Expression of genes involved in progesterone receptor paracrine signaling and their effect on litter size in pigs

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Abstract

Background: Embryonic mortality during the period of implantation strongly affects litter size in pigs. Progesterone receptor (PGR) paracrine signaling has been recognized to play a significant role in embryonic implantation. IHH, NR2F2, BMP2, FKBP4 and HAND2 were proved to involve in PGR paracrine signaling. The objective of this study was to evaluate the expression of IHH, NR2F2, BMP2, FKBP4 and HAND2 in endometrium of pregnant sows and to further investigate these genes’ effect on litter size in pigs. Real-time PCR, western blot and immunostaining were used to study target genes/proteins expression in endometrium in pigs. RFLP-PCR was used to detect single nucleotide polymorphisms (SNPs) of target genes.

Results: The results showed that the mRNA and protein expression levels of IHH, NR2F2 and BMP2 were up-regulated during implantation period (P < 0.05 or P < 0.01). All target proteins were mainly observed in luminal epithelium and glandular epithelium. Interestingly, the staining of NR2F2 and HAND2 was also strong in stroma. SNPs detection revealed that there was a -204C > A mutation in promoter region of NR2F2 gene. Three genotypes were found in Large White, Landrace and Duroc sows. A total of 1847 litter records from 625 sows genotyped at NR2F2 gene were used to analyze the total number born (TNB) and number born alive (NBA). The study of the effect on litter size suggested that sows with genotype CC tend to have higher litter size.

Conclusions: These results showed the expression patterns of genes/proteins involved in PGR paracrine signaling over implantation time. And the candidate gene for litter size was identified from genes involved in this signaling. This study could be a resource for further studies to identify the roles of these genes for embryonic implantation in pigs.

Keywords: Expression, Implantation, Litter size, Pigs, SNPs
to be critical for embryonic development, which operates in an epithelial to mesenchymal manner within the uterus (reviewed in [11]). NR2F2 (nuclear receptor subfamily 2, group F, member 2) has been identified to be a critical regulator in cell differentiation and tissue development as well as angiogenesis and metabolism (reviewed in [12]). IHH and NR2F2 interaction works as HH−NR2F2 axis, which plays a role in transducing an epithelial to stromal signal that initiates embryonic implantation and subsequently decidualization. BMP2 (bone morphogenetic protein 2) and FKBP4 (FK506 binding protein 4) worked as down-stream target genes of HH-NR2F2 axis, which were necessary and sufficient for implantation and decidualization. BMP2 acts via a paracrine mechanism to initiate decidualization after embryonic implantation, and also plays a fundamental role in preparing the epithelium for implantation through the regulation of Fkbps and Wnt ligands. HAND2 is a basic helix-loop-helix (bHLH) transcription factor and a known downstream target of PGR. HAND2 is a critical mediator between active paracrine signaling by PGR signaling and the inhibition of estrogen-induced proliferation within the epithelium, which is critical for embryonic implantation.

Therefore, PGR paracrine signaling is critical for embryonic implantation. Porcine embryos begin to attach to the uterus on pregnancy day 13 and 14, and implantation completes from pregnancy day 18 to day 24 [13]. In this research, we detected the expression level of the genes/proteins involved in PGR paracrine signaling, including IHH, NR2F2, BMP2, FKBP4 and HAND2, in the endometrium on d 13, 18 and 24 of gestation in pigs. SNPs of these genes were detected and the association between the polymorphism and litter size in Large White, Landrace and Duroc pigs was analyzed. The results will provide information towards a better understanding of PGR paracrine signaling, which regulates implantation and subsequently affect litter size in pigs.

**Methods**

**Animal materials**

The Animal Care and Use Committee of China Agricultural University reviewed and approved the experimental protocol used in this study (Code: SYXK (Jing) 2009-0030). Multiparous Large White sows (5th parity) were observed daily for standing heat in the presence of a boar. The sows of the pregnant groups (three groups, three sows each group) were inseminated twice, 12 h and 24 h after heat detection, respectively [14]. The sows of the non-pregnant group (three sows) were treated with inactivated sperm from the same boar [14]. Pregnant sows were slaughtered by electrocution on d 13, 18 and 24 after insemination. Samples of the endometrium attachment sites and inter-sites were taken. Samples were taken from three locations of each uterine horn: proximal (the end, close to the ovaries), medial, and distal (next to the corpus uteri) [14]. Non-pregnant sows were slaughtered on d 13 after insemination. Samples were taken from the comparable locations. Endometrial tissue sampling was carried out according to the procedure of Lord, with minor modifications [15]. The samples used for real time PCR and western-blot were collected immediately, snap frozen in liquid nitrogen and stored at −80 °C. The samples used for immunohistochemistry were collected and placed in a tube containing pre-cooling paraformaldehyde solution (4 %, pH = 7.4) and placed on a rocker overnight for fixation of the tissue. Once the period of fixation was finished, the tissue was rinsed in PBS, and then processed through a series of ethanol washes to displace the water. Then the tissue was infiltrated with and embedded in paraffin. Paraffin-embedded tissues were sliced at 5 μm thickness using a microtome (Leica2016, Germany).

