



Sirtuin 2 promotes cell stemness and MEK/ERK signaling pathway while reduces chemosensitivity in endometrial cancer

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Received: 5 March 2021 / Accepted: 24 August 2021 / Published online: 2 September 2021
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Abstract

Purpose Sirtuin 2 (SIRT2) is functionally important in cancer progression and treatment resistance as an NAD⁺-dependent deacetylase, whereas its role in endometrial cancer (EC) is limitedly investigated. This study aimed to evaluate the regulatory role of SIRT2 on cell stemness and chemosensitivity in EC.

Methods SIRT2 expression was detected in human EC cell lines, including Ishikawa, AN3CA, HEC1A, KLE, and normal human endometrial (uterine) epithelial cells (served as controls). Then, SIRT2 overexpression plasmids (constructed with pcDNA3.1 vector) and knock-down plasmids (constructed with pGPH1 vector) were transfected in Ishikawa cells and KLE cells, respectively to assess the influence of SIRT2 on EC cell stemness and chemosensitivity to cisplatin and paclitaxel.

Results SIRT2 mRNA and protein were both overexpressed in EC cell lines (including Ishikawa cells, AN3CA cells, HEC1A cells, and KLE cells) compared with controls. Upregulation of SIRT2 increased the sphere formation capacity (by sphere formation assay and extreme limiting dilution analysis) and CD133⁺ cells rate in Ishikawa cells, whereas knock-down of SIRT2 reduced the sphere formation capacity and CD133⁺ cells rate in KLE cells. As for chemosensitivity, upregulation of SIRT2 increased relative cell viability in cisplatin-treated and paclitaxel-treated Ishikawa cells. In contrast, SIRT2 knock-down suppressed relative cell viability in cisplatin-treated but not in paclitaxel-treated KLE cells. In addition, SIRT2 overexpression increased, while SIRT2 knock-down reduced p-MEK and p-ERK1/2 levels in EC cells.

Conclusion SIRT2 promotes cell stemness and activates the MEK/ERK signaling pathway while represses chemosensitivity in EC.

Keywords Sirtuin 2 · Endometrial cancer · Stemness · Chemosensitivity · MEK/ERK pathway

Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy, which often occurs in elderly women, whereas the onset of EC in younger women rises in recent years as the epidemic of obesity spreads [1–3]. Most EC cases are postmenopausal women, with 75% diagnosed at an early stage [4]. Except for the scheduled surgery and radiotherapy, taxanes (paclitaxel or docetaxel) and platinum analogs (carboplatin or cisplatin) have been used as first-line chemotherapies for EC patients who always present extremely poor treatment response and clinical outcomes to conventional

chemotherapy with advanced or recurrent disease [5]. It is shown that the stem cell properties of EC cells result in metastatic spread and resistance to cisplatin and paclitaxel. However, the detailed mechanism about the generation of stemness in EC cells remains to be elucidated [2].

Sirtuins (SIRT) are nicotinamide adenine dinucleotide (NAD⁺)-dependent deacetylases that involve in the epigenetics via histone modification, whose implication in the pathogenesis of neurological, cardiovascular, inflammatory diseases, and cancers have been revealed [6]. SIRT2 is one of the seven members of human SIRT located in the cytosol, which directly targets α -tubulin at the microtubules, and regulates cell migration as well as adhesion of the cell mitotic progression [7]. In the process of tumorigenesis, SIRT2 positively modulates anaphase-promoting complex/cyclosome (APC/C) via deacetylation, and its expression has been found to be upregulated in human HCC and NSCLC tissues [8, 9]. Besides, SIRT2 is reported to regulate the

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multidrug resistance via MEK/ERK pathway, then playing a vital role in the generation of stemness and chemoresistance in cancer cells [10–14]. For instance, SIRT2 overexpression in renal cell carcinoma cell lines increases stem cell growth and suppresses fluorouracil-induced apoptosis [15]. However, limited data have been revealed on the role of SIRT2 in EC pathology.

Therefore, the present study evaluated the regulatory role of SIRT2 on cell stemness, chemosensitivity and MEK/ERK signaling pathway in EC.

Methods

Cell culture

Human EC cells Ishikawa (BCRJ, Brazil) were cultured in Dulbecco's Modified Eagle Medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). Human EC cells AN3CA (ATCC, USA) were cultured in Minimum Essential Medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA). Human EC cells HEC1A (ATCC, USA) were cultured in McCoy's 5A Medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and human EC cells KLE (ATCC, USA) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco, USA) supplemented with 10% FBS (Gibco, USA). Normal human endometrial (uterine) epithelial cells (HEEC) (Lifeline[®] Cell Technology, USA) cultured in Lifeline[®] ReproLife[™] Medium (Lifeline[®] Cell Technology, USA). All cells were contained in humidified atmosphere with 5% CO₂ at 37 °C. The expression of SIRT2 in EC cells (with HEEC cells as control) was evaluated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot.

