

# Nitric oxide initiates progression of human melanoma via a feedback loop mediated by apurinic/aprimidinic endonuclease-1/redox factor-1, which is inhibited by resveratrol

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## Abstract

It is well recognized that nitric oxide (NO) is involved in tumor progression, including melanoma. Measurement of proliferative and metastatic capacity by MTS and Matrigel invasion assays, respectively, was done and showed that NO-treated melanoma cells exhibited a higher capacity compared with control, especially metastatic Lu1205 cells. Apurinic/aprimidinic endonuclease-1/redox factor-1 (APE/Ref-1) is a multifunctional protein and its role in tumor biology has attracted considerable attention. To determine whether APE/Ref-1 plays a role in mediating NO stimulation of melanoma progression, we investigated the effect of DETA/NO on levels of APE/Ref-1 and related downstream targets [activator protein-1 (AP-1)/JunD, matrix metalloproteinase-1 (MMP-1), Bcl-2, and inducible nitric oxide synthase (iNOS)] by Western blot and reverse transcription-PCR analysis. Following DETA/NO treatment, APE/Ref-1 and other downstream molecules were induced. Knockdown of APE/Ref-1 or AP-1/JunD by specific small interfering RNA markedly reversed the induction by NO stress of target proteins. These results present evidence for the existence of a functional feedback loop contributing to progression and metastasis of melanoma cells. Resveratrol has been shown to be an APE/Ref-1 inhibitor and significant decreases in AP-1/JunD, MMP-1, Bcl-2, and iNOS protein levels occurred after exposure to resveratrol. This phenolic antioxidant may be an appropriate choice for

combining with other compounds that develop resistance by up-regulation of these molecules. [Mol Cancer Ther 2008;7(12):3751–60]

## Introduction

Nitric oxide (NO) produced during the conversion of L-arginine to L-citrulline by NO synthase (NOS) is an important bioactive agent and signaling molecule required for many physiologic functions (1, 2). Recently, studies of NO function have focused on its role in tumor. A majority of human and experimental tumors are stimulated by NO, which contributes to tumor growth and metastasis by promoting migratory, invasive, and angiogenic properties of tumor cells (3, 4). One such example is that scavenging of endogenous NO resulted in inhibition of melanoma cell growth, which was restored with a NO donor (5).

Melanoma, arising from melanocyte, is a highly metastatic tumor and the incidence has ranked fifth and sixth among the most common cancer afflicting men and women. Although a strong relationship is reported between UV radiation and melanoma with acute sun exposure being implicated in melanomagenesis (6), the mechanisms controlling progression are poorly understood. An increasing number of publications support the notion that UV radiation stimulates the production of NO in human keratinocyte (7–9), causing a series of secondary downstream changes and release of cytokines and other molecules that are of critical importance in the maintenance of normal melanocyte homeostasis and in the modulation of melanocyte proliferation, dendricity, and melanin synthesis (10–12). Therefore, NO generated by the keratinocyte is postulated as being involved in melanomagenesis and progression.

Apurinic/aprimidinic endonuclease-1/redox factor-1 (APE/Ref-1) possesses both DNA repair and redox regulatory activity. It regulates the DNA binding of a number of transcription factors, including activator protein-1 (AP-1), nuclear factor- $\kappa$ B (NF- $\kappa$ B), Pax-5, and Pax-8 in both redox-dependent and redox-independent manners (13–16). APE/Ref-1 is essential for cell survival and proliferation, as mice lacking a functional *APE/Ref-1* gene die during embryonic development (17). Also, antisense APE/Ref-1 overexpression induces a striking increase in cell sensitivity to oxidative stress (18). Thus, APE/Ref-1 has attracted attention on account of its multiple roles, especially in tumors. Several studies show that APE/Ref-1 is very sensitive to the redox disequilibrium that often occurs in tumor cells (19). Additionally, melanoma cells have diminished antioxidant potential compared with

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normal melanocytes, which leads to an accumulation of reactive oxygen species through increased production as well as impaired clearance in different stages of melanoma development (20). Our previous data also showed a role of APE/Ref-1 in melanoma transformation and development of drug resistance (21). Based on this broad spectrum of findings, it has become increasingly cogent to investigate the role of APE/Ref-1 as a pivotal therapeutic target in melanoma.

Resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring compound present in grapes and in red wine, which has been suggested as a potential chemopreventive and antitumor agent based on its striking inhibitory effects on diverse cellular events associated with tumor initiation, promotion, and progression (22). Thus far, the underlying molecular basis for these effects has been elusive, although its antioxidant activity as a reactive oxygen species scavenger clearly is of significance (23). In addition, we have shown that resveratrol is an APE/Ref-1 inhibitor in a previous study (24).

In the current investigation, we present evidence that NO-induced APE/Ref-1 is crucial in initiating a positive functional feedback loop in melanoma cells with involvement of AP-1/JunD, MMP-1, Bcl-2, and iNOS. Resveratrol directed against APE/Ref-1 interrupted this loop, suggesting that it might be an effective inhibitor of progression or enhancer of therapeutic agent activity that is blocked by the high NO level.

## Materials and Methods

### Cell Culture

The metastatic melanoma cell line Lu1205 was cultured in L15/MCDB medium with 5% fetal bovine serum, 5% newborn calf serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. The primary melanoma cell line (Wm3211) was cultured in RPMI 1640 with 5% fetal bovine serum, 5% calf serum, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.01 mg/mL insulin. These cells were generous gifts from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). The passage number for the cell strains used in these experiments was  $\leq 8$ . Cells were treated at 70% confluence with fresh medium and drug was added simultaneously.

