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Mitochondria-Targeted Superoxide Dismutase (SOD2) Regulates Radiation Resistance and Radiation Stress Response in HeLa Cells

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SOD2/Mitochondria/Radiation protection/Oxidative stress/2',7'-dichlorofluorescein/Regulation of gene expression/Pathway analysis.

Reactive oxygen species (ROS) act as a mediator of ionizing radiation-induced cellular damage. Previous studies have indicated that MnSOD (SOD2) plays a critical role in protection against ionizing radiation in mammalian cells. In this study, we constructed two types of stable HeLa cell lines overexpressing SOD2, HeLa S3/SOD2 and T-REx HeLa/SOD2, to elucidate the mechanisms underlying the protection against radiation by SOD2. SOD2 overexpression in mitochondria enhanced the survival of HeLa S3 and T-REx HeLa cells following γ-irradiation. The levels of γH2AX significantly decreased in HeLa S3/SOD2 and T-REx HeLa/SOD2 cells compared with those in the control cells. MitoSox™ Red assays showed that both lines of SOD2-expressing cells showed suppression of the superoxide generation in mitochondria. Furthermore, flow cytometry with a fluorescent probe (2',7'-dichlorofluorescein) revealed that the cellular levels of ROS increased in HeLa S3 cells during post-irradiation incubation, but the increase was markedly attenuated in HeLa S3/SOD2 cells. DNA microarray analysis revealed that, of 47,000 probe sets analyzed, 117 and 166 probes showed more than 2-fold changes after 5.5 Gy of γ-irradiation in control and HeLa S3/SOD2 cells, respectively. Pathway analysis revealed different expression profiles in irradiated control cells and irradiated SOD2-overexpressing cells. These results indicate that SOD2 protects HeLa cells against cellular effects of γ-rays through suppressing oxidative stress in irradiated cells caused by ROS generated in the mitochondria and through regulating the expression of genes which play a critical role in protection against ionizing radiation.

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide anion radical ($O_2\cdot^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) are continually generated as a consequence of aerobic metabolism.[1,2] Excessive accumulation of ROS within cells results in oxidative stress that leads to oxidative damage to DNA, proteins and lipids. Oxidative stress is often initiated by the production of $O_2\cdot^-$, which is converted to potent oxidants such as ·OH.[2–5] ROS are also generated by ionizing radiation in cells. When H_2O, the most abundant intracellular molecule, is exposed to ionizing radiation, decomposition reactions occur, which generate a variety of free radicals such as $O_2\cdot^-$ and ·OH. Previous studies have shown that these ROS also play a critical role in cell death caused by ionizing radiation.[5–7]

On the other hand, endogenous antioxidants in mammalian cells protect cells from the damage induced by ROS. One of the primary families of antioxidant enzymes, superoxide dismutases (SODs), are metalloenzymes which eliminate $O_2\cdot^-$ produced within cells through a dismutation reaction.[1,8,9] There are three SOD isoenzymes present in eukaryotic cells,

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Cu/ZnSOD (SOD1), MnSOD (SOD2) and extracellular SOD.[9,10] SOD1 is located in the cytosol of eukaryotic cells. SOD2 is localized in the matrix of mitochondria, which convert $O_2^{\cdot -}$ to H_2O_2, which is further metabolized to H_2O by glutathione peroxidase.[1,9,10] The cellular redox environment is defined by the balance between the production of ROS and their removal by antioxidant enzymes.

SOD2 plays an important role in protection against cellular damage by ionizing radiation.[11–14] Overexpression of SOD2 in mammalian cells protects them from radiation lethality.[12–16] Epperly *et al*.[13] and Wong[15] previously showed that mitochondrial localization of SOD is required for decreasing radiation lethality. A construct of SOD2 deleting the mitochondrial localization leader sequence was localized in the cytoplasm and did not show radioprotective activity.[13] Furthermore, Takada *et al*.[12] also demonstrated that human ovarian cancer cells overexpressing SOD2 were more resistant to ionizing radiation than the parental cells. They showed that inhibition of p38 mitogen-activated protein kinase and scavenging free radicals blocked the induction of radioresistance by overexpressed SOD2 and also abolished the shortening of the G_2-M duration.[12,15] However, it remains necessary to further elucidate the mechanisms underlying the protection against radiation-induced cellular damage by SOD2. In this study, we showed that SOD2 overexpression in mitochondria also conferred resistance to γ-rays on HeLa cells. The levels of ROS within cells increased with post-irradiation incubation, and this increase was significantly attenuated in HeLa S3/SOD2 and T-REx HeLa/SOD2 cells compared with irradiated control cells. Furthermore, irradiated HeLa S3/SOD2 and T-REx HeLa/SOD2 cells exhibited a more marked decrease of phosphorylated histone H2AX level, compared with irradiated control HeLa S3 and T-REx HeLa cells.

Previous studies demonstrated that induction of SOD2 under different stress conditions could contribute to adaptive responses against ionizing radiation.[11,15–20] In the present study, DNA microarray analysis revealed that, of 47,000 probe sets analyzed, 117 and 166 probes showed more than 2-fold changes after 5.5 Gy of γ-irradiation in control and SOD2-overexpressing HeLa S3 cells, respectively. Pathway analysis revealed different expression profiles in irradiated control cells and irradiated HeLa S3/SOD2 cells. Genes related to the cell cycle or death (Cyclin D1 and Survivin) and stress responses (Heat shock proteins) were upregulated or constitutively overexpressed in irradiated SOD2-overexpressing cells. These results indicate that the mitochondrial SOD2 activity and mitochondria-derived ROS-signaling could regulate radiation-induced gene expression and radioresistance in HeLa S3 cells.

MATERIALS AND METHODS

Cell culture

HeLa S3 cells were grown at 37°C in Dulbecco's modified Eagle's medium containing L-glutamine (D-MEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-Invitrogen) in 5% CO_2-95% air and 100% humidity. T-REx HeLa cells were obtained from Invitrogen and cultured in E-MEM medium supplemented with 10% One Shot fetal bovine serum (Gibco) and 5 μg/ml blasticidin in 5% CO_2-95% air and 100% humidity.

Plasmid construction

Plasmid vectors p3XFLAG-CMV-10 and pcDNA4/TO/myc-HisA were obtained from Sigma and Invitrogen, respectively. To construct a plasmid carrying the human *SOD2* gene, the open reading frame for the *SOD2* gene was amplified by PCR from the HeLa Match Maker cDNA library (Toyobo). Primers (F) 5'-ATATATGAATTCAATGTTGAGCCGGGCAGTGT-3' and (R) 5'-ATATGCTCTAGATTACTTTTTGCAAGCCATGTATCTTTCAGTTA-3' were used for amplification and cloning into p3XFLAG-VMV-10 plasmid vector. Primers (F) 5'-ATATATGAATTCGCCATGGGTTTGAGCCGGGCAGTG-3' and (R) 5'-CCAGCCTCTAGACTTTTTGCAAGCCATGTATCTTTCAGTTAC-3' were used for amplification and cloning into pcDNA4/TO/myc-HisA plasmid vector. The PCR products were digested with *Eco*RI and *Xba*I and ligated into the *Eco*RI/*Xba*I restriction site of the vector plasmids. The sequences were checked to verify that no mutations had been introduced by the PCR. The plasmids obtained were named pSOD2 and pSOD2-myc.

Transfection and stable cell line construction

Stable cell lines overexpressing SOD2 were constructed using Lipofectamine 2000 (Invitrogen). HeLa S3 cells were grown at 37°C to 90–95% confluence in 24-well plates containing D-MEM supplemented with 10% FBS and then transfected with 1–2 μg of pSOD2 or vector plasmid using Lipofectamine 2000. Cells were incubated at 37°C for 24 hr in a CO_2 incubator and diluted at 1:10–1:30 into fresh D-MEM supplemented with 10% FBS containing 600 μg/ml of Geneticin disulfate (G418). The cells were screened for 2 to 3 weeks at 37°C in 5% CO_2-95% air. Several single colonies were selected for stable overexpression of SOD using the two methods described below. The clones were maintained in D-MEM with 200 μg/ml of G418.

