ANTICANCER RESEARCH *40*: 3155-3161 (2020)
doi:10.21873/anticanres.14297

Sirtuin 1 Activation Suppresses the Growth of T-lymphoblastic Leukemia Cells by Inhibiting NOTCH and NF-κB Pathways

SALWA M. OKASHA, MAI ITOH and SHUJI TOHDA

Department of Laboratory Medicine, Tokyo Medical and Dental University, Tokyo, Japan

Abstract. *Background/Aim: The deacetylase sirtuin1 (SIRT1) inhibits tumor suppressor p53 and may promote tumorigenesis; however, SIRT1 effects on leukemia cells are controversial. The aim of this study was to clarify the activity of SIRT1 in leukemia cells. Materials and Methods: The effects of SIRT1 inhibition or activation and SIRT1 knockdown or overexpression were examined in two T cell acute lymphoblastic leukemia (T-ALL) cell lines carrying NOTCH1 mutations and three acute myeloid leukemia (AML) cell lines. Results: The growth of T-ALL cells was promoted by SIRT1 inhibition and SIRT1 knockdown but was reduced by SIRT1 activation and overexpression; however, no effects were observed in AML cells. SIRT1 activation decreased NOTCH, NF-κB, and mTOR signaling and inhibited p53, suggesting that the possible mechanisms of T-ALL growth suppression by SIRT1 are independent of p53. Conclusion: SIRT1 activators acting through the down-regulation of NOTCH, NF-κB, and mTOR pathways can be novel targeted drugs for NOTCH1-mutated T-ALLs.*

Sirtuins (SIRTs) are NAD^+-dependent lysine deacetylases implicated in key cellular processes, including aging and longevity. The SIRT family comprises seven isotypes, SIRT1 to SIRT7 (1); among them, SIRT1, SIRT6, and SIRT7 are predominately localized in the nucleus, where they deacetylate histones and non-histone proteins such as transcription factors and DNA repair proteins (2).

SIRT1 is known to be involved in carcinogenesis, including hematologic malignancies; however, its role in this process is controversial (3). SIRT1 can deacetylate and inactivate the tumor suppressor factor p53, which results in the inhibition of apoptosis and stimulation of oncogenic processes; on the other hand, it promotes DNA repair and genomic stability, acting as a tumor suppressor. Furthermore, Sasca *et al.* (4) have reported that SIRT1 was highly expressed in acute myeloid leukemia (AML) cells, where it prevented p53 activation, whereas Chauhan *et al.* (5) have found that treatment with the SIRT1 activator SRT1720 inhibited the growth and induced apoptosis of myeloma cells. These findings indicate that the functional activity of SIRT1 in hematologic cancers is not fully understood.

The NOTCH1 receptor and its target the MYC oncogene are implicated in the origin of T cell acute lymphoblastic leukemia (T-ALL). More than half of T-ALLs have activating *NOTCH1* mutations, indicating a critical role of NOTCH1 in leukemia development (6). It has also been shown that SIRT1 might interact with NOTCH1 and regulate its localization and function in T cells (7).

In this study, we aimed to elucidate the effects of SIRT1 on the growth of T-ALL and AML cells and disclose the underlying molecular mechanisms by manipulating SIRT1 activity and expression in T-ALL and AML cells using pharmacological and genetic approaches. Our findings indicate that SIRT1 activation suppressed the growth of T-ALL cells harboring *NOTCH1* mutations by inhibiting NOTCH, NF-κB, and mTOR signaling. These findings suggest a clinical potential of SIRT1 activators as novel molecular targeting drugs for *NOTCH1*-mutated T-ALLs.

Materials and Methods

Cell lines and reagents. We used the T-ALL cell lines DND-41 and KOPT-K1 and the AML cell lines THP-1, NB4, and OCI/AML2. DND-41 and KOPT-K1 cells with activating *NOTCH1* mutations (6) were donated by Drs. Harashima and Orita (Fujisaki Cell Center, Okayama, Japan). THP-1, an acute monoblastic leukemia cell line, was obtained from the Health Science Research Resource Bank (Osaka, Japan); NB4, an acute promyelocytic leukemia cell line, was a gift from Dr. M. Lanotte (INSERM, Paris, France), and OCI/AML2, an acute myelomonoblastic leukemia cell line, was a gift from the Ontario Cancer Institute (Toronto, Canada).

EX527, the first selective SIRT1 inhibitor which acts by occupying the nicotinamide site and a neighboring pocket (8), and SRT1720, a small-molecule activator of SIRT1 which lowers the Michaelis constant for acetylated substrates (9), were purchased from SelleckChem (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO) to the final concentration of 20 mM.

