NTP
National Toxicology Program

Molecular Pathology Studies of Mesothelioma in VDC-exposed F344/N Rats

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NTP Technical Reports Peer Review Meeting
October 29th, 2013

NTP Molecular Pathology Studies

- Programmatic effort to add value to the NTP Technical Reports by providing molecular data
- Molecular data is supportive and complementary to the NTP pathology tumor assessment
- Exploratory studies to provide a better understanding of potential mode of action related to chemical treatment in rodents
- Not used for the levels of evidence call

NTP Molecular Pathology Studies

- Investigative Pathology Group uses a variety of techniques to generate informative data

- Molecular characterization of chemically induced rodent tumors
  - Adjunct to pathology assessment to gain additional understanding of chemical carcinogenesis
  - Differentiate chemically induced tumors from background spontaneous tumors
  - Identify similarities to human cancers based on molecular phenotypes

- Generates supplementary and supportive data for the NTP on molecular characterization of chemically induced rodent tumors

NTP Molecular Pathology Studies
Sample Selection

- Sample collection at study laboratory:
  - Collection triggered when treatment-related increase observed
  - Spontaneous and treatment-related tumors > 0.5cm diameter
  - Sectioned in half, one half fixed for histopathology, one half flash frozen in liquid nitrogen
- Sample selection for analysis:
  - Based on tumor size/weight - sample concentration and quality
  - Availability of samples in NTP frozen archives
  - Sample viability/quality as assessed by histopathology
    - Minimal hemorrhage and necrosis

NTP Molecular Pathology Studies

- Molecular/Investigative strategies
  - Gene expression (microarray, qPCR arrays)
  - DNA mutation analysis
  - Epigenetics (methylation arrays, pyrosequencing)
  - Protein analysis (IHC, western blotting)
  - Cell culture (in vitro validation), special techniques (LCM)

- Use of these techniques in NTP studies is important in better understanding of how a chemically-induced tumor is different from spontaneous

NTP Molecular Pathology Studies

- Must first understand biology of spontaneous background tumors
- Genomic databases developed to compare chemically induced tumors
- Hepatocellular carcinoma, pulmonary carcinoma, mesothelioma

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Global Gene Profiling of Spontaneous Hepatocellular Carcinoma in B6C3F1 Mice: Similarities in the Molecular Landscape with Human Liver Cancer

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DOI: 10.1177/0192623312447543

Differential Transcriptomic Analysis of Spontaneous Lung Tumors in B6C3F1 Mice: Comparison to Human Non–Small Cell Lung Cancer

Arun R. Pandiri[1,2], Robert C. Sills[1], Vincent Ziglioli[3], Thai-Vu T. Ton[1], Hue–Hua L. Hong[1], Stephanie A. Lahousse[1], Kevin E. Gerrish[4], Scott S. Auerbach[5], Keith R. Shockley[6], Pierre R. Bushel[6], Shyamal D. Peddada[6], and Mark J. Hoenerhoff[1]

