Published in final edited form as:

Toxicol Pathol. 2014 July ; 42(5): 863–876. doi:10.1177/0192623313501894.

Spontaneous Mesotheliomas in F344/N Rats are Characterized by Dysregulation of Cellular Growth and Immune Function Pathways

Pamela E. Blackshear⁴, Arun R. Pandiri⁵, Thai-Vu T. Ton¹, Natasha P. Clayton¹, Keith R. Shockley², Shyamal D. Peddada², Kevin E. Gerrish³, Robert C. Sills¹, and Mark J. Hoenerhoff¹

¹Cellular and Molecular Pathology Branch, Division of the National Toxicologic Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

²Biostatistics Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

³Microarray Core, Toxicology and Pharmacology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

⁴Integrated Laboratory Systems, Inc., Research Triangle Park, North Carolina

⁵Experimental Pathology Laboratories, Inc., Research Triangle Park, North Carolina

Abstract

Aged male Fischer 344/N rats are prone to developing spontaneous peritoneal mesotheliomas, which arise predominantly from the tunica vaginalis of the testes. A definitive cause for the predominance of this neoplasm in F344/N rats is unknown. Investigation of the molecular alterations that occur in spontaneous rat mesotheliomas may provide insight into their pathogenesis, as well enable a better understanding regarding the mechanisms underlying chemically induced mesothelioma in rodents. Mesothelial cell function represents a complex interplay of pathways related to host defense mechanisms and maintenance of cellular homeostasis. Global gene expression profiles of spontaneous mesotheliomas from vehicle control male F344/N rats from two-year National Toxicology Program carcinogenicity bioassays were analyzed to determine the molecular features of these tumors, and elucidate tumor-specific gene expression profiles. The resulting gene expression pattern showed that spontaneous mesotheliomas are associated with upregulation various growth factors, oncogenes, cytokines, pattern recognition response receptors (PRR) and pathogen associated molecular patterns (PAMP) receptors, and the production of reactive oxygen and nitrogen species, as well as downregulation of apoptosis pathways. Alterations in these pathways in turn trigger molecular responses that stimulate cell proliferation and promote tumor survival and progression.

Keywords

Mesothelioma; F344/N rat; National Toxicology Program; microarray; gene expression; mesothelial cell; Fred-PE cells

Introduction

Mesothelial cells form the lining of the heart, pleural and peritoneal cavities as well as visceral surfaces of organs. The mesothelial lining of the peritoneum in males extends into the scrotum to form the tunica vaginalis, which covers the testes and the epididymis (Hall *et al.* 1990). The mesothelial lining consists of a single layer of two morphologically distinct cell types, 1) flattened cells and 2) dome-shaped cuboidal to polygonal cells, overlying a very thin fibrovascular stroma. The flattened cells are held together by apical tight junctions, adherens junctions, zona occludens, and desmosomes (Antony 2003; Madison *et al.* 1979), and the dome-shaped cells surround pores that connect endothelial cells with the mesothelium, thus providing a direct role for the mesothelial cell in fluid and large molecule transport between the peritoneal cavity and lymphatic system (Hall *et al.* 1990). Both domed and flattened mesothelial cells bear microvilli, which are involved in the absorption of fluid and maintenance of lubrication of peritoneal and pleural cavities. Mesothelial cells are a dynamic cell population, derived from embryonic mesoderm, and provide innate immune mechanisms and multiple cellular functions that aid in maintaining homeostasis for the pleural and peritoneal cavities. Mesothelial cells are capable of pinocytocytosis of small particles as well as phagocytosis of large particles. Their cytoplasm contains microfilaments, which are involved in cell movement and cytoskeletal rearrangement, as well as proper function of mitochondria, smooth and rough endoplasmic reticulum, and pinocytotic vesicles. Mesothelial cells have both epithelial and mesenchymal characteristics; they express a broad range of cytokeratins as well as vimentin, and when activated, they express desmin and smooth muscle actin (Afify *et al.* 2002; Kupryjanczyk and Karpinska 1998; Yung and Chan 2011). Additionally, they produce hyaluronic acid and express a variety of cell surface receptors including CD44 (Nasreen *et al.* 2002; Yung and Chan 2011), pathogen associated molecular pattern (PAMP) receptors (e.g. CD14), and pattern recognition receptors (PRR) such as the toll-like receptors (TLRs), receptors for advanced glycation end products (RAGE) (Jantz and Antony 2008), and mannose receptors.

Mesotheliomas occur rarely in rats and humans, and are characterized by the neoplastic transformation of pleural or peritoneal mesothelial cells. In humans, these tumors have a long latency period (Hall *et al.* 1990) and an insidious nature that makes the diagnosis of mesotheliomas challenging, prognosis poor, and treatment options limited. Mesotheliomas occur at a low incidence in aged male F344/N rats (3.2%, all routes of vehicle exposure, all vehicles, NTP historical controls) (National Toxicology Program 2011), and arise most commonly from the tunica vaginalis (Hall *et al.* 1990). In females, mesotheliomas arise much less frequently (0.17%, all routes of vehicle exposure, all vehicles) (National Toxicology Program 2011), and typically arise from various locations within the peritoneal cavity, including adjacent to the ovary (Hall *et al.* 1990). There are three primary histologic phenotypes of mesothelioma in the rat: epitheliomatous, sarcomatous, and mixed. Most

mesotheliomas in the rat are epitheliomatous, characterized by papillary projections overlying a fibrovascular stalk. Sarcomatous tumors are associated with spindylloid morphology, and mixed tumors have components of both epitheliomatous and sarcomatous phenotypes. All three histologic phenotypes that occur in the rat are also reported in humans (Blobel *et al.* 1985; Mackay *et al.* 1987), with the epitheliomatous subtype the most common (Remon *et al.* 2012). Both peritoneal and pleural mesotheliomas occur in humans, but by far, pleural mesotheliomas are the most common, occurring in up to 80% of all cases (Sekido 2013). Malignant pleural mesothelioma (MPM) is a rare disease in humans with approximately 2000–3000 new cases in the United States diagnosed annually (Kaufman and Pass 2008). The incidence of MPM has steadily increased over the last 20 years, with 70–80% of cases implicating asbestos exposure as the dominant risk factor (Kaufman and Pass 2008; Robinson and Lake 2005). Men have a higher incidence of pleural mesothelioma than women, possibly due to higher occupational exposures of asbestos (Yang *et al.* 2008). The remaining 20–30% of cases are attributed to non-asbestos risk factors, such as germline mutations in BRCA1-Associated Protein 1 (BAP1) (Bott *et al.* 2011; Testa *et al.*), SV40 viral infection (Carbone *et al.* 2003), and exposure to radiation or chemical agents (Carbone and Bedrossian 2006; Yang *et al.* 2008).

Molecular alterations in human mesothelioma associated with asbestos exposure have been an area of intense interest, and a topic of several recent reviews and gene expression profile targeted publications; gene expression profiling and mutation analysis of these tumors in humans has revealed a variety of genetic alterations including mutations in tumor suppressor genes (*TP53*, *WT1*, *NF2* and *PTEN*) or cell cycle genes (*CDKN2A/2B* and *CDKN2C*), and alterations in proteins associated with tumorigenesis (*COL3*, *CCL2*, *LGALS*) (Bott *et al.* 2011; Carbone and Bedrossian 2006; Gordon *et al.* 2005; Gueugnon *et al.* 2011; Romagnoli *et al.* 2009; Sekido 2013; Tomasetti *et al.* 2009; Tsujimura *et al.* 2012). These alterations in gene expression patterns appear to vary between different populations of people, as well as between the various morphological subtypes of human mesothelioma.

The objective of this study was to investigate the gene expression profiles of spontaneous mesotheliomas in the Fischer 344/N rat. Previous studies have characterized the gene expression profiles or molecular features of some chemically-induced mesotheliomas in F344/N rats or F344 crosses (Hu *et al.* 2010; Kim *et al.* 2006). However, global gene expression profiling of spontaneous mesotheliomas in rats has not previously been performed. Moreover, without a database of molecular changes or genetic signatures for spontaneously occurring mesotheliomas, separating changes due to chemical exposure from those inherent in mesothelioma pathogenesis is impossible. Characterization of spontaneous peritoneal mesotheliomas in rats may provide clues into the pathogenesis of chemically induced mesotheliomas, and may also provide useful information as to the utility of the F344/N rat as a hazard identification model for non-asbestos related mesothelioma in humans.

Materials and Methods

Sample collection and histopathology

Spontaneous mesotheliomas were collected from male vehicle control Fischer 344/N rats from three (3) two-year NTP carcinogenicity bioassay studies (Cobalt, Riddelliine, and Codeine). The incidence of malignant mesotheliomas in vehicle control male rats from these studies was 2/50 (4%), 4/50 (8%), and 1/50 (2%), for Cobalt, Riddelliine, and Codeine, respectively. Samples were sectioned in half and one half was fixed in 10% neutral buffered formalin (NBF) and the other half was flash frozen in liquid nitrogen. Five tumor samples were selected for molecular biology based on size from three animals (Riddelliine, peritoneum; Codeine, mesentery; Cobalt, tunica vaginalis, peritoneum, and mesentery) (Table 1), and the morphology of each sample was reviewed histologically to assure minimal necrotic tissue (<20%) and maximum tumor to normal tissue (>80% tumor tissue) per section. . The rarity of large spontaneous mesotheliomas available necessitated using multiple spontaneous tumors from one animal from the Cobalt study. Since isolation of normal mesothelium from rats would not yield adequate material for microarray, we used a non-transformed, immortalized mesothelial cell line (Fred-PE) which was grown and subcultured from the peritoneal cavity of untreated normal F344/N rats (a generous gift from Dr. Yongbaek Kim, NCSU), which has been used as a mesothelium control in previous studies in F344/N rats (Crosby *et al.* 2000; Kim *et al.* 2006).

