The EDN1/EDNRA/β-arrestin axis promotes colorectal cancer progression by regulating STAT3 phosphorylation

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Abstract. Endothelin receptor A (EDNRA) has been reported to play various crucial physiological roles and has been shown to be associated with the pathology of several diseases, including colorectal cancer (CRC). However, the molecular mechanisms of EDNRA in the development of human CRC have not been fully elucidated to date. In this context, the present study was performed to investigate biological functions and novel downstream signaling pathways affected by EDNRA, during CRC progression. First, using public data repositories, it was observed that the EDRNA expression levels were markedly increased in CRC tissues, as compared to normal tissues. Patients with CRC with an increased EDNRA expression exhibited a significantly decreased survival rate in comparison with those with a lower EDNRA expression. Furthermore, a positive correlation between the levels of EDNRA and its ligand, EDN1, was found in CRC tissues. The ectopic expression of EDNRA or its ligand, EDN1, promoted, whereas the silencing of EDNRA or EDN1 decreased cell proliferation and migration *in vitro*. To elucidate the signaling pathways involved in the regulation of EDNRA expression in CRC cells, a phosphokinase array analysis was performed, and it was observed that the knockdown of EDNRA substantially suppressed the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in CRC cells. Of note, STAT3 silencing simultaneously decreased EDN1 and EDNRA expression, with the expression of EDN1 and/or EDNRA appearing to be directly regulated by binding STAT3 to their promoter region, according to chromatin immunoprecipitation and promoter assays, ultimately indicating a positive feedback loop in the expression of EDNRA and EDN1. It was also observed that treatment with an EDNRA antagonist (macitentan), alone or in combination with cisplatin, suppressed cell growth and migration ability, and induced cell apoptosis. Collectively, these data suggest a critical role of the EDN1/EDNRA signaling pathway in CRC progression. Thus, the pharmacological intervention of this signaling pathway may prove to be a potential therapeutic approach for patients with CRC.

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Abbreviations: EDNRA, endothelin receptor A; EDN1, endothelin-1; STAT3, signal transducer and activator of transcription 3; CRC, colorectal cancer; GPCR, G protein-coupled receptor

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Introduction

Colorectal cancer (CRC) is one of the most prevalent malignancies and has the third highest mortality rate worldwide. According to a previous report, >1,880,725 new cases of CRC and 915,880 deaths occurred in 2020 (1). The 5-year relative survival rate for patients with CRC ranges from 90 to 14% for those diagnosed with localized to advanced-stage disease (2). Depending on the cancer stage and tumor location, treatments, including surgery, chemotherapy and radiotherapy can be used in combination (3). However, the complete removal of total CRC cells is often not possible. Over 60% of patients with stage II and III CRC usually undergo adjuvant chemotherapy and/or radiotherapy; however, these treatments have several side-effects due to the low cancer-specific selectivity of chemotherapeutic agents and cytotoxicity. Moreover, a number of patients with CRC relapse even after neoadjuvant therapy (4,5). Over the past decade, in order to overcome low specificity and severe side-effects of classical treatments, molecular targeted therapies have been developed for personalized medicine (6-8). Small molecules and monoclonal antibodies are the major drugs administered as targeted therapies, including vemurafenib, cetuximab, trastuzumab and bevacizumab, and are able to suppress tumor growth, migration and angiogenesis by inhibiting their specific target molecules (9-12). Nevertheless, there are still therapeutic

limitations, including long-term toxicity, target gene mutation, drug resistance and metastasis, as cancer cells may evolve through genetic and epigenetic clonal diversity (13-16). Thus, the identification of novel therapeutic targets and the development of novel approaches to refine or replace existing CRC chemotherapy are urgently required.

Endothelin (EDN) was first isolated from cultured porcine aortic endothelial cells, and was identified as the most potent vasoconstrictor of coronary artery strips (17). In addition, three different EDN peptides, EDN1, EDN2 and EDN3, have been identified in humans and other mammalian species (18). Each EDN peptide is derived from a large precursor of ~200 amino acids that is first processed by furin endopeptidase, producing an intermediate product of 38 amino acids. Finally, EDNs are cleaved by endothelin-converting enzymes to generate the active form of the EDN peptide (19). The biological actions of EDN in mammals have been reported to be mediated by EDN receptors (EDNRs), namely EDNRA and EDNRB. EDNRs belong to the G protein-coupled receptor (GPCR) family (19). Compared to EDN3, EDN1 and EDN2 bind to EDNRA with >100-fold higher affinity. By contrast, EDN1, EDN2 and EDN3 bind to EDNRB with equal affinities (20). Specifically, EDNRA mediates cellular processes by modulating various signaling targets in the MAPK/ERK and cAMP/protein-kinase A pathways by interacting with both Gq and Gs/Gi proteins (21-23). EDN1 and EDNRA are both widely expressed in vascular or non-vascular tissues and are involved in the regulation of various physiological processes, including cardiovascular development, blood pressure regulation, proliferation and migration (24,25). However, the abnormal activation of EDNRA has been shown to be associated with several diseases, including pulmonary arterial hypertension, heart failure and growth retardation. In addition, EDNRA is overexpressed in several types of cancer, including prostate, colon, breast and cervical cancer (21,26,27). In certain studies, increased EDNRA levels stimulated by hypoxia or microRNAs (miRNAs/miRs) have been shown to lead to the promotion of cell proliferation, migration, invasion, metastasis and anticancer drug resistance through G-proteins or β-arrestin downstream signaling pathways (28,29). However, the detailed regulatory mechanisms of EDN1 and EDNRA expression have not yet been completely elucidated and thus warrant further investigation.

In the present study, it was demonstrated that EDN1 and EDNRA promote cell proliferation and migration, and suppress apoptosis in CRC. The activation of oncogenic signal transducer and activator of transcription 3 (STAT3) signaling appeared to be crucial for the concurrent induction of EDN1 and EDNRA expression. Thus, the findings of the present study elucidate the novel, to the best of our knowledge, regulatory mechanism of EDN1/EDNRA expression through the interaction between EDNRA and the STAT3 signaling pathway, leading to the promotion of cell growth and the suppression of apoptosis by the positive feedback loop of STAT3-EDN1-EDNRA in CRC. Therefore, the present study indicates that the EDN1/EDNRA axis may be an emerging therapeutic target for CRC.

Materials and methods

Cell culture and chemical reagents. The human CRC cell lines, HCT116, SW480, SW620 and LS174T, were purchased from the Korean Cell Line Bank (KCLB nos. #10247, #10228, #10227 and #10188, respectively), and cultured in RPMI-1640 medium, supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator. HT29 cells (KCLB no. #30038), which originate from AN adenocarcinoma of the rectosigmoid part of the intestine (30), were cultured in RPMI-1640 medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). KM12SM cells (KCLB no. #80016) were cultured in DMEM supplemented with 10% FBS. For STAT3 inhibitor treatment, the cells were treated with stattic (5 μM; Selleck Chemicals LLC). For EDNRA inhibitor treatment, the CRC cells were treated with macitentan (2 or 10 μM; Merck KGaA).

Plasmid construction and transfection. The coding region of the gene of interest, EDN1 and EDNRA, was subcloned into the pIRES-EGFP vector (Takara Bio, Inc.). In summary, EDN1 and EDNRA gene CDS were amplified by PCR using specific primers (Table SI), and then inserted into the pIRES-EGFP expression vector. To transfect the EDN1 and EDNRA plasmids into CRC cell lines, $1x10^6$ cells were plated on a 60 mm dish. After 24 h, 5 μg plasmid DNA were transfected into the cells using Lipofectamine 2000® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Following 24 h of incubation in a humidified atmosphere of 5% CO_2 and at 37˚C, subsequent experiments were carried out.

siRNA transfection. For knockdown experiments, the sequences of siRNAs (Bioneer Corporation) were used as follows: siRNA for negative control (siNC) sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; siRNA for EDN1 (siEDN1) sense, 5'-CUCGUAGAAGUCUGGUCUA-3' and antisense, 5'-UAGACCAGACUUCUACGAG-3'; siRNA for EDNRA (siEDNRA) sense, 5'-GCACUGGUUGGAUGUGUAA-3' and antisense, 5'-UUACACAUCCAACCAGUGC-3'; siRNA for STAT3 (siSTAT3) sense, 5'-CACAUGCCACUUUGGUGUUUCAUAA-3' and antisense, 5'-UUAUGAAACACCAAAGUGGCAUGUG-3'; siRNA for β-arrestin2 (siβ-arr2) sense, 5'-AAGGACCGCAAAGUGUUUGUG-3' and antisense, 5'-CACAAACACUUUGCGGUCCUU-3'. To transfect the siRNAs, $1x10^6$ CRC cells were plated on a 60-mm dish. After 24 h, the siRNAs were transfected into the cells using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The transfected cells were incubated in a humidified atmosphere of 5% CO_2 and at 37˚C for 48 h, and then subsequent experiments were carried out.

