

## Differential gene expression profile in breast cancer-derived stromal fibroblasts

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Received: 19 June 2007 / Accepted: 31 July 2007 / Published online: 26 September 2007  
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### Abstract

**Background** Breast cancer is characterized by malignant transformation of epithelial cells, but stromal cells also play an important role in tumorigenesis. While tumor-derived fibroblasts display unique phenotypic properties, it is unclear whether they also represent a specific subpopulation.

**Materials and Methods** Stromal fibroblasts deriving from malignant tissue of 10 women with invasive breast cancer, and from normal breast tissue of 10 women with benign breast disorders, were subjected to differential complementary DNA Microarray Analysis by using a 2,400 gene cDNA array. Individual gene expression patterns were confirmed by RT-PCR.

**Results** In a cDNA array that allows to analyze the differential gene expression of more than 2,400 genes, the mRNA expression of 135 genes were increased more than 2 fold in fibroblasts from malignant breast tumors. The majority of these genes encode tumor-promoting cytokines, transcription factors and cell-matrix associated proteins. The mRNA expression of 110 genes decreased to less than 0.5 fold. The remaining 2,155 genes were not significantly altered. RT-PCR performed on individual biopsies from breast cancer and normal breast tissues confirmed the validity of the pooled gene expression signature.

**Conclusion** Breast cancer-derived stromal fibroblasts show a distinctive gene expression pattern that differentiates them from normal breast stroma. Our observation of increased expression of tumor promotion-associated genes even in the absence of adjacent malignant epithelium suggests that tumor stroma is comprised of a fibroblastic subpopulation that provides for a microenvironment which supports tumor growth and invasion.

**Keywords** Breast cancer · Gene expression profile · Stromal fibroblasts

### Introduction

The mammary gland consists of epithelial and stromal cells, and their coordinated function is essential for the development and physiological properties of the female breast. While breast cancer arises primarily from mammary epithelium, it is now recognized that breast stroma also plays an important role in the induction and promotion of breast cancer. Interestingly, many of the stromal factors that have been implicated in the promotion of or protection against breast cancer are also vital for the normal patterning and function of breast tissue [1]. Over the last years, numerous studies have investigated factors that are thought to be involved in stromal-epithelial interactions and to contribute to the growth-promoting environment that is a prerequisite for tumor growth and invasion. Among them, insulin-like growth factor 1 (IGF-1) is prototypical: It mediates the function of growth hormone (GH) and is required by mammary epithelium for proper ductal development [2]. However, high concentrations of IGF-1 are associated with an appreciably increased risk of breast cancer in premenopausal women [3]. Furthermore, IGF-1 protein expression

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in breast cancer cells, is associated with a more malignant biological behavior *in vitro*, and IGF-1 protein overexpression in tumor cells is an established adverse prognostic parameter [4]. TGFbeta, by contrast, is physiologically expressed in mature periductal extracellular matrix and is thought to prevent inappropriate ductal side branching [5]. Disruption of TGFbeta signaling, at least in early cancer stages, results in tumor promotion and local invasion [6]. Other stromal proteins that have been implicated in paracrine stimulation of adjacent malignant epithelium include matrix metalloproteinases (MMPs), integrins and parathyroid hormone-related protein (PTHrP) [7–9].

Although an impressive number of individual molecules have already been studied as factors being potentially involved in stromal-epithelial interactions, it has so far not been possible to fit these components into a model that would encompass the principal mechanisms by which stromal cells support the growth and malignant behavior of adjacent tumor epithelium.

Complementary (c)DNA Arrays provide a powerful tool to overcome this problem and to allow the simultaneous monitoring of hundreds of possible genetic/molecular factors in cells or whole tissues. The present study was undertaken to investigate the differential gene expression signature of stromal fibroblasts from malignant breast tumors. To this end, we have pooled RNA from fibroblast monocultures that had been established from 10 malignant breast tumors and from 10 benign breast tissues in order to identify tumor-specific alterations in stromal gene expression and to identify potential therapeutic targets. Immunohistochemistry in paraffin-embedded tumor sections was then utilized to investigate whether the up-regulation of specific genes in tumoral fibroblasts also translates to protein overexpression in the stromal component of malignant breast tumors.

## Materials and methods

### Fibroblast cultures and RNA isolation

Primary cultures of stromal fibroblasts were established from surgical specimens that had been obtained directly from the malignant tumor of breast cancer patients, or from macroscopically normal breast tissue during surgery for non-malignant breast disease as previously described [19, 20]. The study was approved by the local IRB. In brief, fresh breast tissue was dissected free of fat, minced, and subjected to collagenase/hyaluronidase treatment under constant stirring at 37°C for 6–8 h. The resulting cell suspension was passed through a 50 µm filter, centrifuged, and seeded in a 25 or 75 cm<sup>2</sup> tissue culture flask. After 5–7 days stromal fibroblasts were obtained by differential

trypsinization. The resulting second-passage fibroblasts were then seeded into a separate flask and maintained in DMEM/Ham's F12 supplemented with 10% fetal calf serum and 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 mg/ml amphotericin B (Life Technologies, Rockville, MD).