Extraction and Quantification of RNA

Extraction of RNA was performed using the Invitrogen PureLink Mini Kit (Invitrogen cat no. 12183-081A). Frozen tissue samples were lysed and homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) using a rotary-stator homogenizer. Isolation of RNA was performed according to the mini kit protocol. On-column DNase treatment was performed using the Invitrogen PureLink DNase kit (Invitrogen, Carlsbad, CA) to purify RNA samples. RNA quantification and RNA integrity number were measured on a bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples were aliquoted and stored at -80°C until used.

RNA Labeling, Microarray Hybridization, and Data Processing

Gene expression analysis was conducted using Affymetrix Rat Genome 230 2.0 GeneChip® arrays (Affymetrix, Santa Clara, CA). One hundred ng of total RNA was amplified as directed in the Affymetrix 3' IVT Express kit protocol. 12.5µg of amplified biotin-aRNAs were fragmented and 10µg were hybridized to each array for 16 hours at 45°C in a rotating hybridization oven using the Affymetrix Eukaryotic Target Hybridization Controls and protocol. Array slides were stained with streptavidin/phycoerythrin utilizing a double-antibody staining procedure and then washed for antibody amplification according to the GeneChip Hybridization, Wash and Stain Kit and user manual. Arrays were scanned in an Affymetrix Scanner 3000 and data was obtained using the GeneChip® Command Console Software (AGCC; Version 1.1) using the MAS5 algorithm to generate .CHP files.

Probe intensity data from all arrays were entered into the R software environment (<http://www.R-project.org>) directly from .cel files using the R/affy package (Gautier *et al.* 2003). Data quality was assessed using image reconstruction, intensity histograms and boxplots.

Normalization was performed across all 11 samples (6 Fred-PE and 5 spontaneous mesothelioma samples) using the robust multiarray average (RMA) method to form one expression measure for each gene on each array (Irizarry *et al.* 2003). The RMA method adjusts the background of perfect match (PM) probes, applies a quantile normalization of the corrected PM values, and calculates final expression measures using the Tukey median polish algorithm. Treatment groups (cultured mesothelial cells (Fred-PE), spontaneous mesothelioma) were compared using a t-statistic whose null distribution was derived using 10,000 bootstrap samples by resampling the residuals (Efron and Tibshirani 1993). The bootstrap methodology was implemented in the ORIOGEN software package (Peddada *et al.* 2005). We applied Benjamini-Hochberg procedure (Benjamini and Hochberg 1995) for multiple testing with a nominal FDR of 0.01 to determine differential gene expression.

Core analysis of differentially expressed genes was conducted to determine biologic functions, canonical pathways, and transcription factor activation and over-represented classifications of genes were determined from statistical outcomes by testing for association with gene product relationships from a curated database of biological networks (Ingenuity Pathways Analysis™ (IPA) version 9.0) (www.ingenuity.com). The Ingenuity Pathways Knowledge Base (IPKB) consists of data with known biological relationships between genes and gene products. The significantly differentially expressed genes ($p < 0.001$) in the IPA core analysis were then grouped by pathways to account for upstream and downstream players as well as overlapping pathways. Upstream activation (formerly known as transcription factor activation in earlier versions of IPA) was based on IPA z scores > 2.0 with no bias.

Quantitative real-time PCR (qPCR)

Quantitative gene expression levels were detected using real-time PCR with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and TaqMan MGB probes (FAM™ dye labeled). Primers and probes for all genes analyzed were purchased from Applied Biosystems Assays-on-Demand Gene Expression products. For amplification, diluted cDNA was combined with a reaction mixture containing TaqMan universal PCR Master Mix (Applied Biosystems, Catalog No. 4304437) according to manufacturer's instructions. Samples were analyzed in duplicate, and a sample without RT was included with each plate to detect contamination by genomic DNA. Amplification was carried out as follows: (1) 50 °C, 2 min (for uracil-*N*-glycosylase incubation); (2) 95 °C, 10 min (denaturation); (3) 95 °C, 15 s, 60 °C, 30 s (denaturation/amplification) for 40 cycles. Fold increases or decreases in gene expression were determined by quantitation of cDNA from target samples relative to a calibrator sample (Fred PE passage 8). The 18S RNA gene was used as the endogenous control for normalization of initial RNA levels. To determine this normalized value, $2^{-(C_t)}$ values were compared between target and calibrator samples, where the changes in crossing threshold (C_t) = $C_{t\text{Target gene}} - C_{t18S\text{ RNA}}$, and $C_t = C_{t\text{control}} - C_{t\text{target}}$.

Immunohistochemistry

Protein expression assayed by immunohistochemistry was used to characterize the immunophenotype of spontaneous rat mesotheliomas, and to validate genes associated with mesothelioma development. Formalin fixed, paraffin embedded rat tissues were

deparaffinized and rehydrated and counterstained with hematoxylin, dehydrated, cleared, and coverslipped in a similar manner for all three antibodies. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Antigen retrieval was performed with heat and pressure, using citrate buffer (Biocare Medical, Concord, CA). The sections were incubated with 10% normal donkey serum or normal horse serum (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) for 20 minutes based on the respective antibodies, followed by the Avidin-Biotin Blocking Kit (Vector Laboratories, Burlingame, CA). The sections were incubated with rabbit monoclonal Vimentin antibody (Clone EPR3776, Epitomics, Burlingame, CA) and rabbit IgG (negative control; Epitomics, Burlingame, CA) at 1:250 dilution or Keratin 18 (Clone C-04, Santa Cruz, CA) at a 1:100 dilution and a non-immune mouse serum (negative control) diluted to match protein concentration of Keratin 18 antibody (Jackson ImmunoResearch, West Grove PA) for 60 minutes. Sections were incubated with a donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) or biotinylated horse anti mouse (Vector Laboratories, Burlingame, CA) for 30 minutes at 1:500 dilution. Label incubation was performed using Vector R.T.U. Vectastain Kit (Vector Laboratories, Burlingame, CA) for 30 minutes also. Antigen-antibody complex was visualized using DAB (Dako, Carpinteria, CA) for 6 minutes. EpCAM staining was performed on Discovery XT autostainer (Ventana Medical Systems, Tucson, AZ). Standard OmniMap Kit (Ventana Medical Systems, Tucson, AZ) reagents were used. Sections were incubated with rabbit monoclonal EpCAM antibody (Clone E144, Abcam, Inc, Cambridge, MA) at 1:350 dilution and normal rabbit serum (negative control; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 32 minutes with no heat setting. The sections were incubated with Discovery XT polymer (Ventana Medical Systems, Tucson, AZ) for 16 minutes. Antigen-antibody complex was visualized using DAB (Dako, Carpinteria, CA) for 8 minutes.

Results

Histopathology of spontaneous mesotheliomas in F344 rats

Spontaneously arising mesotheliomas from vehicle control animals (Table 1) were classified as epitheliomatous, characterized by papillary projections of one or more layers of atypical mesothelial cells overlying a fibrovascular stalk (Figure 1). Some tumors had a more solid phenotype, forming sheets of cells interspersed with variable amounts of fibrovascular stroma. Mesotheliomas frequently had mixed to mononuclear inflammatory infiltrates within the fibrovascular stroma. The mixed inflammatory component was variable from sample to sample with either a prominence of eosinophils, mast cells, or neutrophils.

Transcriptomic profiling identifies significant differences in global gene expression profiles between spontaneous mesothelioma and control (Fred-PE) mesothelium

Of the 21,901 genes on the microarray, 15,714 genes were differentially expressed in rat spontaneous mesotheliomas compared to Fred-PE mesothelial cells. Of these genes, 10,294 were mapped in IPA and 5,420 were unrecognized by IPA and therefore designated as unmapped. Of the mapped genes, 7,953 were considered analysis ready according to IPA based on threshold of $p < 0.001$ (Supplementary Table 1). Principal component analysis (PCA) of differentially expressed genes showed clear separation of spontaneous

mesotheliomas from Fred-PE cells in terms of global gene expression (Figure 2). Spontaneous mesotheliomas sampled from different anatomic locations within the same animal (Cobalt) as well as from different anatomic locations and different carcinogenicity studies clustered closely together, indicating that there was little variation in gene expression profiles between these samples based on PCA. However, there was some variability in the gene expression profiles between cell lines based on PCA analysis, which may be attributable to different cell passages or other in vitro cell culture effects. Regardless, hierarchical cluster analysis (HCA) showed distinct clustering of samples within groups based on statistically significant differentially up- and downregulation of genes across the genome (Figure 3). Ingenuity Pathways Analysis (IPA) core analysis was used to group the 7953 significantly differentially expressed genes ($p < 0.001$) into relevant biological functions. Biological functions representative of the top up- and downregulated genes included inflammatory response, cell growth and proliferation, embryonic development, cell death, cancer, carbohydrate metabolism, cell cycle and gene expression. Many of the significantly differentially expressed genes had overlapping biological functions in multiple categories. The microarray accession can be found in the GEO database (accession # GSE47581).

