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cDNA microarray profiling of rat mammary gland carcinomas induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and 7,12-dimethylbenz[*a*]anthracene

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cDNA microarray analysis was used to examine gene expression profiles in normal female Sprague–Dawley rat mammary gland and in carcinomas induced by the cooked meat-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and the potent experimental carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA). Nine tubulopapillary carcinomas (five from PhIP-treated rats and four from DMBA-treated rats) and normal mammary gland from virgin, pregnant and lactating rats were examined on a rat 6.9k cDNA microarray. Although histologically identical, PhIP- and DMBA-induced carcinomas could be distinguished by hierarchical clustering and multi-dimensional scaling analyses of cDNA expression. In addition, the expression of 21 clones was statistically different between PhIP- and DMBA-induced carcinomas (*F*-test, $P < 0.05$). The data indicate that distinct chemical carcinogens induce unique gene expression patterns in mammary gland carcinomas. The specific chemical carcinogen-associated cDNA array profiles found in carcinomas may ultimately be applicable to better understanding cancer etiology. PhIP- and DMBA-induced carcinomas also shared similarities in cDNA expression profiles. By comparing the expression in carcinomas (PhIP plus DMBA induced) with normal rat mammary gland (at any stage of differentiation), 172 clones were found to be differentially expressed. Genes showing increased expression in carcinomas by cDNA microarray analysis (and further validated by immunohistochemistry and western blot analysis) include cyclin D1, PDGF-A chain, retinol binding protein 1, prohibitin and the transcription factor STAT5A. The similarities in gene expression between PhIP- and DMBA-induced carcinomas raise the possibility that several molecular pathways for rat mammary gland transformation are maintained irrespective of the carcinogenic initiating agent.

Introduction

Multiple genetic alterations and molecular pathways have been implicated in human breast cancer; however, the biology of this disease remains incompletely understood (1–3). Several of the genetic alterations include mutations in specific genes such as p53, amplification or over-expression of genes such as cyclin D1 and *erbB2*, and multiple chromosomal deletions detectable as loss of heterozygosity (1–4). The large number of genes potentially involved in cancer development emphasizes the importance of studying multiple genetic alterations in concert. The recent development of large-scale gene expression profiling by cDNA microarrays permits the concurrent analysis of thousands of genes and further knowledge of the biochemical pathways involved in human breast cancer (3,5–8).

Rat mammary gland cancer models have been used for many years to better understand the development of human breast cancer (9). Chemically induced rat mammary gland carcinomas are similar to human breast cancers in various aspects including histopathology, the origin of the cancers from ductal epithelial cells, and the dependency on ovarian hormones for tumor development (9). The experimental polycyclic aromatic hydrocarbon 7,12-dimethylbenz[*a*]anthracene (DMBA) is a potent and well-established mammary gland carcinogen in the rat model (9,10). A single dose of DMBA given to Sprague–Dawley rats at 50 days of age rapidly induces ~100% incidence of mammary gland carcinomas (10). 2-Amino-1-methy-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a mutagenic/carcinogenic heterocyclic amine in cooked meat that has been implicated as a possible etiological factor in human breast cancer (11,12). PhIP was shown to be an effective mammary gland carcinogen in the Sprague–Dawley rat model when administered by multiple oral doses during the period of mammary gland development (13,14).

There are a growing number of recent studies on the molecular alterations in PhIP-induced rat mammary gland carcinomas (15–23). Some of the genetic changes in PhIP-induced carcinomas and/or derived cell lines include mutations in H-*ras*, loss of heterozygosity, microsatellite instability, single nucleotide instability and regions of loss and gain as detected by comparative genomic hybridization (CGH) (15,17,18,20–23). Interestingly, there is evidence that the genetic alterations in PhIP- and DMBA-induced carcinomas are different, with DMBA showing a much lower frequency of allelic imbalance and fewer gross chromosomal aberrations by CGH analysis (22,23).

To better understand the molecular events associated with mammary carcinogenesis, the expression of several genes has been examined in DMBA- and PhIP-induced rat mammary gland cancers, but the data are limited (16,19,24–29). A recent study has provided a more global examination of gene expression profiles in *N*-nitrosomethylurea (NMU)-induced rat mammary gland carcinomas by cDNA microarray analysis and competitive cDNA library screening (30). In the current study, a 6.9k rat cDNA microarray was used to compare the expression profiles in PhIP- and DMBA-induced rat mammary gland carcinomas. This analysis was undertaken to address whether the gene expression profile for a mammary gland carcinoma

Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; MDS, multi-dimensional scaling plot; NMU, *N*-nitrosomethylurea; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