### Reagents and Antibodies

The NO donor DETA/NO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate, was from Alexis Biochemicals. It was dissolved in PBS and used at a concentration of 100  $\mu\text{mol/L}$ . In medium (pH 7.4), DETA/NO spontaneously releases NO with a half-life of  $\sim 20$  h at 37°C. Resveratrol was purchased from Sigma Life Sciences, dissolved in DMSO, and used at a concentration of 50  $\mu\text{mol/L}$ .

The following primary antibodies were used for Western blot analysis: APE/Ref-1 was from Novus Biologicals; AP-1/JunD, MMP-1, Bcl-2, and iNOS were from Santa Cruz Biotechnology; and  $\alpha$ -Tubulin was from Sigma Life Sciences. The following secondary antibodies were

used: horseradish peroxidase-conjugated anti-mouse antibody and anti-rabbit antibody were from Santa Cruz Biotechnology.

### Cell Proliferation Assay

The MTS assay (CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay, Promega) was used for measurement of cell proliferation. Briefly, cells were seeded into 96-well plates at a density of  $1.5 \times 10^4$  per well, incubated overnight, and exposed to DETA/NO. At the indicated time, 20  $\mu\text{L}$  MTS reagent (10 mg/mL in PBS) were added to each well and allowed to react for 3 h at 37°C. Substrate cleavage was monitored by using a microplate reader (Bio-Rad) at 490-nm wavelength and analyzed by using related software. The percentage was defined as  $[(\text{experimental}_{\text{OD}} - \text{blank}_{\text{OD}}) / (\text{control}_{\text{OD}} - \text{blank}_{\text{OD}})] \times 100\%$ , where the  $\text{blank}_{\text{OD}}$  was determined in wells containing medium and MTS only.

### Invasion Assay

The invasiveness of melanoma cell was assessed based on the invasion of cells through Matrigel-coated membrane (pore size, 8.0  $\mu\text{m}$ ). According to the manufacturer's instructions, two groups of cells (control and DETA/NO-treated cells) were collected and reconstituted in serum-free medium at a final concentration of  $4 \times 10^5$  cells/mL. Serum-free medium (750  $\mu\text{L}$ ) was added to each well and 500  $\mu\text{L}$  of prepared cells were added to the upper Matrigel-coated insert (BD Biosciences). After an incubation of 72 h, cells were fixed with ice-cold methanol and then stained with hematoxylin. Subsequently, membranes were cut off, mounted in mounting medium on slides, and visualized microscopically (Olympus Optical Co. Ltd.). The invading cells on each of triplicate membranes were counted and averaged for 10 randomized fields at  $\times 400$  magnification, and a photograph was taken at  $\times 100$  magnification.

### Cell Protein Extraction and Western Blot Analysis

After treatment with DETA/NO or resveratrol for specified times, melanoma cells were collected and lysed as described previously (21). By using a detergent-compatible protein assay kit (Bio-Rad), the protein concentration was precisely measured thrice. Equal amounts of the soluble protein were denatured, subjected to SDS-PAGE, and transferred to nitrocellulose membranes. The specific protein was then detected by the antibodies [anti-APE/Ref-1 (1:3,500), anti-iNOS, anti-Bcl-2 and anti-MMP-1 (1:100), anti-AP-1/JunD and anti- $\alpha$ -Tubulin (1:1,000)] followed by a chemiluminescence detection reagent. Measurement of signal intensity on membranes was done using an imaging densitometer with Multi-Analyst software (Bio-Rad). For statistical analysis, all data were expressed as fold change of the control based on the calculation as the density values of the specific protein bands/ $\alpha$ -Tubulin density values. All figures showing quantitative analysis include data from three independent experiments.

### Quantitative Real-time Reverse Transcription-PCR

To confirm the RNA expression level, total RNA was isolated by using TRIzol according to the manufacturer's protocol (Invitrogen, Inc.). RNA concentration was determined by using spectrophotometer. Equal amounts of

mRNA were amplified using First-Strand cDNA Synthesis for Quantitative Reverse Transcription-PCR kit (Marligen Biosciences, Inc.). Real-time PCR was done with a SYBR Green assay and a MYIQ5 detection system (Bio-Rad). Target cDNA was amplified by using primer pairs: APE/Ref-1, 5'-TGCCACACTCAAGATCTGCTCTTG-3' (forward) and 5'-CCACTGTACCCTTCCTTGTCCTGA-3' (reverse);  $\alpha$ -tubulin, 5'-GCGTGATGGTGGGCATGGGTCAG-3' (forward) and 5'-AGGGGGCCTCGGTCAGCAGCAC-3' (reverse). AP-1/JunD primer was purchased from Santa Cruz Biotechnology. The PCR amplification cycle was as follows: 2 min at 95°C and then 45 cycles of amplification (denaturation at 94°C for 30 s, annealing at 56°C for 35 s, and extension at 72°C for 1 min per cycle). Real-time PCR data was analyzed using the comparative Ct method, where Ct is the cycle number at which fluorescence first exceeds the threshold. The  $2^{-\Delta\Delta C_t}$  showed the difference between the threshold cycles of the target and an endogenous reference ( $\alpha$ -Tubulin).

#### Transient Transfection Studies

Small interfering RNA (siRNA) duplexes directed against APE/Ref-1 or AP-1/JunD were purchased from Invitrogen and Santa Cruz Biotechnology, respectively. Sequences of APE/Ref-1 siRNA were as follows: sense 5'-GUCUGUACGACUGGAGUACC-3', antisense 5'-UACUCCAGUCGUACCAGACCU-3'. We seeded cells at  $1 \times 10^5$  per well in a 6-well plate. After 24 h, the cells were transfected with siRNA to give the final concentration of 60 nmol/L (APE/Ref-1) or 40 nmol/L (AP-1/JunD) according to the manufacturer's directions via Lipofectamine (Invitrogen, Inc.). Six hours later, the medium was replaced with a fresh medium with 10% serum. Thirty hours later, cells were treated with DETA/NO.

