Two naturally derived small molecules disrupt the sineoculis homeobox homolog 1–eyes absent homolog 1 (SIX1–EYA1) interaction to inhibit colorectal cancer cell growth

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Introduction
Colorectal cancer (CRC) is a gastrointestinal cancer that starts in the colon or the rectum.1,2 According to data from GloboCan in 2018, the incidence and mortality of CRC are among the top four cancers in the world.3 CRC is considered a multifactorial disease resulting from genetic instability, epigenetic dysregulation, differentially expressed tumor suppressors and oncogenes, and aberrantly expressed non-coding ribonucleic acids (RNAs).4,5 The combined options of surgery, chemotherapy, and radiotherapy are the basic strategies of CRC treatment; a choice from among these options is made depending on the tumor size, location, and metastatic stages.6,7 Although the 5-year survival rate for CRC patients is over 60%, common chemotherapeutic drugs often lead to resis-

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Abstract
Background: Emerging evidence indicates that the sineoculis homeobox homolog 1–eyes absent homolog 1 (SIX1–EYA1) transcriptional complex significantly contributes to the pathogenesis of multiple cancers by mediating the expression of genes involved in different biological processes, such as cell-cycle progression and metastasis. However, the roles of the SIX1–EYA1 transcriptional complex and its targets in colorectal cancer (CRC) are still being investigated. This study aimed to investigate the roles of SIX1–EYA1 in the pathogenesis of CRC, to screen inhibitors disrupting the SIX1–EYA1 interaction and to evaluate the efficiency of small molecules in the inhibition of CRC cell growth.

Methods: Real-time quantitative polymerase chain reaction and western blotting were performed to examine gene and protein levels in CRC cells and clinical tissues (collected from CRC patients who underwent surgery in the Department of Integrated Traditional and Western Medicine, West China Hospital of Sichuan University, between 2016 and 2018, n = 24). In vivo immunoprecipitation and in vitro pulldown assays were carried out to determine SIX1–EYA1 interaction. Cell proliferation, cell survival, and cell invasion were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, clonogenic assay, and Boyden chamber assay, respectively. The Amplified Luminescent Proximity Homogeneous Assay Screen (AlphaScreen) method was used to obtain small molecules that significantly disrupted SIX1–EYA1 interaction. CRC cells harboring different levels of SIX1/EYAI were injected into nude mice to establish tumor xenografts, and small molecules were also injected into mice to evaluate their efficiency to inhibit tumor growth.

Results: Both SIX1 and EYAI were overexpressed in CRC cancerous tissues (for SIX1, 7.47 ± 3.54 vs. 1.88 ± 0.35, t = 4.92, P = 0.008; for EYAI, 7.61 ± 2.03 vs. 2.22 ± 0.45, t = 6.73, P = 0.005). The SIX1/EYAI complex could mediate the expression of two important genes including cyclin A1 (CCNA1) and transforming growth factor beta 1 (TGFB1) by binding to the myocyte enhancer factor 3 consensus. Knockdown of both SIX1 and EYAI could decrease cell proliferation, cell invasion, tumor growth, and in vivo tumor growth (all P < 0.01). Two small molecules, NSC0191 and NSC0933, were obtained using AlphaScreen and they could significantly inhibit the SIX1–EYA1 interaction with a half-maximal inhibitory concentration (IC50) of 12.60 ± 1.15 μmol/L and 83.43 ± 7.24 μmol/L, respectively. Administration of these two compounds could significantly repress the expression of CCNA1 and TGFB1 and inhibit the growth of CRC cells in vitro and in vivo.

Conclusions: Overexpression of the SIX1/EYAI complex transactivated the expression of CCNA1 and TGFB1, causing the pathogenesis of CRC. Pharmacological inhibition of the SIX1–EYA1 interaction with NSC0191 and NSC0933 significantly inhibited CRC cell growth by affecting cell-cycle progression and metastasis.

Keywords: NSC0191; NSC0933; Sineoculis homeobox homolog 1; Eyes absent homolog 1; Colorectal cancer; Metastasis
tance. Thus, it is necessary to develop new drugs that precisely target key molecules involved in the pathogenesis of CRC, which will provide more options for CRC treatment.

The aberrant expression of tumor suppressors and oncogenes is often controlled by transcription factors. Sineoculus homeobox homolog 1 (SIX1), a member of the sineoculus homeodomain (SIX) family proteins, has been identified as a critical transcription factor that mediates the expression of multiple genes, such as cyclin A1 (CCNA1), glial cell-derived neurotrophic factor (GDNF), solute carrier family 12 member 2 (SLC12A2), and transforming growth factor-beta 1 (TGFB1). SIX1 only contains a deoxyribonucleic acid (DNA)-binding homeodomain but lacks an intrinsic transactivation domain. Thus, it often assembles a transcriptional complex with different members of eyes absent homolog 1 (EYA1) in which EYA members (EYA1–4) function as coactivators. Both SIX1 and EYAs are absent or downregulated post-embryogenesis, while they are re-expressed in multiple cancers such as breast cancer, cervical cancer, Wilms tumor, and liver cancer. SIX1/EYA complex have been shown to control many biological processes, such as cell proliferation and survival, cell migration and invasion, epithelial-to-mesenchymal transitions (EMT), and metastasis. Similarly, SIX1 is also overexpressed in CRC cells and its overexpression (OE) can stimulate angiogenesis and recruit tumor-associated macrophages, thereby promoting metastasis. However, the targets of SIX1 and its associated coactivator in CRC cells are unknown.

