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Gene expression profiling of mammary glands at an early stage of DMBA-induced carcinogenesis in the female Sprague-Dawley rat

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Summary. *Background:* Breast cancer is the most common cause of cancer death among women worldwide and the second leading cause of tumor-related death for women in westernized countries. Most research efforts to find a breast cancer biomarker have focused on the stage after the cancer is diagnosed. To investigate more deeply into mammary cancer prevention, a study of precancerous lesion development seems a priority. Experimentally-induced mammary tumors in rats constitute a powerful tool for studying the pathogenesis of this cancer and the molecular mechanisms involved in neoplastic progression. Furthermore, *in vivo* experimental animal models provide information not otherwise available in human populations. 7,12-dimethylbenz[a] anthracene (DMBA) induced rat mammary carcinomas have several similarities with human breast cancers including: histopathology, origination in the ductal epithelial cells, and hormone dependence. To better understand the molecular events associated with mammary carcinogenesis, we used a time-course high throughput gene expression approach on a DMBA-induced mammary cancer model to identify the early precancerous events as well as new potential diagnostic biomarkers. *Materials and Methods:* Twelve 7 wk-old virgin female Sprague-Dawley rats were randomized into 2 experimental groups: 1) DMBA-treated (40 mg/kg b.w. by intragastric administration (i.g.) in corn oil as the vehicle and 2) treated with corn oil (vehicle) by ig. At 2 and 4 weeks after DMBA administration, 3 animals randomly chosen from each experimental group were sacrificed and necropsied. Total RNA was extracted and the global gene expression patterns from the mammary gland and liver samples collected were used to identify the molecular profile of the precancerous stage genome. Significantly altered genes as evinced by multivariate data analysis were further confirmed by quantitative real time PCR and siRNA knockdown assays. *Results and Discussion:* Genes involved in cancer progression, migration, proliferation and oxidative stress were identified in this study. MARK, *Wnt* and Jak-STAT pathway signaling, known to play a major role at the precancerous stage, were also identified. Two novel less known cancer progression/proliferation related genes, *Pcbd1* and *Ppil1*, upregulated in both liver and mammary glands, were also identified.

Key words: DMBA, gene expression profiling, mammary carcinogenesis, early events, Sprague-Dawley rats, *in vivo* models

«Analisi del profilo dell'espressione genica della ghiandola mammaria nella fase iniziale del processo cancerogenetico indotto da DMBA nella ratta Sprague-Dawley»

Riassunto. *Background:* Il cancro al seno è la causa più comune di morte per cancro tra le donne di tutto il mondo e la seconda causa di morte per tumore per le donne nei paesi occidentalizzati. La maggior parte degli

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sforzi nell'ambito della ricerca scientifica sono indirizzati alla scoperta di biomarcatori dopo la diagnosi. Per indagare più a fondo sulla prevenzione del cancro della mammella, si ritiene prioritario un più approfondito studio sullo sviluppo delle lesioni precancerose. Il modello sperimentale *in vivo* dei tumori mammari indotti in ratti costituisce un potente strumento per lo studio della patogenesi di questo tumore e dei meccanismi molecolari coinvolti nella progressione neoplastica. I modelli animali sperimentali *in vivo*, inoltre, forniscono informazioni non altrimenti disponibili nella popolazione umana. I tumori indotti chimicamente nel ratto con 7,12-dimetilbenz[a]antracene (DMBA) hanno molteplici analogie con tumori al seno umani, tra cui: istopatologia, la sede di origine nelle cellule epiteliali del dotto e l'ormone-dipendenza. Per comprendere meglio gli eventi molecolari della carcinogenesi mammaria, abbiamo usato il modello DMBA associato ad uno studio *time-course high throughput gene expression* per identificare gli eventi biomolecolari precoci del processo neoplastico e nuovi potenziali biomarcatori diagnostici. *Materiali e Metodi:* Dodici ratte Sprague-Dawley di 7 settimane di età sono state randomizzate in due gruppi sperimentali in base al trattamento: 1) trattate con DMBA (via somministrazione intragastrica di 40 mg/kg di peso corporeo) in olio di mais come veicolo e 2) trattate con olio di mais. A 2 e 4 settimane dopo la somministrazione del DMBA, 3 animali scelti *at random* da ciascun gruppo sperimentale, sono stati sacrificati e sottoposti a necroscopia. L'RNA totale è stato estratto e le alterazioni dell'espressione genica globale di campioni di ghiandola mammaria e del fegato sono state utilizzate per l'identificazione del profilo molecolare del genoma nella fase precancerosa. I geni significativamente alterati sono stati selezionati dall'analisi multivariata dei risultati ed ulteriormente confermati mediante indagini di real time PCR e siRNA. *Risultati e Discussione:* Lo studio ha consentito l'identificazione di geni coinvolti nella progressione del cancro, la migrazione, e stress ossidativo. Inoltre sono stati identificati i percorsi molecolari MARK, *Wnt* e Jak-STAT, noti per avere un ruolo importante nella fase precancerosa. Due geni meno conosciuti, correlati a processi di progressione del cancro e proliferazione, *Pcbd1* e *Ppil1*, sono stati ritrovati iper-espressi sia nel fegato che nella mammella.

Parole chiave: DMBA, profilo dell'espressione genica, cancerogenesi mammaria, eventi precoci, ratti Sprague-Dawley, modelli *in vivo*

Abbreviations:

Ahr	Aryl Hydrocarbon Receptor
ATP	Adenosine triphosphate
DAVID	Database for Annotation, Visualization and Integrated Discovery
DMBA	7,12-dimethylbenz[a]anthracene
GAPD	GlycerAldehyde-3-Phosphate Dehydrogenase
GCOS	GeneChip Operating System
Gss	Glutathione synthetase
HIF1-α	hypoxia-inducible factor 1 alpha subunit
Idi1	Isopentenyl-diphosphate delta isomerase
IPA	Ingenuity Pathway Analysis
Jak-STAT	Janus kinase-Signal Transducer and Activator of Transcription
MAPK	Mitogen-activated protein kinase
MDS	Multidimensional Scaling
Mif	Macrophage migration inhibitory factory
NMU	N-nitrosourea
NADPH	Nicotinamide adenine dinucleotide phosphate
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
Nnt	Nicotinamide nucleotide transhydrogenase
Nt5e	5' Nucleotidase, ecto (CD73)
PAHs	polycyclic aromatic hydrocarbons
PCA	principal component analysis
Phgdh	3-Phosphoglycerate dehydrogenase
PIN	Prostatic Intraepithelial Neoplasia
Pla2g2a	Phospholipase A2, group IIA
Ppil1	Peptidylprolyl isomerase (cyclophilin)-like 1
Pcbd1	Pterin 4 alpha carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1) 1
Por	P450 (cytochrome) oxidoreductase
RPL18	Ribosomal Protein L18
SD	Sprague-Dawley
siRNA	Small interference RNA
SNW1/SKIP	SNW domain containing 1/ tribbles homolog 1 (Drosophila)
Srebf1	Sterol regulatory element binding factor 1
Wnt	Wingless-type

Introduction

Breast cancer is the commonest cause of cancer death among women worldwide and the second leading cause of tumor-related death for women in industrialized and westernized countries (1). Apart from cancer of the skin, breast cancer is the most prevalent cancer among US women, accounting for 29% of newly diagnosed cancers (2). About 1 in 8 (12%) US women will develop invasive breast cancer during their lifetime; the American Cancer Society estimated about 231,840 new cases of invasive breast cancer diagnosed for 2015 (3, 4). Despite the plethora of readily available information, about 40,290 women in the U.S. are expected to die for breast cancer every year and full understanding of the mechanisms of mammary tumorigenesis is not yet possessed (3, 4).

