Hydrogen Peroxide Enhances Radiation-induced Apoptosis and Inhibition of Melanoma Cell Proliferation

YUJIANG FANG[1,2], BRADLEY J. MOORE[1], QIAN BAI[1], KATHRYN M. COOK[1], ELIZABETH J. HERRICK[1] and MICHAEL B. NICHOLL[1,2]

[1]Department of Surgery, [2]Ellis Fischel Cancer Center, University of Missouri School of Medicine, Columbia, MO, U.S.A.

Abstract. *The efficacy of radiation therapy (RT) for melanoma is limited in part by its radioresistance. Here, we examined the radiosensitizing effect of hydrogen peroxide (H_2O_2) on a radioresistant melanoma cell line, HTB-65. We found that H_2O_2 synergized with RT to inhibit melanoma cell proliferation and promote apoptosis. The antiproliferative effect of H_2O_2/RT correlated with increased expression of p15 and reduced expression of cyclin D, cyclin E, cyclin-dependent kinase (CDK)2 and CDK4. The pro-apoptotic effect of H_2O_2 /RT correlated with reduced expression of the B-cell CLL/lymphoma (BCL)2. These data highlight the potential of H_2O_2 as a radiation sensitizer for melanoma treatment and show that this warrants further study.*

Melanoma is the most aggressive form of skin cancer and advanced stage melanoma has a very poor prognosis (1). About 70,230 new cases of melanoma and 8,790 deaths were projected to occur in the USA in 2011 (2). Despite efforts to increase early melanoma detection, some patients still present with advanced-stage melanoma. Others diagnosed with early-stage melanoma will go on to develop metastasis (3). Although most patients with early-stage melanoma are cured with surgery alone, the majority of patients with metastatic disease will die of their disease soon after diagnosis.

Even with recent therapeutic breakthroughs (4, 5), the management of advanced melanoma is very challenging, in part because there are few effective treatment modalities (6-8). Although melanoma is resistant to conventional radiation therapy (RT), high dose RT still plays an important role in the palliative management of brain metastases, spinal cord compression and symptomatic bony metastases (1, 9-10). A safe and effective radiosensitizing agent may allow a decrease in bothradiation dose and side-effects associated with RT for advanced melanoma.

Hydrogen peroxide (H_2O_2) plays a pivotal role in cell functions (11-13). This reactive oxygen species (ROS) is produced by certain cell types in response to growth and injury stimuli such as those mediated through epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (11-13). Among ROS, H_2O_2 has the unique capacity for easy cell membrane passage and a relatively low bioactivity, which may facilitate its role as a second messenger for cell signaling associated with cell proliferation and apoptosis (11-13). Combination treatment of H_2O_2 and RT has been reported to enhance apoptosis of human prostate cancer cells (14), suggesting its possible role as a radiation sensitizer. Thus, it is reasonable to hypothesize that H_2O_2 /RT might potentiate the destruction of other types of cancer cells such as melanoma. The current study was designed to test the hypothesis that H_2O_2 sensitizes melanoma cells to RT. Additionally, we report the mechanisms underlying the changes in proliferation and apoptosis observed when H_2O_2 is used in combination with RT.

Materials and Methods

Tumor cell line. HTB-65 cells, derived from human malignant melanoma, were purchased from the ATCC (Manassas, VA, USA). HTB-65 cells (passage 2) were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen). Cultures were incubated at 37°C in a humidified incubator (Fisher Scientific, Pittsburgh, USA) with 5% CO_2. Cells were grown until they reached 70-80% confluence, at which time they were subjected to the designed experimental treatment regimens.

Correspondence to: Dr. Michael B. Nicholl or Dr. Yujiang Fang, Department of Surgery, Ellis Fischel Cancer Center, University of Missouri School of Medicine, M316, Medical Sciences Building, Columbia, MO, 65212, U.S.A. Tel: +1 5738828454, Fax: +1 5738846054, e-mail: nicholllm@health.missouri.edu or fangy@health.missouri.edu

Key Words: Hydrogen peroxide, radiation, apoptosis, proliferation, melanoma.

Treatment of HTB-65 cells with H_2O_2 and RT. At 70-80% confluence, HTB-65 cells were treated with H_2O_2 at the concentration of 50 μM for 24 h, followed by RT at 2 Gy, 4 Gy and 6 Gy, or mock treatment. The concentration and dosage of H_2O_2 and RT was based on previously published data by us and others and our pilot experiments (14-18). All RT was carried out using an XRAD 320 Biological Irradiator (Precision X-ray, North Brandford, CT, USA) as describe before (17, 18). Cells were irradiated at room temperature in 75 cm^2 culture flasks. After RT, cells were further cultured for 72 h.

Clonogenic survival assay. Seventy-two hours after RT, cells were detached and counted in a hemocytometer. Clonogenic survival assay was performed as previously described (17, 18) and cells were stained with 0.05% crystal violet. The number of colonies was counted and expressed as a percentage of total colonies in untreated controls (no RT or H_2O_2). Combination index (CI) was used to evaluate the combination treatment effect of H_2O_2 and RT as described elsewhere (17). CI=1, CI< 1, and CI>1 indicate additive effect, synergism, and antagonism, respectively (17).

Immunohistochemstry (IHC). HTB-65 cells were treated with or without H_2O_2 (50 μM) for 24 h, and then irradiated by RT at 6 Gy, or a mock treatment. Seventy-two hours after RT, cells were spun into slides by a Cytopro cytocentrifuge (Wescor Biomedical Systems, Logan, Utah, USA). IHC staining for proliferating cell nuclear antigen (PCNA), p15, cyclin E, cyclin-dependent kinase (CDK)4 and B-cell CLL/lymphoma (Bcl)2 was described previously (17-20). The concentration used for the primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was 1 μg/ml and for the secondary antibody2 μg/ml. To quantify the staining intensity, the average intensity within the area covered by cells was measured using MetaMorph image analysis software version 6.3r6 (Molecular Devices Analytical Technologies, Sunnyvale, CA, USA). Results are expressed as the average integrated immunostaining intensity of three slides ± SEM relative to that in control cells.

Determination of proliferation with the Quick Cell Proliferation Assay Kit. Besides IHC for PCNA, proliferation was also determined using the Quick Cell Proliferation Assay Kit (BioVision, Milpitas, CA, USA) as previously described (17, 18).

