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Original Article

SOX14 promotes proliferation and invasion of cervical cancer cells through Wnt/β-catenin pathway

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Abstract: SOX14 is a member of the SOXB2 subgroup of transcription factors implicated in neural development. Although SOX14 expression profile and function during development was revealed in various animal model systems, the role of this gene during tumor progression is totally unknown. In this study, the expression of SOX14 increases in four cervical cancer cell lines (HeLa, Caski, HT-3 and SiHa) as revealed by real-time PCR and Western blot analyses. Through knocking down or overexpressing SOX14 in SiHa and HeLa cells, the expression level of SOX14 was found to be positively related to cell proliferation and invasion *in vitro*. Moreover, the TOP-Flash reporter assay and Western blot for β-catenin genes of the Wnt/β-catenin pathway, indicated that SOX14 significantly activated Wnt/β-catenin signaling. Further study showed that the blockage of Wnt/β-catenin pathway by knocking down β-catenin resulted in a significant inhibition of cell proliferation and invasion capacity induced by SOX14. To summarize, these results demonstrate that SOX14 can promote proliferation and invasion capacity of cervical cancer cells by activating the Wnt/β-catenin pathway.

Keywords: SOX14, Wnt/β-catenin pathway, cervical cancer, proliferation, invasion

Introduction

Cervical cancer is the fourth leading cause of death in females worldwide and the second leading cause of mortality among women aged 19-39 years [1], Up to 35% of patients with locally advanced cervical cancer previously treated with surgery or radiation will develop persistent/recurrent/metastatic disease, where platinum-based chemotherapy still represents the gold standard treatment [2]. Abnormal expression of embryonic transcription factors has been linked to cancer development and progression. Inappropriate expression of these transcription factors is thought to reinstitute developmental programs out of context, contributing to tumor initiation and metastasis [3-5].

Wnt/β-catenin signaling pathway plays a decisive role such as morphogenesis, differentiation, and proliferation in the regulation of development [6, 7]. Wnt pathway proteins are a group of evolutionarily conserved intracellular signaling molecules that regulate the cellular fate and are implicated in the tumorigenesis of multiple types of cancers when aberrant activated, such as lung cancer, breast cancer, liver cancer, colon cancer and cervical cancer [8, 9].

Sox genes, a gene family encoding transcription factors involved in a variety of development processes, are found throughout the animal kingdom [10, 11]. The SOX14/Sox14 gene has been identified in many vertebrate species, including human, mouse, chicken, platypus and fish, To date, no SOX14/Sox14 mutations associated with human genetic disorders or animal phenotypes have been described [12]. There is a limited number of studies in various model systems, mostly focused on Sox14 expression during neural development previously, we have cloned and characterized human SOX14 gene and determined its promoter and regulatory elements involved in transcriptional regulation of its expression. Jelena Popovic *et. al*. have also identified transcription factors NF-Y and Foxa2 as positive regulators of SOX14 expression and proposed that the Sonic hedgehog signaling pathway involved in up-regulation of

