

ORIGINAL RESEARCH

Knockdown of *PIMREG* modulates the Wnt/ β -catenin pathway to suppress proliferation and stemness in endometrial cancer

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Abstract

Background: Endometrial cancer is the most common gynecological malignancy, and poses serious threat to health. *PIMREG* (PICALM interacting mitotic regulator also known as *FAM64A*), has been uncovered in promoting tumorigenesis in multiple cancers. But, its regulatory impacts in endometrial cancer remain poorly understood. **Methods:** Cell viability was assessed by the Cell Counting Kit-8 (CCK-8) assay. Protein levels were inspected by Western blotting. Cell proliferation was evaluated by colony formation assay, while stemness was examined by sphere formation assays. **Results:** *PIMREG* expression was found to be upregulated in endometrial cancer. Suppression of *PIMREG* significantly reduced cell viability and suppressed proliferative and stemness capacities. Furthermore, silencing *PIMREG* retarded the evoking of the Wnt/ β -catenin signaling pathway. **Conclusions:** *PIMREG* knockdown suppressed proliferation and stemness by modulating the Wnt/ β -catenin pathway in endometrial cancer. This project hinted that *PIMREG* may be one useful therapeutic target for endometrial cancer treatment.

Keywords

PIMREG; Wnt/ β -catenin pathway; Endometrial cancer; Stemness

1. Introduction

Endometrial cancer is one prevalent gynecological malignancies and is associated with a significant mortality rate [1]. Recently, the occurrence rate of endometrial cancer has shown a rapid upward trend globally [2, 3]. Despite considerable progress in therapeutic strategies, clinical outcomes remain suboptimal for many patients [4]. Therefore, identifying novel molecular targets and elucidating the underlying mechanisms of endometrial cancer progression is imperative to improve treatment options.

Numerous proteins have been identified as potential therapeutic targets in endometrial cancer. For example, EGF-like domain, multiple 6 (EGFL6) has been shown to promote cell migration and proliferation [5]. Proline, glutamate and leucine rich protein 1 (PELP1) suppression impairs ribosomal biogenesis, thereby inhibiting tumor progression [6]. Similarly, myosin IIIB (MYO3B) can facilitate tumor growth [7]. Additionally, silencing of S100 calcium binding protein A2 (S100A2) has been reported to activate the stimulator of interferon genes (STING) pathway, leading to reduced aggressiveness of endometrial cancer cells [8].

PIMREG, also known as *FAM64A*, is a clathrin assembly lymphoid myeloid leukemia gene (CALM) interactor [9]. It has been identified as a key promoter of tumor formation and progression in many cancers. For example, *PIMREG* activates

the nuclear factor kappa-B (NF- κ B) pathway to enhance tumor aggressiveness in breast cancer [10], modulates the DNA damage response to promote glioblastoma progression [11], and is associated with poor survival outcomes and altered immune cell infiltration in lung adenocarcinoma [12]. Notably, high *PIMREG* expression in uterine corpus endometrial carcinoma (UCEC) is linked to reduced overall survival [13]. However, the precise molecular mechanisms through which *PIMREG* contributes to endometrial cancer progression hold unclear.

In this project, we demonstrate that knockdown of *PIMREG* suppresses proliferation and stemness through restraining the Wnt/ β -catenin signaling pathway in endometrial cancer. This finding supplies useful insights into the oncogenic role of *PIMREG* and suggest it may serve as a novel therapeutic target in endometrial cancer.

