Breast Cancer Res Treat
DOI 10.1007/s10549-010-1185-4

PRECLINICAL STUDY

MPA-induced gene expression and stromal and parenchymal gene expression profiles in luminal murine mammary carcinomas with different hormonal requirements

Sebastián Giulianelli · Jason I. Herschkowitz · Vyomesh Patel · Caroline A. Lamb · J. Silvio Gutkind · Alfredo Molinolo · Charles M. Perou · Claudia Lanari

Received: 27 August 2010 / Accepted: 17 September 2010

Abstract Over the past several years, we have been interested in understanding the mechanisms by which mammary carcinomas acquire hormone independence. We demonstrated that carcinoma associated fibroblasts participate in the ligand-independent activation of progesterone receptors inducing tumor growth. In this study, we used DNA microarrays to compare the gene expression profiles of tumors from the MPA mouse breast cancer model, one hormone-dependent (C4-HD) and one hormone-independent (C4-HI), using whole tumor samples or laser-captured purified stromal and epithelial cells obtained from the same tumors. The expression of selected genes was validated by immunohistochemistry and immunofluorescence assays. We identified 413 genes specifically expressed in tumor stroma. Eighty-five percent of these genes were upregulated, whereas the remaining 15% were downregulated in C4-HI relative to their expression in the C4-HD tumor stroma. Several matrix metallopeptidases were overexpressed in the C4-HI tumor microenvironment. On the other hand, 1100 genes were specifically expressed in the tumor parenchyma. Among them, the 29% were upregulated, whereas the remaining 71% were downregulated in C4-HI relative to C4-HD tumor epithelium. *Steap*, *Pdgfc*, *Runx2*, *Cxcl9*, and *Sdf2* were among the genes with high expression in the C4-HI tumor parenchyma. Interestingly, *Fgf2* was one of the few genes upregulated by MPA in C4-HD tumors, confirming its pivotal role in regulating tumor growth in this model. In conclusion, we demonstrate herein a gene expression profile that distinguishes both the epithelial and the stromal cells in mammary tumors with different hormone dependence, supporting the hypothesis that the tumor-associated stroma may contribute to hormone-independent tumor growth.

Keywords Breast cancer · Epithelium · Tumor stroma · DNA microarray analysis

Abbreviations

CAFs	Carcinoma-associated fibroblasts
DCIS	Ductal carcinoma in situ
EASE	Expression analysis systematic explorer
ER	Estrogen receptor
FDR	False discovery rate
GO	Gene ontology
H&E	Hematoxylin and eosin
HD	Hormone-dependent or MPA-dependent tumor
HI	Hormone-independent or MPA-independent tumor

Electronic supplementary material The online version of this article (doi:10.1007/s10549-010-1185-4) contains supplementary material, which is available to authorized users.

S. Giulianelli · C. A. Lamb · C. Lanari (✉)
Laboratory of Hormonal Carcinogenesis, Instituto de Biología y Medicina Experimental (Consejo Nacional de Investigaciones Científicas y Técnicas—CONICET), Vuelta de Obligado 2490, C1428ADN Buenos Aires, Argentina
e-mail: clanari@dna.uba.ar

J. I. Herschkowitz
Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

V. Patel · J. S. Gutkind · A. Molinolo
Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, USA

C. M. Perou
Departments of Genetics and of Pathology and Laboratory Medicine, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA

Published online: 02 October 2010

IDC	Invasive ductal carcinomas
LCM	Laser capture microdissection
MPA	Medroxyprogesterone acetate
NAFs	Normal-associated fibroblasts
PI	Propidium iodide
PR	Progesterone receptor
SAM	Significance analysis of microarrays
s.c.	Subcutaneous

Introduction

Seventy percent of breast cancers express estrogen (ER) and progesterone receptors (PR) and lesions arise mainly in postmenopausal women in which endogenous levels of ovarian hormones are very low. However, most of these tumors will respond to endocrine treatment, suggesting that hormone receptors play a key role driving tumor growth. Different hypotheses have been proposed to explain what activates hormone receptors in these groups of patients (reviewed in [1]). The possibility that ligand-independent activation of hormone receptors could be involved in tumor growth has become an attractive hypothesis in recent years, and accordingly, overexpression of signaling pathways has been related to ER and PR activation [2–4]. This improved sensitivity to growth factor signaling may be due to an increase in autocrine or paracrine factors that may in turn activate hormone receptors [5, 6].

Breast carcinomas consist of a malignant epithelial compartment supported by non-malignant stromal cells. The stromal compartment involves multiple cell types, including fibroblasts, myofibroblasts, myoepithelial cells, adipocytes, endothelial cells, macrophages, and other immune cells [7]. One prevailing view is that these stromal cells are activated by the malignant tumor cells to foster tumor growth [8], and there exists substantial evidence that the tumor microenvironment can modify the proliferation, differentiation and invasive, and metastatic abilities of cancer cells [9–11].

We have been interested in the past several years in understanding the mechanisms by which hormone-dependent (HD) tumors grow in the absence of stimulatory hormones. In medroxyprogesterone acetate (MPA)-induced mouse mammary adenocarcinomas, HD tumors (C4-HD) require the exogenous administration of progestin to grow in vivo, whereas the hormone-independent (HI) variant (C4-HI) can grow in untreated female mice without hormone administration. Regardless of their respective hormone requirements, both tumor types express high levels of ER and PR [12]. In vitro, however, epithelial cells from HD and HI tumors have the same hormone or growth factor requirements, suggesting the involvement of endogenous host factors in regulating the in vivo tumor growth [13]. We have recently demonstrated that carcinoma-associated fibroblasts (CAFs) from HI tumors produce higher levels of FGF-2 in vitro than those obtained from their HD counterparts. Interestingly, FGF-2 is able to activate PR as effectively as progesterone itself, providing a loop by which HI tumors can grow in the absence of exogenous hormone administration [13]. These findings have led us to hypothesize that the stroma from HI tumors actively participates in the acquisition of the HI phenotype by secreting growth factors that may in turn activate steroid receptors in epithelial tumor cells thus bypassing hormone requirements. In addition, we have not yet determined the nature of the factors that the epithelial cells secret to recruit the activated fibroblasts. To further explore this hypothesis, we have now investigated gene expression profiles to detect differences in the stroma and in the tumor parenchyma between the C4-HD tumor and its HI variant, C4-HI, using laser capture microdissection (LCM) and DNA microarray technology.

DNA microarray studies have made great contributions in the breast cancer field, yielding a reclassification of tumors based on their genetic profiles [14]. All of these studies have been performed using whole tissue samples. A similar approach has been utilized in mouse mammary carcinomas from different experimental models [15]. However, because most of these tumor models lack ER and PR expression, the profiling of mouse models of tumors expressing ER and PR is still awaited.

Some recent studies used LCM to select stromal or parenchymal cells for genetic profiling [16–19]. These studies showed that the stroma from aggressive tumors is different from the stroma from normal tissue, and moreover, stromal gene expression may predict clinical outcome [17] and therapy response [20] in breast cancer. However, no studies have evaluated the role of stroma in the acquisition of hormone independence.

In this study, we profiled C4-HD tumors growing in the presence of MPA or when the progestin was removed for 48 h. Only eight genes were upregulated by MPA, including *Fgf2*, supporting our previous findings pointing to a pivotal role for FGF-2 in tumor growth [13]. Using DNA microarrays of laser-captured stromal and epithelial cells from C4-HD and C4-HI tumors, we demonstrated that the stroma from both tumors types have distinct gene expression profiles. Some of the stromal and epithelial genes were validated by immunohistochemistry and immunofluorescence in the same tumors. Several matrix metallopeptidases genes, such as *Mmp3*, *Mmp9*, *Mmp10*, and *Mmp13* were found overexpressed in the C4-HI tumor microenvironment compared to expression in C4-HD tumors. Conversely, some epithelial factors are potential candidates to play a significant role in stromal recruitment,

including PDGFc, CXCL9, and SDF2, which were specifically overexpressed in C4-HI as compared with C4-HD epithelial cells.

Materials and methods

Animals

Two-month-old virgin female BALB/c mice (IByME Animal Facility) were used. Animal care and manipulation were in agreement with institutional guidelines and the Guide for the Care and Use of Laboratory Animals [21].

Tumors

Previously characterized C4-HD and C4-HI tumors from the MPA mouse breast cancer model [12] were used in DNA microarray assays. C4-HD is a HD tumor maintained by serial subcutaneous (s.c.) transplantations in MPA-treated BALB/c female mice (C4-HD +MPA). The HI variant (C4-HI), which was derived from a C4-HD tumor that grew in a mouse that had not been treated with MPA, is maintained by serial s.c. transplantation in BALB/c female mice without progestin treatment. To obtain samples of C4-HD tumors without MPA, the hormone pellets were removed 48 h prior to sacrifice (C4-HD −MPA). C4-HD and C4-HI tumors express high levels of ERα and PR.

Tissue processing and LCM

C4-HD +MPA ($n = 3$) and C4-HI ($n = 3$) tumors of about 60 mm^2 in size were embedded in Tissue-Tek OCT (EMS, PA, USA), frozen and stored at −80°C until sectioning. Eight-micron cryosections from each tumor were obtained, fixed in 70% ethanol (25 s) and washed in RNase-free water followed by quick hematoxylin and eosin (H&E) staining. Finally, sections were dehydrated in increasing concentrations of ethanol (70–100%) for 5 s each, placed in Safeclear II (Fisher Scientific, Pittsburgh, PA, USA) and air-dried. After staining, the sections were analyzed by a pathologist, and cells of interest were located. CapSure LCM Cap (Arcturus, Mountain View, CA, USA) was placed over the target area, and highly enriched epithelial or stromal cell populations from consecutive tissue sections were obtained by using a PixCell IIe LCM system (Arcturus) according to the manufacturer's protocol. Approximately, 4000 laser pulses were used to purify the different cell populations from each tumor sample, and multiple LCM caps containing the isolated cells from each sample were stored in dry ice and immediately processed for RNA extraction.

RNA extraction and linear amplification

Total RNA from whole tumors (C4-HD +MPA $n = 5$, C4-HD −MPA $n = 4$, and C4-HI $n = 7$) and from the common reference sample (equal numbers of C57BL6/J and 129 male and female day 1 pups [15]) was purified using an RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Total RNA (2.5 μg) was reverse-transcribed, amplified and labeled with Cy5-CTP (PerkinElmer, Waltham, MA, USA) for tumor samples and Cy3-CTP (PerkinElmer) for the common reference sample, using a Low RNA Input Amplification kit (Agilent, Santa Clara, CA, USA). The amplified fluorescent-labeled RNA (aRNA) was purified using the QIAquick PCR Purification kit (Qiagen). For microdissected tissue samples, several LCM-caps were pooled into a single tube containing 200 μl of denaturing buffer (GITC: 5.25 M guanidinium isothiocyanate, 50 mM Tris–HCl pH 6.4, 20 mM EDTA, 1% Triton X-100) and 1.6 μl of β-mercaptoethanol. Total RNA was then extracted using a modified protocol of the Stratagene RNA microisolation kit (Stratagene, La Jolla, CA), and amplified with the MessageAmp II aRNA amplification kit (Ambion). Two rounds of RNA amplification were performed. For the second round, cDNA synthesis and purification were performed starting with 0.25 μg of aRNA from the first round using the MessageAmp II aRNA amplification kit. The in vitro transcription reaction and Cy5-CTP labeling were performed using the Low RNA Input Amplification kit, and the aRNA was purified using the QIAquick PCR Purification kit.