Correspondence to: Shuji Tohda, MD, Ph.D., Department of Laboratory Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-Ku, Tokyo 113-8519, Japan. Tel: +81 358035334, Fax: +81 358035629, e-mail: tohda.mlab@tmd.ac.jp

Key Words: Sirtuin1, leukemia, NOTCH, NF-κB, p53.

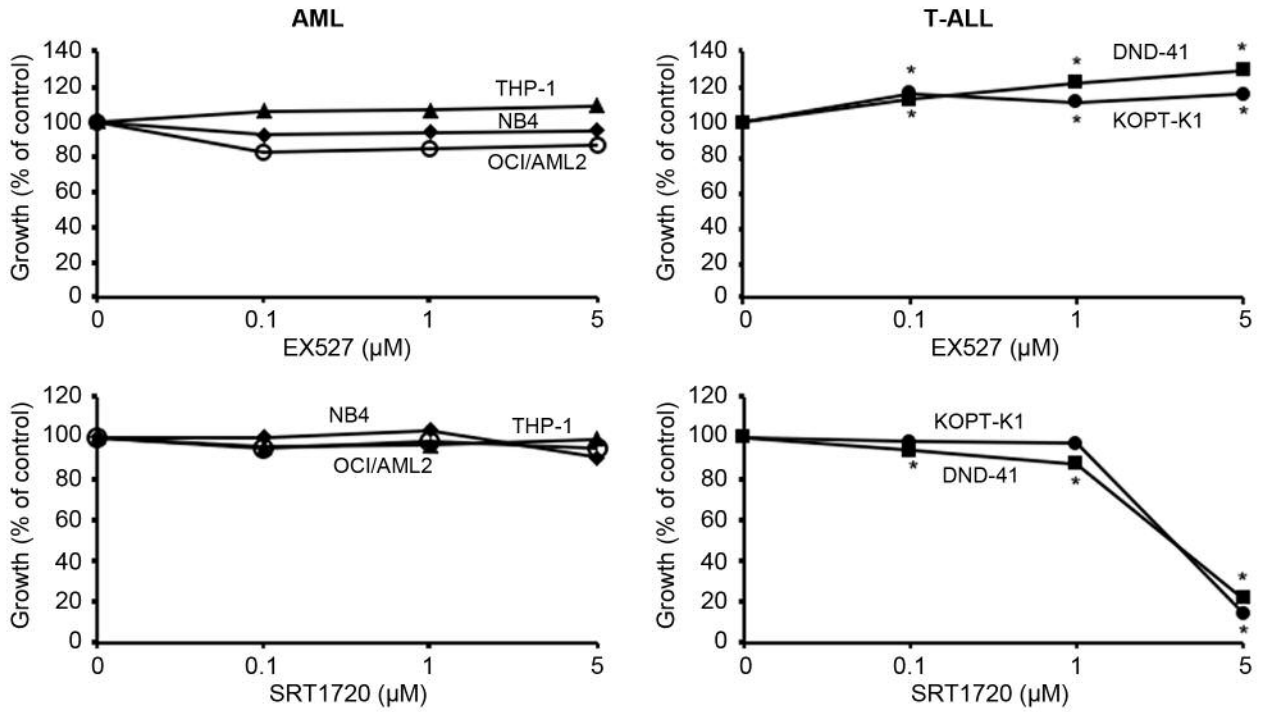

Figure 1. *Effects of the SIRT1 inhibitor EX527 and the SIRT1 activator SRT1720 on leukemia cell growth. Cells were cultured with the indicated concentrations of the compounds for 3 days. Cell growth was evaluated by a colorimetric assay and expressed as the percentage of the mean OD in drug-treated cells to that in control (DMSO-treated) cells. *p<0.05 compared to control.*

Cell growth assay. Cell growth was evaluated by a colorimetric cell counting kit (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and with or without EX527 or SRT1720 in 96-well culture plates in a humidified 5% CO_2 atmosphere. After 72 h, the CCK-8 solution was added and the optical density (OD) was measured. Relative cell proliferation was calculated as the percentage of the mean OD of cells cultured with drugs to that of cells cultured with DMSO as a vehicle control. Statistical significance of the difference was determined by Student's *t*-test. Cell morphology was evaluated under a microscope after staining cytospin preparations with Wright stain.

