BASIC SCIENCE ARTICLE

Circ-ITCH overexpression promoted cell proliferation and migration in Hirschsprung disease through miR-146b-5p/RET axis

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BACKGROUND: Hirschsprung disease (HSCR) is a congenital intestinal disease caused by the abnormal proliferation and migration of enteric nerve cells (ENCC). Research suggested critical roles for circular RNA (circRNA) itchy E3 ubiquitin protein ligase (ITCH) in gastrointestinal malignancies progression. However, the function of circ-ITCH in HSCR remains poorly defined.
METHODS: The related genes expression in 30 HSCR patients and 30 controls without HSCR were detected using qRT-PCR. Cell proliferation was assessed by CCK-8 assay and EdU assay. Cell migration was detected with wound-healing assay and transwell assay. The interactions among circ-ITCH, miR-146b-5p, and RET were confirmed by Dual luciferase reporter assay.
RESULTS: Circ-ITCH and RET expressions were downregulated in HSCR patients and cells, while the miR-146b-5p expression was upregulated. Circ-ITCH overexpression facilitated cell proliferation, migration, and activated MAPK pathway, which were reversed by circRNA-ITCH knockdown. Circ-ITCH negatively regulated miR-146b-5p expression. MiR-146b-5p overexpression abolished the promoting effects of circ-ITCH overexpression on cell proliferation and migration. MiR-146b-5p inhibited RET expression. RET overexpression eliminated the inhibitory effects of miR-146b-5p overexpression on cell proliferation and migration.
CONCLUSION: Circ-ITCH overexpression facilitated cell proliferation and migration in HSCR by regulating miR-146b-5p/RET/MAPK axis.

IMPACT:

- The expressions of Circ-ITCH and RET were markedly reduced in HSCR, while miR-146b-5p expression was increased in HSCR.
- Circ-ITCH overexpression enhanced the proliferative and migratory abilities of SH-SY5Y and 293T cells.
- Circ-ITCH negatively regulated miR-146b-5p expression.

INTRODUCTION

HSCR is also called as intestinal aganglionosis, which has severe features such as obstructive bowel movements, abdominal distension, and vomiting.[1] The incidence of HSCR in live births is about 1/5000[2]. Currently, surgical treatment including removal of the abnormally innervated intestine is the most common treatment for HSCR. However, postoperative defects in bowel function are common.[3] Therefore, it is very urgent to probe the pathogenesis and find the therapeutic targets of HSCR. Up to date, the lack of the enteric nervous system in the distal bowel caused by the migration and proliferation disorders of ENCC has been demonstrated to be the main cause of HSCR.[4,5] Therefore, probing the regulation mechanisms of ENCC proliferation and migration in HSCR is of great significance for HSCR treatment. Herein, 293T and SH-SY5Y cells which were commonly used cell lines to study the cell behaviors of ENCC in HSCR were employed to study the regulatory network of HSCR development.

CircRNAs refer to non-coding RNAs which form loop structures without 5′–3′ polarities and polyadenylated tails.[6] Growing evidence has shown that circRNAs take part in the occurrence and process HSCR.[7,8] For example, circRNA CCDC66 could inhibit cell proliferative and migratory abilities via interacting with miR-488-3p in HSCR.[8] CircRNA ZNF609 was obviously upregulated in HSCR tissues, and circRNA ZNF609 silencing suppressed proliferation and migration of 293T and SH-SY5Y cells.[7] Circ-ITCH is a classic tumor suppressor, which is downregulated in various malignant tumors such as lung cancer, bladder cancer, and ovarian cancer.[9–11] Above evidences indicate that circ-ITCH is involved in regulating the potential biological networks of cancers, which may be a promising diagnostic biomarker and potential target for therapeutic intervention. However, whether and how circ-ITCH is involved in HSCR development remains unclear, and its potential targets have not yet been fully determined. Therefore, it is necessary and urgent to explore the roles and mechanisms of circ-ITCH in HSCR.

Accumulated evidence has demonstrated that circRNAs achieve their biological functions in diseases including HSCR through targeting miRNAs.[12] For instance, circ-PRKCI silence could promote

HSCR progression via targeting miR-1324 (ref. [13]). Bioinformatics prediction revealed circ-ITCH had a potential binding site to miR-146b-5p. Previous study displayed that miR-146b-5p was remarkably upregulated in HSCR patients.[14] However, it remains to be investigated whether circ-ITCH regulates HSCR progression by targeting miR-146b-5p.

REarranged during Transfection (RET) proto-oncogene, which encoded a receptor tyrosine kinase, was reported as one of the most affected genes in HSCR.[15] It has been widely reported that RET has the ability to help nerve rest cells pass through the digestive tract during embryonic development,[16] and RET expression was markedly downregulated in HSCR patients.[17] Additionally, RET was also reported to be able to participate in regulating process of HSCR by regulating its downstream pathway MAPK signaling pathway.[14] Specifically, RET promoted SH-SY5Y cells proliferation by activating the MAPK signaling pathway.[18] However, there is no report about the interaction between RET and miR-146b-5p in HSCR.

Herein, we speculated that circ-ITCH overexpression promoted the proliferation and migration of 293T and SH-SY5Y cells via regulating the miR-146b-5p/RET/MAPK axis, which could suppress HSCR progression. Our study laid the foundation for future research on the mechanisms of HSCR development.

