Deregulated GATA6 modulates stem cell-like properties and metabolic phenotype in hepatocellular carcinoma

Han-Wei Tan1, Carmen Oi-Ning Leung1,2, Kristy Kwan-Shuen Chan1, Daniel Wai-Hung Ho1, Ming-Sum Leung1, Chun-Ming Wong1,3, Irene Oi-Lin Ng1,3 and Regina Cheuk-Lam Lo1,3

1Department of Pathology, The University of Hong Kong, Pokfulam, Hong Kong
2Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hung Hom, Hong Kong
3State Key Laboratory of Liver Research, The University of Hong Kong, Pokfulam, Hong Kong

Introduction

GATA6 belongs to the mammalian transcription factor GATA family of which their conserved tandem zinc fingers bind to the (G/A)GATA(A/T) canonical sequence to regulate gene transcription.1 Physiologically GATA6 regulates lineage decision and specifies mesoderm and endoderm differentiation.2 In the liver, an endoderm-derived organ, GATA6 regulates HNF4α which in turn determines the expression of salient genes defining hepatocytes.3 Induction of GATA6 promotes hepatic fate differentiation in human-induced pluripotent stem cells (iPSC)4 and failure of liver development was observed in embryos derived from GATA6-deficient embryonic stem cells.5 These pieces of evidence show that GATA6 is required for maintenance of hepatocyte differentiation in the liver bud.

Recent studies indicated that the functions of GATA6 extend beyond embryonic development. Deregulation of GATA6 was observed in human cancers and the expression patterns vary among tumor origins. GATA6 overexpression was observed in breast, colorectal, esophageal and gastric cancers6–9 while loss of GATA6 expression was observed in ovarian cancer and glioblastoma.10,11 In addition, GATA6 expression levels vary according to the degree of tumor cell differentiation or histological subtypes. In lung cancer, poorly differentiated tumor cells expressed a lower level of GATA6 when compared to moderately differentiated cells, and expression was further reduced in metastatic tumor versus the primary tumor.12 In the pancreas, a lower GATA6 expression was noted in the basal-like subtype.13,14 Findings gathered from these expression analyses suggest that GATA6 can potentially exhibit oncogenic or tumor suppressive function. In addition, GATA6 expression possibly serves to fine tune the degree of cancer cell differentiation.

As a matter of fact, accumulating evidence has shown that alteration of GATA6 in cancers is linked to cell differentiation.
What’s new?

Dedifferentiation of mature parenchymal cells is a contributing factor in hepatocellular carcinoma (HCC), potentially linked to changes in expression levels of transcription factor GATA6. In this study, GATA6 was found to be downregulated in HCC tissues, with low GATA6 expression correlated with poor tumor cell differentiation and poor disease-free survival in patients. GATA6 downregulation induced tumor cell stemness including metabolic rewiring, with conversion of tumor cells to a glycolytic phenotype. The findings provide additional perspective on the acquisition of cancer stemness in HCC through downregulation of key molecular targets, apart from overexpression of specific markers.

Materials and Methods

Clinical samples

The clinical HCC specimens were obtained from patients who underwent surgical resection at Queen Mary Hospital. The resected specimens were snap-frozen in liquid nitrogen or processed into formalin-fixed paraffin-embedded (FFPE) blocks. The collection and utilization of the clinical samples were approved by the Institutional Review Board (IRB) of the University of Hong Kong.

Cell lines

Human HCC cell lines Huh7, Hep3B, MHCC-97L, MHCC-97H and PLC/PRF/5 were used in this study. Huh7 and PLC/PRF/5 were obtained from Japanese Collection of Research Biological Resources. Hep3B was obtained from American Type Culture Collection. MHCC-97L and MHCC-97H were a gift from Professor Z.Y. Tang, Fudan University, Shanghai, China. Cell lines were maintained in a humidified chamber at 37°C, 5% CO₂. The cell lines were authenticated by short tandem repeat-based assay. The cells were passaged upon reaching 80% confluence.

Establishment of GATA6 knockdown and overexpression clones

Knockdown of GATA6 using the lentiviral approach was performed to suppress the GATA6 expression levels in HCC cell lines. Two plasmids, shGATA6#4 (TRCN0000420674) and shGATA6#5 (TRCN0000423576), as well as a nontarget shRNA control (shNTC), was transfected into 293FT cells to produce recombinant lentiviral particles. Viral supernatant was collected and infected into Huh7 and Hep3B cells. Selection of positively infected cells was performed using puromycin. pcDNA3.1-GATA6 plasmid was a gift from Christine A. Iacobuzio-Donahue and Dr Yi Zhong, Memorial Sloan-Kettering Cancer Center, New York. For the overexpression of GATA6, pcDNA3.1 plasmids (both empty vector and GATA6 construct) were transfected into MHCC-97L cells.

