Twist2 promotes self-renewal of liver cancer stem-like cells by regulating CD24

Allan Yi Liu1, Yao Cai1, Yubin Mao1,2, Yancheng Lin1, Hong Zheng1, Tianjian Wu3, Yangmei Huang3, Xiaoguang Fang3, Shuyong Lin1, Qingzhao Feng1, Zhengjie Huang4, Tianci Yang4, Qi Luo4 and Gaoliang Ouyang1,6

1 State Key Laboratory of Cellular Stress Biology, School of Life Sciences, Xiamen University, Xiamen 361102, China; 2 Department of Pathophysiology in Basic Science, Medical College, Xiamen University, Xiamen 361102, China; 3 Department of Stem Cell Biology and Regenerative Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, USA; 4 Department of Surgical Oncology, First Affiliated Hospital of Xiamen University, Xiamen 361003, China and 5 Center of Clinical Laboratory, Zhongshan Hospital, Xiamen University, Xiamen 361004, China

*To whom correspondence should be addressed. Tel: +86 592 2186091; Fax: +86 592 2181015; Email: oygli@xmu.edu.cn

Correspondence may also be addressed to Qi Luo. Tel: +86 592 2139526; Fax: +86 592 2139526; Email: luqixmzsh@126.com

Twist2 is a highly conserved basic helix-loop-helix transcription factor that plays a critical role in embryogenesis. Recent evidence has revealed that aberrant Twist2 expression contributes to tumor progression; however, the role of Twist2 in human hepatocellular carcinoma (HCC) and its underlying mechanisms remain undefined. In this report, we demonstrate that Twist2 is overexpressed in human HCC tumors. We show that ectopic expression of Twist2 induces epithelial–mesenchymal transition phenotypes, augments cell migration and invasion and colony-forming abilities in human HCC cells in vitro, and promotes tumor growth in vivo. Moreover, we found a higher percentage of CD24+ liver cancer stem-like cells in Twist2-transduced HCC cells. Twist2-expressing cells exhibited an increased expression of stem cell markers Bmi-1, Sox2, CD24 and Nanog and an increased capacity for self-renewal. Knockdown of CD24 in HepG2/Twist2 cells decreased the levels of Sox2, pSTAT3 and Nanog, and reversed the cancer stem-like cell phenotypes induced by ectopic expression of Twist2. Furthermore, Twist2 regulated the CD24 expression by directly binding to the E-box region in CD24 promoter. Therefore, our data demonstrated that Twist2 augments liver cancer stem-like cell self-renewal in a CD24-dependent manner. Twist2-CD24–STAT3–Nanog pathway may play a critical role in regulating liver cancer stem-like cell self-renewal. The identification of the Twist2-CD24 signaling pathway provides a potential therapeutic approach to target cancer stem cells in HCCs.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and one of the most lethal cancers worldwide (1–3). Currently, liver transplantation and hepatic resection are the potential curative treatments for HCC. Although liver transplantation is an alternative option to surgically manage HCC, partial hepatic resections continue to be used as the best method for curative treatment. Within the last decade, surgical resection survival rates have improved due to advances in the early diagnosis and the surgical management of HCC (4). However, HCC remains one of the most severe malignant diseases with limited treatment options; typically, HCC patients are not diagnosed until they have reached the intermediate or advanced stages of disease. Therefore, patients’ long-term survival rates following HCC resection remain unsatisfactory due to high rates of recurrence. Despite tremendous efforts devoted to diagnosing and managing HCCs, the detailed molecular pathophysiology of the tumor remains poorly understood. The heterogeneity of HCC and the absence of well-established and broadly targetable oncogenes are major hurdles in the development of effective therapies against HCC (5). Therefore, it is critical to understand the mechanisms of HCC tumorigenesis, metastasis and recurrence to prevent recurrence and provide effective management after HCC resection.

Current evidence has demonstrated that only a small subset of tumor cells called cancer stem cells (CSCs) or tumor-initiating cells are endowed with the ability to self-renew and seed new tumors. CSCs play a critical role in tumor initiation, growth, metastasis, therapeutic resistance and recurrence (6–8). Recently, tumorigenic liver CSCs have been identified using several markers, including CD133 (9), CD90 (10), EpCAM (11) and CD24 (12). Therefore, strategies aimed at efficiently targeting liver CSCs may offer new avenues of therapeutic intervention of malignant HCCs (13,14).

