

## Original Article

# Transcription factor KLF4 regulates microRNA-544 that targets *YWHAZ* in cervical cancer

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**Abstract:** The deregulation of microRNAs has been demonstrated in various tumor processes. Here, we report that microRNA-544 (miR-544) is decreased in cervical cancer tissues compared with normal cervical tissues. To identify the mechanisms involved in miR-544 deregulation, we studied the regulation of miR-544 expression at the transcriptional level. We first identified the transcriptional start site of miR-544 by 5' rapid amplification of cDNA ends and subsequently determined the miR-544 promoter. We discovered that the transcription factor Krueppel-like factor 4 (KLF4) is involved in the transcriptional regulation of miR-544 through interaction with the miR-544 promoter. In addition, we found that miR-544 directly targets the *YWHAZ* oncogene and functions as a tumor suppressor in cervical cancer cells. miR-544 is involved in cell cycle regulation and suppresses cervical cancer cell proliferation, colony formation, migration and invasion in a manner associated with *YWHAZ* downregulation. In summary, our findings demonstrate that KLF4 upregulates miR-544 transcription by activating the miR-544 promoter and that miR-544 functions as a tumor suppressor by targeting *YWHAZ*. Therefore, miR-544 may be a potential novel therapeutic target and prognostic marker for cervical cancer.

**Keywords:** microRNA-544, *YWHAZ*, KLF4, cervical cancer

## Introduction

microRNAs (miRNAs) are small, non-coding RNAs that modulate gene expression post-transcriptionally either by inhibiting translation or by causing mRNA degradation through binding to the 3'untranslated regions (3'UTRs) of target mRNAs [1, 2]. This interaction leads to the reduced protein expression of miRNA-targeted genes. It has been speculated that miRNAs regulate approximately one-third of all protein-coding genes [3]. Thus, up or downregulation of miRNAs can influence the expression of tumor suppressor genes and oncogenes. Large-scale miRNA expression profiling experiments have been performed in numerous different cancers and have identified a large number of deregulated miRNAs. Recent studies have implicated miRNAs in a wide variety of vital developmental and physiological processes including cell proliferation, differentiation, apoptosis, and organ development [4]. Therefore, it is important to study both the function and regulation of miR-

NAs. However, most studies have focused on the regulatory functions of miRNAs, and few are directed towards how miRNA genes are themselves transcriptionally regulated. Therefore, the underlying mechanisms of aberrant miRNA regulation are still poorly understood.

Cervical cancer is the second most common cancer in women worldwide, with an estimate global incidence of 529,800 new cases and 275,100 deaths per year [5, 6]. Persistent infection of high-risk human papillomavirus (HPV) has been considered as one reason for cervical carcinogenesis [7, 8]. The high-risk HPV genome integrates into the host genome and activates the viral oncogenes E6 and E7, leading to malignant transformation of host cells. A large number of aberrantly expressed miRNAs have been identified in cervical cancer and are closely associated with cervical cancer progression [9-11].

In our study, we found that miR-544 is reduced in cervical cancer, while its target gene *YWHAZ*

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**Table 1.** Primer sequences used in this study

Primer name	primer sequences
RT-miR-544	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACgaactt
RT-RNU6B	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAATAT
qRT-miR-544-F	gccgcgATTCTGCATTTTTAGC
qRT-RNU6B-F	TTCCTCCGCAAGGATGACACGC
qRT-miRNA-R	GTGCAGGGTCCGAGGT
YWHAZ 3'UTR-1-F-XhoI	ccgctcgagGGATACCCAAGGAGACGA
YWHAZ 3'UTR-1-R-NotI	ataagaatgcggccgcCTCAAGTTATTTCCCTGTTA
YWHAZ 3'UTR-2-F-XhoI	ccgctcgagTGACATTGGGTAGCATTA
YWHAZ 3'UTR-2-R-NotI	ataagaatgcggccgcGTTTGGGATTTCAAGTTCT
1YWHAZ 3'UTR-mut-F1	TTGACTACAGCTTGGCCGAGTGTTCCCTTA
1YWHAZ 3'UTR-mut-R1	TAAAGGAACACTCGGCAAGCTGTAGTCAA
1YWHAZ 3'UTR-del-F1	ACCTTTGACTACAGCGTTCCTTTAGAC
1YWHAZ 3'UTR-del-R1	GTCTAAAGGAACACGCTGTAGTCAAAGGT
1YWHAZ 3'UTR-mut-F2	CTCTGGATAAGGGAGCCAAACGTTTACATT
1YWHAZ 3'UTR-mut-R2	AATGTGAACCGTGGCTCCCTTATCCAGAG
1YWHAZ 3'UTR-del-F2	TTACTCTGGATAAGGACGGTTCACATTC
1YWHAZ 3'UTR-del-R2	GAATGTGAACCGTCCCTTATCCAGAGTAA
2YWHAZ 3'UTR-mut-F	CCTCAGTACTTTAGCCGAAACACCAAACA
2YWHAZ 3'UTR-mut-R	TGTTTGGTGTTCGGCTAAAGTACTGAGG
2YWHAZ 3'UTR-del-F	TTCTCAGTACTTAACACCAAACA
2YWHAZ 3'UTR-del-R	TGTTTGGTGTAAAGTACTGAGGAA
qRT-YWHAZ-F	CCTCACTCCCGTTTCCG
qRT-YWHAZ-R	CAGCACCTCCGTCTTT
qRT-GAPDH-F	ATGGGGAAGGTGAAGGTCTG
qRT-GAPDH-R	CTGGAAGATGGTGATGGGA
miR-544 5'RACE outer	ACACAAAACGGATACACACGGTCA
miR-544 5'RACE inner	TGGGTCCAAGACAAGGTTTGATAC
miR-544 promoter (-255)-F-XhoI	ccgctcgagGGCTCTGTGAGATGGATGA
miR-544 promoter (+15)-F-XhoI	ccgctcgagAGGCTGATTCTGGTGAAGG
miR-544 promoter (+256)-F-XhoI	ccgctcgagTTCGGTTTATTGACATGGA
miR-544 promoter (+764)-R-HindIII	cccaagcttAAAGGTTAGCACGGAAAAG
miR-544 promoter (+918)-R-HindIII	cccaagcttCAAGGTTGGACGGTTAGTA
YWHAZ-F-EcoRI	ccggaattcCCACCATGGATAAAAATGAGCTGG
YWHAZ-R-KpnI	cggggtaccTTAATTTCCCTCCTTCTCC
KLF4-F-XhoI	ccgctcgagCCACCATGAGGCAGCCACCTGG
KLF4-R-HindIII	cccaagcttTAAAAATGCCTCTTCATGTGT
qRT-KLF4-F	GCCAAAGAGGGGAAGACGAT
qRT-KLF4-R	CGCAGGTGTGCCTTGAGATG
ChIP qRT-F	ACCTGCCAGTGCTATTGTTT
ChIP qRT-R	CAAAACGGATACACACGGTCA
KLF4-miR-544-mut-F1	ACTCAGCAAGAAGCACGGTATGTGT
KLF4-miR-544-mut-R1	ACACATACCGTGGCTTCTTGCTGAGT
KLF4-miR-544-mut-F2	ATGACTTTAGAAGGAATGATGGCCTCTGG
KLF4-miR-544-mut-R2	CCAGAGGCCATCATTCCTTCTAAAGTCAT
KLF4-miR-544-mut-F3	GCCTCTGGGATATTCCTCTCCTGG
KLF4-miR-544-mut-R3	CCAGGAGAGGAATAATCCAGAGGC
KLF4-miR-544-mut-F4	GGTGCAGTGTAAATAAGGCCAAAAGC
KLF4-miR-544-mut-R4	GCTTTTGGCCTTATTTTACACTGCACC

