

HCRP-1 alleviates the malignant phenotype and angiogenesis of oral squamous cell carcinoma cells via the downregulation of the EGFR/STAT3 signaling pathway

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Abstract. It has previously been reported that human hepatocellular carcinoma-related protein 1 (HCRP-1), which is a tumor suppressor gene, and epidermal growth factor receptor (EGFR) are abnormally expressed in certain solid tumors. Therefore, in the present study, the expression patterns of HCRP-1 in oral squamous cell carcinoma (OSCC) are discussed. Moreover, the present study investigated whether HCRP-1 regulated EGFR expression levels and its downstream effectors to further determine the regulation of tumor cell behavior. Therefore, the expression levels of HCRP-1 in normal oral epithelial and OSCC cells were determined, and the effects of HCRP-1 overexpression on OSCC cell proliferation, migration and invasion were assessed. Moreover, the culture medium from the different groups of OSCC cells was separately supplemented into the human umbilical vein endothelial cell (HUVEC) cultures, and the migration and angiogenesis of the HUVECs were assessed. To determine the roles of EGFR/STAT3 in the regulation of HCRP-1, EGF and colivelin, a STAT3 agonist, were used to treat CAL-27 cells and their effects on the cells were assessed using the aforementioned functional experiments. The results demonstrated that HCRP-1 expression levels were downregulated in OSCC cells and that HCRP-1 overexpression could suppress OSCC cell proliferation, migration and invasion. Moreover, the culture medium from OSCC cells overexpressing HCRP-1 facilitated the migration and angiogenesis of HUVECs. Furthermore, HCRP-1 was demonstrated to function in cells by regulating the EGFR/STAT3 signaling pathway. In summary, the present study indicated that HCRP-1 alleviated

the malignant phenotype and angiogenesis of OSCC cells via the downregulation of the EGFR/STAT3 signaling pathway.

Introduction

Head and neck squamous cell carcinoma (HNSC) is one of the most common types of malignant tumors worldwide, and its onset is closely associated with alcohol consumption, tobacco use and human papillomavirus (1). HNSC has high morbidity and mortality rates, and >90% of patients are susceptible to oral squamous cell carcinoma (OSCC). OSCC is the most devastating and common oral malignancy that accounts for 95% of all oral cancer types and causes 500,000 deaths/year (2). OSCC tends to occur in the tongue, cheeks and gums; however, in the advanced stages, it can also involve the whole tongue, pharynx, jawbone, and vital blood vessels and nerves in the neck and skull base. This results in numbness, pain and the significant impairment of speech and swallowing (3,4). The development of OSCC occurs via a multi-stage process, which is accompanied by invasion, metastasis and precancerous lesions. Furthermore, OSCC development is a consequence of multiple genes, such as HNRNPA2B1, UBE2C and Rab31 (5-7). Moreover, environmental and genetic factors can regulate the occurrence and progression of OSCC; however, the specific etiology of the disease remains unclear (8). Early detection and treatment are important for patient prognosis, and OSCC is considered to be a preventable disease (9). Therefore, it is crucial to deeply understand the underlying mechanisms in the occurrence and progression of OSCC.

Human hepatocellular carcinoma-related protein 1 (HCRP-1), which is also known as vacuolar protein sorting-associated protein 37A, is a subunit of the endosomal sorting complexes required for the transport I protein family, which mediates the internalization process of membrane protein ubiquitination in cells (10). HCRP-1 regulates the cell cycle, proliferation, migration and apoptosis, and maintains the survival of precursor cells prior to cell differentiation. Previous studies have demonstrated that HCRP-1 is a tumor suppressor gene that affects tumor progression, with low expression in various tumors, including prostate (11), breast (12), liver (13) and non-small cell lung (14) cancer. For example, expression levels of HCRP-1 are decreased in

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colon cancer tissues and its knockdown promotes cell invasion and migration (15). Furthermore, HCRP-1 has been reported to significantly inhibit cell proliferation, invasion and the epithelial-mesenchymal transition in esophageal squamous cell carcinoma (16). To date, the expression pattern of HCRP-1 in OSCC and its clinical significance remain to be elucidated.

Epithelial growth factor receptor (EGFR) is widely distributed on the cell surface of mammalian epithelial cells, including fibroblasts and glial cells; it is mainly composed of the extracellular ligand-binding region, the transmembrane region and the intracellular region kinase domain (17). EGFR is a glycoprotein that belongs to the tyrosine kinase-type receptor family and is a receptor for EGF, which is responsible for cell proliferation and signal transduction (18). It has been reported that EGFR is abnormally expressed in certain solid tumors and serves a role in tumor proliferation, angiogenesis, metastasis and apoptosis (19,20).

Therefore, in the present study, the expression pattern of HCRP-1 in OSCC is discussed. Moreover, whether HCRP-1 regulates EGFR expression levels, along with its downstream effectors, is investigated to determine the regulatory mechanism of OSCC tumor cell behavior.

Materials and methods

Cell culture and transfection. Human immortalized oral epithelial cells were purchased from Qingqi (Shanghai) Biotechnology Development Co., Ltd. OSCC CAL-27, Fadu, SCC-4 and SCC-15 cell lines were purchased from the American Type Culture Collection, and immortalized HUVECs were purchased from Cobioer Biosciences Co., Ltd. Cells were incubated in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Shanghai Grammar Biotechnology Co., Ltd.) at 37°C with 5% CO₂. Exogenous EGF (30 ng/ml, Sigma-Aldrich; Merck KGaA) (21) and colivelin (1 µM; Selleck Chemicals) (22) were used to treat CAL-27 cells to activate EGFR and STAT3, respectively.

