Thyroid hormones suppress FOXM1 expression to reduce liver cancer progression

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Abstract. Thyroid hormones (TH) are multifunctional mediators that fine-tune several physiological processes, including metabolic rate, digestive function and tissue development via interactions with type II nuclear thyroid hormone receptors (TR). Upon binding of TH, TRs interact specifically with thyroid hormone response elements of target gene promoter regions to regulate their transcription. Earlier studies suggested a correlation between aberrant TR regulation and hepatocellular carcinoma (HCC). THs are involved in a crosstalk between hepatoma and stromal cells, and disruption of TH signaling is associated with tumorigenesis. Previous cDNA microarray analysis of target gene expression following T_3 treatment of wild-type TR-expressing hepatoma cells led to the identification of forkhead box M1 (FOXM1) as a factor negatively regulated by T_3 and associated with poor prognosis in several cancer types. Increased FOXM1 expression during late stages of HCC was associated with poorer overall and recurrence-free survival in patients with HCC. However, the specific mechanisms underlying FOXM1 activity in liver cancer progression remain to be elucidated. Experiments from the present study showed that TH/TR signaling suppresses FOXM1 mRNA and protein expression. Depletion of FOXM1 induced inhibition of the cell growth rate and a decline in oncogenic cyclin D1, cyclin E and CDK2 expression. Conversely, overexpression of FOXM1 enhanced cell proliferation and expression of oncogenic factors, which was decreased upon FOXM1 depletion. Re-expression of FOXM1 partially rescued suppression of cell proliferation induced by T_3. Collectively, the present findings suggest that TH/TR participates in HCC progression via modulation of FOXM1 expression.

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Introduction

Thyroid hormones (TH) are multifunctional mediators that fine-tune several physiological processes, including metabolic rate, digestive function and tissue development (1,2). T_4, the major TH secreted into the bloodstream by the thyroid gland, is converted to its active form, T_3, by type I and II deiodinases (D1 and D2) that vary among tissues, resulting in tissue-specific distribution of circulating T_3 with the capability to bind nuclear thyroid hormone receptors (TR) (3). Upon binding of TH, TRs interact specifically with thyroid hormone response elements (TRE) of target gene promoter regions to regulate their transcription (4,5).

TRs are type II nuclear receptors encoded by two separate genes, THRA (NR1A1) and THRB (NR1A2). THRA encodes one functional T_3-binding TRα1 while TRα2 and TRα3 have been identified as splice variants with no T_3 binding ability. TRΔα1 and TRΔα2 are truncated variants without DNA binding domains but retain T_3 binding ability and compete with other TRs. THRB genes encode three functional T_3-binding TRβ isoforms, specifically, β1, β2 and β3 (3). Truncated TRΔβ3 lacks the DNA binding domain but retains T_3 binding activity and acts as a dominant-negative antagonist, similar to TRΔα1 and TRΔα2 (6-9). Several studies to date have reported aberrant expression and/or somatic mutations of TR in human cancers (3,10-18), including hepatocellular carcinoma (HCC) (19-28).

HCC is the major histological subtype of primary liver malignancy and one of the major causes of tumor-related mortalities worldwide (29). Liver cirrhosis is the premonitory symptom of HCC, with the majority of cases developing from cirrhotic livers (30).

In 1986, v-erbA, a mutant form of TR devoid of ligand binding ability borne by the avian erythroblastosis virus causing erythroleukemia was identified and subsequently shown to play a role in HCC in transgenic mice (4,31). Mutated or truncated forms of TRα and TRβ are expressed at high frequencies in human HCC (32-36). These mutant TRs display loss of transcriptional activity, along with defects in release and binding of ligand-driven corepressor, and further act as dominant-negative forms, highlighting an association between aberrant TR regulation and HCC (1).

In rat models of HCC generated by a combination of diethylnitrosamine (DEN) and partial hepatectomy, 2-acetylaminofluorene or a High-fat choline-methionine-deficient diet, T_3/TR signaling was shown to suppress carcinogenesis via induction of a preneoplastic hepatocyte differentiation program (2). Disruption of TH signaling promoted tumorigenesis, particularly in HCC (2). Analysis of the genes modulated by T_3/TR in HCC should therefore facilitate elucidation of the mechanisms underlying HCC progression. Previous cDNA microarrays conducted by our group to evaluate target gene expression following T_3 treatment of wild-type TR-expressing hepatoma cells led to the identification of forkhead box M1 (FOXM1), also known as hepatocyte nuclear factor (HNF)-3, HNF-3/fork head homologue-11, membrane palmitoylated protein 2, winged-helix transcription factor or Trident (37,38), as a molecule negatively regulated by T_3.

FOXM1 is characterized by the presence of the DNA-binding domain Forkhead/Winged-helix domain (FKH) (39). Earlier cDNA microarray data suggested that FOXM1 is negatively regulated by T_3 in a TRα1-overexpressing hepatoma cell line (40). Similar to TR, FOXM1 participated in the cellular developmental pathway and maintenance of homoeostasis by activating multiple target genes regulating DNA damage repair, cell proliferation, cell cycle progression, renewal, differentiation, migration, angiogenesis and survival (41). Deregulation of FOXM1 signaling was shown to trigger malignant transformation (42). The mechanisms underlying signal transduction of FOXM1 contributing to tumor growth have been partially elucidated and appear to involve interplay with PI3K, ERK, epidermal growth factor receptor, estrogen receptor, vascular endothelial growth factor and reactive oxygen species (37,41,43), but require further clarification. Data from the current study indicated that TH/TR-modulated FOXM1 participates in HCC progression via affecting downstream gene expression, further highlighting the biological importance of TH/TR homeostasis.

Materials and methods

Cell culture and T_3 depletion. Human hepatoma cell lines J7 (provided by Dr CS Yang, National Taiwan University, Taiwan) (44), Mahlavu, SK-Hep1, HepG2 (American Type Culture Collection) and Huh7 (provided by Dr TY Hsieh, Tri-Service General Hospital, Taiwan) (45) were routinely grown in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (EMD Millipore), L-glutamine, 1% penicillin/streptomycin (PS) and non-essential amino acids (NEAAs). HepG2 cell lines stably transfected with TRα1 (HepG2-TRα1), TRβ1 (HepG2-TRβ1) or vector control (HepG2-neo) have been previously established in our laboratory (46). To generate T_3-depleted FBS (Td-FBS), AG 1-X8 resin (Bio-Rad Laboratories, Inc.) was washed three times with distilled, deionized water (15 min each time), pelleted by brief centrifugation (6,000 x g, 5 min, room temperature) and sterilized by autoclaving. T_3 of FBS (50 ml) was depleted by incubation with 2.2 g AG 1-X8 resin three times each for 5 h and filtered. Cells were cultured in DMEM with 1% PS, L-glutamine, NEAAs and 10% Td-FBS and treated with 0 nM T_3 (control group, Neo T_3 0 nM, FOXM1 T_3 0 nM) or 10 nM T_3 (experimental group, Neo T_3 10 nM, FOXM1 T_3 10 nM) for

24 and 48 h at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. The cell lines were authenticated using the Promega StemElite ID system (Promega Corporation), a short tandem repeat-based assay (25).