2. Materials and methods

2.1 Database analysis

The mRNA expression levels of *PIMREG* (FAM64A) in UCEC tumor tissues and corresponding normal tissues were analyzed using data from The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov>). The mRNA expression profiles of *PIMREG* in UCEC tumor tissues and normal tissues were confirmed through the Gene Expression Profiling Interactive Analysis (GEPIA)

database (<http://gepia.cancer-pku.cn>) where *PIMREG* mRNA expression was assessed both in UCEC and across various cancer types compared to normal tissues. The protein expression of *PIMREG* in endometrial cancer tumor tissues and normal tissues was evaluated using immunohistochemistry (IHC) data available from the Human Protein Atlas (<https://www.proteinatlas.org>). Prognostic relevance of *PIMREG* expression in endometrial cancer patients was verified through the KMplot database (<https://kmplot.com/analysis>), comparing survival outcomes between patients with high and low *PIMREG* expression levels.

2.2 Cell lines and culture

Human endometrial epithelial cells (hEEC) and endometrial cancer cell lines Ishikawa, HEC-1B, KLE and RL-952 were obtained from the American Type Culture Collection (ATCC). Dulbecco's Modified Eagle Medium (DMEM) (12800017, Invitrogen, Carlsbad, CA, USA) was employed for culturing cells. Cells were maintained in one incubator. Cell lines were authenticated by short tandem repeat (STR) profiling and were confirmed to be free of mycoplasma contamination.

2.3 Cell transfection

Short hairpin RNAs targeting *PIMREG* (sh-*PIMREG*#1 and sh-*PIMREG*#2) and a negative control (sh-NC) were bought from GenePharma company (Shanghai, China). Transfections were performed in Ishikawa and KLE cells by Lipofectamine 2000 reagent (11668019, Invitrogen, Carlsbad, CA, USA).

2.4 Western blotting

Total proteins from endometrial cancer cells were drawn through radioimmunoprecipitation assay (RIPA) lysis buffer, and protein concentrations were quantified through the bicinchoninic acid (BCA) Protein Assay Kit (P0011, Beyotime, Shanghai, China). Proteins were separated on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for division. Next, putting proteins to polyvinylidene fluoride (PVDF) membranes (Beyotime, Shanghai, China). The primary antibodies were placed into membranes for 12 h, next the secondary antibodies for 2 h. Protein bands were confirmed through the chemiluminescence detection kit.

The primary antibodies: *PIMREG* (1:1000; ab251896; Abcam, Shanghai, China), c-Myc (1:1000; ab32072), CyclinD1 (1:200; ab16663), β -catenin (1:5000; ab32572), Sex Determining Region Y Box Protein 2 (SOX2) (1:1000; ab92494) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000; ab8245).

2.5 CCK-8 assay

Endometrial cancer cells were placed into 96-well plate at an appropriate density. CCK-8 solution with 10 μ L was put into each well. Cell viability (at 450 nm) was inspected by the spectrophotometer (ND-ONE-W, Thermo Fisher Scientific, Waltham, MA, USA).

2.6 Colony formation assay

Endometrial cancer cells were put in to the 6-well plate. Incubation for 14 days, colonies were got. The fixation and dyeing for colonies were performed. Eventually, colonies were counted.

2.7 Sphere formation assay

Endometrial cancer cells were cultured in DMEM/F12 medium (Gibco, USA) supplemented with 20 ng/mL fibroblast growth factor (FGF), 20 ng/mL epidermal growth factor (EGF), and 1% fetal bovine serum (FBS) was adopted for cell incubation in the ultra-low-attachment culture dishes (Corning, USA). After 14 days, the formed spheres were observed and imaged using a light microscope (CX23, Olympus, Tokyo, Japan).

2.8 Statistical analysis

Data were demonstrated as mean $\pm$ standard deviation (SD). The statistical analysis was executed through GraphPad Prism Software 9 (GraphPad Software, San Diego, CA, USA). The data follow a normal distribution. Three repetitions were performed in each cell experiment. One-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test was employed for comparisons. The statistically significant was affirmed at $p < 0.05$.