Isolation of cell lines expressing tetracycline-inducible SOD2

The pcDNA4/TO/myc-HisA plasmid vector or pSOD2-myc plasmid was transfected using the FuGENE 6 (Roche) into T-REx HeLa, which constitutively express the tetracy-

cline (Tet) repressor. The positive clones were selected in the presence of 100 μg/ml zeocin. The expression level of myc-fused SOD2 was assessed by Western Blotting as described below.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from control HeLa S3 and HeLa S3/SOD2 cells. Five million cells were treated with an RNAqueous-4 PCR kit (Ambion Inc.) and total RNA was isolated according to the manufacturer's recommendations. cDNA was prepared by reverse transcription using M-MuLV Reverse Transcriptase (MBI Fermentas). Gene transcripts were detected by reverse transcription-polymerase chain reaction (RT-PCR). The primers for SOD2 and GAPDH were (F) 5'-ATATATGAATTCAATGTTGAGCCGGGCAGTGT-3', (R) 5'-ATATGCTCTAGATTACTTTTTGCAAGCCATGTATCTTTCAGTTA-3' and (F) 5'-CCATGGAGAAGGCTGGGG-3', (R) 5'-CAAAGTTGTCATGGATGACC-3', respectively. The amplification was performed with denaturation at 95°C for 5 min, then 3 cycles at 94°C for 50 sec, at 48°C for 50 sec, at 72°C for 1 min, and after that 28~32 cycles at 94°C for 50 sec, at 57°C for 50 sec, at 72°C for 1 min, and one final extension at 72°C for 15 min. The RT-PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. The products were quantitated with a Fuji film BAS-2500 Image Analyzer (Fuji Photo Film).

Isolation of mitochondria and western blot analysis

The mitochondrial fraction was isolated to determine the level of SOD2 expression. A ProteoExtract Cytosol/Mitochondria Fraction Kit (Calbiochem) was used to isolate mitochondria and cytosol fractions from 5×10^7 cells. Proteins (30 μg) in the fractions were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Western blot analysis was performed using anti-Superoxide Dismutase II (SOD II) (Abfrontier), anti-OxPho Complex IV-Subunit IV mouse IgG2a monoclonal 20E8 (COX IV) (Molecular Probes) as a mitochondrial marker and anti-Lamin B (C-20) (SC-6216) as a cytoplasmic/nuclear marker. The membranes were then incubated with anti-Rabbit IgG (for SOD II), anti-mouse IgG (for COX IV) and anti-goat IgG (for LaminB) -conjugate with horseradish peroxidase (HRP), respectively, followed by incubation with ECL (GE Healthcare) and being exposed to X-ray films. The content and purity of the mitochondrial fraction were monitored using an antibody directed against COX IV, a mitochondrial marker. Contamination of other cellular components in the mitochondrial fraction was analyzed using antibodies directed against a nuclear marker, Lamin B.

For T-REx HeLa/SOD2 cell lines, whole cell extract was prepared as follows: the cells were harvested using a cell scraper in ice-cold PBS and transferred to test tubes. The cells were spun down and the cell pellets obtained were resuspended in 2 × sample buffer (125 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol, 4% SDS, 10% sucrose, 0.004% bromophenol blue). The sample was sonicated for 15 min using a Bioruptor (Cosmobio) at maximum power, and then boiled for 5 min. Proteins were separated on 12 or 15% SDS-PAGE gel, and transferred to a PVDF membrane. The membrane was incubated with appropriate antibodies, anti-myc conjugated with HRP (Santa Cruz Biotechnology), anti-Rabbit IgG for tubulin and actin (Santa Cruz Biotechnology) or anti-γH2AX (Millipore). Rabbit polyclonal Alexa Fluor 488-conjugated anti-mouse IgG and Alexa Fluor 488-conjugated anti-rabbit IgG were obtained from Santa Cruz Biotechnology.

SOD2 activity assay

The T-REx and T-REx/SOD2 cells were washed once in ice-cold PBS and cell pellets were resuspended in loading buffer (50 mM potassium phosphate pH 7.8, 50% glycerol and 0.004% bromophenol blue). The samples were sonicated for 7.5 sec on and 60 sec off 20 times at 4°C using a Bioruptor at maximum power. Proteins (50 μg) were separated on by electrophoresis on a 12% native polyacrylamide gel. For HeLa S3 and HeLa S3/SOD2 cells, equal volume of mitochondria fraction was applied to 12% native polyacrylamide gel. SOD2 activity was measured as described by Beauchamp and Fridovich.[21] The intensity of each band was determined by image analysis using NIH Image software.

Irradiation and survival assay

Cell survival was assayed by two different methods, a conventional colony formation assay and a high density survival assay.[22,23] Colony formation assay; when the cell density reached about 80% confluence, exponentially growing cells were trypsinized, harvested by centrifugation at 3,000 rpm for 5 min and resuspended in fresh D-MEM supplemented with 10% FBS. The cell suspensions were appropriately diluted and reseeded in 6-cm culture dishes. The cells were exposed to γ-rays at a dose rate of 0.84 Gy/min using a ^{137}Cs γ-Cell 40 Exactor (Nordion International Inc.). After incubation for 12 days, cells were fixed with 0.2% formaldehyde and stained with Giemsa stain reagent. Colonies containing more than 50 cells were counted as survivors under a stereomicroscope. P values were obtained to determine the statistical significance of differences at the 95% confidence level.

The high density survival assay[22,23] was applied for T-REx HeLa cell lines. Five hundred thousand cells were seeded into a 3.5-cm dish and incubated for 12 hours, and 0.1 μg/ml tetracycline (Tet) (Invitrogen) was then added to the EMEM medium containing 10% One Shot FBS. After 12 hours of incubation, the cells were irradiated with γ-rays at a dose rate of 0.84 Gy/min using a ^{137}Cs γ-Cell 40 Exactor. The cells were then incubated for another 24 hours. The cells of

each dish were then detached by use of trypsin/EDTA and diluted at 1:10 into fresh EMEM and seeded into a 10-cm dish containing 10 ml fresh medium, 0.1 μg/ml tetracycline and incubated for 72 hours, then supplemented with 0.1 μg/ml tetracycline in the medium and further incubated for 48 hours. The cells of each dish were again detached by the cells treatment with trypsin and diluted at 1:10 into fresh EMEM medium containing 0.1 μg/ml tetracycline and seeded into 3 6-cm dishes for each point and incubated for 24 hours, and then the surviving cells were trypsin treated and the cell number was counted.

Assay for double-strand breaks in DNA

T-REx HeLa and HeLa S3 cell lines, were seeded in 10-cm dishes (5×10^5 cells per dish) and the cells were grown until the cell density reached > 60% confuency, and then for T-REx HeLa/SOD2 cells, 0.1 μg/ml tetracycline was added to each culture for induction of SOD2-myc-His protein expression and the cells were further incubated for 12 hours. The cells were irradiated with γ-rays at 5 Gy. Unirradiated and irradiated cells were incubated in D-MEM or EMEM at 37°C. The cells were collected and the samples were prepared as described above. The sample was sonicated for 15 min using a Bioruptor (Cosmobio) at maximum power, and then boiled for 5 min. Proteins were separated on a 12 or 15% SDS-PAGE gel, and transferred to a PVDF membrane. The membrane was incubated with anti-γH2AX (Millipore).

Cellular ROS levels after γ-irradiation

One million cells of unirradiated and irradiated cultures were collected by centrifugation and washed twice with PBS. The cell pellets were resuspended in 1 ml of PBS containing 1% FBS and 0.4 mM 2',7'-dichlorofluorescin diacetate (DCFH-DA)[24,25] and incubated at 37°C for 15 min. The cells were washed twice with PBS, and their fluorescence was measured by flow cytometry.

