Adiponectin inhibits KISS1 gene transcription through AMPK and specificity protein-1 in the hypothalamic GT1-7 neurons

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Abstract

Adiponectin secreted from adipose tissues plays a role in the regulation of energy homeostasis, food intake, and reproduction in the hypothalamus. We have previously demonstrated that adiponectin significantly inhibited GNRH secretion from GT1-7 hypothalamic GNRH neuron cells. In this study, we further investigated the effect of adiponectin on hypothalamic KISS1 gene transcription, which is the upstream signal of GNRH. We found that globular adiponectin (gAd) or AICAR, an artificial AMPK activator, decreased KISS1 mRNA transcription and promoter activity. Conversely, inhibition of AMPK by Compound C or AMPKz1-siRNA augmented KISS1 mRNA transcription and promoter activity. Additionally, gAd and AICAR decreased the translocation of specificity protein-1 (SP1) from cytoplasm to nucleus; however, Compound C and AMPKz1-siRNA played an inverse role. Our experiments in vivo demonstrated that the expression of Kiss1 mRNA was stimulated twofold in the Compound C-treated rats and decreased about 60–70% in gAd- or AICAR-treated rats compared with control group. The numbers of kisspeptin immunopositive neurons in the arcuate nucleus region of Sprague Dawley rats mimicked the same trend seen in Kiss1 mRNA levels in animal groups with different treatments. In conclusion, our results provide the first evidence that adiponectin reduces Kiss1 gene transcription in GT1-7 cells through activation of AMPK and subsequently decreased translocation of SP1.

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Introduction

Human puberty is a mystifying process involving a complex series of hormonal events. From a general neuroendocrine perspective, puberty is defined by the full activation of the hypothalamic-pituitary-gonadal (HPG) axis (Ojeda et al. 1986, Grumbach 2002). The onset of puberty is marked by an increase in the secretion of GNRH from the hypothalamus. A crucial role in puberty and reproduction has been recently assigned to a new hypothalamic signaling pathway, the KISS1/G protein-coupled receptor 54 (GPR54) system (Seminara et al. 2003). KISS1 gene encodes a premature 145-amino acid protein called kisspeptin, which is an active ligand of GPR54 (KISS1R) in humans (Kotani et al. 2001, Muir et al. 2001, Ohtaki et al. 2001). The master role of the KISS1/GPR54 system in the central regulation of the HPG axis was first unraveled by the finding that inactivating mutations of the GPR54 gene were associated with the lack of puberty onset and hypogonadotropic hypogonadism both in humans and in mice (de Roux et al. 2003, Seminara et al. 2003), which indicates that KISS1 and GPR54 are the new gatekeepers of reproduction.

Interestingly, hypothalamic KISS1 has been proposed as a key molecular conduit for relaying a number of peripheral signals into the GNRH system in the HPG axis. These include not only feedback actions of androgen and estrogen (Navarro et al. 2004, Smith et al. 2005) but also metabolic cues (Castellano et al. 2006). Castellano et al. (2005) showed an interaction between energy status and the hypothalamic KISS1 system, which may constitute a target for disruption of pubertal development under conditions of negative energy balance. Several neuropeptides and hormones, including leptin, insulin, and kisspeptin, have been reviewed and are involved in communicating energy status to the reproductive axis (Budak et al. 2006, Hill et al. 2008). Besides, an increasing body of evidence indicates that adiponectin secreted from adipose tissues could also directly regulate reproductive functions. For instance, our previous research had demonstrated that adiponectin inhibited GNRH secretion in the hypothalamus (Wen et al. 2008). Adiponectin also inhibits...
both basal and GNRH-stimulated LH secretion in rat and mouse pituitary cells (Rodriguez-Pacheco et al. 2007, Lu et al. 2008). Overexpression of adiponectin impairs female fertility in mice (Campos et al. 2008), and serum adiponectin levels in boys and girls decrease throughout puberty (Bottner et al. 2004). These findings draw immediate attention to the suggestion that changes in the hypothalamic KISS1 gene may be mediated by adiponectin according to metabolic status.

Adiponectin, also termed Acrp30 or AdipoQ, was identified in 1995–1996 as an adipocyte-specific secreted factor with an N-terminal collagenous domain and a C-terminal globular domain (Maeda et al. 1996). Adiponectin activates downstream targets such as AMPK, peroxisome proliferator-activated receptor-A, and MAPK through binding to its two receptors AdipoR1 and AdipoR2 (Yamauchi et al. 2003, Kadowaki & Yamauchi 2005). The roles of adiponectin in antidiabetic (Yamauchi et al. 2001), anti-inflammatory, antiangiogenic, and antiatherosclerotic effects in peripheral tissues (Brakenhielm et al. 2004, Goldstein & Scalia 2004) were reported in the early studies. Recent studies showed that adiponectin participates in the regulation of energy homeostasis (Kubota et al. 2007, Wen et al. 2010) and GNRH secretion through activation of AMPK in the hypothalamus (Wen et al. 2008). However, it has not yet been studied whether and how adiponectin participates in KISS1 expression in the hypothalamus.

