NADPH Oxidase 4 Contributes to Transformation Phenotype of Melanoma Cells by Regulating G2-M Cell Cycle Progression

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Abstract

Generation of reactive oxygen species (ROS) has been implicated in carcinogenic development of melanoma, but the underlying molecular mechanism has not been fully elucidated. We studied the expression and function of the superoxide-generating NADPH oxidase (Nox)4 in human melanoma cells. Nox4 was up-regulated in 13 of 20 melanoma cell lines tested. Silencing of Nox4 expression in melanoma MM-BP cells by small interfering RNAs decreased ROS production and thereby inhibited anchorage-independent cell growth and tumorigenicity in nude mice. Consistently, a general Nox inhibitor, diphenylene iodonium, and antioxidant vitamine E and pyrrolidinedithiocarbamate blocked cell proliferation of MM-BP cells. Flow cytometric analysis indicated that Nox4 small interfering RNAs and diphenylene iodonium induced G2-M cell cycle arrest, which was also observed with another melanoma cell line, 928mel. This was accompanied by induction of the Tyr-15 phosphorylated, inactive form of cyclin-dependent kinase 1 (a hallmark of G2-M checkpoint) and hyperphosphorylation of cdc25c leading to its increased binding to 14-3-3 proteins. Ectopic expression of catalase, a scavenger of ROS, also caused accumulation of cells in G2-M phase. Immunohistochemical revealed that expression of Nox4 was detected in 31.0% of 13 melanoma patients samples, suggesting the association of Nox4 expression with melanoma development. The findings suggest that Nox4-generated ROS are required for transformation phenotype of melanoma cells and contribute to melanoma growth through regulation of G2-M cell cycle progression. [Cancer Res 2009;69(6):2647–54]

Introduction

A growing body of evidence indicates that reactive oxygen species (ROS) generated by NADPH oxidase (Nox) family enzymes [Nox1-5 and dual oxidases (Duox1 and 2)] play critical roles in various physiologic processes in Caenorhabditis elegans as well as mammals (1). These include innate immunity, cell growth, and apoptosis. For example, Nox1 mediates angiotensin II-induced proliferation of vascular smooth muscle cells (2) and platelet-derived growth factor– or epidermal growth factor–dependent mitogenesation of nonphagocytic cells (3, 4). Nox4 is integrated into the receptor systems for insulin-induced glucose transport (5) and lipopolysaccharide-stimulated inflammation (6). The NADPH oxidase consists of multiple subunits including a Nox family protein, the catalytic moiety homologous to phagocytic gp91phox, and regulatory proteins. It catalyzes reduction of molecular oxygen to produce superoxide and its metabolite, hydrogen peroxide. It has been known that ROS are spontaneously generated in malignant cancer cells, but their origin and biological meaning remained obscure (7). Recent studies revealed some aspects of the functional relationships between Nox family genes and increased ROS production in tumor cells. Nox1-generated ROS are functionally required for oncogenic Ras transformation phenotype including anchorage-independent growth and tumorgeneris (4, 8). Nox4 contributes to cell survival of pancreatic cancer cells, and this survival signaling seemed to be mediated by the imparied activities of AKT and its target ASK1 (9, 10). Nox5 has also been implicated in cell viability of prostate cancer cells (11) and Barrett esophageal adenocarcinoma cells (12).

