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Gene expression profiling of early involuting mammary gland reveals novel genes potentially relevant to human breast cancer

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1. ABSTRACT

Mammary gland involution represents one of the most dramatic examples of programmed cell death/apoptosis and tissue regression during development, yet large gaps still exist in the understanding of the mechanisms involved, and the key factors that trigger involution, are not yet identified. With the focus on identifying "novel" genes associated with mammary gland regression, we used microarray analysis to examine differentially expressed genes during early mammary gland involution in the mouse. We then examined the relevance of candidate genes to human tumorigenesis and identified a number of genes not previously implicated or not well characterized in human breast cancer. The expression levels of these genes in human breast cancer were confirmed in breast cancer cell lines and breast tumor tissues. This pilot study demonstrates proof of principle that through the analysis of gene expression during mammary gland involution, it may be possible to identify "novel" genes relevant human breast cancer.

2. INTRODUCTION

Involution, which follows weaning, is the final stage of development in the mammary gland. It is a physiological phenomenon associated with rapid programmed cell death (apoptosis) of mammary epithelial cells followed by the remodeling and regeneration of the tissue (1). Studies indicate that a progressive gain of death signals and loss of survival signals (2,3), tightly control the process. Early involution occurs within the first 24-48 hrs (2,3). Intriguingly, if weaning is re-initiated during early involution, the process of programmed cell death is reversible. Currently, the molecular mechanism of involution is not fully elucidated and the factors, which trigger involution, are poorly understood. It has been suggested that these factors critical to involution exist in the local environment of the mammary epithelial cells (2,3). Thus the early involuting mammary gland is an ideal environment to search for novel growth arrest genes, relevant to mammary tissue. We anticipate that

the expression of genes involved in promoting mammary proliferation will be down regulated and the expression of those genes involved in the suppression/inhibition of mammary proliferation (such as tumor suppressor genes) will be up regulated. Moreover, from a broader perspective, this category of novel genes may include novel apoptosis genes or already known apoptotic genes not previously linked to the mammary gland.

It is also well recognized that in cancer and other disorders of cell growth and differentiation, defects of growth inhibitory pathways play an important role. Such defects may include an ability to resist growth inhibitory signals and to evade apoptosis (4). Therefore we further hypothesized that those genes relevant to growth arrest in the normal mammary gland will be highly relevant to breast cancer.

With the advent of high throughput profiling of gene expression it is now possible to screen thousands of expressed genes simultaneously (5). We applied cDNA microarray to analyze differential gene expression in normal mouse involuting mammary gland, to search for potential growth suppressor genes important to the mammary gland. The mouse mammary gland is a well-established model for studying many aspects of breast development in particular mammary gland involution, which is difficult to study directly in humans (6).

In this study we identified a number of genes that were highly regulated in the early involuting mouse mammary gland, some of which were associated with human tumorigenesis and more specifically, human breast tumorigenesis. Through further database searches we determined that some of these genes have not been previously studied or well studied in the context of human breast cancer. We showed that the expression levels of a number of these genes were altered in human breast cancer cell lines and breast tumors when compared to normal mammary tissues. Our data suggests that these genes warrant further investigation to determine their relevance to human breast tumorigenesis.

3. MATERIALS AND METHODS

3.1. Animals and tissue preparation

Female CD1 mice, 6 weeks of age, were purchased from Charles River Breeding Laboratory (Wilmington, MA) and housed at the University of Manitoba Animal Care Facility. All procedures were approved by the Animal Care Committee of the University of Manitoba, under Canadian Council for Animal Care Guidelines. Virgin female mice were mated with CD1 males. Litter sizes were normalized to six pups, 1-2 days after delivery, with the delivery day considered as day 0 (D0) of lactation. At day ten (D10) of lactation, when lactation was fully established, the pups were forcibly weaned. This time point was deemed day 0 (D0) of involution. Animals were sacrificed and mammary glands were dissected from D0 and D1, involuting mice. Dissected tissue was immediately frozen on dry ice and stored at -70^{o}C.

3.2. Human breast cancer cell lines and human breast tumor specimens

3.2.1. Cell Lines

Human breast cancer cell lines (MCF7, MDA-MB-231, MDA-MB-468, T47D5, T47D, ZR75, BT20) and the human mammary epithelial cell line MCF10A1 were obtained from the American Type Culture Collection. Cells were cultured in DMEM with 5% fetal bovine serum (Life Technologies, Inc., Burlington, ON, Canada) supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin, 2 mM glutamine (Life Technologies, Burlington, Canada), 15 mM sodium bicarbonate and 2mM glucose. Cells were grown at 37^{o} C in an atmosphere of 95% air and 5% CO_2.

3.2.2. Normal breast tissues and breast cancer specimens

Two normal human mammary glands from reduction mammoplasty, were obtained from Clontech (Mississauga, ON) and Ambion Diagnostics (Avotin, TX). A small panel of 13 anonymized fresh frozen human breast tumor specimens were obtained from the Manitoba Breast Tumor Bank (MBTB), which operates with approval of the University of Manitoba Research Ethics board. The tumor panel consisted of a randomized selection of tumors of different grades, stage and estrogen receptor (ER) and progesterone receptor (PR) levels.

3.3. RNA Extraction

3.3.1. Mouse mammary gland

Whole mammary glands were collected from five mice at day 0 of involution (D0, time at which the pups are removed from the dam), and five mice at day 1 of involution (D1, 24hr after pups were removed from dam). Total RNA was extracted from tissue using Trizol reagent (Invitrogen Life Technologies, Burlington, Canada), according to the manufacturer's instructions. The RNA pellets were dissolved in 50µl of diethylpyrocarbonate (depc)-treated water. The purity and yield of the RNA were determined spectrophotometrically. Samples were electrophoresed in 1% agarose gels containing formaldehyde and stained with ethidium bromide in order to verify the integrity of the 18S and 28S ribosomal RNA.

