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ORIGINAL ARTICLE

Hyaluronic acid synthase 2 promotes malignant phenotypes of colorectal cancer cells through transforming growth factor beta signaling

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Abstract

Hyaluronic acid synthase 2 (HAS2) is suggested to play a critical role in malignancy and is abnormally expressed in many carcinomas. However, its role in colorectal cancer (CRC) malignancy and specific signaling mechanisms remain obscure. Here, we report that HAS2 was markedly increased in both CRC tissue and malignant CRC cell lines. Depletion of HAS2 in HCT116 and DLD1 cells, which express high levels of HAS2, critically increased sensitivity of radiation/oxaliplatin-mediated apoptotic cell death. Moreover, downregulation of HAS2 suppressed migration, invasion and metastasis in nude mice. Conversely, ectopic overexpression of HAS2 in SW480 cells, which express low levels of HAS2, showed the opposite effect. Notably, HAS2 loss- and gain-of-function experiments revealed that it regulates CRC malignancy through TGF-β expression and SMAD2/Snail downstream components. Collectively, our findings suggest that HAS2 contributes to malignant phenotypes of CRC, at least partly, through activation of the TGF-β signaling pathway, and shed light on the novel mechanisms behind the constitutive activation of HAS2 signaling in CRC, thereby highlighting its potential as a therapeutic target.

KEYWORDS

colorectal cancer therapy, epithelial-mesenchymal transition, HAS2, malignant tumor, TGF-beta

Kim and Lee contributed equally to this work.

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1 | INTRODUCTION

The number of new cases of colorectal cancer (CRC) is increasing rapidly worldwide, with high mortality and morbidity rates.[1,2] CRC presents a high rate of anticancer therapy failure, with local tumor recurrence or distant metastasis being reported in over 90% of malignant CRC cases.[3-6] Furthermore, the malignant characterization of CRC shows abnormal proliferation, rapid diffuse infiltration and high resistance rate of therapeutic treatment.[6-10] Despite studies on CRC development and molecular characterization of CRC malignancy, therapeutic results are not fully satisfactory.[11,12] Thus, elucidation of CRC malignancy-related target molecules and mechanism are required for developing more effective CRC therapies.

Hyaluronic acid (HA), or hyaluronan, is an essential component of the extracellular matrix (ECM), which regulates tissue stiffness, maintains stroma homeostasis and acts as a signaling component in many types of cells.[13,14] This unbranched heteropolysaccharide is synthesized by HA synthase (HAS), which localizes to the cellular plasma membrane. Three types of HAS have been recognized (HAS1, HAS2 and HAS3) and HA of different sizes are secreted directly into the extracellular space.[15] Recently, the overexpression of HAS has been reported in bladder, lung, ovarian and breast cancers, where it has been related with malignant tumor phenotypes.[16-19] HAS2, in particular, appears to promote tumor proliferation, migration and invasion in many types of tumor.[18,20] Furthermore, HAS2 can modulate the radiosensitivity of CRC through accumulation of DNA damage.[21] Given the established effects of HAS2 in many cancers, it may also contribute to the regulation of CRC malignancy. However, the role of HAS2 expression in CRC malignancy has not been reported. In this study, we investigated the regulatory role of HAS2 in CRC malignancy, especially its effect on the major regulatory steps of metastasis, such as therapeutic sensitivity and epithelial-mesenchymal transition (EMT). We found that HAS2 regulates the expression of transforming growth factor beta (TGF-β), an important tumor malignancy regulatory component, and that the HAS2-mediated regulation of CRC malignancy occurred independently of the HA ligand-mediated pathway. Taken together, our findings suggest the importance of HAS2 in CRC malignancy regulation and its potential as an effective therapeutic target for CRC.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

Antibodies against Slug (SC-10436), Twist (SC-15393) and β-actin (sc-47778) were purchased from Santa Cruz (CA, USA); those against cleaved caspase-3 (9661), cleaved PARP (9541), TGF-β (3711), Smad2 (5339), p-Smad2 (3108), Smad3 (9523) and p-Smad3 (9520) were from Cell Signaling Technology (MA, USA); and those against N-cadherin (610921) and E-cadherin (610182) were from BD, NJ, USA. Anti–HAS2 (ab140671), anti–Vimentin (3634-100) and anti–zeb1 (HPA027524) were from Abcam (MA, USA), Biovision (CA, USA) and Sigma (MO, USA), respectively. Hyaluronan (GLR001, low molecular weight; GLR004, middle molecular weight; GLR002, high molecular weight) was purchased from R&D Systems (MN, USA). 4-Methylumbelliferone (M1381) and SB431542 (S4317) was purchased from Sigma (MO, USA).

2.2 | Cell culture

HT29, WiDr, DLD1, HCT116, SW480, RKO CRC cells were obtained from the American Type Culture Collection. Cell lines were cultured in RPMI-1640 media containing 30 µg/mL gentamicin supplemented with 10% FBS. All cell lines were negative for mycoplasma contamination and were not passaged > 3 months upon thawing. Cells were cultured in a humidified 5% CO_2 atmosphere at 37°C.

2.3 | Western blot analysis

Cell lysates were prepared by incubating with lysis buffer (40 mmol/L Tris-HCl pH 8.0, 120 mmol/L NaCl, 0.1% Nonidet-P40) supplemented with protease inhibitors. Proteins in whole-cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 0.5% BSA in TBS and incubated with primary antibodies overnight at 4°C. Blots were developed with a peroxidase-conjugated secondary antibody, and proteins were visualized using enhanced chemiluminescence (ECL) procedures.

2.4 | Transfection

HCT116, DLD1 and SW480 were plated in 60 or 100 mm dishes in RPMI-1640 complete media. The following day, the complete medium was replaced, and cells were transfected with HAS2 siRNA or pcDNA3.1-HAS2 plasmid vector and negative scrambled control by using TransIT-X2 (Mirus, WI, USA).