SIRT1 knockdown by siRNA. SIRT1 knockdown was performed using pre-designed small interfering RNA (siRNA) targeting *SIRT1* (HSS 118729, 177403, and 177404; Stealth siRNA™, Life Technologies, Carlsbad, CA, USA). Stealth RNAi negative control duplex was used as a control. Cells were transfected with each siRNA using the Neon™ pipette tip chamber-based electroporation system (Life Technologies) and immediately transferred to the culture medium.

Forced expression of SIRT1. The *SIRT1* expression vector was constructed by inserting *hSIRT1* [NM 012238.5] into the pRP[Exp]-EGFP/Puro-CAG plasmid (VectorBuilder Inc., Chicago, IL, USA). Leukemia cells were transfected with the *SIRT1* plasmid and the empty vector using the Neon electroporation system and transferred to the culture medium.

Immunoblotting analysis. Transfected cells and cells treated with EX527 or SRT1720 were lysed and the extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Antibodies against SIRT1 (#2496), NOTCH1 (#3608), cleaved NOTCH1 (#4147), HES1 (#11988), MYC (#13987), p65/RELA (#8242), acetyl-NF-κB p65(Lys310) (#12629), phospho-p65 (S536) (#3033), mTOR (#2938), phospho-mTOR (S2481) (#2974), 4E-BP1 (#9452), phospho-4E-BP1 (#9451), S6K (#2708), phospho-S6K (#9234), LC3B (#3868), p62/SQSTM1 (#39749), acetyl-p53 (Lys382) (#2525), and cleaved caspase-3 (#9664) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phospho-p65 (S276) (#ab183559) were from Abcam (Cambridge, MA, USA). Anti-GAPDH antibodies (Fuji film Wako Pure Chemicals, Osaka, Japan) were used as a loading control. Immunoreactive bands were detected using horseradish peroxidase-linked secondary antibodies and Pierce Enhanced Chemiluminescent Western Blotting Substrate (Pierce Biotechnology, Rockford, IL, USA). Each assay was repeated at least three times to ascertain reproducibility.

Results

Effects of SIRT1 inhibition on leukemia cell growth and signaling proteins. Treatment with the SIRT1 inhibitor EX527 promoted the growth of T-ALL cells in a dose-dependent manner but did not significantly affect that of AML cells (Figure 1). To elucidate the mechanisms underlying the stimulation of T-ALL

cell proliferation, we analyzed the expression of SIRT1-associated signaling proteins by immunoblotting. The results indicated that in DND-41 cells, SIRT1 inhibition up-regulated acetylation of p53 and expression of p21, a p53 downstream target; furthermore, it increased acetylation and phosphorylation of p65/RELA, phosphorylation of mTOR signaling molecules 4E-BP1 and S6K, and expression of HES1 and MYC, key signaling proteins downstream of NOTCH (Figure 2). In p53-deficient KOPT-K1 cells, SIRT1 inhibition promoted p65/RELA acetylation and slightly increased its phosphorylation (Figure 2).

Effects of SIRT1 activation on leukemia cell growth and signaling proteins. SIRT1 activation suppressed the growth of T-ALL cells in a dose-dependent manner but did not significantly affect that of AML cell lines (Figure 1). Evaluation of cytospin preparations revealed nuclear condensation and apoptotic bodies, which suggest induction of apoptosis; in addition, large vacuoles were observed in the cytoplasm of DND-41 and KOPT-K1 cells treated with the SIRT1 activator SRT1720 (Figure 3).

Western blotting analysis indicated that in both T-ALL cell lines, SIRT1 activation induced the deacetylation of p65/RELA, suppressed the phosphorylation of mTOR and its downstream targets 4E-BP1 and S6K, and decreased the levels of cleaved NOTCH1 and MYC (Figure 4). Furthermore, a decrease in the ubiquitin-binding protein p62/SQSTM1 and an increase in the microtubule-associated protein 1 light chain 3 (LC3)-II/LC3-I ratio were observed, suggesting induction of autophagy (10). In DND-41 cells, the phosphorylation of AKT and the expression of cleaved NOTCH1 and HES1 were down-regulated, whereas in KOPT-K1 cells the phosphorylation of p65/RELA was decreased and the expression of cleaved caspase-3 increased, suggesting induction of apoptosis (Figure 4).

Effects of SIRT1 expression on leukemia cell growth and signaling proteins. To confirm that the described effects were due to changes in SIRT1 activity and not to off-target effects, we used genetic approaches to modulate *SIRT1* expression.