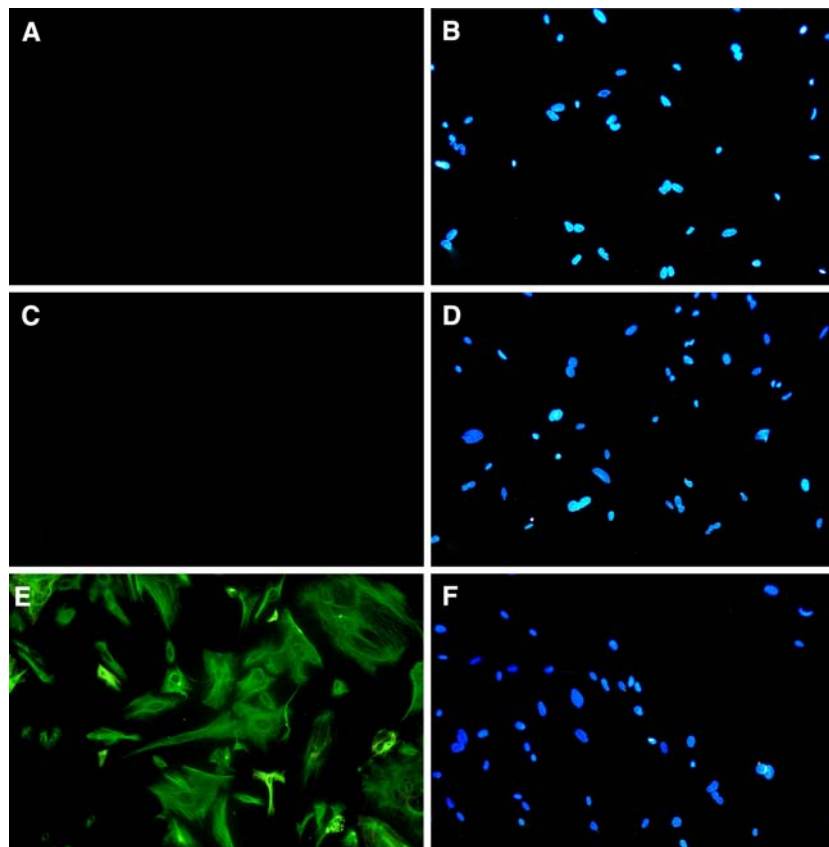
Confluent fibroblast cultures were washed twice in ice-cold PBS before being dissolved in 1,000 µl homogenization buffer containing 0.3 M sodium acetate, pH 6, 0.02 M EDTA, and 1:50 (w/w) vanadyl ribonucleoside complex (GIBCO BRL). After addition of 1 vol. SS-phenol and 1 vol. chloroform:isoamyl alcohol (24:1), the tubes were heated to 55°C for 5 min before being placed into ice for another 5 min. Phases were separated by a 2 min spin at 5,000 g, and the upper phase was removed to a new tube. RNA was precipitated in the presence of 2.5 vol. of absolute ethanol for 10 min on dry ice, and microcentrifuged for 10 min at 4°C. RNA pellets were then washed with 80% ice-cold ethanol and dissolved in RNase-free water. Total RNA concentrations were assessed by UV spectrophotometry (Pharmacia, Uppsala, Sweden).

### Keratin, CD45, and vimentin immunofluorescence

The purity of fibroblast monocultures was assessed by immunostaining for keratin (mouse monoclonal PAN-CK, NeoMarkers, Fremont, CA, dilution 1:100), CD 45 (mouse monoclonal antibody CD45RB, Dako Cytomation, Glostrup, Denmark, 1:100), and vimentin (mouse monoclonal antibody Clone V9, Sigma Chemical, USA, 1:100). A fraction of the cell suspension that was cultured for RNA isolation was plated on LabTec<sup>®</sup> chamber slides (Nunc, Wiesbaden, Germany) and grown until sub-confluent. Cells were then washed with PBS and fixed in ice-cold 0.01% Triton X-100 (Sigma) for 10 min at RT. After two additional washing steps with PBS, the primary antibodies (both diluted 1:100 in PBS with 10 µg/ml BSA) were added and incubated overnight at 4°C. After three washing steps with PBS, the fluorescence-labeled secondary antibody (Alexa Fluor<sup>®</sup> anti-mouse 488, Invitrogen, Carlsbad, CA) was added and incubated for 30 min at RT. After three further washing steps with PBS, the specimens were mounted in glycerol and covered with glass cover slips (Fig. 1). Nuclei were stained with 4'-6-Diamidino-2-phenylindole (DAPI, Sigma). Control cells were incubated with replacement of the primary antibody by PBS (data not shown).

### Microarray probe and hybridization

The MICROMAX<sup>™</sup> human cDNA microarray system I (NEN Life Science Products, Inc., Boston, MA), which