Melanoma is one of the aggressive tumors with a high frequency of metastasis and malignancy in melanoma cells is often accompanied by elevated ROS production through multiple mechanisms (13). Overexpression of the antioxidant enzyme, superoxide dismutase, suppresses the ability of melanoma cells to form colonies in soft agar as well as tumors in nude mice (14). The dietary antioxidant, tocopherol (vitamine E), induces growth inhibition of melanoma cells (15). These observations imply that redox alterations play an important role in the carcinogenic evolution of melanoma. With the respect to involvement of Nox isoymes in these ROS productions, Nox1 has been implicated in acute ROS formation in human keratinocytes elicited by UV A radiation (16), whose role in development of cutaneous melanoma is still being debated. Recently, Nox4 expression was reported to be constitutively up-regulated and partially account for the elevated ROS production in some of melanoma cell lines (17), but the Nox4 action has not been fully elucidated. In the present study, we describe that ablation of Nox4 expression by RNA interference blocks both proliferation of melanoma cells and tumorigeneration derived from melanoma cells in athymic nude mice. This growth inhibition seems to be a consequence of inhibition of G2-M cell cycle transition, which is associated with increased phosphorylation of cyclin-dependent kinase 1 (CDK1), a key mitosis-promoting kinase and hyperphosphorylation of cdc25c. Furthermore, up-regulated expression of Nox4 was detected in some of melanoma samples isolated from patients. Our finding suggests that Nox4-generated ROS exert a mediating role in melanoma cell growth by regulating G2-M cell cycle progression and for the first time.
provides evidence of the involvement of Nox enzymes in cell cycle control.

Materials and Methods

Cell culture and materials. A variety of human melanoma cell lines were obtained from different sources (see Supplementary Table S1) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS). A human epidermal melanocyte cell line was obtained from Cascade Biologies and maintained in Medium 254 (Cascade Biologies) with HGMS (Cascade Biologies). Propidium iodide, 6-hydroxy-2, 5, 7,8-tetramethylchroman-2-carboxylic acid (a water-soluble vitamin E analogue), rabbit anti-catalesta antibodies, and monoclonal anti-p21WAF1/C2P1 antibodies were purchased from Sigma; PDTC, diphenyleneiodonium (DPI), and rabbit anti-phospho CDK1 (tyr-15) antibodies from Calbiochem; and AEC substrate chromagen from Sigma; PDTC, diphenyleneiodonium (DPI), and rabbit anti-phospho CDK1 (tyr-15) antibodies from Calbiochem; and AEC substrate chromagen from Sigma.

Transfection and siRNA constructions. Transfection was carried out by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. DNA oligonucleotides encoding siRNA with loop sequence (TCCAGAGA) were subcloned into the pEGFP-C1 vectors (Clontech) containing Green Fluorescent Protein (GFP-InNox4).

Isolation of stable transfectants. pShlen vector carrying siRNA vectors carrying siNox4-1, siNox4-2, and scrambled siRNA (1 µg) were transfected into MM-BP cells (10⁶). Transfected cells were selected in DMEM supplemented with 10% FBS and 400 µg/mL hygromycin for 2 wk. Isolated colonies were maintained in the presence of 100 µg/mL hygromycin. The presence of stably transfected vectors in the cells was confirmed by PCR using vector-specific primers. We referred to the two cell lines carrying pShlen siNox4-1 as siRNA1-a and siRNA1-b, a cell line carrying pShlen siNox4-2 as siRNA2, and a cell line carrying pSilencer scrambled siRNA as scrambled.

Reverse transcription-PCR and real-time PCR. Total RNAs (5 µg) were extracted from cells with TRIzol (Invitrogen) and reverse-transcribed by Power Script (Clonetech) with Oligo dT primers (Invitrogen). The resultant cDNA was PCR amplified for human NOX1-5 by using human sense and antisense primers:

- sense 5'-GGACGAGAATTGCGGTCAC-3' and antisense 5'-TTGCGTTCCCATTCCGCTG-3' for Nox1; sense 5'-GGAGTTTCAAGTGCGGAAACCT-3' and antisense 5'-GCCAAGACTCAAGTGGAGATGT-3' for Nox2; sense 5'-GGATCGGAGTACCTCCCTGCTG-3' and antisense 5'-ATGACAGCTCTGGGTCAGCTGA-3' for Nox3; sense 5'-CTCAAGGGGAACTACAGCCTTAG-3' and antisense 5'-ATAAGGATGCAATCGGAGTGT-3' for Nox4; sense 5'-CTCATGTGACTACCTGGCACAGC-3' for Nox5

Nox4 expression in various melanoma cell lines was evaluated by real-time PCR (35 cycles) using Taqman Gene Expression assay (Applied Biosystems) with glyceraldehyde-3-phosphate dehydrogenase as an internal control.