Animals used to identify candidate genes for litter size were from Beijing Huadu Swine Breeding Company LTD. All sows were reared and feed in the same condition. Ear tissue samples of 625 Large White, Landrace and Duroc sows were collected in centrifuge tubes (1.5 mL) with 70 % ethanol and stored at 4 °C until DNA extraction. DNA was extracted by phenol and chloroforms (1:1) extraction. There are eight sire families in Large White, eight sire families in Landrace, and seven sire families in Duroc sows. 1847 litters’ records were used for statistical analysis. Litter size records such as total number born (TNB) and number born alive (NBA) were recorded by parity.

**RNA isolation and real time quantitative PCR**

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA, according to the manufacturer’s instructions. For each animal, total RNA consisted of a mix of an equal quantity of total RNA from three locations of each uterine horn: proximal (the end, close to the ovaries), medial, and distal (next to the corpus uteri).

For each sample, first strand cDNA was synthesized using 1 μg of total RNA. M-MLV FIRST STRAND KIT (Invitrogen, Shanghai, China) and oligo (dT)18 primer were used in a total of 20 μL reverse transcription reaction following the supplier’s instruction. Transcript specific primer pairs (see Additional file 1: Table S1) were designed with Oligo 6.0 software. Standard PCRs on cDNA were carried out to verify amplification sizes. Transcript quantification was performed using SYBR Green mix (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) in a Roche LightCycler 480 (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany). The RT-PCR reactions were prepared in a total volume of 20 μL containing 5 μL of cDNA (50 ng, 1:100 dilution), 10 μL of SYBR Green mix, 3 μL water which contained in
the kit and 0.02 μmol/L of both forward and reverse gene specific primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference gene. Cycling conditions were 95 °C for 10 min, followed by 45 cycles of 95 °C (10 s) and 60 °C (10 s) where the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95 °C (10s) followed by 60 °C (1 min) and ramp up to 95 °C with acquired fluorescence during the ramp to 0.2 °C/s. PCR efficiency of each gene was estimated by standard curve calculation using four points of cDNA serial dilutions. Ct values were transformed to quantities using the comparative Ct method, setting the relative quantities of non-pregnant group for each gene to 1 (Qty = 10-ΔCt/slope). Data normalization was carried out using GAPDH as the reference gene. Comparisons of genes expression levels were done using a t-test.

Western-blot
Frozen sections of endometrial samples were prepared and western blotting was performed as previously described with minor modification [16]. Tissues protein was extracted (0.05 mol/L Tris–HCl, NaCl 8.76 mg/mL, 1 % TritonX-100 and 100 μg/mL PMSF) (Sunbio, China) by vortex meter (Kylinbell, China). Total protein concentrations were detected using the BCA Protein Assay Kit (Sunbio, China) according to the manufacturer’s recommendations.

Sample 80–120 μg was separated in a 10 % Tris–HCl polyacrylamide gel in electrophoresis system (Liyuf, China), and protein from the gel was transferred onto a single PVDF membrane (BioRad, USA). After rinsed in TBST for 5 min at room temperature (RT), the membrane was soaked in 5 % skim milk (in TBST) for 1 h. Next, the membrane was immerged into specific dilution (IHH, Santa Cruz Biotechnology, Inc., sc-13088, 1:100; NR2F2, Abcam (Hong Kong) Ltd., ab50487, 1:100; BMP2, Abcam (Hong Kong) Ltd., ab14933, 1:100; FKBP4, Abcam (Hong Kong) Ltd., ab97306, 1:100; HAND2, Biobyt, orb36304, 1:100; β-Actin 1:200) of the primary antibodies, then reacted with primary antibodies (rabbit polyclonal to IHH, Santa Cruz Biotechnology, Inc., sc-13088; rabbit polyclonal to NR2F2, Abcam (Hong Kong) Ltd., ab50487; rabbit polyclonal to BMP2, Abcam (Hong Kong) Ltd., ab14933; rabbit polyclonal to FKBP4, Abcam (Hong Kong) Ltd., ab97306; rabbit polyclonal to HAND2, Biobyt, orb36304; mouse monoclonal toβ-Actin, Santa Cruz Biotechnology, Inc., sc-81178) at 4 °C overnight. Followed by incubation with biotinylated second antibody (Invitrogen, USA) at 37 °C for 25 min, and after being washed in PBS for 15 min three times, the sections were incubated with streptavidin-peroxidase (HRP) (Invitrogen, USA) at 37 °C for 25 min. Finally, the slides were washed with PBS and stained with DAB kit (Invitrogen, USA). After being washed fully with water for 5 min, the slides were stained with hematoxylin and eosin, and then examined by microscope (BH2, Olympus). Instead of primary antibodies, PBS was used as a negative control. Endometrial tissues of non-pregnant sows were used as positive control [17]. ImagePro Plus software was used to measure the level of staining. The gray value of the portion of the picture without tissue was set as 0 to correct the background. Scoring of staining was carried out according to the procedure of Constantine A. Axiotis (1991), with minor modifications [18]. Expression of target protein was determined by assessing the staining intensity and the percentage of stained cells. The staining intensity was rated as follows: weak staining

