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Oral benzo[*a*]pyrene in *Cyp1a1/1b1*(–/–) double-knockout mice: Microarray analysis during squamous cell carcinoma formation in preputial gland duct

Marina Gálvez-Peralta,* Zhanquan Shi,* Jing Chen, Marian L. Miller and Daniel W. Nebert

Department of Environmental Health and Center for Environmental Genetics (CEG), University of Cincinnati Medical Center, Cincinnati OH

Benzo[*a*]pyrene (BaP) is a prototypical polycyclic aromatic hydrocarbon (PAH) found in combustion processes. Cytochrome P450 1A1 and 1B1 enzymes (CYP1A1, CYP1B1) and other enzymes can activate PAHs to reactive oxygenated intermediates involved in mutagenesis and tumor initiation; also, CYP1 enzymes can detoxify PAHs. *Cyp1*(+/+) wild-type (WT) and *Cyp1b1*(–/–) knockout mice receiving oral BaP (12.5 mg/kg/day) remain healthy for >12 months. In contrast, we found that global knockout of the *Cyp1a1* gene (1a1KO) results in proximal small intestine (PSI) adenocarcinoma within 8–12 weeks on this BaP regimen; striking compensatory increases in PSI CYP1B1 likely participate in initiation of adenocarcinoma in 1a1KO mice. *Cyp1a1/1b1*(–/–) double-knockout (DKO) mice on this BaP regimen show no PSI adenocarcinoma, but instead preputial gland duct (PGD) squamous cell carcinoma (SCC) occurs by 12 weeks. Herein, we compare microarray expression of PGD genes in WT, 1a1KO and DKO mice at 0, 4, 8, 12 and 16 weeks of oral BaP; about four dozen genes up- or down-regulated during most critical time-points were further verified by qRT-PCR. In DKO mice, CYP3A59 was unequivocally identified as the BaP-inducible and BaP-metabolizing best candidate responsible for initiation of BaP-induced SCC. Striking increases or decreases were found in 26 cancer-related genes plus eight *Serpin* genes in DKO, but not in 1a1KO or WT, mice on this BaP regimen; of the 26, 8 were RAS-related oncogenes. The mechanism by which cancer-related genes are responsible for SCC tumor progression in the PGD remains to be elucidated.

Cancer Genetics

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in the environment. PAH procarcinogens occur as byproducts of combustion processes such as cigarette smoke, gas- and diesel-engine exhaust, charcoal-grilled food, creosote railroad ties, coal-burning and coke distillation in the petroleum industry. The most thoroughly studied prototypic PAH is benzo[*a*]pyrene (BaP).[1,2] In many different mammalian tissues and cell types as well as the intact animal, metabolically activated BaP and other PAHs are well known to cause cytotoxic, teratogenic, genotoxic, mutagenic and carcinogenic effects.[3–6] Most specifically for BaP, covalent binding of BaP 7,8-*trans*-diol-9,10-epoxide to the N-9 of guanine in DNA is most highly associated with mutations and carcinogenesis.[2] BaP and other PAHs in cigarette smokers are implicated as causative agents in human lung cancer[7,8] and atherosclerosis.[4]

As discussed previously,[9] by far the majority of particles and chemicals in cigarette smoke and other inhaled pollutants are ultimately swallowed; hence, our need is to better understand the pharmacokinetics and pharmacodynamics of ingested PAHs. Moreover, recent studies suggest an increased risk of papillary renal cell carcinoma[10] and exocrine pancreatic cancer[11] among subjects who consume the largest amount of well-done red meat cooked at high temperatures, *i.e.* increased levels of BaP and other food mutagens.

Etiology of PAH-induced cancer is exceedingly complex. By way of aromatic hydrocarbon receptor (AHR), PAHs induce numerous enzymes—involved in both activation and detoxification of PAHs. Traditionally, PAHs are known to be metabolically activated by at least a half dozen Phase I enzymes to reactive intermediates that bind covalently to nucleic acids and proteins[1–5] and detoxified by Phase II (conjugation) enzymes.[12] In addition, PAHs participate in up- and down-regulation of hundreds of other genes *via* both AHR-dependent and AHR-independent mechanisms.[4,5,7,13–16]

Inducible CYP1A1 in the gastrointestinal (GI) tract has been shown to be far more important in detoxication of oral

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Additional Supporting Information may be found in the online version of this article.

*Gálvez-Peralta and Zhanquan Shi contributed equally and should both be considered as first authors.

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Zhanquan Shi's present address is: James L. Winkle College of Pharmacy, University Cincinnati, Cincinnati OH 45267-0004
Correspondence to: Daniel W. Nebert, Department of Environmental Health, University Cincinnati Medical Center, P.O. Box 670056, Cincinnati OH 45267-0056, USA, Tel.: +[513-821-4664], Fax: [513-558-4397], E-mail: dan.nebert@uc.edu

What's new?

Polycyclic aromatic hydrocarbons such as BaP, present in cigarette smoke, among other places, are known to cause cancer. This study investigated the up- and down-regulation of various genes in response to oral BaP exposure in mice. The authors particularly looked at two enzymes, Cytochrome P450 1A1 and 1B1. Mice with both enzymes, and those missing 1B1, resist cancer when exposed to BaP, but mice lacking both CYP1A1 and CYP1B1 soon develop tumors of the preputial gland duct. What other genes are involved? Using microarray analysis, the authors identified a couple of dozen cancer-related genes that showed striking increases or decreases in expression with BaP treatment in the absence of Cyp1A1 and Cyp1B1. The gene encoding CYP3A59 emerged as the strongest candidate for cancer initiation.

BaP than in metabolic activation in wild-type (WT) or *Cyp1b1*(−/−) mice.[17–20] *Cyp1a1*(−/−) knockout mice (1a1KO) receiving oral BaP (125 mg/kg/day) result in immunosuppression and death within ∼30 days.[17] On the other hand, *Cyp1a1/1b1*(−/−) double-knockout mice (DKO), receiving this same high dose of oral BaP, are "rescued" from immunosuppression, *i.e.* they respond more like WT mice.[18] Immunosuppression is most likely prevented in DKO mice because CYP1B1-mediated BaP metabolism is required in immune cells in order to cause BaP-induced immunotoxicity, *i.e.* absence of CYP1B1 leads to negligible immunotoxicity.

In 1a1KO mice receiving a 10-fold lower dose of BaP (12.5 mg/kg/day), an adenocarcinoma of proximal small intestine (PSI) occurs between 8 and 12 weeks.[9] In 1a1KO, but not in WT, mice between 4 and 12 weeks on this BaP regimen, microarray analysis identified specific oncogenes up-regulated and tumor suppressor genes down-regulated that most likely play a role in the carcinogenic process. Moreover, GI tract CYP1B1 levels are markedly increased[9]—suggesting the CYP1B1 monooxygenase might be most likely responsible for creation of reactive intermediates involved in causing the BaP-initiated PSI adenocarcinoma.

DKO mice, receiving this lower dose of oral BaP (12.5 mg/kg/day), result in no PSI adenocarcinoma but instead develop squamous cell carcinoma (SCC) of the preputial gland duct (PGD) epithelium.[9] This finding intrigued us: if a BaP-induced cancer occurs in the absence of CYP1A1 and CYP1B1, what enzyme might be responsible for generation of BaP reactive oxygenated intermediates that are presumably needed for SCC tumor initiation? Alternatively, perhaps some other process is initiating SCC in the PGD; for example, we previously had shown that (non-metabolized) BaP total body burden in DKO is 3-fold higher than that in 1a1KO and 75-fold higher than that in WT.[18] Likewise, what cancer-related genes might be responsible for SCC tumor progression in the PGD? Herein we assess, *via* microarray analysis, the up- and down-regulation of gene expression in the PGD during BaP-induced SCC formation.

