Genomic signature induced by pregnancy in the human breast

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Abstract. We have postulated that the lifetime protective effect of an early pregnancy against breast cancer is due to the complete differentiation of the mammary gland characterized by a specific genomic signature imprinted by the physiological process of pregnancy. For demonstrating this hypothesis we compared the genomic profile of the epithelium and the stroma of normal breast tissues from reduction mammoplasties performed in postmenopausal parous and nulliparous women. The epithelium and the stroma were separately dissected using laser capture microdissection (LCM) and the RNA of each compartment and each sample was isolated, amplified using PCR methodology, and hybridized to cDNA glass-microarrays containing 40,000 human cDNA features. The separation of the epithelial compartment from the interlobular stroma of Lob 1 using LCM allowed us to determine that the epithelial component contained 4,828 genes that were equally expressed in both nulliparous and parous women. There were 73 known genes that included immune-modulation-, DNA repair-, programmed cell death-, chromatin remodeling- and transcription-related genes, whereas in the breast of nulliparous women there were 20 different known genes that were upregulated. Our data provide evidence that breast tissues of postmenopausal parous women express in both the epithelial and the stromal compartments numerous genes that differ significantly from those present in breast tissues of postmenopausal nulliparous women, which could be important contributors to the genomic signature induced by an early full term pregnancy.

Introduction

Worldwide epidemiological data indicate that breast cancer risk is more prevalent in nulliparous women; whereas the lifetime risk is decreased in women who gave birth to a child when they were younger than 24 years of age (1-3). Increasing number of pregnancies and breastfeeding confer additional protection, even in carriers of the breast cancer gene BRCA1 (2).

The protection conferred by pregnancy has been in great part explained by studies in rodent experimental models (4-7), in which the induction of differentiation of the mammary gland by full term pregnancy prevents cancer initiation (4). Interestingly enough, the same preventive effect can be obtained by treatment of virgin rats with the placental hormone human chorionic gonadotropin (hCG) (7), or with the ovarian steroid hormones estrogen and progesterone (8). Our studies have led us to conclude that both parity and the hormones of pregnancy, even in the absence of conception, are important modulators of the pattern of mammary development, creating a permanent genomic signature that is responsible of a lifetime refractoriness to chemical carcinogenesis (9). In women, the first pregnancy stimulates the differentiation of the immature lobule type 1 (Lob 1) into the more developed lobules type 2 and 3 (Lob 2, Lob 3) (9), which attain their maximal development as lobule type 4 (Lob 4) during lactation. At post-weaning all the lobular structures of the breast regress to a resting condition (10). Further regression occurs at menopause, a period of life in which the breast of both nulliparous and parous women contains only Lob 1 (10). However, despite the similarity in the lobular composition of the breast, the risk of developing breast cancer is higher in nulliparous than in parous women. This difference is an indication that Lob 1, which in nulliparous women never completed the process of differentiation, are biologically different because they have retained a high concentration of Stem Cells 1, epithelial cells still susceptible to undergo neoplastic transformation (11,12). Lob 1 found in the breast of early parous postmenopausal women, on the other hand, are composed of Stem Cells 2, an epithelial cell population that is refractory to transformation (11,12). These data provide a powerful rationale for characterizing the genomic signature of normal breast tissues obtained from postmenopausal women with a history of one or more early full term pregnancies for comparison with the gene expression of normal breast tissues from nulliparous postmenopausal women that is the subject of the present work.

Materials and methods

This study was carried out using normal breast tissues obtained from reduction mammoplasties that were performed for cosmetic reasons in postmenopausal parous and nulliparous women (Table I). The parous group consisted of five postmenopausal women free of mammary pathology who had

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Table I. Characteristics of the samples under study.

Patient ID	Age	Reproductive history	Laser capture microdissection	RNA (ng/μl)	aRNA (ng/μl)	aaRNA (ng/μl)
846	56	Parous	Lobule type 1	20.00	58.18	1,690.68
			Interlobular stroma	27.00	93.03	1,500.00
641	60	Parous	Lobule type 1	12.80	105.00	1,927.30
			Interlobular stroma	14.06	92.30	1,955.00
387	58	Parous	Lobule type 1	23.30	178.00	1,284.00
			Interlobular stroma	28.60	179.00	1,472.00
3302	57	Parous	Lobule type 1	21.30	97.00	1,800.00
			Interlobular stroma	39.60	100.00	815.00
33	55	Parous	Lobule type 1	23.00	208.00	1,260.00
			Interlobular stroma	50.70	440.00	1,536.00
426	56	Nulliparous	Lobule type 1	39.68	138.00	1,536.00
			Interlobular stroma	41.70	178.00	1,260.00
527	58	Nulliparous	Lobule type 1	37.20	57.40	892.00
			Interlobular stroma	30.70	51.10	1,460.00

completed a first full term pregnancy (FFTP) before age 24. They ranged in age from 56 to 60 years. The nulliparous group consisted of two postmenopausal women that were 56 and 58 years old, respectively. Postmenopause was defined as at least 1 year since last menses if menopause occurred naturally or if the basal serum follicle stimulating hormone (FSH) was ≥40 ng/ml if the participant was <60 years old. None of these women had a familial history of breast cancer, received hormone replacement therapy, or had had any previous surgical procedures for benign or malignant diseases of the ovaries or the breast. Each sample was obtained after each donor had signed their respective informed consent forms that were approved by the Fox Chase Cancer Center's Human Subjects Protection Committee.

Fragments of breast tissue obtained from reduction mammoplasty specimens were fixed in 70% ethanol within 10 min of the surgical procedure. The tissues dehydrated, embedded in paraffin, sectioned at eight-micron thickness and stained with hematoxylin and eosin, followed by 5 sec dehydration steps in 70, 95 and 100% ethanol. One section was coverslipped for evaluation of the development of the breast and assessment of the ratio of ductal/lobular development and the normality of the tissue, following criteria described elsewhere (10). Additional sections were air-dried and laser microdissected using a PixCell II LCM system (Arcturus Engineering, Mountain View, CA). Epithelial cells from lobule type 1 (Lob 1) and interlobular stroma were obtained in sixtuplicate from each breast sample (Fig. 1).

Total RNA was isolated using TRIzol (Invitrogen, Inc.) separately for each capture and then pooled for each breast sample. The concentration and the quality of the pooled RNA were measured using a spectrophotometric method (Nanodrop Technologies, Inc.). Total RNA from each sample and a human universal reference (Stratagene Inc., CA) was amplified using a dT-T7-RNA amplification method.