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KLF4-miR-544-mut-F5	TGGGTCCTGTAATTTGAGCGGGAA
KLF4-miR-544-mut-R5	TTCCCGCTCAAATTACAGGGACCCA
KLF4-miR-544-mut-F6	TTCCTGAGCGAATTATCATCAGCCC
KLF4-miR-544-mut-R6	GGGCTGATGATAATTCGCTCAGGAA
KLF4-miR-544-mut-F7	CGATGGCTGGAACAACGCTCGTGTG
KLF4-miR-544-mut-R7	CACACGAGCGTTGTTCCAGCCATCG
KLF4-miR-544-mut-F8	GCATCTAATTTGCTTCCAGGACCGC
KLF4-miR-544-mut-R8	GCGGTCCTGGAAGCAAATTAGATGC
KLF4-miR-544-mut-F9	CGTACCAAGTTGTTCAAGGCAGTG
KLF4-miR-544-mut-R9	CACTGCCTTGAACAAACTTGGTACG

Mutant sequences were underlined.

is increased. In addition, we identified the miR-544 transcription start site (TSS) by 5' rapid amplification of cDNA ends (5'RACE) and found the miR-544 promoter sequence. We demonstrate that the transcription factor KLF4 is involved in the transcriptional regulation of miR-544 through interaction with the miR-544 promoter region. Furthermore, we revealed that miR-544 is involved in cell cycle regulation and suppresses cervical cancer cell proliferation, colony formation, migration and invasion. Our study provides comprehensive understanding of how a transcription factor regulates miRNAs and suggests a novel therapeutic strategy for YWHAZ-induced tumorigenesis in cervical cancer.

### Materials and methods

#### *Cell culture, transfection, tumor specimens and reagents*

The human cervical cancer cell lines CaSki and HeLa (ATCC, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) with 10% fetal bovine serum at 37°C with 5% CO<sub>2</sub>. The miR-544 mimic, inhibitor and negative control (NC) were synthesized by Ambion. The siRNA against YWHAZ (siRNA-YWHAZ) and negative control (siRNA NC) were synthesized by Genepharma (Shanghai, China) [12]. The sequences were as follows: siRNA-YWHAZ, 5'-AAAGUUCUUGAUCCCCAAUGC-3' and siRNA NC, 5'-CAGUCGCGUUUGCGACUGG-3'. Transfection was performed using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Twenty pairs of human cervical cancer tissues and matched adjacent noncancerous cervical tissues were collected from Shanghai Changhai Hospital (Shanghai, China). All specimens were obtained from informed patients, and the patients provided written consent. The study was approved by the Ethics

Committee of Fudan University. Polyclonal antibodies use for western blot analysis were anti-beta-tubulin (Abmart, M20005, 1:2000, China), anti-YWHAZ (Santa Cruz, sc-1019, 1:100, USA), HRP anti-rabbit (KPL, 4751-1516, 1:2000, USA) and HRP anti-mouse (KPL, 0751-1809, 1:2000, USA).

#### *RNA isolation and quantitative real-time PCR (qRT-PCR)*

Total RNA was extracted using TRIzol reagent (Ambion, USA). The cDNA was synthesized using the PrimeScript™ RT reagent Kit (TaKaRa, China) according to the manufacturer's instructions. The qRT-PCR was performed using SYBR Premix Ex Taq (TaKaRa) and analyzed using the LightCycler 480 II Real Time PCR system (Roche, Germany). The primers used are shown in **Table 1**. RNU6B and GAPDH were used as internal controls for mature miR-544 and mRNA expression, respectively.

#### *5' rapid amplification of cDNA ends (5'RACE)*

The TSS of the primary transcript for human miR-544 (from HeLa cells) was determined using a 5'-Full RACE kit (TaKaRa) according to the manufacturer's instructions. HL60 total RNA, which was provided in the kit and served as a positive control, was used to analyze the 5' end of human *Prohibitin* (PHB) gene (PCR product, 750 bp). The primary transcripts were determined by nested PCR using outer and inner primers (**Table 1**). The PCR products were separated by agarose gel electrophoresis, purified and sequenced.

#### *Dual-luciferase reporter assays and transcription factor analysis*

For the YWHAZ 3'UTR reporter assay, the wild type (WT) 3'UTR of YWHAZ, mutant type (Mut) and deletion type (Del) including putative miR-

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544 binding sites were cloned into the psi-CHECK-2 luciferase reporter vector (Promega, USA). HeLa cells were cultured in 96-well plates and co-transfected with the miRNA molecules (miR-544 mimic and NC, 30 nM) and 50 ng of the luciferase reporter constructs. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System on a GloMax 96 Microplate Luminometer (Promega). The results are expressed as a normalized ratio of Renilla to firefly luciferase.

For the miR-544 promoter activity assay, the genomic sequences near the miR-544 TSS were amplified and cloned into the pGL3-Basic reporter vector (Promega). HeLa cells were co-transfected with the pGL3-Basic derived reporter vectors and Renilla luciferase plasmid, pRL-TK and pCDNA-KLF4. The cells were lysed at 48 h after transfection, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System on a GloMax 96 Microplate Luminometer (Promega). The activities of firefly luciferase (PGL3-Basic) were normalized to Renilla luciferase (pRL-TK) activities.

### *Chromatin immunoprecipitation (ChIP) assays*

ChIP assays were performed using the EZ-Magna ChIP One-Day Chromatin Immunoprecipitation Kit (Millipore, USA) according to the instruction manual. Approximately  $1 \times 10^7$  HeLa cells were cross-linked with 1% formaldehyde for 10 min, and glycine was used to quench unreacted formaldehyde. Cells were then washed with ice-cold PBS and lysed with cell lysis buffer and then nuclear lysis buffer containing Protease Inhibitor Cocktail II. Chromatin was sheared by sonication to an average size of 200 to 1000 bp. The supernatant was collected in 50- $\mu$ l aliquots. Each 50- $\mu$ l aliquot (containing  $1 \times 10^6$  cell equivalents of chromatin) was diluted in 450  $\mu$ l of dilution buffer. "Input" samples [5  $\mu$ l (1%) of the supernatant] were removed and stored at 4°C. The remaining supernatant was incubated for 4 h with 5  $\mu$ g of anti-KLF4 (RD, AF3640, USA) or normal IgG (negative control) immunoprecipitating antibody and 20  $\mu$ l of protein A magnetic beads. Finally, the immunoprecipitate complexes were collected, washed, eluted, and the cross-links were reversed using proteinase K. DNA was purified using spin columns and analyzed by qRT-PCR.