2.5 | Tissue array

HAS2 expression was quantified using tissue array containing 32 human CRC tissues with corresponding normal tissues (ISU ABXIS, Seoul, Korea). For immunohistochemical analysis, tissues were treated with 0.3% hydrogen peroxide in methyl alcohol for 20 minutes to block endogenous peroxidase activity. After 3 washes with PBS, sections were blocked with 10% normal goat serum (Vector Laboratories, CA, USA) and incubated with anti–HAS2 antibody. After 3 subsequent washes with PBS, sections were incubated with HRP-conjugated secondary antibody (Dako, CA, USA). A diaminobenzidine substrate was used for detection. Quantitative assessment of immunoreactivity was performed with i-solution software.

2.6 | Irradiation

Cells in culture medium were plated in 60 and 100-mm dishes and incubated at 37°C in a humidified 5% CO_2 atmosphere. Cells were

exposed to 10 Gy radiation using a Gammacell-3000 Elan irradiator (137Cs γ-ray source; MDS, ON, Canada).

2.7 | Invasion and migration assays

For the invasion assays, cells were loaded in the upper well of a Transwell chamber (8-μm pore size) that was pre-coated with 10 mg/mL growth factor-reduced Matrigel (BD, NJ, USA). After 48 hours, non-invaded cells on the upper surface of the filter were removed with a cotton swab, and the migrated cells on the lower surface of the filter were fixed and stained with a Diff-Quick Kit (Thermo Fisher Scientific, MA, USA). Invasiveness was determined by counting cells in fields per well, and the extent of invasion was expressed as the average number of cells per microscopic field. Cells were imaged by phase contrast microscopy. For the migration assay, we used Transwell chambers with inserts that contained the same type of membrane but without the Matrigel coating.

2.8 | Animal experiment

Specific pathogen-free (SPF) male Balb/c nude mice (6 weeks old) were obtained from Orient Bio and maintained under SPF conditions at the animal facility of the Korea Institute of Radiological and Medical Sciences (KIRAMS). All mice were housed in a temperature-controlled room with a 12-hour light/dark cycle, and food and water were provided ad libitum. The mice were acclimated for 1 week before experiments and assigned to the following groups (n = 5/group). CRC cells were injected into the spleen. Three weeks later, liver metastasis was analyzed by counting the number of foci on the liver surface using a magnifier. Colon samples of mice were fixed with a 10% neutral buffered formalin solution, embedded in paraffin wax, and sectioned transversely at a thickness of 4 μm for H&E staining. All animal experiments were performed in accordance with the guidelines of and were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences.

2.9 | Human colorectal cancer specimens

Human CRC patient tissues were obtained from the Korea Institute of Radiological and Medical Sciences, Seoul, Korea. CRC tissues were randomly collected from 10 patients diagnosed with CRC between 2017 and experiments were approved by the ethics committee of IRB/Hospital Clinic (Sub IRB No. K-1702-002-059).

2.10 | Apoptosis assay

Cells seeded at a density of 2×10^5 cells per 60-mm dish were left untreated or were treated with 10 Gy radiation or 40 μmol/L oxaliplatin under the indicated experimental conditions. For quantification of apoptosis, cells were trypsinized, washed in PBS, and dually stained with annexin V and propidium iodide. Annexin V-stained cell populations were counted with a FACScan flow cytometer (BD, NJ, USA).

2.11 | Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Following fixation, cells were incubated at 4°C overnight with anti–human HAS2, anti–E-cadherin and anti–Vimentin primary antibodies in PBS with 1% BSA and 0.1% Triton X-100. Stained proteins were visualized using Alexa Fluor 488-conjugated secondary antibodies (Thermo Fisher Scientific, MA, USA). Nuclei were counterstained with DAPI (Sigma, MO, USA). Stained cells were observed with an Olympus IX71 fluorescence microscope (Olympus).

2.12 | ELISA

Concentrations of Hyaluronan in CRC cell culture medium were quantified by a sandwich enzyme immunoassay using the Quantikine ELISA kit (R&D Systems) following the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader (BioTek microplate reader).

2.13 | Statistical analysis

All experiments were repeated 3 times or more. Comparisons between values were performed using unpaired two-tailed Student's *t*-test, or ANOVA for multivariate analysis. The variance was similar between groups that were being statistically compared. All statistical analyses were performed using GraphPad Prism 7.0 and the *P* values < 0.05 were considered significant.

3 | RESULTS

3.1 | HAS2 expression correlates with the malignant phenotype of colorectal cancer tissues and cell lines

To investigate the role of HAS2 in CRC, we first compared the expression levels of HAS2 between neoplastic and non–neoplastic tissues of human CRC patients, by immunohistochemistry (IHC). Importantly IHC analysis revealed that various CRC tissues show higher expression of HAS2 in neoplastic tissues compared to non–neoplastic tissues (Figure 1A). A similar observation was made in the CRC patient tissue samples using western blot analysis (Figure 1B). Further, we analyzed the levels of HAS2 in six different CRC cell lines. Notably, western blot analysis and immunostaining experiments revealed that four cell lines (HD29, WiDr, DLD1 and HCT116) expressed higher levels of HAS2 compared with SW480 and RKO CRC cells (Figure 1C,D). Interestingly, when these CRC cell lines were exposed individually to ionizing radiation (10 Gy) or oxaliplatin (40 μmol/L), an increased apoptotic cell death was observed in SW480 and RKO CRC cells that express comparatively low levels of HAS2 (Figure 1E). Taken together, these results demonstrate that HAS2 levels correlate with the malignant phenotype of CRC.

