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Overexpression of ZFX confers self-renewal and chemoresistance properties in hepatocellular carcinoma

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Zinc finger protein X-linked (ZFX) is a zinc finger protein of Zfy family, which is highly conserved in vertebrates. This transcriptional regulator is not only highly expressed in embryonic stem cells (ESC) and hematopoietic stem cells, but is also upregulated in a number of human cancers where it is functional related to cell proliferation and survival. Hepatocellular carcinoma (HCC) is highly aggressive cancer that commonly resistant to most chemotherapies and displays stemness characteristics. In this study, we examined the expression of ZFX in HCC and its possible functional implications in liver tumorigenesis. Quantitative RT-PCR analysis showed common overexpressions of ZFX in 51.8% HCC tumors when compared with their adjacent non-malignant liver ($n = 43/83$; $p = 0.004$). In line with the pluripotency role of ZFX, we found silencing of ZFX readily inhibited self-renewal capability ($p = 0.0022$), colony formation ability ($p < 0.0001$) and cell proliferation ($p < 0.0001$) through G0/G1 cell cycle arrest of HCC cells ($p = 0.0038$). In addition, suppression of ZFX sensitized HCC cells to chemotherapeutic agent cisplatin ($p < 0.0001$). Further investigations suggested that ZFX bind on the promoter of two important mediators, namely Nanog and SOX-2, activating their expressions in HCC ($p < 0.0001$). Moreover, *in vivo* xenograft study demonstrated that overexpression of ZFX would promote the tumor growth ($p = 0.031$). Taken together, our results show, for the first time, commonly overexpressions of ZFX in HCC, where it likely contributes to the stemness and pluripotent behavior of this highly malignant cancer.

Hepatocellular carcinoma (HCC), which accounts for 80–90% of all liver cancers, is the fifth most common cancer and the third leading cause of cancer-related deaths worldwide.¹ HCC is an aggressive cancer with a high mortality rate; often due to being diagnosed at a late stage when curative therapies are least effective.² Moreover, most HCC patients show disease recurrence that rapidly progresses to advanced stages. It is general believed that the presence of cancer stem-like features is responsible for chemoresistance and recurrence of

HCC.^{3–7} ZFX is a zinc finger protein of the Zfy family that is highly conserved among vertebrates. It is a transcriptional factor encoded on the mammalian X chromosome and contains an acidic transcriptional activation domain, a nuclear localization sequence and a DNA binding domain.^{8,9} Despite elevated levels are detected in pluripotent embryonic stem cells (ESCs) and hematopoietic stem cells (HSCs) where it is required for maintenance of self-renewal ability,⁸ ZFX is also found to be frequently overexpressed in a number of human cancers, including esophageal carcinoma, gastric cancer, prostate cancer, and glioma.^{10–13} Common cancer stemness characteristics, including colony-forming ability, acquisition of drug resistance, cell survival, and cancer metastasis have been further proposed to be induced from ZFX in these cancers. In HCC, the role of ZFX however remains unexplored. The present study hence aims to examine the expression and functional effects of ZFX in the liver tumorigenesis.

Here, we report, for the first time, common upregulation of ZFX in HCC tissues (51.8%). In functional analysis, we showed knockdown of ZFX could induce G0/G1 phase cell cycle arrest and reduce cell proliferation and colony formation abilities of HCC cells. Also, depletion of ZFX could suppress the self-renewal ability and sensitized HCC cells to chemotherapeutic drug cisplatin. Vice versa, ectopic expression of ZFX would promote cell proliferation, and colony formation ability of HCC cells. In addition, ZFX could bind on promoter of transcriptional factors regulatory network

Key words: ZFX, hepatocellular carcinoma, self-renewal, chemoresistance

Additional Supporting Information may be found in the online version of this article.

K.P. and J.C. contributed equally to this work.

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What's new?

Zinc Finger Protein X-linked gene (ZFX) is a member of Zfy family of transcriptional regulators that is often highly expressed in embryonic and hematopoietic stem cells. In this study, the authors find that ZFX is commonly overexpressed in hepatocellular carcinoma, where it promotes the expression of stem cell-associated transcription factors such as Nanog and SOX-2. These findings may shed new light on chemoresistance and frequent recurrence of hepatocellular carcinomas, symptoms attributed to stemness and pluripotent behavior of this cancer.

including SOX-2 and Nanog and influence their expressions. Moreover, the overexpression of ZFX would promote *in vivo* tumor growth. The identification and functional characterization of ZFX in HCC provides new insights into the stemness-associated features of HCC and the possible use of ZFX as a therapeutic target.

Material and Methods**Patients**

Tumorous liver tissues were collected from 83 patients who underwent curative surgery for HCC at Prince of Wales Hospital, Hong Kong. Informed consent was obtained from each patient, and the study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong. A diagnosis of HCC was confirmed on histologic examination by pathologists. The demographic information of HCC patient cohort was shown in Supporting Information Table 1. Six normal livers were used in this study. Three of them were purchased from commercial companies (Ambion and Calbiochem), while remaining three were liver transplantation.

Cell culture

The in-house established human HCC cell lines HKCI-10, HKCI-C2, and HKCI-8 were maintained as described previously.¹⁴ In brief, these cell lines were cultured in RPMI 1640 glutamax medium (Invitrogen) supplemented with 10% fetal bovine serum. The immortalized normal liver cell line L02 was cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum.

RNA extraction, cDNA synthesis, and quantitative RT-PCR (qRT-PCR)

Total RNA from cells and tissues were isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RQ1 Rnase-free Dnase (Promega Corporation, Madison, WI) to eliminate genomic DNA carryover. First-strand cDNA synthesized from random hexamer was subjected to TaqMan assay for target genes in Universal PCR Mix (ABI, Foster City, CA). The emission intensity was detected by iCycler IQ detection system using fluorophore FAM490 (Bio-Rad, Hercules, CA). Threshold cycles were averaged from triplicate reactions. To adjust for variations in starting template, gene expression was normalized against 18S rRNA. Fold change relative to mean value obtained from 6 normal liver controls

was determined by $2^{-\Delta\Delta Ct}$. A relative gene expression of two-fold or more was considered up-regulation.

