**Hippo Coactivator YAP1 Upregulates SOX9 and Endows Esophageal Cancer Cells with Stem-like Properties**


**Abstract**

Cancer stem cells (CSC) are purported to initiate and maintain tumor growth. Deregulation of normal stem cell signaling may lead to the generation of CSCs; however, the molecular determinants of this process remain poorly understood. Here we show that the transcriptional coactivator YAP1 is a major determinant of CSC properties in nontransformed cells and in esophageal cancer cells by direct upregulation of SOX9. YAP1 regulates the transcription of SOX9 through a conserved TEAD binding site in the SOX9 promoter. Expression of exogenous YAP1 in vitro or inhibition of its upstream negative regulators in vivo results in elevated SOX9 expression accompanied by the acquisition of CSC properties. Conversely, shRNA-mediated knockdown of YAP1 or SOX9 in transformed cells attenuates CSC phenotypes in vitro and tumorigenicity in vivo. The small-molecule inhibitor of YAP1, verteporfin, significantly blocks CSC properties in cells with high YAP1 and a high proportion of ALDH1.

Our findings identify YAP1-driven SOX9 expression as a critical event in the acquisition of CSC properties, suggesting that YAP1 inhibition may offer an effective means of therapeutically targeting the CSC population.

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**Introduction**

The Hippo pathway and its transcriptional coactivator Yes-associated protein (YAP1) have emerged as major regulators of organ size and proliferation (1, 2). In mammals, the Hippo pathway consists of a core kinase cascade in which Mst1/2 forms a complex with the adaptor protein Sav1 that phosphorylates the kinases Lats1/2. Lats 1/2 then phosphorylate and repress the transcriptional coactivators YAP1 and TAZ by promoting ubiquitination, degradation, and cytoplasmic retention (3). The importance of YAP1 and deregulation of the Hippo pathway during cancer development and progression is emerging. Studies in mice have demonstrated that conditional deletion of several Hippo pathway proteins, including Mst1/2 and Sav1, can lead to a dramatic increase in organ size and tumor formation at very high incidence (4), effects that are largely dependent on YAP1. YAP1 overexpression and its nuclear localization correlates with poor patient outcome in several cancers (5, 6). Overexpression YAP1 in cancer cell lines can promote EMT and enhance in vitro invasion (7). In transgenic mice, tissue-specific expression of YAP1 results in tissue overgrowth and tumor formation (8). These observations suggest that YAP1 is a key regulator of the cancer cell phenotype and that the Hippo signaling pathway is essential in restricting YAP1 activity in normal tissues. In esophageal cancer, a significant increase in YAP1 cytoplasmic and nuclear localization was reported in both esophageal adenocarcinoma (EAC) and squamous cell carcinoma (SCC; refs. 9, 10). Amplification of the 11q13 locus (containing YAP1) has been reported in EAC, SCC, and liver cancer (9, 11, 12). However, the functional role of YAP1 in driving cancer stem cells (CSC) and its relevant transcriptional targets in esophageal cancer is not known.

SOX9, a high mobility group (HMG) box transcription factor, is required for development and lineage commitment (13). Recently, it was reported that SOX9 is highly upregulated in many premalignant lesions and in tumor tissues and plays an oncogenic role in tumor development (14–16). Coexpression of exogenous SOX9 and Slug serves to convert differentiated luminal cells into mammary stem cells (MaSC); promotes the tumorigenic and metastasis-seeding abilities of human breast cancer cells; and is associated with poor patient survival (17). SOX9 also mediates Wnt/β-catenin activation, which in turn...
induces increased LRP6 and TCF4 expression in breast cancer (18). Taken together, these studies imply that YAP1 and SOX9 are involved in cancer development, but the mechanism by which they coordinately regulate CSCs remains to be defined.

In this study, we provide evidence that YAP1 directly upregulates SOX9 through a conserved TEAD binding site and endows CSC properties onto a wide variety of non-transformed cell types of gastrointestinal origin, including primary esophageal epithelial cells, immortalized embryonic liver cells as well as esophageal cancer cells. In these cells, CSC properties—including tumorsphere formation, propagation, and tumorigenicity—are dependent on YAP1 expression. Our findings suggest that YAP1 regulation of SOX9 is a key modulation of the CSC phenotype and the YAP1–SOX9 axis is potentially an important new therapeutic target in esophageal cancer.

