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ORIGINAL ARTICLE

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Effects of histone deacetylase inhibitor FR901228 on the expression level of telomerase reverse transcriptase in oral cancer

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Abstract We speculated whether or not the expression level of telomerase reverse transcriptase (hTERT) would be modulated by agents targeting epigenetics in oral cancer cell lines. Although hTERT is known to be targeted by epigenetic changes, it remains unclear how chemoagents targeting epigenetics work on hTERT transcription. In the present study, the epigenetic effects of the histone deacetylase (HDAC) inhibitor FR901228 on hTERT transcription in oral cancer cell lines were analyzed by RT-PCR. The mRNA expression of hTERT was upregulated after exposure to FR901228 in hTERT-negative Hep2 cells, and even SAS and KB cells expressed high levels of hTERT. Moreover, cotreatment of protein synthesis inhibitor cycloheximide (CHX) resulted in the induction of hTERT transcription by FR901228. This suggests that the induction of hTERT by FR901228 requires de novo protein synthesis to some extent and is more likely a direct than an indirect effect on epigenetic changes such as histone acetylation/deacetylation. We further examined the effect of FR901228 on c-myc protein, which is one of the main hTERT transcription activators. FR901228 repressed c-myc protein only in the absence of CHX, and depended on the enhancement of de novo protein synthesis. Our results indicate that c-myc protein is repressed indirectly by FR901228 but may not contribute to FR901228-induced hTERT transcription. The present study showed that the HDAC inhibitor FR901228 induced the hTERT gene by a complex mechanism that involved transcription factors other than c-myc, in addition to inhibition of histone deacetylation.

Keywords hTERT · FR901228 · Oral cancer · HDAC inhibitor

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Introduction

Telomerase is an enzyme involved in the de novo synthesis of GGTTAG telomeric DNA onto the ends of chromosomes ends to stabilize the telomeres, resulting in immortality of cancer cells [3, 15, 25]. Telomerase is composed of telomerase reverse transcriptase (hTERT)

[27], telomerase-associated protein (TEP1) [9], and hnRNP A1 [21]. The de novo transcription of the hTERT gene is considered the dominant rate-limiting step in telomerase activation, whereas the expression of hTEP1 and hTR is constitutive [3, 9]. The regulation of hTERT expression is complicated, and it has been reported that hTERT transcription can be activated by overexpression or activation of several transcription activators [8, 17, 20, 24, 34, 36]. Several recent studies have identified c-myc binding sites (E-boxes) in the hTERT promoter and have shown that c-myc positively regulates hTERT expression [12, 30]. The hTERT gene is likely to be regulated epigenetically for example by histone acetylation or promoter methylation. Some studies have shown that the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) can activate hTERT expression in telomerase-negative cells [5, 7, 31, 37]. Hou et al. [13] have reported that TSA and the protein synthesis inhibitor cycloheximide (CHX) induce hTERT mRNA, suggesting that this induction does not require de novo protein synthesis and is likely a direct result of HDAC inhibition at the hTERT gene.

Although it is reasonable to speculate that epigenetic changes such as histone acetylation or promoter methylation are common underlying features in hTERT transcriptional regulation, it remains unclear how chemoagents targeting epigenetics such as HDAC inhibitors work in hTERT mRNA expression. FR901288 is a novel cyclic peptide inhibitor of HDAC isolated from a fermentation broth of *Chromobacterium violaceum* (Fujisawa Pharmaceutical Company, Osaka, Japan), and is currently in phase I clinical trials. FR901228 has a stronger cytotoxic activity than TSA, and is known to be able to increase or decrease the transcriptional levels of several genes following hyperacetylation of histone [26].

In the present study, we focused on the HDAC inhibitor FR901228 and evaluated the effects of FR901228 on hTERT expression and its transcription activator c-myc in oral cancer cells.

Materials and methods

Cell lines and culture

Human oral cancer cell lines (HSC4, HSC3, HSC2, KB, SAS, Hep2 and HO-1-u-1) were obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical Company, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Utah), 100 U/ml penicillin (Meiji Seika, Tokyo, Japan) and 100 μg/ml streptomycin (Meiji Seika) in a CO_2 incubator (Sanyo Electric Company, Osaka, Japan) with an atmosphere comprising 95% air and 5% CO_2 at 37°C.