Western blot analysis

Protein lysates were resolved on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically on to polyvinylidene fluoride membrane. After blocking for nonspecific binding, blots were incubated with specified antibodies against ZFX, SOX-2, Nanog (1:1000; Cell Signaling), or glyceraldehyde-3-phosphate dehydrogenase (1:25,000; Chemicon). After incubation with secondary conjugated to horseradish peroxidase, protein bands were visualized using enhanced chemiluminescence detection (GE Healthcare, Piscataway, NJ).

Lentiviral packaging and transduction

HEK293FT cells were transfected with packaging plasmids pCMV-VSV-G, pRSV-Rev, and pMDLg/pRRE along with pLKO.1-NS Ctrl or pLKO.1-hZFX shRNA (Sigma) using FUGENE transfection reagent (Roche). After 48 hr post-transfection, viral supernatant was precipitated with PEG-it Virus Precipitation Solution in 1:4 ratio to produce concentrated viral stock. HKCI-10 and HKCI-C2 were plated 1 day before transduction. After 48 hr post-transduction, cells were selected in medium containing 2 $\mu\text{g/ml}$ puromycin for 10 days.

Stable expression of ZFX in HCC cell line HKCI-8

HKCI-8 was transfected with pCMV6-empty vector or pCMV6-ZFX (Origene) containing the neomycin resistance gene by using LipofectamineTM 2000 transfection reagent (Invitrogen). After 48 hr post-transfection, cells were selected in medium with 500 $\mu\text{g/ml}$ of Geneticin (Life Technologies) for 3 weeks. The overexpression of ZFX was confirmed by qPCR analysis.

Cell cycle analysis by propidium iodide

Stable transfected cells (3×10^5) were harvested when reached approximately 80% confluence. The cells were trypsinized, washed with PBS and fixed in 3 ml ice-cold 70% ethanol at -20°C overnight. Fixed cells were pelleted by centrifugation at 1,000g for 5 min. Then the cells were washed by PBS twice and stained with 1ml staining solution containing 50 $\mu\text{g/ml}$ propidium iodide and 0.5 $\mu\text{g/ml}$ RNase A for 30 min and subjected to flow cytometric analysis. Flow

cytometric study was performed by BD FACSCalibur™ using CellQuest software. The average value of G0/G1, S, and G2/M phase were averaged from at least three independent experiments.

Cell proliferation assay

Stable transfected cells (1×10^3) seeded on a 96-well microtiter plate with 8 replicate wells were allowed to incubate for different time interval from days 1 to 7. Cell proliferation was measured by MTT assay. Colorimetric product formed will be measured at absorbance 570 nm every day for 7 consecutive days.

EdU cell proliferation assay

DNA replication was measured using a Click-it EdU Flow Cytometry Assay kit, which is based on incorporation of the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) into DNA during replication (Invitrogen). The stable transfected cells (3×10^5) were cultured in complete medium with 10 mM EdU for 2 hr before harvested and washed with 3 ml of 1% BSA in PBS. Then cells were fixed with 100 μ l of Click-iT fixative and incubated for 15 min at room temperature. After washed once with 3 ml of 1% BSA in PBS, the cells were resuspended in 100 μ l of 1X Click-iT saponin-based permeabilization and wash reagent. Then cells were added with 0.5 ml of Click-iT reaction cocktail. After washed and resuspended in 500 μ l of 1X Click-iT saponin-based permeabilization and wash reagent, cells were analyzed on BD FACSCalibur™. The percentages of different fluorescent cells were analyzed using WinMDI Ver.2.9.

Colony formation assay

Stable transfected cells were seeded in 6-well tissue culture plates (200 cells per well). After an incubation period of 7 days, the medium was decanted and each well was washed twice with PBS. The cells were stained with 1% crystal violet (in 100% methanol) for 15 min, followed by detaining. Colonies (more than >20 cells/colony) were counted.

Single-cell clonogenic assay

To assess self-renewal *in vitro*, single-cell clonogenic assay was done as described previously.¹⁵ In brief, a single-cell was seeded into 96-well plates (1 cell per well), and cultured in 10% fetal bovine serum-containing medium. After 1 day incubation, wells containing no cells or more than one cell were excluded. Wells containing only one cell were marked and observed daily under microscope. Cell clones were counted after 7 days when a clone reached ≥ 20 cells.

In vitro cytotoxicity assay

Stable transfected cells (5×10^3) seeded on a 96-well microtiter plate with 8 replicate wells were allowed to incubate for 48 hr with the treatment of anticancer drug cisplatin (20 μ M). After incubation, cell viability was assessed utilizing the thiazolyl blue tetrazolium bromide (MTT) assay. Colorimetric

complex formed dissolved by DMSO was determined for absorbance at 570 nm.

ChIP-PCR

Chromatin immunoprecipitation (ChIP) was performed as previously described,¹⁶ with slightly modification. Briefly, 2 days after transient transfected with ZFX-Flag plasmid, L02 cells were fixed with 1% formaldehyde, and then lysed in RIPA buffer containing 1 \times Protease Inhibitor Cocktail. Cross-linked chromatin were fragmented to approximately 600 bp by sonication. After centrifugation, sonicated chromatin was diluted tenfold with ChIP dilution buffer and pre-cleared with protein G-sepharose for 1 h at 4°C, then incubated with anti-Flag antibody or IgG control at 4°C overnight. After immunoprecipitation, protein G-sepharose beads were used to collect immunoprecipitated complex. The beads were washed once with low salt buffer, once with high salt buffer, once with LiCl washing buffer and twice with TE buffer. Then, protein-DNA complexes were eluted with Elution buffer, followed by cross-linking reversal and protease K treatment. Finally, DNA was purified by QIAGEN PCR purification kit and eluted with 50 μ l sterile water. Real-time ChIP-PCR by SYBR Green-based detection method was performed to detect putative ZFX targets. Primers designed to cover potential ZFX binding sites were listed in Supporting Information Table 2. Each reaction contained equal amount of ChIP or input DNA.

