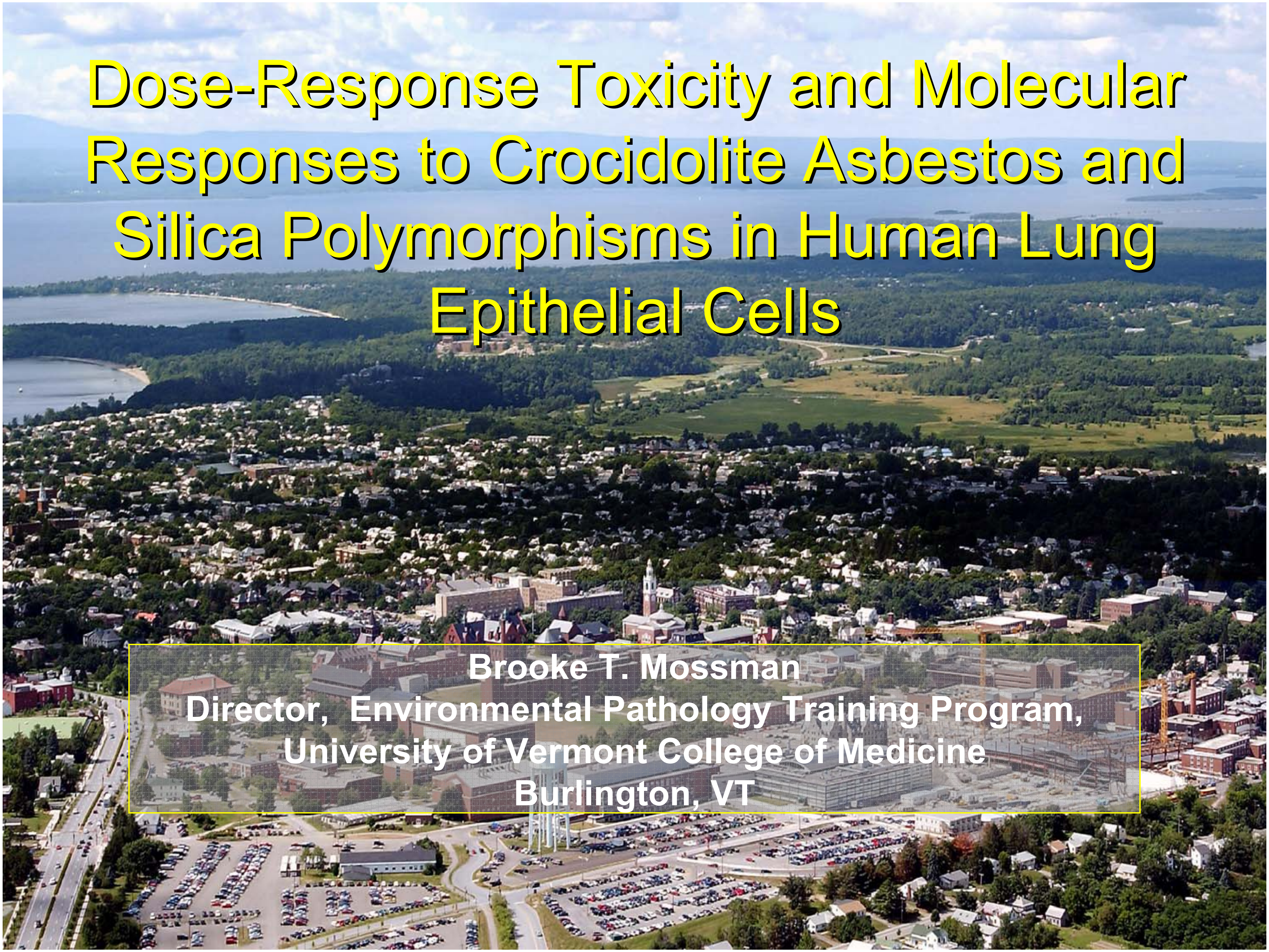

Dose-Response Toxicity and Molecular Responses to Crocidolite Asbestos and Silica Polymorphisms in Human Lung Epithelial Cells

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Outline

- Background
  - Environmental Toxicants (Asbestos and Silica)
  - Silica
    - What is it? Exposure? Health Risks?
  - Silicosis
    - Current mechanisms
- Data
  - Comparative Gene Expression Profiling
    - A model for *in vitro* testing of potentially harmful particulates?

Environmental Toxicants

I. Gases
- Nitrogen Dioxide (NO_2)
- Sulfur Dioxide (SO_2)
- Ozone (O_3)

II. Insoluble Particles and Fibers
- **Asbestos, Silica**
- Airborne Particulate Matter (PM)
- Coal Mine Dust (CMD)

III. Mixtures
- Tobacco Smoke
- Fossil Fuel Exhaust, Fly Ash (ROFA)
- Diesel Exhaust

Relevant Publications (1)

- Shukla A, MacPherson MB, Hillegass J, Ramos-Nino ME, Alexeeva V, Vacek PM, Bond JP, Pass HI, Steele C, Mossman BT: Alterations in gene expression in human mesothelial cells correlate with mineral pathogenicity. *Am J Respir Cell Mol Biol*. **2009** Jul;41(1):114-23.

- Hillegass JM, Shukla A, MacPherson MB, Bond JP, Steele C, Mossman BT: Utilization of gene profiling and proteomics to determine mineral pathogenicity in a human mesothelial cell line (LP9/TERT-1). *J Toxicol Environ Health A*. **2010**;73(5):423-36.

- Hillegass JM, Shukla A, MacPherson MB, Lathrop SA, Alexeeva V, Perkins TN, van der Vliet A, Vacek PM, Gunter ME, Mossman BT: Mechanisms of oxidative stress and alterations in gene expression by Libby six-mix in human mesothelial cells. *Part Fibre Toxicol*. **2010** Sep 11;7:26.

Relevant Publications (2)

- Sabo-Attwood T, Ramos-Nino ME, Eugenia-Ariza M, Macpherson MB, Butnor KJ, Vacek PC, McGee SP, Clark JC, Steele C, Mossman BT: Osteopontin modulates inflammation, mucin production, and gene expression signatures after inhalation of asbestos in a murine model of fibrosis. *Am J Pathol*. **2011** May;178(5):1975-85.

- Perkins TN, Shukla A, Peeters PM, Steinbacher JL, Landry CC, Lathrop SA, Steele C, Reynaert NL, Wouters EF, Mossman BT: Differences in Gene Expression and Cytokine Production by Crystalline vs. Amorphous Silica in Human Lung Epithelial Cells. *Part Fibre Toxicol*. **2012** Feb 2;9(1):6.

