

NANOG Dominates Interleukin-6-Induced Sphere Formation in Prostate Cancer

ABSTRACT

Objective: Identifying the dynamics of prostate tumor aggressiveness is essential to find new therapeutics for the treatment. Cancer stem cells contribute to cancer progression by promoting tumor growth and metastasis, resisting treatment, and evading the immune system. Interleukin 6 (IL-6) is a pleiotropic cytokine that functions in inflammation, immune response, etc. However, dysregulated expression of IL-6 plays a pathological role in such conditions as cancer. In this study, we aimed to elucidate the effect of IL-6 on cancer stemness genes in prostate cancer cells.

Methods: Enrichment of stem-like cells was achieved through the formation of tumor spheres using the DU-145 cell line. Sphere formation was conducted in a medium supplemented with IL-6 and compared to a control group. The number of spheres was quantified, and the resulting pellet was collected for quantitative reverse transcription polymerase chain reaction analysis to assess the impact of IL-6 induction on the expression of stemness-related genes.

Results: Tumor sphere numbers and sizes increased in IL-6-induced environment. NANOG expression elevated in an IL-6-enriched environment compared to the non-treated spheres. Our results demonstrated that IL-6 induction in prostate tumor spheres upregulates NANOG gene expression.

Conclusion: Inducing IL-6 in prostate tumor spheres stimulates stemness biomarker NANOG genes. NANOG may be suggested as a therapeutic target for metastatic prostate cancer.

Keywords: Prostate cancer, tumorsphere, IL-6, NANOG gene

Introduction

Prostate cancer (PCa) is the fourth most common cancer worldwide and one of the main causes of cancer-related deaths in males. An estimated 1.5 million people are diagnosed with PCa and approximately a quarter of it results in death.¹ Prostate cancer is observed in men aged after 50, peaking between the ages of 75 and 79.² In addition to age, family history, race, obesity, and environmental factors are other risk factors for PCa.³ About 5% of men diagnosed with PCa have distant metastases, often to multiple sites like brain, lungs and liver. Another 15% of men have locoregional metastases, which means the cancer has spread to nearby lymph nodes. Men with distant metastases have a poor overall survival rate of only 30% for 5 years. High rates of relapse, metastasis, and drug resistance continue to be a significant concern despite the fact that modern screening programs have enabled earlier identification and treatment.⁴ The background of these concerns in PCa should be clarified to find new therapeutic targets.

In several malignancies, including PCa, a small subpopulation of malignant cells exhibiting stemness characteristics have been found and defined as cancer stem cells (CSCs).⁵ Cancer stem cells, a small cell fraction inside the tumor that is assumed to be responsible

Didem Seven 

Didem Tecimel 

Ömer Faruk Bayrak 

Department of Medical Genetics, Yeditepe University School of Medicine, Istanbul, Turkey

Corresponding author:

Didem Seven

✉ didem.seven@yeditepe.edu.tr

Received: June 23, 2023

Accepted: October 17, 2023

Publication Date: November 20, 2023

Cite this article as: Seven D, Tecimel D, Bayrak ÖF. NANOG dominates IL-6 induced sphere formation in prostate cancer. *Urol Res Pract.* 2023;49(6):376-380.

for heterogeneity, resistance, recurrence, and metastasis of cancers, are characterized by self-renewal, differentiation, long-term culture, and drug resistance potential.⁶ There is growing evidence that CSCs are regulated by *Oct4*, *Sox2*, *Klf4*, and *Myc* (OSKM) genes that are called Yamanaka factors.⁷ The other important collaborator of OSKM genes in pluripotency and stemness is the *NANOG* gene, which is an essential transcription factor for maintaining pluripotency in embryonic stem cells. Aberrant expression of the Yamanaka factors and *NANOG* gene was shown in glioblastoma, breast, lung, colorectal, pancreatic, ovarian cancers, and PCa.⁸⁻¹⁰ *NANOG* is overexpressed in CSCs, and its inhibition can lead to the death of stem cell-enriched population; thus, it is suggested as a therapeutic target.¹¹

