The breast of parous women without cancer has a different genomic profile compared to those with cancer

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Abstract. Our studies are aimed at determining whether pregnancy induces a specific genomic signature in the post-menopausal breast that is responsible for the protective effect elicited by this physiological process. For this purpose we designed a study to compare the gene expression profiles in normal breast tissue from parous postmenopausal women with (case) and without (control) breast cancer. We have used breast samples from 18 parous controls and 41 parous cases. The epithelium and the interlobular stroma were dissected using laser capture microdissection and the RNA of each compartment and each sample was isolated, amplified using PCR methodology, and hybridized to cDNA glass-microarrays containing 40,000 genes, placing the human reference RNA in the green channel (Cy3) and the breast tissue samples in the red channel (Cy5). The normalization and statistical analysis of the expression data were carried out by using the LIMMA software package for the R programming environment which provides functions to summarize the results using the linear model perform hypothesis tests and adjust the p-values for multiple testing. We were able to identify 126 genes that were upregulated and 103 that were downregulated in the parous control group. There were only 56 genes differentially expressed in the interlobular stroma in the parous control group in relation to the other group of women under study. The gene categories that were overrepresented in the breast epithelium of the parous control breast are related to apoptosis, DNA repair, response to exogenous agents and transcription regulation. In the present study we demonstrate that full-term pregnancy imprints a specific genomic signature in the breast epithelium of postmenopausal parous control women that is significantly different from women who have developed cancer. This genomic signature induced by pregnancy could help to predict in which women parity is protective.

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Introduction

Breast cancer is the most common neoplastic disease in women and accounts for up to one third of all new cancer cases in North American women (1). There is substantial evidence that breast cancer risk relates to endocrinologic and reproductive factors. Breast cancer development is strongly dependent on the ovary and on endocrine conditions modulated by ovarian function, such as early menarche, late menopause, and parity (1-4). Epidemiological findings indicate that a lifetime risk decrease has been observed in parous women whose first pregnancy was completed before age 24 (2-6). However, the protection conferred by early first full-term pregnancy does not occur in all women. We have postulated that a cluster of genes associated with early first full-term pregnancy would be absent or modified in the breast of high-risk women, i.e., nulliparous women with cancer and also in parous women who developed cancer (7-9). Furthermore, those genes whose expression may be affected by pregnancy and that can be proven to be functionally relevant in protecting the breast from cancer development could serve as markers for evaluating cancer risk in large populations. In the present study we demonstrate that early first full-term pregnancy induces a genomic signature that is specific for the parous breast and that is different from that in those who are parous and have cancer.

Materials and methods

Sample collection. In this study we have included post-menopausal women who underwent breast biopsies (excluding fine needle biopsies) at FCCC or the participant's hospital

Christiana Care and Somerset Medical Center between October 1, 2002 and December 30, 2006. Each participant had signed their respective informed consent forms that were approved by each Institution's Human Subjects Protection Committee. Women were 50+ years old and postmenopausal, defined as at least one year since last menses if menopause occurred naturally. We have excluded from our study women in which both ovaries were removed or with a history of cancer other than non-melanoma skin cancer, women taking medications that could interfere with the study protocol such as estrogens (including Tamoxifen and Raloxifene), progestins, androgens, prednisone, thyroid hormones, or insulin, and women with Alzheimer's disease or severe cognitive deficit who were unable to give informed consent.

Breast tissue specimens. A total of 59 histologically normal breast tissues from 18 parous controls and 41 parous cases were analyzed and microdissected for the epithelium from type 1 lobules (10,11) and the interlobular stroma by using the laser capture microdissection (LCM) system (12). All the tissues were collected and fixed in 70% ethanol within 10 min of surgical removal. The tissues were embedded in paraffin. Eight-micron sections were obtained and stained with hematoxylin and eosin, followed by 5-min dehydration steps in 70, 95 and 100% ethanol. Once air-dried the sections were laser microdissected with an AutoPix 1000 LCM system from Arcturus Engineering (Mountain View, CA). We captured epithelial cells from the type 1 lobules and interlobular stroma from each breast sample.

RNA isolation, labeling and cDNA human microarrays. The RNA was isolated by placing the LCM cap in 50 μl of Trizol reagent and following procedures previously described (12). The probes were constructed using direct labeling of the random hexamer primer and following protocols described elsewhere (12). The probes were cleaned with a QIA-quick PCR purification kit (Qiagen); PB buffer (500 μl from kit) was added into the Cy3- and Cy5-labeled products. The samples were applied to QIA-quick columns, which were centrifuged at 13,000 rpm (16,000 x g) for 1 min, after which the flow-through was discarded. The column was washed by adding 750 μl of 80% cold ethanol, and spun again for 1 min, and the flow-through was discarded. The washing was repeated twice, and the columns were spun again to remove residual ethanol. The collected material was diluted in 30 μl of DEPC-water heated at 70˚C followed by 3-min incubation at 70˚C. Columns were then centrifuged at 13,000 rpm (16,000 x g) for 1 min, and the elution step was performed only once. The eluted material was partially dried in a vacuum centrifuge and the volume was adjusted to 15 μl of hybridization buffer [containing 20X saline-sodium citrate (SSC) and 0.6 μl of 10% (wt/vol) SDS], then the probes were denatured at 95˚C for 3 min and centrifuged for 3 min at 13,000 rpm. The products were pipetted onto arrays, coverslipped, and the slides were placed in a hybridization chamber (Gene Machine). Arrays were incubated in a water bath at 42˚C for 16-18 h, and subsequently washed with 0.5X SSC, 0.01% (wt/vol) SDS, followed by 0.06X SSC, at room temperature for 10 min each. The slides were spun for 8 min at 800 rpm (130 x g) at room temperature.