CAL-27 cells were transfected with 4 µg specific pcDNA3.1 plasmids (VectorBuilder, Inc.) to overexpress HCRP-1 (ov-HCRP-1 group). Cells transfected with 4 µg empty pcDNA3.1 plasmids were used as negative controls (ov-NC group). Transfection was performed using FuGENE HD reagent [Roche Diagnostics (Shanghai) Co., Ltd.] at 37°C for 24 h and transfection efficiency was confirmed via reverse transcription-quantitative PCR (RT-qPCR) and western blotting. Cells were collected for subsequent experiments after 24 h co-culture at 37°C.

RT-qPCR. Total RNA was extracted from untransfected oral epithelial cells and OSCC cells and complementary DNA was produced using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and the PrimeScript RT Reagent Kit (Takara Biotechnology Co., Ltd.), respectively, according to the manufacturers' protocols. qPCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen China Co., Ltd.). The following thermocycling conditions were used: Initial denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and elongation at 72°C for 30 sec; and a final extension at 72°C for 7 min. Relative

HCRP-1 mRNA expression levels were normalized against GAPDH and quantified using the 2^{-ΔΔC_q} method (23). HCRP-1 forward, 5'-CTGGCTTTTCCCCTGACCA-3' and reverse 5'-AGTGTGAGTTCCGGAGGGA-3'; and GAPDH forward, 5'-GACTCATGACCACAGTCCATGC-3' and reverse, 5'-AGAGGCAGGGATGATGTTCTG-3'.

Western blotting. Proteins were extracted from untransfected oral epithelial cells and OSCC cells or transfected OSCC cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and total protein was quantified using Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.). Total protein (40 µg per lane) was separated on a 10% gel using SDS-PAGE on a polyacrylamide gel and then the separated proteins were transferred to a PVDF membrane [Roche Diagnostics (Shanghai) Co., Ltd.]. The membranes were blocked using skimmed milk for 1 h at room temperature. Subsequently, the membranes were incubated at 4°C overnight with the following primary antibodies against: HCRP-1 (cat. no. ab251760; 1:5,000), MMP9 (cat. no. ab283575; 1:1,000), MMP14 (cat. no. ab51074; 1:5,000), EGFR (cat. no. ab32077; 1:5,000), phosphorylated (p)-EGFR (cat. no. ab40815; 1:2,000), STAT3 (cat. no. ab109085; 1:1,000) and p-STAT3 (cat. no. ab267373; 1:1,000). Following the primary incubation, the membranes were incubated with HRP-conjugated secondary antibody (cat. no. ab6721; 1:5,000) for 2 h at room temperature. All antibodies were purchased from Abcam. Blots were visualized using an ECL detection reagent (MilliporeSigma) and data were analyzed using ImageJ 1.52 software (National Institutes of Health).

Cell counting kit-8 (CCK-8) assay. Transfected CAL-27 cells (3×10³ cells/well) were seeded into a 96-well plate, treated with EGF and colivelin, and incubated for 24, 48 or 72 h. At each time point, each well was supplemented with 10 µl CCK-8 solution (Dojindo Laboratories, Inc.). Absorbance was assessed using a microplate reader (Perlong Medical Equipment Co., Ltd.) at 450 nm following incubation for 2 h.

Colony formation assay. Transfected CAL-27 cells (500 cells/dish) were seeded into culture dishes, treated with EGF and colivelin, and cultured at 37°C for 2 weeks. Subsequently, the cells were fixed with 4% paraformaldehyde (Shanghai Aladdin Biochemical Technology Co. Ltd.) for 15 min and stained with crystal violet (Shanghai Yeasen Biotechnology Co., Ltd.) for 30 min, both at room temperature. Images were captured of the results and a cluster of >50 cells was regarded as a colony. The number of colonies was counted manually.

Wound healing assay. Transfected CAL-27 cells (5×10⁵ cells/well) were seeded into a 6-well plate, treated with EGF and colivelin, and cultured at 37°C until an 80% confluent monolayer formed. A sterile pipette tip was used to generate a wound in the middle of the monolayer. Cells were washed with serum free medium to remove floating cells and then incubated in DMEM supplemented with 1% FBS at 37°C. Images were captured at 0 and 24 h using a light microscope (magnification, x100; Olympus Corporation). The relative migration rate was calculated as (0 h scratch width-24 h scratch width)/0 h scratch width ×100%.

