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Physcion 8-O-β-glucopyranoside exerts carcinostasis ability in Ishikawa cells via regulating lnc-SLC4A1-1/H3K27ac/NF-κB pathway

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This study aimed to investigate whether physcion 8-O-β-glucopyranoside (PG) exerted anti-tumor effects in endometrial cancer cells *via* regulating the long non-coding RNA lnc-SLC4A1-1. The anti-tumor effects of PG on endometrial cancer by evaluating Ishikawa cell growth and metastasis, and the expression of lnc-SLC4A1-1 was determined after PG treatment. Subsequently, the role and regulatory mechanism of lnc-SLC4A1-1 dysregulation in PG-treated endometrial cancer cells were explored. PG treatment resulted in dramatical depression of cell viability, remarkable promotion of cell apoptosis and dramatic suppression of migration and invasion in Ishikawa cells in a dose-dependent way. Moreover, PG decreased the level of lnc-SLC4A1-1, and high levels of lnc-SLC4A1-1 reversed the effects of PG on Ishikawa cells. Furthermore, lnc-SLC4A1-1 was transcriptionally activated by H3K27ac and interacted with NF-κB p65 in Ishikawa cells. PG treatment depressed the NF-κB signal in Ishikawa cells, which were significantly reversed after overexpression of lnc-SLC4A1-1. Our results indicate that PG exerts anti-tumor activity in endometrial cancer cells. Lnc-SLC4A1-1/H3K27ac/NF-κB pathway may be a possible mechanism to mediate the anti-tumor effects of PG, which provide a promising targeted strategy for treatment of endometrial cancer.

1. Introduction

Endometrial cancer remains to be one of the most familiar gynaecological malignancies, with increasing incidence (Morice et al. 2016). Despite great advances in chemoradiotherapy and surgical treatments, some patients will develop recurrence and distal metastasis after treatment (Humber et al. 2005). The majority of affected women have a good prognosis, however, the five-year survival rate of patients decreases significantly after disease recurrence and distal metastasis (Colombo et al. 2016). Therefore, elucidation of the molecular mechanisms of endometrial carcinogenesis and development of effective treatments are still needed.

Long noncoding RNAs (lncRNAs) are a kind of noncoding RNAs longer than 200 bp. Study results have suggested that the aberrant level of some lncRNAs plays a significant role in cancer biology

