Cell culture

The human colon cancer cells(SW480, LoVo),mouse colon cancer cells(MC38),human monocytic cell line(THP1) and 293T cells were purchased from American Type Culture Collection (ATCC, USA) . All cells were cultured in DMEM/high glucose(Gibco, USA) .To induce differentiation into macrophages, THP-1 cells (1×10^6) were treated with 100 ng/mL PMA(MCE,USA) for 24 h. All cells were cultured in a medium supplemented with 10% foetal bovine serum(Gibco, USA) at 37 °C under 5% CO2.

AOM/DSS-induced mouse colon cancer model

To investigate the effect of GABA on tumorigenesis, a colon cancer model was generated using AOM/DSS. Male C57BL/6 mice were injected intraperitoneally with AOM (10 mg/kg) (MCE, USA). 1 week after injection, 2.5% DSS(MCE, USA) feeding was added to water for 1 week, followed by normal water feeding for 2 weeks. This DSS feeding pattern was repeated three times.

Transwell migration assays

5×10^4 cells in medium without FBS were plated in an 8 μm pore Transwell chamber (BD, USA) for the migration assays. And we placed culture medium supplemented with 10% FBS in the lower chambers. Then, the cells were gently wiped away on the top of the filters after incubation for 24 hours for the migration assays. The cells were fixed on the membranes with 5% paraformaldehyde for 20 minutes followed by staining for 15 minutes with 0.1% crystal violet. Lastly, five fields of vision were chosen and the number of cells were calculated under the microscope.

Colony formation assay

SW480 and LoVo cells were cultured in 6-well plates with 500 cells per well and allowed to grow for 14 days in the recommended growth medium. The old medium was replaced with fresh medium every 3 days. Then, the clones were fixed with 4% paraformaldehyde for 20 minutes and stained with 0.1% crystal violet 15 minutes. Finally, five fields of vision were chosen and the total number of colonies were counted to evaluate the results.

Wound healing assays

Cells were plated in 6-well plates overnight. 1 mL pipette tips were used to scratch a straight line when the cells achieved 60-80% confluence. Then cells were cultured with medium without FBS. Then, a picture of the cell wound width was taken under the microscope at 0 and 48 hours.

Immunohistochemistry (IHC)

Tissues were formalin-fixed, dehydrated and paraffin-embedded. Then, the tissue sections were incubated with primary antibodies overnight at 4 ℃. Next day, the tissue sections were incubated with HRP-conjugated secondary antibodies for 1 hour at 37℃. Then sections were further washed with PBS and distilled water, freshly prepared DAB solution (diaminobenzidine) was subsequently used until the tissue sections were ready to observe. On the one hand, we evaluated the intensity of tissue staining. We calculated the percentage of positive tissue staining (graded as 0, < 5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, > 75%). SI score was equal to the product of those two. Two experienced pathologists evaluated all the results from the IHC analysis of the

tissue sections.(The details are listed in Supplementary Table 4)

Immunofluorescence (IF)

Colon cancer cells and PMA-pretreated THP1 cells were placed on a glass slide and fixed with 5% paraformaldehyde when the cells reached 60-70% confluence. Blocked the cells with 5% donkey serum for 1 h and incubated with the primary antibody overnight at 4 ℃. The antibodies used for the IF assay were listed in Supplementary Table S4. The next day, the cells were incubated with the corresponding CY3 secondary antibody (Jackson Immunology Research, Erie, UK) at 37℃for 1 h. After counterstaining the nuclei with DAPI (Sigma, St. Louis, Missouri, USA) for 15 min, the fluorescence images were captured by epifluorescence microscopy (Olympus, Tokyo, Japan).(The details are listed in Supplementary Table 4)

Chromatin immunoprecipitation (ChIP)

ChIP assay was performed using the SimpleChIP® Plus Enzymatic Chromatin IP Kit (CST, USA). Crosslinked the cells with formaldehyde and sonicate to an average size of 300-500 bp. The lysate was added to the EP tube and incubated with the cMYC antibody. Purified cross-linked DNA released from protein-DNA complexes and further evaluated eluted DNA by qRT-PCR. Used both input and IgG to confirm that the detected signal comes from a specific binding between chromatin and MYC. Used JASPAR to predict the binding site between cMYC and the miR-223-3p promoter. All ChIP assays were repeated 3 times independently.

Dual luciferase reporter assay for miRNA binding to 3'UTR

To confirm whether CBLB was a target of miR-223-3p,1×10^5 293T cells were seeded on 96-well plates and contransfected with dual luciferase reporter plasmid containing CBLB 3'UTR and mutated forms with or without 50nM miR-223-3p mimic or mimic-NC.After incubation for 48 hours,cells were lysed with diluted Passive Lysis Buffer.Luciferase Assay Buffer II was added and used to measure firefly luciferase activity.After stopping the reaction with 1XStop & Glo®Reagent,we measured the Renilla luciferase activity.Activity per well=firefly luciferase activity/Renilla luciferase activity.

ELISA

After treatment, blood samples were prepared from C57BL/6 mice. Expressions of Gamma-Aminobutyric Acid (GABA), Norepinephrine (NE), Epinephrine (EPI) and Serotonin(5-HT) in blood were assessed using commercially available ELISA kits (Enzyme-linked Biotechnology Co., Ltd., Shanghai).

Statistical analysis

All data were analyzed by GraphPad Prism5.0 and all assays were repeated at least three times. We used t-test to analyze the frequency of Macrophages in colon cancer tissues and paired adjacent normal tissues. We used the chi-square test to identify the correlation between miR-223-3p and cMYC, CBLB, p-38 and p-ERK in the colon cancer specimens. χ^2 test was used to analyze the relationship between Macrophages frequency and the clinical features of colon cancer. $P < 0.05$ was considered to be statistically significant and all tests were two-sided.