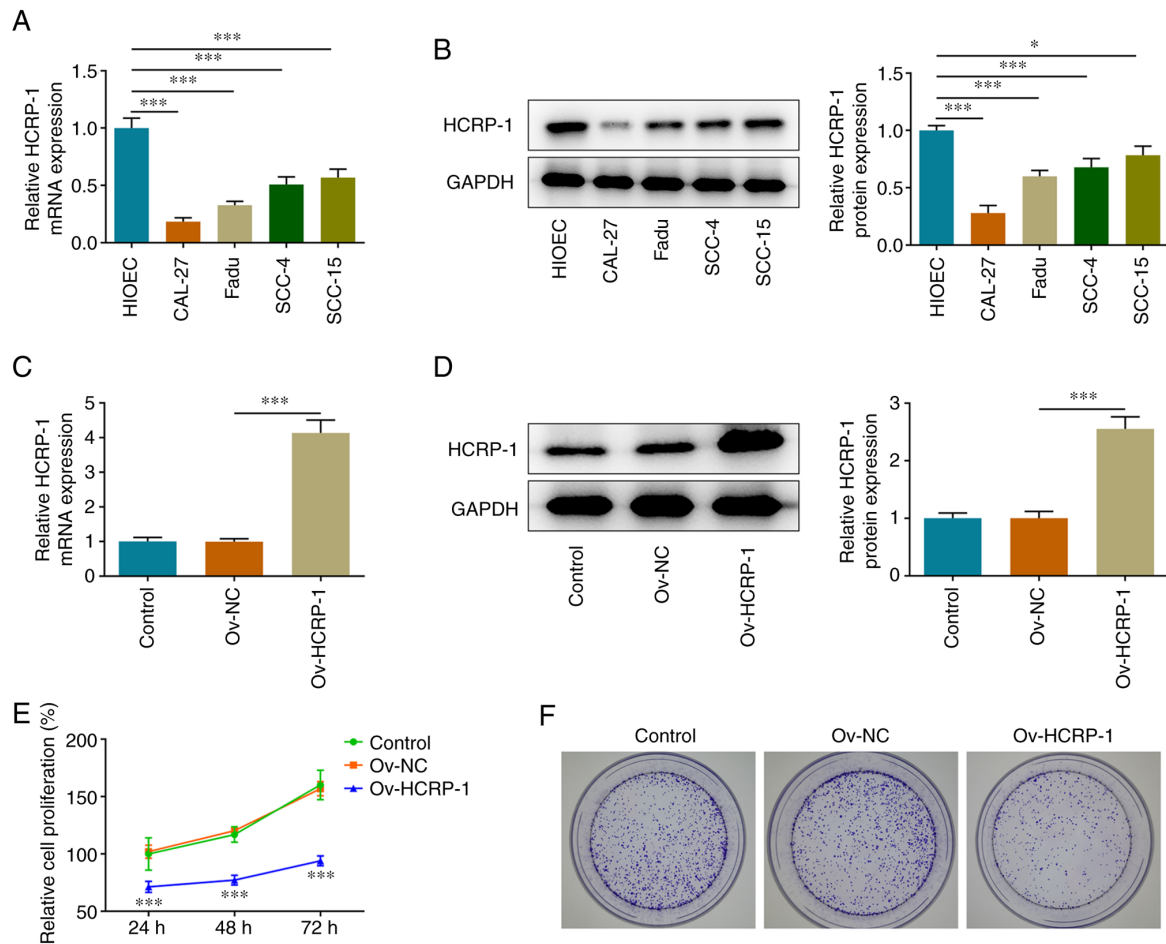


Figure 1. HCRP-1 overexpression inhibits OSCC cell proliferation. HCRP-1 mRNA and protein expression levels in oral epithelial and OSCC cells were determined using (A) RT-qPCR and (B) western blotting, respectively. CAL-27 cells were transfected to overexpress HCRP-1, and (C) RT-qPCR and (D) western blotting were performed to verify transfection efficiency. * $P<0.05$ and *** $P<0.001$. Transfected cell proliferation was assessed using the (E) Cell Counting Kit-8 and (F) colony formation assays. *** $P<0.001$ vs. Ov-NC. HCRP-1, hepatocellular carcinoma-related protein 1; OSCC, oral squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative PCR; Ov-, overexpression; NC, negative control; HIOEC, human immortalized oral epithelial cell.

Transwell assay. To assess the invasion of transfected CAL-27 cells or the migration of HUVECs following EGF and colivelin treatment, a cell suspension (1×10^5 cells) was added to the upper chamber (8 μ m; Corning, Inc.) of 24-well Transwell plates (8.0- μ m PET membrane; Corning, Inc.) in 400 μ l serum-free DMEM precoated with or without Matrigel (BD Biosciences) as described previously (24). RPMI-1640 with 20% FBS was added to the lower chamber. Following 24 h of incubation at 37°C, the cells on the lower surface were fixed with 90% ethanol solution for 30 min at 37°C and stained with 0.1% crystal violet for 10 min at room temperature. Images were captured using a light microscope (magnification, x100; Olympus Corporation).

Angiogenesis assay. HUVECs (8×10^4 cells/well) were seeded into a 24-well plate precoated with Matrigel as described previously (25) and cultured until adherence. The original culture medium was substituted with the culture medium from untransfected CAL-27 cells, CAL-27 cells transfected with Ov-NC, Ov-HCRP-1 plasmids and transfected CAL-27 cells treated with EGF and colivelin followed by incubation at 37°C for 6 h. The structure of the tubes was observed using a light microscope (magnification, x4; Olympus Corporation) and the

images were analyzed using ImageJ 1.52 software (National Institutes of Health).

Statistical analysis. Experiments were independently performed three times. Data are presented as the mean \pm SD and statistical analysis was performed using Prism 8.0 software (GraphPad Software, Inc.). One-way ANOVA followed by Tukey's post hoc test was used to compare the statistical differences between three or more groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HCRP-1 overexpression inhibits OSCC cell proliferation. The expression levels of HCRP-1 in oral epithelial and OSCC cells were determined using RT-qPCR and western blotting. The results demonstrated that HCRP-1 expression levels were significantly downregulated in OSCC cells compared with HIOEC cells. The most significant decline in HCRP-1 expression was noted in CAL-27 cells and these cells were therefore selected for use in the subsequent assays to highlight the underlying role of HCRP-1 (Fig. 1A and B). CAL-27 cells were transfected to overexpress HCRP-1, and RT-qPCR and

