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Induction of Slug by Chronic Exposure to Single-Walled Carbon Nanotubes Promotes Tumor Formation and Metastasis

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Abstract

Carbon nanotubes (CNTs) represent a major class of engineered nanomaterials that are being used in diverse fields. However, their use has increasingly become a concern because of their carcinogenic potential. Accumulating evidence has demonstrated that certain types of CNTs are carcinogenic or tumor-promoting in animal models. However, the underlying molecular and cellular mechanisms are unclear. Here, we report that chronic exposure to single-walled (SW) CNTs results in the induction of Slug, a key transcription factor that induces an epithelial–mesenchymal transition (EMT), in human lung epithelial cells. We show that SWCNT-induced Slug upregulation plays a critical role in the aggressive phenotype of SWCNT-exposed cells, which includes increased cell migration, invasion, and anchorage-independent cell growth. Our *in vivo* studies also show that SWCNT-induced Slug upregulation and EMT activation play a pivotal role in tumor formation and metastasis. Our findings illustrate a direct link between CNT-induced Slug upregulation, EMT activation, and tumor formation and metastasis, and they highlight the potential of CNT-induced Slug upregulation as a target for future risk assessment and prevention of CNT-associated diseases.

Graphical Abstract

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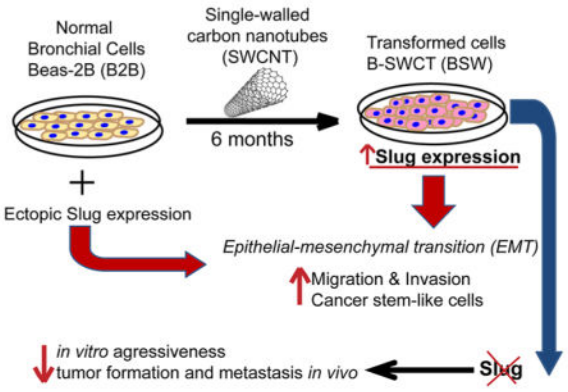
Notes

The authors declare no competing financial interest.



INTRODUCTION

Carbon nanotubes (CNTs) are of great interest due to their unique properties and potential applications in electronics, structural engineering, and medicine. With the rapid increase of CNT production and utilization, potential health risks of CNT exposure have been raised, especially adverse health effects of long-term exposure. The carcinogenic potential of CNTs is of great concern due to their needle-like shape, high durability, and biopersistence, features shared with asbestos. Accumulating evidence from *in vivo* and *in vitro* studies demonstrates the carcinogenic potential of CNTs.[1–11] Our previous studies have also shown that chronic exposure of human lung epithelial cells to noncytotoxic concentrations of SWCNTs induces malignant transformation and promotes the initiation of cancer stem-like cells (CSCs).[12,13] However, the underlying molecular and cellular mechanisms remain to be unraveled, which the current study is beginning to address.

Carcinogenesis is a multistep process typically requiring long-term exposure to various stimuli. This complex developmental process is associated with several phenotypic changes and can be characterized only by the combination of multiple biomarkers. One important hallmark of cancer is the metastatic dissemination of primary tumors.[14] Epithelial–mesenchymal transition (EMT), a process by which epithelial cells undergo a morphological change to a more mesenchymal phenotype, has been linked to tumor metastasis.[15] We have previously shown that short-term exposure to CNTs induces EMT through the TGF-β/Smad2 pathway;[16] however, the effects of long-term exposure and its association to CNT carcinogenesis remain to be elucidated.

EMT enables tumor cells to invade to neighboring tissues and metastasize to different organs. It is induced by several key transcription factors including Twist1, Snail1, and Slug.[17–19] Our previous studies have shown that Slug is highly expressed in lung CSCs and functions to promote metastasis in human lung carcinoma.[20] The main objective of this study was to determine whether Slug expression is upregulated in CNT-exposed lung cells and whether it plays a role in CNT-induced EMT activation and carcinogenesis. Here, we applied our previously developed chronic exposure model in which nontumorigenic human lung epithelial BEAS-2B (B2B) cells were continuously exposed to a low dose of SWCNT in culture over a prolonged period.[1] These SWCNT-exposed (BSW) cells developed

malignant phenotypes, including increased cell proliferation, resistance to apoptosis, and tumor formation in a mouse model.[1,12,13,21] However, the role of EMT in tumor formation and metastasis of BSW cells and the underlying mechanisms are not known. In this study, we demonstrated for the first time that Slug is highly upregulated in chronic SWCNT-exposed lung cells and plays a critical role in EMT activation, tumorigenesis, and metastasis.

