

SP1 Initiates Epithelial-Mesenchymal Transition of CTCs and Inhibits Metastasis in Prostate Cancer

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Research

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Abstract

Background: Circulating tumor cells (CTCs) are the basis of cancer metastasis. Till now, the role of different subtypes of CTCs in metastasis is unclear.

Methods: We used the Canpatrol™ technique to isolate CTCs from 102 prostate cancer (PCa) patients. The EMT markers of CTCs were detected by FISH and classified CTCs into Epithelial (E-CTCs), Mesenchymal (M-CTCs) and Mesenchymal/Epithelial-CTCs (M/E-CTCs). Further, the potential EMT related molecules regulators were predicted by bioinformatics software, and SP1 was identified as the key EMT regulator. Then overexpress SP1 of PCa cells to verify the effect of SP1 on the EMT regulation and PCa metastasis.

Results: The count of Total-CTCs, E-CTCs, and M/E-CTCs in metastatic PCa was significantly higher than that in local PCa. Although M-CTCs was not significantly different between local and metastatic PCa, the ratio of M-CTCs/Total-CTCs in metastatic PCa was markedly lower than that in local PCa. We found that Total-CTCs is an independent risk factor for PCa metastasis. Overexpression of SP1 initiated EMT of PCa cells and enhanced the invasion *in vitro*. Injecting overexpression SP1 PCa cells via tail vein, generating M-CTCs *in vivo*, we found the ability of M-CTCs to form lung metastasis was significantly inhibited compared with that of the control PCa-CTCs.

Conclusion: Our study suggested Total-CTCs >14 predict PCa metastasis. M/E-CTCs might facilitate to PCa metastasis; however, M-CTCs might not. SP1 is an EMT regulator, which has the potential role of regulating the EMT of CTCs, thus changing the proportion of subtypes of CTCs. It is a potential therapeutic target. Trial registration: Chinese Clinical Trial Registry. Registered 30 JUNE 2020, <http://www.chictr.org.cn/registry.aspx>

Background

Prostate cancer (PCa) is the most common cancer in men; it is the second leading cancer-related death among men in the United States [1]. Metastatic PCa has a poor prognosis and is challenging to treat, which is one of the critical factors leading to death. Circulating tumor cells (CTCs) originating from primary tumor sites are considered precursors of tumor metastasis[2]. The increased count of CTCs always indicates metastasis, drug resistance, and poor prognosis[3-6]. Therefore, it is imperative to study the specific role and mechanism of CTCs in metastasis to prevent and treat metastatic PCa.

Further studies confirmed that CTCs were not homogeneous cell populations. According to the expression of Epithelial-mesenchymal transition (EMT) markers, captured CTCs were differentiated into epithelial (E-CTCs), mesenchymal (M-CTCs), and hybrid (M/E-CTCs) phenotypes[7]. EMT might cause CTCs heterogeneity; this transition process is associated with biochemical changes in which epithelial cell markers such as E-cadherin and occludins are down-regulated, which leads to loss of cell-cell adhesion. In contrast, mesenchymal markers such as N-cadherin and vimentin are up-regulated [8, 9]. EMT enables cancer cells to break loose from the primary tumor mass and enter the bloodstream, characterized by

morphological changes in their phenotype from cuboidal to spindle-shaped, promoting metastasis[10, 11]. The specific role of different CTCs in metastasis remains unclear, which hinders drug development for this mechanism.

Canpatrol™ CTCs isolate technology is a simple, economical, and reliable separation technology based on cell size detecting CTCs in peripheral blood[12]. It is generally believed that cancer cells with mesenchymal phenotype are more aggressive and contribute to metastasis^[8, 9]. However, their properties would be much different when cancer cells are intravasation to the circulatory system directly. The ability of M-CTCs to cause metastasis may be pretty different in the circulatory system from that in tissues. It is fascinating that our study found the M-CTCs/T-CTCs ratio was significantly reduced in metastasis PCa. Based on the bidirectional reversibility of EMT, CTCs reduce the proportion of M-CTCs with weak metastasis ability, which may be the mechanism of CTCs enhance metastasis.

The expression of intercellular junction molecules (ICMs) is an essential feature of EMT[13]. When cancer cells start EMT, there are many changes of ICMs. Finding the regulatory genes of these ICMs is of great significance for the subsequent development of drugs to control the epithelial or mesenchymal phenotype of CTCs. Therefore, we used bioinformatics methods to analyze the possible ICMs regulatory genes. A total of 111 EMT-related intercellular junction proteins were collected by manual search. Then, we used bioinformatics software to find out the regulatory molecules of EMT in CTCs. It is deduced that SP1 is one of the critical regulators of these terminal functional molecules. To verify whether this conclusion is effective in CTCs, we detected the expression of SP1 in CTCs of PCa patients and up-regulated SP1 in PCa cells. We found that the increase of SP1 level was related to the rise in M-CTCs and M / E-CTCs ratio of T-CTCs. Further experiments confirmed that overexpression of SP1 initiated EMT of PCa cells and inhibited the formation of lung metastases *in vivo* (Fig. 7).

This study confirmed that different varieties of CTCs have different abilities to cause metastasis, and some kinds of CTCs may have a weak ability to drive metastasis. SP1 initiated EMT of PCa cells, which may regulate cancer cells' ability to lead to metastasis. We believe that our results will contribute to developing anticancer drugs targeting CTCs, transforming CTCs into CTCs with weak or no metastasis ability, and providing new ideas for metastatic PCa treatment.

Methods And Materials

Cell culture and Animals

Human PCa cell lines PC3 were purchased from ATCC (Manassas, VA). PC3 cells were maintained in DMEM medium (Hyclone, Logan, UT) with 10 % FBS, 100 U/ml penicillin, and streptomycin as the previous study described [14].

Eight-week-old male nude mice (22-26g) necessary for the lung metastasis experiments were purchased from the Animal experiment management centre of Chongqing Medical University (BALB/c background, Chongqing, China). All animal experiments were carried out following guidelines set by Chongqing

Medical University (Chongqing, China). We used isoflurane inhalation for anaesthesia. Then, Carbon dioxide asphyxiation followed by cervical dislocation was performed for killing.

Patients

The present study is a prospective study conducted from July 2020 to February 2021, including 102 PCa patients. The primary inclusion criteria were histologically confirmed prostate adenocarcinoma diagnosis; no previous androgen deprivation therapy, chemotherapy, surgery, or radiotherapy. Bone scan, Pelvic MRI, chest computerized tomography (CT) or Positron Emission Tomography-Computed Tomography (PET-CT) were used to diagnose metastatic PCa. Our institution's Ethics and Scientific Committee approved this study, and the enrolled patients provided written informed consent. We registered this study with ClinicalTrials.gov, whose number is ChiCTR2000034274.

