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Redox effector factor-1, combined with reactive oxygen species, plays an important role in the transformation of JB6 cells

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Apurinic/apyrimidinic endonuclease/redox effector factor-1 (APE/Ref-1) is a multifunctional protein involved both in DNA base excision repair and redox regulation. Studies have suggested that abnormal Ref-1 levels and/or activities are associated with tumor progression and sensitivities to treatment, but no direct evidence has yet been published regarding the role of Ref-1 in malignant transformation. We utilized the well-documented tumor promotor-sensitive JB6 mouse epithelial cell model as well as new transformants [by ultraviolet light B (UVB), H_2O_2 or Cd] to study this phenomenon.

Significant increases of reactive oxygen species (ROS) were observed in JB6P+ and all the transformants compared with promotor-resistant JB6P− cells. These increases were paralleled by a sustained elevation of Ref-1 expression. Further analysis exhibited a strong inverse correlation between oxidative DNA lesions [8-oxodeoxyguanosine (8-oxo-dG)] and Ref-1 levels in all JB6 cells. Notably, apoptosis occurred after knock-down of Ref-1 by small interfering RNA (siRNA)] demonstrated by a ∼ 2-fold increase of Annexin V-positive JB6P+ cells. Ref-1 depletion also inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced anchorage-independent growth of JB6P+ by 40% and reduced the colony numbers of JB6P+/H_2O_2 and JB6P+/Cd cells. Mechanistic studies revealed that Ref-1 reduction was associated with an increase of intracellular ROS levels and a marked decrease of activator protein-1 (AP-1) transcription activities in JB6P+/H_2O_2 cells.

This is the first report of the novel role of Ref-1 in cellular transformation. Based on the data presented here, we propose that induction of Ref-1, serving as an adaptive response to elevated ROS, plays a critical role in transformation and protects cells from excess ROS stresses through both DNA repair and activation of transcription factors such as activator protein-1.

Introduction

Epidemiological observations and animal experiments have shown that the process of carcinogenesis involves a complex series of multi-sequence events that result in a cell evolving from a healthy state to a precancerous condition and finally to an early stage of cancer. The 'initiation-promotion-progression' model is a prototype for these stages. Many studies have shown that reactive oxygen species (ROS) are involved in all stages of carcinogenesis. The promotion stage is characterized by the clonal expansion of initiated cells and results in the formation of an identifiable focal lesion and involves reversible processes which require the continuous presence of the tumor promotion stimulus to be maintained. The promotion activity of TPA has been well documented (1) and extensive evidence has shown that tumor promotion by phorbol esters was, to a large degree, due to stimulation of oxygen radical generation and ROS-mediated inflammatory reactions (2,3); radical scavengers exhibited inhibitory effects on tumor promotion (4–6).

Both activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) activation are required for transformation whether induced by TPA or by tumor necrosis factor and blocking ROS-mediated AP-1-mitogen-activated protein kinase (MAPK) and NF-κB activation contributes to the anti-promotion effects of ROS scavengers (7,8). It is also well documented that low levels of ROS can stimulate cell division in the promotion stage and thus enhances tumor growth (9). Agents that interfere with the promotion stage have exhibited chemopreventive potential (10).

The JB6 mouse epidermal cell lines were established in the early 1980s by Nancy Colburn's group and JB6P+ cells (promotion sensitive) represent a well-developed cell culture system which have been extensively utilized as a model to study the molecular events that are associated with tumor promotion (11,12). In the presence of the tumor promoter TPA or epidermal growth factor, JB6P+ cells undergo a response similar to second stage tumor promotion and are associated with anchorage-independent growth and tumourigenic transformation; this does not occur with JB6P− cells. The basis for the transformation resistance has been associated with a deficiency of extracellular signal-regulated kinase signaling and lack of AP-1 activation (12,13).

Redox effector factor-1 (Ref-1) was first recognized by its DNA base excision repair activity and for this reason, it was initially named apurinic/apyrymidinic endonuclease. In addition to its DNA repair potential, Ref-1 also exhibits distinct redox regulation functions that facilitate the DNA-binding activities of many transcription factors (such as AP-1 dimers, NF-κB, p53 and Cellular homologue of avian myeloblastosis virus oncogene (Myb)) both in a redox-dependent and redox-independent manner. Because of its unique multiple functions, Ref-1 has attracted more attention recently and alterations of Ref-1 have been reported in many human tumors (14,15). Ref-1 expression has been shown to be well regulated both at the transcription and post-transcription levels. Apurinic/apyrimidinic endonuclease/Ref-1 is sensitive to alterations in redox status, and serves as a rapid adaptive response to subtoxic levels of a variety of ROS stresses and ROS generators such as H_2O_2, superoxide anion and γ-rays (16–18). Stuart (2004) reported that the reduction of APE1 levels after lowering ROS production by mitochondrial DNA depletion was reversed by H_2O_2 (19,20). Our previous study using human melanoma cells has also demonstrated remarkably elevated nuclear Ref-1 levels that are associated with abnormally increased intracellular ROS levels (15). The regulation of Ref-1 by ROS might occur via transcription activation, as many transcription factors such as AP-1, SP1, CREP and ATF bind to its promoter (14), which are also well known to be sensitive to oxidative stresses. In addition, ROS also affects the functions of Ref-1 by altering the redox status of this protein. Both DNA repair and redox regulation activities of Ref-1 were remarkably reduced either by oxidation of H_2O_2 or cystein replacement by site-directed mutagenesis (21,22).

Many studies have also shown that Ref-1 deficiency rendered a wide range of cells more sensitive to apoptosis induced by alkylating and oxidative agents as well as ionizing radiation (14,23,24). However, the direct involvement of Ref-1 in tumourigenesis or cell transformation remains unclear. In the current paper, we characterized the role of Ref-1 in tumor transformation with the JB6P+/tumor promoter model, and have provided important insights into the mechanisms involved.

Abbreviations: AP-1, activator protein-1; DCF, 2,′7′-dichlorofluorescein; FITC, fluorescein isothiocyanate; HE, dihydroethidium; MAPK, mitogen-activated protein kinase; Myb, Cellular homologue of avian myeloblastosis virus oncogene; NF-κB, nuclear factor kappa B; Ref-1, redox effector factor-1; ROS, reactive oxygen species; SEAP, secreted alkaline phosphatase; si-NS, si-nonsense; si-Ref-1, small interfering RNA against Ref-1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; UVB, Ultraviolet light (290–320 nm); 8-oxo-dG, 8-oxodeoxyguanosine.

