Original Article

Stem Cell Protein PIWIL2 Promotes EMT Process and Stem Cell-Like Properties in MCF7 Breast Cancer Cell Line

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Abstract

Background: Piwi-like RNA-mediated gene silencing 2 (*PIWIL2*) is a member of *AGO/PIWI* gene family, which is enriched in cancer stem cells (CSCs). The purpose of this research was to investigate the overexpression of *PIWIL2* and its role in the induction of EMT and CSC properties in MCF7 breast cancer cell line.

Materials and Methods: MCF7 cells were transfected with the human gene *PIWIL2* (*Hili*) under the control of CMV promoter utilizing the neon electroporation method. Subsequently, the selection was conducted using G418, and doubling time was calculated in the transformed and control cells. RT and real-time PCR were also performed to analyze the expression of epithelial and mesenchymal genes and those related to CSCs.

Results: According to the observations from this study, transfecting MCF7 cells with *PIWIL2* triggered the conversion of epithelial cells to mesenchymal cells and induced the genes specific for breast CSCs, which was coincident with 9-h reduction in the doubling time of the transfected cells. Furthermore, the molecular analyses revealed a significant reduction in the expression of epithelial markers, while a significant increase was detected in the expression of mesenchymal genes and many CSC biomarkers.

Conclusion: PIWIL2 protein acts as a master regulatory protein that is able to manipulate the transcription through specific signaling pathways, which allow the cells to gain stem cell-like properties.

Keywords: Breast cancer, epithelial–mesenchymal transition (EMT), PIWIL2, stem cells

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Introduction

Breast cancer is the most prevalent cancer in women worldwide, and distant site metastasis is the main cause of cancer-related deaths. Therefore, effective cancer therapy requires a perfect understanding of the processes involved in metastasis and presenting approaches to deal with this phenomenon. Metastasis is a complex event that includes a series of consecutive and interrelated steps, and its initiation requires an invasion stage, in which cancer cells undergo a special process called epithelial–mesenchymal transition (EMT).[1] EMT is the main trigger of cancer progression and results from the activation of transcription factors that regulate gene expression to alter cytoskeletal dynamics, which causes loss of cell–cell adhesion and transition from epithelial to mesenchymal morphology.[2] In this stage, spindle, mobile, and flexible cells are created with stem cell-like characteristics, which have a high resistance to cell death and a high ability to migrate and invade.[1] The loss of *E-Cadherin* has been considered a fundamental event in the EMT process. EMT transcription factors (EMT-TF) such as ZEB2, SLUG, and SNAIL suppress the expression of *E-Cadherin* through binding to its promoter region. This, in

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turn, induces EMT and progresses cancer to a more aggressive state. During this process, the expression of mesenchymal markers increases, whereas the expression of epithelial genes decreases via specific signaling pathways.[3] In addition, since EMT is associated with an enrichment of cancer stem (-like) cells (CSCs), the expression of markers specific for stem cells such as *CD44* and *OCT4* is increased in this process.[4] Some EMT-related clinical evidence showed that circulating tumor cells (CTCs) in patients with progressive metastatic breast cancer expressed both epithelial and mesenchymal markers simultaneously. This result suggested a possible association between CTCs and EMT.[5] Accordingly, the expression of EMT-related markers in basal-like breast cancer (BLBC) may be associated with tumor invasions.[6] Piwi-like RNA-mediated gene silencing 2 (*PIWIL2*: alias *Hili* in humans) belongs to the Argonaute/PIWI family and functions in numerous biological processes such as spermatogenesis, controlling self-renewal, maintenance of germline stem cells, apoptosis inhibition, and cell invasion.[7-10] Moreover, *PIWIL2* overexpression has been identified in a variety of human cancers, including breast cancer.[11] It was reported that about 90% of invasive breast cancer-related specimens exhibit the predominant expression of *PIWIL2,* which is connected with the progression of lymph node metastases.[9] Generally, *PIWIL2*, as an oncogene, is highly expressed in CSCs and breast cancers and regulates the proliferation and anti-apoptotic status of these cells through the Stat3/Cyclin D1/Bcl_{xl} signaling pathways.[12] Recently, Kishani *et al*. demonstrated the oncogenic role of PIWIL2 protein in regulating the proliferation, apoptosis, and colony formation of colorectal cancer cells.[13] Also, other studies revealed that the expression of *PIWIL2* is associated with the proliferation and migration of tumor cells in glioma tissues.[14,15] *PIWIL2* plays a critical role in the development, differentiation, and regulation of pre-CSCs (pCSCs).[16] Recently, it was shown that *PIWIL2*-induced CSC (*PIWIL2*-iCSC)-derived exosomes induce cancer-associated phenotype in fibroblasts, promoting proliferation, migration, and invasion in these cells.[17] In addition, a further study revealed that enhanced expression of *PIWIL2*-induced *CD44* in fibroblasts leads to the transformation of these cells and makes them more aggressive.[9] According to this knowledge, *PIWIL2* was hypothesized as an important causal factor in promoting invasion and metastasis. Therefore, this study aimed to investigate the effects of *PIWIL2* overexpression on the induction of EMT process and generation of CSC-like properties in MCF7 breast cancer cells. In addition, different influences of *PIWIL2* ectopic expression on enhancing endogenous *Hili* expression and adjusting its two principal transcripts (V1 and V3), which encode the PIWIL2 protein with 973 amino acids, were also reviewed in this study. Among the most common breast cancer cell lines, we chose MCF7 as a noninvasive cell line, which proves to be a suitable model for breast cancer investigations, including those regarding EMT, invasion, and metastasis. Also, MCF7 cells exhibit characteristics of differentiated mammary epithelial cells; they are positive for epithelial markers and negative for mesenchymal markers, as well as expressing low levels of CD44.

