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p19^{ras} amplifies p73β-induced apoptosis through mitochondrial pathway

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ABSTRACT

p73 and p53 have been known to play an important role in cellular damage responses such as apoptosis. Although p73 is a structural and functional homolog of p53 tumor suppressor gene, much less is known about the mechanism of p73-induced apoptotic cell death. In this study, we demonstrate that p19^{ras} interaction with p73β amplifies p73β-induced apoptotic signaling responses including Bax mitochondrial translocation, cytochrome *c* release, increased production of reactive oxygen species (ROS) and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). Furthermore, endogenous expression of p19^{ras} and p73β is significantly increased by Taxol treatment, and Taxol-enhanced endogenous p73β transcriptional activities are further amplified by p19^{ras}, which markedly increased cellular apoptosis in p53-null SAOS2 cancer cell line. These results have important implications for understanding the molecular events of p19^{ras} to p73 functions in cancer cells.

Apoptosis is a fundamental physiological process which maintains cell homeostasis. It is one of the main types of programmed cell death and involves a series of biochemical events leading to a cellular morphological changes and cell death [1]. Transcription factor p53 exerts its tumor suppressor function, to a large extent, by regulating expression of target genes that control cell-cycle progression and apoptosis [2]. By means of enhancing the expression of pro-apoptotic proteins in cancer cells, many chemotherapeutic agents target p53 tumor suppressor pathway [3].

p73 is a p53-related protein that shares a high degree of amino acid sequence identity with p53 and many of the same structural features [4]. In this regard, p73 can bind to the consensus p53-binding DNA and activate the expression of genes containing such a sequence as well as induction of apoptosis [5]. Recent studies show that p73 is also activated by DNA-damaging agents, including commonly used chemotherapies [6]. Thus, to overcome the resistance to p53-mediated apoptosis, p53 relatives, p73 is candidate for the anti-cancer therapy.

Because p73 is activated in response to some therapeutic agents, it is possible that enhancing p73's transcriptional activity through protein–protein interaction could be more effective strategies to increase apoptosis in many tumor cases. In this aspect, we reported that p19^{ras} is a novel and specific p73β-binding protein [7], which activates transcriptional activity of p73β by blocking MDM2-mediated p73β inhibition. p19^{ras} is an alternative splicing variant of the proto-oncogene H-ras pre-mRNA in conjunction with p21^{ras}. In contrast to p21^{ras}, p19^{ras} does not have a CAAX motif which targets the cellular plasma membrane and is localized in both the cytoplasm and nucleus [8]. Moreover, p19^{ras} has little GTP-binding activity and is devoid of two important GTP-binding sites located in exon E4A of p21^{ras} [8]. This suggests a novel pathway of Ras signaling that occurs in nucleus, involving p19^{ras} and p73β. However, little is known about the precise physiological consequences due to the interaction of p19^{ras} with p73β.

In this study, we found that p19^{ras} interaction with p73β amplifies p73β-induced apoptosis including Bax translocation, cytochrome *c* release, increased production of reactive oxygen species (ROS), and loss of mitochondrial transmembrane potential ($\Delta\Psi_m$). Furthermore, Taxol-enhanced endogenous p73β transcriptional activities are further amplified by p19^{ras}, which markedly increased cellular apoptosis in p53-null SAOS2 cancer cell line. These results might be taken into consideration when p19^{ras} is used as a cancer therapeutic agent to amplify p73's apoptotic activity in p53-inactivated cancer cells.

Materials and methods

Cell culture, transfection, and siRNA. HEK 293 and SAOS2 cells were obtained from the American type culture collection (ATCC: Manassas, VA) and maintained in DMEM or RPMI1640

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supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and penicillin–streptomycin (50 U/ml). Transient transfection was performed by Lipofectamine 2000 (Invitrogen) with different plasmid DNA, according to manufacturer's instructions. p73β siRNA was purchased from Santa Cruz Biotechnology and transfected with Lipofectamine 2000.

