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Post-transcriptional silencing of *Bos taurus* prion family genes and its impact on granulosa cell steroidogenesis

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ABSTRACT

Prion proteins constitute a major public health concern, which has partly overshadowed their physiological roles in several scenarios. Indeed, these proteins were implicated in male fertility but their role in female fertility is relatively less explored. This study was designed to evaluate the role of *SPRN* and *PRNP* prion family genes in bovine follicular steroidogenesis pathways. Post-transcriptional *SPRN* and *PRNP* silencing with siRNAs was established in bovine granulosa cell (GC) *in vitro* culture, and gene expression and progesterone and estradiol concentrations were evaluated. *SPRN* knockdown, led to a down-regulation of CYP11A1 mRNA levels (2.1-fold), and *PRNP* knockdown led to an upregulation of SPRN mRNA levels (2.3-fold). *CYP19A1* expression and estradiol synthesis was not detected in any experimental group. Finally, *SPRN* knockdown led to a mild reduction in progesterone production in GCs and this was the only experimental group that did not exhibit an increment in progesterone levels after 48 h of culture. As a conclusion, it was possible to detect the expression of the *SPRN* gene in bovine GCs, a potential interaction between *SPRN* and *PRNP* regulation, and the impact of *SPRN* expression on *CYP11A1* and progesterone levels. These findings bring new insights into the role of these genes in ovarian steroidogenesis and female reproductive physiology.

1. Introduction

The intriguing and poorly understood role of prion family genes and proteins in reproductive physiology has captured the attention of scientists for decades. Previous research highlighted the role of prion and prion-like genes and proteins in spermatogenesis, sperm motility, zona binding and fertilization [1–9]. The prion gene family comprises four genes: *PRNP*, encoding the cellular prion protein (PrP^C), *PRND* (prion like protein doppel gene), encoding a prion-like protein designated Doppel (Dpl), *SPRN* (shadow of prion protein gene) encoding the Sho (or Shadoo) protein, and *PRNT* (prion protein testis-specific gene), encoding the Prt protein. The PrP^C, Dpl and Sho proteins share structural similarities, and the *SPRN* gene, known to be also expressed in the central nervous system, may be evolutionarily more ancient than *PRNP* [10].

Knowledge on the role of prion gene family in female reproductive biology is even scarcer than in the male. The mammalian ovaries contain many follicles, each one consisting of an oocyte surrounded by granulosa cells (GCs), which are essential somatic cells involved in follicle development, supporting the developing oocyte, proliferating and producing sex steroids and growth factors [11]. In the cow, *PRNP* is expressed in the theca and granulosa cells

Abbreviations: CYP11A1, cytochrome P450 family 11 subfamily A member 1; CYP19A1, cytochrome P450 family 19 subfamily A member 1; E2, 17β-estradiol; GCs, granulosa cells; P4, progesterone; *PRNP*, prion protein gene; *SPRN*, shadow of prion protein gene.

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of ovarian follicles, notably in developing follicles, suggesting a link to the growth of dominant follicles [12]. The *PRNP* encoded protein was detected in the ovary, oviduct, and uterus of pregnant and cyclic ewes [13,14] and is highly expressed in the human placenta [15]. Transgenic reporter mice for *SPRN* showed *SPRN*-LacZ specific expression of the transgene in granulosa cells of ovarian developing follicles, suggesting a role for Shadoo in female reproductive physiology [16]. This expression pattern of two prion family genes in ovarian follicular cells prompts the investigation of its relationship with ovarian steroidogenesis, which is a key regulator of reproductive function.

