Revista Argentina de Clínica Psicológica
2020, Vol. XXIX, N°3, 338-347
DOI: 10.24205/03276716.2020.729

Regulatory Effects of Long-Chain Noncoding RNA PCGEM1 On Invasion and Metastasis of Bladder Urothelial Cell Carcinoma Cells Via the TGF B/Smad Signalling Pathway
Running title: Lncrna And Bladder Urothelial Cell Carcinoma

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Abstract

Background: Long-chain noncoding RNA (lncRNA) highly expressed in prostate cancer cells and acts as prostate cancer gene expression marker 1 (PCGEM1). However, the role of PCGEM1 in bladder urothelial cell carcinoma (BUCC) remains largely unknown.

Objectives: Basic aim of current investigation is to analyse the regulatory effects of lncRNA PCGEM1 on the invasion and metastasis of BUCC cells via the TGF β/Smad pathway.

Materials and Methods: BUCC tissues and normal tissues over 2 cm away were collected from 128 BUCC patients who received surgery from March 2016 to May 2018. The expressions of lncRNA PCGEM1 in BUCC along with surrounding normal tissues as well as different BUCC cell lines were detected by qRT-PCR. LncRNA PCGEM1-silenced T24-siPCGEM1 cells and negative control T24-siNC cells were constructed, using T24 cell line as blank control. Proliferation of T24 cell was examined by MTT and colony formation assays, and invasion and migration were detected by Transwell and scratch assays, respectively. The bioinformatics website starBase was used to predict miRNAs which complementarily bound PCGEM1, and miRNA-targeted binding genes were predicted through the www.microRNA.org website. The expressions of proteins in the TGF β2/Smad2 pathway were measured by Western blotting.

Results: qRT-PCR indicated about the expression of lncRNA PCGEM1 in BUCC tissues, which is higher than the surrounding normal tissues (P<0.05). LncRNA PCGEM1 had the highest expression in T24 cells. The optical density of the T24-siPCGEM1 group at 492 nm significantly decreased than blank control and T24-siNC groups. The numbers of colonies and cells penetrating Matrigel, together with the cell motility and wound healing rate all significantly decreased (P<0.05). LncRNA PCGEM1 complementarily bound miR-148a, and there was a targeted binding site between miR-148a and TGF β2. The expressions of TGF β2 and p-Smad2 in the T24-siPCGEM1 group

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were significantly lower but the expression of miR-148a was higher than those in blank and negative control groups (P<0.05).

Conclusions: LncRNA PCGEM1 is highly expressed in BUCC. High lncRNA PCGEM1 expression may promote the progression of BUCC by down-regulating miR-148a expression and enhancing the TGF β2/Smad2 signaling pathway.

Keywords: lncRNA; PCGEM1; miR-148a; bladder urothelial cell carcinoma; TGF β; Smad

1. Background

Bladder cancer is common malignant tumors in the urogenital system. Among the 20 most common types of malignant tumors worldwide, prostate cancer, bladder cancer and kidney cancer rank 8th, 12th and 17th, respectively (Ferlay, 2015; Heimbach et al., 2018). Bladder cancer is common urological malignancy in China (Cheng, Guan, & Li, 2016; Reed, 2018). Transitional cell carcinoma accounts for 90% of all bladder malignancies, of which about 70% the cases have superficial tumors and 20% have invasive ones. Early diagnosis is crucial in the prognosis of patients with bladder cancer. However, about 5% of the patients have already undergone metastasis before diagnosis (Jamshidian et al., 2014; Samuel et al., 2018). At present, bladder urothelial cell carcinoma (BUCC) is primarily treated by surgery and postoperative chemotherapy, but over 50% of the patients have recurrence and low curability (Hu et al., 2016), so it is urgent to find new therapeutic targets.

Tumor invasion and metastasis dominantly affect the prognosis of cancer. Long-chain noncoding RNA do not involve in encoding proteins, with the lengths of 200~100,000 nt (Z. Zhang et al., 2018). LncRNAs regulate the signaling pathways related with tumor cell cycle, invasion, metastasis and chemoresistance, working similarly to oncogenes or tumor suppressor genes (Yang, Lu, & Yuan, 2014). Expression of PCGEM1 in prostate cancer cells help to promote their proliferation (Y Xue et al., 2013). Nevertheless, the role of PCGEM1 in BUCC remains largely unknown.

