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ORIGINAL RESEARCH

p53 Mediates GnRH Secretion via *Lin28/let-7* System in GT1-7 Cells

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Study Objective: The well-known tumor suppressor transcriptional factor *p53* has been proposed to be one of the central hubs of a functionally related and hierarchically arranged gene network coordinating pubertal timing. Our previous studies revealed that *p53* is involved in the metabolic control of puberty. The current study aimed to investigate the underlying signaling pathway, through which *p53* mediated the metabolic control of puberty.
Design, Setting, Participants, Interventions, and Main Outcome Measures: We engineered the expression of *p53* and/or *Lin28a* in GT1-7 cells to investigate the interaction between *p53* and *Lin28/let-7* system, and their impact on GnRH secretion.
Results: Overexpression of *p53* stimulated, while inhibition of *p53* by pifithrin-α significantly suppressed the GnRH secretion and *GPR54* expression levels in response to kisspeptin stimulation in GT1-7 cells. Furthermore, overexpressed *p53* suppressed *Lin28a* and *c-Myc* expression levels and increased *let-7* expression levels in GT1-7 cell lines. On the other hand, inhibition of *p53* by pifithrin-α upregulated *Lin28a* and c-Myc levels and downregulated *let-7* expression levels. Moreover, *Lin28a* overexpression counteracted the effect of *p53* overexpression in *p53* and *Lin28a* co-overexpression cells, whose GnRH secretion and GPR54 expression levels were not different from controls. Meanwhile, *Lin28a* suppression counteracted the effect of pifithrin-α, and the GnRH secretion and *GPR54* expression levels are not different from controls in *p53* and *Lin28a* co-suppression cells.
Conclusion: These data suggest that *p53* is a central mediator of GnRH secretion in hypothalamus, and this effect is at least partly through the *Lin28/let-7* pathway.
Keywords: pubertal timing, *p53*, *Lin28/let-7* system, gonadotropin-releasing hormone

Introduction

The timing of pubertal onset is affected by complex interactions among genetic, nutritional, environmental, as well as socioeconomic factors.[1,2] Compelling evidence has proved very prominent regulatory roles of nutritional and metabolic signals on the timing of pubertal onset.[3] The secular trends in early pubertal maturation coincide with surging prevalence of overweight and obesity have raised discussion for a possible causal link between greater body fat mass and advanced pubertal age, especially in girls.[4] This association of obesity and advanced puberty has been further supported by observations from animal studies.[5–7] In the previous study, we also observed advanced vaginal opening (VO) in high-fat diet (HFD) rodents.[8]

The pivotal event indicating the onset of puberty is the re-emergence of pulsatile gonadotropin-releasing hormone (GnRH) secretion from the GnRH neuron. The pubertal activation of GnRH neuron is regulated by both trans-synaptic inputs and

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glial inputs.[9] Components of the trans-synaptic network mainly include the KNDy neurons releasing both Kisspeptin and neurokinin B (NKB),[10] GABAergic neurons, opiatergic neurons, and glutamatergic neurons.[11] All these different, yet partially overlapping, sub-systems contribute to the neuroendocrine mechanism controlling the pubertal onset. More importantly, a host of functionally related and hierarchically arranged gene network has been proposed to coordinate all the neuronal and glial inputs in the initiation of pubertal process.[9] This genetic network has been supposed to consist of five central hubs: *CDP/CUTL1, MAF, p53, YY1*, and *USF2*, all of which control the network at the transcriptional level.[9,12] Among these major hubs, *MAF, p53*, and *YY1* have been reported to be involved in obesity and/or metabolic conditions.[13–16]

