

# The histone deacetylase SIRT6 inhibits ovarian cancer cell proliferation via down-regulation of Notch 3 expression

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**Abstract. – OBJECTIVE:** SIRT6 belongs to the NAD<sup>+</sup>-dependent class III deacetylase sirtuin family. Accumulating evidences have supported the critical role of SIRT6 in the proliferation, differentiation, cell cycle progression and apoptosis of cancer cells. The present study aims to determine the expression of SIRT6 in human ovarian cancer tissues and further investigate its biological effect in ovarian cancer.

**MATERIALS AND METHODS:** Real time PCR and western blot were performed to examine the mRNA and protein levels SIRT6 in human ovarian cancer tissues and normal tissues. The proliferation of ovarian cancer cells was determined using MTT methods. Small interfering RNA (siRNA) technology was used to down-regulate the expression of SIRT6 and Notch 3.

**RESULTS:** We found that the SIRT6 expression was significantly reduced in human ovarian cancer tissues compared to the normal tissues. Furthermore, our data showed that overexpression of SIRT6 inhibited the proliferation of ovarian cancer cells SKOV3 and OVCAR3. By contrast, down-regulation of SIRT6 enhanced ovarian cancer cells growth. In addition, our study showed that SIRT6 suppressed the expression of Notch 3 both at the mRNA and protein levels in ovarian cancer cells.

**CONCLUSIONS:** Our findings indicated that SIRT6 inhibited the proliferation of ovarian cancer cells through down-regulation of Notch 3 expression, and might provide novel therapeutic targets for ovarian cancer therapy.

*Key Words:*

SIRT6, Ovarian cancer, Proliferation, Notch 3.

## Introduction

Ovarian cancer is one of the most lethal gynaecological cancers, and the fifth leading cause

of cancer-related death among women in the developed countries<sup>1,2</sup>. The success rate of treatment has remained relatively unchanged, due to its late diagnosis at advanced stages and its resistance to conventional chemotherapy<sup>3</sup>. Statistical analysis shows that the five-year survival rate for women diagnosed with ovarian cancer is only around 40%<sup>4</sup>. Ovarian cancers have been shown to be heterogeneous with distinct genetic mutations and morphological characteristics, and its underlying molecular mechanisms have been incompletely elucidated<sup>5,6</sup>. Although significant improvements have been made in the detection of ovarian cancers in the past decade, more than 20,000 new cases are diagnosed every year<sup>7</sup>. Therefore, it is urgent to investigate the molecular mechanism of tumorigenesis and develop new treatment strategies for ovarian cancer in the clinic.

Sirtuins, or class III histone deacetylases, are NAD<sup>+</sup>-dependent protein deacetylases and/or mono-[ADP-ribosyl] transferases, which comprise seven members<sup>8-10</sup>. Studies demonstrated that sirtuins target a variety of cellular proteins in the nucleus, cytoplasm, and mitochondria for post-translational modification by acetylation (SIRT1, -2, -3 and -5) or ADP ribosylation (SIRT4 and -6)<sup>11,12</sup>. Through such epigenetic modifications, they participated in the tumor cell proliferation, survival and progression<sup>13</sup>. Sirtuin 6 (SIRT6) has been shown to be involved in the regulation of genome stability, longevity, glucose metabolism, and inflammation<sup>14,15</sup>. Recently, SIRT6 was found to play critical roles in the development of various types of cancers, such as colon adenocarcinoma, pancreatic cancer, breast

cancer and liver cancer<sup>16-18</sup>. However, the biological roles of SIRT6 in ovarian cancer have never been investigated. Therefore, the present study aimed to examine the expression of SIRT6 in human ovarian cancer tissues and further investigate its biological function in ovarian cancer cells.

## Materials and Methods

### *Clinical Samples*

The samples from ovarian cancer tissues and normal ovarian tissues were obtained from Obstetrics & Gynecology Hospital between Jan 2011 and June 2013. This study was approved by the Ethic Committee of Obstetrics & Gynecology Hospital, Fudan University, and informed consent for the use of samples was obtained from all the participants.

### *Cell Culture*

The human ovarian cancer cell lines SKOV3 and OVCAR3 were purchased from American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO<sub>2</sub> at 37°C.

