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Carbon Nanotubes Induce Malignant Transformation and Tumorigenesis of Human Lung Epithelial Cells

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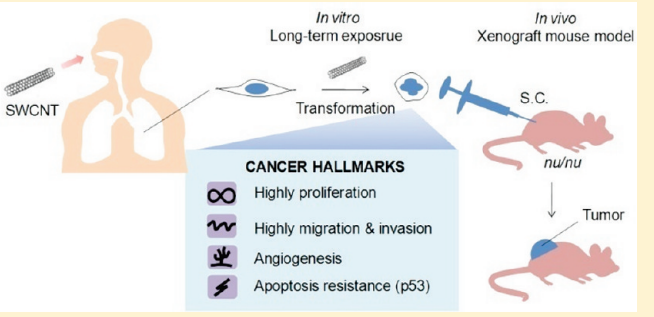
Supporting Information

ABSTRACT: Carcinogenicity of carbon nanotubes is a major concern but has not been well addressed due to the lack of experimental models. Here, we show that chronic exposure to single-walled carbon nanotubes causes malignant transformation of human lung epithelial cells. The transformed cells induce tumorigenesis in mice and exhibit an apoptosis resistant phenotype characteristic of cancer cells. This study provides new evidence for carbon nanotube-induced carcinogenesis and indicates the potential role of p53 in the process.

KEYWORDS: Single-walled carbon nanotubes, lung, carcinogenesis, malignant transformation, apoptosis



Carbon nanotubes (CNTs) rank among the major, newly developed nanomaterials that are currently of interest for a wide range of industrial and biomedical applications.[1,2] Their unique properties, including high tensile strength, flexibility, adsorptivity, durability, and lightweight, have led to the anticipation of a high production volume. In the next decade, high airborne exposure to CNTs by either workers or consumers has been predicted, consequently raising human health concerns about their potential toxicity.[3,4] With their small diameter and high aspect ratio, the physiochemical properties of CNTs have shown a resemblance with asbestos fibers, which are classified as a group I human carcinogen by the International Agency of Research in Cancer.[5,6] Exposure to asbestos has caused a pandemic of lung diseases, including lung cancer, fibrosis, and mesothelioma.[7]

The lung is a major target organ for airborne CNT exposure. Both single-walled carbon nanotubes (SWCNT) and multiwalled carbon nanotubes (MWCNT) have been shown to migrate into the alveolar interstitial compartment of the lung.[8−10] It is anticipated that the low clearance rate from the interstitium would lead to biopersistence of CNTs in the lung, a critical factor in the paradigm of hazardous fiber. Several in vitro studies have suggested the potential carcinogenicity of CNTs. These studies have reported that CNTs can induce cell apoptosis, DNA damage, and activation of mitogen-activated protein kinases (MAPKs), AP-1, NF-κB, and Akt; all of which recapitulate key molecular events involved in asbestos-induced lung cancer and mesothelioma.[11−14] In addition, in vitro exposure of lung epithelial cells to SWCNT induces multipolar mitosis resulting in aneuploidy.[15] In animals, intraperitoneal injection of MWCNT into heterozygous $p53^{\pm}$ mice has been reported to cause mesothelioma similar to that observed with asbestos.[16,17] Furthermore, a short-term abdominal instillation study in C57BL/6 mice reported the induction of asbestos-like granuloma upon receiving MWCNTs.[18]

Currently, there have been no reports on the effects of chronic exposure to CNTs, either in vivo or in vitro. Since carcinogenesis is a multistep process requiring long-term exposure to the carcinogens, such information is necessary for evaluating the epithelial carcinogenic effect of CNTs. We have developed a chronic exposure model in which human lung epithelial BEAS-2B cells were continuously exposed to a low dose of SWCNT in culture over a prolonged time period. After such chronic exposure, the cells were evaluated for malignant transformation in vitro and tumorigenicity in vivo using a xenograft mouse model. The BEAS-2B cells were used in this study because they are from human lung origin and exhibit similar characteristics and cellular responses to carcinogens as the primary or normal lung cells.[15,19,20] They are also

