Research Article   **Open Access**

Effect of Lignin Glycosides Extracted from Pine Cones on the Human *SIRT1* Promoter

Fumiaki Uchiumi[1,2], Haruki Tachibana[3], Steven Larsen[2] and Sei-ichi Tanuma[2,3,4,5]*

[1]*Department of Gene Regulation, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda-shi, Chiba-ken, Japan*
[2]*Research Center for RNA Science, RIST, Tokyo University of Science, Noda-shi, Chiba-ken, Japan*
[3]*Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Noda-shi, Chiba-ken, Japan*
[4]*Genome and Drug Research Center, Tokyo University of Science, Noda-shi, Chiba 278-0022, Japan*
[5]*Drug Creation Frontier Research Center, RIST, Tokyo University of Science, Noda-shi, Chiba 278-0022, Japan*

Abstract

Sirtuins (SIRT1-7) and telomerase (TERT) are known to be involved in the regulation of cellular senescence/aging processes. Resveratrol (Rsv), a natural polyphenol, which has been shown to extend the lifespan of diverse range of species. Furthermore, Rsv has been shown to activate *SIRT1*, the class III NAD$^+$ dependent histone de-acetylases (HDACs), directly in mammalian cells. Here, we examined the changes in promoter activities of the human *SIRT1* and *TERT* genes after treatment with Rsv and lignin glycosidases/lignin carbohydrate complexes (LCC) extracted from pine cones of *Pinus Karalensis*. We constructed Luciferase (Luc) expression vectors, pGL4-SIRT1 and pGL4-TERT, containing 396-bp and 263-bp of the 5'-upstream regions of human *SIRT1* and *TERT* genes, respectively, which were then transfected into HeLa S3 cells. The Luc reporter plasmid assay revealed that treatment with the LCC increases not only *SIRT1* but also *TERT* promoter activities more than that with Rsv. These results suggest that the LCC from pine cones induces expression of both *SIRT1* and *TERT* genes, which is thought to have anti-aging effects.

Keywords: Aging; Cellular senescence; Gene regulation; Lignin glycosidases; Lignin carbohydrate complexs; Resveratrol; *SIRT1*; *TERT*

Introduction

It has been suggested that both cellular senescence and aging of organisms are accelerated by various factors, such as telomere-shortening [1-3] and DNA damaging reactive oxygen species (ROS) that are mainly generated from mitochondria [4,5]. From genetic analyses, it has been shown that several genes, including daf-2 and daf-16, which encode insulin/IGF1 receptor and transcription factor FoxO, respectively, play important roles in controlling the lifespan of *C. elegans* [6]. Studies of budding yeast showed that Sir2, a member of sirtuins having an NAD$^+$-dependent protein deacetylase, has silencing action on chronological aging of yeast cells [7].

Recently, a natural polyphenolic compound Resveratrol (Rsv), which has a stimulating effect on sirtuin enzyme activity, has been shown to increase the lifespan of model animals [8-12]. Previously, we reported that *trans*-Resveratrol (Rsv) activates the human *TERT* promoter thus moderately inducing telomerase activity in HeLa-S3 cells [13]. Moreover, multiple transfection assays showed that promoter activation of the human shelterin protein encoding genes are up-regulated by Rsv treatment [14], suggesting that the natural poly-phenol beneficially affects chromosomal stability by trans-activation of telomere maintenance factor encoding genes. Thus, Rsv and its related poly-phenols have been expected to be one of the candidate drugs for anti-aging therapeutics. However, it should be noted that Rsv has cytotoxic effects inducing cell death by apoptosis especially when it is used at high doses [15-17]. Therefore, in order to develop safe and effective anti-aging drugs, alternative natural compounds that induce *SIRT1* and *TERT* gene expression should be investigated.

We previously showed that lignins and tannins, which are potential inhibitors of poly (ADP-ribose) glycohydrolase (PARG) [18], have many biological effects as antioxydants, antiviral, anticancer, and immuno-potentiating reagents [19,20]. Furthermore, tannic

acid suppressed TPA-induction of HIV promoter activity in Jurkat cells [21,22], suggesting that these natural compounds may affect transcription of various genes. In this study, we examined the effects of lignin carbohydrate complexes (LCC) extracted from pine cones of *Pinus Karakensis* on the promoter activities of the human *TERT* and *SIRT1* genes by transient transfection and Luc reporter assay. Here, we describe the up-regulating effect of the LCC on these two promoter activities, suggesting that it is an attractive candidate for anti-aging drugs.

