Reproduction in Domestic Animals

Reprod Dom Anim doi: 10.1111/j.1439-0531.2011.01968.x
ISSN 0936-6768

Investigation on Association and Expression of *ESR2* as a Candidate Gene for Boar Sperm Quality and Fertility

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Contents

ESR2 is involved in oestrogen-related apoptosis in cell cycle spermatogenesis but their effects have not yet confirmed in pig. Therefore, this study was aimed to investigate the association of *ESR2* polymorphism with sperm quality and boar fertility traits and to analyse the *ESR2* mRNA and protein expressions in boar reproductive tissues. DNA samples from 203 Pietrain (PI) and 100 Pietrain × Hampshire (PIHA) pigs with records of sperm quality [sperm concentration (SCON), motility (MOT), semen volume (VOL), plasma droplet rate (PDR) and abnormal spermatozoa rate (ASR)] and fertility [non-return rate (NRR) and number of piglet born alive (NBA)] traits were available. A SNP in coding region of *ESR2* g.35547A > G in exon 5 was associated with MOT and PDR in the PI and with SCON, VOL, MOT and PDR in PIHA population. For mRNA and protein expression study, a total of six boars were divided into two groups with group I (G-I) and group II (G-II) where G-I characterized for relatively a better sperm quality according to the mean of two groups. mRNA expression was higher in brain and testis than that in all parts of epididymis. Both qRT-PCR and western blot analysis revealed that the *ESR2* gene expression and protein expression were significantly higher in testis collected from G-II compared with that of G-I boars. Moreover, ESR2 protein localization in germ cell, Leydig and Sertoli cells, epithelial cells and spermatozoa was remarkable, which indicated the important role of *ESR2* in spermatogenesis process. These results might shed new light on the roles of *ESR2* in spermatogenesis as candidate for boar fertility, but still the lack of association across populations should be considered.

Introduction

Oestrogens are classically known to play a major role in female reproduction, but there is now compelling evidence that they may also be involved in the regulation of male reproductive functions. Oestrogens are intimately involved in male fertility, and their function is mediated through binding with the oestrogen receptors 1 and 2 (*ESR1* and *ESR2*) (Couse and Korach 1999). It has been speculated that *ESR2* could act as a negative regulatory partner for *ESR1* (Weihua et al. 2000). The lack of *ESR1* leads to reduced epididymal sperm content, reduced sperm motility and fertilizing ability (Couse and Korach 1999), while the overexpression of *ESR2* results in germ cell cycle arrest or apoptosis and infertility (Selva et al. 2004). Aschim et al. (2005) reported a significant association of *ESR2* polymorphism with infertility in human with less sperm concentration, testicular cancer and cryptorchidism. The *ESR2* mRNA was highly expressed in the epididymis of adult mouse, rat, dog, cat and monkey (Hess et al. 1997; Saunders et al. 1997; Nie et al. 2002; Zhou et al. 2002). Rago et al. (2007) and Saunder et al. (2001) localize ESR2 protein in spermatozoa within the testis in human and primates, respectively. However, the *ESR2* gene has scarcely been investigated as a candidate gene for sperm quality and fertility traits in pigs. In pig, *ESR2* is located at the telometric end on the q-arm of SSC1 (Munoz et al. 2004). In this region, QTL for total sperm per ejaculate and sperm motility in boars (Xing et al. 2008) and QTL for number of nipples and age at puberty in sows are reported (Cassady et al. 2001).

Before reaching sexual maturity, a number of germ cells undergo physiological apoptotic death, which has been shown to be controlled by a large number of genes, including the *ESR2* (Delbes et al. 2004). 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. In spermatogenesis, functional male gametes are produced through complex processes in the testis, epididymis and other male reproductive tract (Frungieri et al. 2006). Failure in any of these events leads to disturbances in male fertility. *ESR2* is expressed in the cellular type during normal spermatogenesis but its function is still unknown. For the better understanding of *ESR2* functions in spermatogenesis in pig, the expression and localization of *ESR2* at different parts of reproductive tract including non-reproductive tissues are important. Considering together, it could be speculated that *ESR2* might be a functional as well as a positional candidate gene for male reproduction traits in pigs. But to the author's knowledge, no study was devoted to unravel its association with sperm quality and boar fertility traits, and the functions of *ESR2* in boar spermatogenesis within the reproductive tracts by mRNA and protein expression are poorly understood. Therefore, the aims of this research were to study the association of *ESR2* with boar sperm quality and fertility traits and to investigate the *ESR2* mRNA and protein expressions in sperm and reproductive tissues from boars with divergent phenotype.

Material and Methods

Animals and traits used in association study

Semen samples from Pietrain (PI, n = 203) and Pietrain × Hampshire crossbred (PIHA, n = 100) 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 of more than 31 000 ejaculates were repeatedly collected from these boars. Whole ejaculates were obtained from purebred Pietrain and crossbred Pietrain × Hampshire boars aged between 2 and 5 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 employing light microscopic evaluation according to the guidelines of the World Health Organization. Semen was collected by the vinyl glove hand method twice/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).

