

The SW480 cell line, overexpressing PIWIL2 gene, maintains the expression of stemness and proliferation genes in the mice xenografts

Roya Kishani Farahani¹, Ehsan Nazemalhosseini Mojarad², Hamid Reza Soleimanpour-lichaei¹

¹Department of Stem Cells and Regenerative Medicine, Institute of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

²Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ABSTRACT

Aim: This study aims to confirm previous fundamental in vitro findings about the PIWIL2 gene by investigating the effects of its overexpression on cell cycle, proliferation, apoptosis, and stem cell expression markers in colorectal cancer cells (CRC cells) at in vivo level.

Background: PIWIL2 has a critical role in maintaining cellular stemness and proliferation. PIWIL2 is an oncogene whose expression in CRC is associated with the occurrence, metastasis, and poor prognosis.

Methods: SW480 cells harboring expression vectors with/without PIWIL2 were cultured and inoculated in BALB/c nude mice. Tumor formation and growth were monitored every 3 days. On the 28th day after inoculation, the tumors were harvested for their total RNA extraction, and the expression profiling of the candidate genes was performed by Real-time PCR.

Results: Our results for the expression profiling of the xenografted tumors showed a significant increase in the expression of cancer stem cell markers, including CD24, CD133, and pluripotency marker SOX2 in the PIWIL2 over-expressing xenografts, compared to the control cell line. Moreover, PIWIL2 dramatically promoted the anti-apoptotic pathway by inducing STAT3 and BCL2-L1 genes in the PIWIL2 over-expressing xenografts, along with the up-regulation of Cyclin D1 and Ki-67 genes.

Conclusion: This research supports our prior in vitro findings, highlighting the critical role that PIWIL2 plays in the development of CRC and its substantial promise as a leading candidate for CRC-targeted therapy.

Keywords: PIWIL2, Colorectal cancer, Stemness, Proliferation, Apoptosis.

(Please cite as: Kishani Farahani R, Nazemalhosseini Mojarad E, Soleimanpour-lichaei HR. The SW480 cell line, overexpressing PIWIL2 gene, maintains the expression of stemness and proliferation genes in the mice xenografts. *Gastroenterol Hepatol Bed Bench* 2023;16(1):58-64. <https://doi.org/10.22037/ghfbb.v16i1.2661>).

Introduction

Colorectal cancer (CRC) is the world's second leading cause of cancer death (1), primarily due to metastasis (2). Cancer stem cells (CSCs) are a small subpopulation of tumor cells mainly responsible for tumor initiation, development, recurrence, and metastasis (3). CSCs have some putative markers, such

as CD133, CD24, and SOX2. CD133, a five-transmembrane glycoprotein, has been reported in several studies as a surface marker of CSCs (4). The elevated expression level of CD133 is correlated with tumor progression, lymphatic vascular invasion, recurrence, and metastases (5, 6). The cluster of differentiation 24 (CD24), as another CSCs marker, has been correlated with alterations in some oncogenic signaling pathways (7). It has been reported that CD24+/CD133+ populations in CRC are enriched for cancer stem cells (8). The link between CD24 and lymph node metastasis and the accompanying low survival rate, has previously been established (9, 10). The transcription factor Sox2 is a SRY-related HMG-

Received: 13 July 2022 Accepted: 20 September 2022

Reprint or Correspondence: Hamid Reza Soleimanpour-lichaei, Department of Stem Cells and Regenerative Medicine, Institute of Medical Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.

E-mail: hrs@nigeb.ac.ir

ORCID ID: 0000-0003-0173-958X

box (SOX) family member. Its expression is linked to maintaining an undifferentiated stem cell state and poor prognosis (11).

Unlimited proliferation in CSCs, caused by an imbalance between cell cycle and apoptosis, promotes their survival and stemness. This imbalance between apoptosis and proliferation induces tumor formation, especially in the colon, where apoptosis is a key regulator in intestinal turnover (12).