Chemicals

FR901228 was a gift from Fujisawa Pharmaceutical Company (Osaka, Japan). FR901288, 5-aza-2-deoxycytidine (5-Aza) (Sigma Chemical Company, St. Louis, Mo.) and CHX (Wako Pure Chemical Industries, Osaka, Japan) were diluted in water and added to DMEM to the final concentrations indicated for each treatment.

FR901228 treatment

First, 1×10^6 cells were seeded in 5 ml DMEM in a flask (cat no. 152094; Nalge Nunc International, Roskilde, Denmark). Then, 24 h after seeding, the medium was changed to one containing FR901228 at final concentrations of 0.5 or 1.0 μ*M* and the flask was immersed in a water bath at 37°C (Taitec Company, Saitama, Japan). Cells were also incubated with FR901228 in the presence of 200 μg/ml of CHX, a potent protein synthesis inhibitor. The CHX was added to cells 30 min before the addition of 0.5 μ*M* FR901228. Cells were harvested at the indicated times (4 or 16 h) following FR901228 treatment.

5-Aza-2-deoxycytidine treatment

Stock solutions of 5-Aza were prepared by dissolving the drug at a concentration of 10 m*M* in distilled water no more than 2 h prior to use in an experiment; final treatment concentrations were obtained by diluting the stock solution directly into the tissue culture medium. Cells were incubated with 5-Aza at 4.4 and 8.8 μ*M* for 7 days.

RNA isolation and RT-PCR

Following incubation of the cells with agents under each experimental condition, total cellular RNA was extracted using Trizol reagent (Invitrogen Corporation, Carlsbad, Calif.) according to the manufacturer's instructions. RNA (1.5 μg) was reverse-transcribed with Superscript II reverse transcriptase and oligo dT primers (Invitrogen). cDNAs were amplified under the following PCR conditions: 7 min at 94°C for 1 cycle; then 26 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s; and a final elongation step at 72°C for 10 min. The primers used for the amplification were as follows:

hTERT primers 1

sense 5′-cgg aag agt gtc tgg agc aa-3′

antisense 5′-gga tga agc gga gtc tgg a-3′

hTERT primers 1 amplified a 145 bp product [18, 27].

hTERT primers 2

sense 5′-act ttg tca agg tgg atg tga cgg-3′ (exon 6)

antisense 5′-aag aaa tca tcc acc aaa cgc agg-3′ (exon 10)

hTERT primers 2 amplified a 493 bp product spanning exon 6 to exon 10 [6, 16, 28].

c-myc
sense 5′-aagtcctgcgcctcgcaa-3′
antisense 5′-gctgtggcctccagcaga-3′

GAPDH
sense 5′-gaaggtgaaggtcggagtc-3′
antisense 5′-caaagttgtcatggatgacc-3′

The amplified GAPDH fragment was used as a positive control.

The RT-PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and viewed by UV.

Western blotting

Following incubation of the cells with FR901228, total cellular protein was extracted using 500 µl lysis buffer (1.315 m*M* sucrose, 0.3475 m*M* sodium dodecyl sulfate, 31.25 µl 1 *M* Tris, pH 6.8, 0.125 mg bromophenol blue powder and 25 µl 2-mercaptoethanol, diluted to 500 µl with distilled water). Protein in cell-free extract was loaded at 5 µl per lane and was separated by 10% SDS-PAGE, and the individual proteins transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, Calif.) using a semidry electrophoretic transfer apparatus (LKB-Produkter, Bromma, Sweden) at room temperature. The blotted membranes were blocked for 1 h in TBS-T (containing 0.1% Tween 20) plus 5% powdered skimmed milk and then probed for 2 h with mouse anti-c-myc monoclonal antibody c-myc Ab-5 (clone 67P05) (Neomarkers, Fremont, Calif.) diluted 1:1000 (2 µg/ml) in TBS-T. The membranes were then washed three times in TBS buffer and incubated for 1 h with the appropriate secondary antibody horseradish peroxidase-conjugated AffiniPure goat anti-mouse IgG (H + L) (ImmunoResearch Laboratories, West Grove, Pa.) in TBS-T. Bound antibody was detected using an ECL + kit (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. To quantify the amount of protein loaded per lane, Western blotting for β-actin was used as control. The mouse monoclonal antibody for β-actin (β-actin AC-15-ab6276; Abcam, Cambridge, UK) was diluted 1:5000 in TBS-T and utilized in the same manner.