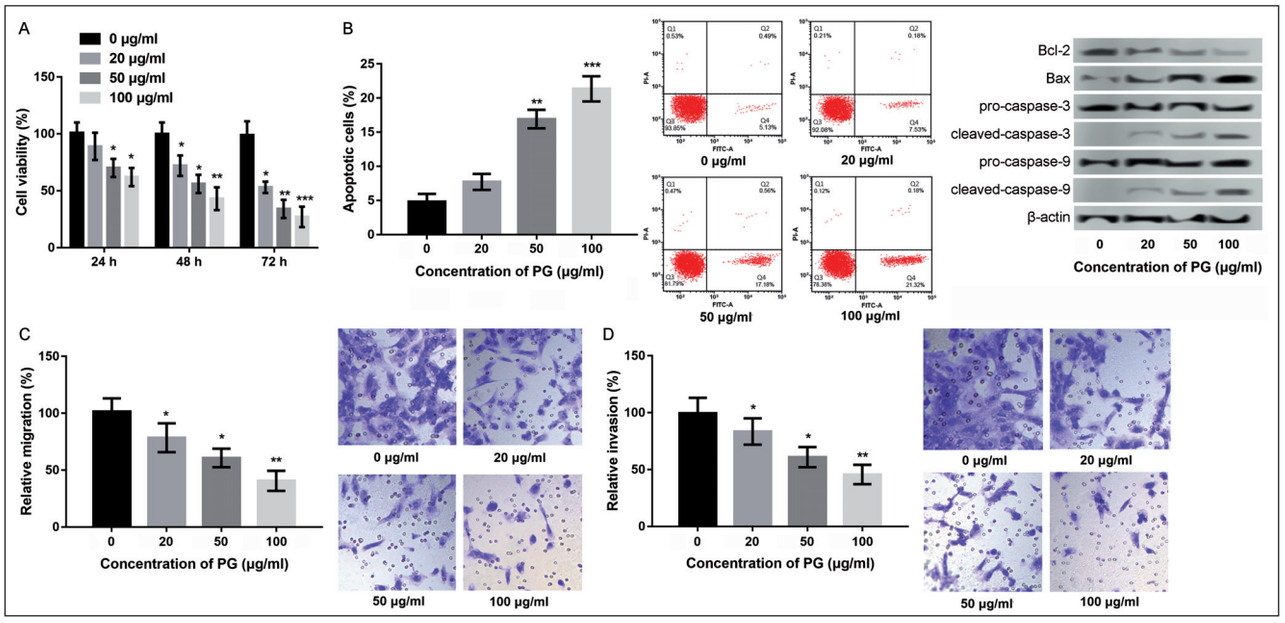

Fig. 1: Treatment of physcion 8-O-β-glucopyranoside (PG) inhibited Ishikawa cell viability (A) promoted apoptosis (B), and suppressed migration (C) and invasion (D) in a dose-dependent manner. Data are presented as mean±SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

(Cheetham et al. 2013; Huarte 2015; Prensner and Chinnaiyan 2011). Several LncRNAs have been recognized as key players in the development of endometrial cancer (Liu et al. 2018, 2019; Qiao and Li 2016; Xie et al. 2018). Recently, lnc-SLC4A1-1 is identified to be implicated in the unexplained recurrent pregnancy loss (Huang et al. 2018). Whether lnc-SLC4A1-1 participates in endometrial cancer is largely unknown.

Physcion 8-O-β-glucopyranoside (PG), a primary active ingredient of *Rumex japonicus* Houtt, is pointed out to be crucial in plenty of pathological processes. Mounting evidence has shown that PG exerts anti-tumor activity in plenty of cancer cells, such as colorectal cancer (Ding et al. 2016), non-small cell lung cancer (Du et al. 2019) breast cancer (Xia et al. 2017), and clear-cell renal cell carcinoma (Wang et al. 2018). However, the effect of PG in endometrial cancer cells has not been disclosed, let alone the possible mechanism involved.

In the present study, we detected the carcinostatic effects of PG by evaluating endometrial cancer cell growth and metastasis, and then determined the expression of lnc-SLC4A1-1 after PG treatment. Subsequently, the role and regulatory mechanism of lnc-SLC4A1-1 dysregulation in PG-treated endometrial cancer cells were explored. This study will lay a theoretical basis for explanation of the pathogenesis of endometrial cancer and provide a new insight for targeted treatment of this disease.

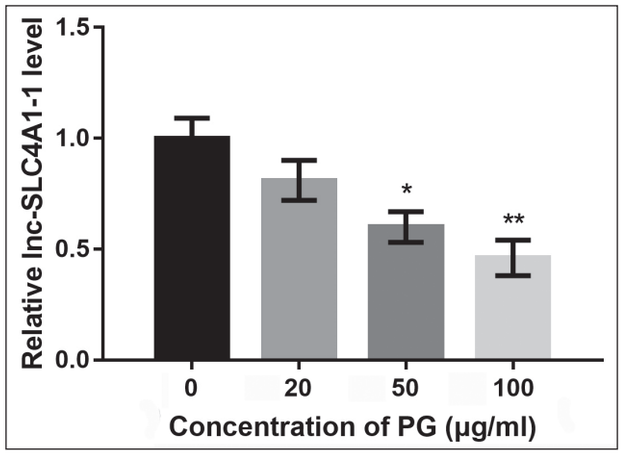

Fig. 2: PG decreased the expression of lnc-SLC4A1-1. Data are presented as mean±SD. * P<0.05, and ** P<0.01.

