARR	19	5.86	
	VRQ/AHQ	7	2.16	
R5	VRQ/ARQ	34	10.49	12.04
	VRQ/ARH	2	0.62	
	VRQ/VRQ	3	0.93	
Unknown	ARQ/ARK	2	0.62	0.62
Total		324	100.0	100.0

Table 8: Allele frequencies of PRNP based on the polymorphisms at codons 136, 154 and 171 within exon 3

Allele	Number	Allele frequency (%)
ARR	247	38.12
ARQ	287	44.29
ARH	11	1.70
AHQ	33	5.09
VRQ	68	10.49
ARK	2	0.31

The objectives of this study were to investigate PRNP expression during prenatal stages and to compare the expression of the gene between scrapie resistant animals and scrapie susceptible animals. So, this study was started at PRNP genotyping in sheep from some commercial farms and the Frankenforst experimental station of the University of Bonn.

DNA from blood samples of 324 sheep of various breeds were sequenced using direct sequencing PCR. The results presented that the percentage of R1, R2, R3, R4 and R5 sheep in this population were 22.8, 1.5, 29.6, 33.3 and 12.0%, respectively. The frequency of ARR/ARQ, ARR/ARR and ARQ/ARQ genotypes were 23.2%, 22.9% and 22.9%, respectively, followed by VRQ/ARQ (10.5%), ARQ/AHQ (6.2%), VRQ/ARR (5.9%), ARQ/ARH (2.5%) and VRQ/AHQ (2.2%). The number of animals other genotypes was less than 1.5% per group. ARH/AHQ and ARH/ARH genotypes were not found in this population. Moreover, of all 324 animals, 2 animals could not be identified with their PRNP genotype (Table 7).

Allele frequencies were calculated from the identified sheep showing that the frequency of ARR, ARQ, ARH, AHQ, VRQ and ARK alleles were 38.1, 44.3, 1.7, 5.1, 10.5 and 3.1%, respectively, as shown in Table 8.

After genotyping, R1 and R5 ewes were selected for further studies. The final number of ewes used in this experiment was 17; n=9 for R1 group and n=8 for R5 group. All the R1 ewes were the Black head breed, whereas seven ewes in R5 group were from the Bentheimer breed and one of them was from the Black head breed. PRNP sequencing was performed to confirm the genotypes in the selected ewes and their foetuses. Breeds

and PRNP genotypes of the sheep used in this experiment are shown in Table 9. The results showed that all the R1 ewes and foetuses used in this experiment were ARR/ARR genotype. Two PRNP genotypes of ARQ/VRQ and VRQ/VRQ genotypes were observed in the R5 group. All R5 ewes were ARQ/VRQ genotype whereas five R5 foetuses carried the ARQ/VRQ and three foetuses carried the VRQ/VRQ genotype. The R1 ram used in this study was the Black head breed, which carried the ARR/ARR genotype, and the R5 ram was from the Bentheimer breed and carried the VRQ/VRQ genotype (data not shown).

Table 9: Breed and PRNP genotypes of sheep used in this study

Month of pregnancy	Breeds	Ewes		Foetuses	
		Genotype	Group	Genotype	Group
1st	Black head	ARR/ARR	R1	ARR/ARR	R1
	Black head	ARR/ARR	R1	ARR/ARR	R1
	Black head	ARR/ARR	R1	ARR/ARR	R1
	Bentheimer	ARQ/VRQ	R5	ARQ/VRQ	R5
	Bentheimer	ARQ/VRQ	R5	ARQ/VRQ	R5
2nd	Black head	ARR/ARR	R1	ARR/ARR	R1
	Black head	ARR/ARR	R1	ARR/ARR	R1
	Bentheimer	ARQ/VRQ	R5	VRQ/VRQ	R5
	Bentheimer	ARQ/VRQ	R5	ARQ/VRQ	R5
3rd	Black head	ARR/ARR	R1	ARR/ARR	R1
	Black head	ARR/ARR	R1	ARR/ARR	R1
	Bentheimer	ARQ/VRQ	R5	ARQ/VRQ	R5
	Black head	ARQ/VRQ	R5	ARQ/VRQ	R5
5th	Black head	ARR/ARR	R1	ARR/ARR	R1
	Black head	ARR/ARR	R1	ARR/ARR	R1
	Bentheimer	ARQ/VRQ	R5	VRQ/VRQ	R5
	Bentheimer	ARQ/VRQ	R5	VRQ/VRQ	R5

4.2 Expression of PRNP mRNA

4.2.1 Expression of PRNP transcripts in ovine preimplantation embryos

The gene expression between the two groups was detected and compared using quantitative real time PCR. This analysis was done in immature oocytes (n=2 per group), 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 foetuses (n=2 per group). The results demonstrated that PRNP mRNA was detected in all stages.

After adjusting the relative expression level using the level of R1 morula as the calibrator, the relative abundance of R1 immature oocytes, mature oocytes, morula-stage embryos and 1-month-old foetuses were found to be 90.7, 5.9, 1.0 and 122.3, respectively. PRNP mRNA level in R1 mature oocyte was significantly lower than the levels in R1 immature oocyte and 1-month-old R1 foetuses ($p \leq 0.05$), whereas the expression levels of the gene compared between immature oocytes and 1-month-old stages were not significantly different ($p > 0.05$).

In the R5 group, the relative mRNA abundance was highest in 1-month-old foetuses, followed by immature oocytes, morula stage embryos and mature oocytes, respectively. The relative abundance levels of PRNP mRNA were 52.6, 1.1, 13.3 and 91.0 for R5 immature ooytes, mature oocytes, morula-stage embryos and 1-month-old foetuses, respectively. The level of transcript abundance in R5 1-month-old foetuses was significantly higher than in the other stages ($p \leq 0.05$) of the same group. The transcript abundance in immature oocytes was also significantly higher than that of *in vivo* embryos and mature oocytes ($p \leq 0.05$); however, no significant difference between morula embryos and mature oocytes was observed ($p > 0.05$).

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 stage ($p > 0.05$, Figure 8).

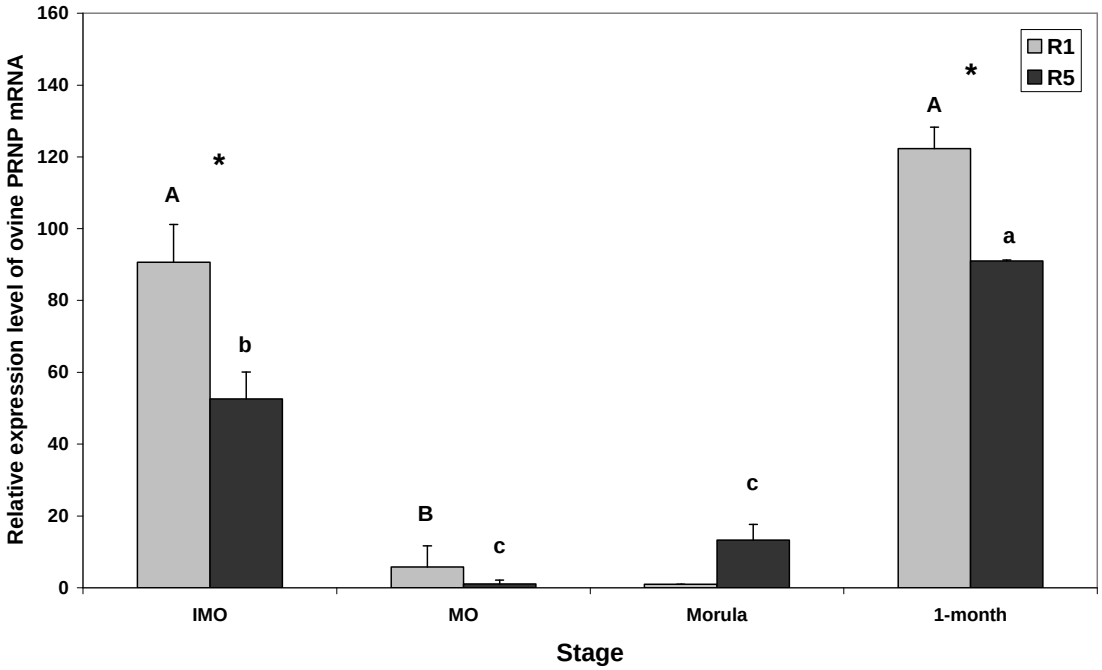

Figure 8: Relative expression levels of PRNP mRNA in *in vivo* ovine immature oocytes (IMO), mature oocytes (MO), morula-stage embryos and 1-month-old foetuses. The relative abundance of mRNA levels represents the amount of mRNA compared to the calibrator (R1 morula stage embryo) which is set to 1. Individual bars show the mean ± standard error (SE). Values with different superscripts (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 * shows a significant difference between two groups at the same stage ($p \leq 0.05$).

4.2.2 Expression of PRNP transcripts in ovine prenatal foetuses

A semi-quantitative expression analysis of PRNP mRNA in various tissues of 2-, 3- and 5-month-old foetuses of R1 and R5 groups showed that the PRNP mRNA was observed at all stages and in all tissues of both groups examined including brain, cotyledon, heart, intestine, kidney, liver, lung and muscle of 2-month-old foetuses (Figure 9), and cerebrum, cerebellum, medulla oblongata, cotyledon, spinal cord, heart, intestine, liver, lung, muscle and spleen of 3- and 5-month-old foetuses (Figure 10 and 11). In this study, 18S rRNA expression level was used as internal standard.

The PRNP mRNA expression of each sample was identified as relative expression level compared to the level of 18S rRNA from the same cDNA source. The gene expression level was identified from the intensity of the PCR product band presented on agarose

gel under the UV light. The results showed that amplification of the 18S rRNA by RT-PCR produces similar quantities of product for all tissues investigated.

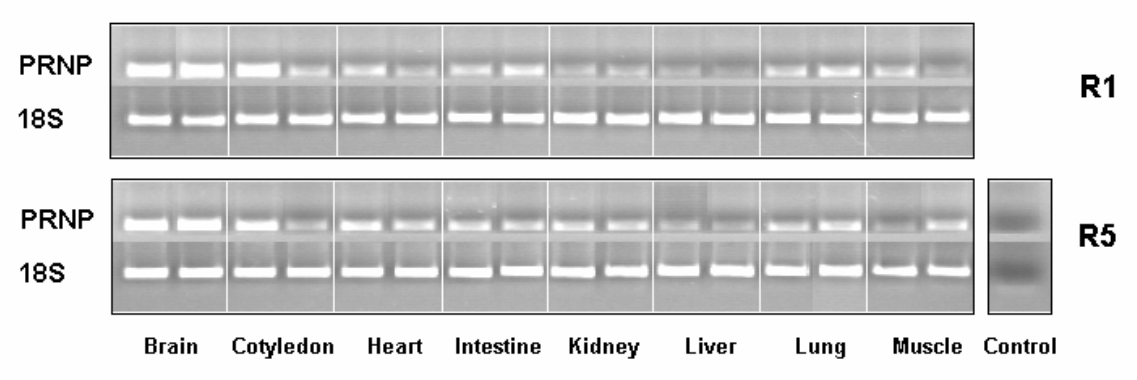

Figure 9: RT-PCR analysis of PRNP and 18S mRNA in various tissues of 2-month-old R1 and R5 foetuses. The negative control reactions were done with no template DNA

In 2-month-old foetuses, PRNP bands were strongest in brain tissue of both R1 and R5 foetuses, resulting that PRNP mRNA abundance was highest in the brain tissue of the foetuses. The PRNP levels were moderate in heart, intestine and lung, and were low in kidney and liver of the two groups. Variation of the gene transcripts in cotyledons and muscle, when compared between two independent samples of each group, were observed. In both R1 and R5 groups, one cotyledon sample showed similar expression level as those of the brain (high level), whereas the other one showed similar level as kidney and liver (low level). In muscle tissue, one sample represent similar expression level as the levels in heart, intestine and lung (moderate level) while the other one presented low expression level as kidney and liver (Figure 9).

PRNP transcripts were observed in all R1 and R5 foetal tissues studied at the 3rd and 5th month after insemination as well. The results of RT-PCR are presented in Figure 10 and Figure 11. Comparison between various tissues at the 3rd month (B) of R1 foetuses shows that the gene expression levels were high in cerebrum, cerebellum, medulla oblongata, cotyledon and spinal cord. The level(s) were moderate in intestine, lung, muscle and spleen, and was low in liver. A low expression level of this transcript was also found in the heart of an individual R1 foetus at this stage, while the level of the other foetus was moderate. At the 5th month after insemination (C), the levels of the gene transcripts could be divided into two groups. High expression levels were found in

cerebrum, cerebellum, medulla oblongata, cotyledon and spinal cord, while the moderate expression levels were detected in the rest of the tissues. Comparing within each tissue between these stages, the gene abundance levels in liver and spleen at the 5th month were higher than those levels at the 3rd month. Whereas it seems to be unchanged through the prenatal stage in the rest of the tissues (Figure 10)

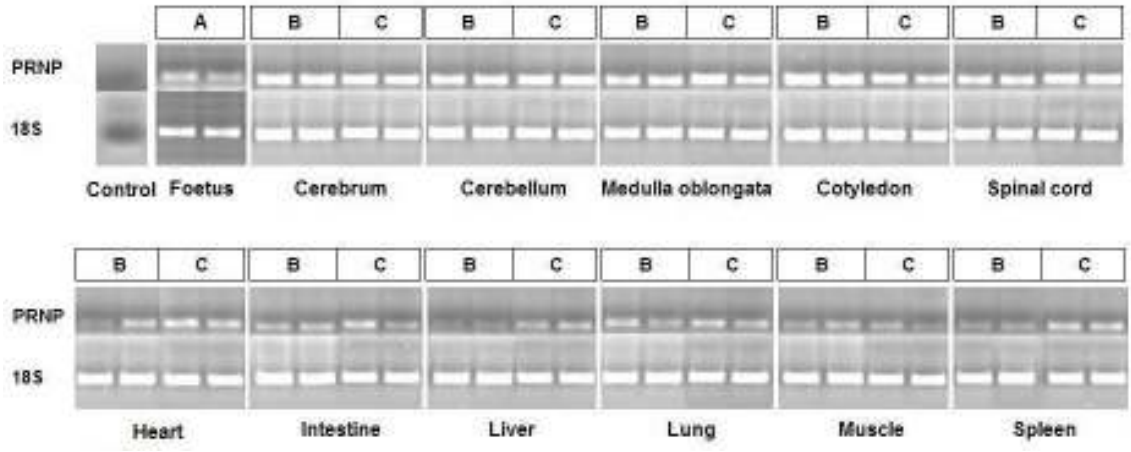

Figure 10: RT-PCR analysis of PRNP and 18S mRNA in 1-month-old R1 foetuses (A) and in various tissues of 3- (B) and 5-month-old (C) R1 foetuses. The negative control reactions were done with no template DNA

