PITX2C increases the stemness features of hepatocellular carcinoma cells by up-regulating key developmental factors in liver progenitor

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Abstract

Background: Tumor cells exhibited phenotypic and molecular characteristics similar to their lineage progenitor cells. Liver developmental signaling pathways are showed to be associated with HCC development and oncogenesis. The similarities of expression profiling between liver progenitors (LPs) and HCC suggest that understanding the molecular mechanism during liver development could provide insights into HCC.

Methods: To profile the dynamic gene expression during liver development, cells from an in vitro liver differentiation model and two paired hepatocellular carcinoma (HCC) samples were analyzed using deep RNA sequencing. The expression levels of selected genes were analyzed by qRT-PCR. Moreover, the role of a key transcription factor, pituitary homeobox 2 (PITX2), was characterized via in vitro and vivo functional assays. Furthermore, molecular mechanism studies were performed to unveil how PITX2C regulate the key developmental factors in LPs, thereby increasing the stemness of HCC.

Results: PITX2 was found to exhibit a similar expression pattern to specific markers of LPs. PITX2 consists of three isoforms (PITX2A/B/C). The expression of PITX2 is associated with tumor size and overall survival rate, whereas only PITX2C expression is associated with AFP and differentiation in clinical patients. PITX2A/B/C has distinct functions in HCC tumorigenicity. PITX2C promotes HCC metastasis, self-renewal and chemoresistance. Molecular mechanism studies showed that PITX2C could up-regulate RALYL which could enhance HCC stemness via the TGF-β pathway. Furthermore, ChIP assays confirmed the role of PITX2C in regulating key developmental factors in LP.

Conclusion: PITX2C is a newly discovered transcription factor involved in hepatic differentiation and could increase HCC stemness by upregulating key transcriptional factors related to liver development.

Keywords: PITX2A/B/C, HCC, Tumorigenicity, Stemness, Developmental factors in liver progenitor

Background

Tumor cells are widely recognized to exhibit phenotypic similarity with lineage progenitor cells. Hepatocellular carcinoma (HCC) is one of the most common solid tumors worldwide with an inferior prognosis [1]. Compared to normal liver tissues, HCC typically expresses markers of liver progenitors (LP) at high levels, including...
alpha-fetoprotein (AFP), cytokeratin 7 (CK7), and cytokeratin 19 (CK19), which are often associated with poor outcome [2, 3]. A variety of growth factors, such as FGF, BMP, HGF, Wnt and TGF-β promote liver progenitor cell migration, proliferation and survival [4]. The Wnt/β-catenin and TGF-β signaling pathway promotes hepatocyte and biliary epithelial cell differentiation during hepatic maturation [5, 6]. These signaling pathways also appear to enhance HCC stemness [7–9]. Evidence suggested that the key factors governing hepatic differentiation in LPs are of critical importance in HCC tumorigenicity and progression.

In our previous study, an in vitro liver differentiation model from human embryonic stem cells (ES) was established [10]. ES could generate definitive endoderm cells (DE) by treating the culture medium with a certain concentration of Activin. Then, the addition of FGF and BMP induced the differentiation of DE differentiate into LPs, which expanded and matured after treatment with a combination of HGF, OSM and dexamethasone. In the last stage of this model, premature hepatocytes (PHs) were acquired [4]. Combining the deep RNA sequencing of cells at four stages with two paired HCC clinical samples transcriptomic data, we screened a group of genes that were actively expressed in LPs and had higher expression levels in HCC than non-tumor tissue. Among these genes, a transcription factor, pituitary homeobox 2 (PITX2) was of interest due to its location in the central of gene regulatory network (Pathway Common) [11], with a unique expression pattern significantly associated with HCC differentiation and poor outcome.

PITX2, a member of the bicoid/paired-like homeobox gene family, is a multifunctional transcription factor [12–14]. PITX2 consists of six transcript variants that are translated into three isoforms: PITX2-V16 (PITX2A), PITX2-V245 (PITX2B), PITX2-V3 (PITX2C). PITX2A/B/C is produced by alternative splicing and transcription [15]. Compared to PITX2A and PITX2B, PITX2C uses an alternative promoter [16, 17]. All isoforms contain dissimilar amino terminus, identical homeodomain and C-terminal domains. Three isoforms differentially regulate the transcription of the target genes. PITX2C is the dominant isoform in developing and adult left atrium [18]. The roles of PITX2 seem to be controversial in cancer research. On the one hand, lower levels of PITX2 expression have been reported in patients with breast cancer, prostate cancer and colon cancer as well as being associated with poor prognosis [19–22]. On the other hand, PITX2 has also been identified as a potential oncogene in thyroid cancer, prostate cancer and ovarian cancer [20, 23–25]. The isoforms of PITX2 may possess diverse functions in tumorigenicity.

