Nrf2-deficiency creates a responsive microenvironment for metastasis to the lung

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The Nrf2 transcription factor is crucial for regulating the cellular defense against various carcinogens. However, relationship between host Nrf2 and cancer metastasis remains unexplored. To address this issue, we examined susceptibility of Nrf2-deficient mice to pulmonary cancer metastasis following implantation of the mouse Lewis lung carcinoma (3LL) cell line. Nrf2-deficient mice reproducibly exhibited a higher number of pulmonary metastatic nodules than wild-type mice did. The lung and bone marrow (BM) of cancer-bearing Nrf2-deficient mice contained increased numbers of inflammatory cells, including myeloidderived suppressor cells (MDSCs), a potent population of immunosuppressive cells. MDSCs can attenuate CD8⁺ T-cell immunity through modification of the T-cell receptor complex exploiting reactive oxygen species (ROS). MDSCs of Nrf2-deficient mice retained elevated levels of ROS relative to wild-type mice. BM transplantation experiments revealed functional disturbance in the hematopoietic and immune systems of Nrf2-deficient mice. Wild-type recipient mice with Nrf2-deficient BM cells showed increased levels of lung metastasis after cancer cell inoculation. These mice exhibited high-level accumulation of ROS in MDSCs, which showed very good coincidence to the decrease of splenic CD8+ T-cells. In contrast, Keap1-knockdown mutant mice harboring high-level Nrf2 expression displayed increased resistance against the cancer cell metastasis to the lung, accompanied by a decrease in ROS in the MDSCs fraction. Our results thus reveal a novel function for Nrf2 in the prevention of cancer metastasis, presumably by its ability to preserve the redox balance in the hematopoietic and immune systems.

Introduction

Cancer metastasis is the primary cause of mortality in cancer patients. Therefore, intense efforts have focused on understanding the details of this complex process in the hope that novel targets for therapeutic intervention can be found. It has been suggested that cancer metastasis is governed not only by genetic abnormalities but also by the host response, which can critically affect cancer cell homing to the metastatic niche.

Nrf2 is a basic region-leucine zipper (bZip) transcription factor, which serves as a key regulator of the cellular defense against oxidative and electrophilic stresses, toxic chemicals and carcinogens (1,2). In the absence of a stress stimulus, Nrf2 is degraded rapidly by the ubiquitin–proteasome pathway through its association with Keap1 (*K*elch-like *E*CH associating protein 1), a substrate adaptor protein of the Cul3-based ubiquitin E3 ligase complex (3,4). Upon exposure to oxidative or electrophilic stress, reactive cysteine residues in Keap1 are covalently modified, liberating Nrf2 from Keap1-

Abbreviations: BM, bone marrow; DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; 8-OHdG, 8-hydroxydeoxyguanosine; MDSC, myeloid-derived suppressor cell; ROS, reactive oxygen species; ROI, region of interest; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labelling.

mediated degradation. This results in Nrf2 stabilization and subsequent nuclear translocation, where it dimerizes with a member of the small Maf family proteins. This complex activates the transcription of a wide range of cytoprotective genes via a *cis*-acting DNA element known as the antioxidant/electrophile responsive element (ARE/EpRE) (1).

Nrf2 is essential for protection against chemically induced carcinogenesis. Molecular basis of this protection can be attributed to increased expression of detoxifying enzymes that enhance the chemical hydrophilicity and excretion of the carcinogen, the removal of reactive oxygen species (ROS), and the clearance of damage caused by ROS (5). Oxidative stress is a common feature of the metastatic microenvironment and is accompanied by chronic inflammation. Such changes induce chemical damage to nucleic acids, proteins, and other cellular and extracellular components within the surrounding tissue (6). Thus, considering the cytoprotective role of Nrf2 and the potential vulnerability of the metastatic microenvironment to oxidative stress, we speculated that Nrf2-deficient mice might be highly susceptible to lung metastasis of cancer cells.

To address this hypothesis, we implanted mouse Lewis lung carcinoma (3LL) cells that are known to have high metastatic potential into Nrf2-deficient mice. When we compared pulmonary cancer metastasis between Nrf2-deficient and wild-type mice, Nrf2-deficient mice reproducibly exhibited increased susceptibility to lung metastasis of 3LL cells, which was associated with greater inflammation. Among the population of inflammatory cell that were increased in cancerbearing mice, the myeloid-derived suppressor cell (MDSC) fraction had significant elevation in ROS levels.

MDSCs are heterogeneous myeloid progenitors of macrophages, dendritic cells and granulocytes. Most importantly, MDSCs have been found to be expanded in many types of cancer patients and exert a suppressive function on both non-specific (innate) and antigenspecific (adaptive) immunity, which is one of the main mechanisms for tumor-immune tolerance (7). A critical determinant of MDSCimmunosuppressive activity is the intracellular ROS level. The accumulation of ROS in MDSCs can potentially suppress the activation and proliferation of CD8⁺ T-cells by introducing peroxynitrite modifications on the T-cell receptor–CD8 complex upon the surface of CD8⁺ T-cells, which can disrupt the interaction with major histocompatibility complex class I molecules on antigen-presenting cells (8). Here, we demonstrate that Nrf2 exerts anti-metastatic activity by regulating the inflammatory status and redox balance of the hematopoietic and immune systems of cancer-bearing mice.

Materials and methods

Cell line

We obtained 3LL and B16-F10 melanoma cell lines (9,10) from the Institute of Development, Aging and Cancer, Tohoku University. For stable transfection, 3LL and B16-F10 cells were transfected with 2 μ g pEF-1-tdTomato (11) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and cultured in selective medium containing 300 μ g/ml G418 (Calbiochem, Berlin, Germany) for 14 days. Bright fluorescent colonies were isolated by means of cloning cup. The cell culture medium was RPMI 1640 (Wako, Osaka, Japan) supplemented with L-glutamine (0.29 mg/ml), 2% penicillin–streptomycin (0.06 mg/ml; Gibco, Carlsbad, CA) and 10% heat-inactivated fetal bovine serum.

Implantation of cancer cells

In metastasis experiments, mice were intravenously injected with 1×10^{6} 3LL or B16-F10 cells in a total volume of 0.2 ml phosphate-buffered saline. The lung metastasis was examined 20 days after 3LL cell inoculation or 16 days after B16-F10 cell inoculation. A lung surface nodule larger than Φ 1 mm was counted as a macroscopic metastatic tumor. In the spontaneous metastasis model, 3LL cells (1×10^{6} in 200 µl phosphate-buffered saline) were inoculated into the right thigh muscle. Weights of the primary tumor, lung and para-aortic lymph node were measured 22 days after inoculation. Numbers of lung surface nodules were calculated after the lung was fixed in Tellyesniczky's solution (12).

