Ti$_{0.8}$O$_2$ Nanosheets Inhibit Lung Cancer Stem Cells by Inducing Production of Superoxide Anion

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ABSTRACT

Recent research into the cancer stem cell (CSC) concept has driven progress in the understanding of cancer biology and has revealed promising CSC-specific targets for drug discovery efforts. As malignancies of lung cancer have been shown to be strongly associated with activities of CSCs, we examined the effects of Ti$_{0.8}$O$_2$ nanosheets on these cells. Here we show that the nanosheets target lung CSCs but not normal primary dermal papilla (DP) stem cells. Whereas Ti$_{0.8}$O$_2$ caused a dramatic apoptosis along with a decrease in CSC phenotypes, in primary human DP cells such effects of nanosheets have been minimal. Nanosheets reduced the ability of lung cancer cells to generate three-dimensional tumor spheroids, lung CSC markers (CD133 and ALDH1A1), and CSC transcription factors (Nanog and Oct-4). Ti$_{0.8}$O$_2$ nanosheets reduced CSC signaling through mechanisms involving suppression of protein kinase B (AKT) and Notch-1 pathways. In addition, the nanosheets inhibited the migration and invasive activities of lung cancer cells and reduced epithelial-to-mesenchymal transition (EMT) markers as N-cadherin, vimentin, and Slug, as well as metastasis-related integrins (integrin-$a$V and integrin-$b$1). Importantly, we found that the selectivity of the Ti$_{0.8}$O$_2$ nanosheets in targeting cancer cells was mediated by induction of cellular superoxide anion in cancerous but not normal cells. Inhibition of nanosheet-induced superoxide anion restored the suppression of CSC and EMT in cancer cells. These findings demonstrate a promising distinctive effect of Ti$_{0.8}$O$_2$ nanosheets on lung CSC that may lead to opportunities to use such a nanomaterial in cancer therapy.

Introduction

Lung cancer has been considered one of the leading life-threatening cancers for several years as its mortality rate is considerably high in comparison with other human cancers; deaths from lung cancer make up nearly 20% of cancer deaths worldwide (Ferlay et al., 2015). Advances in molecular biology as well as translational research have heralded important moves in the comprehension of lung cancer, in particular with regard to the more common non–small cell lung cancer (NSCLC) type, which accounts for 80% of lung cancers. The current therapeutic approaches—surgery, radiotherapy, chemotherapy, and targeted therapy—exhibit various outcomes depending on the cancer subtype and stage. In early stages, a good response is observed in most cases; however, the long-term survival of late-stage patients is negatively impacted by disease relapse (Molina et al., 2008).

Accumulating knowledge has implicitly revealed that NSCLC is heterogeneous in nature with various molecular signatures underlying the hierarchy of cancer stem cell (CSCs) and non-CSC populations (Neelakantan et al., 2015). In general, CSC is a rare specific cancer cell arising in the tumor and referred to as “tumor-initiating cell.” CSCs have been identified in many solid tumors and have been shown to be a key player in cancer initiation, progression, and metastasis (Chen et al., 2013). Increasing evidence from different sources suggests that cells possessing CSC-like features are resistant to current chemotherapies, and their persistence leads to relapse of disease (Zhao, 2016). Therefore, novel therapeutic strategies targeting these cancer-initiating cells are now among the principle endeavors for the improvement of clinical outcomes.

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ABBREVIATIONS: 2D, two-dimensional; AKT, protein kinase B; ALDH1A1, aldehyde dehydrogenase 1 family, member A1; AFM, atomic force microscopy; CD133, prominin-1; CSC, cancer stem cell; DCF, 2’,7’-dichlorofluorescein; DHE, dihydroethidium; EMT, epithelial-to-mesenchymal transition; FBS, fetal bovine serum; GSK3$\beta$, glycogen synthase kinase 3 beta; HPF, 3’-$p$-(hydroxyphenyl) fluorescein; MnTBAP, Mn(III)tetraakis(4-benzoic acid) porphyrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSCLC, non–small cell lung carcinoma; PBS, phosphate-buffered saline; PI, propidium iodide; p-Oct-4, phosphorylation of octamer-binding transcription factor 4; PXRD, powder X-ray diffraction; Rac1, Ras-related C3 botulinum toxin substrate 1; ROS, reactive oxygen species; SEM, scanning electron microscope, microscopy; TEM, transmission electron microscope, microscopy.
Studies have related the process of epithelial-to-mesenchymal transition to CSC phenotypes. They have proven that induction of EMT in cancer could allow several stem cell–like features to concur, including self-renewal and other phenotypes of both normal and cancer stem cells (Abell and Johnson, 2014). This observation suggests that the EMT process may not only provide cancer cells with the ability to become motile and disseminate from the original site but also provide the cancer cells with the self-renewal capability that is crucial for success in clonal formation at metastatic sites (Brabletz et al., 2013; Abell and Johnson, 2014).

Several approaches have been investigated with regard to possible ways to improve cancer therapy. In the last decade, the most important archetype improvement in drug carriage was heralded by the use of nanomaterials (De Jong and Borm, 2008; Lee et al., 2017). Nanotechnology offers a variety of possibilities, including enhancement of drug delivery, facilitation of diagnostics, bioimaging, active implants, and direct therapeutic effects (Nune et al., 2009; Tomisa et al., 2011; Kadam et al., 2012; Laroui et al., 2013; Gmeiner and Ghosh, 2015). Nanosheets are an emerging class of nanomaterial with two-dimensionality (2D) and interesting properties complementing their 1D or 0D analogs (Wang and Sasaki, 2014). The sheets are flexible and highly anisotropic, and molecularly thin but microscale wide. The 2D nature could potentially lead to the effective use of surface atoms otherwise inaccessible to external stimuli. For example, TiO2 nanosheets derived from lepidocrocite titanate microcrystals A1Ti2-yM+yO4 (Maluangnont et al., 2013) are the 2D analog of TiO2, possessing semiconducting, photoactive, and dielectric properties, etc. The chemical exfoliation (i.e., soft chemistry) of the microcrystals can be performed easily on a large scale at room temperature, providing a stable aqueous colloid of negatively-charged nanosheets. Their chemical compositions are derived from the microcrystals, while their lateral sizes can be controlled through exfoliating conditions (Malueangnont et al., 2013). Although nanoparticles and nanotubes of TiO2 have been extensively studied with regard to biomedical applications, (Yin et al., 2013; Kulkarni et al., 2015) studies of TiO2 nanosheets are limited. For example, Song et al. (2014) recently reported in vivo toxicity of TiO2 nanosheets; a slight abnormality of the liver with increasing exposure time was observed. Here, we have performed pioneering experiments to show the selectivity of TiO2 nanosheets (prepared from the potassium zinc titanate precursor K0.8Zn0.4Ti1.6O4) in suppressing CSC but not normal-tissue stem cells, which could be beneficial in the development of nanomaterials for anticancer approaches.