Materials and Methods

Cells and reagents

The human esophageal cancer cell lines FLO-1, SKGT-4, BE3, OE33, JHESO, OACP, YES-6, and KATO-TN were kindly provided by Drs. Mien-Chie Hung and Health Skinner (UT MD Anderson Cancer Center, Houston, TX) and have been previously described (19–21). Mouse embryonic fibroblast (MEF) cells and the immortalized fetal liver cell line B299 were generated by published methods (22). All human cell lines were authenticated and recharacterized in the cell line core facility of UT MD Anderson Cancer Center every 6 months. Verteporfin was purchased from Pharmacoepia.

Doxycycline hyclate was obtained from Sigma-Aldrich. Antibody against YAP1 was purchased from Cell Signaling Technology. CTGF antibody and lentiviral shRNA plasmids directed against SOX9 were from Santa Cruz Biotechnology. SOX9 antibody was from Chemicon. DNA plasmids that encode wild-type human YAP1 (hYAP1, CMV-YAP1) or a mutant protein that no longer be phosphorylated at Ser127 (ref. 23; CMV-S127A-YAP) and TEAD2 (pcDNA2-TEAD2) were obtained from Addgene. Doxycycline-inducible YAP1 lentiviral plasmid (PIN20YAP1) was constructed by inserting flag-tagged YAPI127A cDNA amplified from CMV-S127A-YAP into pINDUCER20 (provided by Thomas Westbrook, Baylor College of Medicine, Houston, TX). Lentiviral shRNA plasmids directed against human YAP1 was kindly provided by Dr. Li Ma (UT MD Anderson Cancer Center) and have been previously described (24).

Primary mouse esophageal epithelial cell isolation and culture

Mouse primary esophageal cells were isolated according to published methods (25, 26). Briefly, esophagi were opened and placed longitudinally, washed in PBS, and then incubated with 1.0 U/mL of Dispase I (Roche) for 15 minutes at 37°C. The mucosa was incubated with 0.05% trypsin/EDTA for 20 minutes at 37°C. Cells were centrifuged and resuspended in BME medium for primary epithelial cells [DMEM/F12 with 10% FBS, 1% Glutamax, insulin (Invitrogen, 10 μg/mL), IGF2 (Preprotech, 30 ng/mL), and EGF (50 ng/mL)].

Protein extraction and Western blot analysis

Protein isolation and Western blot analyses were performed as previously described (27).

Transient transfection and luciferase reporter assays

The SOX9 luciferase reporter was previously described (28). The SOX9 promoter-luciferase construct with mutant TEAD binding site was generated using a site-directed mutagenesis kit (Stratagene). Transient cotransfection with SOX9 luciferase reporter and Renilla vector were performed as previously described (28).

Immunohistochemistry

IHC staining for SOX9 and YAP1 was performed on tissue microarray slides consisting of 113 EAC and nonneoplastic esophageal tissue samples from patients who underwent esophagectomy using antibodies against SOX9 (1:2,000) and YAP1 (1:100) as described previously (28). The staining results were evaluated by a pathologist (D.M. Maru) and a scientist (S. Song) on the basis of the percentage of tumor cell nuclei stained (0, no staining; 1, ≤10%; 2, 10%–50% and 3, >50%) and the staining intensity (0, negative; 1, weak; 2, moderate; and 3, strong). An overall score of 1–5 designated low expression, and an overall score of 6–9 designated high expression for both YAP1 and SOX9 in tissue microarray (TMA) tissues.

Indirect immunofluorescence

Indirect immunofluorescent staining was performed as described (27).

Flow cytometric labeling and FACS

ALDH1+ or ALDH1− JHESO cells were analyzed and collected by FACS according to the ALDEFLUOR Detection Kit as previously described (29).