2. Objectives

Thereby motivated, the aim of current investigation was to assess the effects of PCGEM1 on the proliferation, invasion and migration of BUCC cells, and to explore the underlying mechanism.

3. Materials and Methods

3.1. Tissue Samples

BUCC tissues and normal tissues over 2 cm away were collected from 128 BUCC patients who received surgery from March 2016 to May 2018. Immunohistochemical staining revealed different degrees of deficiency in 20 tissue samples. The remaining 108 samples were confirmed to be primary BUCC by histopathological examination and included in this study. The patients were aged 38-72 years old, with the median of 51. Ethics committee of hospital has approved this study and written informed consents have been obtained from all patients.

3.2. Cells, Main Reagents and Apparatus

Human BUCC cell lines T24, 5637, SCaBER, J82, UM-UC-3 and SW780 were purchased from USA. PCGEM1 and U6 were designed and prepared by Shanghai GenePharma (China). DMEM (D777) and SYBR Green qPCR kit were bought from Toyobo (Japan). Trizol reagent (9009) was obtained from TaKaRa (Japan). Transwell chambers were provided by BD (USA). MTT assay kit was purchased from Beyotime Institute of Biotechnology China. Sanyo MCO-15AC cell incubator (Japan), Nikon Ti-U/Ti-s inverted fluorescence microscope (Japan) and Roche R480 real-time PCR system (USA) were used.

3.3. Cell Transfection

BUCC T24 cells were inoculated into 6-well plates at the density of 1×106/ml (2 ml) and cultured overnight. The cells were transfected with 100 nmol/L siPCGEM1 and negative control using Lipofectamine 2000 (USA). Using T24 as the blank control, T24-siPCGEM1 and T24-siNC cell lines were obtained. T24-siPCGEM1 referred to the T24 cells in which PCGEM1 expression was silenced, and T24-siNC referred to the negative control cells transfected with nonsense sequence.

3.4. Detection of Lncrna Pcgem1 Expression by Qrt-Pcr

Extraction of RNA from cells was carried out by Trizol method and then reverse-transcribed into cDNA according to the instructions of PrimeScrip reverse transcription kit (Japan). Working of PCR system was carried out by following the instructions of SYBR Premix Ex Taq (USA). PCR conditions: Pre-denaturation at 95°C for 10 min, 40 cycles of reaction at 95°C for 10 s, 60°C for 30 s and 72°C for 10 s; 95°C for 5 s, 60°C for 1 min and 95°C for 30 s. Upstream primer for U6: 5'-CTCGCTTCGGCAGCACA-3', downstream primer: 5'-

AACGCTTCACGAATTTGCGT-3'. Upstream primer for lncRNA PCGEM1: 5'-ACTGTCTCCCAACCCTTGTA-3', downstream primer: 5'-GTGCAGGGTCCGAGGT-3'. Upstream primer for miR-148a: 5'-ACTGTCTCCCAACCCTTGTA-3', downstream primer: 5'-GTGCAGGGTCCGAGGT-3'. Relative expression levels were expressed as 2-ΔΔCt. Each sample was tested independently in triplicate.

3.5. Detection of Cell Proliferation by Mtt And Colony Formation Assays

T24, T24-siNC and T24-siPCGEM1 cells were introduced into 96-well plates at the density of 500-1,000/well. After gentle mixing, the cells were cultured in a 37°C incubator. Each of the well was added 20 µl of MTT solution and left still at 37°C in dark for 4 h. After the liquid in each well was discarded, the cells were added 100 µl of DMSO and shaken on a 37°C shaker rapidly for 15 min to dissolve the crystals. Finally, the optical density (OD) of each well at 492 nm was measured by a microplate reader. The above two stably transfected cell lines were cultured in 6-well plates at the density of 5×103/well, and the culture medium was refreshed every other week. After 2 weeks, the cells were stained with Coomassie blue, and the former colonies were counted under a microscope.