MATERIALS AND METHODS
Clinical samples collection
From January 2019 to December 2020, HSCR aganglionic colon segments were collected from 30 children with HSCR, and 30 matched controls were collected children without HSCR. All the patients were diagnosed by barium enema and anorectal manometry evaluation before surgical procedures. After surgery, pathological diagnosis was made for definite diagnosis to verify no ganglion cells were observed. Control colon tissues, without the ischemia or necrosis parts and verified without HSCR, were collected from patients who underwent surgical intervention as a result of intussusception and inguinal hernia. The average age of the control group was 2.45 ± 0.58 years, and the average age of HSCR patients was 2.62 ± 0.72 years. All the subjects and their families agreed to enter the study and signed the informed consent form. The experiment of this work was approved by the Institutional Ethics Committee of Hunan Children's Hospital.

Cell culture
SH-SY5Y and 293T cells were purchased from American type culture collection (ATCC, Manassas, VA). All the cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco) at 37 °C and 5% CO_2.

Cell transfection
In accordance with the manufacturer's instructions, the overexpression plasmid of circ-ITCH (oe-circ-ITCH), overexpression plasmid of RET (oe-RET), short hairpin RNA (shRNA) of circ-ITCH (sh-circ-ITCH), miR-146b-5p mimics, and their negative controls were transfected into cells using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA). The above plasmids and shRNAs were obtained from GenePharma (Shanghai, China).

Cell counting kit 8 (CCK-8) assay
Cells (1×10^4 cells/mL) were seeded in 96-well plates. After 24 h, cells were incubated with 10 μL of CCK-8 solution (Sangon, Shanghai, China) for 3 h. Absorbance at 450 nm was detected with a microplate spectrophotometer
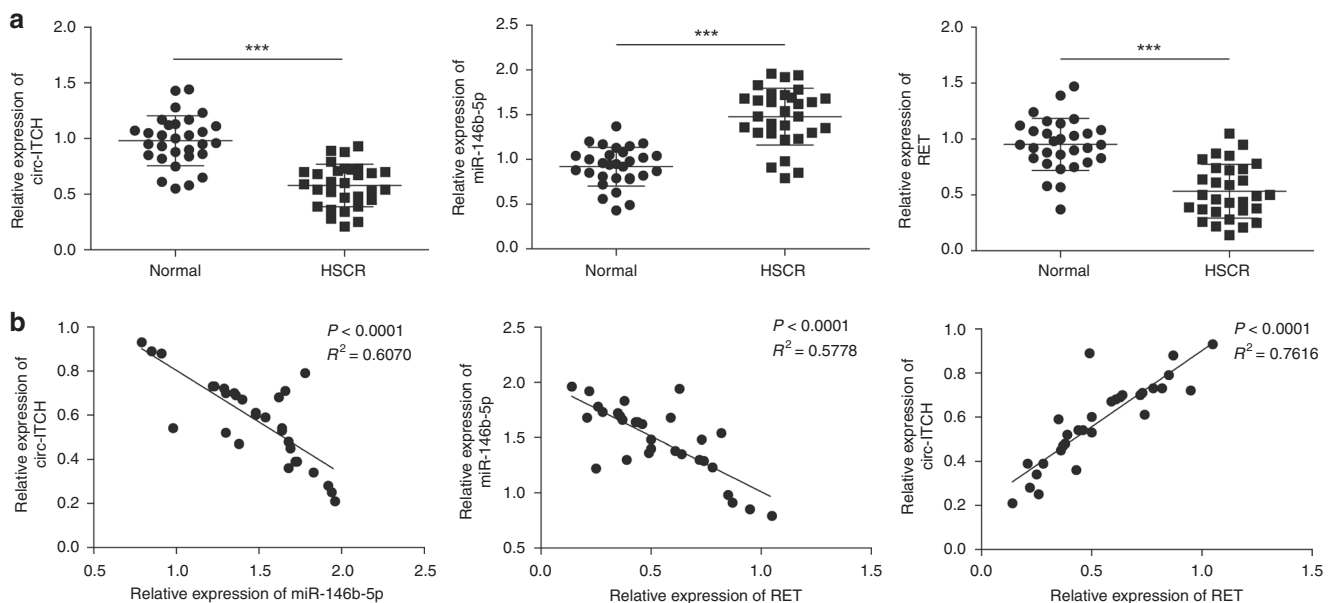
Table 1. Clinical and demographic characteristics.

Clinical characteristics	Control (n = 30)	HSCR (n = 30)	P value
Age (years)	2.45 ± 0.58	2.62 ± 0.72	0.318
Sex			
Male	14	20	0.192
Female	16	10	
BMI	22.43 ± 5.78	20.63 ± 5.49	0.221
Type			–
Short segment	–	15	
Long segment	–	10	
Full colon	–	5	
Family genetic			–
Existent	–	16	
Non-existent	–	14	

Note: *HSCR* Hirschsprung disease, *BMI* body mass index.

