# Podoplanin-mediated platelet activation promotes proliferation and invasion of colon cancer cells

**Weihua Qian** ( weihua2440@126.com )
zhangiagang TCM hospital affiliated to nanjing university od chinese medicine https://orcid.org/0000-0001-5754-1404

**Yonghua Zhang**
zhangjiagang TCM hospital affilited to nanjing university of chinese medicine

**Yingying Pan**
zhangjiagang TCM hospital affiliated to nanjing university of chinese medicine

**Min Tao**
First Affiliated Hospital of Soochow University

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# Podoplanin-mediated platelet activation promotes proliferation and invasion of colon cancer cells


## Weihua QIAN[1,2], Yonghua Zhang[1], Yingying Pan[1], Min Tao[2]

[1]Department of Oncology,Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Suzhou 215600, China.

[2]Department of Oncology, the First Affiliated Hospital of Soochow University, Suzhou 215006, China.

Corresponding to:

Min Tao, M.D., Ph.D., E-mails: mtao@medmail.com.cn; Department of Oncology, the First Affiliated Hospital of Soochow University, Suzhou 215006, China.

Weihua QIAN, M.D., E-mails: weihua2440@126.com; Department of Oncology,Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Suzhou 215600, China.



### Abstract

**Background:** Recent studies have shown that podoplanin is highly expressed in many tumors, suggesting that podoplanin may be related to the invasion and metastasis of malignant tumors. A potential mechanism by which podoplanin promote tumor invasion and metastasis is podoplanin-mediated platelet aggregation and activation.

**Methods:** The expression of podoplanin in colon cancer LOVO cell line and lung adenocarcinoma cell line A549 was detected by immunoblotting. The above cells were co-cultured with washed mouse platelets. The platelet aggregation meter was used to determine the platelet aggregation rate and observe the cell morphology. Immunofluorescence staining and immunoblotting were used to detect the expression of EMT-related factors and the phosphorylation level of Smad2/3 downstream of the TGF-β signal; and then the matrigel-coated transwell chamber invasion test was used to evaluate the invasion ability of tumor cells. Lentiviruses targeting human podoplanin shRNA were constructed to infect colon cancer LOVO cells to generate cell lines with stable podoplanin knockdown, and the above experiments were repeated.

**Results:** The colon cancer LOVO cell line with positive podoplanin expression had platelet aggregation activity, and the platelet aggregation activity of colon cancer LOVO cell line disappeared when anti-podoplanin monoclonal antibody MS-140 was added. However, no podoplanin expression and platelet aggregation activity were detected in lung adenocarcinoma cell line A549. When colon cancer LOVO cell-platelet reactant supernatant was added to colon cancer LOVO cell culture, the morphology of colon cancer LOVO cells showed EMT characteristics. Immunoblot showed that E-cadherin was down-regulated in colon cancer LOVO cells, while Vimentin And N-cadherin expression are upregulated. However, in lung adenocarcinoma A549 cells, there were no changes in EMT morphology and EMT factors. Immunoblot and ELISA showed that

colon cancer LOVO cells with positive expression of podoplanin released TGF-β after aggregating with platelets, activated the TGF-β/Smad2/3 signaling pathway, and induced EMT and increased invasiveness of colon cancer LOVO cells. Treating colon cancer LOVO cells with TGF-β antibodies and TGF-β receptor inhibitors, or using LOVO cell lines with podoplanin knockdown, and repeating the above experiments suggest that platelet aggregation activity disappears and tumor cells EMT are eliminated, and The ability to invade is reduced.

**Conclusions:** Podoplanin-mediated platelet activation plays an important role in colon cancer invasion and metastasis; its possible mechanism is that colon cancer LOVO cells with positive podoplanin expression react with platelets, activate platelets to release TGF-β, and activate TGF-β / Smad2 / 3 Signaling pathway induces EMT in colon cancer LOVO cells and enhances tumor cell proliferation and invasiveness.

**Keywords:** platelets; transforming growth factor-β; podoplanin; colorectal cancer; epithelial-mesenchymal transition.

## 1 Background

Colorectal cancer (CRC) is one of the most common tumors worldwide, ranking third among men and second among women [1]. The main cause of death in most patients with colorectal cancer is local tumor invasion, lymph node metastasis and distant organ metastasis [2].A large amount of experimental evidence shows that platelets, as part of the tumor microenvironment, play an important role in the occurrence, development, invasion, tumor angiogenesis, tumor-related inflammation, immune response, and distant metastasis of cancer. Tesfamariam [3] research found that platelets are involved in the proliferation, invasion and distant metastasis of malignant tumors, and antiplatelet therapy can reduce the occurrence of tumor metastasis.

The occurrence, development, and metastasis of tumors are complex and multi-step processes that are regulated by the interaction of many genes, including podplanin (PDPN). podoplanin mRNA was first identified in the mouse osteoblast cell line MC3Y3-E1 in 1990 [4], originally named E11 antigen [5]; subsequently found on the surface of rat glomerular foot process cells, Because it has the function of regulating the morphology of podocytes, it was renamed as podoplanin [6]. Ordonez [7] and others found that podoplanin is expressed in lymphatic vessels but not in vascular endothelium, so it is used as a specific immunohistochemical marker for lymphatic endothelial cells and lymphatic vessel production. Despite its specific expression in the lymphatic endothelium, high expression of podoplanin has also been found in many different tumors, including tumor cells of squamous cell carcinoma of the lips, mouth, tongue and pharynx [8], malignant mesothelioma [9], thymoma [10], bladder cancer [11], central nervous system germ cell tumor [12], lung squamous cell carcinoma [13], breast cancer [14], etc., suggesting that podoplanin may be associated with malignant tumors Related to invasion and metastasis.

However, recent studies on podoplanin have shown that the expression of podoplanin provides an inhibitory effect on tumorigenesis [15-17]. For example, Dumoff [15] used immunohistochemistry to detect the expression of podoplanin in 138 cases of cervical invasive

squamous cell carcinoma and adjacent tissues. The diffuse or focal immune response of podoplanin was present in 17 ( 12%) and 81 (59%) tumors, while 40 (29%) tumors had no immune response; 56% of tumors had lymphatic infiltration and 29% of tumors had lymph node metastasis; Clinicopathological features, lymphatic vessel infiltration, lymph node metastasis and patient prognosis statistical analysis showed that in tumors with low podoplanin immunoreactivity, lymphatic vessel infiltration and lymph node metastasis were more common (P <0.0001 and P < 0.022). podoplanin immunoreactivity was not associated with any other clinicopathological features (including tumor size, grade, and FIGO stage); in univariate analysis, low podoplanin immunoreactivity was significantly associated with shorter recurrence-free, suggesting podoplanin expression may be a favorable prognostic factor.

The inconsistency of the results of these clinicopathological studies indicates that the podoplanin plays different functions in different organ environments or different malignant tumor cells, it can act as an enhancer in some tumors, and inhibits the progress of other tumors The role of the agent. Therefore, further research is urgently needed to verify the function of Pingpoprotein in tumor cells and intratumor stromal cells in different cancers.

In previous clinical studies [18], we have found that the expression of podoplanin in colorectal cancer tumor tissue is significantly higher than that of non-tumor colorectal tissue; and the mean platelet volume (MPV) is used as platelet activated Indicators can be used as prognostic indicators for patients with rectal cancer. Therefore, combined with the research of scholars at home and abroad, we speculate that tumor cells that highly express podoplanin can undergo aggregation reaction with platelets to activate platelets, thereby promoting tumor cell invasion and metastasis. In order to further demonstrate our speculation, the following research was conducted.

## 2 Materials and methods

### 2.1 Cell culture

Human colon adenocarcinoma LOVO cells were purchased from the Shanghai Chinese Academy of Sciences cell bank and cultured in RPMI-1640 (Nanjing Jiangsu Kaiji Biotechnology Co., Ltd., China) + 10% fetal bovine serum (FBS) medium. Human lung adenocarcinoma A549 cells were purchased from the Cell Bank of the Chinese Academy of Sciences and cultured in F12K (Nanjing Jiangsu Kaiji Biotechnology Co., Ltd.) +10% fetal bovine serum (FBS) medium.

### 2.2 Preparation of mouse platelets

ICR mice were purchased from the Institute of Medical Laboratory Animals, Chinese Academy of Medical Sciences. Anesthetized with chloroform, ICR mouse heart puncture to draw whole blood. After centrifugation at 110 g for 8 minutes, platelet-rich plasma (PRP) was collected from the supernatant of rat whole blood. Prepare washed platelets from the PRP pellet by centrifugation at 500g for 10 minutes, and then proceed with a modified Tyrode solution (137 mM NaCl, 11.9 mM NaHCO 3, 0.4 mM Na 2 HPO 4, 2.7 mM KCl, 1.1 mM MgCl 2, 5.6 mM glucose) Finally wash. The washed platelets were suspended in a modified Tyrode solution containing 2% platelet plasma (PPP)

at a concentration of 2-3×108 platelets/ml. All laboratory animal procedures are carried out in accordance with the regulations of China's laboratory animal ethics.

2.3 Detection of platelet aggregation rate

Tumor cells (5×104 cells) were incubated with washed mouse platelets (5×107 platelets/200 μl) and suspended in Tyrode buffer containing 2% platelet plasma and 250 μM CaCl2. Platelet aggregation rate instrument (LBY-NJ4, Beijing Pulisheng Instrument Co., Ltd., China) was used to obtain the reaction time-aggregation rate curve.

2.4 Preparation of Tumor-platelet aggregation reactant supernatant (SUP)

After incubating platelets with tumor cells at 37°C for 20-30 minutes, the reaction was centrifuged at 20,000 g for 5 minutes. The supernatant was then collected and used in related experiments.

2.5 Western blot

Adjust the pH value of the cells in Lysis Buffer (Lysis Buffer: 0.1M Tris-HCl; 1.4M NaCl; 0.02 M Na2EDTA; 2% CTAB; 0.1% DIECA; 2% PVP K-30 plus 0.2% β-mercaptoethanol) 8.0) Medium cracking. BCA protein content detection kit (China KGI Biotechnology Co., Ltd. KGA902) was used to determine protein concentration, and SDS-PAGE gel preparation kit (China KGI Biotechnology Co., Ltd. KGP113) was used for electrophoretic separation. Then transfer the protein to nitrocellulose membrane (NC) to make "fiber mat-filter paper-gel-NC membrane-filter paper-fiber mat"; put it into the transfer tank, fill the tank with the transfer membrane liquid, and perform mold transfer . Block with 5% skimmed milk powder in blocking solution, and use PBS solution instead of primary antibody as a negative control. Add antibodies against human podoplanin (UK abcam ab236529); E-cadherin (UK abcam ab40772); Vimentin (UK abcam ab92547); N-cadherin (UK abcam ab76011); p-Smad2/3 (UK abcam ab63399); Smad3 (UK abcam ab40854); F-actin (UK abcam ab205); α-SMA (UK abcam ab32575) for immunoblotting. G: BOX EF 2 gel imaging system (Syngene, UK) was used for image acquisition, and Gel-Pro Analyzer 4.0 software (Media Cybernetics, USA) was used for image analysis.

2.6 Enzyme linked immunosorbent assay (ELISA)

Using the human TGFβ1 ELISA detection kit (KGI K107C107b), follow the steps in the instructions, and finally adjust the blank holes to zero and measure the absorbance (OD value) of each well in sequence at 450nm wavelength. The measurement should be performed within 15 minutes after adding the stop solution.

2.7 Virus transfected cells

Entrusted Jiangsu Kaiji Biotechnology Co., Ltd. to construct a lentiviral vector that stably expresses short hairpin RNA (shRNA) targeting human podoplanin/ and TGF-β; co-cultured with human colon adenocarcinoma LOVO cells containing 10% FBS and 2 μg/ ml puromycin medium.Jiangsu Kaiji Biotechnology Co., Ltd. was entrusted to construct a lentiviral vector with

pLVX-PDPN overexpression; co-cultured with human lung adenocarcinoma A549 cells in a medium containing 10% FBS and 2 μg/ml puromycin.

2.8 RNA extraction, reverse transcription and Real time-PCR

Use TRIzol reagent (Invitrogen 15596-026, USA) to extract total RNA from cells; use One Step TB Green™ PrimeScript™ RT-PCR Kit II (TaKaRa RR086B, Japan) kit to reverse transcribe mRNA into cDNA; use ordinary gradient PCR instrument ( US ABI Veriti 96 well Thermal cycler) and fluorescence quantitative PCR cycler (US ABI Step one plus Real time-PCR system) for RT-PCR, PCR reaction program: 95 ℃ pre-denaturation 5min; 95 ℃ denaturation 15s, 60 ℃ annealing 20s, extended 40s at 72℃, 40 cycles. The primer sequence of the target gene was designed using Primer6 software, and Jiangsu Kaiji Biotechnology Co., Ltd. was commissioned to synthesize the primers, and the primers were collected by PAGE purification method. GAPDH serves as an internal reference gene. Relative quantitative 2^-△△Ct method was used for data analysis.

2.9 Transwell cell invasion experiment

The cells in the logarithmic growth phase were digested and inoculated into a six-well plate, and cultured with incomplete medium for 24 hours, and the drugs were added or transfected according to the group, and a negative control group was established. After adding drugs or transfected for 48 hours, use 0.25% trypsin digestion to collect cells; add 30 μL of diluted Matrigel (BD 356234, USA) to the upper chamber of Transwell, take 100 μL of the cell suspension into the upper chamber of Transwell (Corning Incorporated 3422, USA), and add 500 μL of 20% FBS in the lower chamber Culture medium; place the cell culture plate in a 37°C, 5% CO2 incubator for 48 h; remove Transwell, add 500 μL of 0.1% Crystal Violet (Sigma C3886, USA) to the cell culture plate, and take it out after 30 min at 37°C, Wash with PBS, take 3 fields of view in diameter, take a picture (magnification 200×), count.

2.10 Immunofluorescence

Cells were immersed in 4% paraformaldehyde fixative solution for 30 min, and rinsed with PBS; 2 drops of 3% H2O2-methanol solution were added dropwise to each slice, closed at room temperature (15-25°C) for 10 min, and rinsed with PBS; 50-100 ul of goat serum, incubated for 20 minutes at room temperature; primary antibody E-cadherin (abcam ab40772); Vimentin (abcam ab92547); N-cadherin (abcam ab76011); F-actin (abcam ab205); α-SMA (abcam ab32575, UK) 50-100 ul, 37°C, wet box incubation for 2 hours, PBS dip; separately add secondary antibody goat anti-rabbit IgG-HRP (China Jiangsu Kaiji Biotechnology Co., Ltd. KGAA35) or sheep anti-rabbit TRICT (China Jiangsu KGI Biotechnology Co., Ltd. KGAA35) or sheep anti-mouse FITC (China Nanjing Jiangsu KGI Biotechnology Co., Ltd. KGAA35) 50-100 ul, 37℃, incubation for 1h in the dark , PBS dip; cell nuclei were stained with 1 μg/ml Hoechst 33342 (Life Technologies) for 5 minutes. Observe the cell image under a biological inverted microscope (OLYMPUS IX51, Japan), and take three high-expression areas for photo storage.

