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Association study and expression analysis of porcine *ESR1* as a candidate gene for boar fertility and sperm quality

Asep Gunawan[a], Kanokwan Kaewmala[a], Muhammad Jasim Uddin[a], Mehmet Ulas Cinar[a], Dawit Tesfaye[a], Chirawath Phatsara[a,b], Ernst Tholen[a], Christian Looft[a], Karl Schellander[a,*]

[a] *Institute of Animal Science, Animal Breeding and Husbandry Group, University of Bonn, 53115 Bonn, Germany*
[b] *Department of Animal and Aquatic Sciences, Faculty of Agriculture, Chiang Mai University, 50200 Chiang Mai, Thailand*

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ABSTRACT

Male fertility is impaired through the lack of *ESR1* (Estrogen Receptor 1) but little is known about the *ESR1* roles in boar spermatogenesis and fertility. Therefore, this research was aimed at investigating the association with sperm quality and boar fertility traits in a total of 300 boars both from purebred Pietrain and Pietrain × Hampshire crosses. A SNP in coding region of *ESR1* g.672C > T in exon 1 was associated with sperm motility ($P < 0.05$) and plasma droplet rate ($P < 0.01$) while the polymorphism in non-coding region of *ESR1* g.35756T > C in inton 1 was associated with non-return rate ($P < 0.05$). Furthermore, to analyse the mRNA and protein expression of *ESR1* in boar reproductive tissues, a total of six boars were divided into two groups [Group I (G-I) and Group II (G-II)], where G-I had relatively better sperm quality. *ESR1* expression was higher in tissues collected from G-I boars than those of collected from G-II boars, and the difference in mRNA expression was significant ($P < 0.01$) in head of epididymis. The *ESR1* protein expression results from western blot coincided with the results of qRT-PCR. The *ESR1* protein localization observed a strong staining in the cytoplasm of Sertoli cell in the testis, in the epithelial cells in head and tail of epididymis, in smooth muscle in tail of epididymis, and in the post acrosomal region and tail of the spermatozoa. These results will improve the understanding of the functions of the *ESR1* in spermatogenesis within the reproductive tract and will shed light on *ESR1* as a candidate in the selection of boar with good sperm quality and fertility.

1. Introduction

Despite being the 'female' hormone, estrogen is present in low concentrations in male blood and in extraordinarily high concentration in semen (Ganjam and Amann, 1976). The hormone estrogen is mediated through the nuclear estrogen receptor which functions as ligand-dependant transcription factor. The increasing interest in the role of estrogen in the male reproductive tract is mainly due to the demonstration that male fertility is impaired in mice lacking estrogen receptor 1 (*ESR1*) (Eddy et al., 1996). The evidence for *ESR1* in different parts of the male reproductive tract suggests a possible physiological role in spermatogenesis and sperm maturation. The *ESR1* knockout mice have provided evidence for a significant and crucial role of estrogens in maintaining normal spermatogenesis (Eddy et al., 1996). *ESR1* is involved in the reabsorption of luminal fluid during the transit of

* Corresponding author. Tel.: +49 228 732240; fax: +49 228 732284.
E-mail addresses: agun@itw.uni-bonn.de (A. Gunawan), kkae@itw.uni-bonn.de (K. Kaewmala), judd@itw.uni-bonn.de (M.J. Uddin), ucin@itw.uni-bonn.de (M.U. Cinar), dtes@itw.uni-bonn.de (D. Tesfaye), chirawath.p@cmu.ac.th (C. Phatsara), etho@itw.uni-bonn.de (E. Tholen), cloo@itw.uni-bonn.de (C. Looft), ksch@itw.uni-bonn.de (K. Schellander).

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spermatozoa from the testis to the head of the epididymis which is important for their survival and maturation during epididymal storage (Couse and Korach, 1999). The absence of *ESR1* leads to reduced epididymal sperm content, reduced sperm motility and fertilizing ability (Eddy et al., 1996). Several studies have shown a significant association of *ESR1* with semen quality or fertility in humans (Suzuki et al., 2002; Guarducci et al., 2006; Lazaros et al., 2010; Safarinejad et al., 2010). Guarducci et al. (2006) reported a significant association of *ESR1* polymorphism with lower sperm count in men. Suzuki et al. (2002) reported two silent polymorphisms in *ESR1* being associated with azoospermia or severe oligozoospermia in men. To our knowledge, only one study was devoted to analyse the association between *ESR1* polymorphism with litter size in sows but this failed to detect any statistically significant association (Munoz et al., 2007). The *ESR1* mRNA was reported to be highly expressed in the epididymis, testis, pituitary, uterus, kidney and adrenal gland in rats (Kuiper et al., 1996). *ESR1* was localized in head, body and tail of epididymis in the bonnet monkey (Shayu et al., 2004), cat (Nie et al., 2002) and horse (Hejmej et al., 2005; Parlevliet et al., 2006). In pigs, *ESR1* is located on SSC1p24-25, which was evidenced as the QTL region for total sperm per ejaculate and close to the QTL for sperm motility (Xing et al., 2008). Therefore, *ESR1* could be a functional as well as a positional candidate gene for male reproduction in pigs. But no study has yet been devoted to unravelling its association with boar sperm quality and fertility traits.

In spermatogenesis, beside the reproductive tissues testis and epididymis, some other accessory glands and tissues are also involved. Spermatogenesis is the complex process by which immature germ cells undergo division, differentiation and meiosis to give rise to haploid elongated spermatids (O'Donnell et al., 2001). When germ cell development is complete, the mature spermatids are released from the Sertoli cells into the tubule lumen and proceed through the excurrent duct system, known as the rete testis, until they enter the epididymis via the efferent ducts. During passage through the epididymis, the spermatids undergo a series of biochemical changes to become the motile and mature spermatozoa capable of fertilization (O'Donnell et al., 2001). Failure in any of these events leads to disturbances of male fertility. The role of *ESR1* in boar spermatogenesis within the reproductive tract is poorly understood. For the better understanding of *ESR1* roles in spermatogenesis in pigs, the expression of *ESR1* at different parts of reproductive tract, including non-reproductive tissues, are important. Therefore, the aims of this study were to investigate the association of *ESR1* polymorphism with sperm quality and boar fertility traits as well as to highlight the roles of *ESR1* in boar spermatogenesis within the reproductive tract by mRNA and protein expression and immunoreactive *ESR1* localization. This work might be helpful to get further insight into the roles of *ESR1* in spermatogenesis in the boar. The association of *ESR1* polymorphism with boar sperm quality and fertility will support the candidacy of this gene to be used in selection of breeding boars.

