Nrf2 Prevents Initiation but Accelerates Progression through the Kras Signaling Pathway during Lung Carcinogenesis

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Abstract

Nrf2 (Nfe2l2) governs cellular defenses against oxidative and electrophilic stresses and protects against chemical carcinogenesis. However, many cancers have been found to accumulate NRF2 protein, raising questions of precisely how Nrf2 contributes to carcinogenesis. In this report, we explored such questions in an established urethane-induced multistep model of lung carcinogenesis. Consistent with earlier observations, Nrf2-deficient (Nrf2−/−) mice exhibited a relative increase in tumor foci by 8 weeks after urethane administration. However, after 16 weeks, we observed a relative reduction in the number of tumors with more malignant characteristics in Nrf2−/− mice. Furthermore, all Nrf2+/− tumors harbored activated mutations in Kras, whereas Nrf2−/− tumors were rarely associated with similar Kras mutations. Overall, our results established that Nrf2 has two roles during carcinogenesis, one of which is preventive during tumor initiation and the second that promotes malignant progression. These findings establish Nrf2 inhibitors as rational tools to prevent malignant progression in lung cancer, whereas Nrf2 activators are more suited for lung cancer prevention.

Introduction

Nrf2 (Nuclear factor-erythroid derived 2-like 2, Nfe2l2) is a leucine zipper transcription factor and plays an important role in the maintenance of redox balance and cytoprotection against chemical carcinogens (1, 2). When subjected to oxidative and electrophilic stress conditions, Nrf2 is released from Keap1 (Kelch-like ECH-associated protein 1)-mediated rapid degradation. Nrf2 is stabilized, accumulated, and translocates to the nucleus, where Nrf2 dimerizes with a small Maf protein (sMaf). The Nrf2–sMaf heterodimer binds to a specific DNA sequence, referred to as the antioxidative/electrophile response element (ARE/EpRE), and induces the expression of a cohort of cytoprotective enzyme genes, such as Nqo1, HO-1, Gclc, and Gstp1/p2 (1, 2).

Previous reports on chemically induced carcinogenesis have shown that Nrf2-deficient (Nrf2−/−) mice tend to form a larger number of tumors than wild-type (Nrf2+/+) mice, indicating that the perturbation in the carcinogen detoxification system in Nrf2−/− mice leads to cancer susceptibility in various tissues (3–6). However, recent studies have revealed that the NRF2 protein is significantly accumulated in many types of human cancers through several independent mechanisms. For instance, somatic mutations in the interface of KEAP1 and NRF2 (7) or epigenetic modifications in the promoter region of KEAP1 genes (8) gives rise to the accumulation of NRF2. As these changes are often related to accelerated cancer cell growth and poor clinical prognosis, the oncogenic side of Nrf2 function has been attracting considerable attention. Furthermore, we recently showed that the Nrf2−/− mice transplanted with Lewis lung carcinoma (3LL) cells provide a more tumor-permissive immune microenvironment than the Nrf2+/+ mice (9). These observations raise a fundamental question of whether Nrf2 exerts cancer-preventive or -promotive activity in each stage of the carcinogenic process. To address this issue, we used the urethane (ethyl carbamate)-induced lung carcinogenesis model, a well-known multistep murine carcinogenesis model (10).

Urethane has been extensively used as an inducer of chemical lung carcinogenesis (11). Administration of urethane evokes hyperplasia or adenoma formation in the lung and eventually leads to adenocarcinomas in a time-dependent manner (10). Through cytochrome P450 2E1 (Cyp2e1)-mediated oxidation, urethane is converted to vinyl carbamate epoxide, which serves as an ultimate carcinogen by inducing DNA-, RNA-, or protein–adduct formation in airway epithelial cells (12). In the detoxification process, microsomal epoxide hydrolase (mEH) converts vinyl carbamate epoxide into 1,2-dihydroxymethyl carbamate, which subsequently undergoes the glutathione conjugation catalyzed by Gstp1/p2 (13) and is excreted into urine (14).

