Nutlin-3 induces *BCL2A1* expression by activating ELK1 through the mitochondrial p53-ROS-ERK1/2 pathway

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Abstract. Nutlin-3 which occupies the p53 binding pocket in HDM2, has been reported to activate apoptosis through both the transcriptional activity-dependent and -independent programs of p53. Transcription-independent apoptosis by nutlin-3 is triggered by p53 which is translocated to mitochondria. However, we previously demonstrated that the nutlin-3-induced mitochondrial translocation of p53 stimulates ERK1/2 activation, an anti-apoptosis signal, via mitochondrial ROS generation. We report on how nutlin-3-stimulated ERK1/2 activity inhibits p53-induced apoptosis. Among the anti-apoptotic BCL2 family proteins, *BCL2A1* expression was increased by nutlin-3 at both the mRNA and protein levels, and this increase was prevented by the inhibition of ERK1/2. TEMPO, a ROS scavenger, and PFT-μ, a blocker of the mitochondrial translocation of p53, also inhibited *BCL2A1* expression as well as ERK1/2 phosphorylation. In addition, nutlin-3 stimulated phosphorylation of ELK1, which was prevented by all compounds that inhibited nutlin-3-induced ERK1/2 such as U0126, PFT-μ and TEMPO. Moreover, an increase in *BCL2A1* expression was weakened by the knockdown of ELK1. Finally, nutlin-3-induced apoptosis was found to be potentiated by the knockdown of BCL2A1, as demonstrated by an increase of in hypo-diploidic cells and Annexin V-positive cells. Parallel to the increase in apoptotic cells, the knockdown of BCL2A1 augmented the cleavage of poly(ADP-ribose) polymerase-1. It is noteworthy that the augmented levels of apoptosis induced by the knockdown of BCL2A1 were comparable to those of apoptosis induced by U0126. Collectively, these results suggest that nutlin-3-activated ERK1/2 may stimulate the transcription of *BCL2A1* via the activation of ELK1, and *BCL2A1* expression may contribute to the inhibitory effect of ERK1/2 on nutlin-3-induced apoptosis, thereby constituting a negative feedback loop of p53-induced apoptosis.

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Abbreviations: BCL2, B-cell CLL/lymphoma 2; BCL2A1, BCL2-related protein A1; BCLXL, BCL2-like 1; BCLW, BCL2-like 2; CREB1, cAMP response element binding protein 1; ELK1, member of ETS oncogene family; ERK, extracellular signal-regulated kinases; HDM2, human double minute 2; MAPK, mitogen-activated protein kinase; MCL1, myeloid cell leukemia sequence 1; MEK, MAPK/ERK kinase; P-CREB, phospho-CREB1 (S133); P-ELK1, phospho-ELK1 (S383); P-ERK1/2, phospho-ERK1/2 (T202/Y204); P-MEK1/2, phospho-MEK1/2 (S217/221); PFT, pifithrin; ROS, reactive oxygen species; PI, propidium iodide; TEMPO 2, 2, 6, 6-tetramethyl-1-piperidinyloxy

Key words: BCL2-related protein A1, ETS-domain protein Elk-1, extracellular signal-regulated kinase 1/2, Nutlin-3, tumor suppressor protein p53

Introduction

The tumor suppressor protein p53 is a principal modulator of various anti-carcinogenesis effects such as apoptosis, cell cycle arrest, senescence, and DNA repair (1,2). Regarding the induction of apoptosis, it is generally assumed that the activation of the mitochondrial apoptotic pathway by transcriptional target genes of p53 such as NOXA, PUMA, BAX and APAF-1 is the major pathway (3). In addition to transcription-dependent apoptosis by p53, p53 was shown, more than a decade ago, to move to the mitochondria and, when there, trigger intrinsic apoptosis (4). Mitochondrial p53 attenuates anti-apoptotic activity of BCLXL through direct binding to it, thereby leading to the oligomerization of BAK and the subsequent formation of pores in the mitochondrial outer membranes, through which cytochrome c, the pivotal inducer of intrinsic apoptosis, exits to the cytosol (4,5). In skin epidermal cells, TPA directs nuclear p53 to move to mitochondria where p53 interacts with manganese superoxide dismutase (MnSOD), resulting in a decrease of mitochondrial membrane potential which leads to the release of cytochrome c (6). Based on experimental findings demonstrating that the mitochondrial translocation of p53 occurs earlier than the transcriptional induction of p53 target genes, and artificial expression of p53 targeted to mitochondria which lacks transcriptional activity induces apoptosis

efficiently in cancer cells *in vitro* and *in vivo* (7,8), it has been suggested that mitochondrial p53 may have a more important role than nuclear p53 in the induction of apoptosis and may be a sole apoptosis-inducing stimulus. In addition, a recent study reported that mitochondrial p53 triggered mitochondrial permeability transition pore (MPTP) opening by interacting with cyclophilin D (9). The binding of p53 to cyclophilin D was shown to occur under conditions of oxidative stress and to activate necrosis, instead of apoptosis, during ischemia-reperfusion injury of the brain. Therefore, it can be speculated that mitochondrial p53 could induce different forms of cell death depending on the cellular contexts and the nature of the stimuli, and its role as well as the underlying mechanism of mitochondrial p53 in apoptosis induction should be clarified for a complete understanding of p53-induced cancer cell death.

