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The ATP-binding cassette transporter ABCF1 is a hepatic oncofetal protein that promotes chemoresistance, EMT and cancer stemness in hepatocellular carcinoma

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ABSTRACT

ATP-binding cassette (ABC) transporters mediate multidrug resistance and cancer stem cell properties in various model systems. Yet, their biological significance in cancers, especially in hepatocellular carcinoma (HCC), remains unclear. In this study, we investigated the function of ABCF1 in HCC and explored its potential as a therapeutic target. ABCF1 was highly expressed in fetal mouse livers but not in normal adult livers. *ABCF1* expression was upregulated in HCCs. These results demonstrate that ABCF1 functions as a hepatic oncofetal protein. We further demonstrated elevated ABCF1 expression in HCC cells upon acquiring chemoresistance. Suppression of ABCF1 by siRNA sensitized both parental cells and chemoresistant cells to chemotherapeutic agents. Reversely, ABCF1 overexpression promoted chemoresistance and drug efflux. In addition, overexpression of ABCF1 enhanced migration, spheroid and colony formation and epithelial-mesenchymal transition (EMT) induction. RNA sequencing analysis revealed EMT inducers HIF1α/IL8 and Sox4 as potential mediators for the oncogenic effect of ABCF1 in HCC progression. Together, this study illustrates that ABCF1 is a novel potential therapeutic target for HCC treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is currently the fourth leading cause of cancer-related death worldwide [1]. HCC is characterized by tumor heterogeneity, poor liver function, advanced disease at diagnosis and chemoresistance. Tumor recurrence and the development of metastasis is often inevitable and eventually results in patient death. Thus, it remains crucial to improve our understanding of the molecular mechanisms that contribute to therapy resistance and tumor recurrence in HCC, to help develop novel therapeutic options.

Multidrug resistance, either intrinsic or acquired, refers to the resistance of cancer cells to a range of anticancer agents that are structurally and mechanistically diverse. ABC transporters regulate the cellular level of various xenobiotics, lipids, ions and other small molecules. There are 49 human ABC genes, which are divided into 7 subfamilies (subfamily A to subfamily G). The upregulation of ABC transporters, including ABCB1 (P-glycoprotein/MDR1), ABCC1 (MRP1) and ABCG2 (BCRP), has been one of the most established mechanisms of multidrug resistance in cancer cells [2]. ABC transporters have also been reported to have a stem cell-related role in cancers. Perturbation of ABCB1, ABCB5 and ABCG2 expressions were shown to modulate stem cell properties such as differentiation, self-renewal and stem cell

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markers expression in non-small cell lung cancer, melanoma and glioma cells [3–5]. Yet, the clinical significance of ABC transporters remains controversial as clinical trials of inhibitors targeting ABC transporter activity to reverse chemoresistance failed to confer clinical benefits that were demonstrated in initial studies [2,6,7]. Nonetheless, the association between ABC transporters and clinical multidrug resistance has been supported in some settings [8,9]. Further investigations on the importance of individual ABC transporters in cancers and their expression in the physiological settings may facilitate the development of more specific ABC transporter-targeted chemosensitizers [10].

The aim of the current study is to explore novel ABC transporters that play a role in HCC chemoresistance. ABCF1 gene is shown to be highly active in developing mouse embryos and essential for embryonic development [11]. It is involved in regulating protein translation initiation [12–14] and innate immune responses against cytosolic DNA and retroviral infection [15]. Unlike most ABC proteins that are encoded as a functional full or half transporter, members of the ABCE and ABCF protein subfamilies are composed of 2 nucleotide binding domains (NBDs) and have no transmembrane domain. They are therefore, predicted to differ in functions to most ABC transporters [10]. Little is known on the functional significance of ABCF1 in cancer, particularly in HCC. In this study, we showed the upregulation of ABCF1 expression in drug-selected chemoresistant HCC cells and illustrated its role in promoting chemoresistance, migration, epithelial-mesenchymal transition (EMT) and cancer stemness in HCC cells.

2. Materials and methods

2.1. Patient samples, animal specimens and cell culture

Patient samples, animal specimens and cell culture details were provided in Supplementary Materials and Methods. The study was approved by ethics committee with standard care in accordance with institution guidelines.

2.2. Gene/protein expression analysis

Immunofluorescence and confocal microscopy, flow cytometry and gene/protein expression analyses, as well as subcellular fractionation and cell surface protein isolation analyses were specified in Supplementary Materials and Methods.

2.3. Functional assays and statistical analysis

Apoptosis assays, doxorubicin exclusion assays, wound healing assays and transwell migration assays, as well as colony/spheroid formation assays and statistical analysis were detailed in Supplementary Materials and Methods.

3. Results

3.1. ABCF1 is a hepatic oncofetal protein with a potential role in mediating HCC chemoresistance

ABCF1 promoter was reported to be highly active during embryogenesis and is essential for mouse embryonic development [11]. To understand the significance of ABCF1 in liver development, the expression profile of ABCF1 in mouse liver during development was studied. Mouse livers were collected from prenatal (embryonic day (E) 13.5, E15.5 and E17.5), neonatal (postnatal day (D) 1, D3 and D7) and adult ICR mice. *ABCF1* mRNA was expressed at a higher level in early developing livers (E13.5: 2.91 ± 0.48 fold; E15.5: 2.07 ± 0.52 fold) and gradually decreased to minimal expression in adult livers (Fig. 1A). Similarly, the well-established hepatic oncofetal marker *AFP* was strongly expressed during liver development but not in adult liver tissues. Robust protein expression of ABCF1 was also detected in mouse fetal livers (Fig. 1B and C). IHC staining detected predominant expression of ABCF1 at the cytoplasm of most cells in fetal livers (E13.5, E15.5 and E17.5). As reflected by parallel AFP staining, ABCF1-expressing cells during early liver development belong to the hepatic lineage (Fig. 1C). The intensity of ABCF1 expression dropped significantly in neonatal livers and was largely undetectable in the parenchyma of adult liver tissues. The results underline the significance of ABCF1 in liver development. Next, we demonstrated *ABCF1* expression in normal hepatocytes MIHA and in all 7 human liver cancer cell lines examined. In particular, *ABCF1* in the Hep3B cell line was 6-fold higher than that in MIHA (Fig. 2A). Hep3B-derived 5FU-resistant cells (Hep3B-5FUR), doxorubicin-resistant cells (Hep3B-DoxoR) and cisplatin-resistant cells (Hep3B-CisR), which have been described previously [16], were selected respectively by 5-fluorouracil, doxorubicin and cisplatin. All 3 chemoresistant populations showed enhanced expression of *ABCF1* compared to their parental cells (Fig. 2B). The expression of ABCF1 in HCC cell lines and its elevated expression in Hep3B-5FUR, Hep3B-DoxoR and Hep3B-CisR cells was confirmed at protein level (Fig. 2A–B). *ABCF1* level was evaluated in 10 pairs of HCC and adjacent non-tumor liver tissues and 8 normal liver tissues. Higher *ABCF1* transcript levels were observed in tumor compared to non-tumor and normal liver tissues ($P = 0.008$) (Fig. 2C). Subsequent analysis of *ABCF1* expression in HCC tissue and normal tissue samples from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) datasets using the GEPIA platform [17] also showed that ABCF1 was upregulated in HCC tissues ($P < 0.01$) (Supplementary Fig. 1). Kaplan-Meier survival analysis of the Cancer Genome Atlas (TCGA) dataset by the Human Protein Atlas (HPA) platform [18] show that HCC patients with high expression of ABCF1 had poorer overall survival (log rank test, $P < 0.041$). Due to the scarcity of human fetal liver specimen, the anatomical and physiological similarity between human and mouse livers, the expression of ABCF1 in liver development was focused in mouse. Nonetheless, our results that demonstrated low expression of ABCF1 in adult mouse livers is consistent with the results observed in normal human liver tissues and non-tumor liver tissues (Fig. 2C and Supplementary Figs. 1A and B). Together, these results suggest that ABCF1 is a hepatic oncofetal protein with a potential role in HCC chemoresistance.