Among the three *SIRT1*-specific siRNAs, HSS 177404 (5'-UGGGACAUGCCAGAGUCCAAGUUUA-3') was the most potent in decreasing SIRT1 protein levels (data not shown) and its effects on cell growth and signaling proteins were investigated in detail. Transfection with HSS 177404 significantly increased the proliferation of DND-41 and KOPT-K1 cells to 117±6% and 107±1% of control siRNA-tranfected cells after culture for 48 h, respectively (p<0.05). In both cell lines, *SIRT1* knockdown up-regulated p65/RELA acetylation. In DND-41cells, it slightly increased the expression of cleaved NOTCH1 and MYC (Figure 5).

SIRT1 overexpression resulted in a significant decrease of T-ALL cell proliferation compared to control plasmid-transfected cells: to 40±1% and 48±7% for DND-41 and

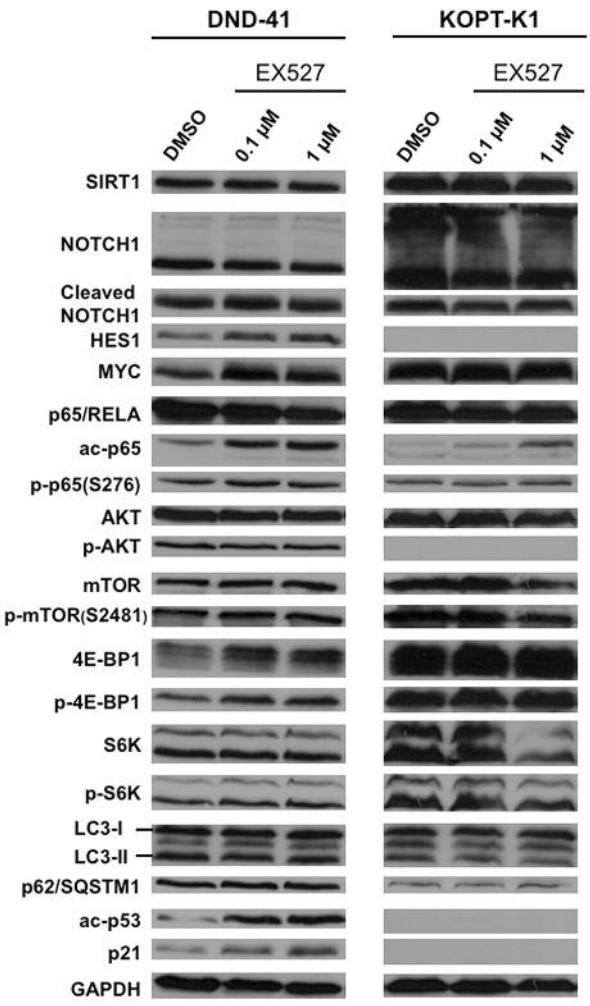

Figure 2. *Effects of EX527 on signaling proteins in DND-41 and KOPT-K1 cells. Cells were cultured with 0.1 or 1 μM EX527 for 24 h and analyzed for the expression of the indicated proteins by immunoblotting. DMSO was used as a vehicle control.*

KOPT-K1 cells after culture for 24 h, respectively (p<0.05). Furthermore, SIRT1-overexpressing cells showed a decrease in p65/RELA expression and p65 phosphorylation and acetylation, as well as in mTOR phosphorylation and expression of mTOR-related molecules and NOTCH1 signaling proteins (Figure 6). Changes in autophagy-associated proteins p62/SQSTM1 and LC3-II/LC3-I were also observed in both cell lines (Figure 6). In DND-41 cells, a decrease in AKT phosphorylation and p53 acetylation was detected.

Discussion

Our results indicate that SIRT1 inhibition promotes, whereas its activation suppresses, the growth of T-ALL cell lines carrying activating *NOTCH1* mutations, which is confirmed