Transfection

The 0.8 µg negative control (NC) and 0.8 µg SIRT2 (Access number: NM_012237.4) overexpression plasmids, which were constructed with pcDNA3.1 vector (Genomeditech, China), were transfected into 2 × 10⁵ Ishikawa cells. After transfection, the cells were termed into pcDNA-NC group and pcDNA-SIRT2 group, respectively. The 0.8 µg NC and 0.8 µg SIRT2 knock-down plasmids, which were constructed with pGPH1 vector (GenePharma, China), were transfected into 2 × 10⁵ KLE cells. And the cells were divided into pGPH1-NC group and pGPH1-SIRT2 group after transfection, accordingly. The transfection was completed with the use of HilyMax (Dojindo, Japan). The target sequences for NC and SIRT2 were: NC, 5'-AATTCAAGTCGUGUCACGUTT-3'; SIRT2, 5'-GATCCGAUCACCUGUAUCUATT-3'.

At 24 h (h) after transfection, the expression of SIRT2 in each group was evaluated by RT-qPCR and western blot.

Sphere formation assay and extreme limiting dilution analysis (ELDA)

To complete the sphere formation assay, the cells were cultured in DMEM/F12 medium containing 2% B27, 20 ng/ml bFGF and 20 ng/ml EGF and plated in ultra-low attachment plates; after 7 days, the number of the spheres with diameter > 50 µM was counted [16]. For ELDA, cells at different densities (10, 100, and 1000/well) were cultured in the medium described in the sphere formation assay and cells at each density were cultured for 24 wells; after 7 days, the number of wells with at least one sphere (diameter > 50 µM) was counted and calculated by ELDA online program (<http://bioinf.wehi.edu.au/software/elda/>) [16].

Flow cytometry

At 48 h after transfection, the cells were collected and stained with Alexa Fluor[®] 488 Conjugated CD133 mouse monoclonal antibody (CST, USA). After the staining, the cells were sorted with a flow cytometer (BD, USA) and analyzed with FlowJo 7.0 (BD, USA).

Cisplatin and paclitaxel treatment

At 48 h after transfection, the cells were treated with different concentrations of cisplatin (Sigma, USA) or paclitaxel (Sigma, USA) for another 48 h. The concentration ranges of cisplatin (Sigma, USA) cultured with Ishikawa cells and KLE cells were 0–32 µM and 0–80 µM, respectively. And the concentration ranges of paclitaxel (Sigma, USA) cultured with Ishikawa cells and KLE cells were 0–8 nM and 0–80 µM. Then the cell viability was evaluated by cell counting kit-8 (Dojindo, Japan) according to the kit's instruction. The relative cell viability was calculated using the following formula: (absorbance value at each concentration/absorbance value at 0 concentration) × 100%.

Pathway detection

In the previous studies, the SIRT2 is reported to regulate the multidrug resistance via MEK/ERK pathway in cancers [10, 17]. Meanwhile, the MEK/ERK pathway plays an important role in the regulation of stemness and chemosensitivity in cancers [11–14]. Therefore, the expression of MEK1, phosphate-MEK1 (p-MEK1), ERK1/2 and p-ERK1/2 in cells was detected by western blot at 48 h after transfection.

RT-qPCR

The RNA expression of SIRT2 was detected by RT-qPCR. Briefly, total RNA was extracted using TRIzol™ Reagent (Thermo, USA) and reversely transcribed to cDNA using ReverTra Ace® qPCR RT Kit (Toyobo, Japan). Then, PCR was performed using SYBR® Green Realtime PCR Master Mix (Toyobo, Japan), from which the relative expression of SIRT2 was calculated with β -actin as the internal reference. The primers sequence is listed in Supplementary Table 1.

Western blot

The protein expressions were detected by western blot. The cells were suspended by RIPA Lysis and Extraction Buffer (Thermo, USA) and quantified by Pierce™ BCA Protein Assay Kit (Thermo, USA). After collecting from the supernatant from centrifugation, the proteins underwent electrophoresis on SDS PAGE GEL: NuPAGE Bis–Tris Gels 4–12% (Thermo, USA), and were transferred to polyvinylidene fluoride membrane (PALL, USA). Then, the membrane was incubated with primary antibodies for a night and incubated with secondary antibodies for 1.5 h at room temperature. The Novex™ECL Chemiluminescent Substrate Reagent Kit (Invitrogen, USA) was used for chemiluminescence, and the proteins were visualized on X-ray film (Kodak, USA). The antibodies applied in western blot are listed in Supplementary Table 2.

