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ORIGINAL ARTICLE

Aberrant hedgehog signaling is responsible for the highly invasive behavior of a subpopulation of hepatoma cells

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Hepatoma exhibits a series of heterogeneous subpopulations in its cell surface markers, tumorigenicity, invasion and metastatic capability. We previously demonstrated that the $CD133^-/EpCAM^-$ hepatoma subpopulation was more metastatic than its counterpart; however, the controlling mechanisms are unexplored. The present study aimed to delineate the significance of aberrant hedgehog (Hh) signaling in the mediation of metastases. Fluorescence-activated cell sorting-enriched $CD133^-/EpCAM^-$ (double negative, DN), Huh-7 cells underwent a transwell selection for metastatic cells (transwell-selected, TS). The TS cells displayed much greater metastatic activity as evidenced by an increased invasion rate, extremely upregulated expression of matrix metalloproteinase (MMP)-1/2/9 genes compared with DN and double-positive (DP) subpopulations. In contrast to DP cells, TS cells lost E-cadherin and were all vimentin-positive as shown by immunocytochemistry. There was a transitional increase in Gli-1/2 gene expression levels from DP, DN to TS subpopulations, which was consistent with elevated Gli-1/2 or Twist-1 protein levels in the nuclear fraction. Furthermore, truncated Gli-1 (tGli-1), which transactivates molecules involved in metastasis, was detected in the highly invasive Huh-7 cell subpopulation, but not in less metastatic hepatoma cells or normal hepatocytes. The enhanced metastatic features with increased expression of MMPs as well as the presence of twist and snail genes in TS Huh-7 cells were reversed by LDE225, a potent Smoothened antagonist. In conclusion, the highly metastatic capability of a unique TS subpopulation was highly attributed to significant epithelial–mesenchymal transition, enhanced Hh activity and aberrant occurrence of a tGli-1 variant, which appears to be responsible for the highly invasive behavior.

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INTRODUCTION

In the United States, the incidence of hepatocellular carcinoma (HCC) has steadily increased during the last two decades, and it will keep rising in the next 2–3 decades because of the high incidence of hepatitis B virus and hepatitis C virus infections, and non-alcoholic fatty liver disease.[1,2] HCC has become a leading cause of cancer-related death worldwide. The only effective therapies, such as surgical removal and liver transplantation, are available with a considerable relapse rate in the remaining liver or grafts within 3 years.[3] For non-resectable HCC, the efficacy of adjuvant therapies is not promising and the 5-year survival rate is still very poor.[4] Invasion and metastases are two fundamental properties of the malignancy, which determine the prognosis of HCC patients,[5] especially poorly differentiated HCC. This category of HCC is often insensitive to chemotherapy, metastasizes at an early stage and possesses an unfavorable prognosis.[6] Patients with this type of HCC are often classified as refractory to currently available regimens. Thus, seeking molecular targets that could improve the treatment outcome is intriguing.

The hedgehog (Hh) signaling pathway is highly conserved, and has a crucial role in embryogenesis, adult tissue homeostasis and carcinogenesis.[7] Transcription factors Gli-1, Gli-2 and Gli-3 constitute the Gli family.[8,9] Gli-1/2 have been implicated to be pivotal for positively transactivating target genes, such as snail, Gli-2, c-myc and B-cell lymphoma-2, and cyclin D and so on.[10] Hh signaling is activated following binding of Sonic, Desert or Indian Hh ligands to their transmembrane receptor Patched, leading to the release of Gli transcription factors by derepressing Smoothened (Smo). Gli-1/2 translocate to the nucleus, where they bind to the Gli-binding consensus element in target genes resulting in their activation.[11] Overexpression and aberrant activation of Hh signaling molecules have been demonstrated in 50–70% of hepatoblastomas, primary HCC and cholangiocyte carcinomas,[12,13] and the Smo antagonists, cyclopamine and GDC-0449 have been used to block the growth of hepatoma cell-derived xenografts in animal experiments.[12,14] However, no study is available focusing on whether overexpression of Hh molecules or enhancing Hh signaling activity is responsible for the highly metastatic behavior of HCC.

Epithelial–mesenchymal transition (EMT) is characterized as the loss of epithelial morphology and development of a highly mobile mesenchymal phenotype.[15,16] During cancer progression, EMT promotes the metastatic capability of cancer cells. In our previous study, we enriched subpopulations of hepatoma cells according to their surface markers (CD133, EpCAM, ALDH (aldehyde dehydrogenase)), and demonstrated that HLF, HLE and Huh-7 $CD133^-/ALDH^{low}$ or $CD133^-/EpCAM^-$ are highly metastatic, resistance to

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chemotherapeutic agents and acquired EMT as reflected by negative E-cadherin but positive vimentin expression.[17] At the same time, these double-negative (DN) subpopulations possessed high Hh signaling activity as reflected by enhanced expression of the Hh receptor Patched-1, increased nuclear Gli-2 protein content and increased Hh signaling activity and so on.[17] However, no direct evidence that Hh modulates metastases was provided because of the fact that the surface marker expression profile was unstable over passaging. In this study, we enriched a transwell-selected (TS) subpopulation, which stably retained negative CD133/EpCAM expression profile over multiple passaging, and displayed a highly metastatic behavior to delineate the underlying molecular interactions responsible for this highly metastatic behavior. Moreover, an Hh signaling inhibitor, LDE225, the specific inhibitor of Smo at nM levels with a high bioavailability,[18] was used to investigate the crucial role of Hh signaling in the mediation of metastasis. Our findings suggest that aberrant Hh signaling activity and the occurrence of truncated Gli-1 (tGli-1) have a crucial role in mediating the highly metastatic capability by activating EMT transcription factors and matrix metalloproteinases (MMPs) in this unique subpopulation.