Table I shows the RNA concentration (ng/μl) before and after PCR amplification. For each sample the quality and integrity of the amplified RNA (aRNA) were verified by real-time RT-PCR, using GAPDH and 18S genes as reference. Probes were constructed using indirect labeling with amino-allyl-d/TP method using 5-10 μg of aRNA. The probes were cleaned with a Qia-quick PCR purification kit (Qiagen Inc., Valencia, CA) and pippeted onto microarrays, coverslipped, and the slides were placed in a hybridization chamber (Corning, Corning, NY). Arrays were incubated in a 42˚C water bath for 16-24 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 dried by centrifugation for 7 min at 800 rpm (130 g) at room temperature. Arrays were read with an Affymetrix 428 fluorescent scanner (MWG, CA). The resulting images were analyzed using IMAGENE version 5.5 and GeneSight version 4.2 software (Biodiscovery, CA). The glass microarrays were hybridized in the red channel (Cy5) with the sample and in the green channel (Cy3) with the human universal RNA reference (Stratagene Inc.). Gene expression was studied in the seven samples by triplicate using cDNA microarrays, which were prepared by robotically spotting 40,000 human cDNA on glass microscope slides (Fox Chase Cancer Center-NCI supported Microarray Facility). The cDNA included approximately 28,000 genes representing characterized human proteins and 10,000 identified by ESTs; the remaining spots contained control genes. The identities of the cDNA have been sequence-verified. The intensity of the spots was measured using IMAGENE software, which consists of a collection of algorithms, each one performing a step in the intensity extraction process to produce results that are reliable and robust, giving one confidence in the data that are imported to the data analysis software. The algorithm used in this particular work was the most conservative of error between universal error model and propagated error. Spots were defined

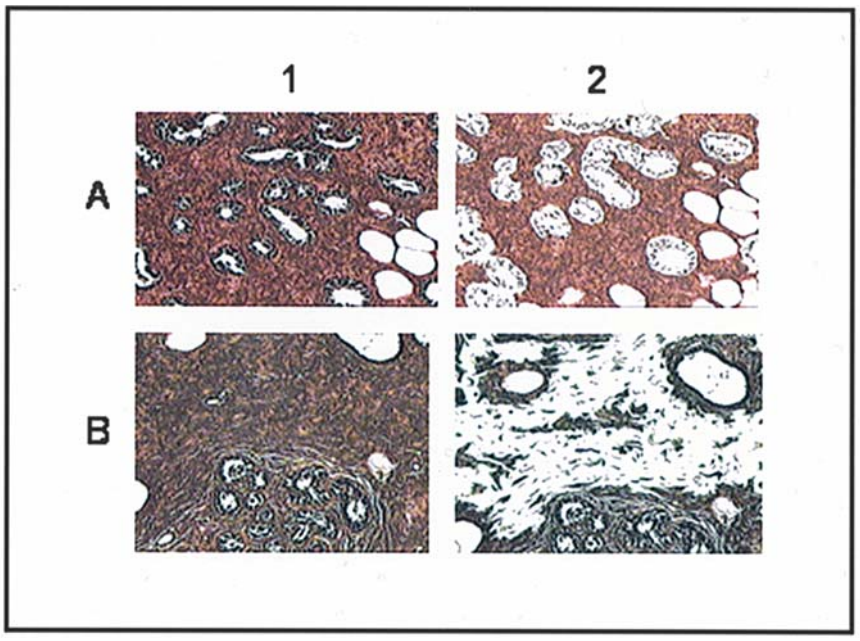

Figure 1. Laser capture microdissection (LCM) of the human breast. A-1, Ductules of lobules type 1 before LCM; A-2, Ductules of lobules type 1 after LCM; B-1, Interlobular stroma of Lob 1 before LCM; B-2, Interlobular stroma of Lob 1 after LCM. Sections stained with hematoxylin and eosin. x10.

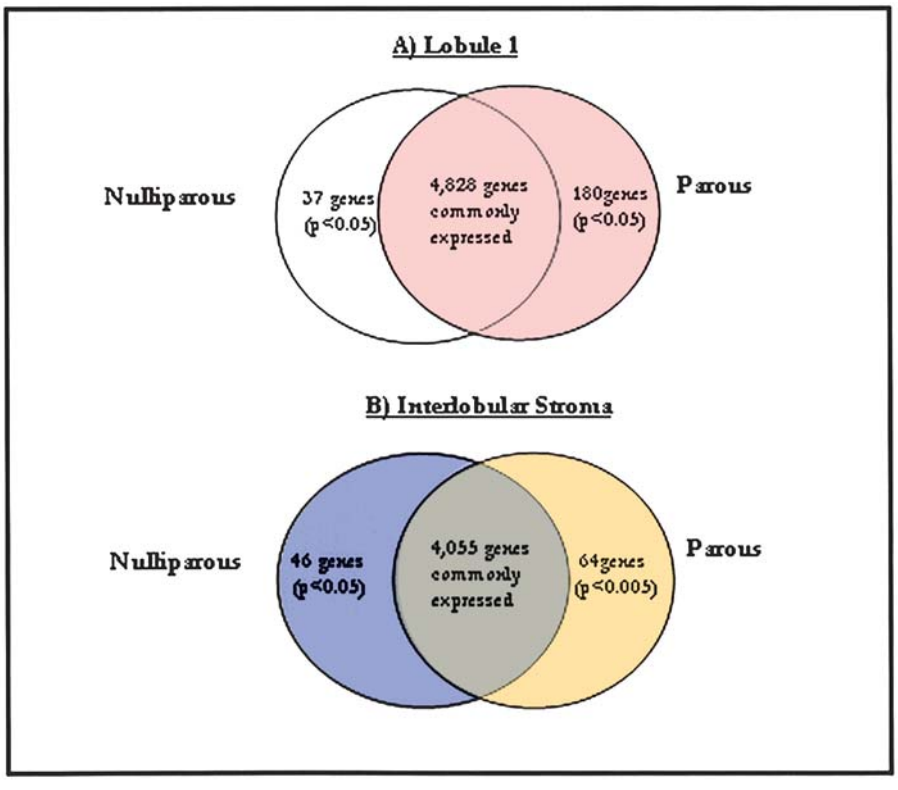

Figure 2. Genes differentially expressed between nulliparous and parous women. A, Number of genes expressed in epithelium from lobule 1. Overlapping circles, genes commonly expressed; left circle, unique genes expressed in the nulliparous breast and right circle, unique genes expressed in the parous breast. B, Number of genes expressed interlobular stroma.

