Pulmonary Cell Proliferation: *The Missing Link* in PSLTP-induced Lung Cancer *A pathologist's perspective*

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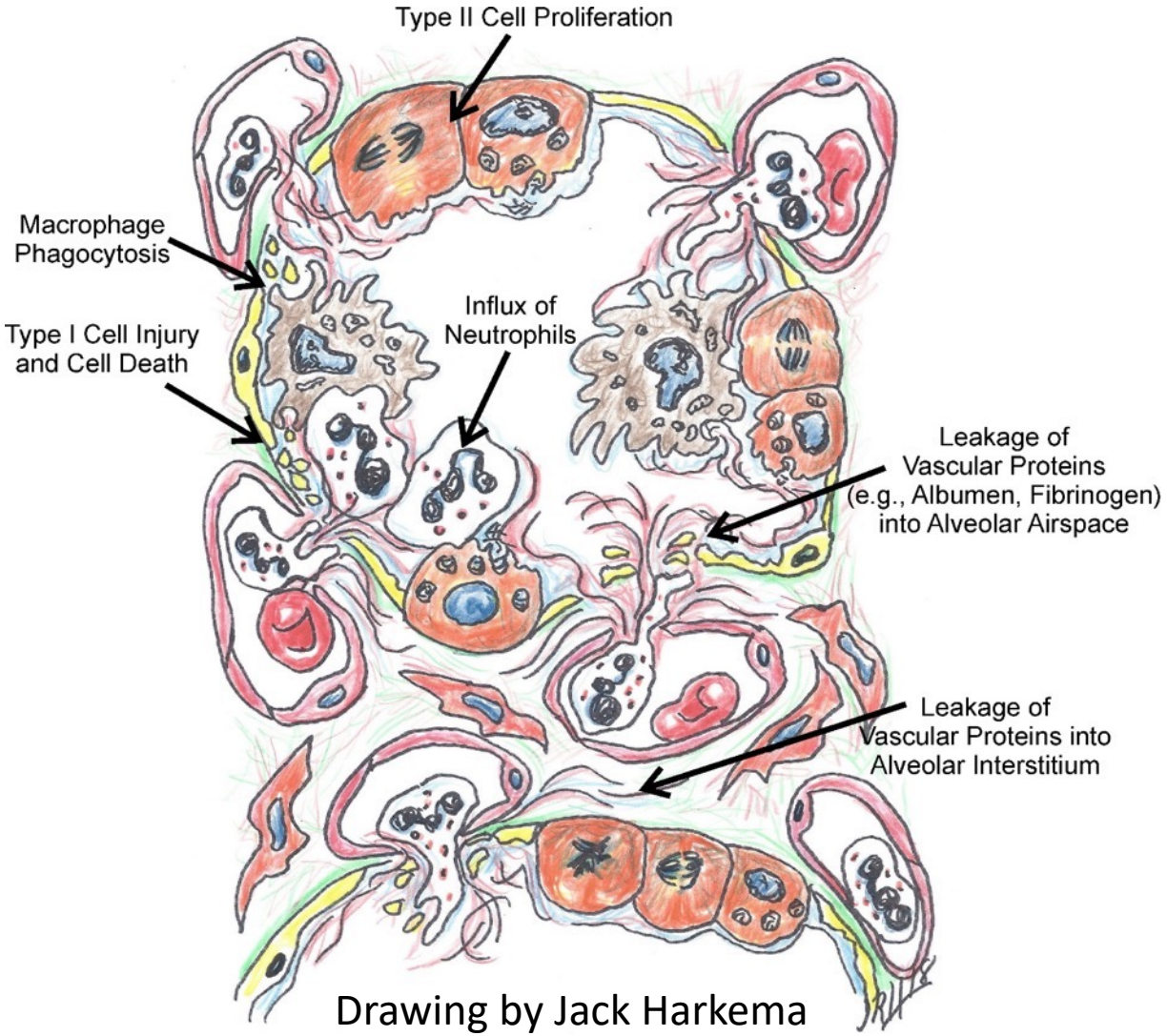
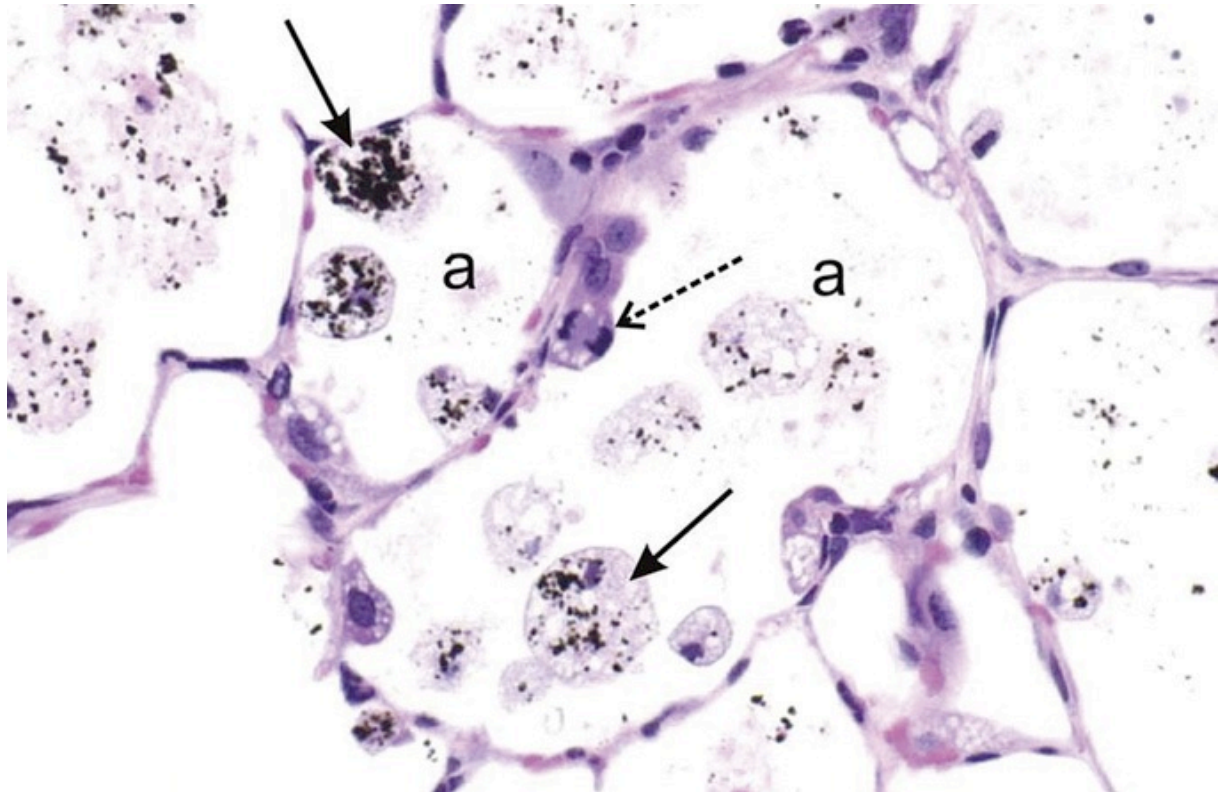
Outline

- Epithelial cell proliferation in toxicant-induced lung injury, repair, adaptation and cancer.
- Carbon Black Particle-induced proliferative and inflammatory responses (lesions) in rodent lungs
- Short-term bioassay for predicting pulmonary cancer potential of PSLTPs
- Ancillary methods to better elucidate the respiratory toxicity/cancer potential of inhaled PSLTPs

Acute Alveolar Injury, Inflammation (alveolitis) and Repair



Drawing by Jack Harkema



Light photomicrograph by Jack Harkema

Alveolar Epithelial Injury and Repair Mechanisms (Evans, 1975)

EXPERIMENTAL AND MOLECULAR PATHOLOGY 22, 142–150 (1975)

Transformation of Alveolar Type 2 Cells to Type 1 Cells Following Exposure to NO_2 [1, 2]