Major improvements have been made in early detection and treatment of breast cancer in recent years. Indeed, although global breast cancer incidence rates continued to increase through the late 1990s, breast cancer deaths over the past 25 years have been stable or slightly decreasing in some North American and European countries. These slight reductions have been attributed to advances in the breast cancer awareness campaign, progress in techniques for earlier detection and improved treatments (2). In contrast, mortality rates continue to increase in many Asian countries such as Japan and Korea, most likely due to lifestyle changes associated with westernization and the delayed introduction of effective breast cancer screening programs (5).

The majority of the breast cancer biomarker discovery studies have focused on the stage after the cancer has been diagnosed. To investigate more deeply into mammary cancer prevention, study of precancerous lesion development seems to be a priority. Early breast cancer detection will not prevent tumors, but it can help find them when the likelihood of successful treatment is greatest. More than 90% of women diagnosed with breast cancer at the earliest stage survive their disease for at least 5 years compared to around 15% of women diagnosed with the most advanced stage of disease (6). Earlier diagnosis would enhance survival, provide better quality of life for cancer patients and reduce medical costs. Improving our knowledge of the molecular pathways involved in the early events of the mammary carcinogenesis process would anticipate identification of the pre-neoplastic lesions and open the door to more specific, targeted therapeutic treatment (7).

Molecular analysis of pre-invasive breast disease, and in particular differential gene expression analysis by microarrays, in the last decade has proved an extremely useful tool in biomedical and clinical research for improving knowledge and clarifying the multistep model of breast carcinogenesis (8, 9). Efforts focusing on the analysis of genetic alterations of pre-invasive breast cancer in women have provided new insights in the biology of such lesions in women. Ductal carcinoma *in situ* shares many molecular genetic alterations with invasive carcinoma and is for this reason considered a pre-invasive stage of malignant lesions in women (8-11). Epidemiological or clinical studies require large numbers of people involved and long years of observation. For this reason, the use of an experimental model that mimics the human situation may provide important information that is not otherwise available in the human population (12-14). Comprehensive molecular profiling of *in situ* ductal carcinoma has been partly limited by the small size of such lesions as well as the few suitable experimental models available to recapitulate human disease, i.e. xenograft or genetic modified models (9, 15, 16).

Although there are marked anatomical differences in the mammary gland across and within species, its development and function in the female has been considered generally similar across all mammals (17). For this reason, rat mammary gland cancer models have been used for many years and are considered well suited to studying *in situ* and invasive lesions and breast cancer mechanisms in general (18, 19). In particular chemically induced rat mammary gland adenocarcinomas represent one of the most valuable and useful models for this purpose (12-14, 19). Chemically induced rat mammary gland adenocarcinomas are similar to human breast cancers in various aspects including histopathology, the origin of such cancers from ductal epithelial cells, and the dependency on ovarian hormones for tumor development (13, 18-22).

The two most widely used active chemical inductors of mammary carcinogenesis in female rats are 7,12-dimethylbenz[a]anthracene (DMBA) and N-

methylnitrosurea (NMU). The more commonly used of the two is DMBA. The susceptibility of the rat mammary gland to DMBA is strongly age-dependent and is maximal when carcinogens are administered to virgin females between 40 and 60 days of age (12, 20).

Rodents have been widely used to investigate mammary gland carcinogenesis (23-25) and the DMBA-induced mammary gland adenocarcinoma model in female Sprague-Dawley rats is commonly utilized due to its similarity with human breast cancer development (13, 18, 20-22). Exposure to 40 mg/kg b.w. of DMBA in 7 week-old female virgin Sprague-Dawley rats induces hormone-dependent mammary gland cancer in nearly 100% of animals treated (18, 20, 26-28). DMBA is in the class of polycyclic aromatic hydrocarbons (PAHs) which are a widespread and diverse class of environmental pollutants that are genotoxic and known to be important initiators of lung, breast, colon, and other types of cancers (29). The early steps in mammary DMBA tumorigenesis, as well as other PHAs, involve the aryl hydrocarbon receptor (AhR), which activates the signal transduction pathway cascade that leads to AhR-dependent induction of cytochromes P450s which in turn mediate the transformation of DMBA into its mutagenic epoxide intermediate which readily forms DNA adduct, triggering DNA damage and causing mammary transformation (13, 21). It has been demonstrated that DMBA causes mammary cancer by damaging the highly proliferating undifferentiated cells of the terminal end buds, the most immature structure in the mammary gland at the time of carcinogen administration (13, 28).

Exposure to PAHs, it has been suggested, has an important role in late-onset breast cancer in the United States (30). PAHs are formed by the incomplete combustion of coal, oil and gas, as well as grilled meats, tobacco smoke and other substances. For these reasons people's occupational and living environment are the two major sources of human exposure to PAHs. Active and passive smokers, coke oven workers, firefighters and foundry workers are found to be exposed to high levels of PAHs and several studies have been done to discover biomarkers for such exposure (31-35).

Recent studies have employed the gene expression profiling technique to identify potential breast cancer diagnostic and/or prognostic markers. Unfortunately, most published data have focused on either the terminal cancer signature or perinatal exposure. In this study, we used a high throughput gene expression profiling technique to identify the transcriptome in the early events of the rat mammary neoplastic process induced by DMBA.

The aim of the study focused on identification of the specific genes involved in early events in mammary carcinogenesis induced by DMBA in the rat model, as well as study of cancer progression biology for the detection of potential early tumor biomarkers. Furthermore, since DMBA like other PHAs is metabolized by cytochromes P450s in the liver, the principal site of drug metabolism, it was decided to evaluate gene expression profile analysis of the liver together with the mammary gland.

Materials and methods

Animals, treatment and sample collection

Virgin Female Sprague-Dawley (SD) rats were purchased for this experiment from Charles River (Charles River, Frederick, MD, USA) and housed at the animal facility of the Laboratory Animal Sciences Program at NCI in Frederick, MD, USA. After one week of acclimatising inside the animal facility, the rats (106 animals for the whole experiment) were uniquely identified by ear punch (by Jackson Laboratory procedures) and randomly distributed in 2 experimental groups described as follows: (1) treated by gavage with DMBA dissolved in corn oil, and (2) receiving corn oil (vehicle) alone. All animals were provided with NIH Lab Chow (Diet D12450B, Research Diets Inc., New Brunswick, New Jersey) and water *ad libitum* and housed (two *per* cage) on a 12 hour light and dark cycle. All the animals were clinical examined once a week for palpable mammary tumors and the incidence was recorded weekly as from administration of DMBA (beginning of the experiment). Mean feed and water consumption and body weight were measured and recorded weekly from the beginning of the experiment. Mammary gland malignant tumors were chemically induced by gavage of a single dose of 40 mg/kg b.w. DMBA (Sigma-Aldrich, St. Louis, MO, USA) at

7 weeks old (day 0). The original protocol included 6 interim sacrifice time points from DMBA administration to the following 23 weeks and a total of 106 female rats included. Interim sacrifices were performed at 2, 4, 6, 8, 13 and 18 weeks after the beginning of the study, in which 7 animals randomly chosen from each experimental group were euthanized by CO_2 overexposure and submitted to partial necropsy. The present report deals with study results concerning mammary and liver gene expression analysis in a limited number of animals (No. 3) sacrificed at the first two time points (2 and 4 weeks after from DMBA administration) of the whole time-course study together with their age-matched controls. It was decided to analyze samples harvested at the first two time-points because the aim of the study was identify the earliest genes altered in mammary carcinogenesis.

Two weeks (T2) and 4 weeks (T4) after DMBA administration, three animals were euthanized by CO_2 overexposure and mammary gland and liver samples were collected. In particular, the mammary glands were microdissected using an optic microscope and any lymph nodes or excess fat tissue were discarded. Age-matched vehicle controls were designated as N2 (week-2) and N4 (week-4) respectively. All specimens were collected using RNA-free precautions. All the tissues were snap-frozen in liquid nitrogen at the time of necropsy and stored at -80°C prior to RNA extraction. The study was performed under an approved animal study protocol (ASP# 05.081.MI, Sept 22nd, 2005), and animal care was in accord with NCI Animal Care and Use Committee guidelines.