Results

Comparison of hTERT expression among oral cancer cell lines

The relative levels of hTERT mRNA among seven oral cancer cell lines were determined by RT-PCR (Fig. 1). The majority of oral cancer cell lines contained detectable amounts of hTERT transcript, and HSC4 and SAS cells showed especially high levels of mRNA. Hep2 showed no evidence of hTERT message under the specific experimental conditions employed here.

Comparison of hTERT expression before and after FR901228 or 5-Aza treatment

The hTERT-downregulated cell line Hep2 was incubated with FR901228 at concentrations of 0.5 and 1.0 µ*M*. The hTERT-expressing cell lines SAS and KB were also analyzed as controls. Substantial amounts of hTERT mRNA were present, as shown in Fig. 2a. Following incubation of the cells with FR901228, very dense bands were detected, suggesting the induction of hTERT transcripts in Hep2 cells. The SAS and KB cells also showed upregulation of hTERT expression following FR901228 treatment.

To test whether hTERT gene expression was suppressed by hypermethylation of its promoter or not, hTERT-downregulated Hep2 cells were exposed to 5-Aza, an inhibitor that prevents methylation of newly synthesized DNA. The Hep2 cells regained their ability to produce high levels of hTERT mRNA following exposure to graded doses of 5-Aza (Fig. 2a).

These findings suggest that, in both cases, epigenetic-targeted agent-mediated transactivation of the hTERT gene might result from localized hyperacetylation of histones or CpG methylation at the hTERT promoter.

Effects on hTERT expression of treatment with FR901228 combined with CHX

To determine whether the FR901228-mediated induction of hTERT expression was a direct effect or indirect effect, hTERT-downregulated Hep2 cells and hTERT-

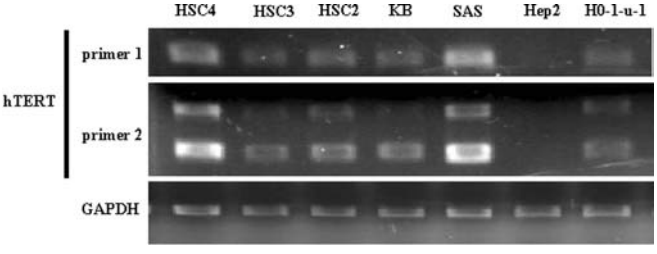

Fig. 1 Comparison of hTERT expression among oral cancer cell lines. Extracted RNA was reverse-transcribed to cDNA and the relative levels of hTERT mRNA among seven oral cancer cell lines (HSC4, HSC3, HSC2, KB, SAS, Hep2 and HO-1-u-1) were determined by RT-PCR. The hTERT primers 1 amplified a 145 bp product and the hTERT primers 2 amplified a 493 bp product spanning exon 6 to exon 10. The two middle bands amplified by primer 2 might have resulted from partially spliced hTERT transcripts. The majority of the oral cancer cell lines contained detectable amounts of hTERT transcript, and HSC4 and SAS cells showed especially high levels of mRNA, as indicated by the very dense bands in the relevant lanes. The gels clearly show that Hep2 cells contained no hTERT mRNA under the specific experimental conditions employed here