In the ovary, follicular growth and maturation is triggered by the interaction of gonadotropins and autocrine/paracrine factors. Pituitary follicle-stimulating hormone (FSH) binds to receptors on granulosa cells and stimulates the expression of enzymes responsible for estrogen synthesis contributing to oogenesis and follicular development, namely cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*), also known as cytochrome P-450scc, which promotes cholesterol side chain cleavage, and cytochrome P450 family 19 subfamily A member 1 (CYP19A1), which is responsible for aromatizing and cleaving the methyl group of testosterone to synthesize estradiol [17]. Recent studies also reported an interaction between Growth Hormone/Insulin-like growth factor 1 (GH/IGF-I), androgen and ovarian bone morphogenetic protein (BMP) in the regulation of granulosa cell steroidogenesis [18–20]. Moreover, the involvement of the transforming growth factor (TGF) family, Notch and Hippo signaling pathways in the control of granulosa cell proliferation and steroidogenesis was recently reported [18,21]. This reveals a complex and intricate steroidogenic regulatory mechanism involving several players, which is still not fully understood.

One of the most direct ways to find out the phenotype associated to a gene is to evaluate the impact of abrogating its expression. Given the difficulty of applying gene knockout technologies to species other than mice, gene silencing techniques have been widely used as valuable tools to investigate gene function and identify potential therapeutic targets [22].

Herein, we evaluated the putative regulatory role of *SPRN* and *PRNP* prion family genes, in bovine follicular granulosa cell steroidogenesis, by conducting their post-transcriptional gene silencing and quantifying the expression of two main steroidogenic genes, *CYP11A1* and *CYP19A1*. The concentrations of progesterone (P4) and 17β-estradiol (E2), the end-products of these biosynthetic steroidogenic pathways, were also evaluated.

2. Materials and methods

2.1. Establishment of bovine granulosa cell cultures

Bovine ovaries were collected *postmortem* in a slaughterhouse and transported to the laboratory in phosphate buffer saline (PBS) solution supplemented with kanamycin (Sigma-Aldrich, USA). Granulosa cells were obtained from the follicular fluid aspirated from follicles with 2–9 mm of diameter, as previously described [23]. Each session, of a total of three, consisted of a pool of ovarian cells from 30 cows. Briefly, the follicular fluid was centrifuged at 200×*g* for 5 min and the concentration of viable cells was determined using a hemocytometer, following cell staining with Tripan Blue (0.4% w/v). The viable cell number was then adjusted to 5×10^5 cells/well in M199 culture medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich, USA) and antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin; Sigma-Aldrich, USA) in 6-well cell culture plates (Nunc®, Denmark), allowing the formation of an 80%–90% confluent monolayer, before performing the silencing experiments. The cell culture supernatants were collected 48 h after siRNA transfection and freshly used for LDH enzymatic activity measurement and stored at −20 °C until progesterone (P4) and 17β-estradiol (E2) measurement assays.

2.2. SPRN and PRNP siRNA design and transfection

The siRNA target sites were selected from *Bos taurus* SPRN (NM_001080321.1) and PRNP [consensus sequence from the transcript variants 1 (NM_001271626.2), 2 (NM_181015.3), and 3 (NM_001271625.2)] mRNA sequences. The siRNAs were designed and synthesized by Dharmacon (GE Healthcare, USA) and the respective sequences are presented in the Supplementary Table 1. The control siRNA used as a negative control was obtained from Dharmacon (GFP Duplex I, P-002048-01-20).

For the siRNAs silencing experiments, 125 pmol/well of the respective siRNA was transfected with 12.5 μL/well DharmaFECT1 transfection reagent (T-2001-02; Dharmacon, USA) (DH1) and incubated for 48 h. Transfections were repeated in three independent experiments.

2.3. RNA extraction and synthesis of complementary DNA (cDNA)

Cell cultures were harvested by follicular puncture, snap frozen in liquid nitrogen, and stored at −80 °C before processing. Total RNA was extracted using the NZY Total RNA Isolation kit (NZYTech, Portugal), according to the manufacturer's protocol, and quantified using a NanoDrop Spectrophotometer (ND-2000c, Peqlab GmbH, Germany). To exclude possible amplification of contaminating genomic DNA, an additional step of DNase digestion was performed with the RNase-free DNase Set (Qiagen Inc. Hilden, Germany). Then, total RNA (200 ng) was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (4368814; Applied Biosystems, USA) and random RT primers, following the manufacturer's instructions. The prepared cDNA was stored at −20 °C until analysis.

