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

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Abstract

Lung cancer is the leading cause of cancer-related death in people and is mainly due to environmental factors such as smoking and radon. The National Toxicology Program (NTP) tests various chemicals and mixtures for their carcinogenic hazard potential. In the NTP chronic bioassay using B6C3F1 mice, the incidence of lung tumors in treated and control animals is second only to the liver tumors. In order to study the molecular mechanisms of chemically induced lung tumors, an understanding of the genetic changes that occur in spontaneous lung (SL) tumors from untreated control animals is needed. The authors have evaluated the differential transcriptomic changes within SL tumors compared to normal lungs from untreated age-matched animals. Within SL tumors, several canonical pathways associated with cancer (eukaryotic initiation factor 2 signaling, RhoA signaling, PTEN signaling, and mammalian target of rapamycin signaling), metabolism (Inositol phosphate metabolism, mitochondrial dysfunction, and purine and pyrimidine metabolism), and immune responses (FcγR-mediated phagocytosis, clathrin-mediated endocytosis, interleukin 8 signaling, and CXCR4 signaling) were altered. Meta-analysis of murine SL tumors and human non–small cell lung cancer transcriptomic data sets revealed a high concordance. These data provide important information on the differential transcriptomic changes

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in murine SL tumors that will be critical to our understanding of chemically induced lung tumors and will aid in hazard analysis in the NTP 2-year carcinogenicity bioassays.

Keywords

lung cancer; B6C3F1 mouse; microarray; gene expression analysis; comparative genomics

Introduction

Lung cancer is the leading cause of cancer-related death in people, with women having higher incidence than men. Each year in the United States, about 170,000 new cases of lung cancer will be diagnosed with a 5-year survival rate of <15%. However, in many cases, early diagnosis assures about a 60% 5-year survival rate compared to about 5% 5-year survival rate in late diagnosis. Tobacco smoking is said to be responsible for ~85% of lung cancer incidence and the remaining ~15% may be due to occupational and environmental exposure to carcinogens such as asbestos and radon as well as poorly understood genetic factors (Doll 2000). Based on histopathological criteria, lung cancer is classified into two major categories: non-small cell lung cancer (NSCLC) and (SCLC) small cell lung cancer (de Seranno and Meuwissen 2010). SCLC includes neuroendocrine tumors that are highly aggressive and readily metastasize and accounts for about 20% of all lung tumors. Due to the high rate of metastasis, surgery is generally not an option for SCLC and combination chemotherapy is the standard of care. NSCLC includes adenocarcinomas, squamous cell carcinoma, and large cell carcinomas that account for approximately 80% of all lung cancers. These tumors are treated by surgery and radiotherapy in early stages but combination chemotherapy in cases of metastatic disease. In addition to morphological differences, NSCLC and SCLC are dichotomous in their biologic behavior, genetic alterations, and response to treatment (Nikitin et al 2004).

The mouse develops spontaneous lung (SL) tumors (not treatment related) that are morphologically similar to NSCLC adenocarcinomas in humans. Mouse SL tumors are primarily adenocarcinomas of papillary, acinar, solid, or a mixture of these phenotypes. They do not metastasize as readily as human NSCLC. Mouse lung tumors have some differences in tumor biology with human NSCLC but there are significant similarities in the pathogenesis and cancer biology between the two species (Nikitin et al. 2004; Meuwissen and Berns 2005). Given these similarities, the murine lung tumors may provide a great model to study human NSCLC.

In addition to the chemically induced and genetically modified mouse models of lung cancer, several strains of mice develop SL tumors. The most sensitive strains include A/J (onset at 3–4 months and 100% by 18–24 months) and SWR mice; strains of intermediate susceptibility include Swiss (23%, life span), CD-1 (22%, 18–23 months), and BALB/c strains (52%, 24 months), CBA, and C3H; and very resistant strains include C57BL/6 (8%, 24 months) and DBA (Meuwissen and Berns 2005). The National Toxicology Program's (NTP) B6C3F1 mouse strain likely corresponds to the intermediate susceptible strain. This

relative level of susceptibility to lung tumor development in the above mouse strains is also maintained in chemically induced lung tumor incidence (Malkinson 1991).

The B6C3F1 (C57BL/6J $\times$ C3H F1) mouse strain is used in the NTP to assess the carcinogenic potential of several chemicals and chemical mixtures. In the 2-year NTP bioassay, where the mice are exposed to chemicals for 2 years, the evidence of carcinogenicity is most common in the liver (57%) followed by the lung (22%; Grace Kissling, Personal communication). In some NTP studies, the incidence of lung tumors is almost 100% when exposed to certain chemicals. The incidence of SL tumors in control B6C3F1 mice (all routes, all vehicles) at the end of the 2-year bioassay is 26% and 9% in males and females, respectively (*NTP historical control database, 2011*). Given this background tumor incidence, it is critical to comprehensively evaluate the transcriptomic changes of SL tumors in the B6C3F1 mouse strain. This will provide a background of genomic events in SL tumors that may aid in elucidating the molecular “genetic signature” of tumors resulting from chemical exposure in the chronic NTP bioassay. More importantly, it may also provide information on comparative molecular genetics of murine lung tumors and human NSCLC that will provide information relevant to human exposures and their carcinogenic risk. We have recently published on the differential transcriptomic changes within spontaneous hepatocellular carcinomas from untreated control B6C3F1 mice from chronic NTP bioassay that has provided a critical database for evaluating genomic changes in liver tumors due to chemical exposure, and their relevance to the disease in humans (Hoenerhoff et al. 2011).

In this study, we have evaluated the differential gene expression and predominant cancer pathways in SL tumors compared to age-matched normal lung (NL) samples from untreated/control B6C3F1 mice. In addition, the SL microarray data set was compared to a human NSCLC data set to evaluate the differential gene expression and cancer pathways in murine and human lung tumors.

Materials and Methods

Animals and Tissue Sampling

SL tumors were obtained from untreated control B6C3F1 mice at terminal sacrifice of a 2-year inhalation NTP bioassay. At necropsy, lung tumors larger than 0.5 cm diameter were sectioned in half and the one half was fixed in 10% neutral buffered formalin for histological evaluation and the other half was flash frozen in liquid nitrogen for molecular analysis. Also NLs without tumors from age-matched untreated mice were collected for histological evaluation and molecular analysis as described above. After 24 hr of formalin fixation, the tissues were transferred to 70% ethanol and were routinely processed in graded alcohols, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic analysis. All samples for molecular analysis were paired with formalin-fixed paraffin-embedded histopathology samples for morphologic analysis and immunohistochemical localization of proteins.

Extraction and Quantification of RNA, Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

RNA was extracted from six flash frozen lung tumors (four males and two female) and six NL tissues (four males and two female) using the Invitrogen PureLink Mini kit (Invitrogen cat# 12183-018A, Carlsbad, CA) according to the manufacturer's protocol. Frozen tissue samples were lysed and homogenized in TRIzol reagent (Invitrogen) using a rotor-stator homogenizer. Isolation of RNA was performed according to the mini kit protocol. On-column deoxyribonuclease (DNase) treatment was performed using the Invitrogen PureLink DNase kit (Invitrogen) to purify the RNA samples. RNA concentration and quality were measured on a Bioanalyzer (Agilent Technologies, Santa Clara, CA). Samples were aliquoted and stored at -80°C until they were analyzed.

RNA Labeling and Microarray Hybridization

Gene expression analysis was conducted using Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA). One microgram of total RNA was amplified using the Affymetrix One-Cycle Complementary Deoxyribonucleic Acid (cDNA) Synthesis protocol. Fifteen micrograms of amplified biotin-complementary RNA were fragmented and 10 mg were hybridized to each array for 16 hr at 45°C in a rotating hybridization oven using the Affymetrix Eukaryotic Target Hybridization Controls and protocol. Array slides were stained with streptavidin/phycoerythrin using a double-antibody staining procedure and then washed using the EukGE-WS2v5 protocol of the Affymetrix Fluidics Station FS450 for antibody amplification.

Data Processing

Arrays were scanned in an Affymetrix Scanner 3000 and data were obtained using the GeneChip Command Console Software (AGCC, version 1.1). Fluorescent pixel intensity measurements were acquired from the arrays. Gene expression data were normalized across all samples using the robust multiarray analysis (RMA) methodology (Irizarry et al. 2003) in the R statistical software environment. RMA subtracts background and adjusts signal intensities across arrays by applying quantile (ranked based) normalization at the probe set level. Finally, RMA analysis fits a linear additive model to the log-transformed perfect match values using median polish to produce the gene expression measures used for the statistical analyses presented here. Microarray data files (.cel) and associated annotations have been submitted to the Gene Expression Omnibus (GEO) database (GSE31013).

Identification of Differentially Expressed Genes

RMA-normalized data were used for statistical analysis. For each probe set, pairwise comparisons were made using a bootstrap t test while controlling the mixed directional (FDR) false discovery rate (Guo, Sarkar, and Peddada 2010). This methodology controls for the overall FDRs for multiple comparisons as well as directional errors when declaring a gene to be upregulated or downregulated. The bootstrap methodology does not make any assumptions of normality and is robust to the underlying variance structure.