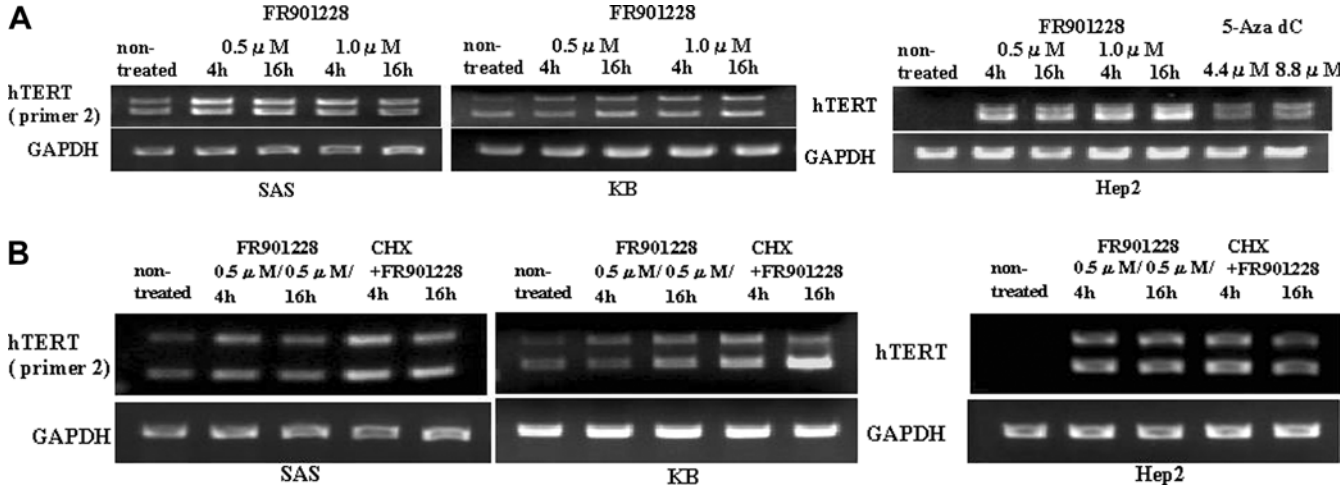

Fig. 2 Epigenetic-targeted agent-mediated transactivation of the hTERT gene. **a** Comparison of hTERT expression before and after FR901228 or 5-Aza treatment. Cells of the hTERT-downregulated cell line Hep2 cells were incubated with FR901228 at 0.5 or 1.0 μ*M*. The cell lines SAS and KB expressing high levels of hTERT were also analyzed as controls. Substantial amounts of hTERT mRNA were determined by RT-PCR. The gels clearly show induction of hTERT expression after 4–16 h of treatment with FR901228 in all three cell lines. Additionally, hTERT-downregulated Hep2 cells were incubated for 7 days with 5-Aza, an inhibitor that prevents methylation of newly synthesized DNA, at concentrations from 4.4 to 8.8 μ*M*. The Hep2 cells regained their ability to produce high levels of hTERT mRNA. **b** Effects on hTERT expression of treatment with FR901228 combined with CHX. To determine whether FR901228-mediated induction of hTERT expression was a direct effect or not, Hep2, KB and SAS cells were incubated with FR901228 in the presence of 200 μg/ml of CHX and were then analyzed for hTERT mRNA by RT-PCR. The SAS, KB and Hep2 cells showed slight induction of hTERT expression; however, cotreatment with CHX enhanced hTERT expression in comparison with FR901228 alone

expressing SAS and KB cells were incubated with FR901228 in the presence of 200 μg/ml of CHX, a potent protein synthesis inhibitor, and were then analyzed for hTERT mRNA (Fig. 2b). Following FR901228 treatment, cells of all three cell lines showed induction of hTERT expression; however, cotreatment of FR901228 with CHX enhanced hTERT expression in SAS and KB cells. This clearly reflects a direct role for the HDAC inhibitor in the transcriptional activation of the hTERT gene to some extent, and implies that de novo protein synthesis is also involved in hTERT regulation.

Comparison of c-myc expression before and after FR901228 treatment

We also examined whether or not FR901228 had an effect on the hTERT transcription activator, c-myc. Figure 3a, b shows that FR901228 reduced endogenous expression of c-myc in SAS, KB and Hep2 cells. We further analyzed for c-myc mRNA in cells incubated with FR901228 in the presence of CHX (Fig. 3c). SAS, KB and Hep2 cells exposed to FR901228 alone showed lower levels of c-myc mRNA than when incubated with both FR901228 and CHX. Interestingly, in Hep2 cells, CHX treatment even seemed to upregulate baseline expression of c-myc, and this was apparently dependent upon the absence of new protein synthesis in the presence of CHX. The rapid reduction in c-myc mRNA following FR901228 treatment clearly reflects an indirect role for the HDAC inhibitor in the reduction of this gene, but both direct and indirect effects are at play.