3.2. ABCF1 expression confers chemoresistance in HCCs in vitro and in vivo

Since ABCF1 expression is enhanced in the chemoresistant populations of HCC cells, we determined the role of ABCF1 in HCC chemoresistance by suppressing the expression of ABCF1. Hep3B and Hep3B-5FUR cells were transfected with 3 independent *ABCF1*-targeted siRNAs. All 3 siRNAs significantly suppressed *ABCF1* mRNA levels (Fig. 3A). Apoptosis was assessed by Annexin V/7-AAD staining. Decrease in *ABCF1* level increased 5FU-induced apoptosis in both Hep3B cells (4.7% ± 1.5%–11.7% ± 1.5% net increase in apoptotic populations) and Hep3B-5FUR cells (0.4% ± 0.6%–22.2% ± 4.9%) (Fig. 3B). It is noted that a more efficient siRNA knockdown (siABCF1#3) resulted in higher apoptosis upon 5FU treatment.

Next, we studied the correlation between ABCF1 level and resistance of HCC cells to other common chemotherapeutic agents used against HCCs. Hep3B and HepG2 cell lines with stable ABCF1 overexpression were established using retroviral-based vectors. Overexpression of ABCF1 in Hep3B-ABCF1 and HepG2-ABCF1 cells compared to empty vector controls were confirmed using real-time quantitative PCR, Western blot and flow cytometry (Fig. 3C and Supplementary Fig. 2). ABCF1 overexpression resulted in reduction of cisplatin-induced apoptosis in Hep3B-ABCF1 (18.1% ± 8.2% –10.3% ± 4.8%; $P = 0.022$) and HepG2-ABCF1 cells (21.2% ± 11.0%–7.9% ± 11.4%; $P = 0.004$) compared to their respective vector controls (Fig. 3D). Similarly, a significant decrease in apoptosis was observed in Hep3B-ABCF1 (34.4% ± 10.0%–9.4% ± 5.9%; $P = 0.020$) and HepG2-ABCF1 cells (23.0% ± 1.1%

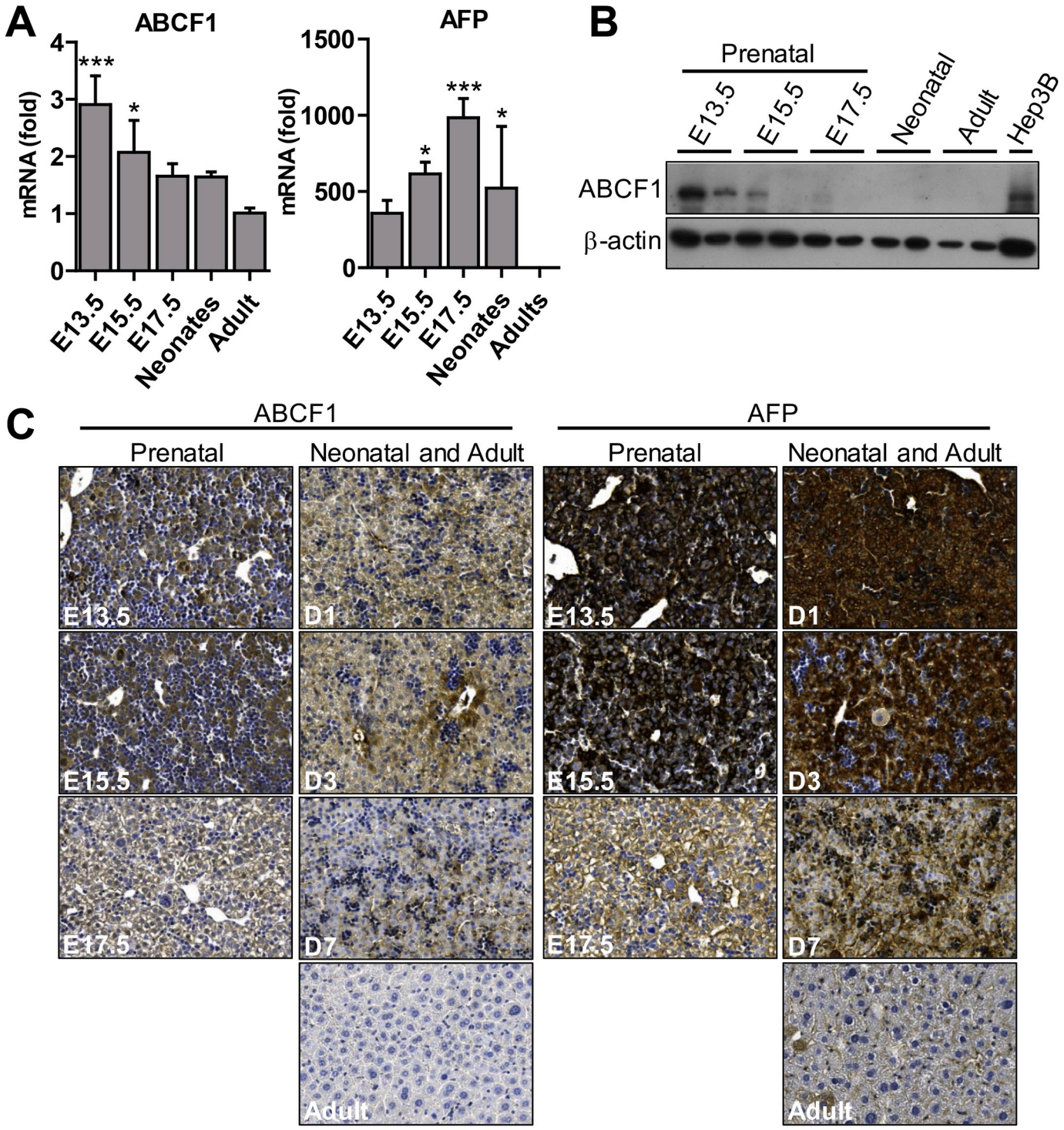

Fig. 1. Expression of ABCF1 in fetal liver development. (A) RT-qPCR analysis of *ABCF1* and *AFP* expression in developing mice livers collected at prenatal (E13.5, E15.5 and E17.5), neonatal (D1, D3 and D7) and adult stage. Data represent fold change relative to the expression of mRNA in adult mouse liver (mean ± SD). 18s rRNA was used as the reference for normalization. (B) Representative images of ABCF1 protein expression in developing and adult mouse liver (n ≥ 5). β-Actin was used as a normalization control. (C) Immunohistochemical analysis of ABCF1 and AFP protein in developing mouse livers throughout development. Representative images (100x) at each stage was shown (n ≥ 3).

