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DNA Damage Induced by Multiwalled Carbon Nanotubes in Mouse Embryonic Stem Cells

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ABSTRACT

Carbon nanotubes (CNTs) have shown promise as an important new class of multifunctional building blocks and innovative tools in a large variety of applications, ranging from nanocomposite materials through nanoelectronics to biomedical devices. Because of their unusual one-dimensional hollow nanostructure and unique physicochemical properties, CNTs are particularly useful as novel drug delivery tools and imaging agents. However, such biomedical applications will not be realized if there is no proper assessment of the potential hazards of CNTs to humans and other biological systems. Although a few reports on the cytotoxicity of CNTs have been published, very little is known about the toxicity at the molecular level, or genotoxicity, of CNTs in mammalian cells. We have for the first time assessed the DNA damage response to multiwalled carbon nanotubes (MWNTs) in mouse embryonic stem (ES) cells. We found that MWNTs can accumulate and induce apoptosis in mouse ES cells and activate the tumor suppressor protein p53 within 2 h of exposure. Furthermore, we also observed increased expression of two isoforms of base excision repair protein 8-oxoguanine-DNA glycosylase 1 (OGG1), double strand break repair protein Rad 51, phosphorylation of H2AX histone at serine 139, and SUMO modification of XRCC4 following the treatment with MWNTs. A mutagenesis study using an endogenous molecular marker, adenine phosphoribosyltransferase (Aprt), showed that MWNTs increased the mutation frequency by 2-fold compared with the spontaneous mutation frequency in mouse ES cells. These results suggest that careful scrutiny of the genotoxicity of nanomaterials is needed even for those materials, like multiwalled carbon nanotubes, that have been previously demonstrated to have limited or no toxicity at the cellular level.

Carbon nanotubes (CNTs) with their unique one-dimensional hollow nanostructure and unusual properties are emerging as an important new class of multifunctional building blocks for the development of nanoscience and nanotechnology.[1] Following the early 1990s great need for the macroscopic preparation of CNTs as innovative tools in nanoscience, the recent rapid development in nanotechnology has renewed the pressing demand for large scale production of CNTs for potential applications in commercial products, including nanocomposite materials, nanoelectronics, and biomedical devices (e.g., gene and drug carriers).[2−4] The number of industrial scale facilities for the relatively low-cost production of multiwalled carbon nanotubes (MWNTs) continues to grow, and with that the professional and public exposure to MWNTs is expected to increase significantly in the coming years. Therefore, it is essential to ascertain the potential hazards of CNTs, particularly MWNTs, to humans and other biological systems. So far, only a few studies on the biocompatibility and cytotoxicity of CNTs have been reported and the results are somewhat conflicting. For instance, it has been demonstrated that CNTs can induce inflammatory and apoptosis responses in human T-cells.[5−7] Furthermore, gene expression analysis indicated that MWNTs activated genes involved in cellular transport, metabolism, cell cycle regulation, and stress response in human skin fibroblasts.[8] More recently, Magrez et al. found evidence of cytotoxicity for carbon-based nanomaterials, although MWNTs were the least toxic among the carbon nanotubes, carbon nanofibers, and carbon nanoparticles tested.[9] In a somewhat related, but independent study, Dumortier et al. demonstrated that water-soluble CNTs functionalized with polyethylene glycol chains did not have toxic effects when tested in a wide variety of immune system cell types.[10] Although the health effects of CNTs have attracted considerable attention, the scientific community has thus far focused primarily on the studies of CNT toxicity at the cellular level. Information concerning possible CNT-induced DNA damage and mutagenic effects at the molecular level is largely lacking.

