Hypoxia promotes vasculogenic mimicry formation by the Twist1-Bmi1 connection in hepatocellular carcinoma

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Abstract. Aggressive tumor cells can mimic embryonic vasculogenic networks and form vasculogenic mimicry (VM). Preliminary studies demonstrated that hypoxia can promote VM formation; however, the underlying mechanism remains unclear. The present study aimed to investigate the role of the Twist1-Bmi1 connection in hypoxia-induced VM formation and the underlying mechanism. In the *in vitro* experiments, western blot analysis demonstrated that hypoxia upregulated the expression of Twist1, Bmi1, epithelial-mesenchymal transition (EMT) markers, stem cell markers and VM-associated markers. The 3D culture assay showed that hypoxia promoted VM formation in hepatocellular carcinoma (HCC) cell lines. Using transfection and *in vitro* cell experiments, the Twist1-Bmi1 connection was confirmed to have an important role in inducing EMT, cell stemness and VM formation. In the *in vivo* experiments, the murine hypoxia models were established via incomplete femoral artery ligation and the mechanism by which hypoxia promoted Twist1 and Bmi1 expression and led to VM formation was demonstrated by immunohistochemistry staining and endomucin/periodic acid Schiff double-staining. In conclusion, hypoxia upregulate the expression of Twist1 and Bmi1, and these two proteins have an important role in inducing EMT and cancer cell stemness, which contributed to VM formation.

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Abbreviations: $CoCl_2$, cobalt chloride; CSCs, cancer stem cells; DMEM, Dulbecco's modified Eagle's medium; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; MR, migration rate; PAS, periodic acid Schiff; VE-cadherin, vascular endothelial-cadherin; VM, vasculogenic mimicry

Key words: hypoxia, Twist1, Bmi1, epithelial-mesenchymal transition, stemness, vasculogenic mimicry

Introduction

Hepatocellular carcinoma (HCC) is a prevalent malignancy and the most common malignant tumor of the liver. It requires an adequate blood supply to support its growth *in vivo*. Vasculogenic mimicry (VM) is an alternative pathway for the blood supply. VM is formed by tumor cells that mimic endothelial cells to form cell extracellular matrix-rich channels [periodic acid Schiff (PAS)-positive], which are sinusoidal structures that surround clusters of tumor cells. Studies have demonstrated that tumors, including HCC, may develop alternative pathways, such as VM and mosaic vessels (1,2).

Hypoxia has an important role in various functional responses in tumor cells, including apoptosis and angiogenesis (3,4). A previous study has demonstrated that hypoxia is critical in VM formation (5). Hypoxia can directly upregulate the expression of HIF-1 (SETD2), and HIF-1 can further combine to the promoter of Twist1 and promote its transcript expression. Twist1 is a basic helix-loop-helix transcription factor that can form homo- or hetero-dimers and bind the Ndel E-box element and activate or repress its target genes. Previous evidence has revealed the important role of Twist1 in cancer metastasis, as shown by its overexpression in human cancers, induction of epithelial-mesenchymal transition (EMT) and association with a more malignant phenotype (6).

Previous studies identified that Bmi1 is directly regulated by the EMT regulator Twist1 (7). Bmi1 is a polycomb group family member that maintains self-renewal and is frequently overexpressed in human cancers (8). Twist1 and Bmi1 are mutually essential in promoting cell stemness, and the co-overexpression of Twist1 and Bmi1 can promote the tumor-initiating capability of cancer cells (7). This functional connection between Twist1 and Bmi1 provides a novel insight into the common mechanism mediating EMT and cancer stemness.

Although hypoxia has been reported to promote VM (5), the underlying mechanism remains unclear. In the present study, we hypothesized that hypoxia may lead to Twist1 and Bmi1 upregulation, and the interaction between Twist1 and

Bmi1 may promote VM formation by inducing EMT and cell stemness.

Materials and methods

Plasmid. The plasmid pEGFP-Twist1 was as previously described (9). The HepG2 cells were transfected with pEGFP-Twist1 or pcDNA control vector (pcDNA-negative).

Small interfering RNAs (siRNAs) encoding oligos against human Twist1 were as previously described (9). A non-silencing small interfering RNA sequence (target sequence, AATTCT CCGAACGTGTCACGT) was used as the negative control, as previously described (10). siRNA was used to block the Twist1 expression in Bel7402 cells.

Western blot analysis. Cells were washed using phosphate-buffered saline (PBS), and 10% sodium dodecyl sulfate (SDS) was used to lyse the cells. The whole cell lysates were resolved via SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Temecula, CA, USA). The membranes were blocked with skimmed milk powder and incubated with primary antibodies, followed by incubation with a secondary antibody, goat anti-rabbit (ZF-2301), goat anti-mouse (ZF-2305) or rabbit anti-goat (ZF-2305) immunoglobulin G-horseradish peroxidase (1:2,000; Zhongshan Chemical Co., Beijing, China). Protein expression was measured using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The primary antibodies for Twist1 (SC-15393; 1:200), E-cadherin (SC-7870; 1:200), Oct4 (SC-8629; 1:200) and cluster of differentiation 44 (CD44; SC-53298; 1:200) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), Bmi1 (Ab14389; 1:1,000), vimentin (42011; 1:500; Epitomics), vascular endothelial-cadherin (VE-cadherin; AB-33168; 1:200) and β-actin (ab8226; 1:2,000) were purchased from Abcam (Cambridge, MA, USA), and fms-related tyrosine kinase 1 (FLT1; RB-1527; 1:200) and kinase insert domain receptor (KDR; RB-1526; 1:200) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Cell culture and hypoxia treatment in vitro. Human liver cell lines HepG2 and Bel7402 were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). Cobalt chloride ($CoCl_2$) was used to simulate hypoxic conditions. Cells were seeded in dishes or plates at 300 cells/mm^2 and grew for 24 h in complete medium. The medium was removed, and the cells were washed with PBS. The cells were treated with 100 μM $CoCl_2$ and were subsequently incubated for different times as required.

