Inhibition of let-7b-5p contributes to anti-tumorigenic macrophage phenotype through SOCS1/STAT pathway in prostate cancer

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Abstract

Background

Dysfunction of microRNAs (miRNAs) is a major cause of aberrant expression of inflammatory cytokines and contributes to macrophage polarization. Previous studies have shown that miRNA let-7 is associated with cell differentiation, and the expression of let-7b-5p is significantly increased in M2 macrophages. However, the mechanism by which let-7b-5p regulates macrophage differentiation in prostate cancer (PCa) remains largely unknown.

Methods

Human macrophages were induced by the blood monocytes from healthy male donors. M1 macrophages were polarized by stimulating overnight with 100 ng/ml lipopolysaccharides and 100 ng/ml IFN-γ, while conditioned medium from PC-3 cells were used to induce prostatic macrophages (M-CMs) in vitro, and then let-7b-5p mimics or inhibitors were transfected into M1 and M-CMs for 72 hours respectively. The expression of cluster of differentiation 206 (CD206) in each group was detected by a High Throughput Connotation of Imaging System. Quantitative real-time polymerase chain reaction (qRT-PCR) was executed to examine the expression of inflammatory cytokines IL-10, IL-12, IL-13, TNF-alpha and let-7b in macrophages. SOCS1 protein level was evaluated by ELISA, and the phosphorylation difference of STAT family member proteins was analysized by CST signal pathway chip. Phagocytosis of macrophages and the effect of macrophages on the proliferation of prostate cancer cell PC-3 were evaluated by phagocytosis assay or Cell Counting Kit-8 (CCK-8). The relationship between SOCS1 and let-7b-5p was confirmed with the dual-luciferase reporter.

Results

The expression of cluster of differentiation 206 (CD206), an M2-like macrophage surface molecule, was significantly increased in M1 macrophages treated with let-7b-5p mimics, while CD206 expression was decreased in M-CMs treated with let-7b-5p inhibitors. Overexpression or knockdown of let-7b-5p significantly affected the expression of inflammatory factors in macrophages including interleukin 10 (IL-10), IL-12, IL-13, and tumor necrosis factor alpha. Let-7b-5p downregulated the expression of suppressor of cytokine signaling 1 (SOCS1) and increased the phosphorylation of signal transducer and activator of transcription 1 (STAT1), STAT3, and STAT5a proteins in M-CMs and M1 macrophages with let-7b-5p mimics compared with the other groups. In addition, with high expression of let-7b-5p, the phagocytosis of macrophages showed a significant decrease. As a result, M-CMs treated with let-7b-5p inhibitors reduced the proliferation of PC-3 PCa cells.

Conclusions

Collectively, these data indicate that let-7b-5p may regulate M2 polarization through the SOCS1/STAT pathway, and reversal of M2 differentiation by let-7b-5p inhibitors can enhance macrophage phagocytosis, ultimately inhibiting the proliferation of PCa cells.

Trial registration

Not applicable

1. Background

Prostate cancer (PCa) is the second most common cause of cancer-related death in men in the western world[1,2]. Recent advances have revealed that the tumor microenvironment (TME) is an important determinant of tumor behavior, and cancer cells are confronted with various types of stromal and immune cells across all stages of disease progression. Increasing evidence has indicated that the “cross-talk” between tumor and stromal cells can modify cellular compartments, leading to the co-evolution of tumor cells and the TME. Macrophages play a prominent active role in the TME, infiltrating tumors and actively contributing to the initiation and progression of cancer. To date, there have been many reports that macrophages infiltrating tumors have proangiogenic activity and are generally associated with high vascular density[3], and increased infiltration of tumor-associated macrophages (TAMs) has been associated with worse pathological characteristics and poor prognosis in breast, colon, and bladder cancer[4,5]. Gollapudi[6] demonstrated that the number of TAMs was highest in PCa, followed by prostatic intraepithelial neoplasia and benign tumor tissue, suggesting the potential role of TAMs in PCa development[6]. For example, the number of macrophages infiltrating PCa is reportedly associated with Gleason degree and is also a useful predictive factor of PCa progression after hormonal therapy[7,8].

Macrophages are classified into classically activated M1-like or alternatively activated M2-like macrophages based on their functional status induced by the microenvironment. The majority of evidence indicates that TAMs within the primary TME are considered to be similar to M2 macrophages and play a tumor-promoting role that inhibits inflammation and promotes tumor invasion and metastasis[9]. Moreover, macrophages account for most of the infiltrating immune cells in the TME, comprising up to 50% of the tumor mass[10].

A recent study revealed that the distribution and function of TAMs differ considerably in different microregions of the neoplastic tissue, and macrophages have a mixed phenotype expressing both M1- and M2-like markers[11]. Different signals from particular components of the TME seem to influence the activation of TAMs, and many inflammatory signaling pathways reportedly play a role in macrophage polarization; however, how these signals stop or decay is unknown. Suppressor of cytokine signaling (SOCS) proteins are negative feedback regulators of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) or receptor tyrosine kinase pathways[12], which mediate cytokine-induced immunological responses. It has been demonstrated that abnormal expression of SOCS is involved in the occurrence and progression of human cancers. For example, SOCS1 exerts growth-inhibitory functions through the downregulation of cyclins and cyclin-dependent kinases in PCa. They also act as regulators of innate and acquired immunity, negatively regulating the state of macrophages and dendritic cells (DCs)[13].

MicroRNAs (miRNAs) are master epigenetic regulators that are involved in the initiation and progression of various tumors[14,15]. In immune cells infiltrating the tumor, miRNAs can exert effects on cell function and phenotype and then enhance or suppress anti-tumor immunity by mediating some immune regulatory molecules[16]. The let-7 miRNA family regulates developmental timing and cell proliferation, mediates immune responses, and adjusts inflammation[17,18]. It has been identified as regulators of immune escape by directly or indirectly modulating the expression of immune-regulating molecules, especially important cytokines such as interleukin 6 (IL-6), IL-10, and tumor necrosis factor alpha (TNF-α)[19]. However, the relationship between let-7 and SOCS/STAT in macrophage differentiation remains unclear.

Our previous results indicated that conditioned media from PC-3 PCa cells can induce macrophages to differentiate into M2 macrophages, a process that is significantly associated with the high expression of let-7[20]. Therefore, we focused on let-7b-5p targeting SOCS1/STAT in macrophages and its role in the progression of PCa. Our results showed that let-7b-5p has the capacity to promote macrophage differentiation to M2 by regulating the SOCS1/STAT pathway, and reversal of M-CMs by let-7b-5p inhibitors leads to significantly increased phagocytosis and suppresses the proliferation of PCa cells.