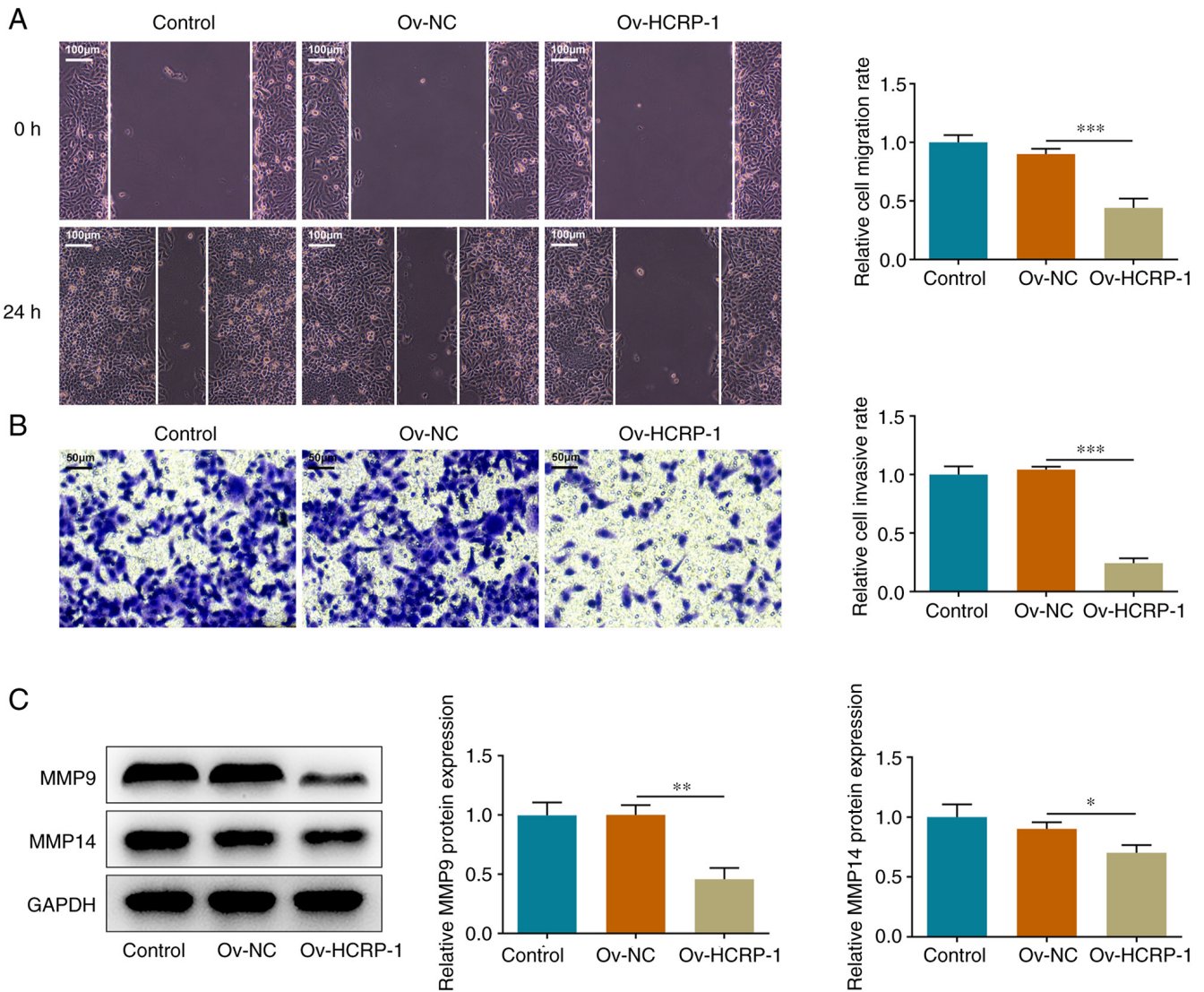


Figure 2. HCRP-1 overexpression inhibits oral squamous cell carcinoma cell migration and invasion. Effects of HCRP-1 overexpression on cell migration and invasion were assessed using (A) wound healing (scale bar, 100 μ m) and (B) Transwell (scale bar, 50 μ m) assays. (C) Protein expression levels of MMP9 and MMP14 were determined via western blotting. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. HCRP-1, hepatocellular carcinoma-related protein 1; Ov-, overexpression; NC, negative control.

western blotting were performed to verify transfection efficiency (Fig. 1C and D). The proliferation of transfected cells was subsequently assessed using the CCK-8 and colony formation assays. HCRP-1 overexpression significantly reduced the proliferation of CAL-27 cells (Fig. 1E) and the number of colonies formed was also markedly reduced compared with the ov-NC group (Fig. 1F).

HCRP-1 overexpression inhibits OSCC cell migration and invasion. The effects of HCRP-1 overexpression on cell migration and invasion were subsequently assessed. The migration rate was quantified using the wound healing assay. The results demonstrated that cell migration in the Ov-HCRP-1 group was suppressed compared with that in the Ov-NC group (Fig. 2A). The results from the Transwell assay also indicated that HCRP-1 overexpression suppressed the invasion ability of the CAL-27 cells (Fig. 2B). Furthermore, the protein expression levels of MMP9 and MMP14 were determined using western blotting. Compared with the

Ov-NC group, the protein expression levels of MMP9 and MMP14 were significantly decreased in the Ov-HCRP-1 group (Fig. 2C).

HCRP-1 overexpression inhibits HUVEC migration and angiogenesis. The culture medium from the control, Ov-NC and Ov-HCRP-1 groups of CAL-27 cells was separately supplemented into the HUVEC culture and the migration ability of the HUVECs was assessed using the Transwell assay. HUVECs in the culture medium from the control group possessed a relatively strong migratory capacity, whereas the migratory capacity of the cells in the culture medium from the Ov-HCRP-1 group was inhibited (Fig. 3A). Moreover, the tube formation ability in these groups of HUVECs was assessed using the angiogenesis assay. Similar to the aforementioned results, the culture medium from the control group promoted the angiogenesis of HUVECs, whereas the culture medium from the Ov-HCRP-1 group markedly suppressed angiogenesis, which resulted in a smaller number of junctions (Fig. 3B).

