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Functional role of SIRT1-induced HMGB1 expression and acetylation in migration, invasion and angiogenesis of ovarian cancer

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Abstract. – OBJECTIVE: Ovarian cancer is a commonly occurred tumor in females. High motility group box-1 protein (HHMB1) is a chromosome-related protein with multiple functions. A recent study revealed critical roles of HMGB1 in occurrence and progression of ovarian cancer. Sirtuin 1 (SIRT1) is a recently ide[illegible] novel molecule, which regulates acetyla[illegible] HMGB1. Whether SIRT1 is involved in migr[illegible]n, invasion or angiogenesis of ovarian cancer is[illegible] clear. This study aims to investigate the role[illegible] SIRT1-induced HMGB1 acetylati[illegible] migratio[illegible] invasion, and angiogenesis in[illegible]ancer.

PATIENTS AND METHO[illegible] In o[illegible]an cancer cell line, SIRT1 exp[illegible]on wa[illegible]otentiated. Western blot and imm[illegible]fluor[illegible] used to measure H[illegible]B1 [illegible]n, ace[illegible]tion level, and nucl[illegible]ranslo[illegible] Scratch assay and transwell [illegible]mber metho[illegible]re used to examine cell m[illegible]and invasio[illegible]tency. A mouse model [illegible]n ov[illegible]cancer cell transplantation was [illegible]nerated to [illegible]sure induced nitric oxide sy[illegible]se (iNOs) and [illegible]5 expression.

RES[illegible]S: Compared to [illegible]jacent tissues, ova[illegible] cance[illegible]ssues had significantly decre[illegible] SIR[illegible]expression. In ovarian cancer cells, [illegible]ver-exp[illegible]ion decreased HMGB1 [illegible] ace[illegible]on le[illegible], and SIRT1 knockdown [illegible]ated [illegible]Expression and acetylation. SI[illegible] over-e[illegible]sion also suppressed nuclear [illegible]oslocatio[illegible] of HMGB1. Meanwhile, SIRT1 [illegible]s, migration and angiogenesis of [illegible]arian ca[illegible]er cells via HMGB1.

CONCLUSIONS: SIRT1 over-expression effectively inhibited HMGB1 expression and acetylation, thus inhibiting ovarian cancer migration, invasion and angiogenesis. HMGB1 modulated behaviors of ovarian cancer via SIRT1. Therefore, SIRT1 might work as a treatment target for managing ovarian cancer migration.

Ke[illegible]rds:
[illegible]T1, HMGB1, Ovarian cancer.

[illegible]troduction

Ovarian cancer is derived from female ovary [illegible] and 90-95% of these cases belong to pri[illegible]ncer[1]. Due to the lack of typical early symptoms and limited efficiency of screening, diagnosis is extremely difficult at early stage. Patient diagnosis is further compromised due to strong potency of metastasis at advanced stage. Therefore, the investigation of treatment approaches to inhibit migration or invasion of ovarian cancer cells and tumor angiogenesis presents high priority. Early study showed critical roles of high motility group box-1 protein (HHMB1) in ovarian cancer pathogenesis[2]. HMGB1 is a pluripotent chromosome related protein, which participates in various biological functions including nuclear DNA rearrangement, repair and transcription, thus playing important roles in maintaining chromosome stability[3]. HMGB1 is one highly conserved nuclear protein that can regulate gene transcription, and can maintain nuclear body structure[4]. In addition, HMGB1 can work as inflammatory cytokine to be related from necrosis cells or being actively secreted by stress cells[5]. A recent study showed that HMGB1 also worked as mediator playing important roles in multiple diseases. In lipid denaturation, inflammatory response, fibrosis and tumor occurrence, HMGB1 expression was significantly increased[6-8]. In recent years, HMGB1 over-expression has been reported

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yet, and has been shown to facilitated migration, invasion and angiogenesis of tumor cells[9].

Mammalian sirtuin 1 (SIRT1) is a NAD-dependent histone deacetylase, and plays critical roles in multiple physiological processes including gene transcription, cell aging, energy metabolism, oxidative stress and inflammatory response[10,11]. Other researches showed that certain microRNA can mediate migration and invasion behaviors of ovarian cancer cells via mediating SIRT1[12]. In recent years, SIRT1 is newly found to regulate acetylation and release of HMGB1. In pyaemia and fatty liver disease, SIRT1 expression was inhibited[13,14], thus elevating acetylation level of HMGB1 to potentiate its activity. However, whether SIRT1 participates in HMGB1-dependent migration, invasion and angiogenesis regulation in ovarian cancer is still unclear. Thus, we aimed to investigate the functional role of SRIT1-induced HMGB1 acetylation in migration, invasion and angiogenesis of ovarian cancer. We firstly utilized ovarian cancer cell line to up-regulate SIRT1 expression by cell transfection, followed by detection of HMGB1 expression, acetylation level change and nuclear translo[illegible]n of HMGB1, to substantiate modulatory [illegible] SIRT1 on HMGB1. We next examined the c[illegible]e on ovarian cancer cell line migration and i[illegible] sion by HMGB1 acetylation mediated by SIR[illegible] Lastly, we introduced mouse m[illegible]h ovaria[illegible] cancer xenograft transplanta[illegible] to [illegible]mine the effect of SIRT1 mediated [illegible]GB1 ac[illegible]ation on ovarian cancer angiogene[illegible]