For each probe set, pairwise t tests were performed between the groups of samples. The null hypothesis is that none of the pairwise contrasts is different from 0 versus at least one pairwise difference is not equal to 0. If for a given probe set, the null hypothesis is rejected, (mdFDR) multidimensional false discovery rate (Guo, Sarkar, and Peddada 2010) is determined in a “post hoc” manner to control for multiple testing and to account for the errors made in assigning the direction of differential change (i.e., positive or negative). If no significant probe sets are detected, then for each pairwise contrast where for a given probe set the null hypothesis is rejected, the standard FDR (Benjamini and Hochberg 1995) is determined to control for multiple testing. The level of significance was set at $p < .05$ and the mdFDR was set at 5%.

The statistical analysis in this study was very conservative since three different pairs of samples were compared and mdFDR correction was applied to each of the three comparisons, that is, SL tumors were compared to NL, chemically induced lung tumors were compared to NL, and SL tumors were compared to chemically induced lung tumors. This approach was taken to compare the SL tumors in the context of chemically induced tumors and the data from the latter two comparisons will be presented in a subsequent manuscript. In order to confirm that this overly conservative (three pairwise comparisons with corresponding mdFDR corrections) statistical analysis did not change the significant biological categories, we reran the analysis including only one pairwise comparison (with mdFDR correction), that is, SL tumors and NL tumors. The significance of this reanalyses of data will be examined in the result and discussion section (see Table 1).

Principal Component Analysis (PCA) and Unsupervised Hierarchical Clustering

Partek Genomics Suite, version 6.4 (release date September 22, 2009; Partek, St. Louis, MO), was used to perform PCA on the normalized data and to generate heat maps to compare the NL and SL tumor samples for differentially expressed probe sets ($p < .05$). PCA uses a linear transformation to reduce the dimension of the data from n probe sets to k PCs. The first three PCs that capture the majority of the variation in the data were used to visualize the spatial relationship of the NL and SL tumor samples. For hierarchical clustering, gene expression measures were first standardized across samples in a gene-specific manner in which the mean expression level of a gene is subtracted from each gene expression value and the quantity is divided by the standard deviation of values across all samples. After standardization, the genes (rows) and samples (columns) of the data matrix holding gene expression measures for significant genes were subjected to agglomerative hierarchical clustering, where Pearson’s dissimilarity was used as the distance measure and Ward’s method was used to evaluate distance between clusters (Ward 1963).

Determination of Overrepresented Canonical Pathways

Ingenuity Pathway Analysis (IPA) 9.0, application build-124019, version-11631407, was used to evaluate the most statistically significant overrepresented canonical pathways. These canonical pathways are based on the Ingenuity knowledge base. The significant biological canonical pathways were derived from IPA and the statistical significance was assumed at a $p < .001$ (Fisher’s exact test). The entire catalog of IPA canonical pathways was evaluated and classified into four broad categories, that is, (1) *cancer pathways* (cancer, cell cycle

regulation, intracellular and second messenger signaling, cellular stress and injury and apoptosis, cellular growth, proliferation and development, growth factor signaling, and organismal growth and development); (2) *metabolic pathways*; (3) *Immune response pathways* (cytokine signaling, cellular immune response, and humoral immune response); (4) *IPA xenobiotic pathways* (Ingenuity toxicity list pathways, nuclear receptor signaling, and xenobiotic metabolism).

Comparison of Murine SL Tumors and Human NSCLC

NextBio tool was used to compare the transcriptomic data sets of murine SL tumor to human NSCLC and evaluate pathway enrichment in both systems. NextBio is a curated and correlated repository of experimental data derived from an extensive set of public sources (e.g., ArrayExpress and GEO) that allows the user to compare patterns of gene expression in their experiment to thousands of genomic signatures derived from published data sets. Statistical analysis is carried out using rank-based enrichment analysis to compute pairwise correlation scores of the uploaded data set and all studies contained in NextBio. The statistical analysis method used by NextBio is referred to as a “Running Fischer” and is very similar to the gene set enrichment analysis (Subramanian et al. 2005). A detailed explanation of the analysis protocol used by NextBio is described elsewhere (Kupersmidt et al. 2010).

NextBio software was utilized to compare the differential transcriptomic changes common to human NSCLC (curated by NextBio) and the murine SL tumors. The top ranked upregulated and downregulated genes, as well as functional categories (termed biogroups by Nextbio) such as canonical pathways and predicted microRNA (miRNA) targets (from TargetScan), that are common to murine SL tumors and human NSCLC were compared. The prediction of miRNA targets by NextBio is based on TargetScan (Lewis, Burge, and Bartel 2005). A biogroup’s score is based on the overall statistical significance and consistency of the enrichment, or overlap, between the set of genes that make up the biogroup and each of the queried biosets. The most significant biogroup receives a score of 100. All other biogroup scores are normalized to the top ranked biogroup (Kupersmidt et al. 2010).

Reverse Transcription and Quantitative Real-Time PCR

To remove genomic deoxyribonucleic acid (DNA), RNA samples were incubated with 1 U of ribonuclease-free DNase I (Invitrogen) per microgram of RNA for 15 min at room temperature. DNase I was then inactivated by the addition of 2.5 mM ethylenediaminetetraacetic acid (pH 8.0) and heated at 65° C for 10 min. To generate the cDNA, reverse transcription of RNA using Murine Leukemia Virus reverse transcriptase (Applied Biosystems, CA, USA) was carried out according to the manufacturer’s instructions using oligo-deoxythymidine primers (Invitrogen). As a negative control, a sample containing RNA but no reverse transcriptase was also included. A 1:10 dilution with DNase-free water of this cDNA was used for realtime PCR analysis in ninety-six well plates.

Quantitative gene expression levels were detected using real-time PCR with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) and TaqMan 3’–Minor groove

binder-DNA (MGB) probes (FAM [Flourescein amidite] 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(R) universal PCR Master Mix (Applied Biosystems, catalog no. 4304437) according to manufacture's instructions. Samples were analyzed in duplicate, and a sample without reverse transcriptase was included in each plate to detect contamination by genomic DNA. Amplification was carried out as follows: 50°C for 2 min (for uracil-*N*-glycosylase incubation), 95°C for 10 min (denaturation), 95°C for 15 s, and 60°C for 30 s (denaturation and amplification) for forty cycles. Fold increases or decreases in gene expression were determined by quantitation of cDNA from tumor samples relative to NL samples. 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 SL tumors and NLs, where the changes in crossing threshold (C_t) = $C_{t\text{Target gene}} - C_{t18S \text{ RNA}}$, and $C_t = C_{t\text{Tumor}} - C_{t\text{Normal}}$.

Immunohistochemistry

Immunohistochemistry was performed on unstained formalin fixed, paraffin embedded sections using an avidin-biotin-peroxidase system (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. The 5 μ m sections were collected on charged glass slides and were deparaffinized in xylene and rehydrated through a decreasing graded ethanol series. Antigen retrieval was performed by immersing the slides in 200 ml of 1 $\times$ citrate buffer and pressure cooked in a decloaker for 5 min. Endogenous peroxidases were quenched with 3% (vol/vol) hydrogen peroxide for 15 min followed by 1 $\times$ PB S wash. Nonspecific binding sites were blocked with 4% (vol/vol) normal donkey serum in 1 $\times$ PBS (Phosphate buffered saline) for 1 hr at room temperature in a humidified chamber. Negative controls received the antisera from the same animal species as the source of the secondary antibody. Positive controls included tissues known to exhibit positive expression of proteins of interest. The source and working dilutions of antioat polyclonal antibodies that were used to validate the protein expression included Inhibitor of DNA binding 2 (ID2; C-20 at 1:200, Santa Cruz Biotechnology Inc, Santa Cruz, CA), high mobility group AT-hook 1 (HMGA1; ab4078 at 1:250, Abcam Inc, Cambridge, MA), Survivin (Aab469 at 1:500, Abcam Inc, Cambridge, MA), serine threonine kinase 39 (STK39; ab71825 at 1:10, Abcam Inc, Cambridge, MA), and Hepatocyte nuclear factor 4a (HNF4a; H-171 at 1:25, Santa Cruz Biotechnology, Inc, Santa Cruz, CA). All the sections were incubated with the corresponding primary antibodies for 1 hr in humidified chambers. After being washed in 1 $\times$ PBS, biotinylated secondary donkey antioat Immunoglobulin G (1:500 in blocking solution) was incubated on the sections for 1 hr at room temperature. After further washes, sections were incubated with avidin-biotin-peroxidase complex (ABC) solution following the manufacturer's instructions (Vector Laboratories). After the primary antibody and secondary antibody treatment with multiple intervening washes, the antigen was visualized by peroxidase enzyme reaction with 3,3-diaminobenzidine chromogen. Slides were rinsed in tap water, counterstained with modified Harris hematoxylin, dehydrated through graded ethanol series and xylene, and finally glass coverslips were applied.