2.11 Statistical analysis

Use Statistical Package for the Social Sciences version 18.0 (SPSS, Chicago, IL, USA) to analyze the experimental data. The results are expressed as mean ± standard deviation. Use Mann-Whitney U test or Student's t test or one-way analysis of variance to analyze the statistical significance of the differences. N.S. means Not Significant. Not statistically significant; $P<0.05$ is considered statistically significant.

## 3 Results

### 3.1 Tumor cell lines with positive expression of podplanin can induce platelet aggregation

We first investigated the potential of a tumor cell line with positive expression of podplanin to induce platelet aggregation. Immunoblotting was used to detect the expression of podplanin in tumor cell lines; as shown in Figure 1a, the expression of podplanin was detected in the colon cancer LOVO cell line, but no podplanin was detected in the lung adenocarcinoma cell line A549 Protein.

Colon cancer LOVO cells and lung adenocarcinoma A549 cells ($5\times10^4$ cells) were incubated with washed mouse platelets ($5\times10^7$ platelets/200 μl), and the platelet aggregation rate was measured using a platelet aggregation meter, as shown in Figure 1b, the colon cancer LOVO cell line positively expressing podplanin showed platelet aggregation activity, while the lung adenocarcinoma cell line A549 did not show platelet aggregation activity.

On this basis, the two groups were separately added anti- podplanin monoclonal antibody (mAb) MS-140, and the platelet aggregation rate was measured again using a platelet aggregation meter; as shown in Figure 1b, the platelet aggregation activity of colon cancer LOVO cell line suppressed. It shows that colon cancer LOVO cell line can induce platelet aggregation may depend on the expression of podplanin.

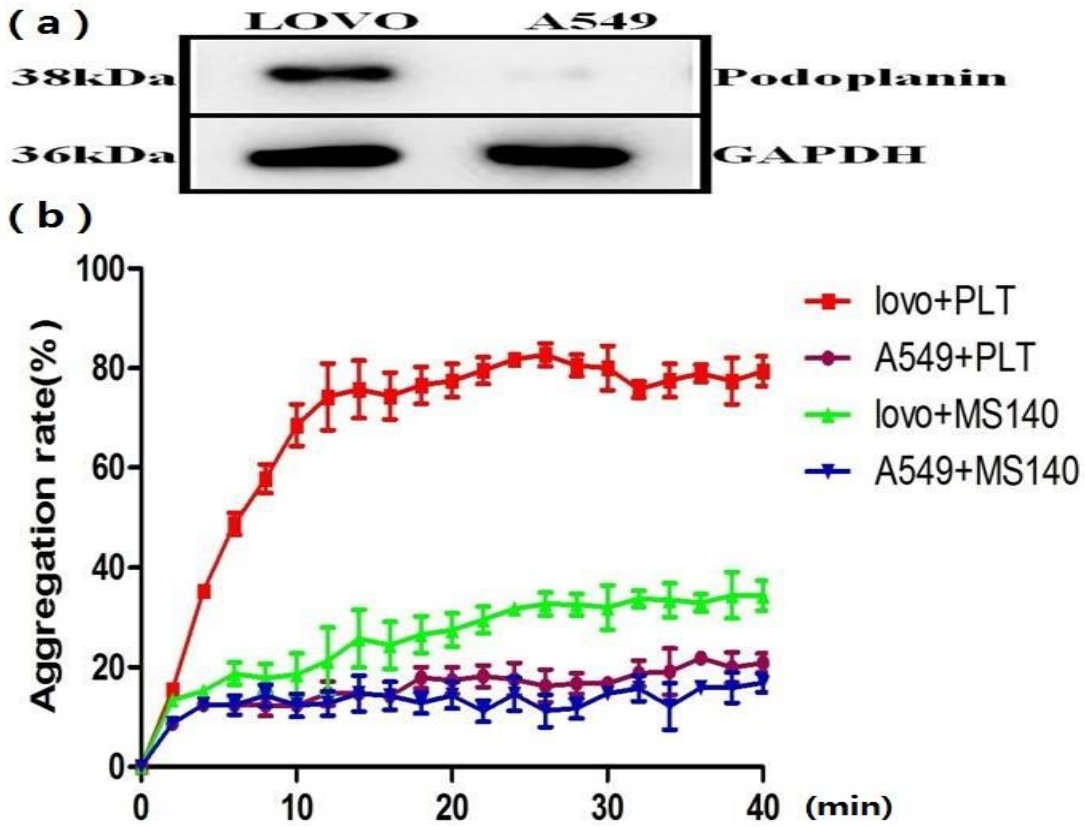



**Figure 1: Colon cancer LOVO cells with positive expression of podplanin can induce platelet aggregation.** (a) Western blot analysis showed that podoplanin expression in colon cancer LOVO cells, but no podoplanin expression in lung adenocarcinoma A549 cells. GAPDH is the internal reference. (b) Incubate colon cancer LOVO cells and lung adenocarcinoma A549 cells ($5\times10^4$ cells) with washed mouse platelets ($5\times10^7$ platelets/200 μl), and determine the platelet aggregation rate using a platelet aggregation meter; On this basis, monoclonal antibodies (mAb) MS-140 against podplanin were added to the two groups, and the platelet aggregation rate was measured again using a platelet aggregation meter.

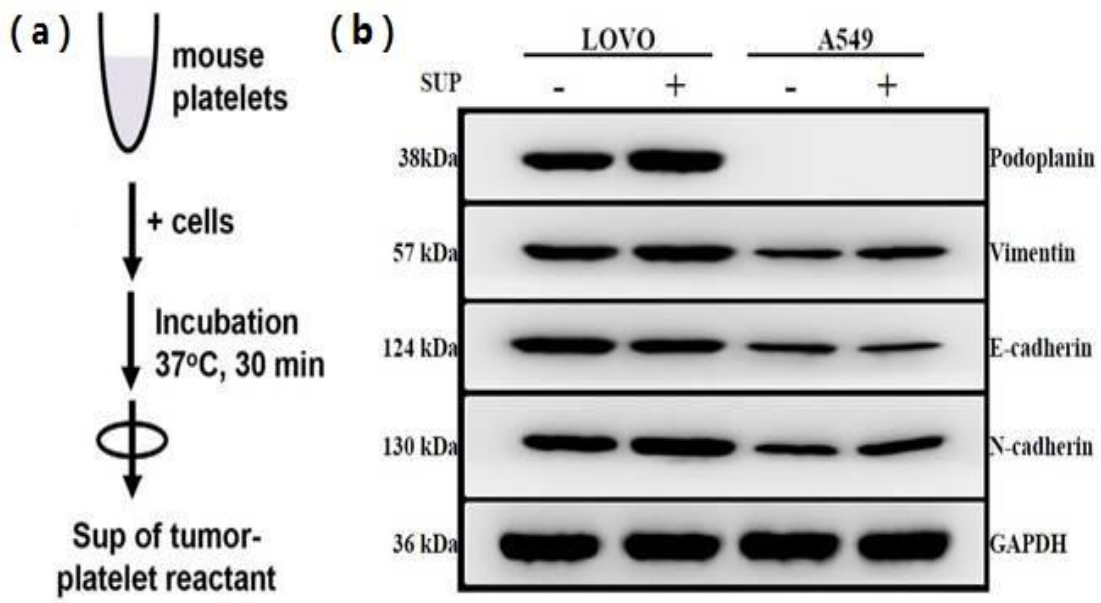
3.2 Factors released by tumor-platelet aggregation reaction induce tumor cell EMT

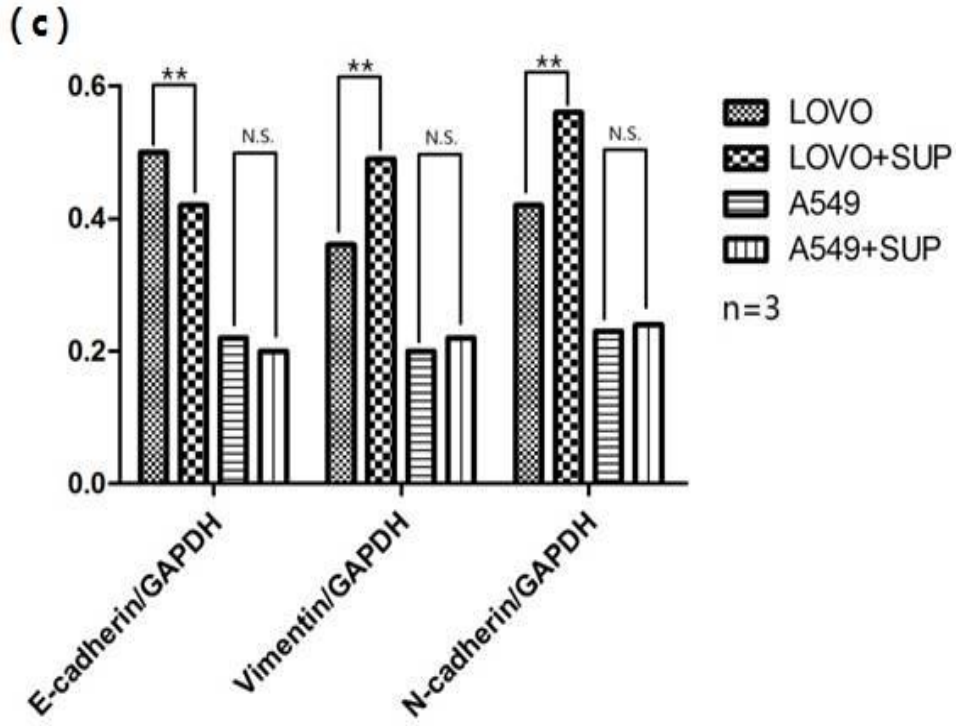
Platelets store many cytokines and growth factors in cytoplasmic granules, which are released after forming aggregates with cancer cells, leading to platelet activation [19-23]. Therefore, we next investigated the role of soluble cytokines released after podplanin-induced tumor-platelet aggregation. To eliminate the potential confounding effect of direct contact between tumor cells and platelets, we prepared tumor cell-platelet reactant supernatant (Figure 2a).

The supernatant of colon cancer LOVO cells-platelet reactant was added to the colon cancer LOVO cell culture dish, it can be observed that the morphology of colon cancer LOVO cells appearing as clustered colonies in the culture dish is exposed to tumor cell-platelets The supernatant of the reactant then becomes a dispersed configuration (Figure 2d), and this morphological change is one of the characteristics of EMT. We use immunofluorescence staining and immunoblotting to detect EMT-related factors, including: α-SMA, E-cadherin, Vimentin and N-cadherin. Immunoblotting showed that E-cadherin was down-regulated in colon cancer LOVO cells, while

Vimentin and N-cadherin expression were up-regulated (Figure 2b, 2c). Immunofluorescence showed that reduced expression of E-cadherin was observed in colon cancer LOVO cells, while up-regulation of α-SMA and Vimentin expression (Figure 2d).

The supernatant of lung adenocarcinoma A549 cell-platelet reactant was added to the lung adenocarcinoma A549 cell culture dish, and the above test was repeated. The lung adenocarcinoma A549 cell showed no EMT morphological changes and changes in EMT-related factors (Figure 2b, 2c, 2d). These results indicate that tumor-platelet aggregation must occur before releasing certain soluble cytokines into the supernatant, inducing tumor cell EMT.



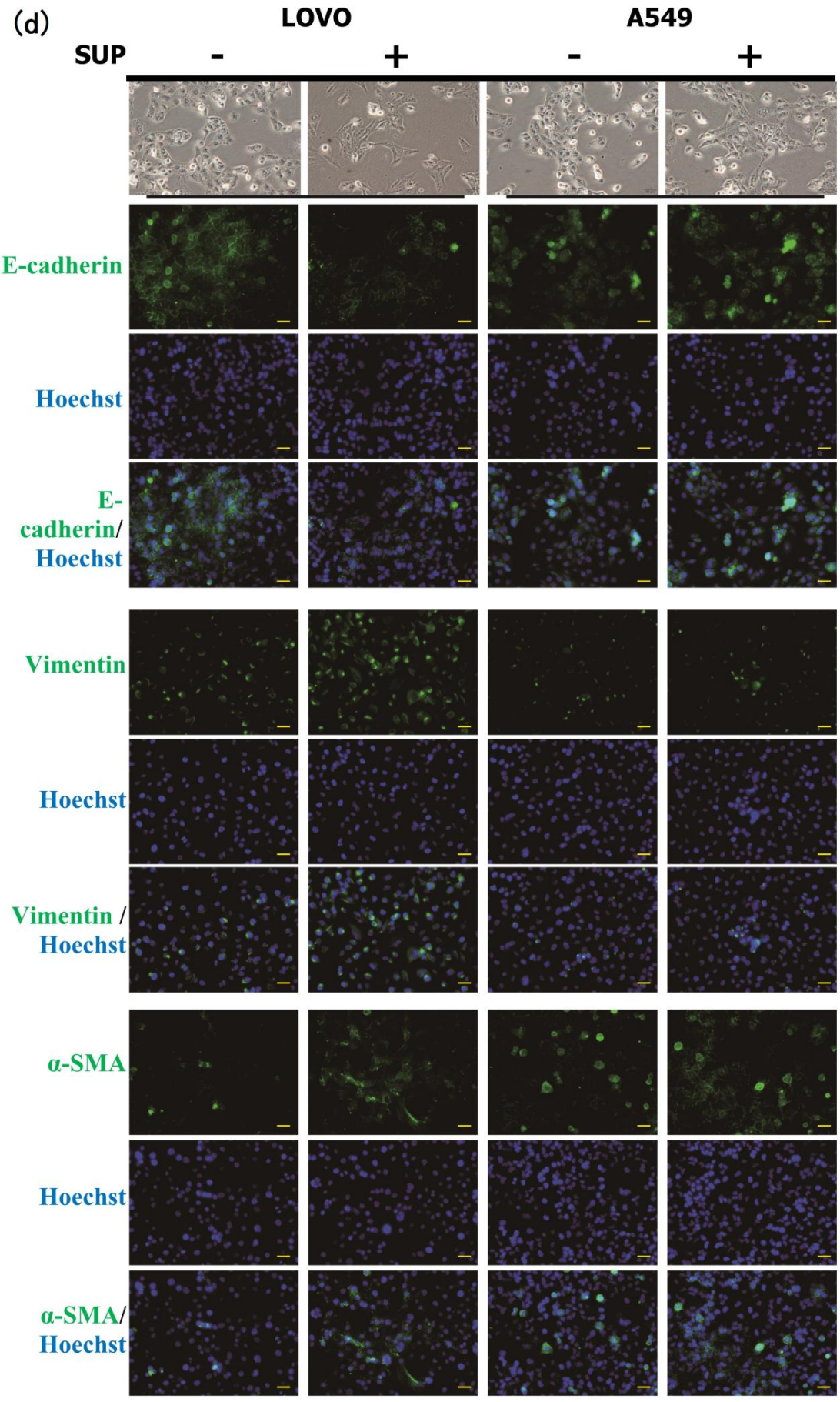

(d)
LOVO
A549
SUP
-
+
-
+
E-cadherin
Hoechst
E-cadherin/ Hoechst
Vimentin
Hoechst
Vimentin / Hoechst
α-SMA
Hoechst
α-SMA/ Hoechst

**Figure 2: Factors released by the tumor-platelet aggregation reaction induce tumor cell EMT.** (a) Schematic diagram of preparation and collection of tumor-platelet reactant supernatant. (b) Immunoblotting to detect the expression of podoplanin, E-cadherin, Vimentin and N-cadherin. GAPDH is the internal reference. (c) Use the Gel-Pro32 software to perform gray-scale analysis on the results of E-cadherin, Vimentin, N-cadherin and GAPDH. All data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistics Analysis; NS means No statistically significant; ** means $P<0.05$. (d) Immunofluorescence technology to detect E-cadherin (green), α-SMA (green) Vimentin (green) and nuclear DNA (blue, Hoechst); use phase contrast microscope (top) and fluorescence microscope (bottom) to capture images . Scale bar represents 50μm.