Reverse transcriptase-polymerase chain reaction (RT-PCR). Cells were treated with or without H_2O_2 for 24 h, and then irradiated by RT at 6 Gy, or a mock treatment. Seventy-two hours after RT, cells were harvested. Cells were washed with phosphate buffered saline (PBS), centrifuged and homogenized in TRIzol (Invitrogen). RNA was extracted and its concentration was determined. Subsequently, 1 μg RNA was reverse transcribed as previously described (17-20). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to verify that the same amount of RNA was amplified. Primer sequences for *GAPDH*, pro- and anti-proliferative molecules (*p15, p18, p21, p27, p53*, Cyclin B, Cyclin D, Cyclin E, CDK2 and CDK4) and pro- and anti-apoptotic molecules (FAS, FASL, TRAILR1, TRAIL, BAX, FLIP, BCL2) have been described elsewhere (17). Survivin primers were: sense: 5'-AGCCCTTTCTCAAGGAC CAC-3', antisense: 5'-GCACTTTCTTCGCAGTTTCC-3'.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining. Apoptosis was determined by TUNEL assay using an Apoptag kit (Chemicon, El Segundo, CA, USA) as previously described (17-20). To quantify the number of apoptotic cells, all cells in 5-6 randomly selected high power fields (magnification: ×400) were manually counted using image analysis software MetaMorph. TUNEL+ cells were expressed as a percentage of total cells.

Measurement of caspase-3 activity. Cellular caspase-3 activity of PC-3 cells recognizing the sequence DEVD (Asp-Glu-Val-Asp) was measured using a caspase-3/CPP32 colorimetric assay kit (BioVision) as described elsewhere (17-20).

Statistics. All experiments were repeated at least two times. Statistical analysis of data was performed using an unpaired two-tailed Student's *t*-test or the Mann-Whitney rank sum test. A p-value<0.05 was considered significant.

Results

H_2O_2 sensitized melanoma cells to RT-induced inhibition of proliferation and growth. To investigate the effect of H_2O_2 on melanoma radiosensitivity, 70-80% confluent HTB-65 cells were treated with H_2O_2 (50 μM) for 24 h, followed by RT at escalating doses as described in the Material and Methods. Cell survival was evaluated by clonogenic survival assay 72 h after RT (Figure 1A). In the absence of H_2O_2, the percentage of colonies of HTB-65 after RT was 93±4% (2 Gy) and 89±7% (4 Gy), respectively, which was comparable to that in the control group. Although there was a significant difference in the percentage of colonies compared to the control group, three quarters (75±6%) of the colonies survived when cells were treated at the dosage of 6 Gy, suggesting that HTB-65 was resistant to RT. Interestingly, When HTB-65 cells were treated with H_2O_2 alone, the percentage of colonies of HTB-65 decreased significantly to 58±6%. The percentage of colonies of HTB-65 after H_2O_2/RT decreased to 41±7%, 19±5% and 8±2% for 2 Gy, 4 Gy and 6 Gy), respectively. Combination index CI was also used to evaluate the combination effect of H_2O_2 and RT as previously described in detail (17). It indicated that H_2O_2 exerted synergistic effect with RT (CI<1) on inhibition of proliferation and growth of HTB-65 cells, suggesting that H_2O_2 sensitized melanoma cells to RT-induced inhibition of proliferation and growth.

This sensitization was also evident by analysis of staining intensity for PCNA, a cell proliferation marker (Figure1 B and C). Similar results were obtained when a Quick Cell Proliferation Assay Kit was used to analyze cell proliferation as described in the Materials and Methods (Figure 1D). These results strongly indicate that H_2O_2 synergizes with RT to inhibit cell proliferation and reduce the survival of melanoma cells.

Effect of H_2O_2/RT on the expression of pro- and antiproliferative molecules in HTB-65 cells. p15, p18, p21, p27 and p53 are important antiproliferative molecules,

