

Gene expression profiling of mouse mammary gland: transition from pregnancy to lactation*

Tadeusz Malewski¹, Stanisław Kamiński², Lech Zwierzchowski¹

¹ Polish Academy of Sciences Institute of Genetics and Animal Breeding,
Jastrzębiec, 05-552 Wólka Kosowska, Poland

² Department of Animal Genetics, University of Warmia and Mazury in Olsztyn,
Oczapowskiego 5, 10-718 Olsztyn, Poland

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Gene expression analysis comparing pregnant and lactating mouse mammary gland was performed with Mouse Panorama Apoptosis cDNA macroarray containing 243 genes. Transition from pregnancy to lactation led to repression of 15 and induction of 24 genes, while 12 genes were found to change their expression more than twice. Expression of 16 genes was shown, not described so far in the mammary gland of mouse. Simultaneous analysis of 243 genes made it possible to begin arranging them into the gene regulation network of the mammary gland.

KEY WORDS: mouse / mammary gland / gene expression / cDNA macroarray

Unlike most mammalian organs, which develop primarily during embryonic and foetal stages, development of the mammary gland occurs also in the postpubertal period. There are six defined stages involved in development of the mammary gland including: foetal, prepubertal, pubertal, pregnancy, lactation and involution. These stages can be characterized further as a series of highly orchestrated transitions, or switches, in which critical developmental decisions are made concerning cell differentiation, pattern formation and cell function. Each of these stages involves a complex interaction of hormones, growth factors, and signal transduction pathways, leading to expression of

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developmentally regulated genes [Cunha and Hom 1996, Medina 1996, Visvader and Lindeman 2003].

Near midpregnancy, the alveolar epithelium acquires the capacity to produce milk proteins (the stage I transition of lactogenesis) but secretory function is inhibited. At parturition, inhibition of secretory function is released and these cells begin to secrete large volumes of milk (the stage II transition of lactogenesis). Milk protein gene expression has been investigated by numerous authors [Marti *et al.* 1999, Rosen *et al.* 1999, Rijnkels 2002]. There is, however, no comprehensive analysis of other genes expression during transition from pregnancy to lactation.

Microarray analysis has had a major impact on our understanding of the transcriptional basis of complex biological systems. The few microarray studies of normal mouse mammary gland have either focused on early stages in the developmental cycle [Master *et al.* 2002, Visvader and Lindeman 2003] or have used the mammary data to illustrate methods of data analysis [Lemkin *et al.* 2000, Phang *et al.* 2003]. One of our long-term goals is to elucidate genes and pathways associated with lactation. To begin, we compared gene expression profiles of lactating mouse mammary tissue *versus* pregnant on Panorama macroarray. In the present study we applied a macroarray approach to study the transcriptional expression of 243 mouse genes in a mammary gland during transition from pregnancy to lactation.

Material and methods

Animals and tissues

Mammary glands of MIZ mice on day 16 of pregnancy and day 1 of lactation were used. Mammary gland samples were excised immediately after cervical dislocation. Mammary tissues were cleared from most adjacent muscles, fat and connective tissues, frozen at -25°C and stored at -75°C until use.

RNA extraction

Total RNA from frozen tissues was extracted with TRI Reagent (SIGMA-ALDRICH, Inc.) according to the manufacturer protocol. Briefly, up to 100 mg of frozen tissue was homogenized in 1 ml of TRI Reagent. Next, 0.2 ml of chloroform was added, shaken vigorously and incubated 15 min. at room temperature. The mixture obtained was centrifuged at 12 000 g for 15 min. at 4°C. Aqueous phase was collected, and transferred into fresh tube. Isopropanol (0.5 ml per ml of TRI Reagent) was added and the mixture was incubated for 10 min. at room temperature. It was then precipitated and RNA pellet washed with 75% ethanol. In order to quantify the amount of total RNA extracted, the optical density was determined with DU-68 Spectrophotometer (BECKMAN, Inc.). RNA integrity was electrophoretically verified on agarose gel stained with 0.5 µg/ml ethidium bromide.