3.3 TGF-β released by tumor-platelet aggregation reaction comes from platelets instead of tumor cells

In order to study which cytokines released after the tumor cell-platelet aggregation reaction caused tumor EMT, we used ELISA to analyze the levels of several soluble factors commonly found in the supernatant of tumor cell-platelet reactant; among them, transforming growth factor ( TGF)-β is one of the most effective and common inducers of EMT. It can induce spindle-shaped cell morphology, inhibit cell proliferation, and promote the movement and invasion of tumor cells. The results showed that in the supernatant of colon cancer LOVO cell-platelet reactant, the concentration of TGF-β was significantly higher than that of lung adenocarcinoma A549-platelet reactant (Figure 3a); importantly, In the supernatant of colon cancer LOVO cell-platelet reactant treated with monoclonal antibody (mAb) of podoplanin (MSb), platelet aggregation activity was inhibited (Figure 1b), and the concentration level of TGF-β decreased (Figure 3a) .

In order to exclude that colon cancer LOVO cells release TGF-β itself to affect the concentration level of TGF-β in the tumor-platelet reactant supernatant, we prepared TGF-β-silent colon cancer LOVO cells (Figure 3b). We used the best silencing LOVO-shTGF-β2 cell line and platelets to incubate at 37℃ for 20-30 minutes, collected the supernatant, and detected the concentration of TGF-β by ELISA. The experimental results showed that TGF-β silenced colon The concentration of TGF-β in the cancer LOVO-shTGF-β2 cell line-platelet supernatant is equivalent to the concentration of TGF-β in the normal LOVO cell-platelet supernatant. Silencing the TGF-β of LOVO cells does not affect the release when platelet aggregates TGF-β levels. These results indicate that when the tumor cell-platelet aggregation reaction occurs, it is platelets rather than tumor cells that release a large amount of TGF-β (Figure 3c)。

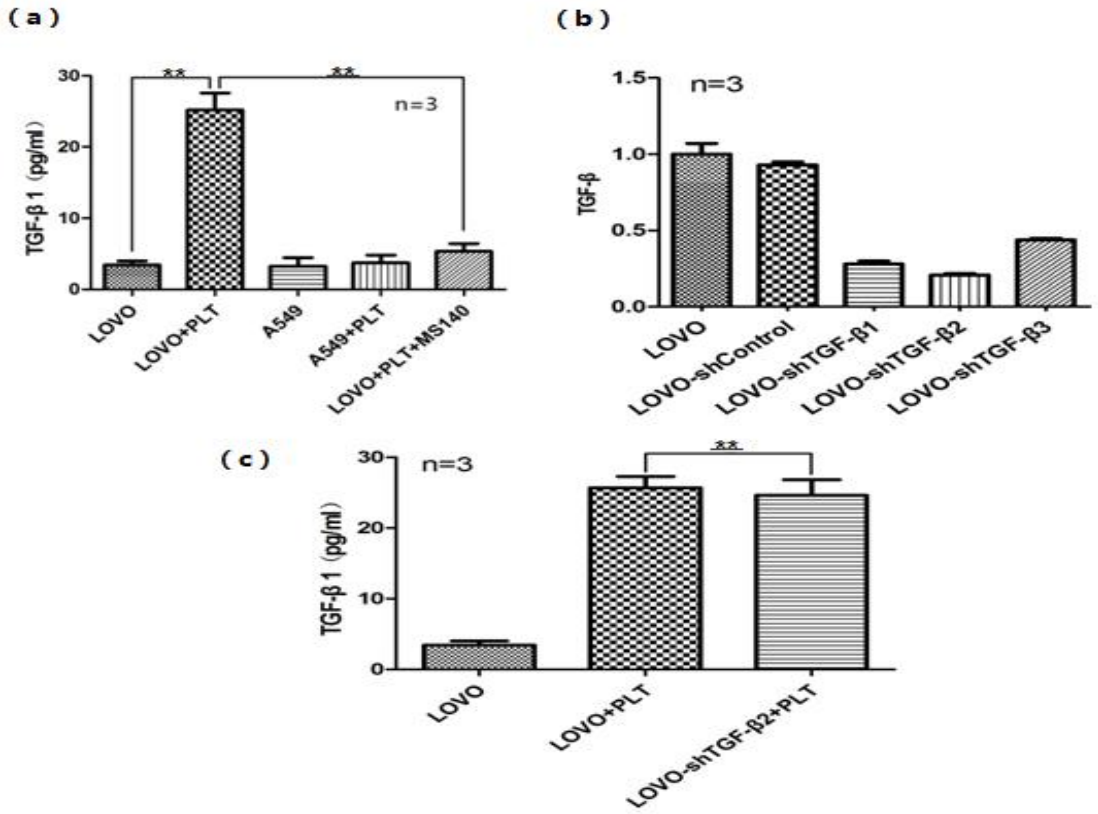



**Figure 3: TGF-β released by tumor-platelet aggregation reaction comes from platelets rather than tumor cells.** (a) ELISA method was used to analyze the concentration of TGF-β1 in each group; all data were expressed as mean±standard deviation (n=3); error bars indicated standard deviation. Student's T test was used for statistical analysis; N.S. means No statistical significance; ** means P<0.05. (b) Silencing the expression of TGF-β in colon cancer LOVO cells using RNA interference technology, and using RT-PCR method and 2^-△△Ct method to detect the expression of TGF-β. (c) ELISA method was used to analyze the concentration of TGF-β1 in each group; all data were expressed as mean±standard deviation (n=3); error bars indicated standard deviation. Use Student's T test for statistical analysis; N.S. means No statistical significance; ** means P<0.05.

3.4 TGF-β released by tumor-platelet aggregation reaction induces EMT of tumor cells

In order to detect the reactivity of tumor cells to TGF-β, colon cancer LOVO cells and lung adenocarcinoma A549 cells were treated with purified active TGF-β1. Immunoblotting showed that the expression of E-cadherin was down-regulated in both cell lines, while the expression of Vimentin and N-cadherin was up-regulated (Figure 4a, 4b); immunofluorescence showed that both in colon cancer LOVO cells and lung adenocarcinoma A549 cells E-cadherin expression was observed to be reduced, while α-SMA and Vimentin expression were up-regulated (Figure 4c); indicating that TGF-β1 induced EMT in colon cancer LOVO cells and lung adenocarcinoma A549 cells. Both colon cancer LOVO cells and lung adenocarcinoma A549 cells responded to TGF-β1, however treatment with supernatant of lung adenocarcinoma A549-platelet reactant could not induce A549 cells EMT

(Figure 2), which means that podoplanin induction The release of TGF-β related to platelet aggregation is crucial for EMT.

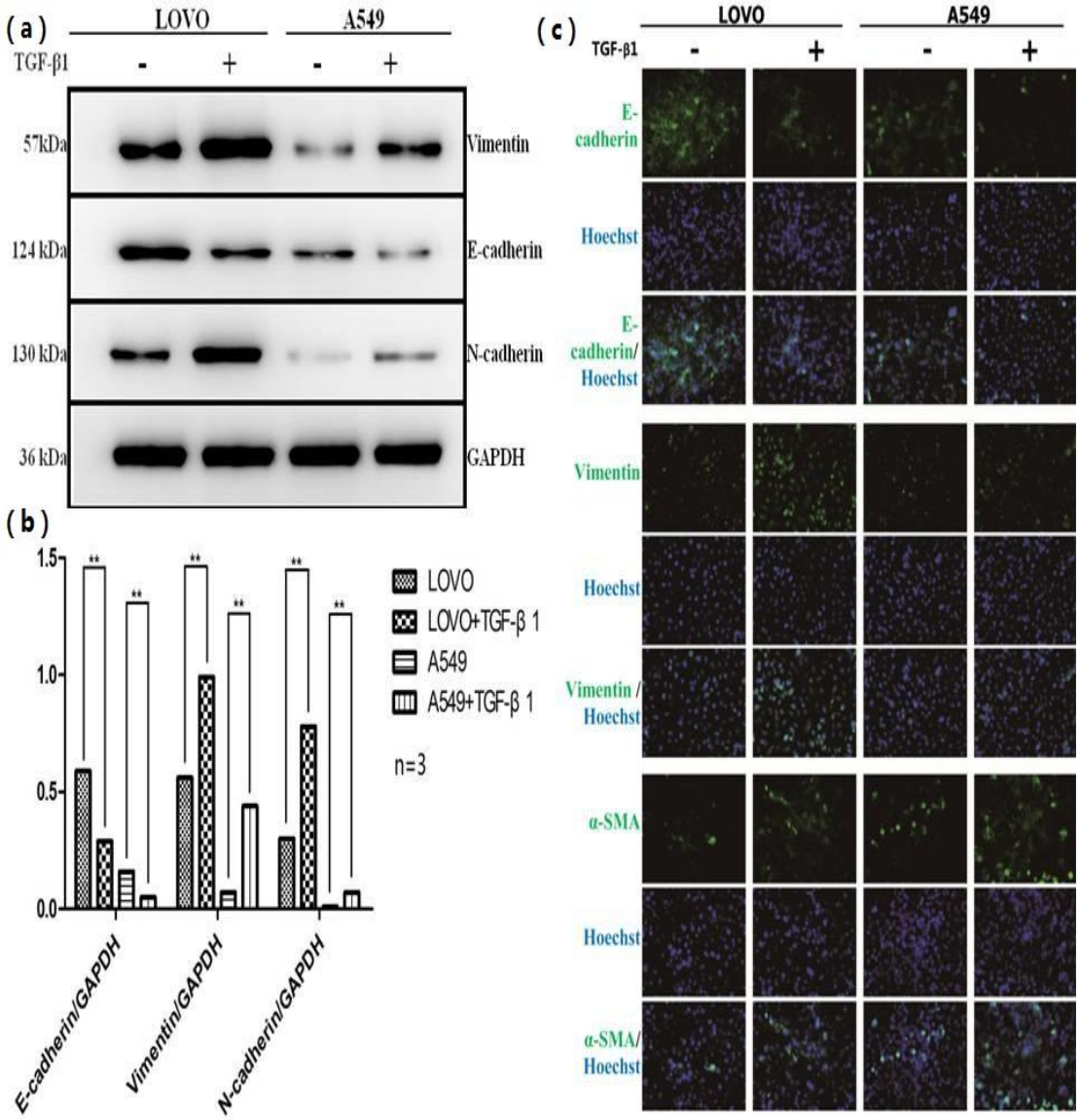



**Figure 4: TGF-β released by tumor-platelet aggregation reaction induces EMT of tumor cells.** (a) Immunoblotting to detect the expression of E-cadherin, Vimentin and N-cadherin. GAPDH is the internal reference. (b) Use Gel-Pro32 software to perform gray-scale analysis on the results of E-cadherin, Vimentin, N-cadherin and GAPDH, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistics Analysis; NS means No statistically significant; ** means P<0.05. (c) Immunofluorescence technology to detect E-cadherin (green), α-SMA (green) Vimentin (green) and nuclear DNA (blue, Hoechst); fluorescence microscope to capture images. Scale bar represents 50μm.

In order to detect the status of the TGF signaling pathway, we used immunoblotting to detect the phosphorylation level of Smad2/3 downstream of the TGF-β signaling pathway. We tested 8 groups

of specimens, the divisions are: 1, LOVO; 2, LOVO + platelets (200ul); 3. LOVO + tumor-platelet reactant supernatant (200ul); 4, LOVO + TGF-β1 (3ng/ml) ; 5, A549; 6, A549 + platelets (200ul); 7, A549 + tumor-platelet reactant supernatant (200ul); 8, A549 + TGF-β1 (3ng/ml). The results showed that the phosphorylation of Smad2/3 downstream of Group 2, Group 3, Group 4, and Group 8 that had tumor cell-platelet aggregation reaction was up-regulated, while the phosphorylation of Smad2/3 in other groups was not up-regulated (Figure 5a, 5b). The above results indicate that TGF-β is released after tumor cell-platelet aggregation occurs, TGF-β/Smad2/3 signaling pathway is activated, and EMT of tumor cells is induced.

