

# Bone marrow mesenchymal stem cells promote head and neck cancer progression through Periostin-mediated phosphoinositide 3-kinase/Akt/mammalian target of rapamycin

Chuanxia Liu | Xiaoxia Feng | Baixiang Wang | Xinhua Wang | Chaowei Wang | Mengfei Yu | Guifen Cao | Huiming Wang 

Affiliated Hospital of Stomatology, Medical College, Zhejiang University, Hangzhou, China

## Correspondence

Huiming Wang, Affiliated Hospital of Stomatology, Medical College, Zhejiang University, Hangzhou, China.  
Email: whmwhm@zju.edu.cn

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Bone marrow mesenchymal stem cells (BMMSC) have been shown to be recruited to the tumor microenvironment and exert a tumor-promoting effect in a variety of cancers. However, the molecular mechanisms related to the tumor-promoting effect of BMMSC on head and neck cancer (HNC) are not clear. In this study, we investigated Periostin (POSTN) and its roles in the tumor-promoting effect of BMMSC on HNC. In vitro analysis of HNC cells cultured in BMMSC-conditioned media (MSC-CM) showed that MSC-CM significantly promoted cancer progression by enhancing cell proliferation, migration, epithelial-mesenchymal transformation (EMT), and altering expression of cell cycle regulatory proteins and inhibition of apoptosis. Moreover, MSC-CM promoted the expression of POSTN and POSTN promoted HNC progression through the activation of the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway. In a murine model of HNC, we found that BMMSC promoted tumor growth, invasion, metastasis and enhanced the expression of POSTN and EMT in tumor tissues. Clinical sample analysis further confirmed that the expression of POSTN and N-cadherin were correlated with pathological grade and lymph node metastasis of HNC. In conclusion, this study indicated that BMMSC promoted proliferation, invasion, survival, tumorigenicity and migration of head and neck cancer through POSTN-mediated PI3K/Akt/mTOR activation.

## KEYWORDS

bone marrow mesenchymal stem cells, epithelial mesenchymal transition, head and neck cancer, Periostin, PI3K/Akt/mTOR

## 1 | INTRODUCTION

Head and neck cancer (HNC) ranks sixth among malignancies worldwide, afflicting >500 000 individuals each year. Approximately 40% of them occur in the oral cavity. Oral squamous cell carcinoma

(OSCC) is the most common histological type, with frequency observed in the literature of approximately 90%. The majority of OSCC are diagnosed at a late phase, in stages III or IV, which decreases survival rate and reduces quality of life of the patients significantly. The current available therapeutic strategies include

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resection of malignant tissues or a combination of radiotherapy or chemotherapy, but the 5-year survival rate is still around 50%.<sup>1,2</sup> In addition, a high percentage of patients have a poor response to therapy and have a high recurrence rate. In particular, tongue cancer is characterized by a high potential for regional metastasis, even in early stages.<sup>3,4</sup> As OSCC severely affects the appearance, swallowing, breathing and psychological state of patients, the application of accurate and efficient biomarkers for early diagnosis and prognostic prediction is urgent. OSCC arises from the accumulation of genetic and epigenetic changes or abnormalities in cancer-associated signaling pathways, causing the acquisition of cancer-related phenotypes that have previously been summarized by Hanahan and Weinberg.<sup>5</sup> Interactions between tumor cells and cells in the tumor microenvironment also play important roles in malignant transformation.<sup>6</sup>

The tumor microenvironment undergoes extensive changes during the growth and progression of a tumor. Cells in the tumor microenvironment, including carcinoma-associated fibroblasts (CAF), bone marrow-derived mesenchymal stromal cells, tumor-associated macrophages, other inflammatory cells and vascular cells, all contribute to the hallmarks of cancer and the cancer ecosystem.<sup>7,8</sup> Mesenchymal stem cells (MSC) were initially identified within the stromal compartment of bone marrow and characterized by the potential of multidirectional differentiation. They have stem cell-like characteristics and are able to differentiate into osteogenic, adipogenic or chondrogenic lineages when placed in appropriate environments.<sup>9</sup> Bone marrow mesenchymal stem cells (BMMSC) have been shown to be recruited to the hypoxic tumor microenvironment and the site of metastasis where they integrate into the tumor microenvironment and provide a source for cells, such as cancer-associated fibroblasts (CAF).<sup>10,11</sup> Growth factors and cytokines secreted by tumor cells together with endocrine factors of inflammatory tissues surrounding tumors can attract BMMSC to tumor stroma.<sup>12</sup>

A number of studies have shown that BMMSC play important roles in tumor progression and promote invasion and metastasis in various cancers.<sup>13,14</sup> In the tumor microenvironment, upon interaction with MSC, breast cancer cells showed altered biological functions of certain gene clusters, hence increasing stemness of tumor cells, migration ability, angiogenesis, and drug resistance.<sup>15</sup> Li et al<sup>16</sup> showed that MSC were mainly distributed in the tumor stroma after i.v. injection and a low frequency MSC were found to be merged into hepatocellular carcinoma tissue. Rappa et al<sup>17</sup> found that spontaneous formation of bone marrow-derived multipotent stromal cells-breast cancer cell hybrids was a potential mechanism of the generation of invasive/metastatic breast cancer cells. Salo et al<sup>18</sup> found that MSC promoted the invasion of tongue carcinoma cells by inducing the expression of components associated to chemokine signaling, epithelial plasticity, cell motility and invasion, potentially promoting invasion favoring changes in cancer cells and in the tumor microenvironment.

Periostin (POSTN), also known as osteoblast-specific factor-2, is a disulfide-linked 90-kDa secretory protein expressed in bone tissues as well as an extracellular matrix protein. As an adhesion protein,

POSTN regulates cell adhesion and differentiation of osteogenic cells.<sup>19,20</sup> POSTN is up-regulated and plays important roles in a wide variety of cancers.<sup>21,22</sup> The effect of POSTN in ovarian cancer stroma cells activated the Akt pathway in the tumor and was correlated with clinical late stage and tumor recurrence.<sup>23,24</sup> Moreover, recombinant purified POSTN supported the adhesion and migration of ovarian epithelial cancer cells by interacting with integrin receptors  $\alpha V\beta 3$  and  $\alpha V\beta 5$ . Also, molecular actions in the Akt pathway were shown to be an important determinant of POSTN in tumor progression.<sup>25</sup> Meanwhile, POSTN contributed to the acquisition of multipotent stem cell-like properties in human breast cancer cells and played important roles in the effect of BMMSC.<sup>26,27</sup> However, whether POSTN could activate the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway in head and neck cancer was not clear.

In the present study, we investigated the effect of BMMSC on HNC and explored associated molecular mechanisms, detected the expression of POSTN and showed the functional roles of POSTN in the growth, proliferation, metastasis and epithelial mesenchymal transition (EMT) in head and neck cancer.

