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, but a second which 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). Upon 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). Nrf2-sMaf heterodimer binds to a specific DNA sequence, referred to as the antioxidant/electrophile response element (ARE/EpRE), and induces the expression of a cohort of cytoprotective enzyme genes, such as \textit{Nqo1}, \textit{HO-1}, \textit{Gclc} and \textit{Gstpl/p2} (1, 2).

Previous reports on chemically induced carcinogenesis have demonstrated that \textit{Nrf2}-deficient (\textit{Nrf2}–/–) mice tend to form a larger number of tumors than wild-type (\textit{Nrf2}+/+) mice, indicating that the perturbation in the carcinogen detoxification system in \textit{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 demonstrated that the \textit{Nrf2}–/–-mice transplanted with Lewis lung carcinoma (3LL) cells provide a more tumor-permissive immune microenvironment than did the \textit{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 employed the urethane (ethyl carbamate)-induced lung carcinogenesis model, a well-known multi-step 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-dihydroxyethyl 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 micro-nodules in the early phase after the urethane-administration. However, in the later stages \(Nrf2^{+/+}\)-mice developed a higher number of Kras-mutated adenocarcinomas than did \(Nrf2^{-/-}\)-mice. These results demonstrate that Nrf2-deficiency leads to an increased susceptibility to chemical carcinogens and resultant high-level tumor initiations, while 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−/−-mice with an ICR/CD-1 genetic background (outbred) were used in this study (2, 15). Age-matched (5~9-weeks) Nrf2+/−-mice were used as concurrent controls. The mice were maintained in a facility free of specific pathogens (SPF). Nude mice (8~9-weeks) were purchased from CLEA Japan. All animal experiments were performed 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) (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 2nd 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 stereo-microscope. The lung tumors and non-tumor regions of Nrf2+/+ and Nrf2−/− 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). Heat maps 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 biological and toxicological functions was performed 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’s exact test. The GEO accession number for the microarray data is GSE46048.

Flow cytometry

Analyses of the bone marrow (BM) cells were performed using FACS-Caliber (BD Pharmingen). Quantification of ROS level with 2,7-dichlorodihydrofluorescein diacetate (DCFDA), separation of MDSCs, and T cells has been described (9).

Immunoblotting analysis
Nuclear extracts were prepared from NIH3T3 cells that were treated with the indicated concentrations of urethane for 6-h. The mouse lung nuclear extracts were prepared from the $Nrf2^{+/+}$-mice administered either vehicle (PBS) or urethane (1-g/kg body weight) for the indicated time periods. Immunoblotting analysis was performed 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’s $t$-test or the Mann-Whitney $U$ test. The values for either the incidence of lung nodules or large tumors were analyzed using the Fisher’s exact probability test. $P$-values < 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

While 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 μM for 6 hours (-h)). Upon treatment with urethane, Nrf2 accumulated in nuclear fraction of NIH/3T3 cells (Fig. 1A). We also found that Nrf2 accumulated in the lung tissues 3-h after intraperitoneal injection of urethane (1-g/kg body weight) into Nrf2+/+-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 demonstrate 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, HO-1 and Gclc, were markedly induced in the Nrf2+/+-mice in a time-dependent manner (Fig. 1D). In contrast, inducible expression of the Nrf2 target genes was attenuated in the Nrf2+/--mice. These observations demonstrate 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 Cyp2e1 level in Nrf2+/−-mouse lungs. Basal Cyp2e1 mRNA level did not differ substantially between Nrf2+/− and Nrf2+/+ mice, and induced Cyp2e1 mRNA expression after the urethane administration was comparable between the two genotypes (Supplementary Fig. S1B). Cyp2e1 immunoreactivity in the airway epithelial cells was also comparable between the two genotypes regardless of the urethane treatment (Supplementary Fig. S1C). These results indicate that the basal and induced expression of Cyp2e1 is independent of the Nrf2 activity. We also examined mRNA expression of the enzymes involved in the detoxification process of vinyl carbamate epoxide. We found that urethane-induced expression of mEH was diminished in Nrf2+/−-mice in comparison with those of Nrf2+/+ mice (Supplementary Fig. S1D). Gstp1/p2 expression in Nrf2+/−-mice at both basal and urethane-induced states was lower than that in Nrf2+/+ mice. Together, these observations support our contention that both maintained Cyp2e1-mediated oxidation and attenuated mEH-Gst detoxification in Nrf2+/−-mice lead to the accumulation of a higher amount of vinyl
carbamate epoxide than that in $Nrf2^{+/+}$-mice upon the urethane treatment.

