Identification of SPINT3 as a Metastatic Suppressor in Gastric Cancer Through Induction of TGF-β1 Secretion

Haifeng Wang
Zhejiang University
Li-song Teng (Li-songlsteng@zju.edu.cn)
Zhejiang University
Haohao Wang
Zhejiang University

Research Article

Keywords: SPINT3 (serine peptidase inhibitor, Kunitz type, 3), gastric cancer, TGF-β1 secretion, tumor metastasis

Posted Date: July 29th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-745710/v1

Abstract

Background: Cancer metastasis is responsible for most cancer-related deaths. *SPINT3* (serine peptidase inhibitor, Kunitz type, 3) is a newly identified gene, and its relationship with gastric cancer has not been well studied.

Methods: We first designed an sgRNA sub-library to screen for metastatic suppressors, and chose *SPINT3*. We then used QPCR and western blotting to detect the expression level of SPINT3 in gastric cancer tumor tissues. In addition, we used annexin V/propidium iodide (PI) to detect changes in apoptosis. PI staining and wound healing assays were used to detect the cell cycle and changes in cell migration, respectively. Finally, CyTOF and T cell proliferation experiments were performed to detect changes in immune cells in the tumor microenvironment.

Results: SPINT3 sgRNA (based on the sgRNA library used) was present in every metastatic tumor. The expression of SPINT3 was low in gastric cancer tissues, and this increased the metastatic ability of gastric cancer cells. In addition, high expression of SPINT3 induced cell apoptosis, downregulated cell proliferation, and impaired cell migration. Furthermore, low expression of SPINT3 induced the secretion of TGF-β1 and an immunosuppressive signal, which could be blocked by apatinib and SB431542.

Conclusion: We confirmed that SPINT3 is a metastasis suppressor in gastric cancer.

1. Introduction

Gastric cancer is the fifth most common cancer in the world after lung, breast, colorectal, and prostate cancer [1]. In 2012, there were approximately 952,000 new cases and 723,000 gastric cancer-related deaths worldwide [2]. Gastric cancer is characterized by geographical distribution, and is common in East Asia, Central Europe, and South America [3]. The number of gastric cancer-related deaths in China in 2012 was 320,000, accounting for 45% of total deaths in the country [4]. This shows that the disease burden of gastric cancer is extremely heavy, and it makes the search for new biomarkers and therapeutic targets a top priority.

SPINT3 (serine peptidase inhibitor, Kunitz type, 3) is a relatively new gene, and its function might be related to serine-type endopeptidase inhibitor activity. In this study, we chose *SPINT3* as our candidate gene owing to the wide presence of SPINT3 sgRNA in a metastatic mouse model. Studies have shown that SPINT3 is expressed at low levels in gastric cancer tissues, and that downregulation of *SPINT3* activates tumor cell metastasis and generates an immunosuppressive signal.

2. Materials And Methods

2.1 Ethics Statement

This the study was carried out in compliance with the ARRIVE guidelines. All experiments were approved by the First Affiliated Hospital of Zhejiang University. All the patients involved in this study provided informed consent, and all the sample extraction protocols followed the Declaration of Helsinki and were authorized by the Institutional Ethics Board of The First Affiliated Hospital, School of Medicine, Zhejiang University. All mouse experiments were performed at the First Affiliated Hospital of Zhejiang University in accordance with the relevant guidelines and regulations formulated by the hospital. Mice were anesthetized using avertin and sacrificed by applying excessive carbon dioxide.

2.2 Patient samples

Tumor tissues and adjacent normal tissues of five cases of gastric adenocarcinoma were collected from The First Affiliated Hospital, School of Medicine, Zhejiang University. The samples were divided into two parts and subjected to QPCR and western blotting, depending on the size of the sample.

2.3 Mouse experiment

The SCID, nude, and 615 mice used in this study were housed in the animal facility of the First Affiliated Hospital of Zhejiang University. (1) For the library experiment, nude mice (6-8 weeks old) were injected subcutaneously with the CON or SPINT3 KO MKN-45 cell line, and then the primary tumors were harvested, cut into 1 mm × 1 mm × 1 mm slices, and implanted in the gastric mucosa of SCID mice. (2) To detect the tumor microenvironment, 5 million CON or SPINT3 KO MFC cells were injected subcutaneously into 615 mice; after 2 weeks, the primary tumors were harvested and digested into single cells. For, Mice were anesthesia by avertin in the implantation of tumor cells, and sacrificed by excessive carbon dioxide inhalation.

2.4 In silico analysis

In this study, all in silico analyses were completed using R 3.4.3 software. (1) Extraction of 22-gene list: the transforming growth factor beta binding pathway (GO:0050431) was selected, and the genes involved in this pathway were extracted using AnnotationDbi and annotated using the org.Hs.eg.db package. (2) Other analysis: TCGA-STAD (Stomach adenocarcinoma dataset from The Cancer Genome Atlas Program) data were downloaded and normalized using the GDCRNATools package, and the gene expression data, follow-up time, and survival status of patients were extracted and analyzed using the univariate COX algorithm. For pathway enrichment analysis, samples were first divided into SPINT3 low and high expression groups based on the expression of SPINT3; then, the GSEA function from the clusterProfile package was used to detect pathway enrichment in SPINT3 low expression samples and compare it with that in SPINT3 high expression samples.

2.5 Sub-library design

(1) Twenty-two genes belonging to GO:0050431 were selected, and the sgRNA that target these genes were designed; the sgRNA oligo sequences are listed in Table 1. (2) Oligos were cloned into the

lentiCRISPR v2 vector (Addgene 52961). (3) The vectors were packaged as lentiviruses using the 2nd lentivirus package system.