Genotyping of ESR2 SNP

As a single nucleotide polymorphism, the arginine (A)-to-guanine (G) transversion of *ESR2* at g. 35547A > G in exon 5 reported by Munoz et al. (2007) was further investigated in this study. For PCR amplification, the forward (5′-cttccttggatttagcc-3′) and reverse (5′-atgctctccttcttcggtga-3′) primer pairs were designed covering exon 5 of porcine *ESR2* genomic sequence (GenBank accession No. ENSSSCG00000005632) 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 $MgCl_2$), 0.25 mM of each dNTP, 5 pmol of each primer and 0.1 U of Taq DNA polymerase (GeneCraft). The PCR were 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 57°C, 30 s at 72°C and final elongation of 10 min at 72°C. After checking the PCR products in 1.5% (w/v) agarose gels, genotyping was carried out following the restriction fragment length polymorphism (RFLP) analysis. The digestion of restriction enzyme *Fat*I (New England BioLabs, Ipswich, MA, USA) was carried out in 10 μl of reaction mixture of each sample and incubated overnight at 65°C. Restriction fragment length polymorphisms of 304 boars were detected by electrophoresis in 3% (w/v) agarose gels.

Statistical analysis for sperm quality traits

The association of the *ESR2* genotypes with sperm quality and quantity traits was carried out using mixed model (PROC MIXED) in the SAS software package (SAS Institute Inc., ver. 9.2, Cary, NC, USA) as described by Kaewmala et al. (2011).

$$y_{ijkl} = \mu + \text{season}_i + \text{genotype}_j + \text{age}_k + \text{ejaculation}_l + \epsilon_{ijkl} \text{ [Model 1]}$$

where y_{ijkl} is the sperm quality traits (SCON, VOL, MOT, PDR and ASR); μ is the overall population mean; season_i is the fixed effect of the i-th season ($i = 1$ through 8; four seasons/year, in total eight seasons within 2 years from January 2000 to December 2001); genotype_j is the fixed effect of the j-th genotype ($j = 1, 2$ and 3); age_k is the effect of boar age (covariable); ejaculation_l is the permanent environmental effect of the l-th boar (random); and ϵ_{ijkl} is the residual error. As ejaculation was recorded as repeated measurement, it was considered as random effect in the statistical model.

The association analysis between *ESR2* and the fertility traits was carried out using the following generalized linear model (PROC GLM) in SAS (Kaewmala et al. 2011).

$$y_{ij} = \mu + \text{genotype}_i + \text{year}_j + \epsilon_{ij} \text{ [Model 2]}$$

where y_{ij} is the boar fertility trait (NRR and NBA); μ is the overall population mean; genotype_i is the fixed effect of the i-th genotype ($i = 1$, 2 and 3); year_j is the fixed effect of the j-th boar year of birth ($j = 1$ through 3: boar born before 1996, in 1996–97 and in 1998–99); and ϵ_{ij} is the residual error.

The distribution of the genotype was tested for Hardy–Weinberg equilibrium by chi-square (χ^2) test. Least-square mean values for the *ESR2* genotypes were compared by t-test and p-values, adjusted by the Tukey–Kramer correction.

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) and non-reproductive tissues (brain, muscle and liver) from six breeding boars with divergent phenotypes were collected from the AI station (SuisAG, Sempach, Switzerland) for mRNA and protein study as described earlier by Kaewmala et al. (2011). For differential expression study between reproductive and non-reproductive tissues by reverse transcription PCR (RT-PCR) study, mRNA from all six boars were 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 RT-PCR, qRT-PCR and western blot, respectively. For these purposes, the six boars were divided into two groups based on extreme phenotypes (high/low SCON, SMOT and SVOL). Correlation between sperm quality traits was carried out using correlation analysis (PROC CORR) in SAS. 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. Therefore, grouping was made on the basis of SCON, SVOL and SMOT (Table 1). The six boars were selected and equally divided into group I (G-I) with high SCON ($> 262.32 \times 10^6$ ml), high SMOT ($> 76.59\%$) and low SVOL (< 215.24 ml/ejaculation) and group II (G-II) with low sperm concentration and motility and high sperm volume (Table 1). The differ-

Table 1. Means, standard deviation (SD), number of boars and ranges of traits selected for mRNA and protein expression study

	Selected animals (n = 6)		G-I (n = 3)		G-II (n = 3)		
Traits	Mean	SD	Mean	SD	Mean	SD	p-value
SCON (10^6/ml)	262.32	87.97	335.94	50.78	188.70	22.54	<0.05
SVOL (ml)	215.24	34.93	185.07	16.33	245.40	7.42	<0.01
SMOT (%)	76.59	3.71	79.03	1.89	74.14	3.60	0.12

ence 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 ($p = 0.12$).

Reverse transcription PCR

Total RNA was isolated using TRI Reagent (Guanidinium thiocyanate-phenol-chloroform extraction) (Sigma-Aldrich, Munich, Germany) from different reproductive and non-reproductive tissues of breeding boars mentioned earlier. RNA was purified using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was treated using on-column RNase-Free DNase set (Promega, Mannheim, Germany) and quantified spectrophotometrically (ND8000; Nano Drop, Thermo Scientific, Wilmington, DE, USA). Furthermore, RNA integrity was checked by 2% agarose gel electrophoresis. First-strand cDNA were synthesized from individual RNA using Superscript II enzyme (Invitrogen, Darmstadt, Germany).

cDNA amplification was performed by using specific forward and reverse primers (forward: 5′-ggccccatatatatccctcc-3′ and reverse: 5′-gagttggccacaacacattg-3′) derived from porcine *ESR2* sequence (GenBank accession AF164957). 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 58°C for 1 min and 72°C for 1 min, on the PCR Thermal Cycler (BioRad, Munich, Germany). PCR products were electrophoresed on a 1.5% agarose gel and visualized upon staining with ethidium bromide. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as housekeeping gene.