In the present study, we found that Nrf2−/−-mutant mice developed a large number of urethane-induced lung micronodules in the early phase after urethane administration.
However, in the later stages, Nrf2<sup>+/−</sup> mice developed a higher number of Kras-mutated adenocarcinomas than did Nrf2<sup>−/−</sup> mice. These results show that Nrf2 deficiency leads to an increased susceptibility to chemical carcinogens and resultant high-level tumor initiations, whereas Nrf2 serves as an oncogenic factor that accelerates malignant progression of Kras-mutated adenocarcinomas in the later stages of lung carcinogenesis.

**Materials and Methods**

**Experimental animals**

Nrf2<sup>−/−</sup> mice with an ICR/CD-1 genetic background (out-bred) were used in this study (2, 15). Age-matched (5–9 weeks) Nrf2<sup>+/−</sup> mice were used as concurrent controls. The mice were maintained in a facility free of specific pathogens (SPF). Nude mice (8- to 9-weeks old) were purchased from CLEA Japan. All animal experiments were conducted with the approval of the Tohoku University Animal Care Committee.

**Lung carcinogenesis experiments**

Mouse lung tumors were induced by the intraperitoneal injection of urethane (1 g/kg body weight; refs. 16, 17). For the enumeration of lung surface tumors, the lungs were removed and the total number of lung surface nodules per mouse was counted macroscopically.

**Kras mutation analysis**

PCR-amplified DNA samples from the urethane-induced lung tumors and intact stromal tissues were subjected to sequencing analysis to detect Kras mutations. The primers amplifying the nucleotide sequences in the second exon of Kras gene, which contains codon 61, are listed in Supplementary Table S1.

**Microarray analysis**

Surface lung tumors were excised, and surrounding tissues were carefully removed under a observation via a stereo-microscope. The lung tumors and nontumor regions of Nrf2<sup>+/−</sup> and Nrf2<sup>−/−</sup> mice that had been treated with urethane (8 mice/group) were pooled and subjected to a whole-mouse genome microarray analysis (4 × 44 k; Agilent Technologies). The expression data were analyzed with GeneSpring software (Silicon Genetics). Heatmaps were generated using Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/) and JAVA Treeview 159 (http://jtreeview.sourceforge.net/). The classification of the selected genes according to their biologic and toxicologic functions was done using Ingenuity Pathway Analysis (IPA) software (Ingenuity system). P value, represented as the negative log ratio of the IPA results, is the probability based on Fisher exact test. The GEO accession number for the microarray data is GSE46048.

**Flow cytometry**

Analyses of the bone marrow cells were conducted using FACS-Caliber (BD Pharmingen). Quantification of reactive oxygen series (ROS) level with 2,7-dichlorodihydrofluorescein diacetate (DCFDA), separation of myeloid-derived suppressor cells (MDSC), and T cells has been described previously (9).

**Immunoblotting analysis**

Nuclear extracts were prepared from NIH3T3 cells that were treated with the indicated concentrations of urethane for 6 hours. The mouse lung nuclear extracts were prepared from the Nrf2<sup>+/−</sup> mice administered either vehicle (PBS) or urethane (1 g/kg body weight) for the indicated time periods. Immunoblotting analysis was conducted using anti-Nrf2 and anti-lamin B antibodies (Santa Cruz Biotechnology) as described previously (9).

**Statistical analyses**

The data are expressed as the mean ± SD. The statistical differences were determined using Student t test or the Mann–Whitney U test. The values for either the incidence of lung nodules or large tumors were analyzed using the Fisher exact probability test. P values less than 0.05 were considered significant.

See Supplementary Materials and Methods for further details.