2.6 QRT-PCR

(1) Firstly, cells were lysed with 500 μL TRIzol and centrifuged at 12000 rpm for 10 min to remove the cell fraction. (2) The solution was added to 200 μL chloroform, shaken up and down for 15 s, and centrifuged at 12000 rpm for 15 min. (3) 100 μL of the supernatant was drawn, mixed with 100 μL isopropanol, and centrifuged at 12000 rpm for 10 min. (4) The precipitate was washed with 75% ethanol, and the concentration of total RNA was detected using the OD at 260 nm/280 nm. (5) Total mRNA was reverse transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (R047A, Takara Bio) and TB Green Premix Ex Taq (Tli RNase H Plus) (RR420A, Takara Bio) to detect the expression of the internal reference gene and target gene; the primer sequences used in this study were human SPINT3 F-5'-GATCTCCTCCCAAATGTATGCGC-3', R-5'-TCCGCAGCCTCCGTAAGCAAAT-3', and human GAPDH F-5'-GTCTCCTCTGACTTCAACAGCG-3', R-5'-ACCACCCTGTTGCTGTAGCCAA-3'. The relative expression level of SPINT3 was determined using the $2^{-\triangle\triangle CT}$ value.

2.7 Western blotting

(1) Bulk tissue was lysed using RIPA buffer on ice for 30 min, mixed with loading buffer, and boiled for 15 min. (2) 20ug of samples were loaded on polyacrylamide gels and proteins were separated based on molecular weight using electrophoresis. (3) The proteins were then transferred to a PVDF membrane and blocked with 5% BSA solution. (4) the membrane was labeled with different primary antibodies, including GAPDH (ab8245), TGF-β1 (ab215715), TGF-β type 1 receptor (ab31013), TGF-β type 2 receptor (ab186838), Smad3 (ab40854), and p-Smad3 (ab52903), which were all purchased from Abcam. The SPINT3 antibody was synthesized by Beyotime Company (Shanghai, China), dilution is 1:1000. After culturing overnight, the membrane was washed thrice and labeled with secondary antibodies, and protein expression was quantified using ECL solution.

2.8 Cell apoptosis, proliferation, migration, and cell cycle

For cell apoptosis, proliferation, and cell cycle experiments, MKN-45 cells were first transfected with the CoN and SPINT3 overexpression vectors for 48 h. (1) Cell apoptosis was detected using the APC-conjugated Annexin-V/propidium iodide (PI) kit (BD Biosciences, San Jose, CA, USA). Cells were firstly harvested and washed with PBS, and then suspended in annexin V binding buffer; 5 μL of annexin V APC antibody and 5 μL of PI were added, and after culturing for 15 min, the BD Calibur machine was used to detect positive cells from the FL2 and FL4 channels. The ratio of apoptotic cells was quantified using the percentage of APC-positive cells. (2) Cell proliferation was detected using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Rockville, MD, USA); firstly, cells were dispersed in a 96-well plate, each well was seeded with 2000 cells, 10 μL of CCK-8 solution was added, and the cells were cultured for 2 h. Then, a microplate reader was used to measure the OD at 450 nm, and the proliferation index was quantified using the ratio OD450 nm at other time point/OD450 nm at 0 h. (3) Cell migration: Cells were

firstly dispersed in a 24-well plate, and each plate was seeded with 100000 cells; after cell attachment, white tips were used to scratch the bottom, and pictures were taken at the 0 and 12 h time points. (4) Cell cycle: After transfection of the SPINT3 vector for 48 h, cells were washed with PBS and fixed in 70% ethanol overnight. The cells were then stained with PI and cultured for 20 min; the BD Calibur machine was used to detect changes in the cell cycle.

2.9 CyTOF experiment

(1) Firstly, single cells from primary tumors were isolated and the CyTOF experiment was completed by Novogene (Beijing, China). The markers used in CyTOF analysis included LY6G, CD8A, CD45, CD11B, CD28, CD3E, LY6C, CD19, CD24, CD14, F4-80, and CD326. (2) After obtaining the FCS file, the data were analyzed using R 4.0.2 software. The FCS file was read using the flowCore package, and analyzed using the Rtsne package; the conditions used for the Rtsne function were tsne_expr, check_duplicates = FALSE, pca = T,pca_scale = T,perplexity = 120. Finally, the results were visualized using the ggplot2 package.

2.10 T cell proliferation

2.10.1 Isolation of myeloid-derived suppressor cells (MDSCs) and T cells

(1) Firstly, the spleen was ground and washed off with PBS, and then red blood cells were lysed and filtered with a 40 μm filter. (2) Simultaneously, the primary tumor was washed with PBS and lysed with red blood cells. (3) The single-cell suspension of the primary tumor was treated with CD11b and GR1 antibodies, and then with CD3, CD4, and CD8 antibodies. After 30 min of ice labeling, PBS was used for washing. (4) CD11b+ GR1+ cells were isolated from bone marrow single-cell suspensions using a BD aria III sorting instrument, and CD3+ CD4- CD8+ T cells were obtained from the spleen single-cell suspension.

2.10.2 Detection of T cell proliferation

(1) CFSE was added to the T cell suspension. After labeling, the cells were washed with PBS. (2) The labeled T cells were divided into two parts. One part was used to detect the expression of CFSE by flow cytometry, and the other part was used for co-culture with MDSCs. (3) After counting the MDSC cell suspension, the supernatant of the CoN group or SPINT3 sgRNA group was used for re-suspension, and 2 folds of CD8+ T cells were added. (4) CFSE expression in CD11b- cells was detected using flow cytometry. Cells with a lower fluorescence intensity at 0 h were used as T cells in the proliferation phase.

2.11 Statistical analysis

The significance levels in this study were determined using the Student's t-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3. Results

3.1 Identification of SPINT3 as metastatic suppressor using CRISPR library

In this study, to determine the link between TGF-β binding and gastric cancer metastasis, we first extracted 22 genes from the transforming growth factor beta binding pathway (GO:0050431), and then synthesized an sgRNA library to target these 22 genes, as shown in Figure 1A. After 12 weeks, all the SCID mice were dissected, and the presence of the GFP signal was detected. Among the 20 SCID mice implanted with gastric cancer tissue from the sgRNA library group, 12 developed lung metastasis, and 5 SCID mice implanted with MKN-45 primary tumor tissue did not have any metastatic tumors, suggesting increased invasion upon library treatment (Figure 1B). Then, we checked the expression of sgRNA in the lung; using PCR, we confirmed that all the 12 library mice were positive for SPINT3 sgRNA. Moreover, VASN and TWSG1 were present in 5 mice (Figure 1C, D).Based on these results, we chose *SPINT3* as our candidate gene for further analysis.