Results

Murine Lung Tumors Share Morphologic Similarities with Human Non–Small Cell Lung Tumors

Murine SL tumors, in almost all cases, resemble human NSCLC, adenocarcinoma. The most common tumor morphology was a papillary adenocarcinoma with occasional solid areas (Figure 1A). Neoplastic cells exhibited moderate anisocytosis, anisokaryosis, nuclear atypia, and a mild to moderate mitotic index. The tumors were large expansile masses that compressed the adjacent pulmonary parenchyma. Multifocally, the tumors were locally invasive, but there was no evidence of metastasis in any of the animals examined.

Comparison of Transcriptomes from B6C3F1 Mouse Lung Tumors and Age-Matched NL Tissue

Analysis of SL tumors compared to age-matched NL tissue using Affymetrix Mouse Genome 430 2.0 GeneChip arrays indicated 7,119 of the 21,901 annotated genes were differentially expressed at $p < .05$, mdFDR 5%, and a fold change >1 or <-1 . PCA captured 84.5% of data variation in all the differentially expressed genes and indicated tight clustering of the control lung tissues away from the SL tumors (Figure 2). Clustering among the SL tumors was not as tight as the NL sample since 2/6 SL tumors clustered farther from the other 4 SL tumors. The unsupervised agglomerative hierarchical cluster analysis of the differentially expressed genes within the SL tumors and NL tissue demonstrated significant differences in gene expression (Figure 3).

Significantly overrepresented canonical pathways (at $p < .001$) derived from IPA are represented within Table 1. This table also provides the ratio of number of genes within the differentially expressed gene list compared to the curated and catalogued genes in that particular canonical pathway within the IPA database as well as the $-\log(p \text{ value})$ as determined by Fisher's exact test. The significant *cancer pathways* include eukaryotic initiation factor 2 (eIF2) signaling, $-\log(p \text{ value}) = 12.356$, axonal guidance signaling (8.825), mammalian target of rapamycin (mTOR) signaling (8.144), regulation of eIF4 and p70S6K signaling (8.132), RhoA signaling (7.347), tight junction signaling (5.997), phosphatase and tensin homolog (PTEN) signaling (5.681), ephrin receptor signaling (5.574), integrin signaling (4.065), and Integrin Linked Kinase (ILK) signaling (4.025); significant *metabolic pathways* include inositol phosphate metabolism (5.534), pyrimidine metabolism (4.394), purine metabolism (4.169), oxidative phosphorylation (3.954), and citrate cycle (3.157); significant *immune response signaling* include FcγR-mediated phagocytosis in macrophages and monocytes (4.78), clathrin-mediated endocytosis signaling (4.239), interleukin 8 (IL-8) signaling (3.805), CXCR4 signaling (3.801), and Caveolar-mediated endocytosis signaling (3.312); and significant *IPA xenobiotic pathways* include mitochondrial dysfunction (5.352), androgen signaling (4.705), retinoic acid receptor (RAR) activation (3.724), estrogen receptor signaling (3.274), aldosterone signaling in epithelial cells (3.007), and NF-E2-related factor 2 (Nrf2)-mediated oxidative stress response (2.968).

Reanalyzing the transcriptomic data using a single pairwise comparison between the murine SL tumors and NL indicated that 13,054 of the 21,901 annotated genes were differentially

expressed at $p < .05$, mdFDR 5%, with a fold change of >1 or <-1 . Reanalyzing this bigger data set using IPA revealed the same overrepresented canonical pathways and in addition, identified three more significant ($p < .001$) canonical pathways, namely Aryl hydrocarbon receptor signaling (4.979), cyclins and cell cycle regulation (4.207), and antiproliferative role of TOB (Transducer of ERBB2, 1) in T cell signaling (3.371; Table 1).

Common Genes and Pathways in Murine SL Tumors and Human NSCLC

Using NextBio, the differentially expressed murine SL tumor data set was compared to the most similar human lung cancer data set (from the NextBio curated data sets as well as National center for Biotechnology Information's GEO database). The human NSCLC data set (GSE19804) that was most concordant with the murine SL tumor data set (with a NextBio score of 78%) is from stage 3 lung cancer tissue from nonsmokers compared to adjacent NL tissue using Affymetrix Human Genome U133 Plus 2.0 Array (Lu et al. 2010).

Comparison of the 7,119 differentially expressed murine SL tumor genes to the 10,438 differentially expressed human NSCLC genes resulted in an overlap of 3,284 genes (p value = $9.5E-253$) with 1,262 upregulated genes (p value = $3.3E-199$) and 1,345 downregulated genes (p value = $3.0E-307$) genes (Figure 4). Top concordant genes (Table 2), canonical pathways (Table 3), and predicted miRNA targets (Table 4) from TargetScan that are common to murine SL tumors and human NSCLC are presented.

Validation of Selected Genes by Real-Time RT-PCR and Immunohistochemistry

Some of the differentially altered genes identified from the microarray data sets of murine SL tumors were validated by RT-PCR (Table 5). In addition, some additional genes identified from other chemically induced murine lung tumor microarray data sets were also validated using the same RNA samples from the murine SL tumors. Some of the genes (*Cdkn2a*, *Hnf4a*, *Hmga1*, and *Braf*) that were not considered statistically significant ($p < .05$, mdFDR at 5%) within the microarray data set are differentially altered by RT-PCR (with no mdFDR correction). The expression of six proteins relevant for carcinogenesis was validated by immunohistochemistry. The HMGA1 is an architectural transcription factor that binds to A-T rich DNA sequences and participates in enhanceosome formation, chromatin remodeling, and regulation of transcription of several genes resulting in functions associated with cell growth and differentiation. All the nuclei within the tumor stained intensely with the HMGA1 antibody (Figure 1B). STK39 is activated by cellular stress and activates p38 mitogen-activated protein kinase (MAPK) pathway. There was marked nuclear expression and moderate cytoplasmic expression of STK39 within the tumor cells (Figure 1C). HNF4a is a transcription factor that regulates expression of several genes involved in metabolism and cell growth. It is highly expressed within the nucleus and has mild to moderate cytoplasmic expression within the tumor cells (Figure 1D). ID2, dominant negative helix-loop-helix is a transcription factor that inhibits H-L-H transcription factors and negatively regulates cell differentiations. The tumors cells exhibited moderate cytoplasm and plasma membrane staining (Figure 1E). Baculoviral IAP repeat containing 5 (BIRC5 or Survivin) is a member of IAP family and negatively regulates apoptotic cell death. The tumor cells exhibited moderate nuclear staining and mild cytoplasmic staining (Figure 1F).

Discussion

In this study, using differential transcriptomic analysis of B6C3F1 SL tumors, we have identified several dysregulated canonical pathways related to carcinogenesis, metabolism, immune responses, and IPA xenobiotic pathways. All the murine SL tumors were histologically very similar and were consistent with the phenotype of alveolar bronchiolar papillary adenocarcinomas (Dixon et al. 1999; Nikitin et al. 2004). However, the PCA plot (Figure 2) does not demonstrate a tight clustering within the murine SL tumor samples compared to NL samples that cluster very tightly. All the SL tumor samples were reevaluated for this heterogeneity (loose clustering) by examining the individual animal records as well as blinded histopathology evaluation but no apparent differences could be noted. Thus, this heterogeneity may be explained by the existence of molecular subtypes within the histologically similar murine SL tumors or due to a higher variation in the gene expression data of the SL tumor samples compared to the control NL samples with low variance. This is an interesting finding and deserves further study with a greater sample size especially since the molecular heterogeneity of human NSCLC adenocarcinomas is well known.

