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# Exploring stemness gene expression and vasculogenic mimicry capacity in well- and poorly-differentiated hepatocellular carcinoma cell lines

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ABSTRACT

Vasculogenic mimicry (VM) is the phenomenon where cancer cells mimic endothelial cells by forming blood vessels. A stem cell-like phenotype has been proposed to be involved in this tumor plasticity. VM seems to correlate with metastasis rate, but there have been no reports on the effects of pro-metastatic and pro-angiogenic factors or hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) on VM formation of hepatocellular carcinoma (HCC) cells. Here, we determine VM capacity and expression of stemness genes (Oct4, Sox2, Nanog and CD133) in well- and poorly-differentiated HCC cell lines. The poorly-differentiated cell line SK-Hep-1 with mesenchymal features (high invasiveness and expressing Vimentin, with no E-cadherin) could form VM *in vitro*, while the well-differentiated cell line HepG2 did not form VM. There was no correlation between expression of stemness genes and intrinsic VM capacity. However, HGF but not VEGF, could induce VM formation in HepG2, concomitant with epithelial–mesenchymal transition (EMT), de-differentiation and increased expression of stemness genes. Our results show that the role of stemness genes in VM capacity of HCC cells is likely to depend on differentiation status.



## 1. Introduction

Maniotis et al. [1] demonstrated the existence of non-endothelial microcirculation in tissue sections of human uveal melanoma, which they termed vasculogenic mimicry (VM) for vessels formed by melanoma cells without an endothelial cell lining. *In vitro* studies revealed that aggressive human uveal melanoma cell lines can form tubular networks on Matrigel, similar to endothelial cells. VM was recognized in several cancer types including hepatocellular carcinoma (HCC) [2]. The presence of VM leads to problems in cancer therapy, because conventional angiogenesis inhibitors fail to inhibit VM formation in melanoma cells [3]. Moreover, the inhibition of endothelial angiogenesis may unintentionally promote VM formation in tumors [4], as recently evidenced by a report showing increased VM channels in tumor-bearing mice receiving short-term treatment of anti-VEGF monoclonal antibody bevacizumab [5]. Thus understanding the mechanism of VM formation may improve cancer therapy.

VM capacity was proposed to result from tumor cell plasticity acquired by de-differentiation of cancer cells to an embryonic- or stem cell-like phenotype, allowing expression of several endothelial-specific genes in VM-forming cancer cells [6]. The cancer stem cell (CSC) concept has been applied to VM [7], and recently observed in glioblastoma [8]. However, the relationship between stem cell-like phenotype and VM formation in HCC was proposed but not yet reported.

VM in HCC was associated with invasion and metastasis rate [2,9]. The epithelial–mesenchymal transition (EMT) is a cellular plasticity process, whereby epithelial cells reduce epithelial characteristics, such as decreased cell–cell contact and down-regulation of E-cadherin, while simultaneously acquiring mesenchymal properties including fibroblast-like shape, increased cell motility and up-regulation of mesenchymal markers such Vimentin [10]. EMT occurs during embryonic development, but also contributes to malignant progression of carcinoma through invasion-metastasis and de-differentiation [10]. Normally, EMT is transient, but permanent

*Abbreviations:* VM, vasculogenic mimicry; HCC, hepatocellular carcinoma; EMT, epithelial–mesenchymal transition; CSCs, cancer stem cells; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; MMPs, matrix metalloproteinases; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; G6Pase, glucose-6-phosphatase.

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EMT may exist in cancer cells, due to genetic changes or signaling maintenance [11]. Aberrant expression of EMT regulators was found in VM forming cancer cell lines, Twist1 in HCC cells [9], and ZEB1 in colorectal carcinoma cells [12]. Moreover, hypoxia can enhance VM capacity of HCC cells through increased Twist expression [13,14].

Hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), present in HCC tumor microenvironment, play important roles in hepatocarcinogenesis and tumor progression [15]. HGF is a mesenchymal-derived cytokine inducing cell migration and morphogenesis in epithelial cells during embryonic development and wound repair [16]. Recent studies show association between high levels of pre-operative serum HGF, c-Met expression and tumor recurrence or metastasis in HCC patients [17]. VM in HCC is associated with invasion and metastasis, but it is unclear whether HGF participates in VM formation.