3.3.2. Breast cancer cell lines and tumor tissues

For cell lines, semi confluent 75 mm^3 flasks of cells were trypsinized and cell pellets collected. RNA was isolated from cell pellets and frozen breast tumor tissue sections with Trizol reagent and purity and yield determined as described above.

3.4. Microarray analysis

Pooled RNA from D0 mice and from D1 mice, (five mice per time point), was compared for differential gene expression by microarray analysis conducted by Paradigm Array Labs (Research Triangle Park, North Carolina, USA). The Affymetrix GeneChip Mouse Genome 430 Plus 2.0 Array (Affymetrix, Santa Clara CA. USA) was used. The array contained 45,100 probe sets (The

Table 1. Primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')	No [1]
Mouse primer sequences used for validation of microarray data by quantitative real time PCR			
CLDN1	AGGAAAGGCCCTTCAGCAGAGCAA	GTGCCCCCTCTTGACTCATGCAAC	BC008536
ANGPTL4	CCCAATGGCCTTTCCCTGCCCTTC	GCTCCTGCCGTTGCCGTGGGATAG	NM_020581
SOX4	CCCCAGCAAGAAAAGAAGCCAAGC	AACGCATCACCCCAGAGCCTTCTT	NM_009238
CLDN4	CTCTTGCCATTACGCCCCGTGTT	CCCTCAAACCCGTCCATCCACTC	NM_009903
SYNJ2	TGAAAGGCAGGGCCGTGAAGATT	GGCTGGCCAAACCCATCCACTAA	NM_011523
ZFAND2A	ACCTCAGCTCCAGGGGCAAATGA	AGCTCCTCACTGGTGGGCTCTGG	NM_133349
TNFRSF12A	TTCCAGACAGAAGCCGAGCCAGAC	ACTGCCAGGACTTGTGGGGAACAA	NM_013749
ANXA3	TGAGAACAAATGGGGCACAGACGA	GGCCAGCAGCAGGTCTTCAAAATG	AW702161
STMN1	ATCTGTCCCCGATTTCCCCCTTTC	CCTCCTTCTCATGCTCCCGCTTC	BC010581
SAA2	TCCATGCTCGGGGGAACTATGATG	GGCAGGCAGTCCAGGAGGTCTGTA	NM_011314
CACC	TAGGAGCCGATGTCACAGCCATCA	TTGCCTGGACACGCACTTTTAGGC	AF108501
SOCS3	GAGTGGCAAGGGGTGACCTGAAGA	TGTTGATCTGCCTCCCTTCGGTGT	NM_007707
ANXA1	CAGTCCCCGGGAAGACAAGCAAAT	CTCACTGCTGACCCAGGACCACCT	NM_010730
CLDN3	AGTGCTTTTCCTGTTGGCGGCTCT	ATCGCGGCGCAGAATAGAGGATCT	NM_009902
SLC34A2	CTCCTATTCGTCCCTGCCCCAGAG	CCCCAACCATGTGTCTCTCCATCC	NM_011402
KLF15	AGGAAGCAAGCCATGCTGGGAAA	GGAGGCTGGTGTCTGGTGTCTGG	NM_023184
GOS2	ACGCAAGGACGCCTGACCTTCTC	CGTCTCAACTAGGCCGAGCACCA	NM_008059
STEAP	CGAGCTTCAGCACACGCAGGAAT	GGGTAATGGCGACCATTGGCAAG	AF297098
NETO1	GACTTTGCAGACGTGGCGGAAGA	GGATGAGGGGCTTGACTGGCTGT	AF448840
FGF18	GTCCTGGGCCGTAGGATCAGTGC	CCACGAGCTTGCCTTTTCGGTTC	NM_008005
PTHLP	ACTCCAAACCTGCTCCCAACACCA	GGCAGACCGAGTCCTTCGCTTCTT	BC058187
Human primer sequences			
ANGPTL4	CATGCGTTGCCTCCTGAGATCGAG	CCCAGGGGACCTACACACAACAGC	BC023647
SOX4	TTTTTCCTTCACCGATGCTCCATCC	GGAGACGTAAAATGGCGTGGGTAACA	NM_003107
TNFRSF12A	GGGGAACCTTCCAAGGTGTCTGGTT	CCCGTGGTGGAATTTCAGGACATCT	NM_016639
SAA2	TCCATGCTCGGGGGAACTATGATG	TCTCTGCCACTCCTGCCCCATTTA	NM_030754
SLC34A2	CAACATCTCCGGCATCTTGCTGTG	GGAGTCGGAGGCACAGTACCAGGA	NM_006424
GOS2	AAGCGCAAGGGGAAGATGGTGAAG	CGTGTCCTGCTGCTTGCCTTTCTC	BC009694
STEAP	CACCAAACAGCCCATGCTGATGAAT	CCAATGCCAAGAGAGTGATGGAAACC	NM_012449

[1] GenBank Accession Numbers

Affymetrix probe-set identificator) and represented 39,000 transcripts. The Affymetrix Statistical Algorithms, implemented in the Affymetrix Microarray Suite 5.0 software, were used in the expression analysis of GeneChip probe arrays. Differentially expressed genes were those having signal log ratios (SLR) significantly different ($p<0.05$) from a value of zero (fold change = 2^{SLR}). Hence, SLR = 0 denotes no change, SLR = 1 denotes 2-fold induction and SLR = -1 denotes 2-fold reduction in expression. These differentially expressed genes were further analyzed using database resources available via Genbank (www.ncbi.nlm.nih.gov). Pathways were analyzed using the Ingenuity Pathway Analysis (IPA, Ingenuity Systems), PathwayAssist™ (Ariadne Genomics Inc, Rockville, MD) and OntoExpress (Detroit, MI). The GEO DataSet accesssion number is GSE5317.

