Peroxiredoxin-2 Represses Melanoma Metastasis by Increasing E-Cadherin/β-Catenin Complexes in Adherens Junctions

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Introduction

Melanoma is the most dangerous type of skin cancer owing to its high metastatic potential and resistance to therapeutic agents (1, 2). Melanoma cells are derived by malignant transformation of melanocytes that produce melanin to protect the skin cells against UV damage. In normal skins, UV stimulates keratinocytes to secret growth factors, such as stem cell factor, fibroblast growth factor, and hepatocyte growth factor. These factors stimulate melanocytes for melanin production as well as for proliferation and differentiation. However, melanocytes undergo malignant transformation in the individuals who either have a genetic defect in melanin production, that is, albinism, or are intermittently exposed to intense UV light. In the early stages, the melanocytes grow and form benign naevi near the epidermis–dermis junction. As the disease progresses, the melanoma cells grow radially and then vertically at later stage showing growth pattern of pagetoid spread. The vertical growth of melanoma leads to metastatic malignancy, whereby melanoma cells infiltrate into blood vessels and lymphatic system. Melanoma diagnosed at early stage is usually cured by a local surgical excision; however, metastatic malignant melanoma has a poor prognosis even after surgery. Therefore, it is necessary to develop a therapeutic agent to treat the late stages of melanoma, especially targeting metastatic dispersion.

The intense UV radiation can damage genomic DNAs directly by formation of thymine dimer adduct or indirectly by intracellular production of reactive oxygen species (ROS). Indeed, melanoma involves various genetic mutations in the oncogenes and tumor suppressors (3). Among the mutated genes are NRAS, BRAF, PTEN, AKT3, and CDKN2A. Because these genes are involved in various cellular functions including cell cycle, proliferation, and survival, their gain-of-function or loss-of-function mutation is a key for the metastatic malignancy of melanoma cells. An important consideration is that these gene products are linked to cellular redox homeostasis. For example, the Ras activation has been known to induce robust production of cellular ROS (4, 5). The lipid phosphatase activity of PTEN protein was regulated by reversible oxidation of the active site cysteine residue (6, 7). Recently, Jenkins and colleagues showed that p16INK4a regulates the cellular ROS level in skin cell types including keratinocytes and melanocytes (8). Furthermore, the NADPH oxidases, which produce superoxide anion by transferring an electron to molecular oxygen, have been shown to influence melanoma cell cycle and growth.
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(9, 10). Although the involvement of ROS in melanoma formation and metastasis was proposed (11), the endogenous antioxidant enzyme has not been studied in relation to melanoma pathophysiology.

Mammalian cells have well-defined endogenous antioxidant enzymes that include superoxide dismutases, catalase, glutathione peroxidases, and peroxiredoxins (Prx). Among these enzymes, Prx is the most abundant enzyme and is known to be involved in receptor tyrosine kinase–mediated signal transduction (12). Interestingly, it has recently been shown that one of the Prx family members Prx2 (gene loci, PRDX2), which belongs to 2-cys Prx subfamily and reduces the peroxides in the presence of NADPH by coupling with thioredoxin/thioredoxin reductase system, is downregulated in melanoma cell lines and particularly in metastatic malignant types (13, 14). However, the function of the Prx2 enzyme in the melanoma cell generation and metastasis remains unknown.

In this study, we investigated the role of the Prx2 peroxidase in in vitro cellular function and in vivo metastasis of melanoma cells. The level of Prx2 expression inversely correlated with in vitro proliferatory and migratory activities and in vivo metastatic potential of melanoma cells. We also elucidated a novel Prx2-mediated signaling pathway for suppression of melanoma metastasis, which involves a synergistic collaboration of the extracellular signal–regulated kinase (ERK)-dependent E-cadherin expression and the Src-dependent β-catenin retention in the adherens junctions. Furthermore, a natural compound mimicking the Prx2 peroxidase activity prevented lung metastasis of melanoma cells, which implicates a small molecule mimic for Prx2 as a therapeutic agent to inhibit melanoma metastasis.

Materials and Methods

Reagents, RNA interference, and cell culture

U0126 was purchased from Cell Signaling Technology. PD98059, PP2, and PP3 were from Calbiochem. Gliotoxin (GT) was from Sigma-Aldrich. Zonula Adherens Sampler Kit was kindly provided by BD Transduction Laboratories. Antibodies against α-tubulin, p-ERK (Thr-202/Tyr-204), ERK1, p-Src (Y416), Src, p-Akt, and Akt were from Cell Signaling Technology. Anti-p-β-catenin (Y654) antibody was from Abcam. Anti-phosphotyrosine (4G10) was from Upstate Biotechnology. Alexa Fluor 568 donkey anti-mouse, Alexa Fluor 488 donkey anti-rabbit, Alexa Fluor 488 donkey anti-mouse, and Alexa Fluor 568 donkey phosphotyrosine (4G10) was from Upstate Biotechnology.