Array scanning. Arrays were read with an Affymetrix 428 fluorescent scanner (MWG, CA) at 10-μm resolution and variable photomultiplier tube (PMT) voltage settings to obtain the maximal signal intensities with <1% (wt/vol) probe saturation. The resulting images were analyzed using ImaGene software version 4.2 (Biodiscovery, CA). The glass microarrays were hybridized in all the cases placing the amplified RNA from the breast samples in the red channel (labeled with Cy5) and the amplified RNA from human universal reference (Stratagene, Inc, CA) in the green channel (labeled with Cy3). We studied gene expression in the 59 patients by triplicate using cDNA microarrays, which were prepared by robotically spotting 40,000 human cDNA on mirror glass slides (NCI-supported Microarray Facility from Fox Chase Cancer Center). The cDNA included approximately 28,000 different genes that represented characterized human proteins and 10,000 identified by ESTs, the rest were controls and blank spots. The identities of the cDNA had been sequence-verified. Each hybridization compared Cy5-labelled cDNA reverse transcribed from amplified RNA isolated from each patient with the Cy3-labelled cDNA reverse transcribed from a universal human reference amplified RNA sample. The reference sample was used in all the hybridizations employing the same probe for all of them in order to have an equal and common reference for the experiment. The fluorescent probes were performed in triplicate and after checking the quality, replicates from the same sample were combined and re-distributed into 3 separate tubes in order to have an identical replicate. Equal amounts of fluorescent probes were used to hybridize the cDNA microarrays.

Data analysis. Normalization and statistical analysis of the expression data were carried out by using the LIMMA software package for the R programming environment (13,14). This package contains a number of analysis methods not found in other software. Local background subtraction usually produces log-ratios that are very variable at low intensities. Because it was desired to detect differential expression for genes that might not necessarily be highly expressed, filtering out low-intensity spots was avoided. Instead, a strategy of background correction was used that avoids exaggerated variability of log-ratios for low-intensity spots. Background correction was performed by using the 'normexp' method in LIMMA to adjust the local median background estimates. This strategy is similar to the background correction method used by the popular RMA software for Affymetrix data (15). It avoids problems with background estimates that are greater than foreground values and ensures that there were no missing or negative corrected intensities. An offset of 100 was used for both channels to further damp down the variability of log-ratios for low-intensity spots. The resulting log-ratios were normalized by using the print-tip group Lowess method with a span of 0.4, as recommended by Smyth and Speed (16). Here and elsewhere, the small number of spots that were manually marked as 'bad' on visual inspection of the scanned arrays were filtered out of the analysis, while spots that were flagged as 'not found' by GENESIGHT were kept in the analysis but downweighted. The arrays used in these experiments were from four different print runs that were all printed with the same elements but with slightly different print layouts. The

arrays from the different print runs were therefore normalized separately and the normalized expression data were combined and aligned by probe for subsequent analysis. After normalization of the data, each microarray had similar distributions in order to eliminate the microarray effect once we detected the gene expression of certain genes.

The basic statistic used for significance analysis is the moderated t-statistic, which is computed for each probe and for each contrast. This has the same interpretation as an ordinary t-statistic except that the standard errors have been moderated across genes, i.e., shrunk towards a common value, using a simple Bayesian model. This has the effect of borrowing information from the ensemble of genes to aid with the same inference about each individual gene (17). Moderated t-statistics lead to p-values in the same way that ordinary t-statistics do except that the degrees of freedom are increased, reflecting the greater reliability associated with the smoothed standard errors. The most popular form of adjustment is 'fdr' which is Benjamini and Hochberg's method to control the false discovery rate (19). The meaning of fdr adjusted p-values is as follows. If all genes with a p-value below a threshold, e.g. of 0.05, are selected as differentially expressed, then the expected proportion of false discoveries in the selected group is controlled to be less than the threshold value, in this case 5%. The B-statistic is the log-odds that the gene is differentially expressed (15). For example, if B=1.5, the odds of differential expression (1.5) =4.48; i.e., ~4.5:1. The probability that the gene is differentially expressed is 4.48/(1+4.48)=0.82. A B-statistic of zero corresponds to a 50-50 chance that the gene is differentially expressed. The B-statistic is automatically adjusted for multiple testing by assuming that 1% of the genes, or some other percentage specified by the user referring to empirical Bayes (17-19), is expected to be differentially expressed. The p-values and B-statistics will normally rank genes in the same order. In fact, if the data contains no missing values or quality weights, then the order will be precisely the same. As with all model-based methods, the p-values depend on normality and other mathematical assumptions which are never completely precise for microarray data. It has been argued that the p-values are useful for ranking genes even in the presence of large deviations from the assumptions. The B-statistic probabilities depend on the same assumptions but require in addition a prior estimate for the proportion of differentially expressed genes. The p-values may be preferred to the B-statistics because they do not require this prior knowledge (20).