RNA isolation

Total RNA from left axillary mammary gland and liver samples were isolated by acid guanidinium isothiocyanate–phenol–chloroform extraction (TRIzol Reagent, Invitrogen, Rockville, MD, USA) (1 ml of solution *per* 100 mg of tissue) and further purified by the RNeasy Midi Cleanup Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturers' protocols. Total RNA concentrations were determined by spectrophotometric evaluation of absorbance at 260 nm, and RNA quality and integrity were confirmed by 260/280 nm ratio (Nanodrop2000, UV-Vis Spectrophotometer, Thermo Scientific, Wilmington, DE, USA) and 1% agarose RNA gel electrophoresis respectively. The RNA quality of random samples was also tested on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Microarray assay

The microarray assay was performed using the Affymetrix GeneChip® Rat Genome 230 2.0 (Santa Clara, CA, USA) Array platform which is able to analyze the expression level of approximately 31,000 (Affymetrix probesets) rat genes. The manufacturers' recommended protocols were followed for probe synthesis, hybridization, and scanning of arrays as described in Takahashi (36) and Padovani (37). The signal values and detection calls of the probe sets were determined using Affymetrix GCOS (ver. 1) software. Relative intensity variation across arrays was normalized by scaling to an average signal level of 500 counts, excluding low 2% and high 2% signals.

Multivariate analysis

GeneChip data were exported to BRB-array Tools (38) for multivariate statistical analysis. A non-supervised learning hierarchical clustering approach was chosen for data set exploration. Various linkage methods and distance measuring algorithm combinations were utilized to cluster the samples to ensure the presence of a constant dendrogram (clustering pattern). Multidimensional Scaling (MDS) analysis was also performed, allowing us to visually analyze the data set in a hyperdimensional view.

Class comparison analysis

The class comparison module in BRB-ArrayTools (38) was utilized in order to identify genes that significantly altered their expression levels at both time points between control and DMBA-treated mammary gland samples. Microarray data from control (vehicle) and DMBA-treated samples from two time points (week 2 and week 4) were subjected to a two-sample *t*-test (with a random variance model). Type I error correction was adopted for all pairwise comparisons.

Pathway and ontology analyses

Gene lists winnowed from the multivariate analysis were exported to Ingenuity Pathway Analysis (IPA) (39) and DAVID (40) for gene ontology and pathway analysis.

Quantitative real time polymerase chain reaction

Total RNA from mammary glands and liver were reverse-transcribed into cDNA by PowerScript cDNA synthesis strip (Clontech, Mountain View, CA, USA) following the standard protocol recommended by the manufacturer. Primer pairs for selected target genes (Table 1) were designated with RefSeq (NIH) sequences by Primer3 (41). All Real Time PCRs were designed to follow a universal real time PCR condition: 94°C 2 min, 45 cycles of 94°C 30 sec and 60°C 30 sec with IQ SYBR Green Supermix reagent (Bio-Rad, Hercules, CA, USA) or TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Specificity of the amplicons was checked on a 2.2% agarose gel (FlashGel System, Lonza, Rockland, ME, USA) to make sure there were no non-specific amplicons or formation of primer-dimer. The delta-delta-Ct approach was adopted for relative expression calculations. Amplification efficiencies for all primers were checked against the housekeeping genes (GAPD and RPL18) to ensure that signals were detected at the PCR exponential phase and all primers had similar amplification efficiencies.

Cell culture

SD rat mammary gland adenocarcinoma cell line NMU and SD rat normal liver cell line Clone 9 were obtained from the America Type Culture Collection (Manassas, VA, USA). NMU cells were incubated in F-12K medium (ATCC) with 10% fetal calf serum and Clone 9 cells were incubated in Eagle's minimum essential medium (ATCC) with 10% fetal calf serum. Both cell lines were incubated at 37°C with 5% CO_2. Cells were washed with 1X PBS before being harvested with trypsin. Cell densities were counted using a hematocytometer and visually examined to ensure the confluency was below 70%.

RNA interference

Small interference RNA (siRNA) induced gene knockdown for the *Ppil1* (ID# s159959, s159960 & s159958) and *Pcbd1* (ID# s132049, s132047 & s132048) genes were obtained from Applied Biosystems (Foster City, CA, USA). Both genes were knocked down by a pool of three pre-designed Select siRNAs (Applied Biosystems, Foster City, CA, USA) to increase the efficiency. All siRNA assays were run in triplicate and included Positive Control siRNA (*Gapd*) (Applied Biosystems, Foster City, CA, USA), Negative Control siRNA (Applied Biosystems, Foster City, CA, USA), a transfection reagent only for Negative Controls, and an untreated (medium only)

Table 1. Primer Sequences. Real time PCR primer pair sequences. Primer pair designs are based on the RefSeq sequences (download from NCBI, NIH) and designed with Primer3 (MIT). All amplicons are checked on gel electrophoresis to ensure no non-specific amplification. Amplification efficiency of all genes was measured and it was made sure they were comparable to housekeeping genes prior to the relative expression level calculation.

Gene Symbol	Forward Primer	Reverse Primer
GAPD	AAGGGCTCATGACCACAGTC	GGATGCAGGGATGATGTTCT
RPL18	AAGATCCTGACCTTCGACCA	GGACATAGGGTTTGGTGTGG
GSS	AGCTGGCTGGCACTAAGAAG	TCTTCACCCATGTCCAGTGA
MIF	CCATGCCTATGTTCATCGTG	ACGTGCACTGCGATGTACTG
PCBD1	CAGGCTGAGTGCTGAGGAAC	CCAAAAGCCCTGTTGAAGTC
PPIL1	GCTGGAGCTGTACTGGAAGC	ACCTCGACCTGTGCCTGTC
SREBF1	CCTTACACACCCAGGTCCAG	AGTGTGGCTGCAGTACAACG
IDI1	ACTGGCAGGAGTGATTGGAT	GAACACAGCGATTCCAGACA
NNT	ACACTGTCTGGGGAGTGACC	CCAACTGCAGTCAAACCTGA
NT5E	TGAAGATGCAGCCATCAAAG	AGGTTTCCCATGTTGCACTC
PHGDH	CATCCAGGTGGTGACACAAG	TCTCTCAGAAGGCCGACAAT
PLA2G2A	GCTATGGCTTCTACGGTTGC	TCATGAGTCACACAGCACCA

Control. siRNAs were transfected into the cells with Lipofectamine 200 (Invitrogen, Rockville, MD, USA) according to the manufacturer's protocol. Optimal siRNA conditions were determined by incubating the cells at various siRNA concentrations and cell densities for up to 96 hours in 96-well plates (data not shown). In order to generate sufficient total RNA for the subsequent quantitative real time PCR, up-scaled siRNA assays were performed on 6-well plates.

Cell proliferation assay

The impact from siRNA transfection was determined by measuring the cell growth rate. The cell proliferation rate of NMU and Clone 9 cells were measured by a fluorometric AlamarBlue (Invitrogen, Rockville, MD, USA) assay. Briefly, 5 x 10^3 cells suspended in 100 μl of culture medium with 10% AlamarBlue (V/V) were added to each well of a 96-well plate. siRNA and the controls mentioned above were added 24 hours after seeding. Fluorescent signals were measured at the following time points: 0, 1, 2, 3, 4, 5, 6, 24, 48, 72 and 96 hours.

Gene knockdown efficiency

The *Ppil1* and *Pcbd1* gene knockdown efficiency induced by siRNAs was ascertained by the quantitative real time PCR method mentioned above. Additional TaqMan probes (*Ppil1*: Rn01428073_m1 and *Pcbd1*: Rn01433678_m1 (Applied Biosystems, Foster City, CA, USA) for these two genes were also utilized to determine gene knockdown efficiencies.

Statistical analysis for assays other than microarray

Statistical analyses were performed with Prism ver. 4 (Graphpad Software, La Jolla, CA, USA). Differences between groups' mean values were determined by the unpaired *t*-test and one factor analysis of variance (ANOVA), followed by Fisher protected least significant *post-hoc* analysis for pairwise comparison of means.