Experimental animals and bone marrow transplantation

*Nrf*2-null (*Nrf*2^{-/-}) and *Keap1*-knockdown (*Keap1*^{t/t}) mice on C57BL6/J genetic background (1,13) were used in this study. Weight matched (14.5–27.0 g) and age matched (5–12 weeks) mice were used with concurrent control mice. For bone marrow (BM) transplantation, 2×10^6 BM cells from 8–12 week old *Nrf*2^{+/+} or *Nrf*2^{-/-} (C57BL/6-CD45.1) mice were injected into the tail vein of lethally irradiated (7.2 Gy) *Nrf*2^{+/+} or *Nrf*2^{-/-} mice of 10–12 week old (C57BL/6-CD45.2). After an 8 week recovery period, the chimerism of donor mice was examined by the CD45.1^{+/}(CD45.1⁺ plus CD45.2⁺) cell ratio of peripheral blood mononucleated cells. The recipient mice with full donor chimerism (>90%) were selected for subsequent metastasis experiments. The mice were executed with the approval of the Tohoku University Animal Care Committee.

Cell sorting analysis

Single cell suspensions from cancer-bearing mouse lungs were prepared as described previously (14). Sorting and analyses of BM and lung cells were performed using FACS-Aria and FACS-Caliber (BD Pharmingen, San Diego, CA). For the separation of MDSCs, the cell suspension from BM or lung was incubated with allophycocyanin (APC)-conjugated Mac1 and phycoerythrin (PE)-conjugated Gr1 monoclonal antibodies (BD Pharmingen). For quantification of ROS levels, BM and lung cell suspensions were incubated with 5 μ M 2',7'-dichlorodihydrofluor-escein diacetate (DCFDA; Invitrogen) at 37°C for 30 min (15). For the separation of CD8- or CD4-single-positive T-cells, cell suspension from spleen was incubated with fluorescein isothiocyanate (FITC)-conjugated CD8 and phycoerythrin-conjugated CD4 monoclonal antibodies (BD Pharmingen). Fluorescence activated cell sorting analysis was performed using FACS-Diva 6.0 (BD Bioscience, San Diego, CA) and FlowJo (TOMY Digital Biology) software.

Quantitative real-time RT-PCR

Total RNA was extracted from Mac1⁺Gr1⁺ MDSCs using ISOGEN-LS (Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized from total RNA using random hexamers and a Superscript III polymerase kit (Invitrogen). Real-time RT-PCR was performed using an ABI PRISM 7300 sequence detector system (phycoerythrin-Applied Biosystems, Foster City, CA) and 2× SYBR Green PCR master mix (Invitrogen). The primer sequences for Nrf2, heme oxygenase-1 (HO-1), γ -glutamylcysteine ligase regulatory subunit (γ -GCLR) and β -actin were described previously (16). The abundance of each cDNA was determined based on C_t (threshold cycle) values and experimentally determined by the amplification efficiency for each primer set, then normalized to the abundance of β -actin.

Fluorescent imaging with IVIS

Ex vivo biofluorescent imaging was performed with an IVIS imaging system IVIS100 with Living Image Software ver. 2.5 (Caliper Life Science, Hopkinton, MA). The region of interest (ROI) was set around the detectable td-Tomato fluorescence in whole lung tissue. The light intensity from the ROI was measured in units of photons per second.

Histological analysis

A piece of right lung was fixed in 4% paraformaldehyde at 4°C overnight and processed for frozen sections. Five micrometers thick sections were stained with hematoxylin–eosin using standard techniques. Sections were examined under an All-in-one Type Fluorescence Microscope (BZ-8000; Keyence). Lung tumor area (>0.1 mm²) in the maximum section of each lung was measured using the BZ Analyzer Software (Keyence, Osaka, Japan).

Immunoblotting analysis

Nuclear extracts were isolated from lung of the mice harboring experimental 3LL metastasis using the Dignam method (17). Immunoblotting analysis was performed using anti-Nrf2 (18) and anti-LaminB antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) as reported previously (19).

Immunohistochemistry

Cellular proliferation was analyzed by using Ki67 antibody staining (DAKO, Los Angeles, CA). Apoptotic cells in cancer tissue was detected by terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) staining (Takara In Situ Apoptosis Detection Kit; Takara Bio, Otsu, Japan). Ki67-positive or TUNEL-positive 3LL cancer cells were counted within cancer metastatic nodules larger than Φ 150 µm (minimal size histologically diagnosed as metastatic tumor) from three arbitrarily selected sections from one mouse. The proliferative or apoptotic index of each group was determined by quantifying the relative proportion of Ki67- or TUNEL-positive cells per total metastatic cell population. An anti-mouse Mac1 monoclonal antibody (Cedarlane, Hornby, Ontario, Canada) was used for the detection of infiltrating macrophages and granulocytes. A rabbit polyclonal anti- β -galactosidase antibody (Abcam, Cambride, MA) and an anti-8-hydroxydeoxyguanosine

(8-OHdG) monoclonal antibody (Japan Institute for Control of Aging, Shizuoka, Japan) were used for the detection of a nuclear Nrf2–LacZ fusion protein and oxidative stress-induced DNA damage, respectively.

Statistical analyses

Data are expressed as the mean \pm SD. Statistical differences were determined using the Student 's *t*-test or the Mann–Whitney *U*-test. The values for either the incidence of total metastatic lung nodules or large metastatic colonies (>0.1 mm²) were analyzed using the Fisher's exact probability test. *P*-values <0.05 were considered statistically significant.

Results

Nrf2-deficient mice display the increased susceptibility to metastasis by lung cancer cells

Nrf2 is crucial for regulating the cellular defense against various carcinogens. In unstressed conditions, Nrf2 is degraded rapidly through the association with Keap1 (Figure 1A, left panel). However, upon exposure to carcinogens reactive cysteine residues of Keap1 are modified and this liberates Nrf2 from the degradation, resulting in Nrf2 stabilization and subsequent nuclear translocation (Figure 1A, middle panel). In the nucleus, Nrf2 dimerizes with a member of small Maf proteins and activates the transcription of cytoprotective enzyme genes to attain cancer chemoprevention (Figure 1A, right panel). However, relationship between host Nrf2 and cancer metastasis remains unexplored. To examine the anti-metastatic function of hostderived Nrf2, we first utilized 3LL cells. Monitoring of the metastatic status of the implanted 3LL cells was facilitated by the generation of a 3LL cell line that stably expressed td-Tomato fluorescent protein (3LL-td-Tomato). The 3LL-td-Tomato cells displayed a bright red fluorescence in culture (supplementary Figure 1A and B is available at Carcinogenesis Online).

Twenty days after the intravenous injection of 3LL cells, we observed a significantly higher incidence of metastatic lung tumors in $Nrf2^{-/-}$ mice relative to control $Nrf2^{+/+}$ mice. Indeed, all 16 $Nrf2^{-/-}$ mice presented with macroscopic lung tumors ($\Phi > 1$ mm; 100%, n = 16), in comparison with only six $Nrf2^{+/+}$ mice ($\Phi > 1$ mm; 28.6%, n = 14, P < 0.01; Figure 1B–D, Table I). In addition, $Nrf2^{-/-}$ mice exhibited a higher number of lung surface nodules (11.0 ± 11.8 ; n = 16) than $Nrf2^{+/+}$ mice (2.1 ± 3.8 ; n = 14, P < 0.01; Figure 1D and Table I). The size of the metastatic tumor tended to be larger in $Nrf2^{-/-}$ than $Nrf2^{+/+}$ mice. In fact, eight mice (50.0%) in the $Nrf2^{-/-}$ recipient group (n = 16) exhibited large palpable nodules ($\Phi > 5$ mm), whereas only two mice (14.2%) in the $Nrf2^{+/+}$ group (n = 14) exhibited nodules of comparable size (red dots in Figure 1D).

