


# MicroRNA 26b promotes colorectal cancer metastasis by downregulating phosphatase and tensin homolog and wingless-type MMTV integration site family member 5A

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Invasion and metastasis are crucially important factors in the survival of malignant tumors. Epithelial-mesenchymal transition (EMT) is an early step in metastatic progression and the presence of cancer stem cells is closely related to tumor survival, proliferation, metastasis, and recurrence. Herein we report that ectopic overexpression of microRNA 26b (miR-26b) in colorectal cancer (CRC) cell lines promoted EMT and stem cell-like phenotypes in vitro. Furthermore, miR-26b directly targeted and suppressed multiple tumor suppressors, including phosphatase and tensin homolog (PTEN) and wingless-type MMTV integration site family member 5A (WNT5A). Notably, miR-26b is markedly upregulated in tumor samples from patients with lymphatic metastases. These results indicate that miR-26b promotes CRC metastasis by downregulating PTEN and WNT5A, and may represent a therapeutic target for metastatic CRC.

## KEYWORDS

colorectal cancer, invasion and metastasis, miR-26b, PTEN, WNT5A

**Abbreviations:** CRC, colorectal cancer; CSC, cancer stem cells; EMT, epithelial-mesenchymal transition; miR-26b, microRNA 26b; OS, overall survival; PTEN, phosphatase and tensin homolog; RT, reverse transcription; WB, Western blot; WNT5A, wingless-type MMTV integration site family member 5A.

Fan, Lin, Zhang and Zhong contributed equally to this study.

## 1 | INTRODUCTION

Metastasis is responsible for over 90% of cancer-related deaths.<sup>1</sup> The process consists of multiple continuous and interlinked steps, including the initial transformation of cancer cells, angiogenesis, intravasation, survival in the circulation, arrest and adherence at the

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capillary bed, extravasation, proliferation of a metastatic focus, and defense against host immune responses.<sup>2</sup> EMT is an important step in the initial transformation of metastatic cancer.<sup>3</sup> It includes morphological changes of the tumor cell, decreased intercellular adhesion, increased cell motility, and other processes.<sup>4</sup> An increasing number of studies have shown that abnormal activation of EMT promotes the progression of malignant tumors.<sup>5-7</sup> Previous studies have also shown that Wnt/ $\beta$ -catenin signaling plays an important role in the development and promotion of EMT and cancer metastasis.<sup>8</sup>

Cancer stem cells, also known as tumor-initiating cells, are a subset of tumor cells with the capacity for self-renewal and unlimited proliferation. Many studies have shown the relationship between CSC and tumorigenesis and subsequent tumor development; in addition, the ability of CSC to migrate makes metastasis possible.<sup>9,10</sup> Therefore, CSC play an important role in tumor survival, proliferation, metastasis, and recurrence.

A previous study on head and neck squamous cell carcinoma found that increased expression of miR-26b correlated with poor prognosis.<sup>11</sup> It has also been reported that miR-26b expression was upregulated in tissues and serum upon exacerbation of inflammatory bowel disease-associated carcinogenesis.<sup>12</sup> However, studies have shown that the expression of miR-26b in CRC tissue is lower than that in normal tissue, suggesting that miR-26b depletion may play a role in the tumorigenesis of CRC.<sup>13</sup> Therefore, the role of miR-26b in CRC metastasis remains under debate.

Phosphatase and tensin homolog is a tumor suppressor gene that antagonizes PI3K/Akt signaling and is frequently silenced in numerous tumor types.<sup>14,15</sup> By inhibiting PI3K/Akt signaling, PTEN can inhibit  $\beta$ -catenin activity, ultimately affecting EMT.<sup>14</sup> In addition, previous studies in prostate cancer showed that by inhibiting the PI3K/Akt pathway, PTEN also significantly inhibits the formation of CSC.<sup>16</sup>

Wingless-type MMTV integration site family member 5A, a member of the non-canonical Wnt family, suppresses the motility and invasiveness of cancer cells by CK1 $\alpha$  activation, increasing formation of the  $\beta$ -catenin/E-cadherin complex at the cell membrane and inhibiting the Wnt/ $\beta$ -catenin signaling pathway, ultimately affecting EMT.<sup>17</sup> WNT5A plays an important role in inhibiting the formation of various cancer types, such as CRC, thyroid cancer, and acute lymphoblastic leukemia.<sup>18-20</sup>

Here we report that a human miRNA, miR-26b, promotes EMT and CSC formation by suppressing the expression of PTEN and WNT5A, consequently promoting CRC metastasis.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell culture

The CRC cell lines Caco2 and DLD1 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The human embryonic kidney cell line HEK293T was purchased from the ATCC. Cells were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Life Technologies), 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Pen Strep; Life Technologies).

### 2.2 | Transient transfection

The miR-26b mimic, inhibitors, and corresponding control oligonucleotides were purchased from RiboBio (RiboBio Co., Ltd, Guangzhou, China) and were transfected into Caco2 and DLD1 cells cultured in 6-well plates using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions.

