Inhibition of NADPH oxidase 2 induces apoptosis in osteosarcoma: The role of reactive oxygen species in cell proliferation

KAZUMASA KITAMOTO[1*], YUJI MIURA[2*], SIVASUNDARAM KARNAN[3], AKINOBU OTA[3], HIROYUKI KONISHI[3], YOSHITAKA HOSOKAWA[3] and KEIJI SATO[1]

[1]Department of Orthopedic Surgery, Aichi Medical University School of Medicine;
[2]Department of Pathophysiology, Aichi Medical University School of Nursing;
[3]Department of Biochemistry, Aichi Medical University School of Medicine, Nagakute, Aichi 480-1195, Japan

Received July 23, 2015; Accepted July 12, 2016

DOI: 10.3892/ol.2018.8291

Abstract. Osteosarcomas (OS) are aggressive tumors that are characterized by dysregulated growth and resistance to apoptosis. Reactive oxygen species (ROS) are thought to be important signal transduction molecules in the regulation of cell growth. ROS-generating nicotinamide adenine dinucleotide phosphate oxidase (*NOX*) family enzymes have previously been suggested to be involved in neoplastic proliferation. To examine whether *NOX*-mediated generation of intracellular ROS confers anti-apoptotic activity, and thus a growth advantage, the current study first analyzed the mRNA expression of *NOX* family members by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in five human OS cell lines. RT-PCR analysis revealed that *NOX2* and *NOX4* mRNAs were expressed in all the OS cell lines examined, whereas little or no *NOX1* and *NOX3* mRNAs were detected. By RT-qPCR, *NOX2* mRNA expression levels were demonstrated to be higher than *NOX4* mRNA expression levels. The viability of OS cells decreased in a dose-dependent manner with treatment of diphenylene iodonium (DPI), an inhibitor of flavoprotein-dependent oxidase. DPI treatment was observed to reduce intracellular ROS levels by ~50%, and increase the frequency of apoptosis by 30%. Notably, small interfering RNAs (siRNAs) targeting *NOX2* significantly suppressed ROS generation; ROS depletion by DPI or *NOX2* siRNAs induced apoptosis in OS cells. Together, the results of the present study indicate that *NOX2*-mediated ROS generation promotes cell survival and ROS depletion leads to apoptosis, thus highlighting the *NOX2*-ROS signaling pathway as a potential therapeutic target for OS treatment.

Correspondence to: Professor Yoshitaka Hosokawa, Department of Biochemistry, Aichi Medical University School of Medicine, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan
E-mail: hosokawa@aichi-med-u.ac.jp

*Contributed equally

Abbreviations: DCF, dichlorofluorescin; Duox, dual oxidase; DCFH-DA, 2', 7'-dichlorodihydrofluorescein diacetate; DPI, diphenylene iodonium; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; OS, osteosarcoma; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; ROS, reactive oxygen species; RT-PCR, reverse transcription quantitative-polymerase chain reaction

Key words: osteosarcoma, NADPH oxidase, reactive oxygen species, RNA interference, apoptosis

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and young adults, affecting three to five people per million annually (1,2). OS typically presents in the bones around the knee (60% of cases), accounting for ~5% of the pediatric malignancies (1,2). The second most common site for OS to present is the pelvis; other initial presentation sites have also been reported, including the end of the humerus, skull and clavicle (3-5). OS usually occurs in teenagers (60%): The majority of OS cases are diagnosed before 20 years of age, a total of 75% (6,7).

The treatment outcome of OS has improved markedly over the course of several years. The current standard treatment for OS is a combination of surgery and chemotherapy: Preoperative (neoadjuvant) chemotherapy, limb sparing surgery and postoperative (adjuvant) chemotherapy. In the late 1970s, the 5-year survival rate was 10-20% due to apparent lung metastases post-surgery; however, 5-year survival has improved to the current rate of 50-80% (8-12). In addition to improvements in surgical and diagnostic techniques, the introduction of intensive chemotherapy has reduced the rate of lung metastases. The majority of patients today receive the same drugs as 25 years ago, including doxorubicin, cisplatin, high-dose methotrexate and ifosfamide in varying combinations (13,14). In order to further improve the survival rate, the development of novel anticancer agents is also necessary. Genetic studies have been performed to identify novel therapeutic targets for OS; however, the underlying molecular mechanisms have not yet been completely established (15-17). Candidate gene studies and a recent genome-wide association study have identified several common single nucleotide polymorphisms associated with OS (18,19).

The recently-discovered epithelial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (*NOX*) family of enzymes mediate critical physiological and pathological processes, including cell signaling, inflammation and mitogenesis through the generation of reactive oxygen species (ROS) (20). The role of the ROS produced by these enzymes in specific tissue types and distinct cellular compartments has been the subject of several recent investigations (21,22). Cancer cells, like non-malignant tissues, produce ROS; in tumors, reactive oxygen metabolites may act as signaling molecules to promote cell survival over apoptosis (23,24). *NOX* enzymes and the mitochondria are a major source of cellular ROS (25). There are currently seven identified enzymes in the NADPH family, including five *NOX* enxymes (*NOX1-5*) (26,27). *NOX* enzymes have a fundamental role in numerous cell functions, including signal transduction, differentiation, proliferation and cell death (25,26). However, current understanding of the roles of the *NOX* family members in the development and growth of human cancers remains limited (27-31).

In the present study, it was hypothesized that *NOX*-mediated ROS generation conferred anti-apoptotic activity, and, thus, a growth advantage to OS cells. It was demonstrated that treatment with a flavoenzyme inhibitor, diphenylene iodonium (DPI), and the knockdown of *NOX2* significantly suppressed ROS generation in OS cells, which induced apoptosis, indicating that *NOX2*-mediated ROS may transmit cell survival signals and provide a potential clinical approach for OS treatment.

Materials and methods

Cell culture and materials. Five OS cell lines (HOS, MOS, MG-63, NOS-1 and HuO 9N2) were used in this study. The MOS and NOS-1 cell lines were kindly provided by Dr Masahiko Kanamori (School of Medicine, University of Toyama, Toyama, Japan) (32-36). Three cell lines (HOS, MG-63, and HuO 9N2) were obtained from the Japanese Collection of Research Bioresources cell bank (Ibaraki, Osaka, Japan). Cells were maintained at 37˚C containing 5% CO_2 atmospheric air in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine, 200 U/ml penicillin and 100 μg/ml streptomycin. DPI was purchased from Calbiochem (EMD Millipore, Billerica, MA, USA).