MATERIALS AND METHODS

Materials

Elemental analysis of the supplied SWCNTs (CNI, Houston, TX) by nitric acid dissolution and inductively coupled plasma-atomic emission spectrometry (ICP-AES, NMAM #7300) showed that the SWCNTs were 99% elemental carbon and 0.23% iron. The specific surface area was measured at –196 °C by the nitrogen adsorption–desorption technique (Brunauer–Emmett–Teller method) using a SA3100 surface area and pore size analyzer (Beckman Coulter, Fullerton, CA). The diameter and length distribution of the SWCNTs were measured by field emission scanning electron microscopy as previously described.[22] The surface area of the SWCNTs was 400–1000 m^2/g, and the length and width of individual SWCNTs were 0.1–1 μm and 0.8–1.2 nm, respectively.

Preparation of SWCNTs

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 of sonication (Sonic Vibra Cell Sonicator, Sonic & Material Inc., Newtown, CT).

Cell Culture

Human bronchial epithelial BEAS-2B cells were cultured with SWCNT (0.02 $\mu g/cm^2$) for 6 months to generate SWCNT-transformed bronchial epithelial cells (BSW), as previously described.[1] This cell model has been reported as an appropriate model for *in vitro* lung carcinogenesis studies.[23,24] BSW cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco, Gaithersburg, MA). Cells were grown at 37 °C in a humidified 95% air/5% CO_2 incubator.

Inhibition of Slug by RNA Interference

Knockdown of Slug by RNA interference was performed as previously described.[20] Briefly, lentiviral transduction particles carrying a short hairpin RNA sequence against human *SNAI2* or control nontarget sequence (Sigma-Aldrich, St. Louis, MO) were used to infect BSW cells. Cells were incubated with lentiviral particles in the presence of hexadimethrine bromide (8 μg/mL) for 36 h. Infected cells were allowed to recover for 48 h and were cultured for 28 days in puromycin-containing medium (1 μg/ mL). The stable Slug knockdown cells (BSW-shSlug) and control cells (BSW-shCtrl) were identified by immunoblotting using an anti-Slug antibody (Cell Signaling Technology, Beverly, MA) and were cultured in puromycin-free DMEM.

Plasmid Transfection and Overexpression

BEAS-2B cells were transfected with SNAI2 (Addgene, plasmid 31698) or GFP (Invitrogen, Carlsbad, MA) plasmid by nucleofection (Amexa Biosystems, Cologne, Germany), according to the manufacturer's instructions. The transfected cells were cultured for 72 h and used in the experiments.

Immunoblotting

Cells were collected and incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride, and 1× complete EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) at 4 °C for 20 min. The protein concentration in the lysates was determined by BCA spectrophotometry. Proteins (40 μg) were resolved under denaturing conditions by sodium dodecyl sulfate polyacrylamide (7.5–12%) gel electrophoresis (SDS-PAGE) and transferred to polycarbonate (PVDF) membranes (Merck Millipore, Tullagreen Carrigtwohill, Ireland). Membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween 20) and incubated with the appropriate primary antibodies at 4 °C overnight. Membranes were rinsed three times with TBST for 10 min and incubated with HRP-coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were visualized by enhanced chemiluminescence.

Cell Proliferation

BSW-shCtrl and BSW-shSlug cells (2×10^3) were seeded in 96-well plates, and cell proliferation was evaluated at the indicated time points using a CellTiter 96 AQueousOne kit (Promega, Madison, WI).

Cell Migration and Invasion

In vitro cell migration and invasion were measured using a 24-well Transwell unit with PVDF filters (8 μm pore size) (BD Biosciences Discovery Labware, Bedford, MA). A PVDF membrane coated with Matrigel was used in the invasion assay, and a membrane without Matrigel coating was used in the migration assay. 3×10^4 cells/well (invasion) or 1.5×10^4 cells/well (migration) were seeded onto the upper chamber of the Transwell unit in serum-free medium. The lower chamber was filled with normal growth medium. Chambers were incubated at 37 °C in a 5% CO_2 atmosphere for 48 h. The nonmigrating or noninvading cells were removed from the inside of the inset with a cotton swab. Cells that migrated or invaded to the underside of the membrane were fixed and stained with 10 μg/mL Hoechst 33342. Inserts were visualized by fluorescence microscopy (Leica Microsystems Inc., Deerfield, IL), and cell numbers were quantified in a minimum of three independent experiments for each group. Cells that had migrated or invaded to the underside of the membrane were subsequently stained with crystal violet (0.2% w/v in 2% v/v methanol), and images of the migrated or invaded cells (purple stained) were taken at 100× total magnification.

Soft Agar Colony Formation Assay

Briefly, 0.5% w/v agar medium was obtained by mixing Difco agar, 15% v/v FBS, and 2× concentrate EMEM (Lonza, Walkersville, MD) at 44 °C. The same amount of each growth factor from the BEGM SingleQuots kit (Lonza, Walkersville, MD) was added to the warm agar. Next, cells were suspended in 0.33% agar at 1×10^4 cells/well and slowly layered onto precast agar in triplicate in six-well plates. After 2 weeks of incubation, colonies were examined and photographed under a light microscope. Only colonies exceeding 50 μm in diameter were scored as positive in five random fields per well. A minimum of three independent soft agar assays was performed for each experiment.

