TGFβ-induced FOXS1 controls epithelial–mesenchymal transition and predicts a poor prognosis in liver cancer

Kevin Bévant ,[1,2] Matthis Desoteux ,[1,2] Gaëlle Angenard,[2] Raphaël Pineau ,[1] Stefano Caruso ,[3] Corentin Louis ,[1,2] Panagiotis Papoutsoglou ,[1,2] Laurent Sulpice ,[1,2] David Gilot ,[1] Jessica Zucman-Rossi ,[3,4] and Cédric Coulouarn [1,2]

Transforming growth factor beta (TGF-β) plays a key role in tumor progression, notably as a potent inducer of epithelial–mesenchymal transition (EMT). However, all of the molecular effectors driving TGFβ-induced EMT are not fully characterized. Here, we report that forkhead box S1 (FOXS1) is a SMAD (mothers against decapentaplegic)–dependent TGFβ-induced transcription factor, which regulates the expression of genes required for the initial steps of EMT (e.g., snail family transcription repressor 1) and to maintain a mesenchymal phenotype in hepatocellular carcinoma (HCC) cells. In human HCC, we report that FOXS1 is a biomarker of poorly differentiated and aggressive tumor subtypes. Importantly, FOXS1 expression level and activity are associated with a poor prognosis (e.g., reduced patient survival), not only in HCC but also in colon, stomach, and kidney cancers. *Conclusion:* FOXS1 constitutes a clinically relevant biomarker for tumors in which the pro-metastatic arm of TGF-β is active (i.e., patients who may benefit from targeted therapies using inhibitors of the TGF-β pathway). (*Hepatology Communications* 2022;6:1157-1171).

Liver cancer is the sixth most common cancer worldwide in terms of incidence and the fourth leading cause of cancer-related death with more than 780,000 deaths annually.[1] Hepatocellular carcinoma (HCC) accounts for 75%-85% cases of malignant liver primary tumors.[1] HCC is associated with a rising incidence and limited curative treatments due to a late diagnosis and tumor heterogeneity.[2] For advanced HCC, only multikinase inhibitors demonstrated moderate survival benefits, either in first-line (e.g., sorafenib) or in second-line (e.g., regorafenib) treatments.[2] Large-scale functional genomics studies addressed HCC heterogeneity and identified clinically relevant HCC subgroups.[3,4] Notably, a poor prognosis molecular subtype characterized by a gene-expression signature reflecting the functional duality of the transforming growth factor beta (TGF-β) pathway was highlighted.[3-6]

TGF-β is a pleiotropic cytokine involved in processes frequently deregulated in cancer, including cell proliferation, differentiation, and migration. However, the actions of TGF-β in cancer are complex, as TGF-β exhibits either onco-suppressive or pro-tumorigenic properties, depending on the tumor stage.[7,8] At an early stage, TGF-β exerts anti-proliferative properties in premalignant epithelial cells; at an advanced stage, TGF-β promotes tumor growth and metastatic progression, notably as a potent inducer of epithelial–mesenchymal

Abbreviations: ADAM17, ADAM metallopeptidase domain 17; AFP, alpha-fetoprotein; APOE, apolipoprotein E; BMF, Bcl2 modifying factor; BTG3, BTG anti-proliferation factor 3; C5, complement C5; CCNE2, cyclin E2; CDH1, E-cadherin; CEBPG, CCAAT/enhancer-binding protein gamma; COL1A1, collagen type I alpha 1 chain; CTNNB1, catenin beta 1; EIF2A, eukaryotic translation initiation factor 2A; EMT, epithelial-mesenchymal transition; F7, coagulation factor VII; FBG, F-box protein; FOX, forkhead box; FOXA1, forkhead box A1; FOXS1, forkhead box S1; FZD5, frizzled class receptor 5; GOF, gain of function; GSEA, gene-set enrichment analysis; HCC, hepatocellular carcinoma; HDAC1, histone deacetylase 1; HIF1A, hypoxia inducible factor 1 subunit alpha; HPH, human primary hepatocyte; ID2, inhibitor of DNA binding 2; ITGA6, integrin subunit alpha 6; ITGB6, integrin subunit beta 6; KO, knockout; LOF, loss of function; LOXL2, lysyl oxidase like 2; MAP2K1, mitogen-activated protein kinase kinase 1; MET, mesenchymal–epithelial transition; MMP9, matrix metallopeptidase 9; mRNA, messenger RNA; NC, negative control; PCR, polymerase chain reaction; RASSF1, Ras association domain family member 1; RB1, RB transcriptional corepressor 1; SERPINA1, serpin family A member 1; SERPINE1, serpin family E member 1; siNC, negative control siRNA; siRNA, small interfering RNA; SMAD3, mothers against decapentaplegic homolog 3; SNAI1, snail family transcription repressor 1; SNAI2/SLUG, snail family transcriptional repressor 2; ST, surrounding nontumor tissue; TCF7, transcription factor 7; TCGA, The Cancer Genome Atlas; TGF-β, transforming growth factor beta; TGFB2, transforming growth factor beta 2; TJP1, tight junction protein 1; VIM, vimentin.

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transition (EMT).[7,8] EMT is a dynamic and reversible process associated with the disruption of cell–cell junctions and cytoskeleton reorganization, favoring cell motility, metastasis, and chemoresistance. At the molecular level, EMT is tightly executed by transcription factors, including those from the snail family transcription repressor 1 (SNAI), TWIST and ZEB families, regulating the expression of epithelial (e.g., E-cadherin [CDH1]) and mesenchymal (e.g., vimentin [VIM]) markers.[9-11] Characterizing the effectors of the TGF-β pathway involved in the switch of its actions from tumor suppression toward EMT and tumor promotion could lead to identify clinically relevant biomarkers to select patients who may benefit from interference with the TGF-β pathway.

Forkhead box (FOX) proteins are transcriptional regulators characterized by a conserved forkhead DNA-binding domain.[12] So far, more than 40 FOX proteins classified into 19 subfamilies (FOXA to FOXS) have been discovered in human.[13] They exhibit a functional diversity acting in cell proliferation, apoptosis, metabolism, differentiation, and migration.[12] In this study, we first identified TGF-β-regulated genes in HCC cell lines, including *FOXS1*, highly expressed in cells with a mesenchymal-like phenotype. Next, we focused on determining the role and the clinical relevance of FOXS1 in cancer. FOXS1 shares a high degree of similarity with EMT-related transcription factor FOXC2, which promotes HCC cell invasion.[14] Thus, we hypothesized that FOXS1 could mediate pro-metastatic actions of TGF-β in poor-prognosis HCC.

Materials and Methods

CELL CULTURE

Cell lines were purchased from ATCC (www.lgcstandards-atcc.org) and cultured as previously described.[15] ATCC performed cell line authentication by STR DNA profiling. Freshly isolated human primary hepatocytes (HPHs) were from Biopredic International (St. Grégoire, France) and cultured in William's E medium. Cells were treated with 1 ng/mL recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) and 10 μM LY2157299 (Sigma-Aldrich, St. Louis, MO) after overnight serum starvation.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep4.1866/suppinfo.

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Transcript profiling: Data have been deposited into the public Gene Expression Omnibus database under the accession number GSE148795.