Fig. 1 Circ-ITCH and RET were downregulated in HSCR patients and miR-146b-5p was upregulated. a The expressions of Circ-ITCH, miR-146b-5p, and RET were detected using qRT-PCR. **b** Pearson correlation analysis between circ-ITCH expression, miR-146b-5p expression, and RET expression. The measurement data were presented as mean ± SD. $n = 30$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
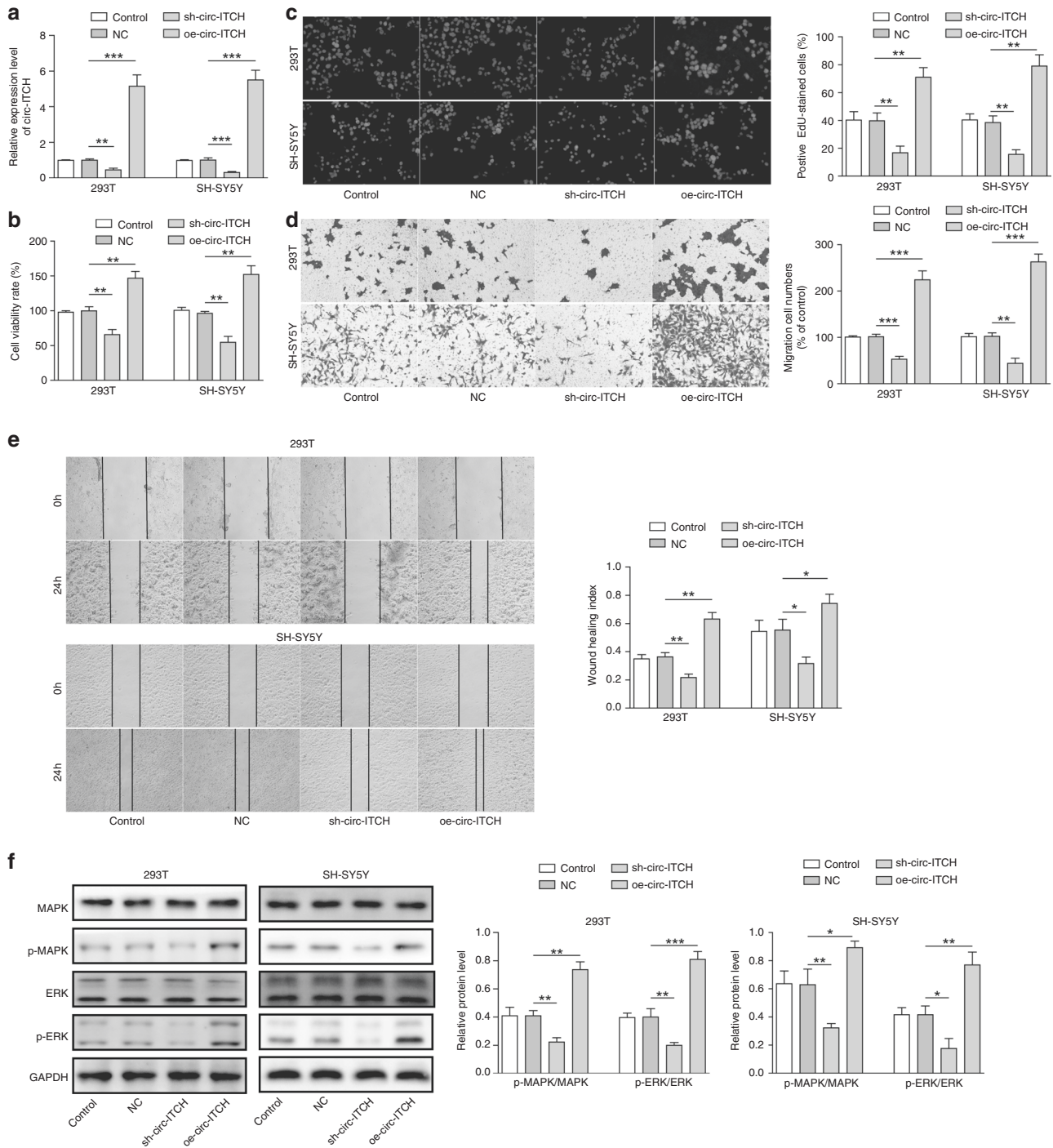
Fig. 2 Circ-ITCH overexpression promoted cell proliferation and migration of 293T and SH-SY5Y cells. The 293T and SH-SY5Y cells were transfected with sh-circ-ITCH or oe-circ-ITCH. **a** Circ-ITCH expression was determined using qRT-PCR. **b, c** Cell proliferation was assessed by CCK-8 assay and EdU assay. **d, e** Transwell migration assay and wound-healing assay were performed to determine cell migration. **f** MAPK, p-MAPK, ERK, and p-ERK levels were assessed using western blot. The measurement data were presented as mean ± SD. All of the tests in this study were conducted for three times. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(Thermo Fisher Scientific, Waltham, MA). Cell viability was analyzed and the cell viability of control group was normalized to 100%.

5-Ethynyl-2′-deoxyuridine (EdU) assay
Cells were plated into six-well plates (5×10^4/well) and cultured for 24 h. Then cells were incubated with 10 μM EdU (Beyotime, Shanghai, China) for 2 h. Subsequently, cells were fixed with 4% paraformaldehyde (Sigma-

Aldrich, Saint louis, MO) for 30 min. Cells were stained with click additive solution 30 min and then stained with DAPI for 5 min. The images were taken with a microscope (Olympus, Tokyo, Japan).

Wound-healing assay
Cells (5×10^5/well) were plated into six-well plates and starved for 24 h. Wounds were created by passing a plastic tip across the monolayered

a

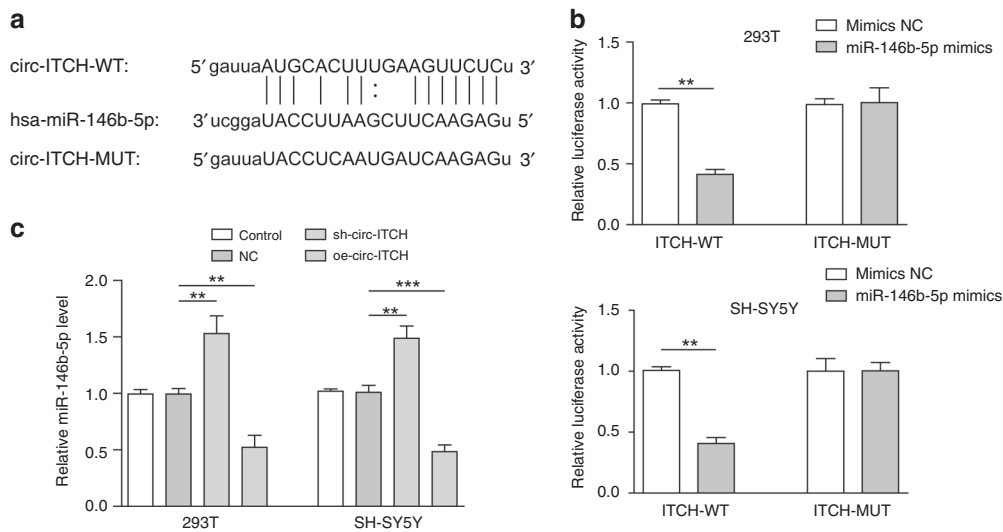| circ-ITCH-WT: | 5′ gauuaAUGCACUUUGAAGUUCUCu 3′ |
| hsa-miR-146b-5p: | 3′ ucggaUACCUUAAGCUUCAAGAGu 5′ |
| circ-ITCH-MUT: | 5′ gauuaUACCUCAAUGAUCAAGAGu 3′ |