### *Cell proliferation and colony formation assays*

For cell proliferation assays, CaSki and HeLa cells were plated in 96-well plates at 3000 cells per well and transfected with the indicated miRNA mimics (30 nM) and plasmids (100 ng) and maintained in culture media. After 24 h, the cells were incubated with 20  $\mu$ l MTT (0.5 mg/ml) at 37°C for 4 h. Then, the medium was removed, and 100  $\mu$ l of DMSO was added to dissolve the precipitated formazan. The absorbance at 490 nm was read using a plate reader (BioTek, USA). For colony formation assays, 200 cells were plated in 24-well plates and incubated for 10 days. The colonies were fixed with methanol and stained with 0.1% crystal violet. Colonies containing more than 50 cells were counted.

### *Migration and invasion assays*

*In vitro* migration and invasion assays were performed using 8- $\mu$ m pore size 24-well transwell chambers (Corning, USA). For the migration assays,  $5 \times 10^4$  cells were resuspended in 0.1 ml of serum-free DMEM and added to the transwell inserts. For the invasion assays, the transwell inserts were coated with 150  $\mu$ g of Matrigel (BD, USA), and  $1 \times 10^5$  cells in serum-free DMEM were added to the inserts. DMEM with 10% FBS was added to the bottom wells. After incubation for 20 h, the cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed with methanol and stained with 0.1% crystal violet. The migrated or invaded cells were counted using an  $1 \times 51$  inverted microscope (Olympus, Japan).

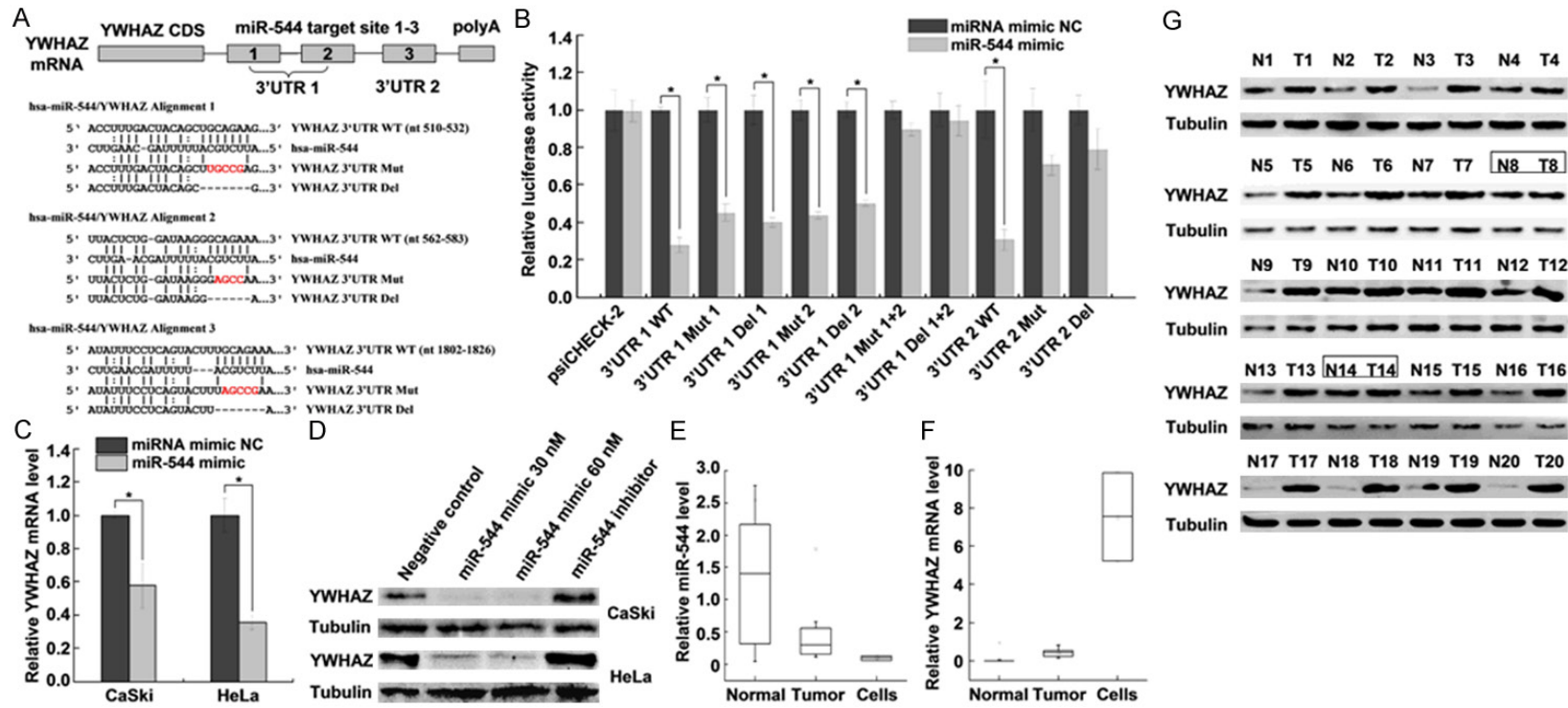
### *Flow cytometry assays*

For cell cycle analysis, cells were collected and fixed in ice-cold 75% ethanol overnight. The fixed cells were washed and resuspended in propidium iodide staining solution containing 50  $\mu$ g/ml propidium iodide, 50  $\mu$ g/ml ribonuclease A, and 0.2% Triton X-100 in phosphate-buffered saline for 30 min in the dark. The cells were then analyzed using a FACSCalibur Flow Cytometer (BD, USA). The data were analyzed using the Modfit software.

### *Statistical analyses*

The results are representative of three independent experiments and presented as the mean  $\pm$  standard deviation (SD). The data were analyzed using Student's t-test to evaluate sig-

