SIRT1 negatively regulates the expression of Prl2C3, a senescence-associated protein

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ABSTRACT

SIRT1 is a mammalian homologue of yeast longevity protein Sir2. SIRT1 deacetylates transcription factors, cofactors, and histones in an NAD+ dependent manner, and promotes cell survival, anti-oxidative function, and DNA repair. Although some studies have indicated that SIRT1 is involved in longevity, the function of SIRT1 for preventing aging and senescence is still unclear. In mouse embryonic fibroblasts (MEFs), we found that SIRT1 expression decreased by aging and SIRT1 reciprocally regulated the expression level of Prl2C3, one of the prolactin-like peptides. In young MEFs, purified Prl2C3 inhibited the growth and increased the number of senescence-associated β galactosidase-positive cells with enlarged and flattened shapes. Moreover, immunostaining of human skin sections showed the expression of Prl2C3 in the basal cells of the epidermis. These results indicate that SIRT1 negatively regulates a senescence-associated protein Prl2C3.

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Key words: Prl2C3, SIRT1, senescence, MEF

1. Introduction

Sirtuins are mammalian homologues of Sir2, which is implicated in the longevity of yeast. Increases in the Sir2 gene dosage promote longevity, whereas yeast with decreases in Sir2 activity shows a short lifespan1). Of the seven mammalian sirtuins, SIRT1 is an NAD+-dependent protein deacetylase that plays crucial roles in metabolism, cell survival, oxidative stress reduction, DNA repair and other cellular responses2). Although the global SIRT1 overexpression in mice does not extend their lifespan, decreases of glucose homeostasis, bone mass, wound-healing and neuro-muscular function are ameliorated in aged SIRT1-transgenic mice3). Brain-specific overexpression of SIRT1 promotes longevity in mice, resulting in approximate 10% increase in median lifespan4). Accordingly, the lifespan of the homozygote SIRT1-knockout mice is reduced5). Resveratrol (3,4,5’-trihydroxy-trans-stilbene), a polyphenol found in grapes and red wine, has been found to activate SIRT16). Resveratrol opposes the effects of high-calorie diets and extends the lifespan of mice fed with such diet7). Dietary restriction has been shown to prolong mean and maximum lifespan in various organisms8) and induces high expression levels of SIRT19). However, SIRT1-knockout mice do not show any lifespan extension through dietary restriction10). Thus, SIRT1 is closely related with improvement of healthy aging and longevity.

Because SIRT1 controls gene expression by deacetylation of various transcription factors, coactivators, corepressors, and histones2), we have hypothesized that some proteins affecting aging may be regulated by SIRT1 activity. Using DNA microarray experiments we identified Prl2C3, one of the prolactin-related proteins. Prl2C3 was induced in aged mouse embryonic fibroblasts (MEFs) and negatively regulated by SIRT1 activity. Prl2C3 was expressed in basal cells of the epidermis, the expression level of which increased in aged persons.
2. Materials and Methods

2. 1. Cell culture

MEFs were isolated from ddY mice purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). After the head and visceral tissues were removed from the embryos, the remaining bodies were finely chopped using a razor blade, which was followed by trypsinization at 37°C for 20 min. Tissues and cell clumps then were dissociated by pipetting and the cells were plated in a 10-cm culture dish (Passage 1). In the 3T3 protocol, cells were trypsinized and counted every 3 days, which was followed by replating of 300,000 cells. MEFs were cultured in Dulbecco modified Eagle medium (DMEM; Wako) supplemented with 10% FBS at 37°C in a humidified incubator with 5% CO2. C2C12, COS7, and B16F1 cells were grown at 37°C in 5% CO2 in DMEM containing 10% FBS (C2C12 and COS7) or 5% FBS (B16F1 cells).

2. 2. siRNA transfection

Cells were transfected with siRNA (50 nM) using RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Mouse SIRT1 siRNA (SASI_Mm01_00105675) and siRNAs for negative control were purchased from Sigma Genosys. Knockdown was confirmed by RT-PCR and immunoblot 48 hr after transfection.