Fig. 3 **Circ-ITCH targeted miR-146b-5p and negatively regulated its expression. a** The putative binding site between circ-ITCH and miR-146b-5p was predicted by Starbase. **b** Dual luciferase assay was employed to verify the binding relationship between circ-ITCH and miR-146b-5p. **c** MiR-146b-5p expression was evaluated using qRT-PCR. The measurement data were presented as mean ± SD. All of the tests in this study were conducted for three times. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

cells. Cells were cultured, and the images were taken at 0 and 24 h with a microscope (Olympus). Cell migration distance was analyzed by the software Image J (National Institutes of Health, Bethesda, MD).

Transwell migration assay
Cells (1×10^4) were suspended in serum-free medium. Then, cells were cultured in the upper compartment, and 600 μL complete medium was placed in the lower compartment. After 24 h, the migration cells on the membrane bottom surface were stained with hematoxylin (Sigma-Aldrich) for 30 min. Cells were photographed with a microscope (Olympus). The numbers of migrating cells were counted by the software Image J (National Institutes of Health) and the numbers of control group were normalized to 100%.

Dual luciferase reporter gene assay
The binding sites of target gene were predicted by online website Starbase (http://starbase.sysu.edu.cn/). Wild-type (WT) reporter plasmids of ITCH 3′-UTR sequences containing miR-146b-5p-binding sites were cloned into PGL3 vector (GenePharma) to construct ITCH-WT vectors. Site-directed mutagenesis of the miR-146b-5p-binding site in the fragment of circ-ITCH was performed using a site-directed mutagenesis kit (Stratagene, San Francisco, CA) to construct ITCH-MUT vectors. Then, Lipofectamine™ 3000 (Invitrogen) was used to co-transfect the corresponding plasmids, miR-146b-5p mimics, and mimics NC into cells. The luciferase activity was evaluated using the dual luciferase assay system (Promega, Madison, WI) after 24 h. The same method was used to verify the binding relationship between miR-146b-5p and RET.

Quantitative real-time polymerase chain reaction (qRT-PCR)
Total RNA of cells and tissues was extracted by using TRIzol reagent (Thermo Fisher Scientific). RNA was reverse transcribed into cDNA with HiFiScript cDNA synthesis kit (Life Technologies, Carlsbad, CA). Then, an Ultra SYBR Mixture kit (Thermo Fisher Scientific) was employed for qRT-PCR assay. GAPDH was used as the reference gene for mRNA, and U6 was served as the miRNA reference. Data were analyzed with the $2^{-\Delta\Delta CT}$ method. The primers were displayed as follows (5′–3′):
Circ-ITCH (F): CCTTGAGCAAGAAGACTATGCCAAT;
Circ-ITCH (R): CCGCATTCTGTGGTAAGCAATCA;
miR-146b-5p (F): GATGAGAAGGTATTTCTGCT;
miR-146b-5p (R): GAGAAATTGAAGGTCATAAA;
RET (F): GATGGCACTAACACTGGG;
RET (R): ACCTGGGAACTGAACACG;
GAPDH (F): GGAGCGAGATCCCTCCAAAAT;
GAPDH (R): GGCTGTTGTCATACTTCTCATGG;
U6 (F): CTCGCTTCGGCAGCACA;
U6 (R): AACGCTTCACGAATTTGCGT.

Western blot
The proteins from cells were extracted with RIPA (Thermo Fisher Scientific), and the concentrations of protein were quantified by a BCA Kit (Beyotime). Then, proteins were transferred to a PVDF membrane (Millipore, Boston, MA). Subsequently, membranes were incubated with primary antibodies including MAPK (Abcam, 1:1000, ab205926), p-MAPK (Abcam, 1:1000, ab74032), RET (Abcam, 1:1000, ab134100), extracellular regulated protein kinase (ERK) (Abcam, 1:1000, ab53277), p-ERK (Cell Signaling Technology, 1: 1000, #4370), and GAPDH antibody (Sigma-Aldrich, 1:10,000, SAB2701826). Then, membranes were incubated with the corresponding secondary antibodies (Abcam, 1:10,000, ab7090, ab97035) after washed with PBST three times. The membranes were visualized and imaged by GEL imaging system (Bio-Rad, Hercules, CA). The quantification of proteins was analyzed with the software Image J (National Institutes of Health).

Statistical analysis
SPSS 19.0 (IBM, Armonk, NY) software package was applied for statistical analysis and the measurement data were expressed as means ± standards deviation (SD). The differences among two groups were analyzed by Student's t-tests. Analysis of variance (ANOVA) followed by Turkey's post-test was employed to evaluate the differences among multiple groups. $P < 0.05$ was considered as significant difference. All the tests conducted in this work were repeated at least three times.