Results

Identification of differentially expressed genes in breast epithelium. Genes whose expression changes were considered to be statistically significant using established algorithms, and whose expression changed by at least 1.2-fold as a result of being a case, were selected for further analysis (19). This combined analytic approach has previously been shown to be capable of identifying differentially expressed genes with high sensitivity and specificity (19). We were able to identify gene sequences differentially expressed (t-test with false discovery rate $p<0.05$) in epithelium from the parous breast control compared to that from the parous breast cancer cases. We

Table I. Upregulated genes in the parous control breast epithelia.

Apoptosis- GO:0006915; GO:0042985	
BAX	2.6500
TIA1	1.5600
TRAF1	1.7200
TRADD	1.4200
CRADD	1.8900
PPM1F	1.3500
Cell adhesion- GO:0007155; GO:0016337; GO:0007160	
SEMA5A	1.810
ICAM3	1.7000
EVA1	1.2500
FBLN5	1.7900
FNBP4	1.2900
SDK1	1.2600
NRP1	1.2500
Signal transduction- GO:0007165; GO:0007242; GO:0016055; GO:0008277; GO:0007186	
EMR2	1.5100
ANK2	1.2500
IRS1	1.2900
CNIH2	1.2500
CHN2	1.3000
LRP5	1.4000
GIT1	1.3500
GALR2	1.2000
Cell cycle and growth- GO:0000067; GO:0000074	
DNAJA2	1.5600
HIPK2	1.8700
RBBP6	1.5800
DCTN1	1.4800
Response to exogenous agents- GO:0008152; GO:0006805; GO:0045454; GO:0042113; GO:0006725; GO:0006950; GO:0006979; GO:0006954; GO:0006952	
RDH11	1.6428
EPHX1	1.7800
TXNRD1	1.9200
IGBP1	1.3800
CBARA1	1.3800
TIRAP	1.3800
SCARA3	1.3900
GSTT1	1.2400
C10orf59	1.9300
NAT2	1.5000
Cell transport- GO:0006810; GO:0006811; GO:0006812; GO:0015031; GO:0005794; GO:003001; GO:0006826; GO:0006813; GO:0006406	
TPR	1.5300
TLOC1	1.6300
GALNT10	1.4700
ARMC1	1.6500

Table I. Continued.

TRPM1	1.2400
CLCN6	1.3500
HRB	1.4000
SHKBP1	1.6300
TTYH1	1.6000
SLC19A3	1.6400
SLC22A9	1.5600
Chromatin modification- GO:0016568; GO:0007001	
HIST1H2AC	1.2700
SETD1A	1.8700
Development-morphogenesis- GO:0007275; GO: 0030154; GO:0007399	
DVL2	2.0669
LTBP4	1.9901
DOPEY2	2.5697
EFNB3	1.6402
DGCR14	2.3211
FGF11	2.2619
DNA repair- GO:0006284; GO:0006281; GO: 0000731; GO:0006298; GO:0006310	
RAD51L3	1.9200
ERCC8	1.2500
POLD3	1.5000
ANKRD17	1.780
TSN	1.940
NTHL1	1.9200
TREX1	1.5400
Miscellaneous process- GO:0005887; GO:0016043; GO:0006936; GO:0005887; GO:00031012	
CEACAM4	1.6500
DIAPH3	2.2219
SSPN	1.5900
THSD4	1.8500
RNA processing- GO:0006396; GO:0006364; GO:0007600; GO:0008033; GO:0000245	
DDX17	3.024
EIF4A3	1.710
TTC8	1.8722
PUS7	1.4400
POP7	1.540
SIP1	1.7400
Metabolism- GO:0030201; GO:0008152; GO:0007206; GO:0016042; GO:0006559; GO:0006796	
HS3ST4	1.980
DBT	2.9821
HOMER1	2.0195
SIDT2	1.2718
FAH	1.2900
DHDDS	1.9600
ACSS1	1.7600
PTPRB	1.2600

Table I. Continued.