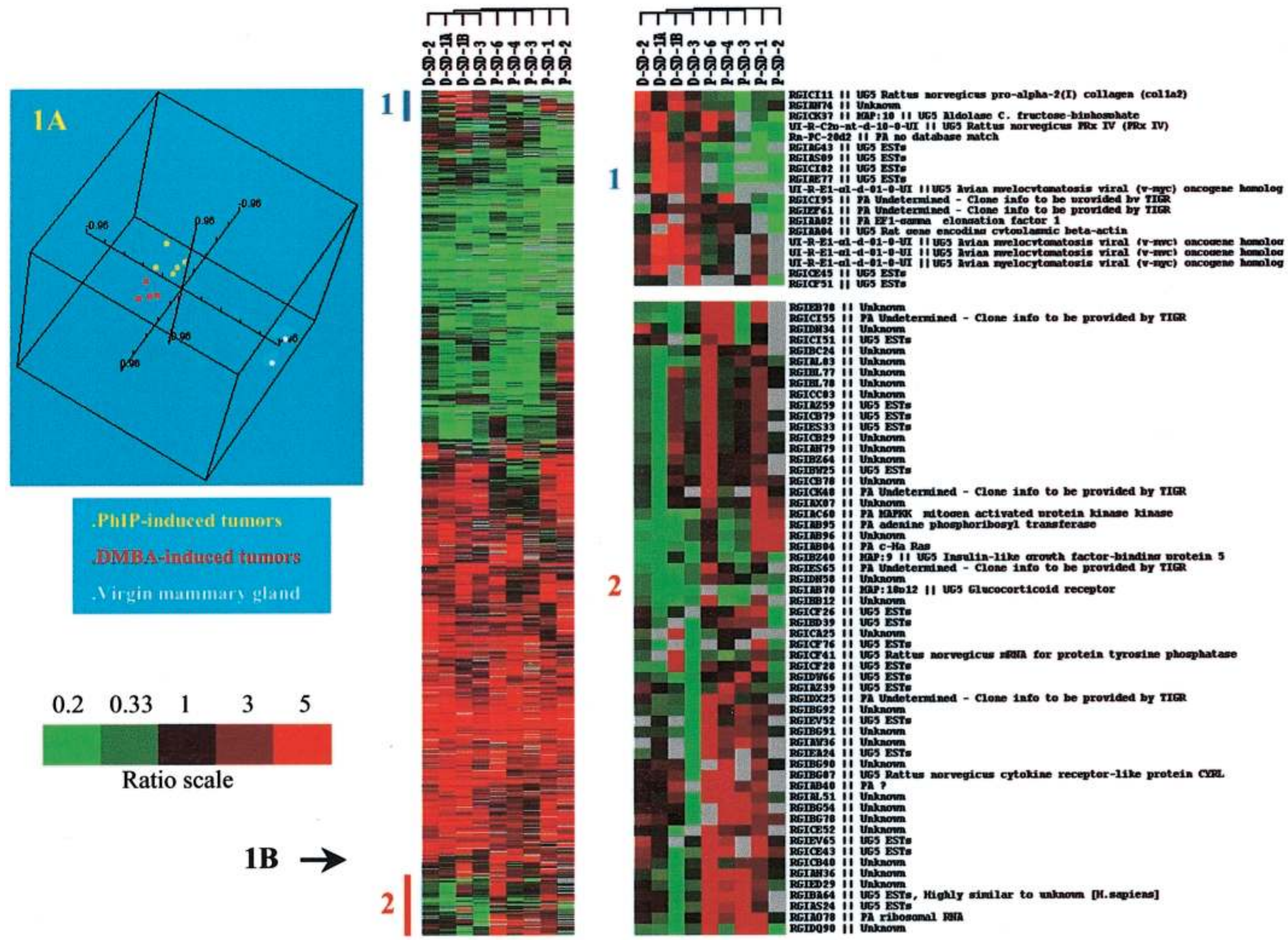

Fig. 1. Clustering of gene expression data from PhIP- and DMBA-induced rat mammary gland carcinomas. (**A**) MDS plot of expression data in the carcinomas induced by PhIP (yellow) and DMBA (red) and in normal virgin mammary gland (white). MDS was carried out with 3498 clones that passed the quality control filters with both Cy3 and Cy5 channels (signal/background ⩾2.0, spot size ⩾25 mm, and signal intensity ⩾500). B. Hierarchical clustering of carcinomas and gene expression array matrix for carcinomas induced by PhIP (P) and DMBA (D). A total of 869 clones were used in the cluster analysis. Two clusters were identified and shown expanded on the right with corresponding clone information. Cluster 1 contains clones with higher expression in DMBA-induced carcinomas, and cluster 2 contains clones with higher expression in PhIP-induced carcinomas. In all cluster figures, each column represents one sample and each row represents a distinct clone. Green squares, clones with low relative expression (ratio less than 1); black squares, clones with expression similar to reference (ratio of approximately 1); red squares, clones with high relative expression (ratio greater than 1); gray squares, insufficient data.

reflects a specific etiological agent. In addition, this study compared the molecular profiles in normal mammary gland (virgin, pregnant and lactating rats) to PhIP- and DMBA-induced carcinomas to gain insight into the gene expression alterations associated with rat mammary gland carcinogenesis that are in common for both carcinogens.

Materials and methods

Animals, treatment and mammary gland samples

Female Sprague–Dawley rats were obtained from the NIH animal supply (Animal Production Area, FCRDC, Frederick, MD). All animals were provided NIH Lab Chow and water *ad libitum* and housed in the NIH animal facility on a 12 h light/12 h dark cycle. Mammary gland carcinomas were induced by PhIP–HCl (Toronto Research Chemicals, North York, ON) and DMBA (Sigma, St Louis, MO) following established protocols that have been described previously in detail (10,13,14). Briefly, PhIP (75 mg/kg, p.o.) was administered to 43-day-old virgin rats once per day over a 12 day period (13,14). DMBA (Sigma) was administered as a single oral 75 mg/kg dose to 50-day-old virgin rats. Twenty-four hours after carcinogen administration, rats were placed on a defined high-fat diet until necropsy (13). Carcinomas were collected over an 8–13 week period after PhIP and DMBA treatment. Mammary gland tumor samples were fixed in formalin or 70% ethanol for histological or immunohistochemical examination, or snap frozen and stored at –80°C prior to RNA isolation. Tubulopapillary carcinomas, the major type of carcinoma induced by PhIP and DMBA in the Sprague–Dawley rat, were selected for array analysis. Histological classification was made according to the criteria outlined previously (31). Only tubulopapillary carcinomas were used for array analysis in order to avoid the possible variation in expression arising from histologically different tumor subtypes. Carcinomas were histologically indistinguishable between the two carcinogen treatment groups. Each carcinoma used in this study was from a separate rat.

Normal mammary gland tissue was collected from 50-day-old virgin, mid-pregnant (10–15 days) and lactating rats (with 10–12-day-old pups). For reference RNA in the array analysis, mammary gland tissue (abdominal and inguinal glands) was collected from five virgin Sprague–Dawley rats that were age-matched to the experimental rats, sham dosed with vehicle only, and provided the same defined high-fat diet after 55 days of age. Total RNA was isolated from the mammary gland from each rat, and an equal amount of RNA from each was pooled to provide the reference RNA for all cDNA microarray hybridizations.

RNA isolation

Total RNA was isolated using TRIzol extraction reagent (Gibco BRL, Rockville, MD) and as needed, samples were further purified using the Qiagen

Table I. Differentially expressed clones between PhIP- and DMBA-induced carcinomas[a]

Gene description	Clone ID
Predominantly expressed clones in PhIP-induced tumors	
cytokine receptor-like protein	RGIBG87
guanine deaminase	RGIEK95
HLA-B associated transcript 3	RGIAP25
phosphodiesterase 4B, cAMP-specific	RGIBB95
ESTs	RGIAW7
ESTs	RGIAX19
ESTs	RGICA89
ESTs, weakly similar to K2C8	RGIEF76
ESTs	RGIAF74
ESTs	RGIAG43
unknown	RGIAL51
unknown	RGIBZ46
unknown	RGICD40
unknown	RGIBG54
unknown	RGIBG78
unknown	RGIBG90
Predominantly expressed clones in DMBA-induced tumors	
CD24 antigen	RGIAA59
dual specificity phosphase	RGIAD87
PIPK2 (phosphatidylinositol 5-phosphate 4-kinase alpha)	RGIBA80
ESTs, moderately similar to S43424 zipper-containing protein	RGIAZ92
undetermined	NCI-ATC-Rn:1005e10

[a]Expression of clones is statistically different between PhIP- and DMBA-induced carcinomas (*F*-test, $P < 0.05$). Statistical analysis included all clones with permutation at 1000.