In different cancer types and xenograft and transgenic mouse models, knockdown (KD) of SIX1/EYA complexes can greatly inhibit cancer cell growth and tumor progression, which implies that inhibitors targeting these complexes may ultimately result in promising effects on cancer therapy. Based on this notion, a small molecule known as NCGC00378430 has recently been identified using the amplified luminescent proximity homogeneous assay screen (AlphaScreen) method and it shows a strong effect on decreasing the SIX1–EYA2 interaction. NCGC00378430 can reverse transcriptional and metabolic profiles mediated by SIX1 and can inhibit transforming growth factor beta (TGF-β) signaling, thus repressing tumor metastasis. In addition, EYA family proteins also show phosphatase activity. Several EYA2 phosphatase inhibitors, such as benz bromarone and N-(aryliden)e benzohydrazide-containing compounds, have been discovered to show moderate half maximal inhibitory concentration (IC50) values to inhibit cell motility and angiogenic tubulogenesis. This study aimed to investigate the roles of SIX1–EYA1 in the pathogenesis of CRC, to screen inhibitors disrupting the SIX1–EYA1 interaction and to evaluate the efficacy of small molecules in the inhibition of CRC cell growth.

Methods

**Ethical approval and tumor sample collection**

Cancerous colon tissues and their adjacent non-cancerous tissues were collected from 24 CRC patients who underwent surgical treatments from June 2016 to December 2018 in the Department of Integrated Traditional and Western Medicine, West China Hospital of Sichuan University. All patients gave their informed consent, and the study protocol was reviewed and approved by the ethical board of West China Hospital of Sichuan University (No. 2016668HA). Basic information (age, gender, and stages of the tumor, nodes, and metastases [TNM]) of these 24 patients is summarized in Supplementary Table 1, http://links.lww.com/CM9/A745. The animal experiments were performed following a protocol (No. 2017039MA) approved by the Institutional Animal Care and Use Committee (IACUC) of West China Hospital of Sichuan University.

**Cell lines, cell culture, and transfection**

The source and growth conditions of human colon epithelial cells (HCEC), namely HCEC-1CT, were the same as described previously. The sources and growth conditions of seven CRC cell lines including HT29, HT55, HCT-15, HCT-116, HCA-24, SW620, and T84 were the same as described previously. For short hairpin RNA (shRNA) transfection, two independent MISSION shRNA lentiviral transduction particles of SIX1 (#TRCN0000015235 and #TRCN0000015237) and EYA1 (#TRCN0000303462 and #TRCN0000315624) were purchased from Sigma-Aldrich (St. Louis, MO, USA). These particles and pLKO.1-puro (Control) were individually transfected with FuGene 6 (Roche Diagnostics Corp., Indianapolis, IN, USA, #E2691) into cells following the manufacturer’s protocol. The transfected cells were selected with puromycin (1 μg/mL) for 48 h and single cells were picked out to examine messenger RNA (mRNA) and protein levels of the target proteins. We generated two independent KD cell lines of each gene and one Control-KD cell line harboring pLKO.1-puro in both HT-29 and HCA-24 backgrounds. The verified KD cells were subjected to the required experiments. For transfection using OE plasmid, the pCDNA3.2-Flag (empty vector), pCDNA3.2-Flag-SIX1, and pCDNA3.2-Flag-EYA1 vectors were transfected into cells using Lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA, USA, #11668019) according to the method provided by the manufacturer. We generated one OE cell line of each gene and one Control-OE cell line harboring pCDNA3.2-Flag empty vector. After 48 h, the cells were subjected to the required experiments.

**Western blotting**

CRC cancerous tissues and their adjacent non-cancerous tissues and cultured CRC cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher Scientific, #89901) mixed with protease inhibitor (ThermoFisher Scientific, #78425). Equal amounts of total cell extracts were resolved in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. After transferring onto polyvinylidene fluoride membranes and blocking with 5% milk for 1 h, proteins were incubated with primary antibodies including anti-SIX1 (Sigma-Aldrich, #HA001893), anti-EYA1 (Sigma-Aldrich, #HPA028917), anti-CCNA1 (Sigma-Aldrich, #SAB1409961), anti-TGF-β
Cells (1 × 10⁷) were lysed in 2 mL RIPA buffer containing a protease inhibitor, and 0.2 mL cell lysates were taken out as input. The other 1.8 mL cell lysates were incubated with protein A agarose (Abcam, #ab193254) at 4°C for 2 h, followed by supplementing with anti-SIX1 or anti-EYA1. The input cell extracts, immunoprecipitated SIX1 complex, and EYA1 complex were subjected to examinations of protein levels of SIX1 and EYA1, respectively.

**Luciferase assay**

The wild-type (WT) promoters of CCNA1 and TGFβ1 and their mutants (deletion of myocyte enhancer factor 3 [MEF3] consensus) were cloned into pGL3 firefly vectors. These vectors were co-transfected with Renilla reporter vector into Control-KD, SIX1-KD, Control-OE, and SIX1-OE cells, respectively. The luciferase activity was determined using the Dual-Luciferase Report Assay Kit (Promega, Madison, WI, USA, #E1910). The firefly/Renilla ratio in Control-KD cells was defined as one-fold and the ratios in other cells were normalized to Control-KD cells.