### 2.3 | Plasmids and generation of stably engineered cell lines

The miR-26b expression plasmid was generated by cloning the genomic pre-miR-26b gene, with a 300-bp sequence on each flanking side, into the lentiviral transfer plasmid pSin-puro (Takara Bio Inc., Kusatsu, Shiga, Japan) to generate pSin-miR-26b, which was cotransfected with psPAX and pMD2.G plasmids into HEK293T cells using standard calcium phosphate transfection as previously described.<sup>21</sup> Thirty-six hours after cotransfection, the supernatants were collected and incubated with cells to be infected for 24 hours in the presence of polybrene (2  $\mu$ g/mL). After infection, puromycin (1.5  $\mu$ g/mL) was used to select stably transduced cells over a 14-day period. PTEN and WNT5A coding sequences were generated by PCR amplification and cloned into the retroviral transfer plasmid pMSCV-puro (Clontech Laboratories Inc.). The 3' UTR of PTEN and WNT5A were amplified and cloned downstream of the luciferase gene in a modified pGL3 control vector.

### 2.4 | RNA extraction, RT, and real-time RT-PCR

Total RNA was extracted from cultured cells using TRIzol (Ambion; Life Technologies), and cDNA was synthesized with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (FSQ-301; Toyobo, Osaka, Japan). Expression of miRNAs was analyzed by real-time RT-PCR using the Bulge-Loop<sup>TM</sup> miRNA qRT-PCR Assay kit (RiboBio Co., Ltd). Detection of mRNA was carried out with Power SYBR Green PCR Master Mix (Applied Biosystems [Waltham, MA, USA], Thermo Fisher Scientific) according to the manufacturer's instructions. Sequences of the primers were as follows:

PTEN forward, 5'-CGGTGCATAATGTCTTTCAGC-3';  
 PTEN reverse, 5'-TGAAGCGTATACAGGAACAAT-3';  
 WNT5A forward, 5'-AGCCTGTAAGTGTCATGGAGT-3';  
 WNT5A reverse, 5'-CGCGGCGCTATCATACTTCT-3';  
 LgR5 forward, 5'-GTTTCCCAGCAAGACGTA-3';  
 LgR5 reverse, 5'-CAGCGTCTTACCTCCTACC-3';  
 Bmi1 forward, 5'-TCGTTGTTGATGCATTCT-3';  
 Bmi1 reverse, 5'-CTTTCATTGTCTTTCCGCC-3';  
 ALDH forward, 5'-CCACTACTGAATCATGCCA-3';  
 ALDH reverse, 5'-GCACGCCAGACTTACCTGTC-3';  
 CD44 forward, 5'-CACGTGGAATACACCTGCAA-3';  
 CD44 reverse, 5'-GACAAGTTTTGGTGGCAGC-3';  
 CD133 forward, 5'-TTTGATTTCATATGCCTTCTGT-3';  
 CD133 reverse, 5'-CCATTGGCATTCTCTTTGAA-3';  
 CD166 forward, 5'-AGGTACGTCAAGTCGGCAAG-3';

CD166 reverse, 5'-CTTCTGCCTCTTGATCTCCG-3';  
 GAPDH forward, 5'-ATTCCACCCATGGCAAATTC-3';  
 GAPDH reverse, 5'-TGGGATTCCATTGATGACAAG-3'.

## 2.5 | Western blot analysis

Western blots were carried out as described previously.<sup>22</sup> The following primary antibodies were used: anti-GAPDH, anti-PTEN, anti-WNT5A, and anti-N-cadherin (all from Abcam, Cambridge, UK; ab128915, ab32199, ab72583, and ab76011, respectively). IRDye 800CW goat anti-rabbit IgG and IRDye 680CW goat anti-mouse IgG (both from LI-COR Biosciences, Lincoln, NE, USA) were used as secondary antibodies.

## 2.6 | Wound healing, cell invasion, and migration assays

Indicated cells were plated in 6-well plates. When confluent, scratches were created in the monolayer with pipette tips, and the progression of migration was imaged 24, 48, and 60 hours after wounding. Cell invasion and migration assays were analyzed using Transwell chambers (Costar; Corning Inc., Corning, NY, USA), with or without Matrigel coating (BD Biosciences, San Jose, CA, USA). The lower chamber of the Transwell device was filled with 500  $\mu$ L DMEM supplemented with 10% FBS and the upper chamber was filled with serum-free DMEM. After 24 hours of incubation, cells that had migrated to the bottom side of the inserts were fixed, stained, imaged, and quantified by counting the cells in 5 random high-power fields per sample.

## 2.7 | Luciferase reporter assay

Cells (6000/well) were seeded in 3-6 replicates in 48-well plates and incubated for 24 hours before transfection with 200 ng of the indicated reporter plasmids (pGL3-PTEN-3' UTR and pGL3-WNT5A-3' UTR) plus 20 ng pRL-TK *Renilla* plasmid using Lipofectamine 2000 (Life Technologies). After 36 hours, a Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA) was carried out according to the manufacturer's instructions.

## 2.8 | Statistics

All statistical analyses were carried out using SPSS 19.0 statistical software. Continuous data were compared using Student's two-tailed *t* test. In all cases,  $P < .05$  was considered statistically significant.

