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Restoration of miR-34a in p53 Deficient Cells Unexpectedly Promotes the Cell Survival by Increasing NFκB Activity

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ABSTRACT

Upregulation of miR-34a by p53 is recently believed to be a key mediator in the pro-apoptotic effects of this tumor suppressor. We sought to determine whether restoration of miR-34a levels in p53 deficient cells could rescue the response to DNA damage. Compared with the p53 wildtype U2OS cells, miR-34a expression was much lower in p53 deficient Saos2 cells upon cisplatin treatment. Unexpectedly, delivery of miR-34a in Saos2 cells does not increase the cell sensitivity to apoptosis. This effect was mediated by direct downregulation of SirT1 expression by miR-34a, which in turn increased the NFκB activity. Inhibition of NFκB activity in Saos2 cells by Aspirin sensitized the miR-34a overexpressing cells to cell death. Thus, in tumors with p53 deficiency, miR-34a restoration alone confers drug resistance through Sirt1-NFκB pathway and combination of miR-34a and NFκB inhibitor could be considered as a promising therapeutic strategy. J. Cell. Biochem. 113: 2903–2908, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: SIRT1; miR-34a; p53; NFκB; CISPLATIN; APOPTOSIS

Cancer has emerged as the key reason for death, which is due to genomic mutations that produce oncogenes with dominant gain of function and tumor suppressor genes with recessive loss of function [Hanahan and Weinberg, 2000]. The p53 protein is a transcription regulator that is frequently altered by mutation in multiple cancers, including osteosarcoma, colon cancer, and lung cancer [Fearon and Vogelstein, 1990; Berman et al., 2008]. Lots of the downstream effectors of p53 have been identified, and the

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different sets of genes induced by p53 determine the switch between a pro-survival and a pro-apoptotic fate [Wee et al., 2009]. Restoration of the pro-apoptotic downstream molecules is thought to be promising in cancer therapy in p53 deficient cells.

MicroRNAs (miRNAs) are a class of noncoding, single-stranded small RNAs (approximately 22 nucleotides) that modulate the expression of a network of mRNAs through binding the imperfectly complementary sequence in the target. In other words, a single, aberrantly expressed miRNA might target multiple gene networks that are essential for cancer development. Reconstitution of tumor-suppressive miRNA, or sequence-specific knockdown of oncogenic miRNAs by "antagomirs" has produced favorable antitumor outcomes in experimental models [Tong and Nemunaitis, 2008]. Recently, miR34a has been shown to be one of the key mediators and downstream factors of p53 [Chang et al., 2007; Raver-Shapira et al., 2007]. It is reported that miR-34a induces cell cycle arrest and apoptosis by down-regulating cell cycle and apoptosis related proteins such as NMYC, CDK6, Cyclin D1, Cyclin E2, E2F, and SIRT1 [Tazawa et al., 2007; Fujita et al., 2008; Sun et al., 2008; Wei et al., 2008; Yamakuchi et al., 2008]. Although miR-34a plays a tumor suppressor role in multiple cells, its tumor suppressor role is context dependent. It is found that the tumor suppressor role is dependent on an intact p53 pathway [Merkel et al., 2010]. In another study, miR34a has even been found to act as an oncogene in myc overexpression cells through direct repressing myc expression [Sotillo et al., 2011]. Some of the miRNA targets functions bidirectionally in a context dependent manner. Take SIRT1 (Silent mating type information regulation 2 homolog 1) for an example, which is an NAD-dependent histone/protein deacetylase, is implicated in diverse cellular processes including apoptosis [Michan and Sinclair, 2007]. The anti-apoptotic activity of SIRT1 is implicated in tumorigenesis, since SIRT1 expression is elevated in a variety of cancer cell lines and tumors [Saunders and Verdin, 2007]. SIRT1 deacetylates pro-apoptotic proteins such as p53 and promotes cell survival under genotoxic and oxidative stresses. However, SIRT1 was also reported to deacetylateNFκB at certain lysine sites and inhibit its activity [Yeung et al., 2004; Chen et al., 2005; Yang et al., 2007; Rajendrasozhan et al., 2008]. Nuclear factor-kappa B (NFκB) controls the expression of gene products that affect important cellular processes, such as adhesion, cell cycle, angiogenesis, and apoptosis [Karin and Lin, 2002]. Acetylation of RelA/p65 is a dynamic process where the acetylation status of specific lysine residues affects both the DNA-binding ability and transcriptional activity of the protein [Chen et al., 2002; Kiernan et al., 2003].