2. Materials and methods

2.1. Phenotypes

Samples and phenotypes from 200 purebred Pietrain (PI) and 100 Pietrain × Hampshire crossbred (PI × HA) boars were used for association analysis in this study. These animals were used for AI in commercial pig herds in North-Western Germany. Details of the populations and phenotypes were described previously by Wimmers et al. (2005), Lin et al (2006) and Kaewmala et al. (2011). In brief, sperm samples from more than 39,000 ejaculates were repeatedly collected from these boars. Whole ejaculates were obtained from purebred Pietrain and crossbred Pietrain × Hampshire boars age between two and five years with an average age of 3.5 years. Sperm quality traits included sperm concentration (SCON [$\times 10^8$ ml]), semen volume per ejaculate (VOL [ml]), sperm motility (MOT [%]), plasma droplets rate (PDR [%]) and abnormal spermatozoa rate (ASR [%]) and were obtained from each ejaculate with light microscopy according to the guidelines of the World Health Organization. Semen was collected by the vinyl glove hand method twice per week. For each boar, the repeated measurements of sperm quality traits were available. Fertility data (non-return rate data [NRR] at 42 days after insemination [%] and number of piglet born alive [NBA] per litter) of each boar were available as the deviation from the population means within sow breed, parity of sow, farm and season classes as described earlier by Lin et al (2006) and Kaewmala et al. (2011). The general description of sperm quality traits and boar fertility traits are shown in Table 1.

2.2. Polymorphism study

Two single nucleotide polymorphisms detected by Munoz et al. (2007) were used in this study: a cytosine (C) transversion to a thymine at g.672C>T in exon 1 and a thymine (T) to a cytosine (C) transversion at g.35756T>C in intron 1 of *ESR1*. For PCR amplification, the forward (5′-gttcaaatccctggttgcat-3′) and reverse (5′-ctaggcgtctccccagattag-3′) primers were designed from the exon 1 and the forward (5′-gacagcttccctgcagattc-3′) and reverse (5′-ttcatcatgcccacttcgta-3′) primers were designed from the intron 1 of porcine *ESR1* genomic sequence (GenBank accession No. ENSSSCG00000004083) using the Primer3 tool (Rozen and Skaletsky, 2000). Polymerase chain reactions (PCR) were performed in a 20 μl volume containing 100 ng of porcine genomic DNA, 1 × PCR buffer (with 1.5 134 mM MgCl2), 0.25 mM of each dNTP, 5 pmol of each primer and 0.1 U of Taq DNA polymerase (GeneCraft). The PCR reaction was performed under the following conditions: initial denaturation at 95 °C for 5 min followed by 35 cycles of 30 s at 95 °C, 30 s at 52 °C, 30 s at 72 °C and final elongation of 10 min at 72 °C for the polymorphism in exon 1. The PCR conditions were the same for the polymorphism in intron 1 except the annealing temperature of 30 s at 56 °C. After checking the PCR products in 1.5% (w/v) agarose gels, genotyping was done following the restriction fragment length polymorphisms (RFLPs) analysis. The digestion of restriction enzyme was done using BstNI and

Table 1
Means, standard deviation (S.D.), sample size, ranges of traits in Pietrain and Pietrain × Hampshire.

Population	Traits	Sample size	Mean	S.D.	Minimum	Maximum
Pietrain (PI)	SCON (10^8/ml)	29,161	3.03	0.94	1	6
	VOL (ml)	30,772	237.03	57.32	25	522
	MOT (%)	30,118	84.72	4.37	65	95
	PDR (%)	30,239	5.41	3.33	0	15
	ASR (%)	30,528	6.53	4.18	0	20
	NRR42 (%)[a]	200	0.28	7.06	−24.07	17.68
	NBA (per litter)[a]	200	0.02	0.57	−1.69	1.37
Pietrain × Hampshire (PIHA)	SCON (10^8/ml)	9123	2.95	0.97	1	6
	VOL (ml)	9673	297.50	81.62	56	546
	MOT (%)	9395	85.46	4.03	70	95
	PDR (%)	9409	5.76	3.14	0	15
	ASR (%)	9543	4.95	4.00	0	20
	NRR42 (%)[a]	100	0.97	4.18	−12.23	13.79
	NBA (per litter)[a]	100	0.05	0.52	−2.97	0.83
PI and PIHA	SCON (10^8/ml)	38,284	3.00	0.95	1	6
	VOL (ml)	40,445	256.87	75.54	25	546
	MOT (%)	39,513	85.01	4.37	65	95
	PDR (%)	39,648	5.51	3.28	0	15
	ASR (%)	40,071	5.99	4.26	0	20
	NRR42 (%)[a]	300	0.41	6.23	−24.07	17.67
	NBA (per litter)[a]	300	0.01	0.55	−2.97	1.37

[a] Fertility (NRR42, NBA) corrected with factors: parity, farm, season and breed.

*Alu*I for g.672C > T and g.35756T > C, respectively (BioLabs). The digestion was carried out in 10 μl of reaction mixture of each sample and incubated overnight at 37 °C for g.35756T > C and 65 °C for g.672C > T. Detection of RFLPs of 300 boars was carried out by electrophoresis in 3% (w/v) agarose gels.

2.3. Statistical analysis for sperm quality traits

The association of *ESR1* with boar sperm quality traits was analysed by variance analysis (PROC MIXED) using the SAS software package (ver. 9.2; SAS Institute Inc., Cary, USA) as described earlier by Kaewmala et al. (2011).

$$y_{ijklm} = \mu + \text{breed}_i + \text{season}_j + \text{genotype}_k + \text{age}_l + \text{ejaculation}_m + \varepsilon_{ijklm} \quad \text{[Model 1]}$$

where y_{ijklm} are the sperm quality traits (SCON, VOL, MOT, PDR, ASR); μ is the overall population mean; breed_i is the fixed effect of the i-th breed (i = PI; PIHA, PI and PIHA); season_j is the fixed effect of the j-th season (j = 1 through 8; four seasons per year, in total eight seasons within 2 years from January 2000 to December 2001); genotype_k is the fixed effect of the k-th genotype (k = 1, 2 and 3); age_l is the effect of boar age (covariable); ejaculation_m is the permanent environmental effect of the m-th boar (random) and ε_{ijklm} is the residual error.