Microarray experiments and data analysis

Two micrograms of each amplified experimental sample was co-hybridized with 2 μg of the amplified reference to Agilent Mouse Oligo Microarrays (G4121B) at 60°C in an overnight incubation. The microarrays were washed in 2× SSC/0.005% Triton X-102 buffer, then in 0.1× SSC buffer and finally put in Stabilization and Drying Solution (Agilent). The arrays were then scanned on an Axon GenePix 4000B scanner and analyzed using GenePix 4.1 software. Areas of the array with evident defects were manually flagged and excluded from subsequent analyses. All microarray raw data tables were uploaded into the UNC microarray database (UMD, https://genome.unc.edu/), where background subtraction and Lowess normalization are automatically performed to adjust the Cy3 and Cy5 channels [15]. Genes were filtered to select those that had a Lowess normalized signal intensity of at least 30 units in both channels. The $\log_2$Cy5/Cy3 ratio was obtained for each gene. Only those genes with reported values in 70% or more of the samples were included, and the genes were

centered by medians. All data have been deposited into the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE23214. A two-class significance analysis of microarrays (SAM) was performed to identify significantly differentially expressed genes between whole tumor samples and LCM tumor samples [22]. The SAM δ values were adjusted to obtain the largest gene list that gave a false discovery rate (FDR) of less than 5%. Hierarchical clustering using gene lists obtained by SAM, was performed with Cluster v2.12 software [23]. The results were visualized using JavaTreeview v1.0.8 software [24]. Expression Analysis Systematic Explorer analysis (EASE, http://david.abcc.ncifcrf.gov/) [25] was used to interpret the gene lists derived using SAM and to convert the gene list into biological themes. Highly enriched gene ontologies were defined if the threshold of EASE score, a modified Fisher exact probability, had a P value <0.05.

Immunohistochemistry

Sections (5 μm) of formalin-fixed, paraffin-embedded tumor tissues were reacted with different antibodies using the avidin–biotin peroxidase complex technique (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA). Briefly, microwave antigen retrieval (four cycles of 5 min each in 0.1 M citrate buffer) using a 750-W Philips M902 microwave oven was performed before endogenous peroxidase activity inhibition (10% H_2O_2 in distilled water). Blocking solution (10% normal fetal bovine serum) was used before hybridization with specific antibodies. Polyclonal antibodies to PDGFc (H-125) and TF (FL-294), both from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and to RUNX2 (Ab23981) and PTEN (Ab32199), both from Abcam (Cambridge, MA, USA), were used at 1:100 dilutions and incubated overnight at 4°C. After biotin-conjugated secondary antibody (1:100 dilution) incubation for 1 h at room temperature, the reaction was developed using the DAKO Liquid DAB + Substrate Chromogen System (K3468, DAKO) according to the manufacturer's protocol under microscopic control. Specimens were lightly counterstained with hematoxylin, dehydrated and mounted.

Immunofluorescence

Frozen sections (15 μm) were formalin-fixed, blocked with 10% normal fetal bovine serum, and incubated with primary antibodies recognizing MMP13 (H-230) and MMP9 (C-20, both from Santa Cruz Biotechnology), αSMA (Ab5694, from Abcam) and cytokeratin (pancytokeratin, from DAKO) at a 1:100 dilutions overnight at 4°C. After FITC-conjugated secondary antibody (1:100 dilution) incubation for 1 h at room temperature, the nuclei were counterstained with propidium iodide (PI). Sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and analyzed under Nikon Eclipse E800 Laser Confocal Microscope and EZ-C1 2.20 software.

Results

Gene expression changes mediated by MPA in C4-HD tumors

The hormone-dependent tumor C4-HD only grows in vivo in the presence of MPA. Hormone removal induces complete tumor regression [26]. To locate MPA-regulated genes in this tumor model, we used DNA microarrays. We compared the gene expression patterns of C4-HD tumors growing in the presence of MPA (C4-HD +MPA, $n = 5$) to those in tumors in which MPA had been removed 48 h before tumor dissection (C4-HD −MPA, $n = 4$). SAM [22] with an FDR of less than 5% was used to find significant differences in gene expression between MPA-treated or untreated C4-HD tumors (Table 1). Only 8 genes were found to be upregulated by MPA, whereas 104 genes were downregulated by the progestin. To visualize these expression changes, we combined the SAM-supervised lists of significant genes and performed an unsupervised hierarchical clustering analysis (Fig. 1a). The dendrogram shows two primary branches, one grouping C4-HD +MPA tumors and one including C4-HD −MPA tumor samples. Table 2 shows the top 50 highest regulated genes by MPA in C4-HD tumors (see Supplementary data file 1). Interestingly, the first gene on the list was *Fgf2*, a growth factor that has been

Table 1 Number of genes significantly altered in tumor samples as determined by SAM

Tumor samples comparison	No. of significant genes			No. of false significant	FDR (%)
	Total	Up	Down		
C4-HD +MPA vs C4-HD −MPA	112	8	104	5.52	4.9
C4-HI vs C4-HD +MPA	5242	2696	2546	225.6	4.3
C4-HI vs C4-HD −MPA	4534	2024	2510	191.94	4.23

SAM significance analysis of microarrays, *FDR* false discovery rate, *up* upregulated genes, *down* downregulated genes

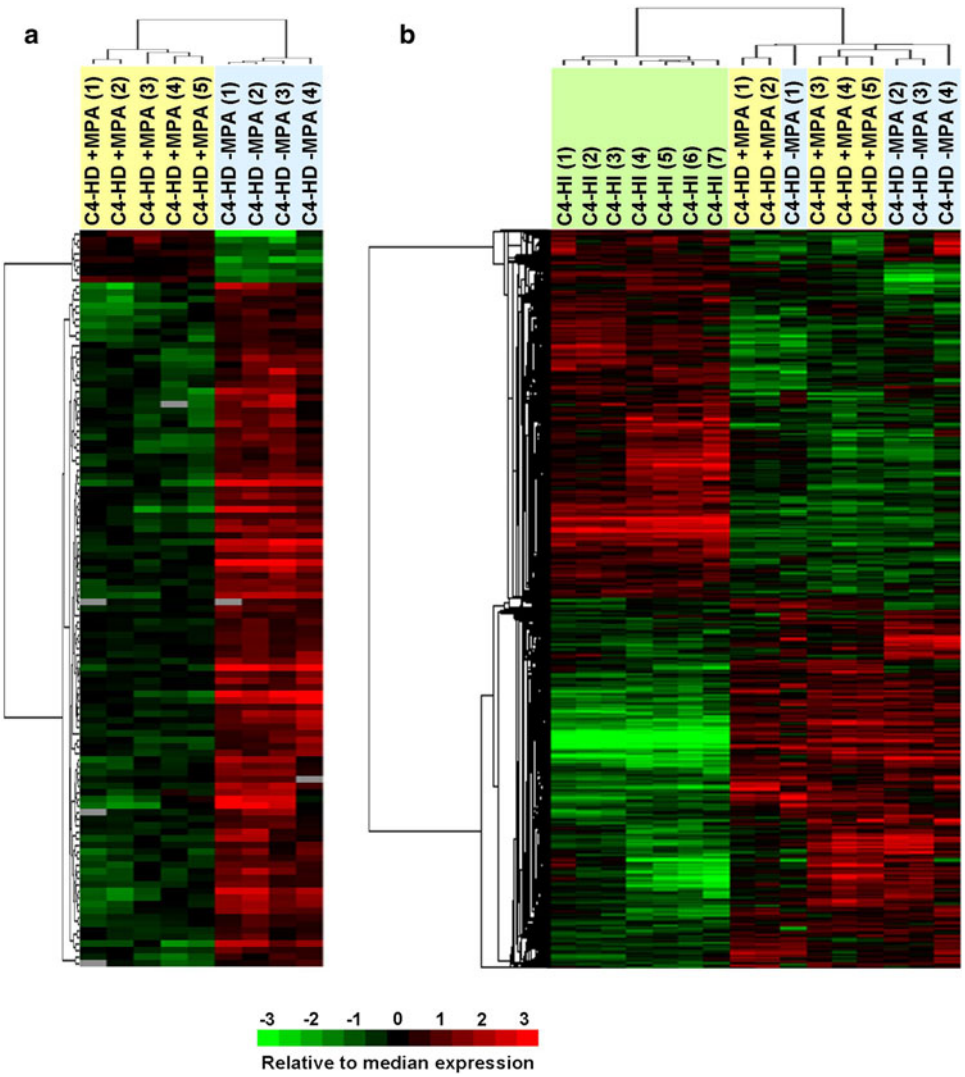

Fig. 1 Gene expression patterns for genes significantly altered in C4-HD and C4-HI whole tumors. **a** Complete cluster diagram using 112 significantly expressed genes (Table 1) identified by SAM with an FDR $< 5\%$ in C4-HD tumors growing with MPA (C4-HD +MPA) or when the progestin was removed 48 h before tumor dissection (C4-HD −MPA); **b** two-dimensional unsupervised hierarchical clustering of the data matrix consisting of 6418 nonredundant genes in 16 samples from C4-HI and C4-HD tumors. In both cases, the fold change relative to the median expression value across all tumors is shown; *rows* represent genes and *columns* represent samples. The *color scale* is shown at the *bottom*

demonstrated to replace MPA in stimulating C4-HD tumor growth [13]. Aquaporin 5 (*Aqp5*), carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8 (*Chst8*), and relaxin 1 (*Rln1*) were the highest-expressed genes in MPA-deficient C4-HD tumors. In addition, most of the genes upregulated in the absence of the progestin are involved in growth arrest and apoptosis (*Gadd45a*, *Gadd45g*, *Bcl-2l14*), response to hypoxic stress and remodeling of the vascular endothelium (*Hif1a*, *Id2*, *Id3*, *Angptl4*).

Genes differentially expressed in C4-HD and C4-HI tumors

In an attempt to find relevant genes involved in the acquisition of hormone independence, we also used C4-HI tumors growing s.c. in different mice ($n = 7$). They were analyzed by DNA microarrays, and their gene expression profile was compared to that obtained for C4-HD tumors (+MPA or −MPA). Table 1 shows the number of significant differentially expressed genes after comparing gene expression profiles of C4-HI tumors and C4-HD +MPA tumors or C4-HD −MPA tumors using SAM. Tables 3 and 4 show the top 50 genes differentially regulated in C4-HI tumors relative to C4-HD +MPA and C4-HD −MPA tumors, respectively (see Supplementary data file 2 for the complete gene lists).

Differentially expressed genes in both tumor types (Table 1) were combined into a nonredundant list of 6418 genes and used to cluster the tumors by their gene expression patterns. Figure 1b, shows the unsupervised hierarchical clustering analysis, by which the samples were sorted in two primary clusters of tumors. The right group of the dendrogram contains all the C4-HD tumor samples, and the left group contains all the C4-HI tumors, showing the similarity of the replicates in each group regarding their gene expression patterns.

