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Long noncoding RNA HOTTIP is associated with male infertility and promotes testicular embryonal carcinoma cell proliferation

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Abstract

Background: It has been proposed that lncRNAs, widely transcribed from genomes, play pivotal regulatory roles in a variety of biological processes, but their function in regulating spermatogenesis in human males is rarely reported.

Methods: QRT-PCR was adopted to detect *HOTTIP* expression level in testicular tissues from hypospermatogenesis (Hypo) patients or controls. The proliferation levels of NT2 and 293T were measured *via* CCK-8 and EdU detection. Meanwhile, luciferase reporter gene assay and bioinformatics analysis were carried out to identify a target of *HOTTIP*. Additionally, the underlying mechanism of *HOTTIP*'s function was investigated using western blotting and RIP analysis.

Results: The research results manifested that the expression of *HOTTIP* in testicular tissues from Hypo patients was prominently reduced in comparison with that in control testicular tissues. Interestingly, it was noted that *HOTTIP* exhibited a high expression in testicular embryonal carcinoma cell line NT2 compared with that in normal control cell line 293T. It was denoted in cell function evaluation that cell proliferation was impeded by downregulated *HOTTIP* but evidently stimulated by overexpressed *HOTTIP*. Moreover, *HOTTIP* was capable of positively modulating *HOXA13* expression *via* the competitive binding to miR-128-3p.

Conclusion: Therefore, *HOTTIP* acting as ceRNAs to promote testicular embryonal carcinoma cell proliferation.

KEYWORDS
cell proliferation, *HOTTIP*, hypospermatogenesis, NT2, spermatogenesis

1 | INTRODUCTION

As a global healthy issue in the reproductive system, infertility occurs in about one-fifth couples planning for pregnancy (Szkodziak et al., 2016). Half infertility cases ascribe to male factors, 75% of which resulted from unknown factors due to the unclear molecular mechanisms of the defects (Chalyi, Akhvlediani, & Kharchilava, 2016). Male infertility is often clinically characterized by nonobstructive azoospermia (NOA) or severe oligozoospermia and uniform testicular maturation arrest (MA) (Esteves, 2015; Ferras et al., 2004; Oud et al., 2017), so unfolding the potential pathogenesis of hypospermatogenesis (Hypo) may be conducive to the therapeutic effects of these patients.

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As a complicated development process, spermatogenesis sustains the production of spermatozoa and fertility during the whole life of an adult male. A complex transcriptional network strictly modulates the three main phases of spermatogenesis, namely mitotic proliferation of spermatogonia, haploid differentiation of spermatids, and meiosis of spermatocytes. During spermatogenesis, various ncRNAs, including microRNAs (miRNAs) (Kotaja, 2014), endogenous small interfering RNAs (siRNAs) (Neto, Bach, Najari, Li, & Goldstein, 2016), and long noncoding RNAs (lncRNAs) (Dianatpour & Ghafouri-Fard, 2017) apart from protein-coding messenger RNAs, also exert crucial regulatory effects on genes. However, how lncRNAs regulate spermatogenesis still remains elusive.

LncRNAs are a kind of RNAs with length exceeding 200 nucleotides, whose capacity to encode proteins is limited (Forrest & Khalil, 2017). Reports in recent years have pointed out that lncRNAs function in the pathogenesis of different diseases, such as reproductive system diseases, nervous system diseases, and different tumors (Kondo, Shinjo, & Katsushima, 2017; Quan, Zheng, & Qing, 2017; Shen & Zhong, 2015). According to studies, lncRNAs are capable of adjusting gene expression at transcriptional and posttranscriptional levels (Weidle, Birzele, Kollmorgen, & Ruger, 2017). *HOTTIP* (Gene ID: 100,316,868), an lncRNA on chromosome 7p15.2, can promote the proliferation ability of A, B, and C cancer cells (Deng, Zhao, Wu, & Song, 2017; Li et al., 2016; Yu, Nangia-Makker, Farhana, & Majumdar, 2017). Nevertheless, the specific mechanism by which *HOTTIP* plays a role during spermatogenesis is not clear.

Through investigation, this research showed that *HOTTIP* was evidently decreased in testicular tissues of NOA patients with Hypo. Subsequently, a train of assays in vitro were performed to explore the underlying role of *HOTTIP* in the process of spermatogenesis. In general, the research results indicated that *HOTTIP* may participate in the mechanism of Hypo by influencing *HOXA13* expression, which gives a new insight into studying Hypo from the perspective of lncRNA.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

The research was approved by the Ethics Committee of Jiangsu Provincial Hospital of Traditional Chinese Medicine.

2.2 | Human testicular samples

In lncRNA microarray, testicular samples were obtained from Jiangsu Provincial Hospital of Traditional Chinese Medicine. In this study, 16 patients with Hypo and 16 control subjects receiving orchiectomy for prostate carcinoma were enrolled. The control samples were harvested from patients with normal spermatogenesis confirmed by histological examination and no meiotic defect history or infertility in the urology department. Informed consent was gained from all patients, and the Ethics Committee in Jiangsu Provincial Hospital of Traditional Chinese Medicine approved this study.

