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Article

Tea domain transcription factor TEAD4 mitigates TGF-β signaling and hepatocellular carcinoma progression independently of YAP

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Tea domain transcription factor 4 (TEAD4) plays a pivotal role in tissue development and homeostasis by interacting with Yes-associated protein (YAP) in response to Hippo signaling inactivation. TEAD4 and YAP can also cooperate with transforming growth factor-β (TGF-β)-activated Smad proteins to regulate gene transcription. Yet, it remains unclear whether TEAD4 plays a YAP-independent role in TGF-β signaling. Here, we unveil a novel tumor suppressive function of TEAD4 in liver cancer via mitigating TGF-β signaling. Ectopic TEAD4 inhibited TGF-β-induced signal transduction, Smad transcriptional activity, and target gene transcription, consequently suppressing hepatocellular carcinoma cell proliferation and migration *in vitro* and xenograft tumor growth in mice. Consistently, depletion of endogenous TEAD4 by siRNAs enhanced TGF-β signaling in cancer cells. Mechanistically, TEAD4 associates with receptor-regulated Smads (Smad2/3) and Smad4 in the nucleus, thereby impairing the binding of Smad2/3 to the histone acetyltransferase p300. Intriguingly, these negative effects of TEAD4 on TGF-β/Smad signaling are independent of YAP, as impairing the TEAD4–YAP interaction through point mutagenesis or depletion of YAP and/or its paralog TAZ has little effect. Together, these results unravel a novel function of TEAD4 in fine tuning TGF-β signaling and liver cancer progression in a YAP-independent manner.

Keywords: TGF-β, TEAD4, p300, signaling regulation, transcriptional activity, hepatocellular carcinoma

Introduction

Transforming growth factor-β (TGF-β) is a secreted polypeptide belonging to the TGF-β cytokine superfamily, which comprises TGF-β1, TGF-β2, TGF-β3, activins, nodal, inhibins, bone morphogenetic proteins, growth differentiation factors, and anti-Müllerian hormone (also known as MIS) (Heldin and Moustakas, 2016; David and Massague, 2018; Derynck and Budi, 2019). TGF-β initiates a classic cell membrane-to-nucleus signaling process by first binding to two types of single-pass membrane receptors that bear intrinsic Ser/Thr kinase activity, i.e. type I (TβRI) and type II (TβRII) receptors. Then, activated TβRI recruits receptor-regulated Smads (Smad2/3) and phosphorylates them at the extreme carboxyl terminal SXS motif, leading

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to the oligomerization of Smad2/3 with the common Smad protein (Smad4). The heteromeric Smad complexes accumulate in the nucleus, bind to DNA, and regulate gene transcription by associating with other transcription factors and cofactors. TGF-β is engaged in the regulation of a plethora of cellular processes, such as cell proliferation, apoptosis, differentiation, and migration, and extracellular matrix organization, amongst others (Morikawa et al., 2016; Pickup et al., 2017; Zhang et al., 2017). Fine-tuning of TGF-β signaling plays a pivotal role in the homeostasis of adult tissues, including the liver (Fabregat et al., 2016; Hata and Chen, 2016; Morikawa et al., 2016). Not surprisingly, dysregulation of TGF-β signaling has been connected to the initiation and progression of liver cancer, including hepatocellular carcinoma (HCC), the most prevalent form of primary liver cancers and one of the leading life-threatening cancer types (Fabregat et al., 2016; Dituri et al., 2019; Zhang et al., 2020). Although acting as a growth inhibitor in normal hepatocytes and early-stage cancer cells, TGF-β switches to an oncogenic factor during liver cancer progression by promoting cancer cell proliferation, migration, invasion, and metastasis, in addition to modifying the tumor microenvironment (Fabregat et al., 2016; Katz et al., 2016; Dituri et al., 2019; Yu and Feng, 2019; Zhang et al., 2020).

The biological actions of TGF-β signaling also depend on its intimate interplays with other intracellular signaling pathways, including the Hippo pathway, which exerts a critical tumor-suppressive function by inactivating the transcriptional coactivators Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) (Attisano and Wrana, 2013; Zhang et al., 2013). The Tea domain transcription factors, TEAD1–TEAD4, have been demonstrated to be the major binding partners of YAP/TAZ mediating their oncogenic roles in cancer (Zhao et al., 2008; Varelas, 2014; Lin et al., 2017). Considerable evidence indicates that the TGF-β/Smad pathway and Hippo signaling crosstalk with each other at different levels through multiple mechanisms (Attisano and Wrana, 2013; Noguchi et al., 2018). For instance, in embryonic stem cells, TAZ has been evidenced to associate with the Smad2/3–Smad4 complex in a TGF-β-reliant manner, promoting the nuclear accumulation and Smad transcriptional activity (Varelas et al., 2008). YAP/TAZ, TEADs, and Smads have been reported to form a protein complex on DNA and consequently regulate the expression of specific genes, depending on the cofactors recruited (Ben Mimoun and Mauviel, 2018). In cancer cells, nuclear YAP/TAZ and TEADs cooperate with TGF-β-activated Smad2/3 to increase the expression of some protumor genes and consequently drive cancer progression (Fujii et al., 2012; Hiemer et al., 2014). In contrast, in embryonic stem cells, YAP/TAZ have been shown to associate with TEADs, Smad2/3, FoxH1, and Oct4 on the promoters of some mesendodermal genes, thereby shutting off their expression by recruiting the NuRD corepressor complex and maintaining pluripotency (Beyer et al., 2013). It is noteworthy that cytoplasmic YAP/TAZ have been suggested to sequester Smads and inhibit TGF-β signaling under certain conditions (Varelas et al., 2010; Chen et al., 2013; Grannas et al., 2015). Of relevance, the Hippo pathway scaffold protein RASSF1A could be recruited to TGF-β receptors upon TGF-β stimulation, resulting in RASSF1A degradation, Hippo signaling inactivation, and the nuclear entry of the YAP–Smad2 complex (Pefani et al., 2016).

Although much progress has been made in understanding the crosstalk mechanisms between TGF-β signaling and the Hippo pathway, whether TEAD4 plays a YAP-independent role in regulating TGF-β signaling and liver cancer progression remains elusive. Here, we demonstrate that TEAD4 protein can mitigate TGF-β signaling and its oncogenic functions in HCC by interacting with Smad2/3 and impeding their transcriptional activity. Intriguingly, YAP and TAZ are dispensable for the inhibitory functions of TEAD4 on TGF-β signaling. These results shed new light on TGF-β signaling regulation and the interplay between Smads and TEADs in the context of liver cancer.

Results

Ectopic TEAD4 protein antagonizes TGF-β signaling and its tumor-promoting functions in HCC

In order to explore the roles of TEAD proteins in TGF-β signaling, we first carried out luciferase reporter assays in HEK293FT cells with three classical TGF-β/Smad-responsive reporters, i.e. CAGA-luciferase, ARE-luciferase, and 3TP-luciferase (Wrana et al., 1992; Huang et al., 1995; Dennler et al., 1998). Stimulation with human TGF-β recombinant protein was able to induce the expression of the three reporters remarkably. Intriguingly, ectopic expression of either TEAD3 or TEAD4 led to a dramatic decrease of the stimulatory effects of TGF-β (Figure 1A). This result was somehow different from those obtained from previous studies reporting that TEAD4, YAP, and Smad2/3 cooperate to stimulate the expression of specific genes in cancer cells, prompting us to further explore the relationship between TEADs and TGF-β/Smad signaling in liver cancer. Because the four members of the TEAD family share striking sequence identity and functional conservation (Lin et al., 2017; Huh et al., 2019), TEAD4 was tested as a representative in the following experiments. Consistent with the above observations, overexpression of TEAD4 led to the attenuation of TGF-β signaling in the HCC cell line HCCLM3, as assessed by different reporter assays (Figure 1B). In addition, TEAD4 exerted similar effects when tested in three other HCC cell lines, MHCC97H, MHCC97L, and SMMC-7721, with either CAGA-luciferase reporter (Figure 1C) or ARE-luciferase reporter (Figure 1D). Together, these results suggest that TEAD4 could act as an inhibitor of TGF-β signaling in liver cancer.