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ARTICLE INFORMATION:

From the [1]Inserm, Univ Rennes, UMR_S 1242, Chemistry, Oncogenesis, Stress Signaling, Centre de Lutte contre le Cancer Eugène Marquis, Service de Chirurgie Hépatobiliaire et Digestive, CHU Rennes, Rennes, France; [2]Inserm, Univ Rennes, Inrae, UMR_S 1241, NuMeCan (Nutrition, Metabolisms and Cancer), Rennes, France; [3]Centre de Recherche des Cordeliers, Inserm, Sorbonne Université, Université de Paris, Université Paris 13, Functional Genomics of Solid Tumors Laboratory, Paris, France; [4]European Hospital Georges Pompidou, AP-HP, Paris, France.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Cédric Coulouarn, Ph.D.
Inserm, UMR_S 1242, Chemistry
Oncogenesis, Stress Signaling
Centre de Lutte contre le Cancer Eugène Marquis
Rue de la Bataille Flandres Dunkerque, Bat D
F-35042 Rennes, France
E-mail: cedric.coulouarn@inserm.fr
Tel.: +33-2-2323-3881

GAIN AND LOSS OF FUNCTION

FOXS1 small interfering RNA (siRNA) were from Ambion: siFOXS1#1 (5′-AAUCGGCCCAU GAUGUAGCgg-3′), siFOXS1#2 (5′-CAUGCAA AAAUUCAGUGCCgg-3′), and negative control siRNA (siNC; AM4620). Silencing of mothers against decapentaplegic homolog 3 (SMAD3; #4088; Dharmacon Inc., Lafayette, CO), SMAD4 (#4089; Dharmacon), and FOXA1 (4392420; Ambion, Inc., Austin TX) was performed according to manufacturer's instruction. Cells were transfected at 70% confluency with 50 nM siRNA using lipofectamine RNAiMAX and Opti-MEM. Gain of function (GOF) was performed by infecting cells with a HA-tagged FOXS1-expressing lentivirus (Lenti-pReceiver-Lv120; GeneCopoeia). FOXS1 knockout (KO) was performed using custom CRISPR-Cas9 (Sigma-Aldrich). Stable cell lines were generated after puromycin selection for 2 weeks and validated by quantitative real-time polymerase chain reaction (PCR), western blot, and sequencing of *FOXS1* locus.

GENE-EXPRESSION PROFILING

Gene-expression profiling was performed using a low-input QuickAmp labeling kit and human SurePrint G3 8x60K microarrays (Agilent Technologies, Santa Clara, CA). Data sets were deposited into the Gene Expression Omnibus (GSE148795). Integration of transcriptomic data was conducted using publicly available data sets (GSE1898 and GSE4024) as previously described.[16] The Cancer Genome Atlas (TCGA) RNA-sequencing (RNA-seq) data were used as validating data sets (https://portal.gdc.cancer.gov). In total, three independent cohorts of HCC patients were analyzed (697 cases). Quantitative real-time PCR was performed using a SYBR Green (Applied Biosystems, Carlsbad, CA) and analyzed as previously described.[17] The list of primers is provided in Supporting Table S1.

IN SITU HYBRIDIZATION

FOXS1 riboprobes were generated by *in vitro* transcription (IVT) from PCR products incorporating the promoter of T7 RNA polymerase. IVT was performed with 40 units T7 in the presence of 0.35 mM digoxygenin-11-UTP (Roche Diagnostics, Meylan, France). *In situ* hybridization was performed on the Discovery Automated stainer (Ventana Medical Systems, Tucson, AZ).[18]

PROTEIN EXPRESSION ANALYSIS

Protein analysis by immunoblotting was as previously described.[19] EMT antibody sampler kit (#9782) was from Cell Signaling. For immunofluorescence studies, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at room temperature. The list of antibodies is provided in Supporting Table S1.

IN VIVO STUDY

Wild-type and FOXS1 KO SNU-449 cells were modified to express GL261-Luc (CMV-Firefly luciferase lentivirus [Neo], PLV-10064-50; Cellomics Technology LLC, Halethorpe, MD). A total of 10,000,000 cells (100 µL in PBS + 100 µL matrigel) were implanted on the flanks of immunodeficient NOD scid gamma mouse (NSG; *NOD Cg-Prkdcscid Il2rgtm1Wjl/SzJ*) 8-week-old male mice (Charles River Laboratories, Wilmington, MA). All animal procedures met the European Community Directive guidelines (Agreement B35-238-40, Biosit Rennes, France; DIR #7163) and were approved by the local ethics committee, ensuring the breeding and the daily monitoring of the animals in the best conditions of well-being according to the law and the 3R rule (Reduce-Refine-Replace). Mice were housing in a ventilated rack cage system from Techniplast and bedding in poplar shavings. Enrichment was performed with cottons and/or poly-sulfone tubes. Tumor growth was evaluated by a direct measurement of tumor size using caliper and by bioimaging (PhotonIMAGER systems, BIOSPACE LAB). After 93 days, mice were sacrificed and tumors were subjected to molecular analysis.

HUMAN HCC

Human HCC and surrounding nontumor tissues (ST) were obtained from freshly resected specimen (Biological Resource Center; BB-0033-00056). The research protocol fulfilled national laws and regulations and was approved by the local ethics committee and the institutional review board of Inserm (IRB00003888). Precision-cut tissue slices (300 µm) were performed with a Vibratome Leica VT1200S and maintained in culture in William's E medium.

STATISTICAL ANALYSIS

Statistical analyses were performed using R-3.5.1 and GraphPad 7.0. Differentially expressed genes were identified by a two-sample univariate *t* test and a random variance model.[16] For group comparison of quantitative variables, *t* testing was applied. Categorical data were analyzed by chi-squared testing. Survival was estimated by the Kaplan-Meier method and log-rank test.

Additional information about assays on cell proliferation, colony formation, cell viability, cell migration/invasion, chromatin immunoprecipitation–based experiments, promoter reporter assays, and meta-analysis of public gene-expression data sets are provided as supporting data.

Results

TGF-β-REGULATED GENES IN SNU-449 LIVER CANCER CELLS

By integrative transcriptomics, we previously highlighted early (cytostatic) and late (metastatic) TGF-β signatures, respectively, associated with onco-suppressive and pro-oncogenic properties of TGF-β in cancer.[5] Notably, the metastatic TGF-β signature predicts a poor prognosis in patients with HCC and invasiveness in HCC cell lines, including SNU-423, SNU-449, and SNU-475.[5] To get insights into the molecular mechanisms involved and to identify key transcriptional regulators, we profiled the SNU-449 cell line following TGF-β exposure and identified 80 differentially expressed genes (Fig. 1A and Supporting Table S2). This signature established by using stringent statistical criteria ($P < 0.001$ and fold change >2) included well-known TGF-β target genes such as Bcl2 modifying factor (*BMF*), collagen type I alpha 1 chain (*COL1A1*), platelet-derived growth factor subunit A (*PDGFA*), SKI-like proto-oncogene (*SKIL*) and *TGF-BI* (Supporting Table S2), but also novel candidates, including *FOXS1*, a transcription factor of the FOX family, which shares a high degree of similarity with EMT-related factor FOXC2, known to promote HCC cell invasion.[12] FOXS1 ranked #9 in the top genes up-regulated by TGF-β (Supporting Table S2). Up-regulation of *FOXS1*, *COL1A1*, *SKIL*, and *BMF* by TGF-β was validated by quantitative real-time PCR in SNU-449 (Fig. 1B). Thus, FOXS1 was selected for further functional investigation, as it may regulate important genes down-stream of TGF-β.