In vivo xenograft study

1×10^7 of ZFX-stable expressed HKCI-8 cells were resuspended in 100 μ l PBS and were injected on the dorsal surface of the 7 weeks old nude mice (control: left side, ZFX: right side). Tumor volumes were monitored every 5 days over the course of treatment by caliper measurement of the length and width and calculated using the formula of $(\text{width})^2 \times \text{length}/2$. Mice were sacrificed after 3 weeks of treatments then the tumors were removed and measured.

Statistical analysis

Expression of ZFX in HCC tumors was compared with adjacent nontumoral livers by paired Student's *t* test and to normal livers by Mann-Whitney test. Statistical correlations of ZFX expression and clinicopathological parameters were carried out by unpaired Student's *t* test. The effect of ZFX in functional studies was compared using the paired Student's *t* test. $p < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 3.02 (GraphPad Software Inc., San Diego, CA).

Results

ZFX is commonly overexpressed in human HCC

The expression of ZFX in HCC was determined by qRT-PCR in a cohort of 83 HCC tumors (T) and paired adjacent nontumoral livers (TN), and 6 normal liver tissues (NL). Quantitation of ZFX levels showed significant up-regulation in HCC

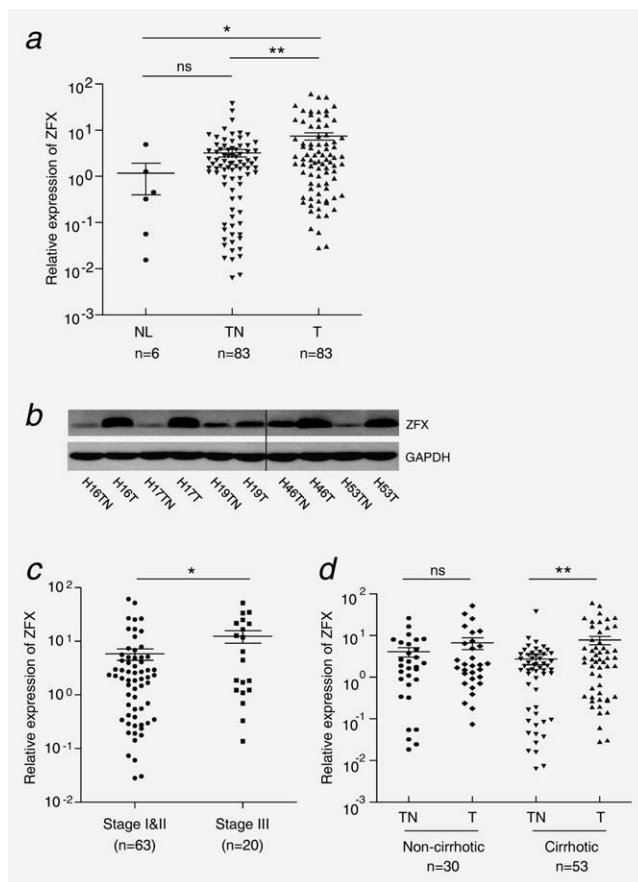


Figure 1. Overexpression of ZFX in HCC. (a) qRT-PCR showed the upregulation of ZFX in HCC tumoral tissues ($n = 83$) ($*p = 0.036$; ns, not significant) as compared to normal livers ($n = 6$) and in 43 tumors when compared with their adjacent nonmalignant livers (T/TN>2) (51.8%) ($**p = 0.0041$). (NL: normal liver tissues, TN: paired adjacent nontumoral livers, T: HCC tumors). (b) Western blot analysis demonstrated the upregulation of ZFX protein level in HCC tumors as compared to their adjacent nonmalignant livers. (c) qRT-PCR analysis showed a higher expression of ZFX in stage III tumors as compared to stages I and II HCCs ($*p = 0.0348$). (d) qRT-PCR showed a significant higher expression of ZFX in cirrhotic HCC tumoral tissues as compared to noncirrhotic HCC tumoral tissues ($**p = 0.0099$; ns, not significant).

tumors as compared to normal livers (Mann-Whitney test $p = 0.036$), and in 43 tumors when compared with their adjacent nonmalignant livers (51.8%) (paired Student's t test $p = 0.0041$) (Fig. 1a). In five cases that showed increased ZFX mRNA expression, Western blot also concurred in indicating higher protein level of ZFX in tumors compared to matching nonmalignant liver (Fig. 1b). We also attempted to correlate expression of ZFX in tumoral tissues with clinicopathological features of patients. Correlative analysis did not suggest statistical significance between ZFX expression and parameters, including gender, age, HBV status, histology grade, number of lesions, macrovascular invasion, and microvascular invasion. However, advanced Stage III tumors exhibited higher ZFX expressions as compared to early stages I and II HCCs (unpaired Student's t test $p = 0.0348$) (Fig. 1c), suggesting

ZFX expression is related to tumor staging. On further segregation of HCC tumors arising from a cirrhotic or noncirrhotic background, we found a significantly higher expression of ZFX in cirrhosis-associated tumors compared to their non-malignant counterpart (paired Student's t test $p = 0.0099$). Since tumor adjacent cirrhotic liver is often considered a pre-malignant lesion of HCC, our finding would suggest ZFX plays a role in predisposing the risk of HCC development (Fig. 1d).

