Heliyon 9 (2023) e21519

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

TGF-β/SMAD canonical pathway induces the expression of transcriptional cofactor TAZ in liver cancer cells

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ARTICLE INFO

Keywords:
TGF-β cytokine
Hippo-pathway
TAZ cofactor
HepG2 cells
Liver cancer

ABSTRACT

The TGF-β and Hippo pathways are critical for liver size control, regeneration, and cancer progression. The transcriptional cofactor TAZ, also named WWTR1, is a downstream effector of Hippo pathway and plays a key role in the maintenance of liver physiological functions. However, the up-regulation of TAZ expression has been associated with liver cancer progression. Recent evidence shows crosstalk of TGF-β and Hippo pathways, since TGF-β modulates TAZ expression through different mechanisms in a cellular context-dependent manner but supposedly independent of SMADs. Here, we evaluate the molecular interplay between TGF-β pathway and TAZ expression and observe that TGF-β induces TAZ expression through SMAD canonical pathway in liver cancer HepG2 cells. Therefore, TAZ cofactor is a primary target of TGF-β/SMAD-signaling, one of the pathways altered in liver cancer.

1. Introduction

The TGF-β and Hippo pathways have a physiological relevance in health and disease, as they regulate multiple homeostatic processes from development to adulthood. In the liver, a major crosstalk between TGF-β and Hippo pathways is critical for organ size control, regeneration, and hepatocarcinogenesis. TGF-β and Hippo pathways are tumor suppressors in hepatocytes, as both inhibit cell proliferation and maintain homeostasis, and are essential players in liver regeneration, although they may promote hepatocarcinogenesis when their signaling pathways become altered [1,2].

TGF-β cytokine signals through a complex of transmembrane Ser/Thr kinase receptors. Once the type II receptor (TβRII) phosphorylates and activates the type I receptor (TβRI or ALK5), the receptor complex signals through SMAD2 and SMAD3 downstream effectors. The phosphorylated SMAD2 and SMAD3 form heterotrimeric complexes with co-SMAD4 for subsequent translocation to the nucleus, and the SMAD complexes bind to specific DNA sequences (AGAC or GTCT) called SBE (SMAD-Binding Elements), allowing the expression of TGF-β target genes [3]. In contrast, Hippo pathway negatively regulates the function of the transcriptional cofactors YAP (Yes-Associated Protein 1) and TAZ/WWTR1 (Transcriptional co-activator with PDZ-binding motif/WW domain-containing

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https://doi.org/10.1016/j.heliyon.2023.e21519
Received 6 April 2023; Received in revised form 20 October 2023; Accepted 23 October 2023
Available online 31 October 2023

Transcription Regulator 1), through a phosphorylation cascade initiated by MST1 and MST2 kinases to phosphorylate LATS1 and LATS2 (Large Tumor Suppressors 1 and 2) kinases [4]. The activated LATS1/2 kinases phosphorylate and inhibit YAP and TAZ, causing their nuclear exclusion and retention in the cytoplasm, and also promote their ubiquitination and degradation via the ubiquitin-proteasome system [4]. While YAP and TAZ have similar structures, regulations, and functions, they may have distinct roles. The inhibition of Hippo pathway promotes that YAP/TAZ proteins localize to the nucleus and act mainly through TEAD family transcription factors to stimulate the expression of many genes, such as *CYR61*, *CTGF*, *AXL*, *BIRC5*, and *AREG*, which are involved in cell proliferation and apoptosis [5]. In addition, YAP/TAZ cofactors interact with other transcription factors, such as SMADs, RUNX2, P73, and TBX5, to mediate cellular context-dependent gene regulation [4,5].

TGF-β and Hippo pathways are part of a signaling network that orchestrates numerous biological processes. The crosstalk between TGF-β and Hippo pathways occurs under different physiological and pathological conditions, varying in a context-dependent manner [6]. In response to TGF-β, YAP/TAZ promotes the accumulation of SMAD proteins in the nucleus, where they synergize transcriptionally [2,7]. Thus, YAP and TAZ provide a mechanism to couple the TGF-β/SMAD and Hippo pathways to cell confluence sensing [6]. Intriguingly, the promoters of several genes harbor both SBE and TEAD-binding elements; consequently, YAP/TAZ, TEAD, and SMAD2/3 may form complexes that regulate gene transcription. Thus, YAP/TAZ participate as transcriptional coregulators of TGF-β pathway; for instance, YAP/TAZ proteins, in cooperation with TGF-β signaling, stimulate the production of fibrogenic factors (CTGF and PAI-1) and extracellular matrix proteins [8]. In cancer, TAZ is upregulated in many primary tumors, including liver cancer [9], and interestingly, TGF-β signaling and YAP/TAZ may endow stem cells with tumorigenic phenotypes [7,8,10].

TAZ is ubiquitously expressed during embryogenesis and in adulthood, and regulates the expression of many genes associated with cell proliferation, stem cell differentiation, migration, and epithelial-mesenchymal transition (EMT). Also, it is expressed in many cancerous cells, such as those from liver, breast, pancreas, glioma, and neuroblastoma [9]. TAZ and YAP are thought to share similar regulatory mechanisms as they exhibit high similarity in their protein sequence; however, they may also have differential regulation. In the case of TAZ, its expression is regulated at the transcriptional level by different factors: MRTF, HIF1, SMAD3, and STAT3; whereas several miRNAs control *TAZ* mRNA translation. The TAZ protein stability and activity can be regulated by post-transcriptional modifications, such as phosphorylation, acetylation, O-GlcNacylation, ubiquitination, and methylation [11].

In contrast to *YAP* gene, the regulation of *TAZ/WWTR1* gene is poorly studied. Recently, there is evidence that TGF-β pathway regulates the expression of *TAZ/WWTR1* gene through different mechanisms. Thus, a non-canonical p38/MRTF-dependent and SMAD3-independent TGF-β pathway increases endogenous TAZ protein levels in fibroblasts-type and epithelial cells, as well as the expression of a transfected human TAZ reporter gene [12]. Likewise, the TGF-β/SMAD3 and IL-6/STAT3 pathways cooperate and synergize to induce mouse *TAZ* gene expression in $CD4^+$ lymphocytes to promote their differentiation towards Th17 cells, while TAZ expression deficiency promotes regulatory T cell (Treg) differentiation. In this scenario, TGF-β increases TAZ mRNA levels in a SMAD3-dependent manner, although its effect on TAZ protein levels has not been tested [13].

As mentioned, TGF-β pathway regulates human and mouse TAZ expression through distinct mechanisms, depending on cell-context and specie-specific gene promoter [12,13]. These reports demonstrate that TGF-β mainly regulates *TAZ/WWTR1* gene expression through SMAD-independent pathways or in synergy with other cytokine pathways. However, it was unclear if TAZ was a direct target gene of the canonical TGF-β pathway. Here, we reported that TGF-β through its canonical SMAD pathway induces TAZ expression, and subsequently increases TAZ protein levels in HepG2 cells, a model of cancerous hepatocytes. These findings demonstrate that TAZ transcriptional cofactor is a primary target of TGF-β/SMAD signaling, one of the main pathways altered in liver cancer. Furthermore, the consequent establishment of the TGF-β/SMAD/TAZ axis may generate specific genetic signatures in hepatocarcinogenesis.