Mate[illegible]nd Meth[illegible]

Major Ma[illegible]nals and [illegible]gent

SIRT1 [illegible]MGB1, β-act[illegible]d induced nitric oxide [illegible]nase (iNOs) antibo[illegible] were purchased fro[illegible]oVisio[illegible]753-100, Mountain View, CA, USA[illegible]rs[illegible]sh perc[illegible]dase (HRP) labeled goat anti-ra[illegible]condar[illegible]body and FITC-labeled [illegible]anti-[illegible]t fl[illegible]escent secondary antibody we[illegible]purcha[illegible]om ZSJQ Biotech. (Beijing, Ch[illegible] Eosin, [illegible]ematoxylin-staining solutions [illegible]d from Qiwu Biotech (Shanghai, [illegible]ina). Total protein extraction kit was purchased [illegible]m Kaiji Biotech (Shanghai, China). Western [illegible]lysis buffer and bicinchoninic acid (BCA) pro[illegible]in quantification kit were provided by Beyotime (Shanghai, China). Immunohistochemistry kit and diaminobenzidine (DAB) lysis buffer were purchased from ZSJQ Biotech. (Beijing, China).

Major Equipment

Ultrapure workstation was provided by Boxun (Changsha, China). Gel imaging system UVP Multispectral Imaging System (UVP, Sacramento, CA, USA). Model PS-9 semi-d[illegible]sfer electrophoresis was purchased fr[illegible]ngh[illegible] (Nanjing, China). CO_2 chamber an[illegible]ermo-354 microplate reader were purchased[illegible] Thermo Fisher Scientific (Waltham, MA, USA[illegible]

Sample Collection

A total of 20 tumor[illegible]e and[illegible]cent tiss[illegible] were collected from c[illegible]n c[illegible]r patie[illegible]ts (older than 18 years) t w[illegible]uited f[illegible]Jinan Maternity and [illegible]d Care H[illegible]l. Ti[illegible] patients with cogni[illegible]sfunctions,[illegible]recurrence or other p[illegible]ess[illegible]ease, or w[illegible] systemic infection and severe di[illegible]rs were excluded. After infor[illegible]nsents we[illegible]ned, ovarian cancer tis[illegible] and adjacent tissu[illegible] (within 3 cm from c[illegible]er tissues) were collected during surgery and [illegible] immediate[illegible]tored in liquid nitrogen for f[illegible]r assays. T[illegible] study was approved by the Eth[illegible]Comm[illegible] of Jinan Maternity and Child Care H[illegible]

[illegible]ine and Culture

[illegible]an cancer cell line Hey was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured within Dulbecco's modified eagle Medium (DMEM) containing 10% sterile fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA) in a 37°C chamber with 5% CO_2.

Construction of SIRT1 Over-Expression Lentiviral Plasmid and siRNA Knockdown Plasmid and Cell Transfection

SRIT1 over-expression lentiviral plasmid and siRNA knockdown plasmid were designed and synthesized by Gimma (Shanghai, China). 24 h before transfection, cells were passed within 24-well plate until reaching 30-50% confluence. 1.25 μl small interfere RNA (siRNA) stock solution (20 μM) or over-expression plasmid (20 μM) was dissolved into 100 μL Option minima essential medium (Opti-MEM) medium as solution A. 1 μl Lipofectamine 2000 or Lipofectamine™ RNAi-MAX was dissolved into Opti-MEM medium as solution B. After incubation for 5 min, solution A and B were mixed, and kept still for 20 min before adding culture plate. After 4 h incubation, DMEM medium containing 10% fetal bovine se-

rum (FBS) was used. SIRT1 expression was measured to calculate transfection efficiency.