Immunohistochemistry

FFPE tissue sections were used for immunohistochemical (IHC) staining. Tris-EDTA (pH 9.0) and heat antigen retrieval and was performed for GATA6 and glucose transporter 1 (GLUT1), respectively, followed by inhibition of endogenous peroxidases using 3% hydrogen peroxide. The slides were blocked using serum-free protein block (Dako) and incubated with the primary antibody, GATA6 (1:25, R&D Systems, Minneapolis, MN, AF1700) and GLUT1 (1:500; Abcam, Cambridge, MA, ab15309), at room temperature for 3 hr (GATA6) and 4°C overnight (GLUT1), respectively. Subsequently, the tissue sections were washed in Tris-buffer saline with tween-20 (TBST) and incubated in horseradish-peroxidase (HRP)-conjugated secondary antibody. The tissue sections were then developed using the DAB+ substrate-chromogen system (Dako). The slides were counterstained with Mayer’s hematoxylin and examined by a pathologist.

Immunofluorescent staining

The frozen tissue sections slides from clinical samples were permeabilized with methanol and blocked with 3% bovine serum albumin (BSA) in TBST. The slides were incubated with the primary antibody GATA6 (1:50; R&D Systems, Minneapolis, MN, AF1700) at 4°C overnight. The slides were washed with TBST before incubation with the fluorescent-conjugated secondary antibody (1:500; AF488) and DAPI. Huh7 cells were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature before permeabilization using 0.1% TritonX100/TBST for 10 min and blocking with 3% BSA in TBST for 1 hr at room temperature. The cells were
incubated with the primary antibodies, GATA6 (1:100; R&D Systems, Minneapolis, MN, AF1700) and HNF4α (1:100; Abcam, Cambridge, MA, ab41898) at 4°C overnight. The cells were washed before incubation with the fluorescent-conjugated secondary antibodies (1:500; AF488 and AF594) and DAPI. The tissues/cells were imaged under the LSM700 microscope (Carl Zeiss) and representative images were overlaid using the Zen software (Zeiss).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRizol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized by reverse transcription of 1 μg of the total RNA. The mRNA expression of various target genes was examined using the Power SYBR® Green PCR Master Mix Kit (Applied Biosystems, Foster City, CA) by quantitative polymerase chain reaction (qPCR) using the ABI7900HT Fast Real-Time PCR Detection System. HPRT gene was used as the housekeeping gene for normalization purpose. The primer sequences are listed in Supporting Information Table S1.

Western blotting

Protein lysates were collected using RIPA lysis buffer with protease inhibitors (Roche). SDS loading dye was added to the protein lysates and boiled at 95°C for 10 min. All denatured protein samples, together with the PageRuler™ prestained protein ladder (Thermo Fisher Scientific, Waltham, MA), were separated by SDS-polyacrylamide gel electrophoresis. The resolved protein gel was transferred to a PVDF membrane (Merck Millipore), prewetted in methanol. Following the transfer step, the membrane was blocked with 5% nonfat milk in TBST at room temperature for 1 hr. The blocked membrane was then incubated with the primary antibody overnight, at 4°C with shaking, based on the recommended dilutions in the manufacturer's datasheet. The following day, the membrane was washed with TBST, before incubation with the secondary HRP-conjugated antibodies at room temperature with shaking for 1 hr. Subsequently, the protein signals were probed using the WesternBright™ enhanced chemiluminescence (ECL) kit (Advansta) and the protein bands were visualized on the X-ray films. The antibodies used are listed in Supporting Information Table S2.

Transwell migration and invasion assays

For transwell migration assay, 8 x 10⁵ HuH7, 2 x 10⁵ Hep3B and 1.5 x 10⁵ MHCC-97L HCC cells suspended in serum-free medium were added to the upper chamber of the polycarbonate membrane transwell inserts of 8.0 μm pore size (Merck Millipore). For invasion assay, the transwells were first precoated with matrigel (BD Biosciences, Heidelberg, Germany). Complete medium containing 10% FBS was added to the lower chamber to serve as chemoattractant for the setup of HuH7 and Hep3B cells whereas conditioned medium was used for the setup of MHCC-97L cells. After incubation, the transwell membranes were fixed in methanol and stained with 1% crystal violet (Sigma Aldrich, St. Louis, MO). Random fields of the membranes were captured under the microscope and the migrated or invaded cells were quantified.

