Original Research

Overexpression of *p53* accelerates puberty in high-fat diet–fed mice through *Lin28/let-7* system

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Impact statement

High-fat intake and subsequent obesity are associated with premature onset of puberty, but the exact neuroendocrine mechanisms are still unclear. The transcriptional factor *p53* has been predicted to be a central hub of the gene networks controlling the pubertal onset. Besides, *p53* also plays crucial roles in metabolism. Here, we explored *p53* in the hypothalami of mice fed a high-fat diet (HFD), which showed an up-regulated expression. Besides, we also revealed that overexpressed *p53* may accelerate hypothalamo-pituitary-gonadal (HPG) axis activation partially through the *c-Myc/Lin28/let-7* system. These results can deepen our understanding of the interaction between metabolic regulation and puberty onset control, and may shed light on the neuroendocrine mechanisms of obesity-related central precocious puberty.

Abstract

High fat intake is one of the most important reasons of the surging prevalence of childhood obesity all over the world. Obesity and high fat intake have been revealed to cause premature activation of hypothalamo-pituitary-gonadal axis and central precocious puberty. The onset of puberty is controlled by neuroendocrine mechanisms containing overlapping and interacting gene networks. The latter contains five major transcriptional level hubs, among which the transcriptional factor *p53*, a well-established tumor suppressor protein, also plays a crucial role in obesity and metabolic disorders. In the current study, we repeated prior observations that high-fat diet advances vaginal opening in rodents and extended these findings by demonstrating that high-fat diet mice had higher expression of *p53* in hypothalami than mice fed with normal chow. More importantly, in high-fat diet mice, hypothalamus-specific overexpression of *p53* can make vaginal opening much earlier, while inhibition of *p53* expression relatively delayed vaginal opening. The *c-Myc* and *Lin28b* levels increased, while *let-7a* mRNA levels decreased in the high-fat diet mice. Overexpression of *p53* reduced *c-Myc* and *Lin28b* mRNA and protein levels, whereas elevated let-7a mRNA levels in high-fat diet mice. Inhibition of p53 expression by pifithrin-α elevated *c-Myc* and *Lin28b* but reduced *let-7a* levels in high-fat diet mice. In conclusion, high fat intake can accelerate the onset of puberty by up-regulation of *p53* expression in hypothalamus. Overexpressed *p53* may accelerate hypothalamo-pituitary-gonadal axis activation partially through the *c-Myc/Lin28/let-7* system.

Keywords: High-fat diet, obesity, puberty onset, central precocious puberty, *p53*, *Lin28/let-7* system

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Introduction

Obesity has become one of the major public health concerns in the 21st century. The prevalence of childhood obesity has increased worldwide since 1975.[1] In the past 30 years, the incidence of childhood obesity has increased from less than 5% to approximately 20% in the United States.[2] In China, a similar trend of pediatric obesity prevalence was also observed, from less than 3% in 1985 to 19.2% in 2010.[3] Increased fat intake, other than declined physical activity, has been considered as one of the most important reasons contributing to pediatric obesity.[4]

Previous studies have revealed a crucial role of nutritional status and hormonal cues in pubertal activation of the hypothalamo-pituitary gonadal (HPG) axis.[5,6] Epidemiologically, there is a coincidence of trends of childhood obesity and early puberty onset worldwide.[7–9]

Premature activation of HPG axis earlier than expected for the normal population is defined as central precocious puberty (CPP).[5] It has been observed that obesity occurs at a high rate among children with CPP.[6] Besides, in obese children, the earlier sexual development was found to be correlated with higher body mass index (BMI). [10,11] Those who develop earlier tend to be more obese, with a trunk-oriented distribution pattern.[12] Thus, obesity and nutrition excess are associated with early puberty onset, but the neuroendocrine mechanism underlying this phenomenon has not been fully explored.[13,14]

It has been proposed that the onset of puberty is controlled by neuroendocrine mechanisms containing overlapping and interacting gene networks endowed with elements of hierarchical and scale-free features.[13–15] The five major transcriptional level hubs in the network were predicted to be *CDP/CUTL1*, *MAF*, *p53*, *YY1*, and *USF2* via cis-regulatory analysis of shared transcription factor binding sites. Interestingly, *MAF*, *p53*, and *YY1* are involved in obesity and metabolic conditions,[16–19] among which *p53* has been proved to play a crucial role in obesity and related metabolic disorders.[18,19] Besides, Lin28/let-7 axis has been shown to regulate both puberty and weight in a sex-specific manner.[20]

In this study, we hypothesized that *p53* has a role in the advancement of puberty onset induced by metabolic factors, the mechanism under which is partially through *Lin28/let-7* axis. To test this hypothesis, we examined the expression levels of *p53* and components of *Lin28/let-7* axis in the hypothalami of mice fed with high-fat diet (HFD). Next, we manipulated the hypothalamus-specific expression levels of *p53* in mice, observed the vaginal opening in these mice, and explored the possible underlying mechanisms via *Lin28/let-7* axis.

