



Demethylation of the NRF2 Promoter Protects Against Carcinogenesis Induced by Nano-SiO₂

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Nano silicon dioxide (Nano-SiO₂) has been widely used in industries such as the field of biomedical engineering. Despite the existing evidence that Nano-SiO₂ exposure could induce oxidative stress and inflammatory responses in multiple organ systems, the carcinogenicity of Nano-SiO₂ exposure has rarely been investigated. Thus in this study, two types of human bronchial epithelial cell lines (16HBE and BEAS-2B) were selected as *in vitro* models to investigate the carcinogenicity of Nano-SiO₂. Our results revealed that Nano-SiO₂ induces a malignant cellular transformation in human bronchial epithelial cells according to the soft agar colony formation assay. The carcinogenesis induced by Nano-SiO₂ was also confirmed in nude mice. By using immunofluorescence assay and high-performance capillary electrophoresis (HPCE), we observed a genome-wide DNA hypomethylation induced by Nano-SiO₂. Besides the reduced enzyme activity of total DNMTs upon Nano-SiO₂ treatment, altered expression of DNMTs and methyl-CpG binding proteins were observed. Besides, we found that the expression of NRF2 was activated by demethylation of CpG islands within the NRF2 promoter region and the overexpression of NRF2 could alleviate the carcinogenesis induced by Nano-SiO₂. Taken together, our results suggested that Nano-SiO₂ induces malignant cellular transformation with a global DNA hypomethylation, and the demethylation of NRF2 promoter activates the expression of NRF2, which plays an important role in protecting against the carcinogenesis induced by Nano-SiO₂.

Keywords: Nano-SiO₂, malignant transformation, carcinogenesis, DNA methylation, NRF2

INTRODUCTION

Nano silicon dioxide (Silica nanoparticles, Nano-SiO₂), due to its special characteristics of large surface area and optical transparency, has been widely used in various fields including biomedical imaging, drug delivery, cosmetics, and electronics industry (Vivero-Escoto et al., 2012; Sweeney et al., 2016; Mohajerani et al., 2019). Meanwhile, its widespread applications raise potential health risks to humans through occupational and environmental exposure. Recent studies have demonstrated that Nano-SiO₂ exposure in a short term could induce oxidative stress, mitochondria dysfunction, and inflammatory responses in multiple organ systems

(Song et al., 2009; Napierska et al., 2010; Fruijtier-Polloth, 2012; Lu et al., 2013), among which respiratory system is the primary site that exposed to the airborne Nano-SiO₂ particles (Song et al., 2009; Yu et al., 2015).

Long-lasting nanomaterials in certain tissues have been reported to cause chronic adverse effects such as carcinogenesis due to their special physicochemical properties (Hirose et al., 2011), and nano-sized particles might be more carcinogenic than micron-sized particles concerning long-term exposure (Gebel, 2012). Inhalation of TiO₂ nanoparticles was shown to increase the risk of lung cancers in rats (Bermudez et al., 2004) and carbon nanotube exposure through inhalation is carcinogenic to the lungs of male and female rats (Kasai et al., 2016). Although SiO₂ has been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC) in 1997 (Iarc., 1997), there is limited data on carcinogenicity following chronic exposure to Nano-SiO₂. Nano-SiO₂ can be localized to the nucleus thereby affecting nuclear integrity and causing DNA damage (Chen and von Mikecz, 2005; Wang et al., 2007).

Alterations in DNA methylation, a typical epigenetic modification, have been reported in cells exposed to Nano-SiO₂. A global genomic hypomethylation induced by Nano-SiO₂ was observed in HaCaT cells and the CpGs in the promoter of PARP1 gene were demethylated, resulting in DNA repair dysfunction (Gong et al., 2010, 2012b). Abnormal DNA methylation occurs frequently in the process of carcinogenesis (Miousse et al., 2018; Dreval et al., 2019), through devastating genome stability and activating aberrant transcription (Gama-Sosa et al., 1983). Decreased global DNA methylation and increased expression of DNA methyltransferases (DNMTs) were demonstrated in the early stage of Nano-SiO₂-induced malignant cellular transformation (Seidel et al., 2017), indicating that DNA methylation is involved in the Nano-SiO₂-induced carcinogenesis.

Nuclear factor erythroid-2-related factor 2 (NRF2) is a key transcription factor involved in cellular responses to stresses induced by electrophiles, oxidants, and chemicals (Motohashi and Yamamoto, 2004). In addition to stress responses, NRF2 plays a contradictory role in cancers (Wu et al., 2019). Loss of NRF2 ultimately leads to malignant cellular transformation in the prostate gland of murine models (Frohlich et al., 2008). NRF2 dysfunction in mice accelerates the acetylhydrolase-induced hepatocarcinogenesis (Marhenke et al., 2008). On the contrary, accumulating evidence indicated that increased activation of NRF2 frequently occurs in multiple types of tumors, which promotes cancer cell growth and metastasis formation (Liu et al., 2016; Zhang et al., 2016; Lu et al., 2017). The hypomethylation of CpG islands within the NRF2 promoter region was reported in colorectal cancer (Kang et al., 2014; Zhao et al., 2015). Decreased NRF2 expression due to hypermethylation of CpG islands within the NRF2 promoter region was found to be associated with prostate cancer (Yu et al., 2010; Khor et al., 2014).

In the present study, we demonstrated that Nano-SiO₂ induces a malignant cellular transformation and a global DNA hypomethylation in human bronchial epithelial cells. Intriguingly, we found that demethylation of CpG islands within

the NRF2 promoter region increases the NRF2 gene expression, which inhibits the carcinogenesis induced by Nano-SiO₂.

MATERIALS AND METHODS

Chemicals and Reagents

The 15 nm Nano-SiO₂ particles were purchased from Wan Jing New Material Co., Ltd. (Hangzhou, Zhejiang, China) and were characterized as previously described (Gong et al., 2012a). Nano-SiO₂ samples were sonicated to distribute in the solution as evenly as possible. Dosing solutions were prepared by dissolving the calculated amount of Nano-SiO₂ in the cell culture medium, following the approved standard operating procedures for handling toxic agents. All other reagents were obtained from commercial sources and were of the highest available grade.

Cell Culture

Human bronchial epithelial cell lines (16HBE and BEAS-2B) and adenocarcinomic human alveolar basal epithelial cell line (A549) were purchased from the Cell Bank in Chinese Academy of Sciences Cell Bank in Shanghai. Frozen cells were thawed and expanded in MEM medium supplemented with 10% fetal bovine serum (FBS), 10 U/ml penicillin, and 10 U/ml streptomycin. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C and passaged at about 80% confluence.

Nano-SiO₂ Treatment

Human bronchial epithelial cell lines were treated with Nano-SiO₂ for several passages and harvested at certain time points as shown in **Figure 2A**. In detail, 16HBE and BEAS-2B cells were grown in complete MEM medium until they reached a confluence of about 70–80%. Cells were then treated with Nano-SiO₂ (10.0 μg/ml for 16HBE cells, 40.0 μg/ml for BEAS-2B cells) for 24 h and changed into the complete MEM medium until they are ready for subculture, these cells treated with Nano-SiO₂ for one passage are referred to P1. Accordingly, cells treated as above for n passages are named as P(n). Cells cultured in complete MEM medium for the same passages without any Nano-SiO₂ treatment are taken as the negative control. A549, an epithelial cell line derived from human lung carcinoma, was used as a positive control.

