Animal Science Papers and Reports vol. 24 (2006) no. 2, 119-127
Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

Gene expression profiling in the mouse mammary gland cell line EpH4 during duct-like structure formation on collagen gel*

Tadeusz Malewski, Lech Zwierzchowski, Zofia Szymańczak

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

(Received January 31, 2006; accepted June 5, 2006)

Differentially expressed genes were investigated in mouse mammary gland epithelial cell line EpH4 using cDNA microarrays. In this preliminary study differences were found in gene expression between cells growing on plastic substrate and in collagen gel. Eighty-three genes were shown to be up-regulated and 49 down-regulated. Up-regulated expression of estrogen receptor, *CREB*, and cyclin D1 genes suggest that they may be important for milk duct development.

KEY WORDS cell culture / gene expression / mammary gland / microarray / mouse

The postnatal development of mammary gland involves a tightly scheduled sequence of morphological processes, which include elongation, branching and subsequent budding of alveoli from the growing ducts [Daniel and Silberstein 1987]. These events can be recapitulated *in vitro* by growing mammary epithelial cells within reconstituted three-dimensional matrices. Thus, when embedded in collagen gels, a number of immortalized mammary epithelial cell lines have been reported to form histotypic structures resembling branching ducts or alveoli [Berdichewsky *et al.* 1995, Soriano *et al.* 1995, Montesano *et al.* 1998].

Lumen formation and ductal branching are fundamental events in the morphogenesis of the mammary gland. However, quite little is known about the mechanisms that control these biological processes [Zhang *et al.* 2003, Jackson-Fisher *et al.* 2004,

* Supported by the Ministry of Education and Science, grant 3 P06D 017 23

Morris *et al.* 2006]. Advances in microarray methods allow obtaining expression data of many genes and generating a global expression profile [Chung *et al.* 2002, Steinman and Zamwil 2003]. Microarray analysis has been also a fruitful strategy for the identification of functional genes and used for global gene expression profiling to identify candidate genes and to map growth, metabolic, and regulatory pathways that control important production traits [Cogburn *et al.* 2003].

The present study was undertaken to determine which genes could regulate growth and branching of milk ducts. To address this question we have used EpH4 murine epithelial cell line that forms duct-like structures growing in collagen gel. Expression profiling allowed to select genes that can regulate this process.

Materials and methods

Culture of EpH4 cells

EpH4 cells were grown in tissue culture flasks (Corning) in Dulbecco's modified Eagle's medium (DMEM, GIBCO-Invitrogen) supplemented with 5% calf serum (GIBCO-Invitrogen) and 2 mM L-glutamine (the medium is further referred to as complete medium). For collagen gel cultures, EpH4 cells were harvested with tripsin-EDTA, centrifuged, and suspended in three-dimensional collagen gel as described by Montesano *et al.* [1991]. Briefly, 8 volumes of collagen type I solution (ROCHE) (approximately 1.5 mg/ml) were mixed with 1 volume of 10 × concentrated minimum essential medium (GIBCO) and 1 volume of sodium bicarbonate (11.76 g/ml) in a sterile flask kept on ice to prevent premature collagen gelation. Cells were resuspended in the cold mixture at concentration 1×10^4 cells/ml and 2 ml aliquots were dispensed into 35 mm dishes. After 10 min incubation at 37°C to allow collagen gelation, complete medium was added and changed every 2 days. Cells were grown for one week.

Synthesis of labelled cDNA

Total RNA from fresh cells was extracted with TRI Reagent (SIGMA-ALDRICH, St Louis, MO, USA) according to instructions of the manufacturer. Five μg of total RNA from cells growing in tissue culture flask or in collagen gel were used as a template for reverse transcription (RT) reactions. Amino-modified first-strand cDNA was synthesized using BD Atlas PowerScript fluorescent labelling kit (BD BIOSCIENCES, Alameda, CA, USA) and purified using QuickClean resin to remove protein contaminants. Second-strand cDNA synthesis was performed with oligo$(dT)_{15-18}$ primers using PCR thermal cycler with the following steps: 5 min at 70°C, 5 min at 20°C, 65 min at 42°C, 5 min at 70°C, and 20 min at 37°C. Resulting cDNAs from cells growing on plastic dishes and on collagen gel were differentially labelled with Cy3 and Cy5 dyes as described by manufacturer. Removal of unincorporated dye was performed using FluorTrap Matrix (BD BIOSCIENCES, Alameda, CA, USA).