Tumor Sphere Assay

Tumor spheres were formed under nonadherent and serum-free conditions as previously described for stem cell-selective conditions.[25,26] Briefly, cells were resuspended in 0.8% methylcellulose-based serum-free medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 20 ng/mL epidermal growth factor (BD Biosciences, San Jose, CA), 20 ng/mL basic fibroblast growth factor, and 4 mg/mL insulin (Sigma-Aldrich, St. Louis, MO) and seeded into 24-well ultralow-attachment-surface plates at 5×10^3 cells/well in 0.5 mL of medium. Cells were cultured for 2 weeks and examined by light microscopy. Only spheres exceeding 50 μm in diameter were scored as positive in five random fields per well. A minimum of three independent tumor sphere assays was performed for each experiment.

Xenograft Mouse Model

Animal care and procedures described in this study were in accordance with the Guidelines for Animal Experiments at West Virginia University and approved by the Institutional Animal Care and Use Committee (IACUC no. 12-0502). Immunodeficient NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (Jackson Laboratory, Bar Harbor, ME), commonly known as NOD scid gamma (NSG), were maintained under pathogen-free conditions at the institutional animal facility. Food and water were given *ad libitum*. Mice were subcutaneously injected with 1×10^6 BSW-shCtrl or BSW-shSlug cells suspended in 100 μL of ExtraCel hydrogel (Advanced BioMatrix, San Diego, CA). The tumor size was measured weekly by external caliper, and tumor volume was calculated using the following formula: tumor volume [mm^3] = 1/2 (length [mm]) × (width [mm]^2). At the end of experiments, mice were euthanized and tumors were dissected and weighed. Tumor specimens were sliced into 5 μm sections and analyzed by immunohistochemistry (IHC) to determine the expression of Slug. Tumor metastasis to liver and lung tissues was analyzed by H&E and IHC staining. Tissue sectioning and H&E staining were carried out by the West Virginia University Pathology Laboratory for Translational Medicine.

Immunohistochemistry

Lung sections (5 μm thick) were deparafinized and rehydrated using several washes in xylene and decreasing concentrations of alcohol, following standard histological protocols. After quenching of endogenous peroxidase activity using a solution of 0.3% H_2O_2 in methanol, slides were incubated in BLOXALL blocking solution for 1 h at room temperature and then incubated with anti-Slug and anti-human mitochondria (Millipore,

Temecula, CA) primary antibodies overnight at 4 °C. After three rinses in TN buffer (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5), sections were incubated with biotin-conjugated secondary antibodies for 30 min at room temperature. After three rinses in TN buffer, slides were incubated with Streptavidin-HRP solution for 30 min at room temperature. HRP activity was measured with a DAB substrate kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin.

Statistical Analysis

The two-sided Student's *t* test was used to compare two groups. Data are reported as the mean values ± SEM from multiple determinations. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Role of Slug in SWCNT-Induced EMT

Our previous studies showed that chronic exposure of human lung epithelial cells to noncytotoxic concentrations of SWCNTs induced malignant transformation and triggered lung epithelial cells to initiate CSCs.[1,12,13] However, the detailed cellular and molecular mechanism remains obscure. EMT plays a crucial role in tumor cell migration and invasion, and increasing evidence supports the association between EMT induction and the emergence of CSCs. To investigate whether EMT is involved in SWCNT-induced cell transformation and CSC initiation, we first compared the protein expression levels of several EMT markers between passage-matched control BEAS-2B (B2B) cells and chronic SWCNT-exposed B2B (BSW) cells. As shown in Figure 1A, although changes in Vimentin and α-SMA levels were limited, the epithelial markers ZO-1, E-cadherin, and Claudin-1 were significantly downregulated in BSW cells, whereas the level of the mesenchymal marker N-cadherin increased. ZO-1, E-cadherin, and Claudin-1 are major proteins responsible for cell–cell junctions of epithelial cells, which will be disrupted during the EMT process due to the downregulation of these proteins.[27,28] N-cadherin, present at relatively high levels in various tumors, mediates less stable cell–cell adhesion and contributes to stromal interaction and invasion of carcinoma cells.[29–31] Our results suggest that EMT activation has been induced by chronic SWCNT exposure. Next, we evaluated the effect of chronic SWCNT exposure on the expression of three core EMT regulatory factors: Twist1, Snail1, and Slug. Within this group, we found that Slug was most significantly upregulated (Figure 1B). To determine whether Slug is essential for SWCNT-induced EMT, we generated BSW cells expressing short hairpin RNA directed against Slug (BSW-shSlug) and tested the effect of Slug knockdown on EMT. As shown in Figure 1C, Slug knockdown strongly upregulated the epithelial markers E-cadherin and Claudin-1 and downregulated the mesenchymal marker N-cadherin with minimal effect on ZO-1. These results suggest that Slug upregulation in BSW cells plays a critical role in their EMT activation.