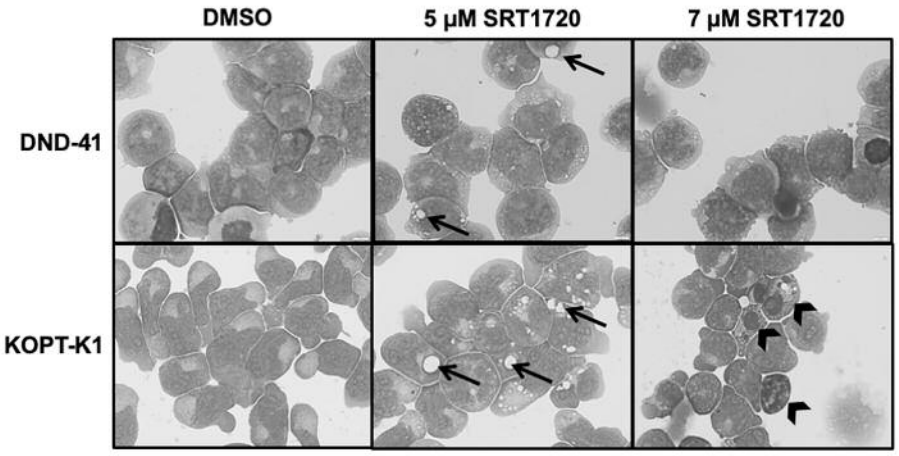

Figure 3. *Morphological changes of DND-41 and KOPT-K1 cells treated with SRT1720. Cytospin preparations from cells cultured with SRT1720 for 6 h were stained with Wright stain (original magnifications, ×600). Arrows and arrowheads point on vacuoles and apoptotic bodies, respectively.*

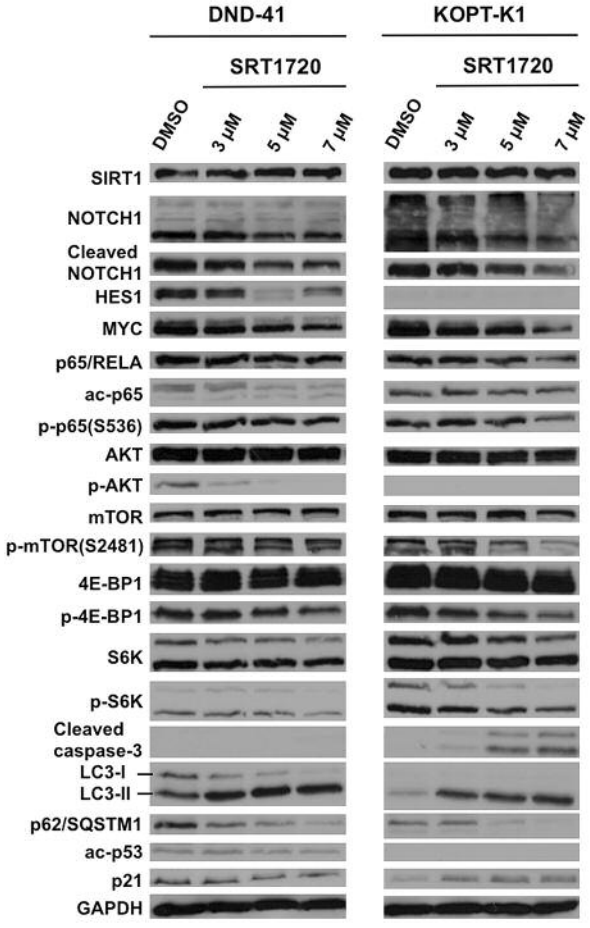

Figure 4. *Effects of SRT1720 on signaling proteins in DND-41 and KOPT-K1 cells. Cells were cultured with the indicated concentration of SRT1720 for 24 h and analyzed for protein expression by immunoblotting. DMSO was used as a vehicle control.*

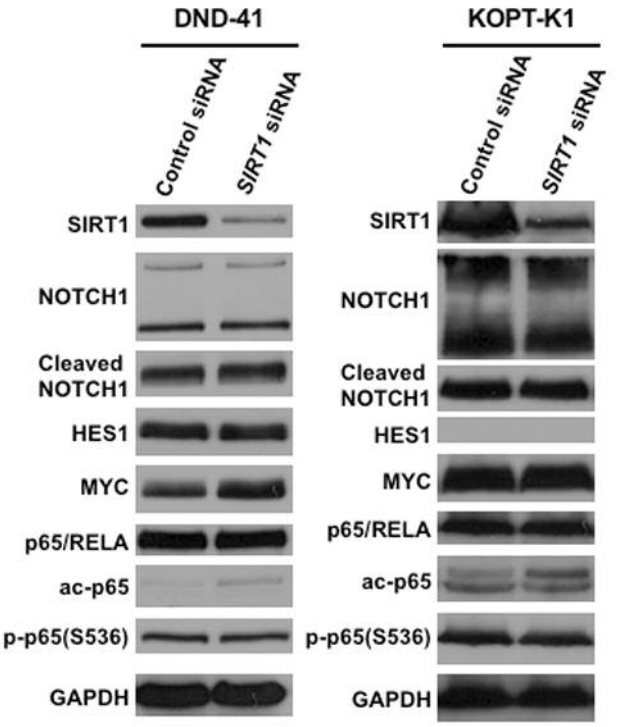

Figure 5. *Effects of SIRT1 knockdown on signaling proteins in DND-41 and KOPT-K1 cells. Cells transfected with SIRT1 siRNA and control siRNA were cultured for 48 h and analyzed for the expression of indicated proteins by immunoblotting.*

in the experiments with SIRT1-deficient and SIRT1-overexpressing cells. These findings led us to suggest a possible role of SIRT1 as a tumor suppressor in *NOTCH1*-mutated T-ALLs.