–13.4% ± 2.1%; $P = 0.028$) when treated with doxorubicin. The results suggest that ABCF1 regulates chemoresponse in HCC cells. The effect of ABCF1 expression on HCC chemoresponse *in vivo* was further examined. Hep3B-ABCF1 or Hep3B-Vector cells were inoculated subcutaneously in the right dorsal flank of mice (3×10^6 cells/mouse). When tumors reached approximately 0.3 cm^3 in volume, mice were randomized (n ≥ 4 per group) for treatment with saline or cisplatin (3.75 mg/kg/week) for 3 weeks. Hep3B-ABCF1 xenografts had overexpression of ABCF1 compared to Hep3B control xenografts (Supplementary Fig. 3) corroborating their *in vitro* counterparts. Hep3B-ABCF1 xenografts were significantly larger than the vector control group when treated with cisplatin; similar difference was observed in the saline-treated group though the difference had not reached statistical significance (Fig. 3E). Hep3B-ABCF1 xenografts showed increased

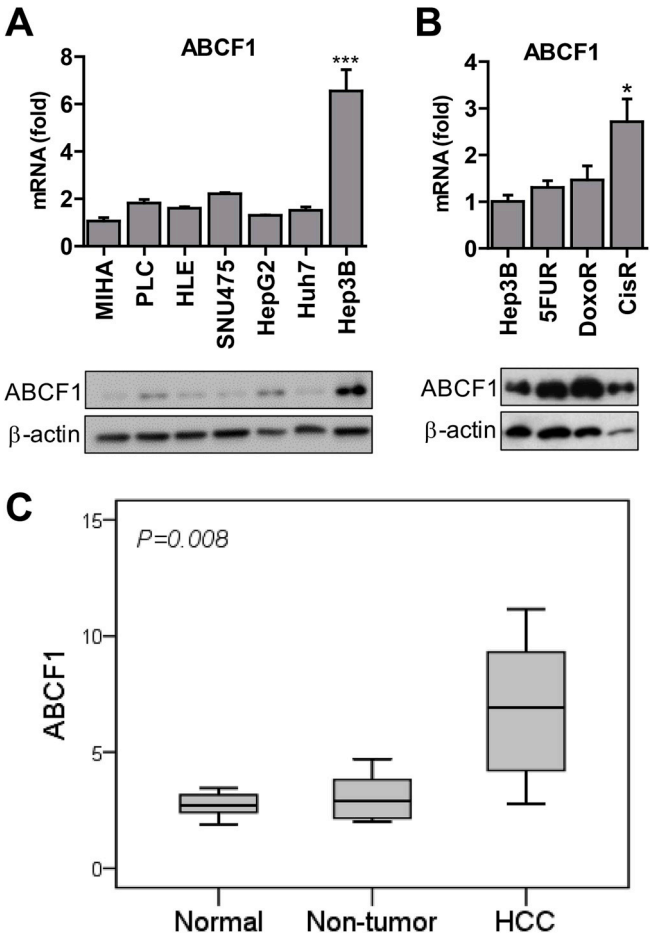

Fig. 2. Elevated expression of ABCF1 in primary HCC and chemoresistant populations of HCC cells. (A–B) mRNA and protein expression of ABCF1 in normal liver cell line and established human liver cancer cell lines and in the chemoresistant Hep3B cell lines selected against 5- fluorouracil (5FUR), doxorubicin (DoxoR) and cisplatin (CisR). RT-qPCR data are presented as fold difference (mean ± SD) relative to MIHA non-tumor hepatocyte cell line (A) and parental Hep3B (B), respectively. 18s rRNA was used for normalization. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when compared to control. (C) *ABCF1* mRNA is significantly upregulated in HCC compared with paralleled adjacent non-tumor liver tissues (n = 10) and normal liver tissues (n = 8) ($P = 0.008$, one-way analysis of variance).

proliferating cells as revealed by Ki-67 immunohistochemistry staining after treatment with cisplatin (Supplementary Fig. 3). Nonetheless, cisplatin inhibited tumor growth (Fig. 3E) but did not showed significant effect on apoptosis as demonstrated by cleaved-caspase-3 immunohistochemistry (Supplementary Fig. 3). The results suggest that ABCF1 expression rendered xenografts more resistant to cisplatin. In summary, suppression of ABCF1 level sensitized HCC cells and their resistant populations to chemotherapeutic agents with enhanced apoptosis whereas overexpression of ABCF1 increased chemoresistance in HCC *in vitro* and *in vivo*.

3.3. ABCF1 enhances drug efflux in HCC cells with elevated cell surface ABCF1 expression

High expression of multiple ABC transporters has been known to promote drug efflux and hence multidrug resistance in cancer cells. In line with its lack of a transmembrane domain, ABCF1 has been reported to be localized in the cytoplasm and nucleus and possess functions distinct from most ABC transporters. Given the functional redundancy of the highly conserved ABC transporter family, we investigated the effect of ABCF1 overexpression on the drug efflux ability of HCC cells. Doxorubicin accumulation was determined as the DOX fluorescent index. ABCF1 overexpression significantly impeded doxorubicin accumulation in Hep3B (12.1 ± 1.1 to 4.9 ± 1.8; $P = 0.003$) and HepG2 (8.2 ± 3.1 to 4.7 ± 0.9; $P = 0.042$) cells compared to their respective vector controls, revealing the regulatory function of ABCF1 on drug efflux in HCC cells (Fig. 4A). The subcellular localization of ABCF1 was then studied by analyzing the subcellular fractions of HCC cells Hep3B, HepG2 and Huh7 with immunoblotting (Fig. 4B). ABCF1 was detected in both cytoplasmic and nuclear fractions of HCC cells, which is consistent with the confocal images of GFP-tagged ABCF1 in Hep3B and HepG2 cells (Supplementary Fig. 4B). Notably, a portion of ABCF1 proteins was expressed in the membrane fractions. Furthermore, cell surface protein isolation by extracellular biotinylation of Hep3B cells demonstrated the membrane-associated localization of ABCF1 specifically at the cell surface membrane (Fig. 4C). To further examine whether the membrane-associated localization of ABCF1 has a role in supporting drug transport, we compared the cell surface expression of ABCF1 in the Hep3B-ABCF1 and HepG2-ABCF1 cells to their vector controls. FACS analysis showed that surface ABCF1 level is 3-fold higher in Hep3B-ABCF1 cells (16.7% ± 3.2%–51.9% ± 5.8% surface ABCF1 expressing cells; $P = 0.002$) and 2-fold higher in HepG2-ABCF1 cells (15.1% ± 8.7%–36.4% ± 3.9%; $P = 0.002$) (Fig. 4D). Confocal microscopy validated the increased localization of ABCF1 at the cell surface membrane of a subset of Hep3B-ABCF1 and HepG2-ABCF1 cells (Fig. 4E). These results suggest that ABCF1 is a novel drug transporter that facilitates drug efflux and hence, multidrug resistance in HCC cells.