Material and Methods

Chemicals

BaP was purchased from Sigma (St. Louis, MO). All other chemicals and reagents were bought from either Aldrich Chemical Company (Milwaukee, WI) or Sigma Chemical Company as the highest available grades. Corn oil was purchased from the local grocery store.

Mice

Generation of the 1a1KO[21] and DKO[18] mouse lines was previously described. These two genotypes have been backcrossed onto the C57BL/6J background for eight generations—ensuring that the 1a1KO or DKO genotype resides in a >99.8% C57BL/6J genetic background.[22] Age-matched C57BL/6J *Cyp1*(+/+) WT mice, purchased from The Jackson Laboratory (Bar Harbor, ME), can therefore be used as comparative controls. Experiments with the WT, 1a1KO, and DKO genotypes were carried out using males only. All animal experiments (protocol no. 04-05-10-01) were conducted in accordance with the National Institutes of Health standards for the care and use of experimental animals and the University Cincinnati Medical Center Institutional Animal Care and Use Committee.

Treatment

All experiments in the present study used oral BaP at 12.5 mg/kg/day. Feeding experiments with male mice were begun at age 6 ± 1 weeks.[9] Rodent chow (Harlan Teklad; Madison, WI) was soaked for at least 24 hr in BaP-containing corn oil (1.0 mg/ml) before presentation to the mice. By knowing the weight of food ingested daily by a 20-g mouse and by using [^{3}H]BaP in earlier experiments,[23] we estimated that the daily oral BaP dose was ∼12.5 mg/kg/day. To start Day 1 of the experiment, mice (after having been fasted overnight) were presented with the BaP-laced food. Fresh food was replaced weekly. Mice eat corn oil-soaked chow eagerly. Experiments in this study included time-points of 0, 4, 8, 12 and 16 weeks.

Biohazard precaution

BaP is highly toxic and regarded as a human carcinogen. All personnel were instructed in safe-handling procedures. Lab coats, gloves and masks were worn at all times, and contaminated materials were collected separately for disposal by the Hazardous Waste Unit of the University Cincinnati Medical Center or by independent contractors. BaP-treated mice are housed separately, and their carcasses are treated as contaminated biologic materials.

Mouse tissue preparation

Following 0, 4, 8, 12 or 16 weeks of oral BaP, the PGD was isolated from tissues under the skin of the penis; surrounding fat

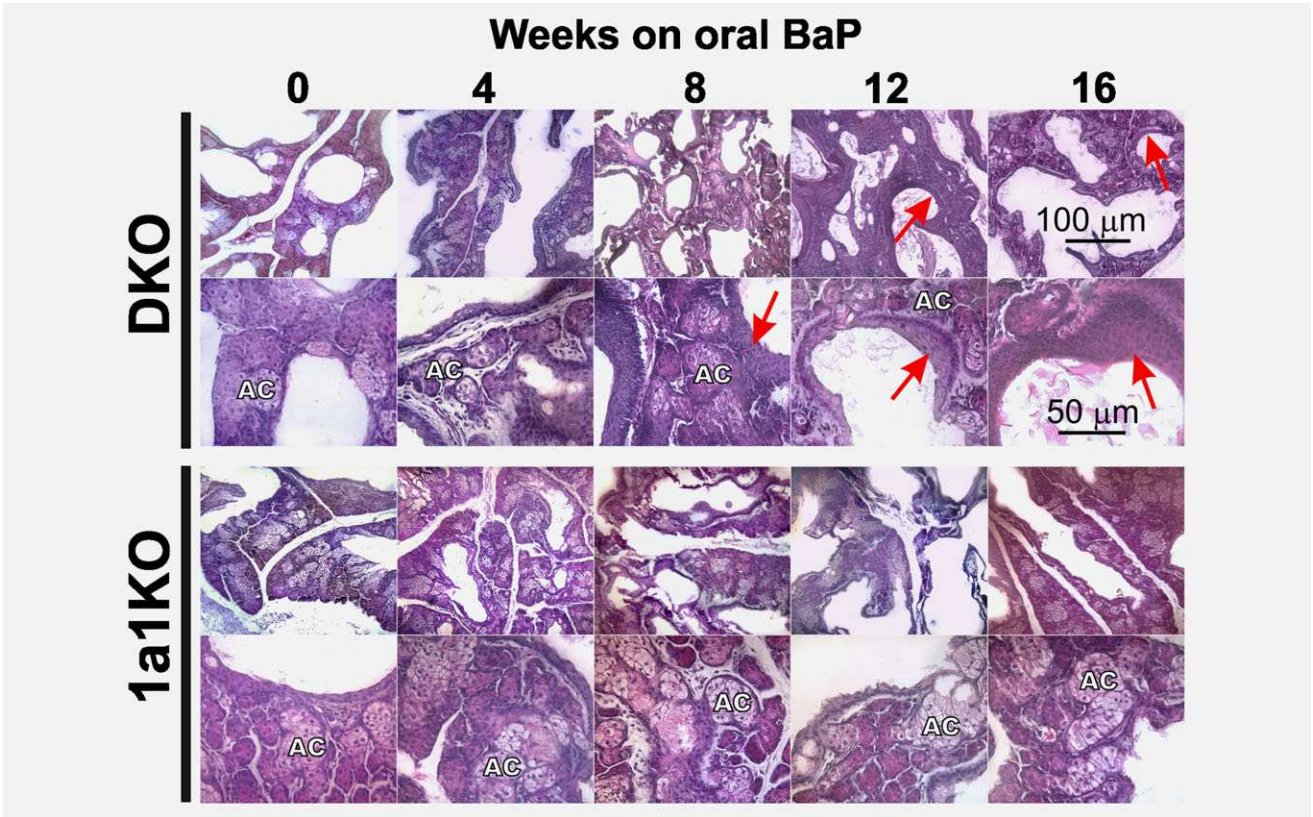

Figure 1. Comparison of PGD histology in Cyp1a1/1b1(–/–) double-knockout (DKO) mice receiving oral BaP (12.5 mg/kg/day) from 0 to 16 weeks, top two rows, with that in Cyp1a1(–/–) single-knockout (1a1KO) mice, bottom two rows. Magnification is same in first and third rows, and higher in second and fourth rows. Arrows denote squamous dysplasia at 8-week time-point, squamous cell neoplasia at 12- and 16-week time-points. Invasion of cancerous cells into connective tissue (not shown) occurred at 16-week time-point; these changes were seen only in the DKO and not in the 1a1KO (bottom two rows) nor in WT mice (data not shown). AC, acinar cell (four of five panels in 2nd row; all five panels in 4th row). Normal PGD morphology with stratified squamous cell epithelial lining of the larger ducts and cuboidal holocrine secretory acinar cells is seen at 0 and 4-week time-points. Thickening of the squamous epithelium (arrow) and increased keratin production can be seen in DKO at 8 weeks. Excessive accumulation of keratin within ducts and resulting dilatation, and an epithelial morphology characteristic of SCC (arrows) are observed in DKO at 12 and 16 weeks.

was carefully separated so that the preputial gland could be harvested without breaking, as well as with virtually no other attached tissue (surrounding skin, cartilage, collagenous tissue or muscle). In addition to gross inspection, paraformaldehyde-fixed tissues were dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin; the sections were photographed for histology at several magnifications.

Because tumor formation was not seen in all DKO mice, we began with groups of 15; those appearing histologically "most typical" were selected for each time-point ("normal" at 0 and 4 weeks, evidence of dysplasia at 8 weeks, neoplasia at 12 weeks and metastasis at 16 weeks). Three groups of $N = 2$, for each of the three genotypes, were used for microarray analysis in triplicate.