FOXS1 IS A CANONICAL SMAD-DEPENDENT TGF-β TARGET

FOXS1 expression was next evaluated at basal level and following short-term TGF-β exposure (16 hours) in a panel of eight cell lines representative of HCC heterogeneity (Supporting Table S3), as well as in freshly isolated HPHs. Neither a basal expression nor a significant induction by TGF-β was observed in HPH or in PLC/PRF/5, HepG2-C3A, HuH6, and Hep3B HCC cell lines, all associated with the onco-suppressive properties of TGF-β (Fig. 1C). However, *FOXS1* was expressed and induced by TGF-β in SNU-423, SNU-449 and SNU-475, which on the contrary were all associated with the metastatic properties of TGF-β (Fig. 1C). Accordingly, *FOXS1* and serpin family E member 1 (*SERPINE1*), a well-known TGF-β transcriptional target, were down-regulated in SNU-449 and SNU-475 cultured in the presence of LY2157299 (galunisertib), a selective TGF-β receptor type I kinase inhibitor, both at basal level and following TGF-β exposure (Fig. 1D). Time-course analysis and co-treatments of cells with TGF-β and cycloheximide, an inhibitor of translation, demonstrated that *FOXS1* is an early and direct target of the TGF-β pathway (Supporting Fig. S1A,B). *FOXS1* induction by TGF-β was also observed in cholangiocarcinoma and pancreatic cancer cell lines, and in LX2 hepatic stellate cell line (Supporting Fig. S1C). Similar to *SERPINE1*, a SMAD-dependent induction of *FOXS1* by TGF-β was demonstrated. Induction was abolished in the presence of siRNAs targeting SMAD3 and/or SMAD4 (Fig. 1E and Supporting Fig. S1D). SMAD2/3 were not shown to directly bind the proximal promoter of *FOXS1* in SNU-449 (Supporting Fig. S1E). However, chromatin immunoprecipitation (ChIP) assays demonstrated that SMAD2/3 bind on a putative enhancer located about 10 kb downstream of *FOXS1* transcription starting site, which is supported by the presence of H3K27ac and H3K4me1 active histone marks (Supporting Fig. S1F,G). More importantly, *FOXS1* was induced in human-tissue slices (HCC and ST from patients undergoing surgery) exposed to TGF-β (Fig. 1F and Supporting Fig. S2). Altogether, these data demonstrate that *FOXS1* is a SMAD-dependent target of the canonical TGF-β pathway.

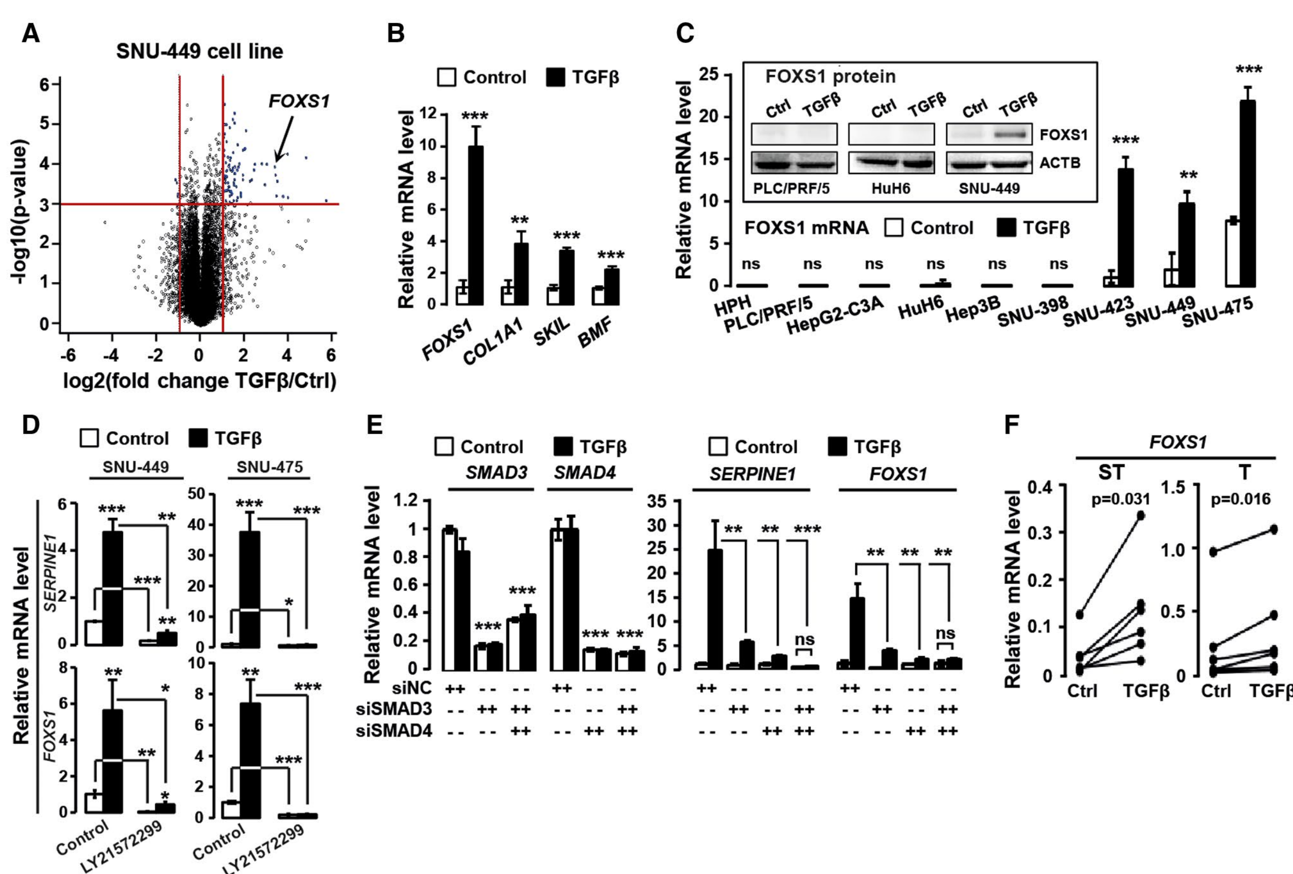


FIG. 1. FOXS1 is a SMAD-dependent TGF-β target. (A) Volcano plot of genes differentially expressed by TGF-β in SNU-449. (B) Quantitative real-time PCR of *FOXS1* and well-known targets in SNU-449 (1 ng/mL TGF-β; 72 hours). (C) Quantitative real-time PCR and western blot (inset) analysis of *FOXS1* expression in HPHs and eight HCC cell lines (1 ng/mL TGF-β; 16 hours). (D) Quantitative real-time PCR of *SERPINE1* and *FOXS1* in SNU-449 and SNU-475 cells (1 ng/mL TGF-β; 10 μM LY2157299; 16 hours). (E) Quantitative PCR analysis of *SMAD3*, *SMAD4*, *SERPINE1*, and *FOXS1* in SNU-449 cells transfected with siSMAD3 and siSMAD4, alone or in combination (50 nM each), or a siNC (50 nM) (1 ng/mL TGF-β; 72 hours). (F) Quantitative real-time PCR of *FOXS1* in human ST (left panel; n = 6 patients) or tumor (T, right panel; n = 7 patients) tissue slices derived from patients after surgical resection (1 ng/mL TGF-β; 48 hours). Statistical analysis was performed by a Wilcoxon matched-pairs signed-rank test. In (B)-(E), statistical analysis was performed by a Student *t* test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns *P* > 0.05; n ≥ 3 replicates). Abbreviations: Ctrl, control; mRNA, messenger RNA.