Materials and methods

Cell culture

The JB6P− and JB6P+ cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). The JB6P+/UVB, JB6P+/Cd and JB6P+/H_2O_2 subcell lines were developed by our laboratory with long term (>2 months) repeated (twice a week) UVB (10 mJ/cm^2) or sustained exposure to $CdCl_2$ (1 μg/ml) or H_2O_2 (10 μM). Cells were seeded into 60 mm dishes at low density and changed with fresh medium. After 2 months, cell colonies exhibiting vertical growth potential were separated. The irreversibility of the transformation was confirmed by soft agar growth independent of TPA. All cells were cultured in Eagle minimum essential medium supplemented with 4% fetal bovine serum, 2 mM L-Glutamine (Cellgro®, Midiatech, Herndon, VA) and 25 μg/ml gentamicin (Invitrogen Corporation, Carlsbad, CA) and incubated with 5% CO_2 at 37°C. The cells were split with 0.25% Trypsin–ethylenediaminetetraacetic acid and kept at low density (1.5×10^4 cells per ml) and the fresh medium was changed every 2 days. All the chemical reagents were purchased from Sigma, St Louis, MO.

Anchorage-independent cell transformation assay

Cell transformation in the presence or absence of TPA was investigated by soft agar colony formation analysis. Cells (8×10^3 per ml) were exposed to TPA (10 or 20 ng/ml) in 1 ml of 0.33% basal medium Eagle agar containing 10% fetal bovine serum. The cultures were incubated in a 5% CO_2 atmosphere at 37°C for 14–18 days (11) and the colonies (containing >50 cells) were quantified using a light microscope at ×40 magnification.

Cellular protein extraction and immunoblot analysis

Nuclear extracts from cells were prepared as described previously with minor changes (25). Briefly, cells were scraped and suspended in cold phosphate-buffered saline, spun and cell pellets re-suspended in buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 10 mM KCl, 0.150 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride) and allowed to swell on ice for 10 min. Ten percent NP-40 was added and cells were lysed by vortex. After 30 s of centrifugation, buffer A was aspirated and the nuclei pellet suspended in buffer C (20 mM HEPES, 20% glycerol, 0.42 M NaCl, 0.15 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice with shaking occasionally for >15 min. After centrifugation for 30 min at 14 000*g*, the supernatant was removed and diluted with buffer D (20 mM HEPES, 20% glycerol, 50 mM KCl, 0.5 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride). Whole-cell lysis extracts were prepared according to the directions of the manufacturer (Cell Signaling Technology, Danvers, MA). Briefly, after washing with cold phosphate-buffered saline, cells were incubated with 1× lysis buffer on ice for 5 min, sonicated briefly and extracts spun for 10 min at 14 000*g* and the supernatants collected for analysis. Using a Bio-Rad Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA), protein concentrations of all the samples were precisely measured to confirm equivalent loading; the correlation coefficient of the standard curve was at least 0.99. Samples were stored at −80°C.

Equal amounts of protein extracts (25 μg of nuclear extracts, 50 μg of whole-cell lysis) were loaded and electrophoresis was performed in a 10% polyacrylamide separating gel/5% stacking gel. Proteins were transferred to polyvinylidene difluoride membrane and incubated in blocking solution for 2 h. The membrane was incubated with primary mouse anti-human Ref-1 antibody (1:2000, Novus Bio, Littleton, CO) at 4°C overnight. Blots were washed in 0.1% Tween-20/Tris-Buffered Saline (TBST) and incubated with second antibody (peroxidase-conjugated anti-mouse IgG, 1:4000, Santa Cruz, Santa Cruz, CA) for 1 h at room temperature. After extensive washing, the second antibody was visualized by chemiluminescence reagents. Tubulin expression as an equal loading control was also performed with a mouse anti-human tubulin antibody (Sigma).

Probes of ROS

Relative hydrogen peroxide and superoxide anion levels were determined using 6-carboxy-2′7′-dichlorodihydrofluorescein diacetate (5 μM) and dihydroethidium (HE) (10 μM), respectively (Molecular Probes, Eugene, OR). 6-Carboxy-2′, 7′-dichlorodihydro-fluorescein diacetate diffuses through cell membranes and is hydrolyzed by intracellular esterase to the fluorescent 2′,7′-dichlorofluorescein (DCF). This molecule remains trapped within cells, reacting with hydrogen peroxide and generates a fluorescent signal. HE is the sodium borohydride-reduced form of ethidium bromide that is permeable to viable cells. HE is directly oxidized to ethidium bromide by superoxide anion, which then fluoresces. Cells were incubated with the appropriate probe for 15 min, harvested and analyzed by flow cytometry using a Becton-Dickinson FACScan with CellQuest software.

Oxidative DNA damage (8-oxo-dG) analysis

Oxidative modified guanine derivatives 8-oxo-dG is one of the typical mutagenic DNA lesions induced by ROS and serves as the most commonly used biomarker of oxidative DNA damage. Avidin has a high affinity for 8-oxo-dG, which is structurally similar to biotin. Binding of avidin–fluorescein isothiocyanate (FITC) (Sigma) to JB6 series cells was performed as described previously with minor modifications (26). Briefly, cells were fixed in methanal (20 min, −20°C), permeabilized in Tris-buffered saline (containing 0.1% Triton X-100; 15 min, 25°C), blocked for non-specific binding (Tris-buffered saline containing 15% fetal calf serum; 2 h, 25°C) and stained with 15 μg/ml FITC-conjugated avidin (1 h, 37°C). After staining, cells were collected for flow cytometry analysis.

Transient transfection of Ref-1 siRNA in JB6 cells

The 21-nucleotide RNAs used for decreasing Ref-1 in JB6 cells were ordered from Invitrogen. Sequences of the double-stranded siRNA were as follows: sense, 5′-GUCUGGUACGACUGGAGUACC-3′; antisense, 5′-UACUCCAGUCGUACCAG ACCU-3′ (23). Transfection of siRNA was carried out with Lipofectamine 2000 according to the directions of the manufacturer (Invitrogen), and was applied to cells ∼50% confluent in six-well plates to give the final concentration of 60 nM or 120 nM. After 6 h, the medium was replaced with fresh Eagle minimum essential medium +4% serum. Cells were analyzed 24 h, 48 h or 72 h, respectively, after transfection.

Analysis of apoptosis by Annexin V/propodium iodide staining

Annexin V assay was performed according to the manufacturer's protocol (BD PharMingen, San Diego, CA). Briefly, cells were harvested, washed twice in 1× phosphate-buffered saline and re-suspended in binding buffer at a concentration of 1×10^6 cells per ml of which 100 μl was incubated with 5 μl of Annexin V conjugated to FITC and 10 μM propidium iodide for 15 min. Cells were analyzed by flow cytometry using a Becton-Dickinson FACScan. The proportion of apoptotic cells was estimated by the percentage of cells that stained positive for Annexin V, while remaining impermeable to propidium iodide.