Cell Viability Measurement by MTT Assay

Cell viability was determined by MTT assay. 16HBE and BEAS-2B cells growing at the exponential phase were seeded in 96-well plates with a density of 5×10^4 cells/ml. After treated with various dosages of Nano-SiO₂ (0, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 μg/ml for 16HBE, 0, 1.0, 5.0, 10.0, 20.0, 40.0, 80.0, and 160.0 μg/ml for BEAS-2B) for 24 h, 50 μl MTT solution was added to each well and incubated for another 4 h. After adding 150 μl DMSO, the absorbance of each well was measured at 490 nm using a spectrophotometer. Each treatment group has at least three replicates. Cell viability was obtained as a percentage of the value of viable cells in the control groups.

Anchorage-Independent Cell Growth Measured by Soft Agar Colony Formation Assay

Anchorage-independent growth of Nano-SiO₂-treated human bronchial epithelial cells (16HBE and BEAS-2B) was measured by soft agar colony formation assay. Cells treated with Nano-SiO₂ for various passages (P0, P8, P16, and P32 of 16HBE cells, P0, P15, P30, and P45 of BEAS-2B cells) were trypsinized. Cells were resuspended at a density of 1×10^4 /ml in MEM medium with 0.3% agar and plated over 3 ml of a solidified complete MEM culture medium containing 0.5% agar. Cells were then incubated in a humidified atmosphere with 5% CO₂ at 37°C for 3 weeks. The colonies were photographed and counted by using ImageJ software.

Tumorigenicity in Nude Mice

The tumorigenicity of the Nano-SiO₂-treated human bronchial epithelial cells (16HBE and BEAS-2B) was determined in nude mice. Balb/c-nu male nude mice (4 weeks old) purchased from the Laboratory Animal Center in Shanghai, were housed in ventilated microisolators with sterile food and water. After one week of acclimation, mice were randomly divided into eight treatment groups, with three mice in each group. Specifically, four groups of mice were injected with different passages of 16HBE cells (P0, P8, P16, and P32), another four groups of mice were injected with different passages of BEAS-2B cells (P0, P15, P30, and P45). Nude mice injected with cells cultured in complete MEM medium for the same passages without any Nano-SiO₂ treatment were taken as the negative control. Cells were harvested by trypsinization, washed, and resuspended in PBS. Cell suspensions were then injected (0.2 ml) subcutaneously to mice at a density of 1×10^7 cells/ml. Tumor growth was monitored for 90 consecutive days after injection by measuring the major diameter of the tumor externally with a slide caliper. Mice were sacrificed by cervical dislocation if the major diameter of its tumor reaches about 2 cm. Mice were kept for more than

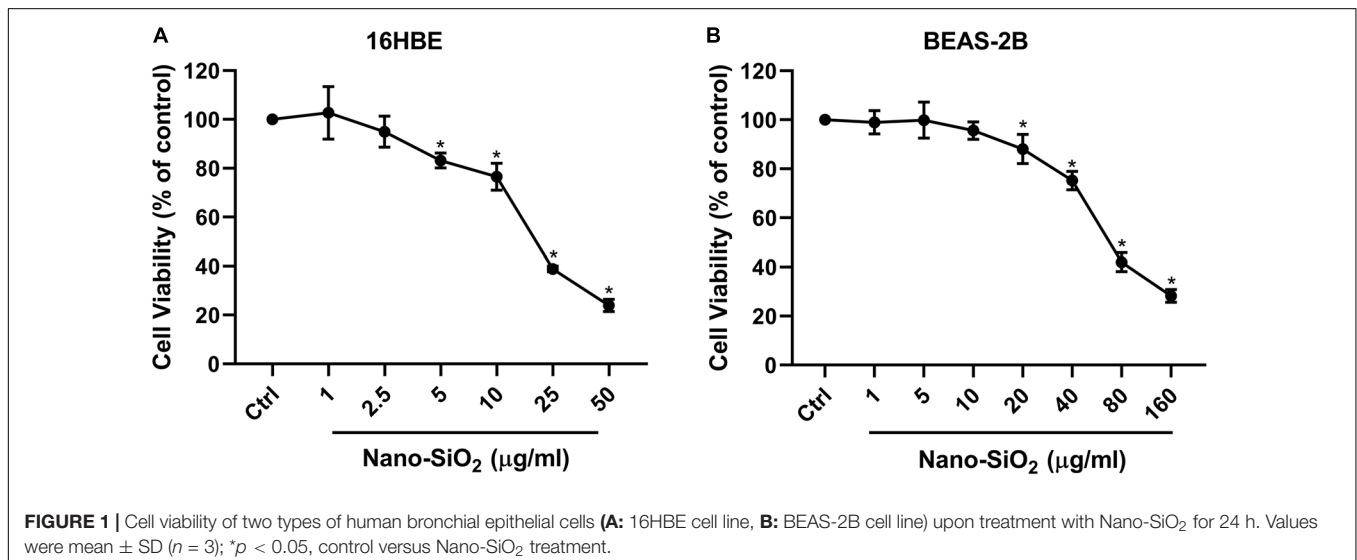
90 days after injection. The tumor sizes were measured at the fifth week after injection and the incidence and latency period of tumorigenesis were recorded. The incidence was presented as the number of mice with a tumor at the site of injection versus the total number of mice. The latency period is defined as the time required for a tumor to be able to visually detect.

Quantitative Reverse Transcription-PCR (qRT-PCR) Assay

Total RNAs from three cell lines (16HBE, BEAS-2B, and A549) were isolated and reverse transcribed into cDNAs by using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio Inc., Japan). Four genes related to the NRF2 signaling pathway were selected for qRT-PCR assay. Primers are listed in **Supplementary Table S1**. All qPCR reactions were performed on an Applied Biosystems™ StepOne™ Real-time PCR system using iTaq™ Universal SYBR Green Supermix, with three technical replicates. The amplification procedure was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. Relative quantification of target genes was performed using the $\Delta\Delta C_t$ method with GAPDH as a reference gene.

Western Blotting Assay

Cells were washed with ice-cold PBS and lysed on ice with a protease inhibitor cocktail. Protein concentrations were measured by BCA method. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. The membranes were probed with NRF2 (1:500), HO1 (1:1000), SOD1 (1:1000), GST (1:1000), DNMT1 (1:500), DNMT3a (1:500), DNMT3b (1:500), MeCP2 (1:500), MBD2 (1:500), GAPDH (1:10000) antibodies (Santa Cruz Biotechnology, Inc., Texas, United States) at 4°C overnight. The bands were visualized after incubation with a chemiluminescent substrate. Quantification of the band density was determined by densitometric analysis.



