Supplementary materials for

Tumor-derived exosomal miR-425-5p and miR-135b-3p enhance colorectal cancer progression through immune suppression and vascular permeability promotion

Feng CZ *et al*. Exosomal miR-425-5p & miR-135b-3p's Roles in CRC

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Author contributions: Feng CZ, Zhong SQ, Ye SW, Zheng Z, Sun H and Zhou SH designed the study; Feng CZ performed the research; Zhong SQ and Ye SW performed validation and data curation; Zheng Z carried out data analysis; Sun H handled visualization; Feng CZ and Zhou SH wrote and reviewed the manuscript; all authors approved the final version of the article.

Supportive foundation: None.

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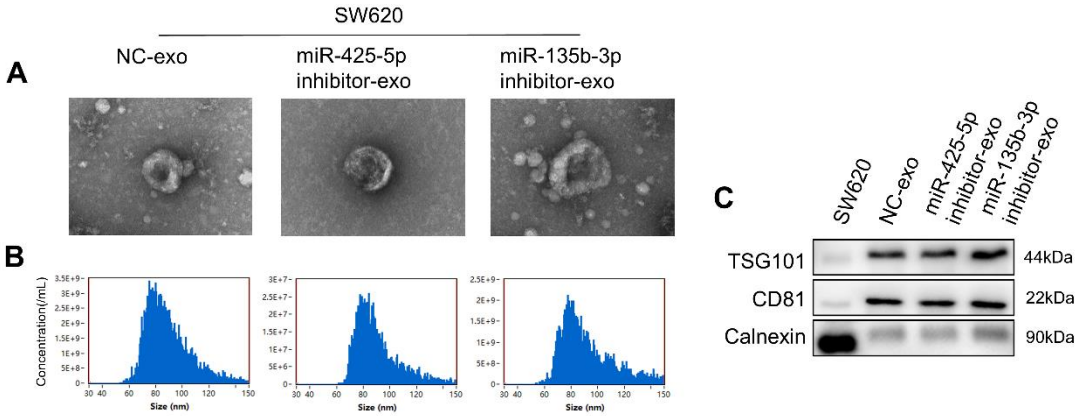
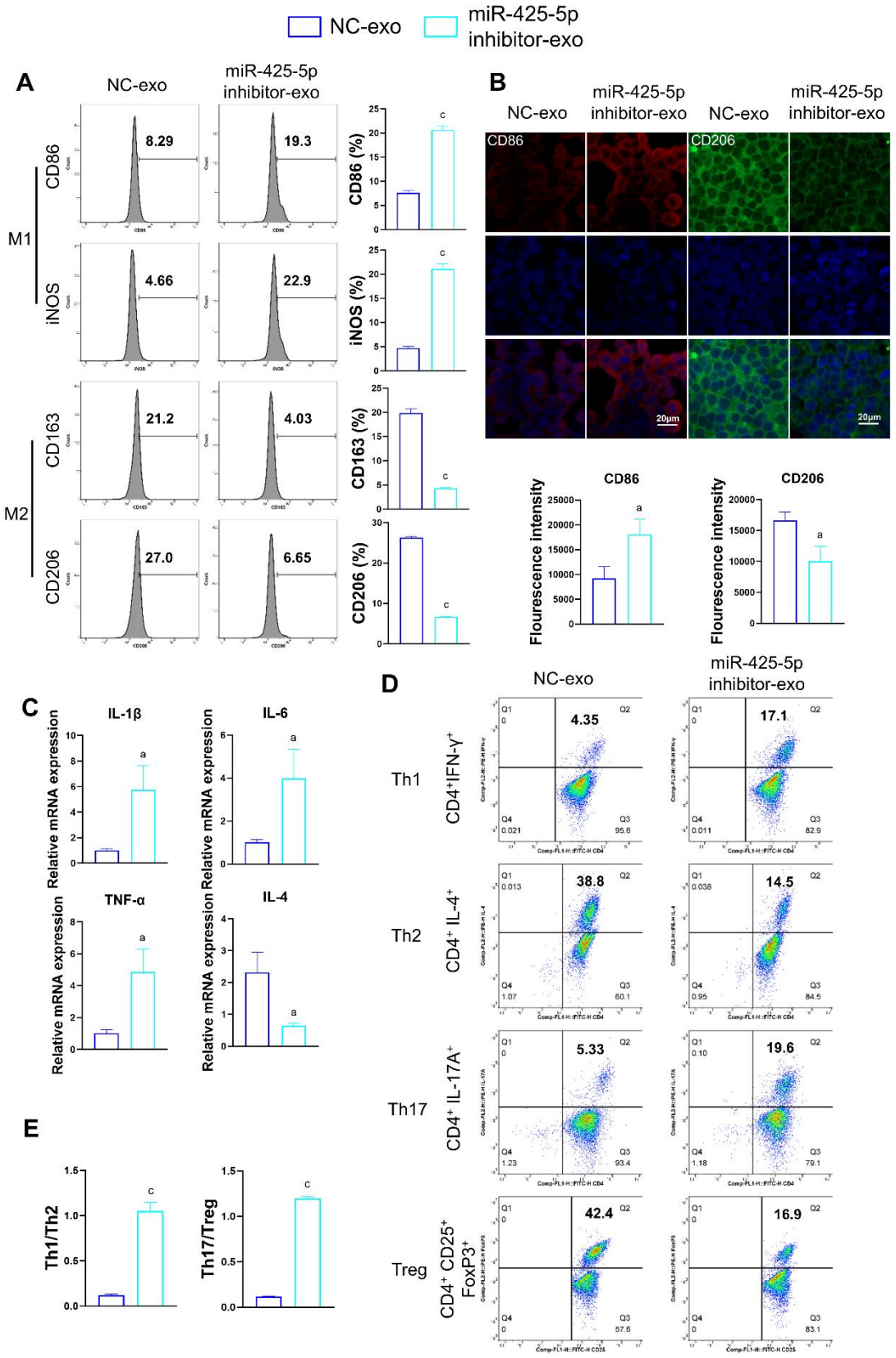
Supplementary figures:

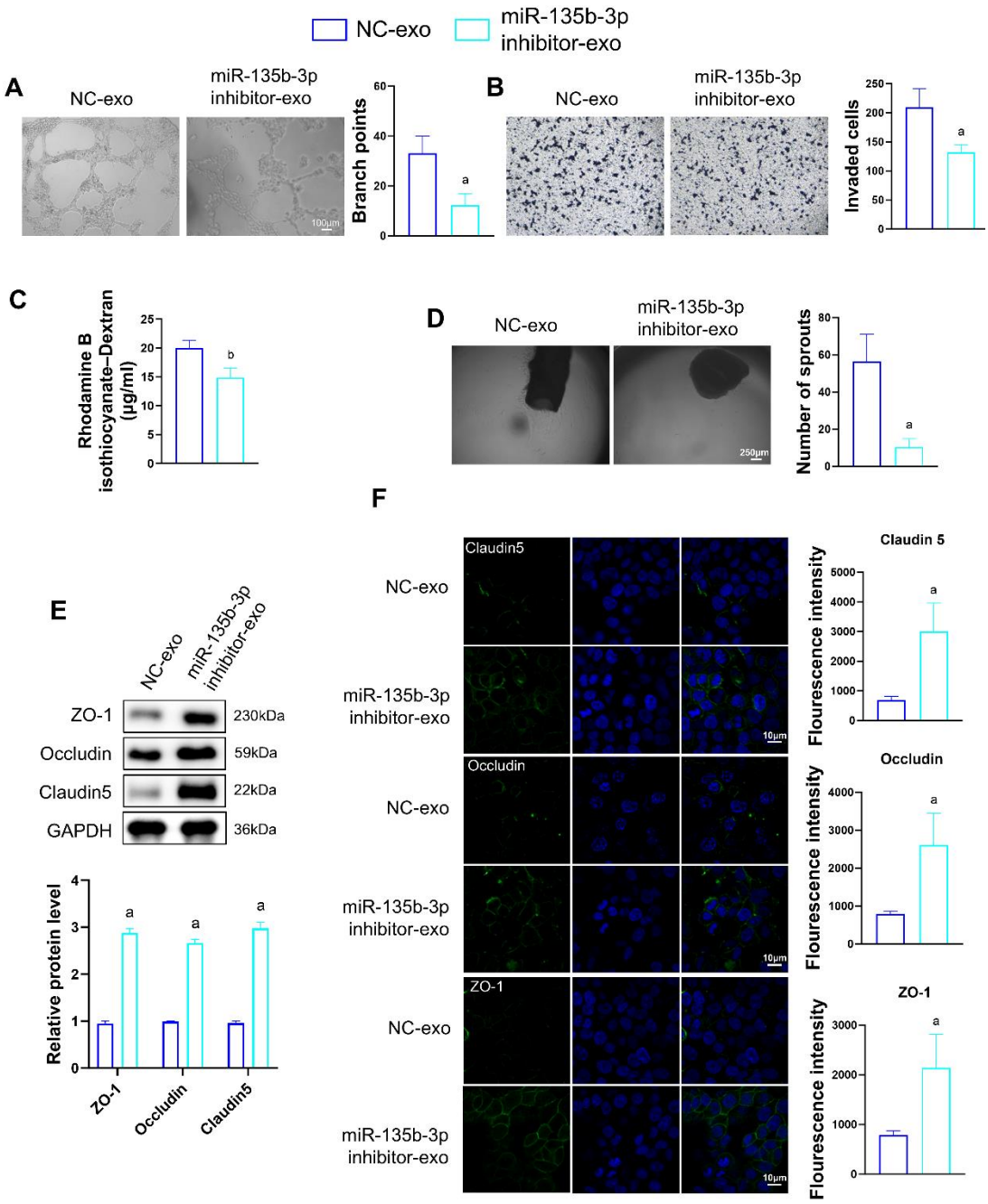


Supplementary Figure 1 Characterization of exosomes from SW620 cells transfected with miRNA inhibitors or negative control. A: Transmission electron microscopy images and B: Nanoparticle tracking analysis of exosomes isolated from SW620 cells. C: Western