It was clear that KISS1 can be regulated at the transcriptional level by specificity protein–1 (SP1) in different cells (Mitchell et al. 2006, Li et al. 2007, Mitchell et al. 2007). SP1 is a ubiquitously expressed, prototypic C2H2-type zinc-finger-containing DNA binding protein that can activate or repress transcription through GC-rich elements in response to physiological and pathological stimuli. SP1 can be phosphorylated by various kinases at different sites, and the effects of these modifications can lead to its translocation from the cytoplasm to the nucleus, followed by the regulation of gene expression (Majumdar et al. 2006, Solomon et al. 2008, Tan & Khachigian 2009). A report from Chu & Ferro (2005) shows that AMPK downstream signaling molecules, such as ERK, can mediate the phosphorylation of transcription factor SP1.

In this study, we investigated the intracellular signaling mechanisms and AMPK activation in adiponectin-treated GT1-7 cells, an immortalized hypothalamic KISS1 gene-positive neuron (Chu and Ferro 2005, Li et al. 2007). We hypothesized that adiponectin dynamically regulated KISS1 gene transcription through activation of AMPK and sequential subcellular compartmentalization of SP1 in GT1-7 cells. To test this hypothesis, we evaluated KISS1 gene transcription levels in response to adiponectin, as well as AMPKζ1-SiRNA, 5-aminimidazole-4-carboxamide riboside (AICAR), and Compound C, which regulate the activity of AMPK specifically or nonspecifically. In addition, we conducted mechanistic studies aiming to disclose the mechanism by which adiponectin regulates KISS1 gene transcription.

### Materials and Methods

#### Culture of neurons

Immortalized hypothalamic neurons (GT1-7 cells) were provided gratis by Shanghai Clinical Center for Endocrine and Metabolic Diseases, Shanghai Jiaotong University School of Medicine (Shanghai, China). GT1-7 cells were maintained in 4:5 g/l glucose DMEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin (at 37 °C in a humidified atmosphere of 5% CO2). Cells were deprived of serum overnight by transferring them to a serum-free medium for incubation before pharmaceutical exposure. Then cells were exposed to globular adiponectin (gAd) at concentrations 0.1, 0.2, 1.0, and 5.0 μg/ml, respectively, for 2, 6, 9, 18 and 24 h, AICAR (0.1 and 1.0 mM) for 2 or 24 h, Compound C (20 and 40 μM) for 2 or 24 h, and mithramycin A (100 and 200 nM) for 24 h. Mouse gAd (Gingras et al. 2009) was purchased from R&D systems (Minneapolis, MN, USA), AICAR and Compound C from Sigma, and mithramycin A from AppliChem (Ottoweg, Darmstadt, Germany).

#### Animals

Female Sprague Dawley rats (5–6 months) were obtained from SLAC Laboratory Animal, Inc., Shanghai, China. All animals were housed under controlled environmental conditions (12 h light:12 h darkness cycles; 20–24 °C; humidity 55 ± 10%) with free access to food and water. The female rats having had two consecutive estrous cycles were bilaterally ovariectomized and allowed to recover in 14 days. Animals were anesthetized with i.p. injection of pentobarbital and given an i.c.v. injection (−0.8 mm posterior, 4.0 mm ventral to the bregma, and 1.5 mm lateral from the midline) of colchicines at 75 μg/15 μl using a protocol adapted from Adachi et al. (2007). Two days later, the rats were randomly assigned into four groups (n = 5) and given i.c.v. injection of PBS/DMSO, gAd, AICAR, or Compound C respectively. The method is described in Table 1. Three hours later, animals were perfused with 200 ml of 0·1 M PBS and 200 ml of cold 4% paraformaldehyde in 0·1 M PBS (pH 7·4) through left atrium in succession. Brains were immersed in paraformaldehyde for 12 h followed by 25% sucrose/0·1 M PBS for 2 days. The fixed brain tissue was sectioned coronally at 30 μm using a sliding microtome. All procedures were performed via free access.

#### Table 1 The methods of i.c.v. for the four groups

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approved by the Animal Care Committee of Fujian Medical University in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Isolation of RNA and cDNA synthesis
GT1-7 cells, as well as hypothalamic tissues from female Sprague Dawley rats, were used in this process. For Sprague Dawley rats, their brain tissues were immediately removed after decapitation and kept on ice and the tissue of the ARC area was then dissected out of the brain with a microknife. RNA extraction was conducted according to the manufacturer’s instructions using TRIZOL reagent (Invitrogen). All RNA samples went through DNase enzymatic digestion (Qiagen) to eliminate DNA contamination. In a subsequent analysis, cDNA was synthesized based on 1 μg total RNA from each sample using the TaqMan RT kit (Promega) following the manufacturer’s condition (60 min at 42°C, 5 min at 95°C, and 5 min at 4°C).