Flow cytometry and DNA fragmentation. After cells were trypsinized approximately 1×10^6 cells were collected by centrifugation at 1000*g* for 5 min. Cells were then washed in phosphate-buffered saline (PBS), followed by resuspension and fixation in 70% ethanol for approximately 2 h. Cellular DNA was then stained by the addition of 10 μg of propidium iodide (PI), and cells were analyzed by FACScan flow cytometer using Cellquest software (Becton–Dickinson, Franklin Lakes, NJ). Cultured cells were washed twice with PBS and lysed in buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K) at 37 °C for 18 h. DNA was extracted with equal volumes of phenol/chloroform (1:1) and was precipitated at −70 °C. DNA fragments were visualized under ultraviolet light.

In vivo binding assay and Western blotting. SAOS2 cells were seeded in 100 mm plates at an initial density of 2×10^6 cells and allowed to grow for 12 h. The cells were treated with 50 nM Taxol for 48 h, and lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.1% SDS, 1% Nonidet P-40, and 1 mM PMSF. For immunoprecipitation assays, the supernatants were pre-cleaned with 20 μl of protein A/G agarose bead (50% slurry) and then incubated at 4 °C overnight with 30 μl of protein A/G bead in the presence of appropriate antibodies. Boiled samples were analyzed by Western blotting using the appropriate antibodies to detect protein expression. The FLAG and $p19^{ras}$ protein were detected by each primary antibody (FLAG: purchased from Santa-Cruz, $p19^{ras}$: polyclonal antibodies were raised from rabbit). The polyclonal β-actin, p73, COX IV, Bax antibodies were from Santa Cruz Biotechnology.

Immunocytochemistry. HEK 293 cells were grown on poly-D-lysine-coated coverslips in 6-well plates. Cells were pre-treated with 100 nM MitoTracker CMXRos during the last 30 min of treatment, then were washed with PBS, and were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. The coverslips were soaked in a blocking solution (PBS containing 5% bovine serum albumin (BSA) and 0.2% Triton X-100) for 30 min, incubated with anti-Bax (1:300) overnight at 4 °C and then incubated with Alexa-488 anti-rabbit IgG antibodies (1:400) for 30 min in the blocking solution before mounting. Fluorescence was analyzed by confocal microscopy.

Measurement of ROS and mitochondrial transmembrane potential ($\Delta\Psi_m$). Intracellular ROS generation was monitored by flow cytometry based on fluorescence produced from the oxidation of dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR) to DCF. Cells were detached by trypsinization after incubation with 5 μM DCFH-DA during the last 30 min of treatment. To measure mitochondrial transmembrane potential ($\Delta\Psi_m$), cells were incubated with 100 nM $DiOC_6$ (Molecular Probes Inc.) during the last 30 min of treatment. Cells were then harvested by trypsinization, washed with 1× PBS, and resuspended in PBS (for $DiOC_6$) or PBS containing 25 μg/ml PI (for rhodamine 123). Fluorescence intensity was determined by flow cytometry (Becton–Dickinson), with a shift to the left indicating that fewer cells retained $DiOC_6$ in the mitochondria.

Luciferase assay. The luciferase assay was adapted as described previously [9]. Cells were cultured in 60 mm dishes and transfected with the firefly luciferase Bax reporter gene (0.1 μg) and pCMV-β-galactosidase (0.1 μg) together with pcDNA-FLAG-$p19^{ras}$ or pcDNA-FLAG-$p19^{ras}$C1 using Lipofectamine 2000. After 24 h of transfection, cells were lysed in reporter lysis buffer (Promega). Luciferase activities of the Bax-luciferase vector were normalized based on the β-galactosidase activity of the co-transfected vector.