blot analysis of exosomes isolated from SW620 cells. CD81: CD81 antigen; TSG101: Tumor susceptibility gene 101 protein.



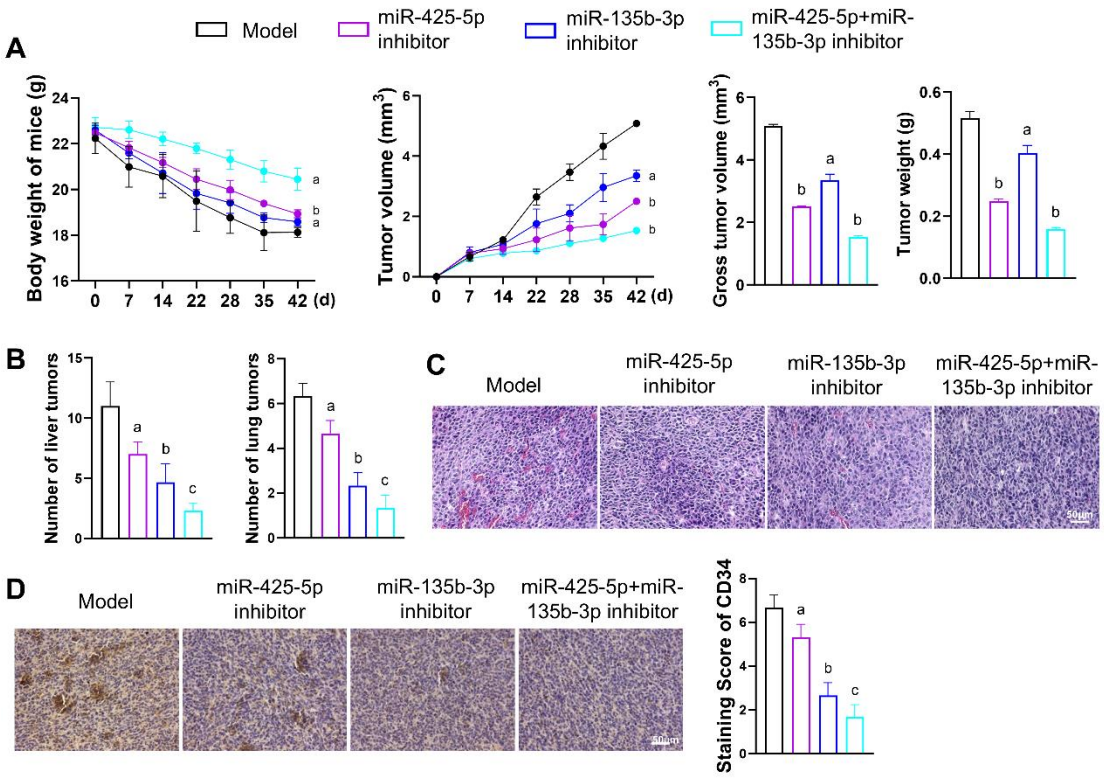
Supplementary Figure 2 Validation of exosomal miR-425-5p inhibition promoting macrophage M1-like polarization and proinflammatory T cell