cDNA macroarray

Synthesis of labelled cDNA. For cDNA synthesis equal amounts of RNA extracted from five 16-days pregnant or 1-day lactating mice were pooled. To confirm the validity of the assay, cDNA synthesis was performed in duplicate. The cDNA labelling reactions were performed in two steps according to the manufacturer's protocol. In the first step Mouse Apoptosis Labelling Primers were annealed to RNA template. Then, the reverse transcriptase was added to initiate cDNA synthesis reaction (Panorama Mouse Apoptosis cDNA Labelling and Hybridisation Kit). To 2 µg of total RNA added were 4 µl Mouse Apoptosis cDNA labelling primers, and water to 14.5 µl final volume. RNA was denatured at 90°C 2 min. and Mouse Apoptosis cDNA primers were annealed to RNA template for 20 min. at 42°C. After annealing, reverse transcriptase buffer, dATP, dGTP and dTTP to final concentration of 333 mM, 20 U ribonuclease inhibitor, 50 U reverse transcriptase, and 40 µCi[α -³²P]dCTP were added. Reaction mixture was incubated at 42°C for 3 h. The unincorporated radiolabelled nucleotide was removed from labelled cDNA by purification over Sephadex G-25 gel-filtration column according to manufacturer instructions.

Hybridization and analysis of array. Nylon array (Panorama Mouse Apoptosis Gene Arrays, Sigma-Genosys, The Woodlands, TX) was rinsed in 50 ml 2×SSPE at room temperature for 5 min, and pre-hybridized in 5 ml of hybridization solution for 1 h at 65°C. Labelled probes were denatured at 90°C for 10 min and added to 3 ml of hybridization solution. Probes were hybridized to a nylon array at 65°C overnight. After hybridization, the nylon membranes were washed three times for 2-3 min at room temperature with 50 ml 0.5×SSPE + 1% sodium dodecyl sulphate (SDS), and twice with 50 ml 0.5×SSPE + 1% SDS at 65°C for 20 min. On the next step, membranes were washed once with 50 ml 0.1×SSPE + 1% SDS at 65°C for 20 min., and then exposed to Phosphorscreen (KODAK, Japan) for 24 hours. The screens were scanned by Bio-Rad FX Scanner at a maximum resolution of 25 µm. Results from three independent hybridizations were obtained for each probe. Images were analysed by Quantity One (BioRad) software. Each image was overlaid with grids so that signal intensities of individual spots could be assessed. Local background for each membrane was calculated on the basis of 10 positions with no DNA spotted area. Expression levels of individual genes are represented in arbitrary units after subtracting background. Intensity-based global normalization was then performed.

Results and discussion

Macroarrays provide a powerful tool for analysing complex biological systems because they can extract patterns of gene expression from a significant proportion of the total genomic content of an organism. In this study we simultaneously analysed 243 transcripts to determine basic expression patterns in adult mammary gland during the transition from pregnancy to lactation. Autoradiographs of arrays hybridized with [³²P-cDNA] from 16 day of pregnancy and 1 day of lactation are presented in Photo