Interleukin 6 (IL-6), one of the important cytokines, is involved in the regulation of cellular stemness by increasing transcriptional factors.¹² Interleukin 6 levels are normally quite low in the typical homeostatic condition, whereas a wide range of cells release IL-6 in response to abnormalities such as inflammation. Interleukin 6 is a main component that is ubiquitously found and known to be dysregulated in cancer. Various PCa research studies have associated IL-6 increment with tumor aggressiveness. Most of the PCa cells show elevated levels of IL-6 and exhibit malignant potential.¹³

Overexpression of IL-6 is associated with tumor aggressiveness in PCa. The majority of PCa cell lines express IL-6; especially, the cell lines that are devoid of androgen receptor exhibit increased malignant potential due to elevated levels of IL-6.¹⁴ In this study, we aimed to investigate the IL-6-induced stemness properties of PCa cells.

Material and Methods

Cell Culture

The DU-145 PCa cells were obtained from American Type Culture Collection (Manassas, Va, USA). Considering the cells were provided commercially, no ethical approval is required for this study. The cells were cultured in RPMI1640 Medium (GIBCO, Grand Island, NY, USA) medium with 10% FBS (GIBCO, USA) and 1% penicillin–streptomycin (GIBCO, USA) in a humidified incubator maintained at 37°C with 5% CO₂ in air. The cells were shown to be mycoplasma free. When they reached 80% confluency, the cells were trypsinized. The medium was changed 3 times a week.

MAIN POINTS

- *Elevated levels of interleukin 6 (IL-6) in tumor microenvironment are associated with tumor growth, invasiveness, and metastasis.*
- *Cancer stem cells can be mimicked by using tumorspheres.*
- *A small number of single prostate cells are adequate to can form tumor spheres in an environment where IL-6 is present. These tumor spheres are also larger than those formed in an environment without IL-6.*
- *The expression of stemness biomarkers is upregulated in prostate cancer spheres when they are exposed to IL-6.*
- *The promotion of stemness in prostate tumorspheres by the addition of IL-6 cytokine is driven by an increase in NANOG gene expression.*

Sphere Formation

A total of 30000 DU-145 single cells were seeded in RPMI medium containing EGF (10 ng/mL) (GIBCO, USA) and bFGF (10 ng/mL) (GIBCO, USA), 2% B27 (GIBCO, USA), 1% N2 (GIBCO, USA), and 1% penicillin–streptomycin in ultra low-attachment 6-well plate (Corning Inc., Corning, NY, USA). To assess the sphere-forming potential of a population of cells, between 500 and 2000 cells were seeded in each well of a 24-well plate. Sphere medium was added on the third day and was changed once in a week. Spheres were counted after 10 days. Human recombinant IL-6 protein (Gibco, Thermofisher Scientific) at a concentration of 10 ng/μL was added to the sphere medium to detect IL-6-induced effect. The microscopic images were recorded for each day and the experiments repeated in triplicate.

Ribonucleic Acid Extraction and Reverse Transcription

Spheres were collected from 6-well plates on day 10 for RNA extraction. The media was collected in a centrifuge tube and spin down for 5 minutes at 1200 rpm. The supernatant was removed, and the pellet was stored for RNA extraction. Total RNA was isolated from each sphere pellets by using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, Calif, USA). The purity and yield of RNA were measured by using NanoDrop ND-1000 spectrophotometer. 1000ng of total RNA was reverse-transcribed into cDNA in 20 μL final volume using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland).

