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Supplemental Information

Tumor-derived exosomal microRNA-106b-5p activates EMT-cancer cell and M2-subtype TAM interaction to facilitate CRC metastasis

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Supplemental Figures and Legends

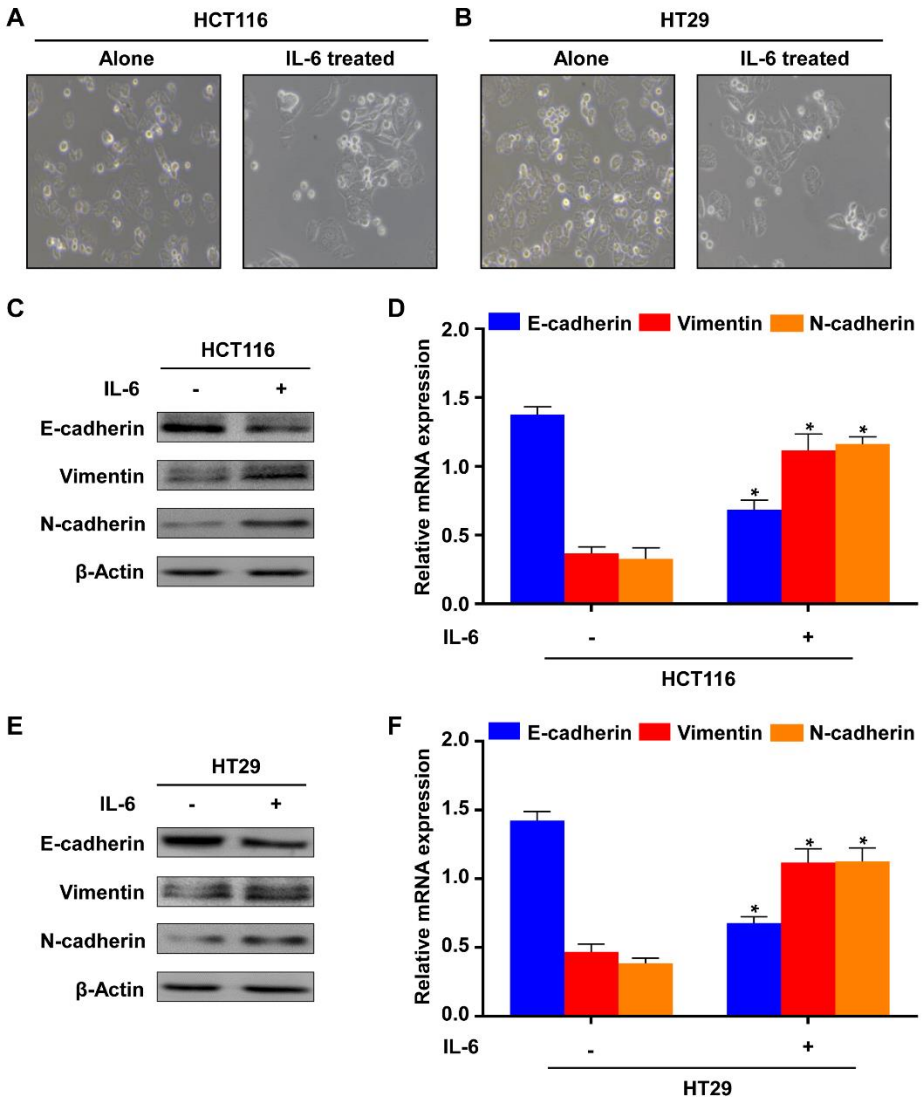

Figure S1. (A-B) The representative bright-field images of HCT116 and HT29 treated by IL-6 for 48 h (magnification, ×200). Western blot and RT-qPCR detected the expression of epithelial marker (E-cadherin) and the mesenchymal marker (N-cadherin and vimentin) in HCT116 (C-D) and HT29 cells (E-F) that treated without or with IL-6 treatment.