In the present study, the functions of the three isoforms of PITX2 were characterized in HCC. The mRNA level of PITX2C was associated with AFP and differentiation in HCC clinical samples. PITX2C overexpression increased the stemness-related characteristics of HCC in vitro and in vivo functional assays, while mechanistic studies revealed that PITX2C rather than PITX2A/B could activate TGF-β signaling by regulating the transcription of RALYL. Furthermore, PITX2C was found to regulate and cooperate with key factors related to hepatic differentiation and HCC tumorigenicity.

**Materials and methods**

**HCC samples and cell lines**

Two cohorts of patients with HCC were carefully evaluated histologically and included in this study. One cohort for checking the mRNA expression level by qRT-PCR included 93 pairs of primary HCC tissues and their matched non-tumor tissues collected after surgical resection at Sun Yat-sen University Cancer Center (Guangzhou, China) between 2001 and 2005 (Cohort-1). The surgical specimens (both tumor and adjacent non-tumor tissue) were processed immediately after the operation and snap-frozen in liquid nitrogen for RNA extraction. The other cohort for pathological study of tissue microarray (TMA) is a retrospective cohort of 132 patients with HCC who also underwent hepatectomy at Sun Yat-sen University Cancer Center (Guangzhou, China) between 2001 and 2008 (Cohort-2). The dissected tumor tissues were embedded in paraffin block and used for tissue microarray (TMA) construction. None of these patients were diagnosed with autoimmune diseases and received chemotherapy or radiotherapy before operation. The mean age of these enrolled HCC patients was 48 years (range, 21–79 y). The median follow-up period was 34.5 months (range: 6–96.3 mo). Over 85% of these patients had hepatitis B virus infection. About 20% of patients had cirrhosis. Moreover, the clinical characteristics of these patients included age, sex, serum AFP, serum HBsAg, differentiation, tumor size, metastatic status [26]. The status of differentiation was determined by histopathologists according to the pathological properties of tumor tissues [27]. Differentiation (II-III) was defined as well-differentiated tumor tissues, whereas differentiation (III-IV) was defined as poorly-differentiated ones. The samples used in this study were approved by the committees for Ethical Review of Research Involving Human Subjects at the Sun Yat-Sen University Cancer Center. Human immortalized hepatic cell lines, MiHA, LO2, and HCC cell lines (i.e. MHCC97L, H2P, PLC-8024, SNU-475, SNU-449, Hep3B and HepG2) were tested for mycoplasma contamination. STR DNA profiling analysis
was conducted for cell line authentication. Details about these cell lines was found in our previous study [26, 28].

In vitro and vivo functional assays
In vitro and vivo functional assays was reported in our previous study [26]. A detailed description could be found in the Supplementary Materials and Methods.

Immunohistochemical staining (IHC) and antibodies
IHC staining was performed according to the standard procedure. Details could be found in Supplementary Materials and Methods. The antibodies used for IHC included PITX2 from Sigma-Aldrich (St. Louis, MO), proliferating cell nuclear antigen (PCNA) from Immunoway Biotechnology Company (Plano, TX, USA), and c-Myc, NANOG, CD133 and EPCAM from Cell Signaling Technology (Danvers, MA, USA).

Luciferase reporter assays
The pGL3.0 luciferase expression system was purchased from Promega Corporation (Australia) and was performed according to the manufacturer’s instruction.

Chromatin immunoprecipitation
A chromatin immunoprecipitation (ChIP) assay was performed as previously described [29]. Antibodies for ChIP included FLAG and H3K27Ac from Cell Signaling Technology (Danvers, MA, USA). The ChIP products were amplified using specific primers (Supplementary Table 1A). Details were described in Supplementary Materials and Methods.

Statistical analysis
Statistical analysis was conducted using the SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Pearson’s Chi-square test was used to analyze the association of PITX2 expression with clinicopathological parameters as well as RALYL expression. Kaplan-Meier plots and log-rank tests were used for survival analysis. Data are presented as the mean ± SD of three independent experiments. A P value less than 0.05 was considered statistically significant.

Results
Identification of PITX2C in an in vitro hepatocyte differentiation model
An in vitro hepatocyte differentiation model was previously established from ES cells into DE, LP and PH cells [26]. Transcriptome sequencing was performed to identify the gene profiling of four stages in the vitro hepatocyte differentiation model. The heatmap of the expression profiles together with the qRT-PCR results demonstrated that the marker genes were highly specifically expressed in their corresponding stages [10, 26]. Thereafter, the expression pattern of four hepatic developmental stages (ES, EN, LP and PH) and two paired HCC clinical samples was analyzed (Fig. 1A). To identify the key factors involved hepatic differentiation and maturation regulation, a group of genes encoding nuclear proteins that are specifically expressed in the LP and PH stages were screened (Supplementary Fig.1A). Pathway Commons was used to analyze the regulatory and interaction networks between selected nuclear proteins. As a result, we found that PITX2 was located at the center of the network (Supplementary Fig.1B). Full-length of PITX2A/V3 contained varying 5’ sequences (Supplementary Fig.1C). Specific forward primers were designed to detect the six transcript variants, which were translated into three isoforms (PITX2-V16: PITX2A; PITX2-V245: PITX2B; PITX2-V3: PITX2C) (Supplementary Table 1). The expression patterns of the three PITX2 isoforms in the hepatic differentiation model were further confirmed by qRT-PCR. Surprisingly, only PITX2C showed its peak expression. Kaplan-Meier plots and log-rank tests were used for survival analysis. Data are presented as the mean ± SD of three independent experiments. A P value less than 0.05 was considered statistically significant.