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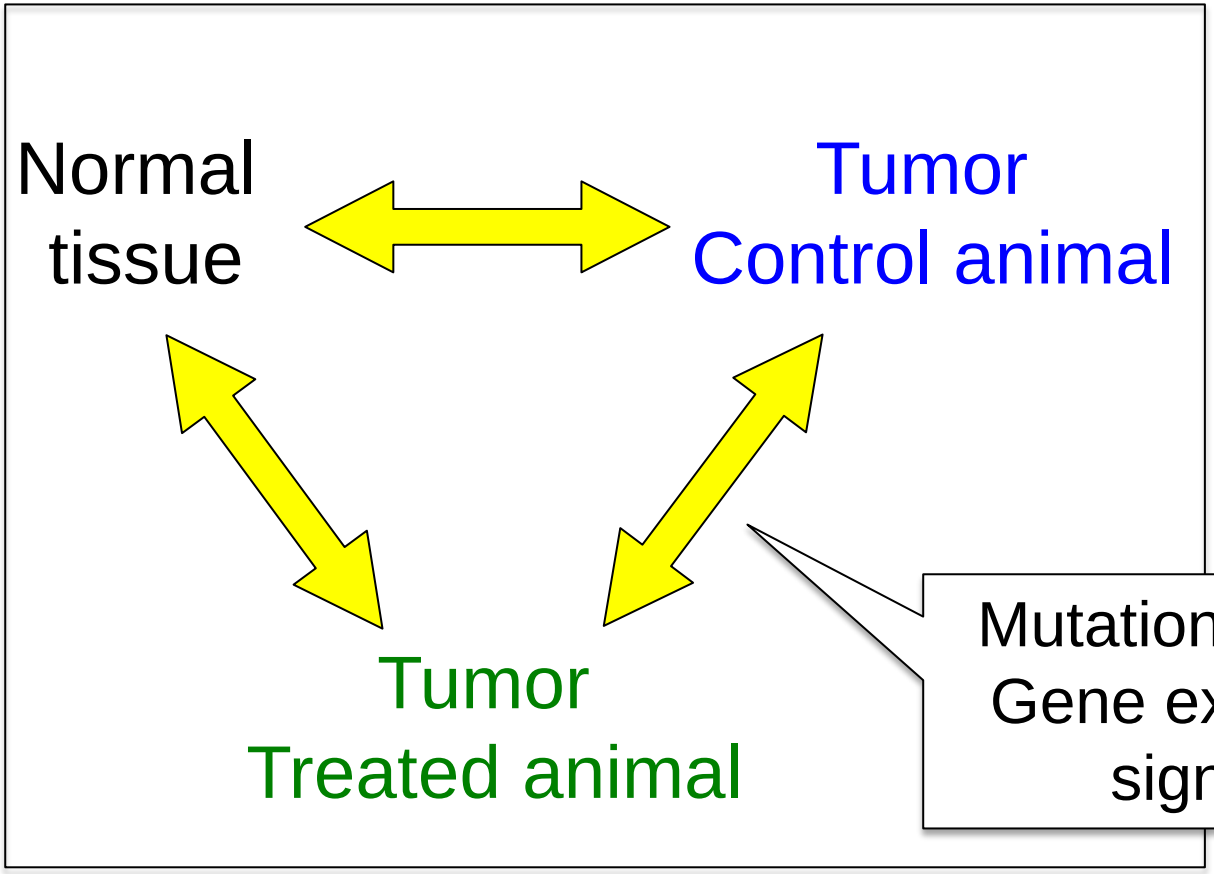
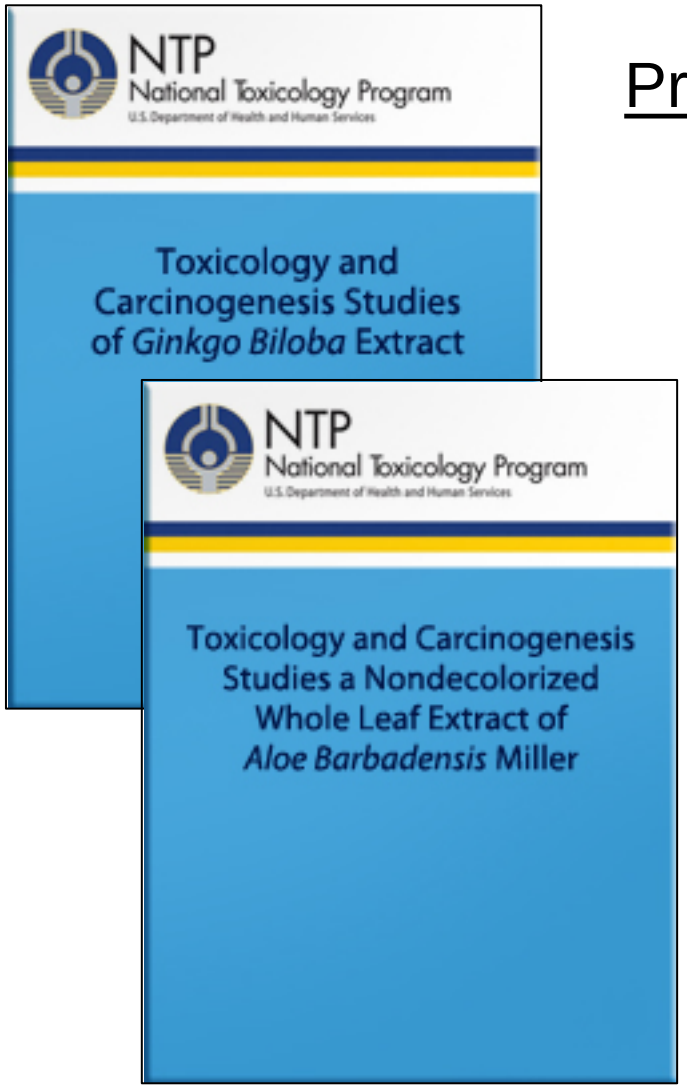
Gene expression alterations observed in human cancer

NTP Molecular Pathology Studies



What molecular changes occur in response to chemical exposure? Relevant to human cancer?