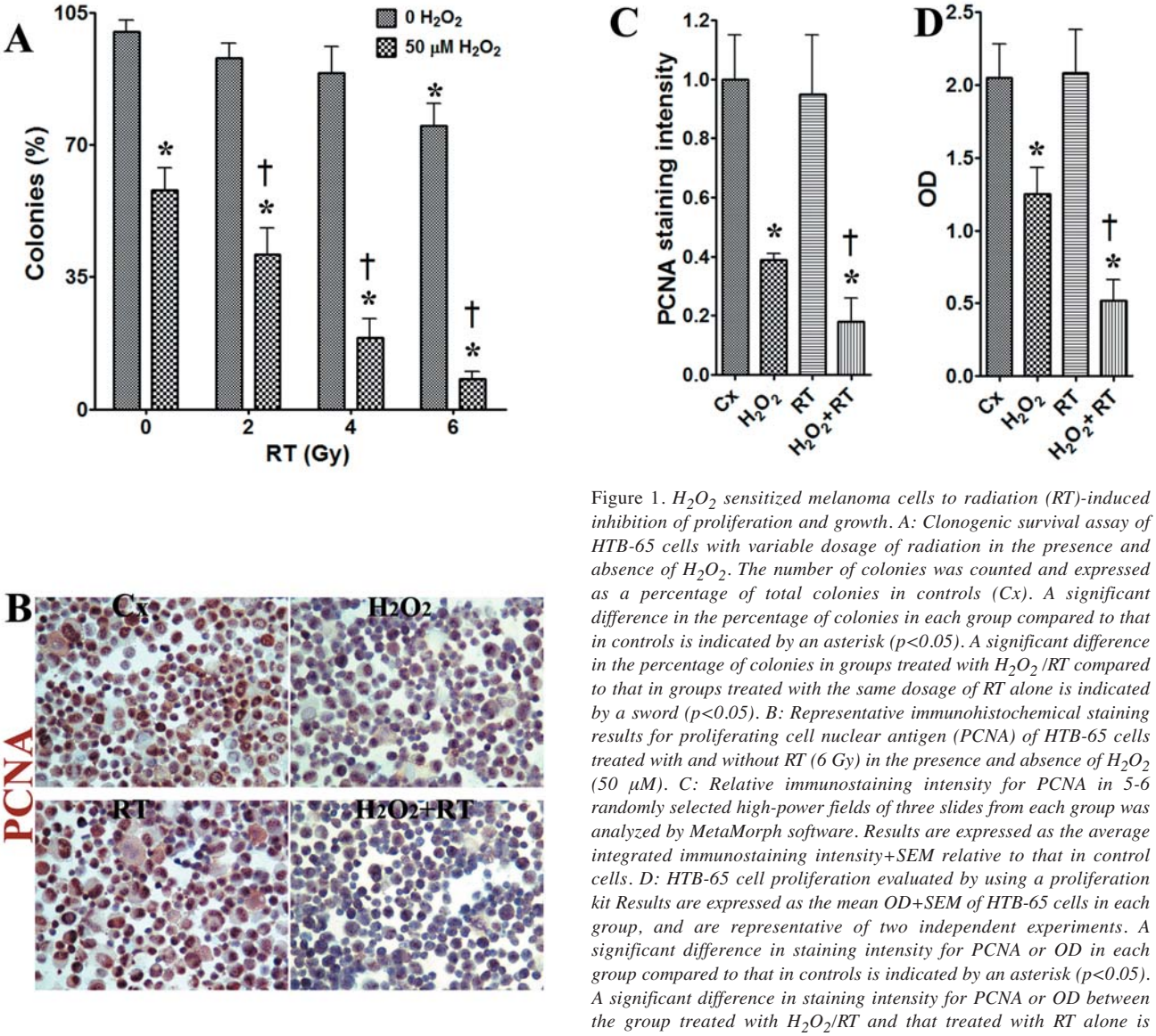

Figure 1. *H_2O_2 sensitized melanoma cells to radiation (RT)-induced inhibition of proliferation and growth. A: Clonogenic survival assay of HTB-65 cells with variable dosage of radiation in the presence and absence of H_2O_2. The number of colonies was counted and expressed as a percentage of total colonies in controls (Cx). A significant difference in the percentage of colonies in each group compared to that in controls is indicated by an asterisk ($p<0.05$). A significant difference in the percentage of colonies in groups treated with H_2O_2 /RT compared to that in groups treated with the same dosage of RT alone is indicated by a sword ($p<0.05$). B: Representative immunohistochemical staining results for proliferating cell nuclear antigen (PCNA) of HTB-65 cells treated with and without RT (6 Gy) in the presence and absence of H_2O_2 (50 μM). C: Relative immunostaining intensity for PCNA in 5-6 randomly selected high-power fields of three slides from each group was analyzed by MetaMorph software. Results are expressed as the average integrated immunostaining intensity+SEM relative to that in control cells. D: HTB-65 cell proliferation evaluated by using a proliferation kit Results are expressed as the mean OD+SEM of HTB-65 cells in each group, and are representative of two independent experiments. A significant difference in staining intensity for PCNA or OD in each group compared to that in controls is indicated by an asterisk ($p<0.05$). A significant difference in staining intensity for PCNA or OD between the group treated with H_2O_2/RT and that treated with RT alone is indicated by a dagger ($p<0.05$). Original magnification in B: ×400.*

while cyclin B, cyclin D, cyclin E, CDK2 and CDK4 are important pro-proliferative molecules (21-25). The normal balance between these molecules plays a pivotal role in cell proliferation and survival (21-25). To determine if these molecules are involved in the synergistic effect of H_2O_2 with RT in inhibiting cell proliferation and reducing survival of HTB-65 cells, mRNA expression of these molecules with and without RT (6 Gy) in the presence and absence of H_2O_2 (50 μM) was determined by RT-PCR. mRNA expression of the antiproliferative molecule *p15* was significantly higher and that of the pro-proliferative molecules cyclin D, cyclin E, CDK2 and CDK4 was significantly lower in cells treated with H_2O_2/RT than that in cells treated with RT alone. mRNA expression of pro-proliferative molecule cyclin B was comparable in cells treated with H_2O_2/RT to that in cells treated with RT alone. Surprisingly, the mRNA expression of *p18, p21, p27* and *p53* was reduced in cells treated with H_2O_2/RT compared to that in cells treated with RT alone. The reason for this is unknown. Consistent with the mRNA expression patterns of *p15*, cyclin E and CDK4 shown in Figure 2, the relative immunostaining intensity for p15 was higher in cells treated with H_2O_2/RT than that in cells treated with RT alone (Figure 3 A and B), whereas the relative

immunostaining intensity for cyclin E and CDK4 was stronger after H_2O_2/RT than that cells treated with RT alone (Figure 3 A, C and D). Taken together, these results indicate that the effect of H_2O_2/RT in inhibiting cell proliferation and reducing survival of melanoma cells correlated with the increased expression of antiproliferative molecule *p15* and reduced expression of pro-proliferative molecules cyclin D, cyclin E, CDK2 and CDK4.

H_2O_2/RT promotes apoptosis of HTB-65 cells. The lower percentage of colonies in cells treated with H_2O_2/RT might also be due to the increased apoptosis of HTB-65 cells. To address this, we further examined the effect of H_2O_2/RT on apoptosis of HTB-65 cells. Apoptosis was evaluated by TUNEL staining (Figure. 4 A and B). There were few TUNEL+ cells in the RT group, whereas 26±5% HTB-65 cells were TUNEL+ in the H_2O_2/RT group and the difference in TUNEL+ cell between them was significant ($p<0.05$). Similar results were obtained when caspase-3 activity of HTB-65 cells was assayed (data not shown). These results suggest that H_2O_2/RT promotes apoptosis of melanoma cells.

Effect of H_2O_2/RT on the expression of pro- and antiapoptotic molecules in HTB-65 cells. Similar to the critical role of the normal balance between pro- and antiproliferative molecules in cell proliferation and survival, the normal balance between pro- and antiapoptotic molecules also plays an important role in cell survival (19, 20, 26-29). FAS, FASL, TRAILR1, TRAIL and BAX are important pro-apoptotic molecules, while, FLIP and BCL2 are important anti-apoptotic molecules (19, 20, 26-29). To address their roles in the promoted apoptosis of HTB-65 cells by H_2O_2/RT, mRNA expression of these molecules with and without RT (6 Gy) in the presence and absence of H_2O_2 (50 μM) was determined by RT-PCR (Figure 5). Unexpectedly, mRNA expression of the pro-apoptotic molecules FAS, TRAILR1 and TRAIL was significantly lower in cells treated with H_2O_2/RT than that in cells treated with RT alone. mRNA expression of pro-apoptotic molecules FASL and BAX and anti-apoptotic molecules FLIP and survivin was comparable in cells treated with H_2O_2/RT to that in cells treated with RT alone. Of particular interest, the mRNA expression of anti-apoptotic molecule BCL2 was significantly lower in cells treated with H_2O_2/RT compared to that in cells treated with RT alone. Consistent with the mRNA expression of BCL2, the relative immunostaining intensity for BCL2 was also much lower in cells treated with H_2O_2/RT than that in cells treated with RT alone (Figure 6 A and B). Thus, the increased apoptosis in cells treated with H_2O_2/RT compared to control group correlated with reduced expression of BCL2.