According to the literature, the function of SPINT3 includes serine-type endopeptidase inhibitor activity; however, the exact role of SPINT3 in gastric cancer remains unknown. Hence, we first extracted the gastric cancer dataset from the TCGA database and found that the expression of SPINT3 (relatively fold change) in healthy donor and gastric cancer tissues is 8.13 ± 1.49 vs. 6.23 ± 1.82; using the t-test, we confirmed that SPINT3 expression was significantly lower in gastric cancer tissue than in healthy tissue (Figure 1E). In-house data also showed lower SPINT3 expression in 5 cases of gastric cancer compared with normal tissue at the mRNA level (Figure 1F); furthermore, SPINT3 protein expression was low in the tumor tissue (Figure 1G). From a clinical perspective, patients with low SPINT3 expression would have a poorer prognosis, further proving that SPINT3 is a possible tumor suppressor in gastric cancer (Figure 1H).

3.2 Low expression of SPINT3 increases metastasis in gastric cancer cell line

Given that SPINT3 was consistently expressed at low levels in gastric cancer tissue, we infected the MKN-45 cell line with the CoN or SPNT3 sgRNA lentivirus (Figure 2A), and then used the model in part 1 to detect the changes in CoN and SPINT3 sgRNA cell metastasis. Lung metastasis nodules (Figure 2B) and GFP intensity (Figure 2C) in SPINT3 KO mice were significantly more than those in the CoN group. In addition, SPINT3 expression was lower in the lung metastasis nodules(Figure 2D), proving the link between low expression of SPINT3 and increased lung metastasis.

3.3 Relation between expression of SPINT3 and biological characteristics of gastric cancer cells

To further validate the role of SPINT3 in gastric cancer, we designed a SPINT3 overexpression vector and transfected it into a gastric cancer cell line. Using western blot, we confirmed that the level of SPINT3 expression was elevated in the SPINT3 cDNA-transfected group (Figure 3A). Using annexin V/PI double staining, we found that apoptosis was induced after transfection of SPINT3 for 48 h (Figure 3B); moreover, cell growth was inhibited in SPINT3-overexpressing cells (Figure 3C, D). Furthermore, cell migration was inhibited after transfection of SPINT3 cDNA (Figure 3E). These results indicated that inhibition of SPINT3 was critical for the survival and migration of gastric cancer cell lines.

Using in silico analysis, we concluded that in the TCGA-STAD dataset, the TGF-β1-related pathway was highly enriched in SPINT3 low expression samples (Figure 3F). Using western blots, we also confirmed that overexpression of SPINT3 decreased the TGF-β1 signal, indicated by downregulated TGF-β1, TGF-β receptor 1, and downstream signal Smad3 (Figure 3G). SPINT3 knockout also increased the secretion of total TGF-β1 and active TGF-β1 (Figure 3H), suggesting that the deficiency of SPINT3 might modify the tumor microenvironment via the secretion of TGF-β1.

3.4 Modification of tumor microenvironment by SPINT3 KO

In the previous section, we stated that knocking out SPINT3 could increase the secretion of TGF-β1. TGF-β1 is a well-known factor that regulates cell proliferation [5], EMT [6], and cancer metastasis [7], as well as the tumor microenvironment [8]. In the TGF-β-rich tumor microenvironment, CD8+ T cell proliferation and maturation of T helper cells are inhibited [9]. In our study, to determine the changes in immune cells in the primary tumor, we chose 615 mice owing to their complete immune system. Firstly, we infected the MFC cell line with the CON or SPINT3 sgRNA lentivirus (Figure 4A), and then injected CON, SPINT3 shRNA, and SPINT3-overexpressing MFC cells into 615 mice subcutaneously. Consistent with previous results, knockout of SPINT3 increased tumor cell proliferation in vivo after 2 weeks (Figure 4B). Single cells from the primary tumor were subjected to CyTOF analysis (Figure 4C), and we confirmed that after SPINT3 was knocked out, CD8+ CD28+ T cells decreased in number, whereas M2 macrophages, PMN-MDSCs ($CD11b^{+}Ly6G^{+}Ly6C^{low/int}$), and MO-MDSCs ($CD11b^{+}Ly6G^{-}Ly6C^{high}$) increased in number (Figure 4D). We further validated this change using FACS, and proved the decrease in the number of CD8+ T cells (Figure 4E) and increase in the number of PMN-MDSCs (LY6G+) and MO-MDSCs (LY6C+) (Figure 4F). Moreover, the levels of active markers of MDSCs, including NOS2 and ARG1, significantly increased in SPINT3 knockout tissues (Figure 4G).

MDSCs are a bone marrow-derived heterogeneous cell population with immunosuppressive activity, and recent studies have shown that the expansion of MDSCs is capable of inhibiting T cell proliferation in vivo and in vitro. Therefore, in our experiment, we sorted MDSCs from the primary tumors in the CON and SPINT3 knockout groups, co-cultured them with T cells from the spleen of 615 mice, and found that the activity of the MDSCs was higher in the SPINT shRNA group than in the CON group; the activity was quantified using the percentage of amplified T cells (Figure 4H). These results suggested that, in SPINT3 knockout primary tumors, the anti-tumor immune response was compromised, reducing cancer cell recognition and clearance, and promoting tumor cell invasion.

3.5 Restoration of T cell proliferation by inhibitor

To inhibit the increase in tumor cell proliferation and invasion caused by SPINT3 deficiency, we investigated a drug for target therapy, and confirmed that the expression of SPINT3 in SPINT3 shRNA cells was reversed by treatment with apatinib and the TGF-β1 inhibitor SB431542 (Figure 5A). Further experiments proved that cell apoptosis and proliferation were inhibited (Figure 5B,C), and in the primary tumors from 615 mice, the percentage of PMN-MDSCs and MO-MDSCs decreased after treatment (Figure

5D). The activity of MDSCs decreased in the treatment group, as shown by the T cell proliferation experiment (Figure 5E), and treatment with apatinib and SB431542 in vivo successfully blocked the metastasis process in 615 mice (Figure 5F).