We have previously demonstrated that MWNTs generated reactive oxygen species (ROS) (e.g., superoxide anions, hydroxyl radicals, hydrogen peroxide) after having been introduced into certain mammalian cells.[11] Free radicals can

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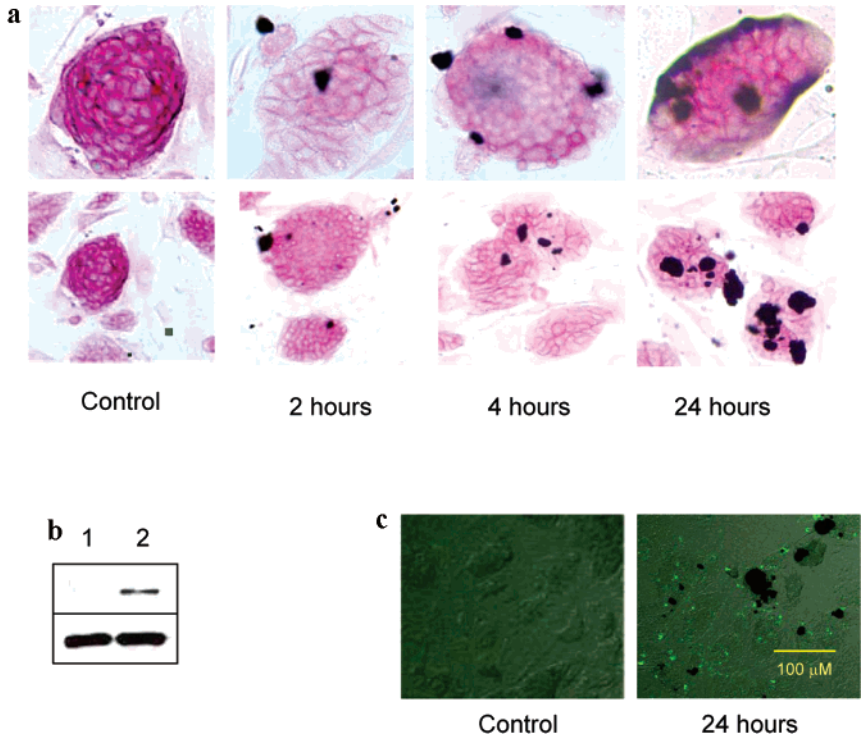

Figure 1. The images of MWNT uptake and induce differentiation and apoptosis in mouse ES cells following MWNT treatment. (a) Mouse ES cells were treated with 100 μg/mL of MWNTs for 2, 4, and 24 h. ES cells (control and exposed) were washed with 1X PBS and fixed with 4% paraformaldehyde. AP stain results showed that some ES cells began to lose AP turning to white in color compared with the untreated cells, indicating that the MWNT reduced the stem cell marker expression. The images were captured by inverted microscope (Olympus CK2) at 10× (bottom row) and 20× (top row) magnification via QCapture Pro imaging software. (b) The stem cell factor Oct4 protein expression 48 h after MWNTs treatment. Equal amounts of cell lysate were analyzed by Western blot with Oct4 antibody. Lane 1, untreated cell lysate; Lane 2, MWNTs-treated cell lysate. (c) Annexin V−FITC-staining result of ES cells untreated and treated with 100 μg/mL of MWNTs for 24 h. ES cell were washed with binding buffer and stained with the Annexin V−FITC. Images were acquired with a Fluoview laser scanning confocal microscope mated to a Zeiss Axioplan upright microscope using 10× magnifications.

chemically alter DNA bases and cause DNA damage within the cell. In the present study, we chose mouse embryonic stem (ES) cells as a sensitive assay to assess MWNT-mediated DNA damage, as ES cells have previously been shown to be susceptible to DNA damaging agents.[12−14] In response to DNA damage, eukaryotic cells, including ES cells, have developed several mechanisms to protect genomic integrity. In the presence of damaged DNA for instance, the p53 protein is activated by protein phosphorylation as a master guardian that activates cell cycle checkpoints and triggers cell cycle arrest to provide time for the DNA damage to be repaired.[15,16] Enhanced expression of p53 may also trigger cell death by apoptosis if the DNA damage is beyond repair,[17] while under normal conditions (absence of DNA damage) p53 is expressed at low levels. The close relationship between p53 activation and DNA damage makes p53 the molecular marker of choice for assessing the genotoxicity of MWNTs to mouse ES cells.