Global gene expression profiling of spontaneous mesotheliomas in F344/N rats identifies dysregulation of genes associated with cell proliferation and tumorigenesis

Up-regulation of genes (Table 2) associated with cell proliferation, cell motility and cell migration, cell invasion, and cell survival included growth factors (*Tgfa*, *Tgfb1*), transporter molecules (*Abcg1*), solute carrier molecules (*Slc7a9*, *Slc7a7*, *Slc28a2*) adhesion molecules (*Epcam*, *Cdh22*), bioactive lipids receptors (*Sp1r1*), and oncogenes (*v-Mafb*, *Lyn* (*v-yes1* homolog)), Allograft inflammatory factor 1 (*Aif1*), pattern recognition receptors (*Tlr8*, *Tlr7*, *Mannose receptor-1*) and C-type lectins (*Clec4a3*, *Clec4A*, *Clec7A*, *Clec10A*), and embryonic genes (*Plac8*, *Plau*, *Wnt4*, *Gata5*). In addition there was the upregulation of biomarkers for mesothelial cells and mesothelial cell hyperplasia (*Ck18*, *Vim*, *Wt*, *Des*, *Thbd*). Genes associated with up- and downstream regulation of transcription factor pathways with a Z score of >2 included interferon alpha and gamma (*Ifna*, *Ifng*), signal transducer and activator of transcription 1 (*Stat1*), and interleukin 10 (*Il10*). There was downregulation of critical human tumor suppressor genes (*Pten*, *Tp53*, *Rassf1*, and *Cdkn2a/2b*), cell cycle regulators (*p21*, *p27*) and genes associated with cell proliferation (*Myc*, *Fos*, *Jun*). In addition, there was upregulation of anti-apoptotic factors such as *Bcl2a1* and downregulation of pro-apoptotic factors such as *Bax*. These alterations in gene expression represent dysregulation of cell growth and proliferation pathways in mesothelial cells leading to transformation and oncogenesis in F344/N rat spontaneous mesotheliomas.

Alterations in inflammatory and immune cell function are overrepresented in global gene expression profiles of spontaneous F344/N rat mesotheliomas

The top up- and downregulated genes from the 7953 analyzed were associated with inflammatory/immune response (Table 3), and included cytokines (*Il18*, *Il10*, *Il24*) and chemokines (*Ccl11*, *Cxcl13*, *Ccl5*), inflammatory pathways/products of inflammatory cells (arachidonic acid metabolism, *Mcpt8*, *Lyz1/2*, *Chi3l1*), major histocompatibility complexes (*Hla-dra*, *Hla-dqa1*, *Hla-dqb1*), the complement pathway (*Cfh*, *C1qb*, *Serping1*), integrins

and adhesion molecules (*Cdh22*, *Itgb2*), and oxidative stress/response to reactive oxygen species (ROS) (*Duox2*, *Gpx2*). Many of the genes with greater than 5 fold change in expression were inflammatory mediators associated with macrophage activation and maturation, lymphocyte (T and B cell), leukocyte, and NK cell activation and recruitment, and immune evasion (*Il18*, *Il24*, *Il10*, *Cd40*, *Col6a1*, *Cd68*, *Cd163*, *Cd53*, *Cd74*, *Stab1*). Select differentially expressed genes were validated by quantitative RT-PCR (qPCR) (Table 4). Genes were selected based on known involvement with cancer progression, embryonic development, cell survival/decreased apoptosis, mesothelial cell function, immune response or for their role as a tumor biomarker. In all cases, RNA expression of selected genes matched the same directional change observed in the microarray.

Downstream protein expression of spontaneous F344/N rat mesotheliomas confirm a biphasic phenotype and share protein markers with human mesothelioma

Spontaneous mesotheliomas in the current study were evaluated immunohistochemically with antibodies to cytokeratin 18 (CK18) and vimentin (VIM) (Figure 4). Rat spontaneous mesotheliomas strongly expressed both vimentin (VIM) and cytokeratin 18 (CK18) (Table 1), and protein expression correlated positively with directional fold change in gene expression on the microarray. Expression of CK18 was restricted to the cell membrane in mesotheliomas, similar to expression in positive controls (rat salivary gland) compared to negative controls. Vimentin was co-expressed in the cytoplasm of mesothelioma cells, and expression was similar to positive controls (rat kidney). Mesotheliomas expressed epithelial cell adhesion molecule (EpCAM) (Table 1), a protein involved in the maintenance of tight junctions that is overexpressed in a variety of human cancers including mesothelioma. However, while expression of EpCAM was restricted to the cell membrane in positive controls (rat gastrointestinal tract mucosa), in spontaneous rat mesotheliomas, there was translocation of protein to the cytoplasm and nucleus, suggesting dysregulation of this protein (Figure 4).

Discussion

Our study has characterized the genomic profile of spontaneously occurring peritoneal mesotheliomas in male Fisher 344/N rats. In their roles as a primary barrier to pathogens and maintenance of peritoneal homeostasis, mesothelial cells have a wide range of initiation and response mechanisms associated with cellular growth, cytokine and growth factor release, regulation of pattern recognition receptors, and pathogen-associated molecular patterns (Izzi *et al.* 2012; Wu *et al.* 2010; Yung and Chan 2007). Carcinogenesis is a very complex process involving dysregulation of multiple cell growth and proliferation pathways, metabolic perturbations, inflammation and immune dysfunction, and hormonal factors, and consistent with this, we have shown that the genomic profiles observed in spontaneous F344/N rat mesotheliomas are similarly complex, involving dysregulation of numerous intersecting and interrelated pathways related to, cell growth and proliferation, contributions by inflammatory mediators, and interactions with reactive oxygen species and free radicals, all well-known and contributory mechanisms in carcinogenesis.

Oncogenes, Tumor Suppressor Genes, and Apoptosis Mediators

Alterations in tumor suppressor genes and oncogenes are key genetic factors related to tumor development and progression. Spontaneous F344/N rat mesotheliomas showed downregulation of several tumor suppressor genes (*Tp53*, *Pten*, *Gadd45A*, *Cdkn2b*, *Cdkn1a*, *Cdkn1b*) and overexpression of oncogenes (*v-MafB*, *v-Yes*). MAFB is overexpressed in human mesothelioma (Gordon *et al.* 2005), and *c-YES* is a mediator of cell proliferation in human malignant mesothelioma cells (Sato *et al.* 2012). In addition, a variety of tumor suppressor genes (*CKDN2A*, *TP53*, *PTEN*, *NF2*, *BAP1*) and their downstream targets are similarly affected in human mesothelioma (Bueno *et al.* 2010; Gordon *et al.* 2005; Gueugnon *et al.* 2011; Tsujimura *et al.* 2012). While promoters of cell cycle progression and cellular proliferation such as *FOS*, *MYC*, *JUN* and *CCND1* are typically upregulated in asbestos associated human mesothelioma, these genes were downregulated in spontaneous rat mesothelioma in this study. Lastly, spontaneous rat mesotheliomas were associated with an anti-apoptotic phenotype characterized by the overexpression of anti-apoptotic *Bcl* mediators (*Bcl2a1*), downregulation pro-apoptotic *Bcl* members (*Bax*) and *Fadd* and *Fas* mediators, and upregulation of *Fas* apoptotic inhibitory molecule 3 (*Faim3*). Alterations in tumor suppressor genes, oncogenes, and apoptosis mediators have a profound effect on the expression of downstream cellular growth factors and the inflammatory response, two other significantly represented categories of altered gene expression in this study.

Growth Factor Pathways

Dysregulation of cellular growth is an integral part of carcinogenesis. In spontaneous F344/N rat mesotheliomas, there was upregulation of mediators in a number of growth factor pathways, including the transforming growth factor-alpha and -beta (*Tgfa/β*), insulin-like growth factor (*Igf*), p38 mitogen-associated protein kinase (*p38 Mapk*) and nuclear factor kappa-B (*Nfκb*) pathways. TGFβ was originally discovered as a mediator involved in tumor cell survival (Abbas and Lichtman 2003), regulates insulin-like growth factor-1 (IGF1) and inhibits cell adhesion and enhances cell-collagen interactions associated with invasion and metastasis. TGFα is associated with positive regulation of cell proliferation, differentiation and development, and negatively regulates apoptosis. Upregulation of *TGFA/β* is associated with a variety of cancers, including breast, liver, kidney, lung, pancreatic, and hematologic cancers. In some cancers, *TGFβ* plays conflicting roles in transformation and tumor progression; for example, it has been shown to act as a tumor suppressor in early stages of breast cancer development, but acts to promote invasion and metastasis late in the course of disease (Akhurst and Derynck 2001; Tang *et al.* 2003). *TGFA* and β are also involved in a large number of physiologic processes, and interface with other growth factor pathways such as the MAP-kinase (MAPK) and NFκB signaling pathways. In addition, *TGFβ1* is produced by and regulates cells of the immune system (Letterio and Roberts 1998; Yang *et al.* 2010), representing another overlapping component of the interlacing pathways associated with mesotheliomas in this study. Several members of the insulin like growth factor (*Igf*) pathway were upregulated in spontaneous mesotheliomas (*Igf1*, *Igfbp1*, *Igfbp2–7*), similar to a variety of human cancers including mesothelioma, where they are associated with high-grade or poorly differentiated tumors (Gullu *et al.* 2012; Jean *et al.* 2012; Whitson and Kratzke 2006). The p38 mitogen activated protein kinase (*p38 Mapk*) pathway is regulated by a wide variety of molecules and conditions including growth

factors, cytokines, cytosolic proteins and environmental stresses (Cuadrado and Nebreda 2010). Several mediators within the *p38 Mapk* pathway (*p38 Mapka/β/γ*, *Stat1*, *Tgfβr1*, *Tgfβ1*, *Tnfrs11b*, *Tnfrs14*, *Tnfrs25*) were up regulated in spontaneous mesotheliomas. Mediators in this pathway are involved in cell survival and proliferation, and there is moderate crosstalk between other growth pathways such as the NFκB, WNT, JNK and AKT pathways (Cuadrado and Nebreda 2010). Moreover, *p38 Mapk* is activated in rat mesothelial cells exposed to asbestos (Swain *et al.* 2004), and regulates cell proliferation in rat pleural mesothelioma cell lines (Zhong *et al.* 2011). The various *p38 Mapk* isoforms act across many pathways, and may be activated by environmental stress (oxidative stress, UV irradiation, hypoxia, ischemia) and inflammatory cytokines such as *IL1* and *TNFα* (Cargnello and Roux 2011).

