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A Study on the Expression of CCL5,CXCR4 and Angiogenic Factors by Prostate Cancer Stem Cells

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ABSTRACT

Prostate cancer is the second most common cancer in men worldwide. Prostate Cancer Stem cells have been shown to be responsible for relapse in prostate cancer and the development of Castration Resistant Prostate Cancer (CRPC). As Prostate Cancer Stem cells are not eliminated by traditional drugs, it is important to study them and their interactions to target them with greater efficiency. We isolated Prostate Cancer Stem cells from the PC-3 cell line by treating the cells with 5-Fluorouracil. These stem cells expressed the stem cell markers CD133 and CD44. We showed that Prostate Cancer Stem cells express the chemokine CCL5 and the chemokine receptor CXCR4. These chemokines and receptors were expressed at a significantly higher level by the Prostate Cancer Stem cells in comparison to PC-3 cells. CCL5 and CXCR4 have been shown to be involved in the tumor growth, progression and tumor angiogenesis. We also showed that Prostate Cancer Stem cells express VEGF, c-Src and HIF-1αall of which are involved in tumor angiogenesis. We conclude that Prostate Cancer Stem cells help not only in the resurgence of prostate cancer but also actively participate in the maintenance of the tumor microenvironment. CCL5 could be a potential therapeutic target for Prostate Cancer Stem cells.

Keywords
Prostate Cancer Stem cells, Chemokines, CCL5, CXCR4, VEGF, c-Src and HIF-1α

Introduction

Prostate Cancer Stem cells play an important role in cancer growth and relapse. Prostate Cancer Stem cells (PCSCs) are resistant to most drugs that are used to treat Prostate Cancer. This makes it important to develop better methods to target and eliminate them. We are interested in studying the functions that PCSCs perform in the tumor microenvironment. We believe that along with helping the tumor cells proliferate PCSCs actively participate in several signaling pathways. We decided to investigate the function of PCSCs in chemokine signaling. We also investigated whether PCSCs contribute to the angiogenic pathways in the tumor microenvironment.

Chemokine signaling has been shown to play a crucial role in the tumor microenvironment. CXCR4 and SDF1(CCL12) expression has been shown to increase with tumor grade. CXCR4 was expressed in neo-vessel endothelial cells. CXCR4 and SDF1 colocalized to regions in tumors that contributed to angiogenesis.[1] SDF-1 was shown to stimulate the migration of bone marrow cells in a manner similar to VEGF. SDF-1α stimulated BM cell chemotaxis and promoted the interaction of these cells with functional tumor vessels. Tumors treated with anti-VEGF therapy may utilize an alternate SDF-1 mediated angiogenesis pathway to sustain growth and expand neovasculature[2]. SDF-1 was shown to recruit endothelial progenitor cells (EPCs) and increase blood flow and perfusion in cases of ischemia[3]. The expression of SDF-1 and CXCR4 can be

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used as a predictor of the negative pathological characteristics of a tumor. Studies suggest that SDF-1 levels should be screened for in accessory pathomorphological tests. This could help with more accurate prognosis of cancer. SDF-1 was shown to stabilize newly formed blood vessels by recruiting Smooth Muscle Progenitor Cells that express CXCR4, PDGFR and c-kit[4]. This shows the importance of SDF-1 and CXCR4 in vascular remodeling. SDF-1 induced the secretion of VEGF-A, IL-6, IL-8 and tissue inhibitor of metalloproteinase-2 in prostate cancer cells. All of these factors play important roles in tissue remodeling and vascularization. It has been shown that despite high levels of SDF-1 and VEGF-A secretion by vascular tumor cells, it is SDF-1 that is the main inducer of angiogenesis.[5]