Table 2 The top 50 genes differentially regulated by MPA in C4-HD tumors

Gene	Description	Fold change
Upregulated genes		
Fgf-2	Fibroblast growth factor 2	3.5
Kcnmb4	Potassium large conductance calcium-activated channel, subfamily M, beta member 4	2.5
Loc381796	Similar to carbohydrate (chondroitin 4) sulfotransferase 13	2.4
Oxct1	3-oxoacid coa transferase 1	2.1
Solt	Soxlz/Sox6 leucine zipper binding protein in testis	1.9
Glt25d1	Glycosyltransferase 25 domain containing 1	1.8
Bc005764	Cdna sequence BC005764	1.6
Prss16	Protease, serine, 16 (thymus)	1.6
Downregulated genes		
Aqp5	Aquaporin 5	10.4
Chst8	Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8	7.3
Rln1	Relaxin 1	6.1
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	4.9
Cnr2	Cannabinoid receptor 2 (macrophage)	4.8
Tmem86a	Transmembrane protein 86A	4.2
Ctns	Cystinosis, nephropathic	4.1
Ggtla1	Gamma-glutamyltransferase-like activity 1	3.8
Cyp3a25	Cytochrome P450, family 3, subfamily a, polypeptide 25	3.7
Stc2	Stanniocalcin 2	3.6
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma	3.5
Hs3st3b1	Heparan sulfate (glucosamine) 3-*O*-sulfotransferase 3B1	3.4
Hs3st6	Heparan sulfate (glucosamine) 3-*O*-sulfotransferase 6	3
Prkcm	Protein kinase C, mu	2.9
Gsn	Gelsolin	2.6
Slc12a4	Solute carrier family 12, member 4	2.6
Rkhd3	Ring finger and KH domain containing 3	2.6
Padi2	Peptidyl arginine deiminase, type II	2.6
Myh14	Myosin, heavy polypeptide 14	2.6
Aspa	Aspartoacylase (aminoacylase) 2	2.6
Fuca1	Fucosidase, alpha-L- 1, tissue	2.6
Abcc3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	2.5
Angptl4	Angiopoietin-like 4	2.4
Trem2	Triggering receptor expressed on myeloid cells 2	2.4
Rab27b	RAB27b, member RAS oncogene family	2.4
Gng12	Guanine nucleotide binding protein (G protein), gamma 12	2.4
Greb1	Gene regulated by estrogen in breast cancer protein	2.4
Jundm2	Jun dimerization protein 2	2.3
Hif1a	Hypoxia inducible factor 1, alpha subunit	2.2
Krt2-4	Keratin complex 2, basic, gene 4	2.2
Rasl11b	RAS-like, family 11, member B	2.2
Tuba8	Tubulin, alpha 8	2.2
Psen2	Presenilin 2	2.2
Furin	Furin (paired basic amino acid cleaving enzyme)	2.1
Per2	Period homolog 2 (Drosophila)	2.1
Sh3d19	SH3 domain protein D19	2.1
Kif16b	Kinesin family member 16B	2.1
Mras	Muscle and microspikes RAS	2.1
Spg20	Spastic paraplegia 20, spartin (Troyer syndrome) homolog (human)	2.1
Pcca	Propionyl-Coenzyme A carboxylase, alpha polypeptide	2.1
Memo1	Memo1 mediator of cell motility 1	2
Myh9	Myosin, heavy polypeptide 9, non-muscle	2

Gene expression changes in the stromal and epithelial compartments from C4-HD and C4-HI tumors

We have already demonstrated that in spite of being very different in their hormone requirements for in vivo growth, both C4-HD and C4-HI tumors have the same hormone or growth factor requirements in vitro [12, 13, 27], suggesting the involvement of host factors in regulating in vivo tumor growth. We have also demonstrated that in vitro, CAFs from C4-HI tumors produce more FGF-2 than those from HD tumors and thus induce a higher increase in epithelial cell proliferation, providing the first demonstration of differences between CAFs from HD and HI tumors [13]. Thus, our hypothesis is that the stromal compartment has a pivotal role in the acquisition of the HI phenotype. To address this premise and to broaden the scope of our in vitro observations, we used DNA microarrays to compare gene expression profiles of purified stromal and epithelial cells obtained by LCM (Fig. 2) from C4-HD (+MPA: $n = 3$) and C4-HI ($n = 3$) tumors. To produce a more

Table 3 The top 50 genes differentially expressed in C4-HI tumors relative to C4-HD +MPA tumors

Gene	Description	Fold change
Upregulated genes		
Sostdc1	Sclerostin domain containing 1	127.4
Mela	Melanoma antigen	31.5
Slc47a1	Slc47a1 solute carrier family 47, member 1	12.9
Aqp5	Aquaporin 5	9.4
Xist	Inactive X specific transcripts	8.6
Gpr177	G protein-coupled receptor 177	8.5
Tnfrsf19	Tumor necrosis factor receptor superfamily, member 19	8.1
Cp	Ceruloplasmin	7.6
S100a8	S100 calcium binding protein A8 (calgranulin A)	7.4
Pdgfc	Platelet-derived growth factor, C polypeptide	6.9
Fbp2	Fructose bisphosphatase 2	6.4
Robo1	Roundabout homolog 1 (Drosophila)	6
Insig1	Insulin induced gene 1	5.6
Adcy2	Adenylate cyclase 2	5.5
Steap1	Six transmembrane epithelial antigen of the prostate 1	5.5
Serpina3g	Serine (or cysteine) peptidase inhibitor, clade A, member 3G	5.4
Tmprss2	Transmembrane protease, serine 2	5.1
Lphn2	Latrophilin 2	4.9
Gpm6b	Glycoprotein m6b	4.9
Saa3	Serum amyloid A 3	4.9
Nbea	Neurobeachin	4.8
Cdkn1b	Cyclin-dependent kinase inhibitor 1B (P27)	4.7
Plk2	Polo-like kinase 2 (Drosophila)	4.6
Slc2a12	Solute carrier family 2 (facilitated glucose transporter), member 12	4.5
Igf2r	Insulin-like growth factor 2 receptor	4.3
Downregulated genes		
Clca3	Chloride channel calcium activated 3	60
Cyp2f2	Cytochrome P450, family 2, subfamily f, polypeptide 2	44.3
Tmem16a	Transmembrane protein 16A	36.6
Defb1	Defensin beta 1	26.4
Akp2	Alkaline phosphatase 2, liver	25.9
Mgp	Matrix Gla protein	21.2
Pyy	Peptide YY	20.2
Mum1l1	Melanoma associated antigen (mutated) 1-like 1	18.7
Oit1	Oncoprotein induced transcript 1	16.3
Rhox2	Reproductive homeobox 2	16.3
Calca	Calcitonin/calcitonin-related polypeptide, alpha	16.1
Fgg	Fibrinogen, gamma polypeptide	15
Myl7	Myosin, light polypeptide 7, regulatory	14.4
Krt1-15	Keratin complex 1, acidic, gene 15	13.3
Scin	Scinderin	12.9
Itlna	Intelectin a	12
Man1a	Mannosidase 1, alpha	11.8
Kcnmb4	Potassium large conductance calcium-activated channel, subfamily M, beta member 4	11.8
Stra8	Stimulated by retinoic acid gene 8	10.7
Trim29	Tripartite motif protein 29	10.4
Aldh1a7	Aldehyde dehydrogenase family 1, subfamily A7	10.3
Dmn	Desmuslin	10.1
Pcolce	Procollagen C-endopeptidase enhancer protein	9.1
Cyp3a25	Cytochrome P450, family 3, subfamily a, polypeptide 25	8.8
Slco3a1	Solute carrier organic anion transporter family, member 3a1	8.4

reliable result, we delimited the study of the LCM samples by using the list of 6418 differentially expressed genes among the previously described whole tumors. Within this gene list, we identified upregulated or downregulated genes in LCM C4-HI tumor samples relative to LCM C4-HD tumor samples. Table 5 shows the number of genes significantly altered in stroma and epithelium LCM samples after SAM and the complete lists of genes obtained can be found in Supplementary data file 3.

We next compared the 1112 significantly altered genes in stromal samples with the 1799 significantly expressed genes in epithelial samples with the aim of finding specifically expressed genes in each compartment. A total of 413 and 1100 genes were exclusively expressed by stromal and epithelial cells, respectively. This is shown in the Venn diagram displayed in Fig. 3a. We then separated each specific stromal or epithelial gene list by upregulated and downregulated genes in C4-HI tumors relative to C4-HD tumors (Fig. 3b, see Supplementary data file 3). To visualize these expression changes, we combined the supervised lists of significantly specifically expressed genes for stromal and epithelial samples and performed a hierarchical clustering analysis (Fig. 3c). Table 6 shows the top 40 specifically expressed genes in the parenchyma and in the tumor stroma from C4-HI tumors relative to C4-HD tumors (see Supplementary data file 3). Specific genes that were

Table 4 The top 50 genes differentially expressed in C4-HI tumors relative to C4-HD −MPA tumors

Gene	Description	Fold change
Upregulated genes		
Sostdc1	Sclerostin domain containing 1	105.8
Mela	Melanoma antigen	29.5
Slc47a1	Slc47a1 solute carrier family 47, member 1	18
S100a8	S100 calcium binding protein A8 (calgranulin A)	7.5
Gpr177	G protein-coupled receptor 177	7
Cp	Ceruloplasmin	7
Fbp2	Fructose bisphosphatase 2	6.6
Robo1	Roundabout homolog 1 (Drosophila)	6
Insig1	Insulin induced gene 1	5.9
Tnfrsf19	Tumor necrosis factor receptor superfamily, member 19	5.4
Steap1	Six transmembrane epithelial antigen of the prostate 1	5.3
Saa3	Serum amyloid A 3	5.1
Cdkn1b	Cyclin-dependent kinase inhibitor 1B (P27)	5
Lrrn6a	Leucine rich repeat neuronal 6A	5
Pdgfc	Platelet-derived growth factor, C polypeptide	4.9
Xist	Inactive X specific transcripts	4.9
Arg1	Arginase 1, liver	4.6
Cmkor1	Chemokine orphan receptor 1	4.6
Neto2	Neuropilin (NRP) and tolloid (TLL)-like 2	4.5
Adcy2	Adenylate cyclase 2	4.3
Dixdc1	DIX domain containing 1	4.3
Nbea	Neurobeachin	4.2
Cited1	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1	4.2
Cpxm2	Carboxypeptidase X 2 (M14 family)	4.1
Crabp1	Cellular retinoic acid binding protein I	3.7
Downregulated genes		
Clca3	Chloride channel calcium activated 3	77.9
Tmem16a	Transmembrane protein 16A	36.7
Cyp3a25	Cytochrome P450, family 3, subfamily a, polypeptide 25	32.8
Cyp2f2	Cytochrome P450, family 2, subfamily f, polypeptide 2	25.5
Mum1l1	Melanoma associated antigen (mutated) 1-like 1	20.8
Man1a	Mannosidase 1, alpha	20.3
Akp2	Alkaline phosphatase 2, liver	19.8
Pyy	Peptide YY	18
Rhox2	Reproductive homeobox 2	17.1
Scin	Scinderin	15.1
Krt1-15	Keratin complex 1, acidic, gene 15	14.7
Chst8	Carbohydrate (N-acetylgalactosamine 4-0) sulfotransferase 8	12.8
Oit1	Oncoprotein induced transcript 1	12.1
Slc35f3	Solute carrier family 35, member F3	12.1
Prkcm	Protein kinase C, mu	12
Myl7	Myosin, light polypeptide 7, regulatory	11.8
Rln1	Relaxin 1	11.8
Cgnl1	Cingulin-like 1	10.4
Glul	Glutamate-ammonia ligase (glutamine synthetase)	10.3
Defb1	Defensin beta 1	10.1
Pcolce	Procollagen C-endopeptidase enhancer protein	10
Stra8	Stimulated by retinoic acid gene 8	9.7
Trfr2	Transferrin receptor 2	8.8
Lyplal1	Lysophospholipase-like 1	8.8
ZDHHC2	Zinc finger, DHHC domain containing 2	8.8

more strongly induced in C4-HI tumor stroma included numerous matrix metallopeptidases, such as *Mmp3*, *Mmp9*, *Mmp10*, and *Mmp13*, whereas *Steap*, *Cxcl9*, *Pdgfc*, *Runx2*, *Sdf2*, and *Plk2*, among others, were specifically induced in the C4-HI tumor parenchyma. Conversely, genes such as *Pten*, *Calca*, and *Defb1*, presented a higher expression in C4-HD epithelial cells.

Several genes identified herein in the tumor parenchyma are known to be PR-regulated in the murine uterus and in normal or transformed mammary epithelial cells. These include *Steap* [28], *Calca* [29–32], *Defb1* [32], *Plk2* [28, 29], and *Pdlim1* [29, 33], thus demonstrating the confidence and quality of our data.