Results

$p19^{ras}$ interaction with p73β potentiates a synergistic activation of p73β-induced apoptosis

Previous results in our group showed that $p19^{ras}$ was a direct interacting partner of p73β in the nucleus and activated p73β-dependent transcriptional activity [7]. To investigate whether $p19^{ras}$ interaction with p73β effectively induced apoptotic cell death, HEK 293 cells were co-transfected with the $p19^{ras}$ and GFP-p73β expression plasmid. When we examined the cellular DNA content profile by flow cytometric analysis, co-expression of cells with $p19^{ras}$ and p73β resulted in a prominent increase in apoptotic DNA patterns with 36.01% (Fig. 1A). Moreover, when

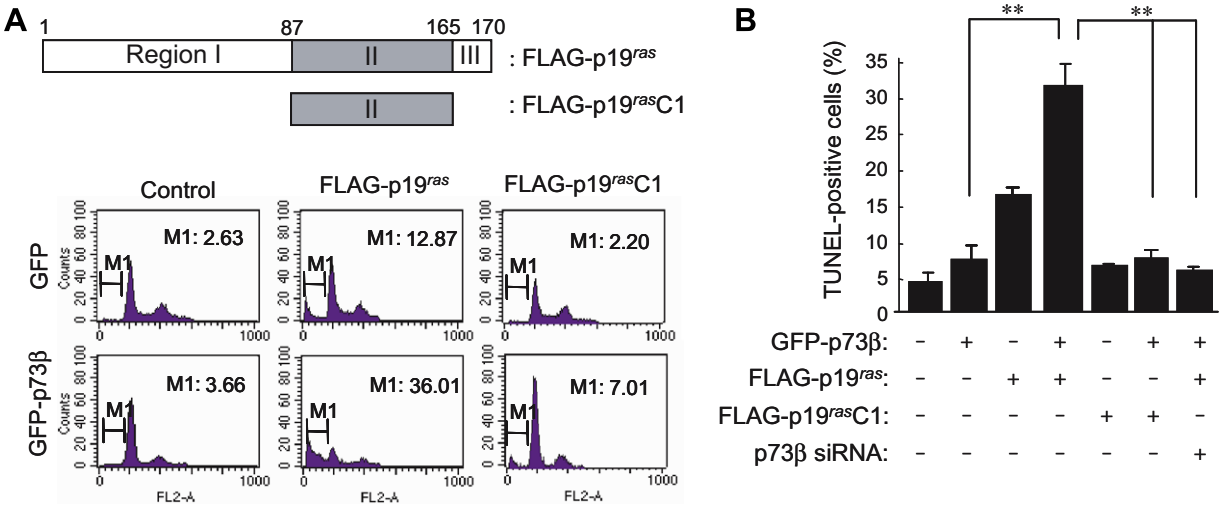

Fig. 1. A synergistic activation of p73β-induced apoptosis through $p19^{ras}$ interaction with p73β. (A) Apoptotic cells were analyzed by flowcytometry. The DNA content of M1 was examined, and the percentage of change is shown in graphic profiles. Apoptosis (M1, sub-G1 phase peak) was measured by PI staining. (B) Apoptotic cells were quantified by TUNEL assay before and after transfected with $p19^{ras}$ and p73β expressing plasmids. All values are expressed as means ± SD. Statistically significant differences in apoptosis were determined by Tukey's post-hoc test, indicated by asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$). $P < 0.05$ was considered to imply statistical significance.

we assessed genomic DNA fragmentation, p19^{ras} interaction with p73β strongly caused apoptotic DNA fragmentation (S1A). For further confirmation, we also performed TUNEL assay to determine the internucleosomal cleavage that occurs in apoptosis. TUNEL-positively labeled apoptotic cells caused by p19^{ras} interaction with p73β were increased about 26% in comparison with those of controls (Fig. 1B, lane 4). In order to investigate amplified apoptosis by the interaction of p19^{ras} with p73β are specifically modulated though p73β, we used p73β-siRNA transfectants, in which the expression of p73 proteins was almost abrogated (S1B). The ratio of p19^{ras}/p73β-amplified apoptotic cells (TUNEL positive population) were significantly reduced after p73β-siRNA transfection (Fig. 1B, lane 7). Taken together, these results indicate that p19^{ras} interaction with p73β potentiates a synergistic activation of p73β-induced apoptosis in HEK 293 cells.

p19^{ras} amplified p73β-triggered Bax translocation into mitochondria and mitochondrial cytochrome c release

