Animal Reproduction Science 114 (2009) 157–166

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Doppel gene polymorphisms in Portuguese sheep breeds: Insights on ram fertility

R.M. Pereira[a,*], P. Mesquita[a], M. Batista[b], M.C. Baptista[a], J.P. Barbas[a], J. Pimenta[a,b], I.C. Santos[d], M.R. Marques[c], M.I. Vasques[a], M. Silva Pereira[a], F. Santos Silva[a], M.C. Oliveira Sousa[a], C.M.G. Fontes[b], A.E.M. Horta[a], J.A.M. Prates[b], C.C. Marques[a]

[a] *L-INIA, Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal*
[b] *Faculdade de Medicina Veterinária - CIISA, Universidade Técnica de Lisboa, Av. da Universidade Técnica, 1300-477 Lisboa, Portugal*
[c] *Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*
[d] *Faculdade de Engenharia de Recursos Naturais, Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal*

ARTICLE INFO

Article history:
Received 22 April 2008
Received in revised form 17 September 2008
Accepted 3 October 2008
Available online 11 October 2008

Keywords:
Prion-like protein Doppel
Ram
Fertility
Polymorphism
Genetic variability

ABSTRACT

Transgenic knockout of the gene encoding the prion-like protein Doppel leads to male infertility in mice. The precise role of Doppel in male fertility is still unclear, but sperm from Doppel-deficient mice appear to be unable to undergo the normal acrosome reaction necessary to penetrate the zona pellucida of the oocyte. The objective of this study was to characterize Doppel (*Prnd*) gene polymorphisms in eight Portuguese sheep breeds and to determine a possible relationship between these polymorphisms and ram fertility. Ovine genomic DNA of 364 animals of different breeds (Bordaleira entre Douro e Minho, Churra Badana, Churra Galega Mirandesa, Churra Mondegueira, Merino da Beira Baixa, Merino Branco, Saloia and Serra da Estrela) were analysed by multiple restriction fragment-single-strand conformation polymorphism (MRF-SSCP). This analysis revealed a synonymous substitution G → A in codon 26 of *Prnd* gene. Churra Galega Mirandesa and Saloia breeds were more polymorphic ($P = 0.005$ and $P = 0.04$, respectively) than the overall population, while Serra da Estrela and Merino Branco animals were less polymorphic ($P = 0.007$ and $P = 0.04$). No polymorphism was found in Churra Mondegueira

* Corresponding author. Tel.: +351 243 767 380; fax: +351 243 767 307.
E-mail address: rosalnp@gmail.com (R.M. Pereira).

0378-4320/$ – see front matter
doi:10.1016/j.anireprosci.2008.10.003

breed. Semen from 11 rams of Churra Galega Mirandesa breed (7 homozygous wildtype GG and 4 heterozygous GA) routinely used in the Portuguese Animal Germoplasm Bank was collected and frozen for fertility tests. A classification function was estimated, using data from post-swim-up semen motility and concentration and Day 6 embryo production rate, allowing the identification of the Doppel homozygous GG genotype with 86.7% of accuracy. This preliminary study detected the presence of only one polymorphism in codon 26 of *Prnd* gene in the Portuguese sheep breeds. In the polymorphic Churra Galega Mirandesa breed, GG genotype could be characterized through a model using three fertility traits, suggesting a relationship with male reproduction. Any future research should investigate not only AA genotype and its influence on ram fertility but also the possible consequences of the European Community selection program to eradicate Scrapie on the Prnd genotypes and indirectly on sheep breed's viability and preservation.

1. Introduction

Prion protein (PrP^C) is critically involved in the transmission and pathogenesis of transmissible spongiform encephalopaties (TSEs) which include scrapie in sheep, variant and sporadic Creutzfeldt-Jakob disease in humans and bovine spongiform encephalopathy in cattle. The normal, cellular PrP^C protein encoded by the prion protein gene (*Prnp*) is required for susceptibility to TSE (Chesebro, 2002). Analysis of *Prnp*-knockout mouse lines suffering from loss of Purkinje cells and ataxia syndromes led to the discovery of another gene, Doppel (an acronym derived from downstream prion protein like), whose locus is located about 16 kb downstream of that of the *Prnp* (Moore et al., 1999). The *Prnd* gene coding for Doppel is thought to result from the ancestral duplication of *Prnp* and is evolutionary conserved from human to sheep and cattle, suggesting an important function for Doppel (Tranulis et al., 2001; Rivera-Milla et al., 2006).

Unlike PrP^C, Doppel is poorly expressed in the brain, and *Prnd*-knockout mice are susceptible to scrapie, implying that Doppel is not needed for prion propagation (Behrens et al., 2001). Nonetheless, the apparent co-regulation of PrP^C and Doppel suggests a functional link between them (Moore et al., 2001).

Interestingly, females lacking Doppel are viable and fertile but males without Doppel are sterile. Male *Prnd*-knockout mice had normal sexual behaviour with reduced or normal sperm concentrations but its spermatozoa (spz) were unable to perform the acrosome reaction and fertilize the oocyte (Behrens et al., 2002; Paisley et al., 2004). Moreover the localization of Doppel on both Sertoli and germ cells (Peoc'h et al., 2002; Espenes et al., 2006) strongly suggests that this protein may play a major role in male fertility.