RESULTS

Verification of cell surface markers after multiple passages of Huh-7 subpopulations and the TS subpopulation

Fluorescence-activation cell sorting (FACS)-enriched subpopulations have unique properties in terms of their tumorigenicity, sensitivity to chemotherapeutics and metastases. However, the cell surface markers and their properties change with passaging. To obtain a subpopulation displaying a highly invasive capability, we used a transwell approach to select cells from a FACS-enriched $CD133^-/EpCAM^-$ (DN) subpopulation, and obtained a subpopulation, which exhibited highly invasive capability. At the same time, the cell surface marker profile remained as $CD133^-/EpCAM^-$ (96.9%) even after seven passages (Figure 1d). In contrast, Huh-7 DP and DN subpopulations could not maintain their cell surface marker profile after multiple passages (Figures 1b and c), and tended to change back to the profile of cells without FACS enrichment (Figure 1a).

Enhanced invasive capability in the TS $CD133^-/EpCAM^-$ Huh-7 subpopulation

Cell migration capability of Huh-7 subpopulations was evaluated using a transwell Matrigel invasion assay. As shown in Figures 2c and e, a marked difference in invasive capacity was found between different subpopulations with the highest rate of migration in the TS $CD133^-/EpCAM^-$ Huh-7 subpopulation. To determine the molecular basis for the highly metastatic property of this TS population, we evaluated the gene expression of MMP-1, 2 and 9 in various subpopulations, and found that the mRNA levels of MMP-1, MMP-2 and MMP-9 were extraordinarily increased in the TS Huh-7 subpopulation (4294-, 74- and 36-fold) compared with the double-positive (DP) subpopulation, although a tendency of increased MMP gene expression existed from the

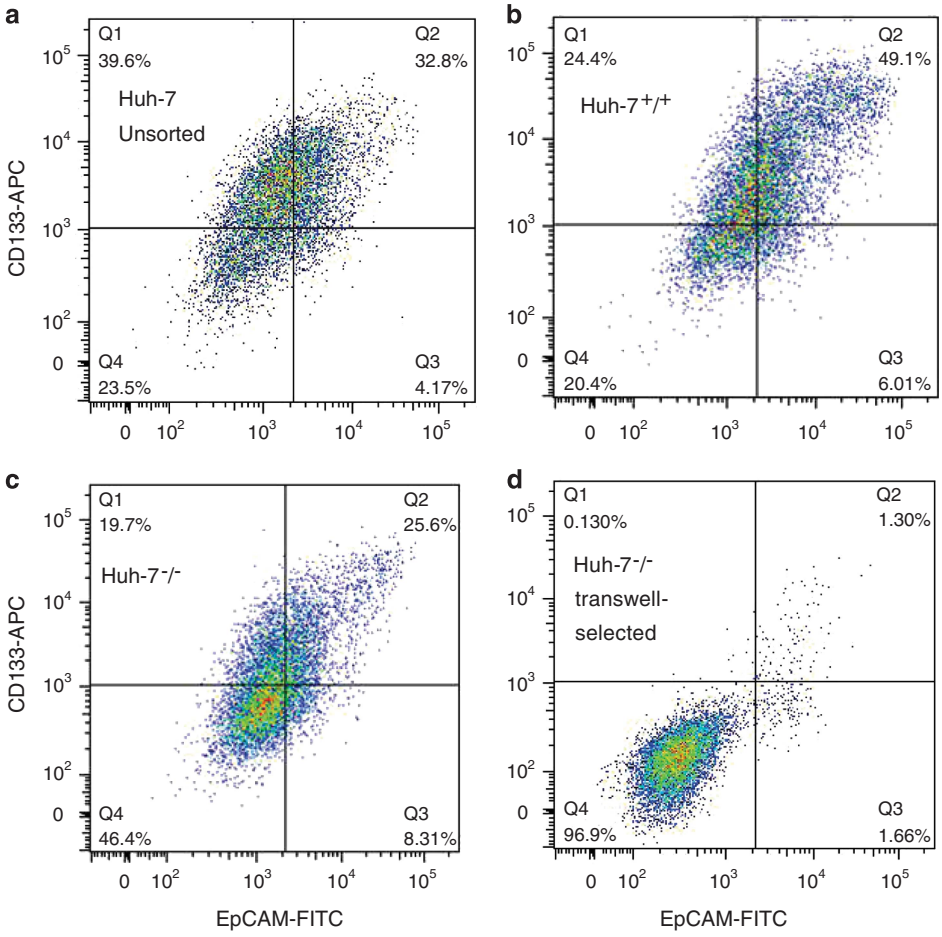

Figure 1. FACS-enriched subpopulations were grown for seven passages after transwell selection or FASC enrichment. Surface marker expression profile was verified in the corresponding subpopulations by flow cytometry. (**a**) Huh-7 unsorted; (**b**) Huh-7 $CD133^+/EpCAM^+$ (double positive, DP) subpopulation; (**c**) Huh-7 $CD133^-/EpCAM^-$ (double negative, DN) subpopulation; and (**d**) Transwell-selected $CD133^-/EpCAM^-$ subpopulation.