Generated SP1 overexpress stable cell lines

PC3 cells with stable overexpress of SP1 (Lenti-GV-SP1) and respective control cells (control) were generated using GV-492-GFP-Pur lentiviral vector (10^8 p.f.u.) (JiKai, Shanghai China). The stable Lenti-GV-SP1 cell line was developed as the previous study [14]. Briefly, PC3 cells were cultured into 6-well plates and then infected by lent virus solution with polybrene (Sigma-Aldrich) for 16 hours. Three days later, transfection efficiency was tested through GFP expression and subjected to 4 $\mu\text{g}/\text{ml}$ puromycin (Life Technologies, Grand Island, NY) selection until GFP was expressed in all cells.

Bioinformatics analysis of regulatory molecules of EMT

We collected about 111 EMT-related molecules by manual search combined with our knowledge background (the list of the genes is available in supplement table1). Online Metascape software ([HTTP://metascape.org/gp/index.html#/main/step1](http://metascape.org/gp/index.html#/main/step1)) was used to analyze the pathway enrichment, Protein-protein interaction (PPI), and upstream gene regulation of these genes [15, 16].

Cancer cells invasion detected by Transwells

Invasion assay was performed using matrigel invasion chambers from BD Biosciences as in a previous study [17]. Briefly, we used 24 wells invasion chamber with 8 μm pores coated with Matrigel to detect the invasion. PC3 cells were seeded on the top chambers, then the invaded cells in the lower membrane surface were fixed with eosin staining (Olympus, Japan).

Lung metastasis analysis *in vivo*

We randomly divided nude mice into two groups—each group has 5 mice—for the administration of PC3 cells. Cells (0.5×10^6) suspended in sterile normal saline were injected (i.v.) to each group of mice separately via the tail vein. In all experiments, mice were assessed for metastasis at 2 weeks after PC3 cells were administered. Then lung metastasis was measured by India ink staining as the previous study [14].

Western blot analysis

Western blotting was performed as described previously [18]. Briefly, the membrane was incubated with primary antibodies against SP1, E-cadherin, N-cadherin (1: 1000 dilution), and β -actin (1: 5000 dilution). After that, the membrane was incubated with secondary antibodies (1: 5000, Abcam, Cambridge, MA). After developing the membrane with ECL reagent (Life Technologies, Grand Island, NY), the protein expression was observed.

Statistical Analysis

All the data are expressed as Mean \pm SD and were calculated from multiple independent experiments. PSA and Total-CTCs' cutoff value was calculated by Receiver Operating Curve (ROC) curve analysis (Medcalc version 9.6.2.0). The Student's two-tailed *t*-test was used to determine significant differences among groups using SPSS 11.0 software. Regression analysis was used to analyze the independent risk factors of metastatic PCa using SPSS 11.0 software. Data with $p < 0.05$ were considered to have significant differences.

Results

1 The number of CTCs in patients with metastatic prostate cancer was significantly higher than that in patients without metastasis.

The separation of CTCs is difficult and expensive, which seriously hinders the research in this field. Many studies have proved CanpatrolTM CTCs technique to be a safe, economical, and effective method [12]. Although this method is still imperfect, it can fully meet the needs of this study. To verify the method's reliability, we tested three healthy people for CTCs, and the study confirmed that CTCs in healthy people were indeed very low (Fig. 1A-B).

Although the better way to diagnose metastatic PCa is to use ⁶⁸Ga-PSMA-PET/CT, it is costly, and it is challenging to ensure that every patient has the examination. In this study, bone scan, chest CT and Pelvic MRI, or PET-CT were used to determine whether the patient was metastatic PCa. We enrolled 102 PCa patients in the present study. We found that the number of Total-CTCs in patients with metastatic PCa was significantly higher than that in patients without metastasis ($p < 0.01$, Fig. 1C-D). The number of CTCs in patients with organ metastasis was significantly higher than that in patients with bone metastasis ($p < 0.01$, Fig. 1E-F).

2 There were more M/E-CTCs and less M-CTCs in patients with metastatic prostate cancer

This study designed all the epithelial cells' markers as red and mesenchymal markers as green respectively (Fig. 2A). This mixed Fluorescence in situ hybridization (FISH) avoids the tedious process of repeated in situ hybridization and can identify CTCs as M-CTCs, E-CTCs, and M/E-CTCs quickly and accurately.

Our study found that the number of E-CTCs and M/E-CTCs in patients with metastatic PCa was significantly higher than that in local PCa (Fig.2C-D, $p < 0.01$). Because of the significant difference between the two groups in the total number, we further calculated the proportion of E-CTCs and M/E-CTCs in the Total-CTCs. We found that the ratio of E-CTCs in the total-CTCs was significantly lower in patients with metastasis, while the proportion of M/E-CTCs in the total-CTCs in patients with metastasis was significantly higher than that in local PCa patients (Fig.2F-G, $p < 0.01$). There was no significant difference in the absolute number of M-CTCs between patients with metastasis and local PCa, but the proportion of M-CTCs/T-CTCs in patients with metastatic PCa was significantly lower than that in patients in local PCa (Fig.2B,2E, $p < 0.01$).

3 The number of CTCs is an independent risk factor for prostate cancer metastasis

Bone scan, MRI, PET-CT, and even ^{68}Ga -PSMA-PET/CT are needed to determine the metastasis of PCa. However, PET-CT and ^{68}Ga -PSMA-PET/CT are expensive, and patients often do not examine these because of the cost. However, judging whether PCa patients with metastasis have essential value for treatment selecting. Therefore, we intend to explore the predictive effect of CTCs on PCa metastasis

We used regression analysis to analyze the predictive effects of age, MRI, Risk stratification, tumor volume, Gleason score, PSA levels, and Total-CTCs on PCa metastasis. Our study found that PSA and Total-CTCs are independent risk factors for PCa metastasis (Fig.3 A, $p < 0.01$). Through ROC curve analysis, we found that PSA and T-CTCs were good predictors of PCa metastasis. If the count of Total-CTCs > 14 , it predicts PCa cells metastasis, in which the sensitivity was 90.48%, and the specificity was 96.67%, more sensitive than PSA (Fig.3 B-C).

4 SP1 is an essential regulator of cell-cell junction proteins analyzed by bioinformatics software

The change of cell surface molecules is a critical feature of EMT[8]. So far, researchers have reported a large number of EMT surface markers. Complex net mechanisms should regulate these EMT markers. We retrieved 111 reported EMT-related molecules by manual search in Pubmed to find the main regulatory molecules among numerous cell surface molecules.

We found that these molecules are mainly extracellular matrix organization, cell junction organization, and cell-substrate adhesion molecules by cluster enrichment analysis (Fig.4A). Through the study of protein-protein interaction (PPI), these molecular interactions were divided into three categories according to the degree of interaction, namely, E-Cadherin (CDH1) group molecules, Claudins (CLDNs) group molecules, and FN group molecules (Fig.4B).