Epithelial cell adhesion molecule (*Epcam*) is a trans membrane p-glycoprotein reported to have multiple roles including cell signaling, migration, proliferation, and differentiation (Patriarca *et al.* 2012). It is a well-known epithelial tumor marker that is overexpressed in breast, bladder, colorectal, pancreas, prostate and ovarian cancer, and epitheliomatous mesotheliomas in humans (Ryan *et al.* 1997; Shield *et al.* 1994). Alterations in EpCAM interfere with cadherin-mediated cell to cell adhesion (Litvinov *et al.* 1997), and cytoplasmic and nuclear translocation of its intercellular domain (EpICD) is associated with stimulation of cell proliferation (Maetzel *et al.* 2009). In addition to up regulation of *Epcam* at the gene expression level, there was cytoplasmic and nuclear translocation of EpCAM protein in spontaneous rat mesotheliomas in this study (Figure 4); the EpCAM antibody used in this study recognizes the C terminus on EpICD (Patriarca *et al.* 2012), confirming the nuclear translocation of the intercellular domain and implicating EpCAM as mediator of cell proliferation and mesotheliomagenesis in both F344/N rats and humans. It is well known that mesothelial cells have a biphasic nature, expressing a broad spectrum of low and high molecular weight epithelial keratins and vimentins (Mullink *et al.* 1986; Whitaker *et al.* 1980). As such, spontaneous mesotheliomas in this study expressed both vimentin and CK18 by immunohistochemistry.

Inflammatory Pathways and Immune Dysfunction

Inflammation and immune dysregulation are mechanisms central to many neoplastic processes, including the development of mesotheliomas, and are considered the seventh hallmark of cancer (Colotta *et al.* 2009; Hanahan and Weinberg 2011). In fact, a key component of early stage asbestos-related mesothelioma in humans is a non-specific inflammatory response (Boutin and Rey 1993). Given the nature of the disease in humans, a chronic inflammatory response is not surprising. What is interesting however, is the predominance of genomic changes associated with altered immune function and inflammation in spontaneous rat mesotheliomas, which arise in the absence of any initiating chemical or physical agent. This response could be attributed to chronic inflammation, cancer-related inflammation, dysregulation of mesothelial cell function, and/or intrinsic genetic events. Mesothelial cell proliferation may be stimulated through the induction of proinflammatory pathways by activated macrophages or mesothelial cells, with the subsequent production of proinflammatory cytokines (TNF, IL-1) and growth factors (IL6, GM-CSF), upregulation of prostaglandins, nitrous oxide (NO), angiogenic factors, and

adhesion molecules. Research on the etiology of mesotheliomas in humans (asbestos) and animal models (multiwalled carbon nanotubes, MWCNT) suggest that a major pathophysiologic factor in the development of these tumors is frustrated phagocytosis leading to biopersistence of the initiating agent, which results in chronic oxidative stress and inflammation (Walker *et al.* 1992; Yang *et al.* 2008). Similarly, rat spontaneous mesotheliomas in this study were associated with upregulation of a variety of cytokines and cytokine receptors associated with chronic inflammation (*Il6r*, *Tnfrs11b*, *Tnfrs14*, *Tnfrs25*, and *Il1b*), including *Il10*, which promotes cell survival and abrogation of immune surveillance. It is known that asbestos in fact can cause alterations in normal cells that result in decreased tumor immunity (Matsuzaki *et al.* 2012). Whether these are contributing factors in the induction and pathogenesis of this tumor in rats, or merely secondary to the presence of the neoplasm and the tissue damage associated with its invasion is uncertain. Certainly, the chronic tissue damage and inflammation associated with asbestos exposure in human mesotheliomagenesis is due to the persistence of the asbestos fiber and its interaction with cellular components within the lung parenchyma, ultimately leading to generation of reactive oxygen species and secondary DNA damage (Matsuzaki *et al.* 2012). In terms of non-asbestos etiologies in human mesothelioma, many of these are also associated with inflammatory processes in the lung (SV40 viral infection, exposure to radiation or chemical agents (Carbone and Bedrossian 2006; Yang *et al.* 2008); whereas others, such as germline mutations in BRCA1-Associated Protein 1 (BAP1) (Bott *et al.* 2011; Testa *et al.*), arise without such associated tissue damage and inflammation. Similarly, mesothelioma in vehicle control F344/N rats arises spontaneously without an inciting cause related to tissue damage or inflammation, and may represent an inherent genetic susceptibility that could be influenced by chemical exposure.

Many of the receptors associated with innate immunity that are constitutively expressed in mesothelial cells were upregulated in spontaneous mesotheliomas in this study. Pleural and peritoneal mesothelial cells express a wide range of pattern recognition receptors (PRR), such as the toll-like receptors (TLRs), which are triggered by pathogen activated molecular patterns (PAMPs), including components of bacteria, fungi, or viruses, and are therefore used in defense systems employed by the mesothelium (Takeuchi and Akira 2010). Toll-like receptors are pattern recognition receptors that trigger activation of the NFkB pathway and/or MAPK activation, and increased production of proinflammatory cytokines and interferons (Takeuchi and Akira 2010). The genomic profile of spontaneous rat mesotheliomas is highlighted by the prominent upregulation of pattern recognition receptors (*TLR1*, *2*, *7*, *8*, *Mrc1*) that trigger activation of the TLR-NFkB and interferon pathways, with subsequent release of inflammatory cytokines and other inflammatory mediators. Endogenous TLR ligands also have an increasing role in tumorigenesis through promotion of cancer cell survival and proliferation, immune system evasion, and enhancement of metastasis and chemoresistance (Yu *et al.* 2012). Endogenous molecules that are released from mesothelial cells following tissue injury can act as danger associated molecular pattern receptors (DAMPs) and activate TLRs (Yu *et al.* 2012). Several mediators that act as DAMPs (*Mrc1*, *S100a8/9*, *hyaluronan*, *Lyve1*, *Stab1*), were upregulated in spontaneous rat mesotheliomas; these mediators can promote tumorigenesis and invasion through the activation of various TLRs (Turovskaya *et al.* 2008; Voelcker *et al.* 2008; Yu *et al.* 2012).

Spontaneous rat mesotheliomas were also associated with upregulation of interferon pathway genes, associated with immunomodulation (*Ifi135*, *Psm8*, *Ifitm1*, *Tap1*, *Aif1*, *Irf1/7/9*). Overexpression of some of these immunomodulatory genes are also associated with cell proliferation, inhibition of apoptosis, and tumor growth (Liu *et al.* 2008), and upregulation of the interferon receptor pathway results in stimulation of a variety of other cell growth signaling networks including the Jak/Stat pathway, glucocorticoid pathway, and immune function and cytokine expression. This suggests a significant inflammatory component to the process of spontaneous mesothelioma development in these animals.

Evidence supporting the hypothesis that the immune system plays an important role in modulating cancer has been increasing over the last decade. Indeed, in addition to their role as activators of the immune response, protumorigenic activity has been described for upregulated TLRs in cancer cells or tumor associated macrophages (Yu *et al.* 2012). Moreover, TLR stimulation has been associated with tumor promotion and invasion in rodents and humans (Ochi *et al.* 2012; Xie *et al.* 2010). Mediators expressed by activated and/or tumor associated macrophages have been shown to have tumor promoting qualities (Traves *et al.* 2012). Tumor associated macrophages (TAMs) are present in variety of cancer types and are associated with tumor progression, tumor cell invasion, metastasis, and angiogenesis (Pollard 2004). Cell surface markers associated with TAMs in rat spontaneous mesotheliomas in the current study included *Cd163*, *Cd68*, *Cd53*, and C type lectins (*Cd93*), many of which play roles in cell-cell and cell-matrix interactions (Bos *et al.* 2012), cell adhesion, migration, and inflammation (Greenlee-Wacker *et al.* 2012), pro-survival signaling through the AKT/PIK3 pathway (Yunta and Lazo 2003), or are expressed by tumor cells and TAMs in a variety of human cancers (Maniecki *et al.* 2012; Shabo and Svanvik 2011; Ch'ng *et al.* 2013; Feng *et al.* 2012; Jia *et al.* 2008). The upregulation of these cell surface markers in spontaneous rat mesotheliomas may be due to overexpression by mesothelioma cell themselves, or due to the presence of activated or tumor-associated macrophages within these tumors.

In this study, a complex interaction of dysregulated mesothelial cell processes including growth, differentiation, and immune system function was observed. It is clear that mesothelioma in humans (Yung and Chan 2007) and rats (Xu *et al.* 2012) is associated with risk factors of chronic inflammation; however, in spontaneous mesothelioma, the prominent inflammatory phenotype is unexpected, and it is unclear as to whether the prominent immune cell response was a contributing factor for the development of mesothelioma, or merely a response to the presence of the neoplasms in these animals.