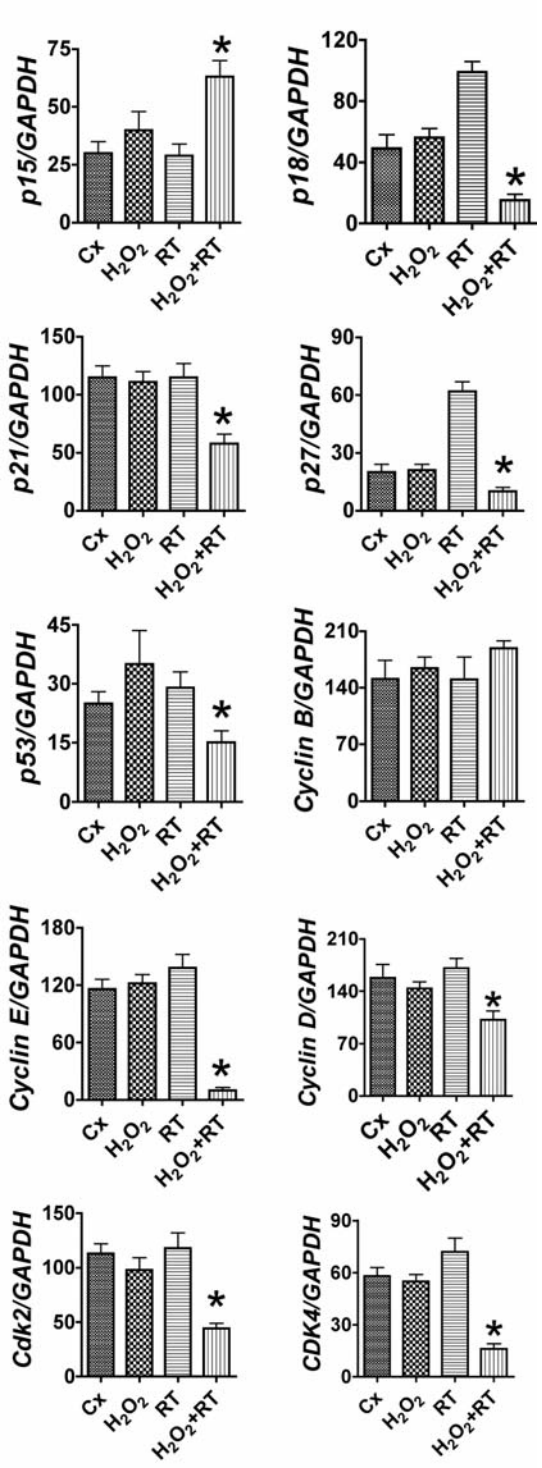

Figure 2. *Effect of H_2O_2/RT on the expression of pro- and antiproliferative molecules in HTB-65 cells evaluated by reverse-transcriptase-polymerase chain reaction (RT-PCR). mRNA was extracted as described in the Materials and Methods. Experiments were carried out in triplicate and results are expressed as the mean ratio of pro- and antiproliferative molecule in densitometric units/GAPDH+SEM (×100), and are representative of two or three independent experiments. A significant difference between the group treated with H_2O_2/RT and that treated with RT alone is indicated by an asterisk ($p<0.05$).*