#### Electrophoretic Mobility Shift Assay

The AP-1 DNA-binding activity was determined by Gel Shift Assay Systems (Promega) with optimizations. In brief, nuclear extracts from Lu1205 melanoma cells, with or without DETA/NO treatment, were incubated in a final volume of 10  $\mu$ L containing 2  $\mu$ L of Gel Shift binding buffer (five times) and 1  $\mu$ L of  $^{32}$ P-labeled AP-1 consensus oligonucleotides. The specificity of binding was determined by competition with preincubation with a 50-fold excess of unlabeled oligonucleotide probes for 20 min before the addition of hot probes. The reaction mixture was separated on 5% nondenaturing polyacrylamide gels. Through autoradiography, the shift bands were visualized.

#### Statistical Analysis

Data are presented as the mean  $\pm$  SD from three independent experiments. Student's *t* test was used to compare two groups, with a *P* value of <0.05 considered statistically significant. All tests were two sided.

## Results

### DETA/NO Potentiates the Proliferative and Metastatic Capacities of Melanoma Cells

To investigate the effect of NO on proliferative and metastatic capacities of melanoma cells, the MTS and invasion

assays were used. Cell viability was measured on days 1, 2, 3, and 4. After a 24-hour exposure to DETA/NO, Lu1205 cell viability initially showed a slight decrease followed by an evident increase. By the 4th day, the viability of DETA/NO-treated Lu1205 cells was  $\sim$ 1.5 times greater compared with control cells. Primary Wm3211 cells were stimulated, although less than that of metastatic Lu1205 cells (Fig. 1A).

Matrigel invasion assay confirmed that the invasiveness of treated cells increased, particularly the metastatic Lu1205 melanoma cells (Fig. 1B). The average number of treated Lu1205 cells invading the underside of the membrane was  $\sim$ 5.5 times higher than that of control cells. Following DETA/NO treatment, primary Wm3211 cells also showed an elevated but less invasive capacity, about a 2-fold increase.

### NO Induces APE/Ref-1 and Other Downstream Molecules in Melanoma Cells

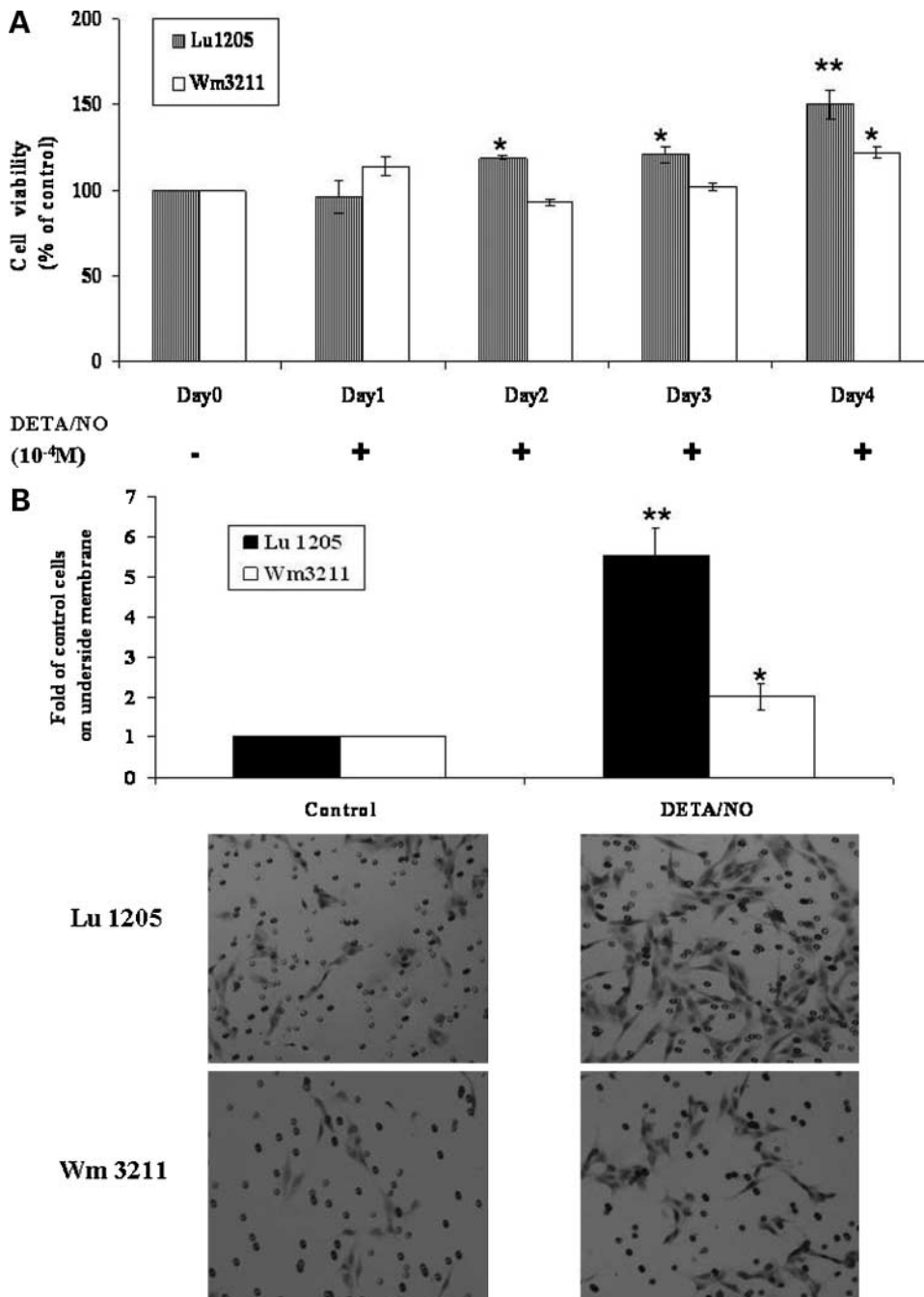
APE/Ref-1 was induced by NO stress. Two peaks occurred in Lu1205 cells as determined by imaging densitometer quantification: The first peak was 2.3-fold higher than control after 2-hour treatment and the second occurred after an exposure of 12 hours with a 3.8-fold increase and then lasted until 36 hours (3.1-fold increase; Fig. 2A). The elevation was not so significant in Wm3211 cells at a 1.3- and 1.9-fold increase, respectively (Fig. 2B). Also, APE/Ref-1 protein level increased in metastatic A375, c81-46A, and c81-61 melanoma cells treated with DETA/NO, which were all higher than Wm3211 (data not shown). Furthermore, the downstream molecules were induced after 24-hour treatment: There was a 3.0-fold (AP-1/JunD), 2.8-fold (MMP-1), 3.3-fold (Bcl-2), and 1.7-fold (iNOS) increase in Lu1205 cells and a 1.4-fold (AP-1/JunD), 1.4-fold (MMP-1), 1.6-fold (Bcl-2), and 1.3-fold (iNOS) increase in Wm3211 cells. Subsequently, real-time PCR analysis of APE/Ref-1, AP-1/JunD (Fig. 2C), and iNOS (data not shown) in Lu1205 cells also confirmed the induction by NO stress at the mRNA level.