Growth curve. Cells (10⁴) were grown in the presence or absence of antioxidants and DPI, and the numbers of five cells were determined by trypan blue exclusion. Alternatively, cells were inoculated and labeled with [³H]thymidine (0.1 µCi; GE Healthcare) in the presence or absence of the above chemicals. Labeled cells were precipitated with 10% chilled trichloroacetic acid for 10 min, and the incorporated radioactivity was determined by nitrocellulose filtration, followed by scintillation counting.

Soft agar assay. The assay was performed as described previously (4). The appearance of colonies was monitored for up to 12 d. The numbers of colonies larger than 4 mm were counted.

Immunoblotting. Cells were lysed in radioimmunoprecipitation assay buffer [PBS, 0.1% SDS, 1% Na-deoxycholate, 1% Triton X-100, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium PPI leupeptin (10 µg/mL), pepstatin A (10 µg/mL), and phenylmethylsulfonyl fluoride (1 mmol/L)]. Extracted proteins were separated by SDS-PAGE and subjected to immunoblotting with indicated antibodies.

Tumorigenecity analysis. To determine tumor formation by each transfected cell line, trypsinized cells (10⁴) were suspended in 0.2 mL of PBS, and the cell suspensions were inoculated s.c. into athymic mice. All of the animals were observed for the formation of tumors for up to 3 wk. The size of tumor was measured at the 12th day with an external caliper, and volume was calculated.

Preparation of anti-Nox4 antibodies. The COOH-end peptides of Nox4 (CLSNQNSYGTTRFYUNKESFS) cross-linked to keyhole limpet hemocyanin

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NOTE: Melanoma tissues isolated from patients were processed for immunohistochemistry using anti-Nox4 antibodies as described in Materials and Methods.

Abbreviations: ALM, acral lentiginous melanoma; rt. arm, right arm; LN, lymphnode; SSM, superficial spreading melanoma; NM, nodular melanoma.


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were immunized into rabbits, and the antiserum was affinity purified. In some experiments, mouse monoclonal antibodies against Nox4 (gift by Dr. B. Goldstein, Department of Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA) were used.

**Measurement of intracellular ROS.** Forty-eight hours after plating, cells were incubated with HBSS (Invitrogen) containing 10 μmol/L 2,7′-dichlorodihydrofluoresceindiacetate (Molecular Probes) for 10 min at 37°C. Images were obtained with a Zeiss confocal microscope. Fluorescence was quantitated on an arbitrary unit and the data represent the fluorescence intensity of 50 random cells obtained from three separate fields.

**Determination of the cellular DNA content by fluorescence-activated cell sorting analysis.** MM-BP cells were plated at 1 × 10⁵ cell density per 60-mm dish, starved for 48 h in DMEM containing 0.5% serum, and then maintained for 48 h in DMEM containing 10% serum. Trypsinized cells (10⁶–10⁷) were subjected to cell cycle analysis using propidium iodide as described (18). Relative DNA content of nuclei was measured by a fluorescence-activated cell sorter (Becton Dickinson).

**Immunohistochemistry.** Primary or metastatic melanoma tissues were obtained from informed patients. Immunohistochemistry was performed on 4-μm thick formalin-fixed paraffin-embedded tissue. Paraffin sections were dewaxed in xylene and rehydrated with distilled water. The tissue sections were activated in Tris-EDTA buffer (pH 9) in the microwave oven for 25 min. The slides were subsequently incubated with the purified rabbit anti-human Nox4 antibody, followed by HRP-conjugated anti-rabbit IgG antibodies. For controls, the antibodies preadsorbed with Nox4 peptide antigens were used. Staining was visualized with AEC substrate chromogen, followed by hematoxylin counterstaining. Indirect immunofluorescence staining was performed and samples were observed under a confocal microscope (LSM510; Carl Zeiss) as described previously (8).