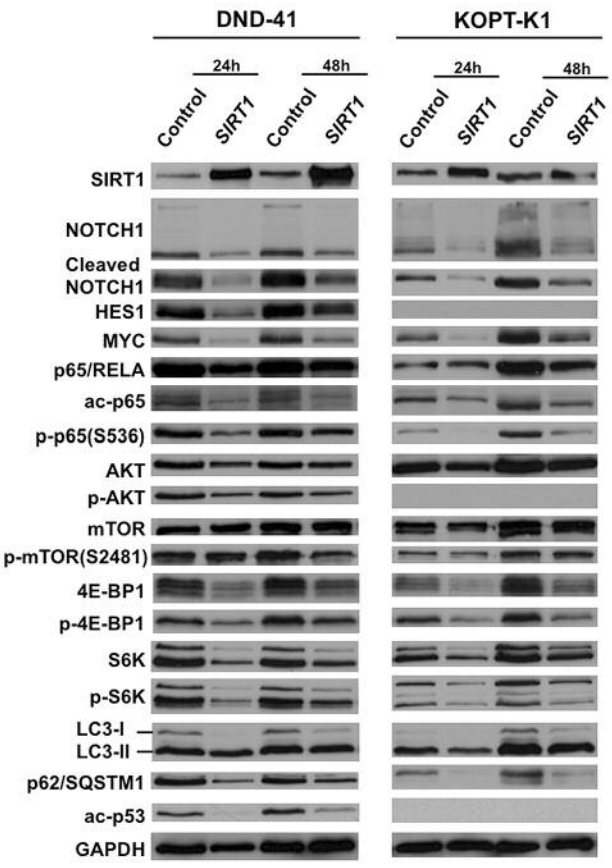

Figure 6. *Effects of forced SIRT1 expression on signaling proteins in DND-41 and KOPT-K1 cells. Cells transfected with the SIRT1 expression vector and vehicle plasmid (control) were cultured for 24 and 48 h and analyzed by immunoblotting.*

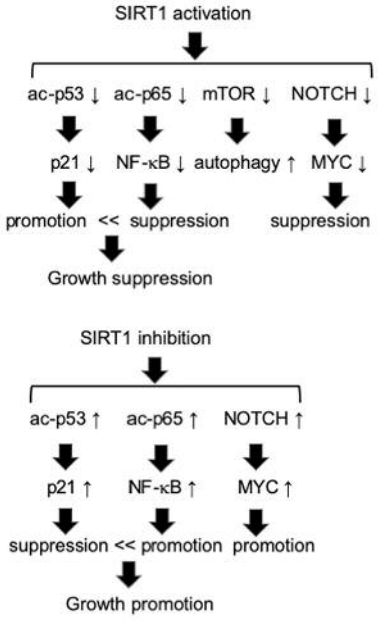

Figure 7. *Schematic presentation of the effects of SIRT1 activation and inhibition in T-ALL cells carrying NOTCH1 mutations. ↑: up-regulation; ↓: down-regulation.*

To examine the underlying molecular mechanisms, we analyzed acetylation of p53 and p65/RELA, both of which are SIRT1 substrates (11, 12). Indeed, the SIRT1 inhibitor EX527 up-regulated, whereas the SIRT1 activator SRT1720 down-regulated p53 and p65/RELA acetylation. However, these results were in conflict with the effects on the growth of T-ALL cells: EX527 promoted and SRT1720 suppressed cell proliferation despite the increase and decrease in acetylated (activated) p53, respectively, which was observed in DND-41 cells (KOPT-K1 cells are p53-deficient). One explanation is that p53 may have a weak biological activity in DND-41 cells, which consequently would not be affected by SIRT1-dependent p53 deacetylation (11).