### *Plasmid Construction, siRNA and Transfection*

The cDNA fragment encoding SIRT6 and Notch3 was isolated with Takara RNA PCR kit (Takara, Japan) using total RNAs from ovarian cancer cells. The primers used were designed by Primer 5.0 and PCR products were cloned into pcDNA3.1 (+) (Invitrogen, Carlsbad, CA). SIRT6 siRNA was purchased from Invitrogen (Carlsbad, CA, USA). Cells were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

### *MTT Assay*

Ovarian cancer cell proliferation was evaluated by MTT assay. To be brief, cell density of 3x10<sup>4</sup> (cells/well) was seeded into 96-well plates and left to adhere overnight. 10 ml of 5 mg/ml MTT was added and incubated in dark at 37°C for 2 h. The absorbance was determined with the wavelength of 490 nm.

### *Real-Time PCR*

Total RNAs were isolated from tissues or cells by TRIzol reagent, and reversely transcribed by Takara RNA PCR kit (Takara, Orsu, Shiga, Japan) according to the manufacturer's instructions. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Tokyo, Japan) on ABI 7500 system (Applied Biosystems, Foster, CA, USA). Amplification protocols were followed: 95°C for 3 min; 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 30 s. The transcript levels of interest genes were normalized to the  $\beta$ -actin.

### *Western Blot Analysis*

Total cells extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on a nitrocellulose filter membrane. The membranes were blocked in PBS/0.1% Tween-20 with 5% nonfat dry milk, and incubated with primary antibodies against SIRT6, Notch 3 and  $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA).  $\beta$ -actin was used as a loading control. The proteins were visualized with the enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

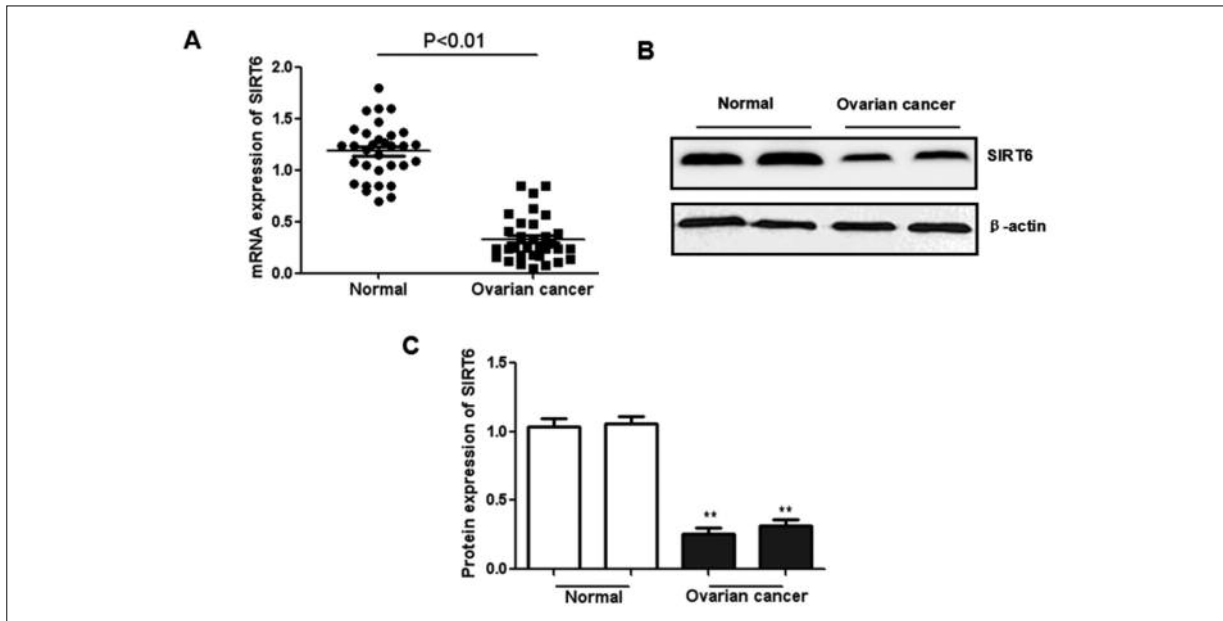
### *Statistical Analysis*

All data were presented as mean  $\pm$  SD of at least three independent experiments, and treated for statistics analysis by SPSS program (SPSS Inc., Chicago, IL, USA). Comparison between groups were determined by Student's *t*-test or ANOVA and statistical significance was indicated as \**p* < 0.05.

