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Inhibition of NAMPT pathway by FK866 activates the function of p53 in HEK293T cells

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ABSTRACT

Inactivation of p53 protein by endogenous and exogenous carcinogens is involved in the pathogenesis of different human malignancies. In cancer associated with SV-40 DNA tumor virus, p53 is considered to be non-functional mainly due to its interaction with the large T-antigen. Using the 293T cell line (HEK293 cells transformed with large T antigen) as a model, we provide evidence that p53 is one of the critical downstream targets involved in FK866-mediated killing of 293T cells. A reduced rate of apoptosis and an increased number of cells in S-phase was accompanied after knockdown of p53 in these cells. Inhibition of NAMPT by FK866, or inhibition of SIRT by nicotinamide decreased proliferation and triggered death of 293T cells involving the p53 acetylation pathway. Additionally, knockdown of p53 attenuated the effect of FK866 on cell proliferation, apoptosis, and cell cycle arrest. The data presented here shed light on two important facts: (1) that p53 in 293T cells is active in the presence of FK866, an inhibitor of NAMPT pathway; (2) the apoptosis induced by FK866 in 293T cells is associated with increased acetylation of p53 at Lys382, which is required for the functional activity of p53.

1. Introduction

The tumor suppressor protein p53 safeguards the integrity of a cell against stress stimuli such as oncogene activation, or DNA damage. In several cases of cancers, with no described genomic mutation of the *TP53* loci, the tumor suppressor function of p53 can be circumvented by several other mechanisms [1]. The large T-antigen is an oncoprotein encoded by the genome of SV-40 virus, and is largely involved in the transformation of the infected host cells. A possible mechanism by which large T-antigen transforms cells is by its interaction with host tumor-suppressor protein, like p53. Interaction of p53 with large T-antigen blocks the recruitment of p53 to the promoter of its target genes relevant in tumor suppressor functions [2,3]. This leads to abrogation of p53's tumor-suppressor function [4]. The abolishment of p53 interaction with SV40 T-antigen using small molecules is considered a potential approach to rescue the function of p53 [5]. Evidence exists that in SV-40 transformed cells, a small fraction of free p53 which is not bound to large T-antigen is metabolically active [6]. This offers the possibility of activating the unbound fraction of p53 and reconstituting its defense mechanism in SV-40 transformed cells. The variety of post-translational modifications provide p53 protein with the ability to coordinate a complex network of cellular proteins relevant in tumor suppression [7]. Acetylation of lysine residues in the C-terminal domain of p53 regulates several distinct functions, which includes transactivation, stabilization and translocation.

The histone deacetylase (HDAC) inhibitors (HDACi) are a new class of accessory therapeutic agents which exhibit anti-tumor activity on multiple human cancers [8]. Recently, class III HDACs, sirtuins (SIRTs), have gained attention due to their ability to deacetylate important histone and non-histone targets. To mediate deacetylation reactions, SIRT relies on the co-factor NAD^+ which is synthesized by the enzyme NAMPT [9,10]. Deacetylation of p53 by SIRT1 abrogates the ability of p53 to activate the transcription of target genes involved in tumor suppression and induce apoptosis after exposure of cells to genotoxic stress [11–13].

The 293T cell line is a variant of the HEK293 cell line, generated by overexpression of SV-40 large T-antigen. In this study, we provide evidence for the presence of free p53 in 293T cells, which was functionally active in the presence of FK866, an inhibitor of NAMPT pathway. Further, we have demonstrated that the induction of

