

A Toxicogenomic Assessment of Primary Human Cells Exposed to Single-Walled Carbon Nanotubes



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TOXICOGENOMICS

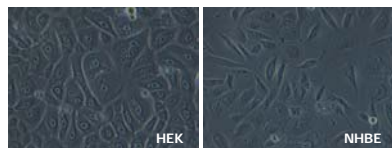
Abstract

Engineered nanomaterials are characterized as having at least one dimension of 1 – 100 nm. Early reports show contradicting results as to the toxicity of unmodified single-walled carbon nanotubes (SWNT). Here, an in vitro toxicogenomics approach is used to assess the toxicity of these nanomaterials. Human epidermal keratinocytes (HEK) and normal human bronchial epithelial (NHBE) cells were cultured for 24 hours with cytotoxic doses of SWNT, silica (SiO₂), and carbonyl iron (CI). Cells from individual treatments were harvested at each time point and snap frozen to -80° C. Total ribonucleic acid (RNA) was isolated from these cell pellets and complementary ribonucleic acid (cRNA) probes were synthesized and hybridized against gene expression microarrays. All microarray experiments were done in triplicate. To compare expression profiles from the two cell systems, only the 3,464 common unique genes were considered in this work. Gene expression profiles of significantly expressed genes (2-fold change or greater) from HEK cells were nearly 1 – 2 orders of magnitude greater than that of the NHBE cells. Using hierarchical clustering and principal components analysis (PCA), the largest variation in the gene expression values were between the skin and lung cells, regardless of nanomaterial treatment. Also, PCA showed profiles from SWNT exposure to be similar to untreated samples for both cell systems. A cytotoxic exposure with CI showed highest gene activity in the HEK cells while treatment with SiO₂ gave the highest activity in the NHBE cells. HEK cells treated with SWNT showed a 4-fold increase in expression for the gene that encodes for human IL-8. This is in agreement with previous citations for SWNT exposure. Two potential biomarkers (NAT10 and ZHX2) have been identified for SiO₂ exposure.

Introduction

Single-walled carbon nanotubes are engineered materials which have at least one dimension measuring < 100 nm [1]. These materials possess enhanced properties due their small size and large surface to volume ratios. They have several applications: semiconducting electronics, energy, composite materials, aerospace, chemical and biological sensors, drug delivery agents and other medical applications, etc. The increased use of such nanomaterials in consumer products and medical applications has called for a concern for any adverse effects it may have on humans and the environment. The current results available have conflicting and contradicting views about the adverse effects of SWNT [2,3]. To date, little is known about the molecular and cellular mechanism of cytotoxicity of this class of nanomaterials [4,5]. In this work we compare the gene expression profiles from two different primary human cell lines.

Materials and Methods



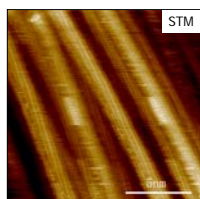
Randomly proliferating cells are treated for 24 h at their pre-confluency state with a cytotoxic dose of **SiO₂**, **SWNT**, and **CI**. The total RNA is isolated from the cells post treatment, its complementary cRNA's made, labeled and hybridized onto GE CodeLink microarrays. Each biological sample is run on triplicate arrays for good statistical analysis. Every sample also has a time matched 0 h control for computing expression fold changes

- GE CodeLink arrays (single color)
- Uniset Human I Bioarray (for HEK expression)
- Uniset Human 20K I Bioarray (for NHBE expression)

$$\text{Magnitude of study} = \left\{ \begin{array}{l} \{3 \text{ compounds} + \text{Untreated}\} \times \{2 \text{ time points}\} \\ \times \{ \text{Triplicate arrays} \} \times \{2 \text{ Cell systems}\} \end{array} \right\} = 48 \text{ arrays}$$

Single-Walled Carbon Nanotubes

Image & Spectrofluorimetric Analysis



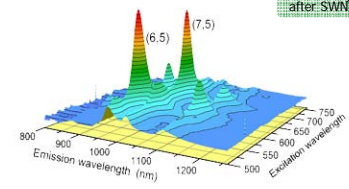
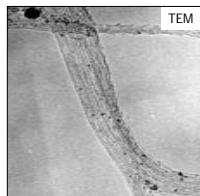
Unique CoMoCAT method of manufacturing SWNT

- CO disproportionation on a silica supported catalyst containing low Co:Mo proportion at 700- 950 °C in a tubular fluidized bed reactor
- SWNT produced is of high quality, high specificity and very narrow size distribution

Elemental Analysis

Element	Percentage
C	92.31
O	7.07
N	0.05
Si	0.05
Co	0.04
Mo	0.48

Low heavy metal carry over after SWNT purification



SWNT from CoMoCAT process shows only 2 types of semiconducting nanotubes (6,5) and (7,5) while HiPCO process shows several different populations

Gene Expression Data Preprocessing

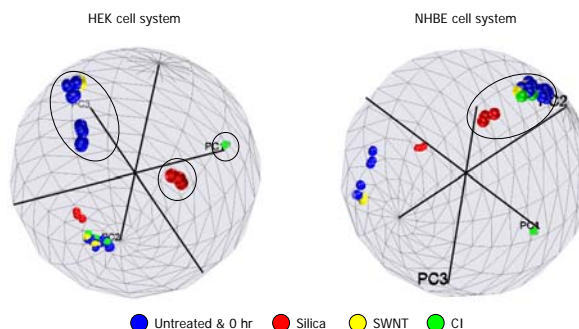
- 10K Bioarray → 9,970 **discovery** genes + positive, negative and fiducial
- 20K Bioarray → 20,012 **discovery** genes + positive, negative and fiducial
- Common genes between the two cell systems → 3,464
- Quality control → genes marked *G* (good intensity signal)
- Signal intensity → **median normalization** on each array
- Process variability → validated with **triplicates** for each sample/treatment
- Common Discovery genes with *G* tag across both cell systems = **3,464**