2.3 | Cell culture

Tumor cell lines (NT2, NCCIT, TE1, A549, and SY5Y) and a human normal cell line (293T) were bought from ATCC (Manassas, VA, USA) and cultured with RPMI1640 or DMEM containing 10% FBS (Hyclone, UT, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen, USA) in the environment with humidity and 5% carbon dioxide at 37°C. According to the manufacturer's protocol, all the cells were transfected with *HOTTIP* siRNAs, *HOTTIP* overexpression (*HOTTIP* OE) plasmids, miR-128-3p inhibitors, and miR-128-3p mimics synthesized by GenePharma (Shanghai, China) using Lipofectamine 3,000 (Invitrogen, CA, USA).

2.4 | RNA separation and quantitative real-time PCR (qRT-PCR)

TRIzol reagent (Life Technologies, CA, USA) was applied to extract total RNAs, whose purity was determined after the measurement of A_{260} and A_{280} using a UV spectrophotometer. Then, the extracted total RNAs were stored in a −80-C refrigerator for standby application. With reference to the instructions of the Reverse Transcription Kit (Takara, Tokyo, Japan), RNAs were subjected to reverse transcription into cDNAs and stored at −20°C for standby application. The primers used were diluted as required, and qRT-PCR was performed to examine the expressions of lncRNAs, mRNAs and miRNAs on the ABI 7900HT (Applied Biosystems, CA, USA). *HOXA13* primer sequences: F: 5'-CTGCCCTATGGCTACTTCGG-3', R: 5'-CCGGCGGTATCCATGTACT-3'. $2^{-\triangle\triangle Ct}$ method was adopted for measuring the relative concentration of the samples to be tested. Each assay was repeated 3 times, followed by averaging.

2.5 | Cell proliferation assessment

Following inoculation on 96-well plates with DMEM free of serum, cells were incubated for required time, and each well was added with 10-μl Cell Counting Kit-8 (CCK-8) reagents (Beyotime, Nantong, China). At 1 hr after incubation, TECAN infinite M200 multimode microplate reader (Tecan, Mechelen, Belgium) was applied to evaluate A_{450}, and 5-Ethynyl-2'-deoxyuridine (EdU) assay was carried out for evaluation of cell proliferation capacity. The same experiment was repeated 3 times at least.

2.6 | Location of subcellular fractionation

Based on the protocol, RNAs in the cytoplasm and nucleus were separated using the PARIS Kit (Life Technologies, USA). Total RNAs separated from each fraction were determined *via* qRT-RCR, with GAPDH as a cytoplasmic marker and U6 as a nuclear control transcript.

2.7 | Dual-luciferase reporter assay

The following fragments were constructed after the binding site between *HOXA13* and *HOTTIP* was inserted into the KpnI and SacI sites of pGL3 promoter vectors (Realgene, Nanjing, China): pGL3-*HOXA13*-WT, pGL3-*HOXA13*-MUT, pGL3-*HOTTIP*-WT, and pGL3-*HOTTIP*-MUT. Then the cells were cultured on 24-well plates until the cell density reached 50%–60% and co-transfected with 50 nmol/L miR-128-3p mimics, 5 ng pRL-SV40, 80 ng plasmids, and NC *via* Lipofectamine 2000 (Invitrogen, Shanghai, China). In line with the manufacturer's protocol, the luciferase activity change at 36 hr after incubation was assessed through the dual-Glo luciferase assay system (Promega, Madison, WI, USA). Each experiment was separately repeated 3 times.