**Urethane elicits acute inflammatory response in Nrf2-deficient mouse lung**

$Nrf2^{+/+}$-mice are susceptible to a number of oxidative or electrophilic insults, including butylated hydroxytoluene and bleomycin, and the mice are more susceptible to pneumonia, fibrosis, and inflammatory cell infiltration than $Nrf2^{+/+}$-mice (19). Given this, we next examined inflammatory status of the lungs after the urethane treatment. We observed a large number of inflammatory cell foci in the pulmonary perivascular region of the $Nrf2^{-/-}$-mice 24-h after urethane administration, while such inflammatory cell infiltration was not observed in $Nrf2^{+/+}$-mouse lungs (Supplementary Fig. S2A). These cell clusters mainly consisted of CD3$^+$ T-lymphocytes. Pan-cytokeratin$^+$ epithelial cells were also involved frequently (Supplementary Fig. S2B). Of note, we observed a greater increase in Ki67-positive cells in the cell clusters of $Nrf2^{+/+}$-mouse, which presumably represent increased proliferation of the epithelial cells (Supplementary Fig. S2B). Hence, 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 four different observation periods: A, very short-term observation (4-weeks), B, short-term observation (8-weeks), C, middle-term observation (16-weeks), and D, long-term observation (24-weeks) (Supplementary Fig. S3). At 4-weeks after the intraperitoneal urethane injection, \( Nrf2^{-/-} \)-mice showed much larger number of microscopic nodules (average 11.7, \( n=3 \); \( P<0.05 \)) than the \( Nrf2^{+/+} \)-mice (average 1.67, \( n=3 \); Fig. 2A and B), while both groups rarely showed gross surface tumors. At 8-weeks after the urethane administration, all the urethane-treated \( Nrf2^{-/-} \)-mice developed macroscopic (\( \phi >0.5 \) mm) lung surface tumors, whereas only half of the \( Nrf2^{+/+} \)-mice developed the gross surface tumors (Fig. 2C and D, Table 1A). Furthermore, \( Nrf2^{-/-} \)-mice formed much higher number of lung surface nodules than \( Nrf2^{+/+} \)-mice. These results indicate that Nrf2 contributes to the prevention of urethane-induced carcinogenesis at the early tumorigenic stages (4- or 8-weeks).

\( Nrf2^{-/-} \)-mice are resistant to urethane carcinogenesis at middle-term observation period

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

Nrf2-deficient mice are resistant to urethane carcinogenesis at long-term observation period

An observation at even late stage revealed more significant difference in the cancer progression between Nrf2<sup>−/−</sup> and Nrf2<sup>+/−</sup>-mice. At 24-weeks after the urethane administration (Supplementary Fig. S3D), Nrf2<sup>−/−</sup>-mice exhibited a markedly reduced number of tumors compared to the Nrf2<sup>+/−</sup>-mice (Fig. 3A, B and Table 1C). In addition, the size of the tumors was larger in Nrf2<sup>+/−</sup>-mice than in Nrf2<sup>−/−</sup>-mice. Indeed, 60% of mice in the Nrf2<sup>+/−</sup>-group exhibited large palpable nodules (φ > 2 mm), including very large ones (φ > 5 mm), while no Nrf2<sup>−/−</sup>-mice formed such large tumors (Fig. 3B). Consistent with this observation, average tumor diameter of Nrf2<sup>+/−</sup>-tumors was larger than that of Nrf2<sup>−/−</sup>-mice (1.2 mm vs. 0.8 mm, respectively; Fig. 3C). Importantly, the heterozygous (Nrf2<sup>+/−</sup>)-mice showed an increased number of lung surface tumors compared with the Nrf2<sup>−/−</sup>-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, histological examinations revealed that the Nrf2<sup>+/−</sup>-mice developed malignant adenocarcinomas at higher frequency than the Nrf2<sup>+/+</sup>-mice (Fig. 3D and E). Tumors in the Nrf2<sup>+/+</sup>-mice exhibited extensive invasion into the surrounding tissues, but Nrf2<sup>−/−</sup>-tumors were minimally invasive with a clear border. Tumors in the Nrf2<sup>+/+</sup>-mice appeared to show a higher number of Ki67-positive cells than those in the Nrf2<sup>−/+</sup>-mice (Fig. 3D and F). Nrf2<sup>+/+</sup>-tumors exhibited accumulation of mucosubstances stained with periodic acid-Schiff (PAS; Fig. 3D), which correlates with progression to adenocarcinoma (21). Taken together, these observations demonstrate that Nrf2 plays important roles for the malignant transformation of lung adenomas in the late stage of carcinogenesis.