2.4. Statistical analysis for fertility traits

The association analysis between *ESR1* and the fertility traits was carried out using the following generalized linear model (PROC GLM) in SAS (Kaewmala et al., 2011).

$$y_{ijkl} = \mu + \text{breed}_i + \text{genotype}_j + \text{year}_k + \varepsilon_{ijkl} \quad \text{[Model 2]}$$

where y_{ijk} is the boar fertility traits (NRR and NBA); μ is the overall population mean; breed_i is the fixed effect of the i-th breed (i = PI, PIHA, PI and PIHA); genotype_j is the fixed effect of the j-th genotype (j = 1, 2 and 3); year_k is the fixed effect of the k-th boar year of birth (k = 1 through 3: boar born before 1996, in 1996–97 and in 1998–99), ε_{ijk} is the residual error.

A chi-square (χ^2) test was conducted to test the populations for Hardy–Weinberg equilibrium. Least square mean values for the *ESR1* genotypes were compared by *t*-test and *P*-values were adjusted by the Tukey–Kramer correction.

2.5. Selection of animals for mRNA and protein expression

The reproductive (testis, head, body and tail of epididymis, vas deferens, bulbourethral gland, vesicular glands and prostate gland), non-reproductive (brain, muscle and liver) tissues from 6 breeding boars with divergent phenotypes were collected from the AI station (SuisAG, Sempach, Switzerland) for mRNA and protein study. For differential expression study between reproductive and non-reproductive tissues by semi-quantitative PCR study, mRNA from all 6 boars was pooled together according to the tissues. On the other hand, the differential mRNA and protein expression study in different reproductive tissues from two divergent groups of animals was performed by

Table 2
Means, standard deviation (S.D.), number of boars and ranges of traits selected for mRNA and protein expression study.

Traits	Selected animals (n = 6)		G-I (n = 3)		G-II (n = 3)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
SCON (10^6/ml)	262.32	87.97	335.94	50.78	188.70	22.54
SVOL (ml)	215.24	34.93	185.07	16.33	245.40	7.42
SMOT (%)	76.59	3.71	79.03	1.89	74.14	3.60

semi-quantitative PCR, qRT-PCR and western blot, respectively. For these purposes, the 6 boars were divided into two groups: group I (G-I) and group II (G-II), each group containing 3 boars on the basis of SCON, SVOL and SMOT (Table 2). The SCON (average sperm concentration) was highly negatively ($r = -0.8$) correlated with SVOL (average semen volume), whereas SCON was highly positively ($r = 0.7$) correlated with SMOT (average sperm motility). Moreover, SVOL was highly negatively ($r = -0.8$) correlated with SMOT. For the animals of G-I the SCON was higher than the mean (262.32×10^6 ml), SMOT was higher than the mean (76.59%) and SVOL was lower than the mean (215.24 ml/ejaculation) of respective parameters. For the animals of G-II these parameters were vice versa. The significant difference between the two groups was calculated using proc *t*-test in SAS. There were differences for SCON ($P < 0.05$) and for SVOL ($P < 0.01$) between G-I and G-II, whereas for the SMOT the difference was not significant ($P = 0.12$).

2.6. Semi-quantitative PCR

Total RNA was isolated using TRI reagent (Sigma) from different reproductive and non-reproductive tissues of breeding boars mentioned above (Section 2.5). RNA was purified using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated using on-column RNase-Free DNase set (Promega) and quantified sphectrophotometrically (Nano Drop, ND8000). Furthermore, RNA integrity was checked by 2% agarose gel electrophoresis. First-strand cDNA were synthesized from individual RNA using Superscript II enzyme (Invitrogen).

cDNA amplification was performed by using specific forward and reverse primers (forward: 5′-agggagaggagtttgtgtg-3′ and reverse: 5′-tctccagcagcaggtcatag-3′) derived from porcine *ESR1* sequence (GenBank accession AF035775). Amplification was performed with an initial heating at 95 °C for 5 min followed by 35 cycles of 95 °C for 45 s, annealing temperature at 60 °C for 1 min and 72 °C for 1 min, on the PCR Thermal Cycler (BioRad). PCR products were electrophoresed on a 1.5% agarose gel and visualized upon staining with ethidium bromide. Amplification of Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as housekeeping gene.

2.7. Quantitative Real-Time PCR (qRT-PCR)

For qRT-PCR, total RNA was isolated using TRI reagent (Sigma) from different reproductive tissues of the two divergent groups of animals (G-I and G-II) as previously described (Section 2.5). Total RNA isolation and cDNA synthesis was described in the previous section (Section 2.6). The same primer pair used in semi-quantitative PCR was also used in qRT-PCR. Nine-fold serial dilution of plasmid DNA was prepared and used as a template for the generation of the standard curve. In each run, the 96-well microtiter plate contained each cDNA sample, plasmid standards for the standard curves and a no-template control. To ensure repeatability of the experiments, each sample was run in three replications. qRT-PCR was set up using 2 μl first-strand cDNA template, 7.6 μl deionized H_2O, 0.2 μM of upstream and downstream primers and 10 μl 1× Power SYBR Green I master mix with ROX as reference dye (BioRad). The thermal cycling conditions were 3 min at 94 °C followed by 40 cycles of 20 s at 94 °C and 1 min at 60 °C. Experiments were performed using the ABI prism®7000 (Applied Biosystems) qRT-PCR system. An amplification-based threshold and adaptive baseline were selected as algorithms. The housekeeping gene *GAPDH* (forward: 5′-acccagaagactgtggatgg-3′ and reverse: 5′-acgcctgcttcaccaccttc-3′) derived from porcine sequence (GenBank accession No. AF017079) was used for the data normalization. Final results were reported as the relative abundance level after normalizing with mRNA expression level of the housekeeping gene. Differences in *ESR1* mRNA expression were analysed with the simple *t*-test in SAS software (SAS Institute Inc., ver. 9.2). Values of $P < 0.05$ were considered to indicate statistically significant differences.

