# High glucose induces epithelial-mesenchymal transition and results in the migration and invasion of colorectal cancer cells

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**Abstract.** Diabetes mellitus (DM) is associated with an increased risk of colorectal cancer (CRC). Hyperglycemia, a chronic abnormality in diabetes, is an independent predictor of cancer-associated mortality in CRC. However, the underlying biological mechanism of hyperglycemia in CRC cells is largely unknown. In the present study, HCT-116 and HT-29 cell proliferation, apoptosis, migration and invasion were assessed. In addition, the expression of epithelial (E)-cadherin, vimentin and high-mobility group A protein 2 (HMGA2) were assessed using western blotting. The results demonstrated that high glucose (HG; 30 mmol/l) caused CRC cells to lose their epithelial morphology, with a decrease in E-cadherin and an increase in vimentin, suggesting epithelial-mesenchymal transition (EMT). Furthermore, HG significantly enhanced the cell migration and invasion of CRC cells and the expression of HMGA2. Transfection with HMGA2 small interfering RNA reversed the HG-induced changes to CRC cells. In addition, HG promoted CRC cell proliferation and suppressed apoptosis. The results of the present study suggest that hyperglycemia promotes EMT, proliferation, migration and invasion in CRC cells and may provide novel insights into the link between HG and CRC.

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*Abbreviations:* DM, diabetes mellitus; CRC, colorectal cancer; EMT, epithelial-mesenchymal transition; HMGA2, high-mobility group A protein 2; DMEM, Dulbecco's modified Eagle's medium

*Key words:* colorectal cancer, epithelial-mesenchymal transition, high glucose

## Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors and the leading cause of cancer-associated mortality in humans (1). CRC is the third most commonly diagnosed cancer in males and the second in females, with an estimated 1.4 million cases and 693,900 deaths occurring worldwide in 2012 (2). In the USA, CRC is the third leading cause of cancer-associated mortality (3), while tumor invasion and metastasis are the leading causes of patient mortality (4). Many CRCs are metastatic at the time of diagnosis (5). Diabetes mellitus (DM) is a metabolic disorder characterized by increased blood glucose levels (6) and is considered to be one of the most important health problems worldwide (7). It has been demonstrated that DM is associated with an elevated risk of CRC in both men and women (8). A meta-analysis of 8 studies identified a positive correlation between type 2 (T2)DM with a 1.21-fold increased risk of CRC (9). Patients with colorectal cancer and DM have an increased risk of cancer-specific mortality and have worse disease-free survival than those who do not have DM (10,11). DM has also been reported to be a risk factor for CRC, although this remains controversial (11-14).

Epithelial-mesenchymal transition (EMT) is the morphological transformation of epithelial-like cancer cells to an elongated mesenchymal phenotype (15). During EMT, cancer cells stop expressing adhesion proteins, including epithelial (E)-cadherin and claudin-1, and increase the expression of mesenchymal phenotype markers, including vimentin, neural (N)-cadherin and Snail (16). EMT serves an important role in the invasion and metastasis of CRC (17) and is able to induce circulating tumor cell properties in transformed colorectal epithelial cells (18). Furthermore, EMT is highly prognostic for colon cancer recurrence (19). High glucose (HG) induces EMT in breast cancer cells (20) and human peritoneal mesothelial cells (21); however, this effect has not been studied in CRC.

The aim of the present study was to investigate the association between HG and the migration, invasion and apoptosis of colorectal cancer cells. The expression of EMT-associated proteins was detected and the underlying mechanisms were investigated.

## Materials and methods

*Cell culture and transfection*. The human CRC cell lines HCT-116 and HT-29 were obtained from American Type Culture Collection (Manassas, VA, USA). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Genom Biotech Pvt., Ltd., Bhandup, Mumbai) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA), 100 unit/ml penicillin, 100 $\mu$g/ml streptomycin with normal glucose (NG; 5.5 mmol/l) or HG (30 mmol/l). Cultures were maintained at 37˚C in a humidified atmosphere containing 5% $CO_2$.

*Human samples*. A total of 6 CRCs with or without T2DM in this study were histologically and clinically diagnosed at Ningbo Urology and Nephrology Hospital between October 2015 to March 2016 and the tissues were collected immediately following surgical resection for diagnosis. The inclusion criteria was as follows: i) Patients had to be diagnosed with CRC by preoperative pathological biopsy using a colonoscope; ii) aged between 18 and 75 years; iii) exhibit no distant metastasis; and iv) with or without diabetes. Patients were excluded if they: i) Received radiotherapy and chemotherapy prior to surgery; ii) exhibited acute infection; or iii) had a history of abdominal surgery or other malignant tumors. The specimens were then stored at -80˚C. The present study was approved by Ningbo Yinzhou Ethics Committee and signed informed consent was obtained from the patients or their family. Patient data is summarized in Table I.