NTP Molecular Pathology Studies - Previous



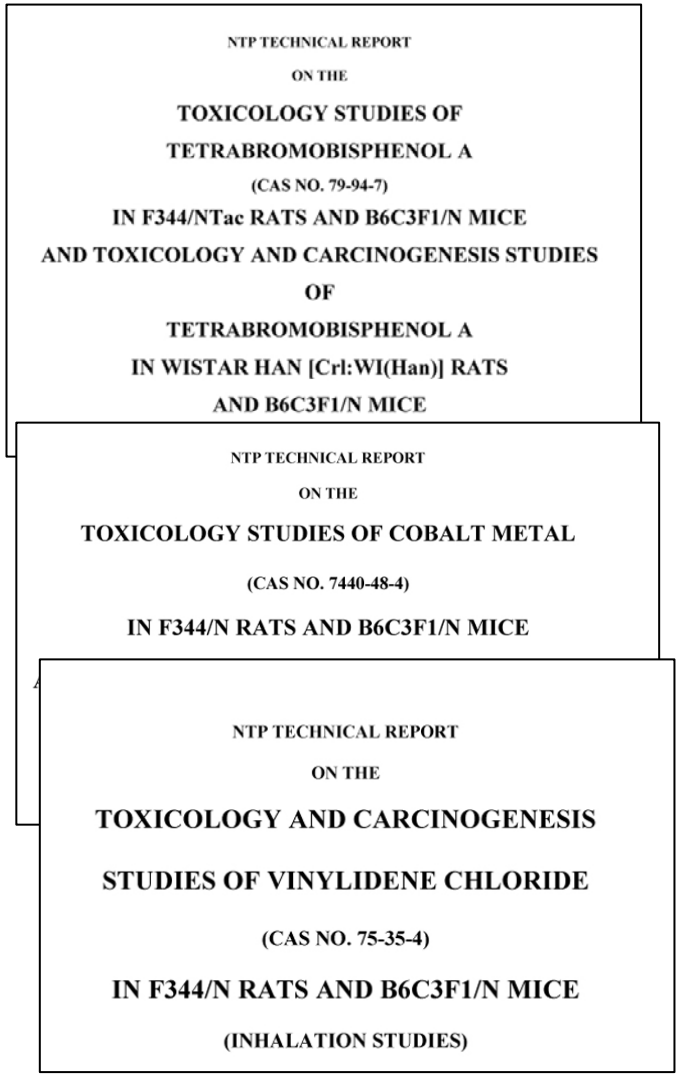
Previous NTP Molecular Studies

Herbal Supplements

Aloe vera extract (TR577)
- Colorectal tumors (rats)
- qPCR gene expression arrays

Ginkgo biloba extract (TR578)
- Hepatocellular carcinoma (mice)
- Gene expression microarray

NTP Molecular Pathology Studies – Current


NTP TECHNICAL REPORT
ON THE
TOXICOLOGY STUDIES OF
TETRABROMOBISPHENOL A
(CAS NO. 79-94-7)
IN F344/NTac RATS AND B6C3F1/N MICE
AND TOXICOLOGY AND CARCINOGENESIS STUDIES
OF
TETRABROMOBISPHENOL A
IN WISTAR HAN [Crl:WI(Han)] RATS
AND B6C3F1/N MICE

NTP TECHNICAL REPORT
ON THE
TOXICOLOGY STUDIES OF COBALT METAL
(CAS NO. 7440-48-4)
IN F344/N RATS AND B6C3F1/N MICE

NTP TECHNICAL REPORT
ON THE
TOXICOLOGY AND CARCINOGENESIS
STUDIES OF VINYLIDENE CHLORIDE
(CAS NO. 75-35-4)
IN F344/N RATS AND B6C3F1/N MICE
(INHALATION STUDIES)

