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The Developmental Changes and Correlation of Adiponectin, Adiponectin Receptors and Hormones of the Hypothalamic-Pituitary-Ovarian Axis in Growing Wannan Spotted Gilts

Yun Li, Jie Zhou, Linqi Zhang, Kang Shao, Fugui Fang and Juhua Wang

College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, P. R. China

Abstract

Adiponectin(Adp) is an adipocyte-derived hormone that plays an important role in lipid metabolism and glucose homeostasis, and could preserve reproductive functions by stimulating hypothalamic-pituitary-gonadal axis activities at different levels. To investigate and verify the relationship between the Adp and the hypothalamic-pituitary-ovarian axis (HPO axis) in Wannan spotted gilts, serum reproductive hormones, serum Adp and mRNA expression of Adp, AdpR1, AdpR2, GnRH, GnIH, GnRHR, LH, FSH, FSHR, and CYP19 in HPO axis of 1, 30, 45, 90, and 180-day-old Wannan spotted gilts were measured with ELISA and quantitative RT-PCR using β-actin as an internal standard, respectively. The developmental pattern of serum FSH, and LH levels reaching the peak at 30d, followed by a significant decline on 45d. Serum Adp showed an opposite developmental pattern. The mRNA levels showed a similar relationship between serum hormones and Adp in Wannan spotted gilts. Thus we postulated that Adp may inhibit the secretion of some hormones in HPO axis through endocrine pathways and its action is mediated by AdpR during the prepubertal stages in Wannan spotted gilts.

Keywords: Wannan spotted gilt; Adiponectin(Adp); Adiponectin Receptors (AdpRs); reproductive hormone; HPO axis

Corresponding author: Jie Zhou

zhoujie@ahau.edu.cn

College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, P. R. China

Tel: 0086-551-65786328

Fax: 0086-551-65785027

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Introduction

Adipokines, secreted by adipose tissue, participate in the regulation of thermogenesis, feeding, and reproduction [1]. As an adipokine, adiponectin (Adp), also known as AdipoQ [2], Acrp30 [3], apM-1 [4], and GBP28 [5], is a homotrimer of three, 30 kDa subunits [6], and is widely expressed in adipose tissue, heart, muscle, and placenta [7-10]. Adp circulates as a multimer in plasma, at concentrations from 8 to 25 μg/ml in humans, and exhibits sexual dimorphism, with higher levels observed in females [11]. It has been shown that serum Adp levels are lower in obesity. However, there is little information regarding the effects of adiponectin on reproduction. Accumulating evidence indicates that Adp plays an important role in the regulation of food intake [12] and energy homeostasis [4], as well as in reproduction [13-17]. The biological role of Adp is mediated by three Adp receptors (AdpRs): Adp receptor 1 (AdpR1), Adp receptor 2 (AdpR2), and T-Cad [18]. Expression of AdpR1 and AdpR2 is widespread, and had been identified not only in muscle, liver, and adipocytes, but also in the hypothalamus, pituitary, and ovary of humans and rodents [14,19-21].

The hypothalamic-pituitary-ovarian axis (HPO axis) is central to the female mammalian reproductive system. The hypothalamus releases gonadotropin-releasing hormone (GnRH) in pulses and these stimulate the pituitary to secrete both luteinizing hormone (LH) and follicle-stimulating hormone (FSH), via the interaction between GnRH and the gonadotropin-releasing hormone receptor (GnRHR). FSH then acts on the follicle-stimulating hormone receptor (FSHR) to stimulate the ovary to secrete estradiol (E2). The enzyme aromatase P-450, encoded by the CYP19 gene, is responsible for a key step in estrogen

biosynthesis. Recent findings indicate that GnRH is not the sole hypothalamic regulatory neuropeptide of vertebrate reproduction, and gonadotropin-inhibitory hormone (GnIH) also plays a key role in the suppression of reproduction [22-24]. There is limited information regarding the modulatory effect of Adp on reproductive functions at different levels of the gonadal axis in pigs, and most studies have focused on the effects of Adp on the ovary [25-27]. The effects of Adp on overall endocrine function of the HPO axis in pigs remains unclear.

