PIASy mediates hypoxia-induced SIRT1 transcriptional repression and epithelial-to-mesenchymal transition in ovarian cancer cells

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Summary

Epithelial-mesenchymal transition (EMT) has an essential role in organogenesis and contributes to a host of pathologies, including carcinogenesis. Hypoxia (low oxygen supply) aids tumor metastasis in part by promoting EMT in cancer cells. The underlying mechanism whereby hypoxia orchestrates EMT remains poorly defined. Here we report that SIRT1, a multifaceted player in tumorigenesis, opposed ovarian cancer metastasis in vitro and in vivo by impeding EMT. Hypoxic stress downregulated the expression of SIRT1, primarily at the transcriptional level, by reducing the occupancy of the transcriptional activator Sp1 on the proximal promoter of the SIRT1 gene in a SUMOylation-dependent manner. Further analysis revealed that the SUMO E3 ligase PIASy (also known as PIAS4) was induced by hypoxia and prevented Sp1 from binding to the SIRT1 promoter. Conversely, knockdown of PIASy by small interfering RNA (siRNA) restored Sp1 binding and SIRT1 expression in cancer cells challenged with hypobaric hypoxia, reversed cancer cell EMT, and attenuated metastasis in vivo in nude mice. Importantly, analysis of human ovarian tumor specimens indicated that PIASy expression was positively, whereas SIRT1 expression was inversely, correlated with cancer aggressiveness. In summary, our work has identified a new pathway that links downregulation of SIRT1 to hypoxia-induced EMT in ovarian cancer cells and, as such, sheds light on the development of novel anti-tumor therapeutics.

Key words: SIRT1 transcription, Ovarian cancer, EMT, PIASy, SUMOylation, PIAS4

Introduction

Metastasis refers to the migration and settlement of cancer cells to secondary sites and contributes substantially to the morbidity of cancer patients (Eccles and Welch, 2007). Cancer cells gain invasiveness in a dynamic and reversible process known as epithelial-to-mesenchymal transition (EMT), which is characterized by the loss of one set of genes commonly found in epithelial cells (e.g. E-cadherin, ZO-1 and occludin) and the possession of another set of genes typical to mesenchymal cells (e.g. N-cadherin, fibronectin and vimentin) (Kalluri and Weinberg, 2009). EMT has pivotal roles in early embryogenesis and when aberrantly activated, contributes to cancer metastasis (Kang and Massagué, 2004). A host of extrinsic and intrinsic pathways that regulate EMT have been identified, including hypoxia, TGF-β and Wnt–β-catenin (Haase, 2009; Scheel et al., 2011; Vincent et al., 2009). Regardless of the nature of the stimuli, different pathways driving EMT seem to converge on a panel of transcriptional repressors that include Snail, Slug, Twist and ZEB (Thiery et al., 2009). For instance, hypoxia-induced cancer metastasis relies largely on the transcription factor HIF-1x; upon stabilization by low oxygen tension, HIF-1x directly activates Twist (Yang et al., 2008), ZEB1 or ZEB2 (Krishnamachary et al., 2006) and Snail (Evans et al., 2007) to shut down the expression of epithelial markers. TGF-β, on the other hand, primarily signals through Smad proteins that directly induce mesenchymal-specific genes and indirectly repress epithelial-specific genes by upregulating Snail, Slug, Twist and/or ZEB (Zavadil and Böttinger, 2005). Despite recent advances in understanding the molecular maneuvers in EMT, many details remain to be revealed.

SIRT1 belongs to the mammalian sirtuin family and is an ortholog of the yeast Sir2 protein implicated in a range of biological events including cell proliferation, metabolism and longevity (Finkel et al., 2009). The biological functionalities of SIRT1 are intimately tied to its enzymatic activity as a lysine deacetylase; among its fast-growing list of substrates, many have clear implications in cancer metastasis. The precise function of SIRT1 in tumorigenesis, and particularly in metastasis, however, is obscure and constantly being contested. On the one hand, several reports position SIRT1 as a tumor suppressor that safeguards the organism from oncogenic stress. For instance, mice with a heterozygotic deletion of the Sir1 allele in a Tp53 haploinsufficient background experience increased genomic
instability and develop tumors in multiple tissues spontaneously, as early as 5 months after birth (Wang et al., 2008). In addition, SIRT1 deacetylates and destabilizes the proto-oncogene Myc to stall tumor cell outgrowth (Yuan et al., 2009). On the other hand, SIRT1 activation attenuates p53-dependent apoptosis and/or senescence, and promotes the survival of cancer cells (Chen et al., 2005), essentially indicating that SIRT1 could function, at least under certain circumstances, as a tumor-promoting factor. Thus far, no direct evidence has linked SIRT1 to cancer metastasis.