Although the mechanisms underlying the metabolic control of puberty remain to be fully elucidated, there have been multiple studies addressing this issue. Kisspeptins, a family of structurally related peptides, have been recognized as a pivotal neuroendocrine regulator of GnRH neurons.[17,18] Accumulating evidence has revealed that kisspeptin neurons convey metabolic information to the control center of puberty onset.[19] The well-known adipose hormone leptin, levels of which are proportional to fat mass, has been implicated to regulate GnRH neuron via indirect mechanisms.[20] A leptin-kisspeptin-GnRH pathway has been proposed based on the fact that exogenous leptin can induce *Kiss1* mRNA expression in the ARC of leptin-deficient mice.[21] Moreover, leptin is also supposed to control puberty onset indirectly through mTOR (mammalian target of rapamycin) and its downstream effectors.[17,22] Besides, Sirtuin 1 (SIRT1), a fuel-sensing deacetylase, has recently been reported to mediate obesity and nutrient-dependent perturbation to timing of puberty onset via epigenetic control of *Kiss1* expression.[23]

p53, a well-known tumor suppressor protein and one of the central hubs in the gene network controlling pubertal onset, has long been regarded as a regulator of metabolism. Human studies have shown the P27R polymorphism of *p53* predisposes to obesity and metabolic dysfunction.[15] Animals and in vitro studies also revealed the roles of *p53* in diet-induced obesity.[16] In the previous study, we revealed that HFD mice have higher expression of *p53* in hypothalamus than mice fed with normal chow. More importantly, in HFD mice, hypothalamus-specific overexpression of *p53* can make VO much earlier, while inhibition of *p53* expression relatively delayed VO.[8]

Lin28/let-7 axis has been proposed as a subordinate node of the gene network controlling puberty onset.[9] In this study, we hypothesized that the impact of p53 was via *Lin28/let-7* axis. To test this hypothesis, we manipulated the expression of *p53* and *Lin28a* in GT1-7 cells, which are murine hypothalamic GnRH neuronal cells, and explored the interaction between *p53* and *Lin28/let-7* axis, as well as their impact on kisspeptin-stimulated GnRH secretion function in these cells.

Materials and Methods

Ethical Approval

All procedures performed in the present study were in accordance with the ethical standards of the ethical committee of Children's Hospital of Soochow University. The ethical committee of Children's Hospital of Soochow University approved this study and the use of GT1-7 cell lines kindly provided by Shanghai Ruijin Hospital.

Lentiviral Vectors, Transfection, and Expression

For mouse *p53* overexpression, the lentiviral vector (CL1128_PDS159-MUS-p53) was designed and constructed as previously described.[8] The *p53* was prepared via mouse cDNA library using RT-PCR. Then, we subcloned the *p53* cDNA sequence 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). For high titer lentiviruses collection, recombinant lentiviruses were produced by transient transfection in 293T cells. Infectious particles were harvested at 48 h after transfection, filtered through 0.45-μm-pore cellulose acetate filters, concentrated by ultracentrifugation (50,000g for 2h), redissolved in 1 mL sterile DMEM, aliquoted, and stored at −80°C. For mouse *Lin28a* overexpression and knockdown, the lentiviral vectors CL1126_ PDS159-MUS-lin28a and CL1127_ PDS19-MUS- SH-lin28a, respectively, were designed and constructed.

Cell Transfection

GT1-7 cells (2×10^5, passage number 7) were inoculated in each well of 96-well plates, and transfection was then conducted by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). GT1-7 cells were infected with different lentiviruses described above. The effect of *Lin28a* knockdown, as well as *Lin28a* and *p53* overexpression were validated

by measuring their mRNA levels by qRT-PCR. Cell lysates were harvested for further qRT-PCR and Western blot (WB) analysis.

GnRH Secretion Studies

Cells were treated with Metastin (45–54) amide (kisspeptin-10, M2816, Sigma-aldrich, France) at final concentrations of 100nM for 1h. RIA for GnRH was performed by the RIA kit (Phoenix pharmaceuticals, RK-040-02) according to the manufacturer's instructions. Protein content was consistently equivalent between wells (≤10% variation) (P0012, Beyotime Biotechnology, China). GnRH content in 200μL of media from each treated plate was harvested for quantification of GnRH secretion. The GnRH concentration in each media was also assayed by Western blot as described below.

qRT-PCR

We used qRT-PCR to detect the mRNA levels in the in vitro experiments, with mus β-actin mRNA expression level as the internal control. Total RNA from GT1-7 cells was isolated using TRIzol reagent (Invitrogen). The qRT-PCR was performed by a CFX96TM Real-Time System (Bio-Rad) using fluorescent SYBR Green technology (CS7561, Invitrogen). Each qPCR contained 10μL chamQ SYBR QPCR master Mix (Q311-02, vazyme), and a final primer concentration of 200 nM. The melting curve analysis was used to verify the specificity of the amplification products. The primer sequences were all summarized in Supplementary Table 1.