Assays of cell growth and migration. CRC cells were seeded into 96-well plates, and cell viability was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Inc.) reagent after 6, 24, 48 or 72 h. For the Transwell migration assay, $3x10^5$ cells were seeded in the upper chamber (8 μm; Corning Life Sciences) without FBS medium, and the bottom chambers were filled with 500 μl medium containing FBS. The migrated cells were fixed with 4% formaldehyde and 100% methanol at room temperature, and after 24 h, stained with 0.1% crystal violet (cat. no. V5256, MilliporeSigma) at room temperature, and counted.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For total RNA extraction, the NucleoZol reagent (Macherey-Nagel GmbH) was used following the manufacturer's instructions. cDNA was synthesized using the PrimeScript RT reagent kit (cat. no. RR037A, Takara Bio, Inc.). EDN1, EDNRA and GAPDH expression was quantified by SYBR-Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) using gene-specific oligonucleotide primers (Table SI) on a StepOne Plus Real-Time System (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed at 95˚C for an initial 3 min followed by 40 cycles of 10 sec at 95˚C and 1 min at 59˚C. The expression levels of *GAPDH* were used to normalize the expression levels of *EDN1* and *EDNRA*. All reactions were analyzed by the comparative $2^{-\Delta\Delta Cq}$ method (31).

Western blot analysis. The cells were harvested and lysed using RIPA lysis buffer (Thermo Fisher Scientific, Inc.) with protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). The cell lysates were centrifuged at 12,000 x g at 4˚C for 15 min, and a Pierce BCA Protein Assay kit (cat. no. 23227, Thermo Fisher Scientific, Inc.) was used for the quantitation of protein lysates. Subsequently, the lysates were boiled for 10 min. For electrophoresis, 20 μg protein samples were separated on a 10% polyacrylamide SDS-PAGE gel and transferred to PVDF membranes (MilliporeSigma). For blocking, the membranes were incubated with 3% BSA (cat. no. A7906, MilliporeSigma) in PBS containing 0.1% Tween-20 (cat. no. P1379, MilliporeSigma) at room temperature for 1 h with agitation. The membranes were incubated with the primary antibodies [anti-EDNRA (1:1,000; cat. no. ab85163, Abcam), anti-EDN1 (1:1,000; cat. no. ab2786, Abcam), anti-phosphorylated (p-) STAT3 (1:1,000; cat. no. 9145, Cell Signaling Technology, Inc.), anti-STAT3 (1:1,000; cat. no. 12640, Cell Signaling Technology, Inc.), anti-β-actin (1:2,000; cat. no. AbC-2004, AbClon, Inc.), anti-caspase-3 (1:1,000; cat. no. 9662, Cell Signaling Technology, Inc.), anti-phosphorylated (p-)p70S6K (1:1,000; cat. no. 9204, Cell Signaling Technology, Inc.) and anti-p70S6K (1:1,000; cat. no. 9202, Cell Signaling Technology, Inc.)] at 4˚C overnight. The membranes were washed with PBST and then incubated with a specific secondary antibody, which was an anti-mouse IgG HRP-linked antibody (1:10,000; cat. no. 7076, Cell Signaling Technology, Inc.) or anti-rabbit IgG HRP-linked antibody (1:10,000; cat. no. 7074, Cell Signaling Technology, Inc.) at room temperature for 1 h. After washing with PBST, the Proteins were detected by chemiluminescence using an ECL Prime Western Blotting System (cat. no. RPN2232, Cytiva). The band densities were determined using ImageJ software (version 1.53k; National Institutes of Health).

Phosphokinase array. For the phosphokinase assay, the Proteome Profiler Human Phospho-Kinase Array kit (cat. no. ARY003B, R&D Systems, Inc.) was used. Briefly, the HCT116 cells were transfected with negative control siRNA (siNC) or siRNA for EDNRA (siEDNRA). The cells were lysed with lysis buffer 6 (R&D Systems, Inc.) and incubated for 30 min on ice, after which the protein lysates were centrifuged at 14,000 x g for 5 min at 4˚C. The BCA assay (cat. no. 23227, Thermo Fisher Scientific, Inc.) was used for the quantitation of protein lysates. The membranes were incubated with 2 ml of diluted cell lysates on a rocking platform shaker at 4˚C overnight and then incubated with detection antibodies (DAC-A or DAC-B, provided with the kit by R&D Systems, Inc.). Proteins were detected by chemiluminescence using an ECL Prime Western Blotting System (Cytiva, Inc.). Spot densities of the phosphokinase array were determined by ImageJ software.

Cellular apoptosis assay. For flow cytometric analysis, the HCT116 cells were seeded and transfected with siRNA or chemotherapeutic agents, including cisplatin and macitentan, for 48 or 72 h. The cells were harvested and suspended at $1x10^5$ in 100 μl of PBS with binding buffer (BD Biosciences). Subsequently, the cells were stained with 5 μl of Annexin V conjugated to fluorescein isothiocyanate (FITC) and 5 μl of propidium iodide (PI; BD Biosciences) at room temperature, for 15 min. Apoptosis analysis was performed using a FACSVerse flow cytometer (BD Bioscience). For another cellular apoptosis assay, HCT116 cells were seeded at $1x10^5$ in white 96-well plates. After 24 h, HCT116 cells were treated with 100 nM ET-1 and 2 μM or 10 μM macitentan for 6 h at 37˚C, and the cells were then equilibrated to room temperature for 30 min, and 100 μl of Caspase-Glo 3/7 reagent (cat no. G8090, Promega Corporation) was added to each well and gently mixed by hand tapping. The plate was measured using a Centro XS3 Luminescence Microplate Reader (Berthold Technologies GmbH & Co.KG).

Chromatin immunoprecipitation (ChIP) assay. The STAT3-binding motif in the promoter region of EDNRA was identified by JASPAR (http://jaspar.genereg.net/). The sequence of the promoter region was confirmed in the Eukaryotic Promoter Database (EPD) database (https://epd.epfl.ch/). ChIP was performed using Dynabeads Protein A and G (Thermo Fisher Scientific, Inc.). A total of $1x10^7$ cells were cross-linked with 1% formaldehyde for 10 min at 25˚C with continued agitation, and the crosslinking reaction was stopped by adding glycine to a final concentration of 0.125 M for 5 min at 25˚C with continued agitation. The cells were then resuspended and lysed for 10 min at 4˚C with ChIP lysis buffer. The lysate was sonicated using an ultrasonicator (Sonics & Materials, Inc.). Fragment sizes were sized approximately at 250-750 bp. The samples were diluted with ChIP lysis buffer and pre-cleared with 50 μl of Dynabeads Protein A and G for 1 h at 4˚C. Primary antibodies were added to the precleared supernatants, and the mixtures were incubated overnight at 4˚C. The antibodies used for the ChIP assay included anti-STAT3 (dilution 1:100; rabbit polyclonal; cat. no. 9139S; Cell Signaling) and IgG (dilution 1:1,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.). Subsequently, 50 μl of Dynabeads Protein A and G were added to the samples, and the mixtures were incubated for 2 h at 4˚C. The beads were subsequently washed with wash buffers (low-salt RIPA, high-salt RIPA, LiCl, and TE), and the precipitated chromatin was eluted in 100 μl elution buffer with 0.1 M $NaHCO_3$ (MilliporeSigma) and 1% SDS (MilliporeSigma) for ≥15 min at 65˚C. The chromatin was then treated with RNase A for 1 h at 37˚C, and proteinase K was added to each sample for 1 h at 65˚C. Reverse cross-linking was performed overnight at 65˚C, and DNA was purified using a QIAquick PCR Purification kit (cat. no. 28104, Qiagen, Inc.). ChIP-PCR assays were

performed using PCR Master Mix (cat. no. 4309155, Thermo Fisher Scientific, Inc.) and 1% agarose gel (cat. no. 50004, Lonza Group, Ltd.) electrophoresis.

Plasmid construction for promoter assay. The EDN1 and EDNRA promoter regions (from -1,000 to +100 bp) were amplified using the primers listed in Table SI. Subsequently, the PCR products were purified with a QIAquick PCR Purification kit (Qiagen, Inc.). To construct the vector, the promoterless pGL4 luciferase vector (Promega Corporation) was modified. Briefly, the SV40 promoter and PCR product were ligated with the pGL4.10 basic vector, and the sequences were then confirmed by Sanger sequencing using RVprimer3 (Promega Corporation).

Promoter assay. HCT116 cells were co-transfected with the pGL4.10-EDN1 promoter region or PGL4.10-EDNRA promoter region, and pRL-TK vectors with 50 nM siNC or siRNA for STAT3 (siSTAT3) using Lipofectamine 2000® (Thermo Fisher Scientific, Inc.). After 24 h, a luciferase assay was performed using the Dual-Glo Luciferase Assay System (Promega Corporation) according to the manufacturer's instructions. Firefly and *Renilla* luciferase activities were measured using the Centro XS3 Luminescence Microplate Reader (Berthold Technologies GmbH & Co.KG).

Tissue microarray. For the tissue microarray, paraffin-embedded slide glasses, including 29 paired colon tumor/adjacent normal samples, were purchased from Biochain. For hematoxylin and eosin (H&E) staining, the tissue sections were deparaffinized, and stained with hematoxylin (cat. no. S3309, Dako; Agilent Technologies, Inc.) for 1 min at room temperature and eosin (cat. no. CS701, Dako; Agilent Technologies, Inc.) for 3 min at room temperature. The slices were dehydrated, and the tissues were then sealed with mounting solution. Briefly, for the immunohistochemical staining of EDN1 and EDNRA, tissue sections were deparaffinized, incubated with 3% H_2O_2 at room temperature for 10 min, and washed with PBS three times. The tissues were incubated with 5% normal goat serum at room temperature for 1 h with anti-EDNRA (1:200; cat. no. ab117521, Abcam) or anti-EDN1 (1:200; cat. no. ab2786, Abcam) at 4˚C for 12 h. ImmPRESS® HRP anti-rabbit IgG polymer kit (cat. no. MP-7451, Vector Laboratories, Inc.) or ImmPRESS® goat anti-mouse IgG polymer kit (cat. no. MP-7452, Vector Laboratories, Inc.) was added as a secondary antibody for 1 h, followed by rinsing. Then, 3,3'-diaminobenzidine, a DAB substrate (cat. no. SK-4105, Vector Laboratories, Inc.), was added for 2 min at room temperature to develop color.