In view of the above data, it is interesting to test whether restoration of miR-34a in p53 deficient cells could rescue the p53 deficiency. In this study, we found that miR-34a expression was significantly repressed in p53 deficient Saos2 cells. Unexpectedly, restoration of miR-34a in Saos2 cells did not decrease the cell survival rate. Molecularly, miR-34a increased the NFκB activity through down-regulation of SIRT1. Abrogation of NFκB activity by Aspirin greatly increased the cell death in miR-34a expressed Saos2 cells. In conclusion, our study demonstrates a strong synthetic role of combining miR-34a and Aspirin in inducing p53 deficient cells to death.

MATERIALS AND METHODS

ANTIBODY AND REAGENTS

Anti-caspase-3 was obtained from Sigma and anti-α-tubulin, anti-Bcl2, anti-sirt1 were from Santa Cruz. The protease inhibitor Cocktail was obtained from Calbiochem and Aspirin was obtained from Sigma. Penicillin/streptomycin, trypsin/EDTA, and PBS were obtained from Gibco. Synthetic miR34a mimics and the control were synthesized in Genepharm in Shanghai and dissolved in DEPC-treated H_2O at a concentration of 20 nM as a stock. Cisplatin and nicotinamide were bought from Sigma. All other reagents were purchased from domestic companies.

CELL CULTURE

Osteosarcoma cell lines U2OS and Saos2, obtained from ATCC, were cultured in 1640 medium (Invitrogen), supplemented with 2 mM glutamine, 0.06 g/L penicillin, 0.1 g/L streptomycin, and 10% fetal bovine serum (FBS) (Sijiqing, Hangzhou, China) at 37°C in a humidified atmosphere of 5% CO_2.

REPORTER ASSAY

Cells were plated into 24-well plates at a density of 70% confluence (unless otherwise indicated). Twenty-four hours later, cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions, with the designated combination of miRNA mimics or the control, 0.2 μg 3 × κB firefly luciferase reporter plasmid, and 0.1 μg pRL-TK (Promega) for transfection normalization. Twenty-four hours after transfection, cells were lysed using passive lysis buffer and analyzed for firefly and Renilla luciferase activities using the dual-luciferase reagent assay kit (Promega) according to the manufacturer's instructions.

WESTERN BLOT ANALYSIS

Cell culture monolayers were washed twice with ice-cold PBS and lysed with nuclear protein extraction kit or whole cell lysis buffer. Protein concentration was determined by the BCA protein assay (Pierce Chemical Co., Rockford, IL). Equal amounts of cell lysates were separated on SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were then incubated in blocking solution (5% nonfat-milk in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) (TBS-T), followed by incubation with the indicated antibodies at 4°C overnight. The membranes were then washed in TBS-T and incubated with HRPO-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemiluminescence reaction.

RT–PCR

Cells with indicated treatments were harvested for isolation of RNA using Trizol reagent (Invitrogen) according to manufacturer's instructions. First-strand cDNA synthesis was performed using random primers catalyzed by murine leukemia virus (M-MLV) reverse transcriptase for mRNA detection. For miRNA detection, miRNAs were reversely transcribed using miScript Reverse Transcription Kit (Qiagen). RNA abundance was detected by qPCR. Real-time PCR was performed using AB 7500 system. The conditions were 10 μl of SYBR Green I (Takara), 0.5 μM ofeach 5′

and 3′ primer, and 2 μl of sample and H2O to a final volume of 20 μl. Samples were amplified for 45 cycles with a denaturation at 95°C for 5 s, annealing and extension at 60°C for 34 s. SYBR green fluorescence was measured to determine the amount of double-stranded DNA. To discriminate specific from nonspecific cDNA products, a melting curve was obtained at the end of each run. Relative mRNA levels were normalized to GAPDH levels and compared with the control using the $2^{-\Delta\Delta Ct}$. Relative miR-34a levels were normalized to U6B levels. Primers for the tested genes were listed in Table I.