Western Blot

12 h after cell transfection, culture medium was completely removed. Cells were washed in phosphate-buffered solution (PBS) for three times. 10 μl phenylmethanesulfonyl fluoride (PMSF, 100 mM, Amersham Biosciences, Little Chalfont, Buckinghamshire, England) were added into each 1 ml lysis buffer. Within 6-well plate, each well was added with 100 μl lysis buffer, and was processed on ice for 5-10 min. Cells were hanged on one side of the culture well by a swab, and cell debris and lysis buffer were removed into pre-cold Eppendorf (EP) tubes. Cell lysate was centrifuged at 12000 r/min for 5 min at 4°C. The supernatant was collected as total protein solution. Western blot was performed following previous literature[15]. Extracted total proteins were quantified by bicinchoninic acid (BCA) approach to unify concentrations. After adding load buffer, protein mixture was boiled for 5 min for complete denature. Loading samples were separated by electrophoresis in 10% sodium dodecyl sulfate polyacrylamid al electrophoresis (SDS-PAGE) until targeted was separated with adjacent proteins. Sep d protein samples were transferred to polyviny ne difluoride (PVDF) membrane by 300 mA fo h. Rabbit anti-HMGB1, SIRT1 and iNO antibody (1:1000 dilutions) ad for 4°C overnight incubation. The brane washed in Tris-buffered saline an een-2 for three times, and h erad idase (HRP)-conjugated goat rabbit s ary antibody (1:1000) was add 2 h 37°C i ion. Chemiluminescen app was emplo d for visualize prot in bands.

Acet on Assay for HM B1

E ssion of acetylated HMGB1 was mea by mune-p cipitation approach. Firstly, su amou HMGB1 antibody were l int proteins, and were incubated ov ght at with gentle vortex. Immunocomple as captur d by adding 25 μl protein A+G followed by 4°C gentle mixture for h. The mixture was centrifuged at 1500 × for 5 n at 4°C. Precipitants were washed for three ti- in cold phosphate-buffered saline (PBS), and we resuspended in 1 × loading buffer, followed by 5 min boiling. Immunocomplex was then dissociated from beads. Supernatant was collected by centrifugation for Western blot.

Immunofluorescence

Cultured cells were inoculated into 6-well plate. 24 h after transfection, cells were fixed within 10% formaldehyde for 24 h, and were blocked in normal goat serum for 20 min. Primary of HMGB1 or CD105 (1: 100) was ad for 4 overnight incubation. On the next d cells were incubated for 30 min at room tem re. After PBS washing, fluorescein isothiocya (FITC) labeled secondary antibod s added ark incubation, and cells we ashed in 4',6- dino-2-phenylindole (1) sta buffer 10 min. After washing w observe under an inverted micro e.

Cell Migra ssay

All cell re d in 6-well te until fully attached growth and fection. 12 h later, normal was added en cells reached 90% co ence, 200 μl pipette p were used to draw p llel scratch lines in the middle of plate bot- Scratched s were washed away and the p was obser under an inverted microscope. es w aken at certain positions with equal w cratch lines across different wells. 12 h later, images were taken at the same plate for ing cell migration conditions.

Transwell Assay for Cell Invasion Potency

Transwell chamber and assay apparatus were pre-cold at 4°C fridge one night before assay. Extracellular matrix (ECM) gel was thawed at 4°C fridge. Transwell chamber was placed into 24-well plate, which was laid flat in iced box. ECM gel was diluted and added into transwell chamber (50 μl per well), which was incubated at 37°C for 4 h still incubation. Residual liquid was removed and the chamber was air-dried for further use. Cholangiocarcinoma cells were harvested for 12 h, and were then prepared into cell suspensions by adding trypsin. After adjusting cell concentration, all groups of cells were added into the upper chamber, whilst lower chamber was filled with culture medium containing 10% fetal bovine serum (FBS). The experiment consisted of normal control group, SIRT1 knockdown group and SIRT1 over-expression group, each of which contained 3 replicated wells. After adding liquids, transwell chambers were extracted at specific time points, and were stained by 0.1% crystal violet, and were observed under an inverted microscope. A total of five fields were samples from upper, lower, left, right and middle sites for enumeration.