A consensus that has gained wide attraction recently recognizes the importance of tumor microenvironment in metastasis. The so-called ‘pro-metastatic niche’ is characterized by hypoxia and chronic inflammation (Joyce and Pollard, 2009). By virtue of deacetylating and deactivating HIF-1α (Lim et al., 2010), which mediates the cellular hypoxic response, and NF-κB, a key node in the pro-inflammatory circuit (Ben-Neriah and Karin, 2011), SIRT1 presents itself as a promising druggable target that can effectively fend off cancer metastasis.

Here we report that SIRT1 activation favors an epithelial-like phenotype of ovarian cancer cells blocking metastasis. Hypoxia suppresses SIRT1 expression as part of its pro-metastasis program in a SUMOylation-dependent manner. Therefore, restoration of SIRT1 expression by targeting the cellular SUMOylation pathway might yield a viable strategy in combating ovarian cancer metastasis.

**Results**

**Activation of SIRT1 prevents cancer metastasis**

We first evaluated the role of SIRT1 in ovarian cancer metastasis in vitro and in vivo. Ectopic expression of wild-type (WT) but not enzyme-deficient (HY) SIRT1 blocked the migration and invasion of an ovarian cancer cell line SKOV3 in vitro (supplementary material Fig. S1A,B). By contrast, knockdown of SIRT1 promoted cell migration and invasion (supplementary material Fig. S1D,E). These observations were recapitulated in cells treated with SIRT1 agonist and antagonists (supplementary material Fig. S1G,H). Interestingly, activation of SIRT1 upregulated epithelial cell signature genes [CDH1 (encodes E-cadherin) and OCLN (encodes occludin)] and downregulated mesenchymal signature genes [FN1 (encodes fibronectin) and VIM (encodes vimentin)], whereas suppression of SIRT1 exerted the opposite effect (supplementary material Fig. S1C,F,I), indicating that SIRT1 regulates ovarian cancer cell EMT.

Hypoxia is known to promote cancer metastasis in part by initiating EMT (Cooke et al., 2012; Sahlgren et al., 2008). WT, but not HY, SIRT1 antagonized hypoxia-induced migration and invasion of SKOV3 (Fig. 1A,B; supplementary material Fig. S2A,B) and A2780 (supplementary material Fig. S2C,D) cells. Concomitantly, expression of epithelial markers was upregulated, whereas expression of mesenchymal markers was repressed at both mRNA (Fig. 1C) and protein (Fig. 1D) levels. Interestingly, the anti-metastatic property of SIRT1 seemed to be unique because other members of the mammalian sirtuin proteins failed to have an impact on cancer cell migration or invasion (supplementary material Fig. S2E,F). Alternatively, the SIRT1 agonist (supplementary material Fig. S2G-J) exerted similar effects to SIRT1 overexpression. We also assessed the hypothesis that SIRT1 antagonizes cancer migration and invasion in vivo. Intraperitoneal injection of the SIRT1 agonist resveratrol (RSV) for 4 weeks in nude mice significantly attenuated the metastasis...
of ovarian cancer (Fig. 1E; supplementary material Fig. S2K). In addition, SKOV3 cells overexpressing WT but not HY SIRT1 were less prone to spread in vivo (supplementary material Fig. S2L,M). Together, these data illustrate that activation of SIRT1 contributes to alleviation of ovarian cancer metastasis in vitro and in vivo by blocking cancer cell EMT.