Conclusion

This study shows that there is dysregulation of numerous genes in overlapping pathways including those associated with cellular proliferation and survival, inflammation and immune system dysregulation, inhibition of apoptosis, and dysregulation of oncogenes, tumor suppressor and embryonic genes in the process of spontaneous mesotheliomagenesis. Importantly, this dataset now provides a point of reference to better understand the events that occur in chemically induced mesothelioma in the F344/N rat, and how these events may be relevant to human hazard characterization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge Norris Flagler, Eli Ney, and Beth Mahler for their imaging expertise and assistance with figures. We would like to thank the CMPB Histology and Immunohistochemistry Core Laboratories, and the NIEHS Microarray Core for their technical expertise.

Author's note: This work was supported by the National Institutes of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), and The Division of the National Toxicology Program (DNTP). This article may be the work product of an employee or group of employees of the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health (NIH), however, the statements, opinions or conclusions contained therein do not necessarily represent the statements, opinions or conclusions of the NIEHS, NIH, or the United States government.

References

- Abbas, AK.; Lichtman, AH. Cellular and Molecular Immunology. Philadelphia: Saunders; 2003.
- Afify AM, Al-Khafaji BM, Paulino AF, Davila RM. Diagnostic use of muscle markers in the cytologic evaluation of serous fluids. *Appl.Immunohistochem.Mol.Morphol.* 2002; 10:178–182. [PubMed: 12051638]
- Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. *Trends Cell Biol.* 2001; 11:S44–S51. [PubMed: 11684442]
- Antony VB. Immunological mechanisms in pleural disease. *European Respiratory Journal.* 2003; 21:539–544. [PubMed: 12662014]
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. 1995:289–300.
- Blobel GA, Moll R, Franke WW, Kayser KW, Gould VE. The intermediate filament cytoskeleton of malignant mesotheliomas and its diagnostic significance. *American Journal of Pathology.* 1985; 121:235–247. [PubMed: 2414994]
- Bos SD, Lakenberg N, van der BR, Houwing-Duistermaat JJ, Kloppenburg M, de Craen AJ, Beekman M, Meulenbelt I, Slagboom PE. A genome-wide linkage scan reveals CD53 as an important regulator of innate TNF-alpha levels. *European Journal of Human Genetics.* 2012; 18:953–959. [PubMed: 20407468]
- Bott M, Brevet M, Taylor BS, Shimizu S, Ito T, Wang L, Creaney J, Lake RA, Zakowski MF, Reva B, Sander C, Delsite R, Powell S, Zhou Q, Shen R, Olshen A, Rusch V, Ladanyi M. The nuclear deubiquitinase BAP1 is commonly inactivated by somatic mutations and 3p21.1 losses in malignant pleural mesothelioma. *Nat Genet.* 2011; 43:668–672. [PubMed: 21642991]
- Boutin C, Rey F. Thoracoscopy in pleural malignant mesothelioma: a prospective study of 188 consecutive patients. Part 1: Diagnosis. *Cancer.* 1993; 72:389–393. [PubMed: 8319170]
- Bueno R, De Rienzo A, Dong L, Gordon GJ, Hercus CF, Richards WG, Jensen RV, Anwar A, Maulik G, Chirieac LR, Ho KF, Taillon BE, Turcotte CL, Hercus RG, Gullans SR, Sugarbaker DJ. Second generation sequencing of the mesothelioma tumor genome. *PLoS One.* 2010; 5:e10612. [PubMed: 20485525]
- Carbone M, Bedrossian CW. The pathogenesis of mesothelioma. *Seminars in Diagnostic Pathology.* 2006; 23:56–60. [PubMed: 17044196]
- Carbone M, Pass HI, Miele L, Bocchetta M. New developments about the association of SV40 with human mesothelioma. *Oncogene.* 2003; 22:5173–5180. [PubMed: 12910254]
- Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev.* 2011; 75:50–83. [PubMed: 21372320]
- Ch'ng ES, Tuan Sharif SE, Jaafar H. In human invasive breast ductal carcinoma, tumor stromal macrophages and tumor nest macrophages have distinct relationships with clinicopathological parameters and tumor angiogenesis. *Virchows Arch.* 2013; 462:257–267. [PubMed: 23283409]

- Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A. Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis*. 2009; 30:1073–1081. [PubMed: 19468060]
- Crosby LM, Hyder KS, DeAngelo AB, Kepler TB, Gaskill B, Benavides GR, Yoon L, Morgan KT. Morphologic analysis correlates with gene expression changes in cultured F344 rat mesothelial cells. *Toxicology and Applied Pharmacology*. 2000; 169:205–221. [PubMed: 11133343]
- Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. *Biochem J*. 2010; 429:403–417. [PubMed: 20626350]
- Efron, B.; Tibshirani, R. *An Introduction to the bootstrap*. 1993.
- Feng F, Wu Y, Zhang S, Liu Y, Qin L, Yan Z, Wu W. Macrophages facilitate coal tar pitch extract-induced tumorigenic transformation of human bronchial epithelial cells mediated by NF-kappaB. *PLoS One*. 2012; 7:e51690. [PubMed: 23227270]
- Gautier L, Bolstad BM, Irizarry RA. Affy-Analysis of Affymetrix GeneChip probe level data. *Bioinformatics*. 2003; 20:307–315. [PubMed: 14960456]
- Gordon GJ, Rockwell GN, Jensen RV, Rheinwald JG, Glickman JN, Aronson JP, Pottorf BJ, Nitz MD, Richards WG, Sugarbaker DJ, Bueno R. Identification of novel candidate oncogenes and tumor suppressors in malignant pleural mesothelioma using large-scale transcriptional profiling. *American Journal of Pathology*. 2005; 166:1827–1840. [PubMed: 15920167]
- Greenlee-Wacker MC, Galvan MD, Bohlson SS. CD93: recent advances and implications in disease. *Curr Drug Targets*. 2012; 13:411–420. [PubMed: 22206251]
- Guegnon F, Leclercq S, Blanquart C, Sagan C, Cellier L, Padieu M, Perigaud C, Scherpereel A, Gregoire M. Identification of novel markers for the diagnosis of malignant pleural mesothelioma. *Am J Pathol*. 2011; 178:1033–1042. [PubMed: 21356356]
- Gullu G, Karabulut S, Akkiprik M. Functional roles and clinical values of insulin-like growth factor-binding protein-5 in different types of cancers. *Chin J Cancer*. 2012; 31:266–280. [PubMed: 22313597]
- Hall, WC.; Boorman, G.; Eustis, S.; Elwell, MR.; Montgomery, CA.; MacKenzie, WF. *Pathology of the Fischer Rat*. San Diego, CA: Academic Press; 1990. Peritoneum, Retroperitoneum, Mesentery, and Abdominal Cavity.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011; 144:646–674. [PubMed: 21376230]
- Hu Q, Akatsuka S, Yamashita Y, Ohara H, Nagai H, Okazaki Y, Takahashi T, Toyokuni S. Homozygous deletion of CDKN2A/2B is a hallmark of iron-induced high-grade rat mesothelioma. *Lab Invest*. 2010; 90:360–373. [PubMed: 20065947]
- Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Research*. 2003; 31:e15. [PubMed: 12582260]
- Izzi V, Masuelli L, Tresoldi I, Foti C, Modesti A, Bei R. Immunity and malignant mesothelioma: from mesothelial cell damage to tumor development and immune response-based therapies. *Cancer Lett*. 2012; 322:18–34. [PubMed: 22394996]
- Jantz MA, Antony VB. Pathophysiology of the pleura. *Respiration*. 2008; 75:121–133. [PubMed: 18332619]
- Jean D, Daubiac J, Le Pimec-Barthes F, Galateau-Salle F, Jaurand MC. Molecular changes in mesothelioma with an impact on prognosis and treatment. *Arch Pathol Lab Med*. 2012; 136:277–293. [PubMed: 22372904]
- Jia J, Bai Y, Fu K, Sun ZJ, Chen XM, Zhao YF. Expression of allograft inflammatory factor-1 and CD68 in haemangioma: implication in the progression of haemangioma. *British Journal of Dermatology*. 2008; 159:811–819. [PubMed: 18647307]
- Kaufman AJ, Pass HI. Current concepts in malignant pleural mesothelioma. *Expert Rev. Anticancer Ther*. 2008; 8:293–303. [PubMed: 18279069]
- Kim Y, Ton TV, DeAngelo AB, Morgan K, Devereux TR, Anna C, Collins JB, Paules RS, Crosby LM, Sills RC. Major carcinogenic pathways identified by gene expression analysis of peritoneal mesotheliomas following chemical treatment in F344 rats. *Toxicology and Applied Pharmacology*. 2006; 214:144–151. [PubMed: 16460773]