VEGF is a major angiogenesis regulator of human endothelial cells. VEGF and its receptors appear to contribute to VM formation in some cancer types including ovarian carcinoma, osteosarcoma and melanoma [18–20]. However, VEGF did not show any effect on VM formation of Ewing sarcoma cells [21] and the effect of VEGF on VM formation of HCC cells is still unknown.

Here, we have explored the relationship between VM capacity and stem-like and endothelial phenotypes in HCC cells, by analyzing expression of genes essential for endothelial angiogenesis, stemness and EMT markers, in well- and poorly-differentiated human HCC cell lines. The effects of HGF and VEGF on VM formation of HCC cells were also studied.

## 2. Materials and methods

### 2.1. Chemicals

The list of chemicals and reagents are shown in Supplementary data.

### 2.2. Cell culture

SK-Hep-1 and HepG2 were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science Walkersville (Walkersville, MD, USA). Details of culture media are provided in Supplementary data.

### 2.3. Matrigel tube formation assay

Matrigel tube formation assay was performed as described in Supplementary data. Briefly, cell suspension was seeded into Matrigel-coated 96-well plate and incubated at 37 °C in 5% $CO_2$. Effect of HGF or VEGF on tube formation was studied by adding test growth factor into the wells immediately after the cell seeding, to a final concentration of 50 ng/ml.

### 2.4. Invasion and migration assay

Invasion and cell migration were assayed in Transwell chambers (Corning Costar, MA, USA) as previously described [22], as detailed in Supplementary data. Briefly, cell suspension in serum-free media was seeded into the inserts, while the lower chambers were filled with the same media with or without test growth factor (50 ng/ml). The invasion assay was performed in the same manner as the migration assays, except that Matrigel-coated inserts were used in the invasion assay. At the end of incubation, invaded or migrated cells were stained with crystal violet solution.

### 2.5. Gelatin zymography

Matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) in conditioned media of the HCC cells were detected by gelatin zymography [23], as described in Supplementary data. Briefly, the conditioned media obtained from culturing cells in serum-free media for 24 h were subjected to electrophoresis in gelatin-incorporated SDS–polyacrylamide gel under non-reducing conditions. After electrophoresis, the gel was incubated for 24 h at 37 °C with incubation buffer. The gel was stained with Coomassie Brilliant Blue. Bands of MMPs activity were visualized as clear bands on a blue background.

### 2.6. qRT-PCR

First-strand cDNA synthesis, quantitative determination of expression of selected genes by real-time PCR, and primer sequences were described in Supplementary data. Data are expressed as gene expression level relative to β-Actin. In HGF-treated experiments, the relative change in gene expression compared with untreated conditions was expressed as fold change, calculated by $2^{-\Delta\Delta Cp}$ method.

### 2.7. Statistical analysis

Data were analyzed for statistical significance of differences between groups using the Student's *t*-test, with a *p*-value lower than 0.05 being considered significant.

## 3. Results

### 3.1. VM capacity correlates with metastatic potential of human HCC cell lines

Tube formation on Matrigel is an assay used to determine *in vitro* angiogenesis of endothelial cells and to determine *in vitro* VM capacity of cancer cells [1]. Two human HCC cell lines were used, with different differentiation status, a well-differentiated HCC cell line (HepG2) and a poorly-differentiated HCC cell line (SK-Hep-1) [24]. SK-Hep-1 tube formation was first observed at 6 h and completed after 18 h after seeding. The SK-Hep-1 tubular organizations resembled the *in vitro* angiogenesis of endothelial cells, HUVECs (Fig. 1A), while HepG2 did not form tubes (Fig. 1A).

Invasion and cell migration were assayed using Transwell chambers in serum-free media without chemoattractant. After 24 h incubation, SK-Hep-1 showed large numbers of invaded and migrated cells, while HepG2 showed low numbers of such cells (Fig. 1B).