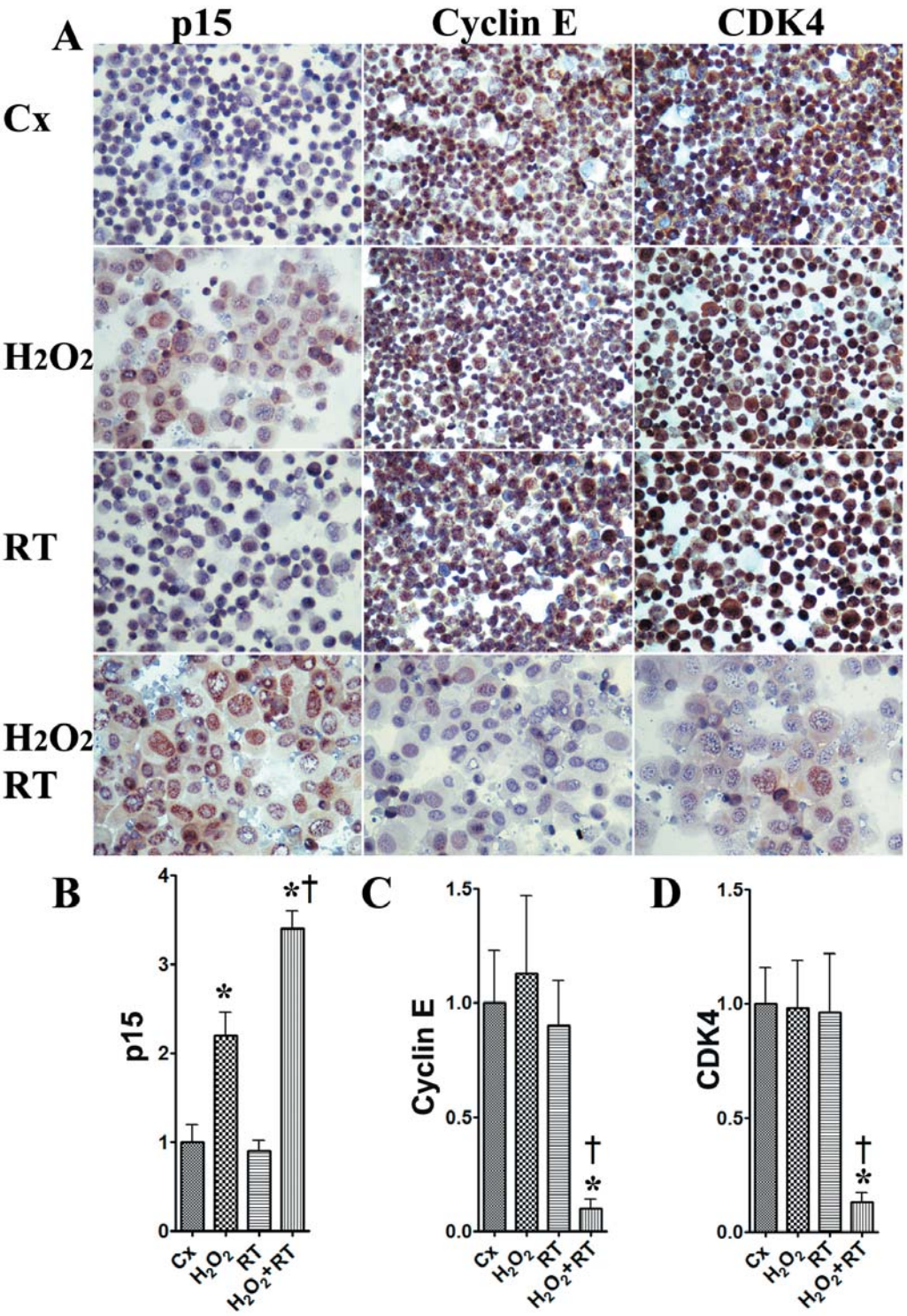

Figure 3. *Effect of H_2O_2/RT on the expression of pro- and antiproliferative molecules in HTB-65 cells evaluated by immunohistochemistry (IHC). A: IHC for p15, cyclin E and cyclin-dependent kinase (CDK)4 of HTB-65 cells treated with and without RT (6 Gy) in the presence and absence of H_2O_2 (50 μM). B-D: Relative immunostaining intensity for each protein in 5-6 randomly selected high-power fields of three slides from each group was analyzed by MetaMorph software. Results are expressed as the average immunostaining intensity+SEM relative to that in control cells. A significant difference in immunostaining intensity in the groups compared to that in controls (Cx) is indicated by an asterisk ($p<0.05$). A significant difference in immunostaining intensity between the group treated with H_2O_2/RT and that treated with RT alone is indicated by a dagger ($p<0.05$). Data are representative of three independent experiments. Original magnification: A: ×400.*

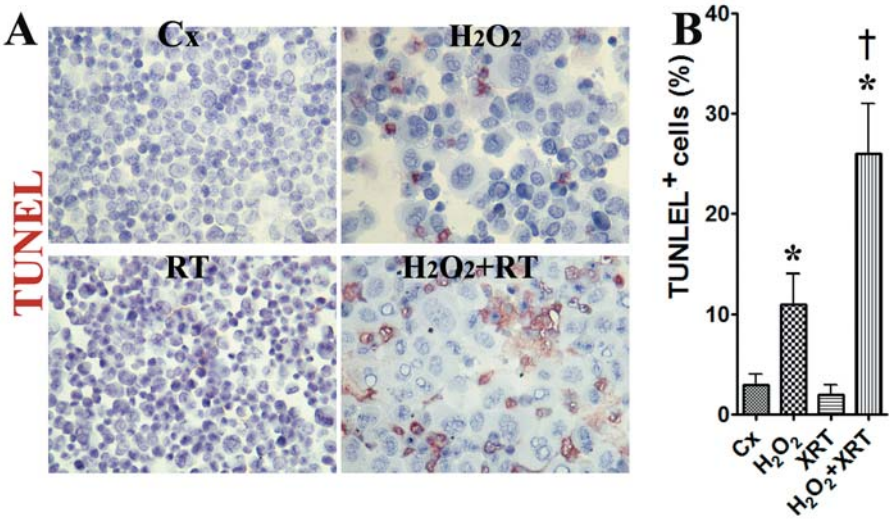

Figure 4. *Effect of H_2O_2/RT on apoptosis of HTB-65 cells. A: Representative Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining of HTB-65 cells is shown. B: TUNEL+ cells (red) in 5-6 randomly selected high-power fields of three slides were counted. A significant difference in the percentage of TUNEL+ cells in the treated groups compared to that in controls (Cx) is indicated by an asterisk ($p<0.05$). A significant difference in the percentage of TUNEL+ cells between the group treated with H_2O_2/RT and that treated with RT alone is indicated by a dagger ($p<0.05$). Data are representative of three independent experiments. Original magnification: A: ×400.*

Discussion

RT has a small but important role in melanoma treatment (1, 10). In part, the relative radioresistance of melanoma limits the efficacy of RT (1, 9). Improving the effecacy of RT may allow its expanded use for melanoma while reducing the potential side effects associated with RT. In this study, we demonstrate that H_2O_2 synergizes with RT to inhibit proliferation and promote apoptosis of a melanoma cell line. The antiproliferative effect of H_2O_2/RT treatment correlated with increased expression of the antiproliferative molecule p15 and reduced expression of pro-proliferative molecules cyclin D, cyclin E, CDK2 and CDK4. The proapoptotic effect of H_2O_2/RT correlated with reduced expression of the antiapoptotic molecule BCL2. To our knowledge, this is the first study to demonstrate the synergistic effect of H_2O_2 and RT on melanoma *in vitro*. This is also the first study to investigate the detailed molecular mechanisms by which H_2O_2/RT inhibits the survival of melanoma cells. Kariya *et al*. showed that H_2O_2 enhances RT-induced apoptosis of human PC-3 prostate cancer cells (14). A major limitation of this work in prostate cancer is the lack of a molecular mechanism to explain the observed phenomena. Our study expands the work of Kariya *et al*. by showing that the proapoptotic effect of H_2O_2 on cancer cells is not limited to prostate cancer and that the tumoricidal effect of H_2O_2/RT on melanoma cells is not only due to a pro-apoptotic effect, but also due to an antiproliferative effect.

The rationale for H_2O_2/RT in melanoma treatment is supported by molecular findings specific to melanoma. Melanoma cells have been shown to have lower catalase levels compared to melanocytes and there is also increased expression of antioxidants in melanoma cell lines, including of superoxide dismutase and ferritin (30-32). Several groups have attempted to use ROS as a melanoma treatment strategy (33-35). Ogawa *et al*. have proposed the use of H_2O_2 as a radiation sensitizer to treat human tumors including melanoma (36, 37). Our data further provides a rationale for this approach in humans by strongly suggesting a radiosensitizing role for H_2O_2 in melanoma. Radiosensitization of melanoma may allow for reduction of effective radiation dose and side-effects associated with RT.