Twist1 and Twist2 are highly conserved basic helix-loop-helix transcription factors that play a critical role in embryogenesis and tumorigenesis (15–17). Twist1 and Twist2 are significantly overexpressed in various human solid tumors and contribute to tumor progression (18). Twist1 is overexpressed in human breast cancer and can confer stem cell-like properties to human mammary epithelial cells and breast cancer cells via the epithelial–mesenchymal transition (EMT) program (19–21). Twist2 contributes to breast cancer progression by promoting the EMT and the self-renewal of cancer stem-like cells (22). Twist1 overexpression is involved in human HCC metastasis through induction of the EMT phenotype (23–25). However, the function and the precise mechanism underlying the effects of Twist2 in human HCCs remain poorly understood.

In this report, we provide evidence that Twist2 is highly expressed in HCC tissue samples, whereas Twist2 is not expressed in most matched non-tumor liver tissue samples. Overexpression of Twist2 induces the EMT phenotypes in human HCC cells in vitro and promotes tumor growth in vivo. Ectopic expression of Twist2 confers CSC-like properties in HCC cells. Furthermore, CD24 is essential to Twist2-induced CSC-like properties to human HCC cells. Our results suggested that Twist2 promotes an EMT phenotype and augments cancer stem-like cell self-renewal via upregulation of CD24 expression in human HCC cells.

Materials and methods

Cell culture and generation of cell lines

Human HCC cell HepG2 and packaging cells GP2-293 and 293T were generous gifts from Prof. K. Luo (University of California at Berkeley, Berkeley, CA). The cells expressing either Twist2 or empty vector were generated by retroviral infection as reviewed in ref. (22). CD24 was stably knocked down in HepG2/Twist2 cells using lentivirus delivery of either shRNAs targeting CD24 (shCD24-1 and shCD24-2) or the non-target control. The sequences for the sh-RNAs targeting CD24 (Forward: 5′-TGAATGTTGGCTTGAGAAATTTCAAGATTTCTTCAACGGCACAATCTTTTITTC-3′, Reverse: 5′-TCGAGAAAAGAGAATGTGGC TTGAGAATATCTTCTGAATATTTCTCAAGCCATTTCA-3′; Forward: 5′-TGGTTTCTACATGGTGACCTTCAAGATAGTACCTCCAA CAATGAAACCCTTTTITC-3′, Reverse: 5′-TCGAGAAAAAGGTTPFACA TTGTTGAGCTATCTTCTGGAA TAGCTCAAACATGATACCAAC-3′) were cloned into pL3.7 (Hpla/Ixhd). The lentivirus-mediated packaging system contained four plasmids pL3.7-shCD24, pMDL, REV and VSVG at the ratio of 5:5:2:3 in quantity. The transfection and lentiviral infection process was similar to that described in ref. (22).

Immunoblotting and immunofluorescence staining

Western blot and immunofluorescence assays were performed as described in ref. (22). The primary antibodies used included antibodies against E-cadherin, c-Myc, Nanog, Twist2, Bmi-1, CD24, CD133, CD90, EpCAM, Sox2, and E-cadherin.
α-catenin, N-cadherin, fibronec tin, CD24 (BD Bioscience), α-SMA (Sigma–Aldrich), vimentin, Sox2 (R&D System), STAT3, pSTAT3 (Y705), EpCAM (Cell Signaling), Bmi-1, Nanog and β-actin (Millipore). The data for immuno- 
fluorescence were quantified using ImagePro Plus.

Wound healing assay

Wound healing assay was performed as described in ref. (22).

Migration and invasion assays

For transwell migration assay, 1×10^4 cells were plated on the top chambers of 8 μm pore size Transwell plates (Corning), and complete media were added to the bottom chambers. After 36 h, the cells on the upper surface of the filters were removed, and the bottom cells were fixed and stained with crystal violet. Then the migrated cells were counted. For Matrigel-coated Transwell invasion assay, the procedures and the analyses were the same as those for the Transwell migration assays except for the presence of the Matrigel on the upper surface of the chambers and fibronec tin on the bottom surface of the chambers. All of the experiments were performed at least three times in triplicate.

Colony formation assay

Colony formation assay was performed as described in ref. (22).

Oncomine analysis

The Oncomine database was searched for Twist2 gene. The data sets containing expression data for Twist2 were filtered to display upregulation in hepatocellular carcinoma versus normal liver tissue with P < 0.05.