Real-Time Polymerase Chain Reaction Experiment

Quantitative reverse transcription polymerase chain reaction was performed by using a StepOnePlus instrument (Applied Biosystems, Foster City, Calif, USA). TaqMan gene expression assays were used to detect OCT4 (Hs04260367_gH), SOX2 (Hs04234836_s1), KLF4 (Hs00358836_m1), and c-MYC (Hs00153408_m1) genes. Polymerase chain reaction (PCR) was set up by mixing 0.5 μL TaqMan Assay, 5 μL TaqMan Universal Master Mix (Applied Biosystems, Rotkreuz, Switzerland), 50 ng of cDNA, and the final volume was completed to 10 μL with 2.5 μL of PCR-grade water. Amplification and real-time data acquisition were run using the following cycle conditions: 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C. The threshold cycle values was used to evaluate the fold change. The expressions of genes were normalized against those of housekeeping genes *YWHAZ* (Hs01122445_g1) and *TBP* (Hs00427620_m1). The 2^{−ΔΔCT} method was used to calculate fold change.¹⁵ The experiments were carried out in triplicate.

Statistical Analysis

Graphpad Prism 8.4.3 (GraphPad Software, Inc. CA, US) was used to analyze the data. *t*-test was applied to compare sphere formation and Wilcoxon rank test was used to analyze fold change of the genes. A *P*-value less than .05 value was considered significant.

Results

We observed DU-145 sphere formation under light microscopy. Both DU-145 spheres and IL-6-induced prostate tumor spheres were successfully generated. In addition, the size of the spheres was larger in the IL-6-treated group (Figure 1). To check the self-renewal capacity of the spheres, we seeded different amounts of single cells in IL-6-treated medium and counted the spheres after 10 days. We showed that PCa cells could generate at least 10 spheres when we initiated with 500 K cells. Starting with 1,000 to 2,000 cells resulted

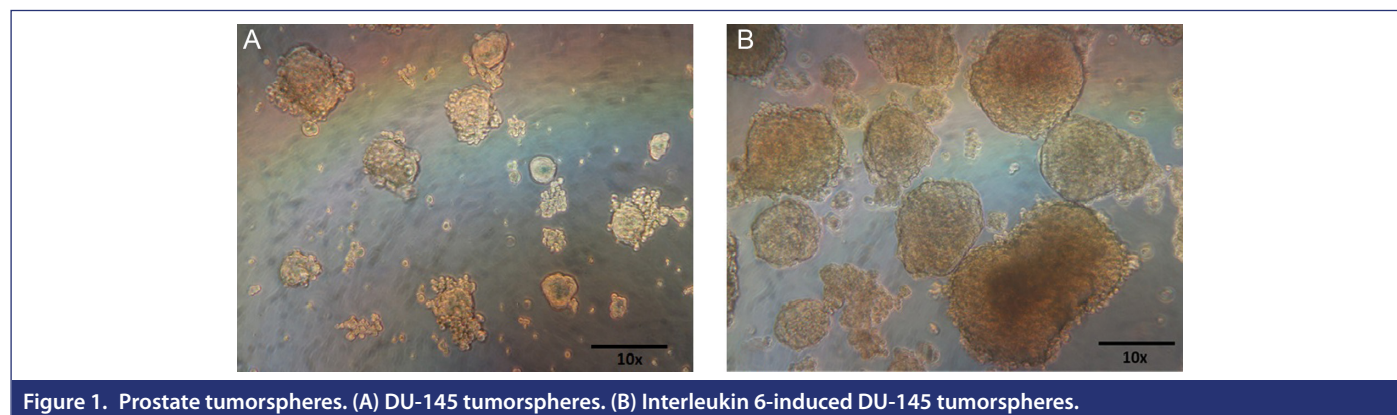


Figure 1. Prostate tumorspheres. (A) DU-145 tumorspheres. (B) Interleukin 6-induced DU-145 tumorspheres.

in a significant increase in the number of tumor spheres formed. The addition of IL-6 to the medium with an initial cell population of 1000 cells yielded approximately 20 spheres, whereas the increase in cell count to 2000 cells corresponded to doubling in the number of spheres formed. A notable upregulation was observed from the initial cell count of 1000 cells onward (Figure 2).