The eukaryotic cell cycle is tightly regulated (21-25). The balance between pro- and antiproliferative molecules plays an important role in cell proliferation. Cyclin D and cyclin E, as well as CDK2 and CDK4, play major roles in proliferation. Down-regulation of cyclin D delays or inhibits entry to the S phase and overexpression of cyclin D shortens the G_1 phase (21-25). Cyclin E is active in late G_1 phase and is maximal at the G1-S transition. p15 is an important antiproliferative molecule which exerts its effect through inhibition of CDKs (21-25). In this study, we found that the antiproliferative effect of H_2O_2/RT correlated with increased expression of p15 and reduced expression of cyclin D, cyclin E, CDK2 and CDK4. These results are also in line with our previous study showing that resveratrol synergized with RT to inhibit proliferation of PC-3 prostate cancer cells by up-regulation of p15 and down-

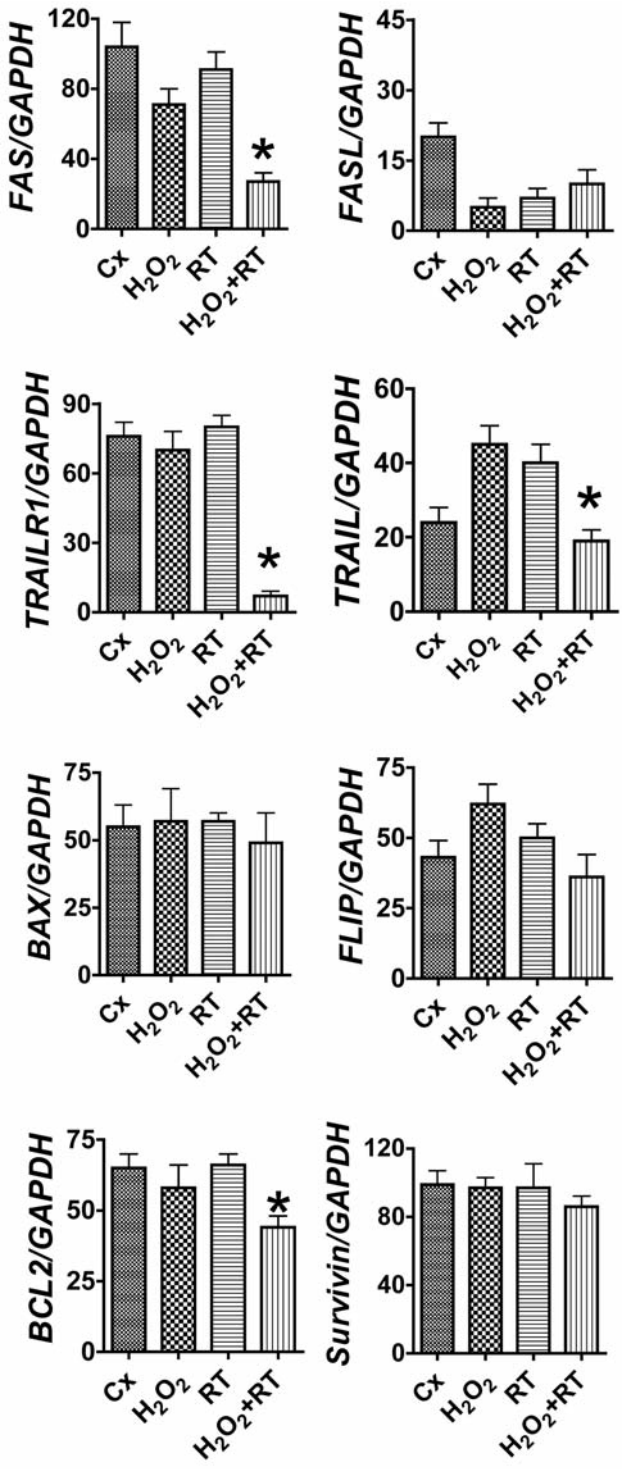

Figure 5. *Effect of H_2O_2/RT on the expression of pro- and antiapoptotic molecules in HTB-65 cells evaluated by RT-PCR. mRNA was extracted as described in the Materials and Methods. Experiments were carried out in triplicate and results are expressed as the mean ratio of pro- and antiapoptotic molecules in densitometric units/GAPDH+SEM (×100), and are representative of two independent experiments. A significant difference between the group treated with H_2O_2/RT and that treated with RT alone is indicated by an asterisk ($p<0.05$).*

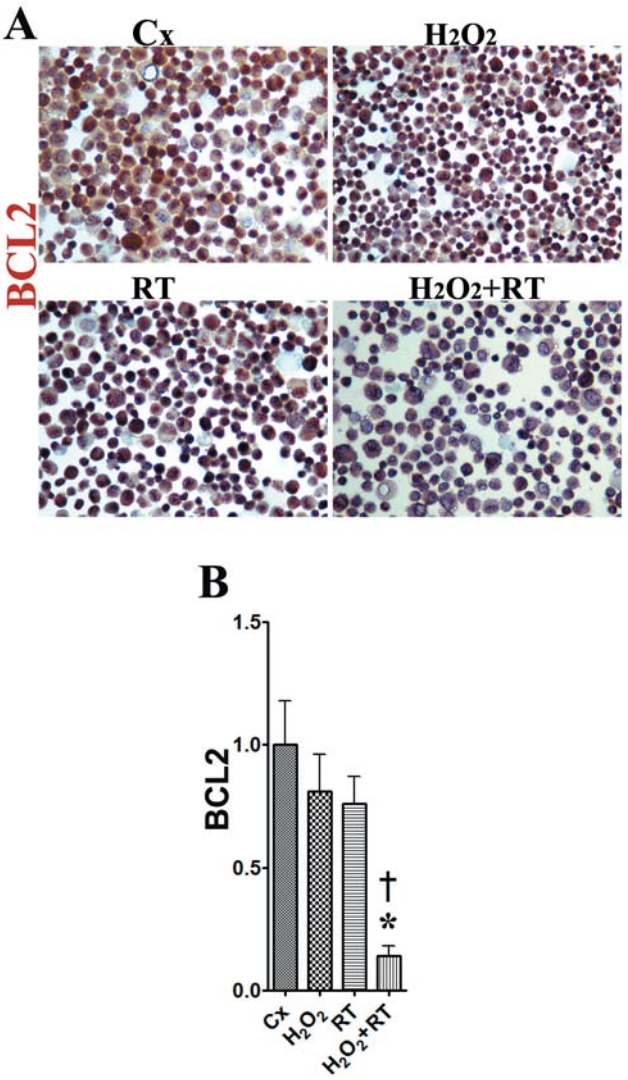

Figure 6. *Effect of H_2O_2/RT on the expression of BCL2 in HTB-65 cells evaluated by IHC. IHC for BCL2 of HTB-65 cells treated with and without RT (6 Gy) in the presence and absence of H_2O_2 (50 μM). B: Relative immunostaining intensity for BCL2 was analyzed by MetaMorph software. Results are expressed as the average immunostaining intensity+SEM relative to that in control cells (Cx). A significant difference in immunostaining intensity in groups compared to that in controls is indicated by an asterisk ($p<0.05$). A significant difference in immunostaining intensity between the group treated with H_2O_2/RT and that treated with RT alone is indicated by a dagger ($p<0.05$). Data are representative of three independent experiments. Original magnification: A: ×400.*