3.5. Reverse Transcription and Real Time PCR

All reagents were purchased from Invitrogen Life Technologies, except where indicated. Total RNA was reverse transcribed using Superscript II reverse transcriptase enzyme. Briefly, 1 μg oligo $(dT)_{16,}$ was added to 3 μg of total RNA and annealed at 70°C for 10 min, followed by cooling to 45°C for 10 min. One hundred units Superscript II reverse transcriptase was then added in the presence of 50 mM Tris-HCL, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 1 mM of each deoxynucleotide, 10 mM DTT, and 20U RNase Inhibitor (GE Healthcare), in a final volume of 20 μl, and incubated at 42°C for 80 min. The samples were subsequently diluted 1:3 with depc-treated water to a concentration of 50 ng/μl of total RNA.

Using the accession numbers supplied by Affymetrix for each gene, we located the cDNA sequence in the NCBI database. Primers were then designed for optimal hybridization kinetics (see Table 1) using Primer 3 (http://frodo.wi.mit.edu/primer3/primer3_code.html) and the Oligo Primer Analysis Software, version 5, (National Bioscience Inc., Plymouth MN).

PCR amplification mixtures contained 1μl of RT reaction, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.5uM of forward and reverse primers, 1 μl of DMSO, 0.2 mM each dNTP, 1 U of Platinum Taq DNA polymerase, Sybr green 1, 1/50,000 (Sigma) and fluorescein, 1/100,000 (BioRad). Amplification was carried out in duplicate (for each sample and each gene) in 96 well microtitre plates using the icycler (BioRad). The cycle profile consisted of denaturation at 94°C for 2 min., followed by 40 cycles of 94°C for 15s, 67°C for 5s, and 72°C for 15s. The icycler iQ Optical System Software Version 3.0 a (BioRad Resource Guide) was used to determine the cycle threshold (C_T) for each reaction. For each gene analyzed, the difference between the mean C_T at D0 involution and the mean C_T at D1 involution (SLR value) was determined. This value was corrected for any difference in the mean C_T of the cyclophilin E reaction, $[(C_{T.}\,D0 - C_{T.}D1)_{gene} - (C_{T.}\,D0 - C_{T.}D1)_{cyclophilin}]$. Also, the difference between the mean C_T of each breast cancer cell line sample and the mean C_T of the reference cell line MCF10A1 was determined. This value was then corrected for any difference in the mean C_T of the cyclophilin E reaction, $[(C_{T.}\,MCF10A1 - C_{T.}cell\ line)_{gene} - (C_{T.}\,MCF10A1 - C_{T.}\,cell\ line)_{cyclophilin}]$. Similarly the difference between the mean C_T of the normal mammary gland samples and the mean C_T of each breast tumor sample was determined for each gene $[(C_{T.}normal\ mammary\ gland - C_T\ breast\ tumor)_{gene} - (C_{T.}\,normal\ mammary\ gland - C_{T.}\,breast\ tumor)_{cyclophilin}]$.

Table 2. Validation of microarray results by real time PCR

	Microarray Signal Log Ratio	PCR Signal Log Ratio[1]
Upregulated Genes		
Claudin 1(CLDN1)	7.5	7.5
Fibrinogenangiopoietin related protein(ANGPTL4)	5.8	5.8
SRY-box containing gene (SOX4)	5.4	6.0
Claudin 4(CLDN4)	5.1	6.4
Synaptojanin 2(SYNJ2)	5.1	0.2
zinc finger, AN1-type domain 2A (ZFAND2A)	3.9	2.9
Type1 transmembrane protein Fn14(TNFRSF12A)	3.7	3.3
Annexin A3(ANXA3)	3.6	3.4
Leukemia-associated gene(STMN1)	3.5	5.4
Serum amyloid A2 (SAA2)	3.5	5.2
Ca(2+)-sensitive chloride channel 2 (CACC)	3.5	4.8
Cytokine inducible SH2-containing protein 3(SOCS3)	3.4	3.8
Annexin A1 (ANXA1/lipocortin 1)	3.3	5.2
Claudin 3(CLDN3)	2.9	3.9
Downregulated Genes		
Solute carrier family 34 (sodium phosphate) member 2(SLC34A2)	-7.3	-7.7
Kruppel-like factor 15 (KLF15)	-5.5	-5.1
GoG1 switch gene 2 (GOS2)	-5.1	-4.6
Six-transmembrane epithelial antigen of the Prostate(STEAP)	-4.6	-5.1
Neuropilin and tolloid like-1 (NETO1)	-4.3	-3.1
Fibroblast growth factor 18 (FGF18)	-4.2	0.3
Parathyroid hormone-like peptide(PTHLP)	-4.1	-3.5
Control Genes		
Cyclophilin E	1.5[2]	0

Twenty-one genes were randomly selected from the microarray analysis dataset for validation by real time PCR analysis. The icycler iQ Optical System Software Version 3.0a was used to determine the cycle threshold (C_T) for each reaction. The difference in the C_T between Day 1 and Day 0 of involution is comparable to the signal log ratios (SLR, fold change=2^{SLR}) defined in the microarray analyses. The correlation between the microarray results and the real time PCR was significant ($p<0.0001$). [1]values represent the mean of duplicate reactions for each gene.[2]Not statistically significant as determined by Affymetrix Microarray Suite 5.0 software.

3.6. Gene selection strategy

Genes identified as significantly differentially expressed were placed in order of most highly up regulated and most highly down regulated, based on their signal log ratio (SLR) value. We then focused on the more highly regulated genes, those with an SLR value of 3 (8 fold change in gene expression) or higher, for further study. Known genes were separated from expressed sequence tags (ESTs) and grouped according to molecular and biological functions. Using IPA, we examined those genes either previously reported to be associated with cancer or shown to be deregulated during tumorigenesis, for example, those involved in functions such as motility, adhesion, apoptosis, proliferation, angiogenesis, stress response, signaling. Furthermore, we were particularly interested in those with human homologs and had little or no previous link with breast cancer. Using the PathwayAssist™ we also examined other genes and known signaling pathways with which these genes are known to interact.