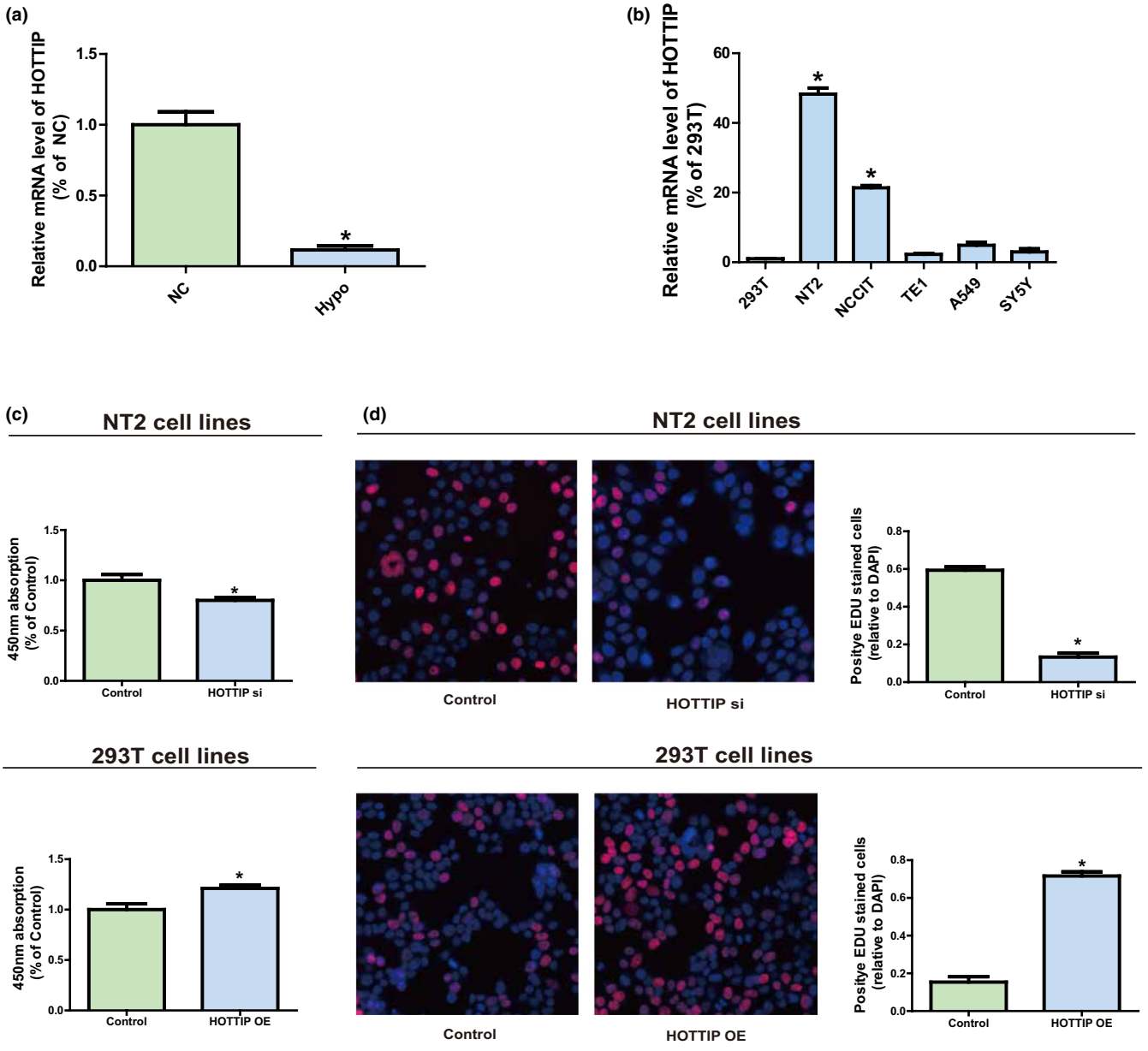

FIGURE 1 The role of *HOTTIP* in Hypo. (a) QRT-PCR confirms *HOTTIP* is down-regulated in Hypo patients. (b) Expression levels of *HOTTIP* in tumor cell lines (NT2, NCCIT, TE1, A549, and SY5Y) and a human normal cell line (293T). (c) Effect of transfection with *HOTTIP* siRNAs or OE plasmids on cell proliferation level of human cell lines NT2 and 293T observed *via* CCK-8 assay. (d) Effect of transfection with *HOTTIP* siRNAs or OE plasmid on cell proliferation level of human cell lines NT2 and 293T observed *via* EdU assay. Data are presented as mean ± *SD*. *$p \leq .05$, Student's *t*-test

2.8 | RNA immunoprecipitation (RIP)

With reference to the protocol of the manufacturer, RIP assay was carried out using anti-AGO2 (Abcam, ab32381, Cambridge, MA, USA) and the Magna Nuclear RIP™ (Native) Nuclear RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA).

2.9 | Western blotting

RIPA was conducted to extract total proteins, and the appropriate concentration of SDS-PAGE gel was chosen in light of the molecular weight of target proteins. The proteins were transferred to a PVDF membrane and stained on the basis of normal immune staining after SDS-PAGE. Anti-*HOXA13* and anti-GAPDH antibodies (Abcam, Cambridge, MA, USA) were diluted at 1:500 and added for incubation at 4°C overnight. Diluted at 1:1,000, secondary antibodies were utilized for 2 hr at 37°C, followed by enhanced chemiluminescence. Finally, the color was fixed and pictures were taken.

2.10 | Statistical analysis

Data were analyzed by means of GraphPad Prism 6.0 (GraphPad Software Inc., CA, USA) and SPSS 20.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± *SD*. Statistical differences between data sets in line with normal distribution were investigated using the Student's *t*-test, whereas the differences were analyzed using nonparametric tests in case the data were not in line with normal distribution. The difference was considered to be statistically significant when $p < .05$.

3 | RESULTS

3.1 | *HOTTIP* expression changes in Hypo patients

QRT-PCR analysis confirmed *HOTTIP* was downregulated in Hypo patients (Figure 1a). *HOTTIP* expression level was measured in tumor cell lines (NT2, NCCIT, TE1, A549, and SY5Y) and a human normal cell line (293T) by qRT-PCR. It could be obviously seen from Figure 1b that compared with 293T, the *HOTTIP* level in testicular embryonal carcinoma cell line NT2 was markedly increased.

3.2 | The role of *HOTTIP* in NT2 and 293T

HOTTIP expression silencing was achieved through transfection of NT2 with *HOTTIP* siRNAs to figure out