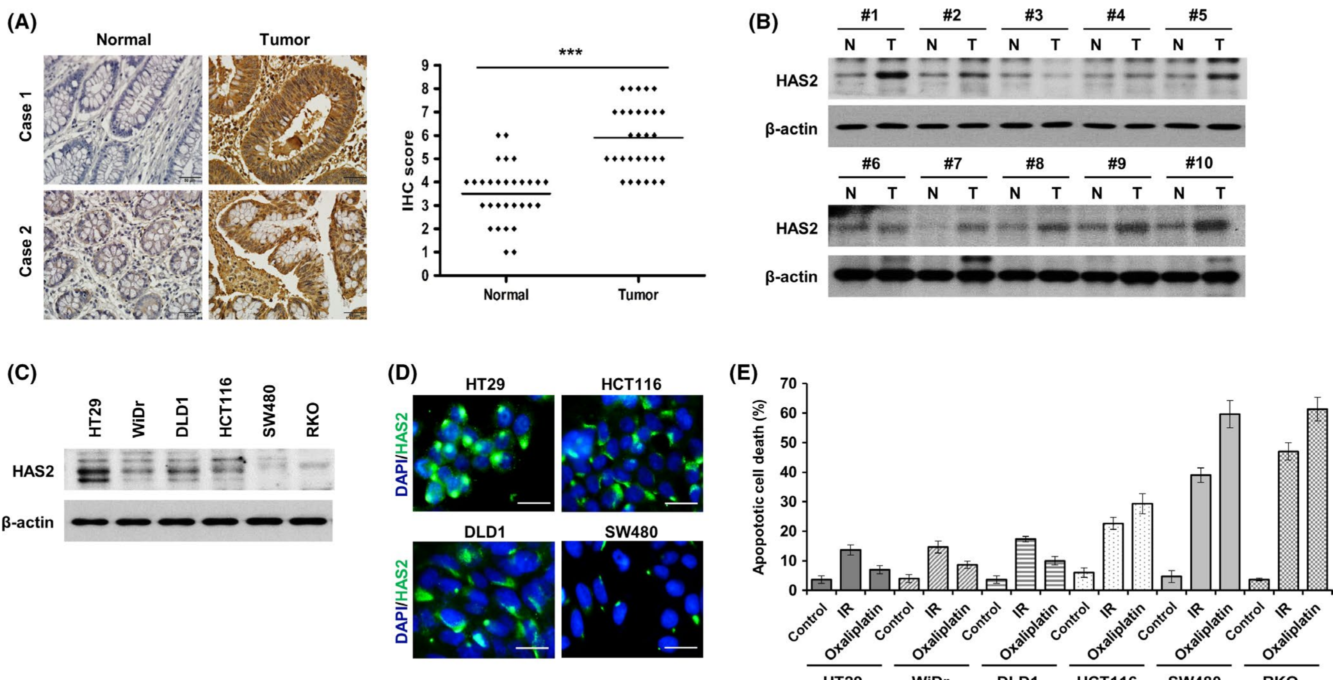

FIGURE 1 Correlation of hyaluronic acid synthase 2 (HAS2) with the colorectal cancer (CRC) tissues and cell lines. A, Immunohistochemistry of HAS2 expression in non–neoplastic and CRC tissue samples (n = 32) and scores for HAS2 staining. Scale bars: 50 μm. B, Western blot analysis of HAS2 expression levels in 10 paired CRC and tumor-free tissue samples. (C) Western blot analysis and (D) immunofluorescence staining for comparison of HAS2 levels in CRC cell lines. E, Flow cytometric analysis of the apoptosis rate in CRC cell lines after therapeutic treatment (irradiation = 10 Gy, Oxaliplatin = 40 μmol/L). Data are presented as mean ± SD from one of three independent experiments with similar results. *P < 0.05 vs control; **P < 0.01 vs control; ***P < 0.001 vs control

3.2 | Knockdown of HAS2 sensitizes colorectal cancer cells to anticancer treatment

Because high levels of HAS2 were related to apoptosis of CRC in response to anticancer treatment, we postulated that HAS2 might have a role in CRC malignancy. Hence, we depleted endogenous HAS2 molecule by siRNA transfection system in the HCT116 and DLD1 cell lines, which express high levels of HAS2 (Figure 2A). Importantly, knockdown of HAS2 reduced the growing ability (Figure 2B) and colony formation efficiency of the malignant CRC cell lines (Figure 2C). Colony forming ability could be suggestive of cell responses such as proliferation, differentiation and cell death.[22] Therefore, we tested whether HAS2 depletion could sensitize CRC cells to radiation and oxaliplatin. By FACS analysis using Annexin V and propidium iodide (PI) double staining, measurement of apoptosis of CRC cells after transfection with HAS2 siRNA followed by treatment with radiation or oxaliplatin indicated an increase in apoptotic cell death (Figure 2D). In agreement with these results, the cleaved forms of caspase-3 and PARP, the hallmarks of apoptosis,[23] were increased in HAS2-depleted CRC cells compared with that in the control cells (Figure 2E). Collectively, these data suggest that HAS2 depletion sensitizes CRC cells to anticancer treatment.

3.3 | Knockdown of HAS2 suppresses metastatic ability of colorectal cancer cells through epithelial-mesenchymal transition

To further investigate the role of HAS2 in CRC malignancy regulation, we next examined the effect of HAS2 on the metastatic ability of CRC. To this end, we first investigated migration and invasion of HCT116 and DLD1 CRC cells after transfection with HAS2 siRNA. By Boyden chamber assay, we observed that siRNA-mediated HAS2 depletion effectively suppresses migration and invasion of these CRC cells (Figure 3A). Many studies suggested that the migration and invasion properties of cancer cells are associated with the EMT program.[24] To examine the role of HAS2 in EMT, we analyzed EMT markers and transcriptional activators after treatment with HAS2 siRNA. Our western blot analysis indicated that HAS2 is a critical EMT regulator in CRC cells, as evidenced by expression levels of EMT markers (E-cadherin, N-cadherin and Vimentin) and its master transcription factors (Zeb1 and Snail) (Figure 3B). A similar observation was obtained by immunocytochemical analysis in which E-cadherin was increased, while Vimentin was decreased by HAS2 depletion (Figure 3C). Based on these in vitro data, we attempted to validate the effect of HAS2 on in vivo metastasis of CRC. HCT116 cells were transfected with HAS2 shRNA or scrambled control shRNA prior to injection into the spleen of athymic nude mice. Three

FIGURE 2 Hyaluronic acid synthase 2 (HAS2) is required for therapeutic resistance of colorectal cancer (CRC) cell lines. A, Western blot analysis for validation of HAS2 siRNA efficacy. B, Growth of HCT116 and DLD1 CRC cell lines transfected with siRNA against HAS2 (for 4 d). C, Quantification of colony formation in HAS2 knockdown HCT116 and DLD1 cells. D, Apoptosis assay after therapeutic treatment of HCT116 and DLD1 CRC cells transfected with siHAS2. E, Western blot analysis for the expression of cleaved caspase3 and cleaved PARP in HAS2-depleted HCT116 and DLD1 cells treated with irradiation and oxaliplatin. Data are presented as mean ± SD from one of three independent experiments with similar results. *$P < 0.05$ vs control; **$P < 0.01$ vs control; ***$P < 0.001$ vs control