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

One of the standard approaches testing the tumorigenicity of cancer cells is to transplant the cells into immuno-deficiency mice (22). When highly metastatic 3LL cells were transplanted into Nrf2<sup>−/−</sup>-mice, Nrf2-deficiency generated a more permissive microenvironment for cancer cell growth than that in Nrf2<sup>+/+</sup>-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<sup>−/−</sup>-mice. To compare the cell autonomous proliferative ability of Nrf2<sup>+/+</sup>- and Nrf2<sup>−/−</sup>-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 5-months observation period, Nrf2–/–-tumors failed to engraft and grow in the nude mice, while Nrf2+/+-tumors grew progressively to approximately 50-fold increase in volume (Fig. 3G and 3H). These results demonstrate 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 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 performed microarray analyses using the comparable size of lung tumors (1.0 mm < φ < 1.5 mm) and intact non-tumor 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, while 32 genes were increased in both tumor genotypes (Fig. 4B and C; Supplementary Tables S2A, B, and C for details). Lung adenocarcinomas tend to be associated with the 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 Nmyc (29) in the Nrf2+/-tumors compared with the Nrf2+/+-tumors (Fig. 4D). It has been reported that Sox9 accelerates the 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+/-tumors than in the Nrf2+/+-tumors and this observation is consistent with the notion 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+/+-tumor 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 the
cancer progression (Supplementary Fig. S4A and B).

**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 not been evaluated. As urethane is known to evoke constitutive-active mutations in Kras, particularly at codon 61 (CAA→CGA; Gln→Arg) in the 2nd exon (33), we sequenced the 2nd 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 the codon 61 substitution, while only 1 out of 13 *Nrf2*−/−-tumors showed the *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.

*Spp1* (Secreted phosphoprotein 1 or osteopontin) is a secreted glycoprotein highly expressed in several types of cancers and precancerous lesions. The high-level expression of *Spp1* frequently associates with the 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<sup>−/−</sup>-tumors than in Nrf2<sup>+/+</sup>-tumors (Fig. 4D). A microarray analysis revealed that a number of other lung cancer-related genes, such as Grin2A (36), Itgav (37), and Ptk6 (38), were preferentially induced in the Nrf2<sup>−/−</sup>-tumors. These data suggest that the Nrf2<sup>−/−</sup>-cancer cells develop through Kras-independent oncogenic pathways.

**Nrf2-deficient cancers show reduced potency for activation of MDSCs**

Myeloid-derived suppressor cells (MDSCs) are potent immune suppressor cells, which increase in many types of cancer hosts and create immune tolerance to cancers (39). The intra-cellular 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<sup>−/−</sup>-host mice, which led to the generation of a tumor-permissive microenvironment (9). To delineate changes in the host immune microenvironment after the urethane treatment, we examined ROS levels in the MDSCs fraction (MDSCs-ROS) and population of CD8<sup>+</sup>-T-cells at 16-weeks after the urethane administration, when the urethane-induced tumor numbers were comparable between Nrf2<sup>−/−</sup>- and Nrf2<sup>+/+</sup>-mice. The tumor-bearing Nrf2<sup>+/+</sup>-mice showed
increased MDSCs-ROS levels compared with the concurrently vehicle-treated mice (Fig. 6A). However, the MDSCs-ROS levels in the tumor-bearing Nrf2+/+ -mice were only slightly increased relative to the vehicle-treated mice.

