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mRNA Expression of Genes Associated with Puberty Onset in the Adipose and Hypothalamic Tissues of Anhui White Goat

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Authors' Contributions
SC and QB conceived the project, and supervised the study. DZ obtained the animal samples. WH, JT, and QB conducted laboratory processing. WH wrote the article.

Key words
Goat, Puberty onset, Gene expression.

ABSTRACT

Puberty is the process of physical maturation of an animal with the capability of sexual reproduction. In domestic animals, age at attainment of puberty is an event that contributes significantly to lifetime reproductive efficiency. Anhui white goat is a native Chinese breed, with the characters of early time puberty onset and high fecundity. However, the molecular mechanism of puberty onset has not been well understood. It has been demonstrated leptin act as a critical metabolic cue linking adipose and the onset of puberty. In the present study, we first assessed the morphological changes in adipose tissue at pre-puberty and puberty onset stages of goats, and then determined the role of leptin to activate signaling pathways which modulate the expression of hypothalamic genes involved in reproduction and metabolism. We found that leptin significantly upregulated the LEPR in the adipose tissue of goat at the puberty onset stage. Moreover, we observed that LEPR, JAK2, STAT3 ($P<0.05$), KISS1 ($P<0.05$) were also upregulated in the hypothalamic tissue, except NPY, which was downregulated. Taken together, it is, therefore, inferred that probably leptin secreted in the adipose tissue via its receptor leptin receptor through the JAK2/STA3 signal pathways, promote the upregulation of KISS1, which activates TRPC5, and, inhibit the release of NPY, thereby promoting the release of GnRH, thus hastening the onset of puberty in goat. During this process, leptin, LEPR and its signal pathways may act as a link between the adipose and puberty onset in goat. However, further more studies need to be carried out to verify this point.

INTRODUCTION

Puberty is the process of physical maturation of an animal with the capability of sexual reproduction (Dorn *et al*., 2011). In animal, puberty is defined as age at first estrus when an animal will stand for breeding. Therefore, in domestic animals, age at attainment of puberty is an event that contributes significantly to lifetime reproductive efficiency (Haldar *et al*., 2014).

In fact, the onset of puberty is a complex phenomenon, which is known to be controlled by genetic, environmental and endogenous factors (Burns *et al*., 2010). Factors such as body weight, growth and body fat are important regulators of age at onset of puberty (Dunger *et al*., 2006; Rosales Nieto *et al*., 2013; Chan *et al*., 2015; Bohlen *et al*., 2016). Accumulating studies have been reported that adipose are crucial endogenous factors that affect the timing of sexual maturation (Dunger *et al*., 2006; Martos-Moreno *et al*., 2010; Sanchez-Garrido and Tena-Sempere, 2013; Roa and Tena-Sempere, 2014; Bohlen *et al*., 2016). Pubertal onset may be advanced by fatness, with leptin potentially acting as a permissive factor (Barash *et al*., 1996; Chehab *et al*., 1996; Sanchez-Garrido and Tena-Sempere, 2013). However, the mechanism how leptin exerts its effect is still a matter of debate.

The hypothalamus receives neural and endocrine input from these systems to appropriately activate the pituitary-ovarian axis under conditions favorable for successful pregnancy to occur. Accumulating evidence demonstrates that the effects of leptin to regulate the onset of puberty are thought to be mediated mainly by its receptor LEPR action on hypothalamic neurons (Bellefontaine *et al*., 2014; de Luque *et al*., 2007; Sanchez-Garrido and TenaSempere, 2013; Sheffer-Babila *et al*., 2013). Therefore, to unravel Leptin receptors (LepR) are highly expressed in arcuate nucleus (Arc) neurons, where they partially colocalize with kisspeptin, one of the most potent regulators of the

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reproductive axis (Zuure *et al*., 2013). It has been reported that mutations of LEPR are associated with the time of puberty onset (Haldar *et al*., 2014; Juengel *et al*., 2015; Day *et al*., 2016). Kiss1 gene, encoding the kisspeptins that bind its receptor Gpr54, has been recognized as indispensable molecule in the neuroendocrine control of puberty and reproduction (Pinilla *et al*., 2012; Oakley *et al*., 2009; Navarro and Tena-Sempere, 2011). It has been reported kisspeptin neurons were depolarized by leptin via activating TRPC Channels in guinea pig (Qiu *et al*., 2010, 2011).