Flow cytometry and cell sorting

Identification of CD24+, CD44+, CD133+ and CD90+ cells was performed using anti-CD24-PE (Clone:ML5), anti-CD44-FITC (Clone:G4-H126) (BD Biosciences), anti-CD133-PE (Clone:AC133) (Miltenyi Biotec) and anti-CD90-PE (Clone: 53-2.1) (eBioscience) antibodies. For double staining CD24 and CD133, we used anti-CD24-APC-eFluor780 (Clone:SN3, A5-2H10) (eBioscience) and anti-CD133-PE antibodies. The cells were labeled and these stem markers were analyzed using a BD LSRFortessa™ cell analyzer (BD Biosciences). For cell sorting, the labeled cells were filtered through 300 mesh filter and sorted with MoFlo XDP Cell Sorter (Beckman Coulter).

Tumor growth assay

All experiments using animals were performed in accordance with a protocol approved by the Animal Care and Use Committee of Xiamen University. Tumor growth assay was performed as described in ref. (22). For tumor growth of HepG2/Vector and HepG2/Twist2, mice were euthanized at 6 weeks after cell injection and examined for the growth of subcutaneous tumors. For tumor growth of HepG2/Twist2 shCtrl, HepG2/Twist2 shCD24-1, HepG2/Twist2 shCD24-2, HepG2/Twist2 CD24+ and HepG2/Twist2 CD24- cells, mice were euthanized at 4 weeks after cell injection and examined for the growth of subcutaneous tumors.

Tumorsphere culture and serial passage assay

Tumorsphere culture was performed as described in ref. (22). For primary tumorsphere culture, cells were seeded at 4000 cells per well. After 7 days, primary tumorspheres were centrifuged (1000 r.p.m.), dissociated with 0.05% trypsin-EDTA (Invitrogen), and then sieved through a 40 μm sieve to obtain single cells. The single cells from primary tumorspheres were seeded to obtain 3000 cells per well for secondary tumorspheres, and the single cells from secondary tumorspheres were seeded at 2000 cells per well for tertiary tumorspheres.

Limiting dilution assay

Cell numbers were adjusted to give a starting concentration of 10 000 cells/ml from which serial dilutions were made. Final cell dilutions ranged from 100 cells per well to 1 cell per well in 0.2 ml aliquots. The number of tumorspheres was scored 7 days later, depending on the spheroid growth rates.

Luciferase reporter assay

The CD24 promoter (1896 bp) and its mutants were amplified from human genomic DNA by PCR and cloned into the pGL3 vector upstream of the luciferase reporter (Promega). The E-cadherin promoter was cloned as described previously in ref. (22). Promoter assays were performed in 24-well plates containing 50 000 cells. HepG2/Vector or HepG2/Twist2 cells were co-transfected with pGL3-CD24 and Renilla control plasmid. Luciferase activity was measured 48 h after transfection using the Dual-luciferase reporter assay system (Promega).

Immunohistochemical staining of HCC specimens

Immunohistochemical staining of HCC specimens was performed as described in ref. (22). X-linked chromatin immunoprecipitation assay

HepG2/Twist2 cells were cross-linked with 1% formaldehyde for 10 min and reaction was stopped by adding glycine. Cells were lysed and sheared by sonication to make chromatin an average size of 500–1000 bp. Chromatin was precipitated with Twist2 and Histone H3 (Santa Cruz) as positive control. IgG purified from mouse serum were used as negative controls. The antibody–chromatin–beads complex was washed, eluted, reverse cross-linked and extracted. The purified DNA was analyzed by PCR using CD24-specific primers (Forward: 5′-CAGCTGACCTGGAATTGCG-3′; Reverse: 5′-CAGGGACCTGGCAATCTACCC-3′).

Statistical analysis

All data (mean ± SD) were analyzed by Student’s t-test. A level of P < 0.05 was considered statistically significant.