Hybridization and analysis of array

Glass array (Mouse 1.0 BD Atlas Glass Microarray, BD BIOSCIENCES Clontech, Palo Alto, CA, USA) contains probes for 1081 genes. Absorbances of Cy3 and Cy5 labelled probes were measured on spectrophotometer DU-68 (BECKMAN Instruments, Fullerton, CA, USA) at wavelength 550 nm and 650 nm, respectively. Equal amount of Cy3 and Cy5 labelled probes (0.01 OD) was added to hybridization solution. Hybridization was performed in an Atlas Glass Hybridization chamber (BD BIOSCIENCES, Alameda,CA, USA). Warmed up to 50°C GlassHyb Hybridization Solution (1.82 ml) and labelled cDNAs were transferred into hybridization chambers and hybridized overnight at 50°C. After hybridization, the microarray slides were washed once, for 10 min, in GlassHyb Wash Solution and two times in GlassHyb Wash Solution with 0.1 × SSC. Next, the slides were rinsed in 0.1 × SSC and in distilled water, and dried by centrifuging in the Beckman GS-3 centrifuge at 1200 rpm for 6 min. Immediately after hybridization and washing the slides were scanned with a ScanArray Lite scanner (PERKIN-ELMER, Boston, MA, USA) to detect Cy3 and Cy5 fluorescence with excitation wavelengths 543 and 633 nm and emission filter wavelengths 570 and 670 nm, respectively. Laser power was kept constant for Cy3/ Cy5 scans. Results from two independent microarrays were obtained. QuantArray software (PACKARD BIOSCIENCE, Billerica, MA, USA) was used for processing microarray images, for spot location, and for creation reports of raw spot intensities. Intensity-based global normalization was done to remove dye-specific bias, and background correction was performed by subtracting the normalized median pixel intensity of the background value from the normalized median pixel intensity of the spot. Images for each spot on the array were quantified and stored in an Excel spreadsheet, then merged with the address file for identification. Ratio of means (the ratio of the arithmetic mean intensities of each feature for each wavelength to the median background subtracted) was calculated for every spot. Genes with two-fold changes in expression were considered to be up- or down-regulated.

Results and discussion

EpH4 is a nontumorigenic cell line derived from spontaneously immortalized mammary gland epithelial cells [Fialka *et al.* 1996]. Growing in collagen gels EpH4 cells forms three-dimensional structures similar to milk ducts. Prelimary analysis showed 83 genes to be up-regulated (Tab. 1) and 49 down-regulated (Tab. 2). Among the up-regulated were estrogen receptor gene, *CREB*, cyclin D1, *p53*, *Mdm2* and Cathepsin D genes.

Estrogens induce cell proliferation in target tissues by stimulating progression through the G(1) phase of the cell cycle. Induction of cyclin D1 expression is a critical feature of the mitogenic action of estrogen. In EpH4 cells growing in the collagen gel up-regulated is expression of estrogen receptor and cyclin D1 genes. Sabbah *et al.* [1999] showed the presence of cAMP response element in the cyclin D1 promoter