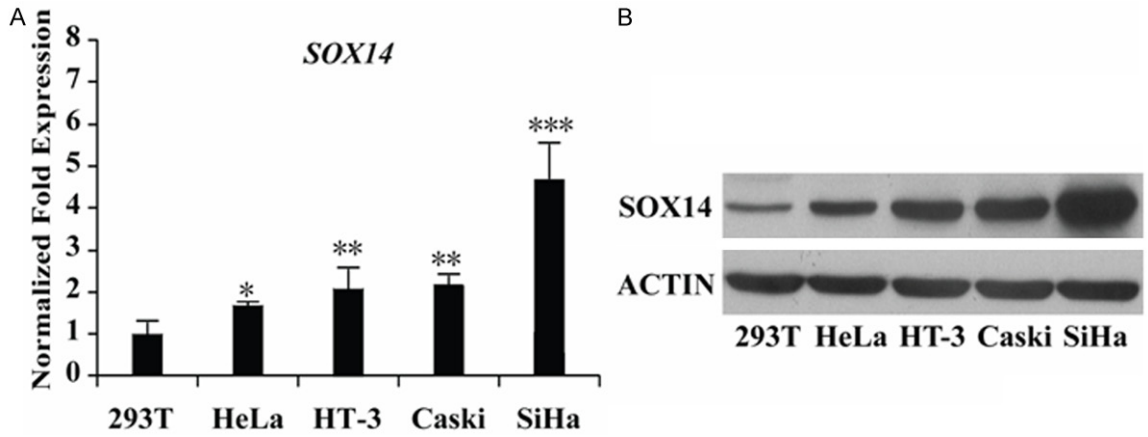

Figure 1. Expression of SOX14 is higher in cervical cancer cell lines than 293T cells. A. Expression of SOX14 at mRNA level. B. Western blot showed expression of SOX14. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

SOX14 expression might be, at least in part, mediated by FOXA2 [13]. It's well known that sonic hedgehog signaling pathway plays critical roles in the development of cervical cancer [14, 15], recently published paper showed ectopic SOX14 expression downregulates SOX1 in HeLa cells [16]. But whether SOX14 was expressed aberrantly during the development of cervical cancer and whether SOX14 functioned in this process are still totally unknown.

In this study, we studied the expression of SOX14 in cervical cancer cell lines. We demonstrate that SOX14 increases in four cervical cancer cell lines (HeLa, Caski, HT-3 and SiHa) as revealed by realtime PCR and western blot analyses. Then we further examined the function of SOX14 in cervical cancer. Through knocking down or overexpressing SOX14 in SiHa and HeLa cells, the expression level of SOX14 was found to be positively related to cell proliferation and invasion in vitro. Moreover, the TOP-Flash reporter assay and Western blot for β-catenin genes of the Wnt/β-catenin pathway, indicated that SOX14 significantly activated Wnt/β-catenin signaling. Further study showed that the blockage of Wnt/β-catenin pathway by knocking down β-catenin resulted in a significant inhibition of cell proliferation and invasion capacity induced by SOX14.

Materials and methods

Plasmid construction

The complete SOX14 coding sequence was amplified by PCR from cDNA of SiHa cells, using primers 5'-gatcGCTAGCatgtccaaaccttcagaccac-3' (forward) and 5'-gatcGCGGCCGCttacatggccgtagcgtgg-3' (reverse). The PCR product was purified and cloned into pCDH lentivirus vector using NheI/NotI. The selected clone was fully sequenced in order to verify that no mutations were introduced by PCR. The SOX14 RNAi sequences are: 5'-ggaaacttgcaaacgttatgt-3'. The CTNNB1 RNAi sequences are: 5'-GGTATTTGAAGTATACCATAC-3'. Scramble sequences are: 5'-CAAGATGAAGAGCACCAAA-3'.

Real-time RT-PCR

Quantitive RT-PCR analysis was used to determine the relative expression level of SOX14 in different cervical cancer cells. Total RNA was extracted from cells using Trizol (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized by using Reverse Transcription Kit (Fermentas). The expression of SOX14 was detected by RT-Real Time PCR (BioRad). Primer sequences are: 5'-TACGTGGTGCCCTGTAACTG-3' (forward) and 5'-GGGTCTATGCCAGTCTTGGT-3' (reverse). Each sample in each group was measured in triplicate and the experiment was repeated at least three times.

Cell culture

HeLa cell lines were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. Caski cell lines were cultured in RPMI1640 containing 10% fetal bovine serum (Hyclone,