Results

Twist2 is overexpressed in human HCC and induces EMT in human HepG2 cells

We used immunohistochemistry to determine whether Twist2 is overexpressed in 29 specimens of human primary HCC tumor tissue samples compared with their matched non-cancer liver tissue samples. As determined by immunohistochemistry, seven tumors and their matched non-cancer liver tissues were positive for the staining of Twist2, whereas most tumor tissue samples (22 of 29) were positive for Twist2 staining. Among these 22 Twist2-positive tumor tissue samples, 15 matched non-tumor liver tissues were negative for Twist2, whereas the levels of Twist2 in other 7 matched non-tumor liver tissue samples were similar to their matched tumor tissues (Figure 1A). We also analyzed cDNA microarray data in Oncomine (26) to further confirm the role of Twist2 in HCC pathogenesis. The data from the database Wurmbach liver (GEO/GSE6764), which contains both normal liver and HCC tissues (27), revealed that the mRNA level of Twist2 was overexpressed in HCCs compared with normal liver tissues (P < 0.05), whereas the expression level of Twist2 in cirrhosis and liver dysplasia samples showed no significance with normal liver tissues (Supplementary Figure 1A is available at Carcinogenesis Online).

To further elucidate whether Twist2 plays a role in the tumorigenesis of HCC, we first stably overexpressed Twist2 in human HepG2 cells with nearly undetectable endogenous expression of Twist2 (Figure 1B). We found that Twist2 was located in both the nuclei and the cytoplasm of HepG2/Twist2 cells (data not shown). Overexpression of Twist2 resulted in morphological changes in HepG2 cells (Figure 1C). Increased levels of mesenchymal markers α-SMA, vimentin and fibronectin were observed in HepG2 cells overexpressing Twist2 by immunofluorescence assay and quantitative analysis (Figure 1D–F; Supplementary Figure 1B–D, available at Carcinogenesis Online). Conversely, the level of epithelial marker α-catenin was decreased (Figure 1G; Supplementary Figure 1E, available at Carcinogenesis Online). Loss of E-cadherin is a common event during the EMT. We also investigated whether Twist2 could decrease E-cadherin expression by immunofluorescence and western blot analyses. E-cadherin level was downregulated when Twist2 was expressed in HepG2 cells (Figure 1H and I; Supplementary Figure 1F, available at Carcinogenesis Online). We further used a reporter plasmid containing the luciferase gene under the control of an 887 bp fragment of the human E-cadherin promoter to determine the role of Twist2 on E-cadherin expression at transcriptional level. We found that the luciferase reporter activity was significantly repressed in HepG2/Twist2 cells compared with the vector cells, indicating that Twist2 decreases E-cadherin transcription in HepG2 cells (Figure 1J). Therefore, ectopic overexpression of Twist2 triggers an EMT and results in acquisition of a mesenchymal phenotype in HCC cells.

Twist2 enhances cell migration, invasion and colony formation of HepG2 cells in vitro and tumorigenicity in vivo

To test whether ectopic Twist2 overexpression enhanced cell motility, we examined the motility of HepG2 cells in a wound healing assay and found that the Twist2-expressing cells exhibited a greater...
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Fig. 1. Twist2 is overexpressed in human HCC tumor tissues and induces EMT in HepG2 cells. (A) Expression of Twist2 in two representative cases of human HCCs was detected by immunohistochemical assay. (B) HepG2 cells expressing either Flag-tagged Twist2 or empty vectors were determined by immunoblot analysis. (C) Twist2 overexpression induced a mesenchymal morphology in HepG2 cells. Immunofluorescence analysis revealed that overexpression of Twist2 in HepG2 cells increased α-SMA. (D) Vimentin (E) and fibronectin (F) and decreased α-catenin (G) and E-cadherin (H). (I) Western blot analyses showed that E-cadherin was decreased in HepG2/Twist2 cells. (J) Twist2 significantly decreased the promoter activity of E-cadherin in HepG2 cells (**P < 0.01).
migratory potential than the HepG2/Vector cells (Figure 2A). Further evidence of the ability of Twist2 to promote EMT was measured by Transwell chamber assays, and we found that HepG2/Twist2 cells had an increased ability for cell migration (Figure 2B) and invasion (Figure 2C), compared with control vector cells. We next investigated whether Twist2 could increase the colony-forming activity of HepG2 cells in vitro. As shown in Figure 2D, after cultured for 14 days, HepG2/Twist2 cells formed more colonies than the control vector-transfected cells. To obtain further insight into the biological relevance of Twist2 overexpression, we analyzed its effect on the tumorigenic properties of HepG2 cells. Both the empty-vector controls and the cells overexpressing Twist2 formed solid, bulky tumors at the injection site (Figure 2E); however, the volumes and weights of the tumors derived from the Twist2-expressing cells were larger than their counterparts (Figure 2F and G). Therefore, Twist2 promotes cell migration, invasion and colony formation in vitro and tumorigenesis.