Nutlin-3, a *cis*-imidazoline analog which upregulates the p53 protein by disrupting interactions between p53 and HDM2, is capable of inducing p53-dependent apoptosis in various cancer cells including leukemia and multiple myeloma cells (10,11). To be consistent with the mitochondrial trafficking of p53 by anticancer therapeutics and p53-overexpression plasmids, nutlin-3-upregulated p53 also moves to mitochondria and triggers the intrinsic apoptotic pathway (12,13). This mitochondrial p53 induced by nutlin-3 was found to be sufficient to induce apoptosis, and moreover, the inhibition of transcriptional activity of p53 potentiates nutlin-3-induced apoptosis, suggesting that the mitochondrial translocation of p53 may be the primary and major initiator for nutlin-3-induced apoptosis.

In a previous study, however, we reported that mitochondrial p53 stimulates the activation of the MEK1/2-ERK1/2 pathway in cancer cells treated with nutlin-3 (14). This activation of MEK1/2 and ERK1/2 was attributed to an accumulation of mitochondrial ROS caused by mitochondrial p53, and was found to suppress nutlin-3-induced apoptosis, suggesting the possibility that mitochondrial p53 can induce cell survival pathways, thus counteracting p53-induced apoptosis, depending on the type of cancer cells. Although the inhibition of ERK1/2 was shown to potentiate nutlin-3-induced apoptosis, the mechanism how ERK1/2 suppresses apoptosis in nutlin-3-treated cells remains unclear. To address this issue, we report on attempts to identify a member of the anti-apoptotic BCL2 family which is expressed as a function of the level of ERK1/2 activity and inhibits nutlin-3-induced apoptosis as well.

Materials and methods

Reagents. Nutlin-3 and U0126 were purchased from Selleckchem (Houston, TX, USA) and Tocris (Ellisville, MO, USA), respectively. All the other chemicals were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA), unless specified otherwise. The reagents were of molecular biology or cell culture tested grade.

Cell culture. The human osteosarcoma cells U2OS and SAOS were maintained in DMEM (Hyclone, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (Hyclone), 100 U/ml penicillin (Hyclone) and glutamine (Invitrogen, Carlsbad, CA, USA) at 37˚C in a 5% CO_2-humidified incubator.

Antibodies. Rabbit anti-BCL2A1 and other rabbit antibodies against ERK1/2, phospho-CREB1, and phospho-MEK1/2 were purchased from Abcam (Cambridge, UK) and Cell Signaling Technology (Boston, MA, USA), respectively. All the other antibodies were also commercially obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA, mouse antibodies against phospho-ERK1/2, phospho-ELK1, and p53), Epitomics (Burlingame, CA, USA, rabbit anti-MEK1/2 and anti-CREB1), Merck (Billerica, MA, USA, chicken anti-GAPDH), Sigma-Aldrich Inc. (HRP-conjugated anti-rabbit or -mouse IgG) and KPL (Gaithersburg, MD, USA, HRP-conjugated anti-chicken IgG).

Transfection of small interfering RNAs (siRNAs). SiRNA against p53 was obtained from Santa Cruz Biotechnology, and siRNAs against *CREB1* and *BCL2A1* were obtained from Sigma-Aldrich Inc. *ELK1* siRNA was purchased from Bioneer (Daejeon, Korea). Transfections of siRNAs were carried out using Lipofectamine™ RNAiMAX (Invitrogen), following the manufacturer's instructions.

Immunoblot analysis. Cells treated as described in the figure legends were lysed in RIPA buffer supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland), and were subjected to immunoblot analysis. Briefly, 20 μg aliquots of lysates were separated on SDS-polyacrylamide gels and then, were transferred to nitrocellulose membranes (Merck). After submerging in 5% skim-milk/TTBS (Tris-buffered saline containing Tween-20 0.025%) for 30 min, the membranes were incubated in 3% BSA/TTBS containing primary antibodies, washed with TTBS and then incubated with HRP-conjugated anti-IgG. The protein bands that reacted with antibodies were then detected using enhanced chemiluminescence reagents (ECL, GE Healthcare, Buckinghamshire, UK).

Quantitative real-time RT-PCR (QRT-PCR). Total RNA extracted using RNAiso Plus (Takara Bio Inc., Shiga, Japan) was subjected to QRT-PCR. Briefly, cDNA was generated using PrimeScript™ RT reagent kit (Takara Bio Inc.) and QRT-PCR was performed using SYBR FAST qPCR kit (Kapabiosystems, Woburn, MA, USA). All reactions were performed in triplicate by the ABI 7300 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). Relative changes in transcript level normalized by GAPDH mRNA were calculated by the $\Delta\Delta$Ct method (15)

Apoptosis assay. Apoptosis was determined by ApoScan kit (BioBud, Gyunggido, Korea) according to a previous report (16). Briefly, cells treated as indicated in the figure legends were stained with Annexin V-Fluos at room temperature for 15 min, washed and resuspended in binding buffer. Propidium iodide (PI, 1 μg/ml) was added and stained cells were then analyzed using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). For the measurement of hypo-diploidic cells, cells were incubated in 70% ethanol solution for 2 h, and then stained with propidium iodide for 15 min, followed by flow cytometric analysis (17). Cell cycle distribution of the PI-stained cells was analyzed by the CellQuest and Modfit software following the manufacturer's instructions.