Gene Ontology (GO) analysis using EASE [25] was conducted to interpret the specific gene lists identified for stroma and epithelium and convert these lists into biological themes. Significantly enriched GO terms ($P < 0.05$) that describe the biological process, molecular function, cellular component, GenMAPP pathway, KEGG pathway, and BBID pathway represented by these genes are listed by statistical rank in Tables 7 and 8, showing the top 30 GO terms enriched in tumor stroma and tumor parenchyma, respectively (see Supplementary data file 4 for the complete GO term lists). Among these categories, collagenase activity and wounding response, which include a group of

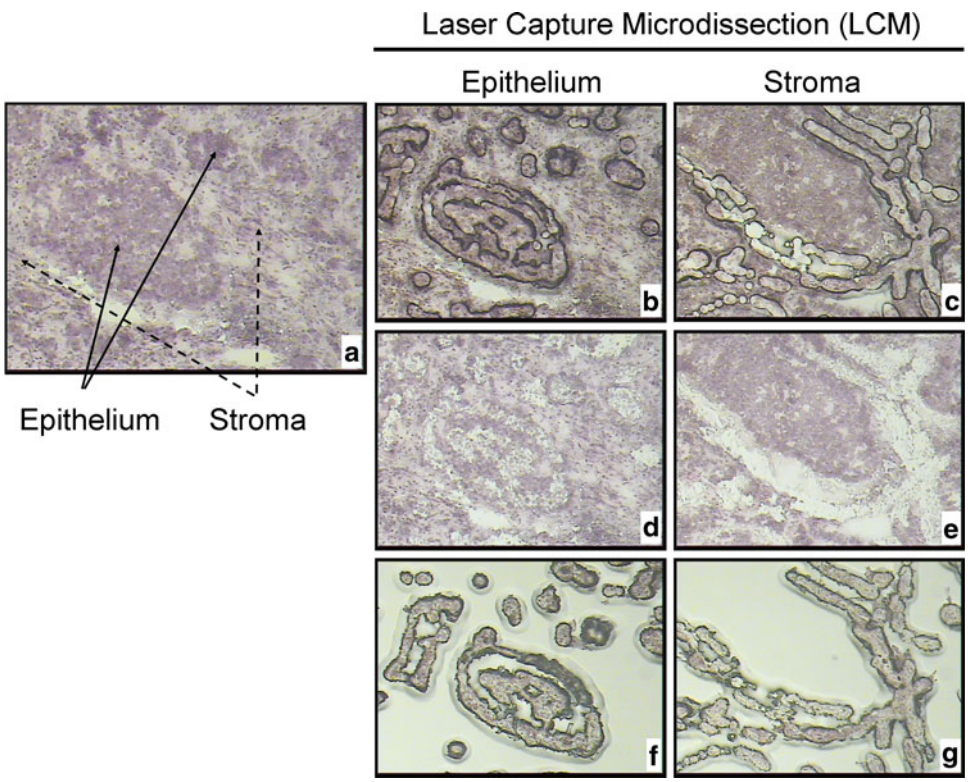

Fig. 2 LCM of epithelial and stromal cells. Example of the tumor microenvironment and tumor parenchyma compartment from C4-HI tumors targeted by LCM. **a** Representative epithelial (*solid arrow*) and stromal (*dotted arrow*) areas to be captured from H&E stained sections. **b**, **c** Images of selected epithelial and stromal areas, respectively, captured by LCM. **d**, **e** Remaining tissue after LCM capture. **f**, **g** Epithelial and stromal cells, respectively, captured on CapSure LCM transfer film

Table 5 Number of genes significantly altered in LCM samples as determined by SAM

LCM samples comparison	No. of significant genes			No. of false significant	FDR (%)
	Total	Up	Down		
Stroma C4-HI vs Stroma C4-HD	1112	735	377	53.86	4.84
Epithelium C4-HI vs Epithelium C4-HD	1799	703	1096	80.67	4.48

SAM significance analysis of microarrays using the 6418 differentially expressed genes among whole tumors, *FDR* false discovery rate, *up* upregulated genes, *down* downregulated genes

genes that are known to be expressed in stromal components and during breast cancer progression, are probably also important in C4-HI tumor stroma [16, 19, 34]. Additionally, the categories of electron transporter activity and antigen processing are prominent GO terms in the C4-HI tumor parenchyma.

Validation of selected differentially expressed genes

To confirm the results generated by gene microarray analysis, we used immunohistochemistry (IHC) and immunofluorescence (IF) assays on C4-HD and C4-HI tumor sections. We selected a subset of six genes based on our interest in the function of their encoded proteins and on antibody availability. In our microarrays data, *Runx2* and *Pdgfc* presented increased expression in C4-HI tumor parenchyma. *Tissue factor* (*Tf*) and *Pten* were upregulated in C4-HD tumors, and the latter (*Pten*) was specifically expressed in epithelial cells; whereas *Mmp9* and *Mmp13* were strongly induced in C4-HI stromal cells (Fig. 4). By IHC, we studied the expression of the selected proteins RUNX2, PDGFc, TF, and PTEN proteins (Fig. 5). We found that C4-HI tumors presented an elevated expression of RUNX2 and PDGFc proteins relative to C4-HD tumors. In addition, we confirmed that these two proteins were preferentially expressed in epithelial cells. On the other hand, TF, which is a progestin-regulated gene in breast cancer cells [33, 35], was highly expressed, as determined by IHC in C4-HD tumors treated with MPA. Similarly, PTEN was specifically expressed in C4-HD epithelial cells, confirming the gene expression data (Fig. 5). PTEN is a negative regulator of Akt activity, and the higher expression of this gene in C4-HD tumors is in agreement with recent work by our group showing that C4-HI tumors exhibit elevated activation of Akt compared with that in C4-HD tumors [36].

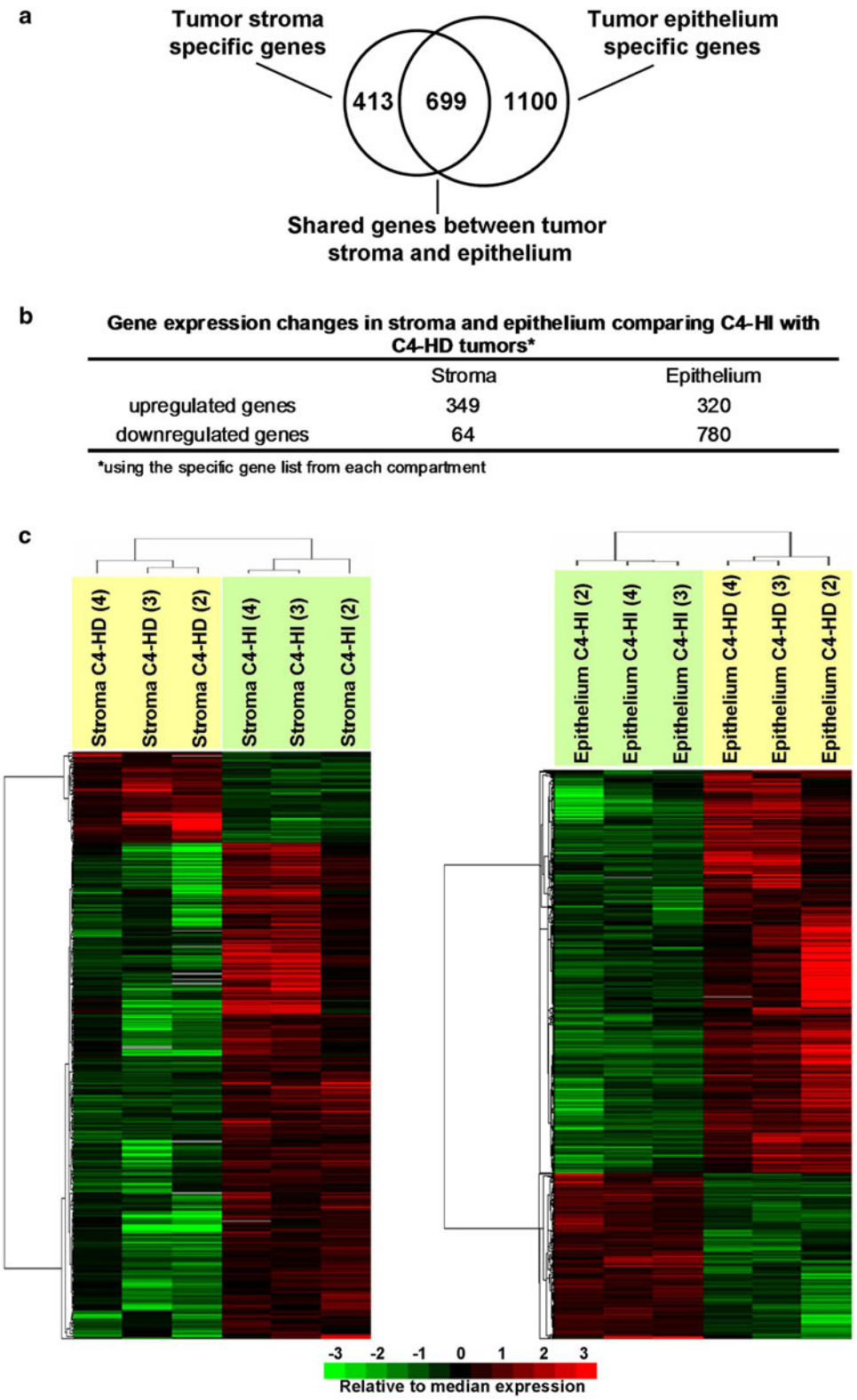

Fig. 3 Gene expression patterns for specific stromal or epithelial genes significantly altered in C4-HI and C4-HD tumors. **a** Venn diagram showing the number of specifically expressed genes in the stromal and epithelial compartments after comparing the 1112 significantly altered genes in stromal LCM samples with the 1799 significantly expressed genes in epithelial LCM samples (Table 5; Supplementary data file 3). **b** Table showing the numbers of genes upregulated and downregulated in stroma and epithelium from C4-HI tumors relative to C4-HD tumors using the specific gene lists from each compartment shown in **a**. **c** Complete cluster diagram using the 413 specifically expressed genes in stromal LCM tumor samples (*left*) and the 1100 specifically expressed genes in epithelial LCM tumor samples (*right*). *Rows* represent genes and *columns* represent samples. The fold change relative to the median expression value across all tumors is shown. *Color scale* is shown at the *bottom*

To study the expression of MMP9 and MMP13 proteins, we used IF experiments. We found that both matrix metallopeptidases were preferentially expressed in the stroma of C4-HI tumors (Fig. 6), again confirming the gene microarray results. The myofibroblast marker α-smooth muscle actin (αSMA) and the epithelial marker cytokeratin (CK) were used to confirm the stromal expression of the MMPs.