Altered Canonical Cancer Pathways in Murine SL Tumors

Several canonical pathways involved in cancer, metabolism, immune response, and IPA xenobiotic pathways were dysregulated within the murine SL tumors (Table 1). The eIF2, $-\log(p \text{ value}) = 12.356$, eIF4 and p70S6K (8.32), and mTOR (8.144) signaling were among the most significantly altered canonical cancer pathways within murine SL tumors. These pathways are activated by stress, heat shock, hypoxia, and amino acid starvation and play critical roles in translational regulation (Koumenis et al. 2002; Pestova et al. 2001) and have been reported to be activated within human NSCLC (Rosenwald 2004). Cellular stress activates EIF2AK2 (*Eif2ak22*, 2.201) and EIF2AK4 (*Eif2ak4*, 2.078) resulting in phosphorylation of EIF2A that subsequently causes termination of global protein translation and induces apoptosis (Gingras, Raught, and Sonenberg 1999). The cellular phenotype of neoplastic cells is the result of a complex interplay of growth- and apoptotic-signaling pathways. The mTOR pathway is one of the major growth pathways and signals through the PI3K/AKT signal transduction pathway (Wulfschleger, Loewith, and Hall 2006). The PI3K/AKT signal transduction pathway may be activated by the MAPK-signaling pathway or by Insulin receptor substrate 1 (*Irs1*, 2.405; Farrar, Houser, and Clarke 2005). The p85 regulatory subunit of PI3K binds phosphorylated IRS and results in activation of the p110 catalytic subunit. PI3K activates 3-phosphoinositide-dependent protein kinase 1 (PDK1) that causes phosphorylation and activation of AKT (*Akt1s1*, 1.449). AKT phosphorylates mTOR directly or indirectly through the action of the inhibitory Tuberous sclerosis genes (*Tsc2*, -1.256), a tumor suppressor gene (Inoki, Zhu, and Guan 2003; Jozwiak et al. 2005). Activation of AKT and mTOR pathways is frequently observed in NSCLC and preneoplastic bronchial lesions (Balsara et al. 2004). Hypoxia stimulates the stress-induced protein DNA-damage-inducible transcript 4 (*Ddit4*, 2.514) resulting in inhibition of tuberous sclerosis 2 (TSC2). The inactivated TSC2 fails to inactivate the RHEB-GTP (Ras homolog enriched in brain-Guanosine-5'-triphosphate) that can activate mTOR (Zhang et al. 2003). In addition, increased phosphatidylcholine-specific phospholipase D1 (*Pld1*, 2.523) is

associated with increased mTOR signaling. mTOR signaling enhances the activation of Hypoxia inducible factor 1A (*Hif1α*, 3.389) that is increased by hypoxia (Hudson et al. 2002). HIF1A is frequently overexpressed within several cancers including lung cancer (Zhong et al. 1999).

Several tightly interrelated pathways involved in maintaining cytoskeletal architecture, microtubule dynamics, adhesion, cell migration, cell–cell and cell–extracellular matrix interactions, and cell cycle progression, such as RhoA signaling, $-\log(p \text{ value}) = 7.347$, axonal guidance signaling (8.825), tight junction signaling (5.997), ephrin receptor signaling (5.574), and integrin signaling (4.065) were significantly altered within murine SL tumors. RhoA is a member of the Ras superfamily of small GTPases and is activated by a variety of growth factors like insulin-like growth factor 1 (Igf1, 2.83), adhesion molecules, integrins, and G-proteins (Ephexin, 1.597). RhoA is important for organization of stress fibers and regulation of cytoskeleton. A number of genes involved in axonal guidance-like semaphorins (*Sema3a*, -4.025 ; *Sema3b*, -3.373 ; *Sema3c*, -4.432 ; *Sema3d*, -3.017 ; *Sema3e*, -4.147 ; *Sema3f*, -3.338 ; *Sema3g*, -15.23 ; *Sema4a*, 2.826; *Sema4d*, 2.201; *Sema5a*, -3.019 ; *Sema6a*, -4.763 ; *Sema6d*, -2.233 ; *Sema7a*, -2.819), Netrins (*Ntn1*, -6.122 ; *Ntn4*, -4.355), and ephrins (*Efna4*, 2.912; *Efnb1*, -2.01) also play important roles in cancer. Semaphorins like SEMA3A, SEMA3C, and SEMA3F play an important role in lung morphogenesis during development and SEMA3B, SEMA3F, and SEMA6A act as tumor suppressors in lung cancer (Potiron, Roche, and Drabkin 2009). Downregulation of *Sema3b* is mediated by methylation in lung cancer cell lines (Tomizawa et al. 2001). SEMA3B and SEMA3F are targets of the tumor suppressor p53, suggesting that they could be activated during DNA damage or other stress responses (Futamura et al. 2007; Ochi et al. 2002). *Sema4b* and *Sema4d* are upregulated in lung cancer and lung cancer cell lines. SEMA4B promotes migration and metastasis in H460 NSCLC cell line, and SEMA4D stimulates EC chemotaxis and may promote tumor angiogenesis (Basile et al. 2006; Nagai et al. 2007). Netrin 1 and 4 (*Ntn1* and *Ntn4*) acts as a tumor suppressor of NSCLC by regulating angiogenesis and promoting tumor cell death (Delloye-Bourgeois et al. 2009; Nacht et al. 2009). Ephrin receptors and ephrin ligands are capable of bidirectional (forward and reverse, respectively) signaling and recognizing extracellular signals and influencing cell–cell interaction, cell migration, angiogenesis, and tumor-igenesis (Surawska et al. 2004). *Efna4* is upregulated and *Efnb1* is downregulated in murine SL tumors as well as human cancers (Pasquale 2010).

The PTEN pathway is significantly, $-\log(p \text{ value}) = 5.681$, dysregulated and *Pten*, a bonafide tumor suppressor gene is downregulated (-1.71) within the murine SL tumors (Yanagi et al. 2007) as well as several human cancers including lung cancer (Teng et al. 1997). PTEN functions as both a dual specificity protein phosphatase and an inositol phospholipid phosphatase and regulates signaling pathways such as FAK-CAS, Ras-Raf-MAPK, and PI3K/AKT that influence cell growth, migration, and apoptosis (Gu, Tamura, and Yamada 1998). Phospholipase C-signaling and protein ubiquitination pathways are also significantly, $-\log(p \text{ value}) = 5.545$, and 5.241, respectively, altered within the murine SL tumors. These two signaling pathways play an important role in several molecular pathways relevant to carcinogenesis such as transcriptional regulation, DNA repair, endocy-tosis, cell

motility, apoptosis, immune, and inflammatory responses (Spataro et al. 1998; Viallet and Sausville 1996).

Altered Canonical Metabolic Pathways in Murine SL Tumors

In a recent review article, Hanahan and Weinberg (2011) have added two additional hallmarks of cancer, that is, reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg 2011). In murine SL tumors, several energy metabolism pathways are altered, such as Inositol phosphate metabolism, $-\log(p \text{ value}) = 5.534$, oxidative phosphorylation (3.954), and purine metabolism (4.169), pyrimidine metabolism (4.394), and citrate cycle (3.157). Inositol phosphate metabolism is altered in several cancers and they regulate chromatin remodeling (Steger et al. 2003), and it is altered in rat fibroblasts that are transformed by v-src (viral-sarcoma tyrosine kinase), serum withdrawal, and chronic endothelin treatment (Mattingly et al. 1991). The purine and pyrimidine metabolism is accelerated in tumors due to increased demand for nucleotides and nucleic acid replication. Tumor cells commonly outgrow their vascular supply and become hypoxic. Hypoxia-inducible factor (*Hif1a*, 3.389) is activated as a result of homeostatic response to hypoxia and aids the cell in increasing its glucose uptake by upregulating the glucose transporter solute carrier family 2 (facilitated glucose transporter), member 1 (*Slc2a1*, 3.896) and in its glucose phosphorylation by upregulating hexokinase (*Hk1*, 1.661; *Hk2*, 4.414). Thus, increased glycolysis leads to increased pyruvate levels. However, HIF1A can downregulate oxidative phosphorylation by upregulating pyruvate dehydrogenase kinase 1 (*Pdk1*, 4.392) that phosphorylates and inhibits pyruvate dehydrogenase conversion of pyruvate to acetyl-CoA resulting in suppression of the Krebs cycle and mitochondrial respiration (Kim et al. 2006; Papandreou et al. 2006). HIF1A can upregulate lactate dehydrogenase (*Ldha*, 3.62) and convert the excess pyruvate within the cytosol into lactate that is shunted to extracellular space, regenerating NAD (Nicotina-mide adenine dinucleotide) for continued glycolysis (Papan-dreou et al. 2006). Also, this excess pyruvate within the cell may be directed toward lipid synthesis that is required for membrane assembly and lipid-modified signaling molecules (Gogvadze, Orrenius, and Zhivotovsky 2008). A key enzyme linking glucose metabolism to lipid synthesis is ATP (Adenosine triphosphate) citrate lyase (*Acl*, 2.21), which catalyzes the conversion of citrate to cytosolic acetyl-CoA, and inhibition of ACL leads to suppression of tumor growth and induction of differentiation (Hatzivassiliou et al. 2005). The citrate cycle is significantly altered within the murine SL tumors. In addition, the suppression of pyruvate oxidation through HIF1A-mediated PDK1 upregulation may protect cells from production of cytotoxic amounts of ROS (Reactive Oxygen species) from mitochondria (Kim et al. 2006).