ES cells are a unique cell population with the ability to undergo both self-renewal and differentiation with the potential to give rise to all cell lineages and an entire organism.[18−20] It has been shown that ES cells are highly sensitive to DNA damaging agents.[12−14] The sensitivity of ES cells to DNA damage prompted us to study the genotoxicity of MWNTs in mouse ES cells using p53 as a molecule marker. To study the cellular uptake and response of MWNTs to mouse ES cells, the ES cells derived from the inner cell mass of 3.5 day-old blastocysts were fixed and stained with an alkaline-phosphatase (AP) detection kit after administering MWNTs and incubation for periods of 2, 4, and 24 h. AP is a stem cell marker that is only expressed on the ES cell's surface. The undifferentiated ES cells that express AP turn into red colonies after staining with Naphthol/Fast Red Violet Solution, while the differentiated colonies lacking the stem cell marker are colorless. Figure 1a shows that the ES cells began uptake of MWNTs after 2 h exposure and continued to accumulate throughout the time course of this study. The AP-staining results showed that MWNTs reduced the red color of the AP-stained ES cells, suggesting that MWNTs reduced the stem cell marker expression. We have further confirmed the MWNT-induced the stem cell differentiation by examining the stem cell factor Oct4 expression (Figure 1b). Furthermore, as shown in Figure 1c, Annexin V−FITC-staining result of the ES cells treated with 100 μg/mL of MWNTs for 24 h (right image) with respect to the untreated cells (left image) indicated that MWNTs-induced ES cells undergo apoptosis through one of the molecular mechanisms to maintain the ES cell genomic integrity in response to DNA damage.[13]

To investigate the DNA damage response to MWNTs, we analyzed p53 expression levels by Western blot following the MWNT treatment. The results clearly showed that p53

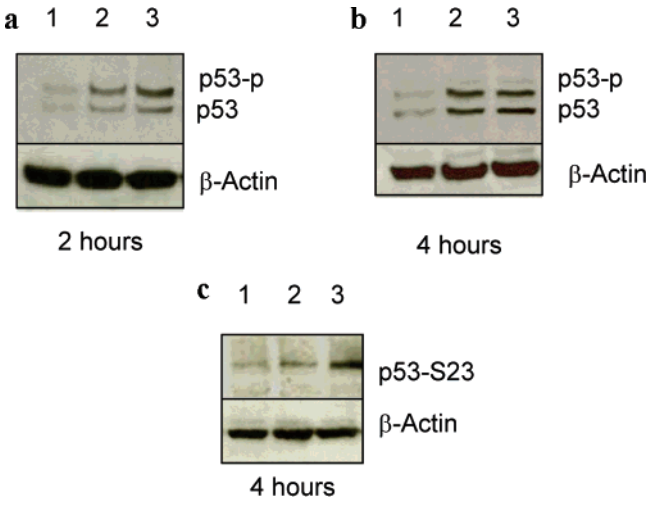

Figure 2. MWNT induced p53 protein expression and modification. The ES cells were lysed with RIPA buffer, and the cells lysates were analyzed by Western blot with a p53 monoclonal antibody. (a) Two hours after the MWNT treatment. Lane 1, untreated; Lanes 2 and 3, subjected to 5 and 100 μg/mL MWNTs, respectively. (b) Four hours after the MWNT treatment. Lane 1, untreated; Lanes 2 and 3, subjected to 5 and 100 μg/mL MWNTs, respectively. (c) Cell lysates were analyzed using a phosphor-specific antibody to p53-ser-23. Lane 1, untreated; Lanes 2 and 3, subjected to 5 and 100 μg/mL MWNTs, respectively. β-actin was used as a loading control.

protein expression level increased within 2 h after the cells were exposed to MWNTs (Figure 2a). Furthermore, the p53 expression levels proportionally increased with the amount of MWNTs used in the treatment (Lanes 2 and 3 in Figure 2a). Activation of p53 is known to be modulated by protein phosphorylation, which transforms the p53 protein from a latent to an active conformation. As can be seen in Figure 2a,b, two bands were observed using the p53 monoclonal antibody. Using the phospho-specific antibody to p53-Ser-23 to examine p53 phosphorylation, we confirmed that MWNTs indeed induced p53 phosphorylation by the checkpoint protein kinase 2 (Chk2) (Figure 2c). The above observations suggest that MWNTs could cause DNA damage, as evidenced by the induction and accumulation of the p53 tumor suppressor protein.