Table 6 The top 40 specific expressed genes in epithelium and stroma from C4-HI tumors relative to C4-HD tumors

Gene	Description	Fold change
Stroma		
Upregulated genes		
Mmp10	Matrix metallopeptidase 10	6
Cpxm2	Carboxypeptidase x 2 (m14 family)	5.7
Pdcd1	Programmed cell death 1	5.3
Klra2	Killer cell lectin-like receptor, subfamily a, member 2	5.1
Angpt2	Angiopoietin 2	4.9
Tcrg	T cell receptor gamma chain	4.7
Cxcr6	Chemokine (c-x-c motif) receptor 6	4.4
Ehd3	Eh-domain containing 3	4.4
Cd300le	Cd300 antigen like family member e	4.2
Cd48	Cd48 antigen	4.1
Klrd1	Killer cell lectin-like receptor, subfamily d, member 1	4.1
Mmp13	Matrix metallopeptidase 13	3.9
Rarres2	Retinoic acid receptor responder (tazarotene induced) 2	3.9
Ch25h	Cholesterol 25-hydroxylase	3.9
Il12rb1	Interleukin 12 receptor, beta 1	3.9
Nid2	Nidogen 2	3.9
Clec4a3	C-type lectin domain family 4, member a3	3.8
Mmp9	Matrix metallopeptidase 9	3.8
Col13a1	Procollagen, type xiii, alpha 1	3.8
Emcn	Endomucin	3.8
Downregulated genes		
Stra6	Stimulated by retinoic acid gene 6	13.8
Hs3st3b1	Heparan sulfate (glucosamine) 3-*O*-sulfotransferase 3B1	8.3
Kif5c	Kinesin family member 5c	7.4
Glycam1	Glycosylation dependent cell adhesion molecule 1	6.1
Tmem42	Transmembrane protein 42	5.1
Fem1c	Fem-1 homolog c (c.elegans)	4.1
Trrap	Transformation/transcription domain-associated protein	3.7
Myom3	Myomesin family, member 3	3.2
Pcsk2	Proprotein convertase subtilisin/kexin type 2	3
Mapk8ip3	Mitogen-activated protein kinase 8 interacting protein 3	2.9
Dscr6	Down syndrome critical region homolog 6 (human)	2.8
Add2	Adducin 2 (beta)	2.8
Lama2	Laminin, alpha 2	2.7
Tekt2	Tektin 2	2.4
Thrap2	Thyroid hormone receptor associated protein 2	2.4
Wnk4	Wnk lysine deficient protein kinase 4	2.4
Rasgef1a	Rasgef domain family, member 1a	2.2
Hsd3b3	Hydroxysteroid dehydrogenase-3, delta < 5>-3-beta	2.2
Socs2	Suppressor of cytokine signaling 2	2.1
Golt1b	Golgi transport 1 homolog b (s. Cerevisiae)	2.1
Epithelium		
Upregulated genes		
Xist	Inactive x specific transcripts	17
Gpr177	G protein-coupled receptor 177	9.2
Steap1	Six transmembrane epithelial antigen of the prostate 1	6.8
Sytl2	Synaptotagmin-like 2	6.1
Cxcl9	Chemokine (c-x-c motif) ligand 9	6
Pdgfc	Platelet-derived growth factor, C polypeptide	5.6
Tm4sf1	Transmembrane 4 superfamily member 1	5.6
Slc11a1	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	5.3
Stc2	Stanniocalcin 2	5.3
Tmprss2	Transmembrane protease, serine 2	4.8
Rkhd3	Ring finger and kh domain containing 3	4.5
Runx2	Runt related transcription factor 2	4
Ngef	Neuronal guanine nucleotide exchange factor	3.8
H2-dmb1	Histocompatibility 2, class ii, locus mb1	3.8
Dixdc1	Dix domain containing 1	3.7
Ltb4dh	Leukotriene b4 12-hydroxydehydrogenase	3.5
Tmsb4x	Thymosin, beta 4, x chromosome	3.4
Lmcd1	Lim and cysteine-rich domains 1	3.2
Plscr4	Phospholipid scramblase 4	3.2
Rufy1	Run and fyve domain containing 1	3.2
Downregulated genes		
Man1a	Mannosidase 1, alpha	17.8
Calca	Calcitonin/calcitonin-related polypeptide, alpha	17.2
Slco3a1	Solute carrier organic anion transporter family, member 3a1	15.8
Defb1	Defensin beta 1	15.4
Nup62	Nucleoporin 62	14.3
Flt1	Fms-like tyrosine kinase 1	14
Slc1a7	Solute carrier family 1 (glutamate transporter), member 7	13.9
Cpne8	Copine viii	12.6
Itlna	Intelectin a	11.5
Lrrc28	Leucine rich repeat containing 28	11.4

Table 6 continued

Gene	Description	Fold change
Ppargc1b	Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	11
Ormdl3	Orm1-like 3 (s. Cerevisiae)	10.9
Dicer1	Dicer1, dcr-1 homolog (drosophila)	9.9
Pfpl	Pore forming protein-like	9.5
Als2cr15	Amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 15	8.9
Sdccag1	Serologically defined colon cancer antigen 1	8.8
Proz	Protein z, vitamin k-dependent plasma glycoprotein	8.4
Prkcn	Protein kinase c, nu	8.2
Eif3s10	Eukaryotic translation initiation factor 3, subunit 10 (theta)	8.1
Pgm5	Phosphoglucomutase 5	8

Discussion

C4-HD tumors only grow in BALB/c mice in the presence of an exogenous progestin supply. Variants that can grow in untreated mice have been generated, including C4-HI tumors. Interestingly, these tumors express high levels of ER and PR, and in primary cultures, they behave as C4-HD epithelial cells. This suggested that these HI tumors might recruit a different microenvironment that could be responsible for bypassing the exogenous hormone effect in vivo. There is no human situation that exactly mimics the growth of C4-HD tumors, because they grow only under continuous exogenous hormone administration. However, we believe that the C4-HI tumor may be compared with luminal human breast carcinomas that highly express ER and PR because they have a ductal histology, they express keratins 8 and 18 (see Supplementary data file 2), they grow in untreated mice and they are ER- and PR-dependent (the blockage of either of these receptors inhibits tumor growth) [37, 38]. In addition, C4-HI tumors metastasize in axillary lymph nodes similarly to most human breast cancers [12, 39], and they are able to acquire hormone resistance [38].

As our first approach, we focused on the MPA-regulated genes in C4-HD tumors. Because C4-HD tumors only grow in the presence of MPA, we had to stimulate growth and then remove the MPA pellet. Histological images confirmed the effectiveness of hormone depletion, characterized by increased apoptosis, cytostasis, and stromal tissue, as previously shown [26]. Our work provides the first analysis of MPA-regulated genes in an in vivo breast tumor model, and reveals novel progestin-regulated genes. We detected eight genes that were highly expressed in MPA-treated tumors compared to their expression in untreated mice, and *Fgf2* was first on the list. This is in absolute agreement with our previous reports indicating that FGF-2 can replace MPA effects both in vitro [27] and in vivo [13]. The fact that progesterone is able to induce FGF-2 synthesis was reported in granulose and in uterine cells in the rat [40, 41].

Table 7 The top 30 gene ontology terms enriched in tumor stroma

Gene category	List hits[a]	Probability[b]
Specific genes for stroma upregulated in C4-HI tumors		
Mm_matrix metalloproteinases	6	1.59E−05
Defense response	28	3.03E−05
Collagenase activity	4	7.77E−05
Immune response	23	0.00012
Lytic vacuole	10	0.00013
Lysosome	10	0.00013
Response to biotic stimulus	28	0.00014
Collagen catabolism	4	0.00025
Purine nucleotide metabolism	7	0.00029
Vacuole	10	0.00035
Rho interactor activity	2	0.00042
Purine ribonucleotide metabolism	6	0.00082
Response to pest/pathogen/parasite	14	0.0010
Response to wounding	11	0.0011
Aromatic amino acid transporter activity	2	0.0012
Thymocyte differentiation	2	0.0014
Purine ribonucleoside triphosphate metabolism	5	0.0015
Ribonucleoside triphosphate metabolism	5	0.0015
Ribonucleotide metabolism	6	0.0016
Hs_g13 signaling pathway	2	0.0017
Specific genes for stroma downregulated in C4-HI tumors		
Basal lamina	2	0.0013
Heparin-glucosamine 3-O-sulfotransferase activity	1	0.0033
Insulin-like growth factor receptor binding	1	0.0033
Atpase activity—uncoupled	1	0.0033
Proprotein convertase 2 activity	1	0.0033
Growth hormone receptor binding	1	0.0033
Positive transcription elongation factor activity	1	0.0033
Vitamin E metabolism	1	0.0033
Vesicle-mediated transport	4	0.0058
Basement membrane	2	0.0062

Significant enriched Gene Ontology (GO) terms as determined by EASE software analysis, using specific stromal genes upregulated or downregulated in C4-HI tumors relative to C4-HD tumors: [a] the number of genes in each category, [b] the threshold of EASE Score, a modified Fisher exact probability ($P < 0.05$)

Table 8 The top 30 Gene Ontology terms enriched in tumor epithelium

Gene category	List hits[a]	Probability[b]
Specific genes for epithelium upregulated in C4-HI tumors		
Electron transporter activity	14	0.000003
Perinuclear space	4	0.00010
MHC class II receptor activity	3	0.00026
Cytoplasm	85	0.00074
Oxidoreductase activity—acting on NADH or NADPH	5	0.0015
Late endosome	3	0.0019
Antigen processing	3	0.0034
Intracellular	118	0.0037
Antigen presentation	3	0.0053
NADH dehydrogenase activity	3	0.0069
Icosanoid metabolism	3	0.0078
Organelle organization and biogenesis	13	0.0091
Electron transport	13	0.0095
Oxidoreductase activity—acting on NADH or NADPH—other acceptor	3	0.0098
Prostanoid metabolism	2	0.012
Specific genes for epithelium downregulated in C4-HI tumors		
Procollagen-lysine 5-dioxygenase activity	3	0.00028
Oxidoreductase activity	5	0.00051
Catalytic activity	199	0.00086
Amino acid derivative metabolism	8	0.0016
Establishment of tissue polarity	2	0.0017
Secondary metabolism	4	0.0022
Mitotic cell cycle	20	0.0023
Protein metabolism	111	0.0026
Vitamin binding	5	0.0027
Glycine hydroxymethyltransferase activity	3	0.0035
Small monomeric gtpase activity	12	0.0044
Activation of MAPKKK	2	0.0052
Carboxypeptidase A activity	4	0.0071
Metallocarboxypeptidase activity	4	0.0071
Mitotic chromosome condensation	3	0.0072

Significant enriched gene ontology (GO) terms as determined by EASE software analysis, using specific epithelial genes upregulated or downregulated in C4-HI tumors relative to C4-HD tumors: [a] the number of genes in each category, [b] the threshold of EASE Score, a modified Fisher exact probability ($P < 0.05$)

We expected to find the upregulation of a plethora of genes related to cell proliferation in MPA-treated mice as compared with those in which MPA was removed. However, the regressing tumors showed more general upregulation of genes (104 genes). *Aqp5* was the first gene on the list, but because it is also highly expressed in growing

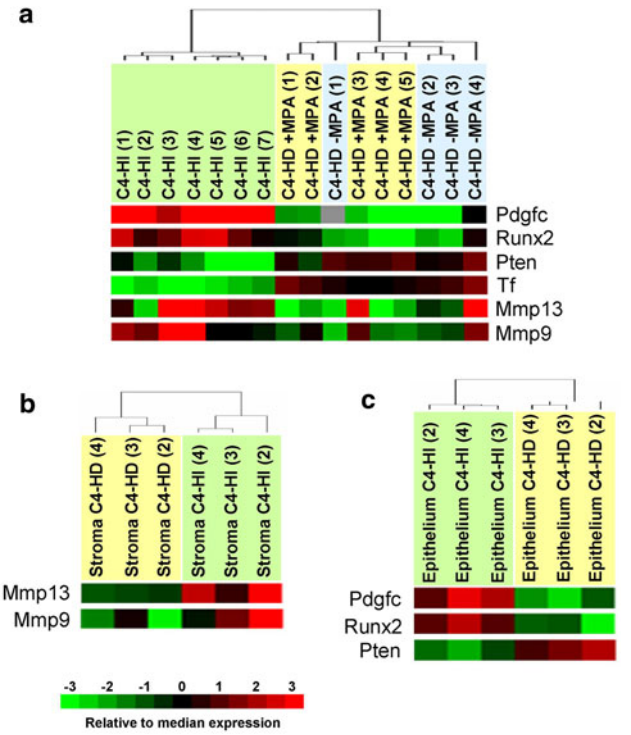

Fig. 4 Selected genes for validation. **a** DNA microarrays obtained data for *Pdgfc*, *Runx2*, *Pten*, *Tf*, *Mmp9*, and *Mmp13* genes after comparing the gene expression profiles of C4-HD and C4-HI whole tumor samples, **b** DNA microarrays obtained data for *Mmp9* and *Mmp13* genes specifically expressed in stromal LCM tumor samples, or **c** for *Pdgfc*, *Runx2*, and *Pten* genes specifically expressed in epithelial LCM tumor samples. In all cases, the fold change relative to the median expression value across all samples is shown; *rows* represent genes, and *columns* represent samples. *Color scale* is shown at the *bottom*