We next asked whether TEAD4-mediated inhibition of TGF-β signaling could lead to functional consequences in HCC. To address this, HCCLM3 cells stably expressing TEAD4 or the control lentivirus vector were established and verified by both immunoblotting (IB) and quantitative real-time polymerase chain reaction (q-PCR) (Figure 1E). Then, the migratory ability of these stable cell lines was measured by

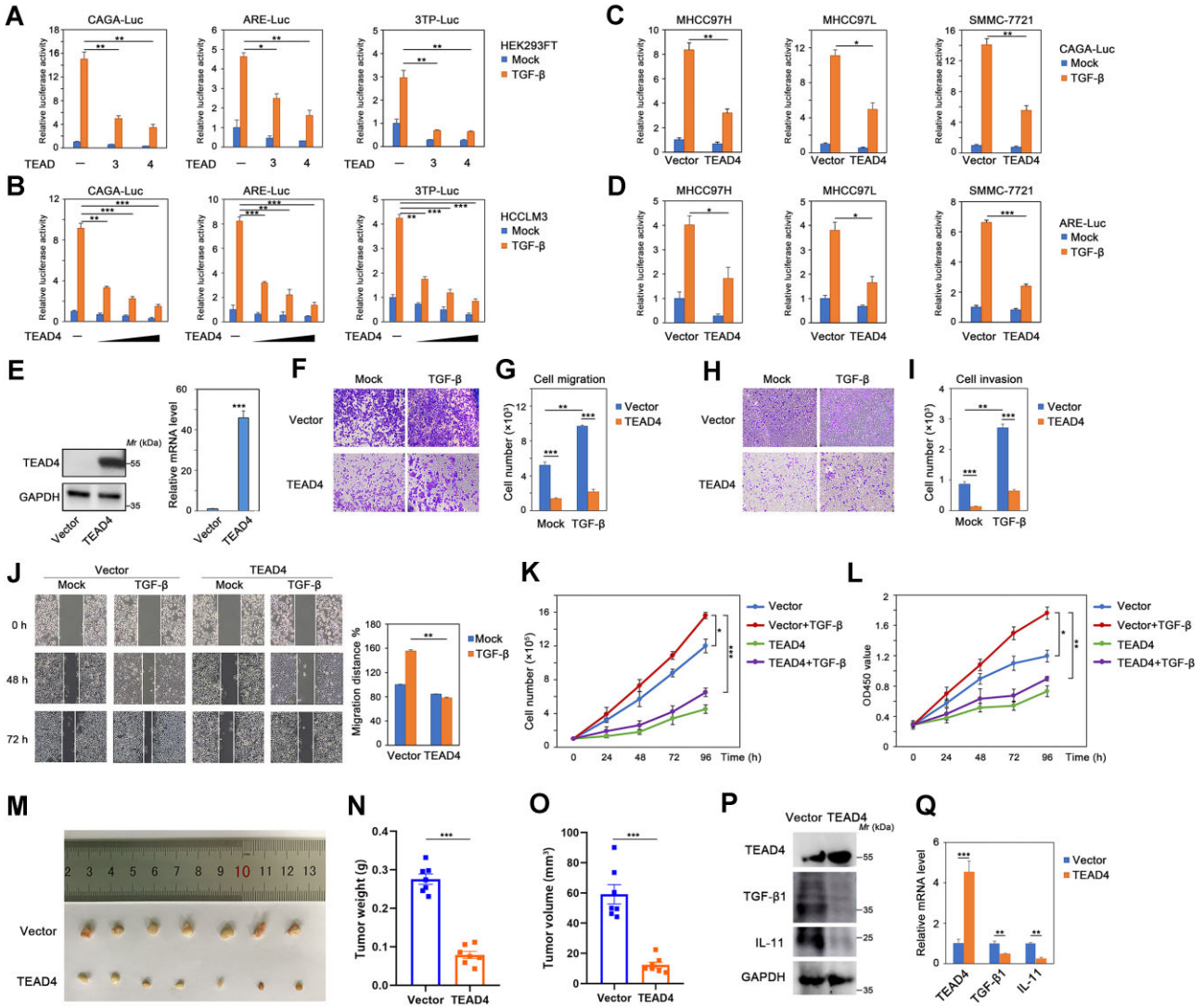

Figure 1 Ectopic TEAD4 protein restrains TGF-β signaling and HCC development. (**A**) HEK293FT cells were transfected with constructs encoding CAGA-luciferase, ARE-luciferase, or 3TP-luciferase reporter (200 ng each), together with TEAD3- or TEAD4-expressing plasmids (100 ng each). Renilla-luciferase (50 ng) was also transfected as a normalization control, and empty vector was transfected to ensure equal total amounts of plasmids in each sample. At 20 h post-transfection, cells were treated with or without 100 pM recombinant TGF-β protein. After another 20 h, cells were harvested for luciferase activity measurement. (**B**) HCCLM3 cells were transfected with different luciferase reporter plasmids as indicated (200 ng each), together with Renilla-luciferase (50 ng) and TEAD4-expressing plasmids (50, 100, and 200 ng) or empty vector, and then were treated with or without 100 pM TGF-β before luciferase activity determination. (**C** and **D**) CAGA-luciferase (**C**) and ARE-luciferase (**D**) reporter assays were performed in MHCC97H, MHCC97L, and SMMC-7721 cells, respectively. (**E**) HCCLM3 cells stably expressing TEAD4 or the control lentivirus vector were examined by IB and q-PCR, respectively. In IB, GAPDH expression served as a loading control. In q-PCR, gene expression level was normalized to that of GAPDH, and each experiment was performed in triplicate. (**F**–**I**) Transwell migration (**F** and **G**) and matrigel invasion (**H** and **I**) assays were performed in TEAD4- or vector-expressing HCCLM3 cells treated with or without 200 pM TGF-β in 2% FBS-containing DMEM. Cell number was quantified based on three biological repeats per group (**G** and **I**). (**J**) HCCLM3 cells expressing TEAD4 or vector were treated with or without 200 pM TGF-β in 2% FBS-containing DMEM and subjected to wound-healing assay. Cell migration was monitored by following up the narrowing of the wound gap at the indicated time points under a phase-contrast microscope. (**K** and **L**) HCCLM3 cells expressing TEAD4 or vector were treated with or without 100 pM TGF-β, and cell growth was followed at the indicated time points by direct cell counting (**K**) or CCK-8 assay (**L**), respectively. (**M**) Representative images of nude mouse xenograft tumors derived from HCCLM3 cells stably expressing TEAD4 or vector. (**N** and **O**) Xenograft tumors were quantified in terms of weight (**N**) and volume (**O**), respectively. (**P** and **Q**) Xenograft tumor tissues were subjected to IB (**P**) and q-PCR (**Q**).

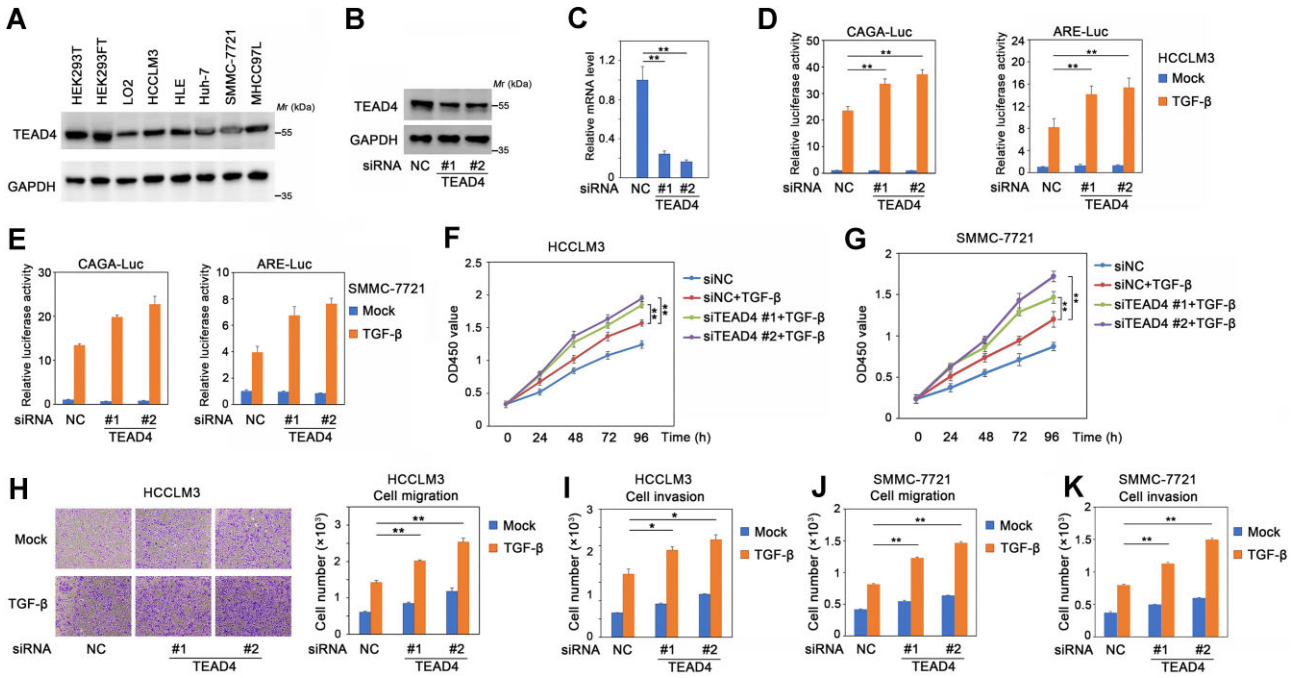

Figure 2 Depletion of endogenous TEAD4 facilitates TGF-β responsiveness in liver cancer cells. (**A**) TEAD4 protein expression was examined by IB with a TEAD4-specific antibody in different HCC cell lines. GAPDH expression served as a loading control. (**B** and **C**) Efficacy examination of TEAD4-specific siRNAs. Two TEAD4-targeting siRNAs and a non-specific control (NC) siRNA were transfected into HCCLM3 cells. TEAD4 expression was examined by IB (**B**) and q-PCR (**C**), respectively. (**D** and **E**) HCCLM3 (**D**) and SMMC-7721 (**E**) cells were transfected with constructs encoding CAGA-luciferase or ARE-luciferase reporter (200 ng each), together with Renilla-luciferase (50 ng) and siRNAs as indicated. Cells were treated with or without 100 pM TGF-β before luciferase activity measurement. (**F** and **G**) HCC cells transfected with the indicated siRNAs were treated with or without 100 pM TGF-β, and cell growth was monitored by CCK-8 assay. (**H**–**K**) HCC cells transfected with siRNAs were treated with or without 200 pM TGF-β in 2% FBS-containing DMEM, followed by transwell migration (**H** and **J**) and matrigel invasion (**I** and **K**) assays.