**Statistical analysis.** The statistical analysis was performed with Student’s t test. For multiple treatment groups, a factorial ANOVA followed by Bonferroni’s t test was applied. Differences with P values of <0.05 were considered to be statistically significant.

**Results**

**Effects of antioxidants and DPI on melanoma cell growth.** To explore the functional role of ROS-generating enzymes in malignant melanoma, we first examined whether proliferation of melanoma cells requires ROS production. Human melanoma MM-BP cells (19) were treated with increasing amounts of a dietary antioxidant vitamin E and a thiol-containing antioxidant pyrrolidine dithiocarbamate (PDTC). Consistent with other reports (15), vitamin E decreased the growth of MM-BP cells in liquid culture (Fig. 1A) and PDTC also effective in inhibiting the cell growth (Fig. 1A). Furthermore, DPI, a flavoprotein-dependent oxidase inhibitor, showed the growth inhibitory effect (Fig. 1A), whereas rotenone, a mitochondrial oxidase inhibitor, had little or no effect (data not shown). The inhibitory effects of vitamin E, PDTC, and DPI were also confirmed by cell proliferation assay using [3H] thymidine incorporation (Fig. 1B). These results suggest that Nox

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**Figure 1.** Effect of antioxidants and DPI on MM-BP cell growth and Nox4 expression in melanoma cell lines. A, cells were cultured in the presence or absence of vitamin E, PDTC, and DPI until live cells were harvested at the indicated times and the number of live cells was determined. Points, mean in triplicated dishes; bars, SD. B, alternatively, MM-BP cells (10⁵) were metabolically labeled with [3H]thymidine for 48 h in the presence of 2 μmol/L vitamin E, 1 μmol/L PDTC, or 1 μmol/L DPI. The radioactivity incorporated were determined. Columns, mean (n = 4); bars, SD. P*, P**, and P† < 0.05 versus none. C, The levels of Nox4 expression in various melanoma cell lines were examined by real-time PCR. Columns, mean of duplicated assays; bars, SD.
family enzymes participate in growth control involving ROS in MM-BP cells. To further study the source of ROS generation, we examined the expression of Nox family mRNAs in MM-BP cells. Reverse transcription-PCR analysis revealed that a relatively high amount of Nox4 mRNA was expressed in MM-BP cells compared with other Nox family homologues including Nox1, Nox2, Nox3, and Nox5 (Fig. 2A). No appreciable Nox4 messages were detected in melanocytes under the condition used (Fig. 2A). In addition, real-time PCR showed that significant levels of Nox4 mRNA were expressed in 13 of 20 melanoma cell lines with different origins (Supplementary Table S1; Fig. 1C). Accordingly, it is conceivable that up-regulation of Nox4 is not a particular characteristics of MM-BP cells but a frequent event among melanoma cell lines. In the subsequent study, we therefore focused on the Nox4 action in MM-BP cells.

**Nox4 is involved in ROS generation in melanoma cells.** To probe the functional role of Nox4 in melanoma, we stably transfected the expression vectors carrying siRNAs for Nox4 into MM-BP cells. With the isolated clones, RT-PCR and immunoblotting analysis showed that loading with siRNA1 or siRNA2 significantly decreased the amounts of Nox4 mRNAs (Fig. 2A) and Nox4 proteins (the data for a cell line, siRNA1-b, are not shown; Fig. 2A) compared with those in the scrambled siRNA preparation, which indicated that introduced Nox4 siRNAs effectively repressed the Nox4 expression. To test whether Nox4 mediates intracellular ROS production, generation of H2O2, a dismutated metabolite of Nox4-derived superoxide was measured in Nox4siRNA-transfected cell lines after addition of the fluorescent oxidant indicator dye 2′,7′-dichlorodihydrofluorescein diacetate. Although intracellular DCF fluorescence was detected in scrambled siRNA-transfected MM-BP cells, this fluorescence was sharply attenuated in siRNA1-a, siRNA1-b, and siRNA2 cells (Supplementary Fig. S1; Fig. 2B). The results indicate that Nox4 siRNAs inhibit spontaneous oxidant generation in MM-BP cells, suggesting the involvement of Nox4 in ROS production in melanoma cells.