To find these EMT molecules' regulatory molecules, we used TRRUST online software to calculate these molecules' potential upstream regulatory molecules. We discovered that SP1 is the second most involved in EMT regulation, second only to Snail 1. Since many studies reported Snail1 (Snai1) function on EMT already, we choose to explore the role of SP1 in the regulation of EMT of CTCs in PCa.

5 SP1 promotes the transformation of CTCs into mesenchymal CTCs via EMT.

SP1 is an essential regulatory molecule of EMT, which can initiate EMT in cancer cells[19]. However, the regulation effect of SP1 on EMT in CTCs is less studied, and the specific role is still not cleared. In this study, FISH was used to detect the SP1 level of CTCs. In the actual measurement, SP1 was given a purple mark. To show clearly with DAPI, we converted its color to white (Fig.5A).

According to the different expression levels of SP1 in CTCs, we divided them into negative, weakly positive (+), and strongly positive (+ +). We found that in patients with metastatic PCa, the proportion of strongly positive SP1 CTCs increased (Fig.5B). The number of SP1+ cells increased significantly in metastatic PCa compared with local PCa; however, there was no significant difference in the proportion of SP1 positive cells /Total-CTCs between metastatic PCa and Local PCa. Further data analysis showed that SP1 + + cells/Total-CTCs were significantly decreased in metastatic PCa compared with local PCa [Fig.5 C-E, $p < 0.05$].

If SP1 can activate the transformation of CTCs into mesenchymal cells, then when the expression of SP1 increases, the distribution of different varieties of CTCs will change in theory. We divided CTCs into E-CTCs, M -CTCs, M/E-CTCs, and M-CTCs+ M/E-CTCs. The number of weakly and strongly positive SP1 cells was calculated when these cell varieties changed significantly [Fig.5 F]. We found that with the increase of SP1 + and SP1++, CTCs tended to transform to M/E-CTCs and M-CTCs+ M/E-CTCs [Fig.5 F]. Correlation analysis showed no significant correlation between SP1 + + expression and M -CTCs count. However, there was a good correlation between the number of SP1 positive cells and M-CTCs+ M/E-CTCs count ratio (Fig.5 G-J, $R = 0.91$).

6 Overexpression of SP1 initiated PCa cells EMT, enhanced cancer cell invasion, but inhibited lung metastasis.

The results of CTCs only implied that SP1 might be involved in the regulation of EMT in PCa cells. To verify this conclusion, we constructed a PCa cell line, PC3 overexpressing SP1.

Our study confirmed that overexpression of SP1 could reduce the expression of E-Cadherin and increase the expression of N-Cadherin in PCa cell line PC3 (Fig.6 A-D, $p < 0.05$). Overexpression of SP1 in PC3 cells significantly enhanced the invasiveness of PC3 cells *in vitro* (Fig.6 E-F, $p < 0.05$). However, we found that overexpression of SP1 significantly inhibited lung metastasis in mice lung metastasis model *in vivo* (Fig.6 J-H, $p < 0.05$).

Discussion

Circulation Tumor cells (CTCs) are essential for cancer metastasis. A large number of studies have confirmed that EMT of cancer cells is a bidirectional reversible process. We found that among E-CTCs, M-CTCs, and M/E-CTCs, the ability of M-CTCs to induce metastasis is weak. If it promotes CTC's transformation to the type with weak or almost no metastatic ability, it will be a new strategy for treating

metastatic cancer. Our study found that overexpression of SP1 initiated the EMT process of CTCs and promoted the transformation of CTCs to M-CTCs, hence inhibiting the metastasis of PCa. The results of this study enrich our knowledge of CTCs and are beneficial for the drug development of metastatic PCa.

Studies have proved that the amount of CTCs is closely related to cancer metastasis, chemotherapy drug resistance, cancer recurrence, and prognosis [20, 21]. Our study also confirmed that the number of CTCs in patients with metastatic PCa was significantly higher than that in patients without metastasis (Fig.1 A-D). Bone is the most common target organ for PCa metastasis; organ metastasis indicates patients with lung, liver, brain, and kidney metastasis in PCa [22]. The prognosis of prostate cancer patients with organ metastasis is often worse than that of bone metastasis. In patients with metastatic PCa, the quantity of Total-CTCs in patients with organ metastasis is higher than that in patients without organ metastasis (Fig.1 E-F, $p < 0.05$). These results suggest that the further increase of CTCs may lead to organ metastasis.

The treatment of metastatic PCa is different from that of local PCa. It has essential values to predict whether PCa patients with metastasis. We used regression analysis to analyze the predictive effects of age, MRI, Risk stratification, tumor volume, Gleason score, PSA levels, and Total-CTCs on PCa metastasis. We found that Total-CTCs is an independent risk factor for PCa metastasis, and its number greater than 14 is highly suggestive of metastasis (Fig.3 A-C). This result may reduce unnecessary PET-CT or ^{68}Ga -PSMA-PET/CT examination in patients with PCa. However, it needs further study in the future.

EMT is an important mechanism to regulate the metastasis and invasion of cancer cells [23]. The occurrence of epithelial-mesenchymal transition (EMT) in CTCs results in epithelial- (E-CTCs), mesenchymal- (M-CTCs), and hybrid-subtypes of CTCs (M/E-CTCs). So far, the specific role of different varieties of CTCs in metastasis is still unclear. Our study found that the number of E-CTCs and M/E-CTCs in patients with metastatic PCa was significantly higher than that in local PCa (Fig.2C-D, $p < 0.01$), suggesting these two kinds of CTCs might be facilitated to PCa metastasis. Unlike *in vitro* experiments, it is generally believed that the migration and motility of epithelial cancer cells will be weakened [24]. With the deepening of research, it has also been reported that the overexpression of epithelial marker molecules in cancer cells is necessary for cell metastasis, which implies that Epithelial-CTCs may contribute to metastasis [25].

Many studies reported that cancer cells transformed into mesenchymal cells would obtain more vigorous invasion and have a more substantial metastasis potential *in vitro*. However, in the circulatory system, the role of M-CTCs may be different from that of *in vitro* experiments. Moreover, as known to us all, cells' invasiveness is different from patients' metastatic ability. In animal experiments, few studies suggested that M-CTCs may have a weak ability to cause metastasis [26]. This study found no significant difference in the absolute number of M-CTCs between patients with metastasis and local PCa. However, if further comparing the ratio of M-CTCs, we found that the proportion of M-CTCs in Total-CTCs of patients with metastatic PCa was significantly lower than those in local PCa (Fig.2B,2E, $p < 0.01$). The main reason why there was no statistical difference in the absolute number of M-CTCs between the local and metastatic PCa was that the absolute number of M-CTCs was too low to distinguish statistically. Combined with the

results of animal experiments in this study, the lung metastasis caused by M-CTCs was significantly lower than that caused by E-CTCs. Based on these results, we speculate that there may be some mechanism to reduce the proportion of M-CTCs in T-CTCs in patients with metastatic PCa, and increase the absolute number of E-CTCs and M/E-CTCs on the premise of increasing T-CTCs, to promote the PCa metastasis.