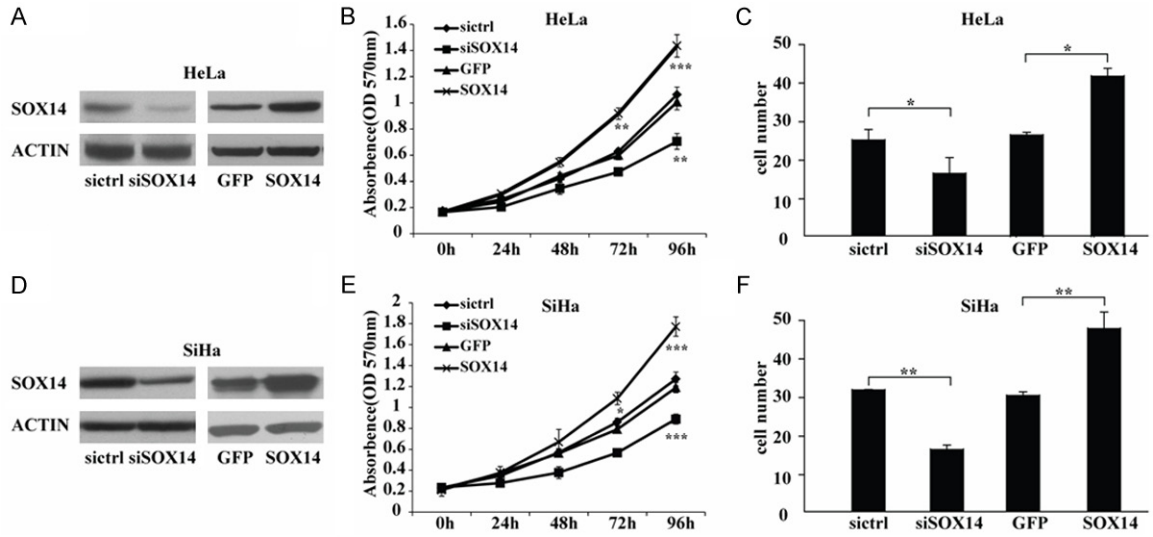

Figure 2. SOX14 promotes proliferation and invasion of cervical cancer cells in vitro. A. Western blot assay was used to characterize the expression of SOX14 in SOX14-knockdown and -overexpressing HeLa cells. B. The cell growth curve of HeLa cells is shown. C. The number of HeLa cells passed through the tranwell membrane is shown. D. Western blot assay was used to characterize the expression of SOX14 in SOX14-knockdown and -overexpressing SiHa cells. E. The cell growth curve of SiHa cells is shown. F. The number of SiHa cells passed through the tranwell membrane is shown. Data are expressed as the mean numbers of independent triplicate experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. SiHa cell lines were cultured in Eagle's Minimum Essential Medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. HT-3 cell lines were cultured in McCoy's 5a Medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO_2.

Western blotting

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 30 μg samples were loaded into each lane of 12% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto nitrocellulose membranes (PALL) by electrophoretic transfer. The membrane was incubated with rabbit polyclonal antibodies against-SOX14 (Abcam, Cambridge, UK, ab49047, diluted 1:400), Rabbit monoclonal [E247] to beta-Catenin (Abcam, Cambridge, UK, ab32572), mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 16 h at 4°C. The specific protein-antibody complex was detected by using horseradish peroxidase conjugated rabbit anti-mouse or rabbit anti-mouse IgG. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control.

Cell proliferation assay

Cells were transfected with siRNA or were infected with virus overexpressing SOX14, respectively. 48 h after transfection or infection, cells were seeded in 24-well plates at low density (2×10^4), and allowed to attach overnight. Then cells were cultured for indicated times. Twenty microliters MTT (5 mg/ml) (Sigma, St. Louis, MO, USA) were added into each well at 0, 24, 48, 72 and 96 h, and the cells were incubated for further 4 h. The absorbance was recorded at A570 nm with a 96-well plate reader after the DMSO addition.

In vitro invasion assay

Cells were transfected with siRNA or were infected with virus expressing SOX14, respectively. For invasion assays, the cells (2×10^5 cells/well) were seeded in the top of an 8.0-mm-pore membrane chamber (Corning Costar