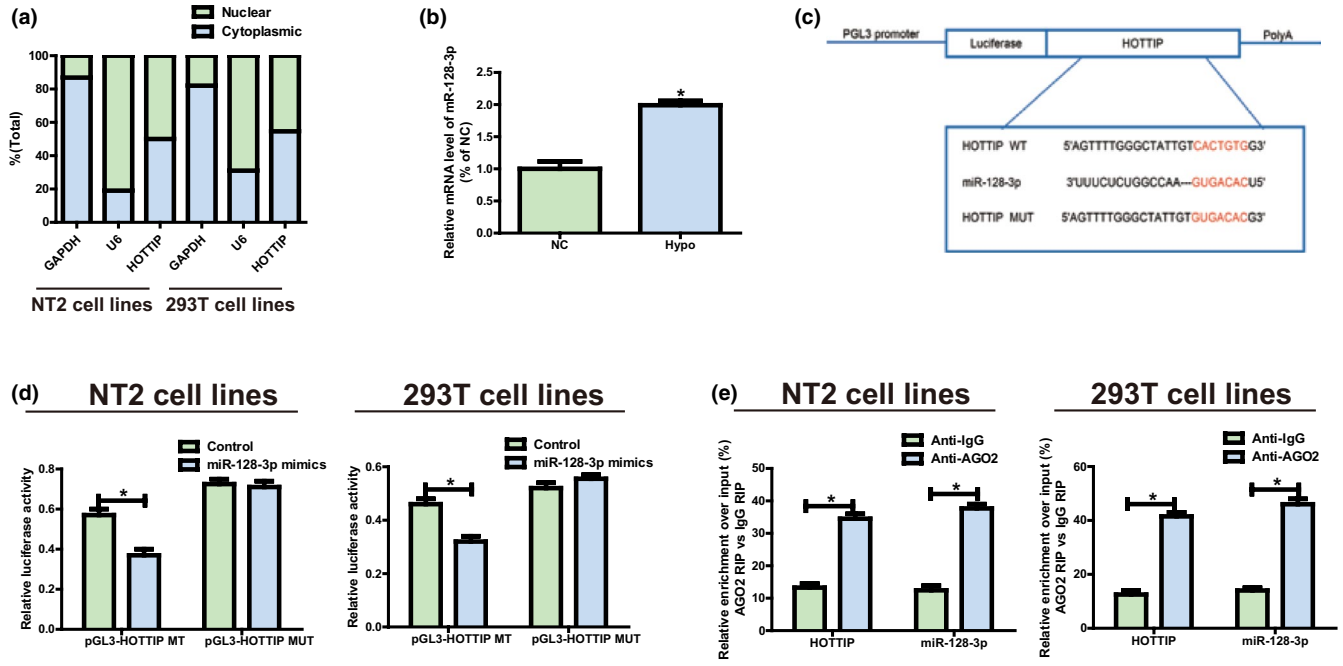

FIGURE 2 *HOTTIP* directly interacts with miR-128-3p. (a) Distribution characteristics of *HOTTIP* in NT2 and 293T detected *via* qRT-PCR. (b) MiR-128-3p expression level is decreased in Hypo patients compared to the normal controls. (c) Bioinformatics evidence that miR-128-3p binds to *HOTTIP*'s 3'-UTR. The speculated miRNAs recognition sites that are cloned into the luciferase gene downstream are named as pGL3-*HOTTIP*-WT. Bottom: Mutant *HOTTIP* sequences that produce the mutant luciferase reporter constructs gain the name of pGL3-*HOTTIP*-MUT. (d) Detection of luciferase activity in NT2 and 293T following co-transfection with plasmids (pGL3-*HOTTIP*-WT or pGL3-*HOTTIP*-MUT) and miRNA mimics *via* dual-luciferase reporter assay. (e) RIP assays are performed in NT2 and 293T to figure out whether *HOTTIP* is present in ribonucleoprotein complexes with miRNAs. QRT-PCR is adopted to measure miR-128-3p and *HOTTIP* expression levels. Data are presented as mean ± *SD*. *$p \leq .05$, Student's *t*-test

HOTTIP's biological function in NT2 and 293T. Besides, 293T was transfected with *HOTTIP* OE plasmids so as to elevate *HOTTIP* expression. The results manifested that *HOTTIP* expression was substantially blocked after NT2 was transfected with *HOTTIP* siRNAs (Supplementary Figure S1a), and *HOTTIP* level was raised after 293T was transfected with *HOTTIP* OE plasmids (Supplementary Figure S1b). Subsequently, CCK-8 assay and EdU assay showed that downregulation of *HOTTIP* could evidently reduce the proliferation ability of NT2. On the contrary, *HOTTIP* OE can significantly enhance the proliferation capacity of 293T (Figure 1c,d). In brief, it can be seen that *HOTTIP* may play a regulatory role in the proliferation of NT2 and 293T to certain extent.

3.3 | Subcellular localization of *HOTTIP*

Subcellular localization of lncRNAs determines their biological functions. As such, cellular localization of *HOTTIP* was verified through isolation of NT2 and 293T into the nucleus and the cytoplasm, with U6 and GAPDH as controls. U6 is mainly found in the nucleus, while GAPDH is primarily distributed in the cytoplasm. As shown in qRT-PCR results, *HOTTIP* was monitored in the cytoplasm of NT2 (45.4%) and 293T (49.1%) (Figure 2a). These findings imply that *HOTTIP* may be involved in the pathogenesis of Hypo through both transcriptional- and posttranscriptional-level regulations.

3.4 | MiR-128-3p targets *HOTTIP*

The results showed that *HOTTIP* exhibited an obvious increase in NT2, and it was able to boost proliferation, but the specific mechanism remains not clear. It was hypothesized that in biological processes, *HOTTIP* might be considered as a ceRNA since *HOTTIP* is partially located in the cytoplasm. RegRNA 2.0, Starbase was applied for bioinformatics predictions, which unfolded that miR-128-3p was closely matched with the sequence in *HOTTIP*'s 3'-UTR. MiR-128-3p expression level was lowered in normal controls compared to the Hypo patients (Figure 2b), which is opposite to the features of *HOTTIP* expression. *HOTTIP* fragments (pGL3-*HOTTIP*-WT and pGL3-*HOTTIP*-MUT) with predicted or mutated target sites were established into the firefly luciferase gene downstream to figure out the interaction between *HOTTIP* and miR-128-3p (Figure 2c). Treatment of NT2 and 293T with *HOTTIP* WT and miR-128-3p mimics remarkably weakened the intensity of luciferase compared to the control group, but the intensity did not change following co-transfection with miR-128-3p mimics and *HOTTIP* MUT (Figure 2d). Moreover, to clarify whether *HOTTIP* was present in ribonucleoprotein complexes containing mRNAs, RIP assays were carried out in NT2 and 293T. Subsequently, compared with that in immunoglobulin G (IgG) controls, the relative RNA expression in immunoprecipitates was detected *via* qRT-PCR, and it was discovered that anti-AGO2 antibodies could enrich *HOTTIP* in NT2 and 293T, which was similar