2. Materials and methods

2.1. Materials and reagents

All reagents for cell culture were obtained from GIBCO-Invitrogen. Recombinant human TGF-β1 (TGF-β) was from PeproTech. Actinomycin D (ActD), SB431542 (SB43), and Verteporfin (VP) were from Tocris-Bioscience. Cycloheximide (CHX) was from Millipore. Anti-TAZ, anti-SMAD2, anti-phospho-SMAD2 antibodies were from Cell Signaling Technology; and HRP-conjugated anti-HA antibodies were from Roche. HRP-conjugated secondary antibodies were from Invitrogen, Jackson-ImmunoResearch or Santa Cruz Biotechnology, whereas anti-β-actin antibodies were from Santa Cruz Biotechnology.

2.2. Gene expression analysis using public datasets

Public datasets of normal liver and hepatocarcinoma (HCC) samples were obtained from TCGA (https://www.cancer.gov/tcga) or GTEx (https://getexportal.org), and the expression of genes encoding for TGF-β and Hippo pathway components were analyzed using two web servers: UALCAN (http://ualcan.path.uab.edu) [14] or GEPIA (https://gepia.cancer-pku.ca) [15]. A set of human liver cancer cell lines was analyzed using the CCLE (Cancer Cell Line Encyclopedia) database (https://portals.broadinstitute.org/ccle) [16]. These data were obtained as normalized RNA-seq values ($\log_2$ TPM+1) (TPM: Transcripts Per Million), and from a RPPA (Reverse Phase Protein Array) expressed as signal $\log_2$. The liver cancer cell lines analyzed were sorted as epithelial (E), epithelial-mesenchymal (E/M) or mesenchymal (M) according to previous classification [17].

2.3. Cell culture

Human HepG2 cell line was obtained from ATCC (HB-8065), and maintained in Minimal Essential Medium with 10 % FBS, 1 mM pyruvate, plus 1 % antibiotics (penicillin/streptomycin), under a 5 % CO_2 atmosphere at 37 °C. Cells were serum-starved for 12 h prior to any treatment.

2.4. Total RNA isolation and RT-PCR

Total RNA (2 μg), isolated using TRIzol (Invitrogen), was used for cDNA synthesis with random hexamers (Roche) and M-MLV Reverse transcriptase (Invitrogen), and PCR was carried out using Taq PCR Master Mix Kit (Qiagen) using specific primers for *TAZ/WWTR1* (Fw: GAGCACTGACAGTGGCCTG; Rv: CTCTACATCATTGAAGAGGGG), *CYR61* (Fw: CGAGGACTGCAGCAAAAC; Rv: AGTTCTTGGGGACACAGAG), *SMAD7*, *GAPDH*, and *β-ACTIN* (Supplementary Table S1), following the program: 95 °C × 5 min; 95 °C × 45 s; 60, 58 or 55 °C × 30 s; and 72 °C × 1 min.

2.5. Immunoblots

Cells were lysed with TNTE buffer as described previously [18]. Total proteins (50–100 μg) were separated by SDS-PAGE and detected by immunoblots, using an ECL kit (Millipore). Densitometry was carried out with FIJI software.

2.6. Bioinformatic analysis

The promoter region of the *TAZ/WWTR1* gene was obtained from the human genome database UCSC Genome browser (https://genome.ucsc.edu/). The genome assembly GRCh38/hg38 was used to analyze *TAZ/WWTR1* gene (genomic coordinates: chr3:149,517,235−149,658,025), and to identify the promoter region by using specific tracks for CpG-islands, DNase I hypersensitive sites (DHS), transcriptional start site (TSS), multiple transcription factor binding sites (Txn factor), and specific histone marks, using public data from Encyclopedia of DNA Elements (ENCODE) portal (PMID: 31713622; PMCID: PMC7061942). Moreover, the positions of *TAZ/WWTR1* gene exons were delimited according to NCBI reference sequence NM_015472.6 for *TAZ* mRNA (transcript variant 1). Thus, a region of ~1.13 kb (genomic coordinates: chr3:149,657,951−149,659,084) from −1059 to +75 from TSS (+1) was defined as *TAZ/WWTR1* (hTAZ) gene promoter region and analyzed to predict putative SBE motifs using two web servers: ALGGEN-PROMO [19] and Jaspar [20]. In addition, public ChIP-seq data were obtained from the Gene Expression Omnibus (GEO) data listings for ENCODE to identify the interacting sites of SMAD2/3/4 transcription factors in different cell lines, including HepG2, HUES-8 (human embryonic stem cells), HASMC (human aortic smooth muscle cells), and HUVEC (human umbilical vein endothelial cells) (Supplementary Fig. S1).

2.7. Cloning of human TAZ/WWTR1 gene promoter and plasmid constructs

Two fragments (1.13 kb and 407 bp) of *TAZ/WWTR1* gene promoter were amplified by PCR from human genomic DNA obtained from freshly isolated blood cells using Quick DNA Miniprep plus kit (Zymo Research). PCR was carried out using Phusion Hot Start II High-Fidelity DNA polymerase and specific primers flanked by *Kpn*I and *Sac*I restriction sites (Fw(hTAZ-1.13): GATAggtaccGGGCAAGGCCTCCTCCT; Fw(hTAZ-407): GGGCgagctcAGCCTGTTTCATCAAGG; Rv: TAGAgagctcGGCCTAAGGCGCTAGTG) (Supplementary Table S1). The PCR program used was: 98 °C × 30 s; 98 °C × 10 s; 65 °C × 20 s; 72 °C × 30 s; and 72 °C × 5 min, during 30 cycles. These *TAZ/WWTR1* gene promoter fragments were located at position −1059/+75 (1.13 kb) and −332/+75 (407 bp) from TSS (+1), and were cloned into pGEM-t easy, and then subcloned into *Kpn*I and *Sac*I sites of pGL3-Basic vector to obtain the following reporter genes: hTAZ(1.13)-Luc and hTAZ(407)-Luc. Mutated hTAZ(1.13mutTRE)-Luc reporter plasmid was generated by site-directed mutagenesis on the hTAZ(1.13)-Luc template using specific primers (Fw: tagttaccAATTGAGAGCCAGGCAGC; Rv: gagcagattGGTGCCCAGAAAGTGCCT) and the Q5 site-directed mutagenesis kit (New England BioLabs). In hTAZ promoter region, the wild-type sequence 5'CGTCTGTGCTCTAGCAGACAA3' (located at −636 to −616 from TSS+1) harboring two SBEs (underlined) was mutated to 5'Caatc−TGCTCTAGttacCAA3', where 8 bases (in lowercases) were changed, and 1 base (−) was deleted. All constructs were sequence-verified.

2.8. Cell transfection and reporter gene assay

HepG2 cells were transiently transfected with each pGL3-basic/hTAZ-Luc reporter plasmids with or without any of the following plasmids: pCMV5/TβRI(WT)-HA (ALK5 wild-type or ALK5(WT)), pCMV5/TβRI(T204D)-HA (constitutively activated ALK5 or ALK5 (TD)), pCMV5/Flag-SMAD2 (S2), pCMV5/Flag-SMAD3 (S3), or pCMV5/HA-SMAD4 (S4), or in the presence of 3XFlag-pCMV5-TOPO-TAZ(S89A) (No. 24815) from Addgene [7] or pBABE-puro-H-RasV12 (No. 9051) from Addgene. Luciferase activity was measured using a kit (Promega) and a Luminometer $20/20^n$ (Turner Biosystems) [18].

