Ape1 regulates WNT/β-catenin signaling through its redox functional domain in pancreatic cancer cells

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Abstract. Apurinic/apyrimidinic endonuclease 1/redox factor-1 (Ape1/Ref-1, Ape1) is a multifunctional protein that is upregulated in human pancreatic cancer. Ape1 redox domain plays an essential role in regulating the effects of reactive oxygen species (ROS) generated during physiological metabolism and pathological stress. In the present study, we explored whether Ape1 and ROS affect WNT/β-catenin signaling. We used E3330, a small molecule inhibitor of the redox activity of Ape1, and a siRNA approach to knock down *Ape1*, in two human pancreatic cancer cell lines. Inhibition of Ape1 resulted in growth suppression of pancreatic cancer cells, increased ROS levels, upregulation of β-catenin and c-myc and downregulation of cyclin D1. Consistent with these data, overexpression of Ape1 in pancreatic cancer cells reduced ROS and c-myc levels and increased cyclin D1 levels. Moreover, treatment of pancreatic cancer cells with H_2O_2 to induce oxidative stress resulted in upregulated ROS levels, decreased Ape1 at both the mRNA and protein level, and alterations in WNT/β-catenin pathway components. Finally, treatment of pancreatic cancer cells with the WNT/β-catenin inhibitor IWR-1 resulted in growth inhibition, which was greatly enhanced when combined with E3330 treatment. In summary, our results demonstrate that ROS is an important intracellular messenger that can modulate WNT/β-catenin signaling. The present study provides interesting new insight into crosstalk between the redox function of Ape1 and WNT/β-catenin signaling in cancer cells. Furthermore, our data show that the combination of Ape1 and WNT inhibitors enhanced the inhibition of pancreatic cell proliferation. These results provide a promising novel therapeutic strategy for treating pancreatic cancer in future.

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Abbreviations: AP-1, activator protein 1; Ape1/Ref-1, apurinic/apyrimidinic endonuclease 1/redox factor-1; BER, base excision repair; Cdk5, cyclin-dependent kinase 5; CTCs, circulating tumor cells; Dvl, dishevelled; E3330, (2E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)methylene]-undecanoic acid; EMT, epithelial-mesenchymal transition; eNO, endothelial nitric oxide; HIF-1α, hypoxia inducing factor; IWR-1, 4-(1,3,3a,4,7,7a-hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-benzamide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NPM1, nucleophosmin; NRX, nucleoredoxin; PCP, planar cell polarity; ROS, reactive oxygen species; TRF2, telomeric repeat-binding factor 2; VEGF, vascular endothelial growth factor

Key words: Ape1, ROS, E3330, IWR-1, β-catenin

Introduction

Pancreatic cancer is a leading cause of cancer-related death, largely due to metastatic dissemination, and it has the worst prognosis of any major tumor type, with a 5-year survival rate of ~5% (1). Patients with pancreatic cancer respond poorly to existing chemotherapeutic agents and radiation due to the high degree of hypoxia in pancreatic tumors (2,3). Hypoxia-inducible

factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) are ROS-related proteins that are both regulated by Ape1 redox signaling (4). Furthermore, ROS have been shown to switch on hypoxia-dependent epithelial-mesenchymal transition (EMT) in cancer cells (5). Abnormal activation of the WNT/β-catenin pathway is also associated with pancreatic cancer (6). Because ROS are important intracellular messengers that can modulate WNT/β-catenin signaling by a redox mechanism (7), we hypothesized that there may be crosstalk between Ape1 redox signaling and WNT/β-catenin signaling in pancreatic cancer cells.

Ape1 is a multifunctional protein that regulates a wide variety of important cellular functions (8). It functions as an apurinic/apyrimidinic endonuclease in base excision repair (BER) of DNA lesions and as a redox-modifying factor in eukaryotic transcriptional regulation (9-13). For example, the redox function of Ape1 can stimulate the DNA-binding activity of HIF-1α, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activator protein 1 (AP-1), and p53 (12-16). A recent report also showed that Ape1 is an essential factor stabilizing telomeric DNA. Its deficiency is associated with telomere dysfunction and segregation defects in immortalized cells maintaining telomeres by either the alternative lengthening of telomere pathway or telomerase expression. The DNA repair and N-terminal acetylation domains are required for Ape1 function at telomeres. Ape1 associates with telomere proteins in U2OS cells, and Ape1 depletion causes dissociation of telomeric repeat-binding factor 2 (TRF2) from telomeres (17). Ape1 also regulates endothelial nitric oxide (eNO) production and vascular tone (18), and interacts with nucleophosmin (NPM1) within nucleoli, where it not only plays a role in the rRNA quality control process, but also where its BER activity is stimulated in cells (19-21). Furthermore, Ape1 can be phosphorylated by cyclin-dependent kinase 5 (Cdk5) complexes, thus, reducing its apurinic/apyrimidinic (AP) endonuclease activity, which results in the accumulation of DNA damage and contributes to neuronal death (22). Accumulating evidence suggests that Ape1 is upregulated in pancreatic cancers (23), and it can affect WNT/β-catenin signaling target genes, such as *cyclin D1* and *c-myc*, in different ways (24,25). In addition, small interfering RNA targeting Ape1 enhances the sensitivity of human pancreatic cancer cells to gemcitabine *in vitro* (26). Single nucleotide polymorphisms (SNPs) in the *Ape1* gene are also associated with breast cancer risk in a Chinese population (27), suggesting Ape1 plays a role in multiple types of cancer. Nevertheless, the functional role of Ape1 in tumor pathogenesis and progression remains unclear.

In the present study, we define a critical pathway by which Ape1 regulates WNT/β-catenin signaling through its redox function. Inhibition of the redox functional domain of Ape1 by E3330 resulted in the upregulation of β-catenin and its target gene c-myc, but not cyclin D1. The level of cyclin D1 was positively correlated with Ape1, but not c-myc. Treating cells with a combination of an Ape1 inhibitor and a WNT inhibitor was more effective at inhibiting pancreatic cell proliferation, compared with either inhibitor alone. These data indicate that using a combination of these inhibitors will enhance their efficacy in pancreatic cancer therapy.

Materials and methods

Cell lines. SW-1990 and Panc-1 were purchased from, and authenticated by the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained at 37˚C in 5% CO_2 and grown in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA).