We next examined the expression pattern of anti-apoptotic and pro-apoptotic in Bcl-2 family to determine the mode of apoptosis at the molecular level. As demonstrated in Fig. 2A, Bcl-2 expression was decreased in co-transfected HEK 293 cells with p19^{ras} and p73β, whereas, the expression of Bax was strongly increased after the co-transfection. Concomitant with increased Bax expression level, the released mitochondrial cytochrome *c* was strongly detected in the cytoplasm of p19^{ras} and p73β co-transfected cells. To determine whether p19^{ras} interaction with p73β induces mitochondrial translocation of Bax during apoptosis, we further examined the subcellular localization of Bax proteins in co-transfected cells with p19^{ras} and p73β. Bax proteins were significantly increased in the mitochondrial portion and decreased in the cytosolic fractions of co-transfected cells (S2A). The expression of cytochrome *c* oxidase (COX) IV was used as a control [10]. For further corroborating evidence, the interaction of Bax with voltage-dependent anion channel (VDAC) was also examined [11]. The interaction of Bax with VDAC increased remarkably in p19^{ras}/p73β co-transfected cells, compared to the cells transfected with either p19^{ras} or p73β alone (S2B). Bax translocation into mitochondria was further confirmed by immunofluorescent staining with Bax (green) and MitoTracker (red; mitochondrial marker) (Fig. 2B). FITC-Bax was primarily localized in cytosol, however, it was distributed in both of cytosol and mitochondria in co-transfected cells with p19^{ras} and p73β. These results demonstrate that p19^{ras} amplified p73β-triggered mitochondrial damage influenced by the increased level of Bax translocation to mitochondria and the release of cytochrome *c*, thereby leading to activating apoptotic cell death.

p19^{ras} interaction with p73β induces the production of reactive oxygen species (ROS) and the loss of mitochondrial transmembrane potential ($\Delta\Psi_m$)

Elevated intracellular ROS may play an important role in triggering mitochondrial cell death by inducing cytochrome *c* release and Bax translocation [11]. Therefore, we investigated production of ROS in p73β-induced apoptotic responses by measuring DCFH-DA fluorescence. Compared to those of p73β or p19^{ras} only transfected cells, significantly increased ROS production was detected in co-transfected cells with p19^{ras} and p73β (Fig. 3A, left). To determine whether changes in $\Delta\Psi_m$ influenced by p19^{ras} interaction with p73β are coincident with the ROS production during apoptosis, cells were incubated with $DiOC_6$ as an indicator of the energy state of the mitochondria (Fig. 3A, right). The fluorescence in co-transfected cells with p19^{ras} and p73β was significantly decreased compared to those of transfected cells with p73β only, or p73β plus p19^{ras}C1. However, the increased fluorescences were specifically suppressed by the pre-treatment with thiol anti-oxidant *N*-acetyl-L-cysteine (NAC) that can scavenge reactive oxygen intermediates (S3A). The NAC treatment also strongly reduced TUNEL-positive cells increased in cells co-transfected with p19^{ras} and p73β (Fig. 3B). Furthermore, p73β siRNA did markedly inhibit ROS production increased by co-transfection with p19^{ras} and p73β (S3B), suggesting that activated apoptosis by p19^{ras} interaction with p73β may be specifically modulated through the p73β-related pathway. Taken together, these results indicate that p19^{ras} interaction with p73β enhance p73β-mediated ROS production and loss of $\Delta\Psi_m$ in apoptotic cell death.

Anti-cancer drug Taxol enhances the p19^{ras} interaction with p73β in p53-null osteosarcoma SAOS2 cells

p73β was reported to be induced by microtubule dynamic inhibitor Taxol, which upregulates both p73 mRNA and stability of p73 [6]. To examine the expression levels of p73β and p19^{ras} proteins in Taxol-treated cells, we examined Western blotting using specific anti-p73β and anti-p19^{ras} antibodies. This result showed that endogenous p73β and p19^{ras} proteins were noticeably induced by Taxol treatment in a time-dependent manner (S4A). Because Taxol induces both p73β and p19^{ras}, we next examined the