**Binding of Sp1 to the SIRT1 promoter is disrupted by hypoxia**

Next, we tackled the possibility that hypoxia might regulate SIRT1 expression to promote cancer cell EMT. 1% O₂ downregulated mRNA (Fig. 2A) and protein (Fig. 2B) levels of SIRT1 in SKOV3 cells. We then examined whether suppression of SIRT1 expression by hypoxia was due to decelerated transcription. To this end, cells were transfected with reporter plasmids under the control of the human SIRT1 proximal promoter harboring serial deletions. Hypoxic stimulus potently repressed the activity of the SIRT1 promoter similar to the extent where SIRT1 expression was reduced (Fig. 2C). All the promoter constructs were repressed equally well, indicating that the hypoxia-response element is located within the minimal promoter region (−115/+58). A closer examination of this region revealed a conserved Sp1 site (boxed) (Fig. 2D).

**Fig. 2. Binding of Sp1 to the SIRT1 proximal promoter is disrupted by hypoxia.** (A,B) SKOV3 cells were harvested at different time points following exposure to 1% O₂. mRNA (A) and protein (B) expression were assessed by qPCR and western blot analysis, respectively. (C) SIRT1-promoter–luciferase constructs harboring serial deletions were into SKOV3 cells followed by exposure to 1% O₂. Luciferase activities were expressed as a percentage of the control. (D) Alignment of proximal promoter sequences of the SIRT1 genes highlighting a conserved Sp1 site (boxed). (E) SKOV3 cells were exposed to 1% O₂ and harvested at the indicated time points. ChIP assays were performed using anti-Sp1 antibodies. (F) HA-tagged Sp1 (WT or KR) constructs were transfected into HEK293 cells followed by exposure to 1% O₂. Proteins were immunoprecipitated by anti-HA antibodies and SUMOylation was revealed by western blot analysis. (G) Sp1 constructs were co-transfected with a SIRT1 reporter plasmid into SKOV3 cells followed by exposure to 1% O₂. Luciferase activities were expressed as a percentage of the control.
region revealed a conserved binding site for the transcriptional activator Sp1 (Fig. 2D), raising the possibility that hypoxia disrupts the binding of Sp1 to the SIRT1 promoter. Indeed, ChIP assays demonstrated that hypoxia attenuated Sp1 binding to the SIRT1 promoter (Fig. 2E).

Previous investigation has implicated a SUMOylation-dependent mechanism as a means of dampening Sp1 activity (Spengler and Brattain, 2006). Thus, we examined the effect of hypoxia on Sp1 SUMOylation. Hypoxia significantly increased SUMOylation levels of wild-type (WT) but not SUMOylation-defective (K16R) Sp1 (Fig. 2F). Conversely, the KR mutant of Sp1 was refractory to hypoxia in terms of SIRT1 transcriptional repression (Fig. 2G). Together, these data suggest that a SUMOylation-dependent mechanism dictates the repression of SIRT1 transcription in response to hypoxia.

**Disruption of SUMOylation restores expression of SIRT1 in ovarian cancer cells in response to hypoxia**

To further explore the possibility that SUMOylation has a role in SIRT1 transcriptional repression and cancer cell EMT, we exploited a dominant-negative Ubc9 (Ubc9 DN) that can disrupt the second step, namely the conjugation reaction, of SUMOylation (Mo et al., 2005). Indeed, Ubc9 DN blocked Sp1 SUMOylation, reversed downregulation of Sp1 binding on the SIRT1 promoter by hypoxia and normalized SIRT1 expression (Fig. 3A–D). Consequently, cancer cells underwent morphological alterations by regaining expression of epithelial-specific genes and shedding off mesenchymal markers (Fig. 3C,D), and became less aggressive in migration and invasion (supplementary material Fig. S3A,B,E,F). To determine whether Ubc9 altered cancer cell EMT by targeting SIRT1, we employed two types of SIRT1 inhibitors, nicotinamide (NAM) and sirtinol. Treatment with either SIRT1 inhibitor abrogated the restoration of epithelial marker expression, re-activated mesenchymal gene expression and rendered cancer cells more migratory and invasive (Fig. 3E–G). In a similar manner, shRNA-mediated silencing of endogenous SIRT1 (supplementary material Fig. S3C–E) favored a mesenchymal phenotype of ovarian cancer cells in the presence of Ubc9 DN.