Protein biosynthesis and metabolism- GO:0006470; GO:0008104; GO:0006464; GO:0006468; GO:0006412; GO:0006457	
PTPRC	1.3209
VPS13C	2.3952
LOX	3.6726
TTLL5	3.2004
PTPN21	1.2500
MGC42105	1.2400
RPL9	1.2600
GRPEL1	1.2500
BACE2	1.2400
Proteolysis and ubiquitination- GO:0006508; GO:0006511; GO:0006512; GO:0006397; GO:0006398; GO:0016567	
CTSB	2.1571
DPP3	1.5400
PEPD	1.420
EDD1	1.6632
RNF44	1.3200
ATE1	1.2600
HNRPR	1.2500
GEMIN4	1.3000
Transcription- GO:0000122; GO:0006355; GO:0006350; GO:0006357; GO:0006366	
BPTF	2.000
SUPT5H	2.150
SOX10	1.9300
PCAF	1.2500
FOXK2	1.2550
KCNIP3	1.3000
PIAS1	1.3100
RFXAP	1.2400
ZNF16	1.2340
ID4	2.100
GTF2B	1.539
ZNF26	1.534
ZNF498	2.0074
ZNF544	1.2500
ZNF710	1.9023
BAZ2A	1.2450
HOXD1	1.2600
HIRA	1.2600
TLE3	1.2500
ZNF268	1.5000
ZNF275	1.2800
Biological process unknown- GO:0000004	
FBLN2	1.835
DHX57	2.9587
ZDHHC9	1.2400
WDR44	1.8603
ENTPD3	1.2600
ORMDL1	3.0238
ANKRD12	1.8890
TMEM27	1.7801
DKFZP434A0131	1.2500

found that 126 genes were upregulated (Table I) and 103 were downregulated (Table II) in the parous control group, with respect to the parous breast cancer group.

Gene functional category analysis. There are four major biological processes that were overrepresented in the parous control group; apoptosis, DNA repair, response to exogenous agents and transcription regulation. The apoptosis process in the parous control group contained six genes: *BAX*, *BCL2-associated X protein; TIA1, cytotoxic granule-associated RNA binding protein; TNF receptor-associated factor 1; TRADD, TNFRSF1A-associated via death domain; CRADD, CASP2 and RIPK1 domain containing adaptor with death domain;* and *PPM1F, protein phosphatase 1F (PP2C domain containing)* (Table I). However only two genes were down-regulated in the parous control group; *Transformed 3T3 cell double minute 4-p53 binding protein (mouse)* (*Mdm4)* and *Programmed cell death 5 (PDCD5)*. There are also two anti-apoptotic genes, *Baculoviral IAP repeat-containing 6 (apollon)* and *BCL2-associated athanogene*, that were downregulated in the parous control group (Table II). This indicates that the programmed cell death is a signature prevalently expressed in the parous control group.

The DNA repair process was also overrepresented in the parous control breast epithelia containing seven genes that were significantly upregulated (Table I): *RAD51-like 3 (S. cerevisiae); Excision repair cross-complementing rodent repair deficiency, complementation group 8; Polymerase (DNA-directed), Δ 3, accessory subunit; Ankyrin repeat domain 17; Translin; Nth endonuclease III-like 1 (E. coli);* and *Three prime repair exonuclease 1*.

The third biological process overrepresented in the parous control group was the cluster of genes related to cell response either in the immunosurveillance category or response to genotoxic agents (Table I), such as *Retinol dehydrogenase 11 (all-trans/9-cis/11-cis) (RDH11); Epoxide hydrolase 1, microsomal (xenobiotic)(EPHX1); Thioredoxin reductase 1 (TXNRD1); Immunoglobulin (CD79A) binding protein 1 (IGBP1); Calcium binding atopy-related autoantigen 1 (CBARA1); Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP); Scavenger receptor class A, member 3 (SCARA3); Glutathione S-transferase θ 1 (GSTT1); Chromosome 10 open reading frame 59 (C10orf59); and N-acetyltransferase 2 (arylamine N-acetyltransferase) (NAT2)*.

Another biological process that was significantly over-represented in the parous control breast epithelia is that controlling gene transcription/gene transcription-regulation in which 21 genes were upregulated and 12 were downregulated (Tables I and II). Among these genes the *Suppressor of Ty 5 homolog (SUPT5H)*, *Homeo box D1 (HOXD1)*, *p300/CBP associated factor (PCAF)* and *Inhibitor of DNA binding 4 (ID4)* were highly upregulated in the control parous breast epithelia (Table I).

Genomic signature of the breast interlobular stroma. There were only 56 differentially expressed genes in the interlobular stroma from parous control women (Table III). The selection criteria was based on a B-value >0 and a $p<0.05$. We found 30 genes that were differentially overexpressed and 26

Table II. Downregulated genes in the parous control breast epithelia.

Apoptosis- GO:0006915	
PDCD5	-2.1500
MDM4	-1.2500
Antiapoptosis- GO:0006916	
BIRC6	-1.2600
BAG4	-1.2700
Cell adhesion- GO:0007154; GO:0007155; GO:0016337	
DSCAM	-2.1000
DLG5	-1.8000
COL16A1	-1.7800
LAMC1	-2.9100
Cell cycle and growth- GO:0007049; GO:0007050; GO:0000082; GO:0000074; GO:0000076; GO:0000086	
KATNA1	-2.6600
TACC1	-2.1700
SESN3	-1.9000
GSPT1	-3.5000
PPP2R1B	-2.3500
LATS1	-1.9800
TMCO7	-1.6000
Cell signaling- GO:0007267	
Signal transduction- GO:0007165; GO:0007264; GO:0000160; GO:0000079; GO:0000186; GO:0005794	
NPY1R	-1.460
RAPGEF6	-1.4000
RAB27A	-1.8454
CCDC132	-3.8868
BCCIP	-1.3792
SCYE1	-3.8681
NPSR1	-1.4600
ANKDD1A	-2.3100
EDNRA	-1.4500
GIPC1	-1.5500
DDEF2	-1.3000
Cell transport - GO:0006886; GO:0006811; GO:0006817; GO:0006810; GO:0005794; GO:0006118; GO:001503; GO:0007242	
STON2	-1.7224
GABRB3	-2.3657
FCN1	-1.6600
G3BP	-3.8192
FREQ	-1.4241
ACOX1	-1.5300
CYB5R4	-1.7000
RAP1B	-1.8100
DBNDD2	-1.3100
SLC20A2	-1.7100
KLHL2	-2.0500
SNX11	-1.4600
Development-morphogenesis- GO:0009653; GO:0007275; GO:0007517; GO:0000188; GO:0007399; GO:0030326; GO:0007275; GO:0009790	
TWIST1	-1.2500