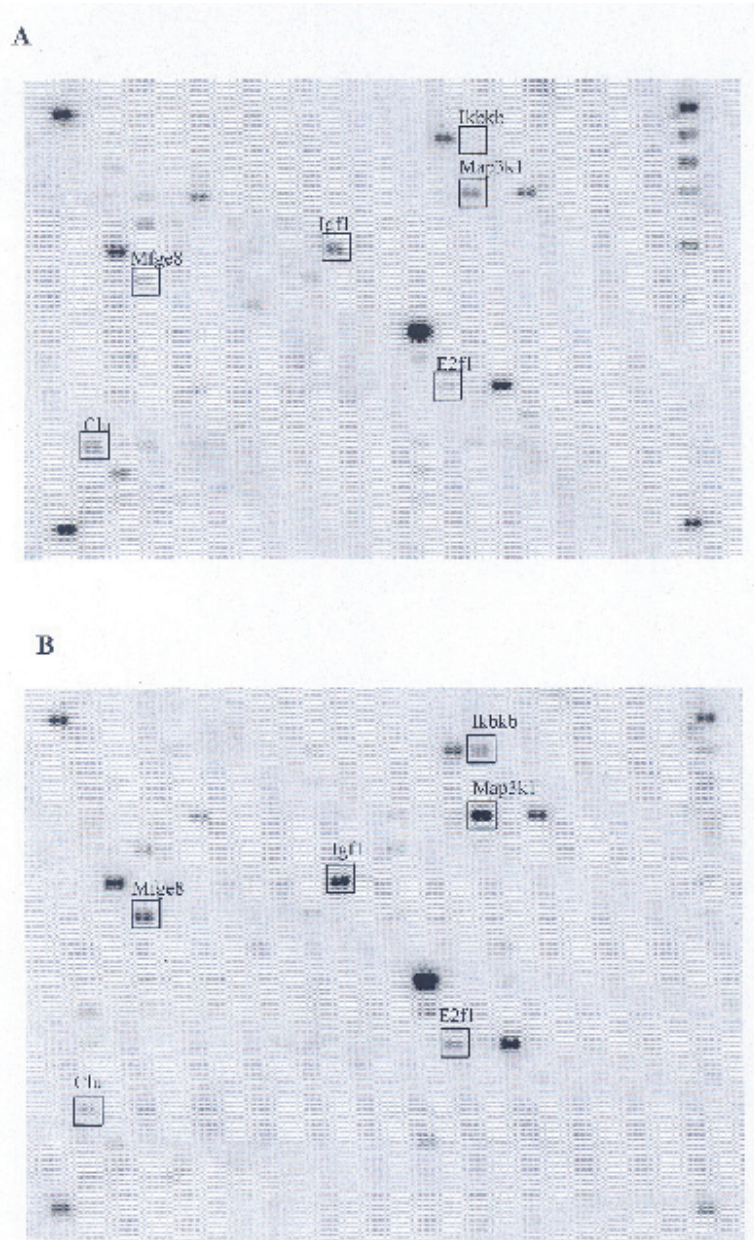


Photo 1. DNA array hybridization of 16 day pregnant (A) and 1 day lactating mice (B). Hybridizations were performed using radioactive ^{32}P -labelled probes prepared from pregnant and lactating mice mammary gland of five animals each. Selected genes changing expression during transition from pregnancy to lactation are indicated.

1-A and 1-B. Gene expression signals were quantified by Quantity One software (Bio-Rad). Analysis of these data show that during transition from pregnancy to lactation 15 genes were repressed (Tab. 1), 24 genes induced (Tab. 2), and expression of 12 genes changed more than twice (Tab. 3). Macroarray analysis confirmed the previously described expression of several genes in the mammary gland: IGF1 and IGF2 receptor [Boutinaud *et al.* 2004], PTEN [Moorehead *et al.* 2003], and transforming growth factor beta [Ewan *et al.* 2002].