Quantitative real-time PCR

For real-time PCR, total RNA was isolated using TRI reagent (Sigma; Sigma-Aldrich, Munich, Germany) from different reproductive tissues of two divergent groups of animals (G-I and G-II) as described in aforesaid section. cDNA synthesis was carried out as described in the previous section. The same primer pairs used in RT-PCR were also used in qRT-PCR. Ninefold serial dilutions of plasmid DNA were prepared and used as template for the generation of the standard curve. In each run, the 96-well microtitre plate contained each cDNA sample, plasmid standards for the standard curves and no-template control. To ensure the repeatability of the experiments, each plate 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, Carlsbad, CA, USA) qRT-PCR system. 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. Glyceraldehyde 3-phosphate dehydrogenase was quantified twice as technical replication and the average was used for the normalization of target gene ESR2. Final results were reported as the relative abundance level after normalizing with mRNA expression level of the housekeeping gene. Differences in *ESR2* 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.

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 three G-I boars were pooled together and proteins from three G-II boars were pooled together according to the tissues. The proteins extracted from tissues were separated by SDS-PAGE (gradient 4–18%). Subsequently, the proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). After blocking in blocking buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 1% polyvinylpyrrolidone) at room temperature for 1 h, the membrane was incubated with the anti-ESR2 antibody purified from goat polyclonal antibody (Cat.nr. Sc6822; Santa Cruz, Heidelberg, Germany) 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-goat IgG (Cat.nr. Sc2020; Santa Cruz) was used as the secondary antibody (diluted 1 : 50 000). 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 chemiluminescence was detected by using the ECL plus western blotting detection system (Amersham Biosciences) and was visualized by using Kodak BioMax XAR film (Kodak, Stuttgart, Germany). Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control and for normalization. The membrane was stripped by incubation in 2% SDS, 100 mM Tris–HCl and 0.1% β-mercaptoethanol for 30 min at 60°C and reprobed with GAPDH antibody (Cat.nr. Sc20357; Santa Cruz).

Protein localization by immunofluorescence

Owing to the limitations of fresh samples from G-I and G-II boars, we collected different fresh reproductive

tissues from a healthy breeding boar after slaughtering for protein localization by immunofluorescence (Kaewmala et al. 2011). Immunofluorescence staining was performed on 8-μm cryostat sections of snap-frozen tissues. All sections were kept at −80°C for further analysis. To block unspecific staining, sections were incubated for 30 min at room 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 ESR2 goat polyclonal primary antibody (Cat.nr. Sc6822; Santa Cruz) diluted at 1 : 50 in PBST followed by six times (10 min to time) washing with PBS. Then, the sections were incubated for 1 h at room temperature with the biotinylated donkey anti-goat IgG-B conjugated with fluorescein isothiocynate (FITC)-reactive water-soluble fluorescent dye (Cat nr. Sc2090; Santa Cruz) (dilution 1 : 200) as a secondary antibody for ESR2 followed by six times (10 min to time) washing with PBS. Finally, the samples were counterstained with vectashield mounting medium (Vector Laboratories, Loerrach, Germany) containing 4,6-diamidino-2-phenyl indole (DAPI) and covered with a cover glass slip. The staining was observed by confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). In case of negative controls, PBS was used instead of the primary antibody.

Results

Association study

The polymorphism of *ESR2* at g. 35547A > G in exon 5 was used to genotype the PI and PIHA population by PCR–RFLP. The DNA restriction fragments obtained for the *ESR2-Fat*I polymorphism were 284, 146 and 28 bp for the AA genotype, 284, 174, 146 and 28 bp for the AG genotype and 284 and 174 bp for the GG genotype. The genotype and allele frequencies of porcine *ESR2* gene calculated are shown in Table 2. Homozygote AA was more frequent, and homozygote GG was rare in both populations. The chi-square test revealed that the locus of *ESR2* was in Hardy–Weinberg equilibrium in both populations (Table 2).

The general descriptions of sperm quality and fertility traits are shown in Table 3. Means of VOL, MOT and PDR traits in the crossbred PIHA were higher compared with those in the PI populations. The NRR42 was higher in PIHA compared with PI, while the NBA was similar in both PI and PIHA populations.

Association analysis of g.35547A > G with sperm quality and fertility traits revealed significant ($p < 0.01$) association with MOT and PDR and suggestively ($p = 0.06$) with NBA in PI population. SCON, VOL, MOT and PDR were found to be significantly ($p < 0.05$) associated with the g.35547A > G genotype in the PIHA population (Table 4). This association with MOT and PDR was consistent in PI and PIHA populations. The genotype AA is different from AG/GG genotype. The genotypes AG and GG were associated with lower MOT and higher PDR than the animals having a AA genotype in the PI population (Table 4). In the PIHA population, the animals AG and GG genotypes were associated with lower SCON and MOT, but higher VOL and PDR than animals having AA genotype. The polymorphism g.35547A > G of *ESR2* showed highly additive effect on MOT ($p < 0.01$) in PI population. The results also indicated higher additive effect on SCON and VOL ($p < 0.05$) in PIHA population (Table 4).