Table II. Continued.

PIAS2	-1.5000
MAP1B	-2.6405
DUSP22	-1.6400
TPM3	-2.2300
CRIM1	-1.3500
SHFM1	-1.7200
HLF	-1.5000
BRUNOL4	-1.3400
DNA replication- GO:0006260; GO:0000067	
UBE1	-1.250
SMC2	-1.6100
Metabolism- GO:0005975; GO:0046677; GO:0008152	
AMY1A	-1.2500
ACSL3	-1.2500
Miscellaneous processes- GO:0007596; GO:0046677; GO:0008152	
ANXA5	-1.3938
LACTB	-1.8100
Protein biosynthesis and metabolism- GO:0006470; GO:0006412; GO:0006487; GO:0006461; GO:0006468; GO:0006457; GO:0018347	
STYXL1	-2.6900
MRPS16	-1.6700
EIF2B1	-1.4500
WARS2	-1.7200
LIPC	-2.1300
PARD3	-1.5500
TRPC4AP	-1.5700
CAPZA1	-1.7800
COL4A3BP	-1.6700
MRPS11	-1.6700
EIF1AY	-2.1500
FNTA	-1.7200
GRPEL2	-1.6100
Protein degradation and ubiquitination- GO:0016567; GO:0006508; GO:0006511; GO:0006512	
SAE1	-1.5842
AFG3L2	-2.1800
TRIAD3	-2.0472
USP30	-1.7700
ARIH1	-2.1400
UBE2E1	-2.0700
RNA processing- GO:0000398; GO:0007046	
SFRS10	-2.2000
BMS1L	-1.6900
BXDC2	-2.1000
Transcription- GO:0000122; GO:0006355; GO:0006350; GO:0006306	
HDAC8	-2.2000
ZNF425	-1.6200
PKNOX2	-1.6400
MBD3	-3.1705
GTF3C4	-1.8700
RNF12	-1.2500
DPF2	-1.690
SOX3	-2.114

Table II. Continued.

POU6F1	-1.3700
MLLT6	-1.4100
RORA	-1.4000
GATAD2A	-1.9700
Biological process unknown- GO:0000004	
PHACTR1	-1.9118
MGC4562	-1.8500
HNRPM	-1.7300
RB1CC1	-1.300
DOCK5	-1.3700
DDX46	-2.1900
DOK5	-1.2500
PGRMC2	-1.2500
BCL7A	-1.3000
ZNF320	-1.5000
NRSN2	-4.8500
TMEM32	-4.4300
ZRANB1	-1.8500
VKORC1L1	-1.6600
FAM57A	-3.8600
MCPH1	-3.9400

genes that were downregulated in the parous control group (Table III). There was no biological process overrepresented and most of the Gene Ontology revealed unknown biological function.

Discussion

In the present study we demonstrate that early first full-term pregnancy imprints a specific genomic signature in the breast epithelia of postmenopausal women that is significantly different from that of women that also have had an early full-term pregnancy but have developed cancer. The genomic signature is made up of 126 upregulated and 103 down-regulated genes. The gene ontology categories that were overrepresented in the breast epithelia of the parous control breast are related to apoptosis, DNA repair, response to exogenous agents, and gene transcription/gene transcription-regulation.

There are 10 genes that control apoptosis that were differentially expressed in the breast epithelia of the parous women. Among them six were upregulated such as the *BCL2-associated X protein* (*BAX)* that belongs to the BCL2 protein family. *BAX* is a pro-apoptotic gene whose transcription is stimulated by active p53, including the pro-apoptotic gene p21, a cell cycle regulator (21-23). This protein forms a heterodimer with BCL2, and functions as an apoptotic activator (24). The expression of this gene is regulated by the tumor suppressor P53 and has been shown to be involved in P53-mediated apoptosis (21,25). *Programmed cell death 5* (*PDCD5*) and *Transformed 3T3 cell double minute 4* (*Mdm4*) were down-regulated in the parous breast epithelia. The *Mdm4* gene is amplified and overexpressed in a variety of human cancers and encodes structurally related oncoproteins that bind to the

Table III. Gene expression profile of the stroma of the human breast.