**Protein purification and in vitro pulldown assay**

The full lengths of human SIX1 and EYA1 were cloned into pGEX-6P-1 and pET28a empty vectors, respectively. The pGEX-6P-1-SIX1 and pET28a-EYA1 plasmids were transformed into BL21 (DE3.0) to express glutathione S-transferase (GST)-SIX1 and histidine (His)-EYA1 fusion proteins, with the induction of 1 mmol/L isopropyl β-D-thiogalactoside (Sigma-Aldrich, #I6758) at 16°C for 16 h. The GST-SIX1 and His-EYA1 proteins were purified with Glutathione Sepharose 4B beads (GE Healthcare, Chicago, IL, USA, #GE17-0756-01) and nickel-nitrilotriacetic acid (Ni-NTA) beads (ThermoFisher Scientific, #ND-2000), a 1°C nickel-nitrilotriacetic acid (Ni-NTA) beads (ThermoFisher Scientific, #ND-2000), and the ratios in other cells were normalized to Control-KD cells.

**Immunoprecipitation (IP) assay**

Cells (1 × 10⁷) were lysed in 2 mL RIPA buffer containing a protease inhibitor, and 0.2 mL cell lysates were taken out as input. The other 1.8 mL cell lysates were incubated with protein A agarose (Abcam, #ab193254) at 4°C for 2 h, followed by supplementing with anti-SIX1 or anti-EYA1. The input cell extracts, immunoprecipitated SIX1 complex, and EYA1 complex were subjected to examinations of protein levels of SIX1 and EYA1, respectively.

**Immunofluorescence (IF) and immunohistochemistry (IHC)**

The IMF and IHC assays were performed following previous protocols. To elucidate, 5-μm-thick tumor tissues and the fixed HT29 cells were blocked with 1% bovine serum albumin for 30 min at room temperature. The slides were incubated with anti-SIX1 (Abcam, #ab252224) overnight at 4°C, followed by incubation of AlexaFluor-488 goat anti-rabbit secondary antibody (Abcam, #ab150077) for 1 h at room temperature. After processing antigen retrieval and washing in PBS buffer, slides were incubated with anti-EYA1 (Sigma-Aldrich, #HPA028917) for 1 h at room temperature, followed by incubation of rhodamine red X-AffiniPure donkey anti-rabbit immunoglobulin (IgG) (Jackson ImmunoResearch
The ChIP assay was carried out as described previously.\(^{18}\) For IHC assay, 5-μm-thick paraffin sections were deparaffinized, and antigens were unmasked and probed with anti-SIX1, anti-EYA1, anti-CCNA1, and anti-TGF-β. The information on these antibodies was the same as that in the western blotting assay. The slides were then incubated with biotin-labeled secondary antibodies (Abcam, #ab207995 and #ab6788) for 1 h. After staining with the Vectastain avidin-biotinylated enzyme complex kit (Vector Laboratories, Burlingame, CA, USA, #PK6100) and the diaminobenzidine peroxidase substrate kit (Sigma, #391A), images were photographed with a fluorescence microscope (Nikon, #TE2000-S).

**AlphaScreen assay**

The AlphaScreen assay was performed using GST-SIX1 and His-EYA1 proteins in the same small molecule pool as described previously.\(^{19}\) In brief, equal amounts (7.5 μL) of GST-SIX1 and His-EYA1 proteins were mixed with 5 μL glutathione donor beads and 5 μL nickel chelate acceptor beads (PerkinElmer, Waltham, MA, USA, #6760603M). After 30 min, equal volumes (2 μL) of individual compounds were added into the protein mixture and incubated at 16°C for 2 h. The AlphaScreen signals were collected by reading plates in an Envision Multilabel Reader (PerkinElmer, #2105-0010), and compounds that caused signal values to decrease significantly (<5000) were selected as candidates.

**Chromatin immunoprecipitation (ChIP)**

The ChIP assay was carried out as described previously.\(^{18}\) To elicitate, cells (1 × 10⁶) were cross-linked with 1% formaldehyde for 12 min, followed by quenching with 0.125 mmol/L glycine for 10 min. After rinsing twice with PBS buffer, cells were sonicated 15 × 30 s on ice in 4 mL lysis buffer provided by the Millipore ChIP Assay Kit (Millipore, Burlington, MA, USA, #17295). About 0.4 mL lysed cells were taken out as input, and the other 3.6 mL cells were equally divided into three parts and were then subjected to ChIP procedures following the manufacturer’s method using anti-SIX1, anti-EYA1, and IgG (negative control). The purified input and output DNA were used for RT-qPCR analyses with the following primers: CCNA1 promoter forward: AGACAGACAGGGTTTCACCATG; reverse: ATATCTACACTGAGGCGG; TGFB1 promoter forward: AGAGACTGTCAGAGCAG; reverse: CTCTGTGACCTCAACAC.

**Tumor xenograft model**

Six-week-old male C57BL/6 mice were injected subcutaneously with equal volumes of Control-KD, SIX1-KD, EYA1-KD, Control-OE, SIX1-OE, and EYA1-OE cells (8 × 10⁴; n = 5 for each cell line). Tumor length and width were measured with fine calipers every 5 days and tumor volumes were calculated by the formula volume = (length × width²)/2. For the administration of small molecules in mice, equal volumes of NSC0191 (10 and 20 μmol/L) and NSC0933 (80 and 160 μmol/L) were mixed with HT29 cells (8 × 10⁴), respectively, and were then injected into male C57BL/6 mice (n = 10 for each concentration). The same concentrations of small molecules were further injected into mice every 5 days. Mice were euthanized following the IACUC protocol and tumors from all animals were excised and subjected to western blotting, IP, and IHC experiments.