## 3 | RESULTS

### 3.1 | Expression of miR-26b is related to lymphatic metastasis of CRC

To investigate the relationship between miR-26b expression and CRC metastasis, we extracted CRC microRNA expression profiles from the Gene Expression Omnibus public microarray database

(Series GSE29623). This analysis showed that in CRC patients, miR-26b expression was significantly higher in the presence of lymphatic metastasis (N1/N2 groups), than in its absence (N0 group;  $P = .01$ ; Figure 1). This suggests that miR-26b expression may be associated with the development of CRC metastasis.

### 3.2 | Overexpression of miR-26b promotes the invasiveness and migration of CRC cells

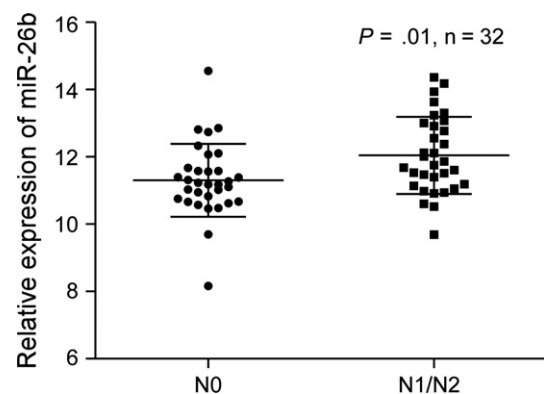
To understand the biological effects of miR-26b on the metastasis of CRC cells, *in vitro* gain-of-function analyses were carried out using an overexpression strategy. Human CRC cell lines Caco2 and DLD1 with stable expression of miR-26b (Caco2-miR-26b and DLD1-miR-26b) were generated, and upregulation of miR-26b in these cells was confirmed by RT-PCR, compared to cells transfected with corresponding control vectors (Caco2- and DLD1-vectors; Figure 2A).

As shown in Figure 2B, overexpression of miR-26b in CRC cells led to a significant increase in the mesenchymal marker N-cadherin (Figure 2B), suggesting that miR-26b might promote EMT. Consistent with this idea, Matrigel-coated (for invasion) and -uncoated (for migration) Transwell assays showed that miR-26b overexpression significantly increased the invasion and migration of DLD1 cells (Figure 2C,D). Furthermore, wound-healing assays showed that miR-26b overexpression enhanced the migratory speed of DLD1 and Caco2 cells compared with control cells (Figure 2E). Collectively, these results suggest that miR-26b greatly contributes to the development of CRC metastasis.

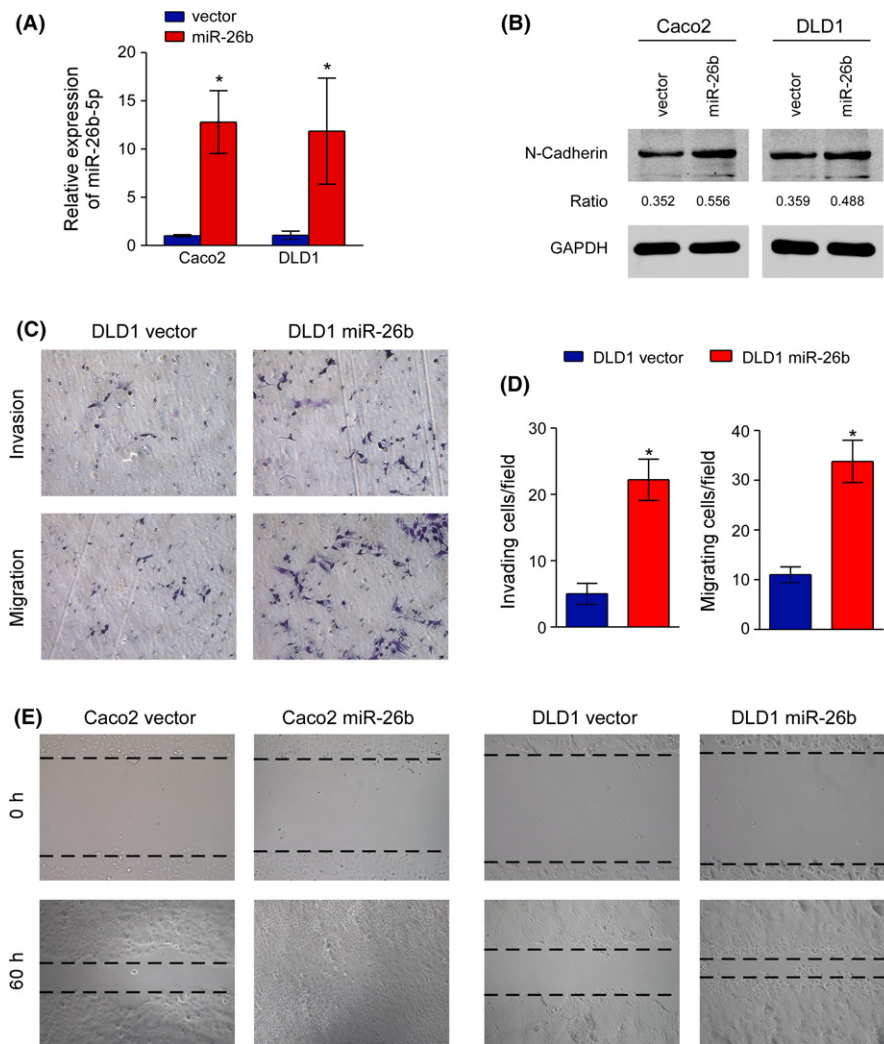
### 3.3 | Overexpression of miR-26b promotes a stem cell-like phenotype in CRC cells