Western Blot

The cellular protein lysates were obtained from the GT1-7 cells. The expression levels of β-actin, GAPDH and tubulin were used as the internal controls. In brief, protein (40μg each) from cell lysates 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), Lin28a (ab46020, abcam), Kiss1 (ab19028, abcam), p53 (#32,532, cell signaling technology), p21 (ab188224, abcam), GRP54 (ab100896, abcam), β-actin (bs-0061R, Bioss, Beijing, China), and GAPDH (bsm-0978M, Bioss, Beijing, China). Then, the goat anti-rabbit secondary antibody (BV-S8008, Biovol Biotech, Shanghai, China) was used to incubate the membranes and an enhanced chemiluminescence Western blotting substrate kit (Pierce Rockford, IL, USA) was used to detect the signals.

Statistical Analysis

Statistical analyses were assessed by SPSS 22.0. Differences among groups were analyzed by one-way ANOVA, followed by post hoc Tukey's test. Each experiment was repeated at least three times and data are expressed as mean ± SD. The $P<0.05$ was considered significant.

Results

Overexpression of *p53* Suppressed *Lin28a* Expression in GT1-7 Cell Lines

The effect of overexpression of *p53* on gene expression of *Lin28/let-7* pathway was measured by qRT-PCR and Western blot (Figure 1). We found that GT1-7 cells with *p53* overexpression had significantly lower levels of *Lin28a* and *c-Myc* (Figure 1A–D). Besides, *p53* overexpression significantly elevated the *let-7a* levels, while *Lin28a* overexpression decreased the *let-7a* levels in GT1-7 cells (Figure 1E). Moreover, the levels of *Lin28a* and *c-Myc* were not different from controls in *p53* and *Lin28a* co-overexpressed cells (Figure 1A–D).

Inhibition of *p53* Elevated *Lin28a* Expression in GT1-7 Cell Lines

The gene expression of *Lin28/let-7* pathway was measured in GT1-7 cells with *p53* inhibition via qRT-PCR and Western blot (Figure 2). Pifithrin-α was used to suppress the expression of *p53*. We found that the expression levels of *Lin28a* and *c-Myc* significantly increased after *p53* suppression (Figure 2A–D). Additionally, sh-*Lin28a* significantly downregulated *Lin28a* and *c-Myc* expressions in GT1-7 cells (Figure 2A–D). Besides, pifithrin-α suppressed but sh-*Lin28a* elevated the levels of *let-7a* (Figure 2E). The use of pifithrin-α in GT1-7 cells together with sh-*Lin28a* make the expressions of *Lin28a* and *c-Myc* not different from normal controls (Figure 2A–D).

GnRH Secretion and *GPR54* Expression Levels in Response to Kisspeptin Stimulation in GT1-7 Cells

The mRNA and protein expression levels of the *GPR54* gene, as well as GnRH concentrations in the culture media were assessed in different GT1-7 cell groups (Figures 3 and 4). After kisspeptin stimulation, significantly higher GnRH