Cell proliferation assay

The cell proliferation assay was performed using the direct cell counting method. 1 x 10⁴ HCC cells were seeded in a 12-well plate and incubated in a humidified chamber at 37°C with 5% CO₂ for 6 days. The total viable cell number was counted at day 2, 4 and 6 using trypan blue and a hemocytometer under the light microscope.

Tumorsphere formation assay

The assay was performed as we previously described. Twenty-five hundred HCC cells, unless otherwise specified, were suspended in DMEM/F12 GlutaMAX™ medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen, Carlsbad, CA), 4 μg/ml insulin (Sigma-Aldrich), 20 ng/ml of epidermal growth factor (Sigma-Aldrich), 20 ng/ml of basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO) were seeded onto a 24-well plate, coated with 1% polyHEMA (Sigma-Aldrich, St. Louis, MO) for 8–10 days. Subsequently, the tumorspheres were photographed under the light microscope and the total number of spheres formed was counted. For the secondary propagation of primary tumorspheres, the primary tumorspheres were dissociated to single cells suspension using trypsin and resuspended in DMEM/F12 medium with supplements.

Glucose uptake assay

The assay was performed as we previously described. Twenty-two HCC cells were incubated with 0.1 mg/ml of 2-NBDG (Invitrogen, Carlsbad, CA) in DMEM without glucose medium (Invitrogen, Carlsbad, CA) at 37°C for 15 min in dark, unless otherwise specified. The cells were washed with 1 ml of cold PBS before re-suspending in 300 μl of cold PBS in the flow tubes. The glucose uptake rate of the cells was measured using FACScalibur flow cytometer and CellQuest Pro software (BD Biosciences, Heidelberg, Germany). The data were analyzed using FlowJo software (v10.0.7, Tree Star Inc., LLC, Ashland, OR).

Lactate production assay

5 x 10⁵ HCC cells were incubated in phenol red-free medium at 37°C, 5% CO₂ for 1 hr. The cells were spun down and the supernatant medium was collected. The lactate secreted by the cells was measured using the Lactate Colorimetric/Fluorometric Assay Kit according to the manufacturer’s protocol (BioVision, Inc.).

Chromatin immunoprecipitation assay

The assay was performed as we previously described. Chromatin immunoprecipitation (ChIP) assay was performed according to the kit and protocol provided by the manufacturer (Merck Millipore). In brief, HCC cells, upon reaching...
80% confluence in culture dishes, were cross-linked with 1% formaldehyde, lysed with SDS lysis buffer and sonicated on ice using the Soniprep 150 sonicator (MSE). The supernatant was precleared with Protein A agarose and immunoprecipitated with the GATA6 antibody (R&D Systems, Minneapolis, MN) and normal goat IgG overnight. The pull-down DNA was purified and quantified using qPCR. The primer sequences are listed in Supporting Information Table S3.

**Dual-luciferase reporter assay**

HCC cells were seeded in a 12-well plate, at a density of 1 x 10^5 per well. The assay was performed as we previously described. The pGL3-basic plasmids (Promega, Madison, WI) containing the wild-type or mutant promoter region of the PKM gene, together with the pRL-SV40 plasmids (Promega, Madison, WI), were cotransfected into the HCC cells. Luciferase activity was detected using the Dual-Luciferase® Reporter Assay Kit (Promega, Madison, WI). The primer sequences are listed in Supporting Information Table S4.

**In vivo mouse model**

The use of animals and the study protocol were approved by the Committee of the Use of Live Animals in Teaching and Research (CULATR) at The University of Hong Kong. All the animal experimental procedures were carried out in full concordance according to the Animals (Control of Experiments) Ordinance (Cap. 340). The limiting dilution assay was performed through the xenotransplantation of Huh7 shNTC and shGATA6#5 clones into the posterior flanks (both left and right sides) of the NOD/SCID mice. In this study, 1,000 cells and 5,000 cells of each clone were subcutaneously inoculated into the mice. The mice were placed under close observation for tumor growth. Upon reaching the end point, the mice were sacrificed and the tumor bulks were harvested for subsequent secondary serial transplantation.

**Methylation-specific polymerase chain reaction (MSP)**

The assay was performed as we previously described. Genomic DNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA). Bisulfite treatment of the DNA was then performed using sodium metabsulfite, sodium hydroxide and hydroquinone. The treated DNA was desalted and cleaned up using GeneJET gel extraction kit (Thermo Scientific, Waltham, MA). Purified DNA was desulfonated with sodium hydroxide and neutralized with ammonium acetate. The clean-up of DNA was repeated. The treated DNA was subjected to polymerase chain reaction using methylated and unmethylated MSP primer sets targeting the same CpG sites. Methylation status of the promoter region was determined by a semiquantitative analysis of the intensity of the MSP products.