3D culture assay. VM formation *in vitro* was evaluated using 3D culture. In the 3D culture assay, Matrigel (BD Biosciences, San Jose, CA, USA) was thawed at 4˚C and 30 μl was quickly added to each well of a 96-well plate and allowed to solidify for 12 h at 37˚C in a humidified 5% CO_2 incubator. Tumor cells in complete medium were subsequently seeded onto the gel and incubated at 37˚C for 24 h. The formation of capillary-like structures was observed under a phase-contrast microscope (magnification, x200). Each experiment was performed in triplicate.

Wound-healing assay. In wound-healing assays, different groups of cells were plated in 24-well culture plates and allowed to grow for 24 h. A micropipette was used to create a straight scratch in the center of each well. Cell migration ability was assessed by measuring the movement of cells into the scratch. The migration rate (MR) was monitored after 12, 24, 36 and 48 h. The following formula was used to calculate the MR at different time-points: MR = (d - d')/d (d is the length of the wound at 0 h, and d' is the length at other different time-points).

Invasion assay. In the invasion assay, 1x10^5 cells in 100 μl of DMEM without FBS were seeded in the upper 24 wells coated with Matrigel matrix (1 mg/ml; BD Biosciences) containing polyethylene terephthalate filters with 8-μm porosity (Invitrogen). The lower chamber was filled with medium containing 10% FBS. The cells were incubated for 48 h and the non-invading cells on the upper surface of the membrane were removed. The cells that invaded through the Matrigel matrix and attached to the bottom surface of the membrane were fixed by methanol and stained by 0.5% crystal violet. The number of invading cells was counted using an inverted light microscope (Nikon, Tokyo, Japan).

Sphere culture assay. The sphere culture method was as described previously (11). Briefly, single HCC cells were seeded in 12-well plates coated with poly(2-hydroxyethylmethacrylate) (poly-HEMA; Sigma-Aldrich, St. Louis, MO, USA) at a density of 1x10^4 viable cells/ml in a serum-free medium (DMEM-F12 1: 1 media; Gibco, Grand Island, NY, USA) supplemented with 1xB27 (Invitrogen), 10 ng/ml epidermal growth factor and 20 ng/ml fibroblast growth factor 2 (both from Peprotech, Rocky Hill, NY, USA), and 1% pen/strep (Invitrogen). The culture medium was replaced or supplemented with additional growth factors every 3 days.

Clone formation assay. In the clone formation assay, each well of a 6-well plate was seeded with 1,000 cells. The plates were incubated at 37˚C and 5% CO_2 for 12 days, fixed with methanol and stained with 0.5% crystal violet. The number of clones was counted under the microscope.

Murine xenograft model. A total of 40 five-week-old male BALB/c nude mice were purchased from HuaFukang Biological Technology Co., Ltd, Beijing, China. All the studies on mice were carried out in accordance with the Guidelines of the Laboratory Animal of Tianjin Medical University (Tianjin, China). The mice were divided into two groups; the HepG2 and Bel7402 groups. All the animals received anesthesia with 2% pentobarbital sodium (60 mg/kg) prior to surgery. The overall surgical mortality rate was 0%. Through a 1-1.5-cm incision in the right groin, the femoral artery was carefully separated from the vein and nerve fiber, and ligated by 8-0 silk with a needle placed along the vessel during ligation. The needles were 0.25 mm in diameter and were removed following ligation. The left limb was opened without femoral artery ligation as a control. Subsequently, 10^7 cells suspended in 0.1 ml of PBS were injected intramuscularly into both sides. The mice were monitored, and tumor sizes were measured daily using a Vernier caliper. The