Plasmid construction. Cysteines 65 and 93 in human Ape1 were mutated using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) to generate Ape1 (C65/93A). Ape1 (ΔNLS) encodes Ape1 with a 20-aa deletion of the putative N-terminal nuclear localization signal. All mutations and deletions were verified by DNA sequencing. Wild-type Ape1 (WT), Ape1 (C65/93A), and Ape1 (ΔNLS) were cloned into the pDsRed-N1 expression vector by standard cloning methods, as previously described (18).

Cell transfections. SW-1990 cells were transfected with each Ape1 plasmid (2 μg) using Lipofectamine 2000 (Invitrogen). Forty-eight hours after transfection with plasmids, stable transfected cell lines were selected for by growing the cells in G418 (800 μg/ml) for one month. The cells were imaged with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The stable transfected cells were then harvested for analysis by RT-PCR, western blot analysis and flow cytometry.

RNA interference. SW-1990 and Panc-1 cells were transfected with 40 nM siApe1 using Lipofectamine 2000 (Invitrogen), as described by the manufacturer. The siRNA sequence targeting human Ape1 was previously described (17). The transfected cells were maintained for 48 h, and then harvested for analysis by RT-PCR and western blot analysis.

RNA isolation and RT-PCR. SW-1990 and Panc-1 cells were harvested after incubating with E3330 and/or IWR-1 for 48 h. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized in a 20 μl final volume containing 1 μg of total RNA, 4 μl of 5X reverse transcriptase buffer, 4 μl of 2.5 mM dNTP, 1 μl of oligo-dT (100 pmol/μl), 1 μl of RNase inhibitor (4 units/μl), and 1 μl of AMV reverse transcriptase (RT) (5 units/μl) at 42˚C for 1 h. Then, the reaction mixture was boiled for 5 min to inactivate the RT and quickly chilled on ice. The gene-specific primers used for PCR amplification are listed in Table I. PCR was performed using a thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed by electrophoresis on a 1.5% agarose gel.

In vitro cell growth assay. *In vitro* growth assays were performed with SW-1990 and Panc-1 cells that were exposed to varying concentrations of E3330 and/or IWR-1. Briefly, either SW-1990 or Panc-1 cells were cultured in 96-well plates (4,000 cells/well) for 12 h, treated with E3330 and/or IWR-1 which Diluted with DMSO at the indicated dose for 48 h, and then the number of viable cells was determined using the nonradioactive Cell Counting kit-8 (CCK-8; Dojindo, Kyushu, Japan). All assays were repeated five times.

Table I. Primers for RT-PCR.

Gene	Primer sequence	Size (bp)	Temperature (˚C)
Ape1	Forward: ACTTCAGGAGCTGCCTGGACT Reverse: AATGCAGGTAACAGAGAGTGGGA	564	56
β-catenin	Forward: TGATGGAGTTGGACATGGCCATGG Reverse: CAGACACCATCTGAGGAGAACGCA	570	62.5
c-myc	Forward: GCGTCCTGGGAAGGGAGATCCGGAGC Reverse:TTGAGGGGCATCGTCGCGGGAGGCTG	328	62.5
Cyclin D1	Forward: GAGAACAAACAGATCATCCGCA Reverse: GCTTCGATCTGCTCCTGG	242	56
GAPDH	Forward: GAAGGTGAAGGTCGGAGTCA Reverse: TTCACACCCATGACGAACAT	402	60

Western blot analysis. Cells were treated with E3330 and/or IWR-1 for 48 h and then lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA). Protein concentrations were measured using the BCA protein assay kit (Thermo Fisher Scientific). Samples were resolved through a 10% SDS-PAGE gel and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked in Tris-buffered saline containing 0.05% Tween-20 with 5% non-fat skim milk for 1 h at room temperature, and then the membrane was incubated with primary antibody overnight at 4˚C. After three washes in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After three washes in TBST, the membrane was visualized by enhanced chemiluminescence using the ChemiDoc XRS+ imaging system (Bio-Rad Laboratories). The following antibodies were used: GAPDH, Ape1, β-catenin, cyclin D1 (Cell Signaling Technology, Danvers, MA, USA), and c-myc (Abcam, Burlingame, CA, USA).

Detection of reactive oxygen species by flow cytometry. Cells were treated with E3330 and/or IWR-1 for 48 h, washed with PBS, and resuspended in DMEM. Then, the cells were incubated in 0.5 μM DCFH-DA (Beyotime Institute of Biotechnology, Jiangsu, China) for 30 min at 37˚C. ROS fluorescence intensity was determined by flow cytometry with an excitation at 488 nm and an emission at 525 nm using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Detection of cell cycle by flow cytometry. Cell cycle analysis was performed on SW-1990 and Panc-1 cells following a 48-h incubation with E3330 and/or IWR-1. The cells were fixed in chilled methanol overnight before staining with 50 μg/ml propidium iodide (Nanjing KeyGen Biotech, Co., Ltd., Jiangsu, China) in the presence of 20 μg/ml RNase (Beyotime Institute of Biotechnology) and 0.1% NP-40 (Sigma). Analysis was performed immediately after staining using a FACSCalibur flow cytometer (Becton-Dickinson).

Statistical analysis. Statistical analysis was performed using a GraphPad Prism v5.0 statistical software package, and a Student's test was used to test the probability of significant differences between samples. The cut-off for statistical significance was set at $P<0.05$.

Results

Inhibition of Ape1 redox function with E3330 suppresses the growth of human pancreatic cancer cell lines through cyclin D1 downregulation. To determine the function of the Ape1 redox domain in human pancreatic cancer cells, we treated SW-1990 and Panc-1 cells with E3330, a small molecule inhibitor of the Ape-1 redox domain. We measured the *in vitro* growth of SW-1990 and Panc-1 cells that were exposed to increasing doses of E3330 using the Cell Counting kit-8 (CCK-8) assay (Fig. 1A). There was a significant inhibition in the growth of SW-1990 and Panc-1 cells at doses of E3330 >10 μmol/l. Based on this result, we analyzed the level of the cell proliferation-related gene cyclin D1, and we found that cyclin D1 was reduced at both the mRNA and protein level with increasing concentrations of E3330 (Fig. 1B-E). These alterations in cyclin D1 levels were accompanied by the upregulation of ROS (Fig. 1F).