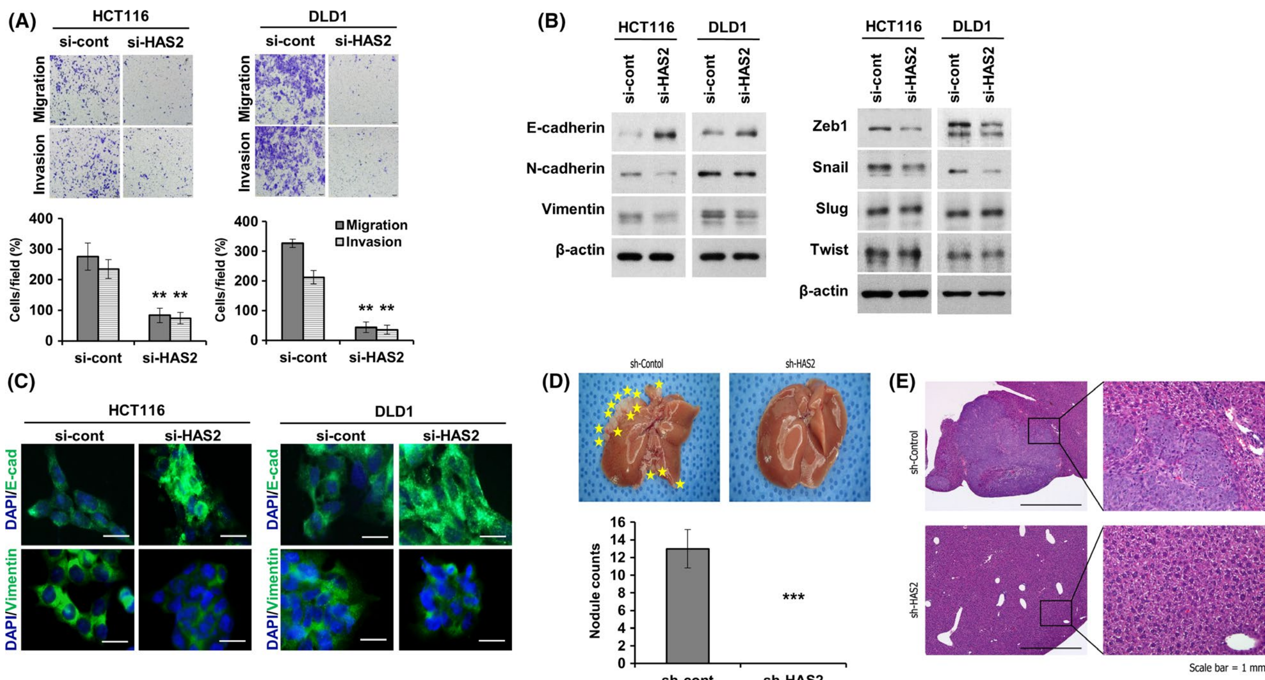

FIGURE 3 Hyaluronic acid synthase 2 (HAS2) promotes colorectal cancer invasiveness by inducing epithelial-mesenchymal transition (EMT). A, Migration and invasion assay in HCT116 and DLD1 colorectal cancer (CRC) cells transfected with control or HAS2 siRNA. B, Western blot analysis for EMT markers (E-cad, N-cad, VIM) and regulators (ZEB1, SNAIL, SLUG, TWIST) in HCT116 and DLD1 CRC cells that were transfected with siRNA against HAS2. C, Immunocytochemistry of HCT116 and DLD1 CRC cells treated with siRNA against HAS2 for EMT markers. D, Representative images and quantification of liver metastatic foci in mice after spleen injection of HCT116 CRC cells transduced with HAS2 shRNA as indicated. E, H&E staining of liver tissue sections after mammary spleen injections in HCT116 cells transduced with HAS2 shRNA. Data are presented as mean ± SD from one of three independent experiments with similar results. *$P < 0.05$ vs control; **$P < 0.01$ vs control; ***$P < 0.001$ vs control

weeks later, these mice were sacrificed and the metastasis was analyzed. Notably, control shRNA-transfected HCT116 cells were easily metastasized into the liver; however, HAS2-depleted HCT116 cells were not metastasized (Figure 3D,E). Collectively, these data suggest that HAS2 depletion suppressed the metastatic ability of CRC cells through EMT regulation.

3.4 | Overexpression of HAS2 confers malignancy on colorectal cancer cells

In addition to HAS2 knockdown, we overexpressed HAS2 in SW480 colorectal cancer cell lines. The overexpression of HAS2 was confirmed by western blotting (Figure 4A). As expected, overexpression of HAS2 resulted in resistance to therapeutic treatment. FACS analysis revealed that 10 Gy dose γ-radiation and 40 μmol/L oxaliplatin treatment reduced apoptotic cell death over 20% in HAS2 overexpression SW480 cell line compared with that in control groups (Figure 4B). Levels of cleaved caspase3 and PARP followed a similar pattern (Figure 4C). Additionally, cells overexpressing HAS2 acquired EMT-related metastatic ability. Transwell assay revealed that HAS2 overexpression dramatically increased migratory and invasive properties (Figure 4D) In parallel, exogenous expression of HAS2 decreased epithelial cell marker E-cadherin and increased mesenchymal markers N-cadherin and vimentin. Furthermore, EMT regulators Zeb1 and Snail were also upregulated in HAS2-overexpressed SW480 CRC cell lines. (Figure 4E) These results indicate that HAS2 is a critical factor of CRC malignancy regulation.