**Results**

**Urethane induces accumulation of Nrf2 and detoxification enzymes in the lung**

Although urethane exerts carcinogenic activity through electrophilicity of its metabolites (18), it remains unclear whether urethane induces Nrf2 accumulation. To address this question, we examined Nrf2 accumulation in NIH/3T3 cells treated with urethane (10 or 50 μmol/L for 6 hours). Upon treatment with urethane, Nrf2 accumulated in nuclear fraction of NIH/3T3 cells (Fig. 1A). In addition, we found that Nrf2 accumulated in the lung tissues 3 hours after intraperitoneal injection of urethane (1 g/kg body weight) into Nrf2<sup>+/−</sup> mice (Fig. 1B). As the Nrf2 accumulation could be monitored by the immunohistochemistry for Nrf2–β-galactosidase fusion protein expressed from the Nrf2-targeted allele (9), we conducted anti-β-galactosidase antibody staining with paraffin-embedded lung sections. The Nrf2–β-galactosidase fusion protein predominantly accumulated in the bronchial epithelium of the urethane-treated mice (arrows in Fig. 1C). These results show that urethane induced Nrf2 accumulation in the nucleus of airway epithelial cells.

To clarify downstream events upon the urethane treatment, we next examined mRNA expression of Nrf2 target genes in the lung. Expression levels of Nrf2 target genes, i.e., Nqo1, H0-1, and Gclc, were markedly induced in the Nrf2<sup>+/−</sup> mice in a time-dependent manner (Fig. 1D). In contrast, inducible expression of the Nrf2 target genes was attenuated in the Nrf2<sup>−/−</sup> mice. These observations show that urethane-treatment increased the expression of Nrf2-target genes through the induction of Nrf2 protein accumulation in normal lung tissue.

**Cyp2e1 expression is independent of Nrf2 activity**

It has been shown that Cyp2e1-mediated oxidation plays an essential role in urethane-induced carcinogenicity (Supplementary Fig. S1A) and, indeed, Cyp2e1-deficient mice are resistant to urethane-induced lung carcinogenesis (14). To clarify whether the Nrf2-deficiency affects the urethane bioactivation to its carcinogenic metabolite, we examined the
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Urethane elicits acute inflammation in urethane treatment. We found that urethane-induced expression of Nrf2 and its downstream genes is independent of the Nrf2 activity. In Cyp2e1−/− mice, which presumably represent increased proliferation of the epithelial cells (Supplementary Fig. S2B). Therefore, we surmised that Nrf2−/− mice were susceptible to the urethane-induced inflammatory lung injury; thereafter, the inflammation-induced proliferating pulmonary epithelial cells might give rise to high frequency of lung tumorigenesis.

Nrf2−/− mice are susceptible to urethane-induced lung carcinogenesis in early stage

To examine susceptibility to lung cancer, we adopted urethane-induced carcinogenesis experiments with Nrf2−/− and Nrf2+/+ mice in the following 4 different observation periods: (i) very short-term observation (4 weeks); (ii) short-term observation (8 weeks); (iii) middle-term observation (16 weeks); and (iv) long-term observation (24 weeks; Supplementary Fig. S3). At 4 weeks after the intraperitoneal urethane administration, which presumably represent increased proliferation of the epithelial cells (Supplementary Fig. S2B). Notably, we observed a greater increase in Ki-67-positive cells in the cell clusters of Nrf2−/− mice, which presumably represent increased proliferation of the epithelial cells (Supplementary Fig. S2B). Notably, we observed a greater increase in Ki-67-positive cells in the cell clusters of Nrf2−/− mice, which presumably represent increased proliferation of the epithelial cells (Supplementary Fig. S2B). Therefore, we surmised that Nrf2−/− mice were susceptible to the urethane-induced inflammatory lung injury; thereafter, the inflammation-induced proliferating pulmonary epithelial cells might give rise to high frequency of lung tumorigenesis.