RNease Mini Kit (Qiagen, Valencia, CA) according to the manufacturers' protocols.

cDNA microarrays and statistical analysis

The rat cDNA microarray, printed in the Advanced Technology Center, National Cancer Institute, contained 6900 cDNA clones selected from the Rat Gene Index of the Institute of Genomic Research essentially as described previously (32). Thirty micrograms of total RNA were used for cyanine 3-dUTP (Cy3) and 60 μg for cyanine 5-dUTP (Cy5) labeling. The labeling was performed by oligo(dT)-primed polymerization using SuperScript II reverse transcriptase and the labeled Cy3 and Cy5 cDNA probes were cleaned up using a Microcon column YM-30 (Millipore, Bedford, MA). The microarray was hybridized to the probe overnight at 65°C. After washing of the microarray slides, the signal intensity was detected using the Axon GenePix 4000 scanner and analyzed using GenePix Pro 3.0 software (Axon, Union City, CA). The image and data were deposited into NCI microarray database system (http://nciarray.nci.nih.gov) supported by the Center for Information Technology of NIH for data interpretation and visualization. To ensure the reliability of data, all samples were also reciprocally labeled in all hybridization experiments. Each sample was hybridized at least twice using both Cy3 and Cy5 labeling. The average ratio from the reciprocal replicates was used to evaluate the expression level. Clones with inconsistent expression in reciprocal hybridizations, implying the data were not reproducible, were excluded from the analysis. The data were analyzed using hierarchical clustering (M.Eisen *et al.*, Stanford's Cluster and TreeView Program), multi-dimensional scaling plot (MDS) and BRB Array Tools. These tools were available from the NCI microarray database system web site (http://nciarray.nci.nih.gov). BRB Array Tools used in the statistical comparison of expression of PhIP- and DMBA-induced carcinomas (supervised *F*-test) were developed by Richard Simon and Amy Peng (Biometric Research Branch of NCI) (http://linus.nci.nih.gov).

Immunohistochemical analysis

Immunohistochemistry was performed using antibodies against cyclin D1 (R-124, Santa Cruz, Santa Cruz, CA), STAT5A (L-20, Santa Cruz), RBP 1 (clone G4E4, NeoMarkers, Fremont, CA, 1:400) and PDGF-A (N-30, Santa

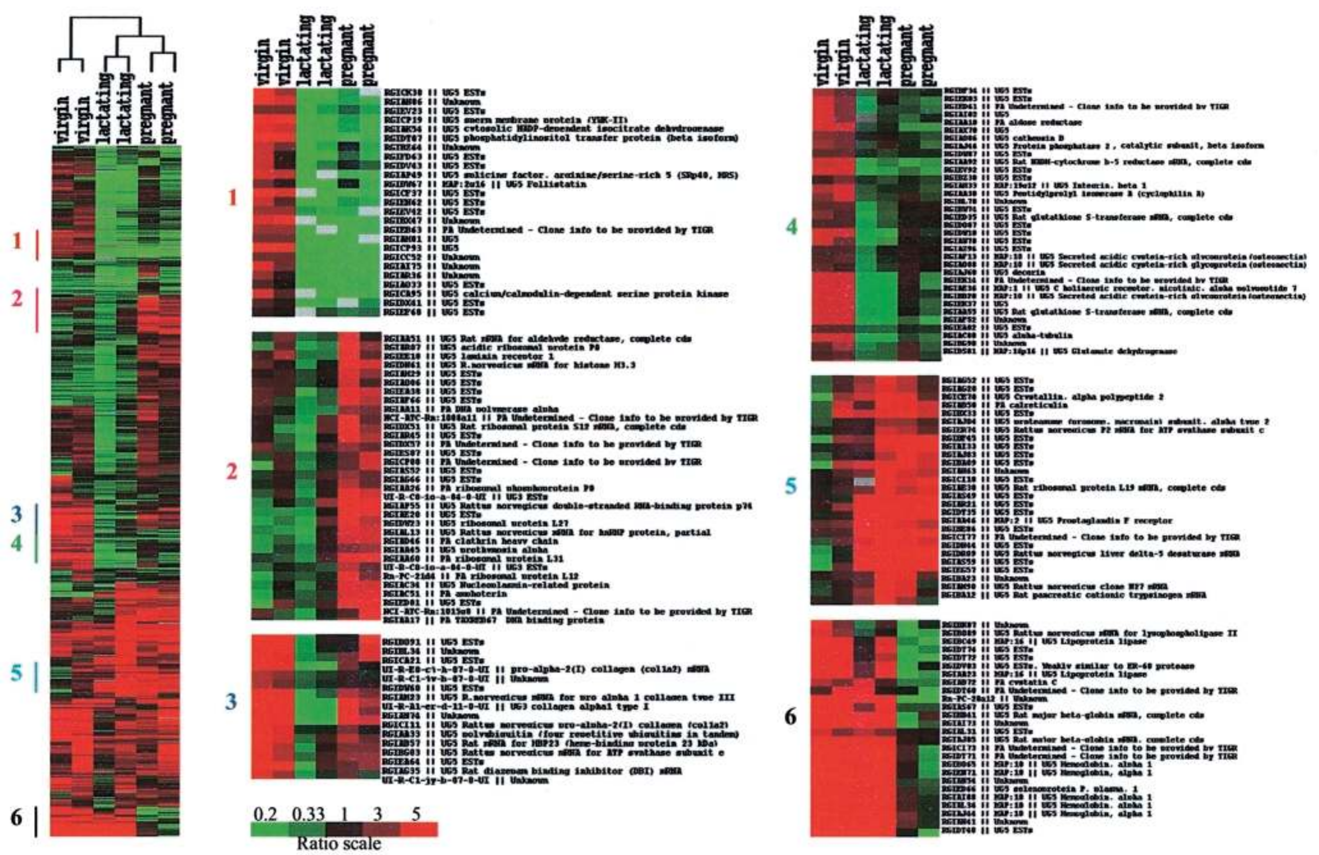

Fig. 2. Clustering of gene expression data from normal mammary gland from virgin, lactating and pregnant rats. Hierarchical clustering and gene expression array matrix containing 371 clones is shown on the left. Six clusters were identified and are shown expanded with corresponding clone information to the right. Clusters 1 and 4 are distinct in the magnitude of the difference in clone expression between the virgin and lactating or pregnant glands. β-casein, -lactalbumin, and whey acidic protein, genes that are known to be differentially expressed with mammopoesis, were not on the cDNA array.