In addition to invasiveness and metastasis, we examined whether miR-26b overexpression contributed to the promotion of a stem cell-like phenotype in CRC cells. We found that the mRNA expression levels in the pluripotency- and stem cell-associated markers Lgr5, Bmi1, ALDH1, CD44, CD133, and CD166 were significantly increased in CRC cells after upregulation of miR-26b ( $P < .05$ ; Figure 3A).

In addition, we conducted a tumor sphere formation assay to examine the effect of miR-26b overexpression on the self-renewal of



**FIGURE 1** Relationship between microRNA 26b (miR-26b) expression and lymphatic metastasis in colorectal cancer microarray data from the Gene Expression Omnibus



**FIGURE 2** Overexpression of microRNA 26b (miR-26b) promotes invasion and migration of colorectal cancer cell lines in vitro. A, Verification of miR-26b expression in cell lines with stable expression of miR-26b and corresponding vector controls by RT-PCR analysis. B, Expression of the mesenchymal cell marker N-cadherin was examined by Western blot analysis. GAPDH was used as a loading control. C, Typical images of indicated invading and migrating cells in Matrigel-coated and -uncoated Transwell assays, respectively. D, Quantification of indicated invading and migrating cells in 5 random fields from Matrigel-coated and -uncoated Transwell assays, respectively. E, Representative micrographs from wound-healing assays of the indicated cells. Wound closures were photographed 0 and 60 h after wounding. Experiments in A-E were repeated at least 3 times with similar results, and the error bars in A and D represent the mean  $\pm$  standard deviation. \* $P < .05$ . Original magnification: C,  $\times 200$ ; E,  $\times 100$

spherogenic CRC cells. Notably, the miR-26b-upregulated CRC cells formed approximately 2- to 3-fold higher cell content in single spheres compared with vector control cells (Figure 3B). Sphere formation rate (Figure 3C) and sphere density (Figure 3D) were also higher in miR-26b-upregulated CRC cells. Collectively, these results indicate that overexpression of miR-26b promotes a stem cell-like population in CRC cells.

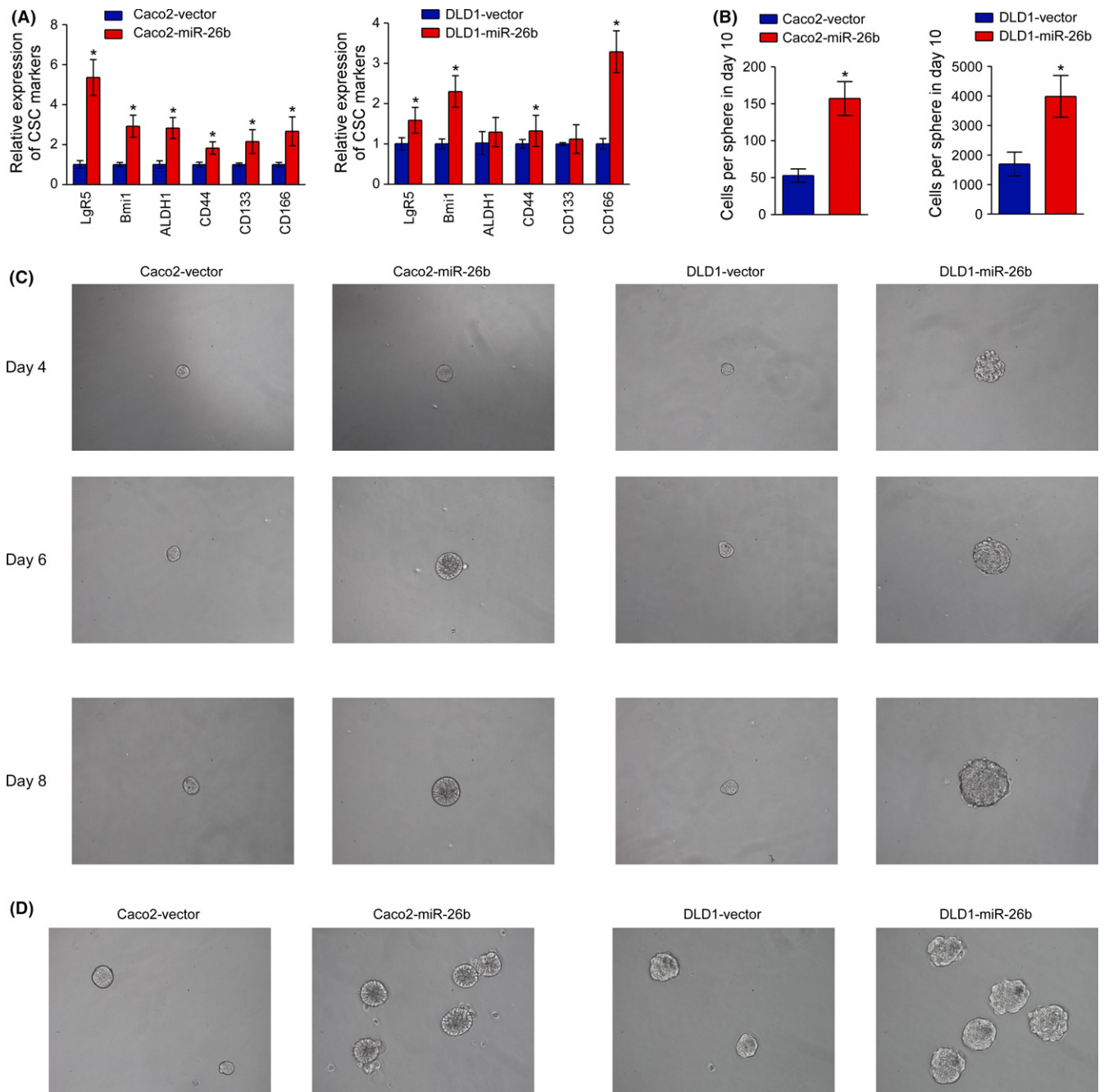
### 3.4 | Tumor suppressors PTEN and WNT5A are directly repressed by miR-26b