### Depletion of APE/Ref-1 with siRNA Reverses the Induction by NO Stress of AP-1/JunD, MMP-1, Bcl-2, and iNOS

We used siRNA to transiently knock down APE/Ref-1. Figure 3A indicated that APE/Ref-1 was successfully depleted in Lu1205 cells after transfection, especially after 72 hours. In siRNA-transfected cells, the induction by NO stress of AP-1/JunD, MMP-1, Bcl-2, and iNOS was inhibited (Fig. 3B), indicating that APE/Ref-1 might mediate the NO stimulation in melanoma cells.

### Role of AP-1/JunD in NO-Stimulated Melanoma Cells

To study the possible role of AP-1/JunD for involvement in NO-stimulated melanoma cells, we first treated Lu1205 cells with DETA/NO and subjected the nuclear extracts to electrophoretic mobility shift assay. Figure 4A (lane 1) showed that specific AP-1 DNA binding was competed by unlabeled AP-1 consensus oligonucleotide ( $\times$ 50). The binding activity of AP-1 was enhanced by NO stress, especially after 2-hour (lane 4) and 12-hour (lane 6) treatments, which coincided well with the two peaks of APE/Ref-1 induction by NO stress (Fig. 2A). Next, we knocked down AP-1/JunD with siRNA (Fig. 4B, a).



**Figure 1.** NO potentiates proliferation and invasive capacity of melanoma cells. **A**, cellular proliferation of melanoma cells in response to DETA/NO treatment. **B**, different invasive capacity between control and DETA/NO-treated cells. Representative pictures were taken from the underside of the membrane at  $\times 100$  magnification. The level of control was set at a value of 1. Data are fold change of control. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus control. Columns, mean of three independent experiments; bars, SD.

Depletion of AP-1/JunD blocked the induction by NO stress of MMP-1, iNOS, APE/Ref-1, and Bcl-2 (Fig. 4B, b and c), further supporting the existence of a feedback loop and demonstrating that AP-1/JunD was one of the crucial elements involved.

#### Resveratrol Pretreatment Inhibits APE/Ref-1 Expression Accompanied by Decreases of AP-1/JunD, MMP-1, Bcl-2, and iNOS

We studied molecule expression changes after resveratrol and DETA/NO treatment in three groups of mel-

noma cells (control cells, cells pretreated with resveratrol for 24 hours followed by a 24-hour incubation of fresh medium without resveratrol, and cells pretreated with resveratrol for 24 hours followed by a 24-hour incubation of DETA/NO with fresh medium). As shown in Fig. 5, resveratrol pretreatment resulted in a reduction in APE/Ref-1 level accompanied by decreases of AP-1/JunD, MMP-1, Bcl-2, and iNOS. Moreover, resveratrol-pretreated cells that were reincubated with DETA/NO and fresh medium produced no induction of these