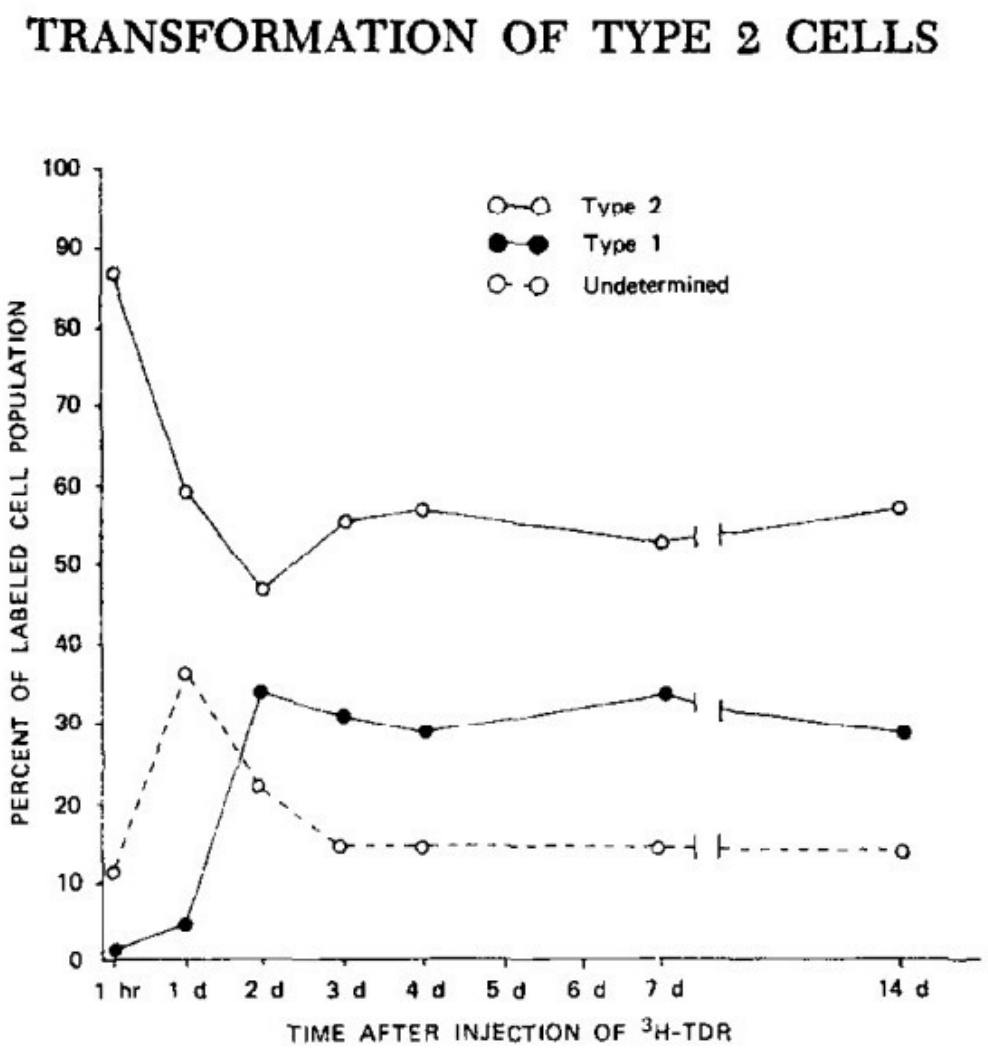
MICHAEL J. EVANS, LINDA J. CABRAL, ROBERT J. STEPHENS, AND GUSTAVE FREEMAN

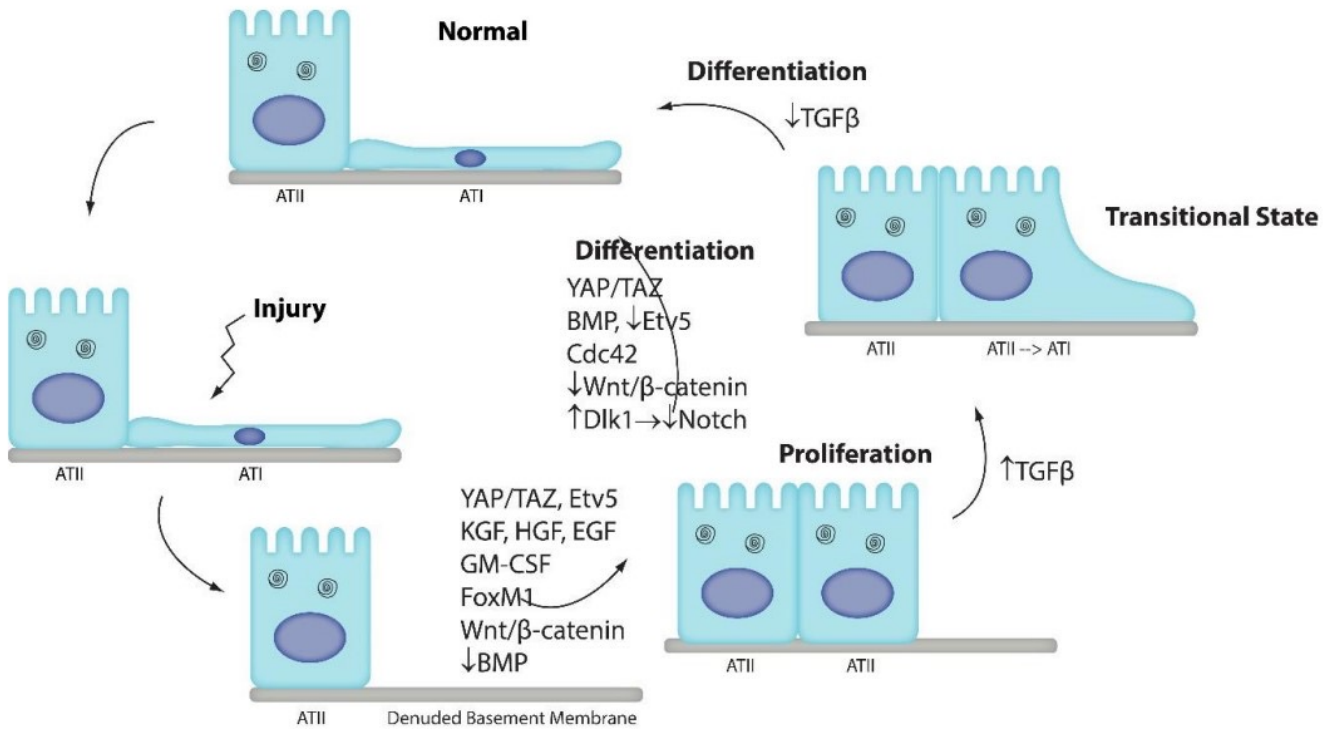
Life Sciences Division, Stanford Research Institute, Menlo Park, California 94025

Received July 11, 1974

This research was undertaken to study the fate of Type 2 cells after they have divided. To accomplish this, male rats were exposed to NO_2 to increase the number of dividing Type 2 cells. Dividing cells were labeled with ^{3}H-TdR and studied with autoradiographic techniques in the electron microscope for up to 14 days after labeling. The results show that initially most of the ^{3}H-TdR labeled cells were Type 2. However, by 2 days there was a decrease in frequency of labeled Type 2 cells and a large increase in labeled Type 1 cells. The new frequencies of labeled alveolar epithelial cells were stable from 2 through 14 days. This evidence supports the interpretation that Type 2 cells may transform into Type 1 cells. In addition, it was shown that under the conditions of this experiment: (1) the time for transformation was about 2 days, and (2) during this process an intermediate cell type was present.

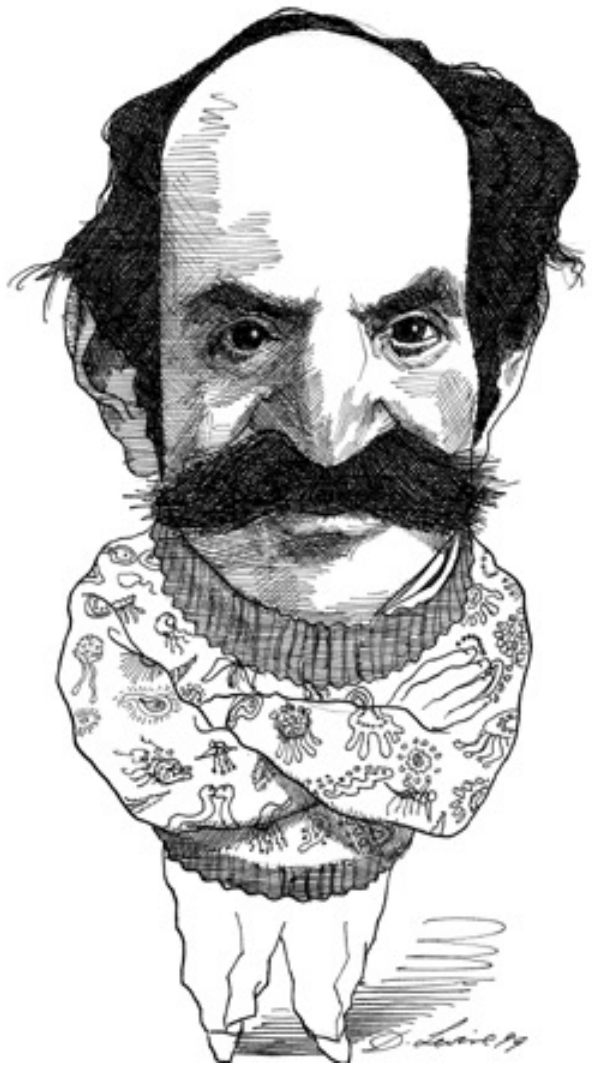
Alveolar Epithelial Injury/Repair Mechanisms (Evans, 1975; Zemans, 2020)

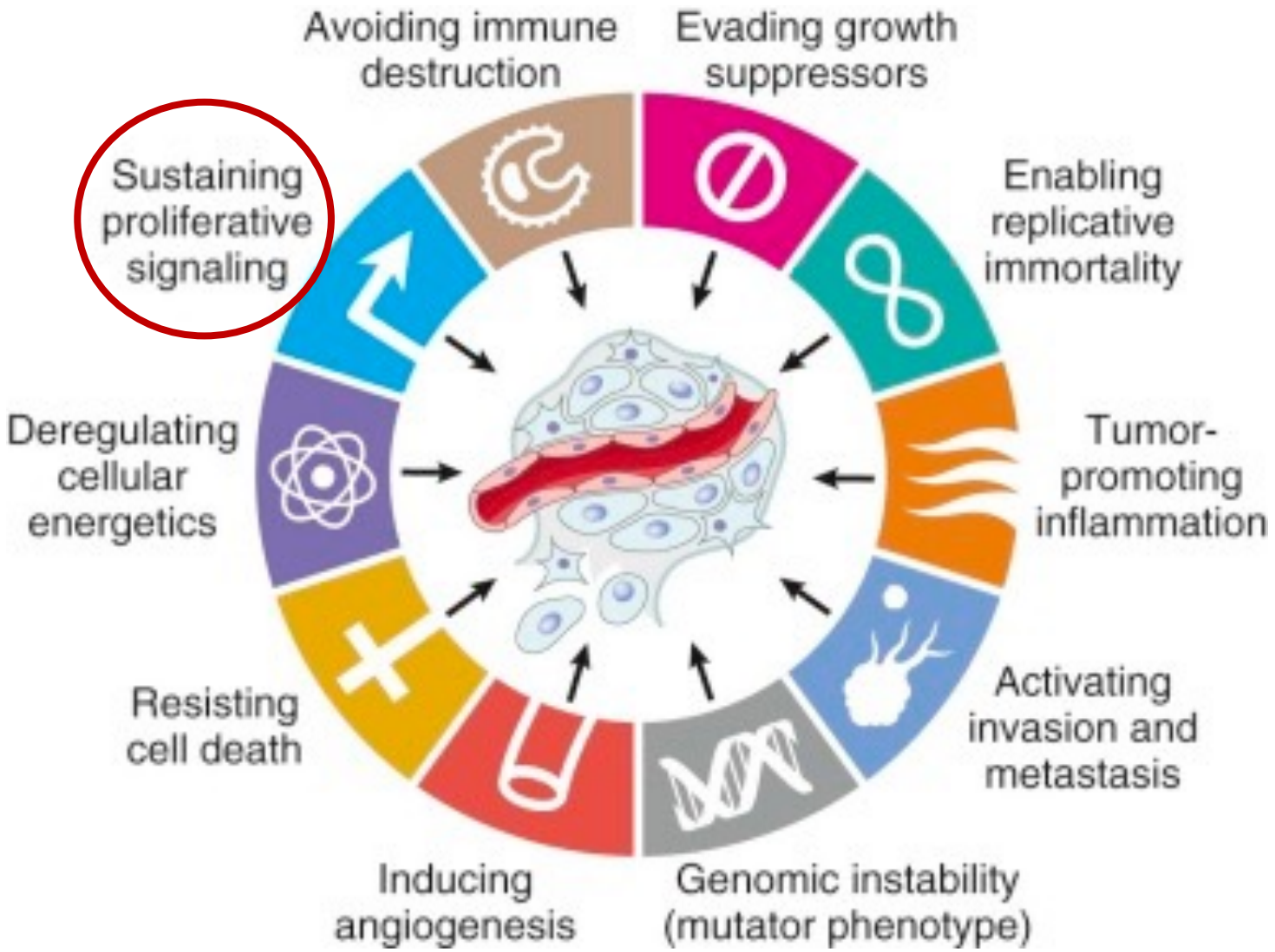




Evans MJ et al. Exp Mol Pathol 22:142, 1975; Aspal M & Zemans RL. Int J Mol Sci 21:3188, 2020

Molecular and Cellular Hallmarks of Cancer





From *Robbins and Cotran: Pathologic Basis of Disease, 9th edition, 2015*
(Adapted from Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144:646.)