**Statistical analysis**

All data were collected from at least three independent experiments and shown as the mean ± standard deviation. Statistical analyses were performed using a two-sided Student’s t test with Statistical Package for the Social Sciences software (IBM Corp., Armonk, NY, USA, version 26). Significance was set at P < 0.05.

**Results**

**SIX1 and EYA1 were overexpressed in CRC cancerous tissues and cells**

Similar to many cancer types, OE of SIX1 has also been observed in CRC.\(^{16}\) To further solidify this observation, we collected 24 pairs of cancerous tissues and their adjacent non-cancerous tissues from 24 CRC patients. Using these samples, we measured the mRNA levels of SIX1 members including SIX1, 2, 3, 4, 5, and 6. The RT-qPCR results showed that only SIX1 was significantly increased in the cancerous tissues (CRC) compared to non-cancerous tissues (Control) (7.47 ± 3.54 vs. 1.88 ± 0.35, t = 4.9, P = 0.008), but the other five SIX members were not (for SIX2, 1.74 ± 0.73 vs. 1.79 ± 0.78, t = 0.92, P = 0.422; for SIX3, 1.98 ± 0.72 vs. 1.93 ± 0.85, t = 0.78, P = 0.237; for SIX4, 1.86 ± 0.73 vs. 1.94 ± 0.82, t = 1.09, P = 0.181; for SIX5, 1.98 ± 0.81 vs. 1.91 ± 0.69, t = 0.68, P = 0.228; and for SIX6, 1.85 ± 0.75 vs. 1.77 ± 0.72, t = 0.47, P = 0.428) (Figure 1A and Supplementary Figure 1A – D). Given that SIX1 couples with EYAs to activate gene expression, we next sought to examine the mRNA levels of EYA members. Among 4 EYA members, we observed the increased expression of EYA1 (7.61 ± 2.03 vs. 2.22 ± 0.45, t = 6.7, P = 0.003), EYA3 (4.05 ± 1.87 vs. 2.33 ± 0.94, t = 2.83, P = 0.016), and EYA4 (3.21 ± 1.48 vs. 2.01 ± 0.67, t = 2.16, P = 0.034) in cancerous tissues compared to controls, but not EYA2 (2.16 ± 0.79 vs. 2.04 ± 0.65, t = 0.79, P = 0.334) (Figure 1B and Supplementary Figure 1F–H). Thus, we speculated that SIX1 and EYA1 played a dominant role in CRC and we therefore focused our current study only on revealing their roles instead of the other SIX and EYA members.

We next sought to determine the expression levels of several SIX1 target genes (CCNA1, TGFB1, GDNF, and SLC12A2) that have been reported in other cancer types.\(^{10,11,12,13}\) Using 24 pairs of CRC tissues, our results indicated that the expression levels of CCNA1 and TGFB1 increased 3.12-fold and 6.04-fold, respectively (Figure 1C and 1D), while both GDNF and SLC12A2 only increased...
1.93-fold in the cancerous tissues compared to the non-cancerous tissues [Supplementary Figure 1A and 1B, http://links.lww.com/CM9/A745]. Based on the important roles of CCNA1 and TGFB1 in cell-cycle progression and tumor metastasis, we only used these two genes as representative targets of SIX1 in the following study. Except for mRNA levels, we also examined the protein levels of SIX1, EYA1, CCNA1, and TGFB1 in three paired tissues extracted from patients with TNM stage 3. The immunoblot results showed that all these four proteins were significantly increased in the cancerous tissues in comparison to their adjacent healthy tissues [Supplementary Figure 2A and 2B, http://links.lww.com/CM9/A745]. The relative levels of these proteins in CRC tumor samples were increased to (2.73 ± 0.24)-fold (SIX1, P = 0.008), (2.45 ± 0.17)-fold (EYA1, P = 0.007), (2.68 ± 0.21)-fold (CCNA1, P = 0.005), and (3.68 ± 0.44)-fold (TGFB1, P = 0.009) [Supplementary Figure 2B, http://links.lww.com/CM9/A745].

To examine the expression levels of SIX1, EYA1, CCNA1, and TGFB1 in CRC cells, we selected one non-cancerous cell line (HCEC-1CT) and seven CRC cell lines including HT29, HT55, HCT-15, HCT-116, HCA-24, SW620, and T84. Our results showed that the expression patterns of SIX1, EYA1, CCNA1, and TGFB1 were consistent in all seven CRC cells. Of these seven CRC cells, SIX1, EYA1, CCNA1, and TGFB1 were mostly increased in HT29 cells but were observed to be the least increased in HCA-24 cells. The relative mRNA levels of SIX1, EYA1, CCNA1, and TGFB1 were increased to (4.34 ± 0.29)-fold (P < 0.001), (4.13 ± 0.39)-fold (P < 0.001), (6.88 ± 0.54)-fold (P < 0.001), and (10.25 ± 0.89)-fold (P < 0.001) in HT29 cells, respectively. In contrast, their expression levels were only increased to (2.31 ± 0.25)-fold (P = 0.004), (1.85 ± 0.15)-fold (P = 0.021), (2.94 ± 0.19)-fold (P = 0.006), and (4.11 ± 0.42)-fold (P < 0.001) in HCA-24 cells, respectively [Figure 1E–H]. Consistently, we also observed similar patterns of SIX1, EYA1, CCNA1, and TGFB1 protein levels in these seven cell lines [Supplementary Figure 2C and 2D, http://links.lww.com/CM9/A745].