Anhui white goat is a native Chinese goat breed, well known for its characters of higher fertility and earlier age at puberty onset, although its physique is relatively smaller, compared with other Chinese native goat breed (Chen *et al*., 2009). However, the possible molecular mechanisms underlie the body mass and the capability of earlier puberty onset remains unclear.

Herein, the present study was designed to investigate the relationship between adipose and puberty onset. The morphological changes of adipose were analyzed by histological analysis in goat at pre-puberty and puberty onset stages. Furthermore, mRNA expression of Leptin, LEPR, JAK2, STAT3, KISS1 and TRPC5 were detected in the adipose and hypothalamic tissue of pre-puberty and puberty onset goats, respectively, by Real-time PCR, which was aimed to see whether the changes of adipose affect the ability of leptin to activate signaling pathways and modulate the expression of hypothalamic genes involved in reproduction and metabolism.

MATERIALS AND METHODS

Ethic statement

The animals were handled in strict accordance with Animal Ethics Procedure and Guidelines of the People's Republic of China. All experiments protocol described here were approved by the Institutional Animal Care and Use Committee of Animal Science and Veterinary institute, Anhui Academy of Agriculture Science.

Determination of age at puberty onset

The age at puberty onset was determined according to the method described by Haldar *et al*. (2014) with some modifications. The data of Anhui white ewes were recorded when she was born, including birth date, weight, litter size, as well as the weaning weight and data at weaning. Age of puberty onset was determined using crayon marking by a vasectomized ram fitted with a mating harness. Estrus detection was performed daily from 60 days (served as pre-puberty stages), when ewe lambs were exposed to such rams. During which time herdsmen observed them for estrous behavior. The puberty age was defined as the date in which the first standing estrus was detected. Then, marked ewe was removed from the group and monitoring continued for all unmarked ewes.

Procedure adopted

Anhui white goat included in the study including four puberty onset female lambs and four pre-puberty counterparts. The animals were euthanized and the tissues were sampled immediately after death. The hypothalamus and abdominal adipose tissue were removed and quickly snap frozen in liquid nitrogen before being used for RNA extraction or fixed in 4% buffered formalin for histological evaluation.

Histological evaluation

Intra-abdominal (visceral) fat pads were fixed in Formalin solution and embedded in paraffin. Histological sections (5 µm) were stained with hematoxylin and eosin (H&E) stain according to standard procedures. Images were analysed by an inverted fluorescent microscope (Nikon Ti-s, Japan).

RNA extraction and cDNA synthesis

Total RNA was extracted and purified using the Tiangen RNAprep pure Tissue Kit (Tiangen, Beijing, China) with a genomic DNA removal step as per manufacturer's protocol. RNA quality and concentration levels were determined using a photometer (Nanodrop2000, Thermofisher, USA), and RNA integrity was verified via electrophoresis. cDNA was synthesized with equal amounts of RNA samples using one-step reverse transcription kit (Tiangen, Beijing, China) according to the manufacturer's instructions.

Real-time PCR

Expression of the target genes, (*viz*. Leptin, LEPR, JAK2, STAT3, KISS1, TRPC5) were analyzed through quantitative real-time PCR (qRT-PCR) and using the cDNA of various tissues in different stages of puberty as templates. The GAPDH was selected as an internal control based on its expression stability.

According to sequences obtained from GenBank, the primers for each target gene listed in Table I, were designed by Primer premier 5.0 and synthesized by Boshang biotechnology company (Shanghai, China). Real-time PCR was performed using the 7500TM system (Applied Biosystems) under the following condition: denaturation at 94°C for 4min, followed by 35 cycles of 94°C for 15s, annealing at 55-60°C for 20s, and extension at 72°C for 20s, and a final extension step of 10 min at 72°C. Each reaction was carried out in a total volume of 20µL, consisting of 12.5µL SYBR® Premix Ex, 0.5µL each primer

Table I.- Primers used for real-time polymerase chain reaction.