Anti-Prx2 antibodies were from AbFrontier. Fluor 488 donkey anti-rabbit were from Invitrogen. Anti-Prx1 anti-rabbit, Alexa Fluor 488 donkey anti-mouse, and Alexa Fluor 568 donkey phosphotyrosine (4G10) was from Upstate Biotechnology. The cell lines used in this study were purchased from American Type Culture Collection.

Immunoblot analysis

The cells were rinsed once with ice-cold PBS and then lysed in an extraction buffer containing 20 mmol/L HEPES (pH 7.0), 1% Triton X-100, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L dithiothreitol, 5 mmol/L Na3 VO4, 5 mmol/L NaF, 1 mmol/L 4-(2-aminoethyl) benzenesulfon fluoride, aprotinin (5 μg/mL), and leupeptin (5 μg/mL). After centrifugation at 12,000 × g, the clarified cell extract was used for immunoblotting. To control for loading, the membranes were stripped by shaking them for 30 minutes at 60°C in 67 mmol/L Tris (pH 6.7), 2% SDS, 100 mmol/L 2-mercaptoethanol, and reprobed with the appropriate pan antibody.

Immunocytochemistry

The cells were grown on glass cover slides and fixed with prewarmed 4% paraformaldehyde solution for 15 minutes. The fixed cells were washed twice with PBS and permeabilized with 0.2% Triton X-100 for 30 minutes at room temperature. After permeabilization, the cells were then blocked with 2% bovine serum albumin (BSA) in PBS (blocking buffer) for 1 hour and incubated at 4°C overnight with the indicated primary antibodies diluted in a blocking buffer: anti-phosphotyrosine (clone 4G10; 1:100), anti-Prx1 (1:300), anti-Prx2 (1:300), anti-β-catenin (1:200), and anti-E-cadherin (1:200). The cells were washed three times with a blocking buffer and incubated with Alexa Fluor 568–conjugated or Alexa Fluor 488–conjugated secondary antibody for 30 minutes. The coverslips were then washed three times with a blocking buffer and mounted. The fluorescence images were taken by an LSM 510 META confocal laser-scanning microscope (Zeiss).

Measurement of intracellular H2O2

Intracellular hydrogen peroxide (H2O2) level was assessed with an oxidation-sensitive fluorescent dye 5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-DCFH-DA; Invitrogen). The human epidermal melanocytes, SK-MEL-5, SK-MEL-28, A375, and G361 cells (3 × 103) were cultured in 35-mm dishes and infected with retroviruses for 24 hours. The cells were then deprived of serum for 18 hours and stimulated with 20% FBS in phenol red–free media for 10 minutes. After stimulation, the cells were rinsed quickly with Krebs–Ringer solution and incubated for 5 minutes with 5 μmol/L CM-DCFH-DA. The 2′,7′-dichlorodihydrofluorescein (DCF) fluorescence was collected for 10 seconds with an inverted Axiovert 200 fluorescence microscope (Zeiss). The relative DCF fluorescence was calculated by averaging the fluorescence intensities of the 60 to 80 cells after background subtraction in each image using ImageQuant software (GE Healthcare). Note that the detached round cells were omitted from quantification.
The retroviruses encoding human Prx2 were generated using a bicistronic pLXIN vector and a Retro-X Q vector system (Clontech). First, the coding sequence for human Prx2 wild-type (WT) was inserted by PCR cloning into pLXIN. The resulting vector was used for viral production by stably transducing a NIH3T3-based dualtropic packaging cell line RetroPack PT67. These viruses were mainly used for the Prx2 overexpression in melanoma cells. Second, the production of retrovirus encoding myc-tagged human Prx2 WT and inactive CS mutant (Cys51Ser/Cys173Ser) using the Retro-X Q vector system was previously described (16). The viral titers were determined (10^7) viral particles/mL) and the aliquots were stored at −70°C. For retroviral infection, the viral aliquots were thawed in warm water bath and mixed with 10 µg/mL polybrene.