Wannan Spotted pig is the local breed in Huangshan, China, and has the characteristics of early maturity, a high reproduction rate, disease resistance and high-quality pork. In order to understand the interrelationship between Adp and the HPO axis in Wannan Spotted gilts, we measured serum levels of reproductive related hormones and mRNA expression of these genes. To the best of our knowledge, this study is the first to identify the effects of Adp on developmental changes in the HPO axis of gilts and to demonstrate developmental patterns of GnIH expression in pigs. The aim of this study was therefore to investigate the modulatory action of Adp on reproductive functions at different levels of the HPO and to examine the role of Adp in endocrine regulation in gilts. This information will be of great significance for the further development and utilization of this breed.

Materials and Methods

Animals

All animal experiments were approved by the local Animal Care Committee. Twenty-five healthy, Wannan Spotted gilts were supplied by the Animal Husbandry and Veterinary Medicine Bureau of Yi County, Huangshan, China. Five gilts were humanely slaughtered for blood and tissue sampling at each of 1, 30, 45, 90 and 180 days of age. Blood was collected into serum separator tubes (BD Microtainer 365967) and centrifuged at 6000 g for 90 s; resultant serum supernatant was stored at -80°C until hormone assays were conducted. Hypothalamus, pituitary, and ovary were snap frozen in liquid nitrogen and maintained at -80°C until required.

Measurement of serum hormones

Serum was assayed for the presence of Adp, GnRH, GnIH, FSH, LH, and E2 using pig ELISA kits (R&D, USA). The sensitivity of the ELISA assays for Adp, GnRH, GnIH, FSH, LH, and E2 was given as 25 pg/mL, 1.0 mIU/ml, 1.0 pg/mL, 0.1 mIU/ml, 0.1 mIU/ml, and 1.0 pg/mL, respectively.

Quantitative RT-PCR (qRT-PCR) of tissue samples

Total RNA was extracted from tissues using Trizol Reagent (Takara, China) following the manufacturer's directions. RNA (1 mg) was converted into cDNA using QIAGEN Quantitect kit and qRT-PCR performed in triplicate using cDNA from 100 ng RNA as the starting material for all reactions. PCR reactions (25 µl) were performed using SYBR®Premix Ex TaqTM II (Takara, China). All qRT-PCR reactions were measured using the Rotor-Gene 6000 Quantitative Real-time PCR instrument (Corbett, Australia), using thermal cycling conditions recommended by the manufacturer (40 cycles of 10 s at 95°C, 20 s at 60°C, and 15 s at 72°C). qRT-PCR was performed using intron-spanning primers and all primers were designed using Primer 5.0 software and Primer-BLAST-NCBI. The 2-ΔΔCT (cycle threshold) method was used to calculate fold changes and β-·actin was employed as an internal standard. Primer pairs (Shanghai Sangon, China) used for specific amplification of porcine Adp, AdpR1, AdpR2, GnRH, GnIH, GnRHR, LH, FSH, FSHR, CYP19, and β-Actin are listed in **Table 1**.

Statistical analysis

Each test was repeated in triplicate. Relative quantification of mRNA levels was performed according to the 2-ΔΔCT method [28]. All data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Results were expressed as Mean ± SEM. Statistical analysis and evaluation were performed using one-way ANOVA and Bivariate correlation. A value of $P<0.05$ was considered statistically significant.

Results

Changes in Serum Adp, LH, FSH, and E2 secretion during postnatal development

Mean serum Adp levels were lowest at 30 days of age, compared to all other days during postnatal development ($P<0.05$) (**Figure 1A**). Similarly, E2 levels were lowest at day 30 ($P<0.01$) (**Figure 1B**). In contrast, Serum FSH and LH levels exhibited a different pattern, showing an increase from day 1 to 30 and day 45 to 90 (**Figures 1C and 1D**). FSH, and LH levels were higher at day 30 than other days ($P<0.01$).

Changes in AdpR1, AdpR2, GnRH, and GnIH mRNA expression in the hypothalamus

Adp mRNA levels were very low in the hypothalamus (**Figure 2E**, data not shown). Expression of AdpR1 mRNA in hypothalamus increased between days 1 to 90 and then decreased by day 180

Table 1 Primer sequences and parameter used for real-time quantitative PCR.