3.6. Detection of Cell Invasion by Trans well Assay

Culture medium was converted to serum-free around 12 h before the start of experiment, and 40 µl of Matrigel was coated in each Transwell chamber. Cells were digested and washed two times with 1× PBS. Then 500 µl of complete medium was added into 24-well plate, and 5×105 cells were resuspended. Subsequently, 200 µl of cell suspension was added into each Transwell chamber. The cells were cultured for 24 h in incubator, stained by 500 µl of 0.1% crystal violet solution prepared with methanol and diluted with PBS. The chambers were then rinsed with PBS, wiped with cotton swabs and air-dried. The membrane-penetrating cells were photographed and counted under an inverted fluorescence microscope.

3.7. Detection of Cell Migration by Scratch Assay

T24, T24-siPCGEM1 and T24-siPCGEM1 cells were evenly spread onto 6-well plates at 5×105 and scratched with a 10 µl pipette tip. The cell debris was washed away with PBS, and then culture medium containing 1% serum was added. The plates were photographed and marked under a microscope, with the time recorded as 0 h. Afterwards, the cells were further cultured at 37°C in a 5% CO2 incubator, and were photographed using an inverted microscope at 0 h and 24 h, respectively.

3.8. Bioinformatics Prediction

The bioinformatics website starBase was used to predict miRNAs which complementarily bound PCGEM1, and the corresponding miRNA-targeted binding genes were predicted through the www.microRNA.org website.

3.9. Western Blotting

T24 cells that had been transfected for 48 h were lysed with an appropriate amount of RIPA lysis buffer for 30 min, and then centrifuged at 12000 rpm at 4°C for 10 min. The supernatant was collected to measure the protein concentration with a BCA kit. Buffer was added to protein sample and denatured at 100°C water bath for 5 min and resolved by 5% SDS-PAGE. The product was transferred onto a PVDF membrane at 4°C for 1.5 h. 5% skimmed milk was used to block membrane for 2 h and then incubated with TGF-β and Smad primary antibodies overnight at 4°C, washed by TBST, incubated with HRP-labeled goat anti-rabbit IgG antibody at 37°C for 2 h and analyzed by an automatic gel imaging system using GAPDH as the internal reference.

3.10. Statistical Analysis

SPSS 19.0 was used for data analysis, and quantitative data was expressed as mean ± SD and subjected to the t test. All data were tested for normal distribution. χ2 test was used for the analysis of numerical data. P<0.05 statistically significant parameter.

4. Results

4.1. Expressions of Lncrna Pcgem1 In Bucc And Adjacent Normal Tissues as Well as Different Bucc Cell Lines

qRT-PCR showed that the expression of lncRNA PCGEM1 in BUCC tissues was higher than surrounding normal tissues (P<0.05). LncRNA PCGEM1 had the highest expression in T24 cells which were thus used in subsequent experiments (Figure 1A and B).

4.2. Cell Line Construction

qRT-PCR exhibited that the lncRNA PCGEM1 expressions in blank and negative control T24-siNC groups were similar (P>0.05), which were both higher as compared to that of the T24-siPCGEM1 group (P<0.001). Therefore, lncRNA PCGEM1-silenced cell line T24-siPCGEM1 had been successfully constructed (Figure 1C).

4.3. Effects of Lncrna Pcgem1 On T24 Cell Proliferation

MTT assay confirmed that the OD of the T24-siPCGEM1 group at 492 nm significantly decreased

than blank control and T24-siNC groups (P<0.05) (Figure 1D). Besides, the number of colonies of the T24-siPCGEM1 group significantly decreased (P<0.05) (Figure 2), indicating that the cell proliferative capacity was attenuated after lncRNA PCGEM1 was silenced.

4.4. Effects of Lncrna Pcgem1 On T24 Cell Invasion

The Transwell assay exhibited that cells penetrating Matrigel in the T24-siPCGEM1 group was lower as compared to the blank control and T24-siNC groups (P<0.05) (Figure 3). Accordingly, the cell invasive capacity reduced after lncRNA PCGEM1 was silenced.

4.5. Effects of Lncrna Pcgem1 On T24 Cell Migration

The scratch assay revealed that the cell motility and wound healing rate of the T24-siPCGEM1 group significantly decreased compared with those of blank control and T24-siNC groups (P<0.05) (Figure 4). Hence, the cell migration capacity reduced after silencing of lncRNA PCGEM1.