To determine whether IL-6 has an effect on PCa stemness, we collected both tumor spheres and IL-6-treated sphere pellets. OSKM and *NANOG* gene expression levels were measured. Despite a slight upregulation detected in the *SOX2* gene, the alteration was not significant. The expression of *KLF4*, *OCT4*, and *MYC* genes was not changed. However, a dramatic increase was observed in *NANOG* expression on IL-6-treated PCa spheres. A representative image of the fold changes of the genes is shown in Figure 3.

Discussion

The aggressive environment of metastatic PCa was mimicked by adding IL-6 to tumor spheres in this study. We observed that IL-6 promoted cancer stemness, as evidenced by the dramatic increase in *NANOG* expression.

The 5-year survival rate of metastatic PCa is roughly 30%, despite treatment options such as radiation therapy, chemotherapy, hormone reagents for hormone-sensitive PCa, or combination therapies. Understanding the structure of aggressiveness is important to find new and functional therapeutics.

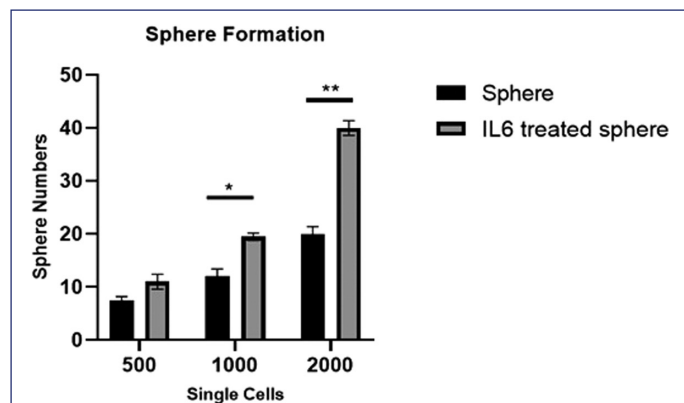


Figure 2. Sphere formation assay.

Cancer stem cells are known to have the ability to differentiate and self-renew. Moreover, they are thought to play a role in cancer relapse, metastasis, and drug resistance.¹⁶ Tumor spheres are 3-dimensional models of CSCs/progenitor cells that are generated from small groups of cancer cells. These stem-like structures ensure to figure out the cancer aggressiveness.¹⁷ Tumorsphere are believed to play a critical role in prostate tumor initiation, progression, and resistance to therapy. In addition, Tumorspheres are thought to better recapitulate the properties of CSCs than traditional 2-dimensional cultures.¹⁸ In our study, we obtain tumor spheres by initiating a low amount of cells, which supports the idea that each sphere originates from a single progenitor cell.¹⁹ Moreover, the addition of IL-6 to the sphere-forming media has resulted in larger, and increased number of spheres, which is thought to cause a more aggressive phenotype. IL-6 is a pro-inflammatory cytokine and acts as a signaling molecule in tumorigenicity. It is well known that both tumor tissue and PCa patients serum have high levels of IL-6.²⁰ It is also demonstrated that IL-6 stimulation boosts PCa proliferation assistant with signal transducer and activator of transcription 3 (STAT3)-activated signaling pathway.²¹ Furthermore, it has a role in regulating stemness, and studies showed that siltuximab treatment, which inhibits STAT3, suppressed the clonogenicity of PCa stem cells.^{13,22}