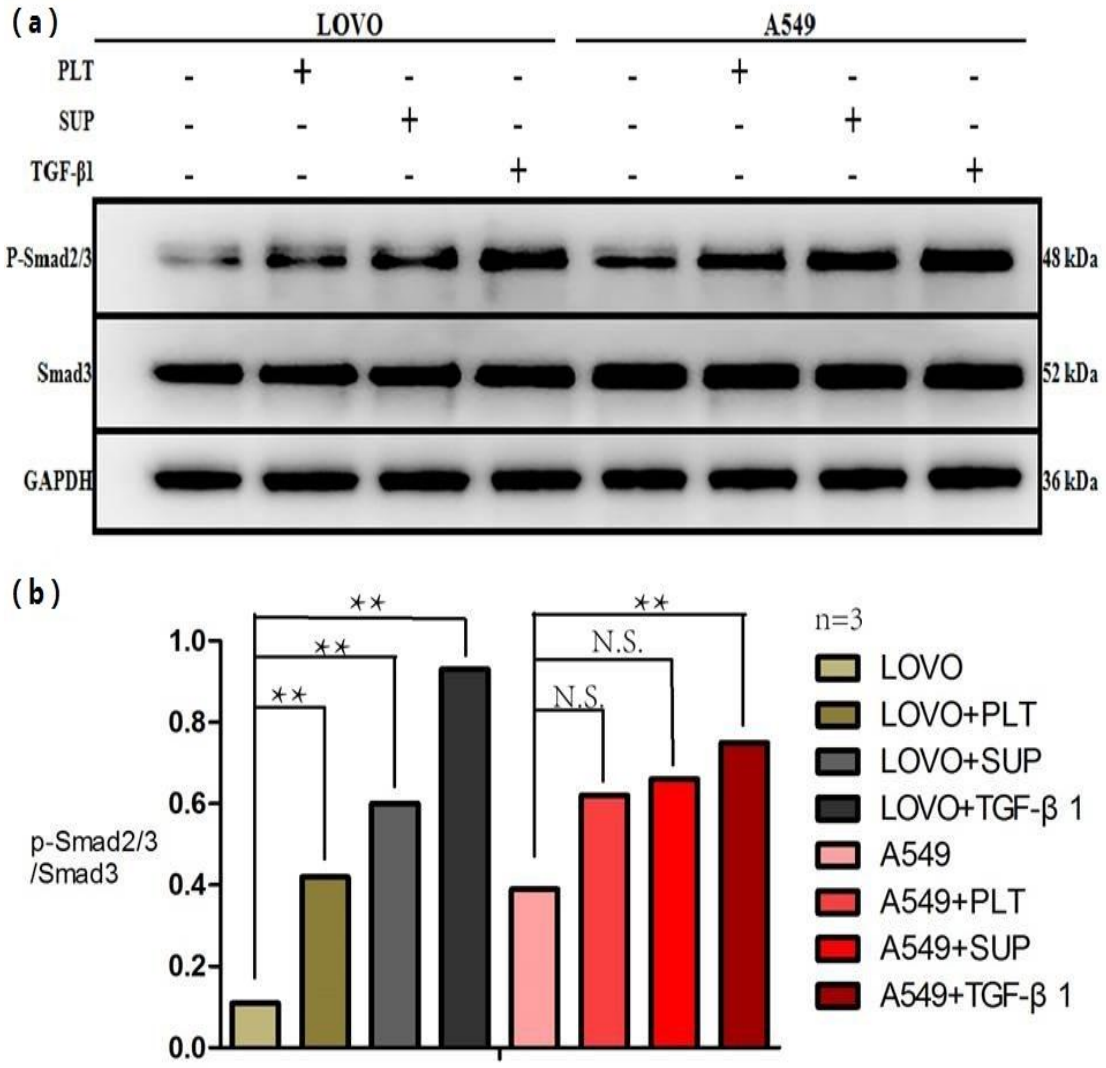



**Figure 5: The tumor cell-platelet aggregation reaction activates the TGF-β/Smad2/3 signaling pathway.** (a) Immunoblotting to detect the expression of p-smad2/3 and smad3. GAPDH is the internal reference. (b) Use Gel-Pro32 software to perform gray-scale analysis of p-smad2/3 and smad3 results, all data are expressed as mean ± standard deviation (n=3); statistical analysis is performed using Mann-Whitney U test; NS indicates No statistically significant; ** means $P<0.05$.

3.5 TGF-β released by tumor-platelet aggregation reaction is essential for the EMT of colon cancer LOVO cells

We used blocking TGF-β signaling to determine the effect of TGF-β on EMT of colon cancer LOVO cells. The activation of TGF-β-mediated signaling pathways is caused by the combination of TGF-β with two different receptors, type I and type II TGF-β receptors (TGFβR1 and TGFβR2) to form a heterodimeric complex. We used TGF-β1 neutralizing antibody (1D11 mAb) and TGF-β receptor inhibitor (LY2157299, targeting TGFβR1 and TGFβR2;) to treat colon cancer LOVO cells for 2 hours, using immunofluorescence double staining and Western blot to detect EMT Related factors; we can observe that after adding the tumor-platelet reactant supernatant to colon cancer LOVO cells, immunofluorescence staining showed that the expression of E-cadherin decreased, and the expression of F-actin increased. Redistribution; and after adding 1D11 mAb or LY2157299 treatment, EMT induced by colon cancer LOVO-platelet reactant supernatant was eliminated (Figure 6a, 6b, 6c, 6d).

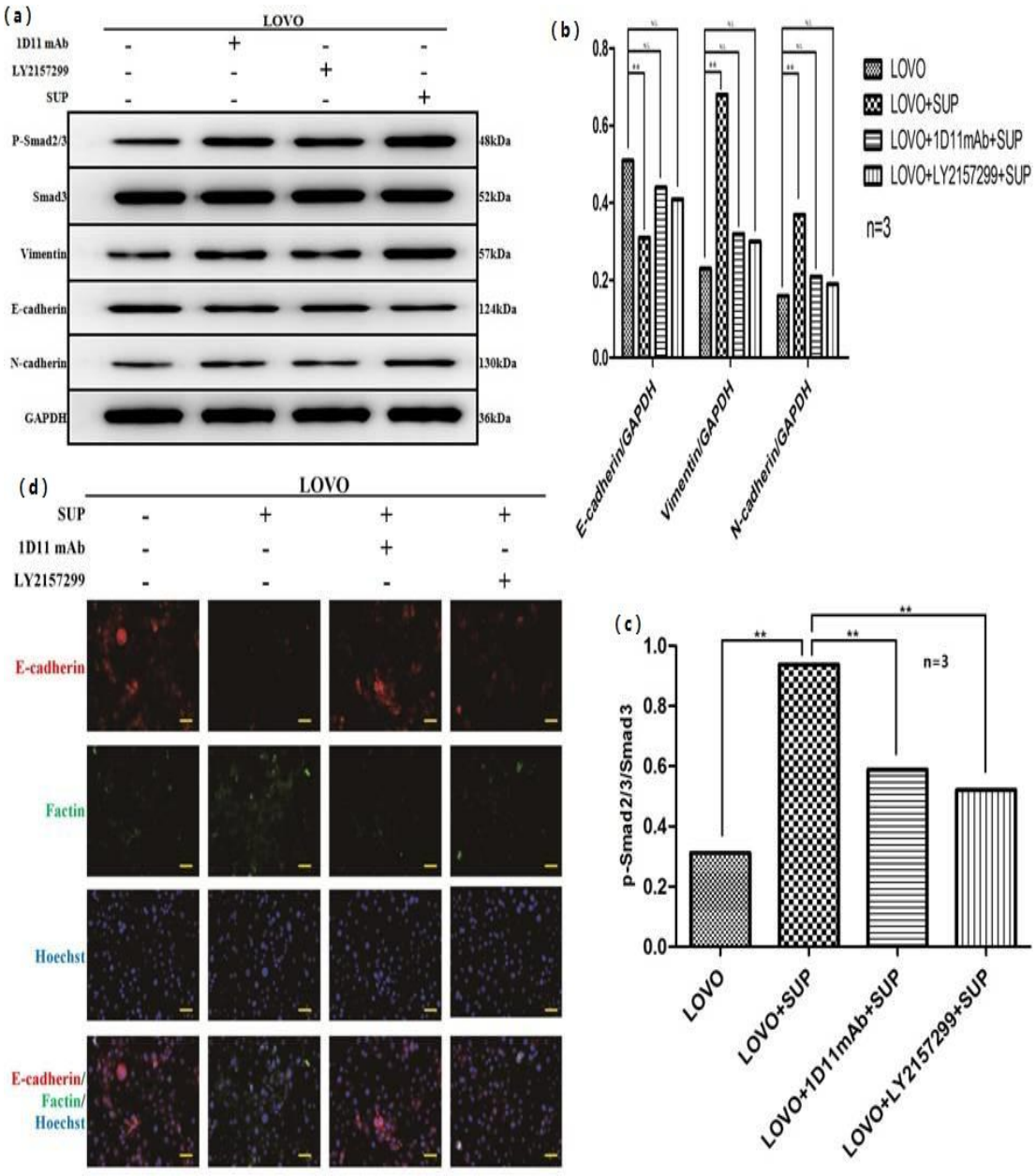


**Figure 6: TGF-β released by tumor-platelet aggregation reaction is essential for EMT of colon cancer LOVO cells.** (a) Immunoblotting to detect the expression of p-smad2/3, smad3, E-cadherin, Vimentin and N-cadherin. GAPDH is the internal reference. (b) Use Gel-Pro32 software to perform gray-scale analysis on the results of E-cadherin, Vimentin, N-cadherin and GAPDH, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistics Analysis; NS means No statistically significant; ** means P<0.05. (c) Use Gel-Pro32 software to perform gray-scale analysis on the results of p-smad2/3 and smad3, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistical analysis;** Means P<0.05. (d) Immunofluorescence technology to detect E-cadherin (red), Factin (green) and nuclear DNA (blue, Hoechst); capture images with a fluorescence microscope. Scale bar represents 50μm.

We used the Matrigel-coated transwell cell invasion test to evaluate the invasion ability of tumor cells. As shown in Figure 7, treatment of colon cancer LOVO cells with the supernatant of colon cancer LOVO cells-platelet reactants increased the invasiveness of tumor cells; whereas colon cancer LOVO cells were first neutralized with TGF-β1 neutralizing antibody (1D11) and TGF-β receptor inhibitor (LY2157299), the supernatant was added, and its invasiveness decreased significantly. These results indicate that the platelet aggregation reaction induced by colon cancer LOVO cells releases TGF-β and the activation of TGF-β signaling is crucial for tumor cell EMT and tumor cell invasion.

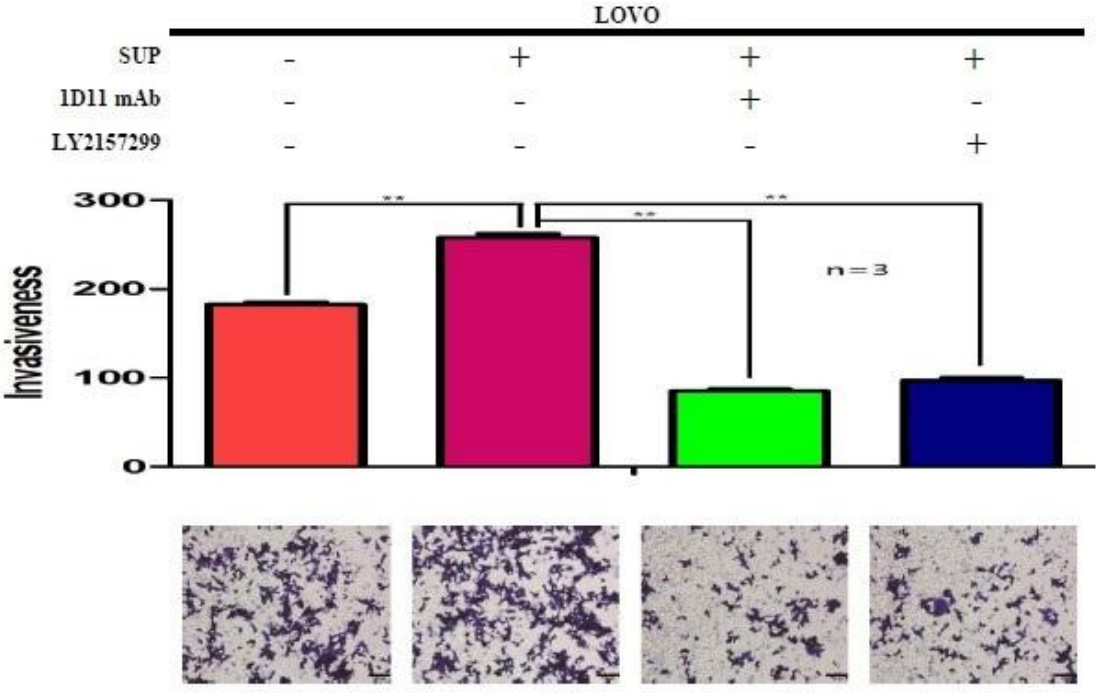
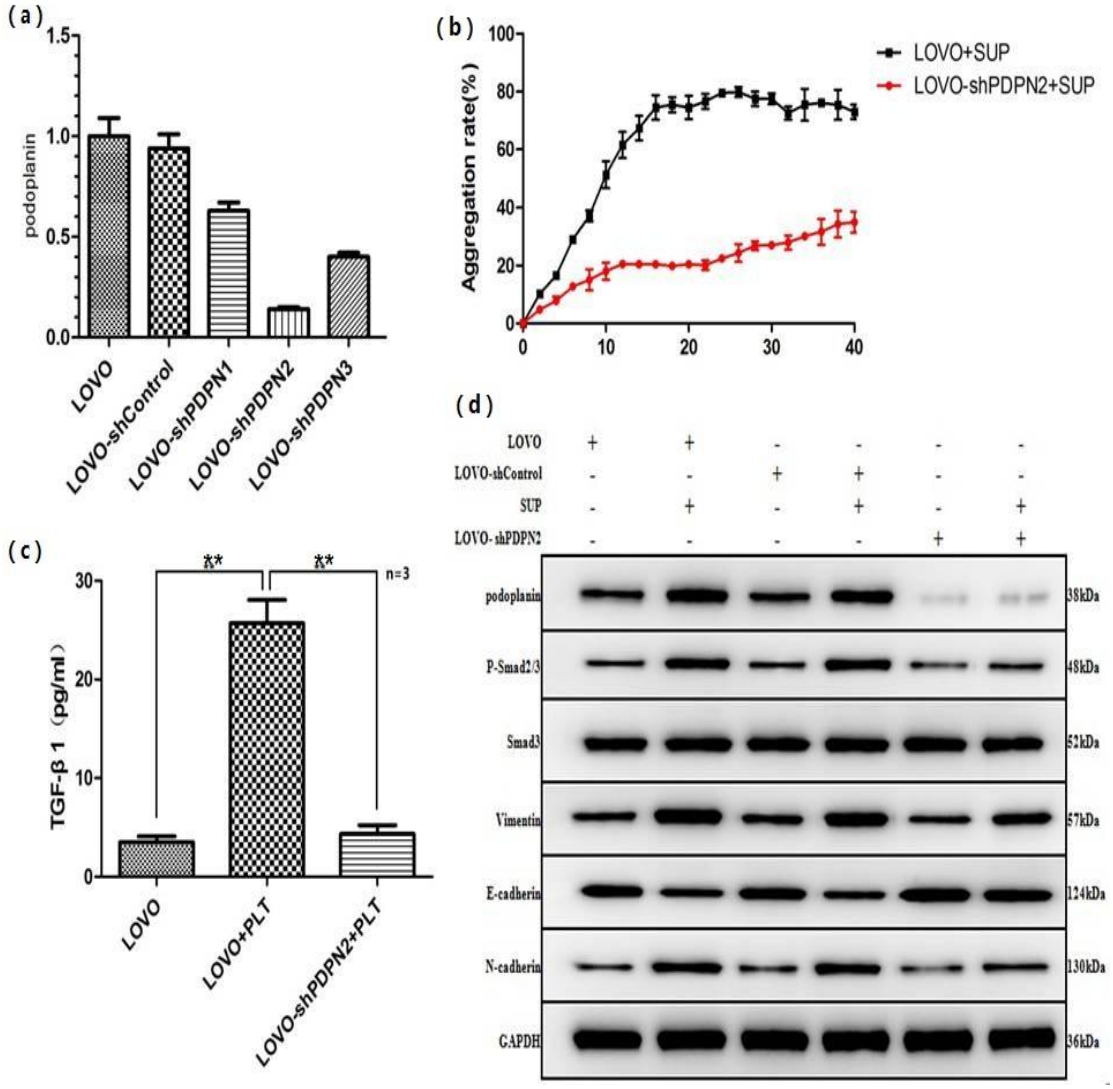