Ethical approval for the present study was obtained from the ethical committee of Aichi Medical University (approval no. 11-039), and informed consent was obtained prior to the start of the study. Heparinized peripheral blood was collected from healthy individuals (n=3). Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Hypaque density centrifugation at 400 x g for 30 min at room temperature.

Based on human *NOX2* and *NOX4* cDNA sequences, small interfering RNAs (siRNAs) were designed as follows (Integrated DNA Technologies, Coralville, IA, USA): 5'-UCAGGGUUCUUUAUUCUCUTT-3' and 5'-AGAGAAUAAAGAACCCUGATT-3' for *NOX2* siRNA-1; 5'-GUACAAUUCGUUCAGCUCCTT-3' and 5'-GGAGCUGAACGAAUUGUACTT-3' for *NOX2* siRNA-2; 5'-GCUGAAGUAUCAAACUAAUUUAGAT-3' and 5'-UCUAAAUUAGUUUGAUACUUCAGCAG-3' for *NOX4* siRNA-1; 5'-GAAUUACAGUGAAGACUUUGUUGAA-3' and5'-UUCAACAAAGUCUUCACUGUAAUUCAC-3' for *NOX4* siRNA-2. Universal scrambled siRNA sequences (Invitrogen; Thermo-Fisher Scientific, Inc., Waltham, MA, USA), which have no significant homology to mouse, rat or human genome databases, were used as controls.

Quantification of NOX2 and NOX4 mRNAs by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was purified with a TRIzol reagent (Thermo-Fisher Scientific, Inc.). The HOS, MOS, MG-63, NOS-1 and HuO 9N2 OS cell lines ($1x10^5$ density) were seeded into 24-well plates and cultured in the presence or absence of *NOX2* and/or siRNAs for 48 h. Cell lysates were prepared in 1 ml TRIzol reagent with adequate mixing. Chloroform (200 μl) was added and the solution was mixed well and centrifuged for 15 min at 12,000 x g at 4˚C. The chloroform and centrifugation steps were repeated. Then, RNA pellet was precipitated with 2-propanol and rinsed with 70% ethanol. Purified RNA was dissolved in 20 μl distilled water. RT was conducted as follows: A total of 8 μl water, containing 1 μg total RNA, was added to 50 ng random primers (Thermo-Fisher Scientific, Inc.) and incubated at 65˚C for 5 min. The samples were chilled on ice and cDNA was prepared with SuperScript III First-Strand Synthesis Supermix (Invitrogen; Thermo-Fisher Scientific, Inc.) according to the manufacturer's instructions. The PCR products created with the NOXs 2 and 4 primers were identified by direct DNA sequence analysis.

RT-qPCR was performed with SYBR Premix Ex Taq II (Takara Bio, Inc., Otsu, Shiga, Japan) in an ABI PRISM 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Briefly, a solution of SYBR Premix Ex Taq II (10 μl) containing sense and antisense primers (10 μM each) was prepared and aliquoted into individual wells of a MicroAmp Optical Plate (ABI-PE; Applied Biosystems; Thermo-Fisher Scientific, Inc.): 2 μl cDNA was added to give a final volume of 20 μl. The cycling conditions for the PCR were 42˚C for 5 min, 95˚C for 10 sec and 40 cycles of 95˚C for 5 sec (denaturation) and 60˚C for 34 sec (annealing/extension). The data were analyzed with Sequence Detector software (version 1.6; ABI-PE; Applied Biosystems; Thermo-Fisher Scientific, Inc.). The quantitative cycle (Cq) during the exponential phase of amplification was determined by real-time monitoring of fluorescent emission by the nuclease activity of *Taq* polymerase. β-*actin* was used as an internal control gene for mRNA expression. Relative transcripts were determined by the formula: $1/2^{(Cq\ target\ -\ Cq\ control)}$ (36). *NOX1, NOX2, NOX3, NOX4, NOX5* and β-*actin* genes were amplified with specific primer sequences (Star Oligo, Rikaken, Nagoya, Japan) according to the NCBI reference sequences (http://www.ensembl.org/Homo_sapiens/index.html). The PCR primer pairs and probes used, were as follows: *NOX1*, 5'-AGCGTCTGCTCTCTGCTTGAA-3' and 5'-GGCTGCAAAATGAGCAGGT-3' (junction between exons 3 and 4); *NOX2*, 5'-TGCCTTTGAGTGGTTTGCAGAT-3' and 5'-ATTGGCCTGAGACTCATCCCA-3' (junction between exons 11 and 12); *NOX3*, 5'-GAACCCTCGGCTTGGAAAT-3' (junction between exons 7 and 8) and 5'-TGGCTTACCACCTTGGTAATGA-3' (junction between exons 8 and 9); *NOX4*, 5'-CCCTCACAATGTGTCCAACTGA-3' (junction between exons 11 and 12)

and 5'-GGCAGAATTTCGGAGTCTTGAC-3'; *NOX5*, 5'-AAGAGTCAAAGGTCGTCCAAGG-3' and 5'-GCTTTCTTTTCTGGTGCCTGT-3' (junction between exons 13 and 14); β-actin, 5'-GATGACCCAGATCATGTTTGAGACC-3' (junction between exons 2 and 3) and 5'-CGGTGAGGATCTTCATGAGGTAGT-3'. Semi-quantitative RT-PCR was performed with 30 cycles (94˚C for 1 min, 55˚C for 1 min, 72˚C for 1 min).

Cell transfection. The cells were transfected with *NOX2*- and/or *NOX4*-specific siRNAs, or scramble RNAs, using Lipofectamine® 2000 (Invitrogen; Thermo-Fisher Scientific, Inc.) according to the manufacturer's instructions.

Assessment of intracellular ROS production. HOS, MOS, MG-63, NOS-1 and HuO 9N2 OS cells ($2x10^5$) were seeded in 6-well plates and treated with 10 μM DPI for 48 h. Following this, the cells were transfected with *NOX2*- and *NOX4*-specific siRNAs and cultured for 48 h. The cells were then incubated with 2.5 μM dihydroethidium (DCFH-DA; Molecular Probes, Thermo-Fisher Scientific, Inc.) for 30 min at 37˚C in the dark. Subsequently, the cells were washed with Hank's buffer and fixed in 1% paraformaldehyde. Fluorescence-activated cell sorting (FACS) was used to measure the fluorescence emission intensities at 488 nm for excitation and at 580 nm for detection. The histograms were analyzed with the BD FACStation System Data Management system (BD Biosciences; Franklin Lakes, NJ, USA). Background fluorescence from a blank sample was subtracted from each reading to normalize the results.