Basic understanding of chemical carcinogenicity

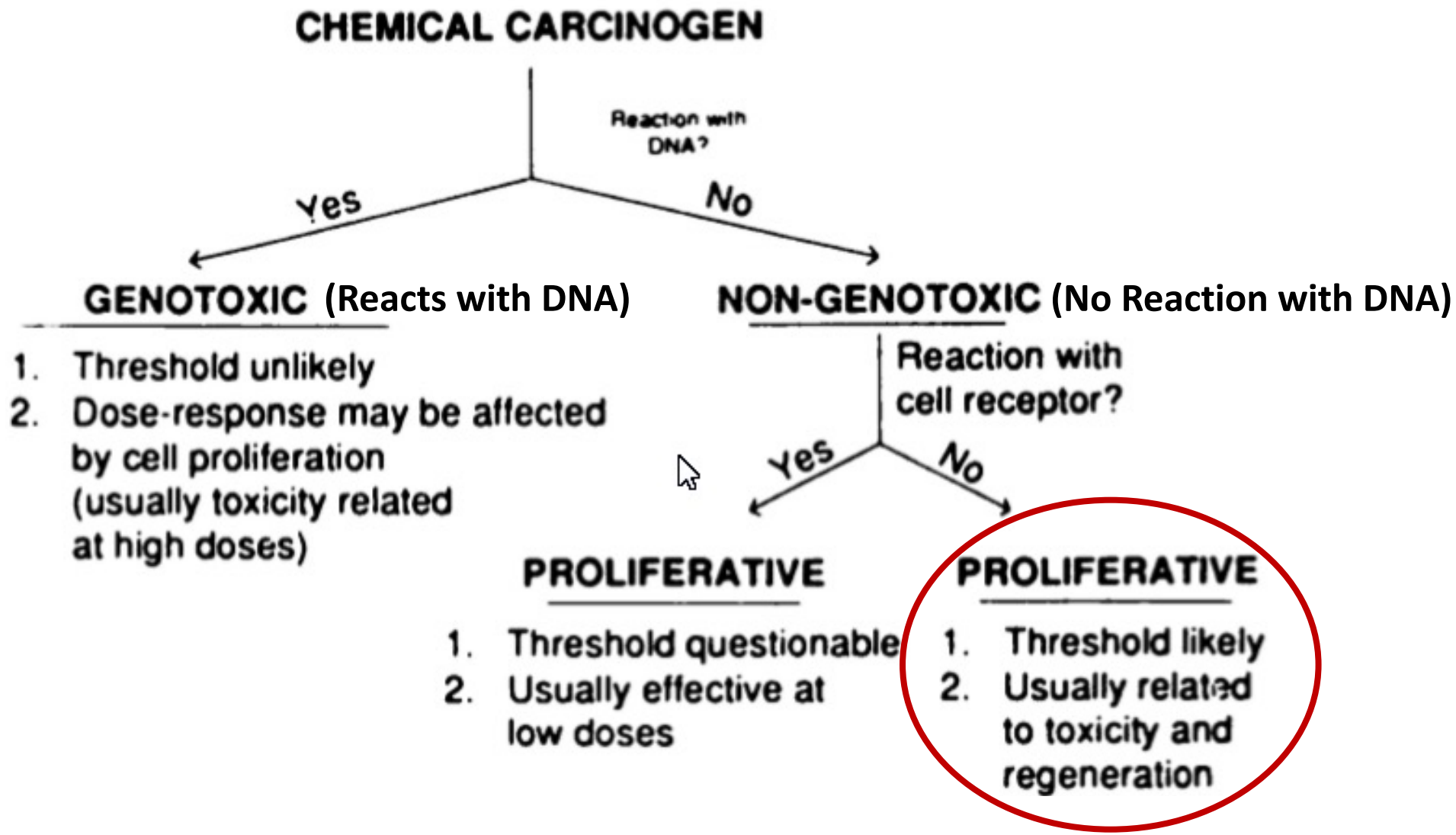
1. Cancer is due to mistakes occurring in the DNA (usually in somatic cells but can be inherited through germ cells).
2. More than one mistake in DNA is necessary
3. All of the mistakes need to accumulate in a single cell (clonal origin of cancer)
4. The cell population at risk are the tissue pluripotential (stem) cells.
5. Every time DNA replicates, permanent mistakes could occur
6. Carcinogenesis is a stochastic process

Two fundamental ways to increase the risk for cancer:

- Damage DNA directly
- Increase the number of stem cell replications

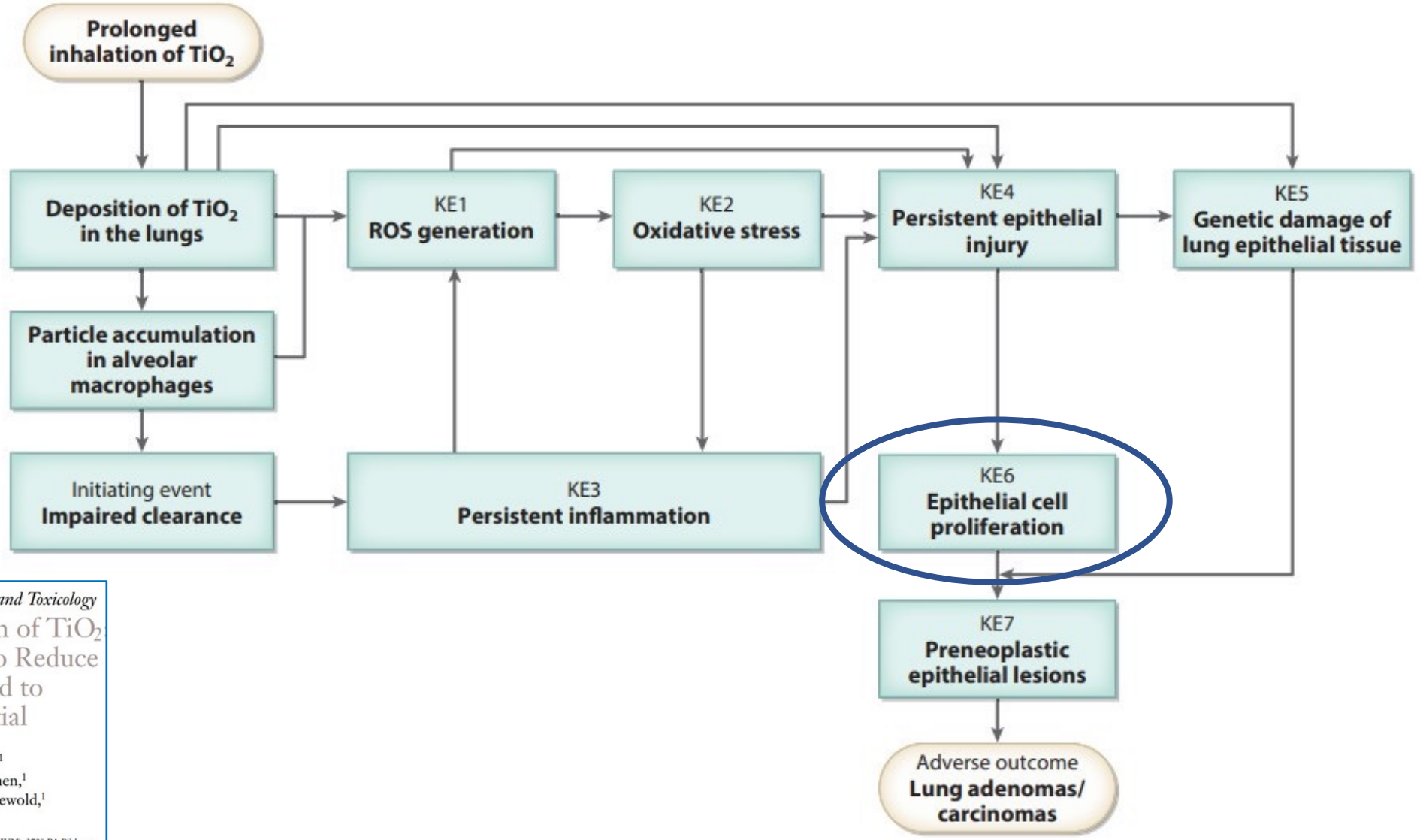
Cohen SM & Ellwen LB, Cancer Research 51, 6493-6505, 1991; Cohen SM et al. Reg Toxicol Pharmacol 103: 100-105, 2019

Classification of Chemical Carcinogens



Cohen SM & Ellwen LB, Cancer Research 51, 6493-6505, 1991; Cohen SM et al. Reg Toxicol Pharmacol 103: 100-105, 2019

Proposed Adverse Outcome Pathway for Poorly Soluble, Low Toxicity Particle-induced Carcinogenicity in the Lung



Annual Review of Pharmacology and Toxicology

Mechanism of Action of TiO_2: Recommendations to Reduce Uncertainties Related to Carcinogenic Potential

Hedwig M. Braakhuis,[1] Ilse Gosens,[1] Minne B. Heringa,[1,2] Agnes G. Oomen,[1] Rob J. Vandebriel,[1] Monique Groenewold,[1] and Flemming R. Cassee[1,3]

[1]National Institute for Public Health and the Environment (RIVM), 3720 BA Bilthoven, The Netherlands; email: hedwig.braakhuis@rivm.nl

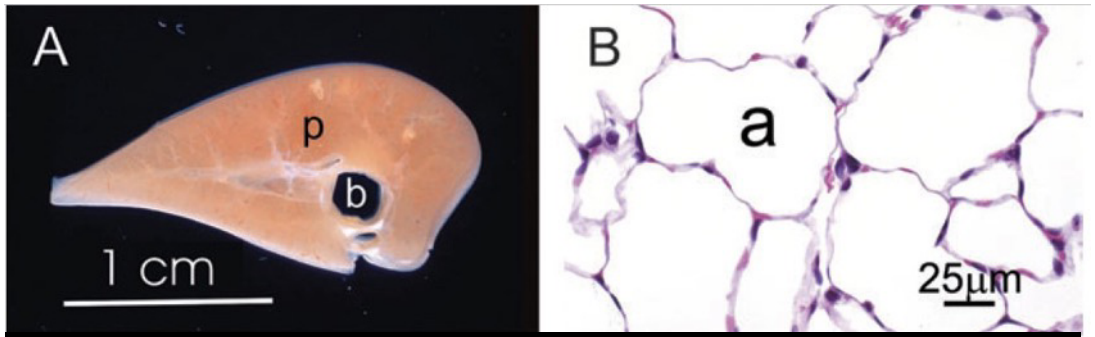
[2]Current affiliation: Reckitt Benckiser, 1118 BH Schiphol, The Netherlands

[3]Institute for Risk Assessment Sciences, University of Utrecht, 3508 TD Utrecht, The Netherlands

Braakhuis H et al. Ann Review Pharmacol Toxicol, 61:203-223, 2021

Normal rat lung (centriacinar region)

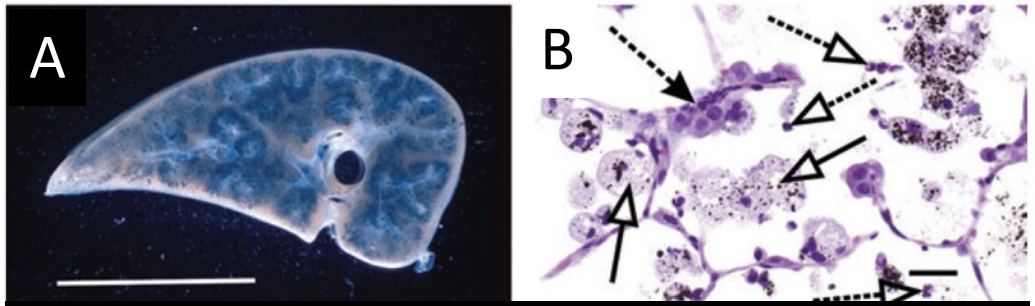
- Bronchiolar epithelium
- Pulmonary artery/arteriole
- Type 1 alveolar epithelial cell
- Type 2 alveolar epithelial cell
- Alveolar septal capillaries
- Alveolar macrophage (resident macrophage)



Elder A et al. Toxicol Sci 88:614-629, 2005

3 months of particle exposure

- Inhaled particles
- Neutrophilic inflammation (type 1 inflammatory/immune response)
- Type 2 alveolar epithelial cell proliferation/hyperplasia
- Macrophage/Monocyte accumulation/activation
- Neutrophilic inflammation; type 1 immune/inflammatory response