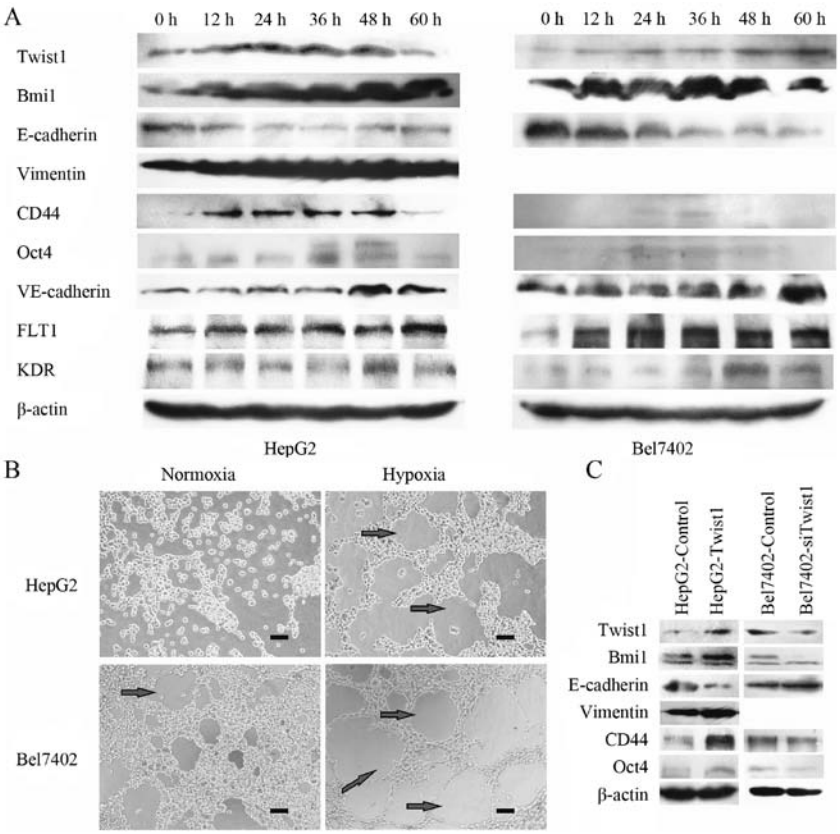

Figure 1. Effects of hypoxia and transfection on hepatocellular carcinoma cell lines. (A) Protein expression levels of Twist1, Bmi1, stem cell, EMT and VM-associated markers in HepG2 and Bel7402 cells after different incubation times of hypoxia (0, 12, 24, 36, 48 and 60 h). β-actin was used as loading control. The two groups demonstrated a significant increase in Twist1 and Bmi1 expression with time under hypoxia. The expression of E-cadherin in the two cell lines was downregulated and the expression of vimentin in HepG2 was upregulated by hypoxia, indicative of EMT. Vimentin expression was not observed in Bel7402 cells. The expression of stem cell markers and VM-associated markers was also promoted by hypoxia. (B) Effect of hypoxia on VM formation in HCC cells cultured on Matrigel matrix (magnification, x100; bars, 100 μm). HepG2 cells did not form typical tube-like structures, whereas Bel7402 cells formed a modest level of tube formation. After hypoxia was administered, VM formation was significantly induced. The arrows show the tube-like structures in the 3D culture. (C) Western blot results showed that the Bmi1 expression level was upregulated following Twist1 transfection and downregulated following siTwist1 transfection. The results also revealed that vimentin expression was increased and E-cadherin expression was decreased in the HepG2 cells following Twist1/Bmi1 upregulation. By contrast, E-cadherin expression was increased in Bel7402 cells following Twist1/Bmi1 downregulation. The stem cell marker CD44 and Oct4 expression was also upregulated by Twist1/Bmi1 upregulation and downregulated by Twist1/Bmi1 downregulation. CD44, cluster of differentiation 44; EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; VM, vasculogenic mimicry.

following formula was used to calculate tumor volume: Tumor volume = $1/2ab^2$ (a is the length and b is the width of the tumor). When the observation was complete, the mice were sacrificed. Tumors were harvested and fixed by formalin.

Immunohistochemistry (IHC) and endomucin/PAS double-staining. Prior to immunostaining, 4-μm paraffin sections were deparaffinized in xylene and rehydrated by a graded series of aqueous ethanol solutions. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 100% methanol for 30 min at room temperature. The sections were pretreated in a microwave, blocked and incubated using a series of antibodies. The staining systems used in the present study were PicTure PV6000 (Zhongshan Chemical Co.). In endomucin/PAS double-staining, the sections were washed with running water for 5 min and were subsequently incubated with PAS for 15 min after IHC for endomucin was performed. Finally, the sections were counterstained with hematoxylin, dehydrated and mounted. PBS was used instead of the primary antibodies for the negative control. The results were quantified according to the method described by Bittner *et al* (12).

Statistical analysis. All the data in the study were evaluated with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Student's t-test was performed to determine differences between two groups in cell functional assays. Paired t-test was performed to compare the hypoxia and control groups *in vivo*. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of hypoxia in vitro

Hypoxia results in Twist1-Bmi1 upregulation. HepG2 and Bel7402 cells were used in the study to verify the association of hypoxia with VM in HCC cells *in vitro*. HepG2 and Bel7402 cells were cultured under hypoxic conditions, and Twist1 and Bmi1 protein expression levels were detected by western blot analysis after 0, 12, 24, 36, 48 or 60 h of hypoxia. The two groups demonstrated a significant increase in Twist1 and Bmi1 expression with time under hypoxia (Fig. 1A).

Hypoxia induces EMT and cell stemness. Accompanied with Twist1/Bmi1 upregulation, the expression of E-cadherin