E3330 treatment upregulates WNT/β-catenin signaling through upregulation of intracellular ROS levels. E3330 treatment of pancreatic cancer cell lines resulted in reduced levels of cyclin D1, a well-known WNT/β-catenin target gene. Therefore, we examined the level of β-catenin, a key member of the WNT/β-catenin signaling pathway, and another WNT/β-catenin downstream target gene, c-myc. β-catenin and c-myc were upregulated at both the mRNA and protein level in an E3330 dose-dependent manner (Fig. 1B-E). ROS have been shown to function as intracellular messengers to augment WNT/β-catenin signaling by modulating the redox-dependent interaction between nucleoredoxin (NRX) and dishevelled (Dvl) (28,29). Therefore, we hypothesize that E3330 augments WNT/β-catenin signaling by inhibiting the redox function of Ape1 and upregulating intracellular ROS levels.

The WNT/β-catenin signaling inhibitor IWR-1 suppresses growth of pancreatic cancer cell lines. IWR-1 is a WNT/β-catenin signaling inhibitor that induces stabiliza-

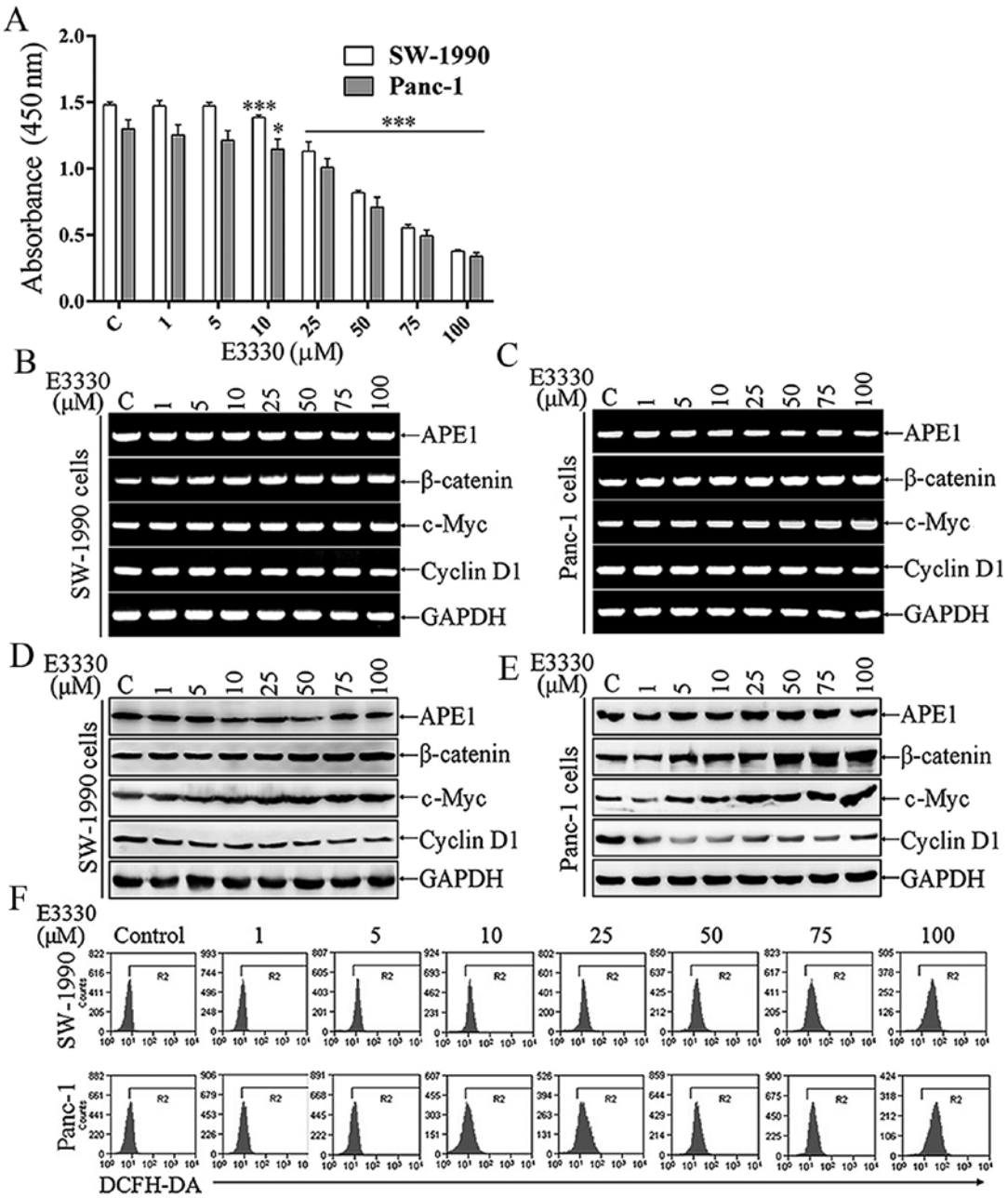

Figure 1. Inhibition of Ape1/Ref-1 redox activity upregulates WNT/β-catenin signaling, but downregulates cyclin D1. (A) The Ape1 redox domain inhibitor E3330 inhibited the *in vitro* growth of pancreatic cancer cells. SW-1990 and Panc-1 pancreatic cancer cells were treated with various doses of E3330 for 48 h, and control was treated with DMSO. The CCK-8 assay was performed to examine cell viability, and the standard deviations (SDs) of five independent experiments were calculated. The statistical significance between each group was calculated using a Student's t-test; $^{*}P<0.05$, $^{***}P<0.001$. (B and C) Total RNA was extracted, and RT-PCR was performed with GAPDH as an internal control. (D and E) Total protein was extracted, and a western blot analysis was performed with GAPDH as an internal control. (F) The ROS levels in SW-1990 and Panc-1 cells that were exposed to E3330 for 48 h were determined using DCFH-DA and flow cytometry.

tion of Axin proteins via a direct interaction, thus leading to enhanced β-catenin destruction (30). To determine the effect of IWR-1 on the *in vitro* growth of SW-1990 and Panc-1 cells, we exposed each cell line to increasing doses of IWR-1 and measured growth effects using a CCK-8 assay (Fig. 2A). There was a significant inhibition in the growth of both SW-1990 and Panc-1 cells at doses of IWR-1 >20 μmol/l. WNT/β-catenin signaling-associated protein levels were examined, and we found that β-catenin, c-myc and cyclin D1 were decreased with increasing doses of IWR-1 (Fig. 2D and E). However, the level of *β-catenin* mRNA was not altered (Fig. 2B and C).

