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RESEARCH ARTICLE

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Knockdown GREM1 suppresses cell growth, angiogenesis, and epithelial-mesenchymal transition in colon cancer

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Abstract

Gremlin 1 (GREM1), as a bone morphogenetic protein (BMP) antagonist and vascular endothelial growth factor receptor-2 (VEGFR2) novel agonist, has been confirmed as overexpressed in colorectal cancer (CRC) tissues but its role in carcinogenesis remains unclear. Here we reported that the GREM1 expression in mesenchymal-like colon cancer cells (SW620 and SW480) was significantly higher than that of epithelial-like colon cancer cells (Caco-2, HTC116, and HT29) and normal colon cell. Simultaneously, we analyzed two series of CRC transcriptomes from Gene Expression Omnibus (GEO) databases and found the great majority of primary CRC tissues expressed high level of GREM1 messenger RNA (mRNA) compared with adjacent normal tissues, and that the GREM1 mRNA expression is correlated with low histological grade development and stage 2 to 3 metastatic recurrence in CRC based on a data analysis of 104 different stage CRC tissue from the GEO databases. Functional studies showed that GREM1 silencing by short hairpin RNA (shRNA) significantly inhibited CRC cells proliferation, migration, the formation of vascular endothelial growth factor (VEGF)-induced capillary structure of human umbilical vein endothelial cells (HUVECs), and epithelial-mesenchymal transition in colon cancer cells by repressing phosphorylation levels of BMP downstream signal Smad1, vascular endothelial growth factor (VEGF) downstream signal matrix metallopeptidase 2 (MMP2), and metastasis-related factor C-X-C motif chemokine ligand 12 (CXCL12) expression. In addition, shGREM1 combined with VEGF inhibitor BAW2881 displayed more effective antiangiogenesis to inhibit the tube formation of HUVEC. Hence, these experiments demonstrated that GREM1 is involved in CRC development and procession and provide a new idea for CRC diagnosis, resistance therapy, and prognosis.

KEYWORDS
colon cancer, epithelial-mesenchymal transition, gremlin 1, metastasis, proliferation

Yan Liu and Yongchao Li are the co-first authors.

1 | INTRODUCTION

According to a cancer statistics report in 2014, colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer death in the United States, and an estimated 71 830 men and 65 000 women will be diagnosed with CRC and 26 270 men and 24 040 women will die of the disease.[1] Although early diagnosis and surgical intervention, along with a combination of chemotherapy, has resulted in improved outcomes, there are still some CRC patients who failed to respond to these treatments. In particular, there are few effective strategies to treat colon cancer once first-line treatments have been exhausted.[2] So improved understanding of the underlying cellular basis of colon cancer and mechanisms of resistance would be critical for the development of novel therapeutics.

In a normal colon, the unit of structure in the colon is the crypt of Lieberkuhn, which is composed of colon stem cells, transit amplifying cells, terminally differentiated goblet cells, enterocytes, and endocrine cells. The stem cells (undifferentiated cells) reside in the bottom and the terminally differentiated cells reside near the top. So these cells continuously cycle from undifferentiated in the bottom of the crypt through the terminally differentiated cells. Moreover, numerous evidence suggested that the intestinal epithelial stem cell renewal and differentiation is determined by the interaction of several key pathways including the wingless-related integration site (WNT), bone morphogenetic protein (BMP), and Notch and Hedgehog (HH) pathways. Among these pathways, Wnt signaling plays a major role in maintaining intestinal stem cell fate and progenitor cell proliferation. In contrast, BMP signaling has been reported to inhibit intestinal stem cell activation and promote intestinal differentiation. Notch signaling is involved with cell fate decisions, as it directed cells toward a secretory lineage in the intestine.[3] Once these key pathways get disordered, they would promote tumorigenesis.

Gremlin 1 (GREM1), is one of the BMP antagonists[4] and a vascular endothelial growth factor receptor-2 (VEGFR2) agonists,[5] similar to BMP family, has been identified that it plays an important role in regulating tissue differentiation, body patterning, and organogenesis.[6] It has been demonstrated that GREM1 is overexpressed in some cancer like lung adenocarcinoma.[7] And some genetic analysis not only found that variants of GREM1, BMP4, and BMP2 might be the cause of the development of familial inheritance of CRC but also confirmed that aberrant epithelial GREM1 expression initiated hereditary-mixed polyposis syndrome (HMPS) and colonic tumorigenesis from the cells outside the crypt base stem cell niche.[8,9] It is noticeable that the secretive GREM1 from cancer stem cells (CSC) could prevent the BMP differentiating effect in glioblastoma,[10] this means GREM1 could be a potent biomarker and be detected in blood serum. In addition, a study from 2015 confirmed that GREM1 is a key factor to keep the ability of self-renewal and multipotency of stem cells and as an identification marker for osteochondroreticular in stem cells.[11] These suggested that GREM1 plays a role in the maintenance of stem cell or cancer stem cell properties, so we inferred GREM1 could be involved with the tumor development and tumor angiogenesis, especially CRC. However, the functional roles of GREM1 in colon cancer remain unclear. Therefore, we carried out experiments to investigate the role of GREM1 in colon cancer and explored, whether or not, increased GREM1 was a major cause of antivascular endothelial growth factor (VEGF) therapy resistance, recurrence, and metastasis in colon cancer. Finally, we found that the expression of GREM1 is higher in mesenchymal-like colon cancer cells (SW620, SW480) than that of epithelial-like colon cancer cells (Caco-2, HTC116, and HT29) and normal colon cell NCM460. Meanwhile, GREM1 silencing by short hairpin RNA (shRNA) not only significantly inhibited proliferation of SW620 and SW480 cells by inhibiting cell cycle in G1 phase but also induced apoptosis. Silencing GREM1 expression of mesenchymal-like colon cancer cells SW620 and SW480 obviously suppressed epithelial-mesenchymal transition (EMT). This study is intended to express the development of drugs that target angiogenesis (inhibition of angiogenesis). GREM1 is a proangiogenic factor. Most of the antiangiogenic drugs are targeted to VEGF. However, this study shows that GREM1 can also bind to VEGFR2 and induce angiogenesis by inducing activation of a pathway similar to VEGF-VEGFR, which in turn activates angiogenesis-related pathways. Moreover, GREM1 can also create a corresponding proangiogenic effect without relying on VEGF.

2 | MATERIALS AND METHODS

2.1 | RNA extraction and quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using TRIzol reagent (Invitrogen, Waltham, MA) according to the manufacturer's instructions. Complementary DNAs (cDNAs) were synthesized using ReverTra Ace qPCR RT Kit (FSQ-101; TOYOBO, Osaka, Japan).[29,30] Real-time PCR analyses were performed with THUNDERBIRD SYBR qPCR Mix (QPS-201; TOYOBO, Osaka, Japan) on a LightCycler 480 detection system (Roche). The samples were transferred to the thermal cycler, and DNA was amplified using the following thermocycling

conditions: 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 30 seconds. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. GREM1 primers: forward primer, 5′-TTAAGCAGACCATCCACGA-3′ and reverse primer, 5′-TGTAGTTCAGGGCAGTTGAGT-3′. All samples were amplified in triplicate according to the manufacturer's instructions. GAPDH primers: forward primer, 5′-GGAGCGAGATCCCTCCAAAAT-3′ and reverse primer, 5′GGCTGTTGTCATACTTCTCATGG3′.