2.9. Chromatin immunoprecipitation (ChIP) on plasmid assay

HepG2 cells were transiently transfected with 0.9 μg of hTAZ(1.13)-Luc, along with or without 0.9 μg pCMV5/TβRI(T204D)-HA or

empty vectors by the Lipofectamine 2000 method. Cells were crosslinked with 1 % formaldehyde for 10 min at room temperature and stopped with 125 mM glycine, and then washed 4X with cold PBS. Cells were lysed with 1 ml of lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 1 % SDS) plus a cocktail of protease- and phosphatase-inhibitors [18,21]. Samples were sonicated on ice for 4 cycles (35 % amplitude) with a Fisher Sonic Dismembrator 300, and diluted 1:5 in a buffer (1 % Triton X100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), and chromatin was pre-cleared in batches; 50 μg chromatin was used per IP using anti-SMAD2 antibodies. DNA was assayed by PCR using specific primers (Fw: CTAGCAAAATAGGCTGTCCC; Rv: CGATTCTGCCCGAAGGCCGG) spanning the region harboring the canonical SBE motifs and also part of the pGL3-basic vector [18,21].

2.10. Statistical analysis

Densitometry analysis was carried out with Image J 2.0 (FIJI) free software. Graphics and statistical analysis were performed using GraphPad Prism version 9.0 software. Data were expressed as mean ± SEM (Standard Error of the Mean). A Student's *t*-test was used to calculate statistical significance; a P value < 0.05 was considered significant.

3. Results

3.1. The expression of hippo- and TGF-β-pathway components are increased in human liver cancer

In order to investigate a relationship between the expression levels of Hippo- and TGF-β-pathway components in human liver cancer, we analyzed public liver hepatocellular carcinoma (LIHC) gene expression datasets from TCGA and GTEx. First, UALCAN was used to analyze the expression levels of the Hippo pathway effector *TAZ/WWTR1*. We also analyzed the levels of TGF-β-pathway components (*TGF-β1*, *TGF-β2*, *TGF-β3*, *SMAD2*, *SMAD3*, and *SMAD4*), and the levels of some EMT-markers (*SNAI1*, *SNAI2*, and *VIMENTIN*) in LIHC, using datasets obtained from TCGA. The heat map showed increased mRNA levels of all these components in liver tumor samples (n = 371) in comparison with normal liver samples (n = 50) (Fig. 1A and Supplementary Fig. S1). We also analyzed public LIHC datasets from TCGA and GTEx using GEPIA. The heat map showed increased mRNA levels of *TAZ/WWTR1*, *TGF-β1*, *SMAD2*, and *SMAD3* in liver tumor samples (n = 369) in comparison with normal liver samples (n = 160) (Fig. 1B). Data showed higher levels of expression of *TAZ/WWTR1*, *TGF-β1*, *SMAD2,* and *SMAD3* in liver cancer in comparison to normal liver.

Then, we focused in analyzing the dataset of 20 liver cancer cell lines obtained from CCLE database. We compared the TAZ mRNA

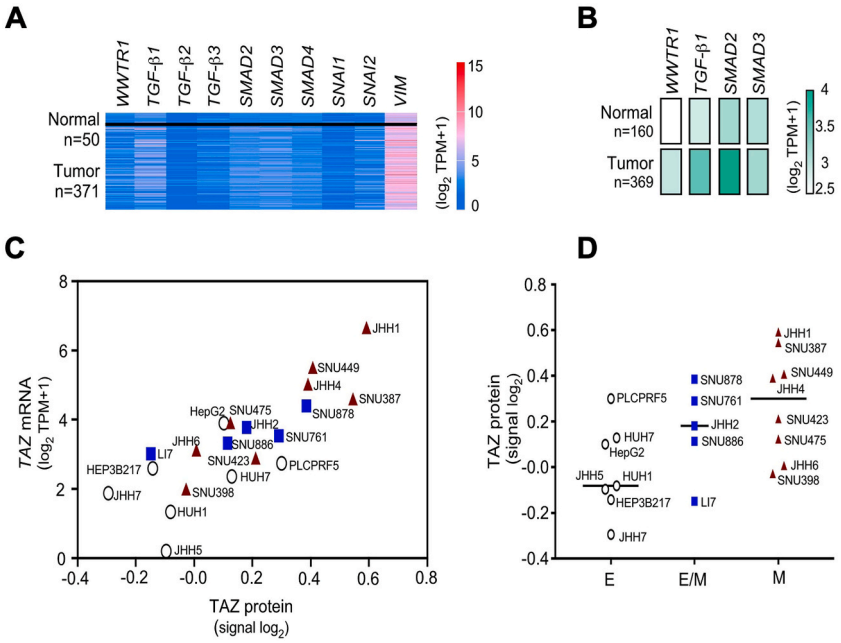

Fig. 1. The expression of TGF-β ligands, SMADs, and TAZ are increased in human liver cancer. **(A)** Heat map for gene expression of TAZ/WWTR1, TGF-β pathway components (TGF-β1, TGF-β2, TGF-β3, SMAD2, SMAD3, and SMAD4), and EMT-markers (SNAI1, SNAI2, and VIMENTIN) was obtained analyzing LIHC dataset from TCGA, using UALCAN. The mRNA levels (TPM) were compared between normal liver samples (n = 50) *versus* tumor samples (n = 371). **(B)** Heat map for gene expression of TAZ/WWTR1, and TGF-β pathway components (TGF-β1, SMAD2, and SMAD3) was obtained by analyzing LIHC dataset from TCGA and GTEx, using GEPIA. The mRNA levels (TPM) were compared between normal liver samples (n = 160) *versus* tumor samples (n = 369). The heat maps were modified from the originals for better visualization. **(C)** Correlation between the levels of TAZ mRNA and protein of 20 HCC cell lines, and **(D)** TAZ protein levels of 20 HCC cell lines sorted by phenotype: epithelial (E) (circles), epithelial-mesenchymal (E/M) (squares) or mesenchymal (M) (triangles). Data were replotted from the original CCLE data for better visualization.

and protein levels among all 20 liver cancer cell lines (Fig. 1C), and also we analyzed TAZ protein levels in all 20 cell lines but according to their sorting by phenotype: E (Epithelial), E/M (Epithelial/Mesenchymal), or M (Mesenchymal) (Fig. 1D). Data showed a correlation between TAZ mRNA and protein levels (Fig. 1C), and higher TAZ protein levels in liver cancer cells with E/M and M phenotype than in those with an E phenotype (Fig. 1D).

3.2. *TAZ expression is increased by TGF-β signals*

YAP/TAZ expression could be regulated through multiple transcriptional factors and mechanisms, including transcriptional and epigenetic regulations [11]. Although YAP has been more studied than TAZ, the exact regulatory mechanisms of YAP/TAZ expression in different tissues or cell lines remain to be defined. Considering the crosstalk between Hippo- and TGF-β-signaling pathways in liver homeostasis and their deregulation in liver cancer [22], and also that the regulation of *TAZ/WWTR1* gene by TGF-β depends on cell-context and is specie-specific, we aimed to investigate the regulation of human TAZ expression by TGF-β/SMAD canonical signaling in a liver cancer cell context using HepG2 cells, an epithelial cell line that retain some normal characteristics of hepatocytes.