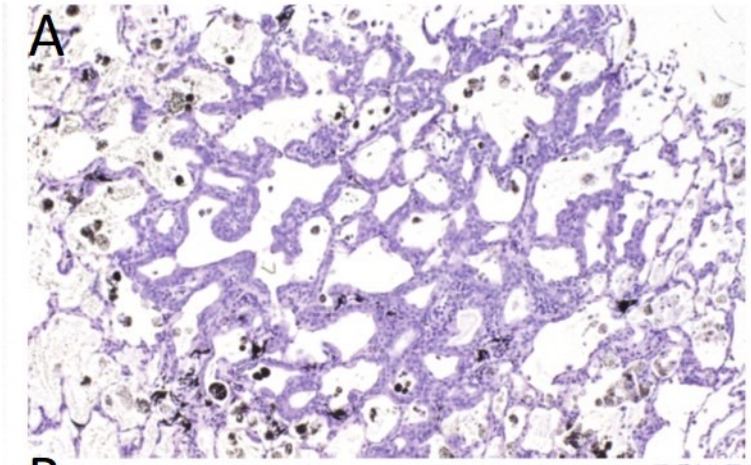


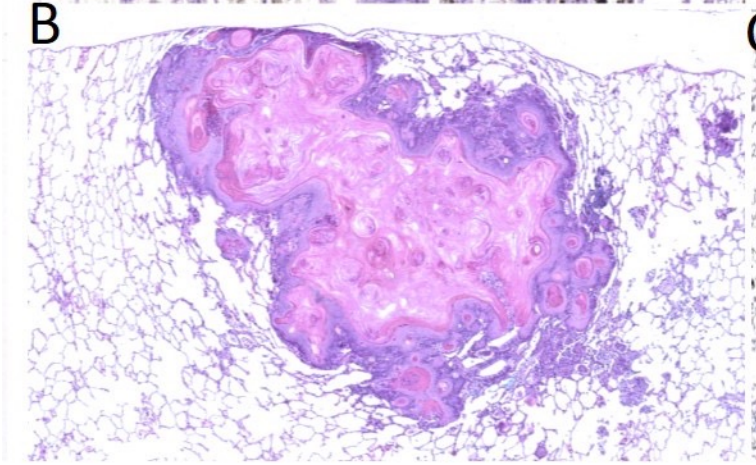
Elder A et al. Toxicol Sci 88:614-629, 2005

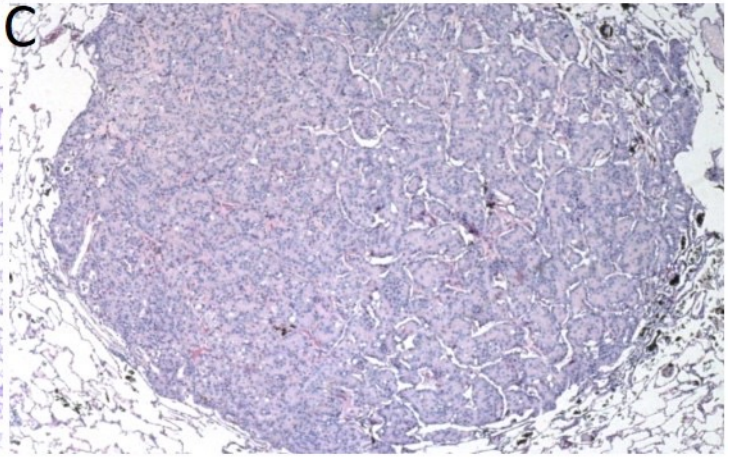
12 months of particle exposure

- Bronchiolar epithelial cell proliferation/hyperplasia
- Hyperplasia/respiratory metaplasia of alveolar type 2 epithelial cells
- Early interstitial fibroblast proliferation and activation (collagen deposition)
- Neutrophilic inflammation
- Macrophage/Monocyte accumulation/activation

24 months of particle exposure







A. Epithelial hyperplasia
B. Squamous cyst
C. Adenocarcinoma

Short-term Bioassays for Chemical Carcinogenesis

- Standard process for evaluating substances for carcinogenic activity is to perform a dose range finding study, usually 90 days, in the test species, followed by a two-year bioassay.
- Now short-term screening assays focus on mode of action and human relevance.
- Ultimately what is needed is a determination as to whether the inhaled agent/particle poses a cancer risk to humans.
- Not an investigation to determine if a chemical poses a cancer hazard in rats or mice.

Proposed Protocol: Short-term Bioassay for Predicting Potential for PSLTP-induced Lung Cancer

- Inhalation study with evaluations at 1, 4 and 13 weeks
- Postexposure evaluation(s) for sustained proliferation
- Microscopic evaluation for cell proliferation: histopathologic examination for alveolar epithelial hyperplasia and immunohistochemistry for DNA synthesis (Ki67; BrdU; PCNA)
- Morphometric analyses for quantitative pathology (labelling index using digital image analysis)
- Detect cell proliferation then develop adverse outcome pathway and evaluate human relevance
  - Transcriptomic analyses: RT-PCR; Bulk tissue RNA sequencing
  - Additional analyses such as single cell RNA sequencing, organoids, or in situ hybridization (RNAscope).

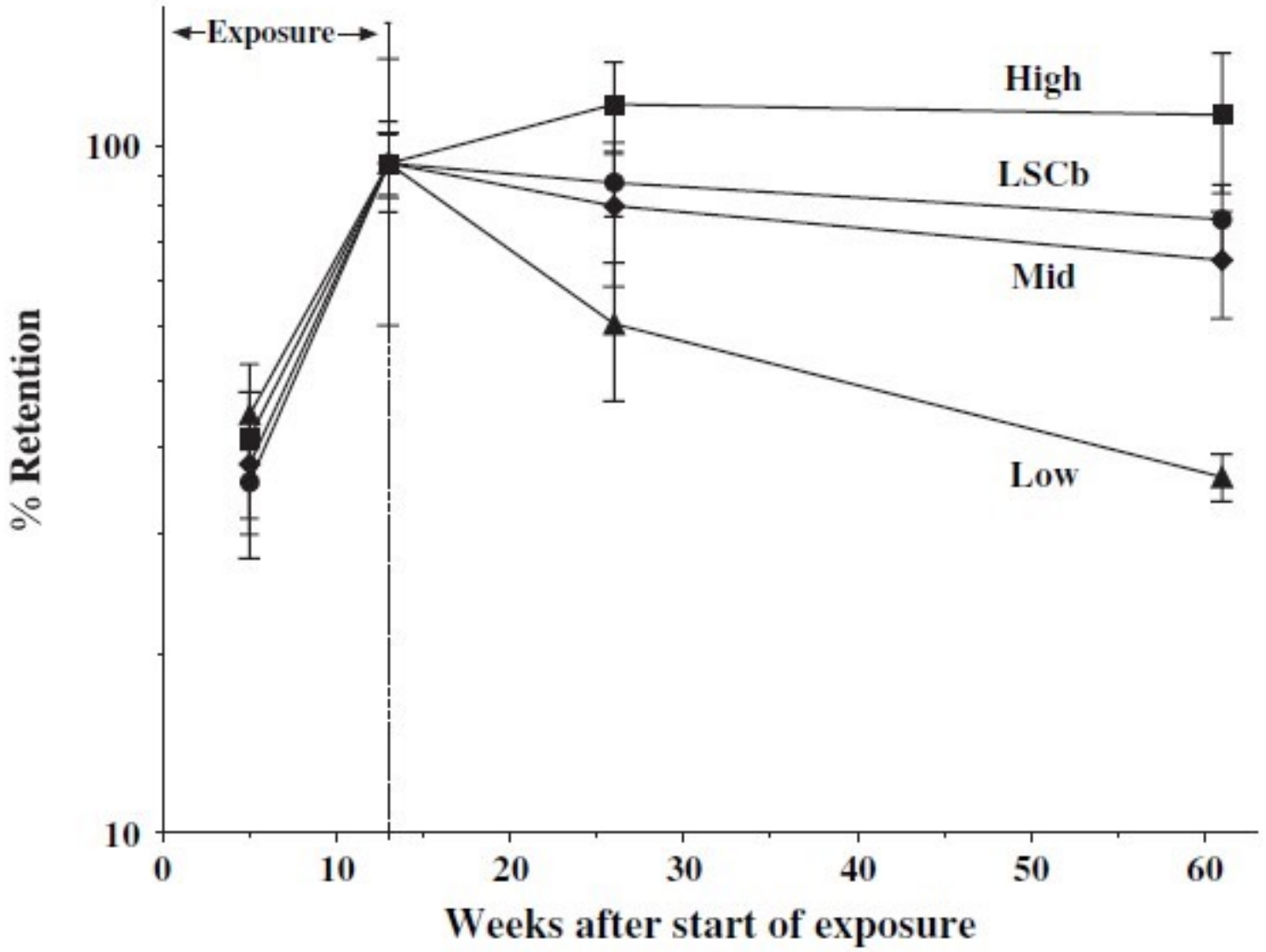
13-wk Carbon Black Inhalation Study: Animal Species and Exposures

Aerosol Concentration and Particle Size During the 65-Day Exposure

	HSCb, Low	HSCb, Mid	HSCb, High	LSCb
Rat				
Chamber concentration	1.1 ± 0.2[a]	7.6 ± 1.9	50.3 ± 5.6	48.2 ± 5.0
MMAD (GSD)	1.4 (2.5)	1.6 (2.7)	1.5 (2.5)	0.8 (3.2)
Mouse				
Chamber concentration	1.1 ± 0.2	13.9 ± 7.1[b]	64.4 ± 15.6[b]	
MMAD (GSD)	2.0 (2.8)	1.6 (2.3)	2.0 (2.5)	
Hamster				
Chamber concentration	1.1 ± 0.2[b]	11.1 ± 4.3[b]	63.0 ± 14.0[b]	
MMAD (GSD)	1.5 (2.5)	1.4 (2.6)	1.4 (2.4)	

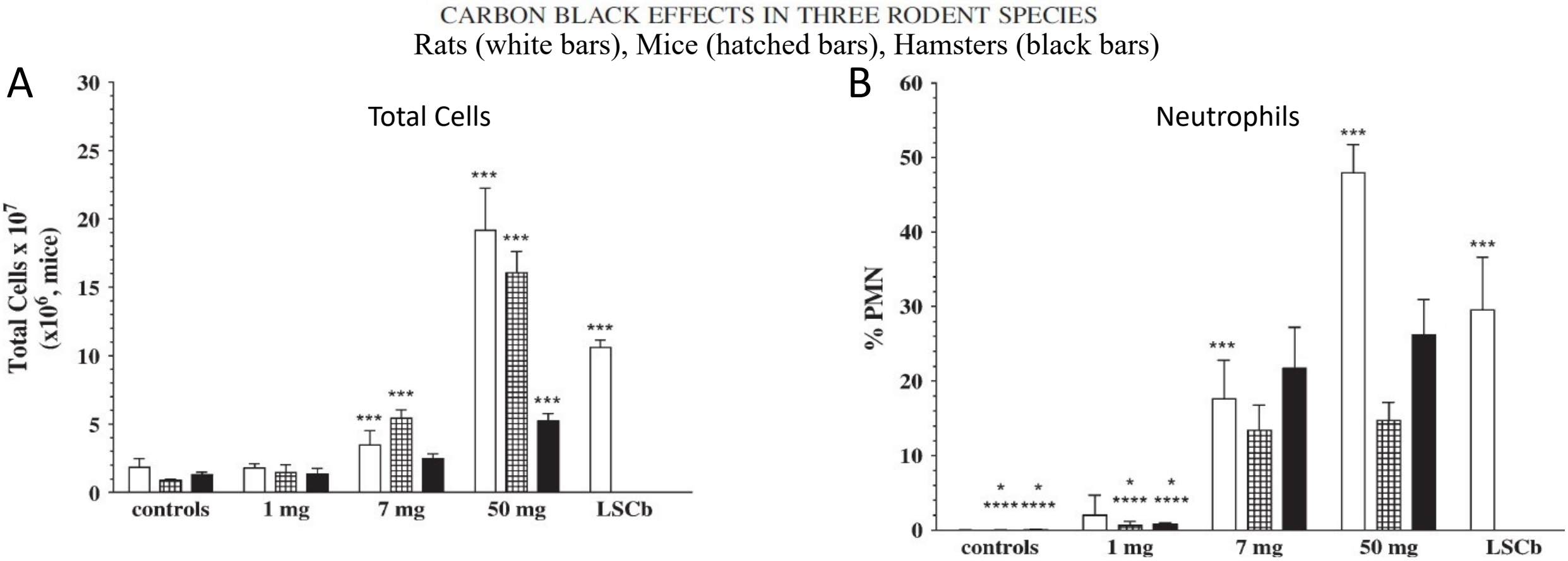
Elder A et al. Toxicol Sci 88:614-629, 2005