Analysis of human colon cancer samples. The Gene Expression database of Normal and Tumor tissues 2 (GENT2) (http://gent2.appex.kr/gent2/) was analyzed for *EDN1*, *EDN2*, *EDN3*, *EDNRA* and *EDNRB* expression in various cancer types including breast, colorectal, and pancreatic cancer. The overall survival data of EDNRA and EDNRB also was analyzed from GENT2 database with Kaplan-Meier plots by median cut-off. Publicly available data from The Cancer Genome Atlas (TCGA) database were analyzed in the present study. Clinical information and mRNA expression data of TCGA samples were downloaded from UCSC Xena (http://xena.ucsc.edu). Patient clinical data of colon cancer were obtained from the TCGA database. Among the colon adenocarcinoma (COAD) TCGA cohort, 471 tumor and 41 normal colon samples were analyzed in the present study. The reads per kilobase of exon per million reads mapped value was used to represent gene expression levels.

Statistical analysis. All statistical data were conducted using GraphPad Prism, version 9.0, software package (GraphPad Software). All data are presented as the mean ± standard deviation, unless otherwise indicated. Depending on the sample size, the Kolmogorov-Smirnov test (when n>50) or Shapiro-Wilk test (when n<50) was performed for the normality test. The unpaired Student's t-test or Mann-Whitney test was used to perform the analysis of differences between two groups. For comparison of multiple groups, one-way ANOVA was performed along with post hoc multiple comparison tests, including SNK, Dunnett's and Tukey's tests. For correlation analysis of each gene, the Spearman's correlation analysis was used. The overall survival from the GENT2 database was analyzed using the log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

EDNRA expression is upregulated in CRC and positively correlates with EDN1 levels. Previous research has demonstrated that EDNRA expression is upregulated in several cancer types, including bladder and gastric cancer (18). Since the EDNRA expression level has yet not been clearly determined in CRC, in the present study, its expression level was first analyzed using the GENT2 dataset (http://gent2.appex.kr/gent2/) based on microarray data collected from 36 different cancer types, including breast, skin and colon cancers. It was observed that EDNRA expression was markedly upregulated in several cancer types, including brain, stomach, thyroid, kidney, bone and pancreatic cancers, as well as CRC (Fig. 1A and Table SII). Similarly, the analysis of an RNA sequencing dataset from the TCGA-COAD cohort also displayed a high expression level of EDNRA in CRC samples (Fig. 1B). By contrast, the expression of the second EDN receptor, EDNRB, was downregulated in CRC tumors compared with normal tissues (Fig. S1A and B). Furthermore, it was determined that a high expression of EDNRA in patients with CRC was associated with a poor patient survival (Fig. 1C), whereas no significant difference in overall survival was observed between patients with increased and decreased EDNRB expression (Fig. S1C).

Subsequently, the *EDNRA* ligands, *EDN1*, *EDN2* and *EDN3*, from the GENT2 and TCGA datasets were analyzed. Although the expression of all three ligands was downregulated in CRC tissues compared to normal tissues (Fig. S2A and B), positive correlations were determined between the *EDN1* and *EDNRA* or *EDNRB* levels (Figs. 1D and S1D). To further examine the protein expression of EDN1 and EDNRA, a tissue microarray analysis was performed, and the increased expression of EDNRA and EDN1 was detected in the tumor tissues compared to the normal tissues (Fig. 1E). Therefore, these results indicated that the interaction between EDNRA and its ligand, EDN1, may be suggestive of the etiology of CRC.

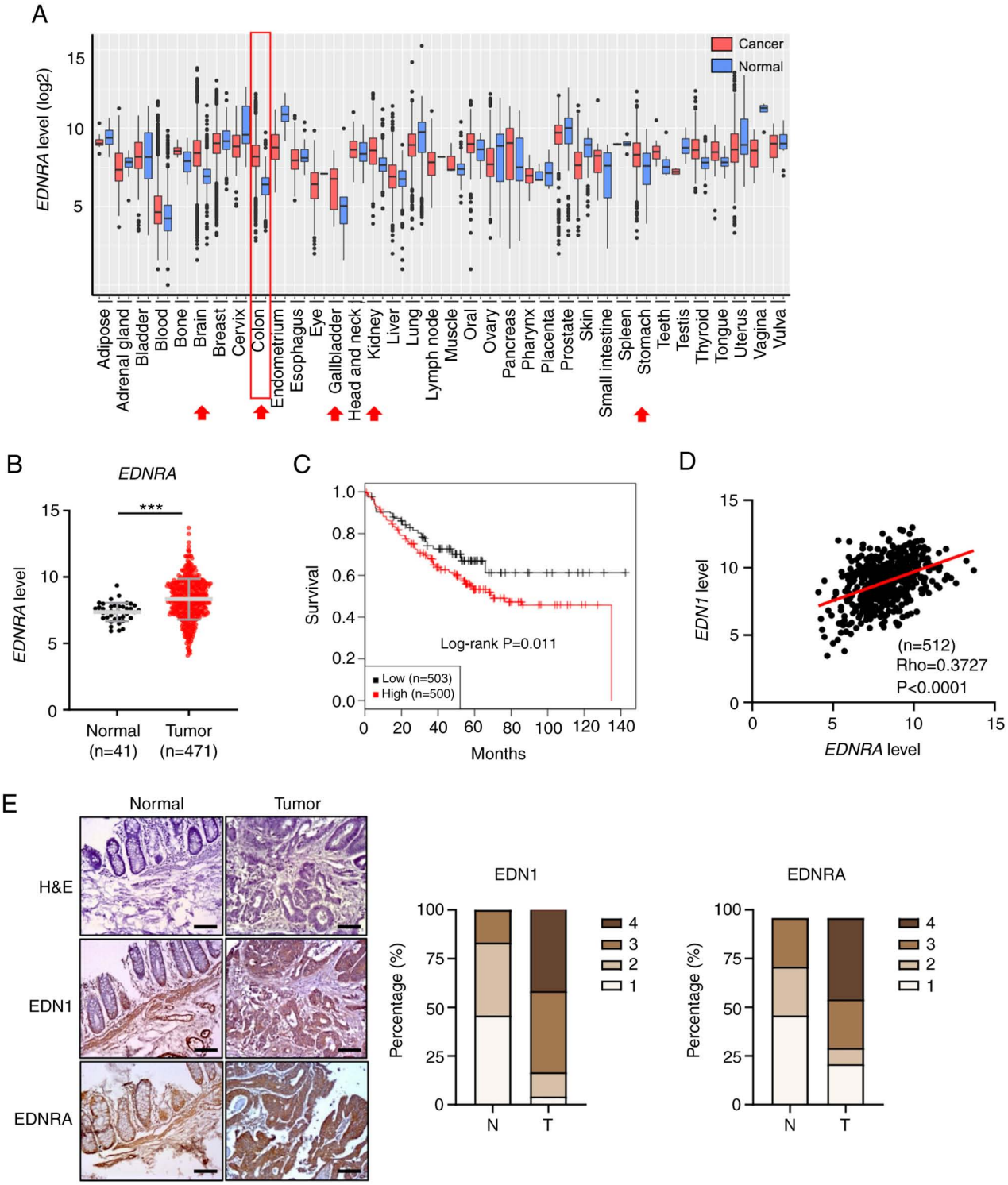

Figure 1. *EDNRA* expression is strongly upregulated in CRC and is positively correlated with *EDN1* levels in the TCGA CRC cohort. (A) *EDNRA* expression in human normal and tumor tissues of the GENT2 database. (B) *EDNRA* expression in human normal (n=41) and tumor tissues (n=471) of the COAD TCGA data set. (C) Kaplan-Meier survival curves of CRC patients according to the *EDNRA* expression level. (D) Correlation analysis between *EDN1* and *EDNRA* levels in the TCGA data set from 41 normal and 471 tumor samples. (E) Representative H&E staining and immunohistochemical staining for *EDN1* and *EDNRA* expression in a tissue microarray of matched pairs of CRC and normal tissues (scale bar, 100 μm). ***P<0.001. *EDNRA*, endothelin receptor A gene; *EDN1*, endothelin-1 gene; CRC, colorectal cancer; TCGA, The Cancer Genome Atlas; COAD, colon adenocarcinoma.

EDN1/EDNRA regulates the proliferative and migratory ability of CRC cells. Based on the results of public data analysis, several functional assays were conducted, in order to elucidate the effects of EDNRA and EDN1 expression in CRC cells. Firstly, the endogenous EDNRA mRNA expression levels were confirmed in six CRC cell lines. Among these, the SW480 and LS174T cells demonstrated the lowest EDNRA expression levels, whereas the highest EDNRA expression levels were observed in the HT29 and HCT116 cells (Fig. S3A). The KM12SM and SW620 cells demonstrated

moderate EDNRA expression levels. The transfection of the EDNRA overexpression vector into SW480 KM12SM cells with a low EDNRA expression increased the mRNA and protein levels of EDNRA (Figs. 2A and S3B). Compared with the control vector, the EDNRA overexpression vector significantly promoted the growth and migratory ability of the SW480 and KM12SM cells (Figs. 2B and C, and S3C and D). The knockdown of EDNRA using siRNA targeting EDNRA (siEDNRA) decreased the EDNRA mRNA and protein levels in HCT116 cells (Fig. 2D). Transfection with siEDNRA significantly reduced the growth of HCT116 cells (Fig. 2E). In addition, the silencing of EDNRA in HCT116 cells resulted in a significantly lower number of migrated cells than that in the negative control cells (Fig. 2F).