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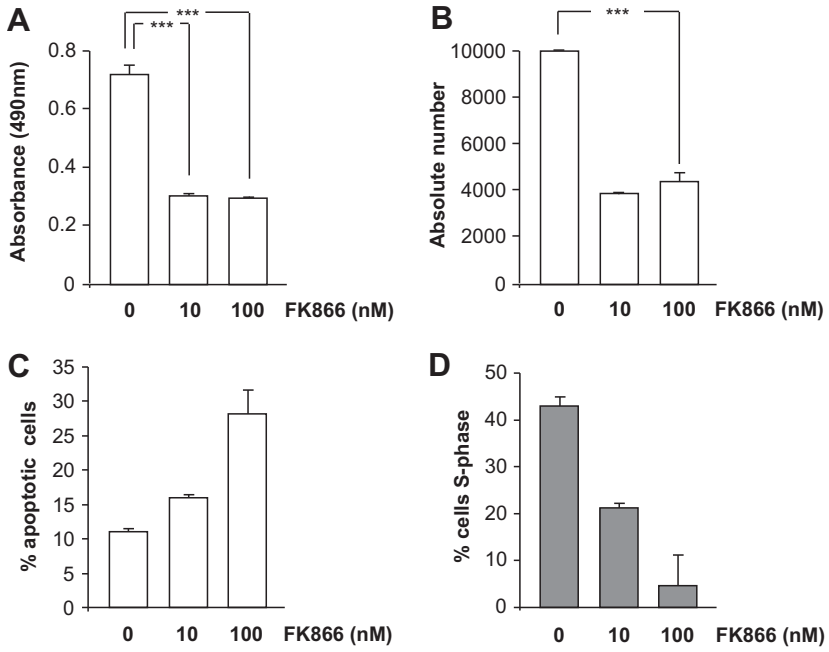

Fig. 1. FK866 reduces cell proliferation by enhanced apoptosis and decreased cell cycle progression. (A) Proliferation of 293T cells, treated with the indicated concentrations of FK866 for 48 h, was measured using CellTiter 96 AQueous reagent at 490 nM. (B) Absolute number of viable 293T cells, treated with indicated concentrations of FK866, was determined after 48 h using trypan blue exclusion assay. (C) Percentage of Annexin V positive cells indicating apoptosis compared to viable cells, was measured 48 h after administration of indicated concentrations of FK866. (D) BrdU/7-AAD cell cycle analysis of 293T cells. Diagram shows the percentage of cells in S-phase 48 h after addition of indicated concentrations of FK866. Measurements of cell cycle and apoptosis were carried out using FACS.

apoptosis in 293T cells by FK866 is associated with increased acetylation of Lysine (Lys-382). The study implicates the role of NAMPT inhibitors in malignancies induced by SV-40.

2. Materials and methods

2.1. Cell lines

HEK 293T cells were cultured in Dulbecco's modified Eagle's medium, high glucose 4.5 g/l, without L-Glu (PAA) supplemented with 10% fetal calf serum; 1% L-glutamine, 1% penicillin and 1% streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cells were passaged every 2 or 3 days in 1:4 or 1:5 ratios depending on the density.

2.2. Transient transfection

Cells (0.01×10^6) in 100 μl medium were seeded in each well of a 96 well plate and after overnight incubation transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. The amount of plasmid used was as indicated in the figures and amount of Lipofectamine used was 0.4 μl for each well. Cells were harvested after 24 h of transfection.

2.3. Virus transduction

Production of viral particles was performed by transfection of 293T cells with target vector, pGagpol vector, pEnv-VSVG vector and REV vector. Supernatant with viral particles was harvested after 36 h. For knockdown of p53 and over expression of NAMPT, cells were seeded in a 24 well plate (0.1×10^6 in 1 ml). 24 h later 50 μL of virus supernatant and 5 μL of polybrene (2 μg/ml) were added. Cells were incubated for 1 h in an incubator and afterwards centrifuged for 2 h at 37 °C and 700*g*. After 16 h in the incubator the medium was replaced. Transduction efficiency was quantified with western blot.

2.4. Plasmids

For silencing of p53 oligonucleotide: 5′ to 3′

Sense: GATCCCCGGCACAGAGGAAGAGAATCTTTCAAGAGAA GATTCTCTTCCTCTGTGCTTTTTGGAAA

Antisense: AGCTTTTCCAAAAAGCACAGAGGAAGAGAATCTTCT CTTGAAAGATTCTCTTCCTCTGTGCCGGG was purchased from Qiagen and cloned into pSUPER (for transient transfection) and further sub-cloned into pRRL.SF.DsYellowEx.pre. (for virus transduction). For overexpression of NAMPT we used the pcDNA3.1 construct containing NAMPT full length cDNA.