differentiation using SW620-derived exosomes. A: Flow cytometry analysis of $CD86^+iNOS^+$ and $CD163^+CD206^+$ cells ratio in THP-1 cells cultured with exosomes isolated from SW620 cells transfected with negative control (NC) and miR-425-5p inhibitor; B: Immunofluorescent staining of CD86 (red) and CD206 (green) in THP-1 cells with DAPI nuclei (blue); C: RT-qPCR analysis of cytokine expression in $CD4^+$ T cells cultured with exosomes; D, E: Flow cytometry analysis of $CD4^+$ T cell subsets. $^a P < 0.05$, $^c P < 0.001$ vs NC-exo (n = 3 per group). CD4: T-cell surface glycoprotein CD4; CD25: Interleukin-2 receptor subunit alpha; CD86: T-lymphocyte activation antigen CD86; CD163: Scavenger receptor cysteine-rich type 1 protein M130; CD206: Macrophage mannose receptor 1; FoxP3: Forkhead box protein P3; IFN-γ: Interferon gamma; IL-1β: Interleukin 1 beta; IL-4: Interleukin 4; IL-6: Interleukin 6; iNOS: Nitric oxide synthase, inducible; Th: T helper cells; TNF-α: Tumor necrotic factor alpha; Treg: Regulatory T cells.