Immunocytochemical staining of mitochondrial superoxide

Cells were seeded in 3.5-cm glass bottom dishes (0.8 or 1×10^5 cells/dish). When the cell density reached about 60% confluency, 0.1 μg/ml tetracycline was added to each culture for induction of SOD2-myc-His protein expression. Twelve hours after the addition of tetracycline, the cells were stained with Mitotracker Red (Molecular Probes) for 30 min in a 37°C humidified incubator containing 5%-CO_2 and stored in the dark until analysis. The cells were washed twice with ice-cold PBS and fixed in methanol/acetone (1:1) for 15 min at 20°C. The cells were soaked in TBST (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 Tween20) for 5 min at room temperature. The cells were incubated with appropriate antibodies (anti-myc and anti-mouse IgG (for myc, Santa Cruz Biotechnology)-conjugated Alexa Fluor 488). The cells treated with antibodies were washed twice with TBST. Samples were observed using an Olympus IX70. For detection of mitochondrial superoxide generation, a specific mitochondrial superoxide indicator, MitoSOX™ Red (Invitrogen Molecular probes), was used according to the manufacturer's instructions. To quantify the amount of mitochondrial superoxide, flow cytometric analysis (BD LSR II) was performed. MitoSOX red was excited by laser at 488 nm and data were collected at 585/42 nm.

DNA microarray and pathway analyses

To identify genes expressed after γ-irradiation, DNA microarray analysis was performed with control HeLa S3 and HeLa S3/SOD2 cells. After post-irradiation incubation in D-EMEM for 12 hours, total RNA was extracted from the cells using an RNeasy Total RNA Extraction Kit (Qiagen) and treated with RNase-free DNase I (30 U/sample; Qiagen) for 15 min at room temperature to remove residual genomic DNA. The extract was treated with phenol-chloroform, and RNA was precipitated with 2.5 volumes of ethanol. Poly(A)-RNA was isolated using an Oligotex RNA kit. Gene expression was analyzed using a GeneChip system with a Human Genome U133 Plus 2.0 Array which was spotted with approximately 47,000 probe sets (Affymetrix). Sample preparation for array hybridization was carried out following the manufacturer's instructions. Hybridization intensity data were converted into a presence/absence call for each gene, and changes in gene expression between experiments were detected by comparison analysis.[26,27]

The data were further analyzed using GeneSpring software (Silicon Genetics) to extract the significant genes. To examine the gene ontology, including biological processes, cellular components, molecular functions and genetic networks, the data were analyzed using Ingenuity Pathways Analysis tools (Ingenuity Systems).[26,27] The gene lists identified by GeneSpring containing Affymetrix gene ID and the natural logarithm of normalized expression signal ratios from GeneChip CEL files were uploaded into the Ingenuity Pathways Analysis. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These so-called focus genes were then used as a starting point for generating biological networks.

RESULTS

Construction of stable transformants of HeLa S3 expressing SOD2, and T-REx HeLa cells lines expressing tetracycline-inducible SOD2

Previous studies showed that overexpression of SOD2 conferred resistance to ionizing radiation in mammalian cells.[11–16] To elucidate the mechanism of the radiation protection, we constructed plasmids bearing the *SOD2* gene and transfected them into HeLa S3 cells and T-REx HeLa cells (which are a Tet-on/off system cell line). The plasmid pSOD2 and pSOD2-myc were introduced into HeLa S3 cells

and T-REx HeLa cells, using Lipofectamine 2000 or FuGENE 6, respectively. After three rounds of extensive screening, we established HeLa S3/SOD2 and T-REx HeLa/SOD2 cells which stably overexpressed SOD2. A stable HeLa S3 cell line and T-REx HeLa cell line containing vector plasmid were also selected as a G418-resistant clone (control HeLa S3 cells, Vector) or zeocin-resistant clone (control T-REx HeLa cells, Vector). Several clones of HeLa S3 cells and T-REx HeLa cells overexpressing SOD2 were obtained.

For the SOD2-overexpressing T-REx HeLa cell line, a zeocine-resistant SOD2 clone was isolated (T-REx HeLa/SOD2). T-REx HeLa cells were incubated with tetracycline at various concentrations (0.1 to 10 μg/ml) for 24 hours, followed by the determination of the amount of SOD2. The expression of SOD2 was significantly induced by adding tetracycline at 0.1 μg/ml to the medium (Fig. 1A). The relative induction was 1.2 and 1.6 times at 1 and 10 μg/ml, respectively. So, we used 0.1 μg/ml of tetracycline during this study. Figure 1A also shows that the induction of SOD2 continued for over 72 hours. The SOD2-myc protein level was increased 3~4 fold compared with the endogenous SOD2. Furthermore, it was evident using a native gel assay that the activity of SOD2 increased 3~3.9 fold beyond the endogenous level of SOD2, while SOD1 activity did not increase (Fig. 1B).

For HeLa S3 cell lines, total RNAs were isolated from selected HeLa S3 cell lines, and used for RT-PCR. The resulting PCR products were separated by electrophoresis on a 1.5% agarose gel. As shown in Fig. 1C, SOD2 was strongly