- Kupryjanczyk J, Karpinska G. Desmin expression in reactive mesothelium: a potential aid in evaluation of gynecologic specimens. *International Journal of Gynecological Pathology*. 1998; 17:123–128. [PubMed: 9553808]
- Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. *Annual Review of Immunology*. 1998; 16:137–161. 137–161.
- Litvinov SV, Balzar M, Winter MJ, Bakker HA, Briaire-de BI, Prins F, Fleuren GJ, Warnaar SO. Epithelial cell adhesion molecule (Ep-CAM) modulates cell-cell interactions mediated by classic cadherins. *Journal of Cell Biology*. 1997; 139:1337–1348. [PubMed: 9382878]
- Liu S, Tan WY, Chen QR, Chen XP, Fu K, Zhao YY, Chen ZW. Daintain/AIF-1 promotes breast cancer proliferation via activation of the NF-kappaB/cyclin D1 pathway and facilitates tumor growth. *Cancer Sci*. 2008; 99:952–957. [PubMed: 18341653]
- Mackay AM, Tracy RP, Craighead JE. Intermediate filament proteins in asbestos-induced mesotheliomas of the rat. *Cancer Res*. 1987; 47:5461–5468. [PubMed: 2443239]
- Madison LD, Bergstrom-Porter B, Torres AR, Shelton E. Regulation of surface topography of mouse peritoneal cells. Formation of microvilli and vesiculated pits on omental mesothelial cells by serum and other proteins. *Journal of Cell Biology*. 1979; 82:783–797. [PubMed: 92474]
- Maetzel D, Denzel S, Mack B, Canis M, Went P, Benk M, Kieu C, Papior P, Baeuerle PA, Munz M, Gires O. Nuclear signalling by tumour-associated antigen EpCAM. *Nat.Cell Biol*. 2009; 11:162–171. [PubMed: 19136966]
- Maniecki MB, Etzerodt A, Ulhøi BP, Steiniche T, Borre M, Dyrskjøt L, Orntoft TF, Moestrup SK, Møller HJ. Tumor-promoting macrophages induce the expression of the macrophage-specific receptor CD163 in malignant cells. *Int J Cancer*. 2012; 131:2320–2331. [PubMed: 22362417]
- Matsuzaki H, Maeda M, Lee S, Nishimura Y, Kumagai-Takei N, Hayashi H, Yamamoto S, Hatayama T, Kojima Y, Tabata R, Kishimoto T, Hiratsuka J, Otsuki T. Asbestos-induced cellular and molecular alteration of immunocompetent cells and their relationship with chronic inflammation and carcinogenesis. *J Biomed Biotechnol*. 2012; 2012:492608. [PubMed: 22500091]
- Mullink H, Henzen-Logmans SC, Alons-van Kordelaar JJ, Tadema TM, Meijer CJ. Simultaneous immunoenzyme staining of vimentin and cytokeratins with monoclonal antibodies as an aid in the differential diagnosis of malignant mesothelioma from pulmonary adenocarcinoma. *Virchows Arch B Cell Pathol Incl Mol Pathol*. 1986; 52:55–65. [PubMed: 2435052]
- Nasreen N, Mohammed KA, Hardwick J, Van Horn RD, Sanders K, Kathuria H, Loghmani F, Antony VB. Low molecular weight hyaluronan induces malignant mesothelioma cell (MMC) proliferation and haptotaxis: role of CD44 receptor in MMC proliferation and haptotaxis. *Oncology Research*. 2002; 13:71–78. [PubMed: 12392154]
- National Toxicology Program, N. NTP Historical Controls Report: All Routes and Vehicles, F344/N Rats. 2011 May.:9.
- Ochi A, Graffeo CS, Zambirinis CP, Rehman A, Hackman M, Fallon N, Barilla RM, Henning JR, Jamal M, Rao R, Greco S, Deutsch M, Medina-Zea MV, Bin Saeed U, Ego-Osuala MO, Hajdu C, Miller G. Toll-like receptor 7 regulates pancreatic carcinogenesis in mice and humans. *J Clin Invest*. 2012; 122:4118–4129. [PubMed: 23023703]
- Patriarca C, Macchi RM, Marschner AK, Mellstedt H. Epithelial cell adhesion molecule expression (CD326) in cancer: a short review. *Cancer Treat Rev*. 2012; 38:68–75. [PubMed: 21576002]
- Peddada S, Harris S, Zajd J, Harvey E. ORIOGEN: order restricted inference for ordered gene expression data. *Bioinformatics*. 2005; 21:3933–3934. [PubMed: 16109745]
- Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat.Rev.Cancer*. 2004; 4:71–78. [PubMed: 14708027]
- Remon J, Lianes P, Martinez S, Velasco M, Querol R, Zanui M. Malignant mesothelioma: New insights into a rare disease. *Cancer Treat Rev*. 2012
- Robinson BW, Lake RA. Advances in malignant mesothelioma. *New England Journal of Medicine*. 2005; 353:1591–1603. [PubMed: 16221782]
- Romagnoli S, Fasoli E, Vaira V, Falleni M, Pellegrini C, Catania A, Roncalli M, Marchetti A, Santambrogio L, Coggi G, Bosari S. Identification of potential therapeutic targets in malignant mesothelioma using cell-cycle gene expression analysis. *American Journal of Pathology*. 2009; 174:762–770. [PubMed: 19218339]

- Ryan PJ, Oates JL, Crocker J, Stableforth DE. Distinction between pleural mesothelioma and pulmonary adenocarcinoma using MOC31 in an asbestos sprayer. *Respiratory Medicine*. 1997; 91:57–60. [PubMed: 9091974]
- Sato A, Sekine M, Virgona N, Ota M, Yano T. Yes is a central mediator of cell growth in malignant mesothelioma cells. *Oncology Reports*. 2012; 28:1889–1893. [PubMed: 22948717]
- Sekido Y. Molecular pathogenesis of malignant mesothelioma. *Carcinogenesis*. 2013
- Shabo I, Svanvik J. Expression of macrophage antigens by tumor cells. *Adv Exp Med Biol*. 2011; 714:141–150. [PubMed: 21506012]
- Shield PW, Callan JJ, Devine PL. Markers for metastatic adenocarcinoma in serous effusion specimens. *Diagnostic Cytopathology*. 1994; 11:237–245. [PubMed: 7532566]
- Swain WA, O'Byrne KJ, Faux SP. Activation of p38 MAP kinase by asbestos in rat mesothelial cells is mediated by oxidative stress. *Am.J.Physiol Lung Cell Mol.Physiol*. 2004; 286:L859–L865. [PubMed: 14617514]
- Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell*. 2010; 140:805–820. [PubMed: 20303872]
- Tang B, Vu M, Booker T, Santner SJ, Miller FR, Anver MR, Wakefield LM. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest*. 2003; 112:1116–1124. [PubMed: 14523048]
- Testa JR, Cheung M, Pei J, Below JE, Tan Y, Sementino E, Cox NJ, Dogan AU, Pass HI, Trusa S, Hesdorffer M, Nasu M, Powers A, Rivera Z, Comertpay S, Tanji M, Gaudino G, Yang H, Carbone M. Germline BAP1 mutations predispose to malignant mesothelioma. *Nat Genet*. 43:1022–1025. [PubMed: 21874000]
- Tomasetti M, Amati M, Santarelli L, Alleva R, Neuzil J. Malignant mesothelioma: biology, diagnosis and therapeutic approaches. *Curr.Mol.Pharmacol*. 2009; 2:190–206. [PubMed: 20021458]
- Traves PG, Luque A, Hortelano S. Macrophages, inflammation, and tumor suppressors: ARF, a new player in the game. *Mediators Inflamm*. 2012; 2012:568783. [PubMed: 23316105]
- Tsujimura T, Torii I, Sato A, Song M, Fukuoka K, Hasegawa S, Nakano T. Pathological and molecular biological approaches to early mesothelioma. *Int J Clin Oncol*. 2012; 17:40–47. [PubMed: 22237727]
- Turovskaya O, Foell D, Sinha P, Vogl T, Newlin R, Nayak J, Nguyen M, Olsson A, Nawroth PP, Bierhaus A, Varki N, Kronenberg M, Freeze HH, Srikrishna G. RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis. *Carcinogenesis*. 2008; 29:2035–2043. [PubMed: 18689872]
- Voelcker V, Gebhardt C, Aeverbeck M, Saalbach A, Wolf V, Weih F, Sleeman J, Anderegg U, Simon J. Hyaluronan fragments induce cytokine and metalloprotease upregulation in human melanoma cells in part by signalling via TLR4. *Experimental Dermatology*. 2008; 17:100–107. [PubMed: 18031543]
- Walker C, Everitt J, Barrett JC. Possible cellular and molecular mechanisms for asbestos carcinogenicity. *American Journal of Industrial Medicine*. 1992; 21:253–273. [PubMed: 1536158]
- Whitaker D, Papadimitriou JM, Walters MN. The mesothelium: a histochemical study of resting mesothelial cells. *Journal of Pathology*. 1980; 132:273–284. [PubMed: 7431162]
- Whitson BA, Kratzke RA. Molecular pathways in malignant pleural mesothelioma. *Cancer Lett*. 2006; 239:183–189. [PubMed: 16216411]
- Wu J, Yang X, Zhang YF, Wang YN, Liu M, Dong XQ, Fan JJ, Yu XQ. Glucose-based peritoneal dialysis fluids downregulate toll-like receptors and trigger hyporesponsiveness to pathogen-associated molecular patterns in human peritoneal mesothelial cells. *Clin Vaccine Immunol*. 2010; 17:757–763. [PubMed: 20200188]
- Xie W, Huang Y, Guo A, Wu W. Bacteria peptidoglycan promoted breast cancer cell invasiveness and adhesiveness by targeting toll-like receptor 2 in the cancer cells. *PLoS One*. 2010; 5:e10850. [PubMed: 20520770]
- Xu J, Futakuchi M, Shimizu H, Alexander DB, Yanagihara K, Fukamachi K, Suzui M, Kanno J, Hirose A, Ogata A, Sakamoto Y, Nakae D, Omori T, Tsuda H. Multi-walled carbon nanotubes translocate into the pleural cavity and induce visceral mesothelial proliferation in rats. *Cancer Sci*. 2012; 103:2045–2050. [PubMed: 22938569]