C4-HI tumors, its downregulation may have been a specific hormonal effect induced by MPA in C4-HD tumors and not necessarily related to cell proliferation. AQP5 is a marker of ductal epithelial cells whose expression is downregulated during pregnancy [42]. A relationship between AQP5 and mammary gland development has been found in C/EBPβ-KO mice [43]. *Rln1* was also highly expressed in regressing tumors, and although its role may be controversial, it has been shown to inhibit MDA-MB-231 and T47D human breast cancer cell proliferation [44], as its expression is regulated by ERα [45]. Interestingly, relaxin 1 induces the expression of MMP2, MMP9, MMP13 and MMP14 [46], which is in line with our previous reports in which we showed increased activity of MMP2 and MMP9 in regressing C4-HD tumors [26]. As opposed to *Aqp5*, *Rln1* expression was also higher in C4-HD tumors without MPA as compared with that in C4-HI tumors, suggesting that the increases in metallopeptidases associated with HI tumor growth may have a different origin than those found in regressing tumors. Similarly, *protein kinase C, isoform*

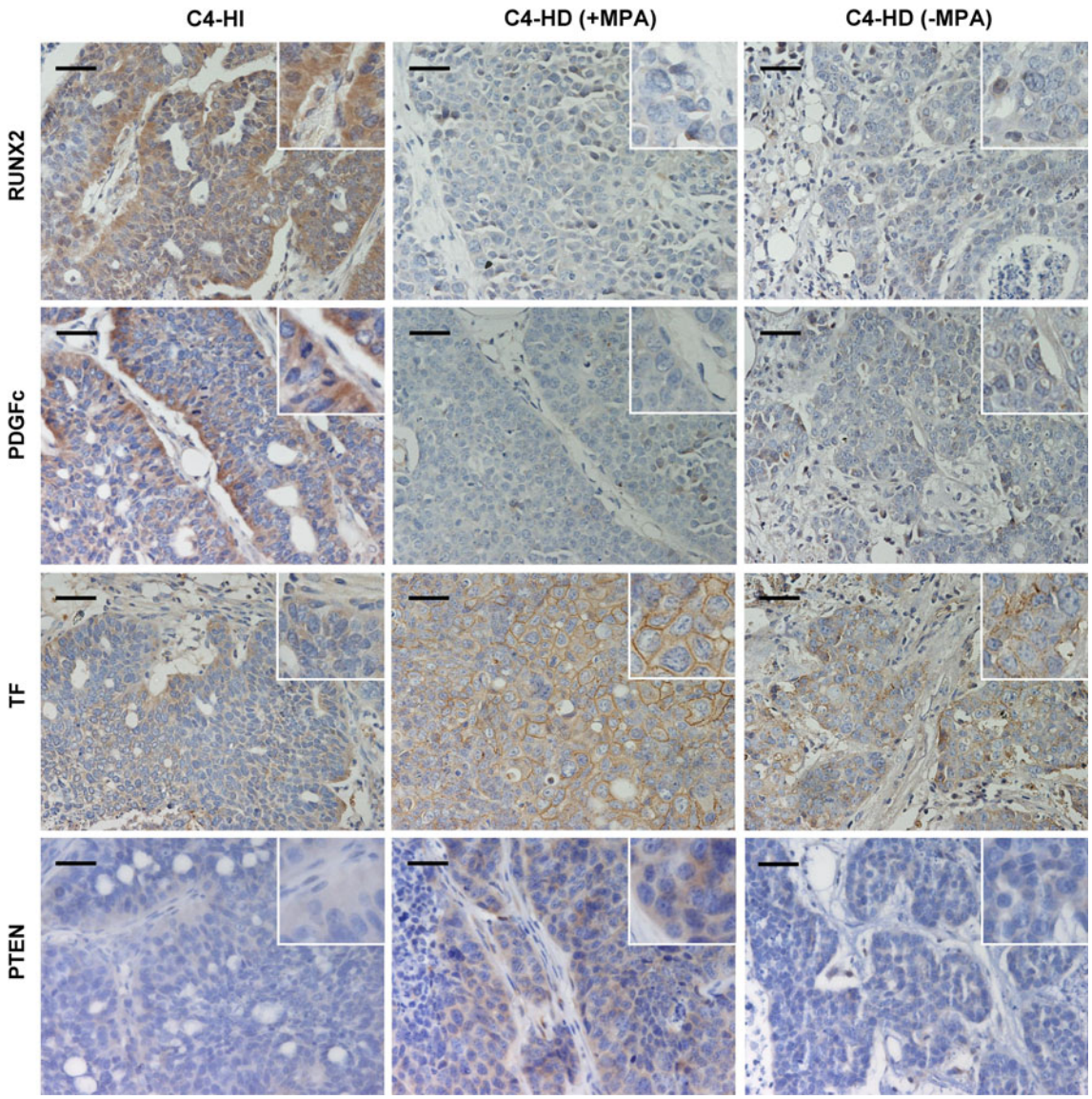

Fig. 5 Validation of epithelial selected genes. Immunohistochemistry showing the expression of RUNX2, PDGFc, TF and PTEN proteins in C4-HI, C4-HD +MPA and C4-HD −MPA tumor sections. Hematoxylin was used for nuclear counterstaining. Resolution: ×400. *Bar*: 50 μm. *Insets* show in detail the specific epithelial staining for RUNX2, PDGFc, TF, and PTEN

mu (*Prkcm*) was highly expressed in regressing tumors as compared with its expression in C4-HD growing with MPA and C4-HI tumors, suggesting that the role of protein kinase Cμ and relaxin 1 in tumor regression should be further studied. Most of the other genes that were upregulated in C4-HD tumors in which MPA was removed were related to quiescence or apoptosis (*Gadd45a*, *Gadd45g*, *Bcl-2l14*) [47–49], response to hypoxic stress and remodeling of vascular endothelium (*Hif1a*, *Id2*, *Id3*, *Angptl4*) [50–56], which is consistent with the lack of proliferative effects mediated by MPA in those HD tumors.

The fact that hormone-induced cell proliferation is related to the downregulation of genes has been also reported by others. 17-β-estradiol induces a 70% downregulation of gene expression in MCF-7 human breast cancer cells [57], and similar effects have been obtained in mouse uterus [58], progesterone-treated mouse mammary glands [31] and monkey's endometrium [59].

After investigating the gene expression profile regulated by MPA in C4-HD tumors, we centered our study on the differences between HD and HI whole tumors and on epithelial and stromal cells captured from each of these mammary carcinomas. It become evident that during the acquisition of hormone independence, many of the patterns of acquired gene expression are probably directly involved with the ability of the tumor to bypass the hormonal requirement, whereas others may be associated with tumor progression or have secondary roles.

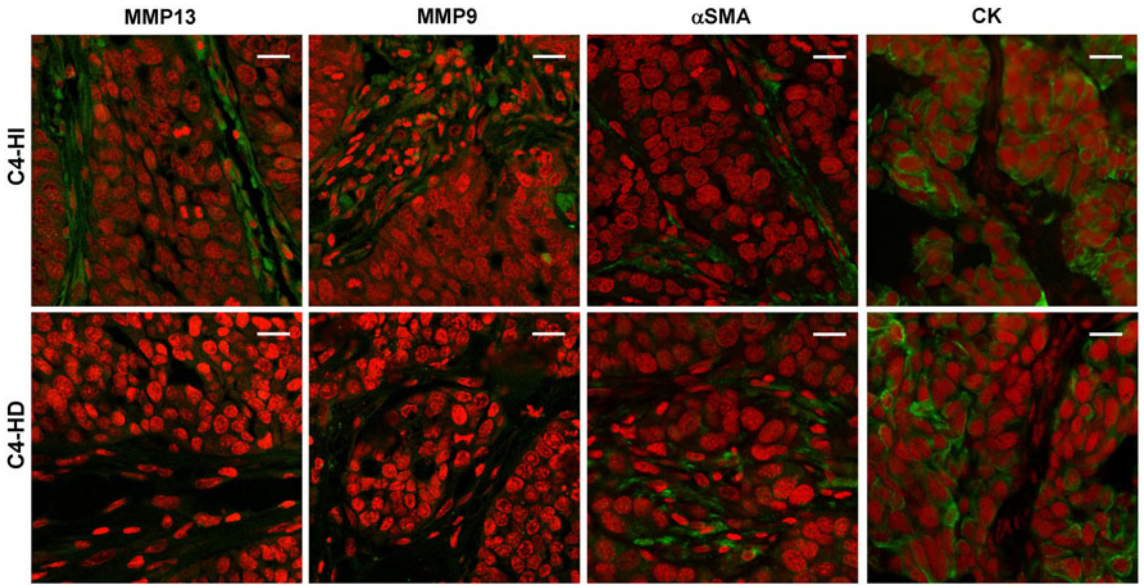

Fig. 6 Validation of stromal selected genes. Immunofluorescence showing the specific stromal expression of MMP9 and MMP13 proteins (*green*, FITC) in C4-HI and in C4-HD +MPA frozen tumor sections. Anti-αSMA antibody (*green*, FITC) was used as a stromal marker, whereas the epithelial cells were visualized with an anti-cytokeratin (CK—green, FITC) antibody. Propidium iodide (*red*) was used for nuclear counterstaining. *Bar*: 30 μm

Our analysis using whole mammary tumors with different hormone dependence clearly identified a subset of genes that distinguish HI from HD tumors. Many of the listed upregulated and downregulated genes that were differentially expressed in C4-HI tumors relative to C4-HD tumors, either in the presence or absence of MPA (Tables 3, 4; Supplementary data file 2), are candidate genes that are possibly involved in the acquisition of the HI phenotype.

During normal mammary development, epithelial cells are exposed to highly regulated signals derived from the stroma. Similarly, during breast tumorigenesis, the microenvironment provides a tumor-promoting atmosphere that supports epithelial tumor growth. Moreover, recent evidence also suggests that the tumor-associated stroma evolves and responds to molecular signals derived from tumor cells to actively support tumor growth [7, 8, 60].

Our results using captured cells obtained by LCM clearly indicate that both the epithelial and the stromal compartments are different in both C4-HD and C4-HI tumors, suggesting that tumor-associated stroma coevolves with the tumor epithelium in the acquisition of hormone independence. No other gene array studies have been performed comparing the stroma or the epithelium between HD and HI breast carcinomas. However, there are some recent works comparing the gene expression profile in each compartment from normal and neoplastic breast tissue, or even from DCIS and IDC. Some of these studies have used primary cultures of fibroblasts [61–64], affinity purification of dispersed myofibroblasts [65], or microdissected tumor stroma or tumor epithelium [16, 17, 19]. Because of this heterogeneity in sample preparation these investigations, are not always be comparable. However, in the majority of these studies, genes involved in or related to extracellular matrix biology have been identified as overexpressed in the stromal compartment of breast carcinomas. Our data are in agreement with those results indicating that collagenase activity (*Timp3*, *Mmp3*, *Mmp9*, *Mmp10*, and *Mmp13*) and wounding response (*Cd8a*, *Fcer1g*, *Cybb*, *Il1b*, *Itgb2*, *Il16*, *Ppicap*, *Ptprc*, *Tnf*, *Pla2g7*, and *Cd244*) are among the upregulated GO categories in the C4-HI tumor stroma. The overexpression of *Timp3* and *Mmp13* was previously observed in human breast tumor stroma [16], whereas *Mmp3* was shown to be upregulated in CAFs compared to expression in NAFs in vitro [62].