To further assess the significance of SIX1 and EYA1 expression levels in CRC, we analyzed the clinical dataset of CRC patient samples from The Cancer Genome Atlas (TCGA; https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). We generated Kaplan–Meier survival curves and discovered that CRC patients with higher expression levels of SIX1 and EYA1 had a worse overall survival than those with lower expression levels of SIX1 and EYA1 [Supplementary Figure 3A and 3B, http://links.lww.com/CM9/A745].

**SIX1 interacted with EYA1 in both CRC cancerous tissues and cells**

Although previous publications have shown that SIX1 can interact with EYA1 in other cancer types,[14-16] and our above results also showed expression patterns which were similar to these, evidence is still lacking for their interaction in CRC cancerous tissues and cells. To determine the interaction of SIX1 and EYA1, we performed IP assays in both CRC cancerous tissues and HT29 cells. Accordingly, we mixed equal weights of three cancerous tissues from CRC patients (TNM stage 3) and then immunoprecipitated with IgG (negative control), anti-SIX1, and anti-EYA1, respectively. The results showed that both SIX1
and EYA1 could pull each other down [Figure 2A]. The same IP assays were also performed in HT29 cells and we also observed that both SIX1 and EYA1 could be pulled down by each other [Figure 2B]. In addition, we also performed co-IP assays to determine the direct interaction between SIX1 and EYA1 in HT29 cells co-transfected with pCDNA3-2 × Flag-SIX1+pCDNA3-6 × Myc-EYA1, pCDNA3-2 × Flag+pCDNA3-6 × Myc-EYA1, and pCDNA3-2 × Flag-SIX1+pCDNA3-6 × Myc. The co-IP assay results showed that SIX1 could directly interact with EYA1 in vitro [Figure 2C]. These results suggested that SIX1 could be assembled as a complex with EYA1 in vivo and in vitro.

To further determine if SIX1 and EYA1 co-localized in CRC cells, we performed an IMF assay using anti-SIX1 and anti-EYA1 specific antibodies in HT29 cells. The IMF results showed that SIX1 and EYA1 co-localized in the nucleus [Supplementary Figure 4A, http://links.lww.com/CM9/A745]. In addition, we also performed an IMF assay in tumor tissue from a CRC patient in TNM stage 3. Similarly, we also observed the co-localization of SIX1 and EYA1 in vivo [Supplementary Figure 4B, http://links.lww.com/CM9/A745]. These results suggested that SIX1 co-localized with EYA1 in vivo and in vitro.

Both CCNA1 and TGFβ1 were the direct targets of SIX1/EYA1 in CRC cells

Although previous publications have shown that both CCNA1 and TGFβ1 were the targets of SIX1 in other cancer types,10,13 direct evidence was still lacking for this conclusion in CRC cells. To verify if CCNA1 and TGFβ1 were also the target genes of the SIX1/EYA1 complex, we generated the Control-KD, two independent KD cell lines of SIX1 (#1 and #2) and EYA1 (#1 and #2), Control-OE, SIX1-OE, and EYA1-OE cell lines in both HT29 and HCA-24 backgrounds. Using these cells, we examined the mRNA and protein levels of SIX1, EYA1, CCNA1, and TGFβ1. The RT-qPCR results showed that KD or OE of SIX1 did not affect the expression of EYA1. However, KD and OE of SIX1 resulted in the downregulation or OE of both CCNA1 and TGFβ1, respectively [Figure 3A and Supplementary Figure 5A, http://links.lww.com/CM9/A745]. Similar expression patterns of EYA1, CCNA1, and TGF-β protein levels were also observed in SIX1-KD and SIX1-OE cells [Figure 3B and 3C, and Supplementary Figure 5B and 5C, http://links.lww.com/CM9/A745]. In EYA1-KD and EYA1-OE cells, we found that KD and OE of EYA1 could not change the mRNA and protein levels of SIX1. However, KD of EYA1 caused decreased mRNA and protein levels of both CCNA1 and TGF-β, and OE of EYA1 resulted in the reverse effect [Figure 3D–F and Supplementary Figure 5D–F, http://links.lww.com/CM9/A745]. Due to the same patterns of SIX1, EYA1, CCNA1, and TGF-β expression levels in both HT29 and HCA-24 cell backgrounds, we only performed experiments in HT29 cells in the following studies.