Additional materials and methods online

Descriptions of total RNA preparation, reverse transcription and quantitative real-time PCR (qRT-PCR) analysis, primer design, microarray hybridization, microarray data analysis (complete raw data available at GEO: GSE40633) and other statistical methods are detailed in our Supporting Information.

Results

Histology of the preputial gland duct

Figure 1 compares typical PGD histology of DKO with that of 1a1KO mice, ingesting daily oral BaP (12.5 mg/kg/day), from 0 to 16 weeks. WT mice responded basically the same as 1a1KO mice, *i.e.* no substantial histological changes seen in the PGD during the 16-week regimen (data not shown). At 0 and at 4 weeks of oral BaP, PGD of DKO mice appeared no different from that of 1a1KO or WT; however, at 8 weeks one could see dysplastic changes in DKO mice with enhanced keratinization, keratin plugs and epithelial thickening.

In DKO but not in 1a1KO or WT, SCC of the PGD became obvious at 12 weeks, with invasion through the basement membrane, and into surrounding tissues, especially at 16 weeks of daily oral BaP (Fig. 1, top two rows, at right). At 8, 12 and 16 weeks of daily oral BaP, PGD of DKO mice showed a general transition from secretory to squamous epithelium—progressing to production of excess keratin and formation of keratin pearls, as well as increases in irregular architecture, relative percentage of keratinized squamous epithelium and

keratinocyte pleomorphic nuclei. The lining of tubular glands became thickened, with abscesses and inflammatory changes; overall architecture became disorganized, and the amount of stroma between glands was increased. Further description of this PGD SCC in DKO mice, receiving 12 weeks of daily oral BaP at this dose, was reported previously.[9]

Strategy of microarray analysis of PGD

We wished to identify genes up- and/or down-regulated that might be associated with SCC formation in the PGD. Similar to what we had done previously for PSI during development of adenocarcinoma by comparing 1a1KO with WT mice,[9] we compared gene expression microarrays at 4-week intervals in DKO *vs.* 1a1KO *vs.* WT mice receiving daily oral BaP.

Figure 2 illustrates the experimental design. Microarray data for each of the five time-points were compared with one another: (4 *vs.* 0 weeks, 8 *vs.* 0 weeks, 12 *vs.* 0 weeks and 16 *vs.* 0 weeks); (8 *vs.* 4 weeks, 12 *vs.* 4 weeks and 16 *vs.* 4 weeks); (12 *vs.* 8 weeks and 16 *vs.* 8 weeks) and (16 *vs.* 12 weeks). Early tumorigenesis processes in DKO mice are expected to be seen at the 4- *vs.* 0-week and 8- *vs.* 0-week comparisons, late tumorigenesis processes at the 12- *vs.* 8-week interval and advanced cancer processes at the 16- *vs.* 12-week comparison. We reasoned that—in the absence of CYP1A1 and CYP1B1—another xenobiotic-metabolizing enzyme (XME), most likely induced by oral BaP, might activate BaP to reactive intermediates responsible for tumor initiation; if induced by oral BaP or increased by some other compensatory mechanism, the gene encoding that enzyme should be detectable as up-regulated—at least at the 4- and/or 8-week time-points. At the 12- and/or 16-week time-points when SCC has occurred, this XME gene might no longer be up-regulated, because dedifferentiation that occurs during progression of neoplasia is anecdotally well known to silence expression of many genes. Finally, this XME gene and enzyme idealistically should appear in DKO but not in 1a1KO or WT groups that do not exhibit neoplasia.

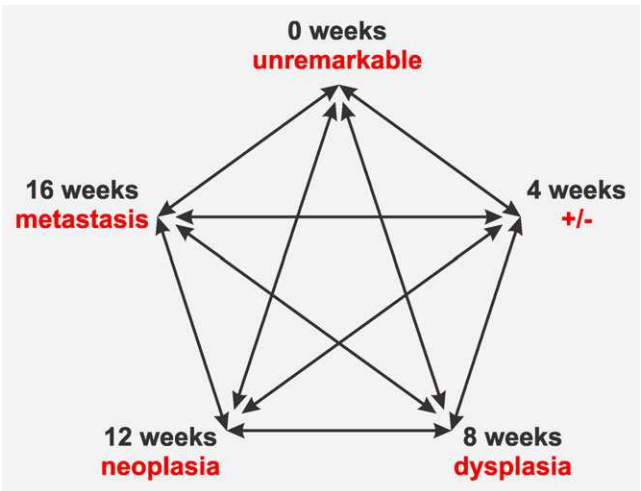

Figure 2. Microarray scheme of this study. PGD histology at each time-point is described in red font. Compared with "unremarkable" histology at 0 weeks, subtle microscopic changes ("±") could sometimes be seen at 4 weeks, dysplasia was observed at 8 weeks, neoplasia by 12 weeks and invasion into surrounding tissues ("metastasis") by 16 weeks. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Similar reasoning can be applied for cancer-related genes: specific oncogenes might be up-regulated and tumor suppressor genes down-regulated at the 4- and/or 8-week time-points, whereas the inverse might be found at the 12- and/or 16-week time-periods. In addition to comparing 4-minus-0, 8-minus-4, 12-minus-8 and 16-minus-12 weeks, the remaining six comparisons (8-minus-0, 12-minus-0, 16-minus-0, 12-minus-4, 16-minus-4 and 16-minus-8 weeks) should serve as a cross-check and help provide additional confirmation to specific up- and down-regulated genes—related to BaP metabolic activation in the PGD as well as related to SCC development. Moreover, comparing two genotypes (WT and 1a1KO) that show no SCC during this regimen of oral BaP with the double-knockout genotype (DKO) that succumbs to SCC tumorigenesis processes provides a further check-and-balance and additional strength to our microarray analysis.

Overview of statistically significantly up- and down-regulated genes

We chose a conservative cut-off *p*-value of <0.01 (Table 1). At each time-point differential comparison, between 10% and 40% of the genes up- and down-regulated were identified only as: Gene model ("Gm"), *i.e.* unknown putative genes not yet proven to be protein-coding or expressing a functional gene product; having a "RIKEN" number; "pseudogene"; and innumerable olfactory and vomeronasal receptor genes. If a predicted unknown gene ranked among the first in that time-period, it was kept on the list. Otherwise, most of these were removed from the Supporting Information Tables, but anyone who wishes to see the complete gene lists is welcome to request this.

For many of the comparison intervals (Table 1), the DKO PGD showed fewer up-regulated genes than 1a1KO or WT PGD; the number of down-regulated genes at the ten comparison intervals was similar among the three genotypes–except, curiously, the 12-minus-8 and 16-minus-12 week intervals in 1a1KO. For each of the three genotypes alone plus the three comparisons between the genotypes, all ten comparison time-points are provided in Supporting Information Tables S1–S24. These tables include only the top three dozen or fewer genes up-and down-regulated, plus the top two dozen most highly significant GO-KEGG (gene ontology; Kyoto Encyclopedia of Genes & Genomes) categories for up- and down-regulated genes. *p*-Value cut-offs for the genes and categories shown in the various tables can be seen to vary widely, ranging from <0.01 to $<10^{-21}$ (Supporting Information Tables S1–S24).

Gene expression in the three genotypes alone, and comparison between genotypes

Table 2 is an overview of the most highly ranked KEGG and GO categories listed in Supporting Information Tables S2, S4, S6, S8, S10, S12, S14, S16, S18, S20, S22 and S24.