**Effects of Nox4 siRNAs on melanoma proliferation.** Because DPI or antioxidants treatment blocked proliferation of MM-BP cells (Fig. 1A–D), we next examined whether suppression of the Nox4 activity by Nox4siRNAs induces dysregulation of cell proliferation. Soft agar assay as an *ex vivo* model of tumorigenicity shows significantly decreased the amounts of Nox4 mRNAs (Fig. 2A) and Nox4 proteins (the data for a cell line, siRNA1-b, are not shown; Fig. 2A) compared with those in the scrambled siRNA preparation, which indicated that introduced Nox4 siRNAs effectively repressed the Nox4 expression. To test whether Nox4 mediates intracellular ROS production, generation of H2O2, a dismutated metabolite of Nox4-derived superoxide was measured in Nox4siRNA-transfected cell lines after addition of the fluorescent oxidant indicator dye 2′,7′-dichlorodihydrofluorescein diacetate. Although intracellular DCF fluorescence was detected in scrambled siRNA-transfected MM-BP cells, this fluorescence was sharply attenuated in siRNA1-a, siRNA1-b, and siRNA2 cells (Supplementary Fig. S1; Fig. 2B). The results indicate that Nox4 siRNAs inhibit spontaneous oxidant generation in MM-BP cells, suggesting the involvement of Nox4 in ROS production in melanoma cells.

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that Nox4siRNAs caused a significant reduction in anchorage-independent growth of MM-BP cells compared with that caused by scrambled siRNAs (Fig. 2C). The inhibitory effect of Nox4siRNAs on cell growth was detected in liquid culture, as well (Fig. 2C). These data indicate that Nox4-generated ROS are required to sustain growth of melanoma cells.

Suppression of tumor formation by Nox4siRNAs. Inhibition of anchorage-independent growth of MM-BP cells by Nox4siRNAs (Fig. 2C) suggests that silencing of Nox4 by Nox4siRNAs suppresses transformation phenotype of the cells. To test whether MM-BP cells become less tumorigenic through overexpression of Nox4siRNAs, the cells transfected with either scrambled or Nox4siRNAs were implanted into athymic nude mice. Although scrambled siRNA-transfected cells formed significant tumors in 2 weeks, introduction of Nox4siRNAs markedly decreased tumor growth (Fig. 2D). Thus, the results indicate that Nox4siRNAs inhibit tumorigenicity of MM-BP cells, suggesting the critical mediating role of Nox4-derived ROS in development of some types of melanomas.

Because tumor progression is often associated with augmented cell motility and invasiveness of cancer cells, we compared scrambled siRNA-transfected cells with Nox4siRNA-transfected cells for the cell migration activity by Matrigel assay, but the scrambled siRNA-transfected cells with Nox4siRNA-transfected MM-BP cells, suggesting the critical mediating role of Nox4-derived ROS in development of some types of melanomas.

