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•Original article•

# Fu-Zheng-Yi-Liu Formula inhibits the stem cells and metastasis of prostate cancer *via* tumor-associated macrophages/C-C motif chemokine ligand 5 pathway in tumor microenvironment

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**[ABSTRACT]** Prostate cancer (PCa) is the second most common malignancy among men globally. The Fu-Zheng-Yi-Liu (FZYL) Formula has been widely utilized in the treatment of PCa. This study investigates whether the FZYL Formula can inhibit PCa by targeting the TAMs/CCL5 pathway. We conducted *in vitro* co-cultures and *in vivo* co-injections of PCa cells and TAMs to mimic their interaction. Results showed that the FZYL Formula significantly reduced the proliferation, colony formation, subpopulations of PCSCs, and sphere-formation efficacy of PCa cells, even in the presence of TAM co-culture. Additionally, the Formula markedly decreased the migration, invasion, and epithelial-mesenchymal transition (EMT) of PCa cells induced by TAMs. The FZYL Formula also reversed M2 phenotype polarization in TAMs and dose-dependently reduced their CCL5 expression and secretion, with minimal cytotoxicity observed. Mechanistic studies confirmed that the TAMs/CCL5 axis is a critical target of the FZYL Formula, as the addition of exogenous CCL5 partially reversed the formula's inhibitory effects on PCSCs self-renewal in the co-culture system. Importantly, the Formula also significantly inhibited the growth of PCa xenografts, bone metastasis, and PCSCs activity *in vivo* by targeting the TAMs/CCL5 pathway. Overall, this study not only elucidates the immunomodulatory mechanism of the FZYL Formula in PCa therapy but also highlights the TAMs/CCL5 axis as a promising therapeutic target.

**[KEY WORDS]** Traditional Chinese medicine; Tumor-associated macrophages; Prostate cancer stem cells; CCL5; Tumor microenvironment

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These authors have no conflict of interest to declare.

## Introduction

Prostate cancer (PCa) ranked as the second most common cancer and the fifth leading cause of cancer-related deaths among men globally in 2020 [1]. While localized PCa typically presents a favorable prognosis with a 5-year survival rate approaching 100%, the disease often remains asymptomatic in its early stages. Consequently, nearly half of the PCa patients are diagnosed at the metastatic stage, where the 5-year survival rate plummets to only 28% [2, 3, 4]. Androgen deprivation therapy (ADT) is the primary treatment for advanced and metastatic PCa [5]. However, the efficacy of ADT

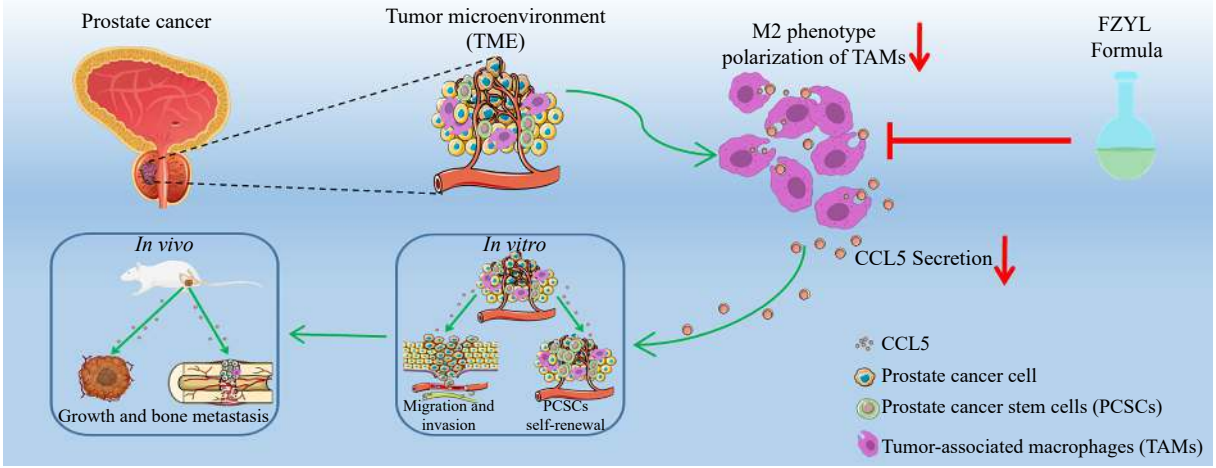



**Fig. 1 FZYL Formula inhibits PCSCs self-renewal and PCa metastasis by suppressing TAMs/CCL5 signaling within the TME (Graphical abstract).**

is generally short-lived, as patients frequently develop resistance, leading to disease progression and metastasis [6, 7]. Therefore, the urgent need to develop new pharmacological targets or drugs to prevent PCa metastasis has become a focal point of increasing research interest.

Traditional Chinese Medicine (TCM) is increasingly recognized as a valuable resource for discovering novel therapeutic drugs for cancer treatment [8, 9]. TCM has reliable efficacies and unique advantages in PCa treatment, such as reducing the incidence of side effects after ADT and reversing its drug resistance [10, 11]. Importantly, the anticancer effects of TCM have been demonstrated through multiple targets [12]. The Fu-Zheng-Yi-Liu (FZYL) Formula, a TCM composition that includes *Panax ginseng C.A.Mey.*, *Epimedium brevicornu Maxim.*, *Astragalus mongholicus Bunge*, *Curcuma longa L.*, *Salvia miltiorrhiza Bunge*, *Paris polyphylla Sm.*, *Lycium barbarum L.*, *Ganoderma lucidum*, and *Buthus martensii Karsch*, has been extensively used for PCa treatment in China for several decades [2]. A clinical study involving 209 advanced PCa patients at the Guangdong Provincial Hospital of Chinese Medicine demonstrated that the FZYL Formula delayed the progression to castration-resistant PCa with a median progression time of 39.62 months and prolonged the overall survival (OS) with a median OS of 47.15 months [2]. The study also indicated significant reductions in serum prostate-specific antigen (PSA) levels and improvements in the quality of life of PCa patients following treatment with the FZYL Formula. Despite its widespread use and reliable efficacy, the molecular mechanisms by which the FZYL Formula inhibits PCa remain under-investigated. Numerous studies have highlighted that TCM Formulas exert anticancer effects by enhancing anti-tumor immune function and remodeling the immunosuppressive tumor microenvironment (TME) [13]. It has also been reported that treatment with the FZYL Formula may enhance the anti-tumor immune response by promoting the activation and survival of dendritic cells within the PCa TME [2]. Given the extensive application and proven therapeutic efficacy of the FZYL Formula, it is clinically significant to further explore its underlying immune-regulation mechanisms to support its clinical use and benefit PCa patients.

TME is characterized by the infiltration of various immunosuppressive cells, including macrophages, regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs) [14]. Macrophages, which constitute approximately 30% to 50% of the tumor mass, are one of the most prevalent immune cell groups within the TME of many cancers [15, 16]. Typically, these macrophages adopt a pro-tumorigenic M2 phenotype that promotes cancer growth and metastasis, although they can be stimulated to transition to the anti-tumorigenic M1 phenotype [17]. Clinical studies have shown that tumor-associated macrophage (TAM) infiltration tends to increase with PCa progression [18] and heightened TAM presence correlates with poorer overall survival, particularly in metastatic cases [19]. TAMs contribute to PCa growth and metastasis by secreting cytokines and chemokines, which serve as intercellular messengers facilitating interactions between PCa cells and TAMs, and by recruiting additional immune cells to establish an immunosuppressive TME [9]. CCL5, notably abundant among the chemokines secreted by TAMs, plays a significant role in this process [20]. Increasing evidence supports the blocking of CCL5-CCR5 signaling as an effective anticancer strategy [21]. Cancer stem cells (CSCs) are fundamental to the initiation and progression of PCa [22, 23]. Our previous research established that CCL5, primarily sourced from TAMs rather than PCa cells, promotes the self-renewal of prostate cancer stem cells (PCSCs) and contributes to PCa bone metastasis [23].

Given these insights, the TAMs/CCL5 pathway emerges as a promising target for PCa therapy. This study investigates whether the anti-PCa activity of the FZYL Formula operates through modulation of the TAMs/CCL5 pathway. By employing a TAMs-PCa co-culture system and PCa xenografts *in vivo*, we have systematically demonstrated that the FZYL Formula can inhibit PCSCs self-renewal and PCa bone metastasis by targeting the TAMs/CCL5 pathway (Fig. 1) .