2.5. Western blotting

Equal number of pelleted cells were washed with ice-cold phosphate-buffered saline (PBS), again pelleted and resuspended in 1.5× laemmli buffer (15% glycerol, 3% SDS, 4.5% ß-mercaptoethanol, 0.2% bromophenol blue in 100 mM Tris/HCl [pH 6.8]), subsequently boiled at 95 °C for 5 min and spun in a microcentrifuge for 5 min. Samples were separated in 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membrane (Amersham Bioscience). The membranes were blocked with 5% non-fat dry milk-TBS-T (10 mM Tris/HCl [pH 8.0], 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature and incubated with primary antibody in TBS-T for 1 h at room temperature or overnight at 4 °C. After washing 4 times for 5 min in TBS-T membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. After washing 4 times for 5 min protein bands were visualized by enhanced chemiluminescence reagent (Pierce) and by exposure to X-ray film (Agfa).

2.6. Quantitative real-time PCR

Cellular RNA was isolated using the RNeasy Micro Kit (Qiagen). Reverse transcription was performed with 500 ng of RNA and re-

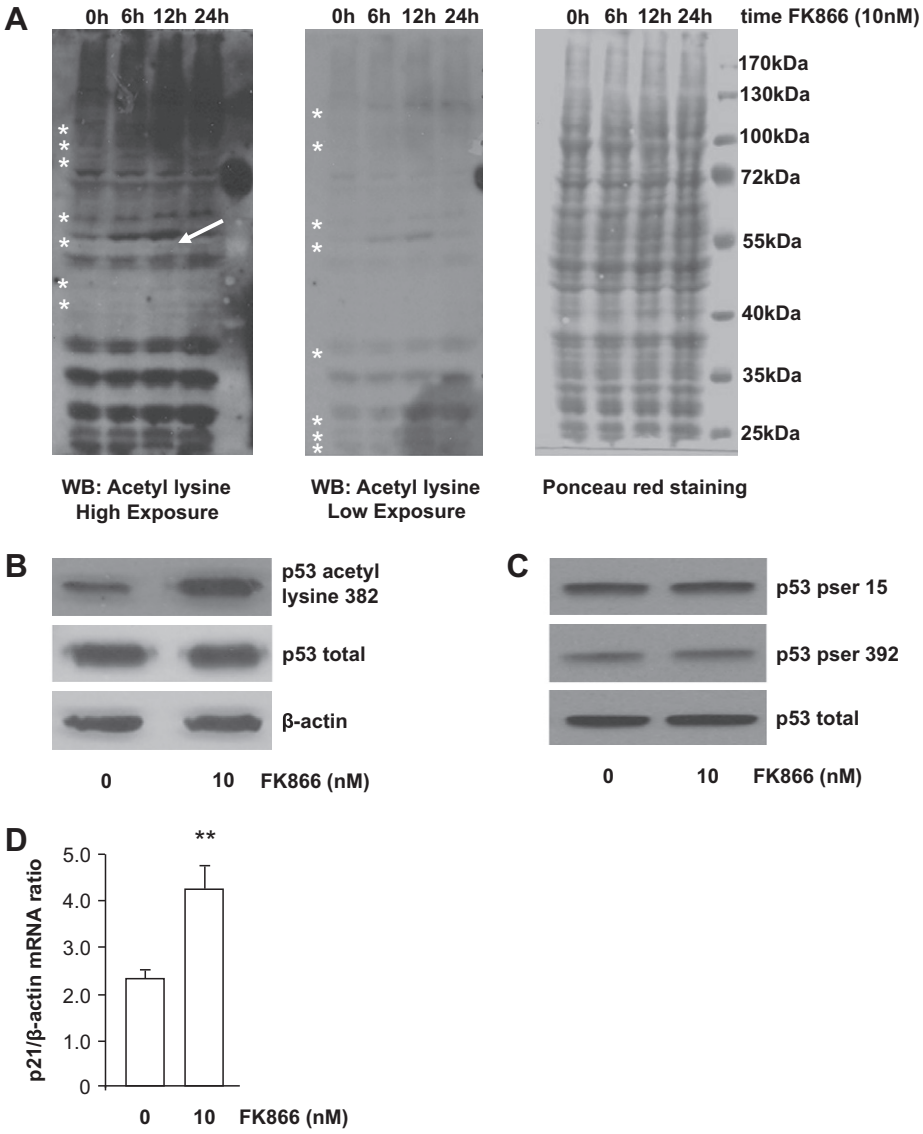

Fig. 2. Exposure to FK866 influences the global acetylation pattern which includes p53. (A) Western blot was performed using acetyl lysine antibody to measure the global acetylation status of proteins in 293T cells after treatment with 10 nM FK866 for indicated time points. The proteins with significantly increased acetylation are highlighted with asterisks. The arrow points at the possible size of p53 band. Both high (left) and low exposure (middle) films are presented and ponceau red staining of the blot (right) is shown to control the loading amounts in each lane. (B) Western blot was performed to measure total amount of p53 and acetylation levels of p53 at lysine 382 in 293T cells after treatment with indicated concentrations of FK866 for 48 h. (C) Western blot was also performed to check the phosphorylation levels of p53 at the critical serine residues 15 and 392. (D) The levels of p21 mRNA after stimulation of 293T cells for 48 h with FK866 were measured with qRT PCR.