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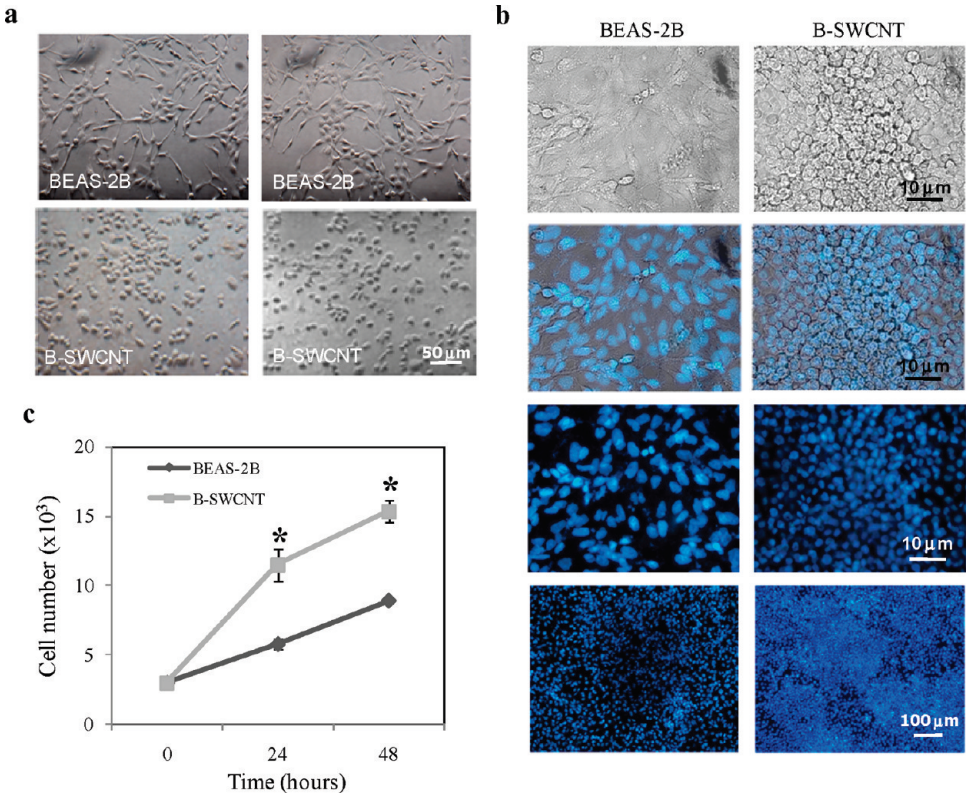

Figure 1. SWCNTs induce morphological changes of human lung epithelial cells. Subconfluent cultures (1×10^5 cells) of lung epithelial BEAS-2B cells in 6-well plates were continuously exposed to 0.02 μg/cm^2 of SWCNT. (a) Phase contrast micrographs of subconfluent monolayers of passage-matched control BEAS-2B cells and SWCNT-treated cells at 12 weeks. (b) Phase, merge, and fluorescence micrographs of control and SWCNT-treated cells stained with Hoechst 33342 dye at 24 weeks. Bottom panel is fluorescence micrographs showing cell mounds. (c) BEAS-2B and B-SWCNT cells were plated in 96-well plates at a density of 3×10^3 cells in growth medium. After 24 and 48 h, cells were incubated with 50 μL of 1× CyQUANT dye binding solution, and cell proliferation was measured at 485/520 nm. Data are means ± standard deviation (SD) (n = 4). *P < 0.05 vs passage-matched control cells.

nontumorigenic and can be grown continuously in culture, thus allowing long-term exposure studies which cannot be achieved using primary lung cells. These cells have also been widely used to define conditions under which various agents and oncogenes cause neoplastic transformation.[21−23] Our results show that chronic exposure to SWCNT causes malignant transformation in vitro and tumorigenicity in vivo upon injection of transformed cells into nude mouse. Such data strengthen the safety concern for CNT exposure and support the prudent adoption of prevention strategies and implementation of exposure control. The in vitro transformation model described here could be used as a screening assay for the carcinogenic potential of other nanoparticles as well as for mechanistic studies which may not be feasible in vivo.

Preparation of Single-Walled Carbon Nanotubes. SWCNTs (CNI, Houston, TX) were produced by the high-pressure CO disproportionation (HiPco) technique, employing CO in a continuous-flow gas phase as the carbon feedstock and $Fe(CO)_5$ as the iron-containing catalyst precursor. The SWCNTs were purified by acid treatment to remove metal contaminates for use in this study. Elemental analysis of the supplied SWCNTs by nitric acid dissolution and inductively coupled plasma-atomic emission spectrometry (ICP-AES, NMAM #7300) showed that the SWCNT was 99% elemental carbon and 0.23% iron. The specific surface area was measured at −196 °C by the nitrogen absorption−desorption technique (Brunauer Emmet Teller method, BET) using a SA3100 surface area and pore size analyzer (Beckman Coulter, Fullerton, CA). The diameter and length distribution of the SWCNT were measured by field emission scanning electron microscopy. The surface area of the SWCNT was 400−1000 m^2/g, and the length and width of individual SWCNT were 0.1−1 μm and 0.8−1.2 nm, respectively. SWCNTs were dispersed by acetone/sonication method as previously described.[24] Briefly, SWCNTs were treated with acetone and placed in an ultrasonic bath for 24 h. The dispersed CNTs were then filtered from the solution using a 20 μm nylon mesh screen followed by a 0.2 μm polytetrafluoroethylene filter. After filter collection, the dispersed CNTs were washed thoroughly with distilled water and suspended in phosphate-buffered saline with 2−3 min sonication (Sonic Vibra cell sonicator, Sonic & Material Inc., Newtown, CT).