Furthermore, in order to ascertain the cellular functions of EDN1, firstly we analyzed the mRNA expression level of EDN1 in six different CRC cell lines (Fig. S4A). The EDN1-overexpression vector was transfected into HCT116 cells (Fig. 2G), and cell proliferation and migration assays were performed. Subsequently, the growth of HCT116 cells significantly increased (Fig. 2H). In addition, a significantly increased number of migrated cells was observed in the EDN1 overexpression group (Fig. 2I). By contrast, the knockdown of EDN1 (Figs. 2J and S4B) significantly inhibited the proliferation and motility of HT29 and SW620 cells (Figs. 2K and L, and S4C and D). Thus, these findings demonstrated that EDN1 and EDNRA may play a crucial role in CRC progression.

Knockdown of EDN1 and EDNRA induces the apoptosis of CRC cells. In order to demonstrate the mechanisms through which EDNRA and EDN1 regulate the growth of CRC cells, flow cytometric analysis was performed, following the transfection of HCT116 cells with siRNA. Annexin V and PI double staining was used to detect apoptotic cells, and the ratio of early and late apoptotic cells was quantified. As depicted in Fig. 3A, the apoptotic cell numbers were increased in the siEDNRA- and siEDN1-transfected cells, as compared to the siNC-transfected cells. Similarly, the caspase-3/7 assay revealed that the knockdown of EDNRA or EDN1 significantly increased caspase-3/7 activity in the HCT116 cells (Fig. 3B). In addition, the transfection of the HCT116 cells with siEDNRA or siEDN1 increased the cleaved caspase-3 form (Fig. 3C). Overall, the inactivation of the EDN1/EDNRA axis suppressed cell growth by inducing cellular apoptosis.

EDNRA modulates the STAT3 signaling pathway in CRC cells. In order to further elucidate the molecular mechanisms underlying EDNRA-induced oncogenic functions, a phosphokinase array assay in HCT116 cells transfected with siNC or siEDNRA was performed. Of note, decreased levels of p-STAT3 (Y705) and p-p70 S6 kinase (T389) in siEDNRA-transfected cells, as compared to siNC-transfected cells were observed (Fig. 4A). To verify the array data, western blot analysis was conducted, using HCT116 cells transfected with siNC or siEDNRA. No significant difference in the phosphorylation of p70S6K between the siNC and siEDNRA groups (Fig. S5A) was observed; however, the p-STAT3 (Y705) level was significantly reduced in siEDNRA-transfected HCT116 cells, while the p-STAT3 levels were highly increased in the EDNRA-overexpressing HCT116 cells (Fig. 4B).

Subsequently, a knockdown experiment of the downstream activator, β-arrestin, was performed in the HCT116 cells to determine the mechanisms through which the p-STAT3 level is regulated by the EDN1/EDNRA axis. For the analysis of these, the EDRNA, p-STAT3 and STAT3 protein levels were evaluated using western blot analysis. The EDNRA protein level was significantly decreased in the siβ-arrestin2 (siβ-arr2)- or siSTAT3-transfected cells (Fig. 4C), suggesting that EDN1-EDNRA-β-arrestin-STAT3 signal transduction may present a positive feedback loop. In addition, the supporting evidence of the positive feedback loop from the COAD TCGA dataset resulted in a significant positive correlation of transcriptional levels between genes EDN1, EDNRA, ARRB1, ARRB2 and STAT3 (Fig. S5B and C).

To confirm that EDN1 and EDNRA are regulated by STAT3, the HCT116 cells were treated with the STAT3 inhibitor, static (32), which dephosphorylates Y705 and S727 of STAT3. Notably, the protein levels of both EDN1 and EDNRA were significantly decreased by the STAT3 inhibitor (Fig. 4D), indicating that EDN1 and EDNRA expression can be modulated by regulating STAT3 phosphorylation.

Induction of EDN1 and EDNRA expression via the STAT3 pathway. The suppression of STAT3 expression using siRNA significantly and concurrently decreased the *EDN1* and *EDNRA* mRNA expression levels in HCT116 cells (Fig. 5A). To further elucidate the mechanisms through which STAT3 regulates EDN1 and EDNRA, the EPD database (https://epd.epfl.ch/EPDnew_database.php) was queried concerning this issue, revealing that STAT3 bound to the EDN1 or EDNRA proximal promoters. In Fig. 5B, the STAT3 consensus binding site with position frequency matrix (Matrix ID: MA0144.2) sequence logo was illustrated from the JASPAR database version 2022 (http://jaspar.genereg.net/analysis). Furthermore, a ChIP assay was performed in the predicted promoter region, using HCT116 cells transfected with siNC or siSTAT3. The ChIP-qPCR results revealed that siSTAT3 significantly decreased the fold enrichment for EDN1 or EDNRA expression compared to siNC (Fig. 5C and D), indicating that STAT3 directly binds to the promoter regions of EDN1 and EDNRA, to regulate their expression.

The role of STAT3 in *EDN1* and *EDNRA* mRNA transcription by constructing luciferase reporter vectors was then examined using the EDN1 or EDNRA gene promoter that contained each pair of putative STAT3-binding sites. The co-transfection of siNC or siSTAT3 with the luciferase reporter vector indicated that the siRNA-mediated suppression of STAT3 decreased the luciferase activity of both the EDN1 and EDNRA constructs (Fig. 5E and F). Taken together, ChIP and promoter assays strongly indicated that STAT3 may directly regulate *EDN1* and *EDNRA* expression at the transcriptional level, by binding to their promoter regions.

Pharmacological inhibition of EDNRA. Macitentan, known as a dual endothelin receptor antagonist, is a Food and Drug Administration (FDA)-approved drug that is being used as a treatment for pulmonary hypertension disease (33-35). Based on the role of EDNRA in the progression of CRC, the possibility that macitentan, which was approved as a drug for pulmonary hypertension by the US FDA in 2013, could be