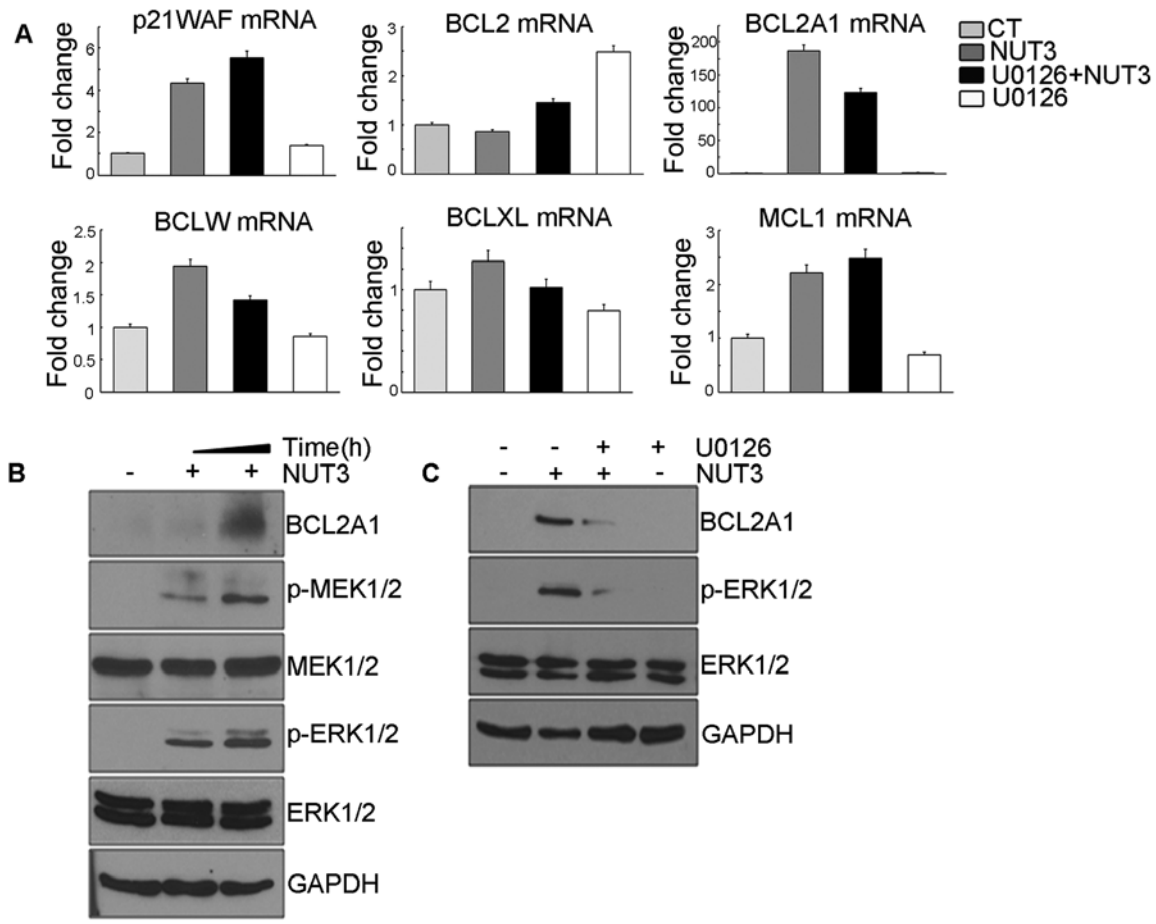

Figure 1. The effect of U0126 pretreatment on nutlin-3-induced expression of *BCL2A1*. (A) U2OS cells were incubated in the presence of 10 μM U0126 for 1 h and treated with 20 μM nutlin-3 for an additional 24 h. Total cellular RNAs were then extracted and subjected to QRT-PCR against the indicated genes as described in Materials and methods. The value of gene expression changes is expressed as the mean ± SD of three independent experiments each of which was measured in triplicate. (B) At 24 and 48 h after U2OS cells were treated with 20 μM nutlin-3, total cell lysates were prepared and subjected to immunoblot analysis against indicated proteins. (C) Total cell lysates of U2OS cells treated as described in (A) were subjected to immunoblot analysis against indicated proteins. GAPDH was used for equal protein loading in immunoblot analysis.

Results

Treatment of nutlin-3 induces the expression of BCL2A1 gene by activating ERK1/2. We attempted to identify genes that are responsible for the anti-apoptotic effect of nutlin-3-induced ERK1/2 activity. To this end, we first compared changes in the expression levels of anti-apoptotic BCL2 family members in nutlin-3-treated U2OS cells. As shown in Fig. 1A, nutlin-3 increased the mRNA levels of *BCL2A1, BCLXL* and *BCLW*. Among these genes, the mRNA increase of *BCL2A1* and *BCLW,* but not *BCLXL* was suppressed by U0126 pretreatment. Although the expression of *BCLW* is also likely to be regulated by ERK1/2, its induction level was <2-fold and so, we analyzed the mechanism involved in the expression of *BCL2A1* and its effect on apoptosis in this model. The expression of *p21WAF1*, a well-known transcriptional target gene of p53, was also induced by nultin-3, but this induction was not reduced by U0126 pretreatment, confirming that U0126 had no effect on the transcriptional activity of p53 and thus, nutlin-3-induced *BCL2A1* expression is robustly regulated by ERK1/2. Consistent with the induction of mRNA by nutlin-3, the expression level of the BCL2A1 protein was increased by nutlin-3 along with the phosphorylation of MEK1/2 and ERK1/2 (Fig. 1B) and this increase was also suppressed by U0126 pretreatment (Fig. 1C). Collectively, these data suggest that nutlin-3-induced ERK1/2 activity should stimulate the expression of *BCL2A1* at both the mRNA and protein levels.