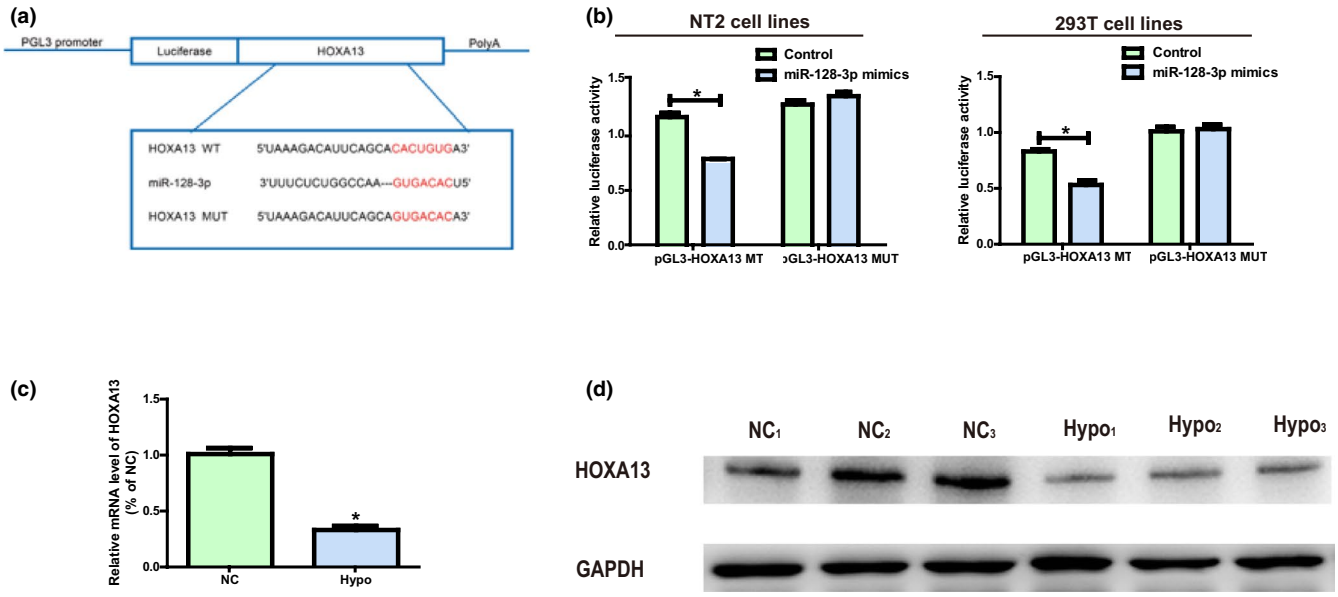

FIGURE 3 *HOXA13* is a miR-128-3p direct target. (a) Prediction of binding site between *HOXA13* and miR-128-3p and construction of *HOXA13*-MUT plasmids. (b) Detection of the luciferase activity in NT2 and 293T after co-transfection plasmid (pGL3-*HOXA13*-WT or pGL3-*HOXA13*-MUT) and miRNA mimics examined *via* dual-luciferase reporter gene experiments. (c) The mRNA level of *HOXA13* in Hypo patients is remarkably decreased. (d) The protein expression level of *HOXA13* in Hypo patients is obviously decreased. Data are presented as mean ± *SD*. *$p \leq .05$, Student's *t*-test