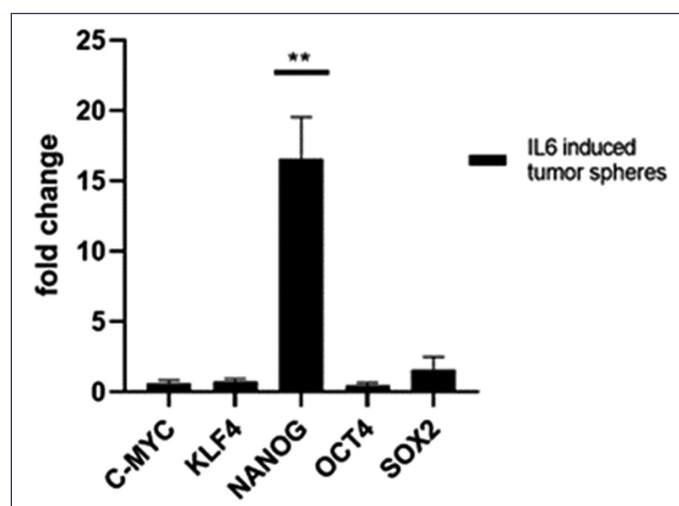


Figure 3. Fold change of the genes in interleukin 6-induced tumor spheres.

In the PCa microenvironment, the pleiotropic IL-6 expression could be the result of any dysregulated mechanisms. Yu et al²³ studied the effect of IL-6 on PC-3 and lymph node carcinoma of the prostate (LNCaP) PCa cells and observed the *SOX2* increment. Drug resistance effect of IL6 on stromal fibroblast was studied by Niu N et al,²⁴ and stemness properties in an IL-6 enriched sphere environment was investigated. According to their results, *NANOG* and *SOX2* were the most affected genes from IL-6 addition. Even though there was a minor increment in *SOX2* expression in our IL-6-induced prostate tumorspheres, it was not significant. When we checked the stemness properties in an IL-6-induced environment, the expression of *OCT4*, *KLF4*, and c-Myc did not change. However, we detected a serious upregulation in *NANOG* expression. The convincing evidence manifested that *NANOG* has a crucial role in self-renewal and differentiation of stem cells, and it could be responsible for the oncogenic process.²⁵ Bao B et al²⁶ previously showed that PCa cells, PC3 and Lncap, increase the level of IL-6 and vascular endothelial growth factor in hypoxic conditions that affect CSCs and increase the level of *NANOG* gene. Shroeder et al²⁷ supported our data by establishing that loss of IL-6 caused the inhibition of CSC population and diminished *NANOG* protein expression. As IL-6 expression upregulate in most of the pathologic conditions, not only hypoxia but also auto-immune and inflammatory situation, we directly induced IL-6 and obtained the results as Bao B et al²⁸ and confirmed the Shroeder et al²⁹ results. The research proved that *NANOG* has a key role in the development of castration-resistant PCa and could be a promising target for the advanced stage of PCa. Considering the significant alteration in gene expression observed only in the *NANOG* gene among the embryonic stemness markers, it can be assumed that IL-6-induced stemness could be dominated by *NANOG*. Recently, it has been demonstrated that there is a strong link between *NANOG* gene and IL-6 signaling in esophageal squamous carcinoma, as *NANOG* is one of the downstream regulators of *STAT3*.³⁰ Therefore, we think that the same mechanism could be responsible for PCa stemness, as *NANOG* promotes the formation of PCa stem cells in an IL-6-rich environment that can be considered as a crucial factor for tumor aggressiveness. Ongoing animal experiments using siNanog or shNanog have shown the promising therapeutic potential of *NANOG* targeting in several types of cancer. Additionally, *NANOG* inhibitors BBI608 or BBI503 were combined with sorafenib in adult patients with hepatocellular carcinoma in a phase II study (NCT02279719).³¹ Promising antitumor effects have been observed in patients with hepatocellular carcinoma who have not undergone systemic chemotherapy previously, and the trial was completed in 2002.

The limitation of this study is that the levels of *NANOG* gene expression was measured in only one type of PCa cell line. In future studies, it is necessary to measure the protein levels of *NANOG* in an IL-6-rich environment.

In this study, *NANOG* is shown to have an important role in the stemness of PCa in an IL-6-enriched environment. Understanding the role of *NANOG* in CSCs has implications for developing targeted therapies and improving cancer treatment outcomes.

Ethics Committee Approval: This is a cell line study that was obtained from a commercial trademark, ethical approval is not required.