Real-time quantitative PCR
Real-time quantitative PCR was carried out to evaluate Kiss1 gene expression levels in the GT1-7 cells and the hypothalamic tissues of Sprague Dawley rats posterior to pharmaceutical exposures (gAd, AICAR, and Compound C). The extraction of RNA and synthesis of first-strand cDNA were as mentioned earlier. The conditions of this real-time PCR were as follows: 20 μl reactions containing 4 μl cDNA, 400 nM primer pairs, and 10 μl SYBR green PCR master were mixed in 96-well plates on an ABI PRISM 7300 sequence detection system (Applied Biosystems). To normalize the quantity of cDNA in the reaction, a housekeeping gene, β-actin, was run in all reactions separately under the same experimental conditions. The primers for the PCR are Kiss1 (forward 5′-GCTGCTGGTTCTCTCCTCTGTG-3′ and reverse 5′-CTGCATACCGCGATTCCTT -3′) and β-actin (forward 5′-GGCTTATCCCTCCTCCATCG-3′ and reverse 5′-CCGGCTTGAACATGGCCATGT-3′). The PCR cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Dissociation curve analysis was also carried out for each gene at the end of the PCR. The raw data from each experiment were used to determine the relative expression levels of the gene by the −ΔΔCT method.

Transient transfection and luciferase assay
Cells were cultured on 24-well plates in DMEM supplemented with 10% FBS for at least 24 h. When cells reached approximately 60% confluence, the transient transfection process was started. PGL3–KISS1 promoter (provided gratis by Shanghai Clinical Center for Endocrine and Metabolic Diseases, and the human KISS1 promoter was cloned from BAC clone RP11-203F10 using primers consisting of XhoI and KpnI sites for ligation into the pGL3-basic vector, as described previously; Mitchell et al. 2007) was transfected with the aid of Lipofectamine 2000 transfection reagent (LF2000; Invitrogen Life Technologies) according to the manufacturer’s protocol. To internally control the transfection efficiency, cells were cotransfected with pRL-TK (Promega), an expression vector for Renilla luciferase, under the control of a thymidine kinase promoter. This TK promoter would nullify any effect of adiponectin on the expression of luciferase. Premixed DNA and Lipofectamine 2000 were applied to the cells for an 18 h term of transfection. Cells were then incubated with gAd, AICAR, Compound C, AMPKz1-siRNA, SP1siRNA, and mithramycin A for a further 24 h term. Cell lysates were prepared using Reporter Lysis Buffer (Promega) and other routine techniques such as centrifugation (18 000 g for 5 min). Aliquots of cell lysates (20 μl) were used to mix with luciferase substrate for luminescent measurement (Berthold Detection Systems, Pforzheim, Baden-Wuerttemberg, Germany).

Western blot analysis
GT1-7 cells were harvested and lysed in radioimmuno-precipitation buffer containing protease inhibitors for total cell protein extract. Nuclear and cytoplasmic proteins were extracted using a kit (Beyotime Institute of Biotechnology, Jiangsu, China). In brief, after the cells were treated as indicated, cytoplasmic proteins were prepared by repeating cycles of freezing and thawing in 0·2 ml of cold buffer A. Nuclear protein was extracted by using ice-cold buffer C. Total cellular extracts (40 μg protein) were separated using 10% SDS–PAGE gels and then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated first with blocking buffer (Tris-buffered saline containing 0·05% Tween 20 and 10% nonfat milk) for 3 h at room temperature. Primary polyclonal antibodies against SP1, β-actin, β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Lamin B1 (Bioworld Technology, Louis Park, MN, USA) were applied overnight at 4°C. After three washes, the membranes were subsequently incubated with secondary antibody conjugated with HRP (Invitrogen) for 2 h at room temperature. The blots were visualized by ECL using Kodak X-OMATLS film (Eastman Kodak). Quantitative data were obtained using a computing densitometer and ImageQuant Software (Piscataway, NY, USA).