In vitro 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. An MTT assay was used to evaluate cell viability after 48 h incubation at 37˚C. OS cells were incubated (in triplicate) in 96-well culture plates at 37˚C in humidified air with 5% CO_2. Three wells that contained OS cells in drug-free DMEM were included to determine the control cell survival rate; another three wells contained only DMEM to calibrate the spectrophotometer. After two days, 10 μl (5 mg/ml) MTT salt (Sigma-Aldrich: St Louis, MO, USA) was added to each well in 96-well culture plates for 6 h. The MTT compound was reduced to colored formazan crystals by the living cells alone. The crystals were dissolved with 100 μl of acidified isopropanol and the formazan crystal production was quantified using a spectrophotometer (562 nm). The optical density (OD) is linearly related to the cell number. Cell survival (CS) was calculated for each drug concentration using the following equation: $CS=(OD_{treated\ well}/OD_{control\ well\ mean})$ x100%.

Measuring apoptosis using flow cytometry. The externalization of phosphatidylserine was measured by flow cytometry with fluorescein isothiocyanate-conjugated Annexin V (BD Pharmingen, San Diego, CA, USA) (37). Flow cytometry analyses were performed using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest Pro Version 4.0.2 (BD Biosciences) software. Cells ($2x10^5$) seeded in 6-well plates were cultured for 48 h following transfection of NOX siRNAs or scramble RNAs, washed and resuspended in 100 μl Annexin-binding buffer [10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 140 mM NaCl and 2.5 mM CaCl2 in PBS], stained with 5 μl Annexin V-Alexa Fluor 488 conjugate for 20 min then analyzed by flow cytometry (FACSCalibur). The cells that stained positive with Annexin V were counted as apoptotic populations.

Statistical analysis. The results of MTT assay, apoptosis assay with siRNA transfection, and RT-qPCR assay were analyzed using one-way analysis of variance with a Turkey Kramer post hoc test using the Statview software (version 5; SAS Institute, Inc., Cary, NC, USA). Results are expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of cell growth and ROS generation by the flavoenzyme inhibitor DPI. Flavoprotein-dependent ROS play a critical role in cytokine-mediated signal transduction in normal tissues and tumor cells. DPI, a flavoenzyme inhibitor, inhibits the membrane-bound, flavoprotein-containing *NOX* enzymes (25,30). The present study examined whether DPI affected cell viability in OS cell lines using an MTT assay. A total of five OS cell lines were treated with various DPI concentrations for 24 h and the representative results for HOS and MOS cells are shown in Fig. 1. As hypothesized, DPI treatment decreased the viability of HOS and MOS cells in a dose-dependent manner ($P<0.0001$ for both cell lines); the IC_{50} values of HOS and MOS cells were 0.5 and 0.8 μM, respectively. The IC_{50} values for the other three cell lines were as follows: 0.9 μM (MG-63), 1.2 μM (NOS-1) and 0.7 μM (HuO 9N2), respectively.

To determine whether DPI affects ROS generation, intracellular ROS levels were evaluated by using flow cytometry. It was observed that untreated HOS cells generated ROS, and DPI treatment eliminated ROS generation in HOS cells (Fig. 2). Therefore, dichlorofluorescein (DCF) fluorescence intensity was reduced from $2.8x10^3$ in untreated cells, to $0.9x10^3$ in DPI-treated cells.

Induction of apoptosis by DPI. The present study examined the effect of DPI on apoptosis in OS cells using Annexin V, and observed that DPI treatment markedly increased apoptosis in HOS (54%), HuO 9N2 (43%) and MG63 (28%) cells (Fig. 3). The results suggest that the depletion of ROS, generated by the *NOX*-like enzymes, triggered apoptosis in OS cells.

Expression of NOX1-5 mRNAs in human OS cell lines. *NOX* family members produce ROS that are pivotal for cell proliferation. To examine the role of the *NOX* family in the proliferation of OS cells, mRNA expression of the *NOX* family members was measured in 5 human OS cell lines by semi-quantitative RT-PCR. *NOX2* mRNA was highly expressed in all of the examined OS cell lines, whereas little or no *NOX1, NOX3* and *NOX5* mRNA was detected (Fig. 4). The OS cell lines also expressed low-moderate levels of *NOX4* mRNA (Fig. 4). To provide a comparison, high levels of *NOX2* mRNA were also detected in human PBMCs (Fig. 4).

NOX2 and NOX4 expression in human OS cell lines. The present study measured the expression levels of *NOX* family mRNAs relative to β-actin by RT-qPCR (Fig. 5). The

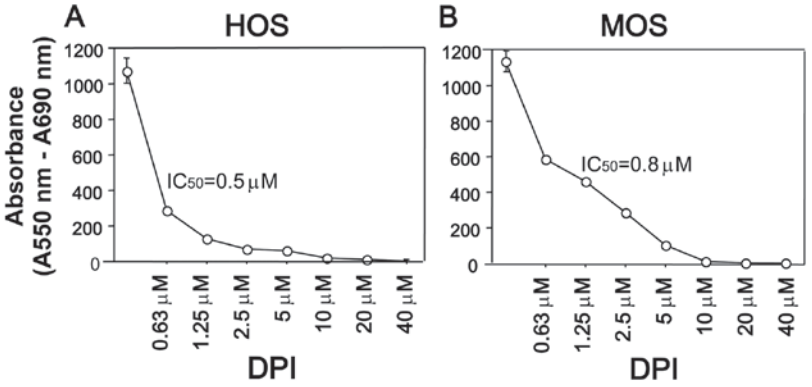

Figure 1. Effect of DPI on the viability of OS cells. DPI treatment reduced the cell viability of OS cells. (A) HOS cells ($1x10^5$ cells) and (B) MOS cells ($1x10^5$ cells) were treated with DPI at the indicated concentrations for 24 h. *In vitro* cell viability was evaluated with an MTT assay. The IC_{50} values (the drug concentrations that induced a 50% inhibition of cell proliferation) were extrapolated from the respective dose-response curves. DPI, diphenylene iodonium; MTT, 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; OS, osteosarcoma.