FOXS1 EXPRESSION IS ASSOCIATED WITH A MESENCHYMAL PHENOTYPE

Next, we characterized FOXS1 negative versus positive HCC cell lines (Fig. 1C) at the cellular and molecular level. FOXS1 negative cell lines (i.e., neither basal expression nor induction evidenced after short-term TGF-β exposure) exhibited an epithelial-like morphology with a cobblestone-like growth pattern, whereas FOXS1-positive cell lines exhibited a mesenchymal-like phenotype with a spreading growth pattern (Fig. 2A). Accordingly, FOXS1 positive cell lines exhibited an increased expression of mesenchymal markers, including *VIM* and *TGF-B2*, together with a decreased expression of hepatocyte differentiation markers, including *FOXA1,* fibrinogen gamma chain (*FGG*), and apolipoprotein E *(*APOE) (Fig. 2B,C and Supporting Table S4). Gene-set enrichment analysis (GSEA) highlighted a positive enrichment of signatures associated with metastasis and poor prognosis (e.g., TGF-β, EMT, migration, cancer recurrence) and a negative enrichment of signatures associated with a well-differentiated hepatocyte phenotype (e.g., liver-specific genes and metabolisms) in the gene-expression profiles of FOXS1 positive cell lines (Fig. 2D). The

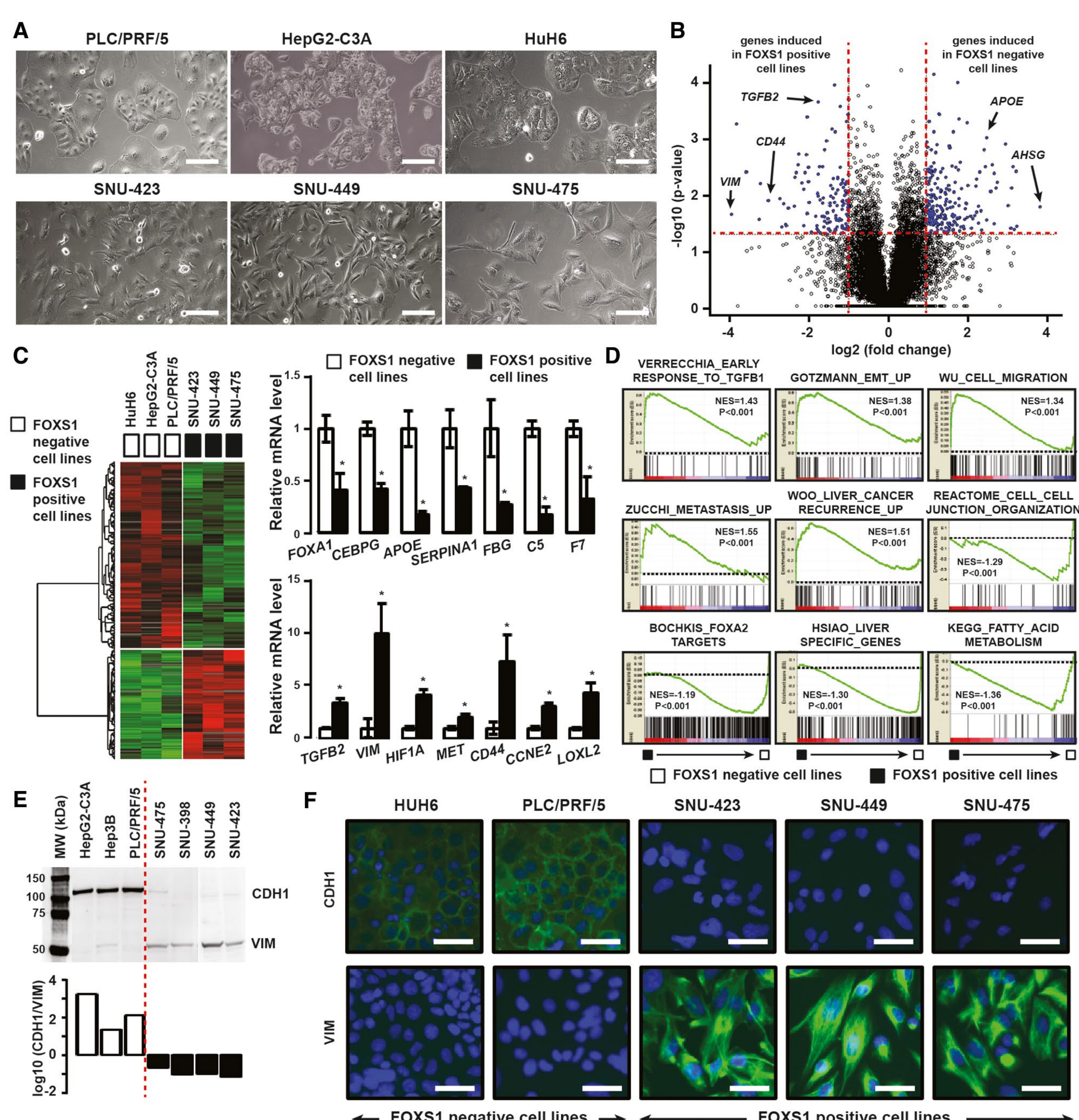


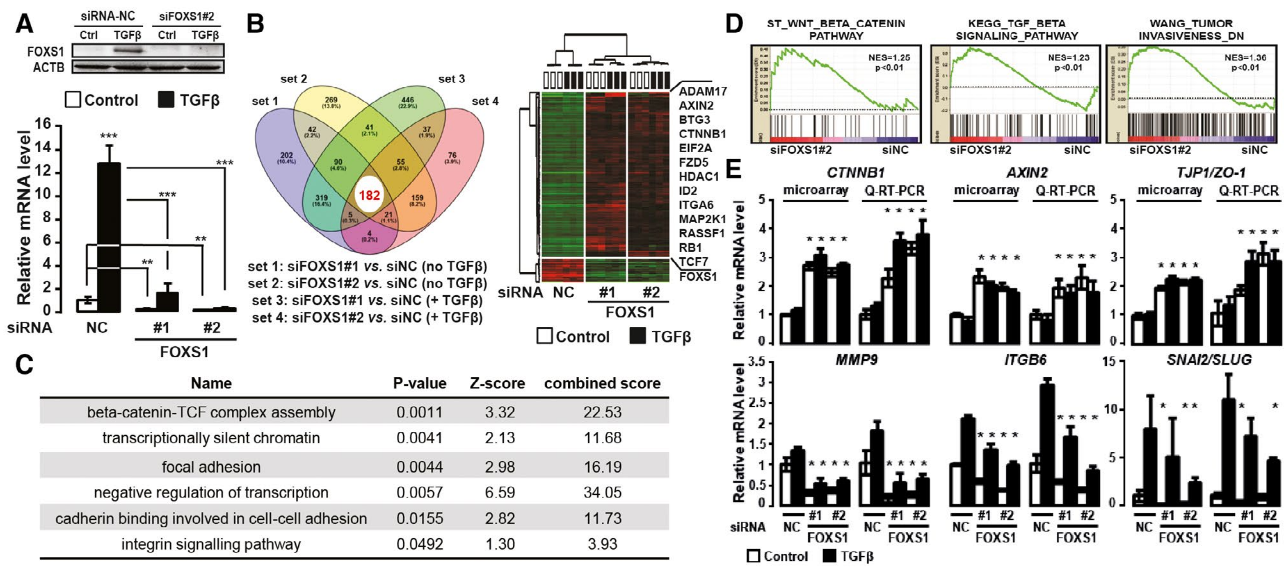
FIG. 2. FOXS1 positive HCC cell lines exhibit a mesenchymal phenotype. (A) Phase-contrast micrographs of representative FOXS1 negative (upper panels) and positive (lower panels) HCC cell lines (scale bar: 100 µm). (B) Volcano plot of genes differentially expressed between FOXS1 negative (PLC/PRF/5, HepG2-C3A, and HuH6) and positive (SNU-423, SNU-449, and SNU-475) cell lines. (C) Hierarchical clustering analysis and examples of genes down-regulated and up-regulated in FOXS1 positive cell lines (*$P < 0.05$). (D) GSEA of signatures significantly enriched ($P < 0.01$) in the gene-expression profiles of FOXS1 positive (black box) and negative (white box) HCC cell lines. (E,F*)* Expression of epithelial marker CDH1 and mesenchymal marker VIM at protein level in HCC cell lines, as determined by western blot (E) and immunofluorescence (F) in FOXS1 negative and positive HCC cell lines. Scale bar: 50 µm.

phenotype of FOXS1 negative and positive cell lines was further confirmed by the expression of CDH1 and VIM at the protein level (Fig. 2E,F). Analysis of *FOXS1* locus suggested that the absence of *FOXS1* expression in well-differentiated HCC cell lines could result from a transcriptional repression by liver-enriched FOXA1

(Supporting Fig. S3A). Although *FOXA1* was induced by TGF-β in FOXS1 negative cells, its silencing was not able to reactivate *FOXS1* expression (Supporting Fig. S3B,C). In addition, the presence of a CpG island on *FOXS1* locus (Supporting Fig. S3D) suggested a possible epigenetic regulation. Accordingly, a statistically significant lower methylation of *FOXS1* locus was observed in human HCC as compared with the ST (Supporting Fig. S3E). However, no significant correlation was observed between the methylation status of *FOXS1* locus and its expression in HCC cell lines (Supporting Fig. S3F). Altogether, these data demonstrate that the basal expression of *FOXS1* and its induction by TGF-β in HCC cell lines correlate with a mesenchymal phenotype.