Combined treatment with E3330 and IWR-1 downregulates cyclin D1 more effectively and blocks G1-to-S progression. E3330 treatment suppressed the growth of pancreatic cancer cells and reduced levels of cyclin D1. However, E3330 treatment also increased WNT/β-catenin pathway genes, such as β-catenin and c-myc. To determine whether inhibition of the WNT/β-catenin pathway can enhance the growth suppressive effects of E3330, we treated pancreatic cancer cells with a combination of E3330 and IWR-1. SW-1990 and Panc-1 cells were exposed to varying doses of E3330 and IWR-1 in combination and growth effects were measured using

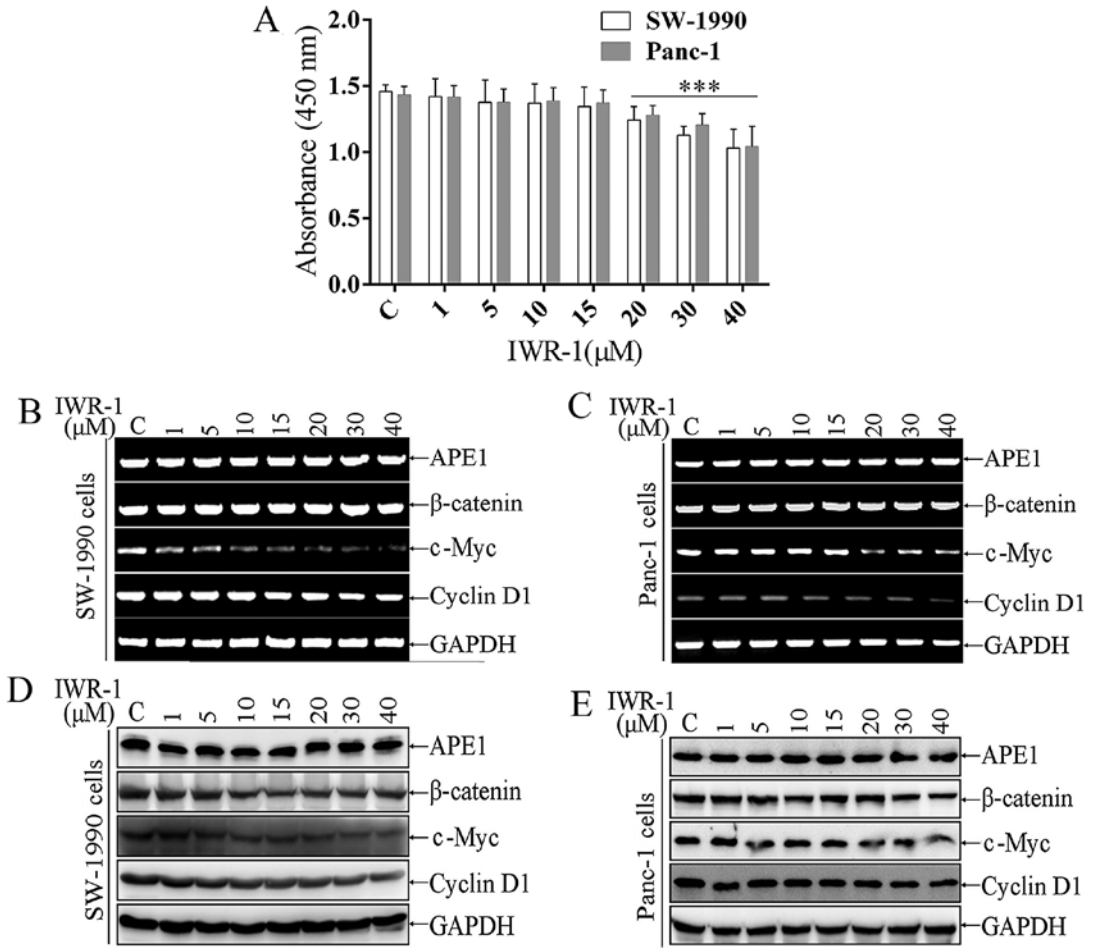

Figure 2. The WNT/β-catenin inhibitor IWR-1 downregulates WNT/β-catenin signaling and suppresses pancreatic cell growth. (A) The WNT/β-catenin inhibitor IWR-1 inhibited the *in vitro* growth of pancreatic cancer cells. SW-1990 and Panc-1 pancreatic cancer cells were treated with varying doses of IWR-1 for 48 h, and control was treated with DMSO. The CCK-8 assay was performed to examine cell viability, and the SDs of five independent experiments were calculated. The statistical significance between each group was calculated using a Student's t-test; $^{**}P<0.01$, $^{***}P<0.001$. (B and C) Total RNA was extracted, and RT-PCR was performed with GAPDH as an internal control. (D and E) Total protein was extracted, and a western blot analysis was performed with GAPDH as an internal control.

the CCK-8 assay. There was a significant inhibition in the growth of SW-1990 and Panc-1 cells that were treated with IWR-1 (15 μmol/l) and E3330 (10 μmol/l) (Fig. 3A). Ape1 and WNT/β-catenin signaling genes were examined by RT-PCR and western blot analysis. The upregulation of β-catenin and c-myc that was previously observed with E3330 treatment was effectively inhibited by the addition of IWR-1. Cyclin D1 downregulation was also enhanced by the combined inhibitor treatment, compared with that observed with individual inhibitor treatments (Fig. 3B-E). In addition, G1-to-S cell cycle progression was blocked with decreasing levels of cyclin D1 (Fig. 3G). These data suggest that E3330 and IWR-1 are promising candidates for a novel combinatorial therapeutic strategy.