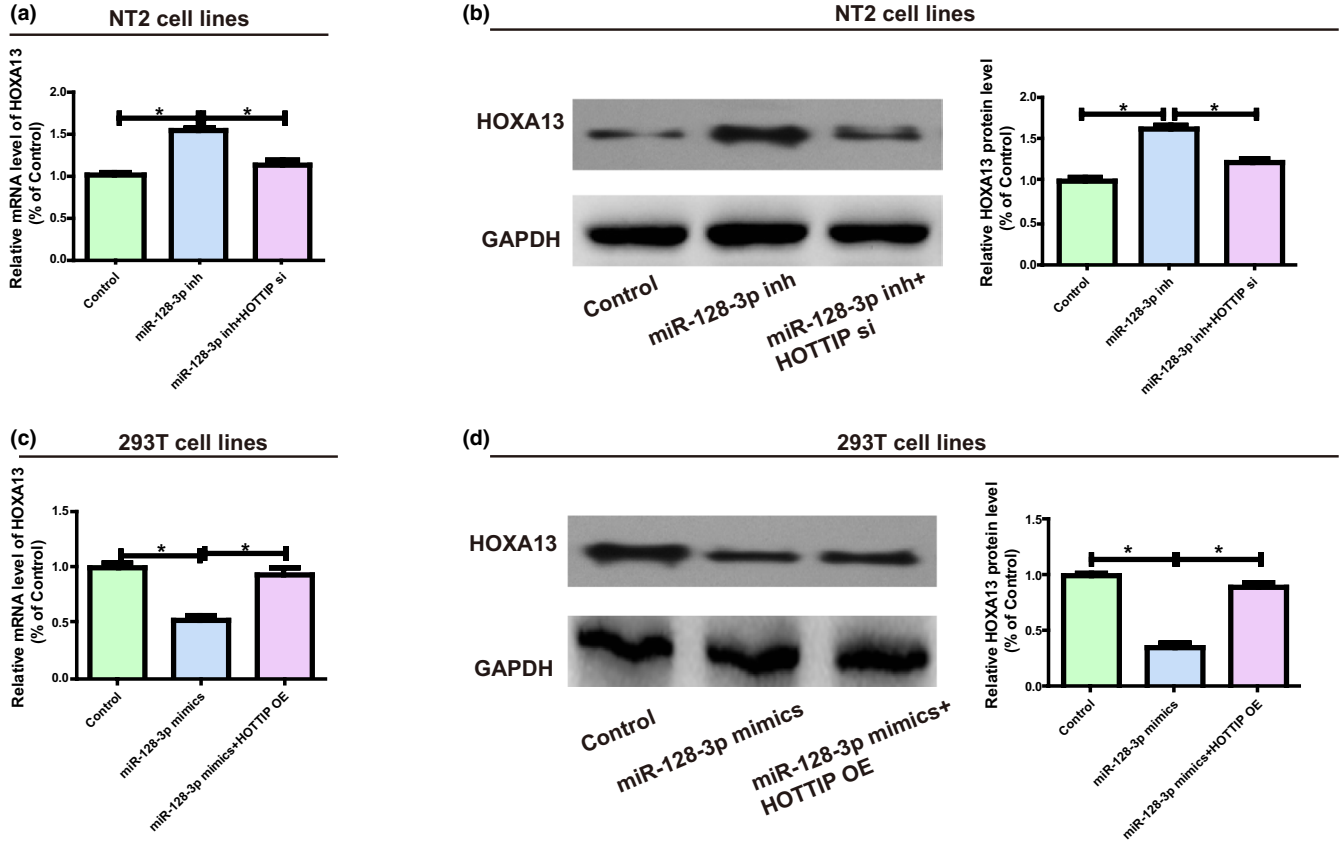

FIGURE 4 *HOTTIP*/miR-128-3p regulatory loop is pivotal for *HOXA13* expression. (a) NT2 is transfected with miR-128-3p inhibitors (with or without *HOTTIP* siRNAs) and the mRNA level of *HOXA13* is assessed *via* qRT-PCR. (b) Following NT2 is treated with miR-128-3p inhibitors (with or without *HOTTIP* siRNAs), western blotting is carried out to detect the *HOXA13* protein level, with GAPDH as a control. (c) 293T is transfected with miR-128-3p mimics (with or without *HOTTIP* OE plasmids), and the relative mRNA level of *HOXA13* is detected using qRT-PCR. (d) 293T is transfected with miR-128-3p mimics (with or without *HOTTIP* OE plasmids), and the relative protein level of *HOXA13* is measured through qRT-PCR. Data are presented as mean ± *SD*. **p* ≤ .05, Student's *t*-test

to the results of miR-128-3p (Figure 2e). The above findings suggest that miR-128-3p can bind to *HOTTIP* in vitro.

3.5 | *HOTTIP* regulates miR-128-3p target *gene, HOXA13*

MiR-128-3p target genes were screened and subjected to intersection through bioinformatics prediction (TargetScan, Starbase, RegRNA) so as to study miR-128-3p molecular mechanism in its biological function. *HOXA13* was ultimately selected for further research. To further prove the binding between miR-128-3p and *HOXA13*, dual-luciferase reporter assay was conducted. pGL3-*HOXA13*-WT and pGL3-*HOXA13*-MUT with WT- or MUT-binding sites were established and subjected to independent co-transfection with NC or miR-128-3p mimics in NT2 and 293T (Figure 3a). It was confirmed that the luciferase activity of the groups co-transfected with miR-128-3p WT plasmids and mimics was inhibited, while that in the groups co-transfected miR-128-3p MUT plasmids and mimics was not blocked (Figure 3b), indicating that *HOXA13* is an underlying miR-128-3p target gene. It was found in Figure 3c that the mRNA level of *HOXA13* in Hypo patients was reduced remarkably. Additionally, western blotting was carried out to measure the protein level of *HOXA13*, and the results identical to those of qRT-PCR were obtained (Figure 3d).

HOXA13 expression after transfection with *HOTTIP* and miR-128-3p was monitored *via* western blotting and qRT-PCR to elucidate whether *HOTTIP* modulated *HOXA13* expression level by binding to miR-128-3p. According to the results, miR-128-3p inhibitors markedly increased *HOXA13* expression in NT2, while co-transfection with *HOTTIP* siRNA could reverse this change (Figure 4a,b). Furthermore, miR-128-3p mimics evidently inhibited *HOXA13* expression in 293T, whereas co-transfection of *HOTTIP* OE plasmids could reverse this change (Figure 4c,d). The above findings generally confirm that *HOTTIP* elevates *HOXA13* expression by directly binding to miR-128-3p.

