



Regulation of SIRT1 determines initial step of endometrial receptivity by controlling E-cadherin expression

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ABSTRACT

Sirtuin 1 (SIRT1), originally found as a class III histone deacetylase, is a principal modulator of pathways downstream of calorie restriction, and the activation of SIRT1 ameliorates glucose homeostasis and insulin sensitivity. We examined the role of SIRT1 in the regulation of uterine receptivity using Ishikawa and RL95-2 endometrial carcinoma cell lines. Exogenous expression of SIRT1 significantly enhanced E-cadherin expression, while small interfering RNA-mediated depletion of endogenous SIRT1 resulted in a significant reduction of E-cadherin expression. A SIRT1 activator resveratrol elevated E-cadherin expression in a dose dependent manner, while SIRT1 repressors nicotinamide and sirtinol exhibited a dose dependent reduction of E-cadherin expression. We also showed that both forced expression of SIRT1 and activation of SIRT1 promote E-cadherin-driven reporter gene constructs, and SIRT1 is localized at E-cadherin promoter containing E-box elements in Ishikawa cells. Using an *in vitro* model of embryo implantation, we demonstrate that exogenous expression of SIRT1 and stimulation of SIRT1 activity resulted in the Ishikawa cell line becoming receptive to JAR cell spheroid attachment. Furthermore, resveratrol enhanced E-cadherin and Glycodelin protein expression at sites of intercellular contact, suggesting an additive role of resveratrol in promoting implantation. The initial step of human reproduction depends on the capacity of an embryo to attach and implant into the endometrial wall, and these results revealed the novel mechanism that activation and increased expression of SIRT1 play an important role in uterine receptivity.

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1. Introduction

After the blastocyst attaches onto the endometrial glandular epithelium, broad adhesion, transient disruption of the uterine epithelium by degradation of extracellular matrix, and penetration into the uterine stroma occurs. The implantation window is hormonally regulated and is characterized by changes in the molecules expressed by uterine epithelial cells [1]. Although numerous cell surface components including adhesion molecules, cytokines, growth factors, and lipids are postulated to be involved in implantation, only a few genes are essential to this process [2,3]. The study of implantation is both technically and ethically difficult to investigate *in vivo*. *In vitro* study using primary tissues possesses many limitations due to the individual variations. Currently endometrial

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated kinase; HDAC, histone deacetylase; NAM, nicotinamide.

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epithelial carcinoma cell lines such as Ishikawa [4] and RL95-2 [5] cells, and trophoblast cell lines have been used to mimic the receptive state of the uterine epithelium in *in vitro* implantation assays, and the *in vitro* implantation assay is regarded as a useful model for studying mechanisms of human implantation [4–6].

E-cadherin would be involved in the initial attachment of embryos because E-cadherin is found on luminal epithelium and on trophoblast [7]. E-cadherin in uterine endometrium was known to be hormonally regulated because E-cadherin expression is significantly enriched at the apical membranes of mouse uterine epithelial cells during the preimplantation stage [8]. Embryos lacking functional E-cadherin by targeted disruption exhibit defective preimplantation development and failure to implant [9]. E-cadherin is known to maintain organized architecture and plays a pivotal role in the regulation of epithelial cell proliferation, differentiation, and survival [10]. In addition, genetic or epigenetic alterations of E-cadherin expression have been often associated with various cancers and a class III histone deacetylase (HDAC), SIRT1, is linked to the E-cadherin expression [11,12].