The European Community has initiated programmes to eradicate Scrapie from its member states and several countries have established breeding programmes to create disease-resistant national flocks with the ram as a major selection target. This allows the introduction of Scrapie-resistant genes into sheep populations within a short period. However, specially in small populations and in breeds with unfavourable ARR allele frequencies, such strategy increases the risk that valuable genetic diversity may be lost due to selective breeding for disease-resistant genotypes (Ehling et al., 2006). In Portugal, the ARR allele is present in all the Portuguese sheep breeds (Gama et al., 2006), although in some of them the frequency is extremely low (0.07 in the Churra Mondegueira breed). Breeding schemes aiming the creation of ARR-homozygous would be possible with intense selection taking for instance at least 11 years in the Churra Mondegueira breed. Nevertheless, this intense selection for the *Prnp* genotype alone would have undesirable consequences in terms of inbreeding, and correlated responses in production and adaptation traits should be evaluated before such a scheme is adopted (Gama et al., 2006). Moreover doubts have been arisen as to whether this program might lead to a blind selection for other genes, specially those related with repro-

duction and/or linked to *Prnp* gene, as the referred *Prnd* (Mesquita et al., 2007a; Lipsky et al., 2008).

The first objective of this study was to characterize *Prnd* gene polymorphisms in 8 Portuguese sheep breeds. Secondly, we aim to determine if there is a relationship between these polymorphisms and ram fertility. Specific functional in vitro assays were designed to test the ability of frozen–thawed spermatozoa from polymorphic rams for the Prnd gene to undergo complex processes such as capacitation, acrosome reaction, oocyte fertilization and embryo development in vitro.

2. Materials and methods

2.1. Animals

A total of 364 animals from eight Portuguese sheep breeds were used in the present study: 50 Bordaleira entre Douro e Minho, 50 Churra Badana, 55 Churra Galega Mirandesa, 19 Churra Mondegueira, 50 Merino da Beira Baixa, 50 Merino Branco, 40 Saloia and 50 Serra da Estrela. These animals were randomly chosen from different flocks at their local regions, all registered in the corresponding flockbook. Some of them belong to the Portuguese Animal Germplasm Bank (BPGA).

2.2. DNA extraction and amplification

DNA was extracted from peripheral blood leukocytes using the Puregene DNA Isolation Kit (Gentra Systems, ref. D5500). The Prnd coding region (located at exon 2) was amplified by PCR using specific primers (DOP1-F: 5′-TCCGACACAATGAGGAAACATCTGGG-3′ and DOP1-R: 5′-TTGATCTCTGTGGCTGCCAACTTGC-3′) designed based on the published ovine Prnd gene sequence (Essalmani et al., 2002). PCR reactions were performed in an UNOII thermocycler (Biometra) according to the following conditions: 50 ng of genomic DNA; 16 pmol of each primer; 1 U Taq DNA polymerase (GE Healthcare, ref. 27-0799-04); 10 mM Tris–HCl, pH 9; 50 mM KCl; 2 mM $MgCl_2$; 200 μM of each dNTP, for a final volume of 25 μL. The amplification included a initial denaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 5 min. The resulting 579 bp amplification fragment was analysed by electrophoresis on a 2% agarose gel containing 0.14 μg mL^{-1} ethidium bromide (Qbiogene, ref. ETBC1001).

2.3. Polymorphism analysis by MRF-SSCP

The amplified fragments were analysed by multiple restriction fragment-single-strand conformation polymorphism (MRF-SSCP): 5–7.5 μL of the amplification products were digested with 3U EcoRV restriction enzyme (GE Healthcare, ref. E1042Y) at 37 °C for 18 h. 22.5 μL of a denaturing solution (containing 95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue) were added to the products of the digestion. The samples were heat-denatured at 95 °C for 5 min, chilled at 0 °C and the total volume was loaded onto an 12% polyacrilamide/TBE gel, with a 2.5% crosslinking, and containing 2.5% glycerol. Each 2 gels were run at 50 W for 9–10 h (6000 Vh) at 20 °C in a DCode TM Universal Mutation Detection System (Bio-Rad), coupled to a refrigeration system. The resulting single-stranded DNA band patterns were detected by silver staining using the PlusOne DNA Silver Staining Kit" (Amersham Biosciences, ref. 17-6000-30).

The region containing the relevant Prnd polymorphism was amplified and sequenced (Applied systems 3730xl and ABI 3700). After identifying the molecular nature and location of the polymorphisms, *Prnd* genotypes were established.

2.4. Semen collection and freezing

Semen was collected from 11 rams of the local sheep breed Churra Galega Mirandesa belonging to the BPGA, by artificial vagina. Only semen with good quality (mass motility >4; individual motility >60%; concentration $>2.5 \times 10^9$ spz mL^{-1}) was refrigerated and frozen in mini-straws (300×10^6 spz)

using a cryoprotective medium (egg yolk 15% and glycerol 6.5%) (Marques et al., 2006). The straws were placed in liquid nitrogen (LN2) vapours during 25 min and then submersed and kept in a LN2 container until the fertility assays were performed.