2. Investigations and results

2.1. Inhibition of cell viability, promotion of cell apoptosis, and suppression of migration and invasion by PG in Ishikawa cells

The effect of PG on endometrial cancer was investigated by determination of Ishikawa cell viability, apoptosis, migration and invasion. PG disposes brought out a dramatically inhibition of cell viability in time- and dose-dependent ways ($P < 0.05$, Fig. 1A). Moreover, 48 h of PG treatment resulted in remarkable promotion of cell apoptosis ($P < 0.05$, Fig. 1B), and dramatic suppression of migration ($P < 0.05$, Fig. 1C) and invasion ($P < 0.05$, Fig. 1D) in Ishikawa cells in a dose-dependent manner. These data confirmed that PG may exert anti-tumor activity in endometrial cancer cells.

2.2. PG decreased the expression of lnc-SLC4A1-1

As presented in Fig. 2, 48 h of PG treatment obviously decreased the expression of lnc-SLC4A1-1 in Ishikawa cells in a dose-dependent way ($P < 0.05$), suggesting that PG may exert anti-tumor activity in Ishikawa cells *via* inhibiting the expression of lnc-SLC4A1-1. Based on these results, 100 μg/ml of PG treatment was used for subsequent experiments.

2.3. Effects of PG on Ishikawa cell growth and metastasis were through decreasing the lnc-SLC4A1-1 expression

To explore whether the effects of PG in Ishikawa cells was achieved by regulation of lnc-SLC4A1-1, Ishikawa cells were treated with PG and then transfected with pc-lnc-SLC4A1-1. The level of lnc-SLC4A1-1 was remarkably enhanced in pc-lnc-SLC4A1-1 group compared to that in pcDNA3.1 group ($P < 0.01$, Fig. 3A), indicating that lnc-SLC4A1-1 was successfully overexpressed in Ishikawa cells after transfection. Moreover, the results showed that overexpression of lnc-SLC4A1-1 significantly promoted cell viability ($P < 0.05$, Fig. 3B), depressed apoptosis ($P < 0.01$, Fig. 3C), and enhanced migration ($P < 0.05$, Fig. 3D) and invasion ($P < 0.05$, Fig. 3E) of PG-treated Ishikawa cells, indicating that overexpression of lnc-SLC4A1-1 reversed the effects of PG on Ishikawa cells.

2.4. Lnc-SLC4A1-1 was transcriptionally activated by H3K-27ac and interacted with NF-κB p65 in endometrial cancer

The potential mechanism of lnc-SLC4A1-1 in endometrial cancer was also investigated. Using a bioinformatic online tool (http://genome.ucsc.edu/), the promoter region of lnc-SLC4A1-1 was highly enriched with H3K27ac (http://genome.ucsc.edu/cgi-bin/