SIRT1, the mammalian homologue of yeast Sir2 (silent information regulator 2), deacetylates multiple targets in mammalian cells [13]. By regulating various molecules, SIRT1 functions as a master regulator of energy homeostasis, transcriptional regulation, heterochromatin formation, genomic stability, p53 function, and cell survival [14]. SIRT1 is associated with the oncogenic functions because SIRT1 promotes cell survival by inhibiting acetylated-p53 dependent apoptosis [15,16]. SIRT1 has been shown to be involved in the maintenance of gene silencing by associating with CpG island of promoter regions in tumor suppressor genes [11]. However, SIRT1 also possesses anti-oncogenic function because SIRT1 inhibits Survivin expression by changing the epigenetic modification of histone H3, and a phytochemical compound resveratrol (trans-3,5,40-trihydroxystilbene) mimic the inhibitory effects of SIRT1, thus serves as an anti-carcinogenic compound [17]. Polyphenols have been known to activate SIRT1 either directly or indirectly, and the deacetylating activity of SIRT1 can be inhibited by nicotinamide (NAM) [13]. Resveratrol is an indirect activator of SIRT1 and has been shown to activate the expression of nicotinamide phosphoribosyltransferase and AMP-activated kinase (AMPK) [18–20]. In addition, SIRT1 and AMPK mutually affect the functions of each other [18,21]. So far, it is not known whether these chemicals are able to modulate the expression of E-cadherin.

To better understand the functional significance and the transcriptional regulation by SIRT1, we studied the effect of the transcriptional regulation of E-cadherin driven by SIRT1. We demonstrate that E-cadherin expression is regulated by SIRT1 in endometrial carcinoma cells. Either activation of SIRT1 or increased expression of SIRT1 plays a key role in the development of human uterine receptivity via inducing E-cadherin expression. These findings establish a principal biological function of SIRT1 in the modulation of E-cadherin function, and further identify SIRT1 as a possible determinant and potential therapeutic target in implantation failure.

2. Materials and methods

2.1. Cell culture and chemicals

Ishikawa human endometrial adenocarcinoma cell line was kindly provided by Dr. M. Nishida (Tsukuba University, Ibaraki, Japan). RL95-2 cells (CRL-1671, human endometrial adenocarcinoma), 293T cells (CRL-11268, human embryonic kidney cells), and JAR cells (HTB-144, human choriocarcinoma cells) were purchased from the American Type Culture Collection (Manassas, VA, USA). Resveratrol, NAM, and sirtinol were from Sigma–Aldrich (St. Louis, MO, USA). AICAR (5-Aminoimidazole-4-carboxamide ribonucleoside) was purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Western blot

To determine the effect of SIRT1 functions, cells were treated with indicated concentrations of resveratrol, sirtinol, NAM, or AICAR. Western blot analysis and immunostaining were performed as described previously [22].

2.3. RNAi

The ablation of SIRT1 and DBC1 was performed by transfection of the Ishikawa cells and RL95-2 cells with small interfering RNA (siRNA) duplex oligos synthesized by Invitrogen (Carlsbad, CA, USA) and Qiagen (Hilden, Germany). Control siRNA (AllStars Negative Control siRNA, 1027281) and DBC1-specific siRNA [DBC1-RNAi: 5' AAACGGAGCCUACUGAACA 3', which covered

mRNA regions of nucleotides 1379–1397 (amino acids 460–466) of DBC1, and KIAA1967-RNAi, SI00461853] were transfected by using HyperFect reagent (Qiagen). Stealth RNAi Duplex (Invitrogen) specific for SIRT1 (Oligo ID: HSS118729, HSS177403 and HSS117404) was transfected by using Lipofectamine RNAi MAX (Invitrogen).

2.4. Luciferase assay

Transfection was performed with Effectene reagent (Qiagen) according to the manufacturer's recommendation. For luciferase assay, indicated expression vectors were cotransfected with E-cad(–108)–Luc or E-cad(–108)Mut–Luc [23]. As an internal control to equalize transfection efficiency, phRL CMV–Renilla vector (Promega Corp., Madison, WI, USA) was also transfected in all the experiments. Individual transfections, each consisting of triplicate wells, were repeated at least three times [22].

2.5. In vitro implantation assay

To generate spheroids of JAR cells for use as blastocyst models, the JAR cells were grown in suspension in petri dishes at a density of 2×10^5 cells/ml, Petri dishes were placed on a slow shaker in 37 °C, 5% CO₂ humidified incubator overnight. During this incubation, JAR cells form spheroids of 50–200 µm in diameter through natural aggregation. Ishikawa cells were seeded in quintuplicate in 24-well plates and incubated until they reached subconfluent monolayers. Thereafter, Ishikawa cells were treated with indicated chemical compounds. On the day of the assay, co-culture of Ishikawa cells and JAR spheroids (approximately 100 spheroids/well) for 24 h was performed. After incubation, the monolayers were gently washed twice with PBS to remove unattached spheroids. Cell monolayers were then examined under light microscope for spheroids numbers.

