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Original Article

YAP promotes epithelial mesenchymal transition by upregulating Slug expression in human colorectal cancer cells

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Abstract: Yes-associated protein (YAP) contributes to the development of multiple tumors, including colorectal cancer (CRC). However, the underlying mechanisms involved in YAP-induced CRC migration and invasion are not fully elucidated. By performing immunohistochemistry (IHC), we found that YAP is highly expressed in CRC tissues and significantly correlated with invasive depth. The expression of YAP was elevated in CRC cell lines. Therefore, we sought to illustrate whether the up-regulation of YAP contributes to CRC the epithelial-mesenchymal transition (EMT). Here migration and transwell assays showed that YAP overexpression promoted migration and invasion in-CRC cells. YAP knockdown inhibited migration and invasion in CRC cells. Furthermore, western blotting showed that CRC YAP overexpression causes the down-regulation of the epithelial marker E-cadherin and the up-regulation of the EMT-related transcription factor Slug, which in turn promotes the EMT in CRC. YAP knockdown inhibited EMT by up-regulating E-cadherin and down-regulating Slug. Furthermore, in YAP-overexpressing CRC cells, Slug knockdown promoted E-cadherin expression and inhibited EMT. In CRC cells with low expression of YAP, high expression of Slug can inhibit E-cadherin expression and promote EMT. Importantly, luciferase assays confirmed that YAP directly transcriptionally activated *Slug* expression. Based on the above results, our study shows that YAP is a driver of EMT in CRC, which inhibits E-cadherin expression by activating transcriptional *Slug* expression.

Keywords: YAP, epithelial-mesenchymal transition, Slug, E-cadherin, CRC

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors and its incidence rate ranks third in the world and China [1], and the incidence rate is increasing year by year. According to statistics, a quarter of newly diagnosed CRC patients have lymph node or distant metastasis. Approximately 90% of deaths of cancer patients are related to metastasis. If patients with metastatic CRC are not treated, the median survival period is only 5-6 months. Therefore, investigating the molecular mechanism of invasion and metastasis of CRC is helpful to find new targets for treatment, which is of great significance for judging prognosis, guiding treatment, and improving patient survival rate.

Epithelial-mesenchymal transition (EMT) is thought to be closely associated with invasion and metastasis in a variety of tumors, including CRC [2, 3]. EMT is an important cellular development process during tumor invasion and metastasis, that allows the polar epithelial cells to convert into transitional mesenchymal cells under certain physiologic or pathologic conditions [4, 5]. When EMT occurs, cell morphology also changes. The shape of epithelial cells gradually changes into fibroblast morphology, and the interaction between cells weakens. The expression of some markers such as E-cadherin in epithelial cells decreases. On the other hand, markers of interstitial cells, such as the expression of N-cadherin and Vimentin, are increased [3, 4]. The above changes cause the epithelial cell-derived tumor cells to lose cell polarity, the intercellular connections become loose, and characteristics of mesenchymal cells are obtained. Thus adhesion ability is decreased, the migration ability increased, and the invasion and metastasis are more likely to occur.

The expression of the marker molecules in EMT is regulated by a series of pleiotropic transcription factors such as *Snail*, *Zeb*, *Twist*, and *Fox* [6]. These transcription factors are called the EMT-related transcription factors (EMT-TF), which have both transcriptional activation and transcriptional repression functions [7]. In addition, the inactivation of E-cadherin is considered a hallmark of EMT [8]. EMT signals converge to activate one or more EMT-TFs. Those TFs, including *Snail* and *Slug* (alternatively termed *Snail2*), directly or indirectly suppress the E-cadherin promoter [6, 9, 10]. Studies have shown that the expression of the transcription factors such as *Snail*, *Zeb1* and *Zeb2* promotes the invasion and metastasis of CRC by regulating EMT, which ultimately leads to a poor prognosis [11]. Although some important transcription factors involved in the regulation of EMT marker molecule expression have been reported, the molecular mechanisms of upstream regulation of these EMT-TFs remain to be elucidated.