Table 2. Genotype, allele frequencies and the chi-square test of the porcine *ESR2* gene in different pig populations

		Genotype frequency			Allele frequency		Chi-square test	
Breed	Number	AA	AG	GG	A	G	χ^2	p-value
Pietrain	203	0.89(176)	0.10(20)	0.01(7)	0.92	0.08	0.01	0.98
Pietrain × Hampshire	100	0.79(77)	0.18(18)	0.03(5)	0.86	0.14	0.02	0.99

Table 3. Means, standard deviation (SD), sample size, ranges of traits in Pietrain (PI) and Pietrain × Hampshire (PIHA)

Population	Traits	Sample size	Mean	SD	Minimum	Maximum
PI	SCON (10^8/ml)	20 077	3.03	0.94	1	6
	VOL (ml)	21 248	237.03	57.32	25	522
	MOT (%)	20 782	84.72	4.37	65	95
	PDR (%)	20 805	5.41	3.33	0	15
	ASR (%)	21 056	6.53	4.18	0	20
	NRR42 (%)[a]	203	0.50	7.00	−24.07	18.62
	NBA (per litter)[a]	203	0.04	0.54	−1.69	1.27
PIHA	SCON (10^8/ml)	7327	2.95	0.97	1	6
	VOL (ml)	7826	297.50	81.62	56	546
	MOT (%)	7610	85.46	4.03	70	95
	PDR (%)	7617	5.76	3.14	0	15
	ASR (%)	7723	4.95	4.00	0	20
	NRR42 (%)[a]	100	0.97	4.18	−12.23	13.79
	NBA (per litter)[a]	100	0.04	0.51	−2.97	1.10

ASR, abnormal spermatozoa rate; MOT, motility; PDR, plasma droplet rate, SCON, sperm concentration; VOL, semen volume. [a]Fertility (NRR42, NBA) corrected with factors: parity, farm, season and breed.

mRNA expression by reverse transcription PCR

ESR2 gene expression was higher in brain and testis, and lower expression was found in the head, body and tail of epididymis. The mRNA expression of *ESR2* was not detectable in accessorial gland (vas deferens, bulbourethral, vesicular and prostate glands), muscle and liver. The RT-PCR result of *GAPDH* showed no remarkable differences among tissues (Fig. 1).

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

The *ESR2* mRNA was expressed in testis, body and tail of epididymis from both the G-I and G-II boars but higher expression was found in testis of G-II than in that of G-I boars by RT-PCR (Fig. 2a). These mRNA expression results of RT-PCR appeared to be consistent with the results of the qRT-PCR. The *ESR2* mRNA expression was higher in testis of G-II compared with that of G-I boars ($p < 0.01$), whereas the difference in expression level was not statistically significant in case of head, body and tail of epididymis between G-I and G-II boars (Fig. 2b). *ESR2* protein with 56 kDa molecular weight was detected in testis, head, body and tail of

Table 4. Association of *ESR2* genotypes with sperm quality and fertility traits

Population	Trait		*ESR2* genotype ($\mu \pm$ SE)			Effect ($\mu \pm$ SE)	
			AA	AG	GG	Additive	Dominance
Pietrain (PI)		No. of observations[g]	17 608	3189	589		
	SCON (10^8/ml)		2.98 ± 0.04	3.03 ± 0.14	2.89 ± 0.28	0.04 ± 0.14	−0.09 ± 0.20
	VOL (ml)		244.37 ± 3.28	247.79 ± 10.15	231.89 ± 19.82	6.23 ± 10.04	−9.66 ± 14.28
	MOT (%)		85.38 ± 0.24[c]	83.76 ± 0.75[d]	81.55 ± 1.46[d]	1.91 ± 0.74**	−0.29 ± 1.05
	PDR (%)		5.04 ± 0.19[c]	6.99 ± 0.59[d]	7.13 ± 1.16[d]	−1.04 ± 0.59	−0.90 ± 0.84
	ASR (%)		6.17 ± 0.20	7.09 ± 0.63	8.05 ± 1.23	−0.93 ± 0.62	0.01 ± 0.88
		No. of boars	176	20	7		
	NRR42 (%)		0.58 ± 0.56	−0.59 ± 1.74	−0.87 ± 4.10	4.22 ± 2.60	−3.05 ± 2.60
	NBA (per litter)		0.04 ± 0.04[e]	0.32 ± 0.13[f]	0.38 ± 0.31[f]	−0.17 ± 0.16	−0.10 ± 0.20
Pietrain × Hampshire (PIHA)		No. of observations[g]	6270	1266	323		
	SCON (10^8/ml)		3.16 ± 0.07[a]	2.98 ± 0.15[a]	2.33 ± 0.33[b]	0.41 ± 0.17*	−0.24 ± 0.22
	VOL (ml)		265.34 ± 6.84[a]	271.85 ± 13.94[a]	360.03 ± 31.19[b]	−47.34 ± 15.97*	40.83 ± 21.17
	MOT (%)		85.13 ± 0.47[a]	82.33 ± 0.97[b]	83.32 ± 2.18[b]	0.90 ± 0.11	1.89 ± 1.48
	PDR (%)		5.43 ± 0.33[a]	7.58 ± 0.68[b]	7.54 ± 1.52[b]	−1.05 ± 0.78	−1.09 ± 1.03
	ASR (%)		6.06 ± 0.40	7.27 ± 0.82	5.84 ± 1.83	0.11 ± 0.94	−1.31 ± 1.24
		No. of boars	77	18	5		
	NRR42 (%)		0.46 ± 0.53	1.86 ± 1.11	3.31 ± 2.49	−1.42 ± 1.24	0.02 ± 1.57
	NBA (per litter)		−0.01 ± 0.06	−0.09 ± 0.14	0.01 ± 0.30	−0.01 ± 0.15	0.12 ± 0.19