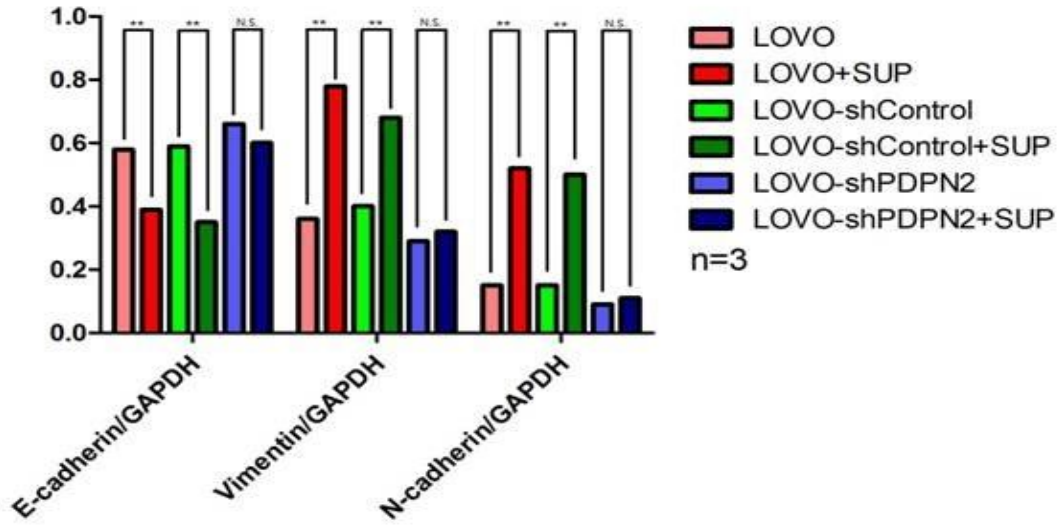

**Figure 7: Transwell cell invasion test using Matrigel coating to detect cell invasion.** Cells that migrated through the membrane were stained with crystal violet, 3 fields of diameter were taken, photographed (magnification 200×), counted, all data are averaged Value ± standard deviation (n=3); scale bar represents 50μm; statistical analysis using Mann-Whitney U test; ** indicates P<0.05.

3.6 podoplanin expression is necessary for tumor cell-platelet aggregation reaction to release TGF-β

In order to prove that the tumor cell line with positive expression of podoplanin is necessary to induce the tumor cell-platelet aggregation reaction to release TGF-β, we used RNA interference technology to construct a stable LOVO cell line with low expression of podoplanin: LOVO-shPDPN2 (Figure 8a); Immunoblotting showed low expression of podoplanin (Figure 8d). We repeated the above experiment using LOVO-shPDPN2 cells, proving that the flat platelet-silencing LOVO cell line had weakened platelet aggregation ability (Figure 8b), TGF-β concentration level was significantly reduced (Figure 8c), and could not induce EMT (Figure 8d, 8e, 8f, 8g), and the invasiveness of the cells also decreased (Figure 8h).



(e)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0.4
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N.S.
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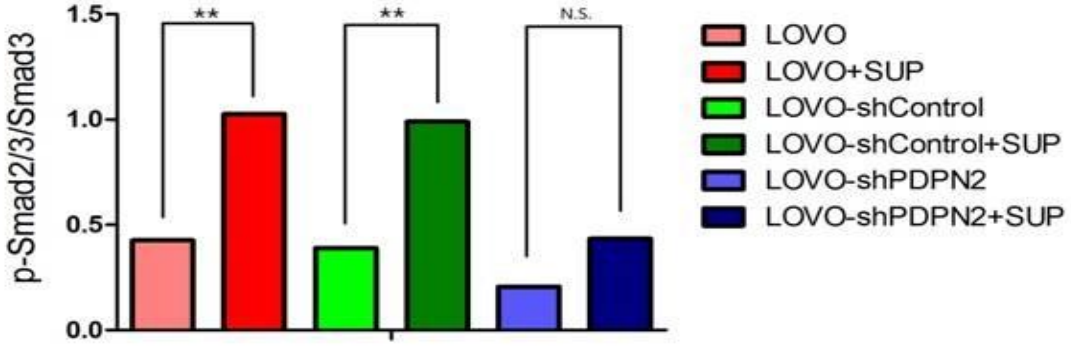
E-cadherin/GAPDH
Vimentin/GAPDH
N-cadherin/GAPDH
LOVO
LOVO+SUP
LOVO-shControl
LOVO-shControl+SUP
LOVO-shPDPN2
LOVO-shPDPN2+SUP
n=3

(f)


p-Smad2/3/Smad3
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N.S.
LOVO
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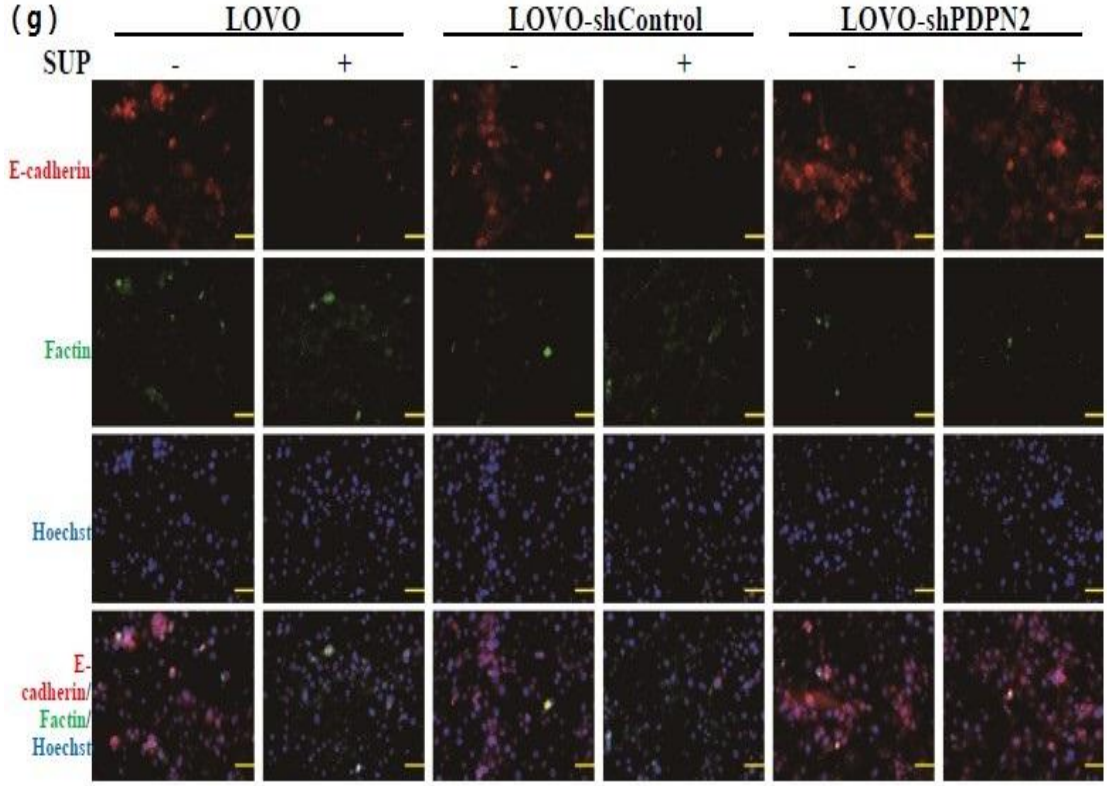
LOVO-shPDPN2
LOVO-shPDPN2+SUP

(g)


LOVO
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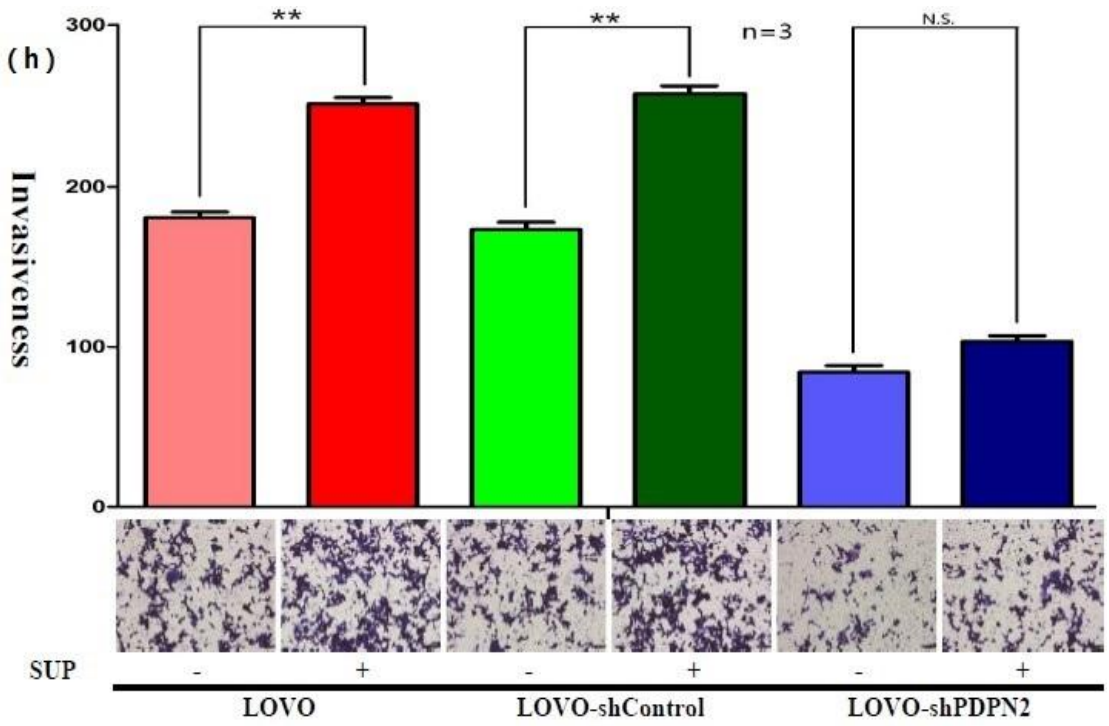


**Figure 8: podoplanin expression is required for tumor cell-platelet aggregation to release TGF-β.** (a) Silencing the expression of podoplanin in colon cancer LOVO cells using RNA interference technology, using RT-PCR method and 2^-△△Ct method to detect the expression of podoplanin. (b) Colon cancer LOVO cells and LOVO-shPDPN2 cells ($5\times10^4$ cells) were incubated with washed mouse platelets ($5\times10^7$ platelets/200 μl), and the platelet aggregation rate was measured using a platelet aggregation meter. (c) ELISA method was used to analyze the concentration of TGF-β1 in each group; all data were expressed as mean±standard deviation (n=3); error bars indicated standard deviation. Student's T test was used for statistical analysis; N.S. means No statistical significance; ** means P<0.05. (d) Immunoblotting to detect the expression of podoplanin, p-smad2/3, smad3, E-cadherin, Vimentin and N-cadherin. GAPDH is the internal reference. (e) Use the Gel-Pro32 software to perform gray-scale analysis on the results of E-cadherin, Vimentin, N-cadherin and GAPDH, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistics Analysis; NS means No statistically significant; ** means P<0.05. (f) Use Gel-Pro32 software to perform gray-scale analysis on p-smad2/3 and smad3 results, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistical analysis; NS indicates No statistically significant; ** means P<0.05. (g) Immunofluorescence technology to detect E-cadherin (red), Factin (green) and nuclear DNA (blue, Hoechst); capture images with a fluorescence microscope. The scale bar represents 50 μm. (h) Transwell cell infiltration test coated with Matrigel was used to detect cell invasiveness. Cells that migrated through the membrane were stained with crystal violet, three fields of view were taken in diameter, photographed (magnification 200×), counted, all data were averaged Value ± standard deviation (n=3); scale bar represents 50μm; statistical analysis using Mann-Whitney U test; NS means No statistical significance; ** means P<0.05.

## 4 Discussion

Platelets are considered to be a multifunctional cell, which not only plays a role in hemostasis and thrombosis, but also participates in autoimmunity and tumor formation [24]. Not only can platelets interact through direct contact with other cell types (such as white blood cells and endothelial cells), but they can also play an indirect role by releasing many factors stored in platelets in the form of dense particles. These platelet effects contribute to tumor cell growth, survival, invasion, angiogenesis, and distant metastasis [25].

A potential mechanism by which podoplanin promote tumor invasion and metastasis is podoplanin-mediated platelet aggregation and activation. The podoplanin expressed on the surface of various tumor cells, the extracellular domain can be combined with C-type lectin-like receptor-2 (CLEC-2[26]) on the surface of platelets, through platelets In the Src, Syk family kinases and phospholipase Cg2, phosphorylate a series of ligands and effector proteins, activate phospholipase γ2 (PLCγ2), induce platelet aggregation, and activate platelets [27].

Akiko Kunita [28, 29] established a wild-type (WT) podoplanin Chinese hamster ovary (CHO) cell line and a podoplanin PLAG domain mutant Chinese hamster ovary (CHO) cell line (PLAG domain Podrin activity is important, mutations in this domain will lose platelet aggregation function [13]), the study found that the expression of podoplanin can induce platelet aggregation and promote lung metastasis in experimental and spontaneous metastasis models without affecting the original The growth of the hair site, these results indicate that the podoplanin helps tumor cells survive in the peripheral circulation by inducing platelet aggregation, promotes tumor cell stagnation in the pulmonary microvascular system, contributes to the formation of metastatic tumors in the lung and reduces mice Survival rate. However, the mutant Chinese hamster ovary (CHO) cell line established by introducing point mutations into the PLAG domain lost platelet aggregation function, and the number of metastatic lung nodules was significantly lower in cloned mice injected with the podoplanin mutant cell line. Clonal mice injected with wild-type podoplanin cell lines; after pretreatment with aspirin at different concentrations (0 to 100 μmol/L) in vitro, platelet aggregation induced by podoplanin was inhibited in a concentration-dependent manner, while It inhibited the formation of lung metastasis in wild-type podoplanin cell lines. These results indicate that the platelet aggregation and activation induced by podoplanin are directly related to tumor lung metastasis. By eliminating platelet aggregation activity, tumor lung metastasis can be suppressed.