agents of Omniscript Reverse Transcription Kit, both according to the manufacturer's protocol. The relative mRNA levels of target genes was measured in triplicate using SYBR Green Master Mix (Applied Biosystems) and the following oligonucleotide primers (Qiagen) were used: ß-actin-sense, 5′-TTCCTGGGCATGGAGTC-3′ and ß-actin-antisense, 5′-AGGTCTTTGCGGATGTC-3′. p21-sense, 5′-ATGTGTCCTGGTTCCCGTCCT-3′ and p21-antisense 5′-CATTGTGGGAGGAGCTGTGA-3′. The mRNA levels were determined using the ΔΔ Ct method and results are shown as mean ± standard error.

2.7. Antibodies and reagents

Rabbit polyclonal antibody to p53, rabbit polyclonal antibody to acetyl-p53-K382 and rabbit polyclonal antibody to NAMPT were obtained from Cell Signaling. Mouse monoclonal antibody to ß-Actin was obtained from Santa Cruz Biotechnology. The antibodies to phospho-p53-S15 and phospho-p53-S392 were obtained from cell signaling. Intracellular staining for flow cytometry analysis was performed with Alexa Fluor 647 Mouse anti p53 (acK382) (#560231) from BD Bioscience and IntraPrep™ Permeabilization Reagent (BeckmanCoulter #A07803) according to manufacturer's protocol. FK866 was obtained from Enzo Life Sciences (#ALX-270-501-M001) and nicotinamide from SIGMA (#N0636-00G).

2.8. Cell proliferation, cell cycle and apoptosis assay

Proliferation of 293T cells was measured using CellTiter 96 AQueous from Promega according to manufacturer's protocol. Absorbance was measured at 490 nm on a GloMax®-Multi 96-well Microplate Multimode Reader. Cells were seeded in a 96 well plate at a density of 5000 cells per well. After treatment, absolute cell counts were quantified using trypan blue cell exclusion assay. All