transwell assays. As shown in Figure 1F and G, TGF-β treatment resulted in significant enhancement of HCCLM3 cell migration. Importantly, stably expressed TEAD4 ameliorated the migratory capability of HCCLM3 cells upon TGF-β stimulation. Similar effect of TEAD4 was also observed in the wound-healing assay (Figure 1J). Furthermore, TEAD4 inhibited TGF-β-mediated invasive capability of HCCLM3 cells in transwell invasion assays (Figure 1H and I). In addition, in cell proliferation evaluations by either direct cell counting or Cell Counting Kit-8 (CCK-8) assay, TGF-β treatment apparently promoted cell proliferation of HCCLM3 cells, whereas TEAD4 counteracted the effects of TGF-β (Figure 1K and L). To explore whether TEAD4 may regulate TGF-β signaling and liver cancer development *in vivo*, we performed a xenograft tumor assay by subcutaneously injecting HCCLM3 cells into nude mice. Stable expression of TEAD4 in HCCLM3 cells led to reduced tumor growth (Figure 1M–O). In addition, the expression levels of TGF-β target genes, such as TGF-β1 itself and IL-11, were significantly attenuated in xenograft tumors overexpressing TEAD4 compared with those expressing the control vector (Figure 1P and Q). In sum, these observations indicate that ectopic TEAD4 can not only mitigate TGF-β-mediated liver cancer cell proliferation and migration *in vitro* but also inhibit liver cancer growth *in vivo*.

Depletion of endogenous TEAD4 facilitates TGF-β signaling

To further investigate the function of endogenous TEAD4 protein on TGF-β signaling, we examined the expression of TEAD4 protein in multiple cell lines. As a result, TEAD4 protein was robustly and similarly expressed in different HCC cell lines and the normal hepatocyte cell line LO2 (Figure 2A). Two independent siRNAs specifically targeting TEAD4 were designed and verified in HCCLM3 cells by both IB and q-PCR (Figure 2B and C). Not surprisingly, depletion of endogenous TEAD4 significantly enhanced TGF-β-induced expression of luciferase reporters in HCCLM3 and SMMC-7721 cells (Figure 2D and E), in addition to HEK293FT cells (data not shown). Furthermore, TEAD4 knockdown promoted the growth-stimulatory effect of TGF-β on HCCLM3 and SMMC-7721 cells (Figure 2F and G) and enhanced TGF-β-mediated migration and invasion of cancer cells (Figure 2H–K). Together, these results demonstrate that TEAD4 can antagonize TGF-β signaling and mitigate TGF-β-elicited

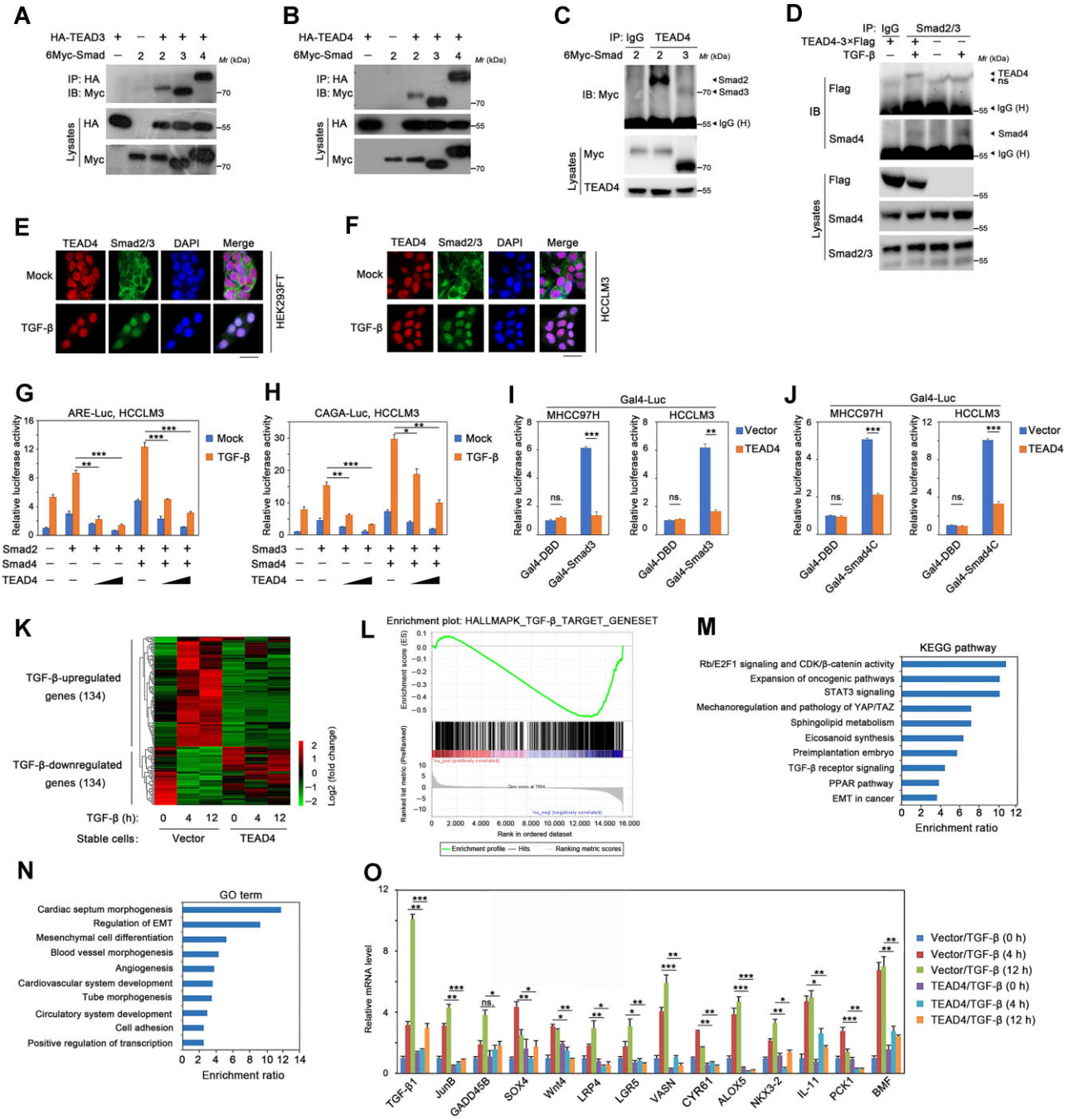

Figure 3 TEAD4 inhibits the transcriptional activity of Smad proteins. (**A** and **B**) HEK293FT cells were transfected with plasmids expressing HA-TEAD3 (**A**) or HA-TEAD4 (**B**), 6Myc-Smad2, 6Myc-Smad3, and 6Myc-Smad4 as indicated. Empty vector was co-transfected where necessary to ensure an equal plasmid amount in each sample. At 40 h post-transfection, cells were harvested for anti-HA IP followed by anti-Myc IB. Protein expression level in cell lysates was determined by IB. (**C**) HEK293FT cells expressing 6Myc-Smad2, 6Myc-Smad3, or empty vector were treated with 200 pM TGF-β for 2 h and then harvested for anti-TEAD4 IP followed by anti-Myc IB. IgG (H), IgG heavy chain. (**D**) HEK293FT cells expressing TEAD4-3×Flag or empty vector were treated with 200 pM TGF-β for 2 h and harvested for anti-Smad2/3 IP followed by anti-Flag IB. ns, non-specific band. (**E** and **F**) HEK293FT (**E**) and HCCLM3 cells (**F**) were treated with or without 100 pM TGF-β for 1 h, followed by anti-TEAD4 (red) and anti-Smad2/3 (green) immunofluorescence, respectively. The nuclei were counterstained by 4,6-diamino-2-phenyl indole (DAPI) (blue). Scale bar, 20 μm. (**G** and **H**) HCCLM3 cells were transfected with plasmids encoding ARE-luciferase (**G**) or CAGA-luciferase (**H**) reporter (200 ng each), together with Renilla-luciferase (50 ng) and Smad2 or Smad3 (100 ng), Smad4 (100 ng), TEAD4 (100 or 200 ng), or empty vector. Cells were treated with or without 100 pM TGF-β for 20 h before

cellular functions in liver cancer in terms of cell proliferation and migration.