This research not only elucidates the immunomodulatory mechanism of the FZYL Formula in PCa treatment but also underscores the potential of TAMs/CCL5 as a viable therapeutic target for eradicating PCSCs and treating metastatic PCa.

## Materials and Methods

### *Preparation and quality control of the FZYL formula*

The FZYL Formula was prepared by extracting a mixture of nine TCM components. These included 9 g of *Panax ginseng C.A.Mey*. [Araliaceae; Ginseng radix et rhizoma] (Barcode: 200702), 9 g of *Epimedium brevicornu Maxim*. [Berberidaceae; Epimedii folium] (Barcode: 20040081), 15 g of *Astragalus mongholicus Bunge* [Fabaceae; Astragali radix] (Barcode: 200800061), 6 g of *Curcuma longa L*. [Zingiberaceae; Curcumae longae rhizoma] (Barcode: 200604071), 15 g of *Salvia miltiorrhiza Bunge* [Lamiaceae; Salviae miltiorrhizae radix et rhizoma] (Barcode: 191101661), 15 g of *Paris polyphylla Sm*. [Melanthiaceae; Paridis rhizoma] (Barcode: 20060408I), 12 g of *Lycium barbarum L*. [Solanaceae; Lycii fructus] (Barcode: 200703931), 12 g of *Ganoderma lucidum* [Polyporaceae; Ganoderma] (Barcode: 20040275I), and 5 g of *Buthus martensii Karsch* [Scorpionidae; Scorpio] (Barcode: 2005001). All ingredients were sourced from the pharmacy at Guangdong Provincial Hospital of Traditional Chinese Medicine. For the preparation of the FZYL Formula, the combined ingredients, totaling 98 g, were ground into a fine powder. This powder was then soaked in pure water using a drug-to-solvent ratio of 1 : 10 for 30 minutes at room temperature. Subsequently, it was refluxed twice with pure water, each session lasting one hour. The mixture was then filtered, and the filtrate evaporated at 50 °C until dryness was achieved, followed by freeze-drying. The yield of the dried extract ranged from 8.1% to 10.4%. Quality control across different batches of FZYL Formula was ensured through high-performance liquid chromatography, utilizing rutin, calycosin 7-*O*-glucoside, ginsenoside Rg1, salvianolic acid B, icariin, and ganoderic acid A (Sigma-Aldrich) as indicators. The relative concentrations of these quality control compounds were semi-quantified by comparing their peak areas in the chromatography fingerprints. This stringent process ensures the consistency and therapeutic efficacy of the FZYL Formula across various batches.

### *Animal experiment*

The animal study was conducted under the approval and oversight of the Institutional Animal Care and Use Committee of Guangdong Provincial Hospital of Chinese Medicine (Approval No. 2018071) and adhered to the institution's guidelines for the use of laboratory animals. Male C57BL/6 mice, aged 6 weeks, were housed in a specific pathogen-free (SPF) environment. RM1 xenografts were established by subcutaneously injecting $2 \times 10^6$ RM1 cells into the tibia regions of the mice. The mice were randomly divided into six groups, each consisting of eight animals: a saline control group; a low-dose (0.5 $g \cdot kg^{-1} \cdot d^{-1}$) FZYL formula group; a high-dose (1.0 $g \cdot kg^{-1} \cdot day^{-1}$) FZYL Formula group; a TAM group (RM1 cells co-injected with Raw264.7-derived TAMs in a 1 : 3 ratio); a TAMs plus high-dose FZYL Formula group; and a TAMs/rCCL5 plus high-dose FZYL formula group (RM1 cells co-injected with Raw264.7/rCCL5-derived TAMs). Treatment with saline or FZYL Formula was administered via oral gavage. Throughout the duration of the experiment, the body weights and tumor diameters of the mice were measured every three days. Bone metastasis of the RM1 xenografts was monitored using X-ray imaging conducted with the IVIS Lumina XR imaging system (PerkinElmer). At the conclusion of the experiment, the mice were euthanized, and their tumors were excised and photographed. Tumor tissues were processed for immunohistochemistry (IHC) and immunofluorescence (IF) assays, as detailed in the Appendix. Additionally, the tibias were collected and subjected to hematoxylin and eosin (HE) staining to assess histological changes. Detailed experimental methods are provided in the Appendix file.

### *Statistical analysis*

Data are presented as the mean ± standard deviation (SD). Statistical significance for pairwise comparisons was determined using one-way analysis of variance (ANOVA) or Student's *t*-test, as appropriate, employing SPSS version 22.0 software. A P-value of less than 0.05 was considered statistically significant.

## Results

### *FZYL Formula remarkably restrains the proliferation and colony formation capabilities of PCa cells even in the presence of TAMs co-culture*

Initially, quality control analysis of the FZYL Formula was conducted by comparing the chromatographic fingerprints across different batches, as shown in Appendix file Figure S1. The concentration results of the quality control compounds, detailed in Appendix file Table S1, indicated consistent stability across batches. Subsequently, the cytotoxic effects of the FZYL formula on PCa cells were assessed using the CCK8 assay. Treatment with FZYL formula (100 to 900 $\mu g \cdot mL^{-1}$) significantly inhibited the proliferation of both human PCa cell lines (DU145 and PC3) and the mouse PCa cell line RM1. The $IC_{50}$ values for the FZYL formula were 533.2 $\mu g \cdot mL^{-1}$ for DU145 cells, 634.4 $\mu g \cdot mL^{-1}$ for PC3 cells, and 541.7 $\mu g \cdot mL^{-1}$ for RM1 cells at 48 h, respectively (Fig. 2A). Additionally, higher concentrations of the FZYL Formula (450 to 900 $\mu g \cdot mL^{-1}$) significantly suppressed colony formation capabilities of PCa cells over a two-week culture period, suggesting a durable suppressive effect on the clonogenic capacities of these cells (Fig. 2B). The TME of PCa is known for its heterogeneity and includes a dominant subpopulation of macrophages that typically exhibit an M2 polarization phenotype, which can promote the growth and metastasis of PCa cells. To mimic the coexistence of PCa cells and TAMs *in vitro*, M2 phenotype macrophages (TAMs) were generated by treating macrophages with the CM of PCa cells.

