Original Article
Normal peripheral prostate stromal cells stimulate prostate cancer development: roles of c-kit signal

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Abstract: Background: To investigated the peripheral stromal cell conditioned medium (CM) -stimulated c-kit-JAK2-STAT1 pathway in prostate cancer. Methods: CM harvested from normal prostate peripheral stromal cells was added to DU145 cells. DU145 cell viability and migration were measured by cell counting kit-8 reagent and Transwell analysis respectively. Colony and sphere formation efficiencies of DU145 cells co-cultured with CM from human prostate stromal cells were also measured. DU145cells were stably transfected with lentivirus-mediated shRNA for c-kit silencing. Results: C-kit expression in prostate cancer was found to be significantly higher than in benign prostatic hyperplasia and positively associated with Gleason scores. The growth, migration and capacity of clonogenic property of DU145 cells significantly increased upon exposure to peripheral stromal CM and then were inhibited after silencing the expression of c-kit. The levels of c-kit, pJAK2 and pSTAT1 were significantly induced by peripheral zone stromal CM compared with controls in serum free medium and the levels of pJAK2 and pSTAT1 decreased after c-kit silencing. Conclusions: C-kit hyper-expression promotes the development of prostate cancer. The peripheral stromal cell CM stimulated c-kit-JAK2-STAT1 pathway in prostate cancer cell viability, migration, and capacity of clonogenic property. This may lead to a greater understanding of the role of c-kit in prostate cancer and provide a potential therapeutic target for prostate cancer.

Keywords: Prostate cancer, stromal-epithelial interaction, c-kit, signaling transduction

Introduction

Prostate cancer is one of the leading cancers diagnosed in men and a major cause of male-related cancer death. The rationale behind the preference of prostate cancer cells for growth in peripheral prostate stromal cells remains unclear. The "Seed and Soil" hypothesis explains the nonrandom trends of breast cancer metastasis and it also has been used to describe the behavior of metastatic prostate cancer cells [1]. Stromal-epithelial interaction is critical to control prostate organ morphogenesis, cell homeostasis, and tissue remodeling during embryonic development and adulthood [2, 3]. The stromal compartment affects epithelial growth and differentiation, and epithelial cell directs stromal growth and differentiation in a mutual manner [4].

C-kit shares structural similarities with the receptors for platelet-derived growth factor and macrophage growth factor [5, 6]. It is well known that gastrointestinal stromal tumors are c-kit-expressing and c-kit-signaling-driven mesenchymal tumors. It is hypothesized that gastrointestinal stromal tumors originate from interstitial cells of Cajal [7, 8]; therefore, c-kit-positive cells in the gut are considered to function as a growth factor of stroma in the gut. In addition, the discovery of constitutive c-kit activation as the central mechanism of gastrointestinal stromal tumor pathogenesis suggested that inhibiting or blocking c-kit signaling might be an important gastrointestinal stromal tumor treatment strategy [9]. Imatinib mesylate represents the standard-of-care frontline drug for the treatment of unresectable and metastatic gastrointestinal stromal tumors [10]. Recently, several reports have shown the existence of c-kit-positive interstitial cells in the prostate [11-13]; Simak et al. [11] first reported the existence of c-kit-positive cells in benign prostatic hyperpla-

Table 1. Correlation of c-kit expression levels with prostate gleason score

Tissue type	Tissue number	c-kit level		
		Low *N* (%)	Medium *N* (%)	High *N* (%)
Normal	20	14 (70)	6 (30)	0
BPH	25	19 (76)	6 (24)	0
Prostate cancer	69	7 (10.1)	26 (37.7)	36 (52.2)
Gleason score				
4-5	10	3 (30)	7 (70)	0
6-7	18	2 (11.1)	10 (55.6)	6 (33.3)
8-9	41	2 (4.9)	9 (22)	30 (73.1)

Footnotes: The tissues of 69 cancer, 20 normal, and 25 BPH prostate were examined by immunohistochemistry methods using c-kit antibody (1:50). The correlations between c-kit expression and prostate Gleason score were evaluated by Fisher's exact test ($P < 0.01$).

sia (BPH). However, the roles of c-kit and c-kit-positive cells in prostate cancer remain unclear.

We propose that c-kit could be associated with growth of the prostate, as in the gut, and could contribute to the pathophysiology of prostate cancer. In this study, we examined the regulation of cell proliferation through a c-kit-mediated mechanism in the prostate and discuss the pathophysiology of this mechanism in prostate cancer. In this work, we used DU145 Prostate cancer cell lines. We determine here the relationship between stromal CM and c-kit in inducing JAK2 and STAT1 in DU145 cells.