Knockdown of ZFX induces G0/G1 cell cycle arrest in HCC cells

To gain insight into functional role of increased ZFX expression in HCC, two HCC cell lines (HKCI-10 and HKCI-C2) with elevated ZFX expression ($p = 0.0091$) (Fig. 2a) were used to establish ZFX-deficient stable clones (clone 1: shZFX-C1, clone 2: shZFX-C2) by lentiviral transfection. The knockdown efficiency of ZFX was determined by qRT-PCR ($p < 0.0001$) and western blotting (Fig. 2b). In cell cycle analysis by PI staining, we found the significant increase in G0/G1 cell population but decrease in both S and G2/M population. The reduction of S + G2/G1 ratio after knockdown of ZFX suggested that depletion of ZFX would induce the G0/G1 cell cycle arrest in HCC ($p < 0.0016$) (Fig. 2c).

ZFX is required for HCC cell growth and proliferation

In addition, the deficiency of ZFX significantly reduced the *in-vitro* proliferation of HCC in MTT assay ($p < 0.0001$) (Fig. 3a) and decreased the amount of proliferating cell population in EdU cell proliferation assay (HKCI-10, shZFX-C1: $p = 0.0038$; shZFX-C2: $p < 0.0001$) (HKCI-C2: $p < 0.0001$) (Fig. 3b). Also, fewer in number and smaller-sized colonies were formed in ZFX-depleted cells from colony formation assay ($p < 0.0001$) (Fig. 3c). Vice versa, ectopic expression of ZFX could promote cell proliferation ($p < 0.0001$) (Supporting Information Fig. 1a) and colony formation ability ($p < 0.0001$) (Supporting Information Fig. 1b) of HCC cells. Our results suggested that ZFX is required for the *in vitro* proliferation and growth of HCC.

ZFX induces stem-like cell properties of HCC cells

Since ZFX plays key roles in the maintenance of pluripotency, we next investigated the potential role of ZFX on regulation of stem-like cell characteristics in HCC. Reasoning that self-renewal and drug resistance are two important features of stemness behavior, we hence assayed for the effect ZFX on these two properties. Indeed, we found that suppression of ZFX could impede the self-renewal ability of HCC cells, which was reflected by fewer colonies and smaller sizes formed in single-cell clonogenic assay in ZFX-depleted HKCI-10 ($p < 0.0001$) and HKCI-C2 ($p = 0.0022$) (Fig. 4a). In addition, we found knockdown of ZFX could sensitize HCC cells to anticancer drug cisplatin, which was reflected by the deduction of IC50 from $16.86 + 1.067 \mu\text{g/ml}$ (shCtrl) to $6.73 + 1.1 \mu\text{g/ml}$ (shZFX-C1), and $7.03 + 1.12 \mu\text{g/ml}$