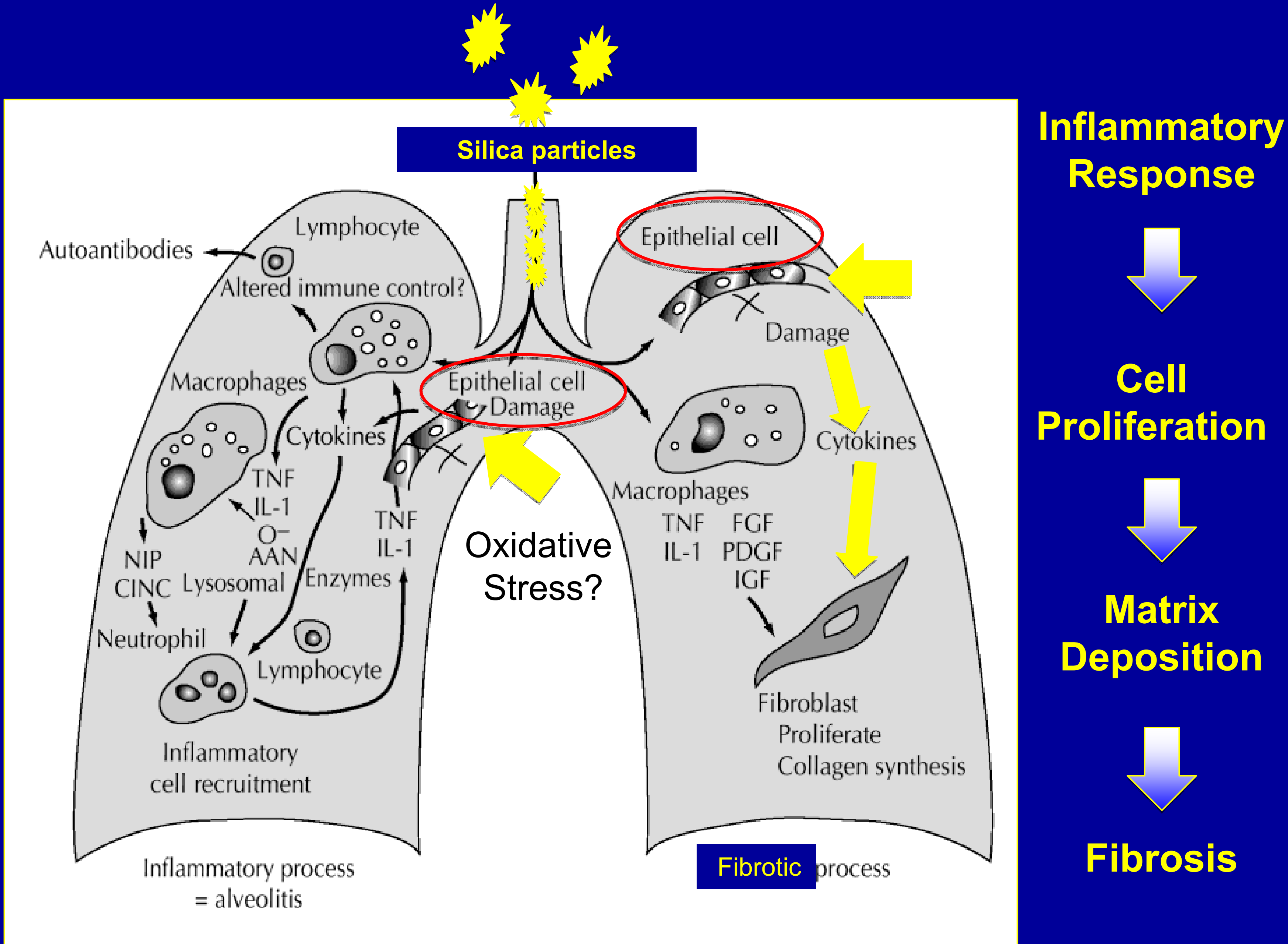
Silica

- ~ 75% of the Earth's crust
- Silicon Dioxide (SiO_2)
- Crystalline (ordered) or amorphous (disordered)
  - Composed of SiO_4 tetrahedral frameworks

- 7 **Crystalline** silica polymorphs; **3** of possible health concern:
  - *Quartz* – granite, sandstones, other common rock types
  - *Cristobalite, Tridymite* – Volcanic rocks (obsidian), opal-CT, flux-calcined diatomaceous earth
- **Amorphous** silica
  - Diatomaceous earth, silica glass, fiberglass, some volcanic glasses, opal-A, synthetic (technology, medicine, etc.)

Health Risks

- Occupational exposure to **crystalline** silica is associated with:
  - o **Silicosis (Pulmonary Fibrosis)**
    - Acute
    - Accelerated
    - Chronic
    - Conglomerate → PMF (*Progressive Massive Fibrosis*)
    - Silico-tuberculosis (increased risk of infection)
  - o Lung Cancer
    - o Crystalline silica classified as Class I carcinogen (IARC 1997)
    - o Highest risk in smokers
  - o Immuno-modulatory Disorders
    - o Rheumatoid Arthritis
    - o Systemic Lupus
    - o Scleroderma



(Adapted from Fujimura, N. *Curr. Opin. Pulm. Med.*, 2000)

Hypothesis

We hypothesized that human lung epithelial cells would exhibit dose-related unique alterations in gene expression and/or cytokine/chemokine release in response to crystalline vs. amorphous silica. These patterns might reveal genes and protein products crucial to initiation and development of silicosis.

Specific Aims

1. Determine the effects of cristobalite and amorphous silica on the viability of human lung epithelial cells (immortalized BEAS 2B cell line), using asbestos fibers as a positive toxic control.

2. Determine gene expression and cytokine/chemokine secretion profiles of BEAS 2B exposed to silica particles.

3. To confirm results of gene expression studies in cultures of primary, normal human bronchial epithelial cells (NHBE).

Silica Particle/Asbestos Fiber Characterization

Particle	Chemical Composition	S.A. (m^2/g)[a]	Mean size (μm)[b]	Source
Cristobalite	SiO_2	5.1	1.5 ± 2.6	C&E Mineral Corp, King of Prussia, PA
Amorphous	SiO_2	2.2	3.5 ± 0.3	University of Vermont, Dept. of Chemistry
Crocidolite	$Na_2Fe^{2+}3Fe^{3+}{}_2Si_8O_{22}(OH)_2$	14.97	7.4 x 0.25*	NIEHS Reference Sample

[a] S.A. = Surface area/ mass (m^2/g)
[b] Mean particle diameter ± geometric standard deviation
* Mean Fiber Dimensions (μm)

- Amorphous silica particles were synthesized by *J. Steinbacher,* by Hydrolysis/Condensation of Tetraethoxysilane. (Adapted from: De, G. et al 2000).

- Cristobalite silica particles were previously characterized (Janssen et al 1992) courtesy of K. BeruBe.