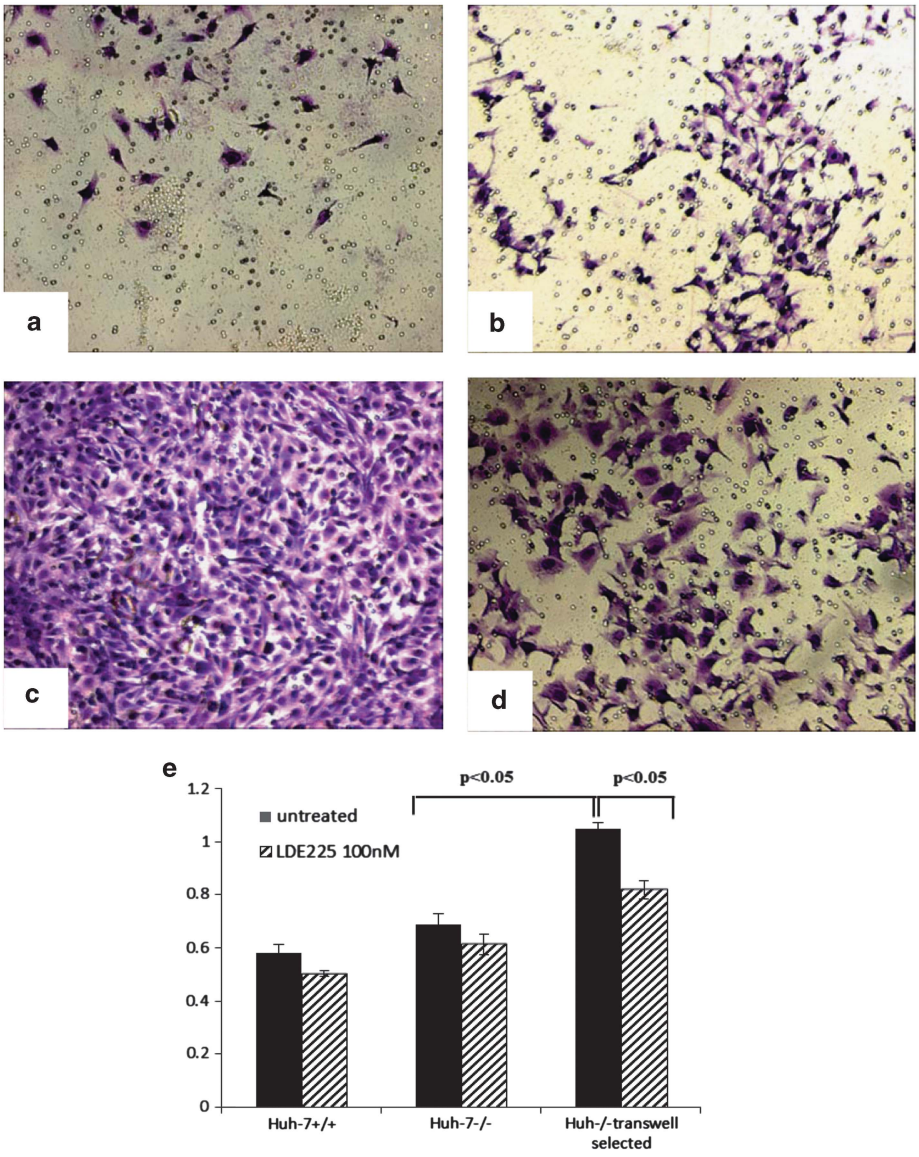

Figure 2. Migration capacity of transwell-selected subpopulation. The metastatic capability was determined by the Transwell Matrigel Invasion assay. Representative images of migrating cells were taken 24 hrs after seeding. The invaded cells were fixed and stained with crystal violet as described in Materials and Methods. (**a**) Huh-7 $CD133^+/EpCAM^+$ (DP) subpopulation; (**b**) Huh-7 $CD133^-/EpCAM^-$ (DN) subpopulation; (**c**) transwell-selected (TS) subpopulation; and (**d**) TS plus LDE225 (100 nM) treatment. (**a**–**d**) Micrographic images were taken at ×200. (**e**) Stained cells were lysed and the lysates were measured spectrophotometrically at 590 nm to reflect the number of migrating cells. All experiments were performed for at least three times with triplicates.

DP, unsorted to DN subpopulations (Figure 3). It is known that MMP-1, MMP-2 and MMP-9 are involved in the cleavage of extracellular matrices by metastatic tumor cells, and elevated MMP levels are indicative of metastatic capability of malignant cells.

Immunocytochemical staining of EMT markers in Huh-7 and HLF cells

We performed immunocytochemical staining for EMT markers including E-cadherin, a cell surface-adhering molecule, and vimentin, a cytoskeleton protein in Huh-7 and HLF subpopulations. Strong E-cadherin staining was observed in the Huh-7 DP subpopulation (Figure 4a), whereas, vimentin staining was negative in the cytoplasm of these cells (Figure 4b). Meanwhile, E-cadherin was negative in the Huh-7 DN subpopulation (Figure 4c), whereas, vimentin staining was positive (Figure 4d). TS $CD133^-/EpCAM^-$ Huh-7 subpopulation completely lost E-cadherin expression (Figure 4e); however, they were vimentin-positive (Figure 4f). The expression profile of EMT markers of the TS Huh-7 subpopulation was similar to poorly differentiated unsorted HLF cells (Figures 4g and h), indicating that there was no difference in the EMT marker expression profile among HLF cells, freshly enriched Huh-7 DN and TS Huh-7 subpopulations, and that the latter two cell subpopulations were in the status of EMT.

Increased expression of Hh signaling and EMT molecules in the highly metastatic Huh-7 subpopulation

To assess the expression level of Hh signaling molecules in Huh-7 subpopulations, we examined mRNA levels of the key signaling molecules: transcription factor Gli-1/2, by a real-time reverse transcriptase PCR (RT–PCR) assay. The results showed that mRNA levels were similar between unsorted and DP Huh-7 subpopulations, but were significantly increased in the DN Huh-7