Statistical analysis

All the data in this study were expressed as mean \pm standard deviation. GraphPad Prism 7.01 (GraphPad, USA) was used to analyze the data and plot the graph. Dunnett's multiple comparisons test was used to compare the difference between control/NC group and other groups. P value < 0.05 was considered as statistically significant. Non-significant was defined as P value > 0.05 and marked as NS.

Results

Expression of SIRT2 in EC cells

The SIRT2 mRNA expression was increased in Ishikawa cells ($P < 0.05$), AN3CA cells ($P < 0.01$), HEC1A cells ($P < 0.001$) and KLE cells ($P < 0.001$) compared with control cells (Fig. 1A). Similarly, the protein expression of SIRT2 was elevated in Ishikawa cells, AN3CA cells, HEC1A cells and KLE cells than in control cells (Fig. 1B).

SIRT2 overexpression and knock-down in EC cells

Since the expression of SIRT2 was the lowest in Ishikawa cells but the highest in KLE cells among the EC cell lines included in this study, SIRT2 overexpression and knock-down plasmids were transfected to Ishikawa cells and KLE cells, respectively, for further experiments. After transfection in Ishikawa cells, the mRNA ($P < 0.001$) (Fig. 2A) and protein expressions (Fig. 2B) of SIRT2 in the pcDNA-SIRT2 group were increased than that in the pcDNA-NC group. In KLE cells, the mRNA ($P < 0.001$) (Fig. 2C) and protein expressions (Fig. 2D) of SIRT2 were reduced in the pGPH1-SIRT2 group compared with the pGPH1-NC group.

The effect of SIRT2 on EC cell stemness

Sphere formation assay revealed that in Ishikawa cells, the sphere number was higher in the pcDNA-SIRT2 group compared to the pcDNA-NC group ($P < 0.05$) (Fig. 3A). In KLE cells, the sphere number was lower in the pGPH1-SIRT2 group than in the pGPH1-NC group ($P < 0.05$) (Fig. 3B). Besides, ELDA assay displayed that the estimated stem cell frequency was higher (1 per 204 cells vs. 1 per 357 cells) in the pcDNA-SIRT2 group compared with the pcDNA-NC group ($P = 0.038$) in Ishikawa cells (Table 1). In KLE cells, the estimated

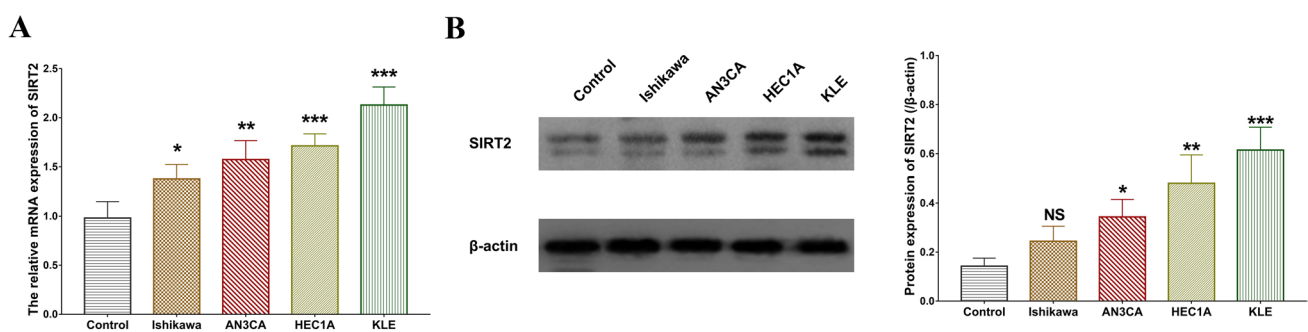


Fig. 1 SIRT2 expression in EC cells. The expression of SIRT2 mRNA in Ishikawa cells, AN3CA cells, HEC1A cells and KLE cells compared with control cells (A). The expression of SIRT2 protein in

Ishikawa cells, AN3CA cells, HEC1A cells and KLE cells compared with control cells (B). SIRT2 sirtuin 2, EC endometrial cancer