TEAD4 impedes the transcriptional activity of Smad proteins

Having established that TEAD4 can mitigate TGF-β signaling, we then asked how TEAD4 exerts this regulatory function. Considering that TEADs have been suggested to interact with Smad3 in a prior study (Fujii et al., 2012), we wonder whether or not TEADs associate with Smad proteins more extensively. Indeed, in co-immunoprecipitation (co-IP) assays, both TEAD3 and TEAD4 associated with Smad2, Smad3, and Smad4, all of which are critical signal transducers in the TGF-β pathway (Figure 3A and B). In addition, ectopic Smad2/3 were observed to interact with endogenous TEAD4 upon TGF-β stimulation, and *vice versa*; meanwhile, TGF-β signaling led to the formation of endogenous Smad2/3–Smad4 complex (Figure 3C and D). TGF-β treatment of HEK293FT and HCCLM3 cells induced translocation of Smad2/3 proteins from the cytoplasm to the nucleus, wherein Smad2/3 colocalized with TEAD4 protein (Figure 3E and F). These results indicate that TEAD4 protein can associate with TGF-β-activated Smad2/3 in the nucleus.

Next, we sought to explore the functional consequence of the Smads–TEAD4 interaction. In HCCLM3 cells, overexpression of Smad2 or Smad3, together with Smad4, activated ARE-luciferase or CAGA-luciferase reporter, respectively, and TGF-β treatment further reinforced the effects of Smads (Figure 3G and H). However, these effects were greatly ameliorated by TEAD4 in a dose-dependent manner, suggesting that TEAD4 might impede the transcriptional activity of Smad proteins. To further confirm this notion, we exploited the Gal4-luciferase reporter system, in which Smad protein is fused with the DNA-binding domain (DBD) of Gal4 and brought to DNA, thereby activating luciferase expression in MHCC97H and HCCLM3 cells (Figure 3I and J). TEAD4 attenuated both Gal4-Smad3 and Gal4-Smad4C activities in HCC cells (Figure 3I and J).

To explore whether TEAD4 affects TGF-β/Smads-induced target gene expression, HCCLM3 cells stably expressing TEAD4 or the control vector were treated with TGF-β for 0, 4, or 12 h, respectively, and then the total cellular RNAs were isolated and subjected to transcriptome sequencing (RNA-Seq). TGF-β stimulation led to significant upregulation of 257 genes and downregulation of 251 genes, respectively. Among them, 134 upregulated genes and 71 downregulated genes displayed attenuated transcriptional responsiveness to TGF-β in the presence of TEAD4 (Figure 3K). Gene set enrichment analysis (GSEA) also revealed that a large portion of TGF-β target genes were reversely correlated with TEAD4-regulated genes in HCCLM3 cells (Figure 3L). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that many of these genes fall into major cellular oncogenic pathways, such as the Rb/E2F1, β-catenin, STAT3, YAP/TAZ, and TGF-β pathways, amongst others (Figure 3M). Gene Ontology (GO) term analysis showed that the most highly enriched genes include those related to mesenchymal cell differentiation, epithelial–mesenchymal transition (EMT), angiogenesis, and tube formation (Figure 3N). Finally, q-PCR experiments verified the accuracy and fidelity of the RNA-Seq data, as TEAD4 was capable of attenuating TGF-β-induced expression of target genes, many of which have been suggested to play a positive role in cancer progression, such as TGF-β1, JunB, SOX4, Wnt4, LGR5, IL-11, etc. (Figure 3O). Therefore, these data demonstrate that TEAD4 can ameliorate gene transcriptional responsiveness to TGF-β in liver cancer.

YAP are dispensable for TEAD4 in regulating TGF-β signaling

YAP and TAZ are not only the most important partners of TEADs in regulating gene transcription, but also play an important role in TGF-β signaling by positively contributing to the activities and functions of Smads in various contexts (Lin et al., 2017; Ben Mimoun and Mauviel, 2018). Therefore, to answer whether TEAD4-mediated inhibition of TGF-β signaling depends on YAP/TAZ or not, we generated three point mutants of TEAD4 (K297A, Y429A, and W299A) that have been reported to lose the ability of interacting with YAP (Chen et al., 2010; Mesrouze et al., 2017). Indeed, co-IP results confirmed that K297A and W299A substitutions in TEAD4 could completely dampen its association with YAP or TAZ, while Y429A mutation reduced the interactions (Figure 4A–C). To support this, overexpression of YAP together with wild-type (WT) TEAD4 led to a robust activation of the TEAD-responsive 8×GTIIC-luciferase reporter (Ota and Sasaki, 2008), whereas the three TEAD4 mutants were disabled in this regard (Figure 4D). However, these TEAD4 mutants retained the ability to associate with Smad3 (Figure 4E and F). Accordantly, these mutants inhibited TGF-β signaling in different reporter assays performed in HCCLM3 cells (Figure 4G) or HEK293FT cells (data not shown). Furthermore, mutant TEAD4 derivatives could still interfere with the transcriptional activity of Smad3 or Smad4, similarly as WT TEAD4 (Figure 4H–K). To further verify the notion that YAP/TAZ are dispensable for TEAD4-mediated inhibition of TGF-β signaling, we depleted YAP and TAZ

Figure 3 *(Continued)* luciferase activity measurement. (**I** and **J**) Gal4-luciferase reporter assays were performed in MHCC97H and HCCLM3 cells, respectively, by transfecting plasmids encoding Gal4-luciferase (pFR-luciferase) (200 ng), Renilla-luciferase (50 ng), Gal4-DBD (100 ng), Gal4-Smad3 (100 ng, **I**) or Gal4-Smad4C (100 ng, **J**), and TEAD4 (100 ng) or empty vector. Cells were treated with or without 100 pM TGF-β for 20 h before luciferase activity measurement. ns, no significance. (**K**–**O**) HCCLM3 cells stably expressing TEAD4 or vector were stimulated with 100 pM TGF-β for 0, 4, or 12 h, respectively, followed by RNA isolation and RNA-Seq. (**K**) Heatmap classification of genes showing attenuated responsiveness to TGF-β stimulation by TEAD4. Color-encoded relative gene expression levels are expressed in log2 scale. (**L**) GSEA enrichment plot showing the enrichment of TGF-β signaling pathway gene signature. (**M** and **N**) KEGG pathway enrichment (**M**) and GO term enrichment (**N**) of TEAD4-affected TGF-β target genes. (**O**) Experimental verification of representative TGF-β target genes by q-PCR analysis.