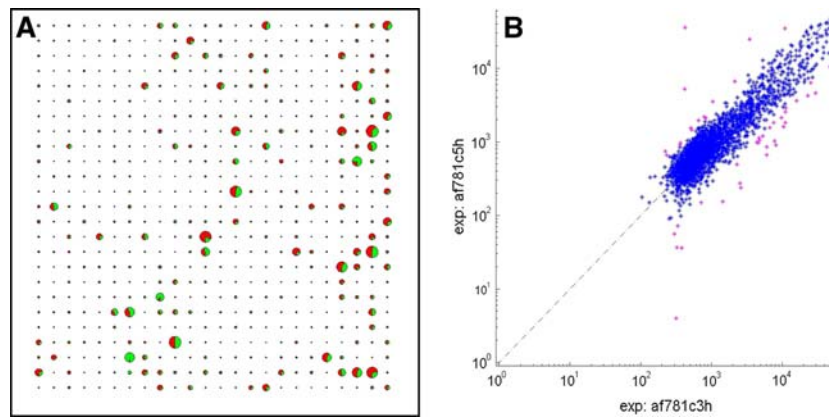
**Fig. 1** Fluorescence immunohistochemistry of monocultures of stromal fibroblasts using pan-keratin (A), CD45 (C) and vimentin (E) antibodies as described in Materials and methods. The corresponding DAPI nuclear staining controls are shown in the right panel (B, D, and F)

contains 2,400 known human cDNA on a slide 1 inch  $\times$  3 inches, was used in this study. Biotin-labeled cDNA was generated from 3  $\mu$ g of total RNA that was created by pooling equal amounts of samples from each of the 10 fibroblast cultures that had been obtained from malignant breast tissue. The samples were pooled in order to minimize unbalanced cDNA expression profiles due to inaccurate pipetting, while at the same time yielding an acceptable “background-to-noise ratio”. Dinitrophenyl-labeled cDNA was generated from 3  $\mu$ g of total RNA that was pooled from 10 fibroblast cultures derived from benign breast tissue. The biotin-labeled cDNA and the dinitrophenyl-labeled cDNA were then mixed, dried, and resuspended in 20  $\mu$ l of hybridization buffer (5 $\times$  standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), and salmon sperm DNA at 0.1 mg/ml (1 $\times$  SSC = 0.15 M NaCl–0.15 M sodium citrate, pH 7)). The mix was layered onto the cDNA microarray and was covered with a coverslip. Hybridization was carried out overnight at 65°C inside a hybridization cassette (Telechem, Inc., Sunnyvale, CA). After hybridization, the microarray was washed with 30 ml of 0.5 $\times$  SSC–0.01% SDS, with 30 ml of 0.06 $\times$  SSC–0.01% SDS, and then with 30 ml of 0.06 $\times$  SSC alone. The hybridization

signal from biotin-labeled cDNA was amplified with streptavidin-horseradish peroxidase and Cy5<sup>TM</sup>-tyramide (NEN Life Science Products, Inc.), and the hybridization signal from the dinitrophenyl-labeled cDNA was amplified with anti-dinitrophenyl-horseradish peroxidase and fluorescent Cy3<sup>TM</sup>-tyramide (NEN Life Science Products, Inc.). After signal amplification the microarray was washed in TNT buffer (i.e., 0.1 M Tris-HCl (pH 7.5)–0.15 M NaCl–0.15% Tween 20), air-dried, and signal amplification was detected with a laser scanner. Laser detection was performed by using the ScanArray3000 confocal laser reader (GSI Lumonics, Watertown, MA). The fluorescence signal intensity and the ratio of the signals from Cy3 and Cy5 for each of the 2,400 cDNAs were analyzed by the software Imagen 3.0 (Biodiscovery Inc., Los Angeles, CA).

#### RT-PCR and agarose gel electrophoresis

0.5–1  $\mu$ g of total RNA extracted from each of the fibroblast monocultures were incubated with random hexamers (Promega, Inc.) and adjusted to 5  $\mu$ l with DEPC (diethyl pyrocarbonate)-treated ddH<sub>2</sub>O, before being heat-



**Fig. 2** (A) Relative gene expression levels are depicted in GenePie™ format for visual evaluation of differential expression. Intensities for each spot are represented as red and green pie slices. The red and green pie sizes are proportional to the percentages of intensities for a particular spot in the green Cyanine 3 (tumor fibroblasts) and the red Cyanine 5 (normal breast fibroblasts) channels respectively. The larger the green pie segment in comparison to the red segment, the higher the relative expression of a gene in tumor-derived fibroblasts. A red pie segment that is larger than the corresponding green segment indicates increased expression of the respective mRNA in non-tumor derived breast fibroblasts. A pie that is all green (red) indicates the presence of mRNA that is only expressed in tumor-derived fibroblasts (in fibroblasts derived from non-malignant breast, respectively). This

segmentation of pies allows to visually evaluate differential expression of genes in the two analyzed samples of RNA. Pie size is proportional to the absolute intensity of the spot on the array and reflects how strongly this particular gene is expressed relative to other genes. Only one representative grid with 600 genes is shown here. (B) The distribution of spots based on their intensity values in the Cyanine 3 and Cyanine 5 channels is shown in a scatter plot. A shift from the diagonal line is proportional to a difference in expression levels between the Cyanine 3 labeled sample and the Cyanine 5 labeled samples. Spots inside and outside the predetermined range of significant differential expression in the assay are depicted in blue and red, respectively

denatured at 70°C for 5 min and chilled on ice. The samples were then added to a reaction mix consisting of 4 µl of 5× RT-buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>), 2 µl dNTP mix stock solution (10 mM each Pharmacia), 1 µl RNase inhibitor (Applied Biosystems, Vienna, Austria), 1 µl dithiothreitol (DTT), and 1 µl MMLV (moloney murine leukemia virus)-RT (200 U/µl, Amersham Bioscience Ltd.). The reaction mix was vortexed and centrifuged briefly, before being incubated at 37°C for 1 h. The reaction was then stopped by a 10 min heat to 80°C and tubes were chilled on ice before they were briefly centrifuged and stored at -20°C.