13wk Carbon Black Inhalation Study: CB Lung Retention



Elder A et al. Toxicol Sci 88:614-629, 2005

13wk Carbon Black Inhalation Study: Inflammatory Cells in BALF

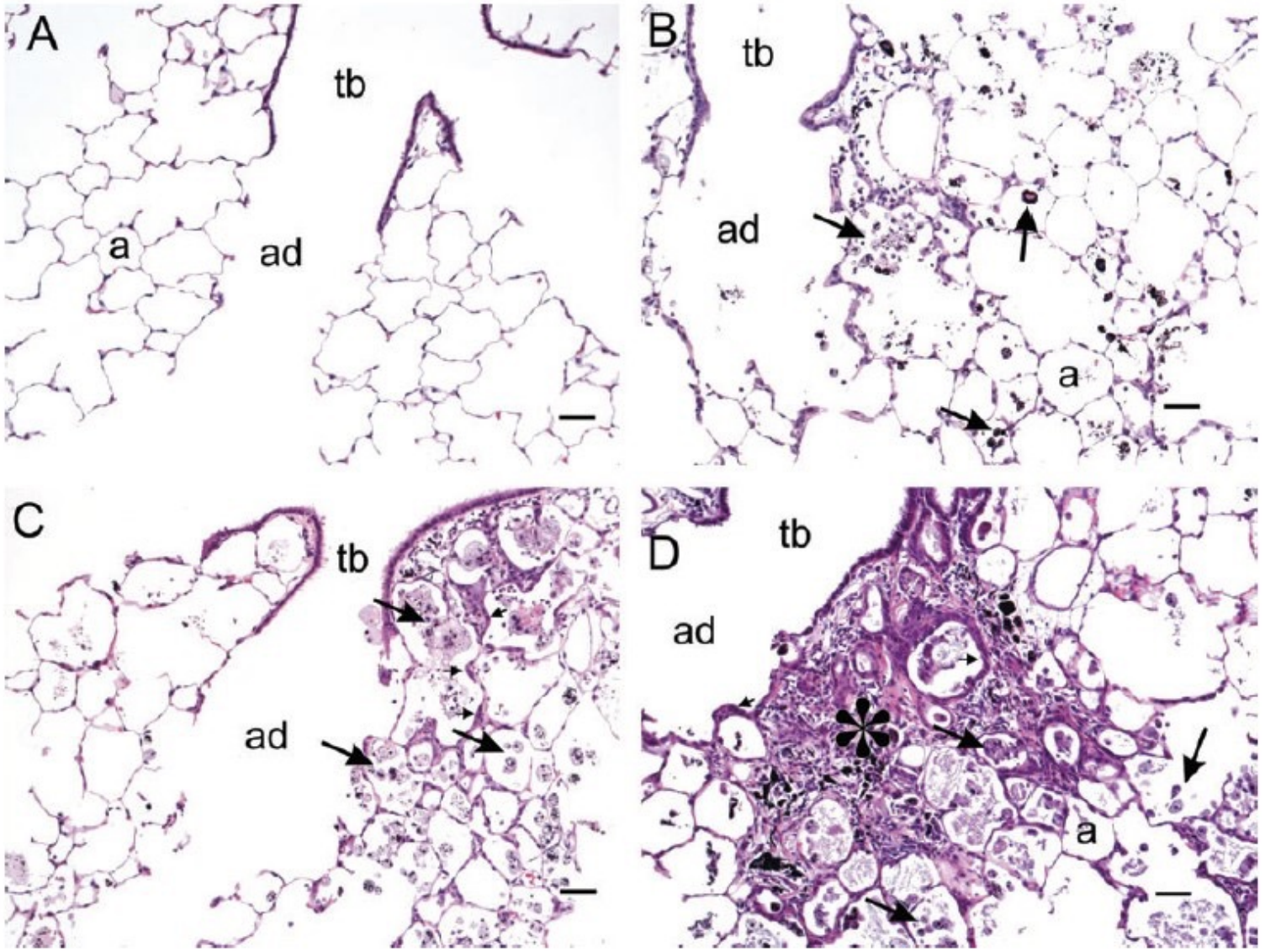


Elder A et al. Toxicol Sci 88:614-629, 2005

13wk Carbon Black Inhalation Study: High Dose HSCB Histopathology

Air Control

End of Exposure

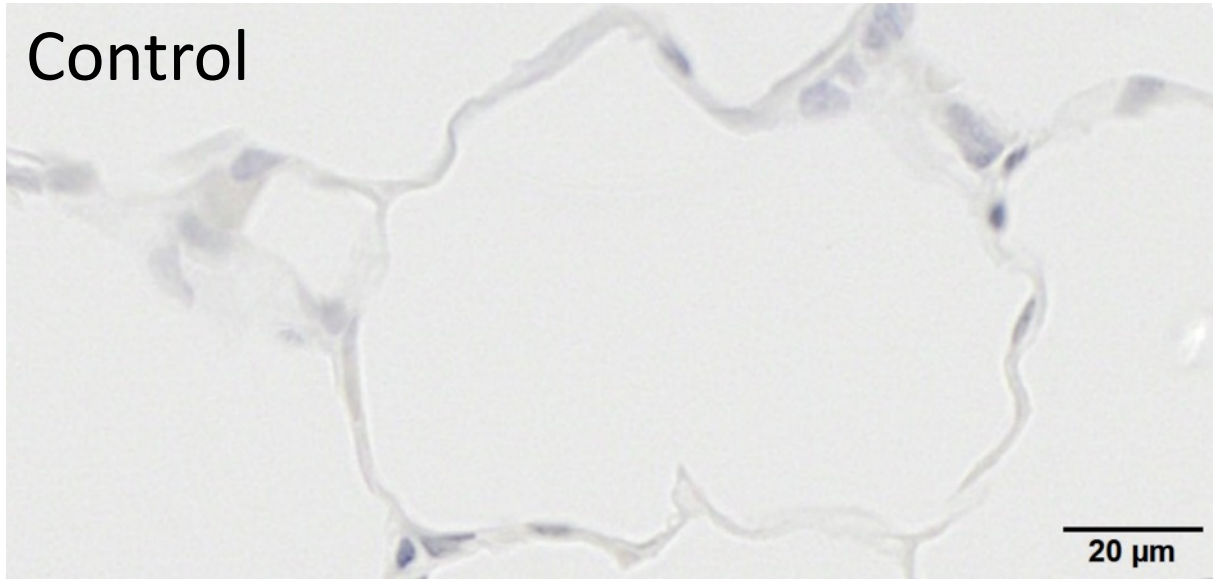


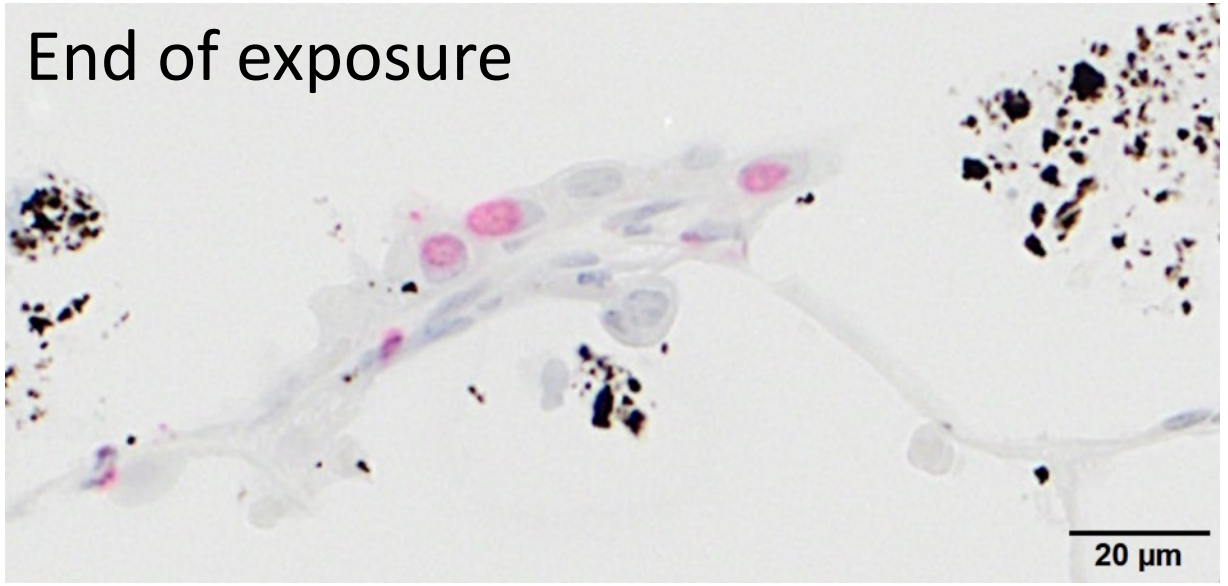
3-month Postexposure

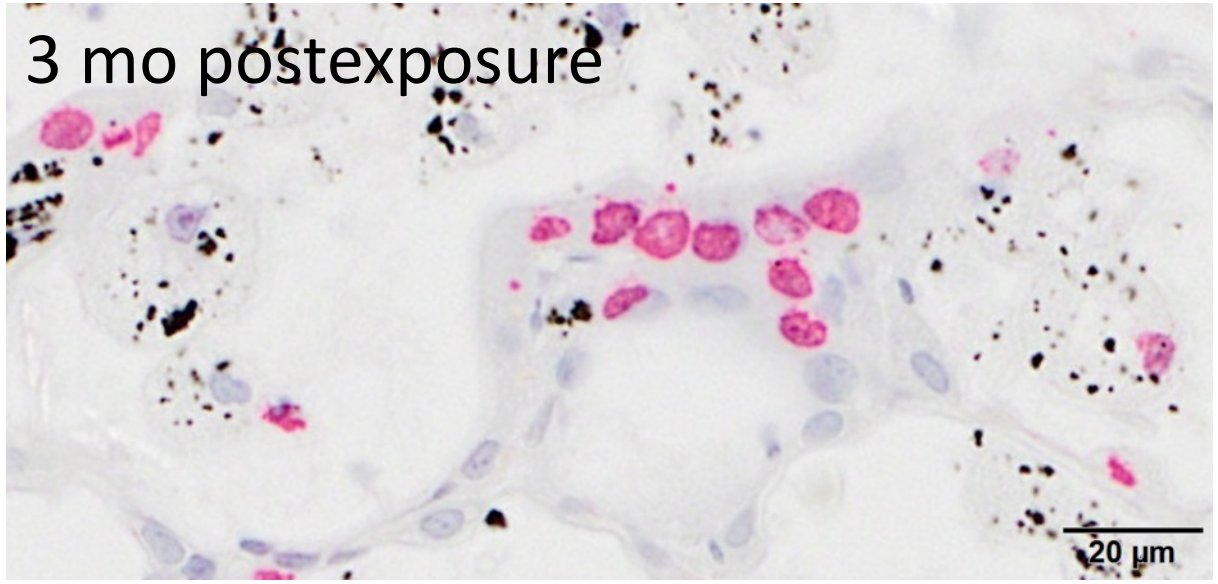
11-month Postexposure

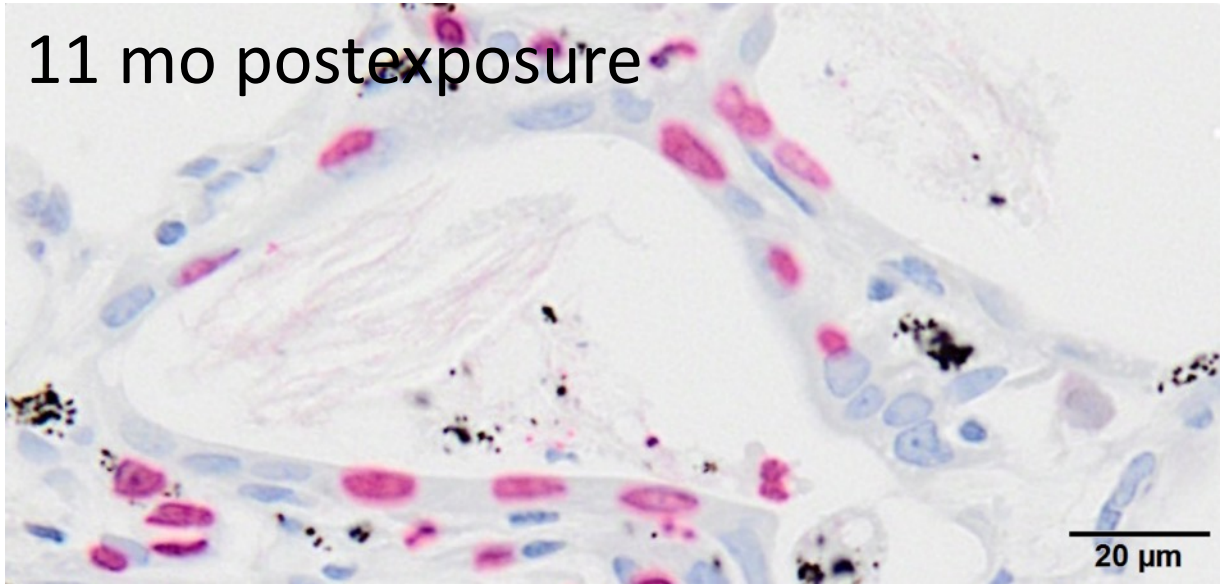
Elder A et al. Toxicol Sci 88:614-629, 2005

HSCB-induced AT2 Cell Proliferation (BrdU, IHC)







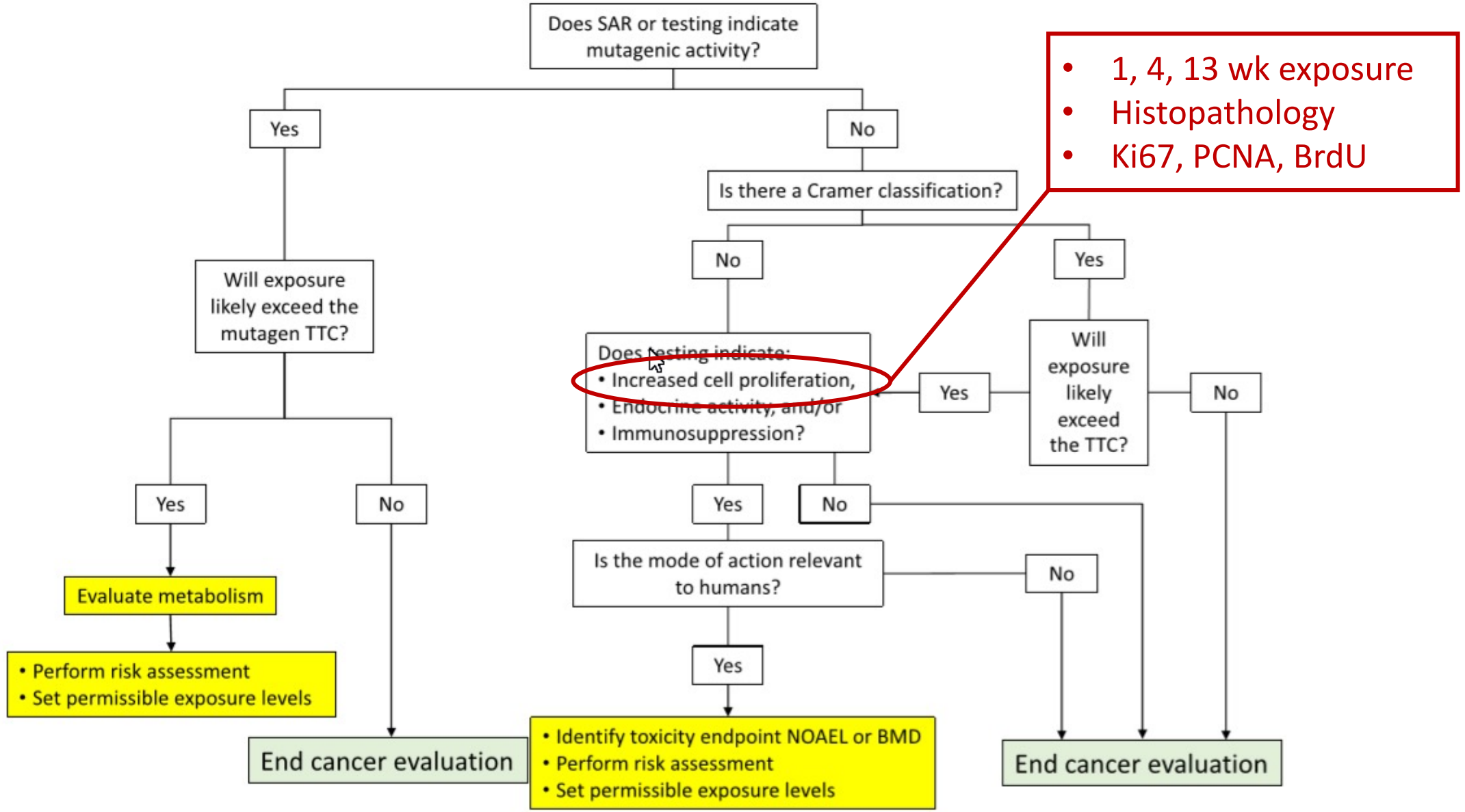


13wk Carbon Black Inhalation Study: AT2 Cell Proliferation

Numeric Densities of Alveolar Type II Cells and Percentages of Cells in S phase in the Lungs of Rats, Mice, and Hamsters Following 13 Weeks of Exposure to Carbon Black

Time post-exposure	Rats	Mice	Hamsters