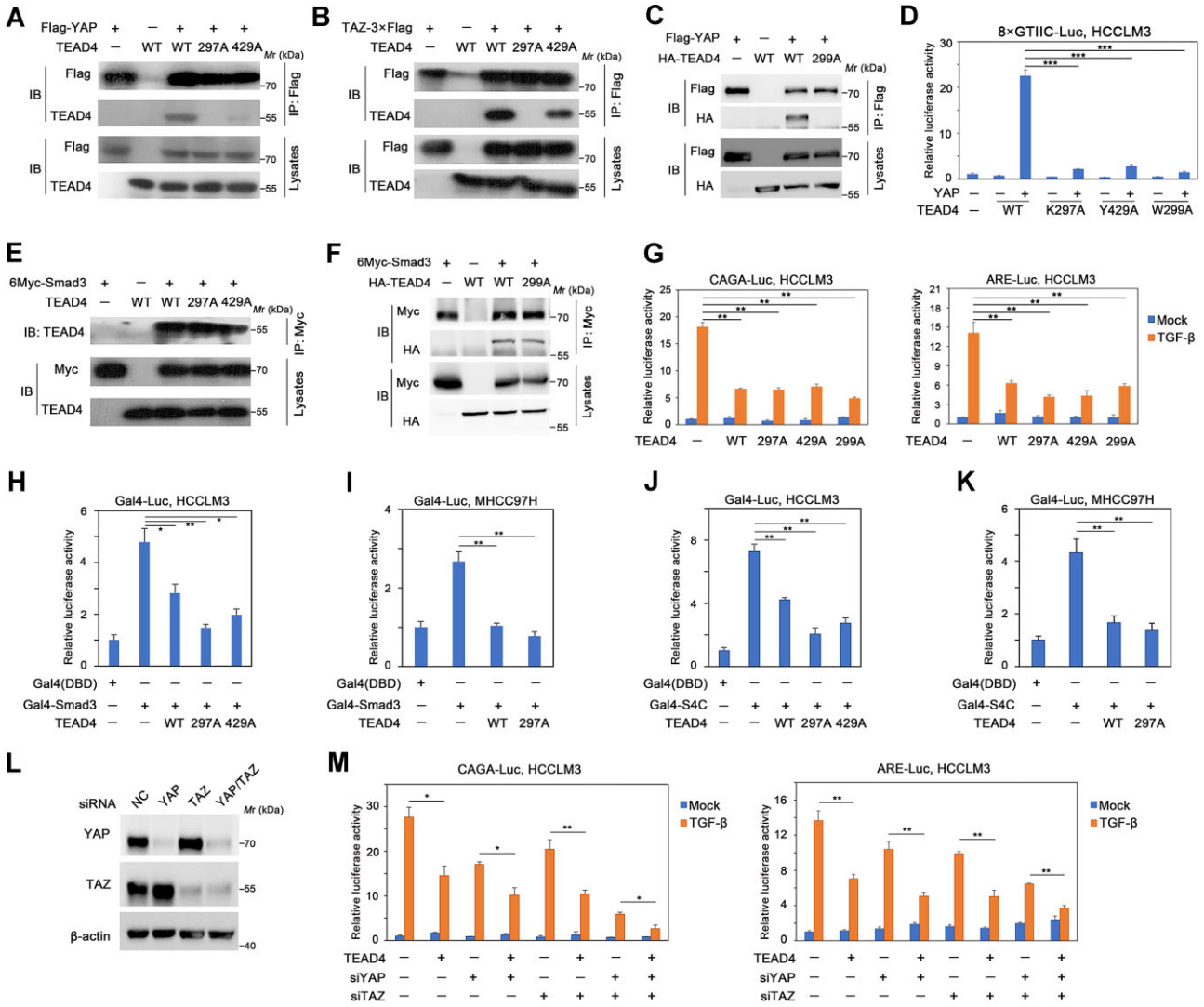

Figure 4 YAP/TAZ are dispensable for TEAD4-mediated inhibition of TGF-β signaling. (**A**–**C**) HEK293FT cells expressing Flag-YAP (**A** and **C**) or TAZ-3×Flag (**B**), together with HA-tagged WT or mutant TEAD4, were subjected to anti-Flag IP and anti-TEAD4 or anti-HA IB. (**D**) HEK293FT cells transfected with plasmids encoding 8×GTIIC-luciferase reporter (200 ng), Renilla-luciferase (50 ng), WT TEAD4 (100 ng) or its point mutation derivatives (K297A, Y429A, or W299A, 100 ng each), and YAP (100 ng) were harvested for luciferase activity measurement. (**E** and **F**) HEK293FT cells expressing 6Myc-Smad3 and HA-tagged WT or mutant TEAD4 were subjected to anti-Myc IP and anti-TEAD4 or anti-HA IB. (**G**) HCCLM3 cells transfected with plasmids encoding distinct luciferase reporters were treated with or without 100 pM TGF-β for 20 h before luciferase activity measurement. (**H**–**K**) Gal4-luciferase reporter assays were done in HCC cells transfected with plasmids as indicated. (**L**) HCCLM3 cells transfected with NC siRNA or siRNAs targeting YAP and/or TAZ were harvested for IB analyses. β-actin expression served as a loading control. (**M**) CAGA-luciferase and ARE-luciferase reporter assays were performed in HCCLM3 cells transfected with siRNAs as indicated and treated with or without 100 pM TGF-β for 20 h.

by siRNAs (Figure 4L). In accordance with the above results, although knockdown of YAP and/or TAZ attenuated TGF-β signaling to a certain degree, TEAD4 could still exert its inhibitory activity toward TGF-β signaling (Figure 4M).

TEAD4 mitigates TGF-β-mediated liver cancer progression independently of YAP/TAZ

HCCLM3 stable cell lines expressing WT TEAD4, the K297A point mutant, and the control vector were established (Figure 5A), and the expression of TGF-β target genes was examined in these cells. In agreement with the results in Figure 4, the K297A mutant exhibited a similar effect as WT TEAD4 in attenuating TGF-β-elicited gene expression (Figure 5B). In addition, both WT TEAD4 and the K297A mutant could attenuate TGF-β-mediated HCC cell proliferation, migration, and invasion (Figure 5C–E). Accordantly, depletion of YAP by siRNAs did not affect the inhibitory effects of TEAD4 on TGF-β-mediated HCCLM3 cell migration (Figure 5F). In line with

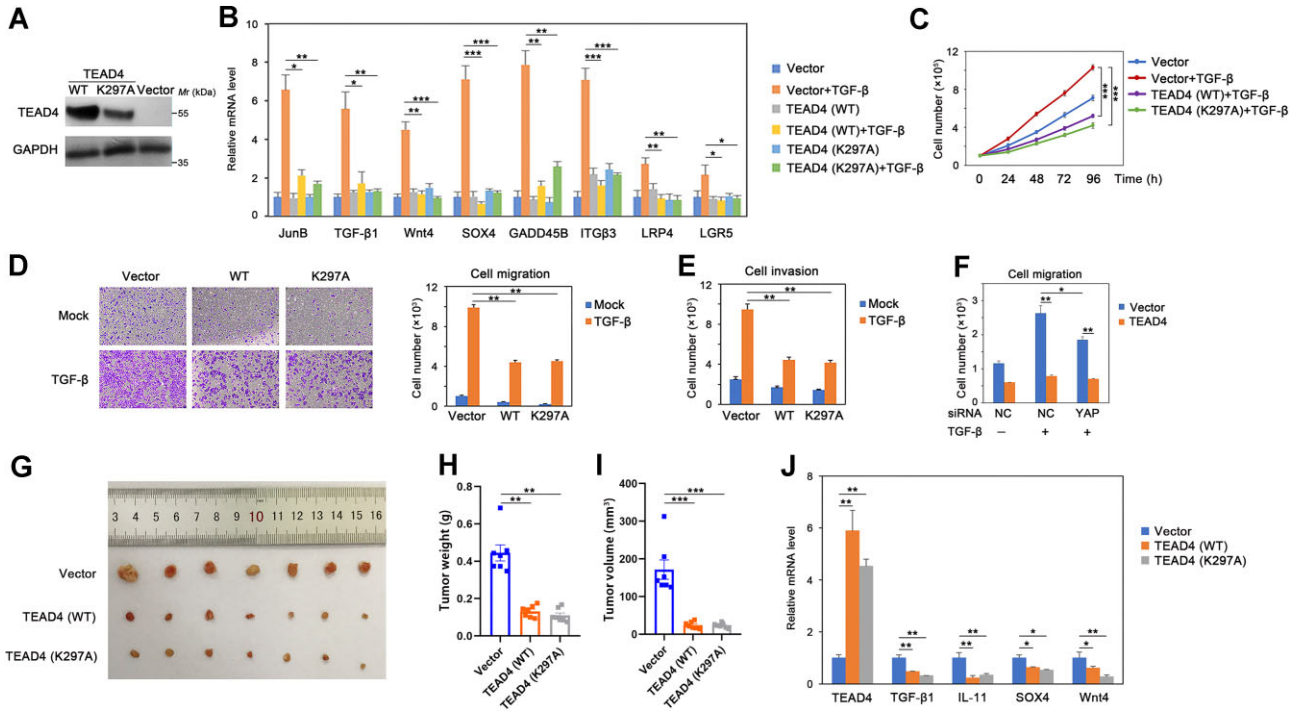

Figure 5 TEAD4 interferes with TGF-β-mediated HCC progression independently of YAP/TAZ. (**A**) HCCLM3 cells stably expressing the control lentivirus vector, WT TEAD4, or its point mutation derivative (K297A) were lysed for IB analysis. (**B**) HCCLM3 stable cells treated with or without 100 pM TGF-β for 4 h were harvested for total RNA isolation and gene expression analysis by q-PCR. (**C**) HCCLM3 cells expressing TEAD4 (WT or K297A) or vector were treated with 100 pM TGF-β as indicated, and cell growth was followed up by direct cell counting at the indicated time points. (**D**–**F**) HCCLM3 stable cells as indicated were treated with or without 200 pM TGF-β in 2% FBS-containing DMEM and subjected to transwell migration and matrigel invasion assays. (**G**) Representative images of xenograft tumors derived from HCCLM3 stable cells as indicated. (**H** and **I**) Xenograft tumor weight (**H**) and volume (**I**) were measured and statistically quantified. (**J**) Gene expression levels in xenograft tumor tissues were analyzed by q-PCR.

these *in vitro* results, overexpression of either WT TEAD4 or its K297A derivative in HCCLM3 cells could inhibit xenograft tumor growth in nude mice (Figure 5G–I). In addition, both WT and mutant TEAD4 decreased the expression levels of TGF-β1, IL-11, SOX4, and Wnt4 in the tumor tissues (Figure 5J), indicative of attenuated TGF-β signaling. In sum, these results demonstrate that TEAD4 can mitigate TGF-β signaling and HCC malignancy in a YAP/TAZ-independent manner.