In 3-month-old R5 foetuses, the gene expression levels of various tissues can be catagorised into 3 groups; high expression levels were found in cerebrum, cerebellum, medulla oblongata, cotyledon and spinal cord, while the levels in heart, intestine, lung and muscle were moderate, and in liver and spleen were low. At the 5th month, the results showed the same trend as those at the 3rd month. However, comparison within each tissue between two stages of foetuses demonstrates that PRNP expression levels in cerebrum at the 5th month were lower than that of the 3rd month, whereas the level in the medulla oblongata at the 5th month was higher than the level at the 3rd month (Figure 11)

Comparisons within each tissue and each stage between the two groups indicates that the PRNP mRNA levels in the heart of R1 foetuses were lower than that of R5 foetuses at the 3rd month while the levels at the 5th month were higher in R1 cerebrum and liver,

and lower in R1 medulla oblongata as compared with those in R5 foetuses (Figure 10 and Figure 11)

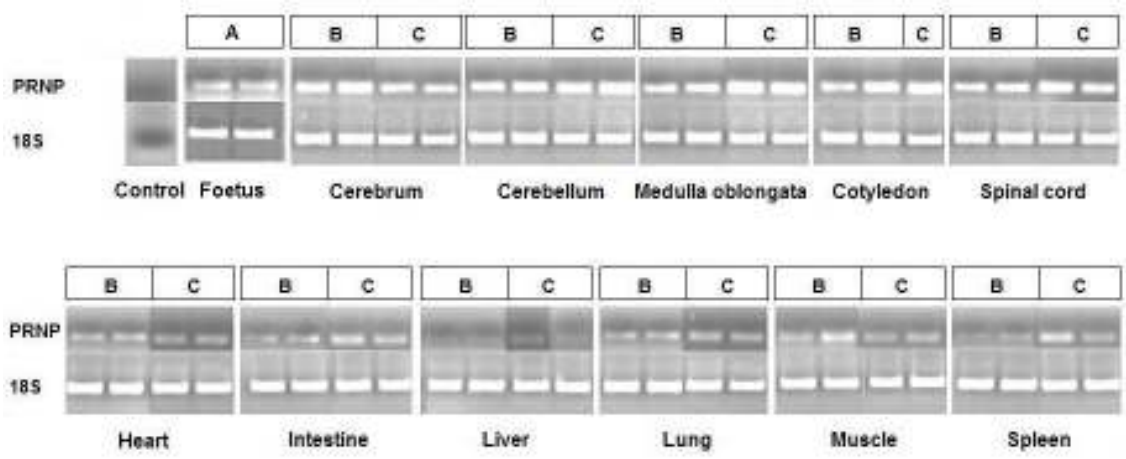

Figure 11: RT-PCR analysis of PRNP and 18S mRNA in 1-month-old R5 foetuses (A) and in various tissues of 3- (B) and 5-month-old (C) R5 foetuses. The negative control reactions were done without template DNA

Following to the results of RT-PCR that showed differences of the gene abundance in heart, liver and spleen of R1 foetuses, and in cerebrum, medulla oblongata, and spleen of R5 foetuses, quantitative real time RT-PCR was performed to define the amount of gene transcripts in cerebrum, medulla oblongata, heart, liver and spleen of both groups using the level of 18S rRNA as an internal standard. The relative levels of PRNP mRNA of each independent sample are shown in Table 10, while mean and standard error of each group are shown in Figure 12.

In 3-month-old R1 foetuses, the relative abundance of the gene transcript was highest in cerebrum followed by medulla oblongata, spleen, heart and liver, respectively. The levels were 47.9, 41.4, 4.3, 2.2 and 5.5 for 3-month-old R1 cerebrum, medulla oblongata, heart, liver and spleen samples, respectively. In 5-month-old R1 foetuses, the levels of PRNP transcript in the tissues mentioned above were 35.1, 50.1, 7.0, 8.0 and 9.4, respectively. The levels in cerebrum and medulla oblongata at this stage were also higher than those of the rest of the tissues.

Table 10: Relative abundance of PRNP mRNA ($x10^{-6}$) in foetal cerebrum, medulla oblongata, heart, liver, and spleen of each individual at the 3rd and 5th month of pregnancy

Tissue	Stage	Group	Relative abundance of PRNP mRNA	
			Animal 1	Animal 2
Cerebrum	3 month	R1	57.33	38.54
		R5	41.54	25.58
	5 month	R1	39.00	31.18
		R5	20.88	28.32
Medulla oblongata	3 month	R1	48.27	34.45
		R5	22.28	41.87
	5 month	R1	81.46	18.77
		R5	78.48	56.04
Heart	3 month	R1	3.00	5.59
		R5	6.05	8.45
	5 month	R1	8.40	5.53
		R5	5.79	9.17
Liver	3 month	R1	2.08	2.22
		R5	2.24	2.45
	5 month	R1	4.66	11.30
		R5	3.38	1.67
Spleen	3 month	R1	6.40	4.62
		R5	3.20	5.37
	5 month	R1	10.33	8.55
		R5	10.41	4.98

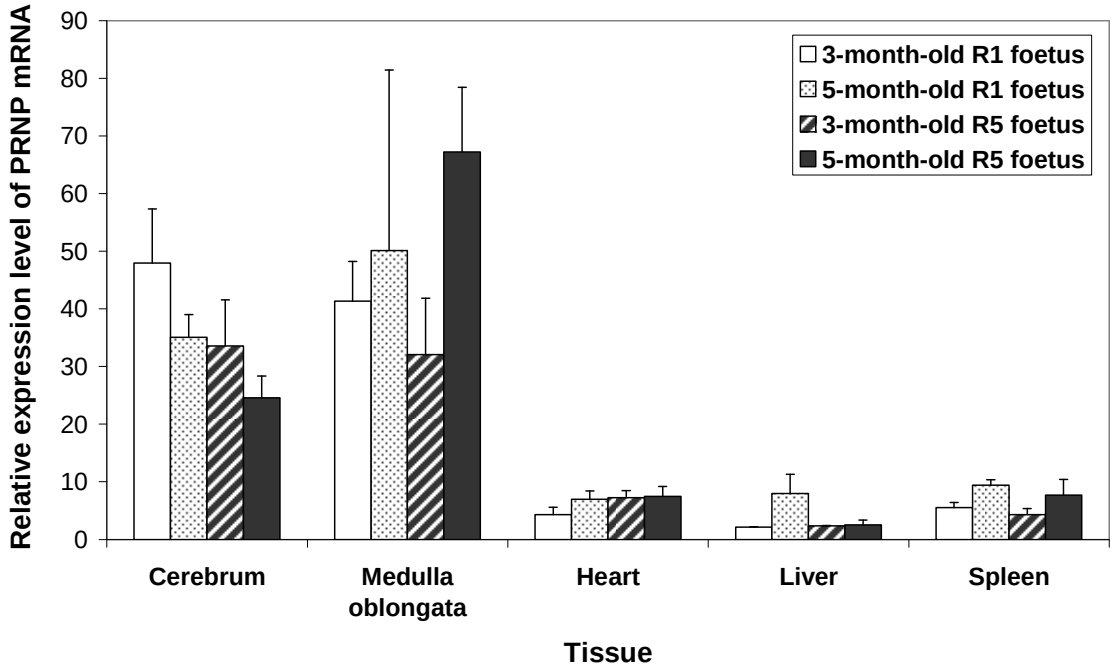

Figure 12: Relative expression levels of PRNP mRNA ($x10^{-6}$) in foetal tissues of scrapie resistant (R1) and susceptible (R5) groups at the 3rd and 5th month of pregnancy. Individual bars show the mean ± SE (n=2 per group).

In R5 foetuses, the relative abundance of the PRNP gene was similar to those of the R1 foetuses. The gene expression levels in medulla oblongata and cerebrum were higher than those in heart, liver and spleen. At the 3rd month of pregnancy, the levels of PRNP transcript in the tissues mentioned above were 33.6, 32.1, 7.3, 2.4 and 4.3, respectively. At the 5th month, the levels in R5 cerebrum, medulla oblongata, heart, liver and spleen were found to be 24.6, 67.3, 7.5, 2.5 and 7.7, respectively.

Comparing between R1 and R5 groups within each tissue of each stage, real time RT-PCR analyses resulted in higher expression level of PRNP gene in R1 cerebrum and R1 spleen at both stages of 3 and 5 months of age compared to R5 cerebrum and R5 spleen, respectively. The levels in the heart of R1 foetuses were lower than those of R5 foetuses at both stages. In medulla oblongata, the expression level of R1 foetuses at the 3rd month was higher than the level of R5 foetuses, but at the 5th month, the level of R1 foetuses was lower than the level of R5 foetuses. These results are opposite to the results in liver that showed lower expression level of the gene in R1 foetuses at the 3rd month and higher expression level at the 5th month compared to the levels of R5 foetuses.

Comparison between the different stages within the same group demonstrated that the relative expression levels of PRNP mRNA in cerebrum at the 3rd month of both R1 and R5 groups were higher than those at the 5th month. The levels in the rest of the tissues were lower at the 3rd month compared to levels at the 5th month.

4.2.3 Expression of PRNP transcripts in reproductive organs of ewes

The results of semi-quantitative RT-PCR in ovine reproductive tissues revealed the presence of the gene transcript in ovary, oviduct, endometrium, myometrium and caruncle of R1 and R5 ewes at all stages (Figure 13 and Figure 14). In this experiment, the expression patterns of the two groups were likely similar. Compared within each group, the highest expression level was found in caruncle tissue at all stages investigated, whereas the relative expression levels of PRNP mRNA in ovary, oviduct, endometrium and myometrium were lower and not different from each other. However, the levels of the gene mRNA abundance in R1 caruncle seems to be unchanged throughout the pregnancy period while it was increased from the 1st month to the 3rd month, and decreased from the 3rd to the 5th month in R5 caruncle. When compared between R1 and R5 groups, the results showed that the expression level of PRNP transcript in caruncle tissue of R1 ewes was higher than that of R5 ewes at the 1st month of pregnancy, while it was lower at the 3rd month. Moreover, at the 1st month, one of the sheep in R1 group showed higher expression level in endometrium compared to the R5 group, while the other one was similar to the levels of the R5 group. The relative abundance of the gene transcript in other tissues was not different compared between R1 and R5 group.

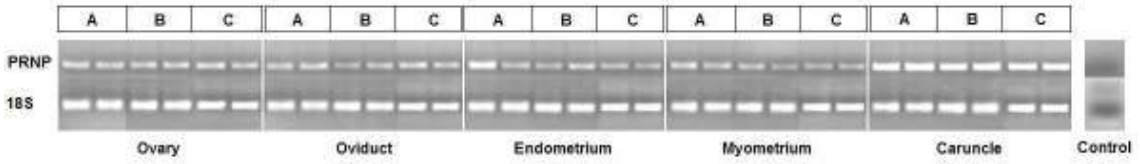

Figure 13: RT-PCR analysis of PRNP and 18S mRNA in reproductive tissues of scrapie resistant ewes at the 1st (A) , 3rd (B) and 5th (C) month of pregnancy. The negative control reactions were done with no template DNA

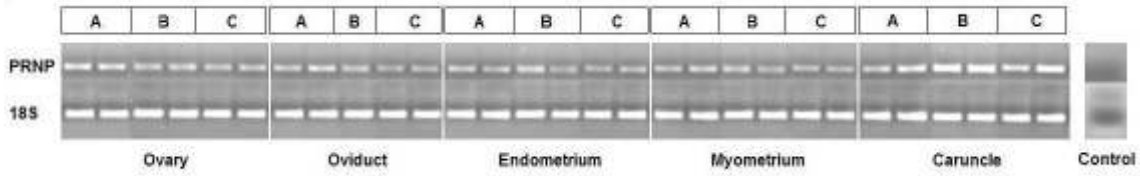

Figure 14: RT-PCR analysis of PRNP and 18S mRNA in reproductive tissues of high susceptible ewes at the 1st (A), 3rd (B) and 5th (C) month of pregnancy. The negative control reactions were done with no template DNA

Real time PCR was carried out to quantify the amount of PRNP transcripts in endometrium of ewes at the 1st month of pregnancy, and in caruncle at the 1st, 3rd and 5th month of pregnancy with three replications using 18S rRNA as an internal standard. The results of real time PCR supported the results obtained by RT-PCR as shown in Figure 15. Relative PRNP expression levels were 25.5 and 5.4 for R1 endometrium, and 10.2 and 13.3 for R5 endometrium. The levels of R1 caruncle at the 1st, 3rd and 5th month were 28.5 and 67.7, 35.8 and 48.1, and 24.0 and 28.6, respectively. While the levels of R5 caruncle were 16.7 and 17.8, 47.4 and 42.3, and 16.3 and 41.1 at the 1st, 3rd and 5th month of pregnancy, respectively. Mean and standard error of the relative abundance of PRNP transcript in R1 and R5 endometrium at the 1st month of pregnancy were 15.5 ± 10.0 and 11.8 ± 1.5, respectively. The levels in caruncle tissue of R1 and R5 ewes were 48.1 ± 19.6 and 17.2 ± 0.6, 42.0 ± 6.1 and 44.9 ± 2.6, 26.3 ± 2.3 and 28.7 ± 12.4 at the 1st, 3rd and 5th month, respectively.