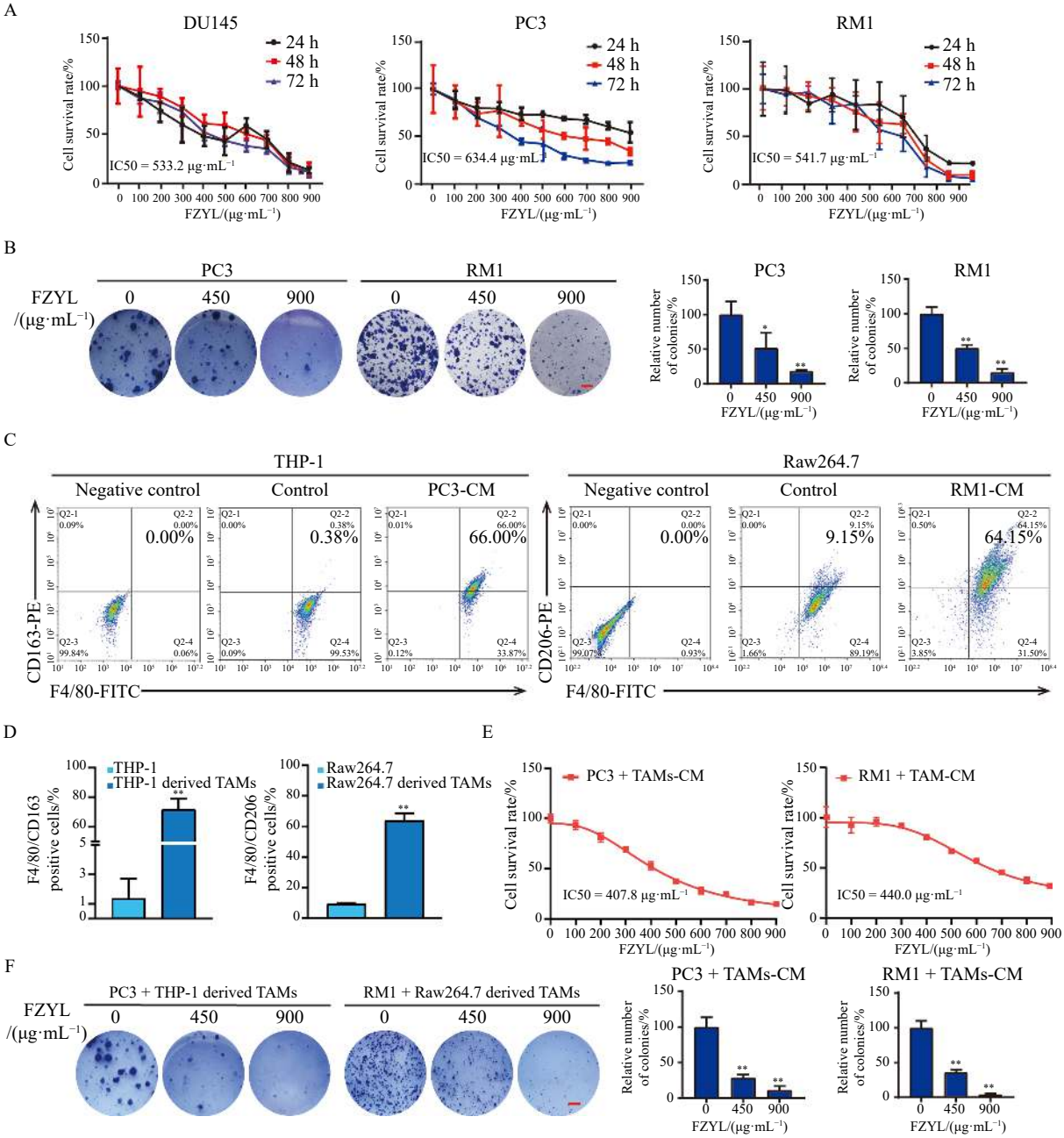



**Fig. 2 FZYL Formula remarkably restrains the proliferation and colony formation capabilities of PCa cells even in the presence of TAMs co-culture. (A) The cytotoxicities of the FZYL Formula in PCa cells were examined by the CCK8 method. (B) FZYL Formula (450–900 μg·mL$^{-1}$) strongly inhibited the colony formation capabilities of PCa cells. (C–D) The conditional medium (CM) of PCa cells successfully induced the M2 phenotype polarization of TAMs. (E) FZYL Formula treatment for 48 h significantly suppressed the proliferation of PCa cells in the TAMs co-culture system. (F) FZYL formula remarkably inhibited the colony formation capabilities of PCa cells in the TAMs co-culture system. *n* = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the control (0 μg·mL$^{-1}$ FZYL).**

The results demonstrated that PC3-CM significantly increased the F4/80$^{+}$/CD163$^{+}$ subpopulation of THP-1 macrophages, while RM1-CM significantly raised the F4/80$^{+}$/CD206$^{+}$ subpopulation of Raw264.7 macrophages (Fig. 2C–2D), indicating successful induction of TAMs. To explore whether the FZYL formula could inhibit the growth of PCa cells in the presence of TAM co-culture, PCa cells were co-cultured with TAMs using a transwell chamber system and treated with various concentrations of the FZYL Formula. It was observed that a 48-hour treatment with the FZYL Formula also significantly suppressed the proliferation of co-cultured PCa cells. The $IC_{50}$ values were 407.8 μg·mL$^{-1}$ for PC3 cells and 440.0 μg·mL$^{-1}$ for RM1 cells in the co-culture system, both significantly lower than those in direct culture conditions (Fig. 2E). Moreover, the FZYL Formula also markedly inhibited the colony formation capabilit-

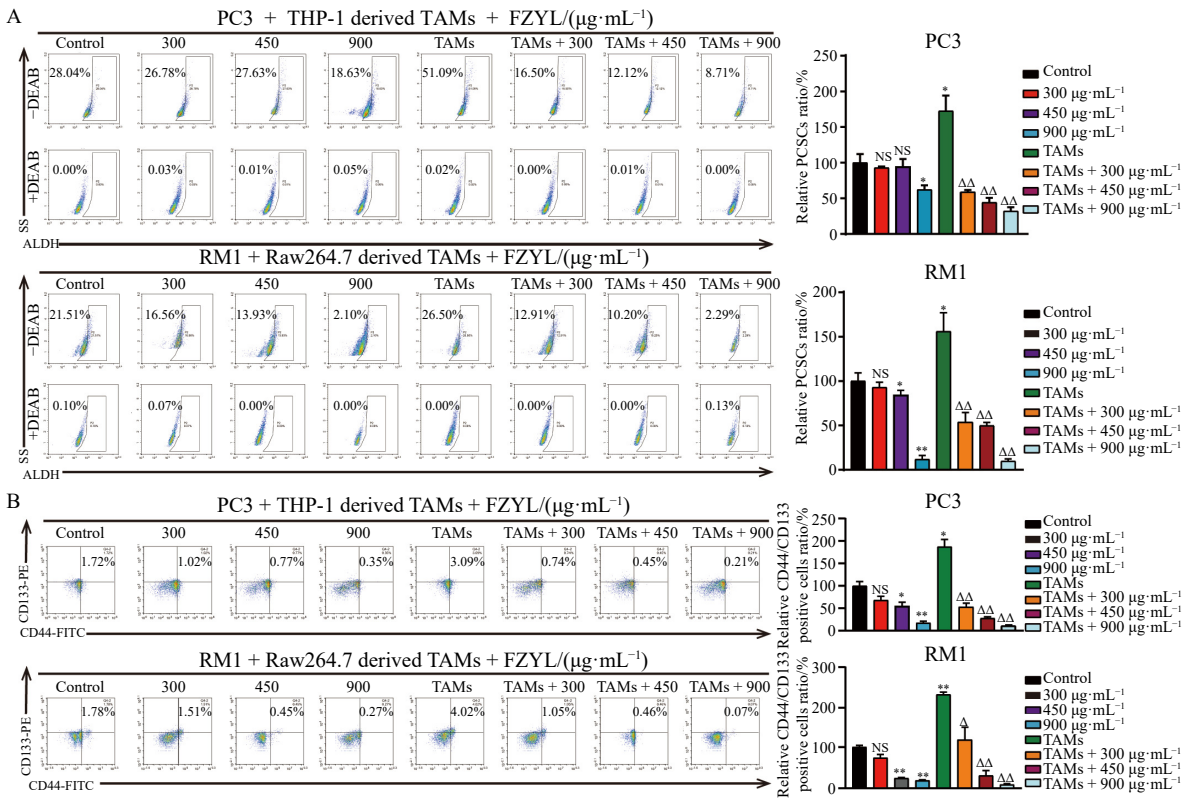
ies of PCa cells in the TAM co-culture system (Fig. 2F). In summary, the FZYL Formula significantly suppresses the proliferation and colony formation capabilities of PCa cells, even in the challenging context of TAM co-culture. This demonstrates the formula's potent inhibitory effects within a simulated TME.

*FZYL Formula suppresses the self-renewal activity of PCSCs induced by TAMs*

The $IC_{50}$ values informed the selection of FZYL Formula concentrations at 300, 450, and 900 μg·mL$^{-1}$ for mechanistic studies, aiming to achieve slight, moderate, or strong inhibitory effects on PCa cells, respectively. PCSCs are pivotal in the initiation and progression of PCa. Therefore, further investigations were conducted to examine the impact of the FZYL Formula on PCSCs. Aldehyde dehydrogenase (ALDH) activity, a specific biomarker for various cancer stem cells, including PCSCs, was significantly reduced by the FZYL Formula treatment [24, 25]. Concurrently, the treatment partly negated the enhancing effect of TAMs-conditioned medium (TAMs-CM) on ALDH$^+$ subpopulations in PCa cells ( Fig. 3A). Additionally, CD44 and CD133, specific surface markers of PCSCs, were examined. PCa cells overexpressing both CD44 and CD133 represent a rare subpopulation with heightened self-renewal capabilities [23]. The FZYL Formula significantly decreased the proportions of CD44$^+$/CD133$^+$ subpopulations, while TAMs-CM had an opposing effect, which was partially reversed by the FZYL Formula (Fig. 3B). The capacity of PCSCs to form non-adherent spherical clusters—a characteristic feature of stemness—was also evaluated. Treatment with the FZYL Formula alone reduced the number of spherical clusters [26], a result contrasted by the effect of TAMs-CM which increased cluster formation. Significantly, the FZYL Formula reversed the promoting effect of TAMs-CM on spherical cluster formation (Fig. 3C). Further analyses focused on β-catenin, a protein typically overexpressed in PCSCs and associated with enhanced stemness [23, 27]. TAMs-CM significantly increased both protein and mRNA levels of β-catenin in PC3 and RM1 cells, an effect that was abrogated by increasing concentrations of the FZYL Formula (Fig. 3D–3E and Appendix file: Figs. S2A–2B). Additionally, epithelial cell adhesion molecule (EpCAM) represents another crucial stemness-related biomarker and is usually used to identify progenitor cells and CSCs [28], was assessed. Western blotting analysis showed that TAMs-CM significantly elevated EpCAM expression, which was partially reversed by the FZYL Formula (Fig. 3F and Appendix file: Figs. S2C–2D). Collectively, these results demonstrate that the FZYL Formula effectively restrains the self-renewal of PCSCs, even in the challenging context of TAMs co-culture, supporting its potential utility in targeting the stem cell-like properties of PCa cells within a TME.