2. Results

2.1 Let-7b-5p promotes expression of cluster of differentiation 206 in macrophages

Cluster of differentiation 206 (CD206) is a mannose receptor on the surface of macrophages and a specific marker for M2 macrophages. Our previous study indicated that let-7b-5p was significantly increased in M2 macrophages, but the relationship between CD206 and let-7b-5p in macrophages has not been evaluated. To address this issue, first, we obtained M0, M1, and M-CMs from human blood monocytes (isolated from healthy male donors) as described in the Materials and Methods, and then transfected let-7b-5p mimics or inhibitors in M1 macrophages or M-CMs, respectively. We confirmed that these reagents could effectively regulate let-7b level (Fig. 1A). Subsequently, we investigated CD206 expression in the macrophages using the High-Throughput Connotation of Imaging System. Our results showed that the expression of CD206 in M-CMs (11.4%) treated with let-7b-5p inhibitors was almost the same as that in M1 macrophages (14.3%), whereas CD206 level in M1 treated with let-7b-5p mimics (80.2%) was similar to that of M-CMs (78.3%) (Fig. 1B). These data indicate that let-7b-5p promotes CD206 expression in macrophages.

2.2 Let-7b-5p regulates the expression of inflammatory cytokines in macrophages

Macrophage subtype switch is thought to involve the secretion of inflammatory cytokines. M1 macrophages are characterized by an IL-1R^{high} and IL-12^{high} phenotype and their capacity to present antigen, but M2 macrophages are characterized by an IL-10^{high} and IL-12^{low} phenotype. To further investigate the role of let-7b-5p in macrophage polarization, let-7b mimics or inhibitors were transfected in M1 and M-CMs, respectively. After 72 h, we analyzed the expression of inflammatory cytokines

including IL-12, IL-13, IL-10, and TNF-α. Our results revealed that after M-CMs were treated with let-7b-5p inhibitors, the expression of TNF-α, IL-10, and IL-13 was significantly decreased (** P < 0.01), similar to M1, whereas IL-12 was significantly upregulated. Meanwhile, in the presence of let-7 mimics, the levels of these cytokines in M1 displayed the opposite results (Fig. 2). These data indicate that let-7b-5p modulates the expression of IL-12, IL-13, IL-10, and TNF-α in macrophage polarization.

2.3 Let-7b-5p directly targets SOCS1 3'-untranslated region

SOCS1 is considered a negative regulator of JAK/STAT or receptor tyrosine kinase pathways involved in cytokine-induced immunological responses. According to the predicted results of TargetScan, SOCS1 was considered the target of let-7b-5p. To demonstrate the relationship between let-7b-5p and SOCS1, first, we detected SOCS1 expression in macrophages. Enzyme-linked immunoassay (ELISA) results showed decreased SOCS1 expression in M1 treated with let-7b-5p mimics, whereas the reverse results occurred in M-CMs treated with let-7b-5p-inhibitors (Fig. 3A; P<0.05). Next, the dual-luciferase reporter assay was used to evaluate whether SOCS1 is a direct target gene of let-7b-5p. Our data demonstrated that let-7b-5p markedly inhibited the activity of firefly luciferase in the wild-type (WT) group compared with miR-negative control (NC), while this inhibitory effect was abolished among cells in the mutant (MUT) group (Fig. 3B, C; P<0.05). These data suggest that let-7b-5p directly targets and regulates SOCS1 expression in macrophages.

2.4 Let-7b-5p activates the STAT signaling pathway

The STAT family is closely related to the secretion of a large number of cytokines involved in inflammation and the immune response. To explore the mechanism underlying let-7b-5p regulation of macrophage polarization, we further analyzed the effects of let-7b-5p on the STAT signaling pathway. After the transfection of M1 and M-CMs with let-7b-5p mimics or inhibitors, respectively, the CST chip was used to observe the changes of some signaling molecules. Our results revealed that let-7b-5p promoted the phosphorylation of STAT1, STAT3, and STAT5A proteins in macrophages (Fig. 4). In M1 transfected with let-7b-5p mimics, the expression of phosphorylated STAT1 (p-STAT1), p-STAT5a, and p-STAT3 was significantly increased (**P<0.01), similar to the M-CM group, whereas the expression of p-STAT1, p-STAT3, and p-STAT5a was significantly decreased in M-CMs transfected with let-7b-5p inhibitors (**P<0.01), similar to that in the M1 group. These results indicate that let-7b-5p activates the STAT signaling pathway.

2.5 Let-7b-5p reduces the phagocytosis of macrophages

Phagocytosis, antigen processing, and presentation of macrophages are an important part of immune surveillance. M2 macrophages or TAMs with M2-like properties display pro-tumor functions by virtue of their poor function in the phagocytosis of dead cells, matrix, cell debris, and pathogens. To address the link between let-7b-5p and macrophage phagocytosis, the phagocytosis test was used to investigate the phagocytosis of macrophage subtypes. The results showed that the ability to phagocytize *Staphylococcus aureus* was significantly decreased in M-CMs expressing high levels of let-7b-5p and in

M1 treated with let-7b-5p mimics (*P<0.01), whereas their ability was significantly increased in M-CMs treated with let-7b-5p inhibitors and M1 (see Fig. 5). These results suggest that let-7b-5p can affect the phagocytosis of macrophages.

2.6 Reversal of M-CMs by let-7b-5p inhibitorssuppresses the proliferation of PCa cells

Macrophages express fairly large amounts of cytokines involved in inhibiting the proliferation of various cancer cells. We further determined if let-7b-5p is involved in pro-tumor functions in macrophages. To this end, we incubated human PCa cells with conditioned media (CM) from macrophages treated with let-7b-5p inhibitors or mimics or negative control. As shown in Figure 6, CM from M1 plus let-7b-5p mimics had more stimulatory effects on PC-3 cells than those from M1. In addition, CM from M-CMs treated with let-7b-5p inhibitors led to the significant suppression of PC-3 cell proliferation compared to that from M-CMs. These results suggest that macrophage differentiation modulated by let-7b-5p plays a critical role in PCa proliferation.

3. Discussion

The mutual shift of M1/M2 is one of the hotspots in the study of the TME. Macrophages can infiltrate tumors and their adjacent normal tissues and become TAMs, which have a phenotype similar to M2[21] including inhibiting inflammation and promoting tissue remodeling and repair. A growing number of studies have revealed that macrophages have high functional plasticity and heterogeneity, and the phenotype and functional shift of macrophages in the TME often affect the occurrence and development of diseases. The reversal of TAMs is critical in promoting anti-tumor immunity, however, how to mediate the M1/M2 transition has remained unclear. This study demonstrated that let-7b-5p modulates macrophage differentiation through interacting with SOCS1, leading to activation of the STAT pathway and secretion of pro-tumor cytokines. Reversal of M2 differentiation by let-7b-5p inhibitors can enhance macrophage phagocytosis and potentially exerts anti-tumor efficacy. These data support the possibility of miRNA-based M1/M2 transition in immunotherapy of PCa.