Overexpression of Ape1 affects WNT/β-catenin signaling and cellular ROS levels. To validate the role of Ape1 in WNT/β-catenin signaling activation, we cloned wild-type Ape1 (WT) and two mutant *Ape1* cDNAs into the pDsRed-N1 expression vector for overexpression studies (Fig. 4A). To generate mutant *Ape1*, we developed a construct with mutated redox-sensitive cysteines (C65/93A), and a construct with a 20-aa deletion of the putative N-terminal nuclear localization signal (ΔNLS) of *Ape1*. Each of these plasmids was transfected into SW-1990 cells. Ape1 (WT) and Ape1 (C65/93A) were largely localized to the nucleus, whereas Ape1 (ΔNLS) was largely localized to the cytoplasm (Fig. 4B). The ROS levels in Ape1 transfected cells were determined using DCFH-DA and flow cytometry. Overexpression of Ape1 (WT) and Ape1 (ΔNLS), but not Ape1 (C65/93A), reduced the level of intracellular ROS (Fig. 4C). We examined the level of Ape1 and WNT/β-catenin signaling genes by RT-PCR and western blot analysis. Overexpression of all of the Ape1 constructs in SW-1990 cells resulted in the downregulation of c-myc, but overexpression of Ape1 (ΔNLS) resulted in the greatest reduction of c-myc. Cyclin D1 was upregulated in SW-1990 cells expressing Ape1 (WT) and Ape1 (ΔNLS), but not Ape1 (C65/93A). These alterations occurred both at the mRNA and protein level (Fig. 4D and E). We believe that c-myc downregulation may be due to the ability of Ape1 to cleave *c-myc* mRNA (31).

Ape1 is required for inhibiting WNT/β-catenin signaling. To confirm the function of Ape1 in WNT/β-catenin signaling, *Ape1* was knocked down by siRNA in SW-1990 and Panc-1

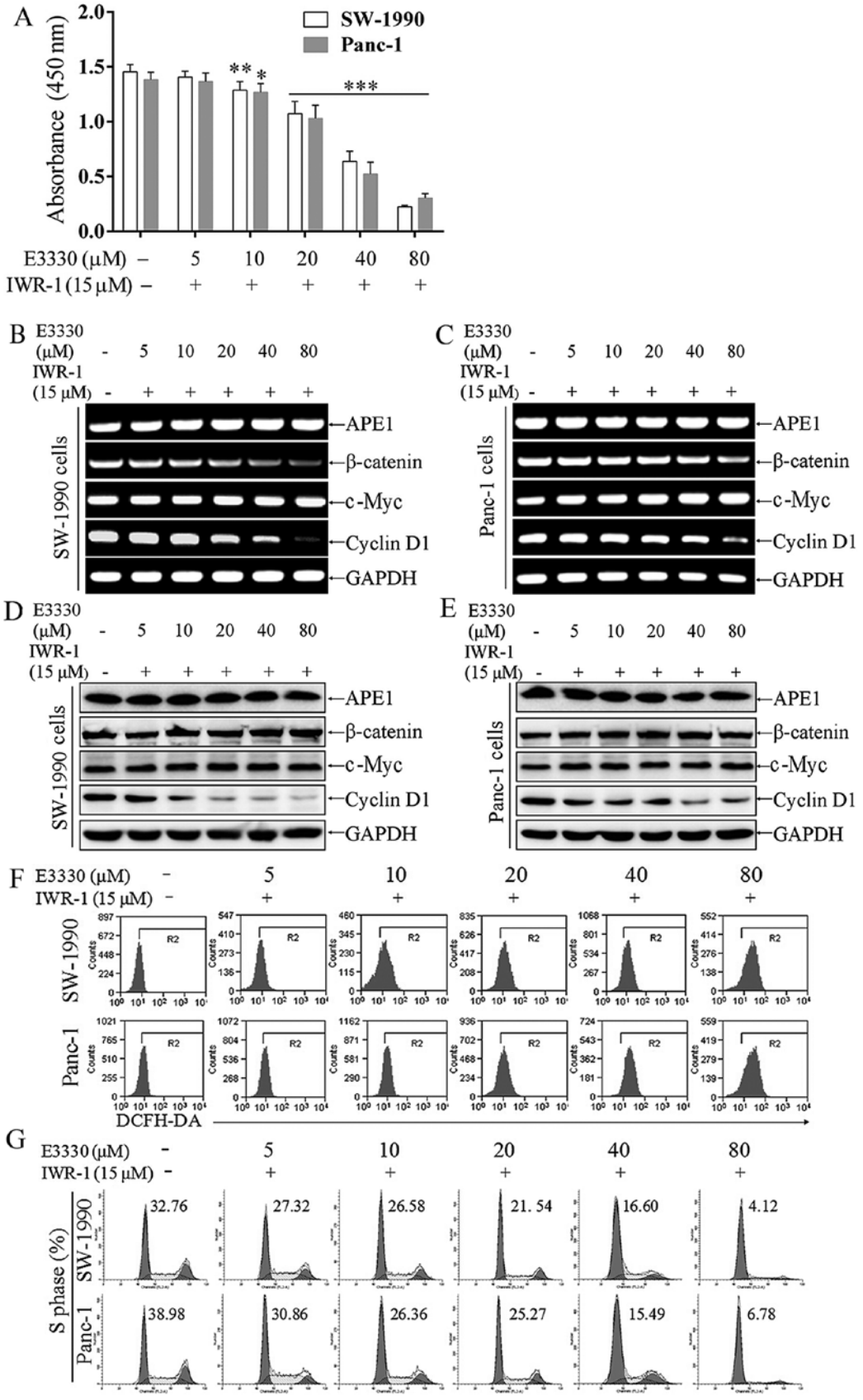

Figure 3. Combined treatment with E3330 and IWR-1 enhances the growth suppression of pancreatic cancer cells. (A) SW-1990 and Panc-1 pancreatic cancer cells were treated with varying doses of E3330 and IWR-1 for 48 h, and control was treated with DMSO. The CCK-8 assay was performed to examine cell viability, and the SDs of five independent experiments were calculated. The statistical significance between each group was calculated using a Student's t-test; *P<0.05, **P<0.01, ***P<0.001. (B and C) Total RNA was extracted, and RT-PCR was performed with GAPDH as an internal control. (D and E) Total protein was extracted, and a western blot analysis was performed with GAPDH as an internal control. (F) The ROS levels in SW-1990 and Panc-1 cells, which were exposed to E3330 and IWR-1 for 48 h, were determined using DCFH-DA and flow cytometry. (G) The DNA content is shown from SW-1990 and Panc-1 cells that were treated with DMSO (as control cells), or with E3330 and IWR-1 at varying doses for 48 h.