HepG2 cells were pre-treated in the absence or presence of 10 μM SB43, and then treated for the indicated times with 0.3 nM TGF-β; cells were also treated only with 10 μM SB43 for the indicated times. Data showed that TGF-β promoted a gradual increase of TAZ protein levels in HepG2 cells through time (Fig. 2A and B), and SB43 (ALK5 receptor inhibitor) inhibited this effect (Fig. 2A and C). Data indicate that TGF-β-signaling pathway induces TAZ expression in liver cancer cells.

To investigate how TGF-β regulates TAZ expression, we first focused on evaluating the effect of TGF-β on *TAZ/WWTR1* gene expression. HepG2 cells were treated with 0.3 nM TGF-β for different times (Fig. 3A and B), or pretreated for 30 min with or without 5 μg/ml ActD, and then incubated with or without 0.3 nM TGF-β for different times (Fig. 3C). The mRNA levels of TAZ, SMAD7 and β-ACTIN were analyzed; SMAD7 was evaluated as a control, as it is a well-known TGF-β-target early gene. Data showed that TGF-β increased TAZ mRNA levels with a slow kinetic, reaching a maximal induction after 12 h of treatment (Fig. 3B); this TGF-β effect was

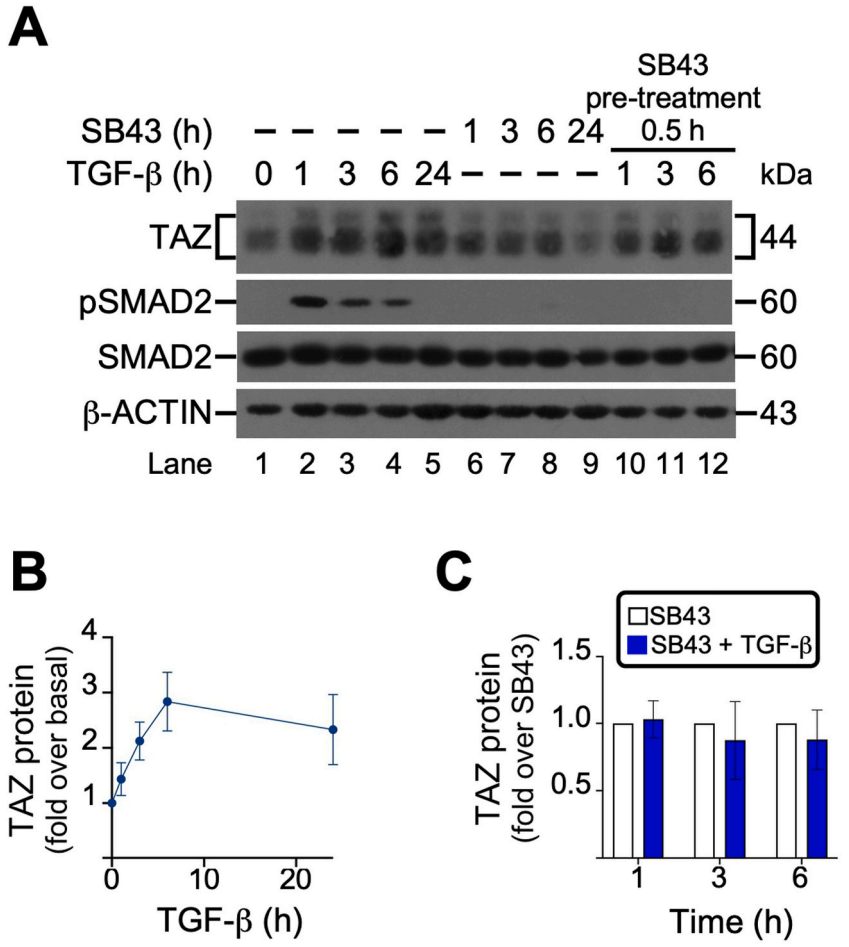

Fig. 2. TGF-β induces TAZ expression in HepG2 cells. **(A)** Cells were treated with 10 μM SB431542 (SB43) for the indicated times, and then treated with 0.3 nM TGF-β for the indicated times; then, TAZ, p-SMAD2, SMAD2 and β-actin protein levels were analyzed by immunoblot (supplementary raw data). **(B)** and **(C)** Graphs show the densitometry values expressed as means ± SEM of 3–4 independent experiments.

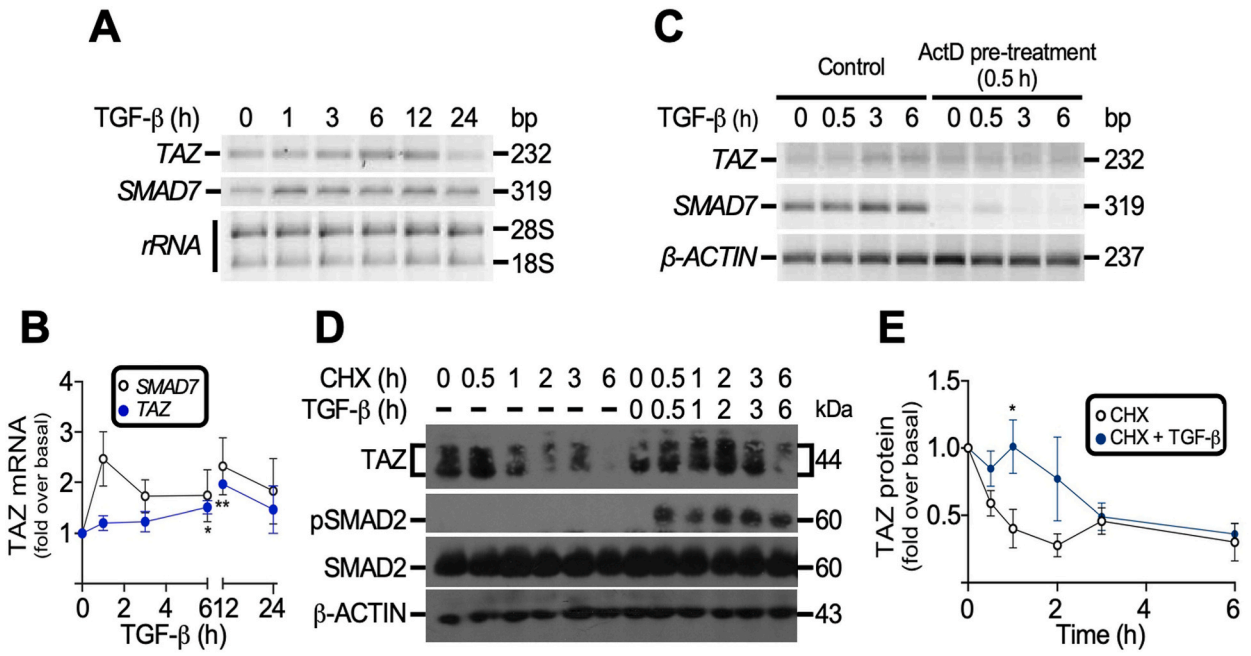

Fig. 3. TGF-β increases TAZ mRNA levels and regulates TAZ protein turnover in HepG2 cells. (**A**) Cells were treated with 0.3 nM TGF-β for indicated times. The mRNA levels of TAZ and SMAD7 were analyzed. The rRNA (28S, 18S) levels were used as a control (supplementary raw data). (**B**) The graph shows the densitometry values expressed as means ± SEM of 3 independent experiments; $P < 0.05$*, $P < 0.0001$**. (**C**) Cells were pre-treated for 0.5 h with 5 μg/ml ActD, and then treated with 0.3 nM TGF-β at indicated times. The mRNA levels of TAZ, SMAD7 and β-ACTIN were analyzed. β-ACTIN mRNA was used as a control (supplementary raw data). Data are representative of 2 independent experiments. (**D**) Cells were treated with 20 μg/mL CHX in the absence or presence of 0.3 nM TGF-β for the indicated times, and TAZ, pSMAD2, SMAD2, and β-ACTIN protein levels were analyzed by immunoblot (supplementary raw data). (**E**) The graph shows the densitometry values expressed as means ± SEM of 3 independent experiments; $P < 0.05$*.