Moreover, the analysis found 16 genes expression of which has not yet been de-

Tab 1-1. Genes repressed in lactation

Gene Bank	UniGene	Abbreviation	Gene name	Ontogenic classification
AF141322	Mm31915	Cant	Caveolin 2	Apoptosis-related factor
NM_008140	Mm1090	Cpx	Glyoxalase peroxidase 1	Apoptosis-related factor
NM_010354	Mm21109	Gen	Gelsolin	Apoptosis-related factor
NM_010447	Mm27927	Hmpal	Histone gene set nuclear ribonucleoprotein A1	Apoptosis-related factor
NM_019682	Mm29908	Dck1	Dynein, cytoplasmic, light chain 1	Apoptosis-related factor
NM_009383	Mm242072	Hall	Cytotoxic granule-associated RNA-binding protein-like 1	Apoptosis-related factor
AE041997	Mm28748	Ptg	Prostaglandin H synthase	Apoptosis-related factor
NM_011143	Mm311918	Pkr	Double stranded RNA-dependent protein kinase	Apoptosis-related factor
AE017337	Mm278701	Smt1	Small regulatory element binding protein-1	Apoptosis-related factor
NM_010849	Mm2444	Myr	Myeloblastosis oncogene	Cell cycle regulator
U04710	Mm24553	Igf1r	Insulin-like growth factor 1 receptor	Cytokines and receptor
AA013499	Mm22541	Tradd	TNFR1A-associated via death domain protein	Signal transducer
NM_009734	Mm488	Bcl2	Bcl2-associated athanogene 1	Mitochondrial associated proteins
NM_011738	Mm332314	Tshah	Tyrosine 3-mono oxygenase/tryptophan 5-mono oxygenase activation protein, alpha polypeptide	Signal transducer
NM_008940	Mm245395	Pan	Phosphatase and tensin homolog	Signal transducer

Table 1. Genes induced in leukemia

Gene Bank	UniGene	Abbreviation	Gene Name	Ontogenic classification
U94_001703	Wnt 109	Cidea	DNA-dependent protein kinase, alpha-subunit-like effector A	Apoptosis-related factors
U94_001610	Wnt 10136	Wnt	Wnt cell adhesion 1	Apoptosis-related factors
U94_001711	Wnt 15701	P131	Putative receptor P131, (unclassified) on chromosome 1	Apoptosis-related factors
U94_001794	Wnt 500	Pcd1	Programmed cell death 1	Apoptosis-related factors
U94_001759	Wnt 717	Pcd1	Programmed cell death 1	Apoptosis-related factors
U94_001610	Wnt 1901	Casp1	Caspase 1	Caspases and their regulators
U94_009819	Wnt 11581	BIRC5	BIRC5 Baculovirus IAP repeat containing gene	Caspases and their regulators
U94_009817	Wnt 101	Apc1	Apoptosis gene related to cell cycle	Cell cycle regulators
AP031161	Wnt 618	Sp1	Transcription factor Sp1	Cell cycle regulators
U94_009804	Wnt 110	Cycl1	Cyclin A1	Cell cycle regulators
U11136	Wnt 1901	Cdk2	Cdk2	Cyclins and receptors
U94_001016	Wnt 11701	Cdk1	Growth associated protein 1	Cyclins and receptors
U94_010887	Wnt 11703	Ikna	Inhibitor of receptor, alpha	Cyclins and receptors
U94_001718	Wnt 1684	Ikna	Inhibitor of receptor, alpha	Cyclins and receptors
U94_001719	Wnt 1159	Cdk2	Colony stimulating factor 1 receptor	Cyclins and receptors
U94_011197	Wnt 1010	Tgfb1	Transforming growth factor, beta 1	Cyclins and receptors
U11191	Wnt 1901	Tyrb1	Tyrosinase protein tyrosinase [tyrosinase]	Cyclins and receptors
U94_001010	Wnt 650	Wnt103	Wnt cell adhesion protein 103	Signal transduction
U94_010905	Wnt 1059	NrasF	Neurofibromin 2-like protein	Signal transduction
AP041910	Wnt 17106	Ikba	Inhibitor of receptor, beta	Signal transduction
U94_001065	Wnt 110	Cdk2-3b	Growth associated DNA-dependent protein kinase 3 beta	Signal transduction
S26660	Neurogenic 1	Narb	Receptor associated protein 1	Signal transduction
U94_011129	Wnt 10191	Turb	TRAP family tumor suppressor 1	Signal transduction
U94_009831	Wnt 103	Tcrl	T cell receptor binding factor 1	T cell receptor related proteins

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Table 3. Changes of gene transcription activity during transition from pregnancy to lactation