Using the bioinformatics tools TargetScan<sup>23</sup> and miRanda,<sup>24</sup> we confirmed that among the predicted targets, tumor suppressors PTEN and WNT5A had multiple highly conserved binding sites for miR-26b

(Figure 4A). To determine whether miR-26b targets PTEN and WNT5A, we transfected miR-26b mimic and inhibitor into established CRC cell lines with high and low expression of miR-26b (Figure 4B). WB analysis consistently showed decreased expression of PTEN and WNT5A in miR-26b-overexpressing cells, whereas miR-26b inhibition increased the levels of these proteins (Figure 4C). Similar trends were observed at the mRNA level (Figure 4D).

Furthermore, when luciferase was linked to the 3' UTR of PTEN or WNT5A, reporter assays showed decreased luciferase activity in miR-26b mimic-transfected Caco2 and DLD1 cells compared with untransfected control cells (Figure 4E). Importantly, mutations introduced into the seed sequence of miR-26b (Figure 4F) abolished its suppressive effects (Figure 4E). Collectively, these data suggest that miR-26b directly suppresses PTEN and WNT5A expression in CRC cell lines.



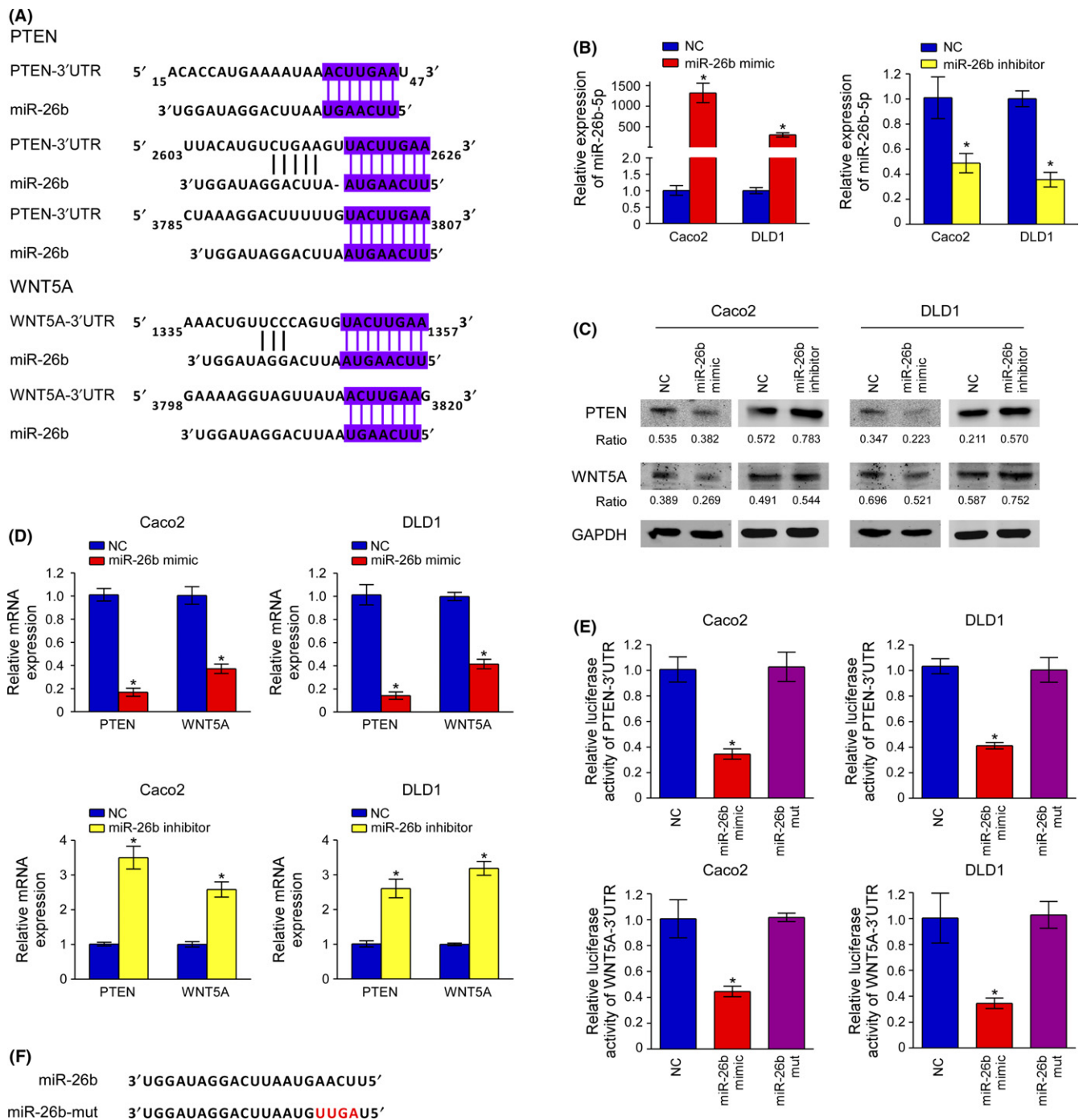


**FIGURE 3** Overexpression of microRNA 26b (miR-26b) promotes a stem cell-like phenotype in colorectal cancer cells in vitro. A, RT-PCR analysis of the expression of the pluripotency- and stem cell-associated markers Lgr5, Bmi1, ALDH1, CD44, CD133, and CD166 in the indicated cells. B, Quantification of single cells in each sphere 10 d after seeding. C, Representative images of the speed of sphere formation. Images were acquired 4, 6, and 8 d after seeding. D, Representative images of the density of spheres formed. Images were acquired 6 d after seeding. Experiments in A-D were repeated at least 3 times with similar results, and error bars in A and B represent the mean  $\pm$  standard deviation. \* $P < .05$ . Original magnification in C and D:  $\times 200$ . CSC, cancer stem cells

### 3.5 | Suppression of PTEN and WNT5A is functionally important for the biological effects of miR-26b

To explore the functional significance of PTEN and WNT5A in the miR-26b-mediated induction of CRC cell line invasiveness, the PTEN and WNT5A coding sequences, without their corresponding

3' UTR, were ectopically overexpressed in miR-26b-transduced Caco2 and DLD1 cells (Figures 5A,B and 6A,B). The stem cell populations of miR-26b-induced CRC cells were partly reduced (Figures 5C and 6C). In addition, reintroduction of PTEN or WNT5A dramatically suppressed the migration and invasiveness of miR-26b-induced CRC cells (Figures 5D and 6D). Moreover, a wound-healing assay showed that overexpression of PTEN or WNT5A repressed