Target Genes	GenBank accession	Primer sequences (5'-3')	Fragment size(bp)
β-Actin	U07786.1	F-CTCGATCATGAAGTGCGACGT S-GTGATCTCCTTCTGCATCCTGTC	114
Adp	AY589691.1	F- CGAGAAGGGTGAGAAAGGAGAT R- ATGCTGAACGGTAGACATAGGC	155
AdpR1	AY578142.1	F- GAGCATCTTCCGCATCCAC S- GAACATCCCAAACACCACCTT	151
AdpR2	AY606803.1	F- GCCACCATAGGGCAGATTG S- GCCAGCCACCACGAAGAT	159
GnRH	NM_214274.1	F- AGCGCTTTGAGTGCACCGCT S- TGCTCGCTGTCAGTGTCTCTGGT	133
GnIH	*GnIH*	F- GAGAGCAGCCCTGGGGCAATAG S-TGAACGCGTGGATTGTTGGAGCA	165
GnRHR	NM_214273	F-AGCCAACCTGTTGGAGACTCTGAT R-AGCTGAGGACTTTGCAGAGGAACT	101
LH-β	NM_214080	F-ATGCTCCAGAGACTGCTGTTGT R-TGCTGGTGGTAAAGGTGATGCAGA	151
FSH-β	NM_213875.1	F-TTGCTGCAATAGCTGTGAGCTC R- TTTCTGGATGTTGGGCCTG	154
FSH-R	NM_214386	F- TCGAGGCAAATGTGTTCTCC S- AAGGTTCTGGAAGGCATCAG	101
CYP19	SSU92246	F- CTCGAGTTTTTCCCCAAGC S- ACTGGCCTTGCTGTGTTTG	190