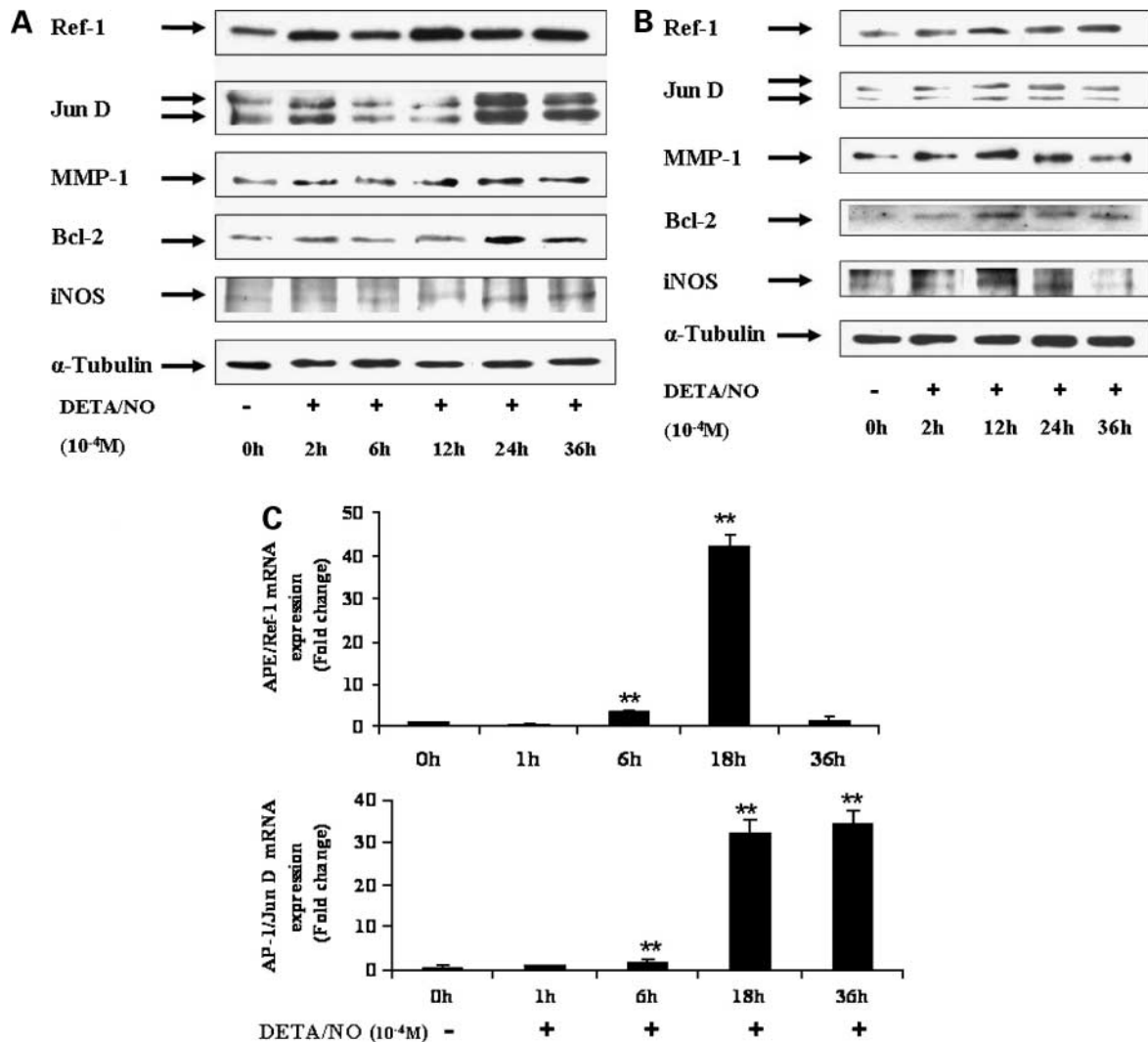
molecules either in Lu1205 (Fig. 5A) or Wm3211 (Fig. 5B) melanoma cells.

## Discussion

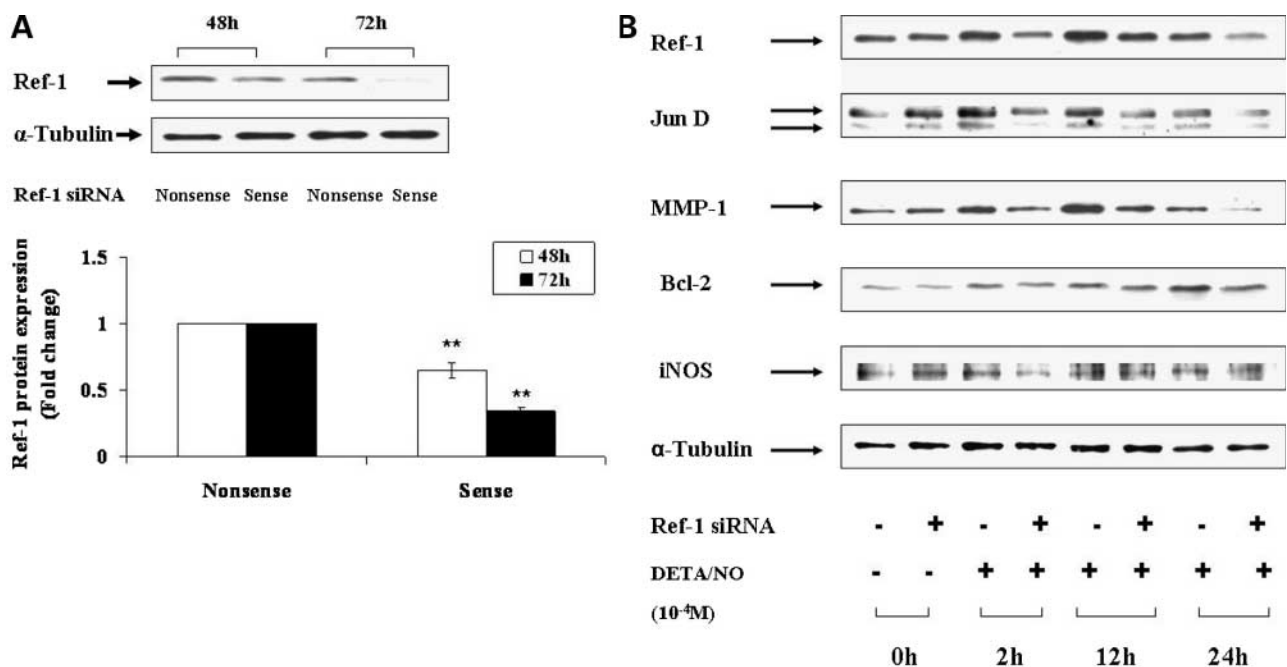
The present study was oriented to understanding the role of APE/Ref-1 in mediating the effect of NO in melanoma progression. The proposed mechanistic schema resulting from our studies is summarized in Fig. 6. NO induces APE/Ref-1, which mediates a series of intracellular signal activations, and the resultant NO production leads to restimulation of APE/Ref-1 in melanoma cells with stronger proliferative and metastatic activities after the enhancements of AP-1/JunD, MMP-1, Bcl-2, and iNOS. Moreover, knockdown of APE/Ref-1 or AP-1/JunD prevented the induction by NO stress of the downstream

targets, supporting the hypothesis that a functional feedback loop existed and APE/Ref-1 induced by NO played a pivotal role. Resveratrol inhibited APE/Ref-1 activity, suggesting that it might act as a reasonable complementary agent in which resistance develops by up-regulating these downstream molecules.