3.6 | *HOTTIP*/miR-128-3p regulatory loop is crucial for cell function

Then, whether miR-128-3p influenced proliferation of NT2 and 293T was determined. Determination of transfection efficiency proved that miR-128-3p inhibitors could impede the expression of miR-128-3p, while miR-128-3p mimics can improve the expression level of miRNA (Supplementary Figure S1c,d). Compared with NC, decreased miR-128-3p in NT2 obviously stimulated cell proliferation ability, and reduced *HOTTIP* could partially reverse the effect of miR-128-3p (Figure 5a,c). Besides, overexpressed miR-128-3p in 293T evidently impeded cell proliferation compared with NC, and upregulation of *HOTTIP* could reverse miR-128-3p's effect to some extent (Figure 5b,d). In conclusion, all the research results reveal the interaction among *HOTTIP*, miR-128-3p and *HOXA13*.

4 | DISCUSSION

As a complicated and highly concerted process, spermatogenesis relies on the loss of germ cells in the processes of meiosis and spermiogenesis and the proliferation activity of spermatogonia (Nishimura & L'Hernault, 2017). Hence, any cause that affects the proliferation capacity of spermatogonia can lead to spermatogenesis process disorders. It has been proved that almost all cellular processes are modulated by lncRNAs, and the imbalance of these noncoding molecules appear to be associated with the development of a variety of diseases (Degirmenci & Lei, 2016). A study of Lu et al. confirmed that downregulation of miR-320a/383 sponge-like lncRNA *NLC1-C* has correlation with male infertility and accelerates the proliferation of testicular embryonal carcinoma cells (Lu et al., 2015). As *HOTTIP* has been verified to exert a regulatory effect in cell proliferation in many diseases, it is hypothesized that *HOTTIP* may participate in the pathogenesis of infection.

The above study revealed that compared with the control testicular samples, *HOTTIP* was downregulated in Hypo patients. In the meantime, it was found that compared with the control normal cell line 293T, the *HOTTIP* level in testicular embryonal carcinoma cell line was evidently increased. These results suggest that *HOTTIP* may participate in the pathogenesis of Hypo by regulating the proliferation of NT2. In tumor-related studies, *HOTTIP* has been proved to participate in the pathogenesis of many tumors including endometrial cancer (Guan, Zhang, Zhang, Liu, & Ren, 2018), ovarian cancer (Zou, Wang, Gao, & Liang, 2018), and renal cell carcinoma (Su et al., 2019) by promoting tumor cell proliferation level. To further verify our hypothesis, the changes in cell function of NT2 and 293T after knocking down and overexpressing *HOTTIP* in vitro were monitored. According to the results, NT2 proliferation was suppressed after *HOTTIP* knockdown, but *HOTTIP* OE sped up 293T proliferation. The above findings evidence that *HOTTIP* participates in the occurrence of Hypo.

By means of diverse mechanisms, lncRNAs are involved in human diseases. The subcellular localization of *HOTTIP* in NT2 and 293T was determined, the results of which displayed that *HOTTIP* showed primary expression in both the nucleus and cytoplasm, implying that *HOTTIP* is capable of modulating gene expression both at transcription and posttranscription levels. Recently, an enormous body of reports has shown that an lncRNA can be regarded as a sponge that adjusts the expression of miRNA targets and binds to miRNAs. For instance, Feng K et al. identified that the viability and invasion of papillary thyroid carcinoma cells can be enhanced by lncRNA *PVT1*, a ceRNA of miR-30a, mediating IGF1 receptor expression (Feng et al., 2018). Yang et al. reported that lncRNA *HOTTIP* accelerates the migration and proliferation of prostate cancer cells by sponging miR-216a-5p (Xiong et al., 2018). After the intersection of bioinformatics prediction results, it was discovered that miR-128-3p was highly consistent with *HOTTIP*'s 3'UTR sequence. To further confirm the correlation between *HOTTIP* and miR-128-3p, dual-luciferase reporter gene assay and RIP assay were conducted. The results manifested that miR-128-3p was capable of decreasing luciferase activity including pGL3-*HOTTIP*-WT sequence, while anti-AGO2 antibodies decrease *HOTTIP* and miR-128-3p. The above results imply that *HOTTIP* can be directly combined with miR-128-3p and considered as a ceRNA. Numerous studies in recent years have confirmed that miR-128-3p can inhibit cell proliferation level (Huang et al., 2015; Huo et al., 2019; Zhou et al., 2018). The current research pointed out that miR-128-3p expression in testicular samples from Hypo patients was notably increased. In addition, the results displayed that miR-128-3p mimics impeded 293T proliferation, while miR-128-3p inhibitors exerted an opposite effect. In conclusion, these findings demonstrate that *HOTTIP* affects the proliferation of NT2 through interaction with miR-128-3p.

For further exploring the exact mechanism of miR-128-3p blocking cell proliferation, *HOXA13* was proved to be a miR-128-3p target gene through dual-luciferase reporter gene assay. Previously found in drosophila, the highly conservative HOX gene family encodes transcription factors that modulate cell differentiation and proliferation and exerts pivotal effects in the development of embryos (Mallo & Alonso, 2013). Abnormal differentiation and proliferation result from dysfunction of HOX proteins due to changed modes of HOX genes (Holland, 2013). In current studies, the expression of HOX gene, especially *HOXA13*, accelerating the proliferation of diverse tumor cells has been investigated (Lin et al., 2017; Qin et al., 2019). According to western blotting and qRT-PCR, *HOXA13* expression in testicular samples from