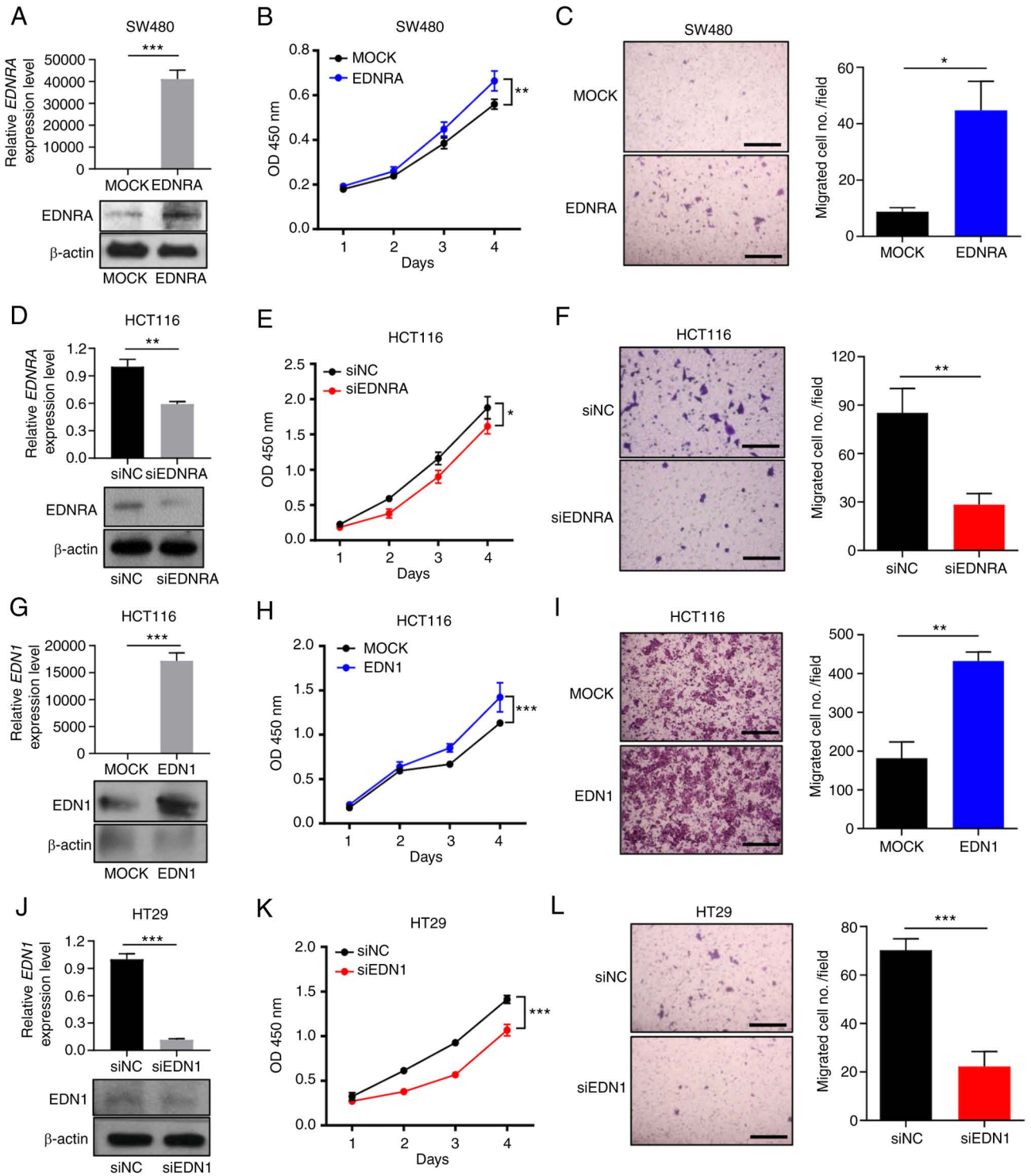

Figure 2. Expression levels of EDN1 and EDNRA regulate the growth and migratory ability of colorectal cancer cells. (A) EDNRA-overexpressing SW480 cells were constructed, and EDNRA expression levels were measured at the mRNA level and protein level using RT-qPCR and western blotting, respectively. EDNRA overexpression increased (B) proliferation and (C) migration compared to mock transfection in SW480 cells. Cell proliferation was measured using CCK-8 assay. Transwell membranes were fixed and stained with crystal violet. The presented images indicate stained migrating cells (scale bar, 500 μm) and the number of relative migrated cells. (D) HCT116 cells transfected with siEDNRA demonstrated decreased EDNRA expression levels compared with siNC-transfected cells. Knockdown of EDNRA decreased (E) proliferation and (F) migration compared to the negative control in HCT116 cells. Cell proliferation was measured by using the CCK-8 assay. Transwell membranes were fixed and stained with crystal violet. The presented images indicate stained migrating cells (scale bar, 500 μm) and the number of relative migrated cells. (G) EDN1-overexpressing HCT116 cells were constructed, and EDN1 expression levels were determined at the mRNA and protein levels using RT-qPCR and western blotting, respectively. The overexpression of EDN1 increased (H) proliferation and (I) migration compared to mock transfection of HCT116 cells. Cell proliferation was measured using CCK-8 assay. Transwell membranes were fixed and stained with crystal violet. The presented images indicate stained migrating cells (scale bar, 500 μm) and the number of relative migrated cells. (J) HT29 cells transfected with siEDN1 displayed decreased EDN1 expression levels compared with siNC-transfected cells. The knockdown of EDN1 decreased (K) proliferation and (L) migration compared to the negative control in HT29 cells. Cell proliferation was measured using CCK-8 assay. Transwell membranes were fixed and stained with crystal violet. The presented images indicate stained migrating cells (scale bar, 500 μm) and the number of relative migrated cells. Data were analyzed using a Student's t-test; *P<0.05, **P<0.01 and ***P<0.001. EDN1, endothelin-1; EDNRA, endothelin receptor A; siNC, negative control siRNA; CCK-8, Cell Counting Kit-8; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

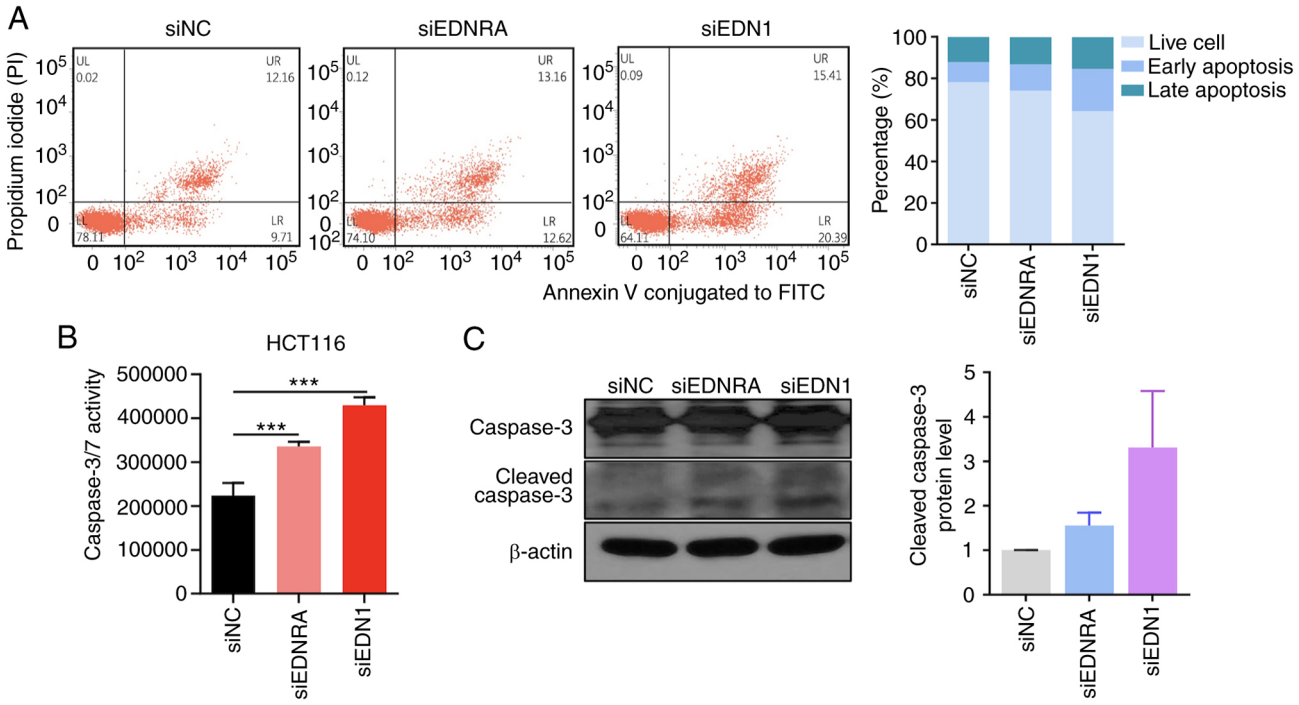

Figure 3. EDNRA or EDN1 silencing induces apoptosis through caspase-3/7 activation. (A) Knockdown of EDNRA or EDN1 by siRNA induced apoptosis levels in HCT116 cells. Cells were double stained with Annexin V conjugated to FITC and propidium iodide followed by FACS analysis. (B) Knockdown of EDNRA or EDN1 by siRNA increased Caspase3/7 luciferase activity at 72 h. One-way ANOVA with Dunnett's test was performed to analyze the differences between groups. (C) EDNRA or EDN1 depletion induced the apoptotic signaling pathway. Western blotting demonstrated that in siNC- and siEDNRA-transfected HCT116 cells, the knockdown of EDNRA or EDN1 increased the cleaved caspase-3 product in comparison with the total caspase-3 expression. One-way ANOVA with Dunnett's test was performed to analyze the differences between groups. ***P<0.001. EDNRA, endothelin receptor A; EDN1, endothelin-1; FITC, fluorescein isothiocyanate.

repurposed as an anticancer drug for CRC, was investigated herein. Treatment of the HCT116 cells with macitentan slightly suppressed cell growth and significantly reduced cell motility (Fig. 6A and B). Furthermore, it was revealed that treatment of the HCT116 cells with macitentan led to a significant increase in the number of apoptotic cells, according to the results of a Caspase-Glo 3/7 assay (Fig. 6C). Furthermore, western blot analysis demonstrated that macitentan reduced the p-STAT3 levels and increased the cleaved caspase-3 levels (Fig. 6D).

To further assess the combined therapeutic effects of macitentan and cisplatin on CRC cells, an apoptosis assay was performed. FACS analysis demonstrated that combination therapy with cisplatin and macitentan increased the apoptotic cell numbers, in comparison to treatment with macitentan or cisplatin alone (Figs. 6E and S6A). Similarly, apoptosis analysis using the Caspase-Glo 3/7 assay revealed that the apoptotic cell numbers were increased in cells treated with macitentan or cisplatin and even further in cells treated with the combination treatment (Figs. 6F and S6B). Collectively, the aforementioned data suggest that the pharmacological EDN1/EDNRA blockade by macitentan, in combination with chemotherapeutic agents, may be a therapeutic option for patients with CRC, by inducing the apoptosis of CRC cells and suppressing STAT3 signaling.

Discussion

GPCRs are membrane receptor proteins that play a crucial role in various cellular processes by transmitting various extracellular signals, triggered by specific peptides, ions, hormones and photons, into cells, in order to regulate cell proliferation, differentiation, and communication. Emerging evidence indicates that abnormally expressed GPCRs are involved in various diseases, including cancers (36). Therefore, blocking GPCRs and their downstream target molecules may be a promising treatment strategy. Moreover, almost 35% of marketed drugs that have been approved by the FDA target GPCRs (37). EDNRA is a well-known GPCR, that regulates blood pressure and acts as a vasoconstrictor when bound to its ligand, EDN1. However, the aberrant expression and activation of EDN1 and EDNRA have been reported to be associated with a number of diseases, including hypertension, diabetes, obesity and cancers. For instance, the importance of EDNRA has been reported in the chemotherapeutic resistance of ovarian cancer, resulting in a high expression of EDNRA related to poor clinical outcomes and chemoresistance by activating the Wnt/β-catenin pathway (38). Consistent with previous reports, demonstrating the overexpression of EDRNA in prostate, colon, breast and cervical cancers (21,27), the present study also observed that EDNRA expression was upregulated in CRC, and the survival rate of patients with CRC with a high expression of EDNRA was decreased. A positive correlation between the *EDN1* and *EDNRA* levels was detected in the COAD TCGA data set. In addition, the *in vitro* experimental results of the present study revealed that silencing EDN1 or EDNRA suppressed cell growth and migratory abilities, suggesting that the suppression of EDN1 and EDNRA may effectively attenuate oncogenic properties; thus, EDNRA