| Target | Non-pregnant | D13 of pregnancy | D18 of pregnancy | D24 of pregnancy |
|--------|--------------|------------------|------------------|------------------|
|        | Attachment sites | Inter-sites | Attachment sites | Inter-sites | Attachment sites | Inter-sites |
| PGR    | 1.01 ± 0.25<sup>a</sup> | −2.33 ± 0.01 | −1.31 ± 0.21 | −2.60 ± 0.21 | −2.12 ± 0.16 | −10.34 ± 0.16<sup>b</sup> | −3.94 ± 0.06<sup>b</sup> |
| IHH    | 1.05 ± 0.33<sup>a</sup> | 1.34 ± 0.34 | 2.67 ± 0.71<sup>b</sup> | 2.12 ± 0.71<sup>b</sup> | 3.92 ± 1.48<sup>b</sup> | 3.48 ± 1.58<sup>b</sup> | 2.60 ± 0.67<sup>b</sup> |
| NR2F2  | 1.02 ± 0.18<sup>a</sup> | 3.80 ± 0.91<sup>b</sup> | 4.77 ± 0.99<sup>b</sup> | 4.42 ± 0.17<sup>b</sup> | 5.32 ± 0.73<sup>b</sup> | 6.18 ± 1.75<sup>b</sup> | 2.18 ± 0.64<sup>b</sup> |
| BMP2   | 1.02 ± 0.18<sup>a</sup> | 2.41 ± 0.19<sup>b</sup> | 3.61 ± 0.50<sup>b</sup> | 4.54 ± 0.94<sup>b</sup> | 4.57 ± 0.97<sup>b</sup> | 3.61 ± 1.52<sup>b</sup> | 3.90 ± 1.70<sup>b</sup> |
| FKBP4  | 1.02 ± 0.21<sup>a</sup> | 1.79 ± 0.39 | 1.50 ± 0.52 | 0.63 ± 0.19 | 0.99 ± 0.15 | 0.35 ± 0.08<sup>b</sup> | 0.76 ± 0.21<sup>b</sup> |
| HAND2  | 1.07 ± 0.14<sup>a</sup> | 1.83 ± 0.33 | 2.09 ± 0.50 | 2.93 ± 0.83<sup>b</sup> | 2.98 ± 0.87<sup>b</sup> | 1.08 ± 0.16 | 1.11 ± 0.16<sup>b</sup> |

<sup>a</sup>, <sup>b</sup> P < 0.05, <sup>A</sup>, <sup>B</sup> P < 0.01
(score = 1), moderate staining (score = 2), strong staining (score = 3). The percentages of positive cells was calculated using ImagePro plus. This formula was used to calculated the final score: \( \sum (\text{percentage of positive cells}) \times (\text{score of positive staining}) \). Average of five different areas per picture was recorded. According to the final score, the protein expressed as follows: <1.0, weak, 1.0–1.5, moderate; >1.5, strong.

**Detection of SNPs and litter size association analysis**

DNA was extracted by phenol and chloroforms (1:1) standard techniques. 18 PCR primer pairs (see Additional file 2: Table S2) were designed to detect SNPs of target genes. PCR amplifications were carried out on an Eppendorf Mastercycler gradient 5331 PCR System (Eppendorf, Germany). The polymerase chain reaction amplification was performed using 50–100 ng of genomic DNA, 25 μL Taq PCR MasterMix (Taq DNA Polymerase: 0.05 units/μL; MgCl2: 4 mM/μL; dNTPs: 0.4 mM/μL), 10 pM of each primers in a 50 μL final volume. All reagents

![Image](image.png)

**Fig. 1** The protein relative abundance of target proteins in endometrium of sows. Note: NP, endometrium of non-pregnant sows; D13a, endometrial attachment sites on d 13 of gestation; D13b, the endometrial inter-sites on d 13 of gestation; D18a, endometrial attachment sites on d 18 of gestation; D18b, the endometrial inter-sites on d 18 of gestation; D24a, endometrial attachment sites on d 24 of gestation; D24b, the endometrial inter-sites on d 24 of gestation.