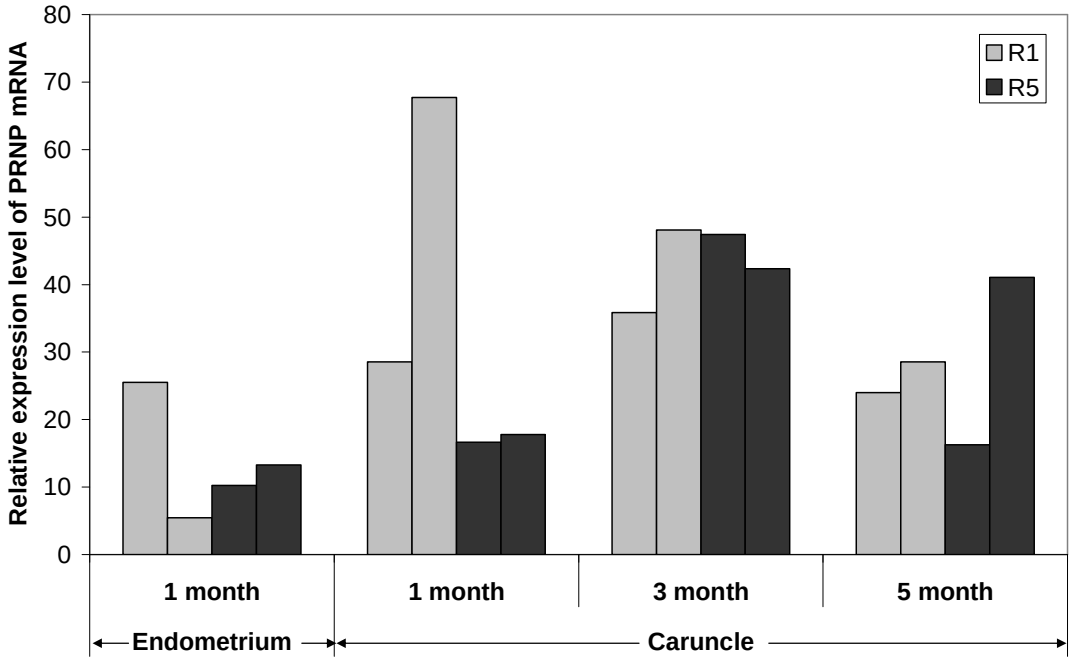

Figure 15: Relative expression levels of PRNP mRNA ($x10^{-6}$) in endometrium and caruncle of resistant (R1) and high susceptible (R5) ewes (n=2 per group).

4.3 Localisation of PRNP mRNA

The results of PRNP mRNA expression analyses showed the presence of the gene transcripts in the total RNA extracted from the whole foetuses at 1 month of age and the ovaries of ewes. However, ovaries comprise of many types of cells which are involved in folliculogenesis such as follicular cells, granulosa cells, theca interna, theca externa, cortical stroma or corpus luteum. So, in this study, fluorescent in situ hybridisation was used as a tool for localisation of the gene mRNA in ovary tissue and in 1-month-old foetuses of R1 and R5 groups. RNA probes used in this study were digoxigenin labelled. After hybridisation, the hybrids were detected by anti-digoxigenin-POD followed by the fluorescein TSA reagent. The nucleus of the cell was labelled with propidiumiodide. Under the fluorescent microscope the fluorescein signals were shown in green while propidiumiodide was shown in red.

4.3.1 Localisation of PRNP mRNA in 1-month-old foetuses

PRNP transcripts were detected in various organs of 1-month-old R1 and R5 foetuses of sheep. The fluorescent signals of PRNP mRNA hybrids were detected in the brain (Figure 16A and B), vertebral column (Figure 16D and E), heart (Figure 16G and H), liver (Figure 16J and K) and kidney (Figure 16M and N) of R1 and R5 foetuses, respectively. Signals were not found in the negative control sections of heart (Figure 16I), liver (Figure 16L) and kidney (Figure 16O), which were hybridised with the sense probe. Weak signals were detected in the control sections of the brain (Figure 16C) and vertebral column (Figure 16F); however, the samples hybridised with antisense probe displayed a higher signal above these background.

4.3.2 Localisation of PRNP mRNA in adult ovaries

In situ hybridisation of the PRNP mRNA was also detectable in the ovary of both R1 and R5 groups. Signals were detected in granulosa cells and theca cells (Figure 17A, E), ovarian cortex (Figure 17B, F), ovarian medulla (Figure 17C, G) and corpus lutuem (Figure 17D, H) of R1 and R5 ovaries, respectively. Signals were not found in the control sections hybridised with sense probe as shown in Figure 17I, J, K and M.

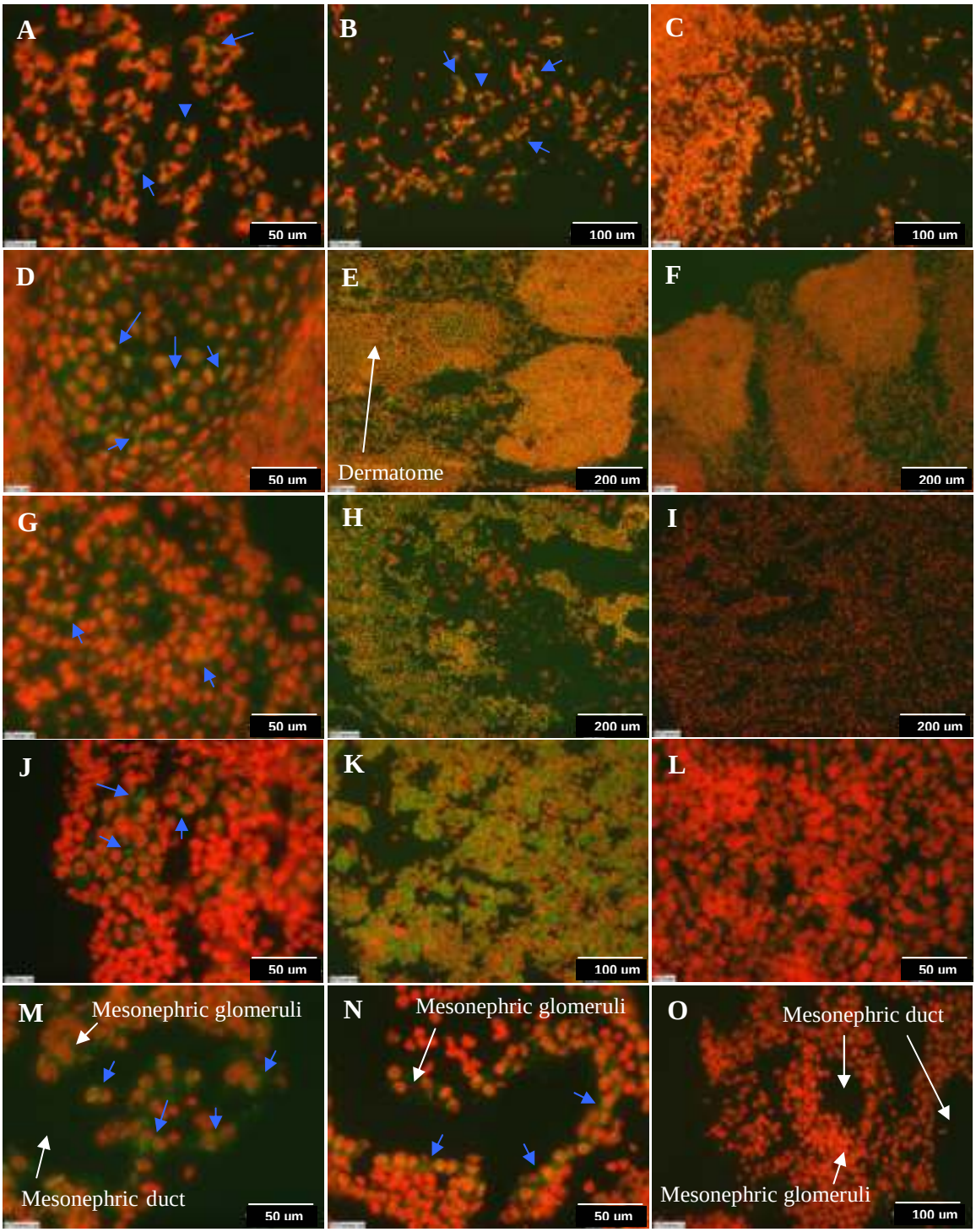

Figure 16: Fluorescent in situ hybridisation of PRNP mRNA with DIG labelled RNA antisense (left and middle column) and sense (right column) probes in 1-month-old R1 (A, D, G, J and M) and R5 (B, E, H, K and N) ovine foetuses. PRNP transcripts, which are shown in green or point with blue arrow, were detected in brain (A and B), vertebral column (D and E), heart (G and H), liver (J and K) and kidney (M and N) of the foetuses compared to the control sections of each organ which are shown in the same line

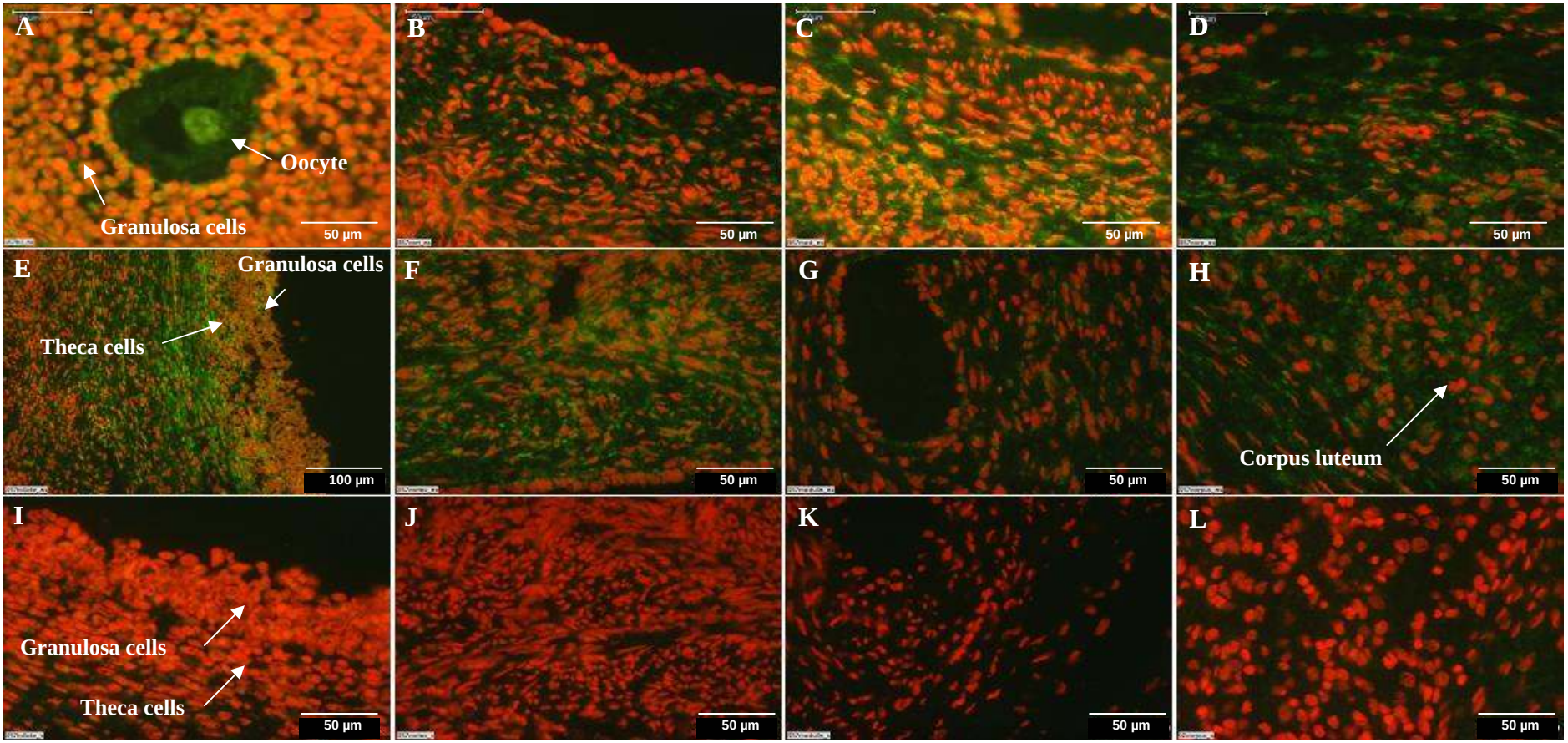

Figure 17: Fluorescent in situ hybridisation of PRNP mRNA with DIG labelled RNA antisense (A-H) and sense (I-L) probes in R1 (A-D) and R5 (E-H) ovaries collected at the 1st month of pregnancy. PRNP transcripts, which are shown in green, were found in oocyte, granulosa cells and theca cells (A and E) as well as in ovarian cortex (B and F), ovarian medulla (C and G) and corpus luteum (D and H) compared to the control sections of each which are shown in the same column

4.4 Expression of PrP^C, western blot analysis

In order to study the PrP^C expression in reproductive tissues of ewes and various tissues of their foetuses, western blot analysis was performed to detect PrP^C in the samples and to compare the expression level of the protein between samples. According to the results of mRNA expression analyses, female reproductive tissues obtained from ewes at the 1st month of pregnancy, the whole foetuses at the 1st month and the foetal tissues at the 2nd month of embryogenesis were used to identify the location of the PrP^C in each tissue, and to compare the protein expression between the two groups.

4.4.1 Expression of PrP^C in ovine prenatal foetuses

To study the expression of PrP^C in the ovine prenatal foetuses, western blot analysis was performed. The proteins extracted from whole prenatal foetuses at 1 month of age and from separated single organs of the foetuses at 2 months of age were used to detect and compare the expression level of the protein between R1 and R5 groups. The results showed that the immunoreactive bands corresponding to the PrP^C were detected in all samples examined. The expression patterns were not different between the two groups. The intensity of the PrP^C 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 high when compared to the levels in heart, intestine, kidney, lung and muscle. The protein expression levels in heart, intestine, lung and muscle were quiet similar. In this study, PrP^C levels detected in cotyledon samples of both groups were very low, even when a double volume of protein extract from this tissue was loaded (Figure 18).

4.4.2 Expression of PrP^C in reproductive organs of ewes

PrP^C of ~33 kDa 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 PrP^C 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 tissue were not different when compared in each tissue

between R1 and R5 ewes, but it was higher in R5 oviduct than that of R1 oviduct (Figure 19).