FOXS1 INDUCES TRANSCRIPTIONAL CHANGES ASSOCIATED WITH EMT AND TUMOR PROGRESSION

Transient loss of function (LOF) was achieved in SNU-449 by two siRNAs, which significantly suppressed endogenous *FOXS1* expression and its induction by TGF-β without affecting the expression of other FOX family members and possible off-targets (Fig. 3A and Supporting Fig. S4). Gene-expression profiling identified 177 nonredundant genes differentially expressed by the two siRNAs in both untreated and TGF-β treated cells (Fig. 3B and Supporting Table S5). Interestingly, 87% genes were induced after FOXS1 silencing, suggesting that FOXS1 may act as a transcriptional repressor (Fig. 3B,C). GSEA demonstrated that FOXS1 silencing impacted genes involved in β-catenin and TGF-β pathways, cell adhesion, and associated with tumor invasion (Fig. 3C,D). The expression of several markers was further validated by quantitative real-time PCR. Cell adhesion–associated genes (e.g., catenin beta 1 [*CTNNB1*], tight junction protein ZO-1) were induced after FOXS1 silencing, while invasion and EMT-associated genes (e.g., *MMP9*, *SNAI2*) were suppressed (Fig. 3E). Gene-expression profiles were further validated in Hep3B cells after *FOXS1* silencing and long-term TGF-β exposure (24 hours and 48 hours) (Supporting Fig. S5). Altogether, these data demonstrate that FOXS1 induces specific transcriptional changes associated with EMT and tumor progression.



Name	P-value	Z-score	combined score
beta-catenin-TCF complex assembly	0.0011	3.32	22.53
transcriptionally silent chromatin	0.0041	2.13	11.68
focal adhesion	0.0044	2.98	16.19
negative regulation of transcription	0.0057	6.59	34.05
cadherin binding involved in cell-cell adhesion	0.0155	2.82	11.73
integrin signalling pathway	0.0492	1.30	3.93

FIG. 3. Specific transcriptional changes evocative of EMT and tumor progression after FOXS1 silencing. (A) Validation of endogenous FOXS1 silencing in SNU-449 cells by western blot (upper part) and quantitative real-time PCR (lower part) (1 ng/mL TGF-β; 72 hours). (B) Venn diagram analysis of genes differentially expressed by FOXS1 silencing, in presence or absence of TGF-β ($P < 0.001$; fold change >1.5). A stringent signature made of 182 probes (177 nonredundant genes) reflecting endogenous FOXS1 silencing was defined. Hierarchical clustering analysis based on the expression of 182 probes highlighted a global gene induction (87% genes). (C) Top enriched pathways in FOXS1 silencing signature. (D) GSEA using gene signatures involved in Wnt/β-catenin and TGF-β pathways, and tumor invasion (down-regulated genes). (E) Validation by quantitative real-time PCR of key genes modulated after FOXS1 silencing from the gene-expression profiling by microarray in SNU-449 (1 ng/mL TGF-β; 72 hours). Statistical analysis was performed by a *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n ≥ 3 replicates). Abbreviation: Q-RT-PCR, quantitative real-time PCR.

FOXS1 CONTROLS EMT AND PROMOTES CELL MIGRATION

To get deeper insight into the molecular mechanisms involved, we generated clonal and stable cell lines with FOXS1 GOF and LOF in PLC/PRF/5 and SNU-449, respectively (Fig. 4A and Supporting Fig. S6). FOXS1 KO by CRISPR-Cas9 resulted in a decreased basal expression of mesenchymal markers (e.g., VIM, SNAI1, SNAI2) and a re-expression of epithelial marker CDH1 evocative of a mesenchymal–epithelial transition (MET) (Fig. 4A). Induction of SNAI1 by TGF-β was abolished in FOXS1 KO cells (Fig. 4A), while activation of endogenous *FOXS1* by CRISPR-Cas9

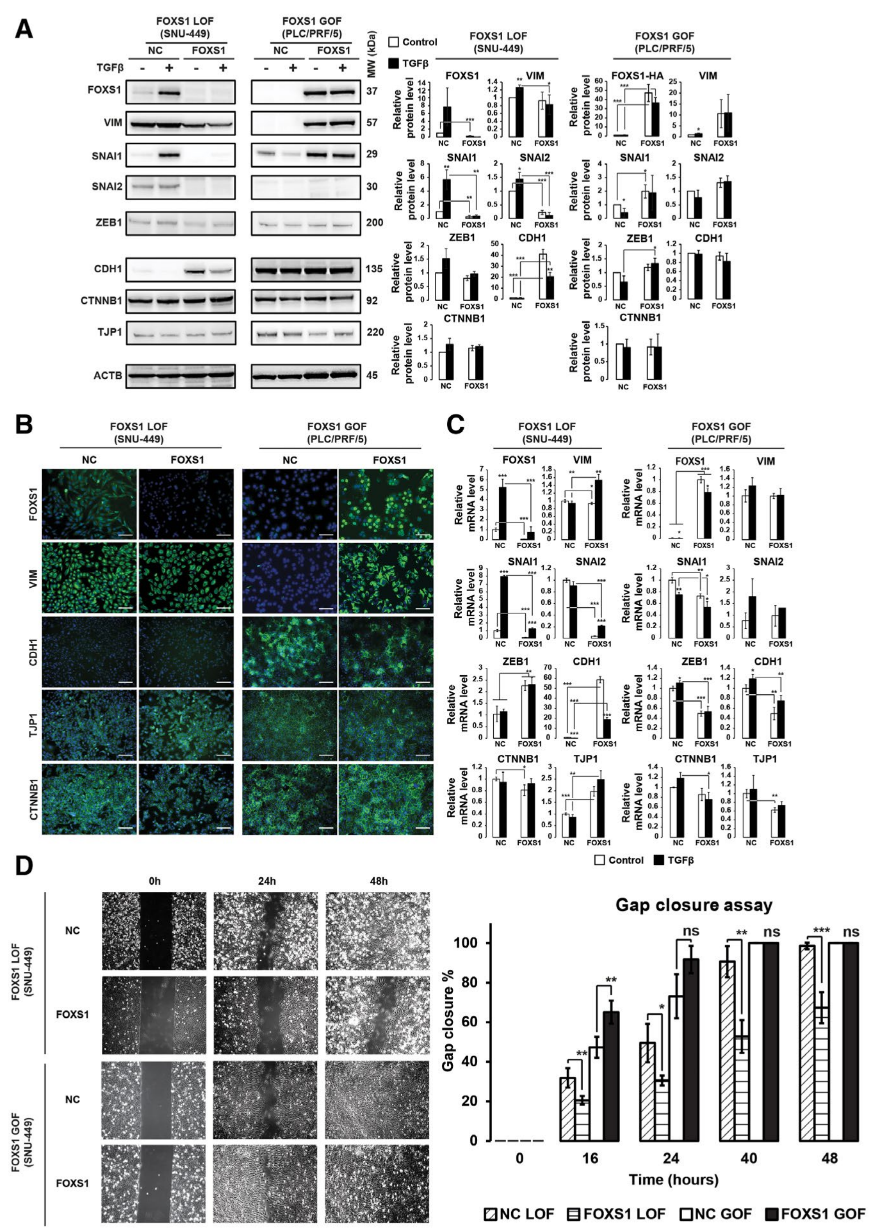


FIG. 4. FOXS1 induces EMT markers and promotes cell migration. (A) Western blot analysis of EMT markers after stable FOXS1 LOF and GOF of function in clonal SNU-449 and PLC/PRF/5, respectively (1 ng/mL TGF-β; 16 hours). (B) Evaluation of EMT markers by immunofluorescence (scale bar: 100 μm). (C) Quantitative real-time PCR of EMT markers (1 ng/mL TGF-β; 16 hours). (D) Migration assay in cells after mitomycin C treatment (10 μg/mL). Statistical analysis was performed by a *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n ≥ 3 replicates). Abbreviation: NC, negative control; ns, not significant.