using a whole spot method rather than measuring the intensity in the center of the spot. Spots with pixels with very low or high intensity were then removed from the defined features and background. Spots with saturated pixels were also flagged. Means and standard deviations were calculated on the remaining inliers pixels within the spot and background. Log ratios of the red and green channels were calculated, as well as a log ratio error and a P-value for each feature. These metrics assessed the level of confidence in order to determine if one gene was or not differentially expressed in this experiment. For gene normalization we selected the rank consistency filter, which has selected genes that fall within the central

tendency of the data by observing consistent trends between the red and green channels. The Lowess normalization method that calculates the normalization curve that measures the potential log ratio bias across the entire range of spot intensities was selected as the normalization method. After Lowess normalization, the data were analyzed using GeneSigh 4.2 software (Biodiscovery, CA), eliminating all the outlier spots with Flags 1-5. Using the confidence analysis with 99% level we selected only those genes that were differentially expressed between same tissue compartments in each group, selecting a cut off of ±1.5 to 2.0 log up or down with respect to the reference.

In Table II are listed the genes whose expression was validated using real time RT-PCR. TaqMan primer and probe sets sequences utilized are described in Table II. A ready to use primer and probe set pre-designed by Applied Biosystems (Assay-on demand Gene Expression Product from ABI) were used for the detection of those genes expression. Commercially available primers and probes for 18S were used for normalization. These probes were labelled with a FAM dye and for avoiding competition in the multiplex PCR reaction tube; the concentrations of the primers were limited. All RT-PCR reactions were performed on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using the fluorescent TaqMan methodology (TaqMan One Step RT-PCR Master Mix Reagents, Applied Biosystems). The PCR cycle at which the fluorescence arises above the background signal is called the cycle threshold (Ct); 100 ng of amplified RNA in a total volume of 50 μl were used for each RT-PCR reaction. Primer and probe concentrations for the target gene were optimized according to the manufacturer's procedure. Each gene was analyzed in triplicate, normalized against 18S as a control gene and expressed in relation to a calibrator sample. As described by Livak and Schmittgen (13), results were expressed as relative gene expression (RGE) using the $\Delta\Delta$Ct method.

Results

The separation of the epithelial compartment from the interlobular stroma of Lob 1 using LCM (Fig. 1) allowed us to determine that the epithelial component contained 4,828 genes that were equally expressed in both nulliparous and parous women (Fig. 2). There were 73 known genes (Table III), and 107 ESTs upregulated in the breast tissues of parous women. In the breast of nulliparous women there were 20 known genes and 17 ESTs that were upregulated. Among the known genes that are differentially expressed between the nulliparous and parous epithelial breast tissue we have separated them according to some putative functional groups (Tables III and IV).

The analysis of the gene expression profile of the interlobular stroma revealed that there were 4,055 genes commonly expressed in both groups. There were 17 known genes and 47 EST upregulated in the breast stroma of parous women. In nulliparous women, there were 6 known genes and 40 ESTs upregulated (Fig. 2, Table IV).

Discussion

Immune surveillance-related gene transcripts. Among five gene transcripts that are involved in immune surveillance, four were upregulated and one down-regulated in the parous epithelial cells (Table III). The Sterile alpha and TIR motif containing 1 (SARM1) (14), known to be instrumental in inducing a signaling cascade upon recognition of specific ligand triggering innate immune responses, the T cell receptor V-beta 1 (TCRB), which recognizes specific cytotoxic T lymphocytes in a major histocompatibility complex (MHC)-unrestricted fashion both in breast tumors (15) and in pure epithelial cell populations obtained by LCM from normal breast tissues. The epithelial origin of this transcript is confirmed by its absence in the stromal component of the same breast tissues. The MHC class I HLA-A24 that is also upregulated in the epithelial component of the parous breast, has the capacity to elicit anti-tumor cytotoxic T lymphocytes (CTL) *in vitro* (16). HLA class I antigen down-regulation in ovarian carcinomas, on the other hand, is associated with worse clinical course, a phenomenon that might reflect an escape of tumor cells from immune recognition and destruction (17). The interleukin 22 receptor, alpha 2 (IL-22), also detected to be upregulated in the cDNA array of parous women's epithelial cells and confirmed by RT-PCR (Table II), is a member of interferon/IL-10 family that plays an important role in immune response through activation of the STAT3 signal transduction pathway (18). In the nulliparous breast there is upregulation of the dendritic cell protein (GA17). Although the role of this protein in the epithelial cells of the nulliparous breast requires further investigation, the finding that primary breast carcinomas produce soluble factors that may attract dendritic cells and their precursors *in vivo*, and promote the differentiation of the latter into Langerhans cells and immature dendritic cells with altered functional capacities may identify a potential role of this protein in cancer development (19). Altogether our findings support the concept that the differentiation of the breast induced by pregnancy stimulates an immuno surveillance process, as supported by findings in the rodent model (6,20).

DNA repair related gene transcripts. Several DNA repair related genes were differentially expressed in breast epithelial cells from parous women (Table III). They included the Excision Repair Cross-Complementing Rodent Repair Group 2 (ERCC2) and 6 (PGBD3), both major DNA repair proteins that are involved in nucleotide excision repair and basal transcription (Tables II and III) (21); the ribosomal protein S3 (RPS3), a multifunctional ribosomal protein that is a structural and functional component of the ribosome, a DNA repair endonuclease involved in the DNA base excision repair pathway and in apoptosis (22), and the RAD51-like 1 that is involved in homologous recombination repair and is an essential process for the maintenance and variability of the genome (Table II) (23). 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 and support previous findings that *in vivo* the ability of the cells to repair carcinogen-induced damage is more efficient in the mammary gland of parous animals (24).

Programmed cell death related gene transcripts. In breast tissues of postmenopausal women four gene transcripts that play a functional role in program cell death were significantly upregulated (Table III). They included the ribosomal protein

Table II. Real time PCR validation.