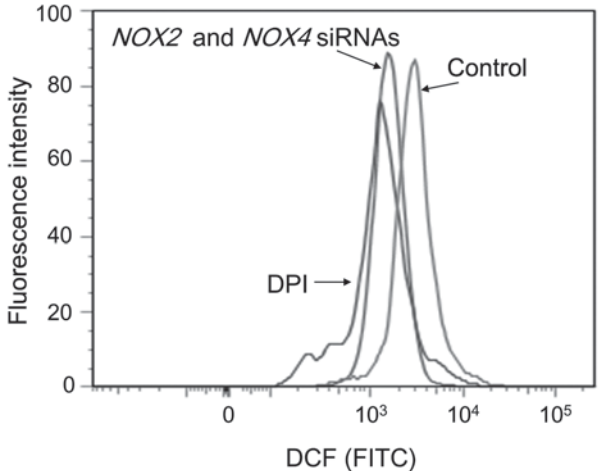

Figure 2. Inhibition of ROS production by DPI or *NOX2* and *NOX4* specific siRNAs. DPI and NOX2 and NOX4-specific siRNA treatment inhibited ROS production. HOS cells ($1x10^5$) were cultured in a 24-well plate for 48 h and subsequently treated with, or without (control), DPI (10 μM) for 24 h. HOS cells were also seeded in 6-well plates ($2x10^5$), cultured overnight and transfected with *NOX2* and *NOX4* specific siRNAs for 48 h. Following cell harvest, the cells were labeled with DCFH-DA and intracellular ROS levels were measured by flow cytometry. DCF fluorescence intensities were normalized to untreated HOS cells. A typical fluorescence profile is exhibited. The x-axis indicates fluorescence intensity with an excitation source of 488 nm and emission wavelength of 580 nm. DPI, diphenylene iodonium; OS, osteosarcoma; ROS, reactive oxygen species; NOX, NADPH oxidase; DCF, dichlorofluorescein; DCFH-DA, 2', 7'-dichlorodihydrofluorescein diacetate; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate.

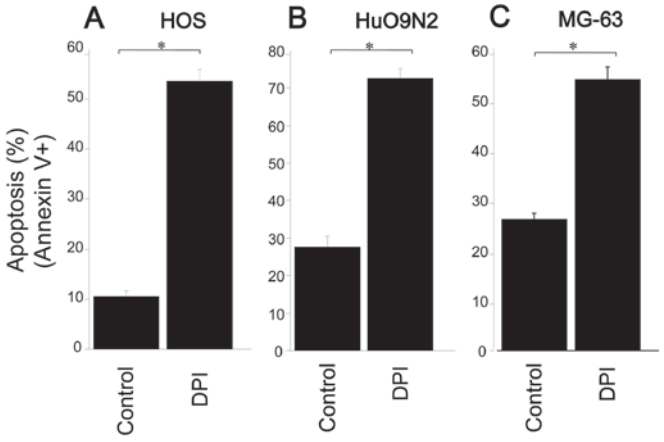

Figure 3. DPI induces apoptosis in OS cells. (A) HOS, (B) HuO 9N2 and (C) MG63 cells. OS cells ($1x10^5$) were cultured in 24-well plates with DPI (10 μM) for 24 h. Following cell harvest, the apoptotic cells were stained with Annexin V and quantitated by flow cytometry. The results are presented as the mean ± standard deviation (n=3). *P<0.05. DPI, diphenylene iodonium.

expression was graded as low (*NOX*/β-actin ratio $<2x10^{-5}$), moderate (ratio $>2x10^{-5}$ and $<50x10^{-5}$) or high (ratio $>50x10^{-5}$). Relative transcripts were determined by the formula: $1/2^{(Cqtarget - Cqcontrol)}$ (36). High-level *NOX2* mRNA expression was observed in all of the examined OS cell lines, with the highest expression detected in MOS cells. Moderate-level *NOX4* mRNA expression was detected in all of the examined OS cell lines. Therefore, *NOX2* mRNA expression was higher than *NOX4* mRNA expression in the included OS cell lines. For comparison, high-level expression of *NOX2* and low-level expression of *NOX4* was detected in human peripheral blood mononuclear cells (Fig. 5).

NOX2 and NOX4 mediate ROS production in OS cells. The present study utilized RNA interference to determine whether *NOX2/4* mediates ROS generation in OS cells. Primarily, the effects of *NOX2* or *NOX4*-specific siRNAs on the endogenous expression of their mRNAs in HOS cells were evaluated (Fig. 6), revealing that their siRNAs functioned effectively (Fig. 6). In order to elucidate how *NOX2* and *NOX4* mRNA expression affects ROS generation, siRNAs targeting *NOX2* and *NOX4* were transiently transfected into OS cells and intracellular ROS levels were evaluated using flow cytometry. Double transfection of *NOX2* and *NOX4* siRNAs reduced DCF fluorescence intensity to $1.1x10^3$ from the untreated control intensity of $2.8x10^3$ (Fig. 2). Thus, the double transfection of OS cells with *NOX2* and *NOX4* siRNAs suppressed intracellular ROS levels (39%) compared with the controls (Fig. 2). The results indicate that *NOX2* and/or *NOX4,* at least in part, are responsible for intracellular ROS generation in OS cells. However, ROS generation was not completely inhibited by *NOX2* and *NOX4* knockdown, suggesting that other *NOX* proteins and mitochondrial components may also contribute to ROS generation.

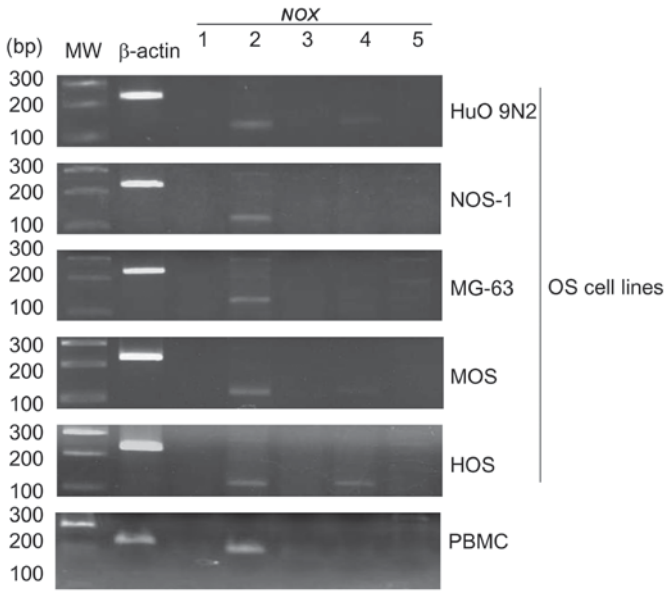

Figure 4. *NOX2* and *4* mRNA expression levels are detected by RT-PCR in human OS cell lines. Total RNAs were extracted from five OS cell lines and the mRNA expression of *NOX* family proteins was analyzed by RT-PCR alongside *β-actin* expression as a normalizing control. OS, osteosarcoma; NOX, NADPH oxidase; PBMCs, peripheral blood mononuclear cells; MW, molecular weight marker; RT-PCR, reverse transcription quantitative-polymerase chain reaction.