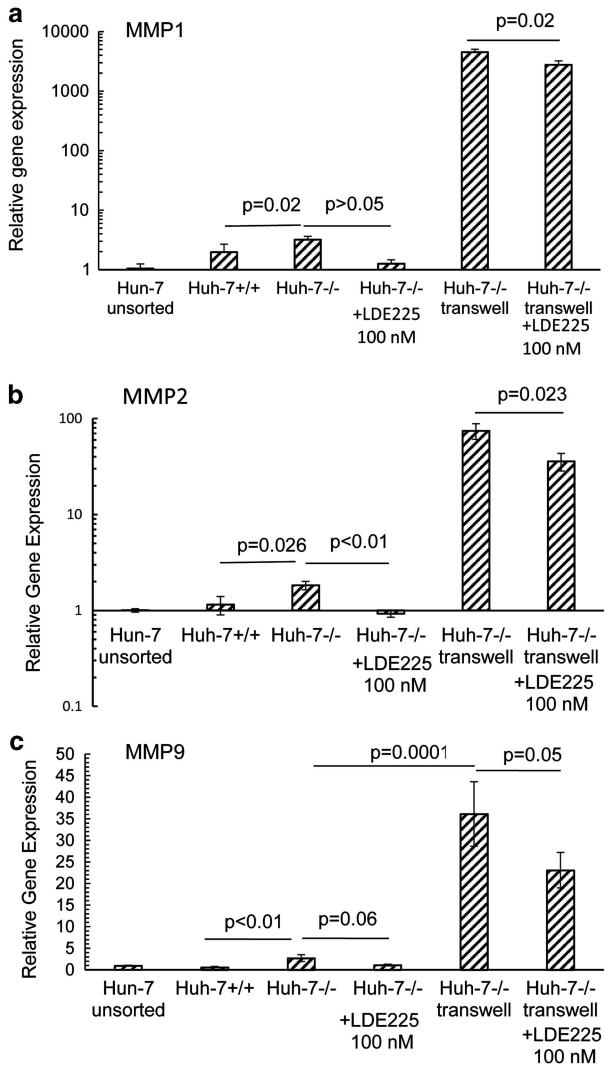

Figure 3. Relative mRNA levels of MMP-1 (**a**), MMP-2 (**b**) and MMP-9 (**c**) in Huh-7 subpopulations and effects of LDE225. RNA was extracted from transwell-selected cells and other Huh-7 subpopulations, and mRNA levels were determined by real-time quantitative RT–PCR using human GAPDH as a house-keeping gene control. Huh-7 DN and transwell-selected cells were exposed to LDE225 for 24 h. In Panel (**a**) and (**b**), log level is shown in the *Y* axis. The data were summarized from three independent experiments.

subpopulation compared with the DP subpopulation. Notably, the expression level of Gli-1/2 was remarkably elevated in the TS Huh-7 subpopulation (Figures 5a and b). Western blotting analysis results were consistent with those of real-time RT–PCR, and it was evident that these two transcriptional factors, Gli-1/2, were increased in the nuclear fraction of DN and TS Huh-7 subpopulations compared with the unsorted or DP Huh-7 subpopulations (Figure 6a).

To evaluate the Hh signaling activity, we transfected Huh-7 subpopulations with a Gli-lux reporter system, and determined firefly luciferase activity 48 h after transfection. It was determined that luciferase activity was much higher in the TS Huh-7 subpopulation than other subpopulations (Figure 6b), whereas cells transfected with mutated Gli-lux did not show any luciferase activity (data not shown). These results confirmed that the TS Huh-7 subpopulation possessed the highest Hh signaling activity, consistent with the highest Gli-1/2 gene expression levels by real-time RT–PCR and western blot analysis.

The tGli-1 variant was detected in highly metastatic hepatoma cells

The novel tGli-1 variant was reported to promote malignant cancer cells with a high migration and invasion rate.[19,20] To investigate tGli-1 expression, we examined the tGli-1 variant fragment in several hepatoma cell lines and primary human hepatocytes by a regular RT–PCR assay, and found that tGli-1 transcripts were negative in normal liver cells and well-differentiated hepatoma cells with a high level of hepatic-specific gene expression, including Hep3B and HepG2 cells. However, tGli-1 was positive in the poorly differentiated HLF line and high metastatic Huh-7 subpopulations, including the TS Huh-7 cells (Figure 6c). The occurrence of the tGli-1 variant in these hepatoma cells indicates that there exists an aberrant Hh signaling mechanism that links the highly metastatic property to the molecular basis of abnormal Hh signaling activity.

Enhanced EMT could well be the mediator for accelerated migration in the TS subpopulation

Consistent with the immunocytochemical staining findings shown in Figure 3, gene expression levels of twist-1 and snail-1, both of which are transcription factors for the occurrence of EMT, in TS Huh-7 cells were much higher than Huh-7 DP and DN subpopulations (Figures 5c and d). This finding was confirmed by protein levels of twist-1 in the nuclear fraction with western blot analysis (Figure 6a). These data confirm that upregulated or aberrant Hh signaling activity in TS cells may utilize EMT as the molecular basis for the accelerated migration by expressing extraordinary MMP genes.

A potent Smo inhibitor, LDE225, suppressed EMT and migration of TS cells

It is obvious that LDE225 was able to inhibit the invasion rate (Figures 2c–e) as well as gene expression of MMP-1, 2 and 9 in TS Huh-7 subpopulation (Figures 3a–c). At the same time, LDE225-treated cells had lower levels of Gli-1 or Gli-2 gene expression than those without the treatment (Figures 5a and b). In LDE225-treated DN subpopulation, snail-1 and twist-1 expression levels were lower than those without treatment; however, the decrease in snail-1 and twist-1 gene expression levels did not reach a statistical significance in the TS subpopulation. Taken together, these data indicate that it was an effective approach to suppress the downstream gene expression by blocking the Hh signaling pathway, as Gli-1/2, twist-1 and snail-1 are the target genes of the Hh signaling pathway.[9,21,22] Moreover, these results further confirmed that Hh signaling is an important pathway in the mediation of the highly invasive behavior of the TS Huh-7 subpopulation. In addition, it appears that LDE225 likely suppresses tGli-1 expression in TS Huh-7 cells (Figure 6c), although a quantitative method is needed to assess the suppressive effects more precisely.

DISCUSSION

HCC develops in a variety of liver diseases with different etiologies, and the clinical presentation, tumor invasiveness, early metastatic features and responses to treatments differ from patient to patient. In the same tumor mass, there are heterogeneous cell populations with different phenotypes. The emergence of diversified tumor cell subpopulations in HCC accounts for their heterogeneous cellular phenotypes and virtually ensures that some tumor cells will ultimately evolve with the most favorable properties for their enhanced abilities to survive, grow, invade and metastasize (tumor progression).[23] Metastasis is a process by which malignant cells move away from the primary tumor through blood or lymphatic vessels and produce metastatic