Discussion

Among those chemoagents targeting epigenetics, HDAC inhibitors are known to exhibit antiproliferative effects on cancer cells through modulating transcription [23, 35], and are considered as potential therapeutic agents against malignancies. Our results showed a clear increase in hTERT expression following FR901228 treatment of oral cancer cell lines. Although our findings are from in vitro studies and the concentrations of FR901228 in our study were higher than those are used in most studies [14, 29], we believe that these data may have important clinical implications for the potential utility of FR901228 in oral cancer therapy. Some studies have shown that the HDAC inhibitor TSA is able to activate hTERT expression in telomerase-negative cells [5, 7, 31, 37]. However, it remains unclear how epigenetics work on hTERT mRNA expression. In previous studies [7, 11], no general correlation has been found between hTERT expression and hTERT promoter methylation status, either overall or at a specific site in cancer cells. Hou et al. [13] have reported that TSA and CHX induce hTERT mRNA. Wang and Zhu [33] have also reported that TSA-induced hTERT transcription and chromatin alterations are not blocked by CHX, suggesting that this induction does not require de novo protein synthesis and is likely a direct result of HDAC inhibition at the hTERT promoter. To characterize whether or not the hTERT induction pathway by FR901228 has a direct effect on chromatin, we treated cells with FR901228 and the protein