Gene	Assay ID	Reporter 1	Context sequence	Real time PCR Nulliparous epithelium	Real time PCR Parous epithelium	cDNA microarray Nulliparous epithelium	cDNA microarray Parous epithelium
p300/CBP-associated factor	Hs00187332_m1	FAM	GCCATGCCCTAG CTGCTCATGTTTC	1.0±0.0	19.4±5.45	-0.36±0.44	1.69±0.03
Fatty acid binding protein 6, ileal (gastrotropin)	Hs00155029_m1	FAM	CATGAAGCTCCT TGGGATCTCCAGC	1.0±0.0	61.3±16.1	0.95±0.22	1.60±0.09
Ubiquitin-conjugating enzyme E2D 2 (UBC4/5 homolog, yeast)	Hs00366152_m1	FAM	TTTCAAAAGTACT CTTGTCCATCTG	1.0±0.0	2.20±0.87	0.98±0.34	1.55±0.22
Excision repair cross-complementing rodent repair deficiency, complementation group 6	Hs00164491_m1	FAM	GCGCGGCCCTG GGTAGTCTGTAGAG	1.0±0.0	9.99±2.34	0.6±0.49	1.67±0.41
Somatostatin receptor 1	Hs00265617_s1	FAM	TGCGTCCCAGAA CGGGACCTTGAGC	1.0±0.0	1.06±0.24	0.61±0.37	1.59±0.23
BCL2-associated X protein	Hs00180269_m1	FAM	GTGCTCAAGGCC CTGTGCACCAAGG	1.0±0.0	16.0±5.70	0.95±0.13	1.91±0.15
RAD51-like 1 (*S. cerevisiae)*	Hs00172522_m1	FAM	TCCCTCACAGAG ATTACAGGTCCAC	1.0±0.0	8.20±2.32	0.79±0.38	2.01±0.06
Cryptic gene 1	C. assay	FAM	CCATGACACCTG GCTGCCCAAGAA	18.60±5.32	1.0±0.0	1.68±0.47	-0.11±0.3
Interleukin 22 receptor, alpha 2	Hs00364814_m1	FAM	CTTACTGGTGTA GCAGGAACTCAGT	1.0	11.87	0.79±0.31	2.49±0.41
LIM domain binding 2	Hs00609711_m1	FAM	GCTCCGCCAGCA GAACCCACAAGGC	1.0±0.0	3.51±1.01	0.92±0.27	1.82±0.11
Odz, odd Oz/ten-m homolog 3 (*Drosophila*)	Hs00216490_m1	FAM	TTTGGCCGCAA GCTCAGGGTTAATG	1.0±0.0	20.38±2.30	0.8±0.17	1.91±0.05
Suppressor of hairy wing homolog 4 (*Drosophila*)	Hs00214302_m1	FAM	TGCCTCCTGGAA CAAAAGTTACTAT	1.0±0.0	0.06±0.12	0.82±0.00	1.50±0.10
Suppressor of Ty 3 homolog (*S. cerevisiae*)	Hs00269211_m1	FAM	CTGCTGAGAGCA CTGCAGCCTGTGG	1.0±0.0	3.31±0.02	0.89±0.03	1.64±0.11

S3 (RPS3), described in the previous section (22), the likely ortholog of the mouse putative IKK regulator SIMPL, the BCL2-associated X protein, and the eukaryotic translation initiation factor 3 (EIF3S5). Overexpression of SIMPL leads to the activation of NF-κB-dependent promoters, and of tumor necrosis factor α (TNF-α), a cytokine that acts as an important mediator of the apoptotic process and that has selective cytotoxicity against malignant breast tumor cells (25). The inactivation of SIMPL inhibits IRAK/mPLK as well as TNF receptor type I-induced NF-κB activity (25). We have confirmed by RT-PCR that the level of expression of BCL2-associated X protein, another gene controlling programmed cell death, is 16-fold increased in the parous epithelial cells (Table II). The transcription of BCL2-associated X protein is stimulated by the active p53, which includes the pro-apoptotic gene p21 (26). Our data indicate that in the breast epithelial cells of parous women there is a selective expression of genes controlling programmed cell death, similar to changes previously reported in the post pregnant rat mammary gland (10,27).

Table III. Genes differentially expressed in the lobules type 1 of the parous and nulliparous women.

Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
	Gene transcripts with immune-surveillance function				
AI300055	Sterile alpha and TIR motif containing 1	SARM1	1.96±0.09	1.00±0.36	0.011
AA909476	T cell receptor V-beta 1	TCRB	1.69±0.09	0.98±0.19	0.0044
AA932515	Interleukin 22 receptor, alpha 2	IL22RA2	2.49±0.41	0.79±0.31	0.0140
AA865910	MHC class I HLA-A24 glycoprotein mRNA, 3' end	HLA-A24	1.59±0.04	-0.41±0.03	0.0001
AA884321	Dendritic cell protein	GA17	0.92±0.35	1.63±0.29	0.055
	Gene transcripts related to DNA repair damage to DNA response to stress				
Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
AA926649	Excision repair cross-complementing rodent repair deficiency, complementation group 2 (*xeroderma pigmentosum* D)	ERCC2	1.62±0.13	0.94±0.32	0.027
AI076461	RAD51-like 1 (*S. cerevisiae*)	RAD51L1	2.01±0.06	0.79±0.38	0.014
AA907277	Excision repair cross-complementing rodent repair deficiency, complementation group 6	PGBD3	1.67±0.41	0.6±0.49	0.044
AA708793	TP53-activated protein 1		-0.87±0.24	1.77±0.1	0.0001
AI027434	*Homo sapiens*, similar to NBR2, clone MGC:5031, mRNA, complete cds	LOC51326	1.60±0.43	0.83±0.18	0.046
AA046713	Ribosomal protein S3	RPS3	1.74±0.11	0.77±0.31	0.014
	Gene transcripts with programmed cell death function				
Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
AI168124	Likely ortholog of mouse putative IKK regulator SIMPL		1.67±0.15	0.98±0.31	0.027
AI565203	BCL2-associated X protein	BAX	1.91±0.15	0.95±0.13	0.0011
AI096618	Eukaryotic translation initiation factor 2, subunit 5 epsilon, 47 kDa	EIF3S5	1.59±0.34	0.5±0.2	0.0052
	Gene with, transcription, translation, and chromatin remodeling function				
Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
AA973690/ AI733278	Transcription elongation regulator 1-like	TCERG1L	2.10±0.34	0.98±0.38	0.019
AI049803	LIM domain binding 2	LDB2	1.82±0.11	0.92±0.27	0.0059
N68754	Heart and neural crest derivatives expressed 2		0.87±0.36	1.52±0.02	0.0003
AI122680	Bromodomain adjacent to zinc finger domain, 2A	BAZ2A	1.69±0.38	0.90±0.16	0.03
AI200090	Processing of precursor 5, ribonuclease P/MRP subunit (*S. cerevisiae*)	POP5	1.50±0.08	0.88±0.26	0.016
AI066778	Zinc finger CCCH type domain containing 5	ZC3HDC5	1.56±0.08	0.83±0.16	0.0023
AI636025	Zinc finger protein and BTB domain containing 11	ZBTB11	1.64±0.22	0.66±0.22	0.0052
AI363907	HMT1 (hnRNP methyltransferase, *S. cerevisiae*)-like 1	HRMT1L1	1.62±0.11	0.65±0.18	0.0013
AI292221	BarH-like homeobox 1	BARX1	1.57±0.26	0.62±0.3	0.015
AI056507	SOX2 overlapping transcript (non-coding RNA)	SOX2OT	1.54±0.07	0.6±0.26	0.0036
AI191937	SRY (sex determining region Y)-box 30	SOX30	1.51±0.23	0.54±0.16	0.0037
AI096618	Eukaryotic translation initiation factor 3, subunit 5 epsilon, 47kDa	EIF3S5	1.59±0.34	0.5±0.02	0.0052
AA703557	BTB and CNC homology 1, basic leucine zipper transcription factor 2		0.93±0.19	1.83±0.28	0.01
AA169339	Integral membrane protein 2B [AT rich interactive domain 5B (MRF1-like)]	ARID5B	0.96±0.23	2.09±0.47	0.0140
N74637	p300/CBP-associated factor	PCAF	1.69±0.03	-0.36±0.44	0.0013
R40322	Suppressor of hairy wing homolog 4 (*Drosophila*)	SUHW4	1.50±0.10	0.82±0.00	0.0003
AA013172	Suppressor of Ty (*S. cerevisiae*) 3 homolog	SUPT3H	1.64±0.11	0.89±0.03	0.0003
AA704582	Nuclear domain 10 protein	NDP52	1.97±0.14	0.97±0.43	0.019
	Gene transcripts related to growth factor and signal transduction pathway				
Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
AI082431	TBC1 domain family, member 16	TBC1D16	1.88±0.06	0.96±0.02	0.0001
AI028704	G protein-coupled receptor 87	GPR87	2.21±0.20	1.00±0.40	0.0094
H98645	Protein kinase C, nu	PRKD3	1.52±0.08	0.99±0.24	0.024
AA962534	Cryptic gene		-0.11±0.3	1.68±0.47	0.0011
AA923583	Odz, odd Oz/ten-m homolog 3 (*Drosophila*)		0.8±0.17	1.91±0.05	0.0004
N67017	Leukemia inhibitory factor receptor	LIFR	1.52±0.31	0.97±0.05	0.041
AI342037	Rho GTPase activating protein 8	ARHGAP8	1.96±0.49	0.66±0.17	0.012
AI129156	Somatostatin receptor 1	SSTR1	1.59±0.23	0.61±0.37	0.018
AI189552	G protein-coupled receptor 30	MGC11257	1.69±0.18	0.57±0.1	0.0006
AA458945	RAB30, member RAS oncogene family	RAB30	1.52±0.27	0.55±0.2	0.0074
H98072	Ran GTPase activating protein 1	RANGAP1	0.92±0.27	1.98±0.19	0.005
AA287380	KH domain containing, RNA binding, signal transduction associated 1	KHDRBS1	0.99±0.33	1.7±0.36	0.068
AI650675	Neuromedin B	NMB	1.62±0.14	0.63±0.23	0.0031

Table III. Continued.

Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
	Gene transcripts related to growth factor and signal transduction pathway				
AA455225	Human clone 23721 mRNA sequence	PTPN11	1.69±0.24	-0.19±0.37	0.0018
AA487575	Calcium and integrin binding protein (DNA-dependent protein kinase interacting protein)		0.62±0.1	1.52±0.13	0.0007
H64261	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit		-0.72±0.21	1.75±0.28	0.0002
AA278852	Thyroid receptor interacting protein 15	COPS2	1.66±0.05	0.94±0.36	0.026
AI039416	Triadin	TRDN	1.69±0.15	0.91±0.12	0.0023
	Gene transcripts related to transport and cell trafficking				
Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
AI183992	Solute carrier family 4, sodium bicarbonate cotransporter, member 9	SLC4A9	1.90±0.30	0.99±0.07	0.0064
AI223142	Calcium channel, voltage-dependent, alpha 2/delta 3 subunit	CACNA2D3	1.76±0.09	0.95±0.13	0.0008
AA278852	Thyroid receptor interacting protein 15	COPS2	1.66±0.05	0.94±0.36	0.026
AI203028	Cytochrome b-245, beta polypeptide (chronic granulomatous disease)	CYBB	1.64±0.13	0.90±0.23	0.0081
AA464566	Low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)	LRP1	1.64±0.48	0.89±0.24	0.074
AA995875	Aquaporin 2 (collecting duct)	AQP2	1.54±0.29	0.86±0.36	0.063
AI263342	ESTs, weakly similar to non-syndromic hearing impairment protein (*Homo sapiens)*		1.57±0.11	0.84±0.11	0.0012
AA999850	Kidney-specific membrane protein Transmembrane protein 27	TMEM27	1.67±0.33	0.81±0.18	0.017
AA973344	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24	SLC25A24	1.59±0.29	0.79±0.19	0.0471
AI218581	ATPase, aminophospholipid transporter (APLT), Class I, type 8A, member 1	APLT	1.82±0.27	0.68±0.28	0.0074
H18668	Vesicle transport through interaction with t-SNAREs homolog 1A (yeast)	VTI1A	1.51±0.02	0.66±0.17	0.0009
AI341839	Solute carrier family 4, anion exchanger, member 2 (erythrocyte membrane protein band 3-like 1)	SLC4A2	1.53±0.21	0.52±0.2	0.0036
AI343847	Solute carrier family 26, member 7	SLC26A7	2.42±0.22	0.3±0.17	0.0002
AI280106	Transient receptor potential cation channel, subfamily C, member 5	TRPC5	1.00±0.2	1.52±0.2	0.034
AA931725	Secreted modular calcium-binding protein 2		0.85±0.23	1.52±0.28	0.033
AA401349	Myosin VB		0.57±0.22	1.58±0.26	0.007
AA864323	Component of oligomeric golgi complex 8		-0.11±0.3	1.57±0.41	0.0047
AI247494	HIV-1 Rev binding protein-like	HRBL	1.64±0.15	0.80±0.32	0.014
	Gene transcripts related to cell proliferation and differentiation				
Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
AI203953	Similar to MAP/microtubule affinity-regulating kinase 1		1.60±0.31	0.89±0.48	0.1
AI080392	Calcyphosphine 2	CAPS2	1.60±0.26	0.98±0.42	0.095
AI208908	Fatty acid binding protein 6, ileal (gastrotropin)	FABP6	1.60±0.09	0.95±0.22	0.0091
AI359037	Fatty acid binding protein 5 (psoriasis-associated)	STX3A	1.55±0.24	0.77±0.06	0.0612
AA464566	Low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)	LRP1	1.64±0.48	0.89±0.24	0.074
AA935809	Male sterility domain containing 1	MLSTD1	1.67±0.1	0.71±0.11	0.1474
AI159884	Neuroligin 3	NLGN3	1.95±0.3	0.76±0.29	0.0766
	Gene transcripts related to protein synthesis and degradation, uptake, and cell metabolism				
Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
300-301 AI248214	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)	UBE2D3	1.55±0.22	0.98±0.34	0.069
AI269774	Phytanoyl-CoA hydroxylase (Refsum disease)		1.55±0.11	0.86±0.49	0.076
AI434448	Galactosamine (N-acetyl)-6-sulfate sulfatase (Morquio syndrome, mucopolysaccharidosis type IVA)		1.57±0.06	0.64±0.15	0.0006
AI146457	3'-phosphoadenosine 5'-phosphosulfate synthase 1	PAPSS1	1.53±0.29	0.61±0.41	0.035
AI077645	Glutathione S-transferase omega 2	GSTO2	1.56±0.12	0.59±0.15	0.001
W69460	Phosphatidylserine decarboxylase		0.89±0.48	2.15±0.46	0.031
AA401477	Glucosidase, beta (bile acid) 2		0.78±0.48	2.24±0.47	0.02
AA401349	Myosin VB		0.57±0.22	1.58±0.26	0.007
AA887547-	Carbohydrate (chondroitin 4) sulfotransferase 13		-0.91±0.14	1.56±0.06	0.0001
AA999850	Kidney-specific membrane protein Transmembrane protein 27	TMEM27	1.67±0.33	0.81±0.18	0.017
AA774044	Thrombospondin 3	THBS3	1.53±0.16	-0.43±0.47	0.0024
R99314	Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3	ALS2CR3	2.33±0.31	-0.09±0.45	0.0015
AI251734	Salivary proline-rich protein 5	PROL3	1.86±0.18	0.82±0.15	0.0015
AI147738	Fucosyltransferase 10 alpha (1,3) fucosyltransferase	FUT10	1.77±0.26	0.73±0.0	0.0796

Table IV. Genes differentially expressed in the stroma of the breast of nulliparous and parous women.

Known genes					
Gene ID	Gene name	Symbol	Parous	Nulliparous	P-value
AA973492	PFTAIRE protein kinase 1	PFTK1	0.98±0.31	1.79±0.13	0.0161
AA621216	Human epididymis-specific 3 beta	FAM12B	0.96±0.50	2.00±0.17	0.0320
AI198232	Ligand of neuronal nitric oxide synthase with carboxyl-terminal PDZ domain	CAPON	0.92±0.34	2.20±0.45	0.0025
AI221925	Homolog of mouse C2PA	RGS3	0.69±0.47	1.92±0.09	0.0304
T91966	Sorting nexin 17		0.27±0.39	1.60±0.41	0.0011
AA461332	Chloride channel 3	CLCN3	0.17±0.52	1.53±0.25	0.0129
AA156964--	Neuropilin 2	NPN2	2.46±0.59	0.98±0.15	0.0282
W37375--	DnaJ (Hsp40) homolog, subfamily C, member 8		2.26±0.58	0.98±0.41	0.0058
AA812973	Chaperonin containing TCP1, subunit 6B (zeta 2)	CCT6B	2.31±0.58	0.95±0.17	0.0290
AA488901	Hypothetical protein FLJ13660 similar to CDK5 activator-binding protein C53	CDK5RAP3	2.56±0.51	0.94±0.49	0.0001
AA022951	Keratin, hair, basic, 5		1.73±0.41	0.91±0.16	0.0296
AA773478	Adaptor-related protein complex 1, mu 2 subunit	AP1M2	2.01±0.49	0.88±0.39	0.0026
AA477283	Kallikrein 11	KLK11	1.81±0.58	0.85±0.27	0.0330
AA465723	Protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	PPM1G	1.90±0.48	0.85±0.27	0.0131
AA866113	Amyloid beta (A4) precursor protein-binding, family B, member 2 (Fe65-like)	APBB2	2.16±0.47	0.84±0.33	0.0037
W47116	Human DNA sequence from clone 71L16 on chromosome Xp11. Contains a probable Zinc Finger protein (pseudo)gene, an unknown putative gene, a pseudogene with high similarity to part of antigen KI-67, a putative Chondroitin 6-Sulfotransferase LIKE gene an	SLC9A7	2.22±0.44	0.81±0.20	0.0095
AA44382	Sialyltransferase 6 (N-acetyllacosaminide alpha 2,3-sialyltransferase		1.96±0.52	0.76±0.42	0.0023
R08311	Nuclear factor I/X (CCAAT-binding transcription factor)		1.97±0.46	0.70±0.40	0.0007
AA426227	Uridine monophosphate synthetase (orotate phosphoribosyl transferase and orotidine-5'-decarboxylase	UMPS	1.66±0.57	0.70±0.18	0.0509
AA045458	M-phase phosphoprotein homolog	ZFR	1.85±0.43	0.68±0.04	0.0351
AA436187	Integrin, alpha M [complement component receptor 3, alpha; also known as CD11b (p170), macrophage antigen alpha polypeptide]		1.98±0.56	0.65±0.19	0.0248
AI057267	Thy-1 cell surface antigen		1.58±0.48	0.57±0.37	0.0039
T99236	Jun B proto-oncogene	JUNB	1.55±0.38	0.56±0.37	0.0000