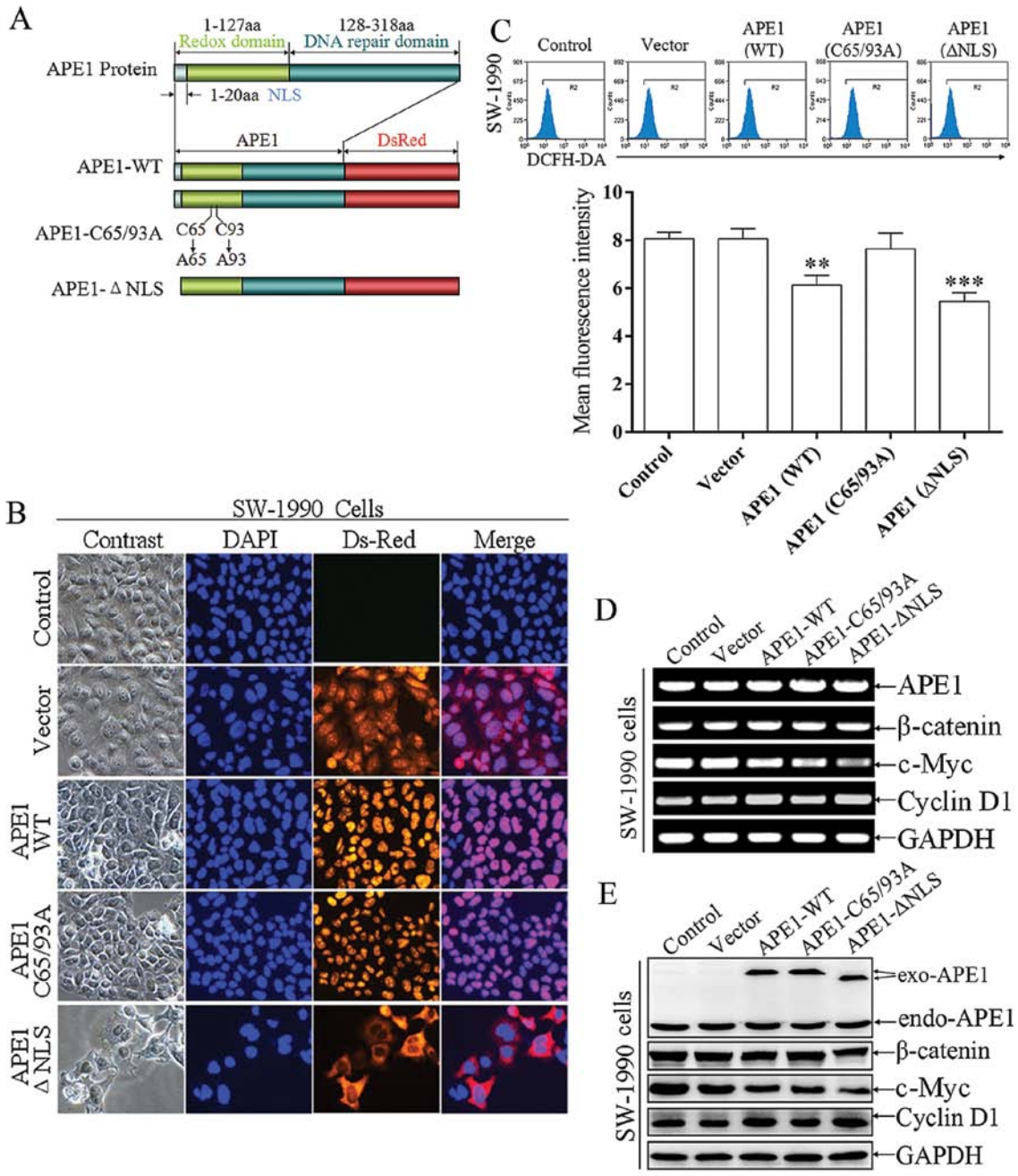

Figure 4. Overexpression of Ape1 in SW-1990 cells alters WNT/β-catenin signaling. (A) This diagram shows the strategy of Ape1 expression vector construction. (B) SW-1990 cells were transfected with each plasmid and selected for using G418 for 1 month. (C) The ROS levels in Ape1 transfected cells were determined using DCFH-DA and flow cytometry, and the SDs of three independent experiments were calculated. The statistical significance between the cells expressing wild-type Ape1 and the mutant forms of Ape1 was calculated using a Student's t-test; $^{**}P<0.01$, $^{***}P<0.001$. (D) Total RNA was extracted, and RT-PCR was performed with GAPDH as an internal control. (E) Total protein was extracted, and a western blot analysis was performed with GAPDH as an internal control.

cells. Ape1 and several genes of the WNT/β-catenin pathway were examined by RT-PCR and western blot analysis, and we found that β-catenin and c-myc were upregulated at protein level more than mRNA level (Fig. 5A and B), whereas cyclin D1 was downregulated both at the mRNA and protein level (Fig. 5A-D). These data further suggest that Ape1 is an inhibitor of WNT/β-catenin signaling.

Ape1 is downregulated by high oxidative stress. To determine the effect of oxidative stress on Ape1, SW-1990 and Panc-1 cells were treated with increasing doses of H_2O_2 for 2 h, and then cultured for 48 h to establish a hyperoxia model. ROS levels were upregulated in SW-1990 and Panc-1 cells, but cell viability was reduced as the concentration of H_2O_2 increased (Fig. 6A). We performed RT-PCR and western blot analysis, and unexpectedly found that Ape1 levels decreased as the concentration of H_2O_2 increased, at both the mRNA and protein level. In addition, β-catenin protein, but not mRNA, was upregulated, c-myc was upregulated at the mRNA and protein level and cyclin D1 was downregulated (Fig. 6B-E). The cyclin D1 promoter contains many transcription factor binding sites, including β-catenin/TCF and NF-κB binding