2.5. Preparation and evaluation of thawed semen

After thawing, sperm motility was immediately examined. Frozen–thawed semen was then incubated at 38.5 °C and 5% CO_2 for 1 h in modified Bracket's medium containing 20% ovine serum for swim-up (Marques et al., 2006). Post-swim-up sperm motility and concentration were determined and aliquots of this semen were used for spz capacitation status evaluation and in vitro fertilization.

2.5.1. Acrosome reaction assay

Acrosome reaction assays were performed on 5 μL aliquots of motile spermatozoa stained using a modified technique described by Perez et al. (1996). Briefly: a chlortetracycline (0.8 mM, Merck ref. K1615512-926) and L-cysteine (7 mM, Merck ref. 1.02838.0025) solution was prepared in a buffer containing 130 mM NaCl and 400 mM Tris. Aliquots (5 μL) of semen were mixed with 5 μL of the staining solution and the process was interrupted after 20 s, using 1 μL of a 12.5% (v/v) glutaraldehyde (Merck ref. 8.20603.0100) solution. Finally, 1 μL of a 0.22 M DABCO (1.4-Diazabicyclo[2.2.2]octane, Merck ref. 8.03456.0100) solution in glycerol was added to prevent fading. Sperm was evaluated under fluorescence within 12 h and spermatozoa classified according to their acrosomal status: non-capacitated, capacitated with intact acrosome and reacted acrosome.

2.5.2. In vitro fertilization

Ovaries collected from sheep at the local slaughterhouse were transported to the laboratory in Dulbecco's phosphate buffer saline (PBS, GibCo, ref. 14040-91) at 37 °C. PBS was supplemented with 0.15% (w/v) of bovine serum albumin (BSA, Fraction V, Sigma, ref. A-7888) and 0.05 mg mL^{-1} of kanamycin (Sigma, ref. K-4000). At the laboratory, the 2–6 mm follicles were aspirated to obtain cumulus–oocyte complexes. These complexes were incubated in maturation medium (TCM-199 Sigma ref. M-4530, 100 μM cysteamine Sigma ref. M-9768, 10 ng mL^{-1} epidermal growth factor Sigma ref. E-4127, 10 μg mL^{-1} estradiol Sigma ref. E-4389 and 10 μL mL^{-1} gentamicin Sigma ref. G-1272) at 39 °C and 5% CO_2 for 22 h. Sheep cumulus enclosed mature oocytes were washed to remove excess cumulus cells and then co-cultured with the spermatozoa (1×10^6 spz mL^{-1}) for 18 h.

Samples of presumptive zygotes (18 h p.i.) were fixed in ethanol:acetic acid prior to staining with 1% aceto-lacmoid to assess fertilization. Oocytes were considered to be fertilized according to the observation, under a light microscope, of a decondensed sperm head, pro-nuclei or zygotes (sincariosis, 1 or 2 nucleus). Polyspermy was defined by the presence of more than two swollen sperm heads, or more than two pronuclei within a single oocyte.

2.5.3. Embryo culture

After fertilization, presumptive zygotes were denuded and cultured in droplets (25 μL) of synthetic oviductal fluid (SOF) enriched with aminoacids (20 μL mL^{-1} BME Sigma M-7145, 10 μL mL^{-1} MEN Sigma B-6766) and bovine serum albumin (6 mg mL^{-1} BSA, Sigma ref. A-7888) at 38.5 °C, under 5% O_2, 5% CO_2 and 90% N_2 in an humidified atmosphere until the stage of 2-4-8 cell embryos. After assessing cleavage, embryo development proceeded until the blastocyst stage in SOF + BSA and 10% FCS.

Cleavage rate was calculated as the number of cleaved embryos per number of inseminated oocytes and D6 embryo rate as the number of morulae and blastocyts at that day per number of cleaved embryos.

2.6. Statistical analysis

Genotypic and allele frequencies were calculated and χ^2 tests were performed for each breed to evaluate possible deviations from Hardy–Weinberg equilibrium, using the Genepop software Version 4.0 (Rousset, 2008).

Data representing 15 and 12 replicates for ram fertility assays of homozygous and heterozygous *Prnd* genotypes, respectively, are expressed as means ± standard error of means (S.E.M.). Each ram was tested three times. In each session, two rams of different *Prnd* genotypes were run simultaneously. After two sessions the two homozygous rams (GG) from the previous sessions were tested together.

The association between *Prnd* genotypes and ram fertility was assessed in two different ways. Firstly, an univariate analysis was performed. Means were compared by analysis of variance considering sessions as a block factor and *Prnd* genotype as fixed treatment effect. Secondly, a stepwise discriminant analysis was performed with all variables to verify the existence of linear discriminations between *Prnd* genotypes and to find a classification function. The variables semen post-thawed motility and capacitation status, fertilization and cleavage rates were eliminated from the model.