2.2 | Data acquisition

GREM1 mRNA expression of primary CRC patient's tissues and normal colonic mucosa tissues were measured with Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Waltham, MA) and downloaded from GSE4107, which contained 22 samples. Meanwhile, GREM1 expression data from different stages of 104 CRCs were measured with the Affymetrix Human Genome U133 Plus 2.0 Array and downloaded from GSE21510 (https://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc = GDS4516). All the gene expression data sets have been processed by a slight normalization method. The protein expression of GREM1, SMAD1, C-X-C motif chemokine ligand 12 (CXCL12), and matrix metallopeptidase 2 (MMP2) in all major tissues and organs in the human body could be found in the Human Protein Atlas database in which those protein has been detected by proteome methods (http://www.proteinatlas.org).

2.3 | Cell lines and culture

All cells were obtained from the Institute of Biochemistry and Cell Biology of Chinese Academy of Science (Shanghai, China). Human colon epithelial cell NCM460 and human CRC cell lines (SW620, SW480, SW620, SW480, and HT29) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum (FBS; Gibco, Waltham, MA) at 37°C in a humidified incubator containing 5% CO_2.

2.4 | Lentivirus construction and cell transfection

To generate shRNA-lentivirus transfected stable cell population, the target sequence of shRNAs against GREM1 was 5′-GCACATCCGAAAGGAGGAA-3′ and the negative control sequence of shRNAs was 5′-TTCTCCGAACGTGT CACGT-3′, and they were constructed using pGenesil1.1 vector. Supernatants containing different lentiviruses generated from HEK-293T cells were collected, all these lentiviruses were constructed by GeneChem Company (Shanghai, China).

For the cell transfection, SW620 and SW480 cells were infected with lentiviruses with a multiplicity of infection (MOI) 30 in the presence of polybrene at a final concentration of 8 μg/mL, then incubated for 72 hours. Most cells expressed green fluorescent protein (GFP) under fluorescence microscopy. Thus, the cell culture medium was refreshed. Quantitative real-time PCR (qRT-PCR) and western blot analysis were used to determine the effectiveness of the shRNA knockdown.

2.5 | Western blot analysis

Cells were harvested and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology, Danvers, MA) with 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Boster, Wuhan, China) and then centrifuged at 14 000*g* for 10 minutes at 4°C. Proteins were measured by bicinchoninic acid assay (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). A total of 50 μg proteins were loaded into sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). After blocking in tris-buffered saline with Tween-20 (TBST) that contained 5% nonfat milk for 60 minutes, the membranes were incubated with the following primary antibodies overnight at 4°C: mouse anti-GREM1 antibody (concentration of 1-5 μg/mL; Abcam, Cambridge, MA); Rabbit anti-pSMAD1/5 (1:1000; Cell Signaling Technology), CXCL12 (1:1000; Cell Signaling Technology), mouse anti-VEGFA antibody, mouse anti-E-Cadherin antibody and mouse anti-Vimentin (1:1000; Abcam), Rabbit anti-MMP2 antibody (1:1000; Cell Signaling Technology), and Rabbit anti-GAPDH (1:1000; Cell Signaling Technology). Then, the membranes were incubated with the secondary antibodies (horseradish peroxidase [HRP]-linked antibody; 1:5000 dilutions; Cell Signaling Technology). After incubating in enhanced chemiluminescence solution (Boster, Wuhan, China), the proteins on the membranes were detected by using Bio-Rad Universal Hood and analyzed by Image III Lab™ software 2.0 (Bio-Rad, Hercules, CA).

2.6 | Immunofluorescence

About 2×10^4 cells were seeded onto a 24-well chamber. After 24 hours, cells were fixed in 4% paraformaldehyde and 0.1% Triton X100 in phosphate-buffered saline (PBS) buffer at 40℃ for 30 minutes. The cells were then washed three times with PBS and incubated with the blocking solution (10% goat serum in PBS). The cells were then incubated with the primary antibodies to E-cadherin and vimentin (1:200; Abcam) overnight, washed three times with PBS plus 0.1% Tween-20 for 15 minutes, and finally

incubated with secondary antibodies (Invitrogen) and DAPI for 2 hours. The slides were washed extensively with PBS and mounted with SlowFade Light Anti fade Kit (Invitrogen). All matched samples were photographed (control and test) using immunofluorescence microscope with identical exposure times.

2.7 | Cell proliferation

Cells were seeded onto 96-well plates (2×10^3 cell/well) and transfected with shGREM lentivirus or NC. The cell proliferation of CRC cell lines was determined by Cell Counting Kit-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan) at the indicated time points (0, 24, 48, 72, and 96 hours) according to the manufacturer's instructions. The groups were carried out in quintuplicate wells.

2.8 | Cell cycle distribution

Forty-eight hours after transfection in 6-well plates, SW620 cells or SW480 cells were harvested and washed with cold 1 × PBS. Then, cells were fixed in 70% ethanol at 4°C overnight and washed with PBS twice, resuspended in 100 μl RNase A, incubated at 37°C for 30 minutes. Staining for DNA content was performed with 400 μl propidium iodide (KeyGen Biotech, Nanjing, China) at 4°C for 30 minutes in the dark, and analyzed by a flow cytometer (Beckman Coulter Epics XL, Brea, CA). The experiments were carried out for at least three times.

2.9 | Apoptosis assay

Seventy-two hours after transfection, SW620 or SW480 cells were harvested, washed, resuspended in the binding buffer, and examined with the Vybrant Apoptosis Assay kit (Invitrogen, Carlsbad, CA). Stained cells were detected by fluorescence-activated cell sorting (FACS) as previously described.[31] Annexin V-positive cells were regarded as apoptotic.

2.10 | Capillary-like tube formation assay

The Matrigel (BD Biosciences, Bedford, MA) was added onto 24-well culture plates (60 μl/well) and allowed to polymerize at 37°C for 30 minutes. Conditioned media was collected from CRC cells transfected with shGREM1 lentivirus or not and was concentrated with Amicon Ultra-15 (Millipore, Billerica, MA) centrifugal filtration device with Ultracel-50 membrane (Millipore, Billerica, MA) to purify the protein. Then human umbilical vein endothelial cells (HUVECs) were resuspended in the conditioned media (2.5×10^5 cells/mL) and were seeded onto the Matrigel and treated with 5 ng/mL VEGF, 50 μl/well extract, and vascular endothelial growth factor (VEGF) inhibitor BAW2881 (Selleckchem, Houston, TX) with 1 nm/L or not, incubated at 37°C for 8 hours, and imaged using an inverted phase contrast microscope.

2.11 | In vitro migration assay

Migration assays were performed using the 24-well Cell Migration with 8 μm pore size polycarbonate membrane (Corning, Corning, NY), according to the manufacturer's instructions. In brief, 24 hours after the transfection, 5×10^4 cells were resuspended in 200 μL serum-free medium and plated in the top chamber. The lower chambers were filled with 0.6 mL of medium containing 10% FBS. Medium with 10% FBS was added to the lower chamber as a chemoattractant. After a 24-hour incubation at 37°C, the cells on the upper surface of the membrane were removed, and the cells on the lower surface were fixed, stained, photographed, and counted under a microscope in five fields.