Mitochondrial p53 is critical in the induction of BCL2A1 expression. Since nutlin-3 is an antagonist of HDM2 which ubiquitylates p53 family proteins such as p63 and p73 as well as p53, nutlin-3 can activate p73-dependent apoptosis in cancer cells which have mutated *p53* gene (18). Thus, we proceeded to determine the role of p53 in this nutlin-3-induced *BCL2A1* expression. In experiments using small interfering RNA against *p53*, nutlin-3 did not increase either *BCL2A1* mRNA or protein in p53-knocked down U2OS cells (Fig. 2A) or in SAOS (human osteosarcoma) cells in which the *p53* gene was mutated (Fig. 2B), confirming the dependency of *BCL2A1* induction on the intact p53 protein. We previously showed that nutlin-3-induced ERK1/2 activation was dependent on mitochondrial ROS generated by mitochondrial p53 (14), which led us to speculate that nutlin-3-induced *BCL2A1* expression would be also dependent on both the mitochondrial

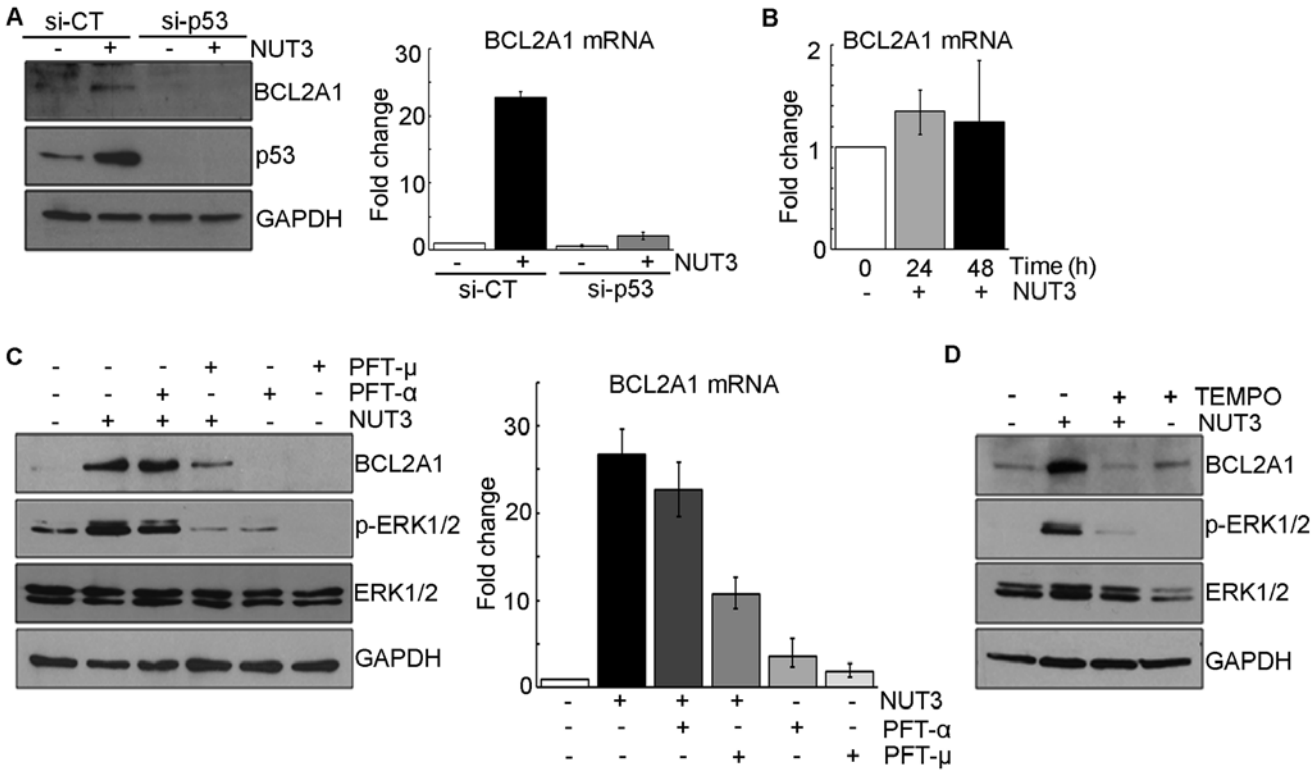

Figure 2. The effect of *p53* siRNA on nutlin-3-induced *BCL2A1* expression. (A) U2OS cells transfected with *p53* siRNA (si-p53) or scrambled siRNA (si-CT) as a negative control for 48 h were treated with vehicle or 20 μM nutlin-3 for an additional 24 h. Total cellular RNAs and whole cell lysates were then extracted and subjected to QRT-PCR against *BCL2A1* and *GAPDH* (right panel), and immunoblot analysis against the indicated antibodies (left panel), respectively. GAPDH was used as an internal reference for QRT-PCR and a loading control of proteins for immunoblot analyses. (B) SAOS cells were treated with indicated concentrations of nutlin-3. After 24 h- and 48 h-treatment, total cellular RNAs were extracted and processed for QRT-PCR of *BCL2A1* with *GAPDH* as an internal reference. (C) U2OS cells were incubated with vehicle, PFT-α or PFT-μ for 1 h and treated with vehicle or 20 μM nutlin-3 for an additional 24 h. Total cellular RNA and whole cell lysates were then prepared and subjected to QRT-PCR (right panel) and immunoblot analysis (left panel) against indicated genes, respectively. GAPDH was used as an internal reference for QRT-PCR and a loading control of proteins for immunoblot analyses. (D) Cell lysates of U2OS cells pretreated with vehicle or 2 mM TEMPO for 1 h and treated with vehicle or 20 μM nutlin-3 for an additional 24 h, were subjected to immunoblot analysis against indicated proteins and GAPDH as a loading control. The value of gene expression changes presented in QRT-PCR result of (A-C) is the mean ± SD of three independent experiments each of which was measured in triplicate.