Type IV collagenases, MMP-2 and MMP-9, are associated with invasion and metastasis. A 92 kDa band of proMMP-9 was detected in SK-Hep-1 in conditioned media, while a very faint 66 kDa band of active MMP-2 was observed in HepG2 conditioned media (Fig. 1C).

Thus, VM-positive SK-Hep-1 was a highly invasive cell line exhibiting both high cell motility and high levels of proMMP-9, while VM-negative HepG2 is a less invasive cell line. These results show a correlation between *in vitro* VM capacity and metastatic potential of the HCC cell lines.

### 3.2. Gene expression analysis of VM-positive and VM-negative cell lines

Expression of three groups of genes, namely stemness and liver differentiation markers, angiogenesis-related genes, and EMT markers was explored in HCC cell lines using qRT-PCR.

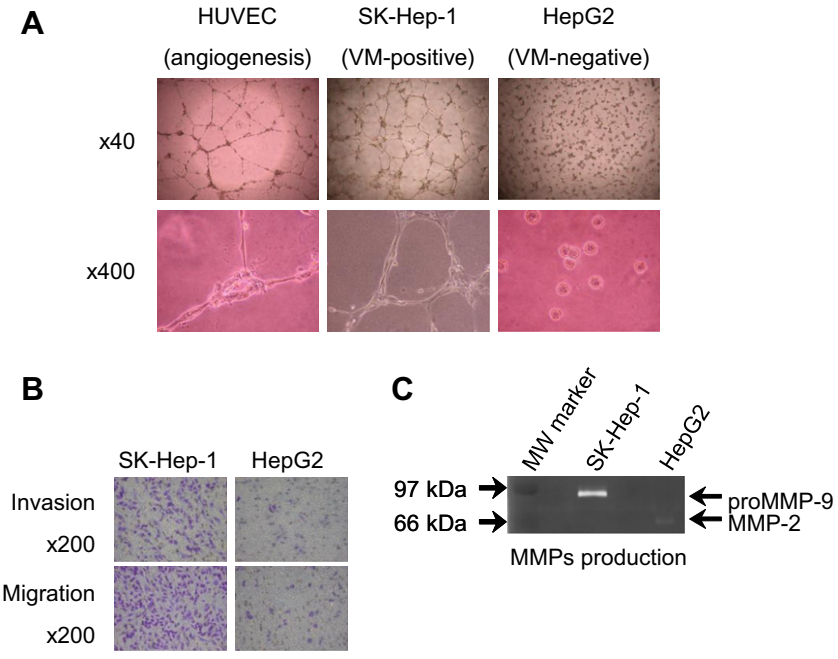


**Fig. 1.** VM capacity and metastatic potential of HCC cell lines. (A) Formation of tubular network structures on Matrigel by endothelial cells HUVEC compared to SK-Hep-1 and HepG2. Cells were seeded into Matrigel-coated 96-well plates and incubated at 37 °C. Photographs were taken after 18 h, with original magnification of 40× or 400× as indicated. (B) Invasion and migration of cell lines after 24 h incubation in Transwell chambers. Invaded or migrated cells attached at the lower surface of the filters were stained with crystal violet. Original magnification was 200×. (C) MMP-2 and MMP-9 production by HCC cell lines were determined by gelatin zymography method. Gelatinolytic activity of MMPs was revealed as clear bands on a blue background. Data are representative of three independent experiments with similar results.

Liver differentiation markers, Albumin and G6Pase, were expressed in HepG2, confirming its well-differentiated status, but not expressed in SK-Hep-1 (Fig. 2A). Oct4, Sox2 and Nanog are transcription factors involved in maintenance of pluripotency of embryonic stem cells [25]. Both cell lines expressed Oct4 and Sox2 at similar levels, but Nanog expression was 7-fold higher in HepG2 compared to SK-Hep-1 (Fig. 2A). However, CD133 – a cell surface glycoprotein used as a CSC marker in several solid tumors, was expressed only HepG2 (Fig. 2A).