2.8. Western blotting

The protein was extracted from different reproductive tissues (testis, head, body and tail of epididymis) from the two divergent groups of breeding boars as used in qRT-PCR. However, for western blot study, proteins from the three G-I boars were pooled together and proteins from the three G-II boars were pooled together according to the tissues. The protein extracted from tissues was separated by SDS-PAGE (gradient 4–18%). Subsequently the proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences). After blocking in blocking buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 1% Polyvinylpyrolidone) at room temperature for 1 h, the membrane was incubated with the anti-*ESR1* antibody purified from rabbit polyclonal antibody (Cat.nr. 543; Santa Cruz) in the blocking medium (diluted 1:500) overnight at 4 °C. Non-specific binding of antibody was washed off with six changes of 0.1% PBST (10 min to time). The horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody (Cat.nr. Sc2004; Santa Cruz) was used as the secondary antibody (diluted 1:5000). The membrane was incubated for 1 h at room temperature with secondary antibody, followed by washing with six changes of 0.1% PBST (10 min to time). The chemiluminesce was detected by using the ECL plus western blotting detection system (Amersham Biosciences) and was visualized by using Kodak BioMax XAR film (Kodak). *GAPDH* was used as a loading control and for normalization. The membrane was stripped by incubation in 2% SDS, 100 mM Tris–HCl, 0.1% β-mercaptoethanol for 30 min at 60 °C and re-probed with *GAPDH* antibody (Cat.nr. Sc20357; Santa Cruz).

2.9. Protein localization by immunofluorescence

Due to the limitations of fresh samples from G-I and G-II boars, we collected different fresh reproductive tissues were collected from a healthy breeding boar after slaughtering for protein localization by immunofluorosence. Immunofluorescence staining was performed on 8 μm cryostat sections of snap frozen tissues. All sections were kept in −80 °C for further analysis. To block unspecific staining, sections were incubated for 30 min at room

Table 3
Genotype, allele frequencies and the chi-square test of the porcine *ESR1* gene.

Polymorphism	Breed	Number of boars (n)	Genotype frequency			Allele frequency		Chi-square test
			CC (n)	CT (n)	TT (n)	C	T	χ^2
g.672C > T	Pietrain	200	0.73 (146)	0.09 (19)	0.18 (35)	0.78	0.22	0.55
	Pietrain × Hampshire	100	0.61 (61)	0.18 (18)	0.21 (21)	0.70	0.30	0.33
	PI and PIHA	300	0.66 (200)	0.15 (44)	0.19 (56)	0.74	0.26	0.38
Polymorphism	Breed	Number of boars (n)	Genotype frequency			Allele frequency		Chi-square test
			TT (n)	TC (n)	CC (n)	T	C	χ^2
g.35756T > C	Pietrain	185	0.83 (154)	0.14 (26)	0.03 (5)	0.90	0.10	0.05
	Pietrain × Hampshire	94	0.80 (75)	0.17 (16)	0.03 (3)	0.88	0.12	0.03
	PI and PIHA	279	0.82 (229)	0.15 (42)	0.03 (8)	0.89	0.11	0.04

temperature with 5% bovine serum albumin in PBS (50 nM sodium phosphate, pH 7.4; 0.9% NaCl). Sections were incubated overnight at 4 °C with the *ESR1* rabbit polyclonal primary antibody (Cat.nr. 543; Santa Cruz) diluted at 1:50 in PBST followed by six times (10 min per time) washing with PBS. Then, the sections were incubated 1 h at room temperature with the biotinylated donkey anti-rabbit IgG-B as a secondary antibody (Cat.nr. Sc2090; Santa Cruz) (dilution 1:200) which was conjugated with fluorescein isothiocynate (FITC). Later on sections were washed six times (10 min per time) with PBS. Finally, the samples were counterstained with vectashield mounting medium (Vector Laboratories) containing 40,6-diamidino-2-phenyl indole (DAPI) and covered with a cover glass slip. The staining was observed by confocal laser scanning microscope (Carl Zeiss). In case of negative controls, PBS was used instead of the primary antibody.

3. Results

3.1. Polymorphism study

Two single nucleotide polymorphisms (SNPs) were confirmed in exon 1 and intron 1 of *ESR1* in the PI and PI × HA populations. Animals of these populations were genotyped at g.672C > T in exon 1 and at g.35756T > C which were the SNP segregating within the populations. The SNPs were confirmed by PCR-RFLP. The DNA restriction fragments obtained for g.672C > T of *ESR1*-B*st*NI polymorphism were: 190, 117, 50, 32 and 17 bp for the CC genotype; 190, 117, 50, 32, 17 and 12 bp for the CT genotype, and 190, 117, 50 and 12 bp for the TT genotype. The DNA restriction fragments obtained for g.35756T > C of *ESR1*-*Alu*I polymorphism were: 93, 48, 40 and 5 bp for the TT genotype; 133, 93, 48, 40 and 5 bp for the TC genotype, and 133, 93, and 5 bp for the CC genotype.

The calculated genotype and allele frequencies of porcine *ESR1* gene are shown in Table 3. In this study, three genotype CC, CT and TT were found for both SNPs at g.672C > T and g.35756T > C in our populations. Homozygote CC was more frequent and homozygote TT was rare at SNP g.672C > T whereas in case of SNP g.35756T > C, the homozygote TT was the more frequent, and homozygote CC was rare in our populations. The chi-square test revealed that the locus of *ESR1* was in Hardy–Weinberg equilibrium in both PI and PI × HA populations (Table 3).

3.2. Association analysis

Association analysis of g.672C > T with sperm quality and fertility traits revealed significant ($P < 0.01$) association with MOT and PDR. Semen from animals with genotype CT had significantly lower sperm motility and higher plasma droplet rate than animals with CC and TT (Table 4). The results also indicated that there were dominant effects ($P < 0.05$) on MOT and PDR in our population. In the case of g.35756T > C, the association analysis showed significant ($P < 0.05$) association with NRR. Animals with the TC genotype had a significantly ($P < 0.05$) higher NRR than those with genotype TT and CC (Table 4).

3.3. mRNA expression by semi-quantitative PCR

ESR1 gene expression was higher in head of the epididymis than in body and tail, moderate in testis, vas deferens, bulbourethral glands and lower expression was found in non-reproductive tissue (brain and liver) except skeletal muscle. The semi-quantitative reverse transcription PCR result of *GAPDH* showed no remarkable differences among tissues (Fig. 1).