3.5 | Hyaluronic acid ligand has no effect on colorectal cancer malignancy

To investigate the role of HAS2 in CRC malignancy regulation, we initially analyzed HA, the enzymatic product of HAS2. HA is a well-known glycosaminoglycan ECM component in most mammalian tissues. This natural component has gained attention as an interesting target molecule for cancer due to its fundamental ability to act as a ligand. First, we examined the HA secretion level in CRC cell lines to check malignancy and hyaluronan correlation. However, ELISA analysis showed that secreted HA has no correlation with the cell malignancy status (Figure 5A). In addition, to identify the direct

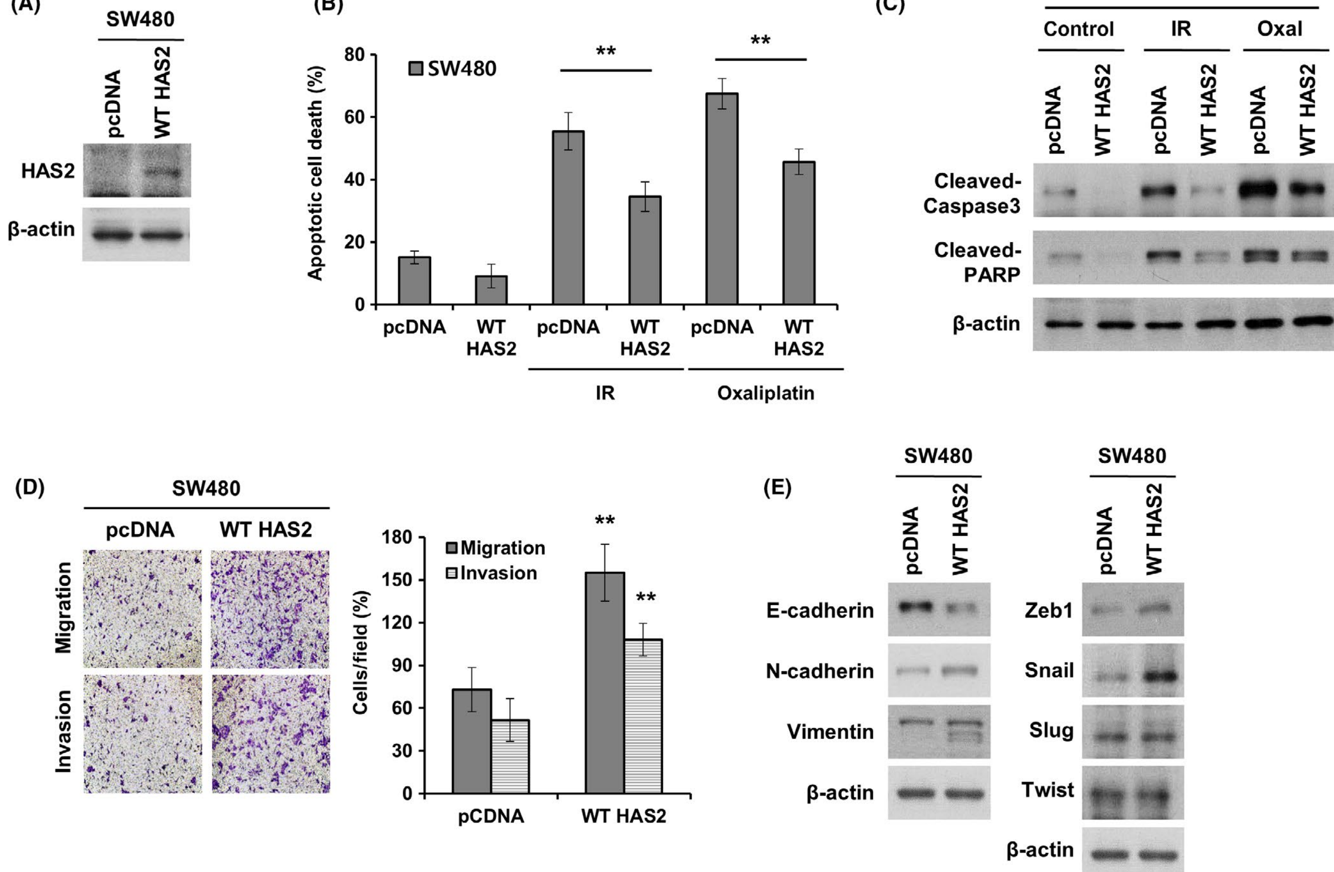

FIGURE 4 Exogeneous expression of hyaluronic acid synthase 2 (HAS2) regulates colorectal cancer (CRC) malignancy. A, Western blot analysis for validation of HAS2 expression vector efficacy. B, Apoptosis analysis after therapeutic treatment of the SW480 CRC cells transfected with overexpression vector targeting HAS2. C, Western blot analysis of cell death indicators cleaved caspase3 and cleaved PARP. D, Migration and invasion assay and (E) western blot analysis for epithelial-mesenchymal transition (EMT) markers and regulators in SW480 CRC cell line. Data are presented as mean ± SD from one of three independent experiments with similar results. *P < 0.05 vs control; **P < 0.01 vs control; ***P < 0.001 vs control

FIGURE 5 Exogenous hyaluronan does not exert any effects on colorectal cancer (CRC) cell lines. A, Quantification of HA in HT29, WiDr, DLD1, HCT116, SW480 and RKO CRC cells by ELISA. B, Apoptosis assay after therapeutic treatment of HCT116 and SW480 CRC cells that are treated with different sizes of recombinant hyaluronic acid (HA) (low, middle, high). C, Migration and invasion assay in HCT116 and SW480 CRC cells treated with different molecular sizes of recombinant HA (low, middle, high). Data are presented as mean ± SD from one of three independent experiments with similar results *P < 0.05 vs control; **P < 0.01 vs control; ***P < 0.0001 vs control

ligand effects of hyaluronan on CRC malignancy, we treated cells with recombinant HA. Contrary to our prediction, recombinant HA had no effect on therapeutic sensitivity or migration and invasion ability (Figure 5B,C). These results suggest that HAS2 promotes the malignancy of CRC independently of the secreted HA-related mechanisms.