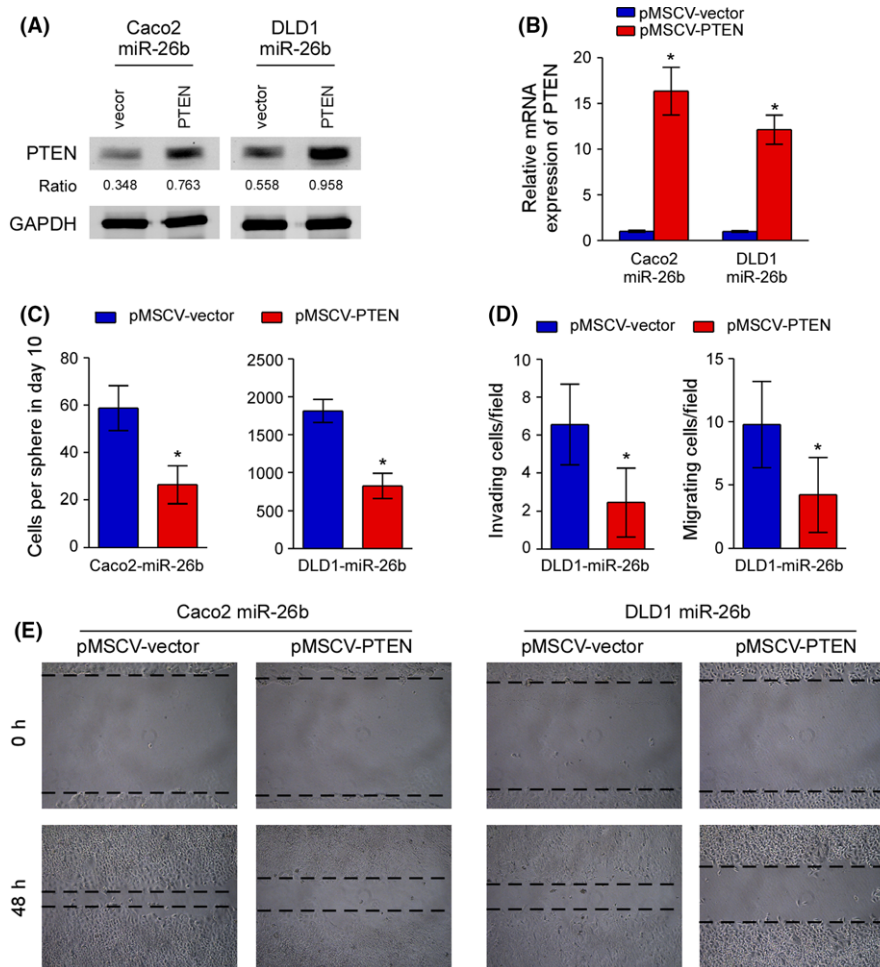


**FIGURE 4** Phosphatase and tensin homolog (PTEN) and wingless-type MMTV integration site family member 5A (WNT5A) are direct targets of microRNA 26b (miR-26b). A, Schematic of putative miR-26b target sites in the 3' UTR of PTEN and WNT5A. B, Real-time RT-PCR analysis of miR-26b expression in colorectal cancer cells transfected with 50 nmol/L miR-26b mimic or inhibitor. C, Western blot analysis of PTEN and WNT5A levels in response to deregulated miR-26b expression in the indicated cells. D, Real-time RT-PCR analysis of the mRNA levels of PTEN and WNT5A in the indicated cells. E, Luciferase assay of pGL3-PTEN-3' UTR and pGL3-WNT5A-3' UTR reporters cotransfected with 50 nmol/L miR-26b mimic or mutant oligonucleotides in Caco2 and DLD1 cells. F, Sequence of miR-26b-mut. Experiments in B-E were repeated at least 3 times with similar results, and error bars in B, D, and E represent the mean  $\pm$  standard deviation. \* $P < .05$ . NC, negative control

the motility of miR-26b-transduced Caco2 and DLD1 cells compared with control cells (Figures 5E and 6E). These results show that PTEN and WNT5A are functionally important for the miR-26b-induced cell stem cell-like phenotype, motility, and invasiveness in CRC cell lines.