Gene	Primer sequences (5'-3')	Annealing temperature (°C)	Expected size (bp)	Reference/ accession no.
GAPDH	F: CAAGTTCAACGGCACAGTCA R: TGGTTCACGCCCATCACAA	59.0	249	XM_005680968.2
Leptin	F: AAGGCTACGGGCACATACA R: AGAGCCCTCAAGTCACTCAAA	60.0	209	XM_005679433.2
LEPR	F: TGAATGGAAGTGGGAGGAT R: CACGAGGAAGACATGGTGC	57.0	209	XR_001295509.1
JAK2	F: CAAAGTGAAAGAGCCTGGTG R: CATAAATTCCGCTGGTGGG	56.0	163	XM_013965950.1
STAT3	F: CTGGGCACAAACACGAAAG R: CACCACCACTGGCAATGAGT	60.0	231	KP343689.1
KISS1	F: CGCTCTGCCCCTCTGAGAG R: CCAGTTGTAGGCGGACACG	55.0	131	NM_001285710
NPY	F: GAAGAAGGGGAGACAATCTAAAA R: AACAAAGGACAAATCAAAAGC	56.5	119	XM_005679293.2
TRPC5	F: CTCAGATGGAGAAAGGGAAAG R: GGAACAGATGCTGGATACGC	58.0	119	XM_005700254.2

(10μ mol/L), 2μL cDNA and 4.5μL ddH_2O. Amplification reaction for each sample was conducted in triplicate. And the levels of transcript were generated from a standard that was simultaneously amplified with the samples. Levels of gene expression were then normalized against GAPDH, which served as internal controls.

Statistical analysis

All data are shown as mean±SEM. The relative mRNA expression levels of goat LEPR, JAK2, STAT3, KISS1, NPY and TRPC5 were calculated by $2^{-\Delta\Delta CT}$ method. Statistical analysis was carried out using SPSS version 17.0. One-way ANOVA test and repeated measure of ANOVA were used for statistical analysis of normalized gene copy number and differences were considered significant at $P<0.05$.

RESULTS

Histological analysis

Histological analysis of selected tissues was clone to assess the morphology of adipose tissue in goat at pre-puberty stage and puberty onset stage. Figure 1A shows vascularization and ECM surrounding at the adipocyte in pre-puberty goat. The morphology of adipocyte is not clear. At puberty onset stage in goat, the adipocyte became mature and bigger (Fig. 1B), is suggested by the comparison of adipocyte area (Fig. 1C). At this stage vascularization and ECM disapear, adipocyte become more uniform, and are mostly unilocular.

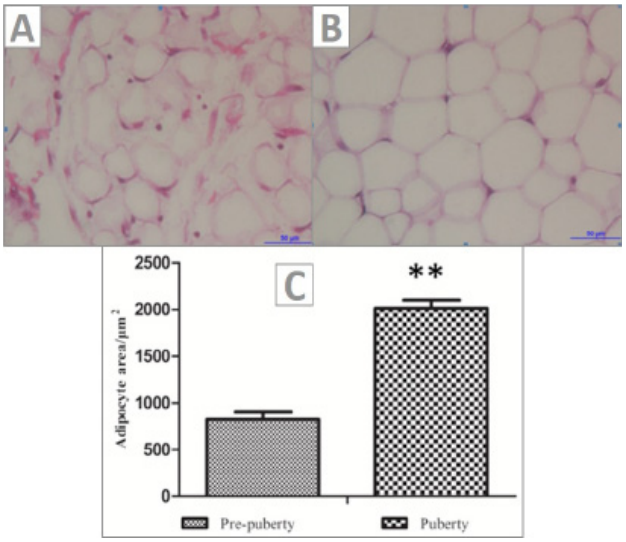

Fig. 1. Histological structure of adipose tissue of goat at pre-puberty stage (A) and puberty onset stage (B). Histological sections were stained with H&E. C, comparison of adipocyte area of goat at the two stages. **:P<0.01 (Puberty group vs .pre-puberty controls).

mRNA expression of Leptin, LEPR, JAK2, and STAT3 in adipose tissue

Figure 2 shows mRNA level of genes of the leptin mediated signal pathways (leptin, LEPR, JAK2, STAT3) in the adipose tissue. Relatively higher expression of these genes was observed in the adipose tissue of goat at the

onset of puberty compared to the pre-puberty stage. LEPR gene showed significantly high over-expression at the onset of puberty compared to the pre-puberty stage ($P < 0.01$).