The Hippo/Yes-associated protein (YAP) signaling pathway was originally found in Drosophila and has been proven to control organ size and maintain tissue cell stemness [12, 13]. Its key components include mammalian sterile 20-like kinases 1/2 (MST1/2), Salvador family WW domain-containing protein 1 (SAV1), large tumor suppressor kinases 1/2 (LATS1/2), YAP, transcriptional co-activator with PDZ binding motif (TAZ), and transcriptional factor domain family members 1-4 (TEAD1-4) [14]. In humans, MST1/2 combines with SAV1 to form an activated complex that initiates LATS1/2 phosphorylation [15]. Once activated, LATS1/2 further promotes the signaling cascade by phosphorylating YAP. Phosphorylated YAP binds to 14-3-3 protein and remains in the cytoplasm for degradation, thus losing its function as a transcription cofactor [16]. Dephosphorylated YAP translocates into the nucleus and binds to transcription factors, which initiates downstream gene transcription to maintain cell proliferation and apoptosis homeostasis [17]. YAP is the key effector of the Hippo pathway. It has been revealed that YAP is involved in the progression of many types of tumors. Therefore, we speculate that YAP contributes to CRC EMT and promotes CRC metastasis.

Here, we investigated the mechanism that could explain the key role of the expression of YAP in driving CRC metastasis, and we show that YAP can regulate CRC EMT by inhibiting E-cadherin expression and by promoting EMT-related transcription factor *Slug* expression in CRC. Thus, our findings provide new insight into the mechanism of YAP-promoted EMT in CRC.

Materials and methods

Cell lines culture

The human CRC stably transfected cell lines (SW620 and HCT116) were cultured in the proper medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. All reagents were stored as recommended by the manufacturer.

Plasmid cDNA and short interfering RNA transfection

YAP shRNA (ShYAP) and YAP cDNA plasmid and their negative controls were from our laboratory. SW620 cells were seeded at 3×10^6 cells/well into 6-well plates 24 h before transfection. Transfection was carried out according to Lipofectamine™6000 (Beyotime Biotechnology) protocol. Then, 12 h post-transfection, medium without penicillin and streptomycin was replaced with complete medium, and 40 h post-transfection, cells were harvested for western blot. Two different double-strand SLUG-targeting siRNA oligonucleotides (GenePharma, China) were used together; the Slug siRNA1 sequences were as follows: 5'-GGACCACAGTGGCTCAGAA-3', and the Slug siRNA2 sequences were as follows: 5'-CCTCACTGCAACAGAGCATTT-3'. The siRNA oligonucleotide showing the highest knockdown efficiency of Slug protein in the SW620 YAP overexpressing cell lines were used for the experiments. The SW620 YAP overexpressing cell lines were divided into two groups: cells transfected with non-targeting as the negative control (NC) group and cells transfected with siSlug as the experimental group. The SW620 YAP knockdown cell lines were divided into two groups: cells transfected with PcDNA3.1 plasmid (Blank) as the negative control group and cells transfected with DDK-Slug (Origene, Rockville, USA) as the experimental group. The cells were cultured in 6-well plates (2×10^6 cells/well). At 24 h following seeding,

until the cells reached 60-80% confluence; they were then transfected with siSlug using Lipofectamine™6000, according to the manufacturer's instructions. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 40 h and then the transfection efficiency was observed using western blotting.

Wound healing assay

For wound-healing assays, cells were seeded at a density of 1 × 10^6 cell/well in six-well plates. An artificial wound was created on the confluent cell monolayer after 12 h using a sterile 10-μl pipette tip. Then the cells were cultured in serum-free medium. The wounds were photographed with a light microscope (Olympus, Japan) every 24 h in five random microscopic regions. Each independent experiment was repeated 3 times, and processed using Image J analysis software. The percentage of wound healing was measured as follows: [1-(empty area X h/empty area 0 h)] × 100.

Invasion assay

For the invasion assay, 5 × 10^5 cells were suspended in 200 μl of culture medium without serum and then seeded on the cell culture insert precoated with 1 μg/μl Matrigel (BD Biosciences, USA). Complete medium was added to the lower chamber to stimulate invasion. After incubation for 48 h, the cells that did not penetrate through the membrane were removed with a cotton swab, while those adhered to the lower surface of the membrane were stained with a 0.1% crystal violet solution. The number of invaded cells in five randomly selected fields was counted under a light microscope (magnification, × 200; Olympus, Tokyo, Japan). All experiments were performed three times.

Real-time RT-PCR

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA integrity, quantity, and purity were examined using a Nano-Drop (Thermo Scientific, Wilmington, DE, USA). cDNA was generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on a CFX96 Touch (BIO-RAD) real-time PCR System (Applied Biosystems) for 39 cycles. After the reaction cycles, the threshold cycle (Ct) values were determined, and the relative mRNA levels were calculated based on the Ct values and normalized to the GAPDH level in each sample. Primer sets for YAP and Slug were purchased from Sangon Biotech (Shanghai, China). YAP-F primer sequence was as follows: AGTGGACTAAGCATGAGCAG, YAP-R primer sequence as follows: TGTTCATTCCATCTCCTTCC. Slug-F primer sequence as follows: CAAGGACACATTAGAACTCACAC, Slug-R primer sequence as follows: CTACACAGCAGCCAGATTC. GAPDH-F primer sequence as follows: TGACTTCAACAGCGACACCCA, GAPDH-R primer sequence as follows: CACCCTGTTGCTGTAGCCAAA. The expression levels of GAPDH were used as internal controls, GAPDH was used for mRNA transcripts. Fold changes in the expression of mRNA among the RNA samples were calculated.