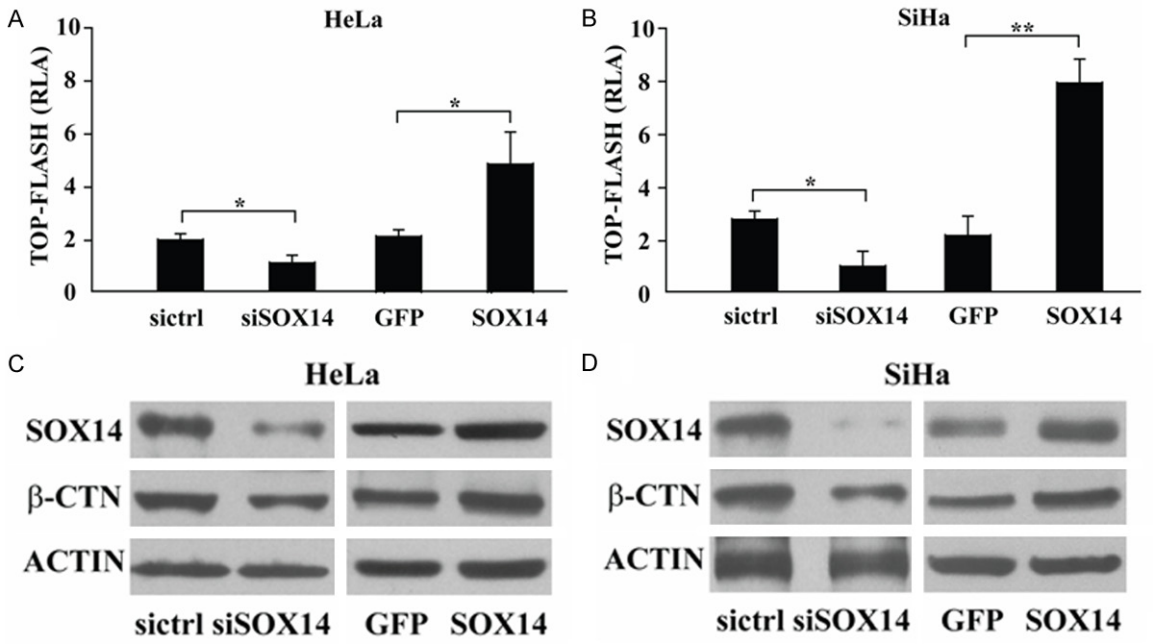

Figure 3. SOX14 enhances the activity of the Wnt/β-catenin pathway. A, B. SOX14-modulated HeLa and SiHa cells were transfected with the TOP-Flash reporter plasmid, and the reporter activities were determined 48 h after transfection by a luciferase assay. SOX14 significantly increased the reporter activity in HeLa and SiHa cells. C, D. The expression of β-catenin (β-CTN) in SOX14-modulated HeLa and SiHa cells was measured by westernblot assay. Data are expressed as the mean numbers of independent triplicate experiments. **P* < 0.05, ***P* < 0.01.

Corp., Cambridge, MA, USA). Following a 6 h incubation period, cells that passed through the membrane to attach to the bottom of membrane were fixed with 4% PFA and stained with crystal violet. Cells were scraped and removed from the top of chamber using soft cotton swab. Cells on the bottom of membrane were counted. The cell invasion was quantified by counting the amount of cells passing through the pores from five different fields per sample at 100× selected in a random manner.

Statistical analysis

Data were analyzed by using SPSS Statistical Package version 16. Independent two group's analyses are used t-test. $P < 0.05$ was considered statistically significant.

Results

SOX14 expression is upregulated in cervical cancer cell lines

To understand whether SOX14 is involved in cervical carcinogenesis, the expression level of SOX14 in cervical cancer was examined, we cultured four human cervical cancer cell lines: HeLa, Caski, HT-3 and SiHa, isolated RNA and reverse-transcripted into cDNA. Then we performed real-time PCR assay. The results showed that, compared with non-malignant cell line 293T, the mRNA expression of SOX14 in all cell lines: HeLa, HT-3, Caski and SiHa are upregulated, SiHa cells have highest expression of SOX14 among these four cell lines (**Figure 1A**). We also collected cell lysates of these cells and performed Western blot. Consistent with realtime PCR data, the expression of SOX14 are higher in HeLa, HT-3, Caski and SiHa cell lines than 293T cell (**Figure 1B**). And SiHa cells have the highest expression of SOX14, so these data showed that SOX14 is upregulated at both mRNA level and protein level in most cell lines of cervical cancer and indicated that SOX14 may function as an oncogene involved in the progression of cervical cancer.