3. Results

3.1 *PIMREG* expression was elevated in endometrial cancer

Analyses of the TCGA and GEPIA databases revealed that *PIMREG* expression was augmented in endometrial cancer tumor tissues (Fig. 1A–C). Consistent with this, IHC data from the Human Protein Atlas further confirmed elevated *PIMREG* protein levels in tumor tissues (Fig. 1D). Moreover, survival analysis using KMplot demonstrated that high *PIMREG* expression can cause poorer overall survival (Fig. 1E). Next, it was verified that *PIMREG* owned the lifted protein expression in endometrial cancer cell lines (Fig. 1F). In general, *PIMREG* expression was elevated in endometrial cancer.

3.2 Suppression of *PIMREG* inhibited cell viability in endometrial cancer

The knockdown efficiency of *PIMREG* by shRNA was confirmed by Western blot analysis (Fig. 2A). Moreover, the cell viability was cut down after *PIMREG* suppression (Fig. 2B). The cell proliferation was restrained after silencing *PIMREG* (Fig. 2C). These results suggest that suppression of *PIMREG* impaired cell proliferation.

3.3 Silencing of *PIMREG* repressed stemness in endometrial cancer

Sphere formation assays showed that stemness capacity was significantly reduced in endometrial cancer cells following *PIMREG* knockdown (Fig. 3A). Consistently, SOX2 protein expression, a key marker of stemness, was also downregulated upon *PIMREG* silencing (Fig. 3B). These findings indicate that