regulation of cyclin D, cyclin E and CDK2. In our current study, H_2O_2 alone has little effect on expression of p21 and p53. This is not consistent with the study by Dr. Manuel Rieber's group, who showed that H_2O_2 up-regulated the expression of p21 and p53 in a mouse melanoma cell line, B16 (38). This difference might be due to the difference in the

concentration of H_2O_2 (50 *vs*. 500 μM) or the differences in species and cell line (human HTB-65 *vs*. mouse B16). The concentration used by Dr. Manuel Rieber's group is 10-folds higher than that used in our study. We also noticed that the expression of p21 and p53 was not upregulated when the concentration of H_2O_2 was reduced to 250 μM in the study by Dr. Manuel Rieber's group. Further studies are needed to address if other factors contribute to this difference. Nevertheless, our data strongly indicate that H_2O_2/RT disrupted the pro-proliferative mechanism and broke the balance between pro- and antiproliferative molecules in melanoma cells, resulting in inhibition of cell proliferation.

Apoptosis is triggered either through a receptor-mediated or mitochondrial-mediated pathway (26, 27). FAS, FASL, TRAILR1 and TRAIL belong to the tumor necrosis factor (TNF) receptor family and have proapoptotic function in receptor-mediated apoptosis (28, 29). FLIP inhibits death receptor-mediated apoptosis by blocking activation of caspase-8 (28, 29). BCL2 and BAX are two important molecules in mitochondrial-mediated pathway. Bcl-2 inhibits apoptosis, while Bax promotes apoptosis through regulation of mitochondrial voltage-dependent anion channels (27, 29). Survivin is a member of the inhibitor of apoptosis family and functions as an antiapoptotic molecule by inhibition of caspsase activation (29-41). In this study, H_2O_2, but not RT, increased apoptosis of melanoma cells (Figure 4) and H_2O_2/RT further increased apoptosis of melanoma cells, suggesting a synergistic effect on apoptosis. This is mainly due to the down-regulation of antiapoptotic molecule BCL2 (Figures. 5 and 6) but not other anti-apoptotic molecules such as FLIP or survivin. It will be of interest to investigate if H_2O_2/RT enhanced-apoptosis can be abrogated when BCL2 is knocked out.

It is necessary to point out that in the analysis of pro- and anti-proliferative and pro- and antiapoptotic molecules, we unexpectedly found some antiproliferative molecules such as p18, p21,p27 and p53 and some proapoptotic molecules, such as FAS, TRAILR1 and TRAIL to be down-regulated by H_2O_2/RT when compared to RT alone. We believe this seemly paradoxical effect may be part of an adaptative response to cell injury to prevent cells from further damage. Similar effects were also noticed in our previous studies (17). Thus, it is reasonable to argue that it is not a specific pro- or antiproliferative and/or pro- and antiapoptotic molecule but the balance between them which dictates the fate of melanoma cells.

Taken together, our results show that H_2O_2 sensitizes melanoma cells to radiation, promotes apoptosis of melanoma when used in conjunction with radiation, and enhances the effect of radiation in inhibiting melanoma proliferation. Further studies may help elucidate the exploitable mechanisms behind the effect of H_2O_2/RT which can be used to increase the efficacy of radiation for melanoma. Preliminary clinical data using H_2O_2/RT supports this as a viable therapy. Our work explores and supports the early clinical results of H_2O_2/RT in patients with melanoma.

Disclosure Statement

The Authors declare that they have no conflict of interest.

Acknowledgements

We thank Chelsea L. Ledgewood and Abdimalik O. Mohamud from the University of Missouri, Columbia, MO, USA for technical assistance.

References

1 Atkins MB: Cytokine-based therapy and biochemotherapy for advanced melanoma. Clin Cancer Res *12*: 2353s-2358s, 2006.

2 Siegel R, Ward E and Jemal A: Cancer statistics, 2011: The impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin *61*: 212-236, 2011.

3 Nicholl MB, Elashoff D, Takeuchi H, Morton DL and Hoon DS: Molecular up-staging based on paraffin-embedded sentinel lymph nodes: Ten-year follow-up confirms prognostic utility in melanoma patients. Ann Surg *253*: 116-122, 2011.

4 Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA and BRIM-3 Study Group: Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med *364*: 2507-2516, 2011.

5 Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A and Urba WJ: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med *363*: 711-723, 2010.

6 Beddingfield FC 3rd: The melanoma epidemic: *res ipsa loquitur*. Oncologist *8*: 459-465, 2003.

7 Balch CM, Soong SJ, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N, Urist M, McMasters KM, Ross MI, Kirkwood JM, Atkins MB, Thompson JA, Coit DG, Byrd D, Desmond R, Zhang Y, Liu PY, Lyman GH and Morabito A: Prognostic factors analysis of 17,600 melanoma patients: Validation of the American Joint Committee on Cancer melanoma staging system. J Clin Oncol *19*: 3622-3634, 2001.

8 Bastiaannet E, Beukema JC and Hoekstra HJ: Radiation therapy following lymph node dissection in melanoma patients: Treatment, outcome and complications. Cancer Treat Rev *31*: 18-26, 2005.

9 Perlis C and Herlyn M: Recent advances in melanoma biology. Oncologist *9*: 182-187, 2004.

10 Atallah E and Flaherty L: Treatment of metastatic malignant melanoma. Curr Treat Options Oncol *6*: 185-193, 2005.

11 Sundaresan M, Yu ZX, Ferrans VJ, Irani K and Finkel T: Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science *270*: 296-299, 1995.