## 4 | DISCUSSION

Colorectal cancer is one of the most common malignant tumors, causing 700 000 deaths every year globally.<sup>25</sup> Benefitting from the rapid development of diagnostic techniques and treatments, the 5-



**FIGURE 5** Phosphatase and tensin homolog (PTEN) is functionally involved in microRNA 26b (miR-26b)-induced migration and invasion of colorectal cancer cell lines. A, Ectopic expression of PTEN was confirmed by Western blot analysis using GAPDH as a loading control. B, Verification of PTEN mRNA levels by real-time RT-PCR analysis. C, Quantification of single cells in each sphere in a sphere-formation assay of PTEN overexpression. D, Quantification of invading and migrating cells with PTEN overexpression in Transwell assays. E, Representative images from a wound-healing assay with PTEN overexpression. Experiments in A-E were repeated at least 3 times with similar results, and error bars in B-D represent the mean  $\pm$  standard deviation. \* $P < .05$ . Original magnification in E,  $\times 100$

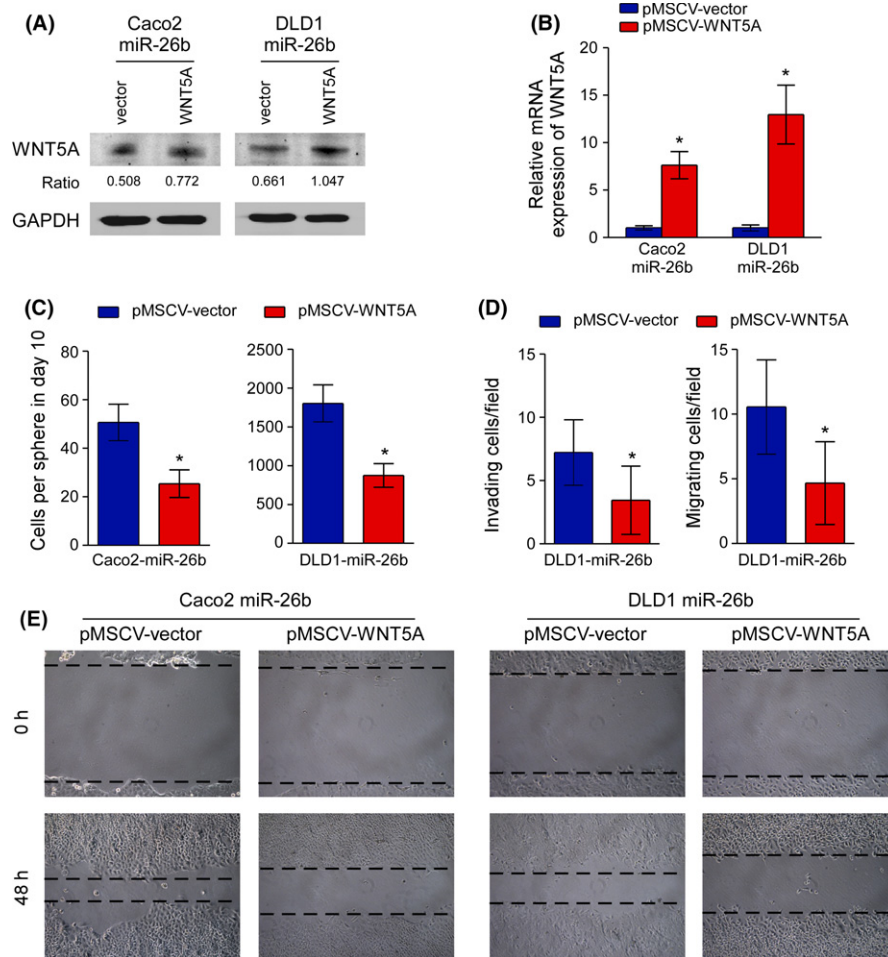
year OS of patients with early-stage, non-lymph node metastatic CRC has reached 90%; however, the OS of patients with distant metastasis is only approximately 10%.<sup>26</sup> Therefore, in-depth study of the mechanisms behind CRC metastasis is crucial to the development of metastatic CRC treatments which would directly benefit these patients.