Synergistic Activation Mediator in SNU-449 cells resulted in SNAI1 induction (Supporting Fig. S7), suggesting that FOXS1 acts upstream to this master EMT transcription factor. However, ChIP assays in genetically engineered SNU-449 cells overexpressing a HA-tagged FOXS1 suggest that EMT-associated transcription factors *SNAI1* and *SNAI2* are not direct targets of FOXS1 (Supporting Fig. S8). An opposite regulation was observed in epithelial-like PLC/PRF/5 after FOXS1 gain of function (GOF), including the expression of mesenchymal markers, as well as the repression of cell junction markers (Fig. 4A). These observations were validated by immunofluorescence (Fig. 4B) and by quantitative real-time PCR (Fig. 4C). Moreover, FOXS1 was found to possibly bind the locus of *VIM* and *CDH1* genes (Supporting Fig. S8). Supporting an association with EMT, *FOXS1* expression positively correlates with *VIM* expression and negatively correlates with *CDH1* expression in several models of EMT and MET (Supporting Fig. S9). The analysis of 34 HCC cell lines also demonstrated that the gene-expression profiles of highly *FOXS1* expressing cells correlate with the expression of EMT markers and are enriched in signatures of cell migration and liver recurrence in patients with HCC[20] (Supporting Fig. S10). Accordingly, FOXS1 GOF and LOF in SNU-449 cells resulted in a significant increase and decrease of cell migration, respectively (no effect was found on cell proliferation and *in vitro* colony formation) (Fig. 4D and Supporting Fig. S11). Altogether, these data suggest that FOXS1 acts as an upstream regulator of EMT and is required to maintain a mesenchymal phenotype.

FOXS1 IMPACTS TUMOR CELL GROWTH *IN VIVO*

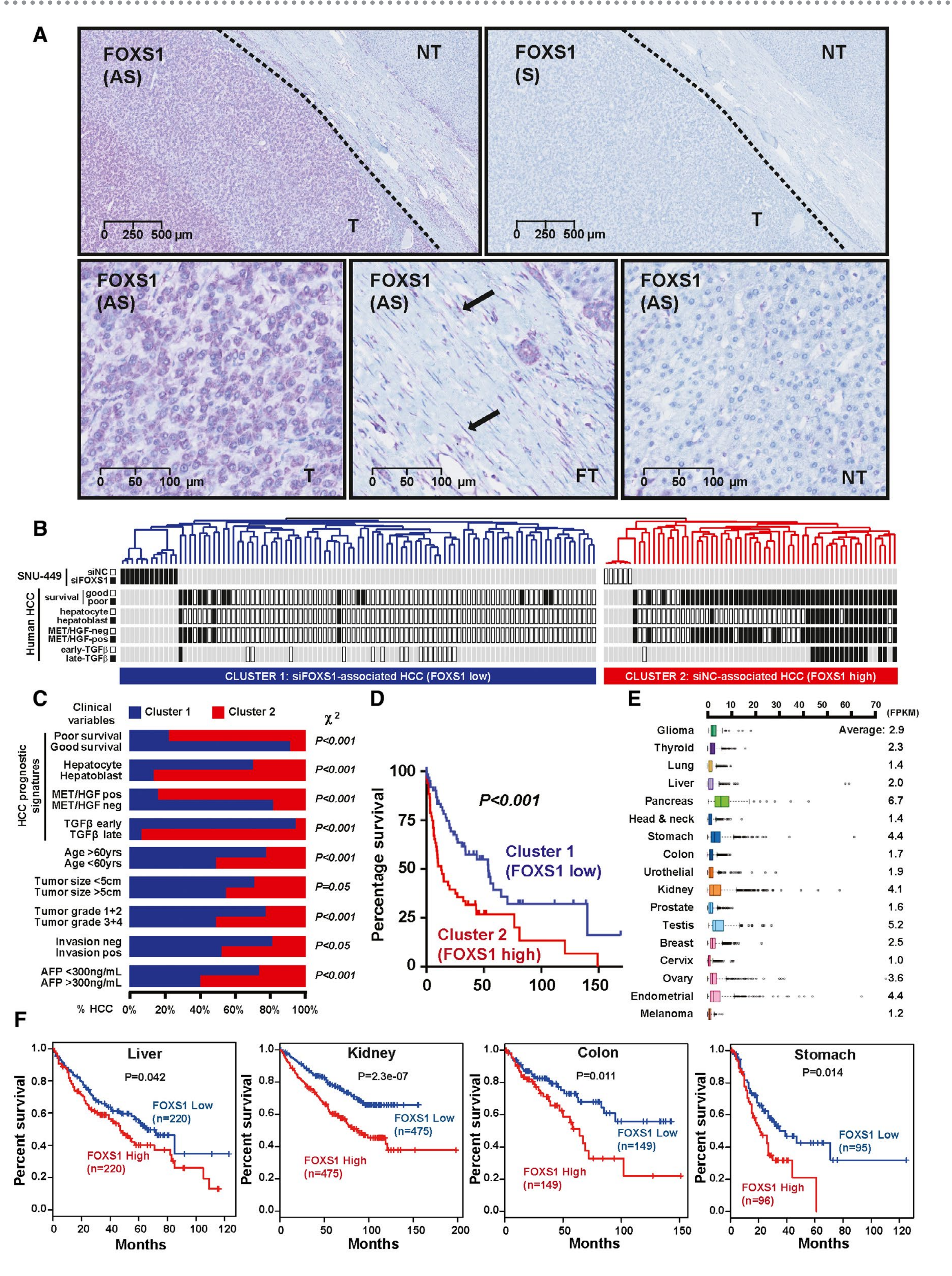
To further address the function of FOXS1 *in vivo* and to follow tumor growth by imaging, *FOXS1* KO SNU-449 cells were genetically engineered to express *luciferase*. Tumor growth was significantly increased in mice xenografted with *FOXS1* KO SNU-449 cells, as evaluated by bioimaging (Fig. 5A) and direct measurement of tumor size (Fig. 5B,C). Molecular analysis of resected tumors highlighted a higher expression of epithelial marker *CDH1* in *FOXS1* KO SNU-449 cell–derived tumors, and a lower expression of mesenchymal markers *VIM* and *SNAI2* (Fig. 5D) recapitulating *in vitro* results (Fig. 4).