Materials and Methods

Cell line and culture conditions

The MCF7 human breast cancer cell line (ATCC® HTB-22™) was obtained from Pasteur Institute, Iran, and was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, UK), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, UK), 100 U/ml of a penicillin/streptomycin mixture (Gibco, UK) at 37°C, and 5% CO2.

Plasmid preparation and transfection

The pcDNA3-PIWIL2 plasmid is an expression vector containing the human *PIWIL2* gene under the control of CMV promoter, which had been previously constructed. Since high concentrations of this construct are required for the plasmid transfection into mammalian cell lines, plasmid maxipreparation was performed using a HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. Next, the plasmid was completely linearized with the PvuI restriction enzyme to establish the stable expression of the desired gene in the transfected cells. Then, it was purified using a QIAquick Gel Extraction Kit (Qiagen, USA). The concentration and quality of the plasmid preparations were controlled by spectrophotometry and gel electrophoresis.

MCF7 cells were electroporated with both plasmids encoding *PIWIL2* (pcDNA3-PIWIL2) and empty pcDNA3 vectors by Neon™ Transfection System (Invitrogen, USA) according to the manufacturer's instructions (pulse voltage: 950 V, pulse width: 40 ms, pulse number: 2, cell density: 5×10^6 cells/ml). For each transfection, 5 µg of total plasmid DNA was used. The MCF7 transfectants were selected by growth in a medium containing 500, 600, and 700 µg/ml of G418 (Geneticin; Sigma, German) for 4 weeks. Two stable cell lines, MCF7-pcDNA3 (control) and MCF7-pcDNA3-PIWIL2, were established for further experiments.

RNA extraction and cDNA synthesis

Total RNA was extracted from MCF7 and MCF7-PIWIL2 cells using RNX-Plus solution (SinaClon, Iran) following the manufacturer's protocol. RNA quantities were measured with the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, USA), and concentrations were adjusted. cDNA synthesis (oligo-dT method) was performed using the First-Strand Synthesis Kit (Thermo Scientific).

In silico sequence analysis

The main transcript variant sequences of *PIWIL2* were acquired from Ensembl [Table 1]. Variant 1 and variant 3 have 23 exons, and the first exons in both variants are noncoding and different in size. However, they share an identical open reading frame (ORF), generating the same peptide (973 aa) (http://atlasgeneticsoncology.org/Genes/GC_PIWIL2.html). Furthermore, to develop the primer sets

for some genes, the specific exon junction spanning primers were designed using AlleleID (version 7.7; PREMIER Biosoft International, Palo Alto, CA, USA) and Oligo7 primer analysis software and checked online by PubMed/blast.

RT-PCR and real-time RT-PCR analysis

After cDNA synthesis, RT-PCR was performed with Taq DNA Polymerase (Amplicon, Denmark) in a Peqlab thermocycler using specific primers for *E-Cadherin*, *ZO-1*, *PCNA*, *PIWIL2*, *Vimentin*, and *Fibronectin* genes. The results were subsequently assessed by agarose gel electrophoresis. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) housekeeping gene was used as the reference gene. Quantitative real-time PCR was also performed to analyze the mRNA expression level of epithelial genes, including *E-Cadherin*, *ZO-1*, Claudin-1, *OVOL2*, and mesenchymal genes, namely *Vimentin*, *Fibronectin*, *ZEB2*, *SLUG*, and *SNAIL*, as well as those related to CSCs such as *CD44*, *OCT4*, *PIWIL2*, *ALDH1A1*, and also *PCNA* as a cell proliferation marker. All quantitative RT-PCR experiments were performed in triplicate using the Power SYBR® Green Master Mix Kit (Thermo, USA) with a Corbett Rotor-Gene System. 100 ng of cDNA was applied as a template DNA for all PCRs. To calculate the relative changes in gene expression, the $2^{-\Delta\Delta^{CT}}$ method was employed and the data were presented as the fold changes in the expression of the target genes in MCF7-PIWIL2 cell line normalized to the reference gene (*GAPDH*) and relative to the MCF7-Mock cell line. The primer sequences are listed in Supplementary File 1.

