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Genome-wide identification and functional analysis of long noncoding RNAs involved in the response to graphene oxide

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ABSTRACT

Long noncoding RNAs (lncRNAs), which are defined as noncoding RNAs having at least 200 nucleotides, can potentially regulate various biological processes. However, the roles of lncRNAs in regulating cellular response to engineered nanomaterials (ENMs) are still unclear. Using Hiseq 2000 sequencing technique, we performed a genome-wide screen to identify lncRNAs involved in the control of toxicity of graphene oxide (GO) using *in vivo Caenorhabditis elegans* assay system. HiSeq 2000 sequencing, followed by quantitative analysis, identified only 34 dysregulated lncRNAs in GO exposed nematodes. Bioinformatics analysis implies the biological processes and signaling pathways mediated by candidate lncRNAs involved in the control of GO toxicity. A lncRNAs-miRNAs network possibly involved in the control of GO toxicity was further raised. Moreover, we identified the shared lncRNAs based on the molecular regulation basis for chemical surface modifications and/or genetic mutations in reducing GO toxicity. We further provide direct evidence that these shared lncRNAs, *linc-37* and *linc-14*, were involved in the control of chemical surface modifications and genetic mutations in reducing GO toxicity. *linc-37* binding to transcriptional factor FOXO/DAF-16 might be important for the control of GO toxicity. Our whole-genome identification and functional analysis of lncRNAs highlights the important roles of lncRNAs based molecular mechanisms for cellular responses to ENMs in organisms.

1. Introduction

Graphene oxide (GO) is a member of graphene family, and contains a single-atom thick, two-dimensional sheet of hexagonally arranged carbon [1]. Due to its specific physiochemical properties, GO is a promising technology with potential biomedical applications including drug delivery, tissue engineering, biosensor, and imaging [2–5]. However, in addition to their industrial and medical applications, engineered nanomaterials (ENMs), including GO, may have negative side effects on human health and organisms in the environment [6–10]. Both *in vitro* and *in vivo* studies have suggested that GO could induce the generation of reactive oxygen species (ROS) in targeted cells, and cause several aspects of adverse effects on organisms such as induction of cell apoptosis, lipid peroxidation, mitochondrial dysfunction, reduced cell adhesion, pulmonary toxicity, and immunotoxicity [11–13]. To describe the molecular mechanisms underlying GO toxicity, Yuan et al. identified 30 differentially expressed proteins in GO treated hepatoma HepG2 cells [14], and Chatterjee et al. identified the dysregulated messenger RNA (mRNA) profiling in HepG2 cells [15]. The data from Qu et al. suggest that GO toxicity may be mediated by activation of toll-like receptor 4 (TLR4) signaling and subsequently in part *via* autocrine tumor necrosis factor-α (TNF-α) production [16].

In order to reduce the toxicity, some specific chemical modifications were developed for GO. For example, PEGylated (PEG = polyethylene glycol) GO exhibited biocompatibility in mice fibroblast cells [17]. In addition, incubation of GO with fetal bovine serum (FBS) or bovine serum albumin (BSA) largely attenuated GO toxicity [18,19]. These data imply that surface chemical modification is a promising strategy against GO toxicity in organisms.

Caenorhabditis elegans (*C. elegans*), a thoroughly studied model animal, is well suited for asking *in vivo* questions on toxicological mechanisms of toxicants with relevance at the organism level [8,20]. The conserved property of basic physiological processes, stress responses, signal transduction pathways, and epigenetic marks between *C. elegans* and humans enables the comparison of molecular mechanisms [21]. So far, *C. elegans* has been successfully

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used for the toxicological study of some carbon-based ENMs [22–25]. In *C. elegans*, previous studies have demonstrated the several aspects of *in vivo* adverse effects of GO on animals, such as the induction of oxidative stress and the toxic effects on the functions of both primary (such as the intestine) and secondary (such as the neuron and the reproductive organs) targeted organs [26–28]. Previous studies on the role of ribonucleic acid (RNA) molecules in regulating GO toxicity have revealed the dysregulated microRNA (miRNA) profiling induced by GO exposure in both *C. elegans* and GLC-82 pulmonary adenocarcinoma cells [29,30]. In *C. elegans*, a previous study has further demonstrated that mutation of *clk-1* gene encoding an ortholog of demethoxyubiquinone hydroxylase required for the biosynthesis of ubiquinone or *isp-1* gene encoding a subunit of mitochondrial complex III induced a resistant property of nematodes to GO toxicity [28].

More recently, long noncoding RNAs (lncRNAs), defined as noncoding RNAs that have at least 200 nucleotides and no (or weak) protein coding ability, have been shown to potentially regulate various biological processes [31–33]. Most of the lncRNAs in *C. elegans* have already been identified [34]. However, the roles of lncRNAs in regulating cellular response to ENMs are still unclear. Thus, in the present study, we employed the *in vivo C. elegans* assay system to perform the genome-wide screen to identify the lncRNAs involved in the control of GO toxicity. Moreover, we examined how the response of lncRNAs to GO is influenced by chemical modifications or genetic mutations in nematodes. Our study suggests an important link lncRNA regulation and GO toxicity in organisms.