*FZYL Formula restrains TAMs-induced migration and invasion of PCa cells*

The influence of the FZYL Formula on the migration and invasion of PCa cells was further investigated using *in vitro* assays. Specifically, the wound-healing assay and the transwell invasion assay were employed to evaluate these cellular behaviors. Results indicated that the FZYL Formula at



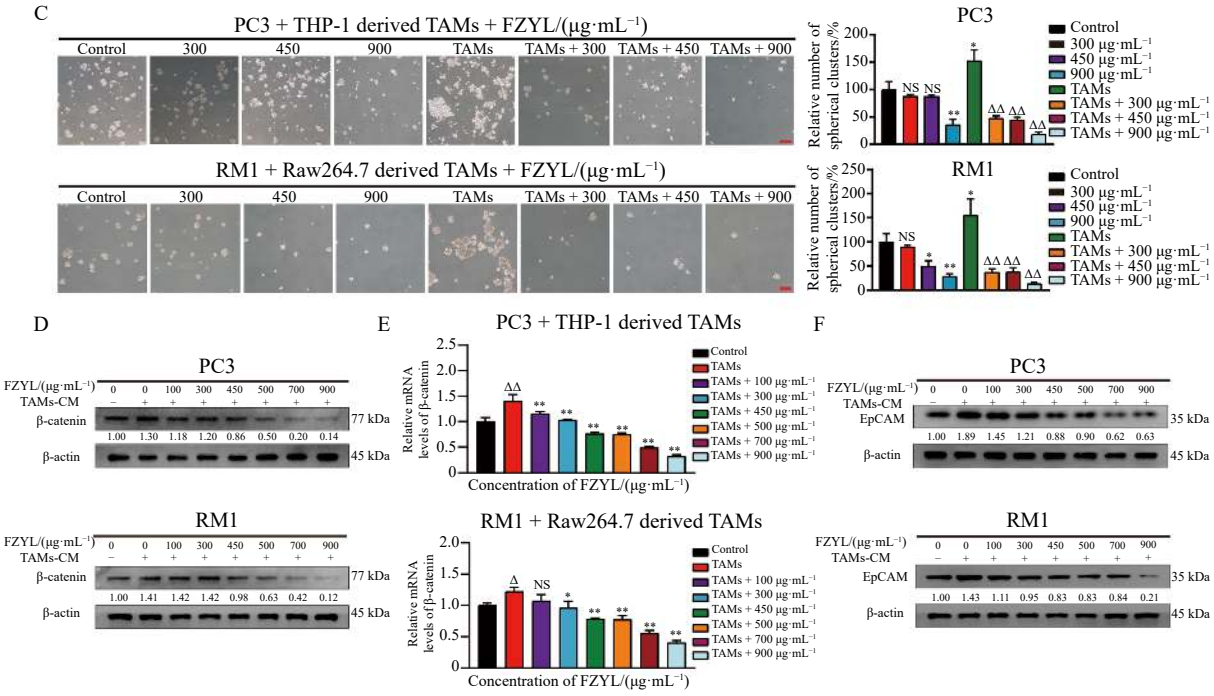



**Fig. 3 FZYL Formula suppresses the self-renewal activity of PCSCs induced by TAMs. (A)The effects of the FZYL Formula on the ALDH$^+$ PCSCs subpopulations in PCa cells cultured alone or co-cultured with TAMs. ALDH represents a biomarker for various CSCs, including PCSCs. DEAB is used to control for background fluorescence in the ALDH staining assay. (B) The effects of the FZYL Formula on the CD44$^+$/CD133$^+$ PCSCs subpopulations in PCa cells cultured alone or co-cultured with TAMs. CD44 and CD133 are the specific surface markers of PCSCs. (C) The FZYL Formula significantly decreases the number of spherical clusters in both PC3 cells and RM1 cells, even in the presence of TAMs co-culture. Scale bar = 50 μm. $^*P < 0.05$, $^{**}P < 0.01$ *vs* the control. $^{\triangle}< 0.05$, $^{\triangle\triangle}P < 0.01$ *vs* the TAMs. (D–E) TAMs-CM significantly elevates the protein (D) and mRNA levels (E) of β-catenin in PCa cells, which can be abrogated by increasing concentrations of the FZYL Formula. β-catenin is a stemness-related marker that is closely related to the elevated stemness of PCSCs. (F) TAMs-CM significantly elevates EpCAM expression in PCa cells, which can be abrogated by increasing concentrations of the FZYL Formula. EpCAM serves as a critical stemness-related marker for progenitor/CSC cells. $n = 3$, $^{\triangle}< 0.05$, $^{\triangle\triangle}P < 0.01$ *vs* the control, $^*P < 0.05$, $^{**}P < 0.01$ *vs* the TAMs.**

concentrations ranging from 300 to 900 μg·mL$^{-1}$ markedly inhibited both the migration and invasion of PCa cells (Figs. 4A–4B). Interestingly, while the TAMs-CM significantly enhanced these processes, the FZYL Formula effectively counteracted this induction, strongly inhibiting the enhanced migration and invasion stimulated by TAMs-CM. Epithelial-mesenchymal transition (EMT) is a critical developmental process that facilitates cell migration and invasion [29]. The effect of the FZYL Formula on EMT was therefore examined, particularly its impact on EMT-related protein expression in PCa cells. The findings, illustrated in Fig. 4C and Appendix file: Fig. S3, showed that FZYL Formula treatment significantly reversed the changes induced by TAMs-CM. This reversal was characterized by an increased expression of E-cadherin, a marker of epithelial phenotype, and a decreased expression of vimentin, a marker typically elevated during mesenchymal transition. In summary, the FZYL Formula effectively suppresses the migration and invasion of PCa cells, even in the presence of pro-migratory and pro-invasive cues provided by TAMs.

*FZYL Formula suppresses the M2 phenotype polarization, CCL5 expression, and secretion of TAMs*

The FZYL Formula has shown remarkable effects in abolishing the induction effect of TAMs-CM on the self-renewal of PCSCs and the migration and invasion of PCa cells. This led to the hypothesis that the FZYL Formula could modulate the activity of TAMs, thereby restraining PCSCs self-renewal and PCa invasion. Initial investigations revealed that treatment with the FZYL formula (100 to 900 μg·mL$^{-1}$) for 48 h exhibited no cytotoxic effects on TAMs derived from THP-1 and Raw264.7 cells, suggesting that the formula's modulatory effects on TAMs do not result from cytotoxicity but potentially through other mechanisms (Fig. 5A). Further analysis focused on the phenotypic polarization of TAMs. F4/80, a specific marker of monocytes/macrophages, along with M2 phenotype markers such as CD163 and CD206, were assessed. The FZYL Formula treatment significantly reduced the F4/80$^+$/CD163$^+$ subpopulation in human THP-1 derived macrophages and the F4/80$^+$/CD206$^+$ subpopulation in mouse Raw264.7 macrophages, indicating a reversal of M2 phenotype polarization in a concentration-dependent manner [30](Figs. 5B–5D). Exploring deeper into the molecular mechanisms, the role of soluble mediators in the transwell co-culture system, which allows only the free exchange of soluble molecules, was considered. It was hypothesized that the FZYL Formula might downregulate pro-tumorigenic cy-