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**Fig. 1** The expression and clinical significance of PITX2. **A** Establishment of an in vitro hepatocyte differentiation model, which induced human embryonic stem (ES) cells into definitive endoderm (DE), liver progenitor cells (LP) and premature hepatocytes (PH). Cells in the four stages of in vitro hepatocyte differentiation model together with two HCC and two adjacent non-tumor tissues were used for deep RNA sequencing (**B**) and qRT-PCR. Surprisingly, only PITX2C showed its peak expression. **C** Heatmap of the expression profiles together with the qRT-PCR results have demonstrated that the marker genes were highly specifically expressed in their corresponding stages [10, 26]. Thereafter, the expression pattern of four hepatic developmental stages (ES, EN, LP and PH) and two paired HCC clinical samples was analyzed (Fig. 1A). **D** The relative expression level of PITX2 and PITX2C in each stage of the in vitro hepatocyte differentiation model was confirmed using qRT-PCR. (**P** < 0.05, **P** < 0.01, independent Student’s t-test). **E** Bar chart of delta Ct (Top: ΔΔCt = ΔCtPITX2/ΔCtGAPDH; Bottom: ΔΔCt = ΔCtPITX2/ΔCtGAPDH) in two immortalized liver cell lines and seven HCC cell lines. GAPDH was used as a reference gene. The higher the ΔCt, the lower expression level of target gene. **F** The relative expression level of PITX2 in HCC clinical samples (Cohort-1) compared to the corresponding non-tumor tissue (Top). ΔΔCtPITX2 = (ΔCtPITX2 in tumor – ΔCtPITX2 in normal) – (ΔCtGAPDH in tumor – ΔCtGAPDH in normal). As 2ΔΔCtPITX2 indicates the fold change of PITX2 in tumor relative to the corresponding non-tumor, PITX2 was up-regulated in 53 out of 93 HCC samples with ΔΔCtPITX2 < 0. **G** Association of PITX2 expression with tumor size in two HCC cohorts. PITX2 expression was determined by qRT-PCR or IHC. **H** Association of PITX2 expression with AFP and differentiation in HCC cohort-1. PITX2 expression is defined as positive in HCC samples when ΔΔCtPITX2 < 0.
Fig. 1 (See legend on previous page.)

A. Activin → FGF+BMP → HGF+OSM+Dex → Deep RNA sequencing

B. PITX2C ** PITX2 *

C. ΔCt_ΔCt2100

D. Non-Tumor HCC-1 HCC-2

E. ΔΔCt_ΔCt2100

F. Overall Survival Disease-free Survival

G. PITX2 expression (Cohort-1) Tumor size (Cohort-2)

H. PITX2C expression (Cohort-1) AFP level (ng/mL) (Cohort-1) Differentiation (Cohort-1)
expression in the LP and PH stages and decreased in non-tumor tissues, as well as HCC tissues (Fig. 1B).

**Clinical significance of PITX2 in HCC**

The expression levels of PITX2 in immortalized liver and HCC cell lines were examined using qRT-PCR and western blotting. These results indicated that PITX2 was expressed at higher levels in 97L, PLC-8024, SNU-475 and SNU-449 cells (Supplementary Fig. 1D). However, compared to *PITX2A* and *PITX2B*, lower mRNA levels of *PITX2C* were detected in cell lines, especially *PITX2C* was almost absent in two immortalized liver cells (LO2 and MiHA) (Fig. 1C). The protein levels of all PITX2 isoforms in a cohort of 132 HCC cases were examined by IHC staining. Higher levels of expression PITX2 were detected in 50.76% (66/132) of HCC tissues (Fig. 1D). Up-regulation of all transcript variants of *PITX2* at mRNA level was also confirmed in 56.9% (53/93) of HCC tissues by qRT-PCR, compared with adjacent non-tumor tissues. However, *PITX2C* was rarely expressed, as the values of ΔCt (Ct *PITX2C* in tumor – Ct GAPDH in tumor) were over 25 in most of HCC cases. Only five cases showed stable expression of *PITX2C* (Fig. 1E). Specific *PITX2C* expression was further determined through Fluorescence in situ hybridization (FISH) staining of HCC and their adjacent non-tumor tissues. Similarly, the transcript of *PITX2C* was rarely detected in non-tumor tissue (Supplementary Fig. 1E). Kaplan-Meier survival analysis showed that higher levels of PITX2 expression were significantly associated with poorer overall survival (OS), but not with disease-free survival (DFS) rates (Fig. 1F). Similar results were observed in the TCGA database (Supplementary Fig. 1F). The clinical pathologic study revealed that PITX2 higher expression was significantly associated with a relatively high AFP level and differentiation (Fig. 1H). Interestingly, the mRNA level of *PITX2C* was associated with a relatively high AFP level and differentiation (Fig. 1H). These findings suggest that although PITX2 may contribute to the tumorigenesis of HCC, PITX2C plays a crucial role in regulating HCC differentiation and stemness.