MTT

To assess cell proliferation, the MTT test was employed. Briefly, cells with different treatments were seeded at a density of 2,000 cells/well in 96-well plates. 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added (100 μg/well) for 4 h at different time points. Formazan products were solubilized with DMSO, and the optical density was measured at 490 nm. All experiments were performed in triplicate.

STATISTICS

All the experiments are done at least in triplicates and the data are expressed as means ± SD. Student's *t*-test or ANOVA is applied for statistics analysis. $P < 0.05$ is considered as significantly different.

RESULTS

LOWER EXPRESSION OF miR-34a IN p53 NULL SAOS2 CELLS

Previously, miR-34a expression was found to be closely related to p53 status [Park et al., 2008]. In this regard, we first analyzed miR-34a expression in p53 wildtype U2OS and p53 deficient Saos2 cells. As expected, miR-34a expression was much lower in Saos2 cells at basal condition (Fig. 1A). Three micrograms per milliliter cisplatintreatment significantly increased the expression of miR-34a in U2OS cells, while the induction of miR-34a was largely compromised in Saos2 cells (Fig. 1A). Next we decided to see whether restoration of miR-34a in Saos2 cells would rescue the insensitivity to apoptosis. Consistent with previous study, miR-34a expression increased U2OS cell death both at basal and cisplatin treatment (Fig. 1B). However, miR-34a expression in Saos2 cells reduced the cell apoptosis induced by cisplatin (Fig. 1C), which was consistent with the total cell number increase as seen from MTT assay (Fig. 1D).

TABLE I. Sequences of the Primer Used or the miRNA Mimics

Name	Sequence
SIRT1	Forward ACCAGAACAGTTTCATAGAGC Reverse TCTGAGGCACTTCATGGGTA
Bcl2	Forward GAGGATTGTGGCCTTCTTTGA Reverse CCGTACAGTTCCACAAAGGCA
GAPDH	Forward GACCTGACCTGCCGTCTA Reverse AGGAGTGGGTGTCGCTGT
miR-34a	Forward TGGCAGTGTCTTAGCTGGTTG
miR-34a mimics	Sense UGGCAGUGUCUUAGCUGGUUGU Antisense AACCAGCUAAGACACUGCCAUU
NC	Sense UUCUCCGAACGUGUCACGUTT Antisense ACGUGACACGUUCGGAGAATT

INCREASED NFκB ACTIVITY IN miR-34a RESTORED SAOS2 CELLS

Previously, miR-34a was also supposed to play a pro-apoptotic role by targeting some targets, such as Sirt1 [Audrito et al., 2011], Bcl2 [Wang et al., 2009]. We thus tested the expression of Sirt1 and Bcl2 in miR-34a restored Saos2 and U2OS cells. In U2OS cells, delivery of miR-34a decreased Sirt1 and Bcl2 expression both at mRNA and protein level (Fig. 2A,C). In contrast, in Saos2 cells, forced expression of miR34a repressed the expression of SIRT1 at both mRNA level and protein level, while an increase rather than the expected decrease of Bcl2 expression was observed (Fig. 2B,C).

Sirt1 was found to deacetylate multiple targets in addition to p53, of which NFκB is another key factor [Rajendrasozhan et al., 2008]. Next, we observed the effects of miR34a on the transcriptional activity of NFκB by reporter assay. miR-34a overexpression enhanced the NFκB reporter luciferase activity in the Saos2 cells (2.9-fold increase) (Fig. 2D). Nicotinamide, a well-known inhibitor of Sirt1 [Rahman and Islam, 2011], significantly reduced the NFκB activity (Fig. 2D), suggesting that miR-34a increasedNFκB activity in a SIRT1 dependent way. In contrast, miR-34a only mildly increased the NFκB activity in U2OS cells (Fig. 2E), perhaps due to the substrate completion by p53.