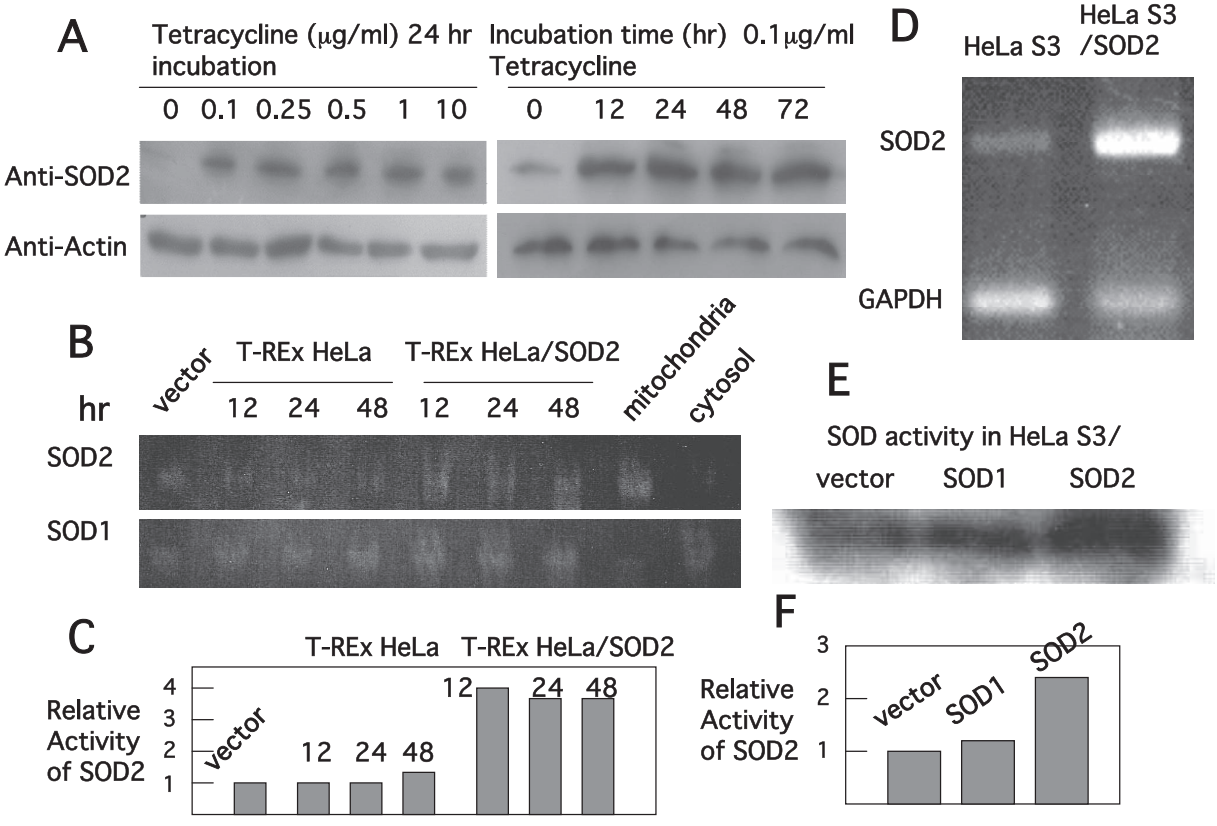

Fig. 1. Overexpression of SOD2 in T-REx HeLa/SOD2 and HeLa S3/SOD2 cells (**A**) SOD2 protein expression in T-REx HeLa/SOD2 cells was induced by adding tetracycline. (left) Tetracycline was added at 0.1, 025, 0.5, 1 or 10 μg/ml to the T-REx HeLa/SOD2 cells, and after 24 hours incubation, the SOD2 protein expression levels were detected using anti-SOD2 antibodies, with anti-actin as a control. (right) 0.1 μg/ml tetracycline was added to the cells and then they were incubated for the indicated periods of times, and SOD2 protein levels were analyzed with anti-SOD antibody, with anti-actin as a control. (**B**) SOD2 protein activities in T-REx HeLa and T-REx HeLa/SOD2 were detected by a nondenaturing gel assay. Tetracycline induction was done for 12, 24 or 48 hours with 0.1 μg/ml tetracycline. The cell extracts were separated on a 12% native polyacrylamide gel. (**C**) Quantitative assay of SOD2 activity. The values represent the ratio to those in control cells. (**D**) Total RNA was isolated from control HeLa S3 and HeLa S3/SOD2. Gene transcripts were detected by RT-PCR. The products were separated by electrophoresis on a 1.5% agarose gel, visualized by ethidium bromide staining and then quantitated with a Fuji film BAS-2500 Image Analyzer. (**E**) SOD activity in mitochondria of (**a**) HeLa S3/vector, (**b**) HeLa S3/SOD1 and (**c**) HeLa S3/SOD2 cells. (**F**) Quantitative assay of SOD2 activity. The values represent the ratio to that in HeLa S3/vector. Quantitative analysis was done using NIH Image Analysis.

expressed in HeLa S3/SOD2 cells compared with the internal GAPDH control. The RT-PCR analysis revealed about a 6-fold increase of SOD2 expression. The activity of SOD2 significantly increased in mitochondrial fraction extract in HeLa S3/SOD2 compared with control HeLa S3 (Fig. 1D).

Endogenous SOD2 is localized in mitochondria.[13,14] To confirm the subcellular localization of overexpressed SOD2 in mitochondria, we followed two independent strategies. For HeLa S3 cell lines, the mitochondrial fraction was isolated from control HeLa S3 and HeLa S3/SOD2 cells. Proteins in the mitochondrial extract were subjected to 12% SDS-PAGE, transferred to a PVDF membrane, and probed with SOD II, COX IV and Lamin B1 antibodies. The results showed that overproduced SOD2 was transported to the mitochondria in HeLa S3/SOD2 cells (Fig. 2A). Compared with control vector cells, the SOD2 protein was increased about 1.8~2.4-fold in HeLa S3/SOD2 cells, and we could not detect any contaminating proteins from other parts of the cells by the use of Lamin as a control.

SOD2-transfected T-REx cell lines had an SOD-myc fusion protein in the cells, which could be detected by immunofluorescence. Whether the SOD2 in T-REx HeLa/SOD2 cells was stably expressed in the mitochondria was determined by using anti-myc antibody (Fig. 2B). Staining of T-REx HeLa/SOD2 showed a clear punctuate staining overlapping with the mitochondrial staining with MitoTracker Red.

Cell survival after γ-irradiation

We investigated whether the activity of mitochondrial antioxidant enzyme SOD2 confers radioresistance in T-REx HeLa and HeLa S3 cells. The results in Fig. 3A show that overexpression of SOD2 but not SOD1 enhanced cellular resistance to γ-irradiation. A similar increase of radiation resistance by the induction of SOD2 was observed using the conventional colony formation assay in T-REx HeLa cells (data not shown). To confirm the radiation protection by SOD2, we used another survival assay, a high-density survival assay for T-REx HeLa cell lines. The high density survival assay is a powerful tool for evaluating cell responses to therapeutic agents.[22] This method is also useful to determine the radiosensitivity of cells that cannot be evaluated by the colony formation survival assay.[22,23] The results are shown in Fig. 3B. It was evident that overexpression of mitochondrial SOD2 conferred resistance to radiation killing in HeLa cells.

Superoxide generation in T-REx HeLa/SOD2 and HeLa S3/SOD2 cells after γ-irradiation

We examined whether ROS levels increased in cells exposed to radiation and whether the superoxide generation in mitochondria was reduced by overexpression of SOD2. HeLa S3 and HeLa S3/SOD2 cells were incubated for up to 72 hours after irradiation with various doses of γ-rays. We used a mitochondrial superoxide indicator, MitoSox™ Red, to assay irradiated T-REx HeLa/SOD2 and HeLa S3/SOD2 cells. Analysis of the cells with the fluorescent probe MitoSox Red revealed that γ-ray exposure resulted in elevated levels of mitochondria superoxide compared to those in unirradiated cells (Fig. 4A). To quantify the mitochondrial

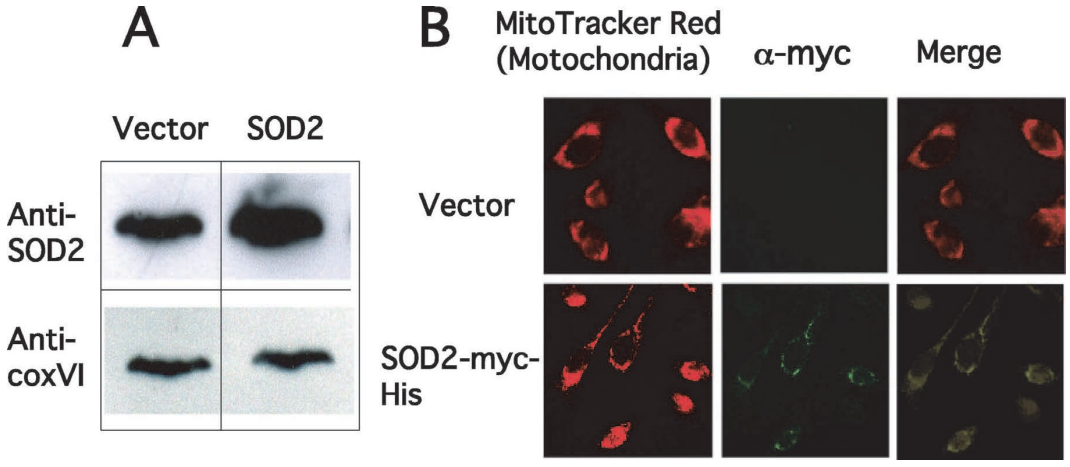

Fig. 2. Induction of SOD2 in mitochondria of HeLa S3/SOD2 and HeLa T-REx cells. (**A**) Proteins prepared from isolated mitochondria of HeLa S3/vector and HeLa S3/SOD2 cells were fractionated by SDS-PAGE and analyzed by western blotting with SOD2 and COX IV antibodies and then quantitated with a Fuji film BAS-2500 Image Analyzer. (**B**) Localization of overexpressed SOD2 -myc fusion proteins in T-REx HeLa/SOD2 cell mitochondria was confirmed by an immunostaining assay. T-REx HeLa and T-REx HeLa/SOD2 cells were grown on 3.5-cm glass bottom dishes (Matsunami) for 36 hours and 0.1 μg/ml tetracycline was added to the medium and the cells were further incubated for 12 hours and then probed with the MitoTracker Red and Anti-myc antibodies. Immunostaining was performed with anti- myc and Alexa Fluor 488-conjugated anti-mouse IgG. The green fluorescence of SOD- myc and red fluorescence of the mitochondrial marker were visualized using confocal microscopy (fluorescence microscopy).