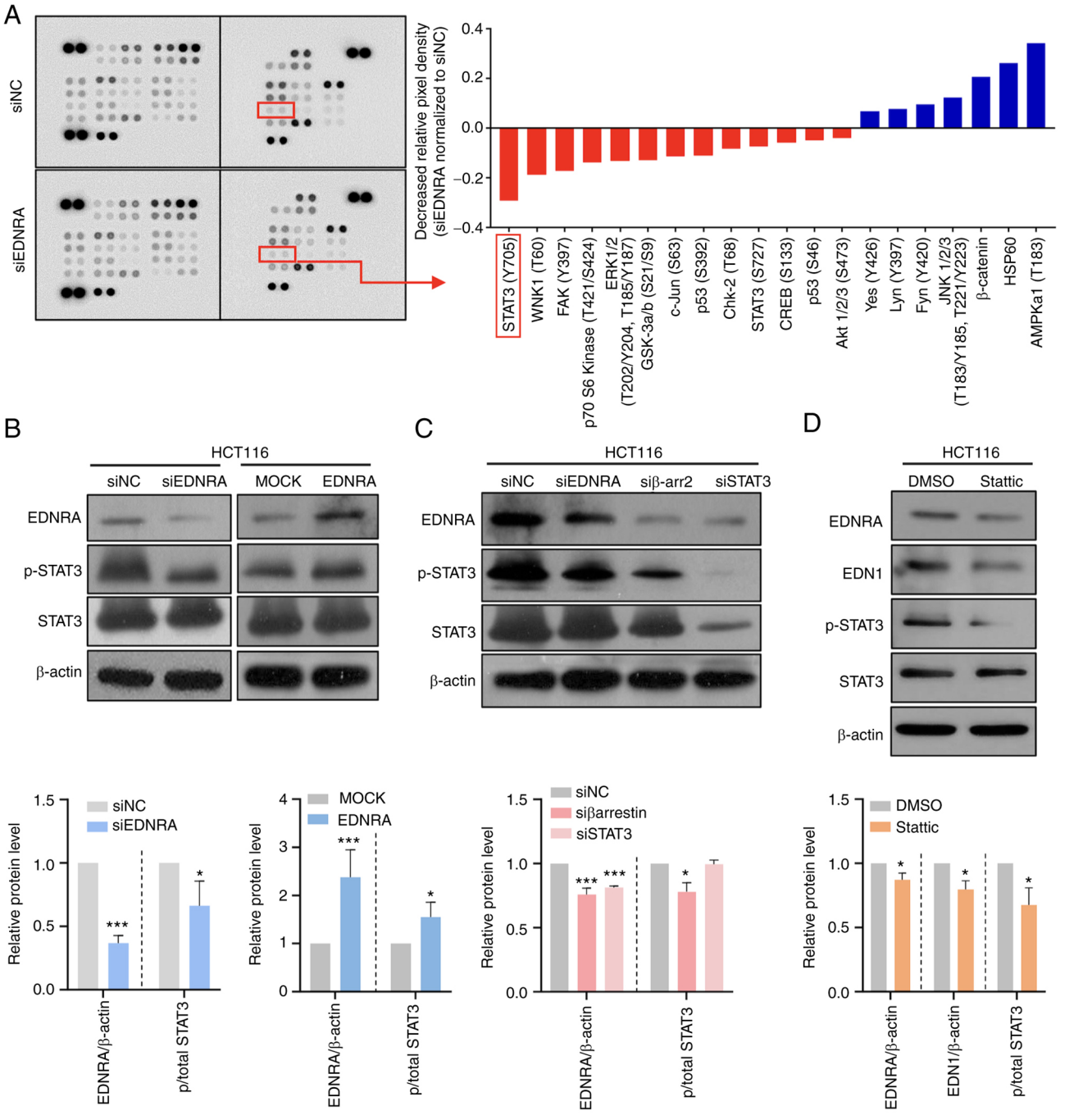

Figure 4. EDNRA modulates the STAT3 signaling pathway in colorectal cancer cells. (A) Phosphokinase array analysis of HCT116 cells transfected with siNC or siEDNRA. Whole-cell lysates were prepared after 48 h of transfection and hybridized with a phosphokinase array kit. Spot densities of phosphoproteins were analyzed using ImageJ software. The fold change in p70S6K and p-STAT3 significantly decreased after siEDNRA transfection compared with siNC transfection. (B) EDNRA-overexpressing HCT116 cells displayed increased phosphorylation levels of STAT3. By contrast, HCT116 cells transfected with siEDNRA exhibited decreased STAT3 phosphorylation compared with siNC-transfected cells. (C) Downregulation of β-arrestin expression by siRNA was performed to determine the signaling pathway, and inhibition of β-arrestin decreased STAT3 phosphorylation in HCT116 cells. The one-way ANOVA with Dunnett's test was performed to analyze the differences between groups. (D) Western blot analysis was used to measure the protein levels of EDNRA, EDN1, STAT3 and β-actin following treatment with DMSO or STAT3 inhibitor in HCT116 cells. Data were analyzed using a Student's t-test; *P<0.05 and ***P<0.001 as compared with siNC or DMSO. EDNRA, endothelin receptor A; STAT3, Signal transducer and activator of transcription 3; siNC, negative control siRNA.

may be an important therapeutic target for blocking CRC progression.

Although the HCT116, SW620 and KM12SM cell lines that have been reported to have high invasiveness or metastatic potential displayed moderate to high expression levels of EDNRA *in vitro*, it could not be concluded that EDNRA expression was strongly associated with CRC progression, as there was no particular association pattern between disease stage and EDNRA expression level *in vivo*, according to the COAD TCGA data set analysis. Animal experiments with the

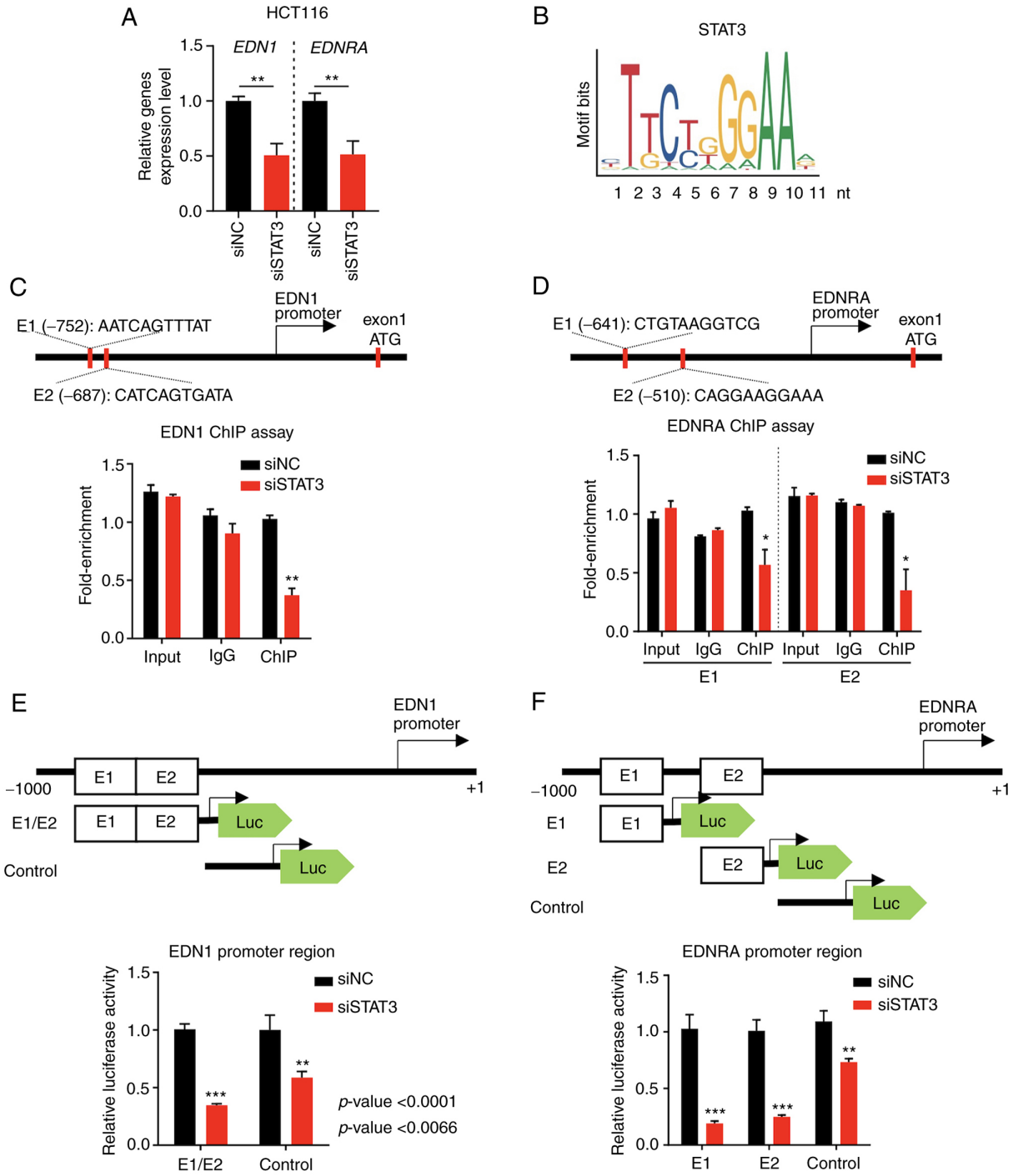

Figure 5. The transcription of *EDN1* and *EDNRA* is regulated by the activity of the transcription factor STAT3. (A) The mRNA levels of *EDN1* and *EDNRA* were decreased in HCT116 cell lines transfected with siSTAT3 compared to siNC. (B) The binding motif of STAT3 for the regulation of the transcription of target genes. (C) The putative STAT3-binding motif of EDN1 was predicted under the condition of a P=0.001, and the ChIP assay confirmed the regulation of *EDN1* transcription by the transcription factor STAT3. Data were analyzed using a Student's t-test. (D) The putative STAT3-binding motif of EDNRA was predicted (P<0.0001), and the ChIP assay confirmed the regulation of *EDNRA* transcription by the transcription factor STAT3. Data were analyzed using a Student's t-test. (E) The *EDN1* promoter region containing the STAT3-binding site was cloned into the pGL4 vector, and luciferase activity was measured. Data were analyzed using a Student's t-test. (F) The *EDNRA* promoter region containing the STAT3-binding site was cloned into the pGL4 vector, and luciferase activity was measured. The results confirmed that *EDN1* and *EDNRA* promoter activity was reduced in siSTAT3 HCT116 cells. Data were analyzed using a Student's t-test; *P<0.05, **P<0.01 and ***P<0.001. EDN1, endothelin-1; EDNRA, endothelin receptor A; siNC, negative control siRNA; ChIP, chromatin immunoprecipitation; STAT3, signal transducer and activator of transcription 3.