Establishment of FOXM1 knockdown and overexpression cell lines. FOXM1 short hairpin RNA (shRNA) TRCN0000015544 (shFOXM1#1; 5'-CCGGGCCCAACAGGAGTCTAATCAAC TCGAGTTGATTAGACTCCTGTTGGGCTTTTT-3') and TRCN0000015547 (shFOXM1#2; 5'-CCGGCGCCGGAACAT GACCATCAAACTCGAGTTTGATGGTCATGTTCCGGC GTTTTT-3') and pLKO.1-shLuc (control group) were purchased from the RNA interference core laboratory (Academia Sinica, Taipei, Taiwan). A total of 5 μg shRNA and 5 μg lentiviral package plasmids were co-transfected in 293TN cells (System Biosciences, LLC) cultured in DMEM with 1% PS, L-glutamine, NEAAs and 10% FBS using a TurboFect reagent kit (Thermo Fisher Scientific, Inc.) to produce viruses. After 24 h, viral supernatant was collected by centrifugation (4,000 x g, 5 min, room temperature) for infection of J7 and Mahlavu cell lines as these cell lines expressed lower levels of FOXM1 following viral infection for FOXM1 depletion. 48 h after infection, cells were transferred to DMEM with 1% PS, L-glutamine, NEAAs, 10% FBS and puromycin for selection. After 48 h of incubation, FOXM1 expression was confirmed via western blotting. The control groups for J7 and Mahlavu (shluc#1 and shluc#2) were established by two independent viral infections of pLKO.1-shLuc. The FOXM1 coding sequence (NM_021953) was cloned into a pcDNA3.1 expression vector (Addgene, Inc.). A total of 5 μg FOXM1-pcDNA3.1 (Neo-FOXM1) and 5 μg control plamid pcDNA3.1 (Neo) were transfected into SK-Hep1 and Huh7 cells with TurboFect reagent for 24 h. The sequences of primer pairs used for FOXM1 were as follows: Forward, 5'-CGCGGATCCGCGATGAAAACTAGCCCCCGTCGGC-3' and reverse, 5'-CCGGAATTCCGGCTACTGTAGCTCAGG AATAAAC-3'.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from HepG2-TRα1, HepG2-TRβ1 and HepG2-neo cells was purified using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the supplier's protocol and reverse-transcribed into cDNA using a Superscript II kit according to the manufacturer's protocol (Thermo Fisher Scientific, Inc.). The reaction was performed in a 25-μl reaction mixture containing 50 nM forward and reverse primers, 1X SYBR Green reaction mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 50 ng cDNA template. The following thermocycling conditions were used for the qPCR: Initial denaturation for 10 min at 95˚C, followed by 40 cycles of 95˚C for 15 sec and final extension at 60˚C for 1 min. Fluorescence emitted by SYBR Green was detected on line using a ABI PRISM 7500 sequencer (Applied Biosystems; Thermo Fisher Scientiic, Inc.). Studies have shown that the initial copy number can be quantified during RT-qPCR analysis based on the threshold cycle (Ct). The Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background. All PCRs were perfomed in duplicate on the same 96-well plate. For quantification of gene expression changes, the $2^{-\Delta\Delta Cq}$ method (47) was used to calculate relative-fold changes normalized to 18S ribosomal RNA expression (18S rRNA) as

described by the manufacturer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primer pairs were used for the qPCR: FOXMI forward, 5'-TCCTCAGCTAGCAGC ACCTTG-3' and reverse, 5'-CCAGGTGTTTAAGCAGCA GA-3' and 18S rRNA forward, 5'-CGAGCCGCCTGGATA CC-3' and reverse, 5'-CCTCAGTTCCGAAAACCAACAA-3'.

Western blot analysis. Total cell lysates of HepG2, J7, Mahlavu, SK-Hep-1 and Huh7-cells were prepared using cell lysis buffer (cat. no. 9803; Cell Signaling Technology, Inc.) and protein concentrations were determined using a Bradford assay kit (Pierce; Thermo Fisher Scientific, Inc.). Western blot experiments were performed as previously described (46). Equal amounts of protein (60 μg/lane) were separated by 8-10% SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes and blocked in 5% milk for 1 h at room temperature. Following blocking, incubation with the following primary antibodies was performed over-night at 4°C: Rabbit anti-human FOXM1 polyclonal antibody (cat. no. sc-502; 1:1,000; Santa Cruz Biotechnology, Inc.) mouse anti-human Cdk2 monoclonal antibody (cat. no. sc-6248; 1:1,000; Santa Cruz Biotechnology, Inc.), rabbit anti-human cyclin E polyclonal antibody (cat. no. 07-687; 1:1,000; Sigma-Aldrich; Merck KGaA), mouse anti-human mono-clonal antibodies against β-actin (cat. no. MAB1501; 1:8,000; Sigma-Aldrich; Merck KGaA) and GAPDH (cat. no. MAB374; 1:8,000; Sigma-Aldrich; Merck KGaA) and rabbit anti-human cyclin D1 monoclonal antibody (cat. no. EPR2241; 1:1,000; Epitomics; Abcam). Following primary antibody incubation, membranes were incubated with the following secondary antibodies for 1 h at room temperature: Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; cat. no. AP124P; 1:5,000; Sigma-Aldrich; Merck KGaA) and peroxidase-conju-gated goat anti-rabbit IgG (cat. no. AP132P; 1:5,000; Sigma-Aldrich; Merck KGaA). Protein bands were visual-ized using Immobilon Western Chemiluminescent HRP substrate (cat. no. WBKLS0500; Millipore; Merck KGaA). Quantification was performed using Image Gauge v3.46 (Fujifilm Holdings Corporation) and relative expression was normalized to b-actin or GAPDH expression.

Dual-luciferase reporter assay. To clone the FOXM1 5'-flanking region for the promoter activity assay, fragments of the FOXM1 promoter (positions -1560 to +22) were amplified with the respec-tive forward (5'-CCGGGTACCTCTCTTCCTCTCTCTCTC TCC-3') and reverse (5'-CCGCTCGAGCAGTTTGTTCCGCTG TTTG-3') primers based on the published nucleotide sequence (GenBank accession no. NT009759) and cloned into a pGL3-Basic vector (Promega Corporation). Two negative TRE (nTRE) motifs, NM23H1 and hTSHβ-N, were applied to Vector NTI advance 11 software (Invitrogen; Thermo Fisher Scientific, Inc.) for the prediction of potential nTREs of the FOXM1 promoter sequence. Serially-deleted FOXM1 promoter fragments were amplified using the following primers: Forward, 5'-CCGGGTACCCCAACTGTT CTGCCCTAATCCA-3' (-1480 to +22); forward, 5'-CCGGGTACC CACACCACACTCCATTCAGGTC-3' (-1300 to +22); forward 5'-CCGGGTACCCTCCAGAGTAGCTGGGACTACAGGCA-3' (-914 to +22); forward, 5'-CCGGGTACCGATTAAAATGTCTGT GCCCCTCTTCC-3' (-728 to +22) and reverse, 5'-CCGCTCGAG CAGTTTGTTCCGCTGTTTG-3'. To determine the transactiva-

tion activity of the TREs on the FOXM1 promoter, HepG2-TRα1 cells (5x10^5 cells/24-well dish) were cultured in DMEM with 1% PS, L-glutamine, NEAAs and 10% Td-FBS and transfected with 0.2 μg pGL3-Basic vector containing FOXM1 promoter sequences using the TurboFect reagent kit. Cells were also transfected with 0.05 μg β-galactosidase expression vector, pSVβ plasmid (Clontech Laboratories, Inc.). At 24 h after transfection, cells were treated with 0 or 10 nM T_3 for an additional 24 h, and were then lysed to measure luciferase and β-galactosidase activities using a Luciferase Assay System (cat. no. E1500; Promega Corporation). Luciferase activity was normalized to β-galactosidase activity.