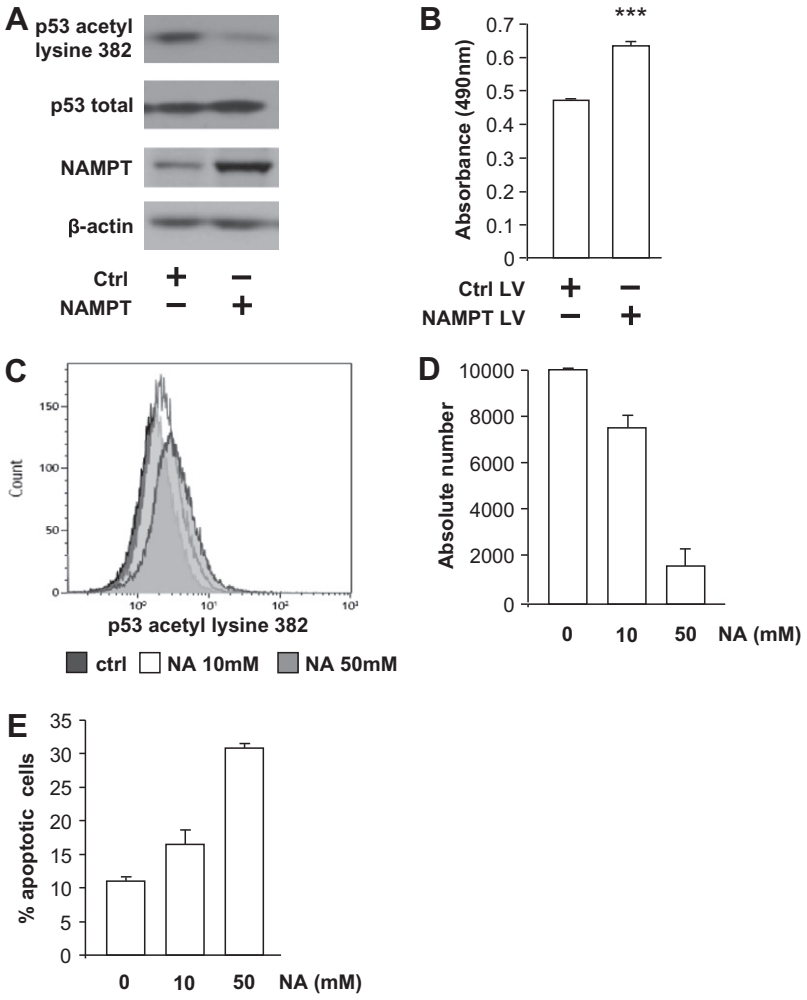

Fig. 3. Overexpression of NAMPT reduces p53 acetylation and increases proliferation of 293T cells. Inhibition of sirtuins by NA leads to increased p53 acetylation and decreases cell proliferation by enhanced apoptosis. (A) Western blot was performed with lysate of 293T cells, transduced with cDNA of NAMPT, to assess the total amount and the acetylation level of lysine 382 of p53. Additionally, NAMPT overexpression was confirmed by western blot. (B) Proliferation of 293T cells was measured in cells overexpressing NAMPT. (C) Acetylation of p53 was measured by flow cytometry with Alexa647 labeled acetyl p53 antibody in presence of indicated concentrations of NA. (D) Absolute number of viable 293T cells, treated with indicated concentrations of NA, was determined after 48 h using trypan blue exclusion assay. (E) Percentage of apoptosis was assessed using Annexin V/7-AAD staining 48 h after exposure to the indicated concentrations of NA using FACS.

reactions were analyzed as triplicates in two independent experiments.

For the cell cycle analysis, 293T cells were incubated for 1 h in 10 μM BrdU solution. Cells were permeabilized, fixed and BrdU- and 7-AAD-stained using the BrdU Flow Kit (BD Pharmingen) according to manufacturer's instructions.

Apoptosis analysis was performed using the Annexin V Apoptosis Detection Kit (BD Pharmingen) according to manufacturer's instructions. Flow cytometry measurements were performed on a Navios (Beckman Coulter).

3. Results

3.1. Pharmacologic inhibition of NAMPT increases apoptosis and cell cycle arrest in 293T cells

FK866, a specific inhibitor of the NAMPT pathway, is known to induce cytotoxic effects in certain types of cancer cells by depletion of the intracellular pool of NAD^+ [14]. FK866-mediated death of 293T cells involves apoptosis (Fig. 1C). In addition to cell death, FK866 decreases the proliferation of 293T cells (Fig. 1A). This decrease in cell proliferation is associated with a reduction in the absolute number of 293T cells (Fig. 1B). To address the mechanism behind the decrease in 293T cell proliferation, we performed BrDU-based cell cycle analysis after challenging the cells with two different concentrations of FK866 (10 nM and 100 nM). We observed a significant dose-dependent decrease in the number of cells in S-phase (Fig. 1D).