**Materials and Methods**

**TiO2 Nanosheets Synthesis.** The TiO2 nanosheets were synthesized through a series of reactions (Malueangnont et al., 2013, 2016) consisting of solid state synthesis, proton exchange, and exfoliation as shown in Fig. 1A. The potassium zinc titanate precursor K0.8Zn0.4Ti1.6O4 was prepared by calcining the stoichiometric mixture of K2CO3, ZnO and TiO2 at 800°C for an hour, followed by grinding and another reheating at 900 °C for 20 hours. The resulting white powder was subject to a repeated proton exchange (three times, overnight each; spent acid was replaced with fresh acid) with 1 M HCl at the solid-to-solution ratio of 1 g to 100 ml. The product was washed with deionized water until it was free from excess acid and was dried at room temperature overnight, giving the proton form H1.6Ti1.6O4. Finally, the exfoliation was accomplished by mechanically shaking the mixture of H1.6Ti1.6O4.0H2O (0.4 g) and diluted tetrabutylammonium hydroxide ([C4H9)4NOH or TBAOH, 1 M; Sigma-Aldrich] (100 ml) at 180 rpm for 14 days, fixing the mole ratio of TBA+ (in the solution) to H+ (in the solid) to 1.

**TiO2 Nanosheets Characterization.** Powder X-ray diffraction (PXRD) measurements were conducted on a Rigaku DMAX 2200Ultima + diffractometer (Cu Kα, 40 kV, 30 mA). The mass-loss curve of the proton lepidocrocite titanate was performed using a Perkin-Elmer thermogravimetric analyzer (Pyris 1) (RT-900°C, N2 gas flowing at 20 ml/min). For the UV-vis measurement, the spectrum at the wavelength = 200–600 nm was recorded using a Shimadzu (PG Instruments) from a diluted colloidal suspension (0.2 ml of the original suspension, diluted to 100 ml with deionized water). The zeta potential of the nanosheets was measured by a Beckman Coulter Delsa Nano C. Microscopic images of the nanosheets were obtained from a JEOL JEM 10 transmission electron microscope. Atomic force microscopy (AFM) images of deposited TiO2 nanosheets were taken with an SPA-400 system (SPA400; Seiko Instruments Inc.) in noncontact mode using a Silicon probe following the reported (Malueangnont et al., 2013) procedure.

**Cell Culture and Reagents.** Human non–small cell lung cancer cell lines, A549, H460, H292, and H23 cells were obtained from the American Type Culture Collection (Manassas, VA). The human keratinocyte cell line (HaCaT) was purchased from Cell Lines Service (Heidelberg, Germany). Human dermal papilla primary cell culture (primary DP1) was purchased from Celprogen (Benelux, Netherlands). Immortalized dermal papilla cells (DP) and human primary hair follicle dermal papilla cells (primary DP2) were purchased from Applied Biologic Materials Inc. (Richmond, BC). H460, H292, and H23 cells were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY), whereas A549, HaCaT, DP, primary DP1, and primary DP2 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco). The medium was supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine and 100 units/ml penicillin and streptomycin (Gibco, MD). Cells cultures were maintained in a 37°C humidified incubator with 5% CO2. Cells were routinely passaged at preconfluent density using 0.25% trypsin solution with 0.53 mM EDTA. RPMI 1640 medium, FBS, l-glutamine, penicillin/streptomycin, phosphate-buffered saline (PBS), trypsin, and EDTA were purchased from Gibco. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide, Hoechst33342, propidium iodide (PI), and bovine serum albumin, dihydroethiodium (DHE), doxorubicin, and cisplatin were purchased from Sigma Chemical, Inc. (St. Louis, MO). Apoptosis Kit (FITC) was purchased from ImmunoTools (Germany). 2’7’-Dichlorofluorescein (DCF); and 3-p-(hydroxyphenyl) fluorescein (HPP) were purchased from Invitrogen. Mn(III) tetras (4-benzoic acid) pyrophyn (MnTRAP) was purchased from Merck (Germany). Antibodies directed against Vimentin, Snail, Slug, protein kinase B (Akt), phosphorylated protein kinase b on Ser473 [p-Akt (Ser473)], phosphorylated glycogen synthase kinase 3 beta on Ser9 (p-GSK3β), and tubulin were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX).

**Cytotoxicity Assay.** For cytotoxicity assay, human lung cancer A549, H460, H292, and H23 cells, and normal cells HaCaT, DP, primary DP1, and primary DP2 cells were seeded onto 96-well plates at the density of 1 × 103 cells/well and were allowed to incubate overnight. Cells were then treated with various concentrations of TiO2 nanosheets for 24 hours at 37°C and analyzed for cell viability using MTT assay according to the manufacturer’s protocol (Sigma Chemical). Cell viability was calculated by dividing the absorbance of
the treated cells by that of the control cells and represented in percentage.

**Nuclear Staining Assay.** Nuclear costaining with Hoechst 33342 (Sigma Chemical) and propidium iodide (Sigma Chemical) was used to determine apoptotic and necrotic cell death. The human lung cancer A549 and H460, and primary DP1 cells were seeded onto 96-well plates at a density of $1 \times 10^4$ cells/well, incubated overnight, and treated with Ti$_{0.8}$O$_2$ nanosheets at various concentration (0–10 mg/ml) for 24 hours at 37°C. Next, cells were incubated with 10 mg/ml of Hoechst 33342 and 5 μg/ml of PI for 30 minutes at 37°C. They were visualized and imaged under a fluorescence microscope (ECLIPSE Ts2; Nikon).

**Cell Apoptosis Analysis.** Apoptosis was determined with Annexin V-FITC Apoptosis Kit (ImmunoTools). The human lung cancer A549 and H460, and primary DP1 cells were seeded in 24-well plates at a density of $1 \times 10^5$ cells/ml, incubated overnight, and treated with Ti$_{0.8}$O$_2$ nanosheets at various concentration (0–10 μg/ml) for 24 hours at 37°C. Cells were detached with trypsin-EDTA (0.25%), washed in PBS, and centrifuged at 1300 rpm for 5 minutes. Cells were suspended in 100 μl of binding buffer and incubated in 5 μl of annexin V-FITC and 1 μl of PI or for 15 minutes at room temperature in the dark. Five hundred microliters of incubation buffer were added, and cells were analyzed by guava easyCyte flow cytometry systems.