2.6. Fluorescence microscopy

Ishikawa cells were grown on 12 mm BD BioCoat (BD Biosciences, Franklin Lakes, NJ, USA) glass coverslips in 6-well plates. Spheroids of JAR cells seeded on the Ishikawa monolayer cells were fixed with PBS containing 4% paraformaldehyde. After blocking, Ishikawa cell and spheroids were incubated sequentially with anti-E-cadherin (610181, BD Biosciences) and anti-Glycodelin (EP870Y, Novus Biologicals, CO, USA) antibodies. Secondary antibodies were Alexa fluor 488 conjugated donkey anti-mouse IgG (A-21428), and Alexa fluor 555 conjugated goat anti-rabbit IgG (A-21202, Invitrogen). The slides were briefly counter-stained and analyzed under the confocal fluorescence microscope (Carl-Zeiss Micro Imaging Inc., Oberkochen, Germany).

2.7. Chromatin immunoprecipitation assay

Preparation of soluble Ishikawa chromatin for PCR amplification was performed essentially as described [24]. Primers to amplify E-cadherin promoter region containing three E-box domains are as follows; forward, 5'-GTGAACCTCAGCCAATCAG-3'; reverse, 5'-TCACAGGTGCTTTGCAGTTC-3'.

3. Results

3.1. E-cadherin expression is regulated by SIRT1 deacetylase

To determine the effect on E-cadherin expression, nonpolar Ishikawa and RL95-2 cells were transfected with siRNA oligos. In contrast to the previous study using breast cancer cell line MDA-MB-231 [11] and prostate cancer cell line PC3 and DU145 [12],

RNAi-mediated knockdown of SIRT1 expression resulted in a decreased expression of E-cadherin in endometrial cancer cell lines (Fig. 1A, C). Thus our data demonstrate that SIRT1 has a critical role in regulating the expression level of E-cadherin in endometrial cancer cells. The forced expression of SIRT1 revealed that the increased expression of SIRT1 resulted in an increased expression of E-cadherin (Fig. 1B, D), confirming that E-cadherin expression was paralleled by the SIRT1 expression. DBC1 is a negative regulator of SIRT1 deacetylase function [15,16], and we investigated the possibility that the regulation of E-cadherin expression by SIRT1 is affected by DBC1. However, DBC1 expression remained unchanged by siRNA-mediated knockdown of SIRT1. The result that SIRT1 is able to stimulate E-cadherin expression led us to examine the role of SIRT1 in the activation of E-cadherin promoter. Transient transfection assays were performed using an E-box-wild type luciferase [E-cad(–108)–Luc] or E-box-mutated [E-cad(–108)Mut–Luc] reporter plasmid, carrying minimum promoter region (–108 to +125 bp) for the E-cadherin expression. The E-box elements (consensus sequence 5'-CANNTG-3') were originally shown to be binding regions of zinc-finger transcription factors such as SLUG and SNAIL that repressed E-cadherin-driven reporter gene constructs and three E-box sites within the promoter have been demonstrated to drive the expression of E-cadherin [23]. Although SIRT1 efficiently elevated the promoter activity of the reporter plasmid in 293T cells in a dose dependent manner, the transactivation function of SIRT1 was not observed by the expression of E-cad(–108)Mut–Luc in luciferase assays (Fig. 1E). To test whether SIRT1 is indeed recruited to E-cadherin promoter, we performed a chromatin immunoprecipitation assay using the E-cadherin gene promoter containing three E-box elements. As expected, clear recruitment of endogenous SIRT1 to the target sequence in the

E-cadherin promoter was observed (Fig. 1F). Thus our results suggest that SIRT1 plays a significant role in the E-cadherin expression, and E-box domains were shown to be crucial for the SIRT1-mediated expression of E-cadherin.