COMBINATION OF miR-34a RESTORATION AND INHIBITION OF NFκB RESCUES THE SENSITIVITY TO CELL DEATH IN SAOS2 CELLS

We have previously shown that NFκB can transcriptionally activate Bcl2 expression [Tan et al., 2009]. It is thus highly possible that increased NFκB might be responsible for the observed increase of Bcl2 (Fig. 2B,C), rather than the supposed miR-34a mediated downregulation Bcl2 expression. In other words, increased NFκB might be a key factor for the pro-survival role of miR-34a in p53 deficient cells.

From the above data, we decide to test whether combination of miR-34a and NFκB inhibitor in Saos2 cells could sensitize the cell to apoptosis. As seen from the cleaved caspase-3, Aspirin alone or miR-34a alone did not lead to cell apoptosis in Saos2 cells, while combination of miR-34a expression and Aspirin significantly increased the apoptosis, as seen from the increased expression of cleaved caspase-3 (Fig. 3A).

MTT assay also showed that combination of Aspirin and miR-34a significantly decreased survival cell number (Fig. 3B), suggesting a synthetic lethality of miR-34a restoration and NFκB inhibition in p53 deficient cells.

DISCUSSION

In this study, we have found that miR-34a expression is much lower in Saos2 cells both in the basal condition and under cisplatin treatment. Restoration of miR-34a alone in Saos2 cells does not rescue the apoptotic response to cisplatin, which is due to increased activation of NFκB. Combination of miR34a with inhibition of NFκB activity by Aspirin leads to significant cell death.