**Scanning Electron Microscopy Morphologic Analysis.** H460-treated cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 1–2 hours. Cells were rinsed twice with phosphate buffer and once with distilled water for 5 minutes each. Cells were dehydrated with a graded series of ethanol (30%, 50%, 70%, 90%, 100%).
and 95% for 5 minutes each and 100% three times, 5 minutes/time), dried, mounted, and coated with gold (spatter coater, Balzers model SCD 040, Germany). After that, Cells were observed under a scanning electron microscope (SEM; model JSM6400; JEOL, Tokyo, Japan).

**Cellular Uptake Analysis.** H460 and primary DP1 cells (1 × 10⁶ cells/ml) were seeded in 10-cm dishes and treated with Ti0.8O2 nanosheets at 1 µg/ml for 24 hours. The treated cells were collected, washed with PBS, and then fixed in 2% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in alcohol, and embedded. Thin sections of resin-embedded H460 and primary DP1 cells were cut, and cellular uptake was observed with a transmission electron microscope (TEM-JEM-1400 (Jeol Ltd.)).

**Migration Assay.** Human NSCLC-derived cells (H460 and A549) and primary DP1 cells were pretreated with Ti0.8O2 nanosheets at nontoxic concentrations (0 to 1 µg/ml) for 48 hours at 37°C. The treated cells were then seeded onto 96-well plates at a density of 3 × 10⁴ cells/well. Monolayer cells were next allowed to migrate after the attached cells were scratched with a P200 micropipette tip to generate a wound space. Four random fields of the wound space were examined and imaged under a phase-contrast microscope (ECLIPSE Ts2; Nikon) at various time points (0, 24, and 48 hours). Relative cell migration was quantified by dividing the percentage change of the wound space in treated cells to that of the control cells. For the Transwell assay, the treated cells (A549, H460, and primary DP1 cells) were seeded at a density of 3 × 10⁴ cells/ml in the upper chamber supplemented with serum-free medium (8-µm pore size) in a 24-well plate. The lower chamber was filled with complete medium containing 10% FBS as a chemoattractant. After 24 hours, the nonmigrated cells in upper chamber were removed by cotton-swab, and the cells that migrated to the underside of the membrane were fixed with cold methanol for 10 minutes and stained with 10 µg/ml of Hoechst 33342 for 10 minutes. The stained cells were visualized and scored under a fluorescence microscope (ECLIPSE Ts2).

**Invasion Assay.** This assay was performed using chambers with 8-µm pore filter inserts in 24-well plates (Corning Life Sciences). The upper chamber of the inserts was coated with 50 µl of 0.5% Matrigel from BD Biosciences (San Jose, CA). Human NSCLC-derived cells (H460 and A549) and primary DP1 cells were pretreated with Ti0.8O2 nanosheets at nontoxic concentrations (0 to 1 µg/ml) for 48 hours at 37°C. The treated cells were seeded at a density of 3 × 10⁴ cells/well in the upper chamber supplemented with serum-free medium (8-µm pore size) in a 24-well plate. The lower chamber was filled with complete medium containing 10% FBS as a chemoattractant. After 24 hours, the noninvasing cells in the upper chamber were removed with a cotton swab and the invading cells in the lower chamber were fixed with cold methanol for 10 minutes and stained with 10 µg/ml of Hoechst 33342 for 10 minutes. The stained cells were then visualized and scored under a fluorescence microscope (ECLIPSE Ts2; Nikon).

**Anchorage-Independent Growth Assay.** Anchorage-independent cell growth was determined by soft agar colony formation assay. Human NSCLC-derived cells (H460 and A549) and primary DP1 cells were pretreated with Ti0.8O nanosheets at nontoxic concentrations (0 to 1 µg/ml) for 48 hours at 37°C. Soft agar was prepared by using a 1:1 mixture of cultured medium containing 10% FBS and 1% agarose. The mixture was allowed to solidify in a 24-well plate to form a bottom layer, after which an upper cellular layer consisting of 3 × 10⁴ cells/ml in the agarose gel with 10% FBS and 0.33% agarose was added. After the upper layer was solidified, the system was added with cultured medium containing 10% FBS and incubated at 37°C. Colony formation was determined after 2 and 3 weeks using a phase-contrast microscope (ECLIPSE Ts2; Nikon). Relative colony number and diameter were determined the values of the treated cells dividing by control cells.

**Spheroids Formation Assay.** Spheroids were grown using the adapted method from Chanvorachote and Luanpitpong (2016), Phiboonchaiyanan et al. (2016), and Powan et al. (2017). Human NSCLC-derived cells (H460 and A549) and primary DP1 cells were pretreated with Ti0.8O2 nanosheets at nontoxic concentrations (0–1 µg/ml) for 48 hours at 37°C. The treated cells at a density of 2.5 × 10³ cells/well were seeded onto an ultralow-attachment plate in 0.8% methylcellulose-based serum-free medium supplemented with 20 ng/ml epidermal growth factor, 4 mg/ml insulin, and basic fibroblast growth factor for 7 days to form primary spheroids. Then the primary spheroids were detached using 1 mM EDTA and suspended into single cells. These cells were grown in a 24-well ultralow-attachment plate at a density of 2.5 × 10³ cells/well in 0.8% methylcellulose-based serum-free medium supplemented with 20 ng/ml epidermal growth factor, 4 mg/ml insulin, and basic fibroblast growth factor for 21 days to form secondary spheroids. At days 14 and 21, the numbers and sizes of secondary spheroids were determined and imaged using a phase-contrast microscope (ECLIPSE Ts2; Nikon).

For CSC-rich population formation, the method was slightly modified from a previously described method (Chanvorachote and Luanpitpong, 2016; Phiboonchaiyanan et al., 2016; Powan et al., 2017). Cells were seeded onto a 24-well ultralow-attachment plate, with approximately 2.5 × 10³ cells/well in 0.8% methylcellulose-based serum-free medium supplemented with 20 ng/ml epidermal growth factor, basic fibroblast growth factor, and 4 mg/ml insulin. The primary spheroids were allowed to form for 7 days. At day 7 of primary spheroid culture, primary spheroids were resuspended into single cells using 1 mM EDTA, and again 2.5 × 10³ cells/well were seeded onto a 24-well ultralow-attachment plate. Secondary spheroids were allowed to form for 14 days.