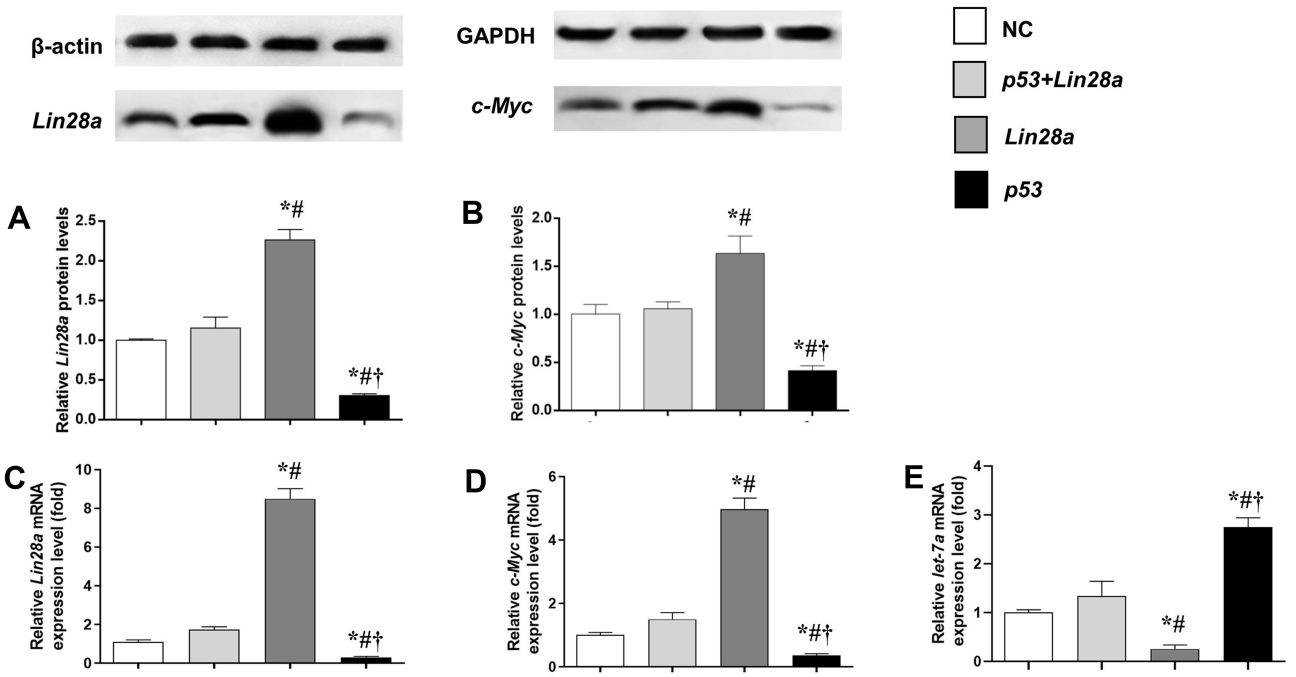

Figure 1 The protein and mRNA expression levels of components of *Lin28/let-7* axis in GT1-7 cells with *p53* and/or *Lin28a* overexpression. (**A, B**) are the relative protein levels of *Lin28a* and *c-Myc*, respectively. (**C**–**E**) are the relative mRNA levels of *Lin28a, c-Myc*, and *let-7a*, respectively. Results are expressed as mean±SD; *p<0.05 vs control; #p<0.05 vs cells with both *p53* and *Lin28a* overexpression; †p<0.05 vs cells with *Lin28a* overexpression.