Proliferation assay

To measure the doubling time intervals of both MCF7-PIWIL2 and MCF7-Mock cell lines, the cells were seeded separately at a density of 7×10^4 cells/ml in 24-well plates at day zero. The plates were incubated in standard laboratory conditions for approximately 5 days. Whenever needed, the cell culture media were replaced with 2 ml of fresh-supplemented DMEM. Every 24 hours after seeding, the culture medium of one well per cell line was removed and 0.25% trypsin/1 mM EDTA solution (Sigma-Aldrich, Germany) was added for 1-2 minutes. After trypsinization, an equal amount of medium (DMEM containing 10% FBS) was added, followed by pipetting up and down a few times to produce a single-cell solution and inactivate the trypsin. Subsequently, all the contents were removed from the well and transferred to a 2-ml Eppendorf tube. Cells were collected by centrifugation (1000 × g for 5 minutes) and then resuspended by gently pipetting to dissociate the clumps. 100 µL of cell suspension was then taken out into a new Eppendorf tube, and 400 µL 0.4% trypan blue was added. Afterward, 100 µL of trypan blue-treated cell suspension was applied to a hemocytometer before counting the live cells. The cell concentration for each cell line was measured at 24-hour intervals. Experiments were performed in four replicate culture wells for each group. Population doubling time (PDT) was calculated by the following equation: $DT = \ln 2 \times T / \ln (N_T/N_0)$, where T is the culture time, N_T is the cell number at the end of a passage, and N_0 is the cell number at the beginning of a passage.

Table 1: The accession number for the *PIWIL2* transcript variant sequences acquired from Ensembl

Transcript variants of human *PIWIL2*	Accession number
Variant 1	(PIWIL2-001; ENST00000356766)
Variant 3	(PIWIL2-003; ENST00000454009)

Statistical analysis

All statistical analyses were assessed using a *t*-test for the differences in gene expression between the two groups by Statistical Package for the Social Sciences (SPSS) version 19 statistical software (SPSS, Chicago, IL, USA). A value of $P < 0.05$ was considered to indicate statistically significant differences.

Results

Establishment of a stable MCF7-PIWIL2 cell line

After transfecting the MCF7 cell line with pcDNA3 plasmids with and without the coding sequence of *PIWIL2* and establishing MCF7-PIWIL2 and MCF7-Mock cell lines, respectively, the overexpression of *PIWIL2* in the transfected cells at the RNA level was confirmed by RT-PCR and qRT-PCR [Figures 1 and 2]. Quantitative analysis of *PIWIL2* gene expression was performed for the main transcript variants of endogenous *PIWIL2* (V1 and V3) and also the endogenous variants together with the exogenous *PIWIL2*, as *PIWIL2* total. The results determined that *PIWIL2* mRNA expression in the MCF7-PIWIL2 cell line had a significant increase compared with the control group (approximately 1200-fold). Our findings also indicated that *PIWIL2* overexpression significantly increased the expression of both main mRNA variants of endogenous *PIWIL2* (V1 and V3) in transfected cells in comparison with the control group [Figure 1].

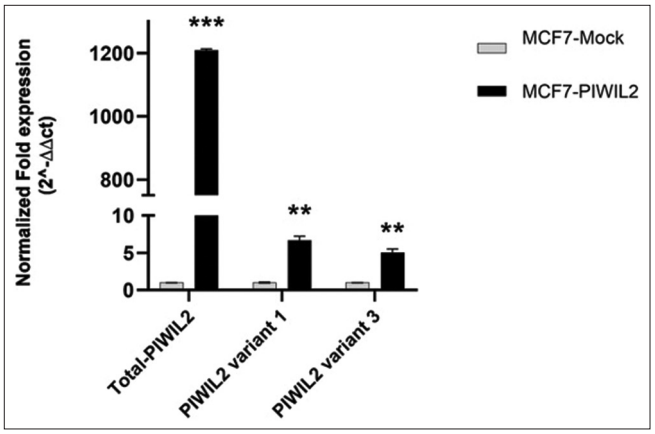

Figure 1: Quantitative expression analysis of *PIWIL2* gene. Overexpression of *PIWIL2* at RNA level in transfected cells was confirmed by qRT-PCR and normalized to *GAPDH* expression. The results of qRT-PCR showed increased expression of main transcript variants of endogenous *PIWIL2*. Data represent mean ± SEM, (***$P < 0.001$, **$P < 0.01$)

The overexpression of PIWIL2 triggers EMT

Ectopic expression of PIWIL2 promotes morphological changes from epithelial to mesenchymal phenotype

The results of morphological investigations in transfected cells (MCF7-PIWIL2) revealed that polygonal epithelial cells were directly converted to mesenchymal spindles due to the overexpression of *PIWIL2*. Furthermore, the indirect transformation of epithelial cells initially into stem-like cells and subsequently into mesenchymal cells was observed [Supplementary File 2]. In fact, cells with indirect conversion became smaller and then switched to circular cells during the selection period with G418 antibiotic. These cells remained in stem-like shape for about 20 days and then suddenly developed into colonies of spindle-shaped cells. In other words, these colonies were obtained from the proliferation and transformation of stem-like cells into mesenchymal cells. It is notable that a significant population of mesenchymal stem-like cells was also observed in this stage. Overall, it can be stated that direct and indirect conversions of the epithelial cells may be related to *PIWIL2* expression levels. Accordingly, it may be concluded that using higher doses of antibiotics resulted in selecting cells with more copy number of the plasmid carrying *PIWIL2* gene, and higher expression of *PIWIL2* in these cells led to the cancer stem-like cell formation, cell differentiation, and eventually production of mesenchymal cells. Therefore, MCF7-PIWIL2 cells lost their cobblestone epithelial shape and gained spindle-like mesenchymal morphology with the manifestation of stem cell-like colonies [Figure 3].