Table 1. Summary of genes up- and down-regulated following daily oral BaP

Time-points (weeks)	Upregulated	*p*-value[1]	Downregulated	*p*-value[1]
***Cyp1a1/1b1*(−/−) [DKO]**				
4–0	84	<0.0098	59	<0.01
8–0	104	<0.01	462	<0.0096
12–0	107	<0.01	18	<0.0086
16–0	121	<0.01	110	<0.01
8–4	129	<0.01	88	<0.009
12–4	255	<0.01	161	<0.01
16–4	142	<0.01	81	<0.01
12–8	100	<0.01	109	<0.01
16–8	100	<0.01	94	<0.0099
16–12	149	<0.01	117	<0.01
***Cyp1a1*(−/−) [1a1KO]**				
4–0	97	<0.01	62	<0.01
8–0	65	<0.01	80	<0.01
12–0	2	<0.0069	3	<0.0098
16–0	67	<0.0099	93	<0.0099
8–4	156	<0.0099	196	<0.01
12–4	255	<0.01	161	<0.01
16–4	864	<0.01	984	<0.01
12–8	2	<0.0084	2	<0.0083
16–8	916	<0.01	1,337	<0.01
16–12	6	<0.0093	3	<0.0083
***Cyp1*(+/+) [WT]**				
4–0	46	<0.0099	88	<0.0098
8–0	169	<0.01	107	<0.01
12–0	49	<0.0098	27	<0.0092
16–0	265	<0.01	220	<0.01
8–4	246	<0.01	211	<0.01
12–4	187	<0.0099	296	<0.01
16–4	276	<0.01	307	<0.01
12–8	204	<0.01	216	<0.01
16–8	442	<0.01	460	<0.01
16–12	154	<0.01	192	<0.01

[1]Instead of a cut-off of false discovery rate (FDR) of <0.10, we used a cut-off *p*-value of <0.01.

Interestingly, gene expression categories in the PGD of oral BaP-treated mice were quite different—depending on genotype (WT *vs.* 1a1KO *vs.* DKO) as well as time of oral BaP exposure. More importantly than studying up- and down-regulation of genes in each of the three genotypes alone (top half of Table 2), comparison of response to oral BaP as a function of time between two genotypes should be more informative (bottom half of Table 2). Specifically, at crucial time-points during the processes of tumor initiation and progression, do we observe changes in DKO-minus-1a1KO, as well as in DKO-minus-WT, which are different from changes in 1a1KO-minus-WT?

GO and KEGG categories in DKO alone differ from those in WT alone or 1a1KO, and these categories in DKO-minus-WT and DKO-minus-1a1KO differ from those in 1a1KO-minus-WT comparisons (top and bottom halves of Table 2, respectively). In particular, keratinization and peptidase inhibitors up-regulated, and lipid metabolism and catabolic processes down-regulated–during the first 8 weeks of oral BaP–were most highly associated with DKO. Curiously, olfactory and pheromone receptor transduction was most highly down-regulated between 8 and 12 weeks, and then most highly up-regulated between 12 and 16 weeks of oral BaP, in DKO which develops the cancer; these patterns were not seen in WT or 1a1KO mice which do not develop the cancer.

P450 gene expression

Among the three possible comparisons of each of two genotypes, Table 3 lists all P450 (*Cyp*) genes significantly up- or down-regulated during each of the ten time-point comparisons. The only unequivocal candidate gene that encodes a BaP-metabolizing enzyme is *Cyp3a59*–strikingly elevated in DKO during the first 8 weeks of oral BaP exposure, and markedly diminished in DKO mice between 8 and 12 weeks of oral BaP exposure; in contrast, *Cyp3a59* is not similarly up- or down-regulated in 1a1KO or WT mice (which do not develop SCC of the PGD).

Cyp4a32 is up-regulated in DKO, but only between 8 and 16 weeks of oral BaP, *i.e.* after dysplasia has occurred. *Cyp2c67* is down-regulated in DKO between 4 and 12 weeks of oral BaP. *Cyp2j13* is down-regulated in DKO between 8 and 12 weeks of BaP. Although CYP2C[24–26] and CYP3A[25–30] enzymes have been shown to metabolize PAHs such as BaP, nothing has been published in this regard about CYP4A or CYP2J enzymes. Moreover, the CYP2C67 and CYP2J13 down-regulated mRNA data were not confirmed by qRT-PCR (Supporting Information Table S25).

Expression of (non-P450) xenobiotic-metabolizing enzyme genes

Similar to our analysis of all P450 genes, we analyzed expression of genes coding for all non-P450 XMEs that were substantially up- and down-regulated (Supporting Information Table S26). One gene of interest in DKO (up-regulation during first 8 weeks of BaP; down-regulation between 8 and 12 weeks of BaP) was *Ugt2a1*. Whereas it is conceivable that UGT2A1 is induced in the PGD by oral BaP, and perhaps UGT2A1 participates in glucuronide conjugation of oxygenated BaP metabolites, we found no published information on this topic. Also, this Phase II enzyme would not be expected to be involved in BaP metabolic activation leading to SCC formation in the PGD.

Prostaglandin G/H synthase-2 (PTGS2; cyclooxygenase-2) is a non-P450 enzyme that is known to metabolize,[31] and

Table 2. Gene expression within each genotypes alone (top) and comparison between two genotypes (bottom) [KEGG and GO categories]

Oral BaP Genotype	Early (0–8 weeks)		Intermediate (8–12 weeks)		Late (12–16 weeks)	
	Up	Down	Up	Down	Up	Down
WT alone	Organ morphogenesis; Cytokine; Immune system; Adhesion	Mitochondria; Oxidative phosphorylation; RNP complex; Organelle structures	Ribosome; Mitochondria; Translation; Oxidative phosphorylation	Pathways in cancer; Intracellular signaling; Transcription; Tube development	Extracellular matrix; Intercelullular signaling; Adhesion; Organ morphogenesis	Mitochondria; Ribosome; Translation; Membrane; Oxidative phosphorylation
1a1KO alone	Lipid metabolism; Lysosome; Alcohol metabolism; Gluconeogenesis	Olfactory and pheromone receptor activity; Neuroactive ligand-receptor; Nucleosome	Olfactory and pheromone receptor activity; Extracellular; Channel activity	Catabolic processes; Organelle; Protein transport; Mitochondria	RNA processing; RNA binding; Spliceosome; Many types of catabolic processes	Olfactory and pheromone transduction and binding; Plasma membrane
DKO alone	Cornified envelope; Olfactory transduction; Keratinization; Response to estrogen stimulus	Mitochondrial and organelle; Catabolic processes; Lipid metabolism; Peroxisome	Chemokine activities; Immune response; Chemotaxis; Defense response	Olfactory and pheromone receptor transduction; Channel activity; Plasma membrane	Olfactory and pheromone transduction and binding; Plasma membrane; Channel activity	Proteosome; Other catabolic processes; Oxidative phosphorylation; Protein transport
1a1KO–WT	Mitochondrial and organelle; Cofactor metabolism; Oxidation-reduction	Olfactory transduction; Transcription factor activity; DNA-binding; Receptor binding	Olfactory transduction; Calcium binding; Transcription factor activity; Membrane	Mitochondria; Organelle; Translation; Ribosome; RNP complex	RNP complex; RNA processing; Ribosome; Translation; Oxidative phosphorylation	Extracellular matrix; Calcium ion Olfactory transduction; Adhesion
DKO–WT	Pheromone and odorant receptor binding activity; Keratinization; Peptidase inhibitor	Lipid and fatty acid metabolism and biosynthesis; Monocarboxylic; Embryo development	Vasculature development; Immune response; Cell proliferation; Pathways in cancer	Ribosome; Pheromone and odorant binding activity; Organelle; Mitochondria; Retinol metabolism	Pheromone and odorant receptor binding activity; Retinol metabolism; Neuro receptor; Peptidase inhibitor	Vasculature development; Enzyme binding; Pathways in cancer; Anchoring junction; Cell cycle
DKO–1a1KO	Olfactory and pheromone receptor transduction; Keratinization; Endopeptidase inhibitor; Defense response	Lipid and cofactor metabolism; Catabolic processes; Organelle; Ketone metabolism	Protein transport; Catabolic processes; Lysosome; Organelle; Envelope; Lipid metabolism	Olfactory and pheromone receptor transduction; Channel activity; Plasma membrane	Olfactory and pheromone receptor transduction; Plasma membrane; Channel activity; Transporter	Spliceosome; RNA processes; RNP complex; Catabolic processes; Oxidative phosphorylation