To further solidify the conclusion that both CCNA1 and TGFβ1 were the downstream targets of the SIX1/EYA1 complex, we analyzed 2000 bp-length promoters of both CCNA1 and TGFβ1 using the consensus sequence of MEF3 [Supplementary Figure 6A, http://links.lww.com/CM9/A745], and we only found one SIX1-binding site in each promoter [Supplementary Figure 6B, http://links.lww.com/CM9/A745]. We then constructed the WT and mutated promoters (deletion of MEF3 site) in the pGL3
Knockdown of either SIX1 or EYA1 significantly decreased the expression of CCNA1 and TGFβ1. (A) The mRNA levels of SIX1, EYA1, CCNA1, and TGFβ1 in SIX1-KD and SIX1-OE cells. (B) The protein levels of SIX1, EYA1, CCNA1, and TGFβ1 in SIX1-KD and SIX1-OE cells assessed by western blotting. (C) Quantified results of SIX1, EYA1, CCNA1, and TGFβ1 protein levels in SIX1-KD and SIX1-OE cells. (D) The mRNA levels of SIX1, EYA1, CCNA1, and TGFβ1 in EYA1-KD and EYA1-OE cells assessed by western blotting. (E) Quantified results of SIX1, EYA1, CCNA1, and TGFβ1 protein levels in EYA1-KD and EYA1-OE cells. (F) Quantified results of SIX1, EYA1, CCNA1, and TGFβ1 protein levels in EYA1-KD and EYA1-OE cells. **P < 0.01, compared with Control-KD; and †P < 0.05; Figure 4A). In addition, we also overexpressed CCNA1 and TGFβ1 in both SIX1-KD1 and EYA1-KD1 cells, respectively [Supplementary Figure 7A and 7B, http://links.lww.com/CM9/A745], and then examined cell proliferation using an MTT assay. The results showed that OE of CCNA1 and TGFβ1 in both SIX1-KD1 and EYA1-KD1 cells could partially reverse the growth defects that were caused by the KD of SIX1 and EYA1 (P < 0.05; Supplementary Figure 7C and 7D, http://links.lww.com/CM9/A745). Except for cell proliferation, we also observed that SIX1-KD and EYA1-KD cells had much lower colony numbers and invaded cell numbers compared to Control-KD cells (P < 0.01), while SIX1-OE and EYA1-OE cells had slightly increased colony numbers and invaded cell numbers in comparison to Control-OE cells (P < 0.05; Figure 4B–E). To evaluate the in vivo role of the SIX1/EYA1 complex, we injected nude mice with Control-KD, SIX1-KD, EYA1-KD, Control-OE, SIX1-OE, and EYA1-OE cells and monitored the formation of tumors. As presented in Figure 4F and Supplementary Figure 8, http://links.lww.com/CM9/A745, mice injected with either SIX1-KD or
EYA1-KD cells had much smaller tumor volumes than mice injected with Control-KD cells ($P < 0.01$). Mice injected with SIX1-OE or EYA1-OE cells had slightly increased tumor volumes in comparison to mice injected with Control-OE cells ($P < 0.01$; Figure 4F and Supplementary Figure 8, http://links.lww.com/CM9/A745). Taken together, the above results suggest that KD of the SIX1/EYA1 complex could inhibit CRC cell growth in vitro and repress tumor growth in vivo.

Two small molecules, NSC0191 and NSC0933, disrupted the SIX1–EYA1 interaction

The significant decrease of cell growth in CRC cells with SIX1 or EYA1 KD suggested that targeting the SIX1/EYA1 complex might be an effective strategy in the treatment of CRC. To screen small molecules that specifically disrupted the SIX1–EYA1 interaction, we firstly purified GST-SIX1 and His-EYA1 proteins and verified their direct interactions using in vitro pulldown assays [Figure 5A]. We then established an in vitro AlphaScreen assay using these two recombinant proteins by binding GST-SIX1 to glutathione donor beads and binding His-EYA1 to the nickel chelate acceptor beads, respectively [Figure 5B]. Using a series of protein concentrations of GST-SIX1 and His-EYA1, we determined the sensitivity and optimal protein concentrations that were required for the AlphaScreen binding reaction [Figure 5C]. Based on this result, we selected 120 nmol/L GST-SIX1 and 100 nmol/L His-EYA1 to perform high-throughput screening. After adding individual compounds ($n = 2000$) into each well containing an AlphaScreen reaction, we screened and obtained two compounds known as NSC0191 and NSC0933 [Figure 5D], which showed strong abilities to decrease the AlphaScreen signal in the first-round screening. We then used a series of concentrations of small molecules to inhibit the binding of SIX1–EYA1. The results showed that NSC0191 decreased protein-binding signals with an IC$_{50} = 12.60 \pm 1.15 \mu$mol/L [Figure 5E], while NSC0933 had an IC$_{50} = 83.43 \pm 7.24 \mu$mol/L [Figure 5F]. These results suggested that NSC0191 had a stronger ability than NSC0933 to disrupt the SIX1–EYA1 interaction. In addition, we also used two concentrations of NSC0191 (10 and 20 $\mu$mol/L) and NSC0933 (80 and 160 $\mu$mol/L) to inhibit the SIX1–EYA1 interaction. The results showed that 10 $\mu$mol/L NSC0191 and 80 $\mu$mol/L NSC0933 caused ~50% inhibition of SIX1–EYA1 interaction signals, while 20 $\mu$mol/L NSC0191 resulted in 80.15% inhibition ($P < 0.01$) and 160 $\mu$mol/L NSC0933 caused 70.33% inhibition ($P < 0.01$) of SIX1–EYA1 interaction signals [Figure 5G].

Treatments with NSC0191 and NSC0933 inhibited the SIX1–EYA1 interaction in CRC cells and repressed the expression of CCNA1 and TGF-$\beta$