Fig. 2. Twist2 enhances cell migration, invasion and colony formation in vitro and tumorigenicity of HepG2 cells in vivo. (A) Twist2 significantly promoted cell migration of HepG2 cells in the wound healing assay. (B) Transwell chamber migration assay showed that Twist2 overexpression enhanced the migration of HepG2 cells. (C) Matrigel-coated Transwell chamber invasion assay revealed that the invasion ability of HepG2/Twist2 cells was increased. (D) Twist2 increased the colony-forming activity of HepG2 cells in vitro. (E) Twist2 promoted the tumorigenic properties of HepG2 cells in vivo. Six weeks after cell injection, mice were euthanized and examined for the growth of subcutaneous tumors. Tumor sizes (F) and weights (G) from the mice in experiment E were measured (***P < 0.01).
of HepG2 cells in vivo. These results demonstrate that Twist2 converts HCC cells into a highly invasive and malignant form.

**Twist2 confers a CSC-like phenotype in HepG2 cells**

A direct link between the EMT program and the gain of CSC competence has been demonstrated in various cancer cells. Therefore, we examined whether stable expression of Twist2 could confer CSC-like properties in HepG2 cells. The formation of tumorspheres in non-adherent serum-free medium is reflective of CSC-like properties and correlates with tumorigenicity in vivo. In Twist2-expressing cells, there was a significant increase in the formation of primary, secondary and tertiary spheroids (Figure 3A and B). Next we investigated the impact of Twist2 on stem cell markers. Compared with the vector control, Twist2 expression increased the levels of stem cell markers Bmi-1, Sox2, Nanog and CD24 (Figure 3C). CD24⁺ HCC cells are critical for the maintenance, self-renewal, differentiation and metastasis of HCCs and significantly impact patients’ clinical outcomes (12). Here, we found that Twist2 expression augmented the subpopulation of CD24⁺ HCC cells from 1.8% to 24.9% ($P < 0.01$) (Figure 3D). We further examined other HCC stem cell markers reported previously such as CD44, CD90, CD133 and EpCAM. Fluorescence-activated cell sorting analysis revealed that ectopic expression of Twist2 increased the CD133⁺ subpopulation but not CD44 or CD90, and CD24⁺ and CD133⁺ had an overlap expression about 3.3% in HepG2/Twist2 cells (Supplementary Figure 2A–D, available at Carcinogenesis Online). Then we examined another HCC stem cell marker EpCAM by western blot. EpCAM was upregulated by Twist2 overexpression (Supplementary Figure 2E, available at Carcinogenesis Online). Then we examined another HCC stem cell marker EpCAM by western blot. EpCAM was upregulated by Twist2 overexpression (Supplementary Figure 2E, available at Carcinogenesis Online).

Fig. 3. Twist2 confers a CSC-like phenotype in HepG2 cells. (A) Morphology analysis showed that Twist2 increased hepatosphere formation in HepG2 cells. (B) Quantification of primary, secondary and tertiary hepatospheres formed by HepG2/Vector and HepG2/Twist2 cells ($***P < 0.001$). (C) Twist2 overexpression increased the levels of stem cell markers in HepG2 cells. (D) Flow cytometric analysis showed that the subpopulation of CD24⁺ cancer stem-like cells was increased in HepG2/Twist2 cells. (E) Tumorsphere assays for HepG2/Vector and HepG2/Twist2 cells were performed by limiting dilution with 100 cells to one cell per well of a 96-well ultra-low attachment plate. The number of hepatospheres was scored at the end of 7 days. This experiment was performed three times, with four wells per cell dilution (N.S: no significance; *$P < 0.05$, **$P < 0.01$).
Carcinogenesis Online). These data indicated that ectopic expression of Twist2 actually enriched the HCC stem cell pool. We further employed limiting dilution assay to determine the stem cell frequency. At 1 cell level, HepG2/Vector and HepG2/Twist2 cells showed no difference in sphere formation. However, at 10, 20, 50 and 100 cells levels, HepG2/Twist2 cells formed more hepatospheres than HepG2/Vector cells, which means a promoted self-renewal state in HepG2/Twist2 cells (Figure 3E). Taken together, these data suggest that Twist2 expression confers a CSC-like phenotype in HepG2 cells.