## Results

### *Decreased Expression of SIRT6 in Ovarian Cancer Tissues*

In order to explore the expression of SIRT6 in ovarian cancers, we measured the mRNA levels of SIRT6 in 32 ovarian cancer tissues and 32 normal ovarian tissues by real-time PCR. Results showed that the mRNA expression of SIRT6 in ovarian cancer tissues was remarkably lower than that in the normal ovarian tissues (Figure 1A). In addition, western blot was performed to determine the protein expression of SIRT6 in ovarian cancers. Consistent with data from real time



**Figure 1.** Down-regulation of SIRT6 in human ovarian cancer tissues SIRT6 expression at the mRNA (**A**) and protein (**B** and **C**) levels was measured by real-time PCR and western blot in human ovarian cancer tissues and normal tissues.  $**p < 0.01$ .

PCR, we also found that the protein level of SIRT6 was obviously decreased in ovarian cancer tissues compared to the normal ovarian tissues (Figure 1B and C).

#### **Overexpression of SIRT6 Inhibited Ovarian Cancer Cells Proliferation**

To explore the biological role of SIRT6 in ovarian cancer, human ovarian cancer cell lines SKOV3 was transfected with plasmids encoding SIRT6 (Figure 2A). We found that up-regulation of SIRT6 markedly suppressed the proliferation of ovarian cancer cells (Figure 2B). Next we silenced the expression of SIRT6 by small interfering RNA (siRNA) in SKOV3 cells, which was confirmed by real time PCR and western blot (Figure 2C-E). As a result, the proliferation rate was significantly elevated after SIRT6 knock-down in SKOV3 cells (Figure 2F). Similar results were also found in OVCAR3 cells. Collectively, these data suggested that SIRT6 could inhibit ovarian cancer cell proliferation.

#### **Inhibition of Notch 3 Expression by SIRT6 in Ovarian Cancer Cells**

We further explored the molecular mechanism underlying the inhibitory effects of SIRT6 on ovarian cancer cells. Intriguingly, real time PCR and western blot showed that the expression of Notch 3 was decreased in SKOV3 cells overex-

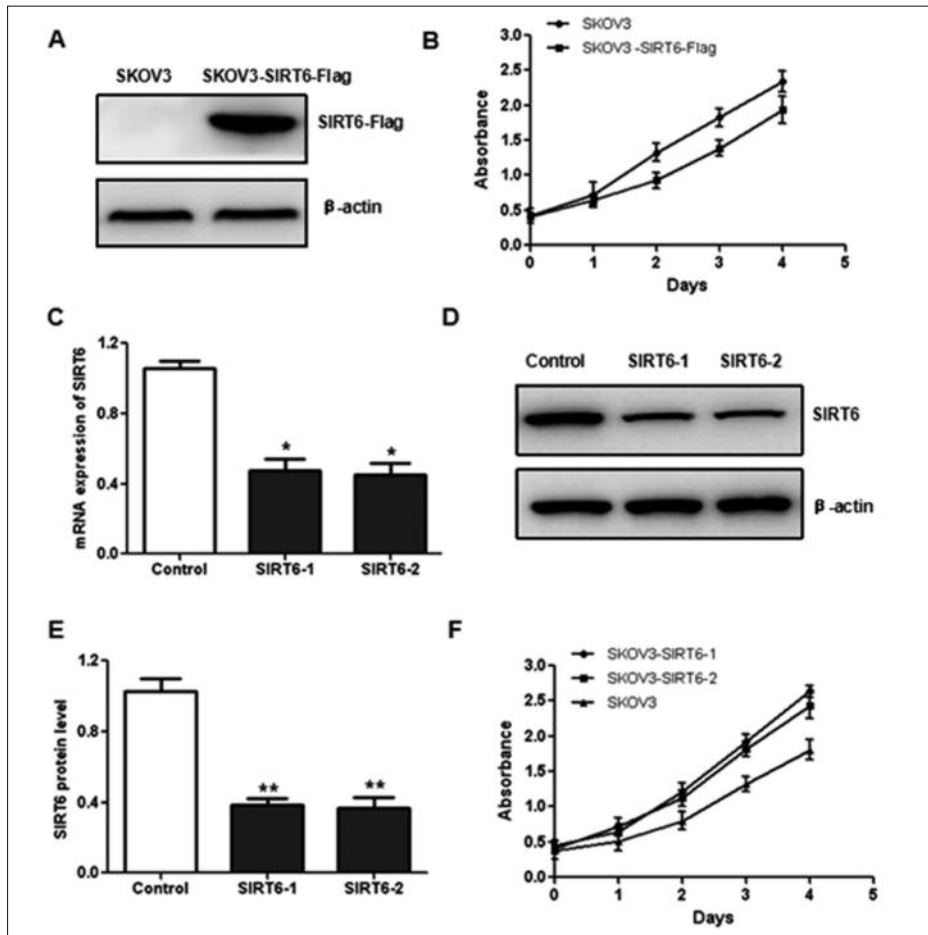
pressing SIRT6 (Figure 3A-C). On the contrary, down-regulation of SIRT6 led to elevated expression of Notch 3 both at the mRNA and protein levels (Figure 3D-F). These results demonstrated that SIRT6 negatively regulated the expression of Notch 3 in ovarian cancer cells.