**PITX2A/B/C has distinct functions in the tumorigenicity of HCC**

To investigate the roles of PITX2A/B/C isoforms in tumorigenicity, the full-length sequences of the three isoforms were cloned into lentiviral vectors and stably transfected into MiHA, LO2, PLC-8024 and Hep3B cells. The ectopic expression of *PITX2A/B/C* was examined at protein level (Fig. 2A). The qRT-PCR results confirmed that the mRNA levels of the three isoforms were also enhanced in the corresponding transfected cells (Fig. 2B). XTT assays showed that the overexpression of *PITX2A* and *PITX2B* promoted cell proliferation. In contrast, *PITX2C* overexpression suppressed cell proliferation compared to that in controls (Fig. 2C). Similar results were obtained in foci formation assays and the colony formation capacity in soft agar (Fig. 2D, E and Supplementary Fig. 2A, B). Moreover, *PITX2* expression was knocked down in PLC-8024 and SNU-449 cells with two short hairpin RNAs (shRNAs) (Fig. 2F and Supplementary Fig. 2C). As expected, *PITX2* knockdown significantly decreased cell proliferation, foci formation and colony formation in soft agar (Supplementary Fig. 2D, E, F).

To further confirm whether *PITX2* could promote HCC tumorigenicity in vivo, subcutaneous tumors induced by *PITX2A*-transfected LO2, MiHA and Hep3B cells and control cells were implanted into the livers of nude mice. The results showed that tumors induced by *PITX2A*-transfected cells were significantly larger (Fig. 2G, Supplementary Fig. 2G-H). In addition, tumor volume was significantly decreased in the *PITX2* knockdown system (Fig. 2H and Supplementary Fig. 2G). Moreover, IHC staining was performed for the orthotopic tumors. A stronger intensity of PCNA was found in tumors induced by higher *PITX2*-expressing cells (Fig. 2I). Taken together, these data suggest that PITX2A/B promotes cell proliferation and tumor growth in HCC, while PITX2C suppresses cell growth.

**PITX2C promotes cell mobility and self-renewal of HCC**

The effect of PITX2A/B/C on cell motility was characterized by cell migration and invasion. Transwell and cell
Fig. 2 (See legend on previous page.)
invasion assays indicated that PITX2A/C significantly enhanced cell motility in both LO2, MiHA and Hep3B cells (Fig. 3A). EMT-related markers were also upregulated or downregulated in PITX2C-transfected cells, which further confirmed that PITX2C could promote cell mobility (Fig. 3B). PITX2 knockdown significantly inhibited HCC cell motility to a lesser extent (Supplementary Fig. 3A).

As PITX2C is actively expressed in LPs and PH which usually exhibit enhanced self-renewal ability, we speculated that PITX2C may regulate HCC differentiation. PITX2C overexpression was found to markedly increase both the primary and secondary spheroid formation capacities in MiHA and PLC-8024, however, no significant difference was observed between PITX2A/B overexpressing cells and controls (Fig. 3C). Conversely, silencing PITX2 decreased the size and number of spheroids formed in the PLC-8024 cells (Supplementary Fig. 3B). The mRNA level of PITX2C was higher in HCC patient-derived organoids than in the corresponding primary tumor tissues (Fig. 3D). Moreover, qRT-PCR revealed that the fold change of two markers of LPs (AFP and Lgr5) were increased in PITX2C overexpressing cells but reduced after PITX2 knockdown (Fig. 3E, Supplementary Fig. 3E).

**PITX2C enhances the chemoresistance of HCC cells in vitro and in vivo**

Chemoresistance is a stemness-related characteristic of tumor cells. Drug resistance allows tumor cells to survive in conventional or targeted therapies, ultimately leading to relapse [30]. The combination of cisplatin, alpha-interferon, doxorubicin and 5-fluorouracil (5-Fu) yielded a promising overall response rate in a phase II trial [31]. As sorafenib is a standard first-line treatment for advanced HCC, clinical trials of sorafenib in combination with conventional chemotherapies in HCC patients are being conducted worldwide [32]. After treatment with 5-Fu at different concentrations, the cell viability of PITX2C-overexpressing cells was significantly higher than that of the controls (Fig. 4A). Consistently, the flow cytometry results revealed that the apoptotic index was lower in PITX2C-overexpressing cells than in the control cells (Fig. 4B, Supplementary Fig. 3C). In addition, the typical molecular indicators of apoptosis, namely caspase-9 and poly (ADP ribose) polymerase were markedly decreased in PITX2C-transfected cells compared with control cells (Fig. 4D). As expected, PITX2 knockdown had the opposite effect (Fig. 4C-D and Supplementary Fig. 3D). However, PITX2C did not contribute to sorafenib resistance in HCC cells (Fig. 4A-B), implying that PITX2C may not regulate the targets of sorafenib, including VEGFR, PDGFR and RAF family kinases.