3.6 | Hyaluronic acid synthase 2 functions as a colorectal cancer malignancy regulator by activating the TGF-β signaling pathway

We next sought out to define the downstream target of HAS2 that regulates malignant features in CRC independently of the HA ligand pathway. Interestingly, western blot analysis showed that the expression level of TGF-β was similar to that of HAS2 in CRC cell lines. Moreover, the phosphorylation of SMAD2 and SMAD3, downstream regulators of TGF-β, increased in HT29, WiDr, DLD1 and HCT116 CRC cell lines (Figure 6A). These findings suggested that HAS2 expression potentially activates TGF-β signaling. To examine whether HAS2 is associated with the regulation of TGF-β, we next tested the effect of downregulation of HAS2 on TGF-β expression. Notably, siRNA-mediated downregulation of HAS2 decreased TGF-β expression and the phosphorylation of SMAD2 and SMAD3 in HCT116 and DLD1 cells (Figure 6B). Next, to confirm that HAS2 regulates CRC malignancy via the TGF-β pathway, we used siSMAD2 and siSMAD3 system (Figure 6C). siRNA-mediated knockdown of SMAD2/3 suppressed migration and invasion ability and increased therapeutic sensitivity mainly in HCT116 and DLD1 CRC cell lines (Figure 6D,E). However, SMAD2 played the major role in this regulation of CRC malignancy. Similar to the HAS2 results, western blot

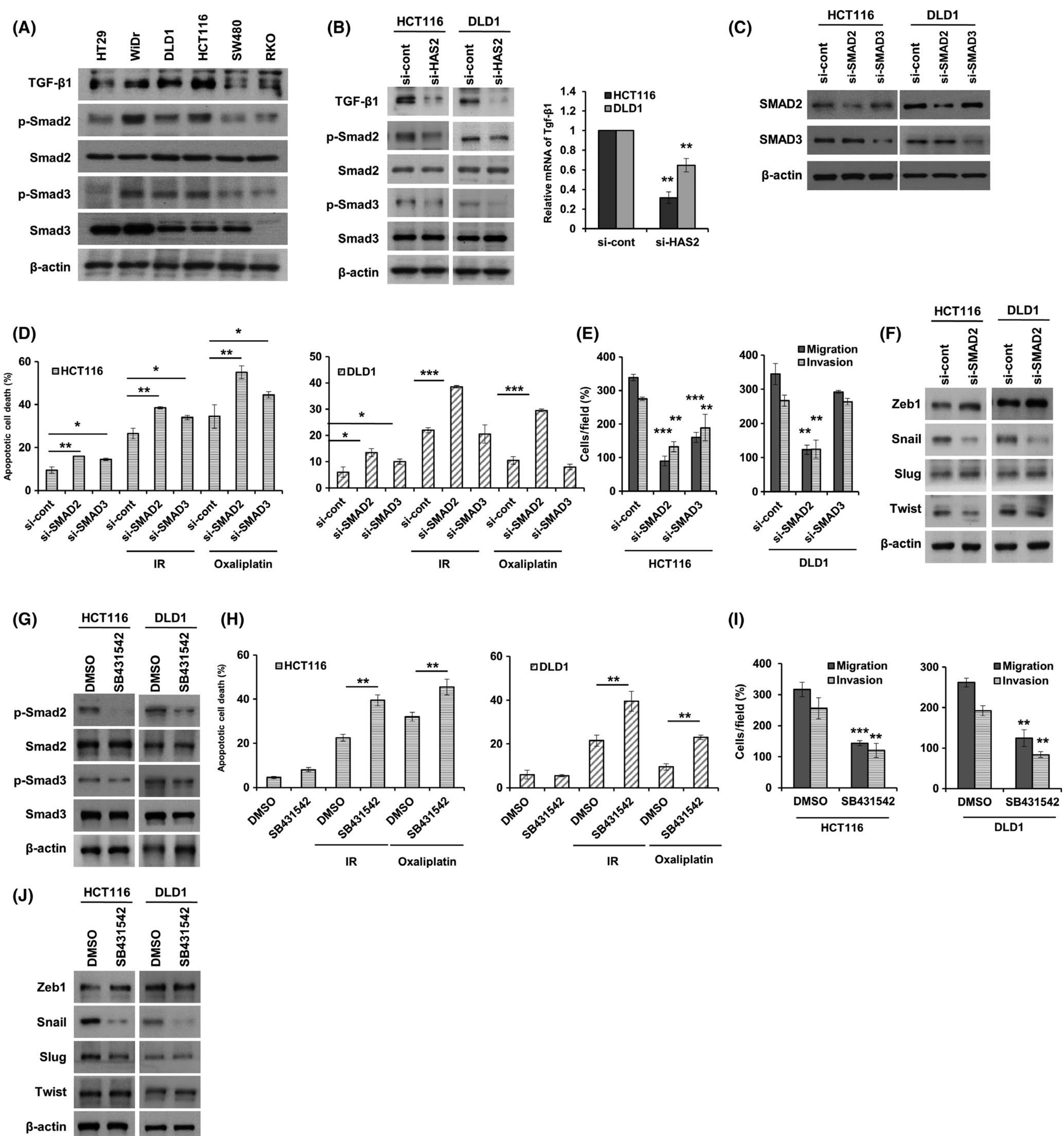

FIGURE 6 Hyaluronic acid synthase 2 (HAS2) activates transforming growth factor beta (TGF-β) signaling in colorectal cancer (CRC) cell lines. A, Western blot analysis for quantification of TGF-β and other components of the signaling cascades in CRC cell lines. B, Western blot and qPCR analyses for determining the expression status of TGF-β and signaling cascades in CRC cells transfected with siHAS2 as indicated. C, Western blot analysis for validation of SMAD2 and SMAD3 siRNA efficacy. D, Apoptosis assay after therapeutic treatment of HCT116 and DLD1 CRC cells that are transfected with siSMAD2 and siSMAD3. E, Migration and invasion assay in HCT116 and DLD1 CRC cells transfected with control or SMAD2, SMAD3 siRNA. F, Western blot analysis for epithelial-mesenchymal transition (EMT) regulators (ZEB1, SNAIL, SLUG, TWIST) in HCT116 and DLD1 CRC cell that were transfected with siRNA against SMAD2. G, Western blot analysis of the TGF-β signaling cascades after DMSO or SB431542 (10 μmol/L) treatment. H, Apoptosis assay after therapeutic treatment of HCT116 and DLD1 CRC cells treated with SB431542 (10 μmol/L). I, Migration and invasion assay in HCT116 and DLD1 CRC cells treated with SB431542 (10 μmol/L). J, Western blot analysis for EMT regulators (ZEB1, SNAIL, SLUG, TWIST) in HCT116 and DLD1 CRC cells after a DMSO or SB431542 (10 μmol/L) treatment. Data are presented as mean ± SD from one of three independent experiments with similar results. *P < 0.05 vs control; **P < 0.01 vs control; ***P < 0.001 vs control