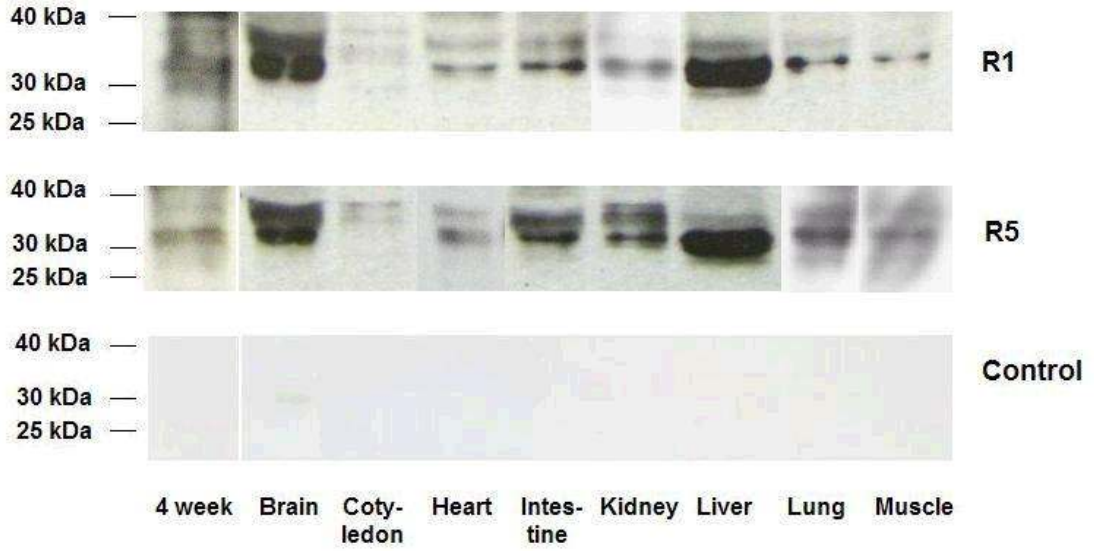

Figure 18: Representative western blot analysis of PrP^C in ovine foetal tissues showed the immunoreactive band corresponding to the PrP^C in 1-month-old foetuses and various tissues of 2-month-old foetuses of both R1 and R5 groups. Negative control membrane was devoid of the PrP MAb incubation

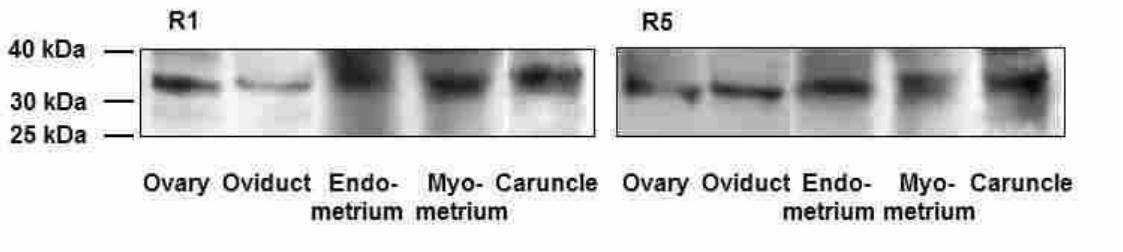

Figure 19: Representative western blot analysis of PrP^C in ovine reproductive tissues showed the immunoreactive band corresponding to PrP^C in ovary, oviduct, endometrium, myometrium and caruncle of resistant (R1) and high susceptible (R5) ewes

4.5 Localisation of PrP^C

As it has been done for PRNP mRNA localisation immunohistochemistry was done to localise PrP^C in ovary tissue and in 1-month-old foetuses of both groups. The technique was optimised firstly using various regions of the brain including cerebrum, cerebellum and medulla oblongata, which served as positive control. In this study, the antigen-antibody binding was visualised using HRP-AEC substrate and the sample was counterstained using haematoxylin. HRP-AEC substrate produced red-rose colour during the enzymatic reaction while haematoxylin produced blue colour on the nucleus of the cell. As a result, PrP^C was detected in every region of the brain examined (Figure 20) with intense staining in the interface between the white and grey matter of cerebrum and cerebellum of R1 (Figure 20 A, D) and R5 (Figure 20 B, E) groups. After optimisation, immunohistochemistry was performed in adult ovaries and 1-month-old foetuses, while adult brain was stained in parallel as positive controls.

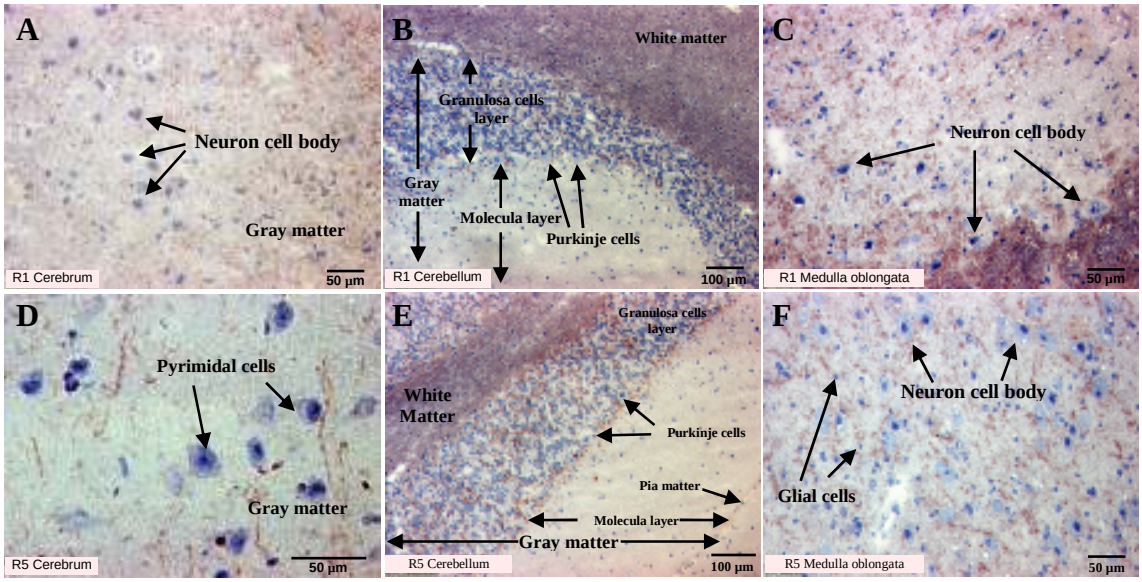

Figure 20: Immunohistochemistry of the prion protein with PrP MAb 12F10 in adult brain of sheep using HRP-AEC substrate which produces the red-rose colour during the emzymatic reaction as a chromogen. PrP^C (shown in red) was detected in the gray matter of cerebrum, interface between the white matter and the gray matter of cerebellum and also in the medulla oblongata. Control sections were devoid of specific staining with the PrP MAb (data not shown)

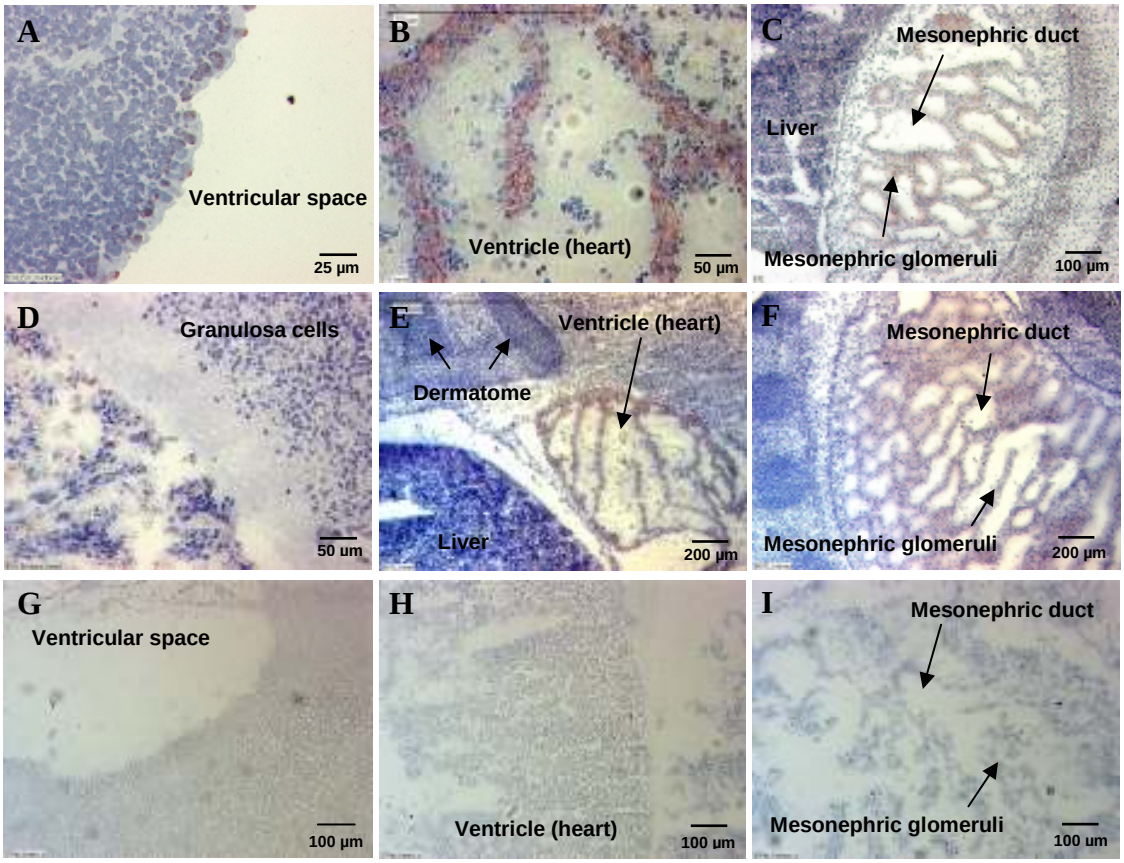

Figure 21: Immunohistochemistry of the prion protein with PrP MAb 12F10 in ovine 1-month-old R1 (A, B and C) and R5 (D, E and F) foetuses using HRP-AEC substrate which produces the red-rose colour during the emzymatic reaction as a chromogen. PrP^C (shown in red) was detected in brain (A and D), heart (B and E) and kidney (C and F). Immunostaining of the PrP^C was not found in liver and dermatome (E). Control sections were devoid of specific staining with the PrP MAb (G, H and I)

4.5.1 Localisation of PrP^C in 1-month-old foetuses

Immunohistochemistry was done in snap-frozen sections of 1-month-old R1 and R5 foetuses. The results presented PrP^C in brain (Figure 21A, D), heart (Figure 21B, E) and kidney (Figure 21C, F) of R1 and R5 foetuses, respectively, compared to the control sections that were devoid of specific staining with the PrP MAb of brain (Figure 21G), heart (Figure 21H) and kidney (Figure 21I). Under the light microscope, the staining was detected around the nucleus of the cells. In this experiment, the intensity of the

PrP^C staining was not different between R1 and R5 foetuses. The staining was found to be more intense in the heart of the foetuses than in kidney and even in the brain. In addition, PrP^C was not detectable in the other organs of the foetuses such as eye (data not shown), liver or dermatome (Figure 21E).

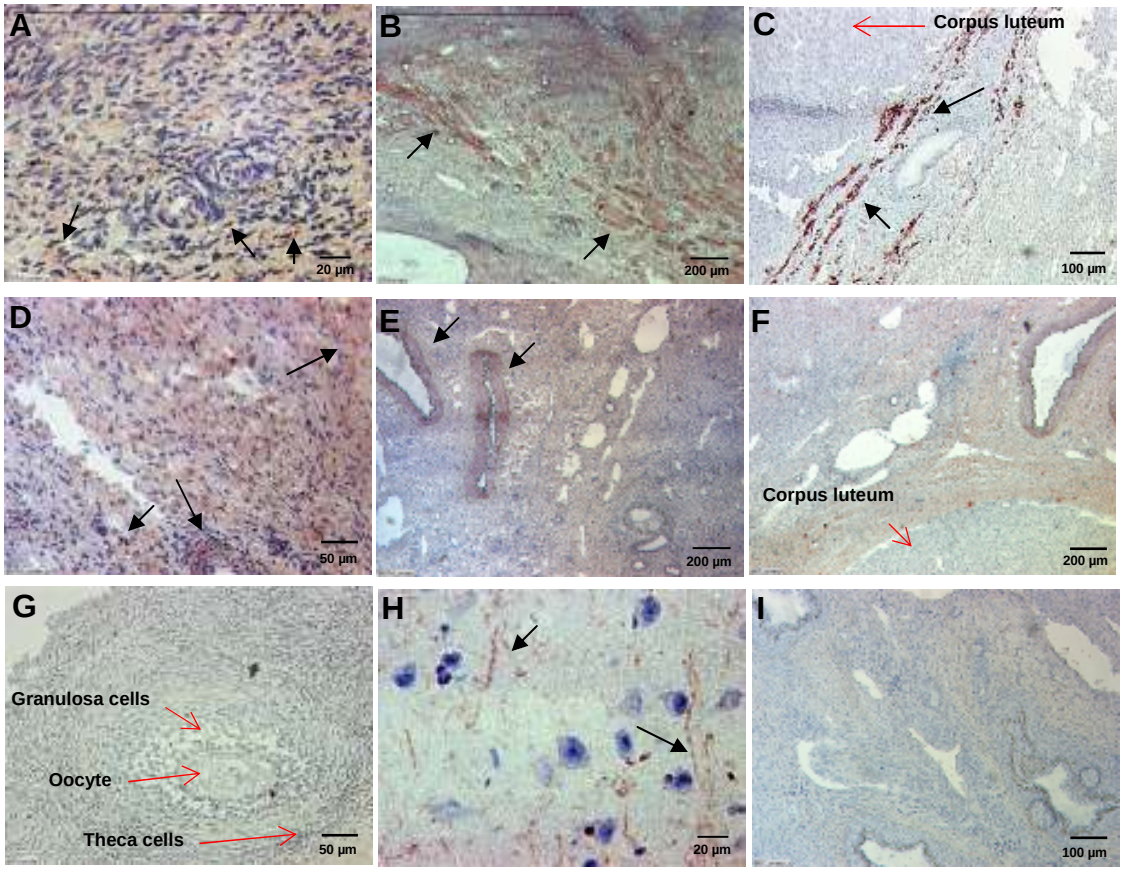

Figure 22: Immunohistochemical localisation of PrP^C in ovine ovaries (black arrow). Strong staining was found in ovarien cortex (A and D) and ovarian medulla (B and E) of R1 and R5 ewes, respectively. PrP^C was not detected in the oocyte, granulosa cells and theca cells of R5 animal (G) and in corpus luteum (C and F) of R1 and R5 ewes, respectively. Ovine brain was stained in parallel as positive control for PrP^C immunostaining (H). Control sections were devoid of specific staining with the PrP MAb (I)

4.5.2 Localisation of PrP^C in adult ovaries

Immunohistochemical localisation was performed in the ovary of both R1 and R5 groups. The results in R1 and R5 ovaries were not different; immunostaining of PrP^C which is shown in red under the light microscope was detectable in cortex (Figure 22A, D) and medulla (Figure 22B, E) of R1 and R5 ovaries, respectively. The staining was not seen in corpus luteum (Figure 22C, F) of both groups, neither in oocyte, granulosa and theca cells (Figure 22G) of R5 ovary in this study. Comparison of the expression level of the protein between ovarian cortex and ovarian medulla demonstrates the higher immune complexes signal in ovarian medulla than that in cortex.