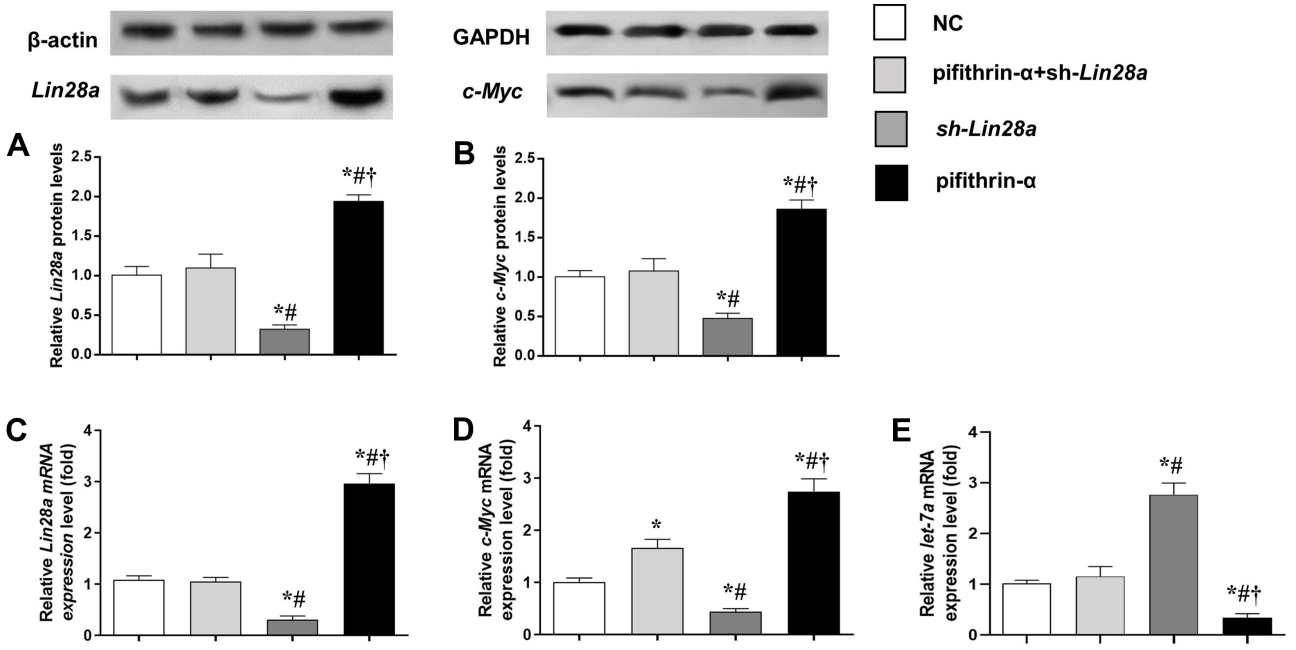

Figure 2 The protein and mRNA expression levels of components of *Lin28/let-7* axis in GT1-7 cells with *p53* and/or *Lin28a* inhibition. (**A, B**) are the relative protein levels of *Lin28a* and *c-Myc*, respectively. (**C**–**E**) are the relative mRNA levels of *Lin28a, c-Myc*, and *let-7a*, respectively. Results are expressed as mean±SD; *p<0.05 vs control; #p<0.05 vs cells with both *p53* and *Lin28a* inhibition; †p<0.05 vs cells with *Lin28a* suppression.

concentration was observed in culture media of *p53* overexpression cells (Figure 3C), while inhibition of *p53* by pifithrin-α significantly suppressed the GnRH secretion of GT1-7 cells (Figure 4C). Upregulation of *Lin28a* significantly suppressed (Figure 3C), while downregulation of *Lin28a* significantly elevated the GnRH secretion of GT1-7 cells (Figure 4C). Co-overexpression or co-down-expression of *Lin28a* with *p53* resulted in similar GnRH

secretion levels as controls (Figures 3C and 4C). The trend of *GPR54* expression levels of GT1-7 cells was similar as the GnRH concentrations in culture media (Figures 3A and B and 4A and B).

Discussion

Although epidemiological studies have demonstrated the pivotal roles of metabolic and nutritional factors in the pubertal onset, the underlying mechanisms of such relationships have not been fully elucidated.[1,2] *p53*, a well-known tumor suppressor protein with metabolic regulating function, has been predicted to be one of the central hubs of a genetic network controlling pubertal onset.[15,16] Our previous study revealed that hypothalamus-specific overexpression of *p53* could induce earlier vaginal opening (VO) in high-fat diet mice, while inhibition of *p53* expression delayed VO.[8] In the present study, we further showed that overexpression of *p53* stimulated, while inhibition of *p53* significantly suppressed GnRH secretion and *GPR54* expression levels in response to kisspeptin stimulation of GT1-7 cells. Moreover, the puberty-controlling function of *p53* is at least partly through the Lin28/let-7 axis.

The GnRH neurons are regulated by a network of excitatory and inhibitory afferents, and the highest level of this intra-network is transcriptionally controlled.[9] Among the five central transcriptional hubs, *MAF, p53*, and *YY1* have been shown participating in obesity and/or