![Image](image.png)

**Fig. 2** Immunohistochemical localization of IHH in pig uterus. GE = glandular epithelium; LE = luminal epithelium; S = stroma. a Negative control; b Immunohistochemical staining of non-pregnant sows uterus with IHH antibody; c Immunohistochemical staining of porcine uterus attachment site with IHH antibody on d 13 of pregnancy; d Immunohistochemical staining of porcine uterus inter-site with IHH antibody on d 13 of pregnancy; e Immunohistochemical staining of porcine uterus attachment site with IHH antibody on d 18 of pregnancy; f Immunohistochemical staining of porcine uterus inter-site with IHH antibody on d 18 of pregnancy; g Immunohistochemical staining of porcine uterus attachment site with IHH antibody on d 24 of pregnancy; h Immunohistochemical staining of porcine uterus inter-site with IHH antibody on d 24 of pregnancy.

| Target | Non-pregnant | D 13 of pregnancy | D 18 of pregnancy | D 24 of pregnancy |
|--------|--------------|--------------------|-------------------|-------------------|
|        | Attachment sites | Inter-sites | Attachment sites | Inter-sites | Attachment sites | Inter-sites |
| IHH    | 0.28 ± 0.10^a | 0.33 ± 0.15 | 0.48 ± 0.11 | 0.48 ± 0.11^b | 1.00 ± 0.02 | 1.03 ± 0.21^b | 0.72 ± 0.03^b |
| N2RF2  | 0.89 ± 0.08 | 0.71 ± 0.05 | 0.99 ± 0.10 | 1.09 ± 0.02 | 1.15 ± 0.06 | 1.16 ± 0.07 | 1.11 ± 0.06 |
| BMP2   | 0.37 ± 0.14^a | 0.77 ± 0.13^b | 0.58 ± 0.01^B | 0.38 ± 0.10 | 0.44 ± 0.11 | 0.33 ± 0.19 | 0.40 ± 0.21 |
| FKBp4  | 0.57 ± 0.14 | 0.66 ± 0.16 | 1.00 ± 0.20 | 0.45 ± 0.19 | 0.66 ± 0.21 | 0.63 ± 0.18 | 0.84 ± 0.16 |
| HAND2  | 0.61 ± 0.03^A | 0.73 ± 0.03^B | 0.71 ± 0.01^B | 0.57 ± 0.06 | 0.78 ± 0.01^B | 0.82 ± 0.03^B | 0.87 ± 0.05^B |

^a, ^b P < 0.05, ^A, ^B P < 0.01
were collected from the National Laboratories for Agrobiotechnology, China Agricultural University. The following conditions of PCR amplification were used:
a denaturation step at 95 °C for 4 min, 30 cycles at 95 °C for 30 s, 52 °C ~ 55 °C for 30 s, and 72 °C for 30 s ~ 1 min 30 s, a final extension step of 72 °C for 10 min. Amplified fragments were separated by 1.5% agarose gel electrophoresis (AGE).

Using pooled DNA amplification and sequencing, several mutations were found. Mutation −204C > A in promoter region of NR2F2 gene caused the deletion of transcription factor binding sites (TFBS) CREB (cAMP-response-element-binding protein).

NR2F2 was selected to be the candidate gene for litter size based on its mRNA/protein expression level during embryonic implantation period and the mutation found in promoter region. PCR- Restriction fragment length polymorphism (PCR-RFLP) was used to detect different genotypes. HaeIII (NEB R0108L, BioLabs Inc.) was used. The PCR products of three genotypes were random selected and sequenced to validate the results.

Alleles and genotypes frequencies of NR2F2 were calculated from the 625 sows, respectively. GLM procedure of SAS 8.02 software was used to compute the least square means of TNB and NBA. According to the
analysis, the effect of sire and dam on litter size was not significant, so the following linear model was used to analyze the genotype effect of NR2F2.

\[ Y_{ijkl} = \mu + \text{HYS}_i + \text{P}_j + \text{G}_k + e_{ijkl} \]

Where \( Y_{ijkl} \) is the traits of TNB and NBA, \( \mu \) is the overall mean, \( \text{HYS}_i \) is the effect of herd-year-season (\( i = 1 \) to 52), \( \text{P}_j \) is the effect of parity (\( j = 1, 2, \geq 3 \) and all parities), \( \text{G}_k \) is the effect of genotype (\( k = 1 \) to 3) and \( e_{ijkl} \) is the random residual. The data was analyzed separately for the first parity, the second parity, the third and following parities, and all parities. The additive effect and the dominant effect were calculated according to the methods of Rothschild et al. [19].

**Results**

mRNA expression in porcine endometrium

The effect of the day of pregnancy on mRNA expression of IHH, NR2F2, BMP2, FKBP4 and HAND2 in sows’ endometrium during implantation period was shown in Table 1. In pregnant sows, the expression of IHH was significantly higher than that of non-pregnant sows on d 18 and d 24 of pregnancy \((P < 0.05)\) (Table 1). The expression of IHH in attachment sites showed an uptrend.
This was consistent with the expression of NR2F2 which was significantly up-regulated during implantation time. The expression of BMP2 was significantly up-regulated (P < 0.05 or P < 0.01) during implantation time (Table 1), which was consistent with IHH and NR2F2. For FKBP4, at attachment sites, the expression of FKBP4 was significantly down-regulated on d 24 of pregnancy (P < 0.01) (Table 1). The expression of HAND2 was the highest on d 18 of pregnancy (P < 0.05) (Table 1).