Informed Consent: No patients was included in this study, that is why informed consent is not required.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – D.S., D.T.; Design – D.S.; Supervision – Ö.F.B.; Resources – Ö.F.B.; Materials – D.S., Ö.F.B.; Data Collection and/or Processing – D.S., D.T., Ö.F.B.; Analysis and/or Interpretation – D.S., D.T.; Literature Search – D.S.; Writing – D.S.; Critical Review – D.S., Ö.F.B.

Declaration of Interests: The authors have no conflict of interest to declare.

Funding: The authors declared that this study has received no financial support.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2021;71(3):209-249. [\[CrossRef\]](#)
2. Grozescu T, Popa F. Prostate cancer between prognosis and adequate/proper therapy. *J Med Life.* 2017;10(1):5-12.
3. Sekhoacha M, Riet K, Motloung P, Gumenku L, Adegoke A, Mashele S. Prostate cancer review: genetics, diagnosis, treatment options, and alternative approaches. *Molecules.* 2022;27(17) [\[CrossRef\]](#)
4. Harris KS, Kerr BA. Prostate cancer stem cell markers drive progression, therapeutic resistance, and bone metastasis. *Stem Cells Int.* 2017;2017:8629234. [\[CrossRef\]](#)
5. Castellón EA, Indo S, Contreras HR. Cancer stemness/epithelial-mesenchymal transition axis influences metastasis and castration resistance in prostate cancer: potential therapeutic target. *Int J Mol Sci.* 2022;23(23) [\[CrossRef\]](#)
6. Nangia-Makker P, Hogan V, Raz A. Galectin-3 and cancer stemness. *Glycobiology.* 2018;28(4):172-181. [\[CrossRef\]](#)
7. Shi Y, Inoue H, Wu JC, Yamanaka S. Induced pluripotent stem cell technology: a decade of progress. *Nat Rev Drug Discov.* 2017;16(2):115-130. [\[CrossRef\]](#)
8. Sánchez-Sánchez AV, García-España A, Sánchez-Gómez P, Font-de-Mora J, Merino M, Mullor JL. The embryonic key pluripotent factor *NANOG* mediates glioblastoma cell migration via the SDF1/CXCR4 pathway. *Int J Mol Sci.* 2021;22(19) [\[CrossRef\]](#)
9. Paranjape AN, Balaji SA, Mandal T, et al. Bmi1 regulates self-renewal and epithelial to mesenchymal transition in breast cancer cells through Nanog. *BMC Cancer.* 2014;14:785. [\[CrossRef\]](#)
10. Du Y, Ma C, Wang Z, Liu Z, Liu H, Wang T. Nanog, a novel prognostic marker for lung cancer. *Surg Oncol.* 2013;22(4):224-229. [\[CrossRef\]](#)
11. Gawlik-Rzemieniewska N, Bednarek I. The role of *NANOG* transcriptional factor in the development of malignant phenotype of cancer cells. *Cancer Biol Ther.* 2016;17(1):1-10. [\[CrossRef\]](#)
12. Kalali D, Hadjiyianni A, Isaakidou A. The use of interleukin-6 as a biomarker of lung cancer: A systematic review and meta-analysis. *J Cancer Res Ther.* 2023.
13. Culig Z, Puhrl M. Interleukin-6 and prostate cancer: current developments and unsolved questions. *Mol Cell Endocrinol.* 2018;462(A):25-30. [\[CrossRef\]](#)
14. Twillie DA, Eisenberger MA, Carducci MA, Hsieh WS, Kim WY, Simons JW. Interleukin-6: a candidate mediator of human prostate cancer morbidity. *Urology.* 1995;45(3):542-549. [\[CrossRef\]](#)
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods.* 2001;25(4):402-408. [\[CrossRef\]](#)
16. Yu Z, Pestell TG, Lisanti MP, Pestell RG. Cancer stem cells. *Int J Biochem Cell Biol.* 2012;44(12):2144-2151. [\[CrossRef\]](#)