Ratio	Gene Bank	UniGene	Abbreviation	Gene Name	Ontogenic classification
3.44	NM_007470	Mm.8924	Cdkn2b	Cyclin-dependent kinase inhibitor 2B	Cell cycle regulator
3.94	AF117340	Mm.15918	Map3k11	Mitogen-activated protein kinase kinase kinase 1	Signal transducer
3.39	NM_007778	Mm.795	Ccfl	Colony-stimulating factor 1	Cytokine and receptor
2.95	NM_008594	Mm.1451	Mfge8	Milk fat globule-EGF factor 8 protein	Apoptosis-related factor
2.69	NM_007891	Mm.18034	E2f1	E2F transcription factor 1	Signal transducer
2.14	NM_010512	Mm.248521	Igf1	Insulin-like growth factor 1	Cytokine and receptor
0.49	NM_009983	Mm.231395	Ccd	Ccna protein D	Apoptosis-related factor
0.47	NM_010784	Mm.22470	Mdm2	Cell division cycle 2	Cell cycle regulator
0.43	U6883	Not assigned, yet	CREB	CREB-binding protein	Cell cycle regulator
0.34	L08235	Mm.200408	Chn	Chordin	Apoptosis-related factor
0.28	AF119383	Mm.28835	Esrr	Estrogen receptor	TNF superfamily
0.21	NM_011440	Mm.1275	Tcn	Thiomidoxin	Apoptosis-related factor

scribed in the mammary gland:

- heterogeneous nuclear riboprotein A1;
- light chain 1 of dynein;
- Tial1, cytotoxic granule-associated RNA-binding protein 1;
- double stranded RNA-dependent protein kinase;
- TNFR1a-associated via death domain protein;
- Bcl2-associated athanogene 1;
- ε chain of tyrosine3-monooxygenase/tryptofan5-monooxygenase activation protein;
- cell death-inducing DNA fragmentation factor, alpha subunit;
- programmed cell death 1;
- programmed cell death 2;
- caspase 2;
- baculovirus IAP repeat containing protein;
- apurinic/apyrimidining nuclease;
- interleukin Il4 receptor alpha;
- TYRO3 protein kinase;
- neutral sphingomyelinase activation associated factor.

Probes present on the Panorama Array cover eight ontologic categories as defined

by the Gene Ontology Consortium <http://www.geneontology.org> [Khatri *et al.* 2002] – Table 4. As it was expected, the biggest category (66 genes) is apoptosis-related factors. The 29 genes (44%) of this group were found to be expressed in the mammary gland at pregnancy and (or) lactation. More than half of them (16 from 29) undergo qualitative or quantitative expression changes during transition from pregnancy to lactation. The second big ontologic category is cell cycle regulators (52 genes). In the mammary gland expressed were 32% of genes of these group. Transition to lactation was associated with induction of 7 genes from the cytokines and receptors group, and 6 genes from signal transduction group. Probes on the Panorama Array representing genes belonging to remaining four ontogenic classes: mitochondrial associated, caspases and regulators, telomerase-related and tumor necrosis factors (41 genes) were rarely expressed in the mammary gland during pregnancy and (or) lactation (9 genes).

Several genes expressed in the mammary gland can be organized in a putative network (Fig. 1). During transition from pregnancy to lactation we showed induction

Table 4. Ontogenic classification of genes expressed in the mouse mammary gland

Category	Number of genes in array	Percent of genes expressed in the mammary gland	Number of genes			
			expressed	induced	changing expression	not changing expression
Apoptosis-related factors	66	44	9	5	2	11
Cell cycle regulators	28	32	1	3	3	3
Cytokines and receptors	52	32	1	7	2	7
Signal transducers	43	35	2	4	2	5
Mitochondrial-associated proteins	14	35	1	0	0	1
Caspases and their regulators	21	10	2	0	0	0
Telomerase-related proteins	3	75	1	0	1	0
TNF α superfamily	3	100	0	0	1	2