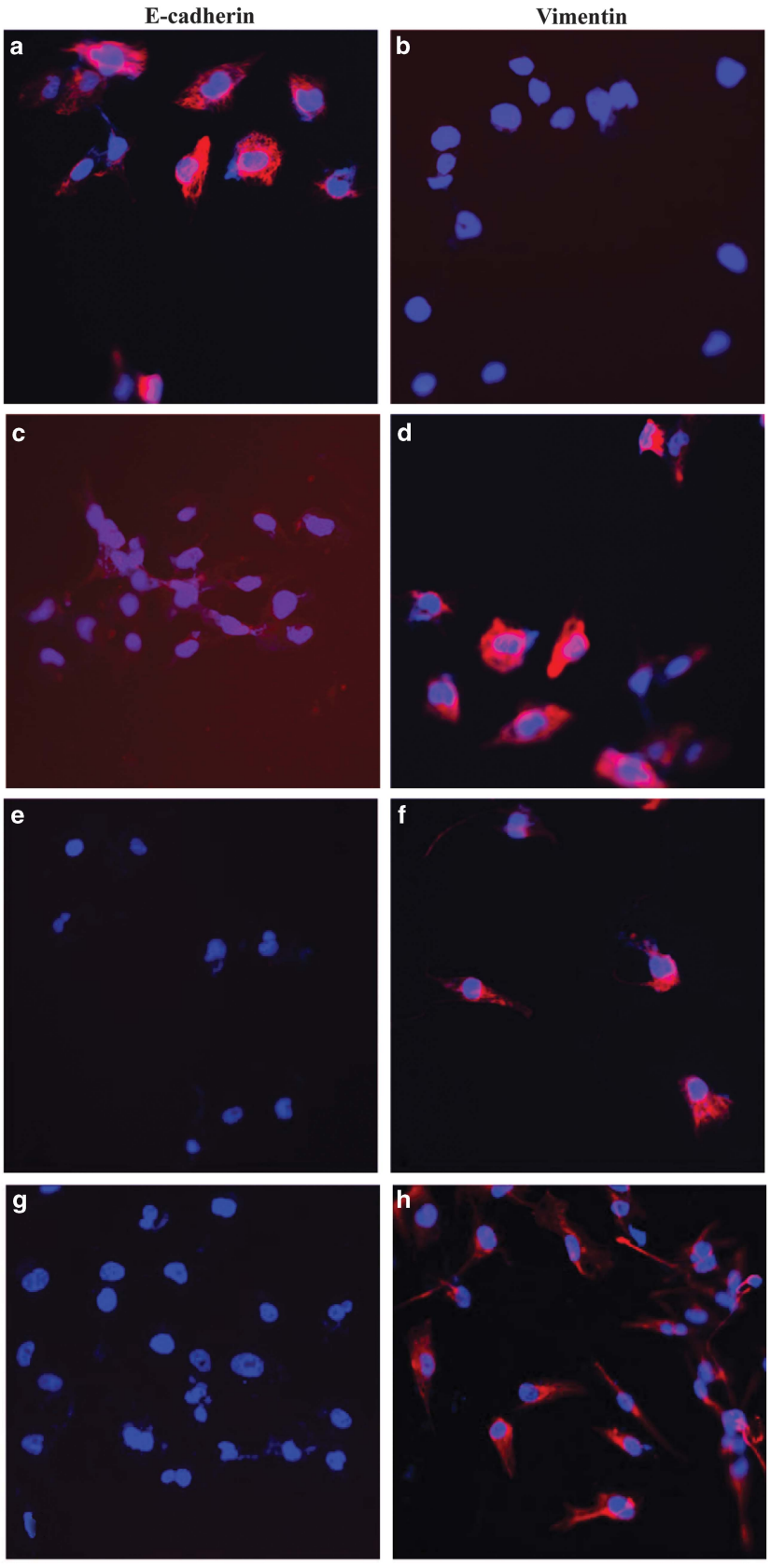

Figure 4. Immunocytochemical staining of HLF and Huh-7 subpopulations. Hepatoma cells were seeded on chamber slides precoated with collagen type I, and after overnight culture, cells were fixed with 4% paraformaldehyde, and stained immunohistochemically as described in the Materials and Methods. All cells were counter stained with DAPI for nuclear imaging. (**a**, **b**) Huh-7 DP subpopulation; (**c**, **d**) Huh-7 DN subpopulation; (**e**, **f**) Huh-7 DN, transwell-selected subpopulation; (**g**, **h**) HLF-unsorted cells. Magnification: ×400.