3.4. ABCF1 promotes cell migration and stemness properties in HCC cells

Previous studies have reported the correlation between *ABCF1* expression and the metastatic potential of soft tissue tumors [19]. We therefore investigated the effect of ABCF1 expression on the migratory ability of Hep3B and HepG2 cells by wound healing assay and transwell migration assay respectively. Overexpression of ABCF1 significantly stimulated cell migration in Hep3B cells while HepG2 cells also exhibited an increase in the number of migrating cells compared with the vector controls (Fig. 5A and B). Western blot analysis of EMT marker expression demonstrated that the ABCF1-induced migratory capacity is concurrent with increased N-cadherin expression in Hep3B and HepG2 cells. In addition, vimentin expression is elevated in Hep3B-ABCF1 cells but not expressed in HepG2 cells (Fig. 5C). Cancer stem cells contribute to treatment failure and tumor recurrence. Since ABCF1 expression is highly associated with early liver development, we investigated whether ABCF1 plays a pathological role in promoting stem cell-like properties in HCCs, by assessing tumorigenic potential and self-renewal ability. Overexpression of ABCF1 significantly increased the number of colonies formed by Hep3B (256 ± 20 to 308 ± 9 colonies, $P = 0.035$) and HepG2 (161 ± 52 to 223 ± 40, $P = 0.041$) cells in culture medium containing 1% FBS; the potentiated colony forming ability was also observed in Hep3B (22 ± 13 to 240 ± 21, $P < 0.001$) and HepG2 cells (137 ± 13 to 183 ± 22, $P = 0.024$) when treated with doxorubicin (Fig. 6A). Spheroid formation assay revealed that Hep3B-ABCF1 ($P < 0.001$) and HepG2-ABCF1 ($P = 0.023$) cells had higher spheroid formation capacity compared to their respective vector controls (Fig. 6B). Consistently, elevated expression of mesenchymal markers *VIM* (vimentin), *ITGA5* (integrin subunit alpha 5) and cancer stem cell markers *EPCAM* and *SOX2* was observed in Hep3B-ABCF1 xenografts after treatment with cisplatin. While it has been reported that epithelial phenotype confers higher tumorigenic capacity than mesenchymal phenotype [20], Hep3B-ABCF1 xenografts in the saline-treated group only showed upregulation of *ITGA5* and no changes in the expression of the stem cell markers were observed in the ABCF1 overexpressing cells *in vitro* (Supplementary Fig. 5). Here, the results demonstrate the plasticity of Hep3B-ABCF1 cells to adopt epithelial states for xenograft formation and to acquire mesenchymal phenotype and

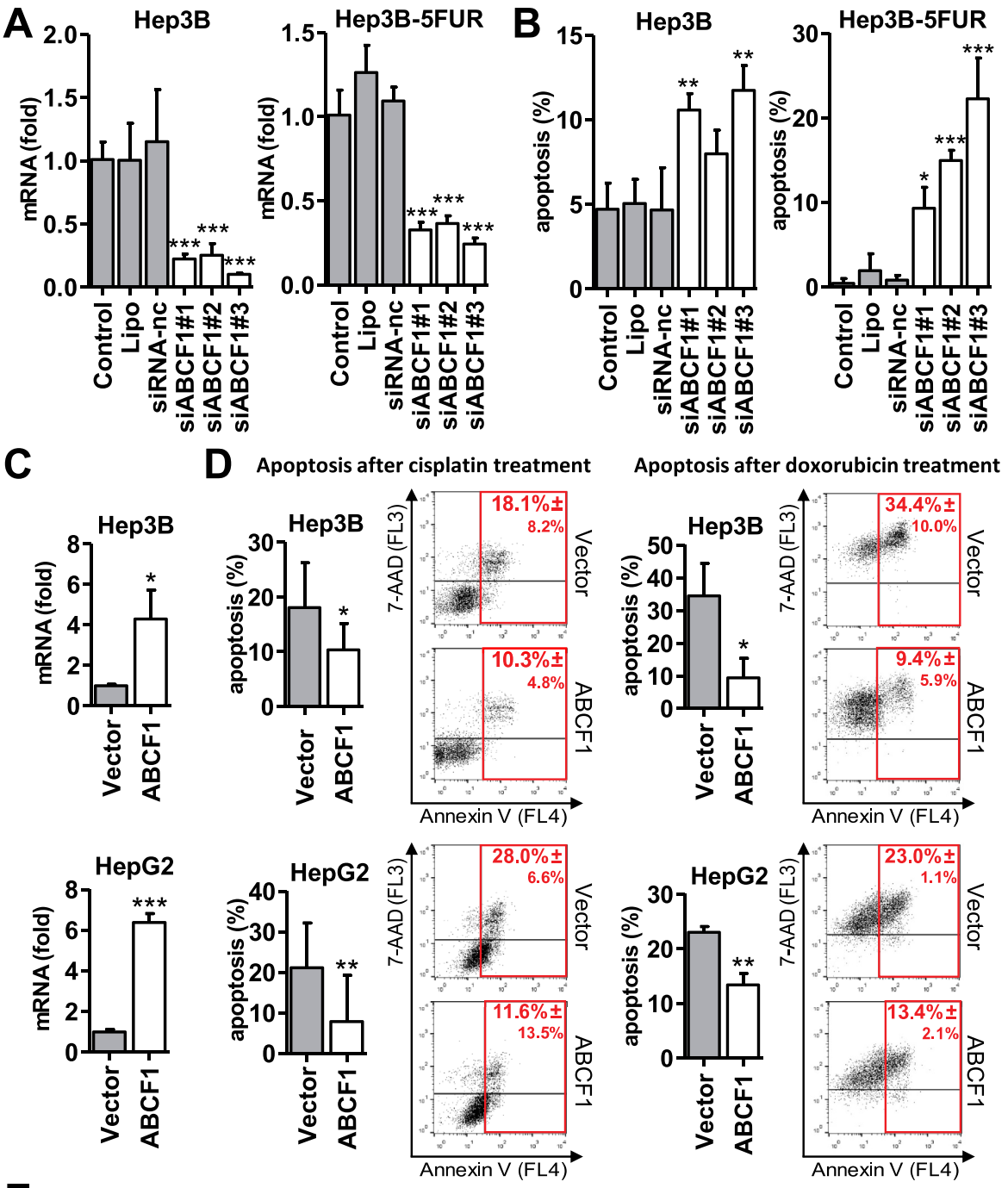

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Fig. 3. ABCF1 expression modulates chemoresponse in HCC cells. (A–B) Efficient suppression of*ABCF1* level by siRNA enhanced apoptosis induced by chemotherapeutic agent 5FU (60 μg/ml). (C–D) Stable overexpression of *ABCF1* in HCC cells reduced sensitivity to cisplatin (10 μg/ml) and doxorubicin (Hep3B: 2 μg/ml; HepG2: 1 μg/ml). Untreated control (Control), lipofectamine control (lipo) and scrambled negative control (siRNA-nc) (A–B) or empty vector control (Vector) (C–D) were included as controls. Mean ± SD of results from representative experiment was shown. (E) Tumor growth curve of mice treated with saline or cisplatin for 3 weeks are summarized (n ≥ 4 per group). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when compared to siRNA-nc control or empty vector control.