3.7. Statistical analysis

Comparison between real time PCR and microarray analysis was performed using Spearman correlation test, Graphpad PRISM, Version 3.02.

4. RESULTS

4.1. Microarray analysis

A total of 5826 probe sets (see "Material and Methods") were identified as differentially expressed genes and of these, 3627 were statistically significantly up regulated and 2199 down regulated as determined by Paradigm Array Laboratories™ (NC) analysis. These probe sets consisted of both known genes and expressed sequence tags (ESTs). Of the up regulated probe sets, 290 were not expressed at day 0 (D0) of involution (i.e. gene expression below the detection level of the analysis), but were "turned on" at D1 of involution. Conversely there were 356 probe sets that were expressed at D0 of involution, but were "turned off" at D1.

4.2. Validation of differential expression of genes by real time PCR

To validate the microarray data, we randomly selected 21 genes exhibiting various levels of differential gene expression and performed real time PCR analysis of their gene expression at D0 and D1 of involution. Reversed transcribed mRNA derived from the pooled RNA samples of the D0 and D1 involuting mouse mammary glands, was used as template. The expression of the reference housekeeping gene cyclophilin, was unchanged. As shown in Table 2, we found a high correlation between the signal log ratios (SLR, see "Material and Methods") of the real time PCR and the microarray ($p<0.0001$), confirming the results obtained by the microarray analysis.

4.3. Gene selection

As described in the Material and Methods, both up regulated and down regulated genes were first ordered according to their SLR values, from highest to the lowest and then separated into categories of known genes and ESTs. Although several ESTs were observed to have high SLR values, all ESTs were eliminated from the list and not followed up further because of a lack of current available information in the public databases regarding these sequences. The remaining known genes were then grouped

Table 3. Apoptosis genes that were differentially expressed at D1 of involution in the mouse mammary gland

Upregulated genes					
Gene	No[1]	Gene	No[1]	Gene	No[1]
BCL2	NM_009741	PDCD5	AF161074	DNASE2A	AK018651
BID	NM_007544	DOCK1	BQ175788	API5	NM_007466
TNFRSF12A	NM_013749	CASP11	NM_007609	SIAH1A	AV008871
GADD45B	AK010420	PEA15	A1323543	TXNL1	B1416051
BCL2L1	NM_009743	PDCL3	BC005601	YARS	BI452674
TRP53INP1	AW495711	CTNNBL1	NM_025680	ROCK1	BI662863
PAWR	BB398886	CASP2	NM_007610	JAK2	BC054807
BAX	BC018228	BNIP2	AV144704	BCL2L13	BC027307
SGPL1	NM_009163	CASP12	NM_009808	TNFSF5IP1	NM_134138
RHOB	BC018275	LTBR	NM_010736	PDCD6	NM_011051
DDX41	BC011308	RAD21	AF332085	TAF10	NM_020024
EGLN3	BB284358	PSEN1	L42177	CASP8	BC006737
DEDD2	AK010701	RYBP	AK010548	SMNDC1	BG071121
APPBP1	BC019163	MCL1	BC003839	HIPK3	BQ076079
ERN1	BG075179	SULF1	BB751459		
APAF1	AK018076	SIRT1	NM_019812		
Downregulated genes					
Gene	No[1]	Gene	No[1]	Gene	No[1]
PAX7	AF254422	DAP	BC024876	GAS2	BM507943
ANK2	BB427897	SIAH2	AA414485	ELMO1	BG066320
TNFSF12	AW909226	BAK1	BG271102	BNIP3	NM_009760
SGK	NM_011361	CIDEA	NM_007702	BAG1	NM_009736
LGALS12	AF244979	PYCARD	BG084230	DAPK1	NM_009736
STK17B	AV173139	AKT1	NM_009652	TRIM35	AB060155

[1]GenBank Accession Number. List was compiled using OntoExpress

according to molecular and biological function. Not surprisingly, the majority of genes fell into the categories of transcription and cell cycle regulation, DNA/protein/ATP binding, as well as transport and metabolism (Figure 1). A large number of apoptotic genes were also identified (Table 3**).** Breast specific genes such as whey acidic protein (WAP) and beta casein were down regulated (data not shown). We further narrowed down our analysis to the more highly up regulated and down regulated genes, those with an SLR value > 3 (Figure 2). To identify genes that are likely to play a role in breast cancer, we focused on genes that displayed functions linked to tumorigenesis (Table 4) such as cell adhesion and migration, proliferation, angiogenesis, stress response and tumor suppression. Some genes identified were only recently being studied in the context of breast cancer and these include claudin 1, 3 and 4 (7) that belong to a family of tight junction proteins and the Rho GTPase activating protein 6 gene, ARHGAP6 (8), which belong to a gene family previously linked to invasion and hyperplastic lesions in developing breast (9).

4.4. Claudin 1 gene expression

The most highly up regulated gene in our dataset was the CLAUDIN 1 (CLDN 1) gene (7). Our laboratory has recently shown that CLDN1 is differentially expressed throughout mouse mammary gland development and is also expressed in mouse mammary tumors of different origins (10).

4.5. Altered gene expression of candidate genes in human breast cancer cell lines

We further selected 7 additional genes, ANGPTL4; TNFRSF12A; SAA2; SLC34A2, STEAP, SOX4 and GOS2, that did not appear to be well studied or not yet implicated in breast cancer and examined their gene expression in a panel of breast cancer cell lines. The "normal" mammary epithelial cell line, MCF10A1, was used to represent baseline expression (control) for each gene. RT/PCR (reverse transcription followed by PCR amplification) of mRNA of the 7 candidate genes confirmed their expression in the human breast cancer cell lines. The results are shown in Table 5.