**Hypoxia activates PIAsy to repress SIRT1 transcription**

The PIA (protein inhibitor of activated STAT) family of E3 ligases are responsible for the last step of SUMOylation reactions (Johnson and Gupta, 2001). Hypoxia upregulated the expression of all four mammalian E3 ligases, while simultaneously downregulating SIRT1 expression (Fig. 2A,B). Unlike other E3 ligases, PIAsy was preferentially recruited to the SIRT1 promoter region in response to hypoxia (Fig. 4A). Moreover, PIAsy neutralized the activation of SIRT1 transcription by Sp1 (Fig. 4B,C) and interfered with its binding to the SIRT1 promoter (Fig. 4D). As expected, the SUMOylation mutant of Sp1 was not responsive to PIAsy, indicating that PIAsy is likely to be the main E3 ligase that regulates SIRT1 transcription in cancer cells. The enzymatic activity of PIAsy was clearly required for suppressing Sp1 occupancy, because a deletion of...
PIASy without the ring-finger domain (ΔRFD) failed to impact the binding of Sp1 on the SIRT1 promoter (supplementary material Fig. S4A).

Overexpression of PIASy repressed SIRT1 mRNA (supplementary material Fig. S4B) and protein (supplementary material Fig. S4C) levels under normoxic conditions. To gain further insight into the role of PIASy in SIRT1 transcription and EMT in response to hypoxia, we silenced endogenous PIASy expression using RNAi. siRNA-mediated elimination of PIASy, but not other PIAS proteins, restored SIRT1 expression in SKOV3 cells cultured under 1% O2 (Fig. 4E,F; supplementary material Fig. S4D). At the transcriptional level, PIASy loss-of-function recovered the binding of Sp1 on the SIRT1 promoter (Fig. 4G). Concomitantly, elevation in migration and invasion of cancer cells in response to hypoxia was diminished (Fig. 5A,B; supplementary material Fig. S4E,F). To ensure that PIASy regulates cancer cell EMT by targeting SIRT1, we used several different SIRT1 inhibitors, nicotinamide (NAM), sirtinol and EX-527. Treatment with SIRT1 inhibitors abrogated the restoration of epithelial marker expression, re-activated mesenchymal gene expression, and rendered cancer cells more migratory and invasive (Fig. 5C–F; supplementary material Fig. S4G–J). In a similar manner, shRNA-mediated silencing of endogenous SIRT1 (supplementary material Fig. S4K–O) favored a mesenchymal phenotype of ovarian cancer cells in the absence of PIASy.

To validate this conclusion, we constructed stable SKOV3 cells in vivo in which PIASy expression was deleted by shRNA (supplementary material Fig. S5A) and then injected these cells into nude mice in an animal model of metastasis. Indeed, PIASy deficiency substantially decreased metastasis of ovarian cancer, as measured by both the number as well as the cumulative weight of nodules (supplementary material Fig. S5B; Fig. 5G,H).
Therefore, PIASy functions as a crucial link in determining cancer cell EMT by modulating SIRT1 transcription. Finally, we measured the expression of SIRT1 and PIASy in ovarian cancer patients with differential diagnosis by both quantitative PCR and immunohistochemistry. As shown in Fig. 6, compared with patients with stage I ovarian cancer, patients with advanced ovarian cancer exhibited dramatically reduced SIRT1 expression and elevated PIASy expression. By contrast, PIAS1 expression was not altered regardless of the diagnosis. In conclusion, our data allude to the potential predictive value of SIRT1 and PIASy expression in the diagnosis of human ovarian cancer.