Table 3. Summary of P450 genes highly up- and down-regulated following daily oral BaP

Weeks of BaP	Up-regulated genes			Down-regulated genes		
	DKO—1a1KO	DKO—WT	1a1KO—WT	DKO—1a1KO	DKO—WT	1a1KO—WT
4–0		*Cyp4a31*	*Cyp1b1*			
8–0	*Cyp3a59*	*Cyp3a59*	*Cyp1b1*			*Cyp3a25*
12–0					*Cyp3a57, Cyp2b19*	
16–0		*Cyp4a12b*	*Cyp2c37, Cyp1b1, Cyp4a12b*			
8–4				*Cyp4a31*	*Cyp4a31*	
12–4				*Cyp2c67*	*Cyp2j5, Cyp2c67*	
16–4			*Cyp2c37*	*Cyp2c39*		
12–8				*Cyp3a59, Cyp2j13*	*Cyp3a59, Cyp2j13*	
16–8	*Cyp4a32*	*Cyp4a32*	*Cyp2c37*			
16–12	*Cyp4f15*	*Cyp2c37*				

thus can be regarded as an XME. Supporting Information Table S27 shows that the pattern of *Ptgs2* expression was completely different from that of *Cyp3a59* expression.

Expression of cancer-related genes

Similar to our treatment of data in Supporting Information Tables 3 and S26, we identified relevant cancer-related genes that were up- and down-regulated in coordinated fashion in both the DKO-minus-1a1KO and DKO-minus-WT comparisons but not seen in the 1a1KO-minus-WT comparison (Table 4): a total of 26 cancer-related genes, plus eight Serpin genes were identified. Possible significance of these cancer-related genes to the oral BaP-induced process of SCC formation is discussed below.

Heat-map analysis

Supporting Information Figure S1 shows heat maps of differentially expressed genes, comparing DKO *vs.* 1a1KO (panels A & B) and DKO *vs.* WT (panels C & D). Three main clusters can be seen in Supporting Information Figure S1A. In the top cluster, gene expression levels were higher in DKO than 1a1KO from 8 weeks onward, the differences peaking at week

Table 4. Summary of putatively relevant cancer-related genes highly up- or down-regulated, following daily oral BaP following daily oral BaP[1]

Weeks of BaP	Up-regulated genes			Down-regulated genes		
	DKO—1a1KO	DKO—WT	1a1KO—WT	DKO—1a1KO	DKO—WT	1a1KO—WT
4–0		***Rab11b, Magea8***	*Pak3*	*Wtap*, ***Rad9b, Jak2***	*Wtap*, ***Jak2, Elk1***	
8–0	*Serpinb3b*	*Serpinb3b*	*Tusc4, Rhod*	***Tnfrsf10b, Mia1***	***Magea2***, *Wtap*	
12–0	*Serpinb3b Serpinb3a, Aim2*	*Serpinb3a, Serpinb3b, Serpinb3d*	*Serpina3i*	*Wtap*	*Wtap*	
16–0		***Rab11b***, *Serpina1b*	*Wtap, Fus*	*Wtap*, ***Magea2, Fus, Pak3***	***Rab7, Magea2***	*Ssxb1, Tnfaip6, Tnfaip3, Fosb*
8–4	*Serpinb3b*, ***Rasa2***	*Serpinb3b*			***Rab11b, Magea8***	
12–4	*Serpinb3b, Aim2*, ***Rap1b***	*Serpinb3d, Serpinb3b*		*Serpina1b*, ***Cage1***	*Tnfaip6*	*Tnfaip6*
16–4	***Rab12, Jak1,*** *Serpinb6a*		*Rerg, Wtap, Aim2*	***Magea2, Diras2, Frat2***	***Ssxb1, Tcta, Frat2, Magea8***	*Ceacam1*
12–8		***Mertk***	*Serpina3n*	***Mrgpra6***, *Serpina6*	*Tnfaip6, Rhod*	*Tnfaip6, Rhod*
16–8	***Mertk, Tnfrsf10b***	***Rab11b, Erg, Mertk, Sla***, *Serpinb9f*	*Aim2*	***Diras2, Rasd1***		*Tusc4*
16–12	*Serpina1b*, ***Tcl1b3***	*Serpina1b*, ***Tcl1b3***	*Aim2*	*Aim2*, ***Fgfr1op2***	***Rab7***	

[1]Those in **bolded font** denote cancer-related genes (other than Serpins) up- or down-regulated in DKO-minus-1a1KO and/or DKO-minus-WT but not in 1a1KO-minus-WT comparisons.

12. In the middle cluster, gene expression levels in DKO were generally less than that in 1a1KO; gene expression in the bottom cluster of Supporting Information Figure S1A does not have a consistent pattern. In Supporting Information Figure S1B, 9 out of 11 genes showed higher and increasing expression levels in DKO than in 1a1KO, especially after 8 weeks of oral BaP. In Supporting Information Figure S1C, three main clusters can be seen. Genes in the top cluster were generally lower in DKO at all times. Genes in the middle and bottom clusters were generally higher in DKO (again, especially after 4 or 8 weeks of BaP); no obvious pattern was observed in the middle cluster, whereas in the bottom cluster a pattern became increasingly clear as a function of length of time of oral BaP. Supporting Information Figure S1D, similar to Supporting Information Figure S1B shows that the majority of genes were expressed more highly in DKO and increased with time, again especially after 4 or 8 weeks of oral BaP.

Discussion

This laboratory has previously shown that WT mice receiving oral BaP (12.5 mg/kg/day) remain healthy and free of cancer[9]; in fact, WT mice receiving ten times this dose remain completely healthy for a year.[23] In contrast, 1a1KO mice receiving oral BaP (12.5 mg/kg/day) develop PSI adenocarcinoma by 12 weeks and show an apparent compensatory increase in CYP1B1 in the PSI, which is likely to be the XME responsible for metabolically activating BaP, leading to PSI tumor initiation.[9] This supposition is also supported by the fact that oral BaP-treated DKO mice, lacking CYP1B1 as well as CYP1A1 globally, are free of PSI adenocarcinoma; however, oral BaP-treated DKO mice instead develop SCC of the PGD.[9] The present study was designed to identify the BaP-metabolizing enzyme in PGD that most likely causes initiation of tumorigenesis, as well as cancer-related genes most likely responsible for progression of this cancer.

Hence, when gene expression of a BaP-metabolizing enzyme in DKO (which develops the SCC) differs substantially from that in 1a1KO and/or WT (having no PGD SCC), especially during the first 8 weeks of oral BaP, that gene would most likely be involved in initiation of tumorigenesis. Likewise, when expression of cancer-related genes in DKO differs substantially from that in 1a1KO and/or WT, especially between 4 and 12 weeks of oral BaP, those genes would most likely be involved in progression of tumorigenesis. Moreover, because 1a1KO mice on this BaP regimen are known to develop PSI adenocarcinoma,[9] it is possible that some systemic/endocrine factors of this cancer might affect gene expression in the PGD of 1a1KO that are not seen in that of the WT or DKO.

Why might rodents develop PGD cancer?