SOX14 promotes proliferation and invasion of cervical cancer cells in vitro

To assess the function of SOX14 in cervical cancer, we firstly studied whether SOX14 plays a role in the proliferation of cervical cancer, stable SOX14-knockdown cekks (HeLa-shSOX14 and SiHa-shSOX14) and stable SOX14-

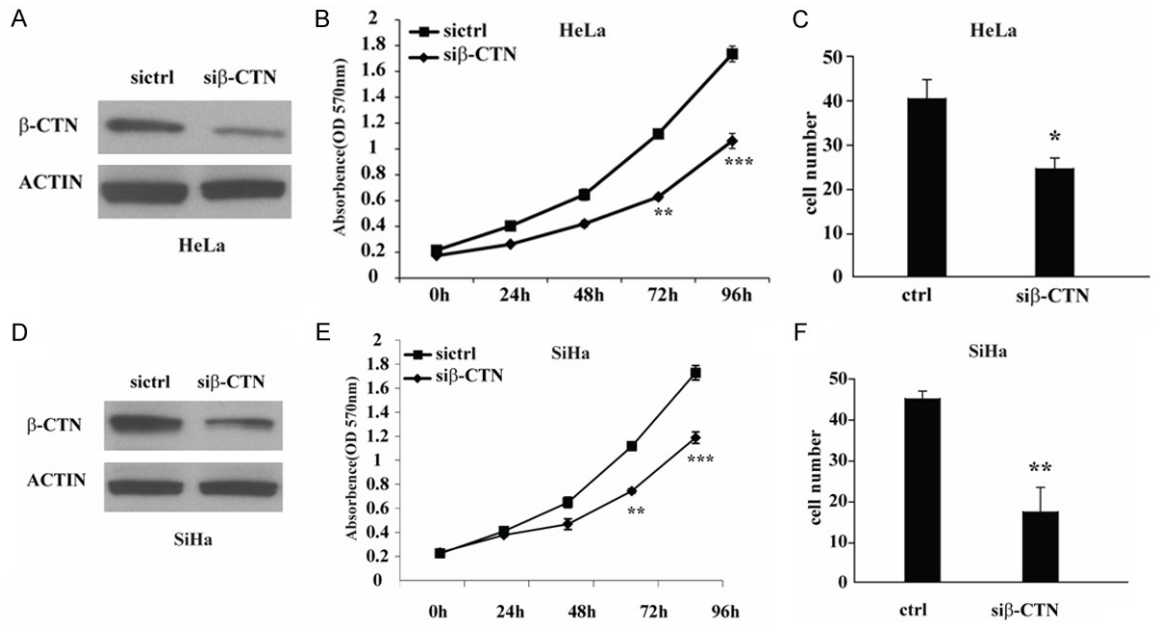

Figure 4. β-catenin knockdown attenuates the increasing proliferation of cervical cancer cells induced by SOX14. A, D. The expression of β-catenin (β-CTN) were shown by Western blot. B, E. The effect of β-catenin on the proliferation of SOX14-overexpressing HeLa and SiHa cells was evaluated by MTT assay. C, F. The effect of β-catenin on the invasion capacity of SOX14-overexpressing HeLa and SiHa cells was measured by transwell assay. Data are expressed as the mean numbers of independent triplicate experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

overexpressing cells (HeLa-SOX14 and SiHa-SOX14) were established by shRNA and plasmid transfection. The expression of SOX14 in the knocked down and overexpressing HeLa and SiHa cells by westernblot Western blot analysis are shown (**Figure 2A**, **2D**). MTT assay was performed *in vitro*. The MTT results showed that SOX14 knockdown in both HeLa and SiHa cells resulted in a significant growth inhibition; However, SOX14 overexpression markedly promoted cell growth in both HeLa (**Figure 2B**) cells and SiHa (**Figure 2E**) cells. These results demonstrate that SOX14 can promote the proliferation of cervical cancer cells.

We studied further the role of SOX14 in controlling the invasion of cervical cancer, The function of SOX14 on the migratory behavior was examined by transwell assay *in vitro*. The results showed that SOX14 knockdown in both HeLa and SiHa cells resulted in a significant downregulation of invasion capacity. However, SOX14 overexpression markedly promoted cell invasion capacity in both HeLa (**Figure 2C**) and SiHa (**Figure 2F**) cells. These results demonstrate that SOX14 can promote the invasion capacity of cervical cancer cells.

SOX14 regulates the Wnt/β-catenin pathway in cervical cancer progression

It has been reported that Wnt/β-catenin signaling regulates cell proliferation and invasion in cervical cancer [8, 9]. However, there are no reports identifying whether SOX14 is able to enhance the proliferation and invasion of cervical cancer cells by activating Wnt/β-catenin signaling.