Western blot

For western blot, total protein was extracted from the cells. Approximately 30 μg of protein was denatured and electrophoresed on 10% SDS-PAGE gels. After electrophoretic separation, proteins were transferred onto polyvinylidene difluoride membranes by electro-blotting and then blocked for 60 min at room temperature in PBS containing 5% skim milk powder; the blots were probed with primary antibodies, and GAPDH was used as an internal control. The blots were incubated with YAP (ABclonal, 1:1000), Slug (Cell Signaling, #9585, 1:1000), Snail (Cell Signaling, #3879, 1:1000), E-cadherin (Cell Signaling, #3195, 1:1000), Vimentin (Cell Signaling, #5741, 1:1000), β-Catenin (Cell Signaling, #8480, 1:1000), Zo-1 (Cell Signaling, #8193, 1:1000), and GAPDH (Beyotime, AF0006, 1:1000) primary antibodies at 4°C overnight. The membranes were washed with TBST and then incubated with secondary antibody (goat anti-rabbit IgG and goat anti-mouse IgG antibody, 1:3000-1:5000 dilution with 5% skim milk powder, Earthox, America) for 1.5 h at room temperature. Finally, images of the western blot bands were collected with an imaging system (Bio-Rad, USA) and quantified by measuring the intensity in each group with Image J software; GAPDH was used as an internal control. The results are expressed as fold-changes, and the data are normalized to the control values.

Dual-luciferase reporter assays

The SW620 stably overexpressing YAP cells and control cells were seeded in 24-well plates.

After 24 h, cells were transfected with the luciferase reporter plasmid (*Slug*-Luc, Addgene) and Renilla luciferase constructs (Renilla-Luc, Addgene) using Lipo6000 transfection reagent. After 40 h, cell extracts were prepared and reporter activity was determined using the dual-luciferase reporter assays system purchased from Promega (E1910, USA). Results are presented as firefly luciferase to Renilla luciferase ratio, after subtracting background contributed from coelenterazine auto-luminescence plus instrument background. Data are representative of three independent experiments.

Statistical analysis

All experiments were performed at least three times. Statistical analysis was performed using the SPSS statistical software package. The significance of the in vitro data was determined using Student's t-test (2-tailed). *P*-value <0.05 was considered significant.

Results

YAP functionally promotes cell migration and invasion ability in CRC cells

To identify the potential role of YAP in cell migration and invasion on CRC progression, we constructed a lentiviral vector overexpressing YAP and a lentiviral vector knocking down YAP. A three-generation lentiviral infection system was used. After packaging the virus, SW620 and HCT116 cells were infected and screened with puromycin for two weeks to obtain stable cell lines. Then, we tested the autonomous migration ability of the CRC cells by using cell wound healing assays. As shown in **Figure 1A**, **1B**, we found that the cell migration ability of CRC SW620 and HCT116 cells overexpressing YAP was significantly enhanced. In contrast, knocking down YAP significantly decelerated the speed of wound closure in both SW620 and HCT116 cells. Consistently, transwell assays further confirmed that YAP overexpression promoted invasion in both SW620 and HCT116 cells (**Figure 1C**, **1D**). We observed that knocking down YAP decreased the number of invading cells in both SW620 and HCT116 cells. These results indicated that overexpressing YAP promoted CRC migration and invasion, but knocking down YAP inhibited CRC migration and invasion.

YAP inhibits expression of E-cadherin in CRC cells

EMT is involved in tumor progression and an aggressive phenotype of a variety of carcinomas. Thus, we hypothesized that YAP promotes CRC cell migration and invasion by inducing the EMT program. To investigate the role of YAP in CRC EMT, we detected whether the expression levels of epithelial markers (E-cadherin and Zo-1), mesenchymal markers (vimentin), EMT transcription factor (Slug and Snail) and other markers (β-catenin) changed under conditions of abnormal YAP expression by western blot assay. As shown in **Figure 2A**, **2B**, YAP overexpression resulted in the down-regulation of E-cadherin and Snail and the up-regulation of vimentin and Slug in SW620 cells and HCT116 cells at different time points. This all suggested that overexpression YAP has a promoting effect on EMT. To further support that YAP regulates E-cadherin expression, we also determined whether the expression levels of epithelial markers and mesenchymal markers changed under knocking down YAP expression. As illustrated in **Figure 2C**, **2D**, western blot showed that knocking down YAP in SW620 cells and HCT116 cells resulted in a gradual down-regulation of slug level as well as vimentin. E-cadherin protein expression was significantly higher than that of negative controls. Our results indicated that knocking down YAP inhibits the occurrence of EMT in CRC stably transfected cells. Taken together, these data suggest YAP inhibits the expression of E-cadherin to promote EMT in CRC cells.