Measuring the binding of activated AP-1 transcription factor

To study AP-1-dependent gene transcription, we used a reporter vector system containing the secreted alkaline phosphatase (SEAP) gene (Clontech Laboratories, Palo Alto, CA). The reporter construct pAP1-SEAP is designed to measure the binding of transcription factors to AP1, providing a direct measurement of activation for this pathway. After transcription activators bind AP1, transcription is induced and the reporter gene is activated. The secreted SEAP enzyme is assayed directly from the culture medium using Great EscAPe Chemiluminescence Detection Kits (Clontech Laboratories). For the transient transfection, we plated different JB6 cells at a density of 1×10^5 per well in a 24-well plate. After transfection with small interfering RNA against Ref-1 (si-Ref-1) or si-nonsense (si-NS), the cells were co-transfected 24 h later with 1 μg of the reporter constructs with the use of the Lipofectamine 2000 reagent according to the manufacturer's directions. Seventy-two hours later, the media was collected for SEAP activity assay, which was performed according to the manufacturer's instructions using a microplate illuminometer.

Statistical analysis

Statistical comparisons utilized the *t*-test and linear regression analysis. Differences were considered statistically significant at the $P < 0.05$ level.

Results

Transformation of JB6P+ cells by UVB radiation, H_2O_2 and $CdCl_2$ stimuli at subtoxic doses

The relative intracellular ROS levels were detected by utilizing the DCF fluorescence probe. As shown in Figure 1A, a one-time exposure of cells to UVB (40 mJ/cm^2) radiation led to a significant increase of ROS production in JB6P+ cells (up to 2.78-fold by 5 h after treatment) compared with that of control (Figure 1A) and the elevation of ROS was maintained in the cells for at least 24 h. Consistently, similar increase of DCF density was also observed with $CdCl_2$ treatment. The peak occurred shortly after 5 min of exposure, followed by quick reduction to normal levels by 30 min, while a notable decrease of intracellular ROS levels were found after 5 h and maintained for 24 h (Figure 1B).

After prolonged exposure (2 month at non-toxic doses) of JB6P+ cells to H_2O_2, $CdCl_2$ or UVB radiation, cells were seeded at very low density and colonies exhibiting vertical growth were selected. Cell

transformation was confirmed by the anchorage-independent growth assay. JB6P+ parent cells formed a low number of colonies in soft agar culture (13.7 ± 3.5 per 20 high-power fields) unless stimulated by TPA (44.7 ± 2.9). Both the number and size of colonies in the new transformants (as shown in Figure 1C) were significantly increased compared with parent JB6P+ cells in the absence of TPA in soft agar culture (104.0 ± 17.3 per 20 high-power fields in JB6P+/UVB; 56.7 ± 15.0 in JB6P+/H_2O_2; 64.3 ± 22 in JB6P+/Cd cells; all $P < 0.05$).

Increase of intracellular ROS levels in promotor-sensitive JB6P+ and transformants compared with promotor-resistant JB6P− cells

As shown in Figure 2, utilizing the molecular probes DCF and HE we measured, respectively, the relative intracellular levels of hydrogen peroxide and superoxide anion in JB6P−, JB6P+, JB6P+/UVB, JB6P+/Cd and JB6P+/H_2O_2 cells. The DCF and HE fluroescences in JB6P+ cells were, respectively, increased to 9.0- and 2.2-fold of JB6P− cells. Increases of DCF and HE florescence were also evident in JB6P+/UVB (3.4- and 1.3-fold), JB6P+/H_2O_2 (6.5- and 1.3-fold) and JB6P+/Cd cells (3.6- and 1.4-fold). Notably, compared with JB6P+ parent cells, all the transformant cell lines exhibited reduction of ROS levels that was still higher than that of JB6P− cells.

Elevated nuclear Ref-1 expression is negatively correlated with cellular oxidative DNA damage lesion levels

Avidin binds with high specificity to the potentially mutagenic oxidized nucleoside, 8-oxo-dG, and to the oxidative modified base, 8-oxoguanine. The Avidin–FITC fluorescence density represents the relative levels of oxidative DNA products. Our results showed a drop of fluorescence staining by 33% in JB6P+ cells compared with that of JB6P− cells. Similar reductions were also obvious in JB6P+/UVB, JB6P+/H_2O_2 and JB6P+/Cd cells (51%, 82% and 57% of JB6P− cells, respectively) as shown in Figure 2B.

As Ref-1 has a critical role in DNA repair, we further studied the relative expression levels of Ref-1 in the JB6 series cells. Nuclear extracts were collected and subjected to western blot assay. The bands were scanned by a densitometer and normalized to actin levels. Our results showed that JB6P+, JB6P+/UVB, JB6P+/H_2O_2 and JB6P+/Cd cells all exhibited higher Ref-1 expression levels than JB6P− (Figure 2C). Ref-1 levels were the highest in UVB-transformed JB6P+ cells (2.6-fold that of JB6P− and increased 47% that of