In this study, bioinformatics technology was used to screen potential EMT regulatory molecules of CTCs. Then we double-checked to verify whether they are EMT-related molecules by cluster enrichment analysis. The results showed these molecules are mainly extracellular matrix organization, cell junction organization, and cell-substrate adhesion molecules (Fig.4A). Through the analysis of PPI, we found that these molecular interactions can be divided into three categories according to the degree of interaction, namely, E-Cadherin (CDH1) group molecules, Claudins (CLDNs) group molecules, and FN group molecules (Fig.4B). The above analyses confirmed that the genes we used were EMT-related once again. Finally, we selected SP1 molecules by TRRUST analysis, which is less studied. SP1 is a zinc finger transcription factor that binds to GC-rich motifs of many promoters. The encoded protein is involved in many cellular processes, including cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodelling. Some studies demonstrate SP1 activates EMT in cancer cells, but few studies on the function of EMT regulated by SP1 in CTCs, especially in PCa. This study found a good correlation between the number of SP1 positive cells and M-CTCs+M/E-CTCs count by correlation analysis (Fig.5 G-J). Along with the increase of SP1 + and SP1++, CTCs tended to transform to M/E-CTCs or M-CTCs+ M/E-CTCs (Fig.5 F). These results suggest that SP1 might be involved in the EMT regulation of PCa CTCs, and the increase of SP1 level may promote the transformation of CTCs cells to mesenchymal phenotype.

To verify the effect of SP1 in PCa patients, we overexpressed SP1 in PC3 cells. We found that overexpression of SP1 reduced the expression of E-Cadherin and increased the expression of N-Cadherin in PC3 (Fig.6 A-D, $p < 0.05$), which confirmed that overexpression of SP1 initiated EMT of PCa cells and promoted the mesenchymal transformation of cancer cells. Functional studies have also demonstrated that the overexpression of SP1 in PC3 cells significantly enhanced the invasiveness of PC3 cells *in vitro* (Fig.6 E-F, $p < 0.05$). However, we found that overexpression of SP1 significantly inhibited lung metastasis in mice lung metastasis model *in vivo* (Fig.6 J-H, $p < 0.05$). Interestingly, the results of *in vivo* and *in vitro* experiments did not match; that is, M-CTCs are not conducive to the formation of cancer metastasis but enhance the invasion of cancer cells. It has been reported that overexpression of twist, one of EMT-transcription factors (EMT-TFs), maintains the cancer cells in mesenchymal phenotype and significantly inhibits cancer cells' lung metastasis formation through tail vein injection [26, 27]. These results indicated M-CTCs are weak to cause metastasis, and up-regulation of SP1 in CTCs could inhibit metastasis.

Conclusion

In conclusion, the count of CTCs is an independent prognostic factor for PCa metastasis. Reducing the proportion of M-CTCs and increasing the count of E-CTCs and M/E-CTCs might promote metastasis. SP1

initiates EMT to inhibit CTCs' metastasis ability. SP1 may become the therapeutic target for PCa patients. Indeed, there are still some deficiencies in this study. The specific roles of E-CTCs and M / E-CTCs in metastasis and related regulatory mechanisms need to be further studied.

Abbreviations

- 1 Circulating tumor cells CTCs
- 2 Prostate cancer PCa
- 3 Epithelial-mesenchymal transition EMT
- 4 Epithelial-CTCs E-CTCs
- 5 Mesenchymal-CTCs M-CTCs
- 6 Mesenchymal/Epithelial-CTCs M/E-CTCs
- 7 Total-CTCs T-CTCs
- 8 Fluorescence in situ hybridization FISH
- 9 Computerized tomography CT
- 10 Positron Emission Tomography-Computed Tomography PET-CT
- 11 Protein-protein interaction PPI
- 12 EMT- transcription factors EMT-TFs

Declarations

Ethical approval and consent to participate

The Ethics Committee approved this study of Chongqing Medical University (Chongqing, China). According to the guideline and regulations for Animal Health and Use, all animal experiments were performed following animal protocols approved by the Chongqing Medical University (National Standardization Administration of China, 2016).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design: Fei Gao; Data production, analysis, and interpretation: Fei Gao, Hui Liu, and Guo Ping Qiu.; writing the manuscript: Fei Gao and Mei Yang. All authors reviewed the manuscript and accepted the content.