MiRNAs are powerful intracellular regulators involved in regulating the development, differentiation, and maturation of immune cells such as T cells, DCs, and macrophages[22]. Data from miRNA-sequencing and microarrays in M1, M2, and monocytes indicate that macrophage polarization is dynamically regulated by several miRNAs[11,23]. As an important family of miRNAs, let-7b-5p participates in the growth, proliferation, invasion, and metastasis of cancer by regulating the expression of a variety of oncogenes and cytokines[24]. Specifically, let-7a, let-7b, and let-7c have been identified as potential tumor suppressors that directly target the mRNAs of genes involved in the cell cycle and signal transduction pathways that contribute to the initiation and development of PCa[25,26]. Let-7c is also associated with macrophage differentiation[27], but the role of let-7b-5p in the shift of M1/M2 in PCa has been largely undefined.

Our previous study showed that let-7b is highly expressed in M-CMs, which have an M2-like phenotype[20]. To further explore the mechanism underlying let-7b-5p regulation of M1/M2 transition, M1/M-CM

macrophages were induced with monocytes from peripheral blood mononuclear cells (PBMCs), and let-7b-5p mimics or let-7b-5p inhibitors were transfected into M1 and M-CMs, respectively, to observe the effect (Fig. 1A). Our results showed that CD206 expression in M-CMs (11.4%) treated with let-7b-5p inhibitors was almost the same level as that in M1 (14.3%), whereas CD206 level in M1 treated with let-7b-5p mimics (80.2%) was similar to that in M-CMs (78.3%) (Fig. 1B). Additionally, we observed that in M-CMs treated with let-7b-5p inhibitors, TNF-α, IL-10, and IL-13 were downregulated, and the IL-12 was upregulated, while in M1 treated with let-7b-5p mimics, the expression of TNF-α, IL-10, and IL-13 was increased, and the expression of IL-12 was decreased (Fig. 2). Due to the change in CD206 and cytokines in the macrophages, we assumed that let-7b-5p modulated the M1/M2 transition via regulating the expression of inflammatory cytokines. These data indicate that let-7b-5p is involved in the regulation of the M1/M2 phenotype transition in PCa.

The inflammatory signaling pathway is considered to be the key pathway of macrophage polarization. Corresponding inflammatory cytokines are also reportedly involved in tumor occurrence and development[9]. Therefore, maintaining the homeostasis of these signal molecules is very important. As an important negative regulator of the signal transduction pathway, the SOCS family mainly regulates the expression of cytokines by inhibiting the JAK/STAT signaling pathway[28].

The JAK/STAT signaling pathway regulated by SOCS1 is a signal transduction pathway stimulated by cytokines and participates in many important biological processes such as proliferation, differentiation, apoptosis, and immune regulation[29,30]. Because SOCS1 is the candidate target gene for let-7b-5p according to the bioinformatics prediction algorithm TargetScan, we compared the levels of SOCSI protein in M-CMs and M1 after let-7b-5p intervention and found that let-7b-5p was negatively correlated with SOCS1 expression. In M-CMs transfected with let-7b-5p inhibitors, the expression of SOCS1 was significantly upregulated as in M1, whereas in M1 transfected with let-7b-5p mimics, the expression of SOCS1 was similar to that in M-CMs (Fig. 3A). Through luciferase assays, we further confirmed that let-7b-5p directly targets the SOCS1 3′-untranslated region (UTR). After co-transfection of let-7b-5p and SOCS1-3′-UTR, the fluorescence intensity of the reporter gene significantly decreased, while the fluorescence intensity of the reporter gene remained unchanged in the MUT group of SOCS1-3′-UTR (Fig. 3B, C).

As a particularly potent inhibitor of JAK1 and JAK2[31], SOCS1 mediates the activities of STAT1[32], STAT3[33], and STAT5a[34], which regulate the expression of a large number of cytokines including IL-9, IL-10, IL-21, IL-22, and TNF-α[35]. For example, STAT3 in TAMs from breast cancer is overactivated, which inhibits the expression of IL-12 and significantly promotes the secretion of TNF-α[36]. To investigate the downstream targets of SOCS1, we next analyzed the phosphorylation level of STAT family proteins in macrophages after let-7b-5p intervention using the CST signal pathway chip. The results showed that let-7b-5p promoted the phosphorylation of STAT1, STAT3, and STAT5A proteins in macrophages (Fig. 4). In M1 transfected with let-7b-5p mimics, the expression of p-STAT1, p-STAT5a, and p-STAT3 was significantly increased (**$P < 0.01$), similar to the M-CM group, whereas the expression of p-STAT1, p-

STAT3, and p-STAT5a was significantly decreased in M-CMs transfected with let-7b-5p inhibitors (**P < 0.01), similar to that in the M1 group. In RAW264.7 cells, Galectin-9 showed similar results, which regulated macrophage polarization through STAT1 and STAT3[37]. These results indicate that let-7b-5p mediates the M1/M2 transition via the SOCS1/STAT signaling pathway.

To further investigate the effects of let-7b-5p on macrophage function, we analyzed the phagocytosis of macrophages after let-7b-5p intervention. As expected, M-CMs transfected with let-7b-5p inhibitors and M1 both showed higher phagocytosis than that in the other groups, and M1 transfected with let-7b-5p mimics had a lower phagocytosis rate similar to M-CMs (Fig. 5). Macrophage cytokines have the ability to enhance the proliferation of normal prostate epithelial cells[38]. Classical activated M1 macrophages, secreting a variety of pro-inflammatory factors such as IL-12 and IFN-*γ*, are considered anti-tumor factors. Instead, TAMs promote the proliferation, invasion, and metastasis of tumor cells. Here, we observed the role of those macrophages in the proliferation of PC-3 cells. Our data demonstrated that CM from M-CMs treated with let-7b-5p inhibitors significantly suppressed the proliferation of PC-3 cells compared to that from M-CMs, suggesting that the inhibition of let-7b-5p can reverse M2 differentiation to some extent and inhibit PCa proliferation.

4. Conclusion

Collectively, these results confirm that let-7b-5p can regulate the M1/M2 transition by regulating the activity of SOCS1/STAT pathway, ultimately affecting PCa proliferation. Our study expands the role of let-7b-5p directly or indirectly in mediating anti-tumor immunity. As a novel regulator of the M1/M2 switch, let-7b-5p is suitable for designing future therapies toward macrophage-mediated pathologies.

