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SIRT1 controls cell proliferation by regulating contact inhibition

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Abstract

Contact inhibition keeps cell proliferation in check and serves as a built-in protection against cancer development by arresting cell division upon cell-cell contact. Yet the complete mechanism behind this anti-cancer process remains largely unclear. Here we present SIRT1 as a novel regulator of contact inhibition. SIRT1 performs a wide variety of functions in biological processes, but its involvement in contact inhibition has not been explored to date. We used NIH3T3 cells, which are sensitive to contact inhibition, and H460 and DU145 cancer cells, which lack contact inhibition, to investigate the relationship between SIRT1 and contact inhibition. We show that SIRT1 overexpression in NIH3T3 cells overcomes contact inhibition while SIRT1 knockdown in cancer cells restores their lost contact inhibition. Moreover, we demonstrate that p27 protein expression is controlled by SIRT1 in contact inhibition. Overall, our findings underline the critical role of SIRT1 in contact inhibition and suggest SIRT1 inhibition as a potential strategy to suppress cancer cell growth by restoring contact inhibition.

Keywords

SIRT1; Contact inhibition; Cell proliferation; Malignant transformation; p27

1. Introduction

Normally dividing cells are halted in their growth when they come into contact with adjacent cells. In doing so, contact inhibition keeps cell proliferation in control and serves to maintain tissue homeostasis [1,2]. Loss of contact inhibition makes cells more susceptible to malignant transformation and hyperplasia, and has been evidenced in cancer cells as well [3,4]. Yet the mechanism behind this loss is mostly obscure.

SIRT1 is an NAD-dependent class III histone deacetylase and has been shown to play a key role in various biological processes including aging, DNA repair, cell senescence, and apoptosis [5–8]. SIRT1 is known to be upregulated in cancer cells [9–12] and its overexpression has been correlated with poor prognosis in many cancer types [13–16]. Recently, we have reported that SIRT1 is an important regulator of p27 [17], a protein

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central to contact inhibition [18–20], but the role of SIRT1 in contact inhibition has not been studied to date.

In this study, we report SIRT1 as a new regulator of contact inhibition. We demonstrate that SIRT1 overexpression overcomes contact inhibition in non-transformed NIH3T3 cells, while knockdown of SIRT1 leads to the restoration of contact inhibition in cancer cells. Furthermore, we show that SIRT1 controls p27 protein expression in contact inhibition, uncovering the role of SIRT1-p27 axis in the regulation of contact inhibition. Through these findings, we demonstrate that SIRT1 controls cell proliferation in a cell contact-dependent manner and that SIRT1 inhibition can repress cancerous growth by restoring contact inhibition.

2. Materials and methods

2.1. Cell lines, plasmids, and antibodies

NIH3T3 fibroblasts were obtained from ATCC (Manassas, VA) and maintained in 1× DMEM with 10% donor calf serum (Sigma-Aldrich, St. Louis, MO). H460 and DU145 cells were also obtained from ATCC and maintained in 1× DMEM with 10% fetal bovine serum (GIBCO-Invitrogen, Carlsbad, CA). The human SIRT1 expression vector pcDNA3.1v5His-SIRT1, vector pcDNA3.1v5His, SIRT1 siRNA expression vector pSUPER.retro.puro-SIRT1, and vector pSUPER.retro.puro-nontargeting siRNA were generated or acquired as previously described [21]. Antibodies to SIRT1 (sc-74504) and p27 (sc-528) were purchased from Santa Cruz Biotechnology (Dallas, TX). Another antibody to SIRT1 (07-131) was purchased from Millipore (Billerica, MA). Antibody to β-actin (A1978) was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Transfection and establishment of stable SIRT1-overexpression cell line

NIH3T3 cells were transfected with pcDNA3.1v5His-SIRT1 (human origin) or pcDNA3.1v5His vector using Lipofectamine 2000 (Invitrogen). The medium was changed after 72 h to include 800 µg/ml Geneticin (Enzo Life Sciences, Farmingdale, NY), which was used to select the successfully transfected cells. After two weeks of selection with Geneticin, cells were pooled and maintained.

2.3. Retroviral infection and establishment of stable SIRT1-knockdown cell lines

The Phoenix packaging cell line was transfected with pSUPER.retro.puro-SIRT1 siRNA expression vector or pSUPER.retro.puro-nontargeting siRNA vector using Lipofectamine 2000. After 48 h, the medium containing retrovirus was collected, filtered, treated with polybrene, and then transferred to H460 and DU145 cell cultures. Successfully infected cells were selected with puromycin, after which the stably infected colonies were pooled.

2.4. Cell proliferation assay

The cells were seeded in triplicates at 5×10^4 cells per well in 100 µl complete media in 96-well plates and cultured for 2–5 days at 37 °C. The cell cultures were sparse after 2 days or confluent after 5 days. 10 µl of the cell counting kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Rockville, MD) was added to the culture media, followed by incubation in the

dark for 1 h at 37 °C, and the absorbance was read at 490 nm on the SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA).