Tumorsphere formation assay

Sphere culture was performed as previously described (28). Briefly, single-cell suspensions of B299, Eso primary and esophageal cancer cells or FACS-isolated ALDH1+ or ALDH1− JHESO cells were seeded in triplicate onto 6-well ultra-low attachment plates (1,000–2,500 cells per well) in serum-free DMEM/F12 supplemented with 20 ng/mL EGF, 5 μg/mL insulin, 0.5 μg/mL hydrocortisone, 2% B27 supplement w/o vitamin A, and 1% N2 Supplement (Invitrogen). After 10 to 20 days of culture, the number of tumor spheres formed (diameter > 100 μm) was counted under the microscope.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation was performed as described previously (30). Briefly, chromatin purified from SKGT-4 DOX−/DOX+ cells was sheared then immunoprecipitated with a YAP1 antibody (Novus Biologicals). The following primer pairs were used for PCR amplification: Sox9 promoter primers (spanning the TEAD binding sequence): forward 5′-GTTCCCGGTGCCGAGAGAGC-3′ and reverse 5′-GGGATCGCAGCCAAAGGGCGGAC-3′; 5′-GGATCTGCAGCAAAAGCCGCGGAC-3′. Sox9 promoter control primers (not spanning the TEAD binding site): 5′-GACCTGAGGTTGACGCGCG-3′. 
In vivo xenograft mouse model
SKGT-4 (PIN20YAP1) cells (1 × 10⁶) without (DOX−) or with (DOX+) YAP1 induction and further knockdown YAP1 (shYAP1) or SOX9 (shSOX9) were inoculated into nude mice (n = 5 per group). Mice in the DOX− groups were provided with drinking water containing 2.5% sucrose and 2.5% doxycycline, whereas mice in the DOX− groups were provided with water containing 2.5% sucrose. Similarly, JHESO cells (1.5 × 10⁶ cells) were subcutaneously injected into nude mice in 2 groups, n = 5 for each group. After 10 days, verteporfin was applied by intraperitoneal (i.p.) injection, 100 mg/kg/mouse, 3 times a week for total three weeks. Control mice were injected with PBS. Tumor sizes were determined by the formula: tumor volume (mm³) = [length (mm) × width (mm)]² × 0.52.

Statistical analysis
Data were analyzed using the Student t test and Fisher exact test (for IHC). A P value of <0.05 was required for statistical significance, and all tests were 2-sided. All tests were done with SPSS 10.1 software (SPSS, Inc.).

Results
Nuclear YAP1 and SOX9 expression are positively correlated in human EAC tissues
Elevated expression and aberrant nuclear localization of YAP1 and SOX9 have been reported in many human solid tumors, including in the lung, colon, and esophagus (10, 31, 32). However, whether elevated YAP1 and SOX9 expression are correlated in these malignancies has not been determined. To determine whether elevated YAP1 expression correlated with SOX9 expression in EAC, immunohistochemistry was performed on a tissue microarray containing 113 cases of EAC using specific YAP1 and SOX9 antibodies. As shown in Fig. 1A, nuclear staining of YAP1 and SOX9 antibodies is weak or absent in normal squamous epithelium. YAP1 and SOX9 showed relatively weak expression in Barrett esophagus tissues, a precursor lesion to EAC. However, YAP1 and SOX9 were positive in a majority of EAC cell nuclei in tumor tissues. Progressively increased expression of YAP1 and SOX9 in normal epithelium, Barrett esophagus, and EAC was observed in tissues from the same patient (Fig. 1A). Furthermore, both YAP1 and SOX9 immunostaining intensity and the combined scores with staining percentage in tumor tissues are highly correlated (Fig. 1B). Highly expressed YAP1 and SOX9 were further confirmed in esophageal cancer tumor cell lines compared with nontumorigenic Barrett cells (CPA, CPC; Supplementary Fig. S1A). These data support the notion that the activation of YAP1 and SOX9 is involved in the transformation of normal esophageal cells to EAC cells.