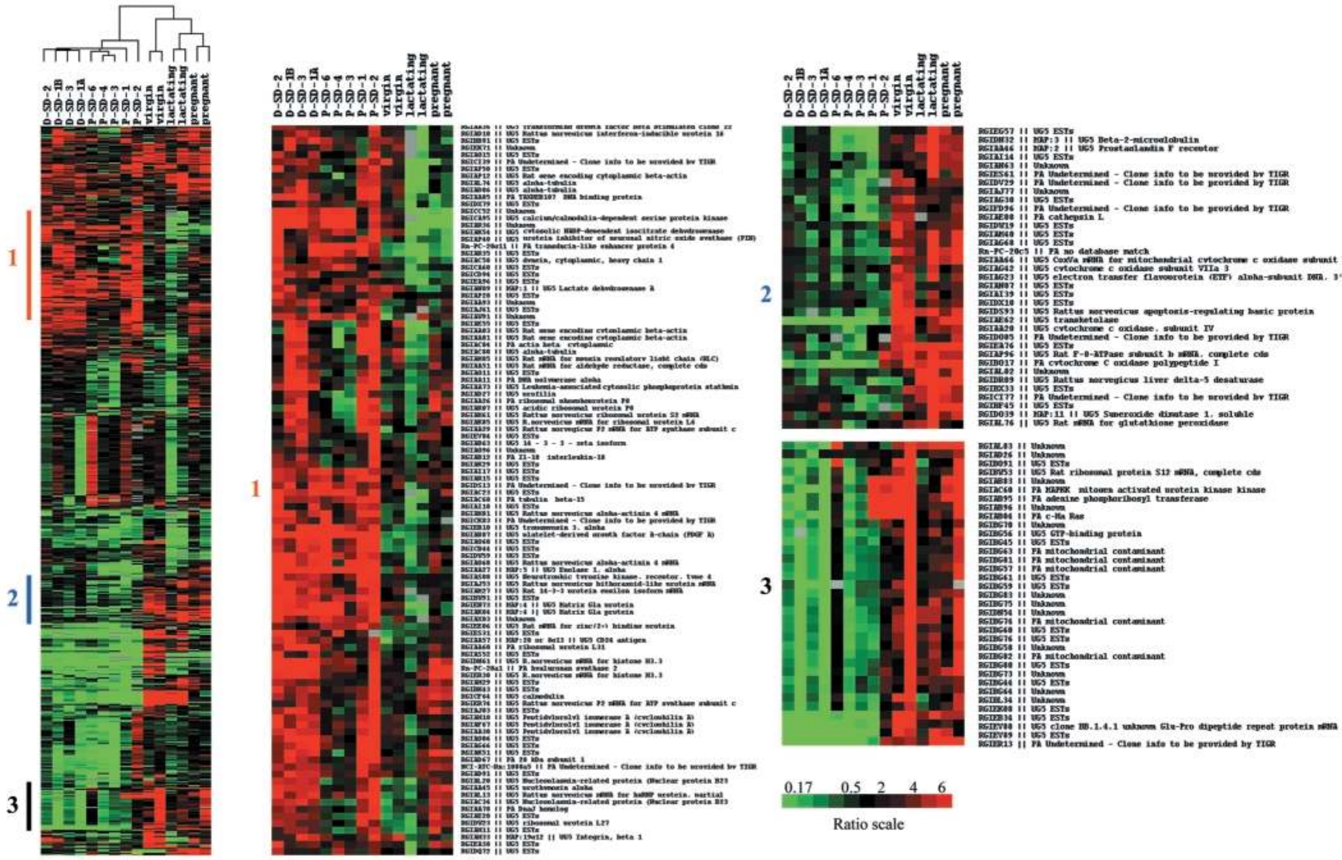

Fig. 3. Gene expression array matrix comparing carcinomas and normal mammary glands. Hierarchical clustering of 605 clones is shown on the left. Three clusters were identified and are shown expanded with corresponding clone information to the right. Cluster 1 contains clones with higher expression in carcinomas in comparison to normal mammary gland, and clusters 2 and 3 contain clones with lower expression in carcinomas in comparison with normal mammary gland. Between clusters 2 and 3, cluster 3 contains clones showing the lowest expression in carcinomas.

Cruz) and Universal DAKO LSAB2 System (DAKO, Carpinteria, CA). Paraffin-embedded sections from the tumors and normal mammary glands were deparaffinized and rehydrated with xylene and ethanol. The endogenous peroxidase activity was blocked by incubation for 40 min in absolute methanol containing 0.3% hydrogen peroxide. Antigen retrieval was performed by immersing the slides in 0.01 M citric acid buffer (pH 6.0) and treated for 5 min, three times using microwave. The slides were then incubated sequentially with primary antibodies overnight at 4°C and HRP-labeled secondary antibody for 2 h at room temperature. Sections were developed using AEC substrate and counterstained with Mayer's haematoxylin.

Results

Comparison of PhIP- and DMBA-induced carcinomas by array analysis

Hierarchical clustering and MDS plotting were used to compare the overall gene expression patterns of PhIP- and DMBA-induced carcinomas (Figure 1A and B). Clustering was based on a total of 869 clones, which satisfied the expression criteria of showing at least a 2-fold change in a minimum of two of nine carcinomas. By hierarchical clustering, clones showing similarities in expression patterns among the samples were grouped. By MDS, tumor samples were placed in a three-dimensional space according to their expression patterns, with the distance between the samples reflecting their approximate degree of correlation. Carcinomas could be separated based on carcinogenic agent indicating that PhIP- and DMBA-induced tumors had distinct expression profiles. It is notable from MDS analysis that the difference between the PhIP- and DMBA-induced carcinomas was clearly smaller than the difference between the carcinomas and the normal virgin mammary gland (Figure 1A). Out of 869 clones, 19 fell within a cluster (cluster 1) showing higher expression in DMBA-induced carcinomas, and 58 (cluster 2) showed a tendency toward higher expression in PhIP-induced carcinomas (Figure 1B). BRB Array Tools analysis further confirmed that 21 clones showed statistically significance differences in expression between PhIP- and DMBA-induced carcinomas (F-test, $P < 0.05$) (Table I).