2.5. Cancer cell 3D culture

5×10^3 SIRT1 knockdown or RNAi control H460 cells were seeded in 3D culture plates (Corning Life Sciences, Tewksbury, MA) and cell colony images were acquired with a digital microscope at 4× magnification over the course of 10 days.

2.6. Immunoblotting assay

Cells cultured in 100 mm dishes were harvested and lysed with 1% Nonidet P-40 lysis buffer (1% NP-40 150 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 8.0, 5 mmol/L EDTA, 1 mmol/L Na_3VO_4) including protease inhibitor cocktail tablet (Cat# 14586500, Roche Diagnostics, Indianapolis, IN). Protein samples were then separated on 8% SDS-PAGE and analyzed with anti-SIRT1 (sc-74504; 07-131), anti-p27 (sc-528), and anti- β -actin (A1978) antibodies.

3. Results

3.1. SIRT1 protein level is negatively associated with contact inhibition

To determine the involvement of SIRT1 in contact inhibition, we tested for the SIRT1 protein level in sparse versus confluent cultures of NIH3T3 cells, an established cell model in the study of contact inhibition due to its high sensitivity to cell density-dependent growth arrest, and H460 and DU145 cancer cells, which are known to lack contact inhibition.

Our western blot result shows that SIRT1 expression level is higher in H460 and DU145 cancer cells than in NIH3T3 cells (Fig. 1A). Furthermore, we found that while the SIRT1 level is decreased when NIH3T3 cells grow from a sparse to confluent culture (Fig. 1B), it remains elevated in H460 and DU145 cancer cells regardless of the culture density (Fig. 1C and D). Thus, down-regulated SIRT1 is correlated with active contact inhibition in NIH3T3 cells while upregulated SIRT1 is correlated with the loss of contact inhibition in cancer cells. These results suggest that SIRT1 may be an important factor in regulating cell contact inhibition and that its upregulation could be a mechanism by which cancer cells lose contact inhibition.

3.2. SIRT1 overexpression overcomes contact inhibition in NIH3T3 cells

Subsequently, we investigated the effect of SIRT1 overexpression on contact inhibition. We overexpressed SIRT1 by transfecting NIH3T3 cells with SIRT1 expression plasmid (Fig. 2A), and compared the growth level of SIRT1 overexpressing cells with that of the vector-transfected cells in sparse versus confluent cultures. We found that SIRT1 overexpression in NIH3T3 cells significantly promotes cell proliferation in the confluent cultures, whereas it does not notably affect the cell proliferation in the sparse cultures (Fig. 2B). This implies that increased SIRT1 expression in NIH3T3 cells can overcome contact inhibition and trigger continual cell growth beyond confluence.

3.3. SIRT1 knockdown restores contact inhibition in cancer cells

Upon observing the effect of SIRT1 overexpression on contact inhibition, we wanted to determine whether SIRT1 knockdown in cancer cells could restore density-dependent growth arrest. Stable SIRT1 knockdown cells were established in both H460 and DU145 cells (Fig. 3A and B). We then compared the proliferation of SIRT1 knockdown cells with that of the RNAi-control cells in sparse versus confluent cultures. Our results indicate that SIRT1 knockdown notably suppresses cell growth of the confluent cultures, resembling the manifestation of contact inhibition in nontransformed NIH3T3 cells, whereas it minimally affects the proliferation of the sparse cultures (Fig. 3C and D). We also observed a cessation in the colony growth of SIRT1 knockdown H460 cells relative to that of the control H460 cells in three-dimensional cell culture (Fig. 3E), which is recognized as a closer representation of *in vivo* systems where cells grow in tighter contact with each other than in 2D culture [22]. Taken together, these results strongly support that SIRT1 upregulation in cancer cells leads to the loss of contact inhibition and imply that SIRT1 inhibition is a potential strategy to suppress cancer cell growth by restoring contact inhibition.

3.4. SIRT1 controls p27 to regulate contact inhibition

p27 has been shown to play an important role in contact inhibition [18–20] and our previous study has shown that SIRT1 regulates p27 expression [17]. We, therefore, aimed to further determine the relationship of SIRT1 and p27 relative to contact inhibition. We performed western blots and found that SIRT1 and p27 are inversely expressed in sparse versus confluent cultures of NIH3T3 cells: p27 is increased when SIRT1 level is decreased upon cell confluence, and vice versa (Fig. 4A). In addition, we studied the regulation of p27 by SIRT1 in confluent cell cultures and found that SIRT1 overexpression reduces p27 level in NIH3T3 cells (Fig. 4B) and that SIRT1 knockdown increases p27 level in H460 and DU145 cancer cells (Fig. 4C and D). Together, these results show that SIRT1 controls p27 expression in contact inhibition, and suggest SIRT1-p27 axis as a regulatory mechanism of contact inhibition.