Differences were considered significant when $P \leq 0.05$ (Statsoft Inc, 1995).

3. Results

3.1. Polymorphism analysis by MRF-SSCP

The PCR fragment of the *Prnd* gene exon 2 analysed by MRF-SSCP was found to be polymorphic in all but not in Churra Mondegueira breed. Under the established conditions, three SSCP patterns were detected (Fig. 1). Sequencing results of the three SSCP patterns were consistent with three genotypes (GG, AG and AA) which resulted from a synonymous substitution G → A in the codon 26 of the *Prnd* gene.

The results of genotype and allele frequencies obtained after MRF-SSCP analysis of the 364 animals from eight Portuguese sheep breeds are represented in Table 1. The frequency of allele A was higher in Churra Galega Mirandesa than in Bordaleira entre Douro e Minho ($P = 0.009$), Merino Branco ($P = 0.001$), Merino da Beira Baixa ($P = 0.02$), Churra Mondegueira ($P = 0.007$) and Serra da Estrela ($P = 0.0001$), but similar ($P > 0.05$) to the frequency observed in Saloia and Churra Badana breeds. This frequency was also higher in Churra Badana and Saloia than in Merino Branco ($P = 0.02$ and $P = 0.003$, respectively),

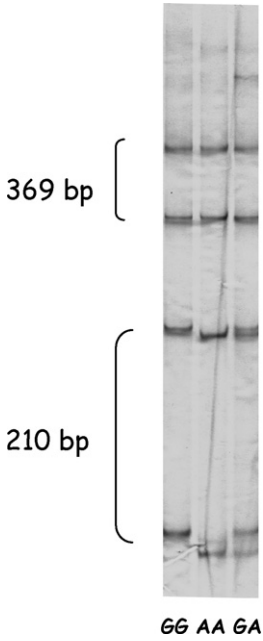

Fig. 1. Single-strand conformation polymorphism of the ovine *Prnd* gene. Three unique SSCP patterns, GG, GA and AA, were detected in this study.

Table 1
Genotype and allele frequencies in the codon 26 of the *Prnd* gene in 8 Portuguese sheep breeds.

Breed	*n*	Genotype frequencies				Allele frequencies		
		GG	GA	AA	χ^2	G	A	χ^2
Bordaleira entre Douro e Minho	50	0.940	0.040	0.020	bc	0.960	0.040	cd
Churra Badana	50	0.800	0.180	0.020	ab	0.890	0.110	abc
Churra Galega Mirandesa	55	0.727	0.255	0.018	a	0.855	0.145	a
Merino Branco	50	0.960	0.040	0.000	c	0.980	0.020	d
Merino da Beira Baixa	50	0.900	0.100	0.000	bc	0.950	0.050	bcd
Churra Mondegueira	19	1.000	0.000	0.000	c	1.000	0.000	d
Saloia	40	0.725	0.275	0.000	a	0.863	0.138	ab
Serra da Estrela	50	0.980	0.020	0.000	c	0.990	0.010	d
Total	364	0.871	0.121	0.008		0.931	0.069	

n, Number of animals; χ^2 test: breeds with different letters are statistically different ($P \leq 0.05$).

Churra Mondegueira ($P = 0.03$ and $P = 0.01$, respectively) and Serra da Estrela ($P = 0.003$ and $P = 0.001$, respectively). Moreover, Saloia breed presented also a higher frequency of allele A than Bordaleira entre Douro e Minho ($P = 0.03$).

When compared with the total population, Churra Galega Mirandesa and Saloia breeds had the highest prevalence of heterozygous ($P = 0.005$ and $P = 0.04$, respectively) and Serra da Estrela and Merino Branco animals were the least polymorphic ($P = 0.007$ and $P = 0.04$, respectively). Churra Mondegueira breed was monomorphic. Homozygous AA animals were found only in Bordaleira entre Douro e Minho, Churra Badana and Churra Galega Mirandesa breeds in frequencies equal or lower than 2.0%. The eight breeds and the overall population were found in Hardy–Weinberg equilibrium ($\chi^2 = 7.171$; df = 12; $P = 0.846$).

Churra Galega Mirandesa rams were chosen for fertility tests due to their highest frequency of heterozygous (25.5%). From the 11 Churra Galega Mirandesa rams belonging to BPGA and trained for semen collection by artificial vagina, 7 were homozygous (GG) and 4 heterozygous (GA) in codon 26 of *Prnd* gene. None of the BPGA rams were AA homozygous.

3.2. Fertility tests

Two Churra Galega Mirandesa rams both homozygous GG in codon 26 of *Prnd* gene were discarded because one was azoospermic and the other presented semen without enough quality for cryopreservation.

No significant differences were observed either for post-thawed and post-swim-up sperm concentration or capacitation status or fertilization rates between homozygous (GG) and heterozygous (GA) Churra Galega Mirandesa rams (Tables 2 and 3). Neither were any significant differences found for embryo production rates (Table 3). Nevertheless using data from post-swim-up semen motility (variable *a*) and concentration (variable *b*) and Day 6 embryo production rates (variable *c*) it was possible to discriminate rams from the Prnd genotypes through a stepwise discriminant analysis. A classification function was estimated ($y = -0.713a - 0.674b + 0.869c$) allowing an identification accuracy of 86.7% for GG and 58.3% for GA genotypes (Table 4).