Fluorescence microscopy
Cells were cultivated on coverslips, washed in PBS three times (5 min each), and fixed in 10% paraformaldehyde (10 min). Cells were washed three times in PBS (5 min each), made permeable in 0·3% Triton X-100 for 15 min, and then washed three times in PBS (5 min each). To block nonspecific antibody-binding sites, cells were incubated in 10% goat serum albumin in PBS for 1 h at room temperature. The cells were then incubated overnight at 4°C with rabbit
polyclonal antibodies to SP1, washed with PBS three times (5 min each), and incubated for 1 h at 37°C with FITC-conjugated goat antibodies to rabbit IgG (ZSGB-BIO, Beijing, China; green; 1:250) in the dark. The cells were washed three times with PBS again and were then stained using 1-aminopyridinium iodide for 30 min in the dark, followed by three washes in PBS. Coverslips were air-dried and mounted with antifading glycerinum, then mounted on glass slides, and finally observed under a fluorescence microscope.

**Immunohistochemistry**

Brain tissues of the killed rats were immersed in paraformaldehyde for 12 h followed by 25% sucrose/0.1 M PBS for 2 days. Fixed brain tissue was sectioned coronally at 30 μm using a sliding microtome. Then the sections through ARC were preserved in ethylene glycol cryoprotectant at −20°C until use. The free-floating sections were rinsed three times (10 min each) in 0.01 M PBS and incubated in 3% H2O2/PBS for 10 min. Before incubation in the primary antiserum, the sections were rinsed and treated in the blocking solution (PBS with 0.3% Triton X-100, 5% sheep serum, and 0.25% BSA) at room temperature for 1 h. Then they were incubated at 4°C for 48 h in anti-KISS1 rabbit polyclonal primary antibody (1:200 000; antiserum 566, a generous gift from Dr A Caraty, Nouzilly, France). After incubation in primary antiserum, the sections were washed and incubated in secondary biotinylated goat anti-rabbit IgG (1:500; Vector Laboratories, Burlingame, CA, USA) for 2 h. Rinsed again three times (10 min each), the sections were incubated for 1 h in ABC Elite Kit (Vector Laboratories) at room temperature. Then the free-floating brain sections were stained with DAB/chromogen solution for 15 min and sequentially rinsed in PBS three times (2 min each). Finally, sections were mounted on gelatinized glass slides, dried, dehydrated in ascending alcohol concentrations, cleared by xylene, and coverslipped with a neutral gum.

**Cell counting**

To quantify kisspeptin immunopositive neurons in the ARC, a Nikon microscope attached to a digital camera (NIKON, ECLIPSE-TS100, Japan) was used. Cell counting was performed by two researchers. Bilateral cell counts for each section were averaged between researchers. Total cell counts of the ARC (eight sections/rat) were summed and analyzed.

**Statistical analysis**

All experiments were repeated at least three times. All analyses were performed using the Statistical Package for Social Sciences (SPSS for Windows, version 16.0; Chicago, IL, USA). Data were log-transformed owing to skewed distributions and were expressed as mean ± S.D. for quantitative values. Unless otherwise noted, data were analyzed using ANOVA followed by Tukey post hoc tests. The significant differences of variate between two groups were assessed by Student’s t-tests. *P<0.05 was considered statistically significant.

**Results**

**gAd and AMPK-inhibited KISS1 gene mRNA transcription in GT1-7 cells**

GT1-7 cells exposed to 0·1, 0·2, 1·0, and 5·0 μg/ml gAd for 2 h have lower transcription of Kiss1 mRNA determined by real-time PCR (shown in Fig. 1A). The inhibition of KISS1 mRNA by adiponectin is concentration-dependent manner with statistical significance (P<0.05).

AMPK is believed to be a major cellular target of adiponectin, and its activation has been reported in GT1-7 cells. In order to discover whether AMPK plays a role in the modulation of KISS1 mRNA transcription, we used artificial means to activate or inhibit AMPK in GT1-7 cells and followed it with the quantification of mRNA transcription in our assay. The results show that AICAR (1 mM), an AMPK

**Figure 1** Regulation of KISS1 mRNA expression under different concentrations of gAd or AMPK. GT1-7 cells were serum starved and then treated with gAd for 2 h at different concentrations (from 0 to 5 μg/ml; A) or treated with 1 mM AICAR or 20/40 μM Compound C for 2 h (B). KISS1 mRNA levels were determined by real-time PCR analysis. Results were representative of at least three replicate determinations for each treatment group. *P<0.05 compared with the control group.

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activator, caused an outstanding decrease in KISS1 mRNA transcription (Fig. 1B; $P<0.001$). Conversely, Compound C (20/40 μM), a relatively specific AMPK inhibitor, promoted the transcription of KISS1 mRNA with a statistical significance in a concentration-dependent manner ($P<0.05$).

gAd inhibited KISS1 gene promoter activity

To examine the transcriptional regulation of the KISS1 gene in the hypothalamus by adiponectin, a 2 kb full-length KISS1 gene promoter ($PGL3$-KISS1 promoter) was transiently transfected into GT1-7 cells. Cells were treated with adiponectin at concentrations 1.0, 5.0, and 10.0 μg/ml for 24 h or at 1.0 μg/ml for 6, 9, 18, and 24 h respectively. The promoter activity was significantly decreased in a time-dependent (Fig. 2A; $P<0.05$) and concentration-dependent manner (Fig. 2B; $P<0.05$). The promoter activity was significantly suppressed by the administration of gAd at the lowest concentration of 1.0 μg/ml for a 6 h term of incubation, and the activity was further reduced as the time extended.