RESULTS
Circ-ITCH and RET were downregulated in HSCR patients and miR-146b-5p was upregulated
To probe whether circ-ITCH was involved in the incidence of HSCR, circ-ITCH expression in 30 HSCR patients was examined. We observed that there was no significant difference between HSCR cases and normal controls in the clinical information, such as age, body mass index (BMI), and sex (Table 1). Results of qRT-PCR displayed that circ-ITCH and RET expressions markedly decreased in HSCR colon tissues compared with control colon tissues, while miR-146b-5p expression remarkably increased ($P < 0.05$; Fig. 1a). Morrover, circ-ITCH expression was negatively correlated with miR-146b-5p expression, miR-146b-5p expression was negatively correlated with RET expression, and circ-ITCH expression was positively correlated with RET expression ($P < 0.01$; Fig. 1b). In summary, circ-ITCH, miR-146b-5p, and RET participated in regulating the progression of HSCR.
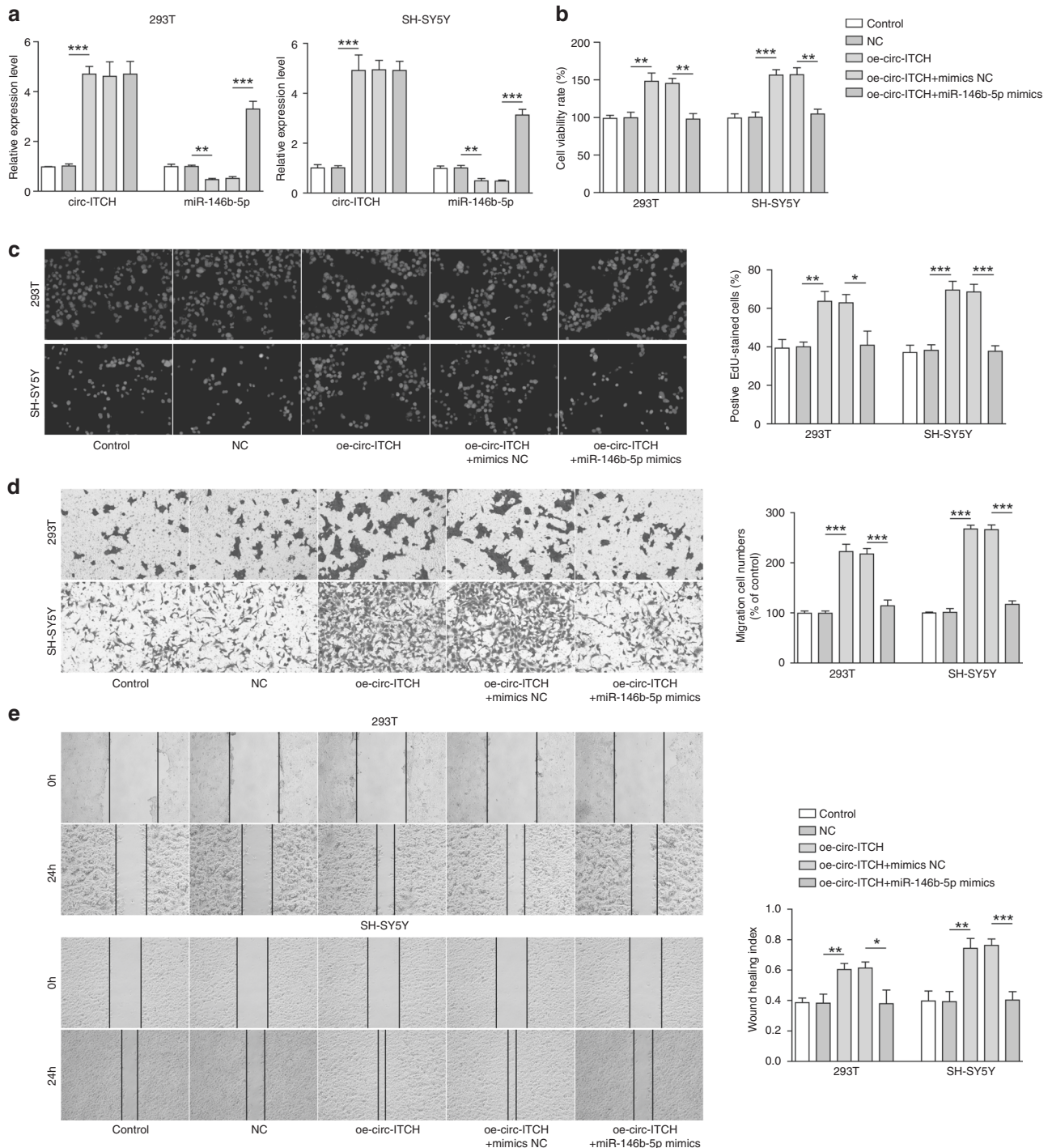
Fig. 4 Circ-ITCH regulated cell proliferation and migration of 293T and SH-SY5Y cells via targeting miR-146b-5p. The 293T and SH-SY5Y cells were transfected with oe-circ-ITCH, co-transfected with oe-ITCH and mimics NC or co-transfected with oe-ITCH and miR-146b-5p mimics. **a** Circ-ITCH and miR-146b-5p expression was determined using qRT-PCR. **b**, **c** CCK-8 assay and EdU assay were employed to evaluate cell proliferation. **d**, **e** Cell migration was assessed by wound-healing assay and transwell migration assay. The measurement data were presented as mean ± SD. All of the tests in this study were conducted for three times. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Circ-ITCH overexpression could promote proliferation and migration of 293T and SH-SY5Y cells