#### **SIRT6 Inhibited Ovarian Cancer Cells Proliferation Via Regulation of Notch 3**

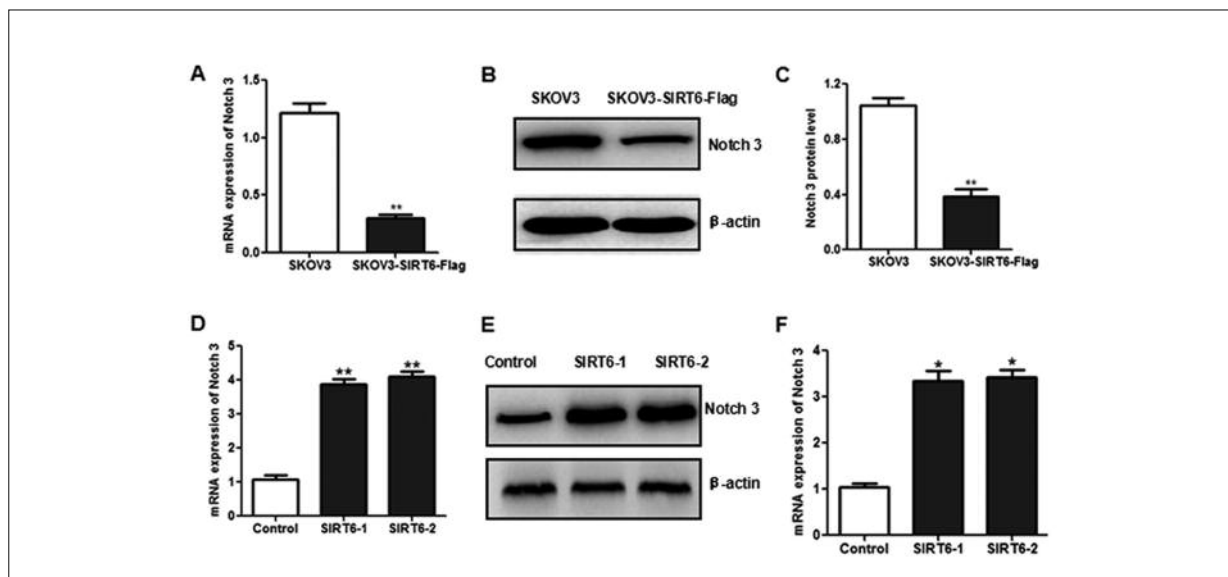
In order to elucidate the anti-proliferative effect of SIRT6 on ovarian cancer cells, we overexpressed Notch 3 in SKOV3 cells by transfection with plasmids encoding Notch 3 (Figure 4A-C). Consequently, the inhibitory effects of SIRT6 on cell proliferation were partially abolished after overexpression of Notch 3 in SKOV3 cells (Figure 4D). These data indicated that SIRT6 suppressed the proliferation of ovarian cancer cells through negative regulation of Notch 3.