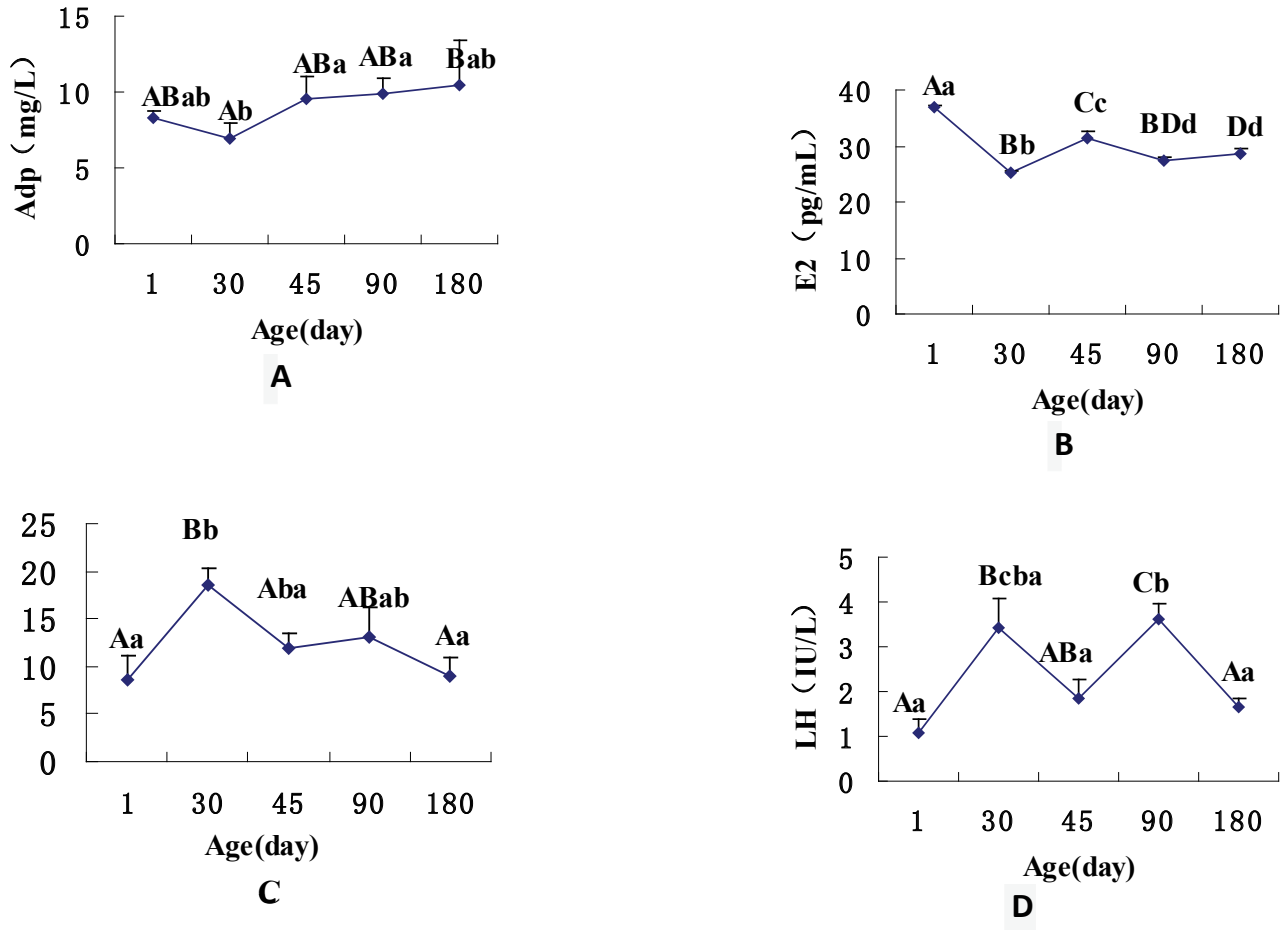

Figure 1 Developmental patterns of serum Adp (A), E2 (B), FSH (C) and LH (D) concentration in Wannan Hua sows by ELISA. The different small letters and capital letters stand for P<0.05 or P<0.01 between ages respectively. The same as follows.Data represent mean±SEM,n=5.

(**Figure 2A**). AdpR2 mRNA expression increased progressively during postnatal development (**Figure 2B**). GnRH mRNA expression was lowest at day 30 compared to all other time points measured (P<0.05) (**Figure 2C**). GnIH mRNA expression decreased between days 1 to 90 and then increased by day 180 (**Figure 2D**).

Changes in AdpR1, AdpR2, FSH, LH and GnRH-R mRNA Expression in the pituitary gland

Adp mRNA levels were also very low in the pituitary gland (**Figure 3E**). AdpR1 and AdpR2 mRNA expression decreased between days 1 to 30, increased at day 45 and then decreased again by day 180 (**Figure 3A**). In contrast, FSH, LH and GnRH-R mRNA expression increased between days 1 to 30, decreased at day 45 and then increased by day 90 (**Figures 3B-3D**). Correlation analyses showed a negative correlation between AdpR2 mRNA levels and GnRHR (r =-0.639, P<0.01), LH-β (r =-0.507; P<0.05), and serum FSH levels (r =-0.460, P<0.05). AdpR1 mRNA levels correlated positively with AdpR2 mRNA levels (r = 0.517, P<0.05) .

Changes in AdpR1, AdpR2, CYP19, and FSH-R mRNA Expression in the ovary

Ovarian AdpR1 mRNA expression increased significantly to reach a peak at 30 to 90 days (P<0.05; **Figure 4A**). The pattern of AdpR2 mRNA expression was similar to that of AdpR1 (P<0.01; **Figure 4B**). CYP19 mRNA expression maintained consistently low levels from days 1 to 45, but was higher on day 90 (**Figure 4C**). FSH-R mRNA expression increased progressively from days 1 to 45 and then decreased to a nadir at day 90 (**Figure 4D**). Correlation analysis showed AdpR1 mRNA levels correlated positively with AdpR2 mRNA levels (r = 0.380; P<0.05), and negatively with CYP19 mRNA (r = -0.472; P<0.05) and serum E2 levels (r = -0.416; P<0.05).

Discussion

Adipose tissue play a crucial role in energy homeostasis, not only by storing triglycerides, but also in response to neural, nutrient, and hormonal signals mediated through adipokine (leptin, adiponectin, and resistin) secretion [1]. It is well known that leptin plays a role in the regulation of energy homeostasis and reproduction, and acts as a mediator in the crosstalk between adipose tissue and the HPO axis [1,29]. Recently, several studies have demonstrated that Adp also participates in the regulation of energy expenditure, thermogenesis, and food intake in the HPO axis [12,14,30].