Inhibition of Nox4 induces cell cycle arrest. To further analyze the mechanism underlying Nox4siRNA-induced growth inhibition, cell cycle distribution of MM-BP cells was analyzed by flow cytometry. Cells were serum starved for 48 h to arrest their growth and refed with the growth medium in the presence or absence of DPI. Of note, treatment of MM-BP cells with DPI for 24 hours was found to induce an accumulation of cells in G2-M phase (Fig. 3A). A marked increase of cell population in G2-M phase was also observed with the cell line, siRNA1-a (Fig. 3A). Similarly, another Nox4-positive melanoma cell line, 928mel (Table 1) displayed cell accumulation in G2-M phase when treated with DPI or transiently transfected with Nox4siRNAs (Fig. 3B). Furthermore, depletion of hydrogen peroxide by overexpression of catalase in MM-BP cells resulted in G2-M cell cycle arrest (Fig. 3C), which is consistent with the notion that Nox4-derived ROS mediate cell cycle progression from G2-M phase.

To characterize the molecular machinery involved in G2-M cell cycle arrest upon ablation of Nox4, the expression of some of cell cycle–controlling genes was examined. A protein kinase CDK1 (cdc2) plays an important role in the regulation of the G2-M cell cycle phase. Activation of CDK1 (dephosphorylation at Tyr-15) is required for transition from the G2-M phase, whereas its inactivation (phosphorylation at Tyr-15) causes G2-M cell cycle block (20). We therefore examined the phosphorylation state of the Tyr-15 of CDK1 by immunoblotting with anti-phospho CDK1 (Tyr-15) antibodies. The level of Tyr-15 phosphorylation was increased in MM-BP cells 24 hours after exposure to DPI (Fig. 4A), which chronologically coincided with induction of G2-M phase cell cycle arrest by DPI treatment. Moreover, introduction of Nox4siRNAs into MM-BP cells resulted in a similar increase in Tyr-15 phosphorylation of CDK1 compared with that in scrambled siRNA-transfected cells (Fig. 4A). The total amount of CDK1 proteins was not changed (data not shown). Likewise, Tyr-15 phosphorylation of CDK1 was induced in DPI-treated or Nox4siRNA-transfected melanoma 928mel cells (Fig. 4A). These data suggest that the G2-M cell cycle arrest was induced as CDK1 was inactivated. p53 and p21WAF1/CIP1, such as CDK1, have been reported to regulate the G2-M transition and be critical in sustaining the G2-M cell cycle arrest after DNA damage through inhibition of CDK1 (21, 22). DPI treatment had no

![Figure 3. Cell-cycle perturbation by DPI, siNox4 RNAs, and catalase. A, parental MM-BP cells were serum-starved for 48 h and treated with or without 2 μmol/L DPI for 24 h (left). Alternatively, the cell lines carrying Nox4siRNAs or scrambled siRNAs were serum-starved for 48 h (right). Cells were then refed with the growth medium for 72 h and subjected to flow cytometric analysis. The proportion of nuclei in each pair of the cell cycle is shown. Similar results were obtained in three separate experiments. Expression of transfected catalase was determined by immunoblotting (IB) with anticatalase antibodies. IP, immunoprecipitation.](image-url)
appreciable effect on either p53 or p21^{WAF1/CIP1} protein levels in MM-BP cells for at least 24 hours, as determined by immunoblotting analysis (Fig. 4B). These results suggest that induction of G_{2}-M arrest by Nox4 inhibition is controlled by a mechanism independent of both p53 and p21^{WAF1/CIP1}.