Table 1. Up-regulated genes in EpH4 cell line growing in collagen gel

GenBank Acc. No	Gene	Ratio collagen/ plastic
M77167	Activating transcription factor 2	2.3
AF030769	Bcl2-like 2	2.6
U20372	Calcium channel beta 3 subunit	2.4
X58995	Calcium/calmodulin-dependent protein kinase IV	2.8
X52886	Cathepsin D	2.2
U06119	Cathepsin H	2.9
X06086	Cathepsin L	2.1
Y15163	Cbp/p300-interacting transactivator	2.4
NM_010786	Murine double minute 2	2.6
D12487	Choline acetyltransferase	2.7
D00472	Cofilin 1	3.1
M85078	Colony stimulating factor 2 receptor, alpha	2.8
S66385	CREB-binding protein	2.5
X84311	Cyclin A1	2.2
Z26580	Cyclin A2	2.4
X66032	Cyclin B2	2.5
S78355	Cyclin D1	2.9
AF091432	Cyclin E2	2.6
Z37110	Cyclin G1	3.1
M59470	Cystatin C	3.4
AF022078	Distal-less homeobox 6	2.6
AB009375	DNA fragmentation factor, alpha subunit	2.8
M38651	Estrogen receptor 1	2.7
U85259	Estrogen related receptor, alpha	2.5
AF097999	Fatty acid amide hydrolase	2.8
L07297	FMS-like tyrosine kinase 1	2.3
M93428	Glycosylation dependent cell adhesion molecule 1	3.3
M33324	Growth hormone receptor	2.5
X61753	Heat shock factor 1	3.1
X73573	Homeobox D3	2.6
U96386	Inhibin beta E	2.1
U04710	Insulin-like growth factor 2 receptor	2.2
X81579	Insulin-like growth factor binding protein 1	2.1
X81582	Insulin-like growth factor binding protein 4	2.1
M60778	Integrin alpha L	3.6

Table 1 continued

GenBank Acc. No	Gene	Ratio collagen/ plastic
M28233	Interferon gamma receptor	2.1
M20658	Interleukin 1 receptor, type I	2.3
M27960	Interleukin 4 receptor, alpha	2.2
J03236	Jun B oncogene	2.7
J04115	Jun oncogene	2.4
X03492	Keratin complex 1, acidic, gene 13	2.3
X03491	Keratin complex 2, basic, gene 4	3.1
X84014	Laminin, alpha 3	3.3
U43298	Laminin, beta 3	2.6
U43327	Laminin, gamma 2	2.9
U42467	Leptin receptor	2.5
X86569	LIM-domain containing, protein kinase	2.7
S67830	Matrix metalloproteinase 9	2.1
L35236	Mitogen activated protein kinase 10	3.5
U14636	Mitogen activated protein kinase kinase kinase 12	2.6
AF039567	Msx-interacting-zinc finger protein	2.7
U35623	Myeloid cell leukemia sequence 1	3.2
M57999	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	2.6
U49185	Occludin	3.9
M10624	Ornithine decarboxylase, structural	2.6
D86599	Oxytocin receptor	2.7
AB010833	Patched homolog 2	2.2
U43144	Phospholipase C, beta 3	2.5
U73488	Potassium channel, subfamily K, member 2	3.4
Z54283	POU domain, class 2, associating factor 1	3.6
U57324	Presenilin 2	2.5
X67914	Programmed cell death 1	2.6
L13593	Prolactin receptor	2.9
X01237	Protein 53	2.3
D45910	RAR-related orphan receptor alpha	2.6
AB004315	Regulator of G-protein signaling 4	2.8
Z27088	Relaxin 1	3.1
S56660	Retinoic acid receptor, beta	2.4
X68837	Secretogranin II	2.2

Table 1 continued

GenBank Acc. No	Gene	Ratio collagen/ plastic
U08378	Signal transducer and activator of transcription 3	2.3
U21103	Signal transducer and activator of transcription 5A	2.1
X68951	Somatostatin receptor 2	2.6
U63933	TATA box binding protein	2.2
X65687	Thymoma viral proto-oncogene	2.6
X62622	Tissue inhibitor of metalloproteinase 2	2.7
J03520	Tissue plasminogen activator	2.4
U59864	TRAF family member-associated NF-kappa B activator	2.1
M29618	Transferrin receptor	2.7
NM_009399	Tumor necrosis factor receptor superfamily, member 11a	2.5
U18343	TYRO3 protein kinase kinase 3	2.8
X51703	Ubiquitin B	2.1
X62701	Urokinase plasminogen activator receptor	2.2
M95200	Vascular endothelial growth factor	2.1

that confers regulation by estrogens in the human mammary carcinoma cells MCF-7. The induction was strictly estrogen-dependent and required the DNA-binding domain as well as both AF-1 and AF-2 domains of the estrogen receptor (ER) alpha. In the current investigation expression of *CREB* transcription factor was up-regulated what is in accordance with proposed mechanism of cyclin D1 regulation.