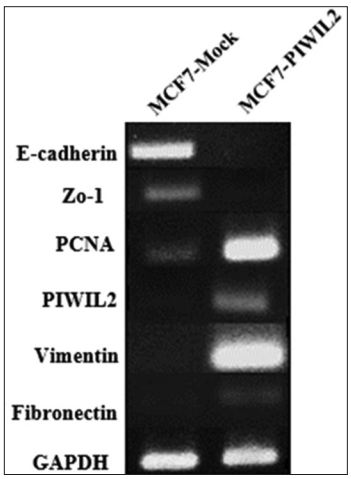

Figure 2: Expression analysis of *E-Cadherin*, *ZO-1*, *PCNA*, *PIWIL2*, *Vimentin*, and *Fibronectin* genes using semi-quantitative RT-PCR. *GAPDH* RT-PCR as a control for RNA integrity

Quantitative expression analysis of EMT biomarkers

To assess whether increased expression of *PIWIL2* can affect the expression of EMT markers, qRT-PCR was performed. The results showed that the overexpression of *PIWIL2* induced a remarkable increase in the expression of mesenchymal genes, and the expression of *SLUG*, *SNAIL,* and *Fibronectin* in MCF7-PIWIL2 cell line was enhanced by approximately 16-, 9.7-, and 3-fold, respectively, in comparison with Mock cells. Surprisingly, forced expression of *PIWIL2* led to a predominant increase in the expression of *Vimentin* and *ZEB2* genes [Figure 4 panels a, b, 2]. Analysis of epithelial gene expression including *E-Cadherin*, *ZO-1*, *OVOL2,* and Claudin-1 was as follows: *E-Cadherin*, *ZO-1,* and *OVOL2* mRNA expression was significantly decreased in MCF7-PIWIL2 cells compared with MCF7-Mock, whereas unexpectedly, Claudin-1 mRNA expression in transfected cells was significantly higher than control (almost 1.6-fold) [Figure 4—panel c, 2]. The expression status of epithelial and mesenchymal markers in MCF7-PIWIL2 as compared to MCF7-Mock cells is shown in Figure 4—panel d.

MCF7-PIWIL2 generates cells with stemness characteristics

As mentioned, the overexpression of *PIWIL2* induced EMT and altered cell morphology toward forming mesenchymal and stem-like cells. In addition to this cellular transformation in the creation of stem-like cells, there were several multinucleated giant cells whose membranes had tended to polarize into spindle-shaped cell structures. Subsequently, further proliferation of these cells and other stem cells, as a distinct subset of cells, led to the formation of dome-shaped cell structures referred to as mammospheres, which are characteristic of breast CSCs [Figure 5a and b].

To examine the stemness of these cells on a molecular level, we analyzed the expression of CSC biomarkers including *CD44*, *OCT4,* and *ALDH1A1* using the quantitative PCR method. The results indicated that the expression of *CD44* and *OCT4* was dramatically increased in MCF7-PIWIL2 cells compared with MCF7-Mock. Moreover, the upregulation of *ALDH1A1* was also observed in MCF7-PIWIL2 cells [Figure 5c].

The effect of PIWIL2 overexpression on cell proliferation

To evaluate the effect of *PIWIL2* on cell proliferation, a doubling time assay was performed to calculate the population

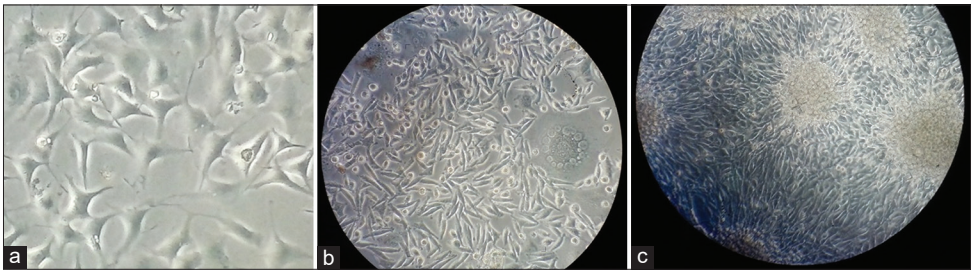

Figure 3: Inverted microscope images of MCF7 cell lines (Mock, PIWIL2). MCF7 cells (a) MCF7-PIWIL2 cells (b) MCF7-PIWIL2 stem cell-like colonies (c)