The CCL5/CCR5 axis was shown to promote VEGF-dependent tumor angiogenesis in tumor microenvironment. This was promoted through the PKC**δ**/c-Src/ HIF-1**α** signaling pathway.[6] CCL5 is a potential therapeutic target in tumor therapy. CCL5 was shown to mediate angiogenesis in a bone marrow independent manner in tumor cells. Angiogenesis could be inhibited by inhibiting CCR5 expressing vasculature, this was proposed as a novel therapy for blocking tumor metastasis.[7] CCL5 exerts pro-angiogenic effects by promoting endothelial cell migration, spreading, neovessel formation, and vascular endothelial growth factor (VEGF) secretion. Moreover, tumor cells, upon CCL5 stimulation, canproduce VEGF or, by secreting CCL5, may recruit CCR5-expressing TAMs. Inhibition of CCL5/CCR5 axis can lead to the development of new therapeutic strategies for treatment of cancer.[89] CCL5 was shown to promote VEGF-dependent angiogenesis through the PI3K/Akt signaling pathway and the downregulation of miR-200b.[10] CCL5 neutralization was shown to inhibit tumor growth and it rendered the tumors more sensitive to PDGFR inhibitor treatment.[11]

Neovascularization is essential for tumor survival and growth. Several angiogenic factors have been shown to be crucial for tumor survival. VEGF is essential for angiogenesis in a tumor and tumor progression[1213]. Tumor cells have been shown to secrete VEGF. This VEGF helps in the formation of new blood vessels[14]. Expression of VEGF in the tumor was correlated with increased microvessel density. Increased expression of VEGF was associated with early relapse[15]. The CXCR4/ CXCL12 signaling axis was shown to induce the production of VEGF and promote angiogenesis[16]. This shows that there is cross talk between the Chemokine pathway and the angiogenesis pathway. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that controls oxygen delivery through angiogenesis and mediates response to hypoxia. HIF-1α is overexpressed in tumors and promotes their growth[17]. HIF-1α has been shown to be crucial for the formation of solid tumors[18]. HIF-1α is required for hypoxia induced VEGF production in tumors and the promotion of angiogenesis[19]. The protein tyrosine kinase c-Src was shown to mediate the expression of VEGF and promote the angiogenic potential of cancer cells[20]. c-Src was shown to be necessary for the vascular permeability activity of VEGF[12] (Eliceri et al. 1999).

Given the importance of chemokine signaling and the role of angiogenic factors in cancer progression, we wanted to study if PCSCs play any role in these pathways.

Methods

1) Culture of PC-3 cells: PC-3 cell line was procured from National Centre For Cell Science, Pune, India. PC-3 cells were cultured in 75 cm^2 cell culture flasks in DMEM medium with 10% Fetal Bovine Serum. 1% antibodies consisting of 100U/mL penicillin (Sigma-Aldrich) and 100 mg/mL streptomycin (Sigma-Aldrich) were added to the cell culture medium. The cell culture medium was replaced every 2-3 days. The cell culture flasks were incubated in cell culture incubators which provided an atmosphere of 5% CO2 at 37°C. When the PC-3 cells reached a confluency of around 80% they were subcultured or

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passaged by trypsinization. The cell culture medium was discarded and the cells were treated with 0.25% trypsin/ 0.02% EDTA medium for 2-3 minutes at room temperature. The treated cells were transferred to new flasks at a 1:5 or 1:10 ratio. For experiments the cells were plated into 6 well plates.