Genes with transcription, translation and chromatin remodeling function. Sixteen genes, the largest group of gene transcripts that was significantly different between the epithelial cells of the parous and the nulliparous breast, belong to the regulators of transcription and translation factors (Table III). They include the LIM domain binding 2, whose proteins contribute to cell fate determination and regulation of cell proliferation and differentiation, phenomena mediated by activation of transcription of cell-type-specific genes that requires cooperation with other nuclear factors (28). Other upregulated genes are SOX2 overlapping transcript and SOX30 or sex determination region Y (SRY), which belong to a family of transcription factors that interacts with various partners, participating in the spatio-temporal order of the occurrence of cell differentiation (29). SOX2 that is expressed by self-renewing and multipotent cells of the embryonic neuro-epithelium is considered to be a marker of stem cells (30). The SOX30 has been shown to be specifically expressed in normal testes, but not in maturing germ cell-deficient testes (31), observations that suggest that the expression of SOX30 in the breast could be a marker of differentiation and part of the signature induced by pregnancy in breast epithelial cells.

In this category might also belong the BarH-like homeobox 1 gene that was significantly upregulated in the parous breast epithelial cells. The BarH1 *Drosophila* is required for the fate determination of external sensory organs in the fly. This gene has also been identified in mice, in which it plays an important role during neurogenesis, and functionally links Barhl1 to the NT-3 signaling pathway during cerebellar development and humans (32). Although there is no knowledge of the function of this gene in the mammary gland, it can be considered that BarH1 could represent another marker of the differentiated Stem Cell 2 in the breast epithelium of the parous woman (9,11,12).

Two transcription factors that are also associated with chromatin remodeling complexes and are significantly up-regulated in the parous breast epithelial cells are the zinc finger and BTB domain containing 11 and the bromodomain adjacent to zinc finger domain 2A (BAZ2A) (Table III). Bromodomain proteins that have been identified as integral components of chromatin remodeling complexes, frequently possess histone acetyltransferase activity (33). Significantly upregulated in the epithelial cells of the parous breast tissue (Tables II and III) is the p300/CBP-associated factor (PCAF), which functions as co-activator of the tumor suppressor p53 and participates in p53's transactivation of target genes through acetylation of both bound p53 and histones within p53 target promoters (34). The fact that in the breast epithelial cells of parous women p300/CBP-associated factor can be considered as a marker of the differentiated Stem Cell 2 (9,11,12), could be similar to the differentiating effect of trans-retinoic acid (ATRA) on breast cancer cells, in which it increases p300 and CBP that suppress the level of histone deacetylase, increases the level of acetylated histone H4, decreasing Bcl-2 and increasing Bax (35).

Among the genes that regulate chromatin organization, the suppressor of Ty homolog 3 has been found to be significantly upregulated in parous breast epithelial cells (Tables II and III). Suppressor of Ty (SPT) genes, originally identified through genetic screening for mutations in the yeast *Saccharomyces cerevisiae*, restore gene expression disrupted by the insertion of the transposon Ty. Classic members of the SPT gene family encode for the histones H2A and H2B, and the TATA-binding protein (TBP). In addition, accumulating evidence suggests that SPT gene products play more diverse roles, including DNA replication, DNA recombination and developmental regulation (36). The fact that this gene that is suppressor of mutations is upregulated in the differentiated breast suggests that this function, in conjunction with that of upregulated DNA repair genes (see above) could provide a significant and unique signature to the parous breast and be functionally involved in the protection conferred by an early pregnancy against cancer in this organ.

Growth factors and signal transduction pathway-related gene transcripts. Among the 18 genes within this category that were analyzed by cDNA microarray, 12 were significantly over-expressed in the epithelial cells of the parous breast (Table III). Relevant among them are the G protein-coupled receptor 30 (GPR30) that is critical for progestin-induced growth inhibition (37) and the leukemia inhibitory factor receptor (LIF-R) a member of the interleukin 6 (IL-6) family of cytokines (38). Many effects have been ascribed to this latter gene, including the maintenance of embryonic stem cell pluripotentiality, hemopoiesis and neural differentiation through physiological activation of STAT3, which in the mammary gland is a key mediator of apoptosis (38).

Cell trafficking-related gene transcripts. There are at 15 gene transcripts that are upregulated and functionally linked to cell trafficking in the breast epithelial cells of the parous women (Table III) Among them are the water channel aquaporin-2 (AQP2) (39), which is an integral membrane protein in channeling the transfer of water and small solutes across the membrane, the solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24, solute carrier family 26, member 7 and solute carrier family 4 member 2, all genes that encode proteins involved in the active transport across biological membranes regulating the absorption, distribution, and excretion of metabolic products and an increasing number of drugs. Thus, these transporters serve as a defense mechanism to protect the tissue against toxic substrates (40). Altogether these gene transcripts must be considered part of the genomic signature induced by pregnancy in the breast epithelial cells although their specific functional role still needs to be elucidated.

Among the ESTs there are few genes whose sequence has been identified to be similar to known genes. The gene identified as AI183969 has been shown to have a similar sequence to the ADP-ribosylation factor that is significantly upregulated in the parous breast tissue. ADP-ribosylation factors (Arfs) are members of a family of Ras-related GTPases that regulate a wide variety of intracellular signaling pathways, including the regulation of membrane traffic and organelle morphology (41). The EST AI269498 has a similarity with the Neurogenic locus Notch protein precursor, which in the *Drosophila* encodes a receptor necessary for cell fate decisions within equivalence groups, such as proneural clusters (42). Whereas the function of these transcripts in the breast of parous women is still not clear, their presence strongly suggests that they may play an important regulatory function by deciding the fate of these cells under hormonal stimulation or genotoxic agents.