Gene expression profiles of normal mammary gland

Normal mammary gland undergoes a process of lobuloalveolar development and differentiation from the virgin state through pregnancy and lactation, a process that has been shown to involve specific changes in gene expression (9,33,34). cDNA microarray analysis of normal virgin, pregnant and lactating rat mammary gland was carried out to examine in more detail the specific genes associated with mammary gland development and mammopoiesis and to facilitate the identification of genes potentially involved in carcinogenesis. Following the criterion of at least a 2-fold change in expression in at least two of the six samples, 371 clones were clustered (Figure 2). Six distinct expression clusters were identified which classified clones showing different expression patterns among the three physiological states of the mammary gland. Generally, the expression was more likely to be similar between the pregnant and lactating rats than between virgin and either pregnant or lactating rats. In three of the six clusters (numbers 1, 4

Table II. Differentially expressed genes in PhIP- and DMBA-induced carcinoma compared with normal mammary gland[a]

Over-expressed genes	Down-expressed genes
Cell growth and cell cycle-related genes	*Cell growth and cell cycle-related genes*
cyclin-dependent kinase 4 (cdk4)	growth response protein (CL-6)
platelet-derived growth factor A-chain (PDGF A)[b]	EGF-like prot. T16
prohibitin (phb)	DNA-binding protein inhibitor ID-3
cyclin D1	lin-7-A
protein kinase SNK (Snk)	*Metabolism-related genes and enzymes*
calcium/calmodulin-dependent serine protein kinase	guanine aminohydrolase (GAH)
macrophage migration inhibitory factor (Mif)	lipoprotein lipase
Signal transduction and transcription-related genes	acyl-coenzyme A:cholesterol acyltransferase
TAXREB107 DNA binding protein	epoxide hydrolase 1 (microsomal xenobiotic hydrolase)
GTPase	cytochrome c oxidase subunit I
tyrosine 3-monooxygenase/tryptophan 5-monooxygenase	cytochrome b5
activation protein, zeta polypeptide	aquaporin 1
leukemia-associated cytosolic phosphoprotein stathmin	aldose reductase
GTP-binding nuclear protein Ran/TC4	adenylyl cyclase type V
signal transducer and activator of transcription 5a	transketolase
transducin-like enhancer protein 4	selenoprotein P, plasma, 1
elongation factor 1-alpha	peptidyl arginine deiminase, type IV
pyrimidine binding protein	xanthine dehydrogenase
phosphatidylethanolamine binding protein	cytochrome c oxidase polypeptide I
diazepam binding inhibitor	*Receptor and hormone regulation-related genes*
Cytoskeleton-related genes	scavenger receptor class B type I
alpha-actinin 4	endothelial differentiation sphingolipid G-protein-coupled receptor 1
190 kDa ankyrin isoform	*Extracellular matrix genes*
tubulin beta-15	alpha 1 type V collagen
tropomyosin 3, alpha	fibronectin 1
annexin 1 (p35) (Lipocortin 1)	desmin
acidic calponin	*Miscellaneous*
keratin (K5)	major beta-globin
ezrin	hemoglobin, alpha 1
Metabolism-related genes and enzyme	clone BB.1.4.1 unknown Glu-Pro dipeptide repeat protein
stearoyl-CoA desaturase 2	mitochondrial dicarboxylate carrier
proprotein convertase subtilisin/kexin type 3	
Na-K-CI cotransporter (Nkcc1)	
mitochondrial malate dehydrogenase (EC 1.1.1.37)	
gamma-glutamyl transpeptidase	
glutathione synthetase gene	
enolase 1, alpha	
brain glucose-transporter protein	
ATPase inhibitor (rat mitochondrial IF1 protein)	
Receptor and hormone regulation-related genes	
lipolysis-stimulated remnant receptor beta subunit	
retinol-binding protein 1	
Extracellular matrix genes	
matrix Gla protein	
Miscellaneous	
Homo sapiens protein KIAA0429	
jacob protein, alternatively spliced isoform delta2 (jac gene)	
neuronal tissue-enriched acidic protein NAP-22	

[a]Normal mammary gland includes virgin, pregnant and lactating glands.
[b]Underlined indicates genes confirmed by western blotting, immunohistochemistry and/or real-time PCR analysis.

and 5), glands from lactating and pregnant rats showed similar expression levels that were distinct from the virgins. Specifically, clones in clusters 1 and 4 were under-expressed during lactation and pregnancy but over-expressed in virgin glands. In cluster 5, clones were over-expressed with lactation and pregnancy, but under-expressed in the virgin state. Other cluster patterns were also observed. Clusters 2 and 6 contained clones showing similarly altered expression levels in the virgin and lactating glands, whereas cluster 3 contained clones having under-expression in the lactating glands, but over-expression in virgin and pregnant states.

Comparison of PhIP- and DMBA-induced carcinomas to normal mammary gland

cDNA microarray analysis was used to compare the expression profile between the carcinomas from PhIP- and DMBA-treated rats and the normal mammary gland at any physiological state, specifically virgin, pregnant and lactating. We hypothesized that the expression profile in carcinomas would be distinct from the profile in normal mammary glands from virgin, pregnant and lactating rats. As the density of epithelial cells is low in the virgin gland relative to carcinomas (~15 versus 90% epithelial cells, respectively), the use of lactating and pregnant rat mammary glands in the comparison array analysis was expected to minimize the epithelial and stromal cell variations that could contribute to the expression differences between carcinomas and normal tissue. PhIP- and DMBA-induced carcinomas were compared with normal mammary gland as a means to determine whether there were changes in expression associated with carcinogenesis that occurred irrespective of the chemical carcinogen and, therefore, may

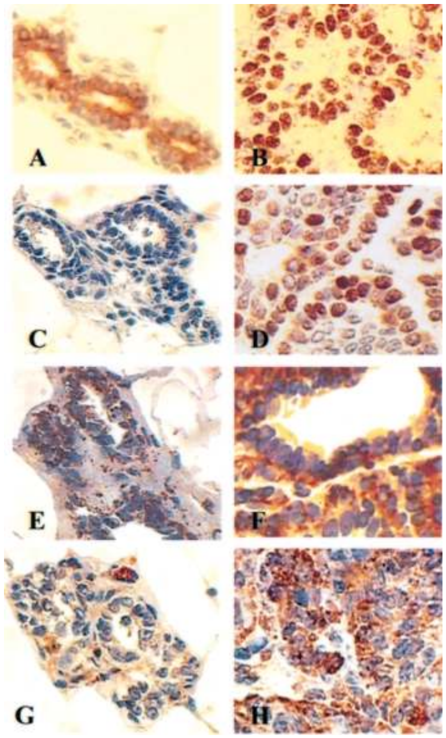

Fig. 4. Representative immunohistochemical analysis of STAT5A (**A**, **B**), cyclin D1 (**C**, **D**,), PDGF-A (**E**, **F**), and retinol binding protein 1 (**G**, **H**) in normal mammary tissue (A, C, E, G) and PhIP-induced carcinomas (B, D, F, H). Magnification, 400×.

represent major conserved pathways for rat mammary gland carcinogenesis. Clustering analysis of 605 clones showing at least a 2-fold change in expression revealed three clusters that were differentially expressed between carcinomas and normal mammary gland at any physiological state (Figure 3). Cluster 1 contained clones showing higher expression in carcinomas than in normal mammary gland, and clusters 2 and 3 contained clones showing lower expression in carcinomas. Comparison between carcinoma and normal mammary gland identified 172 clones including 68 known genes that were either over-expressed or under-expressed in common between PhIP- and DMBA-induced carcinomas (Table II).