We also observed that SIRT1 activation down-regulated NOTCH signaling and its downstream target MYC. Considering that NOTCH activation is a critical event for leukemogenesis (6) and that NOTCH-dependent MYC contributes to the growth of T-ALL cells (13), we propose that SIRT1 inhibits T-ALL proliferation through regulation of these vital oncogenic pathway, just like our previous report showing that *BMI1*-knockdown inhibits T-ALL proliferation through NOTCH and MYC suppression (14). This hypothesis is consistent with reports that SIRT1 acts as an intrinsic negative modulator of NOTCH signaling in endothelial cells (15) and that it regulates cell growth through deacetylation and inhibition of MYC (16).

NF-κB signaling is another important pathway in T-ALL cells and it was shown that SIRT1 could physically interact with and deacetylate the NF-κB subunit p65/RELA at lysine 310, leading to transcriptional inhibition (17). Our results indicated that SIRT1 activation resulted in p65/RELA deacetylation, suggesting that the inhibition of NF-κB activity can augment the anticancer effect of SIRT1.

Furthermore, we observed that SIRT1 activation suppressed AKT/mTOR signaling, which is consistent with a previous report that SIRT1 negatively regulates mTOR (18). Our previous study has revealed that *NOTCH1* knockdown did not reduce AKT/mTOR activity in DND-41 and KOPT-K1 cells (19), suggesting that the AKT/mTOR inhibition caused by SIRT1 activation is not due to decreased NOTCH signaling. It is known that mTORC1 inhibition induces autophagy (20). We showed that SIRT1 activation decreased p62/SQSTM1 and increased the LC3-II/LC3-I ratio, indicating the induction of autophagy in T-ALL cells, which can be associated with AKT/mTOR down-regulation by SIRT1.

In conclusion, our results indicate that activated SIRT1 targets several key oncogenic pathways such as NOTCH, NF-κB, and mTOR. In this scenario, the impact of p53 deacetylation on cancer cell survival might be of lower biological importance (21). Thus, the suppression of T-ALL cell growth by SIRT1 may be due to the down-regulation of NOTCH, NF-κB, and mTOR signaling (Figure 7), which overcomes the pro-survival effects of p53 inhibition.

Our study had some limitations. First, we could not obtain wild-type *NOTCH1*-expressing T-ALL cell lines and, therefore, did not compare T-ALL cells with and without *NOTCH1* mutations to determine whether SIRT1-induced growth suppression was due to mutated *NOTCH1* or was a general characteristic of T-lymphoblasts. Second, we analyzed only p65/RELA as an indicator of NF-κB activity, which may not be enough; other NF-κB-related molecules should be evaluated to confirm our results. Third, we did not perform experiments with samples from T-ALL patients carrying *NOTCH1* mutations and our findings should be verified for correspondence to the clinical situation.

Nevertheless, this study provides evidence that SIRT1 activators such as SRT1720 may have a potential to be novel SIRT1-targeting drugs for *NOTCH1*-mutated T-ALLs.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

SMO and ST designed the study. SMO and MI performed the cell culture and immunoblotting analysis. SMO, MI, and ST wrote the article.

Acknowledgements

The Authors thank Mr. Ryo Sunohara for the experimental support. This study was funded in part by JSPS KAKENHI (Grant Number C: 26460669).

References

1 Yuan H, Su L and Chen WY: The emerging and diverse roles of sirtuins in cancer: a clinical perspective. OncoTargets Ther *6*: 1399-1416, 2013. PMID: 24133372. DOI: 10.2147/OTT.S37750

2 Bonkowski MS and Sinclair DA: Slowing ageing by design: the rise of NAD+ and sirtuin-activating compounds. Nat Rev Mol Cell Biol *17*: 679-690, 2016. PMID: 27552971. DOI: 10.1038/nrm.2016.93

3 Chalkiadaki A and Guarente L: The multifaceted functions of sirtuins in cancer. Nat Rev Cancer *10*: 608-624, 2015. PMID: 26383140. DOI: 10.1038/nrc3985

4 Sasca D, Hähnel PS, Szybinski J, Khawaja K, Kriege O, Pante SV, Bullinger L, Strand S, Strand D, Theobald M and Kindler T: SIRT1 prevents genotoxic stress-induced p53 activation in acute myeloid leukemia. Blood *124*: 121-133, 2014. PMID: 24855208. DOI: 10.1182/blood-2013-11-538819

5 Chauhan D, Bandi M, Singh AV, Ray A, Raje N, Richardson P and Anderson KC: Preclinical evaluation of a novel SIRT1 modulator SRT1720 in multiple myeloma cells. Br J Haematol *155*: 588-598, 2011. PMID: 21950728. DOI: 10.1111/j. 1365-2141.2011. 08888