To further investigate the specific kinds of DNA damage induced by MWNTs, we examined the expression of the key base excision repair pathway enzyme 8-oxoguanine-DNA glycosylase 1 (OGG1) by Western blot analysis following the MWNTs treatment. OGG1 is the major enzyme repairing 8-oxoguanine (8-oxoG), a mutagenic guanine base lesion produced by ROS,[21] through the removal of the oxidized bases via the base excision repair pathway.[22] There are two isoforms of OGG1 encoded by alternatively spliced OGG1 mRNA: a 36 kDa polypeptide in the nuclear extract and a 40 kDa polypeptide in the mitochondria.[23] As shown in Figure 3, the MWNT treatment elevated the expression of both isoforms of OGG1, which suggests the occurrence of both nuclear and mitochondrial DNA damage through a mutagenic guanine base lesion.

The MWNT-induced DNA base modification described above may also cause subsequent breakdown of the DNA

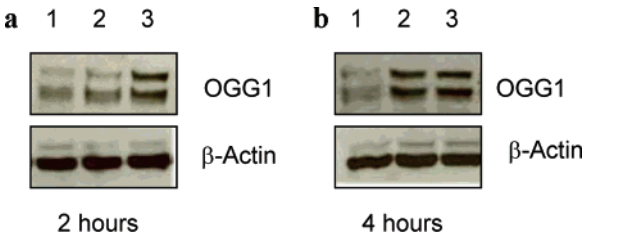

Figure 3. Up-regulation of two isoforms of the key base excision repair protein 8-oxoguanine-DNA glycosylase 1 (OGG1) in response to the MWNT treatment. The ES cells were lysed with RIPA buffer, and cell extracts subjected to Western blots with OGG1 polyclonal antibody. Lane 1, untreated; Lanes 2 and 3, subjected to 5 and 100 μg/mL MWNT treatment, respectively. β-actin was used as a loading control.

double strand.[24] To examine this possibility, we assayed two key double strand break repair proteins: Rad51 and X-ray cross-complementation group 4 (XRCC4) involving in homologous recombination repair and non-homologous end join repair, respectively.[25] As shown in Figure 4, both proteins were up-regulated in response to the MWNT treatment. Furthermore, Western blots revealed that the MWNT treatment increased XRCC4 expression and produced two additional higher molecular weight bands reacting with the XRCC4 antibody (Figure 4b). It has been shown that in response to X-ray treatment, XRCC4 is altered by a small ubiquitin-like modifier (SUMO), which regulates its localization and function in DNA double strand break repair.[26] To investigate the induction of XRCC4 sumolation in the MWNT-treated mouse ES cells, we immunoprecipitated XRCC4 with the XRCC4 antibody and probed it with a SUMO-1 antibody. Figure 4c shows that the 95 kDa and another higher-order adduct are indeed from the SUMO-modified XRCC4, while the major band at 65 kDa corresponds to the unmodified protein as reported by Yurchenko group.[26] As expected, low sumoylation levels of XRCC4 were detected in the untreated samples due to the spontaneous DNA double strand breakage that occurs during the DNA replication or under tissue culture conditions. The increased level of SUMO-modified XRCC4 implicated that MWNTs indeed induced the ES cell DNA double strand breakage. This suggestion was further verified by the immunofluorescent staining with phosphor-specific antibody to Histone H2A-Ser 139 antibody (Figure 4d), as Histone H2A Ser 139 phosphorylation in response to the DNA double-strand breakage has been well characterized.[27]