3.2. Inhibition of NAMPT pathway by FK866 increases the acetylation of p53 protein in 293T cells

To identify the target proteins involved downstream of FK866-mediated signaling, we performed immunoblot assays using acetyl lysine antibody with the protein lysate prepared from 293T cells treated or untreated with FK866. We observed an increased acetylation of several proteins ranging from 20 kDa to 150 kDa (indicated by a star) in the lysates extracted from cells treated with FK866 (Fig. 2A). Interestingly, we also detected a band with increased acetylation at the molecular weight of around 53–55 kDa, which could fit the size of p53 protein. Therefore, we asked if the acetylation level of p53 protein is increased in the presence of FK866. Indeed, using specific antibody against the p53-acetylated lysine 382, we observed increased acetylation at this site in the

presence of FK866, whereas the level of total p53 remained unaffected. Treatment of 293T cells with 10nM FK866 for 24 h inhibited the deacetylation of p53 compared to untreated control cells (Fig. 2B). To rule out that FK866 influences the phosphorylation status of p53, we performed immunoblot assays with an antibody that detects phosphorylated p53 at the sites of serine 15 (ser15) and serine 392 (ser392). We observed no significant difference in the phosphorylation status of p53 at these specific sites (Fig. 2C), indicating that FK866 specifically induces the acetylation of p53. Next, we asked whether FK866 treatment increases the functional ability of p53 to activate target gene expression. Interestingly, treatment of cells with FK866 resulted in an increased expression of endogenous p21 mRNA (Fig. 2D). Thus, we conclude that FK866-mediated inhibition of NAMPT increases the levels of acetylated p53 leading to the potentiation of functional activity of p53 in 293T cells.

3.3. Involvement of NAMPT/SIRT1 in regulation of p53 activity in 293T cells

Acetylation of p53 at lysine residue 382 is important for p53 functions. Previously, it has been shown that an overexpression of NAMPT promotes longevity through deacetylation of p53 protein at lysine residue 382 [15]. Therefore, we asked whether the NAMPT/NAD^+/SIRT1 pathway influences the function of p53 in 293T cells. To begin, we overexpressed NAMPT and evaluated the acetylation status of p53 at lysine 382. The acetylation level of p53 was significantly decreased after overexpression of NAMPT (Fig. 3A). Importantly, this finding correlates with a strong reduction of the p21 mRNA levels (Supplementary Fig. 1A), which is further reflected in an increased proliferation rate of the 293T cells (Fig. 3B). Next, we used nicotinamide (NA), a well known inhibitor of SIRTs, in our experiments to study their involvement in the regulation of p53. Using acetylated p53 antibody labeled with fluro probe Alexa 647, we observed a dose-dependent increase in the acetylation of p53 after inhibition of SIRTs with NA (Fig. 3C). Treatment of 293T cells with NA resulted in a dose-dependent decrease in the total number of cells (Fig. 3D). This decrease in absolute number of 293T cell in the presence of NA is associated with increased apoptosis (Fig. 3E). These results suggest that the NAMPT/SIRT pathway plays an important role in the regulation of p53 acetylation in 293T cells.