**The cancer genome atlas analysis**

Global gene expression profiles by whole-transcriptome sequencing (WTS) and clinicopathological parameters of the cancer genome atlas (TCGA) dataset were obtained from TCGA data portal and http://firebrowse.org (Broad Institute). Data on 50 paired HCC and nontumoral liver samples were retrieved for analysis and subsequent differential gene expression analysis was performed as we previously described. Differential gene expression analysis was carried out by means of edgeR on the raw read counts. Negative binomials were used to capture variance dispersion for WTS read count data, empirical Bayes estimation was adopted for gene-specific variation, and generalized linear models were applicable to general experiments. Promoter methylation status and expression level of GATA6 from 360 HCC samples were retrieved from the TCGA database and analyzed by Spearman correlation.

**Statistical analysis**

The statistical significance of the GATA6 mRNA expression in the clinical HCC samples was calculated using the Wilcoxon signed-rank test in the Prism 6 software (GraphPad Software). The statistical analysis for the clinicopathological correlations were analyzed by the Fisher’s exact test using SPSS Statistics software (SPSS Inc., Chicago, IL). Disease-free survival was computed using the Log-rank test in the Prism 6 software. Univariate and multivariate survival analyses were performed using Cox regression model using SPSS Statistics software. The statistical significance of the experimental assays was determined by the Student’s t-test or Mann–Whitney test in the Prism 6 software and SigmaPlot 10.0 (Jandel Scientific), where appropriate. Statistical significance was defined by p < 0.05.

**Results**

**GATA6 is underexpressed in HCC and associated with tumor cell differentiation and survival outcome**

Initial analysis of the TCGA database revealed a significantly lower GATA6 expression in the tumor tissues from paired HCC clinical samples (Fig. 1a). On further analysis of our in-house clinical cohort of 100 HCC samples, GATA6 was underexpressed in the tumor tissues compared to the corresponding nontumoral liver tissues (p < 0.0001) (Fig. 1b). GATA6 downregulation was identified in 46 cases using a 0.5-fold cutoff (Fig. 1c). By immunofluorescent (IF) and IHC staining in HCC clinical samples, a lower protein expression of GATA6 was observed in tumor cells (Fig. 1d; Supporting Information Figure S1). Upon statistical correlation with clinicopathological parameters, GATA6 underexpression was associated with poorer tumor cell differentiation (p = 0.008) (Supporting Information Table S5) and showed a trend of worse disease-free survival of the patients (Fig. 1e). The correlation with survival outcome was further supported by analyses of the TCGA database. A low GATA6 level was correlated with poorer disease-free survival as derived by log-rank test (Fig. 1f). On univariate analysis by Cox regression model, GATA6 expression and tumor stage were significant predictive markers. Subsequent multivariate analysis revealed
GATA6 expression as an independent prognostic factor for disease-free survival (Supporting Information Table S6).

**Silencing of GATA6 activates EMT in HCC cells**

The expression data from clinical samples prompted us to postulate that reduced GATA6 level in HCC may be linked to aggressive biological behavior. To continue with the investigation, the functional effects of GATA6 in HCC cells were then examined by manipulating the expression of GATA6 in HCC cell lines. With reference to the endogenous expression levels (Fig. 2a), we established GATA6 stable knockdown clones in Huh7 and Hep3B cells using a lentiviral-based approach.

![Figure 1](image-url)
(Fig. 2b). The relatively low endogenous GATA6 expression in metastatic HCC cell lines MHCC-97L and MHCC-97H hinted the potential implication of GATA6 in HCC metastasis. In line with this finding, we observed downregulated E-cadherin and upregulated vimentin upon GATA6 knockdown (Fig. 2c). The functional effects were interrogated by cell motility and cell invasion assays, which showed that knockdown of GATA6 enhanced HCC cell migratory (Fig. 2d) and invasive (Fig. 2e) abilities. On the contrary, overexpression of GATA6 in MHCC-97L cell line suppressed cell migratory and invasive abilities (Figs. 2f and 2g).