Materials and methods

Patients

Human tissues of normal prostate (n = 20) and BPH (n = 25) were obtained from 45 bladder tumor patients receiving radical cystoprostatectomy surgery and tissues of prostate cancer were obtained from 69 patients who underwent radical prostatectomy between October 2009 and October 2013 in our department, and who were subsequently referred to our department for follow-up. Normal human ileum as positive control was obtained from the bladder cancer patients undergoing radical cystoprostatectomy with ileal conduit urinary diversion. Human specimen usage in our study was approved by the ethics committee of the School of Medicine, Shanghai Jiaotong University (Shanghai, China). Diagnoses of normal prostate, BPH or prostate cancer were confirmed by professional pathologists in our hospital. The prostate tissues were fixed by immersing in 10% formaldehyde for 20 hrs and embedded in paraffin for further analysis.

Immunohistochemistry

All tissues were routinely fixed in 10% neutral buffered formalin and embedded in paraffin; 4 mm thick serial sections were taken onto poli-l-lysine-coated slides. The slides were then treated for 10 min at 15°C with methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase activity. Immunostaining for c-kit was performed with a rabbit monoclonal antibody (ab32363; Epitomics, USA) diluted 1:50. The expression of c-kit in stromal and epithelial cells of human prostate was evaluated. Tissue sections of ileum were used as a positive control and stained in the same manner.

Cells, cell cultures and reagents

The androgen-independent prostate cancer DU145 cell line was purchased from ATCC (Manassas, VA, USA). Normal prostate tissues for primary stromal culture were obtained from three consenting donors (aged 22, 23, and 40 years) who had no pre-existing prostate issues and died from other diseases at Shanghai Ninth People's Hospital (Shanghai, China). The peripheral zone tissues of each prostate were dissected and transported to the laboratory within 30 min after resection. The dissected tissues were prepared and processed according to our previous work [14]. Stromal cells in this study were used within 6 passages. Prostate stromal cells from three donors were independently isolated and examined in further experiments. DU145 cells from and prostate stromal cells were routinely cultured in DMEM (GIBCO Cat. No.11965, Carlsbad, CA) with 10% (v/v) heat inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA). The use of fresh prostate tissue specimens for research was approved by the Ethics Committee of the School of Medicine, Shanghai Jiaotong University (Shanghai, China).

Knockdown of c-kit Expression in DU145 cells

To knockdown the c-kit expression in DU145 cells, sub-confluent cultures of cells (in a six well plate) were infected with lentivirus (pur-
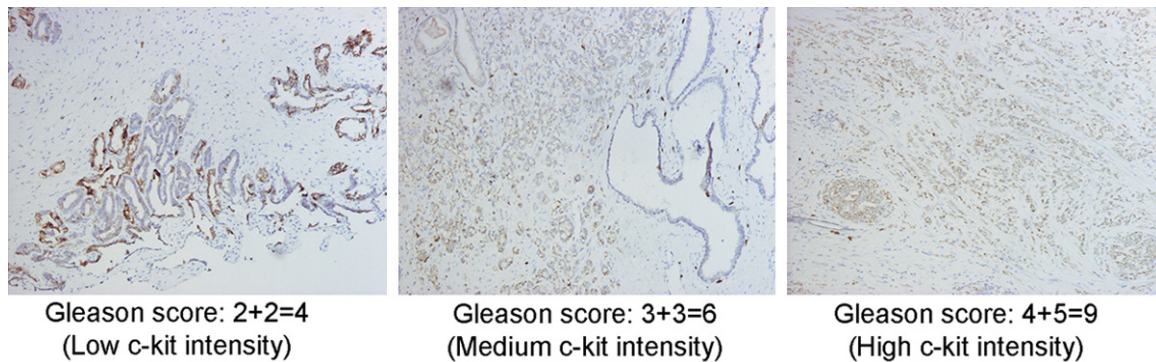
Gleason score: 2+2=4
(Low c-kit intensity)

Gleason score: 3+3=6
(Medium c-kit intensity)

Gleason score: 4+5=9
(High c-kit intensity)

Figure 1. c-kit is highly expressed in advanced tissue. This figure is the representative of low, medium, and high c-kit intensity in prostate cancer tissues with Gleason score 4, 6, and 9.
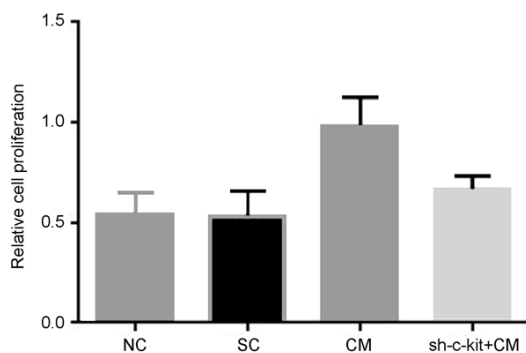

Figure 2. Cell viability assayed by CCK-8 assay. The cell viability of peripheral zone stromal CM treated group was significantly enhanced than SC and NC group; Knockdown of c-kit can decrease the cell viability of CM treated DU145 cells.

chased from Santa Cruz Biotech, Santa Cruz, CA) encoding shRNA to the c-kit gene (sc-29225-V). DU145 cells were infected with the lentivirus encoding a control shRNA (sc-108-080) as a control (SC group). 24 hours after infections of DU145 cells, cells were selected in puromycin (1 µg/ml) for 5 days. Puromycin-resistant DU145 cells were pooled and cell cultures were cultured without the puromycin in the medium for a few days before performing the following experiments.