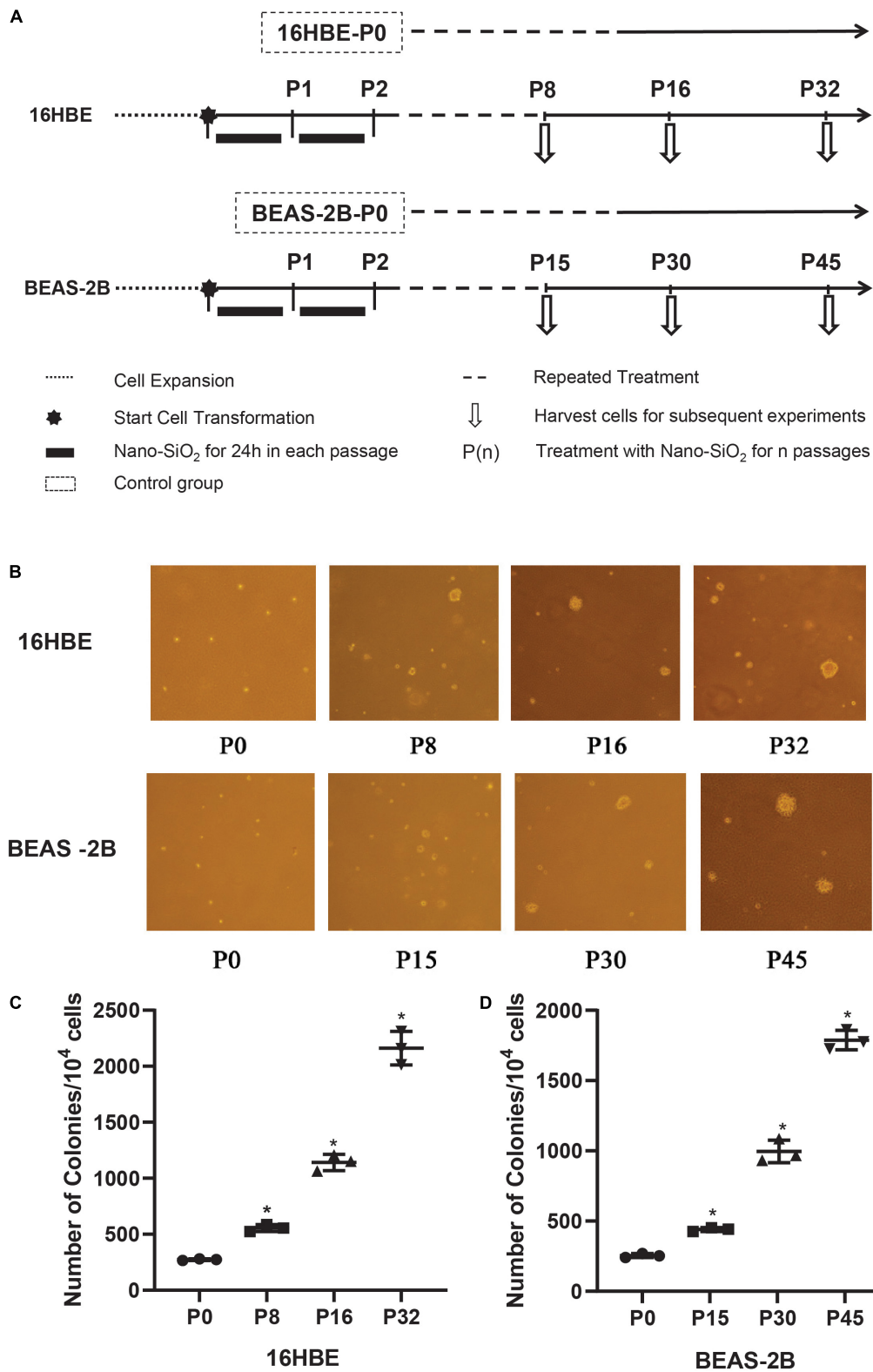


FIGURE 2 | The effects of Nano-SiO₂ on soft agar colony formation of 16HBE and BEAS-2B cells. **(A)** The experimental protocol indicates the Nano-SiO₂ treatment timeline of 16HBE and BEAS-2B cells. **(B)** Representative colonies on soft agar plates. Cells treated with Nano-SiO₂ (10 μg/ml for 16HBE cells, and 40 μg/ml for BEAS-2B cells) or without Nano-SiO₂ (16HBE-P0, BEAS-2B-P0) were grown on soft agar plates after indicated passages. **(C,D)** Dot plots showing the number of colonies developed from 16HBE and BEAS-2B cells. The data are shown as mean ± SD (n = 3); *p < 0.05, control (P0) vs. Nano-SiO₂ treatment.

5-mC Content Measurement by Immunofluorescence Assay

Cells were fixed with 4% paraformaldehyde for 15 min and then fixed with cold formaldehyde for 5 min, washed with PBST, and then incubated with hydrochloric acid at 37°C for 30 min. After blocked with 3% PBST-BSA for 30 min, samples were incubated with anti-5-mC antibody (1:1000) at 37°C for 1 h and incubated with FITC-conjugated secondary antibody (1:400) for 30 min. Finally, coverslips were then incubated with DAPI for double staining and then mounted on glass slides by using Fluoro-Antifading medium II. The fluorescence intensity was detected by using an inverted fluorescent microscope.

Measurement of DNMT Enzymes Activity

Cells treated with Nano-SiO₂ for different passages (P0, P8, P16, and P32 of 16HBE cells, P0, P15, P30, and P45 of BEAS-2B cells) and A549 cells were harvested by trypsinization. Nuclear was extracted and total DNMT enzymes activity was determined by EpiQuik™ DNA Methyltransferase Activity/Inhibition Assay Kit (EpiGentek, United States) according to the procedures provided by the manufacturers.

Qualitative Analysis of DNA Methylation by MSP

Genomic DNA was extracted and treated with bisulfite as previously described (Gong et al., 2012a). After sodium bisulfite conversion, PCR assay was conducted to analyze the DNA methylation level in specific *NRF2* gene loci quantitatively. Primer sets M1 and M2 were designed and synthesized to amplify the methylated DNA, primer sets U1 and U2 were designed and synthesized to amplify the unmethylated DNA. PCR products were separated by electrophoresis at 100 V for 40 min on 2.2% agarose gels. Primers were listed in **Supplementary Table S1**.

Cell Transfection

The cells (16HBE and BEAS-2B) were separately transfected with NRF2 shRNA(h) lentiviral particles (sc-37030-V) or plasmid pEGFP-NRF2 to construct NRF-2 knockdown (KD) or overexpression (OE) cell lines, according to the manufacturer's instructions. For the knockdown of NRF-2, NRF2 shRNA(h) lentiviral particles (shRNA-NRF2) and control shRNA Lentiviral Particles (shRNA-Ctrl) were transfected into cells. After transfected for 24 h, the transfection culture medium was replaced with complete culture medium and incubated for 72 h. Then, 10 µg/ml puromycin dihydrochloride was used to select and achieve stable knockdown cell lines. For the overexpression of NRF-2, pEGFP containing NRF-2 gene sequence (pEGFP-NRF2) or empty pEGFP (pEGFP) were transfected into cells. Full length cDNAs of NRF-2 were amplified through RT-PCR using specific forward 5'-CACCATGGGAATGGACTTGGAGCTGCC-3' and reverse 5'-CTAGTTTTTCTTAACATCTGGCTTCTTAC-3' primers. After transfected for 24 h, cells were selected by G418. Selected cells were harvested for transfection efficiency confirmation and subsequent experiments (**Supplementary Figure S1**).

Statistical Analysis

Data were represented by the means ± SD of at least three independent experiments. Statistical significances among experimental groups were evaluated by ANOVA followed by the Tukey *post hoc* test performed with the GraphPad Prism (version 8.0.2; San Diego, CA, United States).

RESULTS

Nano-SiO₂ Reduces Cell Viability of Human Bronchial Epithelial Cells

Since the respiratory system is the primary site that exposed to the airborne Nano-SiO₂ particles (Song et al., 2009; Yu et al., 2015), two types of human bronchial epithelial cell lines (16HBE and BEAS-2B) were selected as our *in vitro* models. To investigate the cytotoxicity of Nano-SiO₂, we determined the effects of Nano-SiO₂ on the viability of human bronchial epithelial cells by MTT assay. The survival rate of cells treated with Nano-SiO₂ was expressed as the percentage of that of cells in the control group without nano-SiO₂ treatment. For 16HBE cells, no significant change in cell viability was observed when treated with 1.0 or 2.5 µg/ml Nano-SiO₂ for 24 h. After exposure to 10.0 µg/ml Nano-SiO₂ for 24 h, cell viability was reduced to 77.04% of the control group ($p < 0.05$) (**Figure 1A**). For BEAS-2B cells, no significant change in cell viability was observed when treated with 1.0, 5.0, or 10.0 µg/ml Nano-SiO₂ for 24 h (**Figure 1B**). Cell viability was significantly decreased when treated with Nano-SiO₂ no less than 20.0 µg/ml. At a concentration of 40.0 µg/ml Nano-SiO₂, cell viability was reduced to 75.18% of the control group after 24 h ($p < 0.05$) (**Figure 1B**).