TEAD4 dissociates the transcriptional coactivator p300 from Smad2/3

The histone acetyltransferase p300 plays a critical role in TGF-β-induced gene transcription by acting as a transcriptional coactivator of Smads (Gaarenstroom and Hill, 2014; David and Massague, 2018). We first tested whether TEAD4 modulates the effects of p300 on Smad transcriptional activity. Intriguingly, as assessed by CAGA-luciferase and ARE-luciferase reporters, ectopic p300 could markedly strengthen the transcriptional activities of Smad2/3 and Smad4, whereas these effects were greatly ameliorated when TEAD4 was coexpressed (Figure 6A and B). Moreover, Gal4-luciferase reporter assays showed that TEAD4 also ameliorated the stimulatory effects of p300 on the transcriptional activities of Smad2 and Smad3 (Figure 6C). These results prompted us to investigate whether TEAD4 could affect the Smad2/3–p300 association. Indeed, overexpression of TEAD4 was able to attenuate the binding of p300 to Smad2/3 (Figure 6D and E), whereas knockdown of TEAD4 led to enhanced interaction between endogenous Smad2/3 and ectopic p300 or between endogenous p300 and ectopic Smad3 (Figure 6F and G). Reasonably, chromatin immunoprecipitation (ChIP) followed by q-PCR analysis showed that overexpression of WT TEAD4 or the K297A derivative attenuated the binding of p300, but not Smad2/3, to the regulatory regions of TGF-β1 and SOX4 genes (Figure 6H and I). These results together indicate that TEAD4 can inhibit TGF-β signaling by impairing the interaction between Smad2/3 and p300.

Discussion

While playing a pivotal role in maintaining normal liver homeostasis and restraining liver tumorigenesis in the early stage, TGF-β switches to a protumor factor along with liver cancer progression by stimulating cancer cell proliferation, migration, and invasion, amongst others (Fabregat et al., 2016;

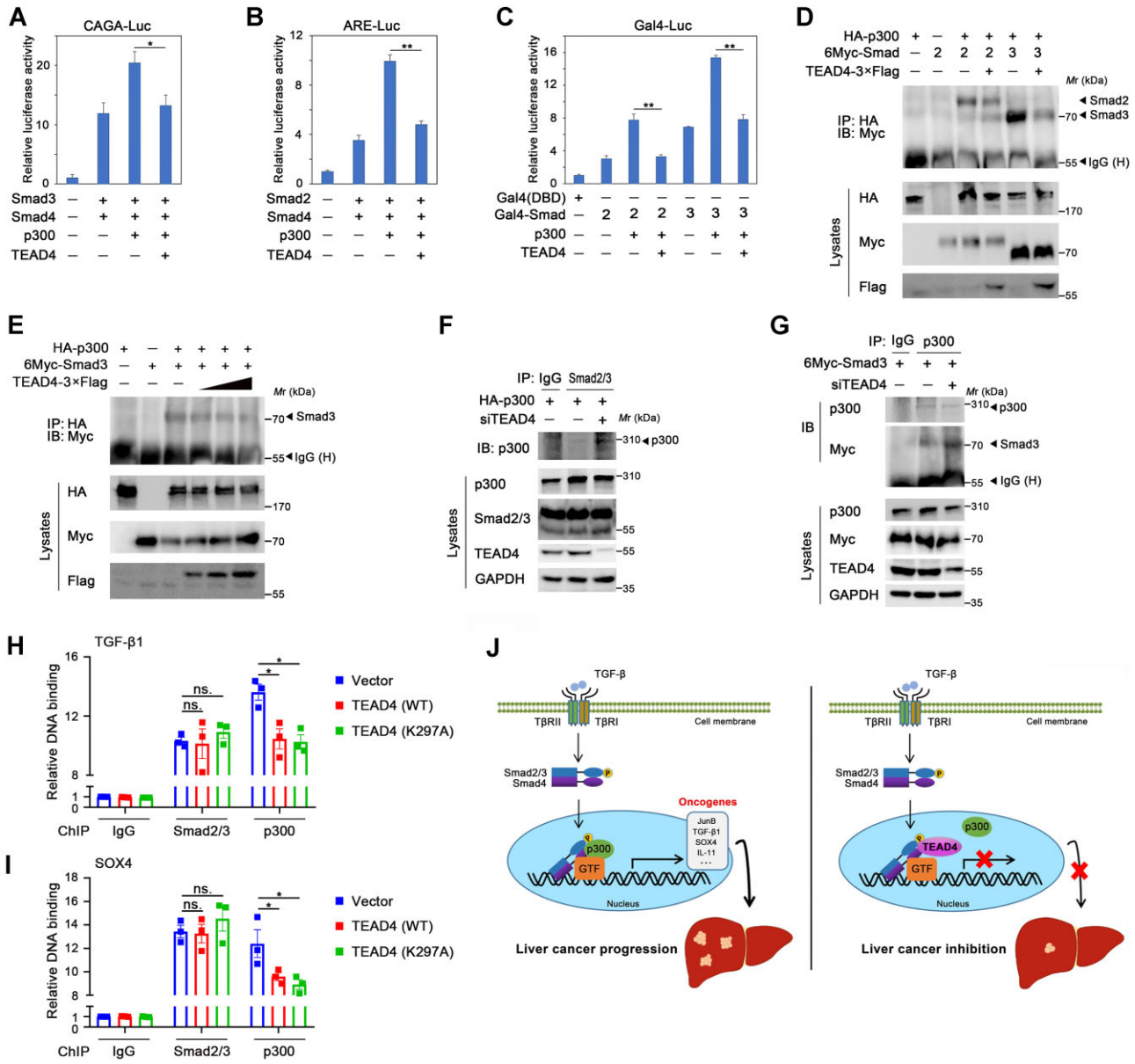

Figure 6 TEAD4 attenuates Smad transcriptional activities by impeding Smad–p300 complex formation. (**A**–**C**) HEK293FT cells transfected with plasmids encoding CAGA-luciferase (**A**), ARE-luciferase (**B**), or Gal4-luciferase (**C**) reporter (200 ng each), Renilla-luciferase (50 ng), and Smad3 (100 ng), Smad4 (100 ng), p300 (50 ng), TEAD4 (100 ng), Smad2 (100 ng), Gal4-DBD (100 ng), Gal4-Smad2 (100 ng), Gal4-Smad3 (100 ng), or empty vector were treated with 100 pM TGF-β for 20 h, followed by luciferase activity measurement. (**D** and **E**) HEK293FT cells were transfected with plasmids expressing HA-p300, 6Myc-Smad2 or 6Myc-Smad3, and/or TEAD4-3×Flag as indicated. Empty vector was co-transfected where necessary to ensure an equal plasmid amount in each sample. At 40 h post-transfection, cells were harvested for anti-HA IP followed by anti-Myc IB. Protein expression level in cell lysates was determined by IB. (**F** and **G**) HEK293FT cells transfected with HA-p300 (**F**) or 6Myc-Smad3 (**G**) and the indicated siRNAs were treated with 200 pM TGF-β for 2 h and subjected to co-IP experiments. (**H** and **I**) Vector- or TEAD4-expressing HCCLM3 cells were treated with 200 pM TGF-β for 2 h, followed by ChIP assays with anti-p300 or anti-Smad2/3 antibodies, with IgG as control. The binding of p300 or Smad2/3 to the promoter of TGF-β1 or SOX4 was analyzed by q-PCR with specific primers. ns, no significance. (**J**) Working model illustrating the regulation of TGF-β signaling by TEAD4. Upon TGF-β signaling, Smads accumulate in the nucleus and bind to DNA. In collaboration with other transcription factors and transcriptional coactivators, including the histone acetyltransferase p300, Smads induce the expression of a group of oncogenes that are involved in liver cancer progression (left). TEAD4 associates with activated Smads in the nucleus in a YAP/TAZ-independent manner and, consequently, attenuates the binding of p300 to Smads and interferes with TGF-β signaling and Smad transcriptional activity (right).