Conditioned medium (CM)

Normal human prostate peripheral stromal cells were grown separately in DMEM with 10% (v/v) FBS (Invitrogen) as described above until 80% confluent. Cells were washed twice with 1 × PBS (Invitrogen) and then switched into serum free (SF) DMEM medium. Conditioned media (CM) were harvested every two days for

two weeks and then all collected media were mixed together. Stromal cells that detached during this culture period were removed along with the medium. CM from the stromal cells was then filtered through a 0.22 µm pore size SteriCup (Millipore Billerica, MA) to remove cells and cell debris. Filtered prostate stromal CM was aliquoted and stored frozen at -80°C until use. We mixed CM half and half (v/v) with SF DMEM medium and treated DU145 cells with the mixture in the CM experiments (CM group). This could ensure the continuous presence of nutrients present in the culture medium. DU145 cells infected with lentivirus encoding shRNA to the c-kit gene (sc-29225-V) were also treated with the CM mixture (sh-c-kit+CM group). DU145 cells treated with only SF DMEM medium were considered as negative-control group (NC group).

Measurement of cell growth viability by CCK-8 assay

The cell proliferation was measured by CCK-8 assay (Dojindo Laboratories, Japan) according to manufacturer instructions. DU145 cells were treated with CM or SF medium over a five day time culture period. The cells were seeded into seven 96-well culture plates at 2.0×10^3/well overnight. After the treatment was completed, 10 µl CCK-8 reagent was added to each well. These plates were then incubated in a humidified CO2 incubator at 37°C for 2 hrs. Finally, optical densities were determined at 450 nm.

In vitro cell migration assay

The migration assay was conducted by using corning transwell according to the manufactur-
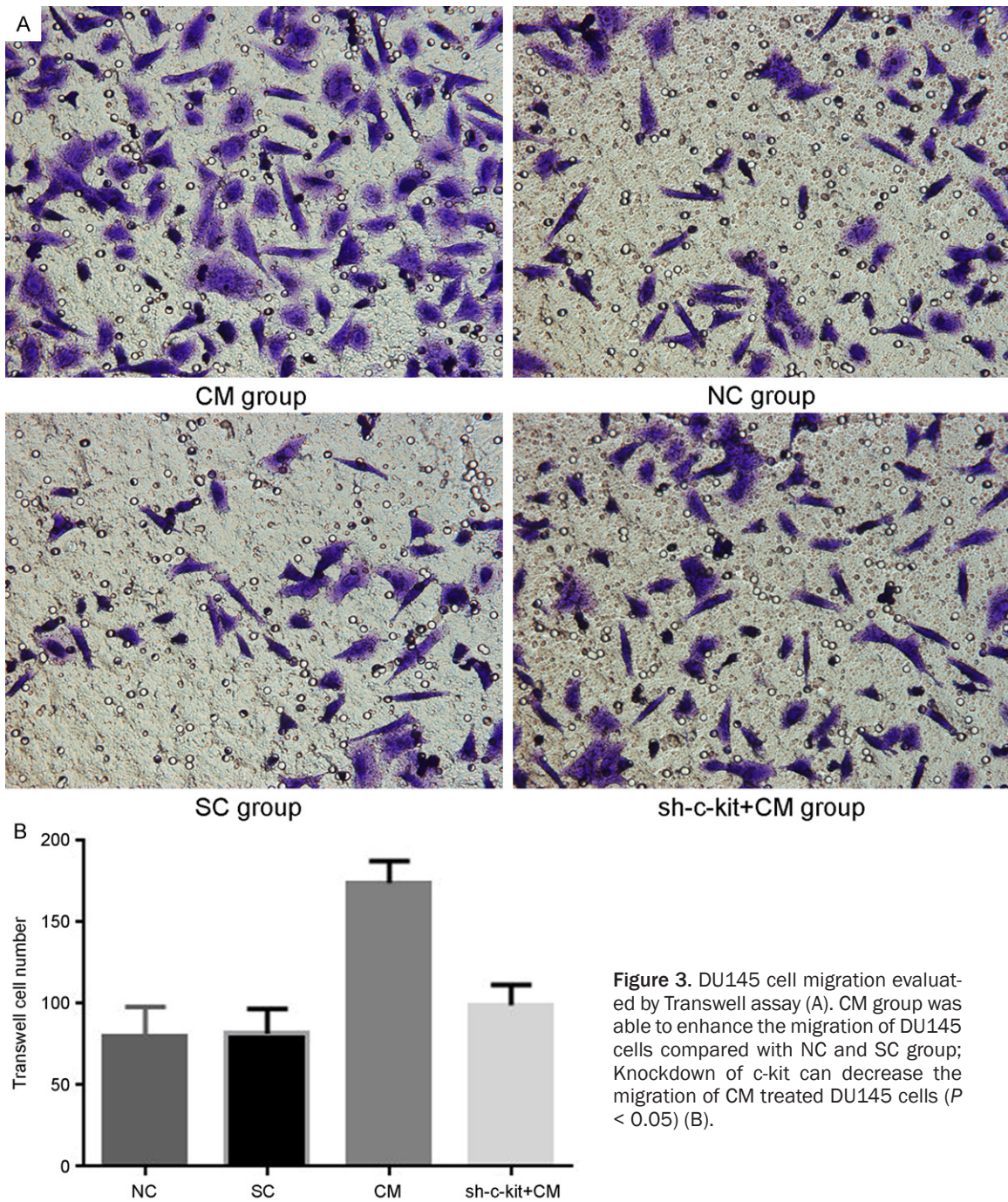
Figure 3. DU145 cell migration evaluated by Transwell assay (A). CM group was able to enhance the migration of DU145 cells compared with NC and SC group; Knockdown of c-kit can decrease the migration of CM treated DU145 cells ($P < 0.05$) (B).

