**Appendices**

**Evaluation of tumorigenic potential of CeO2 and Fe2O3 engineered nanoparticles by a human cell *in vitro* screening model**

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**Appendix A**

**Supplementary Materials and Methods**

***Characterization of ENMs***

nCeO2 and nFe2O3 were manufactured in-house at Harvard University via flame spray pyrolysis using the Harvard Versatile Engineered Nanomaterial Generating System (VENGES) developed by the authors (Demokritou et al. 2012, Sotiriou et al. 2012, Gass et al. 2013). For powdered ENMs, the specific surface area (SSA; m2/g), was determined by the nitrogen adsorption/Brunauer-Emmett-Teller method using a Micrometrics Tristar 3000 (Micrometrics, Norcross, GA, USA) after sample degassing for 1 hour at 150°C in nitrogen. BET equivalent primary particle size was calculated, under a spherical particle assumption, using dBET = 6000/(ρ × SSA), where ρ is the material density. MWCNT Mitsui #7 (Hodogaya Chemical) have previously undergone thorough characterization (Porter et al. 2010),and are known to promote lung adenocarcinoma and serosal lesions consistent with the diagnosis of sarcomatous mesothelioma *in vivo* (Sargent et al. 2014). All materials were characterized using x-ray diffraction diameter, density, and FESEM (Demokritou 2010, Sotiriou et al. 2012, Demokritou et al. 2013, Gass et al. 2013).

***ENM Dispersal, Characterization in Suspension, and Dosimetric Considerations for In Vitro Testing***

Stock solutions of NMOs (0.1 mg/ml) were prepared by dilution of 1 mg/ml stock solutions with sterile MilliQ water. NMO solutions were sonicated in water, while 0.1 mg/ml MWCNT solutions (1 mg/ml) were sonicated in 1.5% v/v bovine serum albumin (BSA) dispersant solution using a SonicCell cup sonicator. Sonication duration was based on the critical delivered sonication energy required for each ENM to achieve a uniform dispersed suspension (Cohen et al. 2014). Immediately following sonication, each dispersed ENM was serially diluted with small airway epithelial cell growth medium (SAGM) to the reported concentrations and characterized for intensity-weighted hydrodynamic diameter (dH), polydispersity index (PdI), zeta potential (ζ), and specific conductance (σ) by dynamic light scattering (DLS) following established protocols (Cohen et al. 2013). The effective density (ρagg) of the formed agglomerates, which plays an important role in the settling and dosimetry *in vitro*, was also measured using the recently developed Volumetric Centrifugation Method (VCM) (DeLoid et al., 2014).

***Dosimetric Considerations for In Vitro Testing***

The authors’ recently developed multi-step, integrated *in vitro* dosimetric platform has been utilized to estimate the delivered cellular dose as a function of time (Cohen et al., 2014, Cohen et al., 2015). In summary, step 1 includes the ENM suspension preparation and characterization as described above (step 1) followed by the use of advanced fate and transport modeling to estimate the deliver to cell dose metrics as a function of exposure time (step 2). The recently developed distorted grid (DG) fate and transport model was utilized (DeLoid et al., 2015) to calculate the concentration profiles across the well (of 96- and 6-well plates) and the fraction of administered particles deposited to the cell surface as a function of time, *f*D(*t*), for test ENMs suspensions. The agglomerate volume-weighted hydrodynamic diameter, *d*H, and the effective density of particle agglomerates suspended in cell culture media, as measured by VCM, were used as inputs to the DG model to calculate the fate and transport of test ENM suspensions. For each ENM the model-derived *f*D(*t*) was fit to the following equation: $f\_{D}\left(t\right)= α\_{1} (1-e^{-α\_{2}t})$.

Relevant *In Vitro* Dosimetry (RID) functions were calculated for both particle suspension in a 6- and 96-well plate experimental condition as described in [Cohen et al. (2014)](#_ENREF_9" \o "Cohen, 2014 #431). In brief, the RID functions were derived from the total mass administered (M), total surface area dose (SA) and total particle number dose (N) by using the equations 1 through 3, as follows:

1. ; where *M*(μg) is the total mass dose, *V* is the volume of exposure media (ml) applied directly to the cells in culture and *γ* (μg/ml) is the mass concentration of the ENM suspension.
2. ; where *N(#)* is the total particle number dose, *rH* (cm) is the hydrodynamic radius, and *ρE*, (g/cm3) is the agglomerate effective density.
3. ; where *SA*(cm2) is the total surface area dose.