PGD tumors are known to occur commonly in male rodents during toxicity and cancer testing with many different environmental chemicals.[32] PAHs such as BaP specifically cause hyperkeratosis of the PGD epithelium. Among the best examples of an XME inducer causing hyperkeratosis is dioxin-induced chloracne in humans.[33] We speculate that BaP-induced keratin-plugging of secretions from the PGD could lead to pruritus, followed by the mouse scratching itself, thereby setting up secondary infections. BaP-induced hyperkeratinization, plus BaP being a well-known tumor promoter, and thus—similar to dioxin—a cause of inflammation,[14,34] all might combine to cause both initiation and promotion of SCC in the PGD. The question remains, however, in the global absence of CYP1A1 and CYP1B1, does some other specific XME take over in BaP metabolic activation to aid in tumor initiation, and which cancer-related genes contribute to cancer progression?

In PGD epithelium, it would appear that CYP1B1 metabolism might be beneficial, *i.e.* effecting BaP detoxication, because these tumors only appear when the *Cyp1b1* gene has been ablated. When body burden of BaP is high, as in the 1a1KO mouse, BaP metabolism by CYP1B1 in the PGD might function to detoxify BaP. When the body burden of BaP is even 3-fold higher, as in the DKO mouse, both CYP1A1 and CYP1B1 are absent in the PGD; thus, neither enzyme can detoxify the PAH parent compound. We thus postulated that either (*i*) there might be compensatory up-regulation of a different gene in the PGD that encodes an XME responsible for metabolic activation of BaP, or (*ii*) chronic irritation and/or inflammation caused by the parent BaP molecule rather than BaP metabolites might lead to SCC formation in the PGD. The present study strongly supports the former hypothesis.

Olfactory and pheromone transduction and receptor binding

Curiously, the KEGG category of "olfactory transduction" and GO categories of "response to pheromone," "pheromone receptor activity," "odorant binding" and "pheromone binding," *en bloc*, are extremely highly significantly up- and then down-regulated in 1a1KO alone (Supporting Information Tables S6 and S8) and DKO alone (Supporting Information Tables S10 and S12), up-regulated in 1a1KO-minus-WT (Supporting Information Table S14), and up- and then down-regulated in DKO-minus-1a1KO (Supporting Information Tables S22 and S24).

It is apparent that loss of CYP1A1, and especially loss of CYP1A1 combined with CYP1B1, intensely sensitizes this *en bloc* of "olfaction and pheromone transduction and receptor binding" genes in the PGD. We conclude that this phenomenon is not directly associated with SCC formation, because the changes are seen in 1a1KO sometimes, as well as DKO mice. However, these data support the findings of many others[35]—suggesting that these receptors in diverse cell types are used not only to sense odors in the nose but also to detect foreign chemicals such as BaP in unexpected tissues such as the PSI[9] and, in this study, the PGD.

Mouse [*Ah*] gene battery

The mouse aromatic hydrocarbon [*Ah*] gene battery[14] comprises a group of chromosomally non-linked genes that are

controlled by AHR and act in many ways similar to that of a bacterial operon responding to specific stimuli. All three *Cyp1* genes and many non-P450 XME genes are included in this battery; many of these [*Ah*] battery enzyme activities have been associated with differences in risk of PAH-induced toxicity, teratogenesis, mutagenesis and cancer.[3,14]

Interestingly, several of these genes (*e.g.*, *Aldh3a1*, *Ugt1@*, *Nqo1*) are strikingly up-regulated—during the first 4 or 8 weeks only–in 1a1KO alone (Supporting Information Table S5), 1a1KO-minus-WT (Supporting Information Table S13) and DKO-minus-WT (Supporting Information Table S17). These data are consistent with the role of CYP1 enzyme(s) controlling the expression of all genes in the [*Ah*] battery; in the absence of CYP1 enzymic activity, an unknown endogenous ligand binding to AHR is not degraded normally, thus leading to persistent activation of AHR and subsequent up-regulation of all [*Ah*] battery genes, as previously reviewed.[14]

Best XME candidate for SCC initiation

Predominant PAH-metabolizing phase I enzymes[36] in diverse vertebrates are well known to include: CYP1A1, CYP1A2 (minor), CYP1B1, CYP2C, CYP2W1 and CYP3A.[1,3,24–30,37,38] This study revealed that CYP3A59 mRNA levels were 2.2-fold decreased ($p = 8.6 \times 10^{-3}$) in 1a1KO at the 8-minus-0 weeks comparison (Supporting Information Table S7), 8.6-fold increased ($p = 5.6 \times 10^{-4}$) in DKO at the 8-minus-0 weeks comparison (Supporting Information Table S9), 4.8-fold decreased ($p = 1.1 \times 10^{-4}$) in DKO at the 12-minus-4 weeks comparison (Supporting Information Table S11), 10.4-fold decreased ($p = 2.1 \times 10^{-5}$) in DKO at the 12-minus-8 weeks comparison (Supporting Information Table S11), 3.9-fold decreased ($p = 5.8 \times 10^{-3}$) in DKO at the 16-minus-8 weeks comparison (Supporting Information Table S11), 10.4-fold increased ($p = 2.2 \times 10^{-3}$) in DKO-minus-WT at the 8-minus-0 weeks comparison (Supporting Information Table S17), 14.5-fold decreased ($p = 4.3 \times 10^{-5}$) in DKO-minus-WT at the 12-minus-8 weeks comparison (Supporting Information Table S19), 19-fold increased ($p = 5.9 \times 10^{-5}$) in DKO-minus-1a1KO at the 8-minus-0 weeks comparison (Supporting Information Table S21) and 13.9-fold decreased ($p = 7.2 \times 10^{-4}$) in DKO-minus-WT at the 12-minus-8 weeks comparison (Supporting Information Table S23). Confirmation of CYP3A59 mRNA levels by qRT-PCR (Supporting Information Table S25) further supports our contention that the *Cyp3a59* gene, encoding CYP3A59, is the strongest candidate for metabolically activating BaP in the PGD and being involved in tumor initiation in this experimental paradigm.

Consideration of PTGS2 (cyclooxygenase-2) as a candidate for metabolic activation

PTGS2 forms reactive intermediates from BaP[31]—at least in part *via* peroxyl radical-mediated metabolism[39]; thus, this was our initial candidate for "the" enzyme that might activate BaP in the PGD of DKO mice having neither CYP1A1 nor CYP1B1. PTGS2 and peroxyl radical-mediated metabolism is primarily responsible for oxidation of (+)-BP-7,8-diol in control animals, whereas the P450 system is primarily responsible for oxidation in PAH-treated animals.[39] Nevertheless, we carefully examined PTGS2 up- and down-regulation. However, the pattern of *Ptgs2* expression was not at all similar to that of *Cyp3a59* expression (Supporting Information Table S27). Hence, we conclude that PTGS2 is not an enzyme responsible for BaP activation in the PGD, which would lead to SCC formation in the absence of CYP1A1 and CYP1B1.

RAS-related candidates for tumor progression

During oral BaP-induced SCC formation in PGD of DKO but not 1a1KO or WT mice, eight RAS-related genes were strikingly up- and/or down-regulated (Table 4). Identification of this oncogene category should come as no surprise, however, because RAS-related genes are well known to be mutated and, hence, critical targets during exposure of chemical carcinogens such as PAHs, *e.g.* Refs. [40–43]).