Estrogen down-regulates glucocorticoid receptor (GR) gene expression by the proteasomal degradation pathway. Estrogen-mediated degradation of GR is coupled to an increase in p53 and *Mdm2*. Chromatin immunoprecipitation assay demonstrated an estrogen-dependent recruitment of ERα to the *Mdm2* promoter, suggesting a role of ER in the regulation of Mdm2 protein expression [Kinyamu and Archer 2003]. Increased expression of p53 can increase expression of cathepsin D gene. Expression of the gene encoding cathepsin D is known to be stimulated by estrogen in mammary cancer cells, and p53 DNA binding site is located in the promoter region of the cathepsin D gene [Ikeguchi *et al*. 2002]. In the present investigation an increased expression of *p53*, *Mdm2*, and cathepsin D encoding gene were observed.

Profiling of gene expression in murine mammary gland epithelial cell line EpH4 growing on plastic support and(or) in collagen gel allowed finding genes that may regulate growth and development of milk ducts. Future research should more precisely estimate expression profile of these genes and their role in growth and branching of milk ducts.

Table 2. Down-regulated genes in EpH4 cell line growing in collagen gel

GenBank Acc. No	Gene	Ratio collagen/ plastic
M12414	Apolipoprotein E	0.44
D90374	Apurinic/apirimidinic endonuclease	0.32
L25602	Bone morphogenic protein 2	0.48
X56906	Bone morphogenic protein 7	0.39
Y13085	Caspase 2	0.45
S69034	Cathepsin B	0.31
U89269	Cathepsin C	0.49
M21952	Colony stimulating factor 1 (macrophage)	0.47
X06368	Colony stimulating factor 1 receptor	0.41
U20636	Cyclin F	0.41
U51001	Distal-less homeobox 1	0.48
S81932	Distal-less homeobox 3	0.37
U67840	Distal-less homeobox 5	0.46
U43512	Dystroglycan 1	0.43
L21973	E2F transcription factor 1	0.49
Z36885	ELK4, member of ETS oncogene family	0.41
U70324	Fyn proto-oncogene	0.40
X95255	GLI-Kruppel family member GLI2	0.44
J03958	Glutatione S-transferase, alpha 2	0.46
M18459	Granzyme B	0.32
L07379	Growth hormone-releasing hormone receptor	0.43
U38505	Guanine nucleotide binding protein, beta 2	0.42
U20370	Homeobox A11	0.38
M92649	Inducible nitric oxide synthase	0.41
X04480	Insulin-like growth factor 1	0.48
U00182	Insulin-like growth factor I receptor	0.43
M14951	Insulin-like growth factor 2	0.46
X81580	Insulin-like growth factor binding protein 2	0.47
X81581	Insulin-like growth factor binding protein 3	0.41
X81584	Insulin-like growth factor binding protein 6	0.46
Z29987	Integrin alpha 2	0.33
M68903	Integrin beta 7	0.45
S69336	Interferon gamma receptor 2	0.44

Table 2 continued

GenBank Acc. No	Gene	Ratio collagen/ plastic
L12120	Interleukin 10 receptor, Ralpha	0.49
X75337	Interleukin 2 receptor, gamma chain	0.49
L28819	Involucrin	0.35
U12147	Laminin, alpha 2	0.37
U37501	Laminin, alpha 5	0.42
M15525	Laminin, beta 1	0.40
X75928	Laminin, beta 2	0.46
U18812	Lepton	0.43
AF072251	Methyl CpG binding protein 2	0.38
AB006787	Mitogen activated protein kinase kinase kinase 5	0.45
AF013632	Neutral sphingomyelinase (N-SMase) activation associated factor	0.49
X17647	Neurotrophic tyrosine kinase, receptor, type 2	0.42
Z32740	Protein tyrosine phosphatase, non-receptor type 13	0.47
Z30970	Tissue inhibitor of metalloproteinase 3	0.43
X57796	Tumor necrosis factor receptor superfamily, member 1a	0.45
M73963	YY1 transcription factor	0.41