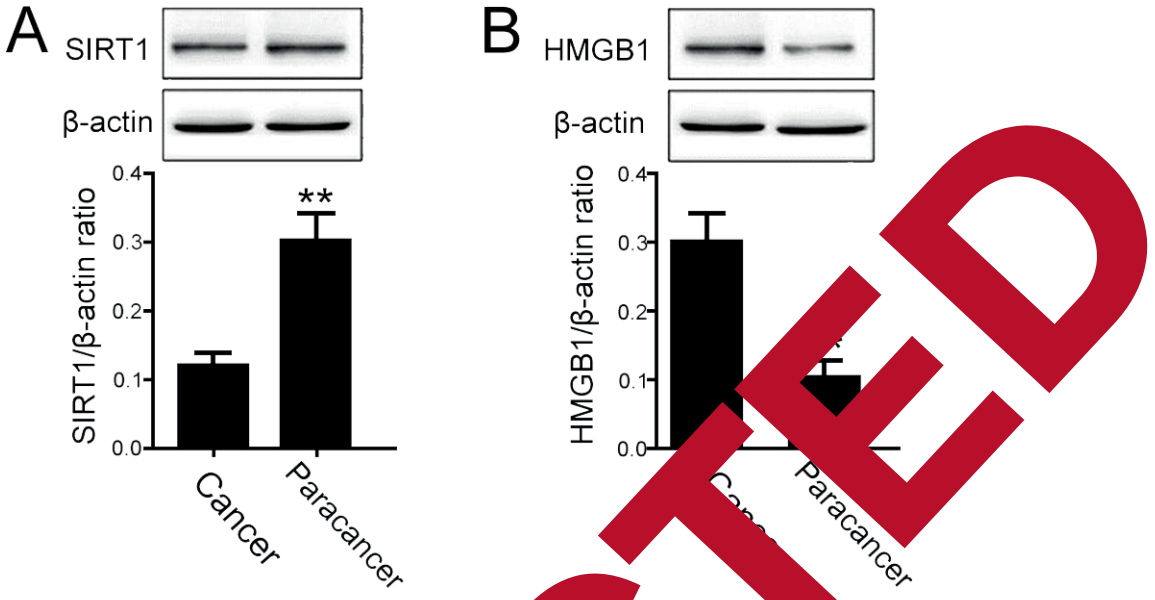

Figure 1. SIRT1 and HMGB1 expression and acetylation in ovaria cer tissues. (A) SIRT1 e ession level. (B) HMGB1 expression level. $^{**}p<0.05$ compared to tumor adjacent tissues.

Animals and Grouping

Male and female BALB/c nude mice we vided by Vital River Lab Animal Techn y Co. Ltd. (Beijing, China). Mice were rando divided into three groups (n=10), including o rian cancer group, SIRT1-inh d SIRT over-expression group. All m were on spe- cific pathology free (SPF) ier an house, with aging between 4 an eeks between 16 and 18 g.

Generation o ian Canc nsplant Model

Ovarian ancer xen mouse model was generate sed on previo ratures[16]. In brief, Hey c were p ssed until 9 confluence. Cells w digest trypsin and centrifuged, and were s ed in P S for adjusting cell density to per 20 . 0.2 ml cell suspension inject bc ously at neck skin of nude mi Genera tions of nude mice were daily ob ed, inclu g motility and food intake. 30 de mice were sacrificed by cervi- l dislocation and tumor tissues were removed. RT1 over-expression and inhibition lentiviral p ids were designed and synthesized by Gim- ma (Shanghai, China). On the same day of tumor implantation, viral particles were infused into mouse by tail vein injection at 50 ng/ml concentration.

Statis alysis

Statistical software SPSS 15.0 was used for ng data (SPSS Inc., Chicago, IL, USA). All s ere presented as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. $p<0.05$ was considered as statistical significance.

Results

SIRT1 and HMGB1 Expressions in Ovarian Cancer Tissues

We obtained ovarian cancer tissues and adjacent controlled tissues from our hospital and performed Western blot to analyze expressional profile of SIRT1 and HMGB1. As shown in Figure 1, SIRT1 expression level was significantly depressed in ovarian cancer tissues ($p<0.05$, Figure 1A), whilst HMGB1 showed opposite patterns and significantly elevated expression in cancer tissues (Figure 1B).

Effects of SIRT1 Knockdown or Over-Expression on HMGB1

We transfected SIRT1 knockdown or over-expression plasmid into cells, and measured SIRT1 and HMGB1 expression, plus HMGB1

acetylation level. As show in Figure 2, comparing to normal control group, SRIT1 knockdown group showed significantly lower SIRT1 expression, and over-expression group revealed elevated transcripts level (Figure 2A). The knockdown of SIRT1 further enhanced HMGB1 expression in cells and culture medium, and over-expression of SRIT1 inhibited intracellular expression and release of HMGB1 (Figure 2B, C). Moreover, we measured expression of acetylated HMGB1 in all groups of cells. We found that SIRT1 knockdown can enhance acetylated HMGB1 expression level inside cells, and SIRT1 over-expression suppressed expression of acetylated HMGB1 inside cells. All these results suggested that SIRT1 negatively regulated HMGB1 expression or acetylation level (Figure 2D, E).

Effects of SIRT1 Over-Expression on HMGB1 Translocation

Recent investigations showed that SRIT1, as an important histone deacetylase, could modulate deacetylation of HMGB1, thus sup... its activation and release towards ... plas... Therefore, this study utilized i... nofluorescence approach to measure the e... of SIRT1 expression on HMGB1 nuclea... trans... n. As shown in Figure 3, cytopla... HMGB... es- sion was significantly e... ted in SIRT... bition group, and SIR... over-ex... ssion sig... ficantly suppressed t... eas... HMGB1 into cytoplasm. Thes... su... ved th... IRT1 could inhibit t... ocation ... MGB... om nucleus toward... lasm.