PCR was performed by adding 20 µl reaction mix to 2.5 µl 10× PCR-buffer, 2 µl dNTP mix (10 mM each), 0.25 µl primer (100 µM), 5 µl TAQ polymerase (5 U/ml) to the respective primers (cadhepsin L: GTT GGG CGT TTA GCG CAT CG and CCC ATG GTC GAG GTT CTT GC; HYL: AAG AAT TCG CGA TGG CGG GGC GAG GCT CT and AAT ATC TCG AGT GGG GTC AGG GCT CCT GGC; osteopontin: TTG CAG CCT TCT CAG CCA A and GGA GGC AAA AGC AAA TCA CTG; cadherin-11: ACC AGA TGT CTG TGT CAG A and GTC ATC CTT GTC ATC TGC A). The housekeeping gene GAPDH (primer sequences: GAA GGT GAA GGT CGG AGT C and GAA GAT GGT GAT GGG ATT TC) was used for normalization. A total volume of 45 µl was reached by adding DEPC-treated ddH<sub>2</sub>O. Cycling conditions were as follows: depending on the primers, 25–35 cycles were

carried out of 94°C for 1 min, 68°C for 2 min, 72°C for 2 min, with an extension of 5 s with each subsequent cycle. Placental total RNA was used in RT-PCR experiments for each of the primers and ddH<sub>2</sub>O was used instead of total RNA for negative controls. Agarose gel electrophoresis was carried out by adding 20 µl of each of the PCR products were subjected to 1.2% NuSieve® agarose gel electrophoresis and in 1 × TBE buffer and were separated by applying a constant voltage at 80 V for 1–2 h. DNA bands were then visualized by ethidium bromide. Band size was determined by a co-loaded DNA size marker.

## Results

### Characterization of fibroblastic monocultures derived from breast stroma

The purity of fibroblastic monocultures was assessed by immunohistochemistry using the pan-leukocyte marker CD45, which also detects macrophages and histiocytes (Fig. 1A). In addition, we used a monoclonal pan-keratin antibody to confirm the absence of epithelial contaminations in our cultures (Fig. 1C). By contrast, cultured cells all stained when using a vimentin antibody as demonstrated in Materials and methods (Fig. 1E). DAPI staining in Fig. 1B, D, and F confirmed the presence of nucleated cells in monocultures.

**Table 1** Genes up-regulated in breast cancer-derived stromal fibroblasts

Signal	Ratio	Difference	Protein/Gene
Growth factors, cytokines and receptors			
Strong	2.90	1306	GM-CSF receptor
Weak	2.76	153	Metabotropic glutamate receptor 7
Medium	2.28	453	Effector cell protease receptor-1 (EPR-1)
Strong	2.25	1111	Pigment epithelium-differentiation factor (PEDF)
Medium	2.20	221	Hepatoma transmembrane kinase ligand (HTK ligand)
Medium	2.16	297	Alpha-2-macroglobulin receptor-associated protein
Strong	2.07	2485	K-ras oncogene protein
Regulation of gene expression			
Strong	2.56	1876	Nuclear-encoded mitochondrial elongation factor Ts (EF-Ts)
Medium	2.55	249	RNA helicase-related protein
Medium	2.52	224	U1 small nuclear ribonucleoprotein 70 kd protein
Strong	2.48	4000	Ribosomal protein S12
Medium	2.23	419	Chromatin assembly factor-I p150 subunit
Medium	2.19	370	Spliceosome associated protein (SAP 145)
Weak	2.07	227	Centrosomal Nek2-associated protein 1 (C-NAP1)
Cell cycle and metabolism			
Strong	5.96	8882	GTP-binding protein alpha q subunit (GNAQ)
Medium	4.29	777	RASF-A PLA2 (phospholipase A2)
Strong	3.67	1420	63 kDa protein kinase related to rat ERK3
Medium	3.05	805	Pescadillo
Medium	2.66	612	CDC2 gene involved in cell cycle control
Strong	2.33	5672	Clock
Medium	2.07	628	Ts11 gene encoding a G-1 progression protein
Medium	2.07	443	Calnexin
Cell–cell interaction, communication and cell structure			
Medium	3.02	566	Osteopontin
Weak	2.47	227	Tight junction (zonla occludens) protein ZO-1
Weak	2.47	210	Aquaporin adipose
Weak	2.40	167	Sperm membrane protein BS-63
Weak	2.26	254	Synaptogyrin 3
Medium	2.18	322	Transmembrane 4 superfamily protein (SAS)
Medium	2.17	391	Tapasin (NGS-17)
Medium	2.04	487	Troponin I fast-twitch isoform
Proteolysis and kinase activation			
Medium	11.21	1970	HYL tyrosine kinase
Strong	4.41	3174	Dihydrodiol dehydrogenase
Medium	3.15	311	Pro-cathepsin L (proteolytic enzyme)
Medium	3.04	333	Beta-D-galactosidase
Medium	2.94	418	Tryptophanyl tRNA synthetase (IFNWRS)
Medium	2.78	558	Rolipram-sensitive 3',5'-cyclic AMP phosphodiesterase
Weak	2.65	186	Sarcomeric mitochondrial creatin kinase (MtCK) gene
Weak	2.31	216	IMP dehydrogenase type 1
Strong	2.27	1053	Protein tyrosine phosphatase (PTPase)