Slug Upregulation Is Responsible for the Transformed Phenotype of BSW Cells

Many studies have demonstrated that EMT is associated with cell migration, invasion, and tumor progression;[32–35] however, it remains controversial whether Slug acts as a tumor promoter or tumor suppressor based on recent reports.[36,37] Our previous studies showed that

SWCNT-transformed cells exhibit aggressive cancer phenotypes, including increased cell migration, invasion, and anchorage-independent cell growth.[1,5,6] To further investigate the potential role of Slug in regulating cancer phenotypes, we first evaluated the effect of Slug knockdown on cell migration and invasion. Slug knockdown in BSW cells significantly reduced the migratory and invasive properties (Figure 2A,B) without having a significant effect on cell proliferation (Figure 2C). These findings are consistent with the role of Slug in promoting EMT activation in SWCNT-transformed cells. We further investigated the effect of Slug knockdown on anchorage-independent cell growth by soft agar colony and tumor sphere formation assays to address its potential role in malignant transformation of BSW cells. As shown in Figure 3A,B, Slug knockdown in BSW cells significantly reduced colony and tumor sphere formation. These findings suggest that Slug upregulation is responsible, at least in part, for the aggressive malignant phenotypes of BSW cells.

Slug Knockdown Reduces Tumor Formation and Metastasis in Mice

Our previous studies showed that SWCNT-exposed cells are capable of forming solid tumors in animals.[1,12,13] To test the potential role of Slug in this process, we injected BSW-shSlug or BSW-shCtrl cells into NSG mice subcutaneously and monitored tumor formation and metastasis over time. At 2 weeks postinjection, tumor formation was observed at the site of injection in both groups of mice. However, by 3 weeks, a substantial reduction in tumor volume was observed in the mice bearing BSW-shSlug cells compared to those bearing BSW-shCtrl cells (Figure 4A). At 4 weeks postinjection, mice were sacrificed and their tumors were examined for tumor weight, morphology, and the expression of Slug. As shown in Figure 4B, tumor weight in mice bearing BSW-shSlug cells was significantly lower than that in mice bearing BSW-shCtrl cells at 4 weeks postinjection. Images of the excised tumors from Slug knockdown and control groups are shown in Figure 4C. Immunohistological analysis of Slug expression in the tumor tissues of mice showed that the Slug level was noticeably lower in the BSW-shSlug group compared to that in the control group (Figure 4D). These results indicate the positive regulatory role of Slug in tumor growth of BSW cells.

We also investigated the effect of Slug knockdown on tumor metastasis. Slug-induced EMT has been shown to play a critical role in tumor metastasis, and our previous studies have demonstrated that Slug is crucial for SOX9-mediated metastasis of lung tumor cells.[20] Thus, we further examined the effect of Slug knockdown on metastasis of BSW cells. Our results showed that at 4 weeks postinjection locally injected BSW-shCtrl cells were able to spread to liver and lung tissues of mice (six of six), whereas minimal dissemination was observed with BSW-shSlug cells (Figure 4E,F). Metastatic nodules were visible in the liver and lung tissues of all mice in the BSW-shCtrl group, but they were not visible in any of the mice in the BSW-shSlug group. Our data support the role of Slug in both tumorigenesis and metastasis of BSW cells. Since Slug controls EMT and the invasive and migratory properties of the cells, it is likely that Slug-induced EMT plays a role in the malignant properties of BSW cells.

Slug Overexpression Promotes EMT and Malignant Transformation of BEAS-2B Cells

To substantiate the role of Slug in SWCNT-induced EMT activation and malignant transformation, we ectopically overexpressed Slug in BEAS-2B cells and then examined its effects on EMT activation, cell migration, and invasion. As shown in Figure 5A, overexpression of Slug significantly reduced the expression of E-cadherin and Claudin-1, but it increased the expression of N-cadherin, confirming its regulatory effect on EMT in these cells. Similarly, we observed increased cell migration and invasion in Slug-overexpressing cells (Figure 5B,C). Tumor sphere formation, which is a key feature of malignant transformation and has been shown to be induced by Slug in human lung carcinoma,[20] also increased in Slug-overexpressing cells, as indicated by the increased number of large floating spheres under nonadherent and serum-starved conditions (Figure 5D,E). Together, these findings support the role of Slug in SWCNT-induced EMT activation and malignant transformation of human lung epithelial cells.