5 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 to better understanding of its function. Semi-quantitative RT-PCR, quantitative real-time PCR and fluorescent in situ hybridisation were used to characterise prion protein gene (PRNP) expression and location, while immunohistochemistry was used to investigate the location of the protein in organs. According to previous studies showing that polymorphisms at codons 136, 154 and 171 of the ovine PRNP associate to the prion disease susceptibility. Animals carrying resistant (R1) or high susceptible (R5) genotypes were used.

5.1 Genotype and allele frequencies of the PRNP

The results of this study showed the genotype and allele frequencies for different amino acids at positions of 136, 154 and 171 of the PRNP in various breeds of sheep. At codon 136 alanine (A) and valine (V) were observed, at codon 154 arginine (R) and histidine (H) were observed, and at codon 171 glutamine (Q), arginine and histidine were commonly detected; two animals from a commercial flock carried lysine (K) at the codon 171. The genotype of these two animals was ARQ/ARK which is rare; a classification in one of the risk groups is not possible. As it has been the case for the other sheep breeds, not all possible allelic and genotypic variants were observed. In this study, six allelic variants and thirteen genotypes were shown. Similar to Acutis et al. (2004) and Gombojav et al. (2003), the allelic variant ARK was observed besides the other five common alleles of ARR, ARQ, ARH, AHQ and VRQ in Italian Biellese breed rams and Mongolian sheep; twenty-one and eleven genotypes were found in those studies, respectively. From the previous studies of Acin et al. (2004) and Tranulis et al. (1999), five common allelic variants were seen in native healthy Spanish and Norwegian sheep; nevertheless, only the ARQ, AHQ and VRQ alleles and six genotypes were shown in Icelandic sheep (Thorgeirsdottir et al. 1999) (Table 11 and Table 12.)

Table 11: Frequencies of PRNP genotypes in sheep of the present study and in Icelandic, Norwegian, Italian, Mongolian and Spanish sheep

Risk group	PRNP genotype	Present study	Icelandic sheep[1]	Norwegian sheep[2]	Italian ram[3]	Mongolian sheep[4]	Spanish sheep[5]
R1	ARR/ARR	22.84	0.0	12.5	1.4	1.8	2.4
R2	ARR/AHQ	1.2	0.0	6.3	0.7	0.4	1.4
	AHQ/AHQ	0.3	0.2	0.8	0.2	0.0	0.0
R3	ARR/ARQ	23.2	0.0	31.7	11.4	18.8	23.2
	ARR/ARH	0.3	0.0	2.1	0.5	0.0	3.1
	ARQ/AHQ	6.2	6.6	10.4	5.5	0.4	3.7
	ARH/AHQ	0.0	0.0	0.0	0.4	0.4	0.0
R4	ARH/ARH	0.0	0.0	0.0	0.2	1.8	0.4
	ARQ/ARH	2.5	0.0	3.8	5.7	8.1	6.6
	ARQ/ARQ	22.8	75.9	16.7	56.3	66.4	50.4
	VRQ/ARR	5.9	0.0	4.6	1.2	0.4	1.5
	VRQ/AHQ	2.2	0.7	1.3	0.3	0.0	0.2
R5	VRQ/ARQ	10.5	16.0	8.3	9.9	1.1	5.7
	VRQ/ARH	0.6	0.0	0.0	0.7	0.0	1.0
	VRQ/VRQ	0.9	0.7	1.7	0.7	0.0	0.5
NC	ARQ/ARK	0.6	0.0	0.0	3.8	0.0	0.0
	ARR/ARK	0.0	0.0	0.0	0.1	0.0	0.0
	ARH/ARK	0.0	0.0	0.0	0.3	0.0	0.0
	AHQ/ARK	0.0	0.0	0.0	0.3	0.0	0.0
	VRQ/ARK	0.0	0.0	0.0	0.2	0.0	0.0
	ARK/ARK	0.0	0.0	0.0	0.2	0.4	0.0
Total		100.0	100.0	100.0	100.0	100.0	100.0

Adapted from: [1/] Thorgeirsdottir et al. (1999), [2/] Tranulis et al. (1999), [3/] Acutis et al. (2004), [4/] Gombojav et al. (2003), [5/] Acin et al. (2004)

NC: Not classified

Table 12: Frequencies of PRNP alleles in sheep of the present study and in Icelandic, Norwegian, Italian, Mongolian and Spanish sheep

PRNP allele	Present study	Icelandic sheep[1]	Norwegian sheep[2]	Italian ram[3]	Mongolian sheep[4]	Spanish sheep[5]
ARR	38.1	0.0	34.8	8.3	11.6	17.1
ARQ	44.3	87.2	43.8	74.4	80.6	69.6
ARH	1.7	0.0	2.9	3.8	6.1	5.6
AHQ	5.1	3.8	9.8	4.1	0.6	3.6
VRQ	10.5	9.0	8.8	6.8	0.7	4.2
ARK	0.3	0.0	0.0	2.5	0.4	0.0
Total	100.0	100.0	100.0	100.0	100.0	100.0

Adapted from: [1/] Thorgeirsdottir et al. (1999), [2/] Tranulis et al. (1999), [3/] Acutis et al. (2004), [4/] Gombojav et al. (2003), [5/] Acin et al. (2004)

The most frequent combination of the three codons in this experiment was ARQ (44.0%), and the high frequent genotypes of more than 20% were ARR/ARQ, ARQ/ARQ and ARR/ARR (23.2, 22.9 and 22.9%, respectively). The animals with ARR/ARQ or ARQ/ARQ are moderately susceptible to scrapie, while the animals with ARR/ARR seem to be resistant to scrapie, as was shown by many studies with naturally or experimentally infected sheep (Belt et al. 1995, Goldmann et al. 1994, O'Doherty et al. 2002, O'Rourke et al. 1997, Thorgeirsdottir et al. 1999). Fortunately, while the allelic variant ARR, which is associated to resistance to scrapie, was represented at a frequency of 38.0%, the frequency of the allelic variant VRQ, which is known to carry a high susceptibility, was represented at 11.0% in the examined group of sheep. These results are in agreement with several investigations, which showed the highest frequency in ARQ allele followed by the ARR allele in Icelandic (Thorgeirsdottir et al. 1999), Norwegian (Tranulis et al. 1999), Italian (Acutis et al. 2004), Mongolian (Gombojav et al. 2003) and Spanish sheep (Acin et al. 2004). The frequency of ARQ allele ranges from 43.8 to 87.2%, and that of ARR, ARH, AHQ and VRQ alleles range from 0.0 to 38.1, 0.0 to 6.1, 0.6 to 9.8 and 0.7 to 10.5%, respectively (Table 12).

The PRNP genotype frequencies in this study were also in agreement with observations made in four healthy major breeds of sheep in Norway that showed the highest

frequency in ARR/ARQ genotype followed by ARQ/ARQ genotype. In contrast, the ARQ/ARQ genotype was more frequent than the ARR/ARQ genotype in Icelandic sheep (scrapie-free regions and scrapie regions), Italian Biellese ram, healthy Spanish and Mongolian sheep (Acin et al. 2004, Acutis et al. 2004, Gombojav et al. 2003, Thorgeirsdottir et al. 1999, Tranulis et al. 1999) (Table 11).

The genetic data on the PRNP polymorphisms offer the possibility of controlling natural scrapie in sheep populations by selecting genotypes carrying scrapie resistant alleles or either by eliminating the PRNP genotypes carrying high susceptible alleles in breeding animals. Breeding programmes for animals carrying genotypes that determine resistance and the elimination of animals with the VRQ allele are already underway in many European (EU) countries. An increase in ARR/ARR genotype frequency was shown in ovine animals from European Commission member states (without United Kingdom, UK) during the period of 2002 to 2005. ARR/ARR genotype frequencies in 2002, 2004 and 2005 were 14.0, 18.0 and 21.0%, respectively (European Commission 2003, 2005, 2006).

In this experiment, sheep were grouped based on the classification system mentioned in Erhardt et al. (2002). This classification system is different from the NSP system used in the UK as shown in Table 13 (European Commission 2004).

Table 13: The PRNP genotypes and risk groups in accordance with the NSP classification system used in the United Kingdom

Risk group	Genotype
NSP1	ARR/ARR
NSP2	ARR/ARQ, ARR/ARH, ARR/AHQ
NSP3 (ARQ/ARQ)	ARQ/ARQ
NSP3 (others)	AHQ/AHQ, ARH/ARH, ARQ/ARH, ARQ/AHQ, ARH/AHQ
NSP4	ARR/VRQ
NSP5	VRQ/ARQ, VRQ/ARH, VRQ/AHQ, VRQ/VRQ

Table 14: PRNP genotypes frequencies in the examined group of sheep in accordance with the NSP classification system used in the United Kingdom

Risk group	Genotype	Number of animals	Genotype frequency (%)	Risk group (%)
NSP1	ARR/ARR	74	22.84	22.84
NSP2	ARR/ARQ	75	23.15	24.69
	ARR/ARH	1	0.31	
	ARR/AHQ	4	1.23	
NSP3 (ARQ/ARQ)	ARQ/ARQ	74	22.84	22.84
NSP3 others	AHQ/AHQ	1	0.31	8.95
	ARH/ARH	0	0.00	
	ARQ/ARH	8	2.47	
	ARH/AHQ	0	0.00	
	ARQ/AHQ	20	6.17	
NSP4	VRQ/ARR	19	5.86	5.86
NSP5	VRQ/ARQ	34	10.49	14.20
	VRQ/ARH	2	0.62	
	VRQ/AHQ	7	2.16	
	VRQ/VRQ	3	0.93	
Unknown	ARQ/ARK	2	0.62	0.62
Total		324	100.00	100.00

The European Commission (2006) reported that, in 2005, the frequencies of NSP1, NSP2, NSP3 (ARQ/ARQ), NSP3 (others), NSP4 and NSP5 risk groups in 190,577 sheep from EU member states were 21.0, 37.0, 26.0, 9.0, 2.0 and 4.0%, respectively. After reclassification of PRNP genotypes in the examined sheep according to the NSP system, the results showed that the frequencies in our population were 22.8, 24.7, 22.8, 9.0, 5.9 and 14.2% for the NSP1, NSP2, NSP3 (ARQ/ARQ), NSP3 (others), NSP4 and NSP5, respectively (Table 14). The NSP4 and NSP5 groups in the population of this study were more frequent compared to those reported by European Commission (2006).

The lower genotype frequency of NSP1 and higher frequency of NSP5 in our sheep population as compared to the recently reported average in EU member states is due to the requirement of sheep with high susceptibility genotypes of this study. The blood samples were collected not randomly, but focused on the breed which is known to carry the high risk genotypes. After classification and selection, the sheep used in this experiment were Blackhead and Bentheimer breeds. All of the Bentheimer sheep used in this study were R5 genotypes, whereas most of the Blackhead sheep carried the R1 genotype. The European Commission (2004) reported that the ARR allele frequency of the Blackhead breed is 70 to 79% and that of the Bentheimer breed is 0 to 9%. According to these findings Blackheads are regarded as a scrapie resistant breed and the Bentheimer are regarded as a scrapie-susceptible breed.

5.2 PRNP mRNA expression in preimplantation embryos

This study is the first report of PRNP mRNA expression in ovine preimplantation embryos. The quantitative gene expression levels in R1 and R5 groups were in the same trend showing that relative expression levels were significantly lower at mature oocyte 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 foetuses were more abundant than those of R5 group. The present results in ovine embryos showed the same trend as the gene levels in bovine embryos; bovine PRNP transcript abundance increased at zygote, was decreased along embryonic development and was elevated at early foetal stage again (own unpublished data). The increasing of PRNP transcript at zygote stage could be due to sperm activity after fertilisation, since PRNP mRNA was found in spermatogenic cells of mice (Fujisawa et al. 2004). Moreover, a C-terminally truncated isoform of PrP^{C} was shown in bovine (Shaked et al. 1999) and ovine (Ecroyd et al. 2004) mature sperm by western immunoblotting. Thus, sperm could be the source of PRNP after fertilisation.

Maternal-zygotic transition (MZT) is a complex phenomenon characterised by the initiation of transcription in the embryo and the replacement of maternal mRNA with embryonic mRNA (Vigneault et al. 2004). 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 (Schultz 1993), occurs at the 4000- to 8000-cell stage in *Xenopus* (Etkin and Balcells 1985) and at the four- to eight-cell stage in humans (reviewed in Telford et al. 1990). 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 (Memili and First 1999). 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 (one-, two-, or four-cell embryos), but a distinctly different pattern is observed in 16-cell embryos and at later stages (reviewed in Telford et al. 1990). Similar to those studies, it seems 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.