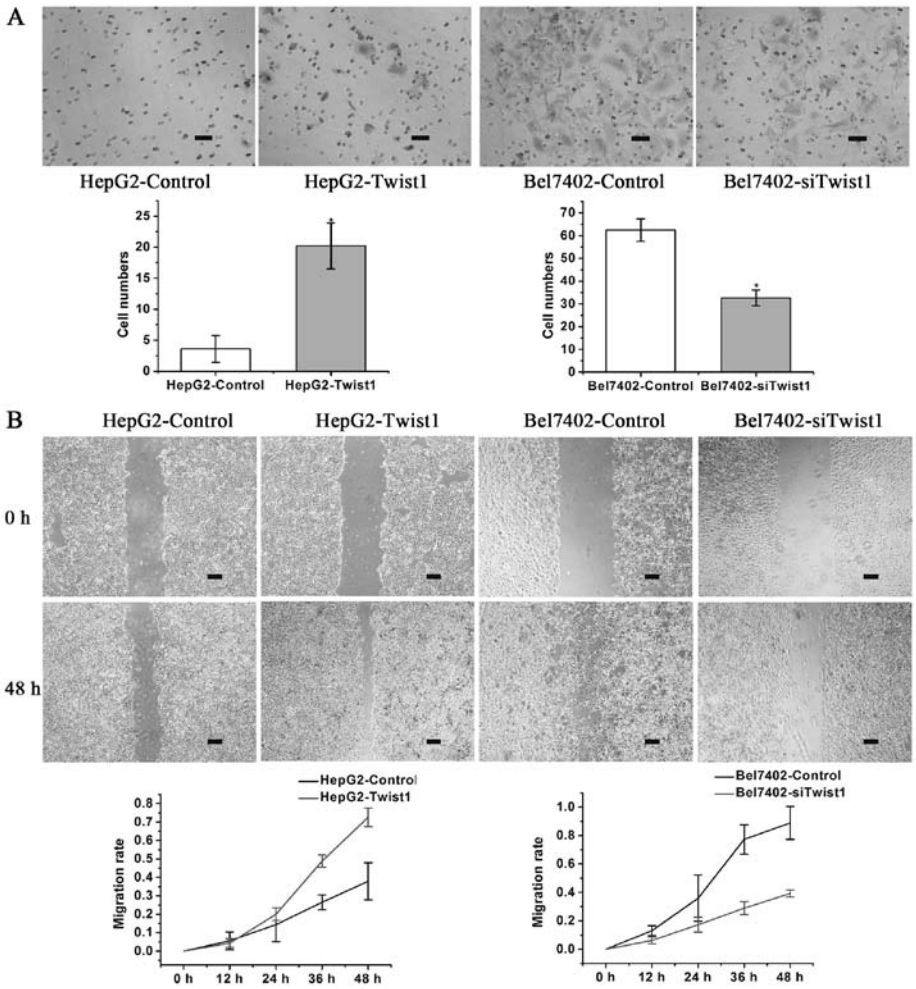

Figure 2. Changes in the capacity of invasion and metastasis of the transfected cells. (A) Number of invasion cells was assessed in the invasion assay (magnification, x200; bars, 50 μm). Results showed that Twist1 upregulation enhanced HepG2 cell invasion and Twist1 downregulation inhibited Bel7402 cell invasion (*P<0.05). (B) Wound-healing assays (magnification, x40; bars, 200 μm) revealed that HepG2-Twist1 cells exhibited a higher motility compared with the HepG2-control cells. Bel7402-control cells exhibited a higher motility compared with the Bel7402-siTwist1 cells. The results were statistically significant (P<0.05).

in the two cells lines was downregulated and the expression of vimentin in HepG2 was upregulated by hypoxia, indicative of EMT (Bel7402 cells did not show any vimentin expression; Fig. 1A). The expression of stem cell markers was also promoted by hypoxia. Stem cell marker Oct4 (OCT3/4, POU5F1) is a member of the POU transcription factor family, and it has a critical role in maintaining cell stemness (13). CD44 is a major extracellular matrix adhesion molecule that is widely used as a cancer stem cell marker in various types of cancer, including HCC (14,15). Western blot analysis showed that hypoxia increased the expression of CD44 and Oct4 in HepG2 and Bel7402 cells (Fig. 1A).

Hypoxia promotes VM formation ability of HCC cells. The expression of VM-associated markers VE-cadherin, FLT1 and KDR were also elevated by hypoxia (Fig. 1A). The ability of VM formation on Matrigel of HepG2 and Bel7402 cells under normoxia and cells that underwent 48 h of hypoxia was assessed. Hypoxia promoted VM formation in the HepG2 and Bel7402 cells (Fig. 1B).

Twist1-Bmi1 cooperation is a critical factor in VM formation

Bmi1 expression is regulated by Twist1. The endogenous expression of Twist1 in the HepG2 cell line was significantly lower compared with the Bel7402 cell line. Thus, HepG2 cells were infected with Twist1 plasmid and the Bel7402 cells with siTwist1 plasmid. Western blot analysis showed that Bmi1 expression was upregulated in HepG2-Twist1 cells and downregulated in Bel7402-siTwist1 cells (Fig. 1C), confirming that Bmi1 expression was regulated by Twist1.

Upregulation of Twist1 and Bmi1 induces EMT and cell stemness. Western blot analysis showed that in HepG2 cells, the upregulation of Twist1 promoted vimentin expression and inhibited E-cadherin expression, indicative of EMT. In Bel7402 cells, the downregulation of Twist1 promoted E-cadherin expression. The expression levels of stem cell marker CD44 and Oct4 were upregulated in HepG2-Twist1 cells and downregulated in Bel7402-siTwist1 cells (Fig. 1C).