YAP1 upregulates SOX9 expression in both normal and transformed cells
The observation that YAP1 and SOX9 are coordinately expressed in EAC tissues led to the hypothesis that YAP1 might regulate SOX9 expression directly or indirectly. To determine this possibility and gain further insight into the relationship between YAP1 and SOX9 expression, we first transduced the esophageal cancer cells SKGT-4 and KATO-TN with a doxycycline-inducible human flag-tagged YAP1S127A cDNA (PIN20YAP1S127A). Successful YAP1 induction in SKGT-4 (PINYAP20) and KATO-TN (PINYAP20) cells by doxycycline at 1 μg/mL increased expression of both SOX9 and CTGF, a known YAP1 target (Fig. 2A). To exclude the possibility that doxycycline itself has any effect on SOX9 expression, several esophageal cancer cell lines (SKGT-4, KATO-TN, and JHESO) were treated with doxycycline at 1 μg/mL for 48 hours. There is no induction of SOX9 expression in these cell lines by doxycycline (Supplementary Fig. S1B). In contrast, shRNA-mediated knockdown of YAP1 in JHESO cells greatly reduces steady-state SOX9 and CTGF protein levels (Fig. 2B). Furthermore, immunofluorescence demonstrates that induction of YAPI increases SOX9 expression in both SKGT-4 and KATO-TN cells (Fig. 2C). These data indicate YAP1 is both necessary and sufficient for SOX9 expression in esophageal cancer cells.

To determine whether SOX9 expression is regulated by Hippo signaling pathway and whether Hippo/YAP1 signaling functions to regulate SOX9 in multiple primary and immortalized cells from diverse lineages, we examined the effect of genetically inactivating Hippo pathway components in primary MEF cells and in immortalized fetal liver cells (B299). Deletion of Lats1/2 in MEFs that contain conditional alleles of Lats1/2 with adenovirus-cre transduction resulted in the upregulation of SOX9 protein levels (Fig. 2D). Similarly, in B299 fetal liver progenitor cells that are homozygous for the Sav1 floxed allele enhanced SOX9 expression (Fig. 2E). Furthermore, transfection of human embryonic kidney (HEK293 T) cells with constitutively active mutant YAP1S127A cDNA or with wild-type YAP1 induced SOX9 expression in concert with YAP1 induction (Fig. 2F). In addition, we have previously demonstrated that conditional deletion of the core Hippo signaling components Sav1 and Mst1/2 results in tumors of the mouse liver through deregulation of YAP1 (4). Real-time quantitative PCR in these mice confirmed the upregulation of SOX9 in tumors from hippoc mutant mice (Sav1 fl/fl or Mst1/2 fl/fl) compared with that of wild-type mice (Supplementary Fig. S2A). IHC staining for YAP1 and SOX9 reveals elevated levels of YAP1 and SOX9 proteins in tumor tissues of alb-cre;Mst1/2 mutant mice compared with normal liver tissues (Supplementary Fig. S2B). These data demonstrate that SOX9 is upregulated in vivo in mouse tumors with inactivating Hippo pathway mutations. Hence, SOX9 expression can be elevated in multiple cell types by overexpression of YAP1 in vitro or by activation of endogenous YAP1 protein that occurs following deletion of Hippo pathway signaling components.