Materials and methods

Animals

All procedures were approved by the Children's Hospital of Soochow University Animal Care and Use Committee. Female C57BL/6 mice were weaned at age 21 days from Shanghai Laboratory Animal Center (Shanghai, China). Upon arrival, all mice were housed 3–4 animals/cage at constant temperature on a 12 L/12D cycle with lights 20 at 0630 h, and were randomly assigned to either a normal chow (10% calories from fat, D12451, Research Diets, New Brunswick, NK, USA) or a high-fat chow (60% calories from fat, D12450B Research Diets, New Brunswick, NK, USA)) fed ad libitum from the day of weaning until the end of the experiment. All the mice are randomly assigned to one of the five groups, normal control (NC, $n=8$) which is fed with normal chow, high-fat diet (HFD, $n=8$), high-fat diet with pifithrin-α (Selleck, S2929) injection (HFD-pifithrin-α, $n=8$), high-fat diet with *p53* overexpression (HFD-*p53*, $n=8$), and high-fat diet with vacant lentivirus control (HFD-C, $n=8$). Mice were weighed each day and checked for vaginal opening (VO). All the mice were killed after VO by cervical dislocation. Hypothalami were collected, weighed, and stored at −80°C until processing.

Lentiviral vectors, infection, and expression

Lentiviral vectors were designed and constructed to produce lentiviruses expressing mouse *p53* (CL1128_PDS159-MUS-p53). The *p53*, prepared via mouse cDNA library using RT-PCR, were subcloned into Nhe I/ASC I restriction enzyme site between the CMV promoter and the IRES-GGFPa1 sequence of the lentiviral expression vector, PDS159_pL6.3-CMV-GFPa1-IRES-MCS (Novobio, Shanghai, China). High titer lentiviruses were produced as described previously.[21] Briefly, recombinant lentiviruses were produced by transient transfection in 293 T cells. Infectious particles were harvested at 48 h after transfection, filtered through 0.45-μm-pore cellulose acetate filters, concentrated by ultracentrifugation (50,000 g for 2 h), re-dissolved in 1 mL sterile DMEM, aliquoted, and stored at −80°C.

Stereotactic intracranial injections

Injections were performed as previously described.[22]Three-week-old C57BL/6 mice were anesthetized with 0.05 mL/kg 1% pentobarbital sodium (Merck KGaA, Germany) and injected with lentivirus (CL1128_PDS159-MUS-*p53* and vacant control, 5×10^7/mL, 4 μL per mouse) or pifithrin-α (0.6 μg/μl, 4 μL per mouse, Selleck, S2929) at a rate of 1 μL/min by a stereotaxic instrument (ZH-LanXing B/S, Huibei, China). Bilateral brain injection coordinates were 1.5 mm posterior to bregma, 0.3 mm lateral to the midline, and 5.8 mm below the cortical surface, based on a calibration study that indicated that these coordinates led to the rostral preoptic area in C57BL/6 strain on our system.[23] The lentiviral vectors or pifithrin-α were delivered using a Hamilton syringe connected to a motorized nanoinjector.

qRT-PCR

The mRNA levels were detected via qRT-PCR. The expression of mus β-actin mRNA was used as the internal control. The RT-PCR was performed by a CFX96TM Real-Time System (Bio Rad) using fluorescent SYBR Green technology (CS7561, invitrogen). The PCR conditions were as follows: cDNA equivalent to 80 ng of total RNA was amplified by PCR for 40 cycles at an annealing temperature of 60°C. Each qPCR contained 10 μL chamQ SYBR QPCR master Mix (Q311-02, vazyme), and a final primer concentration of 200 nM. The specificity of the amplification products was verified by melting curve analysis. Primer sequences used are listed in Supplemental Table 1.