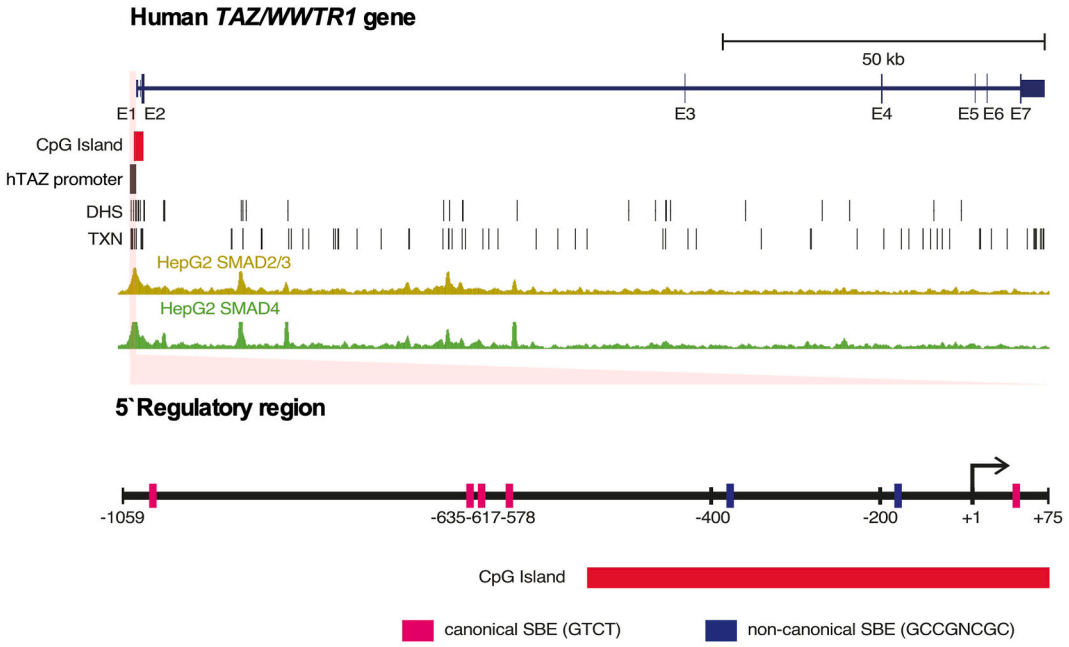

Fig. 4. Human *TAZ/WWTR1* gene promoter harbors SMAD-binding sites. The DNA sequence of TAZ gene obtained from UCSC genome browser and data from ENCODE database were used to elaborate the map of hTAZ gene, using the following tracks: TSS (+1), CpG-islands and specific histone marks. The SMAD2/3/4-interacting regions identified in HepG2 cells by ChIP-seq using GEO data listings for ENCODE are indicated. The positions of predicted canonical and non-canonical SBE motifs on the promoter map are also shown.

prevented with ActD (Fig. 3C). Furthermore, we analyzed the regulation of TAZ protein half-life by TGF-β, when protein synthesis was inhibited with CHX. Thus, HepG2 cells were treated for indicated times with 20 μg/ml CHX (Fig. 3D and E) in the absence or presence of 0.3 nM TGF-β for indicated times. Data showed that TAZ protein had a short half-life (~45 min), and that TGF-β increased TAZ protein half-life (~3 h) (Fig. 3E). Data indicate that TAZ is a high turnover protein and TGF-β signaling is able to regulate both TAZ expression and protein stability in HepG2 cells.

3.3. *TAZ gene promoter is induced by TGF-β/SMAD2 signaling pathway*

To evaluate whether TAZ is a TGF-β target gene in liver cancer cells, we analyzed the *TAZ/WWTR1* gene and its promoter region using the UCSC genome browser (GRCh38/hg38 assembly), identifying CpG-islands, DHS, TSS, and multiple Txn Factor binding sites, as well as an enrichment of specific histone marks at *TAZ/WWTR1* gene promoter (detected in HepG2 cell line) (Fig. 4 and Supplementary Fig. S1). *TAZ/WWTR1* gene was localized at human chromosome 3 (chr3) with the genomic coordinates: chr3:149,517,235–149,658,025 (strand –); the region of ~1.13 kb harboring the *TAZ/WWTR1* gene promoter that was analyzed had the genomic coordinates: chr3:149,657,951–149,659,084, and was located at −1059 to +75 from TSS (+1). We detected several SBE motifs in this region of *TAZ/WWTR1* gene promoter using ALGGEN-PROMO and Jaspar, suggesting a potential regulation by TGF-