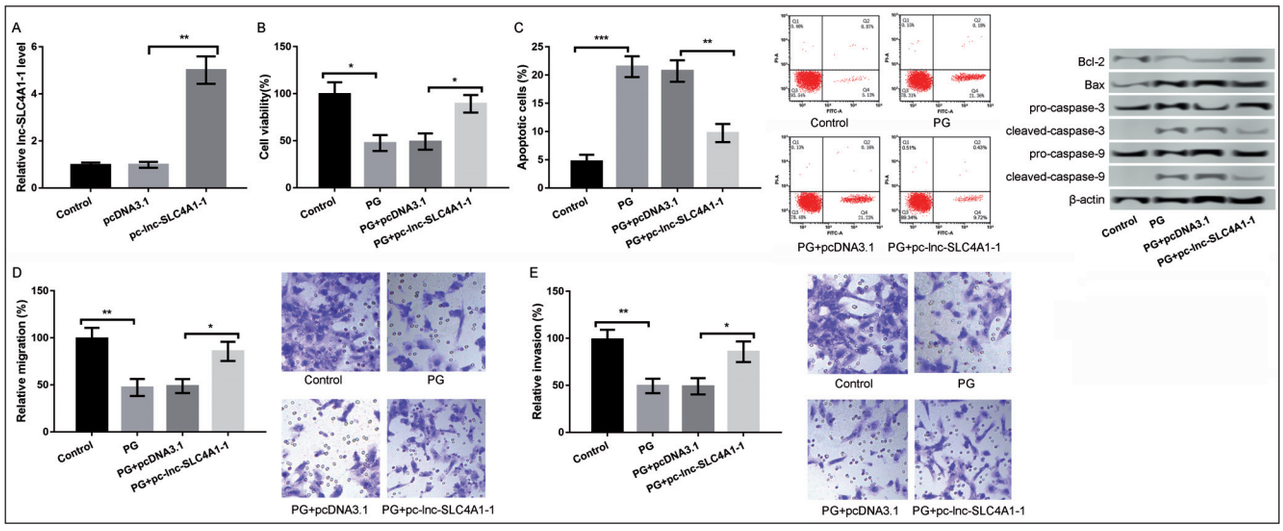

Fig. 3: Lnc-SLC4A1-1 was successfully overexpressed in Ishikawa cells (A), and overexpression of lnc-SLC4A1-1 significantly promoted cell viability (B), inhibited apoptosis (C), and enhanced migration (D) and invasion (E) of PG-treated Ishikawa cells. Data are presented as the mean±SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

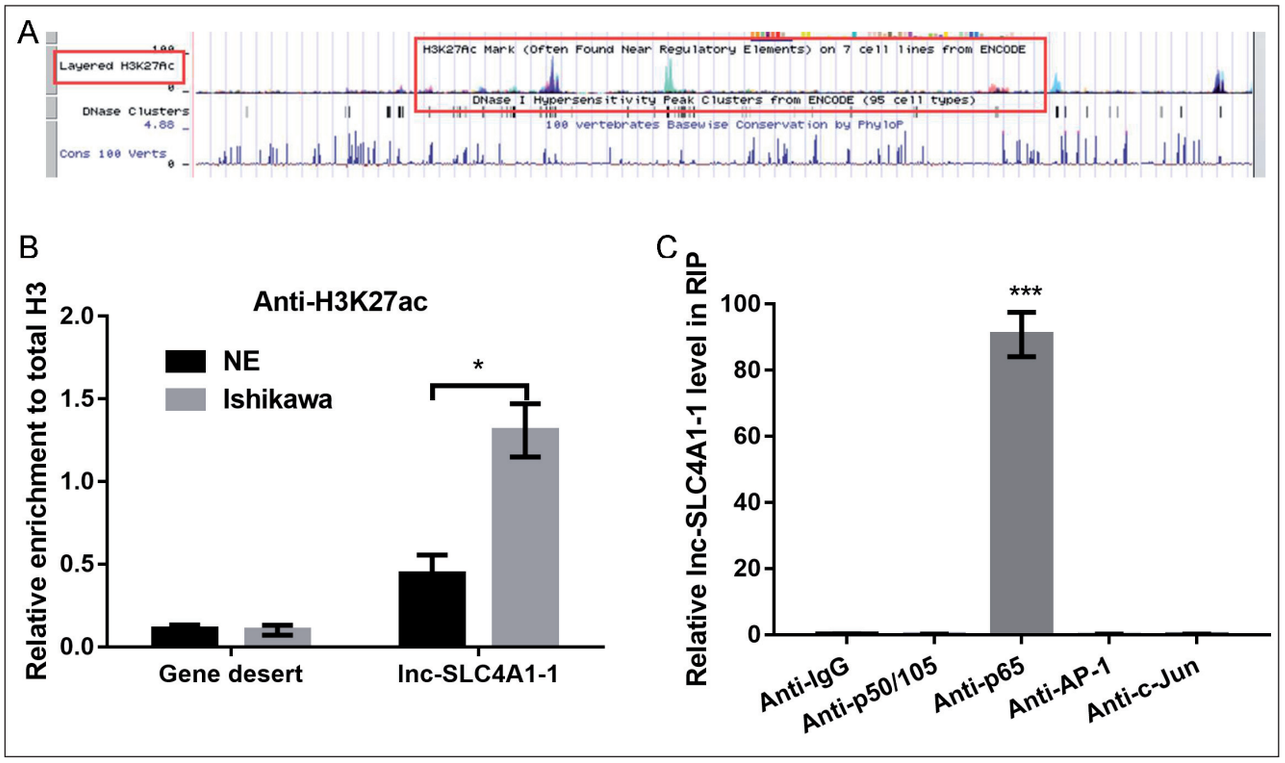

Fig. 4: Lnc-SLC4A1-1 was transcriptionally activated by H3K27ac (A-B) and interacted with NF-κB p65 (C). Data are presented as mean±SD. * $P < 0.05$ and *** $P < 0.001$.

hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr1%3A11102837-11267747&hgsid=720619111_x3CfSGbdpYtmtpMn7WEZfV1us8x2) (Fig. 4A). Further ChIP analysis showed that the acetylation level of H3K27ac in the promoter region of lnc-SLC4A1-1 of Ishikawa cells was remarkably higher than that of normal cycling endometrium (NE) cells ($P < 0.05$, Fig. 4B). Moreover, RIP assay showed that only p65 was significantly enriched ($P < 0.001$), but not p50/105, AP-1, and c-Jun (Fig. 4C). The data implyed that lnc-SLC4A1-1 could interact with NF-κB/p65 in endometrial cancer.

2.5. Effects of PG on Ishikawa cell growth and metastasis were by regulating the NF-κB pathway

The association between PG and NF-κB pathway was investigated to further explore the possbile mechanism involved. The results displayed that PG disposes dramatically decreased the level of p/t-p65 and p/t-IκBα in Ishikawa cells, which were significantly reversed after overexpression of lnc-SLC4A1-1 (($P < 0.01$, Fig. 5). These data indicated that the NF-κB pathway might be a dowstream mechanism to mediate the effects of PG in endometrial cancer cells.

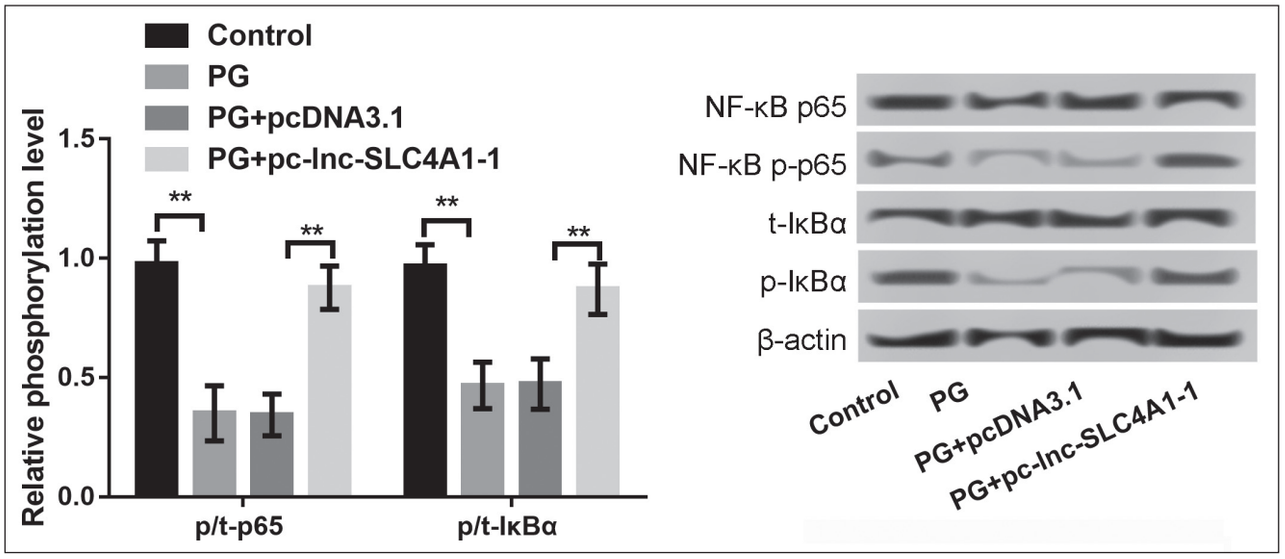

Fig. 5: The protein expression of NF-κB pathway-related proteins, including p/t-p65 and p/t-IκBα in Ishikawa cells after treatment of PG and overexpression of lnc-SLC4A1-1. Data are presented as the mean±SD. ** $P < 0.01$