er's protocol. DU145 cells were treated with CM or SF medium over a five day time culture period. Then the cells (1×10^5) with 100 ml serum-free medium were seeded into the upper chamber of an 8mm pore size Transwell (Corning). The lower chambers in the system were filled with DMEM medium with 10% FBS. After 10 hours of incubation, non-migratory cells in the upper chamber were removed, and the cells migrated through the membrane were fixed with paraformaldehyde solution for 15 min, stained in 0.1% crystal violet for 20 min, and counted by photographing the membrane through the microscope in 10 random high-power fields (200 × magnification).

Colony formation assay

DU145 cells were treated with CM or SF medium over a five day time culture period. Single
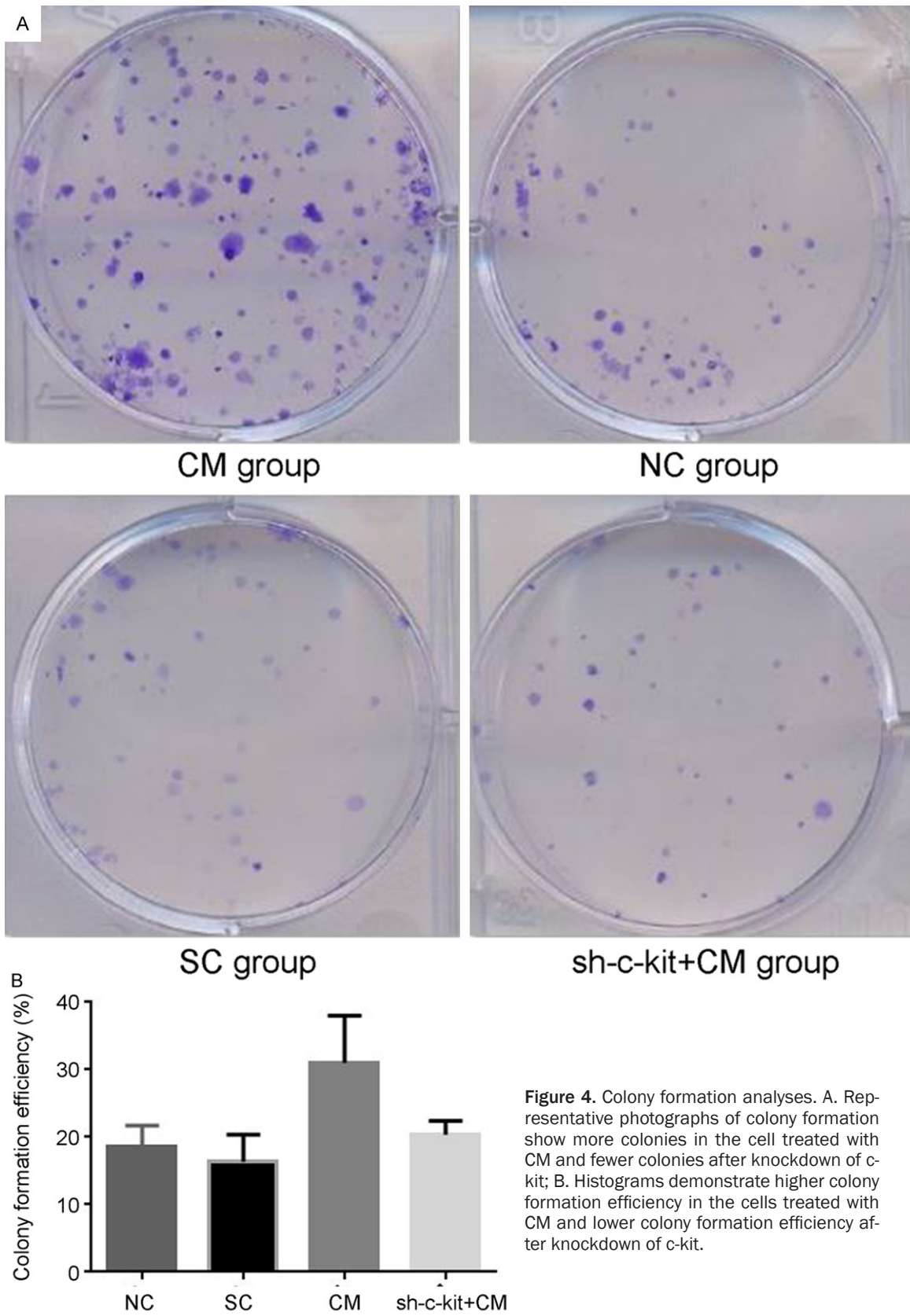
Figure 4. Colony formation analyses. A. Representative photographs of colony formation show more colonies in the cell treated with CM and fewer colonies after knockdown of c-kit; B. Histograms demonstrate higher colony formation efficiency in the cells treated with CM and lower colony formation efficiency after knockdown of c-kit.