ASR, abnormal spermatozoa rate; MOT, motility; PDR, plasma droplet rate, SCON, sperm concentration; VOL, semen volume. [g]Repeated measurements; [a,b]$p < 0.05$; [c,d]$p < 0.01$; [e,f]$p = 0.06$; *$p < 0.05$; **$p < 0.001$.

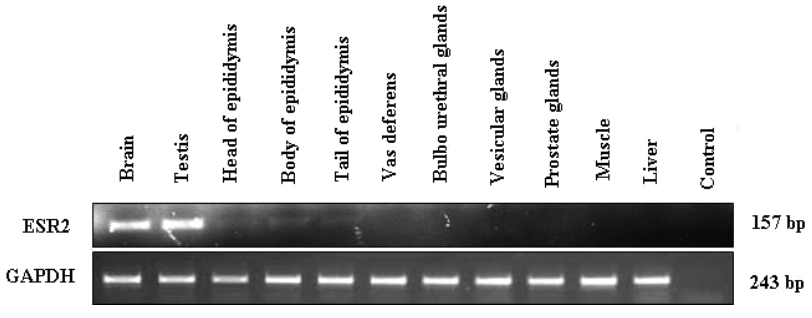

Fig. 1. mRNA expression of *ESR2* in reproductive and non-reproductive tissues by reverse transcription PCR

epididymis in both G-I and G-II boars (Fig. 2c). The western blot result showed that the *ESR2* protein was higher in testis in G-II compared with G-I boars. This protein expression seemed to be consistent with the results of transcription levels.

Localization of ESR2 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 cell-surface ESR2 protein expression (Fig. 3). The Leydig and Sertoli cells in testis and epithelial cells in all parts of epididymis showed signals for ESR2 immunoreactivity (Fig. 3a–d). Immunoreactive ESR2 protein was observed as strong staining in germ cell cytoplasm of Sertoli cells and Leydig cells in testis (Fig. 3a). ESR2 protein was expressed in epithelial cells of head (Fig. 3 b), body (Fig. 3c) and tail of epididymis (Fig. 3d). In case of spermatogenesis, ESR2 protein was expressed in spermatogonia, primary spermatocyte and spermatid (arrow head) within seminiferous tubules (Fig. 3a). The ESR2 protein was expressed in the spermatozoa located within the lumen of the body of epididymis. The ESR2 protein was expressed in the acrosomal cap (arrow) of boar spermatozoa (Fig. 3c).

Discussion

Association of SNP with sperm quality and boar fertility traits

This study revealed an association of *ESR2* with sperm quality and fertility traits in boars. The exonic SNP g.35547A > G was found to be significantly associated with sperm motility and plasma droplet rate and suggestively ($p = 0.06$) associated with number of piglet born alive in PI populations, whereas it was significantly associated with sperm concentration, semen volume, sperm motility and plasma droplet rate in PIHA populations. Sperm motility and plasma droplet rate are consistently associated in both the PI and PIHA populations. It could be seen in both populations that AA genotype contributed significantly to have higher sperm motility and less plasma droplet rate (Table 4). It is important to note that sperm motility and plasma droplet are significantly negatively correlated in our populations. In case of PIHA, genotype AA significantly contributed to higher sperm concentration and lower semen volume (Table 4), which are also in agreement with our correlation results that SVOL and SCON are significantly negatively correlated. In case of *ESR2*, association has been described in sows by Munoz et al. (2004, 2007) but they did not find any statistically