REFERENCES

1. BERDICHEVSKY F., ALFORD D., D'SOUZA B., TAYLOR-PAPADIMITRIOU J., 1994 – Branching morphogenesis of human mammary epithelial cells in collagen gels. ***Journal of Cell Science*** 107, 3557-3568.
2. CHUNG C.H., BERNARD P.S., PEROU C.M., 2002 – Molecular portraits and the family tree of cancer. ***Nature Genetics*** 32, 533- 540.
3. COGBURN L.A., WANG X., CARRE W., REJTO L., PORTER T.E., AGGREY S.E., SIMON J., 2003 – Systems-wide chicken DNA microarrays, gene expression profiling, and discovery of functional genes. ***Poultry Science*** 82, 939-951.
4. DANIEL C.W., SILBERSTEIN G.B., 1987 – Postnatal development of the rodent mammary gland. In: The Mammary gland. Development, regulation and function (M.C. Neville, C.W. Daniel, eds). Plenum Press, New York, p. 3-36.
5. FIALKA I., SCHWARZ H., REICHMANN E., OFT M., BUSSLINGER M., BEUG H., 1996 – The estrogen-dependent c-JunER protein causes a reversible loss of mammary epithelial cell polarity involving a destabilization of adherens junctions. ***The Journal of Cell Biology*** 132, 1115-1132.
6. IKEGUCHI M., SAKATANI T., UETA T., FUKUDA K., OKA S., HISAMITSU K., YAMAGUCHI K., TSUJITANI S., KAIBARA N., 2002 – Correlation between cathepsin D expression and p53 protein nuclear accumulation in oesophageal squamous cell carcinoma. ***Journal of Clinical Pathology*** 55(2),121-126.

7. JACKSON-FISHER A.J., BELLINGER G., RAMABHADRAN R., MORRIS J.K., LEE K.F., STERN D.F., 2004 – ErbB2 is required for ductal morphogenesis of the mammary gland. ***Proceedings of the National Academy of Sciences of the USA*** 101, 17138-17143.
8. KINYAMU H.K., ARCHER T.K., 2003 – Estrogen receptor-dependent proteasomal degradation of the glucocorticoid receptor is coupled to an increase in Mdm2 protein expression. ***Molecular and Cellular Biology*** 23, 5867-5881.
9. MONTESANO R., SCHALLER G., ORCI L., 1991 – Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. ***Cell*** 66, 697-711.
10. MONTESANO R., SORIANO J.V., FIALKA I., ORCI L., 1998 – Isolation of EpH4 mammary epithelial cell subpopulation which differ in their morphogenic properties. ***In Vitro Cellular and Developmental Biology*** 34, 468-477.
11. MORRIS J.S., STEIN T., PRINGLE M.A., DAVIES C.R., WEBER-HALL S., FERRIER R.K., BELL A.K., HEATH V.J., GUSTERSON B.A., 2006 – Involvement of axonal guidance proteins and their signaling partners in the developing mouse mammary gland. ***Journal of Cellular Physiology*** 206, 16-24.
12. SABBAH M., COURILLEAU D., MESTER J., REDEUILH G., 1999 – Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element. ***Proceedings of the National Academy of Sciences of the USA***. 96, 11217-11222.
13. SORIANO J.V., PEPPER M.S., NAKAMURA T., ORCI L., MONTESANO R., 1995 – Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. ***Journal of Cell Science*** 108, 413-430.
14. STEINMAN L., ZAMVIL S., 2004 – Transcriptional analysis of targets in multiple sclerosis. ***Nature Reviews Immunology*** 3, 483-492.
15. ZHANG J., BREWER S., HUANG J., WILLIAMS T., 2003 – Overexpression of transcription factor AP-2alpha suppresses mammary gland growth and morphogenesis. ***Developmental Biology*** 256, 127-145.

Tadeusz Malewski, Lech Zwierzchowski, Zofia Szymańczak

Profilowanie ekspresji genów w mysiej linii komórek EpH4 gruczołu mlekowego podczas hodowli w żelu kolagenowym i tworzenia struktur podobnych do przewodów mlekowych

S t r e s z c z e n i e

Wstępna analiza wykazała co najmniej dwukrotne zmiany w ilości mRNA 132 genów. W przypadku 83 genów ilość mRNA była większa, a w 49 mniejsza w komórkach, które rosły w żelu kolagenowym. Zwiększone ekspresje genu receptora estrogenu, genu *CREB* i genu cykliny D1 sugerują, że geny te mogą pełnić istotną rolę w rozwoju przewodów mlekowych.