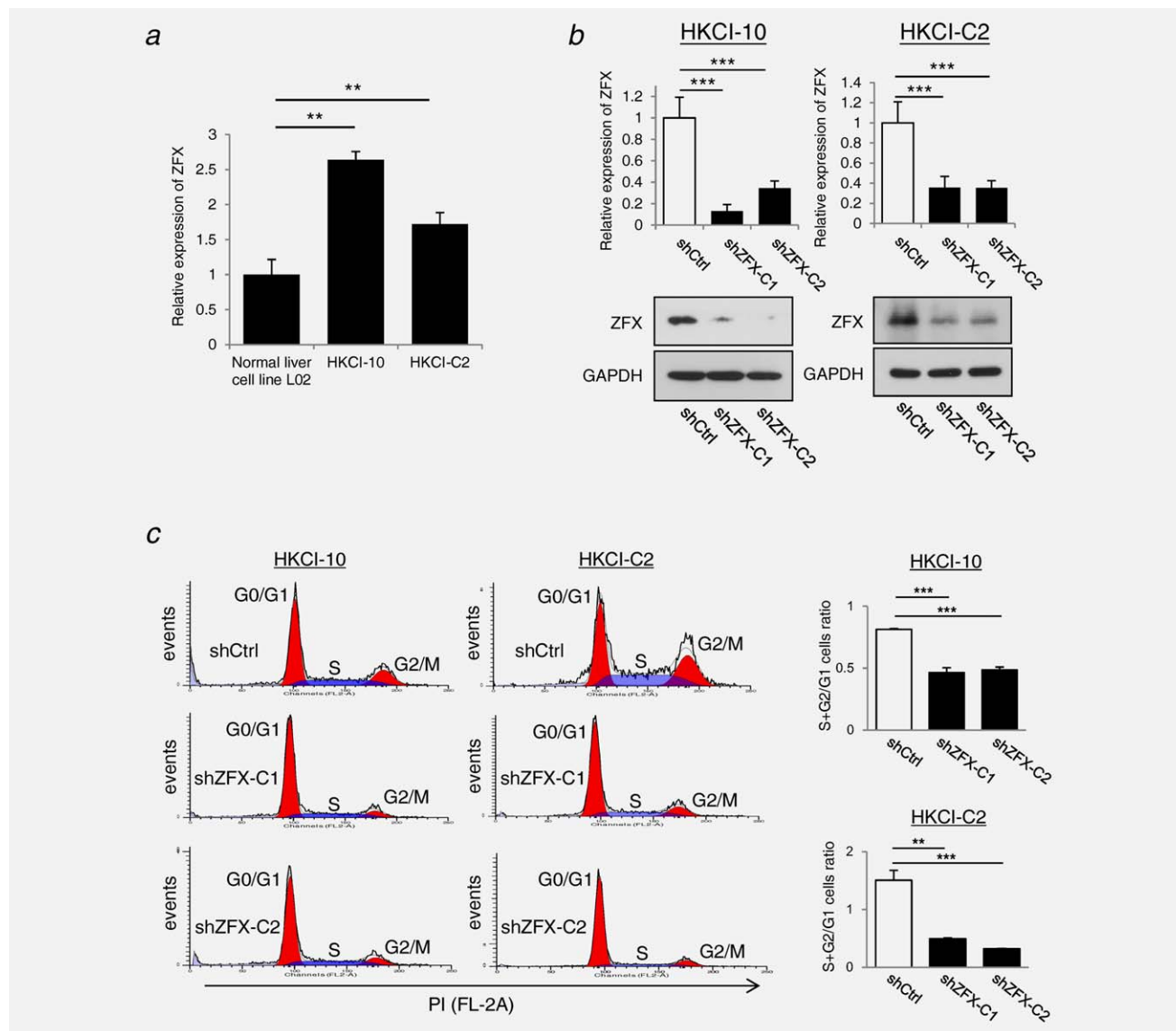


Figure 2. Depletion of ZFX induces G0/G1 cell cycle arrest of HCC cells. (a) HCC cell lines, HKCI-10, and HKCI-C2 showed a high expression of ZFX as compared to normal livers tissues (** $p = 0.0091$). The mRNA expression level of ZFX in normal livers, HKCI-10 and HKCI-C2 was determined by qRT-PCR. (b) Knockdown of ZFX in HCC cell lines. qRT-PCR (upper panel) and western blotting (lower panel) demonstrated the knockdown efficiency of ZFX in ZFX-depleted HKCI-10 (** $p < 0.0001$) and HKCI-C2 (** $p < 0.0001$). (c) Depletion of ZFX induced G0/G1 cell cycle arrest. Propidium iodide (PI) staining demonstrated the induction of G0/G1 cell population but decrease in S and G2/M population (left panel). Right panel showed the reduction of S + G2/G1 ratio after knockdown of ZFX in HKCI-10 (** $p < 0.0001$) and HKCI-C2 (** $p = 0.0016$).

(shZFX-C2) in ZFX-depleted HCC cells ($p < 0.0001$) (Fig. 4b). These findings evidently demonstrated that ZFX confers stem-like properties to HCC cells.

ZFX influences expression of SOX-2 and Nanog in HCC

Transcriptional factors regulatory network including SOX-2, Nanog, OCT-4, and Notch1 have been reported to support and control a set of target genes that play important roles in ESC pluripotency.^{17,18} We then investigated whether ZFX influences the expression of transcriptional factors including SOX-2, OCT4, Nanog, and Notch1. We found a significant

reduction in the mRNA ($p < 0.0001$) and protein expressions of SOX-2 and Nanog (Figs. 5a and 5b) under the depletion of ZFX. However, no apparent change in the expression of OCT-4 and Notch1 was suggested (Supporting Information Fig. 2). In addition, there was significant induction in mRNA of SOX-2 and Nanog after ectopic expression of ZFX in HCC cells ($p < 0.0001$) (Fig. 5c). Moreover, putative ZFX binding sites on SOX2 and Nanog promoters were predicted by JASPAR online database (http://www.jaspar.genereg.net/cgi-bin/jaspar_db.pl) with relative profile score threshold at 85%. Totally six putative sites on SOX2 and three potential