FOXS1 EXPRESSION PREDICTS A POOR PROGNOSIS IN HUMAN CANCER

The clinical relevance of *FOXS1* expression was first determined by analyzing previously published gene-expression cancer data sets. Increased expression of *FOXS1* was observed in numerous cancers, including HCC, where it correlates with tumor stage and *VIM* expression (Supporting Fig. S12). In an independent cohort of 193 HCC, we validated an increased *FOXS1* expression in HCC as compared with STs and a correlation with *VIM* expression. Interestingly, *FOXS1* expression correlated with the degree of fibrosis, *FOXS1* being significantly more expressed in tumors developed on a cirrhotic background (Supporting Fig. S13A,B). A specific increased *FOXS1* expression in the tumor tissue was further demonstrated by *in situ* hybridization on human-resected HCC (Fig. 6A). Interestingly, FOXS1 positive cells were detected in fibrotic areas (Fig. 6A), in agreement with our previous observation of *FOXS1* induction in hepatic stellate cells following TGF-β treatment (Supporting Fig. S1C). The heterogeneous expression of *FOXS1* in HCC tissues (Supporting Figs. S12B and S13A) raised the hypothesis of a possible correlation with clinically relevant HCC subtypes. Supporting this hypothesis, *FOXS1* expression was significantly associated with a previously reported G1-G6 molecular classification[21] with a higher expression in poor prognosis G1 HCC subtype and a lower expression in well-differentiated G5-G6 HCC subtypes with *CTNNB1* mutation. Accordingly, *FOXS1* was significantly induced in tumors with no *CTNNB1* mutation (Supporting Fig. S13C,D). Next, to address the clinical relevance of FOXS1 transcriptional activity, we integrated the endogenous FOXS1 loss-of-function signature generated in SNU-449 cells by the two siRNAs (Fig. 3B) with the gene-expression profiles of 139 cases of human HCC extensively characterized.[22-24] Hierarchical clustering analysis of the integrated data set identified two major clusters (Fig. 6B). Clusters 1 and 2 included human HCC associated with the siFOXS1 (low FOXS1 activity) and the siNC (high FOXS1 activity) SNU-449 samples, respectively. Supporting a correlation with possible clinically relevant HCC subtypes, the biological and clinical parameters of HCC were not

FIG. 5. Impact of FOXS1 on tumor growth *in vivo*. (A,B) Wild-type (WT) control or *FOXS1* KO SNU-449 cells were xenografted on the flanks of NOD scid gamma mouse (NSG) mice. Cells were genetically engineered to express *luciferase*. Evolution of tumor growth was evaluated by bioluminescence (A) and a direct caliper measurement of tumor size (B). (C) Picture of the resected tumors. (D) Quantitative real-time PCR analysis of *CDH1*, *VIM,* and *SNAI2* expression in the tumor tissues. Statistical analysis was performed by a *t* test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

randomly distributed between the two clusters (Fig. 6B,C). Strikingly, cluster 1 included significantly more tumors, which were previously assigned to a better prognosis group, than cluster 2. Conversely, cluster 2 was significantly enriched in HCC previously defined by poor prognosis signatures, including poor survival-associated genes,[(23)] hepatoblast/progenitor traits,[(24)] and activation of a pro-oncogenic MET/hepatocyte growth factor (HGF) pathway[(22)] (Fig. 6B,C). More interestingly, cluster 2 was significantly enriched in HCC harboring the so-called late pro-metastatic TGF-β signature, while cluster 1 was enriched in HCC with the so-called early TGF-β signature associated with the cytostatic properties of TGF-β[(5)] (Fig. 6B,C). In addition, cluster 2 was significantly enriched in less-differentiated (Edmondson grade 3+4) HCC with signs of vascular invasion and occurring in younger patients (age < 60 years). Serum alpha-fetoprotein (AFP) was significantly higher in these FOXS1-high aggressive HCC (Fig. 6B,C). *FOXS1* expression was also induced in portal vein tumor thrombosis, as compared with the primary tumor or the adjacent nontumor tissue (Supporting Fig. S14A). *FOXS1* expression correlated with *SNAI1* expression in pancreatic cancer and liver metastasis (Supporting Fig. S14B). Accordingly, a high *FOXS1* expression was found to predict a reduced survival of patients with HCC



(Fig. 6D). Finally, *FOXS1* expression was directly evaluated in 17 types of human cancer from RNA-seq data generated by TCGA. *FOXS1* was detected across all types of cancer tissues (Fig. 6E). More importantly, a high expression of *FOXS1* significantly ($P < 0.05$) predicted a poor survival in patients with

FIG. 6. FOXS1 predicts a poor prognosis in human cancer. (A) *In situ* hybridization on human HCC samples using FOXS1 sense (S) and antisense (AS) riboprobes. Black arrows: FOXS1 expression in fibroblast-like cells in the interface fibrotic tissue (FT). (B) Clinical relevance of *FOXS1* silencing signature in HCC. Dendrogram overview of *in vitro* experiments using SNU-449 cells transfected with siNC or siFOXS1 integrated with 139 cases of human HCC based on the expression of 177 genes differentially expressed after *FOXS1* silencing. Two major clusters were identified: cluster 1 associated with *FOXS1* silencing and cluster 2 associated with high *FOXS1* expression. Distribution of HCC between previously described subgroups with respect to survival (23) (good vs. poor prognosis), cell origin (24) (hepatoblast vs. hepatocyte), activation of MET/HGF (22) (negative vs. positive), and TGF-β signaling pathway (5) (early vs. late) is indicated on the left side. (C) Statistical analysis of HCC distribution between clusters 1 and 2 based on previous gene signatures and clinical parameters. (D) Kaplan-Meier plots and log-rank statistical analysis revealed a significant decreased overall survival for patients included in cluster 2. (E) Expression of *FOXS1* in 17 cancer types from the TCGA consortium. (F) Kaplan-Meier plots and log-rank statistics analysis revealed a significant decreased overall survival for patients with a high expression of *FOXS1* in liver, kidney, colon, and stomach cancer. Abbreviations: NT, nontumor; T, tumor.

liver, kidney, colon, or stomach cancer (Fig. 6F) and correlated with *VIM*, *SNAI1*, and *TGF-B1* expression (Supporting Fig. S14C). Altogether, these data demonstrate that *FOXS1* expression is a predictor of poor prognosis in human cancer.

Discussion

In this study, we investigated the role and the clinical relevance of FOXS1, a transcription factor of the forkhead box (FOX) family. We report that *FOXS1* is a TGF-β responsive gene whose expression and induction occur in cells with a mesenchymal-like phenotype. We further report that FOXS1 is a positive regulator of EMT, induces cell migration, and predicts a poor prognosis in patients with cancer.

FOX proteins are tightly regulated during embryonic development and are required for the normal specification, differentiation, and function of numerous tissues, including the liver.[12] Early studies reported a critical role of FOXS1 in embryogenesis, especially for normal brain and testis development.[25-27] Interestingly, *FOXS1* was reported to be silenced in most normal tissues, its expression being restricted to the aorta and to a lesser extend to the kidney.[28] Our data suggest that *FOXS1* could be epigenetically silenced in nontumor tissues through DNA hypermethylation. *FOXS1* re-expression in cancer is in agreement with observations reporting hypomethylation-associated up-regulation of *Foxs1* in a mouse model of gonadectomy-induced adrenocortical neoplasia.[29] This specific gene-expression pattern highlights *FOXS1* as a potential oncofetal gene inactivated by methylation at birth and reactivated in cancer, similar to *AFP* in HCC.[30]

We report that *FOXS1* is a direct target of the canonical SMAD-dependent TGF-β pathway in mesenchymal-like cells. SMAD2/3 do not bind directly on *FOXS1* proximal promoter but on a downstream enhancer, suggesting that *FOXS1* expression and induction by TGF-β in specific cells may be controlled by histone marks activating or repressing the enhancer. We hypothesized that a transcriptional repression by liver-enriched FOXA1 could occur in epithelial cells. *FOXS1* repression by FOXA1 was supported by the differential expression of FOXA1 in FOXS1 negative versus positive cell lines both at basal level and following TGF-β treatment. However, FOXA1 silencing was not able to reactivate *FOXS1* expression in negative cell lines, suggesting that transcriptional repression is probably not the main mechanism of *FOXS1* silencing in well-differentiated cells. A transcriptional modulation by the Sonic Hedgehog pathway has been also highlighted, although the reports are conflicting. *FOXS1* was induced by GLI1 in rhabdomyosarcoma and medulloblastoma[31] but repressed by GLI1 in gastric cancer,[32] suggesting a complex interplay between cancer-specific transcriptional or post-transcriptional regulators. In addition, miR-125a-5p was identified as a potent inhibitor of *FOXS1* expression in gastric cancer. Interestingly, miR-125a-5p is a well-known tumor suppressor microRNA in HCC,[33] acting as a potent inhibitor of EMT and cell invasion.[34] Altogether, the data suggest that *FOXS1* expression is tightly regulated by multiple mechanisms occurring probably at epigenetic, transcriptional, and post-transcriptional levels and in a specific cell, tissue, and cancer-specific manner. Additional investigations will be required to fully characterize the regulation of *FOXS1* expression in cancer cells.