YAP up-regulates Slug expression in CRC cells

To further investigate the mechanism by which YAP inhibits E-cadherin, we examined the expression of the upstream EMT-TF Slug of E-cadherin. SW620 cells were transfected with YAP overexpression plasmids for 40 h. Western blot results showed that after SW620 cells were transfected with HA-YAP, Slug expression was up-regulated and E-cadherin expression was down-regulated, but Vimentin and β-catenin were not significantly altered compared with control cells. Similarly, after SW620 cells were transfected with ShYAP, we found Slug expression down-regulation and E-cadherin expression up-regulation compared with control cells (**Figure 3A**). This suggests that YAP up-regulates the expression of Slug in CRC.

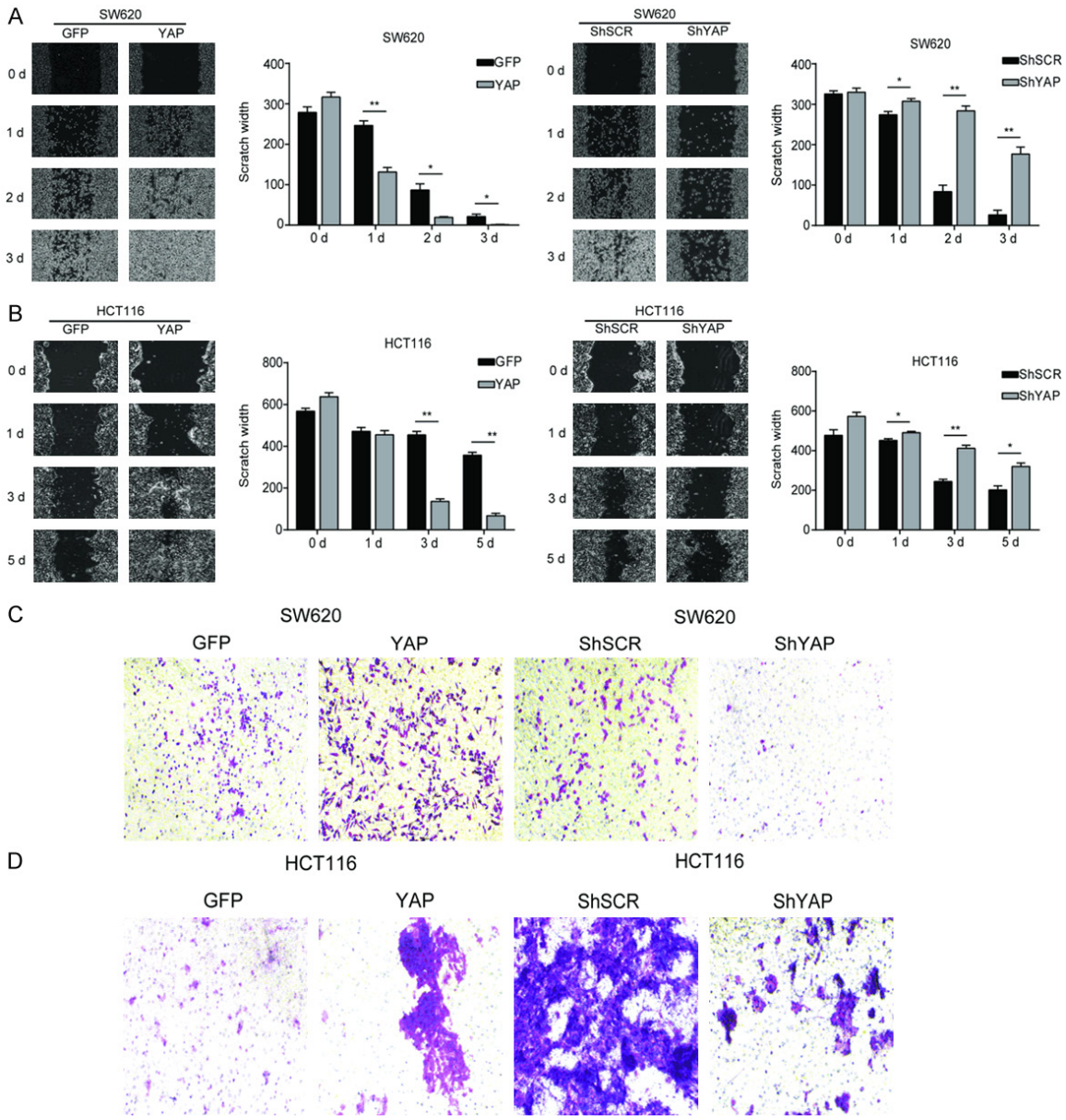

Figure 1. YAP functionally promotes CRC cell migration, invasion, and epithelial-mesenchymal transition ability in vitro. A, B. (left) Representative images from wound-healing assays using overexpressing YAP SW620 cells and HCT116 cells at 0, 1, 2, 3 and 5 d after scratching (migration: × 100 magnification). A, B. (right) Representative images from wound-healing assays using knocked-down YAP SW620 cells and HCT116 cells at 0, 1, 2, 3 and 5 d after scratching (migration: × 100 magnification). C. Representative images of transwell invasion analysis in SW620 YAP and SW620 ShYAP cells compared with respective control cells. D. Representative images of transwell invasion analysis in HCT116 YAP and HCT116 ShYAP cells compared with respective control cells (invasion: × 200 magnification).

YAP inhibits E-cadherin expression via the up-regulation of Slug in CRC cells

To further elucidate the correlation of YAP regulating Slug and E-cadherin, we transfected with Slug siRNA (siSlug) in YAP overexpressing SW620 cells. The Slug-specific small interfering RNA was used to further confirm the role of Slug in CRC EMT. As shown in **Figure 3B** by western blot assay, in SW620 GFP cells after the transfection of siSlug, we found that compared with the NC group, the Slug expression was down-regulated and the E-cadherin expression was up-regulated. Comparing the protein expression changes of SW620 GFP cells and SW620 YAP cells after transfecting with NC