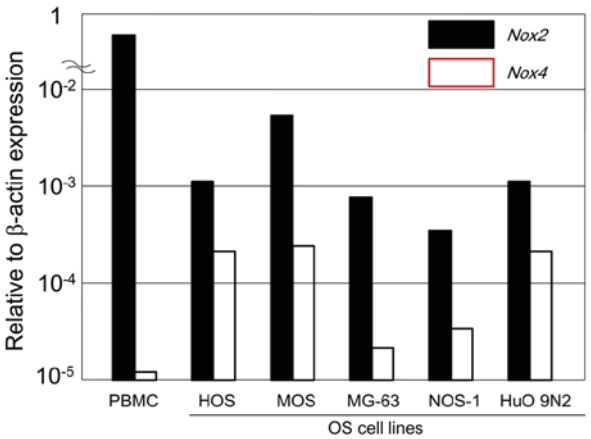

Figure 5. Quantitative analysis of *NOX2* and *NOX4* mRNA expression in human OS cell lines. Quantitative analysis of the mRNA expression of *NOX* family proteins in five human OS cell lines was performed by RT-qPCR using a SYBR Green reagent. The Cq value during the exponential phase of amplification was determined by the real-time monitoring of the fluorescent emission of Taq polymerase nuclease activity. β-*actin* was used as an internal control for normalization. qPCR efficiencies of the target (*NOX2 and NOX4*) and control (β-actin) proteins were approximately equal over a concentration range of 0.1-200 ng total cDNA. The relative transcripts were determined by the formula: $1/2^{(Cq\ target-Cq\ control)}$ (36). The bars represent the mean (n=2). OS, osteosarcoma; NOX, NADPH oxidase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Cq, quantification cycle; PBMCs, peripheral blood mononuclear cells.

NOX2 and NOX4 siRNAs reduce cell viability and induce apoptosis. To explore whether *NOX2* and *NOX4*-mediated ROS affect cell survival, the effect of their knockdown on cell viability and apoptosis was examined. *NOX2* and *NOX4* knockdown significantly reduced HOS cell viability, by 74 and 65%, respectively, relative to that of untreated cells (P<0.0001 for

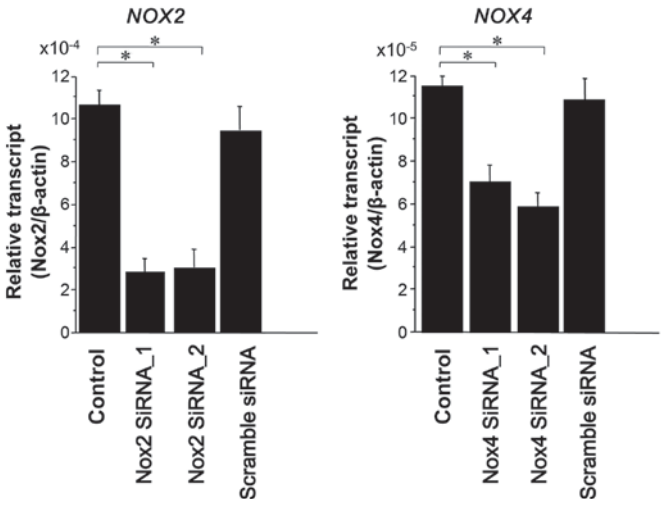

Figure 6. Downregulation of endogenous *NOX2* and *NOX4* mRNA expression by *NOX2* and/or *NOX4* siRNAs. The expression levels of endogenous *NOX2* and *NOX4* mRNAs, relative to β-actin mRNA (control), were evaluated by qPCR 48 h following transfection with NOX2 and/or NOX4 siRNAs, or scrambled siRNAs, into HOS cells. *P<0.05; n=3. OS, osteosarcoma; NOX, NADPH oxidase; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA.

NOX2 knockdown; P=0.0033 for *NOX4* knockdown; Fig. 7A). *NOX2* knockdown in HuO 9N2 cells reduced viability by 61% (P=0.0003; Fig. 7B), while *NOX4* knockdown in the same cell line reduced viability by 11%, which was not statistically significant. Collectively, these results suggest that among *NOX* family members, N*OX2* has a major role in survival of HOS and HuO 9N2 cells.

To verify whether this reduced cell viability was associated with apoptosis, an Annexin V assay was performed and *NOX2* knockdown was observed to markedly induce apoptosis in HOS and HuO 9N2 cells (P=0.0003 for HOS cells; P<0.0001 for HuO 9N2 cells; Fig. 8A and B). Thus, the reduction in cell viability observed with siRNA knockdown was associated with the induction of apoptosis. Therefore, *NOX2* and *NOX4* siRNAs suppressed ROS generation, and the depletion of ROS by *NOX2* knockdown, and DPI treatment induced apoptosis in HOS and HuO 9N2 cells.

Discussion

Cancer cells produce ROS that may act as signaling molecules to promote cell survival and cell growth (23,24,38). It is possible that chronic inflammation may accelerate the development and progression of malignant OS, due to cytokine release and ROS generation.

The present study examined the role of ROS in the viability and apoptosis of OS cell lines. Although ROS are considered to cause stress-induced apoptosis, ROS often confer a survival advantage on cancer cells. The results of the current study demonstrated that suppressing ROS levels by DPI treatment reduced the viability of OS cells. Similar results have been observed in other types of cancer cells, including pancreatic tumor cells (39). The current study also investigated whether the *NOX*2 and 4-mediated ROS generation conferred anti-apoptotic activity and, thus, a growth advantage to OS cells. As such, the expression levels of *NOX* genes in