2. 3. RNA analysis

Isolation of total RNA from cultured cells and reverse transcriptase reaction were performed as previously described. Quantitative PCR was performed using GoTaq qPCR Master Mix (Promega) and the following oligonucleotide primers: for mouse Prl2C3, 5'-CCAATCCAGTAAGCATCTTCC-3' and 5'-GTATCCAGGAGCATGGTTGAATCG-3'; for mouse C2C12, COS7, and B16F1 cells were grown at 37°C in 5% CO2 in DMEM containing 10% FBS (C2C12 and COS7) or 5% FBS (B16F1 cells).

2. 4. Preparation of anti-Prl2C3 polyclonal antibody

The polyclonal antibody for mouse Prl2C3 was raised in rabbits against a synthetic peptide as previously described. Peptide sequence corresponded to amino acid residues 187^207 (DARIHSLYGMISCLDNDFKKV) in the C-terminal region of Prl2C3, which shows identical sequences between human and mice.

2. 5. Human tissue sections and immunohistochemistry

All samples were collected from Japanese patients who underwent outpatient or inpatient care at Sapporo Medical University Hospital. Informed consent was obtained from all patients before collection of the specimens. Approval for this study was obtained from the Institutional Review Board of Sapporo Medical University. Paraffin sections (5 μm thickness) were deparaffinized with xylene and ethyl alcohol and then autoclaved in 0.1 M sodium citrate, pH 6.0 for antigen-activation. Sections were immunostained with an anti-Prl2C3 antibody (1:500 dilution) and anti-SIRT1 antibody (1:500 dilution) as previously described. The bound antibodies were labeled with Alexa Fluor 488 or 594-conjugated goat anti-rabbit secondary antibody (Thermo Fisher Scientific) and examined by confocal microscopy (Radiance 2100, Bio-Rad). Nuclei were stained by Hoechst 33342. Images were analyzed using ImageJ software (National Institutes of Health).

2. 6. Western blotting

Samples from cells were prepared for immunoblot analysis as previously described. The antibodies used for immunoblot analysis were anti-p19 (1:100, Santa Cruz), β-actin (1:10000, Wako Pure Chemical), anti-GAPDH (1:10000, Sigma Aldrich), and anti-α-tubulin (1:10000, Sigma Aldrich).

2. 7. Plasmid construction, expression and purification of Prl2C3

The coding region of mouse Prl2C3 cDNA was obtained by RT-PCR using oligonucleotide primers of 5’-AAGGTATCCATGCTCCCTTCTCC-3’ and 5’-AACTCGAGGCGTAGTATCATATTTT-3’, and inserted into the pIREShrGFP plasmid. The FLAG-tagged Prl2C3 plasmid was transfected into COS7 cells with the ViaFect Transfection Reagent (Promega). Three days after transfection, the
culture medium was incubated with an anti-FLAG antibody resin at 4 °C overnight. Prl2C3-bound resin was collected by centrifugation and washed three times with 50 mM Tris HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl. Purified Prl2C3 was eluted with 100 mM glycine HCl, pH 3.0 and then neutralized using 1 M Tris HCl, pH 8.0. The protein concentration was assayed by the Bradford Reagent (Sigma-Aldrich).

2. 8. Measurements of cellular ATP content

Cellular ATP levels were measured by using the Cell Titer-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s protocol, with an ARVO Light Luminescence Counter (PerkinElmer).

2. 9. SA-β-gal staining

SA-β-gal staining was performed in MEFs using the Senescence Detection Kit (Biovision) according to the manufacturer’s instructions.

2. 10. Reagents

Resveratrol, Hoechst33342 and the anti-FLAG resin were from Wako Pure Chemicals.

2. 11. Statistics

Data from at least three independent experiments are represented as means ± S.D. Comparisons between two groups were made using the Student’s t-test, Welch’s t-test, and Mann-Whitney U test.