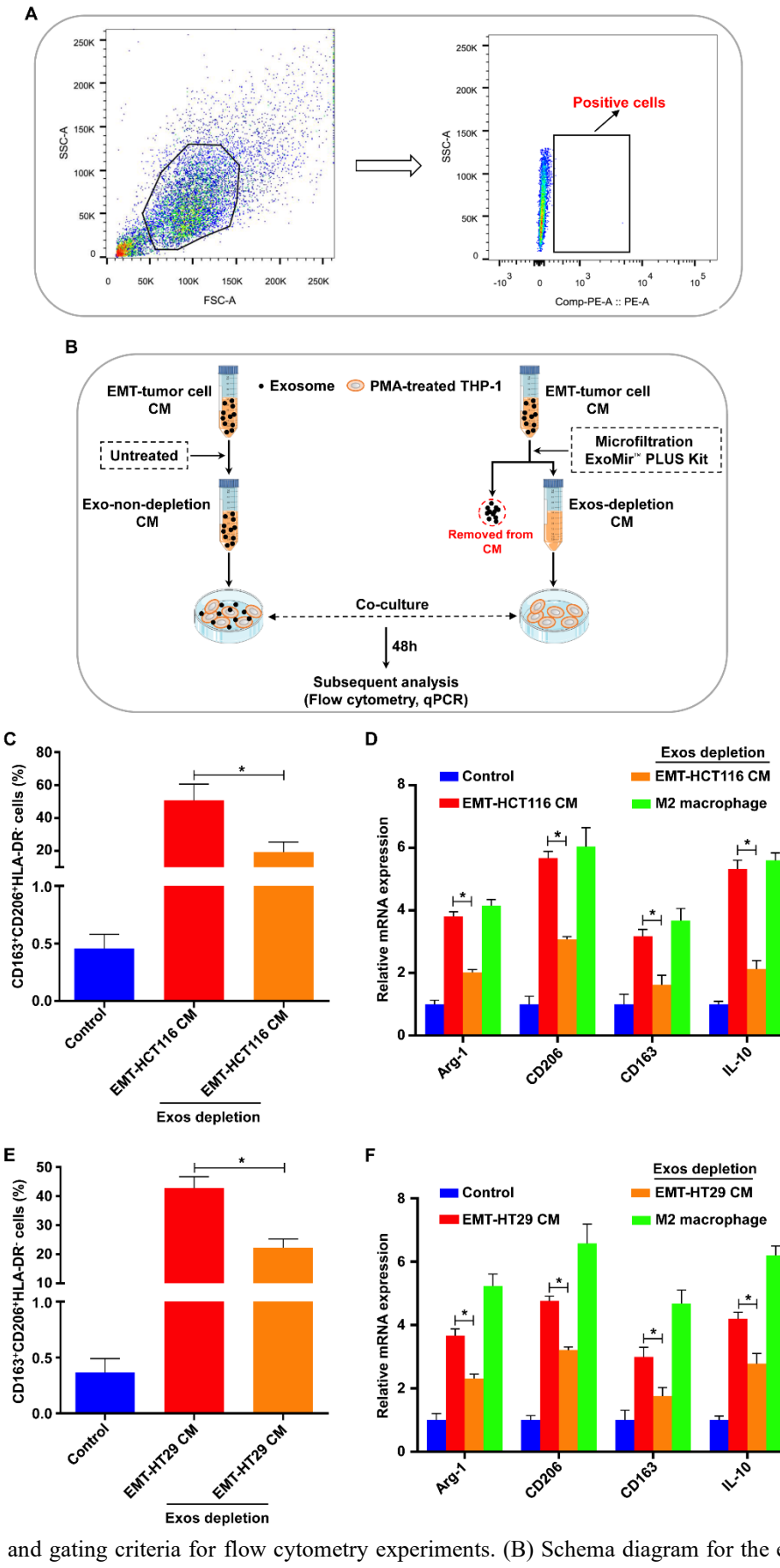

Figure S2. (A) The original plots and gating criteria for flow cytometry experiments. (B) Schema diagram for the experiment procedures. (C-F) Flow cytometry and RT-qPCR analyses of the surface M2 markers (CD163 and CD206) and the expression of M2 markers (Arg-1, CD206, CD163, and IL10) after macrophages were co-cultured with Exos-depletion conditional media of HCT116 and HT29 cells.

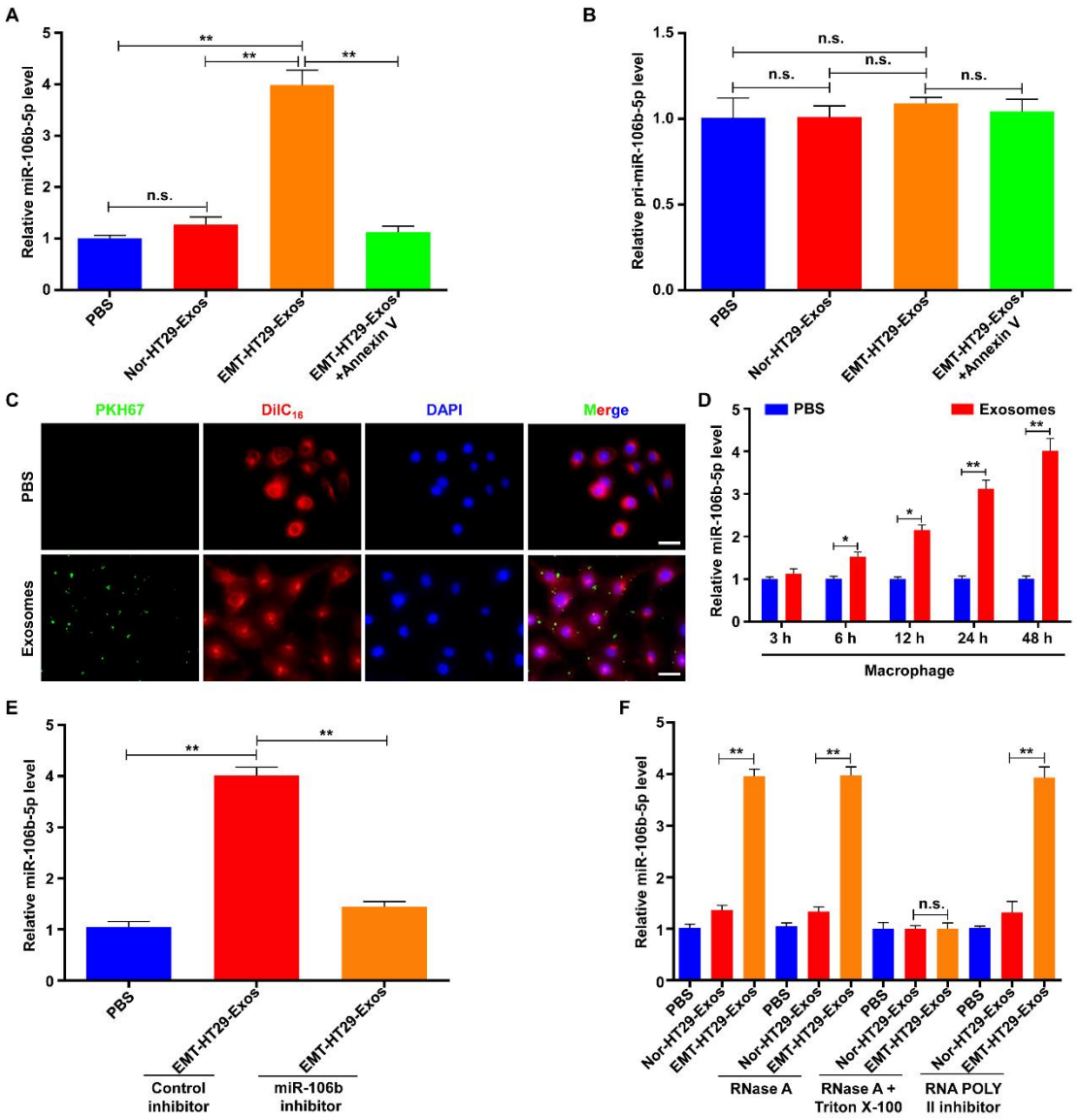

Figure S3. (A-B) RT-qPCR analysis of mature and pri-miR-106b-5p expression in macrophages incubated with PBS, Nor-HT29-Exos, EMT-HT29-Exos and EMT HT29-Exos+Annexin V groups. (C) Representative immunofluorescence images show the internalization of PKH67-labeled EMT-HT29-Exos (green) by DilC16-labeled macrophages (red). Scale bar, 20 μm. (D) RT-qPCR analysis of miR-106b-5p expression in macrophages incubated with EMT-HT29-Exos for 3 h, 6 h, 12 h, 24 h, and 48 h. (E) RT-qPCR analysis of miR-106b-5p expression in macrophages incubated with PBS, EMT-HT29/mock-Exos and EMT-HT29/miR-106b inhibitor-Exos for 48 h. (F) RT-qPCR analyses of miR-106b-5p expression in macrophages incubated with PBS, Nor/EMT-Ht29-Exos that treated by RNase A, RNase A+Triton X-100 or RNA POLY II inhibitor.