2.12 | Statistical analysis

Data are presented as mean ± SD, and each assay was performed with at least three replicates. The multiple comparisons were performed using variance (ANOVA) when there were more than two groups. Differences in GREM1 expression between tumor tissues and normal tissues of human subjects were calculated with a two-tailed independent samples the Student *t* test. Significance was accepted when the two-tailed *P* value was smaller than 0.05. All analyses were performed using Prism version 6.0 (GraphPad Software Inc, San Diego, CA).

3 | RESULTS

3.1 | Aberrant GREM1 expression in CRC and its correlation with low histological grades

To compare the expression of GREM1 mRNA between human primary CRC tissues and normal tissues, we collected a series of 22 samples from the Gene Expression Omnibus (GEO) profiles of GSE4107 datasets to analyze. We found the great majority of primary CRC tissues expressed a high level of GREM1 mRNA than the control tissues, and the median of GREM1 mRNA expression in primary CRC tissues was 7.72-fold higher than that in the healthy control tissues (Figure 1A). Then we proceeded to analyze the correlation between the GREM1 expression and histological grades with the data of 104 CRC patients from GSE21510 database. The results showed that the GREM1 mRNA of stages 2 to 4 was not only

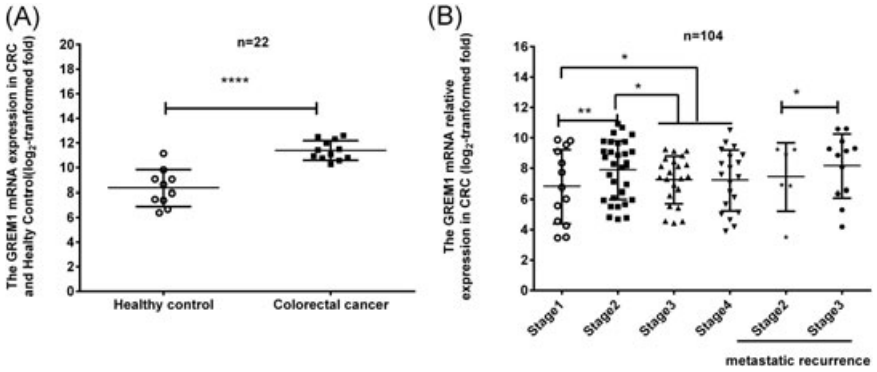

FIGURE 1 The mRNA expression of Grem1 in CRC tissues and healthy controls. A, The GREM1 mRNA expression in primary CRC and healthy controls from GSE4107 that contained 22 samples and measured with Affymetrix Human Genome U133 Plus 2.0 Array. B, The GREM1 mRNA expression in 104 different CRC stages and tumor metastatic recurrence samples from GSE21510 were calculated and analyzed. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. CRC, colorectal cancer; GREM1, gremlin 1; mRNA, messenger RNA

expressed higher than that of stage 1 but also gradually increased in metastatic recurrence tumor with the histological grades procession. Though the GREM1 expression of stages 3 to 4 in primary CRC was lower than that of stage 2, which might be because the number of samples of stages 3 and 4 was too small (Figure 1B). Overall, GREM1 mRNA expression in other CRC stages was higher than that of stage 1 of CRC. These results indicated that GREM1 might be involved in early tumor development and metastatic recurrence in CRC.

3.2 | Silencing GREM1 expression suppresses CRC cells proliferation and migration

Previous studies denoted that GREM1 might be involved in CRC progression, so we decided to investigate the biological effects of GREM1 in CRC cells. First, the GREM1 expression of different CRC cells (SW620, SW480, SW620, SW480, and HT29) and human colon epithelial cell NCM460 in our laboratory were assessed by qRT-PCR. As shown in Figure 2A, GREM1 showed significantly high mRNA expression in mesenchymal-like colon cancer cells (SW620 and SW480 cells) compared with epithelial-like colon cancer cells (Caco-2 and HT29) and normal colon cell NCM460. So we chose mesenchymal-like colon cancer cells SW620 and SW480 cells to carry out the following experiments. Then, we attempted to construct shGREM1 lentivirus to perform the functional study and successfully validated the silencing efficiency of GREM1 mRNA (Figure 2B) and protein (Figure 2C), both could reach 70% after transfection shGREM1 lentivirus with MOI 30 for 72 hours. Then, we attempted to construct a shGREM1 lentivirus to perform the functional study. As demonstrated in Figure 2B, fluorescence microscopy revealed that the transduction efficiency of the GFP-containing shGREM1 lentivirus in both SW620 and SW480 cells after 3 days were greater than 70%, and the virus MOI was 30 for 72 hours. Data from RT-qPCR revealed that the knockdown effects of the shGREM1 lentivirus in SW620 cells and SW480 were, respectively, greater than 75.0% and greater than 80% (Figure 2B) and the Western blot analysis results simultaneously indicated that the shGREM1 lentivirus decreased expression levels of GREM1 in SW620 cells and SW480 were, respectively, 51.4% and 64% compared with control (Figure 2C). These data suggested that the shGREM1 lentivirus was successfully constructed.

To further investigate the effect of shGREM1 on the proliferation and migration of CRC cells, as shown in Figure 3A and 3B, silencing GREM1 expression by shGREM1 significantly inhibited CRC cells proliferation and migration ability compared with those with shNC. These observations, consistent with the GREM1 expressed in CRC tissues, revealed that aberrant high GREM1 expression may promote CRC cells proliferation and migration. Cells were seeded onto a 96-well plate (2×10^3/well) and transfected with shGREM lentivirus or NC. The cell proliferation of CRC cell lines was determined by Cell Counting Kit-8 assay (Dojindo Molecular Technologies) at the indicated time points (0, 24, 48, 72, and 96 hours) according to the manufacturer's instructions. The groups were carried out in quintuplicate wells. In addition, the migration experiment here is to reduce migration, and it is rarely possible to prevent the cell from running. The cell movement is generally controlled by the chemokines on the cell surface. Different cells have different characteristics and migration abilities. Our goal is to place it for 24 hours and see that reducing the number of cell migrations is fine. In these 24 hours, it is generally acceptable to reduce the chance of doubling the number of cells. Generally, the majority of tumor cells, especially solid tumor cells, need to double the number of cells required for 24-25 hours. To