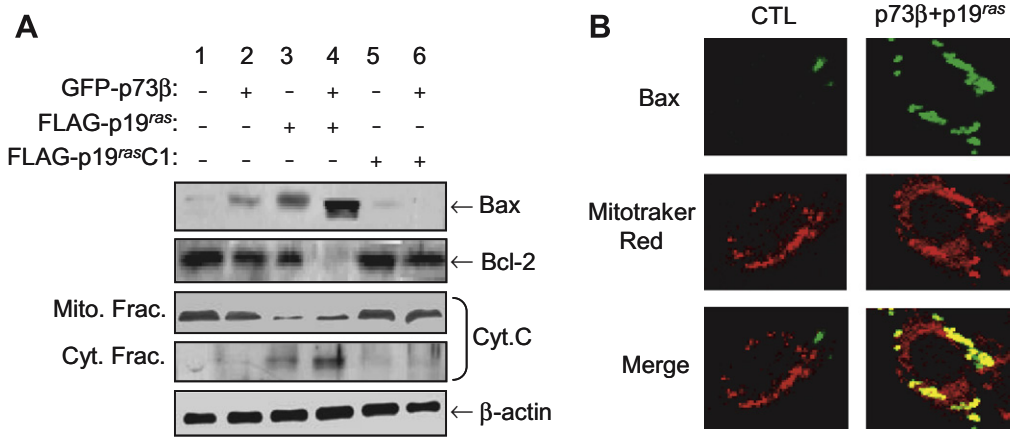

Fig. 2. p19^{ras} amplified p73β -triggered Bax translocation into mitochondria and mitochondrial cytochrome *c* release. (A) Whole cell lysates were separated on SDS–PAGE, followed by immunoblotting using specific antibodies against Bax, Bcl-2, and cytochrome *c*. (B) p19^{ras}/p73β-induced Bax translocation into mitochondria was detected by immunofluorescent staining.

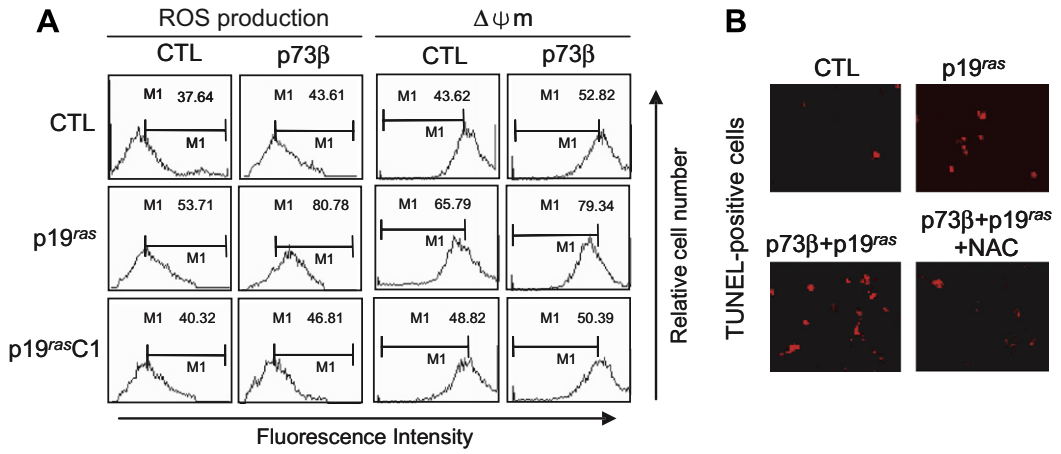

Fig. 3. p19^{ras} potentiates p73β-induced ROS production and loss of $\Delta\Psi_m$. (A) Cells were transfected with p73β-, p19^{ras}-, and p19^{ras}C1-expressing plasmids. Cells were incubated with 5 μM, DCFH-DA for ROS (left), and 100 nM, $DiOC_6$ for $\Delta\Psi_m$ (right). After 45 min, intracellular fluorescence intensity was measured by FACScan flow cytometry. (B) Cells were pre-treated with NAC for 2 h before overexpression of p19^{ras} and p73β. TUNEL assay was carried out 36 h after transfection.