Discussion

Similar to cardiovascular, metabolic and neurodegenerative diseases, cancer is a human pathology that is closely correlated with aging (Herranz et al., 2010). SIRT1, once named the ‘longevity gene’, has proven to be an effective target in treating a range of aging-invoked pathologies including type 2 diabetes, atherosclerosis, coronary heart disease and Alzheimer disease. However, the precise effect of SIRT1 on carcinogenesis remains controversial, as there has been evidence arguing both for an affirmative (Wang et al., 2008) and negative (Chen et al., 2005) role for SIRT1 in this process. One major pitfall of previous studies that tackle the role of SIRT1 in tumorigenesis is that few have explored the connection between SIRT1 and cancer metastasis. Because prognosis for cancer patients with distant metastasis remains poor despite advances in the understanding of cancer biology and the development of novel interventional strategies (Jemal et al., 2009), there is a pressing need to sort out the intertwined circuit that regulates the migration and invasion of cancer cells. Here, we provide evidence that SIRT1 suppresses ovarian cancer cell EMT and that a SUMOylation-dependent pathway dictates downregulation of SIRT1 to aid cancer metastasis. Our data suggest that activation of SIRT1 antagonizes hypoxia-induced EMT in ovarian cancer cells; it remains unanswered at this point how SIRT1 might help to maintain an epithelial-like phenotype. Several lines of evidence indicate that the effect of SIRT1 on EMT could well be multifold rather than linear. First, SIRT1 has been documented to suppress several transcriptional factors that are directly involved in the programming of cellular EMT. HIF-1α is widely believed to be the main mediator of hypoxia-dependent EMT (Haase, 2009). SIRT1 deacetylates HIF-1α at lysine 674 and blocks the interaction between HIF-1α and p300, thereby dampening
HIF-1α activity (Lim et al., 2010). Paradoxically, SIRT1 deacetylates and boosts the activity of HIF-2α (Dioum et al., 2009). Because it has been indicated that unlike HIF-1α, HIF-2α could alleviate rather than promote cancer metastasis (Branco-Price et al., 2012), SIRT1 might influence EMT by tipping the balance between the two different isoforms of HIF. Recently, Rettig and colleagues reported that NF-κB activity was enhanced in VHL low renal cancer cells, leading to accelerated EMT as a result of increased expression of Slug and Twist (Pantuck et al., 2011). Thus, an alternative explanation accounting for diminished EMT following SIRT1 activation can be drawn from the fact that SIRT1 deacetylates NF-κB and paralyzes its ability to bind to DNA, essentially shutting down transcription that is reliant on NF-κB (Yeung et al., 2004). Second, the theory that metabolic disorder is intimately connected to cancer metastasis reveals a whole new level of regulation for EMT and has gained in popularity in the past decade (Hursting and Rettig, 2010). SIRT1, as a master regulator of cellular homeostasis and inhibition of cancer metastasis, by either preventing a hypoxic and/or inflammatory response downstream of the metabolic disorder, or by promoting the synthesis and release of humoral factors that have anti-metastatic properties. Indeed, several known targets of SIRT1, such as adiponectin, whose dysfunction contributes to metabolic syndrome, have been implicated in cancer metastasis (Man et al., 2010). To support this notion are the observations that calorie restriction (CR), a well-studied process known to activate SIRT1, offers some beneficial effects in combating cancer (Bonorden et al., 2009; Lee and Lungo, 2011; van Ginthoven et al., 2010). Given that skewed metabolism is recognized as part of a pro-cancer niche (Cairns et al., 2011), SIRT1 could prevent cancer metastasis by cultivating a metabolically stable internal environment that is unfriendly for EMT. Of note, recent reports have indicated that SIRT1 can both promote (Byles et al., 2012; Eades et al., 2011) and oppose (Nakai et al., 2012; Shirane et al., 2012) EMT depending on the cell type and context. Therefore, it is likely that the cellular makeup and/or microenvironment may alter the impact of SIRT1 on EMT.