stemness properties for chemoresistance when challenged by chemotherapeutic drug. These data suggest that ABCF1 expression promote stemness and migration by modulating EMT activation in HCC cells.

3.5. Gene differential expression analysis of ABCF1-overexpressing HCC cells

To further assess the biological significance of ABCF1 and understand the molecular mechanisms underlying the oncogenic role of ABCF1, we studied the global gene expression changes between Hep3B-ABCF1, HepG2-ABCF1 cells and their vector controls by RNA-Seq. With a cut-off of FPKM ≥20 and Max (FPKM) - Min (FPKM) ≥ 20, 158 transcripts were identified to be differentially regulated by ABCF1 overexpression in both Hep3B and HepG2 cells in a consistent manner; among which, 19 genes were downregulated and 139 genes, including *ABCF1*, were upregulated. Hierarchical clustering showed the differential expression pattern of the identified transcripts parallel to that of *ABCF1* (Fig. 7A and Supplementary Fig. 6). Using GO terms functional classification and overrepresentation analysis, cancer-associated biological functions such as developmental process, angiogenesis, response to drug and stimulus and regulation of cell proliferation were highlighted as predominant processes deregulated by ABCF1 (Fig. 7B and C, Supplementary Table 3). Notably, expression of EMT inducers hypoxia-inducible factor 1 alpha subunit (*HIF1A*), interleukin 8 (*IL8*) and SRY-box 4 (*SOX4*), the mesenchymal marker N-cadherin (*CDH2*), *VIM* and *ITGA5* and the HCC cancer stem cell (CSC) marker *ALDH1A1* were elevated by ABCF1 overexpression. The increased expression of these genes was validated by RT-qPCR (Fig. 7D). Upregulation of ALDH1A1 was validated at protein level (Fig. 7E). These data support the association of ABCF1 with significant biological processes that contribute to cancer stemness, metastasis and drug resistance in HCC.

4. Discussion

The chemoresistant nature of HCC cells impedes treatment response and efficient cancer elimination. ABC transporters such as ABCB1 and ABCG2 (BCRP) mediate the efflux of both conventional chemotherapeutics and novel targeted therapies such as sorafenib [21,22]. The clinical failures of ABC transporter inhibitors present the need to determine which ABC transporters contribute to chemoresistance in different clinical settings. For the first time, we demonstrated that ABCF1 is a hepatic oncofetal protein and its expression modulates the chemoresistant phenotype of HCC cells. Particularly in the context of HCC, the importance of conventional ABC transporters has been controversial. ABCB1 expression was shown to correlate with reduced overall survival of HCC patients after surgical resection whereas another study observed no association between ABCB1 expression and tumor aggressiveness or patient survival [23,24]. On the other hand, ABCC1 (MRP1) was detected at low level or absent in HCC samples with intracellular localization in HCC cells, suggesting that ABCC1 might not contribute to the MDR phenotype of HCC [25]. In addition, protein expression of 14 ABC transporters, including ABCB1, ABCC1 and ABCG2 (BCRP) have been reported in normal adult human liver [26]. Therefore, our data that demonstrated the oncofetal expression of ABCF1 in liver tissues and the association between ABCF1 and HCC chemoresistance would suggest that ABCF1 is a more clinically relevant ABC transporter in HCC and a promising target to restore HCC chemosensitivity. It should also be noted that the transcription levels of ABCF1 did not correlate with the protein levels of ABCF1 in all the HCC cell lines examined, suggesting post-transcriptional regulation such as microRNA regulation of ABCF1 expression. The results might also correlate, at least partially, with the varying results on the clinical significance of ABC transporters in HCC in different studies [23,24]. These results highlight the importance to study the protein expression of ABC transporters to understand their clinical significance. By examining the Human Protein Atlas database [27,28], we confirmed that ABCF1 protein was detected at moderate to high level in 55% (n = 11) of HCC tissues and at low level in normal liver tissues (n = 11) (Supplementary Fig. 1B).

To date, the physiological or pathological significance of ABCF1 remains poorly understood. Interestingly, contrary to protein motif analysis and previous studies [13,29], our experimental data demonstrated a surface membrane-associated expression of ABCF1 and its regulatory function on drug efflux. Among the genes that were identified to be deregulated by ABCF1, significant upregulation of other ABC transporters was not observed. Considering the functional redundancy of the ABC transporter family, our results put forward the putative role of the membrane-tethered ABCF1 proteins in directly mediating drug transport. Though it is likely that ABCF1 induces chemoresistance only partly through drug efflux, given the low abundance of ABCF1 in the membrane fraction. The clinical significance of such localization should be investigated in forth coming studies. As ABCF1 lacks a transmembrane domain but is still involved in driving drug efflux, the potential of ABCF1 on forming heterodimers with other ABC proteins should be explored. Alternatively, it is found that the protein sequence of ABCF1 has a high lysine proportion (11.52%) (Supplementary Table 4). Basic residues such as lysine and arginine in proteins are important in mediating interactions with heparan sulfate (HS). Therefore, ABCF1 might localize to the cell surface membrane through HS binding. Moreover, ALDH1A1, which has been implicated in chemoresistance [27,30], was upregulated in ABCF1 overexpressing cells. Future study will be warranted to elucidate the mechanism of the cell surface localization of ABCF1 and ABCF1 mediated multidrug resistance in HCC cells.