Therefore, the effective strategy for CRC treatment is to explore biomarkers contributing to the survival, proliferation, and self-renewal of CSCs. STAT3 is a key transcription factor strongly linked to CRC initiation and progression by influencing several pathways, such as anti-apoptosis, angiogenesis, invasion, and migration (13). BCL2L1, another main apoptotic inhibitor, was upregulated in 50-70% of CRC samples, which was associated with poorer overall patient survival (14-16). Cyclin D1, as a cell cycle-related gene, regulates the transition from G1 to S phase in the cell cycle (17), while its upregulation drives CRC initiation and development.

Piwi-like RNA-mediated gene silencing 2 (PIWIL2), designated as HILI in humans, is a stem cell-specific protein with central roles in stem cell self-renewal, asymmetric division, gametogenesis, transposon silencing, and chromatin remodeling (18, 19). PIWIL2 belongs to the PIWI family characterized by conserved PAZ and PIWI domains (19, 20). PIWIL2 expression is restricted to male germ cells (21), where the silencing of Piwi protein significantly decreases the rate of their cell division (22). PIWIL2 is reactivated in tumors and plays oncogenic roles in the pathological process of tumor cells. Recent studies revealed that stable PIWIL2 expression was found in the breast's precancerous stem cells (pCSCs), leading to pCSCs development (23). Recently, we established genetically engineered SW480 cells harboring expression vectors with/without the PIWIL2 gene (SW480-PIWIL2 and SW480-control CRC cell lines, respectively) and investigated PIWIL2 influence on some of the key different molecular pathways (24). However, this study aimed to confirm, at in vivo level, the prior fundamental in vitro findings elucidating the role of PIWIL2 overexpression in cell cycle, proliferation, apoptosis, and stemness biomarkers in CRC cells.

Methods

Cell culture and transfection

The SW480 cells were obtained from the National Cell Bank of *Pasteur Institute* (Tehran, Iran). PIWIL2-overexpressing and control SW480 cells were established by transfecting PCDNA3-PIWIL2 and PCDNA3-empty vectors into the SW480 cell line via electroporation, respectively. After 28 days of G418 antibiotic selection (Sigma, 500 μ g/ml), surviving colonies were expanded for further confirmation and analysis (24). The cells were cultured with DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) at 37 °C in a humidified atmosphere of 5% CO₂.

In vivo xenograft experiments

Six BALB/C nude mice were purchased from the Avicenna Research Institute, Shahid Beheshti University (Tehran, Iran). They were randomly divided into 2 groups (three mice per group). All animal procedures were approved by the Experimental Animal Ethics Committee of the National Institute of Genetics Engineering and Biotechnology, Tehran, Iran (approval code: IR. NIGEB.EC.1400.8.17. D). The SW480 overexpressing PIWIL2 or control cells suspensions (1 \times 10⁶ cells) in 100 μ l PBS were subcutaneously injected into the dorsal flanks of nude mice. Tumors growth was examined every three days. After 27 days, tumor samples were carefully removed for further experiments. It should be noted that the three mice from the control group, which were injected with SW480 control cells, did not develop tumor mass after 14 days of injections; hence, these mice were omitted from subsequent research.

After 14 days of injections, tumor mass did not appear in the three mice from the control group, which were injected by SW480 control cells; therefore, these mice were excluded from further experiments.

RNA extraction and cDNA synthesis

The total RNA isolation using High Pure RNA Tissue Kit (Roche, Germany) was applied for RNA extraction, and cDNA synthesis was performed by RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.).

Real-time-PCR

Real-Time PCR was performed using the Real Q Plus 2x Master Mix Green, without ROX

60 Maintains the expression of stemness and proliferation genes in the mice xenografts

(AMPLIQON, Denmark) on Rotor-Gene 6000 HRM Real-Time PCR Thermocycler (Corbett Life Science, Australia). The specificity of the primers was theoretically tested by the BLAST database. The β -actin gene was selected as the internal control. The relative expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ formula. The sequences of all primers are available upon request.

Statistical data analysis

All data were presented as mean \pm SE, and the statistical difference between each group was assessed by Student's t-test using SPSS version 24.0 (SPSS Inc., Chicago, USA). $P < 0.05$ was considered statistically significant as specified for the measurement.