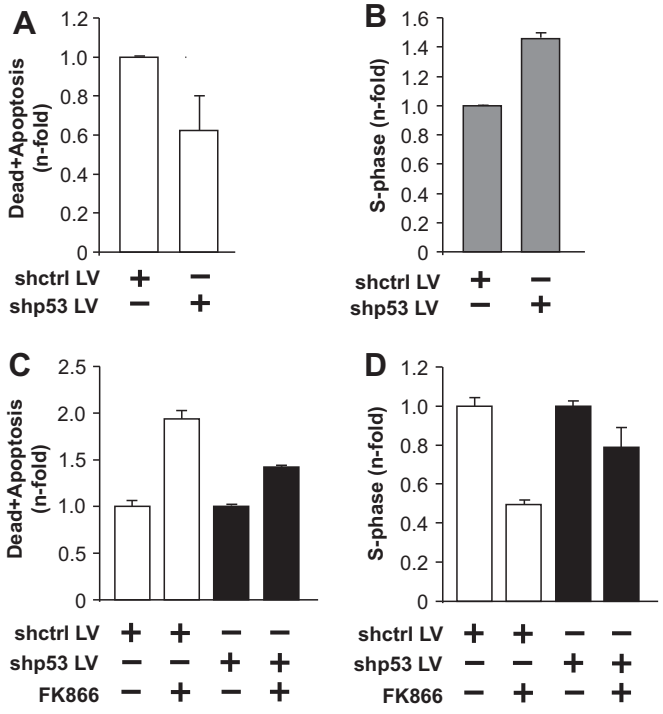

Fig. 4. Evidence of functionally active p53 in 293T cells and direct role of p53 in NAMPT pathway. (A) Percentage of Annexin V/7-AAD cells indicating apoptosis/dead was measured in shp53 LV or empty LV vector transduced cells; (B) BrdU/7-AAD based cycle analysis was performed in 293T cells. Diagrams show the percentage of cells in S-phase in shp53 and empty LV vector transduced 293T cells. Cells transduced with either sh control LV particles or sh p53 LV, were incubated in presence and absence of FK866 for 48 h: Afterwards, (C) The n-fold change of apoptotic/dead cells treated with FK866, indicated by Annexin V/7-AAD staining, compared to untreated cells; and (D) the n-fold change of treated cells in S-Phase, compared to untreated cells was obtained by BrdU/7-AAD cell cycle analysis.

3.4. Knockdown of p53 attenuates the effects of FK866 on apoptosis and cell cycle arrest in 293T cells

In 293T cells, p53 is present in large amounts but is considered to be non-functional due to its interaction with the large T-antigen. Therefore, the next step was to check whether binding to large T antigen completely abolishes p53 functions or to see if a certain fraction of p53 remains functionally active. To demonstrate the functionality of p53 in 293T cells, we performed lentiviral-mediated knockdown of p53 in 293T cells and performed proliferation, apoptosis, and cell cycle assays in these cells. Knockdown of p53 in 293T cells resulted in an increase in cell proliferation (Supplementary Fig. 3A), a decrease in the number of cells undergoing apoptosis (Fig. 4A) and an increase in number of cells entering S-phase (Fig. 4B). In addition, the activity of p53 response element luciferase (Supplementary Fig. 2A), and the endogenous level of p21 mRNA (Supplementary Fig. 2B) were significantly decreased in p53 knockdown cells.

To demonstrate the direct involvement of p53 in the cytotoxic effect mediated by FK866, we performed cell proliferation, apoptosis, and cell cycle assays in p53 knockdown cells in the presence or absence of FK866 and compared it against sh control transduced cells. A decrease in cell proliferation (Supplementary Fig. 3B), increase in apoptosis (Fig. 4C) and a decrease in the number of cells entering S-phase (Fig. 4D) was observed in sh control transduced 293T cells in the presence of FK866. These effects were partially, but significantly, attenuated in the 293T cells after knockdown of p53. These results suggest that p53 is one of the important factors mediating FK866-induced cytotoxic effect in 293T cells.

4. Discussion

Direct binding of SV40 large T-antigen to p53 protein is known to be involved in inactivation of p53 [16]. Although, p53 is inactive, it is present in large amounts in cells overexpressing large T-antigen. It is proposed that association of p53 with T-antigen blocks the ability of p53 to transcribe MDM2, a negative regulator of p53 [17]. It has been previously reported that SV-40 transformed cells also contain free p53 that is not bound to large T-antigen [6]. However, it remains unclear whether this free p53 is functionally active. Activation of p53 pathway in virally transformed cells by non-genotoxic small molecule compounds is a promising approach to target these cells. In the present study, we have demonstrated the presence of functionally active p53 in 293T cells, which are a variant of the HEK293 cell line that exogenously expresses the large T-antigen. This is based on evidence suggesting that knockdown of p53 decreased cell death by decreasing apoptosis and increasing cell proliferation by inhibiting cell cycle arrest (Fig. 4A and B). Besides phosphorylation, acetylation of p53 is crucial in terms of its tumor-suppressor function [18]. SIRT1-mediated deacetylation of p53 abrogates its ability to activate target genes

after exposure to DNA damage [11,12]. We demonstrate that overexpression of NAMPT, an activator of SIRTs, decreases the functional activity of p53 to restrict cell proliferation by downregulating the expression of p21 (Supplementary Fig. 1A) due to decreased levels of p53 acetylation (Fig. 3A). These results additionally support our claim for the presence of functionally active p53 in 293T cells and suggest that the acetylated form of p53 is an active form that exhibits the ability to restrict the uncontrolled proliferation of 293T cells.