cells (400 cells/well) were incubated in six-well plates to allow for cell attachment. Cells were cultivated in SF DMEM. Colony formation was examined after 2 weeks. The cells were fixed with paraformaldehyde solution for 15 min and then stained with 0.1% crystal violet for 30 min. The plates were washed with PBS before micro-scopic colony assessment. Cell cluster with more than 30 cells was assessed as a colony. Colony formation efficiency was evaluated as follows:Colony formation efficiency = colonies/input cells × 100%.

Sphere formation assay

DU145 cells were treated with CM or SF medi-um over a five day time culture period. Single cells (400 cells/well) were seeded in ultralow attachment six-well plates (Ultralow Cluster Plates, Life Sciences). Cells were cultivated in SF DMEM (Invitrogen). Sphere with more than 30 cells was assessed as a full sphere and counted under inverse microscopy. Sphere for-mation efficiency was evaluated as follows: Sphere formation efficiency = colonies/input cells × 100%.

Western blotting

Whole protein extracts from DU145 cells treat-ed with and without CM were resolved on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto polyvinylidene fluoride membranes，which were incubated with anti-bodies of c-kit, 1:500 (ab32363; Epitomics, USA), JAK2, 1:1,000 (D4A8; Cell Signaling, Danvers, MA), and STAT1, 1:1000 (58D6; Cell Signaling, Danvers, MA) overnight at 4°C. Membranes were incubated overnight at 4°C with the primary antibodies diluted in PBS with 1% BSA after blocking for 1 hr at room tempera-ture in 5% milk in phosphate buffered saline (PBS)/0.1% Tween-20. Monoclonal antibody of β-actin (Sigma–Aldrich, St. Louis, MO) was used as internal control. Immunoreactive proteins were visualized with an enhanced chemilumi-nescence detection system (Amersham Phar-macia Biotech, England) after secondary anti-body incubation. Statistical analyses were per-formed using student *t*-test. All experiments were repeated a minimum of three times.