*Immunofluorescence*. CRC tissues were fixed in 4% formaldehyde solution for 2 h at 25˚C and then sectioned into 5-$\mu$M-thick frozen sections. The sections were washed in cold PBS 3 times and subsequently blocked with 2% bovine serum albumin V at 25˚C (BSA-V; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 1 h. Samples were incubated with primary antibodies against E-cadherin (1:20; sc-8426; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) diluted with 1% BSA-V overnight at 4˚C. Following 3 washes with PBS, the sections were incubated with tetramethylrhodamine conjugated goat anti-rabbit secondary antibody (1:1,000; sc-362281; Santa Cruz Biotechnology, Inc.) diluted with 1% BSA-V in the dark for 1 h at 25˚C and washed in PBS again for 3 times. DAPI diluted with PBS was used to stain the nuclei at 25˚C. Images at a magnification of x40 were captured using an inverted fluorescence microscope (Nikon Corp., Tokyo, Japan).

*Western blotting*. Tissues and cells were homogenized in a radioimmunoprecipitation assay lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). A BCA protein assay kit (Cwbiotech, Beijing, China) was used to determine protein concentrations. Proteins (20 $\mu$g) were separated by 10% SDS-PAGE and electrotransferred to polyvinylidene fluoride membranes, which were blocked in 5% nonfat milk for 1 h at 25˚C and probed with primary antibodies against E-cadherin (1:400; #AF0131; Affinity Biosciences, Jiangsu, China), vimentin (1:600; #AF0292; Affinity Biosciences), GAPDH (1:10,000; #T0004; Affinity Biosciences) and high-mobility group A protein 2 (HMGA2; 1:500; 5269s; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. The membranes were subsequently incubated with goat anti-rabbit antibody diluted with 0.3‰ TBST (1:1,000; #SC2004; Santa Cruz Biotechnology, Inc.) or goat anti-mouse antibody diluted with 0.3‰ TBST (1:1,000; #SC2005; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The membranes were scanned with the Tanon 5200 automated image analysis system (Tanon, Shanghai, China) and the ImageJ software (version 1.48U; National Institutes of Health, Bethesda, MD, USA) was used to evaluate the band intensity.

*Scratch assay*. HCT-116 and HT-29 cells (3-5x$10^5$ cells/well) were seeded in 6-well plates and cultured in DMEM containing NG or HG for 4 days at 37˚C. Confluent cultures were scratched with sterile 200 $\mu$l pipette tips and washed gently with PBS to remove floating cells. Then the cells were cultured in DMEM containing NG or HG and 5% FBS. Cells were viewed under an inverted fluorescence microscope (magnification, x40) and images were captured after 0, 24, 48 and 72 h.

*Transwell assays*. HCT-116 and HT-29 cells were cultured in DMEM containing NG or HG for 4 days and then serum-starved for 12 h. The cells (1x$10^5$ cells/well) were seeded into Boyden chambers (EMD Millipore, Billerica, MA, USA) with 8-$\mu$m pore size filter membranes. The inserts were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) for invasion assays or not coated for migration assays. The chambers were then placed in 24-well plates containing DMEM and 10% FBS at 37˚C. After 72 h, the non-invaded cells on the upper side of the filter were removed with a cotton swab and cells attached to the underside of the membrane were fixed in ethanol, stained with crystal violet and images were counted using a microscope (CKX41; Olympus Corporation, Tokyo, Japan; magnification, x40).

*HMGA2 knockdown*. The RNA interference technique was used to downregulate HMGA2 in HCT-116 and HT-29 cells (22). HMGA2 small interfering 400 ng (si)RNAs (siHMGA2-1, 5'-GAAAGCAGAGACCAUUGGATT-3'; siHMGA2-2, 5'-GAAAGCAGAGACCAUUGGATT-3'; Shanghai Genechem Co., Ltd., Shanghai, China) were synthesized and transfected into cells using RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Following 48 h transfection, HMGA2 expression was confirmed by western blotting. Western blotting was then performed as aforementioned.