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Figures

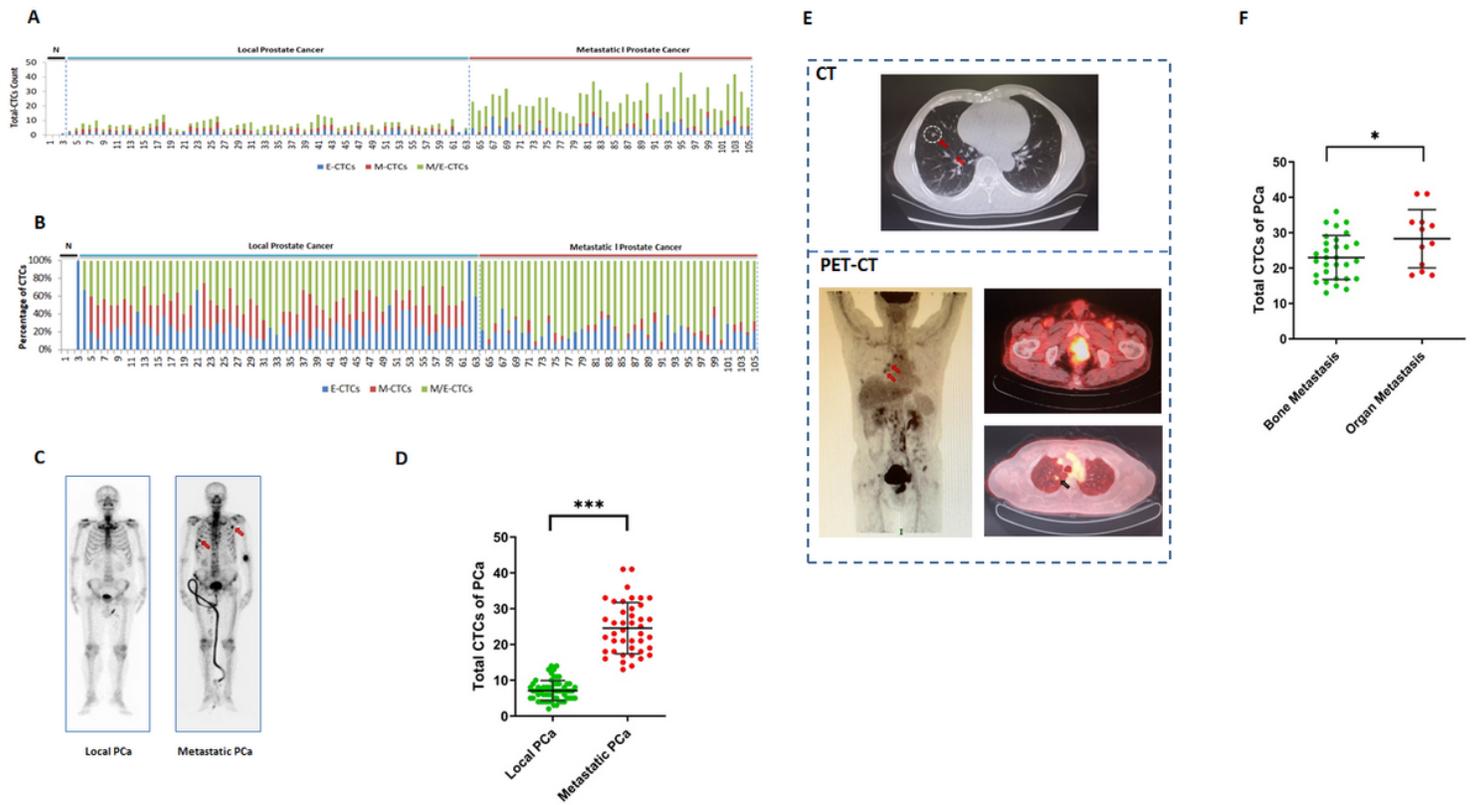


Figure 1

The number of CTCs in patients with metastatic prostate cancer was significantly higher than that in patients without metastasis. A, The number of different types of CTCs in local and metastatic PCa patients. (N represents health people.) B, The overview of the percentage of different CTCs types in patients with local PCa and metastatic PCa. (N represents health people.) C, Bone scan images of local and metastatic PCa patients. The red arrow indicated the metastatic bone site. D, The total CTCs number in local and metastatic PCa patients,***indicated $p < 0.001$, $n = 102$. E, The CT and PET-CT images of metastatic PCa; white circles and arrows indicated metastatic site. F, The total CTCs number in PCa patients with bone metastasis or organ metastasis. *indicated $p < 0.01$, $n = 42$.

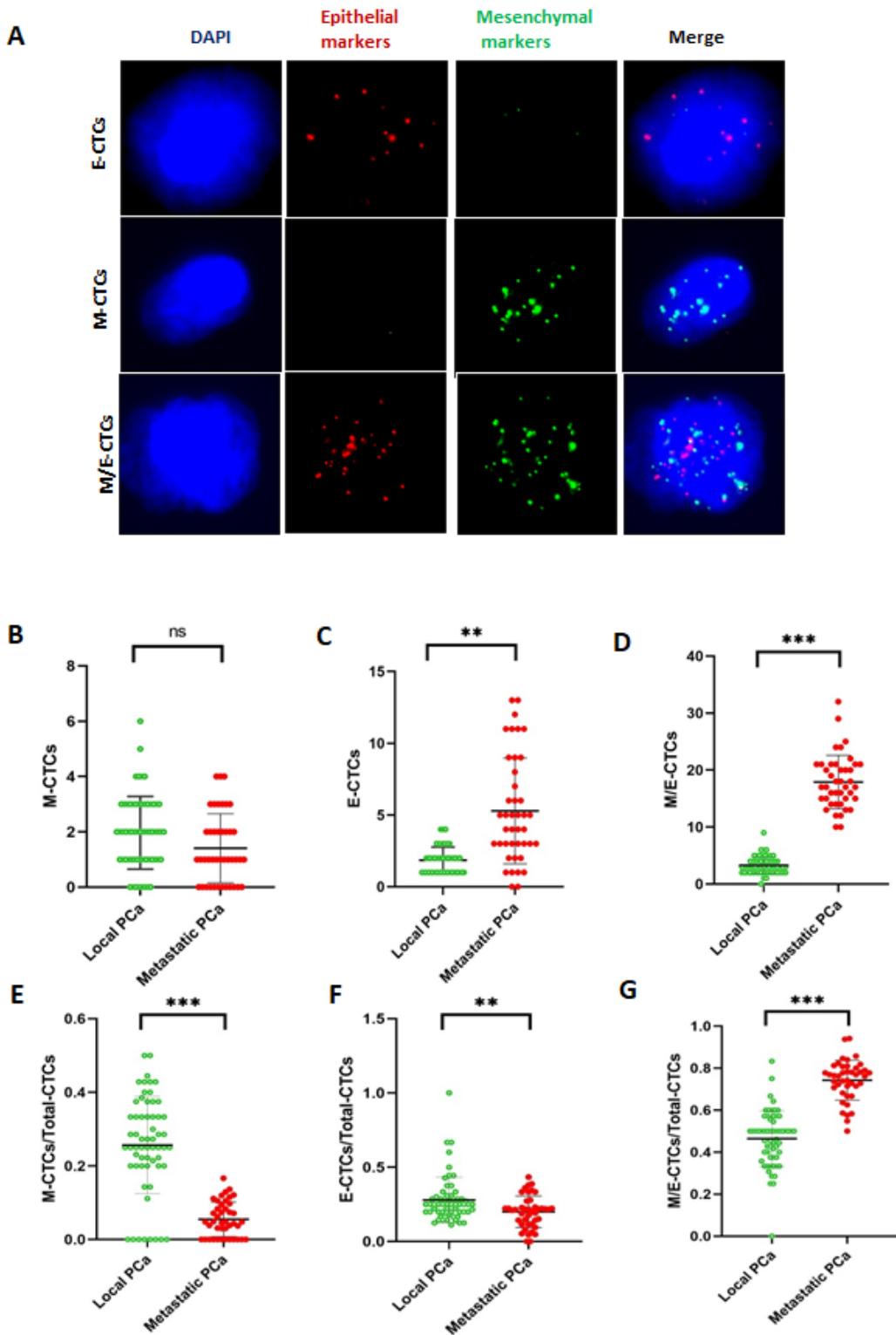
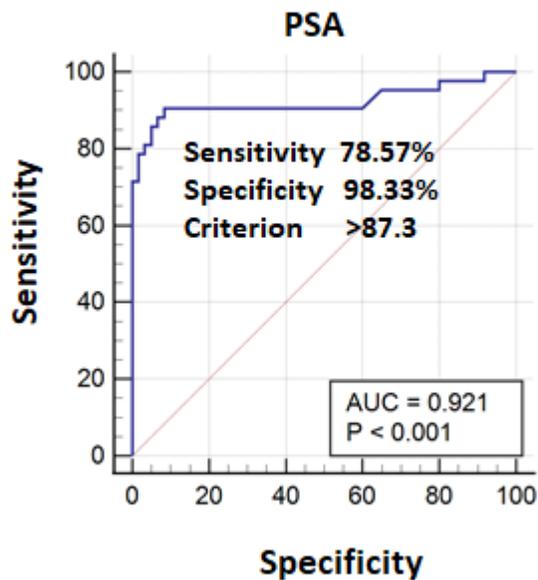
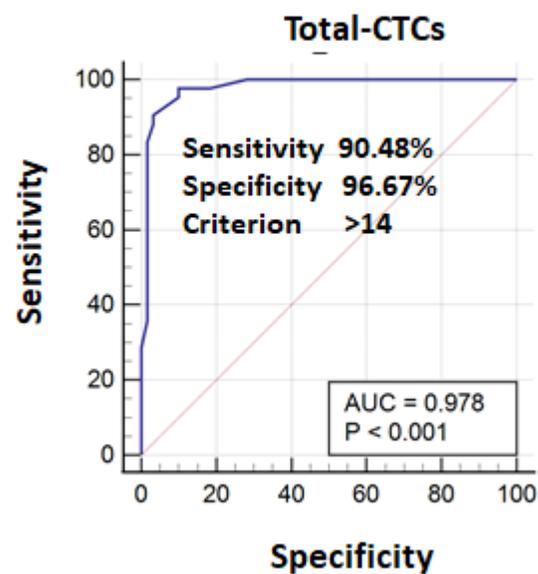