**Retrovirus production**

**Proliferation and migration assays**

The cell proliferation was measured using a WST-1 cell proliferation assay kit (Roche Diagnostics) according to the manufacturer’s protocol. The cell number was expressed as absorbance values at 450 nm averaged from triplicate wells after subtracting the turbidity at 600 nm. The chemotactic migration assays were conducted in 24-well Transwell culture chambers (Costar; polycarbonate membrane insert with 8-µm pore size). The bottom of the insert was coated with gelatin B (1 mg/mL) and air-dried for 1 hour. Melanoma cells (6 x 10^4) were added to the inserts, which contained retrovirus or transfection complex. After 24 hours, the infected or transfected melanoma cells were serum-starved for 18 hours. Solutions of 20% FBS were then added to the bottom chambers with basal media containing 0.5% BSA. The upper chamber wells were filled with each basal media containing 0.5% BSA. Transwell chambers were incubated at 37°C/5% CO2 for 12 hours. After incubation, the nonmigrated cells were removed from the top of the filters. The cells that migrated onto the bottom of filters were fixed and stained with 0.6% hematoxylin and 0.5% eosin. The stained cells were photographed and counted. The number of migrating cells was averaged from triplicate wells.
Wound closure assay

A wound was made on the monolayer of cells by scratching with a pipette tip. The cells were then washed with PBS to remove cell debris and fed with fresh culture medium. Cells were allowed to proliferate and migrate into the wound during the next 12 hours. Migration of cells into the wound was observed using a microscope. The wound width was measured using ImageJ software.

Protein tyrosine phosphatase assay

The control or Prx2 WT retrovirus–infected SK-MEL-28 (5 × 10^5 cells) were serum-starved for 18 hours and stimulated with or without 20% FBS for 10 minutes. After stimulation, the cells were frozen in liquid nitrogen and moved to an anaerobic chamber. The cells were scraped in a hypotonic buffer (2 mmol/L MgCl2, 25 mmol/L KCl, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, and a protease inhibitor cocktail in a 50 mmol/L PIPES buffer, pH6.5) containing 10 mmol/L iodoacetate and 10 mmol/L N-ethylmaleimide. The homogenates were obtained by passing through a 26-gauge needle 15 times and then incubated for an additional 20 minutes to achieve complete alkylation of free thiols. The labeling was quenched using 50 mmol/L DTT. Then, the homogenates were clarified by centrifugation at 12,000 × g and diluted in a 10...
mmol/L DTT-containing reaction buffer. The PTPase activities were measured in a 96-well plate coated with poly-(Glu4-pTyr) peptides, according to the manufacturer's protocol (Universal tyrosine phosphatase assay kit; Takara Bio.; MK-411) and quantified from a standard curve obtained using the recombinant CD45 tyrosine phosphatase.

Lung metastasis of B16F10 melanoma cells in mice

B16F10 cells were transiently transfected with Prx2 siRNA for 24 hours, trypsinized, and then resuspended in Hank’s balanced salt solution (HBSS). The cell suspensions were injected intravenously into C57/BL6 mice (1 × 10^6 cells per mouse). The mice were anesthetized after 10 days, and the lungs were then isolated after transcardiac perfusion-fixation with heparinized saline containing 3.7% formaldehyde. The lungs were paraffin-embedded and sectioned by rotary microtome (Leica RM2255). The two serial tissue sections (4 μm thickness) were stained with hematoxylin and eosin (H&E). A count was made of the metastasized melanoma tumor nodules on the lung’s surface and H&E-stained tissue sections.

To test the effect of gliotoxin, the C57/BL6 mice were intraperitoneally (i.p.) injected with gliotoxin (300 μg/kg) five times during the 10 days following an intravenous injection of melanoma cells.

Statistical analysis

Data were analyzed using Student t test on SigmaPlot 8.0 software, and the P value was derived to assess statistical