It has been known that the activity of CDK1 is regulated by cdc25c phosphatase through dephosphorylation of the Tyr-15 residue, and that the activity of cdc25c is modulated by phosphorylation. To determine whether phosphorylation of cdc25c was regulated by Nox4, we analyzed fractions of cdc25c mobility by immunoblotting. Consistent with the previous report (23), the unphosphorylated form "a" and phosphorylated form "b" (Ser216 phosphorylation) of cdc25c were detected in untreated MM-BP cells (Fig. 4C). In contrast, addition of DPI for 24 hours induced hyperphosphorylation of cdc25c, which is indicated by the appearance of a slower-migrated form "c" of cdc25c (refs. 23, 24; Fig. 4C). Similarly, the level of the hyperphosphorylated cdc25c protein was elevated in a Nox4siRNA-transfected MM-BP cell line, whereas it was not increased in scrambled siRNA-transfected cells (Fig. 4C). Hyperphosphorylation of cdc25c is thought to prolong its remaining in the cytoplasm due to increased binding with 14-3-3 proteins, inhibiting the interaction between cdc25c and CDK1 in the nucleus (19, 25). Indeed, immunostaining showed that exclusion of cdc25c from the nuclei was increased in both DPI-exposed and Nox4siRNA-transfected MM-BP cells (Fig. 4D). Coprecipitation studies showed that Nox4siRNA transfection resulted in enhanced binding of cdc25c with 14-3-3 when compared with scrambled siRNAs transfection (Fig. 4D). Thus, these data suggest that hyperphosphorylation of cdc25c mediates both DPI and Nox4siRNA-induced Tyr-15 phosphorylation of CDK1, leading to cell cycle arrest at G_{2}-M stage.

The growth inhibition is frequently accompanied by induction of apoptosis. By using terminal deoxynucleotidyl-transferase–mediated dUTP nick-end labeling assay, we therefore examined whether or not suppression of the Nox4 activity induces apoptosis. No significant changes in cell survival rate were detected after DPI or Nox4siRNA treatment (Supplementary Fig. S4), which is consistent with the flow cytometric profiles without any sub-G_{1} peak representing apoptotic cell population (Fig. 3A and B). From these data, we conclude that the observed growth inhibitory effects of DPI and Nox4siRNAs in both soft agar and liquid culture are due to cell cycle arrest at G_{2}-M transition rather than apoptosis.

**Figure 4.** Effects of DPI and Nox4 siRNAs on G_{2}-M phase cell cycle controlling proteins. A, MM-BP cells were serum-starved, treated with or without DPI (2 μmol/L), and refed in growth media as in Fig. 3A. Alternatively, the cell line siRNA1-a or scrambled was serum-starved and refed in growth media as above. Then, cell lysates were subjected to immunoblotting with anti-phospho CDK1 (tyr-15) antibodies (top). 928mel cells were serum starved for 48 h, treated with or without DPI (2 μmol/L) for 24 h, and refed in growth media for 72 h. Alternatively, 928mel cells transiently transfected with Nox4siRNAs or scrambled siRNAs were serum starved and refed with growth media as above. Lysates were subjected to immunoblotting using anti-phospho (Tyr-15) CDK1 antibodies (bottom). B, MM-BP cells were incubated with or without DPI (2 μmol/L) for the indicated time periods. Cell lysates were processed for immunoblotting using antibodies to p21^{WAF1/CIP1} or p53. C, DPI or Nox4siRNA-induced hyperphosphorylation of cdc25c. MM-BP cells were serum starved for 48 h and treated with 2 μmol/L DPI for 15 h. Alternatively, cell lines, siRNA1-a or scrambled were serum starved for 48 h and refed with the growth medium. Lysates were subjected to immunoblotting with anti-cdc25c antibodies. Note that form a and b bands partially overlapped due to overloading samples. Protein loading was monitored by β-actin. D, MM-BP cells were treated with or without DPI as in A. Cell lines, scrambled and siRNA1-a were serum starved and refed with complete growth medium for 24 h as in A. Cells were fixed and stained with rabbit anti-cdc25c antibodies and CY3-conjugated anti-rabbit IgG antibodies (Molecular Probes). Right, immunostained patterns. Histograms show means ± SD of 3 separate experiment (average number of counted cells is 80). P < 0.05 (*) and P < 0.05 (**) versus DPI (-) and scrambled, respectively. After serum starvation/refeeding, lysates prepared from scrambled or siRNA 1-a cells were immunoprecipitated with anti–14-3-3 antibodies, followed by immunoblotting with anti-cdc25c antibodies (left). 14-3-3 proteins in lysates and immunoprecipitates were identified by immunoblotting with 14-3-3 antibodies.
Expression of Nox4 in melanoma tissues. To examine whether Nox4 expression is really up-regulated in melanoma tissues, Nox4 expression patterns were evaluated in 13 primary or metastatic melanoma samples isolated from patients by immunohistochemistry using antibodies against Nox4. Immunoblotting analysis showed that the raised Nox4 antibodies recognize Nox4 proteins (Supplementary Fig. S2). The result was then confirmed by showing the staining of the proximal renal tubules of normal kidney with the antibodies as reported earlier (Fig. 5A; ref. 26). Nox4 was expressed in 4 of 13 melanoma tissues, including 5 acral melanomas, 6 superficial spreading melanomas, 1 nodular melanoma, and 1 mucosal melanoma (Table 1). Of these, two acral melanomas and two superficial spreading melanomas expressed Nox4. Frequency of Nox4 expression seemed independent of clinical type. Interestingly, Nox4 was strongly expressed in the nests in epidermis (Fig. 5B) but less in the metastatic lymph nodes. In all of the Nox4-positive melanoma tissues, Nox4 was localized in the cytoplasm or perinucleus. Immunoabsorption of the antibodies with the Nox4 peptide antigen abolished most of the staining, indicating that the staining is Nox4 specific (Fig. 5B). No Nox4 expression was observed in normal skin tissues and cultured melanocytes (data not shown). These observations show that Nox enzyme–mediated signalings link to the cell cycle controlling events. To our knowledge, this is the first observation that Nox enzyme–mediated signalings link to the cell cycle controlling events.