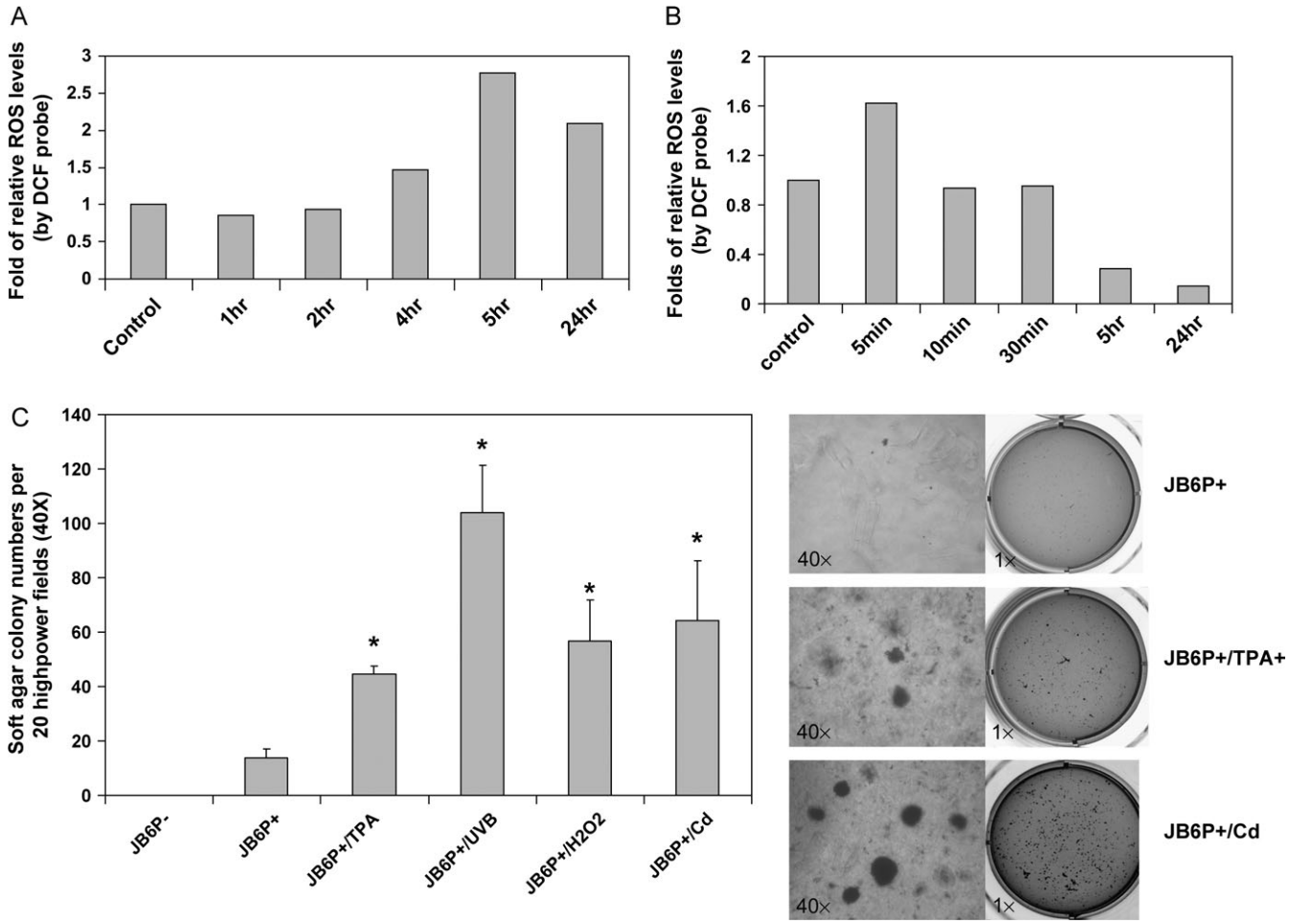

Fig. 1. (**A** and **B**) Increased intracellular ROS levels by UVB radiation and $CdCl_2$ treatment in JB6P+ cells. Cells were exposed to UVB radiation (40 mJ/cm^2) or $CdCl_2$ (1 μg/ml), respectively, for different time, followed by the detection of intracellular content of ROS using DCF fluorescence probing as described in Materials and Methods. The results are expressed as fluorescence intensity of DCF as fold of control, with the control value set at 1.0. (**C**) Prolonged pretreatment of JB6P+ cells with $CdCl_2$, H_2O_2 or UVB induces transformation to anchorage-independent (tumor cell) phenotype. JB6P−, JB6P+, JB6P+/UVB, JB6P+/Cd and JB6P+/H_2O_2 cells were seeded onto 60 mm dishes in medium containing 0.33% agar in the absence or presence of promotor TPA (10 ng/ml) on top of a bed medium containing 0.5% agar. After 16 days, cell colonies were stained by crystal violet/phosphate-buffered saline and counted. The same original magnification was used for taking all pictures as labeled. The data are presented as total number of colonies per 20 randomly selected high-power fields of view (×40), means ± SDs of three independent dishes. * $P < 0.05$ compared with JB6P+ cells. Representative pictures taken with light microscope (×40, ×1) of soft agar colonies were shown next to panel C as labeled.