Figure 2

There was more M/E-CTCs and less M-CTCs in patients with metastatic prostate cancer. A, FISH images of different types of CTCs. B-D, The count of M-CTCs, E-CTCs, and M/E-CTCs in local and metastatic PCa patients. E-G: The ratio of different CTCs in total CTCs of local and metastatic PCa patients. **indicated $p < 0.01$, *** indicated $p < 0.001$, ns indicated $p > 0.05$.

A

	N	Mean \pm SD	Coefficient	t	P value
Age	102	68 \pm 5.96	0.001535	0.408	0.6838
MRI invasion	102	41+/61-	0.1239	1.635	0.1054
Risk stratification	102	L 21 /M 32 /H 49	0.08552	1.210	0.2292
Tumor volume	102	66.55 \pm 33.32	-0.0005016	-0.743	0.4592
Gleason Score	102	7 \pm 1.38	0.02398	0.670	0.5044
PSA Level	102	18.75 \pm 309.02	0.0002276	2.465	0.0155
Total-CTCs	102	10 \pm 9.99	0.02848	8.590	<0.0001

B**C****Figure 3**

The number of CTCs is an independent risk factor for prostate cancer metastasis. A, Regression analysis confirmed that PSA and Total-CTCs were independent risk factors for PCa metastasis. B-C, The cut-off value of PSA and Total-CTCs to predict metastatic PCa by ROC analysis.

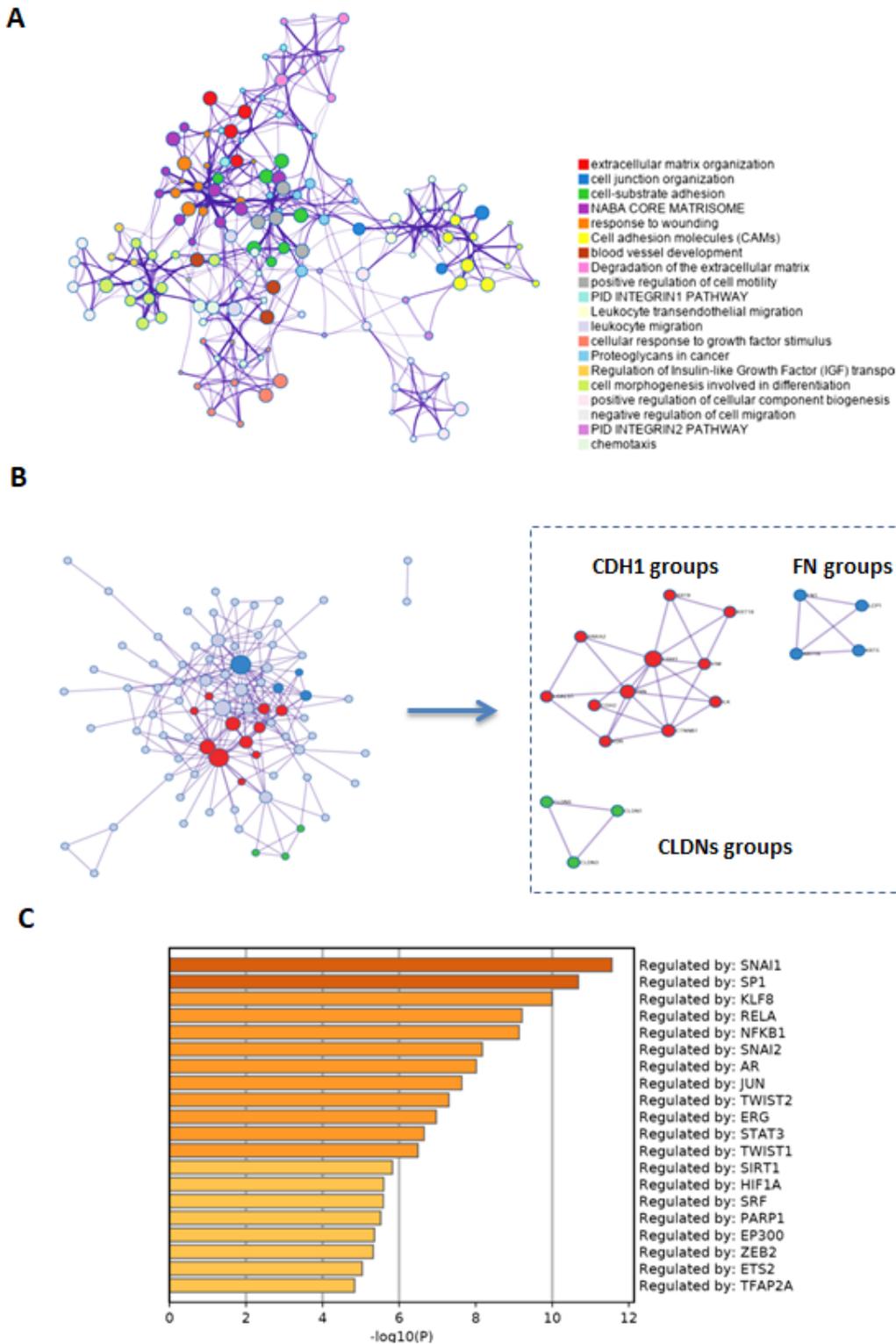


Figure 4

SP1 is an essential regulator of cell-cell junction proteins analyzed by bioinformatics software. A, Signaling pathways of major EMT-related molecules analyzed by Metascape. B, Protein-Protein interaction network of major EMT-related molecules. C, Analysis of upstream regulatory molecules of major EMT-related molecules by TRRUST software.