The surface area of the metastatic tumor from the maximum ordinate level of the right lung was quantified in order to assess severity. The incidence of severe metastatic cases, signified by a tumor >0.1 mm², was significantly higher in $Nrf2^{-/-}$ mice (60.0%; n = 15) relative to $Nrf2^{+/+}$ mice (7.7%; n = 13, P < 0.01; Figure 1C and Table I). Consistently, the host lung weight of tumor-bearing $Nrf2^{-/-}$ mice (0.46 ± 0.27 g; n = 16) was almost 3-fold higher than that of $Nrf2^{+/+}$ mice (0.16 ± 0.24 g; n = 14; P < 0.01), indicating that the $Nrf2^{-/-}$ host lungs were heavily burdened with metastatic tumors (Figure 1D and Table I).

The metastatic lung tumors in $Nrf2^{-/-}$ mice showed bright td-Tomato fluorescence in fresh-frozen sections (supplementary Figure 1C and D is available at *Carcinogenesis* Online). By exploiting the cancer cell-derived td-Tomato fluorescence, we attempted to evaluate the growth and dissemination of metastatic lung tumors by biofluorescence imaging using an IVIS imaging system. Tumor metastasis was measured by quantitative biofluorescence 20 days after cancer cell injection. We defined a metastatic event as a ROI around the detectable td-Tomato fluorescence within whole lung tissue. Consistent with our macroscopic observations (Figure 1B), the ROI in the whole lung tissue was significantly increased in $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice (P < 0.05; Figure 1E). Additionally, we exploited B16-F10 cells, a highly metastatic malignant melanoma cell line, to evaluate the metastatic capacity of cancer cells in $Nrf2^{-/-}$ mice. Upon lateral tail vein injection of B16-F10 cells, several of



Fig. 1. Metastatic lung tumors derived from intravenously injected 3LL-td-Tomato cancer cells. (**A**) The homodimeric Keap1 contacts one Nrf2 molecule through the double glycine repeat (DGR) domain, and the Keap1-Cul3-based ubiquitin E3 ligase complex constantly directs Nrf2 for proteasomal degradation under unstressed conditions (left panel). Oxidative/electrophilic stresses modify reactive cysteine residues in Keap1, leading to the accumulation of Nrf2 (middle panel). Nrf2 dimerized with small Maf activates the transcription of various cytoprotective genes via ARE/EpRE (antioxidant/electrophile responsive element) (see text). (**B**) Metastatic lung surface nodules in $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice; scale bar, 10 mm. *Ex vivo* bioluminescence imaging of metastatic cancers is shown. The relative light intensity was quantified according to the color scale (photons per second) shown on the right. (**C**) Hematoxylin–eosin staining of metastatic ang grous weight of individual lungs was quantified from the $Nrf2^{-/-}$ mouse group (n = 16) and the $Nrf2^{+/+}$ control mouse group (n = 14). Statistical significance of the differences between $Nrf2^{-/-}$ and $Nrf2^{+/+}$ are indicated (**P < 0.01 by Mann–Whitney U-test). Red dots indicate individual mice harboring large tumor nodules ($\Phi > 5$ mm). (**E**) Quantification of bioluminescence signal from metastatic cancer areas (*P < 0.05 by Student's *t*-test). Red bars represent the average.

| Table I. | Experimental | metastasis by | intravenous in | jection of | 3LL cells to | Nrf2+/+ | and Nrf2-/- | mice |
|----------|--------------|---------------|----------------|------------|--------------|---------|-------------|------|
|----------|--------------|---------------|----------------|------------|--------------|---------|-------------|------|

| Genotype | Total cancer | Number of lung | Lung weight (g) | Incidence of tumor | |
|--|---|---|---|---|--|
| | incidence ($\Phi > 1 \text{ mm}$) | surface tumors ($\Phi > 1 \text{ mm}$) | | $\Phi > 5 \text{ mm}$ | $>0.1 \text{ mm}^2$ |
| Nrf2 ^{+/+} Nrf2 ^{-/-} | 6/14 (28.6%) 16/16 ^a (100.0%) | $2.1 \pm 3.8 \ (n = 14)$ 11.0 \pm 11.8 ^a (n = 16) | $\begin{array}{l} 0.16 \pm 0.24 \; (n = 14) \\ 0.46 \pm 0.27^{\rm a} \; (n = 16) \end{array}$ | 2/14 (14.2%) 8/16 (50.0%) ^b | 1/13 (7.7%) 9/15 (60.0%) ⁶ |

 $^{a}P < 0.01$ compared with wild-type mice.

 $^{b}P < 0.05$ compared with wild-type mice.

the $Nrf2^{-/-}$ mice exhibited higher numbers of lung metastatic nodules in comparison with $Nrf2^{+/+}$ mice, although the difference in susceptibility was not so significant as in the case of 3LL cells (supplementary Figure 2 and Table IA are available at *Carcinogenesis* Online). These results demonstrate that Nrf2 activity is indispensable for preventing lung metastasis by 3LL lung cancer cells, whereas in the case of B16-F10 cells, the Nrf2 status is of lesser importance.

Nrf2-deficient mice are susceptible to spontaneous metastasis from a distant primary tumor

We next examined the role Nrf2 plays in the prevention of spontaneous lung metastasis from a distant primary tumor. To this end, 3LL cells were implanted into the right thigh muscles of $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice, generating a primary tumor. Mice were killed 22 days after the inoculation, and the primary tumor, para-aortic lymph nodes and lung were analyzed. The incidence of metastatic lung tumors was not significantly different between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice (Table II) and the weight of the primary tumors in thigh muscle and para-aortic metastatic lymph nodes of $Nrf2^{-/-}$ mice were not significantly increased in comparison with $Nrf2^{-/-}$ mice were not significantly increased in comparison with $Nrf2^{+/+}$ control mice (Table II and supplementary Figure 3 is available at *Carcinogenesis* Online). In contrast, we clearly observed a significant increase in the number of spontaneous metastatic tumor nodules on the lung surface of $Nrf2^{-/-}$ mice (191.0 ± 243.0, n = 10) compared with $Nrf2^{+/+}$ control mice (46.1 ± 47.2, n = 18, P < 0.05; Figure 2A and B and Table II).