**Suppression of GATA6 enhances tumorigenicity and self-renewal in HCC**

We first examined the expression of HNF4α, a well-characterized hepatocytic differentiation marker, upon silencing of GATA6 in Huh7 and Hep3B cells. A lower expression of HNF4α was noted in the knockdown cells as compared to shNTC (Fig. 3a). With IF staining, the colocalization of GATA6 and HNF4α in Huh7 cells could be demonstrated (Fig. 3b). This is coherent with our findings on the statistical correlation between GATA6 expression and tumor cell differentiation from clinical samples. Furthermore, we examined the expression of stemness markers upon manipulation of GATA6 level in HCC cells. Silencing of GATA6 resulted in upregulation of nanog, Oct4 and Sox2; whereas these three markers were downregulated upon GATA6 overexpression (Fig. 3c). In light of this, we further examined the functional effects upon manipulation of GATA6 levels. With tumorsphere formation assay, knockdown of GATA6 promoted the self-renewal of HCC cells as evidenced by augmented tumorsphere-forming ability. Both the number and size of tumorspheres were increased upon silencing of GATA6 (Fig. 3d). The reverse effects were observed upon GATA6 overexpression, which led to inhibited tumorsphere-forming ability of HCC cells (Fig. 3e). The tumorsphere assays were also performed at clonal dilution and consistent findings were observed (Supporting Information Fig. S2). Moreover, suppression of GATA6 facilitated, whereas overexpression of GATA6 inhibited, cell proliferation of HCC cells (Fig. 3f).

To clarify the above findings, the functional effects in vivo were also tested by a subcutaneous inoculation NOD/SCID mouse model with serial transplantation using 1,000 & 5,000 Huh7 cells. In line with the in vitro results, knockdown of GATA6 increased tumorigenicity and self-renewal in vivo. In the primary generation, tumor incidence was increased (from 3/10 to 10/10 with 1,000 cells; from 3/9 to 8/9 with 5,000 cells) and tumor latency period was reduced (Fig. 4a). The effects were propagated in the secondary animal recipients injected with 1,000 cells as reflected by the tumor incidence (shNTC: 3/10 vs shGATA6: 9/10) and tumor latency period (shNTC: 36 days vs shGATA6: 32.3 days) (Fig. 4b).

**A low GATA6 level triggers glycolysis by activating pyruvate kinase M2 transcription**

The above results suggest that downregulation of GATA6 gives rise to enhanced stem cell-like phenotypic features in terms of tumorigenicity, self-renewal and metastatic potential. Next, we proceeded to interrogate whether GATA6 is also taking part in reprogramming the metabolism of HCC cells. To this end, we examined the expression profiles of major metabolic pathways in the HCC cells harvested from the tumorspheres (Supporting Information Fig. S3). Interestingly, the mRNA levels of key molecular targets in the glycolytic pathway namely pyruvate kinase M2 (PKM2), GLUT1 and hexokinase 2 (HK2) were all elevated in GATA6 knockdown groups compared to shNTC control, with the alteration in PKM2 consistently reaching statistical significance (Fig. 5a). The upregulated expression of PKM2 was then confirmed at protein level by Western blotting (Fig. 5b). The functional effect of GATA6 on HCC glycolysis was then demonstrated by an increase in glucose uptake upon GATA6 knockdown (Fig. 5c). This link between GATA6 and glucose metabolism was could also be observed by examining GLUT1 expression in the primary HCC tumor tissues harvested from the mice (Fig. 4) using IHC. In line with the in vitro findings, a higher degree of GLUT1 expression was observed in the tumor tissue from the GATA6-knockdown group compared to control group (Fig. 5d). To confirm the above findings on the regulatory role of glucose metabolism by GATA6, validation experiments were performed by an overexpression system with MHCC-97L cells. Consistently, overexpression of GATA6 inhibited expression of PKM2 at both transcript and protein levels (Fig. 5e), and suppressed glucose uptake together with lactate production (Fig. 5f; Supporting Information Fig. S4).