Table 2
Post-thawed sperm quality parameters evaluation of Churra Galega Mirandesa Portuguese sheep breed according to Doppel genotype (homozygous GG or heterozygous GA in the codon 26 of Prnd gene).

Ram	*n*	Thawed	Post-swim-up		Capactitation status		
		Motility (%)	Motility (%)	Conc. ($\times 10^6$ mL^{-1})	NCap (%)	Cap (%)	AR (%)
GG	15	42.7 ± 2.1	49.0 ± 2.3	16.1 ± 3.4	52.8 ± 2.8	21.7 ± 1.9	26.2 ± 2.6
GA	12	44.6 ± 3.1	52.9 ± 4.2	21.5 ± 7.1	52.4 ± 3.1	20.0 ± 2.6	27.4 ± 3.4

Motility, individual spermatozoa motility; conc., spermatozoa concentration; NCap, non-capacitated spermatozoa; Cap, capacitated spermatozoa with intact acrosome; AR, acrosome reacted spermatozoa.

Table 3
IVF parameters evaluation of Churra Galega Mirandesa Portuguese sheep breed according to Doppel genotype (homozygous GG or heterozygous GA in the codon 26 of Prnd gene).

Ram	Fertilization[a]			Embryo production		
	Inseminated oocytes	Fertilization (%)	Polyspermic (%)	Inseminated oocytes	Cleavage (%)	D6 embryos (%)
GG	198	63.3 ± 3.3	3.5 ± 1.3	674	52.8 ± 2.8	21.7 ± 1.9
GA	139	54.4 ± 5.5	1.4 ± 0.7	554	52.4 ± 3.1	20.0 ± 2.6

[a] Oocytes fixed prior to staining with aceto-lacmoid 18 h post-insemination.

Table 4
Number of rams in which Doppel genotypes in the codon 26 were correctly or incorrectly assigned on the basis of discriminant function of post-swim-up semen motility and concentration and Day 6 embryo production rates (classification matrix).

Ram genotype	Correct classification (%)	Assigned genotype	
		GG	GA
GG	86.7	13	2
GA	58.3	5	7
Total	74.1	18	9

4. Discussion

Present results showed that the examined 8 Portuguese sheep breeds are poorly polymorphic for *Prnd* gene. The only identified polymorphism was a synonymous substitution G → A in codon 26 of the *Prnd* gene. Churra Badana, Churra Galega Mirandesa and Saloia breeds were characterized by a higher frequency of the rare allele A for this polymorphism, while Serra da Estrela and Merino Branco breeds were less polymorphic. Churra Mondegueira breed seems to be monomorphic. Regarding geographic/origin of the breeds, Serra da Estrela have frequencies between Merino Branco and Churra Mondegueira, the two breeds from which Serra da Estrela arises.

As referred, the discovery of Doppel gene in mice by Moore et al. (1999) resulted from the analysis of *Prnp*-knockout mouse lines that suffered from loss of Purkinje cells and ataxia syndromes. Later on, the isolation and structural organization of the bovine and ovine *Prnd* genes were achieved by Comincini et al. (2001). *Prnd* maps to the same chromosomal region as *Prnp*, namely OA13q17/18 and three polymorphisms (R50H, N110H and R132Q) were revealed in the cattle *Prnd* coding region. In sheep only two synonymous substitutions (I12I, A26A) were found.

It is well established that the ovine PrP^C coding region is highly polymorphic and that some polymorphisms, namely those at codons 136, 154 and 171, strongly affect scrapie susceptibility (Baylis and Goldmann, 2004). As shown here, the situation with *Prnd* appears different because we only detected a synonymous substitution. The same occurred in Spain with Alvarez et al. (2006). Interestingly, goats are clearly differentiated from sheep. Goats are less polymorphic at the codons of *Prnp* gene that modulate the susceptibility/resistance to scrapie in sheep (Vaccari et al., 2006) but previous studies from our laboratory (Mesquita et al., 2007b) and from Uboldi et al. (2005) identified several synonymous and non-synonymous substitutions in *Prnd* gene in Portuguese and Italian goats. None of these polymorphisms were significantly relatable to TSE status. Also in bovine and ovine the detected Doppel gene polymorphisms did not show any clear association with BSE or scrapie diseases (Comincini et al., 2001).