Materials and Methods

Preparation of LCC from *Pinus Karalensis* pine cones

Our preliminary experiments demonstrated that none of the active material from pine cones of *Pinus karalensis* was ethanol soluble, all of the following extractions were performed with extensive alcohol washing and acetone drying to remove fatty materials [19,20]. Pine cones thus prepared were extracted with 0.3 N NaOH over night. The pine cone extracts were neutralized by 2 N HCl to pH 5.5, and then an equal volume of ethanol was added. The ethanol precipitates were collected and dialyzed against water. The dialyzates were lyophilized and purified by Sepharose LC-4B and Toyopal HW 40-F column chromatographies. The fractions were evaluated by pGL4-SIRT1

reporter plasmid transfection and Luciferase (Luc) assay, and *SIRT1* promoter activating fractions were collected. The partial structures of the purified LCC were analyzed by NMR (Figure 1). The molecular weight of the LCC is 8,000 to 10,000 containing uronic acid (10 to 20% of total sugar residues) and neutral sugars, including glucose, galactose, mannose, and arabinose (80 to 90% of total sugar residues).

Cell culture

Human cervical carcinoma (HeLa S3) cells were grown in Dulbecco's modified Eagle's (DME) medium (Nacarai, Tokyo, Japan), supplemented with 10% fetal bovine serum (FBS) (Sanko-Pure Chemical, Tokyo, Japan) and penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2.

Construction of Luc reporter plasmids

The Luc reporter plasmids carrying the human *TERT* promoter region was designated as described previously and named pGL4-TERT [13,23]. The 5'-flanking region of the human *SIRT1* gene was obtained by PCR with Prime Star Taq polymerase (Takara, Kyoto, Japan) and genomic DNA from HeLa S3 cells as a template. The sense and antisense primers used for PCR are hSIRT1-8570; 5'-TCGG-TACCAAAACTTGAGCTGTTCCGGCGG-3', And AhSIRT1-8965; 5'-ATCTCGAGGGCCGCCTCGTCCGCCATCTTC-3', respectively. PCR products were digested with *Kpn I* and *Xho I* and then ligated into the multi-cloning site of the pGL4.10 [luc2] vector (Promega, Madison, WI), as described previously [13,23,24]. The resultant plasmid, containing 396-bp of 5'-upstream region of the human *SIRT1* gene, was named pGL4-SIRT1.

Transient transfection and luc assay

Plasmid DNAs were transfected into HeLa S3 cells by the DEAE-dextran method [22,23]. The DNA-transfected cells were divided into at least four dishes. After 24 h of transfection, pine cone LCC, *trans*-Resveratrol (Cayman Chem., Ann Arbor, MI) or other natural compounds were added to the culture medium. After a further 24 h of incubation, cells were collected and lysed with 100 μL of 1 X cell culture lysis reagent, containing 25 mM Tris-phospate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, and 1% Triton X-100, then mixed and centrifuged at 12,000 x g for 5 sec. The supernatant was stored at – 80°C. The Luc assay was performed with a Luciferase assay system (Promega) and relative Luc activities were calculated as described previously [22,23].

Results

Isolation and characterization of the 5'-flanking region of the human *SIRT1* gene

The nucleotide sequence contained in the pGL4-SIRT1 revealed that the 0.4-kb region is identical to that of GenBank Accession No. NT_030059.13 (nucleotide No. 20448570 to 20448964) and the presence of the sequence containing the most 5'-upstream end of the cDNA (Accession No. NM_012238.4: GENE ID. 23411SIRT1). TF-SEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html) was executed for the 0.4-kb region and characteristic recognition sequences of several known transcription factors were found (Figure 2). Although, no sequence similar to a TATA or CCAAT box was found, putative binding sites for CREB (-288 to -281), GATA-1/2/3 (-72 to -63, -55 to -46, +50 to +59), c-Myc (-59 to -48), C/EBPβ (-270 to –256), USF/SREBP (-141 to –127, -59 to -48), Sp1 (-83 to -75, -65 to -57) and c-Ets (-182 to -172) are located in the 0.4-kb region (Figure 2).