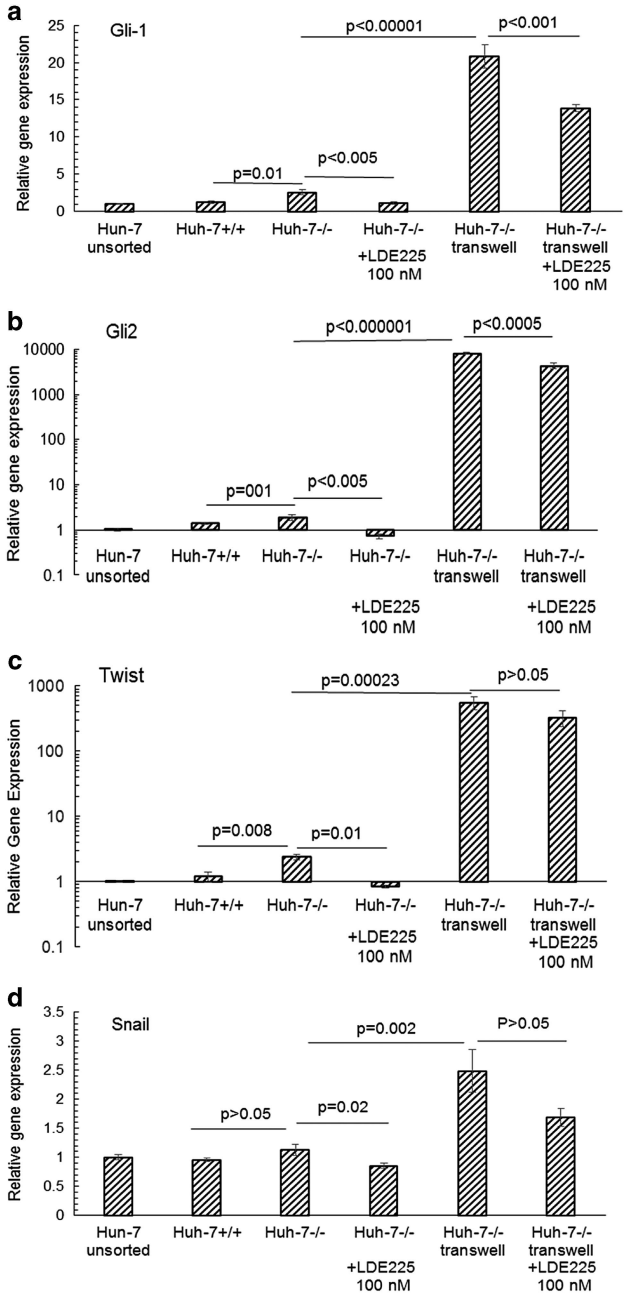

Figure 5. Relative gene expression levels of Gli-1, Gli-2, twist-1 and snail-1 in Huh-7 subpopulations and effects on LDE225. Hepatoma Huh-7 subpopulations were cultured and treated with or without LDE225 for 24 h. Total RNA was extracted, and mRNA levels of Gli-1 (**a**), Gli-2 (**b**), twist-1 (**c**) and snail-1 (**d**) were determined by real-time quantitative RT–PCR using the GAPDH gene as a house-keeping control. In Panel (**b**) and (**c**), relative gene expression levels were expressed as a log scale in the *Y* axis. The data were summarized from at least three independent experiments.

nodules at secondary sites. Metastasis takes place as a complex multistep process involving adhesion, invasion, migration, reseeding and colonization of cancer cells in distal sites.[24] We previously found CD133 and EpCAM are useful markers for Huh-7 subpopulations, and the $CD133^{-}/EpCAM^{-}$ Huh-7 subpopulation possesses a profound invasive ability comparing with their DP counterparts.[17] In the supplementary data, we showed that three highly metastatic hepatoma cell lines, SMCC-7721, MHCC-97H and MHCC-97L, from the Shanghai region,[5,25] were all CD133-negative and EpCAM-negative by flow cytometric analysis (supplementary Figure 1). These DN hepatoma cell lines express high levels of Gli2 and MMP-1/2 (60–155-fold increase) compared with unsorted Huh-7 cells (supplementary Figure 2). Freshly FACS-enriched subpopuplations were used in previously studies. However, after several passages, CD133 and EpCAM expression profile of Huh-7 subpopulations had a tendency to return to the surface profile of unsorted Huh-7 cells, and the phenotype was changeable too. To establish a stable subpopulation with more metastatic ability, we obtained a highly metastatic Huh-7 subpopulation by transwell selection. After that, we examined the surface marker expression profile of the TS Huh-7 subpopulation by flow cytometry, and found that these cells kept the surface marker profile similar to that after FACS enrichment, that is CD133 and EpCAM DN, even after several passages (Figure 1d). Next, we further characterized the property of this subpopulation, and confirmed its metastatic features by performing the transwell invasion assay again in comparison with unsorted or other FACS-enriched subpopulations as shown in Figures 1b and c. Moreover, these cells exhibited the striking features of EMT, manifested by the loss of E-cadherin expression, and being positive for vimentin expression in their cytoplasm (Figure 4). In association with a markedly enhanced metastatic capability, the mRNA levels of MMP-1, MMP-2 and MMP-9 in the TS subpopulation were much higher than in the DN, DP and unsorted Huh-7 cells (Figure 3). Therefore, the highly metastatic feature of the TS subpopulation is attributed to the release of MMPs, which have been suggested to be critical factors, destroying the extracellular matrices for vascular invasion of metastatic cells.[22]

In our previous study, we have demonstrated that Hh signaling is responsible for the drug resistance and metastatic feature of DN Huh-7 subpopulation, and the occurrence of EMT may well be the molecular basis for these characteristics seen in the DN subpopulation.[17] However, we did not have direct evidence to demonstrate that the Hh signaling actually governs the metastatic feature through EMT. This highly metastatic Huh-7 subpopulation offered an opportunity to explore the critical role of Hh signaling in this process. We first determined gene expression levels of Gli-1/2, as well as two transcription factors for EMT, twist-1 and snail-1, in Huh-7 subpopulations. As shown in Figure 5, the subpopulation of DN and TS Huh-7 cells expressed much higher levels of Gli-1/2, twist-1 and snail-1 compared with unsorted, DP and DN Huh-7 subpopulations. The high Gli-1/2 transcription factor expression was in agreement with increased luciferase activity after the transfection of a Gli-lux reporter plasmid, in which the firefly luciferase gene is under the control of the Gli promoter.[26] Therefore, we assume that a higher level of Hh Gli-1/2 expression led to the significantly increased expression of twist-1 and snail-1 in the TS Huh-7 subpopulation comparing with other subpopulations. These findings demonstrated that the expression of Hh transcription factors, Gli-1/2, in the TS Huh-7 subpopulation may be responsible for the acquisition of EMT in these cells, as twist and snail have been confirmed as downstream target genes of these two Gli transcription factors,[22] both of which have been demonstrated to act as promoting factors in the metastasis of HCC or other cancers.[27,28]

A novel tGli-1 isoform in which 41 amino acids are deleted, corresponding to an alternative splicing of the entire exon 3 and part of exon 4 of the gene was reported in glioblastoma and breast cancer.[19] Hh signaling regulates the expression of Gli-1 isoforms.[29] Most notably, the tGli-1 isoform behaves as a gain-of-function of Gli-1 and induces expression of genes that are normally not regulated by Gli-1, and it is known to foster more aggressive cancer phenotypes. tGli-1 has a greater tendency than Gli-1 to promote glioblastoma and breast cancer cell migration and invasion by enhancing the expression of MMP-2 and MMP-9.[20] Enhanced Hh signaling has been identified in ~70% of human HCC specimens;[30] however, no previous study has