Katz et al., 2016; Dituri et al., 2019; Zhang et al., 2020). This is consistent with the observations that TGF-β is produced in large amounts by liver cancer cells as well as stromal cells, and elevated TGF-β expression level correlates well with malignant progression and bad clinical outcomes (Fabregat et al., 2016; Dituri et al., 2019). The multifaceted roles of TGF-β in the liver can be attributed to, at least in part, the fine-tuning of signaling robustness and duration, as well as the contextual regulation of Smad transcriptional activity (Fabregat et al., 2016; Zhang et al., 2020). Deregulation of TGF-β/Smad signaling is closely associated with liver tumorigenesis and malignant progression (Fabregat et al., 2016; Zhang et al., 2020). For instance, tumor suppressors, like CXXC5, KLF2/4, FHL1, ELF, and PTPN3, and oncoproteins, such as EVI, Axl, and Snail, have all been identified to converge on regulation of TGF-β signaling and, consequently, modulation of liver cancer progression (Kitisin et al., 2007; Ding et al., 2009; Reichl et al., 2015; Yasui et al., 2015; Moon et al., 2017; Yan et al., 2018; Yuan et al., 2019; Li et al., 2020). In the present study, we demonstrate that TEAD4 associates with Smad proteins (Smad2/3 and Smad4) in the nucleus, leading to mitigation of TGF-β signaling and Smad transcriptional activity, attenuation of the transcriptional responsiveness of a large cohort of protumor genes to TGF-β, and amelioration of TGF-β-mediated HCC functions *in vitro* and HCC xenograft tumor growth in nude mice. These results unveil a novel role of TEAD4 in the regulation of TGF-β signaling during liver cancer progression.

As the downstream transcriptional regulators of the Hippo pathway, both TEADs and YAP/TAZ have been found to modulate the activities and functions of TGF-β-activated Smads (Attisano and Wrana, 2013; Ben Mimoun and Mauviel, 2018; Noguchi et al., 2018). As a matter of fact, on the promoters of many TGF-β target genes, there exist binding sites for both Smads and TEADs (Fujii et al., 2012; Beyer et al., 2013; Hiemer et al., 2014). Not surprisingly, Smads, YAP/TAZ, and TEADs have been reported to form different protein complexes on gene promoters, thereby regulating cancer progression, tissue fibrosis, and stem cell differentiation by recruiting distinct transcriptional cofactors (Fujii et al., 2012; Beyer et al., 2013; Hiemer et al., 2014; Noguchi et al., 2018). Intriguingly, we observed that YAP/TAZ are dispensable for TEAD4-mediated inhibition of TGF-β signaling and its protumor functions in HCC. First, although not able to interact with YAP/TAZ, point mutation derivatives of TEAD4, including the K297A, Y429A, and W299A mutants, retain the capability of associating with Smads, inhibiting their transcriptional activity, and ameliorating TGF-β-mediated tumor-promoting functions during HCC progression, both *in vitro* and *in vivo* (Figures 4A–K and 5). Second, depletion of YAP and/or TAZ by siRNAs attenuated TGF-β signaling to some extent, which is in agreement with previous results that YAP and TAZ positively contribute to TGF-β signal transduction and Smad activity (Varelas et al., 2008; Hiemer et al., 2014; Pefani et al., 2016). However, TEAD4 is still able to mitigate TGF-β signaling after knockdown of YAP/TAZ (Figures 4L, M and 5F). In line with our results, several other studies also support the notion that TEADs may function in the absence of YAP/TAZ. TEAD4 has been reported to drive colorectal cancer cell EMT and malignant progression by transactivating TCF4 or upregulating the transcription of EMT-related genes, especially vimentin (Liu et al., 2016; Jiao et al., 2017). TEAD4 was also shown to promote the transcriptional activity of MyoD and drive myoblast terminal differentiation (Feng et al., 2019), as well as reinforce the occupancy of glucocorticoid receptor and C/EBPβ on the promoter of PPAR-γ2 during adipogenesis (Park et al., 2019). All the above actions of TEAD4 seem to be independent of YAP and TAZ (Liu et al., 2016; Jiao et al., 2017; Feng et al., 2019; Park et al., 2019).

It is interesting that TEAD4 antagonizes TGF-β/Smads-induced expression of several luciferase reporters that encompass the classic binding sites for Smads but not those for TEADs (Wrana et al., 1992; Huang et al., 1995; Dennler et al., 1998). These results implicate that TEAD4 may regulate Smad activity via novel mechanisms, probably by acting as a transcriptional corepressor, although not yet determined directly. In support of this notion, TEAD1 has been evidenced to associate with serum response factor, thus inhibiting its transcriptional activity in luciferase reporter assays and ameliorating the expression of smooth muscle-specific genes (Liu et al., 2014). Similarly, TEADs can antagonize Runx2 transcriptional activity and interfere with osteoblast differentiation (Suo et al., 2020). In addition, early studies by using Gal4 reporter systems also revealed that TEADs display transcription-repressive effects when fused with the DBD of Gal4 (Xiao et al., 1991; Yockey et al., 1996).

The histone acetyltransferase p300 has been well documented to be broadly involved in gene transcriptional activation via acetylation of histones and transcription factors, including Smads (Gaarenstroom and Hill, 2014). Indeed, in agreement with previous studies (Inoue et al., 2007; Tu and Luo, 2007), p300 could associate with Smad2/3, potentiate their transcriptional activity, and bind to the regulatory regions of TGF-β target genes, including TGF-β1 *per se* and SOX4, but all these effects were impaired by TEAD4 (Figure 6). Although this is able to explain how TEAD4 ameliorates TGF-β-induced gene upregulation, it remains unanswered and deserves future investigation as to how TEAD4 attenuates the transcriptional responsiveness of TGF-β-downregulated genes. In sum, our study unveils a novel mechanism by which TEAD4 mitigates TGF-β signaling and Smad transcriptional activity in liver cancer.

Materials and methods

Cell culture and transfection

HEK293FT, HEK293T, the normal hepatocyte cell line LO2, and human HCC cell lines including Huh-7, HLE, SMMC-7721, MHCC97H, MHCC97L, and HCCLM3 were cultured in Dulbecco's modified Eagle's medium (DMEM, Corning) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in a humidified, 5% CO_2 incubator. Plasmid transfection into mammalian cells was conducted with Lipofectamine 2000 (Invitrogen) or Vigofect (Vigorous Biotechnology).

Plasmids, gene silencing, and reagents

Mammalian expression constructs of p300, YAP, TAZ, TEAD3, and TEAD4 (WT and the K297A, Y429A, and W299A mutants), fused with HA or Flag tag, were generated based on the pcDNA3.1(+) vector. The luciferase reporters, including CAGA-luciferase, ARE-luciferase, 3TP-luciferase, Gal4-luciferase (pFR-luciferase), and Renilla-luciferase, and constructs encoding Gal4-DBD, Gal4-Smad3, Flag-Smad3, Flag-Smad4, 6Myc-Smad2, 6Myc-Smad3, and 6Myc-Smad4 were described previously (Yan et al., 2016, 2018).

Double stranded siRNAs targeting human YAP, TAZ, or TEAD4 and the non-specific control siRNA were purchased from RiboBio (China). siRNAs were transfected using the siTran™ siRNA Transfection reagent (OriGene). The sequences of siRNAs used in this study were as follows: YAP siRNA, 5′-CCACCAAGCTAGAT AAAGA-3′; TAZ siRNA, 5′-CCGCAGGGCTCATGAGTAT-3′; TEAD4 siRNA #1: 5′-GTATGCTCGCTATGAGAAT-3′; and TEAD4 siRNA #2: 5′-GGAACAAACUGUGCCUGAATT-3′. Recombinant human TGF-β1 peptide was purchased from R&D Systems. In this study, TGF-β treatments all refer to recombinant TGF-β1 peptide.

Luciferase reporter assay

Luciferase reporter assay was carried out as described previously (Yan et al., 2016).

IP, IB, and immunofluorescence

IP and IB were conducted as previously described (Yan et al., 2018).

Antibodies used in IP and IB are as follows: anti-TEAD4 (12418-1-AP) and anti-Myc (16286-1-AP) antibodies were bought from Proteintech; anti-HA (390025) and anti-Flag (700002) antibodies were purchased from ZEN BIO; anti-Smad4 (46535S), pan-TEAD (13295S), anti-YAP (12395S), anti-TAZ (4883S), and anti-p300 (86377S) antibodies were bought from Cell Signaling Technology; and anti-GAPDH (sc-47724), anti-β-actin (sc-8432), and anti-Smad2/3 (sc-133098) antibodies were purchased from Santa Cruz Biotechnology.

For immunofluorescence, cells were sequentially treated with 4% paraformaldehyde for 10 min, 0.5% Triton X-100 for 5 min, 0.5% bovine serum albumin for 30 min, the primary antibody overnight at 4°C, and finally the secondary antibody for 1 h in the dark. The nuclei were counterstained with DAPI (Sigma). Images were obtained with a confocal ZEISS LSM 800 microscope. Anti-Smad2/3 (Cell Signaling Technology), anti-HA (ZEN BIO), and anti-TEAD4 (Proteintech) antibodies were used for immunofluorescence.