3.4. mRNA and protein expression study in tissues from G-I and G-II boars

The *ESR1* mRNA was expressed in testis, body and tail of epididymis from both the G-I and G-II boars but the higher expression was shown in head of epididymis of G-I than that of G-II boars in semi-quantitative PCR (Fig. 2a). These mRNA expression results of semi-quantitative PCR appeared to be consistent with the results of the qRT-PCR. The *ESR1* mRNA expression was higher in head of epididymis of G-I boars ($P < 0.01$), whereas the expression

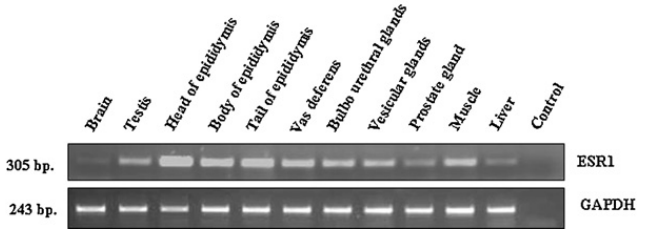

Fig. 1. mRNA expression of *ESR1* in reproductive and non-reproductive tissues by semi-quantitative PCR.

Table 4
Association between *ESR1* genotypes with sperm quality and fertility traits.

Polymorphism	Trait	*ESR1* genotype ($\mu \pm$ S.E.)			Effect ($\mu \pm$ S.E.)	
		CC	CT	TT	Additive	Dominance
	Sperm quality	Number of observations[#]				
		26,078	5971	7507		
g.672C > T	SCON (10^8/ml)	3.00 ± 0.04	2.98 ± 0.09	2.95 ± 0.08	0.02 ± 0.04	-0.01 ± 0.10
	VOL (ml)	258.90 ± 3.76	258.59 ± 7.30	258.80 ± 6.74	0.05 ± 3.76	-0.26 ± 8.13
	MOT (%)	85.02 ± 0.27^{a}	83.47 ± 0.53^{b}	84.73 ± 0.49^{ab}	0.14 ± 0.27	$1.40 \pm 0.59^{*}$
	PDR (%)	5.34 ± 0.20^{c}	6.88 ± 0.40^{d}	5.97 ± 0.37^{cd}	-0.31 ± 0.20	$-1.22 \pm 0.45^{*}$
	ASR (%)	6.18 ± 0.23	6.98 ± 0.55	6.66 ± 0.41	-0.24 ± 0.23	-0.56 ± 0.50
	Fertility	Number of boars			Additive	Dominance
		200	44	56		
	NRR42 (%)	0.64 ± 0.44	-0.64 ± 1.13	-2.24 ± 2.60	1.42 ± 1.30	-1.41 ± 1.65
	NBA (per litter)	0.20 ± 0.11	0.10 ± 0.09	0.02 ± 0.08	-0.09 ± 0.14	-0.09 ± 0.11
Polymorphism	Trait	*ESR1* genotype ($\mu \pm$ S.E.)			Effect ($\mu \pm$ S.E.)	
		TT	TC	CC	Additive	Dominance
	Sperm quality	Number of observations[#]				
		26,015	5837	637		
g.35756T > C	SCON (10^8/ml)	3.02 ± 0.04	3.21 ± 0.10	2.96 ± 0.23	0.02 ± 0.11	-0.21 ± 0.15
	VOL (ml)	257.27 ± 3.49	253.35 ± 7.67	257.45 ± 7.24	-0.09 ± 0.70	4.01 ± 1.59
	MOT (%)	84.67 ± 0.24	84.93 ± 0.54	85.87 ± 1.20	-0.62 ± 0.60	0.31 ± 0.80
	PDR (%)	5.72 ± 0.18	5.93 ± 0.41	4.47 ± 0.91	0.63 ± 0.46	-0.80 ± 0.61
	ASR (%)	6.45 ± 0.21	6.26 ± 0.47	5.76 ± 0.21	0.35 ± 0.53	-0.14 ± 0.71
	Fertility	Number of boars			Additive	Dominance
		230	41	8		
	NRR42 (%)	0.11 ± 0.48^{e}	2.50 ± 1.02^{f}	0.20 ± 0.28^{a}	-0.14 ± 1.73	-3.24 ± 2.18
	NBA (per litter)	0.01 ± 0.04	0.04 ± 0.09	0.25 ± 0.06	-0.01 ± 0.10	-0.06 ± 0.11

[#]Repeated measurements.
[a,b]$P < 0.01$; [c,d]$P < 0.001$; [e,f]$P < 0.05$. *$P < 0.05$.

differences were non-significant in the testis, body and tail of epididymis in between G-I and G-II boars (Fig. 2b). *ESR1* protein with 66 kDa molecular weight was detected in testis, head, body and tail of the epididymis in both of G-I and G-II boars (Fig. 2c). The western blot result showed that the *ESR1* protein expression was higher in head of the epididymis in G-I boars. This protein expression seemed to be consistent with the results of transcription levels.

3.5. Localization of ESR1 protein in boar reproductive tissues by immunofluorescence

Sections of testis, head, body and tail of epididymis were stained through the same optical panel for the *ESR1* protein expression (Fig. 3). Immunoreactive *ESR1* protein was observed as strong staining in cytoplasm of Sertoli cells but poorly in germ cells and Leydig cells in testis (Fig. 3a). Spermatozoa within the lumen of the epididymis head were found to be positively immunostained with *ESR1* (Fig. 3b). Similarly, spermatozoa within the lumen of the epididymis body and tail were positive to immunoreactive *ESR1* protein (Fig. 3c and d). The immunofluorescence of *ESR1* protein was specifically localized in the post acrosomal region (arrow) and tail of the sperm (arrow head) when spermatozoa within the epididymis were examined (Fig. 3e). More precisely, the epithelial cells in the head and tail of the epididymis expressed higher immunoreactive *ESR1* protein (Fig. 3b and d), whereas the epithelial cells in the epididymis body poorly expressed the target protein (Fig. 3c). In the case of the epididymis head and body, the sterocilia cells were remarkably higher immunostained while in the epididymis tail the protein was strongly localized in the smooth muscle (Fig. 3b–d).