Chromatin immunoprecipitation (ChIP) assay. HepG2-TRa1 cells (4x10^6) were cultured in DMEM with 1% PS, L-glutamine, NEAAs and 10% Td-FBS and treated with 0 or 10 nM T_3 24 h, then cross-linked by adding 1% (v/v) formaldehyde for 10 min at room temperature. The reaction was terminated by treatment with 0.125 M glycine. Following washing with ice-cold PBS, cells were resuspended in RIPA buffer [50 mM Tris, pH 8.0, with 0.1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 150 mM NaCl and 5 mM EDTA] containing protease inhibitors (1 mM each of PMSF, aprotinin and leupeptin). Solutions were soni-cated using a Misonix Sonicator 3000 homogenizer (Mandel Scientific Company Inc.). All samples were precleared by the addition of 60 ml protein A/G-agarose (Sigma-Aldrich; Merck KGaA) for 1 h at 4°C. Next, an anti-TR antibody (3 μg/ml) (provided by Dr SY Cheng, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA) or anti-IgG antibody (3 μg/ml; cat. no. MAB1101; R&D Systems, Inc.) was added and incubation proceeded at 4°C overnight before the addition of 80 ml protein A/G-agarose suspension for pulldown of relevant DNA/protein complexes. A fragment containing the predicted negative TRE (nTRE) of the FOXM1 promoter was amplified using the forward primer, 5'-CAACATTTGTTT GTTTTGGAGACGG-3' and reverse primer, 5'-AAAAATTAG CCGGGCGTGGT-3'. A fragment lacking the nTRE of GAPDH was detected using the forward primer, 5'-CAAGGCTGAGAA CGGGAAGC-3' and reverse primer 5'-AGGGGGCAGAGA TGATGACC-3', which served as a negative control.

Cell proliferation assay. The proliferative capacity of the aforementioned FOXM1 knockdown and overexpression cell lines was assessed by proliferation assay. Cells were seeded at a density of 3x10^4 cells/six-well plate routinely grown in DMEM with 1% PS, L-glutamine, NEAAs and 10% FBS. Trypsin was used to isolate cells from culture plates after 1, 3 and 5 days. Cells were then resuspended in 1 ml DMEM with 1% PS, L-glutamine, NEAAs and 10% FBS. A total of 10 μl cell suspension were stained with equal parts of 0.4% trypan blue, and live cells were counted using a LUNA-II™ Automated Cell Counter (Logos Biosystems). To assess the proliferatve capacity of T_3-mediated FOXM1 expression, 5 μg FOXM1-pcDNA3.1 (Neo-FOXM1) and 5 μg control plamid pcDNA3.1 (Neo) were transfected into SK-Hep1 and Huh7 cells with TurboFect reagent for 24 h, and cells were seeded at a density of 3x10^4 cells/six-well plate routinely grown in DMEM with 1% PS, L-glutamine, NEAAs and 10% Td-FBS and treated with 0 or 10 nM T_3 for 24, 72 and 120 h (days 1, 3 and 5). Trypsin was used to isolate cells from culture plates at indicated timepoints. Cells were resuspended in 1 ml DMEM
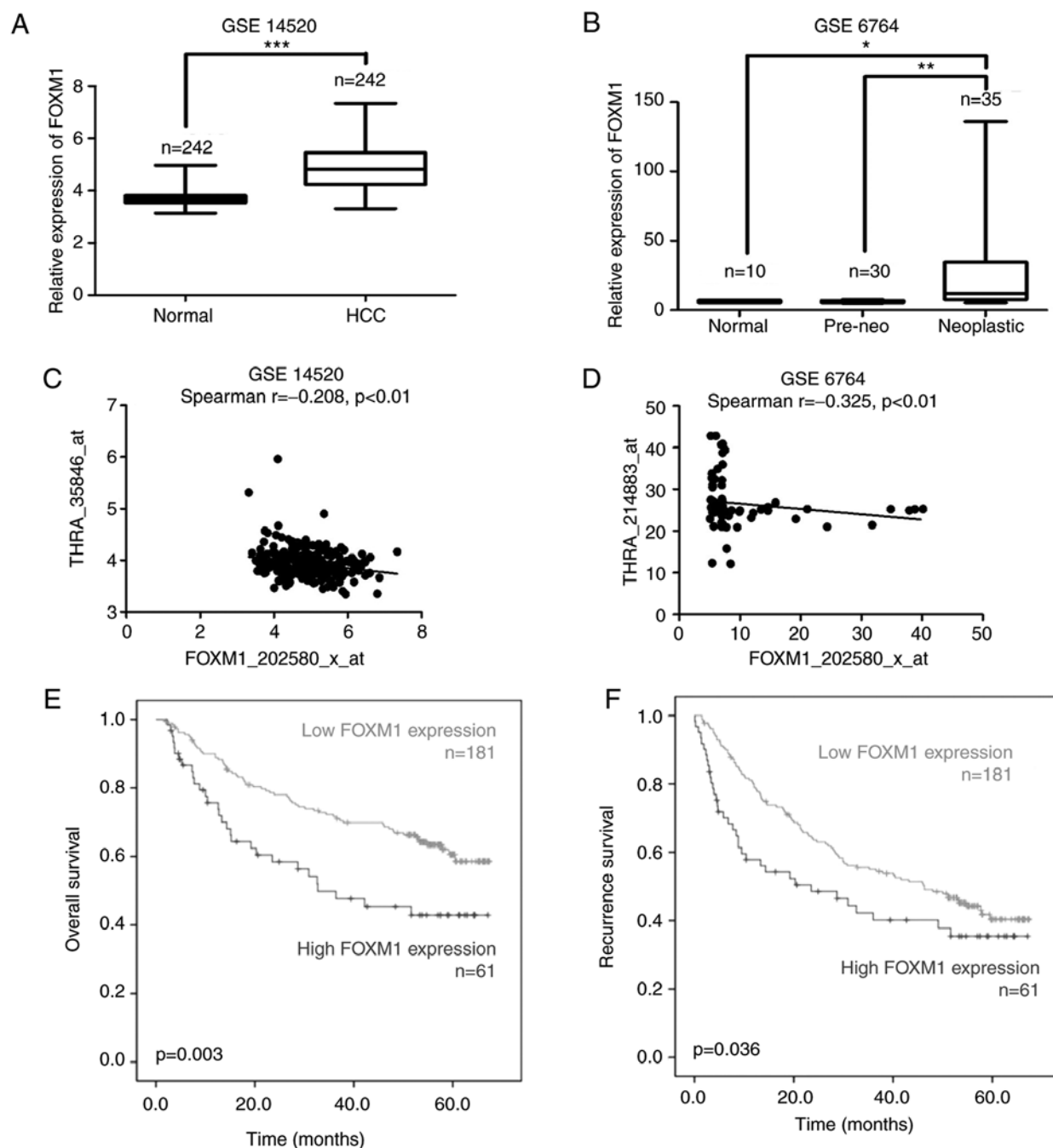
Figure 1. High expression of FOXM1 is associated with poor prognosis in HCC. Comparison of FOXM1 expression between normal and HCC tissues in two independent cohorts (A) GSE14520 and (B) GSE6764. Spearman's correlation analysis of THRα and FOXM1 expression in (C) GSE14520 and (D) GSE6764. (E) Kaplan-Meier survival analysis of FOXM1 in GSE14520. (F) Recurrence Kaplan-Meier survival analysis of FOXM1 in GSE14520.

with 1% PS, L-glutamine, NEAAs and 10% Td-FBS. A total of 10 μl cell suspension were stained with equal parts of 0.4% trypan blue, and live cells were counted using a LUNA-II™ Automated Cell Counter (Logos Biosystems).