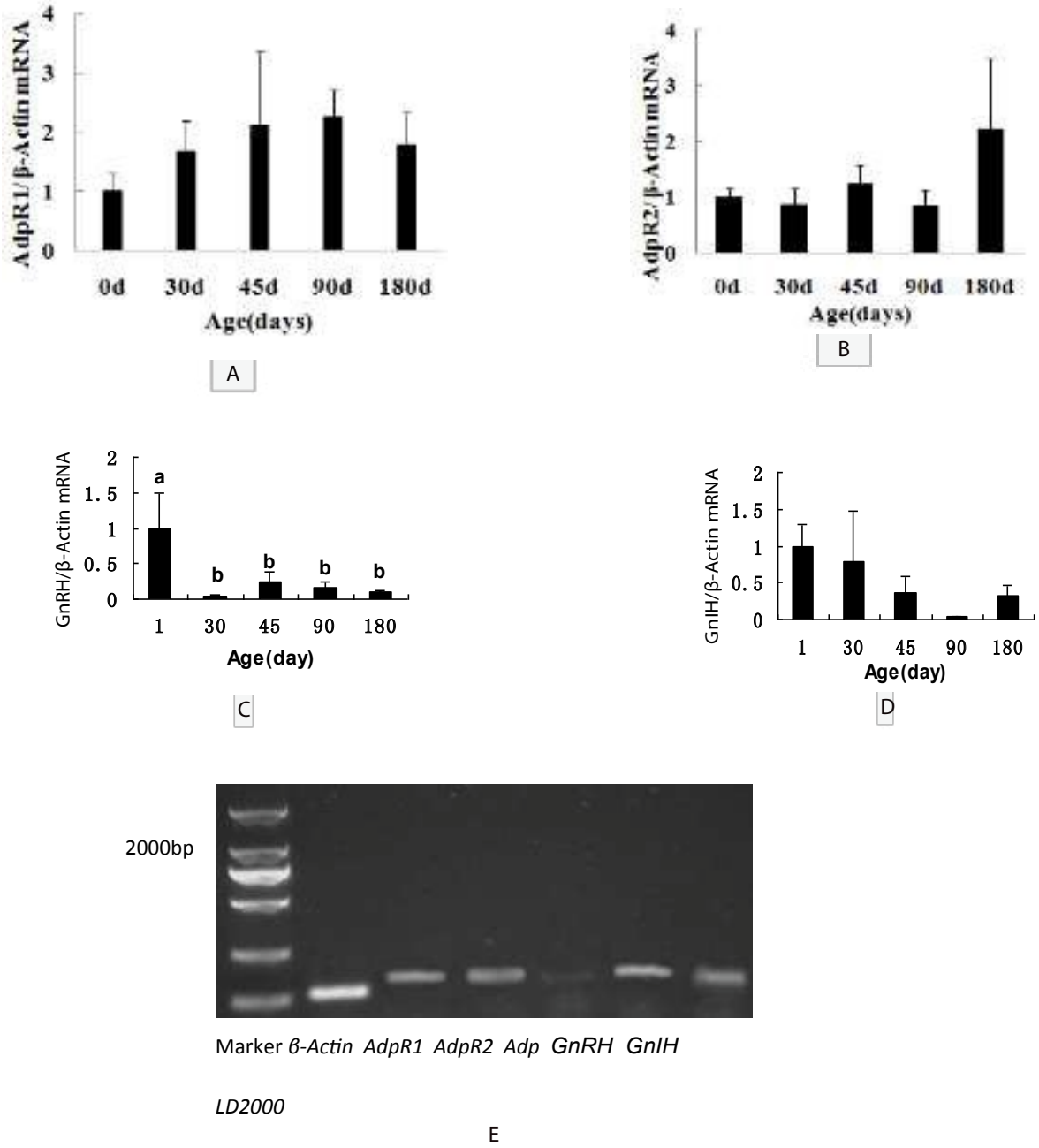

Figure 2 Developmental changes of *AdpR1*(A), *AdpR2*(B), *GnRH*(C), *GnIH*(D) mRNA expression in the Wannan Hua sow hypothalamus. (A, B, C and D) Representative Quantitative RT-PCR products for mRNA *AdpR1*(**a**), *AdpR2*(B), *GnRH*(C), and *GnIH*(D) mRNA level expressed as arbitrary units relative to β-actin mRNA, respectively. (E) Representative agarose gel electrophoresis of RT-PCR production of cDNA. The different small letters and capital letters stand for P<0.05 or P<0.01 between ages respectively. The same as follows. Data represent mean ± SEM, n=5.