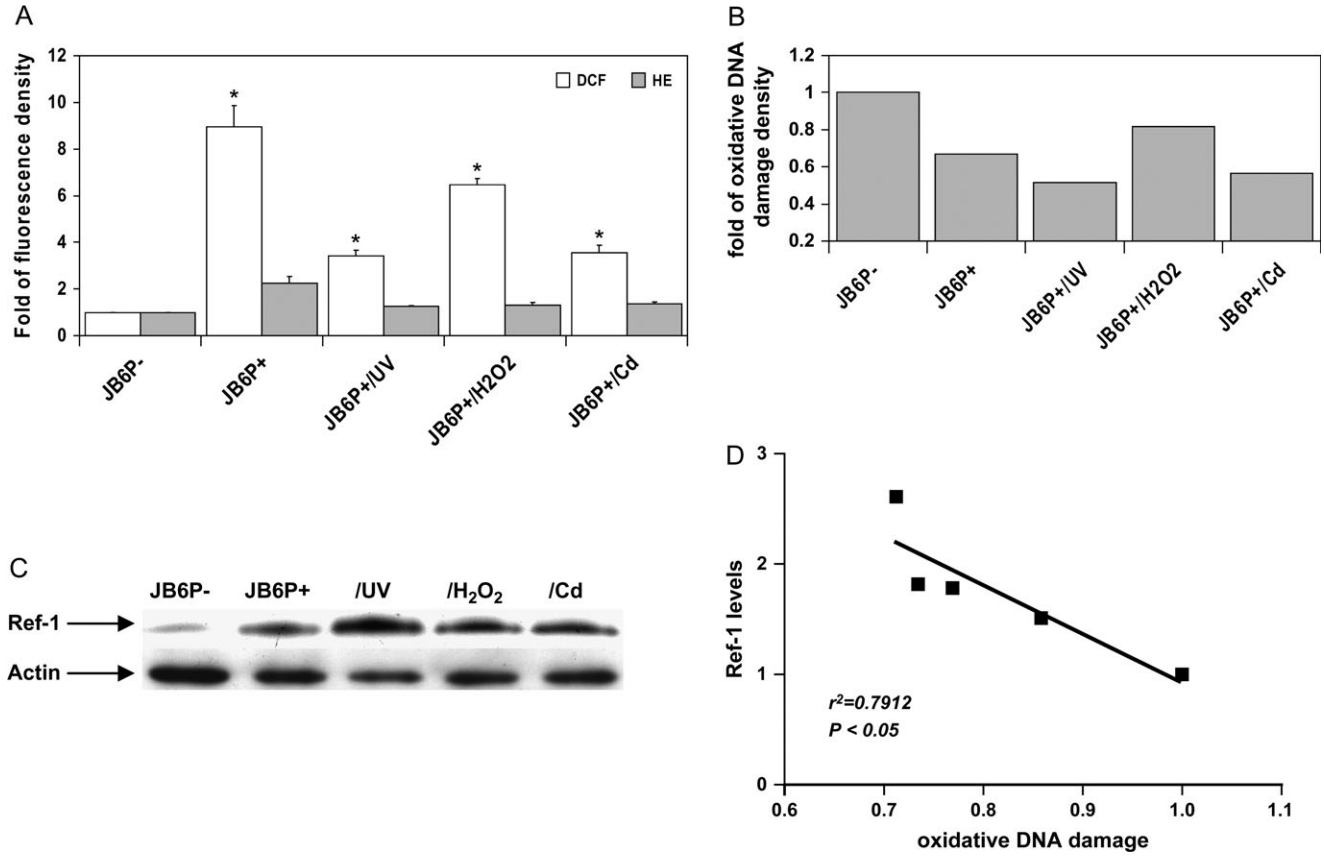

Fig. 2. Elevated Ref-1 protected cells from extensive ROS stresses. (**A**) Attenuation of ROS levels following transformation of JB6P+ cells with UVB, H_2O_2 or $CdCl_2$. Intracellular contents of peroxide and superoxide were estimated as indicated in the Materials and Methods, and are expressed as fluorescence intensity as fold of control, with the control value set at 1.0. Data are presented as fold of control intracellular DCF (white bar) and HE (gray bar) fluorescence, respectively, means ± SDs for three independent experiments. Significant difference from control DCF is $P < 0.05$ *. (**B**) Relative intracellular oxidative DNA damage levels in different JB6 series cells. Relative oxidative DNA damage product 8-oxo-dG levels were detected by Avidin-FITC by Flow cytometry as described in Materials and Methods. (**C**) Elevation of nuclear Ref-1 expression levels in JB6P+ cells transformed by UVB, H_2O_2 or $CdCl_2$. Nuclear extracts of different cells were collected and subjected to western blotting using antibodies against human Ref-1. Equal loading was adjusted by actin staining. (**D**) Correlation between Ref-1 expression levels and oxidative DNA damage. Correlation analysis of relative Ref-1 levels (standardizing Ref-1 level in JB6P− cells as 1.0) and relative oxidative DNA damage levels (standardizing fluorescence density in JB6P− cells as 1.0).

JB6P+). There were no significant differences of Ref-1 expression in $CdCl_2$- and H_2O_2-transformed cells (1.82- and 1.76-fold, respectively) compared with JB6P+ (1.78-fold).

Additional statistic analysis revealed a strong negative correlation ($P < 0.05$, $r^2 = 0.7912$) between relative Ref-1 expression and labeled fluorescence density of oxidative DNA lesions (Figure 2D). Higher Ref-1 levels were associated with a reduced level of oxidative DNA damage products.

Knock-down of Ref-1 inhibits tumor promoter-induced cell transformation in JB6P+ cells and proliferation of JB6P+/UVB, JB6P+/H_2O_2, JB6P+/Cd cells

To further delineate the role of Ref-1 in cell transformation and proliferation, a si-Ref-1 was introduced into the cells. The efficiency of siRNA (60 nM) in knocking down Ref-1 after transfection was confirmed by western blotting analysis with whole-cell lysates. The decrease of Ref-1 occurred as early as 24 h after transfection, reached maximal inhibition by 3 days and returned to normal levels after 5 days (data not shown). As shown in Figure 3A, Ref-1 protein levels in different cells declined in excess of ∼80–90% (varied among cell lines) 3 days after si-Ref-1 transfection compared with si-NS.

Next, we investigated whether Ref-1 depletion affected malignant cell transformation. After 24 h of transfection with si-NS or si-Ref-1 (60 nM), JB6P+ cells were collected and reseeded in soft agar in the presence of TPA (20 ng/ml). After 15 days, the number of colonies were counted and analyzed (Figure 3B). There was no significant difference between lipofectamine-negative control cells (standardized as 1.0) and cells transfected with si-NS (1.04 ± 0.20; #, $P > 0.05$), while a substantial reduction was demonstrated in Ref-1-depleted cells (0.59 ± 0.14, ∗, $P < 0.05$ compared with si-NS).

To determine if Ref-1 depletion can reverse the transformation in stably transformed cells, we further studied colony formation potential of the transformants in soft agar after knock-down of Ref-1. Depletion by si-Ref-1 significantly suppressed colony formation of JB6P+/H_2O_2 and JB6P+/Cd cells (Figure 3C). The mean numbers of colonies decreased by 45 % and 50%, respectively, compared with si-NS-transfected cells ($P < 0.05$). However, in JB6P+/UVB cells, knock-down of Ref-1 only produced a marginal reduction of colony formation ($P > 0.05$, data not shown).

Depletion of Ref-1 induced apoptosis in transformed cells, associated with elevated intracellular ROS levels

We next examined whether the inhibitory effects of Ref-1 depletion on transformation was due to induction of apoptosis. Flow cytometry analysis was performed following si-Ref-1 transfection (120 nM). The results showed that in Ref-1-depleted JB6P+ cells, the fluorescence density of Annexin V-FITC binding was increased (3.4 folds of lipofectamine alone control cells), while cells