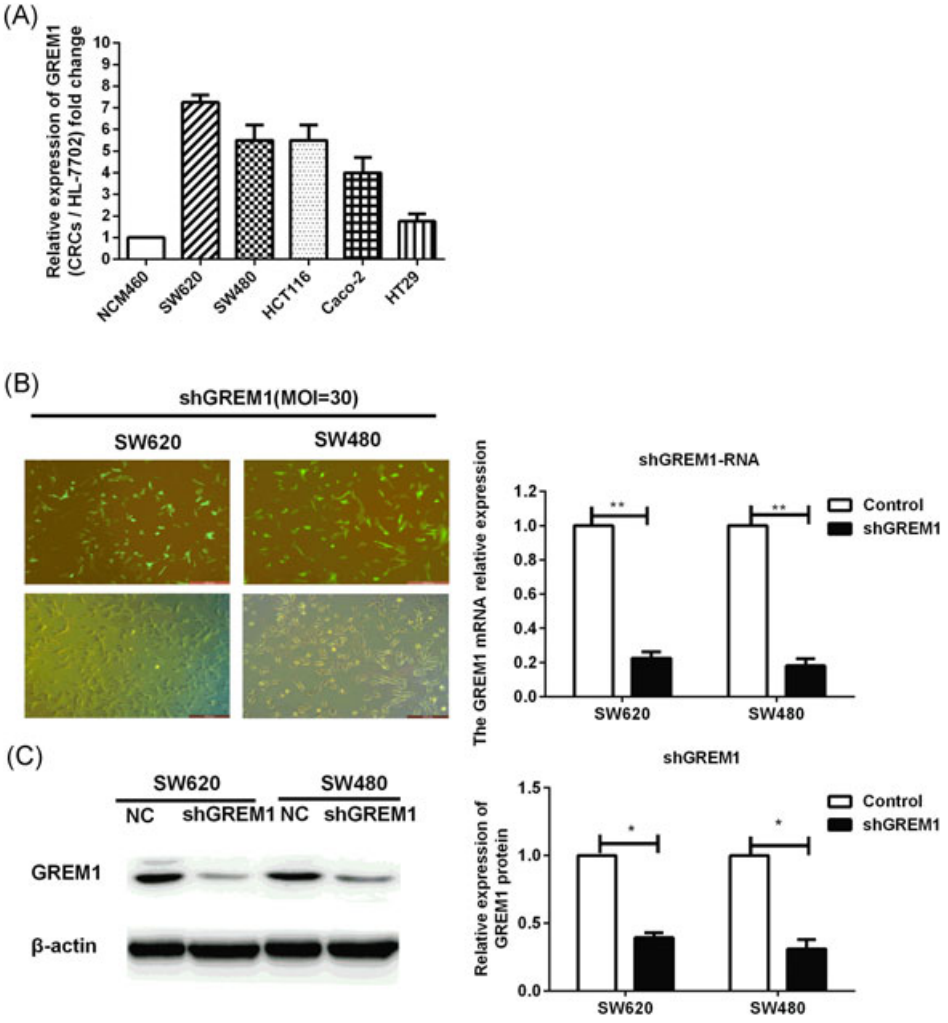

FIGURE 2 GREM1 expression in CRC cells and the validation of shGREM1 lentivirus. A, GREM1 expression in different CRC cells (SW620, SW480, HCT116, Caco-2, and HT29) compared with the human normal colon cell NCM460, which were analyzed by real-time qRT-PCR. B, GREM1 mRNA of CRC cells after transfected by shGREM1 lentivirus with MOI 30 for 72 hours was measured by qRT-PCR. C, Protein level of the GREM1 in CRC cells after transfection with shGREM lentivirus for 72 hours. Similar results were obtained in three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$. CRC, colorectal cancer; GREM1, gremlin 1; MOI, multiplicity of infection mRNA, messenger RNA

further investigate the effect of shGREM1 on the proliferation and migration of CRC cells, as shown in Figure 3A and 3B silencing GREM1 expression by shGREM1 significantly inhibited CRC cells proliferation and migration compared with those of shNC (migration: SW620, NC 207 ± 13.36 vs shGREM1 108 ± 8.08, inhibit the rate of migration: 52.27%; SW480, NC 439 ± 26.21 vs shGREM1 218 ± 10.38, inhibit the rate of migration: 49.74%).

3.3 | Silencing GREM1 repress tumor cell growth by inhibiting cell cycle and inducing apoptosis

To analyze whether the inhibition of shGREM1 on tumor cell growth was related to the cell cycle and apoptosis. As shown in Figure 4A, the number of apoptotic cells of SW620 and SW480 transfected with shGREM1 lentivirus for 72 hours, respectively, significantly increased compared with their controls ($P < 0.01$). Cell cycle analysis on the CRC cell lines indicated that shGREM1 slightly blocked cell cycle progression of SW620 cells at the G0/G1 phase and G2/M phase while for SW480 cells at G0/G1 phase ($P < 0.01$; Figure 4B). These results signified that GREM1 repressed cancer cells growth by inducing apoptosis and blocking cell cycle progression.

It is well known that cell proliferation and apoptosis are linked by cell cycle regulation, so we analyzed whether the inhibition of shGREM1 on tumor cell growth was related to the cell cycle and apoptosis. So we used FACS to analyze the cell cycle and apoptosis rate of SW620 and SW480 cells, which were passaged and cultured for 2 days after constructing stable shGREM1

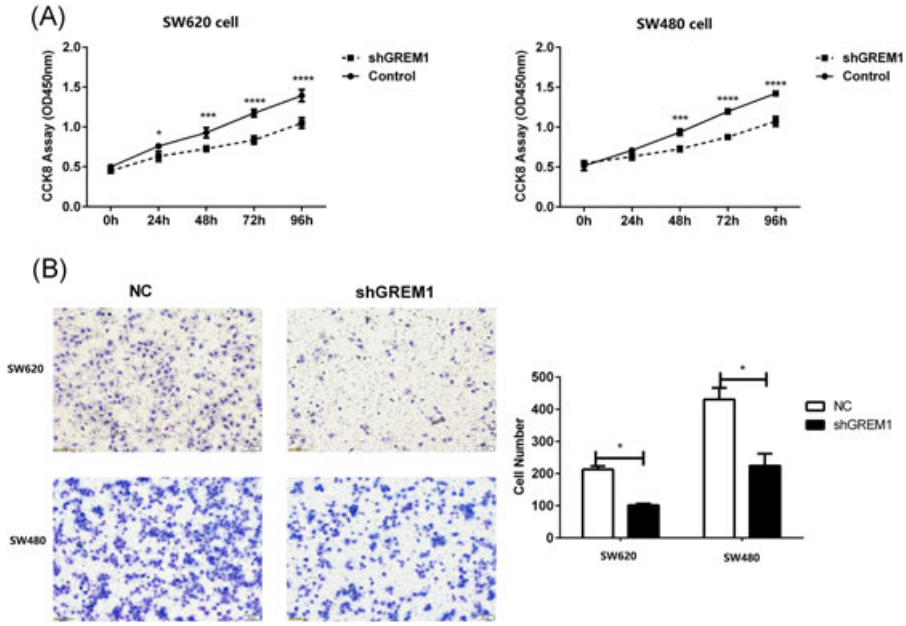

FIGURE 3 Silencing GREM1 suppresses CRC cell proliferation and migration. A, The proliferation of CRC cells (SW620 cells and SW480 cells) were evaluated with Cell Counting Kit-8 assay after transfection with shGREM1 at the indicated time points. B, Transwell migration assays in SW620 and SW480 cells transfected with shGREM1 or NC duplex. The number of cells was calculated with crystal violet staining. Data are shown as mean ± SD ($n = 4$) of one representative experiment. Similar results were obtained in three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, and ****$P < 0.0001$. CRC, colorectal cancer; GREM1, gremlin 1; NC, negative control

CRC cells. As shown in Figure 4A, silencing GREM1 expression in SW620 and SW480 cells induced apoptosis (SW620: shGREM1 14.85 ± 0.35% vs NC 7.75 ± 0.21%; SW480 shGREM1 16.15 ± 0.73% vs NC 8.50 ± 0.42%; Figure 4A). As shown in Figure 4B, flow cytometer further analyzed the influence of GREM1 on cell cycle and found that the cell cycle percentage of SW620 cells, which GREM1 blocked in G1 phase, was higher than the control group (71.92 ± 0.91 vs 64.39 ± 1.45), while in G2/M phase was less (4.23 ± 0.68 vs 9.66 ± 0.62) even after being cultured for 48 hours. Likewise, the result of SW620, the cell cycle percentage of SW480 cells, which GREM1 blocked in G1 phase, was higher than the control group (74.12 ± 1.45 vs 66.31 ± 0.75). These results indicated that suppressed GREM1 expression in CRC cells inhibited tumor cell proliferation by inducing cell cycle distribution blocked in G0/G1 phase.