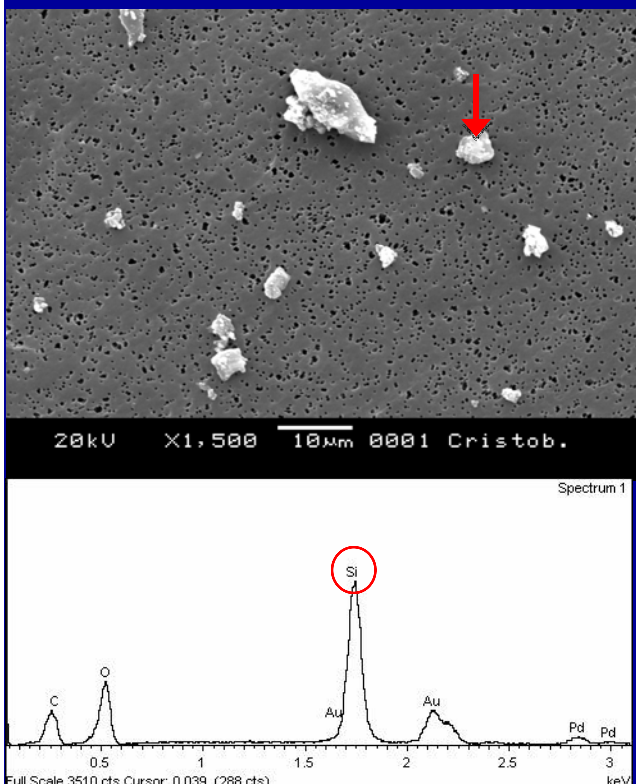
- Crocidolite asbestos fibers (NIEHS reference samples) were also previously described (Campbell et al 1980).

Surface areas were determined by BET nitrogen adsorption analysis. (*J. Steinbacher)*

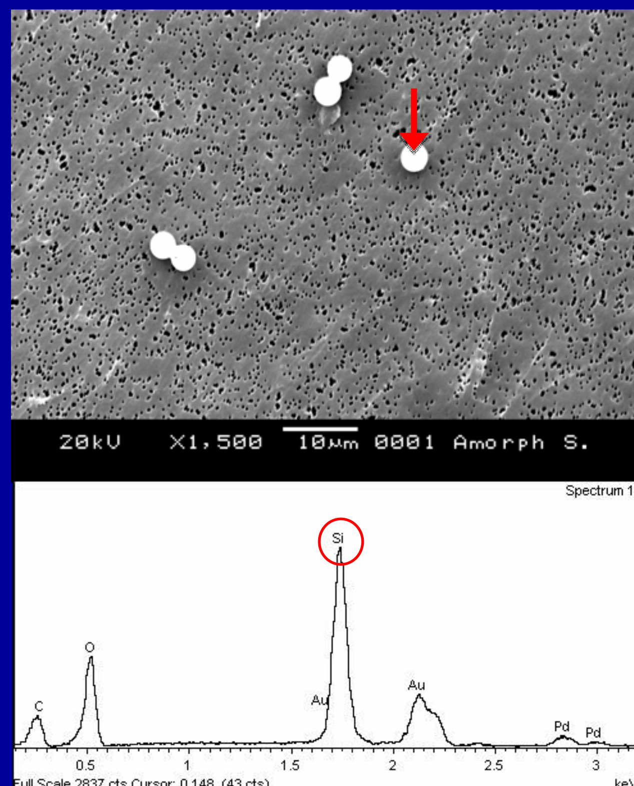
Perkins *et al*. *Particle and Fiber Toxicology* 2012, 9:6

Particle/Fiber Detection

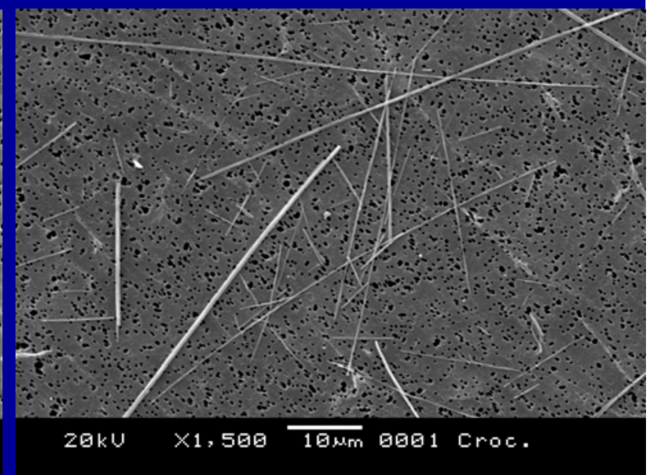
A. Cristobalite Silica



B. Amorphous Silica



C. Crocidolite Asbestos



Unpublished Data

SEM/EDS: S.L. Macura

Study Design

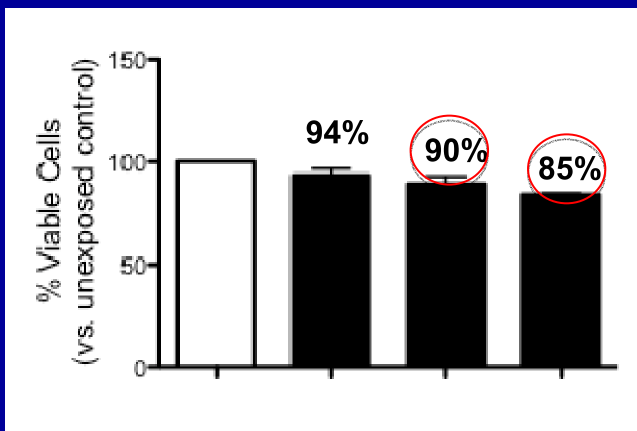
- BEAS 2B (Ad12-SV40 hybrid immortalized) or normal human bronchial epithelial cells (NHBE) were exposed to:
    - Cristobalite silica
    - Amorphous silica
    - Crocidolite asbestos
        - 3 S.A. concentrations (15, 75, and 150 $x10^6 \mu m^2/cm^2$) – 24 hours

- N = 3 for each exposure group and controls, in 3 independent experiments

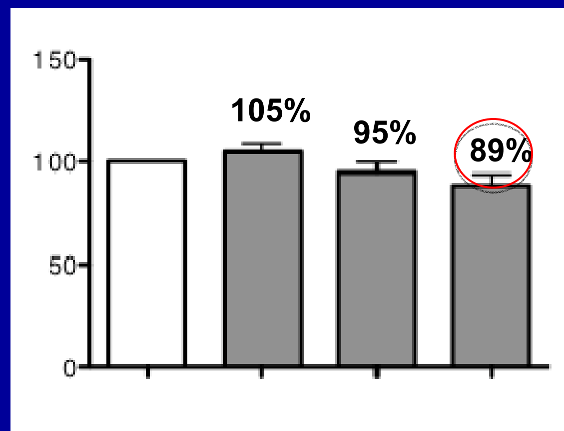
- Viability of cells post-exposure was determined by Trypan blue exclusion assay.