Flame Retardants

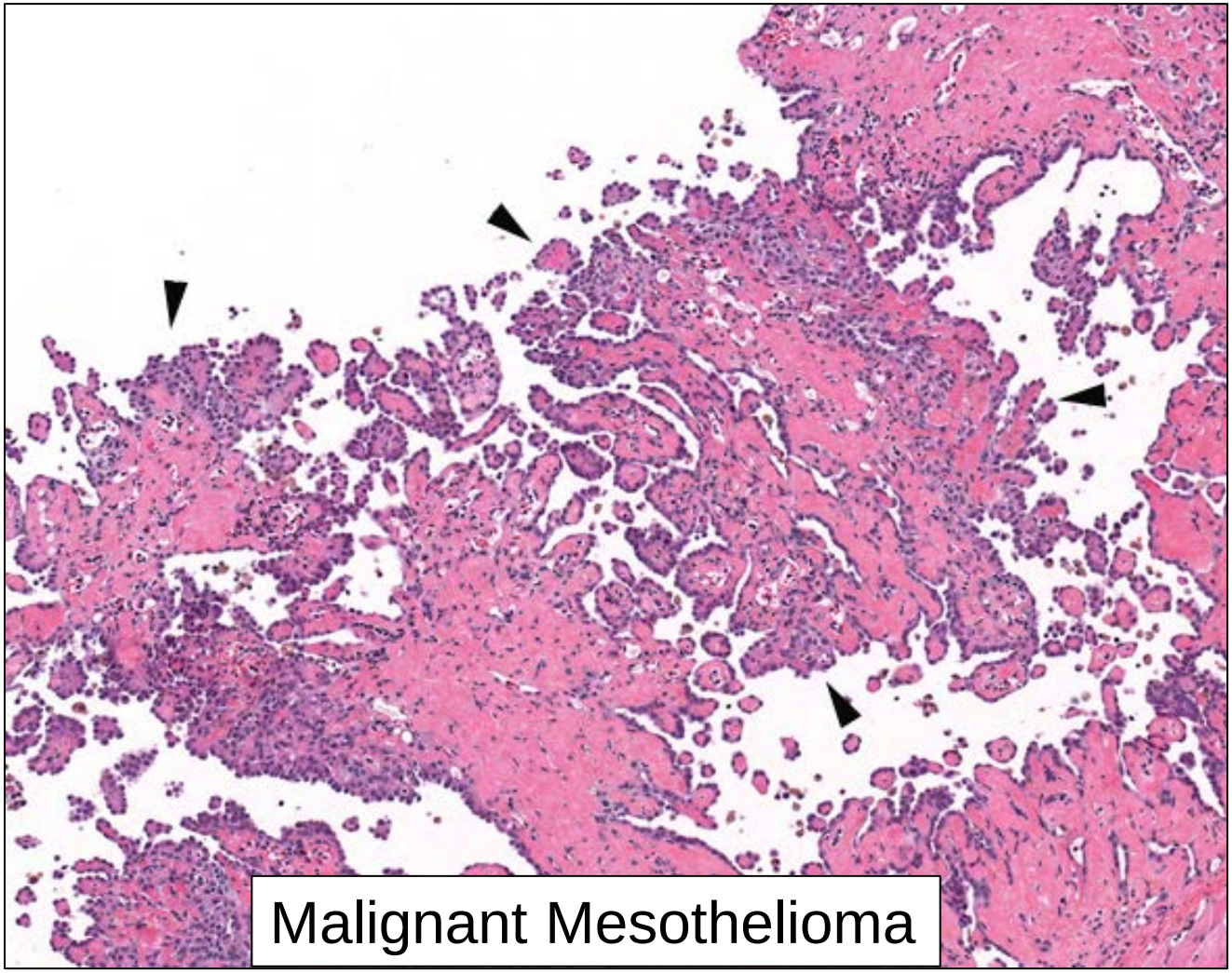
Tetrabromobisphenol A (TR587)
- Uterine carcinoma (rats)
- Gene mutation analysis

Occupational Hazards

Cobalt metal dust (TR581)
- Pulmonary carcinoma (mice)
- Large scale gene mutation analysis

Vinylidene chloride (TR582)
- Mesothelioma (rats)
- Global gene expression microarray

Gene expression studies of mesothelioma in VDC-exposed F344/N rats



Malignant Mesothelioma – F344/N Rats

- Low incidence of spontaneous mesothelioma
  - Males: All routes: 26/699, 3.7%; Inhalation studies: 1/200, 0.5%
  - Females: All routes: 0/700, 0%; Inhalation studies: 0/200, 0%

- May be chemically induced by a variety of compounds
  - o-Nitrotoluene, Bromochloroacetic acid, fibers

- Molecular features of rat mesothelioma:
  - Oncogenes (*Mafb, Myc, v-Yes*), tumor suppressor genes (*Tp53*, *Pten, Rassf1*)
  - Cell cycle dysregulation (*p21, p27, p16*)
  - Growth pathway activation (*Tgfβ*, *Igf1*, *Akt*, *Ctnnb1, Ras/Mapk*)

-Kim *et al.*, Toxicol Appl Pharmacol 2006; Blackshear *et al.*, Toxicol Pathol, 2013

Malignant Mesothelioma – Research Approach

- Characterize gene expression profiles in mesotheliomas from VDC-exposed F344/N rats

- Compared global gene expression profiles by microarray
  - VDC-exposed mesotheliomas (n = 8)
  - Spontaneous mesotheliomas from three other NTP studies (n = 5)
  - Fred-PE immortalized non-transformed cell line as control (n = 6)

Malignant Mesothelioma – Vinylidene Chloride Sample Selection

Dose (ppm)	Animal No.	Location
25	236	Peritoneum
50	401	Mesentery
50	402	Mesentery
100	601	Testes capsule
100	613	Testes capsule
100	632	Testes capsule
100	640	Testes capsule
100	646	Testes capsule

- Eight tumor samples used in final analysis

Malignant Mesothelioma – Spontaneous
Sample Selection

Study	Route	Animal No.	Location
Riddelliine	Gavage	5	Peritoneum
Codeine	Feed	19	Mesentery
Cobalt	Inhalation	34	Mesentery
Cobalt	Inhalation	34	Peritoneum
Cobalt	Inhalation	34	Testes capsule