translocation of p53 and ROS. As expected, the nutlin-3-induced expression of both *BCL2A1* mRNA and protein was suppressed by pretreatment with PFT-μ and TEMPO, an inhibitor of the mitochondrial translocation of p53 and a radical scavenger, respectively, which prevented the phosphorylation of ERK1/2, but not in the case of pretreatment with PFT-α, an inhibitor of the transcriptional activity of p53 (Fig. 2C and D and data not shown). Accordingly, these data suggest that nutlin-3-induced *BCL2A1* expression is also dependent on the mitochondrial translocation of p53 and ROS generation which are critical regulators of ERK1/2 activation in nutlin-3-treated U2OS cells.

Induction of BCL2A1 expression is mediated by ELK1. Next, we attempted to identify the signaling molecules downstream of ERK1/2 that induce the expression of *BCL2A1*. It is well known that ERK1/2 stimulates the activity of several transcription factors, including ELK1 and CREB1 by directly or indirectly phosphorylating them, which leads to the transcriptional induction of a variety of genes (19,20). In this model, nutlin-3 also increases the phosphorylation of CREB1 and ELK1, which prompted us to analyze the effect of these transcriptional factors on *BCL2A1* expression (Fig. 3A). In experiments using siRNAs to knock down *ELK1* and *CREB1*, nutlin-3-induced *BCL2A1* expression was suppressed by the knockdown of *ELK1* (Fig. 3C) but not *CREB1* (Fig. 3B), suggesting that the ELK1 protein is required for the nutlin-3-induced transcription of *BCL2A1*. Moreover, compounds such as U0126, PFT-μ, and TEMPO which prevented both the nutlin-3-induced phosphorylation of ERK1/2 and the expression of *BCL2A1* also inhibited the phosphorylation of ELK1 (Fig. 3D-F). In contrast, PFT-α, an inhibitor of the transcriptional activity of p53, which did not prevent either the nutlin-3-induced phosphorylation of ERK1/2 or the expression of *BCL2A1*, also failed to inhibit the phosphorylation of ELK1 (Fig. 3E). These results collectively suggest that ROS generated by the nutlin-3-induced mitochondrial translocation of p53 activates ERK1/2, which in turn activates ELK1, finally leading to the transcriptional induction of *BCL2A1*.

BCL2A1 inhibits nutlin-3-induced apoptosis. Finally, we analyzed the effect of BCL2A1 protein expression on nutlin-3-induced apoptosis. As shown in Fig. 4A and C, the nutlin-3-induced accumulation of both hypo-diploid cells in the sub-G1 phase and Annexin V-positive cells was potenti-