3.2. Stimulation of SIRT1 function increases the expression level of SIRT1 and E-cadherin

We next hypothesized that expression of E-cadherin would be regulated by small molecules that govern SIRT1 function. In concordance with the previous report, SIRT1 expression was also stimulated by resveratrol (Fig. 2A, B). Contrary to this, Ishikawa cells exposed to sirtinol (Fig. 2C) and NAM (Fig. 2D) exhibited decreased expression of E-cadherin in a dose-dependent manner. AICAR is an AMPK activator and AMPK enhances SIRT1 activity by increasing cellular NAD⁺ levels [21]. We also showed that AICAR stimulated E-cadherin and SIRT1 expression in Ishikawa and RL95-2 cells, but the extent of increase by AICAR was relatively modest compared to that by resveratrol (Fig. 2E). We further examined the ligand-induced transactivation function of E-cadherin promoter, and the resveratrol-induced transactivation was dose-dependent with a roughly estimated concentration value required for one-half maximal activation (EC₅₀) of about 10 μ M (Fig. 2F).

3.3. SIRT1 expression and activity affect the spheroid attachment to the Ishikawa cell monolayer

We next examined whether the expression of SIRT1 and its activity affected the implantation capacity of Ishikawa cells using an *in vitro* model of embryo attachment. Forced expression of SIRT1 in Ishikawa cells exhibited a 1.3-fold increased number