Figure 1. Urethane administration activates Nrf2 and its downstream genes in vitro and in vivo. A and B, Nrf2 expression was detected by Western blotting in NIH3T3 cells and urethane-treated mouse lungs. Diethyl maleate (DEM) and PBS were used as positive and negative controls, respectively. Anti-lamin B antibody staining was used for equal nuclear protein loading control. C, induction of Nrf2-LacZ protein by urethane treatment in lung epithelial cells in Nrf2−/− and Nrf2+/+ mice. Arrows indicate positive cells. The black box indicates a high magnification region. Black scale bar, 20 μm; red scale bar, 5 μm. D, induction of Nrf2-target genes by urethane treatment in lung. mRNA expression of Nrf2-target genes was examined by qRT-PCR analysis using lung tissues from Nrf2−/− and Nrf2+/+ mice after the urethane treatment. The expression level of each mRNA was normalized to the β-actin abundance. The data are presented as the mean ± SD. The significant differences determined by Student t test are indicated (*, P < 0.05 and **, P < 0.01).
microscopic nodules (average 11.7, \( n = 3; P < 0.05 \)) than the \( \text{Nrf2} /{+/-} \) mice (average 1.67, \( n = 3; \) Fig. 2A and B), whereas both groups rarely showed gross surface tumors. At 8 weeks after urethane administration, all the urethane-treated \( \text{Nrf2} /{-} \) mice developed macroscopic (\( \phi > 0.5 \text{ mm} \)) lung surface tumors, whereas only half of the \( \text{Nrf2} /{+/-} \) mice developed gross surface tumors (Fig. 2C and D, Table 1, A). Furthermore, \( \text{Nrf2} /{-} \) mice had a much higher number of lung surface nodules than \( \text{Nrf2} /{+/-} \) mice. These results indicate that Nrf2 contributes to the prevention of urethane-induced carcinogenesis in the early tumorigenic stages (4 or 8 weeks).

**Nrf2 /{-} mice are resistant to urethane carcinogenesis in the middle-term observation period**

Given the oncogenic function of NRF2 in human cancers (20), we hypothesized that \( \text{Nrf2} /{-} \) tumor cells might have a lower proliferative potency than \( \text{Nrf2} /{+/-} \) tumor cells. To test this hypothesis, we conducted a middle-term observation (16 weeks; Supplementary Fig. S3). The total number of gross surface tumors (\( \phi > 0.5 \text{ mm} \)) per mouse was comparable between 2 genotypes (Fig. 2F and Table 1, B). However, when we determined the diameter of the largest tumors in the individual lung, we found that, while all \( \text{Nrf2} /{+/-} \) mice developed large tumors (\( \phi > 1.5 \text{ mm} \)), only 1 out of the 8 \( \text{Nrf2} /{-} \) mice harbored such large tumors (Fig. 2E and G). Furthermore, when we measured all tumors in the individual lung, the average diameter of \( \text{Nrf2} /{+/-} \) tumors tended to be larger (average 1.2 mm) than those of \( \text{Nrf2} /{-} \) mice (average 0.9 mm; Fig. 2H). These results show that the \( \text{Nrf2} /{-} \) mice showed a lower susceptibility to urethane-induced carcinogenesis than the \( \text{Nrf2} /{+/-} \) mice at 16 weeks after the urethane administration.

**Nrf2-deficient mice are resistant to urethane carcinogenesis in the long-term observation period**

An observation at an even late stage revealed more significant difference in the cancer progression between \( \text{Nrf2} /{-} \) and \( \text{Nrf2} /{+/-} \) mice. At 24 weeks after the urethane administration (Supplementary Fig. S3D), \( \text{Nrf2} /{-} \) mice exhibited a markedly reduced number of tumors compared with the \( \text{Nrf2} /{+/-} \) mice (Fig. 3A and B and Table 1, C). In addition, the size of the tumors was larger in \( \text{Nrf2} /{+/-} \) mice than in \( \text{Nrf2} /{-} \) mice. Indeed, 60% of mice in the \( \text{Nrf2} /{+/-} \) group exhibited large palpable nodules (\( \phi > 2 \text{ mm} \)), including some that were very large (\( \phi > 5 \text{ mm} \)), while no \( \text{Nrf2} /{-} \) mice formed such large tumors (Fig. 3B). Consistent with this observation, the average tumor diameter of \( \text{Nrf2} /{+/-} \) tumors was larger than that of \( \text{Nrf2} /{-} \) mice (1.2 vs. 0.8 mm, respectively; Fig. 3C). Importantly, the heterozygous (\( \text{Nrf2} /{-/-} \)) mice showed an increased number of lung surface tumors compared with the \( \text{Nrf2} /{-} \) mice (Fig. 3B), suggesting that the Nrf2 abundance is a critical determinant of the lung cancer growth.