To further confirm the role of PITX2C in the chemoresistance of HCC in vivo, nude mice with xenograph tumors formed by PITX2C- and control-transfected PLC-8024 cells were treated with 5-Fu (50 mg/kg body weight) regularly. When the subcutaneous tumor reached a similar size of approximately 5 mm in diameter, 5-Fu was administrated via intraperitoneal injection every 5 days. Tumors induced by PITX2C-transfected cells grew faster and were larger than those in the control group (Fig. 4E). Intriguingly, PITX2C-expressing cells were significantly enriched in tumors treated with 5-Fu (Fig. 4F, Supplementary Fig. 4).

**PITX2C enhances stemness by up-regulating key developmental factors in liver progenitor**

To characterize the downstream targets regulated by PITX2C in HCC, ChIP assay was carried out using anti-flag antibody in PITX2C-flag transfected PLC-8024 and MiHA cells. Primers were designed to selectively amplify the promoters of genes that are specifically highly expressed in the LP (Supplementary Fig. 1A). ChIP-PCR revealed PITX2C could bind to the promoter region of RALY RNA binding protein like (RALYL) (Fig. 5A). In the pGL3-promoter dual luciferase reporter system, the overexpression of PITX2C rather than PITX2A/B significantly enhanced the luminescent signals in the PITX2C transfected groups compared with the control groups (Fig. 5B). Our previous study confirmed that RALYL is a liver progenitor specific gene that enhances the stemness of HCC [26]. PITX2C overexpression or silencing increased or decreased the expression levels of molecular indicators of apoptosis, namely caspase-9 and poly (ADP ribose) polymerase were markedly decreased in PITX2C-transfected cells compared with control cells (Fig. 4D). As expected, PITX2 knockdown had the opposite effect (Fig. 4C-D and Supplementary Fig. 3D). However, PITX2C did not contribute to sorafenib resistance in HCC cells (Fig. 4A-B), implying that PITX2C may not regulate the targets of sorafenib, including VEGFR, PDGFR and RAF family kinases.

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Fig. 3 (See legend on previous page.)
of RALYL respectively (Fig. 5C). Furthermore, the presence of RALYL was associated with that of PITX2C in 72 paired HCC clinical samples (P = 0.014, Pearson Chi-square test, Fig. 5D). The co-transfection of PITX2C and RALYL promoted cell growth and self-renewal in PLC-8024 and MiHA (Fig. 5E). Surprisingly, compared to cells transfected with PITX2C alone, RALYL-transfected cells showed stronger stemness, which may be due to the significantly higher levels of RALYL expression caused by its own transfection than by PITX2C overexpression. The expression level of RALYL was relatively low compared to PITX2 expression in LP, and was almost absent in mature hepatocytes (Supplementary Fig. 1A) [26]. We speculated that RALYL expression could trigger stemness-related abilities in HCC even at very low level. Moreover, PITX2 was further silenced in RALYL overexpression system. However, the self-renewal abilities of cells were not significantly decreased (Fig. 5E). The exogenous expression of RALYL is so powerful to maintain the self-renewal of HCC cells that PITX2 silencing failed to reverse the effects. As RALYL could sustain the stability of TGF-β2 mRNA, PITX2C transfection also increased the expression levels of TGF-β signaling related targets, such as p-AKT, p-STAT3 and c-Jun (Fig. 5F).

To further investigate the whole genomic binding signature of PITX2C in HCC, high-throughput sequencing of ChIP products of PITX2C was performed. From the sequence results, high-score PITX2C binding motifs were identified. Interestingly, PITX2C shared similar binding motifs with key transcriptional factors in liver development, suggesting that PITX2C may cooperate with these transcriptional factors (e.g., FOXA1, FOXP1, HNF4A, LEF1, GATA3) to regulate hepatic differentiation (Supplementary Fig. 5A). Top enriched gene ontology (GO) analysis further showed that PITX2C binding targets participated in biological processes, including extracellular matrix organization, liver development, angiogenesis, TGF-β, and Wnt signaling (Fig. 5G and Supplementary Table 2). Moreover, PITX2C binding sites were found at the promoters of genes that regulated the expression of hepatic genes and were specifically expressed in the LP and PH stages (Supplementary Fig. 5B-C). These results further support the notion that PITX2C transcriptionally regulates and cooperates with the key factors in liver development.