Twist2 drives CSC self-renewal through upregulation of CD24

CD24 is overexpressed in human HCC tissues and can be regarded as a liver CSC marker (12). Here we found that ectopic expression of Twist2 upregulated CD24 expression and increased the CD24+ subpopulation of HepG2 cells. Then we tested whether CD24 knockdown could reverse the CSC-like phenotype induced by ectopic expression of Twist2. A lentiviral-based approach was used to knockdown CD24 in HepG2/Twist2 cells. The knockdown efficiency was confirmed by western blotting (Figure 4A). Previous report has revealed that CD24 activates the phosphorylation of STAT3 (Y705) to drive the overexpression of Nanog in HCC cells and that CD24 drives tumor formation and self-renewal via STAT3-mediated Nanog expression (12). Therefore, we performed western blotting to detect the level of stem cell markers Sox2, Nanog and the downstream factor of CD24, STAT3 and phosphorylated STAT3. We found that knockdown of CD24 in HepG2/Twist2 cells dramatically decreased the levels of Sox2, Nanog and phosphorylated STAT3 (Figure 4A). We further examined the sphere formation ability of shCD24 HepG2/Twist2 cells and their non-target control cells. We found that upon knockdown of CD24, HepG2/Twist2 cells exhibited smaller and fewer hepatospheres than the knockdown control cells in primary, secondary and tertiary tumorsphere formation (Figure 4B and C). Similar data were obtained by an in vitro limiting dilution sphere formation assay. We detected no sphere formation at 1 cell level by knockdown CD24 in HepG2/Twist2 cells, whereas the non-target control cells could form sphere. Compared with HepG2/Twist2 shCD24 cells, the non-target control cells also exhibited more sphere formation and higher frequency of stem cells at 10, 20, 50 and 100 cell levels (Figure 4D). So knockdown CD24 can dramatically inhibit CSC phenotype induced by Twist2 in vitro. Furthermore, we performed in vivo tumor formation assay to assess whether knockdown CD24 can repress tumor growth in vivo. As shown in Figure 4E–G, knockdown CD24 in HepG2/Twist2 cells impaired tumor growth with smaller tumor size and lower tumor weight. We also sorted the CD24+ and CD24− subpopulations from HepG2/Twist2 cells with an in vivo tumor formation assay. Interestingly, the CD24+ HepG2/Twist2 cells formed tremendously large tumors, whereas CD24− HepG2/Twist2 cells only gave rise to very tiny tumors (Supplementary Figure 3A–C, available at Carcinogenesis Online). These data reinforced the hypothesis that CD24 is a key downstream target of Twist2 in regulating stemness of CSC. In all, these results indicate that Twist2 promotes cancer stem-like cell self-renewal in HCC cells in a CD24-dependent manner.

Twist2 regulates the expression of CD24 by directly binding CD24 promoter

Next, we further investigated whether Twist2 could regulate CD24 expression at transcription level. Basic helix-loop-helix family transcription factors binds to the E-box sequence (CANNTG) and undergoes transcriptional regulation (28); hence, we mapped and detected three putative E-box sequence on the 1896bp CD24 promoter region (Figure 5A). We cloned and transfected the wild type and deletion mutants into HepG2/Vector and HepG2/Twist2 cells to analyze the reporter activities. As shown in Figure 5B, Twist2 could activate CD24 transcription and the secondary E-box region between −518 and −513bp is critical for CD24 activation. To confirm whether this E-box was essential for Twist2 binding, we used the site-directed mutation to obliterate this site (Figure 5C) and found that Twist2 could not activate the mutant CD24 promoter in the reporter assay, indicating that the secondary E-box is necessary for Twist2 binding (Figure 5D). To further confirm the direct interaction between Twist2 and CD24, we performed the chromatin immunoprecipitation assay. PCR amplification was performed using specific primers to human CD24 promoter region encompassing the identified E-box site. Our data showed the preferential binding of Twist2 to CD24 compared with IgG control (Figure 5E), indicating that Twist2 can regulate the expression of CD24 by directly binding to the E-box region in CD24 promoter.

Discussion

Tumorigenesis is a complex process in which normal cells acquire a series of traits to evolve to a neoplastic state (29,30). EMT is a multifaceted pathological program that endows cancer cells with abilities of invasion, resisting apoptosis and dissemination. EMT-inducing transcription factors such as Twist1, Snail and Slug are aberrantly expressed in human cancers and are responsible for a poor prognosis in patients (31,32). Here, we demonstrate that Twist2 is overexpressed in HCC tumors and that overexpression of Twist2 confers an EMT phenotype and malignant capability to HepG2 cells, indicating that Twist2 may play a potential role in HCC progression.