4.6. Bioinformatics Prediction Results

The bioinformatics website starBase presented that lncRNA PCGEM1 complementarily bound miR-148a, and the www.microRNA.org website showed that there was a targeted binding site between miR-148a and TGF β2 (Figure 5).

4.7. Relationship Between Lncrna Pcgem1 And Tgf B2/Smad2

TGF β2 and p-Smad2 expression in the T24-siPCGEM1 group were lower but of miR-148a was higher as compared to blank and negative control groups (P<0.05) (Figure 6), indicating that lncRNA PCGEM1 facilitated the expressions of these proteins in the TGF β2/Smad2 pathway.

5. Discussion

BUCC has complex early symptoms lacking obvious characteristics, so the diagnosis and treatment are often delayed (Yijun Xue et al., 2013). Therefore, it is of great importance to find markers for the progression and metastasis of BUCC.

Some lncRNAs have abnormal expressions in tumors and work similarly to oncogenes or tumor suppressor genes (Han, Ma, Yourek, Park, & Garcia, 2011). Until now, no marker LncRNA that can directly predict BUCC has been found. In prostate cancer cells, LncRNA PCGEM1 is specifically expressed and located at the chromosome 2q32 (Kim & Kang, 2020). Wang et al. found by RT-PCR that the expression of PCGEM1 in colon cancer tissue was higher as compared to the surrounding normal tissue (K. Zhang et al., 2018). Additionally, Chen et al. reported increase in the expression of lncRNA PCGEM1 in the tissues and serum of patients having ovarian cancer (Chen et al., 2018). The expression of lncRNA PCGEM1 significantly increased during the differentiation of ovarian cancer cells. After silencing of lncRNA PCGEM1, the cell proliferation was weakened, and the expressions of proliferation-related genes also decreased, suggesting that lncRNA PCGEM1 promoted the proliferation of ovarian cancer cells. In current study, we have observed that the lncRNA PCGEM1 expression in BUCC tissues is higher as compared surrounding normal tissues, indicating that lncRNA PCGEM1 can play an important role in the onset and progression of BUCC. To this end, we constructed a PCGEM1-silenced cell line. The invasive and migration capacities of T24 cells decreased significantly after LncRNA PCGEM1 expression was down-regulated.

It has previously been reported that miRNA bound target genes through incomplete complementation of bases, thereby affecting the apoptosis, migration and metastasis of tumor cells and triggering the necrosis and apoptosis of surrounding cells (Kou, Liu, Tang, & Kou, 2018). Meanwhile, lncRNA works largely depending on miRNA. Herein, starBase predicted that lncRNA PCGEM1 complementarily bound miR-148a. Li et al. investigated that expression of miR-148a in esophageal cancer tissues was lower as compare to normal surrounding tissues, and up-regulating such expression facilitated cell proliferation and migration, verifying that miR-148a promoted the progression of esophageal cancer as an oncogene (Li, 2017). In this study, the expression of miR-148a decreased after silencing of lncRNA PCGEM1, suggesting that lncRNA PCGEM1 promoted proliferation probably by down-regulating miR-148a expression. Moreover, there was a targeted binding site between miR-148a and TGF β2, as predicted by the www.microRNA.org website. After the expression of miR-148a increased, that of TGF β2 protein dropped. The TGF β2/smad2 signaling pathway dominantly participates in the onset and progression of tumors, which allows the signal transduction of intracellular pathways by phosphorylating transcription factors Smad proteins including Smad1-Smad9 (Deng et al., 2018). When the phosphorylation\of Smad2 is inhibited or even silenced, the biological function of the TGF β2/Smad2 signaling pathway changes, then suppressing tumor cell proliferation (Zhao et al., 2015). Herein, the phosphorylation level of Smad2 in the T24-siPCGEM1 group was significantly attenuated. Yu et al. reported that down-regulating

lncRNA PCGEM1 expression modulated that of TGF β2 to suppress the invasion and migration of colorectal cancer (Yu et al., 2018). Hence, lncRNA PCGEM1 may influence cancer progression by regulating the TGF β2/Smad2 pathway.

In summary, high expression of lncRNA PCGEM1 may promote the progression of BUCC by down-regulating miR-148a expression and enhancing the TGF β2/Smad2 signaling pathway. The findings pave the way for the development of novel biopharmaceuticals based on lncRNA PCGEM.