APE/Ref-1 regulates the activation of numerous transcription factors that coordinate the cellular adaptation to a wide array of environmental stimuli. Recent findings have implicated that APE/Ref-1 is involved in different stages of carcinogenesis and forms a unique link between the DNA base excision repair pathway, transcription factor regulation, redox balance, and tumorigenesis (25). Data from our previous study also uncovered a role of APE/Ref-1 in melanoma transformation. In this present study, APE/Ref-1 was induced by NO stress both in protein and



**Figure 2.** NO stress induces APE/Ref-1 and other downstream molecules in melanoma cells. Cells were treated with DETA/NO (100  $\mu$ mol/L) for different times and lysates were examined by Western blot analysis for expression. APE/Ref-1, AP-1/JunD, MMP-1, Bcl-2, and iNOS were induced by NO stress. **A**, Lu1205 melanoma cells. **B**, Wm3211 melanoma cells. **C**, real-time PCR analysis of mRNA obtained from Lu1205 cells treated with DETA/NO. Data are fold of control that was set at a value of 1. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus control. Columns, mean ( $n = 3$ ); bars, SD.



**Figure 3.** Depletion of APE/Ref-1 with siRNA inhibits the induction by NO stress of AP-1/JunD, MMP-1, Bcl-2, and iNOS in Lu1205 melanoma cells. **A**, efficacy of APE/Ref-1 knockdown by transfecting siRNA for different periods. **B**, Western blot analysis of APE/Ref-1, AP-1/JunD, MMP-1, Bcl-2, and iNOS. Ref-1-siRNA transfection effectively reversed the induction of molecules by NO stress. Representative blots from three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus control. Columns, mean ( $n = 3$ ); bars, SD.

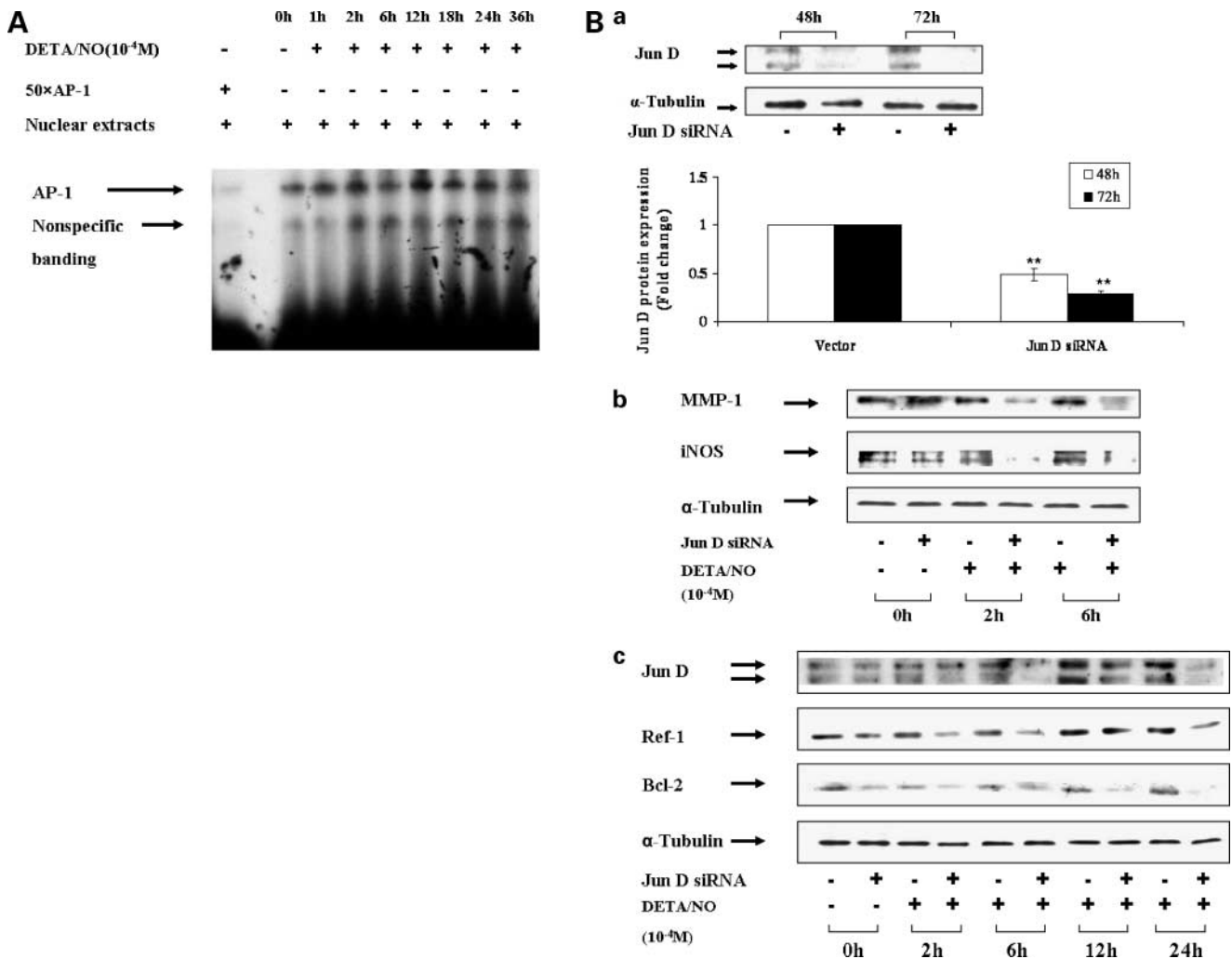
mRNA levels in melanoma cells. Furthermore, knockdown of APE/Ref-1 by siRNA reversed the induction of downstream molecules, indicating that induced APE/Ref-1 should be the major mediator of NO stimulation and occupy a significant position in melanoma.