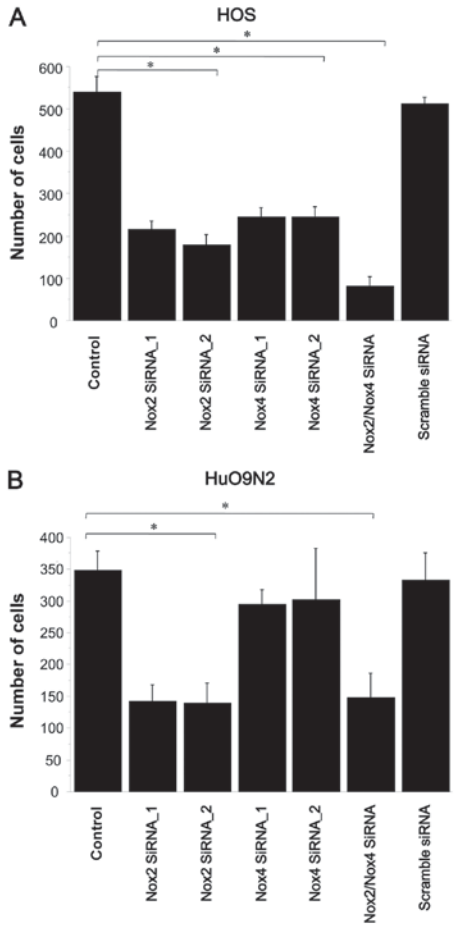

Figure 7. Inhibition of viability of OS cells by *NOX2* and/or *NOX4* siRNAs. (A) HOS cells and (B) HuO 9N2 cells. OS cells ($5x10^6$) were transfected with *NOX2* and/or *NOX4* siRNAs, or scrambled siRNAs. The viability of control and siRNA-treated cells was evaluated by an MTT assay. *NOX2* and/or *NOX4* siRNAs significantly inhibited cell viability of OS cells. The error bars represent the standard deviation (n=3). *P<0.05. OS, osteosarcoma; NOX, NADPH oxidase; siRNA, small interfering RNA; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SD, standard deviation.

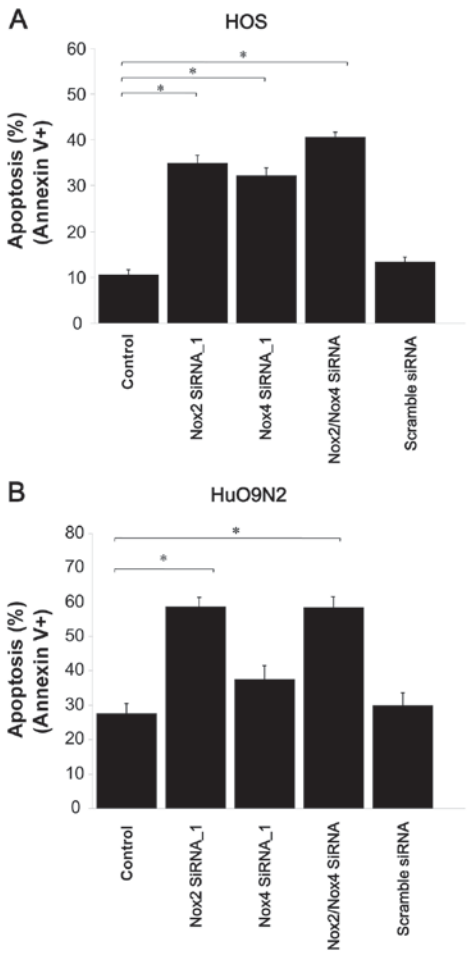

Figure 8. Induction of apoptosis by *NOX2* and/or *NOX4* knockdown. *NOX2* and/or *NOX4* siRNA significantly induced the apoptosis of OS cells. (A) HOS cells and (B) HuO9N2 cells. OS cells ($2x10^5$) were cultured for 48 h following transfection with *NOX2* and/or *NOX4* siRNAs, or scramble siRNAs. Apoptotic cells were quantified with Annexin V staining. The error bars represent the standard deviation (n=3). *P<0.05. OS, osteosarcoma; NOX, NADPH oxidase; siRNA, small interfering RNA; SD, standard deviation.

five human OS cell lines were examined. RT-PCR analysis revealed that *NOX*2 and *NOX*4 mRNAs were expressed in the OS cell lines; however little or no *NOX*2, *NOX*3 and *NOX*5 mRNAs were detected. RT-qPCR revealed that *NOX*2 and *NOX*4 mRNAs were expressed in OS cells at high and moderate levels, respectively. In all the examined OS cells, *NOX*2 mRNA exhibited the highest expression levels. *NOX*2 siRNAs significantly reduced intracellular ROS generation and OS cell viability. Concordantly, ROS depletion by DPI treatment or *NOX*2 knockdown induced apoptosis. The results of the present study suggested that NOX2-mediated ROS generation promotes the production of cell survival signals and that ROS depletion induces apoptosis in OS cells.

*NOX*4 mRNA overexpression has previously been reported in primary breast, ovarian, prostate, melanoma and glioblastoma cancer cell lines (40-42). *NOX*4 expression was moderate-high in two of the four tested ovarian cancer cell lines. Notably, high-level acquired resistance to cisplatin was associated with a marked decrease in the *NOX*4 mRNA levels in A2780/DDP cells (30). Previously, NOX4 was demonstrated to be an oncoprotein localized in mitochondria (43). In the current study, *NOX*4 knockdown induced apoptosis in a subset of OS cells. Considering that *NOX*2 knockdown significantly reduced cell viability and induced apoptosis in all the examined OS cell lines, it is possible that *NOX*2-mediated ROS generation has a major role in promoting the survival of OS cells. The present study raises the possibility that NOX2 may act as an oncoprotein in the pathogenesis of OS, similar to NOX4 in other types of cancer. Therefore, NOX2 and NOX2-associated signaling molecules may be good candidates for the targeted therapy of OS. NOX2 has been previously reported to be involved in a variety of physiological and pathological conditions, including prion disease (44-47). NOX2 has also been implicated in cancer biology (48-50) as NOX2 was established to serve a pro-survival role in human leukemia and be involved to promote apoptosis in human glioma (48,50). Considering the possible role of *NOX*2 in cancer development, it may be of interest to investigate the correlation between *NOX*2 expression and OS prognosis in further studies.

In conclusion, the present study demonstrated that *NOX*2-mediated ROS generation promotes the survival of OS cells and that ROS depletion through *NOX*2 knockdown and DPI treatment leads to apoptosis. The current study raises the possibility of the *NOX*2-ROS signaling pathway being used

as a novel therapeutic target for OS. For example, antioxidant treatments targeted to this signaling pathway have the potential to enhance the therapeutic index of cisplatin-based therapies. Further studies are required to contribute to the development of targeted therapies for OS.

Acknowledgements

The authors would like to thank Dr Masahiko Kanamori and Dr Taketoshi Yasuda (Department of Orthopedic Surgery, School of Medicine, University of Toyama) for providing the OS cell lines.