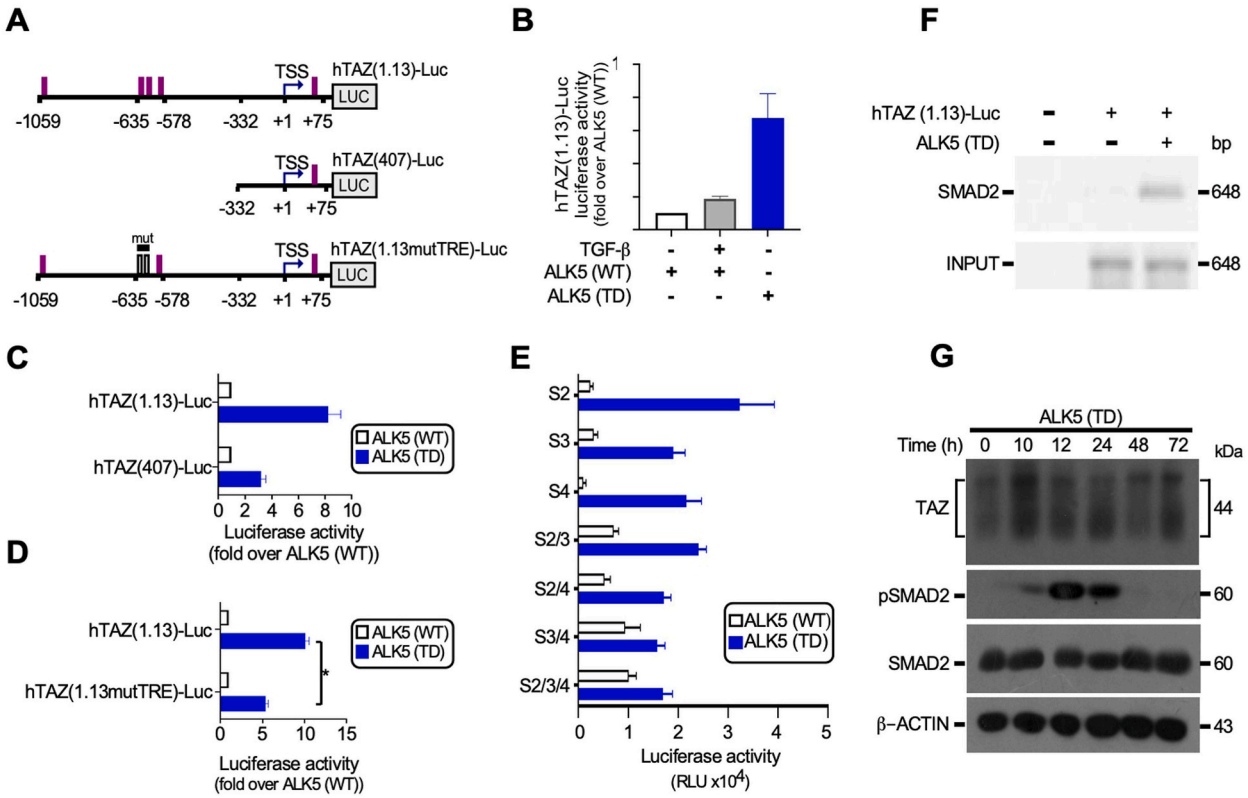

Fig. 5. Human *TAZ/WWTR1* gene promoter is responsive to canonical TGF-β/SMAD pathway. **(A)** The pGL3 constructs containing the different DNA fragments of the hTAZ promoter and one mutant are shown. **(B)** HepG2 cells were transiently transfected with hTAZ(1.13)-Luc along with ALK5(WT) or ALK5(TD) for 48 h, and then treated or not for 24 h with 0.2 nM TGF-β. At 72 h post-transfection, luciferase activity was measured. Data are representative of 2 independent experiments in triplicate. **(C)** HepG2 cells were transiently co-transfected with hTAZ(1.13)-Luc or hTAZ (407)-Luc reporters along with ALK5(WT) or ALK5(TD), and then luciferase activity was measured at 72 h post-transfection; values are expressed as means ± SEM of 2 independent experiments in sextuplicate. **(D)** HepG2 cells were transiently transfected with hTAZ(1.13)-Luc or hTAZ(1.13-mutTRE)-Luc constructs, each in co-transfection with ALK5(WT) or ALK5(TD), and luciferase activity was measured at 72 h post-transfection; values are expressed as means ± SEM of 2 independent experiments in sextuplicate. $P < 0.05$*. **(E)** HepG2 cells were co-transfected as indicated, with pGL3-basic/hTAZ(1.13)-Luc along with plasmids bearing SMADs full-length cDNA: pCMV5/Flag-SMAD2 (S2), pCMV5/Flag-SMAD3 (S3) or pCMV5/HA-SMAD4 (S4), and ALK5(WT) or ALK5(TD). Then, cells were lysed after 72 h post-transfection, and luciferase activity was analyzed. Raw RLU (Relative Light Units) values are expressed as means ± SEM of 2 independent experiments in quadruplicate. **(F)** HepG2 cells were transiently co-transfected with hTAZ(1.13)-Luc alone or with ALK5 (TD); then, ChIP on plasmid assay was carried out using anti-SMAD2 for IP. PCR was performed using primers spanning the canonical SBE region (648 bp) and a part of the pGL3-basic vector (supplementary raw data). Data are representative of 3 independent experiments. **(G)** HepG2 cells were transiently transfected without or with ALK5(TD) for indicated times, and then TAZ, pSMAD2, and SMAD2 protein levels were evaluated by immunoblot; β-ACTIN was used as a loading control (supplementary raw data). Data are representative of 2 independent experiments.

β/SMAD pathway. We found five canonical SBE motifs (GTCT), and two non-canonical SBE motifs (GCCGNCGC). Three nearby canonical SBE motifs were located around −635 to −578 from TSS (+1). Intriguingly, all predicted canonical SBE co-localize with the SMAD2/3/4-interacting regions detected by ChIP-seq analysis using ENCODE database, as in HepG2 cells (Fig. 4) as in other cell lines like HUES8, HASMC, and HUVEC (Supplementary Fig. S2). As expected, an enrichment of SMAD2/3-interacting sites was observed in the *TAZ/WWTR1* gene promoter region. These data strongly suggest that *TAZ/WWTR1* gene promoter harbors multiple SMAD-binding sites that may be regulated for TGF-β/SMAD canonical pathway in distinct cell types.

To determine the contribution of those SBE motifs, we generated reporter genes by cloning two DNA fragments (1.13 kb and 407 bp) of this *TAZ/WWTR1* gene promoter region into pGL3 reporter vectors (Fig. 5A). First, HepG2 cells were transiently transfected with hTAZ(1.13)-Luc plasmid along with plasmids encoding ALK5(WT) or ALK5(TD) receptor (Fig. 5B). At 48 h post-transfection, cells were treated or not with 0.2 nM TGF-β for 24 h, and luciferase activity was measured at 72 h post-transfection. We observed that TGF-β and ALK5(WT) induced lower reporter activity than the constitutively active ALK5(TD) receptor (Fig. 5B). Reporter gene assays were also performed transfecting hTAZ(1.13)-Luc or hTAZ(407)-Luc reporters in the absence or presence of ALK5(WT) or ALK5(TD) (Fig. 5 C). Data showed that ALK5(TD) mainly increased the activity of hTAZ(1.13)-Luc reporter in comparison with hTAZ(407)-Luc reporter, indicating that the hTAZ(1.13)-Luc reporter harbors the main SBE responsive to TGF-β/ALK5 signaling.

To determine if the cluster of SBE motifs located around −635 to −617 from TSS (+1) was required by SMAD proteins to regulate human *TAZ/WWTR1* gene promoter, the mutant hTAZ(1.13mutTRE)-Luc construct was transfected along with ALK5(WT) or ALK5 (TD) (Fig. 5D), and data showed that ALK5(TD) induced a lower activity of mutant reporter gene than of wild-type reporter gene. Data suggest that the cluster of SBE motifs located around −635 to −617 from TSS (+1) represent the main TGF-β-responsive element (TRE) at human *TAZ/WWTR1* gene promoter. Our results correlated with the previous identification of a similar TRE on mouse *TAZ* gene promoter that also harbors crucial SBE motifs regulated by SMAD3 [13].

It was previously reported that TGF-β signaling could regulate *TAZ/WWTR1* gene expression through SMAD-independent pathways [12,13]. Thus, in order to evaluate the participation of SMAD proteins in the regulation of *TAZ/WWTR1* gene expression, HepG2 cells were co-transfected with hTAZ(1.13)-Luc and with single or combined plasmids encoding full-length cDNA for SMAD2 (S2), SMAD3 (S3), or SMAD4 (S4), as well as with the plasmids encoding for ALK5(WT) or ALK5(TD) (Fig. 5E). Data showed that all SMAD, alone (S2, S3, S4) or combined (S2/3, S2/4, S3/4, S2/3/4), were able to increase the TAZ gene reporter activity induced by ALK5(TD) in comparison with ALK5(WT).