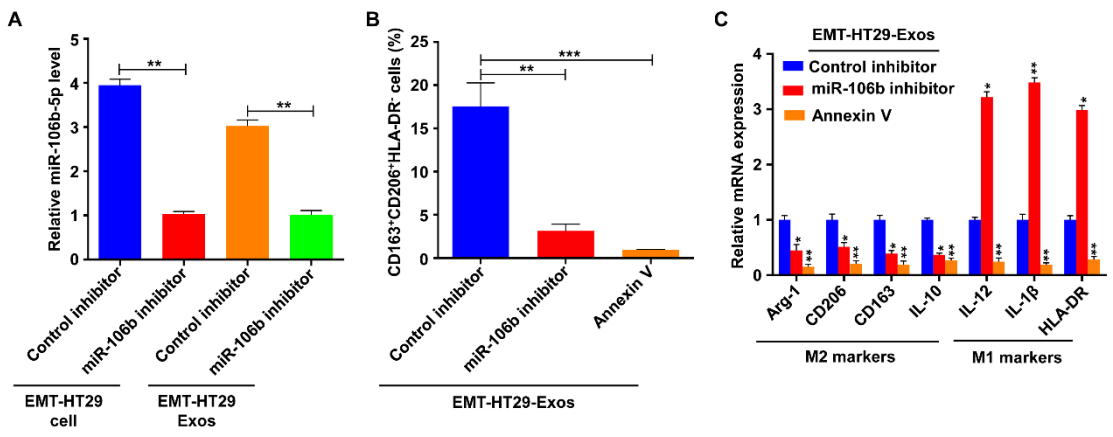

Figure S4. (A) RT-qPCR analysis of miR-106b-5p expression in cells and exosomes of EMT-HT29 transfected by control inhibitor or miR-106b-5p inhibitor. (B) Flow cytometry for analyzing the population of CD163highCD206highHLA-DRlow macrophages incubated with EMT-HT29/mock-Exos, EMT-HT29/miR-106b inhibitor-Exos and EMT-HT29-Exos+Annexin V groups. (C) RT-qPCR analyses of the macrophage-associated markers in macrophages co-cultured with EMT-HT29/mock-Exos, EMT-HT29/miR-106b inhibitor-Exos and EMT-HT29-Exos+Annexin V groups. (d-e) ELISA for analyzing the secretion of IL-10 and IL-12 in macrophages incubated with EMT-HT29/mock-Exos, EMT-HT29/miR-106b inhibitor-Exos and EMT-HT29-Exos+Annexin V groups.