Assessment of BEAS 2B viability after exposure to particles/fibers (24 h)

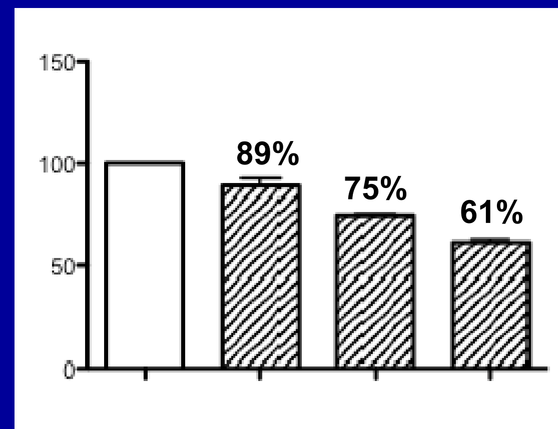
A. Cristobalite



B. Amorphous



C. Crocidolite



	A				B				C			
[A]Surface Area:	Control	15	75	150	Control	15	75	150	Control	15	75	150
[B]Mass:	Control	2.9	14.7	29.4	Control	6.8	34.1	68.2	Control	1.0	5.0	10.0

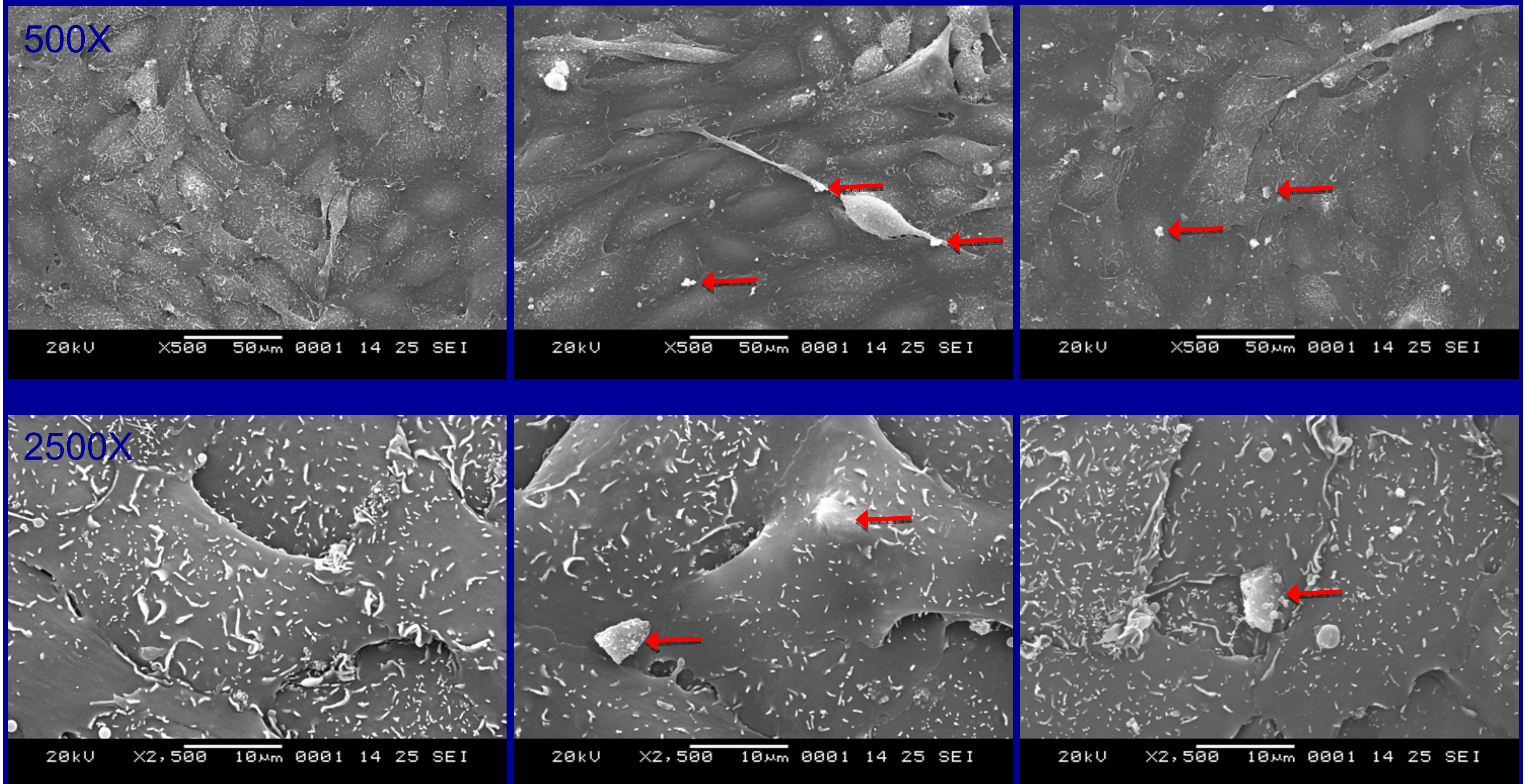
[A] ($x10^6 \mu m^2/cm^2$)

[B] ($\mu g/cm^2$)

A and B: Perkins *et al*. *Particle and Fiber Toxicology* 2012, 9:6 **C: Unpublished Data**