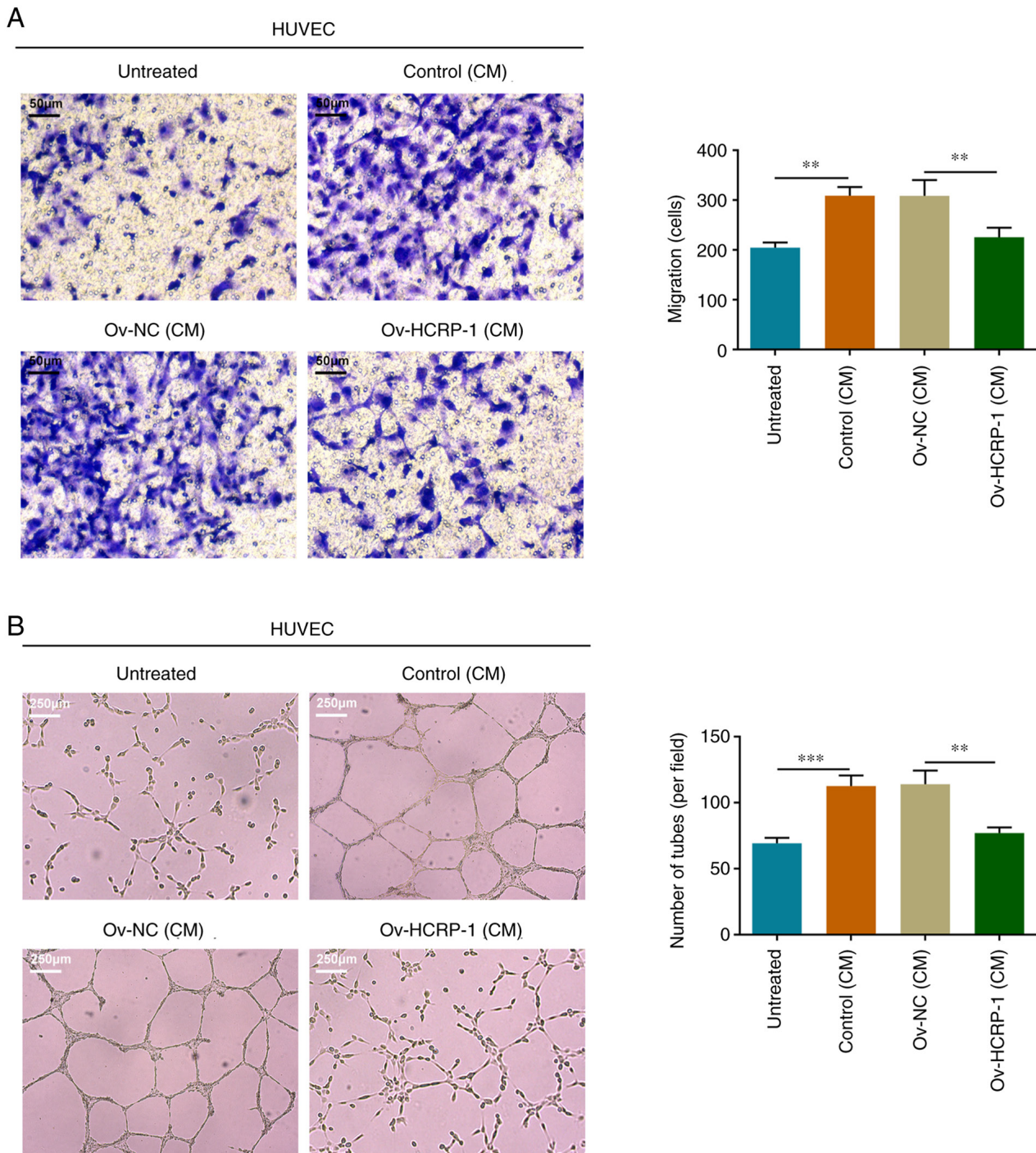


Figure 3. HCRP-1 overexpression inhibits HUVEC migration and angiogenesis. (A) CM from the control, Ov-NC and Ov-HCRP-1 groups of CAL-27 cells were separately supplemented into the HUVEC CM, and the migration of HUVECs was assessed using the Transwell assay (scale bar, 50 μ m). (B) Angiogenesis capacity in each group of HUVECs was assessed using the angiogenesis assay (scale bar, 250 μ m). ** P <0.01 and *** P <0.001. HCRP-1, hepatocellular carcinoma-related protein 1; Ov, overexpression; NC, negative control; CM, culture medium.

HCRP-1 overexpression inhibits the EGFR/STAT3 signaling pathway in OSCC cells. The expression levels of proteins associated with the EGFR/STAT3 signaling pathway were determined using western blotting. The protein expression levels of p-EGFR and p-STAT3 were significantly decreased in the Ov-HCRP-1 group, whereas EGF treatment reversed this decrease (Fig. 4A). To assess the roles of EGFR and STAT3 in the regulation of HCRP-1, EGF and colivelin were used to treat CAL-27 cells. The proliferation of cells was determined

using the CCK-8 and colony formation assays. EGF and colivelin treatment both promoted proliferation compared with the Ov-HCRP-1 group (Fig. 4B and C).