The single three-dimensional spheroid formation assay cells were then allowed to form primary and secondary spheroids as detailed above. After 14 days, secondary spheroids were dissociated into single cells with 1 mM EDTA, and again 2.5 × 10³ cells/well were seeded onto a 24-well ultralow-attachment plate. Secondary spheroids were allowed to form for 14 days.

**Enzyme-Limiting Dilution Assay.** This study was slightly modified from previously described methods (Slika and Ahmed, 1996). Cells were plated in gradually decreasing number from 100 to 1 cell/well onto a 96-well ultralow-attachment plate and cultured for 14 days, and the number of spheroid was recorded. The treated spheroids were calculated in all wells compared with the control.

**Cell Aggregation Behavior Evaluation.** Cells were seeded in a 24-well plate at 6 × 10³ cells/well and incubated overnight for cell attachment. The cells were then treated with various concentrations of TiO₂ nanospheres (0 to 1 µg/ml) for 72 hours after exposure to the treatments, aggregation behavior of cells was determined at 72 hours. Aggregation behavior of cells was photographed by a phase-contrast microscope (ECLIPSE Ts2; Nikon).

**Intracellular Reactive Oxygen Species Determination.** The intracellular reactive oxygen species (ROS), hydroxyl radical, and superoxide anion production were determined by flow cytometry using DCF, HPF, and DHE as fluorescent probes. In brief, cells were seeded into 24-well plates at a density of 3.0 × 10⁴ cells/well and treated with 0.5 and 1 µM DCF, 10 µM HPF, or 10 µM DHE for 30 minutes for the detection of intracellular ROS, hydroxyl radical, and superoxide anion, respectively. The fluorescence intensity of DCF, HPF, and DHE were detected by flow cytometric analysis.

**Western Blot Analysis.** After TiO₂ nanospheres treatment, human NSCLC-derived cells (H460 and A549) and primary DP1 cells were incubated with lysis buffer containing 20 mM TrisHCl (pH 7.5), 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche Molecular Biochemical) for 30 minutes on ice. The cellular lysates were collected and their protein content was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein from each
sample were separated by SDS-PAGE and transferred to 0.45-μm nitrocellulose membranes (Bio-Rad). The resulting blots were blocked for 1 hour with 5% nonfat dry milk in TBST (Tris-buffer saline with 0.1% Tween containing 25 mM Tris-HCl, pH 7.5, 125 mM NaCl, and 0.1% Tween 20) and incubated with specific primary antibodies against Vimentin, Snail, Slug, Akt, p-Akt (Ser473), GSK3β, p-GSK3β (Ser 9), Nanog, p-Oct-4, β-catenin, Notch-1, ALDH1A1, Rac1, Integrin αv, α5, β1 and β3, and β-actin at 4°C overnight. After three washes in TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 hours at room temperature. Finally, protein bands were detected using an enhanced chemiluminescence substrate (Supersignal West Pico; Pierce) and exposed to film.

**Flow Cytometry Analysis.** Cells were harvested by centrifugation after which the resuspended cells were incubated on ice with a rabbit anti-CD133 antibody or an anti-ALDH1A1 antibody for 1 hour. Next, the primary antibodies were removed and the cells were washed and incubated for 30 minutes with Alexa Flour 488 (Invitrogen)-conjugated goat anti-rabbit IgG (H+L) secondary antibody. After a washing, the fluorescence intensity was determined by flow cytometry using a 488 nm excitation and a 519-nm band-pass filter. The mean fluorescence intensity was quantified using guava easyCyte flow cytometry systems.

**Immunofluorescence.** Cells were seeded onto 96-well plates at the density of 1 × 10^4 cells/well. After treatment for 48 hours, the cells were fixed with 4% (w/v) paraformaldehyde for 30 minutes and permeabilized with 0.1% (v/v) Triton-X for 20 minutes. The cells were next incubated with 3% (w/v) bovine serum albumin for 30 minutes, washed, and incubated with an anti-N-cadherin or anti-E-cadherin antibody overnight at 4°C, washed, and incubated with Alexa Flour 488 (Invitrogen)-conjugated goat anti-rabbit IgG (H+L) secondary antibody for 1 hour at room temperature in the dark. Thereafter, the cells were washed with PBS, contrasted with 10 μg/ml Hoechst 33342, and visualized and imaged using fluorescence microscopy (ECLIPSE Ti2; Nikon).

**Statistical Analysis.** Data from three or more independent experiments are presented as mean ± S.D. Multiple comparisons for statistically significant differences between multiple groups were performed using analysis of variance, followed by individual comparisons with Scheffe’s post-hoc test. Statistical significance was considered at *P* < 0.05.

**Results**

**Characterization of K_{0.8}Zn_{0.4}Ti_{1.6}O_{4} and H_{1.6}Ti_{1.6}O_{4}·0.8H_{2}O Nanosheet.** The PXRD patterns of K_{0.8}Zn_{0.4}Ti_{1.6}O_{4} and H_{1.6}Ti_{1.6}O_{4}·0.8H_{2}O in Fig. 1B are characteristic of compounds with a lepidocrocite structure, (Sasaki and Watanabe, 1998; Maluangmont et al., 2016, 2017), thereby confirming a successful synthesis. As shown in Fig. 1A, K_{0.8}Zn_{0.4}Ti_{1.6}O_{4} is built from the sheets of double edge–shared (Ti/Zn)O₆ octahedra interleaved by K⁺ ions. The distance *d*₂₀₀ is the intersheet separation. The shift of the *d*₂₀₀ from *d* = 0.78 nm (2θ = 11.3°) to *d* = 0.90 nm (2θ = 9.8°) as a result of proton exchange indicates (Sasaki and Watanabe, 1998) the notable interlayer expansion resulting from the incorporation of a water molecule between the sheets.