Once these three metrics have been calculated, the RID functions for delivered dose metrics can now be computed using equations 4 through 6 and using as input the material-media specific parameters obtained from the fate and transport algorithm (*α*, deposition constant and *t*, exposure duration), as follows:

1. Delivered to cell mass (RIDM, μg): 
2. Delivered to cell particle number (RIDN, number of particles): 

(6) Delivered to cell surface area (RIDSA, cm2):  It is worth noting that these RID functions are material and media dependent based on the conditions used here and cannot be used for other conditions. For the latter, the two step approach has to be re-run in order to derive new RID functions.

***ENM Uptake and Cellular Localization***

Uptake and localization analysis of ENM in pSAECs (Lonza) was performed using enhanced darkfield imaging (CytoViva) following a 24 h exposure, as previously described (Wang et al. 2014). Briefly, pSAECs were seeded onto conditioned coverslips in a 24-well plate, and exposed in duplicate to administered doses of either dispersed 0.06 µg/cm2 NMO or 0.06 µg/cm2 MWCNT for 24 h. Next, cells were PBS rinsed, fixed in 10% neutral-buffered formalin, stained with 1% toluidine blue, mounted onto particle-free glass coverslips, and imaged on an Olympus BX-41 scope with a high resolution Dage Excel digital camera (Michigan City, IN). Experiments were independently performed three times. ENM intracellular localization was assessed via transmission electron microscopy (TEM) using previously described methods (Snyder-Talkington et al. 2013, Ma et al. 2011). Briefly, pSAECs (1x106) were seeded in duplicate in 60 mm plates overnight, exposed to all ENMs (0.6 µg/cm2) for 24 h, harvested in ice-cold PBS, fixed, embedded, dehydrated,, stained with uranyl acetate/lead citrate, sectioned (70 nm), and examined with TEM (JEOL 1220, Tokyo).

***Cancer Cell Hallmark Assessment***

***Cell proliferation.*** Since carcinogenesis is a multistep process in which exposed cells display enhanced cancer cell-like behaviors, ENM-exposed pSAECs were assessed for several different cancer cell hallmark phenotypes at 6 and 10 weeks of exposure duration following methods previously described (Wang et al. 2014). Following cessation of ENM exposure, cells were held in particle free growth medium for 3 days prior to cancer cell hallmark assessment. First, pSAECs, from both ENM-treated and controls, were seeded (5x103/well) in sextuplet to a 96-well plate, incubated in particle-free culture medium for 24 – 72 hours. Mitochondrial metabolism, a marker for cell proliferation, was determined by WST-1 (Roche) spectrophotometric assay. At each time point, cells were incubated with WST-1 (Roche) for 3 h followed by absorbance determination at 450 and 650 nm according to the manufacturer’s protocol. To assess transient *vs.* persistent effects on proliferation ability, 10 week exposed cells were cultured for 8 days post-exposure in particle-free medium and then assayed for WST-1 metabolism. In addition, reverse phase contrast cell photographs were taken using an inverted Olympus scope at 48 h post-seeding (5x104) in 6-well plates to qualify cell proliferation and gross morphology comparison.

***Invasion.*** Treated pSAECs, in duplicate per particle treatment, were suspended in normal SAGM or growth factor-free medium, seeded (3x104 cells) into the upper chamber of a Matrigel® invasion inserts (BD Biosciences), and then immediately placed into a well containing SAGM growth media supplemented with 5% FBS as a chemoattractant for 48 h. Next, attached cells on the underside of insert were fixed, stained, and dried via DiffQuik protocol, and photographed using bright field microscopy. Cell counts per field of view were performed on five replicate photos per insert. Four independent experiments were conducted and results were pooled for statistical analyses.

***Soft agar colony formation***. To ascertain attachment-independent growth ability, exposed pSAEC cells (4.3x104) were mixed 1:2 in agar/media containing Difco agar, 15% FBS, 2x minimal MEM medium, Clonetics Growth Supplements and 1% gentamicin. Suspended cells (3x104) were slowly layered onto cooled agar/media in triplicate 6-well plates and allowed to solidify. Colonies from three independent experiments were photographed using dark field inverted microscopy at 14 d and 21 d. Colonies ≥ 50 µm in diameter from five replicate photographs per well were counted.