Western blot

The protein levels were detected via WB. The expression of GAPDH and β-acting was used as the internal controls. In brief, proteins (40 μg each) from tissue homogenate were subjected to gel electrophoresis on SDS-polyacrylamide gel, and separated proteins were transferred onto nitrocellulose membranes and probed with rabbit antiserum against c-Myc (ab32072, abcam), Lin28b (ab191881, abcam), p53 (#32532, cell signaling technology), GAPDH (bsm-0978M, Bioss, Beijing, China), and β-acting (bs-0061R, Bioss, Beijing, China). Subsequently, membranes

were incubated with goat anti rabbit secondary antibody (BV-S8008, Biovol Biotech, Shanghai, China) and signals were detected using an enhanced chemiluminescence Western blotting substrate kit (Pierce Rockford, IL, USA).

Statistical analysis

Each experiment was repeated at least three times and data were expressed as mean ± SD. Statistical analyses were performed by SPSS 22.0. The Mann–Whitney U test was applied to check the data distribution. One-way ANOVA was used to compare data from more than two groups, followed by *post hoc* Tukey test. $P < 0.05$ was considered significant.

Results

Effect of hypothalamus-specific overexpression and inhibition of *p53* on puberty onset of high-fat diet mice

As shown in Figure 1, the weight and body length were not different among all mice groups throughout the study. All mice fed with HFD were significantly younger at VO than mice fed with normal chow. VO was significantly delayed in the HFD-pifithrin-α mice compared to the NC, HFD, and HFD-C mice. More importantly, VO was significantly advanced in the HFD-*p53* mice compared to the NC, HFD, and HFD-C mice. The VO age of HFD-C mice was similar to that of HFD mice.

Effect of hypothalamus-specific overexpression and inhibition of *p53* on *c-Myc*/*Lin28*/*let-7* system in the hypothalami of high-fat diet mice

The effect of hypothalamus-specific overexpression and inhibition of *p53* on gene expression levels were measured by qRT-PCR (Figure 2) and Western blot (Figure 3). The mRNA and protein levels of *p53* were significantly elevated in the hypothalami of HFD-fed mice compared to the NC mice. The *c-Myc* and *Lin28b* levels were also higher in the HFD group than those in the NC group. Overexpression of *p53* significantly decreased *c-Myc* and *Lin28b* mRNA and protein levels in the HFD-*p53* mice compared to NC, HFD, and HFD-C mice. On the other hand, inhibition of *p53* expression by pifithrin-α significantly elevated *c-Myc* and *Lin28b* levels in the HFD-pifithrin-α mice compared to NC, HFD, and HFD-C mice. The *let-7a* mRNA levels were significantly lower in HFD-fed mice compared to those in mice fed with normal chow. Overexpression of *p53* significantly elevated *let-7a* mRNA levels in the HFD-*p53* group compared to NC, HFD, and HFD-C groups.

Discussion

In the present study, we repeated prior observations that HFD advances VO in rodents and extended these findings by demonstrating that mice fed with HFD had higher expression levels of *p53* in the hypothalami compared to NC mice. More importantly, in the HFD mice, hypothalamus-specific overexpression of *p53* induced earlier VO, while inhibition of *p53* expression delayed VO.