Fig. 2 SIRT2 expression in EC cells after transfection. The effect of SIRT2 overexpression on SIRT2 mRNA (A) and protein (B) levels in Ishikawa cells. The effect of SIRT2 knock-down on SIRT2 mRNA (C) and protein (D) levels in KLE cells. SIRT2 sirtuin 2, EC endometrial cancer

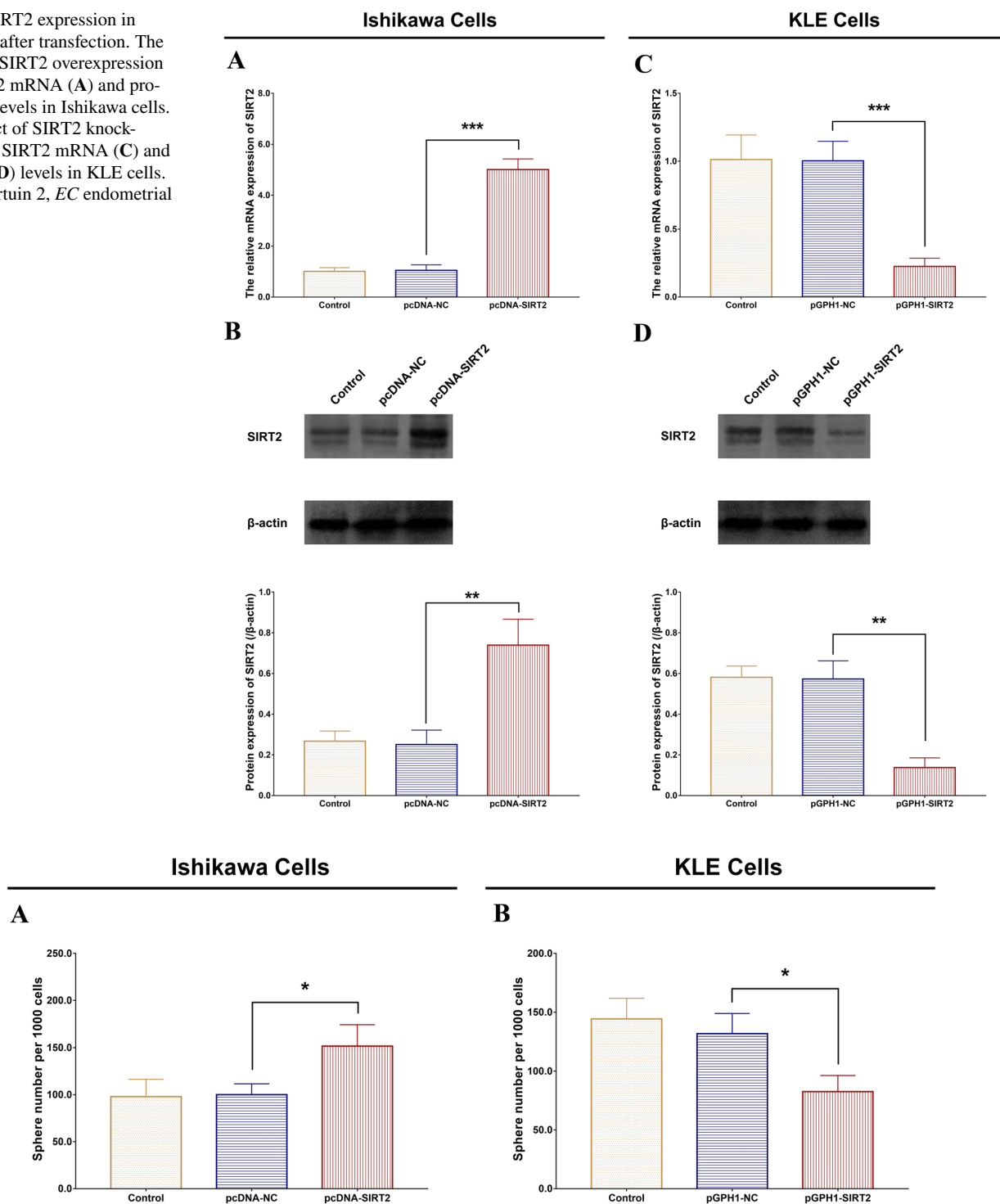


Fig. 3 Effect of SIRT2 on sphere formation ability in EC cells. The effect of SIRT2 overexpression on sphere formation ability in Ishikawa cells (A). The effect of SIRT2 knock-down on sphere formation ability in KLE cells (B). SIRT2 sirtuin 2, EC endometrial cancer

stem cell frequency was lower (1 per 221 cells vs. 1 per 128 cells) in the pGPH1-SIRT2 group compared with the pGPH1-NC group ($P = 0.040$). In addition, the Ishikawa CD133⁺ cells rate in the pcDNA-SIRT2 group was higher

than that in the pcDNA-NC group ($P < 0.01$) (Fig. 4A, B), while the KLE CD133⁺ cells rate was decreased in the pGPH1-SIRT2 group compared with the pGPH1-NC group ($P < 0.05$) (Fig. 4C, D). These findings suggested