Particle-cell interactions imaged by SEM

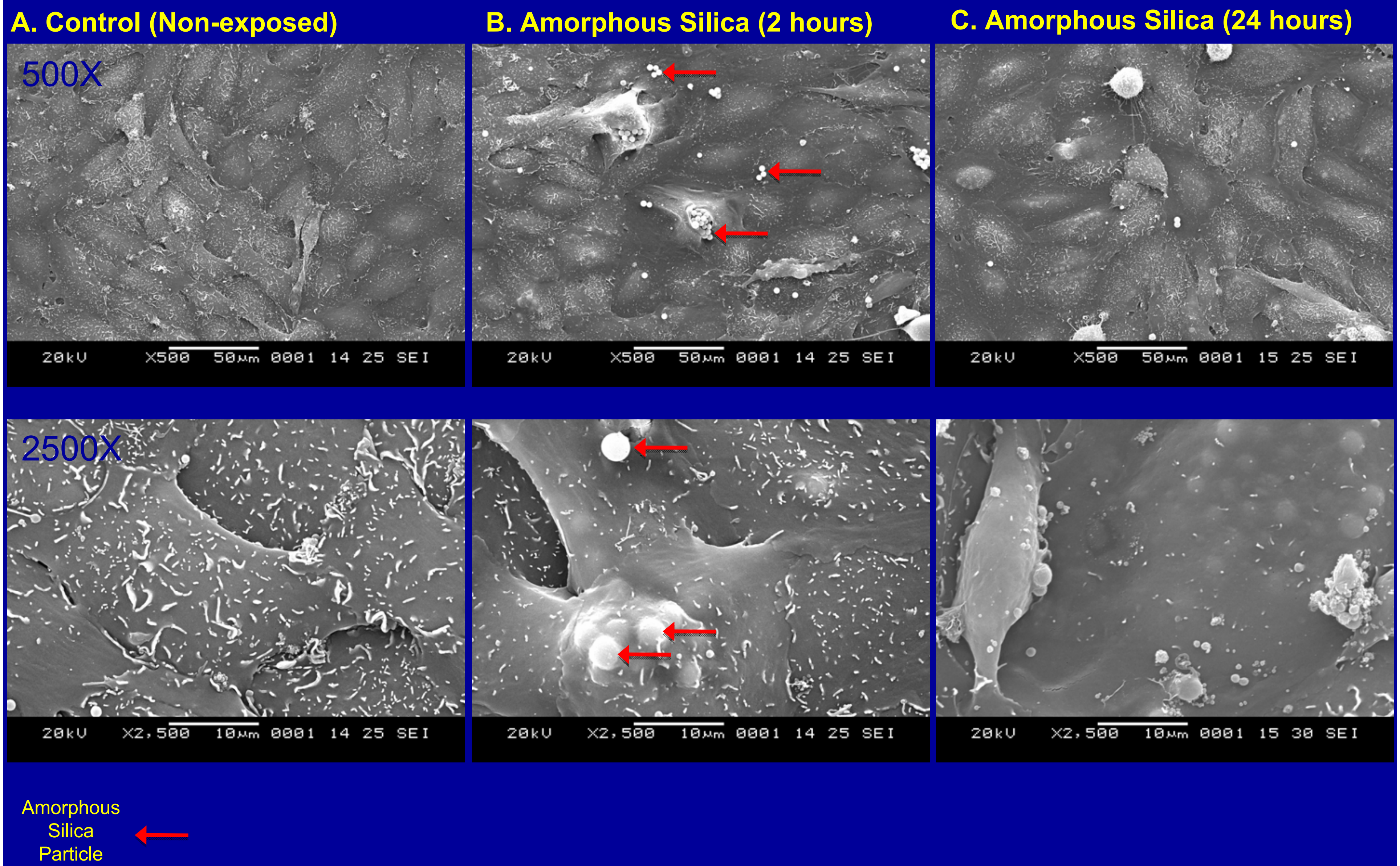
A. Control (Non-exposed) | B. Cristobalite Silica (2 hours) | C. Cristobalite Silica (24 hours)



Cristobalite Particle

Perkins *et al. Particle and Fiber Toxicology* 2012, 9:6

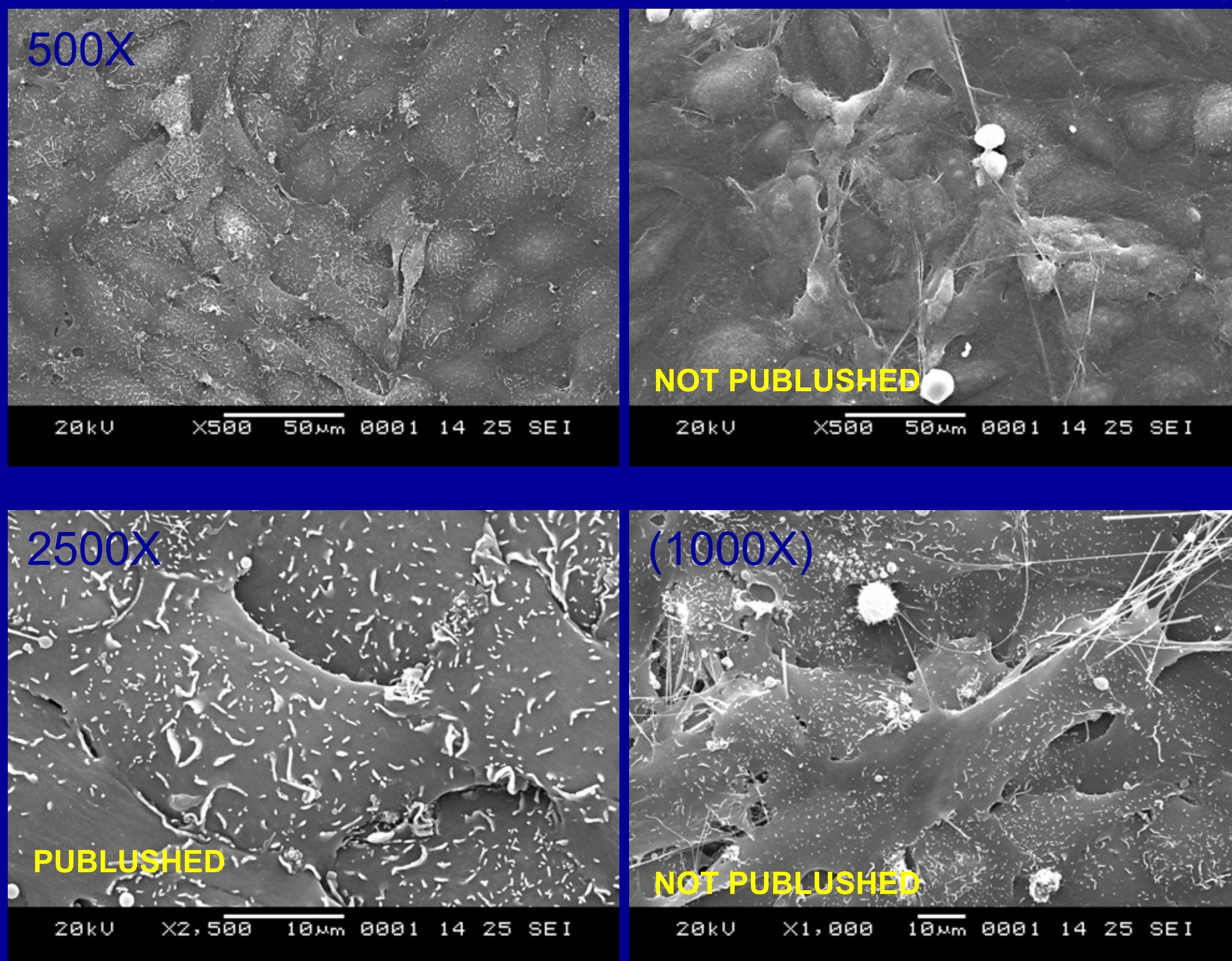
Particle-cell interactions imaged by SEM