- Yang H, Testa JR, Carbone M. Mesothelioma epidemiology, carcinogenesis, and pathogenesis. *Curr.Treat.Options.Oncol.* 2008; 9:147–157. [PubMed: 18709470]
- Yang L, Pang Y, Moses HL. TGF-beta and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends Immunol.* 2010; 31:220–227. [PubMed: 20538542]
- Yu L, Wang L, Chen S. Exogenous or endogenous Toll-like receptor ligands: which is the MVP in tumorigenesis? *Cell Mol Life Sci.* 2012; 69:935–949. [PubMed: 22048194]
- Yung S, Chan TM. Mesothelial cells. *Peritoneal Dialysis International.* 2007; 27(Suppl 2):S110–S115. S110–S115. [PubMed: 17556288]
- Yung S, Chan TM. Pathophysiology of the peritoneal membrane during peritoneal dialysis: the role of hyaluronan. *J Biomed Biotechnol.* 2011; 2011:180594. [PubMed: 22203782]
- Yunta M, Lazo PA. Apoptosis protection and survival signal by the CD53 tetraspanin antigen. *Oncogene.* 2003; 22:1219–1224. [PubMed: 12606948]
- Zhong J, Lardinois D, Szilard J, Tamm M, Roth M. Rat mesothelioma cell proliferation requires p38delta mitogen activated protein kinase and C/EBP-alpha. *Lung Cancer.* 2011; 73:166–170. [PubMed: 21227534]

Figure 1.

Spontaneous mesothelioma, abdominal cavity, F344/N rat. Spontaneous mesotheliomas in F344/N rats exhibited an epitheliomatous phenotype, characterized by papillary projections of plump mesothelial cells overlying a fibrovascular stalk (A, H&E, 10X). Cells were polygonal to cuboidal, with scant to moderate faintly eosinophilic cytoplasm and a central to eccentric round to oval hyperchromatic nucleus and an occasional prominent nucleoli (B, H&E, 40X).

Figure 2.

Principal Component Analysis (PCA) comparing global gene profiles of cultured Fred-PE mesothelial cells (Meso-Normal) and spontaneous mesotheliomas from control F344/N rats (CTL). PCA analysis shows clear clustering of samples within groups, indicating significant similarities in global gene expression of samples within experimental groups, and clear separation of experimental groups in space, indicating significant differences between groups in terms of their global gene expression.

Figure 3.

Unsupervised hierarchal cluster analysis (HCA) comparing global gene expression profiles of Fred-PE mesothelial cells (Meso-Normal) and spontaneous mesotheliomas from control F344/N rats (CTL). HCA analysis indicates significant differential expression of upregulated (red) and downregulated (green) genes across the genome between Fred-PE mesothelial cells and spontaneous F344/N rat mesotheliomas.

Figure 4.

Immunohistochemical validation of mesothelioma markers in spontaneous mesotheliomas from control F344/N rats. Neoplastic cells in spontaneous mesotheliomas (A, C, E, 10X; B, D, F, 40X) showed strong membrane immunoreactivity with anti-cytokeratin 18 antibody (A–B), strong cytoplasmic immunoreactivity with anti-vimentin antibody (C–D), and strong cytoplasmic and nuclear immunoreactivity for antibodies to epithelial cell adhesion molecule (E–F) (hematoxylin counterstain).

Table 1

Select Differentially Expressed Genes Dysregulated in Spontaneous Mesotheliomas in F344/N rats.

Gene Name	Gene Symbol	Fold Change
Transforming Growth Factor		
transforming growth factor, alpha	<i>Tgfa</i>	12.45
transforming growth factor, beta 2	<i>Tgfb2</i>	-17.61
transforming growth factor, beta receptor 1	<i>Tgfb1</i>	2.14
transforming growth factor, beta-induced, 68kDa	<i>Tgfb1</i>	43.39
Platelet Derived Growth Factor		
platelet derived growth factor D	<i>Pdgfd</i>	1.88
platelet derived growth factor C	<i>Pdgfc</i>	-1.75
Vascular Endothelial Growth Factor		
vascular endothelial growth factor A	<i>Vegfa</i>	-2.03
vascular endothelial growth factor B	<i>Vegfb</i>	-2.58
vascular endothelial growth factor C	<i>Vegfc</i>	-7.65
epidermal growth factor		
epidermal growth factor receptor	<i>Egfr</i>	1.53
Fibroblast Growth Factor		
fibroblast growth factor 13	<i>Fgf13</i>	-10.61
fibroblast growth factor receptor 2	<i>Fgfr2</i>	-5.11
Insulin-like Growth Factor		
insulin-like growth factor 1 (somatomedin C)	<i>Igf1</i>	17.48
insulin-like growth factor 2 binding protein 1	<i>Igf2bp1</i>	-2.73
insulin-like growth factor 2 binding protein 2	<i>Igf2bp2</i>	-5.19
insulin-like growth factor 2 binding protein 3	<i>Igf2bp3</i>	-9.54
insulin-like growth factor binding protein 6	<i>Igfbp6</i>	10.20
insulin-like growth factor binding protein 3	<i>Igfbp3</i>	32.46
Cell cycle		
cyclin-dependent kinase inhibitor 1A (p21, Cip1)	<i>Cdkn1a</i>	-4.53
cyclin-dependent kinase inhibitor 1B (p27, Kip1)	<i>Cdkn1b</i>	-5.69
cyclin D1	<i>Ccnd1</i>	-1.90
Oncogenes/Protooncogenes		
v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	<i>Ma1b</i>	25.86
v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	<i>Mycn</i>	-29.75
FBJ murine osteosarcoma viral oncogene homolog	<i>Fos</i>	-9.62
jun B proto-oncogene	<i>Junb</i>	-4.69
v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	<i>LYN (v-yes)</i>	5.12
RAS pathway		
RAS, dexamethasone-induced 1	<i>Rasd1</i>	39.86
Rho family GTPase 1	<i>Rnd1</i>	-13.89
Rho family GTPase 3	<i>Rnd3</i>	-13.90

Gene Name	Gene Symbol	Fold Change
Tumor suppressor genes		
cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	<i>Cdkn2b</i>	-3.77
tumor protein p53	<i>Tp53</i>	-2.25
phosphatase and tensin homolog	<i>Pten</i>	-1.41
Wilms Tumor 1	<i>Wt1</i>	3.18
Ras association (RalGDS/AF-6) domain family member 1	<i>Rassf1</i>	-1.34
Adhesion Molecule, Integrins, catenins		
epithelial cell adhesion molecule	<i>Epcam</i>	22.65
cadherin 22, type 2	<i>Cd22</i>	128.56
catenin (cadherin-associated protein), beta 1, 88kDa	<i>Ctnnb1</i>	-1.41
integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	<i>Itgb2</i>	22.11
Protein Kinase		
protein kinase C, beta	<i>Prkcb</i>	73.34
mitogen-activated protein kinase 12	<i>Mapk12</i>	5.33
mitogen-activated protein kinase-activated protein kinase 3	<i>Mapkapk3</i>	15.96
Reactive Oxygen Species (ROS)		
dual oxidase 2	<i>Duox2</i>	104.54
glutathione peroxidase 2 (gastrointestinal)	<i>Gpx2</i>	102.71
Collagen		
collagen, type VI, alpha 2	<i>Col6a2</i>	66.60
collagen, type VI, alpha 1	<i>Col6a1</i>	64.48
Transporters and Solute Carriers		
solute carrier family 7, member 9	<i>Slc7A9</i>	12.70
solute carrier family 7, member 7	<i>Slc7A7</i>	14.15
solute carrier family 28, member 2	<i>Slc28A2</i>	14.08
ATP-binding cassette, sub-family A (ABC1), member 4	<i>Abca4</i>	33.23
Mesothelial cell marker		
cytokeratin 18	<i>Krt18</i>	162.94
cytokeratin 19	<i>Krt19</i>	511.14
thrombomodulin	<i>Thbd</i>	28.22
desmin	<i>Des</i>	24.19
Embryonic/cell development (cell migration, differentiation)		
placenta-specific 8	<i>Plac8</i>	39.53
wingless-type MMTV integration site family, member 4	<i>Wnt4</i>	11.04
plasminogen activator, urokinase	<i>Plau</i>	18.00
GATA binding protein 5	<i>Gata5</i>	15.36
Hormone receptors		
progesterone receptor	<i>Pgr</i>	8.10
estrogen receptor 1	<i>Esr1</i>	2.15
estrogen receptor 2 (ER beta)	<i>Esr2</i>	-1.23
androgen receptor	<i>Ar</i>	1.69

Gene Name	Gene Symbol	Fold Change
Growth Arrest, Apoptosis		
growth arrest and DNA-damage-inducible, beta	<i>Gadd45b</i>	-11.25
growth arrest and DNA-damage-inducible, alpha	<i>Gadd45a</i>	-2.01
BCL2-associated X protein	<i>Bax</i>	-1.53
BCL2-related protein A1	<i>Bcl2A1</i>	12.93
Fas apoptotic inhibitory molecule 3	<i>Faim3</i>	9.96
Fas ligand (TNF superfamily, member 6)	<i>Faslg</i>	2.17

Table 2

Select Differentially Expressed Genes Associated with Immune Response in Spontaneous Mesotheliomas in F344/N rats.