5.3 PRNP mRNA and PrP^C expression in prenatal foetuses

In order to investigate the expression profile of PRNP transcripts, prenatal stage whole foetuses at the 1st month of pregnancy and various tissues of 3- and 5-month-old foetuses were used. In addition and for scale up the number of tissues examined, various tissues of 2-month-old foetuses (R1 and R5 groups) including brain, cotyledon, heart, intestine, kidney, liver, lung and muscle were added into the experiment to identify the activation of PRNP mRNA and protein in single organs during this stage. The results showed that PRNP mRNA was found in all tissues of 2-month-old foetuses examined.
At the 3rd and 5th month of pregnancy, the expression patterns of ovine PRNP mRNA showed a similar trend in R1 and R5 groups. Comparison of the levels in each tissue between the 3rd and 5th month stages shows similar expression in the R5 group. These results showed high expression levels of the PRNP mRNA in cerebrum, cerebellum, medulla oblongata, cotyledon and spinal cord, while moderate levels were detected in lung, heart, intestine and muscle, and low expression levels were detected in liver and spleen. This pattern was also similar to the pattern of R1 foetuses at the 5th month of pregnancy; nevertheless, it was different from the pattern at the 3rd month which showed the same level of the transcript abundance in liver and spleen as in lung, heart, intestine and muscle. The higher expression levels in neuron nerval compared to those of the

other tissues were in agreement with previous studies (Han et al. 2006, Harris et al. 1993, Manson et al. 1992, Kubosaki et al. 2000, McLennan et al. 2001, Tichopad et al. 2003). On the other hand, in non-nerval tissues, the results in this study were not in agreement with Han et al. (2006) and Ning et al. (2005) that showed similar expression level of the ovine and murine PRNP transcripts in liver as in heart, lung and spleen. Moreover, Tichopad et al. (2003) showed a higher abundance of bovine PRNP in liver of adult cattle than in lung.

The ovine PRNP expression levels were stable throughout the prenatal stage in most of the foetal tissues investigated. However, the gene abundance levels were decreased in cerebrum, and were increased from 3 to 5 months of age in medulla oblongata, heart, liver and spleen of both groups. Quantitative real-time PCR was performed to quantify the amount of the gene transcripts in these tissues. The results by real time PCR supported those results obtained by RT-PCR.

For the PrP^C expression, Amselgruber et al. (2005) presented that PrP^C was detected in pancreas of bovine prenatal embryos ranging from 7 to 86 cm in crown-rump length. The present study demonstrated, for the first time, that the PrP^C is expressed in brain, cotyledon, heart, intestine, kidney, liver, lung and muscle of ovine prenatal embryos since the 2^{nd} month of embryogenesis. Since PrP^C expression levels in various tissues of R1 and R5 foetuses were in the same trend, and comparison within each tissue shows similar level between the two groups, it could be concluded that there is no relationship between PRNP genotype and the tissue expression of the PrP^C in prenatal foetuses at this developing period.

PrP^C is a glycoprotein present at high level in the brain. It is also found in the heart, kidney and lung at intermediate levels, but is not detectable in the liver (Bendheim et al. 1992, Horiuchi et al. 1995). Moudjou et al. (2001) have revealed the presence of PrP^C in skeletal muscle, uterus, thymus, tongue and liver other than in brain, heart and lung of ewes. By a two-size enzyme immunometric assay of that study, the lowest concentration of PrP^C was shown in the liver. PrP^C in brain was found to be between 564-16,000 fold more abundant than in liver, and between 20-50 fold more abundant than in the rest of tissues mentioned (Moudjou et al. 2001). This present study supports previous studies that PrP^C was detectable at moderate level in heart, intestine, kidney, lung and muscle; however, the level of PrP^C detected in liver was as high as in brain in our study. Ikeda et al. (1998) demonstrated that PrP^C expression was negligible in normal liver tissue, but

was found to increase dramatically in diseased livers associated with stellate cell activation. It is reported that activated stellate cells undergo metabolic alterations which influence the production of nerve-related proteins such as N-CAM (Knittel et al. 1996) or GFAP (Niki et al. 1996), respectively, in rats. Moreover, Manson et al. (1992) have suggested that PrP^C may function as a neural cell receptor and directing and/or maintaining the architecture of the nervous system. Therefore, the demonstration of high PrP^C levels in brain and liver by western blot is consistent with these views.

Prion protein is a cell surface glycosylated protein with two N-glycosylation sites; therefore, forms of mature PrP^C or glycoforms are found with different degrees of glycosylation. The glycoform signature of PrP^C in the brain, as obtained with most anti-PrP antibodies described in the literature, is in general characterised 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 (Moudjou et al. 2001). In this study, PrP^C could be detected by loading protein extracts from only 1 mg of tissue per lane of almost all tissues except cotyledon. The PrP^C reactive bands detected in cotyledon tissue of both 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 foetuses (R1 and R5 groups). The results clearly revealed the existence of PrP^C with the molecular weight of ~ 30 kDa in cotyledon of 3- and 5-month-old R1 and R5 foetuses, and assured the presence of PrP^C in the 2-month-old foetuses (Figure 23). Interestingly, most of foetal tissues examined produced mainly the biglycosylated isoform, and minimally the monoglycosylated isoform of PrP^C. Nevertheless, the cotyledon of both R1 and R5 foetuses produced only the monoglycosylated isoform. These suggest that the PrP^C in foetal cotyledon is differentially glycosylated from other foetal tissues, and could be involved in different physiological functions of the protein.

The study of PrP^C expression in prenatal foetuses established that the levels of PrP^C in some tissues were not related to the levels of the gene transcript. At 2 months of age, PRNP mRNA level(s) were high in brain and cotyledon, and low in liver. On the other hand, the PrP^C was highly expressed in brain and liver, and was very low in cotyledon. Liver is an organ which plays a major role in detoxification, blood protein formation and several metabolism functions. In the foetus, blood leaving the placenta via the umbilical vein passes through the ductus venosus and other veins of the foetal liver

(Valadian and Porter 1977). Since PrP^C was found on peripheral blood mononuclear cells (Halliday et al. 2005), the accumulation of PrP^C in foetal liver might have resulted from the circulation of PrP^C from its maternal placenta. Our finding suggested more that organs, for which infectivity has been demonstrated (brain and intestine), contain more PrP^C than tissues shown not to support infection (cotyledon, heart, lung and muscle) (Hadlow et al. 1982).

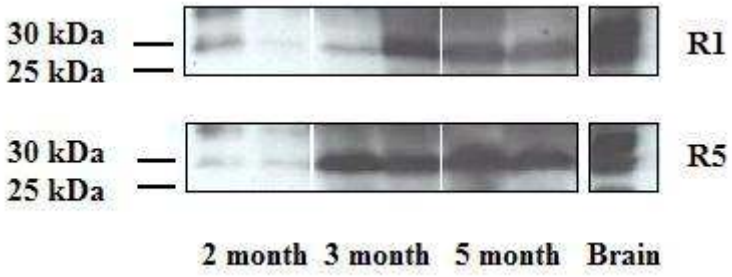

Figure 23: Western blot analysis, revealing the immuno reactive bands of PrP^C in foetal cotyledon of scrapie-resistant (R1) and high susceptibility (R5) groups at 2, 3 and 5 months of pregnancy. Protein extracts from 2 mg of each sample were loaded per lane. The foetal brains were used as positive control

5.4 In situ analysis of PRNP mRNA and PrP^C expression in 1-month-old foetuses

Localisation of the mRNA and protein in tissues is an important tool to understand the function of target genes at the cellular level. In the present study, fluorescent in situ hybridisation and immunohistochemistry were used as a tool for localisation of the PRNP transcript and the PrP^C, respectively, in 1-month-old foetuses and ovaries.

The results presented here demonstrate for the first time that the ovine PRNP mRNA is distributed not only in neural cells, but was also detected in many non-neural organs such as dermatome, vertebral column, heart, liver and kidney of sheep foetuses since the 1 month of age. The in situ hybridisation signals were detected in the brain and liver of control sections which were hybridised with sense RNA probe as well; however, the signals in the sections with antisense probe were intensely labelled above the control one. At this time of age, PrP^C was localised in developing brain, heart and kidney. These results correlate well with several studies demonstrating a distribution of PRNP mRNA in mouse and chicken foetuses during embryonic development. PRNP transcripts were found in developing brain and spinal cord of embryonic day 13.5 as

well as in ganglia and nerves of the central and peripheral nervous system of embryonic day 16.5 in mice. The gene transcripts were also detected in specific non-neuronal cell populations such as intestine, dental lamina, tooth bud and kidney of the 13.5 and 16.5 day embryos, and in extra-embryonic tissues from day 6.5. No PRNP transcript could be detected in 6.5- or 9.5-day-old mouse embryos (Manson et al. 1992). In contrast to the study of Manson and co-workers, Miele et al. (2003) presented the activation of PRNP transcription since the day 8.5 and 9.5 of mouse embryogenesis. Eventhough the gene transcript was not found at day 8.5, it was widespread throughout all regions of the developing brain such as telencephalic, mesencephalic and metencephalic vesicles, and the developing neural tube at day 9.5. During chicken embryogenesis, the mRNA for a chicken PRNP was found in the brain and spinal cord as early as embryonic day 6 (the earliest stage examined). This transcript was also detectable in dorsal root ganglia, retina, aorta, gizzard, intestine and heart of embryonic day 11 (Harris et al. 1993). The expression levels of PRNP mRNA increased 8-fold between days 1 to 42 of postnatal development in mouse brains. The PrP^{C} level has also increased over this period, most significant from days 1 to 10 (Miele et al. 2003). PRNP mRNA and protein continue to be expressed at high level in the central nervous system of adult animal (Bendheim et al. 1992, Han et al. 2006, Harris et al. 1993, Kubosaki et al. 2000, Manson et al. 1992, McBride et al. 1992, McLennan et al. 2001, Tichopad et al. 2003). The mRNA was predominantly localised in the brain within pyramidal cells of the hippocampus, large neurons of the thalamus and neocortex and Purkinje cells of the cerebellum (Tanji et al. 1995). Furthermore, the regulation of PRNP mRNA expression by nerve growth factor (NGF) has been shown in a clonal cell line, PC12 cells, (Wion et al. 1988) and many regions of postnatal mouse brain (Mobley et al. 1988). The expression of PRNP mRNA and PrP^{C} in developing brain and neural tube started at the early stage of foetal development and its continued expression in adult brain demonstrate that PRNP might be critical to neuronal differentiation or survival during the development of the central and peripheral nervous system.

5.5 PRNP mRNA and PrP^C expression in reproductive tissues of ewes

The expression patterns of PRNP transcript in reproductive organs of R1 and R5 ewes were not different at all stages studied. Comparison among tissues within each group displays the similar expression level of PRNP transcript in ovary, oviduct, endometrium and myometrium, whereas the highest level, as high as in brain (data not shown), was found in caruncle of both groups. Between R1 and R5 ewes within each stage, the expression level of PRNP mRNA in ovary, oviduct, endometrium and myometrium were not different, while the levels in R1 caruncle were higher than that in R5 caruncle at the 1st month of pregnancy. However, western blot analysis revealed a similar abundance of PrP^C in R1 and R5 caruncle, but higher abundant when compared to the level in other tissues. Moreover, lower expression levels of PrP^C were detected in oviduct as compared to ovary, endometrium and myometrium of both groups. The ruminant placenta is classified as synepitheliochorial (Wooding and Flint 1994) and has a cotyledonary organization in which both foetal and maternal villi are discernible as discrete structures (placentome) on the uterine epithelium. Intimate contact between maternal and foetal tissue occurs only in the placentome (comprising the foetal cotyledon and maternal caruncle), which is the most highly vascularized portion of the placenta. This study also found that the ovine PRNP mRNA and PrP^C levels were highest in caruncle compared to other tissues investigated. Moreover, the PrP^C was abundant at low level in the cotyledon of 2-month-old foetuses, while the levels were high at the 3rd and 5th month of pregnancy. These results were in agreement with Kubosaki et al. (2000) who showed the stronger signals of PRNP mRNA in sheep caruncle than the signals in myometrium by in situ hybridisation. Similarly, Tuo et al. (2001) showed higher expression level of PrP^C in caruncular endometrium than in intercaruncular endometrium, myometrium, oviduct and ovary by western blot analysis. The results from this study suggest that the sheep placenta, caruncle especially, may be an important organ for the conversion of PrP^C to PrP^{Sc}, and also substantiate the probability that placenta plays an important role in natural transmission of scrapie. Even Onodera et al. (1993) displayed the undetectable level of PrP^{Sc} in the placenta of Corriedale and Suffolk sheep at the 3rd and 5th month of pregnancy, respectively, a significant amount of scrapie agent has been isolated from the placenta of pregnant ewes by Race et al. (1998).

The present study has demonstrated for the first time that the PRNP transcript was localised in oocytes, granulosa cells, theca cells, ovarian cortex, ovarian medulla and corpus luteum. Interestingly, the PrP^{C} was detectable only in ovarian cortex and ovarian medulla, but not in the oocyte, granulosa and theca cells. In sheep, the ovum is released from the ovary at estrus and reaches the uterus through the oviduct within 3 to 4 days. The embryo will not hatch from the zona pellucida until it is in the uterus. Tuo et al. (2001) and Hadlow et al. (1979) demonstrated that PrP^{C}, but not the PrP^{Sc}, was present in the ovary and oviduct. These findings prove the recent observations on the prevention of TSE transmission (maternal-vertical) in bovine and ovine preimplantation embryos and foetuses using embryo transfer (Foote et al. 1993, Foster et al. 2004, Wang et al. 2002, 2001, Wrathall et al. 2002). The prevention of the TSE transmission by embryo transfer procedures, which are neither transmitted to offspring via the embryo nor to recipient animals, even when the embryos are collected from the TSE-infected donors, might have resulted from the lack of PrP^{C} in oocytes, the protection of zona pellucida and the lack of PrP^{Sc} in ovary and oviduct. In addition, the fact that PrP^{C} was not expressed in oocyte, granulosa and theca cells is meaningful for the use of reproduction biotechnologies, which aim at the application of these cells (embryo transfer, in-vitro-fertilisation/production). In the uterus of non-pregnant ewes, PrP^{C} was shown in caruncular endometrium and myometrium. The level of this protein was increased in the pregnant caruncular endometrium as compared to non-pregnant one (Tuo et al. 2001). Throughout the pregnancy period, PRNP mRNA expression level was increased in the caruncle. Moreover, both RT-PCR and western blot analysis revealed the higher expression levels of the mRNA and protein in the caruncle than those of other tissues studied. Since PrP^{Sc} was detected only in caruncular endometrium and cotyledonary chorioallantoic of the placentome from pregnant infected ewes (Tuo et al. 2001), it appears that PrP^{Sc} is present in tissue known to express high level of PrP^{C}. In addition, PrP^{C} was not detectable in the amnion during pregnancy period (Tuo et al. 2001); therefore, the foetus might be separated from the PrP^{Sc}-rich placental tissues by the PrP^{C}-free amnion.