HCRP-1 functions via the regulation of the EGFR/STAT3 signaling pathway. Subsequently, the effects of EGF and colivelin on the migration and invasion of CAL-27 cells were assessed. The results from the wound healing and Transwell assays demonstrated that EGF and colivelin treatment

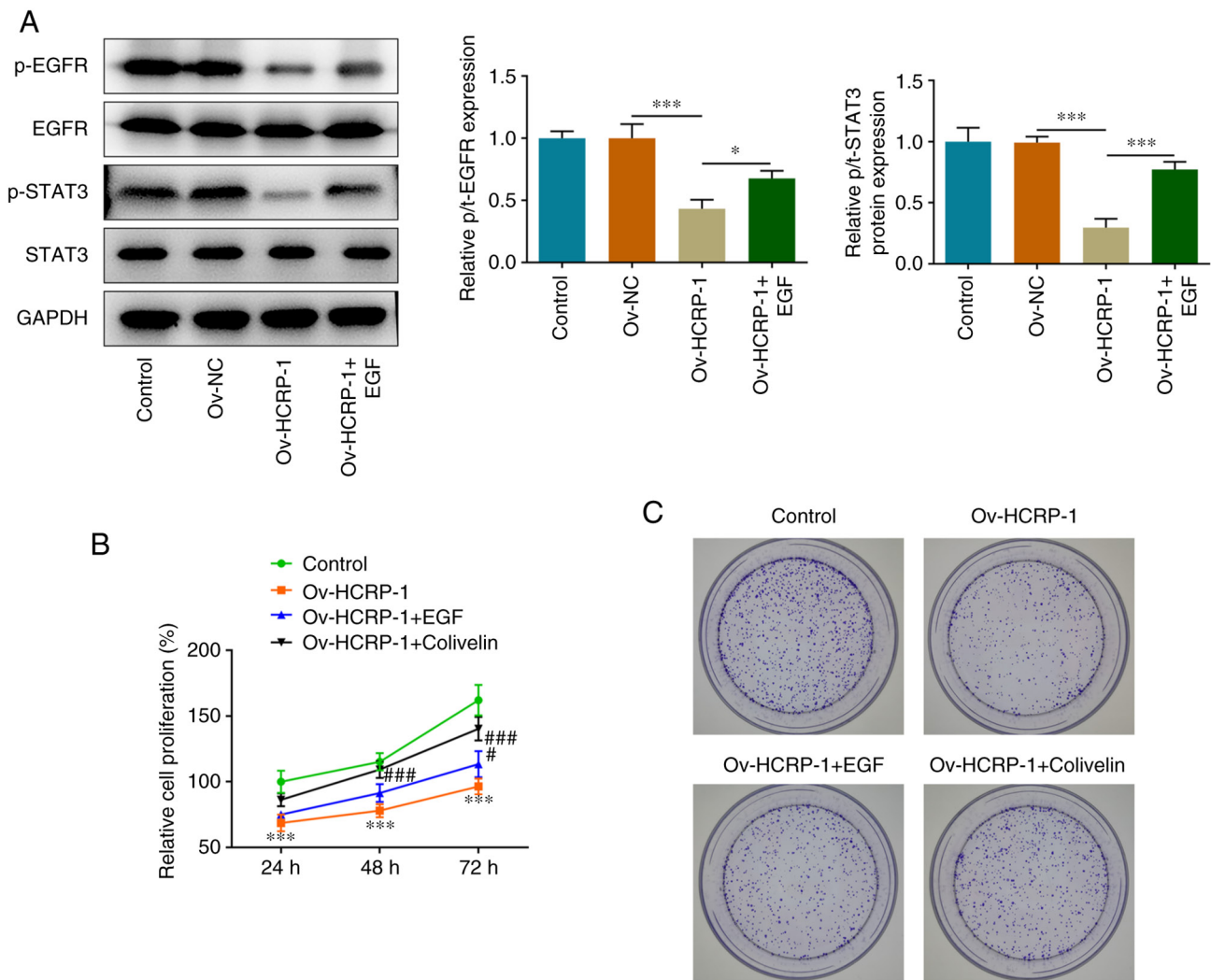


Figure 4. HCRP-1 overexpression inhibits the EGFR/STAT3 signaling pathway in oral squamous cell carcinoma cells. (A) Western blotting was used to determine the protein expression levels of proteins associated with the EGFR/STAT3 signaling pathway. * $P<0.05$ and *** $P<0.001$. Cell proliferation was determined using the (B) Cell Counting Kit-8 (* $P<0.001$ vs. control; * $P<0.05$ and *** $P<0.001$ vs. Ov-HCRP-1) and (C) colony formation assays. HCRP-1, hepatocellular carcinoma-related protein 1; Ov, overexpression; EGFR, epidermal growth factor receptor; p/t-, phosphorylated/total.

accelerated cell migration and invasion (Fig. 5A and B). These results were supported by the increase exhibited in the MMP9 and MMP14 protein expression levels (Fig. 5C). Furthermore, the migration and angiogenesis in each group of HUVECs were assessed. EGF and colivelin treatment facilitated the migration and angiogenesis of HUVECs, which indicated that the activation of EGFR/STAT3 signaling reversed the effects of HCRP-1 overexpression on cells (Fig. 6).

Discussion

The special anatomical structure and environment of the oral cavity, the abundant blood supply of the maxillofacial region, and the abundant lymph node tissue of the maxillofacial region and neck have led to the limitation of conventional surgical treatment and radio-chemotherapy for OSCC (26). The 5-year survival rate of patients has not significantly improved and remains at only 50% (27,28). Moreover, for patients with advanced OSCC with recurrence or distant metastasis, the 5-year survival rate is <50% (29). With the

development of molecular targeted therapy and individualized treatment, clinical multidisciplinary comprehensive treatment has gradually emerged, which provides a novel opportunity for the treatment of OSCC (30-32). A recent study by Yokokawa *et al* (33) reported that, according to data from 208 patients with OSCC following post-surgical treatment, EGFR overexpression can be regarded as an indicator to evaluate patient prognosis. Moreover, the molecular targeted drug cetuximab has also been approved for the clinical treatment of OSCC and has previously been shown to achieve significant efficiency (34). These aforementioned studies have therefore demonstrated the use of EGFR as an important therapeutic target.