12 Griendling KK and Harrison DG: Dual role of reactive oxygen species in vascular growth. Circ Res *85*: 562-563, 1999.
13 Dalton TP, Shertzer HG and Puga A: Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol *39*: 67-101, 1999.
14 Kariya S, Sawada K, Kobayashi T, Karashima T, Shuin T, Nishioka A and Ogawa Y: Combination treatment of hydrogen peroxide and X-rays induces apoptosis in human prostate cancer PC-3 cells. Int J Radiat Oncol Biol Phys *75*: 449-454, 2009.
15 Medina VA, Massari NA, Cricco GP, Martín GA, Bergoc RM and Rivera ES: Involvement of hydrogen peroxide in histamine-induced modulation of WM35 human malignant melanoma cell proliferation. Free Radic Biol Med *46*: 1510-1515, 2009.
16 Morgado S, Granados MP, Bejarano I, Lopez JJ, Salido GM, Gonzalez A and Pariente JA: Role of intracellular calcium on hydrogen peroxide-induced apoptosis in rat pancreatic acinar AR42J cells. J Appl Biomed *6*: 211-224, 2008.
17 Fang Y, DeMarco VG and Nicholl MB: Resveratrol enhances radiation sensitivity in prostate cancer by inhibiting cell proliferation and promoting cell senescence and apoptosis. Cancer Sci *103*: 1090-1098, 2012.
18 Fang Y, Herrick EJ and Nicholl MB: A possible role for perforin and granzyme B in resveratrol enhanced radiosensitivity of prostate cancer. J Androl *33*: 752-760, 2012.
19 Fang Y, Wei Y, DeMarco V, Chen K, Sharp GC and Braley-Mullen H: Murine FLIP transgene expressed on thyroid epithelial cells promotes resolution of granulomatous experimental autoimmune thyroiditis. Am J Pathol *171*: 875-887. 2007.
20 Fang Y, Sharp GC, Yagita H and Braley-Mullen H: A critical role for TRAIL in resolution of granulomatous experimental autoimmune thyroiditis. J Pathol *216*: 505-513, 2008.
21 Fang Y, Yu S and Braley-Mullen H: TGF-β promotes proliferation of thyroid epithelial cells in IFN-$\gamma^{-/-}$ mice by down-regulation of p21 and p27 *via* AKT pathway. Am J Pathol *180*: 650-660, 2012.
22 Johnson DG and Walker CL: Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol *39*: 295-312, 1999.
23 Sherr CJ: G1 phaseprogression: Cycling on cue. Cell *79*: 551-555, 1994.
24 Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM and Pagano M: Human cyclin E, a nuclear protein essential for the G_1-to-S phase transition. Mol Cell Biol *15*: 2612-2624, 1995.
25 Sherr CJ: D-Type cyclins. Trends Biochem Sci *20*: 187-190, 1995.
26 Thompson CB: Apoptosis in the pathogenesis and treatment of disease. Science *267*: 1456-1462, 1995.
27 Zhang H, Xu Q, Krajewski S, Krajewska M, Xie Z, Fuess S, Kitada S, Pawlowski K, Godzik A, and Reed JC: BAR: An apoptosis regulator at the intersection of caspases and BCL2 family proteins. Proc Natl Acad Sci USA *97*: 2597-2602, 2000.
28 Griffith TS, Brunner T, Fletcher SM, Green DR and Ferguson TA: Fas ligand-induced apoptosis as a mechanism of immune privilege. Science *270*: 1189-1192, 1995.
29 Fang Y and Braley-Mullen H: Cultured murine thyroid epithelial cells expressing transgenic Fas-associated death domain-like interleukin-1β converting enzyme inhibitory protein are protected from Fas-mediated apoptosis. Endocrinology *149*: 1121-1129, 2008.
30 Picardo M, Grammatico P, Roccella F, Roccella M, Grandinetti M, Del Porto G and Passi S: Imbalance in the antioxidant pool in melanoma cells and normal melanocytes from patients with melanoma. J Invest Dermatol *107*: 322-326, 1996.
31 Baldi A, Lombardi D, Russo P, Palescandolo E, De Luca A, Santini D, Baldi F, Rossiello L, Dell'Anna ML, Mastrofrancesco A, Maresca V, Flori E, Natali PG, Picardo M and Paggi MG: Ferritin contributes to melanoma progression by modulating cell growth and sensitivity to oxidative stress. Clin Cancer Res *11*: 3175-3183, 2005.
32 Bravard A, Cherbonnel-Lasserre C, Reillaudou M, Beaumatin J, Dutrillaux B and Luccioni C: Modifications of the antioxidant enzymes in relation to chromosome imbalances in human melanoma cell lines. Melanoma Res *8*: 329-335,1998.
33 Yu J and Kim AK: Effect of taurine on antioxidant enzyme system in B16F10 melanoma cells. Adv Exp Med Biol *643*: 491-499, 2009.
34 Kolarova H, Nevrelova P, Bajgar R, Jirova D, Kejlova K and Strnad M: In vitro photodynamic therapy on melanoma cell lines with phthalocyanine. Toxicol In Vitro *21*: 249-253, 2007.
35 Posen Y, Kalchenko V, Seger R, Brandis A, Scherz A and Salomon Y: Manipulation of redox signaling in mammalian cells enabled by controlled photogeneration of reactive oxygen species. J Cell Sci *118*: 1957-1969, 2005.
36 Ogawa Y Ue H, Tsuzuki K, Tadokoro M, Miyatake K, Sasaki T, Yokota N, Hamada N, Kariya S, Hitomi J, Nishioka A, Nakajima K, Ikeda M, Sano S and Inomata T: New radiosensitization treatment (KORTUC I) using hydrogen peroxide solution-soaked gauze bolus for unresectable and superficially exposed neoplasms. Oncol Rep *19*: 1389-1394, 2008.
37 Ogawa Y, Kubota K, Ue H, Tadokoro M, Matsui R, Yamanishi T, Hamada N, Kariya S, Nishioka A, Nakajima H, Tarutani M and Sano S: Safety and effectiveness of a new enzyme-targeting radiosensitization treatment (KORTUC II) for intratumoral injection for low-LET radioresistant tumors. Int J Oncol *39*: 553-560, 2011.
38 Gomez Sarosi LA, Rieber MS and Rieber M: Hydrogen peroxide increases a 55-kDa tyrosinase concomitantly with induction of p53-dependent p21/waf1 expression and a greater Bax/Bcl-2 ratio in pigmented melanoma. Biochem Biophys Res Commun *312*: 355-359, 2003.
39 Sah NK, Khan Z, Khan GJ and Bisen PS: Structural, functional and therapeutic biology of survivin. Cancer Lett *244*: 164-171, 2006.
40 Yamamoto H, Ngan CY and Monden M: Cancer cells survive with survivin. Cancer Sci *99*: 1709-1714, 2008.
41 Altieri DC: Survivin, cancer networks and pathway-directed drug discovery. Nat Rev Cancer *8*: 61-70, 2008.

Received March 18, 2013
Revised April 11, 2013
Accepted April 12, 2013