YAP1-induced SOX9 transcription requires an intact TEAD binding site in the SOX9 promoter
Having established that YAP1 is both necessary and sufficient for SOX9 expression in multiple cellular contexts and in an in vivo mouse model, we next examined whether this regulation was direct or indirect. Analysis of the human and
murine SOX9 proximal promoter regions reveals a conserved TEAD (CATTCC) binding site located approximately 280 base pairs upstream of the transcription start site. As YAP1 is known to bind to TEAD transcription factors, we investigated whether YAP1 and TEAD can transactivate a SOX9 promoter-luciferase construct in esophageal cancer cells. A wild-type SOX9 promoter (28) containing approximately 1 kb from the transcription start site fused to a luciferase cDNA was transfected into 3 esophageal cancer cell lines-SKGT-4, YES-6, and KATO-TN that contain a stably integrated doxycycline-inducible YAP1S127A cDNA. Upon YAP1S127A induction by doxycycline (DOX +) administration, a 3- to 5-fold induction of luciferase activity was observed (Fig. 3A). This effect is not limited to esophageal cancer cell lines, as SOX9 promoter–directed luciferase activity was increased by about 10 fold upon cotransfection with either activated YAP1 (S127A) or wild-type YAP1 cDNA into 293T cells (Supplementary Fig. S3A). In contrast, knockdown of YAP1 in JHESO cells reduced SOX9 promoter activity significantly (Fig. 3B). Cotransfection of both YAP1 and TEAD2 further enhanced SOX9 transcriptional activity in HEK293T cells, suggesting that TEAD activities are required for maximal SOX9 promoter-luciferase activation (Fig. 3C). To determine whether the TEAD binding site in the SOX9 promoter is crucial for induction of SOX9 by YAP1 and Tead2, a mutation of the TEAD binding site in the SOX9 promoter was generated using site-directed mutagenesis as depicted in Fig. 3D (inset). Induction of SOX9 transcriptional activity by YAP1 and TEAD2 was greatly diminished when mutations of the TEAD binding site in the SOX9 promoter were introduced (Fig. 3D). To further determine whether YAP1 is recruited to the SOX9 promoter in esophageal cancer cells, chromatin immunoprecipitation (ChIP) assays were performed in SKGT-4 cells with or without YAP1 induction by doxycycline (1 μg/mL). PCR analysis was performed using a pair of SOX9 promoter primers spanning the TEAD binding site and a second pair of control primers that do not contain TEAD binding site (primer locations depicted in Fig. 3E and Supplementary Fig. S3B). As demonstrated in Fig. 3F, immunoprecipitation of YAP1 associated chromatin selectively enriches DNA fragments of the SOX9 promoter that contain the TEAD binding site, whereas...
no clear DNA band was amplified using control primers. These findings further confirm the specificity of the ChiP assay, and that the interaction between YAP1 and SOX9 promoter was further enhanced when YAP1 S127A was induced (DOX+) in SKGT-4 cells. Similar findings were seen in B299 cells with YAP1 induction (data not shown). These data indicate that YAP1 induces SOX9 transcription through association at the TEAD binding site of the SOX9 promoter.

**YAP1 endows primary mouse esophageal epithelial cells and immortalized mouse fetal liver cells with self-renewal capacity**

Having determined that SOX9 is a direct transcriptional target of YAP1, we next sought to explore the consequences of activating YAP1 expression in primary esophageal epithelial cells. To that end, murine esophageal cells (Eso) were isolated from wild-type mice and transduced with YAP1 S127A lentiviral...
Figure 3. YAP1 induces SOX9 transcription and requires an intact TEAD binding site. A, SOX9 luciferase promoter activity was determined by transient transfection of SOX9 luciferase promoter reporter in SKGT-4, YES-6, and KATO-TN EC cells with or without induced YAP1. B, SOX9 promoter activity was detected in JHESO cells with or without YAP1 knockdown. C, SOX9 promoter activity was detected in 293T cells after co-transfection of YAP1, NICD, or the combination of YAP1 and TEAD2. D, SOX9 promoter activity was determined in 293T cells after cotransfection of wild-type (wt) or mutant SOX9 promoter luciferase with either YAP1 or YAP1 in combination with TEAD2. E, ChIP assay was performed using YAP1 and normal IgG pull down of chromatin from SKGT-4 with (DOX+) or without (DOX-) YAP1 induction using primers that amplify SOX9 promoter containing the TEAD binding site and primers that amplify a control promoter region that does not contain the TEAD binding site (control site). F, SOX9 promoter primers designed for ChIP assays in the SOX9 promoter containing a TEAD binding site.
particles followed by selection for stable integration with G418. Primary Eso cells show a typical epithelial cobblestone morphology and initially proliferated in culture and could be passaged several times in vitro. However, by passage 10, they were unable to be passaged further and exhibited signs of replicative senescence (Fig. 4A, left). In contrast, primary Eso cells that expressed YAP1S127A (DOX+) have a mesenchymal morphology and do not show signs of replicative senescence in that they can be cultured for more than 20 passages without reduced proliferative capacity (Fig. 4A, right). To determine whether YAP1 expression can confer stem cell–like properties in normal mouse Eso cells, sphere assays on ultra-low-attachment plates were performed in defined media (see Materials and Methods). In the absence of exogenous YAP1S127A (DOX−), Eso cells were unable to form spheres under these conditions, whereas YAP1S127A–induced cells (DOX+) gain the capacity to form spheres (Fig. 4B). This indicates that YAP1 expression in primary mouse esophageal cells allows them to bypass senescence and acquire immortalized or transformed properties. Similarly, induction of YAP1S127A in immortalized, nontransformed B299 cells (PIN20YAP1) endows B299 cells with the capacity to form spheres, whereas B299 cells do not form spheres without YAP1 induction (DOX−; Fig. 4C and D). These YAP1S127A B299 cells can be propagated as spheres with DOX induction for at least 10 generations, suggesting the presence of bona fide stem cells in this population. At each passage, spheres were enzymatically dispersed into single cells and then seeded in 2 groups, one with doxycycline (YAP1 induction, DOX+) and the other without (no induction of YAP1, DOX−). Strikingly, the cells with YAP1 (DOX+) consistently form large spheres at all passages by 10 days of culture, whereas cells without YAP1 induction (DOX−) never form spheres (Fig. 4C–E). The sphere number and sphere-forming frequency per 2,500 cells in each generation are shown in Fig. 4E. Immunoblotting confirmed that the expression of YAP1 and SOX9 was higher in the doxycycline-induced B299 cells (DOX+) than in noninducible B299 cells (DOX−; Fig. 4F). Furthermore, we tested the tumorigenicity of B299 cells with (DOX+) or without (DOX−) YAP1 induction by subcutaneous injection into nude mice. Mice were transplanted with B299 cells and administered doxycycline-containing drinking water for DOX+ group. B299 DOX− cells generated no detectable tumors in all groups. However, B299 DOX+ cells formed tumors even after the injection of as few as 1 × 10⁶ cells. The tumorigenicity of different cell numbers transplanted is displayed in Fig. 4G. These results indicate that YAP1 expression confers CSC properties onto untransformed mouse cells, namely the ability to form spheres under sphere culture conditions, serial passage of spheres, and tumor-forming ability when transplanted into nude mice.