2) 5Fluorouracil treatment of PC-3 cells: PC-3 cells were plated into 6 well plates at 1×10^6 cells per plate. 13 milligrams of 5-Fluorouracil was added to 100 ml of distilled water to prepare 1 molar solution. This solution was diluted to produce a stock solution with a concentration of 1 millimolar. The stock solution was further diluted to produce solutions of 1µM, 2µM, 5µM, 10µM and 20µM. The above concentrations of 5-FU were used in the treatment of PC3 cells.
3) FACS: Drug resistant Prostate Cancer Stem cells that survived the 5-FU treatment were fixed using a 1% paraformaldehyde in PBS solution for 10 mins at room temperature. The cells were then stained with anti-CD133 or anti-CD44 primary antibodies for 2 hours at room temperature. Cells were then stained with donkey anti-rabbit fluorescein secondary antibody for 30 minutes at room temperature. The cells were then washed twice and resuspended in FACS buffer (1% BSA in PBS with 0.1% NaN3). A minimum of 20,000 events per sample were collected on a FACS Calibur flow cytometer (Beckton Dickinson) and analyzed with CellQuest Pro software (Beckton Dickinson). The filters used were 575/26 BP for PE and 530/28 BP for FITC with a 488-nm Ar laser to stimulate both FITC and PE.
4) Isolation of CD133 and CD44 expressing cells: The Mini-Midi MACS system (manufactured by MiltenyiBiotec, Singapore) was used to separate CD133, CD44 expressing cells from the cell culture samples that were treated with 5-FU. Cells were passed through MACS Microbead columns containing anti-CD133 antibodies. Cells positive for CD133 get attached to the microbeads because of the strong magnetic field. These cells are then eluted from the microbead column. The CD133 positive cells are then passed through a microbead column containing anti-CD44 antibodies. Cells positive for CD44 get attached to the microbeads column and are eluted from the column. We were able to isolate CD133 and CD44 expressing PCSCs using this method.
5) Western Blotting: Drug resistant Prostate Cancer Stem cells that survived the 5-FU treatment were used in western blotting. Proteins were extracted from whole cell lysates. These were probed for the expression of MDR1, CD133, CD44, CCL5, CXCR4, VEGF, c-Src and HIF-1α. After addition of chemiluminescent reagents the blots were exposed for developing and images were captured. The blots were stripped and reprobed for anti-β-actin to determine equivalent loading. β-actin was also used as the control housekeeping protein.
6) Statistical Analysis:Data were analyzed by one-way ANOVA followedby Dunnett's t-test using GraphPad InStat version 3.05 (GraphPad software Inc., San Diego, CA, USA). All P-values <0.05 were considered to be statistically significant.

RESULTS

Generation of 5-Flurouracil resistant Prostate Cancer stem cells

PC3 cells were cultured until they reached about 80% confluence. The cells were then treated with 5-Fluorouracil. 5-Fluorouracil was chosen as a cancer drug because it has been shown to be effective against most cancer cells, but also because it was cost effective and readily available. Cultures of PC-3 cells were treated with different concentrations of 5-Fluorouracil (1µm**,** 5 µm,

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10 µm, 20 µm) for a period of 48 hours. It was observed that treatment of PC-3 cells with 5 µm 5-Fluorouracil was sufficient to induce significant cell death. It was decided that for all further experiments PC-3 cells would be treated with 5 µm 5-Fluorouracil for a period of 48 hours. The cells that survived the 5-Fluorouracil treatment at the end of the 48 hours were deemed to be prostate cancer stem cells. To ascertain that the surviving cells were in fact prostate cancer stem cells and to determine the percentage of cells that were stem cells, FACS analysis for stem cell markers was performed on these cells. CD44 and CD133 were used as stem cell markers as both markers have been shown to be expressed on cancer stem cells. FACS analysis revealed that around 95% of the cells expressed both CD44 and CD133. This shows that the cells that survived the 5-Fluorouracil treatment were prostate cancer stem cells. The rest of the experiments in the study were performed on these 5-flurouracil treated cells, which will henceforth be referred to as Prostate Cancer Stem cells.