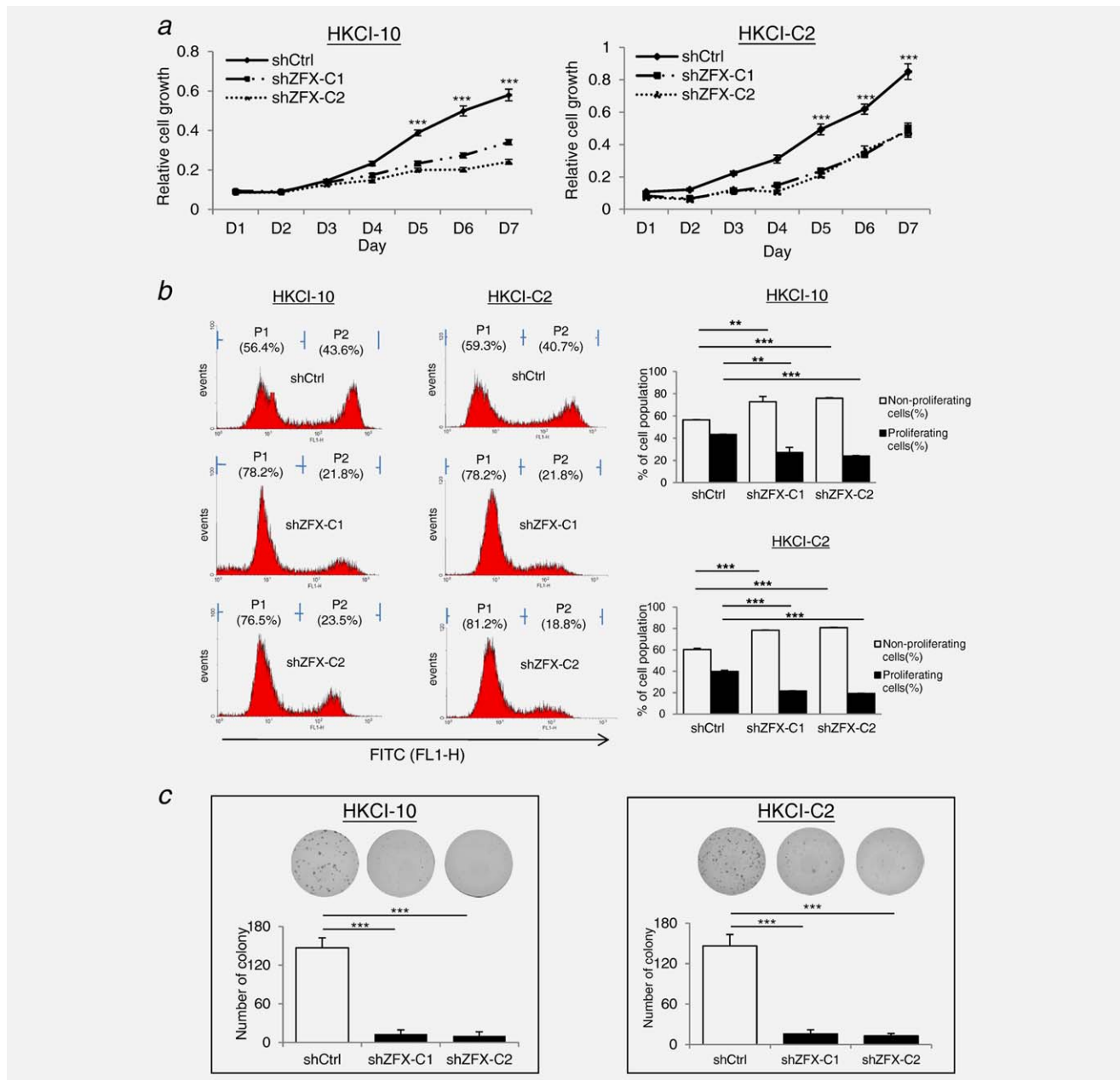


Figure 3. Knockdown of ZFX suppresses *in vitro* proliferation of HCC cells. The cell proliferation of ZFX-depleted cell was measured by (a) MTT assay ($***p < 0.0001$) and (b) EdU cell proliferation assay [left panel showed the population of nonproliferating cell (P1) and proliferating cell (P2). Right panel showed the change in percentage of nonproliferating cell and proliferating cell under ZFX-depletion ($**p = 0.0038$, $***p < 0.0001$)]. (c) Colony formation assay showed the reduction of colony formation ability after knockdown of ZFX ($***p < 0.0001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

sites on Nanog had been identified within their 2k promoter regions (Fig. 5d).¹⁸ To confirm whether ZFX bind to promoter regions of SOX-2 and Nanog, we designed different sets of primers (Supporting Information Table 2) that covered the putative binding sites of ZFX. Using ChIP-PCR, we found that the ZFX-pull down DNA enriched the binding site on the promoter regions of SOX-2 ($p < 0.012$) (Fig. 5e). Although a significant enrichment on the promoter regions of Nanog was not suggested, qPCR analysis demonstrated a

positive correlation between the expression of ZFX and Nanog in the 83 pairs of HCC primary cases ($p = 0.031$) (Fig. 5f). Our data highlights the likelihood of Nanog and SOX-2 as downstream targets of ZFX.

ZFX promotes *in vivo* tumor growth

Finally, the tumorigenicity of overexpressed ZFX was examined using nude mouse model. Our result demonstrated that overexpression of ZFX in HCC cells could promote the