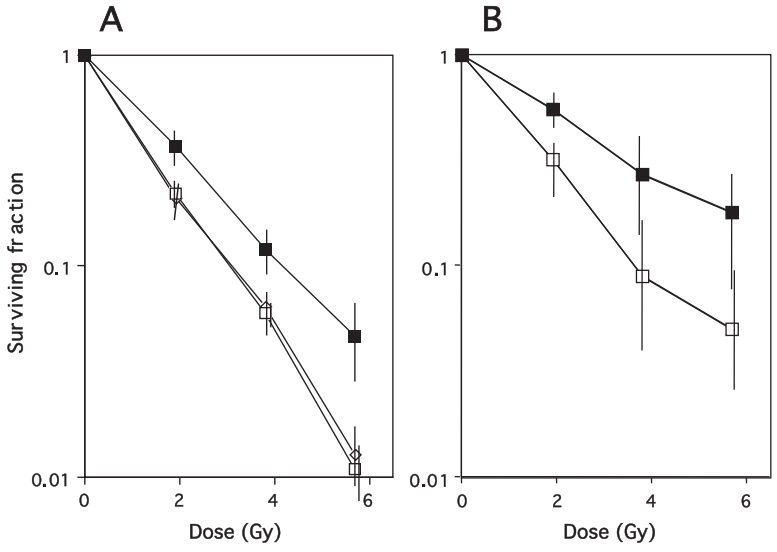

Fig. 3. Survival of T-REx HeLa and HeLa S3 cells following exposure to γ-rays. (**A**) Exponentially growing cells were appropriately diluted and reseeded in 6-cm culture dishes. The cells were exposed to γ-rays at room temperature. After incubation for 12 days, colonies containing more than 50 cells were counted under a stereomicroscope to estimate survival. The values represent the mean ± standard deviation. (n = 4). P values were obtained to determine the statistical significance of differences between surviving fractions. The P values were < 0.05 for HeLa S3/SOD2 cells compared to HeLa S3 cells, indicating a significant difference at the 95% confidence level. -□-, control HeLa S3; -◇-, HeLa S3/SOD1; -■-, HeLa S3/SOD2. (**B**) Exponentially growing cells were appropriately diluted and reseeded in 3.5-cm culture dishes at 5×10^5 cells/dish and incubated for 12 hours. 0.1 μg/ml tetracycline was added to the medium and the cells were further incubated for 12 hours. The cells were exposed to various doses of γ-rays and incubated for 72 hours and 0.1 μg/ml tetracycline was added again. The cells were further incubated for another 48 hours. Subsequently, 1/10 of the cells of each dish were seeded into 3 new 6-cm dishes and incubated for a further 24 hours. Then the total number of cells in each dish was counted using the trypan blue dye exclusion test. The values represent the mean ± standard deviation (n = 3). P values were obtained to determine the statistical significance of differences between surviving fractions. The P values were < 0.05 for the cells irradiated with 2 Gy, indicating a significant difference at the 95% confidence level. -□- T-REx HeLa/vector; -■- T-REx HeLa S3/SOD2.

superoxide, flow cytometric analysis of the stained cells was also performed (Fig. 4B). The quantitative data (Fig. 4C) showed the changes in mean fluorescence intensity. The amount of mitochondrial superoxide was increased after γ-ray irradiation in control HeLa T-REx/vector cells, but the overexpression of SOD2 significantly suppressed the superoxide generation in T-REx HeLa.

Levels of ROS in HeLaS3/SOD2 cells after γ-irradiation

Next we examined whether the levels of ROS in the cytoplasm/nuclear fraction were influenced by overexpression of SOD2 in mitochondria. Flow cytometry with DCFH-DA is frequently used to determine the levels of ROS generation in mammalian cells by measuring the fluorescence intensity of its oxidized derivative. Non-fluorescent DCFH-DA is converted to its derivative 2',7'-dichlorofluorescin (DCFH) by the action of esterases and peroxidases in cells.[24,25] DCFH is easily oxidized by peroxides, including H_2O_2, to generate fluorescent 2',7'-dichlorofluorescein. To assess ROS generation as a marker of the status of oxidative stress after irradiation, control and HeLa S3/SOD2 cells were irradiated with various doses of γ-rays and incubated at 37°C in D-MEM with 10% FBS for up to 24 hours. At appropriate intervals, an aliquot of the cell suspension was withdrawn and subsequently incubated with 0.4 mM of DCFH-DA at 37°C for 15 min, followed by measurement of the fluorescence using flow cytometry. The results are shown in Fig. 5. The fluorescence intensity increased with post-irradiation incubation. The increase in the ROS level depended on the radiation dose. It was also evident that the oxidative stress level was significantly lowered in HeLa S3/SOD2 compared with that in control HeLa cells.