Table II. Spontaneous metastasis by intramuscular injection of 3LL cells to $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice Lung cancer Genotype Number of Primary tumor Lymph node Positive cells/3LL cells Lung incidence lung surface Weight (g) Ki67 (%) TUNEL (%) tumors Nrf2+/+ 17/18 (94.0%) 46.1 ± 47.2 0.18 ± 0.07 9.6 ± 1.13 0.1 ± 0.09 15.2 ± 12.7 32.1 ± 15.3 (n = 18)(n = 18)(n = 18)(n = 18)(n = 9)(n = 9) 10.3 ± 7.9^{b} 41.2 ± 22.0^{b} Nrf2^{-/-} 10/10 (100.0%) 191.0 ± 243.0^{a} 0.20 ± 0.12 9.1 ± 1.10 0.1 ± 0.01 (n = 10)(n = 10)(n = 10)(n = 10)(n = 10)(n = 10)

 $^{a}P < 0.05$ compared with wild-type mice.

 $^{b}P < 0.01$ compared with wild-type mice.



Fig. 2. Spontaneous lung metastasis from primary tumor cells in thigh muscle. (**A**) Representative gross observation and hematoxylin–eosin sections of metastatic lungs. Arrows and arrowheads indicate each nodule; scale bars, 1 mm. (**B**) The number of lung nodules in each lung. Note that $Nrf2^{-/-}$ mice exhibit a significantly increased number of metastatic lung nodules (*P < 0.05 by Mann–Whitney *U*-test). (**C**) The difference in mean lung weight was not statistically significant. Red bars indicate average. (**D**) Ki67-positive cancer cells are more abundantly observed in $Nrf2^{-/-}$ mouse lung compared with $Nrf2^{+/+}$ control mouse lung (upper panels). TUNEL-positive cell population is decreased in $Nrf2^{-/-}$ mouse lung compared with $Nrf2^{+/+}$ control mouse lung (upper panels). (**E**) The proportion of Ki67-positive cells in a metastatic 3LL lung tumor from $Nrf2^{-/-}$ and $Nrf2^$

The mean lung weight of $Nrf2^{-/-}$ mice $(0.20 \pm 0.12 \text{ g}, n = 10)$ was marginally increased compared with that of $Nrf2^{+/+}$ control mice $(0.18 \pm 0.07 \text{ g}, n = 18)$, but statistical significance could not be

verified (Figure 2C and Table II). These observations strongly support the notion that Nrf2 is important for the prevention of spontaneous metastasis of distant primary tumors to the lung.



Fig. 3. Metastatic lung cancer cells induce oxidative stress and nuclear Nrf2 accumulation in the inflammatory cells. (A) Mac1 immunostaining of cancer-bearing lung. (a and b) Mac1-positive cells (green) were abundantly recruited around metastatic tumors in $Nr/2^{-/-}$ mice compared with cancer-bearing $Nr/2^{+/+}$ control mice. td-Tomato fluorescence (red) indicates a metastatic cancer tumor. White arrowheads; Mac1-positive cells; scale bars, 100 µm. (c) Nrf2 accumulation in the nuclear extract of tumor-bearing host lung. Each lane represents individual mouse lung. Nrf2 accumulation is increased in tumor-bearing Nrf2^{+/+} host lung compared with vehicle (phosphate-buffered saline)-injected control. Nrf2^{-/-} host lung, which is highly infiltrated with metastatic tumors, had almost undetectable Nrf2 accumulation. Equal protein loading was confirmed using anti-LaminB antibody. (B) Immunostaining for Nrf2-LacZ fusion protein in tumor-bearing Nrf2-/host lung using anti- β -galactosidase antibody. The dotted circle indicates a metastatic tumor. (a) Phosphate-buffered saline-injected Nrf2^{-/-} control mice showing no LacZ-positive cells in host lung. (b) Positive staining for Nrf2-LacZ fusion protein is predominantly observed in small inflammatory cells around metastatic tumor in $Nrt2^{-/-}$ host lung (arrow heads in right two panels). (c) Higher magnification of inlet in (a). (d) Higher magnification of inlet in (b). Scale bars, 100 µm in (a and b) and 10 μ m in (c and d). (C) Representative flow cytometry dot plot of MDSCs in BM (upper panels) and lung (lower panels) from $Nrf2^{-t}$ or $Nrf2^{+t}$ mice with or without 3LL cancer metastasis. Percentages of MDSCs (Gr1+Mac1+ fraction in blue rectangle) are indicated. (D) Quantification of percentages of the MDSCs fraction in BM (upper panel) and lung (lower panel). Data were presented as means \pm SDs (*P < 0.05; **P < 0.01 by Student's *t*-test; n = 3 in each group). (E) Representative histogram of ROS level analysis in MDSCs from BM (upper panels) and lung (lower panel) using DCFDA. Right panels indicate the mean intensity of DCFDA signal. Data were presented as means \pm SDs (*P < 0.05; **P < 0.01 by Student's *t*-test; n = 3 in each group). (F) Messenger RNA (mRNA) quantification of γ -glutamylcysteine ligase regulatory subunit (γ -GCLR) and heme oxygenase-1 (HO-1) expression in MDSCs fraction by SYBR Green real-time qRT–PCR. Data were presented as means \pm SDs (**P < 0.01 by Student's *t*-test; n = 7 in each group). (D–F) red, $Nrf2^{+/+}$; blue, $Nrf2^{+/+}$ with 3LL metastasis; green, $Nrf2^{-/-}$ and orange, $Nrf2^{-/-}$ with 3LL metastasis.



Fig. 4. (A) Schematic diagram of BM transplantation experiment (see Materials and Methods). (B) $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ mice ($Nrf2^{+/+}$ recipient mice transplanted with $Nrf2^{-/-}$ BM cells) exhibit an increased number of metastatic lung nodules (**P < 0.01 by Mann–Whitney *U*-test). The difference in mean lung weight was not statistically significant. Red bars indicate average. (C) BM MDSC fraction of cancer-bearing $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ mice accumulated more ROS than cancerbearing $Nrf2^{+/+} \rightarrow Nrf2^{+/+}$ mice. The DCFDA histogram of MDSC fraction was derived from mixture of bone marrow cells either from $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ mice (n = 7; red) or $Nrf2^{+/+} \rightarrow Nrf2^{+/+}$ mice (n = 7; black). (D) A model of T-cell tolerance induced by MDSCs in cancer-bearing animal. Cancer cell-derived factors stimulate MDSC expansion in hematopoietic tissues and around tumor site, and induce accumulation of intracellular ROS. MDSC directly contacts with CD8⁺ T-cell via the molecular interaction between the major histocompatibility complex (MHC) class I molecule and the T-cell surface, which severely attenuates CD8⁺ T-cell responsiveness to subsequent antigen-presenting cell (APC)-mediated stimulation. (E) The splenic CD8⁺-single-positive T-cell population was lower in cancer-bearing $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ mice, whereas the CD4-single-positive T-cell population was equivalent between both types of recipient mice. (a) Representative flow cytometric dot plots of spleen cells. (b and c) Quantification of CD4⁻CD8⁺ cells and CD4⁺CD8⁻ cells in spleen either from $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ mice (n = 7) or $Nrf2^{+/+}$ mice (n = 7) (*P < 0.05 by Student's *t*-test).