Consistent with the increase of MDSCs-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 the tumor progression. The number of CD4+ -T-cells was almost equivalent irrespective of a 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 underscore 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 GM-CSF, G-CSF, M-CSF, 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 demonstrated that \( \text{Nrf2}^{-/-} \)-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 the urethane carcinogenesis the \( \text{Nrf2}^{+/+} \)-mice developed much less malignant cancers than the control \( \text{Nrf2}^{+/+} \)-mice did. These results indicate that the Nrf2 activity accelerates the malignant transformation of benign adenoma to adenocarcinoma. Molecular basis of the Nrf2 contribution to the urethane carcinogenesis is summarized in Supplementary Fig. S5.

The constitutively active \( \text{Kras} \) mutations have been identified in many types of human cancers, including lung, pancreas and colon, as well as in experimental cancer models of rodents (42). Urethane is known to cause \( \text{Kras} \) codon 61 mutations in multiple mouse strains (33). An important observation is that Kras activates Nrf2 and leads to the cancer malignant transformation (32). Therefore, in the \( \text{Nrf2}^{+/+} \)-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 \( \text{Kras} \) mutations. Such carcinogens include N-nitrosobutyl (4-hydroxybutyl) amine, azoxymethane, and 7,12-dimethyl-benz[a]anthracene. Of note is that these carcinogens are found to provoke cancers more abundantly in the \( \text{Nrf2}^{+/+} \)-mice than \( \text{Nrf2}^{+/+} \)-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 the $Nrf2^{-/-}$-tumors than in the $Nrf2^{+/+}$-tumors, suggesting that Spp1 contributes to lung tumorigenesis in $Nrf2^{-/-}$-mice. Because forced expression of Nrf2 in a chondrocyte cell line significantly decreased $Spp1$ mRNA expression (44), Nrf2 may act as a negative regulator for $Spp1$ gene expression. These results thus imply that $Spp1$ 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 demonstrated for the treatment of chronic kidney disease and diabetic nephropathy in a large clinical trial (47). Our results unequivocally demonstrated 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 heavy smoking and inhalation of asbestos. On the other hand, treatment with Nrf2 inhibitors through the lung cancer-targeted drug delivery system would
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reduce the cancer lesions associated with oncogenic Kras mutation. Over all, 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.
Authors’ contributions

Study concept and design: HS, TM

Acquisition of data: HS, JT, ME

Analysis and interpretation of data: HS, TM, MY

Writing, review and/or revision of the manuscript: HS, TM, MY

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Figure legends

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 employed 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 indicates 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 RT-qPCR 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’s t-test are indicated (*, P < 0.05 and **, P < 0.01).

Figure 2.

Urethane-induced lung tumorigenesis in the short- and middle-term observation protocols.

A-D, Microscopic and macroscopic examination of precancerous nodules at 4-weeks and 8-weeks after the urethane-administration. A, Histological examination of precancerous lesions in the lung of Nrf2+/- and Nrf2+-mice. Lung sections from the mice at 4-weeks after
the urethane administration were stained with HE. Scale bar, 100 μm. B, Number of microscopic nodules in Nrf2+/+ and Nrf2/−-mice 4-weeks after the urethane-administration. C, Representative gross observation of lung surface tumors in Nrf2+/+ and Nrf2/−-mice. Lungs from mice at 8-weeks after the urethane-administration were examined. Arrowheads show the surface tumors. Scale bar, 10 mm. Lower panels show representative HE-stained sections. Scale bar, 100 μm. D, Number of lung surface tumors in each mouse of both genotypes. E-H, Macroscopic examination of surface tumors at 16-weeks after urethane-administration. E, Representative gross pictures of lung surface tumors in Nrf2+/+ and Nrf2/−-mice. Arrows indicate the lung surface tumors. Scale bars, 10 mm. F, Numbers of surface tumors in lungs of Nrf2+/+ and Nrf2/−-mice. Each dot represents total number of macroscopic tumors (φ > 0.5 mm) in individual mouse. G, Number of large-size nodules (φ > 1.0 mm) in lungs of Nrf2+/+ and Nrf2/−-mice. The color of dots indicates size of the largest tumor in each mouse lung. H, Average of tumor diameters in lungs of Nrf2+/+ and Nrf2/−-mice. The significant differences by Student’s t test are indicated (*, P < 0.05 and **, P < 0.01).