However, the application of cetuximab still has certain difficulties including mutations, toxicity/side effects, drug resistance and an optimal dosage to administer (35-38). Furthermore, EGFR level detection needs to be performed prior to the administration of cetuximab. At present, each detection method has both advantages and disadvantages, and there is no unified standard, which leads to different results in the same patient (39). Alternatively, downstream

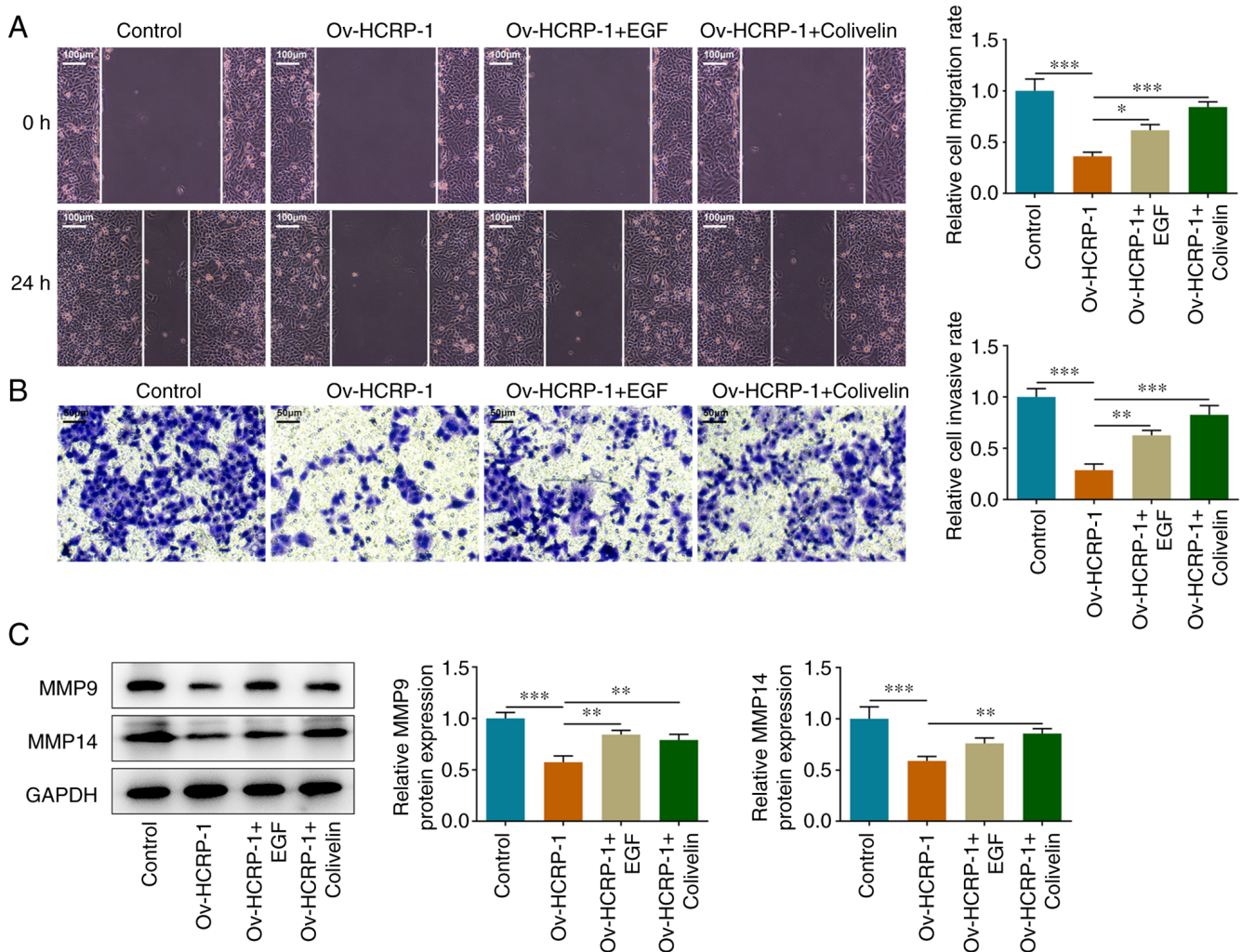


Figure 5. HCRP-1 in CAL-27 cells functions via the regulation of the EGFR/STAT3 signaling pathway. Effects of EGF and colivelin on the migration and invasion of CAL-27 cells were assessed using the (A) wound healing (scale bar, 100 μ m) and (B) Transwell assays (scale bar, 50 μ m). (C) Western blotting was performed to determine the protein expression levels of MMP9 and MMP14. * P <0.05, ** P <0.01 and *** P <0.001. HCRP-1, hepatocellular carcinoma-related protein 1; EGF, epidermal growth factor; Ov-, overexpression.

or upstream regulation from a known target can also be used as a potential approach. Therefore, in the present study, compared with that in oral epithelial cells, the expression of HCRP-1 was downregulated in OSCC cells and HCRP-1 overexpression could inhibit cell proliferation, migration, invasion and angiogenesis. These results suggested that HCRP-1 may potentially act as a tumor suppressor in OSCC as well as in the aforementioned types of cancer (prostate, breast, liver, colon and non-small cell lung cancer). Subsequently, it was demonstrated that HCRP-1 overexpression inhibited EGFR phosphorylation and that EGF treatment markedly increased EGFR expression in cells, which reversed the effects of HCRP-1 overexpression. EGFR signaling is involved in the malignant process of OSCC (40) and it can therefore be hypothesized that HCRP-1 can alleviate the malignant phenotype of cells via the inhibition of EGFR.