The tissue distribution of PrP^{C} in sheep was first studied by Horiushi et al. (1995). In 2001, Mohammed et al. displayed the presence of PrP^{C} in some additional tissues from sheep with each ARR/ARQ, ARR/AHQ or ARQ/VRQ genotypes. Unfortunately, due to the low number of sheep tested, the relationship between animal genotype for the PRNP

locus and tissue expression of PrP^C could not be determined. However, studies with permissive cells expressing two different PRNP variants (ARR and VRQ) indicated that cell biological properties of ovine PRNP can vary with natural polymorphisms and raise the possibility that differential interactions of PRNP variants with the cellular machinery may contribute to permissiveness or resistance to prion multiplication (Sabuncu et al. 2005). Moreover, several studies demonstrated that the accumulation of PrP^{Sc} in placenta of ewes is determined by the foetal PRNP genotype and the pregnancy status of scrapie-infected ewes. The PrP^{Sc} was detected in placentomes of ewes with 171QQ conceptuses, but not in those with 171QR conceptuses or in non-pregnant uterus of infected ewes (Andreoletti et al. 2002, Tuo et al. 2002). While the accumulation level of PrP^{Sc} in the uterus was decreased throughout the pregnancy period, it was increased in the caruncle (Tuo et al. 2002). PrP^C is a primary substrate for the conformation change of the PrP^C into PrP^{Sc}. The amount of PrP^C is a rate-limiting step in the development of the prion disease. This present study showed an effect the PRNP variants on the gene expression level in caruncle during the gestation period; PRNP mRNA levels were increased in R5 caruncle, while it seems to be stable in R1 caruncle. Nevertheless, no relationship between the gene variants, and the normal protein distribution was observed in the present study. Interestingly, all female reproductive organs examined in this study showed only bands of the biglycosylated isoform. This contrasts to previous studies that PrP^C reactive bands of either bi- or hyperglycosylated isoformes were mostly found (Ikada et al. 1998, Mohammed et al. 2001, Pammer et al. 1998, Tuo et al. 2001).

In conclusion, knowledge about the switch-on period of the prion protein gene both on mRNA and protein levels during prenatal stage of mammalian embryos was not available so far. In order to understand the mechanism and function of PrP^C in embryogenesis, this information is of a great importance. This study is the first to report the expression of PRNP mRNA and PrP^C during prenatal stages of sheep. The relative abundances of PRNP mRNA and protein have been documented in various tissues of prenatal embryos as well as reproductive tissues of ewes. The mRNA and protein were found in many tissues of preimplantation stages foetuses as early as 1 month of age, and was detected in all tissues studied at the 2^{nd} month; however, the levels of those activities were different between tissues. In 1-month-old foetuses, PRNP mRNA was expressed in developing brain, heart, kidney, liver, vertebral column and dermatome,

while the PrP^C was detectable only in the brain, heart and kidney. Both mRNA and protein were detected in all foetal tissues examined including brain, cotyledon, heart, intestine, kidney, liver, lung, muscle and spleen since at the 2nd month (Table 15). With the exception of the foetal cotyledon and liver, the levels of PrP^C correlate well with the relative mRNA expression levels in sheep tissues. This study displayed that the mRNA was high in cotyledon, but was low in liver of the foetuses. In contrast, PrP^C were found at high level in liver but low level in cotyledon. These might be involved in the function of PRNP gene. In female reproductive organs of pregnant ewes, both PRNP mRNA and PrP^C were found at similar level when compared between ovary, oviduct, endometrium and myometrium. The mRNA and the protein were highest and increased throughout the gestation period in R5 caruncle. In addtion, the studies in R1 and R5 genotypes sheep showed no relationship between PRNP genotypes and the mRNA and protein expression levels in the prenatal foetuses. Since the levels of PRNP mRNA in R5 caruncle were higher than that of R1 caruncle, the PRNP genotype (according to the polymorphisms at codons 136, 154 and 171) may have an effect on the gene expression level in caruncle tissue of ewes.

In the present study, the expressions of PRNP transcripts were observed as early as at the preimplantation developmental stage. The level was increased immediately after fertilisation compared to the oocyte stage in cattle (own unpublished data). These suggest that the minor transcription activity of prion protein occur at zygote stage, and the PrP^C might have role in normal embryo development. The increase of the gene transcript at zygote stage might have resulted from sperm activity, since a C-terminally truncated PrP^C isoform was found in bovine (Shaked et al. 1999) and ovine (Ecroyd et al. 2004) mature sperm by the western immunoblotting; thus, sperm could be one source of PRNP in the zygote. The PRNP mRNA expression profiling should be performed in some additional stages of ovine preimplantation embryos to clarify the minor and major embryonic activation of this gene. The protein expression profiling in those preimplantation embryos, and, moreover, silencing the gene at zygote stage might give us the clues to its physiological role. In addition, PRNP expression both at mRNA and protein levels could be investigated in the earlier stages of before 1 month of age *in vitro* in order to define the exact timing and localisation of PRNP transcriptional and translational activations during the prenatal stage.

Table 15: PRNP mRNA and PrP^C expression in 1- 2- and 3-month-old foetuses

Month of age	Brain	Cotyledon	Spinal cord	Heart	Intestine	Kidney	Liver	Lung	Muscle	Spleen	Vertebral column	Eyes
Ovine PRNP												
-1 month	+	nd	nd	+	nd	+	+	nd	nd	nd	+	+
-2 months	+	+	nd	+	+	+	+	+	+	nd	nd	nd
-3 months	+	+	+	+	+	nd	+	+	+	+	nd	nd
Ovine PrP^C												
-1 month	+	nd	nd	+	nd	+	-	nd	nd	nd	-	-
-2 months	+	+	nd	+	+	+	+	+	+	nd	nd	nd

+ present

- absent

ns = not done

6 Summary

This study was carried out with the main objectives (i) to determine whether the polymorphisms at codons 136, 154 and 171 of PRNP locus affect the gene expression, (ii) to observe the exact timing of PRNP transcript and protein activation, and (iii) to investigate the expression profile of the PRNP in preimplantation embryos, reproductive organs of ewes and various tissues of their foetuses during prenatal stage. To full fill these objectives, sheep with scrapie resistance genotype (R1 group; ARR/ARR) and high susceptible to scrapie genotypes (R5 group; VRQ/ARQ, VRQ/ARH, VRQ/VRQ) were used as an animal model. The samples were immature and mature oocytes, morula stage embryos, 1-month-old foetuses, various tissues such as brain, cotyledon, spinal cord, heart, intestine, liver, lung, muscle and spleen from 3- and 5-month-old foetuses as well as ovary, oviduct, endometrium, myometrium and caruncle from 1, 3 and 5 months pregnant ewes.

For experiments using real time PCR, mRNA has been isolated using oligo (dT)25 attached magnetic beads from three independent pools containing 15 to 20 immature oocytes, or three members of each mature oocytes or embryos. mRNA was reverse transcribed to cDNA with oligo dT(12)N primer. Real time RT-PCR was done using those samples and 1-month-old foetuses (n=2). The results displayed expression of the gene in all samples examined. The relative abundance of the gene transcript as compared to the level of the ovine Histone H2a was higher in immature oocytes and 1-month-old foetuses and lower in mature oocytes and morula stage embryos of both groups ($p \leq 0.05$). Significant effects of the PRNP genotype on the mRNA level were shown in immature oocytes and 1-month-old stages as indicated by the higher levels in R1 samples than those of R5 samples.

To identify the gene transcript and to compare the expression level among tissues, stages and risk groups (n=2 for each), semi-quantitative RT-PCR was performed by using 18S-rRNA as an internal standard. Total RNA was isolated from samples using TRIZOLE reagent and then subjected to DNase digestion. Finally, 1 µg of total RNA was reverse transcribed to cDNA with oligo dT(12)N and random primers. The results revealed that PRNP mRNA was detectable in all maternal and foetal tissues throughout the gestation. The gene mRNA level was highest in caruncle, whereas the levels, which seem to be equal and stable during the gestation, were low in ovary, oviduct,

endometrium and myometrium. Interestingly, while the mRNA level in R1 caruncle was stable through the pregnancy period, the level increased from the 1st to 3rd months, and decreased at the 5th month in R5 caruncle. To confirm these changes, real time PCR was performed. The quantitative expression levels in R1 and R5 caruncle support the results by RT-PCR. Mean and standard error in R1 caruncle at the 1st, 3rd and 5th months of pregnancy were 48.1 ± 19.6, 42.0 ± 6.1 and 26.3 ± 2.3, respectively, and that of R5 caruncle were 17.2 ± 0.6, 44.9 ± 2.6 and 28.7 ± 12.4, respectively.

In both R1 and R5 prenatal foetuses, PRNP transcript levels were high in spinal cord, cotyledon and all regions of the brain such as cerebrum, cerebellum and medulla oblongata. Moderate levels were found in heart, intestine, lung and muscle, and low levels were observed in liver and spleen. The level of the gene transcript from 3 to 5 months of age was increased in spinal cord, but stable in cerebellum, intestine, lung and muscle of both groups. Interestingly, while the level was stable in R1 cerebrum and R1 medulla oblongata, it was changed through the gestation in those of R5 foetuses. Moreover, a differentiation in the gene expression pattern between R1 and R5 foetuses was observed in the heart, liver and spleen. Therefore, real time PCR was used to quantify these differences. The results showed that PRNP levels relative to the levels of 18S-rRNA in R1 cerebrum, medulla oblongata, heart, liver and spleen were 47.9, 41.4, 4.3, 2.2 and 5.5, respectively, at the 3rd month of pregnancy, while 35.1, 50.1, 7.0, 8.0 and 9.4, respectively, at the 5th month. From real time PCR analysis results, it seems likely that PRNP expression level was decreased through the pregnancy period in R1 foetal cerebrum, while it was increased in the rest of tissues. Similar to R5 foetuses, at the 3rd month of pregnancy, PRNP mRNA relative abundance levels in cerebrum and medulla oblongata were higher than those in heart, liver and spleen. The levels at this stage were 32.1, 33.6, 7.3, 2.4 and 4.3, respectively. At the 5th month, the level in R5 medulla oblongata (67.3) was also higher than those of cerebrum (24.6), heart (7.5), liver (2.5) and spleen (7.7).

Since the gene transcript was detected in all tissues of 3-month-old foetuses, RT-PCR was carried out in brain, cotyledon, heart, intestine, kidney, liver, lung and muscle of 2-month-old foetuses of both groups. Results revealed the presence of the gene transcripts in all tissues investigated. The gene expression patterns were in the same trend as that of 3- and 5-month-old foetuses as indicated by the high expression level in brain and

cotyledon, moderate level in heart, intestine, kidney, lung and muscle, and low level in liver.

Western blot was performed in reproductive tissues of 1-month pregnant ewes and those tissues from 2-month-old foetuses to define whether the prion protein (PrP^C) is activated at this stage. The results displayed the immuno reactive bands corresponding to the PrP^C in all tissues investigated of both groups. Interestingly, while the PrP^C level in most tissues correlated well with the level of PRNP transcript, the level of PrP^C in foetal cotyledon was very low and the level in foetal liver was as high as that in brain.

As PRNP transcript and protein were detected in 1-month-old foetuses and ovaries, fluorescent in situ hybridisation (FISH) and immunohistochemistry (IHC) were performed to localise the gene mRNA and protein, respectively, on these samples. FISH was carried out on 7 to 10 µm cryostat sections of snap frozen tissues. A non-radioactive probe with a reporter molecule digoxigenin (DIG) detected by indirect method with the help of tyramide signal amplification (TSA, fluorophore system) was applied in this experiment. The control sections hybridised with sense RNA probe were done in parallel. On the other hand, IHC was performed on 5 to 7 µm cryostat sections with the help of avidin-biotin complex (ABC). The immuno-complexes were visualised using 3-amino-9-ethylcarbazole (AEC) substrate. Sections devoid of specific staining with the PrP antibody were used as control for the IHC. Staining intensity is a function of the enzyme activity and improved sensitivity can be achieved by increasing the number of enzyme molecules bound to the tissue. The precipitation of the tyramide (a phenolic compound) after oxidation and the multiple binding sites between the tetravalent avidin and biotinylated antibodies (bound to the antigen) are ideal for achieving this amplification. Both of FISH and IHC were pre-optimised in adult brains which were done in parallel as positive control through out the experiment. The results showed that both of PRNP mRNA and PrP^C were localised in developing brain, heart and kidney of 1-month-old foetuses of both groups, whereas only the mRNA, but not the PrP^C, was found in liver, vertebral column and dermatome. The gene transcript was also localised in oocytes, granulosa and theca cells, and dispersed in ovarian cortex, medulla and corpus luteum. However, the PrP^C was detected only in the ovarian cortex and medulla of 1-month pregnant ovaries of both groups.

In conclusion, this study is the first report of PRNP mRNA and PrP^C expression in ovine preimplantation embryos and prenatal foetuses. The levels of PRNP transcript in

immature oocytes and 1-month-old foetuses were significantly higher ($p \leq 0.05$) than those in mature oocytes and morula stage embryos. The PRNP mRNA is activated in brain, vertebral column, dermatome, heart, liver and kidney as early as the 1st month of embryogenesis. At this time, PrP^C is produced in the brain, heart and kidney. Furthermore, the protein activation in cotyledon, intestine, liver, lung and muscle occur in between the 1st and 2nd months of foetal development. Throughout the prenatal stages, the mRNA levels were high in spinal cord, cotyledon and all regions of brain of the foetuses. Moderate levels were shown in intestine, lung and muscle, and low levels were detected in liver and spleen. In reproductive organs of ewes, high expression level of the gene transcript was found in caruncle of ewes, while the level in ovary, oviduct, endometrium and myometrium were moderate. With the exception in foetal cotyledon and liver, the level of PrP^C correlates well with the relative mRNA expression level in sheep tissues. The results of this study revealed a correlation between the reported PRNP polymorphisms and the gene mRNA level in immature oocytes and 1-month-old stages. Moreover, it seems likely that these polymorphisms have an effect on the gene expression level in caruncle of ewes.