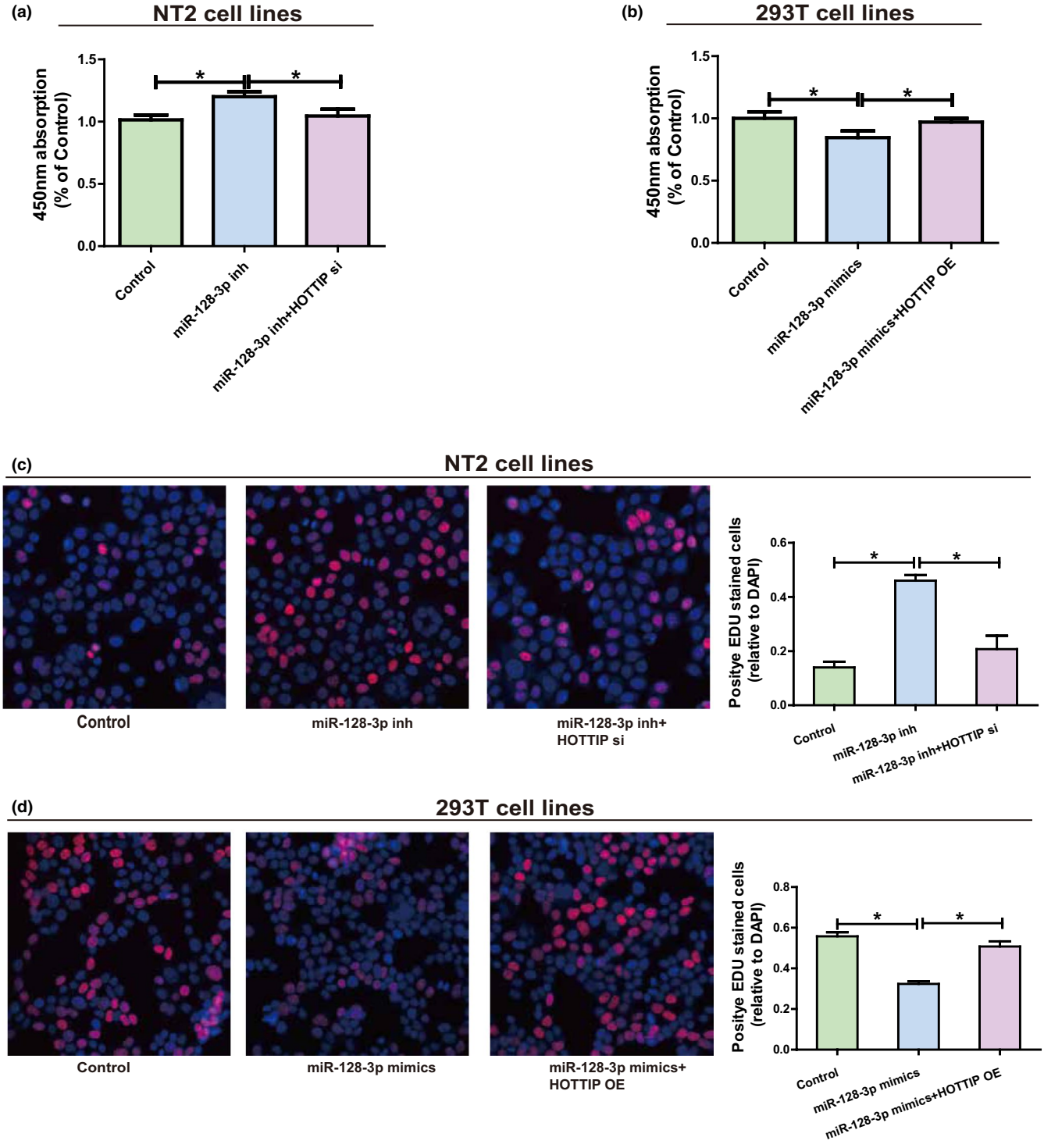

FIGURE 5 *HOTTIP*/miR-128-3p regulatory loop is important for cell function. (a) Following NT2 is transfected with miR-128-3p inhibitors (with or without *HOTTIP* siRNAs), the cell proliferation level is measured by CCK-8. (b) After miR-128-3p mimics (with or without *HOTTIP* OE plasmids) are transfected into 293T, the cell proliferation level is determined by CCK-8. (c) Following NT2 is transfected with miR-128-3p inhibitors (with or without *HOTTIP* siRNAs), the cell proliferation level is measured by EdU. (d) Following transfection of 293T with miR-128-3p mimics (with or without *HOTTIP* OE plasmids), the cell proliferation level is determined by EdU. Data are presented as mean ± *SD*. *$p \leq .05$, Student's *t*-test

Hypo patients was markedly elevated compared with control. However, whether there is interaction among *HOXA13*, *HOTTIP,* and miR-128-3p still needs further verification. It was also discovered that transfection with *HOTTIP* siRNAs can reverse the increase of *HOXA13* caused by miR-128-3p inhibitors. As such, it is speculated that *HOTTIP* is involved in the pathogenesis of male infertility to adjust *HOXA13* expression *via* the competitive binding to miR-128-3p.

5 | CONCLUSION

The current research denotes that the expression level of *HOTTIP* in testicular samples from Hypo patients is decreased, and the downregulation of *HOTTIP* inhibits the cell proliferation ability of NT2. Furthermore, the above experiments eventually prove that *HOXA13* expression can be adjusted by *HOTTIP* sponging miR-128-3p in NT2 as a ceRNA. In summary, this research demonstrates that lncRNA *HOTTIP* has correlation with male infertility and stimulates the proliferation of testicular embryonal carcinoma cells.

ACKNOWLEDGMENTS

None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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