Another substantial finding that originated from the present study is that a SUMOylation-dependent pathway regulates EMT and cancer metastasis by targeting SIRT1 transcription. The involvement of SUMOylation in metastasis has been actively pursued for quite some time now, and several key proteins in EMT have been identified as targets for SUMOylation, including the type I TGF-β receptor (Kang et al., 2008), SnoN (Netherton and Bonni, 2010), and Smad (Long et al., 2003). However, the investigations have yet to settle down on a unified model, reflecting the complex nature of this process. Our data presented here suggest that the SUMO E3 ligase PIASy determines SIRT1 expression in ovarian cancer cells. To the best of our knowledge, this is the first demonstration that a specific PIAS protein can control cancer EMT by targeting SIRT1. It is noteworthy that PIASy-null mice were born normal with a minor immunological anomaly, suggesting that PIASy loss-of-function is fully compensated by other family members under physiological conditions (Roth et al., 2004). Furthermore, a human fibroblast overexpressing PIASy exhibits increased p53 activity and enters premature senescence, phenocopying SIRT1 deficiency (Bischof et al., 2006). Although the possibility that stimulation of p53 by PIASy relies on SIRT1 repression remains to be tested, it is known that deacetylation and SUMOylation within a single protein can forge dialogues fine-tuning the activity of that protein (Brandl et al., 2012; Stankovic-Valentin et al., 2007). Future investigations using tissue-specific PIASy-null mice will probably tighten these loose ends regarding the interplay between PIASy and SIRT1. More recently, Kessler and colleagues have reported that the SUMO-activating enzyme [SAE1 and SAE2 (also known as UBA2)] re-directs part of the Myc-dependent transcriptional program to promote cell growth and viability and that expression of SAE1 and SAE2 is inversely correlated with metastasis-free survival of patients with breast cancer (Kessler et al., 2012), thus affirming our proposal that targeting SUMOylation is an executable strategy in the intervention of malignant cancer.

In summary, our data reinforce the notion that the importance of SIRT1 in cancer metastasis, and EMT in particular, rivals that in cardiovascular and metabolic diseases. Chemical biological methods aiming at the development of small-molecule compounds that inhibit the activity of PIAS proteins will be of tremendous value, both in understanding the role of SIRT1 in cancer metastasis and benefiting patients with advanced-stage cancer.

Materials and Methods

Cell culture and treatment
HEK293 (Invitrogen), SKOV3 (ATCC) and A2780 (ATCC) cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone). These cells have not been authenticated. The SIRT1 agonist (resveratrol and SIRT1720) and antagonists (nicotinamide, sirtinol and EX-527) were purchased from Sigma. Where indicated, hypoxia (1% O2) was achieved by a mixture of ultra-high purity gases (5% CO2, 10% H2, 85% N2) in a 37°C incubator (Thermo Fisher).
Plasmids and transient transfection

The SIRT1-reporter construct was made by fusing the proximal promoter region of the human SIRT1 gene (−972/+58) with the firefly luciferase CDNA in a pA3Luc vector. Expression constructs for SIRT1, Sp1, PIAS1, PIASx, PIASy, PIAS3, Ubc9 and shRNA plasmids targeting SIRT1 have been previously described (Langley et al., 2002; Ryntki and Palvimo, 2009; Vicart et al., 2006; Zhang et al., 2009; Zhou et al., 2008). Small interfering RNA sequences were listed in supplementary Table S1. Transfection were performed with Lipofectamine 2000 (Invitrogen). Luciferase activities were assayed 24-48 hours after transfection using a luciferase reporter assay system (Promega). Experiments were routinely performed in triplicate wells and were repeated three times.

Protein extraction, immunoprecipitation and western blot analysis

Whole cell protein was extracted by resuspending cell pellets in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 0.1% Triton X-100) supplemented with a protease inhibitor tablet (Roche). N-ethylmaleimide was added to the lysis buffer to maximize SUMOylation. Specific antibodies or pre-immune IgGs (P.I.I.) were added to and incubated with cell lysates overnight before being absorbed by Protein A/G-plus Agarose beads (Santa Cruz). Precipitated immune complex was released by boiling with 1×SDS electrophoresis sample buffer. Western blot analyses were performed using anti-FLAG, anti-HA, anti-β-actin, anti-vimentin (Sigma), anti-E-cadherin (BD), anti-SIRT1, anti-Myc, anti-GAPDH, anti-PIAS1, anti-PIASx, anti-SUMO1 (Santa Cruz), anti-PIASy and anti-PIAS3 (Abcam) antibodies.

RNA extraction and real-time PCR

RNA was extracted with the RNeasy RNA isolation kit (Qiagen). Reverse transcription reactions were performed using a SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR reactions were performed on an ABI Prism 7500 system. Primers and Taqman probes were employed for real-time reactions are listed in supplementary material Table S2.