AMPK contributed to KISS1 gene promoter activity

Following our discovery of AMPK’s involvement in KISS1 gene transcription, it is interesting to know whether AMPK functions usually under some known pathways or unusually at the transcriptional level. With these questions unaddressed, we transiently transfected the GT1-7 cells with 2 kb $PGL3$-KISS1 promoter. Then pharmacological regulators of AMPK, AICAR, and Compound C, as well as the sequence-specific interfering RNA of AMPKα1 subunit, were applied to the GT1-7 cell culture. The results showed that activation of AMPK by AICAR decreased the promoter activity of Kiss1 gene (Fig. 2C; $P<0.05$). However, the suppression of AMPK by Compound C or AMPKα1-SiRNA enhanced the promoter activity of KISS1 gene (Fig. 2C and D; $P<0.001$). We also pretreated the GT1-7 cells for 30 min with AICAR (1 μM) or Compound C (20 μM) and then incubated with gAd (5 μg/ml) for 24 h. As shown in Fig. 2E, gAd (5 μg/ml) and AICAR (1 mM) decreased KISS1 gene promoter activity, while Compound C (20 μM) augmented promoter activity of KISS1 gene ($P<0.05$).

Figure 2 Regulation of KISS1 promoter activity by gAd or AMPK. GT1-7 cells were serum starved and then transiently transfected with 2 kb full-length KISS1 promoter ($PGL3$-2k) along with a Renilla luciferase to control for transcription efficiency. After 18 h of transfection, cells were treated with gAd (1-0 μg/ml) for different times (A) or at different concentrations for 24 h (B), treated with 1 mM AICAR or 20 μM Compound C (C) or AMPK1α1-SiRNA (D), or pretreated with AICAR (1 mM), Compound C (20 μM) for 30 min before incubation with gAd (5-0 μg/ml) for 24 h (E). Luciferase expression level driven by KISS1 promoter was measured and normalized to Renilla luciferase activity. Results were representative of at least three replicate determinations for each treatment group. *$P<0.05$ compared with the control group.
Adiponectin inhibits KISS1 gene transcription

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Adiponectin inhibits KISS1 gene transcription

gAd regulated subcellular redistribution of SP1 in GT1-7 cells

SP1 functions as an important transcriptional factor by binding to GC-rich elements in response to physiological and pathological stimuli, and it can either activate or repress gene transcription. Previous studies have shown that the mechanism of its gene regulation partially lies in the translocation of SP1 triggered by its posttranslational modifications. This translocation, from the cytoplasm to the nucleus, is more commonly described as subcellular compartmentalization.

This experiment is to investigate whether the sequential subcellular compartmentalization of SP1 is involved in the regulation of KISS1 gene in GT1-7 cells after gAd administration. Western blotting was used to observe the changes in SP1 content before and after subcellular compartmentalization. As shown in Fig. 3, the nuclear fraction of SP1 decreased by 50% compared with the control group; however, the cytoplasmic fraction increased 1.2-fold after global adiponectin incubation (10.0 μg/ml; P<0.05), and this increase corresponds to the concentration-dependent manner of gAd administration. Total SP1 content in whole cells remains constant in GT1-7 cells (P=0.55).

AMPK regulated subcellular redistribution of SP1 in GT1-7 cells

From the above-mentioned experiments, it is clear that both AMPK and SP1 are playing roles in regulating KISS1 gene transcription. Then our curiosity leads us to a question whether AMPK regulates the procedure of subcellular compartmentalization of SP1 or vice versa. We treated the GT1-7 cells with AICAR, Compound C, and AMPKζ1-siRNA and then we ran western blot to evaluate the changes in SP1 content on subcellular compartmentalization. The results showed that AICAR decreased the nuclear fraction and increased the cytoplasmic fraction of SP1 content (Fig. 4A; P<0.05). Compound C decreased the cytoplasmic SP1 content and increased the nuclear fraction, so did AMPKζ1-siRNA (P<0.05). As shown in Fig. 4, pharmacological regulators of AMPK, AICAR, and Compound C, as well as AMPKζ1-siRNA, had no effect on the total SP1 content in whole GT1-7 cells (P=0.46).