interactions between p19^{ras} and p73 in a p53 independent manner. p53-null SAOS2 cells were transfected with FLAG-p19^{ras} or FLAG-p19^{ras}C1 expressing plasmids and treated with 50 nM Taxol. And then the lysates were co-precipitated with anti-FLAG antibodies. The complexes immunoprecipitated with anti-FLAG (p19^{ras}) contained p73β, however, p19^{ras}C1 does not co-precipitates p73β (S4B). To confirm p19^{ras} interacts with p73β predominantly in the nucleus, subcellular localization was assessed using immunofluorescence staining under the confocal microscopy (S4C). Taxol-induced p73β was located mainly in the nucleus in p19^{ras} or p19^{ras}C1-transfected cells. Taken together, Taxol enhances expression levels of p73β and p19^{ras}, and their interactions distributed in nucleus of p53-null SAOS2 cells.

p19^{ras} significantly amplified Taxol-induced p73β activities in cellular apoptotic responses

Next, to investigate whether p19^{ras} enhances Taxol-induced p73β activities through interaction with p73β *in vivo, we first tested the possible involvement of p19^{ras}* on endogenous p73-activated Bax transcription. p19^{ras} or p19^{ras}C1-transfected cells have treated with 50 nM Taxol, and then performed a luciferase assay using Bax promoter containing reporter plasmids. As shown in Fig. 4A, Bax transactivation induced by Taxol is potently enhanced by p19^{ras}, but not in p19^{ras}C1-transfected cells. This result indicating that p19^{ras} acts as the most potent transcriptional activator of p73β through interaction between them. In this regards, to determine whether p19^{ras} amplifies p73β-mediated apoptosis through mitochondrial pathway, we treated with Taxol in p19^{ras} or p19^{ras}C1-transfected cells, and then cellular Bax levels and cytochrome *c* release were detected by Western blotting (S5). p19^{ras} increased pro-apoptotic Bax expression level and cytochrome *c* release, whereas these changes are not detected in p19^{ras}C1-transfected Taxol-treated or -untreated cells. Moreover, Taxol treatment increases ROS production which accelerated by p19^{ras}-, not in p19^{ras}C1-transfected cells (Fig. 4B). These p19^{ras}-potentiated apoptosis was further confirmed by TUNEL assay in Taxol-treated cells. When SAOS2 cells were transfected with p19^{ras}-expressing plasmids, Taxol treatment sig-