4. Discussion

4.1. Association of SNP with boar sperm quality and fertility traits

This study revealed association of *ESR1* with sperm quality and fertility traits in boars (Table 4). The exonic SNP g.672C > T was found to be significantly associated with sperm quality trait: sperm motility and plasma droplet rate. Association study of *ESR1* is rare in boars, but Lazaros et al. (2010) reported two SNPs of *ESR1* at 397 (T > C) and 351 (A > G) had a positive effect on sperm quality in humans. Moreover, Suzuki et al. (2002) reported a polymorphism of *ESR1* in exon 4 associated with idiopathic azoospermia in humans. The existence of *ESR1* at the post acrosomal sperm head region (Solakidi et al., 2005) indicated a role of

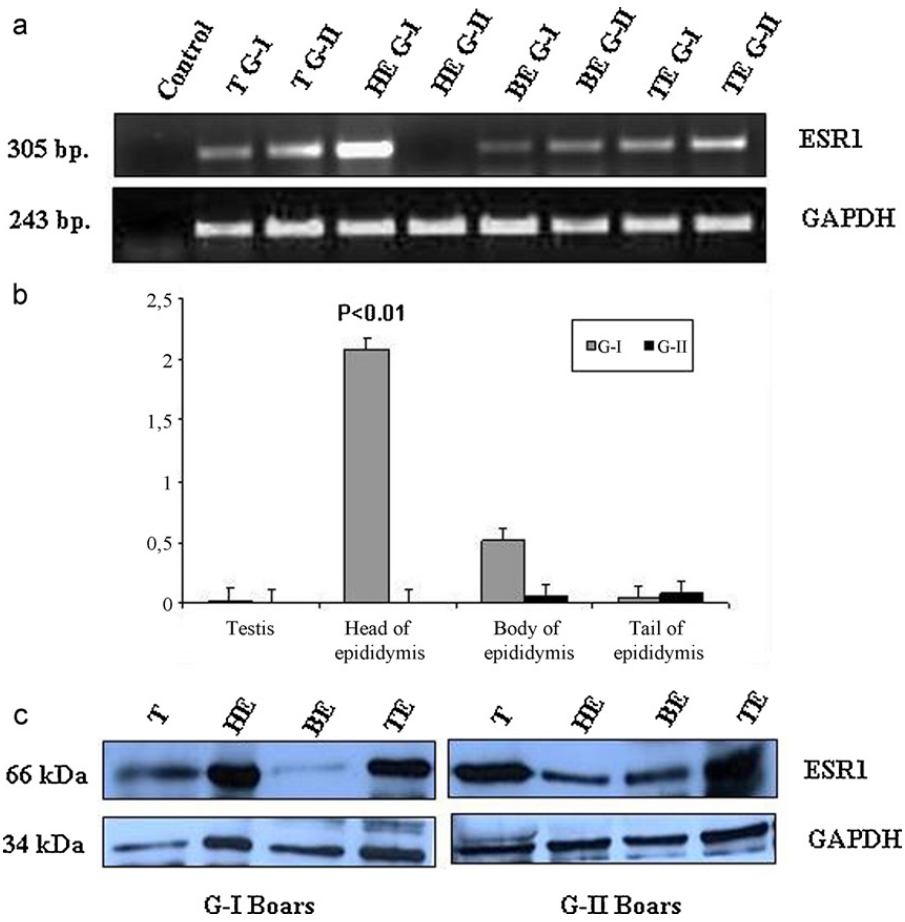

Fig. 2. mRNA and protein expression of *ESR1* in reproductive tissues (testis, head, body and tail of the epididymis). (a) *ESR1* mRNA expression in different reproductive tissues from G-I and G-II boars by semi-quantitative PCR. (b) *ESR1* mRNA expression in different reproductive tissues from G-I and G-II boars by qRT-PCR. (c) Protein expression in different reproductive tissues from G-I and G-II boars by western blotting. (T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis.)

estrogens in male gamete maturation and motility. It has been reported that estrogen produced locally is involved in the regulation of sperm motility (Lazaros et al., 2010). *ESR1* polymorphism may influence these locally acting estrogen levels with effect on sperm motility (Carreau et al., 2002). Abnormal sperm production and reduced fertility have been reported in transgenic male mice lacking *ESR1* (Eddy et al., 1996). Infertility from lacking *ESR1* is reported mainly due to the disruption of fluid reabsorption in efferent ductules which increased the backpressure of the accumulating luminal fluids that leads to a progressive degeneration of the testicular tissue and severely impaired spermatogenesis (Eddy et al., 1996; Hess et al., 1997; Couse and Korach, 1999). The identified SNP is confirmed in exon which may play important role in transcription process. Polymorphism in the coding region could have a direct effect through changing the nucleotide sequence and structure of gene, possibly leading to changes in mRNA synthesis, splicing, maturation, transportation, translation or degradation (Iida and Akashi, 2000).

The intronic SNP g.35756T > C was significantly associated with the fertility trait non-return rate (NRR) (Table 4). Reproductive efficiency of boars is usually measured by non-return rate, which is defined as percentage of sows not appointed for a second insemination within a period of days after the first insemination. Male fertility can be regarded as a result of both fertilizing ability of the sperm cells as well as the number of fertilized oocytes and the viability of the embryo. But, under the field conditions only the outcome of the inseminations can be recorded, i.e. whether or not the females have returned for repeat insemination (Stalhammar et al., 1994). However, little is known about the polymorphism and association of porcine *ESR1* with fertility trait like NRR in pigs. An *ESR1* gene variant at c.1227C > T in exon 5 showed a positive effect on litter size in sows (Munoz et al., 2007). It is important to note that, the detected SNP at *ESR1* g.35756T > C was in intron 1 which is subjected to less functional constraint and may change the primary structure of the *ESR1*. Though 'silent', it could affect *ESR1* function by altering the mRNA stability (Capon et al., 2004). Moreover, there is increasing number of reports describing the role of intron in regulating the expression level of a gene or tissue specific expression pattern (Jiang and Le, 2000; Pagani et al., 2004). Traits related to fertility of boars are of low heritability (h^2 = 0.01–0.06) and are strongly affected by environmental and genetic effects of the boar itself, the sow and the offspring (See, 2000). Therefore, effects of single loci are expected to be low and require a higher number of animals to be identified. In contrast, the sperm quality traits have