**PITX2 regulates the Wnt pathway in HCC**

PITX2 has been reported to interact with canonical and non-canonical Wnt pathways during development. The Wnt signaling cascade controls organogenesis by inducing a wide range of responses for cell proliferation and terminal differentiation during liver development [33]. To confirm the crosstalk between PITX2 and the Wnt pathway in HCC, the correlation between PITX2 and Wnt family members was analyzed using GEPIA [34]. Among the Wnt family members, the expression of PITX2 was positively correlated with that of Wnt5a (Supplementary Fig. 5D). The overexpression of PITX2A/B/C or silencing of PITX2 enhanced or reduced the expression levels of Wnt5a and Wnt5β, respectively (Fig. 6A). Consistently, western blotting showed that Wnt5a/5β secretion in cell culture supernatants was increased in PITX2A/B/C-overexpressing cells (Fig. 6B). Furthermore, PITX2A/B/C overexpression also up-regulated the protein levels of key components of the Wnt signaling pathway, including c-Met, LEF, Frizzled, p-GSK3β and its downstream targets, such as c-JUN, c-Myc and CD44. PITX2 silencing had the opposite effect (Fig. 6C). Taken together, our results demonstrate that PITX2 mediates the Wnt pathway and regulate sequential transcriptional and post-transcriptional events in this pathway.

**PITX2C expression is modulated by H3K27 acetylation in its promoter region**

Three major PITX2 isoforms were generated by alternative splicing and the use of different promoters. PITX2A/B is transcribed using the same promoter region, however, PITX2C employs an alternative promoter located upstream of exon 4 [15]. In contrast to PITX2A/B, PITX2C is expressed at very low levels in HCC, as well as in mature hepatocytes. However, it was also clearly expressed in LP (Supplementary Fig. 1A). Low gene expression is often associated with aberrant methylation and histone deacetylation in promoter regions. Two cell lines, LO2 and MiHA with absent PITX2C, were treated with 5-Aza-dC, a DNA methyltransferase

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**Fig. 4** PITX2C enhances the chemoresistance of HCC cells. A Cell viabilities of PITX2C- and vector-transfected cells were detected by XTT assay after treatment with 5-Fu and Sorafenib at the indicated concentration for 48 h. The apoptotic indexes of vector-, PITX2C-transfected (B) and shCtl- and shPITX2-transfected cells (C) were detected by fluorescence-activated cell sorting-based Annexin V/DAPI double staining after treatment with 5-Fu or Sorafenib at the indicated concentration for 48 h. A-C Values indicate the mean ± SD of three independent experiments with three repeats (*P < 0.05, **P < 0.01, independent Student’s t-test). D The activation of caspase-9, poly (ADP ribose) polymerase were compared between PITX2C-transfected cells (top); shPITX2-transfected cells (bottom) and control cells after 5-Fu treatment for 48 h. GAPDH was used as a loading control. E Subcutaneous tumors induced by indicated cells were treated with 5-Fu. The dose of 5 mg/kg 5-Fu could effectively shrink tumour size in control cells compared to PITX2C-transfected cells. The final tumor volumes are summarized in dot chart. The average tumour volume was expressed as the mean ± SD. P values were calculated using independent Student’s t test. F Representative IHC images show that PITX2 positive cells were enriched in 5-Fu treated cells (400 x, magnification).
Fig. 4 (See legend on previous page.)
inhibitor or trichostatin A, a histone acetylation agent to investigate the effects of DNA methylation and histone acetylation on PITX2C expression. As shown in Fig. 6D, qRT-PCR analysis showed that PITX2C expression could be detected with 100 μM TSA, while it was not significantly affected by 5-Aza-dC treatment. These results imply that histone modification rather than aberrant promoter methylation is responsible for the low expression of PITX2C in HCC and hepatocytes. ChIP assays using H3K27ac antibody in LO2, MiHA, PLC-8024 and SNU-449 cells were performed. Thereafter, the ChIP products were amplified using specific primers targeting the upstream region of PITX2C. The enrichment of H3K27ac at the promoter of PITX2C was identified in PLC-8024 and SNU-449 cells, which expressed PITX2C at relatively higher levels, whereas weak H3K27 acetylation was observed in LO2 and MiHA cells (Fig. 6E). These data suggested that the expression of PITX2C be modulated by H3K27 acetylation in different hepatocytes.