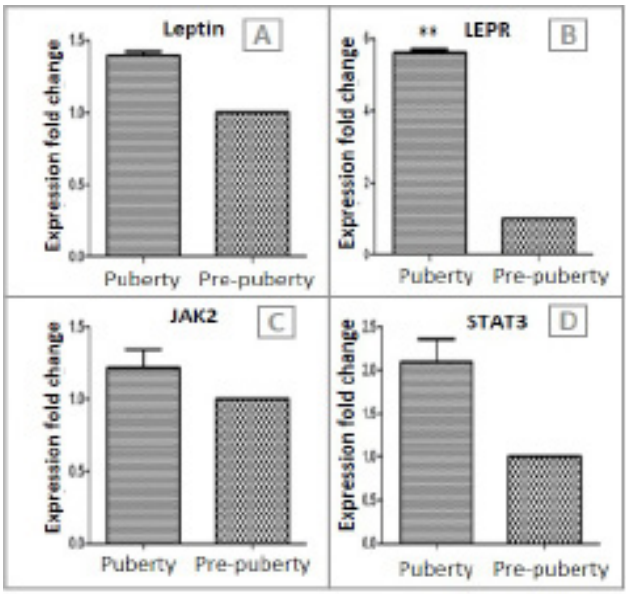

Fig. 2. mRNA levels (in terms of fold change with reference to control) in leptin, LEPR, JAK2, and STAT3 in the adipose tissue of goat. The data shown represent the mean ± SD. *: P<0.05 (Puberty group vs .pre-puberty controls); **: P<0.01 (Puberty group vs .pre-puberty controls).

mRNA expression of LEPR, JAK2, STAT3, NPY, KISS1 and TRPC5 in hypothalamus tissue

Figure 3 shows significantly higher mRNA levels of LEPR, JAK2, STAT3, KISS1, NPY, TRPC5, genes in the hypothalamus puberty at the onset of KISS1, and STAT3 showed substantially increased expression level in the pre-puberty stage (P<0.05). It appears leptin activates KISS1, TRPC5 in the hypothalamus tissue via LEPR through JAK2/STAT3 signal pathways in the hypothalamus of sheep at the onset of puberty. Figure 4 shows a model of interaction of genes of adipose tissue and hypothalamus involved during pre-puberty and onset of puberty in goats.

DISCUSSION

Puberty is a developmental transition for attainment of reproductive capacity in human and animals, which is also the endpoint of a long-lasting developmental continuum and means sexual maturation (Castellano and Tena-Sempere., 2016). Epidemiological data in humans suggest that a critical amount of body fat is required for proper sexual maturation (Frisch, 1985), since adipose tissue plays an essential role in regulating energy balance