To examine the function of circ-ITCH in regulating cell proliferation and migration in HSCR, we knock downed and overexpressed circ-ITCH in 293T and SH-SY5Y cells, respectively ($P < 0.05$; Fig. 2a). Circ-ITCH knockdown remarkably suppressed the proliferation and migration of 293T and SH-SY5Y cells, while circ-ITCH overexpression presented the opposite effects ($P < 0.05$; Fig. 2b–e). Previous study showed that the activation of MAPK signaling pathway could promote ENCC proliferation and migration.[18] Therefore, we further explored whether circ-ITCH could regulate the MAPK signaling pathway. Results demonstrated that the phosphorylation levels of MAPK and ERK were markedly reduced by circ-ITCH silencing, which were reversed by circ-ITCH overexpression ($P < 0.05$; Fig. 2f).
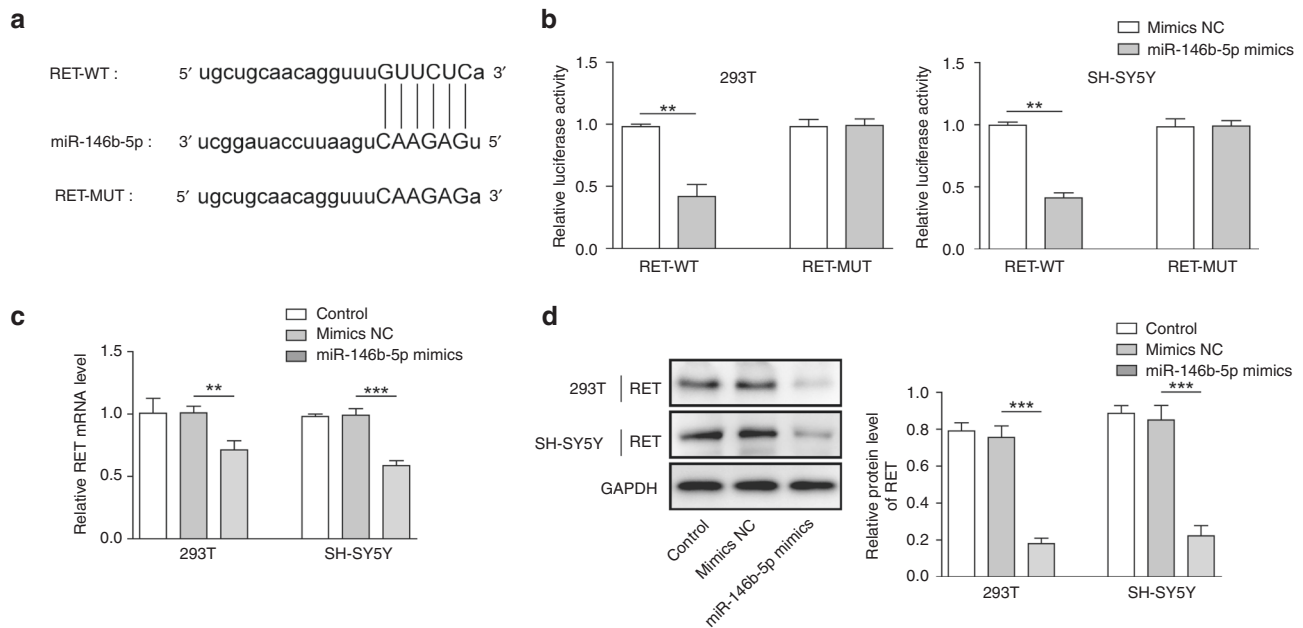
Fig. 5 MiR-146b-5p targeted RET and negatively regulated its expression. a The putative binding site between RET and miR-146b-5p was predicted by Starbase. **b** Dual luciferase assay was employed to verify the binding relationship between RET and miR-146b-5p. **c, d** RET expression in cells after miR-146b-5p overexpression was determined using qRT-PCR and western blot. The measurement data were presented as mean ± SD. All of the tests in this study were conducted for three times. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Collectively, our data demonstrated that circ-ITCH overexpression promoted cell proliferation and migration and activated MAPK signaling pathway.

Circ-ITCH targeted miR-146b-5p and negatively regulated its expression

Starbase predicted that there was the potential binding site between circ-ITCH and miR-146b-5p (Fig. 3a). Subsequently, the dual luciferase reporter gene assay demonstrated that circ-ITCH directly bound to miR-146b-5p ($P < 0.05$; Fig. 3b). Additionally, miR-146b-5p expression was markedly increased after circ-ITCH knockdown, while it was significantly decreased after circ-ITCH overexpression ($P < 0.05$; Fig. 3c). Taken together, circ-ITCH negatively regulated miR-146b-5p expression.

Circ-ITCH regulated proliferation and migration of 293T and SH-SY5Y cells via sponging miR-146b-5p

In order to explore whether circ-ITCH affected HSCR progression by sponging miR-146b-5p, cells were co-transfected with oe-ITCH and miR-146b-5p mimics. Circ-ITCH was significantly elevated and miR-146b-5p expression was reduced by circ-ITCH overexpression alone, while circ-ITCH expression remained unchanged and miR-146b-5p expression was increased after overexpression of circ-ITCH and miR-146b-5p at the same time ($P < 0.05$; Fig. 4a). Cell proliferative ability was enhanced by circ-ITCH overexpression, while it was abolished by miR-146b-5p overexpression ($P < 0.05$; Fig. 4b, c). In addition, cell migration numbers were increased after circ-ITCH overexpression, while miR-146b-5p mimics reversed the effect of circ-ITCH overexpression ($P < 0.05$; Fig. 4d, e). Moreover, co-transfection with oe-circ-ITCH and miR-146b-5p mimics resulted in the phosphorylation levels of MAPK and ERK were reduced in comparison to the oe-circ-ITCH group ($P < 0.05$; Supplemental Fig. 1). In summary, circ-ITCH overexpression could promote 293T and SH-SY5Y cell proliferation and migration via downregulating miR-145b-5p expression.