Figure 3.

Urethane-induced lung carcinogenesis in the long-term observation protocol. A, Gross observation of lungs (left) and HE staining of lung sections (right) of Nrf2+/+ and Nrf2/−-mice. Black scale bar, 10 mm; blue scale bar, 400 μm. B, Number of lung surface tumors (φ > 1 mm)
in the Nrf2+/+, Nrf2+/– and Nrf2–/–-mice. The color of dots indicates size of the largest tumor in each mouse lung. C, Average tumor diameter in each mouse group. D, Representative pathological photomicrographs of the urethane-induced lung tumors in the Nrf2+/+ and Nrf2+/–-mice by HE (upper), Ki67 (middle) and PAS staining (lower). Higher magnification images of each genotype are in the inlets. Black scale bars, 50 μm; blue scale bars 10 μm. E, Number of total adenocarcinomas in a section from each mouse. F, Number of Ki67-positive cells in similar size tumors (φ 100 μm) of Nrf2+/+ and Nrf2+/–-mice. G and H, Nrf2 is necessary for colonization and growth of urethane-induced tumors in nude mice. G, Reduced growth of Nrf2–/–-tumors compared with Nrf2+/+-tumors subcutaneously transplanted in nude mice. H, Representative photographs (upper) and photomicrographs (middle and lower) from each genotype. Black scale bar, 10 mm; blue scale bar, 1 mm; white scale bar, 20 μm.

Figure 4.

Differential gene expression in the urethane-induced lung tumors (4-months after urethane-treatment) of Nrf2−/−-mice. A, Expression of Gpx2 and Mrp4 in the tumors and non-tumor regions of Nrf2+/+ and Nrf2+/−-mice. B, Venn diagram of the differentially expressed genes in the Nrf2+/+ and Nrf2+/−-tumors. The numbers of genes highly expressed in tumors compared with non-tumor tissues of both genotype mice are shown along with the overlap in both genotypes of mice. C, Clustering analysis of differentially expressed genes in...
the lung tumors of \( Nrf2^{+/+} \)- and \( Nrf2^{-/-} \)-mice. Gene expression values (shown below panel B) using the color scheme (green-black-red) indicate low-moderate-high gene expression levels compared to the corresponding non-tumor tissues. Heat map comparisons of genes preferentially expressed in \( Nrf2^{+/+} \) mouse tumors (left panel) and those preferentially expressed in \( Nrf2^{-/-} \)-tumors (right) are shown. D, RT-qPCR analysis of representative mRNAs for cancer-related and lung development-related genes. Data are presented as mean ± SD. The significant differences by Student’s \( t \) test are indicated (*, \( P < 0.05 \) and **, \( P < 0.01 \)).

**Figure 5.**

Constitutively active \( Kras \) mutation frequently observed in the lung tumors of urethane-treated mice. A, Schematic diagram of the Kras-signaling pathway. Blue arrows indicate the genes highly induced in the \( Nrf2^{+/+} \)-tumor. B, Expression of mRNAs coding for representative Kras downstream factors. mRNAs were quantified by RT-qPCR analysis using lung tumors from \( Nrf2^{-/-} \) and \( Nrf2^{+/+} \)-mice 16-weeks after the urethane treatment. C, Left: The A-to-G substitution at the second nucleotide of codon 61 in the 2\(^{nd} \) exon of the \( Kras \) gene in the \( Nrf2^{+/+} \)-tumors. Right: The \( Nrf2^{-/-} \)-tumors showed intact nucleotide sequences of codon 61. D, Frequency of \( Kras \) codon-61 mutation (CAA→CGA; Gln→Arg) detected in the tumors from \( Nrf2^{-/-} \)- or \( Nrf2^{+/+} \)-mice. Significant differences were determined using Fisher’s exact test (**, \( P < 0.01 \)).
Figure 6.