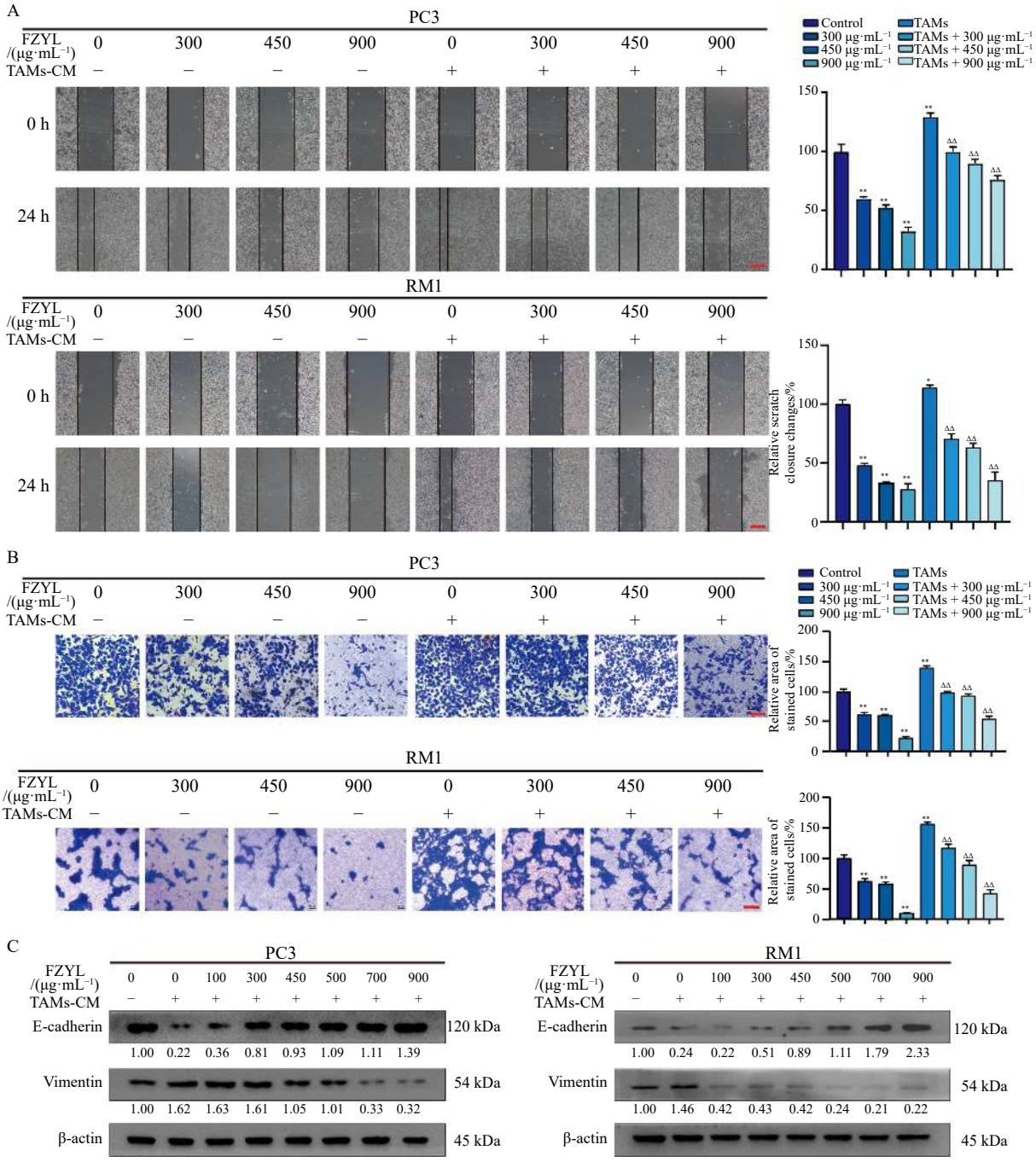



**Fig. 4 FZYL Formula restrains TAMs-induced migration and invasion of PCa cells. (A) The wound-healing assay is performed to investigate the effect of the FZYL Formula (300–900 μg·mL$^{-1}$) on the migration of PC3 and RM1 cells cultured alone or co-cultured with TAMs. 300 μg·mL$^{-1}$ FZYL Formula is a low-toxic concentration for PCa cells. Scale bar = 50 μm. (B) The transwell assay is conducted to determine the influence of the FZYL formula (300–900 μg·mL$^{-1}$) on the invasion of PC3 and RM1 cells cultured alone or co-cultured with TAMs. Scale bar = 50 μm. (C) Western blotting assay was performed to investigate the effect of the FZYL Formula (100–900 μg·mL$^{-1}$) on the EMT activities of PCa cells cultured alone or co-cultured with TAMs. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the control. $^{\triangle\triangle}P < 0.01$ *vs* the TAMs.**

tokines secreted by TAMs, thereby reducing their induction effect on PCSCs self-renewal and PCa invasion. CCL5, previously identified as one of the most abundantly secreted chemokines by TAMs that promotes PCSCs self-renewal and PCa invasion [23, 31], was a focus of this examination. The results confirmed that the FZYL Formula significantly restrained CCL5 secretion from TAMs in a concentration-dependent manner (Fig. 5E). Additionally, the formula also dramatically reduced CCL5 mRNA transcription and protein expression levels in TAMs (Figs. 5F–5G and Appendix file:

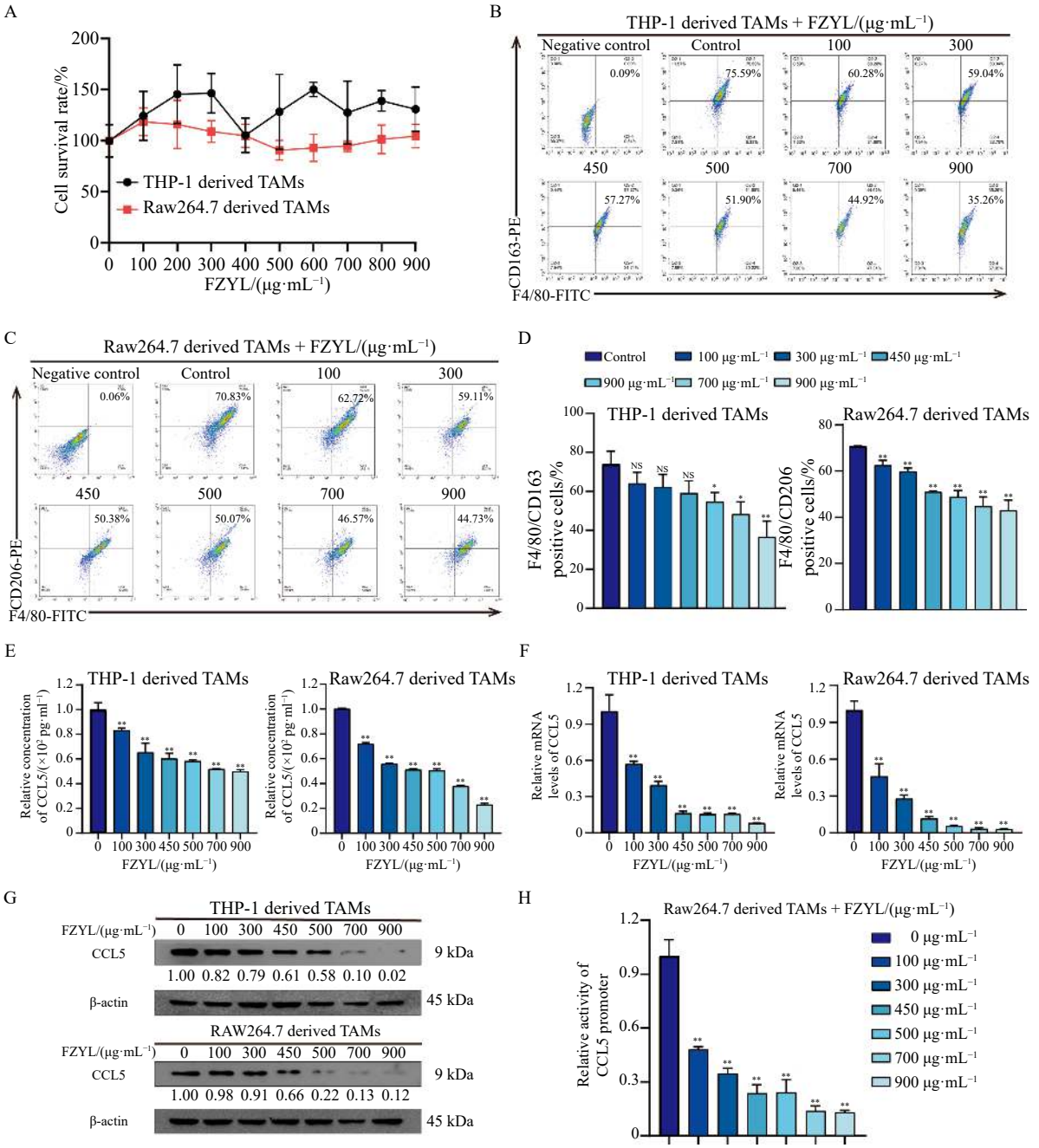



**Fig. 5 FZYL Formula suppresses the M2 phenotype polarization, CCL5 expression, and secretion of TAMs. (A) CCK8 assay demonstrates that the FZYL Formula exhibits little cytotoxicity in two kinds of TAMs. (B–D) FZYL Formula treatment for 48 h dramatically reverses the M2 phenotype polarization of TAMs. F4/80 is a specific surface marker of macrophages. CD163 and CD206 are specific surface markers of M2 phenotype macrophages. (E) FZYL Formula treatment for 48 h remarkably decreased CCL5 secretion from TAMs. (F–G) QPCR and Western blotting results demonstrate that the FZYL Formula treatment for 48 h can dramatically suppress *CCL5* mRNA transcription (F) and protein expression levels (G) in TAMs. (H) FZYL Formula treatment for 48 h suppresses the promoter activity of the *CCL5* gene in Raw264.7 derived TAMs. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the control (0 μg·mL$^{-1}$ FZYL).**

Fig. S4). Further analysis indicated that the FZYL Formula strongly inhibited the promoter activity of the CCL5 gene in TAMs (Fig. 5H). In summary, the FZYL Formula suppresses the pro-tumorigenic functions of TAMs by reversing their M2 phenotype polarization and significantly reducing the expression and secretion of the CCL5 chemokine.