4. Discussion

In this study, we first confirmed that SPINT3 was expressed at low levels in gastric cancer tissues and correlated it with the patient survival rate. Then, using multiple experiments, we proved that SPINT3 expression could influence cell proliferation, migration, and apoptosis. These results show that SPINT3 is a tumor suppressor.

The function of SPINT3 is still unclear, and we deduced that it might be related to the TGF-β1 secretion signal as (1) SPINT3 is involved in the TGF-β1 binding pathway, and (2) in SPINT3 low expression gastric cancer samples, the TGF-β1 secretion and activation pathway was highly enriched. We used ELISA to detect the change in TGF-β1 concentrations in the supernatant of CoN and SPINT3 sgRNA tumor cells, and found that total TGF-β1 and activated TGF-β1 levels increased. TGF-β1 has a significant impact on the recruitment, activation, and function of immune cells; in macrophages, TGF-β1 inhibits the identification and clearance of cancer cells and induces polarization from the M1 to M2 type [10], which further induces the formation of cancer-associated fibroblasts (CAFs) [11]. In NK cells, TGF-β1 can downregulate the expression of NKp30 and NKG2D on the cell surface; it can also inhibit MHC I expression in cancer cells, and suppress the function of NK cells [12]. Elevated TGF-β1 levels in the tumor microenvironment can also regulate the function of myeloid-derived cells, mainly of 3 types: (1) tumor-associated macrophages [13], (2) tumor-associated neutrophils [14], and (3) unmatured CD11B+ GR1+ myeloid cells (myeloid immune suppressor cells, MISCs) or MDSCs [15]. These 3 types of cells are associated with poorer prognosis of cancer patients and resistance to target therapy. On the other hand, most of the recent studies have focused on TGF-β1 secreted by these cells, as opposed to TGF-β1 secreted by tumor cells, and on the changes in the EMT process; for example, SALL4 overexpression in gastric cancer cells can activate TGF-β/Smad and further promote EMT, thus stimulating the metastasis of gastric cancer cells [16].

In this study, we found that the downregulation of SPINT3 increased the number of MDSCs and M2 macrophages in the tumor microenvironment, and that the proliferation of CD8+ T cells was inhibited by the activation of MDSCs. Moreover, the upregulation of SPINT3 using drugs such as apatinib or the TGF-β1 inhibitor SB431542 could inhibit the function of MDSCs in primary tumors. Based on these results, we concluded that SPINT3 could function as a metastatic suppressor in gastric cancer through the secretion of TGF-β1.

Declarations

Conflict of interest

None

Acknowledgments

This work was supported by the Project of regional diagnosis and treatment center of health and Family Planning Commission, title is Construction of Regional Center for diagnosis and treatment of gastrointestinal cancer (NO.JBZX-201903).

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

References

1. Zheng R, Zeng H, Zhang S, et al. Estimates of cancer incidence and mortality in China, 2013[J]. Chinese journal of cancer, 2017, 36(1): 66.
2. Oh J K, Weiderpass E. Infection and cancer: global distribution and burden of diseases[J]. Annals of global health, 2014, 80(5): 384-392.
3. Yang L, Zheng R, Wang N, et al. Incidence and mortality of stomach cancer in China, 2014[J]. Chinese Journal of Cancer Research, 2018, 30(3): 291.
4. Chen H, Lorton B, Gupta V, et al. A TGFβ-PRMT5-MEP50 axis regulates cancer cell invasion through histone H3 and H4 arginine methylation coupled transcriptional activation and repression[J]. Oncogene, 2017, 36(3): 373-386.
5. Yu L, Li J J, Liang X L, et al. PSME3 promotes TGFB1 secretion by pancreatic cancer cells to induce pancreatic stellate cell proliferation[J]. Journal of Cancer, 2019, 10(9): 2128.
6. Tang X, Zhou Y, Liu Y, et al. Potentiation of cancerous progression by LISCH7 via direct stimulation of TGFB1 transcription in triple-negative breast cancer[J]. Journal of cellular biochemistry, 2020, 121(11): 4642-4653.
7. Mouti M A, Pauklin S. TGFB1/INHBA homodimer/Nodal-SMAD2/3 Signalling Network: A Pivotal Molecular Target in PDAC Treatment[J]. Molecular Therapy, 2021.
8. Pedrosa L, Esposito F, Thomson T M, et al. The tumor microenvironment in colorectal cancer therapy[J]. Cancers, 2019, 11(8): 1172.
9. Sivakumar S, Abu-Shah E, Ahern D J, et al. Activated Regulatory T-Cells, Dysfunctional and Senescent T-Cells Hinder the Immunity in Pancreatic Cancer[J]. Cancers, 2021, 13(8): 1776.
10. Sorkin M, Hwang C, Serra Ucer J L, et al. Tuning The Immune Response To Improve Musculoskeletal Extremity Trauma Wound Healing Through Macrophage Directed Tgfb1 Modification[J]. Plastic and Reconstructive Surgery Global Open, 2018, 6(4 Suppl).
11. Jung M, Ma Y, Iyer R P, et al. IL-10 improves cardiac remodeling after myocardial infarction by stimulating M2 macrophage polarization and fibroblast activation[J]. Basic research in cardiology,

2017, 112(3): 33.
12. de Brito W B, Queiroz M A F, Amoras E S G, et al. The TGFB1-509C/T polymorphism and elevated TGF-β1 levels are associated with chronic hepatitis C and cirrhosis[J]. Immunobiology, 2020, 225(5): 152002.
13. Cheng Y, Zhu Y, Xu J, et al. PKN2 in colon cancer cells inhibits M2 phenotype polarization of tumor-associated macrophages via regulating DUSP6-Erk1/2 pathway[J]. Molecular cancer, 2018, 17(1): 1-16.
14. Pan Y, Jia L P, Liu Y, et al. Alteration of tumor associated neutrophils by PIK3CA expression in endometrial carcinoma from TCGA data[J]. Journal of ovarian research, 2019, 12(1): 1-7.
15. Ouzounova M, Lee E, Piranlioglu R, et al. Monocytic and granulocytic myeloid derived suppressor cells differentially regulate spatiotemporal tumour plasticity during metastatic cascade[J]. Nature communications, 2017, 8(1): 1-13.
16. Shao M, Zhang J, Zhang J, et al. SALL4 promotes gastric cancer progression via hexokinase II mediated glycolysis[J]. Cancer Cell International, 2020, 20: 1-13.

Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

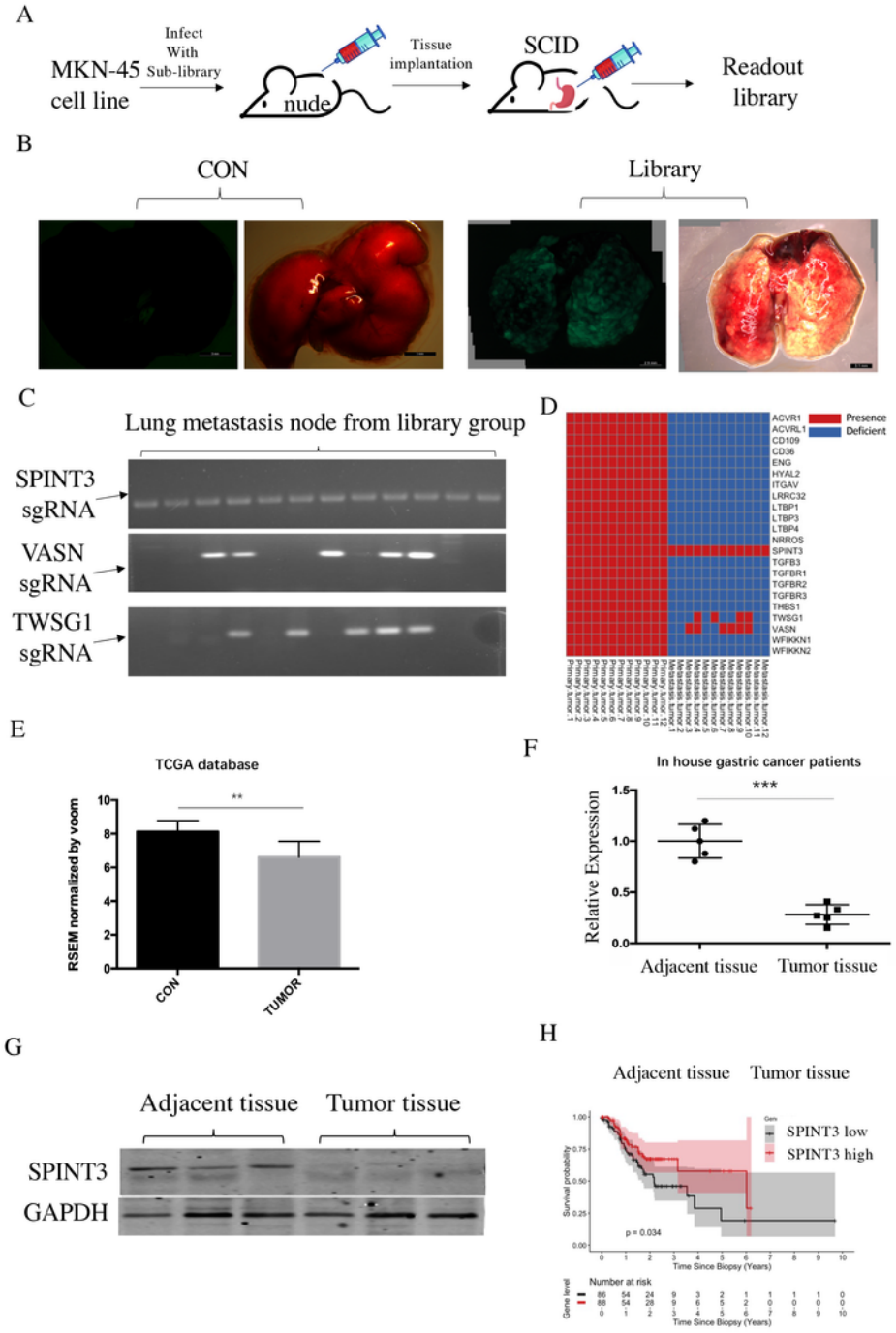

Figure 1

Identification of SPINT3 as metastatic suppressor by CRISPR library (A) Pipeline of implantation of gastric cancer tissue. (B) SCID mice which implant with gastric cancer tissue were dissect, and using fluorescence microscope to detect the GFP intensity in lung. (C) Genome DNA from lung were extracted, and amplified by sgRNA primer. (D) Presence of 22 sgRNAs in all the primary tumor and metastatic tumor tissue. (E) In TCGA-STAD dataset, RNA-seq data of tumor tissue and normal tissue was extracted, and

normalized by VOOM function from limma package. (F) From our in-house data, using QPCR to detect expression of SPINT3 in 5 cases of gastric cancer tissue and adjacent tissue in mRNA level. (G) Using western blot to detect expression of SPINT3 in 5 cases of gastric cancer tissue and adjacent tissue in protein level. (H) In TCGA-STAD dataset, using univariate COX analysis to detect relation of SPINT3 expression and patients' prognosis.