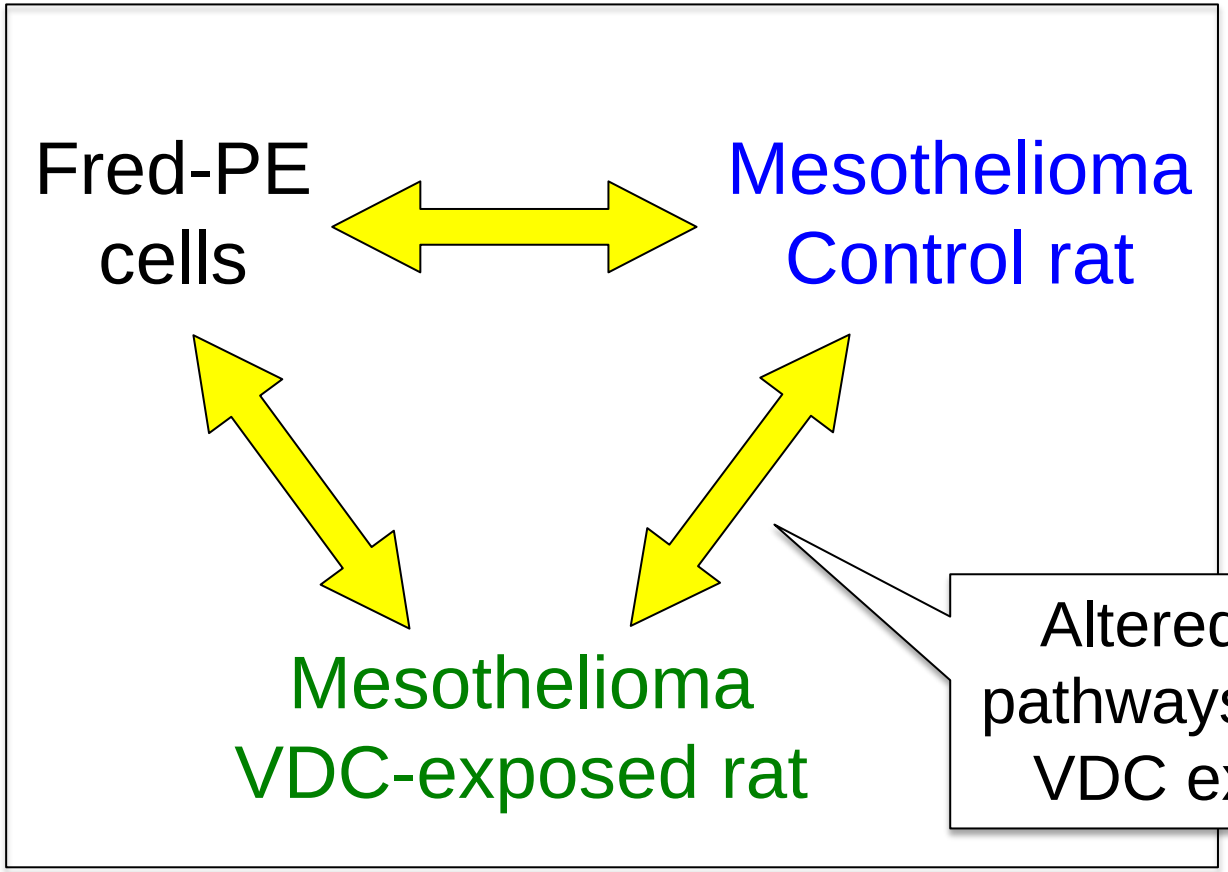
- Five tumors (3 animals) available for analysis
- Uncommon spontaneous tumor
  - (Historical control same route 0% (0/150), all routes 3% (41/1249)

Malignant Mesothelioma – Fred-PE mesothelial cells Sample Selection

- Mesothelial cells cultured from peritoneal cavity of F344 rats
  - Originally isolated by Dr. Fred Angelo, EPA
  - Nontransformed, immortalized mesothelial cell line
  - Commonly used as control tissue in rat mesothelioma studies
  - Mesothelial character confirmed by dual staining with pan-cytokeratin and vimentin