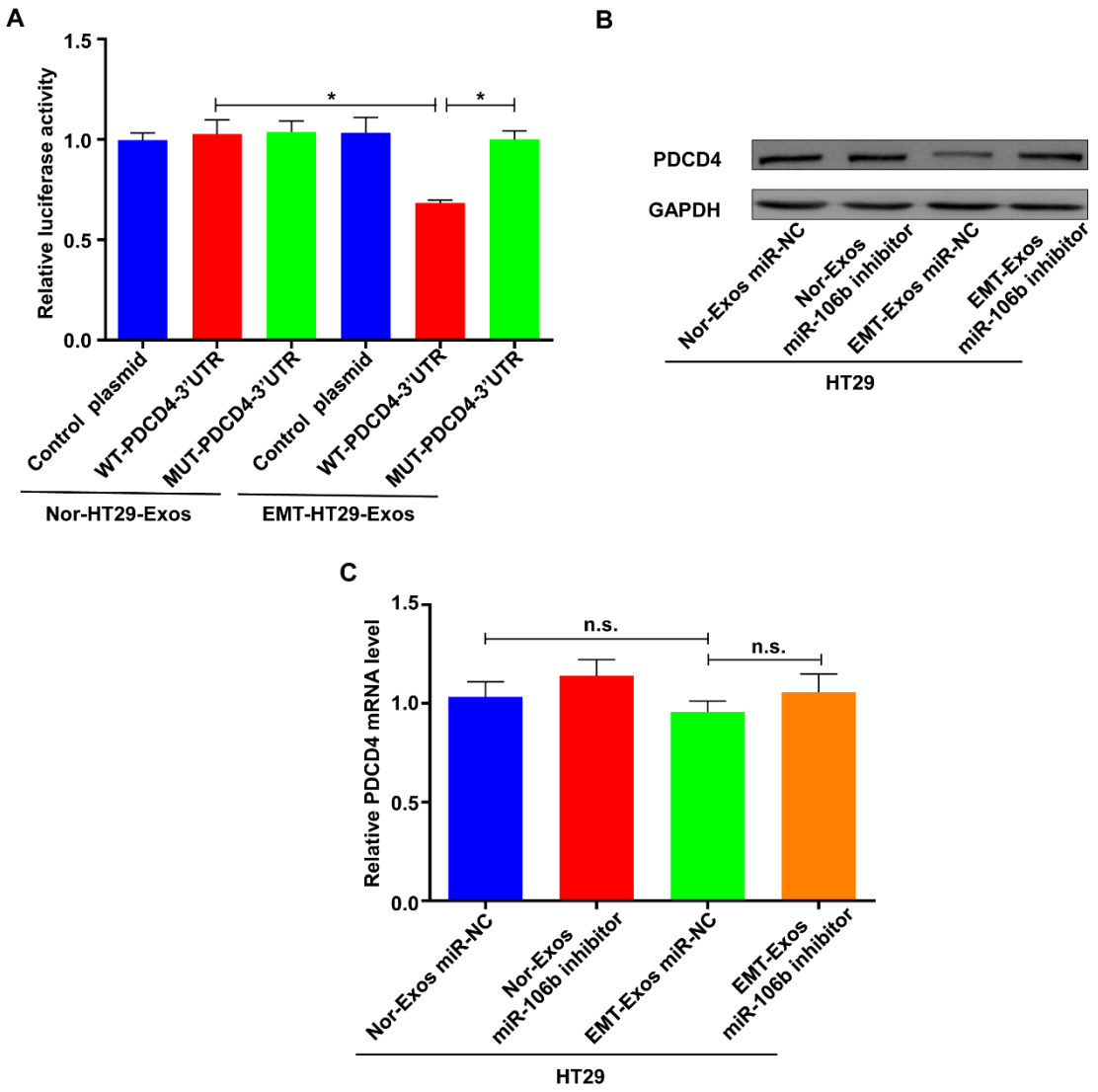

Figure S5. (A) Relative luciferase activity of reporter plasmids carrying wild-type or mutant PDCD4 3′UTR in macrophages treated with Nor-HT29-Exos or EMT-HT29-Exos. (B-C) Western blot and RT-qPCR analysis of the expression of PDCD4 protein and mRNA in macrophages treated with exosomes from normal and EMT-HT29 cells that transfected control inhibitor or miR-106b-5p inhibitor for 48 h.

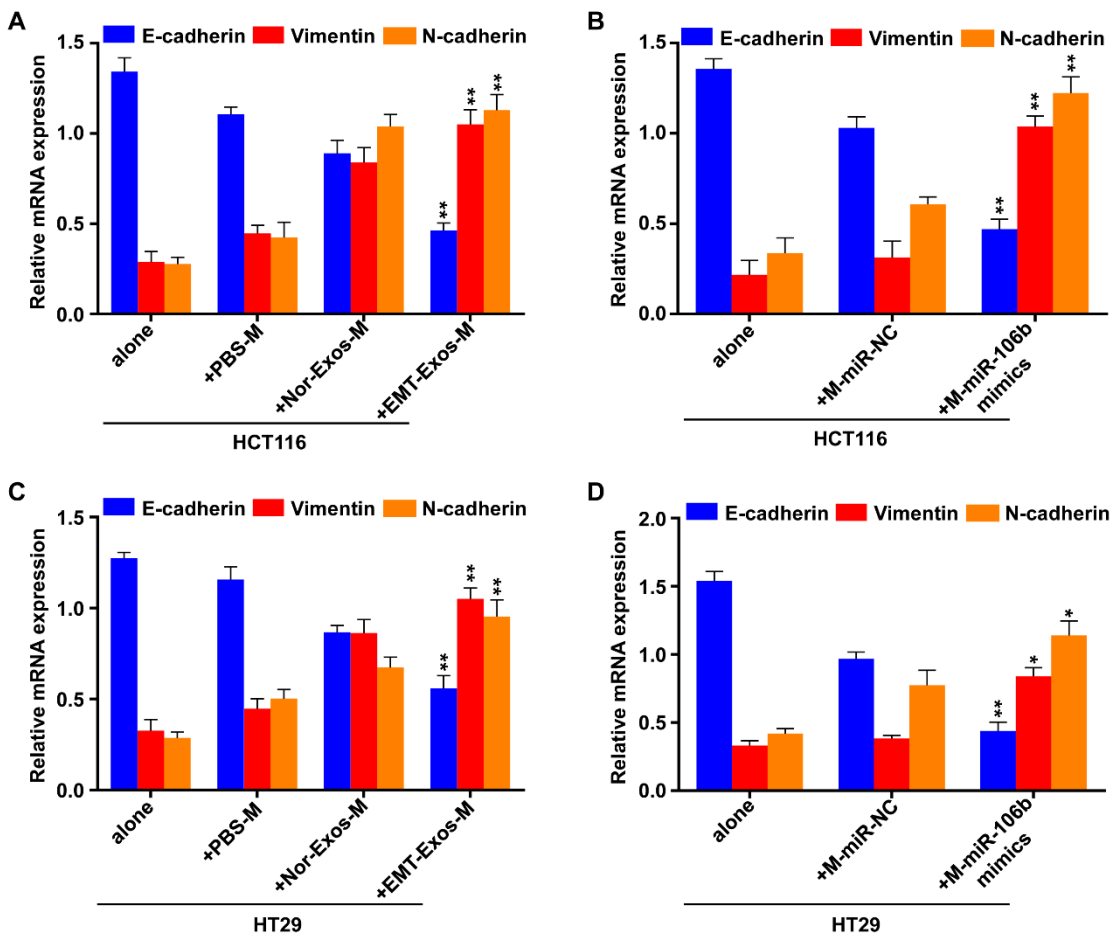

Figure S6. (A-B) RT-qPCR analysis of the expression of epithelial marker (E-cadherin) and the mesenchymal marker (N-cadherin and vimentin) in HCT116 cells that co-cultured with the macrophages treated by EMT-HCT116-Exos or transfected with miR-106b mimics for 48 h. (C-D) RT-qPCR analysis of the expression of epithelial marker (E-cadherin) and the mesenchymal marker (N-cadherin and vimentin) in HT29 cells that co-cultured with the macrophages treated by EMT-HT29-Exos or transfected with miR-106b mimics for 48 h.