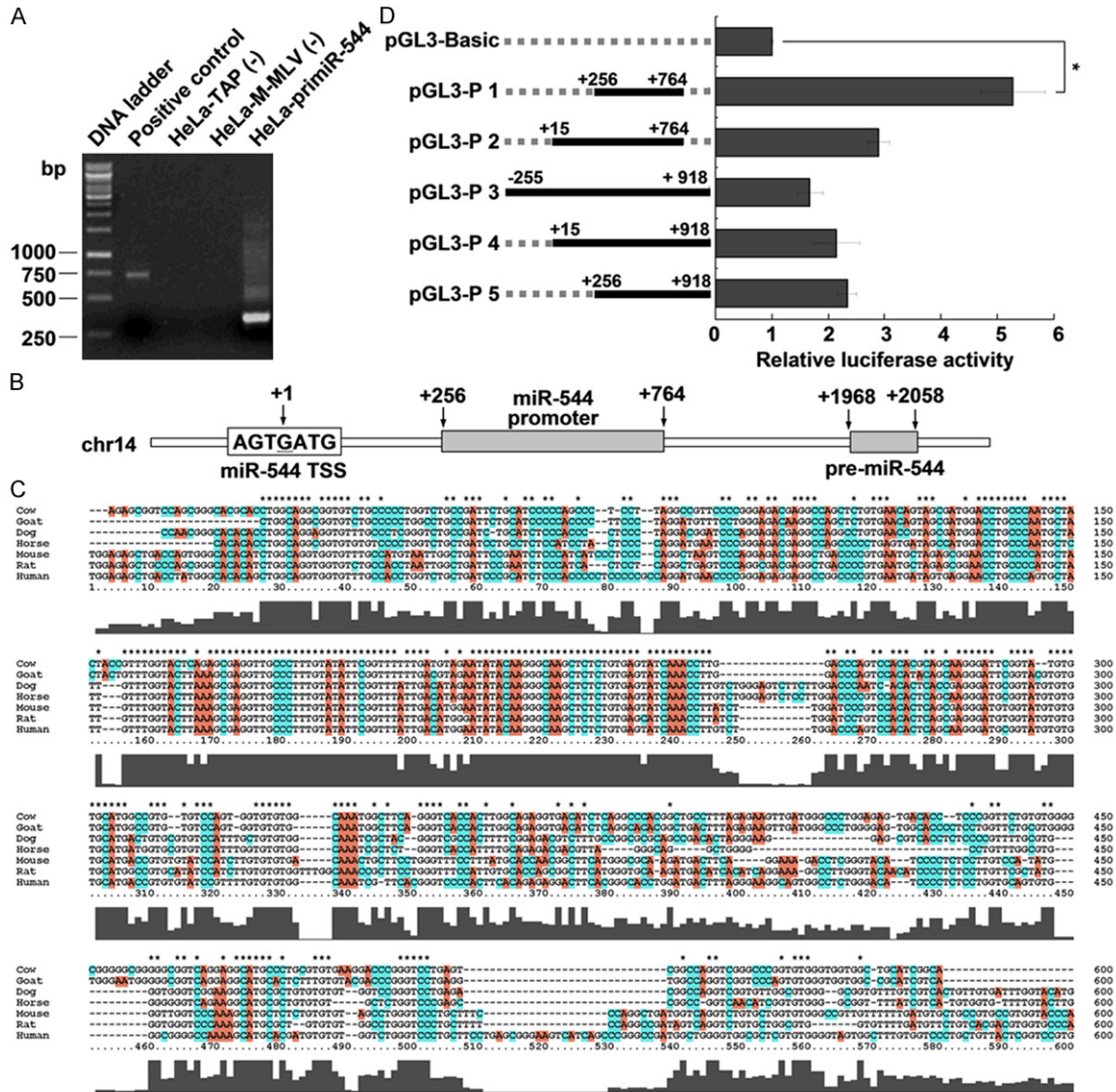
# KLF4 regulate microRNA-544 that targets YWHAZ



**Figure 1.** miR-544 downregulates *YWHAZ* expression by binding to the *YWHAZ* 3'UTR. **A.** Predicted binding sites of miR-544 to the 3'UTR of *YWHAZ*. The wild type (WT), mutated (Mut), and deleted (Del) *YWHAZ* 3'UTR binding sites are shown. The mutant nucleotides of the miR-544 binding sites are indicated in red. **B.** Relative luciferase activity of the *YWHAZ* WT, Mut, and Del reporter in the presence of 30 nM miR-544 mimic or miRNA mimic NC. Renilla luciferase was normalized to firefly luciferase. **C.** qRT-PCR analysis of *YWHAZ* mRNA from CaSki and HeLa cells transfected with miR-544 mimic (30 nM) or miRNA mimic NC (30 nM). GAPDH mRNA was used as an internal control. **D.** Western blot analysis of *YWHAZ* expression in CaSki and HeLa cells transfected with miR-544 mimic, inhibitor or NC. Tubulin was used as a loading control. **E.** qRT-PCR analysis of mature miR-544 expression in 20 paired tumor tissues (Tumor) and adjacent normal cervical tissues (Normal) and cervical cancer cell lines (CaSki and HeLa). RNU6B was used as an internal control. **F.** qRT-PCR analysis of *YWHAZ* mRNA expression in 20 paired tumor tissues (Tumor) and adjacent normal cervical tissues (Normal) and cervical cancer cell lines (CaSki and HeLa). GAPDH mRNA was used as an internal control. **G.** Western blot analysis of *YWHAZ* protein levels in 20 paired tumor tissues (T) and adjacent normal cervical tissues (N). Tubulin was used as a loading control. Two paired samples showing no differences in *YWHAZ* expression are labeled with boxes. The data represent the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ .



# KLF4 regulate microRNA-544 that targets YWHAZ



**Figure 2.** Identification of the miR-544 promoter. A. Agarose gel electrophoresis shows the PCR products of 5'RACE. B. Schematic of the miR-544 promoter location in the genome. The transcription start site of miR-544 is indicated as +1 and the number indicates the relative distance from the miR-544 transcription start site. C. Alignment of the miR-544 promoter sequence between different species. D. Relative luciferase activity of the miR-544 promoter. Luciferase constructs containing the miR-544 promoter (pGL3-P 1 to P 5) and the unmodified construct (pRL-TK) were co-transfected into HeLa cells. Firefly luciferase activity was normalized to Renilla luciferase activity. Data represent the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ .

nificant differences. A  $P$  value  $< 0.05$  was considered significant.