Because chemical modification of DNA bases and the breakage of DNA double strands can introduce a broad spectrum of mutations including mitotic recombination, point mutation, and chromosome loss and translocation, we envisioned that the MWNT treatment might also increase the mutation frequency in ES cells. In this context, we used the endogenous molecular marker Adenine Phosphoribosyltransferase (Aprt)[28,29] as a reporter to examine the mutation frequency in ES cells following the MWNT treatment. The Aprt+/−, heterozygous 3C4 ES cells were treated with MWNTs for 4 h. The Aprt-deficient cells can be selected in the presence of adenine analogues, such as 2-fluoroadenine (FA), whose metabolic products are cytotoxic to Aprt-

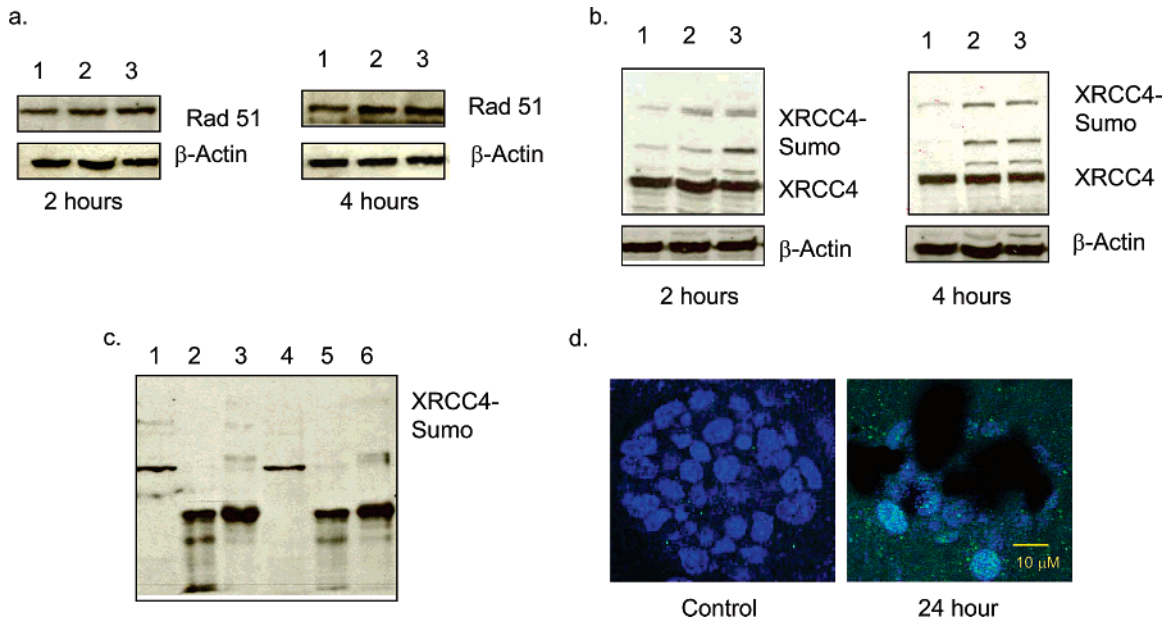

Figure 4. Up-regulation of protein Rad 51, Sumoylation of XRCC4, and phosphorylation of H2A at Ser 139 in response to the MWNT treatment. (a) Cell lysates from samples treated in the same way as for Figure 3 were analyzed with a Rad 51 polyclonal antibody. β-actin acted as the loading control. (b) Cell lysates from samples treated in the same way as for Figure 3 were analyzed with a XRCC4 polyclonal antibody. β-actin was used as the loading control. (c) MWNT-treated cell lysates were immunoprecipitated with the XRCC4 polyclonal antibody, subjected to Western blotting, and probed with a SUMO-1 antibody. Lanes 1−3, untreated samples; Lanes 4−6, after 4 h of the MWNT treatment; Lanes 1 and 4, the whole cell extract; Lanes 2 and 5, protein A/G PLUS-Agarose added to the cell lysate without XRCC4 antibody; and Lanes 3 and 6, protein A/G PLUS-Agarose added to the cell lysate with XRCC4 antibody. (d) Immunofluorescenct staining with phosphor-specific antibody to Histone H2A-ser-139 and costaining with Draf 5 showed that MWNTs can induce the foci formation that indicated MWNTs caused the ES cells DNA double strand breakages. Images were acquired with a Fluoview laser scanning confocal microscope mated to a Zeiss Axioplan upright microscope using 60× oil magnification.

proficient cells. The resistance to the adenine analogue is an indication of the loss of the function of Aprt, as Aprt is known as a purine "salvage enzyme" that converts free adenine into an utilizable nucleotide.[30] Our results indicated that the MWNT treatment increased the mutation frequency by 2-fold compared with the untreated 3C4 ES cells (Figure 5).