DISCUSSION

CNTs are high aspect ratio nanomaterials with high durability and biopersistence similar to asbestos fibers. Accumulating evidence indicates that CNT exposure induces malignant mesothelioma and lung cancer in rat and mouse models,[2–4,6,8] but the underlying mechanisms remain obscure. To mimic the carcinogenic developmental process *in vitro*, we previously developed a chronic exposure model in which human lung bronchial epithelial BEAS-2B cells were continuously exposed to low-dose SWCNTs for a period of 6 months.[1] These cells exhibit malignant properties, as indicated by p53 depletion and CSC initiation, that play critical roles in their tumor-igenicity.[1,12,13] In this study, we further analyzed the effects of chronic SWCNT exposure on EMT, tumor formation, and metastasis, as well as the potential role of Slug in these processes. We demonstrated that chronic exposure to SWCNTs induces an upregulation of Slug in lung epithelial cells and that Slug plays a key role in the aggressive phenotypes and tumorigenicity of the exposed lung cells.

Previous studies by our group and others have shown an important role of EMT in CNT-mediated pulmonary fibrosis.[16,38] EMT also plays a role in cancer cell invasion, resistance to apoptosis, and stem cell properties.[15,32–34] Our previous studies indicated that chronic exposure of lung epithelial cells to noncytotoxic concentrations of SWCNTs not only increased cell migration and invasion but also induced resistance to apoptosis and triggered lung epithelial cells to initiate CSCs.[1,12,13,21] In this study, we further showed that such exposure resulted in the activation of EMT and that such activation was controlled by Slug, as demonstrated by biomarker and functional studies. Although the observed regulation of EMT markers Vimentin and α-SMA was not prominent, that of others including ZO-1, E-cadherin, Claudin-1, and N-cadherin was substantial (Figure 1A). The process of EMT is controlled by a group of transcription factors, including Twist1, Snail1, and Slug. Signaling pathways activated by intrinsic or extrinsic stimuli converge on these transcription factors that regulate the resulting phenotypic changes. Since the Snail family of transcriptional repressors has been linked to the downregulation of epithelial cell markers such as ZO-1, E-cadherin, Claudin-1, and N-cadherin,[39–43] we focused our study on these transcription factors and their role in regulating CNT-induced EMT. We found that chronic SWCNT

exposure induced a robust upregulation of Slug in BSW cells with minimal impact on the expression of other transcription factors (Twist1 and Snail1). A high level of Slug was reported in highly invasive lung cancer cells and tumors, associated with poor survival and cancer relapse.[20,44–46] Consequently, we focused our study on the potential role of Slug in SWCNT-induced malignancies.

Slug is one of the best studied EMT regulators but is not absolutely required for EMT activation in lung cancer cells.[20] Our current study shows that the induction of this transcription factor by chronic SWCNT exposure is associated with E-cadherin and Claudin-1 downregulation, consistent with the accepted role of Slug as a transcriptional repressor of epithelial markers during cancer-associated EMT.[47] Slug is one of the most effective inducers of EMT and is associated with invasion and metastasis of various tumors.[25,42,43,46–52] In accordance with previous studies, our *in vitro* data show that overexpression of Slug in B2B cells induces EMT and malignant transformation.[45,53–56] Others have demonstrated that an over-expression of Slug enhances xenograft tumor growth and increases microvessel counts in an angiogenesis assay.[45] Slug overexpression not only mediates an aggressive phenotype, including enhanced migration activity and anoikis suppression *in vitro*, but also promotes xenograft tumor growth *in vivo*.[54] In this study, we focused mainly on the functional role of SWCNT-induced Slug upregulation in tumorigenesis and metastasis. Consistent with the previous findings by our group and others,[20,37,44,49,50,57] the present study shows that Slug is essential for the aggressive phenotype of BSW cells *in vitro* and is responsible for BSW-induced tumor growth and metastasis *in vivo*. To our knowledge, this is the first demonstration of the effect of chronic CNT exposure on EMT activation and the role of Slug in the process.

The effects of Slug on cell proliferation and tumor formation have been shown to vary among various cancers.[49,58–60] Slug was reported to inhibit the proliferation and tumor formation of human cervical cancer cells.[36] In contrast, Slug promotes cell proliferation and invasion in glioblastoma and prostate cancer cells.[49,61] An alternative study reported that Slug promotes *in vitro* carcinoma cells invasion and *in vivo* tumor growth, without affecting cancer cell migration and proliferation *in vitro*.[45] Our previous study indicated that chronic CNT-exposed BEAS-2B cells exhibited increased proliferative capability.[1] In our current article, we found that Slug knockdown significantly inhibited cell migration and invasion, but not proliferation. It appears that cell proliferation may not be regulated by Slug-mediated EMT but may be correlated with other mechanisms, such as cell adhesion and oxidative status, that are affected by exposure to some types of CNTs.[62–64] Our *in vivo* studies also indicated that Slug knockdown inhibited both tumor formation and metastasis. It is worth noting that tumor formation *in vivo* may not necessarily correlate with cell proliferation *in vitro*. EMT could be a source of CSC enrichment, which increases tumor formation upon transplantation of cancer cells into immunodeficient mice.[65,66] Our data show that Slug plays a critical role in EMT activation and CSC induction, which may at least contribute to tumor formation *in vivo*.