3. Discussion

Rumex japonicus Houtt is used in traditional medicine because of its antimicrobial, anti-inflammatory and anti-tumor activities (Jiang et al. 2007; Zhou et al. 2005). PG as a main active ingredient has shown anti-tumor activity in a variety of cancer cells (Ding et al. 2016; Du et al. 2019; Wang et al. 2018; Xia et al. 2017). In line with these findings, the data of this research show that PG disposes results in significant inhibition of cell viability, remarkable promotion of cell apoptosis and dramatic suppression of migration and invasion in Ishikawa cells in a dose-dependent way, confirming that PG might exert anti-tumor activity in endometrial cancer cells. The study thereby explored the key principles mediating the effect of PG on endometrial cancer. We found that PG decreased the expression of lnc-SLC4A1-1, and overexpression of lnc-SLC4A1-1 reversed the effects of PG on Ishikawa cells. Furthermore, we found that lnc-SLC4A1-1 was transcriptionally activated by H3K27ac and interacted with NF-κB p65 in Ishikawa cells. PG treatment depressed the NF-κB signals in Ishikawa cells, which were significantly reversed after high expression of lnc-SLC4A1-1. These data merit further discussion.

Recently, studies have confirmed that certain lncRNAs with active enhancer regions, could regulate gene expression by interacting with transcription factors (Léveillé et al. 2015; Li et al. 2013; Tsai et al. 2018; Yang et al. 2016). It is reported that H3K27ac is always enriched in active enhancers (Creyghton et al. 2010). Consistent with previous findings (Huang et al. 2018), we found that lnc-SLC4A1-1 was transcriptionally activated by H3K27ac. Moreover, we found that lnc-SLC4A1-1 could interact with NF-κB p65 in Ishikawa cells. NF-κB, a transcription factor, has been pointed out to be the central mediator of the inflammatory process and immune responses (DiDonato et al. 2012). Moreover, NF-κB is recognized as a crucial player in plenty of human cancers (Hoesel and Schmid 2013; Xia et al. 2014). The brisk NF-κB signaling is involved in carcinogenesis (Bours et al. 2000; Michael 2006), including endometrial cancer (Faloppa et al. 2014; Spirina et al. 2012). Our data thereby revealed that PG treatment dramatically decreased the level of p/t-p65 and p/t-IκBα in Ishikawa cells, which were significantly reversed after overexpression of lnc-SLC4A1-1. Based on these results, we assume that PG may exert anti-tumor activity in endometrial cancer via regualting lnc-SLC4A1-1/H3K27ac/NF-κB pathway.

Taken together, our results indicate that PG also exerts anti-tumor activity in endometrial cancer cells. The Lnc-SLC4A1-1/H3K27ac/NF-κB pathway may be a possible mechanism to mediate the anti-tumor effects of PG, which provides a latent targeted strategy for treatment of endometrial cancer. Further studies are still required to confirm our findings.

4. Experimental

4.1. Cell lines and cell culture

The human endometrial cancer cell line of Ishikawa (ATCC, USA) were grown in Minimum Essential Medium Eagle (Sigma-Aldrich, UK) containing 15% FBS (Gibco, Darmstadt, Germany), and then cultured at 37 °C with 5% CO_2. After reaching the logarithmic growth phase, Ishikawa cells were harvested and used for following experiments. To investigate the effect of PG, Ishikawa cells were disposed with plenty of concentrations of PG (0, 20, 50 and 100 μM) for various time points including 24 h, 48 h and 72 h, respectively.

4.2. Cell transfection

The sequence of lnc-SLC4A1-1 was inserted into a pcDNA3.1 vector to construct the overexpression vector, named pc-lnc-SLC4A1-1. For cell transfection, Ishikawa cells were seeded onto 6-well plates and then transfected with pcDNA3.1 and pc-FGD5-AS1 using Lipofectamine 2000 (Invitrogen) based on the manufacturer's control requirements. pcDNA3.1 vector was chosen as negative control.

4.3. Cell viability assay

Cells were grown in 96-well plates. After different treatments, cell proliferation was assessed by a Cell Proliferation Reagent Kit I (MTT) (TaKaRa, Japan) following the manufacturer's protocol.