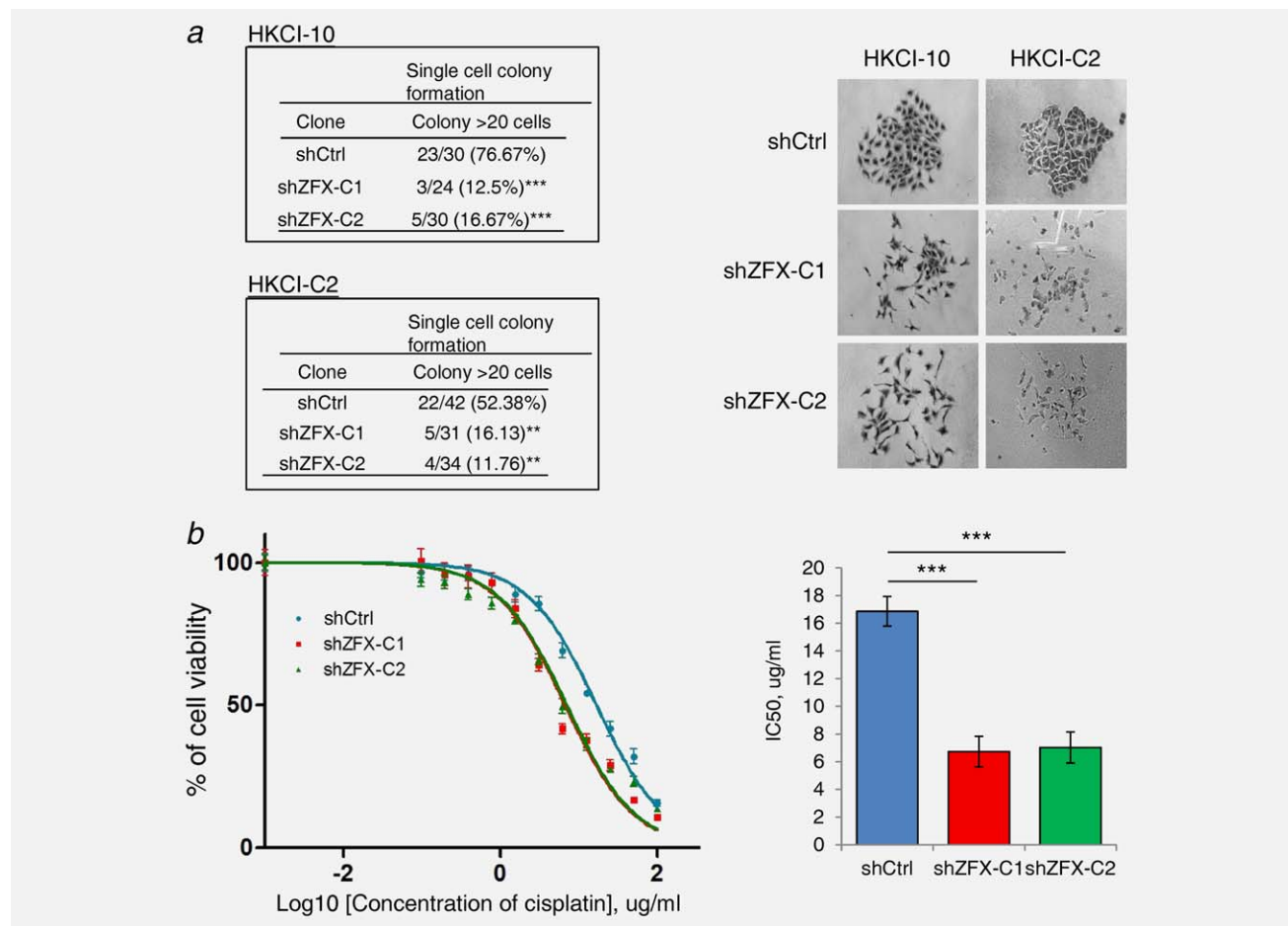


Figure 4. ZFX confers stemness features in HCC cells. (a) Depletion of ZFX reduced the single cell colony formation ability in HCC cells, HKCI-10 ($***p < 0.0001$), and HKCI-C2 ($**p = 0.0022$). Single-cell clonogenic assay (left panel) was used to determine single cell colony formation ability, cell clones reached >20 cells were considered as cell colonies. Representative micrographs (right panel) of HKCI-10 and HKCI-C2 cell colonies in single-cell clonogenic assay. (b) Knockdown of ZFX sensitized HCC to chemotherapeutic drug cisplatin ($***p < 0.0001$). Cell viability of HCC in response to chemotherapeutic drug was determined by MTT assay after treatment of cisplatin (0–100 μM) for 48 h. Left panel was sigmoidal curve showing the percentage of cell viability in response to cisplatin treatment. Right panel showed the deduction of IC₅₀ of cisplatin in ZFX-depleted HCC cells ($***p < 0.0001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

growth of xenografts, suggesting ZFX is required for the *in vivo* growth of HCC tumor ($p = 0.031$) (Fig. 6).

Discussion

Recent studies on ZFX have suggested an important role for this transcription factor in tumorigenesis. Here, we report for the first time the presence of common ZFX up-regulations in HCC and demonstrated the contribution of ZFX in the maintenance of stem-like characteristics of HCC cells. Of interest, we showed a novel association of ZFX overexpression with HCC arising from liver cirrhosis. Currently, about 70–90% of HCC developed from a cirrhotic liver, of which the major etiologic insults included viral hepatitis infections, excessive alcohol consumptions and metabolic syndrome. The finding of elevated ZFX in cirrhosis-associated HCC might imply on its potential contribution in cancer development from the putative premalignant lesion of liver cirrhosis. We also

showed depletion of ZFX could significantly reduce the self-renewal ability of HCC cells. Knockdown of ZFX could also sensitize HCC cells to cytotoxic drug cisplatin, which would in turn suggest ZFX as a potential therapeutic target for more efficacious treatment.

There are growing evidences on different cancer stem cell hypotheses, one of which states that primary tumors are organized in a hierarchy of heterogeneous cell populations and maintained by cancer cells that possess “stem-like” characteristics including unlimited cell division, chemotherapy resistant, and self-renewal in an undifferentiated state.^{19–21} However, this hypothesis of tumor-initiating stem-like cells (TISCs) has much been argued by many scientists who believed that the stem cell-like properties are not limited to a “subpopulation” of cancer cells, and may be a more generalized phenomenon that can be found in the bulk of tumor. In line with the later, our data would infer that stemness