With the increase of Nano-SiO₂ concentration, the reduction of cell viability in both cell lines became more severe, showing a dose-dependent manner. Based on the cell viability results, 10 µg/ml and 40 µg/ml Nano-SiO₂ were used as the treatment

TABLE 1 | Tumorigenicity in nude mice of 16HBE and BEAS-2B cells treated with Nano-SiO₂.

Cell lines	Treatment group	Tumors formed at the injection site		
		Incidence ^a	Latency period ^b (weeks)	Tumor size (mm) ^c Diameter mean (range)
16HBE	P0	0/3	NA	NA
	P8	3/3	5	2 (2–3)
	P16	3/3	3	4 (3–6)
	P32	3/3	1	7 (6–9)
BEAS-2B	P0	0/3	NA	NA
	P15	3/3	5	3 (2–4)
	P30	3/3	3	4 (4–6)
	P45	3/3	2	8 (6–9)

^aThe incidence was presented as the number of mice with a tumor at the site of injection versus the total number of mice. ^bThe latency period is the time required for a tumor to be able to visually detect. No tumor regression was ever observed.

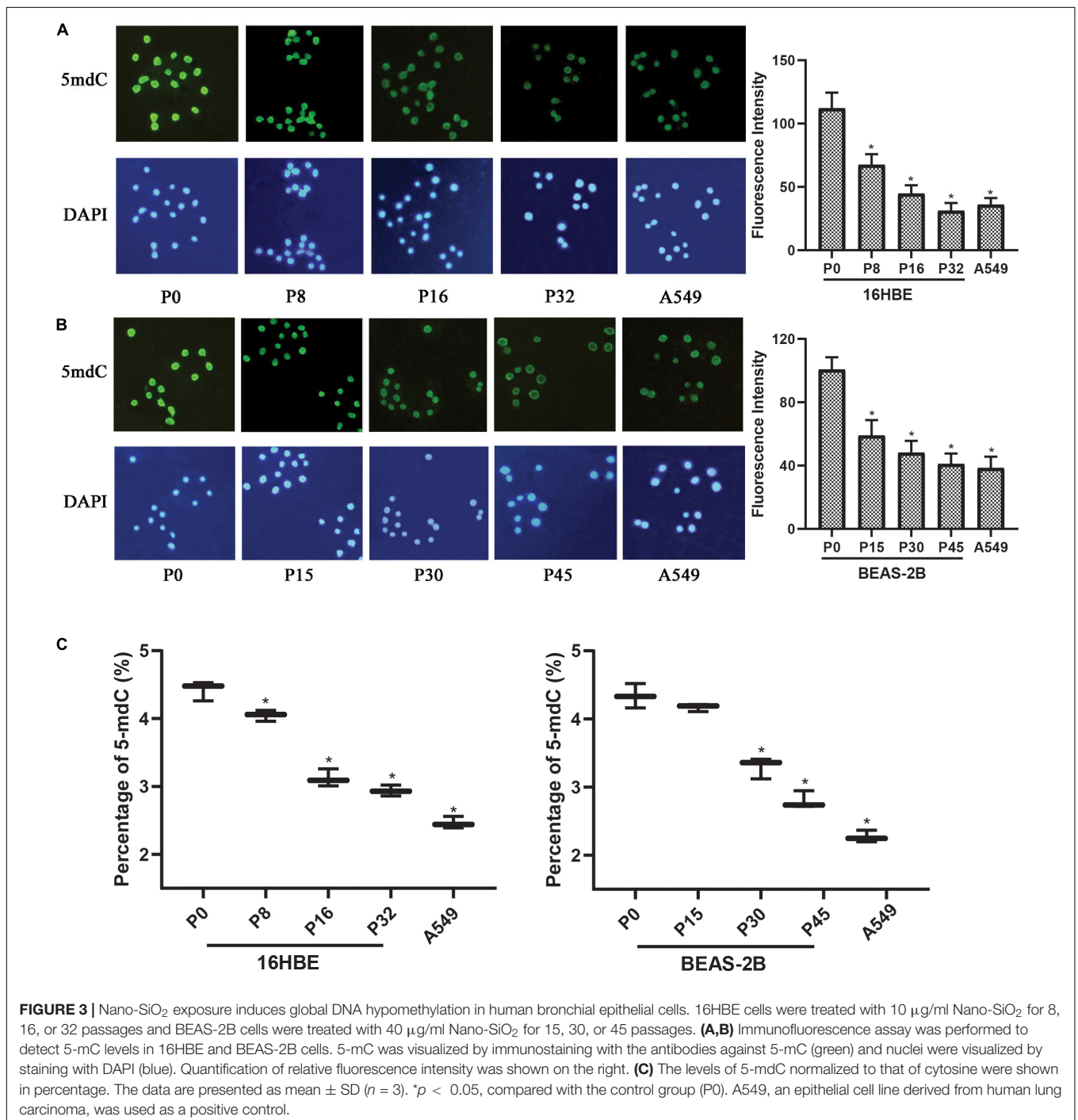
^cThe tumor sizes were measured at the 5th week after injection.

dosage in subsequent experiments for 16HBE and BEAS-2B cells, respectively.

Nano-SiO₂ Induces Malignant Transformation of Human Bronchial Epithelial Cells

To confirm the malignant cellular transformation induced by Nano-SiO₂, a soft agar colony formation assay was

performed to evaluate the ability of Nano-SiO₂-treated cells to grow independently on a solid surface (anchorage-independent growth). The number of colonies grown from 16HBE cells cultured in complete MEM medium (control) for 8, 16, and 32 passages without Nano-SiO₂ exposure was first compared, and no obvious difference was observed (data not shown). Similarly, no obvious difference in the numbers of the colony was observed among the 15th, 30th, and 45th passage of BEAS-2B cells in the



control group (data not shown). Hereafter, when compared with treatment groups, the control group was named as 16HBE-P0 or BEAS-2B-P0.

We observed that with the prolonged exposure to Nano-SiO₂, both 16HBE and BEAS-2B cells developed a significant amount of colonies in the soft agar (**Figure 2B**). Specifically, after treated with 10 μg/ml Nano-SiO₂ for 8 passages, ten thousand 16HBE cells developed 555 colonies in the soft agar, which is approximately twice that from 16HBE-P0 cells which are not exposed to Nano-SiO₂. As the Nano-SiO₂ exposure continued for 32 passages, ten thousand 16HBE cells developed 2161 colonies, which is eight times as many as those growing from 16HBE-P0 cells (**Figure 2C**).

Similarly, after treated with 40 μg/ml Nano-SiO₂ for 15 passages, ten thousand BEAS-2B cells developed 440 colonies, which is approximately twice as many as the colonies growing from BEAS-2B-P0 cells. As the Nano-SiO₂ exposure continued for 45 passages, ten thousand BEAS-2B cells developed 1788 colonies, which is seven times as many as the colonies growing from BEAS-2B-P0 cells (**Figure 2D**). The results indicated that Nano-SiO₂ could induce malignant cell transformation in human bronchial epithelial cells and promote tumorigenesis *in vitro*.