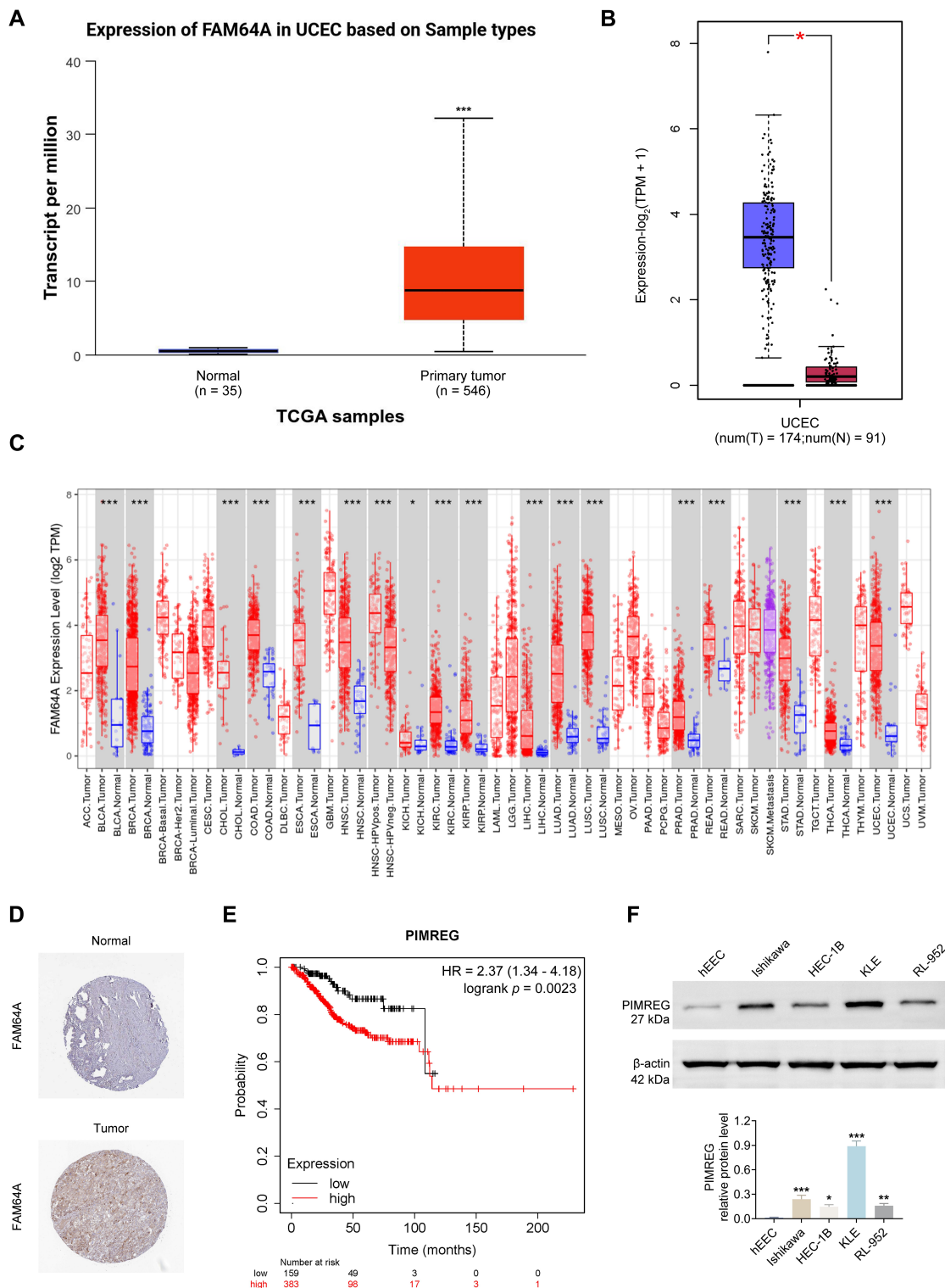


FIGURE 1. *PIMREG* expression was elevated in endometrial cancer. (A) The mRNA expression of *PIMREG* (FAM64A) in UCEC tumor tissues and normal tissues was verified through the TCGA database. (B) The mRNA expression of *PIMREG* in UCEC tumor tissues and normal tissues was verified through GEPIA database. (C) The mRNA expression of *PIMREG* in multiple cancers' tumor tissues and normal tissues was confirmed through the GEPIA database. (D) The protein expression of *PIMREG* in endometrial cancer tumor tissues and normal tissues was examined through IHC assay from the Human Protein Atlas online website. (E) The prognosis of endometrial cancer patients with high or low *PIMREG* expression was obtained from the KMplot tool. (F) The protein expression of *PIMREG* in human endometrial epithelial cell line hEEC and endometrial cancer cell lines (Ishikawa, HEC-1B, KLE and RL-952) through Western blot. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *PIMREG*: PICALM interacting mitotic regulator; UCEC: uterine corpus endometrial carcinoma; GEPIA: Gene Expression Profiling Interactive Analysis; IHC: Immunohistochemistry; TCGA: The Cancer Genome Atlas; TPM: Transcripts Per Million; hEEC: Human Esophageal Epithelial Cells; HR: Hazard Ratio.