Chronic SWCNT Exposure Alters Cell Morphology and Growth Pattern. Subconfluent cultures of human lung epithelial BEAS-2B cells (ATCC; Manassas, VA) were continuously exposed to a subcytotoxic concentration (0.02 μg/cm^2) of SWCNT in culture and passaged weekly. This relatively low concentration was relevant to lung burdens achieved after in vivo exposure of mice to SWCNT, i.e., approximately 0.02−0.08 μg/cm^2 of lung epithelial surface area.[8,9,25] Cells were cultured in normal culture medium without SWCNT for at least two passages prior to further experiments. SWCNT-treated cells

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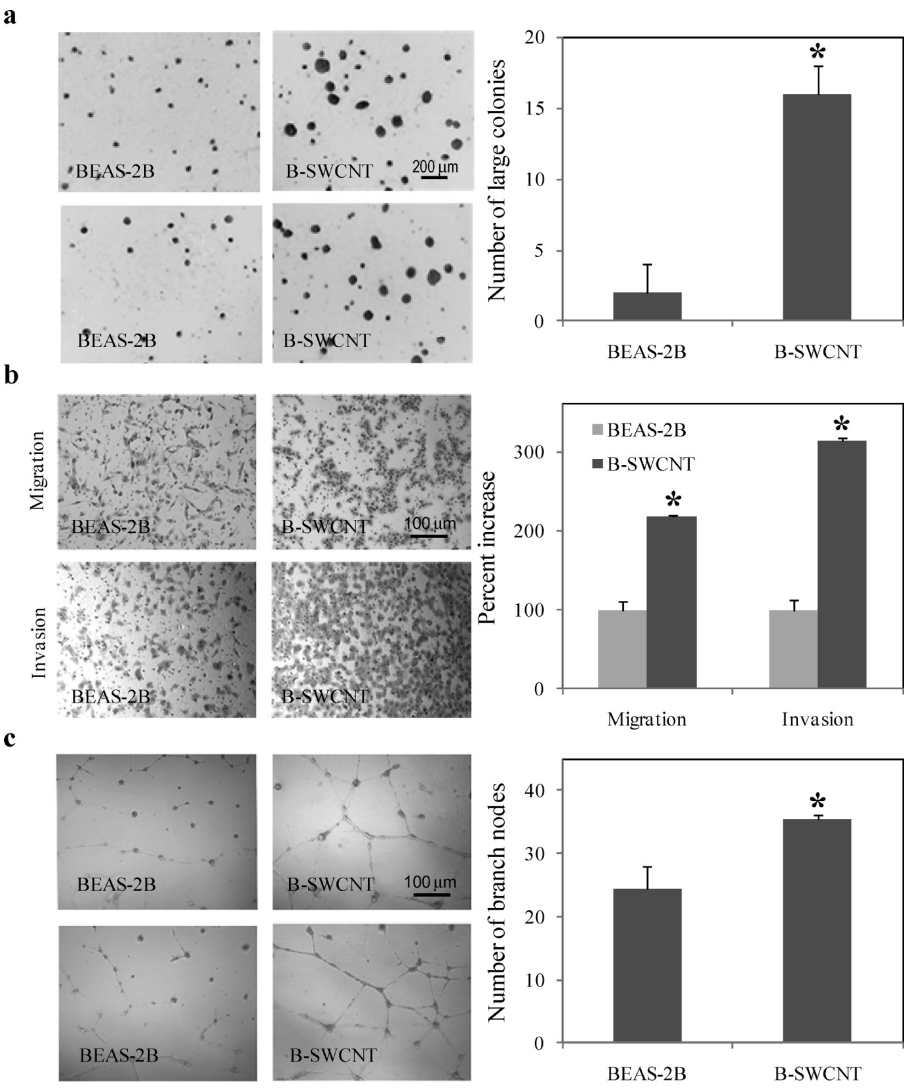

Figure 2. SWCNT induce human lung epithelial BEAS-2B cell aggressiveness. (a) Passage-matched BEAS-2B and transformed B-SWCNT cells (3×10^4 cells) were seeded on 0.5% agar plates. After 2 weeks, large colonies (>50 μm in diameter) were scored under a light microscope. (b) BEAS-2B and B-SWCNT cells, at a density of 1.5×10^4 or 3×10^4 cells per 24-well inserts, were added to control inserts (migration) or inserts coated with Matrigel (invasion) and incubated for 48 h. After diff-quick staining, cells were counted under a microscope, and the average number of migrated or invading cells was plotted. (c) BEAS-2B or B-SWCNT cell supernatants were incubated with HUVEC cells, and endothelial capillary tube formation was scored under a light microscope. Data are means ± SD (n = 4). *P < 0.05 vs passage-matched control cells.

exhibited morphological changes within 12 weeks of exposure. At subconfluent densities, SWCNT-treated cells adopted a small round shape, whereas passage-matched control BEAS-2B cells maintained the generally more expanded and elongated shape (Figure 1a). At 24 weeks of exposure, SWCNT-treated cells began forming cell mounds, indicating a loss of contact inhibition, which is the first indication of malignant transformation. SWCNT-transformed BEAS-2B cells (designated as B-SWCNT) were stained with Hoechst 33342 dye to aid visualization of cell mounding (Figure 1b). To determine whether chronic SWCNT exposure affects cell growth characteristic, the proliferative rate of B-SWCNT and passage-control BEAS-2B cells was compared by CyQUANT cell proliferation assay (Invitrogen, Carlsbad, CA). B-SWCNT cells showed a significant increase in cell proliferation above controls at 24 and 48 h postseeding (Figure 1c).