Statistical analysis

Data are shown as the mean±SD. Data were analyzed using the Student t-test with SPSS 17.0 and Prism 6.0. Statistical significance was considered at < 0.05.
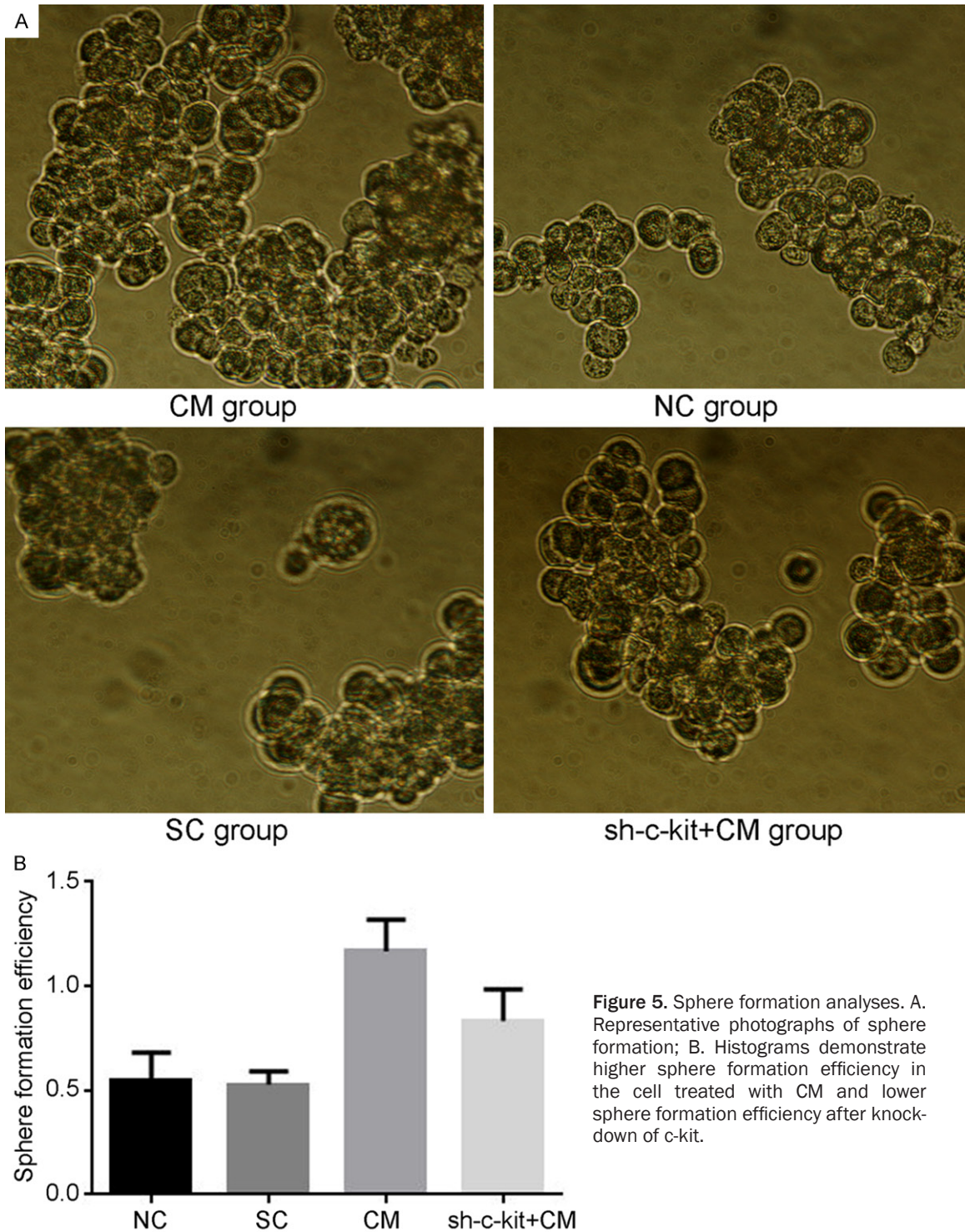
Results

C-kit is highly expressed in advanced prostate cancer tissues

To investigate whether advanced prostate can-cer tissue has a higher c-kit expression level, we performed a c-kit immunohistochemistry assay and analyzed 69 cases of prostate can-cer with various Gleason scores from 4 to 9, 20 normal tissues, and 25 BPH tissues. Based on the intensity of c-kit staining, we divided these specimens into three groups as having low, medium, and high c-kit expression, and our results show that c-kit expression is lower in normal and BPH tissues, while c-kit is highly expressed in advanced prostate cancer speci-mens (**Table 1**). We found that 73.1% of poorly differentiated carcinomas (Gleason score 8-9) showed high c-kit expression, while 33.3% of moderately differentiated (Gleason score, 6-7) and 0% of tumors at Gleason score 4-5 showed high c-kit expression (**Figure 1**; **Table 1**). These results indicate that c-kit expression levels are obviously up-regulated in advanced prostate cancer and c-kit expression level is significantly correlated with prostate tumor Gleason score (Fisher's test, $P < 0.01$). All these results sug-gest a part important role of c-kit in prostate cancer progression.

Stromal CM regulated cell growth and migra-tion in human DU145 cells

The DU145 cell viability was assayed by CCK-8 assay. While no differences were observed between SC group and NC group in normal growth condition, the cell viability of peripheral zone stromal CM treated group was significant-ly enhanced than SC and NC group in normal growth condition; Knockdown of c-kit can decrease the cell viability of DU145 cells ($P < 0.05$, **Figure 2**).

In order to evaluate the metastatic properties of DU145 cells, we carried out Transwell assays. -CM group was able to enhance the migration of DU145 cells compared with NC and SC group; Knockdown of c-kit can decrease the migration of DU145 cells ($P < 0.05$, **Figure 3**), and there were no differences between SC and NC control group.

Figure 5. Sphere formation analyses. A. Representative photographs of sphere formation; B. Histograms demonstrate higher sphere formation efficiency in the cell treated with CM and lower sphere formation efficiency after knockdown of c-kit.