Funding

The present study was supported by the Aichi Cancer Center to Yuji Miura and a grant of Strategic Research Foundation Grant-Aided Project for Private Universities from the Ministry of Education, Culture, Sports, Science and Technology, Japan to Yoshitaka Hosokawa (grant no. AI 52213).

Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

Conception and design, KK, YM, YH and KS; development of methodology, KK, YM, SK, AO and YH; acquisition of data (such as provided cells, provided facilities), KK, YM, HK, YH and SK; analysis and interpretation of data, KK, YM, AO, HK, YH and SK; writing, review, and/or revision, KK, YM, YH and SK; administrative, technical or material support (including reporting or organizing data, preparing vectors), KK, YM, SK, AO, HK, YH and KS; study supervision, AO, HK, YH and SK.

Ethics approval and consent to participate

Ethical approval for the present study was obtained from the ethical committee of Aichi Medical University (approval no. 11-039), and informed consent was obtained prior to the start of the present study.

Consent for publication

Informed consent for publication was obtained prior to the start of the present study.

Competing interests

The authors have no competing interests to declare.

References

1. Raymond AK, Ayala AG and Knuutila K: Conventional osteosarcoma: In Fletcher CDM, Unni KK and Mertens F (eds). World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of Soft Tissue and Bone. Lyon, IARC Press, pp264-270, 2002.
2. Arndt CA and Crist WM: Common musculoskeletal tumors of childhood and adolescence. N Engl J Med 341: 342-352, 1999.
3. Mirabello L, Troisi RJ and Savage SA: International osteosarcoma incidence patterns in children and adolescents, middle ages and elderly persons. Int J Cancer 125: 229-234, 2009.
4. Mirabello L, Troisi RJ and Savage SA: Osteosarcoma incidence and survival rates from 1973 to 2004: Data from the surveillance, epidemiology, and end results program. Cancer 115: 1531-1543, 2009.
5. Anninga JK, Gelderblom H, Fiocco M, Kroep JR, Taminiau AH, Hogendoorn PC and Egeler RM: Chemotherapeutic adjuvant treatment for osteosarcoma: Where do we stand? Eur J Cancer 47: 2431-2445, 2011.
6. Uribe-Botero G, Russell WO, Sutow WW and Martin RG: Primary osteosarcoma of bone. Clinicopathologic investigation of 243 cases, with necropsy studies in 54. Am J Clin Pathol 67: 427-435, 1977.
7. Bielack SS, Kempf-Bielack B, Delling G, Exner GU, Flege S, Helmke K, Kotz R, Salzer-Kuntschik M, Werner M, Winkelmann W, *et al*: Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20: 776-790, 2002.
8. Link MP, Goorin AM, Miser AW, Green AA, Pratt CB, Belasco JB, Pritchard J, Malpas JS, Baker AR, Kirkpatrick JA, *et al*: The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. N Engl J Med 314: 1600-1606, 1986.
9. Anderson P: Chemotherapy for osteosarcoma with high-dose methotrexate is effective and outpatient therapy is now possible. Nat Clin Pract Oncol 4: 624-625, 2007.
10. Ritter J and Bielack SS: Osteosarcoma. Ann Oncol 21 (Suppl 7): vii320-vii325, 2010.
11. Ellegast J, Barth TF, Schulte M, Bielack SS, Schmid M and Mayer-Steinacker R: Metastasis of osteosarcoma after 16 years. J Clin Oncol 29: e62-e66, 2011.
12. Andreou D, Bielack SS, Carrle D, Kevric M, Kotz R, Winkelmann W, Jundt G, Werner M, Fehlberg S, Kager L, *et al*: The influence of tumor- and treatment-related factors on the development of local recurrence in osteosarcoma after adequate surgery. An analysis of 1355 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. Ann Oncol 22: 1228-1235, 2011.
13. Bielack SS: Osteosarcoma: Time to move on? Eur J Cancer 46: 1942-1945, 2010.
14. Federman N, Bernthal N, Eilber FC and Tap WD: The multidisciplinary management of osteosarcoma. Curr Treat Options Oncol 10: 82-93, 2009.
15. Ito M, Barys L, O'Reilly T, Young S, Gorbatcheva B, Monahan J, Zumstein-Mecker S, Choong PF, Dickinson I, Crowe P, *et al*: Comprehensive mapping of p53 pathway alterations reveals an apparent role for both SNP309 and MDM2 amplification in sarcomagenesis. Clin Cancer Res 17: 416-426, 2011.
16. Savage SA, Stewart BJ, Liao JS, Helman LJ and Chanock SJ: Telomere stability genes are not mutated in osteosarcoma cell lines. Cancer Genet Cytogenet 160: 79-81, 2005.
17. Kansara M and Thomas DM: Molecular pathogenesis of osteosarcoma. DNA Cell Biol 26: 1-18, 2007.
18. Savage SA, Mirabello L, Wang Z, Gastier-Foster JM, Gorlick R, Khanna C, Flanagan AM, Tirabosco R, Andrulis IL, Wunder JS, *et al*: Genome-wide association study identifies two susceptibility loci for osteosarcoma. Nat Genet 45: 799-803, 2013.
19. Mirabello L, Koster R, Moriarity BS, Spector LG, Meltzer PS, Gary J, Machiela MJ, Pankratz N, Panagiotou OA, Largaespada D, *et al*: A genome-wide scan identifies variants in NFIB associated with metastasis in patients with osteosarcoma. Cancer Discov 5: 920-931, 2015.
20. Brar SS, Kennedy TP, Sturrock AB, Huecksteadt TP, Quinn MT, Whorton AR and Hoidal JR: An NAD(P)H oxidase regulates growth and transcription in melanoma cells. Am J Physiol Cell Physiol 282: C1212-C1224, 2002.
21. Valko M, Rhodes CJ, Moncol J, Izakovic M and Mazur M: Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160: 1-40, 2006.
22. Rhee SG: Cell signaling. H_2O_2, a necessary evil for cell signaling. Science 312: 1882-1883, 2006.
23. Storz P: Reactive oxygen species in tumor progression. Front Biosci 10: 1881-1896, 2005.
24. Szatrowski TP and Nathan CF: Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 51: 794-798, 1991.