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**Figure 1. GATA6 is underexpressed in HCC and associated with poorer tumor cell differentiation and tumor recurrence.** (a) Analysis of the TCGA database revealed a significantly lower GATA6 expression in the tumor tissues from paired HCC clinical samples (n = 50). (b) Dot-plot diagram indicated that GATA6 mRNA levels were significantly downregulated in the 100 pairs of clinical HCC tumor tissues as compared to the nontumoral tissues (Wilcoxon signed-rank test). HPRT was used as the housekeeping gene for normalization. (c) The waterfall plot diagram showed the log2 ratio of GATA6 mRNA levels in HCC tumor tissue versus NT tissues. The cutoff for downregulation was defined as log2 ratio of HCC/NT ≤ -1. (d) Representative IF (left panel) and IHC (right panel, magnification 400×) images showing the underexpression of GATA6 protein in the tumor sections as compared to nontumoral sections. By IHC, nuclear staining of moderate intensity was observed in NT tissues, while the HCC tissues showed negative staining. (e) A low GATA6 expression level was correlated with poorer disease-free survival of HCC patients (Log-rank test) from both our in-house clinical cohort (left panel—cutoff at 0.5-fold by T/NT fold change; middle panel—in tertiles by T/NT fold change; right panel—upper/middle tertile versus lower tertile by T/NT fold change) and (f) TCGA database (left panel—cutoff at 0.67-fold by T/NT fold change; middle panel—in tertiles by T/NT fold change; right panel—cutoff at Z-score (−1.1 by expression in T). **** p < 0.0001. T: HCC tumor; NT: nontumoral liver.
Figure 2. Silencing of GATA6 activates epithelial–mesenchymal transition in HCC cells. (a) Endogenous GATA6 expression in Huh7, Hep3B, MHCC-97L and MHCC-97H cell lines. (b) mRNA and protein levels of GATA6 in shNTC, shGATA6 #4 and shGATA6 #5 clones (Huh7 and Hep3B). β-actin was used as a loading control. (c) Western blot results indicated that protein levels of E-cadherin were suppressed while that of vimentin was upregulated upon GATA6 knockdown in both Huh7 and Hep3B cell lines. β-actin was used as a loading control. (d) Representative images of the stained cells (Huh7 and Hep3B) in the transwell migration assays and a graph showing the average number of migrated cells for each clone (n = 3, each in duplicates; scale bar: 50 μm). (e) Representative images of the stained cells (Huh7 and Hep3B) in the matrigel invasion assays and a graph showing the average number of invaded cells for each clone (n = 3, each in duplicates; scale bar: 50 μm). (f) mRNA and protein levels of GATA6 in GATA6 OE and EV clones (MHCC-97L). HPRT was used as housekeeping gene and β-actin was used as a loading control. (g) Representative images of the stained cells (MHCC-97L) in the transwell migration and matrigel invasion assays and graphs showing the average number of migrated and invaded cells for each clone (n = 3, each in duplicates; scale bar: 200 μm). Data are presented as mean ± S.D. p-values were calculated using student t-test. **p < 0.01, ***p < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]
Figure 3. GATA6 regulates self-renewal and proliferation of HCC cells in vitro. (a) Relative mRNA expression of HNF4α normalized with HPRT in shNTC and shGATA6 #5 clones (Huh7 and Hep3B) by RT-qPCR (n = 3; student t-test). (b) Representative IF images illustrating the expression and localization of GATA6 and HNF4α in Huh7 shNTC and shGATA6 #5 cells. (c) mRNA expression of stemness markers nanog, Oct4 and Sox2 upon knockdown and overexpression of GATA6 in HCC cells. (d) Representative images and the graphs showing the average number and diameter of the tumourspheres formed by Huh7 and Hep3B (n = 3, each in triplicates; student t-test; scale bar: 50 μm). (e) Representative images and the graphs showing the average number and diameter of the tumourspheres formed by MHCC-97L (n = 3, each in triplicates; student t-test; scale bar: 50 μm). (f) Numbers of HCC cells upon GATA6 knockdown (Huh7 and Hep3B) and overexpression (MHCC-97L) counted at days 2, 4 and 6 (n = 4 for Huh7 and Hep3B & n = 3 for MHCC-97L, each in triplicates; student t-test). Data are presented as mean ± S.D. *p < 0.05, **p < 0.01, ***p < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]
Since GATA6 is a transcription factor, in view of the above results we next speculated that GATA6 is potentially a transcriptional regulator of these molecular targets in particularly PKM2. By means of predictive analysis, we identified three potential GATA6 binding sites at −1,000 bp promoter region upstream of the transcription start site of PKM gene (Fig. 6a). With ChIP assay, the physical binding of GATA6 to site 2 and site 3 of the PKM gene promoter region was confirmed in parental Huh7 cells and MHCC-97L GATA6-overexpressed cells (Fig. 6b). We then carried out luciferase reporter assay and validated the transcriptional regulation of GATA6 at these two sites. Promoter activity was enhanced upon silencing of GATA6 whereas it was suppressed upon GATA6 overexpression. Such effects were abrogated when the assays were performed with mutant constructs (Fig. 6c). Considering that PKM gene is transcribed into both PKM1 and PKM2 with alternative splicing, we also assessed PKM1 mRNA level in the GATA6 clones. Neither knockdown of overexpression of GATA6 significantly altered PKM1 level (Fig. 6d). The overall findings are in support of a transcriptional repressor role of GATA6 on PKM2 and its regulatory function of glycolytic metabolism in HCC cells.