MiR-146b-5p targeted RET and negatively regulated its expression

To explore the underlying mechanism of miR-146b-5p in HSCR, we probed the downstream target of miR-146b-5p. We found that

miR-146b-5p had a binding site to RET using Starbase (Fig. 5a). The result from dual luciferase reporter gene assay displayed that miR-146b-5p directly bound to RET ($P < 0.05$; Fig. 5b). It was also observed that RET expression in 293T and SH-SY5Y cells was significantly reduced by miR-146b-5p overexpression ($P < 0.05$; Fig. 5c, d). In total, miR-146b-5p negatively regulated RET expression via directly binding to RET.

MiR-146b-5p regulated proliferation and migration of 293T and SH-SY5Y cells via targeting RET

To probe whether miR-146b-5p affected HSCR development by regulating RET expression, we performed a rescue experiment: miR-146b-5p mimics + oe-NC group and miR-146b-5p mimics + oe-RET group. MiR-146b-5p overexpression alone resulted in elevated miR-146b-5p expression and reduced RET expression, while the unchanged miR-146b-5p expression and increased RET expression were observed in miR-146b-5p mimics + oe-RET group ($P < 0.05$; Fig. 6a, b). After overexpression miR-146b-5p, cell proliferation of 293T and SH-SY5Y cells was obviously suppressed, while it was promoted by RET overexpression ($P < 0.05$; Fig. 6c, d). Furthermore, co-transfection with miR-146b-5p mimics and oe-RET promoted cell migration compared with the miR-146b-5p mimics ($P < 0.05$; Fig. 6e, f). Subsequently, the phosphorylation levels of MAPK and ERK were decreased following miR-146b-5p mimics transfection, which was eliminated by co-transfection with miR-146b-5p mimics and oe-RET ($P < 0.05$; Supplemental Fig. 2). In summary, miR-146b-5p overexpression inhibited cell proliferation and migration of 293T and SH-SY5Y cells via inhibiting RET expression.

DISCUSSION

HSCR is a kind of congenital digestive tract disease of newborns, which is characterized by insufficient proliferation and migration of ENCC during 5–12 weeks of embryogenesis.[19] HSCR is a multigenic disease. As reported, many coding genes and non-coding coding genes participate in regulating HSCR development.[20] Herein, we studied the mechanisms of circ-ITCH in HSCR.

Owing to the difficulty of using ENCC, neuroblastoma cell lines (such as SH-SY5Y cells, SKN-SH, etc.) and 293T cells were
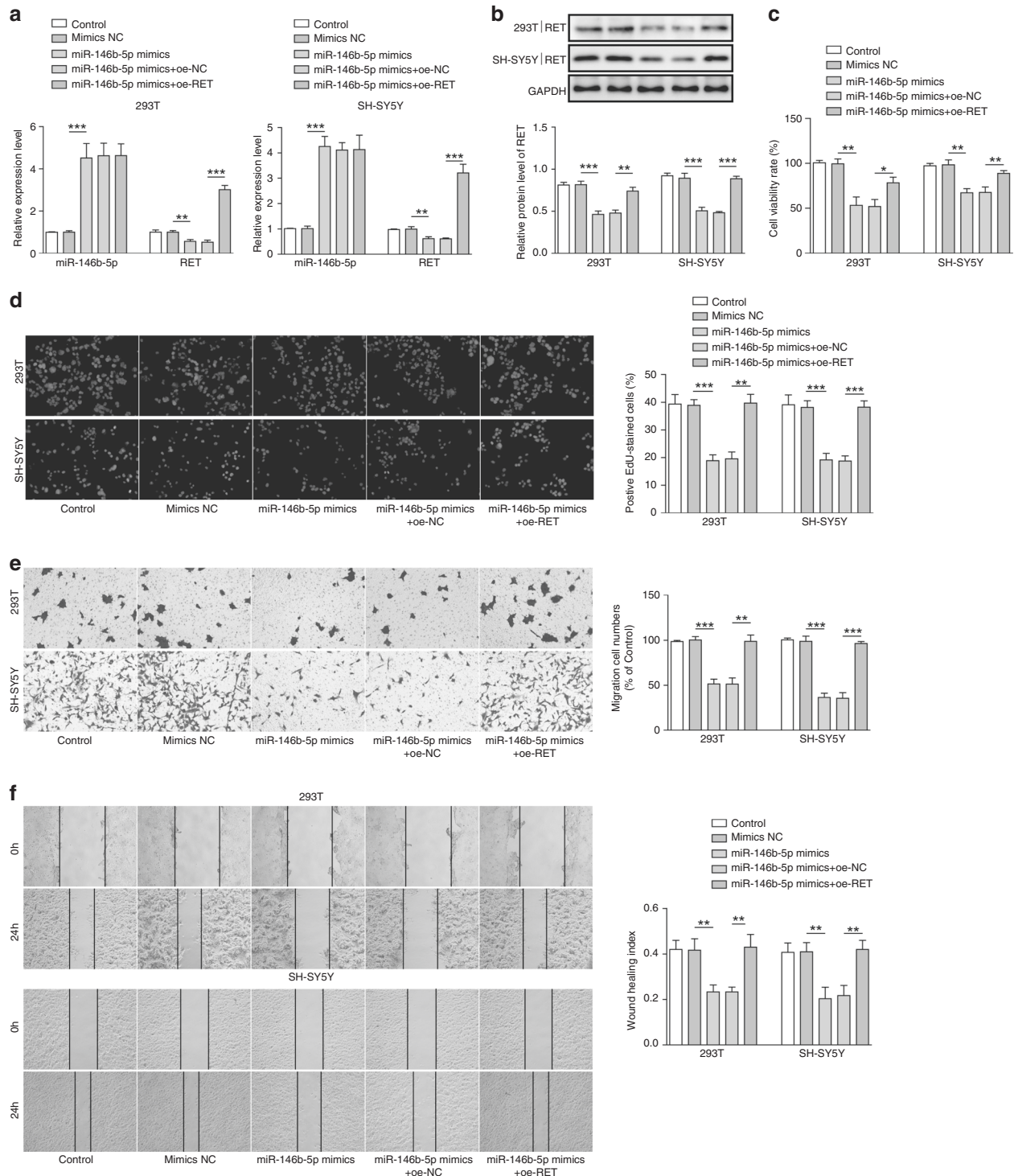
Fig. 6 MiR-146b-5p regulated cell proliferation and migration of 293T and SH-SY5Y cells via targeting RET. The 293T and SH-SY5Y cells were transfected with oe-RET or co-transfected with oe-RET and mimics NC or co-transfected with oe-RET and miR-146b-5p mimics. **a**, **b** qRT-PCR and western blot were used to evaluate the expressions of miR-146b-5p and RET. **c**, **d** Cell proliferation was assessed by CCK-8 assay and EdU assay. **e**, **f** Cell migration was determined using transwell migration assay and wound-healing assay. The measurement data were presented as mean ± SD. All of the tests in this study were conducted for three times. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