Adp has been found in porcine follicular fluid at concentrations equivalent to that found in serum [25], although, Adp mRNA has not been previously identified in porcine ovarian or brain tissue [27]. In the present study, we found that porcine Adp mRNA was detected at very low levels in the hypothalamus, pituitary, and ovary. AdpRs mRNA is expressed widely in peripheral tissues, the brain, and granulosa cells in pigs [25,31]. In this study, we demonstrated that AdpRs mRNA is expressed in the HPO axis and shows no significant changes during postnatal development. We also found that serum Adp concentrations ranged between 5 to 15 mg/L over the study period. Based on these results, we speculate that Adp produced in adipose tissue plays a role as an endocrine modulator in the reproductive function of the HPO, mediated through AdpR. Although we did not investigate how Adp reaches the brain, a previous study has shown that Adp does not cross the blood-brain barrier [19]. Thus, the mechanism through which Adp regulates the reproductive functions of the HPO axis requires further investigation.

The hormones of the HPO axis play an important role in HPO

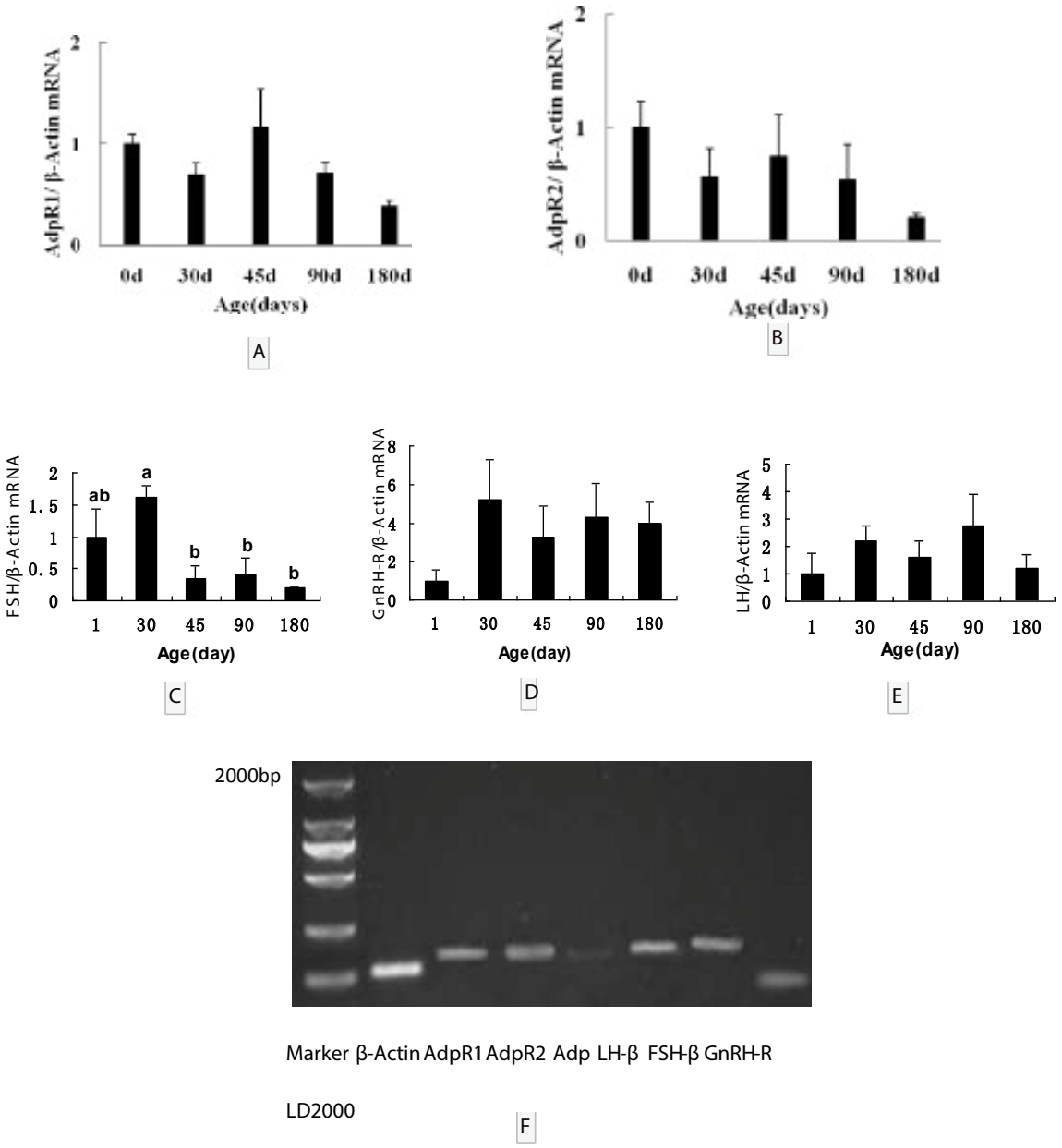

Figure 3 Developmental changes of *AdpR1*, *AdpR2, FSH*, *LH*, *GnRH-R* and *Adp* mRNA expression in the Wannan Hua sow pituitary. (A, B, C, D, E and **F**) Representative Quantitative RT-PCR products for mRNA *AdpR1*(**a**), *AdpR2*(**B**)*, FSH* (**C**), *GnRH-R* (**D**), and *LH* (**E**) mRNA level expressed as arbitrary units relative to β-actin mRNA, respectively. (**F**) Representative agarose gel electrophoresis of RT-PCR production of cDNA. The different small letters and capital letters stand for $P<0.05$ or $P<0.01$ between ages respectively. The same as follows. Data represent mean ± SEM, n=5.

axis development. Serum Adp levels have been shown to be inversely correlated with androgen levels in humans [32-34] and Adp inhibits both basal and GnRH-stimulated LH secretion in short-term treated rat pituitary cells [14]. At the level of the rat hypothalamus, Adp also influences oxytocin-secreting neuron excitability, perhaps explaining the increased oxytocin secretion observed in the obese human population [35]. Lu et al. demonstrated that Adp activated adenosine monophosphate protein kinase and decreased LH secretion in mouse LβT2-immortalized gonadotropic cells [13]. Furthermore, AdpRs mediate adiponectin to increase production of progesterone and E2 by insulin-like growth factor (IGF) in humans [36]. Adp has also been shown to inhibit progesterone and LH-dependent insulin production by bovine theca cells in vitro [37,38]. These studies suggest Adp does have an effect on reproduction. Here, we observed that the pattern of serum FSH, and LH reaching the peak at 30d, followed by a significant decline on 45d. Serum Adp showed the opposite developmental pattern. These results imply that Adp may be involved in regulation of the early development of the reproductive axis in swine. The down regulation of serum Adp on day 30 may be due to increased levels of reproductive hormones. The longitudinal pattern of hypothalamic and