Tumorigenicity of Nano-SiO₂-Transformed Cells in Nude Mice

To assess the *in vivo* carcinogenicity of Nano-SiO₂-treated cells, twenty-four nude mice received subcutaneous injections of human bronchial epithelial cells with various treatments (three mice in each group). The features of the tumor formed at the site of cell injection are summarized in **Table 1**. We did not observe any tumor when 16HBE or BEAS-2B cells were not exposed to Nano-SiO₂ (P0). In contrast, both 16HBE and BEAS-2B cells treated with Nano-SiO₂ formed tumors in nude mice within five weeks. The latent period of tumorigenesis induced by cells treated with Nano-SiO₂ for a longer period was generally shorter than cells treated for a shorter period. Also, the size of tumors growing in nude mice correlated with the duration of Nano-SiO₂ treatment. Specifically, at the fifth week after injection, P8-, P16-, and P32- 16HBE cells formed tumors with a mean diameter of 2, 4, and 7 mm, respectively. Similarly, at the fifth week after injection, P15-, P30-, and P45-BEAS-2B cells formed tumors with a mean diameter of 3, 4, and 8 mm, respectively. Cells treated with Nano-SiO₂ for a longer time tend to induce larger tumors in nude mice. These results indicated that human bronchial epithelial cells could be malignantly transformed by Nano-SiO₂ exposure and acquire tumorigenicity *in vivo*.

Nano-SiO₂ Induces Global DNA Hypomethylation in Human Bronchial Epithelial Cells

Since global changes in DNA methylation are a hallmark of carcinogenesis, the levels of genome-wide DNA methylation in Nano-SiO₂ treated cells were determined. Immunofluorescence

assay was first performed to estimate the relative intensity of 5-mC in human bronchial epithelial cells (16HBE and BEAS-2B cells) and A549 cells (human lung carcinoma cell line, as a positive control).

A significant decrease in the fluorescence intensity of 5-mC was observed in both 16HBE and BEAS-2B cells treated with Nano-SiO₂. For 16HBE cells, the mean fluorescence intensity of 5-mC was reduced significantly by 39.85%, 60.14%, and 72.18% in P8, P16, and P32 cells, respectively, when compared with the control group (16HBE-P0) (**Figure 3A**). For BEAS-2B cells, the mean fluorescence intensity of 5-mC was reduced significantly by 41.40%, 52.18%, and 59.23% in P15, P30, and P45 cells, respectively, when compared with the control group (BEAS-2B-P0) (**Figure 3B**).

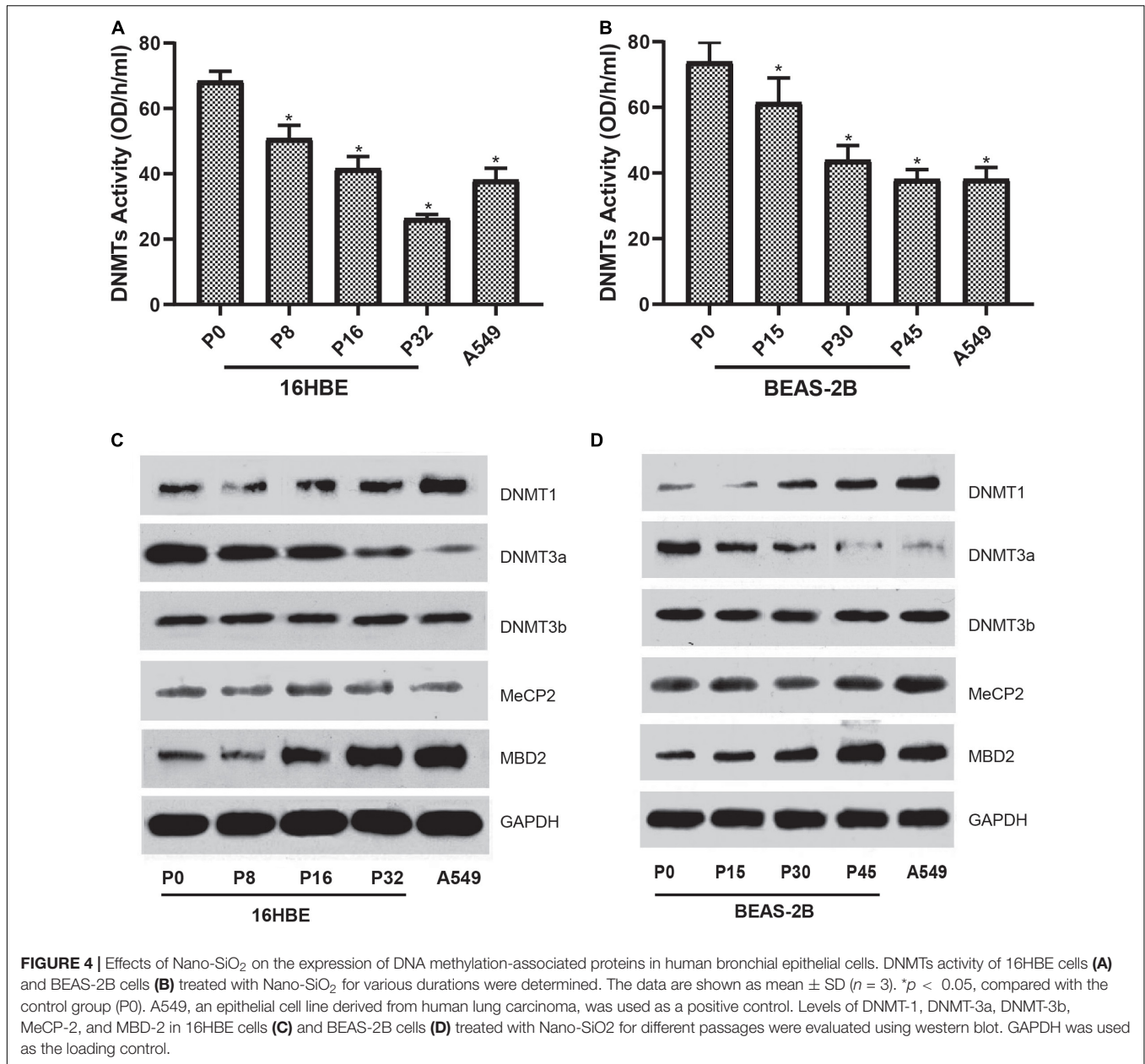
High-performance capillary electrophoresis (HPCE) assays were performed to further investigate the levels of genome DNA methylation in cells treated with Nano-SiO₂. We found that the levels of 5-mdC were reduced in cells exposed to Nano-SiO₂ in both 16HBE and BEAS-2B cells when compared with the non-exposed cells in P0, the control group (**Figure 3C**). Besides, the extent of 5-mdC reduction correlated with the duration of Nano-SiO₂ exposure (**Figure 3C**). These results suggested that Nano-SiO₂ exposure induces genome-wide DNA hypomethylation of human bronchial epithelial cells, and the decrease of DNA methylation is aggravated with prolonged Nano-SiO₂ exposure.

Influences of Nano-SiO₂ on the Expression of DNA Methylation-Associated Proteins

To characterize the global DNA hypomethylation in cells treated with Nano-SiO₂, the total activity of DNA methyltransferases (DNMTs) catalyzing DNA methylation was investigated. We found that the total activity of DNMTs in both cell lines (16HBE and BEAS-2B) was gradually inhibited with the prolonged treatment with Nano-SiO₂ (**Figures 4A,B**). We further determined the levels of DNMTs in cells treated with Nano-SiO₂. According to the results of western blot analysis, we found that the levels of DNMT1 are increased while DNMT3a protein expression is decreased in cells treated with Nano-SiO₂ (**Figures 4C,D**). Further investigation on levels of methyl CpG binding proteins (MeCP-2 and MBD2) revealed that Nano-SiO₂ exposure significantly increases MBD2 protein expression in both 16HBE and BEAS-2B cells. No significant change was observed in levels of MeCP-2 in both cell lines after Nano-SiO₂ treatment (**Figures 4C,D**).