*MTT assay*. HCT-116 and HT-29 proliferation was measured using an MTT assay. Cells were incubated with 0.35 mg/ml MTT solution at 37˚C for 4 h. The medium was removed, 100 $\mu$l dimethylsulfoxide (DMSO) was added and the mixture was vortexed at 112 x g for 10 min at 25˚C. The optical density was read at 490 nm and all experiments were performed 3 times.

*Ki-67 expression and apoptosis analysis*. Cells were seeded in 6-well plates and treated with NG or HG, respectively, for 4 days. Cells were digested using 1 ml trypsin (#C0201; Beyotime Institute of Biotechnology, Beijing, China), washed twice with PBS and incubated in 100 $\mu$l fixation buffer (Biolegend, Inc., San Diego, CA, USA) at room temperature

Table I. Patient data.

| Patients | Number of patients | Age | Sex ratio (F:M) | Comorbidities |
|---|---|---|---|---|
| With diabetes | 3 | 56-64 | 2:1 | No comorbidities |
| Without diabetes | 3 | 60-65 | 2:1 | One with hypertension |

for 15 min. Cells were then washed with 100 $\mu$l permeabilisation buffer (Biolegend, Inc.). Following centrifugation at 1,500 x g for 3 min at 25˚C, the cells were resuspended in 100 $\mu$l permeabilisation wash buffer containing Alexa Fluor 647 mouse anti-Ki-67 antibody (1:100; 561126; BD Biosciences) and incubated at room temperature in the dark for 30 min. A total of 400 $\mu$l permeabilisation wash buffer was added to resuspend the cells for flow cytometric analysis using a FACS flow cytometer (BD Biosciences).

Cell apoptosis was assayed using the Annexin V-phycoerythrin (PE) Apoptosis Detection kit (BD Biosciences). Cells were washed twice with cold PBS and resuspended in Annexin V Binding buffer at a concentration of $1.0x10^6$ cells/ml. Specifically, this suspension (100 $\mu$l) comprised 1 $\mu$l Annexin V-PE, 1 $\mu$l 7-aminoactinomycin D and 98 $\mu$l Binding buffer. The cells were vortexed gently and incubated for 15 min at room temperature in the dark. To each tube, 400 $\mu$l of Binding buffer added and cells were analyzed using a FACS flow cytometer (BD Biosciences) and FlowJo 7.6 software (FlowJo LLC, Ashland, OR, USA).

*Statistical analysis*. Data are expressed as the mean ± standard deviation. One-way analysis of variance was used to test the Homogeneity of variance, then a Mann-Whitney U was used to compare differences between groups. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*HG induces EMT in CRC tissues and cells*. E-cadherin protein expression was measured in tumor tissues from 3 patients with CRC and DM and 3 patients with CRC without DM using immunofluorescence and western blotting. The area of tumor cells with positive E-cadherin staining was increased in patients without DM compared with those with DM (Fig. 1A). Furthermore, the results of western blotting confirmed that the expression of E-cadherin protein was lower in patients with DM compared with patients without DM (Fig. 1B); however, the expression of vimentin protein was significantly higher in patients with DM compared with those without DM (Fig. 1B). HCT-116 and HT-29 cells were exposed to HG for 4 days and it was demonstrated that HG reduced the expression of E-cadherin protein, whereas the expression of vimentin protein was increased (Fig. 1C). These results suggest that HG serves an important role in the EMT of CRC cells.

*HG promotes the migration and invasion of CRC cells*. EMT is characterized by a loss of cell-to-cell adhesion and increased cell migration and invasion (23). As such, the effect of HG on the metastatic capability of CRC cells was investigated. Scratch assays revealed that wound healing was faster in HCT-116 and HT-29 cells grown in HG conditions compared with those grown in NG (Fig. 2A and B). Furthermore, HG accelerated the cells ability to invade and migrate compared with NG (Fig. 2C and D). These results suggest that HG is able to promote the invasion and migration of CRC cells.

*HG promotes EMT by increasing the level of HMGA2 protein*. HMGA2 is known to control the expression of a diverse set of transcription factors associated with the regulation of E-cadherin transcription (24,25). HMGA2 has been reported to regulate EMT in gastric cancer (26,27), tongue squamous cell carcinoma (28) and prostate cancer cells (29). As such, it was hypothesized that HMGA2 may regulate HG-induced EMT and the expression of HMGA2 in CRC cells exposed to HG or NG for 4 days was assessed. HMGA2 was significantly upregulated in HG-stimulated cells compared with those treated with NG (Fig. 3A and B). HMGA2 expression was knocked down in HCT-116 and HT-29 cells and confirmed used western blotting (Fig. 3C and D). The results revealed that HMGA2 knockdown significantly increased E-cadherin protein expression and decreased vimentin protein expression in HG-stimulated cell compared with those treated with NG (Fig. 3E and F).