Discussion
In this study, we deciphered the functional significance of deregulated GATA6, a transcription factor sustaining hepatocytic differentiation, in HCC. Our findings collectively suggest that a high level of GATA6 is fostering tumor suppressive effects in HCC. And underexpression of GATA6 in HCC directs tumor cells to glycolytic metabolism and promotes tumorigenicity, self-renewal and metastasis.

The concept of cell plasticity through which mature hepatocytes acquire progenitor nature was consolidated in major studies. In neoplastic conditions, there is compelling evidence showing the implication of cell plasticity as a prerequisite for HCC biology. In one recent report, the oncogenic reprogramming of hepatocytes was emphasized by lineage-tracing experiments using Sox9, EpCAM and Yap1 as key molecular markers in this process. In this regard, our group previously reported on the in-depth characterization of Sox9 in HCC. Sox9 is a transcription factor and liver progenitor cell marker during embryogenesis and liver regeneration. Sox9 upregulation in HCC tissues was correlated with aggressive clinicopathological features. With functional assays, we demonstrated that Sox9 confers multiple stemness properties in HCC by activating the Wnt/β-catenin pathway and transcriptional modification (acetylation/deacetylation) is possibly involved in the transcriptional regulation (Supporting Information Fig. S5).
While our Sox9 study portrayed how activation of hepatic progenitor cell markers endows a stemness phenotype in HCC, our current study on GATA6 illustrates how downregulation of deterministic transcription factors could revert tumor cells to stem cell-like phenotype in terms of functions and metabolic signature. The latter, as a matter of fact, recapitulates that of embryonic undifferentiated stem cells which shift from glycolysis to oxidative phosphorylation during the process of differentiation to somatic cells. While two previous studies provided indirect clues on the participation of GATA6 in liver cancer, the speculated role of GATA6 based on the findings from the two studies did not appear in line with each other. In one study, miR143 was found downregulated in HCC and that GATA6 was identified as a downstream target. Yet the other study which focused on miR181 showed that GATA6 is a downstream target, and functionally miR181 enhanced EpCAM expression, spheroid formation and in vivo tumorigenicity of HCC. In this present study, we investigated the direct functional effects of independent manipulation of GATA6 in HCC. In addition, we reported expression data of GATA6 in HCC clinical samples together with clinicopathological correlation. We hereby put forward evidence to support GATA6 functions as a potent genetic switch in the oncogenic and metabolic reprogramming of HCC cells and induce stem cell-like phenotypes. It is at the same time a potential prognostic biomarker for liver cancer.

Figure 4. Suppression of GATA6 enhances in vivo tumorigenicity and self-renewal in HCC. (a) In both 1,000 cells and 5,000 cells groups, tumors derived from the GATA6 knockdown group showed increased tumor size and mass compared to the shNTC control (Mann–Whitney test; Scale bar: 1 cm). Tumor incidence was increased and tumor latency period was shortened. (b) In the secondary recipient mice, tumors derived from GATA6 knockdown group similarly demonstrated enhanced tumorigenicity in terms of tumor mass, tumor incidence and latency period (Mann–Whitney test; scale bar: 1 cm). Data are presented as mean ± S.D. *p < 0.05, ***p < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]
Figure 5. A low level of GATA6 triggers glycolytic activity of HCC cells. (a) Transcript levels of GLUT1, HK2 and PKM2 in shNTC, shGATA6 #4 and shGATA6 #5 of Huh7 tumorspheres (n = 4 for primary tumorspheres & n = 3 for secondary tumorspheres; student t-test) by RT-qPCR. (b) PKM2 expression upon GATA6 knockdown in Huh7 and Hep3B by Western blotting with cell lysates from tumorspheres. β-actin was used as a loading control. (c) Relative glucose uptake rate in shNTC, shGATA6 #4 and shGATA6 #5 groups of Huh7 and Hep3B tumorspheres (n = 3, each in duplicates; student t-test). (d) IHC staining of GLUT1 protein in the Huh7 shNTC and shGATA6 #5 tumors. (e) Left panel—Relative mRNA levels of PKM2 normalized with HPRT in the EV and OE clones of MHCC-97L by RT-qPCR (n = 3; student t-test). Right panel—The change in PKM2 was confirmed at protein level by western blotting. (f) Left panel—Relative glucose uptake rate in the EV and OE clones of MHCC-97L (n = 3, each in duplicate; student t-test). Right panel—Lactate production levels in the EV and OE clones of MHCC-97L (n = 3, each in triplicate; student t-test). Data are presented as mean ± S.D. *p < 0.05, **p < 0.01, ***p < 0.001. [Color figure can be viewed at wileyonlinelibrary.com]
To extend our investigation, we overexpressed HNF4α (transcript variant 1, plasmid RC214914 from Origene Technologies, Rockville, MD) in the GATA6-knockdown Huh7 cells and performed cell proliferation assay, migration assay, invasion assay and tumorsphere formation assay. The results showed that HNF4α overexpression did not bring significant different
functional effects (Supporting Information Fig. S6), suggesting that while GATA6 alters HNF4α expression (a marker representing hepatocytic differentiation), HNF4α may not be a major or sufficient effecter for the functions of GATA6 in HCC. Moreover, given there are 12 isoforms of HNF4α, the potential effects of any of the isoform(s) is yet to be defined by adopting individual isoforms in the experiments.