2.4. Reverse transcription quantitative PCR (RT-qPCR)

The RT-qPCR was performed with the StepOne Plus™ Real-Time PCR System, using the Power SYBR® Green master mix (both from Applied Biosystems, Foster City, CA, USA). Reaction mixes of 6.25 μL of master mix, 1 μL of forward and reverse primers (160 nM) Supplementary Tables 2) and 1 μL of cDNA template (diluted 1/10) were pipetted into MicroAmp™ optical 96-well plates and sealed with optical caps (Applied Biosystems, Foster City, CA, USA). After an initial denaturation step at 95 °C for 10 min, a thermocycling program of 15 s at 95 °C, 60 s at 60 °C and 15 s at 95 °C was applied (40 cycles). Total fluorescence data and dynamic well factors were continuously collected to generate background-subtracted amplification curves (StepOne™ Software v2.3, Applied Biosystems, Foster City, CA, USA). The relative gene expression (RE) levels were calculated as a variation of the Livak method [24], corrected for variation in amplification efficiency ($E = 10^{-1/slope}$) [25] (see Supplementary Data). The expression levels of the target genes were normalized against the geometric mean of ribosomal protein P0 (RPLP0), peptidylprolyl isomerase B (PPIB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. All samples were run in duplicates, and no-transcription and no-template controls were included. The specificity of the PCR amplification was confirmed by a melting curve analysis and agarose gel electrophoresis of RT-qPCR products.

2.5. Quantification of progesterone (P4) and 17β-estradiol (E2)

Cell supernatants collected at 48 h after the siRNA silencing protocol and stored at −20 °C were used for quantification of P4 and E2 by a solid-phase, competitive chemiluminescent enzyme immunoassay and a radioimmunoassay (RIA), respectively. P4 concentrations were determined using the Progesterone Kit (L2KPW2, Siemens Healthcare, United Kingdom) and the Immulite 2000 Immunoassay System, both from Siemens Healthcare. Estradiol concentrations were determined by solid phase radioimmunoassay, using the ESTR-US-CT cisbio kit (ESTR-US-CT, Cisbio, France), and the Wallac 1470 Gamma reader (WALLAC, Finland). The sensitivity of the E2 RIA is 2 pg/mL the analytical sensitivity of the P4 immunoassay is 0.1 ng/mL.

2.6. Statistical analysis

Data were analyzed using the PROC MIXED Model of Statistical Analysis System (SAS) version 9.4 (SAS Institute, Cary, NC, USA). In addition, the means and standard errors for each treatment were calculated, and comparisons between groups were performed using the PDIFF test. The session was considered a random effect. The differences were considered statistically significant when $P < 0.05$. Different letters in figures indicate significant differences between groups.

3. Results

3.1. Silencing and quantification of SPRN and PRNP genes in granulosa cells

To knockdown *PRNP* and *SPRN*, two siRNAs per gene (P1 and P2 for *PRNP*; and S1 and S2 for *SPRN*) were designed and tested, alone and combined (P1+P2 or S1+S2). The silencing efficiency was confirmed by quantification of PRNP and SPRN mRNA expression in GCs by RT-qPCR, using specific primers. The P1 and S1 siRNAs showed the greater decrease in mRNA levels, a statistically significant 2.4-fold decrease in mRNA expression in the first case (Fig. 1A) and a statistically significant 2.8-fold decrease in mRNA expression in the second case (Fig. 1B). Therefore, P1 and S1 were the siRNAs selected to be used in subsequent analyses. Moreover, we added two additional siRNA negative controls, Dharmafect transfection reagent without siRNA and no Dharmafect nor siRNA, which showed similar mRNA levels as the siRNA control targeting the green fluorescent protein (GFP) (Supplementary Fig. 1). Finally, we confirmed that the siRNA experiments did not interfere with cell viability, as assessed by LDH quantification (Supplementary Fig. 2). The previous results indicate that we were able to reduce the expression of *PRNP* and *SPRN* in bovine GCs using a siRNA approach without significantly affecting cell viability. Importantly, we detected the expression of the *SPRN* gene in bovine granulosa cells (Fig. 1B) which, to the best of our knowledge, was not reported before.