| Table III. Spontaneous metastasis by intranuscular injection of SEE cens to $M_1 Z^{(n)} \rightarrow M_1 Z^{(n)} \rightarrow M_1 Z^{(n)} \rightarrow M_1 Z^{(n)}$ inde | | | | | | |
|--|----------------------------------|---|---|---|---|--|
| Genotype | Lung cancer incidence | Number of lung surface tumors | Lung | Primary tumor | Lymph node | |
| | | | Weight (g) | | | |
| $Nrf2^{+/+} \rightarrow Nrf2^{+/+}$ Nrf2^{-/-} \rightarrow Nrf2^{+/+} | 15/15 (100.0%) 17/17 (100.0%) | $28.7 \pm 17.2 (n = 15) 47.3 \pm 17.4^{a} (n = 17)$ | $\begin{array}{l} 0.29 \pm 0.13 \; (n = 15) \\ 0.30 \pm 0.08 \; (n = 17) \end{array}$ | $\begin{array}{l} 6.4 \pm 1.04 \; (n = 15) \\ 6.7 \pm 0.47 \; (n = 16) \end{array}$ | $\begin{array}{l} 0.14 \pm 0.06 \; (n = 15) \\ 0.15 \pm 0.06 \; (n = 16) \end{array}$ | |

Table III. Spontaneous metastasis by intramuscular injection of 3LL cells to $Nrf2^{+/+} \rightarrow Nrf2^{+/+}$ or $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ mice

 $^{a}P < 0.01$ compared with wild-type mice.

Metastatic lung cancer cells are fully viable and highly proliferative in Nrf2^{-/-} host mice

To elucidate the molecular and cellular basis for the susceptibility of cancer cell metastasis in $Nrf2^{-/-}$ mice, the proliferation and apoptotic indices of spontaneously metastasized cancer cells were determined. For this purpose, the cancer-bearing lungs from $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice were subjected to Ki67-immunohistochemistry and TUNEL analyses to assess proliferating cells and apoptotic cells, respectively. The proportions of Ki67-positive cells and TUNEL-positive cells were quantified relative to the total cancer cell population in the metastatic tumor (Figure 2D). The number of Ki67-positive cells were significantly increased in metastatic lung tumors from $Nrf2^{-/-}$ mice $(41.2 \pm 22.0\%, n = 10)$ compared with control $Nrf2^{+/+}$ mice $(15.2 \pm 12.7\%, n = 9, P < 0.01;$ Figure 2E and Table II). On the contrary, the proportion of TUNEL-positive cells was significantly decreased in the tumors of $Nrf2^{-/-}$ mice $(10.3 \pm 7.9\%, n = 10)$ when compared with $Nrf2^{+/+}$ control animals (32.1 ± 15.3%, n = 9, P < 0.01; Figure 2F and Table II). Thus, the 3LL cells that had metastasized to the lung showed increased viability and proliferative capacity in $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice, suggesting that Nrf2 deficiency in the host animal provides a favorable growth environment for metastatic cancer cells.

Metastatic lung cancer cells induce oxidative stress and Nrf2 nuclear accumulation in the tumor-localized inflammatory cells of cancer-bearing mice

We attempted to clarify response of the host microenvironment to the metastatic cancer cells. Histological examination demonstrated that a greater abundance of inflammatory cells were recruited to the metastatic tumor sites of $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice (data not shown). Indeed, vigorous infiltration of Mac1-positive inflammatory cells was observed in the metastatic tumors of $Nrf2^{-/-}$ mice relative to cancer-bearing $Nrf2^{+/+}$ control mice [arrow heads in Figure 3A (a and b)], suggesting a profound inflammation had been evoked in $Nrf2^{-/-}$ mice in response to the metastasis.

We also examined the accumulation of oxidative stress damage by immunohistochemical analysis of 8-OHdG, a representative DNA damage marker induced by an OH radical (20). A small population of 8-OHdG-positive cells was consistently observed in the spontaneous metastatic tumor from both $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice (supplementary Figure 4A and B is available at *Carcinogenesis* Online). However, a greater abundance of 8-OHdG-positive cells was observed in the host lung of $Nrf2^{-/-}$ mice relative to $Nrf2^{+/+}$ control mice (supplementary Figure 4 is available at *Carcinogenesis* Online). Higher magnification images show that 8-OHdG-immunoreactivity is predominantly found in infiltrating inflammatory cells such as macrophages and lymphocytes (arrow heads and asterisks in supplementary Figure 4 is available at *Carcinogenesis* Online).

Assuming that the increased oxidative stress is accompanied by Nrf2 accumulation in those infiltrating inflammatory cells, we examined the protein levels of Nrf2 in the tumor-bearing host lung. In immunoblotting analyses of whole lung nuclear extracts, we observed a distinct accumulation of Nrf2 protein in the lung cells of cancerbearing $Nrf2^{+/+}$ mice in comparison with vehicle-injected $Nrf2^{+/+}$ host mice (Figure 3A(c)). Although the $Nrf2^{-/-}$ host lung was highly occupied with metastatic 3LL-tumors compared with $Nrf2^{+/+}$ host mice (Figure 1C), tumor-bearing $Nrf2^{-/-}$ host lung exhibited very weak 3LL-induced Nrf2 protein accumulation (Figure 1C). This ob-

servation suggests that the accumulated Nrf2 protein in $Nrf2^{+/+}$ host lung was mainly derived from the host lung cells, as opposed to the small population of metastasized 3LL cancer cells.

To further clarify this contention we took advantage of the fact that in *Nrf2*-deficient mice the N-terminal domain of Nrf2 forms a fusion protein with the LacZ reporter gene (3). We examined nuclear accumulation of the Nrf2–LacZ fusion protein by immunohistochemistry. As expected, positive nuclear immunoreactivity for β -galactosidase was induced in the cancer-bearing host lung [Figure 3B (b and d)]. Nuclear accumulation of Nrf2–LacZ fusion protein was predominantly observed in the inflammatory cells recruited around metastatic lung tumors in *Nrf2–l*acZ protein in vehicle-injected *Nrf2^{-/-}* mice [Figure 3B (a and c)]. These results collectively suggest that lung cancer metastasis induced the accumulation of Nrf2 in inflammatory cells of the host animal.

Metastatic lung cancer cells induce ROS accumulation in the MDSC fraction of cancer-bearing Nrf2-deficient mice

Given the greatly increased ROS level and nuclear accumulation of Nrf2 protein in the inflammatory cells of cancer-bearing Nrf2-/mice, we next tried to clarify what cellular fraction responded most significantly to the cancer-induced oxidative stress. In this regard, we particularly focused on the MDSC fraction from lung and BM. The percentage of MDSCs (Mac1+Gr1+) in BM was slightly increased by the presence of metastatic 3LL lung tumors in both $Nrf2^{-/-}$ and Nrf2+/+ mice, but statistical significance was not verified (Figure 3C and D). In the vehicle-treated $Nrf2^{+/+}$ lungs, the MDSC population was minimal $(0.035 \pm 0.02\%)$, whereas the vehicletreated Nrf2^{-/-} lung harbored a 5-fold increase in the MDSC fraction $(0.177 \pm 0.122\%)$ (Figure 3C). Of note, the metastatic lung cancer induced a significant expansion of the MDSC fraction in the lung of both $Nrf2^{-/-}$ and $Nrf2^{+/+}$ control mice. In good agreement with the higher number of Mac1-positive cells in the cancer-bearing $Nrf2^{-/-}$ lung section [Figure 3A (a and b)], lung-derived MDSC population was significantly expanded in the cancer-bearing $Nrf2^{-/-}$ mice (Figure 3D).