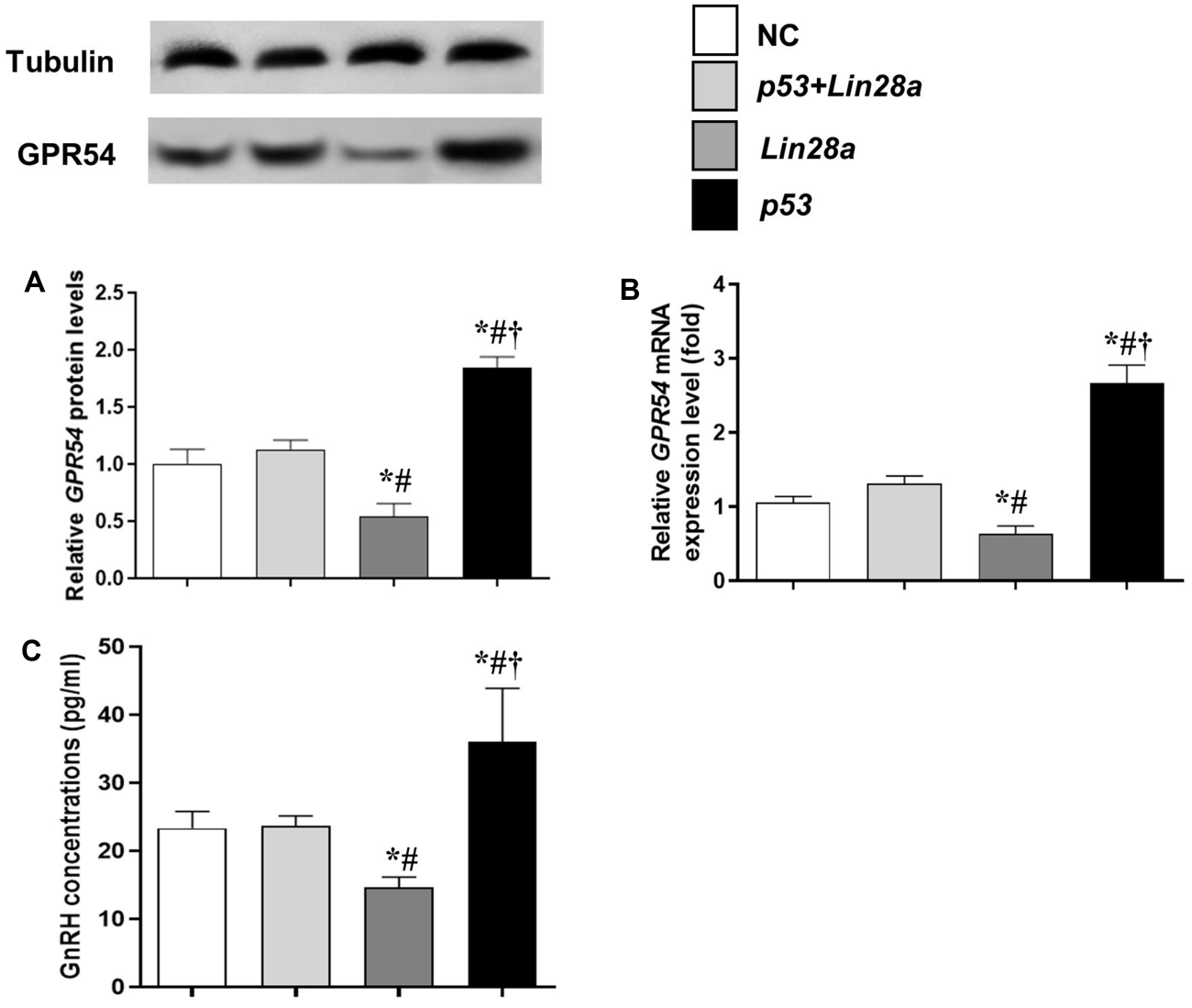

Figure 3 The protein and mRNA expression levels of *GPR54* in GT1-7 cells with *p53* and/or *Lin28a* overexpression after kisspeptin stimulation. (**A, B**) are the relative protein and mRNA levels of *GPR54*, respectively. (**C**) Is the GnRH concentrations in the cell culture media. Results are expressed as mean±SD; *p<0.05 vs control; #p<0.05 vs cells with both *p53* and *Lin28a* overexpression; †p<0.05 vs cells with *Lin28a* suppression.