**YAP1 confers CSC properties on esophageal cancer cells, and pharmacologic inhibition of YAP1 suppresses CSC properties in vitro and tumorigenicity in vivo**

We next sought to determine whether YAP1-mediated SOX9 expression is associated with acquisition of CSC properties in transformed esophageal cancer cells. ALDH1 is a useful CSC marker in many different tumor tissues (33, 34). Consistent with these observations, we found the proportion of ALDH1+ labeling is a consistent marker for CSC properties in esophageal cancer cell lines. KATO-TN esophageal cancer cells have relatively low expression of YAP1 and SOX9, a lower proportion of ALDH1+ cells and fewer ALDH1+/CD44+ double-positive cells (Fig. 5A–C) and form less and smaller tumourspheres. In contrast, JHESO cells contain high YAP1 and SOX9 levels, have a larger proportion of ALDH1+ cells and double (ALDH1+/CD44+) positive cells (Fig. 5A–C), and can easily form tumourspheres concomitant with high ALDH1 and OCT4 levels as demonstrated in Fig. 5D. Importantly, induction of YAP1 by doxycycline (DOX+) in KATO-TN (PIN20YAP1) cells increased the proportion of ALDH1+ cells and double (ALDH1+/CD44+) positive cells, increased expression of both ALDH1 and CD44, and greatly increased tumoursphere numbers and size as demonstrated in Fig. 5E. Conversely, knockdown of YAP1 in JHESO cells decreased the proportion of ALDH1+ cells and double (ALDH1+/CD44+) positive cells, reduced expression of ALDH1 and CD44 in concert with significant reduction of tumoursphere size and number (Fig. 5F). This indicates that the level of YAP1 in esophageal cancer cells dictates their CSC properties.

To further confirm the functional role of YAP1 in regulating CSCs properties, a pharmacologic inhibitor of YAP1 was used. Verteporfin has been identified as a small-molecule inhibitor of TEAD–YAP1 association and a selective means of inhibiting the oncogenic activity of YAP1 (55). As shown in Fig. 6A, verteporfin significantly reduced tumoursphere formation in concert with inhibition of YAP1 and SOX9 expression in JHESO cells (Fig. 6B) but without significantly affecting cell growths in 2-dimensional standard culture conditions at same concentration used. This indicates that verteporfin may affect primarily CSC properties or tumor initiation cells (TIC) by suppressing YAP1 and SOX9. Results from xenograft models further confirmed that verteporfin significantly decreases tumour growth in vivo (Fig. 6C and D) without significantly changing the body weights of the treated mice. Similarly, verteporfin completely blocked tumoursphere formation in B299 cells with induction of YAP1S127A (Fig. 6F). To further define whether verteporfin has differential effects on CSCs cells versus non-CSC cells, ALDH1+ and ALDH1− cells sorted from JHESO cells were treated with verteporfin, as indicated in Fig. 6F. Verteporfin strongly inhibited the tumoursphere-forming capacity of ALDH1+ cells at low concentration (1 μmol/L) compared with a less pronounced effect on ALDH1− cells, although ALDH1+ cells form larger and more numerous tumourspheres than ALDH− cells. These results suggest that verteporfin as a specific inhibitor of YAP1 oncprotein could be very effective in targeting CSCs in esophageal cancer tumors.