We also analyzed ROS levels in the MDSC fraction by means of DCFDA fluorescence as a marker. The intracellular ROS level showed the strongest accumulation in MDSCs compared with the non-MDSC fraction of BM cells (supplementary Figure 5 is available at *Carcinogenesis* Online). Both BM- and lung-derived MDSCs in the vehicle-treated $Nrf2^{-/-}$ mice accumulated a greater level of ROS than those from the vehicle-treated $Nrf2^{+/+}$ mice (Figure 3E). The cancer metastasis induced even greater levels of ROS accumulation in the $Nrf2^{-/-}$ MDSCs relative to the $Nrf2^{+/+}$ MDSCs from both tissues (Figure 3E).

Assuming the increased ROS level in $Nrf2^{-/-}$ MDSCs could be due to a compromised antioxidant defense system, we examined the messenger RNA expression level of two representative antioxidant genes, i.e. *HO-1* and γ -*GCLR*, both are direct target genes of Nrf2 (21). The basal expression levels for both genes in sorted MDSCs were not so significantly different between vehicle-treated $Nrf2^{-/-}$ and $Nrf2^{+/+}$ control mice (Figure 3F). The expression of both genes was highly induced in MDSCs of cancer-bearing $Nrf2^{+/+}$ mice, whereas the expression of these genes was hardly increased in MDSCs of cancer-bearing $Nrf2^{-/-}$ mice (Figure 3F). These results revealed that



Fig. 5. Decreased susceptibility to cancer metastasis in *Keap1*-knockdown (*Keap1*^{f/f}) mice. (A) The number of 3LL lung nodules in a spontaneous metastasis model was lower in the *Keap1*^{f/f} mice (18.3 ± 31.3, n = 7) compared with *Keap1*^{+/+} mice (46.1 ± 47.2, n = 18). Mean lung weight was not significantly different. (B) Representative histogram of ROS level analysis in MDSCs from BM using DCFDA. ROS level in MDSCs fraction of vehicle-treated *Keap1*^{f/f} mice (n = 3; pink) was slightly lower than the vehicle-treated *Keap1*^{+/+} control mice (n = 3; red). Consistently, the cancer-bearing *Keap1*^{f/f} mice (n = 3; light blue) present with significantly lower levels of ROS in the MDSCs fraction when compared with cancer-bearing *Keap1*^{f/f} mice (n = 3; blue). Data were presented as means ± SDs (*P < 0.05 by Student's *t*-test). (C) Resistance to B16-F10 melanoma metastasis in *Keap1*^{f/f} mouse lung. Arrows and arrowheads indicate

Nrf2-mediated antioxidant gene expression is essential for the suppression of ROS production in MDSCs of cancer-bearing animals.

Nrf2-deficient hematopoietic cells are responsible for the higher metastatic susceptibility of $Nrf2^{-\prime-}$ mice

To address the question whether Nrf2-deficiency in the hematopoietic system is responsible for the higher susceptibility to metastatic lung cancer in Nrf2-/- mice, we performed BM transplantation experiments. As schematically depicted in Figure 4A, the hematopoietic system of lethally irradiated Nrf2+/+ mice (CD45.2) was reconstituted by implanting $Nrf2^{-/-}$ or $Nrf2^{+/+}$ BM cells (CD45.1). After 8 weeks of recovery period, the recipient mice transplanted with either $Nrf2^{-/-}$ or $Nrf2^{+/+}$ BM cells (hereafter, we refer these mice to as $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ and $Nrf2^{+/+} \rightarrow Nrf2^{+/+}$, respectively) were subjected to evaluation for spontaneous metastasis of 3LL cells. The number of spontaneous metastatic tumor nodules on the lung surface of $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ recipient mice $(47.3 \pm 17.4, n = 17)$ was significantly increased compared with those $Nrf2^{+/+} \rightarrow Nrf2^{+/+}$ control recipient mice (28.7 ± 17.2, n = 15, P < 0.01; Figure 4B and Table III). Furthermore, we confirmed the increased ROS accumulation in MDSCs from BM of $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ mice compared with $Nrf2^{+/+} \rightarrow Nrf2^{+/+}$ mice, indicating a cell-autonomous function of Nrf2 for the clearance of ROS produced by MDSCs (Figure 4C).

As shown in Figure 4D, the accumulation of ROS in MDSCs has been shown to suppress the activation and proliferation of CD8+ T-cells (8). Therefore, we examined CD4- or CD8-single-positive T-cell populations in the spleen of cancer-bearing $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ and $Nrf2^{+/+} \rightarrow Nrf2^{+/+}$ recipient mice. Splenic CD4- and CD8single-positive T-cells populations were not significantly different between untreated $Nrf2^{-/-}$ and $Nrf2^{+/+}$ control mice (supplementary Figure 6 is available at Carcinogenesis Online). However, splenic CD8-single-positive T-cell population was significantly decreased in cancer-bearing $Nrf2^{-/-} \rightarrow Nrf2^{+/+}$ mice relative to $Nrf2^{+/+} \rightarrow Nrf2^{+/+}$ control mice (Figure 4E). Conversely, the CD4+ T-cell population did not differ much between these mice, indicating that Nrf2-deficient MDSCs provoked the suppression of CD8+ T-cell proliferation utilizing the high-level accumulation of ROS (Figure 4D). These results thus demonstrate that the Nrf2 function in hematopoietic cells is indispensable in preventing aberrant ROS accumulation in MDSCs and for the maintenance of CD8+ T-cell immunity.

Constitutive activation of Nrf2 in host mice leads to resistance to metastatic lung cancer

To further verify the anti-metastatic function of host Nrf2, we adopted a spontaneous metastasis model using 3LL cells injected into Keap1knockdown (Keap1^{f/f}) mice, which retain constitutively elevated levels of Nrf2 (13). We found that the $Keap1^{f/f}$ mice exhibited a marked resistance against the spontaneous metastasis of 3LL cancer cells. The number of 3LL lung surface nodules in the spontaneous metastasis model was significantly decreased in *Keap1*^{f/f} mice (18.3 \pm 31.3, n = 7) in comparison with Keap1^{+/+} mice (46.1 ± 47.2, n = 18, P < 0.05, Figure 5A and Table IV). Furthermore, the *Keap1*^{f/f} mice showed a similar resistance to experimental metastasis by B16-F10 melanoma cells (Figure 5C). The number of B16-F10 lung surface nodules was dramatically decreased in Keap1^{f/f} mice $(8.0 \pm 2.1, n = 5)$ compared with Keap1^{+/+} control recipient mice $(40.4 \pm 11.1, n = 5, P < 0.01)$, Figure 5D; supplementary Table IB is available at Carcinogenesis Online). Lung weights were not significantly different from that of Keap1+/+ controls in either model (Table IV; supplementary Table IB is available at Carcinogenesis Online).