4.6. Altered gene expression of candidate genes in human breast tumors

To further establish the possible relevance of these candidate genes in breast cancer we examined their expression in a small panel of 13 unselected human breast tumors of different grades and stages using real time RT/PCR analysis. The results are presented in Table 6. The majority of the candidate genes exhibited differential gene expression compared to normal breast tissue. Interestingly, overall, the expressions of the candidate genes were mostly down regulated in the breast tumor tissues compared to their gene expression in normal mammary gland tissue. In particular, the ANGPTL4 gene expression (Figure 3), as well as the expression of SAA2, GOS2, SLC34A2 and STEAP were consistently down regulated in every breast tumor sample tested. In contrast, the TNFRSF12A gene expression was up regulated in 12 out of 13 breast tumor specimens analyzed. The small sample size did not allow us to investigate the possible relationship between gene expression and tumor characteristics such as ER/PR status, tumor grade or size.

5. DISCUSSION

Involution of the mammary gland is a complex phenomenon that involves the apoptosis of the vast majority of mammary epithelial cells and is associated with changes in availability of hormones and mechanical stress (1) Although in recent years there has been much progress in our understanding of the pathways and genes involved in the involution process, it is far from complete. For example

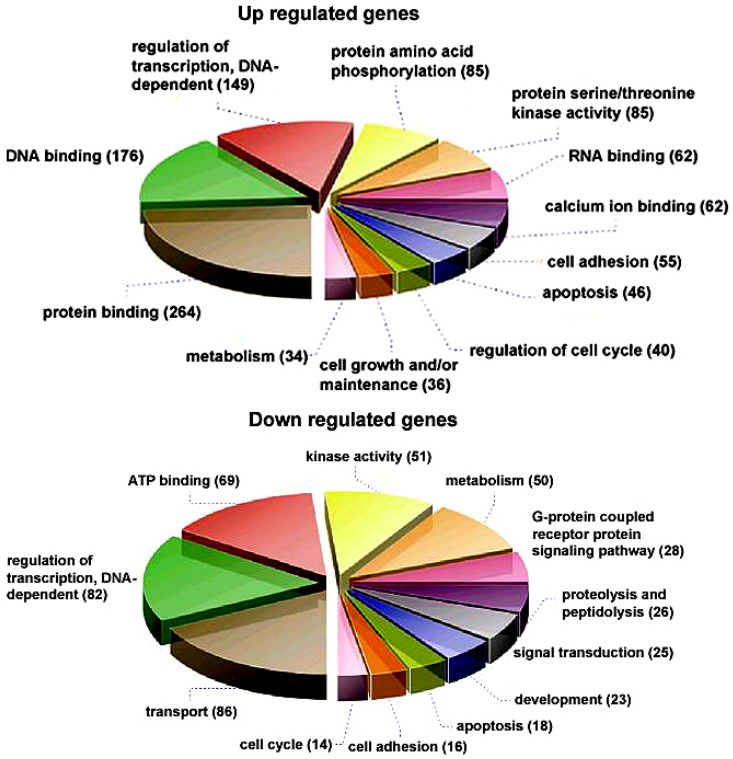

Figure 1. Biological and molecular functions of differentially expressed genes. Numbers in brackets indicate the number of genes in each category, as determined by OntoExpress.

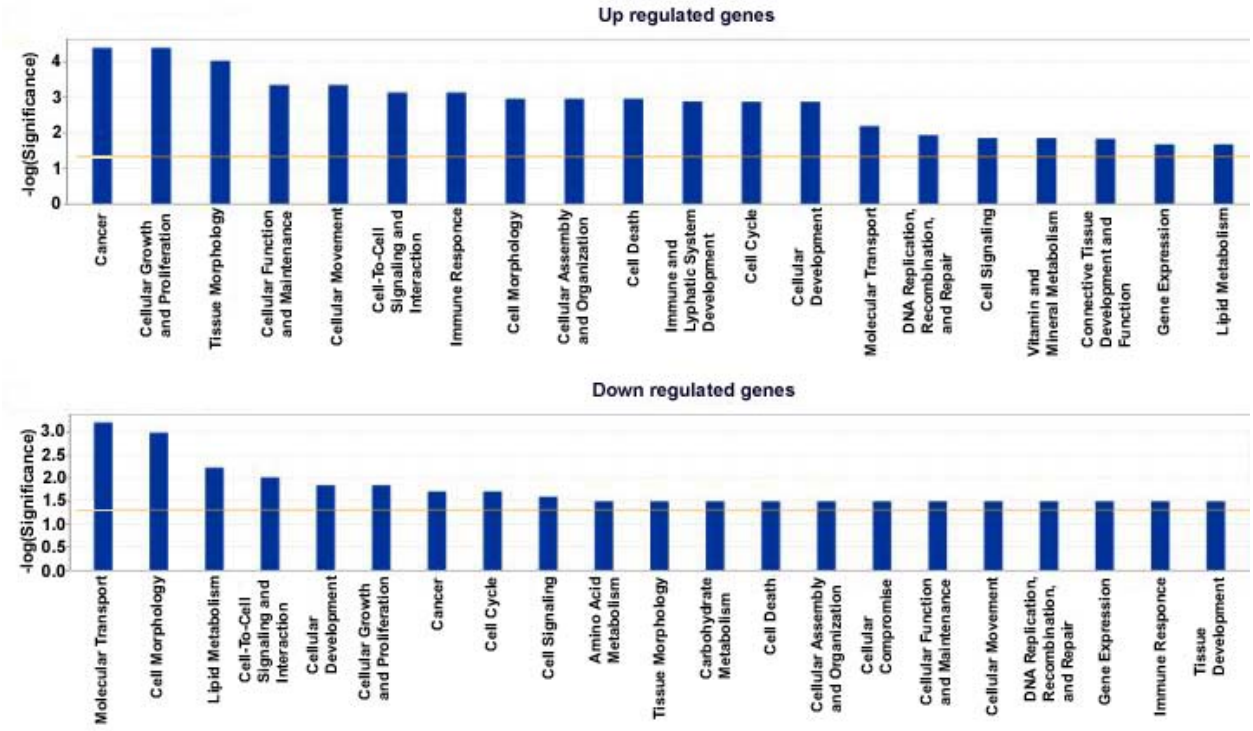

Figure 2. Functional analysis of differentially expressed genes with an SLR>3. The horizontal line represents the threshold value.