Double-strand breaks in γ-irradiated HeLa S3 and T-REx HeLa cells

We determined the amount of double-strand breaks to

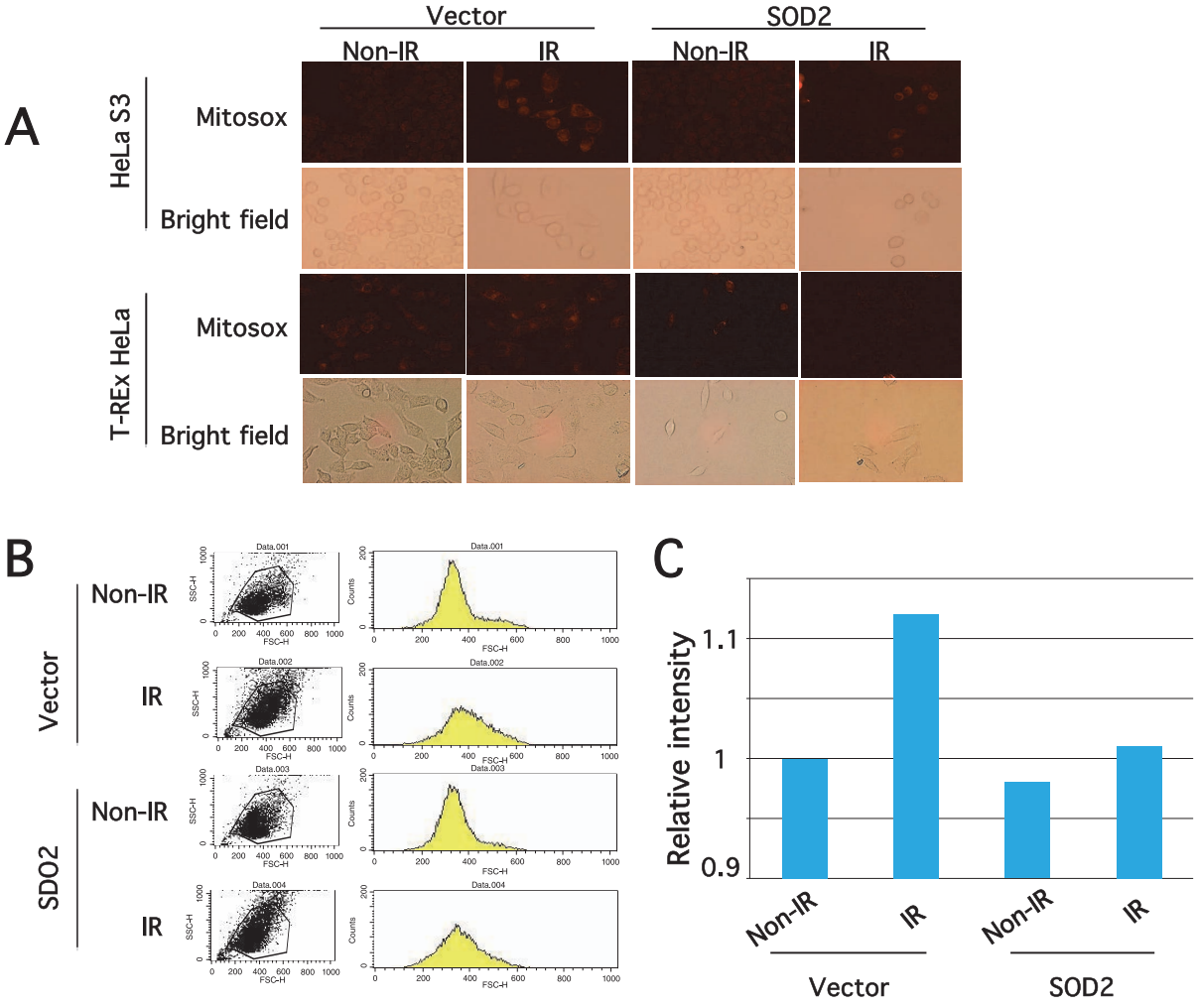

Fig. 4. Effect of high expression of SOD2 on mitochondrial superoxide generation. T-REx HeLa and T-REx-HeLa/SOD2 cells were exposed to 5 Gy of γ-rays and incubated for 72 hours, and then the cells were stained with MitoSox Red. Mitochondrial superoxide generation was visualized using fluorescence microscopy. The levels of superoxide were determined by flow cytometric analysis. MitoSOX was excited at 488 nm and data were obtained at 585 nm. (**A**) Images of fluorescence microscopy. (**B**) and (**C**) Quantitative data of the mitochondrial superoxide levels in γ-irradiated cells. Analysis was done using a flow cytometer. (a) T-REx HeLa/vector (0 Gy), (b) T-REx HeLa/vector (5 Gy), (C) T-REx HeLa/SOD2 (0 Gy), (D) T-REx HeLa/SOD2 (5 Gy). The experiment was repeated three times, and similar results were obtained (a representative data set is shown).

examine whether the level of radiation damage in cellular DNA was affected by the overexpression of SOD2 in mitochondria. We measured the amount of γH2AX, the phosphorylated form of histone H2AX, which correlates well with the double-strand breaks in DNA.[28,29] HeLa S3, HeLa S3/SOD2, T-REx HeLa and T-REx HeLa cells were incubated for 60 min after γ-irradiation. The results are shown in Fig. 6. The amount of γH2AX during post-irradiation incubation was reduced in T-REx HeLa/SOD2 and HeLa S3/SOD2 cells compared with that in control HeLa cells. The suppression of the γH2AX level was observed after 72 hours post-irradiation incubation (data not shown).

Modulation of gene expression by overexpression of SOD2

Irradiation of mammalian cells with ionizing radiation results in the induction of a number of proteins, including SOD2 and proteins involved in signaling pathways, cell death and the stress response.[17–20] Under the present experimental conditions, the expression of SOD2 was increased 2.3~3.1-fold 12 hours after 5.5 Gy-irradiation in HeLa S3 cells. A screen for genes that were upregulated or downregulated by more than 2-fold was performed using GeneSpring software. Genes corresponding to a total 194 of probe sets were found to be differentially expressed between γ-irradiated cells and unirradiated control cells. Among them, 64% were upregulated, and the remainder downregulated. Upreg-

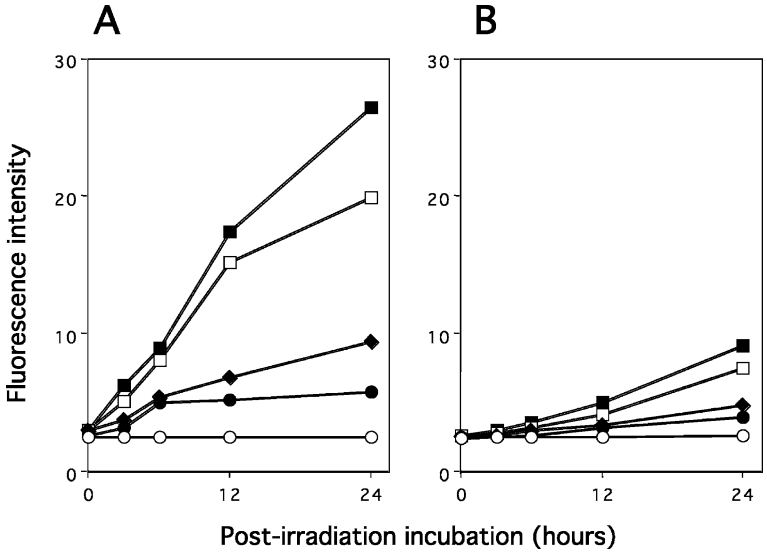

Fig. 5. Relative fluorescence intensity of 2',7'-dichlorofluorescein in control HeLa S3 and HeLa S3 /SOD2 during post-irradiation incubation. One million cells were irradiated with 0 (-○-), 1.8 (-●-), 3.6 (-◆-), 5.5 (-□-) or 11 (-■-) Gy of γ-rays and incubated in D-MEM with 10% FBS at 37°C for up to 24 hours. The cells were centrifuged, washed twice with PBS and then resuspended in 1 ml of PBS containing 0.4 mM 2',7'-dichlorofluorescin diacetate. The suspension was incubated at 37°C for 15 min, followed by measurement of the fluorescence intensity with flow cytometry. The points represent the mean of two replicate experiments. **(A)** control HeLa S3 cells. **(B)** HeLa S3/SOD2 cells.

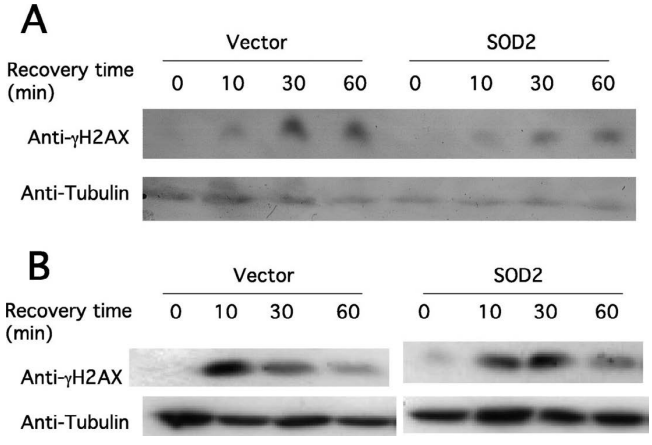

Fig. 6. The amount of γH2AX in γ-irradiated control T-REx/ HeLa, HeLa S3 and T-REx HeLa/SOD2, HeLa S3/SOD2 cells. Growing cells were irradiated with γ-rays at 5 Gy. After incubation at 37°C for the indicated times, the cells were collected, cell extracts was prepared, and total proteins were separated by 12% SDS-PAGE and the proteins were transfer to a 0.2 μm PVDF membrane. The γH2AX was determined with anti-γH2AX antibody and tubulin or actin was used as a control. (**A**) T-REx HeLa cells. (**B**) HeLaS3 cells.

ulated gene products included growth arrest and DNA damage-inducible protein (GADD45), cyclin A, cyclin B1, RAS homolog, NfκB, p21, thioredoxin reductase, cytochrome P450 and GTP binding protein.

Previous studies demonstrated that SOD2 can modulate gene expression, and the SOD2-regulated genes might be involved in protection against cellular damage by ROS.[19,30] In the present study, in unirradiated SOD2-overexpressing cells, 295 probes showed more than a 2-fold increase compared with the expression in control cells. To elucidate the relationship between radiation-responsive genes and SOD2-regulated genes, we compared transcripts from γ-irradiated HeLa S3 with those in SOD2-overproducing cells using GeneChip analyses. Increases in the expression of several transcripts, including GADD45, cyclin A, cyclin B1, p21, Myc, RAS homolog, tumor necrosis factor receptor, NfκB, glucocorticoid receptor and GTP-binding protein were detected in both γ-irradiated cells and SOD2-overexpressing cells.