Table 1 ELDA data

Groups	Limiting dilution (wells)			1/stem cell frequency			P value
	1000	100	10	Lower	Estimate	Upper	
Ishikawa cells							
Control	19/24	12/24	5/24	536	346	219	0.038
pcDNA-NC	18/24	13/24	6/24	562	357	227	
pcDNA-SIRT2	21/24	14/24	9/24	341	204	122	
KLE cells							
Control	22/24	19/24	11/24	193	117	71.1	0.040
pGPH1-NC	22/24	18/24	10/24	212	128	77.0	
pGPH1-SIRT2	18/24	6/24	2/24	366	221	133.6	

ELDA extreme limiting dilution analysis

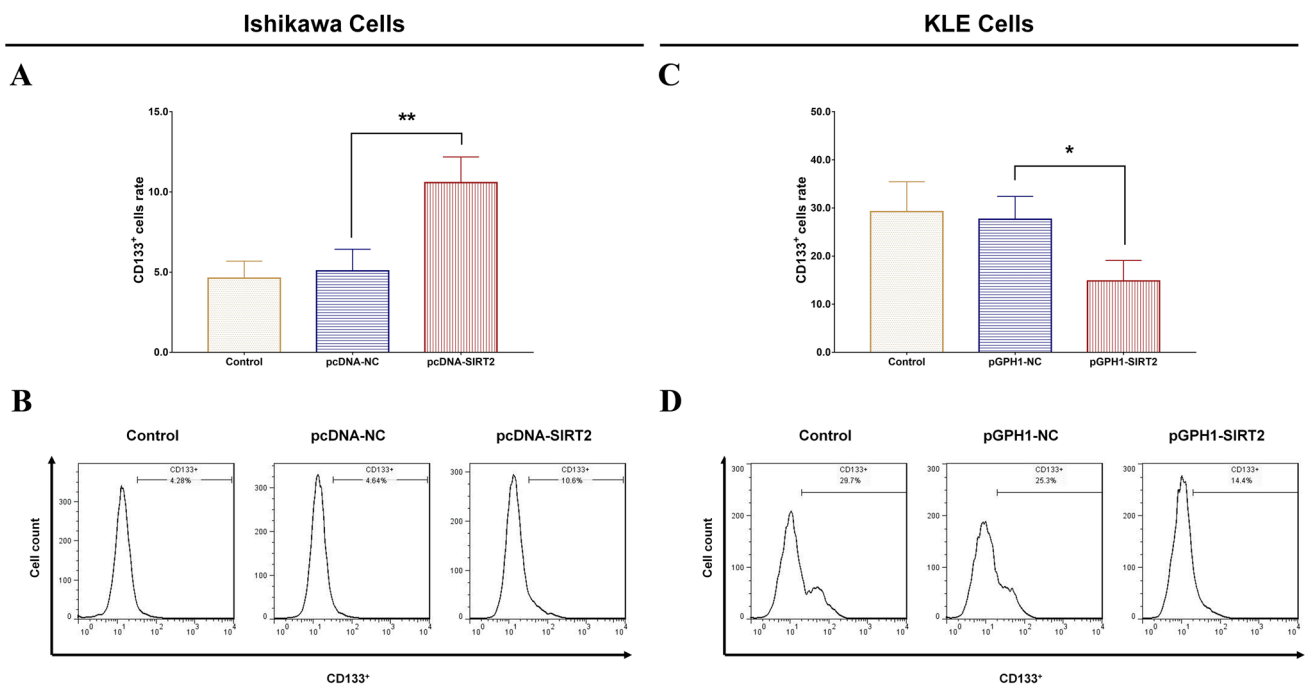


Fig. 4 Effect of SIRT2 on CD133⁺ cells rate in EC cells. The effect of SIRT2 overexpression on CD133⁺ cell rate in Ishikawa cells (**A**, **B**). The effect of SIRT2 knock-down on CD133⁺ cell rate in KLE cells (**C**, **D**). *SIRT2* sirtuin 2, *EC* endometrial cancer

that SIRT2 overexpression increased, while SIRT2 knock-down reduced the stemness of EC cells.