Ratio describes the relative expression of a gene as the ratio signal intensity in tumor-derived stromal fibroblasts/signal intensity in fibroblasts derived from non-malignant breast tissue. Difference indicates the absolute difference of array spot densities (in arbitrary units) between both groups

**Table 2** Genes down-regulated in breast cancer-derived stromal fibroblasts

Signal	Ratio	Difference	Protein/Gene
Medium	0.14	2741	Skeletal muscle alpha-tropomyosin (hTM-alpha)
Medium	0.15	2953	Cytokine-inducible nuclear protein
Medium	0.16	1061	OB-cadherin-1
Weak	0.25	401	Transforming growth factor-beta (tgf-beta)
Strong	0.28	10107	Osteoblast-specific factor-2 (OSF-2p1)
Medium	0.37	245	Heat shock factor 1 (TCF5)

Ratio describes the relative expression of a gene as the ratio signal intensity in tumor-derived stromal fibroblasts/signal intensity in fibroblasts derived from non-malignant breast tissue. Difference indicates the absolute difference of array spot densities (in arbitrary units) between both groups

#### Differential gene expression pattern in stromal fibroblasts derived from benign and malignant breast tissues

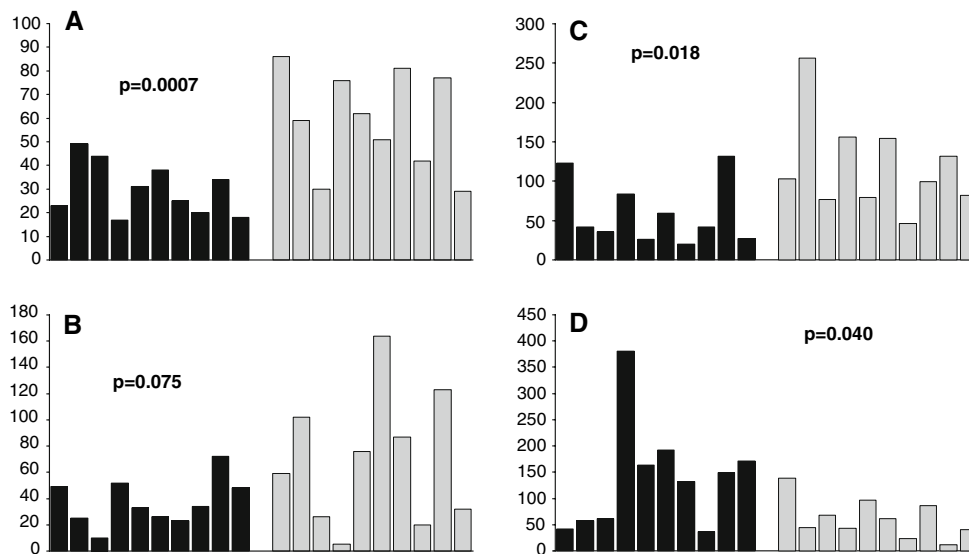
We chose to pool 10 fibroblast samples in each of the two groups in order to minimize unbalanced cDNA expression profiles due to inaccurate pipetting, while at the same time yielding an acceptable “background-to-noise ratio” (the ratio would have increased by pooling a larger number of patient RNAs per group). Figure 2 shows cDNA array hybridization signals on a MICROMAX™ human cDNA microarray system. Relative gene expression levels are depicted in GenePie™ format for visual evaluation of differential expression. Intensities for each spot are represented as red and green pie slices. The red and green pie sizes are proportional to the percentages of intensities for a particular spot in the Cyanine 3 (tumor-derived fibroblasts) and the Cyanine 5 (normal breast tissue-derived fibroblasts) channels respectively. The pie size is proportional to the absolute intensity of the spot on the array and reflects how strongly this particular gene is expressed relative to other genes. One representative grid of four, with 600 genes each, is shown in Fig. 2. The intensity of each spot was measured by densitometry. Expression levels of individual genes in ten pooled fibroblast monocultures derived from malignant breast tissues are shown here in relation to corresponding signal intensities in 10 pooled monocultures that had been established from benign breast tissues. Of the 2,400 mRNA analyzed, 135 genes were upregulated more than 2 fold, and 38 genes were upregulated more than 2.5 fold in tumor-derived fibroblasts. On the other hand, the expression of 110 genes was decreased by at least 2 fold, and of 55 by at least 2.5 fold in fibroblasts derived from malignant breast stroma. About 2,155 genes, were neither up- nor down-regulated.