Gene Name	Gene Symbol	Fold Change
Chemokines		
chemokine (C-C motif) ligand 11	<i>Ccl11</i>	875.96
chemokine (C-X-C motif) ligand 10	<i>Cxcl10</i>	9.45
chemokine (C-C motif) ligand 5	<i>Ccl5</i>	20.40
chemokine (C-C motif) ligand 6	<i>Ccl6</i>	79.68
chemokine (C-X-C motif) ligand 11	<i>Cxcl11</i>	7.27
chemokine (C-X-C motif) ligand 13	<i>Cxcl13</i>	167.52
chemokine (C-X-C motif) ligand 2	<i>Cxcl2</i>	-133.25
Cytokines & Cytokine Receptors		
interleukin 1, beta	<i>Il1b</i>	3.21
interleukin 4 receptor	<i>Il4r</i>	20.95
interleukin 18 (interferon-gamma-inducing factor)	<i>IL18</i>	41.81
interleukin 34	<i>Il34</i>	8.49
interleukin 6 receptor	<i>Il6r</i>	7.20
interleukin 7 receptor	<i>Il7r</i>	8.49
tumor necrosis factor receptor superfamily, member 11a, NFkB activator	<i>Tnfrsf11a</i>	1.90
tumor necrosis factor receptor superfamily, member 11b	<i>Tnfrsf11b</i>	45.94
interleukin 24	<i>Il24</i>	27.76
interleukin 10	<i>Il10</i>	24.21
Interleukin 1 receptor antagonist	<i>Il1rn</i>	10.35
interleukin 23A	<i>Il23a</i>	-5.99
CD40 molecule, TNF receptor superfamily member 5	<i>Cd40</i>	10.29
Jak/Stat Pathway		
signal transducer and activator of transcription 1, 91kDa	<i>Stat1</i>	7.03
signal transducer and activator of transcription 2, 113kDa	<i>Stat2</i>	4.31
signal transducer and activator of transcription 3 (acute-phase response factor)	<i>Stat3</i>	1.77
Janus kinase 2	<i>Jak2</i>	6.86
Janus kinase 1	<i>JAK1</i>	1.60
Histocompatibility Markers		
major histocompatibility complex, class II, DR alpha	<i>Hla-dra</i>	425.51
major histocompatibility complex, class II, DQ alpha 1	<i>Hla-dqa1</i>	172.26
major histocompatibility complex, class II, DQ beta 1	<i>Hla-dqb1</i>	166.25
CD74 molecule, major histocompatibility complex, class II invariant chain	<i>Cd74</i>	564.33
Complement		
complement factor H	<i>Cfh</i>	306.57
complement component 1, q subcomponent, B chain	<i>C1qb</i>	115.07
complement component 1, q subcomponent, A chain	<i>C1qa</i>	132.21
serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	<i>Serping1</i>	303.39

Gene Name	Gene Symbol	Fold Change
Pattern Recognition Receptors		
toll-like receptor 1	<i>Tlr1</i>	3.04
toll-like receptor 2	<i>Tlr2</i>	8.58
toll-like receptor 8	<i>Tlr8</i>	23.83
toll-like receptor 7	<i>Tlr7</i>	10.98
mannose receptor, C type 1	<i>Mrc1</i>	73.69
Interferon Pathway		
interferon, alpha 16	<i>Ifna16</i>	-1.28
interferon gamma receptor 1	<i>Ifngr1</i>	2.33
interferon regulatory factor 8	<i>Irf8</i>	11.31
interferon regulatory factor 7	<i>Irf7</i>	7.42
interferon regulatory factor 9	<i>Irf9</i>	4.02
interferon regulatory factor 5	<i>Irf5</i>	2.27
interferon regulatory factor 3	<i>Irf3</i>	1.43
interferon induced transmembrane protein 1	<i>Ifitm1</i>	21.44
Inflammatory mediators/enzymes/miscellaneous		
allograft inflammatory factor 1	<i>AIF1</i>	189.47
prostaglandin D2 synthase 21kDa	<i>Ptgds</i>	288.68
prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	<i>Ptgs1</i>	27.33
prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	<i>Ptgs2</i>	-101.38
lysozyme 2	<i>Lyz1/lyz2</i>	269.89
mast cell protease 8	<i>Mcpt8</i>	249.84
hemoglobin, alpha 1	<i>Hba1/hba2</i>	241.31
tryptophan 2,3-dioxygenase	<i>Tdo2</i>	212.85
ubiquitin D	<i>Ubd</i>	191.62
carbonic anhydrase IV	<i>Ca4</i>	159.31
DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	<i>Ddx60</i>	149.65
cytochrome b-245, beta polypeptide	<i>Cybb</i>	134.29
phospholipase A2, group IIA	<i>Pla2g2a</i>	96.46
lymphatic vessel endothelial hyaluronan receptor 1	<i>Lyve1</i>	81.91
Activated macrophage products		
chitinase 3-like 1	<i>Chi3l1</i>	149.52
SPARC-like 1 (hevin)	<i>Sparcl1</i>	71.34
complement component 1, q subcomponent, B chain	<i>C1qb</i>	115.07
complement component 1, q subcomponent, A chain	<i>C1qa</i>	132.21
Cell surface markers/receptors		
sphingosine-1-phosphate receptor 1	<i>S1pr1</i>	24.39
Fc fragment of IgG, low affinity IIb, receptor (CD32)	<i>Fcgr2b</i>	198.66
Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	<i>Fcer1a</i>	38.51
Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	<i>Fcer1g</i>	117.78
Fc fragment of IgG, low affinity IIIa, receptor (CD16a)	<i>Fcgr3a</i>	57.05

Gene Name	Gene Symbol	Fold Change
Stabilin 1	<i>Stab1</i>	60.71
Fc fragment of IgG, high affinity Ia, receptor (CD64)	<i>Fcgr1a</i>	30.53
Fc fragment of IgG, low affinity IIa, receptor (CD32)	<i>Fcgr2a</i>	51.26
CD 163 molecule	<i>Cd163</i>	49.51
CD 68 molecule	<i>Cd68</i>	32.09
CD 53 molecule	<i>Cd53</i>	130.44
C-type lectin domain family 4, member a3	<i>Clec4a3</i>	123.54
C-type lectin domain family 10, member A	<i>Clec10a</i>	46.75
C-type lectin domain family 4, member A	<i>Clec4a</i>	36.02
C-type lectin domain family 7, member A	<i>Clec7a</i>	28.97
CD36 molecule (thrombospondin receptor)	<i>Cd36</i>	148.28

Table 3

qPCR validation of select up- and downregulated genes in spontaneous mesotheliomas in F344/N rats.

Entrez Gene Name	Gene Symbol	qRT-PCR	Microarray
GATA binding protein 5	<i>Gata5</i>	604.09	14.2
Interleukin-10	<i>Il10</i>	697.23	24.21
Interleukin-18	<i>Il18</i>	159.52	41.81
Glutathione peroxidase 2	<i>Gpx2</i>	484.44	102.71
Cytokeratin 18	<i>Krt18</i>	1079.19	162.94
Tumor necrosis factor receptor 11b	<i>Tnfr11b</i>	729.76	45.94
Plasminogen activator, urokinase	<i>Plau</i>	278.72	17.9
Wingless-type MMTV integration site family, member 4	<i>Wnt4</i>	7.84	11.19
CD40 molecule, TNF receptor superfamily member 5	<i>Cd40</i>	843.49	10.18
Epithelial adhesion molecule	<i>Epcam</i>	481.96	22.65
Protein kinase C, beta	<i>Prkcb</i>	274.06	73.34
Transforming Growth Factor alpha	<i>Tgfa</i>	12.38	12.45
Cyclin dependent kinase 1a (p21)	<i>Cdkn1a</i>	-2.88	-4.53
Fatty acid binding protein 4	<i>Fabp4</i>	2705.77	68.98
Placenta-specific 8	<i>Plac8</i>	16744.15	39.53
RAS, dexamethasone-induced 1	<i>Rasd1</i>	86.8	39.86
Tumor protein p53	<i>Tp53</i>	-1.11	-2.25

Table 4

qPCR validation of select up- and downregulated genes in spontaneous mesotheliomas in F344/N rats.

Entrez Gene Name	Gene Symbol	qRT-PCR	Microarray
GATA binding protein 5	<i>Gata5</i>	604.09	14.2
Interleukin-10	<i>Il10</i>	697.23	24.21
Interleukin-18	<i>Il18</i>	159.52	41.81
Glutathione peroxidase 2	<i>Gpx2</i>	484.44	102.71
Cytokeratin 18	<i>Krt18</i>	1079.19	162.94
Tumor necrosis factor receptor 11b	<i>Tnfr11b</i>	729.76	45.94
Plasminogen activator, urokinase	<i>Plau</i>	278.72	17.9
Wingless-type MMTV integration site family, member 4	<i>Wnt4</i>	7.84	11.19
CD40 molecule, TNF receptor superfamily member 5	<i>Cd40</i>	843.49	10.18
Epithelial adhesion molecule	<i>Epcam</i>	481.96	22.65
Protein kinase C, beta	<i>Prkcb</i>	274.06	73.34
Transforming Growth Factor alpha	<i>Tgfa</i>	12.38	12.45
Cyclin dependent kinase 1a (p21)	<i>Cdkn1a</i>	-2.88	-4.53
Fatty acid binding protein 4	<i>Fabp4</i>	2705.77	68.98
Placenta-specific 8	<i>Plac8</i>	16744.15	39.53
RAS, dexamethasone-induced 1	<i>Rasd1</i>	86.8	39.86
Tumor protein p53	<i>Tp53</i>	-1.11	-2.25