Chromatin immunoprecipitation (ChIP) assays were performed as described before (Fang et al., 2011; Fang et al., 2009). Briefly, chromatin was cross-linked with 1% formaldehyde. Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with a protease inhibitor tablet. DNA was fragmented into ~500 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 µg of protein were used for each immunoprecipitation reaction. Precipitated genomic DNA was amplified by real-time PCR with the following primers that span the human SIRT1 proximal promoter (−131/+57): forward, 5′-ACCGTAGTGTGTTGGTGG-3′; reverse, 5′-CCTCTGCCGCATCTCC-3′.

Scratch-wound healing and migration assay

Cells were re-suspended in serum-free media. Upon confluence, scratch wound was created by using a sterile micropipette tip. Cell migration was calculated by Image J. Data were expressed as a percentage of migration compared with the control, which was arbitrarily set as 100%.

Boyden chamber invasion assay

24-well inserts (Costar) with 10 µg/ml Matrigel (Sigma) were used for invasion assays. Cells were re-suspended in serum-free media and plated into the upper chamber with the lower chamber filled with complete media. Following exposure to 1% O2, the cells on the upper chamber were removed. Invaded cells were stained with 0.1% crystal violet and counted at ×200 magnification in 10 different fields. Experiments were repeated three times. Data were expressed as a percentage of invasion compared with the control, which was arbitrarily set as 100%.

In vivo metastasis

All animal study protocols were reviewed and approved by the intramural Ethic Committee on Human Care of Experimental Animals. 4–6-week-old male mice were inoculated with SKOV3 (5×105 per mouse, through the peritoneum). The next day, mice were injected peritoneally with RSV (20 mg/kg) or solvent every other day. 25 days after inoculation, mice were killed and metastatized nodules were dissected, counted and weighed.

Human tumor samples and histology

All human studies were reviewed and approved by the intramural Committee on Ethical Conduct of Studies with Human Subjects. Ovarian cancer tissues were collected, under informed consent, from surgical resection specimens of patients who had not undergone radiotherapy or chemotherapy in the Affiliated Hospital of Nantong University (China). Diagnoses of all cases were confirmed by histological examination. Tumor differentiation was graded by the Edmonson grading system. Samples were processed as described previously (Huang et al., 2011). Briefly, paraffin-embedded sections were incubated with anti-SIRT1 monoclonal antibody (1:50, Santa Cruz) or anti-PIASy monoclonal antibody (1:50, Abcam) overnight. Negative control slides were also processed in parallel using a nonspecific immunoglobulin IgG (Sigma).

Statistical analysis

One-way ANOVA with post-hoc Scheffe analyses were performed using an SPSS package. Unless otherwise specified, P values <0.05 were considered statistically significant.

Author contributions

Y.X. conceived the project; L.N.S., H.L., J.L.C., Y.I., T.K. and M.M. performed the experiments and analyzed the data; Y.X., Q.C. and A.G.S. coordinated and supervised the project.

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References


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Supplementary figures: 5
Table I: siRNA or shRNA sequences used in this study