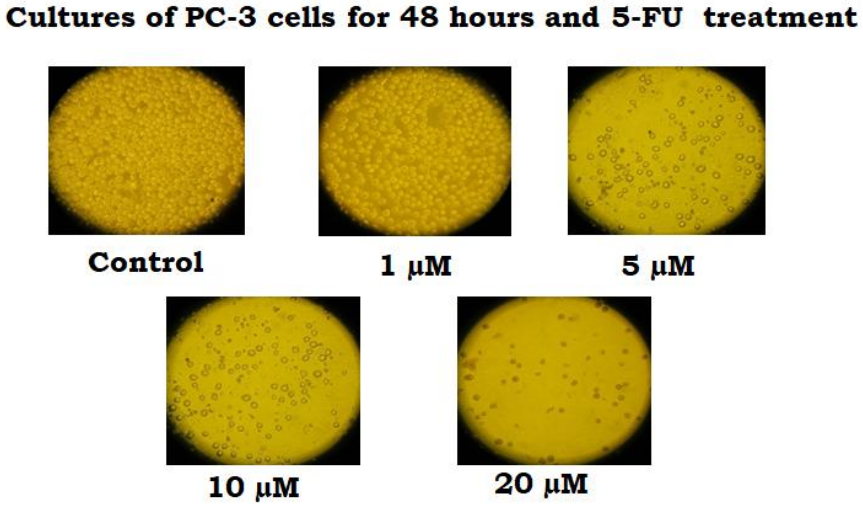
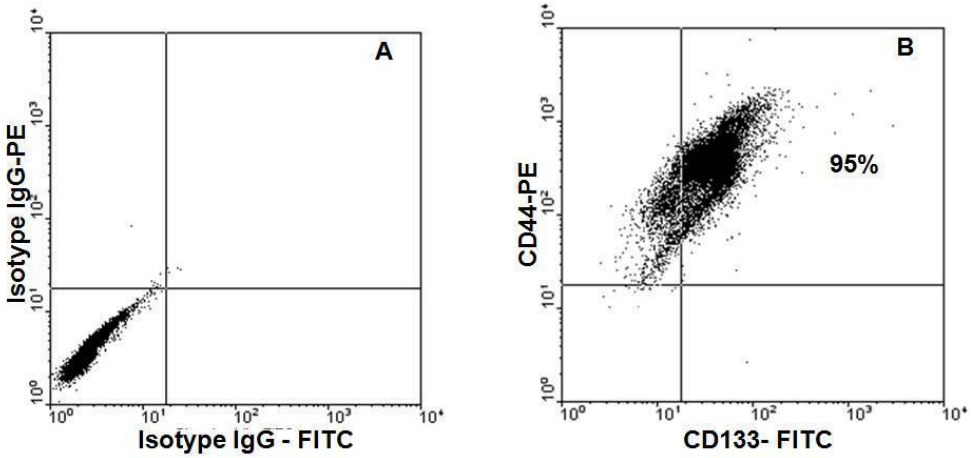


Figure 1 Treatment of PC3 cells with 5- Fluorouracil

PC-3 cells were treated with different concentrations of 5-Fluorouracil for 48 hours. Treatment of cells with 5 µm showed significant cell death. This concentration was used for further experiments.

Figure 2: FACS dot plot of cells expressing CD133 and CD44



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Cells that survived the 5-Flurouracil treatment were subjected to FACS analysis for CD133 or CD44 stem cells markers. FACS analysis showed that 95% cells expressed both CD133 and CD44 markers. These cells were considered Prostate Cancer Stem cells.

Expression of CCL5 and CXCR4 in drug resistant Prostate cancer stem cells

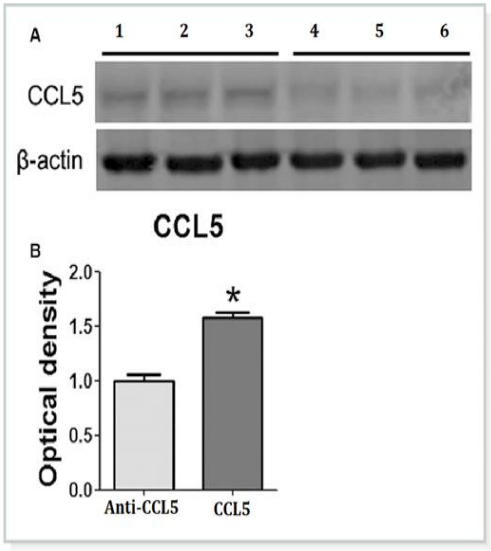
We wanted to study if Prostate Cancer Stem cells express CCL5 and CXCR4 as these chemokines have been shown to play an important role in cancer progression. Western Blot analysis of PC-3 cells showed that PC-3 cells express CCL5 and CXCR4. When CCL5 expression was inhibited using Anti-CCL5 antibodies added in the culture medium, expression of both CCL5 and CXCR4 was diminished in PC-3 cells.