Altered Canonical Immune Response Pathways in Murine SL Tumors

Another important hallmark of cancer includes evading immune destruction by the cancer cells. Several canonical immune-mediated signaling pathways such as Fc γ R-mediated phagocytosis, $-\log(p \text{ value}) = 4.78$, clathrin-mediated endocytosis (4.239), IL-8 signaling (3.805), CXCR4 signaling (3.801), and Caveolar-mediated endocytosis signaling (3.312) are significantly altered within the murine SL tumors. Fc γ R-mediated phagocytosis is one of the host immune responses against the opsonized tumor cells. This Fc γ R-mediated phagocytosis of tumor cells is negatively regulated by several mediators such as interferon- γ , inositol

polyphosphate phosphatase-like 1 (*Inpp1l*, 1.308), and inositol polyphosphate-5-phosphatase, 145 kDa (*Inpp5d*, -2.108; Ai et al. 2006; Backman and Guyre 1994). IL-8 signaling within cancer cells, endothelial cells, neutrophils, and tumor-associated macrophages plays an important role in regulating tumor microenvironment, epithelial-mesenchymal transition, and angiogenesis (Fernando et al. 2011; Rotondo et al. 2009; Waugh and Wilson 2008; Zhu et al. 2004). There is marked upregulation of CX chemokine (C-X-C motif) ligand 1, 2, and 6 (*Cxcl1*, 14.98, *Cxcl2*, 6.859; *Cxcl6*, 6.19) within the murine SL tumors and upon ligand binding, several downstream signaling pathways such as MAPK, NF κ B, AP1, STAT3, and HIF1A may be activated (Waugh and Wilson 2008). The CXCR4/SDF1 axis is also thought to play a major role in activating these pathways especially within NSCLC (Spano et al. 2004). The downregulation of *Sdf1* (*Cxcl12*, -7.27) within murine SL tumors may be related to its feedback inhibition loop. The proplatelet basic protein (chemokine [C-X-C motif] ligand 7; *Ppbp*) is downregulated within both murine (-17.34) and human (-6.48) lung tumors. The CXCL7 is a powerful chemoattractant and activator of neutrophils and downregulation of this important growth factor may be a mechanism, whereby tumor cells evade the host immune response. The plasma levels of CXCL7 are reduced in patients with pancreatic cancer (Matsubara et al. 2011). Caveolins can function as oncogenes or tumor suppressors depending on the stage of oncogenesis and tumor progression, for example, CAV1 is highly expressed in terminally differentiated cells as well as metastatic and drug-resistant tumors but is downregulated in transformed cells allowing increased cell proliferation and anchorage independence. In murine SL tumors, caveolins (*Cav1*, -5.028; *Cav2*, -2.181, *Cav3*, -1.434) are downregulated supporting their role as tumor suppressors in murine lung carcinogenesis (Williams and Lisanti 2005). However, Sunaga et al. (2004) demonstrated that CAV1 is a tumor suppressor in human SCLC but it is a growth promoter for NSCLC (Sunaga et al. 2004).

Altered Canonical IPA Xenobiotic Metabolism Pathways in Murine SL Tumors

Some pathways identified as IPA xenobiotic metabolism pathways were also significantly altered within murine SL tumors, such as mitochondrial dysfunction, $-\log(p \text{ value}) = 5.352$, Ahr signaling (4.979), androgen (4.705) and estrogen (3.724) signaling, and RAR activation (3.724). All these pathways have normal physiological functions in addition to their roles in xenobiotic metabolism. The mitochondrial electron transport chain system is one of the major sources of ROS (superoxide $[O_2^-]$, hydrogen peroxide $[H_2O_2]$, hydroxyl radical $[^{\bullet}OH]$) in the cell. In mitochondrial dysfunction, the ROS can escape the mitochondria and cause damage to different mitochondrial and host cell proteins and DNA that could initiate tumorigenesis and sustain cancer development (Ames and Shigenaga 1992; Lu, Sharma, and Bai 2009). In addition to the cytotoxic, apoptotic, and proliferation inhibition effects, ROS at above and below a certain threshold is involved in tumor promotion and maintenance through activation and transmission of survival and proliferation signals via receptor tyrosine kinase, Ras-mitogen-activated protein kinase (Ras-MAPK), phospholipase C γ (PLC γ), and PI3K/AKT pathways (Guyton et al. 1996; Kamata and Hirata 1999; Preston et al. 2001; Rao 1997). In addition, Polo-like kinase (*Plk2*, 2.127) is upregulated within the murine SL tumors and upregulation of this gene is secondary to ablation of mitochondrial respiration and PLK2 aids in survival of cells with mitochondrial dysfunction (Matsumoto et al. 2009). All of the above pathways are significantly altered within the murine SL tumors.

Significance of Validated Genes in the Context of Cancer

Using quantitative RTPCR, we have validated the fold changes of select genes from the murine SL tumor data set. As indicated within Table 5, some genes like *Hmga1* and *Dusp4* are not documented within the microarray column but are documented within the RT-PCR column. This finding was not entirely surprising and may be explained by the differences in the dynamic range between the RT-PCR and microarray technologies (up to 10^7 -fold in real-time PCR versus a limit of 10^2 to 10^3 -fold in microarrays; Morey, Ryan, and Van Dolah 2006), and the differences in statistics (without and with multiple correction testing, respectively). We have evaluated the protein expression of some well-characterized genes in the context of lung cancer such as HMGA1 (Aiello et al. 2010) and BIRC5 (Adida et al. 1998; Ambrosini, Adida, and Altieri 1997; Hoffman et al. 2002; Monzo et al. 1999; Olie et al. 2000). In addition, we have also identified some proteins that were not so well characterized in the context of lung cancer such as HNF4a, ID2, and STK39 in murine SL tumors. Hepatocyte nuclear factor-4a (HNF4a) is an orphan member of the nuclear receptor superfamily involved in various processes that influence endoderm development, glucose, and lipid metabolism, and it has also been implicated in gastric adenocarcinoma, colorectal cancer, renal cell carcinoma, and hepatocellular carcinoma (Kojima et al. 2006; Tanaka et al. 2006); (Lazarevich et al. 2004; Lucas et al. 2005). In several human cancers, HNF4a levels are downregulated compared to the controls and act as a tumor suppressor. On contrary, in this study, *Hnf4a* was about eighty-fold differentially upregulated and is manifested by intense nuclear staining in multifocal areas within the tumor (Figure 1c). In a mouse colon carcinogenesis model as well as in a colorectal cancer patient cohort, HNF4A is significantly upregulated when compared to adjacent normal tissue (Darsigny et al. 2010). In CCSP-rtTA/(teto)7Stat3C bitransgenic mouse model, Stat3C overexpression caused persistent pulmonary inflammation and adenocarcinomas and *Hnf4a* is one of the genes upregulated by STAT3 (Li et al. 2007; Qu et al. 2009b). HNF4A was upregulated only in human pulmonary adenocarcinomas but not in squamous cell carcinomas (Qu et al. 2009b). It appears that HNF4A can function as a tumor suppressor or a proto-oncogene based on the tumor type. *Hnf4a* is a developmental gene and its upregulation along with other developmental genes like *Smad6*, *Hopx*, *Sox9* and *Mycn* in murine SL lung tumors reinforces the concept of recapitulation of embryonic genes by cancer cells. The exact role of HNF4A in lung cancer needs to be further elucidated. ID2 is a dominant negative H-L-H protein that antagonizes Rb-mediated repression of E2F transcription and thus acts as an oncogene (Iavarone et al. 1994; Lasorella et al. 2000). In addition, ID2 also regulates angiogenesis and apoptosis via transcriptional regulation of VEGF and BCL2 genes, respectively (Lasorella et al. 2005). ID2 plays an important role in lung development and is expressed within the multipotent embryonic progenitor cells in the pseudoglandular and canalicular stage (Rawlins et al. 2009). In this study, ID2 was upregulated (8.123) and the protein expression was limited to the cytoplasm and plasma membrane with a conspicuous absence in the nucleus. In the one paper reported in the literature on ID2 expression in human NSCLC, the cytoplasmic expression of ID2 is seen in well-differentiated adenocarcinomas with good prognosis, and the nuclear expression of ID2 is seen in poorly differentiated NSCLC with poorer prognosis (Rollin et al. 2009). Murine SL tumors are relatively well-differentiated adenocarcinomas and the results may be comparable to human NSCLC. STK39 is a cellular stress gene, and its expression is variably regulated in different

tumor types. It is downregulated in prostate cancer and lymphoma with high rates of metastasis and is upregulated in NSCLC with activating EGFR mutations (Balatoni et al. 2009; Choi et al. 2007; Hendriksen et al. 2006). STK39 displayed very high nuclear staining and mild to moderate cytoplasmic staining within the murine SL tumors, and these results are consistent with human NSCLC.

Comparison of Transcriptomic Data from Murine and Human Lung Tumors

Several of the canonical pathways described above were also altered within the human NSCLC. In addition, metaanalysis by NextBio identified common miRNA targets (Lewis, Burge, and Bartel 2005) based on the differentially expressed genes within the murine and human lung tumors.

Predicted miRNA Targets by TargetScan

Each miRNA can regulate the translation of multiple genes, and many genes may be regulated by multiple miRNAs. Several of the top fifteen TargetScan predicted miRNAs (miRs) based on the murine SL tumor and human lung tumor data sets were described within the context of human cancers, and they include upregulated miRs (putative oncomirs) such as miR-93, 96, 142-5P, 182, 200b, and 381, and downregulated miRNAs (putative mirsupps) such as miR-1, 15, 23, 26, 145, and 181 (Lu et al. 2005; Navon et al. 2009; Yu et al. 2010). miRNA 200b along with miRs such as 21, 486, and 375 were suggested as biomarkers within the sputum of patients with pulmonary adenocarcinoma (Yu et al. 2010). Some miRs such as hsa-miR-93, 182, and 200b were upregulated and has-miR-1, and 126 was downregulated within lung adenocarcinomas compared to normal lung, respectively (Yu et al. 2010). Functional experiments aimed at characterizing these miRs and their targets as well as the roles they play in pulmonary carcinogenesis needs to be determined.