3.2. PRNP silencing induces higher SPRN mRNA expression in granulosa cells

To determine if the knockdown of *PRNP* or *SPRN* prion-family genes could affect each other expression, we analyzed the expression of *PRNP* in *SPRN* knockdown GCs and the expression of *SPRN* in *PRNP* knockdown GCs by RT-qPCR. Although the *SPRN* lower expression (S1) did not affect *PRNP* expression (Fig. 2A), *PRNP* lower expression (P1) led to a significant 2.3-fold increase in *SPRN* expression (Fig. 2B). These results suggest that *PRNP* may be involved in *SPRN* regulation by negatively impacting its expression in GCs.

3.3. SPRN silencing induces a decrease on CYP11A1 mRNA expression in granulosa cells

To evaluate whether *PRNP* and/or *SPRN* could impact the expression of enzymes involved in steroidogenesis, we assessed the expression of two cytochrome P450 family genes by RT-qPCR in *PRNP* and *SPRN* knockdown GCs. *CYP11A1* encodes CYP11A1, an enzyme involved in the conversion of cholesterol to pregnenolone, an early reaction in steroidogenesis (e.g., progesterone, testosterone, and estradiol synthesis), and *CYP19A1* encodes CYP19A1, an enzyme that catalyses the last steps of 17β-estradiol biosynthesis from testosterone. Both enzymes were previously reported to be expressed in GCs [26]. Interestingly, a 2.1-fold decrease in *CYP11A1* gene expression (Fig. 3) was detected in *SPRN* knockdown GCs. Conversely, we did not detect any change in *CYP11A1* expression, related to *PRNP* silencing (Fig. 3) and *CYP19A1* expression was not detected in any sample. Altogether, these results indicate that SPRN may be involved in steroidogenesis through *CYP11A1* expression modulation.

3.4. SPRN silencing has a mild effect on progesterone secretion by in vitro cultured granulosa cells

In order to evaluate the impact of *PRNP* and *SPRN* on the end-product levels of the considered biosynthetic steroidogenic pathways, we determined the concentration of progesterone (P4) and estradiol (E2) in the conditioned culture medium from GCs with and without *PRNP* and *SPRN* knockdown. Estradiol was only detected (439.6 ng/L) in the supernatants obtained before the siRNA transfection (Day zero), but no detection was possible (<1.5 pg/mL) 48 h after the transfection, when the supernatants and cells were collected to be analyzed. On the other hand, the supernatants collected before the siRNA transfection exhibited the

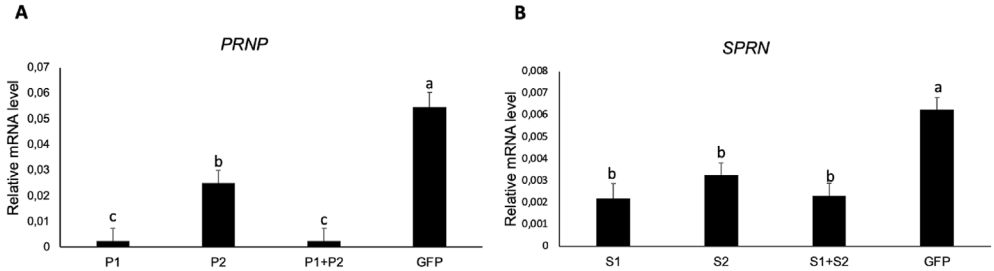

Fig. 1. mRNA expression of *PRNP* and *SPRN* in granulosa cells (GCs) transfected with different siRNA sequences. (A) The mRNA levels of *PRNP* in bovine GCs were detected by RT-qPCR after transfection with PRNP siRNA1 (P1), PRNP siRNA2 (P2), the combination of both (P1+P2), or GFP Duplex I random siRNA control (GFP); (B) The mRNA levels of *SPRN* in bovine GCs were detected by RT-qPCR after transfection with SPRN siRNA1 (S1), SPRN siRNA2 (S2), the combination of both (S1+S2), or GFP Duplex I random siRNA control (GFP).