Gene name	Gene ID	Gene symbol	GO number	Biological function	GO number	Molecular function	Adj. p	Folds
Genes Up-modulated Apoptosis-inducing factor	H18472	AMID	GO:0008637	Apoptotic, mitochondrial	GO:0015036	Disulfide oxidoreductase	0.0354	1.5284
Chromosome 21 open reading frame 45	W72814	C21orf45	GO:0000004	Unknown	GO:000554	Unknown	0.0001	1.2000
Hyaluronoglucosaminidase 1	R44982	HYAL1	GO:0005975	Metabolism	GO:000554	Unknown	0.0001	1.2871
Calcium channel	AA437099	CACNA1D	GO:0006812	Cation transport	GO:0005509	Calcium ion binding	0.0478	1.5600
Hypothetical protein LOC283874	R01257	LOC283874	GO:0006313	DNA transposition	GO:000554	Unknown	0.0001	1.2600
Alcohol dehydrogenase 6 (class V)	H68509	ADH6	GO:0006069	Ethanol oxidation	GO:000554	Unknown	0.0001	1.2400
KIAA0319-like	AA150417	KIAA0319L	GO:0007156	Homophilic cell adhesion	GO:000554	Unknown	0.0219	3.8005
SH3 and multiple ankyrin repeat domains 2	R05837	SHANK2	GO:0007242	Intracellular signaling	GO:000554	Unknown	0.0001	1.3255
Translocase of outer mitochondrial membrane 40 homolog (yeast)	AA443094	TOMM40	GO:0006629	Lipid metabolism	GO:000554	Unknown	0.0354	1.5786
Retinoic acid receptor responder (tazarotene induced) 1	N94424	RARRES1	GO:0008285	Cell proliferation	GO:000554	Unknown	0.0001	1.2400
Protein tyrosine kinase 2	AA447612	PTK2	GO:0006468	Protein phosphorylation	GO:0005524	ATP binding	0.0219	3.6419
Regulator of G-protein signalling 12	W67134	RGS12	GO:0015031	Protein transport	GO:0005096	GTPase activator	0.0483	1.8100
Transcription factor Dp-1	W46439	TFDP1	GO:0006357	Transcription	GO:0003700	Transcription factor	0.0347	1.8023
RNA pseudouridylate synthase domain	H18934	RPUSD1	GO:0006396	RNA processing	GO:0003723	RNA binding	0.0260	2.4640
Colony stimulating factor 2 receptor, ß, low-affinity (granulocyte-macrophage)	AA279147	CSF2RB	GO:0007165	Signal transduction	GO:000554	Unknown	0.0003	1.8900
Distal-less homeobox 5	N74882	DLX5	GO:0001501	Skeletal development	GO:000554	Unknown	0.0000	1.9794
Adenosylmethionine decarboxylase 1	AA504772	AMD1	GO:0006597	Spermine biosynthesis	GO:000554	Unknown	0.0002	1.3800
General transcription factor IIIC	AA429809	GTF3C4	GO:0006350	Transcription	GO:0003677	DNA binding	0.0260	3.1421
Pleiomorphic adenoma gene-like 1	AA463204	PLAGL1	GO:0006350	Transcription	GO:000554	Unknown	0.0001	1.5600
Tripartite motif-containing 24	R38345	TRIM24	GO:0006350	Transcription	GO:000554	Unknown	0.0002	1.5500
Transcription elongation factor A (SII), 2	AA412500	TCEA2	GO:0006350	Transcription	GO:000554	Unknown	0.0003	0.0981
ElaC homolog 1 (E. coli)	AA456439	ELAC1	GO:0006350	Transcription	GO:000554	Unknown	0.0002	1.5600
Transcobalamin II; macrocytic anemia	AA490459	TCN2	GO:0006810	Transport	GO:000554	Unknown	0.0002	1.4400
Similar to CG4502-PA	H17038	FLJ25076	GO:0006512	Ubiquitin cycle	GO:000554	Unknown	0.0478	1.9000
BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)	H09066	BAP1	GO:0006511	Ubiquitin-dependent	GO:000554	Unknown	0.0002	1.6400
Transmembrane and tetratricopeptide repeat	AA447480	TMTC1	GO:0000004	Unknown	GO:0005488	Binding	0.0354	1.5128
Hypothetical protein LOC54103	T82259	LOC54103	GO:0000004	Unknown	GO:000554	Unknown	0.0003	1.2400
C8orfK32 protein	H16974	C8ORFK32	GO:0000004	Unknown	GO:000554	Unknown	0.0260	2.3494
Transcribed locus	N62346		GO:0000004	Unknown	GO:000554	Unknown	0.0354	1.6003
Transcription elongation regulator 1-like	AA009615	TCERG1L	GO:0000004	Unknown	GO:000554	Unknown	0.0406	1.2500
Transcribed locus	AA010383		GO:0000004	Unknown	GO:000554	Unknown	0.0260	2.5121

Table III. Continued.