2. Materials and methods

2.1. Reagents and preparations of GO, GO-PEG, and GO-FBS

GO was prepared from natural graphite powder according to the modified Hummer's method [35,36]. Graphite (2 g) and sodium nitrate (1 g) were added to a 250-mL flask. After addition of concentrated H_2SO_4 (50 mL) on ice, $KMnO_4$ (7 g) was added to the mixture. The temperature of the mixture was raised to 35 °C, and then 90 mL of H_2O was slowly dripped into the paste to cause an increase in temperature to 70 °C. After stirring the diluted suspension at 70 °C for another 15 min, the suspension was treated with a mixture of 7 mL of 30% H_2O_2 and 55 mL of H_2O. The resulting warm suspension was filtered to obtain a yellow-brown filter cake, and this filter cake was washed with a solution of 3% HCl, followed by drying at 40 °C for 24 h. GO was obtained by ultrasonication of as-made graphite oxide in water for 1 h. GO was functionalized with PEG to prepare GO-PEG as described previously [37]. The PEG content in GO-PEG was measured by thermogravimetric analysis (TGA). The chemical structure of PEG-amine was mPEG-CH_2CH_2-NH_2HCl. The molecular weight of PEG-amine was 20000, and the weight % of PEG in GO-PEG was 61.4%. To prepare GO-FBS, 50 μL of 10% (v/v) FBS solution was mixed with 100 μg of GO after sonification (40 kHz, 100 W). The mixture was kept for 2 h at 37 °C for the further centrifugation at 19,000 *g* for 10 min [18].

To determine the stability of GO-FBS, the amount of FBS protein in the supernatant was determined using a Bradford Protein Assay Kit (Tiangen Biotech Co. Ltd., Beijing, China). The amount of protein conjugated to the surface of GO was evaluated by the following equation: $W_{GO} = W_{Total} - W_{Sup}$. The FBS protein conjugating ratio on the surfaces of GO was evaluated using the following equation: $q = (W_{GO} - W_{Sup})/(W_{GO}) \times 100\%$. W_{Total}, W_{Sup} and W_{GO} represent the total amount of protein, the amount of protein in the suspension and the amount of protein conjugated on the surfaces of GO, respectively. The conjugates were resuspended in K-medium. And then, by incubating at 20 °C for 0, 12, 24, 36 and 48 h, the amount of FBS protein in the supernatant was determined.

GO and GO-PEG were sonicated for 30 min (40 kHz, 100 W). Next, GO, GO-PEG, or GO-FBS was dispersed in K medium to prepare a stock solution (1 mg/mL). The stock solution was then diluted in K medium to the working concentration just prior to exposure. All the other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Characterization of GO, GO-PEG, or GO-FBS

The prepared GO, GO-PEG, or GO-FBS was characterized by atomic force microscopy (AFM) (SPM-9600, Shimadzu, Japan), and zeta potential analyzed by a Nano Zetasizer using a dynamic light scattering technique. To perform AFM measurement, a few drops of GO, GO-PEG, or GO-FBS suspension was pipetted on silicon substrates, and then the substrates were air-dried and placed under the AFM tip for morphology analysis. GO and GO-PEG were further characterized by Raman spectroscopy (Renishaw Invia Plus laser Raman spectrometer, Renishaw, UK), and X-ray photoelectron spectrum (XPS) (AXIS Ultra instrument, Kratos, UK).

2.3. C. elegans strain preparation

Some nematode strains used in the present study were wild-type N2, and mutants of *clk-1 (e2519)* and *isp-1 (qm150)*, originally obtained from the *Caenorhabditis* Genetics Center (funded by NIH Office of Research Infrastructure Programs (P40 OD010440)). Nematodes were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20 °C as described [38]. Gravid nematodes were washed off the plates into centrifuge tubes, and lysed with a bleaching mixture (0.45 M NaOH, 2% HOCl). Age synchronous populations of L1-larvae were obtained as described [39]. Exposure to GO, GO-PEG, or GO-FBS was performed from the L1-larvae stage to young adulthood in 12-well sterile tissue culture plates at 20 °C in the presence of food (OP50). The exposed nematodes were used for toxicity assessment with the aid of lifespan, intestinal ROS production, reproduction, and locomotion behavior as the endpoints.

2.4. Toxicity assessment

Lifespan assay was performed basically as described [40]. Hermaphrodite nematodes were transferred daily for the first 4 days of adulthood. Nematodes were checked every day, and would be scored as dead if they did not move even after repeated taps with a pick. Mean lifespan means the time when 50% of animals will die. Forty nematodes were examined per treatment, and three replicates were performed.

The method for ROS production was performed as described previously [41]. The examined nematodes were transferred into 1 μM 5′,6′-chloromethyl-2′,7′dichlorodihydro-fluorescein diacetate (CM-H_2DCFDA; Molecular Probes) in 12-well sterile tissue culture plates and pre-incubated for 3 h at 20 °C in the dark. Then nematodes were mounted on 2% agar pads for examination at 488 nm of excitation wavelength and 510 nm of emission filter with a laser scanning confocal microscope (Leica, TCS SP2, Bensheim, Germany). Relative fluorescence intensity of the intestine was semi-quantified, and the semiquantified ROS was expressed as relative fluorescence units (RFU). Thirty nematodes were examined per treatment, and three replicates were performed.

Reproduction was assessed by the endpoint of brood size as described [42]. To assay the brood size, the number of offspring at all stages beyond the egg was counted. Twenty nematodes were examined per treatment, and three replicates were performed.

Locomotion behavior of nematodes was assessed by endpoints of head thrash and body bend as described [43]. A head thrash was