Effect of LCC on the *SIRT1* promoter

To examine whether human *SIRT1* promoter is affected by natural compounds, including LCC obtained from pine cone extracts (Figure 1), transient transfection and Luc assay were carried out. Luc activities of pGL4-SIRT1 transfected cells were normalized to that of non-treated control cells. As shown in Figure 3A left panel, relative Luc activity of the pGL4-SIRT1-transfected cells was prominently augmented by the addition of Rsv (10 μM) or LCC (100 μg/ml) to the culture medium. In contrast, 100 μM of Quercetin, which is a natural polyphenolic flavonoid [25], moderately up-regulated the *SIRT1* promoter activity (Figure 3A, left).

To examine the dose-dependent response of LCC, HeLa S3 cells were treated with 0 to 100 μg/mL of LCC after 24 h of transfection and collected after further 24 h incubation (Figure 3B, open circle). The results indicated that 10 μg/ml of LCC is enough to induce *SIRT1* promoter activity equal to Rsv (10 μM) treatment. The half maximal effective concentration (EC_{50}) was calculated to be 18 μg/ml. The endotoxin concentration in the LCC was estimated to be 15.7 EU/mg, suggesting that it contains LPS only at low levels.

Effect of LCC on the *TERT* promoter

Similar experiments with pGL4-TERT plasmid were performed (Figure 3A, right). The results indicated that the 263-bp of the human *TERT* promoter region responded to Quercetin (100 μM), Rsv (10 μM), and LCC (100 μg/ml) in a similar manner as the 396-bp of the *SIRT1* promoter (Figure 3A, left). In addition, the LCC-dose response of the *TERT* promoter was almost the same as that of the *SIRT1* promoter (Figure 3B).

Discussion

In this study, we have isolated a 5'-flanking region of the human *SIRT1* gene, and observed that Luc activity under the control of this 396-bp region showed greater response to the treatment with LCC
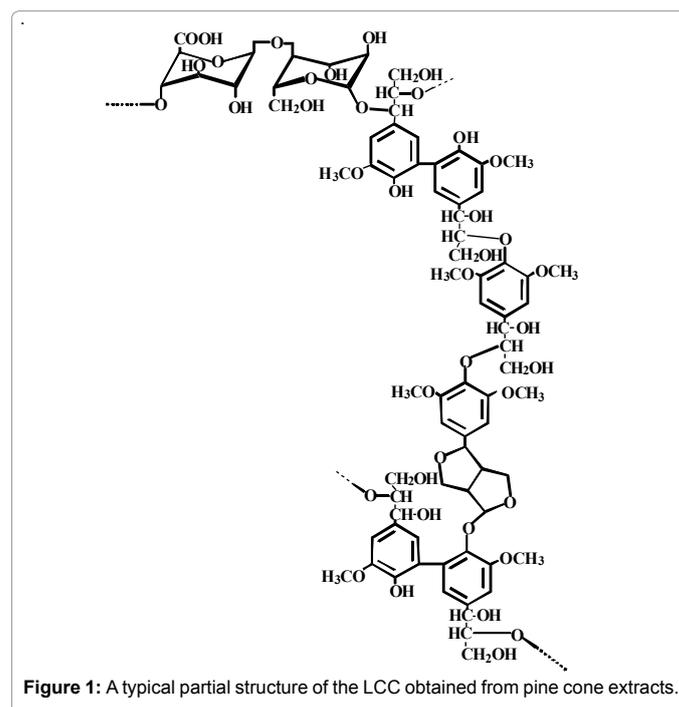
Figure 1: A typical partial structure of the LCC obtained from pine cone extracts.

than that with Rsv or Quercetin in HeLa S3 cells. This region has no apparent TATA-box but contains several well-known transcription factor binding sequences, including CREB, C/EBPβ, c-ETS, USF, SREBP1, Sp1, GATA, and c-MYC binding elements (Figure 2). At present, the LCC- and Rsv-responsive elements in the *SIRT1* promoter region have not been precisely determined. However, the 5'-upstream regions of the *WRN*, *BLM*, *TERT*, *p21* (*CDKN1A*), and *HELB* genes, in which one or more Sp1/GC-box elements are commonly located, positively respond to the Rsv treatment in HeLa S3 cells [13,26]. The GC-box consensus sequence of the Sp1 transcription factor binding site is: 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3' or 5'-(G/T)(G/A)GGCG(G/T)(G/A)(G/A)(C/T)-3' [27]. It has been shown that two GC-boxes, 5'-AGGGCGGGGG-3' and 5'GGGGCGGGTC -3' (Figure 2, -83 to -74 and -66 to -57, respectively), play important roles in the *SIRT1* promoter activity [28]. Therefore, the tandem repeat of the Sp1 binding motif is possibly one of the candidate elements that respond to the LCC treatment. In addition, Rsv up-regulates cAMP level and CREB is known to be regulated by cAMP, and plays important roles in hormonal metabolisms, including that of the insulin signaling system [29]. These observations suggest that the CREB element located in the human *SIRT1* promoter region (-288 to -281) may be also involved in the response to the LCC and Rsv treatments.