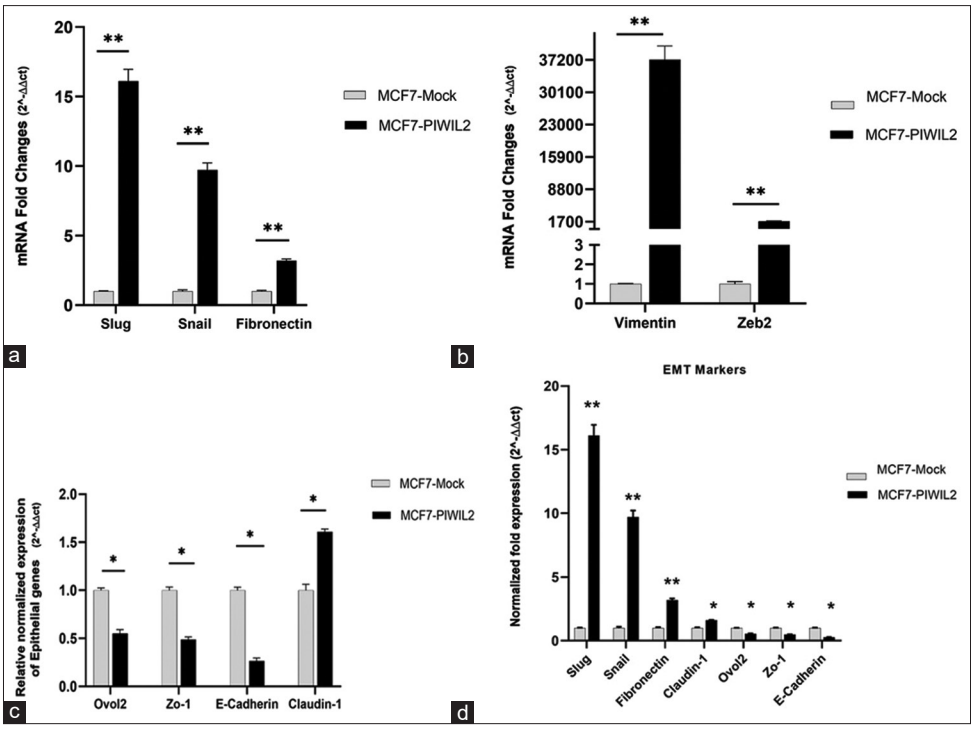

Figure 4: Quantitative expression analysis of EMT biomarkers (a and b) *PIWIL2* overexpression upregulated the expression of mesenchymal biomarkers, including *Vimentin*, *ZEB2*, *SLUG*, *SNAIL,* and *Fibronectin* in the MCF7 cells (***P* < 0.01). (c) The results of qRT-PCR showed decreased mRNA expression of epithelial biomarkers such as *E-Cadherin*, *ZO-1,* and *OVOL2*, while, on the contrary, increased mRNA expression of Claudin-1 was observed in MCF7-PIWIL2 compared with MCF7-Mock cells (**P* < 0.05) (d) Overexpression of *PIWIL2* induced the process of EMT in MCF7 breast cancer cell line (***P* < 0.01, **P* < 0.05)

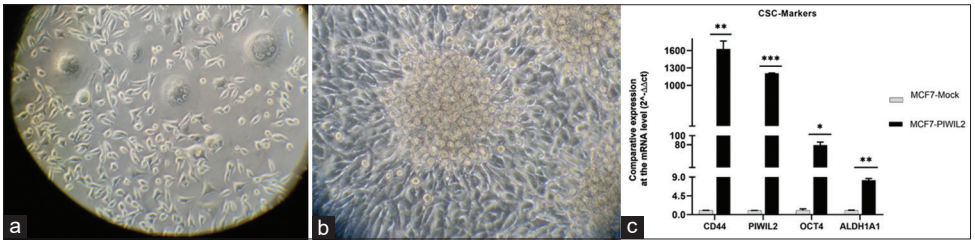

Figure 5: (a) Multinucleated giant cells were generated in MCF7-PIWIL2 cell line (b) Formation of spherical cancer stem-like cell colonies (mammospheres) (c) Quantitative expression analysis of cancer stem cell biomarkers. The results of qRT-PCR showed that the overexpression of *PIWIL2* promoted the expression of cancer stem cell biomarkers including *CD44*, *OCT4*, and *ALDH1A1* in the MCF7 cells (****P* < 0.001, ***P* < 0.01, **P* < 0.05)

kinetics. The cells were harvested at each time point (0, 1, 2, 3, and 4 days), and viable cell numbers were counted with a hemocytometer using trypan blue. According to our results, the cell PDT was decreased from 40.3 hrs in MCF7 control cells to 31.7 hrs in MCF7-PIWIL2 cells and the growth rate increased from 0.017 to 0.021. Therefore, the doubling time of the transfected cells was decreased around 9 hrs and the growth rate increased almost 1.27-fold compared with the control cells. The comparison of doubling time between MCF7 and MCF7-PIWIL2 showed that the cell proliferation of MCF7-PIWIL2 was found to be significantly higher after 24 hrs when compared to MCF7 cells [Figure 6a].

To confirm this result at the molecular level, the expression analysis of *PCNA* as a cell proliferation marker was performed and the results revealed that the expression of *PCNA* was significantly increased (approximately 4.27-fold) in MCF7-PIWIL2 cells compared with MCF7-Mock cells [Figure 6b, 2].