Genes for which mRNAs were up-regulated in pooled fibroblasts derived from breast cancer stroma encoded for growth factors, cytokines and their respective receptors and included GM-CSF receptor, R 2.90; Metabotropic glutamate receptor 7, R 2.76; Effector cell protease receptor-1

(EPR-1), R 2.28; Pigment epithelium-differentiation factor (PEDF), R 2.25; Hepatoma transmembrane kinase ligand (HTK ligand), R 2.20; Alpha-2-macroglobulin receptor-associated protein, R 2.16 and the K-ras oncogene protein, R 2.07. Up-regulated genes that are associated with cell-cell interaction, communication and in the maintenance of cell structures included osteopontin, R 3.02; tight junction (zonula occludens) protein ZO-1, R 2.47; aquaporin adipose, R 2.47; Sperm membrane protein BS-63, R 2.40; synaptogyrin 3, R 2.26; transmembrane 4 superfamily protein (SAS), R 2.18; Tapasin (NGS-17), R 2.17 and Troponin I fast twitch isoform, R 2.04. Genes which encode for proteolytic enzymes and which exhibited an increased expression in tumor-derived fibroblasts were HYL tyrosine kinase, R 11.21; Dihydrodiol dehydrogenase R 4.41; Pro-cathepsin L, R 3.15; Beta-D-galactosidase, R 3.04; Tryptophanyl tRNA synthetase (IFNWRS) R 2.94; Rolipram-sensitive 3', 5'-cyclic AMP phosphodiesterase, R 2.78; Sarcomeric mitochondrial creatin kinase (MtCK) gene, R 2.65; IMP dehydrogenase type 1, R 2.31 and Protein tyrosine phosphatase (PTPase), R 2.27 Table 1.

Up-regulated genes for proteins that are involved in the regulation of gene expression included Nuclear-encoded mitochondrial elongation factor Ts (EF-Ts), R 2.56; RNA helicase-related protein R 2.55; U1 small nuclear ribonucleoprotein (70 kD protein), R 2.52; Ribosomal protein S12, R 2.48; Chromatin assembly factor-I p150 subunit, R 2.23; Spliceosome associated protein (SAP 145), R 2.19 and Centrosomal Nek2-associated protein 1 (C-NAP1), R 2.07. Cell cycle and metabolism-related gene products included GTP-binding protein alpha 1 subunit (GNAQ), R 5.96; RASF-A PLA2 (phospholipase A2), R 4.29; 63 kDa protein kinase related to rat ERK3, R 3.67; Pescadillo, R3.05; CDC2 gene involved in cell cycle control, R 2.66; Clock, R 2.33; Ts11 gene encoding a G-1 progression protein, R 2.07; Calnexin, R 2.07.

Genes that showed decreased expression in pooled breast cancer-derived stromal fibroblasts when compared to pooled fibroblasts derived from non-malignant breast tissues included skeletal muscle alpha-tropomyosin (hTM-



**Fig. 3** Real-time (RT)-PCR for cadhepsin L (A), HYL (B), osteopontin (C), and cadherin-11 (D) was performed on total RNA extracted from fibroblast monocultures deriving from the malignant tissue of 10 women with invasive breast cancer (gray bars) and from

normal breast tissue of 10 women with benign breast disorders (black bars). Primers and cycling conditions for each of the amplified genes are described in Materials and methods

alpha), R 0.14; Cytokine-inducible nuclear protein, R 0.15; OB cadherin-1 (cadherin-11), R 0.16; transforming growth factor beta (TGF-beta), R 0.25; Osteoblast-specific factor-2 (OSF-2p1), R 0.28 and Heat shock factor 1 (TCF5); (Table 2). About 2,155 genes that were differentially up-regulated <2 fold or that were down-regulated <0.5 fold in breast cancer-derived fibroblasts were excluded from further analysis.

RT-PCR confirmation of individual gene expression pattern in stroma derived from benign and malignant breast tissue

To validate differential gene expression levels in the pooled samples and to confirm that mRNA levels of genes were also differentially expressed in each of the individual fibroblast monocultures from benign and malignant breast lesions, RT-PCR analysis was performed in selected, differentially regulated genes. Semi-quantitative mRNA expression of cadhepsin L, HYL, osteopontin and cadherin-11 was determined for each of the 10 patients in both groups. As expected, we observed a significant up-regulation of transcripts coding for cadhepsin L and osteopontin in tumor-derived breast fibroblasts ( $P = 0.0007$ ; and  $P = 0.018$ , respectively, Fig. 3 A and C), while the cadherin-11 mRNA expression decreased in most monocultures (Fig. 3D). While we also observed a trend towards increased levels of HYL mRNA in the 10 breast cancer-derived stromal fibroblasts analyzed, the individual

differences in gene expression pattern did not reach statistical significance ( $P = 0.075$ , Fig. 3B).

## Discussion

Mammary stroma has an active role in the control and regulation of physiological processes in the breast. In breast cancer, however, peritumoral stroma is mainly considered to support the growth and invasion of adjacent malignant epithelium in response to tumor cell-derived paracrine signals [10]. There is, however, accumulating evidence which suggests that mammary stroma is actively involved in the generation of transformed lesions and tumours. It has even been hypothesized that in the absence of pre-existing tumor cells, stromal fibroblasts can promote tumorigenicity in adjacent epithelium by facilitating the development of genomic changes [11].

Several groups have therefore investigated stroma-derived factors that might influence the behavior of adjacent epithelium. We have, for example, previously demonstrated a differential IGF gene expression pattern in tumor-derived fibroblasts when compared to fibroblasts that were derived from neighboring, macroscopically uninvolved stroma of the same patients [12]. While the observed difference in gene and protein expression could be explained by stromal-epithelial interactions that select for a tumor growth-enhancing fibroblast subpopulation, it has been argued that stromal fibroblasts from breast cancer patients represent an entirely distinct population that *per se*

could favor in the process of malignant transformation and tumor progression in neighboring epithelium. This hypothesis is strengthened by findings by Schor et al, who described a characteristic fetal migration pattern in skin fibroblasts from breast cancer patients that was not observed in corresponding cells from healthy controls [13]. The present study also points to the existence of a distinct fibroblast population in breast tissue that specifically expresses tumor-promoting factors.