**shRNA-mediated knockdown of YAP1 or SOX9 inhibits CSC properties in vitro and tumorigenicity in vivo**

To further define whether increased YAP1 and/or SOX9 expression are critical drivers for CSC properties in esophageal cancer cells, shRNA-mediated knockdown of either YAP1 or SOX9 was performed in SKGT-4 (PIN20YAP1) cells with YAP1 induction. The results in Fig. 7A show that YAP1 induction confers high tumoursphere-forming capacity, whereas
depletion of either YAP1 or SOX9 in these cells greatly reduces tumorsphere formation suggesting that tumorsphere formation in esophageal cancer cells is dependent on YAP1 and SOX9 expression (Fig. 7A and B). Similar findings were seen in KATO-TN and B299 cells. Results from in vivo xenograft models further confirm that cells with YAP1 induction (DOX+) significantly increase tumor growth compared with the control group (DOX−; \( P < 0.0001 \)). SKGT-4 (DOX−) cells without YAP1 induction are much less tumorigenic upon injection of \( 1 \times 10^6 \) cells, in that only 1 of 5 mice generated a small tumor after 2 months. However, 5 of 5 mice injected with SKGT-4 cells with YAP1 induction (DOX+) grew large tumors. In this context, knockdown of either YAP1 or SOX9 in YAP1-induced SKGT-4 cells (PIN20YAP1) greatly reduced tumor cell growth as measured by tumor volume and tumor weight (Fig. 7C–E). In addition, expression of YAP1, SOX9, and Ki67 is increased in SKGT4 DOX+ tumor tissues compared with the tumors from SKGT4 DOX−, whereas knockdown of either YAP1 or SOX9 greatly reduced the expression of these markers in concert with inhibition of tumor growth (Fig. 7F). These observations indicate that YAP1 and its target SOX9 are necessary for tumor initiation and tumor maintenance in human esophageal cancer xenografts.

Discussion

In this study, we demonstrate, for the first time, that the Hippo coactivator YAP1 is a molecular conveyor of CSC properties in mouse nontumorigenic primary esophageal cells, immortalized murine fetal cells, and in human esophageal cancer cells. Furthermore, we provide evidence that YAP1 directly regulates SOX9 transcription in concert with its sequence-specific DNA binding partner TEAD2. Genetic and pharmacologic inhibition of YAP1 greatly abolishes tumorsphere-forming capacity in vitro and tumorigenicity in vivo induced by the YAP1–SOX9 axis. Therefore, targeting YAP1–SOX9 axis could be an effective means in combating esophageal cancer.
tumorigenesis (4, 8, 36). YAP1, an effector of the Hippo signaling pathway, has been reported as an oncogene in several tumor types such as hepatocellular carcinoma (HCC), breast cancer, ESCC (7, 9, 12). In breast cancer cells, the activity of TAZ, a transducer of the Hippo pathway, is required to sustain self-renewal and tumor initiation capacities in breast CSCs (37). A recent study (38) suggests that reciprocal regulation of tumor-initiating stem-like cells by TLR4 and TGFβ requires YAP1 and IGF2BP3 in HCC. However, the detailed functional role of YAP1 in acquisition of CSCs properties and in tumor initiation especially in esophageal cancer is unclear thus far.