Discussion

Emerging evidence suggests that the malignant transformation of tumors is associated with the reactivation features of tissue progenitor cells [35]. In liver development, lineage-specific factors often undergo a period of activation, thereafter gradually decreasing until terminal differentiation. The expression levels of some lineage-specific factors (eg. AFP, CD133, Lgr5 and RALYL) are almost absent in mature hepatocytes [26]. HCC exhibits a similar gene expression pattern to that of LPs. Likewise, the epithelial-to-mesenchymal transition (EMT) plays an important role in liver bud morphogenesis as LPs delaminate and migrate into the septum transversum mesenchyme [4]. Liver lineage-specific factors confer progenitor-like features and contribute to the progression of HCC [36]. To investigate the unexplored roles of liver progenitor-specific factors in HCC, we established an in vitro hepatocyte differentiation model [10, 26, 28]. By the transcriptome sequencing of cells at four stages of this model and HCC clinical samples, PITX2 was identified. Previously, studies have reported that PITX2 is highly expressed in the fetal liver at embryonic day 9.5 and activated in the adult liver at 24h after partial hepatectomy, during which LPs are stimulated to initiate liver regeneration [37, 38]. To identify the PITX2 isoform that contributes to liver differentiation, the qPCR targeting specific sequences of the three isoforms was performed. As a result, we found that PITX2C, which is highly expressed in liver progenitors, decreased in premature and normal hepatocytes, but was reactivated in HCC.

PITX2A/B/C has distinct functions in the tumorigenicity of HCC. PITX2C rather than PITX2A/B enhanced both the size and number of primary and secondary spheroids formed by PLC-8024 and MiHA cells. In addition, the overexpression of PITX2C up-regulated the markers of liver progenitors, such as afp and lgr5. Various functions of PITX2 isoforms in tumorigenicity may be responsible for its controversial role in different cancers [19–25, 39]. To characterize the role of PITX2 in the development and progression of cancer, there is a need to confirm the expression level of each PITX2 isoform at first.

Tumor lineage plasticity is considered to be one of the main causes of therapeutic resistance and recurrence. The feature commonly shared between tumors with poor differentiation is the re-expression of specific markers in progenitor cells, which usually have low expression, even completely absent in normal terminal differentiated cells. Among the selected nuclear proteins in our study, PITX2 was predicted to interact or regulate other factors by Pathway Commons. PITX2 has been widely reported to be significantly involved in regulating the Wnt and non-canonical pathways [13, 24, 33, 40, 41]. Similar to previous studies, our study confirmed the role of PITX2A/B/C in the Wnt signaling pathway by promoting Wnt5a/5β secretion in HCC. We further found that only PITX2C could bind to the promoter region of RALYL, which was identified as a stemness-related factor in HCC, as well as maintaining the mRNA stability of TGF-β, while PITX2A/B did not possess this capacity. In addition, both TGF-β and Wnt signaling were enriched in the ChIP-seq of PITX2C. Evidence suggests that signals from the
Fig. 5 (See legend on previous page.)
Fig. 6  H3K27 acetylation in PITX2C promoter region and activation of the Wnt pathway by PITX2. A Relative expression of Wnt5α/β detected by qPCR in transfected cells compared with control cells (*P < 0.05, **P < 0.01, Student’s t test). B The Wnt5α/β secretion level in the cell culture medium was confirmed by western blotting. Total proteins staining with Coomassie Brilliant Blue were used as a loading control. C Western blotting was performed to determine the expression of key components of the Wnt pathway (Wnt5α/β, c-Met, Frizzled, p-GSK3β, GSK3β, LEF, c-JUN, c-Myc and CD44) in cell lysates from PLC-8024 and MiHA cells. β-Actin was used as a loading control. D Detection of PITX2C expression in LO2 and MiHA cells by qRT-PCR after demethylation (5-Aza-dC) and histone acetylation (trichostatin A) treatment. PITX2C expression can be detected when ΔCtPITX2C (referred to CtPITX2C-CtGAPDH) < 30. E The enrichment of H3K27ac at the promoter regions of PITX2C was determined by ChIP-PCR with anti-H3K27ac antibody in LO2, MiHA, PLC-8024 and 449 cells. F The diagram on the left shows the dynamic expression of key markers and transcription factors during liver development and HCC progression. The diagram on the right illustrates the proposed mechanism of PITX2C upregulation in HCC differentiation and progression.
TGF-β and Wnt pathways regulate hepatocyte and biliary epithelium differentiation [4]. Further analysis of ChIP-seq showed that PITX2C shared similar binding sites of liver-enriched transcription factors (FOXA1, HNF1A and HNF4A) which were reported to regulate liver-specific transcripts, suggesting that PITX2C cooperates with them during liver development. Moreover, PITX2C could bind to the promoter regions of these factors, which is in line with these factors functioning in a complex inter-regulatory network (Fig. 6F) [42–44].

Conclusion
The findings presented in this study suggest PITX2C is a newly discovered and important factor in hepatic differentiation. The characterization of PITX2C could provide a basis for the development of HCC therapeutic strategies targeting specific factors in LPs.

Abbreviations
PITX2: Paired like Homeodomain 2; HCC: Hepatocellular carcinoma; AFP: Alpha fetoprotein; ES: Embryonic stem cells; DE: Definitive endoderm; LP: Liver progenitor; PH: Premature hepatocytes; S-Fu: 5-fluorouracil; qRT-PCR: Quantitative real-time PCR; Ct: Cycle threshold; ChIP: Chromatin immunoprecipitation.