Tumor invasion and metastasis are complex, multistep processes controlled by genetic and epigenetic changes in the tumor.<sup>27</sup> However, the regulatory networks that control such molecular alterations are not clearly understood. As a single miRNA can coordinately suppress multiple target genes and thereby modulate multiple steps of metastatic progression in various cancer types,<sup>28</sup> miRNAs are attractive candidates for such regulation. In the present study, we observed that miR-26b is associated with lymphatic metastasis in CRC. Moreover, miR-26b promotes metastasis and invasion in the CRC cell lines Caco2 and DLD1. Furthermore, RT-PCR and sphere formation assays showed that miR-26b promotes a stem cell-like

phenotype in a subpopulation of CRC cells. Thus, our current study provides new insight into this area of research by identifying miR-26b as a promoter of CRC invasion and metastasis.

A class of noncoding small RNAs, miRNAs, are involved in many biological processes, and function by base-pairing with the 3' UTR of a target mRNA, resulting in post-transcriptional inhibition and sometimes mRNA cleavage.<sup>29</sup> Accumulating evidence has expanded the known functions of miRNAs to both physiological and pathological conditions, including cancer.<sup>30</sup> As specific miRNAs are usually capable of targeting the mRNA of multiple different genes, it is of particular interest to identify miRNAs that can simultaneously interact with multiple regulators of the EMT process and CSC formation, and thereby lead to cancer invasion and metastasis.

Importantly, miRNAs are significantly cell- and tissue-specific and, in different cells and tissues, their expression can be significantly different. At present, research exploring the role of miR-26b in the metastasis of CRC cells is greatly lacking. According to previous



**FIGURE 6** Wingless-type MMTV integration site family member 5A (WNT5A) is functionally involved in microRNA 26b (miR-26b)-induced migration and invasion of colorectal cancer cell lines. A, Ectopic expression of WNT5A was confirmed by Western blot analysis using GAPDH as a loading control. B, Verification of mRNA levels of WNT5A by real-time RT-PCR analysis. C, Quantification of single cells in each sphere in a sphere-formation assay of WNT5A overexpression. D, Quantification of invading and migrating cells with WNT5A overexpression in Transwell assays. E, Representative images of a wound-healing assay with WNT5A overexpression. Experiments in A-E were repeated at least 3 times with similar results, and error bars in B-D represent the mean  $\pm$  standard deviation. \* $P < .05$ . Original magnification in E,  $\times 100$

studies, miR-26b expression is downregulated in sporadic CRC compared with normal colorectal mucosa.<sup>13,31</sup> On the contrary, it has also been reported that miR-26b expression was upregulated in inflammatory bowel disease-associated carcinogenesis.<sup>12</sup> However, our study shows that upregulation of miR-26b in CRC cells promotes invasion and metastasis, which seems contradictory. This may be an example of miRNA tissue-specificity: although miR-26b expression is higher in normal cells than in cancerous tissues, miR-26b expression is an indicator of poor prognosis after carcinogenesis. Our studies investigated the effect of different miR-26b expression levels on CRC cells, instead of comparing the expression level of miR-26b between CRC cells and normal colorectal mucosal epithelial cells. The role of miR-26b is diverse in different types of cells, and normal colorectal mucosal epithelial cells and CRC cells can be regarded as two different groups of cells. Therefore, we cannot simply compare the expression of miR-26b in CRC cells and normal cells to determine the role of miR-26b in colorectal cancer. Instead, we should dynamically monitor the expression of miR-26b in the same CRC cell lines.

In summary, our studies show that elevated expression of miR-26b promotes invasiveness, migration, and a stem cell-like phenotype in CRC by simultaneously suppressing the tumor suppressors PTEN and WNT5A. Our future work will involve examining the underlying signaling pathway, carrying out in vivo assays to examine metastasis, and collecting clinical specimens to verify our findings. These may prove clinically useful in the development of a new prognostic biomarker and therapeutic target for CRC stem cell accumulation, invasion, and metastasis.

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## CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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