6 Weng AP, Ferrando AA, Lee W, Morris JP 4th, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT and Aster JC: Activating mutations of *NOTCH1* in human T cell acute lymphoblastic leukemia. Science *306*: 269-271, 2004. PMID: 15472075. DOI: 10.1126/science.1102160

7 Marcel N, Perumalsamy LR, Shukla SK and Sarin A: The lysine deacetylase Sirtuin 1 modulates the localization and function of the Notch1 receptor in regulatory T cells. Sci Signal *10*: 473, 2017. PMID: 28377411. DOI: 10.1126/scisignal.aah4679

8 Gertz M, Fischer F, Nguyen GT, Lakshminarasimhan M, Schutkowski M, Weyand M and Steegborn C: EX-527 inhibits sirtuins by exploiting their unique NAD+-dependent deacetylation mechanism. Proc Natl Acad Sci USA *110*: 2772-2781, 2013. PMID: 23840057. DOI: 10.1073/pnass.1303628110

9 Milne JC, Lambert PD, Schenk S, Carney DP, Smith JJ, Gagne DJ, Jin L, Boss O, Perni RB, Vu CB, Bemis JE, Xie R, Disch JS, Ng PY, Nunes JJ, Lynch AV, Yang H, Galonek H, Israelian K, Choy W, Iffland A, Lavu S, Medvedik O, Sinclair DA, Olefsky JM, Jirousek MR, Elliott PJ and Westphal CH: Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature *450*: 712-716, 2007. PMID: 18046409. DOI: 10.1038/nature06261

10 Mizushima N and Yoshimori T: How to interpret LC3 immunoblotting. Autophagy *3*: 542-545, 2007. PMID: 17611390. DOI: 10.4161/auto.4600

11 Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L and Weinberg RA: hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell *107*: 149-159, 2001. PMID: 11672523. DOI: 10.1016/s0092-8674(01)00527-x

12 Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA and Mayo MW: Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J *23*: 2369-2380, 2004. PMID: 15152190. DOI: 10.1038 /sj.emboj. 7600244

13 Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C, Del Bianco C, Rodriguez CG, Sai H, Tobias J, Li Y, Wolfe MS, Shachaf C, Felsher D, Blacklow SC, Pear WS and Aster JC: c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. Genes Dev *20*: 2096-2109, 2006. PMID: 16847353. DOI: 10.1101/gad.1450406

14 Ohtaka M, Itoh M and Tohda S: BMI1 inhibitors down-regulate NOTCH signaling and suppress proliferation of acute leukemia cells. Anticancer Res *37*: 6047-6053, 2017. PMID: 29061784.

15 Guarani V, Deflorian G, Franco CA, Krüger M, Phng LK, Bentley K, Toussaint L, Dequiedt F, Mostoslavsky R, Schmidt MHH, Zimmermann B, Brandes RP, Mione M, Westphal CH, Braun T, Zeiher AM, Gerhardt H, Dimmeler S and Potente M: Acetylation-dependent regulation of endothelial Notch signaling by the SIRT1 deacetylase. Nature *473*: 234-238, 2011. PMID: 21499261. DOI: 10.1038/nature 09917

16 Yuan J, Minter-Dykhouse K and Lou Z: A c-Myc-SIRT1 feedback loop regulates cell growth and transformation. J Cell Biol *185*: 203-211, 2009. PMID: 19364925. DOI: 10.1083/jcb.200809167

17 Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA and Mayo MW: Modulation of NF-κB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J *23*: 2369-2380, 2004. PMID: 15152190. DOI: 10.1038/sj.emboj. 7600244

18 Ghosh HS, McBurney M and Robbins PD: SIRT1 negatively regulates the mammalian target of rapamycin. PLoS One *5*: e9199, 2010. PMID: 20169165. DOI: 10.1371/journal.pone.0009199

19 Okuhashi Y, Itoh M, Nara N and Tohda S: *NOTCH* knockdown affects the proliferation and mTOR signaling of leukemia cells. Anticancer Res *33*: 4293-4298, 2013. PMID: 24122995.

20 Paquette M, El-Houjeiri L and Pause A: mTOR pathways in cancer and autophagy. Cancers (Basel) *10*: E18, 2018. PMID: 29329237. DOI: 10.3390/cancers10010018

21 Solomon JM, Pasupuleti R, Xu L, McDonagh T, Curtis R, DiStefano PS and Huber LJ: Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. Mol Cell Biol *26*: 28-38, 2006. PMID: 16354677. DOI: 10.1128/MCB.26.1.28-38.2006

Received April 7, 2020
Revised April 18, 2020
Accepted April 20, 2020