In this study, we found that tumor cells with positive expression of podoplanin can induce platelet aggregation, and through tumor cell-platelet interaction, platelets are activated to release a variety of growth factors and cytokines. Tumor cells that do not express podoplanin cannot induce tumor-platelet aggregation, promote platelet activation, and release active factors. On this basis, if the anti-pingpin protein neutralizing monoclonal antibody MS-140 is added, the platelet aggregation activity of colon cancer LOVO cell line disappears, confirming that the colon cancer LOVO cell line induced platelet aggregation depends on the expression of podoplanin (Figure 1). This is consistent with other people's research, and the platelet activation pathway mediated by this podoplanin is different from the physiological condition of platelet activation [30].

A large number of studies have shown that the EMT of cancer cells is a short and reversible process, which can promote the migration and invasion of cancer cells from the primary cancer mass, and transforming growth factor (TGF)-β is one of the most effective inducers of EMT [31 , 32]. TGF-β binds to two different serine/threonine kinase receptors, type I (TGF-βRI) and type II receptor (TGF-βRII). The formation of heterodimers of TGF-βRI and TGF-βRII leads to the activation of TGF-β-mediated signaling pathways [33]. SMAD family proteins play a key role in the process of TGF-β superfamily protein-mediated signal transfer from the cytoplasm to the nucleus. In the classical smad pathway, the activation of TGF-β signaling pathway can lead to the phosphorylation of Smad2 and Smad3, and Transfer them from the cytoplasm to the nucleus and bind with Smad4 to form a Smad complex. This complex induces miRNA expression in the nucleus, inhibits the expression of epithelial marker proteins, promotes the expression of mesenchymal cells, and promotes EMT [33 , 34].

Our research shows that adding the supernatant of colon cancer LOVO cells-platelet reactant to colon cancer LOVO cell culture, it can be observed that colon cancer LOVO cells exhibit EMT morphological changes. Immunoblotting shows that in colon cancer LOVO Medium E-cadherin is down-regulated, while Vimentin and N-cadherin are up-regulated; this indicates that after the tumor cell-platelet aggregation reaction, certain soluble cytokines are released into the supernatant, which induces tumor cell EMT (Figure 2).

The results of Li Yaoyin [35] and others showed that oral squamous cell carcinoma (OSCC) cells with flat feet protein positive through the TGF-β/Smad paracrine pathway and direct co-culture MMP-2/MMP- 14/ITGA5 signaling activates cancer associated fibroblast (CAF), CAFs enhance the invasion and proliferation of OSCC cells through EGFR, AKT and ERK pathways. We hypothesized that TGF-β was released after the tumor cell-platelet aggregation reaction, activated the TGF-β/Smad signaling pathway, and induced tumor EMT. We used ELISA to analyze the level of TGF-β in the supernatant of tumor cell-platelet reactant; the results showed that the concentration of TGF-β in the supernatant of colon cancer LOVO cell-platelet reactant was significantly higher than that of unaggregated Supernatant of lung adenocarcinoma A549-platelet reactant; importantly, platelet aggregation activity was detected in the supernatant of colon cancer LOVO cell-platelet reactant treated with anti-podoplanin neutralizing monoclonal antibody MS-140 Inhibition, the concentration level of TGF-β decreases. In order to exclude the colon cancer LOVO cells releasing TGF-β itself, we prepared colon cancer LOVO cells with TGF-β knockdown (ELISA separately measured the concentration of TGF-β in different substrates and reactants); the experimental results showed that TGF-β Knocked-out colon cancer LOVO cells did not affect the level of TGF-β released during platelet aggregation. These results indicate that TGF-β released during tumor cell-platelet aggregation mainly comes from platelets and not from tumor cells (Figure 3). The results of Labelle [36] and others are consistent.

At the same time, we used immunoblotting to detect the phosphorylation level of Smad2/3 downstream of the TGF-β signal. The results showed that the phosphorylation of Smad2/3 downstream of the tumor EMT group was up-regulated; while the phosphorylation of Smad2/3 in other groups was not up-regulated. The results show that the tumor cells with positive expression of Pingzuo not only promote the formation of tumor-platelet aggregates through the interaction with platelets; but also promote the release of platelet transforming growth factor-β (TGF-β) by activating

platelets. Activate the TGF-β/Smad signaling pathway and induce EMT of tumor cells (Figure 4, Figure 5).

In addition, in this study, we demonstrated that TGF-β plays a key role in the induction of EMT in tumor cells. We can observe that after adding the tumor-platelet reactant supernatant to colon cancer LOVO cells, the expression of E-cadherin decreases, Factin polymerizes and distributes to the cell membrane, suggesting that the cell motility is enhanced, suggesting that induction EMT; while using TGF-β neutralizing antibody 1D11 (abcam ab27969, UK) and TGF-β receptor inhibitor LY2157299 (targeting to bind TGFβR1 and TGFβR2) (abcam ab254448, UK) to treat colon cancer LOVO cells for 2 hours, colon was observed The morphological changes and EMT induced by cancer LOVO cell-platelet reactant supernatant were eliminated (Figure 6). When using the Matrigel-coated transwell cell invasion test to evaluate the invasiveness of colon cancer LOVO cells, the treatment of the supernatant of normal colon cancer LOVO cells-platelet reactants increased the invasiveness of colon cancer LOVO cells, compared with TGF- β-neutralizing antibody 1D11 or TGF-β receptor inhibitor LY2157299 was significantly less aggressive after pre-incubation of colon cancer LOVO cells for 2 hours (Figure 7). These results indicate that the activation of TGF-β signaling is critical for EMT and tumor cell invasion.

We used lentivirus containing shRNA targeting human podoplanin or control (shControl) to infect colon cancer LOVO cells, and constructed a cell line with stable podoplanin knockdown. Repeating the above experiment proved that the platelet aggregation ability of the flat-foot protein knockdown cell line was weakened, TGF-β could not be released, EMT could not be induced, and the invasiveness was reduced (Figure 8).

Previous studies have reported that peripheral blood TGF-β levels in patients with lung cancer, gastric cancer, esophageal cancer, colon cancer, and brain tumors are significantly higher than healthy volunteers. This upregulation of TGF-β in peripheral blood is considered to be caused by tumors or tumor stromal cells Mediated [37]. But our results indicate that tumor-platelet interaction is a new potential source of elevated levels of these factors in the blood. Wicki [38] et al. reported that overexpression of podoplanin promotes the invasion ability of MCF-7 breast cancer cells and enhances the ability of tumor formation and metastasis, but does not induce EMT. Prompt us that podoplanin-induced EMT of tumor cells may be cell type-specific, and different tumor cells have different activation methods; platelet activation mediated by podoplanin-positive tumor cells may induce EMT and podoplanin-negative tumors The induced EMT is different, which requires us to do further research.

In previous clinical studies, we have confirmed that platelet-related indicators combined with podoplanin can be used as prognostic indicators for patients with resectable colorectal cancer. Based on our current research, we suggest that anti-platelet aggregation or antibodies against human feet feet protein or inhibition of TGF-β/Smad2/3 signaling pathway may be a therapeutic strategy for inhibiting tumor invasion and metastasis.

## 5 Conclusions

our research shows that colon cancer LOVO cells with positive expression of podoplanin undergo aggregation reaction with platelets, activate platelets to release TGF-β, activate

TGF-β/Smad2/3 signaling pathway, induce EMT of colon cancer LOVO cells, and enhance Tumor cell proliferation and invasiveness.

## Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Suzhou University and the Medical Ethics Committee of Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine.

## Consent for publication

Not applicable.

## Availability of data and material

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

## Competing interests

The authors claim no conflicts of interest regarding the study or the manuscript.


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## Authors' Contributions

W.Q. and M.T. designed the study. W.Q. gave contribution to perform experiments. W.Q. wrote the first draft. All authors discussedthe results and implications.


### Acknowledgments

Not applicable.


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# Figures

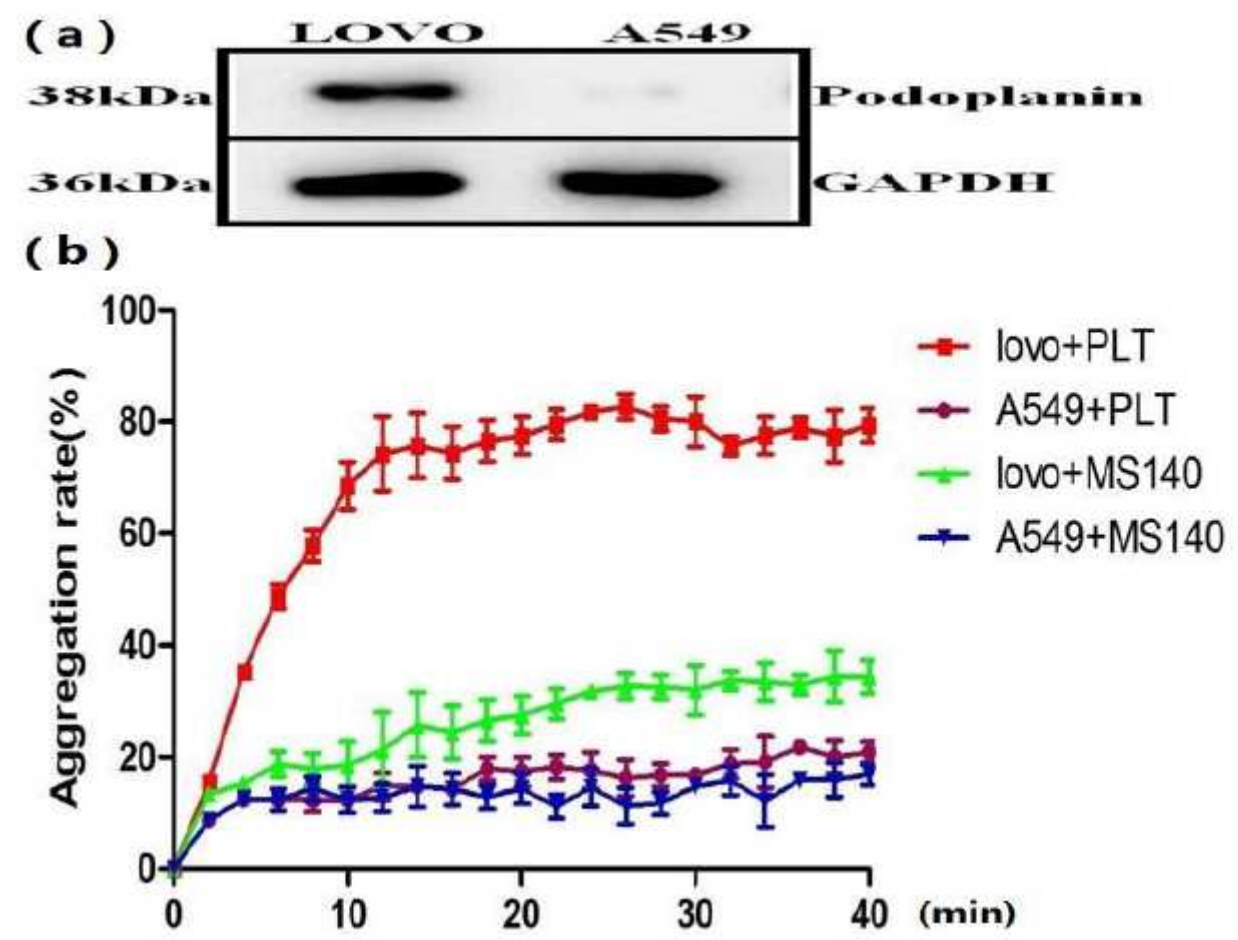
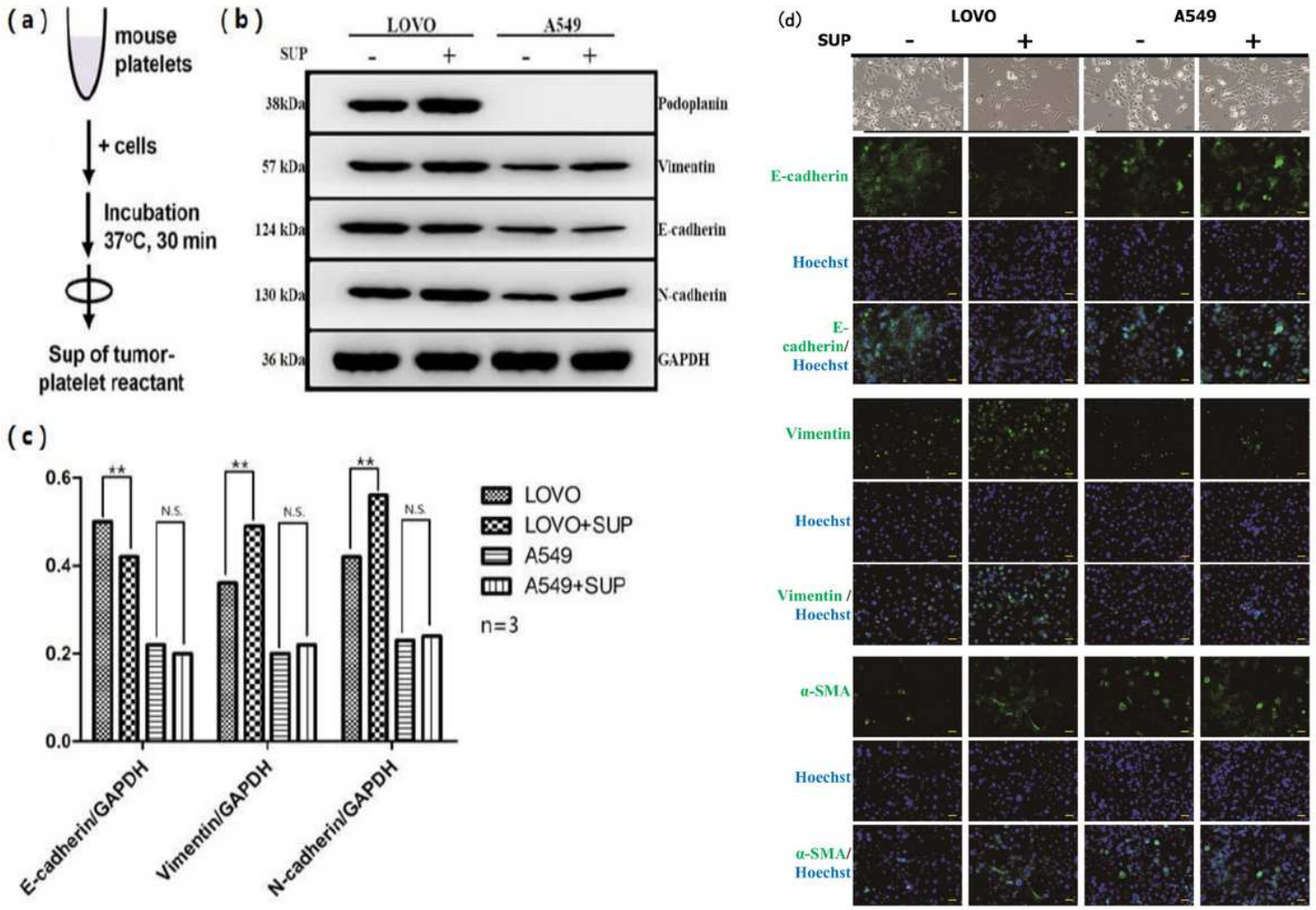