Chronic SWCNT Exposure Induces Malignant Transformation of Lung Epithelial Cells. Carcinogenesis is a multistep process involving DNA alterations, abnormal cell growth, increased cell migration and invasion, evasion of apoptosis, and angiogenesis.[26,27] To assess the carcinogenic potential of SWCNT-exposed cells, we compared several carcinogenic properties of B-SWCNT cells to those of the passage-control BEAS-2B cells. First, anchorage-independent growth, which is the most commonly used in vitro indicator of malignant transformation,[28] was determined by measuring colony formation on soft agar.[29] Since the number of cells in each colony is difficult to determine accurately, we measured the colony size and counted only those with a diameter of 50 μm or more, as previously described.[29] Significant colony formation was observed with B-SWCNT cells as compared to the control BEAS-2B cells as judged by an approximately 8-fold increase in large colony formation

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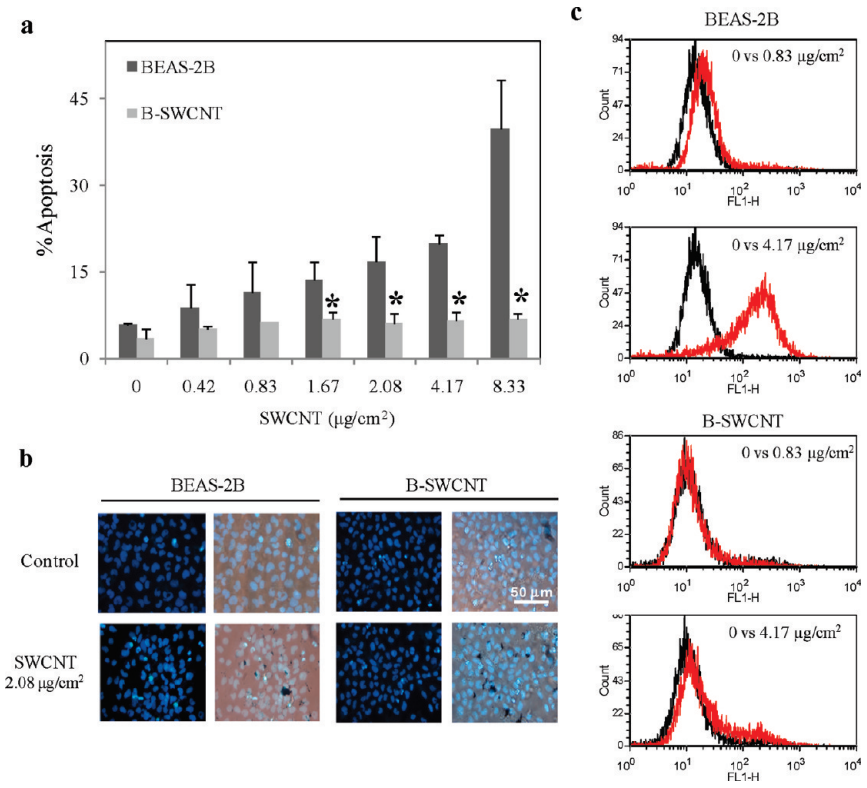

Figure 3. SWCNT induce human lung epithelial BEAS-2B cell apoptosis. (a) Subconfluent monolayers of BEAS-2B and B-SWCNT cells were treated with various concentrations of SWCNT (0−8.33 μg/cm^2) for 24 h and analyzed for apoptosis by the Hoechst 33342 assay. (b) Fluorescence micrographs of SWCNT-treated cells (2.08 μg/cm^2) stained with the Hoechst dye. Apoptotic cells exhibited the condensed nuclei with bright nuclear fluorescence. Data are means ± SD (n = 4). *P < 0.05 vs passage-matched control cells. (c) Representative flow cytometric histograms of annexin V comparing nontreated control and SWCNT-treated cells (0 vs 0.83 μg/cm^2 and 0 vs 4.17 μg/cm^2) in BEAS-2B and B-SWCNT cells (upper and lower panels, respectively). A shift to the right indicates apoptosis of BEAS-2B cell treated with high dose of SWCNT for 24 h.

(Figure 2a). It is noteworthy that BEAS-2B cells which were obtained at autopsy of noncancerous individuals also formed slow-growing colonies on soft agar. This could be due to the fact that BEAS-2B cells are reported to possess a mutated p53^{Ser}47 gene.[21] Furthermore, American Type Culture Collection (ATCC) indicates that the BEAS-2B cell line forms colonies in a semisolid medium but is nontumorigenic in immunosuppressed mice. Therefore, the colony formation observed in the control BEAS-2B cells is due to the indigenous properties of the cell, which represents late-stage transformation and not because of their tumorigenic potential.