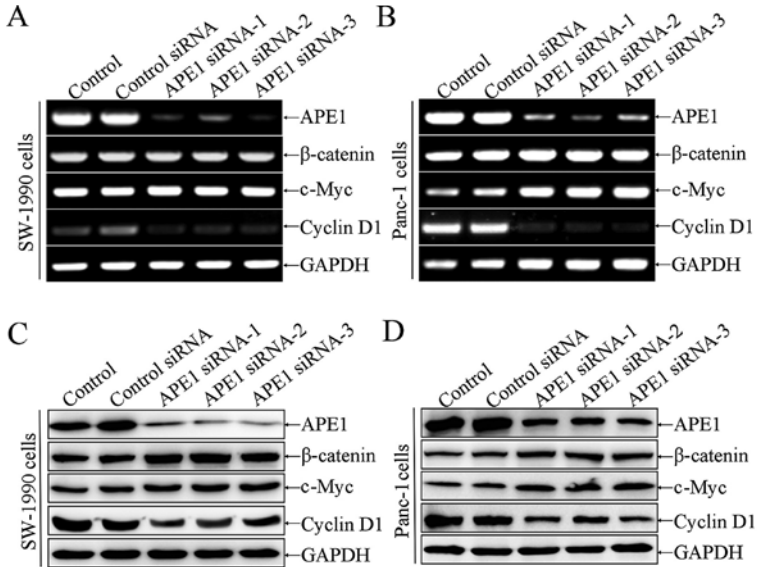

Figure 5. Ape1 knockdown alters WNT/β-catenin signaling. (A and B) SW-1990 and Panc-1 pancreatic cancer cells were treated with the indicated siRNAs for 72 h. Total RNA was extracted, and RT-PCR was performed with GAPDH as an internal control. (C and D) SW-1990 and Panc-1 cells were treated with the indicated siRNAs for 72 h. Total protein was extracted, and a western blot analysis was performed with GAPDH as an internal control.

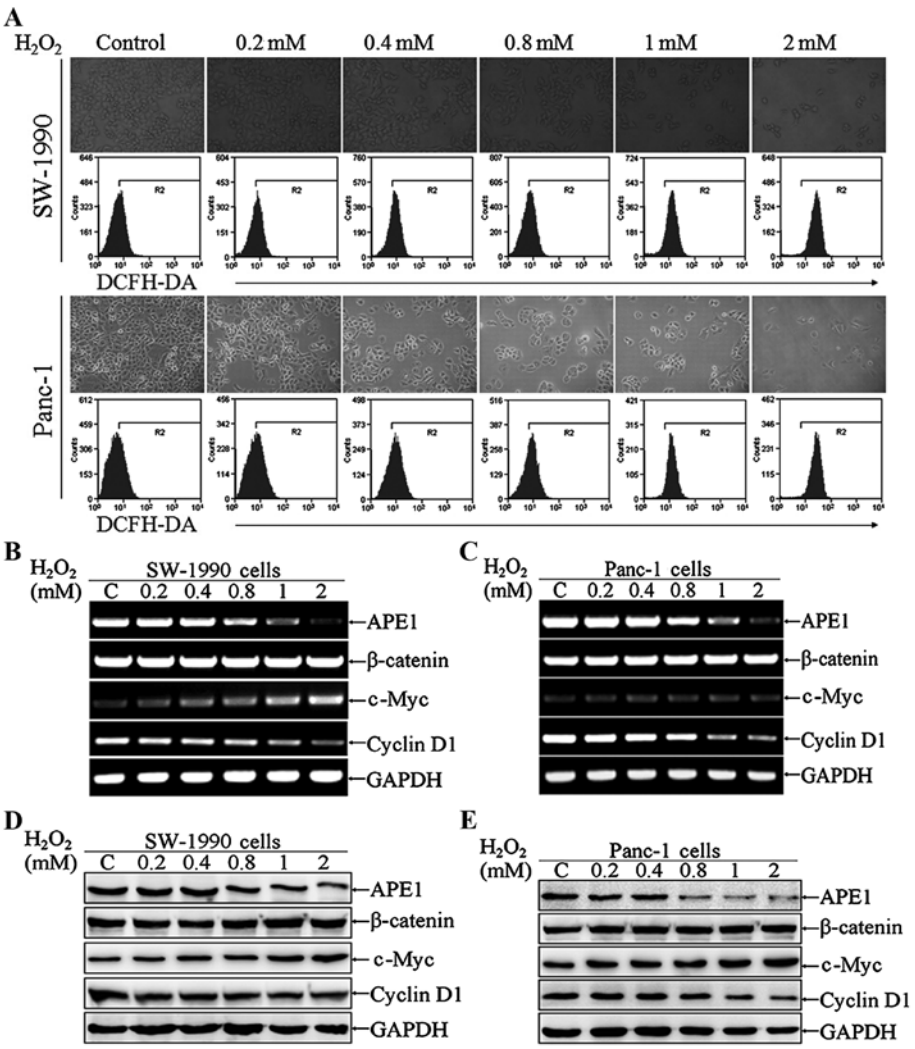

Figure 6. ROS induction by H_2O_2 treatment alters WNT/β-catenin signaling. (A) SW-1990 and Panc-1 pancreatic cancer cells were treated with varying doses of H_2O_2 for 2 h, and then cultured for 48 h. Images were captured using a Nikon eclipse TS100 microscope carrying a pro-microscan camera with ScopePhoto software version 3.0. ROS levels in SW-1990 and Panc-1 cells, which were exposed to H_2O_2 for 2 h and then cultured for 48 h, were determined using DCFH-DA and flow cytometry. (B and C) Total RNA was extracted, and RT-PCR was performed with GAPDH as an internal control. (D and E) Total protein was extracted, and a western blot analysis was performed with GAPDH as an internal control.

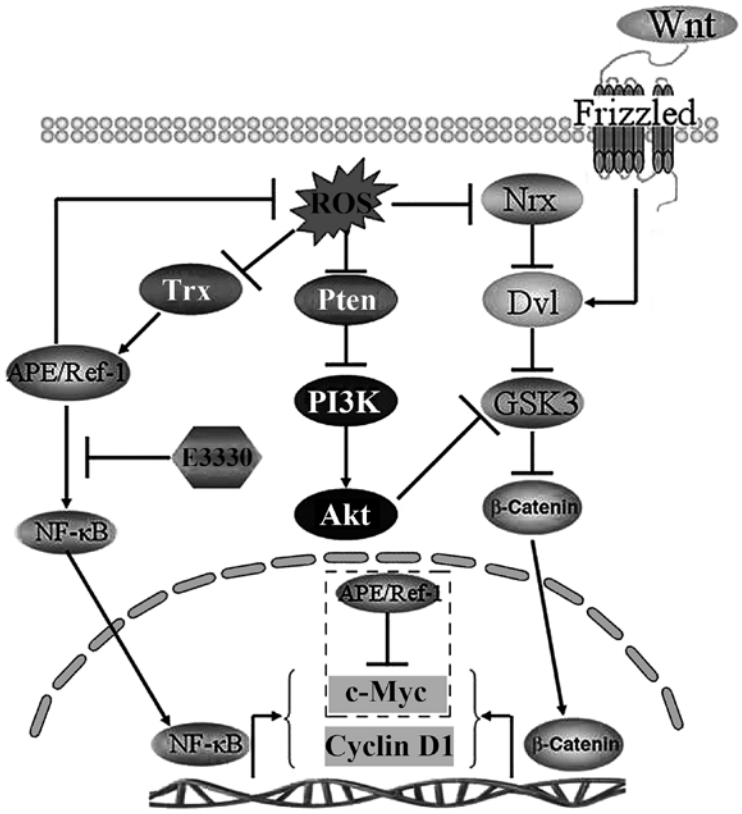

Figure 7. A proposed model highlights the role of Ape1 in regulating WNT/β-catenin signaling.

sequences (7,32). Because Ape1 can activate NF-κB through its redox activity (33), we believe that cyclin D1 expression is mainly controlled by NF-κB signaling in pancreatic cancer cells (Fig. 7).