We next aimed to evaluate the effects of NSC0191 and NSC0933 on the inhibition of SIX1–EYA1 interaction in CRC cells. For this purpose, we treated HT29 cells with two concentrations of NSC0191 (10 and 20 $\mu$mol/L) and NSC0933 (80 and 160 $\mu$mol/L), respectively. We then examined the protein levels of SIX1, EYA1, CCNA1, and TGF-$\beta$. The immunoblot results showed that small-molecule treatments could not change the protein levels.
and molecule-treated cells were immunoprecipitated with EYA1, respectively. The RT-qPCR results showed that both NSC0191 and NSC0933 treatments resulted in the dose-dependent decrease of both SIX1 and EYA1 when small molecule-treated cells were immunoprecipitated with SIX1 and EYA1, respectively [Figure 6D and 6E]. To verify the hypothesis, we next performed IP assays using both anti-SIX1 and anti-EYA1 antibodies in cells treated with or without small molecules. As shown in Figure 6C, the input levels of SIX1 and EYA1 were similar in all cells. However, the same levels of SIX1 or EYA1 in small molecule-treated cells could pull down much less EYA1 or SIX1 than untreated cells. Moreover, we also observed a dose-dependent decrease of both EYA1 and SIX1 when small molecule-treated cells were immunoprecipitated with SIX1 and EYA1, respectively [Figure 6D and 6E]. To verify that the disassociation of the SIX1/EYA1 complex failed to activate the expression of CCNA1 and TGFB1. To verify this hypothesis, we next performed IP assays using both anti-SIX1 and anti-EYA1 antibodies in cells treated with or without small molecules. As shown in Figure 6C, the input levels of SIX1 and EYA1 were similar in all cells. However, the same levels of SIX1 or EYA1 in small molecule-treated cells could pull down much less EYA1 or SIX1 than untreated cells. Moreover, we also observed a dose-dependent decrease of both EYA1 and SIX1 when small molecule-treated cells were immunoprecipitated with SIX1 and EYA1, respectively [Figure 6D and 6E]. To verify that the disassociation of the SIX1/EYA1 complex failed to activate the expression of CCNA1 and TGFB1, we performed ChIP assays using anti-SIX1 and anti-EYA1, respectively. The RT-qPCR results showed that these two small molecules only decreased the occupancy of EYA1 but not SIX1 on the promoter of CCNA1 and TGFB1 [Supplementary Figure 9A and 9B, http://links.lww.com/CMJ/97A745]. These results suggested that both NSC0191 and NSC0933 functioned effectively as inhibitors of SIX1–EYA1 interaction in CRC cells.

Based on the promising results of NSC0191 and NSC0933 in the inhibition of SIX1–EYA1 interaction, we next sought to determine their effect on oncogenic phenotypes. For this purpose, we treated HT29 with NSC0191 (10 and 20 μmol/L) and NSC0933 (80 and 160 μmol/L), respectively, and then determined cell proliferation, colony formation, and cell invasion. Cell proliferation assay results showed that NSC0191 dose-dependently inhibited cell growth. The lower doses of NSC0191 (10 μmol/L) and NSC0933 (80 μmol/L) resulted in a 45–50% reduction of cell proliferation at the 2- to 5-day time points (P < 0.01). Moreover, the higher doses of NSC0191 (20 μmol/L) and NSC0933 (160 μmol/L) resulted in a 72% to 80% reduction of cell proliferation at the 3- to 5-day time points (P < 0.001; Figure 7A). The colony formation assay results also showed that both NSC0191 and NSC0933 treatments caused a dose-dependent decrease in colony numbers [Figure 7B and 7C]. Moreover, a similar dose-dependent inhibition of invaded cells was also observed in cells treated with NSC0191 and NSC0933 [Figure 7D and 7E]. These results suggested that both NSC0191 and NSC0933 had strong cytotoxicities to inhibit CRC cell growth in vitro. To evaluate the in vivo effects of these two compounds, we injected nude mice with HT29 cells and then weekly injected different doses of NSC0191 (10 and 20 μmol/L) and NSC0933 (80 and 160 μmol/L) into mice to inhibit
The injections of small molecules caused a dose-dependent decrease of tumor volumes. The lower doses of NSC0191 (10 μmol/L) and NSC0933 (80 μmol/L) resulted in a 48% to 62% reduction of tumor volumes at the 30-day time point ($P < 0.001$). The higher doses of NSC0191 (20 μmol/L) and NSC0933 (160 μmol/L) resulted in a more than 80% reduction at the 30-day time point ($P < 0.001$; Figure 7F and Supplementary Figure 10, http://links.lww.com/CM9/A745).

In addition, we also measured the protein levels of SIX1, EYA1, CCNA1, and TGF-β in colon tissues from healthy control mice and tumors derived from mice injected with HT29+PBS, HT29+10 μmol/L NSC0191, HT29+20 μmol/L NSC0191, HT29+80 μmol/L NSC0933, and HT29+160 μmol/L NSC0933. The immunoblot and IHC results consistently showed that two small molecules could not change the protein levels of SIX1 and EYA1 [Supplementary Figures 11A, 11B, and 12, http://links.lww.com/CM9/A745]. However, both NSC0191 and NSC0933 treatments resulted in the dose-dependent decrease of CCNA1 and TGF-β in tumors [Supplementary Figures 11A, 11B, and 12, http://links.lww.com/CM9/A745]. Besides, we also performed IP assays using both

Figure 6: NSC0191 and NSC0933 specifically disrupted the SIX1–EYA1 interaction in vivo. (A and B) The effects of NSC0191 and NSC0933 treatments on SIX1, EYA1, CCNA1, and TGF-β. (A) Western blotting results. (B) Quantified protein levels; $^*P < 0.001$, compared with HCEC-1CT; $^{†}P < 0.05$, compared with PBS; $^{‡}P < 0.05$, 10 μmol/L NSC0191 vs. 20 μmol/L NSC0191; and $^{‡‡}P < 0.05$, 80 μmol/L NSC0933 vs. 160 μmol/L NSC0933. (C–E) The treatments of NSC0191 and NSC0933 caused a dose-dependent decrease in the SIX1–EYA1 interaction. Cells were subjected to IP assays using anti-SIX1, anti-EYA1, and IgG, respectively. The input (C), SIX1-immunoprecipitated (D), and EYA1-immunoprecipitated (E) proteins were subjected to western blotting to examine the protein levels of SIX1 and EYA1. CCNA1: Cyclin A1; EYA1: Eyes absent homolog 1; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IgG: Immunoglobulin G; IP: Immunoprecipitation; PBS: Phosphate buffered saline; SIX1: Sineoculis homeobox homolog 1; TGF-β: Transforming growth factor-beta.
anti-SIX1 and anti-EYA1 antibodies in tumor tissues. Our results showed that the same levels of SIX1 or EYA1 in tumors derived from mice injected with small molecules could pull down much less EYA1 or SIX1 than the control tumors [Supplementary Figure 11C–E, http://links.lww.com/CM9/A745]. These results suggested that both NSC0191 and NSC0933 blocked the SIX1–EYA1 interaction in vivo.