Since the dissolution of zinc by acid is well known, the proton exchange of K_{0.8}Zn_{0.4}Ti_{1.6}O_{4} to H_{1.6}Ti_{1.6}O_{4}·nH₂O was first assumed. Here, a 1:1 exchange of protons for K⁺ ions, and a 2:1 exchange for Zn²⁺ ions can be expected. The number of water molecules (*n*) can be calculated from the mass-loss curve (Fig. 1D) assuming the thermal transformations below. By employing formula weights of 156.592 g/mol for H_{1.6}Ti_{1.6}O_{4}·0.8H₂O and 18 for water, the mass loss in both eqs. (1) and (2) equals (0.8 × 18/156.592) × 100% = 9.20%, which is in reasonable agreement with the observed mass losses in the first (9.5%) and second (9.0%) steps.

\[
\text{H}_{1.6}\text{Ti}_{1.6}\text{O}_{4}·0.8\text{H}_{2}\text{O} \rightarrow \text{H}_{1.6}\text{Ti}_{1.6}\text{O}_{4} + 0.8\text{H}_{2}\text{O} \quad (1)
\]

\[
\text{H}_{1.6}\text{Ti}_{1.6}\text{O}_{4} \rightarrow 2\text{Ti}_{0.8}\text{O}_{2} + 0.8\text{H}_{2}\text{O} \quad (2)
\]

The H_{1.6}Ti_{1.6}O_{4}·0.8H₂O powder was next exfoliated with TBAOH. The mixture, initially containing the suspended powder and a clear liquid, gradually transformed into a white translucent colloid, as shown in the left inset of Fig. 1C. This colloid exhibited a Tyndall effect whereby the light was scattered throughout, as shown in the right inset. These observations suggest (Sasaki and Watanabe, 1998; Maluangmont et al., 2013) the infinite separation (i.e., exfoliation) of stacks of layers in the proton-containing lepidocrocite titanate into elementary units. The colloid absorbed light at λₘₚ = 261 nm (Fig. 1C), which is also in good agreement with the literature (Sasaki and Watanabe, 1998). The zeta potential of the nanosheets (~30 mV) confirmed that they are negatively charged, as one would expect considering the stoichiometry of the titanate precursor.

Figure 1E is the representative TEM image showing a flat 2D nanosheet with uniform contrast. The presence of nanosheets was further supported by the AFM image and the corresponding height analysis, shown in Fig. 1, F and G. The 2D objects with a wide distribution of lateral sizes (maximum ~1 × 1 μm) and a height of approximately 1.4 nm can be clearly observed. Although the crystallographic thickness in the b direction (i.e., of the layer) is ~0.75 nm, (Sasaki and Watanabe, 1997) the reported thickness of ~1.4 nm is probably the result of adsorption of water molecules and/or TBA⁺ on the surface.

**Cytotoxicity of Ti_{0.8}O_{2} Nanosheets on Cancerous Human Lung Cells and Normal Cells.** Cells were treated with various concentrations of Ti_{0.8}O_{2} nanosheets (~0–100 μg/ml) and analyzed for cell viability. The results showed that statistically significant cytotoxic effects of Ti_{0.8}O_{2} nanosheets could be found at the concentrations of 0.05–100 μg/ml in H23 cells; 5–100 μg/ml in H460, H292, and HaCaT cells; 10–100 μg/ml in A549 and DP cells; and 50–100 μg/ml in primary DP1 and primary DP2 cells (Fig. 2, A–H). Apoptosis assay revealed that treatment with 10 μg/ml Ti_{0.8}O_{2} nanosheets mediated apoptosis in lung cancer cells (Fig. 2, I, J, L, and M), whereas it had no toxic effect on primary DP1 cells (Fig. 2, K, P). Flow cytometry analysis using annexin V/PI detection as a basis also confirmed that 10-μg/ml Ti_{0.8}O_{2} nanosheets induced dramatic apoptosis in A549 and H460 cells but only had a minimal effect on primary DP1 cells (Fig. 2, N–P). Furthermore, SEM morphologic analysis demonstrated that 10-μg/ml Ti_{0.8}O_{2} nanosheets initially changed the morphology, including the formation of stress fibers (Fig. 2Q). In addition, TEM analysis showed that Ti_{0.8}O_{2} nanosheets can sufficiently enter into the cancer and normal primary DP1 cells (Fig. 2R).

**Ti_{0.8}O_{2} Nanosheets Suppress Cancer Stem Cell–Like Phenotypes.** The ability of cancer cells to form tumor spheroids and grow in an anchorage-independent condition was shown to be a hallmark of cancer stemness as well as metastatic potential (Wang et al., 2017). We further investigated the effect of Ti_{0.8}O_{2} nanosheets on the growth of A549 and H460 cells and primary DP1 under this condition. Figure 3, A–D, demonstrates that treatment of A549 and H460 cells with nontoxic concentrations of Ti_{0.8}O_{2} nanosheets...
Fig. 2. The cytotoxic effect of Ti$_{0.8}$O$_2$ nanosheets on lung cancer and normal cells. Effect of Ti$_{0.8}$O$_2$ nanosheets on cell viability of lung cancer cells (A549, H460, H292, and H23) and normal cells [dermal papilla (DP) cell line, primary DP1, primary DP2, and HaCaT keratinocyte cells] for 24 hours using MTT assays (A–H). Apoptotic and necrotic nuclei in cells treated with Ti$_{0.8}$O$_2$ nanosheets, determined by Hoechst 33342/PI costaining and visualized using fluorescence microscopy (I–M). Annexin/PI costained cells were determined by flow cytometry (N–P). Morphology of H460 cells was determined using SEM (Q). TEM pictures of the cellular uptake of Ti$_{0.8}$O$_2$ nanosheets in H460 and primary DP cells at 24 hours (R). Data are shown as the mean ± S.D. (n = 3). *P < 0.05 vs. nontreated control.
decreased the formation of cancer cell colonies in terms of number and diameter of colonies, in a dose-dependent manner, in comparison with the control. On the other hand, treatment of primary DP1 cells with nontoxic concentrations of Ti$_{0.8}$O$_2$ nanosheets slightly increased colony number and not altered colony size (Fig. 3, E and F).

Our finding was confirmed by a spheroid formation assay showing that the Ti$_{0.8}$O$_2$ nanosheet–treated cells (A549 and H460 cells) exhibited a decreased spheroid-forming ability, with a statistically significant decrease in both spheroid number and diameter in comparison with nontreated control cells (Fig. 3, G–J), but Ti$_{0.8}$O$_2$ nanosheets could not alter spheroid number and diameter of primary DP1 cells (Fig. 3, K and L). Extreme limiting-dilution assay also showed that the nontreated controls exhibited the ability of cells to form spheroids, whereas the nanosheet-treated cells were able to attenuate spheroid formation (Fig. 3, M and N). Further, we found that Ti$_{0.8}$O$_2$ nanosheets decreased the size of the spheroid in both A549 and H460 cells as early as 3 days following the treatment (Fig. 3, O–R).

Having shown that Ti$_{0.8}$O$_2$ nanosheets could suppress CSC-like phenotypes in lung cancer cells, we next investigated the possible mechanism by which such nanosheets could attenuate these features. First, we attempted to explain the CSC-suppressing effect of nanosheets by determining the cellular level of the well known stem cell markers CD133 and ALDH1A1, and stem cell–regulating transcription factors such as Nanog and p-Oct-4. Western blotting revealed that the expression levels of stem cell markers in primary DP1 cells (Fig. 4, A and C) but Ti$_{0.8}$O$_2$ nanosheets did not alter such proteins in primary DP1 cells (Fig. 4E).