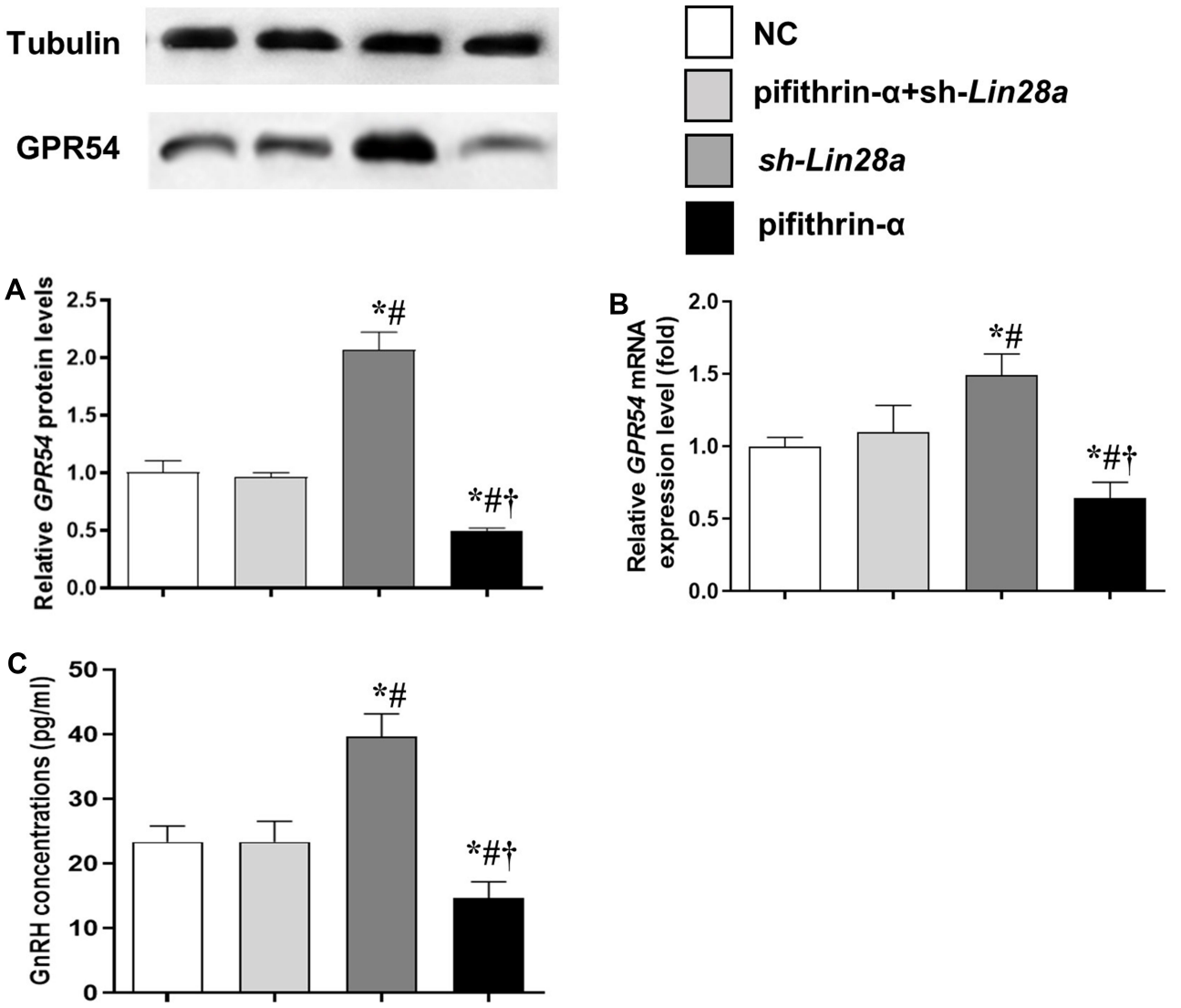

Figure 4 The protein and mRNA expression levels of *GPR54* in GT1-7 cells with *p53* and/or *Lin28a* inhibition after kisspeptin stimulation. (**A, B**) are the relative protein and mRNA levels of *GPR54*, respectively. (**C**) Is the GnRH concentrations in the cell culture media. Results are expressed as mean±SD; *p<0.05 vs control; #p<0.05 vs cells with both *p53* and *Lin28a* inhibition; †p<0.05 vs cells with *Lin28a* suppression.