Gene name	Gene ID	Gene symbol	GO number	Biological function	GO number	Molecular function	Adj. p	Folds
Genes down-modulated Maternally expressed (in Callipyge) 8	N52482	MEG8	GO:0000004	Unknown	GO:000554	Unknown	0.0219	-4.4853
KIAA1128	AA432090	KIAA1128	GO:0000004	Unknown	GO:000554	Unknown	0.0483	-1.8000
Hypothetical protein DKFZp761B107	R52679	DKFZp761B107	GO:0000004	Unknown	GO:000554	Unknown	0.0335	-1.9169
Clone 23688 mRNA sequence	N90403		GO:0000004	Unknown	GO:000554	Unknown	0.0354	-1.5971
Transcribed locus	AA431738		GO:0000004	Unknown	GO:000554	Unknown	0.0481	-1.8700
CDNA clone IMAGE:30404625	H10156		GO:0000004	Unknown	GO:000554	Unknown	0.0406	-1.4100
Coiled-coil domain containing 46	AA406069	CCDC46	GO:0000004	Unknown	GO:000554	Unknown	0.0335	-1.9632
Transcribed locus	AA426065		GO:0000004	Unknown	GO:000554	Unknown	0.0260	-2.6304
Tudor domain containing 10	AA401393	TDRD10	GO:0000004	Unknown	GO:000554	Unknown	0.0499	-1.7300
Transcribed locus	R56233		GO:0000004	Unknown	GO:000554	Unknown	0.0406	-1.1970
Transcribed locus	N48294		GO:0000004	Unknown	GO:000554	Unknown	0.0478	-1.9300
Hypothetical protein P117	AA005401	P117	GO:0000004	Unknown	GO:000554	Unknown	0.0347	-1.7668
Seizure related 6 homolog (mouse)-like	H29013	SEZ6L	GO:0000004	Unknown	GO:000554	Unknown	0.0335	-1.9674
CDNA clone IMAGE:4800096	AA428239		GO:0000004	Unknown	GO:000554	Unknown	0.0260	-2.7245
Transcribed locus	W32296		GO:0000004	Unknown	GO:000554	Unknown	0.0381	-1.4060
Transcribed locus	AA135722		GO:0000004	Unknown	GO:000554	Unknown	0.0406	-1.1710
Thrombospondin, type I, domain containing 1 pseudogene	AA115259	THSD1P	GO:0000004	Unknown	GO:000554	Unknown	0.0393	-1.3400
CDNA FLJ30588 fis, clone BRAWH2008128	T99852		GO:0000004	Unknown	GO:000554	Unknown	0.0260	-2.4091
WD repeat domain 68	AA034041	WDR68	GO:0000004	Unknown	GO:000554	Unknown	0.0260	-2.4248
Component of oligomeric golgi complex 6	W67514	COG6	GO:0000004	Unknown	GO:000554	Unknown	0.0002	-1.3600
5'-nucleotidase domain containing 2	R42815	NT5DC2	GO:0000004	Unknown	GO:000554	Unknown	0.0002	-1.4000
N-acylsphingosine amidohydrolase (acid ceramidase)-like	W47576	ASAHL	GO:0000004	Unknown	GO:000554	Unknown	0.0001	-1.4300
Small proline-rich protein 2C	AA399674	SPRR2C	GO:0000004	Unknown	GO:000554	Unknown	0.0001	-1.5148
Chromosome 16 open reading frame 61	AA181314	C16orf61	GO:0000004	Unknown	GO:000554	Unknown	0.0495	-7.0000

Adj. p, adjusted p-value.

p53 tumor suppressor protein and inhibit p53 activity (26-29). Mice with deleted *Mdm4* die during embryogenesis, and the developmental lethality in this model can be rescued by concomitant deletion of p53 (30). The downregulation of the *MDM4* in the breast of parous epithelia may act as a protective mechanism and be part of the program cell death pathway active in these cells. Altogether this cluster of genes seems to maintain the active programmed cell death pathway in the parous breast epithelia when compared with the parous breast of women with cancer. Supporting evidence for this statement comes from data in the experimental model (31-34) and in the normal breast tissue from reduction mammoplasty specimens of parous women (7-9), in which genes involved in the pathway of apoptosis were significantly upregulated.

We have identified in the present study that upregulation of DNA repair controlling genes is part of the signature induced by pregnancy. This is supported by data generated in the experimental system in which the parous mammary epithelial cells have a higher ability to remove the DNA adducts of 7-12 dimethylbenz (a) anthracene (35,36). The greater ability of the parous mammary epithelial cells to remove the DNA adducts has been the first indication that an improved DNA repair was involved in the protective effect induced by pregnancy. DNA repair is central to the integrity of the human genome and reduced DNA repair capacity has been linked to genetic susceptibility to cancer (37-40). A reduced DNA repair is associated with risk of breast cancer in women (41). The epithelial cells of the breast from parous

control women present DNA repair related genes that are upregulated significantly when compared with the same gene expression in the epithelial cells of the parous women with cancer. *RAD51-like 3* was upregulated in the epithelial cells of the parous breast and it is known to be involved in the homologous recombination and repair of DNA (42-44). Other genes related to the DNA repair process are *Ankyrin repeat domain 17 (ANKRD17)* and *Translin (TSN)*, which encodes a DNA-binding protein that specifically recognizes conserved target sequences at the breakpoint junction of chromosomal translocations (45). These data indicate that the activation of genes involved in the DNA repair process is part of the signature induced in the mammary gland by pregnancy, confirming previous findings that *in vivo* the ability of the cells to repair carcinogen-induced damage by unscheduled DNA synthesis and adduct removal is more efficient in the post-pregnancy mammary gland (35,36).