orthotopic implantation of a pair of parental and engineered cells manipulated to overexpress or knockout EDN1/EDNRA will aid to delineate the inconsistency between *in vitro* and *in vivo* data and, clearly elucidating the role of EDN1/EDNRA in the progression of CRC.

EDNRB functions have been reported as a counter-regulator of EDN1/EDNRA activity via various mechanisms, including nitric oxide production, EDN1 clearance and blocking cell growth in normal cells (39,40). Compared to EDRNA, the detailed mechanisms and functions of EDNRB

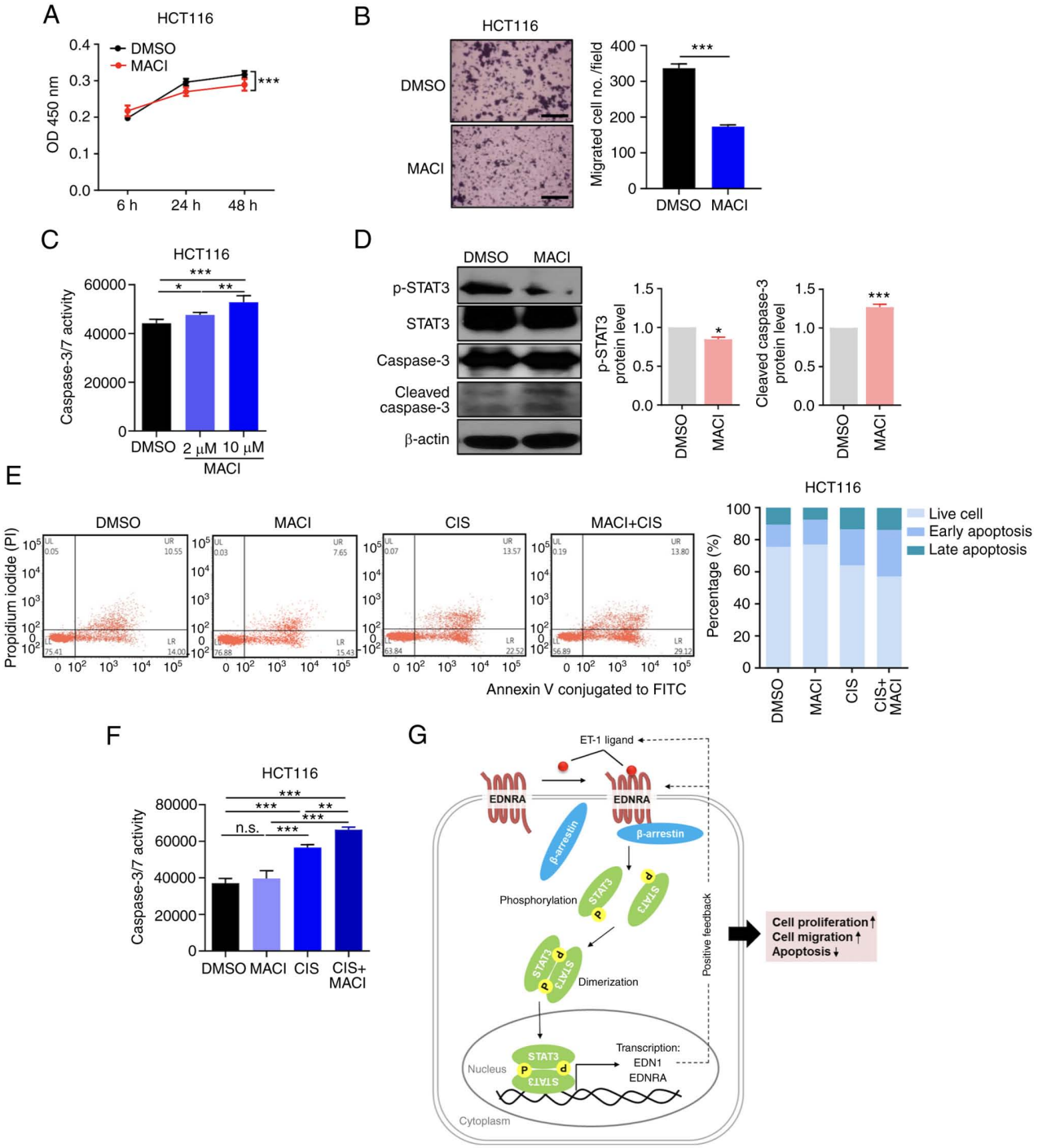

Figure 6. Anticancer effects of combination therapy with MACI and CIS in CRC cells. MACI suppressed HCT116 cell proliferation and migration ability compared to the control (DMSO). (A) Cell proliferation was measured using Cell Counting Kit-8 assay. (B) Transwell membranes were fixed and stained with crystal violet. The presented images indicate stained migrating cells (scale bar, 500 μm) and the number of relative migrated cells. Data were analyzed using a Student's t-test. (C) MACI-treated HCT116 cells demonstrated increased Caspase3/7-Glo luciferase activity at 72 h. One-way ANOVA with the SNK test was performed to analyze the differences between groups. (D) Western blotting of DMSO- and MACI-treated HCT116 cells revealed that the cleaved caspase-3 product increased in relation to total caspase3 expression. MACI induced apoptosis of live HCT116 cells and sensitized HCT116 cells to cisplatin treatment. Data were analyzed using a Student's t-test. (E) HCT116 cells were treated with 10 μM MACI and/or 5 μM CIS for 72 h. The cells were double stained with Annexin V conjugated to FITC and propidium iodide followed by FACS analysis. (F) Caspase-Glo 3/7 assay performed with drug-treated HCT116 cells for 48 h. One-way ANOVA with Tukey's multiple comparisons test was performed to analyze the differences between groups. (G) Mechanism of the EDN1/EDNRA axis in CRC cells. Data were analyzed using one-way ANOVA; *P<0.05, **P<0.01 and ***P<0.001 as compared to the DMSO. CRC, colorectal cancer; DMSO, dimethyl sulfoxide; MACI, macitentan; CIS, cisplatin; FITC, fluorescein isothiocyanate.

have not yet been completely elucidated in cancer research, since the effects of EDN1 on cancer cells are mostly mediated by EDNRA. Although a positive correlation between EDN1 and EDNRB expression was observed (Fig. S1D), the findings that both EDN1 and EDNRB expression levels were significantly lower in CRC tissues, as compared with normal tissues do not support the clinical significance of the correlation data. It has been previously reviewed that EDNRB expression

was upregulated in bladder cancer and was associated with a poor prognosis (41). In addition, Fu *et al* (42) reported that miR-124-3p suppressed cell proliferation and invasion by the direct regulation of EDNRB in bladder cancer. By contrast, EDNRB expression has been shown to be downregulated by aberrant hypermethylation in the promoter site in several tumors, including hepatocellular carcinoma, gastric cancer and CRC (43-45). A reduced EDNRB expression by hypermethylation has been found to be associated with gastric cancer invasion and to be associated with pathological stage in prostate cancer (46). Similarly, the results of the present study also revealed that the expression of EDNRA was more prominent than EDNRB in CRC, indicating that EDNRA may play a critical role in CRC progression.

Although EDNRA expression was upregulated in CRC tumors and had a positive correlation with EDN1 ligand, EDN1 expression was lower in CRC tumors than in normal tissues. This may raise a question whether other ligands exist which are able to bind and activate EDNRA-mediated signaling. It is possible that other ligands, such as EDN2 can also bind to EDNRA with different affinities than EDN1 and can affect EDNRA signaling independently of EDN1 ligand in CRC. In addition, EDN1 could also be derived from cells in the tumor microenvironment including immune cells, fibroblasts and blood vessels and may be used to activate EDNRA signaling in a paracrine manner (47,48). In this regard, single-cell transcriptomic analysis of CRC tumor tissues in the future is expected to provide further information about the role of autocrine and/or paracrine EDN1 in the EDNRA-mediated growth and progression of CRC.