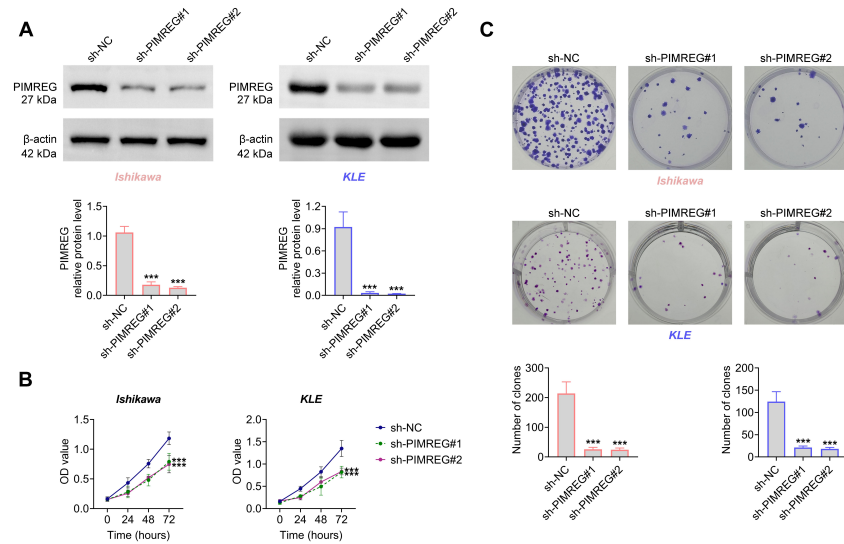


FIGURE 2. Suppression of *PIMREG* inhibited cell viability in endometrial cancer. Groups were separated into the sh-NC, sh-PIMREG#1 and sh-PIMREG#2 groups. (A) The protein expression of *PIMREG* was tested through western blot. (B) The cell viability was examined through the CCK-8 assay. (C) The cell proliferation was determined through colony formation assay. *** $p < 0.001$. *PIMREG*: PICALM interacting mitotic regulator; sh-NC: Short hairpin-negative control; OD: optical density.

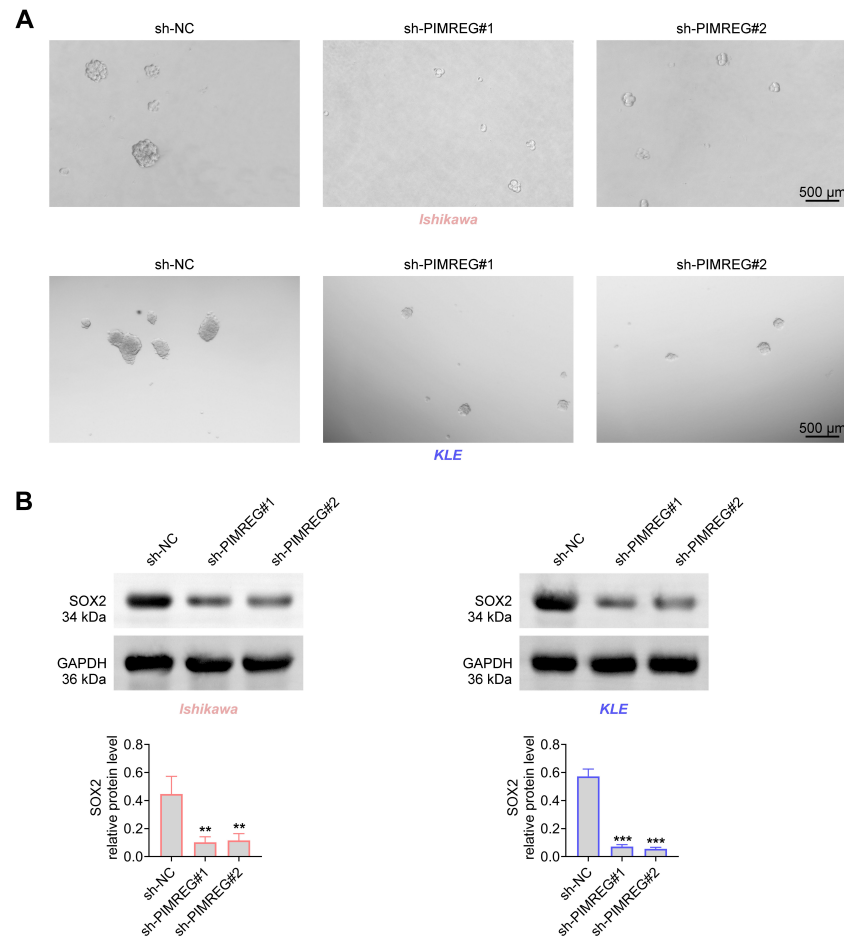


FIGURE 3. Knockdown of *PIMREG* repressed stemness in endometrial cancer. Groups were separated into the sh-NC, sh-PIMREG#1 and sh-PIMREG#2 groups. (A) The stemness ability was confirmed through the sphere formation assay. (B) The *SOX2* protein expression was measured through Western blot. ** $p < 0.01$, *** $p < 0.001$. *PIMREG*: PICALM interacting mitotic regulator; sh-NC: Short hairpin-negative control; *SOX2*: Sex Determining Region Y Box Protein 2; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase.

knockdown of *PIMREG* suppresses the stem-like properties of endometrial cancer cells.