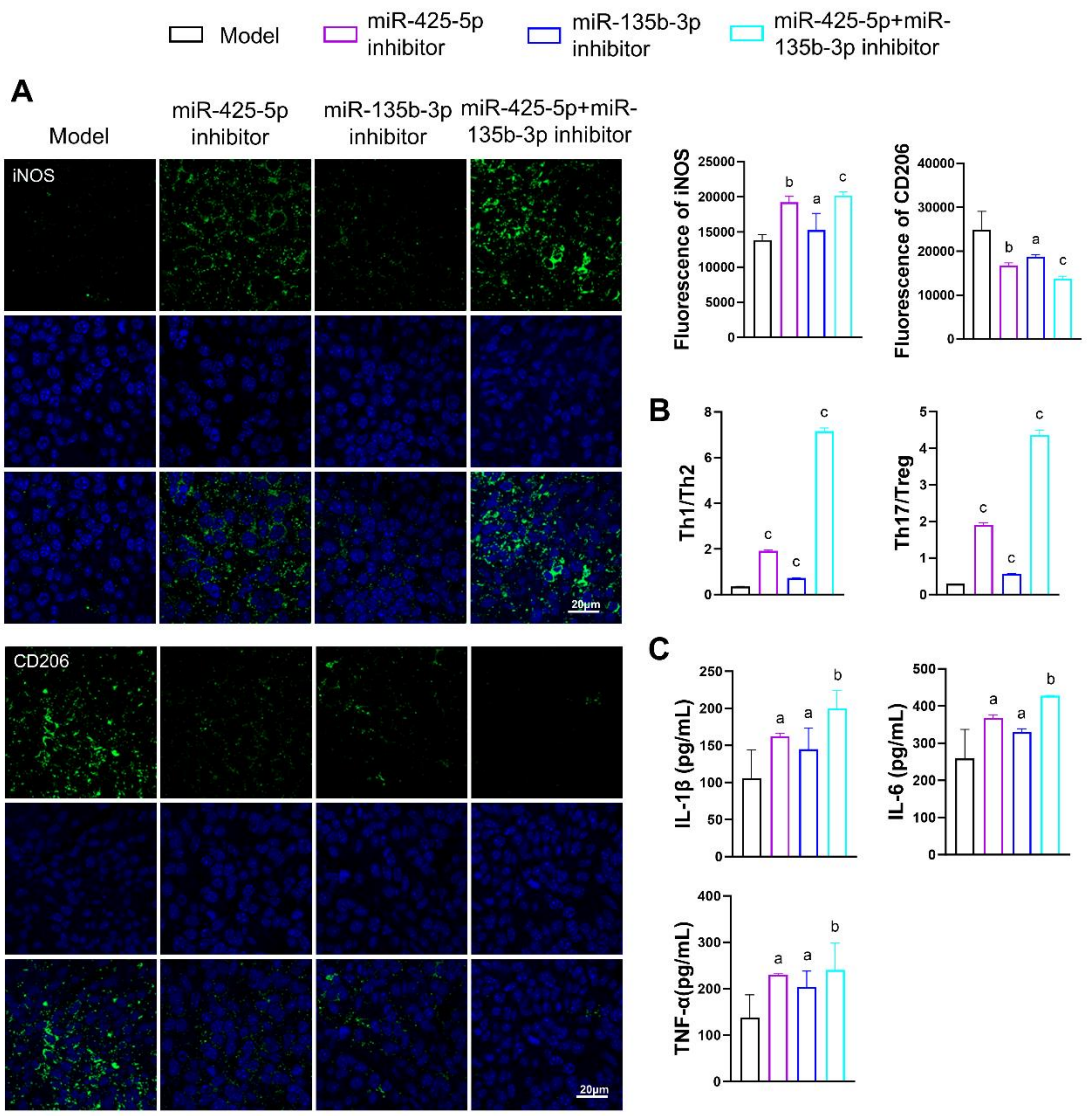


Supplementary Figure 3 Validation of exosomal miR-135b-3p enhancing vascular permeability and angiogenesis using SW620-derived exosomes. A: Representative images and branch points of microscopic observation of angiogenesis in HUVECs cultured with exosomes from SW620 cells transfected with miR-135b-3p inhibitor or negative control (NC); B: Representative images and invaded cells of cell invasion assay; C: Permeability of the HUVEC monolayers to rhodamine–dextran (70 kDa) after exposure to exosomes for 72 h; D: Representative images of the aortic ring and numbers of microvascular

sprouts; E: Western blot analysis of tight junction proteins ZO-1, Occludin, and Claudin-5 in HUVECs treated with exosomes; GAPDH as an internal control; F: Immunofluorescent staining of tight junction proteins ZO-1, Occludin, and Claudin-5 (green) in HUVECs treated with DAPI nuclei (blue). [a] $P < 0.05$, [b] $P < 0.01$ vs NC-exo (n = 3 per group). ZO-1: Tight junction protein 1.



Supplementary Figure 4 Validation of exosomal miR-425-5p and mir-135b-3p on tumor growth, metastasis, and angiogenesis in vivo using SW620 cells. A: Body weight, tumor volume, tumor weight and gross size of tumors from mice treated with SW620 cells; B: Number of liver and lung tumors; C: Representative images of H&E staining in tumor tissues; D: Immunohistochemical staining of CD34 in tumor tissues. [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ vs Model (n = 3 per group). CD34: Hematopoietic progenitor cell antigen CD34.



Supplementary Figure 5 Validation of exosomal miR-425-5p and mir-135b-3p on immune microenvironment modulations using SW620 cells. A: Immunofluorescence staining of M1 marker iNOS and M2 marker CD206 in tumor tissues; B: Flow cytometry analysis of tumor-infiltrating T cells, including $CD4^+IFN\gamma^+$ Th1, $CD4^+IL\text{-}4^+$ Th2, $CD4^+IL\text{-}17A^+$ Th17, and $CD4^+CD25^+Foxp3^+$ Tregs; C: ELISA results of IL-1β, IL-6, and TNF-α in tumor tissues. [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ vs Model (n = 3-5 per group). CD206: Macrophage mannose receptor 1; IL-1β: Interleukin 1 beta; IL-6: Interleukin 6; iNOS: Nitric oxide synthase, inducible; Th: T helper cells; TNF-α: Tumor necrotic factor alpha; Treg: Regulatory T cells.

Supplementary Methods:

Exosome isolation and characterization

Exosomes were isolated from cell supernatants using the exoEasy Maxi Kit (Qiagen, Germany) following the manufacturer's protocol:

1. Conditioned media were centrifuged at 300 × g for 10 min to remove cells.
2. Supernatants were filtered through a 0.22-μm filter (Millipore, USA).
3. Exosomes were pelleted by ultracentrifugation at 100,000 × g for 70 min at 4°C (Beckman Coulter Optima XPN-100 Ultracentrifuge).
4. Exosome pellets were resuspended in PBS and stored at -80°C.