PC-3 cells were treated with 5 µm 5-Fluorouracil for 48 hours, the surviving cells were MACS sorted using the MDR1, CD133 and CD44 stem cell markers. The prostate cancer stem cells that were isolated in this manner displayed a significant expression of CCL5. When these cells were treated with Anti-CCL5 antibody the expression of CCL5 was significantly reduced. This shows that Prostate Cancer Stem cells actively express CCL5.

PC-3 cells were treated with 5 µm 5-Fluorouracil for 48 hours, the surviving cells were MACS sorted using the stem cells markers MDR1, CD44 and CD133. The Prostate Cancer stem cells that were isolated in this manner displayed a significant expression CXCR4. When these cells were treated with Anti-CCL5 antibody the expression of CXCR4 was significantly reduced. This shows that PCSCs actively secrete CXCR4.

These experiments clearly indicate that PCSCs are actively involved in Chemokine signaling. The CCL5 ligand and the CXCR4 receptor are both expressed by PCSCs. The expression of the CXCR4 receptor appears to be dependent on the expression of the CCL5 ligand as the treatment with Anti-CCL5 antibodies reduced the expression of both CCL5 and CXCR4.

Figure 3. Expression of CCL5 and CXCR4 in cultured Prostate cancer stem cells



1. PC-3 Cells in culture
2. MDR1+ PC-3 Cells in culture
3. MDR1+CD133+CD44+ PC-3 Cells in culture
4. Anti-CCL5 to PC-3 cells in culture
5. Anti-CCL5 to MDR1+PC-3 cells in culture
6. Anti-CCL5 to MDR1+CD133+CD44+ PC-3 cells in culture

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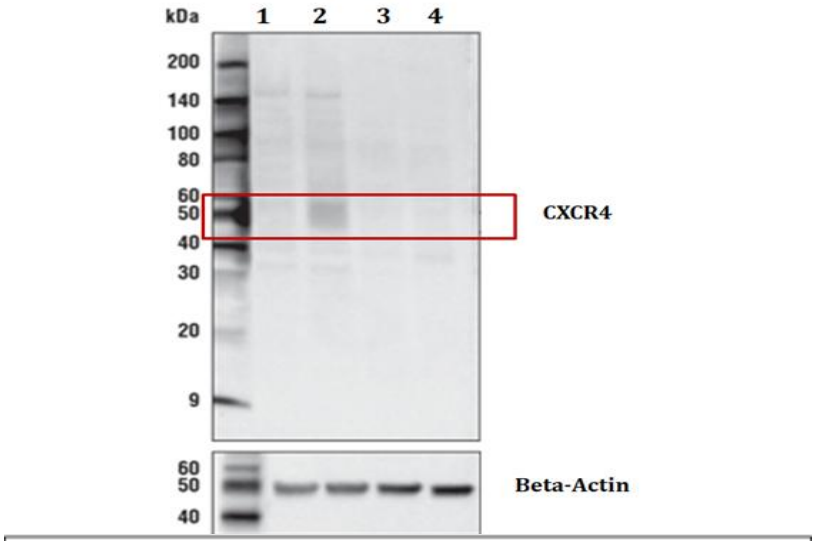

1.PC-3 cells in culture 2. MDR1+CD133+CD44+PC-3 cells in culture
3.Anti-CCL4 to PC-3 cells in culture
4. Anti-CCL4 to MDR1+CD133+CD44+PC-3 cells in culture

Prostate Cancer Stem cells were found to express chemokine CCL5 and the chemokine receptor CXCR4. Thisexpression was significantly higher when compared to PC-3 cells. The expression of CCL5 and CXCR4 was decreased significantly when the Prostate cancer stem cells were treated with anti-CCL5 and anti-CXCR4 antibodies.