Nano-SiO₂ Inhibits the CpG Methylation at the Promoter Region of NRF2 Gene

The transcription factor NRF2 involved in stress response plays a role in cancer development. Expression of the NRF2 gene is known to be influenced by the status of methylation of CpG islands in the promoter region in cancer cells (Kang et al., 2014; Zhao et al., 2015). Therefore, we asked whether DNA methylation

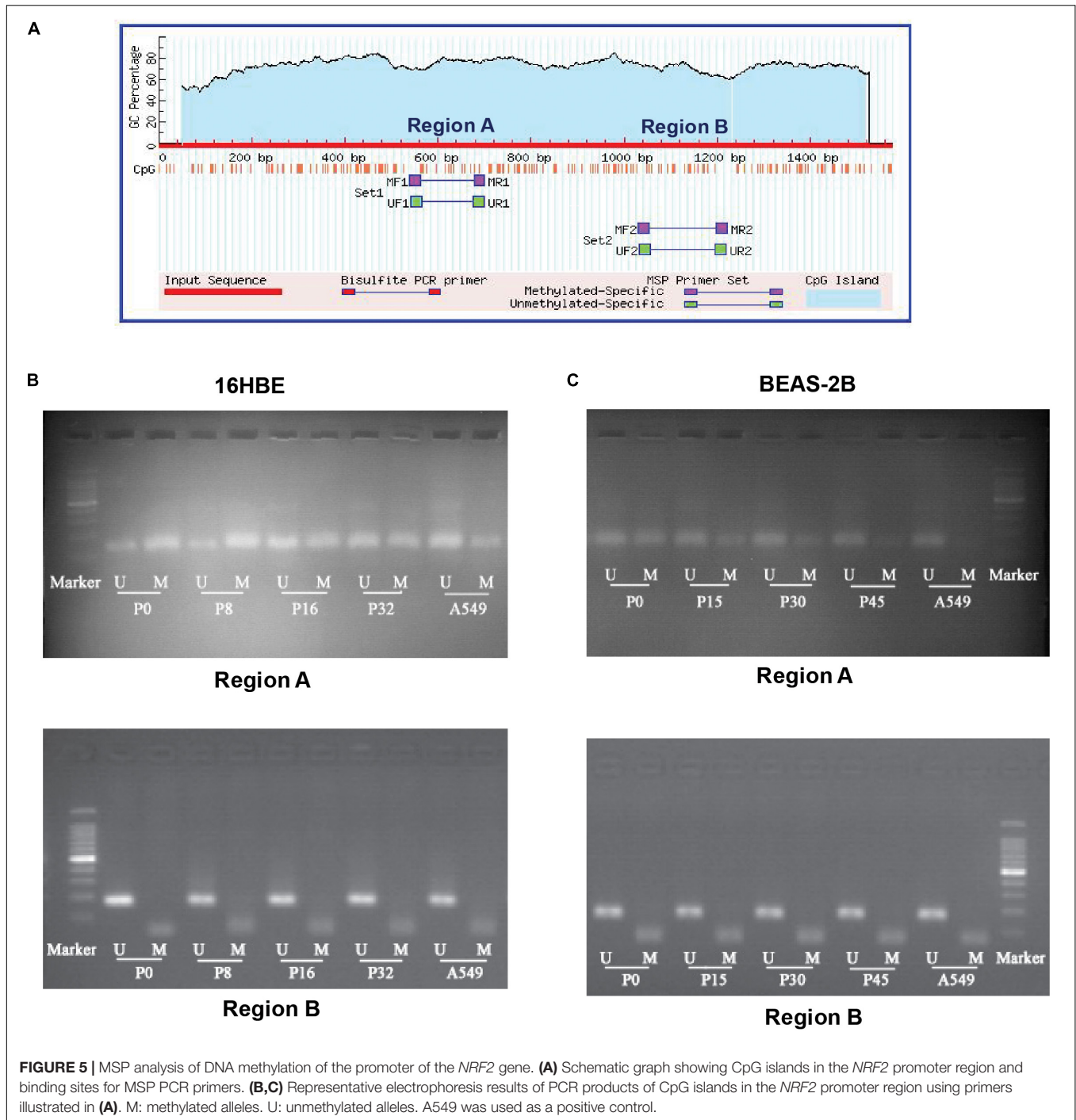


of the promoter region of the NRF2 gene is influenced by Nano-SiO₂ exposure. We conducted a methylation-specific PCR (MSP) to assess the methylation status of CpG sites at the NRF2 promoter region. As illustrated in Figure 5A, two sets of primers (Supplementary Table S2) were designed to detect the methylation status in different CpG islands (region A and region B). The methylation level at region A of 16HBE cells treated with Nano-SiO₂ for 16 (P16) and 32 (P32) passages were decreased when compared with the untreated group (16HBE-P0). Similarly, the methylation level at region A of BEAS-2B cells treated with Nano-SiO₂ for 15, 30, and 45 passages was also reduced when compared with the untreated group (BEAS-2B-P0). However, no obvious changes were found

at region B in 16HBE or BEAS-2B cells after Nano-SiO₂ treatment (Figures 5B,C).

Expression of the NRF2 Gene Is Upregulated in Cells Exposed to Nano-SiO₂

We further investigated the expression of NRF2 gene using qRT-PCR for mRNA and western blot analysis for protein in cells treated with Nano-SiO₂. Our results showed that Nano-SiO₂ exposure significantly increases the mRNA and protein expression of the NRF2 gene in both cell lines (Figures 6A–D), which is consistent with the hypomethylation of global DNA