Bioinformatics. Public microarray data from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) with accession numbers GSE14520 (48) (cohort 1), GSE6764 (49) (cohort 2) and GSE14323 (50) (data not shown) were analyzed. Raw gene expression data were normalized using the Robust Multi-array Average method and global median centering (51). Statistical analysis was performed using SPSS 20 (IBM Corp.).

Statistical analysis. Statistical analysis was performed using SPSS 20 (IBM Corp.). One-way ANOVA was used to compare the results obtained for more than one treatment. Data were analyzed using medians, standard deviations, one-way ANOVA and Tukey's Honest Significant Difference post hoc test. Differences between grpups with two independent variables were analyzed using two-way ANOVA and Bonferroni's post hoc test. Differences between groups were analyzed with Student's t-test. Data are presented the mean ± SD from at least three independent experiments. Expression of FOXM1 and TRα1 in GSE14520 and GSE6764 were analyzed by Spearman's rank correlation. Quartile (Q_3) expression levels of FOXM1 were
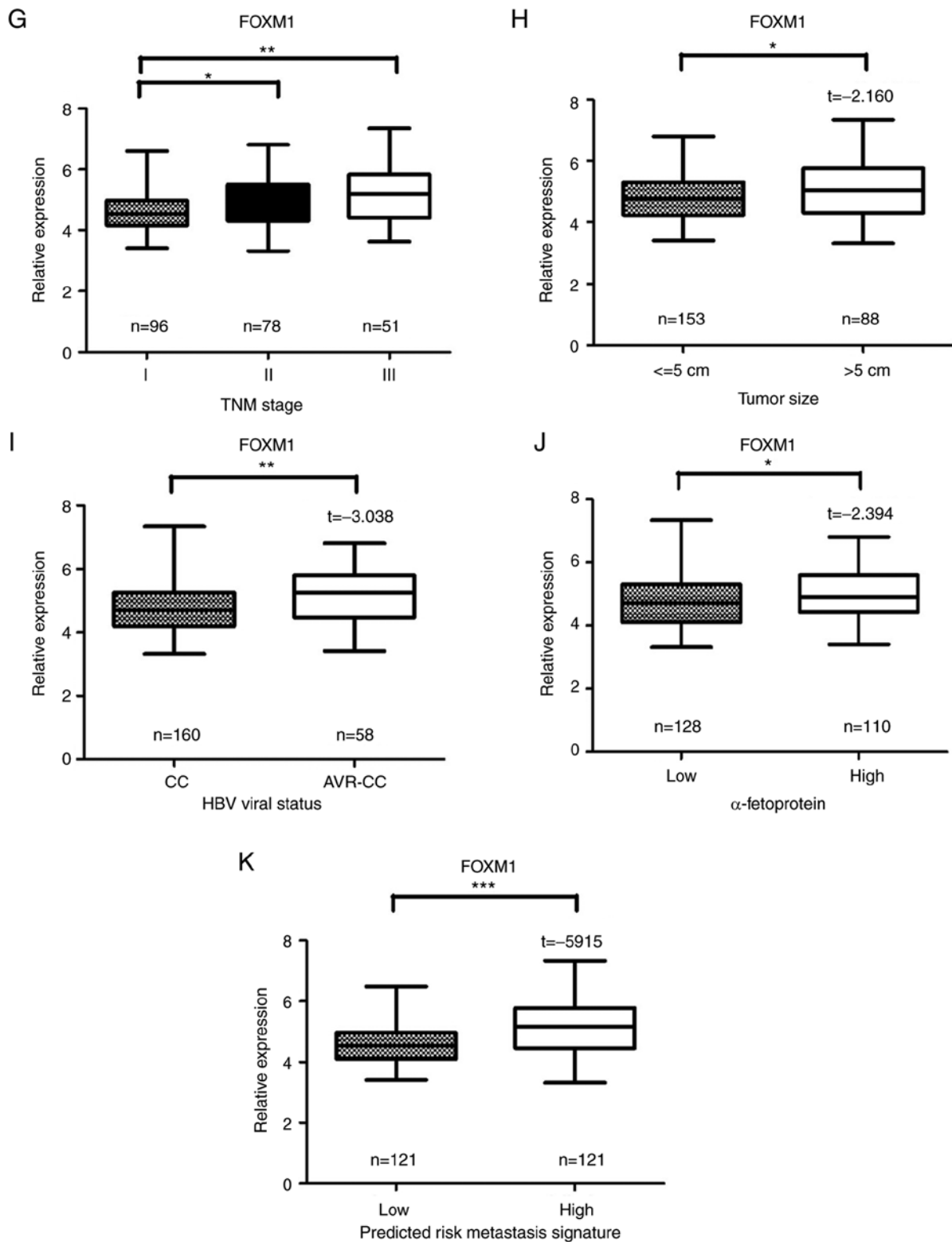
Figure 1. Continued. High expression of FOXM1 is associated with poor prognosis in HCC. (G) Expression analysis of FOXM1 with TNM staging. Analysis of clinicopathological parameters including (H) tumor size, (I) HBV viral status, (J) alpha-fetoprotein and (K) predicted risk metastasis signature of FOXM1 in GSE14520. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. AVR-CC, active viral replication chronic carrier; CC, chronic carrier; FOXM1, forkhead box protein M1; HCC, hepatocellular carcinoma; TNM, TNM Classification of Malignant Tumors; HBV, hepatitis B virus.

used as the cutoff, overall survival and recurrence survival were analyzed using the Kaplan-Meier survival analysis curve for high- or low-FOXM1 expression in 242-paired HCC patients of GSE14520. P-values were determined by the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