Functional and pathway analysis

Furthermore, to determine the biologically relevant networks and pathways of the genes identified in this study, pathway analysis of the up- and down-regulated genes was carried out using the Ingenuity Pathways Analysis Knowledge Base.[26,27] This software was developed based on the knowledge repository of the biological and chemical relationships extracted from thousands of selected scientific literature references. We performed functional and pathway analyses of differentially expressed genes in HeLa S3 and HeLa S3/ SOD2 cells exposed to 5.5 Gy of γ-rays. Significant genetic networks were identified for the differentially expressed

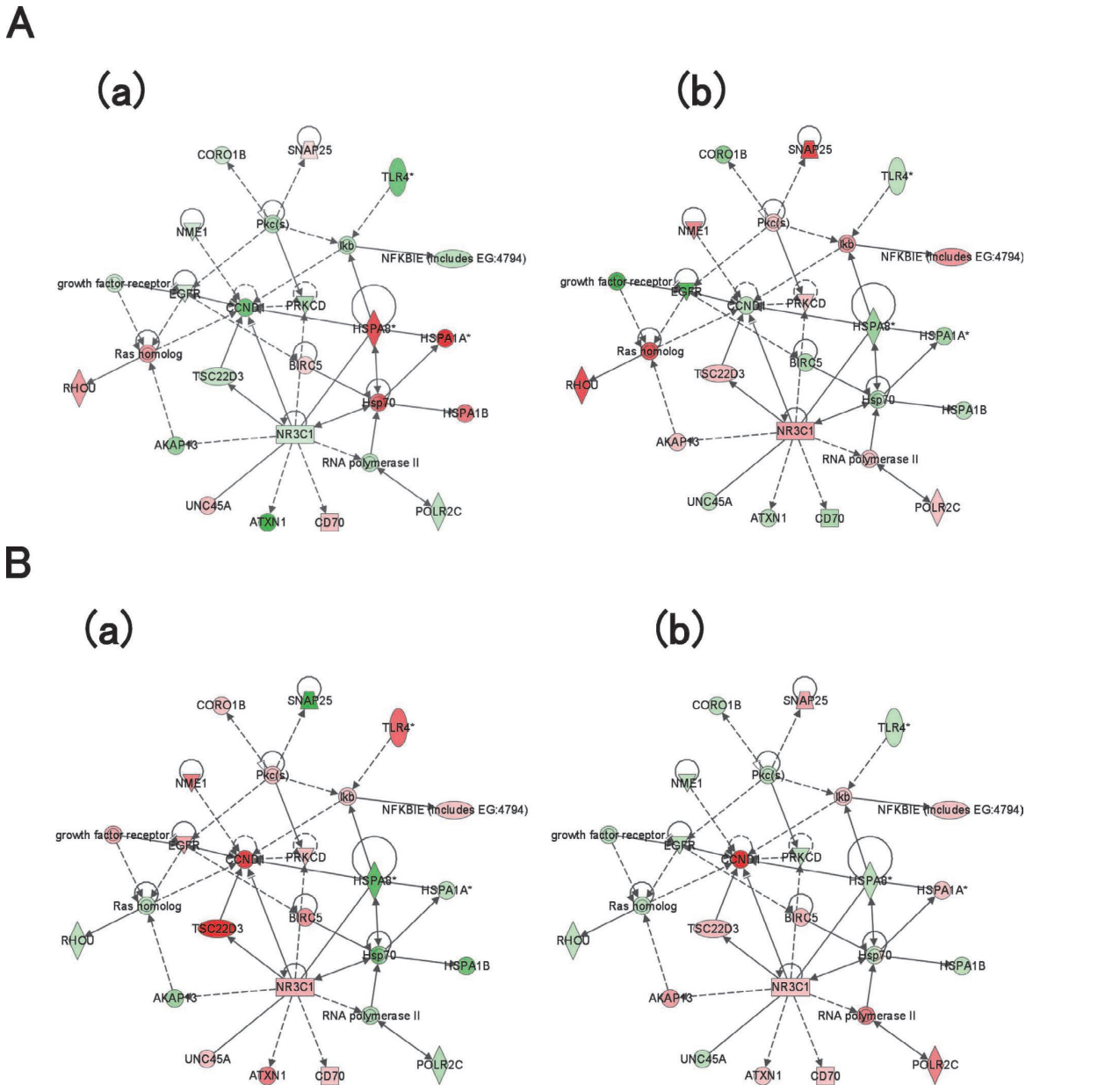

Fig. 7. Networks of genes that were up- and down-regulated in non-irradiated and γ-irradiated control HeLa S3 cells. (**A**) HeLa S3 cells. After post-irradiation incubation for 12 hours, poly(A)-RNA was isolated using an Oligotex RNA kit and gene expression was analyzed using a GeneChip system. The data were analyzed using Ingenuity Pathways Analysis tools (Ingenuity Systems). Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These so-called focus genes were then used as a starting point for generating biological networks. HSPs (heat shock proteins), PKC-mediated SNAP25 and NfκB are stress-response proteins; CD70 is a tumor necrosis factor ligand; NR3CI is a gluocorticoid receptor, CCND1 is cyclin D1 and ATXN1 is ataxin 1. (**a**) unirradiated. (**b**) 5.5 Gy-irradiated. , expression scale in log, > 0.7, 0.3, –0.3 and –0.7 >, respectively. (**B**) HeLa S3/SOD2 cells. The DNA chip analysis and the pathway analysis were performed as described in the legend for Fig. 7**A**. (**a**) unirradiated; (**b**) 5.5-irradiated. , expression scale in log, > 0.7, 0.3, –0.3 and –0.7 >, respectively.

genes affected by γ-irradiation in control and SOD2-overexpressing HeLa S3 cells (Fig. 7). On the basis of the significance and number of genes, the upregulated and downregulated genes affected by γ-irradiation were judged to be involved in cellular growth and proliferation. Genes related to the cell cycle, such as Cyclin D1 and Survivin, and to the stress response, such as Heat shock proteins, were upregulated or constitutively overexpressed in γ-irradiated HeLa S3/SOD2 cells (Table 1).

Table 1. Changes in gene expression caused by SOD2 overexpression and γ-irradiation in HeLa S3 cells

Gene symbol	Changes in gene expression		
	in HeLa S3/SOD2 (non-irradiated)	in HeLa S3/vector (5 Gy-irradiated)	in HeLa S3/SOD2 (5 Gy-irradiated)
HSPs	Decreased	Decreased	Increased
BIRK5	Increased	Decreased	Expressed constitutively at high level
SNAP25	Decreased	Increased	Increased
RAS homolog	Decreased	Increased	Decreased
NFκB	Increased	Increased	Increased
CD70	Expressed constitutively at high level	Decreased	Expressed constitutively at high level
NR3CI	Increased	Increased	Increased
CCNDI	Expressed constitutively at high level	Decreased	Expressed constitutively at high level
ATXN1	Increased	Decreased	Increased

DISCUSSION

The present experiments revealed that overexpression of SOD2 but not SOD1 enhanced cellular resistance to γ-irradiation (Fig. 3). Transfection of the plasmid pSOD2 or pSOD-myc into HeLa S3 or T-REx HeLa cells, respectively, resulted in the overproduction of active SOD2, which was properly transported into mitochondria (Fig. 1). In contrast, SOD1 overproduced in the cytosol did not protect γ-irradiated cells (Fig. 3B).[13] These results indicate that the mitochondrial localization of SOD is required for protection of HeLa S3 cells from the lethal effects of γ-rays. This conclusion was also reported by Epperly *et al.*[13] They investigated the importance of the mitochondrial localization of SOD2 for the protection of hematopoietic cells from radiation lethality. The cells were transfected with a plasmid containing either the native human *SOD1* gene, the native *SOD2* gene, the *SOD2* gene without the mitochondrial localization leader sequence ($SOD2^{-MLS}$), or the *SOD1* gene with the mitochondrial leader sequence attached to the active portion of the *SOD1* gene ($SOD1^{+MLS}$). Radiation survival curves derived for each cell clone showed that SOD1- and $SOD2^{-MLS}$-overexpressing clones had no increase in cell survival compared with the parent cells. In contrast, cell clones overexpressing either SOD2 or $SOD1^{+MLS}$ showed a significant increase in radioresistance.[13] These observations demonstrated that increased SOD activity of either SOD1 or SOD2 must be localized to the mitochondria to decrease radiation-induced lethality.[13]