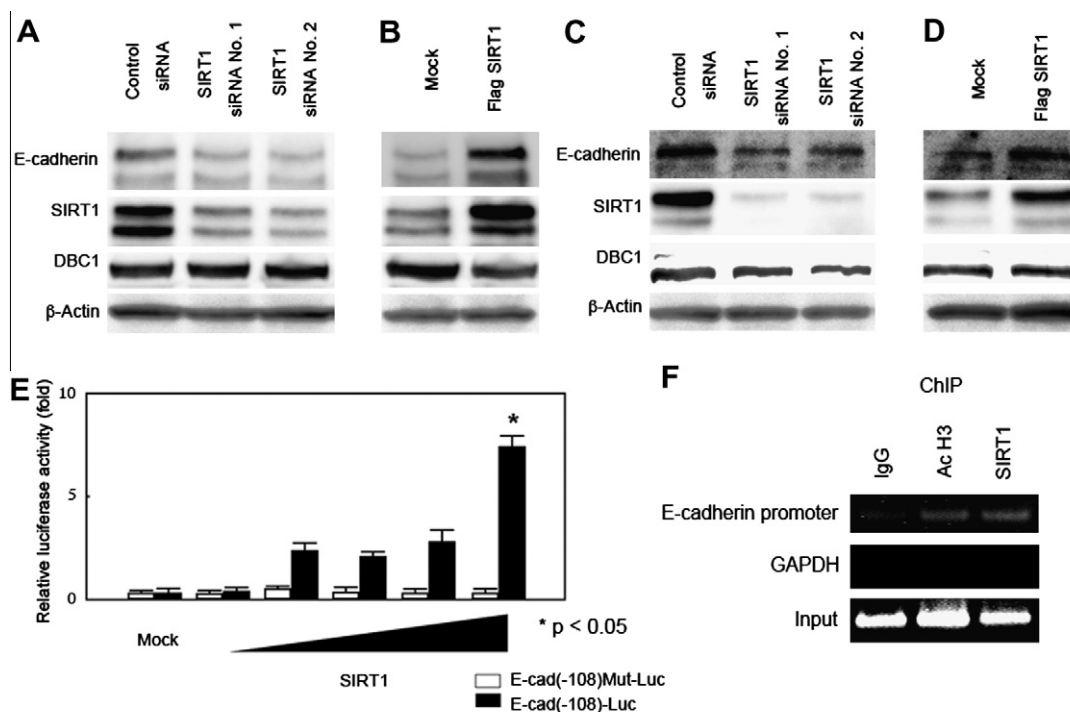


Fig. 1. E-cadherin promoter dependent regulation of E-cadherin expression by SIRT1 deacetylase. Ishikawa (A, B) and RL95-2 (C and D) cells were transfected with expression vector of SIRT1 or indicated siRNA and analyzed by Western blotting. The immunoblotting analysis using anti-SIRT1 antibodies revealed that the expression of SIRT1 paralleled the expression of E-cadherin in cell lysates. (E) 293T cells were transfected with the indicated amounts of SIRT1 expression plasmids (pcDNA Flag SIRT1), and transfected whole cell lysates were assayed for luciferase activity produced from the reporter plasmid [E-cad(–108)–Luc or E-cad(–108) Mut–Luc]. SIRT1 showed a dose-dependent stimulation of the transactivation function of E-cadherin promoter, while E-cadherin promoter possessing mutations within three E-box domains failed to show activation function of SIRT1. (F) Chromatin immunoprecipitation assay was performed to confirm the recruitment of SIRT1 at E-cadherin gene promoter, a region containing three E-box domains. Ach3 denotes acetylated histone H3.

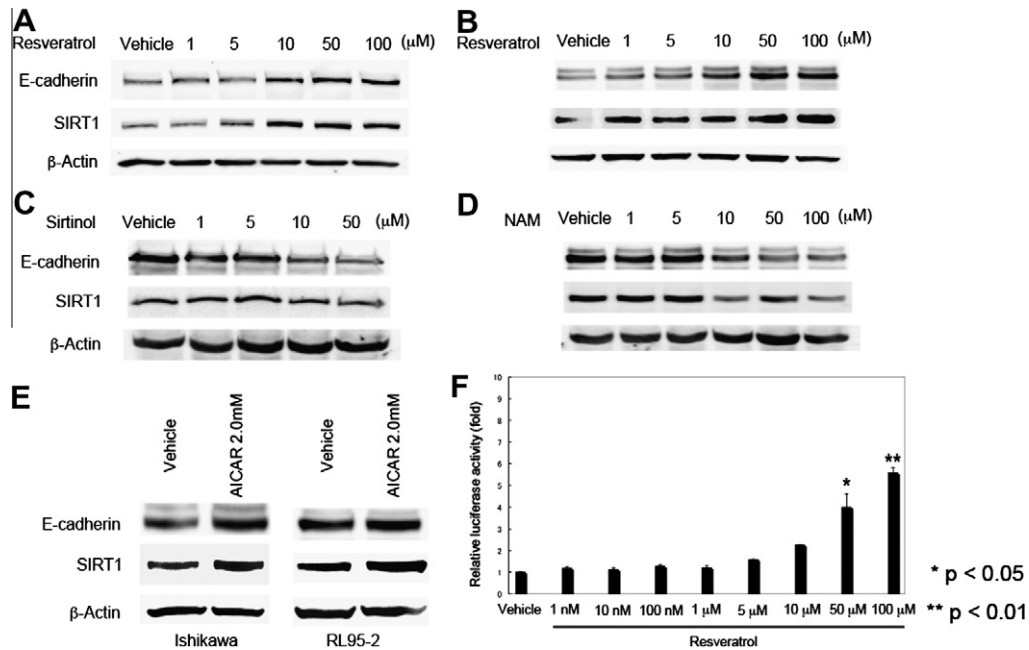


Fig. 2. Activation of SIRT1 using chemical compounds resulted in an increased expression of E-cadherin and SIRT1. Ishikawa (A, C, D, and E left panel) and RL95-2 (B, E right panel) cells were treated with various doses of vehicle, SIRT1 activators (resveratrol and AICAR), and SIRT1 repressors (sirtinol and NAM). Twenty-four hours after treatment, cells were harvested and protein expression of E-cadherin and SIRT1 was analyzed by Western blot. The immunoblotting analysis revealed that SIRT1 activators increased the expression of E-cadherin and SIRT1 in cell lysates, while SIRT1 repressors decreased the expression of E-cadherin and SIRT1 in cell lysates. (F) 293T cells were transfected with E-cad(-108)-Luc plasmids, and treated with various doses of resveratrol. Resveratrol showed a dose-dependent transactivation function of E-cadherin promoter.

of spheroids attached to the cell monolayers, while siRNA-mediated depletion of endogenous SIRT1 resulted in a 4-fold decreased uterine receptivity (Fig. 3B). Then we tested whether activation or inactivation of SIRT1 may modulate the uterine receptivity. Ishikawa cells treated with resveratrol exhibited a 3.5-fold greater number of spheroids attached to cell monolayers compared with cells treated with vehicle alone, while repression of SIRT1 by sirtinol resulted in a 2-fold decrease in spheroid attachment to the Ishikawa cell monolayer (Fig. 3D). Thus, the expression of SIRT1 and its activity influenced on the initial attachment of embryos through the mechanism involved in enhanced human uterine receptivity.