Perkins *et al*. *Particle and Fiber Toxicology* 2012, 9:6

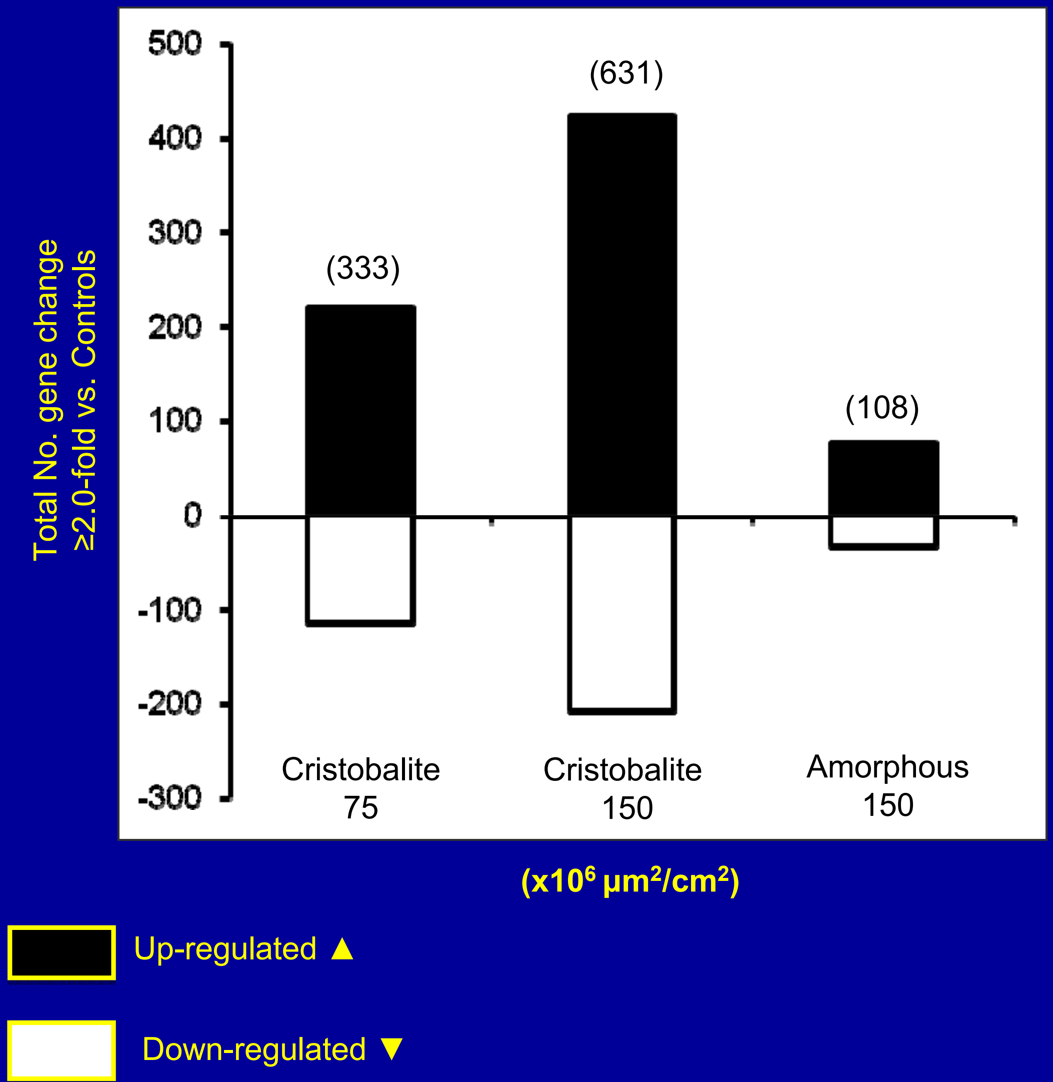
Particle-cell interactions imaged by SEM

A. Control (Non-exposed)　　**B. Crocidolite Asbestos (24 hours)**



A. Perkins *et al*. *Particle and Fiber Toxicology* 2012, 9:6　　**B. Unpublished Data**

Total Significant Gene Changes in BEAS-2B Cells at 24 h



Perkins *et al. Particle and Fiber Toxicology* 2012, 9:6

(GeneSifter Analysis)

Dose-Responses to Cristobalite and Decreased Gene Expression by Amorphous Silica

Gene Name (Abbreviation)	Fold Change mRNA		
	Cristobalite		Amorphous
[BEAS 2B Top 10 genes Up-regulated ▲ by silica particle exposure (24 h)]	75	150	150
Up-regulated ▲			
Myxovirus (influenza virus) resistance 2 (mouse) (MX2)	20.86	73.76	22.33
Matrix metallopeptidase 1 (interstitial collagenase) (MMP1)	16.12	44.68	18.75
Sodium Channel, voltage-gated, type III, beta (SCN3B)	15.33	20.54	NC
2'-5'-oligoadenylate-synthetase 2, 69/71kDa (OAS2)	13.56	34.77	12.17
Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse) (MX1)	9.61	23.33	8.72
Interferon-induced protein 44-like (IFI44L)	7.64	22.82	8.63
Interferon-induced protein 44 (IFI44)	7.51	19.90	8.24
B-cell Linker (BLNK)	7.24	9.32	NC
BTG family, member 2 (BTG2)	7.22	8.56	NC
Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1)	7.19	11.05	NC