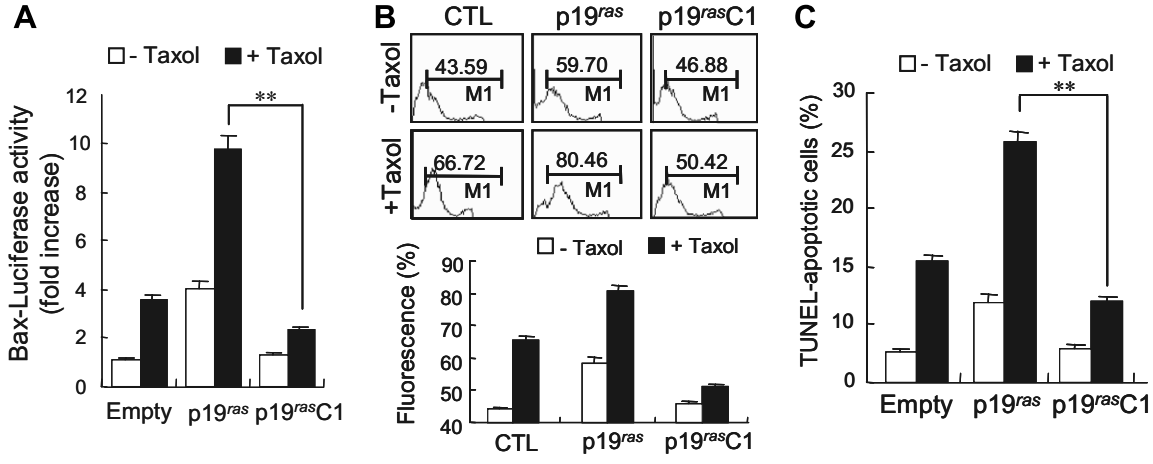

Fig. 4. p19^{ras} amplifies Taxol-enhanced endogenous p73β activities in cellular apoptotic responses. (A) SAOS2 cells were transiently co-transfected with either p19^{ras}-, p19^{ras}C1-expressing plasmids, or corresponding control plasmid together with a luciferase reporter plasmid containing the Bax promoter and pCMV-β-galactosidase. Luciferase activity was measured 36 h after transfection. All data were normalized to β-galactosidase activity. The data are expressed in relative fold increase of luciferase units. (B) Fluorescence intensity of DCF in Taxol-treated/-untreated HEK 293 cells was quantified, and the percentage of change in ROS production was determined in comparison with controls. (C) SAOS2 cells were transfected with either p19^{ras}-, p19^{ras}C1-expressing plasmids, or corresponding control plasmid. They were then treated with 50 nM Taxol for 24 h. The apoptotic ratio of the cells was quantified by the TUNEL assay.

nificantly enhanced the TUNEL-apoptotic cell ratio to 26% compared to the negative control (15%) or $p19^{ras}$C1 (12%) (Fig. 4C). Taken altogether, these results demonstrate that the endogenous expression of $p19^{ras}$ and p73β is significantly amplified by Taxol treatment, and Taxol-enhanced endogenous p73β transcriptional activities are amplified by $p19^{ras}$, by which cellular apoptosis was markedly increased in p53-null SAOS2 cancer cell line.

Discussion

It was previously reported that three *ras* genes (H-*ras*, K-*ras*, and N-*ras*) encode very similar small GTPase proteins, which cycle between active GTP-bound and inactive GDP-bound states [12]. $p19^{ras}$, an alternative splicing variant of c-H-*ras*, is localized in the cytoplasm and the nucleus, in which it associates with GTP for further signaling. Previous results in our group showed that $p19^{ras}$ was a direct interacting partner of p73β in the nucleus and activated p73β-dependent transcriptional activity of a reporter containing p21 promoter. The $p19^{ras}$ also directly interacted with MDM2, a repressor of p73, followed by blocked MDM2-mediated transcriptional repression of p73β leading to the activation of p73β [7].

Several mechanisms by which $p19^{ras}$ interaction with p73β actively induces p73-mediated apoptosis are proposed based on our results. First, in general, both p53 and p73 as p53 family proteins commonly upregulate at least two apoptosis-related proteins that play a role in the mitochondrial apoptotic pathway: Bax and p53AIP1, p53-apoptosis inducing factor 1 [13,14]. In this study we show that the increased Bax activity is efficiently induced by $p19^{ras}$ interaction with p73β to upregulate p73-related apoptotic responses (Figs. 2 and 4). Second, p53AIP1 is localized to the mitochondria and interacts with Bcl-2 to facilitate the release of cytochrome *c* from the mitochondria to cytosol. Previous studies reported that acetylated p73 by p300/CBP activates *p53AIP1* gene transcription [13] and MDM2 disrupts the interaction of p73 with p300/CBP by competing with p73 for binding to the N terminus of p300/CBP [15]. Since we reported that $p19^{ras}$ directly interacted with MDM2 [7] and the $p19^{ras}$/p73β interaction strongly induced the increased cytochrome *c* release in this study, $p19^{ras}$/p73β interaction is cable of facilitating *p53AIP1* gene expression through its blocking effects on MDM2-mediated inhibition of p300/CBP binding to p73, followed by activating the p73 acetylation by p300/CBP. Therefore, $p19^{ras}$ is supposed to be an enhancer of p73-related apoptotic responses.

In this study we demonstrate biological and physiological functionalities of $p19^{ras}$ interaction with p73β, which triggers an effectively increased induction of apoptotic cell death in HEK 293 and SAOS2 osteosarcoma cells. These results support the potential benefit of the association of low doses of Taxol treatment with the gene therapy using $p19^{ras}$. Combining $p19^{ras}$ with chemotherapy based on p73 signaling pathway might be potentiate the effect of chemotherapies reducing the harmful systemic effects mostly occurring during the treatment of cancer. Further investigation of this possibility is warranted.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.06.010.

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