Discussion
Chemotherapy is a major therapeutic strategy for the treatment of CRC, especially for patients with metastasis.\(^6\) Chemoresistance is a major barrier to the favorable outcome of CRC patients.\(^6\) The other disadvantage of traditional chemotherapeutic medicines is the serious side effect on healthy cells and tissues.\(^6\) Thus, developing new medicines that target key molecules involved in CRC metastasis may provide options for CRC treatment. The SIX1/EYA transcriptional complexes are important regulators of tumor progression and metastasis.\(^14\) Importantly, the lower or even absent expression levels of SIX1 and EYA1 in non-cancerous cells and tissues suggest that they are ideal targets, because their inhibition may have limited side effects.\(^17\) In the present study, we identified the OE of the SIX1/EYA1 complex in CRC patients and CRC cells. They can transactivate two important downstream targets, CCNA1 and TGFB1, contributing to tumor progression and metastasis. Thus, targeting the conserved interaction between SIX1 and its activator EYA1 may prevent tumor growth. Based on this notion, we screened and obtained two compounds (NSC0191 and NSC0933) which showed effective abilities to disrupt the SIX1–EYA1 interaction in vitro and in vivo. The disassociation of the SIX1/EYA1 complex failed to transactivate CCNA1 and TGFB1 and caused the inhibition of tumor growth [Figure 8].

In recent years, the importance of the SIX1/EYA complexes in regulating genes involved in tumorigenesis, progression, and metastasis has attracted more attention, with the aim being to develop them as potential targets to screen compounds.\(^14,17\) However, it is still unclear if these complexes are all involved in CRC tumorigenesis, progression, and metastasis. In the current study, we examined all six members of the SIX family and four EYA members in CRC cancerous tissues and we only found increased levels of SIX1, EYA1, EYA3, and EYA4. We only focused our current study on revealing the role of the SIX1/EYA1 complex but not SIX1/EYA3 or SIX1/EYA4. Thus, we cannot exclude that the other two complexes may also contribute to tumorigenesis, progression, and metastasis. To distinguish the roles of the SIX1/EYA complexes, in the future we will perform RNA sequencing (RNA-seq) analyses in EYA1-KD, EYA3-KD, and EYA4-KD cells to obtain aberrantly expressed genes. In addition, we did not
screen the downstream targets of the SIX1/EYA1 complex in the present study because our focus was to screen small molecules to disrupt the SIX1–EYA1 interaction. Thus, CCNA1 and TGFB1 may not be the only two targets of SIX1/EYA1 in the progression and metastasis of CRC. Combining the ongoing RNA-seq results and previously published downstream targets in other cancer types, we may find more downstream targets of the SIX1/EYA1 transcriptional complex in the future and we will evaluate the effects of NSC0191 and NSC0933 on the expression of these targets, which will help us gain a deeper understanding of the molecular changes with these two compound treatments.

The promising effects of both NSC0191 and NSC0933 on inhibiting the SIX1–EYA1 interaction and reversing SIX1/EYA1-mediated cellular phenotypes and tumor growth in a mouse xenograft model consistently support that these two compounds function as specific inhibitors of the SIX1–EYA1 interaction. An important issue for future studies is to investigate the direct binding sites of these two compounds by resolving the SIX1–EYA1 complex structure. In addition, more efforts, such as chemical structure alterations and modifications, are required to improve the inhibitory efficiencies and solubilities of NSC0191 and NSC0933. During our preparation for this paper, Zhou et al. found that a SIX1/EYA2 inhibitor, NCGC00378430, could partially reverse transcriptional and metabolic profiles mediated by SIX1 OE, inhibiting TGF-β signaling and EMT. Comparing the chemical structures of NCGC00378430 with NSC0191 and NSC0933, we did not find any commonalities; this absence of commonalities suggests they may have different binding sites in the SIX1/EYA complexes. If it is possible to synthesize or obtain NCGC00378430 from the original authors, we will compare the inhibitory effects of these three compounds and their different effects on transcriptional and metabolic profiles. Importantly, the conserved regulatory mechanism of SIX1/EYA1 implies that NSC0191 and NSC0933 may also function effectively in the inhibition of cell growth in other SIX1/EYA1 over-expressed cancer cells.

To conclude, we found the OE of the SIX1/EYA1 complex in CRC cells and tissues. This complex can transactivate the expression of CCNA1 and TGFB1, affecting CRC cell-cycle progression and tumor metastasis. Using the SIX1–EYA1 interaction as a target in an AlphaScreen assay, we obtained two compounds, NSC0191 and NSC0933, which can significantly reverse SIX1-mediated transactivation and prevent CRC cell growth in vitro and tumor growth in vivo.

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Conflicts of interest

None.

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