Acknowledgement

This study was financially supported by The Key Support Targets for The University's Talent Pool in 2019 (No. 2019R015).

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Figure Legends

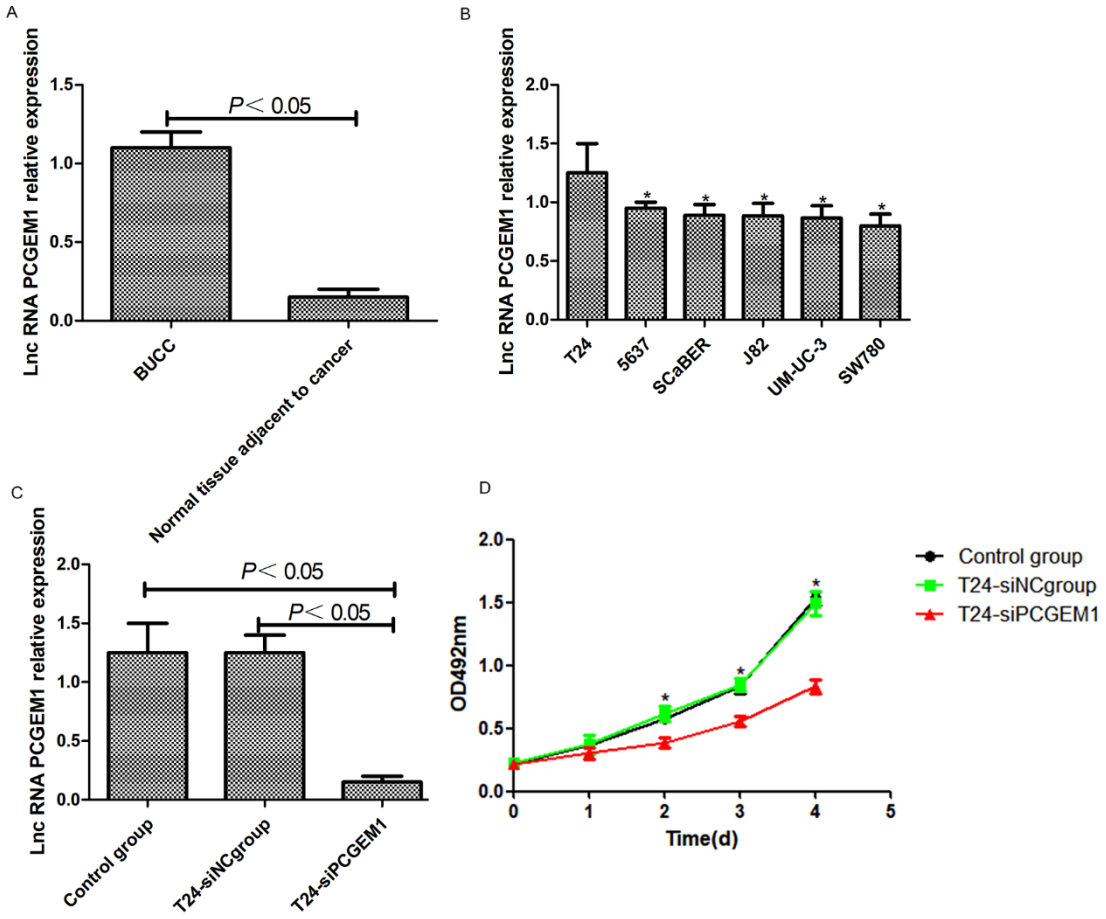

Figure 1. **Lncrna Pcgem1 Expressions In (A) Bucc And Adjacent Normal Tissues as Well As (B) Different Bucc Cell Lines. Compared with T24 Cells, *P<0.05; (C) Lncrna Pcgem1 Expressions in Different T24 Cell Groups; (d) T24 Cell Viability Detected by Mtt Assay. Compared with T24-Sipcgem1 Group, *p<0.05.**