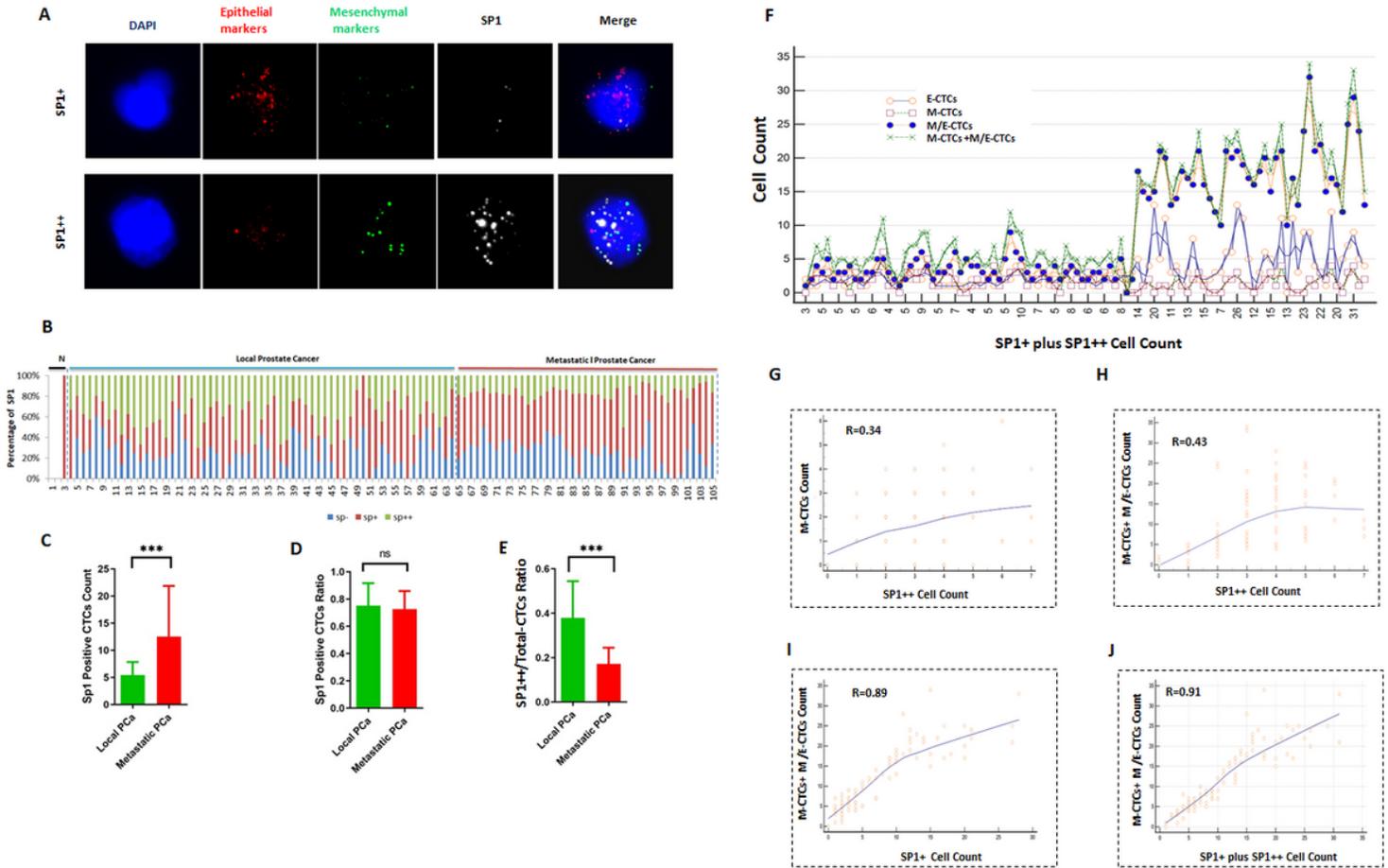


Figure 5

SP1 promoted the transformation of CTCs into mesenchymal CTCs via EMT. A, FISH images of SP1+ and SP1++ CTCs. B, The percentage of different SP1 expression in CTCs of local and metastatic PCa patients. (N represents health people.) C-E, The count of different SP1 expression in CTCs of local and metastatic PCa patients. *** indicated $p < 0.001$, ns indicated $P > 0.05$. F, The distribution of different types of CTCs with the number of SP1+ plus SP1++ positive cells. G-J, Linear regression analysis of the relationship between M-CTCs (or M-CTCs+M/E-CTCs) and the number of SP1 positive cells.