AP-1 consists of a group of structurally and functionally related members of the Jun and Fos protein families. JunD, a broadly expressed member of the Jun family, was reported to protect cells from senescence or apoptotic responses to stress stimuli and lead to a release from cell contact inhibition (26, 27). Our data also confirmed that increased AP-1/JunD level was accompanied by elevated proliferative capacity in DETA/NO-treated melanoma cells. Additionally, some attempts to correlate NO with AP-1 in other cellular system uncovered a positive relationship between them: NO increased AP-1 activity (28, 29) and the inhibition of AP-1 led to the inhibition of NO formation as well (30). In melanoma cells, AP-1/JunD was activated and its DNA-binding activity was enhanced with the trend nearly paralleling that of APE/Ref-1 in response to NO stress. Also, the depletion of APE/Ref-1 suppressed the AP-1/JunD induction by NO stress, thus confirming that APE/Ref-1 regulated the activation of AP-1/JunD by mediating NO stimulation and promoting its binding activity.

The expressions of MMPs are correlated with aggressive behavior and poor prognosis in many types of tumors, including malignant melanoma (31–33). In particular, MMP-1 plays a crucial role at many stages of metastasis. Our data showed that melanoma cells treated with DETA/

NO produced high levels of MMP-1 with stronger invasive capacity, especially in metastatic Lu1205 cells, implying that elevated invasiveness might be attributed to MMP-1 induction by NO stress. Specifically, MMP-1 expression is largely controlled at the transcriptional level with AP-1 binding sites present in its promoter region, suggesting that AP-1 should be involved in regulating its expression (34). Ishii et al. examined the possibility that NO transcriptionally induced MMPs in malignant melanoma and found that the enhancement of MMP-1 transcription was via activation of AP-1 (35). Depletion of AP-1/JunD also resulted in the decrease of MMP-1 in Lu1205 cells. Therefore, it is suggested that the elevated MMP-1 was subjected to AP-1/JunD activation mediated by induced APE/Ref-1.

Bcl-2, an important downstream molecule regulated by NF- $\kappa$ B, is closely tied to apoptosis. Exogenous NO has been reported to prevent apoptosis by inhibiting the drop in Bcl-2 levels (36). Similarly, the protection of keratinocytes and endothelial cells from UV-induced apoptosis was mediated through endogenous NO-stimulated increase in Bcl-2 expression (37), implying that NO affected the expression of Bcl-2. Results from our study showed that Bcl-2 was induced by NO stress in melanoma cells, particularly in metastatic Lu1205 cells. The induction was decreased after APE/Ref-1 siRNA transfection, suggesting that the effect of NO on Bcl-2 might be APE/Ref-1 dependent. Furthermore, APE/Ref-1 regulates the DNA-binding activity of NF- $\kappa$ B as well as AP-1: Up-regulation of APE/Ref-1 results in a notable increase in NF- $\kappa$ B activity and loss of APE/Ref-1 significantly attenuates NF- $\kappa$ B signaling (38). Hence, the stimulation of



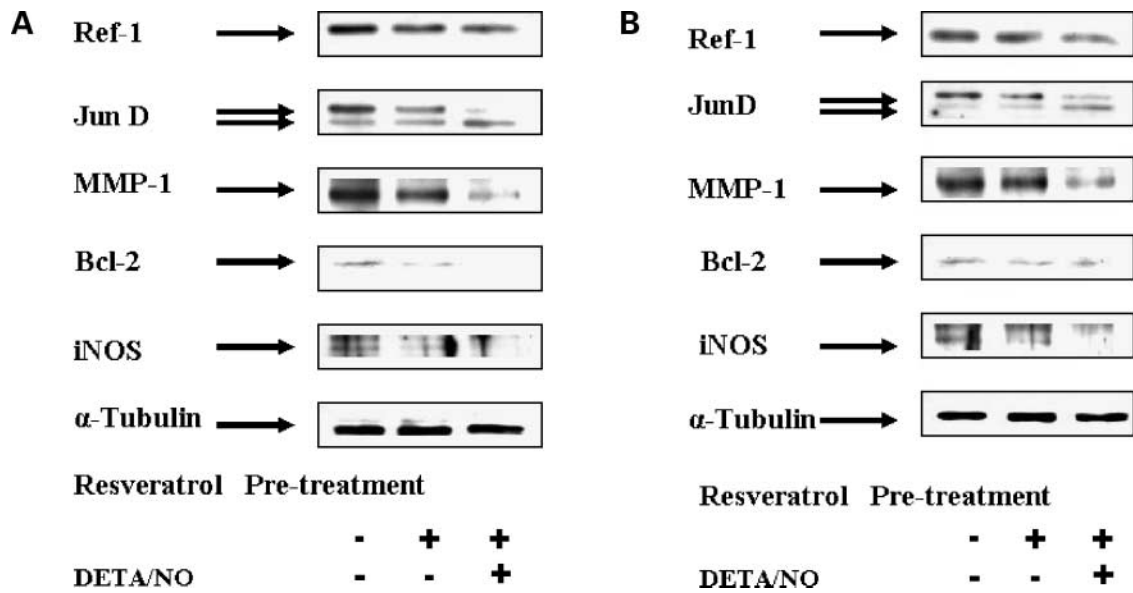
**Figure 4.** Role of AP-1/Jun D in response to NO stress. **A**, effect of DETA/NO on AP-1 binding activity by electrophoretic mobility shift assay. AP-1 transcriptional activity increased, especially after 2- and 12-h treatments (lanes 4 and 6). **B**, knockdown of AP-1/JunD with specific siRNA (**a**) prevented the induction by NO stress of MMP-1, iNOS; (**b**), APE/Ref-1 and Bcl-2; and (**c**) in Lu1205 cells. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus control. Columns, mean ( $n = 3$ ); bars, SD.