Peripheral stromal CM induced colony and sphere formation efficiencies alteration in DU145 cells

The capacity of clonogenic property was investigated by colony formation and sphere forma-

tion assays in DU145 cell lines treated with peripheral zone stromal CM. More colonies were observed in the cells stimulated by peripheral zone stromal CM compared with NC and SC control groups (**Figure 4**). Statistical analyses confirmed a significant increase of colony
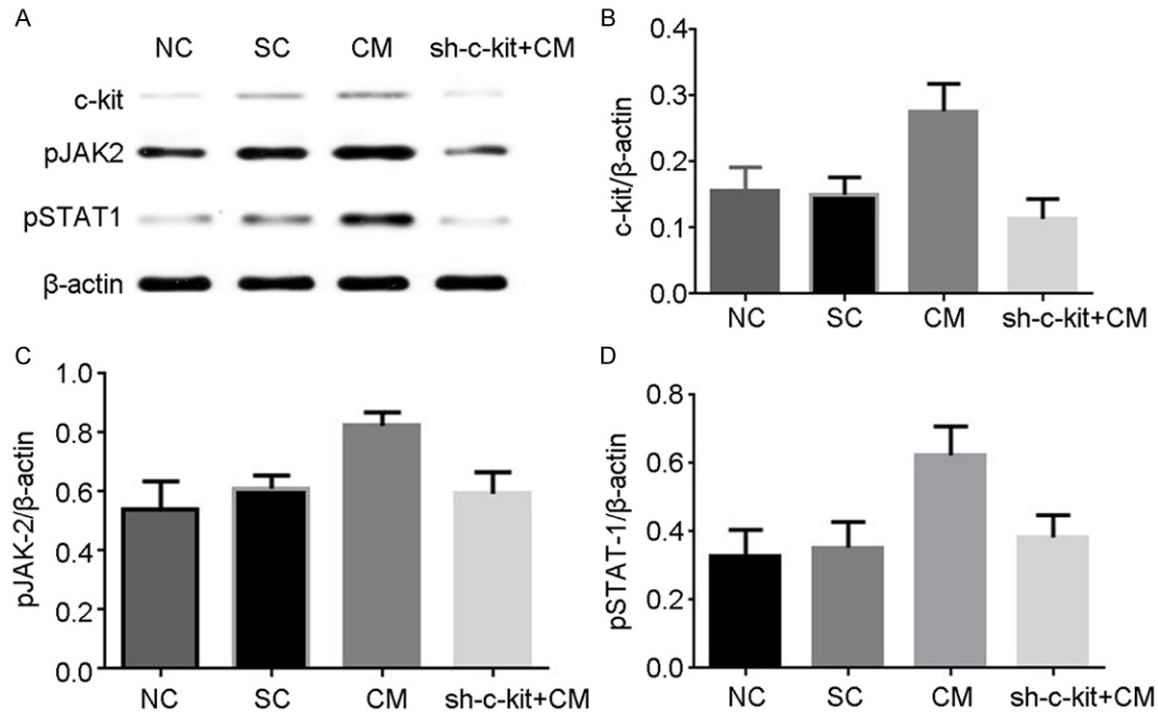
Figure 6. Western blot analysis of the expression of c-kit, pJAK2 and pSTAT1 in DU145 cells. (A) The levels of c-kit (B) pJAK2 (C) and pSTAT1 (D) were significantly induced by peripheral zone stromal CM compared with NC and SC controls ($P < 0.05$, n = 3). Significant decrease of the expression level of c-kit, pJAK2 and pSTAT1 from DU145 cells was observed in CM treated group after c-kit knockdown.

formation efficiency in the DU145 cells treated with peripheral zone stromal CM compared with NC and SC control groups, and knockdown of c-kit can decrease the colony formation of DU145 cells (**Figure 4**).

DU145 cells could form spheres at ultralow attachment plates albeit with low efficiency of sphere formation (**Figure 5**). As shown in **Figure 5**, higher sphere formation efficiency was seen in the cells treated with peripheral zone stromal CM compared with NC and SC group, and significant decrease of sphere formation efficiency was observed after c-kit knock down. The results of colony formation and sphere formation suggest enhancement effects of peripheral zone stromal CM on the clonogenicity of DU145 cells.

Stromal CM modulated c-kit, pJAK2 and pSTAT1 activity in DU145 cells

We performed a western blot analysis using lysates of DU145 cells to evaluate whether the expression level of c-kit, pJAK2 and pSTAT1 from DU145 cells was altered following the treatment with different stromal CM or SF medium. In accordance with the observed colony and sphere formation efficiencies alteration, the levels of c-kit, pJAK2 and pSTAT1 were significantly induced by peripheral zone stromal CM compared with NC and SC controls (**Figure 6**), and significant decrease of the expression level of c-kit, pJAK2 and pSTAT1 from DU145 cells was observed in CM treated group compared with the SC and NC group after c-kit knock down. We concluded from these experiments that DU145 cells are triggered to overexpress c-kit by CM originating from peripheral prostate stromal cells.