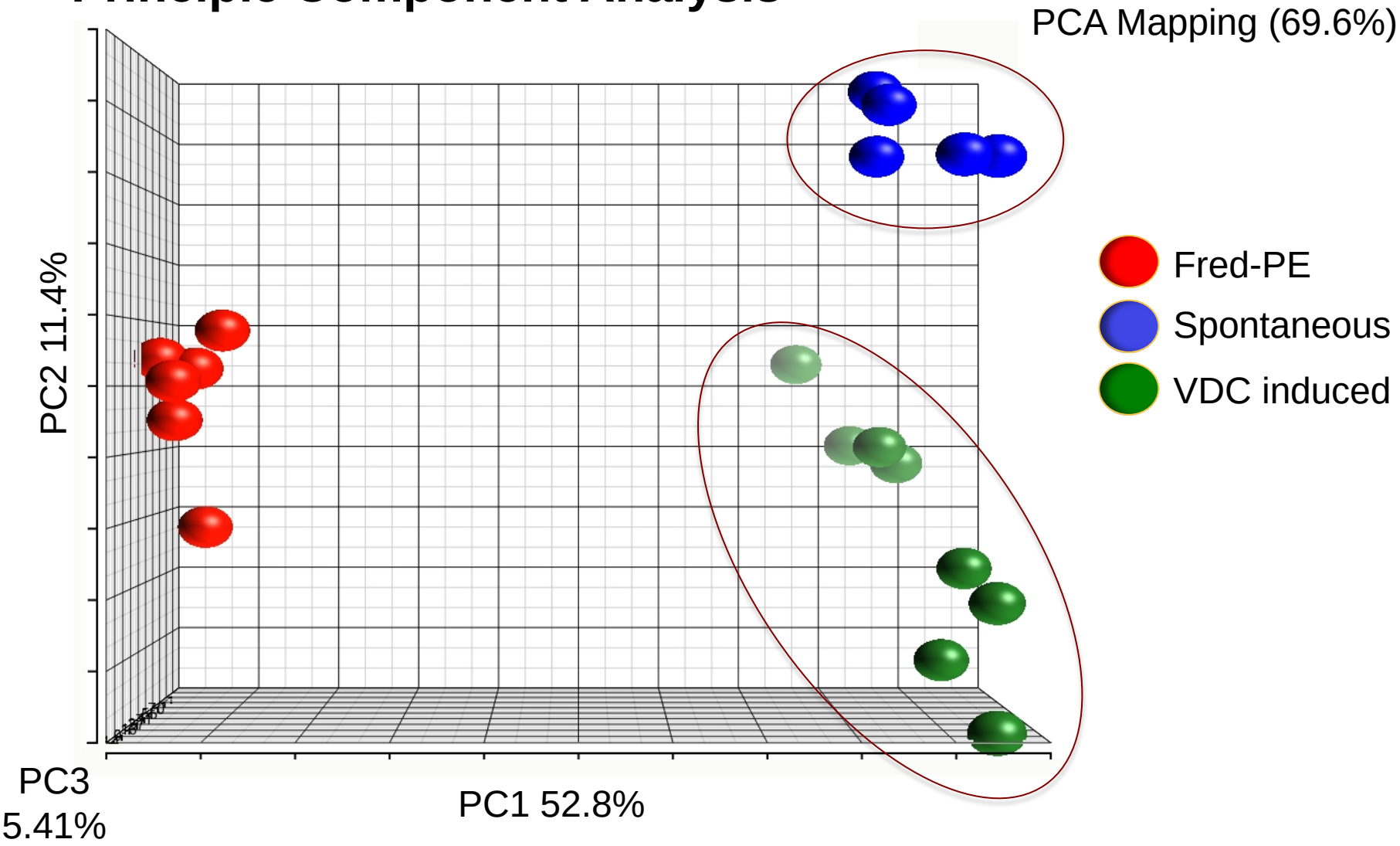
- RNA from these cells was a generous gift from Dr. Yongbaek Kim, North Carolina State University
  - Six samples of varying passages