5. Materials And Methods

5.1 Cell culture

Human PCa cells (PC-3) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (Life Technologies Corporation, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Burlington, ON, Canada) at 37°C in 5% CO_2. Human macrophages were induced by the blood monocytes from healthy male donors. Human monocytes were isolated from PBMCs using anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Purified monocytes ($1x10^6$) were seeded in a 6-well plate and incubated for 7 days in RPMI 1640, supplemented with 10% FBS and 50 ng/mL M-CSF (Peprotech, Rocky Hill, NJ, USA) to obtain macrophages. Subsequently, macrophages were induced with 100 ng/mL lipopolysaccharides (Peprotech) plus 100 ng/mL IFN-γ (Peprotech) or 50% CM from PC-3 cells, respectively, for 48 h to obtain M1 and prostatic M-CM subtype, whereas M0 cells were obtained by treatment with serum-free medium for 48 h. After growing to the logarithmic phase, the cells were cultured in serum-free medium for 24 h. Then the supernatant was collected and centrifuged, and filtered, followed by the addition of 10% FBS to obtain CM.

5.2 Transfection assay

The chemically modified hsa-let-7b-5p inhibitors, mimics, and corresponding NC oligonucleotides were purchased from RiboBio Corporation (Guangzhou, China). M1 macrophages were transfected with 50 nM let-7b-5p mimics, 50 nM NC, or remained untreated using FuGENE® 6 Transfection Reagent (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions, while M-CMs were transfected with 100 nM let-7b-5p inhibitors or 100 nM NC. The cells were harvested after 72 h of transfection for subsequent experiments.

5.3 Immunofluorescence staining

After the supernatants were removed, macrophages in a 96-well plate were washed with PBS three times, after which 200 mL of 4% paraformaldehyde was added to each well for 15 min at room temperature. After the cells were gently washed three times with PBS, the macrophages were labeled with primary antibody (CD206-PE as M2 marker) (eBioscience, San Diego, CA, USA) for 10 min at room temperature. Then stained cells were washed twice with PBS, and RPMI 1640 medium was added. The expression of CD206 was detected by a High Throughput Connotation of Imaging System (Molecular Devices, Silicon Valley, CA, USA).

5.4 ELISA

The whole-cell protein lysates from macrophages of each experimental group were isolated using RIPA, and the protein concentrations were determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). ELISA kits (China Union Biology, Beijing, China) were used to analyze the expression of SOCS1 in each experimental group according to the manufacturer's protocol. The concentration of the SOCS1 was measured by comparing their optical density value with the corresponding standard curve.

5.5 Quantitative PCR

Total RNA was extracted using Invitrogen Trizol Reagent (Life Technologies Corporation, Carlsbad, CA, USA) and then used as a template for cDNA synthesis. For miRNA, 100 ng total RNA was reverse transcribed directly using MicroRNA^{TM}first-strand synthesis (Tokara, Kyoto, Japan) to synthesize cDNA. Quantitative PCR (qPCR) was performed in the Bio-RAD CFX96TM Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the SYBR Green PCR Master Mix (Tokara, Kyoto, Japan) according to the manufacturer's protocol. All primers used in this study are shown in Table 1 (Supplementary data).

5.6 CST signal pathway chip

Macrophage cells of each group were harvested and lysed, and the supernatant was collected. After the protein concentration was determined by the Bio-Rad protein assay, 40 μg cell lysates of each group were used to analyze the expression of signaling proteins of macrophages via the CST signal pathway chip

(Cell Signaling Technology, Inc., Danvers, MA, USA) according to the manufacturer's instructions, and these data were visualized by the Bio-Rad imager (Bio-Rad Laboratories).

5.7 Phagocytosis test

S. aureus was cultured and adjusted to 1×10^9/mL, mixed with an appropriate amount of FITC powder and then kept in 4°C in a dark place for 4 h to obtain FITC-*S. aureus* antigen. After FITC-*S. aureus* antigen was added to the normal cultured macrophages in each group at a ratio of 1:20 (*S. aureus*:macrophages), the mixture was shielded from light and incubated at 37°C for 20 min. Afterward, the supernatant was removed, and the cells were washed three times. Finally, phagocytosis of macrophages in each group was detected and calculated using the High Throughput Connotation of Imaging System. The percentage of phagocytosis (%) = the number of macrophages phagocytizing *S. aureus*/the total number of macrophages counted × 100%.

5.8 Cell Counting Kit-8 assay

Cell viability was detected according to the instructions of the Cell Counting Kit-8 assay (CCK-8; Beyotime Institute of Biotechnology, Beijing, China). PC-3 cells (100 mL) were seeded at 5×10^4/mL in 96-well plates and cultured to 80% confluency. The medium was replaced with CM from the macrophages of different groups, and then incubated for 48 or 72 h. Then 10 mL CCK-8 reagent was added to each well, and cell growth was measured at 490 nm using the SpectraMax M4 Multimode Microplate reader (Molecular Devices). The number of viable cells was presented relative to the control group.

5.9 Dual-luciferase reporter assay

WT containing let-7b-5p binding sites or MUT SOCS1-3'UTR mutated at the let-7b-5p binding sites was inserted into the pmiR-RB-Report™vector (Ribobio Corporation, Guangzhou, China) according to the manufacturer's instructions. Then 50 nM let-7b-5p mimics or mimic control (Guangzhou RiboBio Co., Ltd.) and WT or MUT 3'-UTR of SOCS1 were co-transfected into 293T cells using Lipofectamine® 2000 reagent (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After cells were cultured at 37°C for 48 h, luciferase activities were analyzed using the Dual-Luciferase Reporter Assay system (GeneCopoeia, Inc., Rockville, MD, USA). The activity of firefly luciferase was normalized to the corresponding renilla luciferase activity. Each experiment was performed three times.

5.10 Data processing

Prism 5.0 statistical software was used for statistical analysis. Two-tailed unpaired Student's *t*-tests or one-way analysis of variance was applied to determine statistical significance. Significance values are indicated as *($P<0.05$), ** ($P<0.01$).

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Jiangxi Academy of Medical Sciences (Jiangxi, China), and written informed consent was obtained from all donors. All experimental protocols were in accordance with the approved guidelines for the safety requirements of Jiangxi Academy of Medical Sciences.

Consent for publication

We declare that all the data shown in this article have not been published or submitted elsewhere. All of the authors declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Availability of data and materials

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

Competing interests

We have no competing interest.