In addition to the functional role on chemoresistance, we showed that ABCF1 overexpression enhances chemoresistance, migration and stemness properties in HCC cells. The current data demonstrates that ABCF1 mediates drug efflux in HCC cells but also plays a role in promoting HCC progression, potentially through modulating EMT via the HIF1A/IL8/Sox4 pathway. The EMT program is an integral developmental program which allows epithelial cells to resemble a mesenchymal state and acquire aggressive traits such as cell motility, invasiveness and chemoresistance [31]. In HCC, EMT-related gene expression predicts aggressive local recurrence and tumors with epithelial phenotype were more responsive to sorafenib [32,33]. In the current study, ABCF1 overexpression concurrently promoted chemoresistance, migration and a shift towards the mesenchymal phenotype *in vitro*; ABCF1 overexpressing cells also showed enhanced phenotypic plasticity *in vivo*. Our findings suggest that ABCF1 plays a role in modulating EMT activation, which may facilitate the acquisition of chemoresistance and migratory potential in HCC cells. Given its hepatic oncofetal expression, the physiological role of ABCF1 during liver development might involve in regulating EMT. RNA-Seq results showed

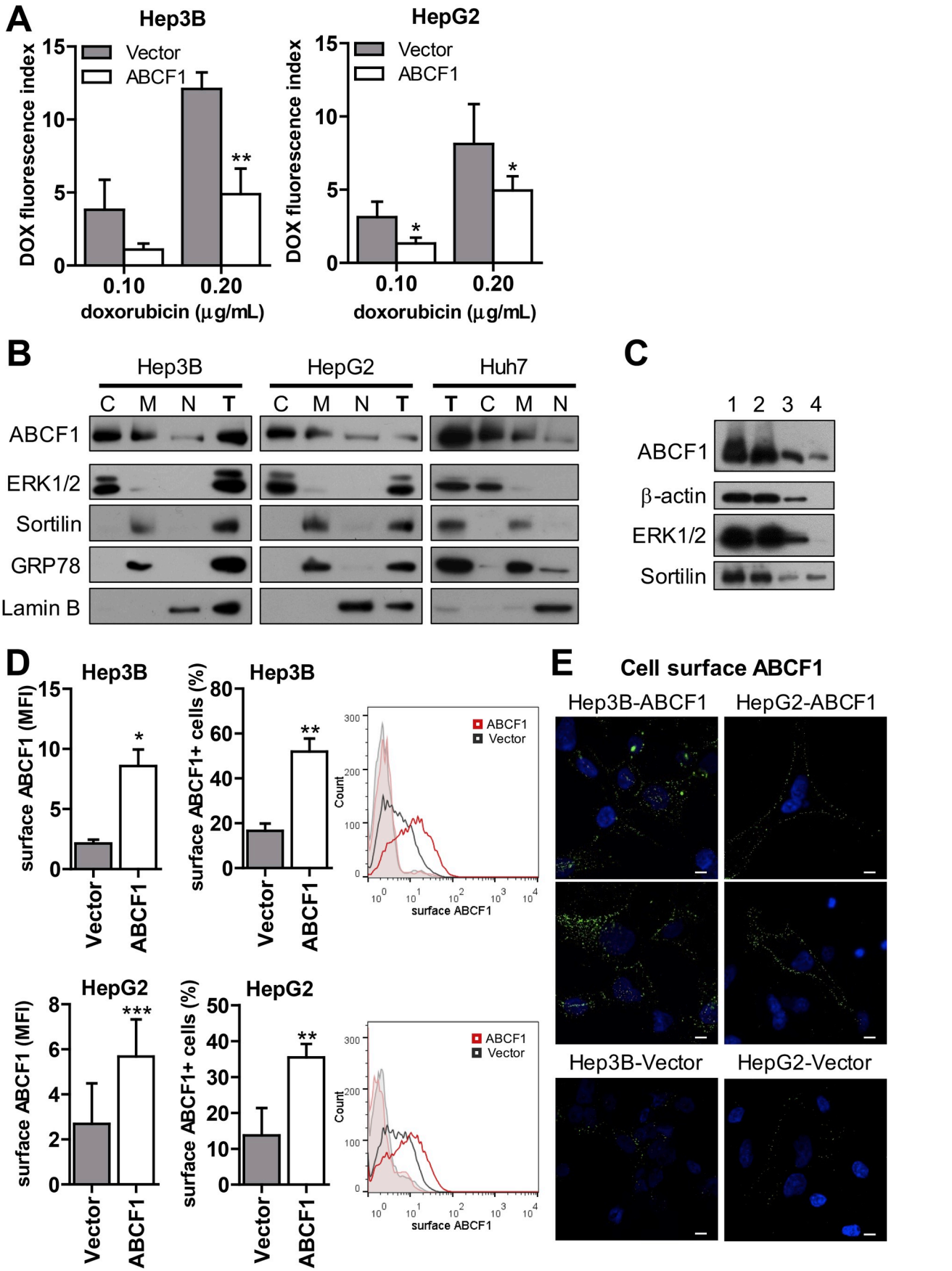

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Fig. 4. ABCF1 expression enhances drug transport and membrane-associated ABCF1 protein. (A) Intracellular doxorubicin accumulation after 4 h of incubation with doxorubicin (0.1 μg/ml or 0.2 μg/ml) was determined. DOX fluorescence index is calculated by mean fluorescence intensity x percentage of doxorubicin positive cells. Mean ± SD of results was shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when compared to vector control. (B) Western blot analysis of subcellular fractions of Hep3B, HepG2 and Huh7 cells. Cytoplasmic [C], membrane [M] and nuclei [N] fractions were loaded and compared with the total [T] lysate. ABCF1 protein was detectable in the membrane fractions of HCC cells. ERK1/2, cytosolic marker; sortilin and GRP78, membrane marker; Lamin B, nuclear marker. (C) Isolation of biotinylated cell surface proteins demonstrated the cell-surface localization of ABCF1 in Hep3B cells. Sortilin and ERK1/2 and β-actin are positive and negative control, respectively, of cell surface protein isolation. 1, before loading to avidin column; 2, flow through from avidin column; 3, wash from the column; 4, elution of the biotinylated cell surface proteins. (D) Flow cytometric analysis showed elevated cell surface expression of ABCF1 in Hep3B-ABCF1 and HepG2-ABCF1 cells. Representative histograms were shown. Red, ABCF1-overexpressing cells; grey, vector controls; tinted area, corresponding signal of isotype control. (E) Confocal microscopy demonstrated cell surface localization of ABCF1 in Hep3B and HepG2 cells with stable ABCF1 overexpression. Cell surface expression were visualized by confocal microscopy at 630x magnification with anti-ABCF1 antibody in non-permeabilized conditions. ABCF1, green; Hoechst 33342, blue. Scale bars, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

upregulation of known EMT inducers *SOX4* and *HIF1A* and the down-stream targets of HIF1α, *IL8*, *TIMP2* and *COL1A1* in ABCF1-over-expressing HCC cells [34–38], highlighting HIF1α and Sox4 as potential mediators of ABCF1-modulated EMT. Further studies would be required to understand how ABCF1 augment the expression of these genes. A recent study introduced the role of ABCF1 in facilitating a third mode of translation. It is believed that m^{6}A-dependent translation permits the translation of specific mRNAs required for cell maintenance and cell survival during stress [39]. Meanwhile, the RNA-Seq results showed an overrepresentation of genes associated with cell response to stress with ABCF1 upregulation. Thus, regulation of translation upon stress might be a mechanism via which ABCF1 modulates chemoresistance, migration and EMT in HCC cells. On the other hand, it has been shown that ABCF1 promote the transition of macrophage TLR4 signaling to the anti-inflammatory TRIF-dependent pathway as an E2 ubiquitin-conjugating enzyme [40]. Induction of TLR4 signaling has been shown to enhance HCC cells proliferation, chemoresistance, migration and invasion and EMT [41–44]. As HIF1A and IL8 are down-stream effectors of the TLR4 signaling pathway [45], ABCF1 might also promote HCC chemoresistance, migration and EMT by modulating TLR4 signaling. It would be interesting to examine the role of ABCF1 in modulating m^{6}A-mediated translation or TLR4 signaling in HCC cells and the functional significance of these modulations. In summary, our findings showed that ABCF1 is a hepatic oncofetal protein with potential to serve as