Results

Expression of colorectal cancer stem cell markers

In this research, we investigated the effects of PIWIL2 on CSCs markers expression in xenograft-PIWIL2 compared to SW480-control cells. CD133 gene expression level was significantly upregulated in PIWIL2-xenografts compared to SW480-control cells (Figure 1A; $P=0.016$). Many researchers reported

CD133 as a poor prognostic CSCs marker (25). CD24, another important CSCs in CRC, was also significantly increased in PIWIL2-xenografts compared to SW480-control cells (Figure 1B; $P=0.016$). SOX2 is a stem cell-related biomarker whose expression is associated with the maintenance of CSCs and poor prognosis in CRC. Our data demonstrated that PIWIL2 significantly upregulated the expression of SOX2 in the tumor xenografts, leading to the augmentation of the cancer stem cell state in the xenografted tumor cells (Figure 1C; $P=0.002$). Indeed, we revealed in this study that PIWIL2 could promote the expression of stem cell-specific biomarkers, including CD24, CD133, and SOX2, which are responsible for driving stemness features in tumor cells.

Effects of PIWIL2 on cell cycle progression and apoptotic pathways

Our results showed that STAT3 expression, as a key regulator of several different signaling pathways, significantly increased in the tumor tissues of PIWIL2-xenografts (Figure 2A; $P=0.01$). BCL2-L1 protein is another key regulator of apoptosis involved in CRC tumor formation and progression. Our findings showed that the BCL2-L1 expression level in PIWIL2-xenografts was significantly higher than that of the

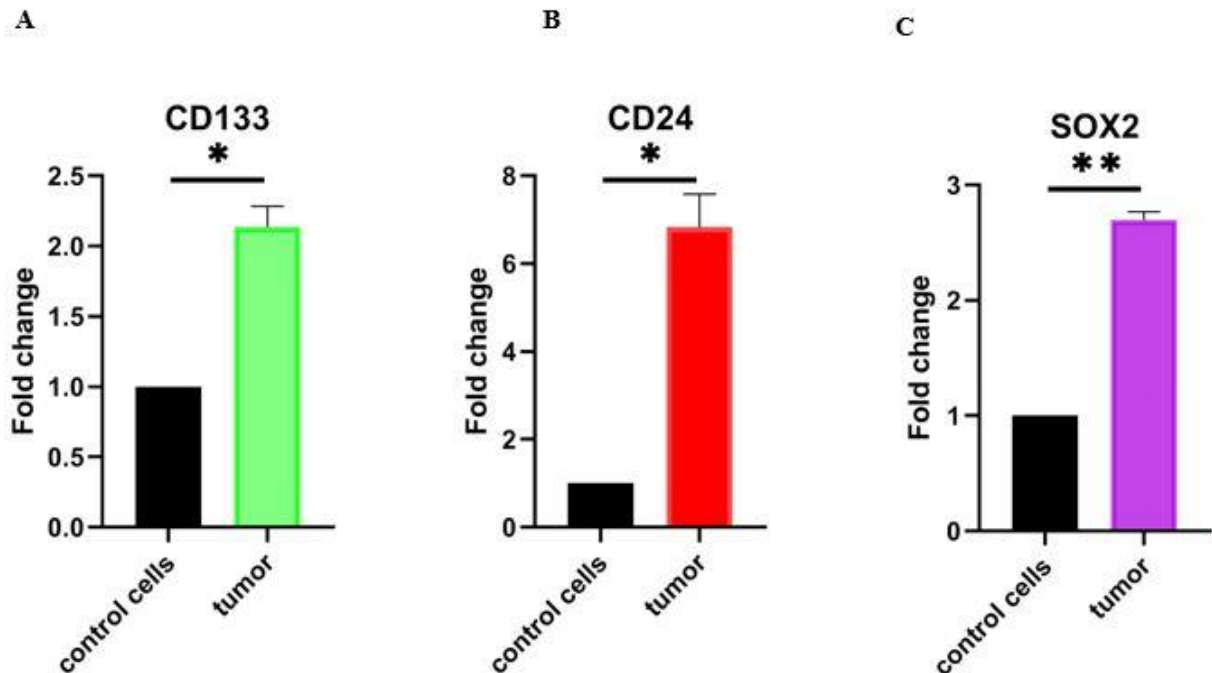


Figure 1. The relative expression level of CSCs markers in the tumor tissues of PIWIL2-xenografts and control cells, including CD133 (A), CD24 (B), and SOX2 (C). * $P \leq 0.05$, ** $P \leq 0.01$.