Showing very good agreement with the macroscopic observations, histologic examinations revealed that the \( \text{Nrf2} /{+/-} \) mice developed malignant adenocarcinomas at a higher frequency than the \( \text{Nrf2} /{-} \) mice (Fig. 3D and E). Tumors in the \( \text{Nrf2} /{-} \) mice exhibited extensive invasion into the surrounding tissues, but \( \text{Nrf2} /{-} \) tumors were minimally invasive with a clear border. Tumors in the \( \text{Nrf2} /{-} \) mice seemed to show a higher number of Ki-67-positive cells than those in the \( \text{Nrf2} /{-} \) mice (Fig. 3D and F). \( \text{Nrf2} /{+/-} \) tumors exhibited accumulation of mucosubstances stained with periodic acid-Schiff (PAS; Fig. 3D), which correlates with progression to...
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Table 1. Summary of urethane-induced lung carcinogenesis experiments

A. Short-term observation (8 weeks) after single urethane administration in Nrf2+/+ and Nrf2−/− mice

<table>
<thead>
<tr>
<th>Tumor size (mm)</th>
<th>Incidence of lung surface tumors</th>
<th>Average number of lung surface tumors per mouse</th>
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<tbody>
<tr>
<td></td>
<td>0.5 &lt; φ</td>
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<tr>
<td>Nrf2+/+ (n = 10)</td>
<td>5/10 (50.0%)</td>
<td>0.8 ± 0.5 (n = 10)</td>
</tr>
<tr>
<td>Nrf2−/− (n = 9)</td>
<td>9/9φ (100.0%)</td>
<td>3.1 ± 1.1φ (n = 9)</td>
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B. Middle-term observation (16 weeks) after single urethane treatment in Nrf2+/+ and Nrf2−/− mice

<table>
<thead>
<tr>
<th>Tumor size (mm)</th>
<th>Incidence of lung surface tumors</th>
<th>Average number of lung surface tumors per mouse</th>
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<tbody>
<tr>
<td></td>
<td>0.5 &lt; φ</td>
<td></td>
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<tr>
<td>Nrf2+/+ (n = 7)</td>
<td>7/7 (100.0%)</td>
<td>18.1 ± 13.3</td>
</tr>
<tr>
<td>Nrf2−/− (n = 8)</td>
<td>8/8 (100.0%)</td>
<td>11.3 ± 7.0</td>
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C. Long-term observation (24 weeks) after four weekly contiguous urethane-treatment in Nrf2+/+ and Nrf2−/− mice

<table>
<thead>
<tr>
<th>Tumor size (mm)</th>
<th>Incidence of lung surface tumors</th>
<th>Number of lung surface tumors per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 &lt; φ &lt; 2.0</td>
<td></td>
</tr>
<tr>
<td>Nrf2+/+ (n = 10)</td>
<td>10/10 (100.0%)</td>
<td>35.7 ± 22.5φ</td>
</tr>
<tr>
<td>Nrf2−/− (n = 5)</td>
<td>5/5 (100.0%)</td>
<td>37.0 ± 10.9φ</td>
</tr>
<tr>
<td>Nrf2−/− (n = 10)</td>
<td>10/10 (100.0%)</td>
<td>16.4 ± 12.6φ</td>
</tr>
</tbody>
</table>

| Tumor size (mm) | 2.0 < φ                          |                                               |
| Nrf2+/+ (n = 10)| 6/10φ (60.0%)                  | 4.9 ± 6.59φ                                  |
| Nrf2−/− (n = 5)  | 2/5φ (40.0%)                   | 0.4 ± 0.55φ                                  |
| Nrf2−/− (n = 10) | 0/10 (0.0%)                    | 0.0 ± 0.0                                    |

n = number of mice.