## 2 | MATERIALS AND METHODS

### 2.1 | Ethics and method statement

The present experiments including animal and human subjects were approved by the Ethics Committee of Zhejiang University, Hangzhou, China. All of the following protocols were approved in advance by the Ethics Committee of Affiliated Hospital of Stomatology, Medical College, Zhejiang University. Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). All human subject research was implemented with reference to the provisions of the Helsinki Declaration of 1975. All the patients involved in this study signed written informed consent in accordance with the institutional guidelines.

### 2.2 | Cell culture and preparation of conditioned medium

Human BMMSC were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were cultured in  $\alpha$ -Minimum Essential Media (MEM; Invitrogen, Waltham, MA, USA) supplemented with 10% FBS (Gibco-BRL, Gaithersburg, MA, USA), 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Human HNC cell lines CAL 27 were obtained from ATCC and HN4 were obtained from NIH. These cells were cultured in DMEM (Gibco-BRL) supplemented with 10% FBS, 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

MSC-conditioned medium (MSC-CM) was obtained from  $1 \times 10^6$  human BMMSC. Cells were washed three times with

10 mL PBS and incubated for 48 hours at 37°C in 10 mL  $\alpha$ -MEM supplemented with 10% FBS. The medium was harvested and centrifuged at 1000 g for 10 minutes at 4°C and the supernatant was stored at -80°C. Control medium was collected in parallel from tissue culture flasks containing no cells.

### 2.3 | Cell proliferation assay

CCK-8 (Dojindo, Japan) assay was used to evaluate cell proliferation according to the manufacturer's instructions. Briefly, after starvation for 6 hours, CAL 27 or HN4 cells were seeded into 96-well plates at a density of 5000 cells in each well with MSC-CM or control medium and 5 duplicates for each group. At 24 hours, 48 hours and 72 hours after seeding, 10  $\mu$ L CCK-8 solution was added to each well and incubated with cells for another 2 hours at 37°C. Optical density was then detected with a microplate reader at a wavelength of 450 nm.

### 2.4 | Cell cycle analysis

CAL 27 or HN4 cells were seeded at  $1 \times 10^5$  cells/dish in 100 mm cell culture dishes. At 24 hours after seeding, the cells were washed with 10 mL PBS 3 times and then 10 mL MSC-CM or control medium was added. After 48 hours,  $1 \times 10^6$  cells were harvested and fixed in ice-cold 70% ethanol for 24 hours. Then the cells were incubated in 10  $\mu$ g/mL propidium iodide solution containing 200  $\mu$ g/mL RNase A. BD FACS Aria II SORP (BD Biosciences, Franklin Lakes, NJ, USA) was used for FACS. For each experiment, 10 000 events were counted, and cell cycle profiles were modeled using Modfit software (Verity Software House).

### 2.5 | Cell apoptosis assay

CAL 27 or HN4 cells were cultured in MSC-CM or control media at 37°C for 48 hours and apoptotic cells were detected using FITC-Annexin V and propidium iodide (BD Biosciences). Briefly, after washing with cold PBS,  $1 \times 10^6$  cells were resuspended in 100  $\mu$ L binding buffer with 5  $\mu$ L FITC-Annexin V and propidium iodide and then incubated for 15 minutes at room temperature. Numbers of apoptotic cells were determined by flow cytometry.

### 2.6 | Wound-healing assay

CAL 27 or HN4 cells were harvested and seeded in 6-well plates in triplicate wells and cultured to confluence in regular MSC-CM or control medium. Forty-eight hours later, the plates were scraped with a P200 pipette tip (Thermo Fisher, Cleveland, OH, USA), and washed with cell culture media 3 times. Then the cells were incubated with serum-free MSC-CM or serum-free control medium. The wounded areas were photographed immediately after wounding (0 hours) and again at the end of the study (24 hours) in 5 random  $\times 100$  fields under a light microscope. Size of the wound area and closure of the wound were analyzed.

### 2.7 | Transwell migration assay

To evaluate the effect of BMMSC on migration of cancer cells, Transwell assay was also used. Briefly, CAL 27 or HN4 cells were cultured with MSC-CM in advance. Forty-eight hours later,  $5 \times 10^4$  cells in 300  $\mu$ L serum-free DMEM were placed in each Transwell chamber (Corning Inc., Corning, NY, USA), and 700  $\mu$ L regular MSC-CM or control medium were placed in the lower chamber. After incubation for 24 hours, the membranes were fixed with paraformaldehyde and stained with crystal violet solution. Cells on the upper surface of the filter were removed and cells that had migrated through the membrane of the inserts were imaged under a light microscope and quantified using Image J software. All experiments were carried out in triplicate and 3 images were processed per membrane.

### 2.8 | RNA extraction and real-time PCR analysis

CAL 27 or HN4 cells were seeded at  $1 \times 10^5$  cells/dish in 100 mm cell culture dishes. Twenty-four hours after seeding, the cells were washed with 10 mL PBS 3 times and then 10 mL MSC-CM or control medium was added. Seventy-two hours later, the cells were harvested and total RNA was extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions and reverse transcribed into cDNA using the PrimerScript RT reagent Kit (Takara, Japan). All the real-time PCR reactions were carried out using an ABI 7500 real-time PCR system (Life Technologies, Carlsbad, CA, USA) and the SYBR Premix Ex Taq reagent kit (Takara). The reaction was carried out according to the manufacturer's instructions in triplicate. mRNA expression was quantified using the delta delta CT method, and GAPDH served as the internal control. The following primers were used:

P16: forward 5'-AACGCACCGAATAGTTACGG-3' and reverse 5'-CACCAGCGTGCCAGGAAG-3'; P21: forward 5'-TGTGATGC GCTAATGGCG-3' and reverse 5'-AAGTCGAAGTCCATCGCTCA-3'; Snail: forward 5'-ATGCCGCGCTCTTCTCGTC-3' and reverse 5'-AGCAGGTGGCCTGGTCGTAG-3'; Twist: forward 5'-GGAGTC CGCAGTCTTACGAG-3' and reverse 5'-TCTGGAGGACCTGGTAGA GG-3'; E-cadherin: forward 5'-GCCGCTGGCGTCTGTAGGAA-3' and reverse 5'-TGACCACCGCTCTCTCCGA-3'; N-cadherin: forward 5'-TCGCCATCCAGACCGACCCA-3' and reverse 5'-TGAGGCGGGT GCTGAATTCCC-3'; Vimentin: forward 5'-ACAACCTGGCCGAGGAC ATC-3' and reverse 5'-AGAGACGCATTGTCAACATCCTG-3'; POSTN: forward 5'-TCACATATTCCGCGAGATCA-3' and reverse 5'-TGCAG CTTCAAGTAGGCTGA-3'; GAPDH: forward 5'-GCACCGTCAAGGCT GAGAAC-3' and reverse 5'-ATGGTGGTGAAGACGCCAGT-3'.