Discussion

It was approved that PIWI proteins are associated with cancer-specific properties, such as cell proliferation, immortality, migration, and invasion so that dysregulated expressions of PIWI proteins and piRNAs have been considered a new therapeutic tool in cancer therapy.[18-20] The human *PIWIL2* gene was originally identified as a stem and testis cell-specific gene. This gene has been highly conserved during evolution and, as a key factor, plays an essential role in the maintenance of self-renewing and differentiation pathways of embryonic and spermatogonial stem cells.[8] As mentioned

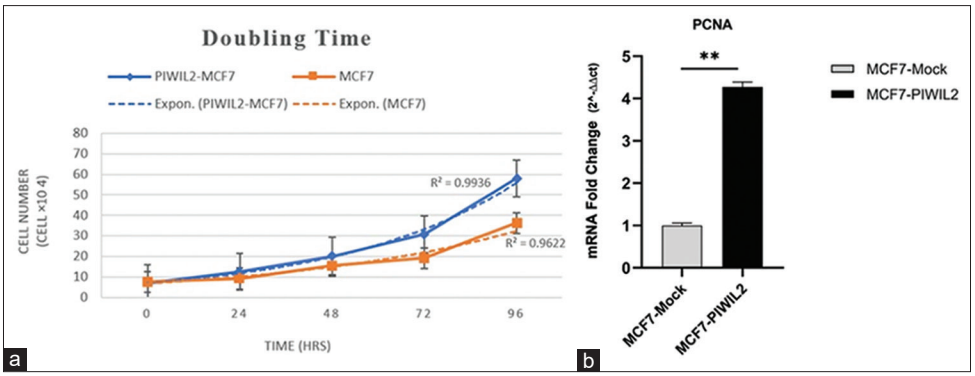

Figure 6: (a) Graph comparing the doubling times of MCF7-PIWIL2 and MCF7 (b) Quantitative expression analysis of *PCNA* showed *PIWIL2* overexpression increased the expression of *PCNA*

before, *PIWIL2* has also been discovered in CSCs, and its oncogenicity has been demonstrated to be essential for the progression of cancer.

To elucidate the potential underlying mechanisms in the EMT process and the effect of *PIWIL2* on the invasive characteristics of breast cancer cells, we used a noninvasive MCF7 cell line and analyzed the expression patterns of EMT-associated biomarkers and some important CSC genes in the *PIWIL2*-overexpressed MCF7 cells. The present study demonstrated that overexpressing *PIWIL2* upregulated the expression of mesenchymal markers and downregulated the expression of epithelial genes, consistent with morphological alterations and generation of mesenchymal CSCs.

Furthermore, unexpectedly, we observed that high expression of *PIWIL2* resulted in increased expression of Claudin-1, which can be supported by the fact that Claudin-1, in interaction with MMPs, participates in EMT and cancer cell migration. This indicates new horizons for the roles of this protein in the invasion and progression of breast cancer. Although decreased expression of Claudin-1 has been reported in luminal-like breast cancer (MCF7), high levels of Claudin-1 describe tumors belonging to more invasive and BLBC.[21] A previous study revealed that knockdown of the *PIWIL2* gene using shRNA significantly reduces invasion and cell migration of prostate cancer by downregulating the expression of MMPs.[22] This evidence and the fact that *PIWIL2* is highly expressed in invasive and metastatic breast cancers indicate potential associations of this protein with cancer cell migration and invasion. Accordingly, it can be of significant support to the hypothesis that aberrant expression of *PIWIL2* promotes EMT and subsequently metastasis in breast cancer cells.

Previous studies demonstrated that mesenchymal-like breast CSCs ($CD44^+/CD24^-$) exhibit phenotypes similar to cells undergone EMT and have a strong ability for invasion, homing, and proliferation at metastatic sites.[23] In general, the presence of these CSC-specific populations is more common in triple-negative breast cancer or basal/mesenchymal cell lines.[24] Another study demonstrated that *PIWIL2* is mainly expressed in the $CD44^+$ population of breast CSCs. Moreover, the *PIWIL2*-expressing cells have high expression rates of pluripotent stem cell markers such as *OCT4* and *NANOG*, which have different cellular characteristics such as stem cell-like colony formation, self-renewal, and differentiation potential.[9] In addition, it has been shown that overexpression of *OCT4* leads to increased expression of EMT-associated genes and invasion of CSCs.[25]

As expected, our findings revealed a significant increase in the incidence of *CD44* and *OCT4* expression. Furthermore, the cell line stably expressing *PIWIL2* generated spherical CSC colonies, which are considered mammospheres. Therefore, overexpressing *PIWIL2* is positively associated with increased levels of mesenchymal-like CSC biomarkers. A relatively few changes in the expression of *ALDH1A1* (which is recognized as an epithelial-like CSC biomarker) were detected. Accordingly, this result can be supported by the fact that breast CSCs display a kind of cellular plasticity that allows them the transfer between EMT ($CD44^+$) and MET ($ALDH1A1^+$) states.[26] Thus, based on the results, it can be suggested that *PIWIL2* as a major stem cell marker could have a reprogramming role in the creation and prevalence of mesenchymal CSCs, which could be a leading cause of cancer invasion through EMT pathways.