NC = no change

Unpublished Data

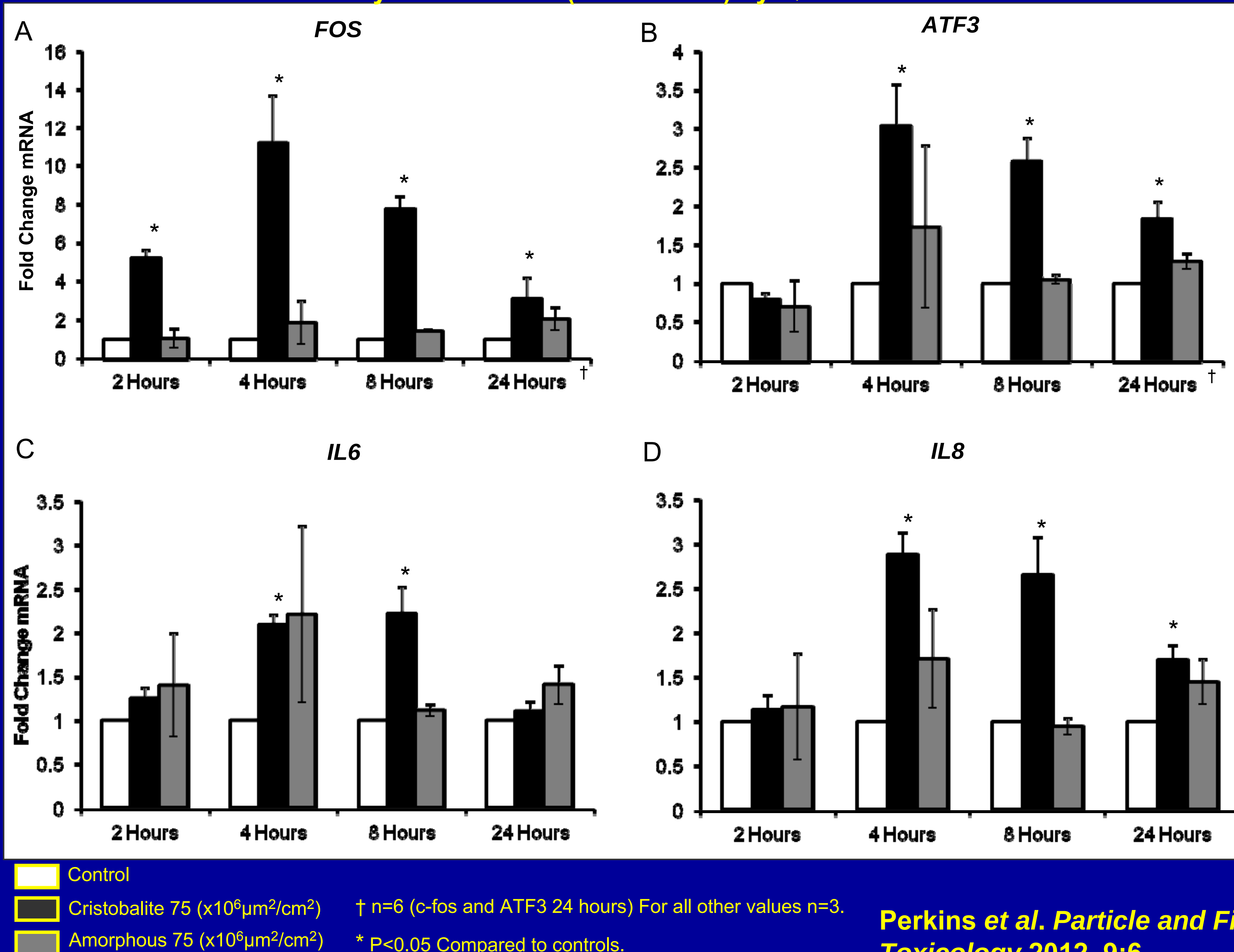
Dose-Responses to Cristobalite and Decreased Gene Expression by Amorphous Silica

Gene Name (Abbreviation)	Fold Change mRNA		
	Cristobalite		Amorphous
[BEAS 2B Top 10 genes Down-regulated ▼ by silica particle exposure (24 h)]	75	150	150
Down-regulated ▼			
Collagen, type I, alpha 2 (COL1A2)	6.08	10.86	2.12
Hypoxia inducible factor 3, alpha subunit (HIF3A)	4.51	7.18	3.94
Collagen, type I, alpha 1 (COL1A1)	4.17	5.52	NC
Methyltransferase like 7A (METTL7A)	4.11	5.67	2.89
Cytochrome P450, family 4, subfamily B, polypeptide 1 (CYP4B1)	3.93	5.55	3.07
Olfactomedin-like 3 (OLFML3)	3.91	5.28	2.06
Mitogen-activated protein kinase kinase 6 (MAP2K6)	3.86	4.78	3.21
Collagen, type XI, alpha 1 (COL11A1)	3.85	5.46	NC
Potassium inwardly-rectifying channel, subfamily J, member 16 (KCNJ16)	3.78	7.11	3.49
Cannabinoid receptor 1 (brain) (CNR1)	3.75	5.44	3.48

NC = no change

Unpublished Data

Steady-State mRNA Levels of Early-Response and Pro-Inflammatory Genes Observed Only in Response to Crystalline Silica (Cristobalite) by QRT-PCR



† n=6 (c-fos and ATF3 24 hours) For all other values n=3.

* P<0.05 Compared to controls.

Perkins *et al. Particle and Fiber Toxicology* 2012, 9:6

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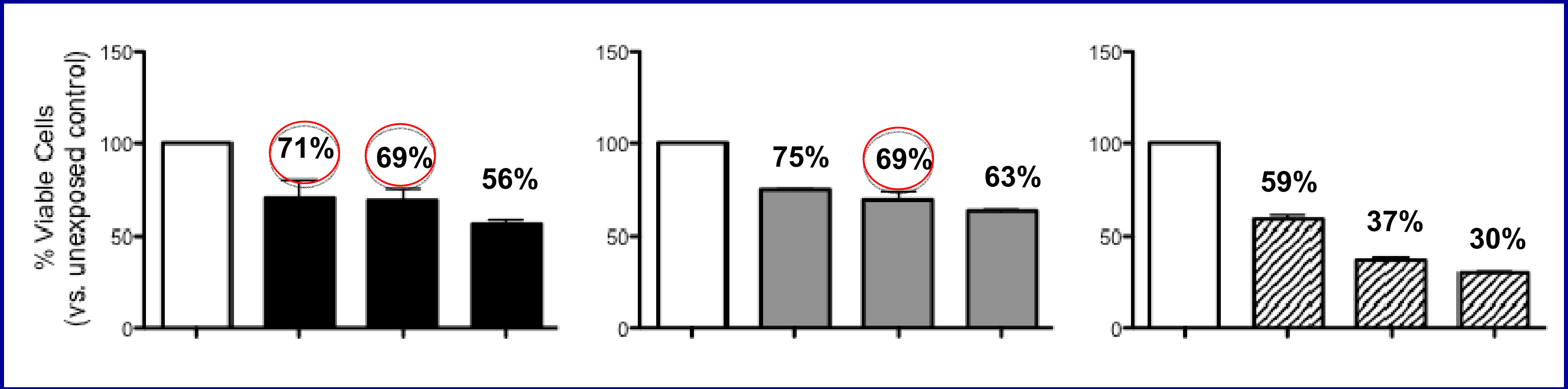
Assessment of primary NHBE viability with exposure to particles/fibers (24 h)

NHBE-17917

A. Cristobalite **B.** Amorphous **C.** Crocidolite



[A]Surface Area:	Control	15	75	150	Control	15	75	150	Control	15	75	150
[B]Mass:	Control	2.9	14.7	29.4	Control	6.8	34.1	68.2	Control	1.0	5.0	10.0