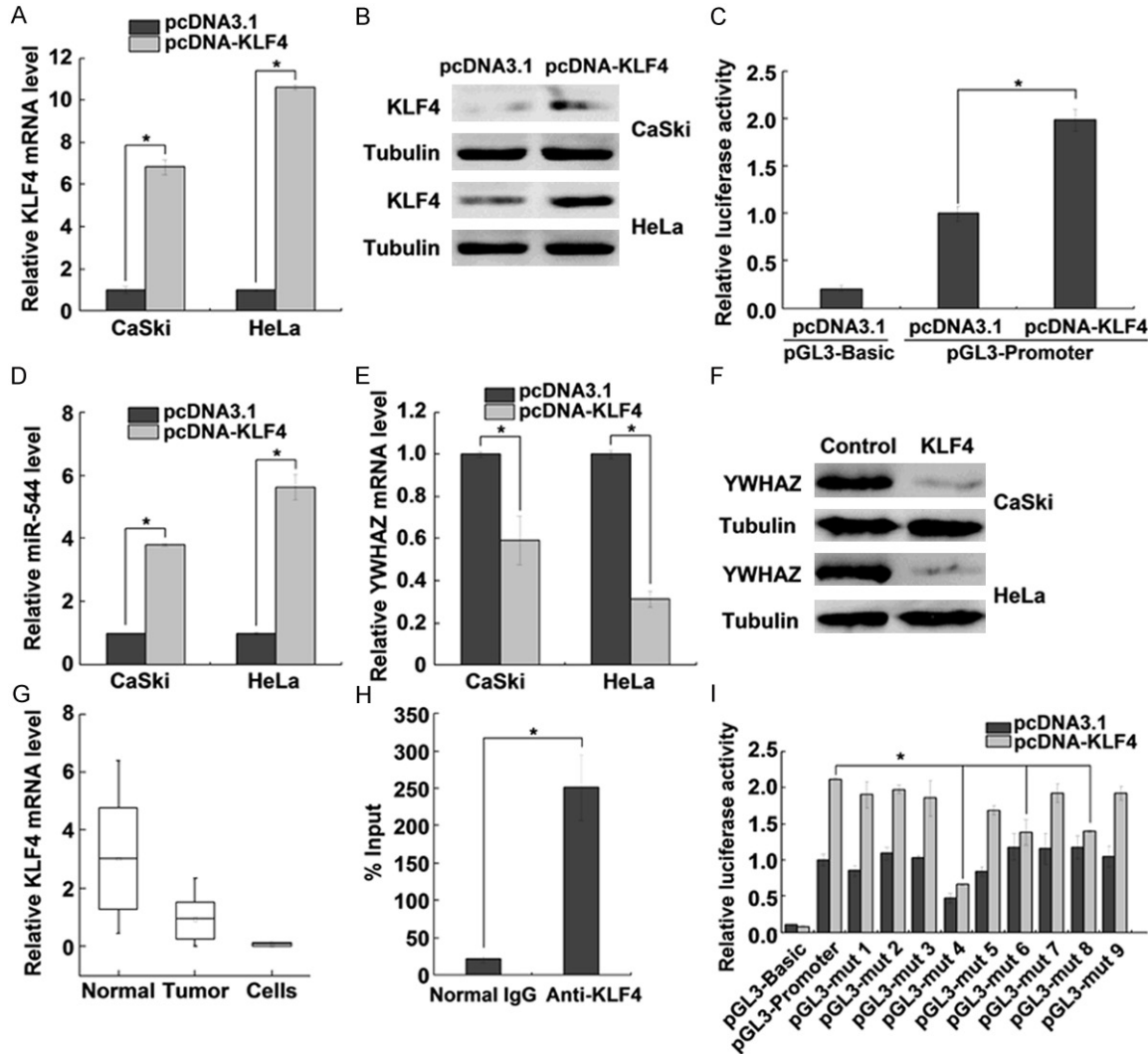
## Results

### miR-544 suppresses YWHAZ expression by targeting the 3'UTR of YWHAZ

Various algorithms (TargetScan, miRanda, and miRecords) were used to predict the potential target genes of miR-544. The results suggest-

ed that there are three predicted miR-544 binding sites in the YWHAZ 3'UTR (Figure 1A). To validate this prediction, we cloned the YWHAZ 3'UTR containing the miR-544 target site (wild type, mutant or deletion) into the luciferase reporter vector psiCHECK-2. The predicted binding sites 1 and 2 were cloned together downstream of the luciferase reporter gene (Figure 1A). For the luciferase reporter assays, the luciferase reporter constructs were co-

## KLF4 regulate microRNA-544 that targets YWHAZ



**Figure 3.** KLF4 promotes miR-544 transcription by activating miR-544 promoter. A and B. qRT-PCR and western blot analysis of KLF4 expression in CaSki and HeLa cells transfected with pcDNA-KLF4. C. Relative luciferase activity of the miR-544 promoter in HeLa cells transfected with pcDNA-KLF4. Luciferase constructs containing the miR-544 promoter (pGL3-Promoter) and the unmodified construct (pRL-TK) were co-transfected with pcDNA3.1 or pcDNA-KLF4 into HeLa cells. Firefly luciferase activity was normalized to Renilla luciferase activity. D. qRT-PCR analysis of miR-544 expression in CaSki and HeLa cells with KLF4 overexpression. E and F. qRT-PCR and western blot analysis of YWHAZ expression in CaSki and HeLa cells with KLF4 overexpression. G. qRT-PCR analysis of KLF4 mRNA expression in 20 paired tumor tissues (Tumor) and adjacent normal cervical tissues (Normal) and cervical cancer cell lines (CaSki and HeLa). H. ChIP qRT-PCR assay were performed to detect the *in vivo* binding of transcription factor KLF4 to miR-544 promoter. HeLa cell chromatin fragments were immunoprecipitated with the specific anti-KLF4 antibody or normal IgG. The immunoprecipitated DNA samples were analyzed by qRT-PCR using primers specific to the miR-544 promoter. I. Relative luciferase activity of the mutant miR-544 promoter constructs in HeLa cells transfected with pcDNA-KLF4. Firefly luciferase activity was normalized to Renilla luciferase activity. The data represent the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ .

transfected with miR-544 mimic or miRNA mimic NC into HeLa cells. The results indicated that miR-544 decreased the relative luciferase activity with the wild type 3'UTR (WT) compared with the control, whereas the relative luciferase activity was not decreased as sharply in mutant (Mut) and deletion (Del) 3'UTRs (Figure 1B).

Furthermore, qRT-PCR and western blot analysis demonstrated that miR-544 dramatically decreased the endogenous mRNA and protein levels of YWHAZ in CaSki and HeLa cells (Figure 1C, 1D). YWHAZ expression levels decreased as the concentration of the transfected miR-544 mimic increased but increased when the

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cells were transfected with the miR-544 inhibitor. These results indicated that miR-544 down-regulated *YWHAZ* expression by directly binding to its 3'UTR target sites 1, 2 and 3.

To determine whether miR-544 expression correlated with *YWHAZ* mRNA expression levels in cervical cancer, we evaluated miR-544 and *YWHAZ* mRNA levels in 20 paired cervical cancer tissues and their adjacent normal cervical tissues as well as in cervical cancer cell lines (HPV 16+ CaSki and HPV 18+ HeLa) via qRT-PCR. Compared with normal cervical tissues, miR-544 was decreased in cervical cancer tissues and cervical cancer cells (**Figure 1E**), whereas *YWHAZ* mRNA expression was increased in cervical cancer tissues and cervical cancer cells (**Figure 1F**). Furthermore, western blot analysis indicated that the *YWHAZ* protein expression was more highly expressed in 18 of 20 paired (90%) tumor tissues (T) compared with normal (N) cervical tissues (**Figure 1G**). Two paired samples that showed no differences in *YWHAZ* expression are labeled with boxes. The results suggested that the aberrant decrease of miR-544 is related to the high expression of *YWHAZ* in cervical cancer.

### Identification of the miR-544 promoter

While our results suggested that miR-544 is decreased in cervical cancer, the reasons for this decrease is unknown. To determine the regulation of miR-544 expression at the transcriptional level, we first determined the TSS of miR-544 by 5'RACE assay. As shown in **Figure 2A**, the 5'RACE PCR products for pri-miR-544 suggested that it was the TSS of miR-544 and was labeled +1 (**Figure 2B**).

Typically, promoter sequences are near TSS and conserved in different species. Therefore, we scanned the sequences including 2 kb upstream and 2 kb downstream of miR-544 TSS with the online tools FirstEF, PromoterScan, softberry-fprom, and Promoter 2.0 Prediction. Unfortunately, no significant results were obtained. Then, we aligned the sequences 2 kb upstream and 2 kb downstream of miR-544 TSS and found a highly conserved region (**Figure 2C**). We hypothesized that this conserved sequence was most likely to be the promoter of miR-544. Later, we cloned the conserved sequence with different lengths into the luciferase reporter vector pGL3-Basic and eval-

uated the promoter activities. As shown in **Figure 2D**, the sequence between +256 to +764 significantly increased luciferase activity (approximately 5 fold). This sequence was considered to be the miR-544 promoter and was used in subsequent experiments.