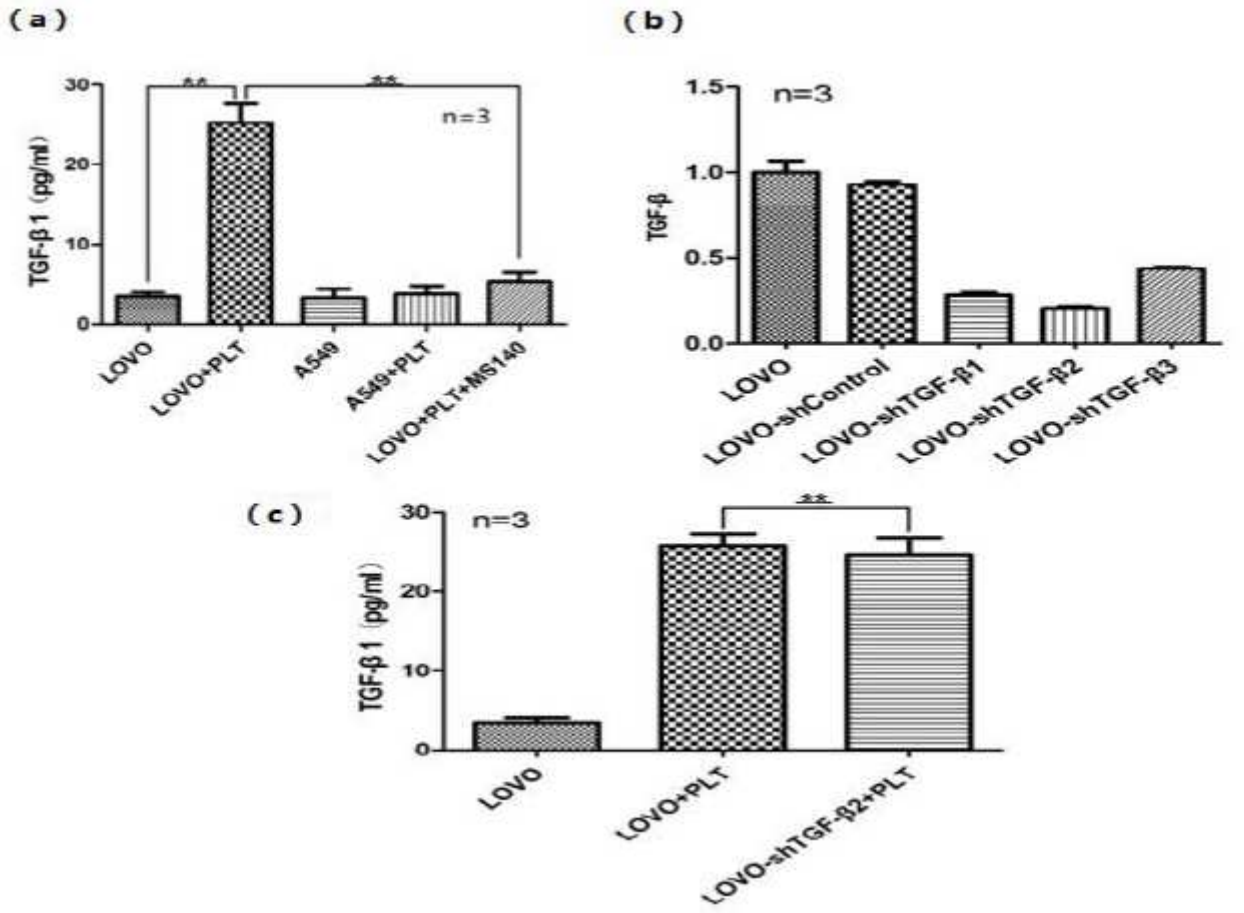

**Figure 1**

Colon cancer LOVO cells with positive expression of podplanin can induce platelet aggregation. (a) Western blot analysis showed that podoplanin expression in colon cancer LOVO cells, but no podoplanin expression in lung adenocarcinoma A549 cells. GAPDH is the internal reference. (b) Incubate colon cancer LOVO cells and lung adenocarcinoma A549 cells (5×104 cells) with washed mouse platelets (5×107 platelets/200 μl), and determine the platelet aggregation rate using a platelet aggregation meter; On this basis, monoclonal antibodies (mAb) MS-140 against podplanin were added to the two groups, and the platelet aggregation rate was measured again using a platelet aggregation meter.



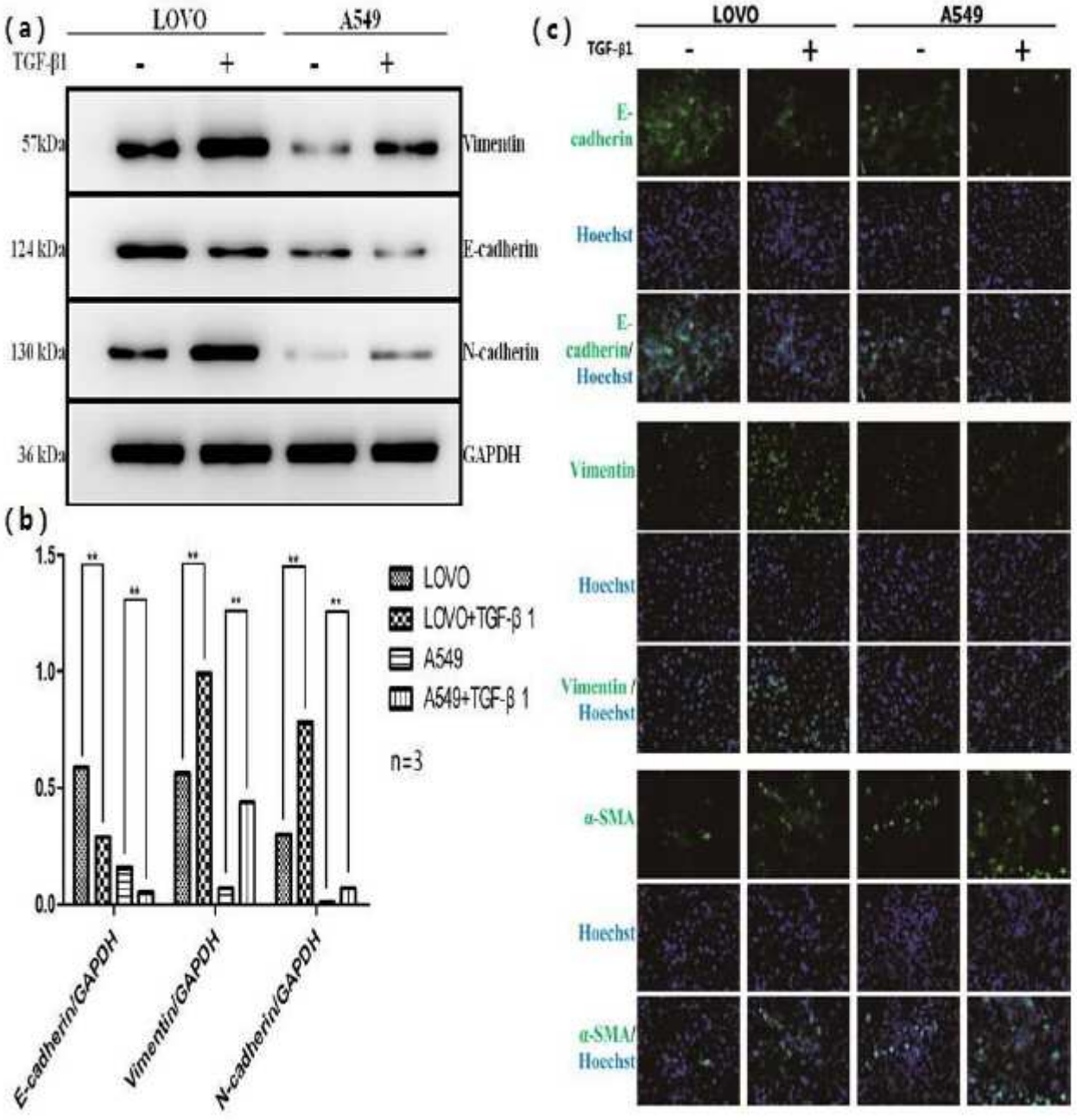

## Figure 2

Factors released by the tumor-platelet aggregation reaction induce tumor cell EMT. (a) Schematic diagram of preparation and collection of tumor-platelet reactant supernatant. (b) Immunoblotting to detect the expression of podoplanin, E-cadherin, Vimentin and N-cadherin. GAPDH is the internal reference. (c) Use the Gel-Pro32 software to perform gray-scale analysis on the results of E-cadherin, Vimentin, N-cadherin and GAPDH. All data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistics Analysis; NS means No statistically significant; ** means $P<0.05$. (d) Immunofluorescence technology to detect E-cadherin (green), α-SMA (green) Vimentin (green) and nuclear DNA (blue, Hoechst); use phase contrast microscope (top) and fluorescence microscope (bottom) to capture images . Scale bar represents 50μm.



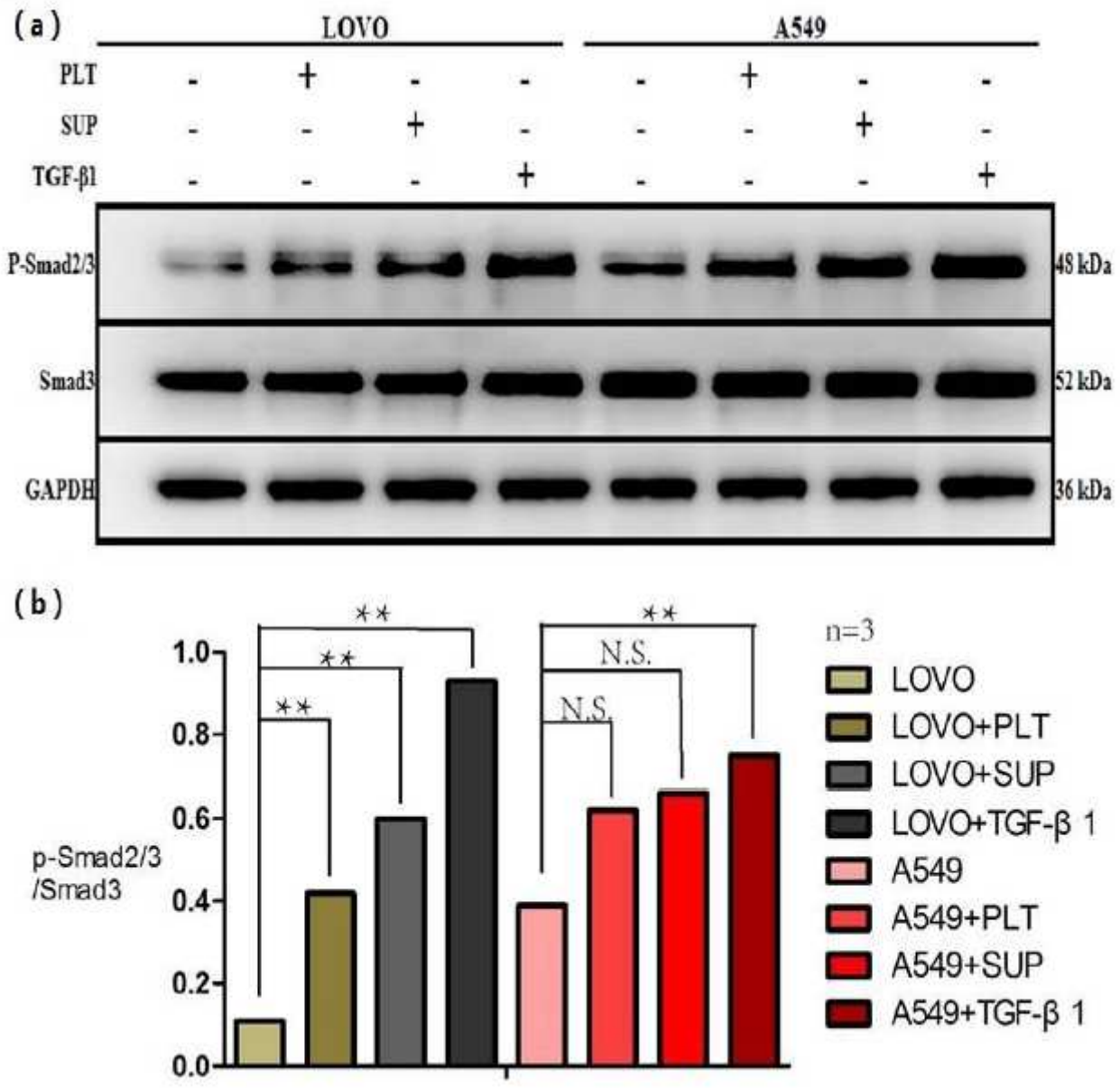

## Figure 3

TGF-β released by tumor-platelet aggregation reaction comes from platelets rather than tumor cells. (a) ELISA method was used to analyze the concentration of TGF-β1 in each group; all data were expressed as mean±standard deviation (n=3); error bars indicated standard deviation. Student's T test was used for statistical analysis; N.S. means No statistical significance; ** means P<0.05. (b) Silencing the expression of TGF-β in colon cancer LOVO cells using RNA interference technology, and using RT-PCR method and 2^-ΔΔCt method to detect the expression of TGF-β. (c) ELISA method was used to analyze the concentration of TGF-β1 in each group; all data were expressed as mean±standard deviation (n=3); error bars indicated standard deviation. Use Student's T test for statistical analysis; N.S. means No statistical significance; ** means P<0.05.