The CSC hypothesis states that solid tumors are hierarchically organized and sustained by a minority of the tumor cell population with stem cell-like properties, such as self-renewal, multilineage differentiation and tumorigenicity. Increasing evidence supports the existence of CSCs in solid tumors such as breast cancer, prostate cancer, HCC and glioma (6,13,14,33,34). In this study, we demonstrated that the expression of stemness genes was upregulated upon ectopic expression of Twist2 in HepG2 cells and that overexpression of Twist2 increased the formation of hepatospheres. These data indicate that overexpression of Twist2 confers a CSC-like phenotype to HepG2 cells. The concept that the EMT generates CSCs has attracted great interest (19,35,36). Twist1 and Twist2 can induce EMT and endow human mammary epithelial cells and breast cancer cells with stem cell-like properties (19,22). Therefore, our current study suggests that overexpression of Twist2 may contribute to HCC progression by activating the EMT program and enhancing the self-renewal properties of cancer stem-like cells.

CD24 is a mucin-like cell surface glycoprotein that is frequently overexpressed in various human cancers and is correlated with poor prognosis (12,37). CD24 is a negative stem cell marker in breast cancer, but recent reports revealed that CD24-positive cells function as CSCs in gastrointestinal cancers, such as colon cancer (39) and HCC (12). Knocking down the expression of CD24 decreases the stem/progenitor cell phenotypes, indicating that CD24 is a functional marker for CSCs in the liver. CD24 can activate Src to phosphorylate STAT3 at Y705; the phosphorylated STAT3 then binds to Nanog promoter and upregulates its expression (12). CD24 overexpression is accompanied by increased Src and STAT3 activities (40). Here, we found that overexpression of Twist2 upregulates CD24 and Nanog levels in HepG2 cells and that Twist2 transcriptionally activates CD24 to promote cancer stem-like cell self-renewal. Using a lentiviral-based RNA interference method, CD24 was confirmed as a downstream target of Twist2 in HCC cells. CD24 knockdown decreased the levels of Sox2, pSTAT3 and Nanog, and reversed the cancer stem-like cell phenotypes induced by ectopic expression of Twist2. Therefore, our data suggest that the Twist2–CD24–STAT3–Nanog pathway plays a critical role in regulating liver CSC self-renewal. Interestingly, Twist2 and Twist1 can function as either transcriptional activators or repressors in a tissue-dependent manner (16). Here, we demonstrated that Twist2 can upregulate CD24 in HCC cells. However, in breast cancer cells, Twist1 downregulates CD24 expression at the transcription level (20), indicating that Twist2 may regulate CD24 expression in breast cancer and HCC cells in a tissue-dependent manner.

In summary, we have shown that Twist2 contributes to HCC progression by promoting the EMT and cancer stem-like cell self-renewal via the upregulation of CD24. The Twist2–CD24–STAT3–Nanog axis...
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Fig. 4. Twist2 drives CSC self-renewal through regulation of CD24. (A) Knockdown of CD24 decreased the upregulated levels of stemness markers that are induced by ectopic expression of Twist2 in HepG2 cells. (B) Upon CD24 knockdown, HepG2/Twist2 cells exhibited smaller and less hepatospheres than the non-target control cells. (C) Quantification of primary, secondary and tertiary hepatospheres formed by HepG2/Twist2 shCD24 cells and non-target control cells. Knockdown CD24 reduced the number of hepatospheres in HepG2/Twist2 cells (**P < 0.01, ***P < 0.001). (D) A limiting dilution was performed on the CD24 knockdown and non-target control HepG2/Twist2 cells from 1 to 100 cells per well. The number of hepatospheres was scored at the end of 7 days. (N.D: not detected; *P < 0.05, **P < 0.01). (E) Knockdown CD24 inhibited tumor formation of HepG2/Twist2 cells in vivo. Four weeks after cell injection, mice were euthanized and examined for the growth of subcutaneous tumors. Tumor sizes (F) and weights (G) from the mice in (E) were measured (**P < 0.01, ***P < 0.001).
A.Y.Liu et al. also provides insight into the molecular basis for the Twist2-induced augmenting of CSCs. Therefore, linking Twist2 with CD24 signaling may provide an attractive therapeutic target against HCC progression and recurrence.

Supplementary material
Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/.

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