Next, we investigated the aggressive behavior of B-SWCNT cells by measuring their invasive and migration activities, which are key determinants of tumor progression[30] using the transwell migration (control inserts) and invasion (inserts coated with Matrigel) assay as previously described.[31] The B-SWCNT cells showed significantly higher migration and invasion rates than the control BEAS-2B cells (Figure 2b), indicating the aggressive malignant phenotype of SWCNT-transformed cells. We also investigated the angiogenic potential of B-SWCNT cells by performing the vascular endothelial tube formation assay. Human umbilical vascular endothelial cells (HUVEC, 4 × 10^4 cells) were incubated with cell-primed supernatants from B-SWCNT or control BEAS-2B cells (0.5 × 10^6 cells/6-well plate) mixed with HUVEC reduced medium (2.5% dialysized FBS) at a ratio of 1 to 1 onto a Matrigel-precoated plate, and the endothelial capillary tube formation was determined. Figure 2c shows that B-SWCNT cell supernatants induced a higher number of branch nodes as well as a more complex pattern of HUVEC tube formation as compared to BEAS-2B cell supernatants. This result indicates an increased angiogenic activity of SWCNT-transformed cells.

Apoptosis Resistance of SWCNT-Transformed Cells. Apoptosis plays an essential role in the removal of mutated or transformed cells, and its disruption contributes to abnormal cell growth and malignancy.[32] To determine the apoptosis phenotype of SWCNT-transformed cells, B-SWCNT and BEAS-2B cells were treated with various concentrations of SWCNT (0−8.33 μg/cm^2 or 0−50 μg/mL) and analyzed for apoptosis by the Hoechst 33342 assay. Cells having intensely condensed and/or fragmented nuclei were considered apoptotic. Treatment with SWCNT induced a dose-dependent increase in apoptosis of the control BEAS-2B cells, whereas it had minimal effect on B-SWCNT cells (Figure 3a and b). This result was confirmed by flow cytometric analysis of apoptosis using the annexin V assay (Figure 3c). To our knowledge, this is the first demonstration of the induction of apoptosis resistance by chronic low-dose exposure to SWCNT, although the induction of apoptosis by acute high-dose CNT treatment has previously been demonstrated.[14,33,34] Since apoptosis resistance is a key characteristic of all cancer cells,[27,32] this finding suggests the potential carcinogenicity of SWCNT-transformed cells,

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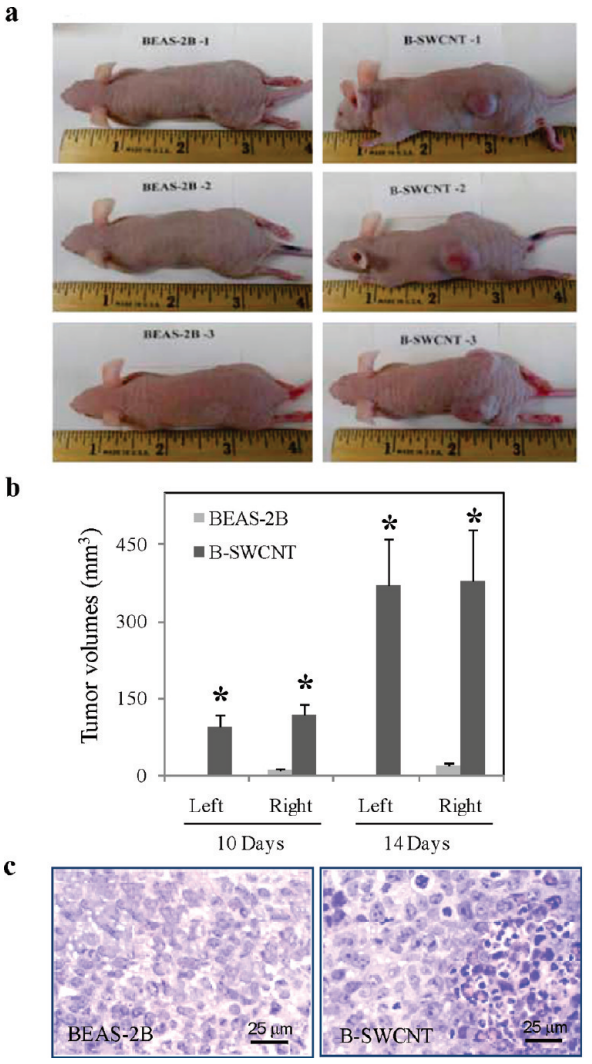

Figure 4. Tumor formation in nude mice injected with SWCNT-transformed cells. Mice were injected subcutaneously with 2×10^6 B-SWCNT or passage-matched control BEAS-2B cells (passage number 60−64). (a) Tumor formation was observed at the site of injections in mice receiving B-SWCNT cells at 14 days postinjection. (b) Tumor size was quantified at day 10 and 14 after the injection. Data are means ± SD of triplicate experiments. $^*P < 0.05$ vs passage-matched control cell group. (c) Tumor tissue samples from B-SWCNT mice and control samples BEAS-2B mice were examined for cell morphology by histological staining. Representative micrographs are shown. Multinucleated cells are evident in the tissue samples from B-SWCNT mice.

consistent with our earlier findings on the increased aggressiveness, anchorage-independent growth, and angiogenicity of B-SWCNT cells.