Inhibition of SIRTs can influence the global acetylation pattern of various cellular proteins that can further influence several cellular processes. Nicotinamide (NA), a precursor of NAD^+, is known to influence the activity of SIRTs. Effect of NA on SIRT1 depends on the dose and the cellular context. In case of granulocytic differentiation, NA is proposed to activate SIRT1 [19]. On the other hand, NA inhibits SIRT1 and induces senescence in context to aging [20]. High concentrations of NA induce death in several cancer cell lines. In line with this observation, we also found that NA at a concentration of 10 mM triggers death in 293T cells by inducing apoptosis (Fig. 3D and E). The electron carrier NAD^+ plays an important role in cellular reactions (i.e. deacetylation, redox reactions, ADP-ribosylation, transcriptional regulation). In particular, cancer cells have a rapid turn over of NAD^+ due to a rapid metabolism. Therefore, they rely on more quickly available NAD^+ as it is synthesized in the three-step NA salvage pathway [21]. Intriguingly, healthy cells using other precursors like nicotinic acid remain unaffected [22], suggesting that FK866 might be a potential agent for use in cancer cells that rely on NA to synthesize NAD^+. One further explanation could be that the low cytotoxicity to healthy cells is due to their ability to effectively convert the oxidized (NAD^+) and the reduced (NADH) forms by enzymes of the catabolic and anabolic pathways. On the other hand, the comparatively slow metabolic rate of healthy cells provides them with an additional protection. These properties of cancer cells suggest that NAMPT could be an attractive target for the treatment of cancers. The dependency of SIRTs on NAD^+ to mediate deacetylation reactions provides an alternative approach to inhibit SIRTs in cancer cells by decreasing the intracellular NAD^+ pool. Based on our observations, we propose that under basal conditions, the low fraction of p53 in 293T cells which is unbound to large T-antigen is not sufficient enough to inhibit uncontrolled proliferation of 293T cells. Treatment with FK866 activates this fraction of p53 by triggering its acetylation, which overrides the T-antigen mediated suppression of p53 in 293T cells. Whether FK866 treatment influences only the function of free p53 that is unbound to T-antigen, and/or interferes with p53 bound to large T-antigen is an interesting topic for future investigations.

Inhibitors of HDACs (HDACi) are a new class of therapeutic agents because they induce cytotoxicity in wide range of cancer cells. Reconstituting the gene expression programme by inhibition of HDACs is a potential underlying mechanism in the efficacy of HDACi. HDACi like SAHA and VPA, which primarily inhibit class I and II deacetylases, exhibit strong anti-tumor activity and have entered phase II clinical trials [8]. The increasing evidence that SIRTs are critical regulators of major tumor-suppressor proteins, like p53 and FOXO3a, has recently lead to the development of drugs which can specifically target SIRTs [23]. The fact that cancer cells require high turnover of NAD^+ to maintain their growth, and SIRTs require NAD^+ to maintain their activity, further highlights the importance of FK866 and its ability to specifically target cancer cells. The presented data contribute towards the understanding of mechanism(s) by which FK866 exerts its anti-cancer effects.

In a recent communication we have shown that FK866 upregulates the acetylation of FOXO3a protein in 293T cells, leading to apoptosis [24]. However, this is the first evidence for the presence of functionally active p53 in 293T cells, a cell line known for the abnormal p53 function due to its interaction with large T-antigen. In conclusion, enhancing acetylation of p53 by inhibiting the NAMPT/SIRT pathway improves functional activity of p53 in cells transformed with large T-antigen, which has broad implications for malignancies characterized by p53 inactivation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.075.

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