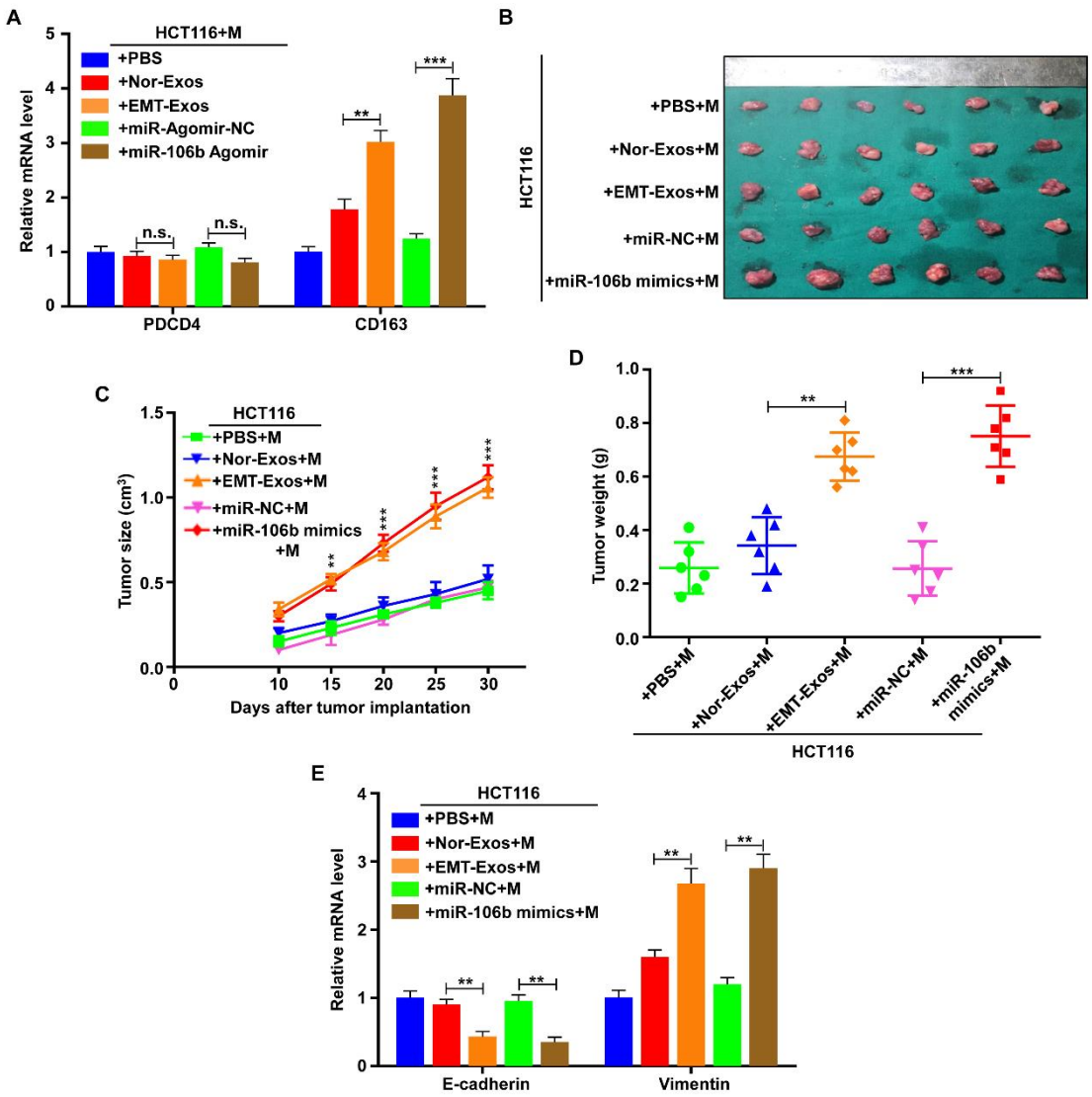

Figure S7. (A) RT-qPCR analyses of the expression of PDCD4 and CD163 mRNA in implanted tumor tissues that received with indicated treatment. (B) Photographs of the dissected tumors in mice that were implanted with HCT116 cells that received with indicated treatment. (C) The growth curves of tumors in different treatment groups. (D). Weights of dissected tumors in different treatment groups. (E) RT-qPCR analyses of the expression of E-cadherin and vimentin mRNA in implanted tumor tissues that received with indicated treatment.