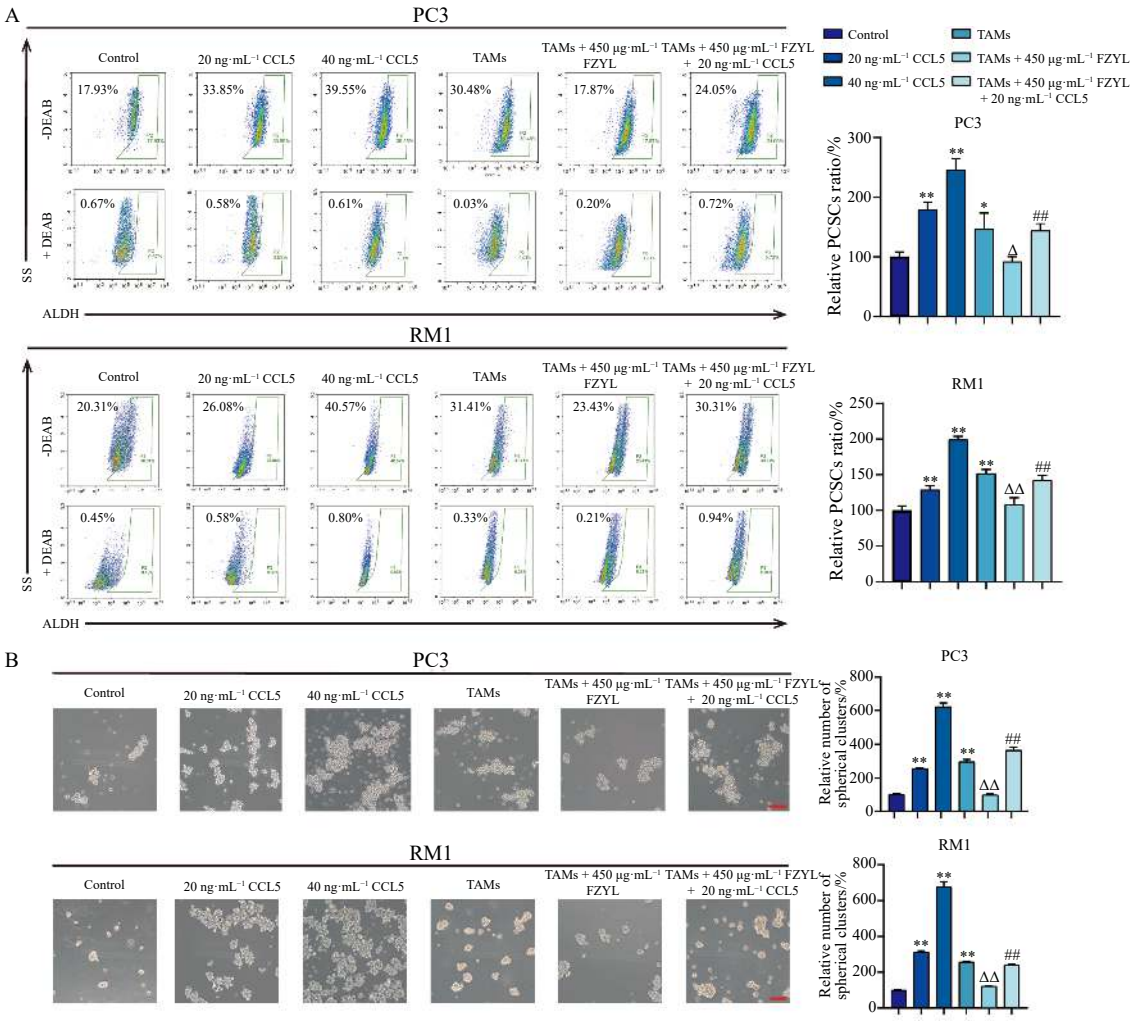
### *FZYL Formula suppresses PCSCs by modulating TAMs/CCL5 signaling*

Given the significant reduction in CCL5 secretion from TAMs by the FZYL Formula, further investigation focused on whether CCL5 is a critical mediator in the FZYL Formula's suppression of PCSCs self-renewal. Experiments re-

vealed that both exogenous CCL5 addition and TAMs-CM significantly increased the ALDH+ subpopulations and the number of spherical clusters in PCa cells, demonstrating CCL5's role in promoting PCSC characteristics (Figs. 6A–6B). Furthermore, the addition of CCL5 partly reversed the suppressive effects of the FZYL Formula on these processes in co-cultured PCa cells, suggesting that CCL5 can counteract the FZYL Formula's inhibitory impact on TAMs-CM-induced self-renewal of PCSCs. Further molecular analyses supported these findings. Quantitative PCR (QPCR) assays and Western blotting showed that CCL5 administration up-regulated the transcription and protein expression levels of β-catenin in PCa cells, a key regulator of stemness, while also reversing the suppressive effects of the FZYL Formula on these parameters in the co-cultured PCa cells (Figs. 6C–6D and Appendix file: Fig. S5). Additionally, both exogenous CCL5 addition and TAMs-CM markedly enhanced the migration and invasion capabilities of PCa cells. Importantly, the presence of CCL5 also partly negated the suppressive effects of the FZYL Formula on these processes. This observation underscores that CCL5 not only influences PCSC self-renewal but also promotes the invasive and migratory behaviors of PCa cells and can mitigate the inhibitory impacts of the FZYL Formula on TAMs-CM-induced changes. To sum up, these results strongly suggest that the FZYL Formula exerts its inhibitory effects on both the self-renewal of PCSCs and the migration and invasion of PCa cells within a TAMs co-culture system by restraining TAMs/CCL5 activity.

*FZYL Formula inhibits prostate tumor growth and bone metastasis in vivo by restraining TAMs/CCL5 pathway*

To validate the suppressive effects of the FZYL Formula on PCa growth and the TAMs/CCL5 pathway *in vivo*, a mouse RM1 xenograft model was utilized. Mice were administered the FZYL Formula orally at dosages of 0.5 to 1.0 $g \cdot kg^{-1} \cdot d^{-1}$, which effectively delayed the growth of RM1 xenografts. In contrast, co-injection with TAMs notably accelerated tumor growth. However, FZYL Formula administration partially negated the enhancement effect of TAMs on RM1 xenograft growth. This reversal was partially mitigated when CCL5 was overexpressed in the co-injected TAMs, suggesting the critical role of the TAMs/CCL5 pathway in this context (Figs. 7A–7B). These findings underscore that the FZYL Formula can inhibit the growth of PCa xenografts by targeting the TAMs/CCL5 activity within TME. Import-