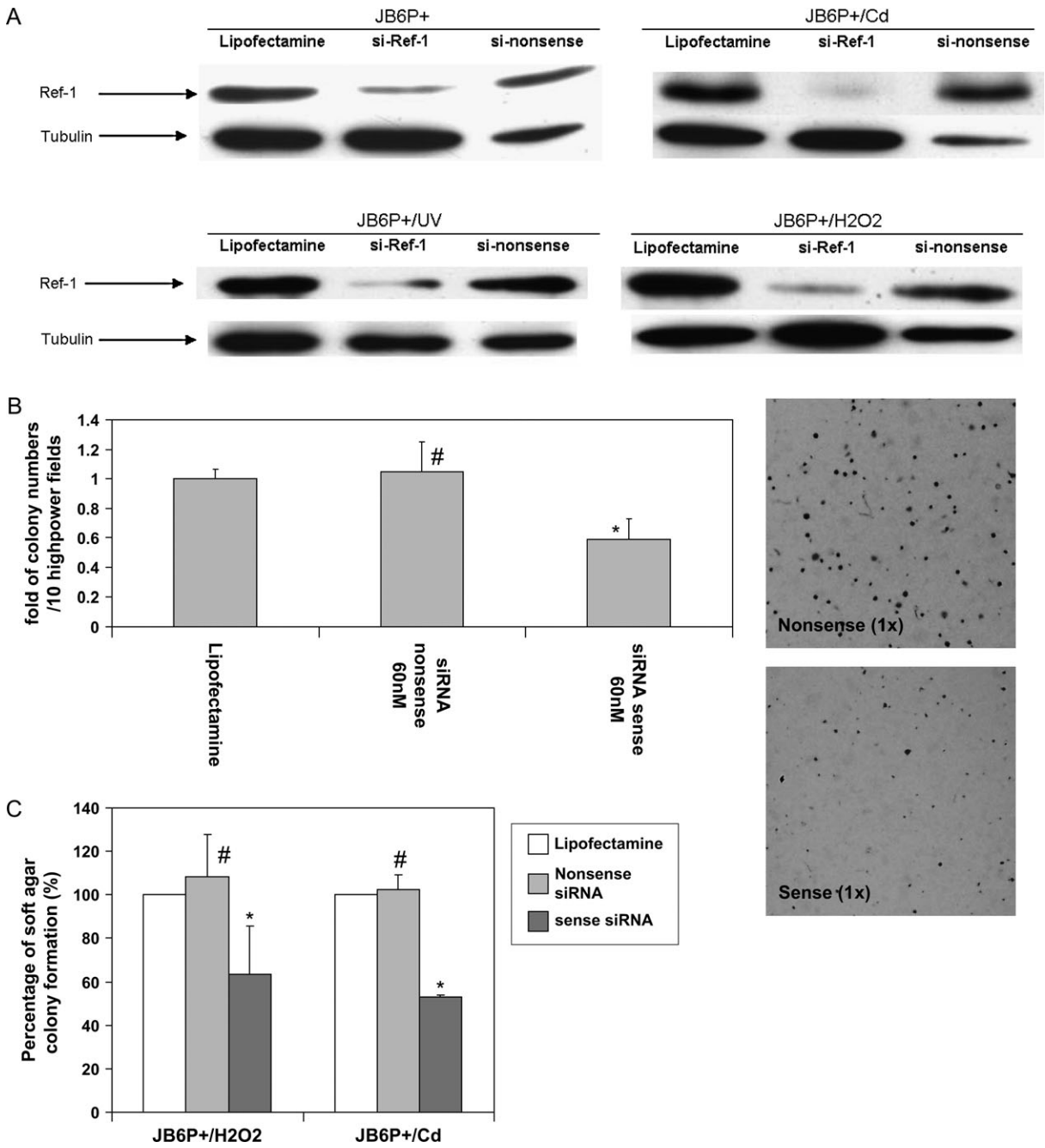

Fig. 3. (**A**) Knock-down of Ref-1 expression by siRNA transfection. Three days after transfection (60 nM), 50 μg whole-cell extracts from different cells were subjected to western blotting using antibodies against human Ref-1. Representative blots are shown. Each blot was re-blotted with antibody to tubulin to standardize equivalent protein loading in each lane. (**B**) Tumor promoter TPA-induced anchorage-independent transformation are inhibited in si-Ref-1-transfected cells. si-NS or si-Ref-1 (60 nM)-transfected cells were used to assess colony formation in a cell anchorage-independent assay. For TPA-induced transformation, JB6P+ cells (8×10^3 per ml) were exposed to TPA (20 ng/ml) in 1 ml of 0.33% basal medium Eagle agar containing 10% fetal bovine serum. The cultures were maintained in a 37°C, 5% CO_2 incubator for 15 days and the colonies scored as described by Colburn *et al.* (11). (**C**) Effect of Ref-1 siRNA on proliferation of JB6P+/H_2O_2 and JB6P+/Cd cells. Cells were grown in soft agar without TPA. * $P < 0.05$, a significantly reduced number of colonies formed by si-Ref-1-transfected cells compared with si-NS-transfected cells; #, $P > 0.05$, number of colonies formed by si-NS-transfected cells compared with lipofectamine-treated cells. Columns, means of three independent experiments; bars, SD.

treated with si-NS exhibited a marginal reduction (Figure 4A). Similar increases in Annexin V-FITC labeling were also evident in JB6P+/UVB (1.7-fold) and JB6P+/Cd (1.9-fold) cells after Ref-1-siRNA transfection; no changes in apoptosis were detected in the tumor promotor-resistant JB6P− cells following knock-down of Ref-1.

In si-Ref-1-transfected JB6P+ cells, Annexin V-positive cells were increased. As shown in Figure 4B, viable cells were reduced from 59.1 to 18.7%, while the apoptosis rate increased from 22.3 to 49.8%. We have also observed this pattern of increased apoptosis in JB6P+/UVB cells but at a less degree. si-Ref-1 decreased cell viability of JB6P+/UVB cells to 39.8% coupled with an elevation of apoptosis to 37.1% compared with 57.0% and 21.4%, respectively, in si-NS cells (data not shown).

We further investigated the effect of Ref-1 depletion on intracellular ROS levels (Figure 4C). Although reduction of Ref-1 by siRNA