The effect of SIRT2 on EC cell platinum and paclitaxel sensitivity

In Ishikawa cells, the relative cell viability was higher in the pcDNA-SIRT2 group compared with the pcDNA-NC group under 4, 8, and 16 μ M cisplatin treatment (all $P < 0.05$) (Fig. 5A); and the relative cell viability was elevated in the pcDNA-SIRT2 group than in the pcDNA-NC group under 2, 4 nM paclitaxel treatment (both $P < 0.05$) (Fig. 5B). In KLE cells, compared with the pGPH1-NC group, the relative cell viability was lower in the pGPH1-SIRT2 group under 10, 20,

40, and 80 μ M cisplatin treatment (all $P < 0.05$) (Fig. 5C). Meanwhile, the relative cell viability was reduced in the pGPH1-SIRT2 group compared with the pGPH1-NC group only under 40 nM paclitaxel treatment ($P < 0.05$) (Fig. 5D). These data indicated that SIRT2 decreased the chemosensitivity to cisplatin slightly but did not affect the chemosensitivity to paclitaxel in EC cells.

The effect of SIRT2 on MEK/ERK pathway in EC cells

In Ishikawa cells, the p-MEK1 and p-ERK1/2 protein expressions were increased in the pcDNA-SIRT2 group than in the pcDNA-NC group (Fig. 6A). However, in KLE cells, the p-MEK1 and p-ERK1/2 protein expressions were

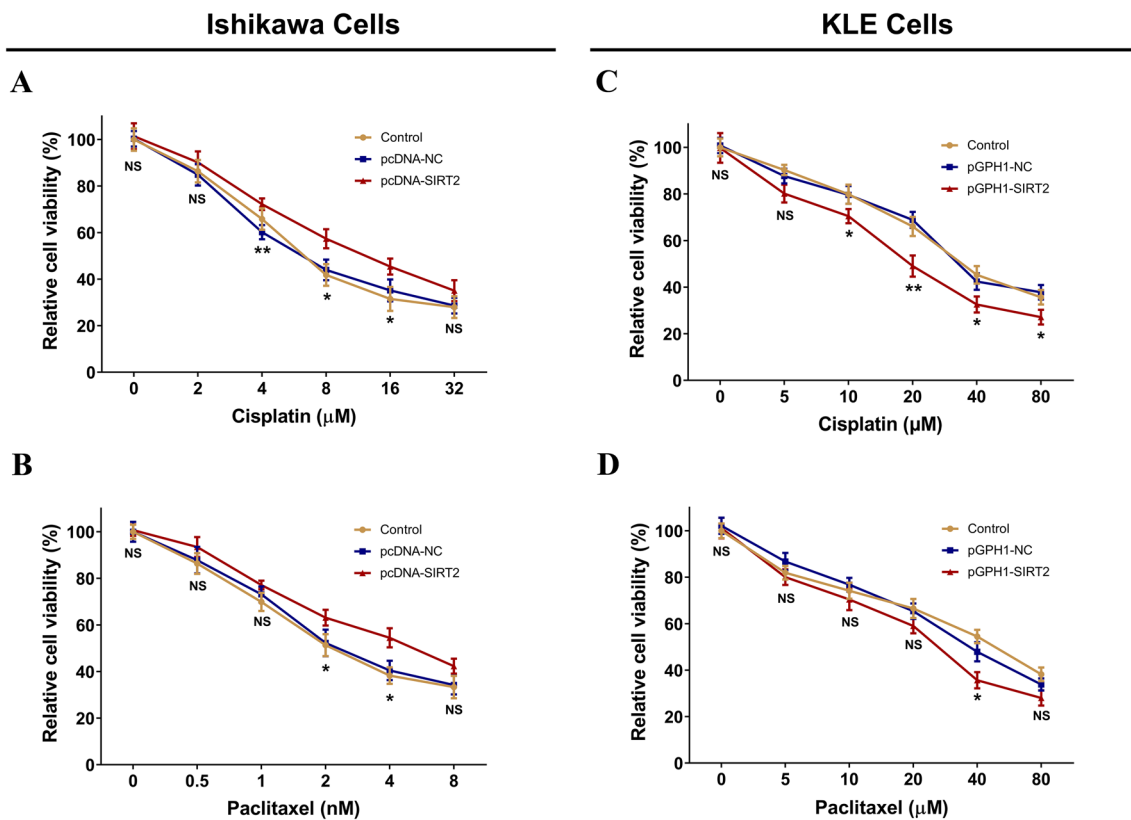


Fig. 5 Effect of SIRT2 on chemosensitivity in EC cells. The effect of SIRT2 overexpression on relative cell viability under cisplatin treatment (A) and paclitaxel treatment (B) in Ishikawa cells. The effect of