High expression of FOXM1 is associated with poor survival in HCC. FOXM1 expression was analyzed using GEO datasets, a web-based microarray database and data mining platform.
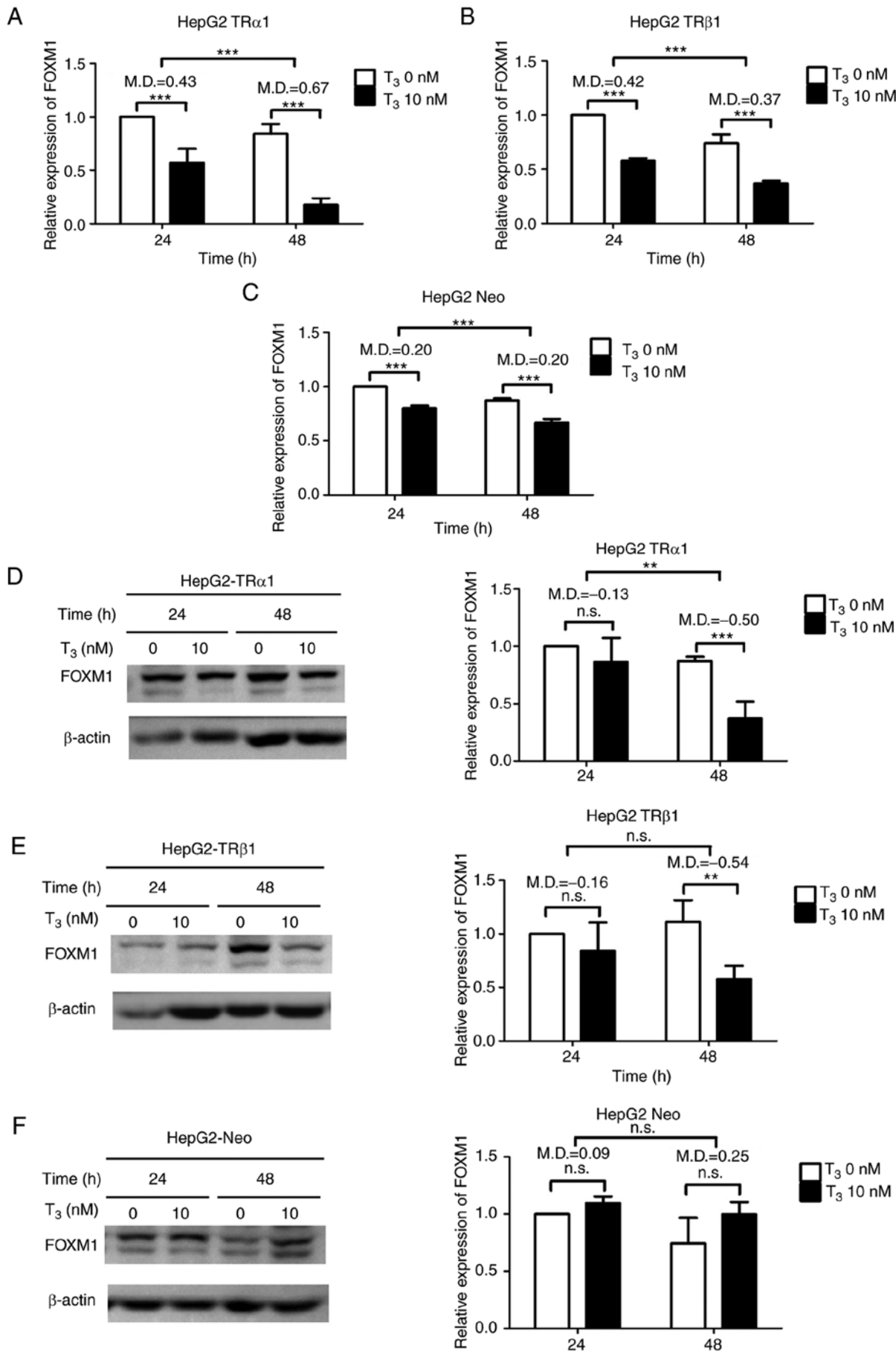
Figure 2. T_3/TR suppresses FOXM1 expression in HepG2 cells. Analysis of FOXM1 mRNA expression in (A) HepG2-TRα1, (B) HepG2-TRβ1 and (C) HepG2-Neo cells after treatment with 0 and 10 nM T_3 for 24 and 48 h. Protein expression of FOXM1 was analyzed in (D) HepG2-TRα1, (E) HepG2-TRβ1 and (F) HepG2-Neo cells after treatment with 0 and 10 nM T_3 for 24 and 48 h. Quantitative results are shown at the right panel.
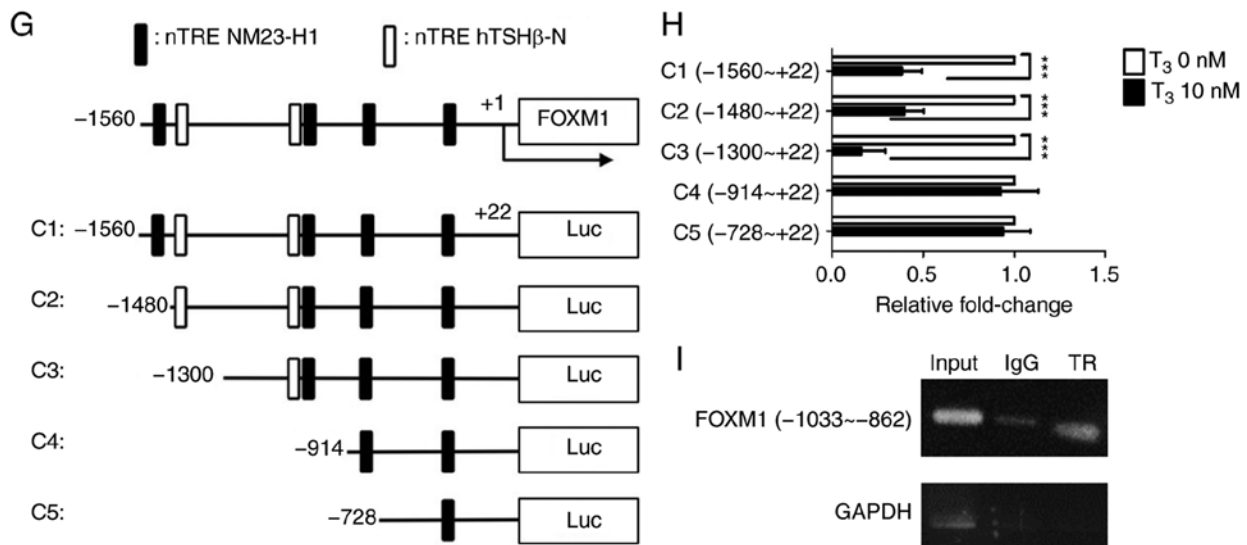
Figure 2. Continued. T_3/TR suppresses FOXM1 expression in HepG2 cells. (G) Illustration of predicted nTREs within the FOXM1 promoter region. (H) Reporter constructs containing serially deleted fragments of the FOXM1 5'-flanking region in a pGL3-luc vector were transfected into HepG2-TRα1 cells treated with or without 10 nM T_3. (I) For the chromatin immunoprecipitation assay, HepG2-TRα1 cell lysates were immunoprecipitated with rabbit nonspecific IgG or antibodies against TR. The promoter region of GAPDH acted as the negative control. Data are presented as the mean ± standard deviation. n=3. **$P<0.01$ and ***$P<0.001$. MD, mean of difference; FOXM1, forkhead box protein M1; TR, thyroid hormone receptors; nTRE, negative thyroid hormone response elements; ns, not significant; IgG, immunoglobulin G; C1, -1560~+22; C2, -1480~+22; C3, -1300~+22); C4, -914~+22); C5, -728~+22, Luc, pGL3-Basic vector containing serially deleted FOXM1 promoter fragments.

Analysis of three independent cohorts revealed significantly higher expression of FOXM1 mRNA in tumor regions of patients compared with normal tissues (Fig. 1A and B). Signifciantly decreased FOXM1 mRNA was accompanied by a concomitant increase in THRA (Fig. 1C and D) in cohort 1 and cohort 2. Although FOXM1 mRNA expression is inversely correlated with THRA mRNA expression, there was no significant difference of FOXM1 mRNA expression between tumor regions and normal tissues of patients in GSE14323 (data not shown). Further analysis of GSE14520 indicated that FOXM1 expression is negatively associated with overall survival (Fig. 1E) and recurrence survival (Fig. 1F). Significantly increased FOXM1 expression was detected at the late stages of HCC (Fig. 1gG and positively associated with specific clinicopathological parameters, including tumor size (Fig. 1H), hepatitis V viral status (Fig. 1I), alpha fetoprotein (Fig. 1J) and predicted metastasis risk signatures (Fig. 1K).