### 2.9 | Western blot analysis

Cells were harvested in SDS lysis buffer (Beyotime, China), and cell lysates (20  $\mu$ g) were electrophoresed through 10% polyacrylamide gels. After transferring the proteins to PVDF membranes, the membranes were blocked with 5% non-fat milk in PBS for 1 hour and

then incubated separately with primary antibodies against E-cadherin, N-cadherin, p-PI3K, p-Akt, p-mTOR (Cell Signaling, Danvers, MA, USA), Vimentin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and POSTN (Abcam, Cambridge, MA, USA). Mouse monoclonal  $\beta$ -actin antibody (1:5000; Sigma-Aldrich, St Louis, MO, USA) was used throughout as a loading control, followed by incubation with HRP-conjugated secondary antibodies (1:5000; Sigma-Aldrich). Antibodies were detected using enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL, USA).

## 2.10 | RNA interference

Commercial POSTN-siRNA oligonucleotides and non-silencing control were obtained from Santa Cruz Biotechnology. Transductions of CAL 27 or HN4 cells with viral and control vectors were carried out according to the manufacturer's instructions. Efficiency of knock-down by POSTN-siRNA was determined by western blot analysis using monoclonal anti-POSTN antibody. Approximately 60% knock-down of POSTN was obtained compared to non-silencing control cells.

## 2.11 | Immunohistochemical analysis

Briefly, paraffin-embedded sections were deparaffinized in xylene, rehydrated through graded ethanols, and then submerged into EDTA buffer for heat-induced antigenic retrieval, blocked with 10% BSA, incubated with primary antibodies at 4°C overnight and developed using the DAKO ChemMate Envision Kit /HRP (Dako-Cytomation, USA) followed by counterstaining with hematoxylin, dehydration, clearing and mounting with neutral gums. Protein expression was determined by randomly selecting 5 cell areas of each specimen under the same conditions using Image-Pro Plus 6.0 software. Primary antibodies were used at the following dilutions: Ki67 (1:500; Abcam), POSTN (1:500; Abcam).

## 2.12 | Animal studies

All surgeries of the mice were carried out under anesthesia with pentobarbital (40 mg/kg) to minimize suffering. The xenograft experiment was implemented in male BALB/C nude mice (4 weeks old) at Laboratory Animal Center, Zhejiang University. The mice were divided into 3 groups with 10 mice in each group. CAL 27 cells were cultured with MSC-CM for 72 hours in advance. CAL 27 in control medium, CAL 27 in MSC-CM, and mixed cells of CAL 27 and MSC were injected into the middle of the tongue of the mice. Diet, weight, health status and growth of tongue tumors were monitored. Two weeks after injection, tumors could be seen in the tongue. The mice were killed 5 weeks after injection. Tongues with tumors were divided into 2 parts. One part was fixed with 4% paraformaldehyde and the other part was preserved in liquid nitrogen. Enlarged lymph nodes were harvested and fixed with 4% paraformaldehyde. H&E staining was done to test pathological changes, Ki67 was done by immunohistochemistry (IHC) to detect

tumor proliferation. Proteins of tumor tissues were extracted and expression of POSTN, E-cadherin and N-cadherin in tumor tissues was detected by western blot.

## 2.13 | Patient samples and clinicopathological data

Fifty cases of tumor tissues and adjacent normal tissues from patients with head and neck cancer treated at the First Affiliated Hospital, Medical College, Zhejiang University were collected from 1 September 2014 to 31 March 2016. Patients were 36 males and 14 females who were not treated before surgery. Median age was 56 years (range 22-81). There were 14 gingival tumors, 13 tongue tumors, 13 buccal tumors, 7 mouth tumors, 1 lip tumor and 2 palate tumors, including 22 cases with lymph node metastasis and 28 cases without lymph node metastasis. Tumor tissues and relatively normal mucosal tissues from more than 2 cm near tumor tissues were fixed by 10% formalin and embedded in paraffin. Protein expression of POSTN and N-cadherin in the specimens was detected by IHC. Relationship between the expression of POSTN, N-cadherin and the biological behavior of oral squamous cell carcinoma was analyzed.

## 2.14 | Statistical analyses

All statistical calculations were done using SPSS 13.0 statistical software. Student's *t* test and one-way ANOVA were used to compare the means of 2 groups or more. All tests were two-sided and *P* values <0.05 was considered to be significant.

# 3 | RESULTS

## 3.1 | BMMSC promoted proliferation and inhibited apoptosis of HNC cells

To examine whether MSC promoted the proliferation of HNC cells, CCK-8 assay was carried out on CAL 27 and HN4 cells. At 48 hours and 72 hours, numbers of HNC cells cultured in MSC-CM were significantly increased compared to cells cultured in control media (Figure 1A). Annexin V and propidium iodide were detected by FACS to determine cell apoptosis. Results showed that the apoptosis rate of CAL 27 and HN4 cells cultured in MSC-CM was significantly decreased compared with the control group (Figure 1B). Propidium iodide staining was used to determine cell cycle. Figure 1C shows that MSC-CM significantly altered the cell cycle distribution of CAL 27 and HN4 cells with a tendency towards an increased percentage of cells in the S phase of the cell cycle. We also assessed mRNA expression levels of two factors that negatively regulate cell cycle P16 and P21. Results of real-time PCR showed that cells cultured in MSC-CM expressed significantly lower mRNA levels of both P16 and P21 compared to the control group (Figure 1D). These results suggest that BMMSC promoted proliferation in HNC cell lines by a mechanism associated with altered cell cycle progression and inhibition of cell apoptosis.

### 3.2 | BMMSC promoted migration of HNC cells

Effects of MSC-CM on the migratory ability of CAL 27 and HN4 cells were assessed using Transwell assay and wound-healing assay. Results showed that culture in MSC-CM significantly enhanced migration of CAL 27 and HN4 cells compared to HNC cells cultured in control media (Figure 2). These data indicate that BMMSC could promote the migration of HNC cells.