SIRT2 knock-down on relative cell viability under cisplatin treatment (C) and paclitaxel treatment (D) in KLE cells. SIRT2 sirtuin 2, EC endometrial cancer

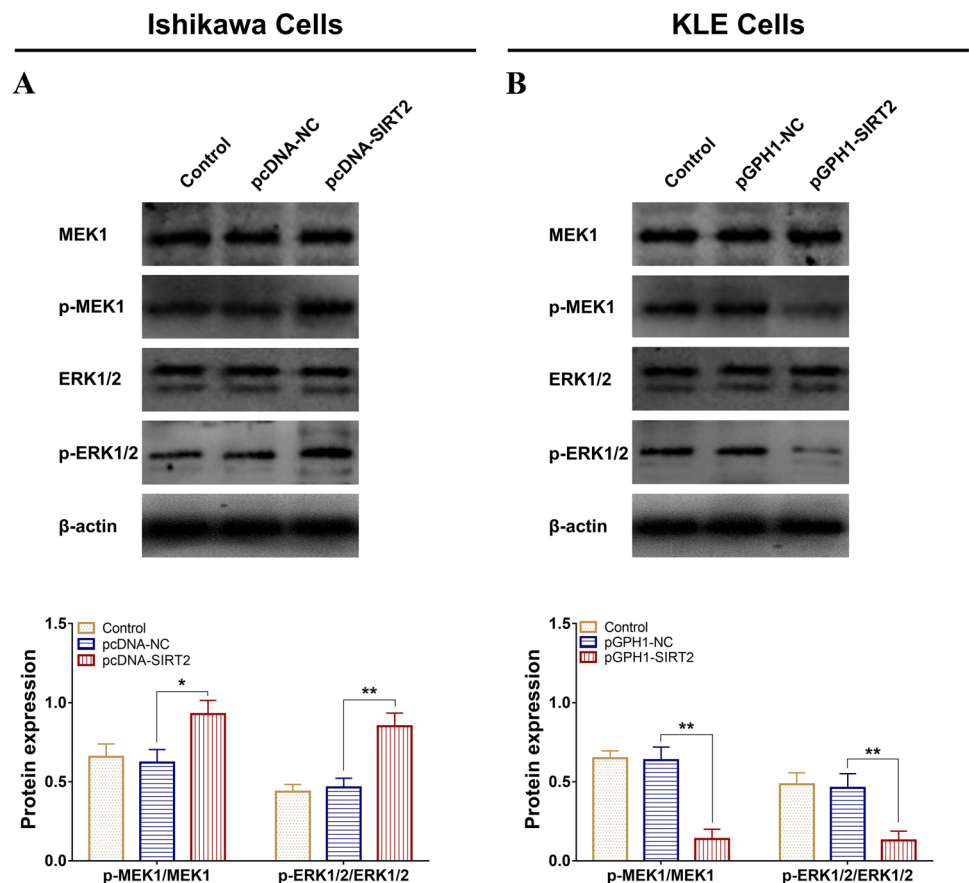
decreased in the pGPH1-SIRT2 group compared with the pGPH1-NC group (Fig. 6B). This finding suggested that SIRT2 overexpression activated, but SIRT2 knock-down suppressed the MEK/ERK pathway in EC cells.

Discussion

SIRT2 is functionally significant in cancer progression as an NAD⁺-dependent deacetylase, but its role in cancers is controversial since its dual functions as tumor promoter and tumor suppressor. For instance, a study reveals that SIRT2 suppresses the growth of NSCLC via targeting Jumonji domain-containing protein 2A. Besides, another study reports that inhibiting SIRT2 facilitates p53 activation in NSCLC. These two studies suggest the controversial role of SIRT2 on the oncogenesis of NSCLC [18, 19]. The same debate has been raised in breast cancer when SIRT2 is shown to shorten recurrence and death in ER-negative breast cancer patients but prolong the tumor recurrence in ER-positive breast cancer patients [20]. Regarding the tumor-inhibiting role of SIRT2, suppression of SIRT2 inhibits cell motility and invasiveness; besides, SIRT2 is upregulated in