Among the genes that were upregulated in breast stroma, HYL (also termed Csk-homologous Kinase CHK or MATK) was found to be increased profoundly in the pooled analysis, a pattern that is somewhat supported by a trend towards an increased HYL gene expression in the 10 individual breast cancer-derived fibroblastic monocultures. The lack of statistical significance in the individual fibroblast samples is most likely explained by the very heterogenous HYL expression within one group of fibroblasts: very high mRNA expression levels in one or more samples of one group—such as the ones seen in Fig. 3B—result in profound differences in the pooled analysis, while an individual analysis of benign versus malignant fibroblasts reveals less impressive overall differences. The tyrosine kinase HYL is able to phosphorylate and inactivate Src family kinases. It is thought to be participate in signalling in breast cancer cells and has been shown to be expressed in breast cancer tissues, while it is absent in adjacent normal tissues [14]. Colony stimulating factor-1 (GM-CSF-1) was another cytokine whose gene expression was upregulated. This growth factor recruits macrophages which, in turn, promote mammary ductal invasion during puberty [15]. CSF-1 is also expressed during pregnancy and appears to mediate lobuloalveolar differentiation [16]. It is, however, also necessary for the progression of mammary tumors to malignancy in a mouse model [17]. Not surprisingly, cathepsin L was also up-regulated in tumoral stroma. It is a protease that catalyzes degradation of the interstitial matrix and basement membranes, thus allowing cancer cells to invade locally and metastasize to distant sites. Our observation is in line with previous findings by Castiglioni et al and Harbeck et al, who also found the protein to be a strong and independent prognostic parameter for DFS *in vivo* [18, 19].

Osteopontin is another protein that was significantly upregulated in breast cancer-derived stromal fibroblasts. It is an integrin-binding protein that has been shown to be involved in physiologic events such as angiogenesis, apoptosis, inflammation, and wound healing. It is, however, also associated with the progression of several cancer types, and to play an important functional role in various aspects of malignancy, particularly tissue invasion and metastasis [20]. Osteopontin is expressed in both fibroblasts and invasive breast cancer cells and has been

demonstrated to stimulate cell motility and nuclear factor kappaB-mediated secretion of urokinase type plasminogen activator through phosphatidylinositol 3-kinase/Akt signaling pathways in breast cancer cells, presumably through paracrine and autocrine mechanisms [21, 22]. Recently, it has been shown that in women with metastatic breast cancer, increases in osteopontin levels over time are strongly associated with poor survival [23].

Among the proteins that were downregulated in breast cancer-derived fibroblasts, TGF-beta is one of the most prominent. Traditionally, the cytokine has been thought to act upon the epithelium as a tumor suppressor, and to be an important factor regulating branching morphogenesis and differentiation by acting on both epithelial and stromal cells [24]. However, it is now also recognized to have a biphasic effect on tumor progression by acting as a tumor suppressor in early stages of cancer yet by promoting invasion and metastasis at later stages [25]. While the biological role of the observed decrease in TGF-beta gene expression *in vitro* is unclear, it could indicate that tumoral stroma favors the early stages of tumorigenesis by suppressing the cytokine that is responsible for inducing cell cycle arrest and possibly apoptosis in adjacent breast epithelium. Cadherin-11 (ob-cadherin), a member of the cadherin family, was also down-regulated in tumoral stroma. Cadherins are essential cell adhesion molecules involved in tissue morphogenesis and the maintenance of tissue architecture in adults, and especially the expression of N-cadherin and cadherin-11 has been shown to be well correlated with the invasive phenotype in cancer cells [26]. Our finding of decreased cadherin-11 expression in tumor stroma is somewhat surprising since the protein is believed to mediate the interaction between malignant tumor cells and other cadherin-11 expressing cell types such as stromal cells or osteoblasts and has been shown to be a molecular marker for the more aggressive, invasive subset of tumors by thus facilitating tumor cell invasion and metastasis [27].

Taken together, we have demonstrated a distinct gene expression profile that is typical for breast cancer-derived stromal fibroblasts and that distinguishes them from fibroblasts obtained from non-malignant breast tissue. The fact that fibroblasts retain their gene expression pattern in the absence of neighboring tumor epithelium and over several culture passages *in vitro*, suggests that tumoral stroma is comprised of a specific fibroblast subpopulation which favors tumor growth and malignant behavior. While *in vivo*, fibroblast gene expression patterns are clearly influenced by adjacent epithelium via paracrine mechanisms, stromal cells are not just responding to epithelium-derived stimuli but also actively influence the fate of adjacent epithelium.



**Acknowledgements** This study was supported by a grant from the Foundation for Breast Health, by a grant from the Austrian Society for Endocrinological Oncology, by a grant from Amgen Inc. and Ebewe Inc., and by a grant from Medizinisch-Wissenschaftlicher Fonds des Bürgermeisters der Bundeshauptstadt Wien.