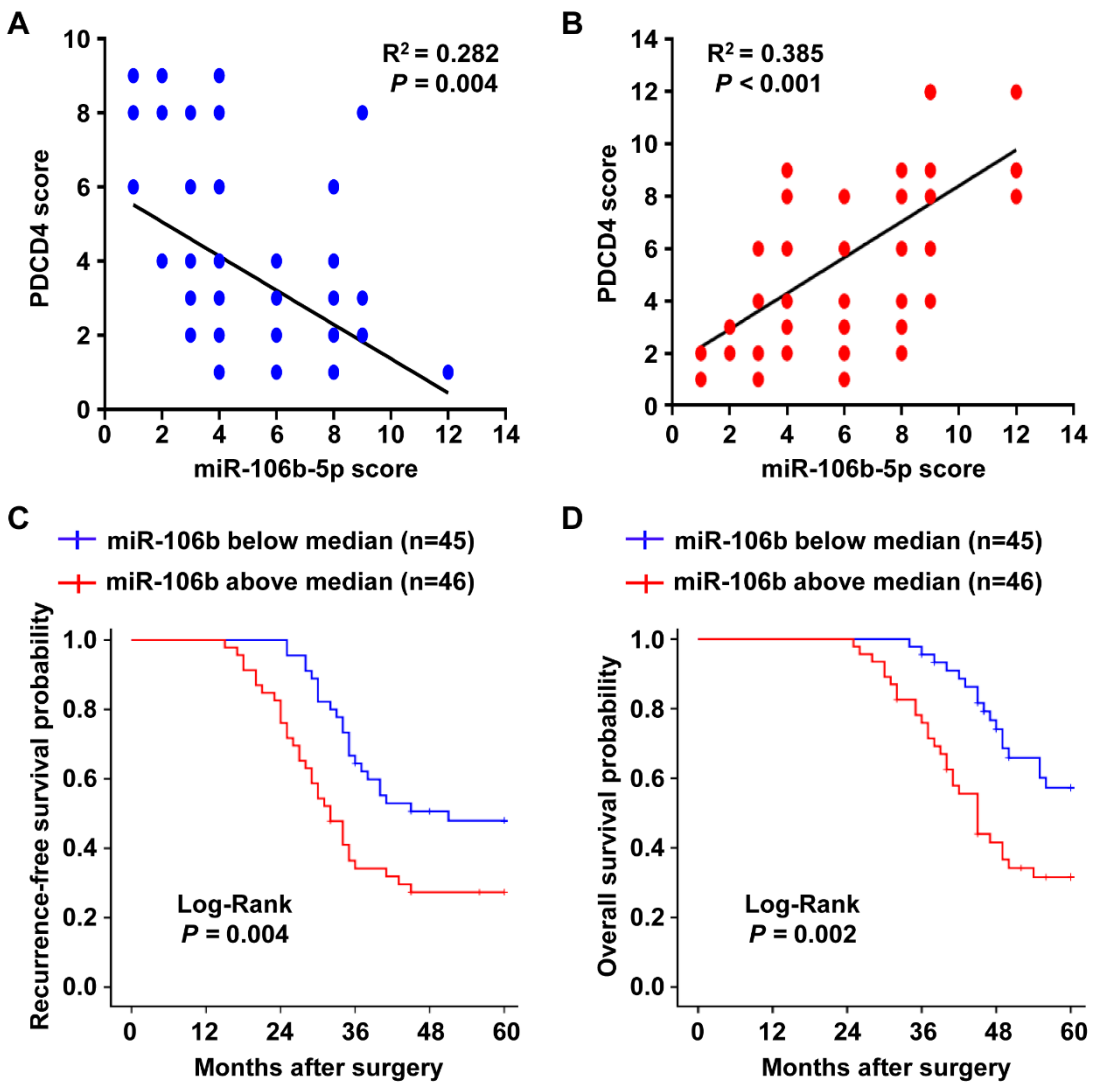

Figure S8. (A) The correlation of miR-106b-5p ISH score and PDCD4 IHC score in CRC tissues. (B) The correlation of miR-106b-5p ISH score and CD163 IHC score in CRC tissues. (C-D) Kaplan–Meier plots of recurrence-free survival and overall survival of 91 patients with CRC, stratified by expression of miR-106b-5p in CRC tissues.

Supplemental Tables

Table S1. Relationship between MiR-106b Expression in Tumor Tissues and Clinicopathological Features of CRC Patients (*n* = 91).

Parameters	No. of patients		miR-106b expression		χ^2 value	*P* value
	No.	%	<median	≥median		
Gender					0.264	0.607
Male	53	58.2	25	28		
Female	38	41.8	20	18		
Age, years					1.323	0.250
<60	39	42.9	22	17		
≥60	52	57.1	23	29		
Tumor site					2.513	0.113
Colon	49	53.8	28	21		
Rectal	42	46.2	17	25		
Tumor size, cm					1.861	0.173
<5	46	50.5	26	20		
≥5	45	49.5	19	26		
Tumor grade					3.965	0.046*
Moderate/Well	41	45.1	25	16		
Poor	50	54.9	20	30		
Lymphovascular invasion					5.078	0.079
Absence	43	47.3	26	17		
Presence	39	42.9	14	25		
Unknown	9	9.8	5	4		
Perineural invasion					4.938	0.085
Absence	46	50.5	28	18		
Presence	38	41.8	14	24		
Unknown	7	7.7	3	4		
Tumor invasion					5.642	0.018*
T1-2	19	20.9	14	5		
T3-4	72	79.1	31	41		
Lymph node metastasis					15.748	<0.001*
N0-1	54	59.3	36	18		
N2-3	37	40.7	9	28		
TNM stage#					8.106	0.004*
I/II	49	53.8	31	18		
III	42	46.2	14	28		
CEA, ng/ml					0.346	0.556
<5	60	65.9	31	29		
≥5	31	34.1	14	17		
Pre-operative CTC status					8.985	0.003*
Negative	22	24.2	17	5		
Positive	69	75.8	28	41		
Overall	91	100.0	45	46	-	-

Notes: *$P < 0.05$; #The 8th edition of the AJCC Cancer Staging Manual. Abbreviations: TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; CTC, circulating tumor cell.

Table S2. Univariate and Multivariate Cox Analyses of Clinicopathologic Factors for Affecting Recurrence-Free Survival and Overall Survival of CRC Patients (*n* = 91).

Parameters	Recurrence-free survival						Overall survival					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value	HR	95% CI	*P* value
Gender (Female vs Male)	1.043	0.614-1.771	0.876	-	-	-	1.012	0.553-1.761	0.905	-	-	-
Age (<60 years vs ≥60 years)	1.173	0.694-1.982	0.551	-	-	-	1.310	0.738-2.234	0.357	-	-	-
Tumor site (Colon vs Rectal)	1.373	0.811-2.323	0.238	-	-	-	1.409	0.792-2.507	0.243	-	-	-
Tumor size (<5 cm vs ≥5 cm)	1.232	0.815-1.864	0.323	-	-	-	1.270	0.808-1.997	0.301	-	-	-
Tumor grade (Well vs Moderate vs Poor)	1.392	1.140-3.222	0.036*	1.511	0.827-2.763	0.180	1.713	1.005-2.781	0.029*	1.667	0.834-2.912	0.143
Lymphovascular invasion (Absence vs Presence)	1.819	1.037-3.194	0.037*	1.716	0.892-3.301	0.106	1.878	1.015-3.472	0.045*	1.628	0.832-3.186	0.155
Perineural invasion (Absence vs Presence)	1.800	1.042-3.111	0.035*	1.229	0.411-1.248	0.712	1.602	0.885-2.898	0.120	-	-	-
Tumor invasion (T1-2 vs T3-4)	1.548	1.069-2.241	0.021*	1.279	0.736-2.221	0.383	1.526	1.016-2.291	0.042*	1.305	0.705-2.416	0.396
Lymph node metastasis (N0-1 vs N2-3)	1.309	1.035-1.654	0.025*	1.314	0.801-2.155	0.279	1.306	1.009-1.690	0.042*	1.538	0.886-2.672	0.126
TNM stage# (I vs II vs III)	2.109	1.236-3.599	0.001*	6.549	2.118-20.254	0.001*	1.715	1.181-2.494	0.005*	1.724	1.135-2.618	0.011*
CEA (<5 ng/ml vs ≥5 ng/ml)	1.512	0.886-2.580	0.130	-	-	-	1.691	0.947-3.021	0.076	-	-	-
Pre-operative CTC status (Positive vs Negative)	1.755	1.098-4.913	0.031*	1.324	1.014-3.653	0.045*	3.786	1.173-12.218	0.026*	7.418	2.214-25.901	0.002*
miR-106b expression (≥ Median vs <Median)	1.747	1.129-2.705	0.006*	2.046	1.059-3.951	0.033*	2.503	1.378-4.549	0.003*	2.265	1.147-4.474	0.019*

Notes: *P< 0.05; #The 8th edition of the AJCC Cancer Staging Manual. Abbreviations: RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis; CEA, carcinoembryonic antigen; CTC, circulating tumor cell.