Like PrP^C, Doppel is a GPI-anchored glycoprotein structured by three α-helices and two β-sheets, yet it has only ~25% aminoacids similarity to PrP^C and lacks the distinctive PrP^C repeats and hydrophobic domain (Silverman et al., 2000). Because its coding locus, *Prnd* lies adjacent to *Prnp*, it was proposed that the two genes are ancient duplicates (Tranulis et al., 2001; Rivera-Milla et al., 2006). However *Prnp* and *Prnd* have different expression patterns, suggesting that the gene products exhibit different biological functions. Whereas PrP^C is an ubiquitous glycoprotein produced in large amounts in the neurons (Kretzschmar et al., 1986), Doppel seems to be tissue specific in mice, sheep and cattle. Unlike PrP^C, Doppel gene transcripts are present only in very small amounts in the brain and are principally found in heart and testis (Moore et al., 1999; Tranulis et al., 2001). Doppel is expressed on both Sertoli and germ cells in mice, rats, swine (Behrens et al., 2002; Serres et al., 2006), humans (Peoc'h et al.,

2002), ovine (Espenes et al., 2006) and bovine (Rondena et al., 2005) where it appears to be involved in male fertility. These authors identified Doppel expression in all the developing stages of germinal cells. However, whereas Doppel is permanently expressed in the Sertoli cells, its expression in the testicular germ cells varies according to species, especially in the ejaculated spermatozoa where its detection was not always possible due to Doppel transient expression (Espenes et al., 2006; Serres et al., 2006).

Moreover, studies with *Prnd*−/− mouse lines showed that males without Doppel are sterile. *Prnd*-knockout mice had normal sexual behaviour with reduced or normal sperm concentrations but its spermatozoa were unable to perform the acrosome reaction and fertilize the oocyte (Behrens et al., 2002; Paisley et al., 2004).

Post-thawed semen quality assessed by general characteristics of spermatozoa, such as sperm motility and sperm morphology, is not always significantly correlated with fertility, especially if it lies within accepted ranges of normality (Morris et al., 2001) as we could expect. Therefore, specific functional in vitro assays were designed to disclose the ability of frozen–thawed ram spermatozoa to undergo complicated processes such as capacitation, acrosome reaction, fertilization of oocytes and embryo development in vitro. Although no differences were found for each fertility test between homozygous (GG) and heterozygous (GA) in codon 26 of *Prnd* gene rams, a discriminant analysis, using data from post-swim-up semen motility and concentration and Day 6 embryo production rate, correctly identified genotype GG in 86.7% of the rams. GG rams are a homogeneous group. In contrast, GA genotype was incorrectly assigned in 41.7% of rams, probably due to their heterozygosity, needing a comparison with both GG and AA rams and the introduction of new fertility traits as variables to optimize the classification equation. Unfortunately none of the rams belonging to the BPGA and trained for semen collection were homozygous AA. We intend to keep looking for this Doppel genotype in the Portuguese sheep breeds to confirm our previous results and help to clarify the importance of the identified polymorphisms in ram reproduction.

Notwithstanding the knowledge that synonymous single-nucleotide polymorphisms (SNP) do not produce altered coding sequences, and therefore they are not expected to change the function of the protein in which they occur, recent studies have indicated that both synonymous and nonsynonymous SNP can affect mRNA stability, mRNA processing, and mRNA maturation, thereby affecting allelic mRNA expression. In fact SNP which are considered "silent" can affect protein folding and its activity/substrate specificity (Kimchi-Sarfaty et al., 2007). For instance, some synonymous mutations in the human dopamine receptor D2 (DRD2) have functional effects. 957T, rather than being "silent", altered the predicted mRNA folding, led to a decrease in mRNA stability and translation, and dramatically changed dopamine-induced up-regulation of DRD2 expression (Duan et al., 2003). A synonymous SNP in the Multidrug Resistance 1 gene results in a gene product P-glycoprotein (P-gp) with altered drug and inhibitor interactions (Kimchi-Sarfaty et al., 2007). These authors reported similar mRNA and protein levels, but altered conformation for the wildtype and polymorphic P-gp. Also recent studies from Carcangiu et al. (in press) found a relationship between the synonymous melatonin receptor 1a gene polymorphism and seasonal reproduction in different Italian goat breeds. Although the identified polymorphism did not induce any aminoacid change, the R/r genotype was present only in Sarda breed and in these animals the reproduction activity was strongly ($P < 0.001$) influenced by photoperiod. Doppel expression studies in the testicular tissue of polymorphic rams could help to establish the spatial expression profile and the function/s of this gene/protein.

In conclusion, we can state that the polymorphism in codon 26 of *Prnd* gene was the only one detected in the Portuguese sheep breeds, under the MRF-SSCP conditions used in this work. In the polymorphic Churra Galega Mirandesa breed, GG genotype could be characterized through a model using three fertility traits, suggesting a relationship with male reproduction. Any future research should investigate not only AA genotype and its influence on ram fertility but also the possible consequences of the European Community selection program to eradicate Scrapie on the Prnd genotypes and indirectly on sheep breed's viability and preservation.

Acknowledgements

The authors express their gratitude to Fundação para a Ciência e Tecnologia - POCI/CVT/57148 and SFRH/BPD/26510/2005 grants and AGRO 438 for supporting this work.

References

Alvarez, L., Fuertes, M., San Primitivo, F., Garcia-Marin, F., Arranz, J.J., 2006. Variabilidad genética en la región codificante del gen Doppel (PRND) en ovejas afectadas de scrapie en Castilla y León. XIII Reunion Nacional de Mejora Genética Animal, 28–30 de Junio, Gijon-Spain (http://www.dcam.upv.es/acteon/CONGRESOS/XIIIReunionMGGIJON/DocsXIII/LAlvarez_XIII.pdf).