Table 4. Examples of genes from our dataset previously linked to tumorigenesis

Putative function	Gene symbol	Gene name	Microarray Signal Log Ratio	Reference
Cell adhesion and Invasion	*CLDN1*	Claudin 1	7.5	7
	CLDN4	Claudin 4	5.1	7
	CLDN3	Claudin 3	2.9	7
	HSP27	Heat shock protein 27	3.4	47
	PTHLP	Parathyroid hormone-like peptide	-4.1	48
	ARHGAP6	Rho GTPase activating protein 6	3.6	8
	SYNJ2	Synaptojanin 2	5.1	49
Proliferation	*MT2*	Metallothionein 2	3.0	50
	CISH3	Cytokine inducible SH2-containing protein 3	3.4	51
	LIF	Leukemia inhibitory factor	3.4	52
	GADD45A	Growth arrest and DNA damage inducible 45 alpha	5.3	53
	TGFB2	Transforming growth factor beta 2	3.4	54
	FGF 18	Fibroblast growth factor 18	-4.2	55
	EGF	Epidermal growth factor	-3.8	56
	KIT	Kit oncogene	3.3	57
	MYT 1	Myelin transcription factor 1	-3.8	58
	GOS2	G0G1 switch gene 2	-5.1	59
Angiogenesis and hypoxia	*THBS1*	Thrombospondin 1	2.4	60
	CTGF	Connective tissue growth factor	3.5	61
	TMSB10	Thymosin, beta10	3.5	62
	ANGPTL4	Angiopoietin-like 14	5.8	29
Apoptosis and Stress	*HSP25*	Heat shock protein 25	2.8	63
	ANXA3	Annexin A3	3.6	64
	CAPN2	Calpain izoenzyme 2	1.6	64
	TNFRSF12A	Tumor necotic factor related type 1 transmembrane protein 12a	3.7	34,35,65
	BID	BH_3 interacting domain death agonist	3.7	66
	BCL2	B-cell CLL/lymphoma 2	3.7	67
	BIK	Bcl-2-interacting killer	3.6	68
Tumor supressor	*ANXA1*	ANNEXIN A1	3.3	69
	SOX4	SRY-box containing gene 4	5.4	70
	KLF15	Kruppel Factor 15	-5.5	71
	JUB	Adjuba	2.9	72
	CDKN1A	Cyclin-Dependent Kinase Inhibitor 1a	1.6	73

Table 5. Real time PCR analysis of candidate gene expression in breast cancer cell lines

Gene	MCF7	MDA-MB-231	MDA- MB-468	T47D5	T47D	ZR75	BT20	MCF 10A1
ANGPTL4	0.4	-0.5	-0.2	0.4	8.0	0.3	3.1	0.0
TNFRSF12A	-0.6	0.6	0.1	1.6	-0.9	3.1	1.3	0.0
SAA2	-11.8	-3.8	-5.0	-8.1	-12.7	2.1	-8.5	0.0
SOX4	3.7	4.1	4.7	2.8	11.0	2.2	1.2	0.0
SLC34A2	-0.8	-3.4	4.0	0.7	-2.9	1.4	-1.1	0.0
STEAP	-3.0	-1.4	-4.0	-2.8	1.9	-0.8	-5.5	0.0
GOS2	-0.1	1.2	-0.4	1.9	-1.1	2.5	0.5	0.0
CYCLOPHILIN E	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

The expression in MCF10A1 cells was used as the baseline and the change in expression of each gene is indicated as the change in cycle threshold between the cell line and MCF10A1, following correction with the human CYCLOPHILIN E (control gene). Values represent the mean of duplicate reactions for each sample and each gene.

Table 6. Real time PCR analysis of candidate genes expression in a panel of human breast tumors

Gene	Breast Tumors													Mean ±S.D.	Expression in D1 involuting mouse mammary gland
	1	2	3	4	5	6	7	8	9	10	11	12	13		
ANGPTL4	-3.7	-3.7	-4.5	-2.0	-2.8	N/A	-3.4	-3.3	-1.4	-3.6	-3.4	-1.3	-5.3	-3.2 ± 1.2	↑
TNFRSF12A	4.1	4.1	2.3	2.8	3.9	4.6	3.6	3.8	3.7	2.5	3.2	1.8	-0.9	3.0 ± 1.4	↑
SAA2	-3.8	-5.7	-10.6	-9.1	-4.8	-5.4	-5.9	-6.5	-7.1	-8.5	-9.2	-10.6	-9.2	-7.4 ± 2.2	↑
SOX4	-0.9	-1.0	-0.4	-0.3	0.7	0.8	0.8	0.6	-0.7	-1.7	-3.3	-1.9	-2.0	-0.7 ± 1.3	↑
SLC34A2	-6.2	-7.2	-7.6	-6.0	-6.3	-7.7	-7.2	-9.2	-7.4	-11.4	-7.5	-10.1	-12.4	-8.2 ± 2.0	↓
STEAP	-3.5	-1.0	-5.1	-1.7	-1.5	-1.3	-1.9	-2.8	-2.8	-3.9	-3.7	-2.5	-1.6	-2.5 ± 1.2	↓
GOS2	-2.8	-6.8	-5.0	-8.1	-8.1	-8.0	-4.2	-2.1	-4.3	-5.8	-3.9	-5.6	-9.8	-5.7 ± 2.3	↓
ER	2.8	41	2	45	103	1.4	96	58	6.1	116	92	140	180		
PR	6.6	23	8.6	67	9.7	0	6.3	6.5	21	97	105	12.5	14.6		

Change in cycle threshold for each gene in each tumor, compared to the cycle threshold for the same gene in normal human mammary glands, with corrections for cyclophilin gene expression (control). Values represent the mean of duplicate reactions for each sample and each gene. ER/PR = estrogen/progesterone receptor values (in fmol/mg of total protein). Tumor specimens are numbered 1-13. N/A: not available.