Supplemental Methods

Cell Culture and Reagents

The human monocyte cell line THP-1, human CRC cell lines (HCT116 and HT29) were purchased from the Chinese Academy of Sciences in Shanghai. Cells were cultured in RPMI 1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C in a humidified atmosphere with 5% CO_2. To induce EMT of CRC cells, cells (1×10^5) were incubated with 100 ng/mL recombinant human IL-6 (R&D Systems) for 48 h as our previously described[1]. To induce differentiation into macrophages, THP-1 cells (1×10^6) were incubated with 100 ng/mL PMA (Sigma-Aldrich, USA) for 24 h. For *in vitro* 'educating' treatment of macrophages, cells were cultured for 48 h in medium containing 100 μg/ml exosomes. Macrophages and CRC cell lines co-cultivation was conducted using the non-contact co-culture transwell system (Corning, USA). Inserts containing THP-1 macrophages were transferred to 6-well plate seeded with CRC cells (1×10^5 cells per well) in advance and co-cultured. After 48 h of co-culture, macrophages or CRC cells were harvested for further analyses. PI3K p110γ inhibitor (TG100-115) was purchased from Med Chem Express, USA.

Cellular Internalization of Exosomes

Purified exosomes were labeled with PKH67 Green Fluorescent mem- brane linker dye (Sigma-Aldrich) according to manufacturer's instructions. Then, the labeled exosome pellets were resuspended and added to the unstained macrophages for exosomes uptake experiments. After incubation for 30 minutes, 6 h, 12 h, 24 h, or 48 h at 37℃, cells were
collected for RNA extraction and RT-qPCR analyses. The macrophages incubated with PKH670-labeled exosomes were stained with DiIC16 (Sigma-Aldrich, St. Louis, MO) and DAPI, and then imaged under a fluorescence microscope.

Plasmid Constructs, MiR Inhibitor/Mimic, and Transfection

Control microRNAs, miR-106b mimic and miR-106b inhibitor were purchased from RiboBio (Guangzhou, China). Transfections were performed by Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions with RNA oligonucleotides at a final concentration of 100 nM. PDCD4 plasmid vector and negative control were chemically synthesized, constructed, sequenced and identified by GeneChem (Shanghai, China). Macrophages were transfected with PDCD4 plasmid vector or negative control using LipoFiter™ reagent (Hanbio Biotechnology Co., LTD. Shanghai, China), according to the manufacturer's instructions. In brief, spread the cells in a six-well plate, and perform transfection when the cell confluence reaches about 50~70%. First, add 4 ul PDCD4 and the control plasmid to 250 ul DMEM and mixed by pipetting. Second, add 10 ul LipoFiter™ reagent to 250 ul DMEM, and mixxing well and leaving at room temperature for 5 minutes. Then, the plasmid solution and LipoFiter solution were mixed and placed at room temperature for 20 minutes. Finally, add the above mixture into a hole of the six-well plate, and mix it evenly. Forty-eight hours after transfection, cells were plated for a functional assay or harvested for RNA and protein analyses.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA of cells or tissues was extracted by TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. A miRcute Plus miRNA First-Strand cDNA Synthesis Kit (TIANGEN, China)

was used to synthesize miRNA cDNA from total RNA of cells and tissues. Prior to extraction of exosomal RNA, a C. elegans miRNA, cel-miR-39 (1 pmol per sample; TIANGEN, China), was spiked into the culture medium (350 μL) or exosomes (100 μg) and used as a exogenous reference. During ethanol precipitation, Dr. GenTLE Precipitation Carrier (Takara, Japan) was added, according to the manufacturer's instructions, as a co-precipitant to enhance the yield of exosomal RNA. Reverse transcription and qRT-PCR were performed as our previously described[1]. In cell and tissue lysates, mRNA levels were normalized against β-actin and miRNA levels were normalized against U6. Moreover, the miRNA levels in culture medium and exosomes were normalized against the exogenous reference cel-miR-39. The primers of miRNAs were purchased from RiboBio (RiboBio, Guangzhou, China).The sequences of primers used for qRT-PCR in this study were listed in Table SEP1.

Protein Extraction and Western Blot

Protein extraction and western blot were performed as our previously reported[1]. In brief, cells were lysed using a RIPA buffer, including a protease inhibitor cocktail (Thermo Scientific, USA). The proteins were separated by SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). After blocking with 5% non-fat milk,, the membranes were incubated with primary antibodies at 4°C overnight. The HRP-conjugated secondary antibodies were used to incubate the membranes for 2 h at room temperature. The membranes were washed and incubated for 1h at room temperature with HRP-conjugated secondary antibodies. Proteins were detected using a Bio-Rad ChemiDoc XRS+System. Bio-Rad Image Lab software was used for densitometric analysis. The following primary antibodies were used: anti-CD9, anti-CD63, anti-CD81, anti-TSG101 and anti-Calnexin (1:500; Abcam, UK); anti-PDCD4 (1:1000; Abcam, UK); anti-E-cadherin, anti-N-cadherin and anti-Vimentin (1:1000; Proteintech, USA); anti-p-PI3K, anti-mTOR, anti-p-mTOR, anti-AKT, anti-p-AKT (1:1000; CST, USA); anti-GAPDH and anti-β-actin (1:5000; Santa Cruz, CA).