### 3.3 | BMMSC promoted EMT of HNC cells

CAL 27 or HN4 cells were treated with MSC-CM or control medium for 72 hours, real-time PCR was used to detect gene expression levels of EMT-related molecules which included Snail, Twist, E-cadherin, N-cadherin and Vimentin. Results showed that the expressions of Snail, Twist, N-cadherin and Vimentin were significantly increased and the expression of E-cadherin was significantly decreased in CAL27, HN4 treated with MSC-CM (Figure 3A). Western blot was also used to detect protein expression levels of E-cadherin, N-cadherin and Vimentin. Figure 3B shows that expressions of N-cadherin and Vimentin were increased significantly, and that expression of E-

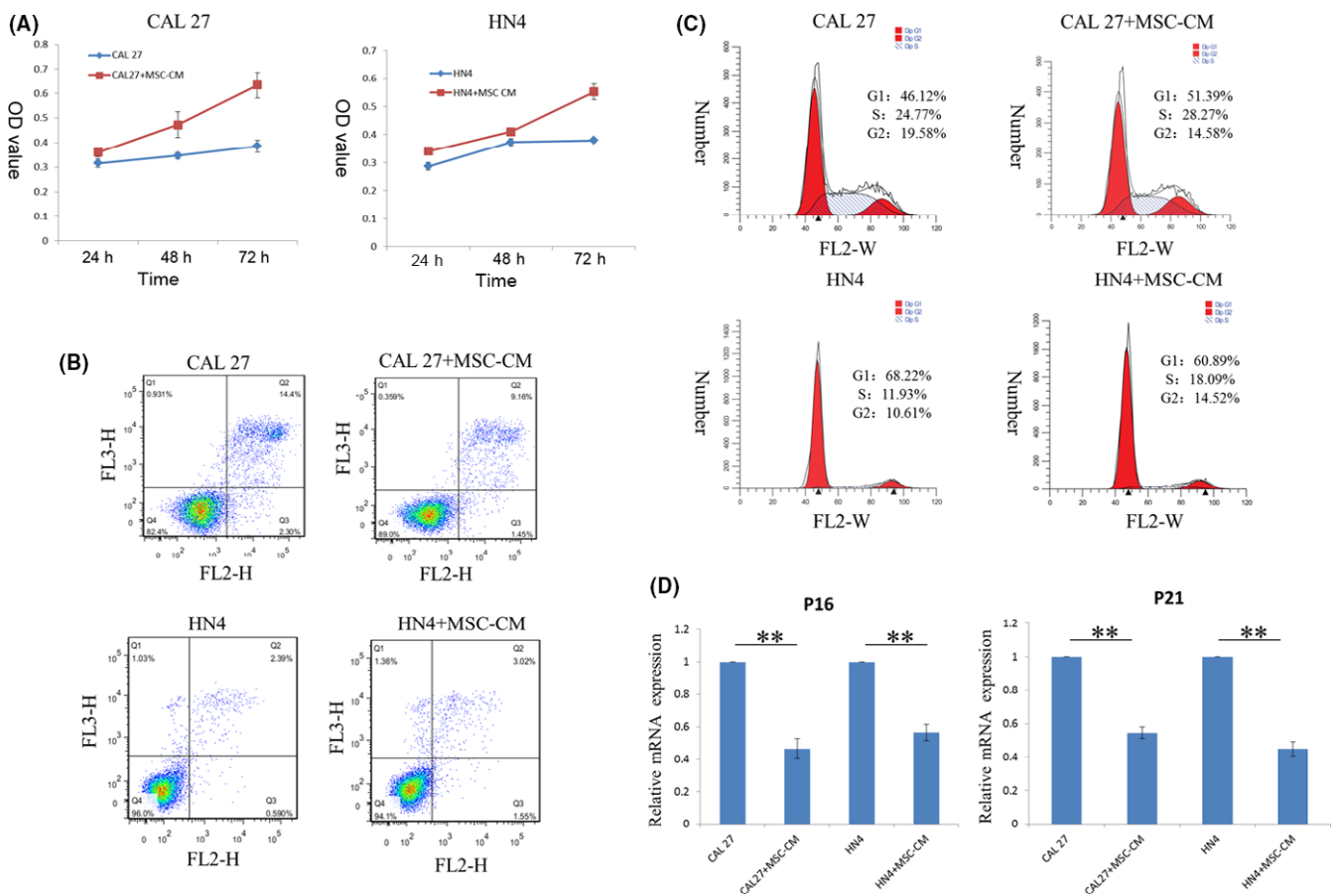
cadherin was decreased significantly when treated with MSC-CM, which suggests that BMMSC could promote EMT of HNC cells.

### 3.4 | BMMSC activated the PI3K/Akt/mTOR pathway in HNC cells

To further investigate the mechanisms by which BMMSC promotes HNC cells, phosphorylated PI3K, Akt and mTOR were analyzed in protein lysates from CAL 27 and HN4 cells cultured in the presence of control media or MSC-CM. Western blot results showed that MSC-CM promoted protein expression of p-PI3K, p-Akt and p-mTOR, which indicated that BMMSC promoted HNC by activating the PI3K/Akt/mTOR signaling pathway (Figure 4A).

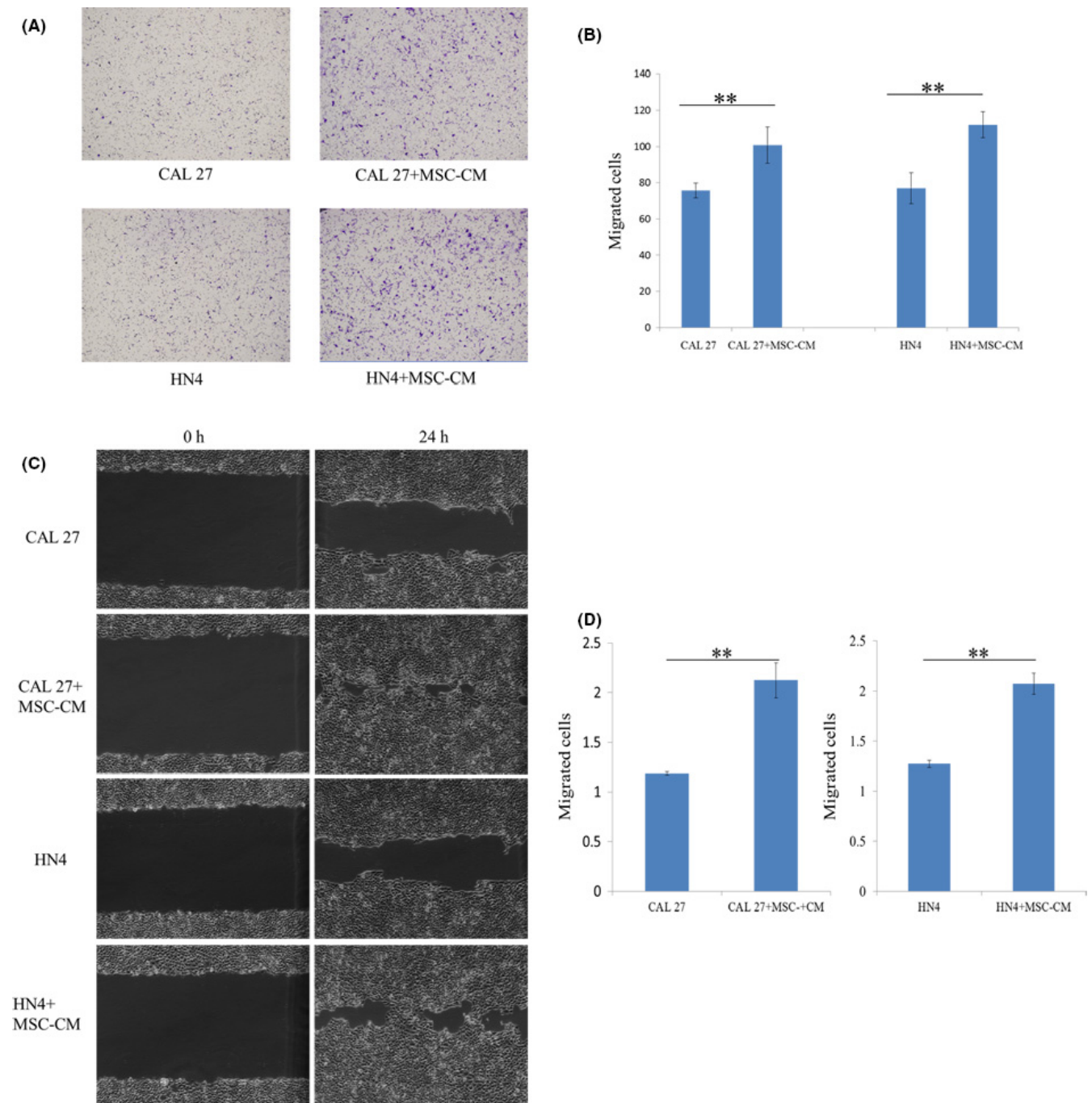
### 3.5 | POSTN activated the PI3K/Akt/mTOR signaling pathway and promoted HNC cells