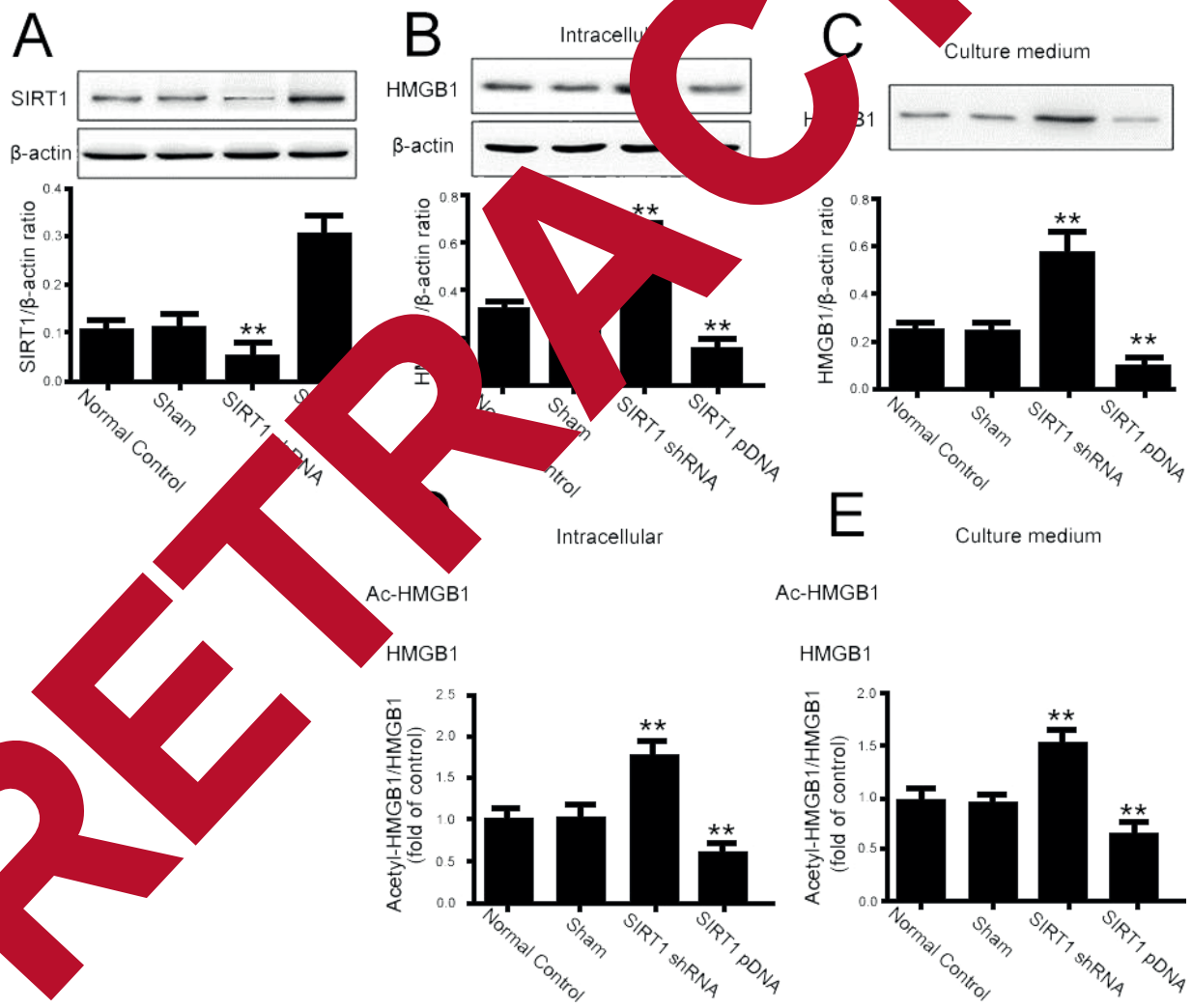

Figure 2. SIRT1 knockdown or over-expression and HMGB1 expression or acetylation. (A) SIRT1 expression. (B) HMGB1 expression inside cells. (C) HMGB1 levels in culture medium. (D) Intracellular expression of acetylated HMGB1. (E) Acetylated HMGB1 in culture medium. $^{**}p<0.05$ compared to normal control group.