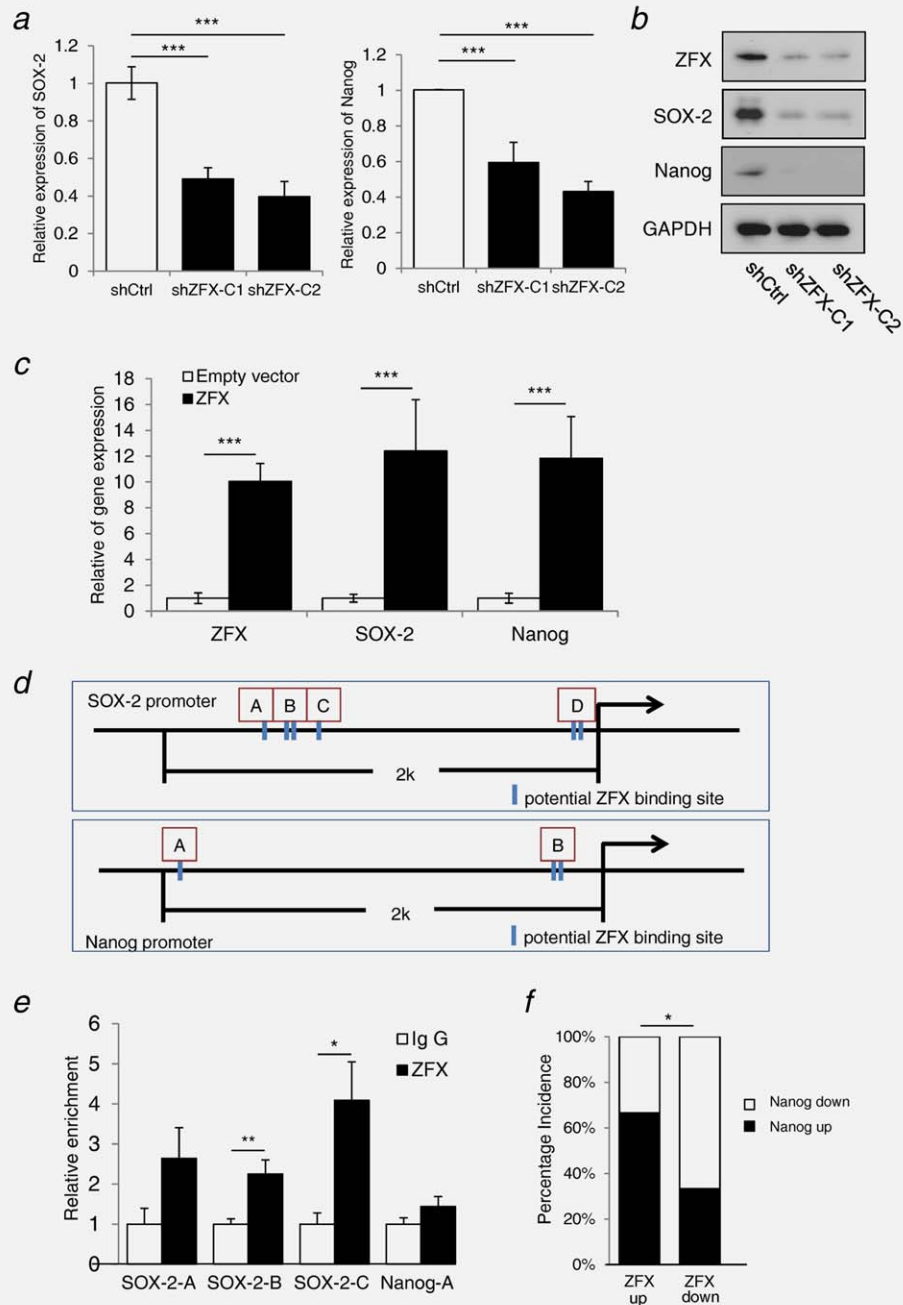


Figure 5. ZFX influences the expressions of transcriptional factors SOX-2 and Nanog in HCC. (a) Decreased in mRNA expressions of SOX-2 and Nanog in ZFX-depleted HCC cells were determined by qRT-PCR ($***p < 0.0001$). (b) The reduction of SOX-2 and Nanog protein expressions level in ZFX-depleted HCC were determined by western blotting. (c) qRT-PCR demonstrated the induction of Nanog and SOX-2 after overexpression of ZFX ($***p < 0.0001$). (d) JASPAR analysis demonstrated six putative ZFX binding sites on SOX-2 promoter and three putative ZFX binding sites on Nanog promoter (highlighted in blue). Red boxes are the primer designed for ChIP-PCR analysis (Supporting Information Table 2). (e) ChIP-PCR analysis of ZFX occupancy in the SOX-2 and Nanog promoters of L02 cells ($*p = 0.012$, $**p = 0.007$). (f) qRT-PCR analysis demonstrated the positive correlation between mRNA expression of ZFX and Nanog in 83 pairs of primary HCC tumoral tissues and adjacent nonmalignant livers ($*p = 0.031$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

features augmented by ZFX can be seen in the major population of HCC cells as suppression of ZFX readily resulted in an extensive reduction in cell growth and self-renewal ability. Moreover, the overexpression of ZFX in HCC could promote

both *in vitro* HCC cell proliferation and *in vivo* growth of xenograft.

In defining the downstream effectors of ZFX in HCC, we explored the influence of ZFX on the expression of stem-like

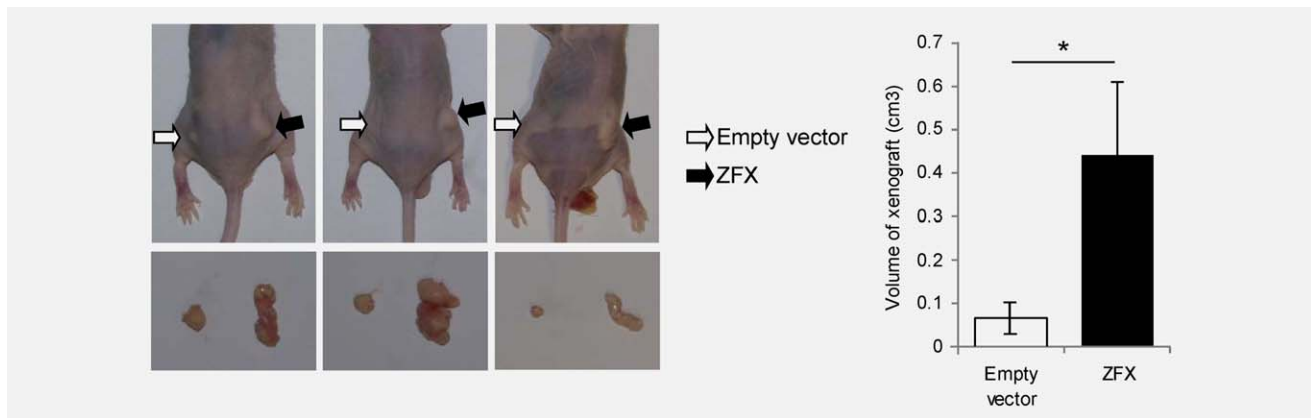


Figure 6. Overexpression of ZFX promote *in vivo* tumor growth. (a) Left panel: three represented nude mice showed the induction of tumor growth by overexpression of ZFX (empty vector control: left side, ZFX: right side). Right panel demonstrated the significant increase in volume of xenograft by ZFX-overexpressed HCC cells ($*p = 0.031$).

promoting genes, including Nanog, Oct-4, Notch1, and SOX-2. Although no apparent changes in the expression of Oct-4 and Notch1 was detected, we found a prominent effect of ZFX on the level of SOX-2 and Nanog expressions. Also, result of ChIP-PCR analysis suggested ZFX would bind on the promoter of SOX-2. Recent studies have indicated that co-expression of SOX-2 and Nanog can reprogram somatic cells into pluripotent embryonic stem-like cells,^{22,23} suggesting that combined expression of stem cell-associated factors in cells with oncogenes could also induce an undifferentiated state in these cells. Furthermore, Nanog and SOX-2 have further been shown to play important roles in the progression of cancers,^{24–26} which might enforce the expression of ZFX,

Nanog and SOX-2 in promoting the tumorigenic potentials of HCC cells. Moreover, it was reported that Nanog expression could significantly increase chemo-resistant oral squamous carcinoma cells and pancreatic cancer cells.^{27,28} Since our data showed that expression of ZFX positively correlated with expression of Nanog, it is plausible that the mechanism underlying drug sensitization in ZFX-depleted HCC cells may be due to the downregulation of Nanog.

In conclusion, this study demonstrates that ZFX is commonly overexpressed in HCC where it confers stemness properties and chemoresistance. Further investigations into the signaling network of ZFX may provide new insights and therapeutic potentials for treatment of HCC.

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