Expression of VEGF, c-Src and HIF-1α in cultured Prostate cancer stem cells

CCL5 and CXCR4 have been shown to be involved in several different pathways that help in cancer cell proliferation and metastases. They have been shown to be involved in angiogenesis. As our results showed that PCSCs express CCL5 and CXCR4, we were interested to investigate if they expressed any factors that are known to be involved in the angiogenesis pathway. We chose to investigate the expression of HIF-1α and c-Src as these are factors involved in the angiogenesis pathway that leads to the production of VEGF. VEGF was investigated as it is an important factor in the angiogenesis pathway that has been shown to be involved in tumor related neo-angiogenesis.

PC-3 cells were analyzed for the expression of HIF-1 α, c-SRC and VEGF using Western Blotting. PC-3 cells expressed HIF-1α, c-SRC and VEGF under normal conditions indicating that they promote the angiogenic pathway. Treatment of PC-3 cells with Anti-CCL5 antibody decreased the expression of HIF-1α, c-SRC and VEGF, indicating that the activation of the pathway and production of VEGF was dependent on the expression of CCL5.

PC-3 cells were treated with 5 µm 5-Fluorouracil for 48 hours, the surviving cells were FACS sorted using the stem cells markers MDR1, CD44 and CD133. Prostate Cancer Stem cells that were isolated in this manner displayed a significant expression of HIF-1α, c-SRC and VEGF. This shows that the PCSCs are involved in the secretion of VEGF and actively promote neo-angiogenesis in the tumor. Treatment of PC-3 cells with Anti-CCL5 antibody decreased the expression of HIF-1α, c-SRC and VEGF, indicating that even in PCSCs, CCL5 regulated the activation of the pathway and the production of VEGF.

These results clearly indicate that PCSCs are actively involved in the production of pro-angiogenic factors and stimulation of neo-angiogenesis in tumors. It is also clear that CCL5 that is expressed by the PCSCs is essential for the production of VEGF. This makes CCL5 a good

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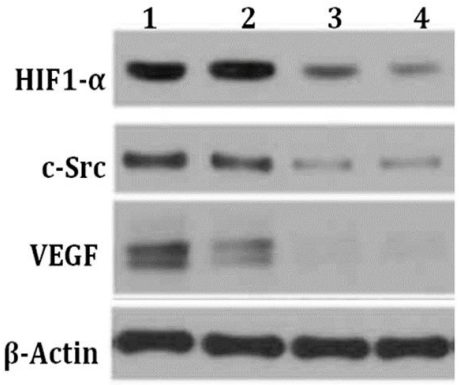
therapeutic target to prevent angiogenesis in tumors. These results also display the importance of PCSCs in maintaining the signaling pathways that are essential to the tumor microenvironment and reinforce the need to eradicate PCSCs to eliminate the tumor entirely.