The differential expression of several known genes was further confirmed by immunohistochemistry. In comparison with normal mammary gland, cyclin D1, PDGF-A, STAT5A and retinol binding protein 1 were over-expressed in carcinomas induced by PhIP or DMBA (Figure 4). Western blot analysis also confirmed the over-expression of these genes as well as the over-expression of prohibitin (data not shown). Prohibitin and protein kinase SNK were further validated as over-expressed in carcinomas by real-time polymerase chain reaction (PCR) (in preparation).

Discussion

The objective of this study was to further characterize the molecular alterations associated with chemically induced rat mammary gland carcinomas through cDNA microarray analysis and to determine if distinct chemical carcinogens induce unique microarray profiles. Using a 6.9k array, 172 clones were found to be differentially expressed between mammary gland cancers and normal mammary gland. Several of the known genes that were identified in our array analysis as showing altered expression in rat mammary gland cancers have also been implicated in human malignancies including human breast cancer. These genes include cyclin D1, PDGF-A, stathmin, matrix Gla protein, prohibitin, peripheral benzodiazepine receptors and STAT family transcription factors (7,35–44). Alterations in expression of many of the same genes in both rat and human breast cancers support the value of the chemically induced rat mammary gland cancer model for elucidating critical genes and signaling pathways involved in the human disease.

In this study, rat mammary gland carcinomas were induced by two diverse chemical carcinogens: PhIP, a heterocyclic amine found in cooked meat, and DMBA, a potent experimental mammary gland carcinogen belonging to the class of polycyclic aromatic hydrocarbons. PhIP and DMBA induce different DNA adducts and unique mutation spectra in the mammary gland (45,46). In addition, the data to date support that carcinomas induced by PhIP and DMBA have different genetic alterations (22,23). In accordance with the data, the findings from cDNA microarray analysis indicate that carcinomas induced by these distinct carcinogens also have unique gene expression profiles. By hierarchical clustering, the carcinomas induced by PhIP and DMBA could be resolved and were further shown to be distinct by MDS plot analysis (Figure 1A). Twenty-one clones were statistically different between carcinomas induced by the two compounds (Table I). Further work is required to clarify the possibly divergent molecular pathways involved in PhIP- and DMBA-induced carcinogenesis. Knowledge of the unique expression profile in carcinomas induced by distinct environmental chemical carcinogens may ultimately be valuable for risk assessment and better understanding cancer etiology in humans.

By cluster analysis of PhIP- and DMBA-induced carcinomas and normal mammary gland, it was also possible to find similar alterations in expression in common between carcinomas induced by different carcinogens (Figure 3). Both PhIP- and DMBA-induced carcinomas showed an increased expression of cyclin D1 and CDK4 relative to normal mammary gland. Our findings concur with previous studies reporting an increased expression of cyclin D1 in DMBA-induced carcinomas (24,26) and studies showing an increased expression and increased protein level of cyclin D1 and CDK4 in NMU-induced carcinomas (30). Therefore, over-expression of cyclin D1 is observed in rat mammary gland carcinomas induced by structurally distinct chemical carcinogens. In human breast cancers, cyclin D1 has been shown to be frequently amplified and its protein over-expressed in >50% of cases (35). The observation that rat mammary gland cancers induced by a diverse group of chemical carcinogens all involve over-expression of cyclin D1 strongly supports the major importance of this gene and the pathways regulating this gene in rat mammary gland carcinogenesis.

PDGF-A is another gene found in this study that was confirmed as over-expressed in both PhIP- and DMBA-induced rat mammary gland carcinomas. In the human mammary gland, PDGF-A is a known mitogen that acts as a survival factor (36,37). It is also over-expressed in a large percentage of human breast cancers, and autocrine and paracrine loops with PDGF-A have been reported in 18 and 38% of human breast cancer cases, respectively (36,37). Additional studies are required to determine if elevated PDGF-A expression in rat mammary gland carcinomas is indicative of an autocrine/paracrine loop that facilitates growth autonomy. Nevertheless, the finding that PDGF-A is over-expressed in both PhIP- and DMBA-induced carcinomas emphasizes the potential importance of this gene in rat mammary gland carcinogenesis.

PDGF as well as other growth factors, cytokines and hormones such as prolactin, a critical hormone for rat mammary gland carcinogenesis (47), in part mediate their effects on mammary gland growth and development via the STAT family of transcription factors (48,49). The results from the current study revealed that STAT5A was over-expressed in rat mammary gland carcinomas induced by PhIP and DMBA. In the mammary gland, STAT5A is known to promote cell proliferation and it is required for mammopoesis and lactation (48,49). Studies in transgenic mice also indicate that STAT5A influences mammary epithelial cell survival and carcinogenesis (50). Phosphorylation and hence activation of STAT5A regulates the activity of many genes including cyclin D1 and the anti-apoptosis gene Bcl-X_L (48,51–53). There is some evidence that STAT proteins are over-expressed or activated in primary human breast cancers and human breast cancer cell lines (7,44,48,54). In addition, a previous study has reported a change in the expression of STAT3 in rat mammary gland carcinomas induced by NMU (30). Although further studies are required, the increase in expression of STAT5A in rat mammary gland carcinomas induced by PhIP and DMBA supports the possibility that STAT5A is relevant for the development of breast cancer. It is tempting to speculate that alterations in the expression and activity of STAT5A may partly account for the elevated cyclin D1 expression in rat mammary carcinomas and may also mediate the cell survival signals imparted by specific growth factors such as PDGF.

In summary, the findings from these studies are consistent with the notion that the profile of gene expression in a mammary carcinoma reflects the specific etiological agent. These specific profiles may ultimately be valuable for better understanding human cancer etiology and for risk assessment. Furthermore, the findings from this study show that there are common alterations in expression profiles among carcinomas that were observed irrespective of the chemical carcinogen. Genes showing similar alterations in expression may represent major molecular pathways associated with rat mammary cancer development. Studies to delineate these pathways in the rat model are expected to provide further insight into the etiology of human breast cancer.