To further investigate the mechanism of how MSC affect HNC cells, gene and protein expressions of POSTN were detected by real-time PCR and western blot. Figure 4B,C shows that MSC-CM significantly enhanced gene and protein expressions of POSTN. In order to



**FIGURE 1** Mesenchymal stem cells conditioned media (MSC-CM) promoted proliferation and inhibited apoptosis of head and neck cancer (HNC) cells. (A) Cell proliferation was assessed using CCK-8 assay. (B) Cell apoptosis assay was assessed by flow cytometric analysis of Annexin V and propidium iodide. (C) Flow cytometric analysis of cell cycle distribution. (D) Real-time PCR analysis of P21 and P16 mRNA expression. (\*\* $P < .01$ )

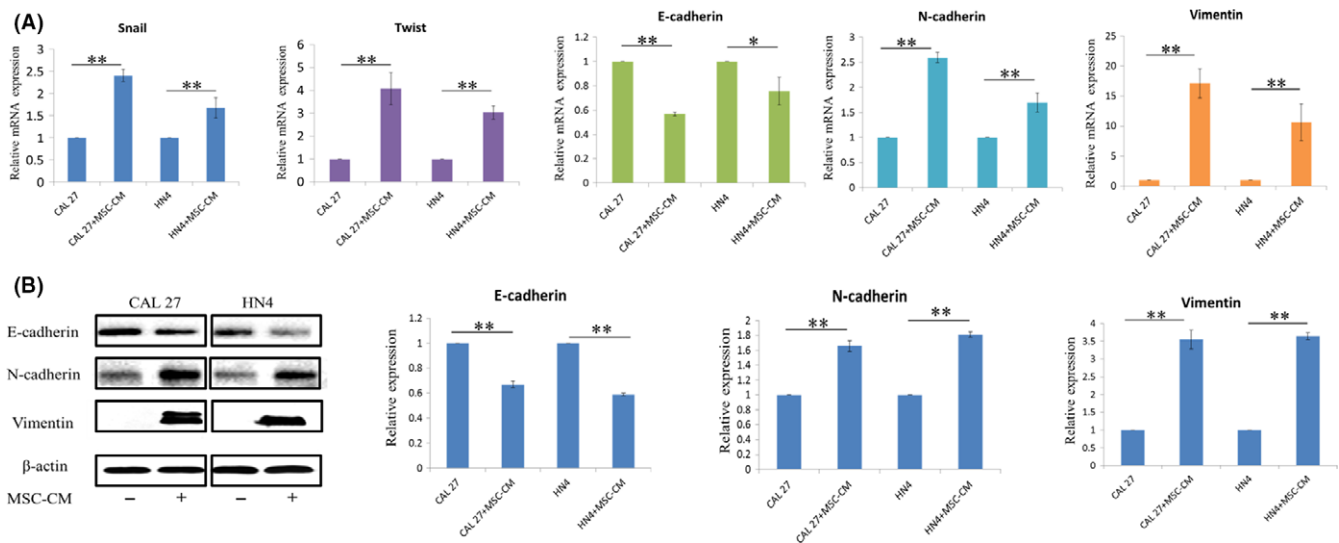




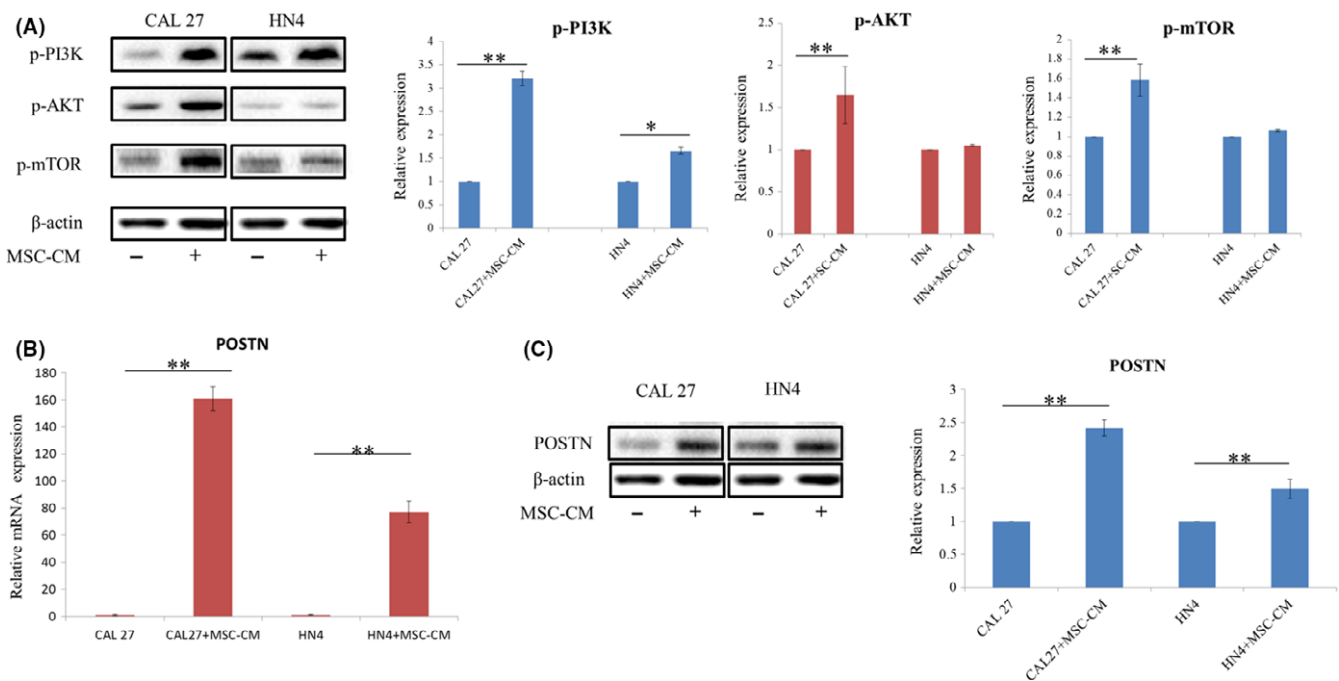
**FIGURE 2** Mesenchymal stem cells conditioned media (MSC-CM) promoted migration of head and neck cancer (HNC) cells. (A,B) Transwell assay shows that MSC-CM promoted the migration of CAL 27 and HN4 cells. (C,D) Wound-healing assay shows that MSC-CM promoted the migration of CAL 27 and HN4 cells. (\*\* $P < .01$ )