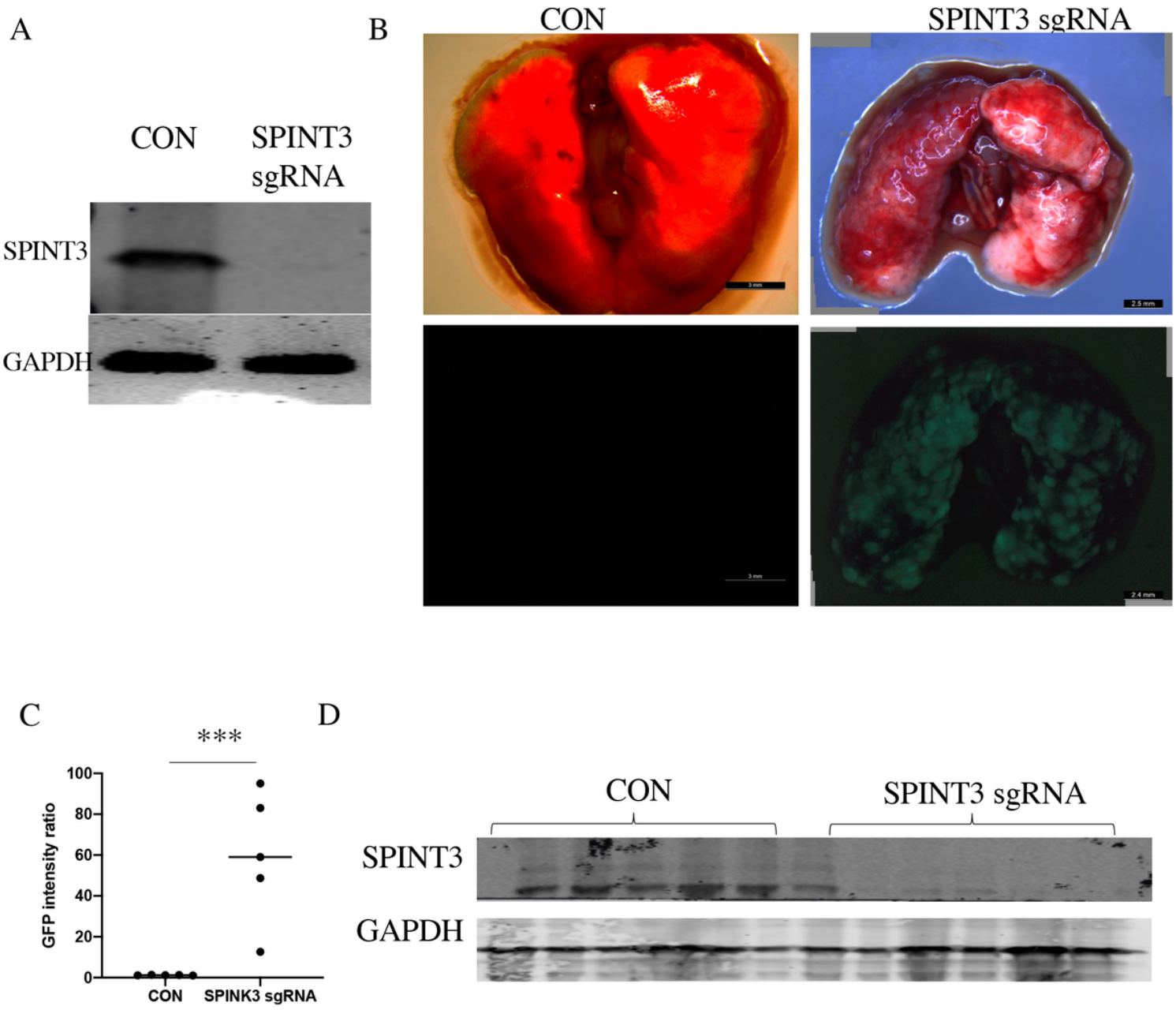

Figure 2

Low expression of SPINT3 increase the metastasis in gastric cancer cell line (A) Gastric cancer cell line MKN-45 were infected with CON and SPINT3 sgRNA for 48h, then using western blot to detect the change of SPINT3 expression in protein level. (B) Metastasis ability of CON and SPINT3 sgRNA cells were detected by the mouse model in Figure 1A. (C) GFP intensity in CON and SPINT3 sgRNA were detected by image J software. (D) Using western blot to detect the change of SPINT3 in CON and SPINT3 sgRNA.