Immunofluorescence analysis supported the regulation of subcellular redistribution of SP1 by AMPK

Other than western blot analysis, we also performed immunofluorescence analysis to directly visualize the effect of AMPK on the subcellular redistribution of SP1 protein. After incubation with AICAR or Compound C for 24 h, changes in SP1 content were assessed using highly specific primary antibodies, which was later coupled with fluorescent tagged secondary antibodies (Witte et al. 1994). As Fig. 5 demonstrates fluorescence, in response to AICAR treatment, SP1 in the cytoplasm increased greatly, but SP1 in the nucleus reduced compared with the control group. To the contrary, Compound C demonstrated a reverse effect on the subcellular localization of SP1. It increased the amount of SP1 in the nucleus and decreased the amount of SP1 in the cytoplasm. These findings were in accordance with the results from the western blot analysis.

SP1 contributed to KISS1 promoter activity

We performed a luciferase assay to demonstrate the important role of SP1 in KISS1 promoter activity. The 2 kb full-length KISS1 gene promoter (PGL3-KISS1 promoter) was transiently transfected into GT1-7 cells. After one passage, we treated the cells with SP1 SiRNA and mithramycin A (known as a pharmacological suppressor of SP1). Figure 6 illustrates that suppression of SP1 resulted in a great decrease in the activity of KISS1 promoter (P<0.05). Our western blot results demonstrated that the SP1 SiRNA we used in the luciferase assay reduced the expression of SP1 dramatically in the GT1-7 cells (P<0.05).
**SP1 promoted KISS1 gene mRNA transcription in GT1-7 cells**

In order to confirm that SP1 definitely has an effect on the expression of KISS1 mRNA, real-time PCR was carried out to detect the KISS1 mRNA levels when SP1 SiRNA was transiently transfected into GT1-7 cells. SP1 protein was definitely knocked down by Sp1 siRNA as shown by western blot (Fig. 6). As demonstrated in Fig. 7, KISS1 mRNA transcription was decreased obviously when the expression of transcription factor Sp1 was knocked down by Sp1 siRNA (*P < 0.05*).

**gAd and AMPK had an effect on the expression of kisspeptin in the ARC area of Sprague Dawley rats**

We performed immunohistochemistry and counted the cell number of kisspeptin immunopositive neurons to demonstrate that gAd and AMPK play an important role in the expression of kisspeptin in the ARC area of Sprague Dawley rats. As shown in Fig. 9, injection of Compound C increased the number of kisspeptin immunopositive neurons in the ARC area of Sprague Dawley rats (Fig. 9D), while injection of gAd or AICAR decreased the number of kisspeptin immunopositive neurons (Fig. 9B and C) compared with control group treated with PBS/DMSO with a significant difference (*P < 0.05*).

**Discussion**

Puberty is a complicated process that is not comprehensively understood by human beings. Lots of factors are believed to play important regulatory roles in this process, which include genetic heritages, environmental effects, neuroendocrinological encounters, metabolic status, and other growth-related factors. Reproductive function is highly sensitive to changes in the hypothalamic-pituitary-gonadal (HPG) axis, and disruption in this axis has been shown to contribute to reproductive dysfunctions, including delayed puberty.
in the metabolic status and energy reserves of an organism, and adverse metabolic conditions are commonly associated with defective reproductive capability involving the alteration in the hypothalamic KISS1 system (Castellano et al. 2006). Kisspeptin, ligand of GPR54, encoded by the KISS1 gene, have recently emerged as major gatekeepers of the gonado-tropic axis. Shahab et al. (2005) showed that KISS1 mRNA levels increase with puberty in both male and female monkeys. In intact females, GPR54 mRNA levels in the hypothalamus increased threefold from the juvenile to midpubertal stage. Hybridization histochemistry indicated robust KISS1 and GPR54 mRNA transcription in the region of the arcuate nucleus. Dhillo et al. (2005) reported that systemic administration of kisspeptin to male volunteers results in a significant increase in plasma LH, FSH, and testosterone. Converging evidence (Navarro et al. 2004, Messager et al. 2005, Kauffman 2009) demonstrates that a key action of KISS1/GPR54 system on the HPG axis occurs directly at the level of GNRH release. It is well established that Gpr54-deficient mice and humans display phenocopy syndromes characterized as isolated hypogonadotropic hypogonadism (de Roux et al. 2003, Seminara et al. 2003). So KISS1/GPR54 represents the molecular ‘gatekeepers’ underlying the onset of pubertal development.