evaluate the roles of POSTN on the effect of BMMSC-promoted HNC cells, siRNA was used to silence gene expression of POSTN. Western blot results showed that protein expression of POSTN was silenced effectively (Figure 5A). Biological activity of HNC cells was then detected after silencing POSTN. Results showed that expressions of p-PI3K, p-Akt and p-mTOR were decreased (Figure 5B), the proliferation ability was decreased (Figure 5C), mRNA level of P16 and P21 was increased and migration ability of CAL27 and HN4 was decreased (Figure 5D,E) after silencing POSTN. Figure 5F shows that

expression of E-cadherin was increased and the expressions of N-cadherin and Vimentin were decreased in CAL27 and HN4 after POSTN silencing. These results indicated that the proliferation, migration, EMT and PI3K/Akt/mTOR pathway were inhibited when POSTN was silent. Meanwhile, the proliferation ability was inhibited, mRNA level of P16 and P21 was increased, migration ability was decreased in CAL 27 and HN4 cells when 20  $\mu\text{mol/L}$  PI3K inhibitor LY294002 (Merck Millipore, Billerica, MA, USA) or 0.1 nmol/L mTOR inhibitor Rapamycin (Sigma-Aldrich) was added to MSC-CM



**FIGURE 3** Mesenchymal stem cells conditioned media (MSC-CM) promoted epithelial-mesenchymal transformation (EMT) of head and neck cancer (HNC) cells. CAL 27 or HN4 cells were treated with MSC-CM or control media for 72 h, then gene and protein expression levels of EMT-related molecules were detected. (A) Real-time PCR assay of Snail, Twist, E-cadherin, N-cadherin and Vimentin. (B) Western blot and quantification analysis of E-cadherin, N-cadherin and Vimentin. (\* $P < .05$  and \*\* $P < .01$ )



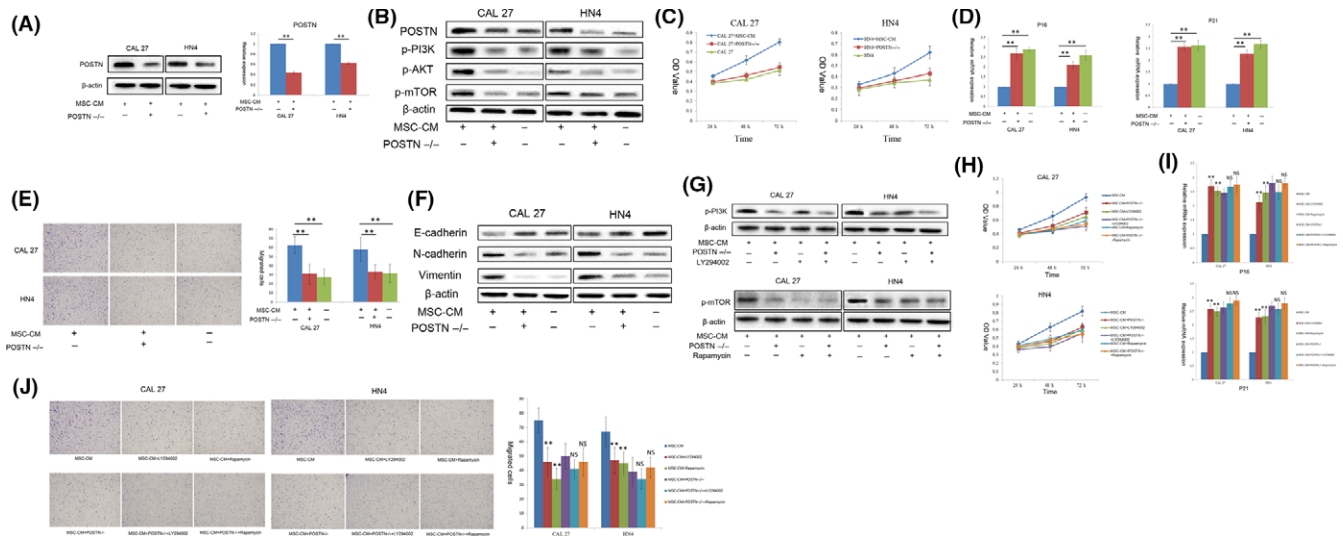
**FIGURE 4** Mesenchymal stem cells conditioned media (MSC-CM) activated the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway and promoted Periostin (POSTN) in head and neck cancer (HNC) cells. CAL 27 and HN4 cells were cultured in MSC-CM or control media for 72 h. (A) Western blot assay of p-PI3K, p-Akt and p-mTOR. (B) Real-time PCR analysis of POSTN mRNA expression. (C) Western blot analysis of POSTN. (\* $P < .05$  and \*\* $P < .01$ )

(Figure 5G-J), which suggests that the POSTN-activated PI3K/Akt/mTOR pathway played important roles in HNC progression.