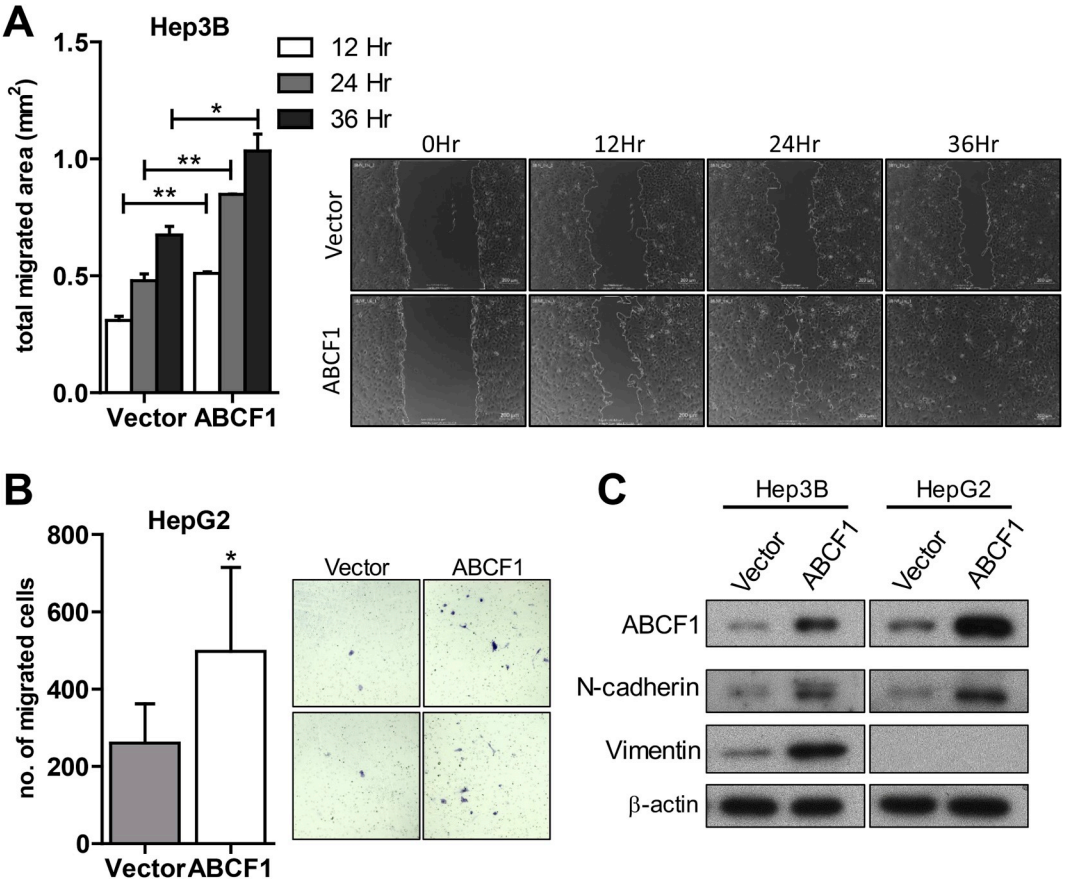

Fig. 5. ABCF1 promotes migration and EMT activation. (A) Wound healing assay in Hep3B cells was assessed by time-lapse imaging. ABCF1 expression augmented cell migration across the scratch. Total area of cell migration across the scratch (mm^2) after 12 h, 24 h and 36 h were determined (mean ± SD). Representative images at the corresponding time point were shown. (B) Transwell migration assay of HepG2-ABCF1 cells. Total number of migrated cells per well (mean ± SD) was plotted. Representative images of the randomly selected fields were shown. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ when compared to empty vector control (Vector). (C) Western blot analysis of HCC cells with ABCF1 overexpression for the ABCF1 and EMT markers expression.