Adiponectin is exclusively expressed and secreted by adipose tissue (Kadowaki & Yamauchi 2005). Except for its regulation effect in energy metabolism and insulin sensitivity, adiponectin has also been proven to be involved in anti-inflammatory, antiangiogenic, and antiatherosclerotic processes (Yamauchi et al. 2001, Brakenhielm et al. 2004, Goldstein & Scalia 2004). An increasing body of evidence indicates that adiponectin could also directly regulate

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**Figure 5** AMPK induces subcellular redistribution of SP1 protein by immunofluorescence analysis (200×). GT1-7 cells were treated with AICAR (1 mM) or Compound C (20 μM) for 24 h, fixed, and immunostained with antibodies to SP1, which was visualized by a secondary antibody coupled to FITC. Nucleus was stained by 1-aminopyridinium iodide (red) and then examined with a fluorescence microscope. SP1 is distributed in the cytoplasm and nucleus of untreated GT1-7 cells (Witte et al.; a). In response to AICAR treatment, SP1 in the cytoplasm increased greatly (Witte et al.; b). SP1 in the nucleus increased greatly after treatment with Compound C (Witte et al.; c). The nucleus was stained by 1-aminopyridinium iodide (red; d, e, and f). Merged figures with graph a and d, b and e and c and f were, respectively, shown as graph g, h, and i. Color of SP1 localized in nucleus merged with nucleus stain shows yellow. The yellow color was more deep in the Compound C group than in the control group and the AICAR group demonstrated the subcellular localization of SP1 (g, h, and i).
reproductive functions. Adiponectin also inhibits both basal and GNRH-stimulated LH secretion in rat and mouse pituitary cells (Rodriguez-Pacheco et al. 2007, Lu et al. 2008).

A study by Campos et al. (2008) suggested that overexpression of adiponectin impairs female fertility in mice. Bottner et al.'s research showed that serum adiponectin levels in boys and girls decrease throughout puberty (Bottner et al. 2004).

In this study, we used gAd to treat GT1-7 cells and found that both transcription levels and promoter activity of KISS1 gene were significantly inhibited. According to the literature, along with the initiation of puberty, adiponectin levels in men decrease dramatically, while the KISS1 gene transcription levels increase rapidly, which also shows that an intrinsic relationship may exist between adiponectin and the KISS1 gene before puberty. AMPK plays a role as an 'energy sensor' in vivo as well as an important signaling molecule mediating the physiological role of adiponectin. Adiponectin facilitates glucose uptake and fatty acid oxidation through activation of AMPK in peripheral tissues (Yamauchi et al. 2002, Wu et al. 2003, Ceddia et al. 2005). Lu et al. (2008) reported that adiponectin could reduce LH secretion in the pituitary gonadotrophic cells in an AMPK-dependent manner.

Additionally, our previous research demonstrated that adiponectin inhibited GNRH secretion via AMPK in GT1-7 cells (Wen et al. 2008). Therefore, AMPK may serve as a conjunction between energy metabolism and central reproduction, as well as a mediator for the regulation of metabolism and reproduction by adiponectin. In order to investigate whether the AMPK signaling pathway is involved in the regulation of KISS1 gene transcription by adiponectin, we used AMPK agonist AICAR as a control to observe its impact on KISS1 gene transcription. The results showed that AICAR also inhibited KISS1 gene transcription and their promoter activity was obvious, and that the AMPK inhibitor Compound C could increase the KISS1 gene transcription and enhance promoter activity. AMPKz1-siRNA by RNA interference reduced the AMPKz1 catalytic subunit protein level and significantly enhanced Kiss1 gene transcription and promoter activity as Compound C did.

A report from Chu & Ferro (2005) showed that AMPK downstream signaling molecules such as ERK can mediate the phosphorylation of transcription factor SP1. SP1 has been proved to be a vital regulator of gene transcription of KISS1 gene (Mitchell et al. 2006, Li et al. 2007, Mitchell et al. 2007). SP1 is a ubiquitously expressed, prototypic C2H2-type zinc finger-containing DNA binding protein. The primary sequence and domain organization of SP1 protein are highly conserved. In addition to three zinc fingers, SP1 possesses two glutamine-rich modules that together make up their transcriptional activation domains (AD). Adjacent to the two AD is a serine/threonine-rich module that may be targeted for post-translational modifications (e.g., phosphorylation or acetylation). Post-translational modifications of SP1 not only regulates its subcellular compartmentalization but also modulates its interactions with other transcription factors to regulate gene expression. For example, phosphorylation of SP1 leads to its enhanced nuclear migration and results in a decrease in SP1 DNA binding and hence suppression of transcription activation of EP4 (PTGER4) gene in...
Therefore, we hypothesized that adiponectin regulates the gene transcription of *KISS1* and the onset of puberty through AMPK and the activity of its downstream signaling molecules.