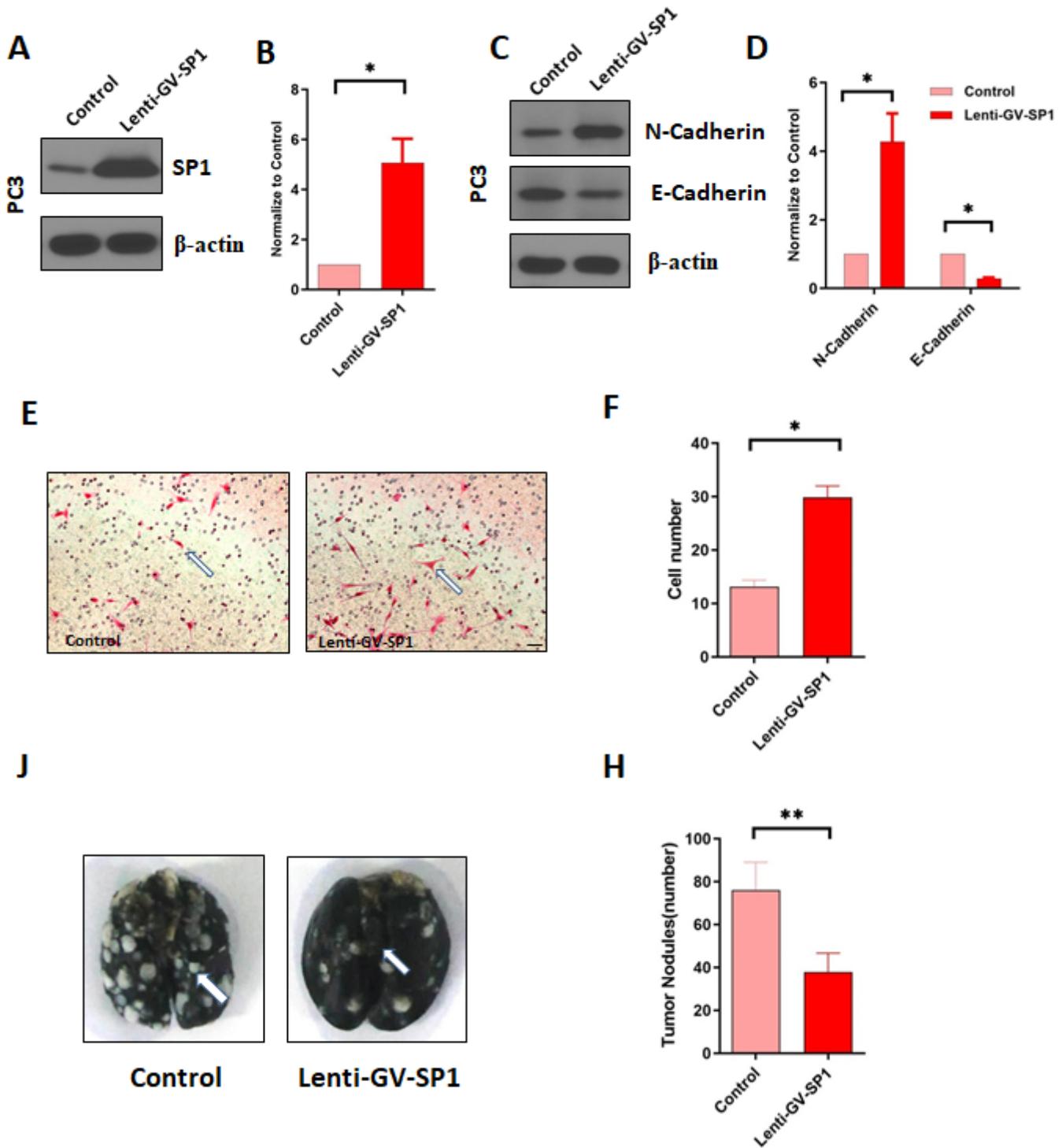


Figure 6

Overexpression of SP1 initiated PCa cells EMT, enhanced cancer cells invasion, but inhibited lung metastasis. A-B, Western blot confirmed that SP1 was overexpressed (*indicated $p < 0.05$, $n = 3$). C-D, Overexpression of SP1 resulted in the increase of N-Cadherin and decrease of E-Cadherin in PC3 cells (*indicated $p < 0.05$, $n = 3$). E-F, Overexpression of SP1 enhanced the invasiveness of PC3 cells (*indicated $p < 0.05$, $n = 6$). J-H, Overexpression of SP1 significantly inhibited lung metastasis of PC3 cells in vivo (**indicated $p < 0.01$, $n = 5$).

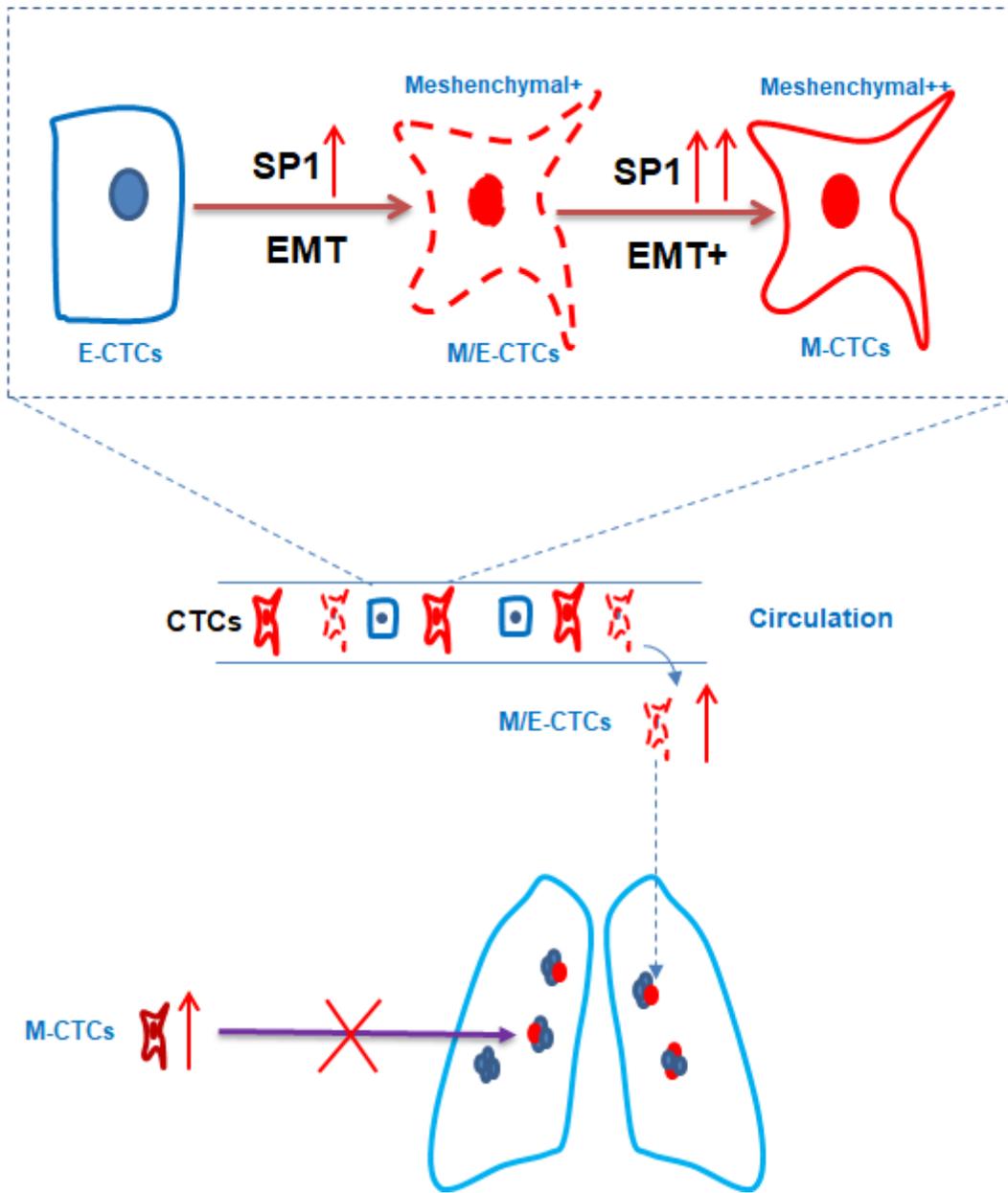


Figure 7

Different types of CTCs have different effects on metastasis. In the circulation system, prostate cancer CTCs could transform from E-CTCs to M-CTCs via the regulation of SP1, and M/E-CTCs is their intermediate state. The ability of lung metastasis was significantly inhibited via over mesenchymal status CTCs.