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<td>Reverse: 5'-CCTTGTGTCCCTGATGTTGC-3'</td>
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Figure S1: (A, B, C) SKOV3 cells were transfected with SIRT1 expression constructs (WT or HY). Wound healing (A) and invasion (B) assays were performed as described under Methods. (C) Expression of epithelial or mesenchymal signature genes was measured by qPCR. (D, E, F) SKOV3 cells were transfected with SIRT1 shRNA construct (shSIRT1) or a control shRNA construct (shLUC). Wound healing (D) and invasion (E) assays were performed as described under Methods. (F) Expression of epithelial or mesenchymal signature genes was measured by qPCR. (G, H, I) SKVO3 cells were treated with indicated chemicals. Wound healing assay (G) and invasion assay (H) were performed and quantified as described under Methods. (I) Expression of epithelial or mesenchymal signature genes was measured by qPCR.
Figure S2: (A, B) SKOV3 cells were transfected with SIRT1 expression constructs (WT or HY) followed by exposure to 1% O2. Wound healing (A) and invasion (B) assays were performed as described under Methods. (C, D) A2780 cells were transfected with SIRT1 expression constructs (WT or HY) followed by exposure to 1% O2. Wound healing (C) and invasion (D) assays were performed as described under Methods. (E, F) SKVO3 cells were transfected with indicated expression constructs for different sirtuin proteins followed by exposure to 1% O2. Wound healing assay (E) and invasion assay (F) were performed and quantified as described under Methods. (G, H) SKVO3 cells were treated with resveratrol (RSV) or DMSO followed by exposure to 1% O2. Wound healing assay (G) and invasion assay (H) were performed and quantified as described under Methods. (I, J) SKVO3 cells were treated with resveratrol (RSV) or DMSO followed by exposure to 1% O2. Expression of epithelial/mesenchymal signature genes was measured by qPCR (I) and Western (J). (K) In vivo metastasis assay was performed in nude mice using SKOV3 cells as described under Methods. Representative pictures of metastasized nodules are shown. (L, M) Stable SKOV3 cells expressing an empty vector (EV), wild type (WT) or mutant (HY) SIRT1 were probed for SIRT1 expression by Western (L). (M) In vivo metastasis assay was performed as described under Methods. Data are expressed metastasized nodules relative to the control group in which EV-expressing stable cells were injected. N=5 for each group.
A

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D

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E

[Graph showing mRNA levels of SIRT1, CDH1, CLDN, CDH2, FN, and VIM under different conditions]
Figure S3: (A, B) SKOV3 cells were transfected with Ubc9 DN and treated with NAM or sirtinol followed by exposure to 1% O\textsubscript{2}. Wound healing (A) and invasion (B) assays were performed as described under Methods. (C, D) SKVO3 cells were transfected with Ubc9 DN, SIRT1 shRNA (shSIRT1), or control shRNA (shLuc) followed by exposure to 1% O\textsubscript{2}. Wound healing assay (A) and invasion assay (B) were performed and quantified as described under Methods. (E) SKVO3 cells were transfected with Ubc9 DN, SIRT1 shRNA (shSIRT1), or control shRNA (shLuc) followed by exposure to 1% O\textsubscript{2}. Expression levels of epithelial/mesenchymal signature genes were measured by qPCR.
Figure S4: (A) HEK293 cells were transfected with HA-Sp1 and indicated PIASy expression constructs. ChIP assay was performed with anti-HA. (B, C) SKOV3 cells were transfected with an empty vector (EV) or indicated PIAS expression constructs. mRNA (B) and protein (C) levels of SIRT1 were evaluated by qPCR and Western. (D) SKOV3 cells were transfected with indicated PIAS-targeting siRNAs or scrambled siRNA (SCR) followed by exposure to 1% O2. Protein levels of PIAS were examined by Western. (E, F) SKOV3 cells were transfected with indicated PIAS-targeting siRNAs or scrambled siRNA (SCR) followed by exposure to 1% O2. Wound healing (E) and invasion (F) assays were performed as described under Methods. (G, H) SKOV3 cells were transfected with siRNA targeting PIASy or SCR and treated with NAM or sirtinol followed by exposure to 1% O2. Wound healing (G) and invasion (H) assays were performed as described under Methods. (I, J) A2780 cells were transfected with siRNA targeting PIASy or SCR and treated with EX-527 followed by exposure to 1% O2. Wound healing (I) and invasion (J) assays were performed as described under Methods. (K, L, M) SKVO3 cells were transfected with indicated siRNAs, shSIRT1, or shLuc followed by exposure to 1% O2. Wound healing assay (K) and invasion assay (L) were performed and quantified as described under Methods. (M) mRNA levels of epithelial/mesenchymal signature genes were measured by qPCR. (N, O) A2780 cells were transfected with indicated siRNAs, shSIRT1, or shLuc followed by exposure to 1% O2. Wound healing assay (N) and invasion assay (O) were performed and quantified as described under Methods.
Figure S5: (A) SKOV3 cells stably expressing a control shRNA (SCR) or shRNA targeting PIASy (shPIASy) were exposed to 1% O₂ for 48 hours. Expression levels of indicated genes were measured by qPCR. (B) Representative pictures of metastasized cancer cells in the peritoneal septum, mesentery, intestinal wall, and internal organs.