Results

Gene expression profiling and clustering analyses in rat mammary gland

We compared the gene expression profiles between age-matched vehicle control (N2-2 week control; N4-4 week control) and DMBA-treated groups (T2-2 week treated and T4-4 week treated) to minimize the background influence such as normal tissue growth and age-related hormonal changes from mammary gland development. One should note that at nine weeks-old rat mammary glands are approaching maturity and are undergoing intensive lobuloalveolar development and differentiation (42).

Non-supervised hierarchical clustering and MDS were used to explore the clusters (Figure 1A, 1B, & 1C) generated by various linkage methods and distance measuring algorithms. Genes having a minimum of 2-fold change and present in over 80% of the samples were submitted for multivariate analysis. Overall, there were approximately 3500 genes (~11%) that passed the filters. For non-supervised hierarchical clustering analysis, 2-week and 4-week samples were always separated into two clusters by various linkage methods (Single, Complete & Average) and combinations of distance-measuring algorithms (Centered Correlation, Uncentered Correlation & Euclidean Distance). It is noteworthy that similar clusters are also found in MDS analysis (Figure 1D). Results from these clusters clearly indicated upon multivariate analysis that mammary gland growth exerts a major influence on the gene expression profile, as is clearly shown in the hierarchical analysis reported in Figure 1.

DMBA altered gene expression in rat mammary gland, analyzed by a microarray experiment

Differentially expressed genes in DMBA-treated groups were filtered and normalized with their age-matched controls: (1) N2 *vs* T2 and (2) N4 *vs* T4. Next step, filtered genes were further adjusted between the two time points, (1) *vs* (2). One thousand and fifty genes significantly and differentially expressed genes were identified by the analysis. Our DAVID pathway analysis on the filtered gene list suggested three ma-

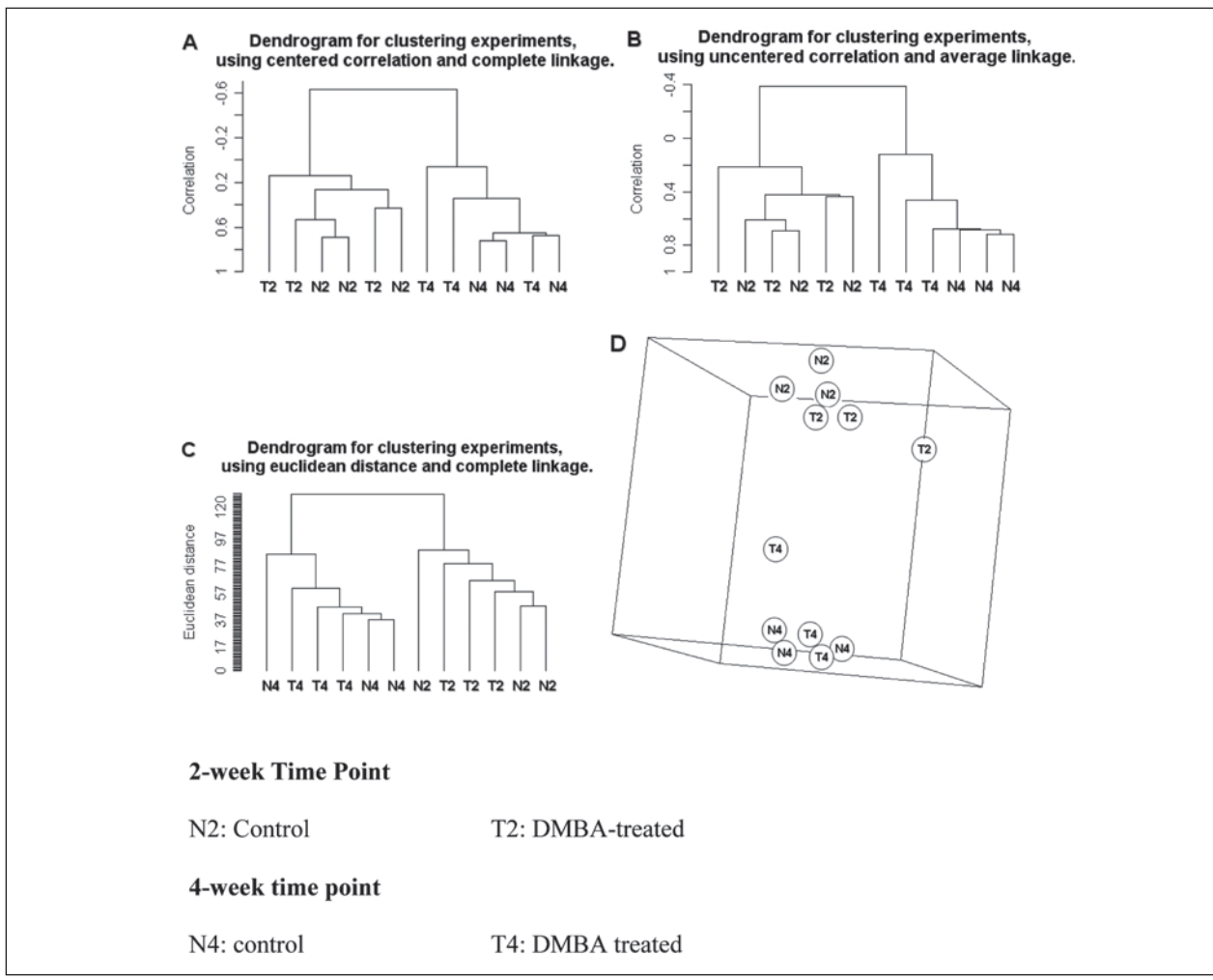

Figure 1. Microarray data: Hierarchical Clustering. Hierarchical Clustering 1A, 1B & 1C. y-axis: relative distance. Samples are clearly separated into 2 clusters according to the time point. Multidimensional Scaling (1D), a 3-dimensional view of the hyper-dimensional data. Samples also clearly separate into two clusters as does hierarchical clustering. T2: 2-week time point DMBA-treated; T4: 4-week time point DMBA-treated, N2: 2-week time point control; N4: 4-week time point control.

jor pathways out of the 51 identified. The three were the following: (1) *MAPK* signaling; (2) *Wnt* signaling and (3) *Jak-STAT* signaling. We also conducted IPA gene networking analysis on the same gene list. *Nnt* and *Srebf1* were found to be associated through Leptin (obesity homologue, mouse) (Figure 2A). *Nt5e* and *Cyp1b1* were associated via *HIF1*-α and *Por* (Figure 2B). Finally, *Mif* and *Ahr* were associated through the cell structure genes' vimentin and lamin A/C (Figure 2C).

Differentially expressed genes at the 2-week stage were identified by Class Comparison analysis. Out of 1050 significantly and differentially expressed genes, 14 genes had a *p*-value lower than 0.001 (Table 2). Genes showing significant up-regulation after DMBA treatment were associated with the following Gene Ontology functions/categories: (1) neurological development and morphology; (2) cellular assembly and organization; (3) cancer; (4) endocrine system disorder; (5) cellular function and maintenance; (6) cell death; (7) cell-to-cell signaling and interaction; (8) genetic disorder; and (9) gene expression. These up-regulated genes are: *Gss*, *Mif*, *Pcbd1*, *Idi1*, *Ppil1*, *Pla2g2a* and *Nt5e*. At the other end, genes down-regulated after DMBA treatment were involved in (1) lipid metabolism; (2) molecular transport; (3) small molecular biochemistry; (4) cellular development; (5) immune sys-