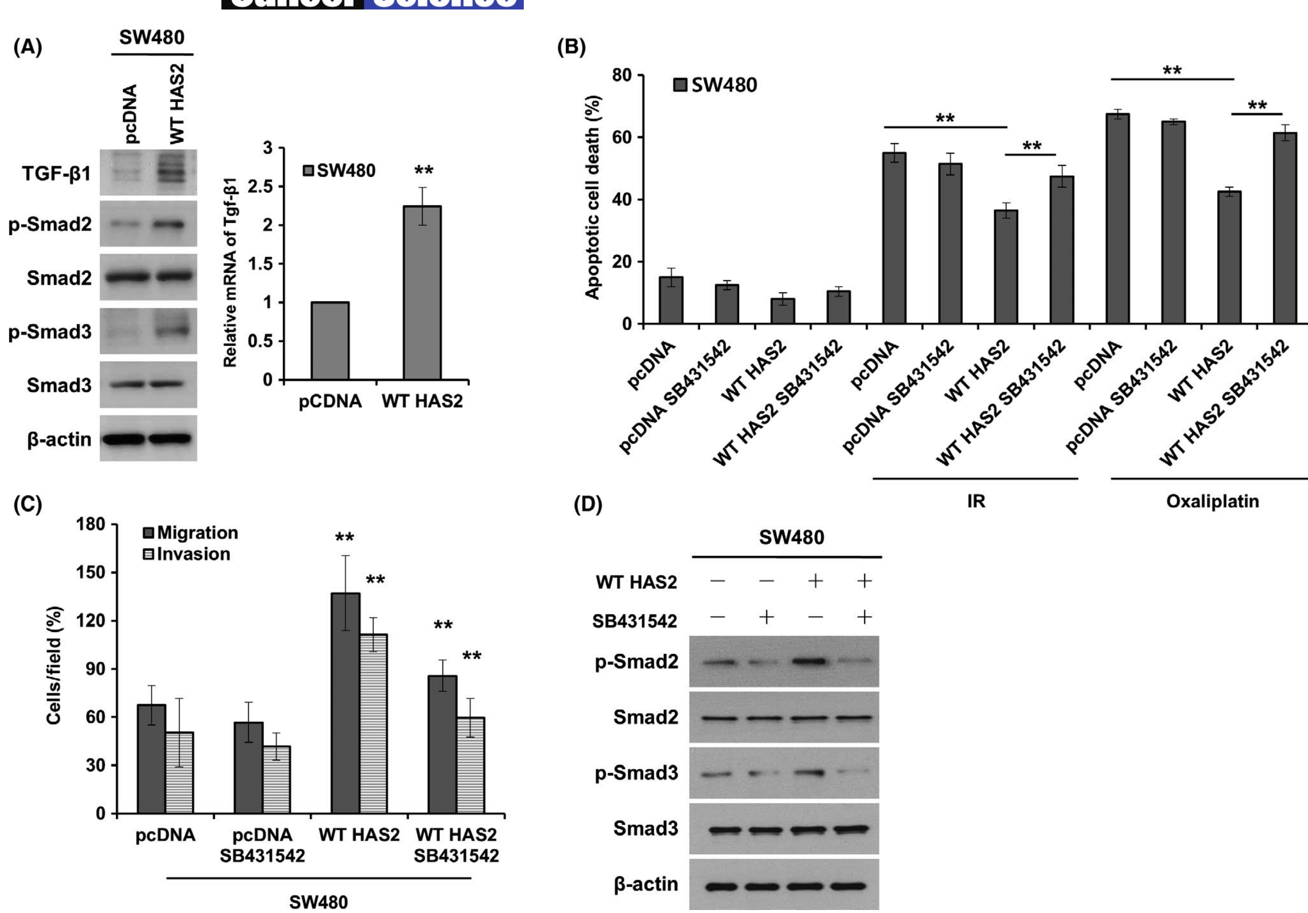

FIGURE 7 Hyaluronic acid synthase 2 (HAS2) mediates colorectal cancer (CRC) malignancy by SMAD2/3 activation. A, Western blot and qPCR analyses showing expression status of transforming growth factor beta (TGF-β) and signaling cascades in CRC cells transfected with WT HAS2 as indicated. B, Apoptosis assay after therapeutic treatment of SW480 CRC cells transfected with WT HAS2 and treated with SB431542 (10 μmol/L) as indicated. C, Migration and invasion assay of SW480 CRC cells transfected with WT HAS2 and treated with SB431542(10 μmol/L) as indicated. D, Western blot analysis showing expression status of TGF-β signaling cascades in SW480 CRC cells transfected with WT HAS2 and treated with SB431542 (10 μmol/L) as indicated. Data are presented as mean ± SD from one of three independent experiments with similar results. **P* < 0.05 vs control; ***P* < 0.01 vs control; ****P* < 0.001 vs control

analysis revealed that Snail, an EMT regulator, was decreased by SMAD2 depletion in CRC cells (Figure 6F). Furthermore, the potent TGF-β inhibitor SB431542[25] (Figure 6G) exerted the same effect on therapeutic sensitivity (Figure 6H) and migration and invasive ability (Figure 6I) of HCT116 and DLD1 cells via Snail regulation. (Figure 6J).