NTP Molecular Pathology Studies

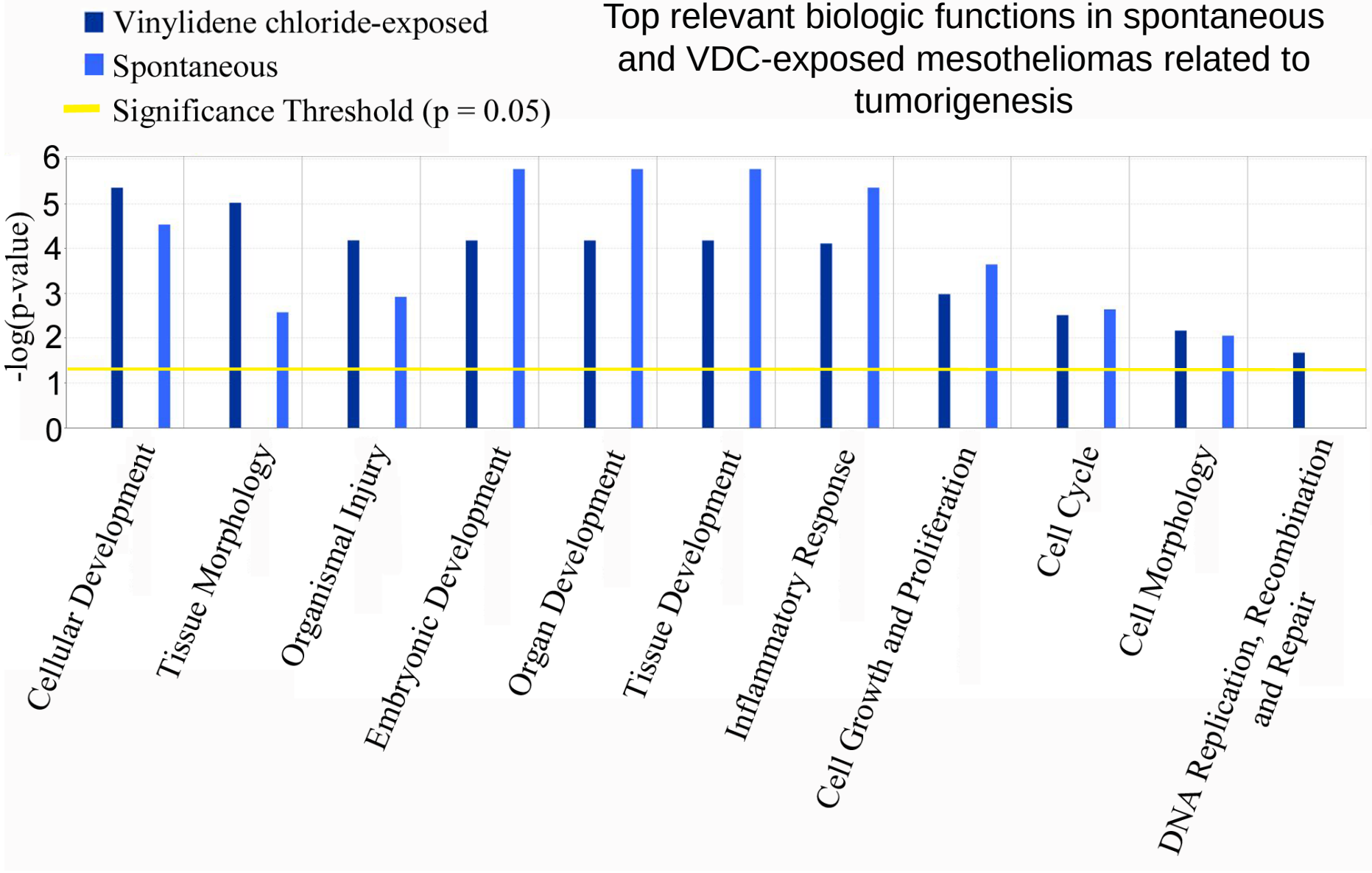


How are mesotheliomas from VDC-exposed animals different from spontaneous?

Malignant Mesothelioma – Vinylidene Chloride
Principle Component Analysis



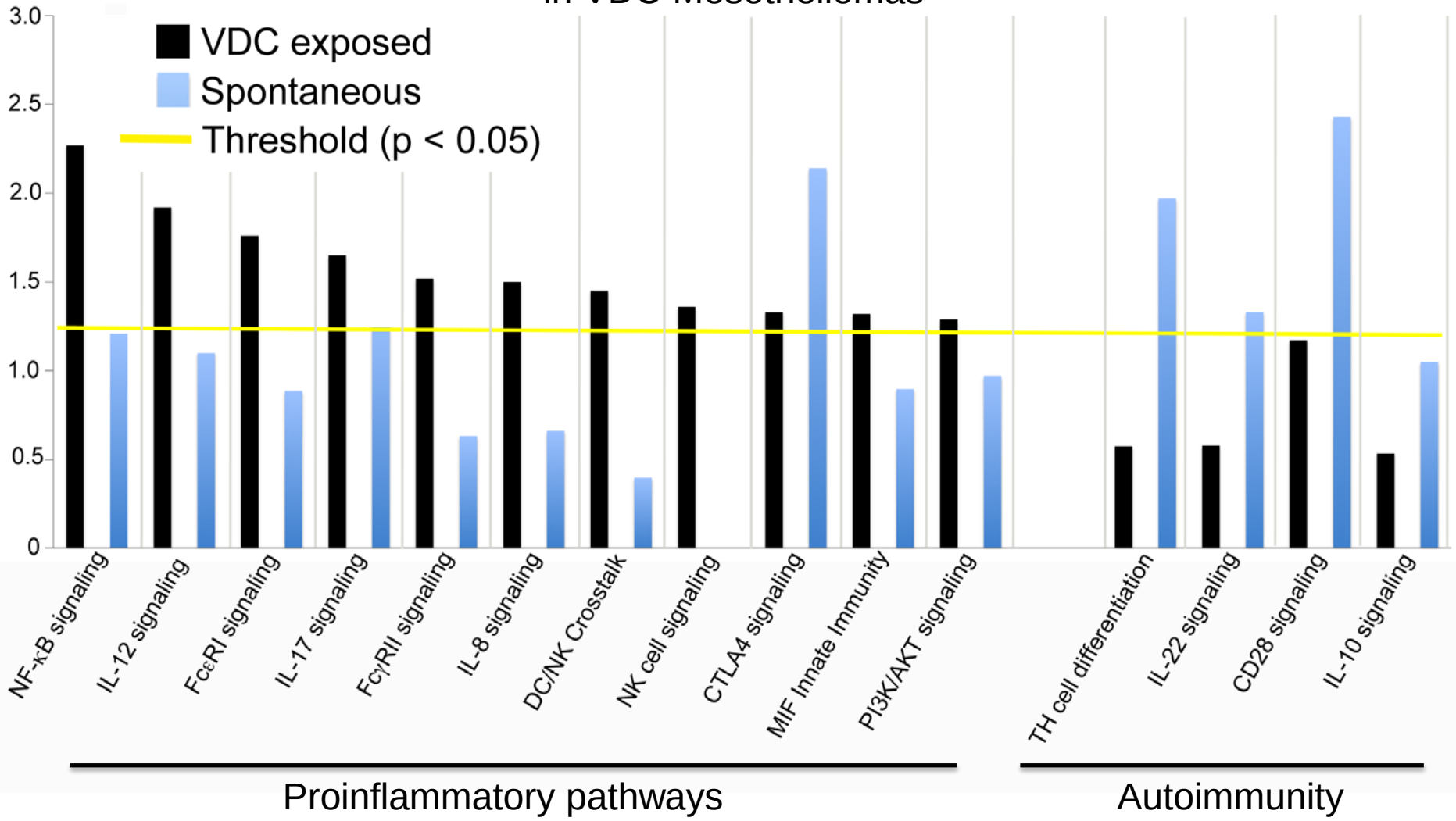
Top relevant biologic functions in spontaneous and VDC-exposed mesotheliomas related to tumorigenesis