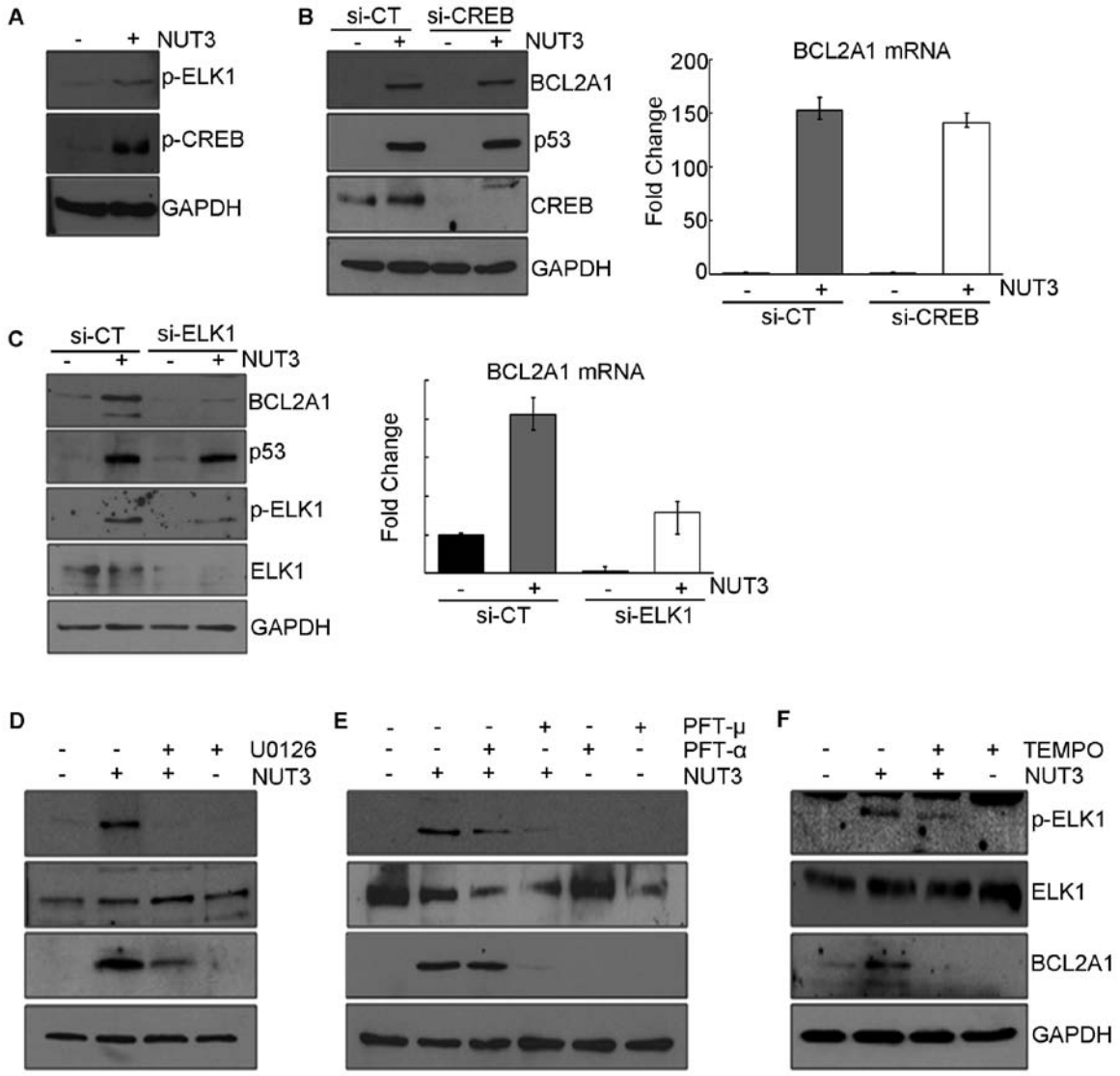

Figure 3. The effect of ELK1 on nutlin-3-induced *BCL2A1* expression. (A) Whole cell lysates of U2OS cells treated with vehicle or 20 μM nutlin-3 for 24 h were subjected to immunoblot analysis against indicated proteins along with GAPDH as a loading control. (B) U2OS cells were transfected with *CREB1* siRNA (si-CREB) or scrambled siRNA (si-CT) as a negative control for 48 h and then treated with vehicle or 20 μM nutlin-3. At 24 h post-treatment, total cellular RNAs and whole cell lysates were extracted and subjected to QRT-PCR against *BCL2A1* and *GAPDH* (right panel) and immunoblot analyses against indicated proteins (left panel), respectively. GAPDH was used as an internal reference for QRT-PCR and a loading control of proteins for immunoblot analyses. (C) U2OS cells were transfected with *ELK1* siRNA (si-ELK1) or scrambled siRNA (si-CT) as a negative control for 48 h and then treated as described in (B). Total cellular RNAs and whole cell lysates were prepared and subjected to QRT-PCR (right panel) and immunoblot analysis (left panel) against indicated proteins, respectively. (D-F) The effect of U0126, PFT-μ, and TEMPO on the nutlin-3-induced ELK1 phosphorylation. Whole cell lysates of U2OS cells pretreated with U0126 (D), PFT-μ or PFT-α (E), or TEMPO (F) for 1 h and treated with 20 μM nutlin-3 for an additional 24 h were subjected to immunoblot analysis against indicated proteins and GAPDH as a loading control.

ated by *BCL2A1* knockdown, suggesting that BCL2A1 has an inhibitory effect on nutlin-3-induced apoptosis. Consistent with the assessment of apoptosis, the knockdown of *BCL2A1* augmented the expression of cleaved poly(ADP-ribose) polymerase-1 (PARP-1) which is a hallmark of apoptosis (Fig. 4B), without affecting the phosphorylation level of ERK1/2 and ELK1. The level of apoptosis induction by the combined treatment of nutlin-3 and siRNA against *BCL2A1* appeared to be close to that for the combined treatment of nutlin-3 and U0126 (Fig. 4C), implying that anti-apoptotic effect of the BCL2A1 protein should be restricted to anti-apoptotic functions of nutlin-3-activated ERK1/2, and that BCL2A1 might be a gene responsible for the anti-apoptotic activity of ERK1/2.

Discussion

Contrary to reports that nutlin-3 induces apoptosis via the mitochondrial translocation of p53 in cancer cells, our previous study concluded that the nutlin-3-induced mitochondrial translocation of p53 stimulated the activation of MAPK such as ERK1/2, JNK and p38 MAPK via the generation of mitochondrial ROS (14,21). This MAPK exerted anti-apoptotic effect, suggesting that MAPK activated by mitochondrial p53 may constitute a negative feedback loop of p53-induced apoptosis. Whereas the JNK and p38 MAPK induced the expression of heme oxygenase-1 (HO-1), an anti-apoptotic protein, we propose that *BCL2A1* may be a downstream gene of activated ERK1/2 and may suppress nutlin-3-induced apoptosis.