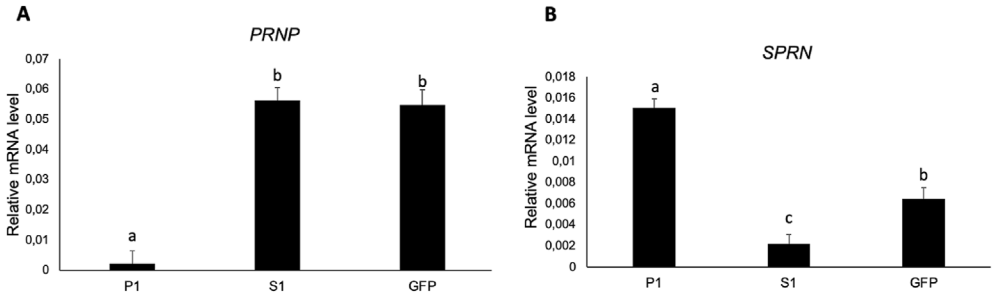

Fig. 2. mRNA expression of *PRNP* and *SPRN* in transfected granulosa cells (GCs). Relative (A) PRNP and (B) SPRN mRNA levels were determined by RT-qPCR after transfection with P1 (PRNP siRNA), S1 (SPRN siRNA), or GFP Duplex I random siRNA control (GFP).

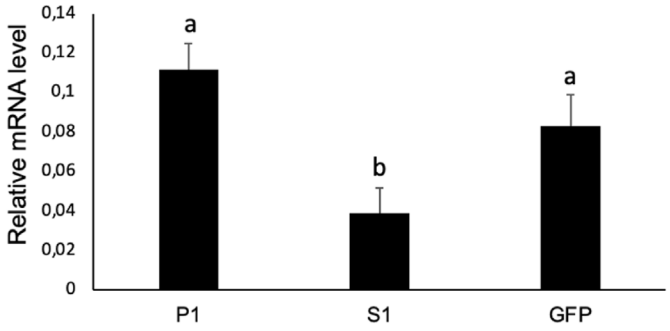

Fig. 3. CYP11A1 mRNA expression in siRNA transfected bovine granulosa cells (GCs). CYP11A1 mRNA levels were detected by RT-qPCR after transfection with P1 (*PRNP* siRNA) and S1 (*SPRN* siRNA), or GFP Duplex I random siRNA control (GFP).

lowest value for P4 (Day zero), comparing to all groups evaluated 48 h after the transfection (Day zero = 29.10 μg/L; after transfection = 182.93–234.10 μg/L; Fig. 4). Moreover, the *SPRN* knockdown GCs (S1), in which we identified reduced *CYP11A1* gene expression (Fig. 3), showed a mild reduction in progesterone levels in the culture supernatants, although not statistically significant (182.93 ± 42.00 μg/L as compared to the GFP control 234.13 ± 42.00 μg/L; Fig. 4). However, culture supernatants from *SPRN* knockdown GCs did not show a statistically significant increase in progesterone levels as compared to day zero either. In all, these results show that *SPRN* knockdown in GCs leads to a mild reduction in progesterone levels, although not statically significant, suggesting a possible role for SPRN in progesterone synthesis regulation through *CYP11A1* gene expression modulation.