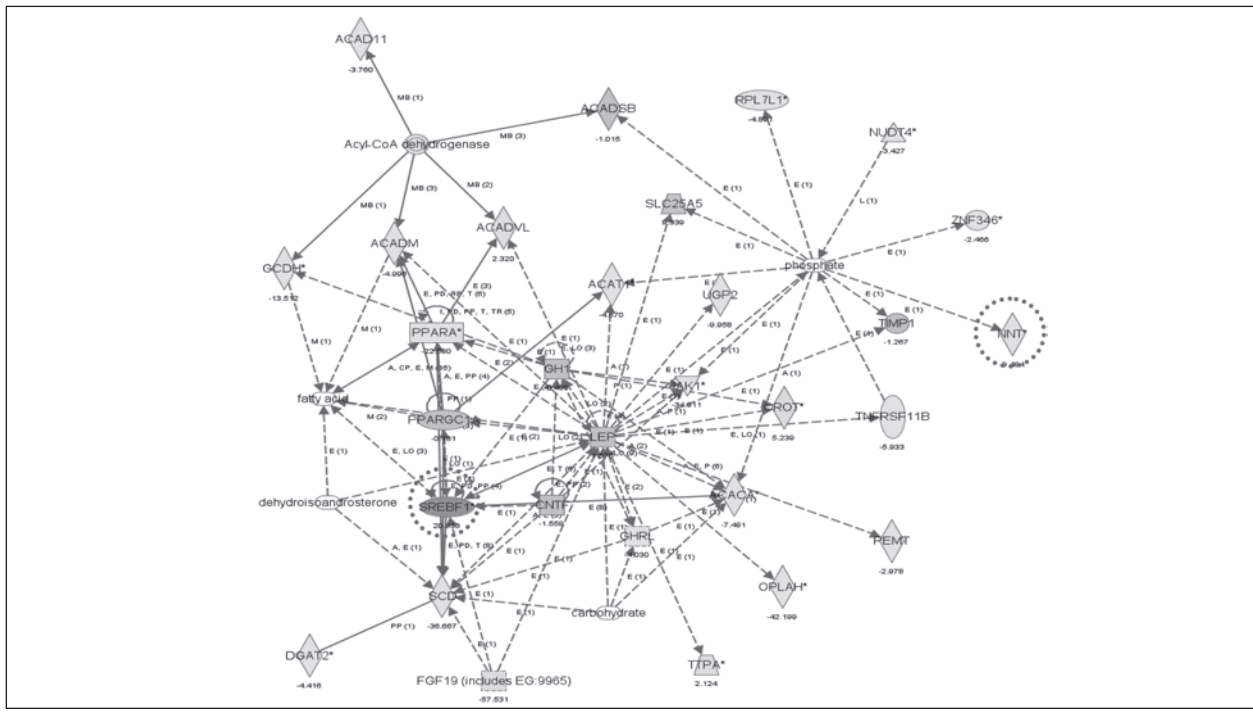

Figure 2A. Microarray data: Pathway Analysis. Ingenuity Pathway Analysis. Significant and differentially expressed genes identified by Class Comparison Analysis were submitted for this gene networking analysis. Here are three representative networks from the analysis. **2A**: indicated that *SREBF1* and *NNT* are associated through the *LEP*.

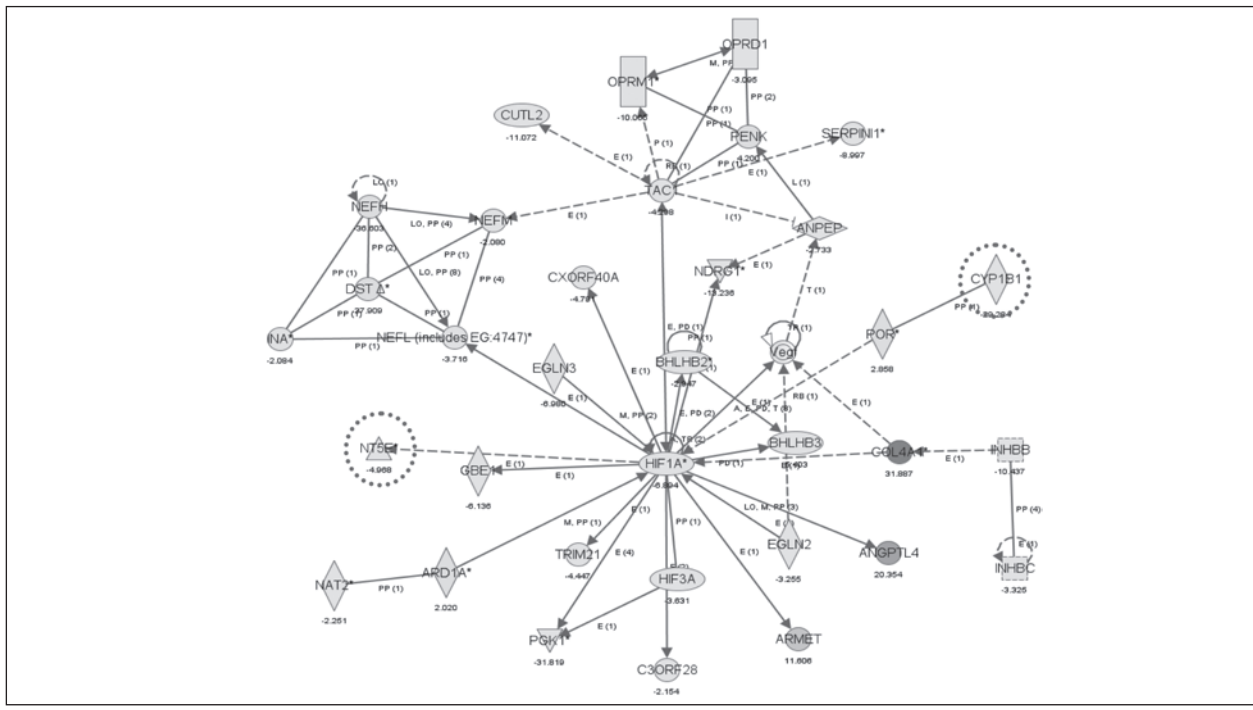

Figure 2B. Microarray data: Pathway Analysis. Ingenuity Pathway Analysis. Significant and differentially expressed genes identified by Class Comparison Analysis were submitted for this gene networking analysis. Here are three representative networks from the analysis. **2B**: the network shows that *CYP1B1* is associated with *NT5E* through *HIF1A* and *POR*.