In light of the results, it can be proposed that overexpression of exogenous *PIWIL2* can promote tumorigenesis, EMT, and invasion via positively regulating endogenous variants. This finding could possibly indicate the nuclear expression of PIWIL2 and positive feedback on its expression. It was reported that shifting PIWIL2 expression pattern from the cytoplasm to the nucleus, as an indicator of tumor progression, can be associated with ki67 expression and the Stat3/Cyclin D1 pathway activation.[9,11] Furthermore, another study identified that PCNA, along with PCNA-associated factor (PAF), has critical roles in the regulation of self-renewal in breast CSCs and the induction of cellular plasticity through the PAF-Wnt pathway.[27] In accordance with the findings, our results confirmed the proliferative role of *PIWIL2* by the upregulation of *PCNA* in BCSCs, as previously reported.[9] The key question in this study is how PIWIL2 regulates the expression of genes related to EMT pathways. In this regard, the combination of PIWIL2 and its associated RNA (piR-932),

as positive regulators in BCSCs, can cause EMT induction and the genesis of CSCs through the methylation of latexin promoter.[28] Moreover, this protein can be participated in the silencing of tumor suppressor genes by interacting with some epigenetic factors, such as heterochromatin protein 1 (HP1).[29] Another mechanism that may be involved in the expression of EMT-related genes is the effects of PIWIL2 on chromatin decondensation and histone acetylation, which can cause genomic instability in different types of cancers, such as ovarian cancer.[30,31]

Since *PIWIL2* could contribute to the induction of EMT and mesenchymal stem cell production, the aberrant expression of *PIWIL2* may result in the activation of SLUG, SNAIL, and ZEB2 transcription factors through various signaling pathways, including TGF-β and Wnt/β-catenin. In fact, the activation of the above factors can lead to suppression of *E-Cadherin* and increase in *CD44* and *Vimentin* expression due to convergence and interference of several signaling pathways and finally the induction of EMT.[2] Overall, this research could significantly enhance our understanding of the EMT process, and it is hoped that the present study will lead to new therapeutic strategies against *PIWIL2* and its molecular functions to inhibit EMT and restore the sensitivity of cancer cells to therapeutic approaches.

Conclusion

In sum, it would be suggested that as a novel EMT inducer, *PIWIL2* can alter transcription programming through specific signaling pathways and activate the EMT process and stem-like properties, thus participating in the invasion and metastasis of tumor cells.

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Conflicts of interest

The authors declare that there are no potential conflicts of interest in this study.

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SUPPLEMENTARY FILE 1

Table 2: List of primer sequences used in RT-PCR and quantitative Real-time PCR

Genes	Forward primer sequence	Reverse primer sequence	Amplicon size
GAPDH	GTCAGTGGTGGACCTGACCT	CACCACCCTGTTGCTGTAGC	256 bp
PIWIL2	AGCATGAGGTTCGGCATGTT	ATGGCATGCATGACATCCAG	428 bp
q-PIWIL2	AGGCAGAGGCCATGTATTTGG	AAGCATTTCCCGTTTCAGAGG	145 bp
PIWIL2 Variant 1 endogenous	CAGCCAAGTATCTGCTACC	CTTCCTCTTTGTCCACGC	123 bp
PIWIL2 Variant 3 endogenous	GTGGGTTGAGCTCGGTCTT	GGGATGGGTGGATAGGAGA	167 bp
E-CADHERIN	GTCAGTTCAGACTCCAGCCC	AAATTCACTCTGCCCAGGACG	254 bp
OVOL-2	ACACAGGCATTCGTCCCTACAA	AGCCGCAATCCTCGCAGA	156 bp
Zo-1	GCAGCTAGCCAGTGTACAGTATAC	GCCTCAGAAATCCAGCTCACGAA	194 bp
VIMENTIN	GGCTCGTCACCTTCGTGAAT	GAGAAATCCTGCTCTCCTCGC	110 bp
SLUG	CACATTAGAACTCACACGGGGG	GTGCAGGAGAGACATTCTGGAG	165 bp
SNAIL	ACCACTATGCCGCGCTCTT	GGTCGTAGGGCTGCTGGAA	115 bp
FIBRONECTIN	AGGAGAGTGGAAGTGTGAGAG	TATTTCCTTGTGTCTTCAGCC	191 bp
CLAUDIN-1	GCGCGATATTTCTTCTTGCAGG	TTCGTACCTGGCATTGACTGG	113 bp
ZEB-2	GAAGAAAATGACCTGCCACCTG	CCTGGCTTGTGTGTCACCATAT	215 bp
CD44	CTGCCGCTTTGCAGGTGTA	CATTGTGGGCAAGGTGCTATT	109 bp
OCT-4	GGGAGATTGATAACTGGTGTGTT	GTGTATATCCCAGGGTGATCCTC	144 bp
ALDH1A1	GCCAGGTAGAAGAAGGAGATAAG	TTGTATAATAGTCGCCCCCTCT	124 bp
PCNA	AGCACCAAACCAGGAGAAAGT	TCACTCCGTCTTTTGCACAG	191 bp