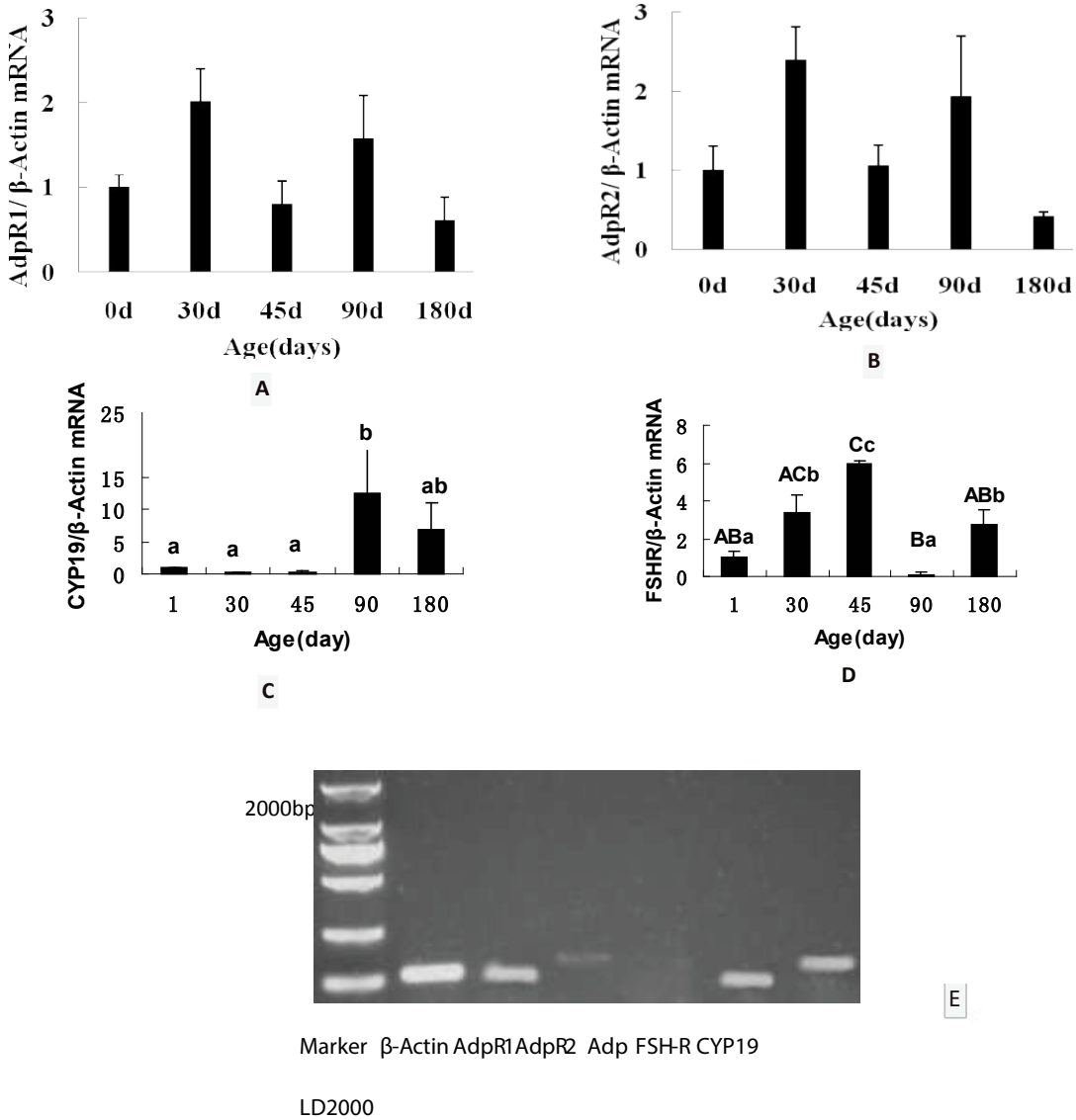

Figure 4 Developmental changes of *Adp*, *AdpR1*, *AdpR2,* CYP19 and FSH-R mRNA expression in the Wannan Hua sow ovarian. (**e**) Representative agarose gel electrophoresis of RT-PCR production of cDNA. (**A**, **B**, **C**, and **D**)Representative Quantitative RT-PCR products for mRNA *AdpR1*(**A**), *AdpR2*(**B**), CYP19 (**C**) and FSH-R(**D**) mRNA level expressed as arbitrary units relative to β-actin mRNA, respectively. The different small letters and capital letters stand for P<0.05 or P<0.01 between ages respectively. The same as follows.Data represent mean ± SEM, n=5.

pituitary mRNA expression showed a similar relationship to serum hormones and Adp. Furthermore, AdpR1 mRNA levels were inversely correlated with CYP19 mRNA and serum E2 in the ovary. These results suggest that Adp inhibited the secretion of hormones in the HPO axis in Wannan Spotted gilts.

Conclusion

In this study we have shown that serum reproductive hormones and Adp levels changed over time and showed reverse developmental changes during prepuberty in Wannan Spotted gilts. We propose that Adp may inhibit the hormones of the HPO axis through endocrine pathways and this action is mediated by AdpR during the prepubertal stages of development. These results benefit the further agricultural development and utilization of Wannan Spotted gilts.

Acknowledgments

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