3.7 | Overexpression of HAS2 mediates colorectal cancer malignancy via SMAD activation

We observed that HAS2 expression correlated with TGF-β/SMAD complexation in malignant CRC cells. In parallel with these findings, HAS2 overexpression increased TGF-β expression and the phosphorylation of SMAD proteins in SW480 CRC cell lines, which showed low malignancy (Figure 7A). Next, to assess the direct effect of HAS2 on TGF-β/Smad2/3 signaling, we treated SB431542 to the SW480 overexpressing cell lines with HAS2. Although SB431542 has no remarkable change of basal SW480 cells because SW480 has still lower activation of SMAD2/3 than malignant CRC cell lines, treatment with these inhibitor mimics reversed HAS2 overexpression-mediated therapeutic sensitivity and malignancy-related events (Figure 7B,C). In addition, treatment with the inhibitors in combination with HAS2 overexpression restored TGF-β and SMAD2/3 activation (Figure 7D). Taken together, these findings suggest that HAS2 promotes the malignant phenotype of CRC cells through the TGF-β/SMAD signaling axis.

4 | DISCUSSION

Despite advancements in anticancer therapeutic strategies, including surgery, chemotherapy using agents such as oxaliplatin, irinotecan, fluorouracil and radiotherapy,[8] the prognosis of CRC remains poor. Many cases of malignant phenotypes of CRC have shown therapeutic resistance, high recurrence rate and metastatic features.[3-5,7] Thus, several new agents against a variety of targets are being developed, which are believed to regulate cancer malignancy characteristics such as proliferation, therapeutic resistance and metastasis. Because cancer cells have to overcome the ECM barrier

before traversing the long route to reach other organs for metastasis, ECM remodeling factors in the tumor microenvironment have gained considerable attention in cancer biology.[26-28] Among these factors, HAS2, the rate-limiting enzyme for HA synthesis, is often elevated in various cancers. However, although its importance is well documented in many cancers, including glioma, breast cancer and squamous cell carcinoma,[18,29,30] its oncogenic role in CRC remains obscure. HA is a major component of the extracellular matrix and regulator of many cell processes such as cell migration, proliferation and differentiation. In normal ECM, HA exerts beneficial effects on tissue homeostasis and the biomechanical integrity, structure and assembly of tissues.[13] In contrast, in malignant tumor tissues, HA is known to promote aggressiveness of cancer cells.[31]

In this study, we observed a correlation between HAS2 levels and malignant phenotypes of CRC cells. HAS2 expression was significantly induced in CRC tissue samples and malignant type CRC cell lines. Furthermore, HAS2 depletion increased apoptosis, therapeutic sensitivity and decreased EMT-related migration and invasive ability of CRC cells. By contrast, HAS2 overexpression promoted malignancy features. EMT is a process in which epithelial cells lose their epithelial characteristics and acquire mesenchymal characteristics. It is a well-known phenomenon that causes the metastatic spread of cancer cells and cancer recurrence.[32] In addition, acquisition of EMT features has been associated with therapeutic resistance.[33]

Hyaluronic acid is a well-known glycosaminoglycan ECM component in most mammalian tissues. This natural component has gained attention as an interesting target molecule for cancer due to its fundamental ability to act as a ligand of CD44 and RHAMM.[34] The interactions of CD44 and RHAMM with HA are well known to be crucial role for tumor cell malignancy via various signaling pathways such as SRC, PI3k and MAPK, in many types of tumor.[35,36] We examined whether the ligand of HA has an effect on CRC by treating cells with recombinant HA. Interestingly, the results revealed that treatment with HA of different sizes had no specific effect on CRC malignancy. This finding is consistent with some recent reports that the HA ligand-related pathway has no effect on regulating cellular behavior.[37,38]

Our results demonstrated that secreted HA ligand has less influence on CRC; however, overexpressed HAS2 regulates CRC malignancy crucially. This led us to postulate that HAS2 has a specific role in CRC malignancy regulation independent of HA synthase. TGF-β is one of the major regulators of cell and tissue behavior such as homeostasis, wound healing, fibrosis, angiogenesis and differentiation.[39] The TGF-β ligand and receptor complex phosphorylates downstream regulator protein SMAD. The phosphorylated SMAD forms a complex with co-SMAD and enters the nucleus to act as a gene transcriptional activator.[40] Previous studies have shown that once carcinogenesis is initiated, the TGF-β signaling pathway promotes cancer malignancy. Furthermore, TGF-β has often been associated with resistance to cancer treatment, increased risk of invasion and metastasis, poor prognosis, and high levels of microsatellite instability.[41-43] Hence, targeting the TGF-β pathway for cancer therapy could be regarded as a logical strategy. In the current study, we found that the TGF-β/SMAD2/Snail signaling axis is closely associated with the malignancy of CRC cell lines and HAS2 regulates TGF-β expression. Both SMAD2 and SMAD3 have malignancy regulation ability. However, SMAD2 activation showed a more dramatic response in the CRC samples and SMAD2 has been reported to be correlated with CRC malignancy.[44] Several studies suggest that intracellular hyaluronan and HAS are involved in physiological events or conditions such as inflammation or cancer.[45,46] In agreement with these studies, we anticipate intracellular HAS2 to be related with CRC malignancy regulation. Furthermore, HAS2 has a multi-pass membrane bound enzyme structure and may interact with other cytoplasmic proteins such as protein kinase C that are involved in TGF-β signaling.[47] In addition, recent studies have reported that TGF-β upregulates HAS2 expression.[38] These previous observations indicate that the expression of TGF-β is maintained at a level similar to that of HAS2 in CRC cell lines and that TGF-β mediates a positive feedback loop between HAS2. However, the molecular mechanisms underlying the oncogenic role of intracellular HAS2 in CRC remain abstruse. In summary, HAS2 is preferentially overexpressed in malignant-type CRC cancer cells compared with that in mild-type CRC. By studying cells with loss-of-function and gain-of-function of HAS2, we demonstrated that HAS2 is a critical regulator for the malignant behavior of CRC such as therapeutic resistance or metastatic ability. Importantly, HAS2 promoted CRC malignancy through HA ligand-independent TGF-β regulation. Therefore, although further investigation is required to identify other mechanisms of HAS2, our findings highlight the potential of HAS2 as a novel therapeutic target for CRC.

DISCLOSURE

The authors declare no conflict of interest.

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