References

1. Beckmann,M.W., Niederacher,D., Schnürch,H.-G., Gusterson,B.A. and Bender,H.G. (1997) Multistep carcinogenesis of breast cancer and tumor heterogeneity. *J. Mol. Med.*, **75**, 429–439.
2. Ingvarsson,S. (1999) Molecular genetics of breast cancer progression. *Semin. Cancer Biol.*, **9**, 277–288.
3. Perou,C.M., Sørlie,T., Eisen,M.B. *et al*. (2000) Molecular portraits of human breast tumors. *Nature*, **406**, 747–752.
4. Kerangueven,F., Noguchi,T., Coulier,F. *et al*. (1997) Genome-wide search for loss of heterozygosity shows extensive genetic diversity of human breast carcinomas. *Cancer Res.*, **57**, 5469–5474.
5. Dangond,F. (2000) Chips around the world: proceedings from the Nature Genetics microarray meeting. *Physiol. Genomics*, **2**, 53–58.
6. Liotta,L. and Petricoin,E. (2000) Molecular profiling of human cancer. *Nature Rev.*, **1**, 48–56.
7. Perou,C.M., Jeffrey,S.S., van de Rijn,M. *et al.* (1999) Distinctive gene expression patterns in human mammary epithelial cells and breast cancer. *Proc. Natl Acad. Sci. USA*, **96**, 9212–9217.
8. Sørlie,T., Perou,C.M., Tibshirani,R. *et al.* (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl Acad. Sci. USA*, **98**, 10869–10874.
9. Russo,J., Gusterson,B.A., Rogers,A.E., Russo,I.H., Wellings,S.R. and van Zwieten,M.J. (1990) Biology of disease. Comparative study of human and rat mammary tumorigenesis. *Lab. Invest*. **62**, 244–278.
10. Huggins,C., Grand,L.C. and Brillantes,F.P. (1961) Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature* **189**, 204–207.
11. Snyderwine,E.G. (1999) Dietary heterocyclic amines and breast cancer. *Women Cancer*, **1**, 21–29.
12. Sinha,R., Gustafson,D.R., Kulldorff,M., Wen,W., Cerhan,J.R. and Zheng,W. (2000) 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, a carcinogen in high-temperature cooked meat and breast cancer risk. *J. Natl Cancer Inst.*, **92**, 1352–1353.
13. Ghoshal,A., Preisegger,K.H., Takayama,S., Thorgeirsson,S.S. and Snyderwine,E.G. (1994) Induction of mammary tumors in female Sprague–Dawley rats by the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine and effect of dietary fat. *Carcinogenesis*, **15**, 2429–2433.
14. Snyderwine,E.G., Thorgeirsson,U.P., Venugopal,M. and Roberts-Thomson,S.J. (1998) Mammary gland carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in Sprague–Dawley rats on high- and low-fat diets. *Nutr. Cancer*, **31**, 160–167.
15. Ushijima,T., Kakiuchi,H., Makino,H. *et al*. (1994) Infrequent mutation of Ha-*ras* and p53 in rat mammary carcinomas induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. *Mol. Carcinogen.*, **10**, 38–44.
16. Davis,C.D. and Snyderwine,E.G. (1995) Analysis of EGFR, TGF-a, neu and c-myc in 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-induced mammary tumors using RT–PCR. *Carcinogenesis*, **16**, 3087–3092.
17. Toyota,M., Ushijima,T., Weisburger,J.H., Hosoya,Y., Canzian,F., Rivenson,A., Imai,K., Sugimura,T. and Nagao,M. (1996) Microsatellite instability and loss of heterozygosity on chromosome 10 in rat mammary tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. *Mol. Carcinogen.*, **15**, 176–182.
18. Roberts-Thomson,S.J. and Snyderwine,E.G. (1997) Effect of dietary fat on codon 12 and 13 Ha-*ras* gene mutations in 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine- induced rat mammary gland tumors. *Mol. Carcinogen.*, **20**, 348–354.
19. Roberts-Thomson,S.J. and Snyderwine,E.G. (2000) Characterization of peroxisome proliferator-activated receptor alpha in normal rat mammary gland and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-induced mammary gland tumors from rats fed high and low fat diets. *Toxicol. Lett.*, **118**, 79–86.
20. Watanabe,N., Okochi,E., Hirayama,Y *et al*. (2001) Single nucleotide instability without microsatellite instability in rat mammary carcinomas. *Cancer Res.*, **61**, 2632–2640.
21. Hokaiwado,N., Asamoto,M., Cho,Y.-M., Imaida,K. and Shirai.T. (2001) Frequent c-Ha-*ras* gene mutations in rat mammary carcinomas induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. *Cancer Lett.*, **163**, 187–190.
22. Yu,M., Ryu,D.-Y. and Snyderwine,E.G. (2000) Genomic imbalance in rat mammary gland carcinomas induced by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. *Mol. Carcinogen.*, **27**, 76–83.
23. Christian,A., Snyderwine,E.G. and Tucker,J.D. (2002) Comparative genomic hybridization analysis of PhIP-induced mammary carcinomas in rats reveals a cytogenetic signature. *Mutat. Res*. (in press)
24. Sgambato,A., Han,E.K.-H., Zhang,Y., Moon,R.C., Santella,R.M. and Weinstein,I.B. (1995) Deregulated expression of cyclin D1 and other cell cycle-related genes in carcinogen-induced rat mammary tumors. *Carcinogenesis*, **16**, 2193–2198.
25. Lu,J., Pei,H., Kaeck,M. and Thompson,H.J. (1997) Gene expression changes associated with chemically induced rat mammary carcinogenesis. *Mol. Carcinogen.*, **20**, 204–215.
26. Jang,T.J., Kang,M.S., Kim,H., Kim,D.H., Lee,J.I. and Kim,J.R. (2000) Increased expression of cyclin D1, cyclin E and $p21^{Cip1}$ associated with decreased expression of $p21^{Kip1}$ in chemically induced rat mammary carcinogenesis. *Jpn. J. Cancer Res.*, **91**, 1222–1232.
27. Trombino,A.F., Near,R.I., Matulka,R.A. *et al.* (2000) Expression of the aryl hydrocarbon receptor/transcription factor (AhR) and AhR-regulated CYP1 gene transcripts in a rat model of mammary tumorigenesis. *Breast Cancer Res. Treat.*, **63**: 117–131.
28. Kim,D.W., Sovak,M.A., Zanieski,G. *et al.* (2000) Activation of NF-kB/Rel occurs early during neoplastic transformation of mammary cells. *Carcinogenesis* **21**, 871–879.
29. Huang,R.Y. and Ip.,M.M. (2001) Differential expression of integrin mRNAs and proteins during normal rat mammary gland development and in carcinogenesis. *Cell Tissue Res.*, **303**, 69–80.