Figure 3. Prx2 knockdown enhances proliferation and migration of the Prx2-expressing melanoma cells. A, selective knockdown of human Prx2 expression in G361 and A375 cells using two different specific siRNAs. B and C, proliferation (B) and migration (C) of G361 and A375 cells with control (con) and Prx2 knockdown. D, proliferation and migration of the A375 cells in response to serum stimulation. Prx1 was knocked down using a specific siRNA. The cells were transfected with control or isoform-specific siRNAs as indicated for 24 hours. The experiments were repeated three times (*, P < 0.01; **, P < 0.005; N.S., not significant).
Figure 4. Prx2 suppresses the serum-induced Src and ERK activation via preserving PTP activity. A and B, serum-induced tyrosine phosphorylation in the control (vec) and Prx2-expressing SK28 cells. The tyrosine phosphorylation was detected by immunoblotting (A) and immunostaining (B) using anti-pTyr antibody (4G10). Representative blots and images are shown (n = 3). C, PTP activities in control and Prx2-expressing SK28 cells. Total PTP activity was measured using poly-(Glu4-pTyr) peptides as described in Materials and Methods. D and E, activation of intracellular signaling molecules in the Prx2-expressing SK28 cells (D) and Prx1/2–depleted G361 cells (E). The G361 cells were stimulated with serum for 30 minutes. The phospho-specific bands were quantified and normalized by the intensities of the corresponding non-phospho protein bands. Data are means ± SD of the relative band intensities from three independent experiments. F and G, proliferation and migration of the SK28 melanoma cells in the presence of specific MEK (F) and Src (G) inhibitors. The SK28 cells were serum-starved for 24 hours and then pretreated with PD98059 (5 μmol/L) or PP2/PP3 (1 μmol/L each) for 1 hour before serum stimulation. PP3 is an inactive analog of PP2. Dimethyl sulfoxide (DMSO) was the control vehicle for untreated sample. The experiments were repeated three times (*, P < 0.01; **, P < 0.005; *, P < 0.001; N.S., not significant).
Figure 5. Prx2 expression increases the E-cadherin/β-catenin complexes in the adherens junctions of melanoma cells. A, expression of adherens junctional proteins in the control (vec) and Prx2-expressing SK28 cells. The intensities of β-catenin and E-cadherin bands were quantified and normalized by the intensities of the corresponding tubulin bands. Data are means ± SD of the relative band intensities from three independent experiments. (*, P < 0.005; N.S., not significant). B, expression of β-catenin and E-cadherin in the Prx2-expressing SK5- and Prx2-depleted G361 cells. C and D, immunostaining of β-catenin and E-cadherin in the Prx2-expressing SK28 cells (C) and Prx2-depleted G361 cells (D). Merged image shows the colocalization of two proteins in the plasma membrane of the Prx2-expressing cells (yellow). The fluorescence intensities were quantified along the white arrows (top right) and expressed as a percentage of the intensity of the indicated fraction versus total intensity (bottom right; n = 33 cells/group; **, P < 0.005; ***, P < 0.001). M1 and M2, plasma membrane; Cyto, cytoplasm. E, reduction of β-catenin Y654 phosphorylation by the Prx2 expression in SK28 cells. The phospho-specific bands were quantified and normalized by the intensities of the corresponding non-phospho protein bands. Data are means ± SD of the relative band intensities from three independent experiments. F, increased β-catenin Y654 phosphorylation by the Prx2 knockdown in G361 cells. The immunoblot shown is a representative of three independent experiments with the same results.
Results

**Prx2 suppresses melanoma cell proliferation and migration**

We immunoscreened the melanoma cell lines using the Prx2-specific antibody for level of Prx2 protein. The SK-MEL-5 (SK5) and SK-MEL-28 (SK28) melanoma cells showed almost no expression of Prx2, whereas the other two cell lines, A375 and G361, expressed the Prx2 proteins (Fig. 1A). The level of Prx2 expression in A375 was comparable with that in the human melanocytes. In contrast, the protein level of the closest isoform Prx1 was similar among the four cell lines. This was also confirmed by immunofluorescence staining (Supplementary Fig. S1A). In addition, the promoter region of the *PRDX2* gene in SK-MEL cell lines, but not A375 and G361 cells, was methylated (Supplementary Fig. S1B). Consistent with previous report (17), this evidence provides a molecular mechanism underlying the silencing of the Prx2 expression. Given that the Prx2 is a cytosolic antioxidant enzyme, we measured the cellular H$_2$O$_2$ level using an oxidation-sensitive fluorescent dye, DCFH-DA. The result showed that the basal H$_2$O$_2$ level was high in SK-MEL cells lacking Prx2, whereas it was low in A375 and G361 cells as well as primary melanocytes where the Prx2 is present (Fig. 1B). This result indicates that the Prx2 is a key antioxidant enzyme regulating intracellular H$_2$O$_2$ level in melanoma cells.