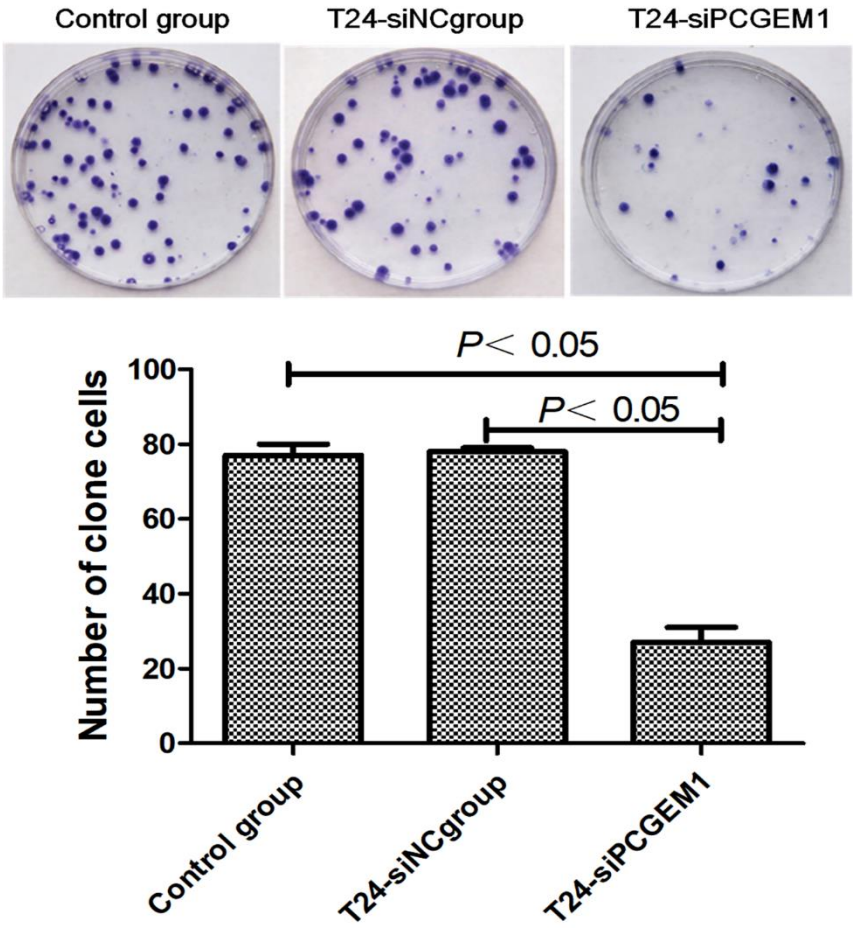

Figure 2. **T24 Cell Proliferation Detected by Colony Formation Assay.**

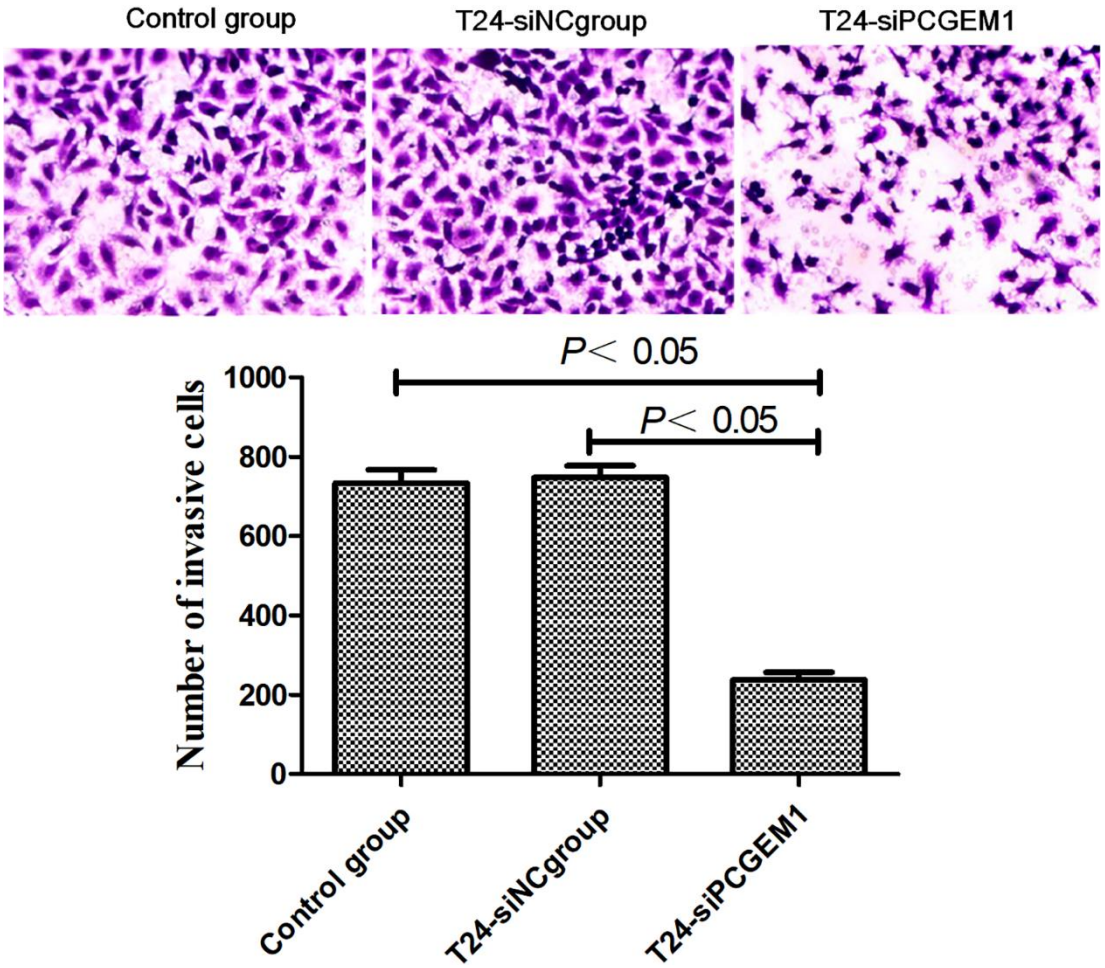

Figure 3. **Effects of lncRNA PCGEM1 on T24 cell Invasion.**

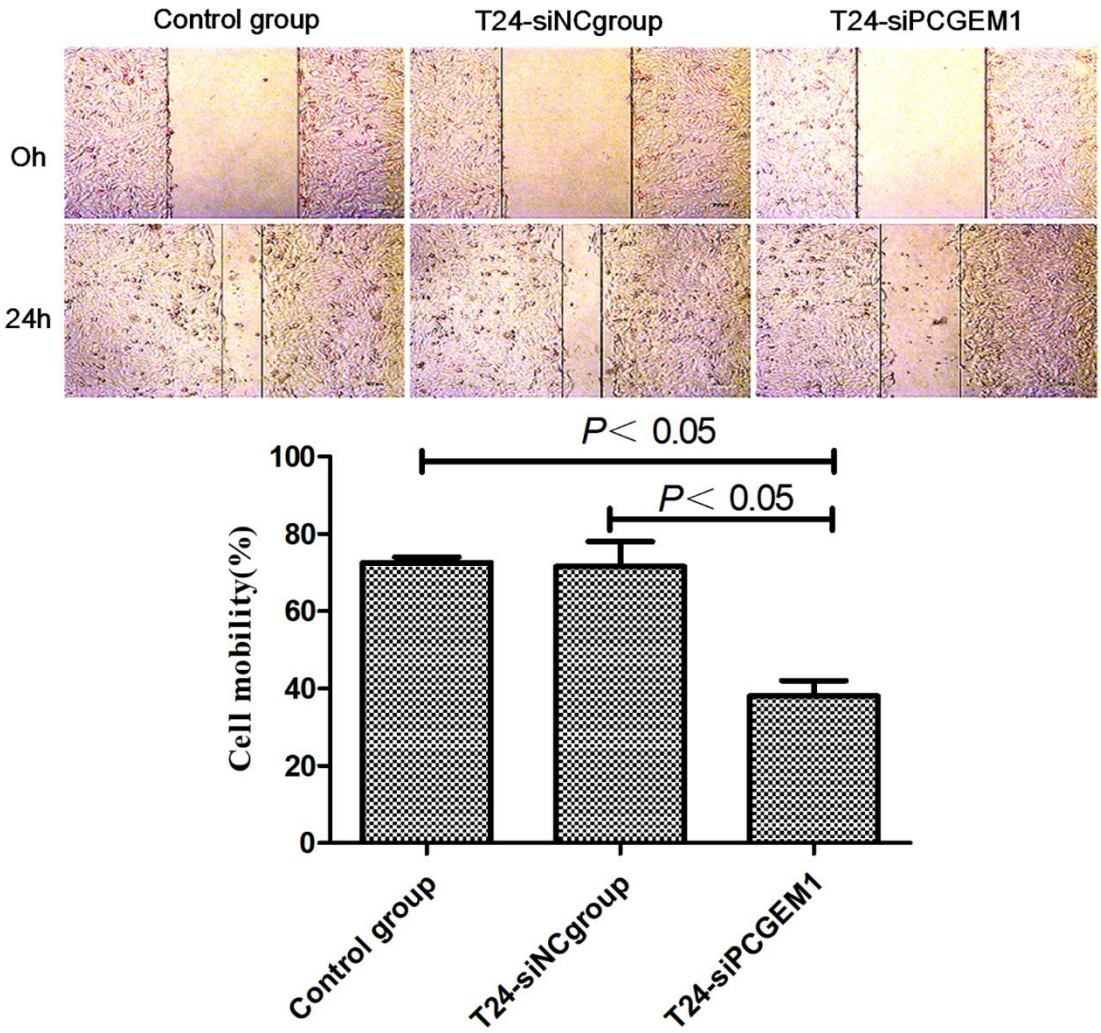

Figure 4. **Effects of lncRNA PCGEM1 on T24 Cell Migration.**

A

miRNA	GeneID	GeneName	GeneType	TargetSite	Alignment