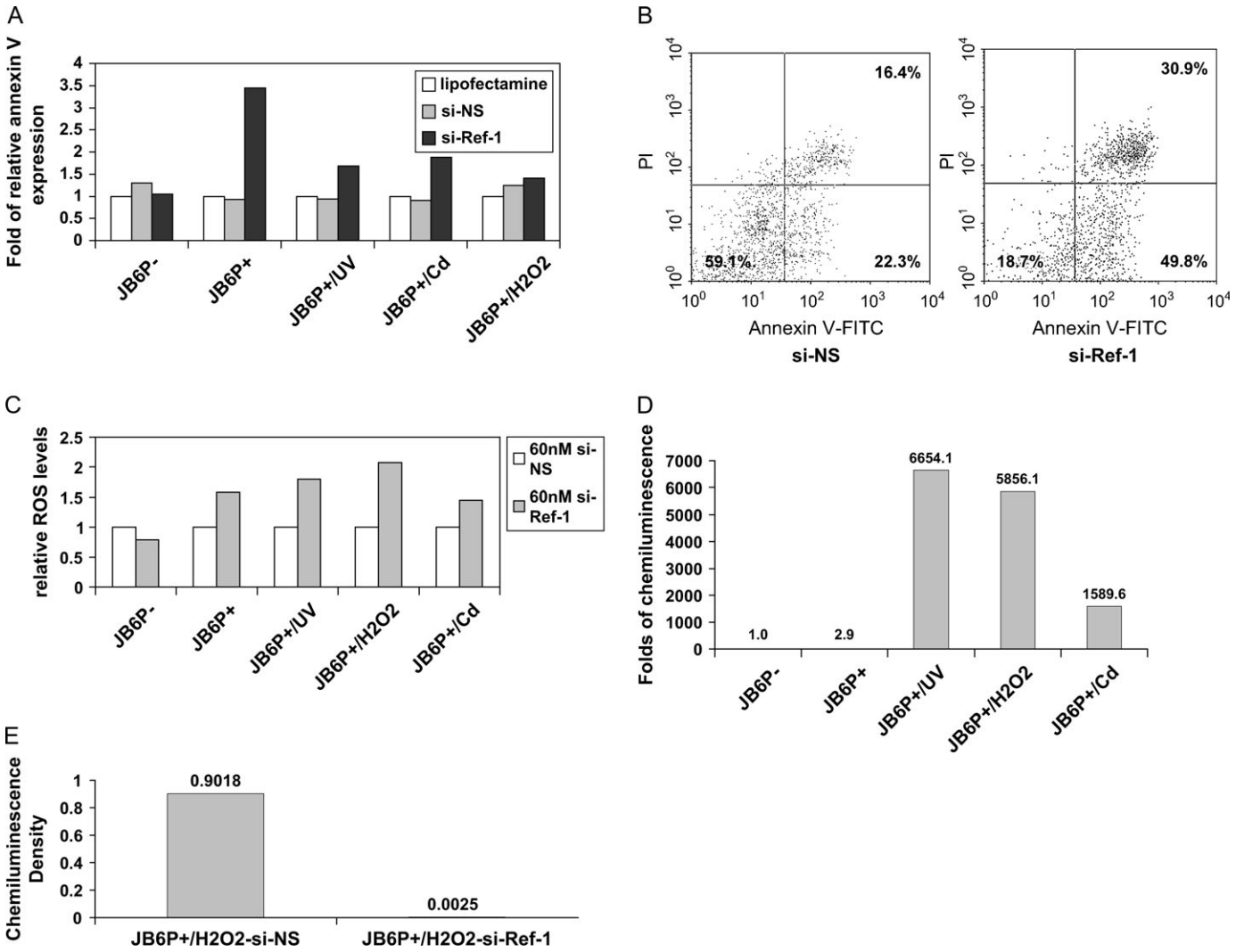

Fig. 4. (**A**) and (**B**) Ref-1 depletion by siRNA significantly induced cell apoptosis. Annexin V labeling of phosphatidylserine externalization was detected by flow cytometry assay, and fluorescence density was captured and analyzed. Cell apoptosis was analyzed by Annexin V-FITC/propodium iodide double staining following different treatments. The percentage of cells undergoing apoptosis was ascertained. Annexin V (+)/propodium iodide (−) represent early apoptosis cells and Annexin V (+)/propodium iodide (+) represent cells undergoing late stage apoptosis and necrosis. Panel B represented apoptotic shift of JB6P+ cells after si-Ref-1 transfection (120 nM). (**C**) Elevation of intracellular ROS levels after Ref-1 depletion. Cells were seeded overnight at the density of 60% for transient transfection of Ref-1 siRNA (60 nM). Forty-eight hours later, cells were collected and stained with DCF fluorescence probe for flow cytometry as described in Materials and Methods. Data are represented as the fold of si-NS control. (**D**) Elevation of AP-1 transcriptional activities in cells transformed by UVB, H_2O_2, or $CdCl_2$ detected by AP-1/SEAP reporter vector. Cells were transfected with AP-1/SEAP vectors containing the consensus AP-1 site. AP-1 binding or transcriptional transactivation results in activation of the SEAP gene and protein activity was quantitated by chemiluminometer. The relative SEAP activity was presented as folds that of JB6P− cells; means of two independent experiments. (**E**) Depletion of Ref-1 inhibited AP-1 transcriptional activity in JB6P+/H_2O_2 cells. Cells were transiently co-transfected with Ref-1 siRNA (120 nM) and AP-1/SEAP vectors as described in Materials and Methods.

was shown 24 h after siRNA exposure, no significant changes of ROS were observed. The notable elevation of ROS occurred by 48 h after transfection before any apoptosis was evident (which occurred 72 h after transfection). Intracellular ROS levels increased by 58% in JB6P+, 79% in JB6P+/UVB, 107% in JB6P+/H_2O_2 and 45% in JB6P+/Cd cells compared with si-NS cells (Figure 4C).

Although we failed to observe a significant reduction of soft agar colony formation in JB6P+/UVB cells with si-Ref-1 transfection, increased Annexin V (+) cells and elevated intracellular ROS levels were evident after Ref-1 depletion. This discordant result might reflect the transient transfection of si-Ref-1 with recovery of Ref-1 levels. Insufficient Ref-1 inhibition may lead only to delay the colony growth or proliferation, which is supported by our observation of marginally reduced soft agar colonies after si-Ref-1 transfection in JB6P+/UVB cells. Further studies using stable expression of short hairpin RNA (shRNA) of Ref-1 will be needed to clarify this issue. Another alterative explanation might be related to the distinct molecular mechanisms of transformation by UVB, $CdCl_2$ and H_2O_2, where the role of Ref-1 might be differentially involved. Our studies have revealed, for example, that AP-1 compositions are differentially regulated in these three transformants (data not shown). As the compositions of AP-1 complexes are critical in determining the downstream target genes involved in different pathways such as proliferation, differentiation and apoptosis, activation of AP-1 DNA-binding by Ref-1 leads to transcription of different down stream target genes. Depletion of Ref-1 alone appears not to be strong enough to inhibit the proliferation of UVB-transformed JB6P+ cells.

Depletion of Ref-1 inhibited AP-1 transcriptional activities

We performed transfection with an AP-1-SEAP reporter vector system containing a synthetic AP-1 element and a SEAP gene. Once AP-1 transcription is induced, SEAP gene is activated and detected. The results showed that basal AP-1 transcription activities were significantly elevated in all transformants (Figure 4D). JB6P+/H_2O_2 cells exhibited

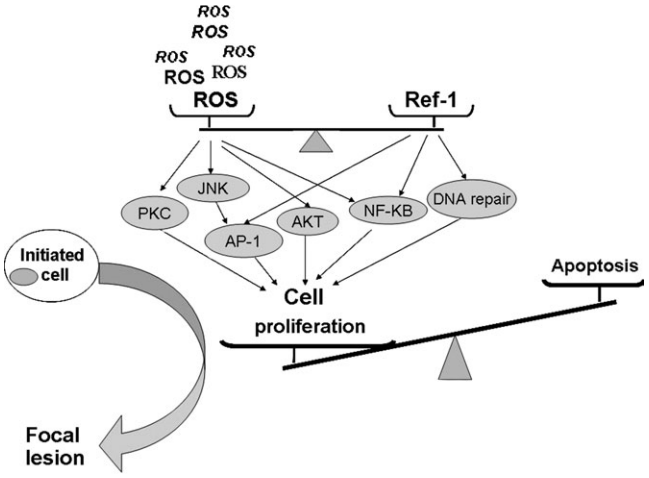

Fig. 5. Schematic diagram of possible roles of elevated intracellular ROS combined with Ref-1 in tumor transformation in JB6P+ cells. Elevated intracellular ROS contributes to tumor promotion process through regulating redox-sensitive anti-apoptotic/proliferation signaling involving protein kinase C, Phosphoinositide 3 kinase, Akt (also known as protein kinase B), Jun N-terminal kinase/AP-1 and NF-κB. As an adaptive response to increased ROS, Ref-1 expression is induced to protect cells from excessive ROS. The mechanisms might be related to its effective activations of many survival/anti-apoptotic signalings in a redox-dependent or redox-independent manner, as well as its critical role in DNA base excision repair, which becomes necessary for cell proliferation and leads to the promotion of initiated cells to focal lesion.

>5000-fold increase compared with JB6P− cells, which was significantly reduced by Ref-1 depletion to a very low level (Figure 4E).

Discussion

It has been well documented in many studies of human cancer that alterations of Ref-1 expression levels and subcellular localization have contributed to progression of tumor cells and development of treatment resistance (27–30). Although a function of Ref-1 in cell transformation and tumor development has been postulated, to date, no direct evidence has yet been published. In the present study, we studied the effect of Ref-1 on cell transformation utilizing the JB6 promotor-transformation model system. Our data suggested that induction of Ref-1, serving as an adaptive response to increased intracellular ROS stresses, plays a critical role in ROS-mediated malignant transformation and protects cells from excess ROS stresses. Depletion of Ref-1 rendered JB6P+ cells more sensitive to apoptosis and inhibited cell transformation as well as proliferation.