The tumor cells that exhibited the ability to undergo EMT also had a greater ability to invade and metastasize. The HepG2-control, HepG2-Twist1, Bel7402-control and Bel7402-siTwist1 cells were subsequently analyzed for functional changes in invasion and migration ability. The *in vitro* invasion assay demonstrated that upregulation of Twist1 in HepG2 cells significantly promoted cell invasion, while downregulation of Twist1 in Bel7402 cells significantly inhibited cell invasion (Fig. 2A). In the wound-healing assay, the MR at

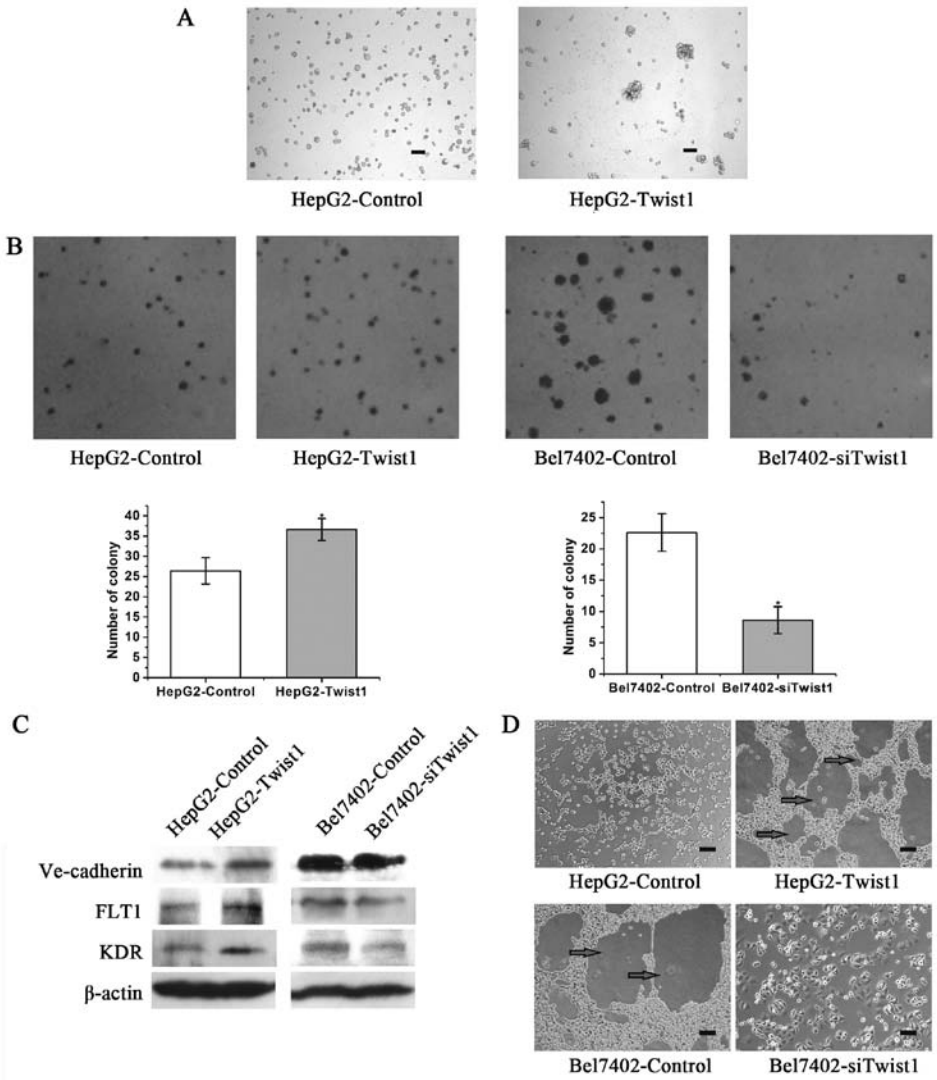

Figure 3. Changes in cell stemness and vasculogenic mimicry formation ability in transfected cells. (A) Viable sphere formation was observed in the HepG2-Twist1 group, however, no spheres were formed in the HepG2-control group (magnification, x100; bars, 100 μm). (B) Clonogenic assay showed increased cell colony formation ability in the HepG2-Twist1 group and decreased cell colony formation ability in the Bel7402-siTwist1 group compared with the control groups (*P<0.05). (C) Western blot results showed that the upregulation of Twist1 promoted the expression of VM-associated markers. By contrast, downregulation of Twist1 inhibited the expression of VM-associated markers. (D) Bel7402-control cells exhibited a higher tube formation capacity compared with the HepG2-control cells in the 3D culture experiments. Twist1 enhanced the tube formation capacity of HepG2 cells, and siTwist1 weakened this capacity in the Bel7402 cells (magnification, x100; bars, 100 μm). VM, vasculogenic mimicry.

the terminal phase (48 h) increased in the HepG2-Twist1 group and decreased in the Bel7402-siTwist1 group compared with the control groups (Fig. 2B).

Sphere culture and clone formation assays were performed to evaluate cell stemness. In the sphere culture assay, HepG2-control, HepG2-Twist1, Bel7402-control and Bel7402-siTwist1 cells were seeded in plates coated with poly-HEMA at a density of $1x10^4$ cells/ml using a commonly used suspension culture containing 1% methylcellulose. After 4-7 days of culture, a viable sphere formation was observed in the HepG2-Twist1 group, however, no spheres were formed in the HepG2-control group (Fig. 3A). Neither Bel7402-control nor Bel7402-siTwist1 group formed spheres (data not shown). In the clone formation assay, Twist1 upregulation in HepG2 cells resulted in the significant promotion of cell colony formation, while in Bel7402 cells, Twist1 downregulation significantly inhibited colony formation (Fig. 3B).