The RAS oncogene superfamily of small GTP-binding proteins[44] includes five gene families: RAS, RAL, RHO, RAP and RAB. These proteins are involved in regulation of a diverse set of essential cellular functions. By cycling between inactive GDP-bound and active GTP-bound conformations, RAS proteins are binary switches that regulate multiple cellular signaling pathways—including those involving cell growth, differentiation and survival. Approximately 30% of all human neoplasms express *RAS* mutations that lock the protein into a constitutively active conformation. RAS activation is regulated by two groups of proteins: [a] guanine nucleotide-exchange factors (GEFs) bind to RAS and enhance exchange of GDP for GTP, thereby activating it; [b] GTPase-activating proteins (GAPs) inactivate RAS by binding to the GTP-bound form and enhancing hydrolysis of the bound GTP back to GDP.[45]

RAB11B (up-, then down-regulated in DKO–WT; Table 4) is a member of the RAB family and plays a critical role in regulating exocytotic and endocytotic pathways. RASA2 (up-regulated in DKO-minus-1A1KO; Table 4) is a member of the GAP1 family; RASA2 stimulates GTPase activity of normal RAS p21 but not its oncogenic counterpart. Acting as suppressor of RAS function, RASA2 enhances intrinsic GTPase activity of RAS proteins–resulting in an inactive GDP-bound form of RAS; this action permits cellular proliferation and differentiation. RAP1B (also up-regulated in DKO-minus-1A1KO; Table 4) is a member of the RAP family and–similar to three members of the RHO family–RAP1B is also regulated by the ubiquitin-proteasome pathway, which plays important roles in controlling cell polarity, migration, cell transformation and actin dynamics.[46] RAB12 (also up-regulated in DKO-minus-1A1KO; Table 4) is a small GTPase that regulates constitutive degradation of endogenous proteins such as the transferrin receptor, presumably from recycling endosomes to lysosomes.[47] RAB7 (down-regulated in DKO-minus-WT; Table 4) is a pivotal regulator in endo-lysosomal trafficking and governs early-to-late endosomal maturation, microtubule endosomal migration and

positioning, and endosome-lysosome transport involving a number of distinct protein–protein interaction cascades.[48]

RASD1 (down-regulated in DKO-minus-1A1KO; Table 4) is a dexamethasone-inducible signal regulator in various functional and protein-interaction screens; RASD1 can promote cell growth and tumor expansion, thus participating in the prevention of aberrant cell growth.[49] PAK3 (down-regulated in DKO-minus-1a1KO; Table 4) is one of six p21 (CDC42/RAC)-activated kinases. PAK proteins foster the loss of focal-adhesion complexes. Binding of these kinases to members of the RHO subfamily are intimately involved in morphological processes, as well as long-term transcriptional events involved in cellular homeostasis, proliferation and cancer. DIRAS2 (down-regulated in DKO-minus-1a1KO; Table 4) is one of three members of the DIRAS (GTP-binding RAS-like) family. DIRAS1 and DIRAS2 have been shown to be located in the plasma membrane and, upon over-expression, they are associated with formation of large intracellular vacuoles.[50]

Besides RAS-related oncogenes, the remaining highly significant cancer-related genes identified in this study are grouped into six additional categories. These are discussed in detail in the Discussion section of our Supporting Information.

Conclusions

In summary, cancer is a complex multi-stage process. An early stage is "tumor initiation," which generally includes one or more mutations and/or DNA damage—caused by enzymatic formation of reactive oxygenated metabolites and/or oxidative stress. Subsequently, "tumor progression" involves oncogene activation and/or loss of tumor suppressor activities; hundreds of genes contribute to the advancement of cancer. The present study describes a paradigm in which daily oral BaP results in SCC of the PGD in *Cyp1a1/1b1*(–/–) double-knockout but not in *Cyp1a1*(–/–), *Cyp1b1*(–/–) or *Cyp1*(+/+) WT mice. Human cancers of most relevance[51] to this mouse paradigm would include SCC of the skin or uterine cervix in which trauma and/or inflammation are contributing factors.

Using microarray analysis over 16 weeks of daily oral BaP, we identified CYP3A59 as the most likely BaP-metabolizing enzyme that becomes up-regulated in the absence of CYP1A1 and CYP1B1 (both of which enzymes metabolize BaP); we conclude that BaP metabolism by CYP3A59 is the strongest candidate for tumor initiation in the PGD of the DKO mouse. This hypothesis can now be tested, because mouse lines having the entire *Cyp3a* cluster ablated[52,53] are available.

Striking up- and down-regulation in expression of 26 cancer-related genes plus eight *Serpin* genes was also observed; eight of the 26 were RAS-related genes. Virtually every one of these 26 cancer-related genes in the SCC of DKO mice differs from those found during development of BaP-induced adenocarcinoma in PSI of 1a1KO mice.[9] Identification of the precise cancer-related gene(s) that participate in progression of SCC in the PGD will require further studies.

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References

1. Pelkonen O, Nebert DW. Metabolism of polycyclic aromatic hydrocarbons: etiologic role in carcinogenesis. *Pharmacol Rev* 1982;34:189–222.
2. Conney AH, Chang RL, Jerina DM, et al. Studies on the metabolism of benzo[*a*]pyrene and dose-dependent differences in the mutagenic profile of its ultimate carcinogenic metabolite. *Drug Metab Rev* 1994;26:125–63.
3. Nebert DW. The *Ah* locus: genetic differences in toxicity, cancer, mutation, and birth defects. *Crit Rev Toxicol* 1989;20:153–74.
4. Miller KP, Ramos KS. Impact of cellular metabolism on the biological effects of benzo[*a*]pyrene and related hydrocarbons. *Drug Metab Rev* 2001;33:1–35.
5. Nebert DW, Dalton TP, Okey AB, et al. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem* 2004;279:23847–50.
6. Ren A, Qiu X, Jin L, et al. Association of selected persistent organic pollutants in the placenta with the risk of neural tube defects. *Proc Natl Acad Sci USA* 2011;108:3100–6.
7. Rubin H. Synergistic mechanisms in carcinogenesis by polycyclic aromatic hydrocarbons and by tobacco smoke: a bio-historical perspective with updates. *Carcinogenesis* 2001;22:1903–30.
8. Yoshino I, Maehara Y. Impact of smoking status on the biological behavior of lung cancer. *Surg Today* 2007;37:725–34.
9. Shi Z, Dragin N, Miller ML, et al. Oral benzo[*a*]pyrene-induced cancer: two distinct types in different target organs depend on the mouse Cyp1 genotype. *Int J Cancer* 2010;127:2334–50.
10. Daniel CR, Cross AJ, Graubard BI, et al. Large prospective investigation of meat intake, related mutagens, and risk of renal cell carcinoma. *Am J Clin Nutr* 2012;95:155–62.
11. Anderson KE, Mongin SJ, Sinha R, et al. Pancreatic cancer risk: associations with meat-derived carcinogen intake in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) cohort. *Mol Carcinog* 2012;51:128–37.
12. Shimada T. Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons. *Drug Metab Pharmacokinet* 2006;21:257–76.
13. Puga A, Maier A, Medvedovic M. The transcriptional signature of dioxin in human hepatoma HepG2 cells. *Biochem Pharmacol* 2000;60:1129–42.
14. Nebert DW, Roe AL, Dieter MZ, et al. Role of the aromatic hydrocarbon receptor and [*Ah*] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochem Pharmacol* 2000;59:65–85.
15. Nebert DW, Karp CL. Endogenous functions of the aryl hydrocarbon receptor (AHR): intersection of cytochrome P450 1 (CYP1)-metabolized eicosanoids and AHR biology. *J Biol Chem* 2008;283:36061–5.
16. Ma C, Marlowe JL, Puga A. The aryl hydrocarbon receptor at the crossroads of multiple signaling pathways. *EXS* 2009;99:231–57.
17. Uno S, Dalton TP, Derkenne S, et al. Oral exposure to benzo[*a*]pyrene in the mouse: detoxication by inducible cytochrome P450 is more important than metabolic activation. *Mol Pharmacol* 2004;65:1225–37.
18. Uno S, Dalton TP, Dragin N, et al. Oral benzo[*a*]pyrene in *Cyp1* knockout mouse lines: CYP1A1 important in detoxication, CYP1B1 metabolism required for immune damage independent of total-body burden and clearance rate. *Mol Pharmacol* 2006;69:1103–14.