commonly used cell models in the study of HSCR.[21–23] In addition, considering that HSCR is characterized by the lack of ganglion cells at the distal end of the digestive tract,[24] we speculate that the relevant primary cells cannot be isolated from HSCR tissues, and there are currently no relevant literature reports on the isolation of primary cells from HSCR tissues. Therefore, herein, SH-SY5Y and 293T cells instead of primary cells were employed to study the pathogenesis of HSCR.

Growing evidence has revealed that circRNAs are dysregulated in HSCR and play key roles in HSCR progression.[7,13] CircRNA-ZNF609 expression was remarkably reduced in HSCR, and circ-ZNF609 knockdown dramatically suppressed cell migration and

proliferation of SH-SY5Y and 293T cells.[7] Herein, circ-ITCH expression was found to be obviously reduced in HSCR tissues, while whether the mechanisms of circ-ITCH in HSCR was never reported before. Therefore, we performed this project to study the mechanisms of circ-ITCH in HSCR. Our results also confirmed that circ-ITCH overexpression could remarkably enhance proliferative and migratory abilities of 293T and SH-SY5Y cells, suggesting that circ-ITCH might have an inhibitory effect on HSCR progression.

Accumulated evidence has shown that circRNAs function as competing endogenous RNAs (ceRNAs) to regulate the expressions of miRNAs, thus affecting the development of many diseases, including HSCR.[8] Therefore, we hypothesized that circ-ITCH regulated HSCR development through regulation of miR-146b-5p expression as a ceRNA. Our results showed that circ-ITCH negatively regulated miR-146b-5p expression. So far, few studies have focused on the function of miR-146b-5p in HSCR. Only one previous study showed that miR-146b-5p expression was elevated in HSCR.[14] However, the specific mechanisms of miR-146b-5p regulating HSCR development remain unknown. Herein, miR-146b-5p expression was markedly elevated in HSCR, and miR-146b-5p overexpression abolished the promoting effects of oe-circ-ITCH on cell migration and proliferation. In conclusion, circ-ITCH could promote cell migration and proliferation in HSCR via downregulating miR-146b-5p, which was reported for the first time.

As we all known, miRNAs play important roles in a series of diseases via directly binding to the 3′-UTR of target genes.[25] Herein, miR-146b-5p was predicted to have a binding site to RET using bioinformatics software. RET was previously considered as an identifiable genetic risk factor in HSCR.[26] It was reported that RET was a key gene to regulate development of ENCC in HSCR.[1] Besides more than 48.4% of HSCR patients had structural or regulatory defects in the RET.[27] However, the specific mechanism of RET in regulating HSCR development remained unknown. Our results suggested that RET was markedly downregulated in HSCR tissues, and dual luciferase reporter assay verified that miR-146b-5p directly suppressed RET expression, which was further confirmed in miR-146b-5p-overexpressing cells. Moreover, RET overexpression eliminated the inhibitory effects of miR-146b-5p mimics on cell proliferative and migratory abilities. Therefore, miR-146b-5p suppressed cell migration and proliferation in HSCR by inhibited RET expression. Recently, RET gene mutation is a research hotspot in the study of HSCR. As previously described, mutation of RET resulted in HSCR.[28] Ohgami also revealed that loss-of-function mutations of RET was associated with HSCR in humans.[29] Our research were involved in the expression and regulation mechanism of RET in HSCR, while it has not yet involved the detection of RET gene mutations in HSCR patients. The specific content needs to be verified after further genetic detection in the future. In addition, this issue has aroused our great interest and provided us with a reference direction for in-depth research.

In total, our work proved that circ-ITCH overexpression promoted cell migration and proliferation in HSCR by activating RET/MAPK axis via suppressing miR-146b-5p expression. Our research clarified the underlying mechanism of circ-ITCH in regulating cell proliferative and migratory abilities in HSCR, and it laid the foundation for future research on the mechanisms of HSCR occurrence and development.

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AUTHOR CONTRIBUTIONS

R.-P.X.: conceptualization, methodology, writing—original draft preparation; F.Z.: visualization; T.-D.M.: investigation; C.-J.Z.: software and validation; G.X.: writing—original draft preparation, writing—reviewing and editing; C.-G.Z.: supervision, writing— reviewing and editing; all the authors approved for the final version.

COMPETING INTERESTS
The authors declare no competing interests.

CONSENT STATEMENT
All participants gave written informed consent and the experiment was approved by the Institutional Ethics Committee of Hunan Children's Hospital.

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