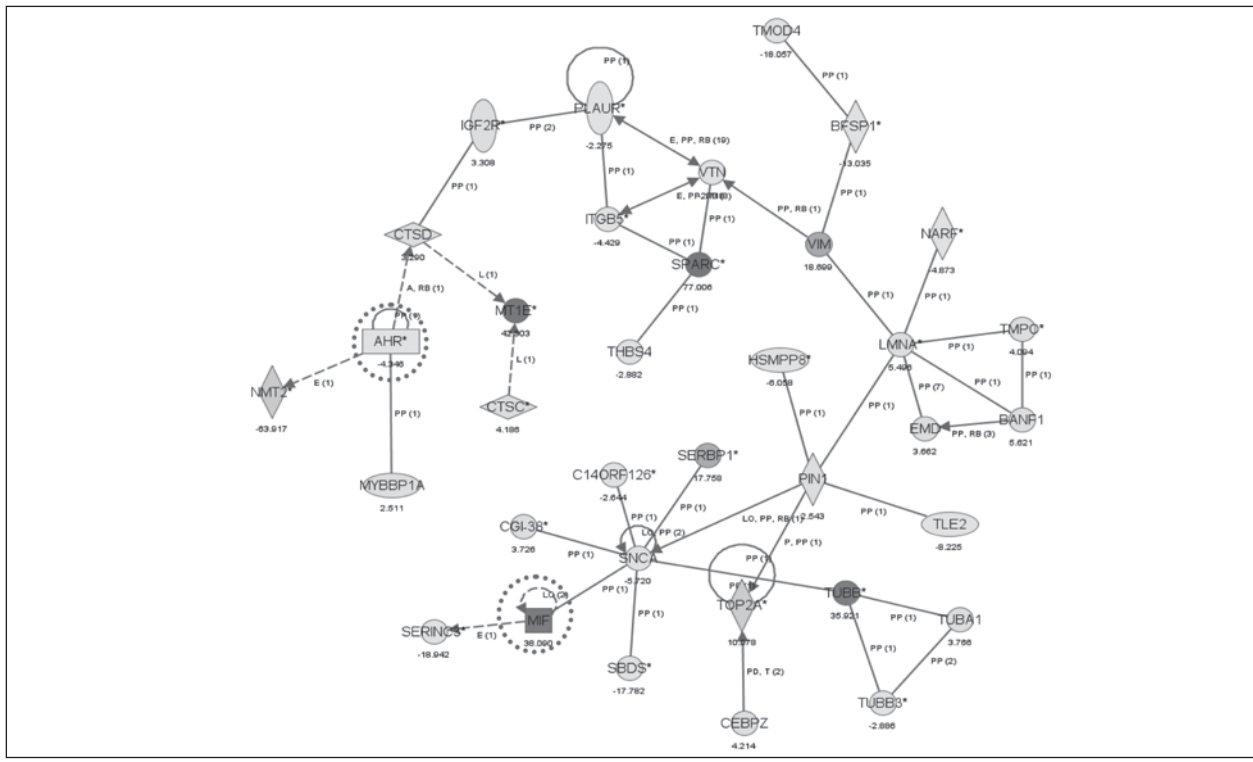

Figure 2C. Microarray data: Pathway Analysis. Ingenuity Pathway Analysis. Significant and differentially expressed genes identified by Class Comparison Analysis were submitted for this gene networking analysis. Here are three representative networks from the analysis. **2C**: *MIF* and *Ahr* are associated via a more complicated network, i.e. *LMNA* and *VIM*.

tem development and function; (6) drug metabolism; (7) endocrine system development; and (8) hematological system development. Genes belonging to this group were: *Nnt*, *Srebf1* and *Phgdh*.

Only 10 (out of 14) genes were identified by Class Comparison Analysis and have annotation available from online databases. Quantitative real time PCR was selected to confirm the alterations in these 10 genes (Figure 3). All primer pairs were specifically designed to eliminate DNA co-amplification by having at least one side of primer binding on an exon-exon junction. Nine genes were shown to have the same expression patterns both in microarray analysis and in real time PCR from the 2-week samples. Moreover, the gene expression pattern results from real time PCR were 100% matched with microarray data from 4-week samples. The small discrepancy between the microarray result and the quantitative real time PCR is probably due to intrinsic differences between the binding sites and/or amplification efficiency inherited from both techniques. Interestingly, half of these 10 genes (*Gss, Mif, Pcbd1, Ppil1 and Phgdh*) have a similar expression pattern in 2-week samples and all are involved in (1) cellular assembly, organization, function and maintenance; (2) cancer; (3) cell death; and (4) gene expression. For the liver samples, since there were no 2-week or 4-week liver microarray data for comparison, we only analyzed the results from the real time PCR. Seven genes in the 2-week and six genes in the 4-week cases showed similar changes in their gene expression as found from mammary gland samples. Notably, transcript levels of *Mif, Pcbd1* and *Ppil1* were elevated while *Phgdh* transcripts had decreased in level among both mammary and liver tissues from both 2-week and 4-week samples.

siRNA knockdown inhibits cell growth and knockdown Ppil1 and Pcbd1 expression

NMU cells (from SD rat mammary gland cells) after 72 hours incubation with siRNAs exhibited a significant slow-down in the growth rate (compared

Table 2. Microarray data: Class Comparison Analysis. Class Comparison Analysis - two-sample t-test: 2-week time point, Control Vs DMBA-treated. The 14 genes identified by Class Comparison Analysis (p-value ≤0.001). The False Discovery Rate (FDR) is also listed for reference. All known gene functions are listed below each gene.

	Gene symbol	Probe set	Description/Function*	Parametric p-value	FDR**
1	Gss	1370365_at	glutathione synthetase	6.64e-05	0.1874132
			Involved in glutathione biosynthesis and sulfur metabolism - provides protection against oxidative stress. Elevated Gss activity inhibits apoptotic signaling pathway after genotoxic exposure.		
2	Mif	1367609_at	macrophage migration inhibitory factor	0.0001567	0.1874132
			A critical proinflammatory cytokine and associated with tumorigenesis and inhibition of apoptosis. Also shown to affect genomic stability and carcinogenesis via the ubiquitin-proteasome system.		
3	Pla2g2a	1368128_at	phospholipase A2, group IIA (platelets, synovial fluid)	0.0002158	0.1935726
			Involved in several inflammatory diseases including Crohn disease, pancreatitis and sepsis. It can suppress gastric adenocarcinoma growth, regulate intestinal multiplicity and be deleted in neuroblastoma.		
4	Phgdh	1367811_at	3-phosphoglycerate dehydrogenase	0.0004608	0.2182939
			Able to affect the selective estrogen receptor modulator (SERM) status.		
5	Srebf1	1388426_at	sterol regulatory element binding factor 1	0.0005815	0.2182939
			Overexpressed in gastric-intestinal stromal tumor and PIN lesions that are hormonal dependent disease/cancer. Also involved in estrogen-induced transcription of high-density lipoprotein receptor gene.		
6	Ppil1_ predicted	1372517_at	peptidylprolyl isomerase (cyclophilin)-like 1 (predicted)	0.0006084	0.2182939
			Overexpressed in colon cancer and affects cell proliferation throug the control of SNW1/SKIP and/or stathmin.		
7	Nnt_mapped	1371483_at	nicotinamide nucleotide transhydrogenase	0.000287	0.2059512
			Responsible for transhydrogenation between NADH and NADP in respiration and ATP hydrolysis		
8	RGD:619899	1370947_at	hypothetical protein RDA279	0.0001151	0.1874132
9	N/A	1378440_at	Transcribed locus	0.0003678	0.2182939
10	Nt5e	1369200_at	5' nucleotidase, ecto	0.0004912	0.2182939
			Catalyzing the dephosphorylation of ribo- and deoxyribonucleotide 5-monophosphates and regulating the availability of adenosine in cell. Promotes invasion, migration and adhesion in human breast cancer (MCF-7) cells. Downregulated by promoter hypermethylation in gastric cancer		
11	N/A	1385832_s_at	Similar to RIKEN cDNA 1200013B08	0.0007262	0.2368732
12	N/A	1383189_at	Transcribed locus	0.0008831	0.2425488

(continued)

Table 2 ***(continued)*****.** Microarray data: Class Comparison Analysis. Class Comparison Analysis - two-sample t-test: 2-week time point, Control Vs DMBA-treated. The 14 genes identified by Class Comparison Analysis (p-value ≤0.001). The False Discovery Rate (FDR) is also listed for reference. All known gene functions are listed below each gene.

Gene symbol	Probe set	Description/Function*	Parametric p-value	FDR**
13 Idi1	1368878_at	isopentenyl-diphosphate delta isomerase	0.0009342	0.2425488
		Involved in biosynthesis of isoprenoid, steroid hormones and cholesterol. Downregulated and accompanied by marked increase of apoptosis in human leukemia cells. Associated with differentiation and/or growth inhibition in mammary gland cancer cell lines.		
14 Pcbd1	1372485_at	pterin 4 alpha carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1) 1	0.0009464	0.2425488
		Involved in cancer progression, regulating the dimerization of HIF1-α and enhancing transcriptional activity of *HIF1*-α.		