4P < 0.01 compared with wild-type mice.

bP < 0.05 compared with wild-type mice.

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adenocarcinoma (21). Taken together, these observations show that Nrf2 plays important roles for the malignant transformation of lung adenomas in the late stage of carcinogenesis.

Urethane-induced lung tumors of Nrf2−/− mice failed to engraft in nude mice

One of the standard approaches in testing the tumorigenicity of cancer cells is to transplant the cells into immunodeficient mice (22). When highly metastatic 3LL cells were transplanted into Nrf2−/− mice, Nrf2 deficiency generated a more permissive microenvironment for cancer cell growth than in Nrf2+/+ mice (9). We, therefore, hypothesized that the less proliferative Nrf2-deficient tumor cells might be able to grow in a tumor-permissive microenvironment in the Nrf2−/− mice. To compare the cell-autonomous proliferative ability of Nrf2+/+ and Nrf2−/− tumor cells by excluding the host environmental factors, we excised lung tumors of approximately equal sizes (φ = 1 mm) from the Nrf2−/− and Nrf2+/+ mice and transplanted these tumors into nude mice. During a 5-month observation period, Nrf2−/− tumors failed to engraft and grow in the nude mice, whereas Nrf2+/+ tumors grew progressively to an approximately 50-fold increase in volume (Fig. 3G and H). These results show that Nrf2−/− tumor cells suffer from a cell-autonomous growth defect.

Nrf2-deficiency decreased the malignancy-risk gene signature

To clarify whether Nrf2 is functionally activated in urethane-induced tumors, we examined the expression of glutathione peroxide (Gpx2) and multidrug resistance–associated protein 4 (Mrp4) mRNAs, as both Gpx2 and Mrp4 contribute to the promotion of cancer (23, 24). The Gpx2 and Mrp4 mRNA expressions were significantly increased in the tumors compared with the normal lung tissues in Nrf2+/+ mice (Fig. 4A), supporting the notion that accumulated Nrf2 contributes to the proliferation of urethane-induced adenocarcinomas. To comprehensively examine changes in the gene expression profile, we conducted microarray analyses using the comparable size of lung tumors (1.0 mm < φ < 1.5 mm) and intact nontumor lung tissues derived either from Nrf2+/+ (n = 8) or Nrf2−/− mice (n = 8) at 16 weeks after the urethane treatment.

Of the 114 genes increased in the lung tumor tissues, 57 genes were increased solely in the Nrf2+/+ tumors and 25 genes
were increased solely in the Nrf2−/− tumors, whereas 32 genes were increased in both tumor genotypes (Fig. 4B and C; Supplementary Tables S2A–S2C for details). Lung adenocarcinomas tend to be associated with increased expression of a variety of lung development-related genes (21, 25). In the expression array data, we noticed upregulation of a series of lung development-related genes, including Sox9 (21, 26), Id2 (21), Nkx2-1 (27), Foxa2 (28), and N-myc (29) in the Nrf2−/− tumors compared with the Nrf2+/+ tumors (Fig. 4D). It has been reported that Sox9 accelerates malignant transformation through an increase in Cdk4 expression (26). Indeed, the increased expression of Sox9 in the Nrf2−/− tumor was accompanied by an increase in Cdk4 expression. Similarly, cyclin D1 expression was more significantly increased in the Nrf2+/+ than in the Nrf2−/− tumors, and this observation is consistent with the theory that many oncogenic signaling pathways converge to elevate cyclin D1 expression at the transcription level (30).

Consistent with these results, we noticed that majority of the genes preferentially expressed in the Nrf2+/+ rather than in the Nrf2−/− tumor are classified into the Cellular Development, Cell Growth and Proliferation, and Embryonic Development categories by the Ingenuity Pathway Analysis (IPA) software analysis. Furthermore, this pathway analysis revealed that the Nrf2−/− tumors were associated with more prominent activation of a number of carcinogenic or stem
cell–related pathways, including Wnt/β-catenin signaling and Notch signaling compared with the Nrf2+/− tumors, suggesting substantial contribution of Nrf2 to cancer progression (Supplementary Fig. S4A and S4B).