On upstream mechanism, it remains a question through what mechanism GATA6 is downregulated in HCC. On literature review, recurrent chromosomal loss of 18q11.2-18q22.2 which encompasses the GATA6 locus (18q11.2) was reported in HCC.33 Hence, loss of heterozygosity (LOH) could possibly contribute to the underexpression of GATA6 in HCC. Apart from this, de novo mutations and deletion of nucleotides in GATA6 gene have been described in congenital heart defects, pancreatic agenesis and diabetes mellitus.34-36 In the context of cancers, loss-of-function mutations in GATA6 gene accompanied with LOH was found in malignant astrocytoma.31 Hence, we resorted to the TCGA data portal for mutations of GATA6 in HCC and found that only 8 out of 353 HCC tumors showed GATA6 gene alteration as a result of fusion, amplification and missense mutation. Moreover, a recent study on gastric cancer reported the regulatory role of GATA2 on GATA6 expression, raising a possibility interaction among various members of the GATA family in cancer model. In view of this, we looked into the correlation of expressions between GATA2 and GATA6 in HCC from the TCGA database, which showed that the correlation was weak (Supporting Information Fig. S7). Besides, while loss of function of GATA4 via germline mutation was recently reported in HCC, its expression was apparently independent of GATA6 from correlation analysis from the database (Supporting Information Fig. S7).

It is worth mentioning that epigenetic regulation of GATA6 expression had been studied in human cancers other than HCC. GATA6 is a target of miR506, miR363 and miR196-b in oral squamous cell carcinoma, colon cancer and lung cancer, respectively.39-41 Hypermethylation of GATA6 promoter region was found in rhabdomyosarcoma, glioblastoma and gastric cancer, and is at the same time an adverse prognosticator for patient survival in the latter two. Epigenetics is an integral part of molecular pathogenesis of HCC. Methylation of different genes takes place in multiple key steps of hepatocarcinogenesis such as DNA repair, apoptosis and cell cycle regulation. Of note, epigenetic regulation is a potent mechanism in controlling transcription pattern which determines cell state and coordinates cell plasticity.46,47 To gain some preliminary insights, we treated HCC cell line PLC/PRF/5, which shows low endogenous GATA6 expression, with demethylating agent 5-Aza-2’-deoxycytidine (5-Aza-dC) and found GATA6 expression was restored (Supporting Information Fig. S8A). By prediction analysis using MethPrimer48 we identified the CpG islands between −2000 bp promoter region and +1155 bp relative to transcription start site (+1) of GATA6. We then performed MSP with reference to the regions of interest in HCC cell lines. Cell line with relatively low endogenous GATA6 level (PLC/PRF/5) showed a more hypermethylated status compared to cell lines with high GATA6 expression (Hep3B and Huh7) (Supporting Information Fig. S8B). Further analysis of the TCGA database revealed an inverse correlation between GATA6 expression level and methylation status in HCC samples (Supporting Information Fig. S9). These findings suggest that promoter site methylation may be a major regulatory mechanism for GATA6 expression in HCC. Translating into clinical implications, demethylating agent to restore GATA6 level in selected patients is a potential therapeutic strategy in HCC.

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Conflict of interest
The authors declare no potential conflicts of interest.

Author contributions
Tan H.W. acquired the data, analyzed the data and wrote the manuscript. Leung C.O. and Chan K.K. acquired and analyzed the data. Ho D.W., Leung M.S. and Wong C.M. acquired the data, analyzed the data and wrote the manuscript. Author contributions. Leung C.O. and Chan K.K. acquired and analyzed the data. Ho D.W., Leung M.S. and Wong C.M. acquired the data. Ng I.O. provided clinical samples. Lo R.C. conceived and supervised the study. Leung C.O. and Chan K.K. acquired and analyzed the data. Ho D.W., Leung M.S. and Wong C.M. acquired the data, analyzed the data and wrote the manuscript. The authors declare no potential conflicts of interest.

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