Flow Cytometry and Enzyme-Linked Immunosorbent Assay (ELISA)

Flow cytometry analysis of macrophages were performed as our previously reported[1]. The following antibodies were used: anti-CD163-PE, anti-CD206-APC, anti-HLA-DR-FITC, anti-CD86-APC-Cy7 (BD Biosciences, USA). Cell culture medium was collected 48 h after the indicated treatment, and the secretion level of IL-10 and IL-12 was quantified by using ELISA kit (Bio-Swamp, USA) as our previously described[1].

Luciferase Reporter Assay

The reporter genes containing pGL3-PDCD4-3′UTR-wildtype (Bio-Asia, China) and pGL3-PDCD4-3′UTR-mut (Bio-Asia) were synthesized. THP-1-derived macrophages were co-transfected with luciferase reporters and miR-106b mimic using Lipofectamine 3000 (Invitrogen, USA). Two days after transfection, the reporter gene activities were measured by a dual luciferase reporter assay kit (Promega, USA) according to the manufacturer's instructions.

RNA immunoprecipitation (RIP) assay

RIP was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, MA, USA) according to the manufacturer's protocol. In brief, cells were collected (at 80-90% confluency) and lysed in complete RIPA buffer containing a protease inhibitor cocktail and RNase inhibitor for each group. The expression of AGO2 protein was measured by western blot, and then the supernatant was

immunoprecipi- tated with antibody to AGO2 with protein A/G magnetic beads at 4 °C overnight. Magnetic bead-bound complexes with AGO2 were immobilized, and unbound material was washed off six times; after digesting proteins with Proteinase-K. Then, the purified RNA was finally suffered qRT-PCR analysis to demonstrate the presence of the binding targets.

Transwell Migration and Invasion Assay

Transwell chambers (8 μm pore size; Corning, USA) with or without Matrigel (Corning, USA) were used to determine the migration and invasion ability of CRC cells. The detailed steps of Transwell migration and invasion assay were performed according to our previously described[1]. In brief, $2x10^5$ cells were suspended in 500 μl RPMI 1640 containing 1% FBS and added to the upper chamber, while 750 μl RPMI 1640 containing 10% FBS was placed in the lower chamber. After 48 h of incubation, Matrigel and the cells remaining in the upper chamber were removed using cotton swabs. Cells on the lower surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Cells in 5 microscopic fields were counted and photographed. All experiments were performed in triplicate.

Immunohistochemistry (IHC) and In Situ Hybridization (ISH)

Tumor samples from CRC patients and xenograft models were used for the preparation of sections. The IHC staining was performed according to the method described in our previous studies[1, 2], Anti-PDCD4 (1:100; Sigma-Aldrich, USA), Anti-CD163 (1:50; Abcam, USA), Anti-E-cadherin and Anti-Vimentin (1:200; Proteintech, USA) were used for IHC staining. For ISH, hsa-miR-106b-5p miRCURY LNA detection probe (Exiqon, Denmark) and hematoxylin (Sigma-Aldrich, USA) was used, and the detail staining procedures were performed according to manufacturer's protocols. Images were captured with Aperio Scan-Scope AT Turbo (Aperio, USA) and assessed with image-scop software (Media Cybernetics, Inc.). The expression score of IHC and ISH staining was judged according to the staining intensity and the percentage of positive cells. Staining intensity: 0 point (negative), 1 point (weak positive, light yellow), 2 point (medium intensity, yellow) and 3 point (strong positive, brown or brown); percentage of positive cells: 0 point (≤5%), 1 point (6~25%), 2 point (26~50%), 3 point (51~75%) and 4 point (≥76%). The final score was defined as the product of the above two scores.

CTC Isolation and Identification

CTC was isolated and identified from blood samples by using the CTCBIOPSY® device (Wuhan YZY Medical Science and Technology Co., Ltd., Wuhan, China) combined with immunofluorescence staining, as our previously described[1-4]. In brief, blood sample was diluted up to 8 ml with 0.9% physiological saline containing 0.2% paraformaldehyde and left for 10 minutes at room temperature, then transferred to ISET tubes with an 8 μm diameter aperture membrane. After filtered by positive pressure from 12 mmHg to 20 mmHg, candidate CTC was adhered to the membrane and were identified by three-color immunofluorescence staining. The following primary antibodies were used: FITC-CK (1:100; Abcam, USA), PE-CD45 (1:100; Santa, USA). Nuclei was stained with Hoechst 33342 (1:500; Sigma, USA).Finally, CTC was imaged and enumerated by using a fluorescence microscopy (IX81; Olympus, Tokyo, Japan).

Table SEP1. The sequences of primers used for qRT-PCR in this study.

Gene name	Primer	Sequence (5′ to 3′)
Arginase 1	Forward	TGGACAGACTAGGAATTGGCA

	Reverse	CCAGTCCGTCAACATCAAAACT
CD163	Forward	TTTGTCAACTTGAGTCCCTTCAC
	Reverse	TCCCGCTACACTTGTTTTCAC
CD206	Forward	GGGTTGCTATCACTCTCTATGC
	Reverse	TTTCTTGTCTGTTGCCGTAGTT
IL-10	Forward	TTAAGGGTTACCTGGGTTGC
	Reverse	CTGGGTCTTGGTTCTCAGCTT
IL-12	Forward	CCTTGCACTTCTGAAGAGATTGA
	Reverse	ACAGGGCCATCATAAAAGAGGT
IL-1β	Forward	TGAACTGAAAGCTCTCCACC
	Reverse	CTGATGTACCAGTTGGGGAA
HLA-DR	Forward	TCTGGCGGCTTGAAGAATTTG
	Reverse	GGTGATCGGAGTATAGTTGGAGC
E-cadherin	Forward	ATTTTTCCCTCGACACCCGAT
	Reverse	TCCCAGGCGTAGACCAAGA
Vimentin	Forward	AGTCCACTGAGTACCGGAGAC
	Reverse	CATTTCACGCATCTGGCGTTC
N-cadherin	Forward	GCGCGTGAAGGTTTGCCAGTG
	Reverse	CCGGCGTTTCATCCATACCACAA
PDCD4	Forward	GCAGAAAATGCTGGGACTGAG
	Reverse	TGTACCCCAGACACCTTTGC
GAPDH	Forward	GCACCACCAACTGCTTAGCA
	Reverse	GTCTTCTGGGTGGCAGTGATG
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT

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