Genomic signature of the nulliparous breast. The number of genes that are upregulated in the breast epithelium of nulliparous women and that define the genomic signature of their nulliparous condition is less numerous than in parous women (Tables II and III). An interesting gene that is significantly upregulated in the nulliparous breast tissue is the cryptic (Cr) gene, which encodes a secreted molecule containing a variant epidermal growth factor-like (EGF) motif (43). In the mouse, Cr is not expressed in adult tissues, whereas Cr-1 is expressed at low level in various tissues, including the mammary gland. Overexpression of Cr-1 in mammary epithelial cells facilitates their *in vitro* transformation; *in vivo* Cr-1-transduced cells produce ductal hyperplasias in the mammary gland. Recombinant mouse or human crypto enhances cell motility and branching morphogenesis in mammary epithelial cells and in some human tumor cells. Expression of crypto is increased several-fold in human colonic, gastric, pancreatic and lung carcinomas, and in a variety of mouse and human breast

carcinomas and premalignant lesions. In mammary epithelial cells, part of these responses may depend on the ability of CR-1 to transactivate erbB-4 and/or fibroblast growth factor receptor 1 through a src-like tyrosine kinase (43). Another gene significantly upregulated in the nulliparous breast epithelial cells is the Odz (odd Oz/ten homolog 3 that is related to the epidermal growth factor family (Tables II and III). The product of the *Drosophila melanogaster* odd Oz (odz)/Tenascin-major (ten-m) pair-rule gene is an evolutionarily conserved function that consists of eight epidermal growth factor (EGF)-like repeats followed by a novel 1,800 amino acid polypeptide stretch unique to proteins of the Odz/Ten-m family (44). Altogether, the upregulation of these two genes in the undifferentiated cells of the nulliparous breast, which have the potential to develop malignancies, might serve as indicators of their greater susceptibility to undergo malignant transformation.

Three transcripts that are functionally connected because of their involvement in the signal transduction pathway and cell proliferation are the Ran GTPase activating protein, the AMP-activated gamma 2 protein kinase (AMPK), which under conditions of hypoxia, exercise, ischemia, heat shock, and low glucose is allosterically activated by rising cellular AMP and by phosphorylation of the catalytic α subunit, and the KHDRBS1 or KH domain containing RNA binding signal transduction associate 1 that could play an important role in cell cycle control (Table III).

An EST weakly similar to ZNF43 gene transcript was significantly upregulated in the nulliparous breast. ZNF43, a multi-zinc finger protein containing the Kruppel-associated box (KRAB) transcriptional repression domain A (45), has been reported to be expressed at high levels in proliferating Ewing-derived EW-1 cells and down-regulated in EW-1 cells induced to differentiate, findings that suggest for this gene a role in the maintenance of embryonary stem (ES) cells in an undifferentiated state (45). The role of this transcript in the breast is not known but we postulate that this could be a marker of undifferentiated cells as part of the signature of the Stem Cell 1 previously described (9,11,12).

Genomic signature of the interlobular stroma of the nulliparous and parous breast. The analysis of gene expression in the laser captured-interlobular stroma of the breast of parous and nulliparous women revealed that a significant number of genes was up- and down-regulated in both groups of women. The parous women's stroma exhibited significant upregulation of the genes listed in Table IV. Among then are:

Neuropilin 2 (Npn2) which belongs to a family of neuropilins and group A plexins. Members of this family are components of receptor complexes for class 3 semaphorins, which help to guide migration of neural progenitor cells and axonal growth cones during development, and guide regenerating axons (46). It has been reported that Npn2 deficiency inhibits VEGF-induced retinal neovascularization (47). Although we observed upregulation Npn2 in the stroma of the parous breast (Table IV), VEGF was not overexpressed, what indicates that the Npn2 gene product acts in the orientation and branching organization of the ductal structures.

Thy-1 cell surface antigen is a marker of haematopoietic progenitor cells and of stem cells in hepatoblastoma (48). The fact that its expression is induced by differentiation of keratocytes to corneal fibroblasts and myofibroblasts, suggesting a potential functional role for Thy-1 in providing a surface marker to distinguish the normal keratocyte from its repair phenotypes and that it could be involved in the maintenance of T cell homeostasis and potentiating antigen-induced T cell responses (48), suggests that in the breast stroma this gene could either increases fibroblast differentiation or acts as an immune modulator in conjunction with the other gene transcripts also upregulated in the epithelium of the Lob 1 of the parous woman (Table III).

JunB belongs to the Jun family of activating protein-1 transcription factors that are important in the control of cell growth, differentiation, and neoplastic transformation (49). The finding of upregulation of JunB in the interlobular stroma of parous women's breast is of great interest because of the important role of this gene in the control of cell proliferation and in epithelial-mesencymal interactions. JunB regulates the number of hematopoietic stem cells (49), regulates mesenchymal-epithelial interactions in the skin through the expression of interleukin-1 (IL-1)-induced keratinocyte growth factor and GM-CSF in fibroblasts, which, in turn, adjust the balance between proliferation and differentiation (49), and in human breast cancer cell lines induced to differentiate by okadaic acid the levels of junB and other early response genes are upregulated (50). These findings suggest that junB may control the stem cells present in the epithelial cell compartment of Lob 1 through its effects on mesenchymal-epithelial interactions. On the other hand, alterations in breast cytoarchitecture, changes in phenotypic properties of breast cells, and increased cell proliferation of breast cancer cells that are induced by estrogens, occur in association with down-regulation of JunB expression (51), further supporting the role ascribed above to this gene.

Among other upregulated genes in the breast stroma of parous women, the presence of integrin, α M, or CD11b, might indicate that the upregulation of this gene at the end of pregnancy (52) is not a transitory event, since it remains upregulated even at postmenopause.

The following transcripts of six known genes have been found to be significantly upregulated in the interlobular breast stroma of nulliparous women (Table IV): Cdc2-related protein kinase, named PFTAIRE protein kinase 1 (PFTK1), human epididymis-specific 3 beta (FAM12B), ligand of neuronal nitric oxide synthase with carboxyl-terminal PDZ domain (CAPON), the homolog of mouse C2PA, sortin nexin 17 and chloride channel 3 (CLCN3). Although the expression and function of these genes have been reported in various organs, their specific function in the human breast and their relationship to nulliparity is unknown.

Altogether data presented here on a limited number of cases provide evidence that breast tissues of postmenopausal parous women express in both the epithelial and the stromal compartments numerous genes that differ significantly in their level of expression from those present in breast tissues of postmenopausal nulliparous women. Although not all the genes identified have a clear function in the protection from breast cancer development conferred by pregnancy, the upregulation of genes related to immune-modulation, DNA repair, programmed cell death, chromatin remodeling and gene

transcription suggest that they are important contributors to the genomic signature induced by an early full term pregnancy.

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