7 Zusammenfassung

Diese Untersuchung wurde mit den folgenden Hauptgesichtspunkten durchgeführt (i) zur Feststellung der Beeinflussung der Genexpression der Polymorphismen an Codon 136, 154 und 171 des PRNP Genortes, (ii) zur Untersuchung der zeitlichen Regulierung der PRNP Transkripte und der Proteinaktivierung und (iii) zur Untersuchung des Expressionsprofils des PRNP in präimplantativen Embryos, reproduktiven Geweben von Mutterschafen und verschiedenen Geweben ihrer Föten während der pränatalen Stadien. Zur Untersuchung wurden Schafe mit Scrapie resistenten Genotypen (R1 Gruppe; ARR/ARR) und für Scrapie hoch anfällige Genotypen (R5 Gruppe; VRQ/ARQ, VRQ/ARH, VRQ/VRQ) als Tiermodell verwendet. Als Proben wurden unreife und reife Oozyten, Embryonen im Morulastadium, einmonatige Föten, verschiedene Gewebe wie Gehirn, Kotyledonen, Rückenmark, Herz, Darm, Leber, Lunge, Muskel und Milz von drei- und fünfmonatigen Föten ebenso wie Ovar, Eileiter, Endometrium, Myometrium und Karunkel von Mutterschaften im ersten, dritten und fünften Trächtigkeitsmonat verwendet.

Für die Experimente unter Verwendung der Real Time PCR wurde mRNA mit Oligo (dT)25 angehefteten magnetischen Beads aus drei unabhängigen Pools mit 15 bis 20 unreifen Oozyten, oder aus drei Teilen von jeder reifen Oozyte oder jedem Embryo, isoliert. Die mRNA wurde entgegengesetzt mit Oligo dT(12)N Primern zu cDNA transkribiert. Die Real-Time RT-PCR wurde mit diesen Proben und den einmonatigen Föten (n=2) durchgeführt. Die Ergebnisse zeigten eine Expression der Gene in allen untersuchten Proben. Die relative Anwesenheit der Gentranskripte war verglichen zum Level des ovinen Histone H2a in unreifen Oozyten und einmonatigen Föten höher und in reifen Oozyten und Embryonen im Morulastadium beider Gruppen niedriger ($p \leq 0,05$). Signifikante Effekte des PRNP Genotyps auf dem mRNA Level konnten, wie durch das höhere Level in R1 Proben verglichen mit den R5 Proben bereits angedeutet, in den unreifen Oozyten und im Ein-Monats-Stadium gezeigt werden. Zur Identifikation der Gentranskripte und zum Vergleich der Expressionslevel zwischen Geweben, Stadien und Risikogruppen (jeweils n=2), wurde eine semi-quantitative RT-PCR, unter Verwendung von 18S-rRNA als internem Standard, durchgeführt. Die gesamt-RNA wurde mit TRIZOL Reagenz aus den Proben isoliert und ein DNase Verdau durchgeführt. Schließlich wurde 1 µg der gesamten RNA entgegengesetzt mit Oligo

dT(12)N und Random-Primern zu cDNA transkribiert. Das Ergebnis zeigte, dass die PRNP mRNA in allen maternalen und fötalen Geweben während der Trächtigkeit detektierbar ist. Das mRNA Level des Gens war am höchsten im Karunkel, während das Level, welches scheinbar gleichbleibend und stabil während der Trächtigkeit war, im Eierstock, Eileiter, Endometrium und Myometrium niedrig war. Während das mRNA Level im R1 Karunkel während der Trächtigkeit stabil war, erhöhte sich das Level interessanterweise vom ersten bis zum dritten Monat und verringerte sich im fünften Monat im R5 Karunkel. Zur Bestätigung dieser Veränderungen wurde eine Real-Time PCR durchgeführt. Die quantitativen Expressionslevel in R1 und R5 Karunkeln sicherten die Ergebnisse der RT-PCR ab. Die Durchschnitte und Standardfehler in R1 Karunkeln im ersten, dritten und fünften Trächtigkeitsmonat waren jeweils 48,1 ± 19,6; 42,0 ± 6,1 und 26,3 ± 2,3 und in R5 Karunkeln jeweils 17,2 ± 0,6; 44,9 ± 2,6 und 28,7 ± 12,4.

In den beiden R1 und R5 pränatalen Föten waren die PRNP Transkript-Level höher im Rückenmark, Kotyledonen und allen Regionen des Gehirns wie Cerebrum, Cerebellum und Medulla oblongata. Mittlere Level wurden im Herz, Darm, Lunge und Muskel gefunden, niedrige Level wurden in Leber und Milz festgestellt. Die Levels der Gentranskripte im Rückenmark stiegen während des Alters von drei zu fünf Monaten, blieben jedoch in Cerebellum, Darm, Lunge und Muskel beider Gruppen stabil. Während das Level im R1 Cerebrum und R1 Medulla oblongata stabil war, veränderte es sich während der Gestation der R5 Föten. Weiterhin wurde eine Differenzierung der Muster der Genexpression zwischen den R1 und R5 Föten in Herz, Leber und Milz gefunden. Daher wurde eine Real-Time PCR zur Quantifizierung dieser Unterschiede durchgeführt. Die Ergebnisse zeigten, dass die PRNP Level relativ zu den Leveln der 18S-rRNA im R1 Cerebrum, Medulla oblongata, Herz, Leber und Milz im dritten Monat der Trächtigkeit jeweils 47,9; 41,4; 4,3; 2,2 und 5,5 waren, während sie während dem fünften Monat der Trächtigkeit jeweils 35,1; 50,1; 7,0; 8,0 und 9,4 waren. Aus den Ergebnissen der Real-Time PCR Analyse schien es, dass das PRNP Expressionslevel während der Trächtigkeitsperiode im R1 fötalen Cerebrum verringert war, während es im restlichen Gewebe erhöht war. Ähnlich der R5 Föten während des dritten Monats der Trächtigkeit waren die Level der relativen Mengen der PRNP mRNA in Cerebrum und Medulla oblongata höher als die in Herz, Leber und Milz. Die Level dieser Stadien waren jeweils 32,1; 33,6; 7,3; 2,4 und 4,3. Im fünften Monat war das Level in R5

Medulla oblongata (67,3) ebenfalls höher als das von Cerebrum (24,6), Herz (7,5), Leber (2,5) und Milz (7,7).

Da das Gentranskript in allen Geweben des dreimonatigen Fötus detektiert wurde, wurde eine RT-PCR im Gehirn, Kotyledonen, Herz, Darm, Niere, Leber, Lunge und Muskel von zweimonatigen Föten beider Gruppen durchgeführt. Die Ergebnisse zeigten die Präsenz der Gentranskripte in allen untersuchten Geweben. Die Muster der Genexpression waren im gleichen Trend wie die der drei- und fünfmonatigen Föten, wie gezeigt durch höhere Expressionslevel in Gehirn und Kotyledonen, mittlere Level in Herz, Darm, Niere, Lunge und Muskel sowie niedrige Level in der Leber.

Ein Westernblot wurde in den reproduktiven Geweben der im ersten Monat trächtigen Mutterschafe und der Gewebe der zweimonatigen Föten zur Feststellung, ob das Prion-Protein (PrP^C) in diesen Stadien aktiviert ist, durchgeführt. Die Ergebnisse stellten die zu dem PrP^C korrespondierenden immunoreaktiven Banden in allen untersuchten Stadien in beiden Gruppen dar. Während das PrP^C Level in den meisten Geweben gut mit dem Level der PRNP Transkripte korrelierte, war das Level des PrP^C im fötalen Kotyledon sehr niedrig und das Level in der fötalen Leber war ebenso hoch wie das im Gehirn.

Aufgrund der Detektion von PRNP Transkript und Protein in einmonatigen Föten und in Ovarien, wurden fluoreszenz in situ Hybridisierung (FISH) und Immunohistochemie (ICH) zur Lokalisation der mRNA und des Proteins des Gens in diesen Proben durchgeführt. FISH wurde in 7 bis 10 µm Cryostat Schnitten von gefrorenem Gewebe durchgeführt. Eine nicht-radioaktive Probe mit dem Reportermolekül Digoxigenin (DIG), detektiert durch eine indirekte Methode mit Hilfe von Tyramide-Signal Amplifikation (TSA), wurde in diesem Experiment angewandt. Die Kontrollschnitte wurden mit sense RNA Proben parallel hybridisiert. Auf der anderen Seite wurde eine ICH auf 5 bis 7 µm Cryostat-Schnitten mit Hilfe von Avidin-Biotin Komplexen (ABC) durchgeführt. Die Immuno-Komplexe wurden mit 3-Amino-9-Ethylcarbazole (AEC) Substraten visualisiert. Die Schnitte ohne spezifische Färbung mit PrP Antikörpern wurden als Kontrolle für die ICH verwendet. Die Intensität der Färbung ist eine Funktion der Enzymaktivität und eine verbesserte Sensibilität kann durch eine Erhöhung der Anzahl von Enzymmolekülen, welche an das Gewebe binden, erreicht werden. Die Ausfällung der Tyramide (phenolische Komponenten) nach der Oxidation und die multiplen Bindestellen zwischen den tetravalenten Avidin und biotinylierten

Antikörpern (binden zu dem Antigen) sind ideal für das Erreichen dieser Amplifikation. Beide, FISH und ICH, wurden in adulten Gehirnen parallel prä-optimiert und als positive Kontrolle während des Experiments verwendet. Die Ergebnisse zeigten, dass beide PRNP mRNA und PrP^C im sich entwickelnden Gehirn, Herz und Niere der einmonatigen Föten beider Gruppen lokalisiert sind, während nur die mRNA jedoch nicht das PrP^C in Leber, Wirbelsäule und Dermatom gefunden wurde. Das Gentranskript war ebenfalls in Oozyten, Granulosa und Thekazellen lokalisiert sowie in der Ovarrinde, im Ovarmark und im Gelbkörper zu finden. Das PrP^C wurde jedoch nur der Ovarrinde und Mark in den Ovarien der Tiere im ersten Trächtigkeitsmonat in beiden Gruppen festgestellt.

Zusammenfassend kann man sagen, dass diese Untersuchung zum ersten Mal die PRNP mRNA und PrP^C Expression in ovinen Präimplantations-Embryonen und pränatalen Föten zeigt. Die Level der PRNP Transkripte in unreifen Oozyten und einmonatigen Föten waren signifikant höher ($p \leq 0,05$) als die in reifen Oozyten und Embryonen im Morulastadium. Die PRNP mRNA ist im Gehirn, Wirbelsäule, Dermatom, Herz, Leber, Lunge und Niere bereits während des ersten Monats der Embryogenese aktiv. Zu dieser Zeit wird PrP^C im Gehirn, Herz und Niere produziert. Darüberhinaus erfolgt die Proteinaktivierung in Kotyledonen, Darm, Leber, Lunge und Muskel während dem ersten und zweiten Monats der fötalen Entwicklung. Während der pränatalen Stadien sind die mRNA Level im Rückenmark, Kotyledonen und allen Regionen des Gehirns der Föten hoch. Mittlere Level wurden im Darm, Lunge und Muskel nachgewiesen und niedrige Level wurden in Leber und Milz detektiert. In den Reproduktionsorganen der Mutterschafe wurden hohe Expressionslevel im Karunkel der Mutterschafe gefunden, während die Level in Eierstock, Eileiter, Endometrium und Myometrium mittel waren. Mit Ausnahme des fötalen Kotyledon und der Leber korrelierten die Level des PrP^C gut mit dem relativen mRNA Expressionslevel im Gewebe des Schafs. Die Ergebnisse dieser Untersuchung ergaben eine Korrelation zwischen den gezeigten PRNP Polymorphismen und dem mRNA Level des Gens in unreifen Oozyten und einmonatigen Stadien. Darüberhinaus, scheint es gut möglich, dass diese Polymorphismen einen Effekt auf das Level der Genexpression im Karunkel der Mutterschafe haben.

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9 Appendix

Appendix 1: Codon-amino acid abbreviations

Codon	Full Name	Abbreviation (3 Letter)	Abbreviation (1 Letter)
TTT	Phenylalanine	Phe	F
TTC	Phenylalanine	Phe	F
TTA	Leucine	Leu	L
TTG	Leucine	Leu	L
TCT	Serine	Ser	S
TCC	Serine	Ser	S
TCA	Serine	Ser	S
TCG	Serine	Ser	S
TAT	Tyrosine	Tyr	Y
TAC	Tyrosine	Tyr	Y
TAA	Termination (ochre)	Ter	X
TAG	Termination (amber)	Ter	X
TGT	Cysteine	Cys	C
TGC	Cysteine	Cys	C
TGA	Termination (opal or umber)	Ter	X
TGG	Tryptophan	Trp	W
CTT	Leucine	Leu	L
CTC	Leucine	Leu	L
CTA	Leucine	Leu	L
CTG	Leucine	Leu	L
CCT	Proline	Pro	P
CCC	Proline	Pro	P
CCA	Proline	Pro	P
CCG	Proline	Pro	P
CAT	Histidine	His	H
CAC	Histidine	His	H

Appendix 1: Codon-amino acid abbreviations (continued)

Codon	Full Name	Abbreviation (3 Letter)	Abbreviation (1 Letter)
CAA	Glutamine	Gln	Q
CAG	Glutamine	Gln	Q
CGT	Arginine	Arg	R
CGC	Arginine	Arg	R
CGA	Arginine	Arg	R
CGG	Arginine	Arg	R
ATT	Isoleucine	Ile	I
ATC	Isoleucine	Ile	I
ATA	Isoleucine	Ile	I
ATG	Methionine	Met	M
ACT	Threonine	Thr	T
ACC	Threonine	Thr	T
ACA	Threonine	Thr	T
ACG	Threonine	Thr	T
AAT	Asparagine	Asn	N
AAC	Asparagine	Asn	N
AAA	Lysine	Lys	K
AAG	Lysine	Lys	K
AGT	Serine	Ser	S
AGC	Serine	Ser	S
AGA	Arginine	Arg	R
AGG	Arginine	Arg	R
GTT	Valine	Val	V
GTC	Valine	Val	V
GTA	Valine	Val	V
GTG	Valine	Val	V
GCT	Alanine	Ala	A
GCC	Alanine	Ala	A

Appendix 1: Codon-amino acid abbreviations (continued)

Codon	Full Name	Abbreviation (3 Letter)	Abbreviation (1 Letter)
GCA	Alanine	Ala	A
GCG	Alanine	Ala	A
GAT	Aspartate	Asp	D
GAC	Aspartate	Asp	D
GAA	Glutamate	Glu	E
GAG	Glutamate	Glu	E
GGT	Glycine	Gly	G
GGC	Glycine	Gly	G
GGA	Glycine	Gly	G
GGG	Glycine	Gly	G
n/a	Aspartate or Asparagine	n/a	B
n/a	Glutamate or Glutamine	n/a	Z

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