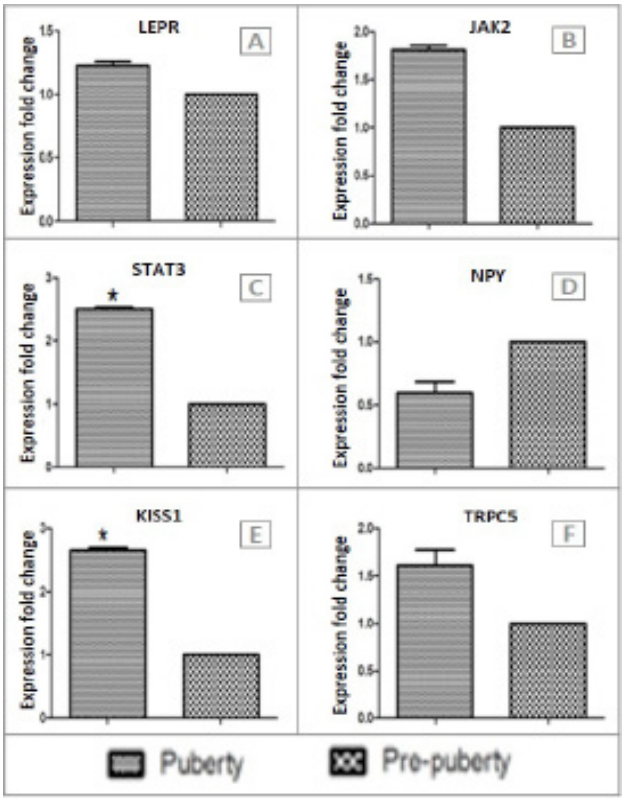

Fig. 3. mRNA levels (in terms of fold change with reference to control) in LEPR, JAK2, STAT3, KISS1, NPY and TRPC5 in the hypothalamus tissue of goat. The data shown represent the mean ± SD. *: P<0.05 (Puberty group *vs.* pre-puberty controls); **:P<0.01 (Puberty group *vs.* pre-puberty controls).

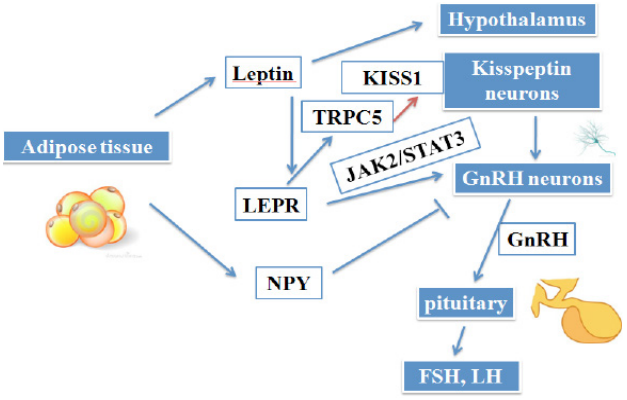

Fig. 4. Model showing inter-relationship of various genes, the expression of which has been studied in the study.

through its metabolic, cellular and endocrine functions during development. Adipose is, therefore, a key factor that affects the time of puberty. In the present study, firstly,

we detected the morphological differences in the adipose tissue at puberty and pre-puberty stages. It has been acknowledged that angiogenesis and adipogenesis are tightly coupled during development (Lee *et al*., 2016). In conjunction with histological differences, it may be indicated that the adipose development is related to the puberty onset. We also detected alteration in expression of genes associated with adipose in goat at pre-puberty and onset of puberty.

Leptin, an adipocyte-derived hormone, is required for normal pubertal maturation in animals and human, therefore, it has been recognized as a critical metabolic cue linking energy stores and the onset of puberty (Cunningham *et al*., 1999; Elias, 2012; Roa *et al*., 2010). Increasing studies demonstrated that humans and mice lacking leptin or LepR are infertile and fail to enter puberty (Barash *et al*., 1996; Chehab *et al*., 1996; Mounzih *et al*., 1997; Moschos *et al*., 2002).

LEPR activates the activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway. Numerous studies have showed evidences that the full effect of leptin in reproduction requires the recruitment of a STAT3-independent pathway (Zuure *et al*., 2013). In the present study, we found that leptin-mediated signaling genes (Leptin, LEPR, JAK2 and STAT3) were up-regulated in the adipose tissue of goat at the onset of puberty compared with pre-pubertal controls. Specifically, LEPR gene experienced a dramatically overexpression level at the puberty onset stage. The data may suggest that the leptin signaling pathways may be strongly activated in the adipose tissue of goat with puberty-onset stage, especially for LEPR.

Furthermore, in order to detect whether leptin from adipose tissue could transduce signals to the brain tissue, we detected LEPR, JAK2, STAT3, NYP, KISS1, and TRPC5 in the hypothalamic tissue of goat at the onset of puberty, and prepubertal stage. The up-regulation of leptin signal pathways, such as, LEPR, JAK2 and STAT3, were likewise observed in the hypothalamic tissue of goat at the puberty onset stage, compared with that of prepubertal goat. It is suggested that the JAK2/STA3 signal is activated during the pubertal onset in goat. LepR are highly expressed in arcuate nucleus (Arc) neurons, where they partially colocalize with kisspeptin, one of the most potent regulators of the reproductive axis (Smith *et al*., 2006; Oakley *et al*., 2009; Colledge, 2009; Pinilla *et al*., 2012).