Figure 4. Expression of VEGF, c-Src and HIF-1α in cultured Prostate cancer stem cells



1. PC-3 Cells in culture
2. MDR1+CD133+CD44+ PC-3 Cells in culture
3. Anti-CCL5 to PC-3 cells in culture
4. Anti-CCL5 to MDR1+CD133+CD44+ PC-3 cells in culture

Prostate Cancer Stem cells were shown to express angiogenic factors VEGF, HIF-1α and c-Src. This expression was found to be dependent on the expression of the chemokine CCL5

Discussion

This study shows that Prostate Cancer Stem cells are actively involved in the signaling pathways that promote the growth and spread of prostate cancer. Prostate Cancer Stem Cells have been shown to play important roles in the survival, growth and metastasis of prostate cancer. This has primarily been associated with the self-renewing capacity of PCSCs. The survival of PCSCs is what leads to remission of prostate cancer and even leads to Castration Resistant Prostate Cancer. This makes it important to study the role of PCSCs in the tumor microenvironment. We are of the opinion that PCSCs do not simply help in the self-renewal of cancer cells but are intimately involved in the signaling pathways that promote the progression of the cancer.

We wanted to investigate the exact methods in which PCSCs are involved in various cancer signaling pathways. This can help us understand the exact functions that the PCSCs play in the tumor microenvironment. Knowing the signaling pathways that PCSCs are involved in will help develop drugs that target these pathways and PCSCs.

Chemokines have been shown to play important roles in every aspect of cancer progression and metastasis. Any interaction that chemokines have with PCSCs will be of great interest in understanding the role of both chemokines and PCSCs in prostate cancer.

Because this interaction can have important implications for the role that PCSCs have in cancer progression. Our study shows that PCSCs express both CCL5 and CXCR4. Both CCL5 and CXCR4 have been shown to play important roles in tumor angiogenesis. CCL5 promotes VEGF-dependent tumor angiogenesis. CXCR4 was shown to play an important role in an alternate SDF-1 mediated angiogenesis pathway to sustain growth and expand neovasculature. Our study shows that PCSCs express both CCL5 and CXCR4. This goes to show that PCSCs play a crucial role in the angiogenesis pathways involved in tumor growth.

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Further experiments will have to be conducted to study whether PCSCs express other chemokines and their receptors. This will provide a better understanding of the role of PCSCs in chemokine signaling.

Our study also showed the PCSCs express VEGF. VEGF is essential for angiogenesis in a tumor and tumor progression. Tumor cells have been shown to secrete VEGF.VEGF helps in the formation of new blood vessels. Expression of VEGF in the tumor is correlated with increased microvessel density. Increased expression of VEGF was associated with early relapse.

Our results show that PCSCs help in the vascularization of prostate cancer in several different ways. They directly express VEGF which is essential for vascularization. PCSCs also secrete c-SRC which is known to induce VEGF. PCSCs also secrete CXCR4 which has been shown to help in the expression of VEGF. It is safe to say that PCSCs play a crucial role in the vascularization of prostate cancer.

This result shows that PCSCs actively contribute to the maintenance and growth of a tumor. Further studies need to be conducted to analyze if PCSCs express other chemokines or their receptors. It is also important to study if they play an active role in other signaling pathways in cancer progression.

This goes to show that PCSCs secrete chemokines and have receptors for them, indicating that they are intimately involved in chemokine signaling in the tumor environment. Our study shows that PCSCs not only help in producing new cancer cells but are involved in cancer signaling pathways. PCSCs are important for maintaining the signaling in the tumor microenvironment. This makes it crucial to target and eliminate PCSCs to completely eradicate prostate cancer.

We speculate that cancer stem cells in other cancers could behave in a way similar to PCSCs and be involved in chemokine signaling and angiogenic pathways.

Conclusion

We have shown that it is possible to isolate stem cell marker expressing cells from the PC-3 cell line by treating the cell line with 5Flurouracil. These cells, which we refer to as PCSCs, were shown to express CD133 and CD44, both of which are stem cell markers. These PCSCs express chemokine CCL5 and the chemokine receptor CXCR4. The PCSCs were also shown to express the angiogenic factors VEGF, HIF-1α and c-Src. PCSCs are involved in tumor angiogenesis. CCL5 is required for PCSCs to perform their role in angiogenesis. These results show that CCL5 is a good therapeutic target for prostate cancer. Targeting CCL5 may help restrict the functioning of PCSCs and help eradicate them.

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