metabolic conditions.[13–16] The extensively expressed transcription factor *c-MAF* is crucial for developmental and cellular differentiation processes, especially in adipose tissue,[24] pancreas,[25] and immune system.[26] It has been proposed as a risk loci of early-onset and morbid adult obesity in the European population by a genome-wide association study (GWAS).[13] The key transcription factor Yin Yang 1 (*YY1*), which is mainly involved in cell proliferation and differentiation, has been revealed mediating hepatic lipogenesis and glucogenesis in animal models.[14,27] More importantly, among all the three central transcriptional hubs, the roles of *p53* in metabolism and obesity are best established.[15,28] Compelling evidence suggests that *p53* in not only related to obesity but also engaged in the glucose homeostasis, insulin resistance, and the development of diabetes.[29] High-calorie diet can upregulate endothelial *p53* expression, while inhibition of endothelial *p53* expression improves dietary metabolic abnormalities.[28] In the previous study, we showed that hypothalamic *p53* expression was involved in the metabolic control of puberty in HFD mice.[8] In the present study, we engineered the expressions of *p53* in GT1-7 cells and found that overexpression of *p53* stimulated GnRH secretion and *GPR54* expression levels in response to kisspeptin stimulation in GT1-7 cells, which further supported the role of *p53* in pubertal regulation.

Previous studies on metabolic control of puberty onset have implicated the regulation effect of leptin, SIRT1, and

mTOR through kisspeptin, a gatekeeper of puberty.[17,20–22] Leptin is a known regulator of *p53* expression in multiple tissues.[30,31] SIRT1 modulates *p53* transcriptional-dependent function via regulating *p53* acetylation.[32,33] Prior studies have revealed the function of *p53* in the regulation of IGF-1/AKT/mTOR pathway.[34] Therefore, we suggest that *p53* might be a central mediator of leptin, SIRT1, and mTOR pathways in the metabolic control of puberty onset.

The heterochronic genes, *Lin28a* and *Lin28b*, were first identified in the nematode *C. elegans*, regulating the timing of larval development.[35] *Lin28a* and *Lin28b* are RNA-binding proteins that have been shown to selectively repress the expression of microRNAs (miRNAs), including those belonging to the *let-7* family.[36,37] They can bind to the terminal loops of precursors of miRNAs in *the let-7* family, inhibiting their maturation.[38] Besides, *Lin28a* and *Lin28b* can derepress *c-Myc* expression by suppressing mature *let-7* synthesis, while *c-Myc* reversely activates both *Lin28a* and *Lin28b* expressions.[39,40] The *Lin28/let-7* axis has been established as a regulator of puberty control.[9] *Lin28b* was suggested to have potential impact on pubertal regulation based on GWASs.[41,42] *Lin28a* was established as a negative regulator of puberty since mice with *Lin28a* overexpression had delayed puberty.[43] Moreover, apparent decrease of *Lin28a, Lin28b*, and *c-Myc* mRNA levels has been observed in the hypothalamus of both male and female rats before/around puberty.[40] Additionally, *Lin28/let-7* axis is a well-known central regulator of metabolism.[44] Hypothalamic ventromedial Lin28a expression positively correlated with energy balance.[45] Therefore, *Lin28/let-7* axis might as well play a role in the metabolic control of puberty onset.

As central nodes of the gene networks of puberty control, we hypothesized that *Lin28/let-7* axis might be regulated by the central hub *p53* to mediate the metabolic control of puberty onset. In HFD mice, we showed that the effect of *p53* on pubertal regulation might be via *Lin28/let-7* axis. In this study, we further explored this observation in GT1-7 cells, and confirmed that *Lin28/let-7* axis can modulate GnRH secretion of GnRH neurons, and this effect was regulated by *p53*.

In conclusion, the transcriptional factor *p53* is a central hub, while *Lin28/let-7* axis is a subordinate node of the gene network controlling puberty. In GnRH neuron, *p53* interacts actively with *Lin28/let-7* axis to regulate the GnRH secretion.

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Disclosure

The authors declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

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