TH inhibits FOXM1 mRNA and protein levels in HCC cells. To determine the potential significance of T_3/TR in modulating FOXM1, HepG2-TRα1, HepG2-TRβ1 and HepG2-Neo (vector-control) cell lines previously established in our laboratory were used for experiments (46). FOXM1 mRNA was quantified after T_3 treatment of cells via RT-qPCR. In the presence of T_3, compared with controls, FOXM1 mRNA expression was significantly decreased in HepG2-TRα1 (Fig. 2A) and HepG2-TRβ1 (Fig. 2B) cells but less reduced in HepG2-Neo cells (Fig. 2C). Western blot analysis further demonstrated a significant decrease in FOXM1 protein expression in HepG2-TRα1 (Fig. 2D) and HepG2-TRβ1 (Fig. 2E) cells following treatment with T_3 for 48 h but not in HepG2-Neo cells (Fig. 2F). To ascertain whether T_3-induced repression of FOXM1 mRNA in HepG2 cells is mediated by direct effects of TR on transcription, the promoter region of FOXM1 with

two nTRE motifs, NM23H1 and hTSHβ-N, was analyzed. Several potential nTREs were identified in FOXM1 promoter regions from positions -1560 to +22 (Fig. 2G). Subsequently, serially-deleted FOXM1 promoter fragments were cloned into a pGL3-Basic vector. The promoter activity of FOXM1 was significantly repressed in fragments encompassing positions -1560 to +22, -1480 to +22 and -1300 to +22 with T_3 treatment compared with controls. However, the effects of T_3 were reduced in fragments -914 to +22 and -728 to +22 (Fig. 2H). Data from ChIP performed to validate the potential binding site of TR suggested that T_3 mediated suppression of FOXM1 promoter activity, with the promoter binding site of TR potentially located between positions -1033 and -862 (Fig. 2I). This indicated that T_3 suppressed both FOXM1 mRNA and protein expression in a concentration- and time-dependent manner, which is positively associated with TR expression.

Manipulation of FOXM1 expression in HCC cell lines affects cell proliferation. To ascertain whether FOXM1 participates in liver cancer progression, the present study established stable J7 and Mahlavu cells with shRNA-mediated FOXM1 knockdown (shFOXM1#1 and shFOXM1#2) and overexpression in SK-Hep1 cells via transient transfection of control pcDNA3.1 vector (Neo#1 and Neo#2) and pcDNA3.1-FOXM1 (FOXM1#1 and FOXM1#2). Depletion of FOXM1 led to significantly reduced growth of both cell lines at day 5 (Fig. 3A and B) compared with their respective controls (shluc#1 and shluc#2). Conversely, overexpression of FOXM1 induced a significant increase in the cell growth rate (Fig. 3C) compared with the vector-transfected control group. The signal transduction pathway associated with alterations in FOXM1 expression were additionally investigated. Depletion of FOXM1 led to significantly reduced expression of positive regulators of cell cycle progression (cyclin D1, cyclin E and CDK2) in both J7
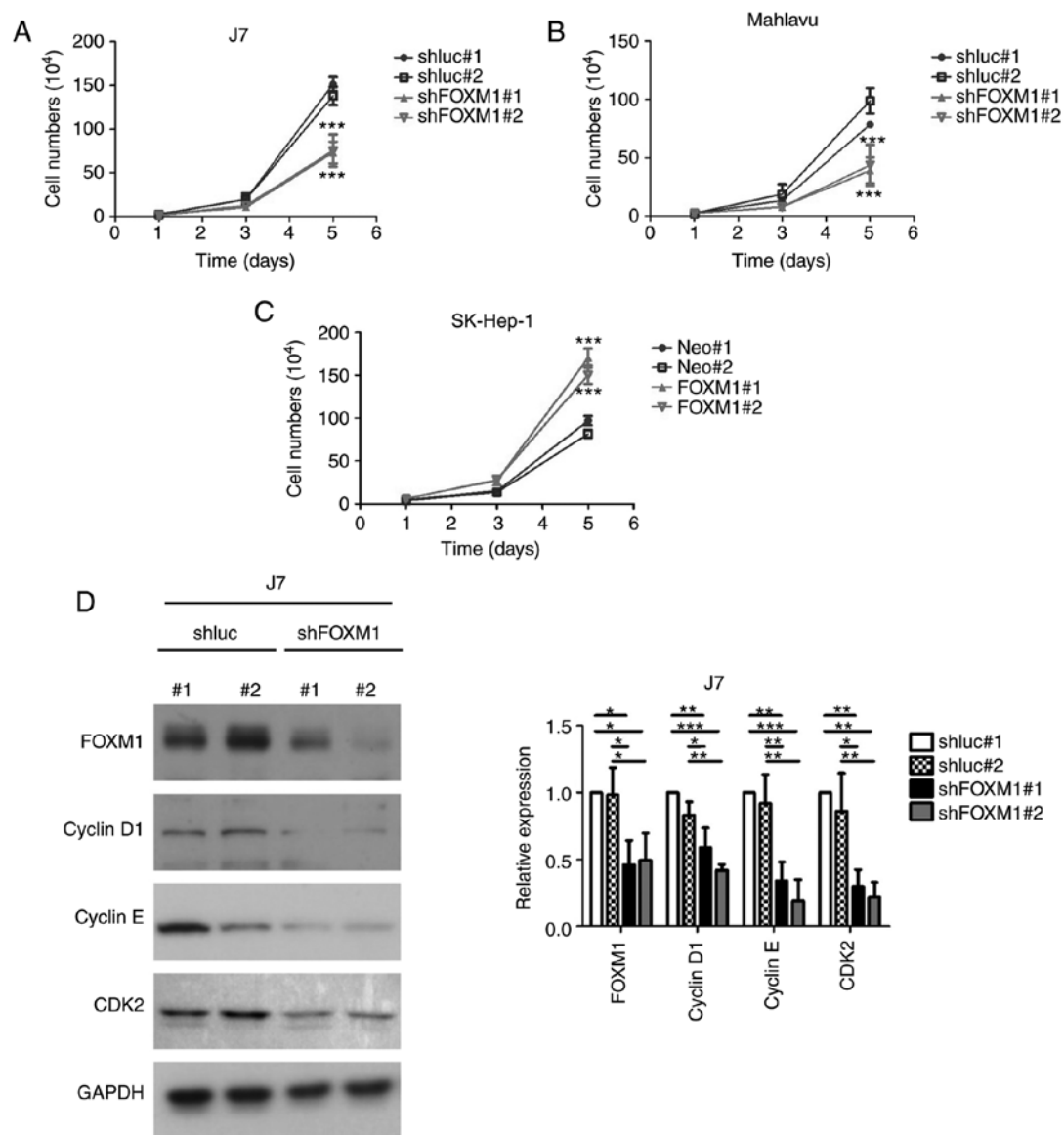
Figure 3. Manipulation of FOXM1 expression affects the proliferation rate of hepatocellular carcinoma cell lines. FOXM1 knockdown (shFOXM1#1 and shFOXM1#2) and control (shluc#1 and shluc#2) (A) J7 or (B) Mahlavu cells were seeded in six-well plates ($3x10^4$ cells) and counted at the indicated times (days 1, 3 and 5). (C) SK-Hep1 cells transfected with pcDNA3.1 vector control (Neo#1 and Neo#2) and pcDNA3.1-FOXM1 (FOXM1#1 and FOXM1#2) were seeded in six-well plates ($3x10^4$ cells) and counted at the indicated times (days 1, 3 and 5). Western blot analysis of cell cycle marker expression (cyclin D1, cyclin E and CDK2) in (D) J7.

(Fig. 3D) and Mahlavu cells (Fig. 3E) compared with their respective controls. Consistent with these findings, enhanced expression of FOXM1 significantly increased the levels of cell cycle-promoting cyclin D1, cyclin E and CDK2 (Fig. 3F). These results collectively demonstrated a tumor-promoting role of FOXM1 in HCC cells, highlighting the role of T_3-mediated regulation of FOXM1 in liver cancer.