Through the combination of the GC/GT box and the interaction with other proteins, SP1 plays an important role in the enhancement or inhibition of gene transcription and has been implicated in the control of a diverse array of cellular processes exemplified by, not limited to, cell growth, differentiation, apoptosis, angiogenesis, and immune response (Tan & Khachigian 2009). A study by Mitchell et al. (2007) indicated that co-expression of SP1 and DRIP-130, which is encoded by human chromosome 6q16.3–q23 and has been proved to be a SP1-coactivator protein, not only rescues *KISS1* expression but also induces inhibition of the invasive and migratory behavior in highly metastatic melanoma cells. Mitchell et al. (2006) have also demonstrated that SP1 directly interacts with activator protein 2α to form transcription complexes at two tandem SP1-binding sites of the *KISS1* gene promoter, which leads to activation of *KISS1* transcription in breast cancer cells. Li et al. (2007) found that there are four proximal GC-rich SP1 binding sites at −188 to −87 of the *KISS1* promoter, and the differential regulation of *KISS1* expression by estradiol in different brain regions can be achieved by the different ratio of SP1 to SP3 in the protein complexes. It can be explained by the regulatory mechanism of SP1: i) SP1 combines with different sites of the *KISS1* gene promoter, resulting in changes in binding affinity; ii) the role of SP1 in the regulation of gene expression depends on its expression level, which varies with cell type during different developmental stages; iii) other factors (as mentioned earlier involving in DRIP-130, AP-2α, SP3, etc.) can be involved in regulation of the transcripational activity of SP1; and iv) as a transcription factor, posttranslational modifications of SP1 play a crucial role in its transcripational regulatory function. Growing evidence indicates that phosphorylation, acetylation, sumoylation, ubiquitylation, and glycosylation are among the posttranslational modifications that influence the transcriptional activity and stability of SP1. Phosphorylation of SP1 that occurs in glutamate-rich region usually enhances transcriptional activation of SP1 in the corresponding pathway, and phosphorylation within the zinc
finger always reduces affinity of SP1 to the DNA, thereby reducing transcriptional activity.

In our study, the nuclear fraction of SP1 decreased in content while the cytoplasmic fraction increased in a concentration-dependent manner after administration of gAd in hypothalamic GT1-7 cells. Meanwhile, we did not find significant changes in the total cell extracts of SP1 after gAd treatment in GT1-7 cells. Also in our study, we discovered that AICAR mimicked gAd in enhancing the cytoplasmic fraction, while Compound C and AMPK-z1-siRNA functioned inversely in the subcellular compartmentalization of SP1 in hypothalamic GT1-7 cells. So we conclude that the changes in subcellular compartmentalization of SP1, instead of the changes in the total cell extracts of SP1, take part in the regulations of Kiss1 gene transcription by gAd and AMPK. Our results showed knockdown of Sp1 expression by Sp1 siRNA induced the inhibitory expression of Kiss1 mRNA in GT1-7 cells. Besides, the luciferase assay demonstrated that SP1 contributed to promote the promoter activity of Kiss1 gene, as both Sp1 siRNA and miθaramycin A, the pharmacological suppressor of SP1, dramatically suppressed the promoter activity of Kiss1 gene. Collectively, these results demonstrated that SP1 could activate Kiss1 promoter activity and promote Kiss1 mRNA transcription. Knockdown of Sp1 induced lower promoter activity of Kiss1, and reduced expression of Kiss1 mRNA. The suppressive effect of gAd and AMPK activator on Kiss1 promoter activity and mRNA transcription could be partly explained by the detained transcriptional factor SP1 in the cytoplasm induced by gAd and AMPK activator, which mimicked the effect on expression of Kiss1 mRNA and promoter activity when expression of Sp1 was knocked down. While Compound C or AMPK-z1-siRNA, which inhibited the activity of AMPK nonspecifically or specifically, played an inverse role as gAd and AICAR did.

Except for the in vitro studies, our results from in vivo experiments demonstrated that injection of gAd and AICAR could inhibit the expression of Kiss1 mRNA and decrease the number of kisspeptin immunopositive neurons in the ARC area of Sprague Dawley rats, while Compound C performed an inverse role as gAd and AICAR did, the transcription of Kiss1 mRNA almost doubled and the number of kisspeptin immunopositive neurons in the ARC area also increased significantly in Sprague Dawley rats treated with Compound C compared with control group. These results further confirmed the results found in in vitro studies.

In summary, our study demonstrated that adiponectin caused the activation of AMPK in hypothalamic GT1-7 neurons and whereby decreased the translocation of SP1 from cytoplasm to nucleus; as a result, the promoter activity and transcription levels of Kiss1 gene are repressed. This may further influence the hypothalamic release of GNRH and the onset of puberty. To the best of our knowledge, the data presented here are the first experimental evidence for the characterization of reproductive regulation by adiponectin, as well as the involvement of AMPK and SP1 pathways in such a process. However, the limitation of this study is that only one kind of cell line was used, and hence the conclusions of the paper seem a little bit thin. Although we have performed some in vivo studies on Sprague Dawley rats, further investigations about the molecular mechanism in vivo will be conducted more completely in our future studies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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