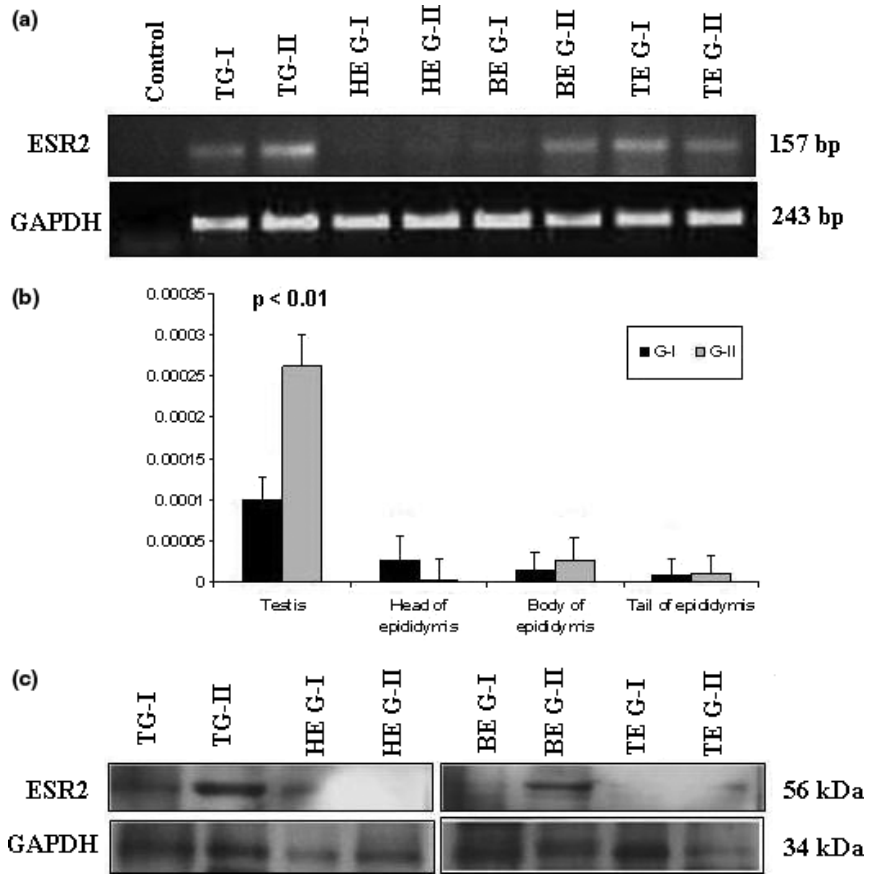

Fig. 2. mRNA and protein expression of *ESR2* in reproductive tissues (testis, head, body and tail of epididymis). (a) *ESR2* mRNA expression in different reproductive tissues from G-I and G-II boars by RT-PCR. (b) *ESR2* mRNA expression in different reproductive tissues from G-I and G-II boars by qRT-PCR. (c) The 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

significant association. However, in human, there are two reported polymorphisms of *ESR2* at 1082 (G > A) and 1730 (G > A) in exons 5 and 8, respectively, but only the SNP at 1082 (G > A) showed an association with male infertility (Aschim et al. 2005). This finding is almost similar to our study describing that the heterozygote AG genotype has negative effect on sperm quality. Aschim et al. (2005) reported a significantly increased frequency of the *ESR2* AG genotype among infertile man, compared with fertile control.

Polymorphism in *ESR2* at g.35547A > G in exon 5 had effect on sperm quality traits in this study. This SNP was observed in the coding region of the *ESR2* gene leading to an amino acid substitution (Met → Val) in the hormone-binding domain, which may be critical for its role as transcription factor (Munoz et al. 2007). Moreover, this alteration involves the replacement of the non-polar amino acid, valine, by the polar sulphur-containing amino acid, methionine, which could modify the secondary and tertiary structures of the protein because of the different ability of these amino acids to form hydrogen and disulphide bonds. The potential deleterious effect of the mutation p.Val317Met located in the ligand-binding domain is suggested by its conserved amino acid position among some mammalian species (*Rattus norvergicus*, GenBank accession number: Q62986, *Mus musculus*: O08537 and *Bos taurus*: Q9XSB5). In human, it has been reported that the *ESR2* polymorphism could have a direct effect through changing the nucleotide sequence and thereby the secondary structure of the *ESR2* mRNA, possibly leading to changes in mRNA synthesis, splicing, maturation, transport, translation or degradation (Iida and Akashi 2000). These data support the possible biological relevance of this amino acid change, and we found association with sperm quality traits. However, we could not detect any significant association of these SNPs with fertility traits. We found a suggestive association of g.35547A > G *ESR2* with number piglet born alive in PI population ($p = 0.06$) but not in PIHA population. Munoz et al. (2004) reported that SNP in *ESR2* are not associated with litter size in Iberian and Chinese–European sows. In addition, sampling additional animals for DNA sequencing may provide detection of other sequence variants in this gene that could be associated with fertility traits. An association study with more individuals should be conducted with this Met/Val substitution.

mRNA and protein expression in boar reproductive tissue

The *ESR2* was highly expressed in brain and testis when compared with other tissues, indicating that this gene might have important functions in these tissues. Repro-