EGFR binds to EGF and undergoes homodimerization or heterodimerization, which results in the phosphorylation of intracellular tyrosine residues. Subsequently activated receptors recruit signaling complexes, activate downstream

signaling proteins, and finally regulate tumor cell proliferation and metastasis (41,42). Therefore, in the present study, the protein expression levels of downstream STAT3 signaling pathway proteins were investigated. The results demonstrated that HCRP-1 also inhibited the phosphorylation of STAT3. Moreover, colivelin treatment reversed the inhibition of HCRP-1 overexpression on cell malignant progression, which suggested that STAT3 signaling potentially mediated the regulatory mechanism of HCRP-1 on cells. The present study therefore demonstrated the regulatory pattern of HCRP-1 in OSCC cells. However, a limitation of the present study is that only *in vitro* experiments were included, and therefore, animal experiments should be performed in future work. In the present study, HCRP-1 was overexpressed to explore its role in OSCC, and knocking it down could be used to verify the present study findings in the future. Data is yet to be located that mentions HCRP-1 expression in patients with OSCC samples in public databases; therefore, this will be an area for further research.

In conclusion, in the present study, HCRP-1 potentially alleviated the malignant phenotype and angiogenesis of OSCC cells via the downregulation of EGFR/STAT3

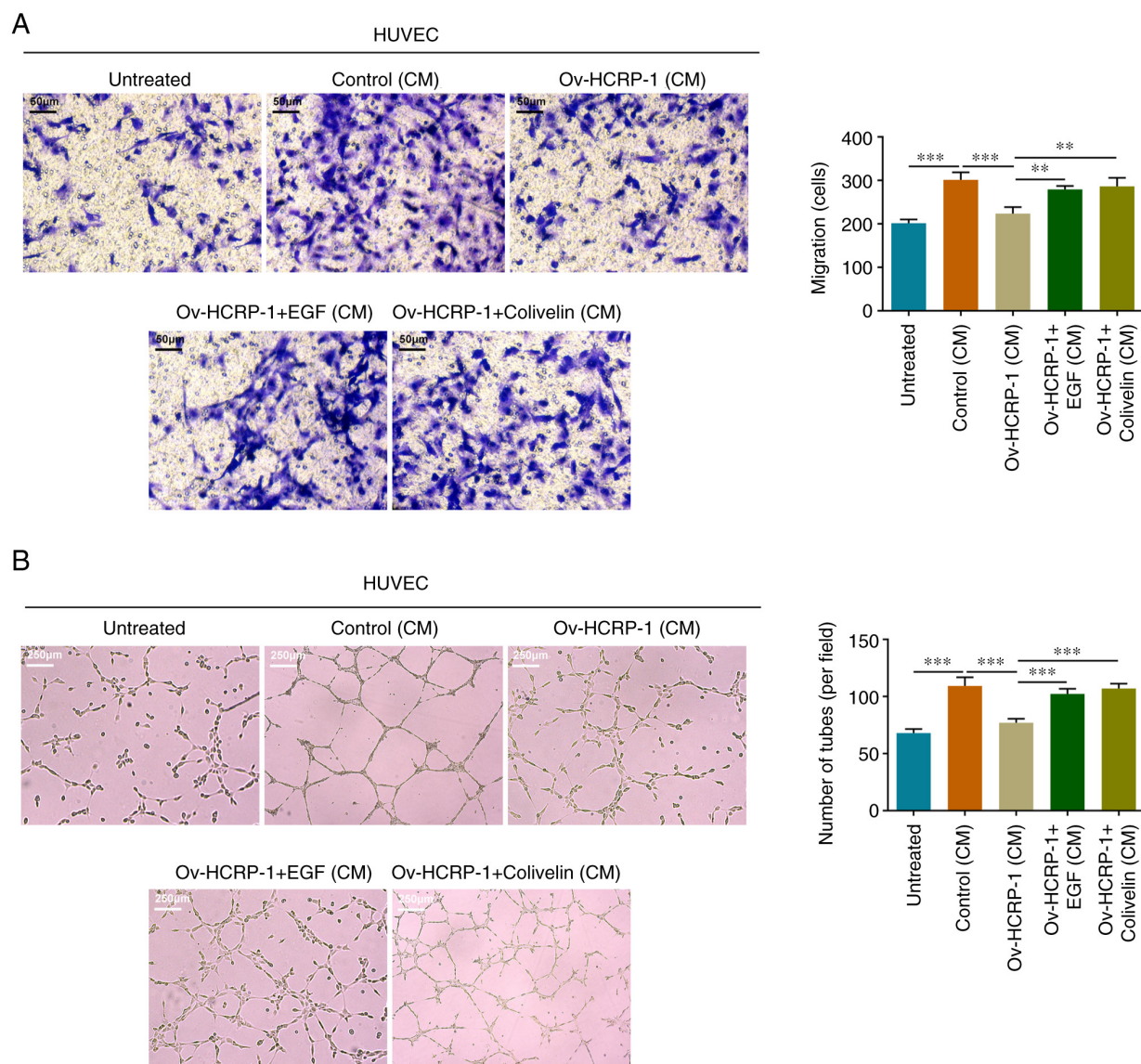


Figure 6. HCRP-1 in HUVECs functions via the regulation of the EGFR/STAT3 signaling pathway. (A) HUVEC migration was assessed using the Transwell assay (scale bar, 50 μ m). (B) Angiogenesis in each group of HUVECs was assessed using the angiogenesis assay (scale bar, 250 μ m). ** P <0.01 and *** P <0.001. HCRP-1, hepatocellular carcinoma-related protein 1; EGF, epidermal growth factor; CM, culture medium; Ov-, overexpression; HUVEC, human umbilical vein endothelial cell.

signaling. The present study may have determined the expression pattern and regulatory pathway of HCRP-1 in OSCC cells and may have further elucidated certain aspects of OSCC pathology.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YC and LH contributed to concept, experiments and analysis. YC contributed to the manuscript draft. YC and LH have read and approved the final manuscript, and confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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