Using HUVECs as positive controls for endothelial cells, the levels of selected angiogenesis-related genes were determined, namely VEGF receptors (Flt-1 and KDR), its downstream mediator endothelial nitric oxide synthase (eNOS), and endothelial-specific cell adhesion molecules (CD31 and VE-cadherin), since lack of any of these genes abrogates tube formation in endothelial cells [26,27]. SK-Hep-1 expressed Flt-1 and VE-cadherin, whereas HepG2 expressed eNOS, CD31 and VE-cadherin (Fig. 2B). Only VE-cadherin was detected in both HCC cell lines, but its expression level in HepG2 was significantly higher than in SK-Hep-1 (Fig. 2B).

The VM-positive cell line SK-Hep-1 exhibited mesenchymal features by showing Vimentin expression with no E-cadherin expression similar to HUVECs, used as positive control of mesenchymal cells (Fig. 2C). The VM-negative cell line HepG2 retained E-cadherin expression but did not express Vimentin. Expression levels of EMT transcription factors, Twist1 and Snail1, differed significantly between the HCC cell lines. SK-Hep-1 expressed Twist1 at a greatly higher level than Snail1 (Fig. 2C). In contrast, HepG2 showed much higher expression level of Snail1 than Twist1 (Fig. 2C). However, both HCC cell lines showed similar expression of c-Met, an HGF receptor (Fig. 2C).

Collectively, the results indicate correlation between VM capacity, de-differentiation status and mesenchymal features, in HCC cell lines. There was no correlation between expression of stemness genes and intrinsic VM capacity, since all stemness genes were detected in well-differentiated HepG2 at similar levels to poorly-differentiated SK-Hep-1. Interestingly, SK-Hep-1 cells could form tubular structures, while lacking several essential angiogenesis-related genes, indicating that the mechanism of SK-Hep-1 tube formation (VM) differs from endothelial tube formation (angiogenesis).

### 3.3. Enhancement of VM capacity of HCC cells by HGF

HGF and VEGF in tumor microenvironment play important roles in metastasis of HCC [15], with HGF acting as an EMT inducer and VEGF acting as an angiogenesis inducer. Thus we examined the effect of HGF and VEGF on *in vitro* VM formation of HCC cell lines.

When enhanced cell motility, a hallmark of EMT activation was used as an EMT functional assay, the results showed that HGF strongly increased cell migration in both HCC cell lines, while VEGF did not affect the migration rate (Fig. 3A).

Then, when HCC cells were cultured on Matrigel in the presence of HGF or VEGF, only HGF could enhance tube formation in both SK-Hep-1 and HepG2, compared with controls, while VEGF did not affect tube formation (Fig. 3B). SK-Hep-1 tube formation could be observed at 4 h after seeding (Fig. 3B), while in HepG2, tubular structures started on day-2 and were clearly seen on day-4 after HGF treatment (Fig. 3B). The HepG2 tubular organizations at enhanced magnification (Fig. 3B) resembled SK-Hep-1 tube formation (Fig. 1A).

HGF, but not VEGF, increased cell migration and enhanced tube formation in both VM-positive and VM-negative HCC cell lines, confirming the correlation between VM capacity and metastatic potential in HCC cell lines. Additionally, EMT inducers such as HGF can modulate VM capacity of HCC cells.

### 3.4. HGF induced changes in EMT and stemness gene expression levels in HepG2 cells

The modulatory effect of HGF on VM capacity was further investigated at gene expression level in HepG2. When the cells were cultured in the presence or absence of HGF for 2–4 days, untreated