* Data from http://www.ncbi.nlm.nih.gov/gene/; http://source-search.princeton.edu/cgi-bin/source/sourceSearch; http://www.ebi.ac.uk/gxa/home; http://www.genecards.org/
** FDR: False Discovery Rate

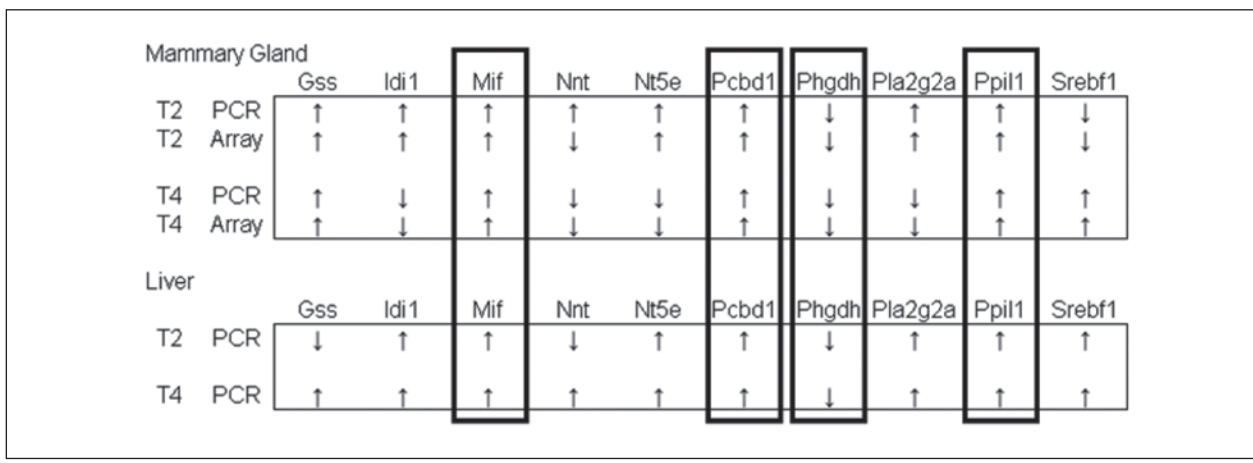

Figure 3. Real Time PCR Results. Quantitative real time PCR confirmation of 2-week and 4-week mammary and liver samples. Most of the mammary real time PCR data are in concordance with the microarray results. Again, *MIF, PCBD1, PHGDH* and *PPIL1* demonstrated consistent expression patterns throughout the mammary and liver tissues at both time points.

to the negative control) (Figure 4) and there was no change in the growth rate of Clone 9 cells (from SD rat liver cells).

siRNA knockdown effects on Ppil1 and Pcbd1 expression levels

The expression levels of *Ppil1* and *Pcbd1* after siRNA knockdown for 72 hours were accessed with SYBR Green based real time PCR and TaqMan real time PCR. The SYBR Green and TaqMan probe quantitative real time PCR results both showed *Ppil1* and *Pcbd1* to be decreased in expression level after the siRNA knockdown (Figure 5).

Discussion

Despite efforts in the last few decades to improve the understanding of the pathophysiology of breast cancer, the factors involved in mammary carcinogenesis progression and the molecular characterization related to differential diagnosis (i.e. *in situ vs* invasive tumors) has still not been clearly identified (9, 15, 16).

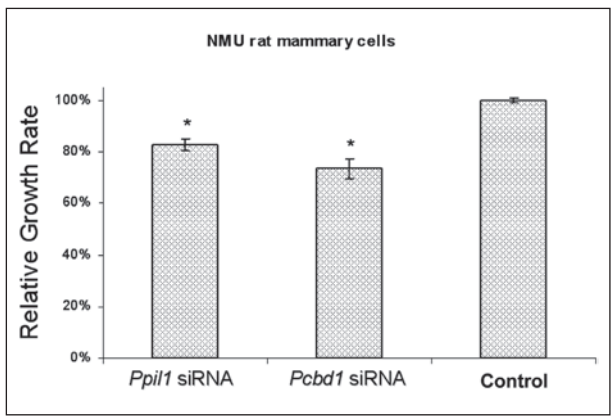

Figure 4. Relative growth rate of NMU cells after siRNA treatment. NMU cells were treated with siRNA targeting *Ppil1* or *Pcbd1* specifically. NMU rat mammary cells were incubated for 72 hours with siRNA. Two tails, two-sample equal variance *t*-test, p-value < 0.05.
* Indicates the p-value < 0.05

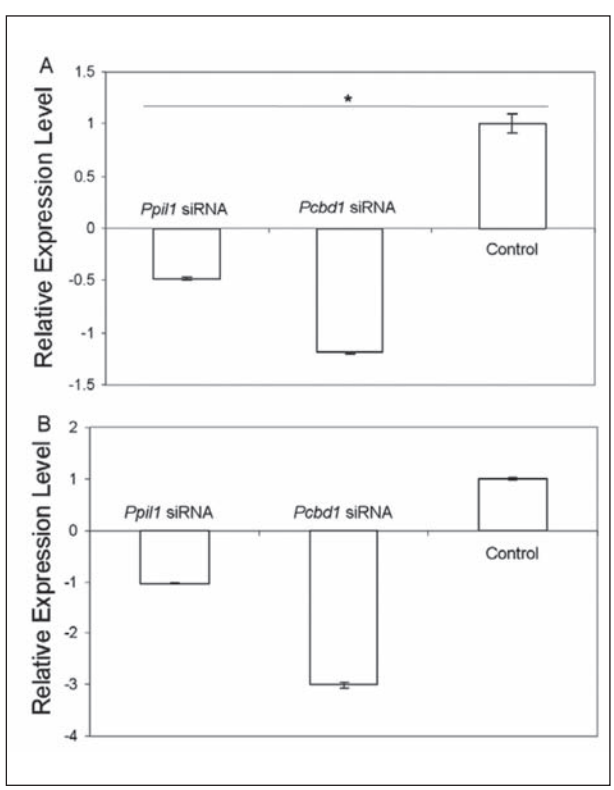

Figure 5. siRNA Knockdown. Relative expression levels of *Ppil1* and *Pcbd1* genes in NUM cells (A) and Clone 9 cells (B) treated with siRNA. Control: negative siRNA. Two tails, two-sample equal variance *t*- test, p-value < 0.05.
* Indicates the p-value < 0.05

In this framework, rodent mammary cancer models have been extremely useful for a better knowledge of the carcinogenic process and its steps, in particular the early stages of the transformation (19). The SD rat DMBA-induced mammary gland tumor model is extensively documented and has been used in the study of carcinogenesis for many years. From perinatal exposure to mammary cancer signatures, numerous studies have utilized this model to clarify the mammary carcinogenesis steps (19, 20, 24, 25, 43).

Chemically-induced mammary tumors develop by a multi-step process. After the pro-carcinogen, DMBA is activated in the mammary tissue by formation of epoxides by P450s metabolic transformation, and the carcinogenic process begins as follows: 1) the initial step is a biochemical lesion caused by interaction of the carcinogenic epoxide with cellular DNA; 2) interaction results in a mutation, chromosomal translocation, inactivation of regulatory genes, or other changes; 3) then the neoplastic development progresses to a stage of autonomous growth, and the lesion acquires the capacity to invade and disseminate metastasis in other anatomic locations (19, 29).

The pathogenesis of DMBA-induced rat mammary adenocarcinomas includes the following steps: undifferentiated terminal end buds affected by the carcinogenic progress to intraductal proliferation (transformed terminal end buds, the earliest lesion which would be detected after DMBA administration), which evolves into *in situ* ductal adenocarcinoma; then, further neoplastic progression causes *in situ* tumor cells to expand and give rise to invasive adenocarcinomas which become metastatic (18, 19, 21). As in rodents, breast cancer development in humans has been considered to be a complex multistep process arising in terminal duct lobular units, which represent the human counterpart of the rat's terminal end buds (10, 19). Furthermore, the multistep carcinogenetic process suggests a transition from normal epithelium to invasive carcinoma via non-atypical and atypical hyperplasia and *in situ* carcinoma. Ductal carcinoma *in situ* shares many molecular genetic alterations with invasive carcinoma and is for this reason considered a pre-invasive stage of malignant lesion in women (8-11).