Protein expression in porcine endometrium
The protein expressions of IHH, NR2F2, BMP2, FKBP4 and HAND2 in the porcine endometrium during the embryonic implantation period were shown in Fig. 1 and Table 2. The protein expression of IHH was significantly up-regulated on d 18 and d 24 of pregnancy (P < 0.05 or P < 0.01) (Fig. 1 and Table 2), which was similar to its mRNA expression. The protein expression of BMP2 was higher on d 13 of pregnancy (P < 0.05) (Fig. 1 and Table 2). For the protein expression of FKBP4, there was not significantly difference between pregnant groups and non-pregnant group (Fig. 1 and Table 2), which was not consistent with its mRNA expression pattern. The protein expression of HAND2 was higher in pregnant sows (P < 0.01) (Fig. 1 and Table 2), except at attachment sites on d 18 of pregnancy.

| Target | Non-pregnant | D 13 of pregnancy | D 18 of pregnancy | D 24 of pregnancy |
|--------|--------------|--------------------|--------------------|--------------------|
|        | Attachment sites | Inter-sites | Attachment sites | Inter-sites | Attachment sites | Inter-sites |
| IHH    | GE LE S GE LE S GE LE S GE LE S GE LE S GE LE S |
| NR2F2  | ++ ++ ± ++ ++ ++ ++ ± ++ ++ ++ ++ ± ++ ++ ++ ++ ++ ± |
| BMP2   | ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± |
| FKBP4  | ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± |
| HAND2  | ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± ++ ++ ± |

GE: glandular epithelium; LE: luminal epithelium; S: stroma ±: weak; +: moderate; ++: strong

Protein localization in porcine endometrium
During implantation period, IHH, NR2F2, BMP2, FKBP4 and HAND2 were observed in luminal epithelium and glandular epithelium (Figs. 2, 3, 4, 5, 6). In stroma, the staining of BMP2 and FKBP4 were weak, but the staining of NR2F2 and HAND2 was strong (Figs. 2, 3, 4, 5, 6). The result was summarized in Table 3.

Detection of SNPs of target genes and association analysis
After analysis samples of 625 sows, several mutations were found (Table 4). Mutation -204C > A in promoter region of NR2F2 gene was found, and this mutation caused the deletion of TFBS CREB (Fig. 7). Synonymous mutation 9619G > A in exon 3 of BMP2 gene was found (Table 4). Seven mutations in FKBP4 gene were found, but no one is missense mutation (Table 4).

NR2F2 was selected to be the candidate gene for litter size based on its mRNA/protein expression level during embryonic implantation period and the mutation found in promoter region. PCR-RFLP was used to detect different genotypes. The representative SNPs sequencing output for genotypes were shown in Fig. 8. The genotype frequencies and allele frequencies at each polymorphic locus in Large White, Landrace and Duroc sows were shown in Table 5. The genotype frequencies of AA, AC and CC in large white were 0.388, 0.414, and 0.198. In Landrace, the genotype frequencies were 0.088, 0.366, and 0.546. In Duroc, the genotype frequencies were 0.358, 0.433, and 0.208. None of the three breeds was found to be in Hardy-Weinberg equilibrium (HWE).

The data for TNB and NBA were observed for the first parity, the second parity, the third and the following parities and all parities. The least square means in Large White, Landrace and Duroc were shown in Tables 6, 7 and 8. In Large White, in the first parity, the sows with AA genotype had an advantage of 0.81 (P < 0.05) NBA per litter over the sows with CC genotype. In the second parity, the sows with CC genotype had an advantage of 1.76 (P < 0.01) and 1.56 (P < 0.01) TNB per litter over the sows with AA and AC, respectively. NBA of CC genotype were...
of 0.99 ($P < 0.05$) more piglets per litter than that of the AA genotype. In the third and following parities, NBA significantly increased for the CC genotype with 0.60 ($P < 0.05$) and 0.85 ($P < 0.01$) more piglets in comparison with the AA and AC genotype, respectively. In all parities, the sows with CC genotype had an advantage ($P < 0.05$) of 0.89 and 0.64 for TNB per litter over the AA and AC genotype sows, respectively. And NBA of CC genotype were of 0.97 ($P < 0.01$) and 0.88 ($P < 0.01$) more piglets per litter than that of the AA and AC genotype, respectively. In all parities, the sows with CC genotype had an advantage of 0.89 and 0.64 for TNB per litter over the AA and AC genotype, respectively. And NBA of CC genotype were of 0.97 ($P < 0.01$) and 0.88 ($P < 0.01$) more piglets per litter than that of the AA and AC genotype, respectively.

In Landrace, in the third and following parities, the sows with CC genotype had an advantage of 0.53 for TNB and 0.61 for NBA per litter over the sows with AA genotype, and 0.53 for TNB over the sows with AC genotype, but not significantly. In all parities, TNB of genotype CC was 1.05 ($P < 0.05$) piglets higher than that of the AA genotype. And the sows with the CC genotype had an advantage of 0.53 and 0.22 for NBA per litter over the sows with AA and AC, but not significantly.