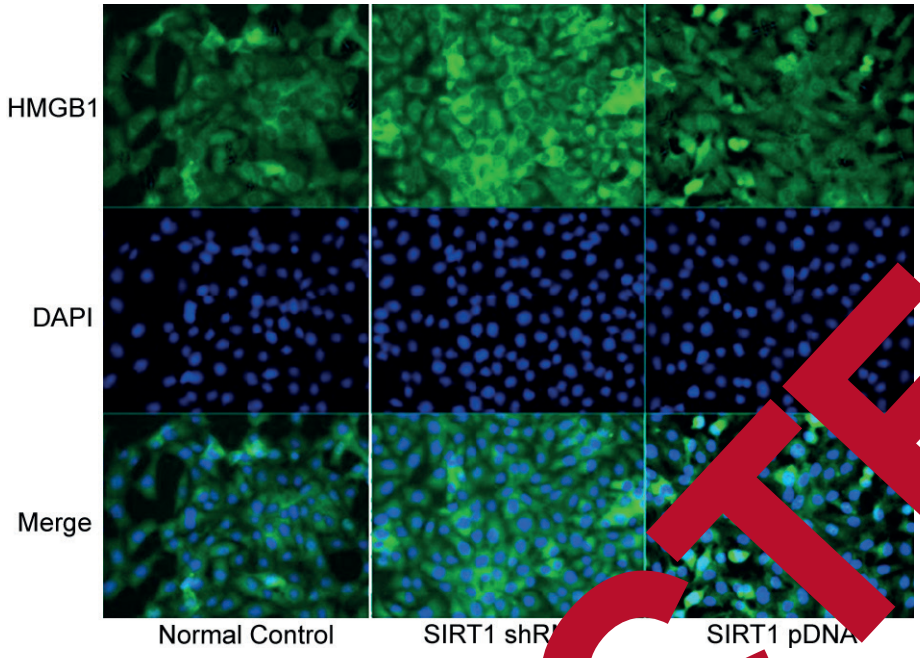

Figure 3. Effects of SIRT1 over-expres n HMGB1 tra ation (200

Effects of SIRT1 Over-Expression on Cell Invasion

To measure cell migration status, we used a-tch assay as previously described[17]. The wid scratch was measured at the same location fro h to 24 h. As shown in Figure 4 betwe cell scratch was significantly T1 inhi- bition group, and such cle signifi tly wide in SIRT1 over-expressio up. showed that SIRT1 exp gnific y inhibited cell mig n.

Effects of S Expressio on Cell In asion

We u transwell cha to measure the chang cell in asion poten By analyzing the nu of cel netrating basal membrane wi- thin und si ficantly increased num- ber of ng cel SIRT1 inhibitor group aring la ontrol group, whilst SIRT1 ov xpress arkably decreased invading cel mber. ese results showed that SIRT1 could prevent cell invasion (Fi- re 5).

ts of SIRT1 Over-Expression on Angiogenesis of Ovarian Cancer

iNOs is the early marker for angiogenesis[18]. In transplanted tumor tissues on nude mice, we measured iNOs expression. As shown in Figure 6A, to blank control group, SIRT1 knockdown mice displayed significantly lower expression, whilst SIRT1 over-expression higher transcript levels. Meanwhile, we measured iNOs expression level in all group and found significant elevation in SIRT1 knockdown group (Figure 6B). In SIRT1 over-expression group, iNOs expression was significantly higher compared to cancer model group, but without significant change with blank control group. These results clearly suggested that up-regulation of SRIT1 expression could facilitate angiogenesis of ovarian cancer tissues. We also used immunohistochemistry staining to measure newly formed vessels using CD105 as the marker. SIRT1 knockdown lentivirus treated mice showed potentiated CD105 staining, and SIRT1 over-expression decreased staining rate of CD105, indicating that SIRT1 over-expression could inhibit angiogenesis (Figure 6C).

Discussion

Ovarian cancer is a commonly occurred malignant tumor in female reproductive organs, and has relatively higher incidence only lower than cervical carcinoma and uterus cancer[19]. Ovarian cancer frequently has early stage metastasis, making it unlikely to completely remove lesions

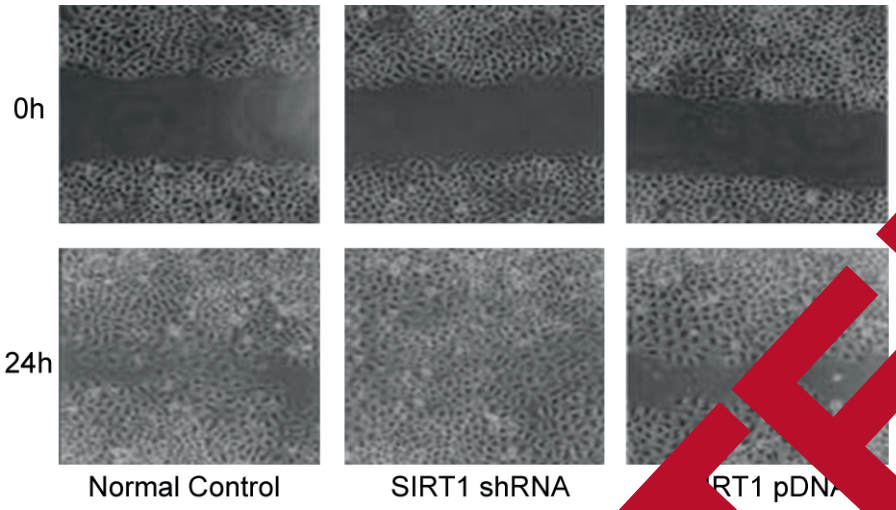

Figure 4. Effects of SIRT1 over-expression on ce[illegible]gratio[illegible]×).