SWCNT-Transformed Cells Induce Tumor Formation in Vivo. Having demonstrated the malignant transformation properties of B-SWCNT cells in vitro, we next tested the potential tumorigenicity of SWCNT-transformed cells in vivo using a xenograft mouse model. Immunodeficient mice (nu/nu; Jackson Laboratory, Bar Harbor, ME) were subcutaneously injected with 2×10^6 B-SWCNT and passage-control BEAS-2B cells suspended in 100 μL sterile medium at left and right flanks. At one week postinjection, small tumors were formed at the injection site in mice receiving B-SWCNT cells, whereas mice receiving control BEAS-2B cells did not develop tumors. At 14 days after the injection, large tumors were formed in the B-SWCNT mice, whereas no or very small lumps were observed in the control mice (Figure 4a). Tumor size was measured at either the left or right flank at 10 and 14 days postinjection by using an external caliper and tumor volume was calculated using the formula: tumor volume [mm^3] = 1/2 (length [mm]) × (width [mm])^2. Figure 4b shows quantitative data for tumor volume measurements of B-SWCNT and control mice. Histological inspection of tumor samples from the B-SWCNT mice showed classic cancer cell morphology, including the presence of multinucleated cells, an indicator of mitotic dysfunction,[35] which is absent in the control mice (Figure 4c). These results demonstrate for the first time the in vivo tumorigenicity of SWCNT-transformed cells.

p53 Mediates Apoptosis Resistance in SWCNT-Transformed Cells. Since apoptosis resistance is a foundation of neoplastic evolution, we further investigated the apoptosis resistance mechanisms in B-SWCNT cells to aid the understanding of SWCNT-induced carcinogenesis. First, protein array analysis was performed on B-SWCNT and control BEAS-2B cells using the human apoptosis array (R&D Biosystems) which detects 35 of the most common apoptosis regulatory proteins by immunoblotting. The B-SWCNT cells exhibit a distinctive expression profile of apoptosis proteins as compared to the control BEAS-2B cells (Figure 5a). A striking difference between the two cell types is the expression of phospho-p53, an indicator of tumor suppressor p53 activation,[36,37] at serine 15, 46, and 392. Densitometric analysis of the phospho-p53 proteins shows a several-fold decrease in the protein expression levels in B-SWCNT cells (Figure 5b), a result that is confirmed by Western blot analysis of the phospho-p53 (Ser15) protein (Figure 5c and d). Since p53 is a key positive regulator of apoptosis, its dysregulation in the B-SWCNT cells suggests its potential role in the apoptosis resistance of the cells. To further investigate the role of p53 in this process, we used two known apoptotic inducers of p53, namely etoposide and TNF-α.[38−40] B-SWCNT and control BEAS-2B cells were treated with various concentrations of etoposide (0−500 nM) and TNF-α (0−100 μg/mL), and their apoptotic responses to these treatments were determined. As compared to control cells, the B-SWCNT cells exhibited a substantial lower apoptotic response to both etoposide and TNF-α treatment (Figure 5e and f), suggesting the involvement of p53 in the apoptosis resistance process.

Phosphorylation of p53 is critical to its function.[41] Several studies have shown that phosphorylation of p53 at serine 15 is a key target during p53 activation and is critical for p53-dependent apoptosis.[42−44] To determine the functional role of p53 (Ser15) in the apoptotic response to the test agents in SWCNT-transformed cells, B-SWCNT and control BEAS-2B cells were treated with etoposide and TNF-α and analyzed for phospho-p53 (Ser15) expression levels by Western blotting. Figure 6a and b shows that etoposide and TNF-α substantially induced p53 (Ser 15) phosphorylation in the control cells but had a much lesser effect in the B-SWCNT cells due to a low p53 basal level. These results are consistent with the apoptosis data and indicate the potential role of p53 in SWCNT-induced apoptosis resistance. The expression of p53 in BEAS-2B cells and its activation (phosphorylation) by the inducers also indicate p53 functionality in these cells. However, due to the likely presence