We surmise that the high-level accumulation of ROS in MDSCs may be responsible for the increased lung cancer metastasis in $Nrf2^{-/-}$ mice. We therefore examined whether the resistance to metastatic cancer in *Keap1*^{f/f} mice correlates with a suppression of ROS in MDSCs. To this end, we collected the Mac1+Gr1+ fraction from cancer-bearing BM of *Keap1*^{f/f} mice and examined intracellular ROS levels using DCFDA. The population of MDSCs was not significantly altered in the *Keap1*^{f/f} compared with wild-type control mice (data not shown). However, the vehicle-treated *Keap1*^{f/f} mice exhibited slightly lower levels of ROS in MDSCs compared with the *Keap1*^{+/+} control mice. Moreover, in the cancer-bearing *Keap1*^{f/f} mice, the ROS levels in the MDSC fraction were significantly lower than the cancerbearing *Keap1*^{+/+} control mice (Figure 5B).

Taken together, the metastatic cancer resistance of $Keap1^{t/f}$ mice demonstrates, in combination with the metastatic cancer susceptibility of $Nrf2^{-/-}$ mice, that the host Nrf2–Keap1 system plays a crucial function in preventing cancer metastasis by regulating the redox balance of cells of the hematopoietic and immune system.

Discussion

In the present study, we demonstrate that mice deficient in Nrf2 exhibit an accelerated colonization and proliferation of metastatic cancer cells in the lung, which is accompanied by a profound recruitment of inflammatory cells. The expanded inflammatory cell population in the cancer-bearing mice displayed an increase in oxidative stress and an accumulation of Nrf2 protein. Thus, these observations suggest that the anti-metastatic activity of Nrf2 may arise from its ability to facilitate an appropriate immunological response against the colonizing cancer cells.

Numerous reports have described the anti-inflammatory activity of Nrf2 under different pathological conditions. In the elastaseprovoked emphysema model, lung inflammation was significantly exacerbated in Nrf2-deficient mice (22). Likewise, lung inflammation augmented by Nrf2-deficiency was associated with enhanced activation of nuclear factor-kappaB in response to lipopolysaccharide or tumor necrosis factor- α administration (23). Consistent with previous reports, the lung tissue of cancer-bearing $Nrf2^{-/-}$ mice harbors a large population of inflammatory cells. Notably, in cancer-bearing Nrf2-/mice, an enhanced recruitment of MDSCs occurs, especially to the lung. These Nrf2-deficient MDSCs display a greater accumulation of intracellular ROS relative to wild-type MDSCs. The expression of inducible antioxidant genes is decreased in the MDSC fraction of cancer-bearing $Nrf2^{-/-}$ mice. These initial observations suggested the involvement of MDSCs and associated cancer immune-tolerance, for the higher susceptibility of $Nrf2^{-/-}$ mice to metastasis.

In mice, MDSCs are associated with the markers $GR1^+Mac1^+$, the putative macrophage, dendritic cell or granulocyte progenitor fraction. These immune cells engulf cancer cells and accumulate ROS through the influence of several cancer-derived factors (24). Indeed, tumor-bearing conditions lead to a significant increase in intracellular ROS levels in MDSCs compared with the non-MDSC fraction of BM cells (supplementary Figure 5 is available at *Carcinogenesis* Online). Of note, even vehicle-treated *Nrf2*^{-/-} mice exhibited a high-level accumulation of ROS in the MDSC fraction. By contrast, vehicle-treated *Keap1*^{f/f}-derived MDSCs had suppressed levels of ROS. These observations suggest that the Nrf2-mediated anti-oxidative response system maintains normal immune status by suppressing the aberrant activation of MDSCs.

It is not only antigen-specific (adaptive) CD8⁺ T-cell immunity that is suppressed by MDSCs, but also non-specific (innate) immunity, the latter of which is the first line of host defense against metastasizing cancer cells. In fact, the increased production of ROS by MDSCs has been suggested to induce the suppression of innate T-cell immunity as well (24). Thus, relatively high

each nodule. Whole lung tissue from two independent mice and representative hematoxylin–eosin staining are shown; scale bars, 2 mm. (**D**) The number of B16-F10 lung surface tumors was significantly decreased in *Keap1^{t/t}* mice (8.0 ± 2.1 , n = 5) compared with *Keap1^{+/+}* control recipient mice (40.4 ± 11.1 , n = 5). The lung weight of cancer-bearing *Keap1^{t/t}* mice was not significantly different from that of *Keap1^{+/+}* control. Red bars indicate average. (*P < 0.05; **P < 0.01 by Student's *t*-test).

| Table IV. Spontaneous metastasis by intramuscular 3LL injection in $Keap1^{\mu\nu}$ and $Keap1^{\mu\nu}$ mice | | | | | | | |
|--|------------------------------|--|--|--|--|--|--|
| Genotype | Lung cancer incidence | Number of lung surface tumors | Lung | Primary tumor | Lymph node | | |
| | | | Weight (g) | | | | |
| Keap1 ^{+/+} Keap1 ^{f/f} | 17/18 (94.0%) 4/7 (57.1%) | $46.1 \pm 47.2 (n = 18) 18.3 \pm 31.3^{a} (n = 7)$ | $\begin{array}{l} 0.18 \pm 0.07 \; (n = 18) \\ 0.14 \pm 0.05 \; (n = 7) \end{array}$ | $9.6 \pm 1.13 \ (n = 18)$ $9.4 \pm 0.9 \ (n = 7)$ | $\begin{array}{l} 0.1 \pm 0.09 \; (n = 18) \\ 0.1 \pm 0.08 \; (n = 7) \end{array}$ | | |

 $^{a}P < 0.05$ compared with wild-type mice.

accumulation of ROS in the MDSC fraction of unchallenged Nrf2-/mice may lead to suppression of innate immunity, which generates a favorable environment for the initial homing of cancer cells. In this regard, MDSCs-mediated suppression of innate immunity might contribute to the defective innate immune response against experimental sepsis, which has been previously reported for Nrf2-deficient mice (21). Meanwhile, the lower levels of ROS in the Keap1^{f/f} MDSCs may prohibit the formation of a favorable niche for metastatic invasion. It would be of particular importance to examine whether the innate immune response of the Keap1^{f/f} mice is more efficiently activated than that of wild-type mice.