Supplementary Information
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Additional file 1: Supplementary Materials and Methods. Supplementary Figure 1. PITX2 selection. (A) Heatmap of the expression profiles of the selected genes which were highly expressed in LP and PH. These genes included the specific genes for LP cells (AFP, GATA3, NKX2.5, FOXE1, CDH2, and the others encoding nuclear protein which showed a similar expression pattern to LP markers. (B) Among those selected nuclear protein genes, PITX2 was located in the central of gene regulatory network (Pathway Common). (C) Screenshot from SeqMan browser (Lasergene software 7.0) showing the varying 5’ sequences of the full-length of PITX2A/B/C (PITX2-V1 and V6 PITX2A; PITX2-V2, V4 and V5 PITX2B; PITX2-V3: PITX2C). (D) Western blotting analysis confirmed the protein levels of PITX2 in immortalized liver cells and HCC cell lines. GAPDH was used as a loading control. (E) Representative images of FISH staining of PITX2 (red) in HCC cases with low, moderate and relative high expression levels of PITX2. DAPI (blue) was used for nuclei counterstaining. (F) Kaplan-Meier overall (left) and disease-free (right) survival curve of two HCC groups in TCGA cohort. PITX2 (+), patients with higher PITX2 expression; PITX2 (-), patients with lower PITX2 expression. Supplementary Figure 2. PITX2A/B/C has distinct function in the tumorigenicity of HCC. Representative images of foci formation assay (A) and colony formation (B) in PITX2A/B/C-transfected and control cells. (C) Two shRNAs targeting PITX2 (shPITX2-1 and shPITX2-2) effectively decreased the mRNA level of PITX2 in PLC-8024 and SNU449 detected by qRT-PCR. Non-transfected cells were used as controls. Data are presented as the mean ± SD of 3 independent experiments. (*) P < 0.05, **P < 0.01, independent Student’s t-test). (D) The cell proliferation between shPITX2-transfected cells and control cells was compared by XTT assay. The results are expressed as the mean ± SD of three independent experiments. (*) P < 0.05, **P < 0.01, independent Student’s t-test). Representative images (left) and summary bar chart (right) of foci formation assay (E) and colony formation in soft agar assay (F) in shPITX2-transfected and control cells. Values indicate the mean ± SD of 3 independent experiments. (*) P < 0.05, **P < 0.01, independent Student’s t-test). (G) Orthotopic tumor formation was performed via intrahepatic implantation experiments using PITX2A-transfected cells and control cells or shPITX2-transfected cells and control cells. The final tumor volumes are summarized in the dot chart. Average tumor volume is expressed as the mean ± SD of 3 mice. The P value was calculated using paired Student’s t-test. (H) Representative images of excised orthotopic tumor formed by intrahepatic implantation experiment using PITX2A-transfected Hep38 cells and control cells. Supplementary Figure 3. PITX2C promotes cell mobility, self-renewal and chemoresistance of HCC. (A) Representative images (top) and bar chart (bottom) of cell migration and invasion abilities in shPITX2-transfected and control cells by Transwell and Matrigel invasion assays. Migration and invaded cells were stained with crystal violet and counted under a microscope. Values indicate the mean ± SD of three independent experiments. (*) P < 0.05; **P < 0.01, independent Student’s t-test). (B) Representative images of sphere formation assay using shPITX2-transfected cells and control cells (left). The numbers of primary and secondary spheroids are calculated in the bar chart (right). Values indicate the mean ± SD of three independent experiments. (**P < 0.01, independent Student’s t-test). The apoptotic indexes of PITX2C-transfected (C), shPITX2-transfected (D) and control cells were detected by fluorescence-activated cell sorting-based Annexin V/AAD double staining after treatment with 5-Fu or Sorafenib at the indicated concentrations for 48 h. (E) The mRNA levels of AFP and Lgr5 were compared by ΔΔCt in PITX2C or shPITX2-transfected and control cells (ΔΔCt = CtshPITX2-CTPITX2C or ΔΔCt = CtshPITX2-CTPITX2C). Supplementary Figure 4. Representatives of IHC staining images with anti-EP-CAM, CD133, c-Myc and NANOG in tumors induced by 8024-Ctrl, 8024-PITX2C cells with 5-Fu treatment. Red arrows indicate cancer stem cells. Supplementary Figure 5. Analysis of the ChIP sequencing data. (A) PITX2C shared similar binding motifs with several key transcription factors in LP. (B) Screenshot from the WashU epigenome browser showing PITX2C binding sites at the promoter of HNF1A, HNF4A, FOXA1, SMAD3, and ARID5B. (C) Heatmap of the expression profile for HNF4A, FOXA1, SMAD and ARID5B in the four stages (ES, EN, LP, PH) of in vitro hepatocyte differentiation model. (D) The expression of PITX2 is positively correlated with that of Wnt5a in GEPIA. Table S1. List of PCR primers for PITX2A/B/C expression. Additional file 2: Table S2. The gene ontology (GO) analysis of PITX2C ChIP-seq.
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