Statistical Analysis

Treatment	% CV	
	HEK	NHBE
0 hr CI	3.69	6.19
0 hr Silica	3.59	5.61
0 hr SWNT	4.73	5.37
0 hr Untreated	3.84	5.45
24 hr CI	4.35	7.85
24 hr Silica	3.61	5.50
24 hr SWNT	5.93	6.41
24 hr Untreated	3.89	5.92

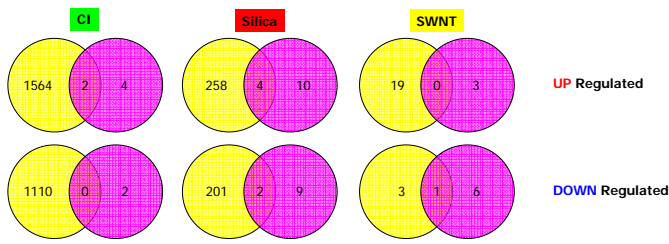
Low % CV → low process variability → maximize biological variability

Principal Component Analysis



- PCA an unweighted way to look at data for similarities in expression profiles
- Maximum difference is observed between the two tissue types and not between treatments
- SWNT in both HEK and NHBE has an expression profile similar to the untreated samples

Significant Gene Expression



GEP of CI >> GEP of Silica >> GEP of SWNT in HEK

Common Significantly Expressed Genes

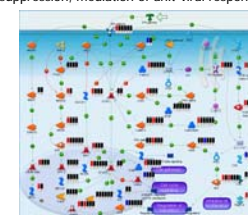
Gene Symbol	Fold Change		Treatment	Function and Processes
	HEK	NHBE		
CLDN4	4.06	2.04	Silica	identical protein binding, structural molecule activity
CRCT1	2.45	2.69	Silica	protein binding
IL8	2.66	3.47	Silica	chemokine activity, immune and inflammatory response,
RHCG	2.68	2.11	Silica	ammonium transport, epithelial cell differentiation
SFRP1	0.19	0.49	Silica	WNT signaling pathway, anti-apoptosis, cell differentiation
TGFB1	0.07	0.36	Silica	cell growth and apoptosis, inflammatory response
DDIT3	3.39	3.59	CI	regulation of apoptosis, response to DNA damage stimulus
AQP3	3.32	2.25	CI	transporter activity, excretion
CXCL14	2.89	5.61	SWNT	immunoregulatory and inflammatory processes

Significant Pathways for Particulate Exposure

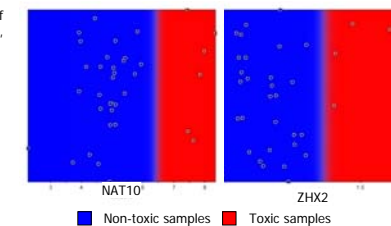
MAP	Map Folders	Cell Process	p-Value	Genes
Ligand-Dependent Transcription of Retinoid-Target genes	Cell signaling/Regulation of transcription Function groups / Transcription factors	transcription, transcription	1.66E-09	85 129
Methionine-cysteine-glutamate metabolism	Metabolic maps / Aminoacid metabolism		6.19E-08	18 18
Insulin regulation of the protein synthesis	Cell signaling / Translation regulation Function groups / Hormones	translation, response to hormone stimulus	7.29E-07	40 55
IFN gamma signaling pathway	Cell signaling / Immune response Function groups / Cyto / chemokines	cytokine and chemokine mediated signaling pathway, immune response	1.29E-06	44 63

Pathway Analysis

Interferon-gamma Signaling: Induces the transcription of IFN-stimulated genes causing inhibition of cell proliferation, tumor suppression, mediation of anti-viral responses



Biomarker Identification



NAT10 – N-acetyltransferase 10; influences activity of histone acetylation and could up-regulate telomerase activity
ZHX2 – Zinc fingers and homeobox 2; major transcriptional mediators of podocyte disease

Temperature bars: RED = UP regulation; BLUE = DOWN regulation
⊕ & ⊙: HEK, NHBE
⊕ & ⊙: HEK, NHBE
⊕ & ⊙: HEK, NHBE – SWNT

Conclusions

- Gene expression microarrays can be used as a tool for screening particulate toxicity
- Expression profiles of SWNT more similar to Untreated samples, therefore it seems to have very little adverse effect on cells
- GEP of HEK and NHBE completely different
- Maximum difference observed between tissue systems rather than treatments
- A comparative study between different tissues is necessary to globally integrate them via a systems biology approach

References & Acknowledgements

[1] Bachilo, S.M., et al. *J. Am. Chem. Soc.* (2003) 125:11186-7; [2] Warheit, D.B., et al. *Toxicol. Sci.* (2004) 77(1):117-25; [3] Lam, C.W. et al. *Toxicol. Sci.* (2004) 77(1), 126-34; [4] Ding, L., et al. *Nano Lett.* (2005) 5(12):2448-64; [5] Cui, D. et al. *Toxicol. Lett.* (2005) 155(1):73-85
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