Supplementary File 2

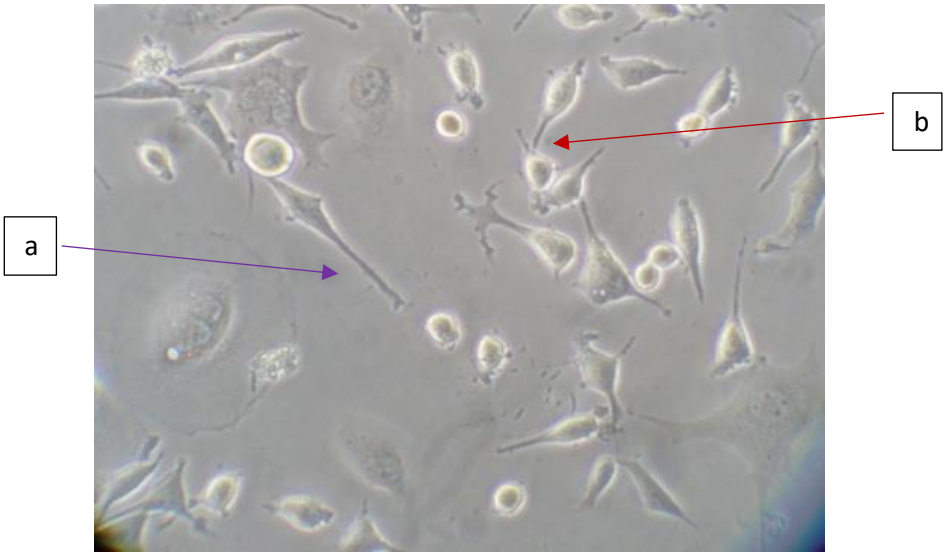

Figure. 7 Direct (a) and Indirect (b) conversion of epithelial cells to mesenchymal cells

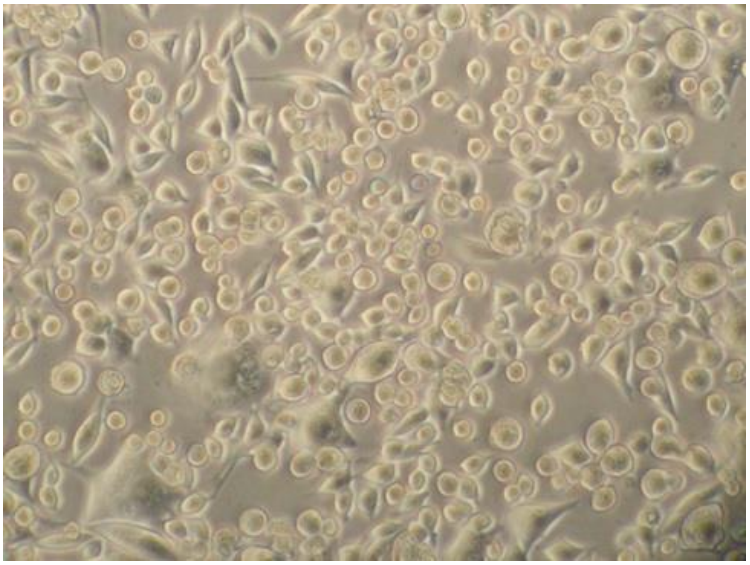

Figure. 8 MCF7-Piwil2 cell line, during selection with G418 (17^{th} day of selection)

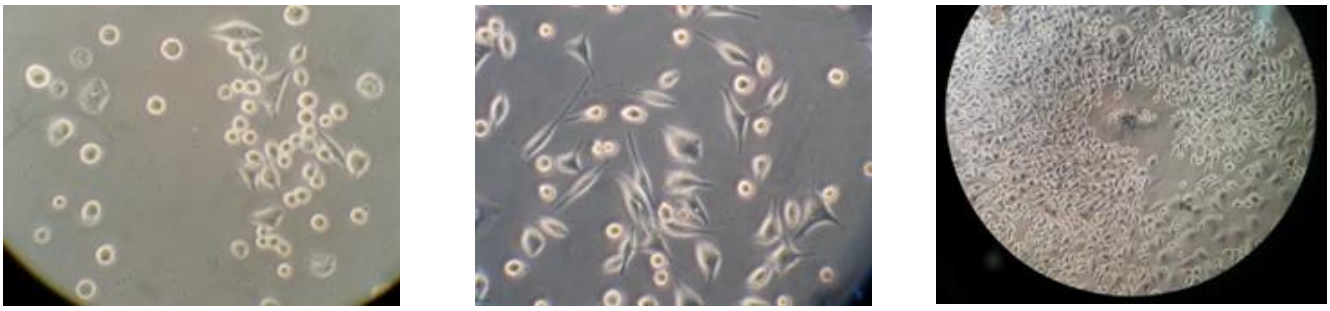

Figure. 9 Cancer stem-like cells formation and their transformation into mesenchymal cells

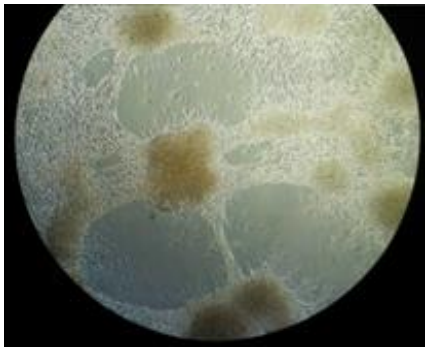

Figure. 10 Establishment of MCF7-Piwil2 cell line and formation of spherical cancer stem-like cell colonies (mammospheres)