Rsv has been shown to affect essential signal transduction factors,
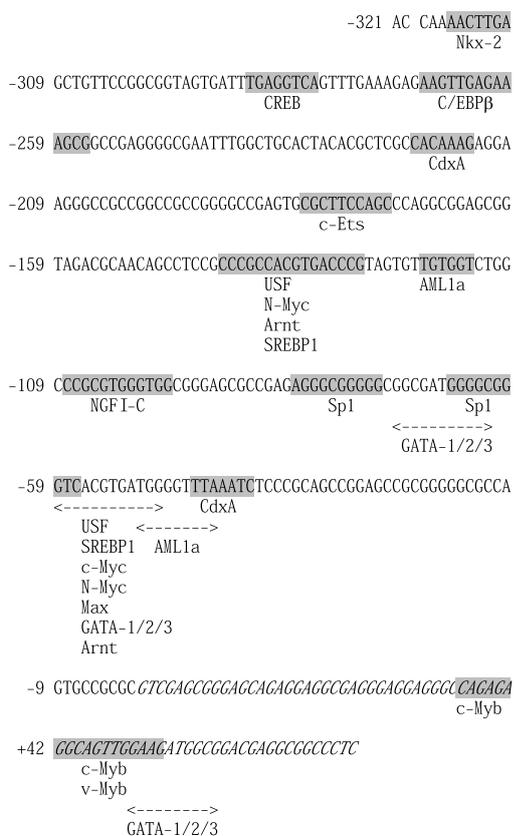
Figure 2: Nucleotide sequence of the 5'-flanking region of the human *SIRT1* gene. The nucleotide sequence of the 0.4-kb fragment obtained by PCR is shown. The most upstream of the cDNA (NM_012238.41) is designated nucleotide +1. Putative transcription factor-binding sites (TFSEARCH score>85) are shaded or indicated by dotted arrows. Bold italic characters represent the cDNA sequence of the *SIRT1* gene (Gene ID: 23411).
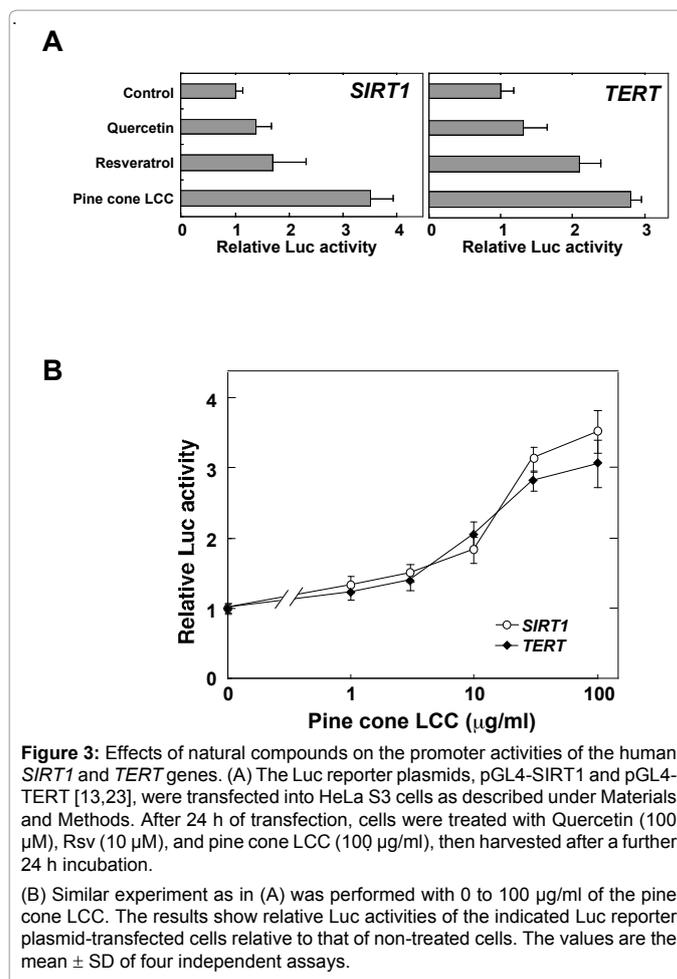
Figure 3: Effects of natural compounds on the promoter activities of the human *SIRT1* and *TERT* genes. (A) The Luc reporter plasmids, pGL4-SIRT1 and pGL4-TERT [13,23], were transfected into HeLa S3 cells as described under Materials and Methods. After 24 h of transfection, cells were treated with Quercetin (100 µM), Rsv (10 µM), and pine cone LCC (100 µg/ml), then harvested after a further 24 h incubation.