3.4. Localization of E-cadherin and Glycodelin to sites of intercellular contact is essential for initial attachment

To further pursue the role of resveratrol, other implantation molecules such as Glycodelin and Survivin were examined. Glycodelin is a progesterone-induced glycoprotein secreted into uterine luminal cavity by endometrial glands in secretory phase [4]. Survivin is an anti-apoptotic molecule and is overexpressed in the majority of human cancers [17], and is upregulated in early gestation [25]. We found significantly higher protein levels for Glycodelin in Ishikawa cells after resveratrol treatment but not for Survivin (Fig. 4A, B). We further investigated whether resveratrol possesses

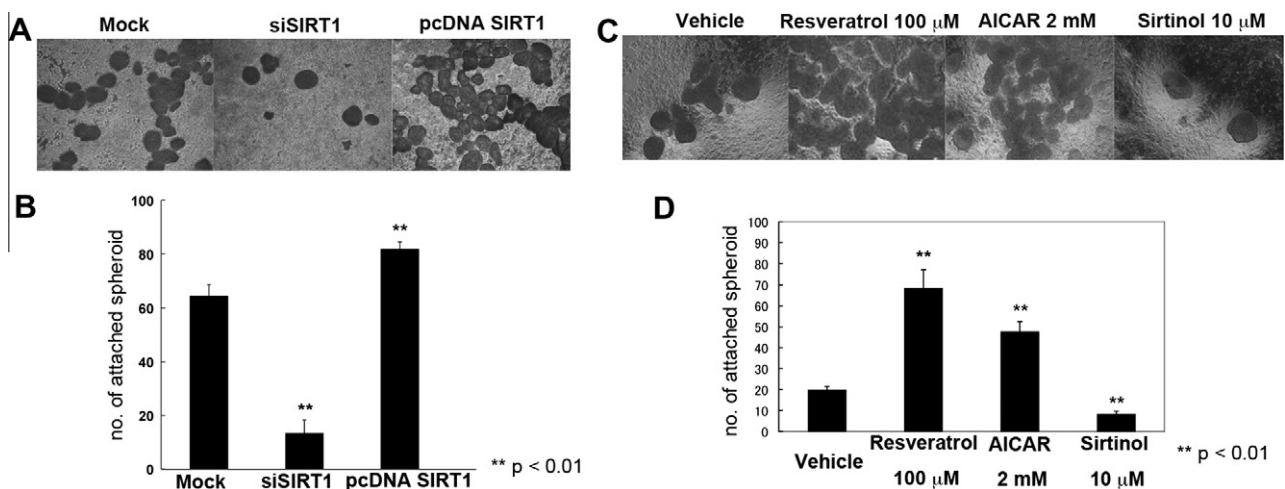


Fig. 3. SIRT1 expression and activity affects uterine receptivity. Spheroid adhesion to Ishikawa cell monolayer was examined by *in vivo* spheroid attachment assay. (A) Representative phase-contrast micrographs of JAR spheroids attached to Ishikawa cell monolayer. (B) Graph showing spheroid adhesion to Ishikawa cell monolayer. Appropriate controls were also analyzed (scramble siRNA and empty pcDNA vector), and no significant difference was observed. (C) Representative phase-contrast micrographs of JAR spheroids attached to Ishikawa cell monolayer. (D) Graph showing spheroid adhesion to Ishikawa cell monolayer after the treatment with chemical compounds.