Then, we compared the *in vitro* cellular activities of four melanoma cell types, that is, proliferation and migration.
Figure 7. A fungal product, gliotoxin (GT), inhibits metastatic ability of melanoma cells. A, basal level of intracellular H₂O₂ in control vehicle [dimethyl sulfoxide (DMSO)] or gliotoxin-treated SK28 melanoma cells. Representative images are shown. Bars in the graph are means ± SD of the relative DCF fluorescence values averaged from 60 to 80 cells (**, P < 0.005). B, serum-induced protein phosphorylation and E-cadherin expression in control vehicle (DMSO) and gliotoxin-treated SK28 cells. C and D, proliferation (C) and migration (D) of control vehicle (DMSO) and gliotoxin-treated SK5 and SK28 cells. The serum-starved cells were pretreated with gliotoxin (100 nmol/L) for 1 hour and then stimulated with serum. The experiments were repeated three times (**, P < 0.005). E, lung metastasis of B16F10 melanoma cells in control vehicle and gliotoxin-injected mice. The mice (7 mice/group) were injected with DMSO or gliotoxin (300 μg/kg, i.p.) five times during the 10 days following intravenous injection of either parental (E) or mPrx2-1 siRNA-transfected (F) B16F10 melanoma cells. As shown earlier, the isolated lungs were examined to count the number of melanoma tumor nodules on the surface.
Because the serum condition is physiologically relevant to tumor metastasis process involving complex factors, we placed the cells on the serum-starved condition and then stimulated with 20% fetal serum to maximize serum-dependent induction of the cellular activities. As a result, the Prx2-lacking SK-MEL cells exhibited higher proliferative activity than that of the other two Prx2-expressing melanoma cells (Fig. 1C). The migratory activities shown in chemotactic migration toward serum and wound closure were much higher in SK-MEL cells than other Prx2-expressing melanoma cells (Fig. 1D and E). The results implicate an inverse correlation between cellular function and the Prx2 level among melanoma cell types.

We then investigated a direct regulatory effect of Prx2 on melanoma cells by exogenous expression or specific knockdown of Prx2. Exogenous expression of Prx2 in the SK-MEL cells was achieved by a retroviral transduction of the human PRDX2 gene (Fig. 2A). When the proliferative activity of the SK-MEL cells was examined, the retroviral expression of Prx2 reduced the proliferation of cells induced by serum stimulation (Fig. 2B). The migratory activities of the SK-MEL cells shown in chemotactic migration and wound closure were more profoundly reduced by Prx2 expression (Fig. 2C and D). Subsequently, we conducted the specific knockdown of the Prx2 expression using two different siRNAs in G361 and A375 cells (Fig. 3A). The Prx2 knockdown significantly increased cell proliferation and migration in both cells responding to the serum stimulation (Fig. 3B and C). In contrast, the knockdown of another cytosolic Prx isozyme, Prxl, did not affect the melanoma cell activities (Fig. 3D). Collectively, the results indicate that the Prx2 enzyme functions as a selective antioxidant suppressor for proliferation and migration of melanoma cells.

**Prx2 negatively regulates c-Src and ERK activation in a H$_2$O$_2$-dependent manner**

To elucidate the molecular mechanism by which Prx2 controls melanoma function, we examined the serum-induced protein phosphorylation in SK28 cells. The serum stimulation induced tyrosine phosphorylation in a timely manner, which was lowered by the Prx2 expression (Fig. 4A). The immunostaining data confirmed that the Prx2 expression directly correlated with the reduced tyrosine phosphorylation in individual cells (Fig. 4B). Because the cellular H$_2$O$_2$ oxidatively inactivates the protein tyrosine phosphatases (PTP; ref. 18), we assessed the PTP activity in SK28 cells. Indeed, the Prx2 expression evidently enhanced the basal PTP activity in unstimulated cells (Fig. 4C). Because the PTPs exhibit broad substrate specificity, we then dissected downstream signaling pathways important for melanoma biology. In particular, the Ras–Raf–MEK–ERK and PI3K–Akt pathways are critical for melanoma cell proliferation, survival, and migration (2). In addition, the Src kinase, which is highly activated in various cancers (19), has also been proven to be involved in melanoma progression (20, 21). Therefore, we examined the serum-dependent activation of c-Src kinase, ERK, and Akt in SK28 melanoma cells. As a result, the c-Src and ERK activation, but not the Akt activation, were markedly attenuated by the Prx2 expression (Fig. 4D). The specific knockdown of Prx2, but not Prxl, in G361 cells inversely increased the c-Src and ERK activation in response to serum (Fig. 4E). Subsequently, we tested whether the activation of these two kinases is sufficient for controlling melanoma cell function. The inhibition of ERK activation was achieved by a selective mitogen-activated protein/extracellular signal–regulated kinase (MEK) inhibitor PD98059, whereas the Src inhibition was achieved by a reversible ATP-competitive inhibitor PP2. PD98059 and PP2 were titrated out to determine the optimum inhibitory concentrations in the SK28 cells. The results indicated that the inhibition of each of MEK/ERK and Src kinases sufficiently blocked melanoma cell proliferation and migration (Fig. 4F and G).