control SW480 cells (Figure 2A; $P=0.006$). Cyclin D1, as a main cell cycle regulator gene, was also investigated in this research. Results showed that PIWIL2 statistically upregulated Cyclin D1 in the tumor tissues of PIWIL2-xenografts compared to control cells (Figure 2B; $P=0.004$). Significantly increased expression of Ki-67, as a specific proliferative biomarker, in PIWIL2-xenografts confirms the above findings that PIWIL2 induction of colorectal cancer is accomplished through cell cycle acceleration inhibiting apoptosis (Figure 2C; $P=0.004$) (26, 27).

Discussion

We previously demonstrated that PIWIL2 is a key component for colorectal cancer stem cell generation, self-renewal, and maintenance (unpublished data). Our findings revealed that PIWIL2 modulated various signaling pathways that contributed to the stemness of colorectal cancer stem cells. Moreover, we have shown that CCSCs need to activate PIWIL2 to maintain self-renewal and obtain pluripotency by upregulating the genes. In addition, our findings demonstrated that PIWIL2 was an essential director for promoting cellular proliferation while inhibiting apoptosis in CRC cells (24). PIWIL2 also induced the expression of the cell cycle regulator genes' expression, leading to the cell cycle's acceleration in vitro. In fact, PIWIL2

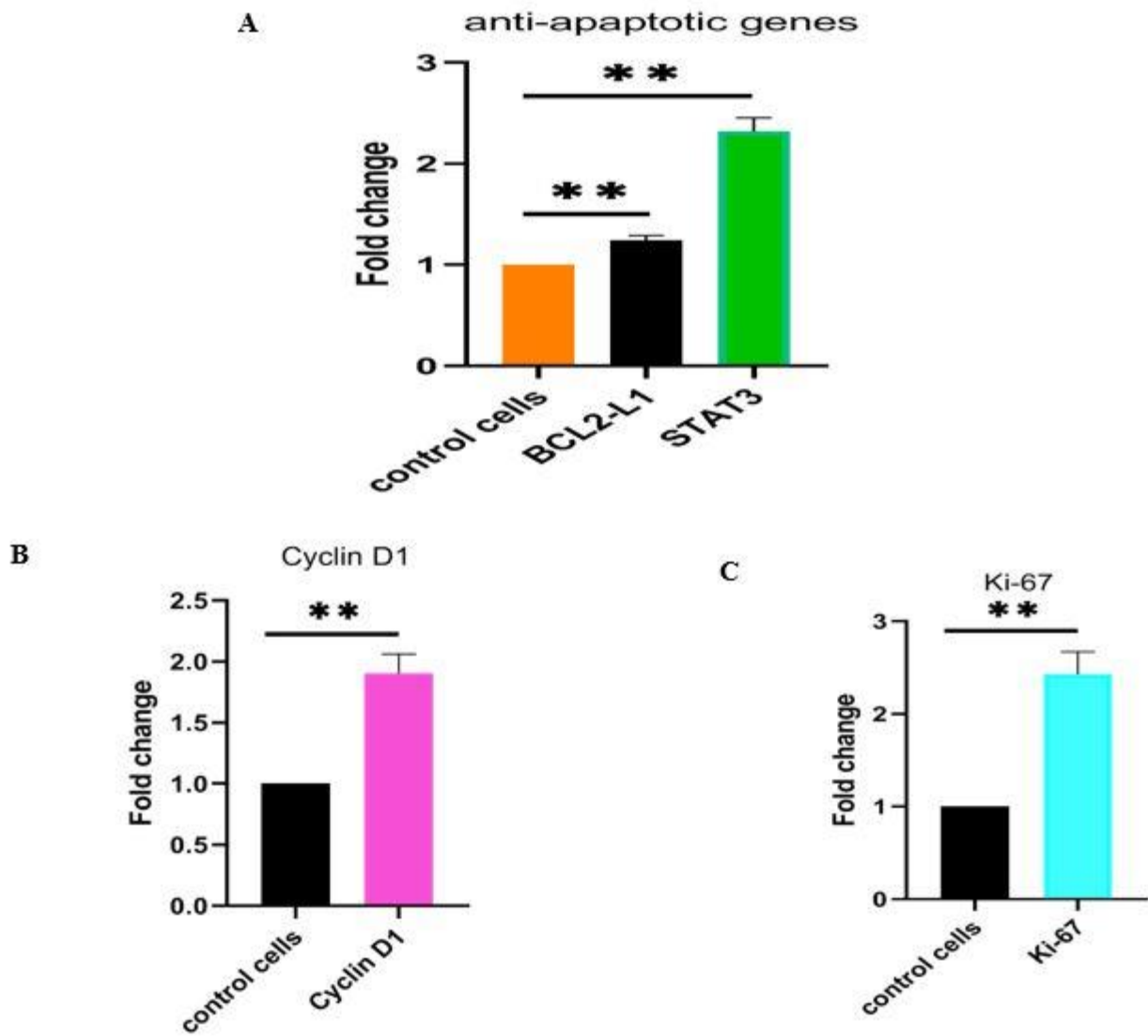


Figure 2. The relative expression level of anti-apoptotic genes (A), cell cycle-related marker (B), and proliferative marker (C) in the tumor tissues of PIWIL2-xenografts and control cells. $^{**}P \leq 0.01$.

62 Maintains the expression of stemness and proliferation genes in the mice xenografts

induces an invasive downstream cascade in CCSCs by regulating multiple signaling pathways that control the survival, proliferation, self-renewal, anti-apoptosis, and cell cycle in CCSCs. To confirm our previous *in vitro* experiments, we inoculated our previously established SW480 cells carrying expression vectors with or without PIWIL2 into BALB/c nude mice. We then analyzed the expression profile of the resulting xenografted tumors. *In vivo* results confirmed our previous findings that PIWIL2 promoted the expression of cancer stem cell markers, pluripotency markers, anti-apoptotic pathways, cell cycle-related genes, and proliferation genes in the tumor tissues of PIWIL2-xenografts. Our results supported the previous reports that the degree of PIWIL2 expression was higher in colorectal carcinomas