3. Results

To examine the role of SIRT1 in cellular senescence and aging, MEFs were examined. Proliferation of MEF cells was severely suppressed after 5 passages. The cell numbers of Passage 5 (P5) and P8 cells were almost identical, indicating that cells progressively became senescent after P5 (Figure 1A). Accordingly, mRNA and protein levels of p19, one of the CDK inhibitors, in P8 MEFs were significantly increased compared with those of P2 MEFs (Figures 1B and 1C). We compared SIRT1 expression levels in MEFs of P2 and P8. SIRT1 mRNA of P8 MEFs was about 60% of that of P2 cells (Figure 1D), whereas Western blot analysis showed that protein levels of SIRT1 in P8 cells significantly decreased to less than 20% of that of P2 cells (Figure 1E). Immunostaining of SIRT1 also showed a significant decrease in the expression level of SIRT1 in P8 cells (Figure 1F).

Resveratrol is an activator of SIRT1. To identify the genes whose expression levels are regulated by SIRT1 activity, resveratrol and SIRT1-siRNA were used. RNAs from C2C12 cells treated with 100 μM resveratrol for 12 hr were compared with those of control cells by DNA microarray. RNAs from SIRT1-knockdown C2C12 cells were also compared with those from control cells. The expression levels of genes up-regulated more than 2-fold or down-regulated less than 0.5-fold by the SIRT1 activator or SIRT1-knockdown, respectively, were examined (data not shown). Among them, the expression level of Prl2C3, one of the prolactin-related proteins, was found to increase by more than 10-fold in C2C12 cells with SIRT1 knockdown. Treatment of cells with resveratrol decreased the Prl2C3 expression level by about one-fourth of that of control C2C12 cells. Importantly, DNA microarray of P2 and P8 MEFs showed that Prl2C3 level increased by 8-fold in P8 MEF cells compared with that of P2 young cells.

Quantitative RT-PCR experiments and Western blot analysis showed that the expression levels of Prl2C3 mRNA and protein increased more than 3-fold in P8 MEFs compared with those of P2 cells (Figures 2A and 2B). Immunostaining also showed a significant increase of Prl2C3 levels in P8 MEFs (Figure 2C). SIRT1 knockdown in MEFs significantly increased Prl2C3 mRNA and protein levels (Figures 2D, 2E, and 2F).

To examine the function of Prl2C3, FLAG-tagged Prl2C3 was expressed in COS7 cells. Prl2C3 was found in the culture medium after transfection (data not shown). Prl2C3 was purified from a culture medium using an anti-FLAG antibody resin. Although purified Prl2C3 was liable to degenerate, it significantly suppressed proliferation of P2 MEFs (Figure 3A).

Cell numbers significantly decreased in MEFs treated with purified Prl2C3, indicating that Prl2C3 suppressed cell proliferation (Figure 3A). Accordingly, ATP content of each well significantly decreased in MEF cells treated with Prl2C3 (Figure 3A). Similar to MEFs, proliferation of B16F1 mouse melanoma cells was also significantly suppressed by Prl2C3 treatment (Figure 3B).
Fig. 1. Decrease in SIRT1 expression level by aging in MEFs

(A) Cumulative population doubling (PD) of MEFs plotted with passage number on the x axis and PD on the y axis. PD = log₂ (collected cell number three days after seeded)/ (seeded cell number). (B) qRT-PCR analysis for p19 on P2 and P8 MEFs. Results were normalized to 18s and expressed as fold change relative to P2. Significance of the difference between P2 and P8 was determined by Student’s t-test (n = 4). (C) Western blot analysis for p19 on P2 and P8 MEFs. Signal intensity was quantified using the ImageJ analysis software. Results were normalized to GAPDH and expressed as fold change relative to P2. Significance of the difference was determined by Welch’s t-test (n = 4). (D) qRT-PCR analysis for SIRT1 on P2 and P8 MEFs. Results were expressed as fold change relative to P2. Significance of the difference was determined by Student’s t-test (n = 4). (E) Western blot analysis for SIRT1 on P2 and P8 MEFs. Signal intensity was quantified using the ImageJ analysis software. Results were normalized to β-actin and expressed as fold change relative to P2. Significance of the difference was determined by Mann-Whitney U test (n = 9). (F) Representative confocal images of P2 and P8 MEFs stained with SIRT1 (green) and Hoechst33342 (blue). SIRT1 staining intensity was quantified using the ImageJ analysis software. Significance of the difference was determined by Student’s t-test (P2: n = 30, P8: n = 10). Scale bar, 30 μm. Statistical significance is represented by asterisks corresponding to *p < 0.05, **p < 0.01.
SIRT1 negatively regulates the expression of Prl2C3, a senescence-associated protein