Expression levels of Twist1 and Bmi1 influences VM formation. Western blot analysis showed that the expression levels of VM-associated markers, VE-cadherin, FLT1 and KDR, were upregulated in HepG2-Twist1 cells and downregulated in Bel7402-siTwist1 cells (Fig. 3C). The formation of capillary-like structures was assessed by the 3D culture assay. HepG2-control cells could not form any tube-like structures, while HepG2-Twist1 formed typical tube-like structures on the surface of the Matrigel medium. Bel7402-control cells showed modest tube-like structures while Bel7402-siTwist1 cells showed no tube-like structures (Fig. 3D).

Effect of hypoxia in vivo

Hypoxia promotes Twist1 and Bmi1 expression and induces EMT and cell stemness in vivo. An incomplete femoral artery ligation murine model was established to investigate the effects of hypoxia *in vivo*. A total of 14 mice carried tumors

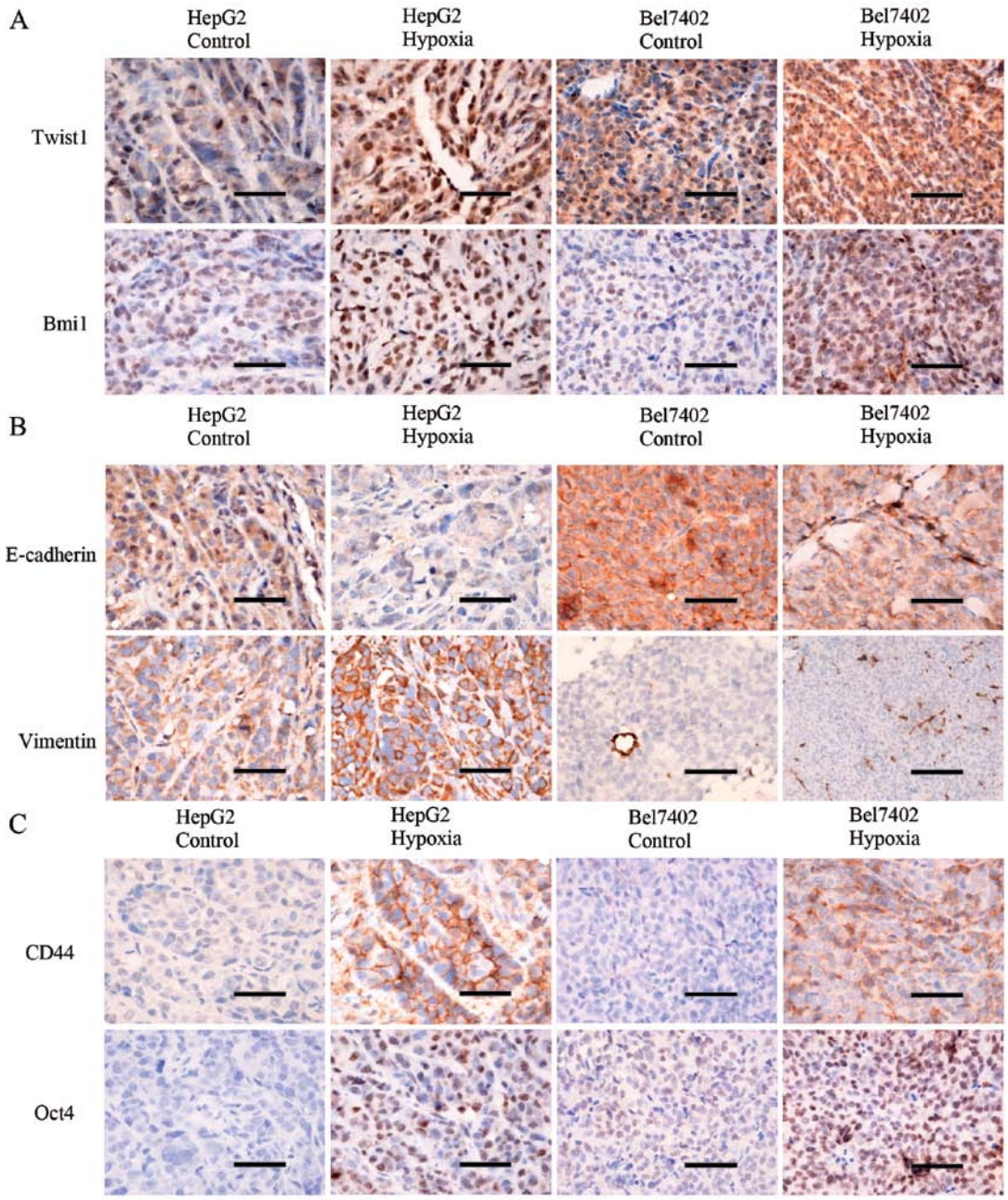

Figure 4. Hypoxia upregulates Twist1 and Bmi1 expression and promotes epithelial mesenchymal transition and stemness *in vivo*. (A) Expression levels of Twist1 and Bmi1 in HepG2- and Bel7402-engrafted tumors (magnification, x400; bars, 50 μm) were detected by IHC staining. Twist1 and Bmi1 expression levels were higher in the hypoxia group compared with those in the control group (P<0.05). (B) Expression levels of EMT markers in HepG2- and Bel7402-engrafted tumors (magnification, x400; bars, 50 μm). The HepG2 hypoxia and control groups exhibited positive staining for E-cadherin in the plasma membrane and vimentin in the cytoplasm. The Bel7402 control and hypoxia groups exhibited positive staining for E-cadherin in the plasma membrane but negative staining for vimentin in tumor cells. The expression of E-cadherin was downregulated and the expression of vimentin was upregulated in the hypoxia groups, but not in the Bel7402 groups (P<0.05). (C) IHC staining also showed that the expression levels of the stem cell markers was higher in the hypoxia group compared with that in the control group (magnification, x400; bars, 50 μm; P<0.05). IHC, immunohistochemistry; EMT, epithelial-mesenchymal transition.