Discussion

Malignant melanoma is the most aggressive form of skin cancer. ROS production has been partly implicated in the development of melanoma and its resistance to radiation and chemotherapy, but the functional roles of ROS are not fully understood. Here, we show that superoxide generation by Nox4 is required for proliferation of melanoma MM-BP cells and formation of tumors derived from MM-BP cells in athymic mice, suggesting a critical role for Nox4 in maintenance of malignant transformation phenotype of melanoma. Furthermore, our studies reveal that Nox4 mediates cell proliferation by regulating G2-M cell cycle progression because inhibition of Nox4 activity by either Nox4-specific siRNAs or DPI caused G2-M cell cycle arrest. This is also supported by the facts that G2-M arrest upon Nox4 inhibition was observed in another melanoma cell line, 928Mel, and that G2-M block was induced by catalase-mediated depletion of hydrogen peroxide, a dismutated metabolite of Nox4-generated superoxide. To our knowledge, this is the first observation that Nox enzyme–mediated signalings link to the cell cycle controlling events.

CDK1 (cdc2) kinase that interacts with cyclin B is a key mitosis-promoting kinase in the control of the G2-M cell cycle progression. Namely, activation of CDK1, complexed with cyclin B, promotes transition from G2 to M phase of the cell cycle. CDK1 is activated by cdc25c phosphatase that dephosphorylates CDK1 at Tyr-15 residues. Our data indicate that both Nox4siRNAs and DPI treatment inactivate cdc25c, causing increased phosphorylation of CDK1 on Tyr-15, a hallmark of the G2-M checkpoint. The activity of cdc25c is believed to be inactivated mainly by two mechanisms: (a) phosphorylation of cdc25c at Thr-47 decreases its phosphatase activity; (b) hyperphosphorylation of cdc25c creates a binding site for 14-3-3 proteins, which sequester cdc25c to the cytoplasm and thereby interfere with its interaction with CDK1 in the nucleus (19, 25). As abrogation of Nox4-generated ROS led to induction of hyperphosphorylated cdc25c, the most-likely possibility is that the G2-M cell cycle arrest was caused via the second mechanism. Although p53 and p21WAF1/CIP1 have also been implicated in regulation of the G2-M cell cycle arrest after DNA damage through inhibition of CDK1 (21, 22), the expression levels of p53 and p21WAF1/CIP1 were unaffected by suppression of the Nox4 activity.
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