Overexpression of FOXM1 in HCC cells leads to partial recovery of response to T_3 treatment. The suppressive effect of T_3 on cell proliferation rate was demonstrated in a previous study by our group (46). To determine whether T_3-mediated FOXM1 decline regulates cancer progression, FOXM1 was overexpressed in Huh7 and SK-Hep-1 cells subjected to T_3 treatment. Expression of FOXM1 was marginally decreased upon T_3 treatment due to low expression of endogenous TR

(Fig. 4A and B). The cell proliferation rate significantly decreased in Huh7 and SK-Hep1 cells (Neo) after T_3 treatment compared with controls. Re-expression of FOXM1 in Huh7 and SK-Hep1 cells (Neo-FOXM1) partially rescued suppression of cell proliferation induced by T_3 (Fig. 4C and D), supporting its role in T_3-mediated regulation of cell proliferation (Fig. 4E).

Discussion

Homeostasis of TH/TR signaling is critical for life processes via regulation of various downstream genes, and disruption of this fine-tuning can lead to malignancies (8). The liver has been identified as one of the targets of TR, with alterations in TH levels shown to trigger HCC (3). However, the underlying mechanisms remain to be elucidated.
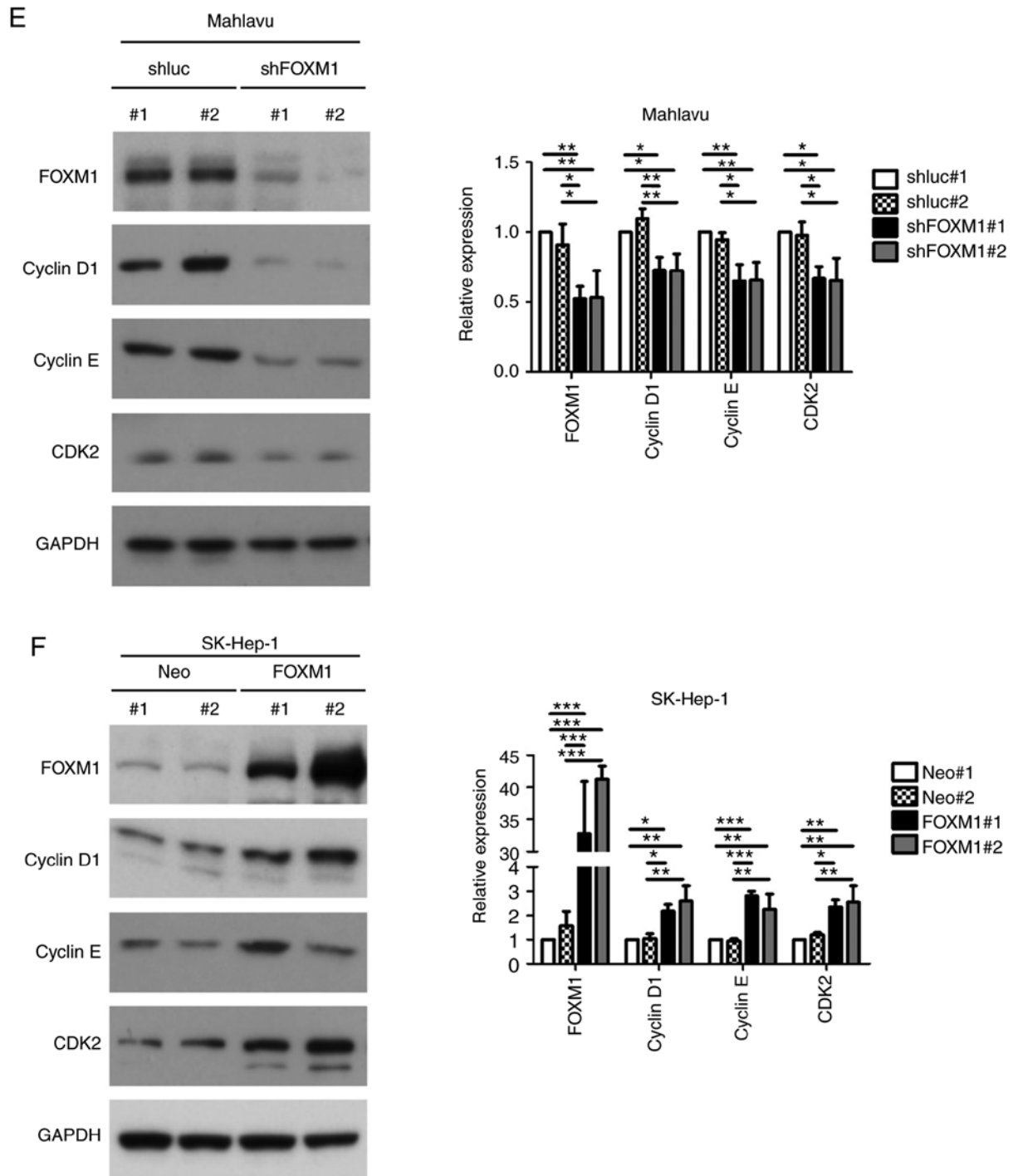
Figure 3. Continued. Manipulation of FOXM1 expression affects the proliferation rate of hepatocellular carcinoma cell lines. Western blot analysis of cell cycle marker expression (cyclin D1, cyclin E and CDK2) in (E) Mahlavu and (F) SK-Hep-1 cells. Quantitative results are shown at the right panel. Data are presented as the mean ± standard deviation. n=3. *P<0.05, **P<0.01 and ***P<0.001. FOXM1, forkhead box protein M1; sh, short hairpin RNA.

FOXM1 is highly expressed in several cancer types, including lung, ovarian, gastric and liver cancer (52). Data from the present study showed that TH/TR inhibited FOXM1 expression in HCC cell lines and FOXM1 and TR are negatively correlated in HCC, providing evidence supporting a tumor suppressor role of thyroid hormone signaling. A promoting role of FOXM1 in HCC development was reported by Kalinichenko et al (39), where depletion of FOXM1 had little effect in normal hepatocytes but suppressed HCC progression in a diethylnitrosamine (DEN)/phenobarbital (PB)-induced HCC mouse model, and depletion of FOXM1