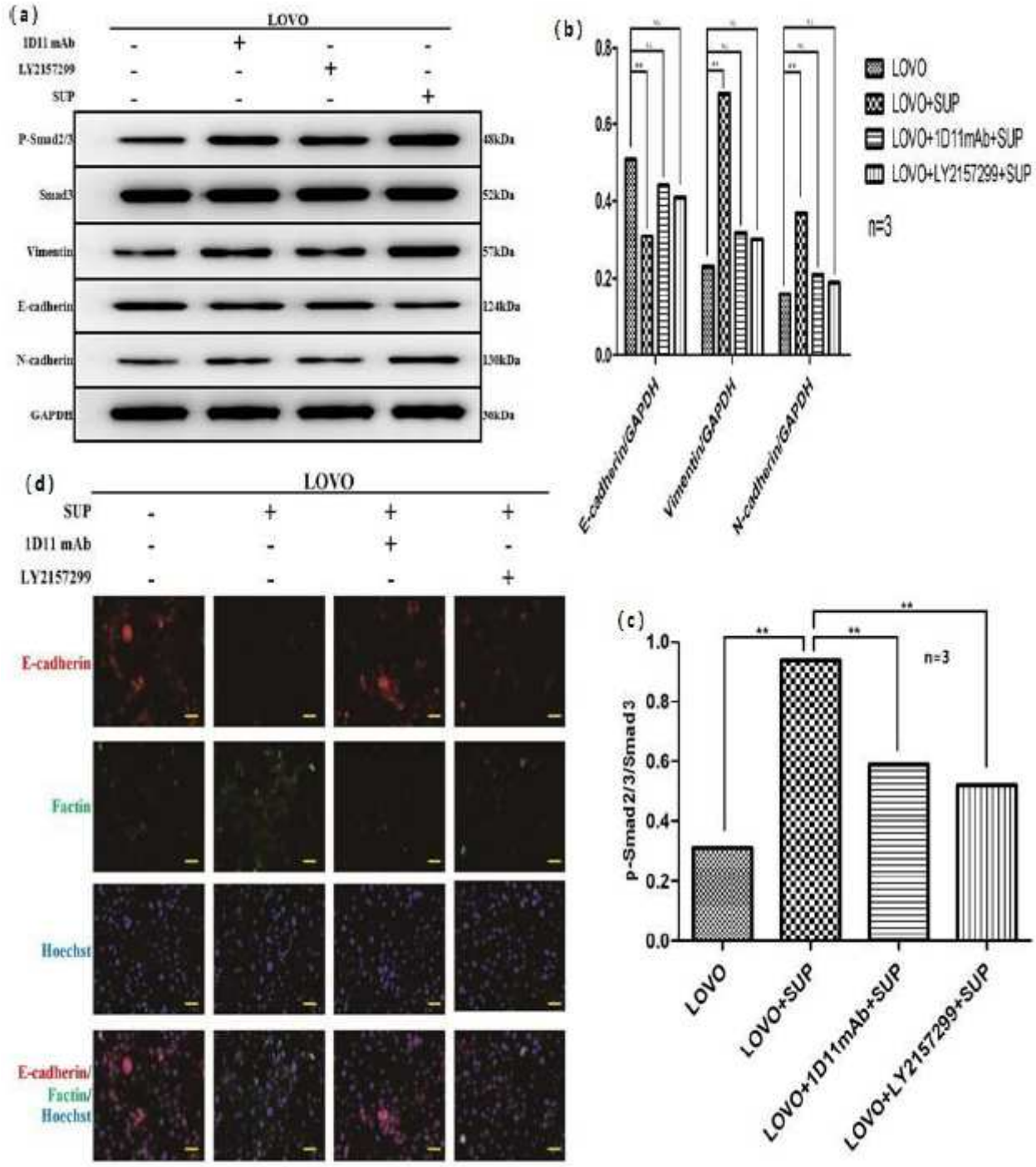

## Figure 4

TGF-β released by tumor-platelet aggregation reaction induces EMT of tumor cells. (a) Immunoblotting to detect the expression of E-cadherin, Vimentin and N-cadherin. GAPDH is the internal reference. (b) Use Gel-Pro32 software to perform gray-scale analysis on the results of E-cadherin, Vimentin, N-cadherin and GAPDH, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistics Analysis; NS means No statistically significant; ** means P<0.05. (c) Immunofluorescence technology to detect E-cadherin (green), α-SMA (green) Vimentin (green) and nuclear DNA (blue, Hoechst); fluorescence microscope to capture images. Scale bar represents 50μm.




## Figure 5

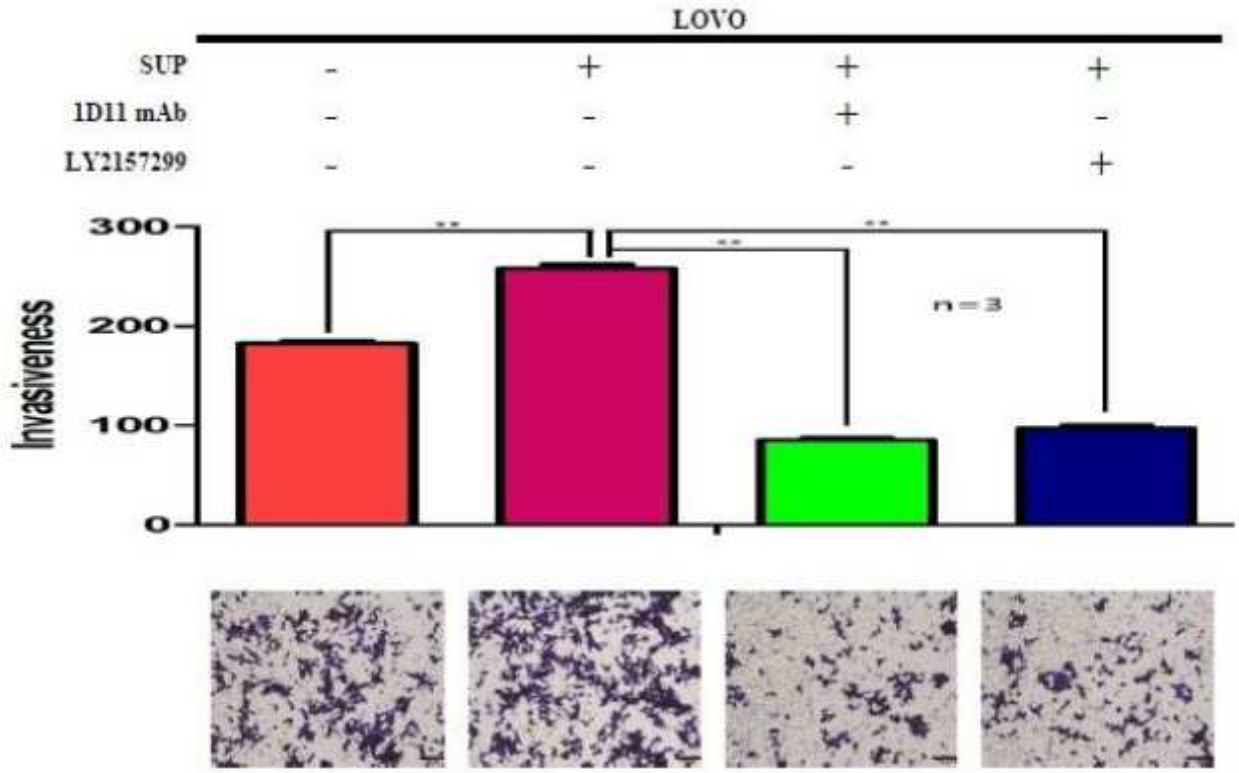
The tumor cell-platelet aggregation reaction activates the TGF-β/Smad2/3 signaling pathway. (a) Immunoblotting to detect the expression of p-smad2/3 and smad3. GAPDH is the internal reference. (b) Use Gel-Pro32 software to perform gray-scale analysis of p-smad2/3 and smad3 results, all data are expressed as mean ± standard deviation (n=3); statistical analysis is performed using Mann-Whitney U test; NS indicates No statistically significant; ** means $P<0.05$.




## Figure 6

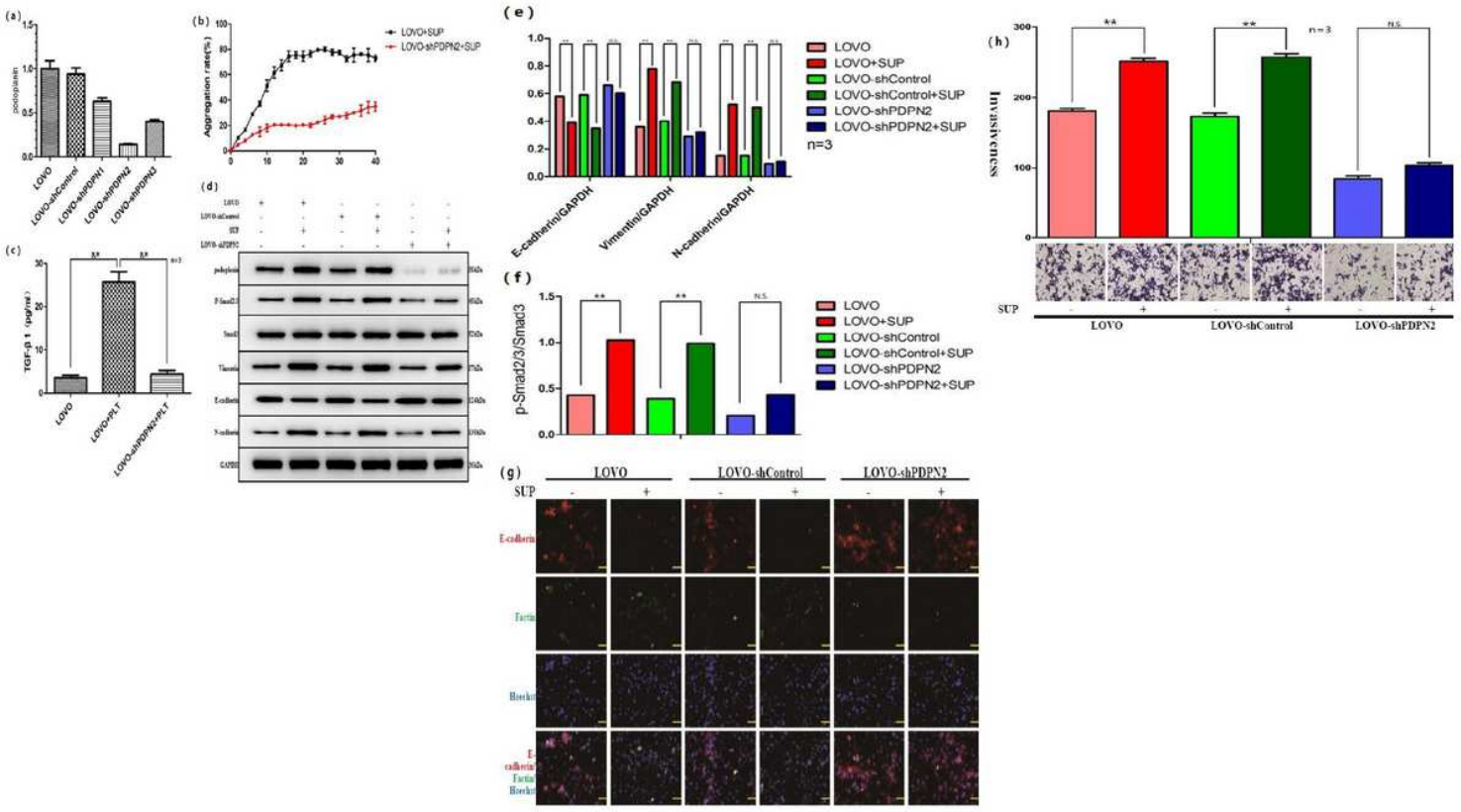
TGF-β released by tumor-platelet aggregation reaction is essential for EMT of colon cancer LOVO cells. (a) Immunoblotting to detect the expression of p-smad2/3, smad3, E-cadherin, Vimentin and N-cadherin. GAPDH is the internal reference. (b) Use Gel-Pro32 software to perform gray-scale analysis on the results of E-cadherin, Vimentin, N-cadherin and GAPDH, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistics Analysis; NS means No statistically significant; ** means $P<0.05$. (c) Use Gel-Pro32 software to perform gray-scale analysis on the results of p-smad2/3 and

smad3, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistical analysis;** Means P<0.05. (d) Immunofluorescence technology to detect E-cadherin (red), Factin (green) and nuclear DNA (blue, Hoechst); capture images with a fluorescence microscope. Scale bar represents 50μm.




## Figure 7

Transwell cell invasion test using Matrigel coating to detect cell invasion. Cells that migrated through the membrane were stained with crystal violet, 3 fields of diameter were taken, photographed (magnification 200×), counted, all data are averaged Value ± standard deviation (n=3); scale bar represents 50μm; statistical analysis using Mann-Whitney U test; ** indicates P<0.05.




## Figure 8

podoplanin expression is required for tumor cell-platelet aggregation to release TGF-β. (a) Silencing the expression of podoplanin in colon cancer LOVO cells using RNA interference technology, using RT-PCR method and 2^-ΔΔCt method to detect the expression of podoplanin. (b) Colon cancer LOVO cells and LOVO-shPDPN2 cells (5×104 cells) were incubated with washed mouse platelets (5×107 platelets/200 μl), and the platelet aggregation rate was measured using a platelet aggregation meter. (c) ELISA method was used to analyze the concentration of TGF-β1 in each group; all data were expressed as mean±standard deviation (n=3); error bars indicated standard deviation. Student's T test was used for statistical analysis; N.S. means No statistical significance; ** means P<0.05. (d) Immunoblotting to detect the expression of podoplanin, p-smad2/3, smad3, E-cadherin, Vimentin and N-cadherin. GAPDH is the internal reference. (e) Use the Gel-Pro32 software to perform gray-scale analysis on the results of E-cadherin, Vimentin, N-cadherin and GAPDH, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistics Analysis; NS means No statistically significant; ** means P<0.05. (f) Use Gel-Pro32 software to perform gray-scale analysis on p-smad2/3 and smad3 results, all data are expressed as mean ± standard deviation (n=3); Mann-Whitney U test is used for statistical analysis; NS indicates No statistically significant; ** means P<0.05. (g) Immunofluorescence technology to detect E-cadherin (red), Factin (green) and nuclear DNA (blue, Hoechst); capture images with a fluorescence microscope. The scale bar represents 50 μm. (h) Transwell cell infiltration test coated with Matrigel was used to detect cell invasiveness. Cells that migrated through the membrane were stained with crystal violet, three fields of view were taken in diameter, photographed (magnification 200×),

counted, all data were averaged Value ± standard deviation (n=3); scale bar represents 50μm; statistical analysis using Mann-Whitney U test; NS means No statistical significance; ** means $P<0.05$.