Baylis, M., Goldmann, W., 2004. The genetics of scrapie in sheep and goats. Curr. Mol. Med. 4, 385–396.

Behrens, A., Brandner, S., Genoud, N., Aguzzi, A., 2001. Normal neurogenesis and scrapie pathogenesis in neural grafts lacking the prion protein homologue Doppel. EMBO Rep. 2, 347–352.

Behrens, A., Genoud, N., Naumann, H., Rülicke, T., Janett, F., Heppner, F.L., Ledermann, B., Aguzzi, A., 2002. Absence of the prion protein homologue Doppel causes male sterility. EMBO J. 21, 3652–3658.

Carcangiu, V., Vacca, G.M., Mura, M.C., Dettori, M.L., Pazzola, M., Luridiana, S., Bini, P.P., in press. Relationship between MTNR1A melatonin receptor gene polymorphism and seasonal reproduction in different goat breeds. Anim. Reprod. Sci., doi:10.1016/j.animreprosci.2007.12.014.

Chesebro, B., 2002. Grand ideas floating freely. In: Conference of the new prion biology: basic science, diagnosis and therapy. Embo Rep. 3, pp. 1123–1126.

Comincini, S., Foti, M.G., Tranulis, M.A., Hills, D., Di Guardo, G., Vaccari, G., Williams, J.L., Harbitz, I., Ferretti, L., 2001. Genomic organization, comparative analysis, and genetic polymorphisms of the bovine and ovine prion Doppel genes (PRND). Mamm. Genome 12, 729–733.

Duan, J., Wainwright, M.S., Comeron, J.M., Saitou, N., Alan, R., Sanders, A.R., Gelernter, J., Gejman, P.V., 2003. Synonymous mutations in the human dopamine receptor D2 (DRD2) affect mRNA stability and synthesis of the receptor. Hum. Mol. Genet. 12, 205–216.

Ehling, C., Rath, D., Struckmann, C., Frenzel, A., Schindler, L., Niemann, H., 2006. Utilization of frozen–thawed epididymal ram semen to preserve genetic diversity in Scrapie susceptible sheep breeds. Theriogenology 66, 2160–2164.

Espenes, A., Harbitz, I., Skogtvedt, S., Fuglestveit, R., Berg, K.A., Dick, G., Krogenaes, A., Tranuli, M.A., 2006. Dynamic expression of the prion-like protein Doppel in ovine testicular tissue. Int. J. Androl. 29, 400–408.

Essalmani, R., Taourit, S., Besnard, N., Vilotte, J.L., 2002. Sequence determination and expression of the ovine Doppel-encoding gene in transgenic mice. Gene 285, 287–290.

Gama, L.T., Carolino, M.I., Santos-Silva, M.F., Pimenta, J., Costa, M.S., 2006. Prion protein genetic polymorphisms and breeding strategies in Portuguese breeds of sheep. Livest. Sci. 99, 175–184.

Kimchi-Sarfaty, C.A., Jung, M.I., Kim, I., Sauna, Z.E., Calcagno, A.M., Ambudkar, S., Gottesman, M.M., 2007. "Silent" polymorphism in the MDR1 gene changes substrate specificity. Science 315, 525–528.

Kretzschmar, H.A., Stowring, L.E., Westaway, D., Stubblebine, W.H., Prusiner, S.B., Dearmond, S.J., 1986. Molecular cloning of a human prion protein Cdna. DNA 5, 315–324.

Lipsky, S., Brandt, H., Lühken, G., Erhardt, G., 2008. Analysis of prion protein genotypes in relation to reproduction traits in local and cosmopolitan German sheep breeds. Anim. Reprod. Sci. 103, 69–77.

Marques, C.C., Barbas, J.P., Baptista, M.C., Vasques, M.I., Pereira, R.M., Cavaco-Gonçalves, S., Mascarenhas, R.M., Horta, A.E.M., 2006. Reproduction in the local ovine Saloia breed—results on seasonal and individual factors affecting fresh and frozen semen performance, in vivo and in vitro fertility. In: Ramalho Ribeiro, J., Horta, A.E.M., Mosconi, C., Rosati, A. (Eds.), Animal Products from Mediterranean Area. EAAP Publications, pp. 325–330.

Mesquita, P., Batista, M., Marques, M.R., Santos, I.C., Pimenta, J., Santos Silva, F., Sousa, C.O., Carolino, M.I., Horta, A.E.M., Prates, J.A., Pereira, R.M., 2007a. Doppel gene polymorphisms and their relation to scrapie susceptibility in Portuguese sheep breeds. In: EAAP (Ed.), Proceedings of 58th Annual Meeting of the European Association for Animal Production. Dublin Ireland, August 26–29, p. 71.

Mesquita, P., Batista, M., Marques, M.R., Santos, I.C., Pimenta, J., Santos Silva, F., Sousa, C.O., Horta, A.E.M., Prates, J.A., Pereira, R.M., 2007b. Doppel gene polymorphisms: differences between Portuguese goat and sheep breeds. In: SPRA (Ed.), Proceedings of VI Symposium of the Portuguese Animal Reproduction Society. Évora, March 16–18, pp. 66–67.