Furthermore, we evaluated the recruitment of SMAD2 protein to *TAZ/WWTR1* gene promoter in HepG2 cells. ChIP on plasmid assay was carried out using anti-SMAD2 antibody for IP (Fig. 5F); PCR was performed using specific primers. Data showed that TGF-β signaling induced the recruitment of SMAD2 protein to *TAZ/WWTR1* gene promoter. As a control for ChIP on plasmid assay, protein

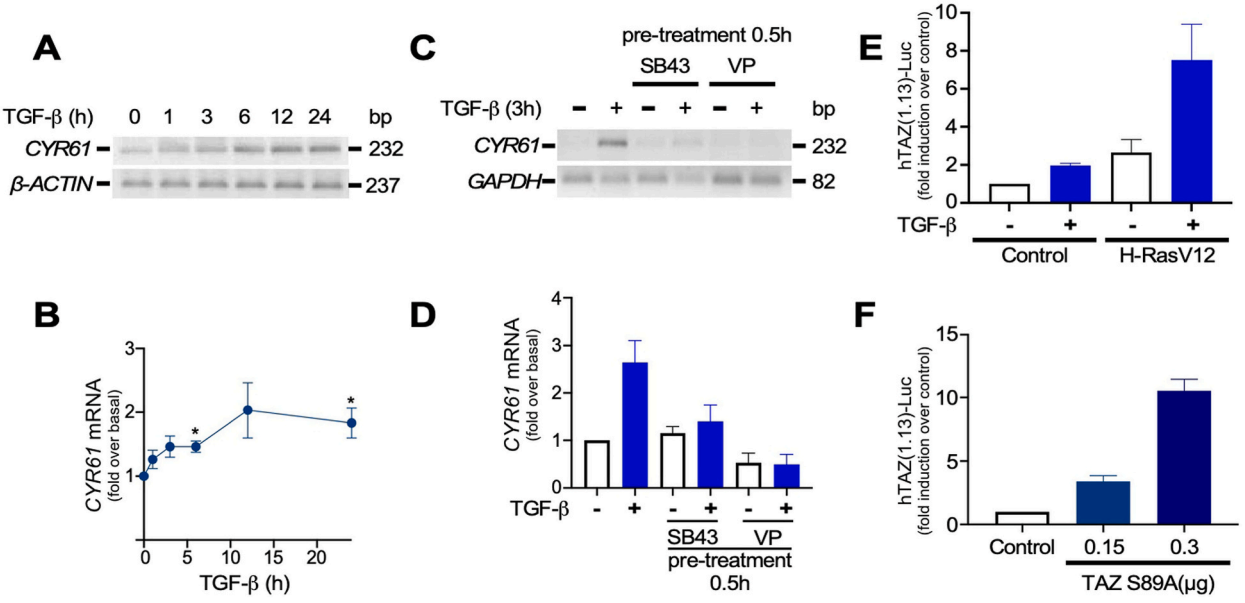

Fig. 6. TGF-β/SMAD and TAZ share target genes. (**A**) HepG2 cells were treated with 0.3 nM TGF-β for indicated times. Total RNA was isolated and mRNA levels of CYR61 were analyzed by RT-PCR with specific primers. β-ACTIN was a control (supplementary raw data). (**B**) The graph shows the densitometry values expressed as means ± SEM of 3 independent experiments; $P < 0.05$*. (**C**) HepG2 cells were pre-treated for 0.5 h with or without 10 μM SB43 or 1 μM VP, and then treated with 0.3 nM TGF-β at indicated times. The mRNA levels of CYR61 were analyzed. GAPDH was a control (supplementary raw data). (**D**) The graph shows the densitometry values expressed as means ± SEM of 3 independent experiments; $P < 0.05$*. (**E**) HepG2 cells were transiently transfected for 72 h with hTAZ(1.13)-Luc alone (control) or along with H-RasV12, and then treated for 24 h with 0.3 nM TGF-β. Luciferase activity was measured at 72 h post-transfection; values are expressed as means ± SEM of 2 independent experiments in triplicate. (**F**) HepG2 cells were transiently transfected for 72 h with hTAZ(1.13)-Luc alone (control) or with indicated concentrations of TAZ S89A, and then luciferase activity was measured; values are expressed as means ± SEM of 2 independent experiments in quadruplicate.

extracts from transiently transfected HepG2 cells were used to evaluate the levels of TAZ and pSMAD2 proteins, which were increased after 10–24 h post-transfection with ALK5(TD) (Fig. 5G). Therefore, data show that the canonical TGF-β/SMAD signaling pathway up-regulates TAZ in liver cancer HepG2 cells.

3.4. TGF-β/SMAD and TAZ share target genes

Furthermore, we aimed to investigate whether the TGF-β/SMAD/TAZ axis was able to regulate the expression of common target genes of Hippo and TGF-β pathways. Thus, we analyzed the expression of *CYR61*, which is relevant in the EMT process and also a key target gene regulated by both pathways [4,5,23]. HepG2 cells were treated with 0.3 nM TGF-β for different times (Fig. 6A and B), or cells were pretreated for 30 min with or without 10 μM SB43 or 1 μM VP (Verteporfin is an inhibitor of TAZ/YAP-TEAD complex), and then incubated with or without 0.3 nM TGF-β for 3 h (Fig. 6C and D). Data showed that TGF-β increased CYR61 mRNA levels with a slow kinetic, reaching a maximal induction after 12 h of treatment (Fig. 6B); TGF-β effect was prevented with SB43 or VP pre-treatment (Fig. 6C and D). Data show that *CYR61* is a common TGF-β and TAZ target gene in HepG2 cells.

TAZ regulates various cellular processes in HCC, such as proliferation, stem cell differentiation, and EMT; intriguingly, EMT appears to promote a self-sustaining mechanism of TAZ activation [24,25]. Therefore, we evaluate TAZ up-regulation by TGF-β in HepG2 expressing a constitutively activated mutant of small GTPase Ras (RasV12A) in order to mimic the EMT context. In this scenario, we observed that 0.3 nM TGF-β increased higher levels of TAZ reporter gene activity in the presence of RasV12A than in control HepG2 cells (Fig. 6E), and as expected, a constitutively activated TAZ mutant (TAZ S89A) was also able to increase TAZ reporter gene activity in a dose-dependent manner (Fig. 6F). Taken together, our data suggest that the cooperation between TGF-β- and Hippo-pathways through the TGF-β/SMAD/TAZ axis probably is enhanced in the context of EMT, a relevant hallmark of cancer cells.

4. Discussion

The TGF-β and Hippo pathways play an essential role in liver physiology from development to adulthood, as they exert cytostatic and pro-apoptotic effects, which are relevant processes for the control of liver mass. Alterations in these signaling pathways are associated with liver pathologies such as chronic liver diseases, fibrosis, and cancer [4,11,22]. In the context of cancer, TGF-β particularly has a dual role, acting as a tumor suppressor in early stages and as a tumor promoter in advanced stages [1]; although, this always depends on the cellular context.

Liver cancer is one of the most common causes of cancer-related death worldwide. In this type of cancer, the tumor microenvironment may foster the tumor-promoting role of TGF-β in the late stages of cancer [1,22]. In 2008, a paper reported TGF-β-dependent temporal (early and late) gene expression signatures established in mouse hepatocytes that predict clinical outcomes in human cancer [26]. These signatures discriminate among distinct subgroups of HCC, showing that a late TGF-β signature (expression of antiapoptotic genes, and EMT- and invasion-related genes) was correlated with increased tumor recurrence and a more invasive phenotype. In contrast, an early signature of TGF-β (antiproliferative and antiapoptotic genes) was correlated with a good prognosis for liver cancer patients [22,26]. Interestingly, *in vitro* attenuation of the TGF-β pathway in human HCC cells does not inhibit proliferation or induce apoptosis, but strongly blocks their ability to migrate and invade, as well as their stemness capacity [22].