17. Rybak AP, He LZ, Kapoor A, Cutz JC, Tang D. Characterization of sphere-propagating cells with stem-like properties from DU145 prostate cancer cells. *Biochim Biophys Acta*. 2011;1813(5):683-694. [\[CrossRef\]](#)
18. Lee CH, Yu CC, Wang BY, Chang WW. Tumorsphere as an effective in vitro platform for screening anti-cancer stem cell drugs. *Oncotarget*. 2016;7(2):1215-1226. [\[CrossRef\]](#)
19. Bahmad HF, Cheaito K, Chalhoub RM, et al. Sphere-formation assay: three-dimensional in vitro culturing of prostate cancer stem/progenitor sphere-forming cells. *Front Oncol*. 2018;8:347. [\[CrossRef\]](#)
20. Ene CV, Nicolae I, Geavlete B, Geavlete P, Ene CD. IL-6 signaling link between inflammatory tumor microenvironment and prostatic tumorigenesis. *Anal Cell Pathol (Amst)*. 2022;2022:5980387. [\[CrossRef\]](#)
21. Lou W, Ni ZY, Dyer K, Twardy DJ, Gao AC. Interleukin-6 induces prostate cancer cell growth accompanied by activation of Stat3 signaling pathway. *Prostate*. 2000;42(3):239-242. [\[CrossRef\]](#)
22. Kroon P, Berry PA, Stower MJ, et al. JAK-STAT blockade inhibits tumor initiation and clonogenic recovery of prostate cancer stem-like cells. *Cancer Res*. 2013;73(16):5288-5298. [\[CrossRef\]](#)
23. Yu D, Zhong Y, Li X, et al. ILs-3, 6 and 11 increase, but ILs-10 and 24 decrease stemness of human prostate cancer cells in vitro. *Oncotarget*. 2015;6(40):42687-42703. [\[CrossRef\]](#)
24. Niu N, Yao J, Bast RC, Sood AK, Liu JS. IL-6 promotes drug resistance through formation of polyploid giant cancer cells and stromal fibroblast reprogramming. *Oncogenesis*. 2021;10(9):65. [\[CrossRef\]](#)
25. Jeter CR, Liu B, Liu X, et al. NANOG promotes cancer stem cell characteristics and prostate cancer resistance to androgen deprivation. *Oncogene*. 2011;30(36):3833-3845. [\[CrossRef\]](#)
26. Bao B, Ahmad A, Kong DJ, et al. Hypoxia induced aggressiveness of prostate cancer cells is linked with deregulated expression of VEGF, IL-6 and miRNAs that are attenuated by CDF. *PLoS ONE*. 2012;7(8):e43726. [\[CrossRef\]](#)
27. Schroeder A, Herrmann A, Cherryholmes G, et al. Loss of androgen receptor expression promotes a stem-like cell phenotype in prostate cancer through STAT3 signaling. *Cancer Res*. 2014;74(4):1227-1237. [\[CrossRef\]](#)
28. Jeter CR, Yang T, Wang J, Chao HP, Tang DG. Concise review: NANOG in cancer stem cells and tumor development: an update and outstanding questions. *Stem Cells*. 2015;33(8):2381-2390. [\[CrossRef\]](#)
29. Zhang K, Fowler M, Glass J, Yin H. Activated 5' flanking region of NANOG 8 in a self-renewal environment is associated with increased sphere formation and tumor growth of prostate cancer cells. *Prostate*. 2014;74(4):381-394. [\[CrossRef\]](#)
30. Deng L, Zhang XP, Xiang XC, et al. NANOG promotes cell proliferation, invasion, and stemness via IL-6/STAT3 signaling in esophageal squamous carcinoma. *Technol Cancer Res T*. 2021;20:15330338211038492. [\[CrossRef\]](#)
31. Hin Tang JJ, Hao Thng DK, Lim JJ, Toh TB. JAK/STAT signaling in hepatocellular carcinoma. *Hepat Oncol*. 2020;7(1):HEP18. [\[CrossRef\]](#)