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

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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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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 MIIZ 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, shook 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.
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**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.
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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-
<table>
<thead>
<tr>
<th>Gene Rank</th>
<th>UniGene</th>
<th>Abbreviation</th>
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<td>XIML</td>
<td>Intein-like protease X</td>
<td>Apoptosis-regulated protease</td>
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**Table 1: Genes involved in apoptosis**
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;
– TNF1a-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 II4 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 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
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 hyperphosphorilation 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.

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


Profilowanie ekspresji genów w gruczołe mlekkowym myszy podczas przejścia od ciąży do laktacji

Streszczenie

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