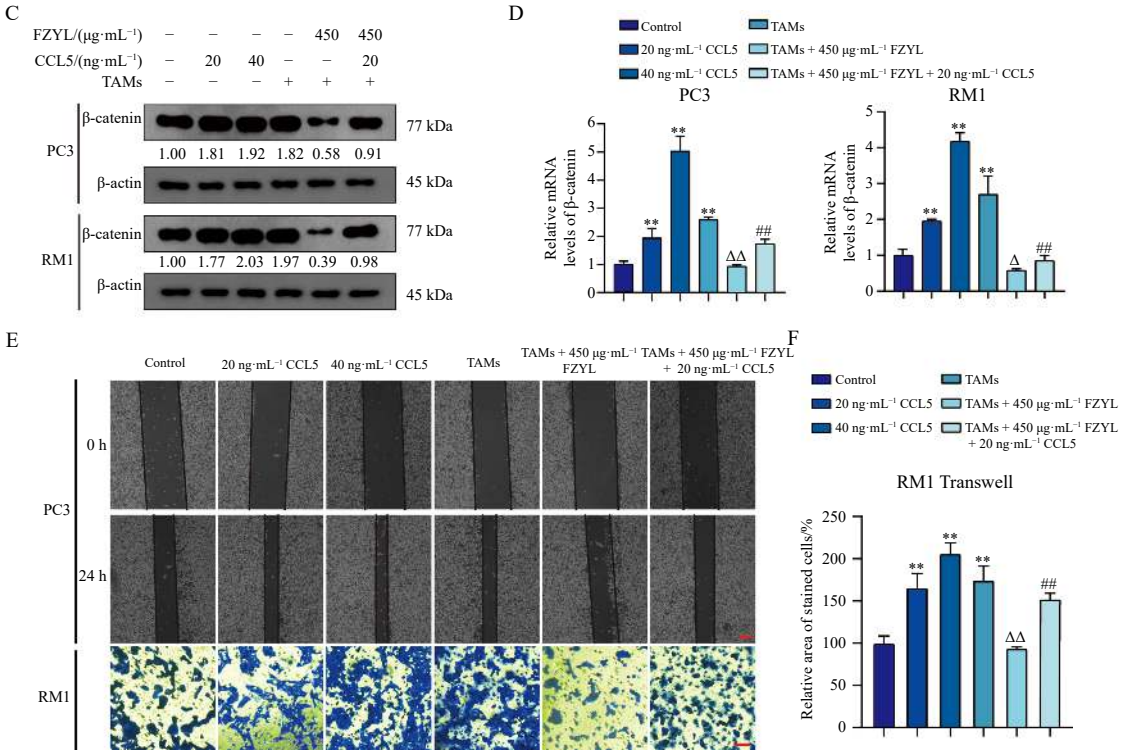



**Fig. 6 FZYL Formula suppresses PCSCs by modulating TAMs/CCL5 signaling. (A–B) CCL5 addition partly abolishes the inhibitory effect of the FZYL Formula on TAMs-CM-induced self-renewal of PCSCs in both PC3 and RM1 cells. The ratio of ALDH$^+$ subpopulations (A) and the number of spherical clusters (B) in PCa cells are detected by PCSC population analysis and sphere-formation assay, respectively. Scale bar = 50 μm. (C–D) CCL5 administration can induce the mRNA transcription (C) and protein expression levels (D) of β-catenin in PCa cells while abolishing the suppressive effect of the FZYL Formula on that in the co-cultured PCa cells. $n$ = 3. (E–F) CCL5 administration can promote the effect of migration (E–F) and enhance the ability of invasion (F) in PCa cells, while abolishing the suppressive effect of FZYL Formula on that in the co-cultured PCa cells. Scale bar = 50 μm. $^*P < 0.05$, $^{**}P < 0.01$ *vs* the control. $^{\triangle\triangle}P < 0.01$ *vs* the TAMs. $^{\#\#}P < 0.01$ *vs* the TAMs + 450 μg·mL$^{-1}$ FZYL.**

antly, there were no FZYL Formula-related mortalities or significant weight losses observed in the mice during the treatment period, indicating minimal toxicity or adverse effects (Fig. 7C). Additionally, both X-ray imaging and HE staining assays demonstrated that the FZYL Formula markedly suppressed the bone metastasis of RM1 xenografts. Co-injection with TAMs significantly induced bone metastasis, but again, the FZYL Formula partially counteracted this effect. This suppression was lessened when CCL5 was overexpressed in the co-injected TAMs, highlighting the formula's efficacy in mitigating TAMs/CCL5-mediated bone metastasis (Fig. 7D). Further, the TUNEL assay indicated significant induction of apoptosis in prostate tumor tissues treated with the FZYL Formula, emphasizing its pro-apoptotic capabilities (Fig. 7E). Moreover, tissue IHC assay showed that FZYL Formula administration remarkably elevated E-cadherin expression but decreased the expression levels of stemness-related proteins (β-catenin and ALDH1A1) and metastasis-related proteins (MMP2 and vimentin) by modulating TAMs/CCL5 activity (Fig. 7F). Finally, tissue IF assays confirmed that the FZYL Formula significantly restrained TAMs/CCL5 activity *in vivo*, even in the presence of TAMs co-injection, reinforcing its therapeutic potential (Fig. 7G). In conclusion, the FZYL Formula demonstrates robust capability to suppress prostate tumor growth, bone metastasis, and PCSCs activity through the inhibition of the TAMs/CCL5 pathway *in vivo*.

## Discussion

Recent advancements in cancer immunotherapy have significantly reshaped the understanding of the immunological interplay between cancer cells and their host [32]. Despite these breakthroughs, PCa remains an immunologically "cold" malignancy, characterized by an immunosuppressive TME. Common immunomodulatory strategies, including chimeric antigen receptor T (CAR-T) cell therapy and immune checkpoint inhibitors, have shown limited clinical success in treating PCa [33]. This underscores the unique challenges posed by the immunosuppressive nature of PCa's TME. TCM offers a promising alternative by targeting and remodeling the immunosuppressive TME, a key mechanism and advantage in cancer treatment. TCM can enhance anti-tumor immune responses within the TME through various pathways. These include remodeling the immunosuppressive phenotypes of regulatory T cells (Tregs) and TAMs, enhancing the maturation and antigen-presentation function of dendritic cells, inhibiting the function of myeloid-derived suppressor cells (MDSCs), and modulating the Th1/Th2 balance [9]. Furthermore, TCM can also influence the interaction between immune cells