The Hippo signaling pathway also plays a critical role in tumorigenesis, as YAP/TAZ deregulation is correlated with several human cancers, although how it promotes cancer progression remains unclear. In liver cancer, aberrant activation of YAP/TAZ has been reported, but the mechanisms that regulate their activities and their possible contribution to liver tumorigenesis are uncertain [11]. Several groups have shown that genetic disruption of Hippo signaling components, such as NF2, WW45 and MST1/2, or the YAP transgene, results in sustained liver overgrowth and tumorigenesis [27–31]. Moreover, 5–10 % of HCCs have genomic amplification of the YAP-containing locus [32], whereas approximately 60 % of human liver cancer is associated with increased YAP/TAZ activity [33,34]. Importantly, TAZ overexpression in HCC is correlated with a lower overall patient survival rate, after liver resection [9]. All this evidence suggests that upregulation of YAP/TAZ may contribute to liver tumorigenesis and also indicates a poor prognosis [4,35–38]. In the case of cancer cell lines, YAP/TAZ cofactors promote proliferation, antiapoptosis, anchorage-independent growth, drug resistance, and stem cell phenotypes [4].

Here, we found a correlation between increased TAZ expression levels and up-regulation of TGF-β ligands, SMAD proteins, and EMT markers (SNAI2 and VIMENTIN) in HCC samples by analyzing public datasets of LIHC. This finding is relevant, since YAP/TAZ signaling in cooperation with TGF-β signaling can induce EMT [39]. In HCC, TAZ regulates cellular proliferation, expression of stem cell markers, and EMT [40,41]. EMT involves the transdifferentiation of cancer epithelial cells to mesenchymal cells to confer aggressive features, such as invasion and resistance to apoptosis, while in advanced stages of the disease it provides cancer stem cell characteristics, anti-apoptosis, and drug resistance [42,43]. Furthermore, EMT can also promote TAZ activation, suggesting that the TAZ-mediated EMT serves as a self-sustaining mechanism of TAZ activation [25]. Our analysis using the CCLE database showed higher TAZ expression in liver cancer cells with epithelial/mesenchymal or mesenchymal phenotypes than in those with an epithelial phenotype. Intriguingly, there is evidence that TGF-β induces EMT in HepG2 cells, an event that may be supported by TAZ upregulation [43,44]; while knockdown of TAZ in HepG2 cells promotes apoptosis.

Mutations in major components of the Hippo pathway are rare, excluding NF2, and activating mutations in YAP/TAZ have not been demonstrated in human cancers. Furthermore, multiple mechanisms of YAP/TAZ activation have been reported in different types of cancer, such as gene amplification. In most tumors, YAP/TAZ signaling can also be turned on or off by many extrinsic signals that are enhanced in cancer tissues, such as mechanical forces, signals from growth factors and inflammatory mediators, as well as by energy stress, endoplasmic reticulum stress, oxidative stress, hypoxia, and altered metabolic conditions [45–49]. However, what triggers

YAP/TAZ activation in liver cancer is poorly investigated.

Because the core components of the Hippo signaling pathway are typically not mutated in liver cancer patients, identification of the network of signaling pathways that regulate YAP/TAZ activities will provide insights on the molecular classification of liver cancer. Of note, YAP/TAZ cofactors work cooperatively with other signaling pathways, including PI3K/AKT, Wnt, and Notch, which may also interact with the Hippo signaling cascade to regulate YAP/TAZ activity in liver cancer [39,50–52]. Thus far, it is clear that TGF-β is a prominent promoter of HCC at later stages, but its molecular targets are poorly identified. TGF-β can regulate the expression of TAZ depending on the cell context; here, we found that TGF-β pathway promotes the up-regulation of TAZ at different levels in HepG2 cells, as it increases TAZ mRNA and protein levels, suggesting that TGF-β can regulate both TAZ gene transcription and TAZ protein stability in liver cancer. Nevertheless, this TGF-β/SMAD/TAZ axis may also play an important role in normal hepatocytes. EMT is known to be an important process during liver regeneration, as it facilitates hepatic cell migration and organization to restore liver architecture [53]. Therefore, the regulation of TAZ expression in normal hepatocytes remains to be investigated.

TAZ expression is regulated at multiple levels (gene, mRNA and protein), and its regulation also depends on the cellular context, which makes it difficult to study. As mentioned, TGF-β can regulate TAZ expression through different mechanisms depending on the cell type, and mainly through SMAD-independent mechanisms [12,13]. Therefore, the regulation of TAZ expression in the HepG2 cell model may partially represent what happens in a tumor. Here, we analyzed the promoter of the human *TAZ/WWTR1* gene and found several canonical and non-canonical SBE motifs, and identified a TGF-β-response element (TRE) highly responsive to TGF-β signals that is composed of canonical SBEs. We mutated two SBEs located in this TRE to obtain the hTAZ(1.13mutTRE) mutant construct, which showed a significantly reduced response to TGF-β. These results were correlated with the regulation of a similar TRE identified in the promoter of the mouse *TAZ* gene that also harbors SBEs crucial for its regulation by TGF-β [13].

Therefore, our results show that the canonical TGF-β/SMAD pathway regulates *TAZ* expression in HepG2 cells through distinct mechanisms: the increase of *TAZ/WWTR1* gene transcription, and the increase of TAZ protein stability. These findings demonstrate that TAZ transcriptional cofactor is a primary target of TGF-β/SMAD signaling, one of the main pathways altered in liver cancer. In addition, the established TGF-β/SMAD/TAZ axis may regulate common target genes, such as *CYR61* and *TAZ/WWTR1*, in a context dependent-manner.

Furthermore, our study suggests that one of the main results of the crosstalk between the TGF-β and Hippo pathways is the establishment of the TGF-β/SMAD/TAZ axis, which could generate specific genetic signatures, useful as potential biomarkers for diagnosis or prognosis. However, therapeutic targeting of the TGF-β/SMAD/TAZ axis in liver cancer can be difficult due to its close relationship with the EMT process. Thus far, the inhibition of EMT in cancer could have a dual effect: positive by reducing metastasis, as a consequence of reducing the migration and invasion of cancer cells, or negative, since it could lead to the activation of the Mesenchymal-Epithelial Transition (MET), a key hallmark related to the establishment of a secondary tumor [54]. Therefore, the implications of the TGF-β/SMAD/TAZ axis in health and disease remain to be investigated.

Funding

This work was supported by PAPIIT/DGAPA/UNAM (grant No. IN208118) and CONAHCyT (grant No. 304023).

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Diana G. Ríos-López: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Angeles C. Tecalco-Cruz:** Investigation, Methodology, Resources, Supervision, Writing – review & editing. **David Martínez-Pastor:** Investigation, Writing – review & editing. **Marcela Sosa-Garrocho:** Investigation, Writing – review & editing. **Gustavo Tapia-Urzúa:** Investigation, Writing – review & editing. **Yuli Aranda-López:** Investigation, Writing – review & editing. **Bibiana Ortega-Domínguez:** Investigation, Writing – review & editing. **Félix Recillas-Targa:** Methodology, Resources, Supervision, Writing – review & editing. **Genaro Vázquez-Victorio:** Funding acquisition, Investigation, Writing – review & editing. **Marina Macías-Silva:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Dr. Marco Briones-Orta and Dr. César Poot-Hernández for helpful discussions, and Dr. Jeff Wrana for kindly provide some plasmids. We thank all members of Unidad de Biología Molecular, and Dr. Ruth Rincón-Heredia and Dr. Abraham Rosas-Arellano for technical support. Diana G. Ríos-López is a Ph.D. student of Posgrado en Ciencias Bioquímicas at UNAM, and received a Ph.D. fellowship from CONAHCyT.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21519.

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