*HG enhances cell viability and suppresses apoptosis in CRC cells*. To characterize the functional roles of HG in cell proliferation, MTT assays were performed and Ki-67 was measured. The results revealed that HG enhances the viability of HCT-116 and HT-29 cells in a time-dependent manner (Fig. 4A and B). Ki-67 is a nuclear antigen present only in proliferating cells and is one of the most widely used proliferation-associated markers in cancer cells (30). Ki-67 staining demonstrated that HG enhances the expression of Ki-67 and therefore the proliferation of HCT-116 and HT-29 cells compared with NG (Fig. 4C and D). The role of HG on apoptosis in HCT-116 and HT-29 cells was also assessed and it was revealed that HG significantly decreased apoptosis compared with HG (Fig. 4E and F).

## Discussion

Impaired metabolism and unlimited growth are two hallmarks of cancer and serve an important role in cancer progression (31) and DM promotes the growth and metastasis of tumor cells (32). The results of the present study demonstrate that HG increases HMGA2 expression and induces EMT in CRC cells.

The invasive and migratory capabilities of CRC cells were significantly enhanced by HG, while transfection with

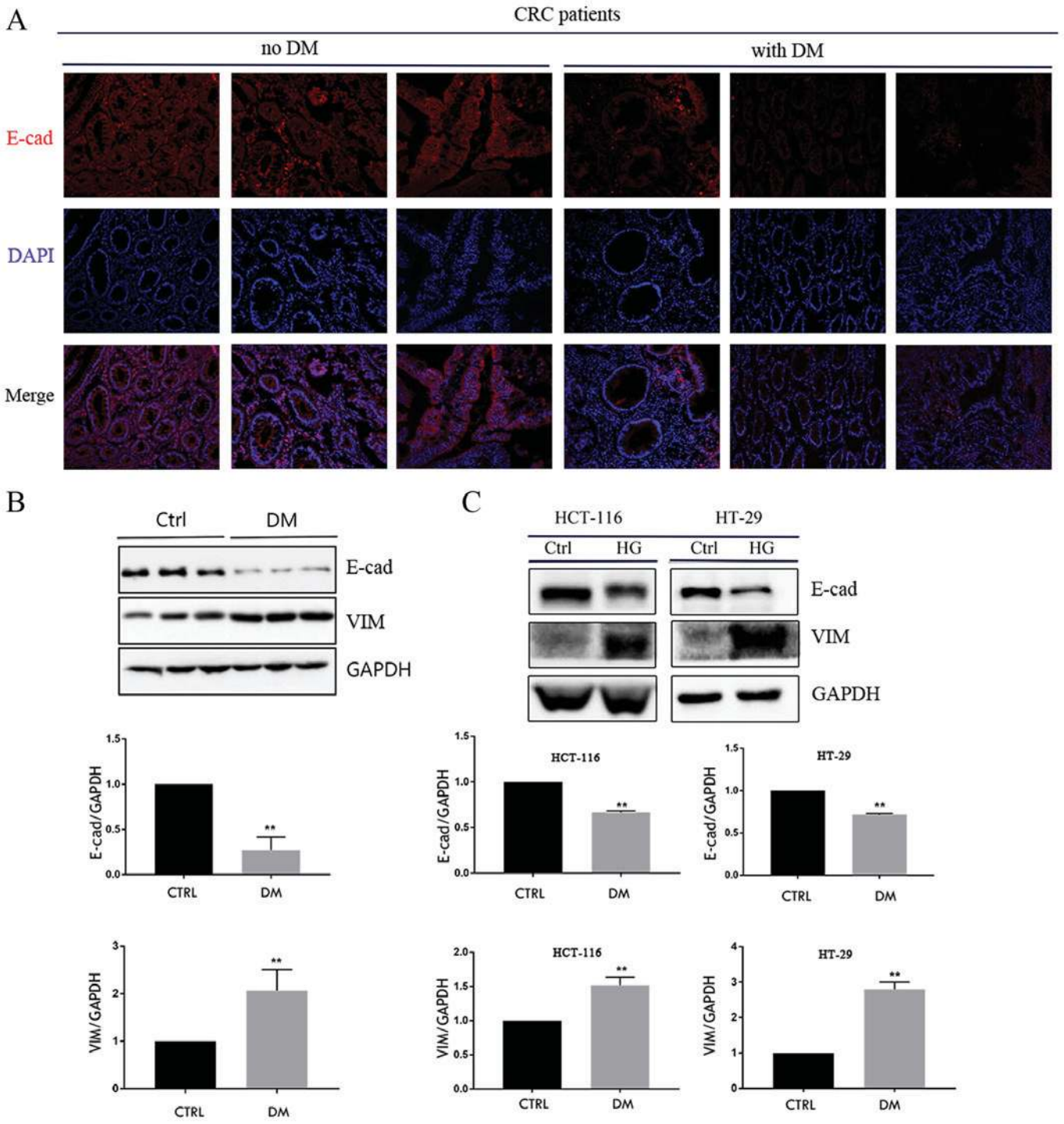