Concordant Gene Expression between Murine and Human Lung Tumors

Several of the statistically significant differentially expressed genes with high fold changes (up and down) within the murine SL tumors were also altered within the human NSCLC. Claudins are important structural proteins of tight junctions (TJs) located apically within the epithelial junctional complex and TJs regulate permeability and polarity to maintain cellular homeostasis. They also play important roles in cancer by promoting promatrix metalloproteinases as well as playing a role in epithelial-mesenchymal transition (Miyamori et al. 2001; Soini 2011). Within the murine SL tumors, several Claudins (*Cldn2*, *Cldn3*, *Cldn4*, *Cldn5*, *Cldn7*, *Cldn10*, *Cldn12*, and *Cldn15*) were altered. Distinct claudin expression appears to correlate with histologic subtypes of lung cancer and *Cldn2* is markedly upregulated (58.52) within murine SL tumors and this appears significantly altered in human NSCLC compared to human SCLC (Moldvay et al. 2007). *Cldn5* is downregulated (-15.34) within murine SL tumors as well as within human NSCLC (Paschoud et al. 2007). Matrix metalloproteinase 12 (*Mmp12*) is a potent proinflammatory and oncogenic molecule (Qu et al. 2009a), and its expression is correlated with local recurrence and metastatic disease in human NSCLC patients (Hofmann et al. 2005). *Mmp12* is markedly upregulated within murine SL tumors as well as human NSCLC. Arginase 1 (*Arg1*) inhibits T-cell proliferation by degrading extracellular arginine, which results in decreased responsiveness

of T cells to CD3/T cell receptor stimulation (Rotondo et al. 2009). The high expression of *Arg1* (30.72) by the murine SL tumors suggests a mechanism, whereby the tumor cells induce anergy within the lymphocytes infiltrating the tumors, and thereby evading the host immune response. ARG1 was slightly down-regulated (−1.6 fold) within the human NSCLC data set. The transmembrane protein (Tmem27, 23.18) plays an essential role in amino acid trafficking within the kidneys (Danilczyk et al. 2006) and is thought to stimulate pancreatic beta cell proliferation (Akpınar et al. 2005); however, its role in lung carcinogenesis is not known. EGL (egalitarian) nine homolog 3 (*Caenorhabditis elegans*; *Egln3*, 17.59) is upregulated within human NSCLC as well as murine SL tumors (Amatschek et al. 2004). EGLN3, an oxygen-sensitive prolyl hydroxylase is induced by hypoxia and negatively regulates hypoxia-inducible factor (HIF) expression (Marxsen et al. 2004). The *Stk39*, a serine/threonine kinase, is activated by hypotonic stress and activates the p38 MAP kinase pathway. The tumor necrosis factor (ligand) superfamily, member 10 (*Tnfsf10*) induces its apoptotic effects by downregulating STK39 (Polek, Talpaz, and Spivak-Kroizman 2006). *Tnfsf10* is downregulated (−9.025) within murine SL tumors. Together, the downregulation of *Tnfsf10* (−9.025) and upregulation of *Stk39* (9.952) result in antiapoptotic effects. Single nucleotide polymorphisms within the *STK39* gene were reported to be prognostic of overall survival in early stage NSCLC (Huang et al. 2009). Involucrin (*Ivl*, 9.44) is closely related to intermediate filaments and is present within well-differentiated pulmonary adenocarcinomas (Ando et al. 1991). Ladinin (*Lad1*, 9.069) may be associated with tumor cell differentiation since it serves as an anchoring filament to bind the epithelial cells to the underlying mesenchyme. Choline dehydrogenase (*Chdh*, 8.994) is upregulated within lung tumors, and it may be related to alterations in choline metabolism in cancers (Ackerstaff, Glunde, and Bhujwalla 2003). The transmembrane protein 100 (*Tmem100*) is markedly downregulated within murine SL tumors (−44.756) as well as human NSCLC tumors (−21.1). The regulation of *Tmem100* appears to be tightly related to the expression of Activin receptor-like kinase 1 (*Acvrl1*, −11.18) since the expression of *Tmem100* was downregulated within the lungs of *Acvrl1*-deficient mice. Both ACVRL1 and TMEM100 were localized within the arterial endothelial cells of developing embryos suggesting their potential role in angiogenesis within tumors (Moon et al. 2010). Mimecan or Osteoglycin (*Ogn*, −38.187) belongs to a family of small leucine-rich proteoglycans (SLRPs) that are secreted into the extracellular matrix and are abundantly expressed within the mouse lung. The expression of *Ogn* appears to be directly related to the expression of p53 in several cancer cell lines (Tasheva et al. 2001), and deficiency of OGN is related to defects in collagen fibril morphology (Tasheva et al. 2002). The antioxidant paraoxonase (PON1) is an endogenous free radical scavenger and has antioxidative and atheroprotective effects (Isik, Ceylan, and Isik 2007). Downregulation of *Pon1* (−33.754) within murine and human lung tumors may suggest a pro-oxidative state of the tumor tissues. Polymorphisms within the *Pon1* gene were noted in a Turkish lung cancer patient population (Aksoy-Sagirli et al. 2011). Glycoprotein M6A (GPM6A) is a transmembrane protein that is present on the cell surface and plays an important role in neuronal differentiation from embryonal stem cells (Michibata et al. 2008) and is dysregulated in viral-induced lymphoid leukemias (Charfi et al. 2011). It is markedly downregulated both within murine SL tumors (−27.284) and human NSCLC (−47.2), and its role in lung cancer needs to be determined. Transcription factor 21 (*Tcf21*, −26.391) is a bonafide tumor suppressor and is hypermethylated in lung cancer

(Smith et al. 2006). In addition to *Tcf21*, *Akap12* (−15.74) and *Syne1* (−3.96) are also coordinately downregulated within the murine SL tumors and human NSCLC tumors (Tessema and Belinsky 2008). Natriuretic peptide (NP) receptors are single transmembrane catalytic receptors with intracellular guanylyl cyclase activity and have physiological roles in fluid homeostasis and were documented in medulloblastomas (Northcott et al. 2011). Natriuretic peptide receptor C/Guanylate cyclase C (*Npr3*) is downregulated within both murine SL tumors (−25.77) and human NSCLC (−2.42), but their role in lung tumor needs to be determined. Indolethylamine N-methyltransferase (*Inmt*, −21.616) is markedly downregulated within both murine and human lung tumors as well as in the developing lung, thereby suggesting embryonic recapitulation of genes by the tumors (Kopantzev et al. 2008). Integrin alpha 8 (*Itga8*, −21.616) regulates mesenchymal cell adhesion and migration, and plays an important role in fetal lung morphogenesis (Benjamin et al. 2009) and is dysregulated in ovarian cancers (Cai et al. 2007). Flavin containing monooxygenases (*Fmo1*, −15.095, *Fmo3*, −21.156) play a major role in xenobiotic metabolism of the lung and are downregulated in both murine and human lung tumors suggesting alterations in the metabolizing ability of the tumor cells (Woenckhaus et al. 2006). In addition, several cytochrome p450 enzymes (*Cyp2B6*, *Cyp2C38*, *CypCD6*, *Cyp2E1*, *Cyp2F1*, *Cyp2J9*, *Cyp2S1*, *Cyp2U1*, *Cyp4B1*, *Cyp4F8*, *Cyp4F16*, *Cyp4F22*) are downregulated within murine lung tumors (Leclerc et al. 2010). In particular, *Cyp4b1* is markedly downregulated in both murine (−17.827) and human (−14.1) lung tumors as well as other lung tumors (Czerwinski et al. 1994). Microfibrillar-associated protein 4 (*Mfap4*, −21.14) is an extracellular matrix protein that binds collagen and contains a C terminal fibrinogen-like domain and a N-terminal located integrin-binding motif and is involved in cell adhesion and intercellular interactions and is downregulated within both murine and human lung tumors. MFAP4 binds to surfactant proteins A (Schlosser et al. 2006) and surfactant protein D (Lausen et al. 1999) and colocalize to extracellular matrix within the lung. It is downregulated within urinary bladder tumors (Zaravinos et al. 2011), but their role in lung cancer needs to be determined. Bone morphogenetic proteins (BMPs) play important roles in TGFβ superfamily and are responsible for myriad effects in normal and cancer cells. Several BMPs (*Bmp1*, *Bmp2*, *Bmp6*) are differentially altered within lung cancer. *Bmp2* is upregulated and stimulates tumor growth via activation of Smad1/5 (Langenfeld Kong, and Langenfeld 2006) where as *Bmp6* is hypermethylated and cross-talks with MAPK-signaling pathway (Kraunz et al. 2005). T-box genes (*Tbx*) encode transcription factors involved in the regulation of embryonic development (Chapman et al. 1996). In several aggressive tumors, the *Tbx* genes are upregulated and they block the p53 pathway and downregulate the tumor suppressor p14ARF (Carlson et al. 2002; Lingbeek, Jacobs, and van Lohuizen 2002). However, *Tbx3* is downregulated within the examined murine (−21.09) and human (−2.92) lung tumor data sets. *Tbx3* is hypermethylated in the gastric cancer cell lines (Yamashita et al. 2006), and their role in lung cancer needs to be determined. Troponin C type 1 (*Tnnc1*) is a regulatory protein of skeletal muscle contraction and is markedly downregulated within murine (−17.78) and human (−18) lung tumors but its role in tumorigenesis needs to be deciphered. Cysteine dioxygenase, type 1 (*Cdo1*), is markedly downregulated within both murine (−16) and human (−8.55) lung tumors. CDO1 is a key enzyme in taurine biosynthetic pathway and taurine inhibits apoptosis (Takatani et al. 2004), and *CDO1* is

methyated within cancers of the breast and lung (Dietrich et al. 2010; Kwon et al. 2011), and thus may act as a tumor suppressor.