In summary, we have demonstrated for the first time that MWNTs can accumulate in mouse ES cells, and that MWNTs that caused DNA damage might be through ROS generation. The DNA damage induced by MWNTs was indicated by Western blot analysis of 8-oxoguanine-DNA glycosylase 1 (OGG1) and by the induction of two key double strand break repair proteins, Rad 51 and XRCC4. Furthermore, the MWNT treatment was found to cause a 2-fold increase in the mutation frequency in mouse ES cells. Therefore, our results indicate the need for careful scrutiny of the toxicity of nanomaterials at the molecular level, or genotoxicity, even for those materials like MWNTs that have previously been demonstrated to cause limited or no toxicity at the cellular level. The results of this study also provide strong support for the use of p53 as a biomarker for preliminary screening of genotoxicity of nanomaterials.

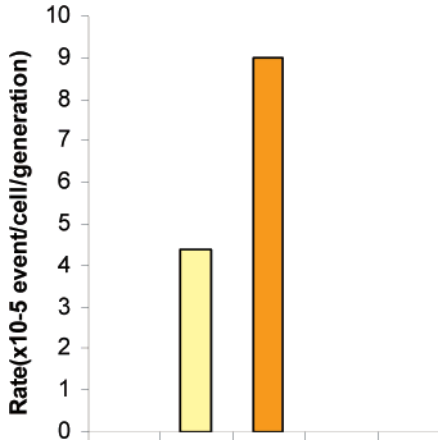

Figure 5. Increase in the mutation frequency in ES cells following the MWNT treatment. The mutation frequencies were determined by Aprt-deficient colonies selected with 2-FA and corrected for colony-forming efficiency. The frequency of spontaneous mutation was determined to be 4.2×10^{-5} (yellow bar), and the mutation frequency for the MWNT-treated (5 μg/mL) cells was 7.8×10^{-5} (magenta bar). Thus, MWNT treatment increased the mutation frequency by approximately 2-fold for the wild-type ES cells.

Materials and Methods. *MWNTs.* The commercially available MWNTs, synthesized by pyrolysis of propylene using iron-based catalyst according to the published method (Supporting Information, S1), were purchased from Tsinghua and Nanfeng Chemical Group Cooperation, China. The MWNTs have been further purified to remove the possible catalyst residues. The detailed procedure was described in the Supporting Information. Both commercially available and purified MWNTs have shown the similar genotoxicity effect.

Cell Culture and MWNTs Treatment. Mouse embryonic stem cells J11 and Aprt+/− 3C4 (a kind gift from Dr. Peter Stambrook, University of Cincinnati) were cultured on a layer of mitomycin C-treated MEF feeder cells at 10% CO_2 and

37 °C. The ES cells were maintained in DMEM supplemented with 15% ES-quality FBS, 1X NEAA (nonessential amino acids), 1X GlutaMax, 100 U/mL penicillin streptomycin, 0.1 μM β-mercaptoethanol, and 50 μM recombinant Leukemia inhibitory factor (LIF, Chemicon) at 10% CO_2 and 37 °C. The purified MWNTs were then UV-sterilized and diluted to a stock solution (5 mg/mL) under ultrasonication. Meanwhile, equal numbers of ES cells (10^5) were placed in gelatin-treated 6-well plates with 3 mL culture medium. Twenty-four hours after the cell culture, 2 mL of culture medium was removed and 0, 1, and 20 μL of the MWNT stock solution was added to each of the wells on the plate containing 1 mL culture medium to achieve a final concentration of 0, 5, and 100 μg/mL, respectively. At 2 and 4 h after incubation, the cells were harvested for immediate use or placed in −20 °C for storage.