Figure 1. HG promotes epithelial-mesenchymal transition in patients with CRC and CRC cells. (A) Immunofluorescence for E-cad in tumors from patients with CRC with or without DM (magnification, x40). Western blot analyses of E-cad and VIM in (B) CRC tissues and (C) HCT-116 and HT-29 cells. $^{**}$P<0.01 vs. Ctrl. HG, high glucose (30 mmol/l); CRC, colorectal cancer; E-cad, epithelial cadherin; DM, diabetes mellitus; VIM, vimentin; Ctrl, control.

HMGA2 siRNA suppressed HG-induced EMT in HCT-116 and HT-29 cells. In addition, HG enhanced the proliferation and reduced the apoptosis of CRC cells. These results suggest that DM causes EMT and promotes metastasis in CRC cells. As such, DM may induce CRC tumor growth.

DM has been reported to have pro-migratory and pro-invasive effects in both normal (33) and cancer cells (34-38). Epidemiological studies have previously established an association between inflammation and DM (39-41). The chronic inflammatory response may contribute to DM development by causing insulin resistance, which in turn intensifies hyperglycemia to promote long-term complications of diabetes (42). Furthermore, inflammation induces EMT in CRC (43,44). Previous research has verified that HG induces EMT in pancreatic and breast cancers (45). Similarly, the results of the present study demonstrate that DM is associated with the downregulation of E-cadherin and upregulation of vimentin in patients with CRC. Meanwhile, HG induces EMT in CRC