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Authors' contributions

All the authors are contributed to this manuscript. Z.W. conceived and designed the experiments; J.R., L.X., Y-Y.H., F.L., Y-Q.Y., H.G. and X.N. performed the experiments; J.R., L.X. and Y-Y.H. analysed the data; Z.W. and L.Z. contributed reagents/materials/analysis tools; and J.R., L.X. Z.W. and H.G. wrote the paper.

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None

Abbreviations

TME, tumor microenvironment; TAM, tumor-associated macrophage; PCa, prostate cancer; SOCS, suppressors of cytokine signaling; JAK/STAT, Janus kinase/signal transducer and activator of transcription; ELISA, enzyme-linked immunosorbent assay; M-CM, macrophages induced by conditioned medium from PC-3 cells

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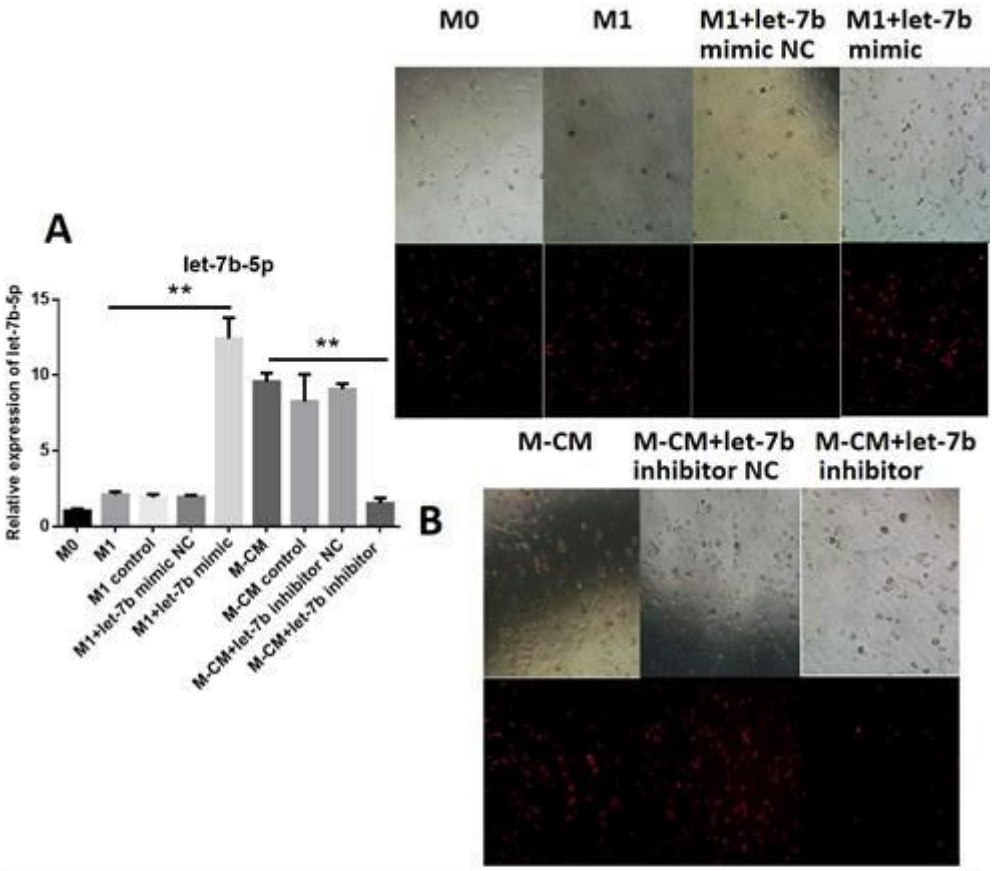
Tables

Table1. A list of all the primers

Primer name	Primer sequences
GAPDH forward primer	5¢-GACCCCTTCATTGACCTCAAC-3¢
GAPDH reverse primer	5¢-CTTCTCCATGGTGGTGAAGA-3¢
TNF-alpha forward primer	5¢-CTGGGCAGGTCTACTTTGGG-3
TNF-alpha reverse primer	5¢-CTGGAGGCCCCAGTTTGAAT-3
IL-12 forward primer	5'-CATTGAGGTCATGGTGGATG-3'
IL-12 reverse primer	5'-CAAGTTCTTGGGTGGGTCAG-3'
IL-10 forward primer	5'-AGAACCAAGACCCAGACATCA-3'
IL-10 reverse primer	5'-GCATTCTTCACCTGCTCCAC-3'
IL-13 forward primer	5'-ATCCTCTCCTGTTGGCACTG-3'
IL-13 reverse primer	5'-CTGGTTCTGGGTGATGTTGAC-3
U6 forward primer	5¢-CTCGCTTCGGCAGCACA-3¢
U6 reverse primer	5¢-AACGCTTCACGAATTTGCGT-3¢
let-7b forward primer	5'- GCGCTGAGGTAGTAGGTTGTG -3'
let-7b reverse primer	5'-GTGCAGGGTCCGAGGT-3'

Figures



Group	Total Cells	Number of CD206⁺(PE)	CD206⁺/total cells Ratio(%)
M0	605	285	47.1
M1	863	123	14.3
M1+let-7b-5p mimic NC	830	115	13.8
M1+let-7b-5p mimic	805	646	80.2**
M-CM	874	684	78.3
M-CM+let-7b-5p inhibitor NC	859	667	77.6
M-CM+let-7b-5p inhibitor	598	68	11.4**

Figure 1

Let-7b-5p enhances CD206 expression in macrophages. (A) Let-7b-5p expression in different macrophages. Human monocytes were isolated from normal donors' blood by using anti-CD14 magnetic beads. M0, M1, and M-CMs were differentiated as described in the Materials and Methods. M-CMs were transfected with let-7b-5p inhibitors, or NC, or remained untreated for 72 h. Instead, M1 macrophages were transfected with let-7b-5p mimics. Relative expression of let-7b-5p was analyzed by qPCR. The expression of miRNA was normalized to U6. Data represent the mean ± standard deviation (SD) of three independent experiments. * P<0.05; ** P<0.01. (B) CD206 expression in macrophages. After M1 and M-CMs were transfected with let-7b-5p mimics or inhibitors, respectively, CD206 expression of subtypes of

macrophages was detected with anti-CD-206 PE (red) by the High Throughput Connotation of Imaging System (original magnification, 100×).