Moore, R.C., Lee, I.Y., Silverman, G.L., Harrison, P.M., Strome, R., Heinrich, C., Karunaratne, A., Pasternak, S.H., Chishti, M.A., Liang, Y., Mastrangelo, Wang, K., Smit, A.F., Katamine, S., Carlson, G.A., Cohen, F.E., Prusiner, S.B., Melton, D.W., Tremblay, P., Hood, L.E., Westaway, D., 1999. Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein Doppel. J. Mol. Biol. 292, 797–817.

Moore, R.C., Mastrangelo, P., Bouzamondo, E., Heinrich, C., Legname, G., Prusiner, S.B., Hood, L., Westaway, D., DeArmond, S.J., Tremblay, P., 2001. Doppel-induced cerebellar degeneration in transgenic mice. Proc. Natl. Acad. Sci. U.S.A. 98, 15288–15293.

Morris, L.H.A., Johnson, W.H., Leibo, S.P., Buckrell, B.C., 2001. Relationship between the characteristics of frozen–thawed ram spermatozoa and in vitro embryo production. Reprod. Fertil. Dev. 13, 193–201.

Paisley, D., Banks, S., Selfridge, J., McLennan, N.F., Ritchie, A.M., McEwan, C., Irvine, D.S., Saunders, P.T., Manson, J.C., Melton, D.W., 2004. Male infertility and DNA damage in Doppel knockout and prion protein/Doppel double-knockout mice. Am. J. Pathol. 164, 2279–2288.

Peoc'h, K., Serres, C., Frobert, Y., Martin, C., Lehmann, S., Chasseigneauc, S., Sazdovitch, V., Grassi, J., Jouannet, P., Launay, J., Laplanche, J., 2002. The human "prion-like" protein Doppel is expressed in both Sertoli cells and spermatozoa. J. Biol. Chem. 277, 43071–43078.

Perez, L.J., Valcarcel, A., de las Heras, M.A., Moses, D.F., Baldassarre, H., 1996. In vitro capacitation and induction of acrosomal exocytosis in ram spermatozoa as assessed by the chlortetracycline assay. Theriogenology 45, 1037–1046.

Rivera-Milla, E., Oidtmann, B., Panagiotidis, C.H., Baier, M., Sklaviadis, T., Hoffmann, R., Zhou, Y., Solis, G.P., Stuermer, C.A., Málaga-Trillo, E., 2006. Disparate evolution of prion protein domains and the distinct origin of Doppel- and prion-related loci revealed by fish-to-mammal comparisons. FASEB J. 20, 317–319.

Rondena, M., Ceciliania, F., Comazzia, S., Pocacquaa, V., Bazzocchia, C., Luvonib, C., Chigionib, S., Paltrinieri, S., 2005. Identification of bovine Doppel protein in testis, ovary and ejaculated spermatozoa. Theriogenology 63, 1195–1206.

Rousset, F., 2008. Genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Mol. Ecol. Resour. 8, 103–106.

Statsoft Inc, 1995. STATISTICA for Windows [Computer program manual]. Statsoft Inc., Tulsa, OK.
Serres, C., Peoc'h, K., Courtot, A.M., Lesaffre, C., Jouannet, P., Laplanche, J.L., 2006. Spatio-developmental distribution of the prion-like protein Doppel in mammalian testis: a comparative analysis focusing on its presence in the acrosome of spermatids. Biol. Reprod. 74, 816–823.
Silverman, G.L., Qin, K., Moore, R.C., Yang, Y., Mastrangelo, P., Tremblay, P., Prusiner, S.B., Cohen, F.E., Westaway, D., 2000. Doppel is an *N*-glycosylated, glycosylphosphatidylinositol-anchored protein. Expression in testis and ectopic production in the brains of Prnp(0/0) mice predisposed to Purkinje cell loss. J. Biol. Chem. 275, 26834–32641.
Tranulis, M.A., Espenes, A., Comincini, S., Skretting, G., Harbitz, I., 2001. The PrP-like protein Doppel gene in sheep and cattle: cDNA sequence and expression. Mamm. Genome 12, 376–379.
Uboldi, C., Del Vecchio, I., Foti, M.G., Azzalin, A., Paulis, M., Raimondi, E., Vaccari, G., Agrimi, U., Di Guardo, G., Comincini, S., Ferretti, L., 2005. Prion-like Doppel gene (PRND) in the goat: genomic structure, cDNA, and polymorphisms. Mamm. Genome 16, 963–971.
Vaccari, G., Di Bari, M.A., Morelli, L., Nonno, R., Chiappini, B., Antonucci, G., Marcon, S., Esposito, E., Fazzi, P., Palazzini, N., Troiano, P., Petrella, A., Di Guardo, G., Agrimi, U., 2006. Identification of an allelic variant of the goat PrP gene associated with resistance to scrapie. J. Gen. Virol. 87, 1395–1402.