and was significantly associated with poor prognosis (28). Following our findings, Li et al. found a significant correlation between PIWIL2 expression and worse clinical and pathological characteristics, as well as poorer five-year metastasis-free survival and overall survival in colon cancer (29). CSCs are mainly responsible for tumor initiation, recurrence, metastasis, and drug resistance. Findings from *in vivo* experiments in this study showed that PIWIL2 could induce cancer stem cell markers and pluripotency genes, such as CD133, CD24, and SOX2, to promote stemness features of CRC tumors. Consistent with our data demonstrating that PIWIL2 can induce CSCs features, Shahali et al. established a cancer stem-like cell line by overexpressing PIWIL2 in the non-cancerous mouse embryonic fibroblasts with higher expression for stem cell markers of CD44 and CD133 (30).

In contrast to our findings, however, their cancer stem cell line expressed a lower level of CD24 than control cells. In addition, another study found that PIWIL2-transfected fibroblasts expressed the pluripotency markers OCT-4, NANOG, SOX-2, KLF-4, and C-MYC (31). Further, in this research, Cyclin D1 and Ki-67 expressions were significantly increased in the tumor tissues of PIWIL2-xenografts, demonstrating the highly proliferative features of these cells. Consistent with our findings, it has been reported that inhibiting Piwil2 reduces cancer cell proliferation and colony formation ability, increases apoptosis in SW620 and SW480 colon cancer cell lines, and inhibits

tumor growth *in vivo* in colon cancer patients (29). In addition, it has been demonstrated that PIWIL2 expression in breast cancers was associated with ER and Ki-67 and cancer development (32). PIWIL2 mediated cell cycle progression by regulating various types of cell cycle-related genes. In non-small cell lung cancer (NSCLC), PIWIL2 regulated the progression of NSCLC cells by controlling CDK2 and Cyclin A (33). Lack of CDK2 and Cyclin A leads to apoptosis (34) which is another mechanism to regulate PIWIL2-associated anti-apoptotic effects in different cancers. In colorectal cancer, we showed that PIWIL2 had anti-apoptotic effects by upregulating STAT3 and Bcl2-L1 both *in vitro* and *in vivo*. Similarly, in breast cancer cells, PIWIL2 silencing suppressed the expression of STAT3, a pivotal regulator of Bcl-X(L) and cyclin D1, whose downregulation reduced cell proliferation and survival (35).