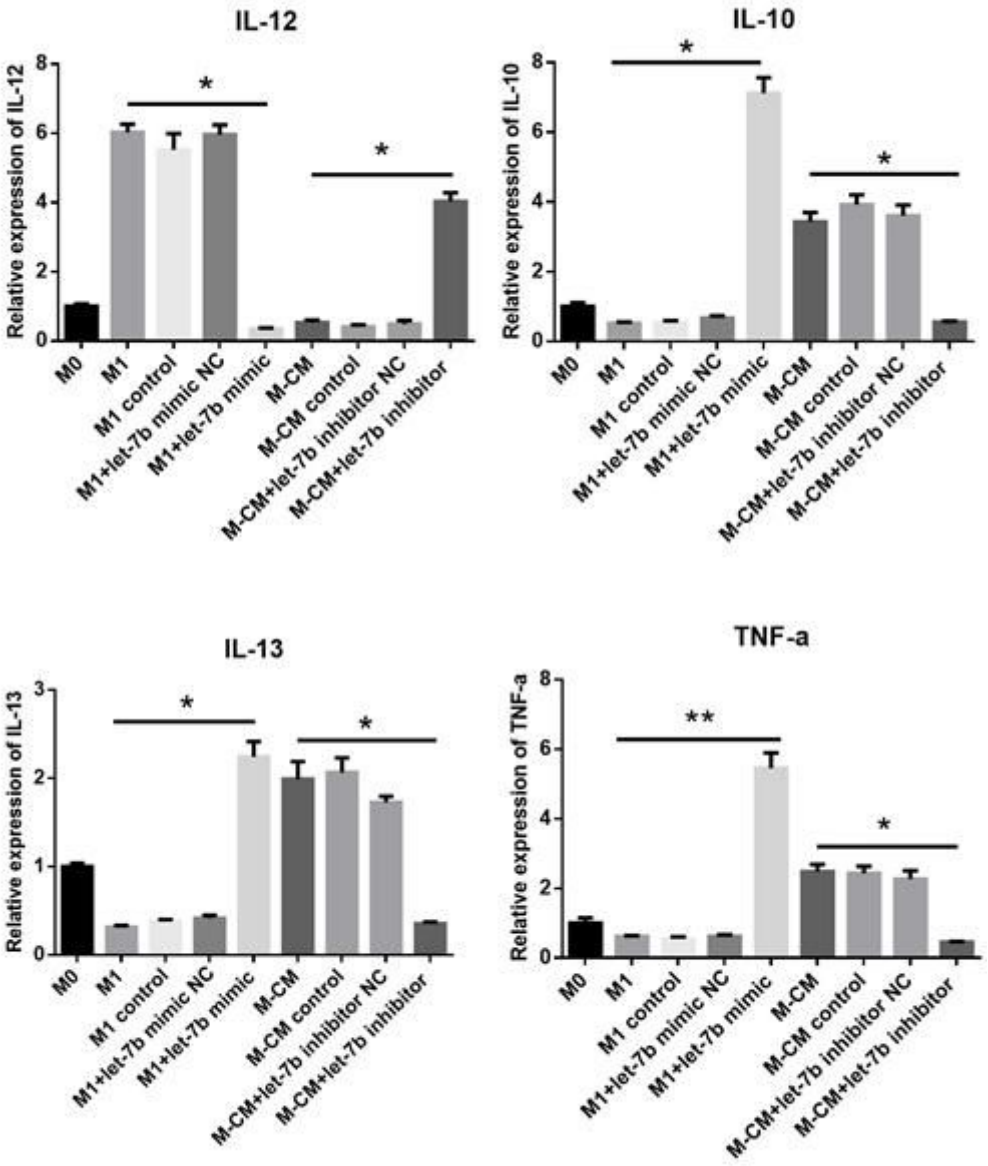


Figure 2

Effect of let-7b-5p on the expression of inflammatory cytokines in macrophages. M0, M1, and M-CMs were differentiated as described in Materials and Methods. M-CMs were transfected with let-7b-5p inhibitors or NC, or remained untreated for 72 h, while M1 macrophages were transfected with let-7b-5p mimics. Expression of IL-12, IL-13, IL-10, and TNF-α was determined by qPCR. Expression of mRNA was normalized to GAPDH. Data represent the mean ± SD of three separate experiments. * $P < 0.05$; ** $P < 0.01$.