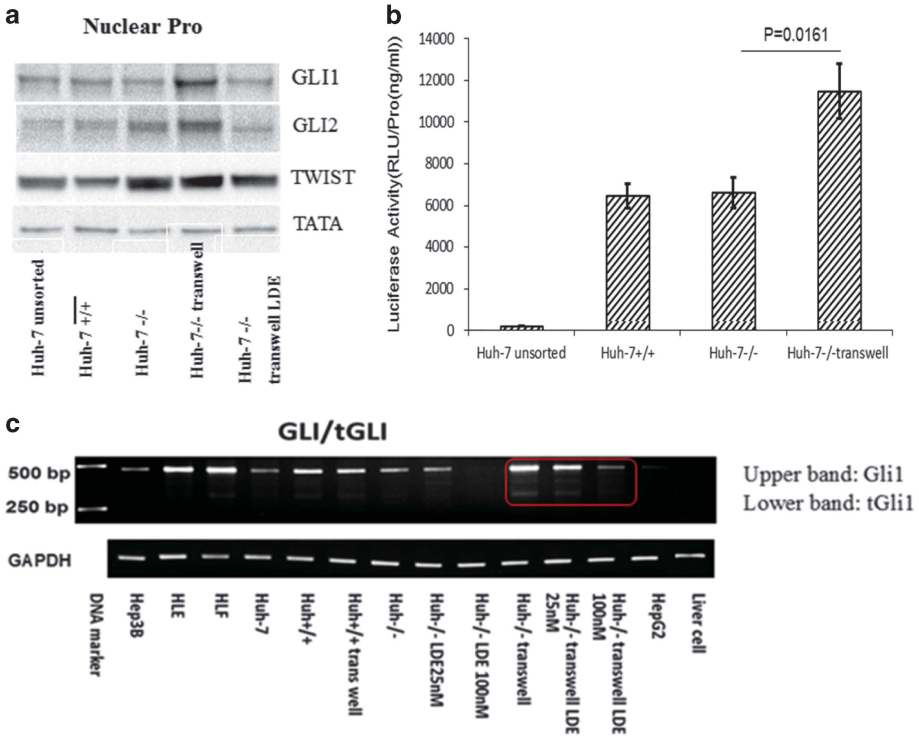

Figure 6. Hh signaling activity and truncated Gli-1 expression in hepatoma cells. (**a**) Western blot analysis of Gli-1, Gli-2 and twist-1 protein levels in nuclear extracts of Huh-7 subpopulations. The TATA box was used as a nuclear fraction loading control. (**b**) Hh signaling activity in Huh-7 subpopulations was determined by transfection of a Gli-Luc reporter construct and expressed as luciferase activity (RLU)/mg protein. Luciferase activity was measured 48 h after transfection. (**c**) Truncated Gli-1 (tGli-1) was detected by a specific primer pair, which amplifies both wild-type Gli-1 and tGli-1, and it is highly expressed in transwell-selected $CD133^{-}/EpCAM^{-}$ Huh-7 cells as indicated in a red frame.

revealed whether tGli-1 is present in hepatoma cell lines or HCC tissue. In the present study, we examined the expression of tGli-1 in different hepatoma cell lines, subpopulations and normal liver cells by regular RT–PCR. The results showed that tGli-1 was present only in poorly differentiated cell lines and the TS Huh-7 subpopulation, in which greatly enhanced MMP-1, MMP-2 and MMP-9 gene expression was documented. It is highly likely that these MMPs contributed to the much accelerated migration of these TS Huh-7 cells. Therefore, the presence of tGli-1 in the highly metastatic TS subpopulation probably provided the extra-driving force in transactivating many downstream target genes, such as Gli-1/2, transcription factors that regulate EMT, such as snail, twist, and other genes, as well as MMPs. The self-amplifying loop of the Hh signaling pathway by activating its own transcription factors, Gli-1/2, leads to the disordered downstream gene expression profile and enhanced tumor cell survival and drug resistance, as well as accelerated metastases. Thus, we have provided convincing evidence demonstrating that the enhanced metastatic property in the TS Huh-7 subpopulation is highly attributed to aberrant Hh signaling activity caused by increased normal Hh signaling molecules and/or the occurrence of the tGli-1 variant in this subpopulation.

Smo inhibitors, such as GDC-0449, LDE225, LEQ-506 and so on, have been evaluated to suppress the progression and metastases of various cancer types, including glioblastoma, pancreatic, ovarian cancer and multiple myeloma in animal models and clinical trials.[14,21,31,32] However, no clinical trials have been undertaken with LDE225 for liver cancer yet. LDE225 has been proven to be effective in reducing the relapse of advanced medulloblastoma and basal cell carcinoma in a Phase I clinical trial, and the adverse effects are acceptable in the treated patients with dose escalation.[31] Our findings documented that exposure to LDE225 at 100 nM levels could suppress the gene expression of Gli-1/2 and MMP-1/2/9, as well as the metastatic capability. Thus, it is conceivable that suppressing Hh signaling activity could be a valuable target in improving the therapeutic outcome of HCC at an advanced stage, and that the high bioavailability after oral administration of this agent makes it potentially useful in patients with HCC.[18]

In conclusion, the findings of the present study demonstrate that TS $CD133^{-}/EpCAM^{-}$ Huh-7 cells are highly metastatic as the result of EMT occurrence, and extraordinary levels of MMP gene expression. These characteristics are attributed to enhanced expression of Hh signaling molecules, such as Gli-1/2, as well as the occurrence of the tGli-1 variant, which is responsible for the abnormal expression of many downstream molecules, such as MMPs. Therefore, Hh signaling molecules, such as Smo or Gli, represent valid targets for the improvement of therapeutic outcomes.