In Duroc, in the second parity, the sows with the CC genotype had an advantage of 0.66 piglets ($P < 0.05$) for TNB and 1.34 ($P < 0.05$) piglets for NBA per litter over the sows with AA genotype. In the third and following parities, the sows with the CC genotype had an advantage of 1.35 piglets ($P < 0.01$) for TNB and 1.34 ($P < 0.05$) for NBA per litter over the sows with AA genotype.

**Discussion**

Expression of genes participated in paracrine signaling in sows endometrium

The embryonic peri-implantation time of pigs is especially longer. During the peri-implantation period of pregnancy, uterine LE and conceptus trophectoderm develop adhesion competency in synchrony to initiate the adhesion cascade within a restricted period of the uterine cycle termed the "window of receptivity" [20–22]. In pigs, this window is orchestrated through the actions of progesterone and estrogen to regulate locally produced cytokines, growth factors, cell surface glycoproteins, cell surface adhesion molecules, and extracellular matrix (ECM) proteins [23]. A fundamental paradox of early pregnancy is that cessation of expression of *PGR* and *ESR1* by uterine epithelia is a prerequisite for uterine receptivity to implantation, expression of genes by uterine epithelia and selective transport of molecules into the uterine lumen that support conceptus development. Thus, effects of *P4* are mediated via *PGR* expressed in uterine stromal and myometrial cells by stromal cell
derived growth factors known as “progestamedins” [24, 25]. As previous indicated, progesterone down regulated the expression of PGR in the uterine epithelia of pigs after d 10 of pregnancy, immediately prior to the time when the endometrium becomes receptive to implantation [26–28]. In pigs, down-regulation of PGR in uterine epithelia is a prerequisite for the expression of genes for uterine secretions and transport of molecules into the uterine lumen that support conceptus development. Down-regulation of PGR is associated with down-regulation of mucin1 (MUC1), as well as up-regulation of the expression of secreted phosphoprotein 1 (SPP1) and insulin-like growth factor binding protein 1 (IGFBP1). During conceptus elongation and the early peri-implantation period, the endometrium increases the release of a number of growth factors and cytokines such as epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor 7 (FGF7), vascular endothelial growth factor (VEGF), interleukin 6 (IL-6), transforming growth factor beta (TGFβ), and leukemia inhibitory factor (LIF) [29, 30]. Some of these genes had been reported to have significant effect on litter size in pigs, such as SPP1, VEGF, MUC1, LIF et al [1, 31–33].

PGR paracrine signaling has been recognized to play a significant role in pregnancy in human and mouse, which have not been studied in pigs [5]. IHH is a progesterone receptor target activated within the epithelium which signals downstream to NR2F2 in the stroma establishing the HH–NR2F2 axis within the dual uterine compartments. Strong evidence exists to propose a role of a HH–NR2F2 axis in the regulation of reproduction in human and mice [12, 34]. Identification of the signaling pathway from stroma to epithelium would aid in the understanding of how the stroma contributes to embryo implantation. Changes in endometrial transcriptome during early stages of conceptus attachment to uterine LE in previous study showed that IHH regulated significantly during pregnancy period in the pigs. In the present study, compared with non-pregnant sows, the mRNA and protein expression of IHH were up-regulated during implantation. The expression of IHH in bovine uterus had been studied. The result showed IHH is modulated by progesterone in bovine uterus, and may be required to be down-regulated to allow expression of genes that drive conceptus elongation in cattle [35]. In pigs, the conceptus elongated rapidly before d 13 of gestation, and the filamentous conceptus continue to elongate but slowly after d 13 of gestation. The expression of IHH did not show significantly changed at d 13 of pregnancy in our result. It may be because the conceptus elongate slowly after d 13 of pregnancy in pigs [36]. The expression of NR2F2 was significantly up-regulated during implantation time and the expression in attachment sites showed an upward trend. This was consistent with previous study, which found NR2F2 up-regulated in d 12 of gestation in Yorkshire pigs [37]. NR2F2 was shown to activate hypoxia-inducible factor 1 alpha (HIF-1α) and HIF-1 is an important mediator of estrogen-induced VEGF expression in the uterus [38, 39]. They thought that the expression of NR2F2 is associated with greater activation of angiogenesis at the stage of implantation in the Yorkshire breed [37]. The expression of IHH and NR2F2 were consistent with their functional role in embryonic implantation and also consistent with previous studies [40–44]. It was reported that HH–NR2F2 axis can transmit the paracrine signaling by PGR from epithelium to stroma [42]. The protein localization of IHH in porcine endometrium showed that IHH mainly observed strongly in luminal epithelium and glandular epithelium. NR2F2 was especially observed strongly in stroma. This confirmed that HH–NR2F2 axis was important in mediating the signal from epithelial to other effect or genes in the stroma.