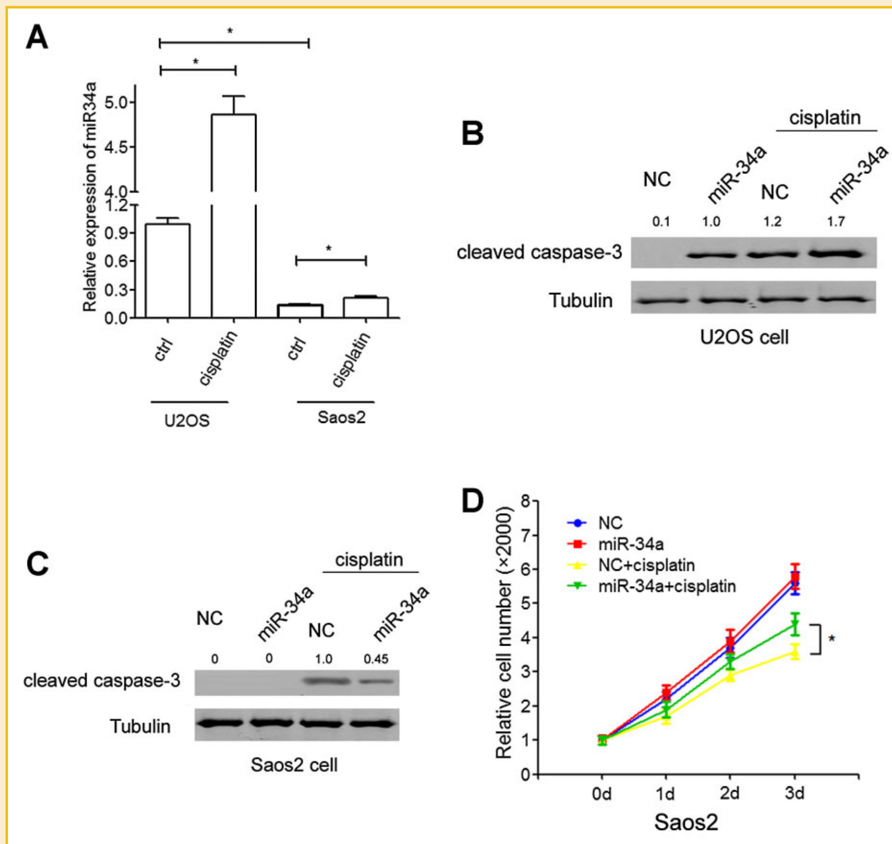

Fig. 1. Lower expression of miR-34a in p53 null Saos2 cells. A: Expression of miR-34a in U2OS and Saos2 cells under vehicle or 3 μg/ml cisplatin treatment was analyzed by miRNA specific qRT-PCR. B: U2OS cells treated with negative control miRNAs (NC) or miR-34a mimics were subjected to vehicle or cisplatin therapy. Cell apoptosis was analyzed by cleaved caspase-3 assay. Data presented here are a representative of three different experiments. Relative expression level of the cleaved caspase-3 was quantified by Image J and indicated above each lane. C: Saos2 cells treated with negative control miRNAs (NC) or miR-34a mimics were subjected to vehicle or cisplatin therapy. Cell apoptosis was analyzed by cleaved caspase-3 assay. Data presented here were a representative of three different experiments. Quantification was done similar as (B). D: Saos2 cells were treated same as the above and cell number was quantified by MTT assay. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Consistent with transcriptional activation of miR-34a expression by p53, we observed lower miR-34a expression in p53 null Saos2 cancer cells. Although restoration of miR-34a repressed the expression of SIRT1, which is consistent with previous studies [Zhao et al., 2010], we did not observe the restored pro-apoptotic role as in p53 wildtype cells [Yamakuchi and Lowenstein, 2009], suggesting that p53 play an essential role in the tumor suppressive function of miR-34a. Besides p53, miR-34a-Sirt1 also targets many other genes, such as p65, which adds another layer of the complexity of miR-34a function. We here found that in the p53 deficient cells, miR-34a-Sirt1-NFκB pathway overwhelms the miR-34a-Sirt1-p53 pathway. Up to now, the detailed mechanism why miR-34a specifically increases NFκB activity in p53 deficient cells is still largely unknown, which is worth further studying. It is highly possible that in the absence of p53, there is enough Sirt1 available for p65 deacetylation, and thus decreased Sirt1 mainly released the suppressed NFκB.

Due to the increased NFκB activity, we did not observe a decrease of Bcl2 expression in miR-34a overexpressed Saos2 cells, as observed in other models [Wang et al., 2009]. All these suggest that the tumor suppressor role of miR-34a function is cell context dependent.

It is important to note that Bcl2 is just one of the factors for the pro-survival role of miR-34a-Sirt1-NFκB pathway in the p53 deficient cells. Besides Bcl2 [Tan et al., 2009], NFκB has been identified to increase some other pro-survival and repress anti-apoptotic genes coordinately, such as cyclinD1, COX2, and IGF-1 [Garrouste et al., 2002]. CyclinD1 is another reported target of miR-34a [Sun et al., 2008], and thus it is highly possible that increased NFκB activity would overwhelm the miR-34a-cyclinD1 regulation.

Chronic inflammation with constitutive activation of NFκB is one of the important characteristics of cancer [Mantovani et al., 2008], including those with p53 mutant. To this end, miR-34a-Sirt1-NFκB regulatory pathway activation, which might overwhelm the tumor suppressor role of miR-34a, might be also true in other cancer models. In this regard, combination of miR-34a and NFκB inhibition should be promising in some p53 deficient cancers.

In summary, restoration of miR-34a alone in p53 deficient Saos2 cells unexpectedly showed a mild prosurvival role, which is due to the increased NFκB activity. Combination of miR-34a restoration