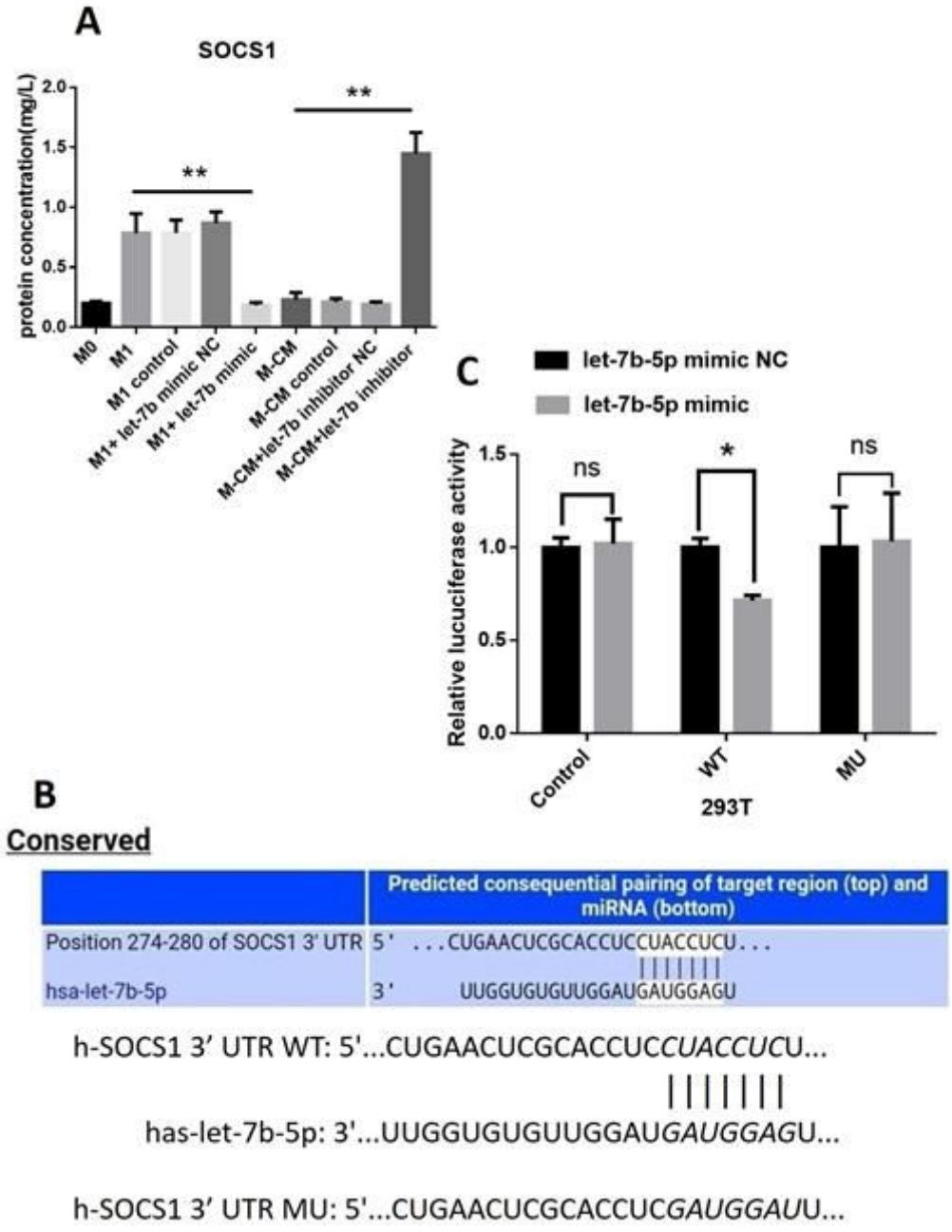


Figure 3

SOCS1 is a direct target gene of let-7b-5p in vitro. (A) Protein expression of SOCS1 in macrophages of each group. Expression of SOCS1 protein was confirmed by ELISA after M1 or M-CMs were transfected with let-7b-5p mimics or inhibitors, respectively, for 72 h, compared with its controls. (B) Illustration of the sequence match between let-7b-5p and SOCS1 3'-UTR determined using TargetScan. (C) Relative luciferase activity was assayed and calculated by the ratio of firefly/renilla luciferase activity following transfection with let-7b-5p mimic compared with transfection with NC in 293T. The data are shown by the mean ± SD of three separate experiments. * $P < 0.05$; ** $P < 0.01$.

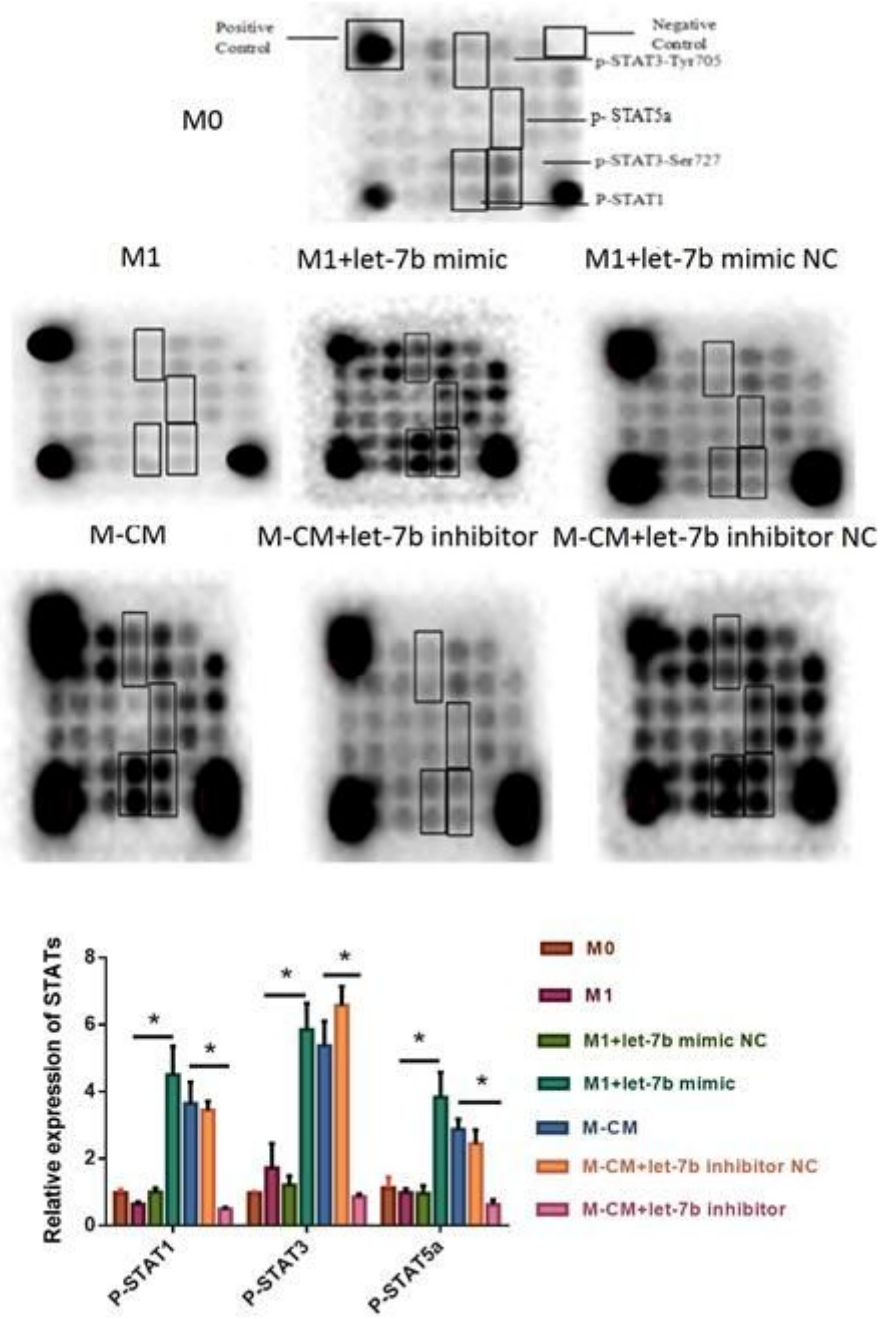
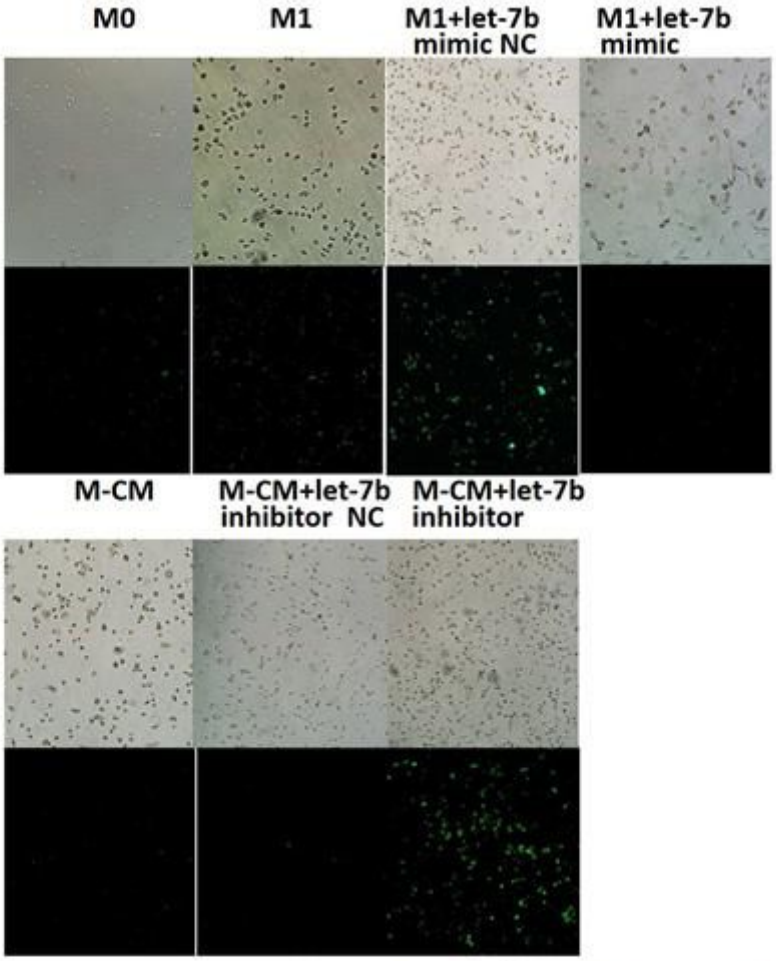