AP and Annexin-V Staining. An AP detection kit (Chemicon) was used to examine the undifferentiated and differentiated ES cells after the MWNT treatment. The cells were placed on a gelatin-treated cover slide 24 h before the MWNT treatment. Both the untreated and MWNT-treated (100 μg/mL) ES cells were fixed with 4% paraformaldehyde after incubation for 2, 4, and 24 h. The ES cells were rinsed with 1X TBST (20 mM Tris-HCl, pH 7.4, 0.15 NaCl, 0.05% Tween-20). The cells were stained with 0.5 mL Naphthol/fast red-violet solution in the dark at room temperature for 15 min. The Annexin V−FITC staining followed the instruction of the manufacturer (BD Pharmacy Inc.). The images were captured by inverted microscope (Olympus CK2) at 10× and 20× (oil) magnification and Olympus Fluoview scanning confocal microscope.

Western Blotting. The harvested cells were lysed in RIPA buffer [0.15 M NaCl, 1% NP-40, 0.05% deoxycholic acid, 1% SDS, 50 mM Tris (pH 8.0)] in the presence of protease inhibitors (Roche, according to manufacturer's instructions). Western blot was used to analyze the stem cell factor Oct4 (Chemicon) and cell cycle checkpoint protein p53 expression level by probing with an anti-p53 monoclonal antibody (Chemicon). p53 phosphorylation was verified by p53-S23 antibody (Cell Signaling Inc.). The cell lysates were also probed with antibodies for DNA repair proteins OGG1, Rad 51, and XRCC4 (Santa Cruz, Inc.).

Immunoprecipitation and Immunofluorescent Staining. The MWNT-treated cells were lysed in RIPA buffer and protease inhibitors (according to Roche manufacturer's instructions) at 4 °C for 1 h with slow rotation. The cell lysates were diluted by adding an equal volume of 1X PBS with protease inhibitors. The XRCC4 polyclonal antibody was added (Santa Cruz, Inc.) and incubated at 4 °C for 1 h with agitation, followed by the addition of protein A/G PLUS-agarose (washed with 1X PBS once before use) and incubation at 4 °C overnight with slow agitation. The agarose was washed three times with an equal volume cold 1X PBST. The samples were examined by Western blotting and probed with SUMO-1 antibody (Santa Cruz, Inc.). For immunofluorescent-staining control, the 24 h MWNT-treated cells were fixed with Formalde-Fresh (formadehyde 4% W/V, methanol 1% W/V), permeabilized in PBS containing 1% NP40, and blocked with 10% rabbit serum for 1 h. The coverslips were stained with Histone H2A-Ser139 phosphor-specific antibody (Cell Signaling Technology, Beverly, MA) at 4 °C overnight. After washing with TBST, the cells were stained with fluorescent-labeled secondary antibody Alexa Fluor 488 (1:400, from molecular probes) and co-stained with Draf 5 for DNA. The coverslips were mounted with Gelmount (Fisher). Images were acquired with a Fluoview laser scanning confocal microscope mated to a Zeiss Axioplan upright microscope using 63× oil magnification.

Mutagenesis Study. The ES cells 3C4, heterozygous at the Aprt locus and derived from F1 blastocytes crossover between 129^{SvEv} $Aprt^{-/-}$ and wild type C3H/Hej, were cultured in ES cell medium.[30] Preexisting mutants were eliminated by culture in the presence of alanosine/adenine for 48 h. A population of 5×10^5 cells were placed in 10 cm plates with mitomycin-C-treated $Aprt^{-/-}$ MEF feeders and 2 μg/mL 2-fluoroadenine (FA). FA was removed after 16 h and the cells were washed three times with the medium. The plates were then incubated under culture conditions for 12 days. Each experiment was done in 10 plates for both control and treated cells to ensure using the enough cell population. The colonies were fixed with methanol and stained with 10% crystal violet. The mutation frequency was calculated as described by Cervantes[29] and corrected for colony forming efficiency.

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Supporting Information Available: MWNTs purification. This material is available free of charge via the Internet at http://pubs.acs.org.

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