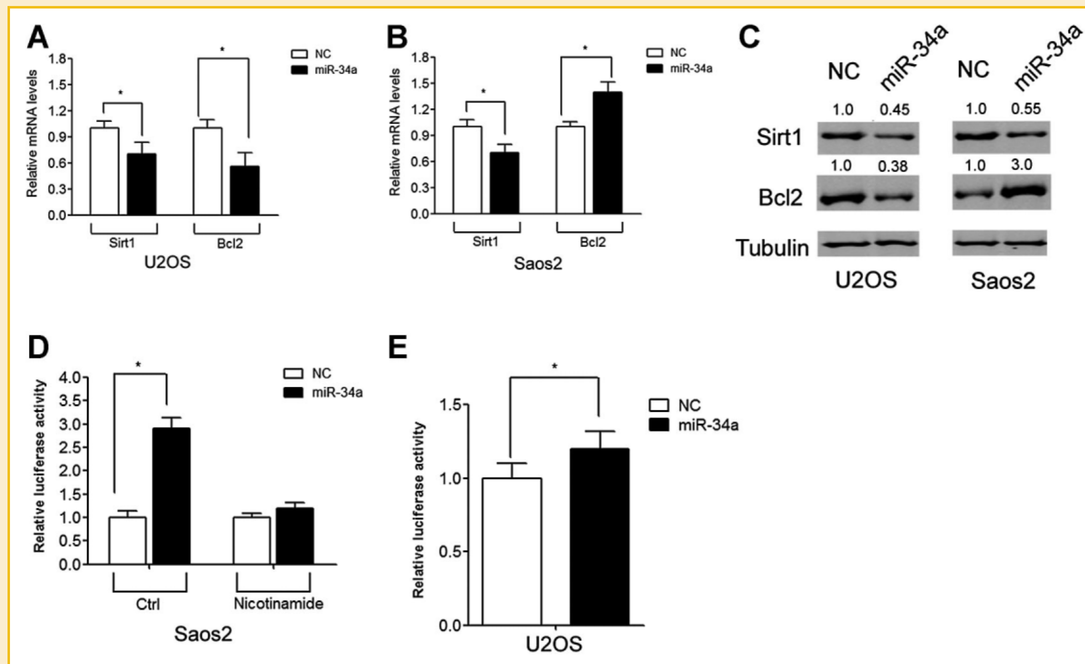

Fig. 2. miR-34a increased the NFκB activity in a Sirt1 dependent manner. Expression of the two known targets (Sirt1 and Bcl2) at mRNA level in miR-34a and NC transfected in U2OS (A) and Saos2 cells (B) were examined by RT-PCR. ($n = 3$). C: U2OS and Saos2 cells were treated same as above and expression of Sirt1 and Bcl2 at protein level was detected by western blot. Relative expression levels of Sirt1 and Bcl2 were quantified by Image J and indicated above each lane. D: Saos2 cells seeded in 24-well plate were co-transfected with 3×κB reporter, phRL-TK control vector, and NC/miR-34a. Cells with the above transfection were additionally treated with vehicle or 2 mM nicotinamide. NFκB activity was tested using the dual luciferase reporter assay. ($n = 3$, $^{*}P < 0.05$). E: U2OS cells seeded in 24-well plate were co-transfected with above reporter vectors and NC/miR-34a. NFκB activity was tested using the dual luciferase reporter assay ($n = 3$).

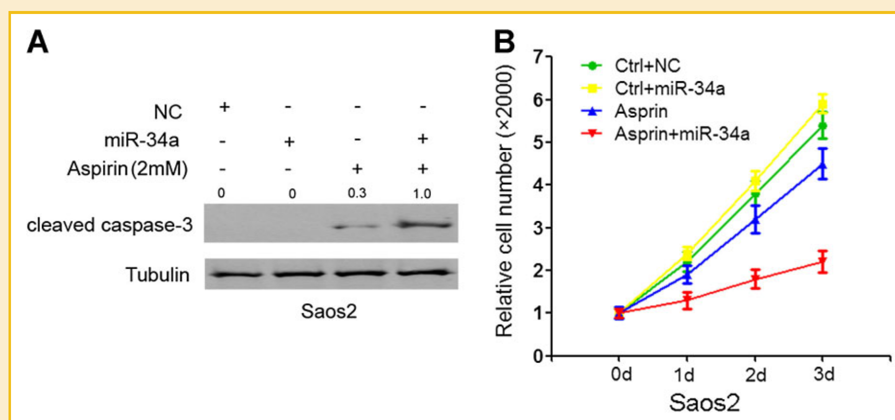

Fig. 3. Combination of miR-34a restoration and inhibition of NFκB rescues the sensitivity of Saos2 cells to apoptosis. A: Saos2 cells with NC or miR-34a transfection were further treated with 2 mM Aspirin or vehicle and cell apoptosis was observed by caspase-3 cleavage. Relative expression level of the cleaved caspase-3 was quantified by Image J and indicated above each lane. B: Cells were treated as indicated. Viable cell numbers were determined at indicated times by MTT assay. The beginning of cell treatment was set as time zero. Data are expressed as means ± SD ($n = 3$). $^{*}P < 0.05$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

and NFκB inhibition holds as a promising therapeutic strategy in p53 deficient cancers.

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