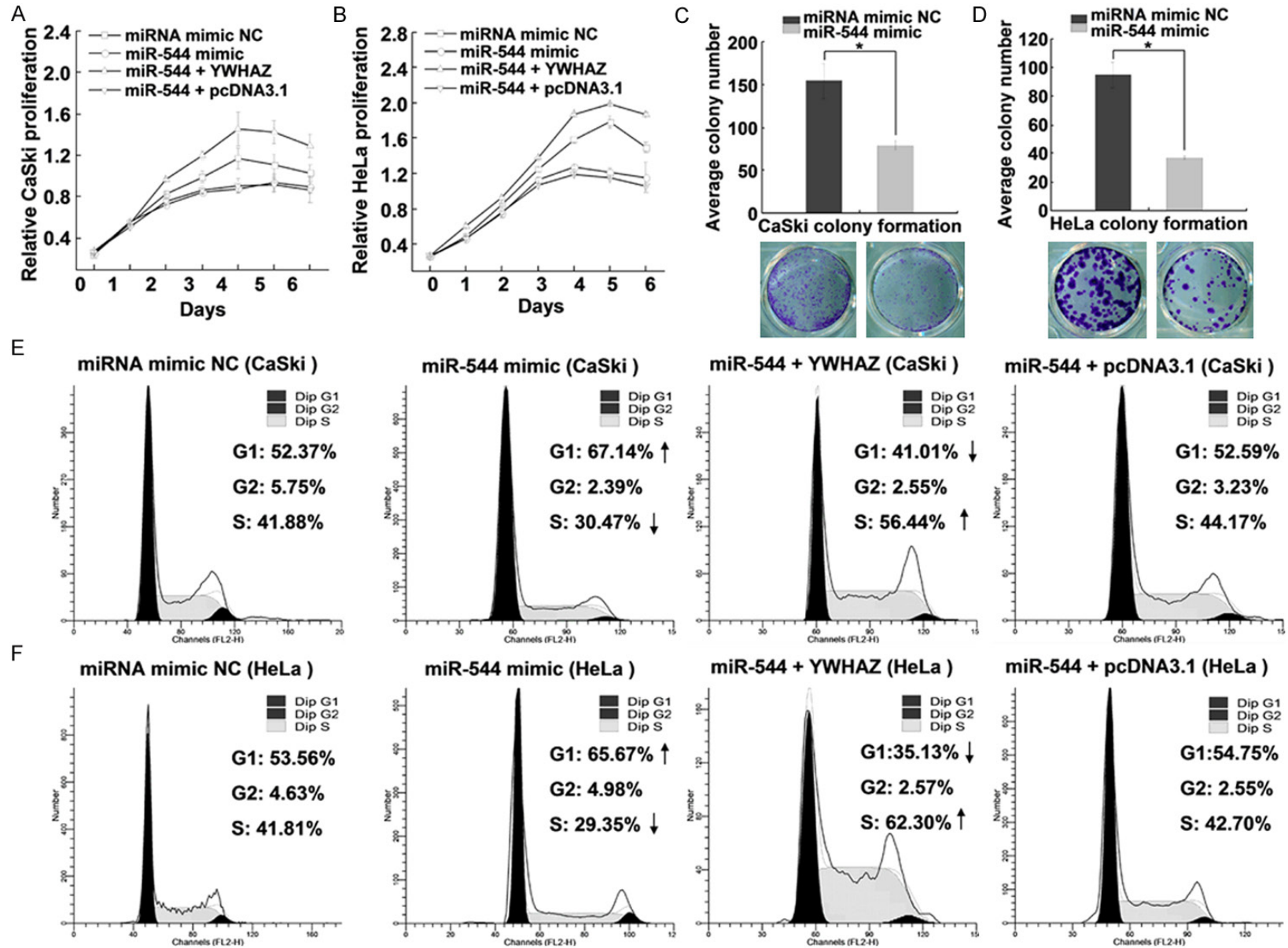
### *KLF4 promotes miR-544 transcription by interacting with the miR-544 promoter*

To determine which transcription factors might influence miR-544 expression, we performed *in silico* analysis of the identified promoter region using TRANSFAC (<http://www.biobase-international.com/product/transcription-factor-binding-sites>) and identified KLF4 as a candidate. KLF4 overexpression was examined by qRT-PCR and western blot, as shown in **Figure 3A, 3B**, and showed that KLF4 mRNA and protein levels were significantly increased when transfected with pcDNA-KLF4 in CaSki and HeLa cells. We then examined the effect of the transcription factor KLF4 on miR-544 promoter-driven luciferase activity and found KLF4 overexpression in HeLa cells activated miR-544 promoter-driven luciferase activity (**Figure 3C**). Consistent with the luciferase assay, endogenous mature miR-544 expression levels were increased by KLF4 overexpression (**Figure 3D**). To determine whether KLF4 regulation of miR-544 can mediate *YWHAZ* expression, we analyzed *YWHAZ* expression by qRT-PCR and western blot. As shown in **Figure 3E and 3F**, *YWHAZ* mRNA and protein levels were reduced in cells transiently transfected with pcDNA-KLF4, strongly suggesting that KLF4 stimulates miR-544 expression, which, in turn, downregulates *YWHAZ* expression. In addition, we examined KLF4 mRNA expression levels in 20 paired cervical cancer samples and their adjacent normal cervical samples as well as in cervical cancer cell lines (HPV 16+ CaSki and HPV 18+ HeLa) by qRT-PCR. The result indicated that the KLF4 expression level was decreased in cervical cancer tissues and cells compared with normal cervical tissues (**Figure 3G**). These results revealed that the low expression of KLF4 may contribute to the decreased expression of miR-544 in cervical cancer.

The interaction between KLF4 and the miR-544 promoter was further confirmed by ChIP qRT-PCR assays. As shown in **Figure 3H**, *in vivo* binding of KLF4 to the miR-544 promoter region was significantly higher than the control.

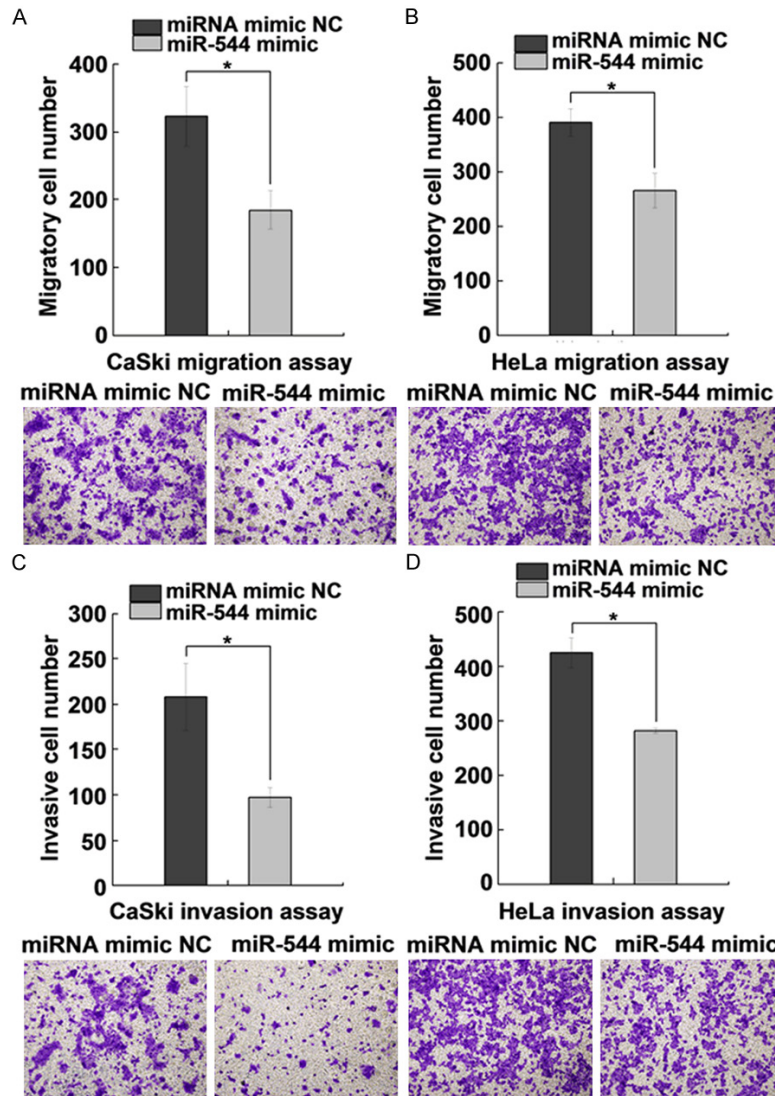


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## KLF4 regulate microRNA-544 that targets YWHAZ