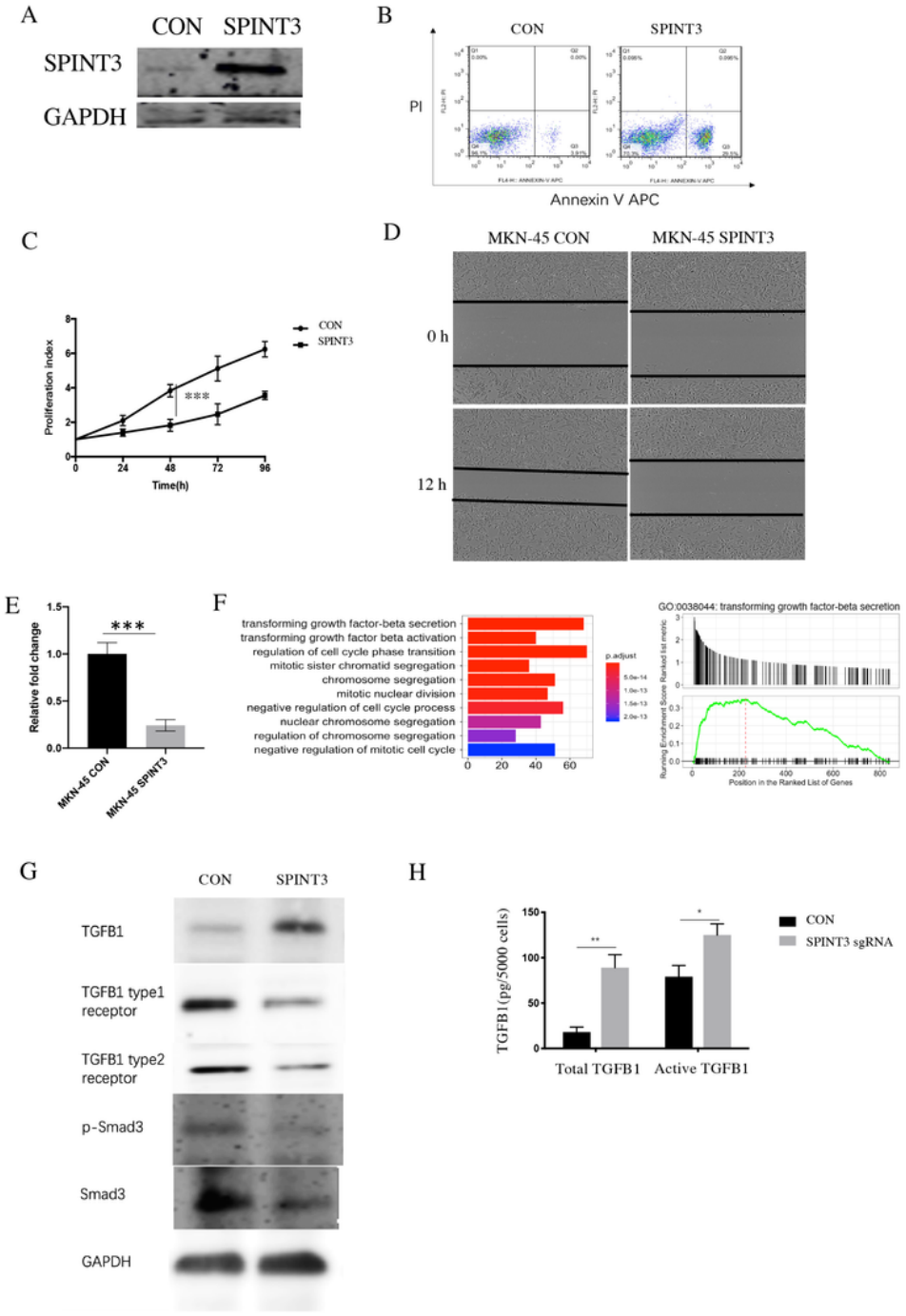

Figure 3

Relation between expression of SPINT3 and biological characteristics of gastric cancer cell. Gastric cancer cells were divided into CON and SPINT3 group, then transfected with CON and SPINT3 cDNA, after 48h. (A) Using western blot to detect the change of expression of SPINT3 in protein level. (B) Using Annexin V APC/PI double staining to detect the change of cell apoptosis. (C) Using CCK8 to detect the change of cell proliferation. (D) Summary of the velocity of migration in (C). (E) Using wound healing assay to detect the change of cell migration. (F) In TCGA database, samples were divided into SPINT3 low and high expression group, then detect pathway enrichment in SPINT3 low expression group. (G) Using western to detect the change of TGFB1 pathway. (H) Using ELISA to determine total TGFB1 and active TGFB1 in the supernatant of CON and SPINT3-shRNA cells.