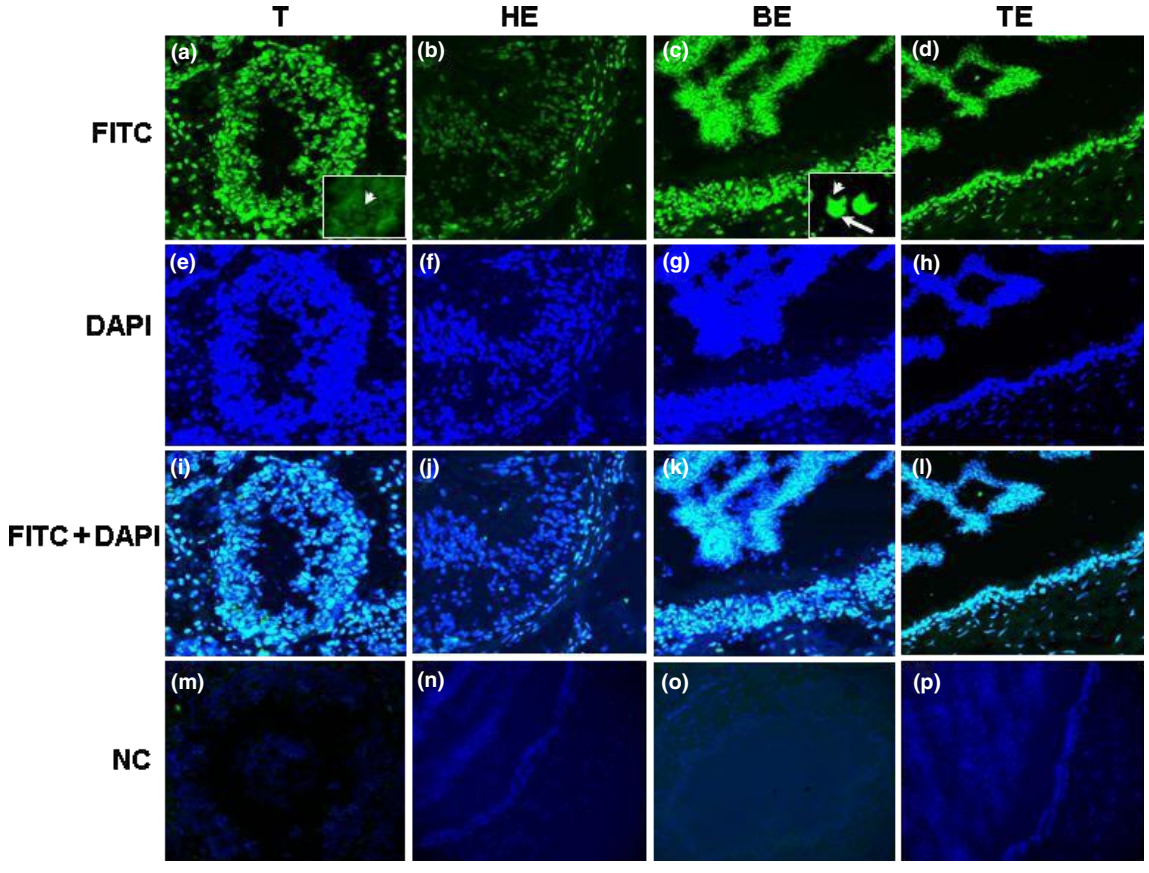

Fig. 3. Localization of ESR2 protein in different parts of the boar reproductive tissues. (a) Immunofluorescence detection of ESR2 in germ cell and in cytoplasm of Sertoli and Leydig cells. Germ cells were stained with ESR2 (arrows), and the nuclei were counterstained with diamidino-2-phenyl indole (DAPI). (b–d) ESR2 protein localization in epithelial cells in the head, body and tail of epididymis. (c) The ESR2 localized in acrosomal cap (arrow) of spermatozoa (arrow head) within the lumen body of epididymis. (e–h) The cell nuclei were counterstained with DAPI. (i–l) Merged images. (m–p) Negative control. Magnification 40×. T, Testis; HE, Head of epididymis; BE, Body of epididymis; TE, Tail of epididymis

ductive processes were maintained by hormone level initiated in the brain (Balthazart and Ball 1997a; Thompson et al. 1998). Enzyme *CYP19* catalyses the oestrogen production in brain, and *ESR2* is reported to express at the same area at brain where *CYP19* (aromatase subfamily 19) is reported to express (Balthazart 1998). It has been reported that testosterone must be transformed into an oestrogen by aromatization to activate the male sexual behaviour (Balthazart and Ball 1997b). Two responses that were known to be oestrogen dependent are the control of aromatase synthesis and the activation of reproductive behaviour (Balthazart 1998).

The expression of *ESR2* gene in the reproductive tissues of G-II was higher than that in G-I boars. The protein expression coincided with mRNA expression for *ESR2*. There were significantly higher *ESR2* mRNA and protein expressions in testis from G-II boars than in the testis from G-I boars. The *ESR2* tended to be higher in G-II, indicating that higher mRNA and protein expressions might have negative effect on boar sperm quality and fertility. *ESR2* is reported to be expressed higher in the semen of infertile males compared with fertile (Bujan et al. 1993). Moreover, the overexpression of *ESR2* is reported to cause apoptosis in cell cycle spermatogenesis (Selva et al. 2004). Another study reported that *ESR2* is involved in oestrogen-related apoptosis of germ cells, and as a consequence, there is a blockade of germ cell growth during foetal and neonatal life (Delbes et al. 2004). *ESR2* plays a role either in regulating the progression of the first meiotic division or in favouring the entrance of primary spermatocytes to an apoptotic pathway (Selva et al. 2004). One of the mechanisms by which *ESR2* might regulate cell cycle is through its direct and specific interaction with the cell cycle spindle assembly checkpoint protein, *Mad2* (Poelzl et al. 2000). In the spermatogenesis process, the over-expressed *ESR2* interacted with *Mad2* in meiosis I in mouse leading to a cell cycle arrest or apoptosis in metaphase I (Wassmann et al. 2003). However, *ESR2* gene has been expressed in diseased as well in normal testis (Makinen et al. 2001). It has been reported in *ESR2* knockout mice (ESR2KO) that *ESR2* is important for maintaining the testicular function (Oliveira et al. 2004). Adult *ESR2* knockout mice study showed that inactivation of *ESR2* affected the cellular composition of the testis (Gould et al. 2007), which may indeed have a direct role in the spermatogenesis. Yet, it is important to note that *ESR2* does not play a role alone in spermatogenesis. *ESR2* regulation is most likely of extratesticular origin, and a plausible candidate for the regulation of *ESR2* in the testis and epididymis would