3.4 | Silencing GREM1 inhibits CRC-induced capillary structure formation and viability of HUVECs

It has been widely demonstrated that GREM1 is a proangiogenesis factor.[5,12,13] So we also detected the effect of tumor-secreted GREM1 on the capillary structure formation of HUVEC. As shown in Figure 5, the ability of CRC-induced capillary structure formation of HUVECs, which was cultured on Matrigel in conditioned media supplemented with 5 ng/mL VEGF and tumor secretion from SW620 and SW480 cells transfected with shGREM1 lentivirus, were both restrained when compared with those of HUVEC cultured in conditioned media supplemented with 5 ng/mL VEGF and tumor secretion from SW620 and SW480 cells transfected with NC. These results suggested that GREM1 is involved with the angiogenesis in CRC.

In fact, the inside of the tumor is a heterogeneous cell group (ie, it contains various cells, such as tumor cells, endothelial cells, and even immune cells). To grow, tumor cells need to generate a capillary network to absorb nutrients. At this time, some cytokines, such as VEGF, PDGF, Gremlin 1, and so on are released to activate the endothelial cells of the tumor microenvironment (including the inside and outside of the tumor) to form a nest near the solid tumor and to form a capillary network to provide nutrition. Our actual tumor cell lines (not the primary tumor tissue) are very pure tumor cells.

3.5 | Silencing GREM1 inhibits spontaneous EMT in colon cancer

Previous studies showed that BMP family has been reported to facilitate EMT, EMT-endowed cells with migratory and invasive properties, induced stem cell properties, prevented apoptosis and senescence, and so on.[14] So we continue to investigate whether GREM1 is involved in the regulation of EMT in colon cancer. Here, we mainly examined the

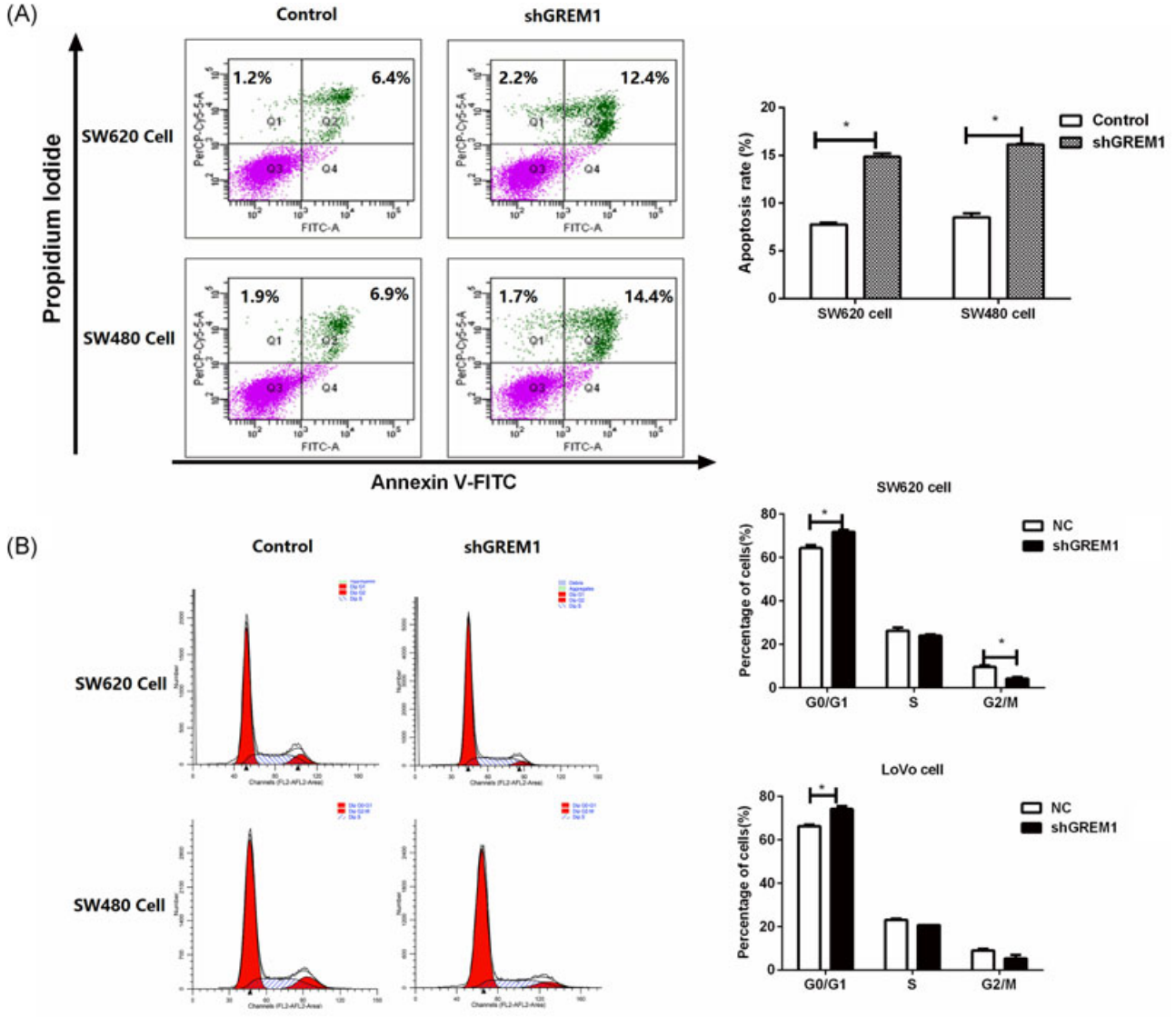

FIGURE 4 Silencing GREM1 induced colorectal cell apoptosis and inhibited cell cycle progression. SW620 and SW480 cells, which were transfected with shGREM1 or NC for 72 hours, were harvested and analyzed by FACS. Tumor cells were stained with annexin V and propidium iodide, and the apoptosis rate (A) measured by FACS. The cell cycle distributions (B) were calculated by FACS and ModFit LT Software (Verity Software House, Topsham, ME). Data are shown as mean ± SD ($n = 3$) of one representative experiment. Similar results were obtained in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. FACS, fluorescence-activated cell sorting; GREM1, gremlin 1

expression of the epithelial cell marker E-cadherin and the mesenchymal marker vimentin in SW620 and SW480 cells after being transfected with shGREM1 lentivirus or not for 72 hours. As shown in Figure 6, the results of cell immunofluorescence and Western blot analysis displayed the expression of E-cadherin, which was significantly upregulated, whereas vimentin was downregulated in both SW620 and SW480 cells even after being transfected with shGREM1 lentivirus for 72 hours. Meanwhile, the cleaved-caspase3 protein was activated both in SW620 and SW480 even after knockdown of shGREM1 with shGREM1 lentivirus. These results demonstrated that GREM1 could modulate EMT positively in human colon cancer cells.