BMP2, as a downstream gene of HH–NR2F2 axis, has demonstrated to be a critical effector for decidualization and the maintenance of pregnancy during post-implantation. BMP2 likely acts as a paracrine signaling factor for the initiation of the proliferative response after embryonic implantation within the uterine stroma. In the present study, the mRNA expression of BMP2 was significantly up-regulated during implantation time, which was consistent with the expression of IHH and NR2F2. In previous study, researchers found that BMP2 and BMP6 can significantly suppress progesterone production in pigs in vitro [45]. So this was consistent with our result, which showed BMP2 up-regulated along with PGR down-regulated during implantation period. The protein expression of BMP2 was significantly up-regulated on d 13 of pregnancy, which demonstrated that BMP2 promotes implantation cooperated with IHH and NR2F2. But on d 18 and 24, the expression did not regulate.

Table 5 Number of alleles (n), allele and genotype frequencies of NR2F2, observed heterozygosity (h)

| Breed       | Sows | Genotype distribution | Genotype frequencies | Allele frequencies | h   |
|-------------|------|-----------------------|----------------------|-------------------|-----|
|             |      | AA   | AC   | CC   | AA   | AC   | CC   | A   | C   |       |
| Large White | 232  | 90   | 96   | 46   | 0.388 | 0.414 | 0.198 | 0.595 | 0.405 | 4.648 |
| Landrace    | 273  | 24   | 100  | 149  | 0.088 | 0.366 | 0.546 | 0.271 | 0.729 | 1.458 |
| Duroc       | 120  | 43   | 52   | 25   | 0.358 | 0.433 | 0.208 | 0.575 | 0.425 | 1.542 |
### Table 6 Effects of the NR2F2 polymorphism on total number born (TNB) and number born alive (NBA) in Large White (LS means ± S.E.)

| Breed    | Genotype | First parity |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
|----------|----------|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|          |          | Litters      | TNB      | NBA      | Litters  | TNB      | NBA      | Litters  | TNB      | NBA      | Litters  | TNB      | NBA      | Litters  | TNB      | NBA      |          |
|          |          |              |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| Large White | AA       | 90           | 11.12 ± 0.25 | 10.33 ± 0.23a | 60       | 11.10 ± 0.40a | 10.21 ± 0.35a | 144      | 11.078 ± 0.40 | 10.09 ± 0.35a | 294       | 10.99 ± 0.32a | 10.23 ± 0.28a |          |          |          |          |
|          | AC       | 96           | 11.43 ± 0.25 | 10.59 ± 0.24  | 66       | 11.30 ± 0.41A | 10.01 ± 0.37  | 190      | 10.96 ± 0.40 | 9.84 ± 0.36A  | 352       | 11.24 ± 0.32A | 10.32 ± 0.29A  |          |          |          |          |
|          | CC       | 46           | 11.72 ± 0.32 | 11.14 ± 0.31b | 26       | 12.86 ± 0.55b | 11.20 ± 0.49b | 93       | 10.69 ± 0.46 | 10.69 ± 0.41bb | 165       | 11.88 ± 0.35b | 11.20 ± 0.32b  |          |          |          |          |

Values with different superscripts show significant levels within columns: a, b P < 0.05, A, B P < 0.01

### Table 7 Effects of the NR2F2 polymorphism on total number born (TNB) and number born alive (NBA) in Landrace (LS means ± S.E.)

| Breed    | Genotype | First parity |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
|----------|----------|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|          |          | Litters      | TNB      | NBA      | Litters  | TNB      | NBA      | Litters  | TNB      | NBA      | Litters  | TNB      | NBA      | Litters  | TNB      | NBA      |          |
|          |          |              |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| Landrace | AA       | 24           | 11.02 ± 0.32 | 10.44 ± 0.31 | 11       | 10.33 ± 0.53 | 10.06 ± 0.47 | 15       | 10.68 ± 0.30 | 10.24 ± 0.33 | 50       | 10.62 ± 0.19A | 10.13 ± 0.23  |          |          |          |          |
|          | AC       | 100          | 11.15 ± 0.25 | 10.54 ± 0.25 | 52       | 10.85 ± 0.39 | 10.25 ± 0.34 | 78       | 11.19 ± 0.23 | 10.79 ± 0.30 | 230      | 10.98 ± 0.15 | 10.44 ± 0.20  |          |          |          |          |
|          | CC       | 149          | 11.36 ± 0.27 | 10.75 ± 0.27 | 97       | 11.26 ± 0.45 | 10.76 ± 0.40 | 151      | 11.21 ± 0.26 | 10.85 ± 0.30 | 397      | 11.67 ± 0.16b | 10.66 ± 0.21  |          |          |          |          |

Values with different superscripts show significant levels within columns: a, b P < 0.05, A, B P < 0.01

### Table 8 Effects of the NR2F2 polymorphism on total number born (TNB) and number born alive (NBA) in Duroc (LS means ± S.E.)