19. Uno S, Dragin N, Miller ML, et al. Basal and inducible CYP1 mRNA quantitation and protein localization throughout the mouse gastrointestinal tract. *Free Radic Biol Med* 2008;44:570–83.
20. Shi Z, Dragin N, Galvez-Peralta M, et al. Organ-specific roles of CYP1A1 during detoxication of dietary benzo[*a*]pyrene. *Mol Pharmacol* 2010;78: 46–57.
21. Dalton TP, Dieter MZ, Matlib RS, et al. Targeted knockout of *Cyp1a1* gene does not alter hepatic constitutive expression of other genes in the mouse [*Ah*] battery. *Biochem Biophys Res Commun* 2000;267:184–9.
22. Nebert DW, Dalton TP, Stuart GW, et al. "Gene-swap knock-in" cassette in mice to study allelic differences in human genes. *Ann NY Acad Sci* 2000;919:148–70.
23. Robinson JR, Felton JS, Levitt RC, et al. Relationship between "aromatic hydrocarbon responsiveness" and the survival times in mice treated with various drugs and environmental compounds. *Mol Pharmacol* 1975;11:850–65.
24. Meehan RR, Speed RM, Gosden JR, et al. Chromosomal organization of the cytochrome *Cyp2c* gene family in the mouse: locus associated with constitutive aryl hydrocarbon hydroxylase. *Proc Natl Acad Sci USA* 1988;85:2662–6.
25. Yun CH, Shimada T, Guengerich FP. Roles of human liver cytochrome P4502C and 3A enzymes in the 3-hydroxylation of benzo[*a*]pyrene. *Cancer Res* 1992;52:1868–74.
26. Bauer E, Guo Z, Ueng YF, et al. Oxidation of benzo[*a*]pyrene by recombinant human cytochrome P450 enzymes. *Chem Res Toxicol* 1995;8:136–42.
27. Sun B, Fukuhara M, Takanaka A. Characterization of benzo[*a*]pyrene metabolism and related cytochrome P-450 isozymes in Syrian hamster livers. *J Toxicol Environ Health* 1995;46: 47–55.
28. Koley AP, Buters JT, Robinson RC, et al. Differential mechanisms of cytochrome P450 inhibition and activation by α-naphthoflavone. *J Biol Chem* 1997;272:3149–52.
29. Fukuhara M, Sun B, Kato K, et al. Cytochrome P450 isoforms catalyzing benzo[*a*]pyrene metabolism in the Chinese hamster liver. *Toxicol Lett* 1999;110:85–93.
30. James MO, Lou Z, Rowland-Faux L, et al. Properties and regional expression of a CYP3A-like protein in channel catfish intestine. *Aquat Toxicol* 2005;72:361–71.
31. Reed GA, Marnett LJ. Metabolism and activation of 7,8-dihydrobenzo[*a*]pyrene during prostaglandin biosynthesis: intermediacy of a bay-region epoxide. *J Biol Chem* 1982;257:11368–76.
32. Mitsumori K, Elwell MR. Proliferative lesions in the male reproductive system of F344 rats and $B6C3F_1$ mice: incidence and classification. *Environ Health Perspect* 1988;77:11–21.
33. Bock KW, Kohle C. Ah receptor**:** dioxin-mediated toxic responses as hints to deregulated physiologic functions. *Biochem Pharmacol* 2006; 72:393–404.
34. Nebert DW, Dalton TP. Role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. *Nat Rev Cancer* 2006;6:947–960.
35. Olender T, Lancet D, Nebert DW. Update on the olfactory receptor (*OR*) gene superfamily. *Hum Genomics* 2008;3:87–97.
36. Guengerich FP. Metabolism of chemical carcinogens. *Carcinogenesis* 2000;21:345–51.
37. Shimada T, Hayes CL, Yamazaki H, et al. Activation of chemically diverse procarcinogens by human cytochrome P-450 1B1. *Cancer Res* 1996;56:2979–84.
38. Wu ZL, Sohl CD, Shimada T, et al. Recombinant enzymes overexpressed in bacteria show broad catalytic specificity of human cytochrome P450 2W1 and limited activity of human cytochrome P450 2S1. *Mol Pharmacol* 2006;69:2007–14.
39. Eling T, Curtis J, Battista J, et al. Oxidation of (+)-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene by mouse keratinocytes**:** evidence for peroxyl radical- and monoxygenase-dependent metabolism. *Carcinogenesis* 1986;7:1957–63.
40. Alguacil J, Porta M, Kauppinen T, et al. Occupational exposure to dyes, metals, polycyclic aromatic hydrocarbons and other agents and *KRAS* activation in human exocrine pancreatic cancer. *Int J Cancer* 2003;107:635–41.
41. Keohavong P, Lan Q, Gao WM, et al. Detection of *TRP53* and *KRAS* mutations in sputum of individuals exposed to smoky coal emissions in Xuan Wei County, China. *Carcinogenesis* 2005;26: 303–8.
42. Chakravarti D, Venugopal D, Mailander PC, et al. The role of polycyclic aromatic hydrocarbon-DNA adducts in inducing mutations in mouse skin. *Mutat Res* 2008;649:161–78.
43. Sparfel L, Pinel-Marie ML, Boize M, et al. Transcriptional signature of human macrophages exposed to the environmental contaminant benzo[*a*]pyrene. *Toxicol Sci* 2010; 114:247–59.
44. Rojas AM, Fuentes G, Rausell A, et al. The RAS protein superfamily**:** evolutionary tree and role of conserved amino acids. *J Cell Biol* 2012;196: 189–201.
45. Yarwood S, Bouyoucef-Cherchalli D, Cullen PJ, et al. The GAP1 family of GTPase-activating proteins**:** spatial and temporal regulators of small GTPase signalling. *Biochem Soc Trans* 2006;34 (Part 5):846–50.
46. Ding F, Yin Z, Wang HR. Ubiquitination in RHO signaling. *Curr Top Med Chem* 2011;11: 2879–87.
47. Matsui T, Itoh T, Fukuda M. Small GTPase RAB12 regulates constitutive degradation of transferrin receptor. *Traffic* 2011;12:1432–43.
48. Wang T, Ming Z, Xiaochun W, Hong W. RAB7**:** role of its protein interaction cascades in endo-lysosomal traffic. *Cell Signal* 2011;23: 516–21.
49. Vaidyanathan G, Cismowski MJ, Wang G, et al. The RAS-related protein AGS1/RASD1 suppresses cell growth. *Oncogene* 2004;23: 5858–63.
50. Kontani K, Tada M, Ogawa T, et al. DIRAS, a distinct subgroup of RAS family GTPases with unique biochemical properties. *J Biol Chem* 2002; 277:41070–8.
51. Maronpot RR, Flake G, Huff J. Relevance of animal carcinogenesis findings to human cancer predictions and prevention. *Toxicol Pathol* 2004; 32 (Suppl 1):40–8.
52. van Herwaarden AE, Wagenaar E, van der Kruijssen CM, et al. Knockout of cytochrome P450 3A yields new mouse models for understanding xenobiotic metabolism. *J Clin Invest* 2007;117:3583–92.
53. Hasegawa M, Kapelyukh Y, Tahara H, et al. Quantitative prediction of human pregnane X receptor and cytochrome P450 3A4-mediated drug-drug interactions in a novel multiple humanized mouse line. *Mol Pharmacol* 2011;80: 518–28.