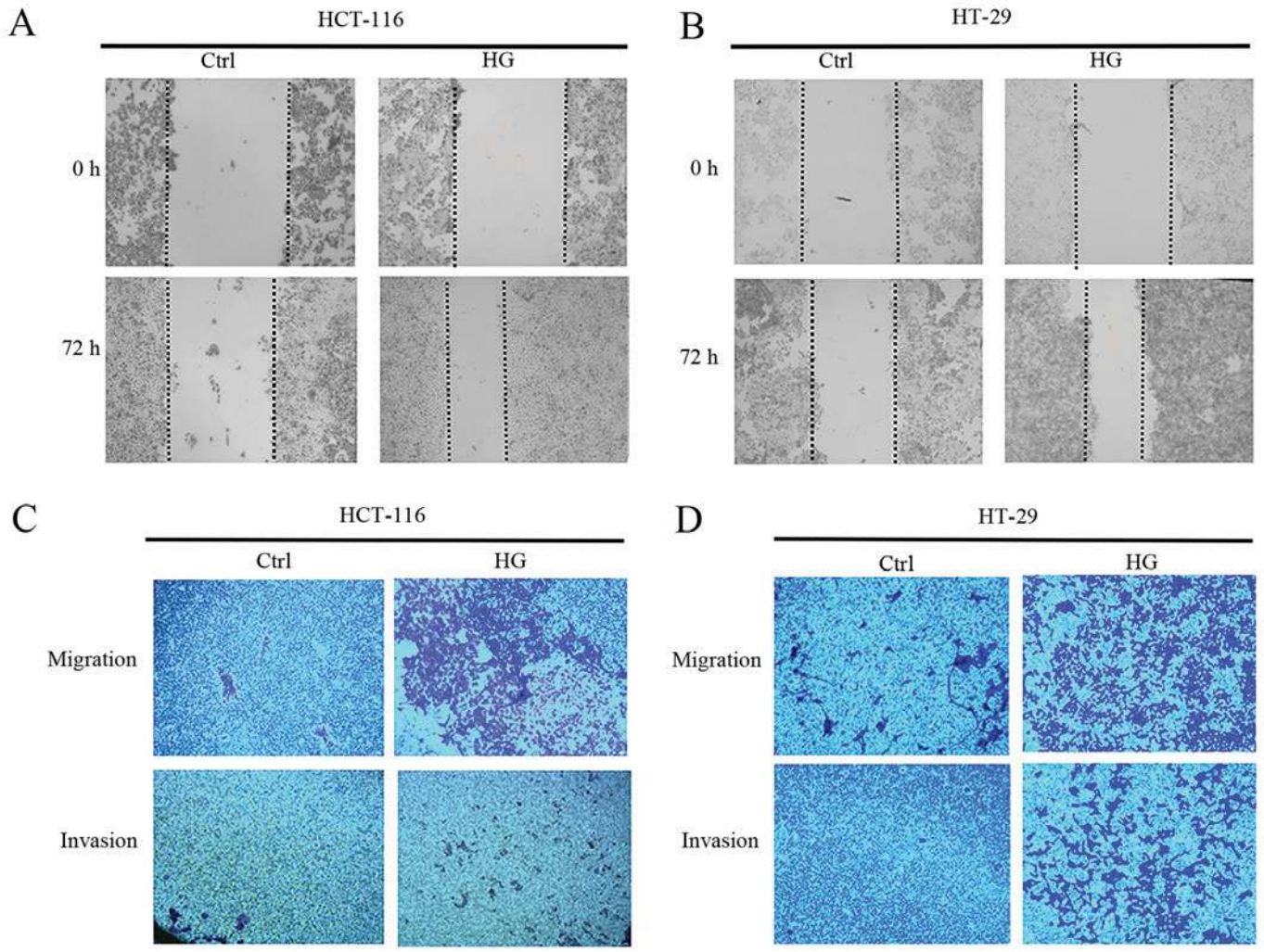


Figure 2. HG promotes the migration and invasion of HCT-116 and HT-29 cells. The migratory abilities of (A) HCT-116 and (B) HT-29 cells were determined using a scratch assay following HG stimulation (magnification, x40). A Transwell assay was also performed to assess the migration and invasion abilities of (C) HCT-116 and (D) HT-29 cells (magnification, x40). HG, high glucose; Ctrl, control.

cells *in vitro*. Downregulated E-cadherin expression is associated with lymph node metastases, poor tumor differentiation and worse prognosis in patients with CRC (46,47). Conversely, increased vimentin expression is significantly associated with lymph node metastasis and poor prognosis in CRC (48). The results of the present study demonstrated that the invasion and migration capabilities of CRC cells were enhanced by the occurrence of EMT. HMGA2 is a chromatin remodeling factor that is able to alter chromatin architecture to activate transcriptional enhancers (49). High expression of HMGA2 is associated with cell proliferation and increased metastasis in a number of cancers (50). The results of the present study are consistent with a number of previous studies in which it was reported that HMGA2 activates EMT in cancer cells (51,52). At least 11 EMT-associated molecular pathways have been reported in the literature about CRC cells, including β-catenin-associated EMT, transforming growth factor-β and Wnt pathway-associated EMT and aberrant NOTCH-1 signaling associated EMT (53) Future studies should aim to elucidate whether there any other signaling pathways are associated with HG-induced EMT.

HG in patients with DM may alter the expression of genes that promote cell proliferation in the colon (32-37,54). The rate of proliferating cell nuclear antigen-positive cells is higher in patients with CRC and DM compared with patients with CRC alone (55). HG conditions enhance cell proliferation via decreasing the population of cells arrested in the G0/G1 phase (56). In accordance with the results of the present study, HG has previously been reported to increase the proliferation of CRC cells (57).

The present study is not without limitations. The effect of HG, which is the main feature of DM, was studied in isolation. T2DM is typically accompanied by other metabolic abnormalities, including hyperlipidemia and hyperinsulinemia (58,59). These abnormalities should be considered in future studies.

In summary, the results of the present study indicate that hyperglycemia is associated with a reduction in epithelial markers and an increase mesothelial markers in CRC. The HG-induced enhanced migratory and invasive abilities of CRC cells may be attributed to EMT via the upregulation of HMGA2. The results of the present study may provide novel insights into the association between DM and CRC.