Kiss1 gene, encoding the kisspeptins, that bind its receptor Gpr54, have been recognized as indispensable molecule in the neuroendocrine control of puberty and reproduction (Pinilla *et al*., 2012; Oakley *et al*., 2009; Navarro and Tena-Sempere, 2011). Loss-of-function mutations in Kiss1 genes cause infertility due to lack of pubertal maturation in mice and humans (d'Anglemont *et al*., 2007; Lapatto *et al*., 2007; Topaloglu *et al*., 2012; de Roux *et al*., 2003). Numerous reports have confirmed that leptin is a significant upregulator of hypothalamic kiss1 expression (Castellano *et al*., 2006; Smith *et al*., 2006; Backholer *et al*., 2010). In the present study, we found that *Kiss1 gene is significantly up-regulated in the* hypothalamic tissue of goat at puberty onset stage, compared with that of prepubertal controls. Our finding is consistent with the studies reported by Cravo *et al*. (2013), who found that prepubertal and leptin signaling-deficient mice display decreased numbers of Kiss1 neurons. Our result may partially suggested that upregulation of leptin may raise Kiss1 during the puberty onset stage.

Since kisspeptin signaling plays a critical role in modulating GnRH neuronal excitability, which controls pituitary gonadotropins secretion and ultimately reproduction. It has been reported that kisspeptin potently depolarizes GnRH neurons primarily through the activation of canonical transient receptor potential channels (TRPC). Qiu *et al*. (2010) and (2011) found that kisspeptin neurons were depolarized by leptin via activating TRPC in guinea pig. Zhang and Spergel (2012) found that kisspeptin activates TRPC though cSrc tyrosine kinase activation, which is a novel signaling pathway for peptidergic excitation of GnRH neurons. In the present study, we found that puberty onset stage witnessed an overexpression of TRPC5 in the hypothalamus of goat, which is suggestive of activation of the TRPC5, by Kiss1 gene which thus releases GnRH, thereby resulting in the onset of puberty.

Neuropeptide Y (NPY) neurons in the arcuate nucleus (ARC) contain the leptin receptor, which are responsive to changes in nutritional status (Baskin *et al*., 1999). It was reported that during the heifers peripubertical stages, increased leptin concentrations, could result in lower hypothalamic NPY release, and greater pulsatile of LH release, thereby hastening puberty onset (Cardoso *et al*., 2014). In the agonadal male, NPY expression pattern from birth to puberty was inversely related to that of pulsatile GnRH release (El Majdoubi *et al*., 2000). In the present study, we found that hypothalamic NPY in the goat of puberty onset stage was lower than that of pre-puberty, suggesting that NPY was negatively associated with the time of puberty onset, which is inversely related to the leptin expression.

CONCLUSION

In conclusion, our study firstly detected the

morphology of adipose tissue during the puberty stage of goat, and found that morphology of adipocytes changed significantly. We next analyzed mRNA expression levels of genes related to leptin and signal pathways in the adipose tissue. It is observed that leptin, LEPR, and JAK2, STAT3 were up-regulated in the puberty onset stage. Furthermore, we analyzed the LEPR, KISS1, TRPC5, NPY, JAK2 and STAT3 in the hypothalamus tissue, we found that expression levels of LEPR, KISS1, TRPC5, JAK2, and STAT3 were upregulated, whereas NPY mRNA expression was downregulated. We gave a hypothesis that Leptin secreted in the adipose tissue via its receptor leptin receptor through the JAK2/STA3 signal pathways, promote the upregulation of KISS1, which activates TRPC5, while in turn, inhibit the release of NPY, thereby promoting the release of GnRH, thus hastening the onset of puberty in goat. However, the differences observed in the onset of puberty were perhaps caused by other neurotransmitters that were not evaluated in our study. It is, therefore, necessary to carry out furthermore studies to identify possible mechanisms of leptin or LEPR that may link the changes in adipose and hypothalamus with the timing of puberty.

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Conflict of interest statement

The authors declare that they have no competing interests.

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