Conclusion

Based upon our findings and previous research, we can conclude that PIWIL2 plays significant role in CRC development and thus has the potential to be an effective candidate for cancer-targeted therapy.

Acknowledgment

This study was supported by a research grant from National Institute of Genetic Engineering and Biotechnology (Grant no. 443).

Conflict of interests

The authors declare that they have no competing interests.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021;71:209-249.
2. Pretzsch E, Bösch F, Neumann J, Ganschow P, Bazhin A, Guba M, et al. Mechanisms of metastasis in colorectal cancer and metastatic organotropism: hematogenous versus peritoneal spread. *J Oncol* 2019;2019.
3. Fahmi MN, Hertapanndika IN, Kusuma F. The prognostic value of cancer stem cell markers in cervical

- cancer: a systematic review and meta-analysis. *Asian Pac J Cancer Prev* 2021;22:4057-4065.
4. Ren F, Sheng WQ, Du X. CD133: a cancer stem cells marker, is used in colorectal cancers. *World J Gastroenterol* 2013;19:2603-2611.
 5. Iinuma H, Watanabe T, Mimori K, Adachi M, Hayashi N, Tamura J, et al. Clinical significance of circulating tumor cells, including cancer stem-like cells, in peripheral blood for recurrence and prognosis in patients with Dukes' stage B and C colorectal cancer. *J Clin Oncol* 2011;29:1547-1555.
 6. Jao SW, Chen SF, Lin YS, Chang YC, Lee TY, Wu CC, et al. Cytoplasmic CD133 expression is a reliable prognostic indicator of tumor regression after neoadjuvant concurrent chemoradiotherapy in patients with rectal cancer. *Ann Surg Oncol* 2012;19:3432-3440.
 7. Panagiotou E, Syrigos NK, Charpidou A, Kotteas E, Vathiotis IA. CD24: a novel target for cancer immunotherapy. *J Pers Med* 2022;12:1235.
 8. Haddock S, Alban TJ, Turcan Ş, Husic H, Rosiek E, Ma X, et al. Phenotypic and molecular states of IDH1 mutation-induced CD24-positive glioma stem-like cells. *Neoplasia* 2022;28:100790.
 9. Belov L, Zhou J, Christopherson RI. Cell surface markers in colorectal cancer prognosis. *Int J Mol Sci* 2010;12:78-113.
 10. Lee J-H, Kim S-H, Lee E-S, Kim Y-S. CD24 overexpression in cancer development and progression: a meta-analysis. *Oncol Rep* 2009;22:1149-1156.
 11. Walcher L, Kistenmacher A-K, Suo H, Kitte R, Dluczek S, Strauß A, et al. Cancer stem cells-origins and biomarkers: perspectives for targeted personalized therapies. *Front Immunol* 2020;11:1280.
 12. Ramesh P, Medema JP. BCL-2 family deregulation in colorectal cancer: potential for BH3 mimetics in therapy. *Apoptosis* 2020;25:305-320.
 13. Gargalionis AN, Papavassiliou KA, Papavassiliou AG. Targeting STAT3 signaling pathway in colorectal cancer. *Biomedicines* 2021;9:1016.
 14. Jin-Song Y, Zhao-Xia W, Cheng-Yu L, Xiao-Di L, Ming S, Yuan-Yuan G, et al. Prognostic significance of Bcl-xL gene expression in human colorectal cancer. *Acta Histochem.* 2011;113:810-814.
 15. Han HS, Park YM, Hwang TS. Differential expression of Bcl-2, Bcl-XL and p53 in colorectal cancer. *J Gastroenterol Hepatol* 2006;21:1108-1114.
 16. Li W, Ma Y, He L, Li H, Chu Y, Jiang Z, et al. Protease-activated receptor 2 stabilizes Bcl-xL and regulates EGFR-targeted therapy response in colorectal cancer. *Cancer Lett* 2021;517:14-23.
 17. Alao JP. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. *Mol Cancer* 2007;6:1-16.
 18. Lee JH, Schütte D, Wulf G, Füzesi L, Radzun H-J, Schweyer S, et al. Stem-cell protein Piwil2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-XL pathway. *Hum Mol Genet* 2006;15:201-211.
 19. Li J, Xu L, Bao Z, Xu P, Chang H, Wu J, et al. High expression of PIWIL2 promotes tumor cell proliferation, migration and predicts a poor prognosis in glioma. *Oncol Rep* 2017;38:183-192.
 20. Sasaki T, Shiohama A, Minoshima S, Shimizu N. Identification of eight members of the Argonaute family in the human genome☆. *Genomics* 2003;82:323-330.
 21. Kuramochi-Miyagawa S, Kimura T, Yomogida K, Kuroiwa A, Tadokoro Y, Fujita Y, et al. Two mouse piwi-related genes: miwi and mili. *Mech Dev* 2001;108:121-133.
 22. Cox DN, Chao A, Lin H. Piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* 2000;127:503-514.
 23. Chen L, Shen R, Ye Y, Pu X-A, Liu X, Duan W, et al. Precancerous stem cells have the potential for both benign and malignant differentiation. *PloS one* 2007;2:293.
 24. Kishani-Farahani R, Soleimanpour S, Golmohammadi M, Soleimanpour-lichaei H. PIWIL2 regulates the proliferation, apoptosis and colony formation of colorectal cancer cell line. *Iran J Biotechnol.* in press.
 25. Akbari M, Shomali N, Faraji A, Shanehbandi D, Asadi M, Mokhtarzadeh A, et al. CD133: an emerging prognostic factor and therapeutic target in colorectal cancer. *Cell Biol Int* 2020;44:368-380.
 26. Sun X, Kaufman PD. Ki-67: more than a proliferation marker. *Chromosoma* 2018;127:175-186.
 27. Richardsen E, Andersen S, Al-Saad S, Rakae M, Nordby Y, Pedersen MI, et al. Evaluation of the proliferation marker Ki-67 in a large prostatectomy cohort. *PloS one* 2017;12:0186852.
 28. Oh S-J, Kim S-M, Kim Y-O, Chang H-K. Clinicopathologic implications of PIWIL2 expression in colorectal cancer. *J Pathol Transl Med* 2012;46:318-323.
 29. Li D, Sun X, Yan D, Huang J, Luo Q, Tang H, et al. Piwil2 modulates the proliferation and metastasis of colon cancer via regulation of matrix metalloproteinase 9 transcriptional activity. *Exp Biol Med* 2012;237:1231-1240.