Low frequency of constitutively active Kras mutations in Nrf2-deficient mice

Kras mutations are strongly associated with the progression of adenocarcinomas (29, 31) and Kras activation induces Nrf2 mRNA expression to exert its oncogenic activity as summarized in Fig. 5A (32). However, frequency of the Kras mutations in the Nrf2−/− cancer cells has been evaluated. As urethane is known to evoke constitutively active mutations in Kras, particularly at codon 61 (CAA→CGA; Gln→Arg) in the second exon (33), we sequenced the second exon of the Kras gene in the Nrf2−/− lung tumors to clarify the somatic Kras mutation status (Fig. 5C). Importantly, all the Nrf2−/− tumors exhibited codon 61 substitution, whereas only 1 out of 13 Nrf2+/− tumors showed a Kras mutation (Fig. 5D). We concomitantly observed an increase of mRNA abundance of Kras signaling pathway genes, including Erk1 and c-Myc, in the Nrf2+/− lung tumors, whereas the mRNA levels of these genes were not significantly increased in the Nrf2−/− tumors (Fig. 5B). These results revealed that the Nrf2-deficient tumors were associated with a decreased frequency of Kras-activating mutations and diminished expression of the Kras pathway genes.

Secreted phosphoprotein 1 or osteopontin (Spp1) is a secreted glycoprotein highly expressed in several types of
cancers and precancerous lesions. The high-level expression of Spp1 is frequently associated with a high-grade malignancy of lung adenocarcinomas and poor clinical prognosis of the patients (34). Spp1-deficient mice are resistant to chemically induced skin tumorigenesis, suggesting the oncogenic function of Spp1 (35). We found that Spp1 expression was 2.5-fold higher in Nrf2+/− tumors than in Nrf2−/− tumors (Fig. 4D). A microarray analysis revealed that a number of other lung cancer-related genes, such as Gmit2A (36), Ilgav (37), and Ptk6 (38), were preferentially induced in the Nrf2−/− tumors. These data suggest that the Nrf2−/− cancer cells develop through Kras-independent oncogenic pathways.

Nrf2-deficient cancers show reduced potency for activation of MDSCs

MDSCs are potent immunosuppressor cells, which are increased in many types of cancer hosts and create immune tolerance to cancers (39). The intracellular ROS level primarily determines the immunosuppressive activity of MDSCs, which decreases the CD8+-mediated cancer immune response through peroxynitrite modification of the T-cell receptor (TCR)–CD8 complex (40). We previously found that transplantation of 3LL cells induced abundant ROS accumulation in the MDSCs population of the Nrf2−/− host mice, which led to the generation of a tumor-permissive microenvironment (9). To delineate changes in the host immune microenvironment after urethane treatment, we examined ROS levels in the MDSCs’ fraction (MDSCs-ROS) and population of CD8−-T-cells at 16 weeks after the urethane administration, when the urethane-induced tumor numbers were comparable between Nrf2−/− and Nrf2−/− mice. The tumor-bearing Nrf2+/− mice showed increased MDSC-ROS levels compared with the concurrently vehicle-treated mice (Fig. 6A). However, the MDSC-ROS levels in the tumor-bearing Nrf2−/− mice were only slightly increased relative to the vehicle-treated mice.

Consistent with the increase of MDSC-ROS levels, the number of splenic CD8−-T-cells was markedly decreased in the tumor-bearing Nrf2+/− mice (Fig. 6C and E). In contrast, the CD8−-T-cell population was not changed substantially in the Nrf2−/− mice regardless of tumor progression. The number of CD4+ T-cells was almost equivalent, irrespective of tumor bearing in both mouse genotypes (Fig. 6C and D). We normalized the population of CD8−-T-cells with that of CD4+ T-cells and further confirmed the preferential reduction of CD8−-T-cell population in the tumor-bearing Nrf2+/− mice (Fig. 6F). These observations emphasize that MDSCs predominantly suppress CD8−-T-cell proliferation in the tumor-bearing Nrf2+/− mice (9).