4. Discussion

Regulation of cell proliferation is central to tissue homeostasis, malignant transformation, and cancerous growth. SIRT1 is a key regulator in the control of cell growth and survival [8], and SIRT1-mediated suppression of apoptosis through p53 deacetylation is known as one of the mechanisms behind the proliferative activity of SIRT1 [23,24]. However, more recent reports have shown that SIRT1 regulates cell growth regardless of p53 status [25,26]. This suggests that other mechanisms exist through which SIRT1 regulates cell proliferation. Our study presents a new mechanism in which SIRT1 regulates cell proliferation through controlling contact inhibition.

Contact inhibition is an important anticancer mechanism, the lack of which unleashes cells to proliferate virtually unchecked. Loss of contact inhibition is observed in most cancer cells, making it one of the hallmarks of malignant transformation [3,4]. Moreover, many studies have evidenced the upregulation of SIRT1 in cancer cells, which is correlated with cancer progression [9–16]. Our findings that SIRT1 overexpression overcomes contact

inhibition while SIRT1 knockdown restores contact inhibition demonstrate that SIRT1 upregulation in cancer cells contributes to their loss of contact inhibition, and suggest SIRT1 repression as a viable method to deter cancerous growth by reestablishing contact inhibition.

The complete mechanism through which contact inhibition operates has been poorly defined, but some participating factors have been identified, such as p27, E-cadherin, p38 α , and YAP [18,27–29]. Moreover, it has been widely accepted that p27 plays a crucial role in the regulation of contact inhibition through its control of cell cycle arrest [18–20]. In this study, we found that SIRT1 and p27 are inversely expressed through SIRT1 control of p27 expression in contact inhibition (Fig. 4), suggesting SIRT1-p27 axis as an important mechanism in controlling contact inhibition. Furthermore, previous studies have evidenced that SIRT1 suppresses E-cadherin, a cell-cell adhesion protein that gets excessively expressed in higher cell densities and induces growth arrest [27,30,31], and that E-cadherin-dependent growth arrest is mediated by p27 [32]. Thus, it would be interesting to have the interactions among these three proteins in the regulation of contact inhibition further studied.

We also found that SIRT1 protein level is decreased when NIH3T3 cells reach confluence (Fig. 1B), whereas it remains elevated in the DU145 and H460 cancer cells regardless of their culture density (Fig. 1C and D). These results suggest that there exists a mechanism in physiological setting that downregulates SIRT1 when cells physically contact each other in order to maintain tissue homeostasis, and that this process is defective in cancer cells. Therefore, the upstream signaling pathway that controls SIRT1 expression in response to cell-cell contact also needs to be further determined.

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Fig. 1. Different expression levels of SIRT1 in sparse versus confluent NIH3T3, DU145, and H460 cultures

Cell extracts were collected and western blot was performed with SIRT1 and β -actin antibodies. **A)** SIRT1 expression level is higher in H460 and DU145 cancer cells than in NIH3T3 cells. **B)** SIRT1 expression level is reduced when NIH3T3 cells reach confluence. **C and D)** SIRT1 expression level has no significant reduction from sparse to confluent culture states of cancer cells DU145 (C) and H460 (D).

Fig. 2. SIRT1 overexpression overcomes contact inhibition

A) Cell extracts were made from SIRT1- and vector-transfected NIH3T3 cells, and immunoblot was performed with a SIRT1 antibody (sc-74504) which only detects ectopically expressed SIRT1 of human origin, rather than SIRT1 in the parental mouse NIH3T3 cells, and β -actin antibody. **B)** Cell proliferation assay was performed on SIRT1- and vector-transfected NIH3T3 cells to record the cell growth levels of sparse and confluent cultures. The data are presented as a percent of the growth obtained with vector control cells (assigned the value of 100). The error bars represent the SEM of three independent experiments (* $p < 0.05$).

Fig. 3. SIRT1 knockdown in cancer cells restores contact inhibition

A and B) Cell extracts were made from SIRT1 knockdown (siSIRT1) and nontargeting RNAi vector-infected (siControl) H460 and DU145 cells, and immunoblot was performed with SIRT1 and β-actin antibodies. **C and D)** Cell growth assay was performed on H460 (C) and DU145 (D) siSIRT1 or siControl cells in sparse and confluent cultures. The data are presented as a percent of the growth obtained with siControl cells (assigned the value of 100). The error bars represent the SEM from three independent experiments (* $p < 0.05$). **E)** Representative images from three independent 3D cultures. H460 siSIRT1 and siControl cells were seeded in 3D culture plates and colony growth was monitored from day 2 to day 10 using a digital microscope at 4× magnification.

Fig. 4. SIRT1-p27 axis in contact inhibition

Cell extracts from confluent cultures were collected and immunoblot was performed with SIRT1, p27, and β -actin antibodies. **A)** SIRT1 and p27 are inversely expressed in NIH3T3 cultures. **B)** SIRT1 overexpression downregulates p27 expression in NIH3T3 cells. **C and D)** SIRT1 knockdown upregulates p27 expression in cancer cells H460 (C) and DU145 (D).