## References

1. Wiseman BS, Werb Z (2002) Stromal effects on mammary gland development and breast cancer. *Science* 296:1046–1049
2. Bonnette SG, Hadsell SL (2001) Targeted disruption of the IGF-I receptor gene decreases cellular proliferation in mammary terminal end buds. *Endocrinology* 142:4937–4945
3. Renehan AG, Zwahlen M, Minder C, O'Dwyer ST, Shalet SM, Egger M (2004) Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *Lancet* 363:1346–1353
4. Kucab JE, Dunn SE (2003) Role of IGF-1R in mediating breast cancer invasion and metastasis. *Breast Dis* 17:41–47
5. Derynck R, Akhurst JR, Balmain A (2001) TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 29:117–129
6. Kaklamani V, Pasche B (2005) Transforming growth factor beta and breast cancer. *Cancer Treat Res* 126:129–156
7. Duffy MJ, Maguire TM, Hill A, McDermott E, O'Higgins N (2000) Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. *Breast Cancer Res* 2(4):252–257
8. Mercurio AM, Bachelder RE, Chung J, O'Connor KL, Rabinovitz I, Shaw LM, Tani T (2001) Integrin laminin receptors and breast carcinoma progression. *J Mammary Gland Biol Neoplasia* 6(3):299–309
9. Torricelli C, Fortino V, Capurro E, Sacchi G, Ponzo P, Pacini A, Muscettola M, Maioli E (2006) Role of PTHrp and PTHrp-engaged pathways in MCF-7 cells migration/invasion. *Matrix Biol* 25(2):104–111
10. Barcellos-Hoff MH, Medina D (2005) New highlights on stroma-epithelial interactions in breast cancer. *Breast Cancer Res* 7(1):33–36
11. Kim JB, Stein R, O'Hare MJ (2005) Tumour-stromal interactions in breast cancer: the role of stroma in tumorigenesis. *Tumour Biol* 26(4):173–185
12. Singer C, Rasmussen A, Smith HS, Lippman ME, Lynch HT, Cullen KJ (1995) Malignant breast epithelium selects for insulin-like growth factor II expression in breast stroma: evidence for paracrine function. *Cancer Res* 55(11):2448–2454
13. Grey AM, Schor AM, Rushton G, Ellis I, Schor SL (1989) Purification of the migration stimulating factor produced by fetal and breast cancer patient fibroblasts. *Proc. Natl Acad Sci U S A* 86:2438–2442
14. Zrihan-Licht S, Lim J, Keydar I, Sliwkowski MX, Groopman JE, Avraham H (1997) Association of csk-homologous kinase (CHK) (formerly MATK) with HER-2/ErbB-2 in breast cancer cells. *J Biol Chem* 272(3):1856–1863
15. Gouon-Evans V, Rothenberg ME, Pollard JW (2000) Postnatal mammary gland development requires macrophages and eosinophils. *Development* 127(11):2269–2282
16. Pollard JW, Hennighausen L (1994) Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proc Natl Acad Sci U S A* 91(20):9312–9316
17. Lin EY, Nguyen AV, Russell RG, Pollard JW (2001) Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* 193(6):727–740
18. Castiglioni T, Merino MJ, Elsner B, Lah TT, Sloane BF, Emmert-Buck MR (1994) Immunohistochemical analysis of cathepsins D, B, and L in human breast cancer. *Hum Pathol* 25(9):857–862
19. Harbeck N, Alt U, Berger U, Thomssen C, Jänicke F, Höfler H, Kates RE, Schmitt M (2001) Prognostic impact of proteolytic factors (urokinase-type plasminogen activator, plasminogen activator inhibitor 1, and cathepsins B, D, and L) in primary breast cancer reflects effects of adjuvant systemic therapy. *Clin Cancer Res* 7:2757–2764
20. Cook AC, Tuck AB, McCarthy S, Turner JG, Irby RB, Bloom GC, Yeatman TJ, Chambers AF (2005) Osteopontin induces multiple changes in gene expression that reflect the six “hallmarks of cancer” in a model of breast cancer progression. *Mol Carcinog* 43(4):225–236
21. Zohar R, Suzuki N, Suzuki K, Arora P, Glogauer M, McCulloch CA, Sodek J (2000) Intracellular osteopontin is an integral component of the CD44-ERM complex involved in cell migration. *J Cell Physiol* 184(1):118–130
22. Das R, Mahabeleshwar GH, Kundu GC (2003) Osteopontin stimulates cell motility and nuclear factor kappaB-mediated secretion of urokinase type plasminogen activator through phosphatidylinositol 3-kinase/Akt signaling pathways in breast cancer cells. *J Biol Chem* 278(31):28593–28606
23. Bramwell VH, Doig GS, Tuck AB, Wilson SM, Tonkin KS, Tomiak A, Perera F, Vandenberg TA, Chambers AF (2006) Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. *Clin Cancer Res* 12(11 Pt 1):3337–3343
24. Wakefield LM, Piek E, Bottinger EP (2001) TGF-beta signaling in mammary gland development and tumorigenesis. *J Mammary Gland Biol Neoplasia* 6(1):67–82
25. Muraoka-Cook RS, Dumont N, Arteaga CL (2005) Dual role of transforming growth factor beta in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 11(2 Pt 2):937s–943s
26. Nieman MT, Prudoff RS, Johnson KR, Wheelock MJ (1999) N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J Cell Biol* 147(3):631–644
27. Pishvaian MJ, Feltes CM, Thompson P, Bussemakers MJ, Schalken JA, Byers SW (1999) Cadherin-11 is expressed in invasive breast cancer cell lines. *Cancer Res* 59(4):947–952