4. Discussion

In the present study, we hypothesized that *SPRN* and *PRNP* prion family genes could have important physiological functions in the regulation of folliculogenesis pathways. A previous study has shown that in pregnant cows, the PRNP mRNA is detectable in reproductive tissues such as the ovary, oviduct, endometrium, myometrium, follicles, and granulosa cells (GCs) [27]. *SPRN* expression in the ovary was also previously reported in the SPRN-LacZ transgenic mouse [16]. Here, we detected PRNP and SPRN mRNAs in GCs cultured *in vitro* for approximately 6 days. To determine a possible role for *PRNP* and *SPRN* in steroidogenesis, we conducted post-transcriptional *PRNP* and *SPRN* gene silencing in *in vitro* cultured GCs, quantified the expression of two main steroidogenic genes, *CYP11A1*, that catalyses the conversion of cholesterol to pregnenolone, the first and rate-limiting step in the synthesis of the steroid hormones, and *CYP19A1* that catalyses the last steps of estrogen biosynthesis [18,19], and analyzed steroid hormone production (P4 and E2).

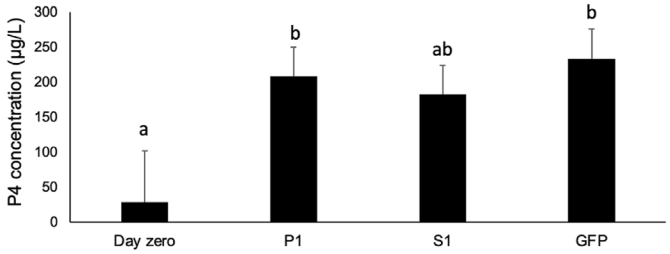

Fig. 4. Progesterone quantification by immunoassay (μg/L) in transfected GCs. Progesterone quantification was performed before siRNA transfection (Day zero) and 48 h after transfection with P1 (*PRNP* siRNA), S1 (*SPRN* siRNA), and GFP (GFP Duplex I random siRNA control).

Silencing of *SPRN* downregulated CYP11A1 mRNA levels and led to a mild reduction in progesterone (P4) production. Moreover, although silencing of *PRNP* upregulated *SPRN* levels, it did not affect CYP11A1 mRNA and P4 levels. It is possible that the levels of progesterone produced by *in vitro* cultured GCs do not fully recapitulate the levels found *in vivo*. Also, the *SPRN* knockdown resulted in a 2.8-fold reduction in SPRN mRNA and the PRNP knockdown resulted in a 2.3-fold higher expression of SPRN mRNA, which may not be enough to detect clear differences regarding progesterone secretion in the former, and CYP11A1 expression and progesterone secretion, in the latter, at least in the presented experimental settings.

We were also not able to detect *CYP19A1* expression and estradiol was only detected until the 4th day of culture before the siRNA transfection. In fact, the expression of *CYP19A1* is essential for estrogen biosynthesis through the conversion of androstenedione and testosterone produced via the Δ5 pathway and entering in GCs [28]. This finding is supported by the expected GC *in vitro* luteinization, which is characterized by a lower E2 and higher P4 secretion. In accordance, and as previously reported by our group [23], we detected P4 at the supernatants of GCs, which was also in agreement with the observed higher expression of *CYP11A1*.

Finally, we have also found that *SPRN* gene expression was increased following *PRNP* knockdown, suggesting that *PRNP* and *SPRN* may be interregulated. Several authors have previously reported an interaction between prion and prion-like family genes in cerebellar neurodegeneration and ataxia syndromes [29] or strongly affecting the degree of susceptibility/resistance to scrapie [30]. Equally, associations between different prion-like genes regulating male fertility were also reported [9]. In the present study, the observed interaction between *PRNP* and *SPRN* expression in bovine GCs also points to a shared regulation in female reproduction.

As a conclusion, the present study unveils the expression of the

SPRN gene in bovine GCs, an association between *SPRN* and *CYP11A1* expression and between *PRNP* and *SPRN* genes in the regulation of steroidogenic pathways. Overall, an important regulatory role for the *SPRN* gene in ovarian steroidogenesis is suggested, which deserves further research.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.02.012.

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