3.6 | ShGREM1 inhibits tumor by downregulation of BMP/SMAD1 and VEGF/MMP2 pathway

To further reveal the functional mechanism of GREM1 in CRC, we first tried to investigate whether silencing GREM1 influenced the downstream signals of the BMP and VEGF pathway in CRC cells. As shown in Figure 7, knockdown GREM1 not only suppressed the phosphorylation of SMAD1/5 but also decreased the metastasis-associated factor CXCL12 protein expression. Moreover, we also found that the shGREM1 suppressed MMP2 protein expression of VEGF pathway downstream signals without any effect on

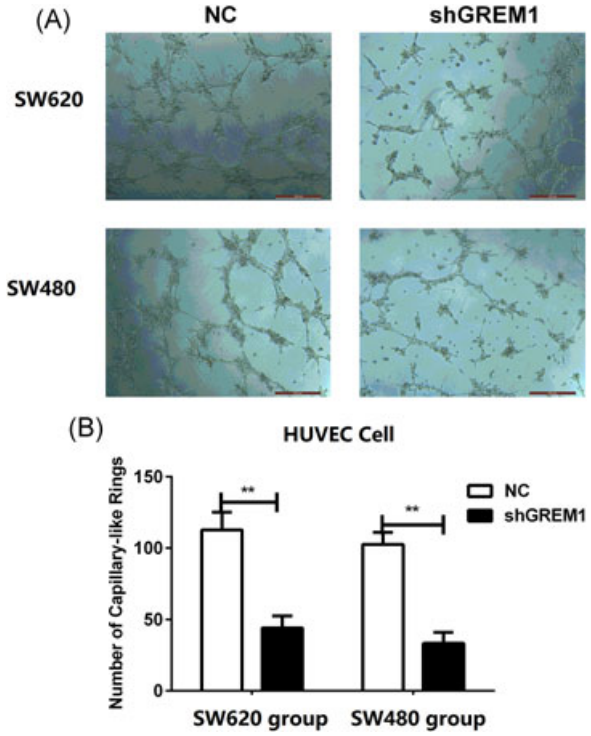

FIGURE 5 Silencing GREM1 inhibited CRC-induced capillary structure formation and viability of HUVECs. HUVECs were cultured on Matrigel in culture-conditioned media supplemented with 5 ng/mL VEGF with tumor extract from the culture medium of SW620 and SW480 cells after being transfected with shGREM1 or NC. The number of capillary-like rings of HUVECs were assessed by the tube formation assays and calculated by the ImageJ software. Representative microphotographs are shown for three different doses. Data are presented as the mean ± standard deviation. $*P < 0.05$ versus the control. CRC, colorectal cancer; GREM1, gremlin 1; HUVECs, human umbilical vein endothelial cells; NC, negative control; VEGF, vascular endothelial growth

the change of VEGF in CRC cells. These data revealed that shGREM exhibited antitumor effect by downregulating the activity of the BMP/SMAD pathway and VEGF/MMP pathway in CRC.

3.7 | Combination treatment of shGREM1 with VEGF inhibitor BAW2881 enhances the inhibition effect of tumor angiogenesis in CRC

Vascular endothelial growth factor (VEGF) has been considered as a very important and powerful proangiogenic factor that involved in primary cancer growth and metastasis process,[11] but the correlation between VEGF with GREM1 has not been investigated in CRC. So, we further explored that knockdown GREM1 combined with VEGF inhibitor would enhance the antiangiogenesis ability in the CRC cells in vitro. Finally, the results showed that combination treatment, shGREM1 with VEGF inhibitor BAW2881 could significantly inhibit the formation of capillary-like rings of HUVECs compared with shGREM1 or BAW2881 alone (Figure 8). These findings hinted that CRC might secrete GREM1 to promote tumor growth and angiogenesis via a VEGF-independent pathway.

4 | DISCUSSION

It is well known that TGF-β/BMP signaling pathway plays an important role in the development of bone and intestinal hemostasis.[15] Although the loss of BMP and increased expression of GREM1 caused the formation of intestinal juvenile polyps in the colon cancer-prone syndrome familial juvenile polyposis,[16,17] little has been known of the significance of GREM1 expression and its relative function in CRC. Here we found the majority of primary CRC tissues expressed a high level of GREM1 mRNA compared with adjacent normal tissues and the GREM1 mRNA expression was correlated with low histological grade development and stages 2 to 3 metastatic recurrences in CRC. Simultaneously, we verified the expression of GREM1 in different colon cancer cells and found that GREM1 was highly expressed in mesenchymal-like colon cancer cells compared with the epithelial-like colon cancer cells and normal colon cell. Functional studies also displayed that shGREM1 could repress CRC cells proliferation, migration, angiogenesis, and EMT by downregulated BMP/SMAD and VEGF-independent pathway. These experiments demonstrated that GREM1 was involved in CRC development and progression, and provided a new idea for CRC diagnosis and resistance therapy and prognosis. There are no endothelial cells at all, and it is impossible to automatically form a microvascular environment.[18,19] These laboratories have specifically tested them. Therefore, experiments using human venous endothelial tube formation experiments to evaluate tumor-induced angiogenesis have been conducted. Venous endothelial cells naturally required growth factors, such as VEGF to promote their proliferation and loop formation. The VEGF added in this experiment was very minimum, and it was directly cultured with the tumor supernatant. There was a concern that there was no factor at all and the cell death was too fast. So this experiment included the addition of VEGF inhibitors later.

In CRC, BMP signaling has been regarded as generally portraying tumor-suppressive capabilities, such as reducing cancer cell proliferation, invasion, motility, and antagonizing EMT.[20-22] In contrast, GREM1, as an antagonist of BMPs, preferentially shifted the differentiation state of