Summary and Conclusions

We have evaluated the differential transcriptomic changes within the murine SL tumors compared to age-matched normal lung tissues and identified several altered canonical pathways associated with cancer, metabolism, immune responses, and IPA xenobiotic pathways. We also described several important differentially expressed genes within the context of human lung cancer. This study provides a background on the transcriptomic changes in murine SL tumors and is expected to be valuable in identifying chemical specific effects in lung tumors and in deciphering the mechanism of actions of pulmonary carcinogens in the NTP's chronic bioassays. Further evaluation of genome-wide epigenetic changes such as whole genome methylation arrays and miRNA arrays within the murine SL tumors will be a valuable adjunct to the transcriptomic data set of the murine SL tumors.

Furthermore, utilizing the NextBio meta-analysis tool, we have compared the differential transcriptomic changes within murine SL tumors and human NSCLC. Despite obvious species differences, several of the canonical pathways as well as genes that are altered within the murine SL tumors are also altered within human NSCLC. These data demonstrate that the murine SL tumors may serve as a valuable model to at least certain types of human NSCLC especially since the murine SL tumor is most similar to NSCLC within a nonsmoking cohort but not to other NSCLC types. Such meta-analysis approaches are very important for evaluating the molecular pathogenesis of lesions common to different species and may aid in better utilization of the model organisms for hazard analysis.

As with most microarray studies, the validation and functional testing through *in vitro* and *in vivo* experiments is needed for confirmation of the molecular mechanisms hypothesized using the transcriptomic data. We have identified some transcription factors like ID2 and HNF4a within murine SL tumors that are not well described within the context of human NSCLC. Some of our future efforts will be aimed at characterizing the roles played by these genes within the context of lung cancer using cell lines. In this study, the transcriptome of murine SL tumors represents a molecular snapshot at the end-stage tumor lesion. Further mechanistic information may be derived by comparing the transcriptomic changes from preneoplastic or benign tumor lesions such as hyperplasias and adenomas in the lung. Such studies involving the transcriptomic changes reflecting the temporal development of lesions will provide valuable information on mechanism of action for chemically induced tumors. In addition to the gold standard histopathology, these high throughput genomic (and epigenomic) technologies will provide valuable information for hazard analysis in NTP studies.

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Abbreviations

BIRC5	Baculoviral IAP repeat containing 5
BMPs	Bone morphogenetic proteins
cDNA	Complementary Deoxyribonucleic Acid
CXCR4	C–X–C chemokine receptor type 4
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EIF2	eukaryotic initiation factor 2
FDR	false discovery rate
GEO	Gene Expression Omnibus
IL-8	Interleukin-8
ID2	Inhibitor of DNA binding 2
IPA	Ingenuity Pathway Analysis
H-L-H	helix-loop-helix
HIF	hypoxia-inducible factor
HMGA1	high mobility group AT-hook 1
HNF4A	Hepatocyte nuclear factor 4a
MAPK	mitogen-activated protein kinase
mdFDR	multidimensional false discovery rate
miRNA	micro RNA
mTOR	mammalian target of rapamycin
NL	normal lung
NSCLC	non–small cell lung cancer
NTP	National Toxicology Program
PC	principal component
PCA	principal component analysis
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction

RAR	retinoic acid receptor
RhoA	Ras homolog gene family, member A
RMA	robust multiarray analysis
SL	spontaneous lung
SCLC	small cell lung cancer
STK39	serine threonine kinase 39
TJs	tight junctions
TSC2	Tuberous sclerosis 2

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Figure 1.

H&E and Immunohistochemistry (IHC) of spontaneous lung tumors. (A), H&E stain demonstrating the pulmonary adenocarcinoma, papillary type. (B), IHC showing intense HMGA1 nuclear staining. (C), Marked HNF4a cytoplasmic and nuclear staining. (D), Prominent STK39 nuclear staining and moderate cytoplasmic staining. (E), moderate ID2 cytoplasmic and plasma membrane staining. (F), Moderate Survivin (BIRC5) nuclear staining and mild cytoplasmic staining. H&E = hematoxylin and eosin; HMGA1 = high mobility group AT-hook 1; HNF4A = hepatocyte nuclear factor 4a.

Figure 2.

Principal component analysis shows that the normal lung samples cluster tightly indicating very similar gene expression profile. Though the spontaneous lung (SL) tumors cluster away from the normal lung samples, SL tumors 2 and 6 do not cluster with the other 4 (1, 3, 4, and 5) tightly clustered SL tumors indicating heterogeneity in global gene expression profile between SL tumors.

Figure 3.

Unsupervised Hierarchical cluster analysis indicates the significant differences between the normal lungs and SL tumors. Please note that spontaneous lung (SL) tumors 2 and 6 cluster separately within the dendrogram. The upregulated and downregulated genes are represented in red and green, respectively.

Murine Spontaneous Lung Tumor (MSLT)

Lung Tumor vs Normal Lung

Human Non Small Cell Lung Cancer (HNSCLC)

Stage 3 HNSCLC vs Adjacent Normal Lung

Common genes (all)

Significance of overlap between gene subsets

Figure 4.

NextBio was used to compare the differential transcriptomes of murine spontaneous lung tumors with the most closely matched human non-small cell lung cancer data set (from the NextBio curated data sets as well as NCBI's GEO database). The human NSCLC (HNSCLC) data set that closely matches the murine SL tumor (MSLT) data set (with a NextBio score of 78%) is from Stage 3 lung cancer tissue from non-smokers compared to adjacent normal lung tissue using Affymetrix Human Genome U133 Plus 2.0 Array (GSE19804; Lu et al., 2010).

Table 1

Most significantly altered canonical molecular pathways within murine spontaneous lung tumors as determined by Ingenuity Pathway Analysis (IPA).

Canonical pathway	Ratio *	–log (p value)
Cancer Pathways		
EIF2 signaling	108/222 (.486)	12.356
Axonal guidance signaling	167/433 (.386)	8.825
mTOR signaling	92/210 (.438)	8.144
Regulation of eIF4 and p70s6 k signaling	77/179 (.43)	8.132
RhoA signaling	59/114 (.518)	7.347
Tight junction signaling	73/164 (.445)	5.997
PTEN signaling	54/124 (.435)	5.681
Ephrin signaling	78/200 (.39)	5.574
Phospholipase C signaling	99/261 (.379)	5.545
Protein ubiquitination pathway	108/274 (.394)	5.241
Cyclins and cell cycle regulation ^a	45/89 (.506)	4.207
Integrin signaling	82/210 (.39)	4.065
Metabolic pathways		
Inositol phosphate metabolism	65/180 (.361)	5.534
Pyrimidine metabolism	62/214 (.29)	4.394
Purine metabolism	108/400 (.27)	4.169
Oxidative phosphorylation	62/159 (.39)	3.954
Citrate cycle	17/57 (.298)	3.157
Immune response signaling		
FcγR-mediated phagocytosis in macrophages	46/102 (.451)	4.78
Clathrin-mediated endocytosis signaling	69/172 (.401)	4.239
IL-8 signaling	72/193 (.373)	3.805
CXCR4 signaling	65/169 (.385)	3.801
Antiproliferative role of TOB in T cell signaling ^a	18/26 (.692)	3.371
Caveolar-mediated endocytosis signaling	35/85 (.412)	3.312
IPA xenobiotic pathways		
Mitochondrial dysfunction	63/175 (.36)	5.352
Aryl hydrocarbon receptor signaling ^a	73/159 (.459)	4.979
Androgen signaling	53/144 (.368)	4.705
RAR activation	71/187 (.38)	3.724
Estrogen receptor signaling	54/136 (.397)	3.724
Aldosterone signaling in epithelial cells	62/170 (.365)	3.007
Nrf2-mediated oxidative stress response	71/192 (.37)	2.968

Note: Statistical Significance is set at $p < .001$ by Fisher's exact test. For each Canonical Pathway, the ratio indicates the number of genes altered within the data set compared to the total genes within that particular Canonical Pathway in IPA.