64 Maintains the expression of stemness and proliferation genes in the mice xenografts

30. Shahali M, Kabir-Salmani M, Nayernia K, Soleimanpour-Lichaei HR, Vasei M, Mowla SJ, et al. A novel in vitro model for cancer stem cell culture using ectopically expressed piwil2 stable cell line. *Cell J (Yakhteh)* 2013;15:250.
31. Zhang D, Wu X, Liu X, Cai C, Zeng G, Rohozinski J, et al. Piwil2-transfected human fibroblasts are cancer stem cell-like and genetically unstable. *Oncotarget* 2017;8:12259.
32. Liu JJ, Shen R, Chen L, Ye Y, He G, Hua K, et al. Piwil2 is expressed in various stages of breast cancers and has the potential to be used as a novel biomarker. *Int J Clin Exp Pathol* 2010;3:328.
33. Qu X, Liu J, Zhong X, Li X, Zhang Q. PIWIL2 promotes progression of non-small cell lung cancer by inducing CDK2 and Cyclin A expression. *J Transl Med* 2015;13:1-10.
34. Katayose Y, Kim M, Rakkar AN, Li Z, Cowan KH, Seth P. Promoting apoptosis: a novel activity associated with the cyclin-dependent kinase inhibitor p27. *Cancer Res* 1997;57:5441-5445.
35. Lee JH, Jung C, Javadian-Elyaderani P, Schweyer S, Schütte D, Shoukier M, et al. Pathways of proliferation and antiapoptosis driven in breast cancer stem cells by stem cell protein Piwil2 in breast cancer stem cells. *Cancer Res* 2010;70:4569-4579.