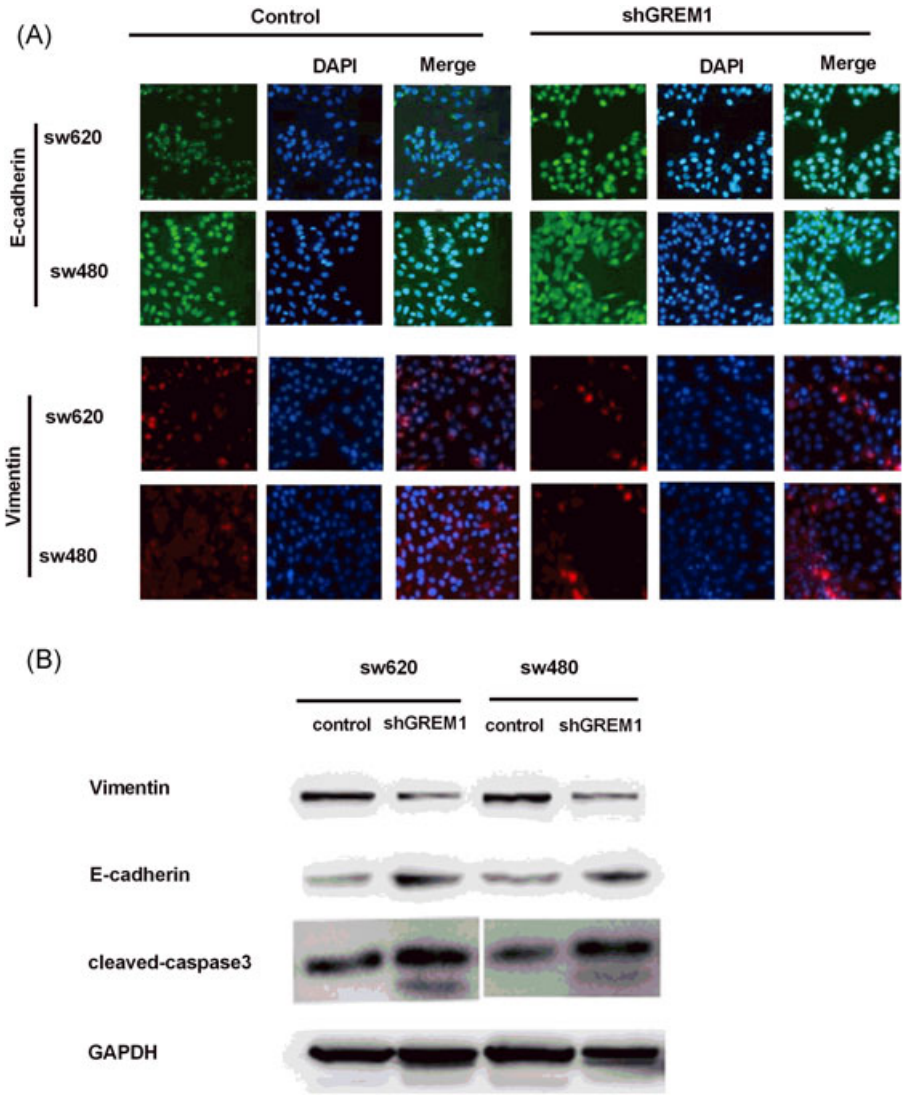

FIGURE 6 Silencing Grem1 inhibited spontaneous EMT in colon cancer. A, The expression of E-cadherin and vimentin in CRC cells, after being transfected with shGREM1 lentivirus or not for 72 hours, was detected by cell immunofluorescence. B, The protein expression of E-cadherin, vimentin, cleaved-caspase3, and GAPDH in CRC cells, transfected with shGREM1 lentivirus or not for 72 hours, was analyzed by Western blot analysis. CRC, colorectal cancer; EMT, epithelial-mesenchymal transition; GREM1, gremlin 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

cancer cells toward a more mesenchymal-like and stem-like phenotype, which might also promote their maintenance within the tumor hierarchy.[4,23] To investigate the problem, our results showed that GREM1 was highly expressed in mesenchymal-like colon cancer cells, such as SW620 and SW480, whereas the expression of GREM1 in the epithelial-like colon cancer cells (Caco-2 and HT29) was less than mesenchymal-like colon cancer cells. These results hinted that GREM1 had the ability of self-renewal in the cancer cell. Moreover, there was a report that showed that secretive GREM1 from cancer stem cells (CSC) could prevent the BMP differentiating effect in glioblastoma.[24] These findings suggested that GREM1 was also involved with the EMT program. According to Singh et al's[25] review, EMT is generally considered to be a relevant molecular event in malignant cancer. Hence, therapies with an anti-EMT potential is a promising direction for the development of glioma therapy.[26] The role of GREM1 in the EMT process of colon cancer cells was investigated in the current study. Our data demonstrated that GREM1 knockdown led to an increased ratio of E-cadherin to N-cadherin in SW620 and SW480 colon cancer cells, which indicated an inhibition in the EMT process. This study aimed to reveal the molecular pathway through which GREM1 played a regulatory role in EMT. It had been demonstrated that EMT-endowed tumor cells with migratory and invasive properties, induced cancer stem cell properties, prevented apoptosis and senescence. So, we further investigated whether silencing GREM1 induced colon cancer cell apoptosis. Consistent with our assumptions, knockdown GREM1 increased the apoptotic rate and cleaved-caspase3 apoptotic protein expression in colon cancer cells. These results were consistent with previous studies, which showed knockdown GREM1 expression impaired the EMT process in cancer cells.

Considering the increasing evidence suggesting that activated VEGFR2 could mediate activation of several protein kinase pathways and thus regulate cell