CONCLUSIONS

We have demonstrated that chronic exposure to SWCNT induces EMT activation through a mechanism that involves Slug upregulation. Such upregulation may play a key role in the aggressive phenotype and tumorigenic and metastatic properties of human lung epithelial cells. Our findings suggest a molecular framework for CNT-induced carcinogenesis, in which CNTs stimulate Slug expression and EMT to induce tumor growth and metastasis. Because of its critical role in the carcinogenic process, Slug may become useful as a mechanism-based biomarker for risk assessment and intervention of CNT-associated malignancies.

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ABBREVIATIONS

CNTs	carbon nanotubes
EMT	epithelial–mesenchymal transition
SWCNTs	single-walled carbon nanotubes
CSCs	cancer stem-like cells
TGF	transforming growth factor
IHC	immunohistochemistry
H&E	hemotoxylin and eosin
BSW	SWCNT-exposed BEAS-2B
B2B	BEAS-2B
α-SMA	α-smooth muscle actin

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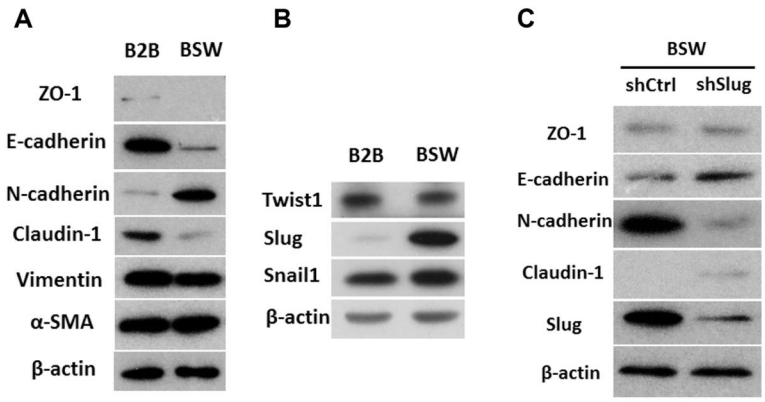

Figure 1.

Slug is upregulated in chronic SWCNT-exposed B2B cells and plays a critical role in SWCNT-induced EMT. Protein expression levels of EMT markers including ZO-1, E-cadherin, N-cadherin, Claudin-1, Vimentin, and α-SMA (A) and key EMT-related transcription factors including Twist1, Snail1, and Slug (B) in B2B and BSW cells were determined by immunoblotting. (C) Immunoblot analysis of ZO-1, E-cadherin, N-cadherin, Claudin-1, and Slug in control (BSW-shCtrl) and Slug knockdown (BSW-shSlug) cells.

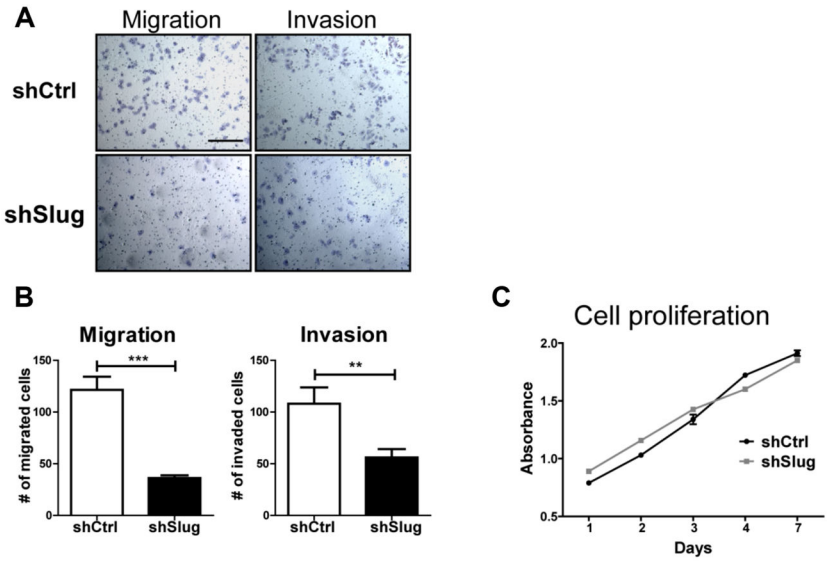

Figure 2.

Slug knockdown decreases cell migration and invasion of chronic SWCNT-exposed B2B cells. (A) Migration and invasion of BSW-shCtrl and BSW-shSlug cells were determined with a transwell migration assay and a Matrigel invasion assay, respectively. Cells that migrated or invaded to the lower side of the membrane were stained with crystal violet; scale bar = 200 μm. (B) Migrating and invading cell numbers were then quantified by counting and depicted as bar charts; ** indicates significant difference from control with $p < 0.01$, *** indicates significant difference from control with $p < 0.001$, and data are the mean ± SEM. (C) BSW-shCtrl and BSW-shSlug cells (2×10^3) were seeded in 96-well plates, and their proliferation rates were evaluated using a CellTiter 96 AQ_{ueous}One kit (Promega, Madison, WI). All values are the mean ± SEM from three independent experiments.