**Figure 4.** miR-544 suppresses cervical cancer cell proliferation and colony formation and is involved in cell cycle regulation by targeting YWHAZ. A and B. The effect of miR-544 on CaSki and HeLa cell proliferation. miR-544 suppresses cervical cancer cell proliferation, and this can be rescued by over expression of YWHAZ. C and D. The effect of miR-544 on CaSki and HeLa cell colony formation. E and F. Flow cytometry analysis of the miR-544 and YWHAZ induced cell cycle checkpoint in CaSki and HeLa cells. Cervical cancer cells with miR-544 and YWHAZ overexpression were stained with propidium iodide and used for cell cycle analysis. The data represent the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ .



**Figure 5.** miR-544 suppresses cervical cancer cell migration and invasion. A and B. Transwell migration assays of CaSki and HeLa cells transfected with miR-544 mimic or NC. Representative images and quantification of migrated cells are shown. C and D. Transwell invasion assays of CaSki and HeLa cells transfected with miR-544 mimic or NC. Representative images and quantification of invaded cells are shown. The data represent the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ .

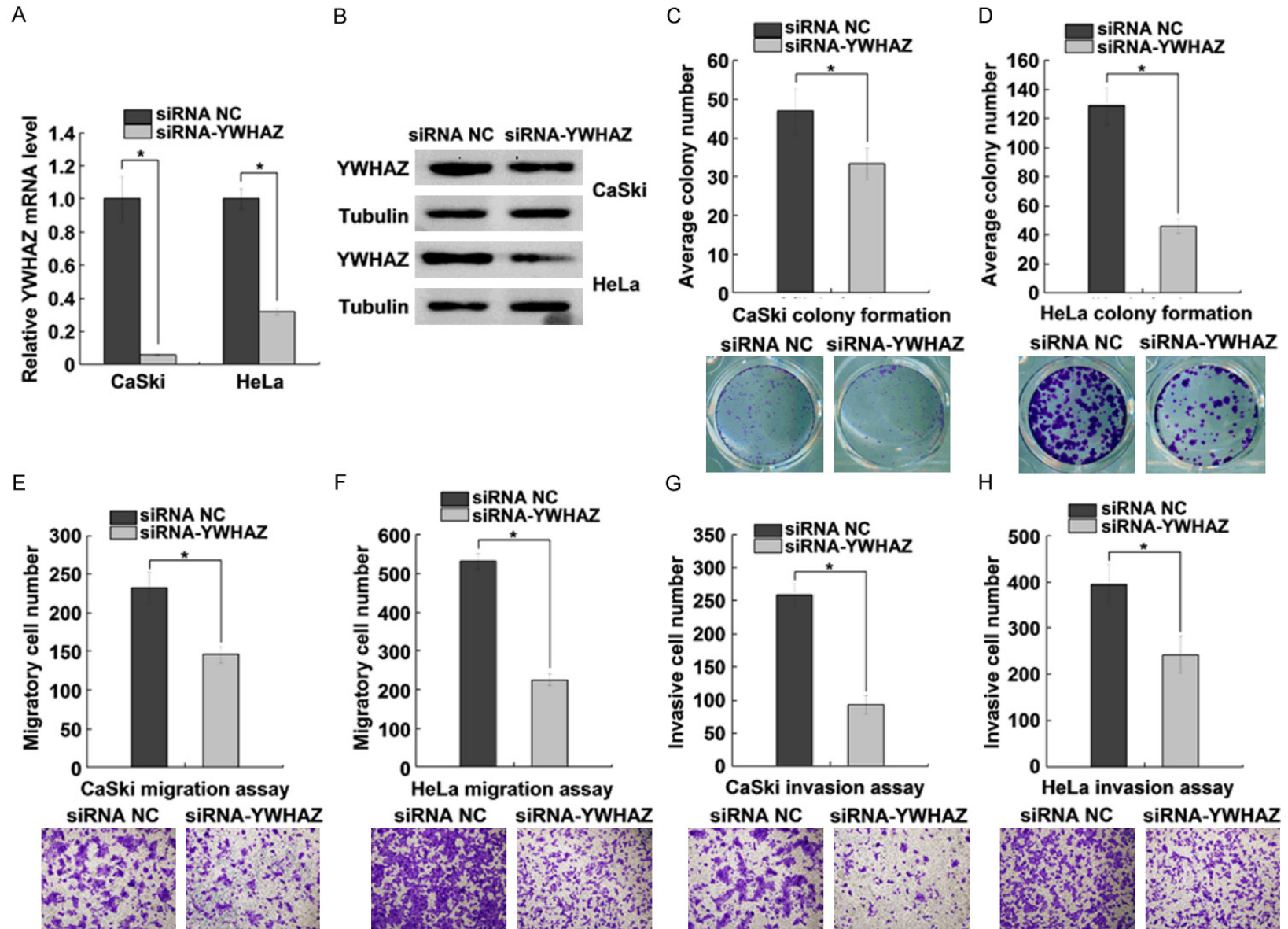
Promoter scanning using TRANSFAC predicted nine potential KLF4 binding sites in the miR-544 promoter region. To identify KLF4 binding sites in the miR-544 promoter, we performed

mutational analysis on the predicted KLF4 binding sites. As shown in **Figure 3I**, mutation of KLF4 binding site 4, 6 and 8 (mut 4, mut 6, and mut 8) abolished the effects of KLF4 on relative luciferase activity, while mutating other binding sites had no significant effect. According to these results, KLF4 regulates the expression of miR-544 by binding to sites 4, 6 and 8. These results indicated that KLF4 is involved in the transcriptional regulation of miR-544 by directly binding to the miR-544 promoter.

*miR-544 suppresses cervical cancer cell proliferation and colony formation and is involved in cell cycle regulation by targeting YWHAZ*

To better understand the role of miR-544 in the development of cervical cancer, we performed cell proliferation assays and found that miR-544 led to a significant decrease in the growth of CaSki and HeLa cells (**Figure 4A, 4B**). However, overexpression of YWHAZ abolished this suppression (**Figure 4A, 4B**). Similarly, colony formation assays showed that cervical cancer cell colony formation abilities were suppressed in miR-544-transfected cells compared with miRNA NC cells (**Figure 4C, 4D**). These results indicated that miR-544 exerts a growth suppression function in cervical cancer cells.