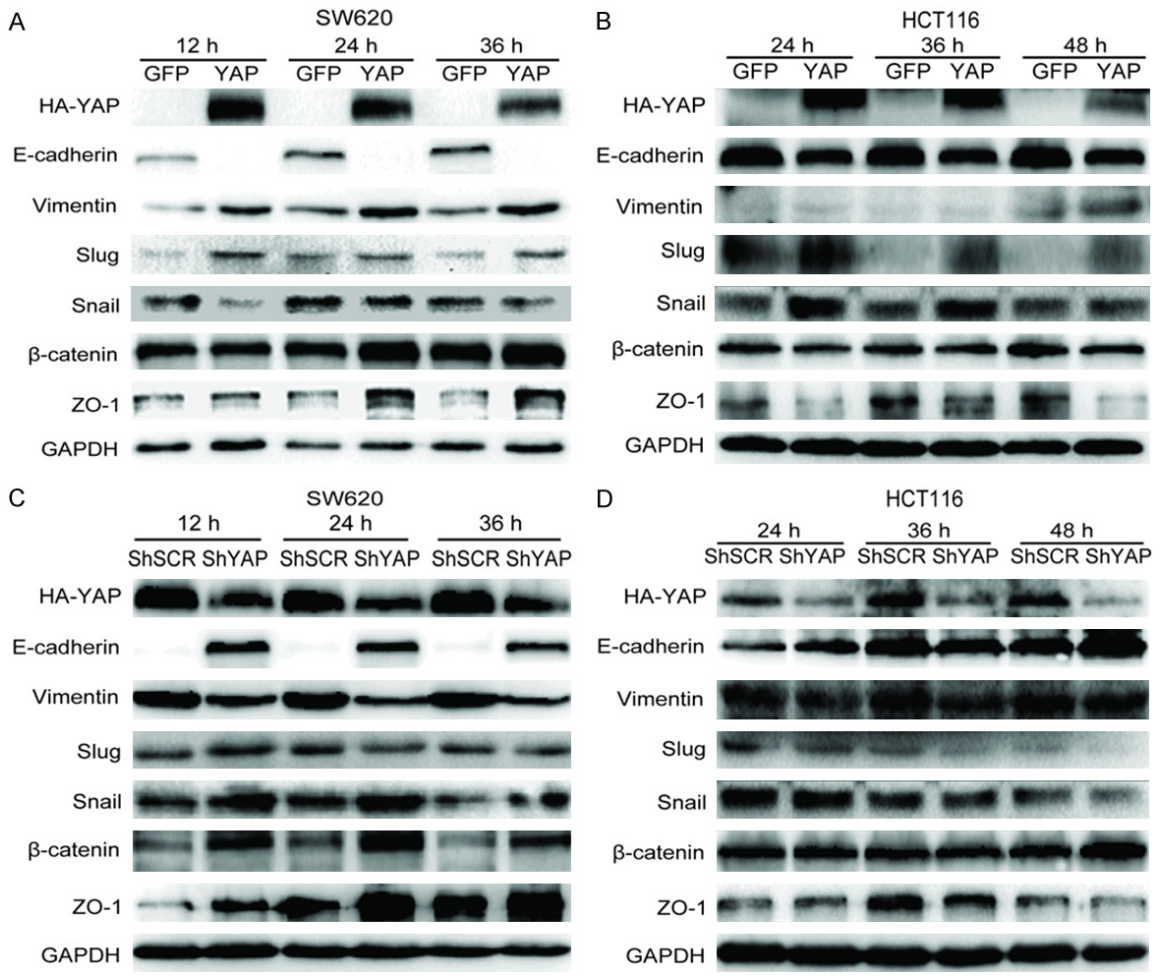

Figure 2. YAP inhibits E-cadherin expression in CRC cells. A, B. Western blot assays show that the overexpression of YAP promotes EMT in SW620 cells and HCT116 cells. C, D. Western blot assays show that knocking down YAP inhibits EMT in SW620 cells and HCT116 cells. GAPDH was used as an internal control. The above experiments were performed at least three times.

plasmid, it was found that in the SW620 YAP overexpressing cells transfected with NC, Slug expression was obviously increased and E-cadherin expression was significantly down-regulated. The SW620 YAP overexpressing cells transfected with siSlug resulted in a significant reduction in Slug protein expression compared with NC treated cells after transfection and E-cadherin expression was significantly recovered. Simultaneously, we respectively transfected with DDK-Slug and its control plasmids in YAP knocking down SW620 cells. As shown in **Figure 3C** by western blot, in SW620 ShSCR cells after the transfection of DDK-Slug, compared with the blank group, the Slug expression was dramatically increased and the E-cadherin expression was decreased. Comparing the protein expression changes of SW620 ShSCR cells and SW620 ShYAP cells after transfecting with the blank plasmid, it was found that in the SW620 YAP knocking down cells transfected with blank, Slug expression was slowly decreased and E-cadherin expression was significantly up-regulated. In addition, transfection with DDK-Slug resulted in a significant rise in Slug protein expression compared with transfecting blank plasmid in the SW620 YAP knock-down cells, and E-cadherin expression was significantly inhibited. These results suggested that Slug mediates the YAP-promoted EMT process in CRC. It also suggests that YAP may inhibit E-cadherin expression by up-regulating Slug.

Slug is a direct transcription target of YAP

Our above results suggested the ability of YAP to promote CRC EMT likely through the activation of Slug expression. Therefore, we per-