Fig. 2. Increase in Prl2C3 expression level by aging and SIRT1-knockdown in MEFs

(A) qRT-PCR analysis for Prl2C3 on P2 and P8 MEFs. Results were normalized to 18s and expressed as fold change relative to P2. Significance of the difference between P2 and P8 was determined by Welch’s t-test (n = 4). (B) Western blot analysis for Prl2C3 on P2 and P8 MEFs. Signal intensity was quantified using the ImageJ analysis software. Results were normalized to GAPDH and expressed as fold change relative to P2. Significance of the difference was determined by Student’s t-test (n = 4). (C) Representative confocal images of P2 and P8 MEFs stained with anti-Prl2C3 antibody (green) and Hoechst33342 (blue). Staining intensity of Prl2C3 was quantified using the ImageJ analysis software. Significance of the difference was determined by Mann–Whitney U test (n = 10). Scale bar, 30 μm. (D) qRT-PCR analysis of SIRT1 mRNA of P2 MEF cells treated with control siRNA or SIRT1-siRNA. (E) qRT-PCR analysis for Prl2C3 of P2 MEF cells treated with control siRNA or SIRT1-siRNA. Results were normalized to 18s and expressed as fold change relative to control. Significance of the difference was determined by Welch’s t-test (n = 4). (F) Western blot analysis for Prl2C3 of P2 MEF cells treated with control siRNA or SIRT1-siRNA. Signal intensity was quantified using the ImageJ analysis software. Results were normalized to α-tubulin and expressed as fold change relative to control. Significance of the difference was determined by Welch’s t-test (n = 4). Statistical significance was represented by asterisks corresponding to *p < 0.05, **p < 0.01.
Fig. 3. Induction of cellular senescence by Prl2C3

(A) Representative images of Hoechst33342 staining in MEFs treated with vehicle (Control) or Prl2C3. Cells were cultured in the absence or presence of Prl2C3 (22 µg/ml) for 120 h. Cell number per one visual field was represented. Significance of the difference was determined by Welch’s t-test (n = 16). ATP contents of cell lysates were expressed as fold change relative to control. Significance of the difference was determined by Student’s t-test (n = 8). (B) Representative images of Hoechst33342 staining in vehicle– (Control) or Prl2C3–treated B16F1 cells. Cells were cultured in the absence or presence of Prl2C3 (22 µg/ml) for 96 h. Cell number per one visual field (control: n = 16, Prl2C3: n = 14), ATP contents (n = 8) were indicated. (C–E) Representative images of control and Prl2C3–treated MEF cells (C), B16F1 cells (D) and C2C12 cells (E). Cell surface area was quantified using the ImageJ analysis software. Results were expressed as fold change relative to control. Significance of the difference was determined by Mann-Whitney U test (n = 30 for MEFs and B16F1 cells, and n = 12 for C2C12 cells). Scale bars, 100 µm. (F) Representative images (left) of SA–β–gal staining in P2 MEFs treated with vehicle (Control) or Prl2C3 (11 µg/ml) for 11 days. The percentage of SA–β–gal–positive cells in a visual field was analyzed (n = 10). Significance of the difference was determined by Mann-Whitney U test. Statistical significance is represented by asterisks corresponding to *p < 0.05, **p < 0.01. Scale bars, 100 µm (A, B, C, D, and F); 10 µm (E).
Aged MEFs were flattened and enlarged compared with young MEFs. Treatment of young P2 MEFs with Prl2C3 induced morphological change, and cell size were significantly increased by Prl2C3 in MEFs (Figure 3C), B16F1 cells (Figure 3D) and C2C12 cells (Figure 3E). Senescence-associated β-galactosidase (SA-β-gal) staining showed that Prl2C3 induced senescence of P2 MEFs (Figure 3F).