in the bilateral hindlimbs in the HepG2 group and there were 7 mice in the Bel7402 groups. Using IHC, Twist1, Bmi1, EMT marker and stem cell marker expression was examined in the engrafted tumors in the HepG2 control, HepG2 hypoxia, Bel7402 control, and Bel7402 hypoxia groups. Cells exhibited positive staining for Twist1 in the nucleus and cytoplasm, Bmi1 in the nucleus, E-cadherin in the plasma membrane, vimentin in the plasma membrane and cytoplasm, CD44 in the plasma membrane, and Oct4 in the nucleus (Fig. 4). The expression of Twist1 and Bmi1 expression in the hypoxia group was stronger compared with that in the control group (P<0.05; Fig. 4A). The expression of E-cadherin was downregulated and the expression of vimentin was upregulated in the hypoxia group (tumor cells in Bel7402-engrafted tumors showed no vimentin expression) (P<0.05; Fig. 4B). The expression of stem cell marker CD44 and Oct4 was also stronger in the hypoxia group compared with that in the control group in the HepG2- and Bel7402-engrafted tumors (P<0.05; Fig. 4C).

Hypoxia promotes VM in vivo. The expression levels of VM-associated markers, including VE-cadherin, FLT1 and

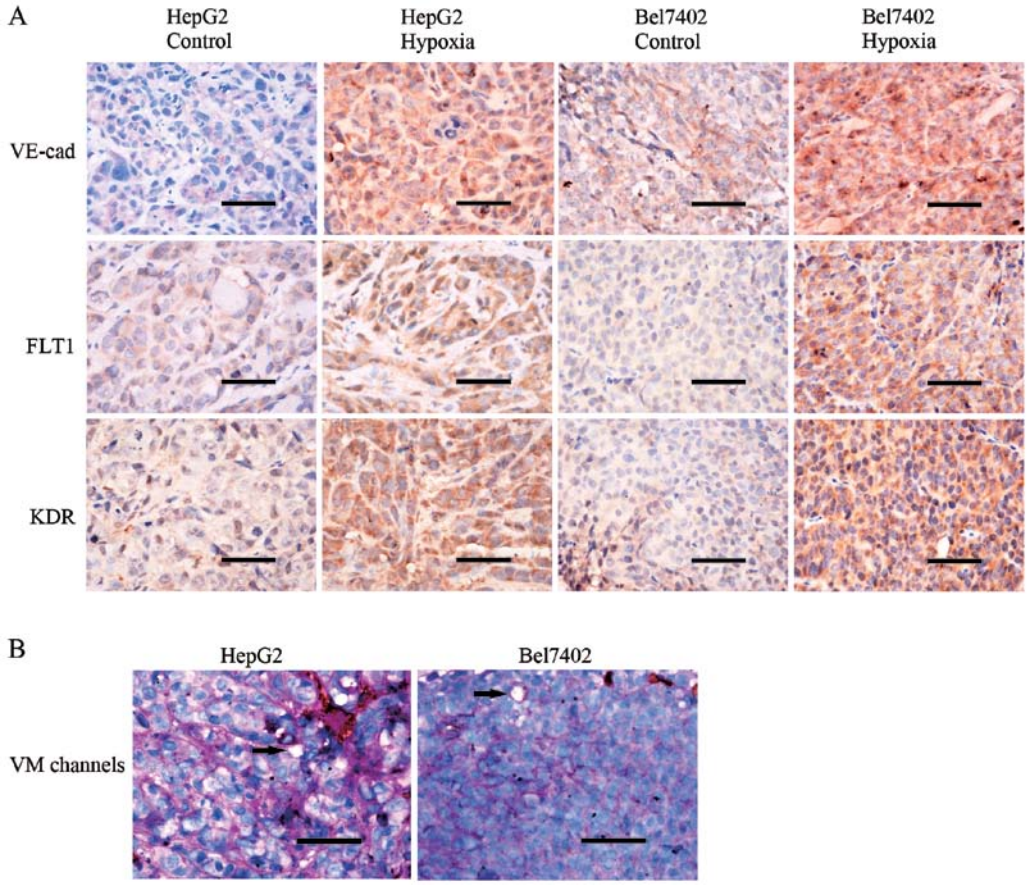

Figure 5. Hypoxia promotes vasculogenic mimicry formation *in vivo*. (A) Expression levels of VM-associated markers in HepG2- and Bel7402-engrafted tumors (magnification, x400; bars, 50 μm). The expression of the VM-associated markers was higher in the hypoxia group compared with the control group ($P<0.05$). (B) Arrows show VM channels in engrafted tumors. VM channels in HepG2- and Bel7402-engrafted tumors formed by tumor cells, which were negative for endomucin; the basement membrane-like structure that lined the channel was positive for PAS (magnification, x400; bars, 50 μm). VM, vasculogenic mimicry; PAS, periodic acid Schiff.