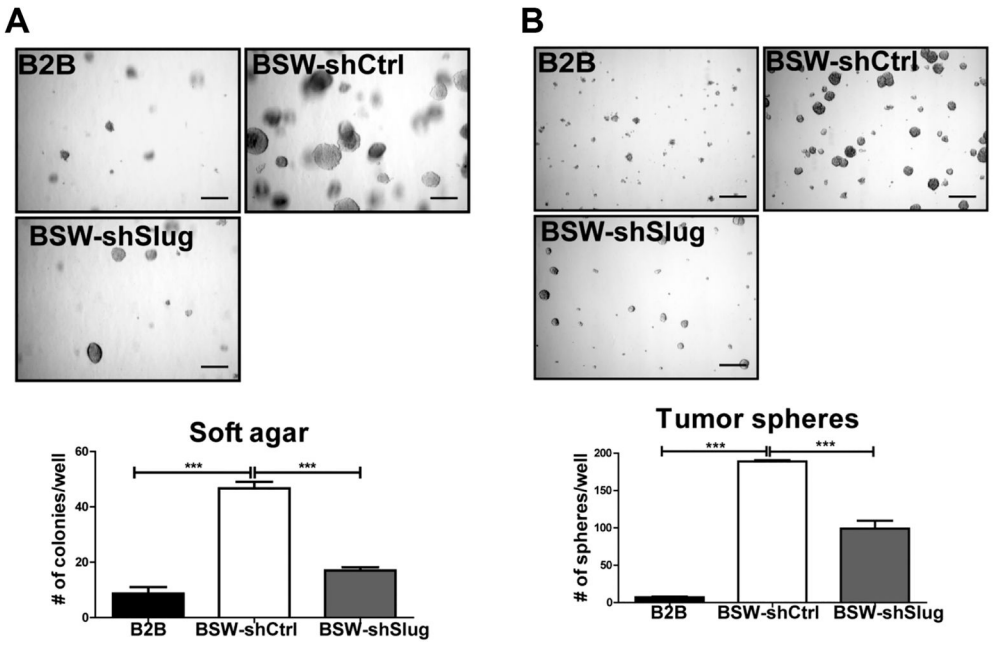

Figure 3.

Slug knockdown decreases malignant transformation of chronic SWCNT-exposed B2B cells. Soft agar colony formation (A) and tumor sphere formation (B) were analyzed in Slug knockdown and control cells after 2 weeks in culture; scale bar = 200 μm. Only colonies with exceeding 50 μm in diameter were scored as positive in five replicate measurements per well; data are the mean ± SEM ($n = 4$). ***$p < 0.001$.

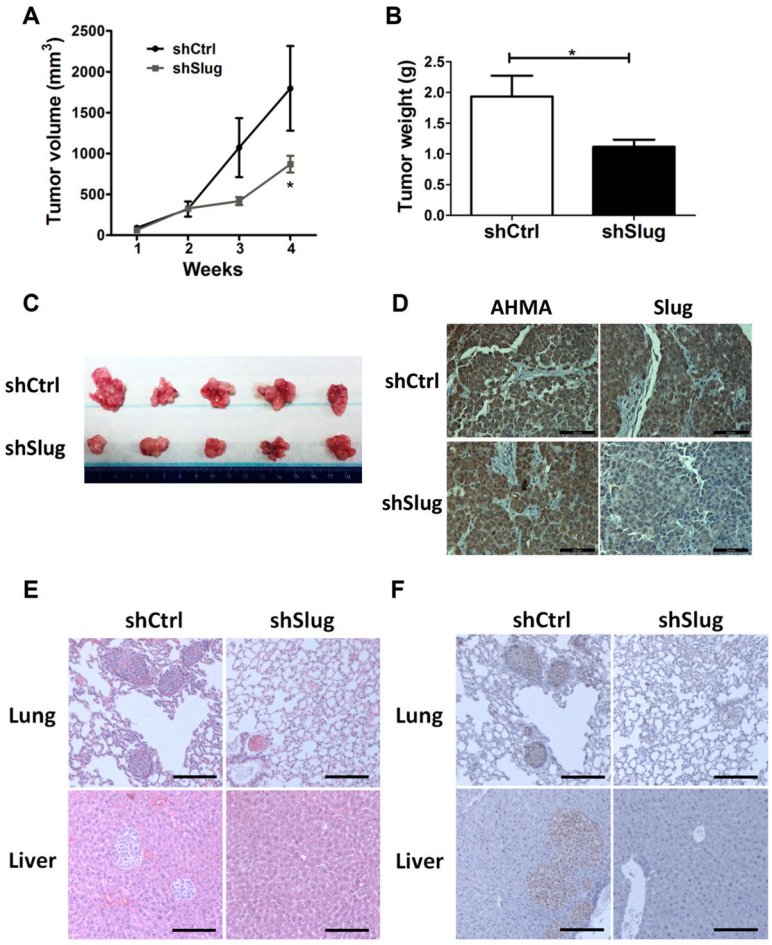

Figure 4.