Discussion

Pancreatic cancer is the fourth most common cause of cancer-related deaths in the United States (34) and the twelfth worldwide. Pancreatic cancer has an extremely poor prognosis: for all stages combined, the 1- and 5-year relative survival rates are 25 and 6%, respectively. Therefore, further study of this disease in cellular biology and pathophysiology may increase the possibility of finding new treatments, and consequently, improving the prognosis and survival of patients. We recently identified aberrant expression of Ape1 in pancreatic cancer cells (23) and colon cancer stem cells (27). In the present study, we begin to elucidate the function of Ape1 in pancreatic cancer cells and demonstrate crosstalk between the Ape1-mediated redox signaling and WNT signaling pathways. Furthermore, we show that treating pancreatic cancer cells with a combination of Ape1 and WNT inhibitors had an enhanced effect on growth inhibition.

Ape1 is a multifunctional protein involved in the maintenance of genomic integrity and in the regulation of gene expression. Pursuing Ape1 inhibition as a potential strategy for cancer cell therapy is justified, based on the following observations. Ape1 expression and activity are upregulated, or dysregulated, in many types of cancer, including prostate, bladder, ovarian, cervical, pancreatic, colon, and non-small cell lung cancer, as well as germ cell tumors. In addition, our previous studies demonstrated the role of Ape1 in regulating cancer cell growth and tumor angiogenesis in both pancreatic cancer (4,23) and colon cancer stem cells (27).

Because WNT signaling is also important in cancer cells, we wanted to determine whether Ape1 regulates WNT/β-catenin signaling in cancer cells. WNT paracrine factors are cysteine-rich glycoproteins that bind to the Frizzled protein, a transmembrane receptor. The binding of WNT to its receptors can stimulate at least three distinct signaling pathways: the β-catenin pathway, the planar cell polarity (PCP) pathway, and the Ca^{2+} pathway (35). β-catenin is a central component of the WNT pathway, and it forms a complex with members of the TCF family of transcription factors in the nucleus to control the transcription of target genes (36). WNT signaling controls critical biological phenomena throughout development and in adult tissues, and it is a highly conserved pathway across all species. In parallel, aberrant WNT signaling underlies a wide range of pathologies in humans (37). Aberrant activation of WNT signaling is involved in the development of several epithelial tumors, including thyroid cancer (38). WNT signaling has been shown to regulate telomerase in cancer cells, and β-catenin has an important role in the maintenance of mitochondrial homeostasis (36). Importantly, WNT/β-catenin signaling is aberrant in pancreatic carcinoma (39). The formation of non-adherent tumor spheres by human pancreatic cancer cells is associated with the upregulation of multiple WNT signaling genes, and pancreatic circulating tumor cells (CTCs) also show enriched expression of WNT signaling genes (40).

Redox balance underlies cellular homeostasis, and cancer initiation and progression has been linked to the disruption

of redox balance and oxidative stress (28,41). In the present study, we demonstrate that ROS can modulate signaling by the WNT/β-catenin pathway. The present study provides interesting new insight into crosstalk between redox and WNT/β-catenin signaling in normal physiology and cancer. The WNT/β-catenin signaling pathway can be regulated by redox signaling through the redox-sensitive association of NRX with Dvl (28). NRX is a thioredoxin-related, redox-regulating protein that inhibits WNT/β-catenin signaling through Dvl (7). Oxidative stress inhibits the interaction between NRX and Dvl, which suggests that treatment with H_2O_2 may activate WNT/β-catenin signaling by releasing the NRX-mediated block on Dvl activity. Therefore, ROS may augment WNT/β-catenin signaling by modulating the redox-dependent interaction between NRX and Dvl (7).

Our previous study demonstrated that Ape1 is highly expressed in pancreatic cancer cell lines, and that inhibition of Ape1 redox activity significantly inhibits pancreatic cell proliferation (23). However, the question of whether Ape1-mediated redox signaling regulated the WNT/β-catenin pathway in cancer cells remained unclear. Further questions regarding whether an Ape1 redox inhibitor could act synergistically with a WNT inhibitor in the growth inhibition of cancer cells also needed to be addressed. In the present study, we identified a regulatory role for Ape1 in WNT/β-catenin signaling through its redox functional domain in pancreatic cancer cells. Our observations suggest that Ape1 acts as an inhibitor of WNT/β-catenin signaling, because β-catenin was upregulated when *Ape1* mRNA was depleted by siRNA. Furthermore, E3330 inhibition of the redox function of Ape1 in pancreatic cancer cells increased intracellular ROS levels and led to the upregulation of β-catenin. Although E3330 inhibited proliferation of pancreatic cancer cells, the increased β-catenin upon E3330 treatment could enhance cancer cell metastasis, which would ultimately result in a worse prognosis for the patient. Therefore, administration of E3330 alone in the treatment of pancreatic cancer is likely not ideal. We found that the combination of E3330 and the WNT/β-catenin signaling inhibitor IWR-1, effectively blocked the upregulation of β-catenin upon E3330 administration, and enhanced the growth suppression of pancreatic cancer cells.

In summary, although E3330 inhibited SW1990 pancreatic cancer cell proliferation in a single dose, it also upregulated β-catenin expression in those cells, which may protect the cells from further growth inhibition. Therefore, a combination of an Ape1 and a WNT/β-catenin inhibitor had a stronger effect in inhibiting pancreatic cell proliferation, indicating that this inhibitor combination may offer a more promising treatment option in pancreatic cancer therapy.

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