Just as in normal tissue stem cells, several cell-signaling pathways have been implicated in mechanisms underlying stemness in CSCs. Protein kinase B (Akt), β-catenin, and Notch signals have been shown to be key regulatory mechanisms underlying stemness in both normal and cancerous stem cells (Hadjimichael et al., 2015; Mohammed et al., 2016; Phiboonchaitayan et al., 2016; Koury et al., 2017). To clarify the possible effects of nanosheets on such stem cell pathways, we determined the levels of related proteins in nanosheet-treated cells. Figure 4, A and C, show that treatment with Ti$_{0.8}$O$_2$ nanosheets decreased the level of Notch1 in A549 and H460 cells (Fig. 4F). To confirm, the CD133 and ALDH1A1-positive cells were evaluated by flow cytometry in A549 and H460 lung cancer cells and in primary DP cells. Although the number of CD133 and ALDH1A1-positive cells was reduced in both types of lung cancer cell by nanosheet treatment (Fig. 4, G and H), the population of those CD133- and ALDH1A1-stained cells was not altered in primary DP1 cells (Fig. 4I). Taken together, these results enabled us to unravel the pivotal data indicating that Ti$_{0.8}$O$_2$ nanosheets attenuate stem-cell properties and signaling in lung cancer cells, but not in normal primary DP cells, via Akt/β-catenin and Notch1 suppression. Moreover, the A549 cells were treated with cisplatin and doxorubicin, standard chemotherapeutic drugs for lung cancer. For comparison, the cytotoxicity of nanosheet, cisplatin, and doxorubicin was determined (Fig. 4J). The result showed that both cisplatin and doxorubicin enriched CSC, as indicated by the increase of CSC markers such as ALDH1A1, Notch1, β-catenin, and p-Akt (Fig. 4K).

### Ti$_{0.8}$O$_2$ Nanosheets Decrease Cell Migration and Invasion

To explore the possibility of using Ti$_{0.8}$O$_2$ nanosheets as a potential therapeutic strategy against cancer, we next investigated whether Ti$_{0.8}$O$_2$ nanosheets could suppress EMT in lung cancer cells. Treatment of the cells (A549 and H460) with Ti$_{0.8}$O$_2$ nanosheets caused a decrease in the level of N-cadherin and an increase in the level of E-cadherin, in a dose-dependent manner (Fig. 5, A and C). Immunofluorescence staining supports our finding that Ti$_{0.8}$O$_2$ nanosheets caused a decrease in the level of N-cadherin and an increase in the level of E-cadherin (Fig. 5, G and H). Ti$_{0.8}$O$_2$ nanosheet caused similar effects on the levels of N-cadherin and E-cadherin in primary DP1 cells (Fig. 5E). In addition, Ti$_{0.8}$O$_2$ nanosheets dramatically reduced the EMT-related proteins of Vimentin, Slug, Snail, and Rac1 in a dose-dependent manner in A549 and H460 cells (Fig. 5, A and C). However, only Snail was found to decrease in primary DP1 cells (Fig. 5E).

It is worth noting that several reports have pointed out the critical role of certain integrins (including αv, α5, β1 and β3) that enhance the metastasis potential of cancer cells (Canel et al., 2013). The levels of the αv, α5, β1, and β3 integrins were therefore evaluated by Western blot analysis. We found that treatment with Ti$_{0.8}$O$_2$ nanosheets decreased the expression of integrins αv and β1 in A549 and H460 cells and primary DP1 cells, whereas the expression of integrins α5 and β3 was not changed (Fig. 5, D and F). Here, we have shown the additional benefits of using Ti$_{0.8}$O$_2$ nanosheets for inhibition of metastasis by suppressing EMT and decreasing the αv and β1 integrins. In addition, the Akt/GSK3β signaling pathway is not only implicated in mechanisms underlying stemness in CSCs but also in regulating the stability and transcription activity of Snail/Slug, repressing E-cadherin, and inducing EMT.

### Ti$_{0.8}$O$_2$ Nanosheets Decrease Cell Migration and Invasion

The ability of cancer cells to migrate and invade has been observed in the characterizations of CSCs, EMT, and metastasis-related integrins. EMT, or transition of an epithelial phenotype to a more mesenchymal phenotype of cancer cell, is another exploitable pathway of augmentation-aggressive activity of cancer. EMT involves the alteration of proteins, including the E-cadherin to N-cadherin switch (Gravdal et al., 2007) and increase of vimentin, Snail, and Slug (Medici et al., 2008; Liu et al., 2015). We next explored whether Ti$_{0.8}$O$_2$ nanosheets could suppress EMT in lung cancer cells. Treatment of the cells (A549 and H460) with Ti$_{0.8}$O$_2$ nanosheets caused a decrease in the level of N-cadherin and an increase in the level of E-cadherin, in a dose-dependent manner (Fig. 5, A and C). Immunofluorescence staining supports our finding that Ti$_{0.8}$O$_2$ nanosheets caused a decrease in the level of N-cadherin and an increase in the level of E-cadherin (Fig. 5, G and H). Ti$_{0.8}$O$_2$ nanosheet caused similar effects on the levels of N-cadherin and E-cadherin in primary DP1 cells (Fig. 5E). In addition, Ti$_{0.8}$O$_2$ nanosheets dramatically reduced the EMT-related proteins of Vimentin, Slug, Snail, and Rac1 in a dose-dependent manner in A549 and H460 cells (Fig. 5, A and C). However, only Snail was found to decrease in primary DP1 cells (Fig. 5E).
Fig. 3. Ti0.8O2 nanosheets decrease anchorage-independent growth and suppress CSC-like phenotypes and CSC growth in CSC-rich populations in lung cancer cells. A549, H460, and primary DP1 cells were treated with Ti0.8O2 nanosheets (0–1 μg/ml) for 48 hours. The treated cells were subjected to anchorage-independent growth assays for 2 and 3 weeks (A, B, and E), and assessed by microscopy (4×). The colony number as a percentage and the size of the treated cell were analyzed and compared with the control (C, D, and F). Ti0.8O2 nanosheets decreased spheroid formation of A549, H460, and primary
metastatic cancer cells (Abell and Johnson, 2014). We further evaluated whether Ti0.8O2 nanosheets could attenuate such aggressive behaviors of cancer cells. Figure 6, A and B, show that Ti0.8O2 nanosheets inhibited cell migration of A549 and H460 cells. Analysis of cell migration and invasion, carried out with Boyden chambers, consistently demonstrated that Ti0.8O2 nanosheets were able to decrease the number of A549 and H460 cells migrating and invading across the Transwell filter within 24 hours in a dose-dependent manner, in comparison with the control, Fig. 4.