Our data has shown that transformation-responsive JB6P+ cells responded to UVB radiation and $CdCl_2$ with induction of intracellular ROS levels. The JB6P+ cells were irreversibly transformed in our laboratory utilizing prolonged low doses of $CdCl_2$ or H_2O_2 or intermittent repeated low doses of UVB. These transformants were able to grow into colonies in soft agar culture with absence of tumor promotor stimulation. It is well documented that UVB radiation, H_2O_2 and $CdCl_2$ increase intracellular ROS levels and/or induces oxidative damages through direct or indirect mechanisms in wide-range types of cells (31–33).

Cd is a highly toxic metal and occupational exposure has been associated with cancers of lung, prostate, pancreas and kidney. Although Cd itself is not a transition metal (it can not generate ROS directly), many studies have shown that carcinogenicity seems to be mediated by the indirect production of various radicals such as superoxide radical, hydroxyl radical and nitric oxide, which contributes to the activation of AP-1/Jun N-terminal kinase and NF-κB signal transduction pathways, in turn leading to the transcription of genes involved in cell growth regulatory pathways, for example, in hepatic cells (32,34,35).

The induction of AP-1 and NF-κB transcription by ultraviolet radiation has also been readily documented (36,37). The phosphorylations of c-Jun and c-Fos by UV-induced Jun N-terminal kinase and p38 MAPKs, respectively, result in remarkable increases of AP-1-DNA binding activities and enhancement of AP1-driven gene expressions (38). In addition, studies also have shown that H_2O_2 treatment induced substantial activation of redox-sensitive AP-1 and NF-κB signaling in different types of cells, resulting in up-regulation of transcription of inflammatory cytokines or other downstream target genes (39). Notably, it is also well studied that UVB radiation-induced oxidative stress is associated with an increase of H_2O_2 production which is positively correlated with lipid peroxidation, protein oxidation and DNA damage (40). Antioxidants effectively attenuated UVB-induced oxidative stress-mediated phosphorylation of extracellular signal-regulated kinase 1/2, Jun N-terminal kinase and p38 proteins of the MAPK family (41). We proposed that the transformations of JB6P+ cells by UVB, $CdCl_2$ and H_2O_2, at least partially, were due to ROS-activated cell proliferation signaling pathways such as AP-1 and NF-κB, which would be synergistically enhanced by Ref-1.

Our results showed a remarkable increase of Ref-1 levels in tumor promoter-sensitive JB6P+ cells compared with transformation-resistant JB6P− cells, which was paralleled by elevated ROS levels in JB6 parental and all transformants; however, higher Ref-1 was associated with a reduced oxidative DNA damage even in the presence of high levels of ROS. Depletion of Ref-1 effectively inhibited TPA-induced transformation and cell proliferation of the transformants. It is well known that the redox states of cellular biological systems are kept within a narrow range under normal conditions. Certain situations (e.g. mitochondrial DNA mutation, melanin oxidation, carcinogen exposure) will stimulate intracellular ROS production (33,42,43). As an adaptive response, increased Ref-1 counteracts intracellular oxidative stress not only by efficiently repairing oxidative DNA damage but also through activating many redox-sensitive signal pathways, such as AP-1 and NF-κB. The decreased relative 8-oxo-dG levels suggested that induction of Ref-1 protected cells from persistent extensive ROS stress through repair of the oxidative DNA damage. Consistent with this explanation, our data indicated that Ref-1 siRNA transfection increased intracellular ROS levels which were associated with the induction of apoptosis. It might contribute, at least partially, to the inhibition of transformation and proliferation by Ref-1 depletion.

Acting hierarchically to regulate many transcription factors (i.e. AP-1 and NF-κB), Ref-1 is critically involved in more than just one pathway. One explanation of the protective role of Ref-1 may also relate to the activating potential of Ref-1 on many transcription factors including AP-1 and NF-κB, which might facilitate or enhance further stimulation by tumor promoters. In supporting this hypothesis, our studies have shown that all transformants exhibited remarkable induction of AP-1 transcriptional activities and a 1.9-fold increase of AP-1 activity was observed in JB6P+ compared with JB6P− cells. Depletion of Ref-1 by siRNA transfection significantly reduced activated AP-1 transcription. Consistently, we have also shown previously that Ref-1 depletion significantly reduced AP-1 and NF-κB DNA-binding activities in human melanoma cells (15). Studies of others have shown elevated p65-specific DNA binding activities in JB6P+ cells and forced expression of p65 restores the transformation response of JB6P− cells to tumor promoters (44). Zhao *et al.* (45) also reported that stimulating AP-1-dependent transcriptional activity by 2-arachidonoylglycerol enhanced epidermal growth factor-induced cell transformation in the same JB6 P+ cells. The presence of elevated Ref-1 might, on the one hand, enhance the ROS-mediated activations of AP-1 and NF-κB induced by UVB, $CdCl_2$ and H_2O_2 stimuli, and on the other hand, protects cells from excess ROS toxicities and facilitate cells transformation. Notably, even when AP-1 activity was reduced to 3% of control, the inhibition of anchorage-independent growth in soft agar was only $\sim$ 40%. As the process of malignant transformation always involves alternations in many signal pathways (12,13,46,47) which form an intricate network in the cells, blocking a specific one might not be sufficient to achieve a complete inhibition of colony growth.

A growing body of evidences has shown that ROS, at low concentration, acts as second messengers in intracellular signaling cascades and induces and maintains the oncogenic phenotype of cancer cells, but on the other hand, at high concentration, produces cellular toxicity. Oxidative stress induces a cellular redox imbalance which has been found to be present in various cancer cells compared with normal cells including human melanoma (48). Stimulation of cellular mitochondrial activity by p43 over-expression consistently induces human dermal fibroblast transformation, associated with significantly elevated ROS production (49). In addition, an increase in ROS by Rac1 mediated matrix metalloproteinase-3-induced malignant transformation and genomic instability (50). The mechanisms involved in ROS-stimulated transformation and promotion include stimulation of mitogen-activated kinases such as MAPK/Erk kinase (MEK) or Erk (extracellular signal-regulated kinase), activation of nuclear transcription factors (such as AP-1) which control cell proliferation and gene mutations induced by oxidative DNA damage (38,51,52). As of the 'two face' character of ROS in cellular biological activities, we hypothesize that adaptively induced Ref-1 protects cells from excessive ROS toxicities and synergistically enhances ROS-mediated promotion of cell growth (Figure 5). The possible mechanisms might be related to Ref-1 activation of many cell survival and proliferation signals mediated by reduction of nuclear transcription factors such as NF-κB and AP-1 that facilitates DNA binding and maintenance of genomic integrity through enhanced repair of DNA lesions. In this regard, we proposed that Ref-1 may be a good target molecule and effective interference of Ref-1-mediated signalings using specific small molecular inhibitors, might achieve better preventive or therapeutic effects in combination with other compounds.

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