^aIndicates new canonical pathways identified after reevaluation of the data using single pairwise comparison.

* Ratio indicates the number of genes from that particular canonical pathway present in the differentially expressed gene data set compared to the total number of genes present in that particular canonical pathway curated in the Ingenuity Pathway Analysis database.

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Table 2

The top concordant upregulated and downregulated genes within murine spontaneous lung tumors and human non-small cell lung cancer as determined by NextBio.

Gene	Description	Murine SLT	Human NSCLC
Top twenty-five upregulated genes			
Cldn2	Claudin 2	58.526	2.82
Mmp12	Matrix metalloproteinase 12 (macrophage elastase)	31.911	18.2
Arg1	Arginase, liver	30.718	-1.6
Tc2n	Tandem C2 domains, nuclear	27.152	2.56
Tmem27	Transmembrane protein 27	23.183	2.01
Areg	Amphiregulin	21.766	-4.06
Egln3	Egl nine homolog 3 (C. elegans)	17.594	2.64
Sertad4	SERTA domain containing 4	16.714	1.97
Cxcl3	Chemokine (C-X-C motif) ligand 3	14.98	-4.07
5730559C18Rik	Chromosome 1 open reading frame 106	12.597	4.39
Rgs5	Regulator of G-protein signaling 5	12.202	-2.1
Gkn2	Gastrophilin 2	10.389	-22.1
Mpzl2	Myelin protein zero-like 2	10.056	2.6
Stk39	Serine threonine kinase 39 (yeast)	9.952	2.78
Ivl	Involucrin	9.441	1.47
Lad1	Ladinin 1	9.069	2.84
Chdh	Choline dehydrogenase	8.994	1.4
Egln3	Egl nine homolog 3 (C. elegans)	8.533	2.64
St8sia6	ST8 sialyltransferase 6	8.265	-1.56
Fabp5	Fatty acid binding protein 5 (psoriasis-associated)	8.1	-2.76
Mpzl2	Myelin protein zero-like 2	7.326	1.46
Frk	Fyn-related kinase	7.285	2.38
Tmem213	Transmembrane protein 213	7.23	1.92
Clu	Clusterin	7.062	-5.69
Gjb1	Gap junction protein, beta 1, 32kDa	6.993	1.68
Top twenty-five downregulated genes			
Tmem100	Transmembrane protein 100	-44.756	-21.1
Ogn	Osteoglycin	-38.187	-9.27
Pcolce2	Procollagen C-endopeptidase enhancer 2	-33.754	-5.62
Pon1	Paraoxonase 1	-32.559	1.24
Gpm6a	Glycoprotein M6A	-27.284	-47.2
Tcf21	Transcription factor 21	-26.391	-14.4
Npr3	Natriuretic peptide receptor C/guanylate cyclase C	-25.777	-2.42
Inmt	Indolethylamine N-methyltransferase	-21.616	-15.8
Itga8	Integrin, alpha 8	-21.616	-5.76
Fmo3	Flavin containing monooxygenase 3	-21.156	-3.21
Mfap4	Microfibrillar-associated protein 4	-21.141	-7.1

Gene	Description	Murine SLT	Human NSCLC
Bmp6	Bone morphogenetic protein 6	-21.097	-2.92
Tbx3	T-box 3	-19.093	-6.39
Cyp4b1	Cytochrome P450, family 4, subfamily B, polypeptide 1	-17.827	-14.1
Tnni1	Troponin C type 1 (slow)	-17.778	-18
Pbbp	Pro-platelet basic protein (C-X-C ligand 7)	-17.34	-6.48
Gcom1	GRINL1A complex locus	-17.292	-8.05
Dpep1	Dipeptidase 1 (renal)	-17.268	1.71
Cdo1	Cysteine dioxygenase, type I	-16	-8.55
Igfbp6	Insulin-like growth factor binding protein 6	-15.856	-2.64
Abi3bp	ABI family, member 3 (NESH) binding protein	-15.835	-11.1
Akap12	A kinase (PRKA) anchor protein 12	-15.736	-6.43
Prx	Periaxin	-15.466	-2.08
Cldn5	Claudin 5	-15.338	-8.06
Fam107a	Family with sequence similarity 107, member A	-15.316	-21.9

NextBio generated top canonical pathways common to murine spontaneous lung tumors as well as human non-small cell lung cancer as predicted by Broad institute's Molecular Signatures Database (*MSigDB*) using the respective transcriptomic data.

Table 3

Top fifteen concordant canonical pathways in murine SLT and human NSCLC	Bioset score	Mouse, $-\log(p \text{ value})$	Common genes	Human, $-\log(p \text{ value})$	Common genes
Cell cycle	36	13.51	35	26.13	54
DNA replication reactome	32	10.52	22	24.80	33
Pyrimidine metabolism	24	14.17	37	13.04	43
Cell Cycle G1 to S control reactome	23	11.20	26	14.77	42
Focal adhesion	22	14.80	59	9.85	90
Smooth muscle contraction	22	13.66	56	10.52	67
Cell communication	21	5.55	12	18.14	38
P53-signaling pathway	21	10.11	20	13.23	28
Purine metabolism	18	10.33	44	9.20	56
Glycolysis and gluconeogenesis	17	8.25	14	10.89	24
Prostaglandin synthesis regulation	16	8.25	8	9.82	17
Glycolysis pathway	14	9.05	6	6.40	7
Citrate cycle	14	10.85	15	4.41	10
Leukocyte transendothelial migration	14	7.17	33	8.00	54
Hemoglobin's chaperone	14	3.30	4	11.85	5

Table 4

Top miRNA targets common to murine spontaneous lung tumors as well as human non–small cell lung cancer as predicted by TargetScan (using NextBio) using the respective transcriptomic data.

Predicted gene targets for	Mouse, $-\log(p \text{ value})$	Common genes	Human, $-\log(p \text{ value})$	Common genes
miR-96	21.00	160	18.00	225
miR-200b	16.55	121	21.92	197
miR-182	18.02	148	18.00	211
miR-20	15.04	144	20.24	214
miR-124a	7.59	183	0.00	305
miR-124u	4.82	123	0.00	225
miR-381	12.19	126	0.00	198
miR-23	5.70	101	16.17	165
miR-15	8.64	132	13.04	192
miR-142-5p	7.19	89	0.00	143
miR-26	7.77	104	12.82	175
miR-1	5.23	79	14.59	143
miR-181	4.48	128	15.05	213
miR-145	6.00	61	13.49	122
miR-93	7.37	88	0.00	138

Table 5

Validation of the microarray differential gene expression data by quantitative real time PCR.

Gene	Entrez Gene Name	Microarray	qRTPCR
Candidate Oncogenes/Growth promoters			
Areg	Amphiregulin	21.77	2.11
Cka1b	CDC28 protein kinase regulatory subunit 1B	5.69	6.7
Clu	Clusterin	7.062	5.772
Ctnnb1	Catenin (cadherin-associated protein), beta 1, 88kDa	1.55	2.31
Egln3	Egl nine homolog 3 (C. elegans)	17.59	112.03
Gjb1	Gap junction protein, beta 1, 32kDa	6.99	3.29
Hmga1	High mobility group AT-hook 1	–	10.46
Id2	Inhibitor of DNA binding 2, dominant -ve H-L-H protein	8.12	9.73
Mmp12	Matrix metalloproteinase 12 (macrophage elastase)	31.91	205.89
Psrc	Proline/serine-rich coiled-coil 1	12.53	13.62
Ros1	c-ros oncogene 1, receptor tyrosine kinase	6.68	15.81
Stk39	Serine threonine kinase 39 (STE20/SPS1 hmlg, yeast)	9.95	10.35
Candidate tumor suppressors			
Akap12	A kinase (PRKA) anchor protein 12	–15.73	–8.58
Bmp6	Bone morphogenetic protein 6	–21.1	–4.02
Cldn5	Claudin 5	–15.338	–1.13
Cyr61	Cysteine-rich, angiogenic inducer, 61	–7.69	–4.56
Dusp4	Dual specificity phosphatase 4	8.17	10.55
Hnf4a	Hepatocyte nuclear factor 4, alpha	—	81.27
Hpgd	Hydroxyprostaglandin dehydrogenase 15-(NAD)	–29.45	–1.78
Id4	Inhibitor of DNA binding 4, dominant -ve H-L-H protein	–7.09	–1.9
Ogn	Osteoglycin	–38.19	–4.26
Rb1	Retinoblastoma 1	–1.77	–1.6
Smad6	SMAD family member 6	–10.87	–1.36
Tcf21	Transcription factor 21	–26.39	–28.27

Note: The fold changes were calculated by the $2^{-(Ct)}$ method.