End of exposure	No. of cells/mm^2 of parenchyma (% of cells with BrdU nuclear incorporation)		
Controls	126 ± 17 (1.3 ± 0.1)	52 ± 6 (13.5 ± 1.1)	19 ± 1 (17.3 ± 1.1)
HSCb, low	184 ± 22 (3.8 ± 0.8)	58 ± 15 (ND)	15 ± 1 (ND)
HSCb, mid	221 ± 13 (5.2 ± 1.8)	58 ± 2 (ND)	20 ± 1[b] (ND)
HSCb, high	288 ± 12[a] (8.3 ± 0.6)[a]	59 ± 5 (13.2 ± 1.4)	26 ± 2*** (19.6 ± 2.0)
LSCb	260 ± 42[a] (7.4 ± 1.5)[a]		
3 Months			
Controls	111 ± 15 (0.7 ± 0.4)	37 ± 2 (6.0 ± 1.3)	12 ± 1 (ND)
HSCb, low	120 ± 12 (2.0 ± 0.8)	39 ± 4 (ND)	11 ± 1 (ND)
HSCb, mid	154 ± 27 (3.3 ± 1.2)	51 ± 2 (ND)	11 ± 1 (ND)
HSCb, high	284 ± 16** (7.0 ± 2.2)[a]	104 ± 13*** (5.1 ± 1.0)	14 ± 1[b]* (ND)
LSCb	261 ± 14** (3.1 ± 1.0)[a]		
11 Months			
Controls	195 ± 23 (2.6 ± 0.3)	18 ± 3 (ND)	12 ± 1 (ND)
HSCb, low	276 ± 21 (3.1 ± 0.6)	21 ± 2 (ND)	10 ± 1 (ND)
HSCb, mid	224 ± 20 (2.4 ± 0.5)	18 ± 3 (ND)	10 ± 1[a] (ND)
HSCb, high	387 ± 33** (12.5 ± 1.4)[a]	12 ± 2 (ND)	12 ± 1[a]* (ND)
LSCb	299 ± 19[a] (7.9 ± 1.2)[a]		

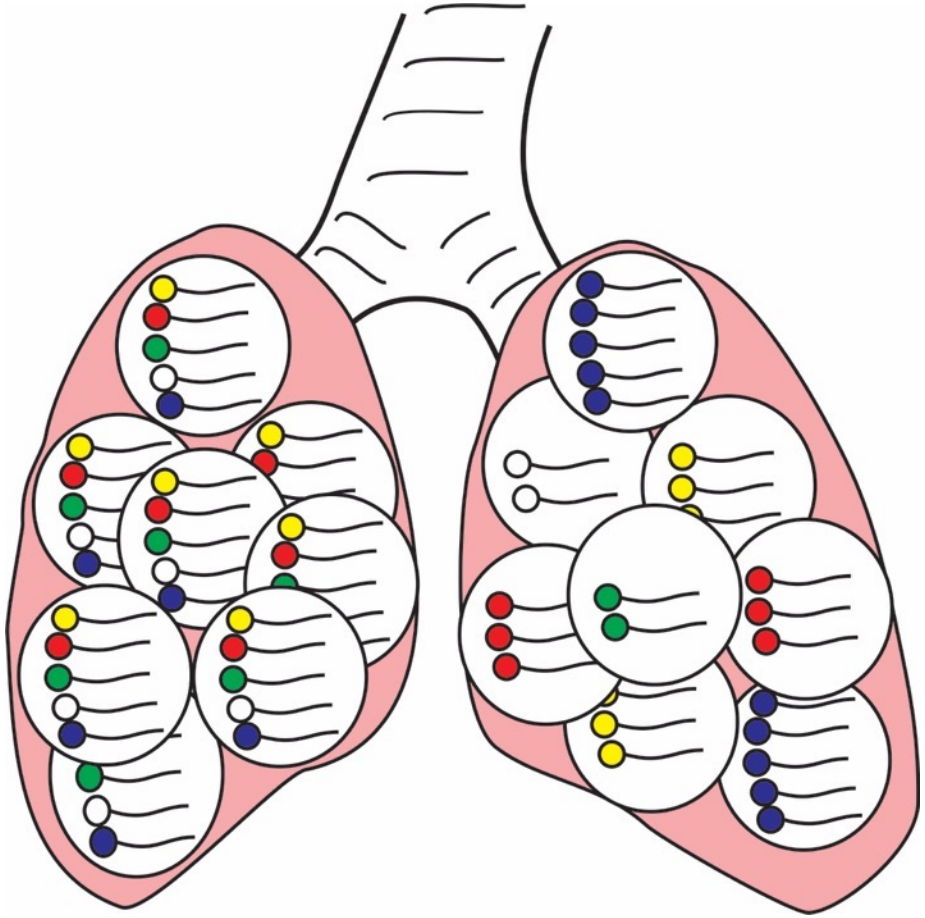
Suggested Carcinogenicity Assessment Process