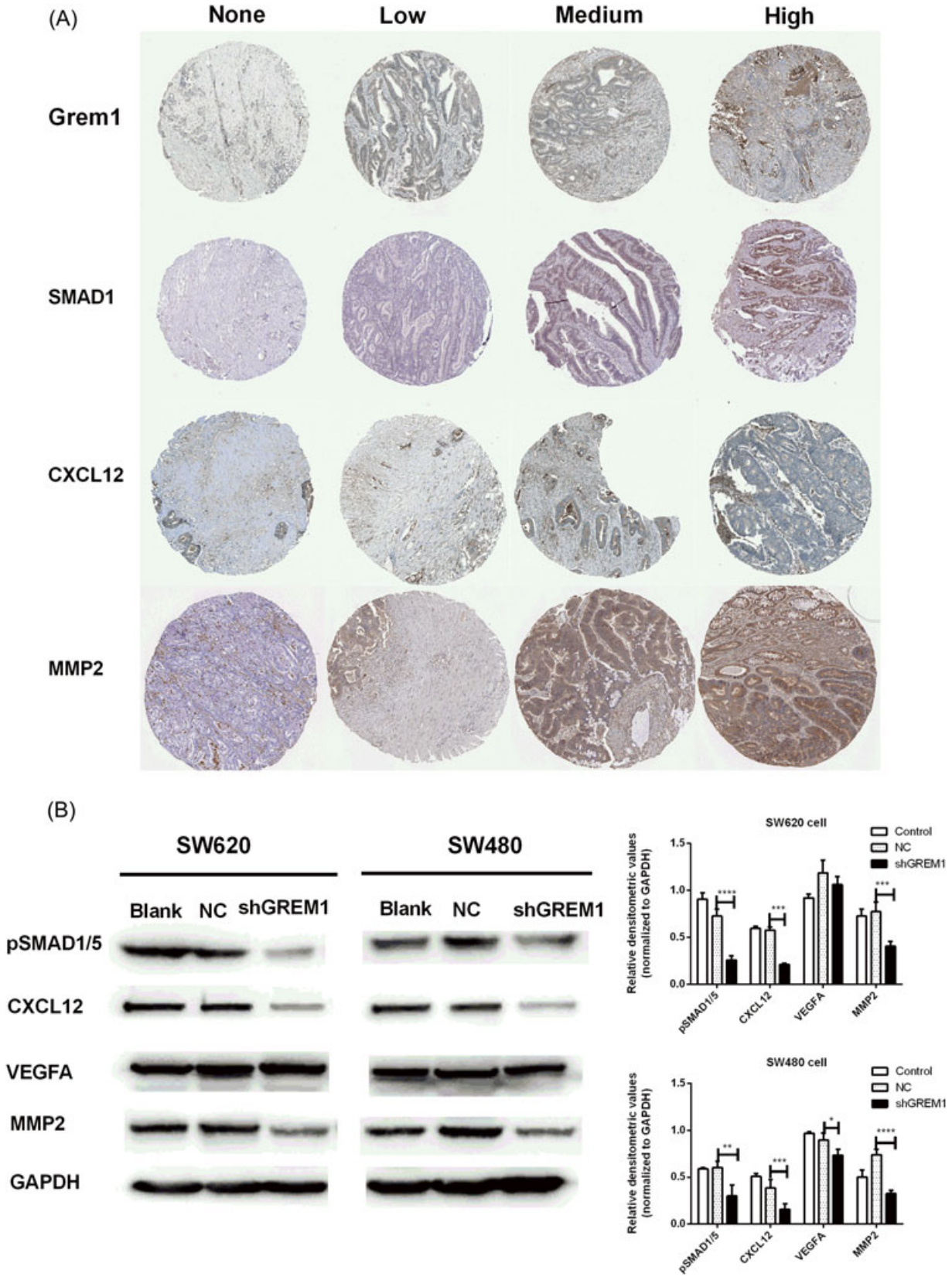

FIGURE 7 Continued.

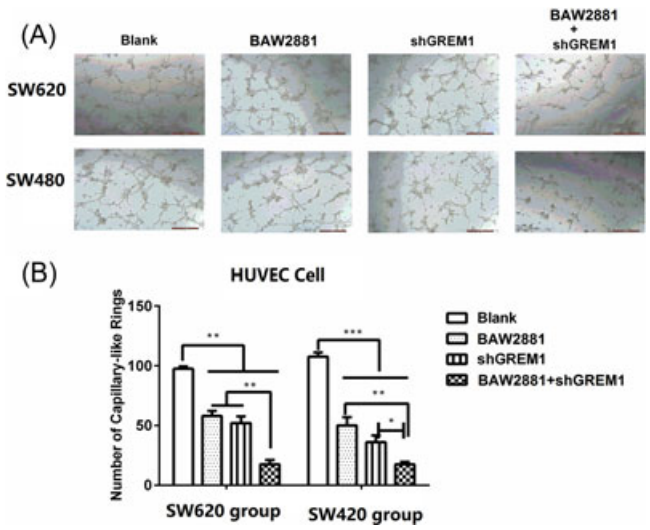

FIGURE 8 shGREM1 combined with VEGF inhibitor BAW2881 enhanced inhibition effect of tumor angiogenesis in CRC. HUVECs were cultured on Matrigel in culture-conditioned media from SW620 to SW480 cells after being transfected with shGREM1 and VEGF inhibitor BAW2881 or alone for 72 hours. The number of capillary-like rings of HUVECs were assessed by tube formation assays and calculated by the ImageJ software. Representative microphotographs are shown for three different doses. Data are presented as the mean ± SD. $^{*}P < 0.05$ versus the control. CRC, colorectal cancer; HUVECs, human umbilical vein endothelial cells

proliferation,[27,28] we could relate the mechanism of GREM1 regulating the development of cancer to BMP/SAMD and VEGF-related downstream pathway. As expected, we found there were obvious changes in pSMAD1 protein expression and metastasis-associated chemokine CXCL12 in SW620 and SW480 cells after being transfected with shGREM1 lentivirus. In contrast, the protein expression of VEGF in SW620 and SW480 cells showed no significant change, whereas MMP2, which was an important VEGF pathway, downstream signals significantly decreased in SW620 and SW480 cells after being transfected with shGREM1 lentivirus. Furthermore, we used VEGF inhibitor BAW2881 combined with shGREM1 to detect the combined effect on antiangiogenesis in CRC. The results demonstrated that combination treatment with VEGF inhibitor BAW2881 and shGREM1 lentivirus on CRC cells could more effectively repress the tube formation of capillary-like rings of HUVECs compared with shGREM1 or BAW2881 alone. These findings hinted that CRC could secrete GREM1 to promote tumor growth and angiogenesis by the VEGF-independent pathway. Our study did not evaluate expression levels of GREM1 in HUVEC cells, but the same cells and the same culture conditions could offset the problem that HUVEC might also autocrine GREM1. However, if HUVEC could secrete factors similar to VEGF, it is estimated that it could grow self-sufficiently. The primary HUVEC, cultured without factoring, using RPMI 1640 + 10% FBS deteriorated in less than a week, and all the cells died.

Originally, the study was focused on GREM1, which was thought to promote angiogenesis and metastasis through unconventional VEGF pathway, and actually began to use targeted drugs. Then, it was found that it did not study the basic functions of colon cancer, and it was part of it. Initially, many targeted treatments for metastatic colon cancer were poor, especially anti-VEGF, a targeted drug, and the cure rate was still poor in 30% to 40% of the patients. Therefore, it was observed that the presence of part of GREM1 caused the anti-VEGF cure rate to be unsatisfactory. And many studies had confirmed that GREM1 was already promoting angiogenesis. In the clinical aspect, in fact, many targeted drugs are not ideal for the later stage. Antiangiogenesis is both beneficial and disadvantageous in the basic theory of tumors. Blocking angiogenesis, although slowing the tumor growth, also leads to some medical treatments, such as chemotherapy, intervention, and sometimes antiangiogenesis; some drugs cannot reach the inside of the tumor. Therefore, the focus here was whether or not GREM1 continued to affect angiogenesis through the bypass pathway. The results showed that the GREM1 mRNA of stages 2 to 4 was not only highly expressed than that of stage 1 but also gradually increased the metastatic recurrence of the tumor with the histological grades progression. Though the GREM1 expression of stages 3 to 4 of primary CRC was lower than that of stage 2, which might be because the number of samples in stage 3 and stage 4 was too small. In this study, we provided novel evidence that GREM1 was highly expressed in mesenchymal-like colon cancer cells compared with epithelial-like colon cancer cells. Silencing GREM1 expression suppressed colon cancer cell proliferation, migration, angiogenesis, and EMT by repressing BMP/SAMD pathway and VEGF-independent pathway. These findings

FIGURE 7 ShGREM1 inhibited CRC by downregulation of BMP/SMAD1 and VEGF/MMP2 pathway. A, The protein expression of GREM1, SMAD1, CXCL12, and MMP2 has been proofed and highly expressed in CRC tissue chips, while hardly being expressed in normal tissue chips base on Human Protein Altas database. B, Protein levels of GREM1 and the indicated BMP/SAMD1 and VEGF/MMP pathway members were analyzed in CRC cells after transfected with shGREM1 or NC for 72 hours. Data are shown as mean ± SD ($n = 4$) of one representative experiment. The images are representative of one experiment. Similar results were obtained in three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$. CRC, colorectal cancer; NC, negative control

suggested a potential clinical use of secretive GREM1 for CRC diagnosis and targeted therapy invention.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest. All authors hereby disclose any commercial associations that may pose or create a conflict of interest with the information presented in this manuscript.

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