Ti0.8O2 nanosheets inhibit the expression of CSC markers and self-renewal transcription-factor proteins. A549, H460, and primary DP1 cells were treated with Ti0.8O2 nanosheets (0–1 μg/ml) for 48 hours. The expression levels of CSC protein markers in A549, H460 (A–D), and primary DP1 (E and F) were determined by Western blotting. CD133 and ALDH1A1 expression levels were measured by flow cytometry (G–I). Effect of Ti0.8O2 nanosheets, cisplatin, and doxorubicin on cell viability of A549 cells (J) and their effects on protein expression levels of CSC protein markers in A549 cells (K).

DP1 cells after 2 and 3 weeks (G, H, and K) according to microscopy assessment (4×). The relative spheroid number and size, compared with those of the untreated controls (I, J, and L). The CSC-rich cells of A549 and H460 cells were treated with Ti0.8O2 nanosheets and plated in decreasing numbers from 100 cells/well to 1 cell/well for 14 days (M and N). The single CSC-rich spheroids were treated with Ti0.8O2 nanosheets for 3, 5, and 7 days (O and Q), and the relative spheroid sizes compared with those of untreated controls were monitored (P and R). Data are shown as the mean ± S.D. (n = 3). *P < 0.05 vs. nontreated control. The relative spheroid sizes were investigated by comparing with nontreated control in each time.

Fig. 4. Ti0.8O2 nanosheets inhibit the expression of CSC markers and self-renewal transcription-factor proteins. A549, H460, and primary DP1 cells were treated with Ti0.8O2 nanosheets (0–1 μg/ml) for 48 hours. The expression levels of CSC protein markers in A549, H460 (A–D), and primary DP1 (E and F) were determined by Western blotting. CD133 and ALDH1A1 expression levels were measured by flow cytometry (G–I). Effect of Ti0.8O2 nanosheets, cisplatin, and doxorubicin on cell viability of A549 cells (J) and their effects on protein expression levels of CSC protein markers in A549 cells (K).
but Ti\textsubscript{0.8}O\textsubscript{2} nanosheets had no effect on the motility of primary DP1 cells (Fig. 6, C and D).

\textbf{Ti\textsubscript{0.8}O\textsubscript{2} Nanosheets Have No Effect on Primary Dermal-Papilla Aggregation Properties.} The data thus far has indicated that DP aggregation patterns reflect the ability to form hair (Sari et al., 2016). Having shown the promising effect of Ti\textsubscript{0.8}O\textsubscript{2} nanosheets in selective suppression of the CSC in lung cancer, we next evaluated whether treatment of normal cells with such a substance affected the viability of normal cells as well as their stem cell properties.

\textbf{Fig. 5.} Effect of Ti\textsubscript{0.8}O\textsubscript{2} nanosheets on EMT. A549, H460, and primary DP1 cells were treated with Ti\textsubscript{0.8}O\textsubscript{2} nanosheets (0–1 \( \mu \text{g/ml} \)) for 48 hours. The expression levels of EMT protein markers and integrins were investigated by Western blotting (A–F). Blots were reprobed with \( \beta \)-actin to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and the mean data from independent experiments were normalized to the results. Data are shown as the mean \( \pm \) S.D. (\( n = 3 \)). *\( P < 0.05 \) vs. nontreated control. Expression of E-cadherin and N-cadherin was analyzed by immunofluorescence staining (G and H).
The human DP cell line and primary DP cells derived from different sources (primary DP1 and DP2) were cultivated in the presence of various concentrations of Ti$_{0.8}$O$_2$ nanosheets (0 to 1 µg/ml) for 72 hours and observed for aggregate formation. Figure 7 shows that Ti$_{0.8}$O$_2$ nanosheets slightly increased the sizes of cell aggregations in comparison with those of the nontreated control, in primary DP2 and DP cells, but such an induction effect did not occur in primary DP2 cells. These results indicate that treatment with Ti$_{0.8}$O$_2$ nanosheets has a minimal effect on the viability of these DP cells as well as their aggregation patterns.

Ti$_{0.8}$O$_2$ Nanosheets Selectively Target Cancer Stem Cell and EMT through Superoxide Anion–Dependent Mechanism. Having shown that Ti$_{0.8}$O$_2$ nanosheets target CSC in lung cancer cells, we searched in addition for the underlying mechanism of their CSC specificity. Intracellular ROS analysis was performed to determine the ROS generation of cancer and normal cells in response to nanosheet treatment. Figure 8 indicates that Ti$_{0.8}$O$_2$ nanosheets selectively induced the generation of intracellular superoxide anion (detected by superoxide anion specific fluorescence dye, DHE) in lung cancer cells; however, nanosheets at same concentration failed...
to induced cellular superoxide anion (Fig. 8, A and C). For hydrogen peroxide and hydroxyl radical, both cancerous and normal DP cells responded alike (Fig. 8, A and C). We further tested whether this nanosheet-induced superoxide anion is in response to the mechanism of CSC and EMT suppression. Cells were pretreated with a specific superoxide anion inhibitor (50 μM of MnTBAP) and treated with Ti0.8O2 nanosheets. The cellular level of CSC and EMT markers were determined by Western blot analysis. We found that inhibition of superoxide anion in the nanosheet-treated cancer cells could restore the decrease in CSC- and EMT-related proteins (Fig. 8B). Unlike the cancer cells, treatment of the primary DP cells with MnTBAP or nanosheet cause no change in the expression of CSC and EMT markers (Fig. 8D). These results revealed that the selectivity of Ti0.8O2 nanosheets may be caused, at least in part, by the induction of superoxide anion in cancer cells.