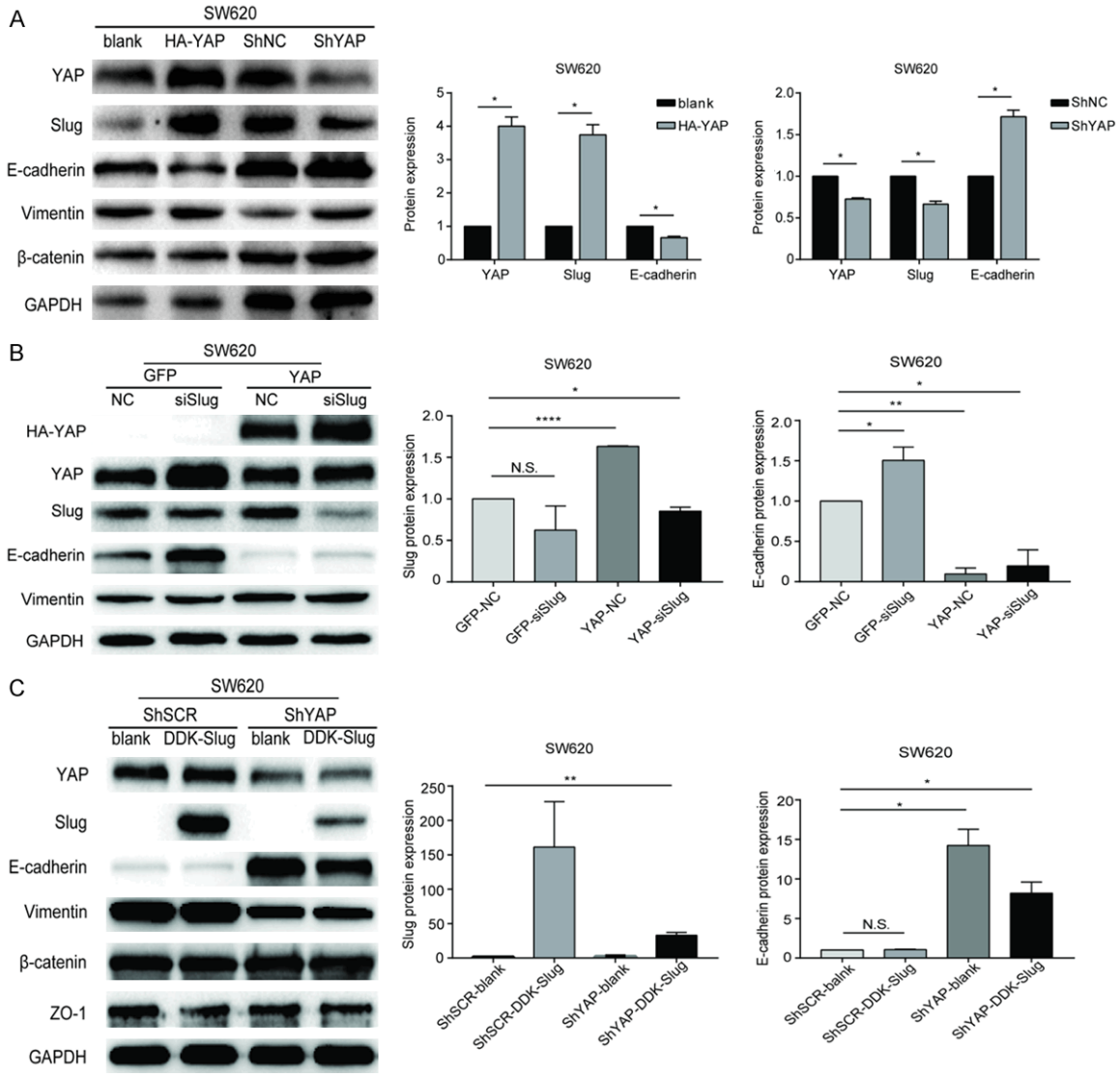

Figure 3. YAP inhibits E-cadherin expression by up-regulation of Slug. A. The primary SW620 cells were respectively transfected with YAP and its control plasmids, ShYAP and its control shRNA for 40 h by western blotting assays. Western blot assays show that YAP promotes Slug expression and reduces E-cadherin expression. B. Western blot is used to detect the changes of E-cadherin expression by transiently transfected interfering Slug siRNA in stable SW620 cell line overexpressing YAP. Western blot assays show that knockdown of Slug promotes the expression of E-cadherin. C. Western blot is used to detect the expression changes of E-cadherin by transiently transfected overexpressing Slug plasmid in stable SW620 cell line knocking down YAP. Western blot assays show that overexpressing Slug inhibits the expression of E-cadherin. GAPDH was used as an internal control. The above experiments were performed at least three times. *P<0.05, **P<0.01, ***P<0.001 vs. CTRL.

formed qPCR to detect *Slug* mRNA expression, and the result suggested that overexpression of YAP increased *Slug* expression and YAP low expression decreased *Slug* expression (**Figure 4A**). Furthermore, we performed dual-luciferase reporter assays. The result showed that overexpressing YAP promoted the luciferase activity of the *Slug*-Luc promoter (**Figure 4B**). This indicates that YAP positively regulated *Slug* expression transcription. Thus, we consider that *Slug* is the target gene of the co-transcriptional activators YAP and contributes to EMT in CRC.

Discussion

The Hippo pathway plays a crucial role in growth control, proliferation, and tumor progression. Hippo signaling has been proposed to be associated with the tumorigenicity of many tumors [18]. YAP, a regulator of cell fate, is up-regulated