(B) Similar experiment as in (A) was performed with 0 to 100 µg/ml of the pine cone LCC. The results show relative Luc activities of the indicated Luc reporter plasmid-transfected cells relative to that of non-treated cells. The values are the mean ± SD of four independent assays.

including AMPK [30,31] and FoxO [32], directly regulating the de-acetylase activity of SIRT1. Other polyphenols, such as Quercetin and Catenins have been reported to moderately activate mammalian SIRT1 or yeast Sir2 enzyme activity [10,25,33]. However, they have no such effect in cellular system [25,33], rather they inhibit SIRT1 activity [33]. Therefore, regulation of lifespan and longevity of organisms might not only be controlled by SIRT1 enzyme activity but also by other factors involved in signal pathways between Rsv and SIRT1. Recently, it was suggested that Rsv has a hormetic effect on cells or organisms, that is to cause a biphasic dose response of various biological events [34]. Here, for the first time, we found the effective up-regulation of the *SIRT1* and *TERT* gene promoter activities by the LCC treatment, suggesting that regulation of some transcription factors, including Sp1, responsible for the LCC may play important roles in control for life spans. Furthermore, the core structures of LCC might be useful to generate lead compounds for novel anti-aging drugs, which could simultaneously activate the *SIRT1* and *TERT* promoter activities.

Acknowledgments

References

1. O'Sullivan RJ, Karlseder J (2010) Telomeres: protecting chromosomes against genome instability. Nat Rev Mol Cell Biol 11: 171-181.

2. Blackburn E (2006) Chapter 1: A history of telomere biology. In: Telomeres (2nd edn), (de Lang T, Lundblad V, Blackburn E, Eds.), Cold Spring Harbor Laboratory Press, NY: 1-19.

3. Vijg J (2007) Chapter 5: Genome instability and accelerated aging. In: Aging of the Genome: The Dual Role of DNA in Life and Death, Oxford University Press, NY: 151-180.

4. Robb EL, Page MM, Stuart JA (2009) Mitochondria, cellular stress resistance, somatic cell depletion and lifespan. Curr Aging Sci 2: 12-27.

5. Benz CC, Yau C (2008) Ageing, oxidative stress and cancer: paradigms in parallax. Nat Rev Cancer 8: 875-879.

6. Kenyon CJ (2010) The genetics of aging. Nature 464: 504-512.

7. Kaeberlein M (2010) Lessons on longevity from budding yeast. Nature 464: 513-519.

8. Stefani M, Markus MA, Lin RC, Pinese M, Dawes IW, et al. (2007) The effect of resveratrol on a cell model of human aging. Ann NY Acad Sci 1114: 407-418.

9. Kaeberlein M (2010) Resveratrol and rapamycin: are they anti-aging drugs? Bioessays 32: 96-99.

10. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, et al. (2003) Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* life span. Nature 425: 191-196.

11. Wood JG, Rogina B, Lavul S, Howitz K, Helfand SL, et al. (2004) Sirtuin activators mimic caloric restriction and delay aging in metazoans. Nature 430: 686-689.

12. Viswanathan M, Kim SK, Berdichevsky A, Guarente LA (2005) A role for SIR-2.1 regulation of ER stress response genes in determining C. elegans life span. Dev Cell 9: 605-615.

13. Uchiumi F, Watanabe T, Hasegawa S, Hoshi T, Higami Y, et al. (2011) The effect of resveratrol on the Werner Syndrome RecQ helicase gene and telomerase activity. Curr Aging Sci 4: 1-7.

14. Uchiumi F, Oyama T, Ozaki K, Tanuma S (2011) Chapter 29: Characterization of 5'-flanking regions of various human telomere maintenance factor-encoding genes. In: DNA Repair (Kruman I, Ed.), InTech-Open Access Publisher, Inc, Rijeka, Croatia: 585-596.