After developing a metastatic tumor, wild-type MDSCs displayed a relatively smaller amount of ROS, which was associated with robust upregulation of antioxidant enzymes. In stark contrast, MDSCs from cancer-bearing $Nrf2^{-/-}$ mice were found to generate abundant ROS compared with wild-type controls. In Nrf2-deficient MDSCs, antioxidant enzyme induction was markedly attenuated. These observations suggest that the normal clearance of ROS by Nrf2-induced enzymes in the MDSC population is directly linked to the prevention of metastatic progression. The functional contribution that Nrf2 makes to the hematopoietic and immune systems in preventing metastatic lung cancer was confirmed by BM transplantation experiments. In wild-type recipient mice that were transplanted with Nrf2-deficient BM cells, the susceptibility to spontaneous metastasis of 3LL lung cancer cells was significantly exacerbated, which is associated with an increased abundance of ROS in donor-derived MDSCs. Activated MDSCs are reported to secrete several angiogenic growth factors, such as Bombina variegata 8 kDa (Bv8) and vascular endothelial growth factor (VEGF) (25), which can aid in the proliferation and vascularization of metastatic cancer cells. Thus, it is quite plausible that the proper function of Nrf2-mediated antioxidant response in MDSCs is important for the prevention of metastatic cancer progression.

Nrf2-deficient mice are susceptible to chemically induced carcinogenesis in many different organ systems (5,26-28). By contrast, an alteration in the Keap1-Nrf2 interaction, which provokes higher Nrf2 activity, leads to multiple advantages for proliferation of cancer cells and this model has been referred to as 'the double-edge sword mechanism' (29-33). These two paradoxical observations indicate that Nrf2-deficient cells are susceptible to cancer initiation, but the Nrf2-deficient pre-cancer cells might have a lower proliferative capacity in comparison with the Keap1-mutated pre-cancer cells. The high frequency of chemical carcinogenesis in the germ-line Nrf2deficient mice might arise from an altered microenvironment, which promotes the vigorous proliferation of cancer cells. Consistent with this hypothesis, somatic loss-of-function mutation of Nrf2 have not been identified in cancer cells thus far.

In summary, our present study demonstrates that Nrf2 activity in the hematopoietic and immune system is crucial for the prevention of cancer cell metastasis. Given that pulmonary metastases is a leading cause of cancer mortality, the administration of chemopreventive agents capable of enhancing Nrf2 activity to boost the antioxidant defenses of the hematopoietic system may prove an effective therapy for the prevention of metastasis, especially to the lung.

Supplementary material

Supplementary Figures 1-6 and Table I can be found at http://carcin .oxfordjournals.org/

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References

- 1. Itoh, K. et al. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun., 236, 313-322.
- 2. Motohashi, H. et al. (2004) Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. Proc. Natl Acad. Sci. USA, 101, 6379-6384.
- 3. Itoh, K. et al. (2003) Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. Genes Cells, 8, 379-391
- 4. Kobayashi, A. et al. (2004) Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol. Cell. Biol., 24, 7130-7139.
- 5. Iida, K. et al. (2007) Nrf2 and p53 cooperatively protect against BBNinduced urinary bladder carcinogenesis. Carcinogenesis, 28, 2398-2403.
- 6. Kasai, H. et al. (2006) Oxidative DNA damage: mechanisms and significance in health and disease. Antioxid. Redox Signal., 8, 981-983.
- 7. Ostrand-Rosenberg, S. et al. (2009) Myeloid-derived suppressor cells: linking inflammation and cancer. J. Immunol., 182, 4499-4506.
- 8. Nagaraj, S. et al. (2007) Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nat. Med., 13, 828-835.
- 9. Sato, T. et al. (1989) Conversion of Lewis lung carcinoma into ascitic form. Oncology, 46, 188-192.
- 10. Poste, G. et al. (1980) In vitro selection of murine B16 melanoma variants with enhanced tissue-invasive properties. Cancer Res., 40, 1636-1644.
- 11. Winnard, P.T. Jr. et al. (2006) Noninvasive optical tracking of red fluorescent protein-expressing cancer cells in a model of metastatic breast cancer. Neoplasia, 8, 796-806.
- 12. Stoner, G.D. et al. (1973) Test for carcinogenicity of food additives and chemotherapeutic agents by the pulmonary tumor response in strain A mice. Cancer Res., 33, 3069-3085.
- 13. Okada, K. et al. (2008) Ursodeoxycholic acid stimulates Nrf2-mediated hepatocellular transport, detoxification, and antioxidative stress systems in mice. Am. J. Physiol. Gastrointest. Liver Physiol., 295, G735-G47.
- 14. Sinha, P. et al. (2008) Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. J. Immunol., 181, 4666-4675.
- 15. Nefedova, Y. et al. (2007) Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. Cancer Res., 67, 11021-11028.

- Kim,H.J. *et al.* (2008) Nrf2 activation by sulforaphane restores the agerelated decrease of T(H)1 immunity: role of dendritic cells. *J. Allergy Clin. Immunol.*, **121**, 1255–1261e7.
- Dignam, J.D. *et al.* (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.
- Watai, Y. et al. (2007) Subcellular localization and cytoplasmic complex status of endogenous Keap1. Genes Cells, 12, 1163–1178.
- Kyhse-Andersen, J. (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods, 10, 203–209.
- Aoki, Y. *et al.* (2001) Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. *Toxicol. Appl. Pharmacol.*, 173, 154–160.
- Nguyen, T. *et al.* (2003) Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annu. Rev. Pharmacol. Toxicol.*, **43**, 233–260.
- Ishii, Y. *et al.* (2005) Transcription factor Nrf2 plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema. *J. Immunol.*, **175**, 6968–6975.
- Thimmulappa, R.K. *et al.* (2006) Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. *J. Clin. Invest.*, 116, 984–995.
- Nagaraj, S. et al. (2008) Tumor escape mechanism governed by myeloidderived suppressor cells. Cancer Res., 68, 2561–2563.
- Shojaei, F. *et al.* (2008) Refractoriness to antivascular endothelial growth factor treatment: role of myeloid cells. *Cancer Res.*, 68, 5501–5504.

- 26. Khor, T.O. *et al.* (2008) Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer. *Cancer Prev. Res. (Phila. Pa)*, 1, 187–191.
- 27. Ramos-Gomez, M. et al. (2001) Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc. Natl Acad. Sci. USA, 98, 3410–3415.
- Xu,C. *et al.* (2006) Inhibition of 7,12-dimethylbenz(a)anthraceneinduced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. *Cancer Res.*, 66, 8293–8296.
- 29. Ohta, T. *et al.* (2008) Loss of Keap1 function activates Nrf2 and provides advantages for lung cancer cell growth. *Cancer Res.*, **68**, 1303–1309.
- Padmanabhan,B. *et al.* (2006) Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. *Mol. Cell*, 21, 689–700.
- 31. Shibata, T. *et al.* (2008) Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology*, **135**, 1358–13681368 e1–4.
- 32. Shibata, T. *et al.* (2008) Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. *Proc. Natl Acad. Sci. USA*, **105**, 13568–13573.
- Singh,A. et al. (2006) Dysfunctional KEAP1-NRF2 interaction in nonsmall-cell lung cancer. PLoS Med., 3, e420.

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