Cell culture and transfection

CRC cell lines (HCT116, SW620, SW480, HT-29) and normal colon epithelial cells (FHC) were cultured in specific media (as described in the Methods) supplemented with 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA).

THP-1 macrophages were differentiated into M0 macrophages using 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, USA) for 48 h.

Primary naive $CD4^+$ T cells were isolated from healthy donor peripheral blood using magnetic beads (Miltenyi Biotec, Germany) and activated with anti-CD3/CD28 beads (Thermo Fisher, USA) for 72 h.

RT-qPCR

1. RNA Extraction: Total RNA from exosomes was isolated using the All-in-One miRNA Extraction Kit (GeneCopoeia, USA), and from cells using TRIzol (Invitrogen, USA).
2. cDNA Synthesis: miRNAs: Reverse transcribed using the miRNA First-Strand cDNA Synthesis Kit (Vazyme, China). mRNAs: Reverse transcribed using MonScript RTIII Super Mix (Monad, China).
3. qPCR Conditions: miRNA qPCR: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. mRNA qPCR: 95°C for 10 min, followed by 40

cycles of 95°C for 15 s and 60°C for 1 min.

4. Primers listed in Table 1 were used at a final concentration of 0.5 μM.

Flow cytometry

1. Cell Staining: THP-1 cells: Surface markers (CD86, CD163, CD206) and intracellular iNOS were stained using BD Cytofix/Cytoperm Kit (BD Biosciences, USA). $CD4^+$ T cells: Surface markers (CD4, CD25) and intracellular cytokines (IFN-γ, IL-4, IL-17A) were stained using BD Cytofix/Cytoperm Kit. Tumor-infiltrating T cells: Cells were stained with Zombie NIR™ viability dye (BioLegend, USA) followed by surface markers and intracellular cytokines.
2. Flow Cytometer: Data were acquired on a CytoFLEX S (Beckman Coulter, USA) and analyzed using Kaluza v2.1 software.

In vitro Functional assays

1. Tube Formation Assay: HUVECs (5×10^4 cells/well) were seeded on Matrigel (Corning, USA) and incubated for 12 h. Images were captured using an Olympus CKX53 microscope.
2. Transwell Migration Assay: HUVECs (1×10^5 cells/well) were seeded in 8-μm transwell inserts (BD Biosciences, USA). Migrated cells were stained with hematoxylin and counted under a light microscope.
3. Permeability Assay: Rhodamine B-dextran (70 kDa, Sigma-Aldrich, USA) was added to the upper chamber, and fluorescence was measured at 544/590 nm using a SpectraMax M5 microplate reader (Molecular Devices, USA).

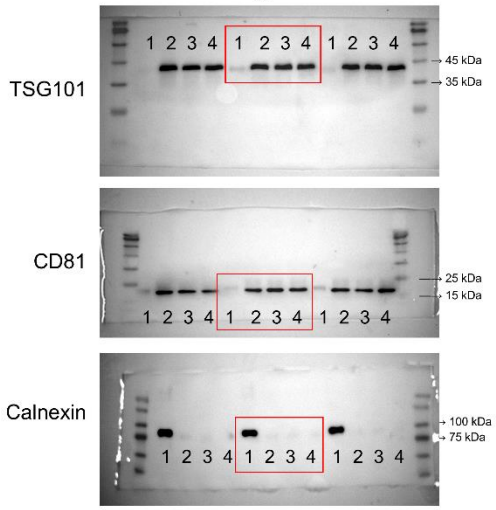
In vivo experiments

1. Xenograft Experiments: Nude mice (4-6 weeks old) were injected subcutaneously with HCT116 cells (2×10^6 cells/mouse) transfected with miRNA inhibitors or NC.

2. Tumor volume was measured weekly using calipers: Volume = (length × width^2)/2.
3. Metastasis Analysis: Livers and lungs were fixed in formalin, sectioned, and stained with H&E to count metastatic nodules.