### 3.6 | BMMSC promoted tumorigenesis in murine models of HNC cells

For the *in vivo* assay, BALB/C nude mice were used to establish a murine xenograft model of HNC. After 14 days, tumors could be

seen in the tongue, and bodyweights of the mice injected with MSC-CM and mixed cells were significantly lower than those in the control group. The mice were killed 5 weeks after injection. Tumors in mice with MSC-CM and mixed cells were bigger than those in the control group. Ki67 staining showed that MSC promoted the formation of xenograft tumors and also increased the extent of tumor invasion (Figure 6A,B). Also, a higher number of mice in the MSC-CM and mixed cells group developed cervical lymph node



**FIGURE 5** Periostin (POSTN)-activated phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway promoted head and neck cancer (HNC) cells. (A) POSTN was silenced effectively by siRNA. (B) PI3K/Akt/mTOR pathway was inhibited when POSTN was silent. (C) Cell proliferation ability tested by CCK-8 assay. (D) mRNA expression of P16 and P21. (E) Cell migration ability tested by Transwell assay. (F) Western blot analysis of E-cadherin, N-cadherin and Vimentin. (G) Western blot assay of p-PI3K and p-mTOR. (H) Cell proliferation ability. (I) mRNA expression of P16 and P21. (J) Cell migration ability. (\*\* $P < .01$ )

metastases than the mice in the control group (Figure 6C). Protein expression of POSTN, E-cadherin and N-cadherin in tumor tissues was detected by western blot. The results showed that the expressions of POSTN in the MSC-CM group and mixed cell group were significantly higher than in the control group (Figure 6D). Expression of E-cadherin was lower and N-cadherin was higher in the MSC-CM group and mixed cell group than in the control group (Figure 6E). These results suggest that BMMSC could promote tumorigenesis and EMT in a murine model of HNC.

### 3.7 | Overexpressed POSTN was related to EMT and tumor progression in HNC clinical samples

IHC was carried out to investigate protein levels of POSTN and N-cadherin in HNC tissues and relatively normal tissues. The results indicated that POSTN was highly expressed in cancer samples compared with normal tissues (Figure 7A). Positive staining of POSTN was mainly located in the cell cytoplasm which showed brown granules. POSTN was positive in most of the cancer tissues, the positive rate being 86% (43/50), whereas POSTN was poorly expressed in normal tissues, the positive rate being 20% (10/50). Figure 7B shows that the positive staining of N-cadherin was also mainly located in the cell cytoplasm. The positive expression rate was 82% (41/50) in cancer tissues and 38% (19/50) in normal tissues, indicating that N-cadherin was highly expressed in cancer samples and that EMT played important roles in HNC.

Expression of POSTN, N-cadherin and their relationship with the biological behavior of HNC were analyzed. There were 30 well-differentiated and 20 moderately or poorly differentiated tumors in the 50 subjects. Expression numbers of each group are shown in Table 1. Difference of both POSTN and N-cadherin between the

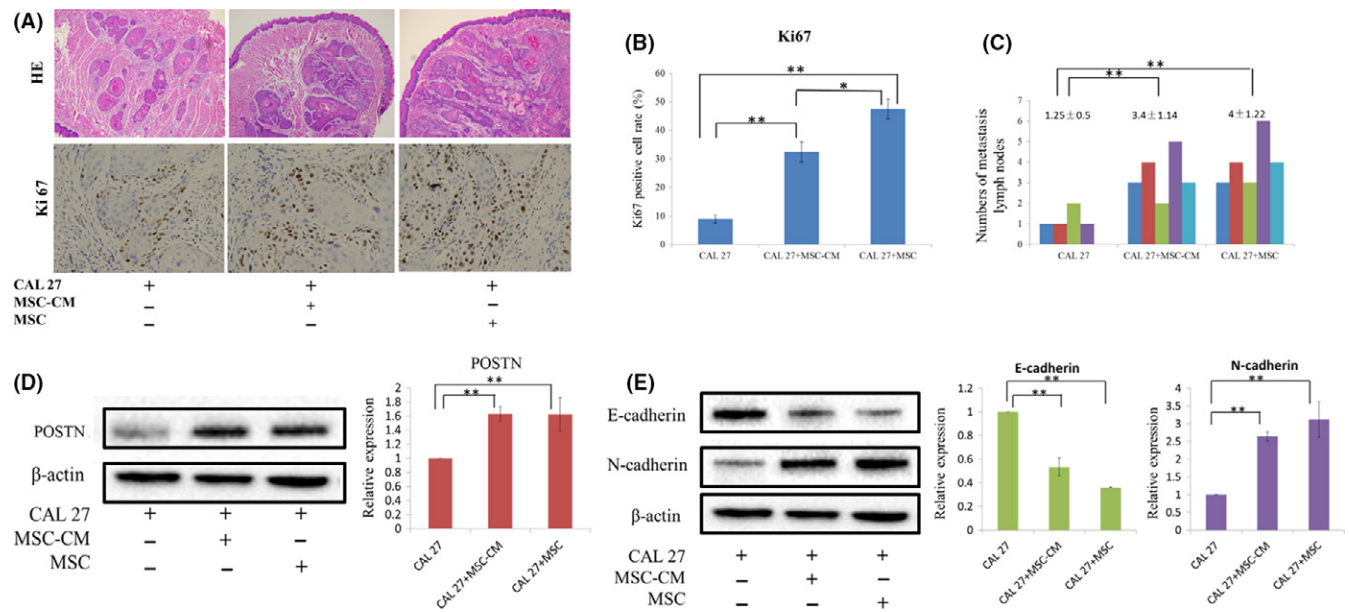
well-differentiated group and the moderately/poorly differentiated group was statistically significant ( $P < .05$ ). According to clinical TNM staging, there were 17 cases in T2 stage, 17 cases in T3 stage and 16 cases in T4 stage. Expression of POSTN and N-cadherin are shown in Table 2, and there was no significant difference between the groups. In the 50 subjects, lymph node metastasis was seen in 22 cases and there were 28 cases with no lymph node metastasis. Expression of POSTN and N-cadherin are shown in Table 3. The difference between the two groups was statistically significant ( $P < .05$ ). The results showed that POSTN and EMT were related to the degree of tumor differentiation and lymph node metastasis, but not related to the TNM staging of the tumors.

Correlation between the expression of POSTN and N-cadherin was also further analyzed. In some cases, expression of POSTN and N-cadherin was consistent. Numbers of both negative, weak positive, positive, and strong positive were 6 cases, 6 cases, 8 cases and 7 cases, respectively. The coincidence rate was 54% (27/50; Table 4), suggesting that the expression of POSTN and N-cadherin was positively correlated and that POSTN was related to EMT in HNC.