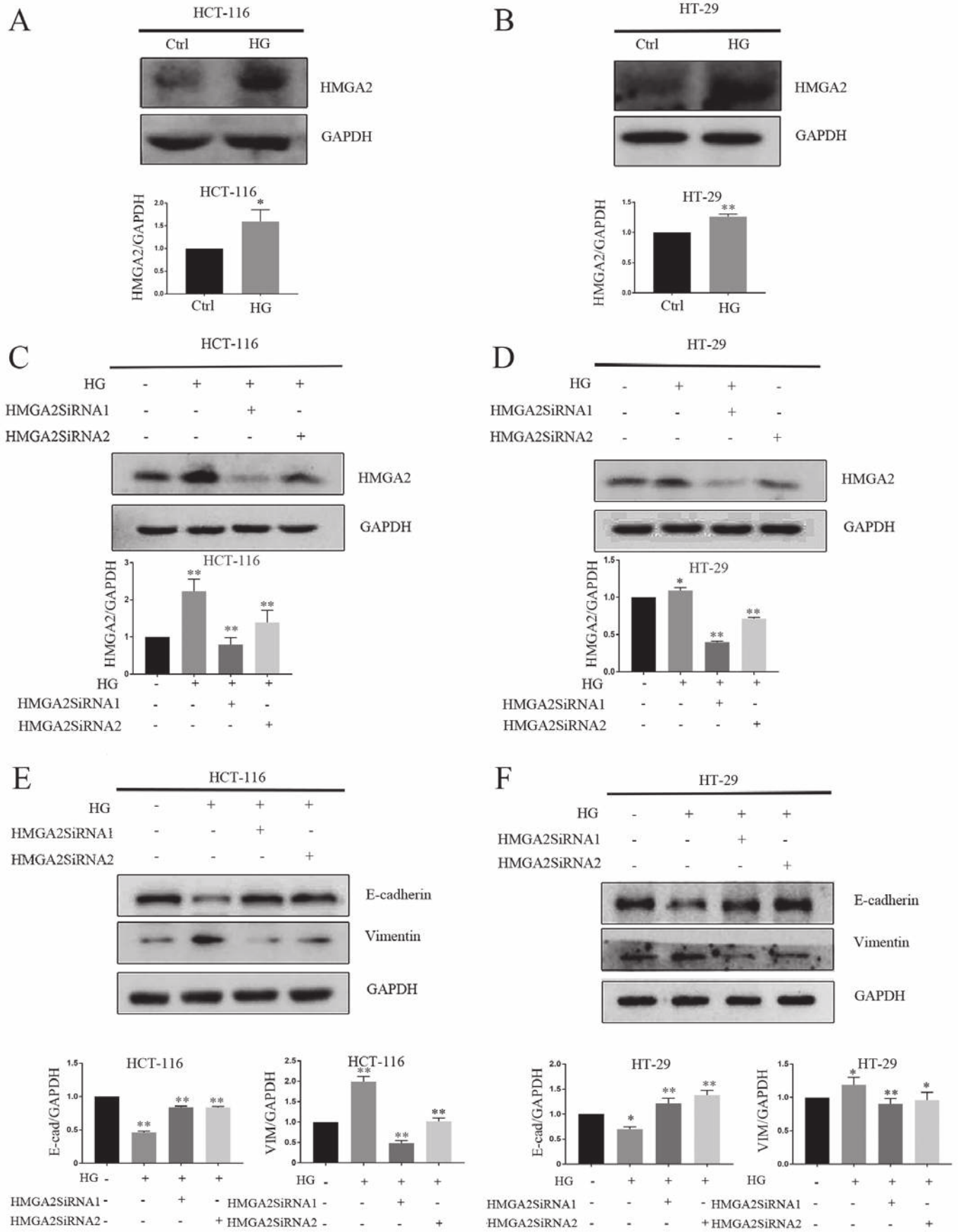


Figure 3. HG promotes EMT by increasing the level of HMGA2 protein. Western blotting was performed to assess the expression of HMGA2 protein in (A) HCT-116 and (B) HT-29 cells. HMGA2 knockdown in (C) HCT-116 and (D) HT-29 cells was confirmed using western blotting. Western blotting analysis revealed that HMGA2 knockdown reversed EMT in (E) HCT-116 and (F) HT-29 cells. $^{*}$P<0.05 and $^{**}$P<0.01 vs. Ctrl. HG, high glucose; EMT, epithelial-mesenchymal transition; HMGA2, high-mobility group A protein 2; Ctrl, control; siRNA, small interfering RNA.