***Morphological Transformation***

Exposed pSAECs were assessed for morphological transformation with the a) colony forming efficiency and b) cell transformation assays using previously described methods with modifications (Wang et al. 2014). First, 6 and 10 week exposed pSAECs were plated at 1 x 103 cells in triplicate per 60 mm dishes in growth medium and cultured for 7 days with a medium change on Day 4. Next, cells were briefly washed in warm PBS, fixed in 10% buffered formalin, stained with 1% crystal violet, triplicate rinsed, and dried. Colony forming unit counts were performed using an AccuCount 1000 (BioLogics) and serves as a quantitative measure of cytotoxicity and ability to survive without significant cell-to-cell signaling (Ponti et al. 2010). Simultaneously, the exposed pSAECs were evaluated for cell transformation frequency. Cells were plated at 1 x 105 cells per 60 mm plate and cultured for 2 weeks with medium changes every 3 days with no passaging. Next, cells were rinsed and held for 7 days in basal medium with no growth factors. Cells were held for 7 days with a medium change every 2 days. Cells were then rinsed, fixed, stained following the above procedure, and qualitatively scored for foci (Type I – III) using previously described scoring criteria (Sasaki et al. 2012).

***Clonal Expansion and Cancer Hallmark Assessment***

 To further evaluate neoplastic transformation in ENM-exposed pSAECs, 10 to 12 soft agar colonies were removed from 10 week exposed treatments, re-established in culture, and evaluated for several cancer hallmarks. Briefly, a small core of agar containing each colony was removed from suspension using the tip of a sterile 1 ml serological pipette and then transferred to a 48-well plate containing fresh SAGM. Colonies were cultured with medium changes every 3 days until clonal cells from the colony reattached to plate bottom and reached >50% confluence. Clonal cells were further expanded and cultured in 24-well and 6-well plates for cancer cell hallmark screening.

 nFe2O3 and MWCNT clones were evaluated for cell proliferation, invasion, and soft agar colony formation as previously described. In addition, apoptosis resistance via either the intrinsic or extrinsic pathway was evaluated by conducting dose-response studies using tumor necrosis factor (TNF-α) and FAS ligand (FasL). Briefly, cells exposed to nFe2O3 for 10 weeks, nFe2O3 clones, and MWCNT clones (1 x104) were plated in triplicate to black, clear bottom 96 well plates (Corning) and exposed to 0 – 50 ng/ml TNF-α, 0 – 30 µM antimycin-α, and 0 – 50 ng/ml FasL for 24 h according to previously described methods (Pongrakhannon et al. 2015). Since unexposed long-term passaged or colony clones of pSAECs were unavailable, SAEC-hTERTs were used as appropriate negative controls. Next, cells were stained with a fluorescent dye cocktail containing Hoescht 33342 (1 µM) and propidium iodide (PI; 10 µg/ml) to evaluate live, apoptotic and necrotic cells. Next, high content imaging was conducted using the ImageXpress Micro XLS system (IMX; Molecular Devices, LLC) at 10x magnification with 4 replicate photos per well. Images were analyzed and quantified using the Multiwavelength Application Model for live, apoptotic and necrotic cells. Data were exported to Excel for further statistical analysis.

***Western Blot analysis of key proteins for iron homeostasis***

 pSAECs and SAEC-hTERTs (5 x105 cells) were plated in the wells of a 6-well plate overnight then exposed to an ENM particle containing medium for 24 or 48 h. Sub-chronic exposed cells were plated at the same density and cultured for either 24 or 48 h. Protein was isolated from all cells following previously described methods (Mishra et al. 2015). Briefly, washed cells were incubated on ice in lysis buffer (Invitrogen) containing PMSF and a protease inhibitor cocktail (Roche Diagnostics) for 30 minutes, scraped off well plates, and briefly sonicated. Collected supernatants were assayed for total protein content using the BCA method with absorbance for each sample measured at 562 nm on a multi-wall plate spectrophotometer (Molecular Devices). Samples were stored at -80 °C until assayed. Samples with equal protein concentrations were loaded into 10% bis-Tris acrylamide gels for electrophoresis separation (Bio-Rad) and then transferred to nitrocellulose membranes. Membranes were blocked in 0.5% non-fat dry milk with Tris-buffered saline with 0.1% Tween (TBS-T) for 1 hour. Primary CD71, FTH1, GAPDH (Cell Signaling) and SCL40A1 (Abcam) antibodies were diluted 1:1000 in blocking buffer while less sensitive rabbit polyclonal DMT1 (Abcam) was diluted to 1:300. Using the SNAP i.d. vacuum manifold apparatus (Millipore), membranes were incubated with each primary antibody twice for 10 minutes, thoroughly rinsed 3x with TBS-T, and incubated with secondary anti-rabbit (sc-2004) or anti-mouse (sc-2005, Santa Cruz Biotechnology) HRP-conjugated antibody for 10 minutes. Following rinsing, membranes were incubated for 5 minutes in chemiluminescent substrate, exposed to X-ray film, and developed. Densitometry was performed on protein bands using ImageJ software (NIH).