Regarding the role of FOXS1 in cancer, there is no consensus in the literature yet. FOXS1 has been reported to exhibit anti-proliferative effects in medulloblastoma.[31] In gastric cancer, conflicting data have

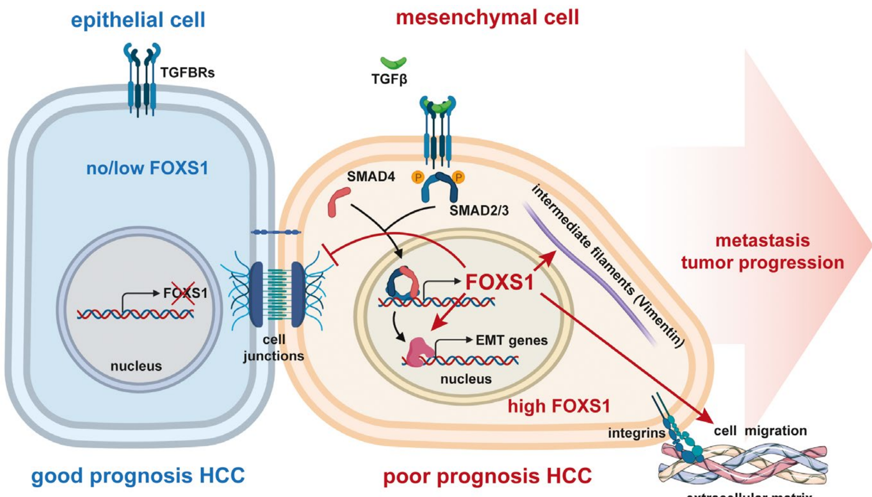


FIG. 7. Functional role and clinical relevance of FOXS1 in HCC.

been reported.[32,35] FOXS1 overexpression inhibits EMT in SNU-216 cells but promotes cell proliferation, migration, invasion, and EMT in SGC7901 and BGC823 cells.[32] These opposite results suggest that FOXS1 function could be highly dependent of cell-specific transcriptional contexts associated to each cancer subtype. It was previously hypothesized that FOXS1 prevent apoptosis of endothelial cells by blocking FOXO3 and FOXO4 action through promoter binding competition.[27] Our data suggest that FOXS1 may indeed act as a transcriptional repressor, given that >80% genes were de-repressed after FOXS1 silencing. If FOXS1 acts as a decoy factor, it could block activators or inhibitors at the same locus, thus changing its action depending on the transcriptional context. Two-hybrid experiments have highlighted a potential interaction between FOXS1 and BMI1.[36] BMI1 is a core component of the polycomb repressive complex 1.[37] This interaction might contribute to the transcriptional repressive activity of FOXS1.

Regardless of previously conflicting reports in gastric cancer and HCC,[38] our data highlight a role of FOXS1 in EMT in HCC, as evidenced by changes in the expression of EMT markers (e.g., VIM and CDH1) and effectors (e.g., SNAI1 and SNAI2) as well as the impact of FOXS1 on cell migration. Those results are in agreement with the reported role of FOXS1 during embryonic development, in which FOXS1 is expressed in migrating neural crest cells.[26] In addition, it was recently reported that FOXS1 up-regulation was related to EMT and temozolomide resistance in glioma cells.[39] Finally, FOXS1 shares a high degree of similarity with EMT-related factor FOXC2, which promotes HCC cell invasion. Thus, our data place FOXS1 in a classical model of EMT, in which an inducible signal (TGF-β) activates EMT-transcription factors (FOXS1, SNAI1, and SNAI2) regulating the expression of EMT markers (VIM and CDH1) (Fig. 7). FOXS1 appears to directly regulate *VIM* and *CDH1* expression through direct fixation on their locus. However, *SNAI1/2* regulation appears to be indirect and probably occurs through interaction of FOXS1 with other signaling pathways (e.g., JAK-STAT), considering that FOXS1 may directly bind the promoter of *IL-6* and the core gene of *STAT3*. EMT occurs in a gradual manner characterized by several states referred to as epithelial, early hybrid, late hybrid, and full EMT states.[9,40] Thus, even if an overactivation of TGF-β signaling can promote a full EMT in different cancers such as HCC,[41] a recent study showed that an inhibition of TGF-β signaling blocks EMT progression at early stages.[9] Total absence of *FOXS1* expression in epithelial cells and reappearance of epithelial features in *FOXS1* KO SNU-449 cells suggest that FOXS1 could not only act as an inducer of EMT but also as a maintainer of the mesenchymal state. Supporting this hypothesis, the TGF-β withdrawal in experimental models of MET results in a decreased expression of *FOXS1* followed by *VIM* down-regulation and

CDH1 up-regulation. Moreover, the reappearance of epithelial features appears to be conserved in tumors derived from *FOXS1* KO SNU-449 cells, which exhibit a more epithelial phenotype than the control tumors. This could explain the increased growth of *FOXS1* KO tumors, considering that progression in EMT states is usually associated with a reduced cell proliferation. Conversely, cells following MET tend to be proliferative but with limited invasiveness potential. This pro-EMT but anti-proliferative action of FOXS1 could explain why its action in cancer is debated. We observed that *FOXS1* GOF or LOF did not affect cell proliferation and colony formation *in vitro*, suggesting that environment factors (e.g., cytokines and growth factors) influence tumor growth *in vivo*. We hypothesize that *FOXS1* LOF results in an increased expression of receptors for pro-proliferative pathways, which could be stimulated by ligands *in vivo*. Accordingly, several genes with pro-mitogenic capacity (e.g., RAS family, mitogen-activated protein kinase kinase 1) were induced after *FOXS1* silencing (Supporting Table S5). However, further studies will be required to fully characterize the underlying molecular mechanisms. Moreover, it was established that some EMT transcription factors like SNAI1 or ZEB1 can in some context reduce cell proliferation and increase resistance to cell death.[11,42] Investigating a potential role of FOXS1 in chemoresistance *in vivo* could also be relevant and be supported by the recent study showing the role of FOXS1 on temozolomide resistance in glioma cells.[39]

As for its role in cancer, the clinical relevance of *FOXS1* expression has been controversial. High expression of *FOXS1* is associated with better relapse-free survival in patients with breast cancer.[31] Conversely, *FOXS1* is enriched in a nine-gene expression signature of poor prognosis in gastric cancer,[43] in agreement with our analysis identifying *FOXS1* as a marker of poor prognosis not only in HCC but also in several other cancers. We also validated the clinical relevance of FOXS1 by using an integrative transcriptomic approach based on a set of genes that reflect FOXS1 activity. Our data highlight FOXS1 as a promising biomarker to identify tumors in which the pro-metastatic arm of TGF-β is active (Fig. 7). Thus, patients who may benefit from targeted therapies using TGF-β inhibitors. This is particularly relevant for treatment personalization in the framework of a phase 2 clinical trial currently evaluating LY2157299 (galunisertib) in patients with advanced HCC (NCT02178358).[44]

In conclusion, our study identified *FOXS1* as a target gene of the canonical TGF-β pathway. FOXS1 controls EMT and cell migration possibly through induction of SNAI1/2, VIM, and decrease of cell junction proteins. *FOXS1* is overexpressed in HCC and predicts a poor prognosis in liver, stomach, colon, and kidney cancers.

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Author names in bold designate shared co-first authorship.

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