KDR, were examined using IHC staining (Fig. 5A). The control and hypoxia groups contained plasma membrane VE-cadherin-, cytoplasmic VEGFR1- and cytoplasmic VEGFR2-positive tumor cells. Tumors in the hypoxia group expressed more of these proteins compared with the control group ($P<0.05$). Using endomucin/PAS double-staining, VM channels (Fig. 5B) were observed in the engrafted tumors. VM channels were formed by tumor cells lined with PAS-positive substances, and these cells were negative for endomucin. Hypoxia promoted VM formation. The HepG2 hypoxia group had 5.14±1.505 VM channels, whereas the control group had 3.00±0.756 VM channels ($P<0.05$). The Bel7402 hypoxia group had 3.29±0.700 VM channels, whereas the control group had 1.43±0.495 VM channels ($P<0.05$).

Discussion

Hypoxia has a pivotal role in the microenvironment, and a hypoxic microenvironment could affect tumor angiogenesis, metastasis, metabolism and the survival of tumor cells (5,16,17). $CoCl_2$ is a hypoxia-mimicking reagent that is commonly used in hypoxia studies *in vitro* (18,19). It mimics hypoxic conditions by inhibiting the activity of prolyl hydroxylase, a critical enzyme in the oxygen-sensing pathway (20). In the present study, $CoCl_2$ was used in cell cultures to simulate constant hypoxia. In the *in vivo* experiments, incomplete femoral artery ligation was performed in nude mice to create hypoxic conditions, as serious gangrene could occur following complete femoral artery ligation and excision in the hindlimb of nude mice (21).

Hypoxia has been proved to promote VM formation (5). VM was first reported in highly aggressive uveal melanoma in 1999 by Maniotis *et al* (22). VM has been found in HCC samples, and studies have reported that VM is associated with metastasis and a shorter survival period in HCC (23). Tumor cells that formed the unique structure of VM channels were directly exposed to the bloodstream, so they could easily metastasize to distant sites by entering the microcirculation. This discovery may explain the elevated risk of metastasis, tumor recurrence and shorter survival period in patients with VM-positive HCC. Hypoxia is a crucial factor in VM formation (5,9).

The EMT regulator Twist1 has been reported to regulate the expression of Bmi1 directly, which provides a novel insight into the association between EMT and cancer stemness (7). The Twist1-Bmi1 link was responsible for inducing EMT and cell stemness under hypoxia conditions. EMT is a pivotal developmental program that is often activated in cancer development (24,25). A number of changes may occur to epithelial cells during EMT, including loss of epithelial characteristics,

acquisition of mesenchymal phenotypes, and ability to invade, resist apoptosis and disseminate. Twist1 is an EMT regulator that regulates EMT in various tumors (26,27), and can be upregulated by hypoxia. Twist1 can repress E-cadherin and promote mesenchymal markers expression (28). Bmi1 is directly regulated by Twist1. Twist1 and Bmi1 are mutually essential in promoting cell stemness, and the co-overexpression of Twist1 and Bmi1 can promote the tumor-initiating capability of cancer cells (7). The present study also showed that the expression of CD44 and Oct4 was upregulated following Twist1-Bmi1 cooperation. Oct4 has an important role in maintaining stemness (29). CD44 is a major extracellular matrix adhesion molecule that is widely used as a cancer stem cell marker in various cancer types, including HCC (14,15). Bmi1 has been reported to be co-expressed with CD44 in cancer stem cells (CSCs) (30,31). Thus, the present study showed that cell stemness was induced by hypoxia. Stemness and EMT are interconnected processes. Twist1/Bmi1-induced stemness may be an important section of the EMT progress. The epithelial cells may first obtain high levels of stemness, and subsequently, they can differentiate toward mesenchymal-like cells. Ectopic expression of Bmi1 in normal nasopharyngeal epithelial cells is sufficient to cause EMT (32). A recent breakthrough in metastasis studies demonstrated that induction of EMT also generates cells with stem-like properties (33,34). The mutual promotion of these two processes is the basis of VM formation.

The present study demonstrated increased VM formation following the induction of EMT and cell stemness. EMT and stemness can induce VM formation. Twist1 can induce EMT, and EMT can contribute to tumor cell plasticity; the general mechanism behind this phenomenon involves inhibiting E-cadherin transcription caused by Twist1 interacting with its promoter and further induction of the expression of downstream mesenchymal markers (6). This process is characteristic of VM formation, which involves tumor cells mimicking endothelial cells, a type of mesenchymal cell, similar to the EMT process. Tumor cell stemness is associated with VM formation; CSCs can undergo asymmetric cell division, which results in tumor cells that promote angiogenesis (35). CSCs may express an endothelial phenotype and form vessel-like networks, thereby mimicking the pattern of vascular networks in the embryo (36,37). Tumor cells expressing high stemness can exist in HCC, and this stem cell population is responsible for VM formation (38). The present study indicated that Twist1-Bmi1 cooperation induced VM formation through induction of EMT and tumor cell stemness. Increased expression of VM-associated markers and VM formation was observed following induced EMT and stemness *in vitro* and *in vivo*.

In conclusion, the present study demonstrated that the effects of hypoxia on VM formation in HCC cells involved the Twist1-Bmi1 connection, which induced EMT and stemness. Studies on VM mechanisms could help us develop appropriate drugs to inhibit tumor growth and metastasis by suppressing VM. Inhibitors could be specially designed to inhibit Twist1, Bmi1 or their associated markers.

Acknowledgements

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