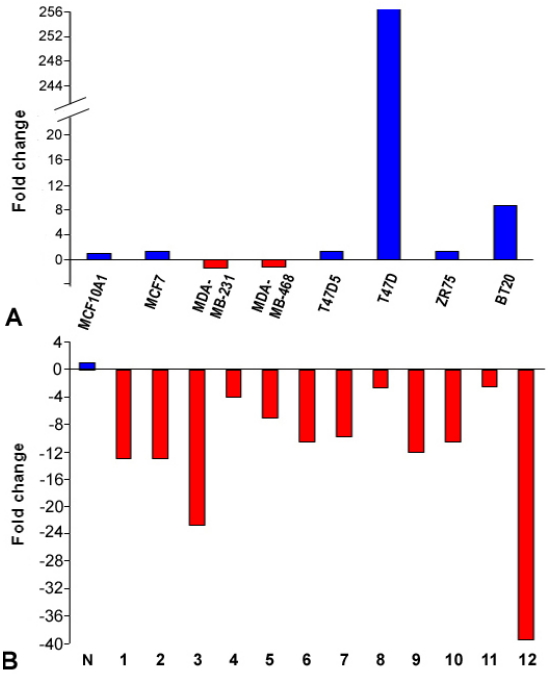

Figure 3. Real-Time PCR analysis of ANGPTL4 in human breast cancer cell lines and breast tumor specimens. A. The change in gene expression is indicated as the change in cycle threshold between each cell line and the MCF10A1 normal breast cells. The ANGPTL4 gene exhibited differential gene expression in the cell lines. B. Numbers on the x-axis indicate different breast tumor specimens. The change in expression is indicated as the change in cycle threshold between each tumor and the mean of 2 samples of normal human mammary gland (N) after correction with the cyclophilin E (control) gene expression. All tumors showed down regulation of ANGPTL4 gene expression relative to the gene expression in the normal gland.

the triggers of mammary gland involution are not yet known. Studies suggest that key players in this process may be found in the local environment of the mammary epithelial cells (2,3). In addition, the actual mechanisms involved in the process are also not fully elucidated. Clearly, a better understanding of target genes and signaling pathways involved not only in involution but in normal mammary gland development is needed, as such lack of understanding has also become a barrier to progress in breast cancer research. Indeed, the abnormal regulation of, or reduction in apoptosis has also been demonstrated to be directly involved in human breast cancer (11-14). Thus apoptosis is highly relevant to breast cancer, and, compounded with the deregulation of proliferation, creates a platform that is both necessary and can be sufficient for cancer (15). Given the above considerations we wanted to examine differential gene expression of the early involuting mammary gland in order to identify novel genes that may be involved in the inhibition of proliferation/suppression of growth and apoptosis of breast tissue. Our long-term goal is to determine the relevance of such genes to breast cancer.

To date, many strategies, including library screening (16), differential display and subtractive hybridization (17), have been utilized to search for new breast specific/breast cancer biomarkers. More recently such approaches have included the use of proteomics (18), genomics (19) and cDNA microarray analysis of gene expression (20). Overall, the emphasis has been to profile gene expression in breast tumors versus normal mammary tissue to identify tumor marker genes involved in the promotion of proliferation, invasion and metastasis. Our novel approach was to use microarray analysis strategies on involuting mammary gland tissue to identify novel genes pertaining to growth inhibition rather than proliferation. Such genes hold promise for the therapeutic management of breast cancer.

We analyzed our data using gene pathway networks. Gene pathway networks generated from screening large amount of data simultaneously are powerful tools to help determine gene interactions and give insight into novel gene function. We found, not surprisingly, several genes involved in metabolism, milk synthesis including lipid synthesis, tissue morphology and architecture, immune responses and protection, mechanical stress and cell survival (see Figure 1,2). Numerous apoptosis related genes were identified including many well known apoptotic genes (BCL2, BID, BNIP3 CASPASE 8) and some lesser known genes (SIRT1 and GAS2) (Table 3). Indeed, there have been an increasing number of studies investigating the gene profiling of differential gene expression during mouse mammary gland involution (21-26). To date, the most comprehensive study reporting on gene expression profiling of the involuting mammary gland has been carried out by Clarkson *et al* (24), who observed that the expression of genes involved in DNA damage and apoptosis, metabolism, and protection and inflammation were highly prominent during involution. In addition, Clarkson *et al* (24) showed that several genes involved in immune responses were differentially expressed in the early involuting gland. In essence, our data is similar to that obtained in studies by other groups who have also examined gene expression in the involuting mouse mammary gland (24,27). However, we have also used the gene expression profile of the early involuting mammary gland to identify new genes that may be associated with the inhibition of breast proliferation and therefore highly relevant to breast cancer with regards to therapeutic management. In this pilot study, we provide evidence that this model is an effective tool in identifying genes whose expression are altered during breast tumorigenesis.

Of the genes that exhibited differential expression, several have previously been implicated in tumorigenesis (Table 4). In this category were genes previously identified as putative suppressors, stress response genes, molecules involved in angiogenesis, cell adhesion, migration, apoptosis, cell cycle and tissue architecture (Table 4). In the latter category was the CLDN1 gene, a member of a family of tight junction proteins whose expression in breast cancer is only recently being investigated (7,28). Studies by Tokes *et al* (7)

demonstrate that the expression of the CLDN1, 3 and 4 genes are altered in human breast tumors and these authors suggest that down regulation of CLDN1 is correlated with increased invasiveness of breast cancer (28). Using tissue microarray analysis we have examined CLDN1 protein levels in a small cohort of human invasive breast tumors of varying ER status. We observed various levels of CLDN1 staining in both ER-ve and ER+ve tumors (data not shown). We could not establish a significant correlation between ER status and CLDN1 levels. However this needs to be explored further in a larger study. More recently, we have demonstrated (10) that the CLDN1 gene is differentially expressed throughout mouse mammary gland development, suggesting that CLDN1 may also have an important role in the developing mammary gland.