be luteinizing hormone (*LH*) because a correlation between *ESR2* and *LHR* (luteinizing hormone receptor) concentration is reported in the male tract (Derecka et al. 1999; Guo et al. 2001). *LH* controls epididymal differentiation, function and sperm maturation through *ESR2*, and lack of *LHR* function results in serious disorders in the development of puberty and fertility (Zhang et al. 1997).

Localization of protein

The concentration of ESR2 appears to be high in germ cell and in cytoplasm of Sertoli and Leydig cells in testis. A number of studies reported that ESR2 is predominant in germ cells of rodents and humans (van Pelt et al. 1999; Jefferson et al. 2000; Makinen et al. 2001; Zhou et al. 2002). A similar expression pattern of ESR2 described in this study suggests that the locally produced oestrogens in these cells might act through ESR2. Furthermore, there are reports of ESR2 expression in other germ cell types such as spermatogonia (van Pelt et al. 1999; Jefferson et al. 2000; Makinen et al. 2001; Zhou et al. 2002) and elongated spermatids (Rosenfeldt et al. 1998). Our observations are in agreement with the results reported in adult rodents, primate goats, dog and cats (Goyal et al. 1997; Nie et al. 2002; Hess and Carnes 2003). Expression of ESR2 seemed rather homogeneous within and between cell types, and there was a little variation in staining intensity. These data revealed on the protein level were confirmed on the mRNA level, indicating that transcription and translation of ESR2 occur in the positive staining cells. Oestrogen activity of pig testis is unknown, but it has been reported that there is a functional linkage between *ESR2* and embryonic growth of pig (Kowalski et al. 2002). In fact, the ESR2 immunostaining pattern in testicular somatic and germ cells as well as in immature and mature gonads suggested that oestrogens might modulate spermatogenesis and testis development via a differential expression of two oestrogen receptor subtypes (Rago et al. 2007).

In this study, the ESR2 immunostaining was found in the epithelial cells from three epididymal regions. The findings of the present study confirmed that ESR2 is expressed in a specific manner in the epididymis and suggest that oestrogens might modulate the epididymal function. Head, body and tail of the epididymis are reported to be involved in morphological and biochemical sperm maturation, in the progression of sperm towards the vas deferens and in its storage (Carpino et al. 2004). We have found ESR2 in the acrosomal cap of pig spermatozoa within the lumen of the body of epididymis. The acrosomal cap is a cellular site closely related to the exocytotic event preceding the oocyte fertilization, and in human, it has been reported that oestradiol is able to influence capacitation and acrosomal reaction of spermatozoa (Aquila et al. 2005). The localization of ESR2 in the acrosomal region implies its involvement in the fertilization process (Solakidi et al. 2005). However, it is important to note that there are more other proteins identified in the post-acrosomal region of sperm, such as equatorin and oscilin, which are important for successful fertilization (Ramalho-Santos et al. 2002; Solakidi et al. 2005).

Conclusion

It might suggest that the higher expression of *ESR2* might have negative effect on boar sperm quality validated through association study and by profiling of mRNA and protein expression in non-reproductive and reproductive tissues. Therefore, with regard to the association, the transcript and differential expression and protein localization depending on boar sperm quality traits provide experimental evidence for the role of ESR2 in male fertility. However, the results of this study have to be validated in another breed/crossbreed population in order to evaluate its potential in genomic selection.

Acknowledgements

This research was financially supported by FBF (Förderverein Biotechnologieforschung e. V., Bonn, Germany). We thank Dr A. Niggemeyer, Dr M. Friedrichs, Dr S. Brüning and Dr A. Riesenbeck (GFS Artificial Insemination Station: Die Genossenschaft zur Förderung der Schweinehaltung, Ascheberg, Germany) and Dr A. Hofer, Dr H. Luther and Dr K. Caspari (SuisAG Artificial Insemination Station, Sempach, Switzerland) for their support during the sample collection and the arrangements of the reproduction records. We are also thankful to Prof Dr C. Knorr (Institute of Veterinary Medicine, Georg-August-University, Goettingen, Germany) for his cooperation.

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

AG performed the experiments and wrote the manuscript; MUC partly supervised the work and revised the manuscript; MJU edited the manuscript; KK performed the experiment with AG; DT was responsible for kits and reagents; CP partly supervised the work; ET was responsible for the statistical analysis; CL revised the manuscript; KS was responsible for the whole experiment and supervised the overall work.

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Submitted: 7 Jun 2011; Accepted: 15 Nov 2011

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