Nrf2-deficient cancers show reduced potency for activation of MDSCs. *Nrf2<sup>−/−</sup>*-mice harboring urethane-induced lung tumors exhibit a lower level of ROS accumulation in MDSCs. **A**, Quantification of ROS levels of the Mac1<sup>+</sup>Gr1<sup>+</sup> MDSCs fraction in the BM cells from *Nrf2<sup>−/−</sup>*- or *Nrf2<sup>+/+</sup>*-mice harboring lung tumors (left panel). Data are presented as the median ± SD (**, *P* <0.01 by Student’s *t*-test; *n*=3 in each group). Representative histogram of ROS level analysis with DCFDA in MDSCs from BM in each group (right panel). **B**, GM-CSF expression level was significantly repressed in the *Nrf2<sup>−/−</sup>*-tumors in comparison with the *Nrf2<sup>+/+</sup>*-tumors. **C-F**, Splenic CD8<sup>+</sup>-T-cell population was decreased in the tumor-bearing *Nrf2<sup>+/+</sup>*-mice compared to the tumor-bearing *Nrf2<sup>−/−</sup>*-mice. **C**, Representative flow cytometric dot plots of spleen cells using CD4 and CD8 antibodies. The green frames indicate the CD8<sup>+</sup>-T-cell population. **D**, The CD4<sup>+</sup>-T-cell population was comparable between both genotype groups. **E**, Splenic CD8<sup>+</sup>-T-cell population was decreased in the *Nrf2<sup>+/+</sup>*-mice bearing tumors more significantly than in the vehicle-treated *Nrf2<sup>+/+</sup>*-mice. **F**, Population of CD8<sup>+</sup>-T cells are normalized with that of CD4<sup>+</sup>-T cells. Data are presented as the median ± SD (*, *P* <0.05 using Student *t*-test; *n*=3 in each group).
Table 1. Summary of urethane-induced lung carcinogenesis experiments.

A. Short-term observation (8 weeks) after single urethane administration in \( \text{Nrf2}^{+/+} \) and \( \text{Nrf2}^{-/-} \) 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>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Nrf2}^{+/+} ) (n=10)</td>
<td>5/10 (50.0%)</td>
<td>0.8±0.5 (n=10)</td>
</tr>
<tr>
<td>( \text{Nrf2}^{-/-} ) (n=9)</td>
<td>9/9(^a) (100.0%)</td>
<td>3.1±1.1(^a) (n=9)</td>
</tr>
</tbody>
</table>

\(^aP<0.01\) compared with wild-type mice. \(^bP<0.05\) compared with wild-type mice.

B. Middle-term observation (16 weeks) after single urethane treatment in \( \text{Nrf2}^{+/+} \) and \( \text{Nrf2}^{-/-} \) 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>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Nrf2}^{+/+} ) (n=7)</td>
<td>7/7 (100.0%)</td>
<td>18.1±13.3</td>
</tr>
<tr>
<td>( \text{Nrf2}^{-/-} ) (n=8)</td>
<td>8/8 (100.0%)</td>
<td>11.3±7.0</td>
</tr>
</tbody>
</table>

\(^aP<0.01\) compared with wild-type mice. \(^bP<0.05\) compared with wild-type mice.

C. Long-term observation (24 weeks) after four weekly contiguous urethane-treatment in \( \text{Nrf2}^{+/+} \) and \( \text{Nrf2}^{-/-} \) 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>( \text{Nrf2}^{+/+} ) (n=10)</td>
<td>10/10 (100.0%)</td>
<td>35.7±22.5</td>
</tr>
<tr>
<td>( \text{Nrf2}^{-/-} ) (n=5)</td>
<td>5/5 (100.0%)</td>
<td>37.0±10.9</td>
</tr>
<tr>
<td>( \text{Nrf2}^{-/-} ) (n=10)</td>
<td>10/10 (100.0%)</td>
<td>16.4±12.6</td>
</tr>
</tbody>
</table>

\(^aP<0.01\) compared with wild-type mice. \(^bP<0.05\) compared with wild-type mice.
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Nrf2 prevents initiation but accelerates progression through the Kras signaling pathway during lung carcinogenesis

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