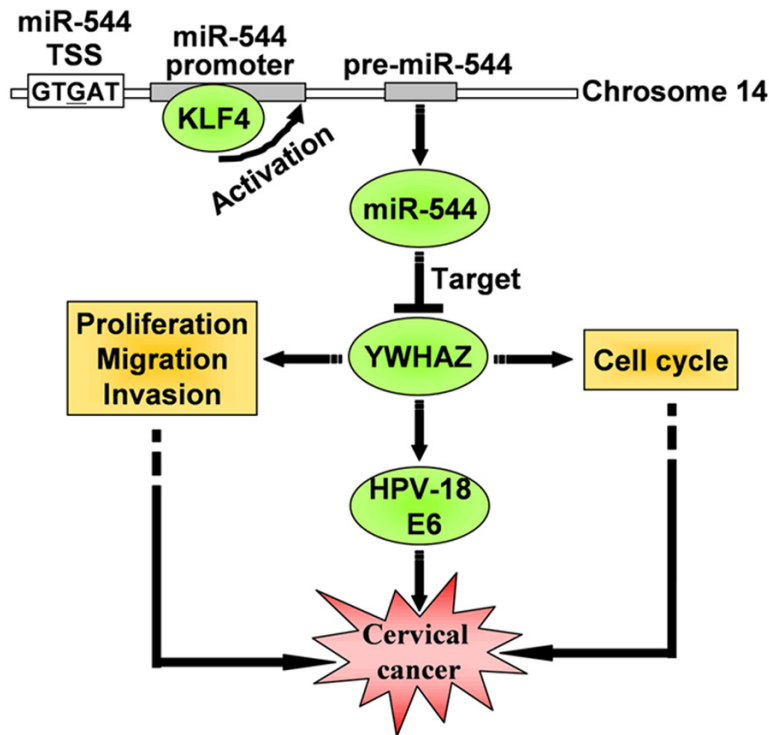
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**Figure 6.** *YWHAZ* knockdown suppresses cell colony formation, migration and invasion. A and B. qRT-PCR and western blot analysis of *YWHAZ* expression in CaSki and HeLa cells transfected with siRNA NC or siRNA-*YWHAZ*. C and D. Effect of siRNA-*YWHAZ* on CaSki and HeLa cells colony formation. Cervical cancer cells were treated with siRNA NC or siRNA-*YWHAZ* and plated in 6-well plates in triplicate. E and F. Transwell migration assays of CaSki and HeLa cells transfected with siRNA NC or siRNA-*YWHAZ*. Representative images and quantification of migrated cells are shown. G and H. Transwell invasion assays of CaSki and HeLa cells transfected with siRNA NC or siRNA-*YWHAZ*. Representative images and quantification of invaded cells are shown. The data represent the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$ .



**Figure 7.** Diagram describing the linear signaling pathway involved in cervical cancer progression.

Meanwhile, we determined whether miR-544 impacts cell cycle progression of cervical cancer cells. Cell cycle analysis indicated that miR-544 overexpression had a higher percentage of G1-phase cells and a lower percentage of S-phase cells (**Figure 4E, 4F**). However, *YWHAZ* overexpression induced the opposite effect. *YWHAZ* overexpression induced a reduction in the percentage of cells in G1 phase and an increase in S phase (**Figure 4E, 4F**). The results indicated that miR-544 is involved in cell cycle regulation by targeting *YWHAZ*.

### *miR-544 suppresses cervical cancer cell migration and invasion in vitro*

To further determine whether miR-544 is associated with cervical cancer progression, we analyzed the effect of miR-544 overexpression

on migratory and invasive ability. As shown in **Figure 5A, 5B**, the migratory ability was significantly suppressed when cervical cancer cells were transfected with the miR-544 mimic. Similarly, miR-544 overexpression dramatically suppressed the invasive ability of CaSki and HeLa cells (**Figure 5C, 5D**). These results demonstrated that miR-544 overexpression contributes to the regulation of cervical cancer cells motility and progression.

### *YWHAZ knockdown by siRNA suppresses cervical cancer cell colony formation, migration and invasion*

The function of *YWHAZ* in cervical cancer cells was analyzed using RNA interference. The results showed that *YWHAZ* mRNA and protein levels were significantly decreased

when cervical cancer cells were transfected with siRNA targeting *YWHAZ* (**Figure 6A, 6B**). *YWHAZ* knockdown significantly suppressed CaSki and HeLa cell colony formation (**Figure 6C, 6D**). Likewise, *YWHAZ* knockdown suppressed CaSki and HeLa cell migration and invasion abilities (**Figure 6E-H**). These results demonstrated that *YWHAZ* functions as an oncogene and may contribute to the tumorigenesis in cervical cancer.

### Discussion

miRNAs are post-transcription regulators of many genes, and their deregulation is related to cancer initiation, development, and progression [4]. Our study demonstrates that miR-544 is decreased in cervical cancer tissues and cells; however, the underlying mechanisms



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involved in the deregulation of miR-544 are still unknown. Therefore, we explored the regulation of miR-544 expression at the transcriptional level. We identified the miR-544 TSS by 5'RACE and identified the miR-544 promoter sequence downstream of the TSS. Typically, the promoters for RNA polymerase I and II are mostly upstream of the TSS, but some promoters for RNA polymerase III lie downstream of the TSS [13]. It has been reported that miRNA genes can be transcribed by either RNA polymerase II or RNA polymerase III into primary miRNA transcripts [14-16]. Therefore, we speculate that miR-544 may be transcribed by RNA polymerase III, as the promoter sequence lies downstream of the TSS.

The protein product of the miR-544 target gene *YWHAZ* belongs to the highly conserved 14-3-3 protein family. *YWHAZ* encodes tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (14-3-3 ζ). Prior studies have suggested that *YWHAZ* is a potential oncogene in various human cancers because it is frequently overexpressed [17-20] and affects multiple pathways involved in cancer [12, 19, 21]. Phosphorylated E6 interacts with *YWHAZ* directly, and knockdown of *YWHAZ* expression results in a significant decrease in the levels of HPV-18 E6 in HeLa cells [22]. A defining feature of cervical cancer is the continued expression of E6 and E7 viral oncoproteins [23, 24]. Abrogation of E6/E7 function or expression suppresses cell growth and induces senescence and apoptosis [25, 26]. Therefore, miR-544 and its target *YWHAZ* can be considered potential targets for anti-cancer therapy in HPV-induced malignancies (Figure 7).

Kruppel-like factor 4 (KLF4) is a zinc finger transcription factor. Previous studies have indicated that KLF4 expression is reduced in different types of cancers [27, 28]. Accumulating clinical evidence also suggests that KLF4 functions as a tumor suppressor gene [29-31]. KLF4 knockdown promotes cell growth, migration and adhesion, and the loss of KLF4 promotes skin tumorigenesis in a mouse model [32]. Our study revealed that KLF4 was decreased in cervical cancer tissues and cells compared with normal cervical tissues. Therefore, the low expression of KLF4 in cervical cancer may contribute to the reduced expression of miR-544 and may also play a crucial role in cervical cancer progression.

In conclusion, miRNAs have emerged as important regulators of diverse biological processes. Aberrant and altered miRNA expression are linked to a multitude of pathological conditions and cancer progression. The cause of the widespread differential expression of miRNAs in pathologic compared with normal cells can be explained by epigenetic mechanisms, miRNA biogenesis machinery and transcriptional regulation of miRNA promoters. Recently, significant advances have been made in miRNA biogenesis and the mode of action and have attracted great attention. Therapeutic approaches based on miRNA-targeted genes may be developed in the future. Therefore, understanding the principles managing miRNA deregulation is essential for the development and successful application of miRNA-based drugs.

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### Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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