Next, we addressed whether the peroxidase activity of the Prx2 is required for melanoma cell function. To this end, we expressed the human Prx2 WT and its inactive mutant (CS), where the two active-site cysteine residues, Cys51 and Cys172, are mutated to serine residues, in SK28 cells by retroviral transduction (Supplementary Fig. S2A). The Prx2 WT eliminated the basal and serum-induced H$_2$O$_2$ levels, whereas the CS mutant did not (Supplementary Fig. S2B). Furthermore, only the Prx2 WT, not the CS mutant, reduced serum-induced tyrosine phosphorylation and cell migration (Supplementary Fig. S2C and S2D). Together, the results indicate that the peroxidase activity of the Prx2 is essential for eliminating intracellular H$_2$O$_2$ and regulating melanoma cell function.

**Prx2 increases the E-cadherin/β-catenin complexes in adherens junctions**

The invasion and metastasis of the tumor cells directly involve the epithelial–mesenchymal transition (EMT; ref. 22). In particular, loss of E-cadherin–β-catenin complexes in adherens junctions is a critical step for acquiring metastatic ability. Therefore, to understand the molecular mechanism underlying the Prx2-dependent mobilization of melanoma cells, we examined the level of adherens junctional proteins in melanoma cells after the Prx2 reexpression or depletion. The SK28 cells expressed various types of cadherins and catenins...
(Supplementary Fig. S3). Among various junctional proteins, the Prx2 expression did not affect the levels of the catenin proteins but evidently increased the E-cadherin expression in SK-MEL cells (Fig. 5A and B). Inversely, the Prx2 knockdown in G361 cells decreased the E-cadherin expression (Fig. 5B). To further seek a connection between Prx2 and catenins, we tested whether Prx2 possibly influences the subcellular distribution of catenin proteins. Surprisingly, the Prx2 expression increased the β-catenin levels in the plasma membrane, particularly in adherens junction as indicated by colocalization with E-cadherin (Fig. 5C). The quantification of the fluorescence intensity along a straight line indicated that the β-catenin moved from cytosol to the plasma membrane. Conversely, the Prx2 knockdown in G361 cells reduced the β-catenin levels in the plasma membrane (Fig. 5D). In addition, the immunostaining data confirmed that the E-cadherin expression was proportional to the Prx2 expression.

To delineate the signaling mechanism underlying the Prx2-dependent reorganization of the adherens junctions in melanoma cells, we explored whether the Src/ERK, which had been downregulated by Prx2, plays a key role in the formation of E-cadherin/β-catenin complexes. It has been reported that the Src phosphorylates β-catenin on tyrosine 654 (Y654), which triggers the release of β-catenin from the adherens junction (23, 24). Indeed, the Src inhibitor PP2, but not the BFAF/MEK inhibitors, blocked the serum-induced Y654 phosphorylation of β-catenin in SK28 cells (Supplementary Fig. S4A) and then resulted in a marked increase in β-catenin level in the plasma membrane of melanoma cells (Supplementary Fig. S4B). Subsequently, we found that the Prx2 reexpression in SK28 cells appreciably inhibited the serum-induced Y654 phosphorylation of β-catenin compared with that in control cells (Fig. 5E), whereas its knockdown in G361 cells increased the β-catenin phosphorylation (Fig. 5F). Thus, the results indicate that the Prx2-induced membrane retention of β-catenin by inhibiting the Src-dependent Y645 phosphorylation. Unexpectedly, we observed that the Src inhibition also increased the E-cadherin level, which might be due in part to the role of Src in the deregulation of E-cadherin by endocytosis (25). Moreover, it was shown that the ERK activation triggers the EMT via inhibiting the E-cadherin expression (26, 27). Indeed, the blockade of ERK and Src pathways by two independent MEK inhibitors (PD98059 and U0126) and PP2, respectively, both increased the E-cadherin level in SK28 cells (Supplementary Fig. S4C). Conclusively, the data show that the ERK and Src pathways were independently involved in the Prx2-mediated augmentation of the E-cadherin/β-catenin complexes.

**Absence of Prx2 enhances lung metastasis of melanoma cells**

On the basis of the regulatory activity of Prx2 in proliferation and migration of melanoma cells, we carried out an *in vitro* experiment using a lung metastasis model of mouse melanoma cells, B16F10. Because the B16F10 cells are known to express WT BRAF (28), we examined whether the Prx2 still regulates the Src/ERK pathways and cellular activity of B16F10 cells. The Prx2 knockdown using mouse Prx2-specific siRNAs increased the Src/ERK activation and the β-catenin phosphorylation, whereas it decreased the E-cadherin level in B16F10 cells (Fig. 6A). Consistently, the Prx2 knockdown increased proliferation and migration of B16F10 cells in response to serum stimulation (Fig. 6B). This result indicates that the function of Prx2 in melanoma cells is perhaps independent of oncogenic BRAF mutation.