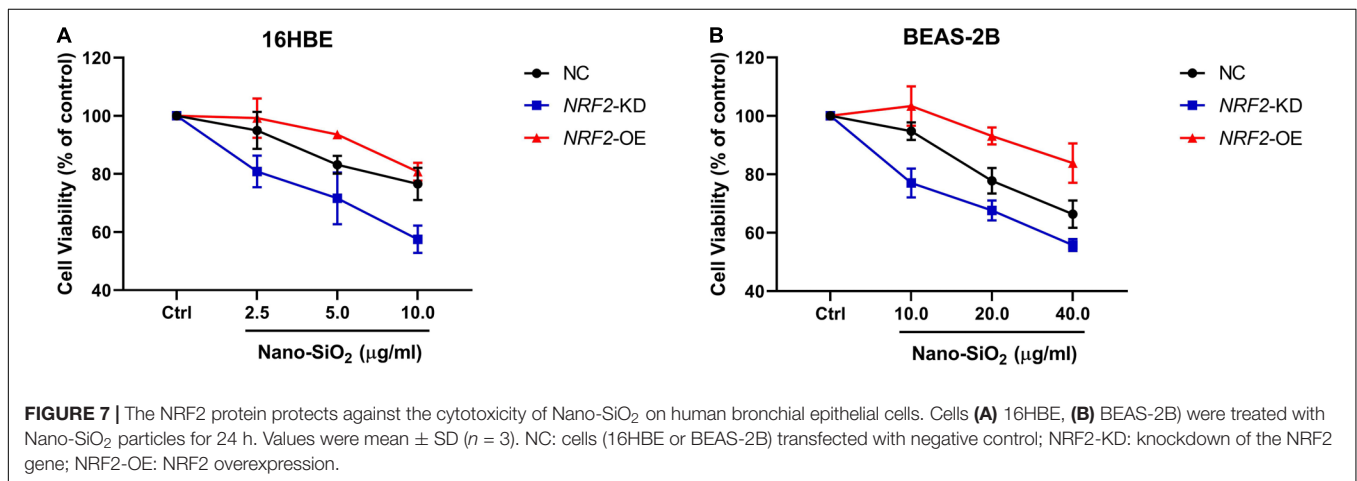
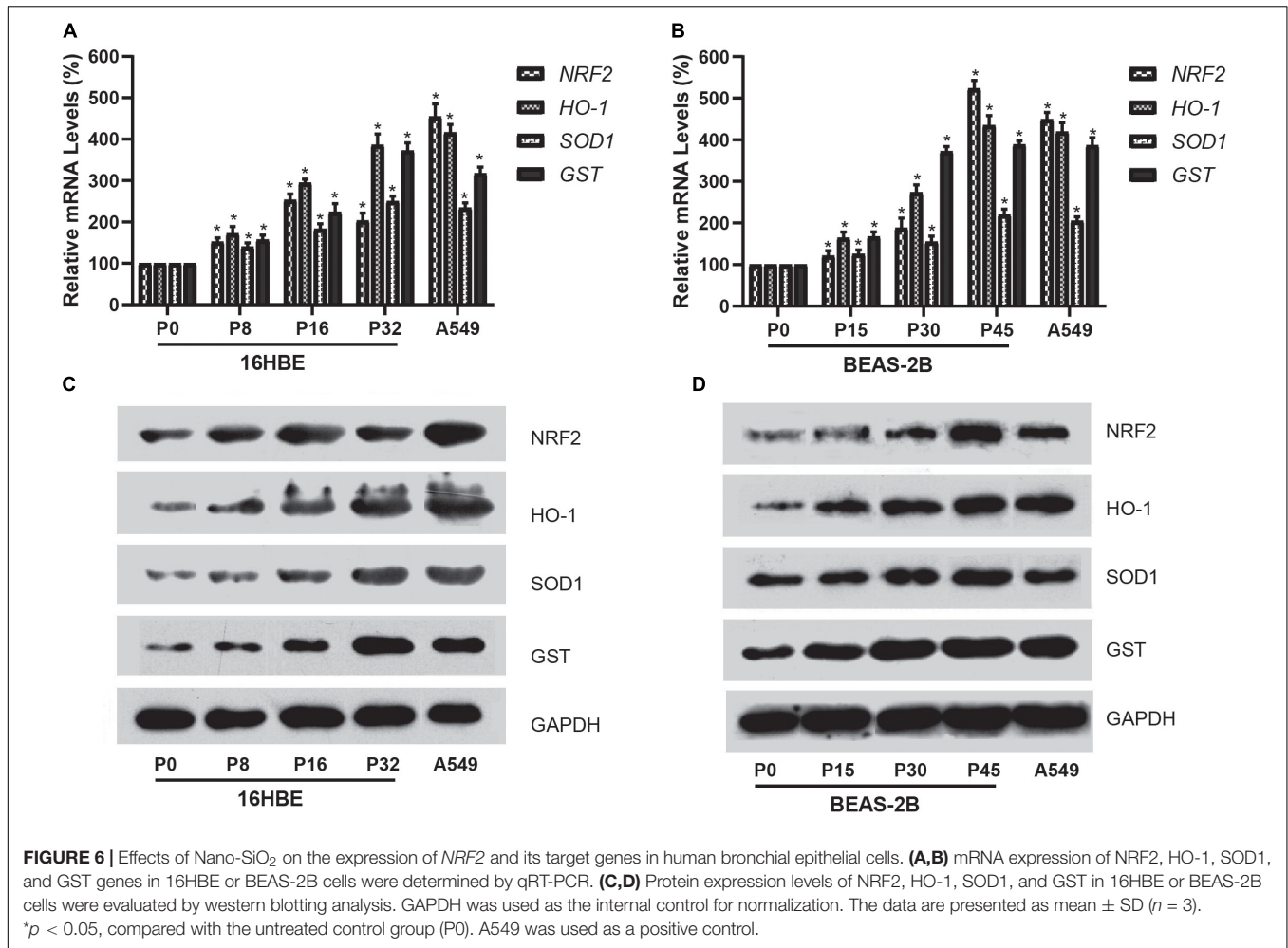


induced by Nano-SiO₂ (Figures 3A,C). Furthermore, we also determined the expression levels of several gene targets (HO-1, SOD1, and GST) of the transcription factor NRF2 upon Nano-SiO₂ exposure. Analysis using qPCR and western blot showed that the mRNA and protein levels of HO-1, SOD1, and GST were also significantly increased by Nano-SiO₂ treatment. The mRNA and protein expression of these anti-oxidative genes regulated by NRF2 largely correlated with the duration of Nano-SiO₂ exposure (Figure 5). These results revealed that

the NRF2 gene and its target genes were up-regulated by Nano-SiO₂ exposure.

NRF2 Inhibits the Carcinogenesis of Human Bronchial Epithelial Cells Induced by Nano-SiO₂

Since the expression of the NRF2 gene is up-regulated by Nano-SiO₂, we further investigated the role of NRF2 in carcinogenesis



induced by Nano-SiO₂ by knockdown or overexpression of the *NRF2* gene. Cells were transfected with vector harboring the *NRF2* gene or the *NRF2* gene shRNA to achieve overexpression (*NRF2*-OE) or knockdown (*NRF2*-KD) of the *NRF2* gene and levels of *NRF2* protein were determined using western blot (**Supplementary Figures S1A–D**). Cell viability assay showed

that neither knockdown nor overexpression of the *NRF2* gene introduces cytotoxicity to 16HBE and BEAS-2B cells (**Supplementary Figures S1E,F**). Thereafter, the effects of *NRF2*-OE and *NRF2*-KD on the viability of cells treated with Nano-SiO₂ were determined using the MTT method. We observed that *NRF2*-OE increases the viability of both 16HBE and BEAS-2B

cells after treated with Nano-SiO₂, whereas NRF2-KD tends to reduce the cell viability upon Nano-SiO₂ treatment (Figure 7). These results suggest that the NRF2 attenuates the cytotoxicity induced by Nano-SiO₂ exposure. To investigate the role of NRF2 protein in the tumorigenicity induced by Nano-SiO₂, the tumorigenicity of cells with NRF2-OE or NRF2-KD exposed to Nano-SiO₂ were determined both *in vitro* and *in vivo*. The 16HBE and BEAS-2B cells with NRF2-OE or NRF2-KD were treated with Nano-SiO₂ (10.0 μg/ml for 16HBE cells, 40.0 μg/ml for BEAS-2B cells) for various passages and followed with soft agar colony formation assay and nude mice injection. With the prolonged Nano-SiO₂ exposure, both NRF2-OE and NRF2-KD cells (16HBE and BEAS-2B) developed a significant amount of colonies in the soft agar (Tables 2, 3). Interestingly, we observed that cells with NRF2-OE formed a lower number of colonies than cells of the control group, while NRF2-KD was able to increase the formation of colonies on soft agar. Consistent with

the *in vitro* assay, both 16HBE (P8, P16, P32) and BEAS-2B (P15, P30, P45) cells with NRF2-OE formed tumors of a smaller size than the control group, while cells with NRF2-KD tend to increase the average tumor size (Figure 8). Taken together, results from both the *in vitro* and *in vivo* assay suggest that the NRF2 protein plays an inhibitory role in the tumorigenesis induced by Nano-SiO₂ exposure.

DISCUSSION

In this study, two types of human bronchial epithelial cell lines (16HBE and BEAS-2B) were selected as the *in vitro* models to investigate the carcinogenicity of Nano-SiO₂. We observed the dose-dependent cytotoxic effects of Nano-SiO₂ on the human bronchial epithelial cells. Also, we showed the tumorigenicity of Nano-SiO₂ in human bronchial epithelial cells.

TABLE 2 | Summary of colony formation in soft agar of 16HBE cells treated with Nano-SiO₂.