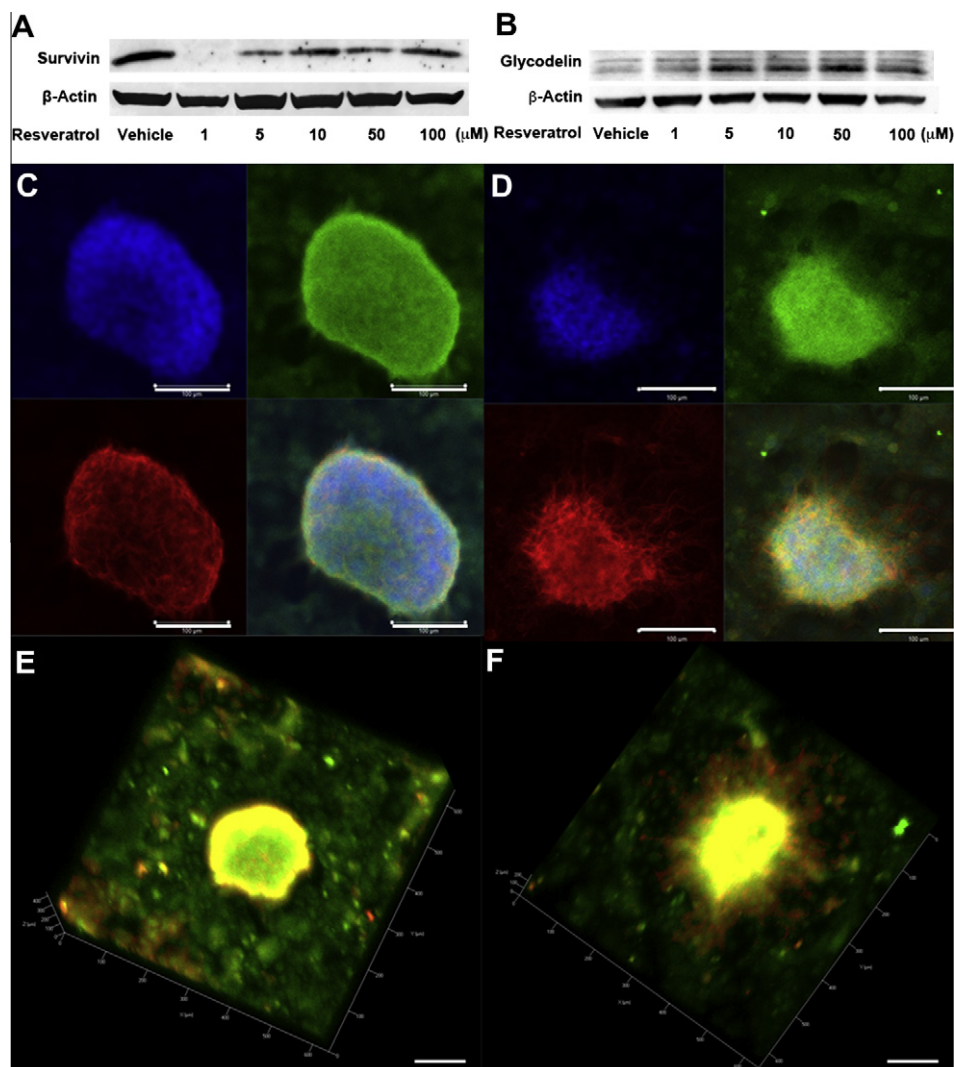


Fig. 4. Expression of implantation-related proteins and colocalization of E-cadherin and Glycodelin in Ishikawa cells. (A, B) Ishikawa cells were treated with various doses of resveratrol and protein expression of Survivin and Glycodelin was analyzed by Western blot. (C–F) *In vivo* spheroid assay, colocalization of E-cadherin and Glycodelin was examined by immunofluorescence study. (C and D) spheroids attached to the cell monolayer at 1 h (C) and at 24 h (D) are shown. (E, F) Three dimension views of attached spheroids at 1 h (E) and at 24 h (F) are shown. Note that the colocalization signal (yellow; merge) is becoming intense. Bars indicate 100 μm. Results shown are representative photographs.