Genes from Top Overrepresented Oncogenic Pathways in VDC and Spontaneous Mesotheliomas

Gene Category	Gene symbols
Growth factors	*Tgfβ2, Tgfβr1, Tgfβi, Vegfc, Fgfr2, Igf1, Igf2bp1, Igfbp3,6*
Cell cycle	*Cdkn1a, Cdkn1b*
Oncogenes	*Mafb, Fos, Junb, v-yes*
Ras/Mapk pathway	*Rasd1, Rnd1, Rnd3, Prkcb, Mapk12, Mapkapk3*
Tumor suppressors	*Tp53, Lats2*
Adhesion molecules	*Epcam, Cdh22, Ctnnb1, Itgb2*
Apoptosis/arrest	*Gadd45b, Bcl2a1, Faim3*
Embryonic genes	*Plac8, Wnt4, Plau, Gata5*
Matrix remodelers	*Epcam, Col6a1, Col6a2, Itgb2*
Transporters	*Slc7a7, Slc7a9, Slc28a2, Abca4*
Mesothelial markers	*Krt18, Krt19, Thbd, Des*
Reactive O2 species	*Duox2, Gpx2*

Overrepresentation of Pro-inflammatory/Immune Pathways in VDC Mesotheliomas



Genes from Overrepresented Pathways in Vinylidene Chloride-Exposed Mesotheliomas

Gene Category	Gene symbols
Chemokines	*Ccl5, Ccl6, Ccl11, Ccl27, Cxcl9, Cxcl11*
Cytokines/receptors	*Il1b, Lk18, Il34, Il6r, Il7r, Tnfrsf11b, Il10, Il24, Il1rn, Cd40*
Jak-Stat Pathway	*Stat1, Stat2, Jak2*
Complement	*Cfh, C1qb, C1qa, SerpinG1*
Pattern Recognition Rcpts	*Tlr2, Tlr7, Tlr8, Mrc1*
Interferon Pathway	*Ifngr1, Irf5, Irf9, Ifitm1*
Inflammatory Mediators/DAMPs	*Aif1, Ptgds, Ptgs1, Ptgs2, Lyz2, Mcpt10, Tdo2, Ubd, Ddx60, Cybb, Pla2g2a, Lyve1*
Activated Macrophages	*Chi3l1, Sparcl1, C1qb, C1qa, S100a8, S100a9*
Cell Surface Receptors	*S1pr1, Fcgr2b, Fcer1a,1g, Fcgr1a,2a,3a, Stab1, Cd163, Cd36, Cd68, Cd53, Clec4a, Clec4a3, Clec7a, Clec10a*

Gene expression suggestive of proinflammatory response and immune dysregulation

Malignant mesothelioma – Vinylidene chloride Discussion

- Differentiated mesothelioma from VDC-exposed and vehicle control rats based on genomic profiling
  - Despite indistinguishable morphology
- Similarities in oncogenic and tissue remodeling pathways
- Overrepresentation of proinflammatory pathways and immune dysregulation in VDC mesotheliomas
- Exposure to VDC: glutathione pathway saturation, reactive VDC metabolites → tissue damage
- Additional studies needed to further define the role of inflammatory signature in the induction of mesothelioma in VDC-exposed rats

Questions?

- NTP Leadership
- NTP Investigative Pathology
  - Arun Pandiri, DVM, PhD
  - Sachin Bhusari, DVM, PhD
  - Lily Hong
  - Kiki Ton
- NTP Toxicologists
  - Michael Wide, PhD (VDC)
  - Mamta Behl, PhD (Cobalt)
  - Michelle Hooth, PhD (Cobalt)
  - June Dunnick, PhD (TBBPA)
- Biomolecular Screening Branch
  - Scott Auerbach, PhD
  - Alex Merrick, PhD
- CMPB Pathologists
- NIEHS Microarray Core
  - Rick Paules, PhD
  - Kevin Gerrish, PhD
- Bioinformatics and Biostatistics
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  - Keith Shockley, PhD
  - Grace Kissling, PhD
- NTP Archives
- NIEHS Core Labs
  - Histology, Immunohistochemistry
  - Laser Capture Microdissection