Slug knockdown decreases tumor formation and metastasis of chronic SWCNT-exposed B2B cells in mice. NSG mice were subcutaneously injected with 3×10^5 BSW-shCtrl or BSW-shSlug cells. (A) Tumor volume was quantified on days 14, 21, and 28 after injection. Data are the mean ± SEM ($n = 6$). *$p < 0.05$. (B) Tumor weight was quantified on day 28 after injection. Data are the mean ± SEM ($n = 6$). *$p < 0.05$. (C) Representative tumors derived from BSW-shCtrl and BSW-shSlug cells. (D) Knockdown of Slug in tumor specimens determined by immunohistochemistry. Anti-human mitochondrial antibodies were used as a human-specific marker and quantification control. Representative micrographs are shown; scale bar = 200 μm. (E, F) Metastatic tumor cells in liver and lung tissues examined by histological staining (E) and IHC staining (F) using an anti-human mitochondrial antibody. Representative micrographs are shown; scale bar = 100 μm.

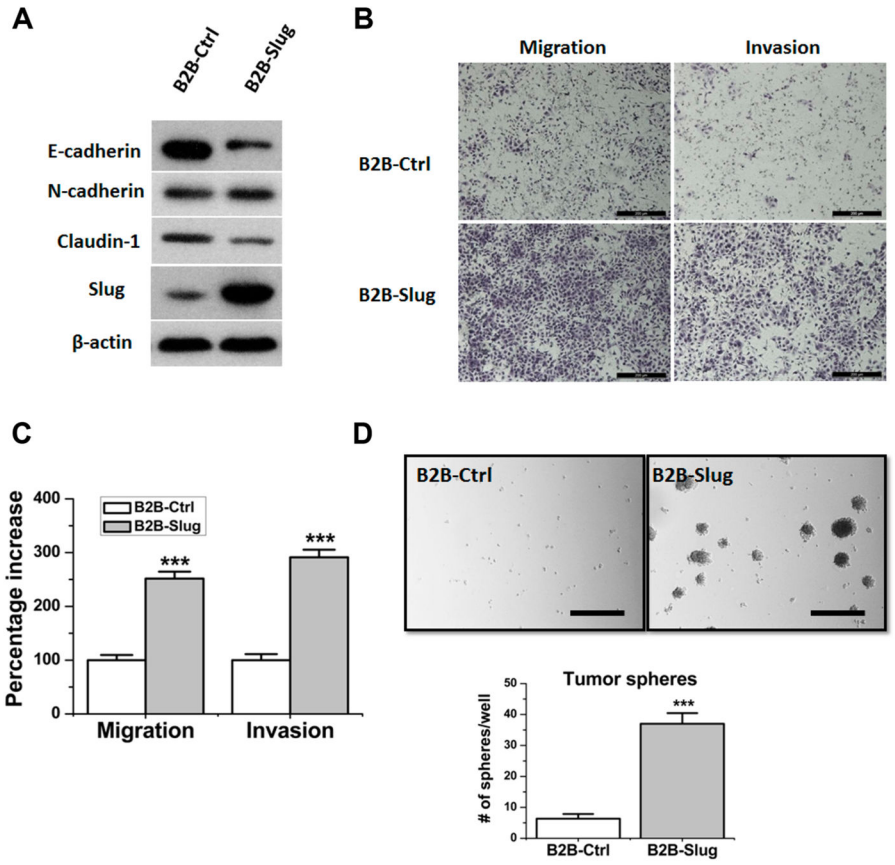

Figure 5.

Slug ectopic overexpression promotes EMT and malignant transformation of B2B cells. Ectopic overexpression of Slug in B2B cells was performed by transient transfection. B2B-Ctrl and B2B-Slug cells were used in experiments 72 h post-transfection. (A) Slug overexpression and its effect on the expression of EMT markers including E-cadherin, N-cadherin, and Claudin-1, as determined by immunoblotting. (B) Migration and invasion of B2B-Ctrl and B2B-Slug cells determined with a transwell migration assay and a Matrigel invasion assay, respectively; scale bar = 200 μm. (C) Number of migrating and invading cells quantified and depicted as bar charts; ***$p < 0.001$; data are the mean ± SEM. (D) Tumor sphere formation of B2B-Ctrl and B2B-Slug cells analyzed after 2 weeks in culture; scale bar = 200 μm. Only colonies exceeding 50 μm in diameter were scored as positive in five replicates per well; data are the mean ± SEM ($n = 4$). ***$p < 0.001$.