To address physiological roles of Prl2C3 in aging, human skin sections were examined by immunohistochemistry. Prl2C3 was expressed in basal cells of epidermis (Figure 4). The intensity of Prl2C3 immunostaining was prominent in basal cells of elderly compared with those of children (Figure 4).

4. Discussion

The SIRT1 protein level of P8 MEFs significantly decreased compared with that of P2 MEFs (Figures 1E and 1F). The expression level of Prl2C3 increased by aging (Figures 2A, 2B, and 2C) and by SIRT1-knockdown in MEFs (Figures 2D, 2E, and 2F). Prl2C3 was identified as a senescence-associated protein, because treatment of Prl2C3 suppressed proliferation and enlarged cell size of MEFs and B16F1 (Figures 3A and 3B) and induced high expression levels of SA-β-gal (Figure 3F).

Previously Prl2C3 was identified as a prolactin-related protein and also referred to as a mitogen-regulated protein (MRP) or proliferin (Plf) showing gestationally specific expression in the placenta. It was suggested that proliferin increased the cell number of hematopoietic and stromal cell lines, microvilli formation and cell proliferation of neuroblastoma cells. There is therefore a discrepancy between previous experiments and our own. The reason for this discrepancy is unknown. Prl2C3 may have dual functions on cell proliferation and cellular senescence, which might be similar to insulin-like growth factor 1 (IGF-1). IGF-1 is a mediator of growth hormones. IGF-1 is released from the liver by growth hormones in the bloodstream and induces cellular proliferation and growth. However, IGF-1 also accelerates aging and increases mortality in various organisms. Hetero-knockout of the IGF-1 gene increased the lifespan of mice by up to 65%.

We found that SIRT1 protein levels were severely decreased after the eighth passage of cells, but the mRNA level of SIRT1 was not comparable to the decrease of the SIRT1 protein level (Figures 1D, 1E, and 1F). Accordingly, a progressive decline of SIRT1 protein levels but not SIRT1 mRNA levels was found in MEFs from mouse ICR strains and human lung fibroblasts (IMR90). PPARγ, a ligand-regulated nuclear receptor, was shown to directly bind SIRT1 and inhibits SIRT1 activity. Although acetylation of PPARγ increased in cellular senescence, further study is necessary to elucidate the decrease in SIRT1 protein levels in senescent MEFs.

Prl2C3 flattened and enlarged MEFs, B16F1 and C2C12 cells (Figures 3C, 3D, and 3E). Moreover, Prl2C3 significantly increased the number of SA-β-gal staining-positive P2 MEFs (Figure 3F). These findings indicate that Prl2C3 induces senescence.

We found that Prl2C3 was expressed in the basal cells of the human epidermis (Figure 4A). Previously, expression of Prl2C3 was reported in mouse hair follicles where Prl2C3 may participate in the hair follicle cycle as a growth factor and/or angiogenesis factor. Prl2C3 might possess another function such as induction of the resting phase of the hair follicle (telogen phase of the hair cycle). Further studies will show the physiological function of Prl2C3 in skin aging.
Author contributions

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SIRT1は老化関連タンパクPrl2C3を負に制御する

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SIRT1は酵母の長寿遺伝子Sir2の哺乳類における相同体である。SIRT1はNAD⁺依存性に転写因子、共同因子、ヒストンを脱アセチル化し、細胞生存、抗酸化機能、DNA修復作用を促進する。SIRT1が長寿に関連することを示す研究報告もあるが、SIRT1の老化予防機能に関しては解明されていない。我々はマウス胚性線維芽細胞（MEF）において、SIRT1の発現が細胞老化によって減少し、SIRT1がプロラクチン類似作用を持つタンパクであるPrl2C3の発現を反的に制御していることを発見した。精製したPrl2C3タンパクは若いMEFの増殖を抑制し、老化を示すβ-ガラクトシダーゼ陽性のMEFを増加させ、細胞を大型で平坦な形態に変化させた。ヒトの皮膚組織の免疫染色において、Prl2C3は主に表皮内の基底細胞に発現していた。これらの結果は、SIRT1が老化関連タンパクであるPrl2C3を負に制御することを示している。