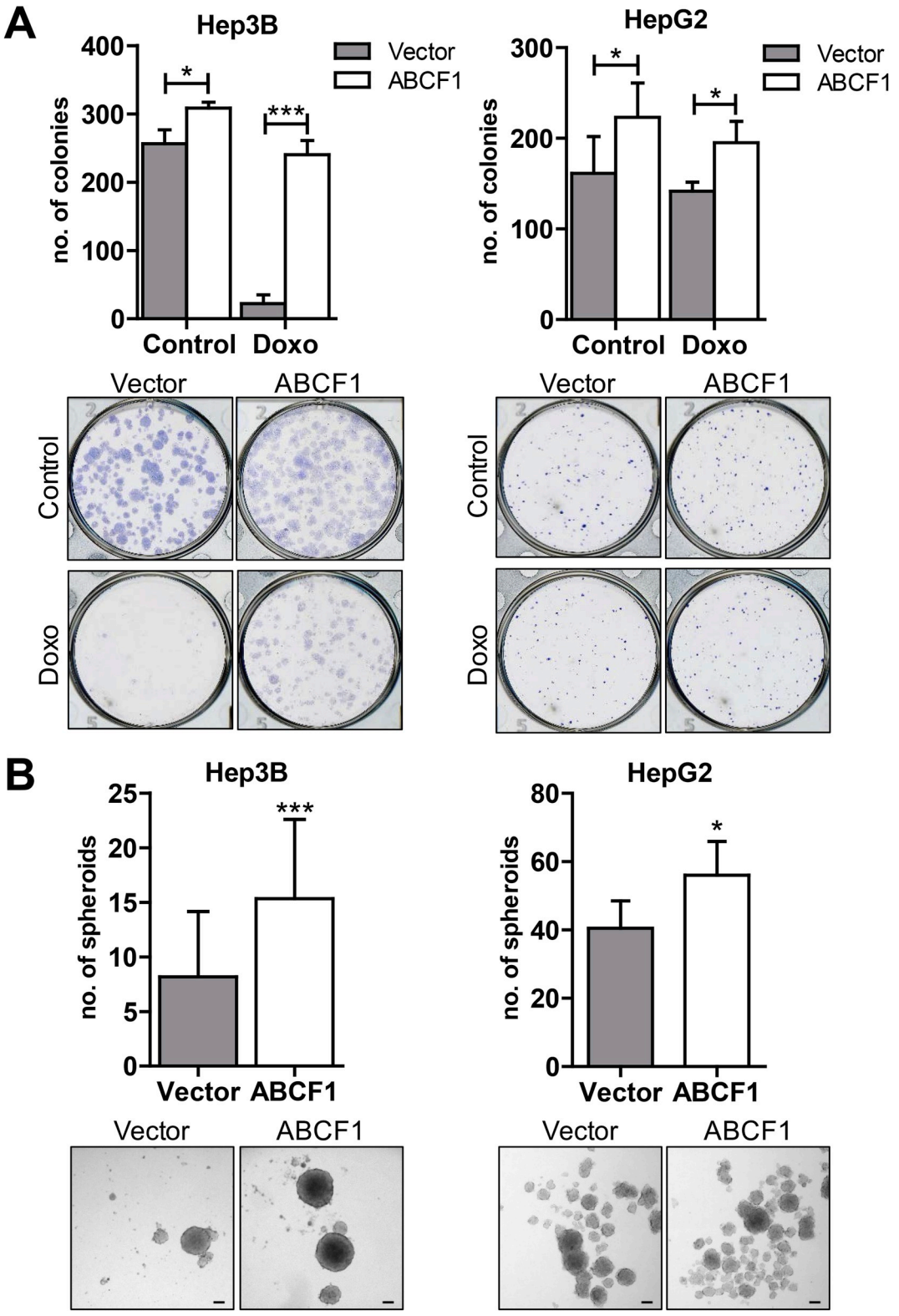

Fig. 6. ABCF1 expression modulates stemness in HCC cells. (A) Colony formation assay and (B) spheroid formation assay for Hep3B and HepG2 cells with ABCF1 overexpression. (A) HCC cells (500 cells/well) were maintained in culture medium containing 1% FBS (control) with or without doxorubicin (Hep3B: 5 ng/ml; HepG2: 10 ng/ml) for colony formation assay. (B) Spheroid formation capacity of ABCF1-expressing HCC cells (Hep3B: 1000 cells/well; HepG2: 200 cells/well) in ultra-low attachment plate were examined. Mean ± SD of number of colonies/spheroids per well was shown (n ≥ 3). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when compared to empty vector control.

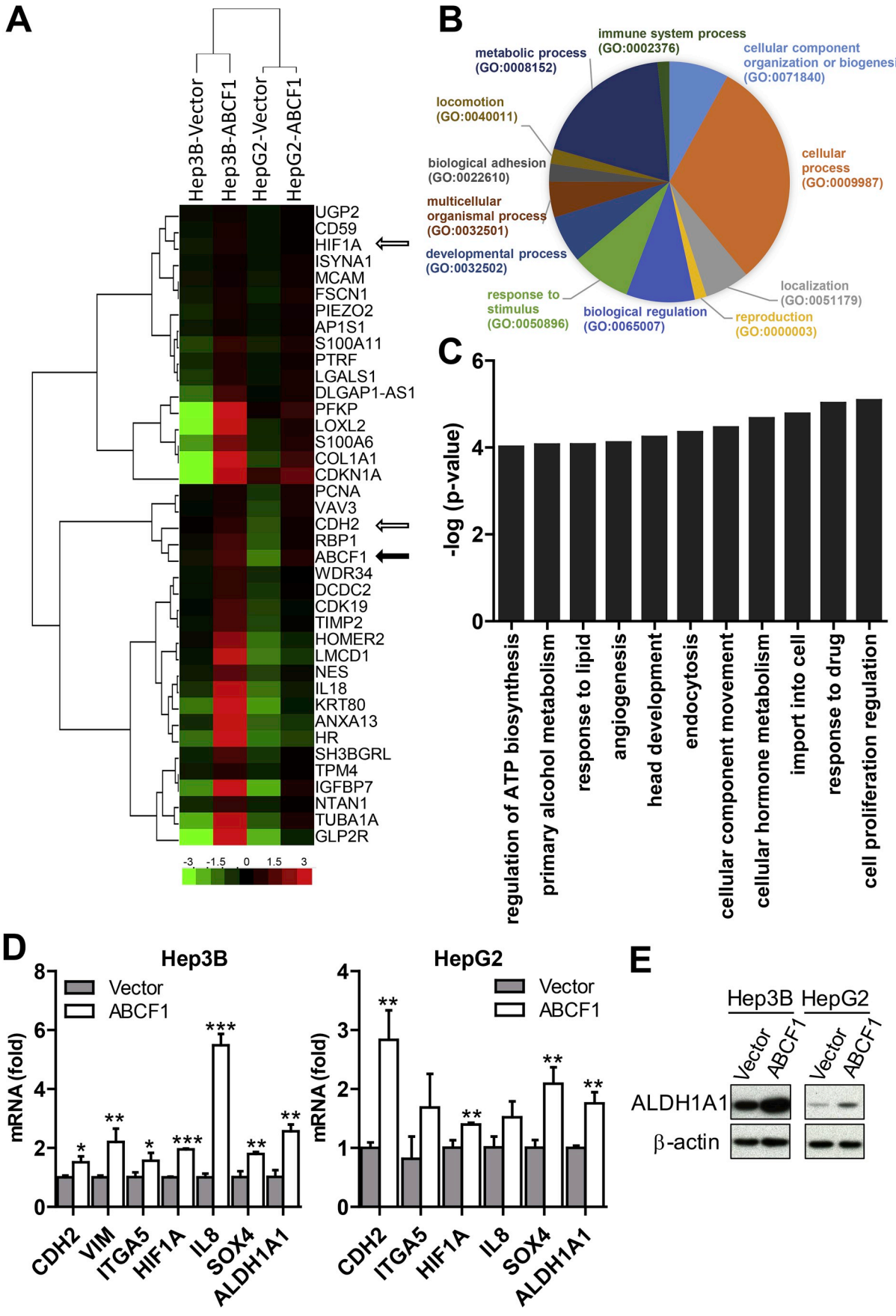

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Fig. 7. ABCF1 transcriptomic analysis. (A) Hierarchical clustering of the FPKM values (log 2 base) by the Pearson's r value shows the top 20 deregulated genes in ABCF1 overexpressing in Hep3B and HepG2 cells. (B) Functional classification of the 157 differentially expressed genes in GO terms for biological processes. (C) Summary of the overrepresented biological processes in differentially expressed genes by PANTHER analysis. (D) Validation by RT-qPCR. The upregulation of *CDH2*, *VIM, ITGA5, HIF1A*, *IL8*, *SOX4* and *ALDH1A1* in Hep3B-ABCF1 and HepG2-ABCF1 was demonstrated. Data are expressed as fold difference relative to the mRNA expression in vector control cells (mean ± SD). 18s rRNA was used for normalization. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ when compared to empty vector control (Vector). (E) Validation by Western blot analysis revealed upregulation of ALDH1A1 by ABCF1. Representative experiment with at least 3 replicates were shown.

therapeutic target, which is important in controlling chemoresistance, migration, stemness phenotype and EMT activation in HCCs.

Conflicts of interest

The authors declared no conflict of interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.05.010.

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