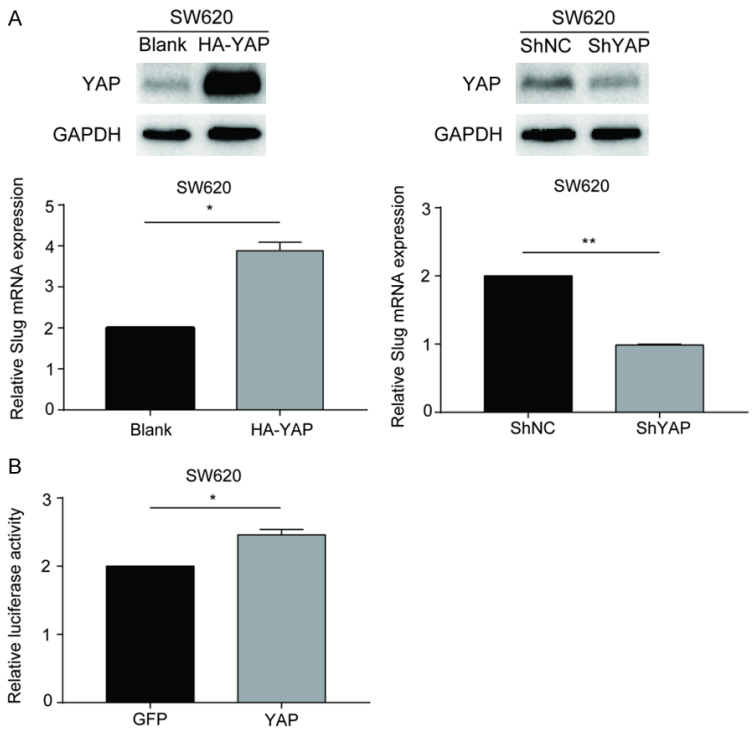

Figure 4. YAP positively regulates Slug transcription. A. Western blot assays show YAP protein expression in different transfection conditions, and qPCR to detect Slug mRNA expression under different conditions of YAP expression. B. Dual-luciferase assay was used to detect up-regulation level of Slug transcriptional activity in stable SW620 cell line overexpressing YAP. Dual-luciferase reporter assays confirm that YAP can positively active the transcription of Slug-luc. *P<0.05, **P<0.01 vs. CTRL.

in multiple cancers and is significantly associated with histologic differentiation, TNM stage, and poor prognosis in colorectal cancer [19, 20]. Recently, Yu et al. found that TEAD-mediated YAP1 promotes the transcription of Slug to induce NSCLC migration and invasion [21]. But whether there is a direct interaction between Slug and YAP that could induce EMT in CRC has not been described previously. In this study, in vitro assays confirmed that overexpression of YAP promoted cell migration and invasion, whereas knocking down YAP significantly inhibited cell migration and invasion, suggesting that YAP is a key regulator of cell migration, invasion, and tumorigenesis in CRC progression.

EMT is thought to be closely associated with invasion and metastasis of a variety of tumors, including CRC. Many studies suggest that EMT is a pivotal step required for epithelial cells to acquire malignant capabilities [22]. E-cadherin, encoded by the CDH1 gene, is a hallmark of EMT [23], a transmembrane glycoprotein that localizes to adjacent cell membranes and is responsible for cell-cell interactions. It has been reported that down-regulation or loss of E-cadherin is associated with the progression of invasion and metastasis of several malignancies, including CRC [24, 25]. Expression of E-cadherin can be regulated by a variety of transcription factors, including Snail and Slug belonging to the Snail family. Snail and Slug can bind to the promoter of E-cadherin and directly inhibit the transcription of the CDH1 gene [17, 26-28]. The previous study revealed that KRAS and YAP1 converge on the transcription factor FOS and activate a transcriptional program involved in regulating the EMT [26]. Down-regulation of YAP/TEAD3 reduced OVOL2 expression and induced high expression of ZEB1 and SNAIL2, inhibited E-cadherin, and up-regulated mesenchymal markers vimentin and N-cadherin, thereby inducing EMT of trophectoderm [29, 30]. Therefore, molecular mechanisms of transcription factors involved in the regulation of EMT marker molecule expression deserve further investigation in the future.

In our study, we found YAP could promote cell migration and invasion. Then we also found that YAP could promote EMT by inhibiting E-cadherin in CRC. YAP overexpression resulted in a decrease in an epithelial marker (E-cadherin) and an increase in a mesenchymal marker (Vimentin), whereas knocking down YAP had opposite effects. We also demonstrated that YAP up-regulates Slug expression in CRC cells. Recently, Zhao et al. suggested that EMT transcription factors (TFs) including SNAI1 and Slug, directly or indirectly suppress the E-cadherin promoter [6]. Our current study supports that YAP directly up-regulated *Slug* expression transcription. Based on these results, suggesting that YAP contributes to the EMT of CRC by up-regulating the transcription of *Slug*, then inhibiting E-cadherin. We believed that YAP and Slug together participate in CRC progression.

In short, we have demonstrated that YAP promotes CRC the EMT by directly regulating the transcription of *Slug*. Our study reveals a previously unrecognized mechanism of YAP and EMT in CRC. We propose that YAP and Slug might be useful markers of CRC metastasis.

Acknowledgements

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Disclosure of conflict of interest

None.

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