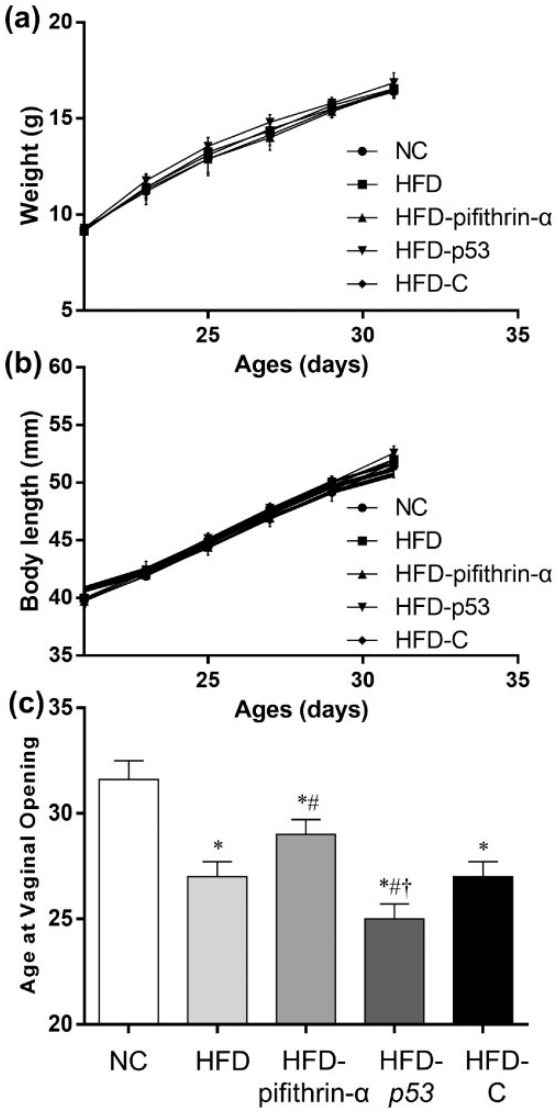

Figure 1. (a) Body weight and (b) body length were not different among groups. (c) Vaginal opening (VO) was advanced in high-fat diet (HFD)-fed mice compared to mice fed with normal chow. Besides, overexpression of *p53* further advanced VO in HFD mice, while inhibition of p53 by pifithrin-α delayed VO. Results are expressed as mean±SD; *$P < 0.05$ versus NC; #$P < 0.05$ versus HFD; †$P < 0.05$ versus HFD-pifithrin-α.

Moreover, the puberty regulation effect of *p53* in the HDF mice may be partially through *Lin28/let-7* system.

The crucial and final step responsible for initiation of endocrine manifestations of puberty is pulsatile secretion of GnRH from hypothalamus, which is regulated by coordinated changes of neuronal and glial networks associated with GnRH neurons.[24] These changes include increase of excitatory inputs from neuronal and glial networks to the GnRH neuronal network, and a reduction of transsynaptic inhibitory tone.[25] The stimulatory control of puberty is mainly induced by glutamatergic neurons, kisspeptin, and a glial component that uses growth factors and small molecules for cell–cell signaling, while the transsynaptic inhibition is produced by GABAergic and opiatergic neurons as well as RFamide-related peptide (RFRP).[15] The molecular mechanisms that provide encompassing

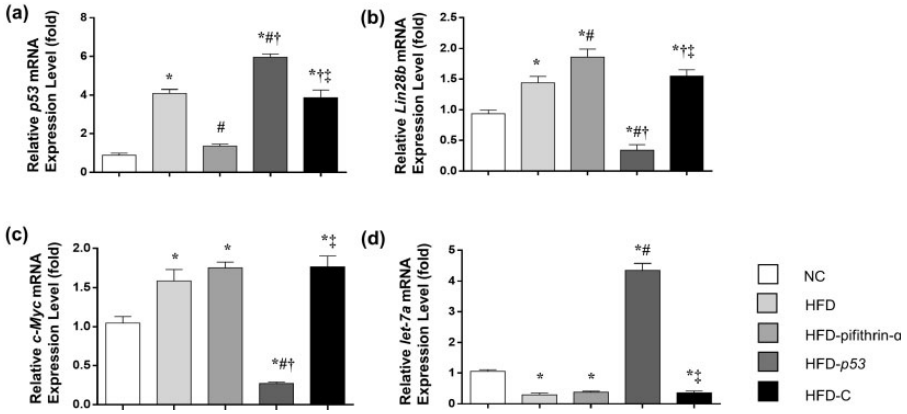

Figure 2. The mRNA expression levels of *p53* and components of *Lin28/let-7* axis in hypothalami of mice. Results are expressed as mean±SD; *$P < 0.05$ versus NC; #$P < 0.05$ versus HFD; †$P < 0.05$ versus HFD-pifithrin-α; ‡$P < 0.05$ versus HFD-*p53*.