15. Calabrese EJ, Mattson MP, Calabrase V (2010) Resveratrol commonly displays hormesis: Occurrence and biomedical significance. Hum Exp Toxicol 29: 980-1015.

16. Gogada R, Prabhu V, Amadori M, Scott R, Hashmi S, et al. (2011) Resveratrol induces p53-independent, X-linked inhibitor of apoptosis protein (XIAP)-mediated Bax protein oligomerization on mitochondria to initiate cytochrome c release and caspase activation. J Biol Chem 286: 28749-28760.

17. Lin HY, Tang HY, Davis FB, Davis PJ (2011) Resveratrol and apoptosis. Ann NY Acad Sci 1215: 79-88.

18. Tanuma S, Tsai YJ, Sakagami H, Konno K, Endo H (1989) Lignin inhibits (ADP-ribose)n glycohydrolase activity. Biochem Int 19: 1395-1402.

19. Sakagami H, Kawazoe Y, Komatsu N, Simpson A, Nonoyama M, et al. (1991) Antitumor, antiviral and immunopotentiating activities of pine cone extracts: potential medicinal efficacy of natural and synthetic lignin-related materials. Anticancer Res 11: 881-888.

20. Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals Botany 91: 179-194.

21. Uchiumi F, Maruta H, Inoue J, Yamamoto T, Tanuma S (1996) Inhibitory effect of tannic acid on human immunodeficiency virus promoter activity induced by 12-O-tetra decanoyl phorbol-13-acetate in Jurkat T-cells. Biochem Biophys Res Commun 220: 411-417.

22. Uchiumi F, Hatano T, Ito H, Yoshida T, Tanuma S (2003) Transcriptional suppression of the HIV promoter by natural compounds. Antiviral Res 58: 89-98.

23. Zhou B, Ikejima T, Watanabe T, Iwakoshi K, Idei Y, et al. (2009) The effect of 2-deoxy-D-glucose on Werner syndrome RecQ helicase gene. FEBS Lett 583: 1331-1336.

24. Uchiumi F, Watanabe T, Tanuma S. (2010) Characterization of various promoter regions of human DNA helicase-encoding genes and identification of duplicated ets (GGAA) motifs as an essential transcription regulatory element. Exp Cell Res 316: 1523-1534.

25. Chung S, Yao H, Caito S, Hwang J-W, Arunachalam G, et al. (2010) Regulation of SIRT1 in cellular functions: role of polyphenols. Arch Biochem Biophys 501: 79-90.

26. Uchiumi F, Higami Y, Tanuma S (2010) Chapter 5: Regulations of telomerase activity and *WRN* gene expression. In: Telomerase: Composition, Functions and Clinical Implications (Gagnon AN, Ed.), Nova Science Publishers, Inc, Hauppauge, NY: 95-103.

27. Wieratra I (2008) Sp1: Emerging roles-Beyond constitutive activation of TATA-less housekeeping genes. Biochem Biophys Res Commun 372: 1-13.

28. Okazaki M, Iwasaki Y, Nishiyama M, Taguchi T, Tsugita M, et al. (2010) PPARbeta/delta regulates the human SIRT1 gene transcription via Sp1. Endocrine J 57: 403-413.

29. Altarejos JY, Montminy M (2011) CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol 12: 141-151.

30. Baile CA, Yang J-Y, Rayalam S, Hartzell DL, Lai CY, et al. (2011) Effect of resveratrol on fat mobilization. Ann NY Acad Sci 1215: 40-47.

31. Canto C, Auwerx J (2010) AMP-activated protein kinase and its downstream transcriptional pathways. Cell Mol Life Sci 67: 3407-3423.

32. Stefani M, Markus MA, Lin RCY, Pinese M, Dawes IW, et al. (2007) The effect of resveratrol on a cell model of human aging. Ann NY Acad Sci 1114: 407-418.

33. de Boer VC, de Goffau MC, Arts IC, Hollman PC, Keijer J (2006) SIRT1 stimulation by polyphenols is affected by their stability and metabolism. Mech Aging Dev 127: 618-627.

34. Calabrese E, Mattson MP, Calabrese V (2010) Resveratrol commonly displays hormesis: Occurrence and biomedical significance. Hum Exp Toxicol 29: 980-1015.