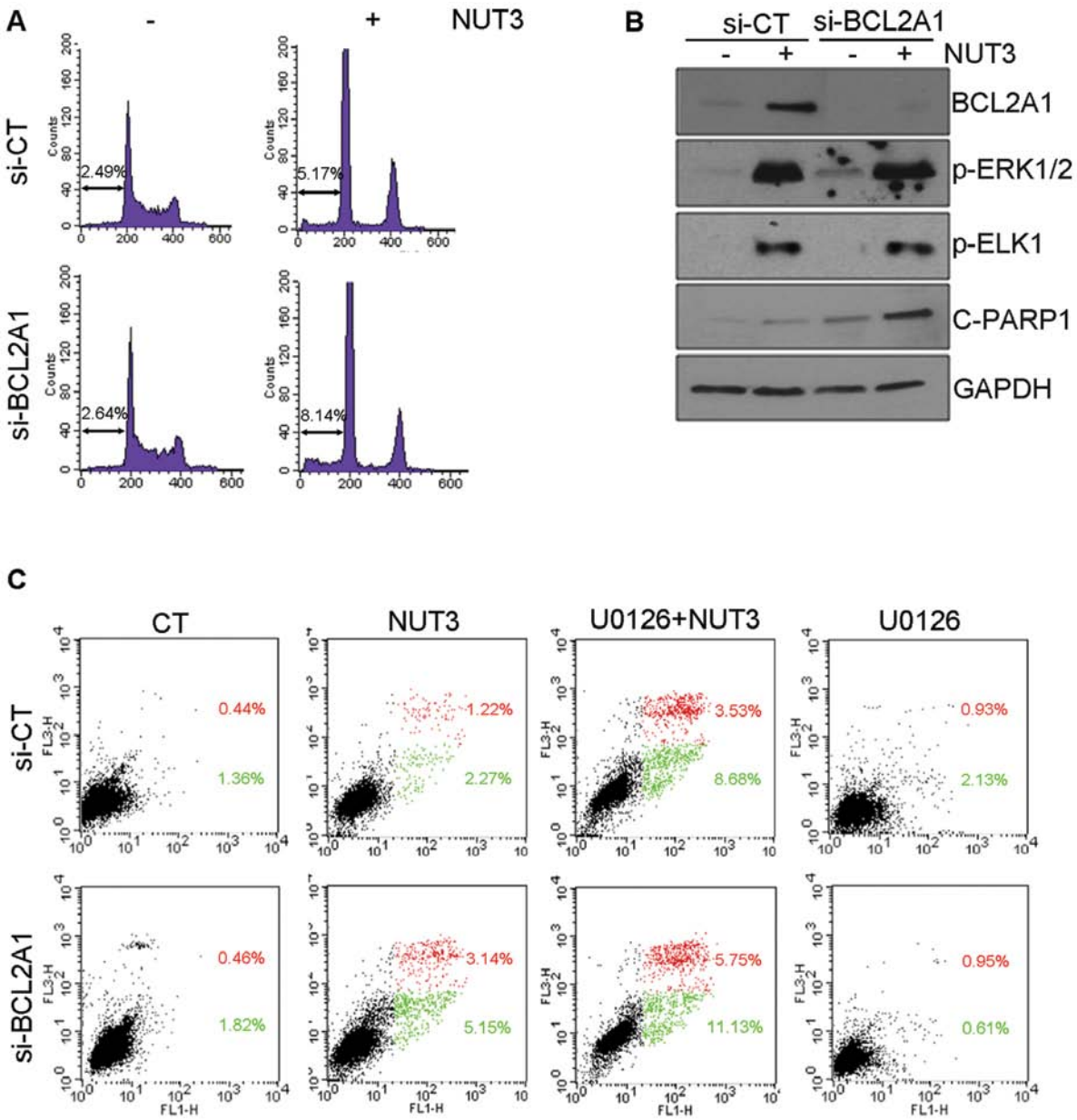

Figure 4. The effect of BCL2A1 protein expression on nutlin-3-induced apoptosis. (A) U2OS cells transfected with *BCL2A1* siRNA (si-BCL2A1) or scrambled siRNA (si-CT) as a negative control for 48 h were treated with vehicle or 20 μM nutlin-3 for an additional 24 h. Cells were then fixed and stained with propidium iodide (PI) for flow cytometric analysis as described in Materials and methods. Cell cycle distribution was analyzed on FACSCalibur. (B) Lysates of U2OS cells transfected with siRNAs and treated with nutlin-3 as described in (A) were subjected to immunoblot analysis against indicated proteins. GAPDH was used for equal protein loading. (C) U2OS cells transfected with *BCL2A1* siRNA (si-BCL2A1) or scrambled siRNA (si-CT) as a negative control for 48 h were incubated in the presence of vehicle or 10 μM U0126 for an additional 1 h, and then treated with vehicle or 20 μM nutlin-3. After 24 h of nutlin-3 treatment, the cells were harvested and stained with Annexin V along with PI, followed by flow cytometric analysis on FACSCalibur. Green dots and red dots indicate early and late apoptotic cells, respectively. C-PARP-1, cleaved poly(ADP-ribose) polymerase-1.