| Breed    | Genotype | First parity |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
|----------|----------|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|          |          | Litters      | TNB      | NBA      | Litters  | TNB      | NBA      | Litters  | TNB      | NBA      | Litters  | TNB      | NBA      | Litters  | TNB      | NBA      |          |
|          |          |              |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
| Duroc    | AA       | 43           | 10.43 ± 0.35 | 9.53 ± 0.37 | 21       | 10.88 ± 0.39A | 9.87 ± 0.35A | 58       | 9.68 ± 0.34A | 9.26 ± 0.39A | 122      | 10.02 ± 0.20 | 9.30 ± 0.24  |          |          |          |          |
|          | AC       | 52           | 10.54 ± 0.30 | 10.07 ± 0.32 | 36       | 11.12 ± 0.39 | 9.98 ± 0.35A | 103      | 10.48 ± 0.25 | 9.97 ± 0.31 | 161      | 10.21 ± 0.16 | 9.63 ± 0.21  |          |          |          |          |
|          | CC       | 25           | 10.60 ± 0.43 | 10.10 ± 0.46 | 11       | 11.54 ± 0.45b | 10.81 ± 0.40b | 40       | 11.03 ± 0.46b | 10.60 ± 0.4b | 76       | 10.17 ± 0.26 | 9.47 ± 0.30  |          |          |          |          |

Values with different superscripts show significant levels within columns: a, b P < 0.05, A, B P < 0.01
significantly. It may be because decidualization did not happen in pigs.

HAND2 was another downstream target of PGR [8]. In the stroma, HAND2 plays an important role in the inhibition of the FGF pathway, a pathway known to be involved in the promotion of epithelial proliferation by estrogen signaling [8]. Therefore, HAND2 is important to inhibit the estrogen-induced epithelial proliferation in the uterus [8]. The inhibition of epithelial proliferation by PGR signaling was possibly via HH–NR2F2 axis. HH–NR2F2 axis then activated HAND2, which caused the inhibition of estrogen signaling and subsequent allowance for proper embryonic implantation. In the present study, the expression of mRNA and protein of HAND2 were both up-regulated on d 13 of pregnancy. This may related with its inhibition of estrogen signaling, and further more promoted the positive role of PGR in implantation. In previous studies, HAND2 had been detected up-regulated at implantation period and late gestation period in pigs [31, 46]. The researchers find HAND2 related with receptivity of uterus and vascular development of placenta [31, 46]. The mRNA of HAND2 was up-regulated on d 18 of pregnancy, but the protein expression was not. Maybe there is regulation mechanism at translation level, which needs further research. The protein localization in porcine endometrium showed that HAND2 observed strongly in luminal epithelium, glandular epithelium, and stroma. This indicated that HAND2 played an important role in transmit the PGR signaling from epithelium to stroma.

The variations of NR2F2 and its association with litter size
Marker-assisted selection (MAS) in conjunction with traditional selection methods is most effective for the traits such as litter size, which are either expressed later in life, are sex-dependent, or are of low heritability [47]. The candidate gene approach has led to notable success in demonstrating reproduction-related genetic markers or major genes, such as ESR, PRLR, the erythropoietin receptor (EPOR) and so on [19, 48–50].

In the present study, we selected NR2F2 as the candidate gene for litter size in pigs, due to its biological function and the interesting mutation. Three genotypes were found: AA, AC and CC. The association with litter size revealed that CC genotype is the favorable genotype. Through analysis using Consite database (http://consite.geneereg.net/cgi-bin/consite?rm=t_input_single), the C→A mutation caused deletion of TFBS CREB (Fig. 7). CREB has been proved played an important role in activation of transcription and regulation of gene transcription [51, 52]. The deletion of CREB may affect the expression of NR2F2 in porcine endometrium and stroma. The effect of NR2F2 on litter size possibly associated with its expression in endometrium during embryonic implantation. This certainly will affect the signal of PGR from endometrium to stroma, in consideration of the PGR-IHH-NR2F2 axis. Subsequently, the embryonic implantation process and litter size was affected.

Conclusions
In current research, the expression patterns of genes/proteins involved in PGR paracrine signaling over implantation time were studied. And candidate gene for litter size was identified from genes involved in this signaling. The present study could be a resource for further studies to identify the roles of these genes for embryonic implantation in pigs.

Additional files

Additional file 1: Table S1. Primers used for Real-time PCR (RT-PCR).
(DOCX 19 kb)

Additional file 2: Table S2. Primer pairs and PCR conditions for SNPs detection. (DOCX 20 kb)

Abbreviations
AGE: agarose gel electrophoresis; CREB: cAMP-response-element-binding protein; D13a: endometrial attachment sites on day 13 of gestation; D13b: the endometrial inter-sites on day 13 of gestation; D13b: the endometrial inter-sites on day 18 of gestation; D13a: endometrial attachment sites on day 18 of gestation; D24a: endometrial attachment sites on day 24 of gestation; D24b: the endometrial inter-sites on day 24 of gestation; HWE: Hardy-Weinberg equilibrium; MAS: Marker-assisted selection; NBA: number born alive; NP: endometrium of non-pregnant sows; PCR-RFLP: PCR-Restiction fragment length polymorphism; PGR: progesterone receptor; TFBS: transcription factor binding sites; TNB: total number born.

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Authors’ contributions
The contributions of the authors are as follows: XC conducted the research, analysis the results and wrote the paper. XC and JLF participated in the animal experiment. AGW was in charge of the whole trail. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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