Lentivirus production and stable cell line establishment

To produce defective lentivirus, HEK293FT cells were transfected using Lipofectamine 2000 with the empty lentivirus vector pL6.3-CMV-GFP-IRES-MCS or its derivatives expressing WT TEAD4 or the K297A mutant, along with the package constructs pCMVΔ8.9 and VSVG. To establish TEAD4- or vector-expressing stable cell lines, HCCLM3 cells were infected by lentivirus particles at a multiplicity of infection of 120 pfu per cell. At 48 h post-infection, the cells were washed with phosphate-buffered saline (PBS), complemented with fresh growth medium containing blasticidin (3 μg/ml). Drug-resistant cells were diluted and dispersed into 96-well plates. Single clones with fluorescence were selected from 96-well plates for expanded culture and subsequently maintained in DMEM in the presence of 10% FBS and blasticidin.

Transwell and wound-healing assays

TEAD4- or vector-expressing stable HCCLM3 cells (1×10^5 or 2×10^5) were seeded in the upper chamber of the transwell system with inserts of 8.0-μm pore size (Corning). As the chemoattractant, 2% FBS-containing DMEM with or without 200 pM TGF-β was added to the lower chamber. After incubating for 24 h, the cells still in the upper chamber were removed with cotton swabs, and the cells already passing through the membrane to invade the lower chamber were fixed with 4% paraformaldehyde and stained using 1% crystal violet. The membranes were cleaned and air-dried, and the cells on the membrane were quantified in five random microscopic fields. For invasion assays, transwell inserts were coated with Matrigel (BD Biosciences), and subsequent steps were performed similarly as above.

For wound-healing assays, cells were plated in 6-well plates. When growing into confluency, the cell monolayer was wounded by scraping with a pipette tip, and then treated with or without 200 pM TGF-β in 2% FBS-containing DMEM. Cell migration was monitored by following up the narrowing of the wound gap width under a phase-contrast microscope at different time points. Experiments were performed in triplicate.

Cellular RNA extraction, RNA-Seq, and data processing

HCCLM3 cells stably expressing vector or TEAD4 were cultured in 35-mm dishes. After being treated with 100 pM TGF-β for 0, 4, or 12 h, respectively, cells were harvested for total cellular RNA extraction with Trizol (Life Technologies). Then, the RNA samples were subjected to RNA-Seq with the Illumina HiSeq 2000 sequencer in BerryGenomics. Transcriptome construction from RNA-Seq raw data and subsequent analyses were conducted similarly as previously described (Yan et al. 2018), with updated databases or softwares. Specifically, raw single-end reads were trimmed of the first 20 bp from each end before mapping. Differentially expressed genes (DEGs) were filtered by the threshold that gene expression levels were altered by a fold change of >1.75 for upregulated genes and <0.57 for downregulated genes, with a P-value <0.05.

q-PCR

Cellular RNA was reverse-transcribed using the PrimeScript RT Reagent Kit (TaKaRa). The resulting cDNAs were used for q-PCR analysis with the Power SYBR-Green PCR SuperMix (Mei5 Biotechnology) in a q-PCR StepOne Real-Time PCR Detection System (Bio-Rad Laboratories). Experiments were performed in triplicate, and gene expression level was normalized to that of GADPH. Primers for human genes used for q-PCR are collected in Table 1.

Table 1 Sequences of primers used in q-PCR.

Gene	Sequence	
JunB	Forward	5′-ACGACTCATACACAGCTACGG-3′
	Reverse	5′-GCTCGGTTTCAGGAGTTTGTAGT-3′
TGF-β1	Forward	5′-ATGACCCTCACCTCTATGTACC-3′
	Reverse	5′-CACAGTTCACAGTTACAATCCCA-3′
GADD45B	Forward	5′-TACGAGTCGGCCAAGTTGATG-3′
	Reverse	5′-GGATGAGCGTGAAGTGGATTT-3′
SOX4	Forward	5′-GACCTGCTCGACCTGAACC-3′
	Reverse	5′-CCGGGCTCGAAGTTAAAATCC-3′
Wnt4	Forward	5′-GTACGCCATCTCTTCGGCAG-3′
	Reverse	5′-GCGATGTTGTCAGAGCATCCT-3′
LRP4	Forward	5′-GTGAGGAGGACGAGTTTCCCT-3′
	Reverse	5′-TCACCGTCGCAGTACCAATG-3′
LGR5	Forward	5′-GAGTTACGTCTTGCGGGAAAC-3′
	Reverse	5′-TGGGTACGTGTCTTAGCTGATTA-3′
VASN	Forward	5′-TCTCACCTATCGCAACCTATCG-3′
	Reverse	5′-CAGACGGAGTAAGTGGCGTT-3′
PCK1	Forward	5′-CATTGCCTGGATGAAGTTTGACG-3′
	Reverse	5′-GGGTTGGTCTTCACTGAAGTCC-3′
BMF	Forward	5′-CAGTGGCAACATCAAGCAGAGG-3′
	Reverse	5′-GCAAGGTTGTGCAGGAAGAGGA-3′
NKX3-2	Forward	5′-CCGCTTCCAAAGACCTAGAGGA-3′
	Reverse	5′-ACCGTCGTCCTCGGTCCTTGG-3′
ALOX5	Forward	5′-GGAGAACCTGTTCATCAACCGC-3′
	Reverse	5′-CAGGTCTTCCTGCCAGTGATTC-3′
CYR61	Forward	5′-GGTCAAAGTTACCGGGCAGT-3′
	Reverse	5′-GGAGGCATCGAATCCCAGC-3′
IL-11	Forward	5′-TCAAGTTCCGTTTGCAGTACC-3′
	Reverse	5′-GGAGGCATCGAATCCCAGC-3′
TEAD4	Forward	5′-GAACGGGGACCCTCCAATG-3′
	Reverse	5′-GCGAGCATACTCTGTCTCAAC-3′
ITGβ3	Forward	5′-CATGGATTCCAGCAATGTCCTCC-3′
	Reverse	5′-TTGAGGCAGGTGGCATTGAAGG-3′
GAPDH	Forward	5′-ACCACAGTCCATGCCATCAC-3′
	Reverse	5′-TCCACCACCCTGTTGCTGTA-3′

Cell proliferation

HCCLM3 cells stably expressing TEAD4 or vector were cultured in 24-well plates and treated with or without 100 pM TGF-β. Cell number was followed up at different time points by direct cell counting. Alternatively, the CCK-8 assay was exploited for cell growth measurement. Cells were seeded in 96-well plates at a density of 1000 cells/well in 100 μl of culture medium. After incubation of the plate for an appropriate length of time, 10 μl of CCK-8 solution was added to each well of the plate, followed by incubation at 37°C for 1–4 h. Then, the absorbance at 450 nm of each sample was determined by using a microplate reader.

ChIP

HCCLM3 cells (3×10^6 cells/10-cm plate) were harvested for the ChIP assay. Briefly, cells were washed with PBS and cross-linked with 1% formaldehyde for 10 min at room temperature. The reaction was stopped by adding 1.25 M glycine for 5 min at room temperature. After washing, nuclear lysis, and sonication, the chromatin was sheared into 250–800 bp fragments. Then, the chromatin fraction was incubated with anti-Smad2/3 (#8685S, Cell Signaling Technology) and anti-p300 (#86377S, Cell Signaling Technology) antibodies or the control IgG (#3900S, Cell Signaling Technology) overnight at 4°C. Chromatin-bound beads were subjected to extensive washing, and the eluted chromatin was de-cross-linked using ChIP elute buffer with proteinase K for 2 h at 65°C. The eluted DNA was purified for subsequent q-PCR analysis. The q-PCR primers were as follows: TGF-β1 forward (−462), 5′-GTCCTGTTGCCCCCTCCT-3′; TGF-β1 reverse (−378), 5′-CCCAGAACGGAAGGAGAGTC-3′; SOX4 forward (+427), 5′-AGCTTCAGCAACCAGCATTC-3′; and SOX4 reverse (+512), 5′-CCCTCTCTCTCGCTCTCTCA-3′.

Xenograft tumor assay

All mouse studies were approved, and all animals were manipulated according to the protocols approved by the Animal Ethics Committee of Nanchang University. Mice were housed in pathogen-free and ventilated cages and allowed free access to irradiated food and autoclaved water ad libitum in a 12-h light/dark cycle, with room temperature at 25°C ± 2°C and humidity between 45% and 65%. Male BALB/c nude mice of 4 weeks of age were purchased from GemPharmatech. Mice were subcutaneously injected with 5×10^6 cancer cells resuspended in 200 μl DMEM into the right flank ($n = 7$ mice per group) and sacrificed on Day 28. Then, the xenograft tumors were dissected, photographed, and weighted. The tumor volume based on caliper measurements was calculated by the formula: 0.5 × (largest diameter) × (smallest diameter).

Statistical analyses

All the experiments were repeated at least in triplicate. The values were presented as mean ± standard deviation, and the significance between two mean values was calculated using Student's *t*-test. A *P*-value <0.05 was considered as statistically significant.

Data availability

The RNA-Seq raw data and processed DEGs have been documented in the NCBI GEO database with accession number GSE168515. All other data are included within the article. The materials and methods in this study are available from the corresponding authors upon reasonable request.

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