To demonstrate the correlation of SOX14 and Wnt/β-catenin signaling pathway, the TOP-Flash reporter assay was used to detect the activity of Wnt/β-catenin signaling in cervical cancer cell lines (**Figure 3A**, **3B**) in which SOX14 was over-expressed or knocked down. The results showed that SOX14 knockdown resulted in a significant inhibition of TOP-Flash reporter activity in both HeLa and SiHa cells, whereas SOX14 over-expression significantly increased the TOP-Flash reporter activity by 3- or 4-fold compared with the control (**Figure 3A**, **3B**). All these results indicate that SOX14 expression is positively related to the activity of the Wnt/β-catenin pathway in cervical cancer cells.

β-catenin is a crucial signaling molecule in Wnt/β-catenin pathway. Therefore, the expression of β-catenin was measured in SOX14-down-regulated and -overexpressing HeLa and SiHa cells. Western blot results showed that the expression of β-catenin in SOX14-knocked down cells was significantly decreased compared with the control cells. In contrast, the expression of these proteins in SOX14-overexpressing cells was significantly increased compared with the control cells (**Figure 3C**, **3D**). These results demonstrate that SOX14 expression is positively associated with the expression of key molecule of Wnt/β-catenin pathway in cervical cancer cells.

To further confirm that SOX14 promotes the proliferation and invasion capacity of cervical cancer cells by Wnt/β-catenin pathway, we designed siRNA that targets β-catenin and transciently transfected into both HeLa and SiHa cells. Western blot result showed about 90% decrease of expression of β-catenin (**Figure 4A**, **4D**). Then we performed MTT and transwell assay. The result showed that deletion of β-catenin resulted in a significant inhibition in cell proliferation (**Figure 4B**, **4E**) and invasion (**Figure 4C**, **4F**) in the SOX14-overexpressing HeLa and SiHa cells, indicating that β-catenin can arrest the cell proliferation and invasion induced by SOX14.

Taken together, these results demonstrate that the SOX14-mediated progression of cervical cancer is mediated by Wnt/β-catenin pathway, so SOX14 can be a target to cervical cancer treatment.

Discussion

Recent evidence has shown that altered patterns of transcription factor expression correlate with various human diseases especially many kinds of cancers. The behavior of transcription factors are complex because they regulate hundreds of targets, resulting in the down regulation of numerous target genes including oncogenes and tumor suppressors. Therefore, exploring their clinical potential is especially worthwhile.

In this study, we first detected the expression of SOX14 in four cell lines of cervical cancer by qRT-PCR and Western blot. We found that the expression of SOX14 was highly up-regulated in most cervical cancer cell lines compared with non-tumorigenic 293T. Further we studied the biological function of SOX14 in the progression of cervical cancer. Through shRNA knockdown or stable plasmid transfection, the SOX14 protein level was found to be positively related to the proliferation and invasion of cervical cancer cells. We noticed that Wnt/β-catenin signaling pathway is very important in the tumorigenesis and metastasis of cervical cancer. In this study, we demonstrated that SOX14 significantly enhanced the activity of the TOP-Flash reporter and increased the expression of target gene of the Wnt/β-catenin pathway in HeLa and SiHa cells. However, it remains inconclusive whether SOX14 promotes cell proliferation and invasion by activating the Wnt/β-catenin pathway. To answer this question, we found that knocking down of β-catenin resulted in a significant inhibition of the cervical cancer cell proliferation and invasion induced by SOX14, suggesting that SOX14 promotes cervical cancer cell proliferation and invasion via activation of the Wnt/β-catenin pathway.

In summary, our data indicated that SOX14 may function as an oncogene by activiating Wnt/β-catenin signaling pathway to regulate proliferation and invasion during cervical cancer progression. However, whether SOX14 directly interacts with the signaling pathway of proliferation and invasion is still unknown. More importantly, whether SOX14 plays the critical role *in vivo* is still unclear. This research may give insight into understanding of cervical cancer development and create an opportunity to approach the diagnosis and treatment of cervical cancer.

Disclosure of conflict of interest

None.

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