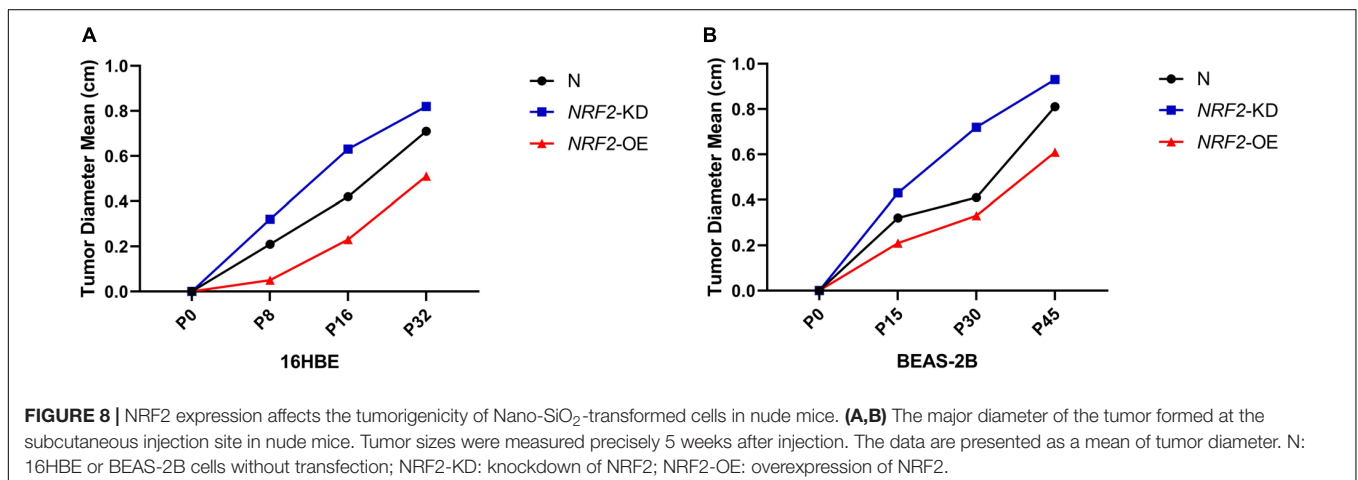
Treatment Group	Cell number	Number of Colonies		
		16HBE	16HBE-ShRNA-NRF2	16HBE-pEGFP-NRF2
P0	1 × 10 ⁴	273 ± 8	284 ± 12	267 ± 10
P8	1 × 10 ⁴	555 ± 32*	730 ± 30* [#]	498 ± 13* [#]
P16	1 × 10 ⁴	1141 ± 73*	1758 ± 108* [#]	846 ± 47* [#]
P32	1 × 10 ⁴	2161 ± 150*	2247 ± 64* [#]	1486 ± 43* [#]

* *p* < 0.05, compared with normal cells without Nano-SiO₂ exposure; [#] *p* < 0.05, compared with normal cells treated with Nano-SiO₂ for the same passages.

TABLE 3 | Summary of colony formation in soft agar of BEAS-2B cells treated with Nano-SiO₂.

Treatment Group	Cell numbers	Number of Colonies		
		BEAS-2B	BEAS-2B-ShRNA-NRF2	BEAS-2B-pEGFP-NRF2
P0	1 × 10 ⁴	254 ± 14	260 ± 8	246 ± 7
P15	1 × 10 ⁴	440 ± 14*	844 ± 53* [#]	324 ± 19* [#]
P30	1 × 10 ⁴	996 ± 80*	1476 ± 51* [#]	705 ± 72* [#]
P45	1 × 10 ⁴	1788 ± 69*	1915 ± 35* [#]	1155 ± 79* [#]

* *p* < 0.05, compared with normal cells without Nano-SiO₂ exposure; [#] *p* < 0.05, compared with normal cells treated with Nano-SiO₂ for the same passages.



As a hallmark of carcinogenesis, alterations in DNA methylation occur frequently in a wide range of cancers (Miousse et al., 2018; Dreval et al., 2019). Our results demonstrated a global genomic hypomethylation in cells exposed to the Nano-SiO₂, which is consistent with the reduction of the total enzyme activity of DNA methyltransferases. Although the total activity of DNMTs is reduced, we found that the expression of DNMT1 protein is elevated and the expression of DNMT3a protein is downregulated. The DNMT1 is responsible for the methylation of tumor suppressor genes (Jair et al., 2006; Ting et al., 2006), the increased expression of which might induce hypermethylation of the tumor suppressor genes, thereby inhibiting the expression of tumor suppressors and promoting carcinogenesis induced by Nano-SiO₂. The DNMT3a protein is known for the *de novo* CpG methylation independent of replication, which is consistent with the global hypomethylation induced by Nano-SiO₂. Loss of DNMT3a has been reported to be associated with leukemia pathogenesis and poor prognosis (Shivarov et al., 2013). Moreover, MBD2 and MeCP2 that can bind to and mediate the repression of methylated tumor suppressor genes (Klose and Bird, 2006; Lopez-Serra et al., 2008; Mian et al., 2011), were also induced by the Nano-SiO₂ exposure.

In addition to the global DNA hypomethylation, we observed a decreased methylation of CpG islands within the NRF2 promoter region in human bronchial epithelial cells exposed to Nano-SiO₂. The NRF2 protein is a key transcription factor involved in cellular defensive mechanisms against stresses induced by electrophiles, oxidants, and chemicals (Motohashi and Yamamoto, 2004). Under the basal condition, NRF2 is localized in the cytoplasm by binding to its cytosolic repressor Kelch-like ECH-associated protein 1 (KEAP1) (Motohashi and Yamamoto, 2004). In response to stress, NRF2 dissociates from KEAP1 and translocates to the nucleus, where it activates the transcription of its target genes to maintain cellular homeostasis (Panieri and Saso, 2019). Short-term exposure to Nano-SiO₂ can increase the expression of the NRF2 gene (Liu et al., 2017), which is consistent with our results that the NRF2 gene expression is up-regulated in both mRNA and protein levels in cells exposed to Nano-SiO₂ exposure. Moreover, our findings revealed that levels of HO1, SOD1, and GST, which are encoded by antioxidative gene targets of the NRF2 protein (Itoh et al., 1997), are increased upon Nano-SiO₂ exposure. These results suggest that NRF2 promotes the stress responses of cells treated with Nano-SiO₂.

Activation of the NRF2 protein is suggested to suppress carcinogenesis, especially in its early stage (Wu et al., 2019). Loss of NRF2 disrupts the antioxidant axis resulting in increased oxidative stress, ultimately leading to DNA damage and the initiation of malignant cellular transformation (Frohlich et al., 2008). In the tumor microenvironment, the tumor suppressor gene BRCA1 activates NRF2 (Gorrini et al., 2013), whereas the

oncogene *Fyn* mediates nuclear export and induces degradation of NRF2 (Niture et al., 2014). NRF2-deficient mice are more susceptible to carcinogens and develop severer tumors in the urinary bladder and liver (Iida et al., 2007; Kitamura et al., 2007).

Collectively, our results showed that NRF2 plays an important role in protecting against the carcinogenesis induced by Nano-SiO₂ exposure. Moreover, hypomethylation at the promoter region of the NRF2 gene contributed to the alterations of NRF2 upon Nano-SiO₂ exposure. It should, however, be noted that histone modifications are also important for NRF2 gene transcription and it cannot be excluded that the alterations of NRF2 at both transcription and protein levels are caused by a more intrinsic mechanism.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Shanghai Municipal Center for Disease Prevention and Control.

AUTHOR CONTRIBUTIONS

DL, GT, and YS jointly conceived this project and supervised the experiments. DL and XW designed the research. DL, XW, PX, QH, JS, and XH performed the experiments and analyzed the experimental results. DL, XW, YS, and GT prepared the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2020.00818/full#supplementary-material>

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Conflict of Interest: YS is employed by the Syngenta (China) Investment Company Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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