3.4 Inhibition of *PIMREG* attenuated the Wnt/ β -catenin pathway

The protein levels of β -catenin, c-Myc and CyclinD1 were lessened after *PIMREG* inhibition (Fig. 4), suggesting that inhibition of *PIMREG* weakened the Wnt/ β -catenin pathway.

4. Discussion

PIMREG has been revealed to own a critical role in tumor formation and progression in multiple cancers [10–14]. However, its functional significance and regulatory mechanisms in endometrial cancer have remained largely unclear. In this project, we demonstrated that *PIMREG* expression is significantly upregulated in endometrial cancer. Importantly, elevated *PIMREG* expression was connected with poor patient prognosis.

Cancer stem cells (CSCs) owns the capacities of anti-apoptosis, self-renewal, tumorigenesis and high metastasis [15]. They are widely regarded as a core driver of tumorigenesis, and their presence has been strongly linked to drug resistance and tumor metastasis [16]. CSCs have been clarified in many solid tumors, and targeting CSCs is considered as the key strategy for effective cancer treatment [17]. In endometrial cancer progression, recent studies have highlighted the importance of stemness regulation. For example, minichromosome maintenance complex component

2 (MCM2) has been shown to enhance CSC-like traits in endometrial cancer [18], while phosphocholine alleviates stemness by suppressing the mammalian target of rapamycin (mTOR)/c-Myc signaling pathway [19]. Furthermore, chromodomain helicase DNA binding protein 4 (CHD4) mutations can stimulate transforming growth factor (TGF)-beta signaling to aggravate endometrial cancer stemness [20]. Additionally, Sirtuin 2 promotes stemness and reduces chemosensitivity, further complicating treatment outcomes [21]. Similarly, in this work, it was also testified that *PIMREG* knockdown significantly reduced sphere formation and downregulated SOX2 expression, indicating that silencing of *PIMREG* repressed stemness.

The Wnt/ β -catenin pathway exhibits importance in the maintenance of CSCs [22]. The accumulated β -catenin can move into the nucleus, and then combine with T-cell factor (TCF) transcription factors, next evoking downstream genes, thereby strengthening stemness and accelerating tumor progression [23]. Thus, focusing on the Wnt/ β -catenin pathway and stemness becomes pivotal. For instance, lysine (K)-specific demethylase 1 (KDM1A) evokes Wnt/ β -catenin signaling to promote stemness in thyroid cancer [24], while Sec62 enhances CSC properties in colorectal cancer via Wnt/ β -catenin activation [25]. In contrast, forkhead box F2 (FOXF2) negatively regulates this pathway to suppress stemness in breast cancer [26]. It has also been clarified that kinesin family member 15 (KIF15) knockdown can weaken stemness to retard the development of endometrial cancer by restraining the Wnt/ β -catenin pathway [27]. In line with these findings, *PIMREG* has been reported to activate the β -catenin