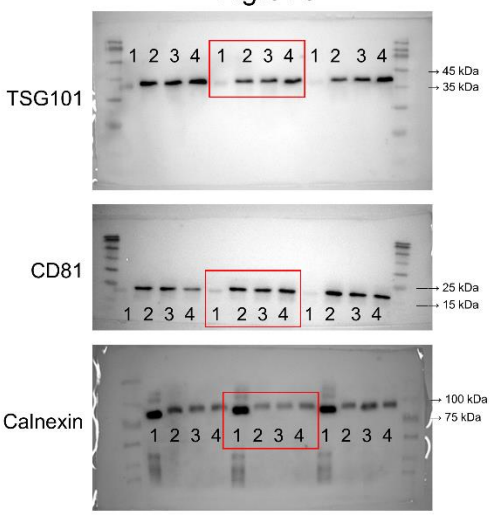
Uncropped blots:

Fig.1G



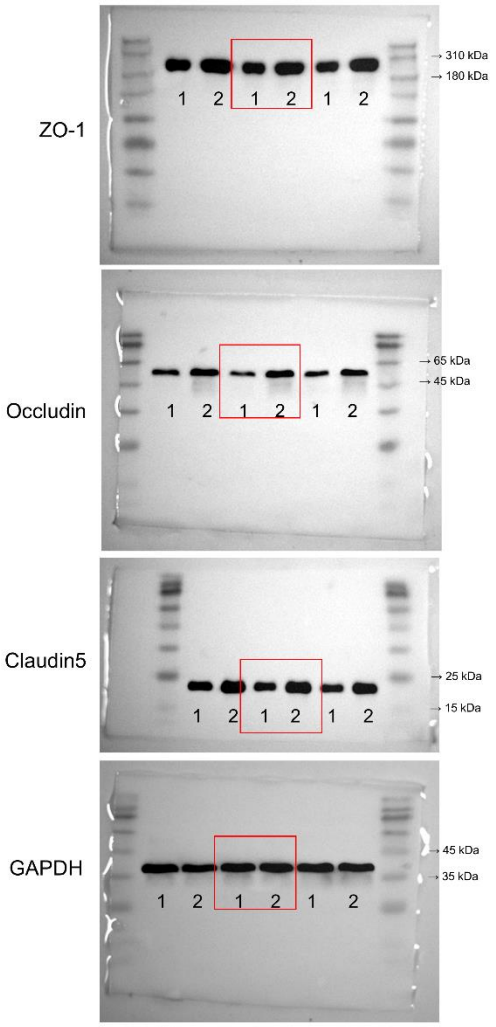
1: HCT116
2: NC-exo
3: miR-425-5p inhibitor-exo
4: miR-135b-3p inhibitor-exo

Fig.S1C



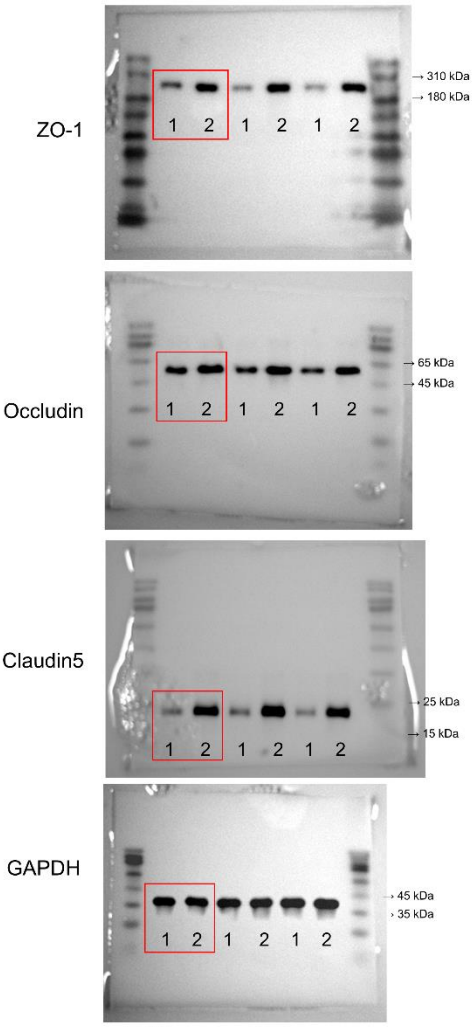
1: SW620
2: NC-exo
3: miR-425-5p inhibitor-exo
4: miR-135b-3p inhibitor-exo

Fig.3E



1: NC-exo
2: miR-135b-3p inhibitor-exo

Fig.S3E



1: NC-exo
2: miR-135b-3p inhibitor-exo