It has been reported that malignant cancers release a set of soluble factors, including granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), interleukin (IL)-3, IL-6, and VEGF, which facilitate the recruitment and ROS accumulation in MDSCs (41). We observed that GM-CSF expression was more highly increased in Nrf2−/− tumors than in Nrf2−/− tumors (Fig. 6B). Overall, these results argue that Nrf2−/− tumors have reduced potency for the induction of ROS accumulation in MDSCs and, therefore, the CD8−-T-cell immunity is barely diminished in the tumor-bearing Nrf2−/− mice.

Discussion

In this study, we have shown that Nrf2−/− mice are sensitive to chemical carcinogens and exhibit a high-level of cancer initiation in the early stages of urethane-induced lung carcinogenesis. In stark contrast, in the late stages of
urethane carcinogenesis, the Nrf2−/− mice developed cancers that were significantly less malignant than did the control Nrf2+/+ mice. These results indicate that the Nrf2 activity accelerates the malignant transformation of benign adenoma to adenocarcinoma. The molecular basis of the Nrf2 contribution to the urethane carcinogenesis is summarized in Supplementary Fig. S5.

The constitutively active Kras mutations have been identified in many types of human cancers, including those of the lung, pancreas, and colon, as well as in experimental cancer models of rodents (42). Urethane is known to cause Kras codon 61 mutations in multiple mouse strains (33). An important observation is that Kras activates Nrf2 and leads to the cancer by malignant transformation (32). Therefore, in the Nrf2−/− mice, even if lung cells suffer a large number of initial genetic hits by the urethane metabolites, subsequent progression of malignant transformation is markedly attenuated because of the lack of Nrf2 activity.

In contrast, several chemical carcinogens are known to rarely induce activating Kras mutations. Such carcinogens include N-nitrosobutyl (4-hydroxybutyl) amine, azoxymethane, and 7,12-dimethyl-benz[a]anthracene. Notably, these carcinogens are found to provoke cancers more abundantly in the Nrf2−/− mice than Nrf2+/+ mice (3, 6, 43). We surmise that these Kras-independent carcinogens evoke a higher number of cancers in the Nrf2−/− mice as a direct consequence of the increased chemical susceptibility and activation of other oncogenic pathways.

Spp1, an integrin-binding and cell transformation-related protein, highly associates with the malignancy of non–small cell lung carcinoma (34). We found that Spp1 is much more abundantly expressed in Nrf2−/− than in Nrf2+/+ tumors,
sugestting that Sppl contributes to lung tumorigenesis in Nrf2−/− mice. Because forced expression of Nrf2 in a chondrocyte cell line significantly decreased Sppl mRNA expression (44), Nrf2 may act as a negative regulator for Sppl gene expression. These results, thus, imply that Sppl may comprise one alternative oncogenic pathway that replaces the Kras-Nrf2 pathway.

Nrf2 activators have been shown to exert preventive effects against various types of carcinogens in animal models and humans (45, 46). In addition, therapeutic efficacy of a potent Nrf2 activator, bardoxolone methyl (CDDO-Me), was shown for the treatment of chronic kidney disease and diabetic nephropathy in a large clinical trial (47). Our results unequivocally showed that Nrf2 is critical for the prevention of the initiation step of lung carcinogenesis. Therefore, preventive treatment that attains Nrf2 activation seems to reduce the cancer initiation and would be of particular value for those who are at high risk of lung cancers, such as those with a history of heavy smoking and inhalation of asbestos. On the other hand, treatment with Nrf2 inhibitors through the lung cancer-targeted drug delivery system would reduce the cancer lesions associated with oncogenic Kras mutation. Overall, this study has revealed that Nrf2 is a prime candidate of personalized cancer treatment in the near future, and development of Nrf2 inhibitors and activators, as well as accurate diagnostic procedures quantifying Nrf2 expression levels in lung cancer cells, are critically important for this purpose.

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

Authors' Contributions
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