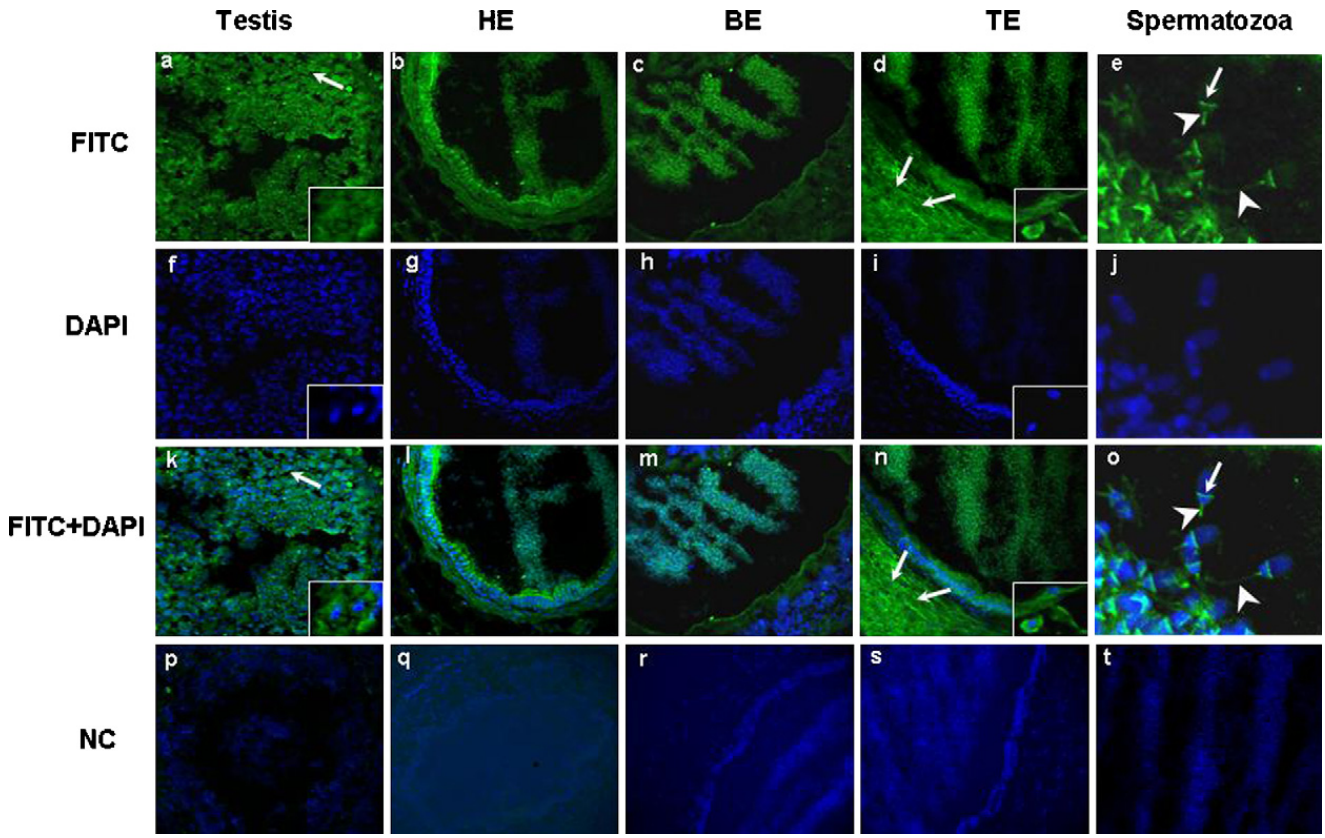

Fig. 3. Localization of *ESR1* protein in different parts of boar reproductive tissues. (a) Immunofluorescence detection of *ESR1* in cytoplasm of Sertoli cells and germ cells. Sertoli cells were stained with *ESR1* (arrows) and the nuclei were counterstained with DAPI (arrow). (b and c) *ESR1* protein localization in epithelial cells in the head and body of the epididymis and in the spermatozoa within the lumen of the epididymis. (d) Localization of *ESR1* protein in epithelial cells, in spermatozoa within the lumen of the epididymis and remarkable expression was in smooth muscle in the tail of the epididymis. Smooth muscle in the tail of the epididymis was stained with *ESR1* (arrows). (e) The *ESR1* localized in acrosomal region (arrow) and tail of spermatozoa (arrow head) within epididymis. (f–j) The cell nuclei were counterstained with DAPI. (k–o) Merged images. (p–t) Negative control. (T: Testis, HE: Head of epididymis, BE: Body of epididymis, TE: Tail of epididymis.)

moderate to medium heritability (h^2 = 0.19–0.37) (See, 2000). However, two SNPs of *ESR1* are identified in both exon and intron which might have an important effects on sperm quality and fertility traits in pig.

4.2. *ESR1* mRNA and protein expression in boar reproductive tissues

The physiological role of estrogens in male reproduction is now extensively revisited (Carreau et al., 2002) but expression study is rare in boars. In this study, mRNA expression analysis by semi-quantitative PCR and qRT-PCR demonstrated that the porcine *ESR1* gene expression was higher in reproductive tissues than in non-reproductive tissues (Fig. 1). Importantly, *ESR1* expressed higher in the head of epididymis collected from G-I boars than that of G-II boars. The highest expression of *ESR1* in the head of the epididymis was in agreement with the previous reports describing that the highest concentration of estrogen receptor was found in the epidydimis head in mice and macaques (Schleicher et al., 1984). It is well documented that male estrogen is in high presence in the efferent ducts in the head of epididymis (Hess and Carnes, 2003). The presence of *ESR1* in the head of the epididymis has immense importance since estrogen plays a crucial function in fluid reabsorption in this region in the male (Hess et al., 1997). This fluid reabsorption is important for sperm maturation and for maintaining the number of sperm in transit (Janulis et al., 1996). Any disruption of the estrogen receptor gene in male mice resulted with decreased fertility and testicular weight and very low sperm counts (Lubahn and Golding, 1993). Moreover, *ESR1* abundance is detected in different parts of male reproductive tract and spermatozoa in mice (Zhou et al., 2002), humans (Aquila et al., 2002) and rodents (Pelletier et al., 2000) which coincides with our findings. The evidence of estrogen in different parts of the male reproductive tract suggests a possible physiological role of estrogen in spermatogenesis and sperm maturation. Furthermore, the lower *ESR1* transcript abundance in the head of the epididymis of G-II boars in this study may indicate a lack of estrogen action which may contribute to the poor sperm quality and fertility. It is known that sperm and its membrane proteins undergo several modifications during maturation within the epididymis. Estrogen antagonist treatment in mice showed that lack of *ESR1* function in the epididymis head causes failure of fluid reabsorption, impaired sperm production and loss of sperm motility (Shayu et al., 2004). Infertility in lacking *ESR1* is reported mainly due to the disruption of fluid reabsorption in efferent ductules which increased backpressure of the accumulating luminal fluids that leads to a progressive