## **Discussion**

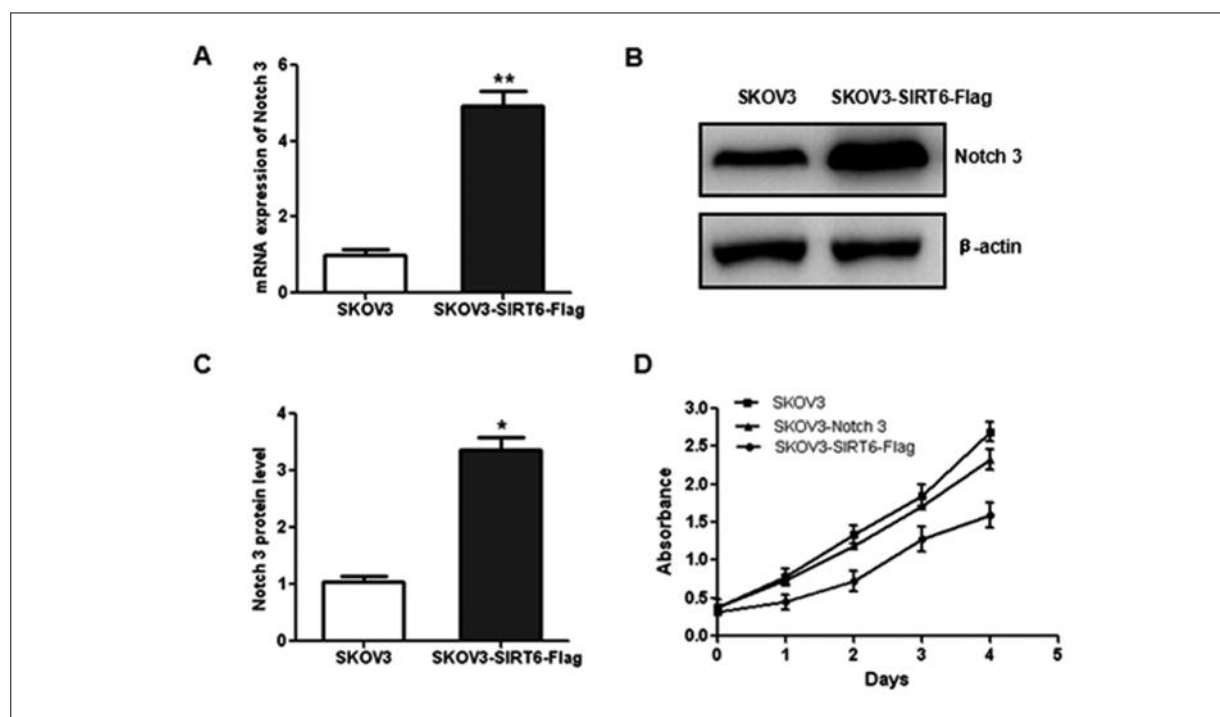
SIRT6 is a member of the NAD<sup>+</sup>-dependent class III deacetylase SIRTUIN family. Accumulating evidences suggest that SIRT6 plays diverse roles in inflammation, metabolism, and carcinogenesis<sup>14,15</sup>. It is suggested that SIRT6 functions as a tumor suppressor which controls aerobic glycolysis in cancer cells. Loss of SIRT6 pro-



**Figure 2.** SIRT6 inhibited ovarian cancer cell proliferation. **A**, The protein level of SIRT6 was determined by western blot in SKOV3 cells transfected with plasmids encoding SIRT6-flag. **B**, The proliferation of SKOV3 cells transfected with plasmids encoding SIRT6-flag was determined using MTT methods. **C-D**, Real-time PCR and western blot were performed to measure SIRT6 expression in SKOV3 cells after transfection with SIRT6 siRNA. \* $p < 0.05$ ; \*\* $p < 0.01$ . **F** MTT assay was used to measure the proliferation of SKOV3 and SKOV3 cells transfected with SIRT6 siRNA.