We have already demonstrated that isolated CAFs from C4-HI tumors express higher levels of FGF-2 compared to CAFs from C4-HD tumors in vitro. CAFs from C4-HI tumors secreting FGF-2 activate PR and induce C4-HI epithelial cell proliferation in vitro. In addition, FGF-2 stimulates, as does MPA, the growth of C4-HD tumors in vivo [13]. However, in the present work, we did not find high expression levels of FGF-2 in the C4-HI tumor stroma. This molecule was overexpressed in both stroma and tumor cells from C4-HD tumors (see Supplementary data file 3). It is possible that the in vitro conditions of culture were responsible for FGF-2 production by C4-HI CAFs. In this study, we analyzed the complete tumor stroma and not purified fibroblasts. Alternatively, the increased levels of MMP3 and MMP13 found in

C4-HI-associated stroma may be responsible for increasing FGF-2 bioavailability, because both peptidases have been shown to promote FGF-2 release by inducing a cleavage of the covalent bonds that bind FGF-2 to heparan sulfate proteoglycans [66]. Breast tumor cells can induce the expression of MMPs by tumor-associated fibroblasts [67], confirming a feedback loop in the epithelial-stromal interaction. The receptors for FGF-2 (FGFR2) were found more highly expressed in C4-HI tumors compared to C4-HD tumors (see Supplementary data file 2), as previously demonstrated [13].

On the other hand, *Xist*, *Gpr177*, *Steap*, *Cxcl9*, *Pdgfc*, *Runx2*, *Sdf2*, and *Plk2* are among the genes that were specifically overexpressed in the C4-HI tumor parenchyma. *SDF2* has been identified as specifically expressed in human ERα+ epithelial breast tumor cells [68]. *PLK2* is a PR-regulated gene in T47D cells [28]. *STEAP* is an estrogen-regulated [69] and progesterone-regulated [28] gene that is highly expressed in epithelial breast tumor cells in addition to prostate cancer, and it has been considered as a potential target for immunotherapy because it is not expressed in normal tissues and is a target of anti-tumor $CD8^+$ immune cells [69, 70]. PDGFc has been shown to participate in the recruitment of CAFs in models of lung carcinoma and melanoma [71, 72]. In addition, PDGFc was upregulated in mouse lymphoma tumors resistant to anti-VEGF therapies [73] and could induce FGF-2 production by stromal fibroblasts [71], which could be inhibited by PDGFR inhibitors [74]. Among these same lines, CXCL9 may participate in stromal recruitment. It has been shown that overexpression of CXCL9 may induce inhibition of tumor growth in a murine experimental model by recruiting activated T cells and natural killer cells [75]. However, in a certain tumor context of non-immunogenic syngeneic tumors, this may serve to favor a propitious microenvironment that helps to recruit a stimulating stroma. RUNX2 is a transcription factor that is involved in gene regulation associated with osteoblast differentiation and bone metastasis in breast cancer via the regulation of MMP9 and MMP13 expression [76–79]. Intriguingly, the FGF-2/FGFR signaling pathway is involved in the expression and activity of RUNX2 [80, 81]. Furthermore, a polymorphism in intron 2 of the *FGFR2* gene has been associated with increased risk of sporadic breast cancer [82], and these single-nucleotide polymorphisms alter the binding of RUNX2 to the *FGFR2* gene and increase its expression, providing an explanation for the risk phenotype [83]. The mechanism by which RUNX2 expression in C4-HI epithelial cells is linked to the stromal increase in MMPs needs to be established.

In this scenario, it may be postulated that PDGFc and other chemotactic factors, such as CXCL9, produced by HI epithelial tumor cells, may participate in the recruitment of activated CAFs that produce high levels of matrix metallopeptidases which in turn participate in the bioavailability of FGF-2 and other factors that induce epithelial tumor growth.

Acknowledgments We are very grateful to Julieta Bolado for excellent technical support and to Laboratorios Craveri (Buenos Aires, Argentina) for providing MPA. SG received an award from Avon Foundation for data presented at the AACR Annual Meeting 2008, and an ICRETT Fellowship from the UICC permitting training in Dr Perou's laboratory. This work was supported by Fundación Sales, SECyT (PICT 2005 and 2007-932), and CONICET (PIP 5351) to CL.

Conflict of interest The authors declare that they have no competing interests.

References

1. Normanno N, Di Maio M, De Maio E, De Luca A, de Matteis A, Giordano A, Perrone F (2005) Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. Endocr Relat Cancer 12:721–747
2. Lange CA, Richer JK, Shen T, Horwitz KB (1998) Convergence of progesterone and epidermal growth factor signaling in breast cancer. Potentiation of mitogen-activated protein kinase pathways. J Biol Chem 273:31308–31316
3. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H (2001) Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem 276:9817–9824
4. Shupnik MA (2004) Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. Oncogene 23:7979–7989
5. Shigemura K, Isotani S, Wang R, Fujisawa M, Gotoh A, Marshall FF, Zhau HE, Chung LW (2009) Soluble factors derived from stroma activated androgen receptor phosphorylation in human prostate LNCaP cells: roles of ERK/MAP kinase. Prostate 69:949–955
6. Yamaguchi Y (2007) Microenvironmental regulation of estrogen signals in breast cancer. Breast Cancer 14:175–181
7. Arendt LM, Rudnick JA, Keller PJ, Kuperwasser C (2009) Stroma in breast development and disease. Semin Cell Dev Biol 21:11–18
8. Shimoda M, Mellody KT, Orimo A (2009) Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression. Semin Cell Dev Biol 21:19–25
9. Studebaker AW, Storci G, Werbeck JL, Sansone P, Sasser AK, Tavolari S, Huang T, Chan MW, Marini FC, Rosol TJ, Bonafe M, Hall BM (2008) Fibroblasts isolated from common sites of breast cancer metastasis enhance cancer cell growth rates and invasiveness in an interleukin-6-dependent manner. Cancer Res 68:9087–9095
10. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, Stanley ER, Segall JE, Condeelis JS (2005) Macrophages promote the invasion of breast carcinoma cells via a colony-stimulating factor-1/epidermal growth factor paracrine loop. Cancer Res 65:5278–5283
11. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell 121:335–348

12. Lanari C, Lamb CA, Fabris VT, Helguero LA, Soldati R, Bottino MC, Giulianelli S, Cerliani JP, Wargon V, Molinolo A (2009) The MPA mouse breast cancer model: evidence for a role of progesterone receptors in breast cancer. Endocr Relat Cancer 16:333–350
13. Giulianelli S, Cerliani JP, Lamb CA, Fabris VT, Bottino MC, Gorostiaga MA, Novaro V, Gongora A, Baldi A, Molinolo A, Lanari C (2008) Carcinoma-associated fibroblasts activate progesterone receptors and induce hormone independent mammary tumor growth: a role for the FGF-2/FGFR-2 axis. Int J Cancer 123:2518–2531
14. Perou CM, Sorlie T, Eisen MB, van de RM, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. Nature 406:747–752
15. Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, Rasmussen KE, Jones LP, Assefnia S, Chandrasekharan S, Backlund MG, Yin Y, Khramtsov AI, Bastein R, Quackenbush J, Glazer RI, Brown PH, Green JE, Kopelovich L, Furth PA, Palazzo JP, Olopade OI, Bernard PS, Churchill GA, Van Dyke T, Perou CM (2007) Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. Genome Biol 8:R76
16. Casey T, Bond J, Tighe S, Hunter T, Lintault L, Patel O, Eneman J, Crocker A, White J, Tessitore J, Stanley M, Harlow S, Weaver D, Muss H, Plaut K (2009) Molecular signatures suggest a major role for stromal cells in development of invasive breast cancer. Breast Cancer Res Treat 114:47–62
17. Finak G, Bertos N, Pepin F, Sadekova S, Souleimanova M, Zhao H, Chen H, Omeroglu G, Meterissian S, Omeroglu A, Hallett M, Park M (2008) Stromal gene expression predicts clinical outcome in breast cancer. Nat Med 14:518–527
18. Ma XJ, Salunga R, Tuggle JT, Gaudet J, Enright E, McQuary P, Payette T, Pistone M, Stecker K, Zhang BM, Zhou YX, Varnholt H, Smith B, Gadd M, Chatfield E, Kessler J, Baer TM, Erlander MG, Sgroi DC (2003) Gene expression profiles of human breast cancer progression. Proc Natl Acad Sci USA 100:5974–5979
19. Ma XJ, Dahiya S, Richardson E, Erlander M, Sgroi DC (2009) Gene expression profiling of the tumor microenvironment during breast cancer progression. Breast Cancer Res 11:R7
20. Farmer P, Bonnefoi H, Anderle P, Cameron D, Wirapati P, Becette V, Andre S, Piccart M, Campone M, Brain E, Macgrogan G, Petit T, Jassem J, Bibeau F, Blot E, Bogaerts J, Aguet M, Bergh J, Iggo R, Delorenzi M (2009) A stroma-related gene signature predicts resistance to neoadjuvant chemotherapy in breast cancer. Nat Med 15:68–74
21. Institute of Laboratory Animal Resources CoLSNRC (1996) Guide for the care and use of laboratory animals. National Academy Press, Washington, DC
22. Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98:5116–5121
23. Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868
24. Eisen MB, Brown PO (1999) DNA arrays for analysis of gene expression. Methods Enzymol 303:179–205
25. Hosack DA, Dennis G Jr, Sherman BT, Lane HC, Lempicki RA (2003) Identifying biological themes within lists of genes with EASE. Genome Biol 4:R70
26. Simian M, Molinolo A, Lanari C (2006) Involvement of matrix metalloproteinase activity in hormone-induced mammary tumor regression. Am J Pathol 168:270–279
27. Lamb C, Simian M, Molinolo A, Pazos P, Lanari C (1999) Regulation of cell growth of a progestin-dependent murine mammary carcinoma in vitro: progesterone receptor involvement in serum or growth factor-induced cell proliferation. J Steroid Biochem Mol Biol 70:133–142
28. Jacobsen BM, Richer JK, Schittone SA, Horwitz KB (2002) New human breast cancer cells to study progesterone receptor isoform ratio effects and ligand-independent gene regulation. J Biol Chem 277:27793–27800
29. Jeong JW, Lee KY, Kwak I, White LD, Hilsenbeck SG, Lydon JP, DeMayo FJ (2005) Identification of murine uterine genes regulated in a ligand-dependent manner by the progesterone receptor. Endocrinology 146:3490–3505
30. Ismail PM, DeMayo FJ, Amato P, Lydon JP (2004) Progesterone induction of calcitonin expression in the murine mammary gland. J Endocrinol 180:287–295
31. Fernandez-Valdivia R, Mukherjee A, Creighton CJ, Buser AC, DeMayo FJ, Edwards DP, Lydon JP (2008) Transcriptional response of the murine mammary gland to acute progesterone exposure. Endocrinology 149:6236–6250
32. Santos SJ, Aupperlee MD, Xie J, Durairaj S, Miksicek R, Conrad SE, Leipprandt JR, Tan YS, Schwartz RC, Haslam SZ (2009) Progesterone receptor A-regulated gene expression in mammary organoid cultures. J Steroid Biochem Mol Biol 115:161–172
33. Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB (2002) Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J Biol Chem 277:5209–5218
34. Kohrmann A, Kammerer U, Kapp M, Dietl J, Anacker J (2009) Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: new findings and review of the literature. BMC Cancer 9:188
35. Kato S, Pinto M, Carvajal A, Espinoza N, Monso C, Sadarangani A, Villalon M, Brosens JJ, White JO, Richer JK, Horwitz KB, Owen GI (2005) Progesterone increases tissue factor gene expression, procoagulant activity, and invasion in the breast cancer cell line ZR-75-1. J Clin Endocrinol Metab 90:1181–1188
36. Polo ML, Arnoni MV, Riggio M, Wargon V, Lanari C, Novaro V (2010) Responsiveness to PI3K and MEK inhibitors in breast cancer. Use of a 3D culture system to study pathways related to hormone independence in mice. PloS ONE 5(5):e10786. doi: 10.1371/journal.pone.0010786
37. Soldati R, Wargon V, Cerliani JP, Giulianelli S, Vanzulli SI, Gorostiaga MA, Bolado J, do Campo P, Molinolo A, Vollmer G, Lanari C (2009) Inhibition of mammary tumor growth by estrogens: is there a specific role for estrogen receptors alpha and beta? Breast Cancer Res Treat 123:709–724
38. Wargon V, Helguero LA, Bolado J, Rojas P, Novaro V, Molinolo A, Lanari C (2009) Reversal of antiprogestin resistance and progesterone receptor isoform ratio in acquired resistant mammary carcinomas. Breast Cancer Res Treat 116:449–460
39. Weigelt B, Peterse JL, 't Veer LJ (2005) Breast cancer metastasis: markers and models. Nat Rev Cancer 5:591–602
40. Peluso JJ, Pappalardo A (1999) Progesterone maintains large rat granulosa cell viability indirectly by stimulating small granulosa cells to synthesize basic fibroblast growth factor. Biol Reprod 60:290–296
41. Rider V, Carlone DL, Foster RT (1997) Oestrogen and progesterone control basic fibroblast growth factor mRNA in the rat uterus. J Endocrinol 154:75–84
42. Shillingford JM, Miyoshi K, Robinson GW, Bierie B, Cao Y, Karin M, Hennighausen L (2003) Proteotyping of mammary tissue from transgenic and gene knockout mice with immunohistochemical markers: a tool to define developmental lesions. J Histochem Cytochem 51:555–565
43. Grimm SL, Rosen JM (2003) The role of C/EBPbeta in mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia 8:191–204