Bcl-2 by NO stress in melanoma cells was likely via APE/Ref-1 pathway based on its regulation of NF- $\kappa$ B.

Recent findings indicated that an increase in iNOS expression was associated with various tumors, including melanoma (39–41), and the up-regulation of this enzyme has been well correlated with tumor progression and poor survival. Remarkable expression has been found in malignant melanomas, especially in the invasive phase (42, 43); in particular, constitutive iNOS leads to intracellular NO production that is directly responsible for supporting melanoma growth (5), which further support the notion that iNOS and the release of NO all play important roles in the progression of metastatic human melanoma. In contrast, transfection with the *iNOS* gene in murine melanoma cells suppressed tumorigenicity and abrogates metastasis (44). This inconsistency might be explained by the distinct difference among species.

Furthermore, the promoter of the *iNOS* gene contains several homologous consensus sequences as binding sites for transcription factors, which are believed to be essential for *iNOS* gene transcription, especially AP-1 (45). In some cases, the inhibition of iNOS expression was found only through the suppression of AP-1 (46). Consistently, our data showed that knockdown of AP-1/JunD prevented the induction of iNOS. Moreover, as we depleted APE/Ref-1 by siRNA, the expression levels of iNOS and AP-1/JunD always paralleled that of APE/Ref-1. Therefore, we proposed that the regulation of iNOS by AP-1 mediated by APE/Ref-1 is involved in modulating the redox balance within the melanoma cell based on our data and related literature (47).

Resveratrol inhibits growth, induces apoptosis, suppresses angiogenesis, and blocks the reactive oxygen species-potentiated invasion of tumor cells (48, 49). In

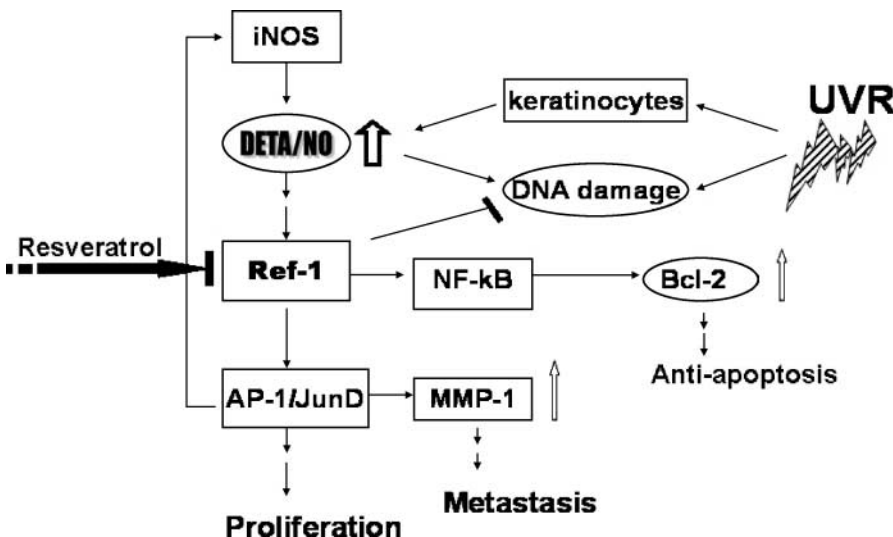


**Figure 5.** Resveratrol pretreatment reduces the expression of APE/Ref-1, AP-1/JunD, MMP-1, Bcl-2, and iNOS in melanoma cells. Three groups of melanoma cells were treated: control cells, cells pretreated with resveratrol (50  $\mu$ mol/L) for 24 h followed by a 24-h incubation of fresh medium without resveratrol, and cells pretreated with resveratrol for 24 h followed by a 24-h incubation of DETA/NO (100  $\mu$ mol/L) with fresh medium. Reduction in APE/Ref-1 expression following resveratrol pretreatment was accompanied by decreased AP-1/JunD, MMP-1, Bcl-2, and iNOS levels, which did not increase after being reincubated with DETA/NO for 24 h, after Western blot. **A**, Lu 1205 melanoma cells. **B**, Wm 3211 melanoma cells.

addition, results from recent studies represented that resveratrol strongly decreased the release of NO by reducing iNOS expression, notably by suppressing the activation of NF- $\kappa$ B that coincides with suppression of AP-1 (50). Our data indicated that resveratrol pretreatment resulted in a reduction in APE/Ref-1 expression accompanied by decreases of AP-1/JunD, MMP-1, Bcl-2, and iNOS. Moreover, as the resveratrol-pretreated cells were reincubated with DETA/NO, no induction of these downstream molecules was found. This activity of resveratrol might involve the follow-

ing mechanism: (a) its scavenging activity against NO, (b) the reduction in iNOS expression, and (c) the suppression of the NF- $\kappa$ B and AP-1 activation mediated by APE/Ref-1.

In summary, the present study shows that APE/Ref-1 induced by NO stress initiates a functional feedback loop contributing to the proliferation and metastatic capacity of melanoma cells, which can be interrupted by resveratrol. This compound may be useful in combination with other agents whose actions are blocked by up-regulation of these downstream molecules.



**Figure 6.** Model for NO-induced APE/Ref-1 and a feedback loop. NO induces APE/Ref-1, which mediates a series of intracellular signal activations and resultant NO production leads to restimulation of APE/Ref-1. As a result, melanoma cells became more proliferative and metastatic with the enhancements of AP-1/JunD, MMP-1, Bcl-2, and iNOS. Furthermore, knockdown of APE/Ref-1 or AP-1/JunD also reversed the induction by NO stress of downstream molecules, confirming the hypothesis that a functional feedback loop existed in which APE/Ref-1 played a central role. Resveratrol is known to inhibit APE/Ref-1 activity, thereby disrupting the loop.



## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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