***Intracellular iron assessment***

 SAEC-hTERTs (2 x106) were plated in triplicate to 10 cm2 culture plates. Cells were then exposed to administered doses of either 0, 0.06, or 0.6 µg/cm2 of each ENM for 48 h. Following exposure, intracellular iron was assessed using the Iron Assay kit according to the manufacturer’s instructions (Sigma Aldrich). Low passage, unexposed pSAECs and sub-chronic-exposed pSAECs were also assessed for iron content. Samples with equal mass ENM without cells were used as particle controls. Briefly, cells were , collected via trypsinization, triple washed, counted, and pelleted, Next, cells were quickly lysed with 100 µl of cold iron assay buffer using micropipette and brief sonication, and then centrifuged at 13,000g for 10 minutes to remove cellular debris, including ENM which were highly visible in the post-centrifuge pellet. Supernatants were split in half to two aliquots, diluted 1:1 v/v with assay buffer, and added in triplicate to a 96-well plate. Iron reducing reagent was added to one aliquot while equal volume of buffer was added to the other aliquot to determine total and ferrous iron, respectively, followed by incubation for 30 minutes. Lastly, samples were incubated with an iron probe for 60 m and absorbance assayed at 593 nm. Absorbance values were corrected from background and PBS buffer controls, then normalized for live cell count (2x106) due to ENM toxicity at 6 µg/cm2 in some treatments.

**Appendix B**

**Figure B.1.** Uptake evaluation of nanometal oxides (NMOs) and MWCNTs in pSAECs using enhanced dark field imaging (CytoViva). Cells plated on glass coverslips were exposed for 48 h to deposited doses of 0.018 or 0.18 µg/cm2 of each NMO, fixed, stained with toluidine blue, and imaged. Cells were exposed to 0.06 µg/cm2 of MWCNT. A) Every nanomaterial co-localized with cells while B) nanometal oxides co-localized with the cytoplasm near the nucleus. White arrows indicate co-localization of MWCNT with cell nuclei.

**Figure B.2.** Transmission electron microscopy imaging of MWCNT uptake in human SAECs. A) MWCNTs were observed to either undergo phagocytosis (black arrows) or puncture the cell plasma membrane (white arrows). B) and C) MWCNTs were observed intracellularly within (black) and puncturing (white) membrane-bound vesicles.

**Figure B.3.** Invasion ability of nanometal oxide-exposed pSAECs at 6 week exposure. Cells were suspended in serum-free medium, seeded at a density of 3x104 cells in upper chamber of a Matrigel Transwell insert, and placed into a 24-well plate containing full growth medium with 5% FBS. Cells were allowed to invade to the bottom side of membrane for 48 h. Cells were fixed, stained, photographed, and counted (n=9). \* and # indicate treatments that were statistically different from saline control or albumin control, respectively (p ≤ 0.05).

**Figure B.4.** Abnormal polynucleated “giant” cells in 6 week MWCNT-exposed pSAECs. A) Black arrows point to the location of “giant” cells. B) and C) Enhanced magnification of representative ‘giant cells’.

**Figure B.5.** Establishment of clones from soft agar colonies following 10 week nFe2O3 exposure to human primary small airway epithelial cells. A) Single colony isolated from soft agar in 48-well plate. B) Loss of colony morphology in favor of attached cells with C) invasive margins.

**Figure B.6.** Superoxide production after 1 h exposure in pSAECs. Cells were co-stained with Hoescht 33342 and MitoSox Red 15 minutes prior to high content imaging.

**Figure B.7.** Reactive oxygen species after 24 h exposure in pSAECs. Cells were co-stained with Hoescht 33342 and DCF for 15 minutes, rinsed with warm PBS, and imaged in fresh PBS.

**Figure B.8.** Live cell proliferation at A) 24 h and B) 48 h of pSAECs following acute exposure to nCeO2 and nFe2O3. Cells were seeded in 96-well plates overnight and exposed to well-dispersed nanometal oxides. Cells were stained with Hoescht 33342 and propidium iodide at each time point, imaged and quantified on a high content imaging system. \* indicate significant difference compared to unexposed cells (p<0.05).

**FigureB.9.** A) and B) Ferritin (FTH1) and C) ferroportin (SLC40A1) exhibited no significant expression change following acute exposure to NMOs in SAEC-hTERTs (p > 0.05).