**Figure 3.** Overexpression of SIRT6 reduced the expression of Notch 3. Real-time PCR and western blot were performed to detect the mRNA (**A**) and protein (**B** and **C**) expression of Notch 3 in SKOV3 cells over-expressing SIRT6. SKOV3 cells were transfected with SIRT6 siRNA and the mRNA (**D**) and protein (**E** and **F**) levels of Notch 3 were measured by real-time PCR and western blot. Relative band intensities of each protein were quantified by densitometry. \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Figure 4.** SIRT6 inhibited the proliferation of ovarian cancer cells through down-regulation of Notch 3. SKOV3 cells over-expressing SIRT6 were transfected with plasmids encoding Notch 3. Then, the mRNA (**A**) and protein (**B** and **C**) levels of Notch 3 were measured by real time PCR and western blot. **D**, MTT assay was performed to determine the proliferation of SKOV3 cells over-expressing SIRT6 and Notch 3. \* $p < 0.05$ ; \*\* $p < 0.01$ .

notes the tumor formation *in vivo* accompanied with elevated glycolysis, suggesting that SIRT6 plays a role in the establishment of cancers<sup>19</sup>. In the present study, we for the first time investigated the biological function of SIRT6 in ovarian cancer tissues.

Sirtuins have been shown to modulate many cellular proteins by acetylation or ADP ribosylation. Their aberrant expression is closely associated with tumor initiation and progression<sup>20,21</sup>. In our study, we found that both the mRNA and protein expression of SIRT6 were significantly reduced in ovarian cancer tissues, implying that SIRT6 may play a critical role in the pathogenesis of ovarian cancer.

A number of studies support that SIRT6 acts as a potential tumor suppressor gene. Chen et al<sup>22</sup> showed that the histone deacetylase SIRT6 inhibited glioma cell proliferation and colony formation *in vitro* and glioma cell growth *in vivo* through binding to PCBP2 promoter region. Investigation of hepatocellular carcinoma demonstrated that SIRT6 suppressed the NF- $\kappa$ B activation and survivin expression, and markedly impairs the initiation and development of cancer

cells<sup>23</sup>. Another study<sup>24</sup> suggested that overexpression of SIRT6 remarkably promoted apoptosis in a variety of cancer cells, which requires the activation of both the p53 and p73 apoptotic signaling cascades. In our study, we found that overexpression of SIRT6 in ovarian cancer cells could markedly suppress cell growth, whereas SIRT6 knockdown with siRNA elevated cell proliferation rate, suggesting that SIRT6 negatively regulated ovarian cancer cells growth.

The Notch pathway is a highly conserved cell signaling system present in most multicellular organisms and has been implicated in a number of pathological conditions, such as cell proliferation, differentiation, apoptosis, and autophagy<sup>25-27</sup>. Multiple studies<sup>28,29</sup> show that the Notch signaling pathway is aberrantly activated in a wide range of cancer, including ovarian cancers. Lu et al<sup>30</sup> have identified Notch 3 as a candidate oncogene which was up-regulated in ovarian cancers compared to normal tissue by using the microarray technique. Another study<sup>31</sup> indicated that the Notch 3 signaling pathway was involved in the tumor progression of ovarian carcinoma, and higher Notch 3 expression may be an indepen-

dent poor prognostic factor. Our study demonstrated that SIRT6 could significantly reduce the expression of Notch 3. Moreover, Notch 3 over-expression abolished the inhibitory effect of SIRT6 on ovarian cancer cells proliferation. These results suggested that the anti-proliferative effect of SIRT6 on ovarian cancer cells was partly mediated by Notch 3.

### Conclusions

The present study for the first time demonstrated that SIRT6 inhibited the proliferation of ovarian cancer cells through down-modulation of Notch 3 expression. Our results might support the rationale for further investigation of SIRT6 as therapeutic targets in ovarian cancer treatment.

### Acknowledgements

This study was supported by Science and Technology Commission of Shanghai Municipality (124119a2900, 114119a2100 to L. Yao), and New Hundred Talents Program of Health and Family Planning Commission of Shanghai (13B122 to L. Yao).

### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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