by surgery, and largely limited application and efficiency of radiotherapy. Therefore, systemic chemotherapy has become a critical auxiliary treatment approach[20], as it plays importa[illegible] les in suppressing ovarian cancer cell mig[illegible] invasion and angiogenesis target for su[illegible]s-sing metastasis of ovarian cancer. High mo[illegible] group box-1 protein (HHMB1) is one polypept[illegible] strand with 215 amino acid re[illegible]ith high[illegible] conserved sequence. On its [illegible]rmi[illegible] there is[illegible] abundantly lysine distribu[illegible] with la[illegible] amoun-ts of positive charges. Its [illegible]rmi[illegible] amounts of glutam[illegible] wit[illegible]e charg[illegible]s. HMGB1 obtained [illegible]ame du[illegible] fast mobility velocity in PAG[illegible]lectrophore[illegible]1, 22]. Seidu et al[9] showed [illegible] up[illegible]lation of [illegible]GB1 mi-ght facilita[illegible] migration[illegible] invasion of ovarian cancer. [illegible]peptide micro[illegible] assay, HMGB1 was fo[illegible] to be the candidate [illegible] SIRT1 substrate. HM[illegible] parti[illegible]es in chromosome remodeling and [illegible]ri[illegible]ial reg[illegible]tion[23]. Within cell secre-tion pr[illegible] HMGB[illegible]n work as an inflamma-[illegible]affin[illegible]le[illegible] marker. Under most sce-na[illegible] macr[illegible], NK cells and dendritic cells ca[illegible]tively sec[illegible]t HMGB1. However, increasing [illegible]ed the involvement of non-immune [illegible]tive cells in secretion of HMGB1, such as those [illegible] hepatocytes and most of tumor cells[23]. Lan et [illegible]s shown that SIRT1 could suppress inflam-ma[illegible]on occurrence or progression in fatty liver disease or hepatic sepsis via mediating HMGB1. Other studies showed that microRNA could af-fect progression of gastric cancer, liver carcinoma a[illegible] ovarian can[illegible] targeting SIRT1[12,14]. Increa-si[illegible]vidence sh[illegible]d the central role of SIRT1 in tu[illegible] thoge[illegible]s. However, whether HMGB1's role in [illegible]ancer invasion, migration and an-giogenesis was under the regulation of SIRT1 has [illegible]n reported.

S[illegible] down-regulation or loss of activity has been found in multiple tumors[9]. Moreover, some potent SIRT1 agonist has been confirmed to exert protective roles in tumors[24]. In this research, we used ovarian cancer tissue samples and adjacent tissues, on which expression of SIRT1 and HMGB1 were measured. We found significantly decreased SIRT1 expression in tumor tissues, and further substantiated such down-regulation in ovarian cancer cells. As consistent with predicted results, HMGB1 level was elevated in ovarian cancer cells. To further substantiate regulatory role of SIRT1 on HMGB1 in ovarian cancer, we established SIRT1

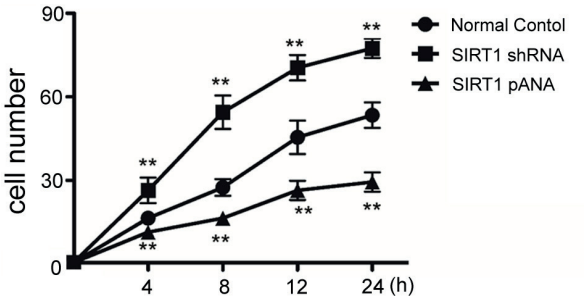

Figure 5. Effects of SIRT1 over-expression on cell invading potency. $^{**}p<0.05$ compared to normal control group.