degeneration of the testicular tissue and severely impaired spermatogenesis (Eddy et al., 1996; Hess et al., 1997; Couse and Korach, 1999). Moreover, swelling and damage of seminiferous tubules and the dilution of the essential proteins secreted from epididymis also contributed to the infertility (Eddy et al., 1996; Hess et al., 1997; Couse and Korach, 1999).

In this study, the protein expression analysis by western blotting showed that *ESR1* antibody recognized a band at 66 kDa in all tissues. The band is similar to the molecular weight of human (66 kDa) and mouse (66 kDa) *ESR1* and very close to that of the stallion (65 kDa). Although, the levels of protein in different tissues from G-I and G-II were not as distinguishable as mRNA, the *ESR1* protein expression was remarkably higher in the head of the epididymis of G-I boars than that of G-II boars (Fig. 2b). The vas deferens in the head of the epididymis are the major sites for *ESR1* expression which regulates the testicular fluid reabsorption and increases the sperm cell concentration as they enter the epididymis (Hess et al., 1997). Moreover, the epididymis is involved in different protein absorption as well as in protein secretion (Syntin et al., 1999). The head and body of the epididymis are reported to secret higher amounts of different proteins in boars in comparison to other farm animals (Syntin et al., 1999). These protein secretions are regulated by estrogen and the proteins are involved in the sperm membrane remodelling, in the initiation of sperm motility, and especially in sperm-egg interaction (Pearl et al., 2007). It has been calculated that 50–90% of the total protein leaving the testis is absorbed by the efferent ducts in the head of the epididymis (Clulow et al., 1994).

4.3. Protein localization of ESR1

ESR1 was found to be expressed strongly in the cytoplasm of Sertoli cells, expressed on germ cells and Leydig cells in testis (Fig. 3a). These results are in agreement with previous results localizing *ESR1* in Sertoli and Leydig cells (Hess and Carnes, 2003) and in Leydig and germ cells in rat (Pelletier et al., 2000). Pelletier et al. (2000) suggested that in the Leydig cells the *ESR1* might be involved in the secretion and maturation of germ cells. Estrogen are reported to be involved in maintaining the Sertoli cell function (O'Donnell et al., 2001) as well as in establishing Sertoli–germ cell adhesion (MacCalman and Blaschuk, 1994). However, the cellular distribution patterns of *ESR1* protein between species could be different.

We localized *ESR1* in the head, body and tail of the epididymis. When sperm are released from their 'nurse' (Sertoli) cells in the testis, they are transported in fluid secreted by the Sertoli cells to a collecting area, the rete testis. From there, the dilute suspension of sperm enter the thin-walled efferent ducts in the epididymis head and the epithelia of efferent ducts express enormous estrogen receptors (Fisher et al., 1997). The sperm pass through the epididymis where they mature and progress toward the vas deferens. Moreover, the *ESR1* in the epididymis are reported to modulate secretion of proteins such as oscilin that promotes the maturity and viability of the spermatozoa (Mowa and Iwanaga, 2001). The most intense signals of *ESR1* are reported in epithelial cells in the head and tail of the epididymis in rats and monkeys and suggested to be responsible for semen concentration (Fisher et al., 1997; Hess et al., 1997). Our study identified a strong signal of *ESR1* in the smooth muscle layer in the tail of the epididymis which is in accordance with a previous immunohistochemical study in human and rabbit which stated that estrogen is essential for transporting the spermatozoa by influencing epididymal smooth muscle contractility as well as for ejaculation with the help of oxytocin (Filippi et al., 2002). It is important to note that the expression of *ESR1* in different parts of the epididymis could vary among species since it has been localized in all parts of epididymis in the rat (Pelletier et al., 2000), mouse (Zhou et al., 2002), bonnet monkey (Shayu et al., 2004), horse (Parlevliet et al., 2006), cat (Nie et al., 2002) and dog (Nie et al., 2002).

We found that the *ESR1* is remarkably localized in sperm within the lumen of the epididymis. *ESR1* was especially immunolocalized in the post acrosomal region and tail of the sperm in this study (Fig. 3e). This result is in good agreement with previous studies which localized immunoreactive *ESR1* in the post acrosomal region of the sperm head (Solakidi et al., 2005) and in the tailpiece of the sperm (Durkee et al., 1998) in humans. The post acrosomal region of sperm is involved in the sperm–egg plasma membrane fusion (Liu et al., 2008). The localization of *ESR1* in the post acrosomal region implies its involvement in the fertilization process (Ramalho-Santos et al., 2002). It is important to note that there are other proteins identified in the post acrosomal region of sperm such as equatorin and oscilin which are important for successful fertilization (Montag et al., 1998; Solakidi et al., 2005). Our localization of *ESR1* in the tail of porcine sperm coincided with previous reports in humans (Durkee et al., 1998; Aquila et al., 2002). Moreover, Aquila et al. (2002) reported that the *ESR1* might be involved in sperm survival and motility. Impaired motility of sperm has been reported in mice lacking functional *ESR1* in sperm (Eddy et al., 1996) and in *ESR1* knockout mice (Hess and Carnes, 2003). Low fertilization rates have been reported in case of immotile spermatozoa (Nijs et al., 1996).

5. Conclusion

Associations of *ESR1* gene polymorphisms with boar sperm quality and fertility traits have been described for the first time, providing evidence that *ESR1* might be an important candidate gene for boar sperm quality and fertility. However, this study has to be validated in other animal populations in order to evaluate its potential in selective breeding. Finally, the *ESR1* might play a role in spermatogenesis validated through association study and by profiling of mRNA and protein expression in non-reproductive and reproductive tissues including spermatozoa.

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