As happens in humans, chemically induced rat mammary tumors are in general hormone-dependent

adenocarcinomas (20, 22, 25); there is a high frequency of adenocarcinomas histologically similar to human breast cancer. This model forms a useful tool for dissecting the multistep process of carcinogenesis and serves as a baseline for testing the carcinogenic potential of chemical or physical agents in risk assessment (19).

Understanding the mechanism, in particular identifying the genes involved in tumorigenesis, will also increase our understanding of the pathogenesis and molecular signaling pathways deregulated in rat mammary gland carcinogenesis as well as in breast cancer (21).

Although considerable progress has been made in elucidating the genetic events in noninvasive and invasive breast cancer, the relationship between premalignant and *in situ* lesions and invasive cancer is not completely established (8, 10, 15, 16).

To fill in this information gap, high throughput gene expression using the whole genome rat Affymetrix GeneChip Rat Genome (>30k genes) was applied in this study. Approximately one tenth of genes passed the filters and showed significant changes in their expression levels compared with their age matched control.

By multivariate analysis tools, consistent dendrogram patterns were identified and confirmed (Figure 1A, 1B & 1C). A non-supervised hierarchical clustering approach was adopted for this study in order to avoid bias/subjective analysis.

As a first result of the study, we observed that mammary gland samples were clearly separated into two main clusters according to their age (Figure 1). Contrary to what was originally assumed, this result suggested that exposure to DMBA has less influence on the transcriptome at the early time point than growth impact (only ~11% of genes had significantly altered expression levels after DMBA exposure) (44). Nonetheless, it is remarkable that the distinctive clustering patterns were also observed in MDS analysis (Figure 1D).

Three main pathways, namely (1) MAP kinase signaling, (2) *Wnt* signaling and (3) Jak-STAT signaling, were identified in early breast cancer development according to the DAVID ontology analysis. The pathways identified by the analysis are vital signaling paths involved in cell survival and defense, demonstrating that the first response of mammary gland cells to the carcinogenic *stimulus* was to try to preserve themselves through activation of self-defense mechanisms.

MAP kinase is involved in a wide variety of cellular processes, namely proliferation, differentiation, transcription regulation and development (45, 46). In estrogen receptor positive breast tumors, MAP kinase pathways can exert "cross talk" affecting estrogen receptor induced transcription as well as the cell cycle (47). The JUN/MAP kinase signaling pathway is known to play an important role in the early G1 cell cycle subphase: inhibition of the JUN/MAPK pathway is able to shorten the early G1, rendering the cell independent of differentiation and apoptotic factors (48).

The Wnt signaling pathway has been well documented for its involvement in carcinogenesis in various cancers via its influence on cell fate, proliferation, migration, and polarity; aberrantly increased *Wnt* signaling is an important factor in mammary oncogenesis (49, 50). Canonical Wnt signaling stimulates cell proliferation and increases carcinogenic potential especially in mammary gland tissue (50).

The Jak-STAT pathway demonstrated its ability to reverse epithelial-mesenchymal transition and promote differentiation in human breast cancer cells (51, 52). The Jak-STAT signal transduction pathway has been observed to play a key role in prolactin signaling transduction in mammary epithelial cells (53).

Changes in proliferation rate and differentiation status were among the earliest molecular alterations induced by DMBA exposure (26). In particular in our study, *Idi1, Pla2g2a*, and *Ppil1* were reported to be involved in this aspect. They showed differential expressions in both mammary gland and liver tissues. *Idi1* and *Pla2g2a* were upregulated earlier (2-week) but down-regulated later on (4-week) in mammary samples. In contrast, both of them were constantly upregulated in liver samples at both time points. Remarkably, *Ppil1* was consistently upregulated in both mammary and liver tissues at both time points.

In our study, treatment with DMBA caused an up-regulation of *Mif* at both time points and in both anatomic districts, mammary gland and liver. This gene encodes a lymphokine involved in cell-mediated immunity, immunoregulation, and inflammation. It plays a role in the regulation of macrophage function in host defense through the suppression of the anti-

inflammatory effects of glucocorticoids. Considering the function of *Mif* gene (Table 2), this provided evidence that exposure to DMBA would cause aberrant changes in apoptotic pathways and could contribute to abnormal cell growth in rat mammary gland (54).

Oxidative stress has been well-documented for its undeniable involvement in various chronic degenerative diseases and in particular in many types of cancers (55), as well as acting as a cofactor contributing to breast carcinogenesis. Thus, *Gss* is involved in many biological processes including protection of cells from oxidative damage by free radicals. In our analysis *Gss* was also found to be overexpressed at both time points in both tissues, mammary gland and liver, suggesting a possible role by DMBA treatment in escaping apoptosis due to elevated *Gss* activity.

DMBA-induced mammary gland cancer is a hormonal-dependent cancer type (22, 43). Certain hormone-inducible genes were found changed in their expression levels after DMBA treatment. *Phgdh* and *Srebf1* were shown to be responsive to estrogen signaling (56, 57) and both of them had altered expression levels after DMBA exposure.

To our surprise, our gene networking analysis discovered that *Nnt,* the gene that is responsible for trans-hydrogenation between NADPH and $NADP^+$ in respiration and ATP hydrolysis, is associated with *Srebf1* via the leptin network (Figure 2A). Nevertheless, there is no documentation to show that *Nnt* is related to estrogenic regulatory pathways.

Genes involved in cancer progression were also detected in mammary gland after DMBA treatment. In our study, the cancer progression gene *Nt5e* is constantly upregulated in the liver at both time points and in mammary tissue at the 2-week time point. On the other hand, *Pcbd1*, which is also involved in cancer progression, is constantly upregulated in both tissues at both time points. *Pcbd1* is involved in cancer progression in that it regulates the dimerization of HIF1-α and enhances the transcriptional activity of *HIF1*-α (58).

Two constantly upregulated genes, *Ppil1* and *Pcbd1* were selected for subsequent RNA interference study. Both genes' transcripts were successfully suppressed by their specific siRNAs. Interestingly, the proliferation rate was only significantly reduced in NMU cells (mammary) but not in the liver. The siRNA assay suggested a tissue specific response and might help to understand why the DMBA-induced cancer site is the mammary gland, not the liver. Further studies including siRNA assays in human mammary cells to confirm the findings achieved in rat cells will be performed to validate the results here presented.

Conclusion

Molecular biology, and in particular the use of the microarray technique, have provided new insight into the different pathways involved in the multistep model of breast carcinogenesis.

Chemical carcinogen induction of mammary tumors in rodents and in particular in SD rats is one of the most widely studied and useful models for the study of mammary carcinogenesis (19, 21). For these reasons we decided to use the time-course high throughput gene expression approach combined with this model to identify the early pre-cancerous changes in the mammary carcinogenesis process. The study here reported is the first investigating and focusing on early stage DMBA-induced mammary gland carcinogenesis in SD rats at transcriptome levels, utilizing a time-course microarray approach. The results of the study suggested that 1) mammary gland samples analyzed at the two time points clearly clustered according to their age, instead of treatment: thus exposure to DMBA has a lesser influence on the transcriptome at the early time point than at maturity and less growth impact (only ~11% of genes altered their expression levels significantly after DMBA exposure); nevertheless among the pathways altered by DMBA 2) important vital pathways, involved in cancer progression, migration, proliferation and oxidative stress were identified in this study and, in our opinion, contribute to breast carcinogenesis. Differentially expressed genes identified were found in concordance with malignancy development in the target tissue. Although more experimental and clinical investigations need to be performed in this direction, the study results allowed us to identify two novel cancer progression/proliferation related genes, *Ppil1* and *Pcbd1*, which might serve as new and promising targets for identification of a pre-

cancerous mammary stage and potentially for new intervention strategies.

The present study provided important information regarding the possibility to assess and monitor early breast cancer development at a molecular level, long before any phenotypic change appears in an *in vivo* experimental model. This information, if confirmed by further studies, would represent an important step forward in the identification of new targets for prevention, such as molecular targets of exposure to certain agents of risk, or specific biomarkers for characterizing and monitoring the various stages of the neoplastic process.

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