hsa-miR-148a	ENSG00000227418	PCGEM1	lincRNA	chr2:193640565-193640585[+]	Target: 5' aggcAUUUCAG-AGUGCACUGu 3' miRNA : 3' uguuUCAAGACAUCACGUGACu 5'

B

hsa-miR-148a/TGFB2 Alignment

```
     3' uguuucaAG-ACAUCACGUGACu 5' hsa-miR-148a
                || || | |||||||
4153:5' ucuuuccUCAUG-AAUGCACUGa 3' TGFB2
```

mirSVR score: -0.8203
PhastCons score: 0.6894

Mouseover a miRNA mature name to see the miRNA/TGFB2 alignment.

Figure 5. **Bioinformatics Prediction of Relationship Between Lncrna Pcgem1 And Tgf β2. A: Prediction Using the Star base Website; B: Prediction Using the www.Microrna.Org Website.**

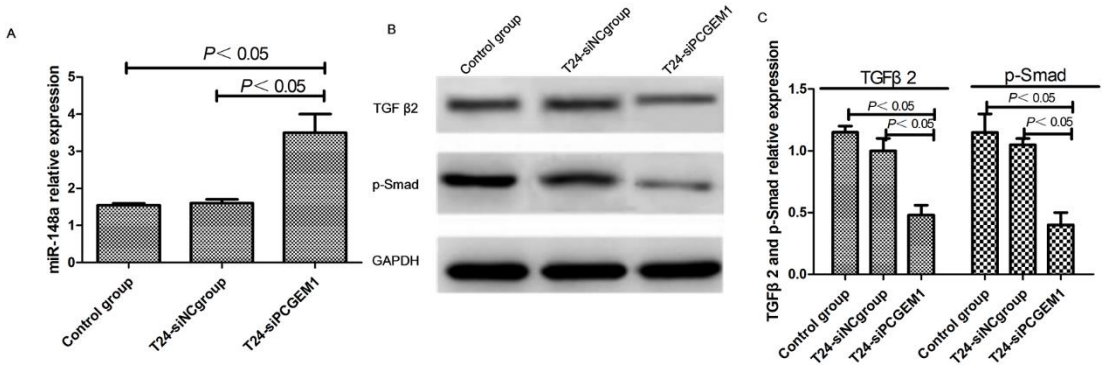

Figure 6. **Relationship Between Lncrna Pcgem1 And Tgf B2/Smad2. A: Expressions of Mir-148a In T24 Cells; B: Tgf B2 and P-Smad2 Expressions Detected by Western Blotting; C: Relative Expressions of Tgf B2 and P-Smad2. Compared with Blank Control Group, *p<0.05.**