For *in vivo* experiment, we used the siRNA-mediated knockdown of the Prx2 expression that guaranteed a stable depletion of the Prx2 expression during lung metastasis assay (Fig. 6C). Then, the B16F10 cells transfected with the mPrx2 siRNA were injected into mice through the tail vein. After 10 days, the mouse lungs were isolated and examined for metastasized tumor nodules. Microscopic examination of both the lung surface and the H&E-stained tissue sections showed that the B16F10 cells with Prx2 knockdown invaded and colonized in lungs more aggressively than the control cells (Fig. 6D and E).

**A fungal product, gliotoxin, inhibits *in vitro* migration and *in vivo* lung metastasis of melanoma cells**

Because a fungal secondary metabolite called gliotoxin was shown to be the first natural compound that exhibits the thioredoxin-dependent peroxidase activity representing a typical Prx activity (29), we tested a therapeutic potential of gliotoxin for prevention of melanoma metastasis. Indeed, the gliotoxin treatment reduced the basal level of intracellular H₂O₂ in the Prx2-lacking SK28 cells at the nontoxic level (Supplementary Fig. S5 and Fig. 7A), which verifies the gliotoxin’s peroxidase activity. Once again, the gliotoxin treatment reduced the Src/ERK activation and the β-catenin phosphorylation, but it increased the E-cadherin expression in SK28 cells (Fig. 7B). Furthermore, gliotoxin reduced the proliferation and migration of both SK5 and SK28 cells in response to serum stimulation (Fig. 7C and D). This result indicates that the gliotoxin acts as a small-molecule substitute for Prx2 in melanoma cells. To examine the therapeutic efficacy *in vivo*, the gliotoxin was intraperitoneally injected into the mice following the injection of B16F10 cells. The gliotoxin treatment evidently reduced lung metastasis of melanoma cells (Fig. 7E). In addition, gliotoxin more drastically reduced the lung metastasis of B16F10 cells in which Prx2 had been knocked down (Fig. 7F). This result reveals the therapeutic potential of a small molecule substituting for Prx2 as an inhibitor of melanoma metastasis.

**Discussion**

Advanced malignant tumors usually accompany with aggressive metastasis. Because the metastatic tumor cells are refractory to the existing chemotherapies, the tumors metastasized to the multiple sites are often fatal. Likewise, the metastatic malignancy is the main reason for melanoma being a deadly skin cancer. Unless metastasized to the lymph node or distant sites, the melanoma lesion can be cleaned by a surgical resection. The tumor metastasis is actually a complex and multistep process where tumor cells with metastatic potential (i) escape from primary site, (ii) circulate through blood and lymphatic vessels, (iii) extravasate to distant secondary organs, and (iv) finally adapt to the new microenvironment.
it is difficult to develop an antimetastatic therapeutic that is powerful by itself or useful in combination with preexisting medications. In this study, we show for the first time that among endogenous antioxidant enzymes the Prx2 specifically downregulates proliferation and migration of melanoma cells in vitro and suppresses their lung metastasis in vivo. The striking mechanism involved is that the Prx2 expression reduced the ERK and Src activation, which in turn resulted in a marked increase of the E-cadherin/β-catenin complexes in the adherens junctions (Fig. 7G).