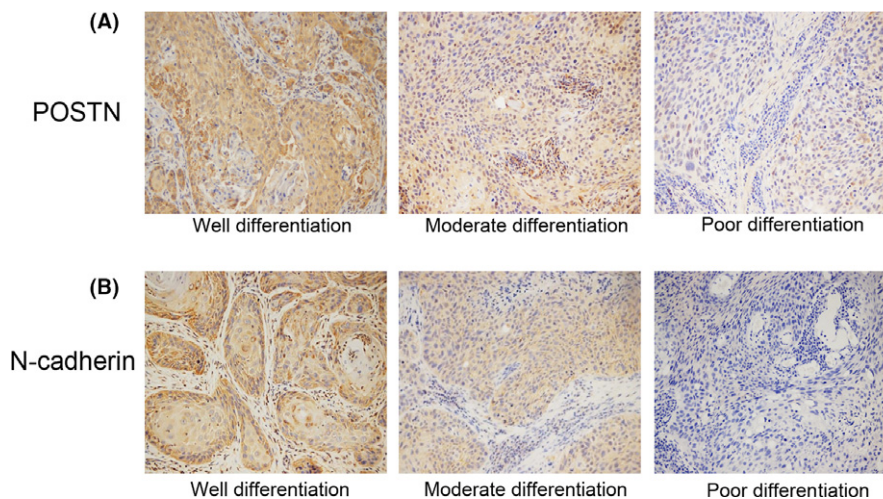
## 4 | DISCUSSION

As an important component of the tumor microenvironment, MSC are associated with tumor development; however, the underlying mechanisms of MSC on HNC remain unexplored and the interactions between HNC and MSC are complex.<sup>7,8</sup> In the present study, we investigated the influence of BMMSC on HNC cells and the associated mechanisms in *in vitro* and *in vivo* experiments. CCK-8 assay showed that BMMSC significantly enhanced the proliferation





**FIGURE 6** Bone marrow mesenchymal stem cells (BMMSC) promoted tumorigenesis in murine models of head and neck cancer (HNC). CAL 27 in control medium, mesenchymal stem cells conditioned media (MSC-CM), and mixed cells of CAL 27 and MSC were injected into the middle of the tongue of BALB/C mice. (A,B) Tumor invasion was assessed using H&E staining and immunohistochemistry (IHC) staining of Ki67. (C) Numbers of neck lymph node metastases were assessed. (D) Western blot analysis of Periostin (POSTN) in tumor tissues. (E) Western blot analysis of E-cadherin and N-cadherin in tumor tissues. (\* $P < .05$  and \*\* $P < .01$ )



**FIGURE 7** Periostin (POSTN) and N-cadherin were highly expressed in head and neck cancer (HNC) cancer samples. Positive staining of POSTN and N-cadherin was mainly located in the cell cytoplasm which showed brown granules. (A) Immunohistochemistry (IHC) staining of POSTN in well, moderate and poorly differentiated HNC tissues. (B) IHC staining of N-cadherin in well, moderate and poorly differentiated HNC tissues

**TABLE 1** Relationship between POSTN and N-cadherin with degree of tumor differentiation

Differentiation	POSTN (cases)				N-cadherin (cases)				Total
	-	+	++	+++	-	+	++	+++	
Well	6	10	8	6	7	12	7	4	30
Moderate or poor	1	2	7	10	2	3	7	8	20
Total	7	12	15	16	9	15	14	12	50

POSTN, Periostin.

of HNC cells. Tumorigenicity and metastatic capacity, hallmarks of advanced tumors, can be induced by stromal cell contact or stimuli in many tumor cells.<sup>28</sup> Likewise, our results showed that BMMSC significantly increased the tumorigenicity and metastatic capacity of HNC cells. Cell cycle analysis showed that BMMSC significantly increased the proportion of S phase cells, and decreased the mRNA and protein levels of P21 and P16, which showed that the ability of BMMSC to promote the proliferation of HNC cells was linked to cell cycle regulators. EMT can increase the migration of epithelial

**TABLE 2** Relationship between POSTN and N-cadherin with TNM staging of tumors

TNM staging	POSTN (cases)				N-cadherin (cases)				Total
	-	+	++	+++	-	+	++	+++	
T2	3	5	5	4	4	7	4	2	17
T3	2	4	6	5	3	4	6	4	17
T4	2	3	4	7	2	4	4	6	16
Total	7	12	15	16	9	15	14	12	50

POSTN, Periostin.

**TABLE 3** Relationship between POSTN and N-cadherin with lymph node metastasis

Metastasis	POSTN (cases)				N-cadherin (cases)				Total
	-	+	++	+++	-	+	++	+++	
No	5	10	9	4	7	12	7	2	28
Yes	2	2	6	12	2	3	7	10	22
Total	7	12	15	16	9	15	14	12	50

POSTN, Periostin.

**TABLE 4** Correlation between the expression of POSTN and N-cadherin in head and neck cancer

POSTN (cases)	N-cadherin (cases)				Total
	-	+	++	+++	
-	6	1	0	0	7
+	3	6	2	1	12
++	0	5	8	2	15
+++	0	2	4	7	16
Total	9	15	14	12	50
P-value	.000				

POSTN, Periostin.

tissue.<sup>29</sup> In the current study, gene and protein expressions of EMT-related molecules Snail, Twist, E-cadherin, N-cadherin and Vimentin were detected, suggesting that MSC could promote EMT in HNC. Taken together, these results strongly suggest that BMMSC promoted the progression of HNC.

PI3K/Akt/mTOR signaling pathway has emerged as one of the most frequently deregulated in HNC.<sup>30</sup> Numerous alterations of various upstream and downstream components have been described. mTOR activation was a potential prognostic biomarker and an inverse predictor of lymph node and distant metastases. The current study also showed that BMMSC up-regulated the levels of phosphorylated PI3K/Akt/mTOR in HNC cells. This indicated that activation of the PI3K/Akt/mTOR signaling pathway played important roles in the ability of BMMSC to promote the progression of HNC.

POSTN plays a functional role in the regeneration of tissues such as bone and heart, promotes wound healing and is frequently up-regulated in various types of human cancers and is implicated in cancer cell proliferation, invasion, and metastasis.<sup>31,32</sup> However, whether POSTN is associated with the effect of BMMSC on HNC had not yet been previously addressed. In this study, we found that MSC-CM could promote the expression of POSTN possibly because BMMSC could secrete many kinds of cytokines into MSC-CM such as interleukin-6 (IL-6), interleukin-10 (IL-10), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF- $\beta$ ) etc. which promoted POSTN expression in HNC.<sup>33-35</sup> The current study also showed that the POSTN-activated PI3K/Akt/mTOR pathway played important roles on the effect of BMMSC on HNC cells. Moreover, BMMSC promoted tumor growth, invasion, formation of metastatic lesions and promoted the expression of POSTN and EMT in tumor tissues in the mouse model of HNC. The positive correlation between the expression of POSTN and EMT in the development of HNC was also further confirmed in HNC clinical samples.

In conclusion, our work showed the relationship between BMMSC and HNC. We showed that BMMSC promoted proliferation, invasion, survival, tumorigenicity and migration of HNC through POSTN-mediated PI3K/Akt/mTOR activation. Further exploration of relative proteins and pathways will help to fully elucidate the tumorigenic effect of BMMSC in HNC.

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## CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

## ORCID

Huiming Wang  <http://orcid.org/0000-0001-5077-7481>

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