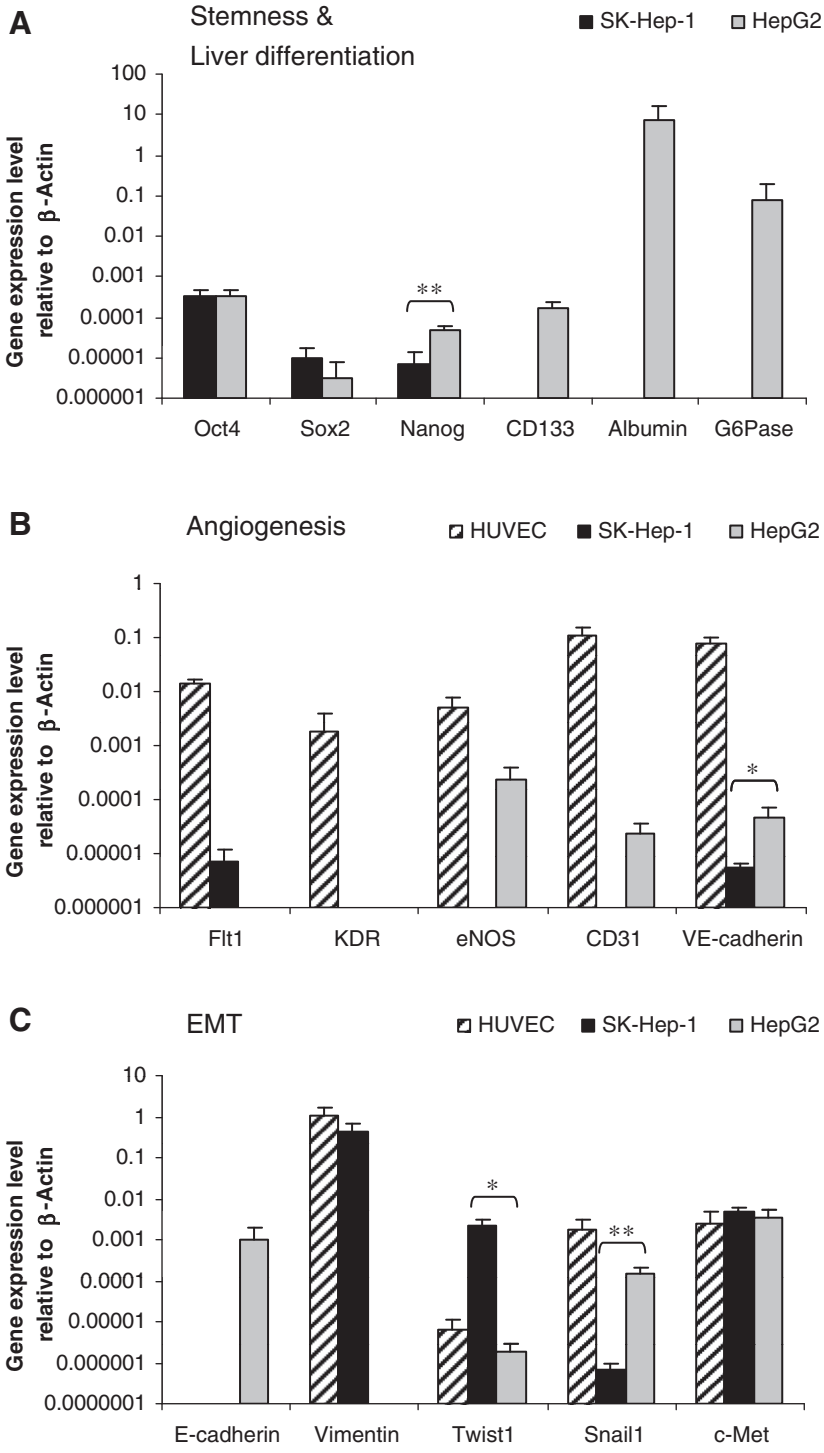


**Fig. 2.** Analysis of gene expression of (A) stemness and liver differentiation markers, (B) angiogenesis-related genes, and (C) EMT markers in HCC cell lines and endothelial cells. Gene expression level was determined by qRT-PCR. Results show gene expression levels relative to β-Actin. Data are expressed as mean + S.D. from three independent experiments, with significant differences between SK-Hep-1 and HepG2 shown by $^{*}P < 0.05$, $^{**}P < 0.01$.

cells exhibited cuboidal shape and compacted colonies, while HGF-treated cells showed fibroblast-like shape and loose colonies, indicating acquisition of EMT shown by acquisition of mesenchymal phenotype and decrease in cell-cell adhesion (Fig. 4A).