Discussion

The pathophysiology of prostate cancer is very complex and not yet fully understood. Cell alterations that include such changes as differentiation, proliferation, apoptosis, and senescence in the stroma and epithelium are implicated in prostate neoplasm pathogenesis. Molecular researches have yielded numerous candidate genes important in the progression of prostate cancer. In this study, we identified c-kit-positive

prostate cancer cells and demonstrated a possible function of c-kit in prostate cancer progression.

Simak et al. [11] found that changed patterns of c-kit expression are associated with BPH. Van der Aa et al. [12] reported that a large number of c-kit-positive cells were expressed between the smooth muscle cells and under the basal layer of the duct system in human prostate, and they suggested that stromal cells could be involved in normal prostate physiology. Shafik et al. [13] demonstrated that c-kit-positive cells in human prostate with morphological and immunological phenotypes were similar to interstitial cells of Cajal of the gut. Imura et al reported that c-kit could regulate cell proliferation in the prostate and play an important role in the pathophysiology of BPH. However, the roles of c-kit and c-kit positive cells in prostate cancer remain unknown [15].

Gastrointestinal stromal tumors are c-kit positive and c-kit signal driven mesenchymal tumors [16]. It is suggested that c-kit positive cells are not only pacemaker cells but also are involved in the growth of stroma in the gut. Just like the gut, the prostate has its embryonic origin in the primitive hind-gut. In our study, we speculated that prostate stromal cells could play a significant role in prostate cancer progression; therefore, we examined the effect of CM of prostate stromal cells on prostate cancer cell proliferation, migration and clonogenic property to determine the role of c-kit in prostate cancer cell growth. Peripheral stromal CM was observed to increase cell proliferation, migration and clonogenic property with upregulation of c-kit, JAK2 and STAT1, while knockdown of c-kit could reverse all these changes in vitro. Proteins of the JAK and STAT families are an important part of signal transduction pathways initiated by interferon receptors and by members of the hematopoietin receptor superfamily [17]. Deberry et al. found that the SCF-c-kit interaction could activate the JAK2-STAT1 signal transduction pathway [18]. Binding of SCF to c-kit could activate multiple pathways, such as JAK/STAT, PLC-gamma, PI3-kinase, MAP kinase, and SRC kinase pathways [7, 8]. This promotes cell cycle activation, cell proliferation, and apoptosis inhibition.

Di Lorenzo et al [19] found a trend to a higher risk of relapse among the c-kit positive pros-

tate cancer although not statistically significant. The c-kit epithelial expression was found in the peripheral zone of prostate. Our study also demonstrated that c-kit mainly exists in the peripheral zone of prostate, so we selected peripheral zone tissues of the prostate to perform this study. In human tissue study, we found c-kit expression in prostate cancer was significantly higher than in benign prostatic hyperplasia and normal prostate, moreover we found c-kit was positively associated with Gleason scores. It may be involved in the prostate cancer progression. Wiesner et al. [20] found an overall trend of increased c-kit expression in prostate cancer bone metastases. They suggested that the bone microenvironment up-regulates c-kit expression on prostate cancer cells, promoting their intraosseous expansion.

Our study suggests that peripheral zone stromal CM may promote prostate cancer progression through the c-kit-JAK-STAT signaling pathway. These results show that activation of the c-kit-mediated mechanism induces prostatic cancer cell proliferation, migration and clonogenic property, and therefore, c-kit may play a significant role in the pathophysiology of prostate cancer, although further investigations of the molecular mechanism of c-kit in prostate cancer is needed. A significant number of patients who have been on imatinib therapy for many years have a reportedly acceptable quality of life. Therefore, imatinib mesylate may be appropriate for medical therapy of prostate cancer and c-kit or c-kit positive cells could be a candidate target of prostate cancer medical therapy.

The limitation of this study is that hormones, inflammation, and other areas may be involved in the pathogenesis of prostate cancer. Further studies will be needed to investigate the mechanism of the c-kit pathway in prostate cancer, and these studies will provide more insight for the future therapy of prostate cancer.

Conclusion

This study indicates that CM from normal human peripheral prostate stromal cell can increase prostate cancer cell viability, migration, and capacity of clonogenic property through c-kit-JAK2-STAT1 pathway. Our findings indicate that c-kit is overexpressed in advanced prostate cancer and c-kit knockdown decreas-

es prostate cancer cell viability, migration, and clonogenic property, and suggest that c-kit may play a critical role in prostate cancer progression and that c-kit inhibition may be a potential therapeutic target for prostate cancer.

Acknowledgements

Disclosure of conflict of interest

None.

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