In addition to CLDN1 we also identified 7 additional known genes, ANGPTL4, TNFRSF12A, SAA2, SLC34A2, STEAP, GOS2, and SOX4, with functional characteristics linking them to tumorigenesis, but not yet implicated or not fully explored in human breast cancer. ANGPTL4, is a fibrinogenangiopoietin related protein whose decreased expression has been associated with decreased tumorigenicity in prostate and gastric cancer (29,30). Le Jan *et al* (31), has also demonstrated that ANGPTL4 is associated with hypoxia in endothelial cells and is a proangiogenic factor produced during ischemia. Anecdotally, ANGPTL4 belongs to the family of angiopoietic factors, the angiopoietins and recently (32) it has been demonstrated that the systemic increase in host angiogensis is increased through estrogen to promote the formation and proliferation of estrogen receptor negative breast tumors. TNFRSF12A is a tumor necrotic factor related type 1 transmembrane protein member 12A, a small protein belonging to the TNF receptor super family that is suggested to play a role in vascular cell migration and angiogenesis (33). Its over expression in NIH 3T3 cells has led to decreased cell adhesion to extracellular matrix proteins, decreased serum stimulated growth and migration (33). Very recently, there has been one report that TNFRSF12A induces mammary epithelial branching and may be pro-tumorigenic in human breast cancer (34). SAA2, the serum amyloid protein A2, is a member of a multigene family associated with high-density lipoproteins that is elevated in response to inflammation and neoplasia (35,36). SLC34A2 is a solute carrier family 34 (sodium phosphate) member 2, a member of the phosphate cotransporter SLC34 family and whose increased expression has been associated with differentiation in ovarian tumors (37). STEAP, a six transmembrane epithelial antigen of the prostate, which is a prostate specific cell surface antigen is highly expressed in human prostate tumors (38) and the G0G1 switch gene 2 (GOS2), is a cell cycle control gene identified in human mammary epithelial cells (39). The SOX4 (SRY-box containing gene 4) was shown to be regulated by ovarian hormones in breast cancer cells (40).

The expressions of these genes were examined in a panel of breast cancer cell lines. The panel of cell lines was comprised of cells of different levels of invasiveness and ER/PR positivity. Using the "normal" breast cell line MCF10A1 as a reference baseline control, differential levels of gene expression was observed in the cell lines (Table 5) for each gene. Indeed, the MCF10A1 is an immortal cell line, and genes involved in proliferation may already be up regulated. Thus, it may not constitute an ideal reference cell line. However it does provide a common point of reference for the expression of these genes in each cell line. SAA2 and STEAP were down regulated in most of the breast cancer cell lines in this study. The highest level of gene expression was observed for ANGPTL4 in the ER+ve T47D cell line (Figure 3). However up regulation of ANGPTL4 gene expression was not observed in two invasive cell lines such as the MDA 231 and MDA468 suggesting that gene expression may not be directly associated with increased invasiveness.

The expression of the selected genes was further studied in human breast tumors. Once again all genes examined showed altered levels of gene expression compared to levels in the normal breast tissue. In this pilot study, the majority of the genes were down regulated in the breast tumors examined. This result is not surprising, as one would imagine that genes whose expression are up regulated during mammary gland involution may be down regulated in breast cancer. However, as we have shown in Table 3, genes linked to tumorigenesis can be either up regulated or down regulated in the early involuting mouse mammary gland. We also observed that STEAP, GOS2 and SLC34A2 were down regulated during involution and also down regulated in breast cancer. Conversely, gene expression of TNFRSF12A was up regulated during mammary gland involution and in breast cancer. Furthermore, TNFRSF12A was the only gene that was consistently up regulated in all but one of the 13 human breast tumor specimen examined. ANGPTL4 and SAA2 were up regulated during involution, but consistently down regulated in our panel of breast tumors. We do not at this time know the significance of these observations, however, one plausible explanation for the observed result could be attributed to the fact that the early involuting mammary gland is comprised primarily of epithelial cells whereas the primary breast tumors are comprised of a heterogeneous population of cells including lymphocytes, adipocytes and fibroblasts. Thus, the expression of these genes within different cell types in the mammary gland may impact on the overall gene expression profile observed. It is also possible that the down regulation of some these genes may be part of the mechanism by which the cells can resist exogenous growth inhibitory signals, proliferate indefinitely and evade cell death. We were not able to establish a relationship between ER/PR status and candidate gene expression. However, this may be easily attributable to the small panel size examined at this time.

It is also of interest to note that some of our candidate genes such as SOX4 (41,42), CLDN1 (43), CLDN4, CDKN1A (44), GADD45 (45), GOS2 (46) and ANGPTL4 (42) were among new marker sets identified for new subsets of human breast cancer identified by other groups. Altogether, our data support the hypothesis that the involuting mammary gland may indeed provide a good

resource for novel developmental stage-specific biomarkers and breast tumor biomarkers.

In summary, we have examined the gene expression profile of the early involuting mouse mammary gland in order to identify potentially 'novel' genes that can report on the proliferative changes in the breast. We hypothesized that the early involuting mammary gland may provide a resource for genes critical to mammary tissue regression and programmed cell death. To our knowledge we are the first to introduce this new concept. We identified a number of candidate genes. Indeed this analysis is not comprehensive; however it does give some insight into a resource that can be utilized further to possibly identify new breast cancer relevant genes. Further characterization of the role of these genes during involution should improve our understanding of mammary tissue regression. The delineation of the roles of these genes in breast tumorigenesis will have implications for breast cancer management as such genes could serve as potentially useful therapeutic targets. In addition, a more comprehensive analysis of our dataset may allow us to identify a larger subset of genes of interest.

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