Since HGF-induced VM formation in HepG2 was observed on day-2 after treatment, gene expression analysis was studied using cells treated for 2–4 days. Induction of EMT by HGF was revealed by decreased E-cadherin and increased Vimentin expression, and these changes were maintained up to 4 days after treatment (Fig. 4B). In HGF-treated cells, Twist1 expression was up-regulated by 2.1–2.5-fold at days 2–4 after treatment, compared with untreated cells, while up-regulation of Snail1 was observed on day-4 after HGF treatment (Fig. 4B). VE-cadherin expression, necessary for VM formation in melanoma cell lines [29], showed a biphasic response to HGF treatment, showing 1.7-fold increase on day-2 and 0.6-fold decrease on day-4 after HGF treatment (Fig. 4B).

HGF also modulated expression of liver differentiation genes in HepG2. In HGF-treated cells, decreases in Albumin and G6Pase expression were maintained up to day-4 after treatment (Fig. 4C),

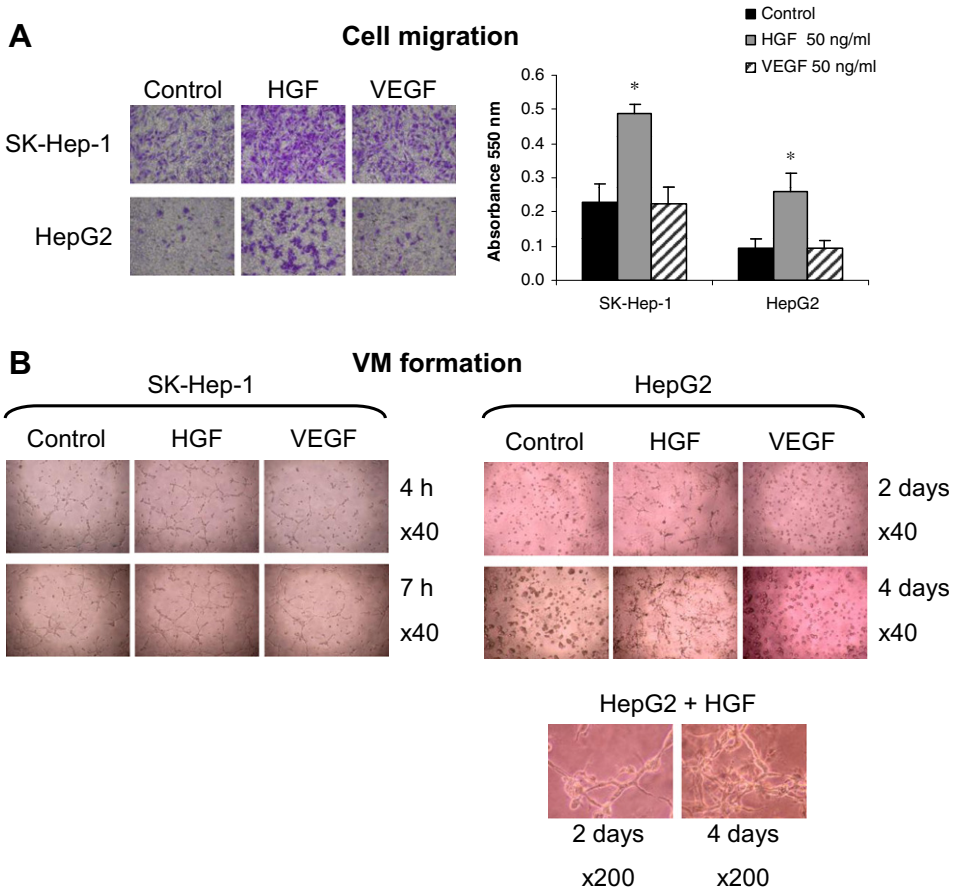


**Fig. 3.** Effect of HGF and VEGF on cell migration and VM formation of HCC cell lines. (A) Migration of HCC cells after incubation in Transwell chambers for 6 h (SK-Hep-1) or for 24 h (HepG2), with lower chambers filled with the tested growth factor (50 ng/ml). Filters were stained with crystal violet, and original magnification was 200×. Cell migration was quantified from absorbance of the dye retained on the filters. Data are expressed as mean + S.D. from two independent experiments, with significant differences from control shown by $^{*}P < 0.05$. (B) VM formation of HCC cells cultured in Matrigel-coated 96-well plates, with culture media containing HGF or VEGF (50 ng/ml). Photographs were taken at indicated times, and original magnification was 40× or 200× as indicated. Data are representative of three independent experiments with similar results.

in parallel with decreased epithelial marker E-cadherin (Fig. 4C), suggesting association between EMT and de-differentiation induced by HGF.