following HCC establishment led to a significant decrease in tumor size. Park et al (42) further reported FOXM1 overexpression in the absence of p19Arf, a potent inhibitor of FOXM1. The researchers generated a bi-transgenic strain in which FOXM1 was expressed from the Rosa26 promoter in an Arf-/- (FOXM1bTg; Arf-/-) background. Mice developed highly metastatic HCC (>70%) following DEN/PB treatment. Metastasis significantly declined in the presence of one copy of Arf (FOXM1bTg; Arf+/- mice), supporting a critical role of FOXM1 in HCC development and its potential application as a therapeutic target.
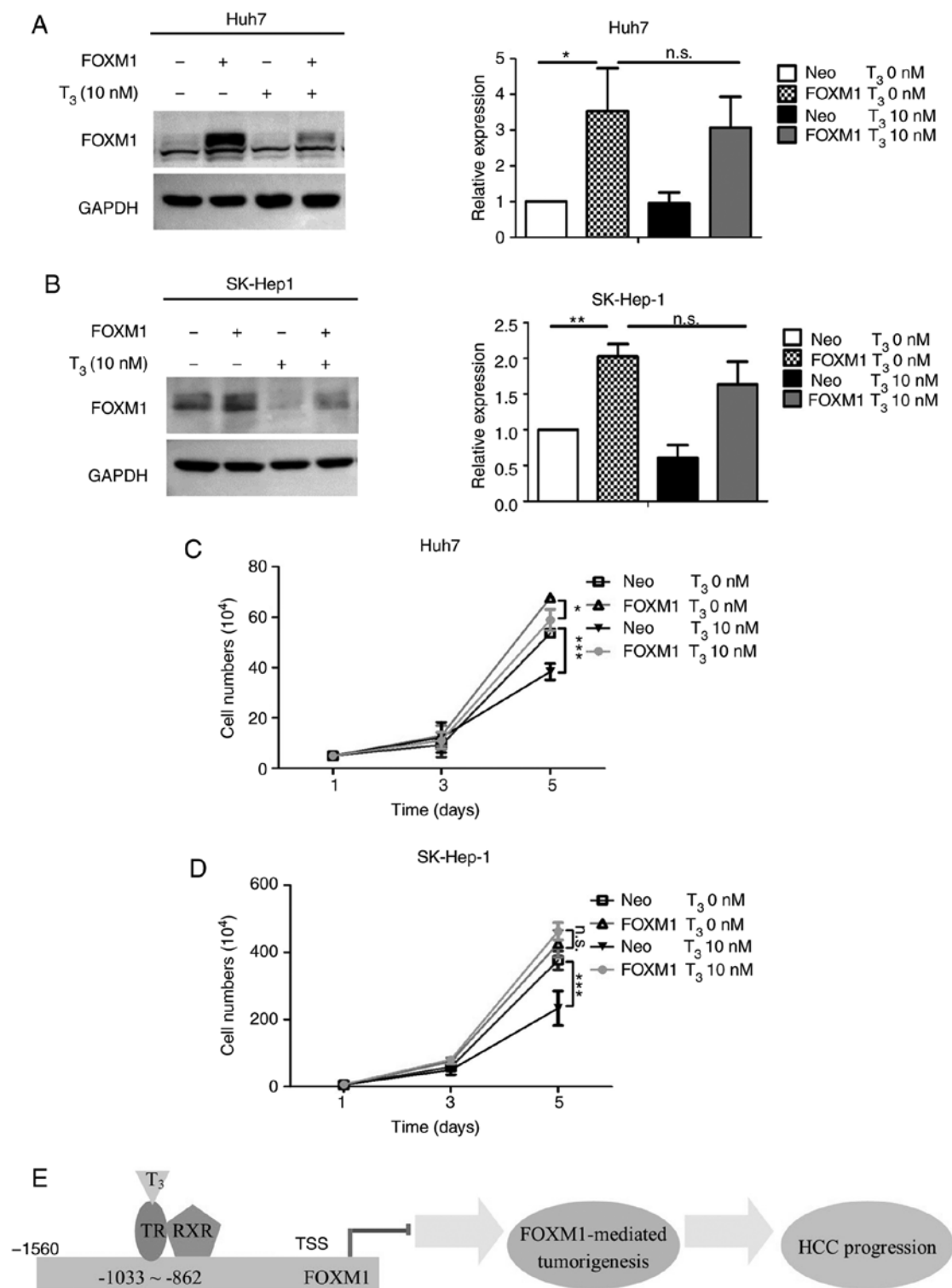
Figure 4. Overexpression of FOXM1 in HCC cells partially rescues suppression of proliferation induced by T_3. FOXM1 expression in (A) Huh7 and (B) SK-Hep-1 control cells (Neo) or FOXM1-overexpressing cells (FOXM1) with T_3 depletion (Neo T_3 0 nM, FOXM1 T_3 0 nM) or 10 nM T_3 (Neo T_3 10 nM, FOXM1 T_3 10 nM) for 24 h. Quantitative results are presented in the right panel. (C) Huh7 and (D) SK-Hep-1 Neo-Td, FOXM1-Td, Neo-T_3 and FOXM1-T_3 cells were seeded in six-well plates and counted at the indicated times (days 1, 3 and 5). (E) Schematic diagram of the mechanism underlying thyroid hormone/TR signaling-mediated suppression of FOXM1 to reduce cancer progression. The red line indicates repression. Yellow arrows indicate promotion. Data are presented as the mean ± standard deviation. n=3. *$P<0.05$, **$P<0.01$ and ***$P<0.001$. FOXM1, forkhead box protein M1; ns, not significant; HCC, hepatocellular carcinoma; TR, thyroid hormone receptors. RXR, Retinoid X receptor; TSS, translation start site.

CDKs and cyclins are involved in cell cycle progression. For instance, phosphorylation of retinoblastoma mediated by cyclin D/CDK4/CDK6 and cyclin E/CDK2 complexes contribute to cell cycle progression (53). Moreover, the cyclin E/CDK2 complex is reported to activate E2F transcription factors and other cell cycle-related genes, facilitating S-phase entry (54). Development of HCC is usually based on a background of chronic hepatitis and occurs as a multistep process

involving dysregulation of multiple cell cycle-related genes, including cyclin E (55). Investigation of the role of CDK2 in hepatocarcinogenesis in Cdk2Δhepa mice revealed significant reduction of tumor load following DEN treatment (56). In liver regeneration studies, Foxm1-/- hepatocytes of Alb-Cre Foxm1 fl/fl mice displayed reduced expression of the cyclin E/CDK2 complex (57), similar to FOXM1-depleted U2OS cells. Several studies reported that the cyclin E/CDK2 complex is critical for early tumorigenesis but dispensable for advanced tumor progression, highlighting the complexity of carcinogenesis and diverse roles of FOXM1 in tumor progression.

Drug resistance is a major challenge in cancer therapy. Several reports indicated that FOXM1 contributes to chemoresistance of various human carcinomas (52). Docetaxel (DTX) is a second-line chemotherapeutic drug for non-small-cell lung carcinoma. Compared to parental A549 cells, FOXM1 expression was significantly elevated in a DTX-resistant A549 cell line, while depletion of FOXM1 promoted DTX sensitivity of these cells (58). Increasing levels of FOXM1 upregulated stathmin to mediate microtubule dynamics, leading to tumor cell escape from DTX-induced apoptosis (59).

N6-methyladenosine has been identified as the most common internal modification of eukaryotic mRNAs, although its specific functions are yet to be elucidated (60). Recently, Zhang *et al* (61) showed that overexpression of the m6A demethylase, α-ketoglutarate-dependent dioxygenase alkB homolog 5 (ALKBH5), is required for proliferation and tumorigenesis of glioblastoma stem-like cells and FOXM1 nascent transcripts are demethylated by ALKBH5. This step promoted the interaction between FOXM1 pre-mRNA with HuR, thereby maintaining FOXM1 expression. ALKBH5-dependent gene expression provided insights into the pivotal role of RNA m6A methylation in cancer progression, supporting a therapeutic strategy involving targeting of RNA epigenetic modulators. In the analysis of the GSE14520 dataset (48) (data not shown), THRA mRNA expression was negatively correlated with the m⁶A methyltransferase, METTL3, but positively correlated with m⁶A demethylase fat-mass and obesity-associated protein (FTO) (data not shown). Additionally, FOXM1 mRNA expression was positively correlated with METTL3 and negatively correlated with FTO, suggesting the possibility that FOXM1 is regulated by TH/TR through RNA modifications (data not shown).

In conclusion, TH/TR signaling suppressed FOXM1 expression, which participated in HCC progression via exerting its effects on downstream gene expression, highlighting the biological significance of thyroid hormone homeostasis in maintenance of physiological cell functions.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CHW designed the study, performed the experiments, analyzed the data and wrote the manuscript. CTY analyzed and interpreted the data. KHL designed the study, analyzed the data, critically revised the manuscript and gave final approval of the version to be published. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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