Our observations in this study yield evidence that YAP1 is able to confer CSC properties onto a wide variety of nontransformed cell types of gastrointestinal origin, including primary isolated esophageal epithelium cells, immortalized embryonic liver cells, as well as in esophageal cancer cells. In all of these cells, tumorsphere formation and propagation were entirely dependent on YAP1 expression. Hence, our findings identify that YAP1 is a molecular determinant of CSC properties in nontumorigenic cells and in esophageal cancer cells.

SOX9 plays a pivotal role in embryonic development and lineage determination (39, 40) and is linked to progenitor cell
status in the gastrointestinal tract including liver and pancreas (13). SOX9 expression is elevated in many human cancers and promotes proliferation, inhibits senescence, and facilitates transformation in vitro (16). Our previous findings suggested that SOX9 is upregulated in esophageal cancer cells by loss of a key TGFβ-signaling pathway adaptor protein that switches TGFβ function from tumor suppression to tumor promotion (28). In this study, we show that inactivation of Hippo signaling or activation of YAP1 results in elevated SOX9 expression in a wide variety of cellular contexts, including in the liver of albumin-cre; Mst1/2-mutant mice, in Lats1/2-deficient MEFs, in nontumorigenic B299 cells depleted of Sav1 or by expressing YAP1 in esophageal cancer cells. SOX9 induction by YAP1 requires an intact conserved TEAD binding site in the SOX9 promoter that is occupied by YAP. Mutation of the conserved TEAD binding site disrupts SOX9 induction by YAP.
and TEAD2, further confirming the requirement of the TEAD binding site for SOX9 induction by YAP1. Hence, SOX9 is likely to be a direct target of YAP1 in a variety of in vitro and in vivo contexts.

Our results indicate that SOX9 induction is an important event in the acquisition of YAP1-induced CSCs self-renewal properties in multiple cell types. Both YAP1 and SOX9 expression have been shown to confer malignant and CSC properties onto nontransformed cells. We have extended these findings to show that YAP1-mediated acquisition of many of these CSC properties requires SOX9 and that YAP1 directly regulates SOX9 expression. Hence, many of the transforming properties of YAP1 may be mediated by SOX9. Alternatively, YAP1 and SOX9 may facilitate transformation by coregulation of downstream targets or by activation of distinct targets that synergize to promote the transformed phenotype. For example, YAP1 has been shown to regulate a number of antiapoptotic factors, including birc5 and members of the BCL family (41), and SOX9...
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...has been shown to regulate BMI1 and INK4a/ARF (16), important regulators of senescence and proliferation. Further studies will be required to define the transcriptional network regulated by YAP1 and SOX9 that confers CSC properties onto non-transformed cells. In addition, we found that both nuclear YAP1 and SOX9 expression are correlated and upregulated in a majority of human EAC tumor tissues. While SOX9 expression has previously been linked to poor survival and metastatic status of EACs (28), and elevated YAP1 expression has been noted in EAC, our current findings provide a mechanistic link between these independent observations. The identification of direct regulation of SOX9 by YAP1 suggests that manipulation of YAP1 activity might be used to selectively target the SOX9-expressing CSC population. Indeed, the small-molecule YAP1 inhibitor verteporfin is effective in vitro to inhibit both YAP1-induced SOX9 expression and tumorsphere-forming ability in vitro and tumor growth in vivo. The inhibition of the self-renewal by verteporfin is more effective in ALDH1+ sorted cells and in cells that express high levels of YAP1. These findings support the idea that verteporfin, as a pharmacologic inhibitor of the YAP1 oncoprotein, could be very useful to target CSCs population in esophageal cancer tumors. The combination of verteporfin (targeting CSCs) and traditional chemotherapy (targeting bulk tumor cells) may lead to a better efficacy in combating esophageal cancer tumors. Additional studies will be necessary to determine whether this approach would be effective in targeting both the bulk tumor and CSC populations in relevant in vivo and preclinical settings.

In conclusion, we have identified YAP1 is a major inducer of CSC properties in nontumorigenic cells as well as in esophageal cancer cells by direct upregulation of SOX9. Thus, the YAP1–SOX9 axis could be an important therapeutic target in esophageal cancer (Fig. 7G). Further studies are warranted to investigate the efficacy of combined verteporfin and cytotoxic chemotherapy in combating both CSCs and bulk tumor cells in esophageal cancer tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S. Song, R.L. Johnson
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