HGF treatment markedly increased expression of stemness genes in a time-dependent fashion. Significantly, CD133 was up-regulated 2 days after treatment (Fig. 4D). Sox2 and Nanog were significantly increased on day-4 after treatment, while Oct4 increased on day-4 after treatment but not significantly (Fig. 4D). Sox2 expression showed 0.4-fold decrease on day-2, but increased to 4.5-fold on day-4 (Fig. 4D). Thus, significant increases in expression of pluripotency transcription factors by HGF treatment occurred after EMT and de-differentiation.

## 4. Discussion

The process of VM formation should be similar to angiogenesis, where endothelial cells migrate into parenchyma, remodel surrounding extracellular matrix using MMPs, differentiate and organize themselves into tubular structures [28]. Most of these steps are also used by cancer cells during invasion. This may explain why VM capacity closely relates to invasiveness of cancer cells. Among the selected angiogenesis-related genes, only VE-cadherin was in both VM-positive and VM-negative cell lines. VE-cadherin is an endothelial-specific cell adhesion molecule necessary for VM formation of aggressive melanoma cell lines [29]. Our results suggested that the VM-negative cell line lacks some critical factors required for VM capacity, such as the ability organize into tubular structures. Although HepG2 possesses VE-cadherin, but if cells were less motile, tubular structures would not be formed or take longer. This was supported by results showing that HGF enhanced the rate of tube formation in SK-Hep-1. In HepG2, HGF induced EMT and enhanced motility of cells, facilitating tubular organization, so EMT might be the missing factor required for VM formation of HepG2.

To our knowledge, this is the first report of exploring stemness gene expression in VM-forming HCC cells, and showing an inducing effect of HGF on VM formation. When well- and poorly-differentiated HCC cell lines are compared, intrinsic VM capacity was associated with mesenchymal features already present in poorly-differentiated SK-Hep-1, rather than with expression levels of stemness genes. But in the case of well-differentiated HepG2, unable to form VM, stemness gene expression was increased during HGF-induced EMT, suggesting that the role of stemness genes in VM modulation of HCC cells is likely to depend on differentiation status of the cells.

CSCs are believed to have greater metastatic potential than non-CSCs. However, our studies showed that pluripotency transcription factor genes Oct4, Sox2 and Nanog were expressed at similar levels in both highly invasive VM-positive and poorly invasive VM-negative cell lines. Surprisingly, CD133 was detected only in the VM-negative cell line. These results indicate that intrinsic VM capacity and invasiveness of HCC cell lines did not correlate with