Cohen SM et al. Reg Toxicol Pharmacol 103: 100-105, 2019

Single cell RNA sequencing – To better understand pathogenesis

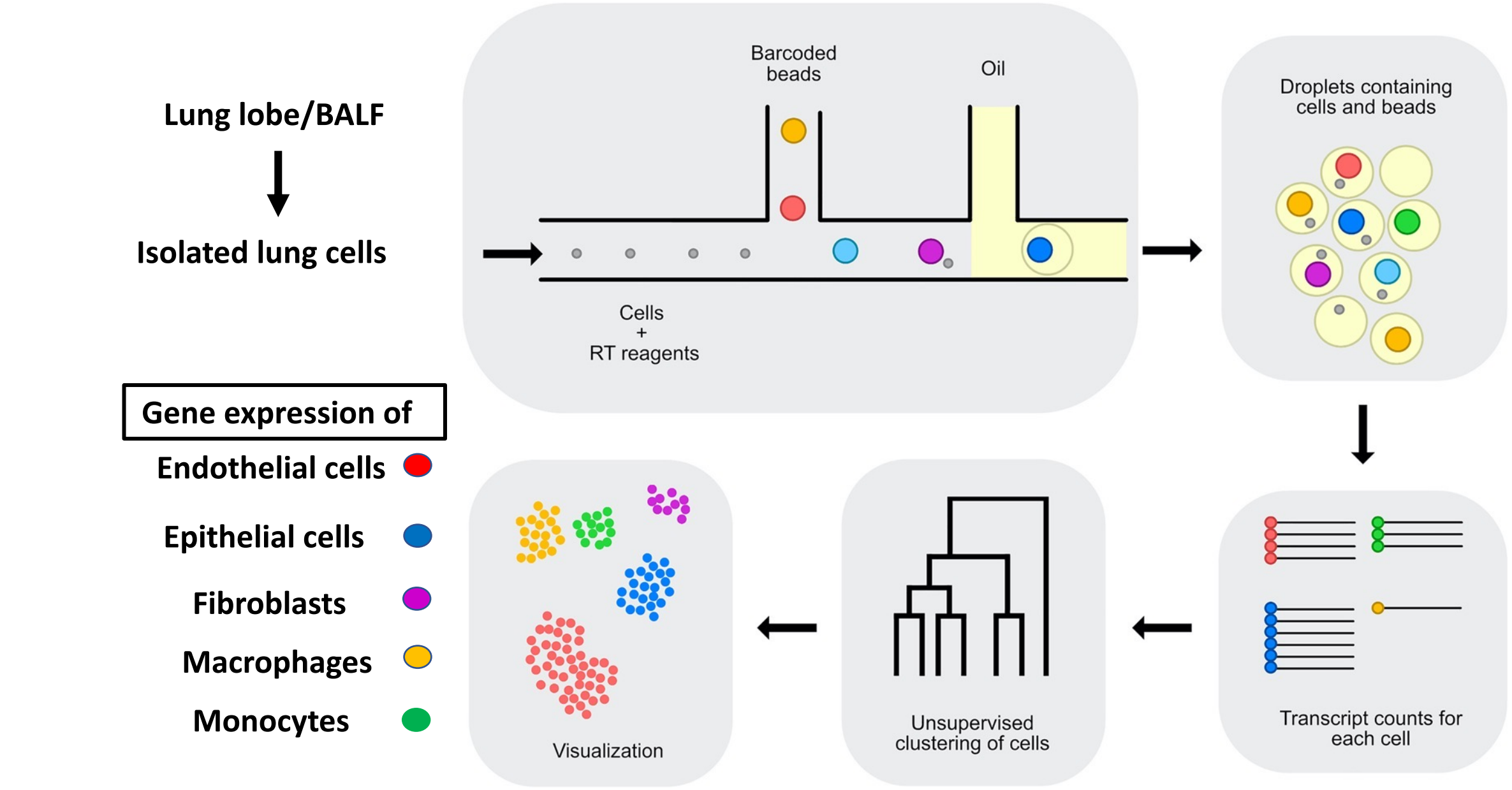
Bulk Tissue RNA Sequencing: Averages gene expression profile



Single-cell RNA Sequencing: Provides single cell gene expression profiling

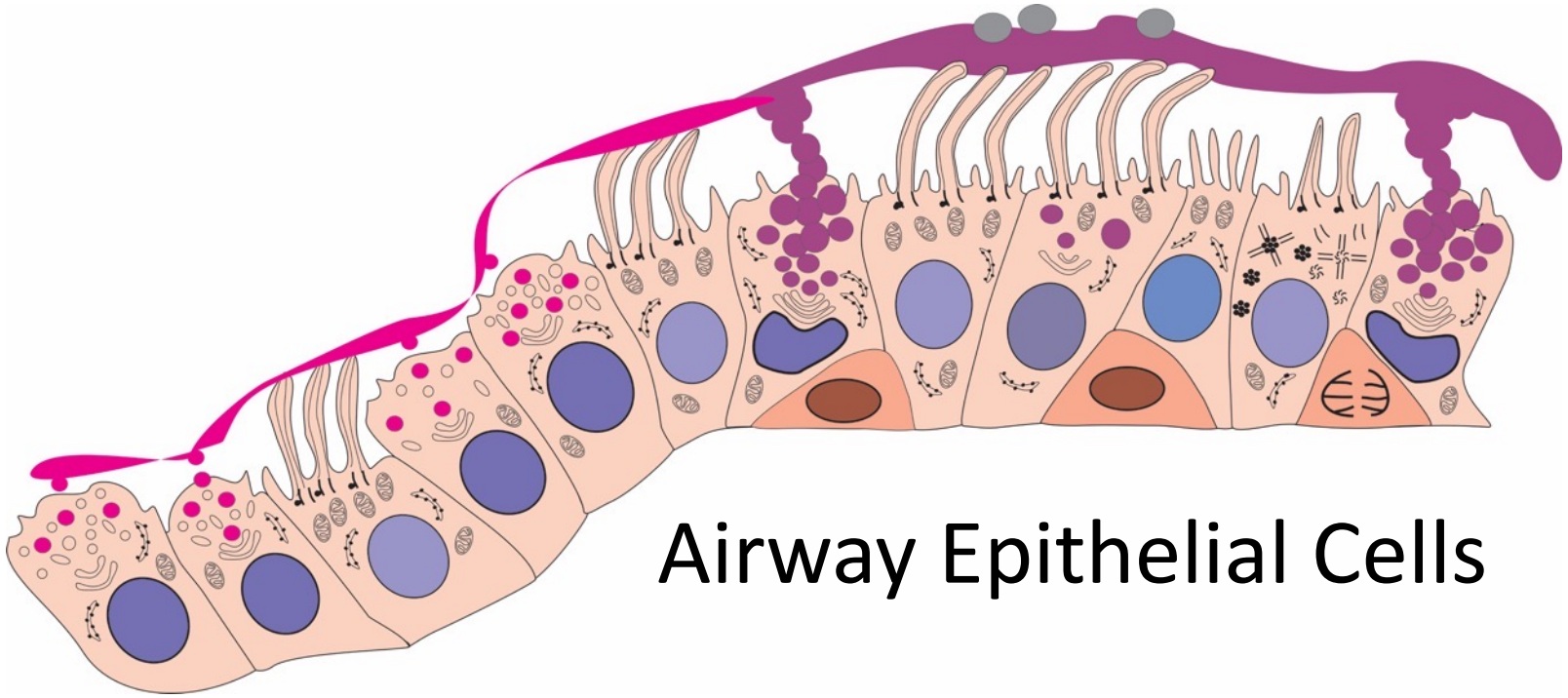
Modified from Chambers DC et al. (2019) Respirology 24, 29-36

Single cell RNA sequencing procedure

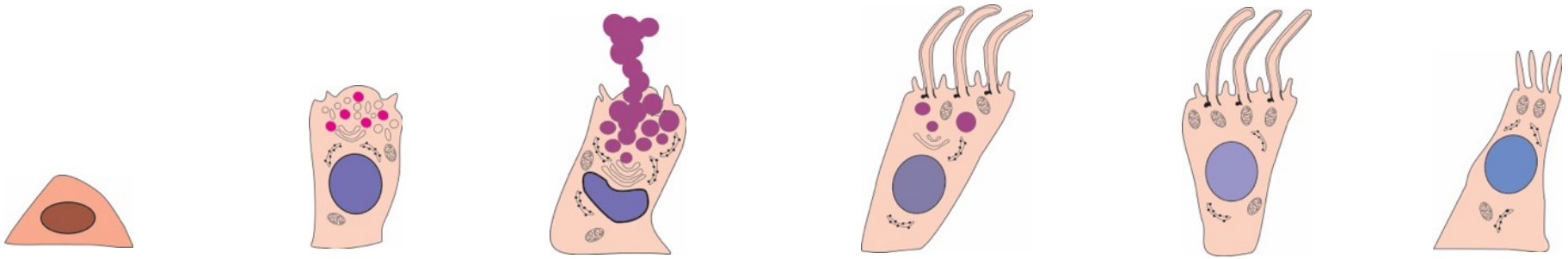


Modified from Chambers DC et al. (2019) Respirology 24, 29-36

Unraveling Airway Cell Lineage by Single Cell RNA Seq



Airway Epithelial Cells



Basal
(KRT5^{+}, TP63^{+})

Club
(SCGB1A1^{+})

Mucous
(MUC5AC^{+})

Mucous/Ciliated
(MUC5AC^{+}, FOXJ1^{+})

Ciliated
(FOXJ1^{+})

Tuft
(ASCL2^{+}, LRMP^{+})

Summary

- ✓Epithelial cell proliferation in toxicant-induced injury, repair, adaptation and cancer.
- ✓Pathogenesis of particle-induced proliferative and inflammatory responses (lesions) in rodent lungs (retrospective - subchronic Cb animal inhalation study)
- ✓Short-term ($\leq$ 3 mo) bioassays for assessing the safety of long-term exposures to inhaled PSLTPs (predicting lung cancer potential)
- ✓Ancillary methods to better assess the respiratory toxicity/cancer potential of inhaled particles after short-term exposures

Acknowledgments

- The Albert C. and Lois E. Dehn Endowed Chair in Veterinary Medicine (Pathobiology), College of Veterinary Medicine, Michigan State University

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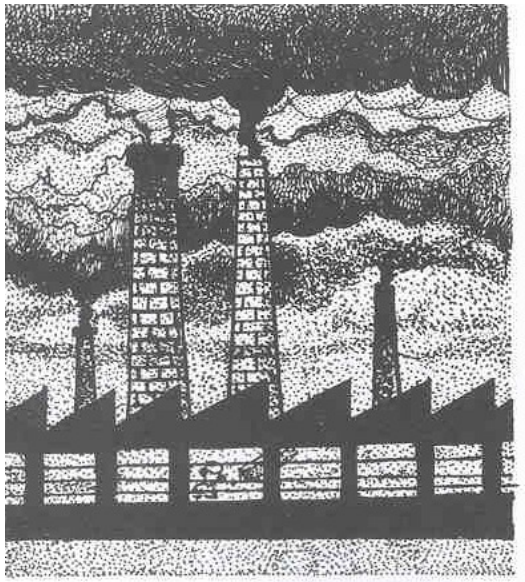
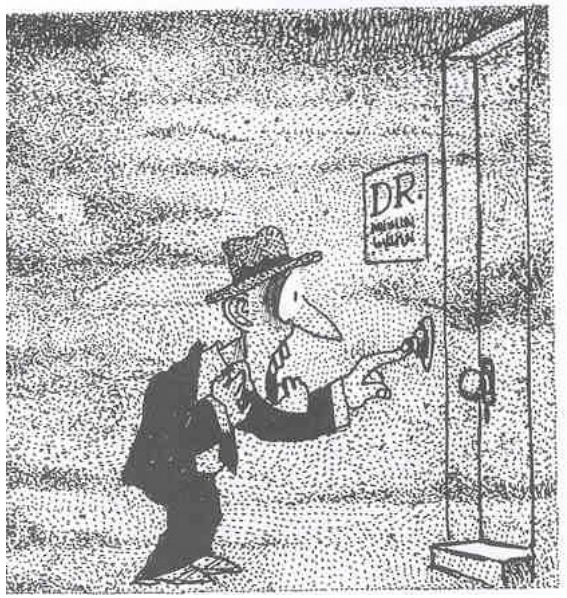
- Flemming Cassee, RIVM/Utrecht University, NL