**Discussion**

Owing to their notable properties, nanomaterials offer more effective drug delivery into cells—particularly into cancer cells—through enhanced permeability associated with nano-size (Nakamura et al., 2016). Furthermore, the use of nanoparticles in cancer cells allows retention of materials at the site of action (Nichols and Bae, 2012). Although the potential of nanoparticles to benefit delivery of drugs as well as genetic material has been intensively investigated, (Yildiz et al., 2011; Posadas et al., 2016) the understanding of unique nanoparticle shapes, especially nanosheets, and their interaction with cancer cells are largely unknown. The shape of a nanoparticle determines the mode of cytotoxicity (Shin et al., 2015). Here, we show for the first time that Ti0.8O2 nanosheets distinctively suppress stem cell properties in lung CSCs but exhibit a lesser effect on noncancerous stem cells. This has been demonstrated in several lines of lung cancer in comparison with two types of primary dermal papilla and a normal keratinocyte. In addition, we have provided supportive information explaining that the cancer cell selectivity of Ti0.8O2 nanosheets may be caused by the generation of a superoxide anion (Fig. 8). Our results indicate that the Ti0.8O2 nanosheets selectively induced superoxide generation in cancer cells but not in normal DP cells (Fig. 8, A and C). The superoxide inhibition in nanosheet-treated cancer cells using a specific superoxide inhibitor further showed that the suppressing effects of Ti0.8O2 nanosheets selectively induced superoxide generation in cancer cells but not in normal DP cells (Fig. 8, A and C). The superoxide inhibition in nanosheet-treated cancer cells using a specific superoxide inhibitor further showed that the suppressing effects of Ti0.8O2 nanosheets on CSC and EMT were superoxide anion–dependent. Consistent with our findings, a previous study had shown that selective targeting of gold nanorods to cancer cells occurred partly through ROS generation (Wang et al., 2011).

Cancer is not a singular entity but in fact comprises multiple cell lineages with different phenotypes (Salk et al., 2010). Although unraveling and understanding the heterogeneity of the disease is at an early stage, the key player in cancer aggressiveness and relapse has been identified and named as “cancer-initiating cells” or CSCs. As a seed of the whole tumor, CSCs are unique cancer cells: They generate other cell lineages to form a tumor and they remain within the tumor and secrete several factors for support (Sengupta and Cancelas, 2010). Therefore, CSCs have become one of the
principle targets in anticancer-drug discovery in recent years (Dragu et al., 2015). The meaningful impact of CSCs in various types of cancer has increasingly been reported. Like other malignant cancers, CSCs of lung cancer have been demonstrated to be crucial for cancer initiation, progression, and metastasis (Templeton et al., 2014). Perhaps more crucially, CSCs display strong resistance to chemotherapy and later on give rise to the regrowth of the cancer cells.

Because responses to anticancer drugs can be poor, the spread of tumor cells to other sites in the patient’s body and cancer relapse have been recognized as important factors accentuating the high mortality rate of lung cancer. CSC-targeted therapy and treatment strategies are urgently needed. CSCs can be recognized by their specific markers, such as ALDH1A1 and CD133, (Miyata et al., 2017) and by their cellular traits, such as colony formation and other aggressive behaviors (Yongsanguanchai et al., 2015; Tiran et al., 2017).

Recent evidence has pointed out the role of integrins, a family of extracellular-matrix transmembrane cell-adhesion proteins, that play a part in the regulation of cancer stem cell–like phenotypes and the potential for metastasis. As solid tumors come from epithelial cells, the integrins expressed by epithelial cells are biologically retained in cancer cells, though their expression patterns and levels may be changed. Most cancerous solid-tumor cells express multiple integrins that have not only been implicated in cancer cell survival, migration and invasion, and growth but also act as receptors in interaction with the tumor stroma (Weis and Cheresh, 2011).

Certain integrins—like integrins β1, β3, α5, and αv—have been associated with poor prognosis in cancer (Rout et al., 2004). We have provided the data indicating that ouabain

Fig. 8. Ti0.8O2 nanosheets target CSC and EMT in human lung cancer through superoxide anion. Effect of Ti0.8O2 nanosheets (0–1 μg/ml) on intracellular ROS induction at 24 hours in A549 and primary DP cells was determined by flow cytometry with fluorescent probes DCF (10 μM), HPF (10 μM), and DHE (10 μM) (A and C). Cell were treated with Ti0.8O2 nanosheets (0 to 1 μg/ml) alone for 24 hours or with the pretreatment of 50 μM MnTBA. Protein expression levels of CSC and EMT markers were detected by Western blot analysis (B and D). Blots were reprobed with β-actin to confirm equal loading of samples. The immunoblot signals were quantified by densitometry and the mean data from independent experiments were normalized to the results. Data are shown as the mean ± S.D. (n = 3). *P < 0.05 vs. nontreated control.
suppresses lung cancer cell migration by decreasing cellular levels of the α4, α5, αv, β3, and β4 integrins (Ninsontia and Chanvorachote, 2014). Likewise, nitric oxide, an important biologic mediator, was shown to have no effect on the increased migratory activity of lung cancer cells via AKT-mediated upregulation of the αv and β1 integrins (Saisongkorh et al., 2016). In lung cancer, integrin αvβ3 has been shown to be a potential biomarker for metastasis and overall survival, and drugs targeting integrins, such as cilengitide (the αvβ3 and αvβ5 inhibitor), are exhibiting promising activity in phase II clinical trials. Cilengitide is currently being tested in a phase III trial in patients, so attenuating these metastasis-associated integrins could be a prospective strategy for reaching improved clinical outcomes. Our results found that treatment of lung cancer cells with Ti0.8O2 nanosheets suppressed the expression of integrins αv and β1, suggesting that the additional nanosheet approach could work against cancer.

All in all, our data contributes to the emerging evidence that the Ti0.8O2 nanosheet exhibits very promising potential, owing to the selective role it plays in suppression of lung CSCs, with minimal cytotoxicity to normal stem cells. Ti0.8O2 nanosheets decreased CSC-like phenotypes and diminished the CSC markers and CSC-related transcription factors. The mechanism underlying how nanosheets decrease CSC phenotypes was found to involve the attenuation of CSC-supportive signaling such as Notch, Akt, and β-catenin. Regarding molecular mechanisms, CSCs enhance and maintain their self-renewal and differentiation activity, and CSC phenotypes, through Notch (Espinoza et al., 2013). Furthermore, Akt signaling has been shown to increase CSC phenotypes by phosphorylation at Ser9 of GSK3β, resulting in the stabilization of the CSC regulatory protein β-catenin (Valkenburg et al., 2011). Consequently, the increased level of β-catenin confers self-renewal and tumorigenic properties on cancer cells (Valkenburg et al., 2011) (Fig. 9). Here, we have shown that Ti0.8O2 nanosheet treatment attenuated CSC properties in these lung cancer cells through the suppression of Akt/GSK3β/β-catenin and Notch-1, and as a consequence, the stemness of the cells was reduced. Further, nanosheets were shown to inhibit other aggressively driven mechanisms, including the process of EMT as well as the expression of certain metastasis-associated integrins (Fig. 9). This novel finding on the role of Ti0.8O2 nanosheets in CSC regulation may have important implications in cancer management.

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