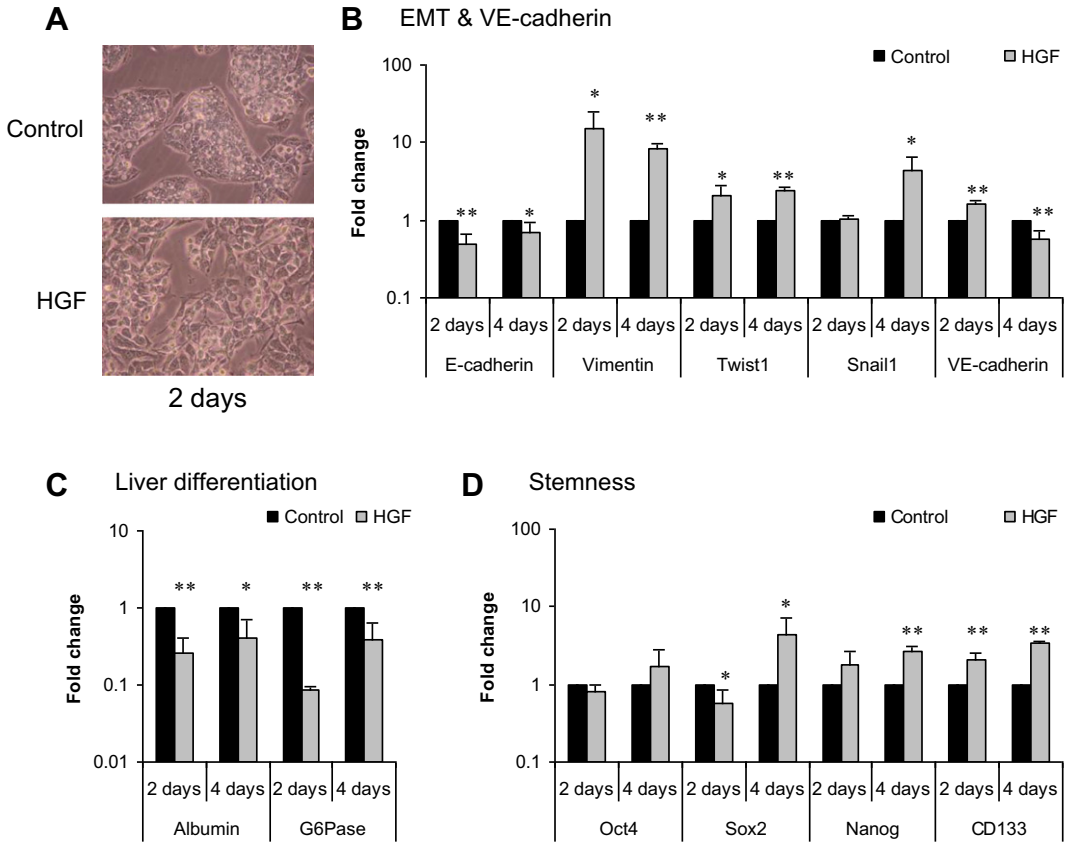


**Fig. 4.** HGF-induced changes in cell morphology and gene expression levels in HepG2 cells. HepG2 cells were cultured in 96-well plates with or without 50 ng/ml HGF for indicated times. (A) Photographs were taken to detect changes in cell morphology, and original magnification was 200×. (B–D) qRT-PCR analysis was used to determine changes in gene expression. Relative HGF-induced change in gene expression compared with control is expressed as fold change calculated by $2^{-\Delta\Delta Cp}$ method. Data are expressed as mean + S.D. from three independent experiments, with significant differences from control shown by $^{*}P < 0.05$, $^{**}P < 0.01$.

basal expression levels of the stemness genes. This agrees with the recent report by Chen et al. [30] showing lack of expression of CSC markers CD133 and EpCAM in poorly-differentiated HCC cell lines, HLE and HLF, which exhibit high invasiveness and show signs of EMT, by expressing EMT regulators without E-cadherin. This suggests that EMT had already been acquired in the poorly-differentiated HCC cell lines and was responsible for stem-like properties such as spheroid formation and chemoresistance.

However, the function of Oct4, Sox2 and Nanog genes in VM formation cannot be ruled out, since they are involved in maintaining pluripotency of embryonic stem cells [25]. It is possible that EMT provides some signals that coordinate with the pluripotency transcription factors needed to maintain the de-differentiation state of cancer cells during EMT activation.

Twist and Snail EMT regulators were shown to independently promote metastasis of HCC [31]. Our data showed that Twist1 expression level was much higher than Snail1 in VM-positive SK-Hep-1. Moreover, increased Twist1 expression paralleled VM formation during HGF treatment of HepG2, while increased Snail1 expression occurred later than Twist1, suggesting that Twist1 is more important than Snail1 in VM formation of HCC cells.

In conclusion, our results show that intrinsic VM capacity of human HCC cell lines was linked to mesenchymal properties of cancer cells, rather than stemness gene expression. Additionally, the VM capacity of well-differentiated HCC cells could be induced by pro-metastatic factors such HGF, through EMT concomitant with increasing stemness gene expression. The finding that VM capacity could be induced by pro-metastatic factors or conditions enhancing cancer cell aggressiveness is important for HCC treatment. Since anti-angiogenic therapy was recently shown to cause tumor hypoxia and promote VM formation in an animal model [5], anti-angiogenic therapy in HCC should be combined with inhibitors of invasion to prevent angiogenesis, VM formation, and metastasis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.009.

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