Accumulating evidence has indicated that EDNRA plays a crucial role in carcinogenesis by regulating several downstream signaling pathways. For example, Cianfrocca *et al* (49) revealed a mechanistic link between EDN1/EDNRA signaling and the β-catenin pathway through a specific interaction with β-arrestin in CRC stem-like cells. Another study demonstrated that EDNRA was directly regulated by miR-200c in gastric cancer (50). The EDNRA 3'-untranslated region encodes a binding site of miR-200c; therefore, EDNRA can be regulated by miRNAs. Notably, Sestito *et al* (51) observed that EDNRA and ZEB1 negatively correlated with miR-200b/c, and the EDNRA/miR-200b/c/ZEB1 circuit promoted epithelial-mesenchymal transition, cell plasticity and metastasis. These results indicated that EDNRA and its associated signaling pathway may be important for tumorigenesis. In the present study, it was also investigated how EDN1/EDNRA may serve its oncogenic role by surveying putative downstream effector molecules that can enhance CRC progression. The phosphokinase array demonstrated that EDNRA silencing decreased the phosphorylation of STAT3 in CRC cells, indicating that EDN1/EDNRA may be involved in STAT3 activation. Therefore, it was hypothesized that EDNRA may promote CRC progression through the activation of STAT3 signaling pathways. STAT3 is a member of the STAT family of transcription factors, and the JAK-STAT signaling pathway is a well-established mechanism that regulates various cell functions, including immunity, cell division and tumorigenesis, through the regulation of the transcription level of its target genes (52). Presenting with these multimodal tumor promoting-activities, it is unsurprising that p-STAT3 levels are increased in CRC (53,54), and its activation has been shown to be associated with cancer progression (55). In the present study, a novel mechanism, to the best of our knowledge, of the EDN1/EDNRA-mediated CRC progression was revealed in that EDNRA expression can promote STAT3 phosphorylation; thus, STAT3-driven tumor-promoting activities may be activated in CRC cells. These oncogenic properties can be further supported by a positive feedback regulatory circuit between STAT3 activation and transcriptional upregulation of EDN1/EDNRA expression. Therefore, the present study provided a strong missing link between STAT3 activation and EDN1/EDNRA for the coordination of tumor progression in CRC.

Additionally, the present study is the first to demonstrate, to the best of our knowledge, that STAT3 simultaneously regulates EDN1 and EDNRA mRNA expression. The inactivation of STAT3 by siRNA or STAT3 inhibitor stattic resulted in the concurrent suppression of EDN1 and EDNRA expression levels, indicating that STAT3 may regulate the expression of EDN1 and EDNRA. By using ChIP-qPCR and promoter assays, it was confirmed that EDN1 and EDNRA were directly regulated by the STAT3 transcription factor. EDN1 has been reported to activate the EDNRA/β-arrestin/STAT3 loop, and this circuit most likely can also be induced by other extrinsic or CRC cell intrinsic signaling pathways, such as the activation of oncogenes and inactivation of tumor suppressors (56,57), that may activate EDN1, EDNRA or STAT3. Once activated, the circuit can be continuously active through its positive feed-forward nature, as many tumor-promoting oncogenes do. Overall, it was suggested that the EDN1/EDNRA signaling pathway promotes various downstream effector proteins, including STAT3; subsequently, these effector proteins may positively regulate the transcriptional levels of EDN1 or EDNRA. To the best of our knowledge, this is the first report demonstrating STAT3-mediated overexpression of EDN1 and EDNRA in CRC and its functional significance. However, the present study has a limitation concerning a lack of a full explanation about how the EDN1/EDNRA axis may be associated with STAT3 activation, since STAT3 can also be activated by several upstream signals, including the inflammation-related receptors gp130, IL-6R, IL-8R, IL-35R and EGFR; thus, further studies are required for the determination of the sophisticated regulatory networks between EDNRA expression and other signaling pathways, including inflammatory signaling in CRC. Moreover, it remains to be fully elucidated whether the exact role of EDN1/EDNRA activation and its downstream STAT3 signaling favor tumor-initiation or tumor-promotion. To delineate this, animal experiments using genetically engineered mice with EDN1 or EDNRA overexpression in the normal intestinal cells need to be performed in future studies, in order to determine whether non-cancerous or pre-cancerous cells may transform into cancer cells.

In addition, other signaling pathways, including with-no-lysine [K] 1 (WNK1) and/or focal adhesion kinase (FAK) signaling can also contribute to the EDRA-mediated progression of CRC, although no experimental evidence was presented in the present study, since the phosphokinase array data analysis of the present study demonstrated decreased phosphorylation levels of WNK1 and/or FAK by EDNRA silencing. WNK kinases have been reported to activate

downstream substrates, including protein odd-skipped-related 1 and STE20/SPS1-related proline-alanine-rich protein kinase to regulate several ion channels and to stimulate angiogenesis by stimulating PI3K-AKT pathway (58). Moreover, WNK kinase regulates Wnt signaling pathways and β-Catenin levels, which are both associated with the development and progression of CRC (59). By contrast, FAK is a well-known multi-functional regulator of cell signaling within the tumor microenvironment (60). It promotes cell proliferation, motility and survival through a kinase-dependent or -independent manner in several cancers, including colon, breast, ovary and prostate cancers (61). Integrin α5 has been reported to promote cell migration and invasion through the activation of FAK/STAT3/AKT signaling pathways (62). In addition to EDNRA, FAK can also activate Src/$ERK_{1/2}$/STAT3 signaling to inhibit E-cadherin expression, thus leading to the promotion of cell motility in melanoma cells (63). Currently, it is not clear whether the activation of STAT3 by EDNRA is FAK-dependent. Moreover, the exact mechanism about the interactions between all signaling molecules that are involved in the EDNRA-mediated CRC progression remains to be fully elucidated. Accordingly, to fully understand the EDNRA-mediated signaling networks and to evaluate the biochemical and clinical significance of the current findings, further experimental evidence is required in the future to delineate biochemical roles of those signaling molecules on the progression of CRC tumors.

Furthermore, considering the findings of the present study in light of recent studies demonstrating that the endothelin receptor antagonist, macitentan, may be used as a therapeutic drug for targeting various cancer types apart from pulmonary hypertension disease (64). Accordingly, previous reports have revealed that targeting endothelin receptors with macitentan represent an effective therapeutic approach for anti-proliferation and anti-angiogenesis in multiple myeloma and high-grade serous ovarian cancer (65,66). Notably, a combination therapeutic strategy with macitentan and chemotherapeutic agents, including oxaliplatin and 5-fluorouracil has been indicated to exert enhanced antitumor effects in CRC (49). Lee *et al* (67) revealed a reduction in cell division and induction of apoptosis by macitentan combined with paclitaxel in breast and lung cancer cells, also significantly increasing overall survival in mice harboring brain metastases. Another group reported that macitentan in combination with temozolomide also leads to glioblastoma regression (68). Similarly, the results of the present study also demonstrated that macitentan effectively inhibited cell growth and migration and induced apoptosis of CRC cells, particularly when used in combination with the chemotherapeutic agent cisplatin. More specifically, combined treatment of macitentan and cisplatin induced tumor cell apoptosis more effectively than treated alone, possibly by blocking different apoptotic signaling pathways, since the mechanism of apoptosis induced by macitentan appears to be mainly mediated by blocking MAPK and/or STAT signaling pathways, whereas cisplatin induces p53 signaling pathway through proapoptotic FasL gene expression (69,70). The clinical significance of the present findings need to be evaluated in the clinical setting, and the data suggested that the EDN1/EDNRA blockade may be possibly used as a as a novel treatment option for patients with CRC.

Although cisplatin is a highly effective anticancer drug widely used for the treatment of several types of cancer in the clinic (71,72), standard chemotherapy including leucovorin, irinotecan and 5-fluorouracil (73,74) but not cisplatin has been used for the treatment of patients with CRC. Therefore, those chemotherapeutics should have been used rather than cisplatin in the present experimental setup to confirm the anticancer effects of combination therapy with EDNRA antagonist and chemotherapeutic agents against CRC. Regardless of the above limitation, the findings of the present study may provide preliminary data presenting the possibility for the EDNRA antagonists being able to improve chemotherapy outcomes, when treated in combination. Additionally, it will be interesting to perform a combination treatment of macitentan with stattic to evaluate whether the blockade of STAT3 and/or EDNRA signaling, alone or in combination, can improve the therapy outcomes for the therapy of patients with CRC. Therefore, further *in vivo* studies to evaluate the therapeutic efficacy of various EDNRA antagonists, and/or STAT3 signaling antagonists, in combination with variety of chemotherapeutic agents for the therapy of patients with CRC are warranted.

In the present study, it was demonstrated that EDN1/EDNRA, being highly expressed in human CRC, promoted CRC cell proliferation and migration, and decreased apoptosis through the β-arrestin-mediated activation of the well-known oncogenic protein STAT3 (Fig. 6G). The activation of STAT3, in turn, continuously activated EDN1/EDNRA signaling by increasing the transcription of several genes associated with tumor progression, including *EDN1*/*EDNRA*, in a positive feedback loop. Based on these findings, considering all data demonstrating that the pharmacological inhibition of EDNRA signaling, in combination with chemotherapy, inhibits the proliferation and migration and induces apoptosis of CRC cells, it was proposed that targeting EDN1/EDNRA signaling may be an effective therapeutic strategy against CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YJL, EJ, JC, TSH and JSK designed the experiments. YJL, EJ, JC, JSH, EJJ and YNR performed the experiments and

collected the data. HSB, SK, SKK and SYK analyzed the bioinformatics data. EJ, HSB, JKM, TSH and JSK analyzed and interpreted the data. YJL, EJ, JC, TSH and JSK drafted the manuscript and revised the manuscript. TSH and JSK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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