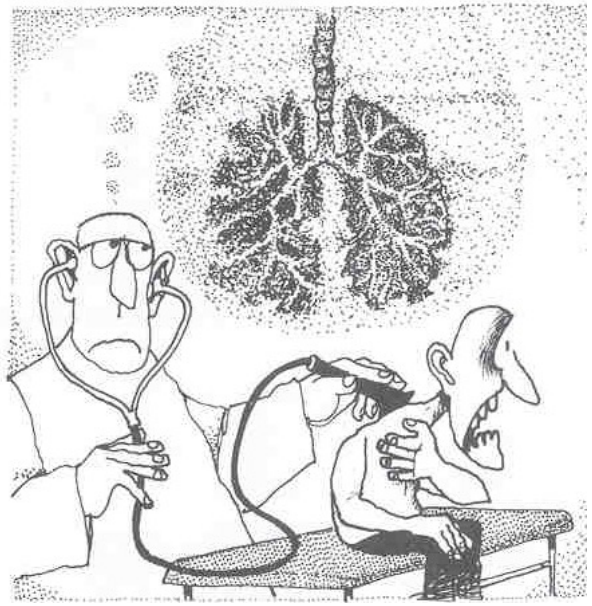
- Bart Westendorp and Gimano Amatngalim, Utrecht University, NL

- Paul Borm and Kevin Driscoll

Thank You



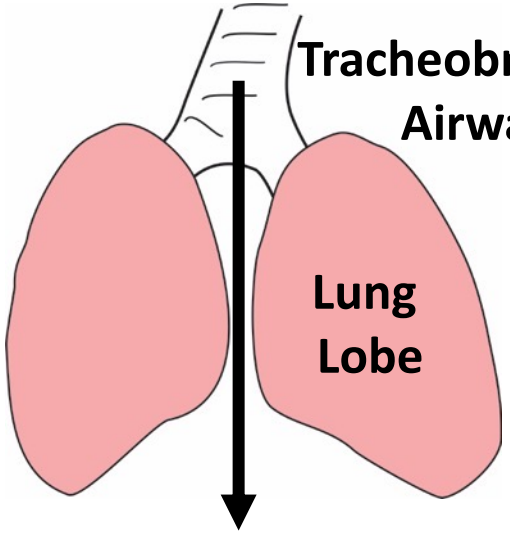




Quino, 2008

Questions?

Human/animal organoids for disease modeling

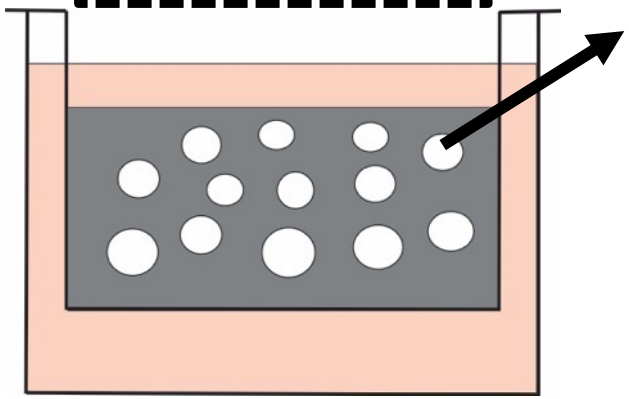


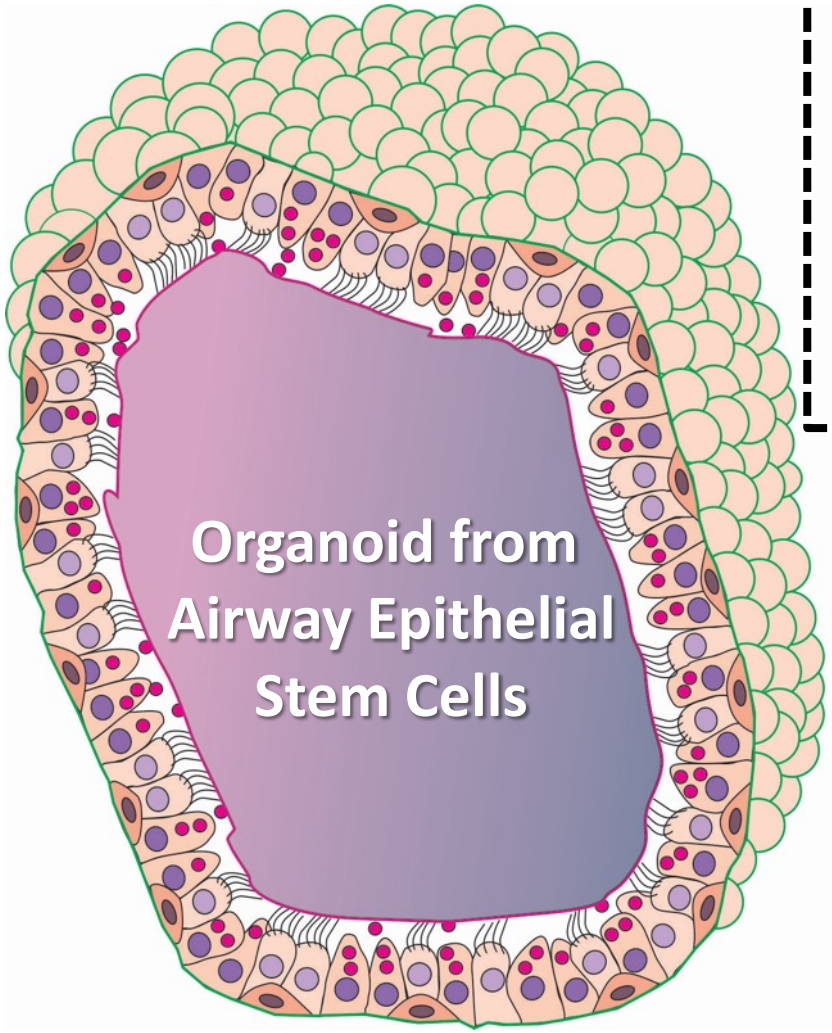
Collection of
Epithelial Cells

Expand cells in
2D Cultures

FAC/MAC
Sorting

3D Cultures with
Matrigel



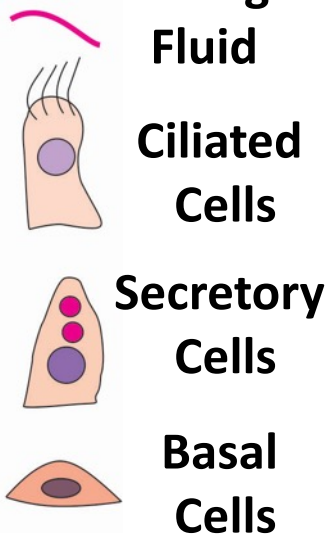


Analyses:

Histology

Morphometry

Gene Expression



Prevalence of Lung Neoplasms in Female Rats Chronically Exposed to DE/CB

Study Design:

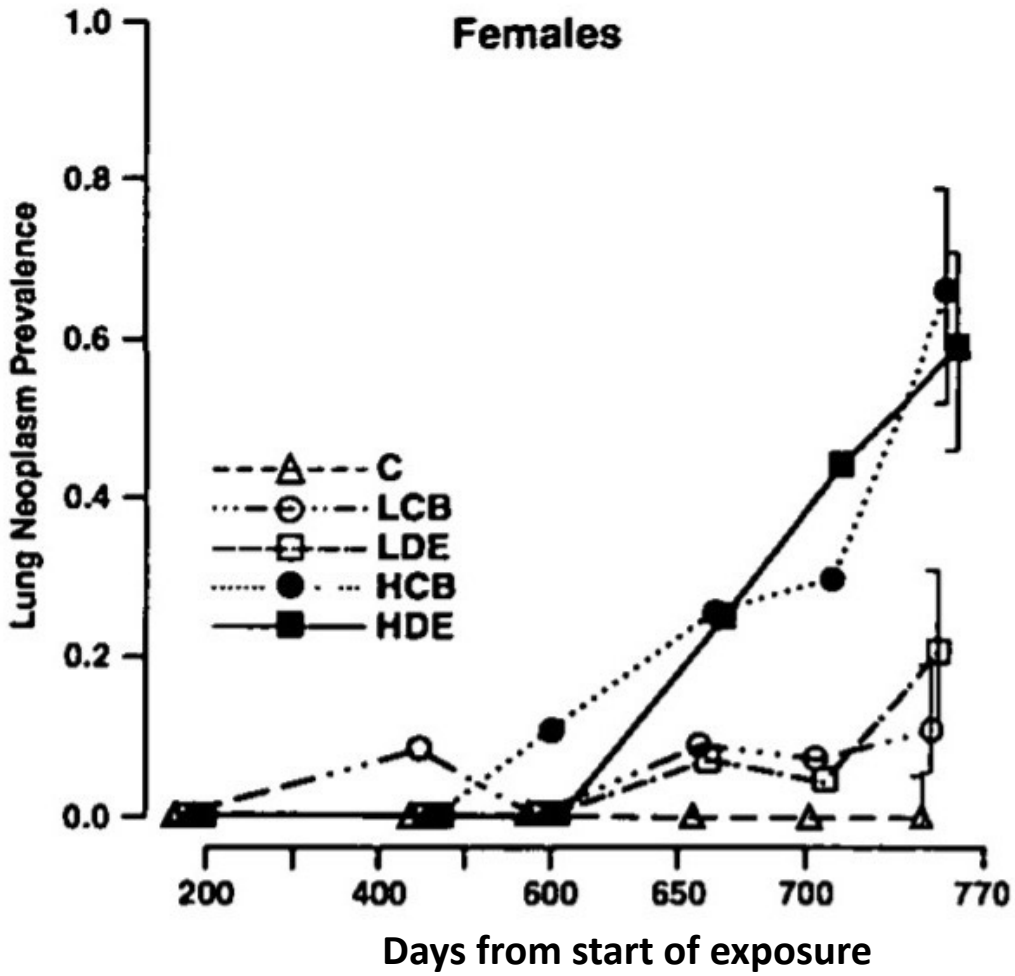
- Male and Female F344 rats
- Chronic DE or CB exposure, 5 days/wk at 0 mg/m3 (control), 2.5 mg/m3 (LDE, LCB) or 6.5 mg/m3 (HDE, HCB)
- Examination after 3, 6, 12, 18 or 23 mo

Results:

- No significant differences between carcinogenic potencies of CB and DE

Conclusion:

- Organic fraction of DE might not play an important role in lung carcinogenicity of DE exposure in rats



Nikula KJ et al. Fund Appl Toxicol 25, 80-94, 1995