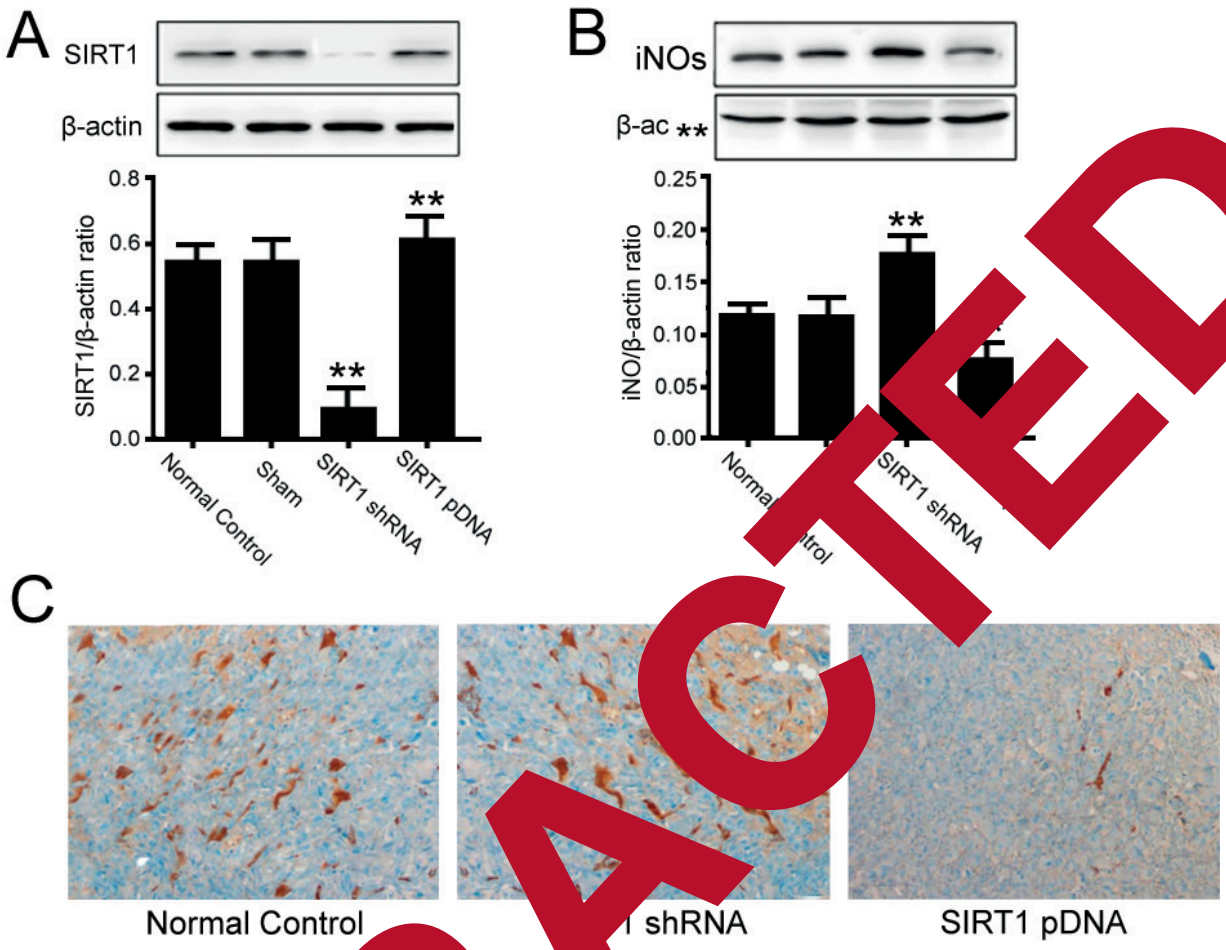

Figure 6. Effects of SIRT1 over-e sion arian can giogenesis. (A) SIRT1 expression level. (B) iNOs expression level. (C) CD105 immunohistoc try stai 200 ×). ** p 05 compared to normal control group.

over-expression an RT1 kno n cell lines, and found decre increase H 1 expression or acety tively. The data demonstrated egulatory f SIRT1 on HMGB1. Meanwhi migration/inva otency of cells, and a genesis ability wer so weakened or enh d with MGB1 down- or up-regulation, resp ly ese dat collectively proved that the reg of cel gration/invasion and angenesis HM was under the direction of T1. We igated the role of SIRT1-HMGB axis on mi ation/invasion and angiogenesis er cells, and demonstrated that the ect of HMGB1 on ovarian cancer cell behaviors s dependent on SIRT1. This work, however, did lustrate whether SIRT1 had full protective role n mice carrying ovarian cancer cell xenograft, or any inhibitory effects on tumor growth and metastasis. Future studies can be performed to evaluate the regulatory role of SIRT1 within ovarian cancer, and possible mechanisms, in addition to its role in mice with ovarian cancer transplantation, all of which require comprehensive and detailed illustration.

Conclusions

We found that SIRT1 over-expression can inhibit HMGB1 expression or acetylation, thus suppressing migration, invasion or angiogenesis of ovarian cancer cells. Modulation on ovarian cancer cell behaviors by HMGB1 requires the involvement of SIRT1. Therefore, SIRT1 can work as therapeutic target for inhibiting ovarian cancer migration.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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