BCL2A1 which belongs to the pro-survival BCL2 family has been reported to be expressed at the level of transcription by inflammatory cytokines, CD40, and oxidative stress in endothelial cells, B lymphocytes, and leukemic cells, respectively (22-24). Elevated BCL2A1 protein prevents apoptosis induced by various stimuli including TNF-α, TRAIL, Fas, and chemotherapeutic agents by directly binding to tBid and BAK (24-29). Regarding the transcriptional induction of *BCL2A1*, it should be noted that NF-κB has been invariably required for all the stimuli reported until now to induce *BCL2A1* expression, indicating that *BCL2A1* is a transcriptional target gene of NF-κB and a possible component of an anti-apoptotic branch of the NF-κB-activated pathways. However, in the present model, the expression of *BCL2A1* was upregulated by ELK1. The phosphorylation of ELK1 seemed to be induced by ERK1/2 which was activated by ROS generated by mitochondrial p53. Therefore, the data presented herein can be summarized as shown in Fig. 5, in which the proposed cell survival pathway is comprised of the sequential induction of mitochondrial p53, ROS generation, ERK1/2 activation, ELK1 activation, and expression of the *BCL2A1* gene. This induction of BCL2A1 protein expression suppressed the acti-

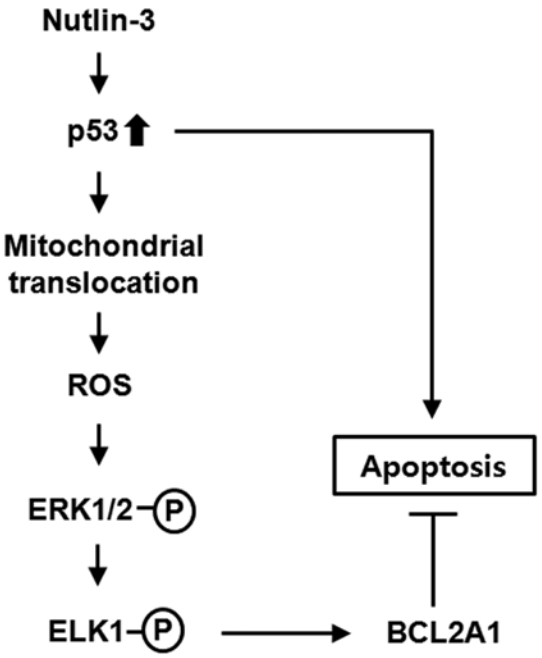

Figure 5. Schematic diagram of the signal transduction pathway leading to nutlin-3-induced *BCL2A1* expression.

vation of the nutlin-3-induced apoptosis program, implying that this pathway might constitute a negative feedback loop of p53-induced apoptosis.

ELK1 is a member of the ternary complex factor (TCF) subfamily and is activated by mitogenic or growth factors such as EGF. ELK1 functions as a transcription factor and regulates the transcription of various genes that are involved in cell growth, differentiation, and survival (30). Among the pro-survival BCL2 proteins, *MCL1* was found to be a transcriptional target of ELK1. For instance, it was reported that EGF and ovarian cancer ascites cause an increase in MCL1 expression by activating ELK1 in breast and ovarian cancer cells (27,31). This ELK1-MCL1 pathway could be a potential target of cancer therapy, as sorafenib, an inhibitor of multiple kinases, induces apoptosis of endometrial carcinoma cells by interfering with ELK1-dependent *MCL1* transcription (32).

In the present model, although nutlin-3 treatment induced ROS accumulation, NF-κB was not activated, and *MCL1* expression was not induced, even in the presence of activated ELK1. Therefore, it can be postulated that intracellular ROS may induce *BCL2A1* expression by directly activating NF-κB and/or by activating ELK1 via the MEK1/2-ERK1/2 pathway. The mechanism involved, regarding which of these two pathways are selected, remains to be clarified.

Nutlin-3 may be a promising anticancer agent, since it specifically activates p53-dependent anticancer programs without genomic DNA damage, which exerts adverse effects on non-transformed cells leading to cancer patients encountering difficulties in adjusting to systemic and conventional anticancer treatments such as radiotherapy and chemotherapy, and even in the case of secondary tumor development (33,34). One of the features of nutlin-3 is that it induces prominent cell cycle arrest with subtle apoptosis in some solid cancer cells such as U2OS cells used in this study, while it induces substantial apoptosis particularly in leukemia and lymphoma cells (35). Although cell cycle arrest can blunt the initiation and progression of cancer, it can also diminish the therapeutic efficacy of anticancer agents (36). The induction of prominent cell cycle arrest by nutlin-3 was suggested to be due to the dramatic expression of p21WAF1 and downregulation of HIPK2 (37,38). The negative feedback loop proposed in this report could thus provide a novel explanation for nutlin-3-induced subtle apoptosis, in part at least, because the blockade of this loop enhanced apoptosis, while no interaction with p21WAF1 and HIPK2 occurred.

A high level of BCL2A1 expression in cancer tissues such as stomach, melanoma, and leukemia has been reported, and its level of expression appears to be correlated with metastasis and the poor prognosis of such types of cancers (39-41). Accordingly, the knockdown of BCL2A1 protein expression was found to sensitize B cell lymphoma cells to apoptosis induced by anticancer chemicals (42). Furthermore, it was recently reported that BCL2A1 confers melanoma and lymphoma cells resistance to vemurafenib (PLX4032), an inhibitor of BRAF, and ABT-737, a small molecule antagonist of BCL2, respectively (43,44). Taking these findings into account, the findings suggest that *BCL2A1* might be expressed via multiple signal transduction pathways including NF-κB stimulated by molecular target therapeutics to cancer, and its expression would diminish their therapeutic efficacy, resulting in the development of an acquired resistance. Therefore, the pathway proposed in the present study comprises the mechanism underlying the induced expression of the BCL2A1 protein by a small molecule in cancer cells and could contribute to the efficient use of small molecular therapeutics against cancer including nutlin-3.

Acknowledgements

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