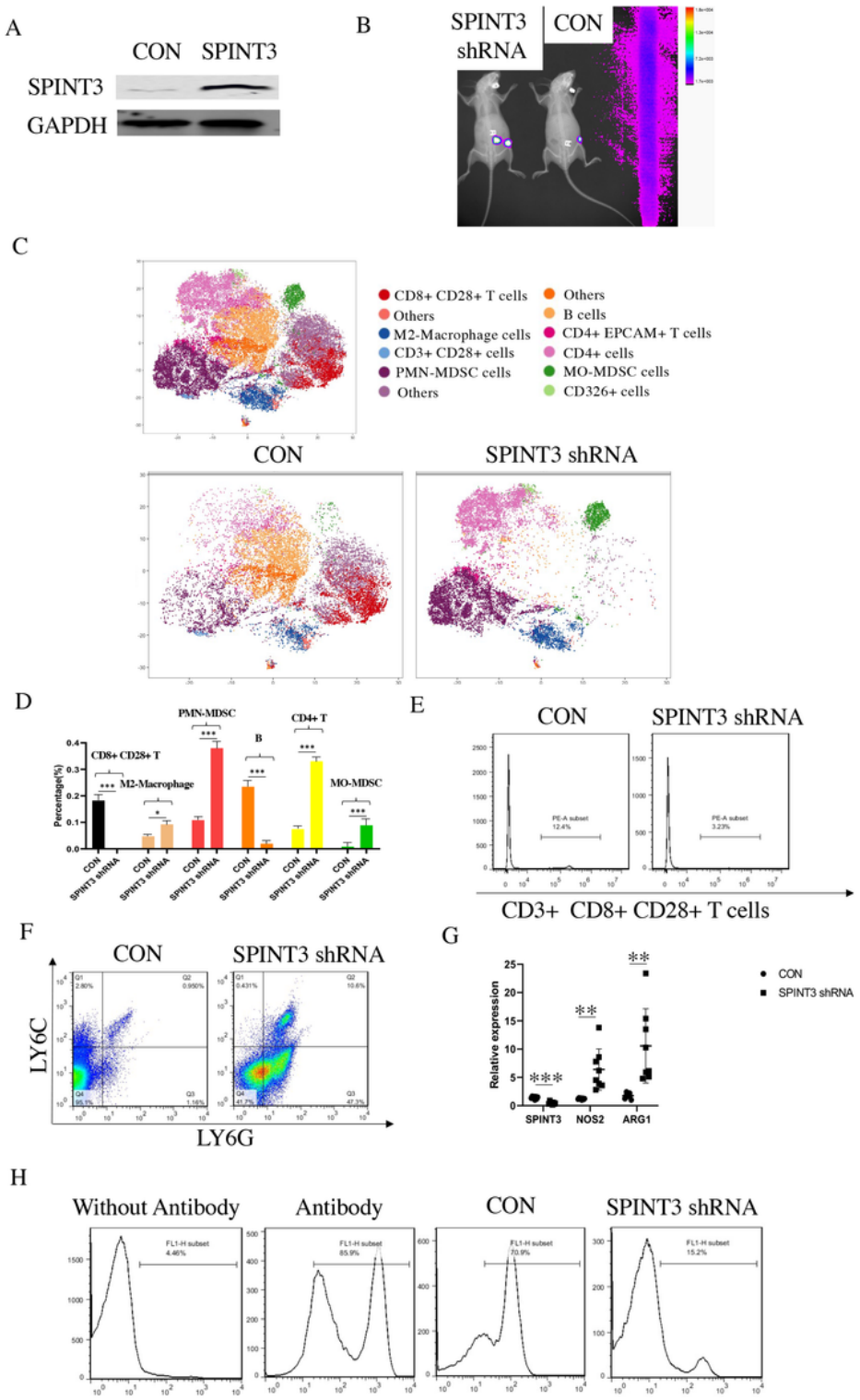

Figure 4

Modification of tumor microenvironment by KO of SPINT3 (A) MFC cell line was infected with CON or SPINT3 shRNA lenti virus, and using western blot to detect the change of SPINT3 in protein level. (B) CON, SPINT3 shRNA and SPINT3 OE MFC cells in 615 mice subcutaneous, in 2 weeks, in vivo image system was used to detect the tumor cell proliferation in vivo. (C) Single cell from primary tumor were analyzed by cytof. (D) Summary figure of the change of immune cells in CD45+ cells. Percentage of

CD8+ T cell(E) and MDSCs(F) from CON and SPINT3 sgRNA primary tumor were further validated by FACS. (G) CD11B+ GR1+ cells were sort out, and using QPCR to detect the change of SPINT3, NOS2 and ARG1. (H) MDSC from CON and SPINT3 sgRNA primary tumor were co-cultured with T cells from spleen, and using CFSE to detect the change of cell proliferation.

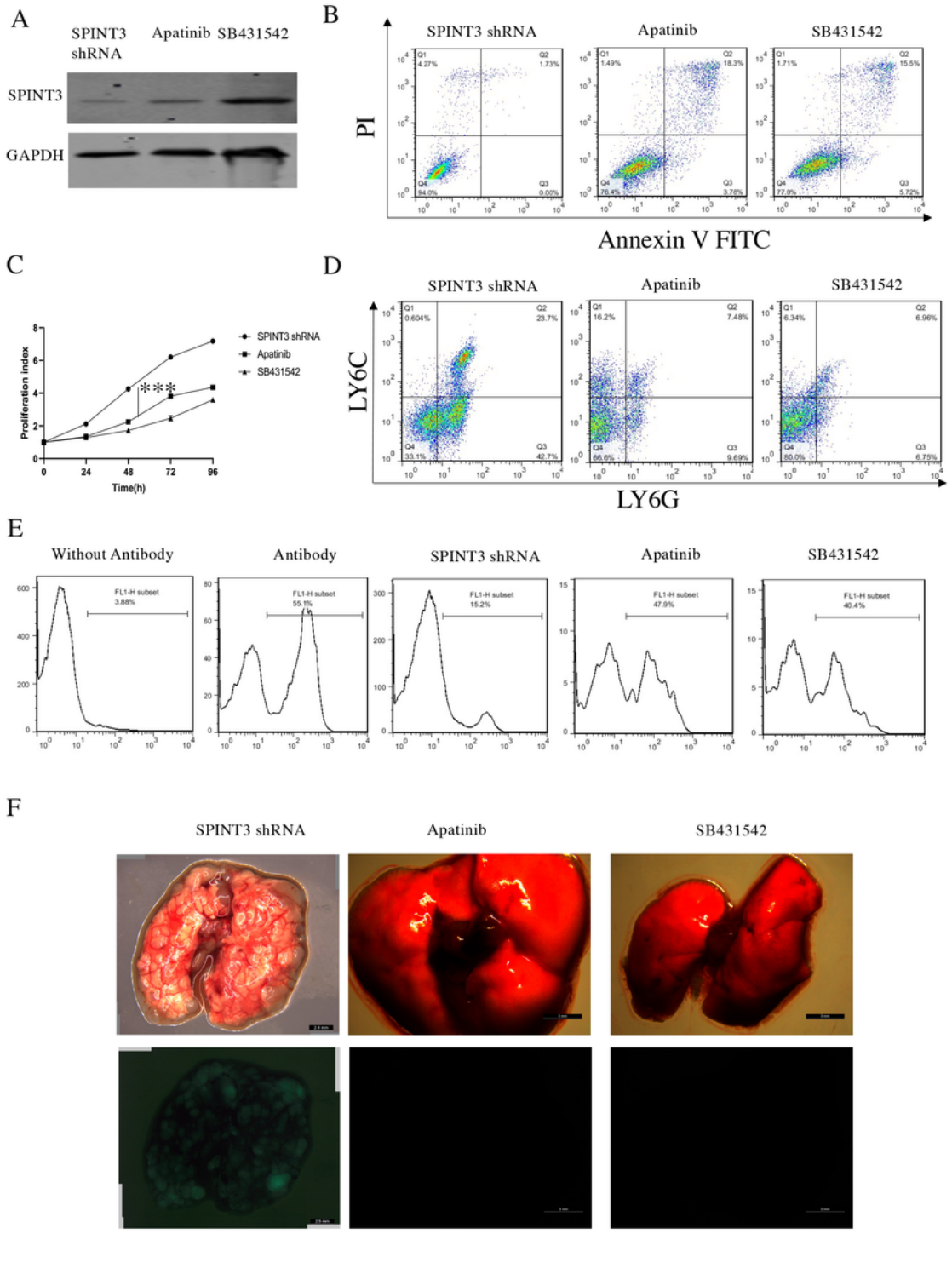

Figure 5

Restoration of T cell proliferation by inhibitor (A) MFC SPINT3 shRNA were treated with Apatinib or SB431542, and using western blot to detect the change of SPINT3 in protein level. Employing Annexin V/PI (B) and CCK8 to measure the change of cell apoptosis and cell proliferation. (D) Using FACS to detect the percentage of PMN-MDSC and MO-MDSC. (E) Using CFSE to detect the change of T cell proliferation in CON, Apatinib treatment and SB431542 treatment group. (F) MFC cell line was infected with SPINT3 shRNA lenti virus and injected in 615 mice, then mice were treated with Apatinib or SB431542, after 3 weeks, tumor metastasis in each group were detected.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Genenameof22genelibrarylist.xls
- supplementaryfile.pdf