4.4. Cell apoptosis detection

After different treatments, Ishikawa cells were reaped and then sustained with double staining with FITC-Annexin V and Propidium iodide (PI) using the FITC-Annexin V Apoptosis Detection Kit (BD Biosciences, USA) following the recommendations of the manufacturer. Flow cytometry (FACScan; BD Biosciences) was chosen for the assessment of the percentage of apoptotic cells via a CellQuest software (BD Biosciences).

4.5. Cell migration and invasion assays

Ishikawa cells exposed to different treatments were suspended in serum-free media and then added into the upper chamber of an insert (8-mm pore size; Millipore, Billerica, MA, USA). Compared to the migration assay, the difference of the invasion assay was that the upper chamber was coated with Matrigel (50 μM). The downside chamber was filled with medium containing 10% FBS. After incubation for 24 h, the migrated or invaded cells across the membranes were stained with methanol and 0.1% crystal violet. Then, IX71 inverted microscope (Olympus, Tokyo, Japan) was chosen for counting the numbers of migrated or invaded cells.

4.6. Quantitative PCR (qPCR)

After different treatments, we isolated total RNA from Ishikawa cells using Trizol (TaKaRa), and then carried out the reverse transcribed to cDNA by the Omniscript RT Kit (TaKaRa). Real-time qPCR reaction was then conducted with a SYBR® Premix Ex Taq™ II kit (TaKaRa) in an StepOnePlus™ (Applied Biosystems) using the following conditions: 20 s at 95 °C, and then 40 cycles of 3 s at 95 °C and 30 s at 60 °C. Relative expression of lnc-SLC4A1-1 was calculated using the $2^{-\Delta\Delta Ct}$ method with. GAPDH as a reference.

4.7. Chromatin immunoprecipitation (ChIP) test

The ChIP assay was carried out as described previously (Qin et al. 2018). In brief, cells were disposed with 1% formaldehyde for 10 min, followed by termination of cross-linking by adding 0.125 M glycine. Short fragments were then generated by sonicating the cells using a Bioruptor (Diagenode, Denville, USA). The chromatin was produced by incubating with antibody to H3K27ac (RRID: AB_2118291) (Abcam, Cambridge, UK) overnight. After incubating with either protein A or G (EMD Millipore, Bedford, USA) beads for 2 h, the he protein-DNA complexes were collected and then reverse crosslinked with Proteinase K at 65 °C overnight. qPCR was then performed to detected the enrichment level of H3K27ac.

4.8. RNA binding protein immunoprecipitation (RIP) assay

For investigation of the association between lnc-SLC4A1-1 and NF-κB signaling, RIP assay was performed. In brief, preparation of lysate and magnetic beads, immunoprecipitation, RNA purification and qPCR analysis were orderly conducted following the recommended protocols of RNA-Binding Protein Immunoprecipitation Kit (17e700, Millipore, USA). Anti-p50/105, Anti-p65, Anti-AP1, anti-c-Jun (Millipore, USA) was employed, with normal mouse Anti-IgG (Millipore, USA) as a negative control.

4.9. Western blot

After different treatments, Ishikawa cells were harvested and lysed with cell lysis buffer (Sangon Biotech, Shanghai, China). After being separated on 12% SDS-polyacrylamide gels, the protein blots were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). The PVDF membranes were then incubated with primary antibodies to apoptotic proteins (Bax, Bcl-2, pro-caspase-3, cleaved-caspase-3, pro-caspase-9, cleaved-caspase-9, NF-κB pathway-related proteins (including p/t-p65 and p/t-IκBα) and β-actin (used as internal control) at 4 °C overnight. All these primary antibodies were acquired from Abcam and diluted to 1:1,000 before use. After incubation with the recommended secondary antibodies, the blots were displayed by the chemiluminescent detection method (Pierce, Thermo Scientific, Waltham, USA).

4.10. Statistical analysis

Statistical analysis was accomplished by the SPSS 16.0 software system (SPSS, Chicago, IL). Three repeats were set up for all experiments. Data are expressed as the mean ± standard error (SD). Student t test was performed to compare the differences between two groups, and one-way analysis of variance (ANOVA) was carried out for more than two groups. $P < 0.05$ is chosen to represent statistically significant.

Conflict of interest: None declared.

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