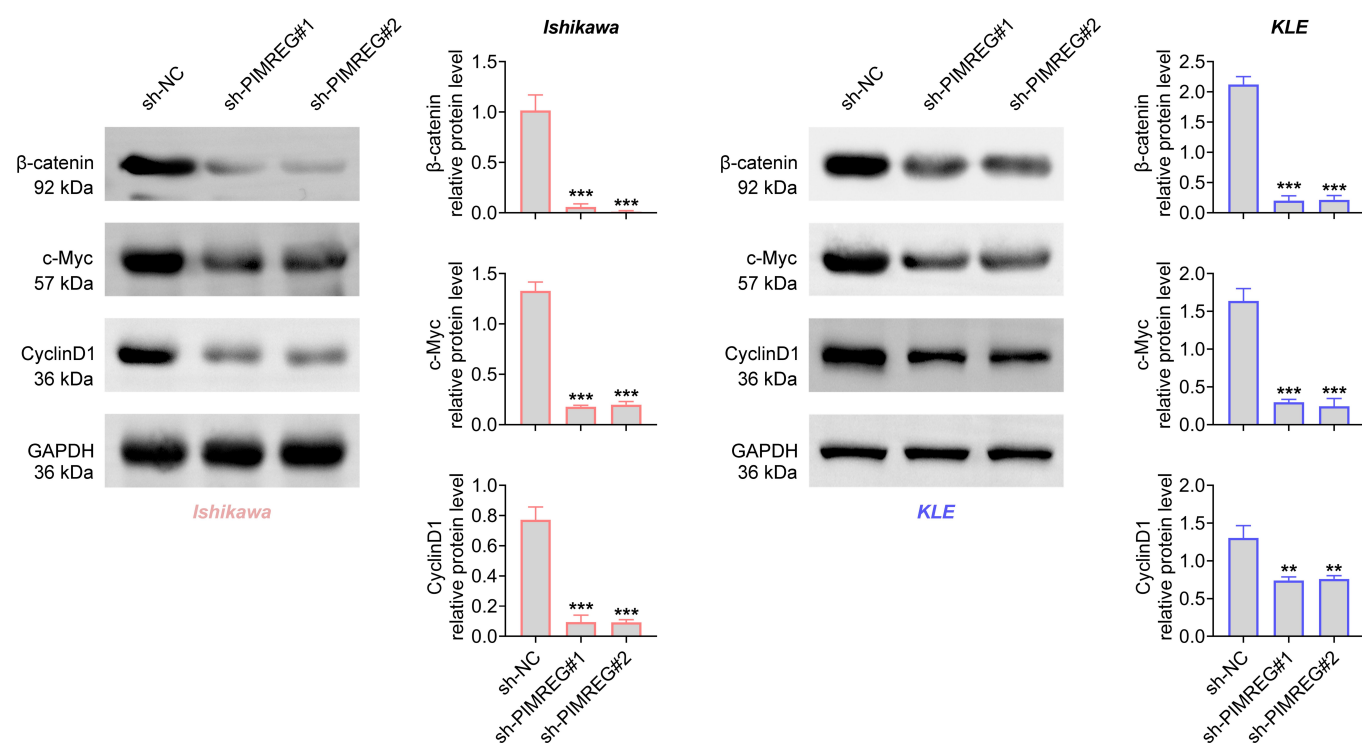


FIGURE 4. Inhibition of *PIMREG* attenuated the Wnt/ β -catenin pathway. Groups were separated into the sh-NC, sh-PIMREG#1 and sh-PIMREG#2 groups. The protein expressions of β -catenin, c-Myc and CyclinD1 were inspected through Western blot. ** $p < 0.01$, *** $p < 0.001$. *PIMREG*: PICALM interacting mitotic regulator; sh-NC: Short hairpin-negative control; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

pathway in glioma, contributing to tumor development [28]. However, in endometrial cancer, the impacts of *PIMREG* on the β -catenin pathway remain elusive. Similar to these above previous studies, in this work, it was also manifested that inhibition of *PIMREG* weakened the Wnt/ β -catenin pathway.

5. Conclusions

Our results uncovered that knockdown of *PIMREG* inhibited cell proliferation and stemness in endometrial cancer via the Wnt/ β -catenin pathway. However, this project has several limitations. The *in vivo* animal models, clinical tissue samples, and comprehensive clinical investigations were not included, which may limit the translational applicability. In future research, further validation using animal models and patient-derived samples, as well as expanded clinical and mechanistic studies, will be essential to fully probe the influences of *PIMREG* on endometrial cancer progression.

AVAILABILITY OF DATA AND MATERIALS

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

AUTHOR CONTRIBUTIONS

ZQH, RHQ—designed the study and carried them out; prepared the manuscript for publication and reviewed the draft of the manuscript. ZQH, QW, JYW—supervised the data collection; analyzed the data; interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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