of TGF- β gene expression (Tab. 2). TGF- β rapidly inhibits *c-myc* expression in a wide variety of cell types [Yue and Mulder 2001], and in the mammary gland too (Tab. 1). TGF- β also downregulates expression of cyclin A and E2F genes in the mammary gland cell line NMuMG [Xie *et al.* 2003], and phosphatase and tensin homolog (PTEN) [Li and Sun 1997, Clarkson *et al.* 2004]. PTEN is a major tumor suppressor that acts by hydrolyzing membrane phosphatidylinositol (PtdIns)-3-phosphates. IGF2 and thioredoxin also regulate expression of PTEN. IGF2 injection into mouse mammary gland significantly increased PTEN expression [Moorehead *et al.* 2003]. Expression of PTEN is downregulated by thioredoxin. Thioredoxin binds in a redox-dependent manner to PTEN to inhibit its PtdIns-3-phosphatase activity [Meuillet *et al.* 2004]

Downregulation of *c-myc* expression can be a cause of induction of caveolin 2 gene during lactation (Tab. 2). It was previously reported that *c-myc* down-regulate

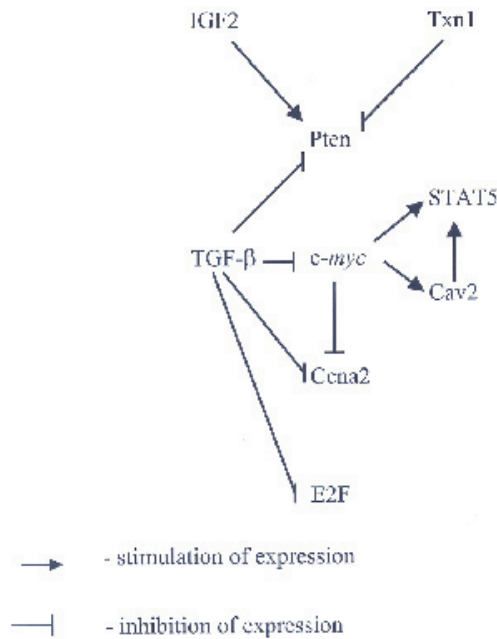


Fig 1. Hypothetical gene network of genes expressed in the mouse mammary gland.

expression of caveolin 1 and activates STAT5 [Blakely *et al.* 2005], E2F1 and cyclin A2 gene expression [Liao *et al.* 2000]. Caveolins are principal structural proteins of caveolae, sphingolipid and cholesterol-rich invaginations of the plasma membrane involved in vesicular trafficking and signal transduction. During caveolae-dependent signalling, caveolin acts as a scaffold protein to sequester and organize multi-molecular signalling complexes involved in diverse cellular activities. Park *et al.* [2001] showed that caveolin-1 expression is significantly downregulated during late pregnancy and lactation. In the presence of lactogenic hormones, recombinant expression of caveolin-1 in HC11 cells dramatically suppresses the induction of the promoter activity and the synthesis of β -casein. Cav-1 null mice show accelerated development of the lobuloalveolar compartment, premature milk production, and hyperphosphorylation of STAT5a at its Jak-2 phosphorylation site (pY694). In addition, the Ras-p42/44 MAPK cascade is hyper-activated [Park *et al.* 2002].

Macroarray analysis of mice mammary gland during transition from pregnancy to lactation allowed finding expression of several new genes and making of genetic network. Future research should more precisely estimate expression profile of genes which expression was found in the mammary gland. Growing number of gene expression profiling should make possible expand the gene regulatory network in the mammary gland.

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Tadeusz Malewski, Stanisław Kaminski, Lech Zwierzchowski

Profilowanie ekspresji genów w gruczole mlekowym myszy podczas przejścia od ciąży do laktacji

Streszczenie

Porównano ekspresję 243 genów w gruczole mlekowym myszy stosując makromacierz Mouse Panorama Apoptosis. Stwierdzono, że podczas przejścia od ciąży do laktacji następuje indukcja 24 genów, represja 16 i ponad dwukrotna zmiana ekspresji 12 genów. Analiza pozwoliła znaleźć 16 nowych genów, których ekspresja zachodzi w gruczole mlekowym myszy. Równoczesna analiza ekspresji 243 genów umożliwiła rozpoczęcie budowania sieci regulacji genów w gruczole mlekowym.