Another cluster of genes that are upregulated in the parous control group are those related to immunosurveillance and detoxification of xenobiotic substances. The concept that an immunological process was involved during pregnancy which is responsible for its protective effect in mammary carcinogenesis has been reported (46,47). In breast epithelial cells of parous postmenopausal women we found that the *Toll-like receptor* gene is upregulated. This gene belongs to the innate immune system recognizing microbial pathogens through Toll-like receptors (TLRs), which identify pathogen-associated molecular patterns (48,49). We have also found that the *regulatory factor X-associated protein* that is part of the major histocompatibility (MHC) class II molecules (50) is upregulated (8). It is a transmembrane protein that has a central role in the development and control of the immune system. These data allow us to postulate that the increased immune-surveillance mechanism has been imprinted during the differentiation cycle induced by pregnancy and could be one of the protective factors induced by the cells against neoplastic initiation or progression.

In addition to this increase in the immune-surveillance mechanism in the breast of parous epithelia there are genes significantly upregulated and involved in the metabolism of xenobiotic substances and oxidative stress. Among them are the *Epoxide hydrolase (EPHX1)* that plays an important role in both the activation and detoxification of exogenous chemicals such as polycyclic aromatic hydrocarbons and the *Thioredoxin reductase 1 (TXNRD1)* that encodes a member of a family of pyridine nucleotide oxidoreductases. TXNRD1 protein reduces thioredoxins as well as other substrates, and plays a role in selenium metabolism and protection against oxidative stress (51,52). *Thioredoxin reductase 1* is one of the major antioxidant and redox regulators in mammals that supports p53 function and other tumor suppressor activities (53,54). *Glutathione S-transferase θ 1 (GSTT1)* is a member of a superfamily of proteins that catalyzes the conjugation of reduced glutathione to a variety of electrophilic and hydrophobic compounds and is upregulated in the parous breast epithelia. The other gene that is also overexpressed is the *N-acetyltransferase 2 [arylamine N-acetyltransferase (NAT2)]* involved in the metabolism of different xenobiotics, including potential carcinogens. The upregulation of these genes is interpreted as an activated system of defense that makes the parous breast cells less vulnerable to genotoxic substances. This contention is supported by data indicating that primary breast epithelial cells from parous women treated *in vitro* with chemical carcinogens do not express phenotypes of cell transformation whereas those from nulliparous women do (55,56).

There are 21 genes encoding proteins controlling gene transcription/gene transcription-regulation that are significantly upregulated in the parous breast epithelia and 12 that are downregulated. This indicates that during pregnancy transcription modifications are important components of the genomic signature induced by this physiological process. Another group of genes associated with their function as coactivator and in chromatin remodeling seems to play an important role in the signature induced by pregnancy in the breast epithelial cells. One of them is the *p300/CBP-associated factor (PCAF)* that is significantly upregulated in the epithelial cells of the parous breast tissue (7-9). *PCAF* is a coactivator of the tumor suppressor, p53. *PCAF* participates in p53's transactivation of target genes through acetylation of both bound p53 and histones within p53 target promoters (57). The role of *p300/CBP-associated factor* in the differentiated breast epithelial cells of parous women could be similar to the effect of trans-retinoic acid (ATRA) treatment on metastatic breast cancer cells that, by increasing the protein levels of the histone acetyl transferases p300 and CBP, suppresses the level of histone deacetylase and increases the level of acetylated histone H4. ATRA also has been shown to decrease Bcl-2 and VEGF and increase *BAX* (58). *BAX* is upregulated in the parous breast epithelial cells. *PCAF* has been considered part of the genomic signature of the Stem cell 2 (7-9).

ID4 (Inhibitor of DNA binding 4) is a member of the Id family of proteins (Id1-Id4), which function as dominant-negative regulators of basic helix-loop-helix transcription factors and are involved in numerous cell processes, including cell proliferation, and differentiation (59). Id4 is constitutively expressed in the normal human mammary epithelium but is suppressed in breast carcinomas and preneoplastic lesions supporting a possible role of Id4 as a tumor suppressor factor in the human breast (59,60). Primary breast cancers have low or no expression of ID4 protein (61) and *ID4* has also been considered a putative tumor-suppressor gene that is methylated in most mouse and human leukemias (62-65).

Altogether our data indicate that early first full-term pregnancy induces in the breast epithelia a specific genomic profile that can be identified in the postmenopausal breast and that makes these epithelial cells different to parous breast tissue from women with breast cancer. This genomic signature allows us to evaluate the degree of mammary gland differentiation induced by pregnancy and it could be the signature postulated for the Stem cell 2 (7-9,66). This signature could help to predict in which woman parity is protective, and it can be used as a biomarker for evaluating preventive agents.

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