25. Lambeth JD: NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 4: 181-189, 2004.
26. Bedard K and Krause KH: The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87: 245-313, 2007.
27. Cheng G, Cao Z, Xu X, Meir EG and Lambeth JD: Homologs of gp91phox: Cloning and tissue expression of Nox3, Nox4, and Nox5. Gene 269: 131-140, 2001.
28. Donkó A, Péterfi Z, Sum A, Leto T and Geiszt M: Dual oxidases. Philos Trans R Soc Lond B Biol Sci 360: 2301-2308, 2005.
29. Geiszt M, Witta J, Baffi J, Lekstrom K and Leto TL: Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. FASEB J 17: 1502-1504, 2003.
30. Juhasz A, Ge Y, Markel S, Chiu A, Matsumoto L, van Balgooy J, Roy K and Doroshow JH: Expression of NADPH oxidase homologues and accessory genes in human cancer cell lines, tumours and adjacent normal tissues. Free Radic Res 43: 523-532, 2009.
31. Wu Y, Antony S, Juhasz A, Lu J, Ge Y, Jiang G, Roy K and Doroshow JH: Up-regulation and sustained activation of Stat1 are essential for interferon-gamma (IFN-gamma)-induced dual oxidase 2 (Duox2) and dual oxidase A2 (DuoxA2) expression in human pancreatic cancer cell lines. J Biol Chem 286: 12245-12256, 2011.
32. Kanamori M, Ohmori K, Yasuda T and Yudoh K: Effects of hyperthermia and differentiation on cultured Dunn osteosarcoma cells. Cancer Detect Prev 27: 76-81, 2003.
33. Yasuda T, Kanamori M, Nogami S, Hori T, Oya T, Suzuki K and Kimura T: Establishment of a new human osteosarcoma cell line, UTOS-1: Cytogenetic characterization by array comparative genomic hybridization. J Exp Clin Cancer Res 28: 26, 2009.
34. Hori T, Kondo T, Kanamori M, Tabuchi Y, Ogawa R, Zhao QL, Ahmed K, Yasuda T, Seki S, Suzuki K and Kimura T: Ionizing radiation enhances tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through up-regulations of death receptor 4 (DR4) and death receptor 5 (DR5) in human osteosarcoma cells. J Orthop Res 28: 739-745, 2010.
35. Kanamori M, Sano A, Yasuda T, Hori T and Suzuki K: Array-based comparative genomic hybridization for genomic-wide screening of DNA copy number alterations in aggressive bone tumors. J Exp Clin Cancer Res 31: 100, 2012.
36. Miura Y, Thoburn CJ, Bright EC, Phelps ML, Shin T, Matsui EC, Matsui WH, Arai S, Fuchs EJ, Vogelsang GB, *et al*: Association of Foxp3 regulatory gene expression with graft-versus-host disease. Blood 104: 2187-2193, 2004.
37. Kumar S, Kain V and Sitasawad SL: Cardiotoxicity of calmidazolium chloride is attributed to calcium aggravation, oxidative and nitrosative stress, and apoptosis. Free Radic Biol Med 47: 699-709, 2009.
38. Diehn M, Cho RW, Lobo NA, Kalisky T, Dorie MJ, Kulp AN, Qian D, Lam JS, Ailles LE, Wong M, *et al*: Association of reactive oxygen species levels and radioresistance in cancer stem cells. Nature 458: 780-783, 2009.
39. Mochizuki T, Furuta S, Mitsushita J, Shang WH, Ito M, Yokoo Y, Yamaura M, Ishizone S, Nakayama J, Konagai A, *et al*: Inhibition of NADPH oxidase 4 activates apoptosis via the AKT/apoptosis signal-regulating kinase 1 pathway in pancreatic cancer PANC-1 cells. Oncogene 25: 3699-3707, 2006.
40. Ushio-Fukai M and Nakamura Y: Reactive oxygen species and angiogenesis: NADPH oxidase as target for cancer therapy. Cancer Lett 266: 37-52, 2008.
41. Shono T, Yokoyama N, Uesaka T, Kuroda J, Takeya R, Yamasaki T, Amano T, Mizoguchi M, Suzuki SO, Niiro H, *et al*: Enhanced expression of NADPH oxidase Nox4 in human gliomas and its roles in cell proliferation and survival. Int J Cancer 123: 787-792, 2008.
42. Yamaura M, Mitsushita J, Furuta S, Kiniwa Y, Ashida A, Goto Y, Shang WH, Kubodera M, Kato M, Takata M, *et al*: NADPH oxidase 4 contributes to transformation phenotype of melanoma cells by regulating G2-M cell cycle progression. Cancer Res 69: 2647-2654, 2009.
43. Graham KA, Kulawiec M, Owens KM, Li X, Desouki MM, Chandra D and Singh KK: NADPH oxidase 4 is an oncoprotein localized to mitochondria. Cancer Biol Ther 10: 223-231, 2010.
44. McCann SK, Dusting GJ and Roulston CL: Nox2 knockout delays infarct progression and increases vascular recovery through angiogenesis in mice following ischaemic stroke with reperfusion. PLoS One 9: e110602, 2014.
45. Li ZY, Jiang WY and Cui ZJ: An essential role of NAD(P)H oxidase 2 in UVA-induced calcium oscillations in mast cells. Photochem Photobiol Sci 14: 414-428, 2015.
46. Sorce S, Nuvolone M, Keller A, Falsig J, Varol A, Schwarz P, Bieri M, Budka H and Aguzzi A: The role of the NADPH oxidase NOX2 in prion pathogenesis. PLoS Pathog 10: e1004531, 2014.
47. Menden H, Welak S, Cossette S, Ramchandran R and Sampath V: Lipopolysaccharide (LPS)-mediated angiopoietin-2-dependent autocrine angiogenesis is regulated by NADPH oxidase 2 (Nox2) in human pulmonary microvascular endothelial cells. J Biol Chem 290: 5449-5461, 2015.
48. Maraldi T, Prata C, Vieceli Dalla Sega F, Caliceti C, Zambonin L, Fiorentini D and Hakim G: NAD(P)H oxidase isoform Nox2 plays a prosurvival role in human leukaemia cells. Free Radic Res 43: 1111-1121, 2009.
49. Jones KJ, Chetram MA, Bethea DA, Bryant LK, Odero-Marah V and Hinton CV: Cysteine (C)-X-C Receptor 4 Regulates NADPH Oxidase-2 during oxidative stress in prostate cancer cells. Cancer Microenviron, Sep 28, 2013 (Epub ahead of print).
50. Li SZ, Hu YY, Zhao J, Zhao YB, Sun JD, Yang YF, Ji CC, Liu ZB, Cao WD, Qu Y, *et al*: MicroRNA-34a induces apoptosis in the human glioma cell line, A172, through enhanced ROS production and NOX2 expression. Biochem Biophys Res Commun 444: 6-12, 2014.