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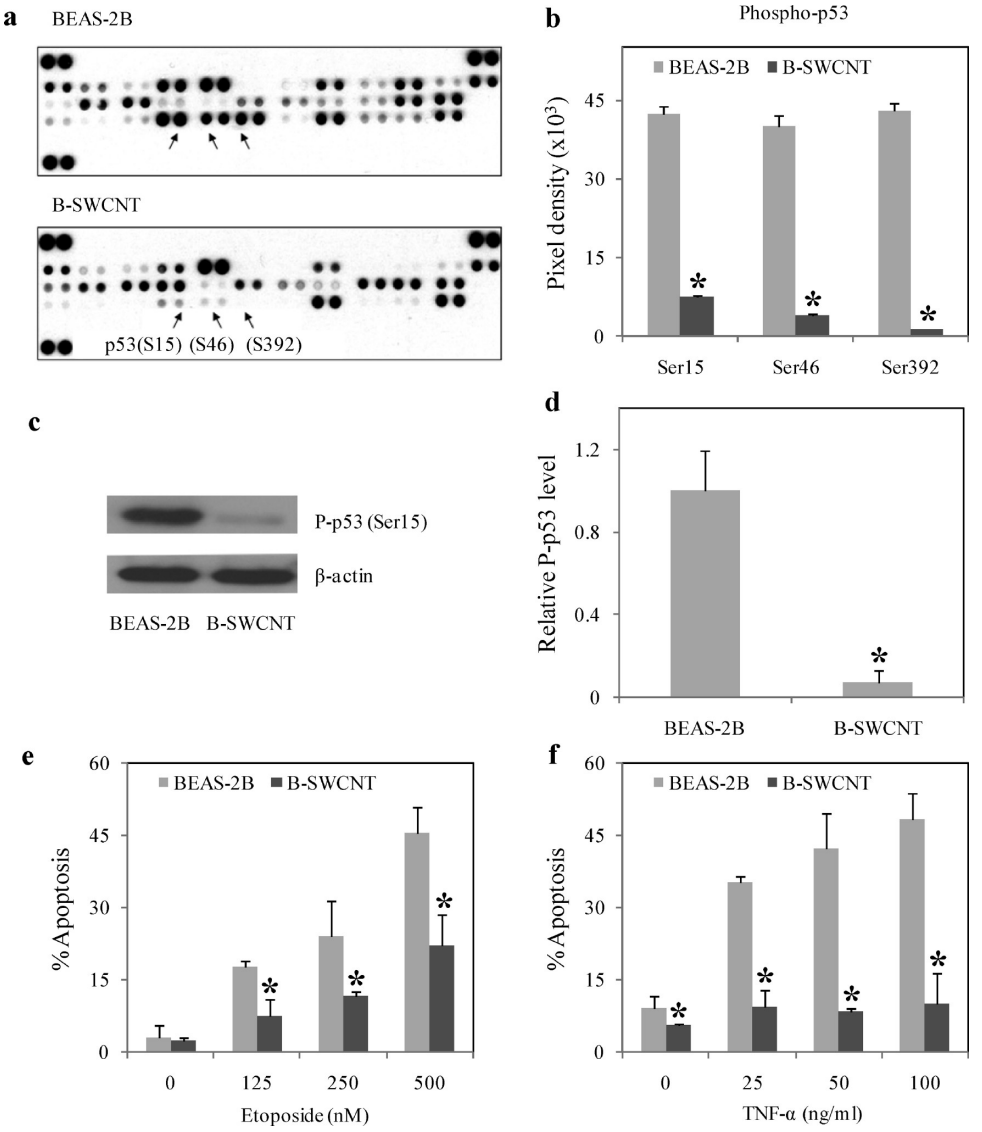

Figure 5. Apoptosis resistance in SWCNT-transformed cells. (a) BEAS-2B and B-SWCNT cell lysates were prepared and analyzed for multiple apoptosis-related proteins by the human apoptotic array (R&D Systems). Arrays were incubated with 500 μg of each cell lysate, and images were collected from the equal exposures to X-ray films. (b) Pixel densities of phospho-p53 (Ser15, Ser46, and Ser392) from the apoptotic arrays are shown. (c) Cell lysates were prepared and analyzed for phospho-p53 (Ser15) by Western blotting. β-actin was used as a loading control. (d) Immunoblot signals of phospho-p53 (Ser15) are shown. (e) BEAS-2B and B-SWCNT cells were treated with various concentrations of etoposide (0−500 nM) for 24 h, and apoptosis was determined by the Hoechst 33342 assay. (f) Cells were treated with TNF-α (0−100 ng/mL) for 12 h, and apoptosis was similarly determined by the Hoechst assay. Data are means ± SD of triplicate experiments. *P < 0.05 vs passage-matched control cell group.

of SV40 viral proteins in these cells (as a result of their immortalization) which could interact with p53, this protein may not be fully functional. To confirm the role of p53 in apoptosis resistance of B-SWCNT cells, the cells were transfected with p53 or control GFP plasmid (Invitrogen), and the effects on etoposide- and TNF-α-induced apoptosis were examined. Figure 6c and d shows that overexpression of p53 reversed the apoptosis resistance of B-SWCNT cells to both treatments. Western blot analysis of the overexpressing cells shows an increased expression of phospho-p53 (Ser 15) (Figure 6e), supporting the role of this protein modification in the apoptosis resistance of SWCNT-transformed cells.

In conclusion, the present study demonstrates that long-term exposure to SWCNT can cause malignant transformation of human lung epithelial cells as demonstrated by their loss of contact inhibition, excessive cell growth, and colony formation as well as increased cell migration, invasion, and angiogenicity; all characteristics of cancer cells. The SWCNT-transformed cells also exhibited an apoptosis-resistant phenotype and induced tumorigenesis in nude mice. These results indicate the carcinogenic potential of chronic SWCNT exposure, which has not been well addressed and is of great concern. Our results also indicate aberrant p53 signaling as a potential mechanism for this SWCNT-induced carcinogenicity, consistent with the previous observations that most human cancers have p53 tumor suppressor gene inactivation.[25,45] A recent demonstration of CNT-induced mesothelioma in p53 knockout mice[16,17] further supports our