Ionizing radiation produces $O_2{\cdot}^-$ in the mitochondria in HeLa cells. Furthermore, ionizing radiation inhibits mitochondrial functions associated with respiratory complexes I (NADH dehydrogenase) and III (cytochrome C reductase), resulting in increased generation of ROS.[31–34] The exposure of isolated mitochondria to radiation resulted in the same pattern of damage to complexes I and III, as shown by assays of electron-transfer activities.[34] Overexpression of SOD2 can reduce the production of $O_2{\cdot}^-$ in the mitochondria, preventing the reaction of $O_2{\cdot}^-$ with DNA, proteins and lipids within the mitochondria. Overexpression of SOD2 has been demonstrated to protect the respiratory chain activity in the mitochondria and prevent the decrease of ATP (which is necessary for repair of DNA damage).[31,35–37] Furthermore, reduction of the amount of ROS by overexpression of SOD2 may decrease the reactions of ROS with mitochondrial components that lead to destabilization of the mitochondrial membrane and release of cytochrome C.[31–33] Some other possible mechanisms by which SOD2 overexpression may protect mitochondrial DNA either during irradiation or from subsequent production of ROS have been proposed.[33,35] Preventing damage to the mitochondrial DNA may protect the mitochondrial functions and prevent apoptosis.[31,36] It is important to clarify whether SOD2 prevents radiation-induced apoptotic cell death. Previously Motoori *et al.*[16] investigated the potential role of mitochondrial SOD2 in protective activity against irradiation by analyzing cell viability by a colony formation assay and by detecting apoptosis in HLE cells stably transfected with the human SOD2 gene. They found that the relative apoptotic index was suppressed in the SOD2-transfected cells compared with HLE, a hepatocellular carcinoma cell line. This fact indicates that SOD2 suppresses radiation-induced apoptosis. We are currently attempting to clarify the role of mitochondrial SOD2 in protecting against radiation-induced apoptosis in HeLa S3 cells.

Experiments utilizing the mitochondria-targeted $O_2{\cdot}^-$-sensitive fluorogenic probe MitoSOX Red[38] revealed that there is a significant increase of $O_2{\cdot}^-$ in mitochondria in irradiated HeLa cells. ROS generated in the mitochondria are not localized in the mitochondria, but rather permeate into the cytoplasm and nucleus of the cell. In fact, the intensity of DCF fluorescence increased with post-irradiation incubation of cells (Fig. 5), indicating that ROS are generated in the

mitochondria and accumulated in the whole cells with post-irradiation incubation. The increase in the level of peroxides is partly due to an increase of H_2O_2 resulting from the overexpression of SOD2. Although DCFH was initially shown to react with H_2O_2, it has been shown to be oxidized to fluorescent DCF by a broad range of intracellular oxidative stresses (ONOO-, NO• and H_2O_2-mediated reactions with peroxidase, SOD and catalase) in addition to H_2O_2 itself. Therefore, the increased fluorescence intensity of DCF represents the oxidation of the probe by such a range of oxidative events, not only by H_2O_2.

It was also noteworthy that the increased levels of oxidative stress were significantly suppressed in HeLa S3/SOD2 and T-REx HeLa/SOD2 compared with those in control HeLa cells. The production of $O_2\cdot^-$ was greatly reduced in the mitochondria by SOD2 overexpression, which in turn reduced the ROS levels in HeLa S3/SOD2 and T-REx HeLa/SOD2 cells. Motoori *et al.*[16] also showed that SOD2 plays an important role in protecting cells against radiation-induced cell death by controlling the generation of mitochondrial ROS in HLE cells. These results suggest that oxidative stress and radiation-induced damage that occur in the mitochondria are a critical role in the development of cellular damage by ionizing radiation.[13,14,31,35,37–39] This notion was supported by our finding that SOD2 overproduction resulted in a reduction of the amount of γH2AX in the nuclear DNA (Fig. 6). Previously, Samper *et al.*[40] also showed that oxidative stress in mitochondria causes chromosomal instability in mouse embryonic fibroblasts.

Previous studies revealed that SOD2 confers protection against free radical-mediated radiation damage through alterations in gene expression (Fig. 7). SOD2 can modulate the gene expression in mammalian cells.[19,30,38] Guo *et al.*[19] and Li *et al.*[38] found that SOD2 regulated the expression profile of several groups of genes related to the stress response, DNA repair and apoptosis in human breast cancer cells. The up-regulated genes included MET, GADD153, CD9, α-catenin and plakoglobin. GADD153 is involved in the cell DNA damage response.[19,38]

The signaling pathways underlying the phenotypic changes in SOD2-overexpressing cells are unknown, although alterations in the activity of several redox-sensitive transcription factors, including AP-1 and NFκB, have been observed.[19,30,38] It is important to determine the downstream signaling molecules involved in the SOD2-induced cell radiation-resistance phenotype. Exposure of mammalian cells to ionizing radiation results in the induction of a number of proteins, including SOD2 and several proteins involved in signaling pathways.[17–20] Under the present experimental conditions, the amount of SOD2 increased 2.3-fold 12 hours after 5.5 Gy-irradiation in HeLa S3 cells. Previously, Akashi *et al.*[18] reported that irradiation markedly increased SOD2 activity and SOD2 mRNA levels. The increase in SOD2 mRNA observed after irradiation occurs through both transcriptional and post-transcriptional mechanisms. Ionizing radiation can trigger the production of cytokines, including tumor necrosis factor and lymphokines.[15,31] The increased cytokine levels may in turn induce the synthesis of proteins that protect against subsequent killing by oxidative stress. Indeed, pretreatment of animals with tumor necrosis factor or lymphokines can protect them against lethal doses of radiation.[15]

Overexpression of SOD2 may mimic the radiation responses in mammalian cells, including the modulation of the expression of SOD2 and certain other genes. To identify the relationship between radiation-induced genes and SOD2-regulated genes, we compared transcripts from γ-irradiated HeLa S3 with those from SOD2-overproducing HeLa S3. Increases in the expression of some genes, including those encoding GADD45, Ataxin 1, Mitogen-activated protein kinase kinase kinase, Zinc finger protein 236 and p21, were detected in common among the genes upregulated in both such kinds of HeLa S3 cells. Pathway analysis revealed that genes related to the cell cycle and stress response were upregulated or constitutively overexpressed in γ-irradiated HeLa S3/SOD2 cells (Table 1). However, further studies will be needed to determine the contributions of each SOD2-regulated gene to SOD2-induced protection against cellular damage by radiation. Accumulating data suggest that SOD2 is involved in regulating cell proliferation.[12,41,42] SOD2 expression by plasmid transfection in tumors or transformed cells leads to reduced clonogenicity.[31,43,44]

Reconstitution of SOD2 in tumor cells induces cell resistance to the cytotoxic effects of tumor necrosis factor, ionizing radiation, and hyperthermia, suggesting that SOD2 functions not only as a tumor suppressor but also as a mediator in the signaling of cell resistance to ROS-induced cytotoxicity.[43–46] Cancer treatment with antioxidant enzymes such as SOD2 is currently under development.[47–49] However, further studies are needed to elucidate the mechanism underlying the protection by mitochondrial SOD2 against radiation and against cellular damage by large amounts of ROS produced in the mitochondria.

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