Figure 4

Overexpression or knockdown of let-7b-5p influences the activity of the STAT signaling pathway. Macrophage cells of each group were harvested and lysed, and the supernatant was collected. After the protein concentration was determined using the Bio-Rad protein assay, 40 μg cell lysates of each group were used to analyze the expression of the signaling proteins of macrophages using the CST signal pathway chip according to the manufacturer's instructions, and these data were visualized by the Bio-Rad imager.



Group	phagocytic rate (%)	phagocytic indices
M0	23.6±2.62	0.53±0.038
M1	39.9±2.49	0.83±0.112
M1+let-7b-5p mimic NC	37.79±1.05	0.82±0.032
M1+let-7b-5p mimic	10.71±1.34**	0.21±0.041**
M-CM	11.33±2.89	0.17±0.265
M-CM+let-7b-5p inhibitor NC	9.51±1.36	0.19±0.072
M-CM+let-7b-5p inhibitor	43.25±3.07***	0.89±0.004***

Figure 5

Let-7b-5p reduces the phagocytosis of macrophages. After the FITC-Staphylococcus aureus antigen was added to the normal cultured macrophages in each group at the ratio of 1:20 (S. aureus:macrophages), the mixture was shielded from light and incubated at 37°C for 20 min. Then the supernatant was removed, and the phagocytosis of macrophages in each group was recorded and quantified by measuring the numbers of macrophages with FITC-S. aureus antigen in the field using the High Throughput Connotation of Imaging System (original magnification, 100×). * $P < 0.05$; ** $P < 0.01$; *** $P<0.001$.

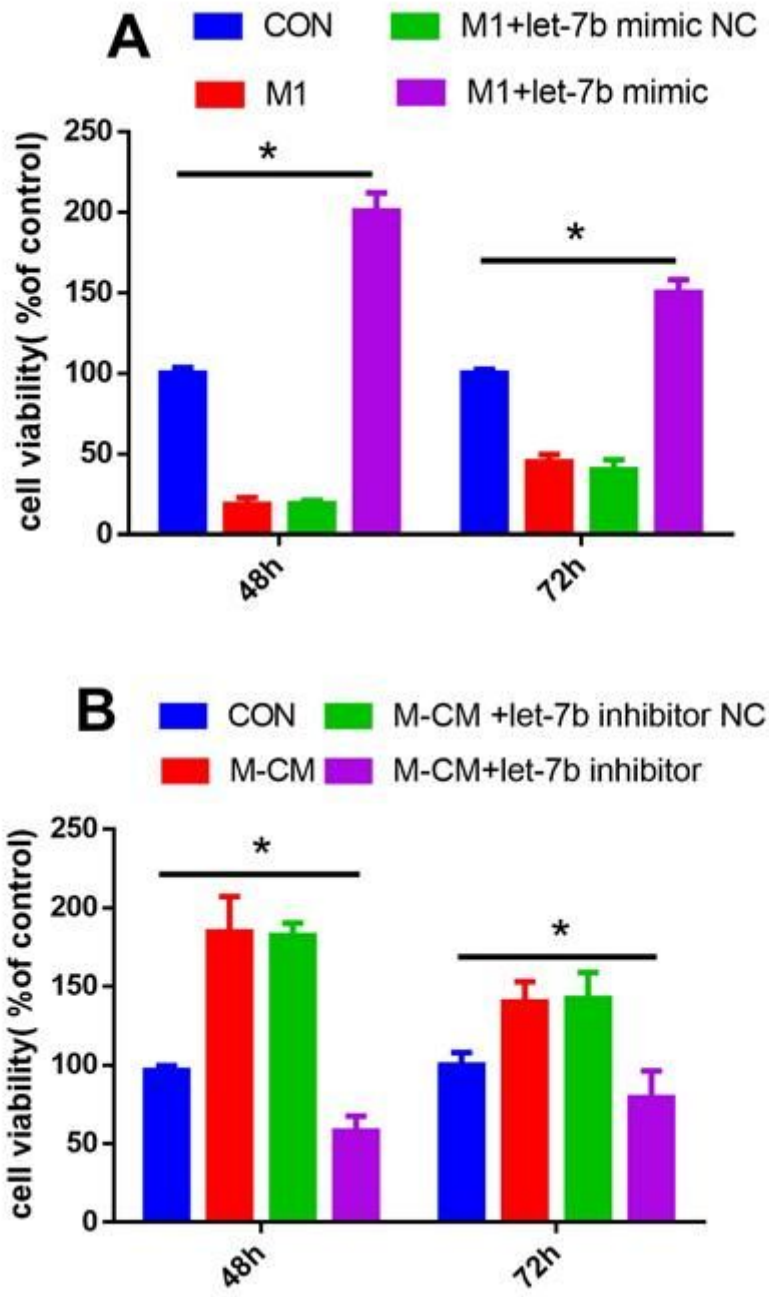


Figure 6

Reversal of M-CMs by downregulation of let-7b-5p inhibits the proliferation of PCa cells M1 and M-CMs were transfected with let-7b-5p mimics or inhibitors, respectively, for 72 h. Then CM from macrophages was collected and added to the PC-3 cells. PC-3 cells were exposed to CM from macrophages of different groups or RPMI 1640 medium as a control for 48 or 72 h. Viability of PC-3 cells was measured by the CCK-8 assay. The number of viable cells was presented relative to the control group. *P < 0.05; ** P < 0.01.