an additional effect on the uterine receptivity compared with SIRT1 alone. Immunofluorescence study was performed to localize E-cadherin and Glycodelin expression at sites of intercellular contact under the confocal microscopy. Although E-cadherin and Glycodelin were expressed at sites of intercellular contact, colocalization signal was not significant in spheroid cells after 1 h incubation (Fig. 4C). However, both E-cadherin and Glycodelin are intensively expressed and the degree of colocalization signal was significantly elevated in spheroid cells showing dispersing morphological changes after 24 h incubation (Fig. 4D), suggesting that E-cadherin stimulates initial step of spheroid attachment and Glycodelin helps to invade spheroids in endometrial lining. Since both SIRT1 expression and SIRT1 stimulation by chemical compounds increase expression of E-cadherin and Glycodelin, these data confirmed that SIRT1 plays pivotal roles in initial step of implantation.

4. Discussion

The studies of SIRT1 in uterine physiology are limited. A recent study examined the SIRT1 and SIRT2 expression and regulation in

human intrauterine tissues [26]. In the present study, we report the possibility that SIRT1 expression and SIRT1 function can regulate human implantation because the stimulation of E-cadherin expression and small molecules that affect SIRT1 activities produced a substantial effect on spheroid attachment ability in an *in vitro* model of endometrial receptivity in nonpolar endometrial cancer cells. Our data was different from the previous observations that SIRT1 is involved in epigenetic silencing of DNA-hypermethylated tumor suppressor genes in breast cancer cells [11], and that SIRT1 serves as a positive regulator of epithelial-mesenchymal transition and metastatic growth of prostate cancer cells [12]. Further investigation should be required to determine the mode of regulation of E-cadherin expression whether this difference could be simply attributed to the difference of cell line.

It is also interesting that resveratrol treatment resulted in an increased expression of SIRT1 protein levels. Our previous study using rat ovarian granulosa cells also demonstrated resveratrol treatment was associated with an increased expression of SIRT1 [14]. The enhancement of SIRT1 expression by resveratrol was considerably potent compared to that by AICAR. This was consistent with the report that the enhancement of SIRT1 expression by AICAR is at best

1.3-fold [27]. Our data provided a novel anti-tumorigenic property of SIRT1 activating chemicals because decreased E-cadherin expression has been identified in a wide variety of malignancies including endometrial cancer [28]. While the underlying mechanism remains elusive, it has been shown that resveratrol has the ability to activate SIRT1 deacetylase activity [29], and resveratrol might have anti-tumorigenic properties. It was reported that resveratrol inhibited the proliferation of a wide variety of human cancer cell lines through the induction of S-phase cell cycle arrest and apoptosis [30] while presenting very low cytotoxicity in animal models. Interestingly, low doses of resveratrol can sensitize cells to low doses of cytotoxic anti-cancer drugs, therefore resveratrol is expected to facilitate the efficacy of anticancer therapy in various human cancers [31].

Suberoylanilide hydroxamic acid (vorinostat), a HDAC inhibitor drug utilized as an anti-cancer drug, has been shown to induce differentiation of endometrial glandular cells and to increase the expression of Glycodelin [4]. Glycodelin was postulated to be secreted from cell that exhibited interactions between spheroids and endometrial lining (Ishikawa cells) because Glycodelin was not found in cultured media from Ishikawa cells [32]. We believe that both Glycodelin protein expression and E-cadherin protein expression synergistically help to improve initial steps of implantation, including attachment, adhesion, and invasion. Considering the teratogenicity of vorinostat in the treatment of infertility, our result is fascinating and may provide a possibility for the practical use of resveratrol because adverse events associated with resveratrol intake were gastrointestinal symptoms alone [33]. The study revealed that repeated administration of high doses of resveratrol resulted in micromolar plasma concentrations. Therefore, our data that elevated E-cadherin expression was observed at a dose of micromolar concentrations of resveratrol can be tolerable and accomplishing dose.

In conclusion, our data indicate that SIRT1 plays an important role in regulating E-cadherin expression. Therefore, SIRT1-activating chemicals including resveratrol and AICAR would be novel therapeutic targets for improvement of initial step of implantation, thereby improvement of assisted reproductive technology success can be expected. However, in view of the difference in the pathophysiology of implantation between cultured cells (*in vitro*) and human (*in vivo*), our data should be interpreted with caution and the present observations should be further verified.

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