44. Radestock Y, Hoang-Vu C, Hombach-Klonisch S (2008) Relaxin reduces xenograft tumour growth of human MDA-MB-231 breast cancer cells. Breast Cancer Res 10:R71
45. Kietz S, Feng S, Agoulnik A, Hombach-Klonisch S (2009) Estrogen and TCDD influence RLN2 gene activity in estrogen receptor-positive human breast cancer cells. Ann N Y Acad Sci 1160:367–373
46. Binder C, Hagemann T, Husen B, Schulz M, Einspanier A (2002) Relaxin enhances in vitro invasiveness of breast cancer cell lines by up-regulation of matrix metalloproteases. Mol Hum Reprod 8:789–796
47. Cretu A, Sha X, Tront J, Hoffman B, Liebermann DA (2009) Stress sensor Gadd45 genes as therapeutic targets in cancer. Cancer Ther 7:268–276
48. Guo B, Godzik A, Reed JC (2001) Bcl-G, a novel pro-apoptotic member of the Bcl-2 family. J Biol Chem 276:2780–2785
49. Zerbini LF, Libermann TA (2005) Life and death in cancer. GADD45 alpha and gamma are critical regulators of NF-kappaB mediated escape from programmed cell death. Cell Cycle 4:18–20
50. Burkitt K, Chun SY, Dang DT, Dang LH (2009) Targeting both HIF-1 and HIF-2 in human colon cancer cells improves tumor response to sunitinib treatment. Mol Cancer Ther 8(5):1148–1156
51. de Candia P, Benera R, Solit DB (2004) A role for Id proteins in mammary gland physiology and tumorigenesis. Adv Cancer Res 92:81–94
52. Hato T, Tabata M, Oike Y (2008) The role of angiopoietin-like proteins in angiogenesis and metabolism. Trends Cardiovasc Med 18:6–14
53. Murata M, Yudo K, Nakamura H, Chiba J, Okamoto K, Suematsu N, Nishioka K, Beppu M, Inoue K, Kato T, Masuko K (2009) Hypoxia upregulates the expression of angiopoietin-like-4 in human articular chondrocytes: role of angiopoietin-like-4 in the expression of matrix metalloproteinases and cartilage degradation. J Orthop Res 27:50–57
54. Poon E, Harris AL, Ashcroft M (2009) Targeting the hypoxia-inducible factor (HIF) pathway in cancer. Expert Rev Mol Med 11:e26
55. Sikder HA, Devlin MK, Dunlap S, Ryu B, Alani RM (2003) Id proteins in cell growth and tumorigenesis. Cancer Cell 3:525–530
56. Stighall M, Manetopoulos C, Axelson H, Landberg G (2005) High ID2 protein expression correlates with a favourable prognosis in patients with primary breast cancer and reduces cellular invasiveness of breast cancer cells. Int J Cancer 115:403–411
57. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS (2003) Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144:4562–4574
58. Hong SH, Nah HY, Lee JY, Gye MC, Kim CH, Kim MK (2004) Analysis of estrogen-regulated genes in mouse uterus using cDNA microarray and laser capture microdissection. J Endocrinol 181:157–167
59. Ace CI, Okulicz WC (2004) Microarray profiling of progesterone-regulated endometrial genes during the rhesus monkey secretory phase. Reprod Biol Endocrinol 2:54
60. Orimo A, Weinberg RA (2006) Stromal fibroblasts in cancer: a novel tumor-promoting cell type. Cell Cycle 5:1597–1601
61. Bauer M, Su G, Casper C, He R, Rehrauer W, Friedl A (2010) Heterogeneity of gene expression in stromal fibroblasts of human breast carcinomas and normal breast. Oncogene 29:1732–1740
62. Sadlonova A, Bowe DB, Novak Z, Mukherjee S, Duncan VE, Page GP, Frost AR (2009) Identification of Molecular Distinctions Between Normal Breast-Associated Fibroblasts and Breast Cancer-Associated Fibroblasts. Cancer Microenviron
63. Singer CF, Gschwantler-Kaulich D, Fink-Retter A, Haas C, Hudelist G, Czerwenka K, Kubista E (2008) Differential gene expression profile in breast cancer-derived stromal fibroblasts. Breast Cancer Res Treat 110:273–281
64. Rozenchan PB, Carraro DM, Brentani H, Carvalho Mota LD, Bastos EP, Ferreira EN, Torres CH, Katayama ML, Roela RA, Lyra EC, Soares FA, Folgueira MA, Goes JC, Brentani MM (2009) Reciprocal changes in gene expression profiles of cocultured breast epithelial cells and primary fibroblasts. Int J Cancer 125:2767–2777
65. Allinen M, Beroukhim R, Cai L, Brennan C, Lahti-Domenici J, Huang H, Porter D, Hu M, Chin L, Richardson A, Schnitt S, Sellers WR, Polyak K (2004) Molecular characterization of the tumor microenvironment in breast cancer. Cancer Cell 6:17–32
66. Whitelock JM, Murdoch AD, Iozzo RV, Underwood PA (1996) The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases. J Biol Chem 271:10079–10086
67. Stuelten CH, DaCosta BS, Arany PR, Karpova TS, Stetler-Stevenson WG, Roberts AB (2005) Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-alpha and TGF-beta. J Cell Sci 118:2143–2153
68. Yang F, Foekens JA, Yu J, Sieuwerts AM, Timmermans M, Klijn JG, Atkins D, Wang Y, Jiang Y (2006) Laser microdissection and microarray analysis of breast tumors reveal ER-alpha related genes and pathways. Oncogene 25:1413–1419
69. Maia CJ, Socorro S, Schmitt F, Santos CR (2008) STEAP1 is over-expressed in breast cancer and down-regulated by 17beta-estradiol in MCF-7 cells and in the rat mammary gland. Endocrine 34:108–116
70. Alves PM, Faure O, Graff-Dubois S, Cornet S, Bolonakis I, Gross DA, Miconnet I, Chouaib S, Fizazi K, Soria JC, Lemonnier FA, Kosmatopoulos K (2006) STEAP, a prostate tumor antigen, is a target of human CD8+ T cells. Cancer Immunol Immunother 55:1515–1523
71. Anderberg C, Li H, Fredriksson L, Andrae J, Betsholtz C, Li X, Eriksson U, Pietras K (2009) Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts. Cancer Res 69:369–378
72. Tejada ML, Yu L, Dong J, Jung K, Meng G, Peale FV, Frantz GD, Hall L, Liang X, Gerber HP, Ferrara N (2006) Tumor-driven paracrine platelet-derived growth factor receptor alpha signaling is a key determinant of stromal cell recruitment in a model of human lung carcinoma. Clin Cancer Res 12:2676–2688
73. Crawford Y, Kasman I, Yu L, Zhong C, Wu X, Modrusan Z, Kaminker J, Ferrara N (2009) PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. Cancer Cell 15:21–34
74. Pietras K, Pahler J, Bergers G, Hanahan D (2008) Functions of paracrine PDGF signaling in the proangiogenic tumor stroma revealed by pharmacological targeting. PLoS Med 5:e19
75. Walser TC, Ma X, Kundu N, Dorsey R, Goloubeva O, Fulton AM (2007) Immune-mediated modulation of breast cancer growth and metastasis by the chemokine Mig (CXCL9) in a murine model. J Immunother 30:490–498
76. Selvamurugan N, Shimizu E, Lee M, Liu T, Li H, Partridge NC (2009) Identification and characterization of Runx2 phosphorylation sites involved in matrix metalloproteinase-13 promoter activation. FEBS Lett 583:1141–1146
77. Selvamurugan N, Kwok S, Partridge NC (2004) Smad3 interacts with JunB and Cbfa1/Runx2 for transforming growth factor-beta1-stimulated collagenase-3 expression in human breast cancer cells. J Biol Chem 279:27764–27773
78. Pratap J, Wixted JJ, Gaur T, Zaidi SK, Dobson J, Gokul KD, Hussain S, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2008)

Runx2 transcriptional activation of Indian Hedgehog and a downstream bone metastatic pathway in breast cancer cells. Cancer Res 68:7795–7802
79. Pratap J, Javed A, Languino LR, van Wijnen AJ, Stein JL, Stein GS, Lian JB (2005) The Runx2 osteogenic transcription factor regulates matrix metalloproteinase 9 in bone metastatic cancer cells and controls cell invasion. Mol Cell Biol 25:8581–8591
80. Park OJ, Kim HJ, Woo KM, Baek JH, Ryoo HM (2010) FGF2-activated ERK mitogen-activated protein kinase enhances Runx2 acetylation and stabilization. J Biol Chem 285:3568–3574
81. Kim BG, Kim HJ, Park HJ, Kim YJ, Yoon WJ, Lee SJ, Ryoo HM, Cho JY (2006) Runx2 phosphorylation induced by fibroblast growth factor-2/protein kinase C pathways. Proteomics 6:1166–1174
82. Hunter DJ, Kraft P, Jacobs KB, Cox DG, Yeager M, Hankinson SE, Wacholder S, Wang Z, Welch R, Hutchinson A, Wang J, Yu K, Chatterjee N, Orr N, Willett WC, Colditz GA, Ziegler RG, Berg CD, Buys SS, McCarty CA, Feigelson HS, Calle EE, Thun MJ, Hayes RB, Tucker M, Gerhard DS, Fraumeni JF Jr, Hoover RN, Thomas G, Chanock SJ (2007) A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. Nat Genet 39:870–874
83. Meyer KB, Maia AT, O'Reilly M, Teschendorff AE, Chin SF, Caldas C, Ponder BA (2008) Allele-specific up-regulation of FGFR2 increases susceptibility to breast cancer. PLoS Biol 6:e108