HCC cell lines and tissues [21]. In addition, SIRT2 inhibitor induces cell cycle arrest in colon carcinoma cells and promotes c-myc oncoprotein degradation [22, 23]. As to EC, there is little information on the role of SIRT2 in its tumorigenesis. In this study, we first observed that SIRT2 was overexpressed in EC cell lines (including Ishikawa cells, AN3CA cells, HEC1A cells, and KLE cells) compared with controls, which suggested that SIRT2 might act as a tumor promoter in EC. Then, further experimental exploration showed that SIRT2 overexpression increased, while SIRT2 knock-down reduced the sphere formation ability and CD133⁺ cells rate in EC cells. The above indicated that SIRT2 increased EC cell stemness, which could be explained from various aspects: (1) inhibition of SIRT2 was reported to induce the differentiation of osteogenic, neuronal, and cancerous cell differentiation [24, 25]. Thus, it was speculated that SIRT2 could facilitate the stemness of EC cells. (2) As validated by our subsequent experiment, SIRT2 activated the MEK/ERK signaling pathway, which increased the expression of stemness-associated cell-surface marker protein CD133⁺; meanwhile, SIRT2 positively correlated with cell-surface glycoprotein CD44 expression, thereby potentially inducing invasion and metastasis, and contributing to chemoresistance

Fig. 6 Effect of SIRT2 on the MEK/ERK pathway in EC cells. The effect of SIRT2 overexpression on the MEK/ERK pathway in Ishikawa cells (A). The effect of SIRT2 knock-down on the MEK/ERK pathway in KLE cells (B). *SIRT2* sirtuin 2, *EC* endometrial cancer



to cisplatin in cancer cells [26]. Despite this, the oncogenic function of SIRT2 in EC needs to be confirmed by clinical exploration.

Cisplatin and paclitaxel belong to the most commonly used chemotherapy regimens for EC treatment. In addition to the influence of SIRT2 on EC cell stemness, we also observed that SIRT2 decreased the chemosensitivity to cisplatin in EC cells. The possible reason was that SIRT2 might promote the adaptive response to chemotherapy-induced stress in EC cells, thereby decreasing the chemosensitivity [27]. SIRT2 was shown to be positively correlated with the stemness of EC cells by our previous experiments. Therefore, it might increase stem cell proportion in EC cells, subsequently reducing sensitivity to chemotherapy. In addition, SIRT2 might regulate the multidrug resistance via the MEK/ERK pathway, which plays an essential role in regulating stemness in cancers [10–14]. What's more, ERK was known to induce HIF1 α -mediated platinum sensitivity by directly targeting PHD2 in ovarian cancer [13]. Hence, SIRT2 might also activate ERK and decrease cisplatin sensitivity in EC cells. Furthermore, the regulatory role of SIRT2 on MEK/ERK was validated in our following exploration.

MEK/ERK signaling pathway is associated with the malignant potential of cancer cells via regulating cell cycle progression, stemness and drug sensitivity, whose mutation

is also frequently observed in various cancers [28]. The activation of the MEK/ERK signaling pathway induces the phosphorylation of proteins (including post-transcriptionally apoptotic regulatory molecules), and reduces sensitivity to doxorubicin and paclitaxel in cancer cells (including breast cancer cells and HCC cells) [11, 28]. Considering that SIRT2 regulates drug sensitivity via MEK/ERK pathway in other cancers [10–14], and the upregulation of MEK/ERK modulates the susceptibility, survival and the recurrence of EC [29], we further evaluated the correlation of SIRT2 with the MEK/ERK signaling pathway in EC cells. It was observed that SIRT2 overexpression activated, but SIRT2 knock-down suppressed MEK/ERK pathway in EC cells, which could be attributed to that SIRT2 might activate Ras protein via lysine defatty-acylation and subsequently activate the downstream MEK/ERK in EC cells [30]. Seeing that the MEK/ERK signaling pathway was associated with chemosensitivity in cancer cells, it could be hypothesized that SIRT2 might reduce chemosensitivity in EC cells via interacting with the MEK/ERK signaling pathway. However, this needed to be validated by further compensative experiments.

In conclusion, SIRT2 promotes cell stemness and activates the MEK/ERK signaling pathway while reduces chemosensitivity in EC, indicating its potential as a target of EC treatment.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00404-021-06216-2>.

Author contributions NZ: project development, data analysis, manuscript editing. YG, PL, YC, YW: data collection, manuscript writing.

Funding This study was supported by Hebei Medical Science Research Project Plan (No. 20201248).

Declarations

Conflict of interest All authors declare no conflict of interest.

Ethical approval Not applicable.

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