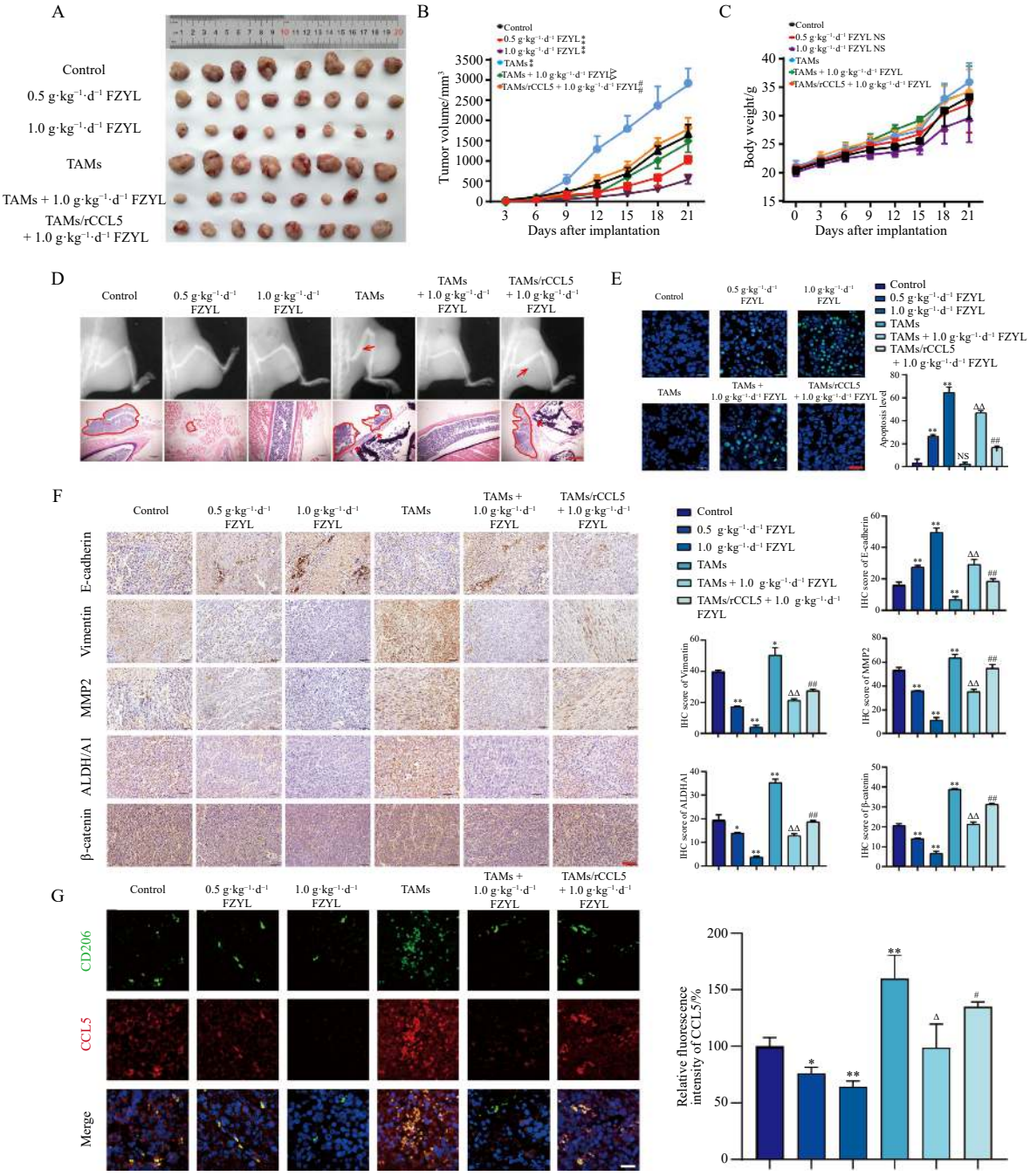



**Fig. 7 FZYL Formula restrains prostate tumor growth and bone metastasis *in vivo* by suppressing the TAMs/CCL5 pathway. (A–B) The FZYL Formula can delay prostate tumor growth by inhibiting TAMs/CCL5 activity. Mice bearing RM1 xenografts receive either saline or FZYL (0.5–1.0 g·kg$^{-1}$·day$^{-1}$) treatment by oral gavage. *n* = 8. (C) The weight changes of mice in various groups. *n* = 8. (D) X-ray imaging assay and HE staining assay suggest that the FZYL Formula significantly suppressed the bone metastasis of RM1 xenografts by down-regulating the TAMs/CCL5 activity. (E) TUNEL assay indicates that FZYL Formula can significantly induce apoptosis in prostate tumor tissues. *n* = 3. Scale bar = 20 μm. (F) The protein expression levels of E-cadherin, vimentin, MMP2, ALDH1A1, and β-catenin in RM1 xenografts are detected by IHC assay. *n* = 3. Scale bar = 50 μm. (G) Tissue IF assay suggests that FZYL Formula could significantly restrain TAMs/CCL5 activity *in vivo*. *n* = 3. Scale bar = 20 μm. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the control. $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ *vs* the TAMs. $^{\#\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* the TAMs + 1.0 g·kg$^{-1}$·d$^{-1}$ FZYL.**

and cancer cells, improving the efficacy of therapies like PD-1 inhibitors by regulating the immune microenvironment [34]. Emerging evidence supports the effectiveness of TCM in managing PCa, particularly in cases of metastatic disease, where conventional treatments often fall short [35]. The FZYL formula, with over 20 years of clinical use in PCa treatment, has demonstrated efficacy in delaying disease progression and extending patient survival [2]. Despite its longstanding

use, the specific mechanisms of action of the FZYL formula remain largely unexplored. This study has elucidated one such mechanism, revealing that the FZYL formula can significantly inhibit PCSCs self-renewal and PCa bone metastasis by targeting the TAMs/CCL5 pathway within the TME. These findings provide valuable experimental support for the clinical application of the FZYL formula in PCa treatment and highlight its potential as a complementary approach in oncology. However, further preclinical studies are necessary to decode the material basis of the FZYL Formula's effects on the TAMs/CCL5 pathway, enhancing our understanding and application of this traditional therapy in modern oncological practice.

CSCs are widely recognized as pivotal in tumor initiation, metastasis, recurrence, and resistance to chemotherapy. Despite the identification and examination of numerous CSC biomarkers and pathways *in vitro*, direct targeting strategies often fail to translate into effective anti-tumor activities *in vivo*, likely due to the complexity and heterogeneity of the TME [6]. CSCs typically reside within a specialized stem cell niche that not only supports their self-renewal and maintenance of stemness but also promotes the formation of an immunosuppressive TME that shields them from immune destruction [36, 37]. This interaction between CSCs and infiltrated immune cells and the mechanisms underlying these interactions present new avenues for cancer treatment [38]. Therefore, a better elucidation of the interaction between CSCs and immune cells, as well as the underlying mechanisms, may provide novel opportunities for cancer treatment [38]. The dialogue between immune cells and CSCs is a promising area for developing novel cancer treatment strategies. Within the CSC niche, immunosuppressive subpopulations such as TAMs, regulatory Tregs, and MDSCs are crucial in maintaining CSCs. For instance, TGF-β from Tregs has been shown to promote glioma growth by facilitating the self-renewal of glioma stem cells (GSCs) [39]. Similarly, prostaglandin E2 (PGE2) secreted by MDSCs has been found to accelerate ovarian cancer growth by boosting the self-renewal capabilities of ovarian cancer stem cells [40]. TAMs, which comprise about 30% to 50% of the tumor mass, are particularly significant due to their abundance and phenotypic plasticity within the TME [41]. Recent studies have indicated that CSCs can modify their niche by attracting monocytes and converting them into TAMs, which, in turn, support CSC self-renewal through the secretion of chemokines and cytokines [42, 43]. Notably, molecules like TNF-α [44] and S100 calcium-binding protein A9 (S100A9) [45] secreted by TAMs have been reported to enhance hepatocellular carcinoma growth by expanding liver CSC populations. Additionally, TAMs play a critical role in protecting CSCs from immune surveillance across various cancer types [46, 47]. However, the specific interactions between TAMs and PCSC remain underexplored. Our findings reveal that CCL5 derived from TAMs can induce PCSCs self-renewal and promote PCa bone metastasis through the activation of β-catenin signaling, positioning TAMs/CCL5 as a viable target for PCa treatment. The FZYL formula has demonstrated the ability to inhibit PCSCs self-renewal and PCa bone metastasis by suppressing TAMs/CCL5 activity within the TME, substantiating the potential of targeting TAMs/CCL5 as a therapeutic strategy for eradicating PCSCs. Given the intricacy of the immunosuppressive elements within the TME, further investigation into the effects of the FZYL Formula on other immune cells and chemokines is essential. This will provide a more comprehensive understanding of the formula's broader regulatory impacts within the TME, enhancing its therapeutic potential against PCa and possibly other malignancies.

Chemokines are a diverse group of small chemotactic cytokines, typically ranging from 7 to 14 kDa in molecular weight, which primarily function as chemoattractants to regulate cell trafficking [30]. More than 50 types of chemokines have been identified and classified into four main subgroups—CC, CXC, CX3C, and C —based on the arrangement of their conserved cysteine residues [48, 49]. Among these, CCL5 is notably secreted in abundance from TAMs rather than directly from PCa cells [20]. Previous research has shown that CCL5 expression is significantly elevated in tumor tissues of PCa patients compared to adjacent non-tumor tissues, with high levels of CCL5 often predicting poor prognosis and increased metastasis in PCa patients [23]. From a molecular standpoint, CCL5 derived from TAMs has been implicated in accelerating PCa growth and bone metastasis by promoting the self-renewal of PCSCs through the activation of the β-catenin/STAT3 signaling pathway [23]. Additionally, CCL5 stimulation enhances the invasion and metastasis of PCa cells by activating the ERK and Rac pathways and by promoting the secretion of matrix metalloproteinases (MMPs) 2 and 9 [30]. CCL5 has also been linked to promoting PCa metastasis by inducing autophagy [50]. Given these multifaceted roles, blocking CCL5 signaling emerges as a potentially effective strategy for treating PCa. Currently, all clinically available drugs that target CCL5 signaling, such as maraviroc, cenicriviroc, anibamine, and DT-13, are antagonists of CCR5, the receptor for CCL5 [51]. The clinical efficacy of these drugs in treating PCa remains to be conclusively determined through clinical trials. In this study, we demonstrated that the FZYL formula can transcriptionally reduce CCL5 expression and secretion from TAMs, thereby mitigating the pro-tumorigenic effects of CCL5 on PCSCs self-renewal. This finding introduces a novel TCM approach to inhibiting CCL5 signaling in cancer therapy. Given the complexity of the TME and the pivotal role of CCL5 in modulating immune and cancer cell interactions, our results support the need for further in-depth investigations to validate and optimize this TCM-based strategy for broader clinical application in PCa treatment.

## Conclusion

In conclusion, the FZYL formula has demonstrated its efficacy in inhibiting PCSC self-renewal and PCa bone metastasis through the suppression of TAMs/CCL5 activity with-

in the TME. This study not only elucidates the immunomodulatory mechanism of the FZYL formula in the treatment of PCa but also highlights the TAMs/CCL5 axis as a promising therapeutic target for the elimination of PCSCs and the broader treatment of PCa.

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

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