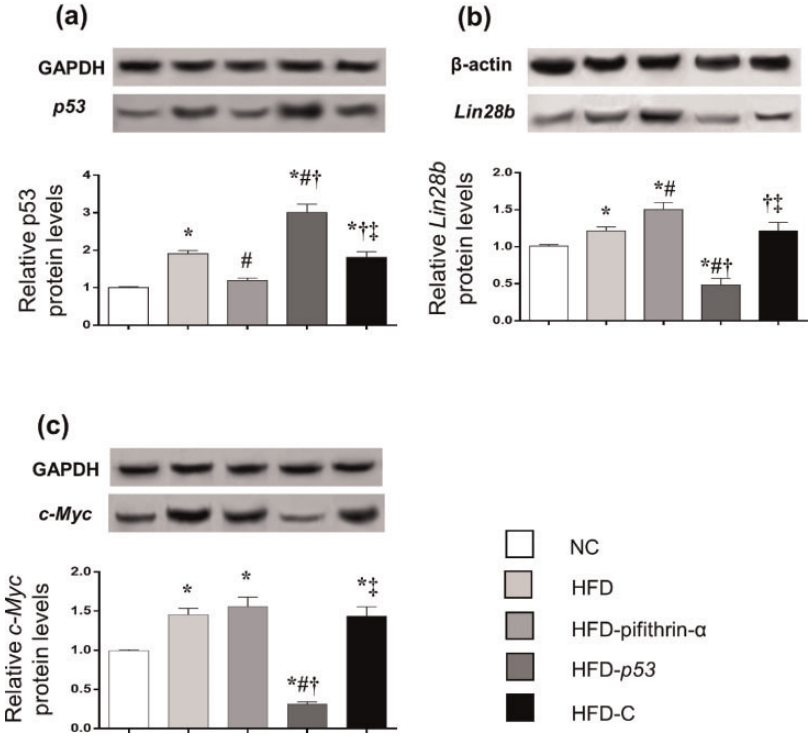

Figure 3. The protein expression levels of *p53* and components of *Lin28/let-7* axis in hypothalamic of mice. Results are expressed as mean±SD; *$P < 0.05$ versus NC; #$P < 0.05$ versus HFD; †$P < 0.05$ versus HFD-pifithrin-α; ‡$P < 0.05$ versus HFD-*p53*.

coordination to this cellular network are not known, but a hierarchically arranged and functionally connected gene network has been postulated via high-throughput approaches and computational methods.[15,25] In this gene network, there are central "hubs," which are robustly interconnected and direct the flow of information throughout the entire network, and subordinate "nodes," which locate at different hierarchical positions of the network.

The transcriptional factor *p53* was proposed as a central hub of the gene network controlling puberty. *p53* is a well-established tumor suppressor protein which exert its tumor-suppressive influence via transactivating over 200 different target genes.[26] Accumulating evidence has revealed that *p53* plays a crucial role in obesity and metabolic disorders. The genome-wide association studies (GWASs) have shown that the most common *p53* single nucleotide polymorphism (SNP) P72R (rs1042522) is associated significantly with increased BMI in humans.[27] Animal studies have also confirmed that R72 mice developed significantly increased fat accumulation and impaired insulin sensitivity.[18] Besides, increased *p53* gene expression had been revealed in adipose tissue and endothelial cells of HFD-induced obese mice,[28] whereas HFD mice with endothelial cell-specific *p53* deficiency showed improvement of insulin sensitivity and less fat accumulation.[29] In our study, we observed higher *p53* levels in the hypothalami of HFD mice compared to those of controls, supporting the impact of *p53* on the early puberty onset of HFD mice. More importantly, overexpression of *p53* further advanced puberty, while inhibition of p53 expression delayed puberty in

HFD mice. These results suggested that p53 is an important regulator of metabolic control of puberty onset.

Lin28/let-7 axis has been proposed as a subordinate node of the gene network controlling puberty onset. This axis was first identified in *C. elegans,* regulating the timing of larval development.[30] In mammals, two *Lin28*-related genes, named *Lin28a* and *Lin28b*, have been reported. Both proteins have been shown to bind to the terminal loops of precursors of *let-7* family of miRNAs, inhibiting their maturation.[31] The potential impact of *Lin28b* on pubertal regulation has been revealed by a series of GWAS studies.[32,33] The expression levels of *Lin28b* and *c-Myc* declined, while the levels of *let-7a* elevated, at puberty in the hypothalami of both male and female rats.[34] Besides, *Lin28/let-7* axis has been reported to have a potential central role in metabolism regulation. Overexpression of *Lin28b* promotes an insulin-sensitized state that resists HFD-induced diabetes. In our study, we found higher expression levels *of c-Myc and Lin28b*, as well as lower *let-7a* levels in the hypothalami of HFD mice. The elevation of *c-Myc* and *Lin28b* levels may be a self-protective measure to compensate the negative effects of HFD on the glucose metabolism. Moreover, overexpression of *p53* drastically inhibited *c-Myc* and *Lin28b* levels, whereas inhibition of *p53* expression elevated *c-Myc* and *Lin28b* levels in the hypothalami of HFD mice. These results further suggested that the roles of *p53* in metabolic control of puberty are partially through *Lin28/let-7* axis.

In conclusion, the transcriptional factor *p53* is a central hub of the gene network controlling puberty. Our study suggests that *p53* might be a crucial mediator between metabolic alterations and pubertal regulation, and this potential effect of *p53* is partially through *c-Myc/Lin28/let-7* system.

Authors' contributions: TC coordinated the project, TC and CC wrote the manuscript, HW, XC, RX, FW, HS, LC, CC, and TC performed the experiment and analyzed the data.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTAL MATERIAL

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