MATERIALS AND METHODS

Cell culture

Hepatoma Hep3B and HepG2 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Huh-7 was kindly provided by Professor Mark Feitelson, PhD from Temple University, Philadelphia, PA, USA. HLE and HLF cells were obtained from the Health Science Research Resources Bank, Tokyo, Japan. SMCC-7721, MHCC-97H and MHCC-97L were obtained from the Institute of Liver Cancer, Fudan University Affiliated Zhongshan Hospital, Shanghai, China. All cell lines were incubated in Dulbecco's modified essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Grand Island, NY, USA) and 1% (v/v) penicillin–streptomycin. Cells were incubated at 37 °C, 5% CO_2 in fully humidified air. LDE225, a special antagonist of Smo, was purchased from Cellagen Technology, San Diego, CA, USA.[33]

FACS enrichment

$CD133^{+}/EpCAM^{+}$ and $CD133^{-}/EpCAM^{-}$ subpopulations were enriched from Huh-7 cells using allophycocyanin-conjugated monoclonal antibodies against human CD133 (Milteny Biotec Inc., Auburn, CA, USA) and

fluorescein isothiocyanate-conjugated monoclonal antibodies against human EpCAM (Dako, North America, Carpentaria, CA, USA) in a BD/Cytopeia Influx Cell Sorter at the UC Davis Flow Cytometry Resource Laboratory, as we previously described.[17] In brief, Huh-7 cells were detached by Cell Dissociation buffer (Life Technologies) and incubated with corresponding antibodies for 45 min. After incubation, cells were enriched by FACS. The resulting subpopulations were cultured in the same medium for several passages before use. CD133 and EpCAM expression profile of the subpopulations after being cultured for seven passages were determined by flow cytometry in BD ISRFortessa with the same protocol as FACS enrichment. Flow cytometric analysis of SMCC-7721, MHCC-97H and MHCC-97 L was performed in a BD FACSCalibur flow cytometer.

TS cells and invasion assay

Freshly enriched Huh-7 DP and DN subpopulations were seeded on the BD Bio Coat Matrigel Invasion Chamber according to the manufacturer's instructions. After 2 days of culture, cells that migrated into the Matrigel layer were digested by trypsin, and cultured as FACS-enriched cells. The invasion rate of FACS-enriched subpopulations and TS cells was determined using BD Bio Coat Matrigel Invasion Chamber according to the manufacturer's instruction (BD Biosciences, Bedford, MA, USA). The invading cells were stained with crystal violet, and the invasion rate was expressed as the percent invasion through the Matrigel matrix membrane relative to the migration through the control membrane.

Immunocytochemical staining

FACS-enriched and TS subpopulations of 10^4 cells were seeded in four-well chamber slides (Nunc Lab-Tek, Rochester, NY, USA), and fixed in 4% buffered paraformaldehyde, as we described previously.[17,34] Primary antibodies used included mouse monoclonal antibodies against E-cadherin (Invitrogen, Carlsbad, CA, USA) and vimentin (Invitrogen). Secondary antibodies were Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen). Cells were counter stained with 4', 6-diamidino-2-phenylindole in mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) for visualizing nuclei. All electronic images were taken under a Keyence fluorescence microscope, as we described previously.[35]

Hh signaling activity by a GLI-Lux reporter system

To determine Hh signaling activity in different subpopulations, we used a Gli-lux reporter system, in which the firefly luciferase gene is driven by the Gli promoter. The Gli-lux reporter system was kindly provided by Dr Hiroshi Sasaki from the RIKEN Center for Developmental Biology, Kobe, Hyogo, Japan.[26] Cells from different subpopulations were transfected with the GLI-lux reporter plasmid using FuGENE 6 (Roche Applied Science, Indianapolis, IN, USA), as described by us previously.[17] Luciferase activity in transfected cells was determined 48 h after transfection as we previously reported.[36]

RNA isolation and quantitative RT–PCR

Total RNA was extracted from various cell types or subpopulations using RNeasy Mini Kit (QIAGEN, Mainz, Germany). Quantitative RT–PCR was performed using the ABI RT–PCR system. In brief, 1 μg of total RNA was used in 20 μl reverse transcription assays.[37,38] Subsequently, 1 μl of the revere transcription product was used as a template for qPCR in a 12 μl volume of PCR reaction (SYBR Green PCR Mix, Applied Biosystems, Foster City, CA, USA) using specific forward and reverse primers listed in Supplementary Table 1. CT values were acquired using the ABI7300 qPCR (Applied Biosystems) instrument, and were normalized to the housekeeping gene, human glyceraldehyde phosphate dehydrogenase. Relative gene expression (%) was expressed as log 2(− ΔΔCT) ($2^{-\Delta\Delta CT}$).

Western blotting analysis

The nuclear protein fraction was extracted from different cell subpopulations using corresponding extraction kits (Pierce Biotech, Rockford, IL, USA). The protein concentration was determined using a bicinchoninic acid protein assay (Pierce Biotech). Western blot assays were performed as reported previously.[39] In brief, equal amounts of protein samples were loaded and separated by 10–12% Tris-Glycine Ready Gel (Invitrogen) and the protein in the gel was transferred to a polyvinylidene fluoride membrane (Novex, Invitrogen). For antibody blotting, the membranes of the nuclear protein were incubated with polyclonal anti-Gli-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-Gli-2 (Abcam, Cambridge, MA, USA) and polyclonal anti-Twist (Santa Cruz Biotechnology, Inc.) in Tris-buffered saline containing 1% BSA for overnight at 4 °C. After washing with Tris-buffered saline with tween 20 for three times, the blotted membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. After being washed for three times with Tris-buffered saline with tween 20, the protein bands were visualized by the western blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.).

Statistical analysis

All the experiments were performed three times with a minimum of triplicates. The data were expressed as means ± s.e.m., and analyzed by student *t*-test for difference between two groups or variance analysis and further Newman–Keuls tests for multiple comparisons among groups when experimental design was more than two groups. $P < 0.05$ was considered as statistically significant.

ABBREVIATIONS

DAPI, 4', 6-diamidino-2-phenylindole; DP, double-positive subpopulation ($CD133^+/EpCAM^+$); DN, double-negative subpopulation ($CD133^-/EpCAM^-$); EMT, epithelial–mesenchymal transition; FACS, fluorescence-activation cell sorting; GAPDH, glyceraldehyde phosphate dehydrogenase; HCC, hepatocellular carcinoma; Hh, hedgehog; TS, $CD133^-/EpCAM^-$ transwell-selected; MMP, matrix metalloproteinase; RT–PCR, reverse transcriptase polymerase chain reaction

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)