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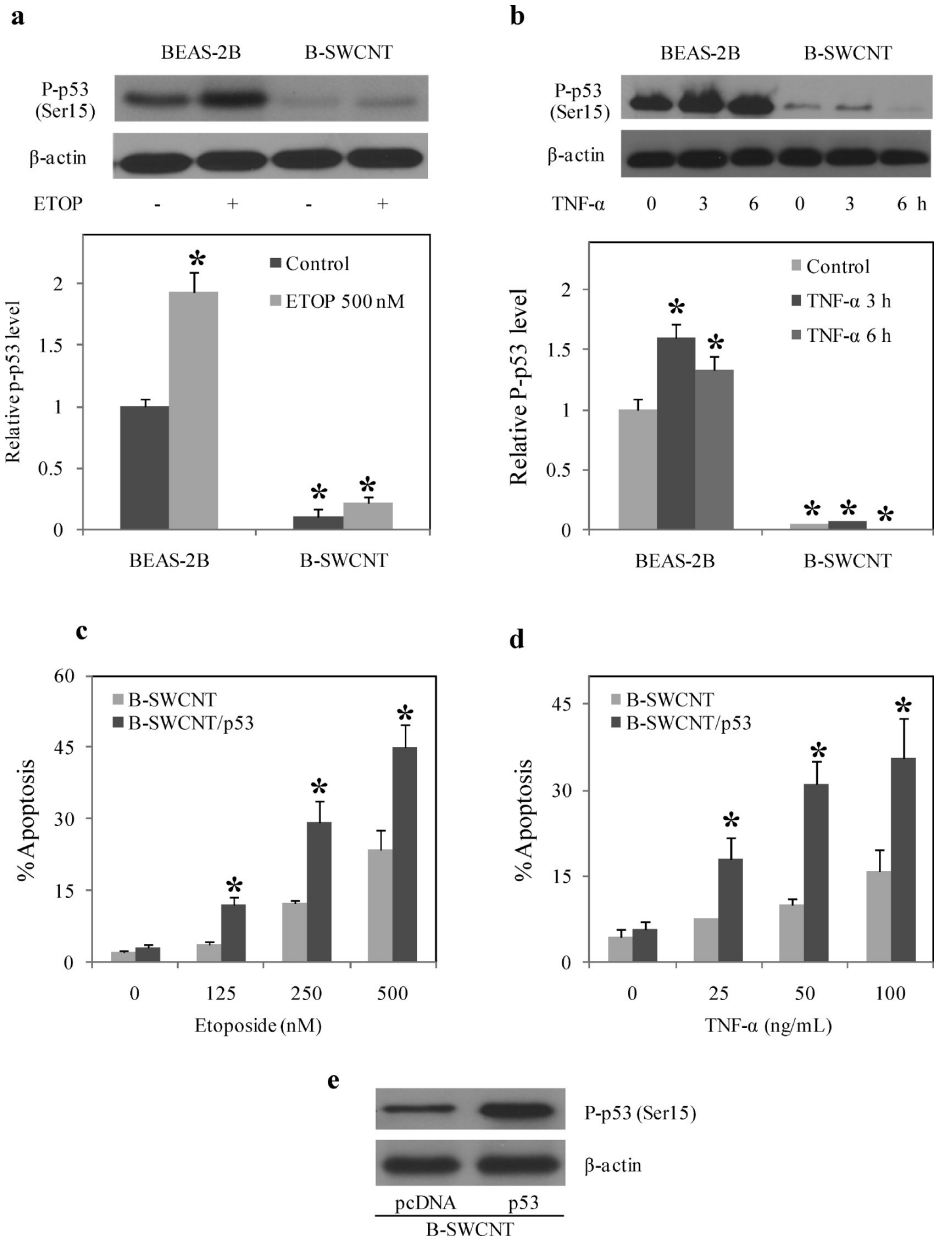

Figure 6. p53-mediated apoptosis resistance in SWCNT-transformed cells. (a) BEAS-2B and B-SWCNT cells were treated with etoposide (500 nM) for 24 h or (b) TNF-α (100 ng/mL) for various times (0−6 h). Cell lysates were prepared and analyzed for phospho-p53 (Ser15) by Western blotting. Immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the result obtained in BEAS-2B cells without treatment (control). (c) B-SWCNT cells were transiently transfected with p53 or control plasmid. Transfected cells were treated with etoposide (0−500 nM) for 24 h, and apoptosis was determined by the Hoechst 33342 assay. (d) Cells were similarly transfected with p53 or control plasmid followed by TNF-α treatment (0−100 ng/mL) for 12 h, and apoptosis was determined by the Hoechst 33342 assay. (e) Cell extracts were prepared and separated on 10% polyacrylamide-SDS gels, transferred, and probed with phospho-p53 (Ser15) antibody. β-actin was used as a loading control.

findings and the role of p53 in CNT-induced carcinogenesis. The development of the chronic in vitro exposure model and in vivo tumorigenesis studies described in this report could aid in mechanistic investigations of the potential carcinogenicity of CNTs and other nanoparticles.

■ ASSOCIATED CONTENT

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

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