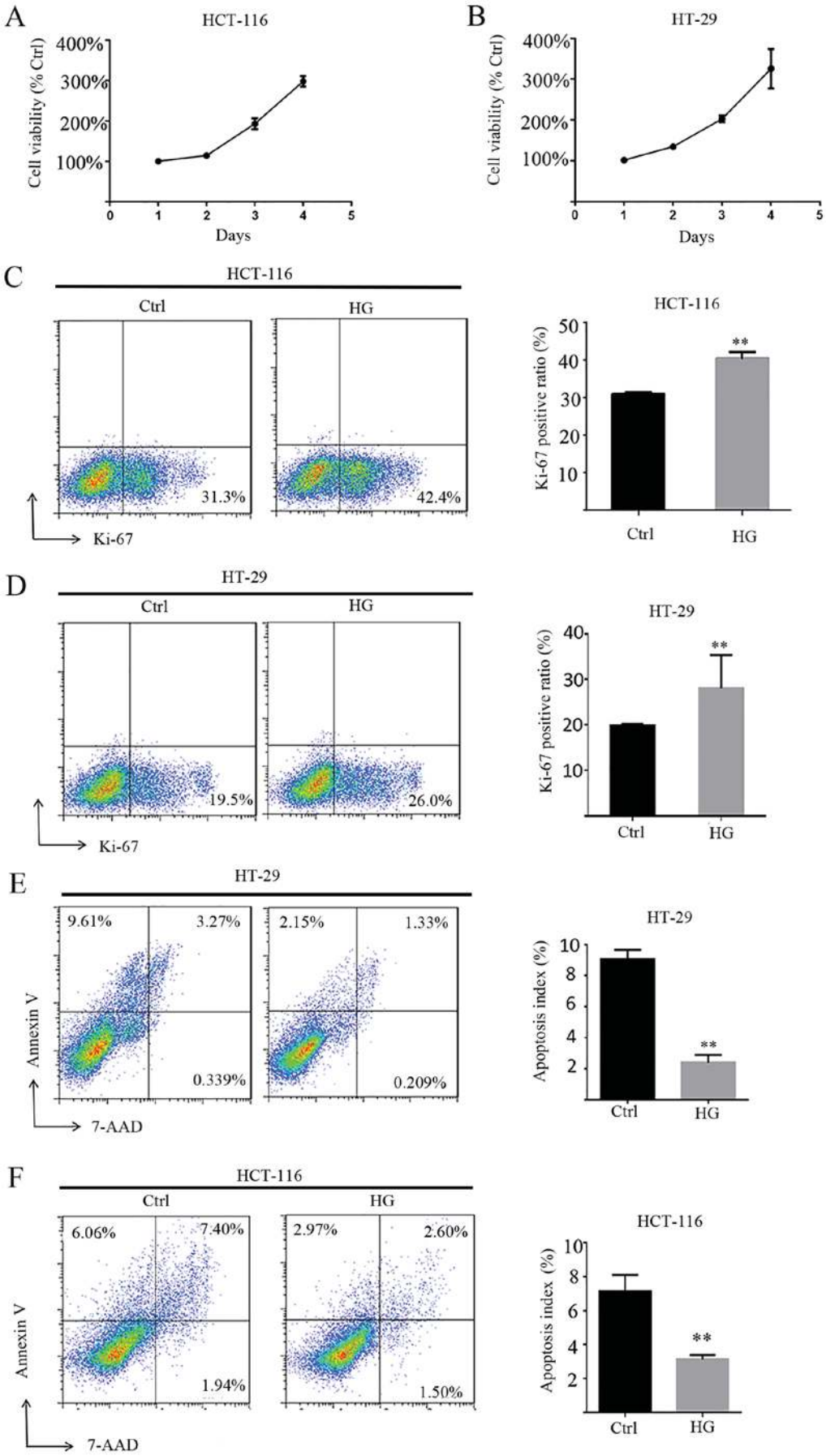


Figure 4. HG promotes cell viability and suppresses apoptosis in colorectal cancer cells. Cell viability was assessed in (A) HCT-116 and (B) HT-29 cells using an MTT assay. Cell viability was also assessed in (C) HCT-116 and (D) HT-29 using flow cytometry. An Annexin V-PE/7AAD assay was performed to measure the number of (E) HCT-116 and (F) HT-29 cells in early apoptosis (lower-right quadrant) and late apoptosis/necrosis (upper-right quadrant) cells. $^{**}$P<0.01 vs. Ctrl. HG, high glucose; PE, phycoerythrin; 7AAD, 7 aminoactinomycin D; Ctrl, control.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

HS collected patient tumor tissue samples, JW and JC performed experimental work and conceived ideas. YX, FW, LL, YZ, XH and SB conceived ideas and evaluated the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The study protocol was reviewed and approved by the Ethics Committee of the Ningbo Urology and Nephrology Hospital and informed consent was taken from all patients.

**Consent for publication**

Patient provided written informed consent for the publication of all associated data and images.

**Competing interests**

The authors declare that they have no competing interests.

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