30. Wang,Y., Hu,L., Yao,R., Wang,M., Crist,K.A., Grubbs,C.J., Johanning,G.L., Lubert,R.A. and You,M. (2001) Altered gene expression profile in chemically induced mammary adenocarcinomas and its modulation by an aromatase inhibitor. *Oncogene*, **20**, 7710–7721.
31. Russo,J. and Russo,I.H. (2000) Atlas and histologic classification of tumors of the rat mammary gland. *J. Mammary Gland Biol. Neoplasia*, **5**, 187–200.
32. Guo,Q.M., Malek,R.L., Kim,S. *et al.* (2000) Identification of c-*myc* responsive genes using rat cDNA microarray. *Cancer Res*., **60**, 5922–5928.
33. Topper,Y.J. and Freeman,C.S. (1980) Multiple hormone interactions in the developmental biology of the mammary gland. *Physiol. Rev*., **60**, 1049–1106.
34. Henninghausen,L. and Robinson,G.W. (1998) Think globally, act locally: the making of a mouse mammary gland. *Genes Dev*., **12**, 449–455.
35. Barnes,D.M. and Gillett,C.E. (1998) Cyclin D1 in breast cancer. *Breast Cancer Res. Treat*., **52**, 1–15.
36. De Jong,J.S., Van Diest,P.J., Valk,P.V.D. and Baak,J.P.A. (1998) Expression of growth factors, growth inhibiting factors and their receptors in invasive breast cancer. I: an inventory in search of autocrine and paracrine loops. *J. Pathol*., **184**, 44–52.
37. De Jong,J.S., Van Diest,P.J., Valk,P.V.D. and Baak,J.P.A. (1998) Expression of growth factors, growth inhibiting factors and their receptors in invasive breast cancer. II: correlations with proliferation and angiogenesis. *J. Pathol*., **184**, 53–57.
38. Curmi,P.A., Noguès,C., Lachkar,S., Carelle,N., Gonthier,M.-P., Sobel,A., Lidereau,R. and Bièche,I. (2000) Overexpression of stathmin in breast carcinomas points out to highly proliferative tumors. *Br. J. Cancer*, **82**, 142–150.
39. Chen,L., O'Bryan,J.P., Smith,H.S. and Edison,Liu. (1990) Overexpression of matrix Gla protein mRNA in malignant human breast cells: isolation by differential cDNA hybridization. *Oncogene*, **5**, 1391–1395.
40. Hough,C.D., Cho,K.R., Zonderman,A.B., Schwartz,D.R. and Morin,P.J. (2001) Coordinately up-regulated genes in ovarian cancer. *Cancer Res*., **61**, 3869–3876.
41. Wang,S., Nath,N., Adlam,M. and Chellappan,S. (1999) Prohibitin, a potential tumor suppressor, interacts with RB and regulates E2F function. *Oncogene*, **18**, 3501–3510.
42. Jupe,E.R., Badgett,A.A., Neas,B.R., Craft,M.A., Mitchell,D.S., Resta,R., Mulvihill,J.J., Aston,C.E. and Thompson,L.F. (2001) Single nucleotide polymorphism in prohibitin 3′ untranslated region and breast-cancer susceptibility. *Lancet*, **357**, 1588–1589.
43. Hardwick,M., Fertikh,K., Culty,M., Li,H., Vidic,G. and Papadopoulos,V. (1999) Peripheral-type benzodiazepine receptor (PBR) in human breast cancer: correlation of breast cancer cell aggressive phenotype with PBR expression, nuclear localization and PBR-mediated cell proliferation and nuclear transport of cholesterol. *Cancer Res*., **59**, 831–842.
44. Watson,C.J. and Miller,W.R. (1995) Elevated levels of members of the STAT family of transcription factors in breast carcinoma nuclear extracts. *Br. J. Cancer*, **71**, 840–844.
45. Yu,M., Jones,M.L., Gong,M., Sinha,R., Schut,H.A.J. and Snyderwine,E.G. (2002) Mutagenicity of 2-amino-1-methyl-6-phenylimidaz[4,5-*b*]pyridine (PhIP) in the mammary gland of Big Blue rats on high and low fat diets. *Carcinogenesis* **23**, 877–884.
46. Manjanatha,M.G., Shelton,S.D., Culp,S.J., Blankenship,L.R. and Casciano,D.A. (2000) DNA adduct formation and molecular analysis of *in vivo lacI* mutations in the mammary tissue of Big Blue rats treated with 7, 12-dimethylbenz[*a*]anthracene. *Carcinogenesis*, **21**, 265–273.
47. Vonderhaar,B.K. (1999) Prolactin involvement in breast cancer. *Endocr. Relat. Cancer*, **6**, 389–404.
48. Bromberg,J. (2000) Signal transducers and activators of transcription as regulators of growth, apoptosis and breast development. *Breast Cancer Res*. **2**, 86–90.
49. Liu,X., Robinson,G.W. and Hennighausen,L. (1996) Activation of STAT5a and STAT5b by tyrosine phosphorylation is tightly linked to mammary gland differentiation. *Mol. Endocrinol*., **10**, 1496–1501.
50. Humphreys,R.C. and Henninghausen,L. (1999) Signal transducer and activator of transcription 5a influences mammary epithelial cell survival and tumorigenesis. *Cell Growth Diff*., **10**, 685–694.
51. Liu,X., Robinson,G.W., Wagner,K.-U., Garrett,L., Wynshaw-Boris,A. and Henninghausen,L. (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev*., **11**, 179–186.
52. Matsumura,I., Kitamura,T., Wakao,H., Tanaka,H., Hashimoto,K., Albanese,C., Downward,J., Pestell,R.G. and Kanakura,Y. (1999) Transcriptional regulation of the cyclin D1 promoter by STAT5: its involvement in cytokine-dependent growth of hematopoietic cells. *EMBO J*., **18**, 1367–1377.
53. Socolovsky,M., Fallon,A.E.J., Wang,S., Brugnara,C. and Lodish,H.F. (1999) Fetal anemia and apoptosis of red cell progenitors in STAT5a$^{-/-}$ 5b$^{-/-}$ mice: a direct role for Stat5 in Bcl-X_L induction. *Cell*, **98**, 181–191.
54. Bowman,T., Garcia,R., Turkson,J. and Jove,R. (2000) STATs in oncogenesis. *Oncogene*, **19**, 2474–2488.

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