Numerous studies have indicated that the cellular antioxidant enzymes including Prxs are highly expressed in cancer tissues (12, 30). In particular, the high level of Prx enzymes may provide a survival benefit on hyperproliferating cancer cells (31–34). However, the specific functions of the two cytosolic Prx isoforms, Prx1 and Prx2, are likely distinguishable in cancer biology. For example, the deletion of PRDX1 gene resulted in the development of malignant tumors in aging mice (35). The following studies have indicated that the absence of Prx2 induces DNA damage and therefore initiates tumorigenesis (36, 37). The expression of Prx1 is transcriptionally regulated by oxidative stress-sensitive Nrf2 transcription factor in cancer cells (38). Thus, the role of Prx1 in cancer cells is supposedly to serve as an antioxidant defense against oxidative stress. In contrast, the Prx2 knockout mice did not show any phenotypes relevant to cancer (39), and the Prx2 expression was relatively low in melanoma and skin cancer versus other cancer types (www.proteinatlas.org). Indeed, it was recently determined that the Prx2 expression is silenced in melanoma and acute myeloid leukemia by the hypermethylation of the CpG islands in the promoter region and epigenetically by histone H3 acetylation (17, 40). Our study now reveals that silencing of the Prx2 expression in melanoma cells results in the accumulation of intracellular H2O2, which in turn leads to the disruption of E-cadherin/β-catenin complexes in adherens junctions by Src/ERK pathways. Moreover, we show that such disrupted junctions lead to an increase in the melanoma cell migration and metastasis (Fig. 7G). Accordingly, the specific silencing of Prx2 and subsequent increase in intracellular ROS level is likely a prerequisite for metastasis of melanoma tumor. Interestingly, there have been supportive documents that ROS promotes tumor metastasis (41, 42) and that Prx2 is involved in cell adhesion by stabilizing E-cadherin/β-catenin complexes (43). Hence, the Prx2 can be a novel metastasis suppressor that induces the stabilization of adherens junctions.

The oncogenic BRAF mutation is predominant in malignant melanoma (44) and leads to the constitutive ERK activation (45). However, our data clearly indicate that the Prx2-dependent regulation of melanoma cell function is regardless of the BRAF mutation because Prx2 regulated the serum-induced ERK activation in both human melanoma cell lines with active BRAF mutation and B16F10 melanoma cells with WT BRAF. We actually carried out additional control experiments to concrete this proposal. To discriminate the WT and oncogenic BRAF, we used a BRAF inhibitor PLX4720 specific to the constitutively active oncogenic mutant (BRAF-V600E). When the effect of this inhibitor on ERK activation was compared with the MEK inhibitor PD98056, the PLX4720 inhibited only the basal, but not the serum-induced, ERK phosphorylation; whereas, the PD98059 inhibited both the basal and serum-induced ERK activation (Supplementary Fig. S4A and S4D). Furthermore, the PLX4720 had no effect on the serum-induced Src/ERK activation enhanced by Prx2 silencing in G361 cells (Supplementary Fig. S4E). This evidence support that the Prx2 effect is independent of BRAF-V600E mutant and the Prx2-dependent H2O2 is thought to be involved in the redox-dependent ERK activation at the downstream of BRAF, possibly through reversible oxidation or proteosomal degradation of mitogen-activated protein kinase (MAPK) phosphatases (46, 47). On the other hand, it is generally accepted that the ROS induces the Src kinase activation (48). More specifically, it was shown that the H2O2 induces the Lyn kinase activation in leukocytes by direct oxidation of a cysteine residue Cys466 (49). Therefore, the intracellular H2O2 level elevated by the Prx2 silencing may be linked to the constitutive Src activation in SK-MEL cells (20, 21). However, considering the classical mechanism of Src kinase activation involving the precedent dephosphorylation of inhibitory tyrosine site Y527, the role of H2O2 in the Src activation is complex. Many studies have indicated that various PTPs, such as T-cell PTP, SHP-1, and RPTP-α, etc., have been known to enhance the Src activation by the Y527 dephosphorylation (48, 50). Given that the PTPs are the best H2O2 targets, the elevation of intracellular H2O2 rather inhibits the Src activation. Thus, the interaction of H2O2 and Src kinase could be a cell type–dependent, and in this context, the molecular mechanism underlying how Prx2 inhibits the Src activation in melanoma cells should be explored further.

In summary, the control of melanoma metastasis via antioxidant enzyme Prx2 provides new insight into antimelanoma strategies for circumventing oncogenic BRAF. More importantly, our in vivo study using gliotoxin implicates the Prx2 mimetic as a promising therapeutic drug for preventing melanoma metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: D.J. Lee, D.H. Kang, S.W. Kang Development of methodology: D.J. Lee, M. Choi, J.Y. Lee, K.W. Lee, S.W. Kang Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.J. Lee, Y.J. Choi, J.H. Park, Y.J. Park Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.J. Lee, D.H. Kang, M. Choi, J.Y. Choi, J.H. Park, Y.J. Park, K.W. Lee, S.W. Kang Writing, review, and/or revision of the manuscript: D.J. Lee, D.H. Kang, S.W. Kang Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.J. Lee, J.Y. Lee, K.W. Lee Study supervision: S.W. Kang

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Peroxiredoxin-2 Represses Melanoma Metastasis by Increasing E-Cadherin/β-Catenin Complexes in Adherens Junctions

Doo Jae Lee, Dong Hoon Kang, Mina Choi, et al.


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