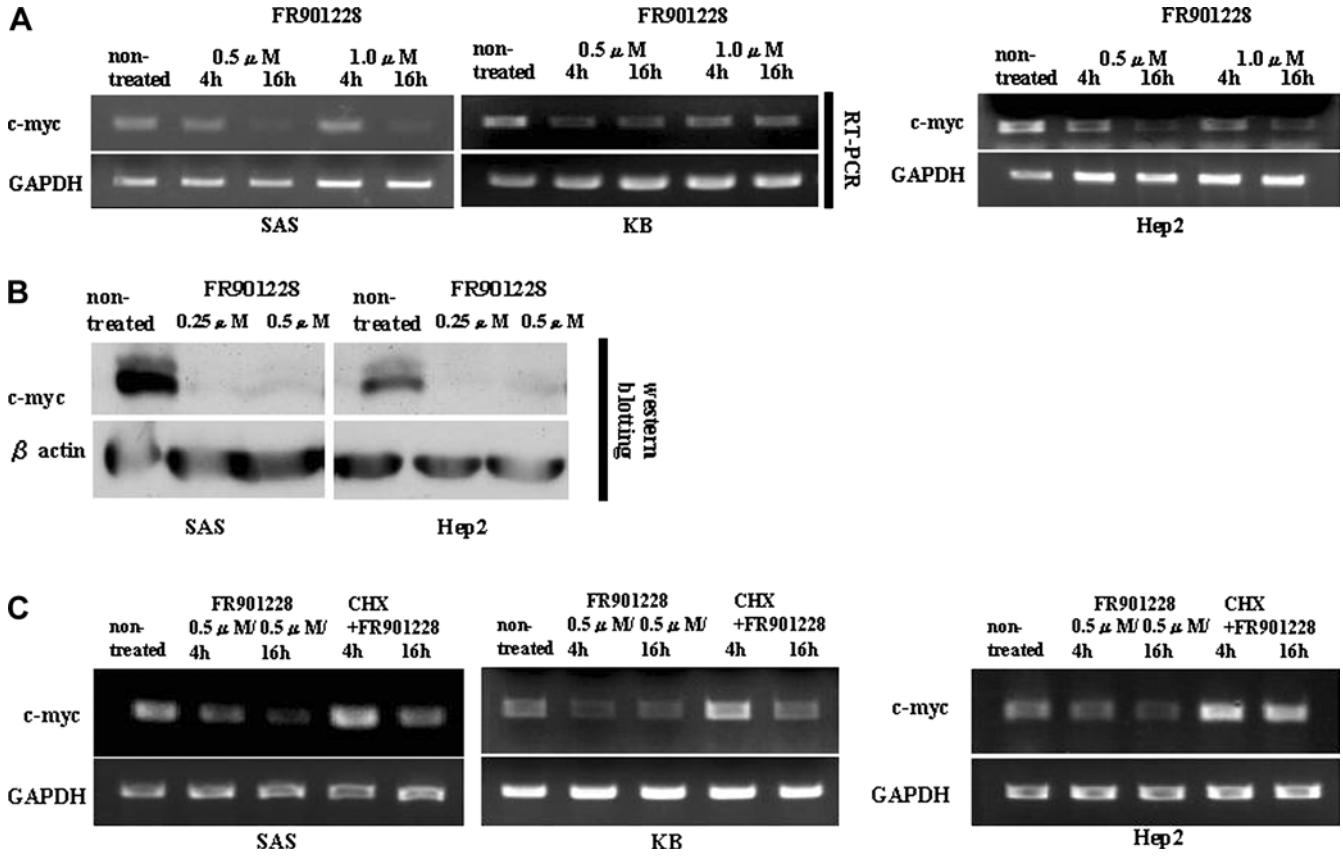

Fig. 3 Comparison of c-myc expression before and after FR901228 treatment. **a** Comparison by RT-PCR of c-myc mRNA before and after FR901228 treatment. **b** Comparison by Western blotting of c-myc protein before and after FR901228 treatment. To examine whether or not FR901228 has an effect on the hTERT transcription activator c-myc, we analyzed c-myc expression before and after FR901228 treatment in SAS, KB and Hep2 cells. FR901228 reduced endogenous expression of c-myc in both cell lines. **c** Effects on c-myc expression of treatment with FR901228 combined with CHX. SAS, KB and Hep2 cells were incubated with FR901228 in the presence of CHX and analyzed for c-myc mRNA by RT-PCR. As when cells were exposed to FR901228 alone, SAS, KB and Hep2 cells showed lower levels of c-myc mRNA than when incubated with both FR901228 and CHX

synthesis inhibitor CHX. In SAS and KB cells, co-treatment with CHX and FR901228 resulted even in the enhancement of the induction of hTERT transcription compared with the result obtained with FR901228 treatment alone. This suggests that the induction of hTERT by FR901228 is more likely a direct effect than an indirect effect on epigenetic changes such as histone acetylation/deacetylation and involves de novo protein synthesis in part.

Overexpression of some transcription factors such as c-myc has recently been shown to play some part in activation of endogenous hTERT transcription [8, 17, 20, 24, 34, 36]. Next, we sought to discover whether or not the effect of FR901228 on hTERT expression is related to a mechanism through one of the main hTERT transcription activators, c-myc, whose repression has been implicated in several apoptosis pathways [32]. FR901228 treatment clearly decreased c-myc expression in oral cancer cell lines. HDAC inhibitors, such as TSA and sodium butyrate, have been reported to decrease c-myc mRNA expression and to increase p21 mRNA [35]. Previous studies have shown that butyrate, known to be an HDAC inhibitor, reduces c-myc mRNA levels [1, 4, 10, 19]. Our results showed that c-myc expression was less reduced in the presence of the protein synthesis inhibitor CHX. This indicates that the c-myc expression is transcriptionally regulated by FR901228 through histone acetylation/deacetylation indirectly, although the precise mechanism by which HDAC inhibitors abrogate c-myc expression remains to be elucidated.

Having demonstrated that FR901228 reduces c-myc expression, the inverse expression patterns of hTERT and c-myc led us to hypothesize that c-myc reduction might play some part in activating hTERT. Li et al. [22] found a complex consisting of BRCA1, c-myc and Nmi that can inhibit hTERT promoter activity in breast cancer cells. Inconsistent with our hypothesis, the combined treatment with FR901228 and CHX resulted in an enhancement of hTERT expression independent of c-myc expression. It is reasonable to speculate that the decreased expression of c-Myc protein that was observed in our study is not required for activation of hTERT expression by FR901228. However, the precise mechanism involved in the interaction between hTERT and c-myc expression remains to be elucidated.

Our study suggested that hTERT expression may be regulated epigenetically through histone acetylation/

DNA methylation to some extent; and other regulatory factors also take part in increasing hTERT expression through an HDAC-independent mechanism. Our study provides some new insights into an endogenous mechanism for hTERT induction by HDAC inhibitors.

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