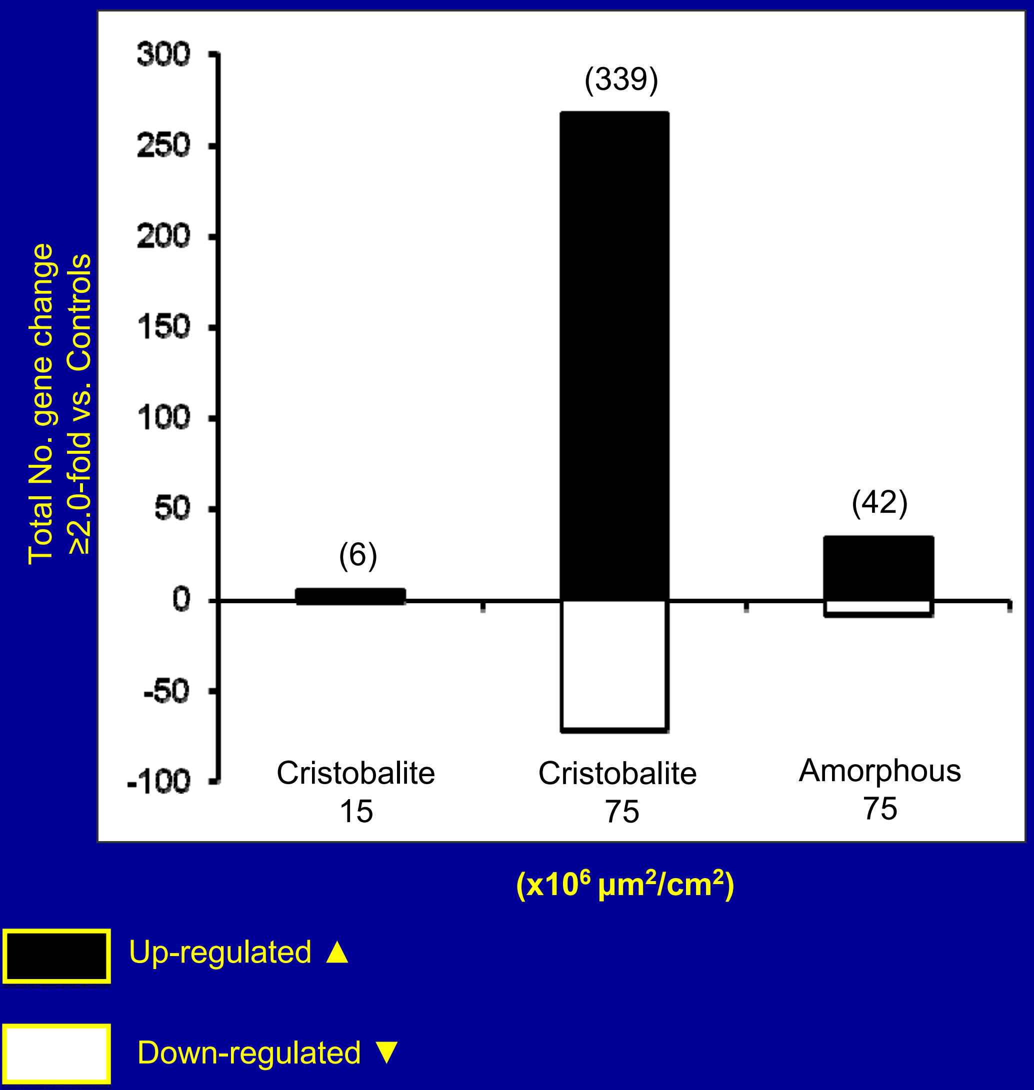
[A] ($x10^6 \mu m^2/cm^2$)

[B] ($\mu g/cm^2$)

A and B: Perkins *et al. Particle and Fiber Toxicology* 2012, 9:6

C: Unpublished Data

Total significant gene changes (▲ or ▼) in NHBE exposed to silica particles (24 h)



Perkins *et al*. *Particle and Fiber Toxicology* 2012, 9:6

(GeneSifter Analysis)

Dose-Response Changes by Cristobalite and Decreased Response to Amorphous Silica in NHBE Cells

Gene Name (Abbreviation)	Fold Change mRNA		
	Cristobalite		Amorphous
Top 10 genes Up-regulated ▲ by silica particle exposure at 24 h	**15**	**75**	**75**
Up-regulated ▲			
Interleukin 8 (IL8)	4.37	28.47	NC
Baculoviral IAP repeat-containing 3 (BIRC3)	2.02	19.81	NC
BCL2-related protein A1 (BCL2A1)	NC	19.27	NC
Tumor necrosis factor, alpha-induced protein 3 (TNFAIP3)	2.33	17.11	NC
Interleukin 1 family, member 9 (IL1F9)	NC	14.14	NC
GTP binding protein over expressed in skeletal muscle (GEM)	NC	12.92	NC
CD83 molecule (CD83)	NC	11.47	NC
Transcribed locus, strongly similar to NP_006463.2 thioredoxin interacting protein	NC	11.09	27.95
Inhibin, beta A (INHBA)	NC	10.25	NC
Thioredoxin interacting protein (TXNIP)	NC	9.74	31.14

NC = no change

Perkins *et al*. *Particle and Fiber Toxicology* 2012, 9:6

Dose-Response Changes by Cristobalite and Decreased Response to Amorphous Silica in NHBE Cells

Gene Name (Abbreviation)	Fold Change mRNA		
	Cristobalite		Amorphous
Top 10 genes Down-regulated ▼ by silica particle exposure at 24 h	15	75	75
Down-regulated ▼			
Sulfatase	NC	3.59	NC
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A)	NC	3.52	NC
Solute carrier family 38, member 4 (SLC38A4)	NC	3.43	NC
Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 8 (ALS2CR8)	NC	3.18	2.00
Growth arrest-specific 1 (GAS1)	NC	2.84	NC
Hexokinase 2 (HK2)	NC	2.69	NC
F-box protein 9 (FBXO9)	NC	2.68	NC
Hairy/enhancer-of-split related with YRPW motif 1 (HEY1)	NC	2.62	NC
CDNA FLJ37852 fis, clone BRSSN2014513	NC	2.61	NC
Growth hormone receptor (GHR)	NC	2.59	NC

NC = no change

Perkins *et al*. *Particle and Fiber Toxicology* 2012, 9:6

Summary/Conclusions (1)

- Cristobalite and amorphous silica particles have equitoxic effects on BEAS 2B and NHBE cell viability at equal surface area doses compared to crocidolite asbestos fibers.

- Cristobalite silica induces a robust and dose-responsive effect on gene expression in BEAS 2B and NHBE cells vs. amorphous silica.

- Gene ontology reveals that most genes altered by cristobalite silica particles are related to *cell signaling* and *proliferation, apoptosis, protein metabolic processes,* and *immune response.*

- Steady state mRNA levels of *early response* and *pro-inflammatory* genes are induced by cristobalite but not amorphous silica.

Summary/Conclusions (2)

- 60 common genes were altered by cristobalite silica exposure in both BEAS 2B and NHBE cells.
- Silica induces expression of a “Macrophage core response module” in both epithelial cell types.
- Gene expression profiling and cytokine/chemokine/growth factor elaboration may be used to determine potential pathogenicity of particulates as opposed to viability and metabolic activity assays.

Future Directions

- Compare effects of cristobalite silica and crocidolite asbestos on gene expression in NHBE cells (Silicosis vs. Asbestosis). *Currently being investigated...*
  - o Differences in gene expression profiles
  - o Ingenuity Pathway Analysis® (IPA)
    - Unique pathways and signaling networks?

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